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ARTICLE

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Auto-transduction in lentiviral vector bioprocessing: A quantitative assessment and a novel inhibition strategy

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Abstract

Lentiviral vectors are highly efficient gene delivery vehicles used extensively in the rapidly growing field of cell and gene therapy. Demand for efficient, large-scale, lentiviral vector bioprocessing is growing as more therapies reach late-stage clinical trials and are commercialized. However, despite substantial progress, several process inefficiencies remain. The unintended auto-transduction of viral vector-producing cells by newly synthesized lentiviral vector particles during manufacturing processes constitutes one such inefficiency which remains largely unaddressed. In this study, we determined that over 60% of functional lentiviral vector particles produced during an upstream production process were lost to auto-transduction, highlighting a major process inefficiency likely widespread within the industry. Auto-transduction of cells by particles pseudotyped with the widely used vesicular stomatitis virus G protein was inhibited via the adoption of a reduced extracellular pH during vector production, impairing the ability of the vector to interact with its target receptor. Employing a posttransfection pH shift to pH 6.7–6.8 resulted in a sevenfold reduction in vector genome integration events, arising from lentiviral vector-mediated transduction, within viral vector-producing cell populations and ultimately resulted in improved lentiviral vector production kinetics. The proposed strategy is scalable and cost-effective, providing an industrially relevant approach to improve lentiviral vector production efficiencies.

KEYWORDS

auto-transduction, cell and gene therapy, lentiviral vector, transfection efficiency, transient transfection, upstream process development

1 | INTRODUCTION

Commonly derived from the human immunodeficiency virus type-1 (HIV-1), lentiviral vectors (LVVs) are used as gene therapy delivery vehicles to transfer and integrate therapeutic transgenes into target tissues and organs to treat various debilitating acquired and inherited diseases (Naldini et al., 1996). Advancements in cell culture technologies, development of highly efficient transfection reagents, and the continued refinement and optimization of processes over the past two decades have enabled transient gene expression (TGE) to

evolve into an attractive industrial technology able to facilitate the large-scale manufacture of biopharmaceutical products, including viral vectors (Gutierrez-Granados et al., 2018; Pham et al., 2006). TGE-based bioprocesses are currently the predominant method for viral vector manufacture, for both clinical and commercial applications (Van Der Loo & Wright, 2016).

The growing demand for viral vector products, as more therapies reach late-stage clinical trials and are commercialized, coupled with a global shortage in contract development and manufacturing organizations (CDMOs) with experienced production capabilities, requires further technical innovation to maximize process efficiency and productivity. With the Milken Institute predicting the approval of 50–75 cell and gene therapies in the United States by 2030 (Choe et al., 2022), it is becoming increasingly clear that new developments and improved viral vector bioprocessing are required to ensure that the industry can meet the growing demand for these products, and that it is achieved at an acceptable cost. While there are many examples of TGE-based manufacturing processes being successfully scaled up to the hundred- and thousand-litre scale (Almo & Love, 2014; Backer, 2022; Girard et al., 2002; Van Lieshout et al., 2023; Tuvesson et al., 2008), several process inefficiencies and challenges remain. One such challenge arises from the widespread use of the vesicular stomatitis virus (VSV) G protein (VSV-G) in LVV production.

The tropism of LVVs is frequently expanded though the utilization of surface glycoproteins derived from other enveloped viruses, a process known as pseudotyping (Joglekar & Sandoval, 2017). VSV-G is extensively used as a pseudotype for LVVs as it confers broad tropism to the vector, can achieve high transduction efficiencies and has been extensively characterized (Perry & Rayat, 2021). The ability of VSV-G pseudotyped LVVs to target a diverse range of cell types is mediated via interactions with its highly ubiquitous target receptor, the low-density lipoprotein receptor (LDLR) (Finkelshtein et al., 2013; Klimatcheva, 1999). This presents a potential challenge to LVV manufacturing processes as viral vector-producing cells which express cell-surface LDLR could act as a major route for particle depletion via unintended transduction (Figure 1). The transduction of viral vector-producing cells by newly synthesized LVVs during manufacturing bioprocesses is a phenomenon known as auto-transduction, also referred to as "retro-transduction" (Ohishi et al., 2007) and "self-transduction" (Klimpel et al., 2023). Auto-transduction appears to be a largely under-recognized, unmeasured yet widespread phenomenon in the industry and potentially constitutes a major process inefficiency. The principal objective of upstream viral vector manufacturing bioprocesses is to maximize the production of vector, within defined tolerances for infectivity, purity, and safety, and any particle loss, by whatever mechanism, is undesirable since the particles can only complete gene delivery on one occasion. Despite the potential issue of auto-transduction, it remains largely unaddressed and warrants further investigation.

In this study, we first quantify how many functional LVV particles are lost to auto-transduction in a typical LVV production process to determine the magnitude of the challenge. Given the magnitude of auto-transduction, we then describe the development of a novel, scalable, and cost-effective approach to inhibit the occurrence of auto-transduction during LVV manufacturing processes.

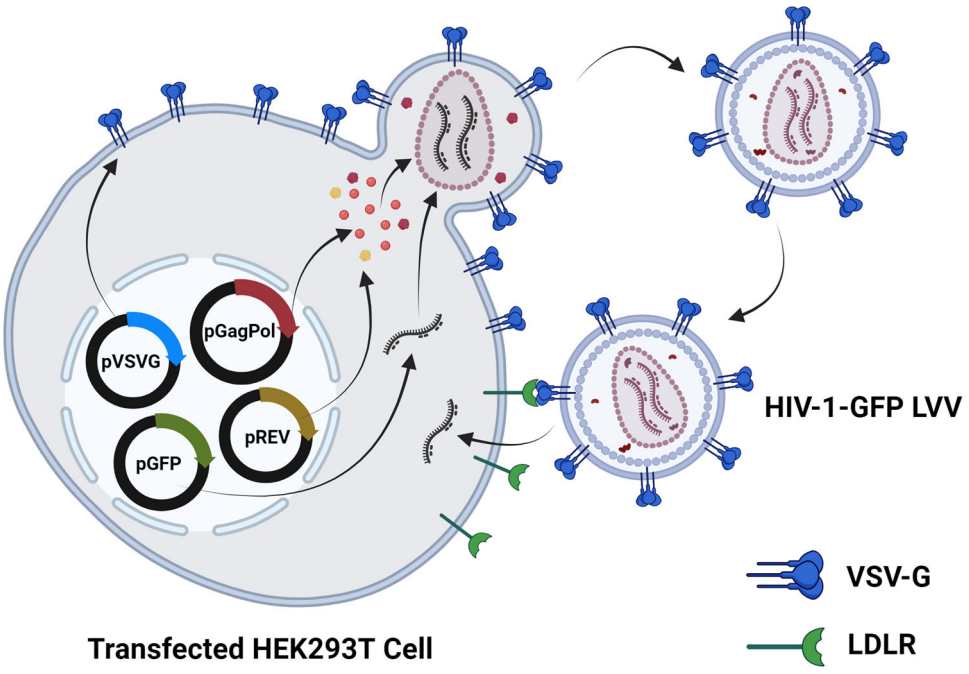

FIGURE 1 The auto-transduction of a, successfully transfected, viral vector-producing cell by a newly synthesized VSV-G pseudotyped LVV particle. GFP, green fluorescent protein; LDLR, low-density lipoprotein receptor; LVV, lentiviral vector; VSV-G, vesicular stomatitis virus G protein.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Suspension-adapted HEK293T cells, provided by Oxford Biomedica (UK) Limited, were routinely passaged (subcultured) in serum-free FreeStyle 293 Expression Medium (Gibco, Thermo Fisher Scientific) and maintained in a shaking incubator (orbital shaking diameter of 25 mm) at 37°C, 300 rpm and 5% CO_2. Cells were cultivated in 24-deep well plates (24-DWPs), Erlenmeyer shake flasks, ambr® 15 (Sartorius AG) bioreactors and ambr® 250HT (Sartorius AG) bioreactors, at working volumes of 3, 25, 13, and 250 mL, respectively.

