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Gene silencing strategies to increase HIV-1 VLP production in HEK 293 cells

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Abstract

The HIV-1 Gag polyprotein, recombinantly expressed in mammalian cell platforms, is self-assembled, generating HIV-1 Gag virus like particles (VLPs). The expression of HIV-1 Gag polyprotein is achieved by DNA/PEI-based transient transfection of HEK 293 cells in suspension. Several strategies to improve the VLP production titers have been already described such as the so-called extended gene expression methodology (EGE) that extends the production phase of the process by the performance of two additional re-transfection rounds to cell culture and medium exchanges, providing a 12-fold improvement of Gag VLP production compared to batch production. The EGE in combination to the addition of chemical production enhancers such as lithium acetate (LiAc), valproic acid (VPA) and caffeine has been reported to increase 15.6 times the Gag VLP production. However it has been demonstrated that these chemical enhancers have a detrimental effect on cell growth and viability. In this study, the use of small interfering RNA (siRNA) has been investigated as an alternative to the use of VPA and caffeine. Two shRNA sequences against HDAC5 and PDE8A genes, cloned in the expression vector containing Gag-GFP gene, were tested. From all tested strategies, only the shRNA substitution of VPA was successfully achieved, nevertheless, by the use of this strategy, the toxic effects on cell growth and viability provided mainly by VPA addition are avoided. Therefore, the optimum strategy is to transfect the cells using the expression vector containing the shRNA sequence against the HDAC5 gene, in combination to the addition of caffeine. This novel strategy has improved 6.65-fold the Gag VLP production without any negative effect over cell growth or viability compared to the standard transient transfection in batch (TGE) mode. Its combination with the EGE methodology showed a 40-fold improvement of Gag VLP production compared to the TGE mode. Therefore, the developed methodology avoids the toxic effects of VPA addition and enables the production of high amount of HIV-1 Gag VLPs. Finally, the impact of this improvement on the corresponding bioprocess development is analysed using several criteria, demonstrating the elevated potentialities of the developed method.

Keywords: Transient transfection · Virus-like particles · Extended gene expression · Gene silencing · Production optimization

1. Nomenclature

VLP	Virus like particles
HIV-1	Human Imunodeficiency Virus -1
HEK 293 cells	Human embryonic kidney 293 cells
Gag	Gag polyprotein
GFP	Green fluorescence protein
PEI	Polyethylenimine
PTM	Post-translational modifications
siRNA	Small interference RNA
shRNA	Short hairpint RNA
PDE	Phosphodiesterasse
HDAC	Histone deacetylase
TGE	Transient gene expression
EGE	Extended gene expression
LiAc	Lithium acetate
VPA	Valproic acid
VCD	Viable cell density
Hpt	Hours post transfection

2. Introduction

Over the last years, mammalian expression systems have become the preferred platform for manufacturing biopharmaceuticals, since these cells are able to produce large and complex proteins with post-translational modifications (PTM) (Dumont, et al. 2016) and human protein-like molecular structure assembly (Zhu, et al. 2012). Even so, the use of mammalian cell lines as expression system for recombinant protein production has the principal disadvantage that lower production titers are obtained in comparison to other expression systems (Brown, et al. 2017). Therefore, further optimisation of recombinant protein production is needed. Virus-like particles (VLPs) are nanostructures which mimic the native structure of viruses, making them recognizable by the immune system. VLPs are composed by the proteins or part of the proteins that are forming the virus capsid in actual viruses, but lacking the genomic material. These type of new generation vaccines are safer than most conventional viral vaccines (like attenuated or inactivated viruses), since the lack of genomic material in VLP particles makes them non-infective (Fuenmayor, et al. 2017a). In this study the protein of interest is the Gag polyprotein from HIV-1, known as the core protein of HIV-1 virus capsid and also one of the three gene products which are encoded by all retroviruses (Göttlinger, et al. 2001). After reaching the cell nucleus and being expressed, Gag polyprotein accumulates at the vicinity of the cell membrane and is self-assembled into VLPs which are released to the media by a budding process. At this point, the virus like particles acquire the lipid envelope from the host cell (Cervera, et al. 2017a). The use of Gag polyprotein as a particle formation machine, combined with the expression of different antigens of interest exposed on the host cell membrane, is a commonly used platform to produce immunogenic VLPs (Göttlinger, et al. 2001).

Transfection is the term given to the process of nucleic acid delivery into host cells, and it is used to facilitate the expression of exogenous products or the regulation of endogenous cellular processes (Brown, et al. 2017). In this work, the production of HIV-1 Gag VLPs is performed by transient transfection of HEK 293 cells in culture. Transient gene expression (TGE) methodology consists of the transfection of cell culture in batch and the transient expression of the protein of interest. Although stable gene expression (SGE) is the most conventional methodology to produce recombinant biopharmaceuticals in mammalian production platforms industrially, TGE is a suitable procedure to produce the protein of interest in sufficient quantities to evaluate recombinant protein candidates in the early stages of its development, in pre-clinical batch productions and early phase clinical trials, or other scenarios that demand rapid production timelines (Baldi, et al. 2007; Stuible, et al. 2018). Furthermore, in the case of genic therapies or personalized treatments, the TGE methodology satisfies the production needs without requiring the generation of stable producing clones (Kim, et al. 2010). Even so, the obtained product titers in TGE are usually low due to dilution effect associate to cell division. The transgene is lost since does not integrate into the cell genome, reducing the production time of TGE and limiting the overall production capacity (Cervera, et al. 2015). To avoid this limitation of TGE procedure, a methodology called extended gene expression (EGE) was developed (Cervera, et al. 2015). In extended gene expression two re-transfections of cell culture were carried out 48 and 96 hours after the first transfection, as well as, a medium exchange every 48 hours. By this approach, the production phase is extended since the cells that were not transfected in the first transfection round, are able to introduce the DNA in second or third transfections. In addition, complete medium exchanges allow the maintenance of culture viability during longer times (10 days). Additionally, in order to enhance the productivity in TGE, some media additives have been used to increase transfection efficiencies (lithium acetate,

DMSO, Nocodazole) (Ye, et al. 2009; Tait et al. 2004) or protein production (butyric acid, valproic acid, caffeine) (Tait et al. 2004; Backliwal, et al. 2008; Ellis, et al. 2011). In previous work, an optimal combination of production enhancers was found for the cell line and VLPs of interest here. This combination, which enabled an improvement of 4 fold of Gag VLPs production, consists of the addition of lithium acetate, valproic acid and caffeine to the cell culture (Cervera, et al. 2015b).

Lithium acetate (LiAc), increases the transportation of DNA/PEI complexes into the cells by changing cell membrane permeability (Ye, et al. 2009). Caffeine has an inhibitory effect over phospodiesterases (PDE) and several kinases including ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein) and DNA-Pks (DNA-dependent protein kinase catalytic subunit) (Ellis, et al. 2011). PDE are enzymes that inactivate the intracellular cAMP and cGMP (Choi, et al. 1988). The mentioned kinases are important signalling proteins involved in the repair of DNA double stranded breaks, and their inactivation has shown a positive effect on recombinant production (Ellis, et al. 2011). Valproic acid (VPA) is shown to have histone deacetylase (HDAC) inhibitory effect (Backliwal, et al. 2008). HDAC enzymes are known to reduce the interaction between histones and DNA by histones deacetylation. Because of that, chromatin remains open and transcription is enhanced. Another observed effect of HDAC inhibitors over cell culture is that cell cycle is arrested in the phase (G1) preventing cell division (Fuenmayor, et al. 2018a; Kaiser et al. 2006). Despite the use of media additives have shown clear enhancement of VLP production in previous work, it has also been observed to have detrimental effect over cell culture growth and viability when performing EGE (Fuenmayor, et al. 2018a). In addition, it is important to realise that its use increases the process production and purification costs. Small interfering RNA (siRNA) could be a feasible alternative to the use of media additives. When a siRNA sequence is encoded in a DNA plasmid is called short hairpin RNA (shRNA), it can be introduced into cultured cells by transfection and suppress specific endogenous gene expression (Fuenmayor, et al. 2018a). Although the shRNA effects over the culture, can last for days, they are transient and limited to cells available for transfection (Lai, et al. 2008). Inside cells, shRNAs are transcribed and then processed by the enzyme DICER resulting in ssRNAs which are complementary to the gene to interfere. These ssRNAs bind the mRNAs of this gene and the RNA-induced silencing complex (RISC) cuts them preventing their translation. Finally, a knock-down for the specific protein is achieved (Lowe, et al. 2011).

Taking into consideration the described background, this work has two main objectives. First, finding an alternative to the use of additives, by using small interfering RNA (siRNA) to improve Gag VLP production while maintaining high cell viabilities when performing TGE. Second, combining the EGE methodology with the novel alternative to the use of additives, and comparing the VLP production obtained in the different systems. In this study, two specific shRNA sequences against HDAC5 and PDE8A genes were tested as candidates to be an alternative to media additives.

