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Polyethylenimine-Based Transfection Method as a Simple and Effective Way to Produce Recombinant Lentiviral Vectors

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Abstract HIV-1-derived lentiviral vectors (LvV) are within the most attractive gene delivery vehicles in the context of both dividing and quiescent cells. LvV is currently produced by the conventional calcium phosphate precipitation method. Nevertheless, this procedure is highly susceptible to variations in pH and impurities, which lead to inconsistencies in LvV production. Here, we present a simple and robust procedure for LvV production using branched 25 kDa polyethylenimine, with a transfection efficiency of over 90% and viral titer yields of about 1×10^7 infective lentiviral particles per milliliter. The procedure outlined is simple, consistent, and as inexpensive as the $CaPO_4$-based method.

Keywords Lentiviral vector · Polyethylenimine · Transfection

Introduction

LvV's derived from the type 1 human immunodeficiency virus (HIV-1) are the vectors of choice for long-term, stable in vitro and in vivo gene transfer. These vectors are attractive because they can transduce both dividing and quiescent cells in a stable manner [1, 2]. Standard LvV production requires co-transfection of three helper plasmids encoding a pseudotyping envelope and the complementing structural genes [3], along with the transgene encoding the lentivector, into human embryonic kidney cells (HEK293-T). Following transfection, lentiviral particles (LvP) are produced and released into the culture supernatant. Stable helper cell lines, transgenic for the constitutive complementation of structural genes and the conditional expression of the toxic vesicular stomatitis virus G

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(vsvg) protein, have been considered as alternatives to quadruple transient transfections [4, 5]. However, their overall production performance did not always meet expectations [6]. Thus, transient transfection is still the preferred technology for the pilot production of LvV in a rapid, reliable, and safe format.

The conventional calcium phosphate precipitation technique [7] is often used for the transient transfection of the HEK293-T cell line to produce LvV. The main drawbacks of this method are the large variability observed in transfection efficiency and viral production and the difficulties in scaling-up the precipitation reactions. Although several transfection protocols have been successfully used for the production of LvV [8–10], most of them involve expensive transfection reagents (e.g., Lipofectamine 2000, Lipofectamine™, SuperFect, FuGENE 6, GeneJammer transfection reagent, FuGENE® HD, Lentifectin™, TransIT®-LT1 Reagent…etc.), which hinder their use in pilot or large-scale production processes. The use of polyethylenimine (PEI) as an alternative transfection method for LvV production was recently described [11]. However, that study focused on the production LvV in serum-free suspension-growing 293 EBNA-1 cells, using linear PEI as the transfection reagent. Although this procedure gave acceptable viral titers in the supernatant of transfected cells, its applicability is limited by the lack of infrastructure and experience of most laboratories to perform suspension cell cultures. Since the transient transfection of adherent cell cultures is still the most widely used procedure for the production of LvV stocks, the purpose of this study was to compare the standard $CaPO_4$-based transfection method with the PEI-based method in producing LvV in these cell cultures. We found that the PEI-based method produced virus titers of about 1×10^7 infective lentiviral particles per milliliter, with a high degree of reproducibility and consistency.

Materials and Methods

Lentiviral Expression System

The 293FT cells and helper plasmids were purchased from Invitrogen-Life Technologies (cat. no. K4950-00). The vectors contained in the ViraPower™ Lentiviral Expression System were used as helper plasmids for virus production. In this system, the pLP1 plasmid contains the Gag and Pol genes; the pLP2 plasmid encodes the Rev protein which interacts with the RRE on pLP1 to induce Gag and Pol expression; and the pLP/VSVG plasmid encodes the vsvg protein for the production of the pseudotyped retrovirus with a broad host range [12]. To generate the pLGW vector, the lacZ expression cassette was removed from the pLenti6/V5-GW/lacZ by digesting with ClaI and Kpn I, and a synthetic DNA fragment containing a central polypurine tract flanked by two multiple cloning sites, was inserted in its place (pL vector). A woodchuck postregulatory element (WPRE) was further inserted into a BamH I site toward the 3′ of the polypurine tract (pLW vector). Finally, a PCR-amplified DNA fragment containing the CMV enhanced promoter and the EGFP gene was cloned into the EcoR V site between the polypurine tract and the WPRE sequence (pLGW vector).