2.2 | LVV production

Recombinant, pseudotyped, replication-incompetent LVVs were produced using Oxford Biomedica's propriety LentiVector® delivery platform. Briefly, HIV-1-based LVVs were produced via the transient co-transfection of suspension-adapted HEK293T cells with third-generation packaging plasmids. Four plasmids were co-transfected in total consisting of a vector genome transfer plasmid encoding green fluorescent protein (GFP) (pOXB-GFP), two separate packaging plasmids: one encoding Rev (pOXB-REV) and one encoding Gag and Pol (pOXB-HSGP) and a plasmid encoding the VSV-G envelope protein (pOXB-VSV-G). Cells were cultured for approximately 24 h before transfection and were transfected with lipoplexes, prepared via the combination of the required plasmids complexed with the cationic lipid, Lipofectamine™ 2000CD (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's guidelines and under ambient conditions. A total of 1.2 µg/mL of total plasmid DNA was added to cultures and a Lipofectamine™ 2000CD: total DNA mass ratio of 4:1 was used. Cultures were transfected at cell densities of 2×10^6 viable cells/mL. Transfected cell populations were supplemented with sodium butyrate (Sigma-Aldrich, Merck, Burlington, MA, USA) to achieve a final concentration of 10 mM, and the HIV-1-based LVV containing supernatant was isolated approximately 48 h posttransfection, clarified through a 0.45 µm filter and stored at −80°C for subsequent analysis.

2.3 | Transfection efficiency analysis

HEK293T cells were removed from vector production cultures approximately 24 h posttransfection and populations analyzed with a 488 nm excitation laser using an Attune NxT acoustic focusing flow cytometer (Thermo Fisher Scientific). Analysis was terminated when 10,000 live cell events had been processed. Subsequent data analysis was performed using FlowJo (FlowJo LLC) and transfection efficiencies determined using Equation (1). The reported GFP production metric was a measure of the intensity of GFP expression for the entire live cell population and was calculated by multiplying the median fluorescence intensity (MFI) of the gated transgene-positive cell population by the percentage of transgene-positive cells (Equation 2). The GFP production metric was used as a measure of the relative level of transgene expression between different cell populations. This method has been used previously by others as a more accurate way to express GFP production; higher MFI values indicate higher GFP production in transgene-positive cells but not necessarily a higher level of GFP expression for the entire cell population (Ruiz De Garibay et al., 2013).

2.4 | Functional vector titer

Functional LVV titer was determined via the transduction of adherent HEK293T cells at a density of approximately 2×10^5 viable cells with HIV-1-based LVV particles in a 1500 µL volume. LVV particle preparations were serially diluted, 400-fold, in DMEM (Sigma-Aldrich, Merck) supplemented with 8 µg/mL polybrene (Sigma-Aldrich, Merck). Two replicate wells of adherent cells were transduced with each sample analyzed and the medium was not changed after transduction. Transduced cells were harvested 72 h following their exposure to the diluted viral vector preparations and subjected to analysis via flow cytometry using an Attune NxT acoustic focusing flow cytometer. The total number of single, live, transgene-positive cells as a percentage of the total number of single, live cells was determined via analysis of the cell populations on FlowJo. Assays were deemed valid if transduced cell populations exhibited a total percentage of GFP-positive cells of <30% for each sample. Equation (3) was used to determine the concentration of functional transducing units/mL.

2.5 | Quantification of vector genome integration events

Following completion of the LVV production phase, end-of-production cells (EOPCs) were sequentially passaged five times over a 16 day period to dilute out residual plasmid DNA. Cell pellets were generated at the end of each passage via the centrifugation of HEK293T cells at 180*g* for 5 min. The supernatant was removed, and cell pellets stored at −20°C for subsequent analysis. DNA was extracted from thawed cell pellets using a QIAamp 96 DNA kit (Qiagen) and a QIAcube®HT (Qiagen), utilizing an automated DNA extraction protocol. Vector integration titer was determined by quantifying the vector packaging signal (Ψ) in extracted samples and normalizing this value to the number of copies of the ribonuclease P RNA component H1 (RPPH1) housekeeping gene (Equation 4), via duplex qPCR using a QuantStudio 7 Pro Real-Time PCR System (Thermo Fisher Scientific).

2.6 | Statistical analysis

Differences between means were evaluated using one-way analysis of variance followed by Tukey's posthoc multiple comparison tests.

Statistical tests were performed using GraphPad Prism V9.1 (GraphPad Software) and values considered to be statistically significant when $p < 0.05$ (*), 0.01 (**), 0.001 (***), 0.0001 (****).

2.7 | Diagrams and schematics

Diagrams and schematics were made using BioRender.com (BioRender). All graphs were produced using GraphPad Prism V9.1.

2.8 | Equations

Transfection Efficiency

$$\text{Transfection Efficiency (\%)} = \frac{\text{Gated single, live, transgene positive cells}}{\text{Gated single, live cells}} \times 100. \quad (1)$$

GFP Production

$$\text{GFP Production} = \text{MFI of gated single, live, trangene positive cells} \times \frac{\text{Transfection Efficiency (\%)}}{100}. \quad (2)$$

Functional Vector Titer

$$\text{Titer (TU/mL)} = \frac{\left[\frac{\text{\% of transgene positive cells}}{100} \times \text{Number of cells prior to transduction} \times \text{Dilution factor}\right]}{\text{Volume of vector added (mL)}}. \quad (3)$$

Functional Vector Integration Titer

$$\text{Titer (TU/mL)} = \frac{\left[\frac{\text{Copy Number}_{\psi}}{\text{Number of cells in PCR reaction}} \times \text{Number of cells prior to transduction} \times \text{Dilution factor}\right]}{\text{Volume of vector added (mL)}}. \quad (4)$$

3 | RESULTS

3.1 | Understanding the dual origins of transgene positivity: Transfection and auto-transduction

Transfection efficiency is a commonly used metric used to determine the degree of transfection and involves confirming the presence and/or expression of the introduced gene of interest in the transfected cell population. It was hypothesized that close to 100% of cells would present as transgene positive, when measured via flow cytometry, in an HIV-1-GFP LVV production process due to the generation of different GFP-positive subpopulations of cells within the culture. These subpopulations were predicted to consist of (1) successfully transfected cells expressing a GFP transfer plasmid, (2) non-transfected cells transduced by a newly synthesized HIV-1-GFP LVV particle, and (3) successfully transfected cells that have also been transduced by LVV particles. To test this hypothesis, suspension-adapted HEK293T cells were either co-transfected with plasmids required to produce an HIV-1-GFP LVV or were transfected with a GFP transfer plasmid in isolation. The percentage of GFP-positive cells in both populations was measured, via flow cytometry, at regular intervals over a 144-h period.