3. Materials and methods

3.1. Cell line, media and culture conditions

The cell line used in this work is HEK293SF-3F6. It is a serum-free medium suspension-adapted HEK293 cell line (kindly provided by Dr.Amine Kamen from the BRI of National Research Council of Canada and McGill University (Montreal, Canada)), derived from a cGMP master cell bank, available for manufacturing of clinical material. The culture medium is Freestyle 293 (Invitrogen, Carlsbad, CA, USA) supplemented with 0,1% Pluronic (Invitrogen, Carlsbad, CA, USA), 1,6 mg/L of r-transferrin (Merck Millipore, Kankakee, IL, USA), 19,8 mg/L of r-insulin (Novo Nordisk Pharmatek , Køge, Denmark.), 0,25 g/L of D-glucose (Merck KGaA, Darmstadt, Germany) and 0.9x of an in-house lipid mixture to maximize cell growth and productivity (Cervera et al.2013). Cells were cultured in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL of culture medium. Flasks were shaken at 130 rpm using an orbital shaker (Adolf Kühner AG, Switzerland) mantained at 37º in a humidified atmosphere of 5% CO_2 in air, inside an incubator (Adolf Kühner AG, Switzerland). Cell count and viability were determined by Nucleocounter NC-3000 (Chemometec, Allerod, Denmark).

3.2. Plasmids and shRNAs

The plasmid pGag-GFP (named pControl in this work) encodes for a Rev-independent HIV-1 Gag protein which was previously fused to the enhanced GFP protein (Hermida-Matsumoto and Resh 2000) to enable an easy quantification of the produced VLPs. The cloning strategy, preparation and purification of Gag-GFP plasmid was previously described (Cervera et al. 2015b). In order to obtain the different pshRNAs, used in this work, the pGag-GFP plasmid was modified. Two specific shRNA sequences against HDAC5 gene (5′CCGGGCCGGGTTTGATGCT GTTGAACTCGAGT TCAACAGCATCAAACCCGGCTTTTT 3′) and PDE8A gene (5′ TGCTGTTG ACAGTGAGCGAGCTAAGATCATGGTTACAAATTAGTGAAGCCACAGATGTAATTTGTAACC ATGATCTTAGCGTGCCTACTGCC TCGGATTTTT 3′) were used (Fuenmayor, et al. 2018a). The sequence of U6 promoter plus each of the shRNAs were obtained from Gen Script (Nanjing, China) and cloned into the pGagGFP in front CMV promoter (Fuenmayor et al. 2018a). The developed expression vectors are represented in Fig 1. The integrity of developed plasmids was confirmed running a 1% agarose gel electrophoresis using SYBR safe DNA gel stain (Invitrogen).

3.3. Transient transfection

Transient transfection was carried out using a 25-kDa linear polyethyleneimine (PEI) (PolySciences, Warrington, PA, USA). Before transient transfection, HEK 293 cells were grown until reaching a cell concentration of $2x10^6$ cells/ mL. A complete medium exchange was performed by centrifuging the cell culture at 300xg for 5 min, prior to transfection. Transfections were performed using 1ug/mL of plasmid DNA and 2ug/mL of PEI (1:2 relation) (Cervera et al. 2013). The PEI/DNA complexes were formed by adding PEI to plasmid DNA previously diluted in fresh culture media without supplementation (10% of the total volume of the culture to be transfected). The mixture was incubated for 15 min at room temperature to allow complex formation before its addition to the cell culture. The addition of production enhancers has been previously described (Cervera et al. 2015b). It was carried out 3 h prior transfection for lithium acetate (20 mM) and 4 hours post transfection for valproic acid (3.36 mM) and caffeine (5.04mM).

3.4. Analysis of the percentage of GFP-positive cells

The percentage of GFP-positive cells was determined in freshly harvested cells every 24 hours for TGE protocols and every 48 hours for EGE protocols. The analysis was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA).

3.5. VLP quantitation

The concentration of Gag-GFP VLPs was assessed by fluorimetry using an in-house developed and qualified quantification assay (Gutiérrez-Granados et al. 2013). VLP-containing supernatants were recovered by cell culture centrifugation at 1000 xg for 5 min. Green fluorescence was measured at room temperature using a Cary Eclipse Fluorescence Spectrophotomer (Agilent Technologies, Santa Clara, CA, USA) set as follows: λex =488 nm (slit 5 nm), λem= 510 nm (slit 10 nm). Relative fluorescence unit values (RFU) were calculated by subtracting fluorescence units (FU) values of untransfected negative control samples. There is a linear correlation between fluorescence intensity and p24 values determined using the INNOTEST ELISA HIV antigen mAb (Innogenetics NV, Gent, Belgium). RFU values can be converted to Gag-GFP concentration values using the following equation:

$$Gag - GFP\ \left(\frac{ng}{mL}\right) = (3.245\ \times RFU - 1.6833\ ) \times 36 \qquad (1)$$

where Gag-GFP is the estimated concentration of polyprotein and RFU is the measured GFP fluorescence intensity in the samples. The first term is the correlation equation between fluorescence values and p24 concentrations determined by ELISA and 36 is a correction factor that takes into account the difference in molecular weight between p24 and Gag-GFP and an underestimation arising from using the p24 ELISA to estimate p55 Gag concentrations. In order to calculate the amount of VLPs per mL, the Gag-GFP concentration is divided by the molecular weight of a Gag-GFP monomer (84 kDa) and then divided by the number of monomers that a VLP contains (2500 monomers/VLP) (Gutiérrez-Granados et al. 2013).

3.6. Cell sorting, total RNA extraction and qPCR analysis

Freshly harvested cells at 48 hpt were sorted into GFP and non-GFP positive cells using a FACSJazz sorter (BD,4480 Biosciences, San Jose, CA, USA). Seventy-five thousand cells were sorted for each population and subsequently centrifuged at 300g for 5 min, re-suspended in PBS, and stored at − 20 °C. RNA extraction from the samples was carried out using the Maxwell® 16 Total RNA Purification Kit (Promega Corporation, Fitchburg, WI). The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) was used to obtain cDNA using 100 ng of total RNA. Relative expression was determined by real-time polymerase chain reaction (PCR) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA levels were quantified with commercial qPCR primers and probes for each studied gene. Single Taqman expression assays (Thermo Fisher Scientific, Waltham, MA) were used to quantify HDAC5 (Hs00608351_m1) and PDE8A (Hs01079617_m1) mRNA levels. β-Actin (Hs01060665_g1) was used as an endogenous control.

3.7. Analysis of VLPs by nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to determine the amount of correctly assembled VLPs of harvest samples using a NanoSight® LM20 device (NanoSight Ltd., Amesbury, UK) at the Service of Preparation and Characterization of Soft Materials located at Institut de Ciència de Materials de Barcelona (ICMAB, CSIC, Campus UAB). The obtained data was analysed with the NanoSight® NTA 2.2 software. Samples were collected and diluted in 0.22 µm-filtered PBS prior to the analysis, in order to obtain a sample with a concentration of 10^8 -10^9 particles/ mL.

3.8. Calculation of transfection protocols performances

- VLPs in total harvest (VLPs):

$$VLPs = \sum([VLP]_i \times V_i) \quad (2)$$

where $[VLP]_i$ is the VLP concentration (VLPs/mL) of harvest round (i) and V_i is the volume (mL) in harvest round (i).

- VLP concentration in total harvest (VLPs/mL):

$$VLP\ concentration = \frac{\sum([VLP]_i \times V_i)}{\sum V_i} \quad (3)$$

- VLP production (VLPs/ hour):

$$VLP\ production = \frac{VLPs}{t} \quad (4)$$

where t is the production phase of the protocol duration in hours.

- VLP volumetric productivity (VLPs/ hour · mL):

$$VLP\ volumetric\ productivity = \frac{VLP\ production}{V_T} \quad (5)$$

where V_T is the total harvest volume (mL).

- Specific productivity (VLPs/ cell):

$$Specific\ productivity = \frac{VLP\ concentration}{VCD \times f_T} \quad (6)$$

where VCD is the concentration of viable cells, expressed in millions of cells/mL, and f_T is the transfection factor which corresponds to the percentage (between 0 and 1) of cells which are transfected, knowing that only transfected cells are able to produce VLPs.

3.9. Statistical analyses

All the protocols developed were compared by a t test in order to establish if the improvement achieved was statistically significant or not. Microsoft Excel (2010) t test function was used to carry out the statistical analyses.

4. Results and discussion

4.1. Comparison of different shRNAs to replace chemical additives in batch culture

The use of shRNA for silencing the genes which are inhibited by the chemical additives, has been proposed as an interesting alternative to avoid the observed detrimental effect of production enhancers on cell growth and viability (Fuenmayor et al. 2018a). In this study specific shRNA sequences against HDAC5 and PDE8A genes have been cloned in the plasmid containing the gene of interest (Gag polyprotein from HIV-1) to evaluate their effect on Gag VLP production. Three different expression vectors were developed as shown in Fig 1, with HDAC5 and PDE8A shRNA sequences cloned either together or alone, in the Gag-GFP containing plasmid.