Preparation of PEI and CaPO4 Stock Solutions

Branched polyethylenimine with a molecular weight of 25 kDa was purchased from SIGMA-Aldrich (cat. no. 408727). To prepare the 18 mM solution, 16.2 mg of the PEI reagent were diluted in 19 ml of dH_2O, adjusted to pH 7 with 1 N HCl, completed to 20 ml with dH_2O and filtered (0.2 μm, Sartorius).

For $CaPO_4$ transfection, to prepare a 2.5-M $CaCl_2$ stock solution, 36.75 g of $CaCl_2$ were diluted in 100 ml dH2O, sterilized by filtration (0.2 μm, Sartorius), divided into aliquotes of 1.5-ml in Eppendorff tubes and stored at –20°C. For the 2× BBS solution [50 mM bromoethanesulfonic acid (BES), 280 mM NaCl, 1.5 mM Na_2HPO_4], 8.18 g of NaCl, 5.33 g of BES (Calbiochem cat. no.391334), and 0.105 g of Na_2HPO_4 were diluted in 450 ml of dH_2O, adjusted to pH 6.95 with 1 M NaOH, completed to a 500 ml volume, filtered through 0.2 μm and stored as 10 ml aliquots at 4°C.

Transfection Procedures

For the PEI-based method, 293-FT cells were seeded at 1.7×10^6 cells per 25 cm^2 flask (Greiner bio-one) about 18 h before transfection. Cells were rinsed and supplemented with a fresh serum-free culture medium (4 ml) just before transfection. The plasmid DNA mix (8 μg per flask) and 8 μl of the polymer solution (1 μl of DNA is 3 nmol of phosphate and 1 μl of 18 mM PEI stock solution contains 18 nmol of amine nitrogen) were each diluted in 50 μl of 5% glucose and placed on a vortex stirrer for 1 min. After ≈10 min, both solutions were mixed, and the resulting solution was again placed on the vortex stirrer for 1 min. After 10 more minutes, the transfection mixture was completed up to 0.5 ml with serum-free culture medium and added to the cells. After 6 h of incubation, the medium was supplemented with 0.5 ml of fetal calf serum reaching a final volume of 5 ml. For the $CaPO_4$-based method, 293FT cells were seeded at 1.7×10^6 cells per 25 cm^2 flask (Greiner bio-one) into precoated poly-L-lysine flasks, about 18 h before transfection. The plasmid DNA mix (8 μg per flask) was added to 175 μl of fresh 0.25 M $CaCl_2$ and mixed by pipetting. A sterile 1 ml pipette connected to a Pipet-Aid was used to bubble air into the tube. While bubbling, 175 μl of the 2× BBS solution were dripped in. The bubbling continued for an additional 5 s to ensure thorough mixing. The mixture was incubated for 15 min at room temperature and then added to the cells which were incubated at 3% CO_2, 37°C overnight (16–20 h) before removing the medium and adding 5 ml of fresh culture medium +2% fetal calf serum. The LvV in the culture medium were harvested 48 h after transfection.

EGFP Assessment by Flow Cytometry

The percentages of EGFP-expressing cells after transfection or lentiviral transduction were determined using a FACScan flow cytometer (Becton Dickinson Biosciences). Cells were released using trypsin and fixed with 2% formaldehyde in phosphate-buffered saline (NaCl 136 mM, KCl 2.6 mM, Na_2HPO_4 8.0 mM, KH_2PO_4 1.47 mM). Just before sorting with the flow cytometer, the cells were strained through a 70-μm nylon cell strainer (BD Falcon, cat. no.352350). During acquisition set-up, the FL1 channel (GFP) was adjusted so that more than 99.99% of the untreated control cells had a fluorescence intensity of less than 10. The median intensity for the control cells was approximately 1.0. For comparison purposes, cells with a fluorescence intensity of 10 or more were scored as positive. Data was analyzed using WinMDI version 2.8 software.