An equivalent percentage of GFP-positive cells were detected in the HIV-1-GFP LVV producing and the GFP-plasmid only populations 24 h posttransfection (corresponding to an overall elapsed process time of 46 h), measured as 46.5% and 46.2%, respectively ($p = 0.8797$) (Figure 2a). However, a significant difference was detected between the populations 28 h posttransfection (corresponding to a total process time of 50 h) where 54.9% and 50.2% GFP-positive cells were measured, respectively ($p = 0.0401$). It was hypothesized that the emergence of a discrepancy between the percentage of GFP-positive cells in the HIV-1-GFP LVV producing and the GFP plasmid only populations after the 24-h time-point indicated that HIV-1-LVV auto-transduction became detectable from this point onward. The delay in the detection of the phenomenon is likely due to the process of auto-transduction requiring the completion of numerous steps including LVV particle production and budding from producer cells, targeting of cells through VSV-G/LDLR binding, internalization of the LVV, reverse transcription of the RNA genome, transfer of the preintegration complex to the nucleus and finally integration into the host cell genome. The difference between the two populations became more pronounced over time with 99.3% GFP-positive cells being detected in the HIV-1-GFP LVV-producing population 122 h posttransfection (corresponding to a total process time of 144 h), compared to 65.3% in the populations transfected with the GFP transfer plasmid in isolation ($p < 0.0001$). Additionally, there was a 1.9-fold increase in GFP production in the HIV-1-GFP LVV-producing cell populations compared to the GFP-producing populations, despite both conditions being transfected with the same amount of GFP transgene plasmid (Figure 2b). The GFP production metric was a measure of the intensity of GFP expression for the entire live cell population and was calculated by multiplying the MFI of the gated transgene-positive cell population by the percentage of transgene-positive cells.

The increased GFP production in the LVV-producing populations is likely due to a combination of expression resulting from both successful uptake of GFP transfer plasmids following transfection and from vector genome integration events into the host cell genome following transduction by newly synthesized HIV-1-GFP LVVs. The amount of Lipofectamine™ 2000CD transfection reagent and GFP transfer plasmid used for transfection were kept constant between the two experimental treatments. To account for the increased total

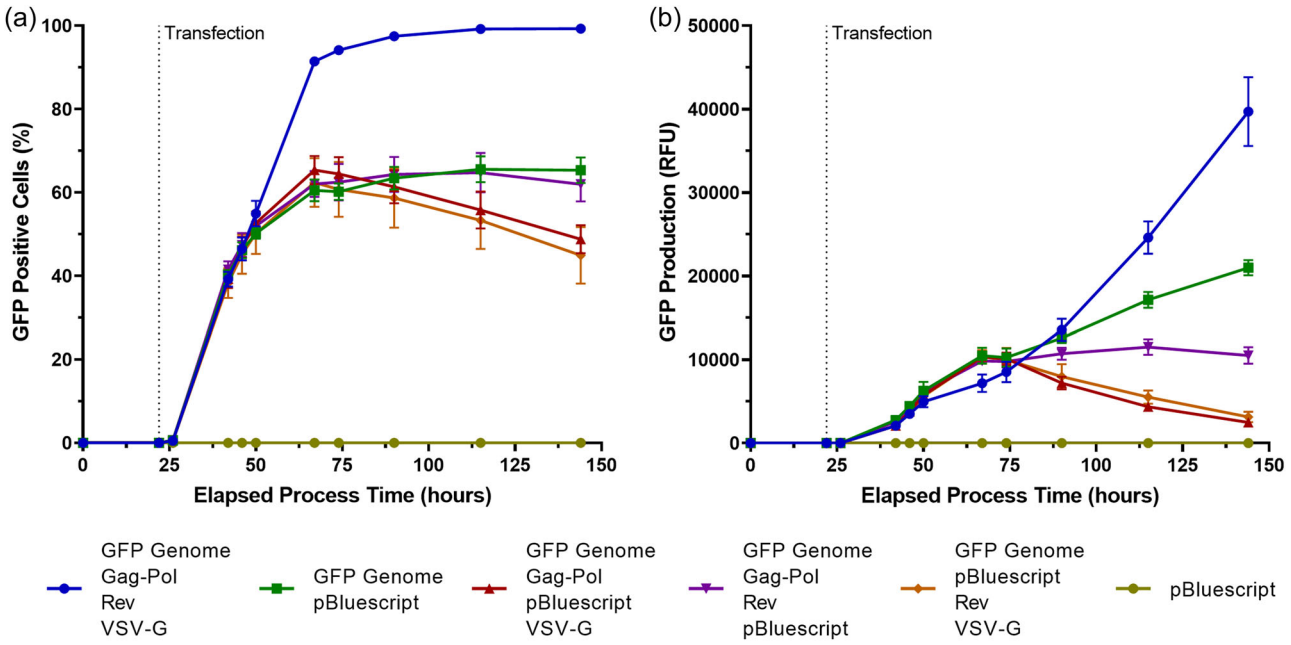

FIGURE 2 Assessing the dual origins of transgene positivity resulting from transfection and auto-transduction. (a) Transfection efficiency and (b) GFP production data measured via flow cytometric analysis of HEK293T cell populations, before and after their transfection with various plasmid combinations (see M&M for GFP production calculation method). Each data point represents the mean ($n = 6$ for the four-plasmid control and $n = 3$ for all test conditions) and error bars are ± one standard deviation of the mean. GFP, green fluorescent protein.

plasmid DNA in the LVV production condition contributed by the helper plasmids, the GFP-only condition was supplemented with a corresponding concentration of a pBluescript plasmid, serving as noncoding "stuffer" DNA, to ensure that the transfection reagent to total plasmid DNA ratio was maintained at the same value for both conditions, since this can impact transfection performance.

To verify that auto-transduction was taking place, additional transfection conditions were investigated, involving the co-transfection of the GFP transfer plasmid alongside two of the three helper plasmids required for HIV-1-GFP LVV production, each time substituting the omitted helper plasmid with pBluescript to maintain the transfection reagent to total pDNA ratio. In each case, the same phenomenon was observed and comparable percentages of cells in the transfected test populations were GFP positive compared to the cultures transfected with all four plasmids required for LVV production when measured 24 h posttransfection (Figure 2a). When longer incubation times were observed, differences in the percentage of GFP-positive cells emerged with the multiplasmid test conditions exhibiting comparable profiles to the cultures transfected with the GFP transfer plasmid only.

3.2 | Auto-transduction accounts for significant LVV loss during production processes

With auto-transduction potentially being detectable as early as 28 h posttransfection, it was hypothesized that a failure to inhibit this phenomenon during upstream viral vector manufacturing processes would result in a significant loss of functional particles. Therefore, the number of LVV genome integration events was determined in HIV-1-GFP LVV-producing cell populations by quantifying the HIV-1 Ψ packaging signal in EOPCs using qPCR. Comparing this value to the number of remaining LVV particles in the supernatant, at the end of the process, allowed the percentage loss to be determined. Following the completion of a transient HIV-1-GFP LVV production process in a suspension 24-DWP cultivation system, EOPCs were removed from the production cultures, transferred into new 24-DWPs, and sequentially subcultured five times over a 16-day period. This was to ensure that residual plasmid DNA retained by cells following their transfection would be diluted out of the populations over time. This was necessary since the GFP transfer plasmid also contained the HIV-1 Ψ packaging signal and would, therefore, erroneously contribute to the quantification metric used to determine the number of vector genome integrations. Vector genome integration events arising from auto-transduction would persist with subsequent cell divisions since the integrated vector DNA would be replicated along with the host cell genome.

