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Optimization of the transductional efficiency of lentiviral vectors: effect of sera and polycations

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Abstract

Lentiviral vectors are widely used as effective gene-delivery vehicles. Optimization of the conditions for efficient lentiviral transduction is of a high importance for a variety of research applications. Presence of positively-charged polycations reduces the electrostatic repulsion forces between a negatively-charged cell and an approaching enveloped lentiviral particle resulting in an increase in the transduction efficiency. Although a variety of polycations are commonly used to enhance the transduction with retroviruses, the relative effect of various types of polycations on the efficiency of transduction and on the potential bias in the determination of titer of lentiviral vectors is not fully understood. Here we present data suggesting that DEAE-dextran provides superior results in enhancing lentiviral transduction of most tested cell lines and primary cell cultures. Specific type and source of serum affects the efficiency of transduction of target cell populations. Non-specific binding of enhanced green fluorescent protein (EGFP)-containing membrane aggregates in the presence of DEAE-dextran does not significantly affect the determination of the titer of EGFP-expressing lentiviral vectors. In conclusion, various polycations and types of sera should be tested when optimizing lentiviral transduction of target cell populations.

Keywords

Lentivirus; transduction; gene transfer; polycation; serum; flow cytometry

INTRODUCTION

Lentiviruses are lipid-enveloped particles comprising a homodimer of linear single-stranded RNA genomes. Unlike retroviruses, lentiviruses rely on an active transport of the pre-integration complex through the nucleopore by the nuclear import machinery of the target cell [1]. This property allows lentiviruses to infect both dividing and non-dividing cells making them attractive candidates for various gene delivery applications in basic research [2–6]. Pseudotyping with amphotropic viral envelope such as the envelope of the vesicular stomatitis virus-G (VSV-G) significantly expands the tropism of lentiviral vector-based gene delivery vehicles and widens the range of possible cellular targets [3]. Since the genetic information required to package a functional lentiviral core in a vector represents only a

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COMPETING INTEREST STATEMENT

The authors declare no competing interests.

fraction of the parental genome “minimal” packaging constructs lacking all genes that are not critical for efficient gene transfer have been adopted [7]. Further increasing vector biosafety, a new generation of “self-inactivating/suicidal” vectors has been developed containing a deletion in the downstream long terminal repeat (LTR) that, upon transduction, results in the transcriptional inactivation of the upstream LTR and substantially diminishes the risk of vector mobilization and recombination [8–10]. The high versatility, transduction efficacy, and the ability to transduce a wide range of cellular and tissue targets, including difficult-to-transfect cells such as the neurons and glial cells, have resulted in a wide-spread use of lentiviral vectors [11–13].

At the initiation of transduction, the binding of virus particles to target cell is mediated by specific interactions between the viral envelope and specific receptors on the cell surface. However, several recent studies have demonstrated that the initial step of virus binding does not involve specific envelope-receptor interactions but rather receptor-independent binding events [14, 15]. The efficiency of this initial event and, consequently, lentiviral transduction is diminished by strong electrostatic repulsion between the negatively charged cell and an approaching enveloped virus [16, 17]. Methods designed to overcome this problem include centrifugation of targets cells with virus at low speeds, co-localization of cells and virus on immobilized proteins, and employing multiple rounds of transduction [17, 18]. Importantly, the addition of positively-charged polycations such as polybrene, DEAE-dextran, protamine sulfate, poly-L-lysine, or cationic liposomes reduces the repulsion forces between the cell and the virus and mediates the binding of retroviral particle to the cell surface resulting in a higher efficiency of transduction [17, 19–25]. Although various polycations are commonly used to enhance the transduction with retroviral and lentiviral vectors [26, 27], few studies have focused on the comparison of the effect of various polycations on the efficiency of lentiviral transduction of established cell lines and primary cell cultures. Importantly, polycationic substances may promote non-specific binding and capture of inactive particles by target cells resulting in an overestimation of transduction efficiency. In this study, we assessed the critical factors affecting the efficacy of transduction with lentiviral vectors and determination of viral titers. We present data demonstrating that the choice of polycation and serum in culture medium significantly affects the resulting efficiency of transduction. Although DEAE-dextran provided highest levels of transduction under the conditions described here, a variety of polycations in combination with various types of sera should be tested when optimizing each system.