Results and Discussion

LvV are classically produced by transient transfection of HEK293-T cells using the calcium phosphate method, since this method is very well established and reagents are inexpensive.

There are many modifications of the basic protocol but they all involve precipitating plasmid DNA in a mixture of calcium chloride and HEPES-buffered saline. HEK293-T cells are typically used because they constitutively express the simian virus 40 (SV40) large T-antigen allowing for the episomal replication of the plasmid vectors containing the origin of replication of the wild-type SV40. Also, HEK293-T cells transfect relatively well by the calcium phosphate method (50–70%). In our laboratory a derivative of HEK293-T cells called 293-FT cells from Invitrogen-Life Technologies are normally used. We have observed much variability in transfecting adherent 293-FT cells by the calcium phosphate method because of two main problems inherent to this method: transfection efficiency is inhibited by reagent impurities (particularly impurities present in calcium chloride) and slight pH changes (0.05 pH units) of the 2× BBS (or 2× HBS) or in the culture medium are found which significantly reduce transfection efficiency. Sometimes, incubation at 3% CO_2 is required to obtain an effective $CaPO_4$ precipitation.

Here, we compared the PEI transfection protocol with the standard procedure based on $CaPO_4$ precipitation for the production of LvV. The pLGW plasmid was used as a packageable vector which contains the EGFP marker under the control of a CMV promoter (Fig. 1A). For both PEI and calcium phosphate transfection protocols, the packageable vector and helper plasmids were transfected in a ratio of 1:2:2:3 of pLP/VSVG:pLP1:pLP2 and pLGW, respectively.

All transfections were performed in 293-FT cells in 25 cm^2 cell culture flasks using 8 μg of DNA per flask. For PEI transfection, branched polyethylenimine with a molecular weight of 25 kDa was used as an 18-mM stock solution. PEI/DNA polyplexes were prepared in a 5% glucose solution using a ratio of PEI nitrogen to DNA phosphate (N/P ratio) of 6 according to the conditions described by Abdallah and co-workers [13]. The PEI-based protocol does not imply a change in the medium, and the LvV can be harvested 48 h after transfection. The $CaPO_4$-based protocol followed the procedure of Tiscornia and co-workers in 2006 [14]. This protocol requires changes in the culture medium 16–20 h after