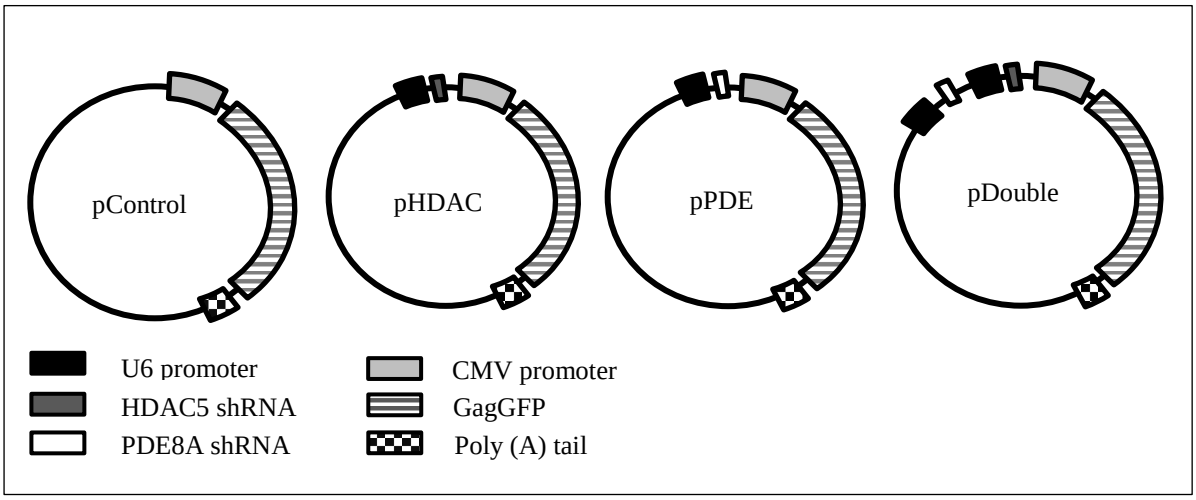

Figure 1: Expression vectors. The plasmid Control is formed by Gag-GFP gene preceded by CMV promoter. All shRNA have been added to pControl, preceded by U6 promoter. In pHDAC the shRNA sequence against HDAC5 gene was added. In the plasmid PDE, was added the shRNA against PDE8A. Finally in pDouble, both shRNA sequences (against HDAC5 and PDE8A) were cloned in the same plasmid.

A standard transient transfection protocol in a 72 hours batch (TGE) (Cervera et al. 2013) was performed to test the three GagGFP plasmids containing the shRNA sequences (shown in Fig 1). Transfections were carried out using: pGagGFP with shRNA against HDAC5 gene cloned in it (pHDAC), pGagGFP with shRNA against PDE8A gene cloned in it (pPDE), pGagGFP with both shRNAs against HDAC5 and PDE8A, cloned together in it (pDouble), pHDAC and pPDE transfected together (pHDAC + pPDE) and pGagGFP (pControl).

The tested pshRNAs had no negative effects on viable cell density and viability of the culture, without significant differences between the different conditions tested. As shown in Fig 2A, the viable cell density reached after 72 hpt, remains between $5x10^6$ cells/ mL and $6x10^6$ cells /mL in all conditions, and the viability was over 90%. For all conditions tested, the percentage of transfected cells increased until 48 hpt, reaching, at the end of the batch, a percentage between 50 and 60% of transfected cells (Fig 2B). As it can be observed in Fig 2C, higher Gag VLP production is obtained when transfecting the cells with pHDAC, in comparison to the other pshRNA combinations tested. As seen in Fig 2D, the enhancement effect over VLP production

of pPDE was nil, in respect to the production observed when transfecting with pControl ($6.82x10^9$ and $7.55x10^9$ VLPs/mL, respectively). The pDouble and the condition of pHDAC and pPDE transfected together, had a similar effect in the improvement of Gag VLP production ($1.00x10^{10}$ and $1.14x10^{10}$ VLPs/mL, respectively), but not as high as the obtained when pHDAC was transfected alone ($2.09x10^{10}$ VLPs/mL). One potential explanation of this observation could be that the pDouble, which encodes for both shRNA sequences (against HDAC5 and PDE8A) in the same plasmid, experiments stearic limitations that prevent the complete transcription of HDAC5 shRNA sequence, therefore, the pDouble design could need to be optimized. In respect to the condition of cell transfection with both plasmids at the same time (pHDAC + pPDE condition), the need to maintain the optimal ratio 1:2 (w:w) between DNA and PEI to prepare the complexes before transfection (Cervera et al. 2013), make not possible to use the same amount of pHDAC DNA as in the condition where pHDAC was transfected alone. Because PEI has showed to have toxic effect in cells (Cervera et al. 2013; Fliedl, et al. 2011), the pHDAC used to transfect the cells was half of the total quantity used for transfect the culture in the pHDAC condition. So, one hypothesis could be that the quantity of pHDAC, used for transfection, is a determinant factor in the finally obtained Gag VLP production titer.

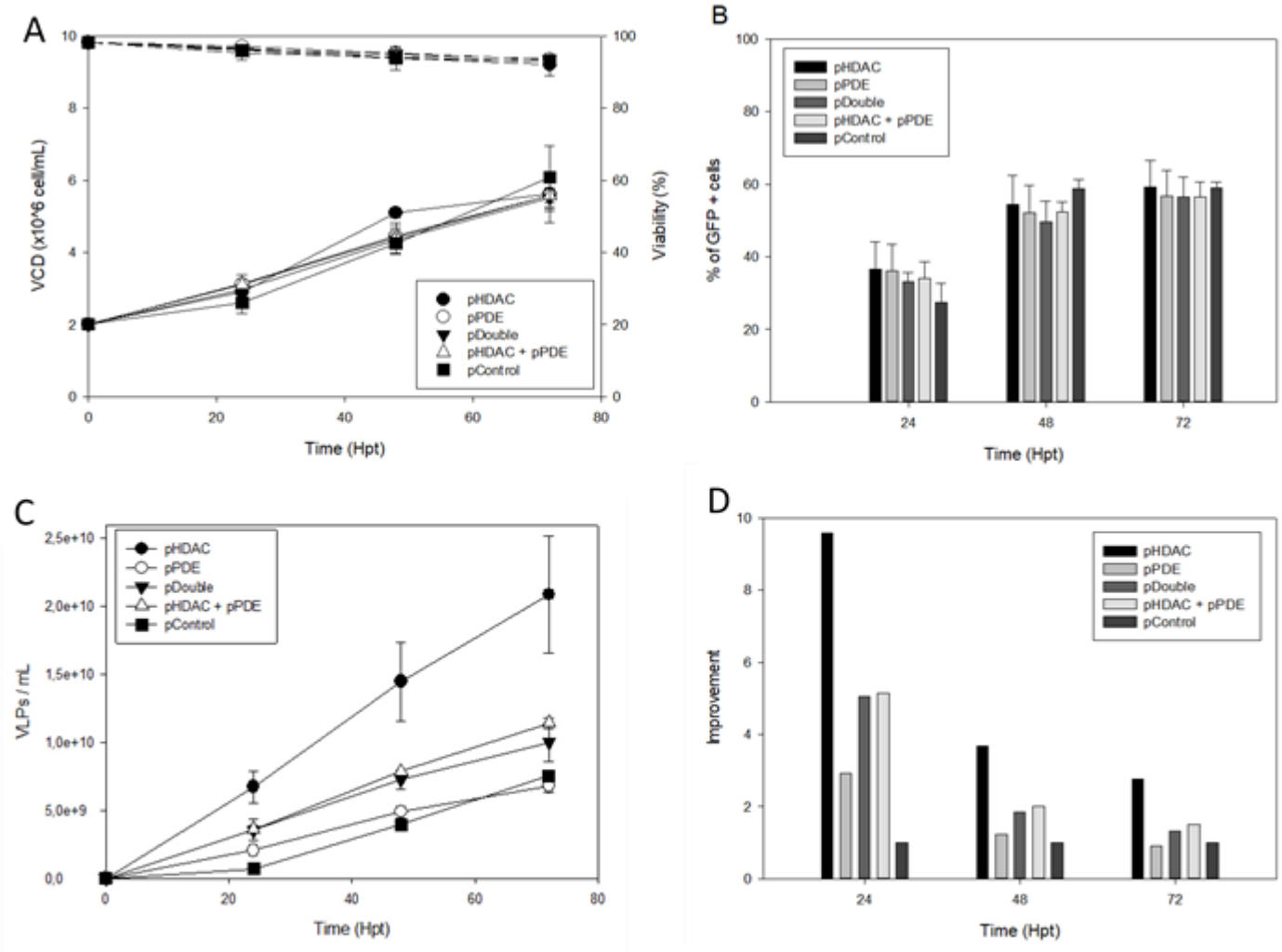

Figure 2: TGE protocols, transfecting cells with pHDAC, pPDE, pDouble, pHDAC + pPDE and pControl. **A)** Viable Cell density (solid line) and viability (dotted line). **B)** Percentage of positive GFP cells. **C)** Obtained Gag VLP production titers. **D)** Fold improvement in Gag VLP production of all conditions tested over the production obtained in pControl.

To prove this hypothesis, another standard transient transfection in batch was carried out. Four conditions were tested in this experiment: using the standardized quantity of 20 µg of pHDAC in complex preparation with PEI (pHDAC), using 10 µg of pHDAC and 10 µg of pControl (0.5pHDAC), using 10 µg of pHDAC and 10 µg of pPDE (pHDAC + pPDE) and using 20 µg of pGagGFP (pControl), all of them using a DNA/PEI ratio of 1:2. Results are shown in Fig 3.