The HIV-1 Ψ packaging signal copy number, measured in extracted host-cell DNA, was very high at the end of the LVV production phase (Figure 3a). An average number of 4465 copies/cell and 4932 copies/cell were detected in cell populations transfected with the GFP transfer plasmid alone and cell populations transfected with the four plasmids required to produce the HIV-1-GFP LVV, respectively. The high copy number is likely attributable to the persistence of residual plasmid DNA within cells following their transient transfection, which would still be present at high levels at this point

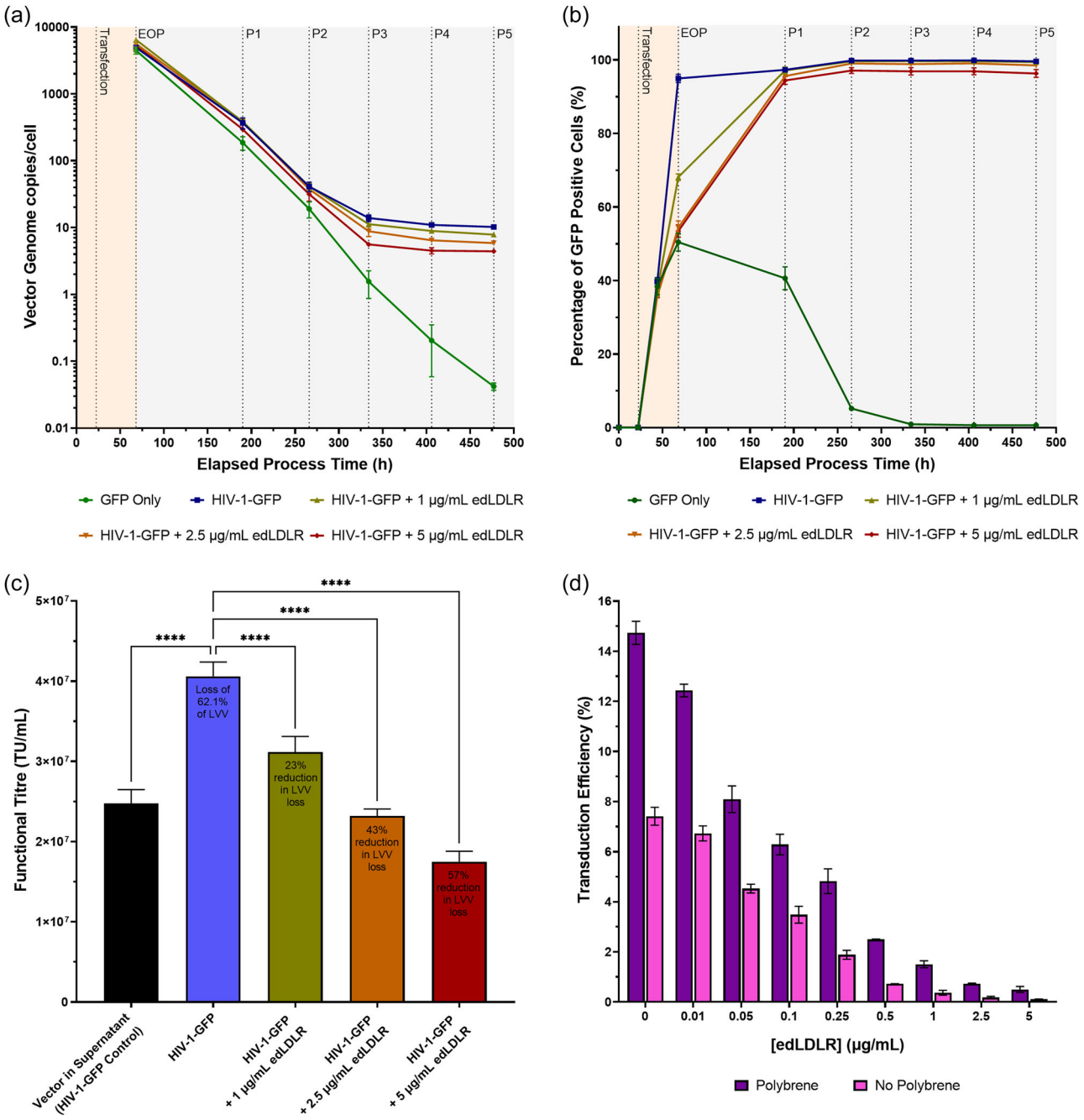

FIGURE 3 Quantifying the magnitude of the effect of auto-transduction in LVV bioprocessing. (a) Quantification of the average number of vector genome copies per cell and (b) the percentage of GFP-positive cells during an LVV production process and in EOPC populations during subsequent passage, both in the presence and absence of edLDLR. (c) Quantification of LVV particles lost to auto-transduction. (d) Assessment of the impact of edLDLR on transduction efficiency of adherent HEK293T cells by VSV-G pseudotyped LVV particles in the presence and absence of polybrene. In all panels, each data point represents the mean and error bars are ± one standard deviation of the mean. In panels a–c, $n = 6$ for the GFP and HIV-1-GFP controls and $n = 3$ for all conditions supplemented with edLDLR. In panel d, $n = 3$ for all conditions. edLDLR, extracellular domain of the low-density lipoprotein receptor; EOP, end of production; EOPC, end-of-production cell; GFP, green fluorescent protein; LVV, lentiviral vector; P, passage; VSV-G, vesicular stomatitis virus G protein.

of the process. As the EOPC cells were passaged, residual plasmid DNA was diluted out of the cell populations and the Ψ copy number declined with each progressive subculture until EOPC subculture 3 (Passage 3, P3) where the Ψ copy number began to stabilize (Figure 3a). At EOPC Passage 5 (P5) a significantly lower average number of Ψ sequences were detected in the cell populations transfected with the GFP transfer plasmid alone (0.04 copies/cell) compared to the cell populations transfected with the four plasmids required to produce the HIV-1-GFP LVV, where an average of 10.2 copies/cell were detected ($p < 0.0001$). The remaining Ψ packing

signals in the cell populations producing HIV-1-GFP LVVs, therefore, likely originated from vector genome integration events following HEK293T auto-transduction. This data aligns with the percentage of GFP-positive cells that were detected in the cell populations transfected with the GFP transfer plasmid in isolation compared to cell populations co-transfected with plasmids required to produce the HIV-1-GFP LVV, where an average of 0.7% and 99.6% of GFP-positive cells were detected in the two populations at EOPC P5, respectively ($p < 0.0001$) (Figure 3b). The number of functional HIV-1-GFP LVV particles present in the supernatant at the end of the vector production process was determined to be 2.48×10^7 TU/mL (Figure 3c). The number of functional vector particles lost to auto-transduction was calculated from the average number of Ψ copies per cell, normalizing the Ψ packaging signal in the transduced HEK293T cells to the RPPH1 housekeeping gene (Equation 4) and was determined to be 4.06×10^7 TU/mL; this represented a 62.1% loss of total functional LVV particles produced during the process.

Treating cultures with a recombinant, 777 amino acid protein comprising only the extracellular domain of the low-density lipoprotein receptor (edLDLR) was hypothesized to block VSV-G pseudotyped LVV auto-transduction by competing with cell surface LDLR for VSV-G binding, thereby disrupting the native protein–protein interaction that mediates transduction. The edLDLR construct was shown to block transduction of adherent HEK293T populations, cultivated in 12-well plates, following a 1-h preincubation of the construct with a diluted VSV-G pseudotyped HIV-1-GFP LVV stock (Figure 3d). The ability of the protein to reduce transduction efficiency was found to be dose-dependent and occurred in both the presence and absence of polybrene. It was hypothesized that the addition of edLDLR to LVV production process would be effective in reducing LVV losses to auto-transduction. Indeed, significant reductions in the Ψ copy number were detected in the cell populations supplemented with the edLDLR 3 h posttransfection ($p < 0.0001$) (Figure 3a). The inhibitory effect was dose-dependent with higher concentrations of edLDLR resulting in reduced Ψ copies per cell; 7.8, 5.8, and 4.4 copies/cell were detected in the cell populations treated with 1, 2.5, and 5 µg/mL sLDLR, respectively. Addition of the edLDLR during vector production was shown to significantly reduce LVV particle loss ($p < 0.0001$). A 23%, 43%, and 57% reduction in HIV-1-GFP LVV loss was calculated in the cell populations treated with 1, 2.5, and 5 µg/mL edLDLR, respectively, compared to the untreated control (Figure 3c).