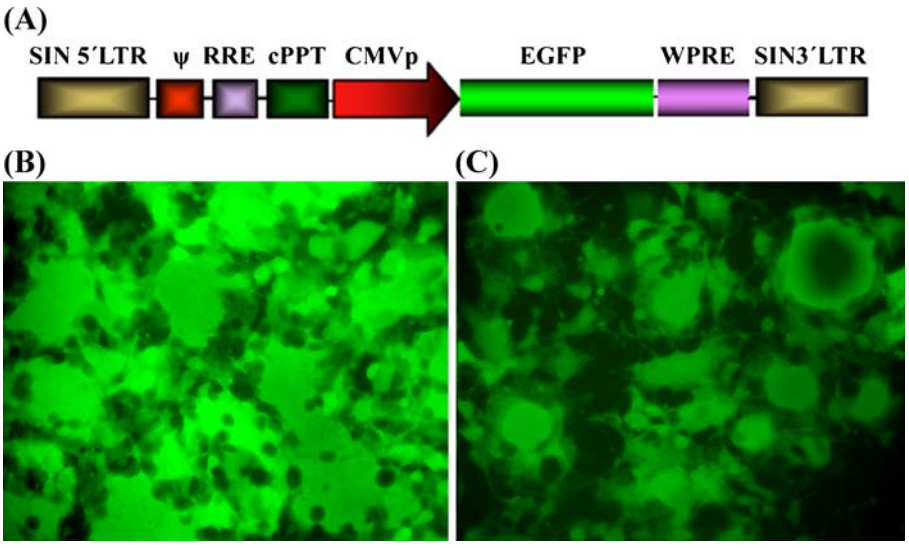

Fig. 1 Transfection performance of the $CaPO_4$ and PEI based method for the production of LvV. **a** pLGW vector. Details of the plasmid construction will be provided upon request. **a** and **b** 293FT cells transfected with PEI- and $CaPO_4$-based methods, respectively, 24 h after transfection

transfection, and the LvV are harvested about 72 h after starting the transfection. Both transfection procedures were performed in triplicate and in three independent experiments, using different stock solutions.

Simple observation under the fluorescent microscope 24 h after transfection revealed a higher density of EGFP-expressing cells in the culture flasks transfected with PEI. Most cells were then forming fluorescent multinucleated syncytia, resulting from the vsvg expression on their surface (Fig. 1b and c). EGFP expression from both the transfected and control flasks was analyzed by flow cytometry. Results show that 94.3%±3.2 of 293FT cells transfected with PEI expressed EGFP, whereas only 66.2%±5.6 of the $CaPO_4$ transfected cells were EGFP positive. The intensity of EGFP fluorescence cells was also higher in the PEI-transfected cells with a median value of approximately 950 versus 210 in the cells transfected by the $CaPO_4$-based protocol.

The concentration of LvP was determined by the quantification of the p24 virus capsid protein in the culture supernatant (p24 assay). p24 levels were quantified using a p24-specific http://www.merriam-webster.com/dictionary/enzyme-linked+immunosorbent+assay enzyme-linked immunosorbent assay according to the supplier's protocol (DAVIH-LAB, La Habana, Cuba). Virus numbers were calculated based on the fact that a LvP contains 2,000 p24 molecules in its envelope [15]. Figure 2a shows the means±SD of three experiments. The average number of LvP/ml for each assay was calculated by combining the data from three flasks. The PEI-based transfection method resulted in LvP production that was 40 to 120 times higher than that obtained with the standard $CaPO_4$-based protocol.

The titers of infective LvP were assessed by their ability to transfer the EGFP gene to the target cells. According to the total LvP concentration, 293FT cells were transduced with 500, 250, and 125 LvP/cell in a 96-well cell culture plate. Figure 2b shows the percentage of GFP-positive cells as determined by flow cytometry for each rate of LvP/cell assayed. The percentage of fluorescent cells in cells transduced with the LvP produced by the $CaPO_4$-based protocol was 1.4–1.8 times higher than in cells transduced with the LvP produced by the PEI-based method. The ratio of the infective to total number of LvP revealed that, under our conditions, 0.93% of the total LvP generated by the CaPO4-based method were infective, while 0.65% of the total LvP produced by the PEI procedure were infective. Although the percentage of infective particles was slightly lower in the PEI-based procedure, the overall yield of infective LvP obtained by this method was about 50 times higher than that obtained by the standard method based on $CaPO_4$ precipitation (Fig. 2c).

As expected, the results of these experiments demonstrated that both methods can be used to generate infectious LvV. However, the PEI-based transfection protocol gave significantly higher viral titers. Furthermore, it is unnecessary to change the culture medium during the viral production phase, which is an additional benefit of this procedure that simplifies work and shortens the time needed for LvV production. In contrast to the $CaPO_4$-based transfection procedure, the solutions required for PEI-based transfection are very simple (18 mM of PEI in dH_2O, pH 6.8–7 and glucose 5%); the method is easy and the overall transfection efficiency is not dramatically altered by pH fluctuations during the formation of DNA/PEI complexes or later in the culture medium.