The viable cell density reached in all tested conditions was practically the same, achieving after 72 hpt, a concentration of approximately $5x10^6$ cells / mL. In addition, culture viabilities remained high during the whole production process (over 90% of viability) (Fig 3A). As observed in the previous experiment, the percentage of transfected cells increased until it reached a plateau at 48 hpt. The final percentage of transfected cells was around 60% in all conditions (Fig 3B).

As it can be observed in Fig 3C, the enhancement effect in VLP production of the pHDAC condition is considerably higher than the effect observed in 0.5pHDAC condition, evidencing that the amount of pHDAC, used in transfection, has a big impact. Furthermore, to support this fact, the enhancement effect observed when transfecting with pHDAC and pPDE together, is the same as when half of the quantity of pHDAC is used (in 0.5pHDAC condition). As already observed in the previous transient transfection experiment carried out, the transfection with pPDE did not increase the Gag VLP production. To endorse the obtained results, the effect of the shRNA sequences over gene expression was analysed by qPCR of GFP positive cells in comparison to the GFP negative population (data not shown). In the GFP positive population the shRNA against HDAC5 gene showed to block a 67% of HDAC5 gene expression, but, in case of the shRNA against PDE8A gene, no significant inhibition effect of PDE8A expression, was observed. As mentioned before, besides the PDE inhibitory effect, it is known that caffeine also inhibits several kinases, including ATM601, ATR and DNA-Pks. In previous work (Ellis, B. L., et al. 2011), inhibition of this mentioned kinases resulted in increased lentiviral titers in HEK 293FT culture. Considering that, in future work it would be interesting to test shRNA sequences against ATM601, ATR and DNA-Pks genes as alternatives to caffeine addition.

After this two TGE series of experiments, it can be concluded that the best pshRNA, is the pHDAC (using 20 µg of DNA for complex preparation), since it is the plasmid that showed the bigger impact in Gag VLP production in transfected HEK 293 culture, providing a 3-fold improvement over the standard TGE (Fig 2D).

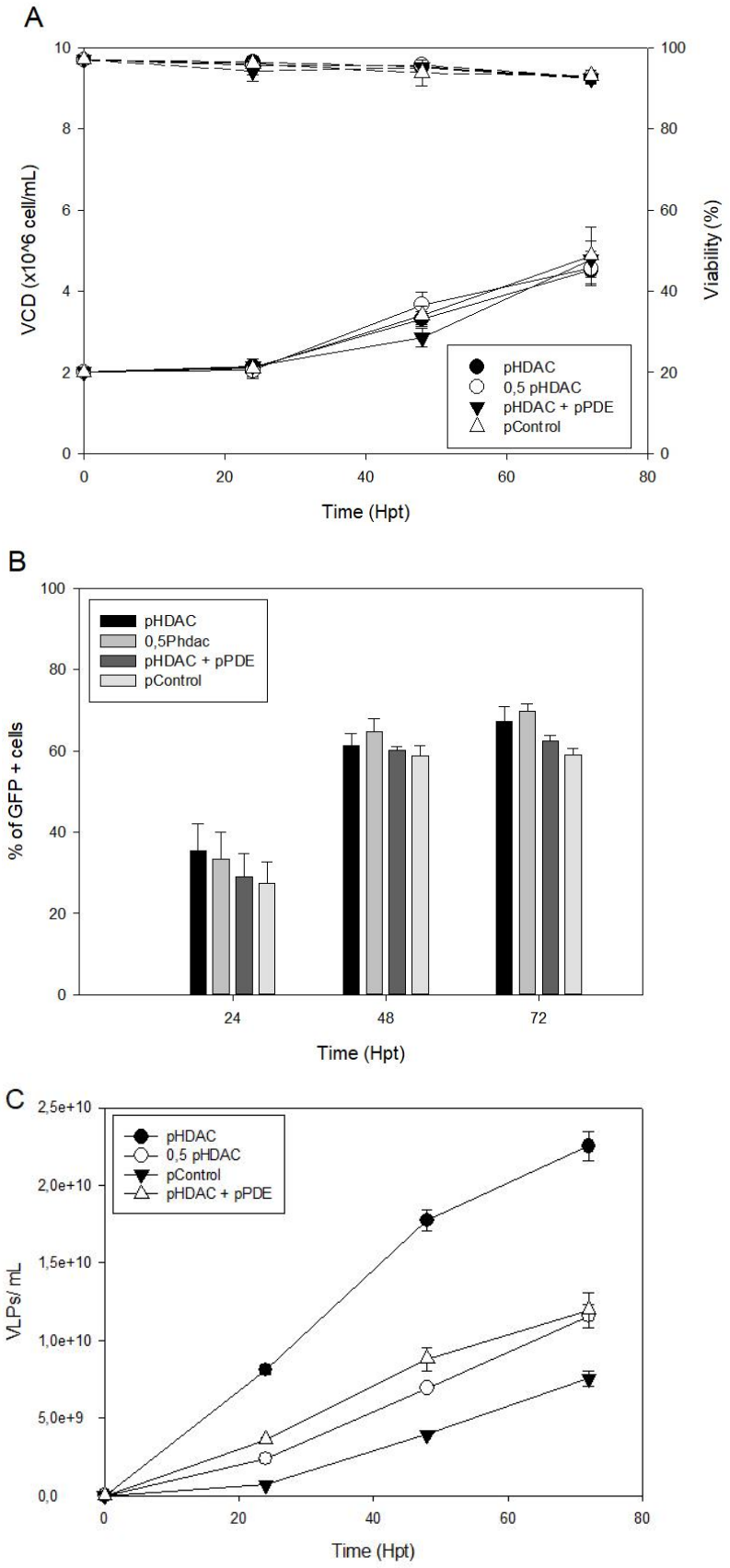

Figure 3: TGE conditions pHDAC, 0.5pHDAC, pHDAC + pPDE and pControl. **A)** Viable Cell density (solid line) and viability (dotted line). **B)** Percentage of positive GFP cells. **C)** Obtained Gag VLP production titers.

4.2. Evaluation of caffeine addition combined with the transfection with the pHDAC plasmid

As previously discussed, pPDE has not any silencing effect over the PDE8A gene, the addition of caffeine (which is reported to inhibit PDE), combined with the transfection with pHDAC, is considered to improve the obtained Gag VLP production titers. In this novel protocol, cells were transfected with pHDAC and caffeine was added 4 hpt. From now on, for simplicity, this novel approach will be referred as (pHDAC + caffeine). This condition was compared with the standard protocol using all additives (Cervera et al. 2015b) (Additives), with pGagGFP with caffeine addition 4 hpt (pControl + caffeine), pHDAC transfected alone (pHDAC) and pGagGFP also transfected alone (pControl).

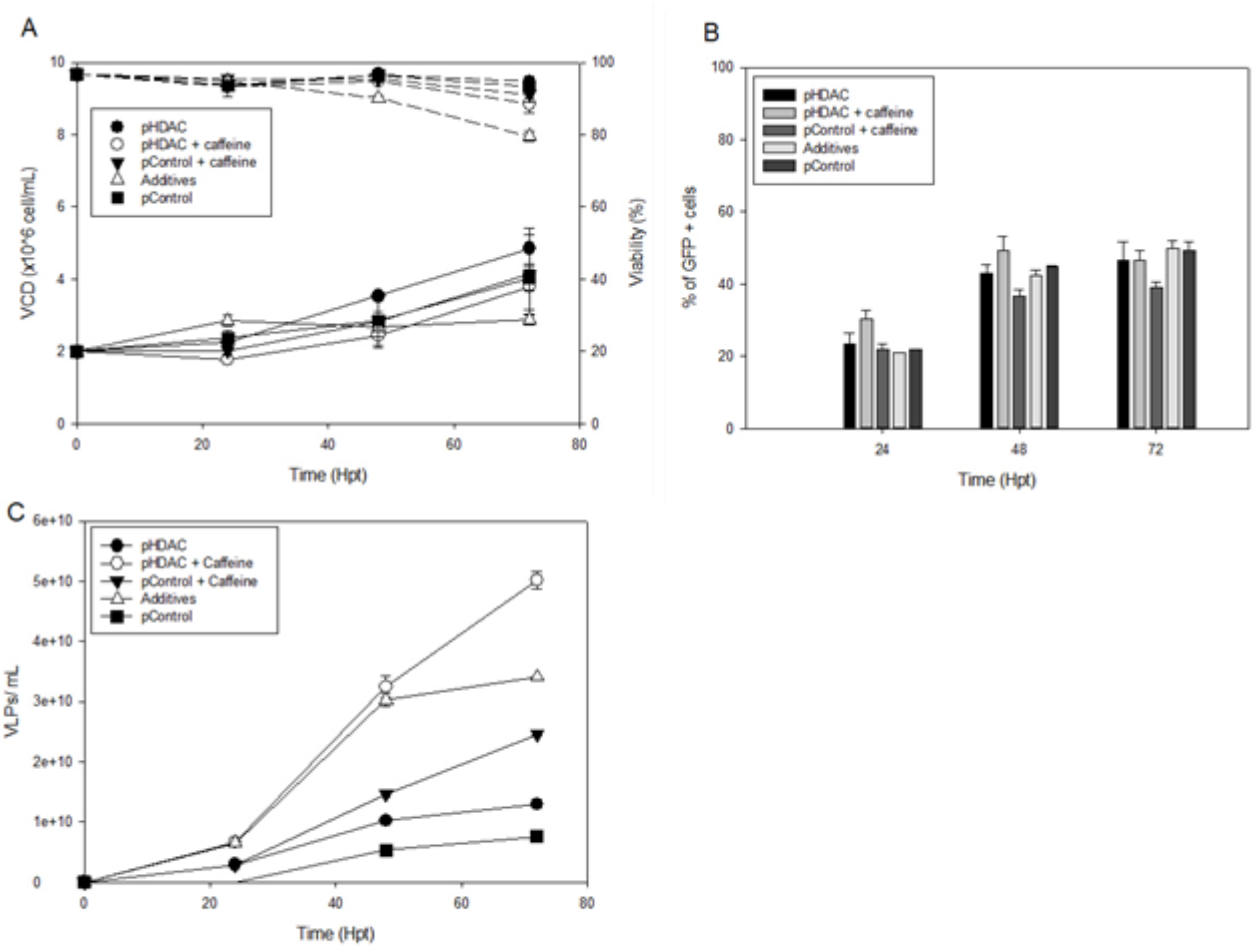

Figure 4: TGE conditions pHDAC, pHDAC + caffeine, Additives, pControl + caffeine and pControl. **A)** Viable Cell density (solid line) and viability (dotted line). **B)** Percentage of positive GFP cells. **C)** Obtained Gag VLP production titers.