3.3 | Transduction efficiency of VSV-G pseudotyped LVVs is compromised by a reduced ability of VSV-G to interact with LDLR below neutral pH

VSV-G interacts specifically with the CR2 and CR3 domains of LDLR, mediating membrane attachment and fusion of VSV-G pseudotyped LVVs (Nikolic et al., 2018). It is able to undergo a reversible, low pH-induced, conformational transformation from a trimeric prefusion state toward a trimeric postfusion state (Beilstein et al., 2020). No interaction was detected between VSV-G and CR2 and CR3 constructs at a pH of 6.00, suggesting that VSV-G can only interact with the LDLR when in its prefusion conformation (Nikolic et al., 2018). We hypothesized that subjecting VSV-G pseudotyped LVV-producing cell cultures to a posttransfection pH shift, sufficient to induce a conformational transformation of VSV-G toward its postfusion state, would reduce auto-transduction. Crucially, the pH-induced conformation transformation is reversible and once the LVV has been isolated from the cell culture, an increase in pH was hypothesized to induce a transition back to the prefusion state, facilitating the transduction of intended target cells.

To determine whether transduction efficiency was dependent on extracellular pH, suspension HEK293T cells were cultivated in ambr15 bioreactors under different pH conditions, ranging from pH 6.60–7.30; cells were treated with an HIV-1-GFP LVV stock, in the absence of polybrene, to achieve a multiplicity of infection of 3 (Figure 4a). Cell samples were taken 18, 40, and 62 h following exposure to the LVV stock to quantify the percentage of GFP-positive cells. High levels of transduction were detected in cell populations maintained at a pH of ≥7.00, and >80% and >99% of cells were found to be GFP positive when measured after an 18 and 62-h incubation, respectively. Conversely, cell populations maintained at a pH <7.00 were more resistant to transduction and transduction efficiency declined rapidly with decreasing pH. When incubated at a pH of 6.60, only 0.4% and 1.1% of cells were found to be GFP positive when measured after an 18 and 62-h incubation, respectively. Culture viability was found to be comparable across the pH range investigated (Figure 4b).

To determine whether the decreased transduction efficiency was due to a reduced ability of VSV-G to interact with LDLR at lower pH, suspension HEK293T cells were transfected with a plasmid encoding VSV-G in ambr15 bioreactors and subjected to a pH shift to either 6.70 (±0.01), 6.80 (±0.01), 6.90 (±0.01), or 7.00 (±0.01), initiated 48-h posttransfection. Cultures were then supplemented with the edLDLR recombinant protein (which had a C-terminal polyhistidine tag) and an antihis antibody with a conjugated R-phycoerythrin fluorophore to measure the ability of edLDLR to bind cell surface VSV-G under different pH conditions (Figure 4c). The MFI of cell populations was found to decrease with decreasing pH (Figure 4d). A 3.2-fold reduction ($p < 0.0001$) in MFI was calculated when reducing the pH from 7.00 to 6.70, suggesting that the ability of VSV-G to successfully interact with LDLR was reduced at values slightly below a pH of 7.00.

To verify that the reduced transduction efficiencies previously observed at low pH were not due to another mechanism, such as the downregulation of cell surface LDLR expression at low pH, an additional experiment was conducted to quantify the relative expression of LDLR at a range of pH values (Figure 4e). Suspension HEK293T cells were cultivated in ambr15 vessels for 48 h at pH set-points ranging from 6.70 (±0.01) to 7.00 (±0.01) and samples were removed after a 48-h incubation and stained with an anti-LDLR antibody. Fluorescence intensity was found to be equivalent in all cell

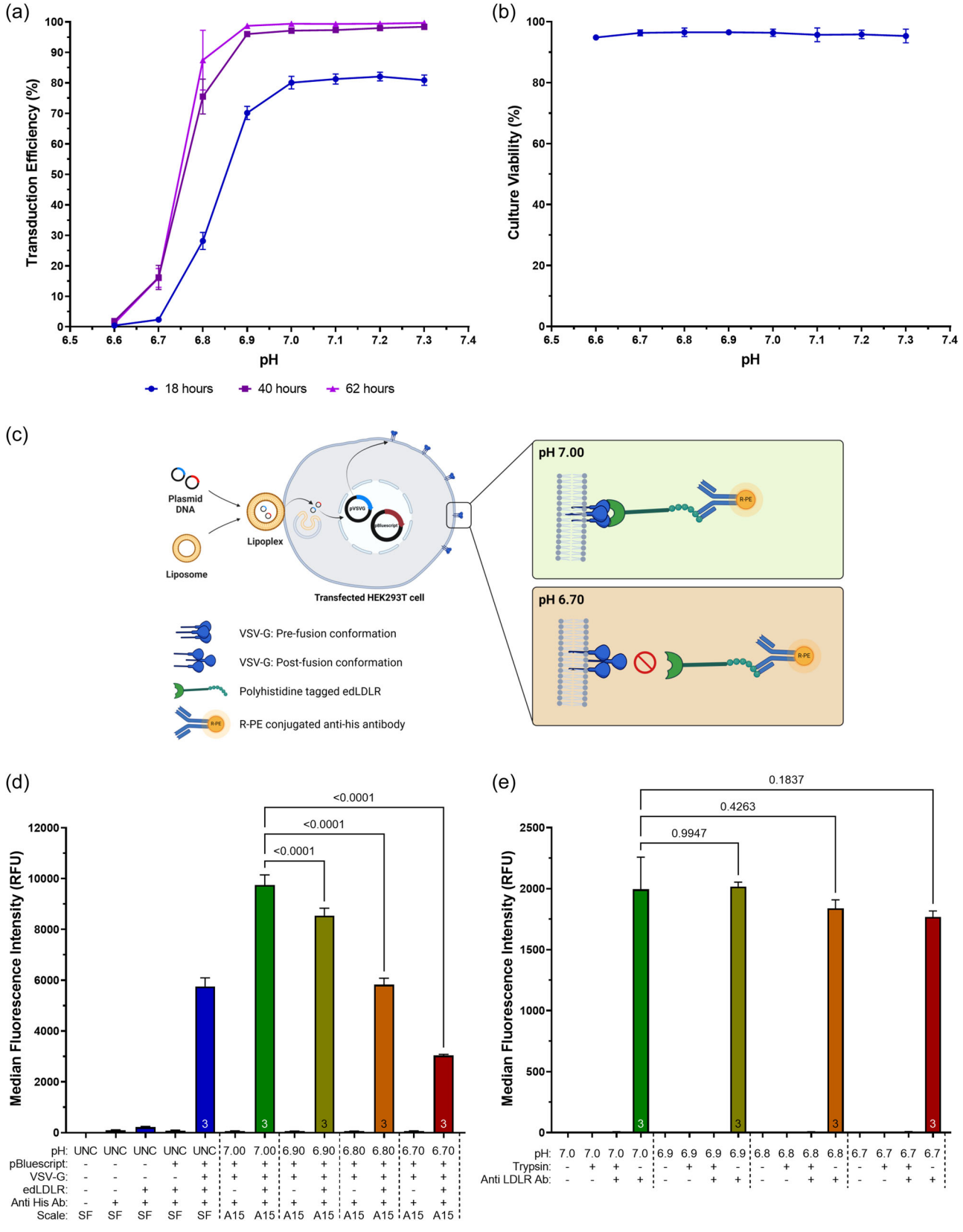

FIGURE 4 (See caption on next page).

populations, indicating an equivalent level of LDLR expression across the range of pHs investigated.