To our knowledge, this is the first report of the use of PEI for generating high titers of lentiviral vectors in adherent cell cultures. The PEI-based transfection protocol outlined here allows for an efficient and reproducible production of high-titer LvV that exhibits robust transduction properties both in vivo and in vitro. With the PEI-based method, we have overcome many of the problems previously found in the consistent production of large volumes of high-titer LvV. For more than a year, our lab has been producing LvV using the 25-kDa branched PEI. We have found that this procedure consistently yields high titer

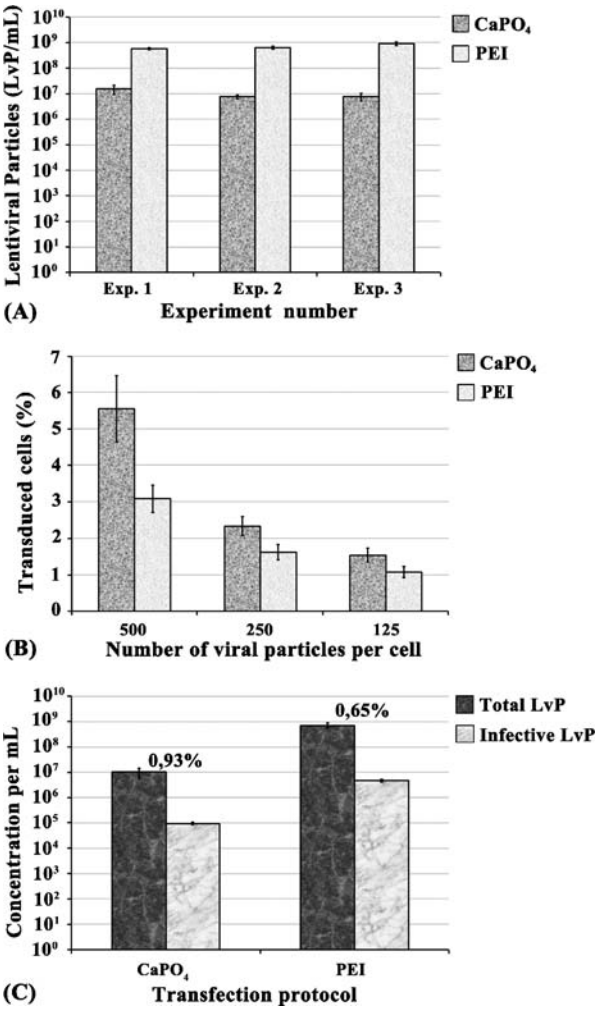

Fig. 2 Influence of the transfection method on the production of total and infective LvP. **a** Total LvP concentration from three separate experiments, calculated by combining data from three different flasks in each experiment. **b** Quantification of infective LvP. Data was calculated by combining the results from nine lentiviral stocks (from three flasks, from three different experiments) for each transfection method. **c** Overall performance of the $CaPO_4$- and PEI-based transfection procedures for the production of LvV. LvP performance is indicated above each pair of bars as the ratio between the infective LvP and total LvP

vector stocks averaging 1.0×10^7 TU/ml; these titers are comparable to vector stocks previously prepared in our lab, using transfection reagents such as SuperFect or Lipofectamine 2000. LvV produced by the PEI-based method has been successfully used for in vitro transduction assays and for the generation of genetically modified mice with transgenesis efficiency ranging from 64% to 87% (articles in preparation). The use of PEI greatly simplified the transfection protocol and significantly reduced costs associated to LvV production. With 100 ml (≈100 g\) of branched 25 kDa PEI from Sigma-Aldrich that can be purchased for approximately $ 52, about 123.45 l of the 18-mM stock solution can be prepared, which is enough to transfect 123.45 g of DNA using a PEI nitrogen to DNA phosphate ratio of 6. Furthermore, the methods can be easily adapted to the large-scale production protocols that are currently being developed for transgenic animal generation approaches or for their potential use in clinical studies.

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