Processing of Lentiviral Vector Pseudotypes Using Anion Exchange and Affinity Chromatography

Yuefei Huang, Michael P. Marino and Jakob Reiser

Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, FDA, Silver Spring, MD, USA

Abstract

In order to simplify the processing of large volumes of lentiviral vector (LVV) supernatants involving LVVs pseudotyped with VSV-G, the measles virus (MV) hemagglutinin (H), or the Tupaia Paramyxovirus (TPMV) H glycoprotein, we previously implemented a concentration and purification strategy based on anion exchange (AEX) membrane chromatography. LVV supernatants processed in this way showed enhanced purity and reduced toxicity compared to vectors concentrated using standard ultracentrifugation protocols.

To improve vector purity, we are currently exploring strategies based on immobilized metal ion affinity chromatography (IMAC) to concentrate LVV pseudotypes bearing hexahistidine (6 x His)-tagged envelope (Env) glycoproteins. Initial experiments focused on LVVs pseudotyped with a 6 x His-tagged, receptor-blind MV H glycoprotein displaying IL-13. Such pseudotypes were captured on HiTrap IMAC HP columns using the AKTA Pure L1 chromatography system and batch eluted using buffers containing imidazole. The recoveries observed were up to 37% of the input samples.

We also tested the ability of the IMAC strategy to capture lentiviral vectors pseudotyped with the commonly used VSV-G Env glycoprotein bearing a 6 x His tag at position 8 of the mature VSV-G glycoprotein (8sHis_VSV-G). The recoveries observed were up to 7% of the input vector sample. The reduced recovery observed using VSV-G pseudotypes compared to MV H pseudotypes is possibly related to limited accessibility of the 6 x His tag present on VSV-G. To improve exposure of the 6 x His tag, we investigated additional sites within the VSV-G ectodomain that can tolerate 6 x His tags while retaining the function of the VSV-G protein. To do this we designed variants with 6 x His tags inserted at positions 2 and 352 of the mature VSV-G glycoprotein (2sHis_VSV-G and 352sHis_VSV-G). Additionally, we explored double-6 x His tags inserted at positions 2, 8 and 352 of the mature VSV-G glycoprotein (2dHis_VSV-G, 8dHis_VSV-G, 352dHis_VSV-G). The data obtained showed that the presence of single or double-6 x His tags inserted at position 2 resulted in vector titers comparable to those obtained using unmodified VSV-G. Vector titers involving VSV-G chimeras with 6 x His tags inserted at positions 8 or 352 resulted in lower titers.

Studies aimed at optimizing the conditions for IMAC purification of LVVs bearing 6 x His tagged Env glycoproteins and improving their recovery and stability are ongoing.

Introduction

The downstream processing of LVVs manufactured on a large scale is challenging due to the lack of simple and selective procedures allowing rapid processing of large volumes of LVV-containing cell culture supernatants.

To simplify the concentration and purification of LVVs pseudotyped with VSV-G or paramyxovirus-derived glycoproteins, we have developed approaches involving Mustang Q anion exchange membrane chromatography. Compared to vectors concentrated using standard ultracentrifugation procedures, vectors processed using Mustang Q chromatography displayed reduced toxicity as well as enhanced purity. (References 1 and 2)

To improve vector purity, we are currently examining the use of immobilized metal ion affinity chromatography (IMAC) to concentrate LVVs pseudotyped with 6 x His-tagged Env-glycoproteins.

Materials and Methods

- **Detection of 6 x His-tagged VSV-G variants in transfected 293T cells:** Transfected cells were exposed to an Anti-His tag -AF647 monoclonal antibody (R&D system) or an isotype control 48 hr after transfection. Cells were analyzed using the FACSCanto™ II system.
- **Determination of lentiviral vector titers:** Functional LVV titers (transducing units, TU) were determined by flow cytometry analysis of HEK 293 cells transduced with different dilutions of EGFP-encoding LVVs (Reference 3).
- **IMAC:** Vector supernatants were collected 72 hr after transfection and passed through a 0.45 µm filter. Ten bed volumes of vector supernatant were mixed with twenty bed volumes of phosphate-buffered saline (PBS) (pH 7.4) and loaded onto HisTrap columns (GE) at a flow rate of 0.5 ml/min using ÄKTA pure 25 L1 system. Columns were washed with 15 column volumes of PBS containing 20 mM imidazole (pH 7.4). LVVs were eluted with PBS containing imidazole ranging from 100 to 500 mM at a rate of 0.5 ml/min. All fractions (input, flow-through, wash, and eluates) were titrated on U251 or HEK 293 cells immediately after IMAC.