3.4 | Introduction of a pH shift during LVV bioprocessing reduces LVV loss to auto-transduction

Cultivating HEK293T cells at a pH below 7.00 was found to be effective in reducing the transduction efficiency of VSV-G pseudotyped LVVs. It was important to evaluate whether exposing LVV-producing cell populations to a reduced extracellular pH would be effective in reducing auto-transduction, thereby increasing the number of LVV particles available for harvest. Three "low pH" bioprocessing conditions were evaluated in ambr250 bioreactors and compared to a control LVV production bioprocess. All bioreactors utilized the same initial pH control strategy where the pH was maintained between a range of 6.90 and 7.50, allowing the cells to drive the pH within this range. Following transfection, the low pH bioprocessing conditions were subjected to a pH shift to either 6.70 (±0.01), 6.80 (±0.01), or 6.90 (±0.01) and the control process was controlled at 7.00 (±0.01) (Figure 5a). Samples were removed from the bioreactors at regular intervals from 38-h posttransfection to 98-h posttransfection to track cell performance and vector production over a prolonged 60-h period. While comparable until the start of the harvest time-course, culture viability was found to decline at an accelerated rate in cultures maintained at pH values of 6.90 and 7.00 during LVV production, compared to cultures maintained at pH 6.70 and 6.80 (Figure 5b); this accelerated decline at higher pH was matched with a faster reduction in the viable cell concentration of these cultures (Figure 5c), and was likely due, in part, to the increased glucose consumption and lactate and ammonium production rates observed in the cultures maintained at higher pH values (Figure 5d–f).

When measured 45-h posttransfection, the percentage of GFP-positive cells in cultures maintained at pH 6.90 and 7.00 were both >96.0%, likely indicating high levels of auto-transduction (Figure 5g). Reduced percentages of GFP-positive cells of 85.8% and 77.5% were measured in cultures maintained at pH 6.80 and pH 6.70, respectively, at the same timepoint. The reduced percentage of GFP-positive cells at pH 6.70 was maintained for the remainder of the process, indicating reduced levels of auto-transduction at the lower pH. This data was matched with greatly increased levels of GFP production in cultures maintained at higher pH set points of 6.90 and 7.00, compared to pH 6.80 and 6.70, likely due to a combination of GFP fluorescence resulting from plasmid expression as well as vector genome integration events resulting from auto-transduction (Figure 5h). EOPCs were removed from all cultures at the end of the production process and sequentially subcultured four times over a 25-day period, to ensure adequate removal of residual plasmid DNA remaining from the transfection unit operation. DNA was extracted from the cell pellets and the number of vector genome integration events quantified via qPCR (Figure 5i). Comparable levels of auto-transduction were measured in the cultures maintained at pH 7.00 and pH 6.90 with 12.1 vector genome copies per cell and 10.7 vector genome copies per cell being measured, respectively ($p = 0.2354$). Significantly lower levels of auto-transduction were detected in the cultures maintained at pH 6.80 and pH 6.70, with 4.1 vector genome copies per cell ($p < 0.0001$) and 1.8 vector genome copies per cell ($p < 0.0001$) being measured, respectively. This corresponded to an LVV loss of 2.7×10^7 TU/mL at pH 7.00, compared to 4.0×10^6 TU/mL at pH 6.70 (Figure 5j). While comparable at the start of the sampling time-course, extracellular concentrations of the p24 capsid protein increased more rapidly with reducing pH (Figure 5k) from the 50-h posttransfection sampling timepoint onward, potentially due to reduced levels of auto-transduction at pH 6.70–6.80. Final concentrations of 2.1×10^6, 2.1×10^6, 1.5×10^6, and 1.1×10^6 pg/mL were calculated in cultures maintained a pH 6.70, 6.80, 6.90, and 7.00, respectively. Equivalent peak functional LVV titers were measured in all conditions, albeit at different points in time, with peak titers being detected earlier in the higher pH conditions compared to the lower pH conditions (Figure 5l). This was potentially due to the increased transfection efficiency (Figure 5g) and higher initial LVV production rates at the start of the production phase (Figure 5l) at higher pH. However, once the peak functional LVV titer was attained in the higher pH conditions, titers immediately declined for the remainder of the LVV production phase, likely due to a combination of high rates of auto-transduction and loss due to temperature-related instability of the vector. Conversely, the peak titer achieved at pH 6.80 and pH 6.70 was maintained at the maximum level for a prolonged period of 36 h, likely due to reduced levels of auto-transduction. This observation was concordant with the percentage of GFP-positive cell data (Figure 5g), GFP production data (Figure 5h), vector genome copies per cell data (Figure 5i) and p24 data (Figure 5k), all of which suggest reduced rates of auto-transduction at lower pH. The peak functional LVV concentrations being maintained for the prolonged 36-h period at pH 6.80 and pH 6.70 resulted in a 1.6-fold ($p < 0.0001$) and a 1.7-fold

FIGURE 4 Assessment of the transduction efficiency of HEK293T cells by VSV-G pseudotyped LVVs and the ability of VSV-G to interact with LDLR under various pH conditions. (a) Transduction efficiency and (b) culture viability measured when supplementing suspension HEK293T cultures with HIV-1-GFP LVV particles at an MOI of three, between pH 6.60 and 7.30. (c) Schematic depicting the hypothesized interaction of VSV-G and LDLR at lower and higher pH. (d) Determining the degree of VSV-G LDLR interaction between pH 6.70 and 7.00. (e) Assessing levels of cell surface LDLR expression in HEK293T cells between pH 6.70 and 7.00. In all panels, each data point represents the mean ($n = 3$) and error bars are ± one standard deviation of the mean. A15, ambr15 bioreactor; GFP, green fluorescent protein; LDLR, low-density lipoprotein receptor; LVV, lentiviral vector; MOI, multiplicity of infection; SF, E125 shake flask; UNC, uncontrolled; VSV-G, vesicular stomatitis virus G protein.