MATERIALS AND METHODS

Cell lines

293FT cells were maintained in a complete DMEM medium (4 mM L-glutamine, 4.5 g/L D-glucose, 0.1 mM MEM non-essential amino acids, 100 U/ml penicillin-streptomycin, and 10 µg/ml G418 (Gibco/Invitrogen, Grand Island, NY)) containing 10% serum as indicated. HT 1080 cells were maintained in a complete DMEM medium with 1 mM sodium pyruvate and 10 % serum. Four different types of sera were tested: Serum 1 - Bovine Calf Serum (BCS, Gibco), heat-inactivated; Serum 2 – Fetal Bovine Serum (FBS; Cellgro, Manassas, VA), not heat-inactivated; Serum 3 – identical to Serum 2 but heat-inactivated; and Serum 4 - FBS (Hyclone, Logan, Utah), heat-inactivated.

Lentiviral vectors

The ViraPower™ Promoterless Lentiviral Gateway Expression system (Invitrogen, Carlsbad, CA) was used to generate lentiviral vectors. The gene encoding enhanced green fluorescent protein (EGFP) was inserted at the position of blasticidin in the pLenti6/R4R2/V5 DEST vector under the control of the SV-40 promoter resulting in the pL-EGFP transfer

vector. To produce the virus, 293FT cells were plated on poly-L-lysine-coated 10-cm plates (Corning, New York) at 6.5×10^6 cells per plate in a complete DMEM medium with 10% serum and 1 mM sodium pyruvate and allowed to adhere for 16 hrs. Transfections were performed as per manufacture's instructions with 4.5 μ g of transfer vector plasmid, 18 μ g packaging mixture (Invitrogen, Carlsbad, CA) and 67.5 μ l of Lipofectamine™ 2000 in serum-free Opti-MEM medium (Invitrogen). 24 hours after transfection the cells were washed with phosphate-buffered saline (PBS); fresh complete DMEM with pyruvate and 10 % FBS (Serum 4) was added and the cells were incubated for an additional 24 hours. Vector supernatants were collected 48 hrs after transfection, filtered through a 0.45 micron syringe filter (Whatman, Clifton, NJ) and concentrated by ultracentrifugation at 22,000 *g* for 2 hr at 4°C.

To establish the titer of viral preparations, 293FT cells were plated at 5×10^4 cells per well in 24-well tissue culture plates, allowed to adhere overnight, and a complete DMEM medium containing lentivirus at various concentrations and supplemented with 6 μ g/ml of polybrene, 6 μ g/ml of DEAE-dextran sulfate, 10 μ g/ml of poly-L-lysine, or 10 μ g/ml protamine sulfate (Sigma-Aldrich, St. Louis, MO) as indicated. Next day, equal amount of fresh media was added and the cells were incubated for an additional 24 hrs. Adherent cells were washed with warm PBS, trypsinized, and resuspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS prior to the analysis by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). Vector titers (IU/ml) were calculated according to the following equation: % *EGFP positive cells* $\times 5 \times 10^5$ / μ l of virus preparation. To ensure maximum accuracy and to compensate for multiple transduction events per cell, the titers were calculated only from transductions with dilution factors resulting in fewer than 25% transduced cells.

Transduction of cell lines and primary cell cultures

Cell lines were plated in a complete media containing 10 % serum and allowed to adhere overnight. Virus was added at the indicated multiplicity of infection (MOI) in the presence of indicated concentration of polycation and the cells were incubated for 24 hrs. After 24 hrs, equal amount of fresh media with no virus was added and the cells were incubated for additional 24 hrs. Adherent cells were washed with warm PBS, trypsinized, and resuspended in 1% paraformaldehyde in PBS prior to the analysis by flow cytometry. Cellular toxicity was determined using a trypan blue staining method.