Results

1. IMAC of lentiviral vectors pseudotyped with measles virus H and F glycoproteins.

LVV stocks pseudotyped with a 6 x His-tagged, receptor-blind measles virus (MV) H glycoprotein displaying IL-13 were prepared using 15-cm tissue culture dishes. Figure 1 shows the combined elution profiles using the ÄKTA pure chromatography system of three independent batches of LVV vectors after capturing onto a 1 ml HiTrap column and batch elution using a buffer containing 125 mM imidazole. Additional batch elution steps involving 250 mM, 500 mM and 1 M imidazole were performed (Figure 1). A total of $1.82 \times 10^7 \pm 1.11 \times 10^6$ TUs were loaded and 33.09±4.01% of the input vector TUs appeared in the 125 mM imidazole fraction.

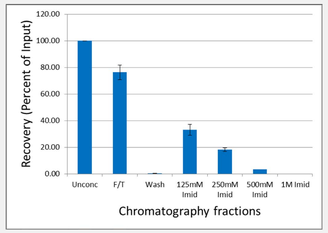

Figure 1. Chromatography of lentiviral vectors pseudotyped with MV-derived H and F glycoproteins by IMAC. Unconc: Vector supernatant; F/T: Column flow through; Wash: 40 mM imidazole (imid).

2. Scaled-up production/IMAC chromatography system for lentiviral vectors bearing 6 x His-tagged MV glycoproteins.

A scale-up of the above system involved 560-ml HYPERFlask vessels. The volumes obtained were 100 ml for each flow through, 75 ml for the wash, and 50 ml for the imidazole elutions. The elution profiles are displayed in Figure 2. A total of $4.82 \times 10^7 \pm 1.58 \times 10^7$ TUs were loaded and 24.24 ± 2.23% of the input vector TUs appeared in the 125 mM imidazole fractions.

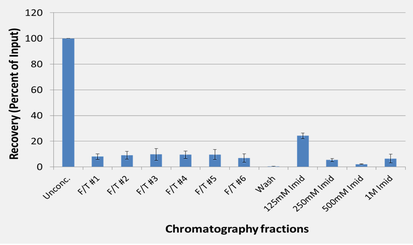

Figure 2. Concentration by IMAC of LVV vectors pseudotyped with MV-derived H and F glycoproteins produced in HYPERFlask vessels using 5 ml HiTrap columns.

3. Testing of accessibility of 6 x His tags present in VSV-G variants

Our next goal was to identify sites within the VSV-G ectodomain that can tolerate the insertion of 6 x His tags while retaining the function of the VSV-G protein. To do this, we designed variants with 6 x His tags inserted at positions 2, 8 and 352 of the mature VSV-G Env. VSV-G variants with 6 x His tag inserted at position 8 are described in Reference 4 and VSV-G variants with 6 x His tags inserted at position 2 and 352 are in Reference 5.

To test the accessibility of the 6 x His tag present on VSV-G variants, recombinant plasmids encoding 6 x His tagged VSV-G proteins were transiently transfected into HEK293T cells. 48 hr later, transfected cells were labelled with an AF647 conjugated anti-His tag antibody (Figure 3A). Anti-His tag antibody binding was analyzed, and percentage of AF647-positive cells and median fluorescence intensities (MedFI) are shown in Figures 3B and 3C, respectively.

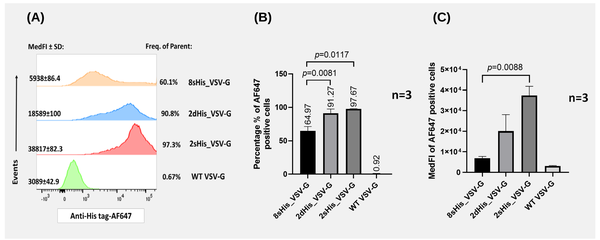

Figure 3. (A) Detection of 6 x His tags on HEK 293T cells transfected with recombinant plasmids encoding 6 x His tagged VSV-G variants. 6 x His tags were detected using anti-His tag antibody conjugated with fluorophore AF647. (B) Percentage of AF647-positive cells and (C) Median fluorescence intensity (MedFI) of AF647 fluorophore (n=3). The statistical analysis involved a paired two-tailed t-test.

4. Testing the impact of the 6 x His tags present in VSV-G variants on lentiviral vector titers

The titers of LVVs pseudotyped with 6 x His tagged VSV-G variants were determined by flow cytometry of transduced HEK293 cells (Figure 4). Unpaired t-tests showed that there were no significant differences among the titers of LVVs pseudotyped with WT VSV-G versus LVVs pseudotyped with 2sHis_VSV-G (p=0.2091) and 2dHis_VSV-G (p=0.1385) (n=3-9).