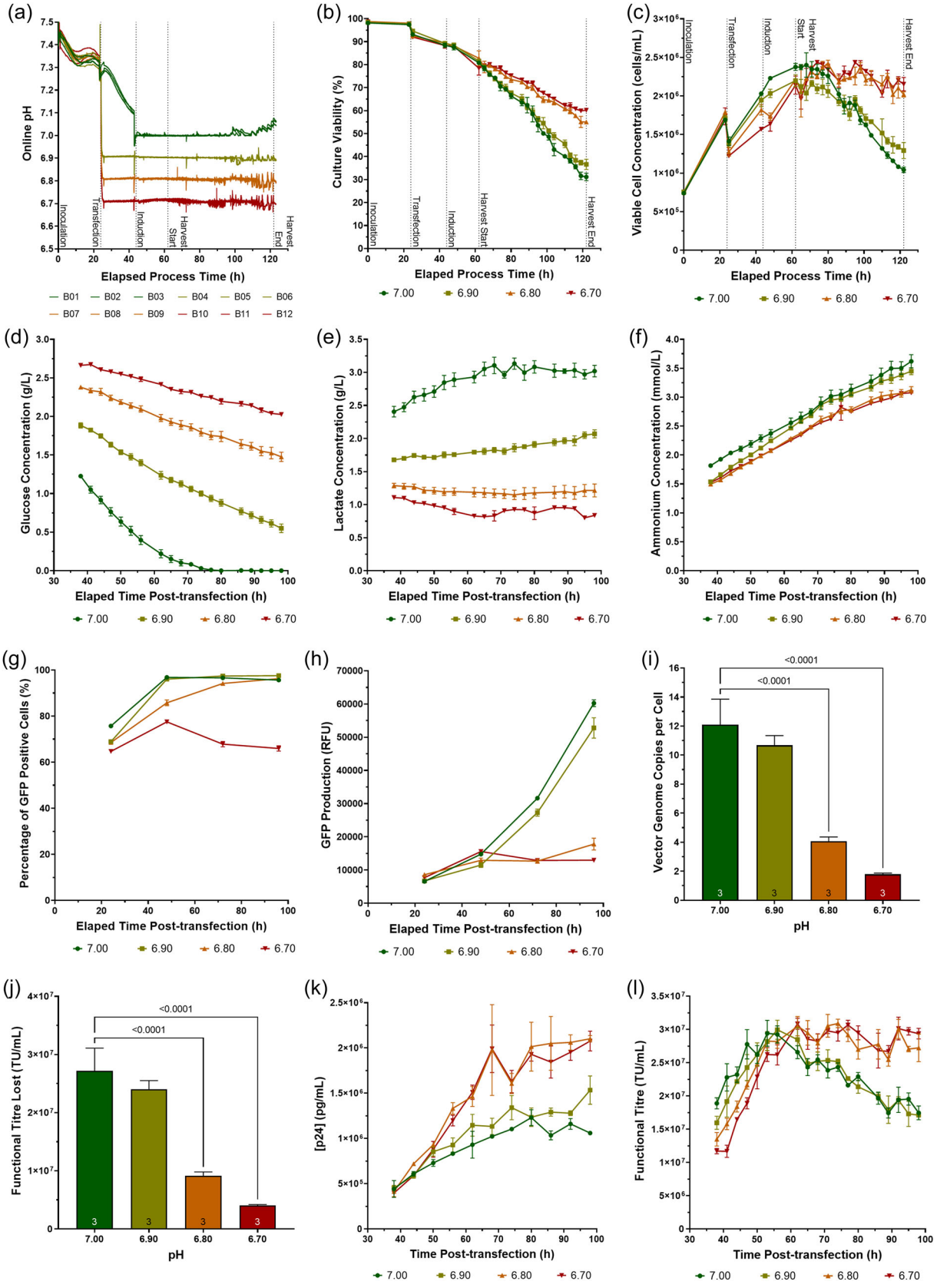

FIGURE 5 (See caption on next page).

($p < 0.0001$) increase, respectively, in the concentration of functional particles at the end of the process compared to the control.

4 | DISCUSSION

LVVs serve as a versatile and efficient tool for delivering therapeutic transgenes into target cells and have become a key tool in the development of cell and gene therapies. Experienced manufacturing capacity for LVVs remains low globally despite huge demand for efficient bioprocesses to ensure the provision of sufficient quantities of vector for clinical and commercial applications (Labbé et al., 2021). Valued at USD 154 million in 2024, the LVV market is anticipated to grow at a compound annual growth rate of 11.2%, reaching a predicted USD 360 million by 2031 (Verified-Market-Research, 2024). Despite huge investment in this area, a number of upstream bioprocessing challenges and inefficiencies persist; a crucial inefficiency involves the loss of newly synthesized LVV particles through the auto-transduction of viral vector-producing cells within the production culture. Auto-transduction has two main implications in LVV bioprocessing: (1) it complicates the calculation of the transfection efficiency metric when assessing process performance and (2) it compromises process productivity through the loss of large quantities of LVV particles.

Transfection efficiency is a common metric used to determine the success of a transfection. We determined that cultures producing an HIV-1-GFP LVV exhibit close to 100% GFP-positive cells at the end of a typical LVV production process. This was significantly higher than cultures transfected with a GFP transfer plasmid in isolation, despite maintaining equivalent transfection conditions, and was likely due to the occurrence of auto-transduction. These findings were concordant with observations previously made by Ohishi and colleagues who conducted a similar experiment and reported 70% GFP-positive cells in HIV-1-GFP LVV-producing HEK293T populations, compared to 15% in populations producing GFP alone, when measured 72 h posttransfection; this was despite observing comparable percentages in the populations when measured 24-h posttransfection (Ohishi et al., 2007). The occurrence of auto-transduction in LVV bioprocesses would likely result in the generation of three distinct subpopulations of GFP-positive cells: (1) successfully transfected cells expressing a GFP transfer plasmid, (2) nontransfected cells transduced by a newly synthesized HIV-1-GFP LVV particle, and (3) successfully transfected cells that have also been transduced by an LVV. Since the determination of the transfection efficiency metric frequently relies on the detection of GFP-positive cells via flow cytometry, it is not possible to effectively discriminate between the three GFP subpopulations as the phenotype is the same in each case. If not appropriately considered, this would result in large overestimations of transfection efficiency and misleading results. Our data suggests that measuring transfection efficiency 24 h post-transfection will avoid overestimations of the metric resulting from the occurrence of auto-transduction. However, it is important to recognize that these experiments also demonstrated that not all successfully transfected cells were detectable 24 h posttransfection, and this approach will likely result in an underestimation of transfection efficiency. Measuring transfection efficiency 24 h post-transfection, therefore, represents a compromise between avoiding an auto-transduction-mediated overestimation of the metric and incurring a potential underestimation, since not all successfully transfected cells are detectable at this timepoint. While neither an overestimation nor an underestimation is ideal, we propose that an underestimation is preferable since measuring the metric at this timepoint provides an accurate snapshot of the initial transfection efficiency and avoids making an overly optimistic estimation of the proportion of transfected cells, which could potentially lead to a misinterpretation of experimental results. This approach still facilitates the capture of a substantial proportion of the transfected cells, allowing distinctions between the performance of different transfection conditions to be reliably made in screening and optimization studies. Conversely, comparing performance of different transfection conditions at later time points, once auto-transduction has occurred, is difficult as near 100% of cells are GFP positive at the end of a production process, making it difficult to determine which conditions may have outperformed others.

An experiment that sought to quantify the magnitude of the auto-transduction effect, via quantifying the number of vector genome integrations in EOPCs, revealed a substantial process inefficiency whereby over 60% of the total functional LVV particles produced during the upstream process were lost. Due to the common and widespread use of the VSV-G pseudotype in LVV bioprocessing and the high ubiquity of its target receptor, the LDLR, it is anticipated that auto-transduction is a widespread issue which affects many LVV production systems. It is highly likely that auto-transduction will also occur when using other vector pseudotypes, provided the corresponding target receptor that mediates vector transduction is expressed by the cell line used to produce the vector. We demonstrated that auto-transduction could be reduced by supplementing LVV production cultures with the edLDLR, likely due to the construct

FIGURE 5 Introduction of a pH shift during LVV bioprocessing to reduce LVV auto-transduction in ambr250 bioreactors. (a) Online bioreactor pH, (b) culture viability, (c) viable cell concentration, (d) glucose consumption, (e) lactate and (f) ammonium production, (g) percentage of GFP-positive cells, and (h) levels of GFP production measured over the course of the LVV production process. (i) Quantification of the number of vector genome integration events in end-of-production cells and (j) calculation of the corresponding number of functional LVV particles lost to auto-transduction. (k) Quantification of the p24 capsid protein and (l) functional LVV particles in supernatant sample taken throughout the process. In all panels, each data point represents the mean ($n = 3$) and error bars are ± one standard deviation of the mean. GFP, green fluorescent protein; LVV, lentiviral vector.

competing with cell surface LDLR for VSV-G binding. This was consistent with previous findings where a soluble, recombinant, LDLR fragment was reported to bind VSV and inhibit VSV-mediated infectivity in human epithelial WISH cells (Finkelshtein et al., 2013). While edLDLR supplementation offered a strategy to inhibit LVV loss to auto-transduction, it did not present as a viable solution for further process development and scale-up since the edLDLR construct must be removed from the vector following harvest to restore particle functionality, which increases process complexity, risk, and cost. Furthermore, the addition of large quantities of recombinant proteins to large-scale manufacturing processes is costly. It was desirable to develop an alternative method to reduce auto-transduction that was both cost-effective and amenable to process scale-up.