The obtained results are shown in Figure 4. Similar viable cell densities and viabilities were observed in all conditions except for Additives condition, were VCD did not increase after 24 hpt (Fig 4A). It has been previously described that the addition of VPA to the cell culture, arrests cells in the phase (G1), avoiding its division (Fuenmayor et al. 2018a; Wu, et al. 2008; Kaiser, et al. 2006). After 24 hpt, viability started to decrease in Additives' condition, due to the toxicity of media additives on cells (Fig 4A). The percentage of transfected cells has no

significant variations between all conditions tested, reaching the maximum number of transfected cells 48 hours post transfection (with 40 to 50 % of transfected cells) (Fig 4B).

In Fig 4C it can be observed that the condition pHDAC + caffeine had the highest VLP production compared to the other tested conditions. Higher VLP production titers were obtained when comparing with the culture transfected with pGagGFP (pControl), ($5{,}02x10^{10}$ VLPs/ mL for pHDAC + caffeine condition and $7{,}55x10^{9}$ VLPs/mL for pControl condition). The pControl + Caffeine condition showed minor enhancement over VLP production than the ones observed in pHDAC+ Caffeine and Additives conditions, indicating that pHDAC has a positive effect on VLP production enhancement, independently of the addition of caffeine. In the Additives condition, where LiAc, VPA and caffeine were added, it can be seen that, until 48 hpt, similar VLP production to pHDAC + caffeine is obtained. Even so, at 72 hpt, VLP production in Additives condition decreases, compared to the obtained when transfecting the cell culture with pHDAC + caffeine. This may be due to the observed decrease in viability after 24 hpt in Additives condition (Fig 4B).

Table 2: Amount of obtained Gag VLPs and specific productivity of the performed TGE protocols. The fold improvement given by the developed protocols is statistically significant (*p value < 0.5*).

	VLPs/ cell*	VLPs		Improvement (VLPs)
Standard	3090	1,51E+11		**1**
Additives	23800	6,82E+11	(*p value < 0,0002)*	**4,52**
pHDAC	9980	4,50E+11	(*p value < 0,002)*	**3**
pHDAC + Caff	26600	1,00E+12	(*p value < 0,00003)*	**6,65**

* Transfected cells

Table 2 summarizes the amount of obtained Gag VLPs and specific productivities of the performed TGE protocols. The use of pHDAC + caffeine is the best option as it provides a 6.65-fold improvement of Gag VLP production in comparison to the standard protocol (where cells were transfected with pControl). When performing the Additives condition, an improvement in VLP production of 4.52-fold was obtained, confirming results from previous work (Cervera, et al. 2015b). The pHDAC + caffeine and Additives protocols showed similar specific productivities, both higher than the specific productivity given by the standard TGE. So, in both conditions, the production capacity of transfected cells was increased by the inhibition of HDAC and PDE expression. Therefore, the VPA's enhancement effect in Gag VLP production, observed in Additives condition, is achieved in this novel condition by the use of shRNA against HDAC5 gene. Moreover, the addition of caffeine in this condition, supplies the lack of an efficient shRNA against PDE8A, increasing the impact over Gag VLP production of the condition of transfection with pHDAC.

Taking into account all the obtained results, the best pshRNA combination to carry out the transient gene expression protocol (TGE) with an enhanced Gag VLP production, is the protocol where the cells are transfected with pHDAC with the addition of caffeine 4 hpt.

4.3. Combination of extended gene expression with pHDAC + caffeine strategy

As previously discussed, extended gene expression protocol (EGE) enables that cells that have not been transfected in first transfection, are able of being transfected in the subsequent transfection rounds. In previous studies, a 12-fold increase in the Gag VLP production in EGE in relation to the production in TGE has been reported (Cervera, et al. 2015a). Even more, it has been previously described a 1.4-fold improvement of Gag VLP production titer when carrying out the EGE protocol with the addition of production enhancers (VPA, lithium acetate and caffeine) (Fuenmayor et al. 2018a). Even so, the use of these media additives made the non-transfected cells not able to incorporate the DNA in second and third transfections, reducing EGE + additives protocol Gag VLP production potential (Fuenmayor et al. 2018a). Since shRNA only cause a gene silencing effect over transfected cells of the culture, the non-transfected cells population is susceptible of being transfected in subsequent transfection rounds, making the use of shRNA a good alternative to the addition of production enhancers. For this reason, the selected pshRNA configuration after the performed TGEs, pHDAC + caffeine strategy, was tested in combination with EGE protocol.

A standard EGE and EGE pHDAC + Caffeine were carried out. As shown in Fig 5, two rounds of re-transfection were performed at 48 and 96 hours post first transfection in both conditions tested, as well as, a medium exchange of the culture every 48 hours. In re-transfection rounds, cells were re-transfected with only 0.5 µg of plasmid DNA/mL instead of the 1 µg of plasmid DNA/mL, used in the first transfection (Cervera, et al. 2015a).

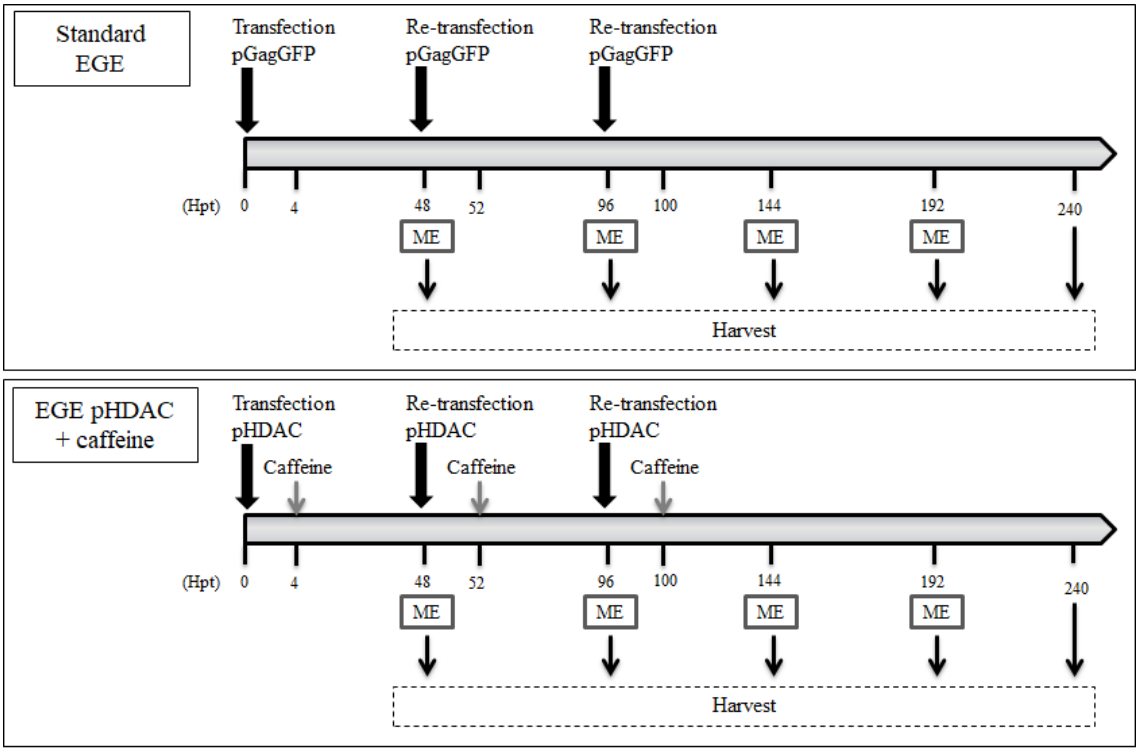

Figure 5: Diagram of performed standard EGE and EGE pHDAC + caffeine protocols, respectively. Where ME means complete medium exchange. For both protocols three transfection rounds are performed at 0, 48 and 96 hpt. In EGE pHDAC + caffeine protocol caffeine is added 4 hpt in every transfection round. Five harvest rounds (with a complete media replacement) are carried out every 48 hours.