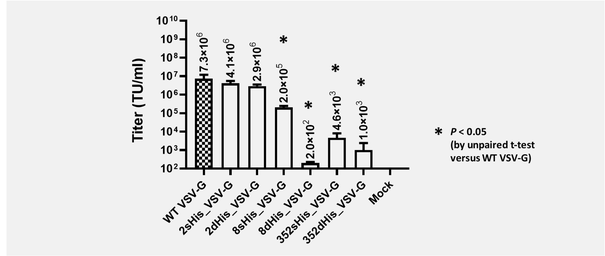

Figure 4. Titers of LVVs pseudotyped with either wild-type VSV-G (WT VSV-G) or 6 x His tagged VSV-G variants. The LVVs used encode EGFP. All titers were determined using HEK293 cells.

5. Investigating the effects of imidazole concentration and freeze-thaw cycle on lentiviral vectors bearing 6 x His tagged VSV-G variants

LVVs were exposed to different concentrations of imidazole for 30 min at room temperature or vectors were subjected to one freeze-thaw cycle. LVVs treated in these ways were used to transduce HEK293 cells and titers were determined by flow cytometry 3 days later (Figure 5).

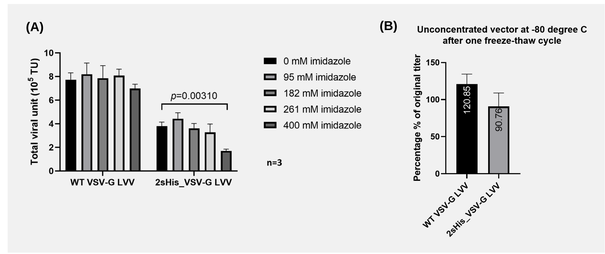

Figure 5. (A) Effects of imidazole concentration on titers of LVVs bearing WT VSV-G or 2sHis_VSV-G. Titers shown represent the averages of three independent experiments (n=3). (B) Stability of unconcentrated LVVs at -80 degree C after one round of freezing and thawing. Values shown are percentages of the original titer from four independent experiments (n=4). Error bars represent standard deviations.

6. IMAC of lentiviral vectors pseudotyped with VSV-G variants bearing 6 x His tag

Small-scale (10 ml) aliquots of lentiviral vectors pseudotyped with the 2sHis_VSV-G variant and/or with unmodified (WT) VSV-G were used for IMAC (Figure 6, panels A-D). Panel A shows the IMAC results obtained using LVVs pseudotyped with WT VSV-G; Panels B and C shows the IMAC profiles of LVVs pseudotyped with WT VSV-G and 2sHis_VSV-G at different ratios; Panel D shows the IMAC profile of LVVs pseudotyped with the 2sHis_VSV-G variant.

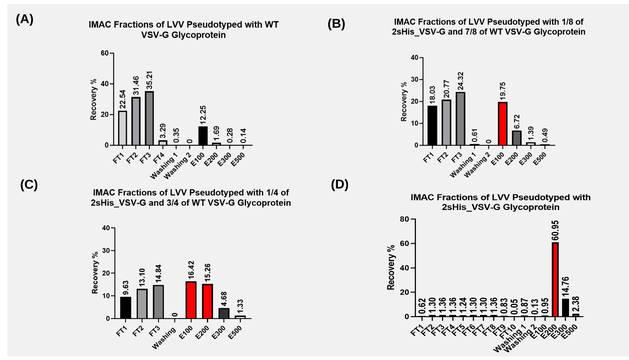

Figure 6. Small-scale IMAC chromatography optimization studies for LVVs pseudotyped with (A) WT VSV-G, (B) 1/8 of 2sHis_VSV-G plus 7/8 of WT VSV-G, (C) 1/4 of 2sHis_VSV-G and 3/4 of WT VSV-G, and (D) 2sHis_VSV-G. A total of 4.26×10^7, 7.32×10^7, 1.04×10^8, and 1.26×10^7 TUs were loaded for samples in panels A, B, C, and D, respectively. The percentage (%) of input vector TUs that appeared in the 100 mM and 200 mM imidazole fractions are indicated.

Conclusions

- **The IMAC results involving lentiviral vectors pseudotyped with 6 x His-tagged paramyxovirus glycoproteins or VSV-G show that such pseudotypes can be captured using HisTrap columns.**
- **Work to demonstrate the specificity of capture of lentiviral vectors by IMAC resin is in progress.**
- **The IMAC results involving lentiviral vectors pseudotyped with the measles virus H protein bearing a 6 x His tag at its C-terminus produced using HYPERFlask vessels demonstrate that the approach is scalable.**
- **The scalability of VSV-G 6 x His-containing lentiviral vectors has yet to be demonstrated.**
- **Work aimed at optimizing desalting and concentration of lentiviral vectors following IMAC will be pursued in the future.**
- **Work to assess the purity of the lentiviral vector pseudotypes bearing 6 x His tags purified by IMAC will be carried out in the future.**

References:

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