To prepare primary splenocytes, spleens from 8–12 w old C57Bl/6 mice were homogenized red blood cells were lysed in 3 ml of RBC lysis buffer (Biolegend, San Diego, CA) and the mononuclear cells were prepared by density gradient centrifugation. Unsorted mononuclear cells were plated at 2×10^6 cells / well in a 24-well plate in RPMI media containing 10% FBS (Hyclone) and 6 μ g/ml of polybrene or DEAE-dextran as indicated. Virus was added at an MOI 9. 48 hours after transduction, cells were resuspended in PBS with 2% FBS and stained with anti-CD4 and CD19 antibodies (BD Biosciences). The percentages of transduced cells were analyzed by flow cytometry.

Data analysis

Data were analyzed using Student's t-test or non-parametric Mann Whitney rank sum test using GraphPad Prism 5 and SigmaStat 3.1 programs. A standard level of statistical significance $p = 0.05$ was employed. Figures were generated using the GraphPad Prism 5 program (GraphPad Software Inc).

RESULTS

In order to optimize the efficiency of lentiviral transduction, the effect of various types of polycations and sera on the transduction efficiency was investigated. As presented on Figure 1, DEAE-dextran was superior to polybrene in mediating transfection of 293FT cells at various multiplicities of infection (MOIs). The difference was statistically significant ($p = 0.04$, 0.03 and 0.001 at MOI 0.07 , 0.7 and 7 , respectively; Student's t -test). This observation is of particular importance since majority of published studies employ polybrene to enhance lentiviral transduction. Furthermore, we found that particular combinations of sera and DEAE-dextran had distinct effects on the transduction efficiency, particularly at mid to high MOI (0.7 to 7). Fetal bovine serum (FBS) provides higher transductional efficiency compared to bovine calf serum. Differences were observed between sera obtained from various manufacturers as well as between specific lots of sera. In contrast, heat inactivation did not exert a discernible effect on the efficiency of lentiviral transduction (Fig. 1; Serum 2 versus Serum 3).

DEAE-dextran appears to be superior to polybrene (PB) in increasing transductional efficiency of most cell lines tested including 293FT (Figure 2A), HT 1080 (Figure 2B), NIH3T3 and other cell lines (data not shown). The effect was observed across a range of concentrations with peak efficiencies at $6-8 \mu\text{g/ml}$ (Figure 2C). There was no or low ($<5\%$; detected using trypan blue staining) toxicity of polybrene or DEAE-dextran on any of the tested cell lines at concentrations of polycations up to $10 \mu\text{g/ml}$. However, as specified in the protocol of the manufacturer of pLenti vectors (Invitrogen), polybrene may be toxic to primary cell cultures such as primary neurons and therefore should be carefully titrated [29]. Since a previously published study suggested that small variances in the pH of transduction media may significantly influence transduction efficiency [16], transduction culture mediums with pH ranging from 7.0 to 7.6 were tested. Optimal transduction of DEAE-dextran-containing cultures typically occurred at pH $7.0 - 7.2$ (Figure 2D); however, the effect of pH appeared to be minor.

To compare the efficacy of DEAE-dextran to that of other polycations used for the facilitation of retroviral transduction, namely protamine sulfate (PS) and poly-L-lysine (PLL), an optimal concentration was determined for each polycation based on the titration using pL-EGFP vector on 293FT cells. When used at optimal concentrations, PB, PS, and PLL were less active compared to DEAE-dextran in mediating the transduction of target cells (Figure 3A). Importantly, as demonstrated on Figure 3B, the presence of DEAE-dextran resulted in an improved efficacy of transduction of primary murine splenocytes resulting in a higher frequency of transduced CD4^+ T cells and CD19^+ B cells compared to cells treated with polybrene.