Similar percentages of transfected cells were observed in the two conditions tested, achieving the maximum values (between 45 and 55 %) after 48 hours post first transfection (as shown in Fig 6A). The achieved VCD when EGE pHDAC + Caffeine protocol was performed was lower than the one reached performing the EGE protocol. As well, culture viability, after 144 hours post transfection, decreases in major extend in EGE pHDAC + Caffeine condition than in EGE condition, dropping under 50% after 240 hpt (Fig 6B). Therefore, the addition of caffeine to the culture has a slightly detrimental effect over cells in comparison to when no additives are added. This observed negative effect was not as significant as the previously observed over cell culture growth and viability when EGE protocol combined with VPA and caffeine addition was carried out (Fuenmayor et al. 2018a).

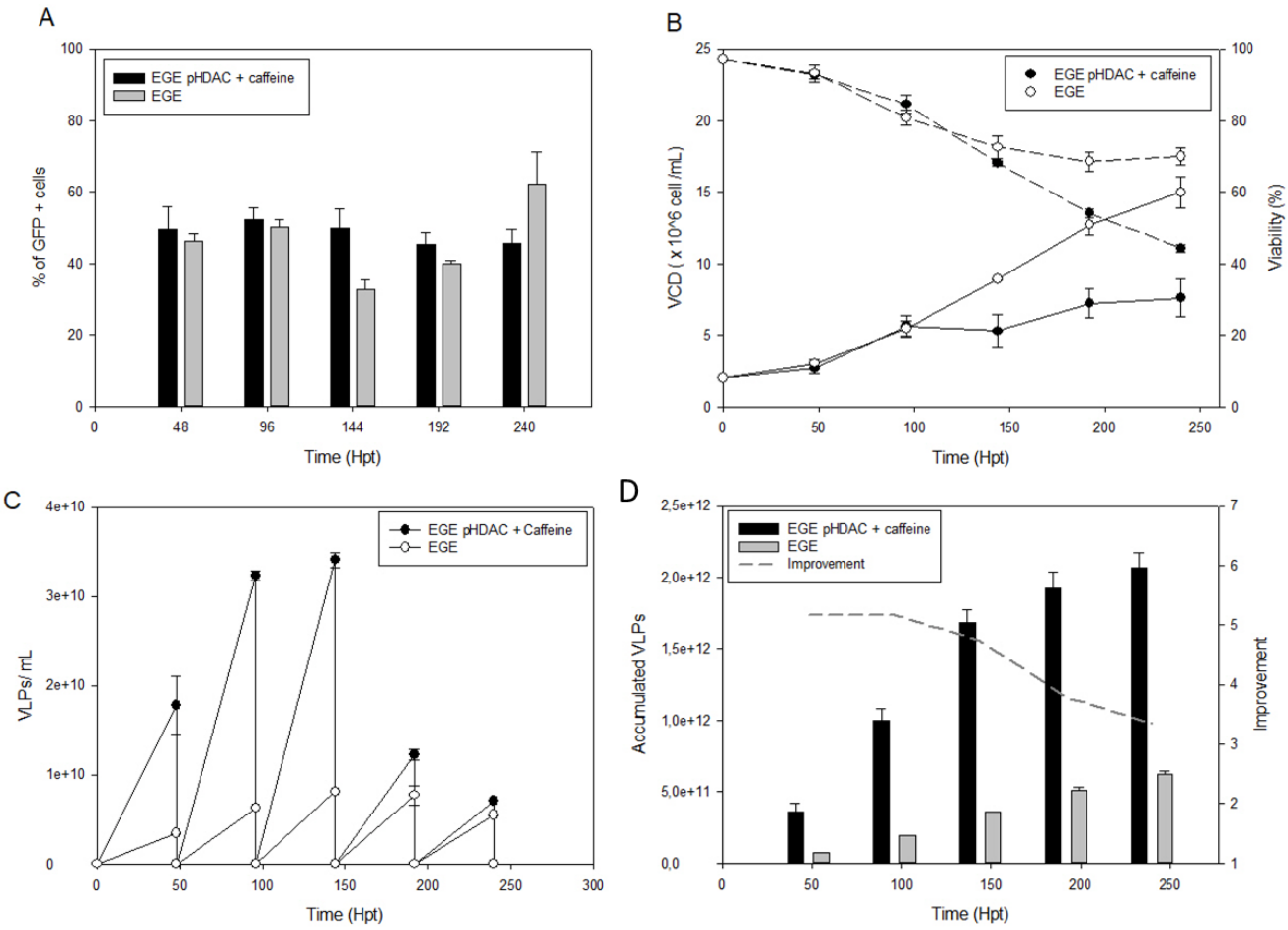

Figure 6: EGE pHDAC + caffeine and standard EGE protocols. **A)** Viable Cell density (solid line) and viability (dotted line). **B)** Percentage of positive GFP cells. **C)** Obtained Gag VLP production titers in every harvest round (performed every 48 hours). **D)** Accumulated VLPs and fold improvement (of obtained VLPs) of EGE pHDAC + caffeine protocol over standard EGE protocol.

Fig 6C shows the Gag VLP production titers for EGE and EGE pHDAC + Caffeine. As it can be seen, the use of pHDAC for transfecting the cells, with the addition of caffeine 4 hours post transfection (EGE pHDAC + Caffeine) had a great impact over Gag VLP production in comparison to production obtained transfecting with pControl (standard EGE). In Fig 6C it can be observed that, until 48 hours after the last re-transfection round (144 hours post first transfection), the VLP production in the condition EGE pHDAC+ caffeine was much higher than the one in EGE condition. After that, a decrease in VLP production titer obtained in EGE

pHDAC + caffeine condition was observed, achieving similar values than in the EGE protocol. Nevertheless, the concentration of VLPs obtained during all the experiment when transfecting with pHDAC and adding caffeine, is higher than the obtained in EGE condition. In Fig 6D it can be observed the total Gag VLPs obtained in the two protocols tested, as well as the fold improvement provided by EGE pHDAC+ caffeine protocol over EGE protocol. At the end of the experiment carried out, a total amount of $2.07x10^{12}$ VLPs was achieved in EGE pHDAC + caffeine condition, in contrast to the $6.20x10^{11}$ VLPs obtained in EGE. In fact, the performed EGE pHDAC + caffeine protocol provided a 3.34-fold improvement compared with the standard EGE protocol. As it can be observed in Fig 6D, the improvement provided by pHDAC + caffeine protocol is higher at the beginning of the experiment and decreases over the time. This observed decrease in VLP production improvement and the decrease in VLP production titer obtained in EGE pHDAC + caffeine (shown in Fig 6C) may be due to two phenomena: In first place, the transfected episomal DNA, which does not integrate into cell genome, is lost over time as a consequence of cell division, when no more re-transfection rounds are performed (Cervera, et al. 2015a); in second place, the adaptation of the transfected cells that, after several days of cultivation, are able to find alternative pathways to bypass the interfered route (silenced HDAC5 gene).

The performance of transfection process carrying out standard EGE and EGE pHDAC + caffeine protocols is summarized in Table 3. The novel EGE pHDAC + caffeine protocol offers clear advantages over the used standard EGE protocol in terms of total VLP concentration (VLPs/ mL), VLPs in total harvest (VLPs), VLP production (VLPs/hour), volumetric productivity (VLPs/h · mL) and specific productivity (VLPs/ cell).

Table 3: Comparison of standard EGE and EGE pHDAC + caffeine protocols transfection performances. The fold improvement given by EGE pHDAC + caffeine protocol is statistically significant *(p value < 0.5).*

	EGE pGag-GFP	**EGE pHDAC + caffeine**	**Improvement**
Total VLP concentration (VLPs/mL) (in total harvest)	6,20E+09	2,07E+10 *(p value < 0,00002)*	**3,34**
VLPs in total harvest (VLPs)	6,20E+11	2,07E+12	
VLP production (VLPs/hour)	2,58E+09	8,63E+09	
Volumetric productivity (VLPs/h · mL)	2,58E+07	8,63E+07	
Specific productivity (VLPs/ cell*)	827	5450	**6,59**

* Transfected cells

The EGE optimized protocol (EGE pHDAC + caffeine), after a production phase that lasted 240 hours, provided an amount of VLPs 3.34 times higher as the one obtained performing standard EGE protocol, during the same production time. In the same way, the observed improvement in total VLP concentration, production and volumetric productivity is also 3.34 since in both performed EGE protocols the used reactor volume (100 mL) and production time (240 hours) are the same. As seen before, when carrying out a TGE protocol in batch, the specific productivity of cells which were transfected with pHDAC + caffeine is higher than in cells transfected with pGagGFP (6.59-folds) (Table 2), proving that the inhibition effect over HDAC

and PDE genes, provided by this optimized protocol, enhances the VLP production potential of each transfected cell. Nevertheless, the specific productivities obtained for EGE protocols are lower than the obtained for TGE protocols. A potential explanation of this fact could be that the cell cultures in EGE protocols are able to achieve higher VCD than in TGE protocols and the used shake flasks system is not able to satisfy the nutritional demands of the culture. As a consequence of that the VLP production potential of transfected cells is being reduced.