The issue of auto-transduction has been recognized by other groups and there have been attempts to generate LDLR knockout (KO) cell lines as a potential method for addressing this process inefficiency. VIVEBiotech, an LVV CDMO, reported a two-fold reduction in the number of auto-transduction events in a HEK293T-based LDLR KO cell line compared to a HEK293T control. However, a corresponding increase in LVV titer was not observed, which the authors attributed to a compromised cholesterol uptake ability in the LDLR KO (Banos-Mateos et al., 2023). In 2019, Miltenyi Biotec GmbH filed a patent for the generation of an LDLR KO packaging cell line engineered to produce VSV-G pseudotyped retroviral vector particles (Schaser et al., 2020), demonstrating a desire within industry to address auto-transduction-related process inefficiencies. The potential for LDLR KOs to address the challenge of auto-transduction is an interesting area of research but it is important to recognize that other LDLR family members serve as alternative receptors to mediate VSV infectivity. Nikolic and colleagues demonstrated that an LDLR KO HAP-1 cell line was susceptible to VSV infection and infectivity was only blocked when these cells were treated with receptor-associated protein (RAP), a protein which blocks ligand binding to all LDLR family-associated receptors with the exception of LDLR itself (Nikolic et al., 2018). Finkelshtein and colleagues demonstrated that LDLR-deficient fibroblasts could still be transduced by a VSV-G pseudotyped LVV and transduction was only completely blocked following supplementation with RAP (Finkelshtein et al., 2013).

VSV-G is a trimeric protein that can undergo a reversible, low pH-induced, conformational transformation from a trimeric prefusion state toward a trimeric postfusion state and has been shown to have a compromised ability to interact with LDLR in the postfusion conformation (Nikolic et al., 2018). We demonstrated that VSV-G pseudotyped LVVs have a reduced ability to transduce HEK293T cells below a pH of 7.00 and minimal transduction was detected at a pH of 6.60, even after a prolonged 62-h incubation. The compromised transduction efficiency appeared to be due to a reduced ability of VSV-G to interact with LDLR at low extracellular pH, presenting a promising method of reducing auto-transduction in LVV bioprocessing. The utilization of posttransfection pH shifts to values of 6.80 and 6.70 during LVV production proved to be highly effective in reducing LVV loss to auto-transduction, resulting in a 6.7-fold reduction in the number of vector genome integration events at the end of the LVV production phase and a twofold increase in the extracellular concentration of p24. Additionally, whilst the peak functional LVV titers achieved in each of the different pH conditions were comparable, titers were maintained at the maximum level for a period of 36 h at pH 6.80 and pH 6.70 and only 3 h at pH 7.00, resulting in a 1.6-fold and a 1.7-fold increase, respectively, in the concentration of functional LVV particles at the end of the process compared to the control. The improved LVV concentration profiles at pH 6.70–6.80 potentially enable the performance of multiple harvests which may result in increased process productivity.

The number of functional LVV particles present in the supernatant represents a balance between rates of vector production and vector loss, with loss likely resulting from a combination of both auto-transduction and temperature-related degradation due to poor LVV stability at 37°C. The reduced levels of auto-transduction at lower pH likely account for the maintenance of functional titers at the maximum concentration for the prolonged period. It is possible that high rates of temperature-related vector degradation over this period meant that increases in functional titer beyond the maximum value detected were not observed. Indeed, the issues around particle thermostability are widely recognized within the industry and HIV-1-derived vector half-life has been reported to be as low as 10 h at 37°C (Higashikawa & Chang, 2001). There are a number of ways in which this could potentially be addressed. Utilization of a perfusion-mediated harvesting strategy, in combination with the employment of a posttransfection pH shift, may offer a complimentary approach effective in addressing concerns related to both auto-transduction and vector thermostability simultaneously. Alternatively, utilization of lower temperatures during the prolonged LVV production phase may increase the stability of the vector, facilitating the accumulation of more functional particles. Several groups have previously reported enhanced stability and production of retroviral vectors at temperatures ranging from 28°C to 34°C, compared to 37°C (Le Doux et al., 1999; Kaptein et al., 1997; Kotani et al., 1994; Lee et al., 1996). Additionally, a number of excipients are used in LVV formulation buffers to enhance vector stability during storage, including sugars such as sucrose and trehalose and proteins such as animal- and human-origin-free recombinant albumin (Carmo et al., 2009; Moreira et al., 2021). While typically used in formulation applications, an assessment of the ability of such supplements to increase vector stability over a prolonged production phase during upstream processing may offer a valuable approach.

It is conceivable that further reductions in extracellular pH may further diminish rates of auto-transduction since transduction efficiency was found to be lower at a pH of 6.60 compared to 6.70, following prolonged incubation periods. However, it is likely undesirable to reduce extracellular pH to values that are much lower than this as we previously reported that LVV production was significantly compromised when cultivating an HIV-1-GFP LVV stable producer cell line (PCL) at a pH of 6.40 compared to a control process where the pH was maintained between 6.90 and 7.10 (Williams et al., 2023). Other groups have previously reported increases in LVV

production when utilizing lower pH set-points; when investigating the effect of pH on LVV yield, Powers and colleagues reported higher titers of a VSV-G pseudotyped LVV in a HEK293T-based stable PCL at pH set-points of 6.60 and 6.80 compared to a pH of 7.00 and 7.20. Efforts to obtain greater improvements in titer by further reducing the pH to 6.40 and 6.20 resulted in decreased LVV yield (Powers et al., 2020), consistent with our previous findings. We propose that a likely explanation for the observed increase in functional titer in the 6.60–6.80 range, at least in part, was due to reduced LVV loss to auto-transduction.

Overall, subjecting VSV-G pseudotyped LVV production cultures to a posttransfection pH shift to 6.70–6.80 proved to be an effective strategy for significantly reducing auto-transduction, resulting in improved LVV production profiles. Importantly, this approach is likely easily amenable for process scale-up to larger scale systems. The thermostability of LVV particles is known to be poor at 37°C and high rates of temperature-related vector degradation likely occurred over the extended harvest period utilized in this work. It is anticipated that the development and implementation of process improvements to enhance particle stability during the upstream process, combined with methods to reduce auto-transduction, will greatly increase LVV process productivity.

AUTHOR CONTRIBUTIONS

Thomas Williams-Fegredo, Lee Davies, and Qasim A. Rafiq conceived the hypothesis. Thomas Williams-Fegredo designed and performed experiments and analyzed data. Thomas Williams-Fegredo, Lee Davies, Carol Knevelman, Kyriacos Mitrophanous, James Miskin, and Qasim A. Rafiq discussed and interpreted data. Thomas Williams-Fegredo wrote the original draft manuscript. All authors revised and contributed to the writing of the final manuscript.

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CONFLICTS OF INTEREST STATEMENT

T. Williams-Fegredo, L. Davies, C. Knevelman, K. Mitrophanous, and J. Miskin were all employees and stock option holders of Oxford Biomedica (UK) Limited at the time the research was conducted. The remaining author declares no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included in the article or are available from the corresponding author upon reasonable request.

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