Importantly, the presence of DEAE-dextran or other positively charged polycations may increase non-specific binding of enhanced green fluorescent protein (EGFP)-containing inactive virions or membrane fractions to target cells. This may result in a shift of fluorescence of cells that are not effectively transduced and thus cause an overestimation of the frequency of genetically modified cells. To distinguish between the DEAE-dextran-mediated augmentation of lentiviral transduction characterized by subsequent de novo synthesis of EGFP versus the non-specific binding of inactive EGFP-containing particles, 293FT cells were transduced with a viral preparation that was either filtered through a $0.45 \mu\text{m}$ syringe filter prior to the ultracentrifugation (1F) or filtered both prior to and following the ultracentrifugation step to remove aggregates formed by membrane fractions (2F). Target 293FT cells were transduced for 6 hrs, washed, and analyzed immediately or incubated for an additional 18 hrs without washing (Figure 4A, B). Control samples were incubated in the presence of an anti-retroviral inhibitor azidothymidine (AZT). Filtration of

viral preparation following ultracentrifugation that removes aggregates formed by membrane fractions did not have a significant effect on relative fluorescent intensity. The significant increase of fluorescence intensity between 6 and 24 hrs of culture confirms de novo synthesis of EGFP in transduced cells ($p < 0.05$ between 6 and 24 hrs for all treatments; Student's t-test). Collectively, these results strongly suggest that binding of EGFP-containing membrane fractions in the presence of DEAE-dextran does not represent a major problem in the determination of viral titers of EGFP-expressing lentiviral preparations.

DISCUSSION

In this study, we assessed the critical factors affecting the efficacy of transduction with lentiviral vectors. We present data demonstrating that, in the settings reported here, DEAE-dextran provides superior results in enhancing lentiviral transduction of cell lines and primary cell cultures compared to polybrene, protamine sulfate, or poly-L-lysine. Although various polycations are commonly used to enhance the transduction with retroviral and lentiviral vectors, only a limited number of studies have addressed the relative effect of various polycations on the efficiency of transduction. Several reports have directly compared the effectiveness of polybrene and protamine sulfate on retroviral vector transduction enhancement with varying results [16, 19, 22, 24, 25, 27]. Lizée et al. (2004) compared the effect of DEAE-dextran and polybrene on the efficacy of lentiviral transduction of human CD40L-induced or EBV-transformed B cells and dendritic cells and reported that polybrene mediated optimal transduction of B cells; however, detailed data were not presented [26]. No other study provides a direct comparison of the effect of DEAE-dextran with other commonly used polycations. Furthermore, the results presented here suggest that the specific type and source of serum affects the efficiency of transduction of target cell population. This is in agreement with previously published observations [16]. Heat inactivation of the serum does not appear to have a major effect on transduction.

Previously published study suggested that small differences in the pH of transduction media may significantly influence transduction efficiency [16, 28]. The data presented here demonstrate that optimal transduction of DEAE-dextran-containing cultures typically occurred at pH 7.0–7.2 (Figure 2D); however, the effect of pH appeared to be minor. This is in contrast with the previously reported optimal conditions for polybrene and protamine sulfate-aided retroviral transduction demonstrating an enhancement of transduction efficiency at pH 7.7 with the benefit of the elevated pH more pronounced in the presence of protamine sulfate [16]. The discrepancy between these results and our observations may be attributed to the differences between the type of viral vectors and polycations used in the respective studies.

The data presented here suggests that contamination by EGFP-containing membrane fraction aggregates does not represent a major confounding factor in the determination of viral titers of EGFP-expressing vectors. Additionally, we show that filtration of viral preparation following centrifugation decreases the resulting viral titer (Figure 4A, B). This is in agreement with previously published data suggesting that partially resuspended pellet of viral aggregates is more efficient in mediating gene transfer compared to free virus preparation [20]. Since omitting filtration following the ultracentrifugation does not increase the background in the viral titer determination assay (Figure 4A, B), this step does not appear necessary and should be omitted as it decreases the resulting transduction efficiency.

Collectively, the presented results suggest that various polycations should be tested when optimizing lentiviral transduction of established cell lines and primary cell cultures. While the DEAE-dextran demonstrated superior activity under the conditions described here,

efficacy of polycation-mediated enhancement of transduction may be influenced by the particular properties of target cell population and a variety of polycations should be tested when optimizing each system [26, 27]. Furthermore, care should be taken when selecting serum for supplementation of culture media for virus-producing cell lines as well as the media supplementing the transduction media. Various types of sera should be tested in order to obtain optimal results.