4.4. Evolution of produced VLP quality

In previous work, when performing transient gene expression, it has been described an increase of free Gag-GFP monomers in supernatant over time (Gutiérrez-Granados, et al. 2013). As culture viability decreases, the disrupted cells release to the media free Gag-GFP monomers and proteases, which are able to degrade assembled VLPs, and therefore, compromise the quality of the product. Because of that, it becomes an important point to stop the production process when the culture viability remains high (Cervera, et al. 2017). So, the determination of optimum extended gene expression (EGE) duration is an agreement between high VLP production titers and quality of produced VLPs.

VLP quality throughout the performed EGE and EGE pHDAC + caffeine, was analysed to determine the best duration time of the novel developed EGE protocol. Released Gag-GFP monomers, as a consequence of cellular lysis, increase the detected fluorescence in supernatant (Gutiérrez-Granados, et al. 2013). For this reason, a nanoparticle tracking analysis (NTA), which can detect nanoparticles according to their size and fluorescence, was carried out and compared to the results obtained from the fluorimetry quantitation assay (shown in Fig 6C) to determine the variation of the percentage of assembled Gag VLPs in supernatant over time (data not shown). Complete Gag VLPs were detected in supernatants of both protocols after NTA assay (Fig 7). As seen in fluorimetry quantification assay (Fig 6D), higher accumulated Gag VLP are obtained when performing EGE pHDAC + caffeine than in standard EGE protocol. For standard EGE, the percentage of Gag VLPs over free Gag-GFP monomers, decreased approximately a 20% since the culture viability dropped under 80%, from 96 hpt to the end of the production phase (240 hpt). In EGE pHDAC + caffeine condition, the percentage of assembled VLPs over free monomers was lower than the one observed in standard EGE condition, mainly due to the lower viability observed during all production phase (Fig 6B). Nevertheless, in this case, a decrease in the percentage of assembled VLPs was not observed until 240 hours post transfection. At this point, the percentage of VLPs decreased roughly a 28%. Therefore, regarding the obtained results about the product quality, it was decided to exclude the fifth harvest round performed at 240 hpt in EGE pHDAC + caffeine protocol, afterwards, the quality of produced VLPs started to decrease.

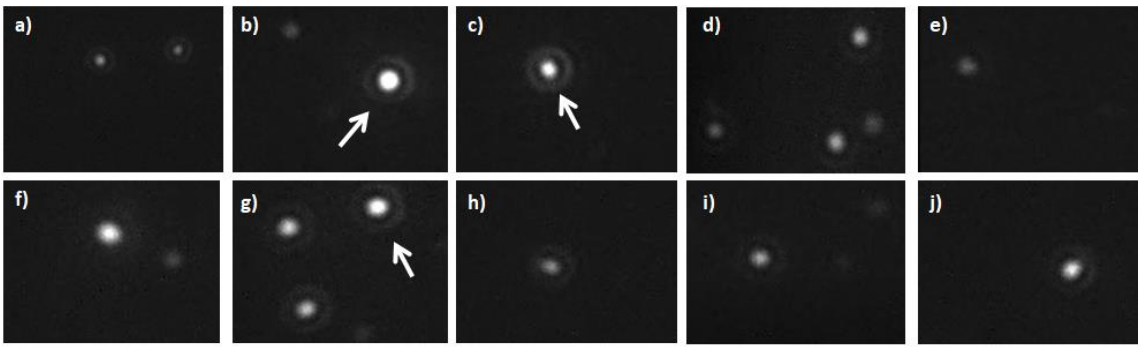

Figure 7: Complete Gag VLPs detected by NTA assay with a mean particle size between 100 and 250 nm (marked with white arrows). The images a), b), c), d) and e) correspond to samples of EGE pHDAC + caffeine protocol, harvested at 48, 96, 144, 192 and 240 hpt, respectively. The images f), g), h), i) and j) correspond to samples of Standard EGE protocol, harvested at 48, 96, 144, 192 and 240 hpt, respectively.

4.5. Production process optimization and implementation

As previously discussed, the determination of the optimum duration of EGE pHDAC + caffeine protocol should balance the final amount of produced VLPs and their quality. Based on the observed results of the evolution of the VLPs' quality over the production phase, it is proposed as design criteria to exclude the fifth harvest round (240 hpt) of the EGE pHDAC + caffeine production protocol, stopping the process at 192 hpt. Nevertheless, as shown in Fig 5A, the obtained VLP production titer in the fourth harvest round (192 hpt) of EGE pHDAC + caffeine protocol was considerably lower than the obtained in the previous harvest rounds (48 hpt, 96 hpt and 144 hpt). Therefore, to optimize the production process it is important to analyse the possibility of eliminating the fourth harvest round, stopping the process at 144 hpt. For this reason, the obtained VLP productivity during one year (VLPs/ mL· year) has been calculated while performing the EGE pHDAC + caffeine protocol carrying out 3 harvest rounds (stopping the process at 144 hpt) or 4 harvest rounds (stopping the process at 192 hpt) (Table 4).

Table 4: Comparison of the performances of EGE pHDAC + caffeine protocol considerin the performance of 3 or 4 harvest rounds. In the calculations it is considered a gap of two days between two consecutive production phases.

	3 harvest rounds	**4 harvest rounds**
Process duration (days)	6	8
VLP concentration/ process (VLPs/ mL) (in total harvest)	2,80E+10	2,41E+10
Required volume/ process (mL)	60	80
Obtained VLPs/ process	1,68E+12	1,93E+12
Number of processes/ year	**45**	**36**
VLP productivity (VLPs/ mL · year)	**1,26E+12**	**8,68E+11**

As shown in Table 4, by carrying out 3 harvest rounds in EGE pHDAC + caffeine protocol, higher number of processes can be performed per year, compared to the case of performing 4 harvest rounds (45 processes/ year instead of the 36 processes/ year). Despite more VLPs per process are obtained by the performance of 4 harvest rounds, the VLP concentration is higher when carrying out only 3 harvest rounds ($2.80x10^{10}$ VLPs/mL instead of $2.41x10^{10}$ VLPs/mL). As a result, the amount of VLPs produced in one year, carrying out only 3 harvest rounds, is higher than the obtained by performing 4 harvest rounds ($1.26x10^{12}$ and $8.69x10^{11}$ VLPs/ mL· year, respectively). As the fourth harvest round did not had a big impact on the final amount of VLPs, it has been decided to also exclude this harvest round of EGE pHDAC + caffeine protocol, performing only 3 harvest rounds and stopping the process at 144 hpt.

The use of the optimized EGE pHDAC + caffeine protocol, could improve the production process, considerably reducing its costs. Fig 8A shows a Gantt diagram of the VLP production process performing EGE pHDAC + caffeine, standard EGE and standard TGE protocols. To produce the same amount of Gag VLPs achieved in EGE pHDAC + caffeine protocol,

performing the standard EGE protocol and considering two days gap between processes for cleaning, sterilization and cell amplification, it would require 4.5-fold the time needed for the EGE pHDAC + caffeine strategy (34 days instead of 6 days). Furthermore, the time required to produce this quantity of VLPs by performing a standard TGE protocol would be 5.6-fold the required in EGE pHDAC + caffeine protocol (54 days instead of 6 days). In addition, the volume of medium needed for this optimized protocol would be more than 6 times lower than the required to carry out standard EGE protocol and 4.3-fold lower than the needed for TGE protocol. Additionally, the plasmid amount to perform all the transfection rounds would be also 3 times lower than in standard EGE protocol (40 ug of plasmid DNA instead of 120 ug) and 6.5 times lower compared to the used in standard TGE protocol (260 ug of plasmid DNA). When it comes to the downstream process, as the product obtained in EGE pHDAC + caffeine protocol is more concentrated than in standard EGE and TGE protocols, it would need less effort to separate it. In addition, the use of a shRNA strategy that avoids VPA addition could further ease downstream process, reducing the number of purification steps.

The development and optimization of transfection methodologies, regarding the large number of variables that affect transfection processes, are commonly carried out at small scale using non-instrumented shake flasks. Once the optimum conditions to perform the transfection protocol are fixed, the next step is the scale-up of the process to obtain the desired production (Gutiérrez-Granados, et al. 2018). Therefore, the implementation of the HIV-1 Gag VLP production by the optimized extended gene expression protocol (pHDAC + caffeine) at bioreactor will be performed in future work. In previous work, EGE to produce HIV-1 Gag VLPs has been successfully performed in a 1.35 L perfusion operated bioreactor (Fuenmayor, et al. 2018b), achieving similar transfection efficiency and VLP production titer, as well as higher specific productivity when comparing to the production in shake flasks. As mentioned before, the used EGE methodology in shake flasks consists in complete media exchange every 48 hours, when cells are centrifuged and separated from the supernatant and fresh medium is fed. Therefore, cells are provided by new nutrients, cell metabolites are removed from the culture media and VLPs are harvested. Nevertheless, centrifugation operation becomes more cumbersome at larger production scales and the risk of contamination of the cell culture increases. Perfusion is the proposed mode of operation for the implementation of this methodology at bioreactor scale, enabling a continuous removal of waste products, feeding of nutrients and VLPs harvest, and as a consequence, prolonging the production phase (Fuenmayor, et al. 2018b). Fig 8B shows a scheme of the optimized HIV-1 Gag VLPs production process run for a perfusion operated bioreactor. In the proposed process the cell culture is previously amplified and seeded into the bioreactor. Then the culture is grown in the bioreactor until reaching the desired VCD ($2x10^6$ cells/ mL), required for optimal cell transfection. The complex formation between pHDAC and PEI is carried out in a separated tank prior to transfection. The DNA/PEI complexes are fed into the bioreactor to transfect the cells in every transfection round. The perfusion is performed at a flow rate of 0.5 vvd (volume of harvest per working volume per day) (considering that in the EGE protocol performed in shake flasks, complete media exchanges were carried out every 48 hours). By this way, the bioreactor is continuously fed by fresh medium and the produced VLPs are harvested in the outlet current and collected in an independent tank. By operating in perfusion mode, the harvest and recovery of produced VLPs is performed at the same time, while the cells are retained inside the bioreactor. Perfusion can be carried out by several devices such as sedimentation (i.e. acoustic settle), filtration (tangential flow filtration (TFF) or alternating tangential flow (ATF) using i.e. Hollow fibre filter [HF]), spin-filter and crossflow microfiltration (Fuenmayor, et al. 2018b; Warkiani, et al. 2014; Clincke, et al. 2013).