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Figure 1. Effect of serum and polycations on the efficiency of transduction

293FT cells were pre-incubated for 16 hrs in a medium containing various types of sera from different sources (as indicated in the Methods section) and transduced with pL-EGFP virus at the indicated MOIs in the presence of either polybrene (PB) or DEAE-dextran (DEX) at 6 μ g/ml. A: Serum 1 - Bovine Calf Serum (BCS, Gibco), heat-inactivated; B: Serum 2 - Fetal Bovine Serum (FBS; Cellgro), not heat-inactivated; C: Serum 3 - identical to Serum 2, heat-inactivated; and D: Serum 4 - FBS (Hyclone), heat-inactivated. The percentage of EGFP-expressing cells was determined 48 hrs post initiation of transduction. The gate in control non-transduced samples (MOI 0) was set to 1 %. Representative results of one of six similar experiments are presented.

Figure 2. DEAE-dextran facilitates higher efficiency of lentiviral transduction compared to polybrene across a range of concentrations

293FT (A) and HT1080 (B) cells were transduced with pL-EGFP lentivirus at indicated MOIs in culture medium containing serum 4 and either polybrene or DEAE-dextran at 6 $\mu\text{g/ml}$. The percentages of EGFP-expressing cells were determined at 48 hrs by flow cytometry. (C) 293FT cells were transduced with pL-EGFP at MOI 7 in the presence of polybrene or DEAE-dextran at indicated concentrations. MOI values of viral preparations used in the experiments presented in panels A, B, and C are based on a pilot experiment determining the MOI in 293FT cell cultures in the presence of 6 $\mu\text{g/ml}$ of DEAE-dextran. Representative results of one of five similar experiments are presented on panels A, B and C. (D) Effect of pH on the efficiency of transduction. 293FT cells were pre-incubated for 16 hrs in a medium with pH adjusted at indicated values. The cells were transduced with 0.02, 0.2, or 2 μl of pL-EGFP virus preparation in the presence of DEAE-dextran at 6 $\mu\text{g/ml}$. The medium was replaced with fresh medium at indicated pH at 24 hrs. The percentage of EGFP-expressing cells was determined 48 hrs post initiation of transduction. Results of one of two experiments are shown.

Figure 3.

(A) DEAE-dextran facilitates higher transductional efficiency than protamine sulfate or poly-L-lysine. 293FT cells were transduced at the indicated MOI in the presence of protamine sulfate (PS; 10 μ g/ml), poly-L-lysine (PLL; 10 μ g/ml), polybrene (PB; 6 μ g/ml) or DEAE-dextran (DEX; 6 μ g/ml). The percentages of EGFP-expressing cells were determined by flow cytometry at 48 hrs post initiation of transduction. One of three similar experiments is presented. **(B) DEAE-dextran enhances the transduction of primary cells.** 2×10^6 primary splenocytes obtained from 8–12 w old C57Bl/6 mice were transduced with EGFP-expressing lentivirus at MOI = 9 in the presence of polybrene (6 μ g / ml) or DEAE-dextran

(6 µg / ml) and 10 % of serum 4. Control samples consist of cells incubated under the same conditions in the absence of lentivirus. 48 hours post transduction, the expression of EGFP in cells gated for CD4⁺ and CD19⁺ populations was analyzed by flow cytometry. Representative results of one of four similar experiments are shown.

Figure 4. Fluorescence shift in DEAE-dextran-containing cultures is not caused by non-specific binding of EGFP-containing membrane aggregates

(A) 293FT cells were transduced with viral preparation that was filtered through a 0.45 μ m syringe filter prior to concentration by ultracentrifugation (1F in the legend table) or filtered both prior and following the ultracentrifugation (2F). Cells were transduced for 6 hrs, washed, and analyzed immediately (6 hrs) or incubated for additional 18 hrs and analyzed 24 hrs post initiation of transduction. In a control set of samples, azidothymidine (AZT; 20 μ M) was present throughout the experiment. (B) Frequency of EGFP-expressing cells in the experiment depicted on panel B. Error bars represent standard error of the means (SEM).

Stars indicate statistically significant difference between 6 and 24 hrs ($p < 0.05$; Student's t-test).