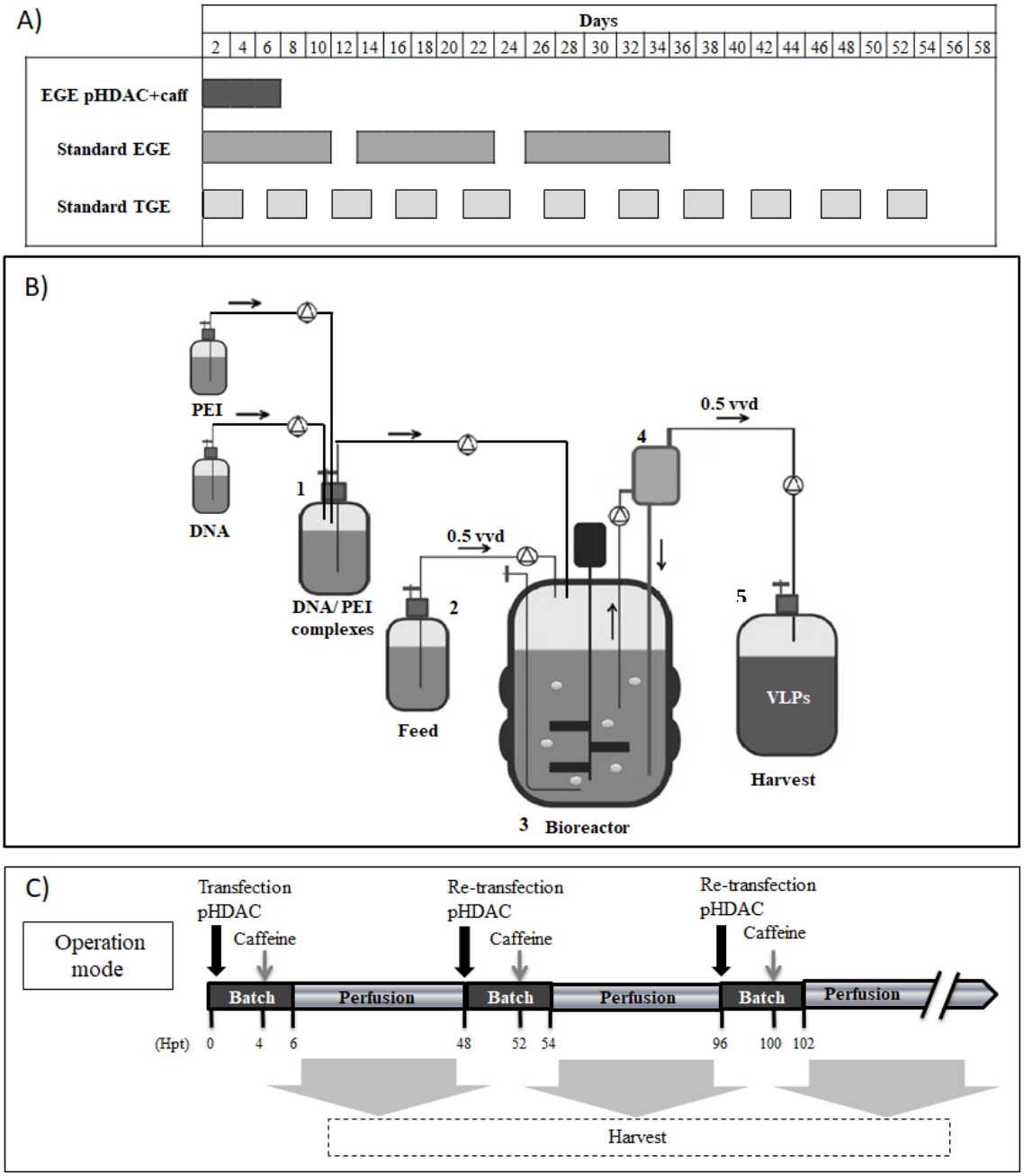

Figure 8: A) Gantt diagram of Gag VLP production process performing EGE pHDAC + caffeine, standard EGE and standard TGE protocols to produce $1.68x10^{12}$ VLPs (the VLP quantity achieved after 144hpt in EGE pHDAC + caffeine protocol). The considered duration of production processes is 144 hours for the EGE pHDAC + caffeine protocol, 240 hours for EGE protocol and 72 hours for TGE protocol. Two days for cleaning sterilization and cell amplification between productions have been considered. **B)** Scheme of the production of HIV-1 Gag VLPs in a perfusion operated reactor. 1- DNA/PEI complex formation tank. 2- Fresh medium continuously fed to the bioreactor at a flow rate of 0.5 vvd. 3- Bioreactor. 4- Perfusion device to retain the cells from the supernatant containing the product. 5- Thank of VLPs harvest in the supernatant at a constant flow rate of 0.5 vvd. **C)** Diagram of the operation mode of the proposed HIV-1 Gag VLP production in a perfusion operated bioreactor.

Fig 8C shows a diagram of the proposed mode of operation of the perfusion reactor. In every transfection round the perfusion would be stopped and would be resumed 2 hours after caffeine addition (which is added 4 hpt), giving time to the DNA/PEI complexes and caffeine to enter

into the cells. In perfusion configuration cells are constantly fed, allowing to maintain higher viability during longer time. In addition, the product is also constantly harvested, reducing its residence time inside the bioreactor. Therefore, the product quality may not be as compromised as in the processes carried out in batch mode (Gutiérrez-Granados, et al. 2018), so, further analysis of produced VLPs quality at bioreactor scale would be needed to determine the optimum process duration. In addition, in previous work (Fuenmayor, et al. 2018b), due to high cell growth, large amount of non-transfected cells were observed while performing EGE in bioreactor, suggesting that more re-transfection rounds could be carried out, increasing the VLP production titers.

5. Conclusion

The use of a shRNA against the HDAC5 gene cloned in pGag-GFP has shown to have a great impact in Gag VLP production while performing TGE methodology. As discussed before, the shRNA against the PDE8A gene did not enhance the VLP production when transfected into cell culture cloned in Gag-GFP plasmid (pPDE). Considering that, it would be interesting to test shRNA sequences against ATM601, ATR and DNA-Pks genes cloned into pGag-GFP as potential candidates to substitute the caffeine addition. Another possibility to study is to clone both shRNA sequences (against the HDAC5 and against one of the mentioned kinases) together in pGag-GFP. So, more work towards this direction is needed to obtain a completely optimized protocol to produce HIV-1 Gag VLP which supplies all of the used production enhancers.

The addition of caffeine when carrying out TGE pHDAC protocol has shown to increase the obtained VLP production titer without any detrimental effect on culture viability or cell growth. Moreover, while performing the EGE pHDAC protocol, the addition of caffeine provided the biggest improvement in produced VLPs in comparison to the produced in standard EGE protocol. Nevertheless, a slight diminution of cell growth and viability, comparing with the standard EGE, was observed due to caffeine addition.

The utilization of gene silencing strategies (shRNA against the HDAC5 gene) combined with caffeine addition in EGE protocol (EGE pHDAC + caffeine) enabled to develop an efficient methodology which is more than four times faster than the formerly used one (standard EGE) and also less intensive in medium and DNA requirements. The proposed strategy provides higher improvement of produced HIV-1 Gag VLPs compared to the improvement achieved when using the EGE protocol combined with the VPA an caffeine addition (3.34-fold and 1.4-fold improvement over standard EGE protocol, respectively) (Fuenmayor, et al. 2018a). In addition, the developed strategy provides a 40-fold improvement of produced VLPs compared to the standard TGE, avoiding the previously mentioned limitations of the addition of production enhancers to the media (Fuenmayor, et al. 2018a).

An optimum time duration of 144 hours has been determined to implement this novel strategy for the production process, with three re-transfections and harvest rounds, according to the observed evolution of product quality over time and the VLP production titer obtained in each harvest round.

Based on the obtained results, the potential of a production process based on the developed methodology has been considered. In a bioreactor of 2 L, a yearly production of $5.6x10^{13}$ VLPs could be attained, with a production phase of 6 days and consuming a total volume of medium

of 8 L (considering that the perfusion is operated at a flow rate of 0.5 vvd). Nevertheless, due to the previously commented benefits of operating in perfusion mode, more re-transfection rounds could be performed and also the production phase could be prolonged, increasing the VLP production potential of the process.

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