Universitat Autònoma de Barcelona

ENHANCEMENT OF VIRUS-LIKE PARTICLE PRODUCTION IN HEK293 CELL CULTURES

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PROGRAMA DE DOCTORAT EN BIOTECNOLOGIA

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PHD THESIS

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PhD thesis presented by Javier Fuenmayor Garcés to apply for the degree of Doctor in Biotechnology by Universitat Autònoma de Barcelona.

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Dr. Francesc Gòdia Dr. Laura Cervera

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ABBREVIATIONS

AAV	Adeno-associated vector
AAVS1	Adeno-associated virus site 1
ADD	Additives
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ATF	Alternating tangential flow
B/IC	Baculovirus-insect cell
CCD	Central composite design
CHO	Chinese hamster ovary cells
CMV	Citomegalovirus
CRISPR	Clustered regulatory interspaced short palindromic repeats
dpt	Day post transfection
EBNA-1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EGE	Extended gene expression
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isotiocyanate
FU	Fluorescence unit
GFP	Green fluorescence protein
GOI	Gene of interest
gp120	Glycoprotein 120
gp410	Glycoprotein 41
HA	Hemagglutinin
HCD	Host cell DNA
HCP	Host cell protein
HDAC	Histone deacetylase
HEK293	Human embryonic kidney cells
HF	Hollow fiber
HIV	Human immunodeficiency virus
hpt	Hour post transfection
HPV	Human papilloma virus
IRNA	interference RNA
M1	Matrix protein 1
mAb	Monoclonal antibody

ME	Medium exchange
NIH	National Institute of Health
NTA	Nanoparticle tracking analysis
OriP	Origen of replication P
p24	Protein 24 (CA subunit from HIV-1 Gag polyprotein)
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PEI	Polyethyleneimine
PI	Propidium iodide
PPP1R12C	Phosphatase 1 regulatory subunit 12C
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
RFU	Relative fluorescence unit
RISC	RNA-induced silencing complex
rpm	Revolutions per minute
SD	Standard deviation
SGE	Stable gene expression
shRNA	Short harping RNA
SIV	Simian immunodeficiency virus
SV40	Simian vacuolating virus 40
SV40ori	Simian vacuolating virus 40 origen of replication
TALENS	Transcription activator-like effector nucleases
TFF	Tangential flow filtration
TGE	Transient gene expression
UC	Ultracentrifugation
VLP	Virus-like particle
VVD	Volume of feed per bioreactor volume per day
ZFN	Zinc finger nuclease
λ_{em}	Emission wavelength (nm)
λ_{ex}	Excitation wavelength (nm)

Resum

Les *virus-like particles* (VLP) tenen un gran potencial com a candidats pel desenvolupament de vacunes. En aquest treball, s'han aplicat diverses estratègies per la millora de la producció de VLPs (basades en la poliproteïna Gag del VIH-1) en HEK293 mitjançant la transfecció transitòria i l'expressió estable. Aquest candidat té aplicacions potencials en el desenvolupament d'una vacuna terapèutica contra la SIDA. Tres capítols del treball descriuen estratègies basades en la transfecció transitòria, mentre que l'últim capítol consisteix en la generació d'una línea HEK293 estable per la producció de VLPs de VIH-1.

En el primer capítol, es va dur a terme la combinació de l'estratègia *extended gene expression* (EGE) amb la complementació del medi amb additius químics. L'EGE (basada en la repetició de diversos recanvis de medi i retransfeccions) ha reportat una millora en la producció de VLPs de 12 cops mentre que la complementació química del medi amb potenciadors de l'expressió gènica comporta una millora de 4 cops. La combinació de l'EGE amb additius químics va resultar en una millora de 1.5 cops en la producció de VLPs de VIH-1 comparat amb l'EGE sola. Com alternativa a l'ús d'additius químics, es va provar l'expressió de shRNA que produeixin el mateix efecte sobre les cèl·lules. Aquesta estratègia innovadora va millorar la producció de VLP en 2.3 cops sense cap detriment sobre la viabilitat cel·lular. Finalment, la combinació dels shRNA amb l'EGE va comportar una millora en la producció de VLPs de 1.3 cops, comparat amb el protocol tradicional d'EGE.

En el segon capítol de la tesi, es va dur a terme l'escalat a nivell de reactor d'EGE, obtenint una producció de VLP comparable a l'erlenmeyer. El bioreactor va permetre l'assoliment de densitats cel·lulars i creixements específics molts més alts. Degut al major creixement en el bioreactor, el percentatge final de cèl·lules GFP positives era considerablement menor que a l'erlenmeyer, la qual cosa podria ser millorada ampliant el nombre de retransfeccions realitzades al cultiu cel·lular. L'anàlisi per quantificació de nanopartícules va revelar un ratio de VLPs respecte a partícules totals més alt en el cas de l'erlenmeyer que en el reactor; possiblement degut a les altes densitat cel·lulars del bioreactor. La metodologia d'EGE va poder ser escala satisfactòriament a nivell de reactor per primer cop, mantenint les produccions de GagGFP.

En el tercer capítol, es va realitzar l'optimització de les concentracions de DNA i PEI usades a la transfecció transitòria per a tres línies HEK293 diferents i tres plasmidis d'expressió. Les concentracions de DNA i PEI optimitzades per a les nou combinacions van

ser molt similar per tots el casos; el que ens indica que l'eficiència de transfecció depèn de la quantitat concreta de DNA i PEI emprada. A més, dues de les tres línies reconeixen orígens de replicació específics. Els orígens de replicació estan inclosos a les seqüències dels vectors per tal de provar la seva capacitat d'augmentar la producció de VLPs. El sistema HEK293E/OriP va ser l'escollit perquè permet una millora de la producció de VLPs de VIH-1 de 3 cops, mantenint les mateixes densitats i viabilitats cel·lulars comparat amb un plasmidi control.

En l'últim capítol d'aquest treball, es va dur a terme la generació d'una línea estable de HEK293 per l'expressió de VLPs. Després d'un procés de selecció basat en la producció, es van seleccionen cinc clons per l'adaptació a suspensió i a medis lliures de sèrum. El clon 10H9 va presentar un temps de duplicació i una densitat cel·lular màxima semblant a la resta de clons i va assolir una la màxima productivitat específica; per tant, va ser seleccionat com a productor de VLPs.

Summary

Virus-like particles (VLP) have a high potential as candidates for vaccine development. In this work, several strategies are tested for VLP production enhancement in HEK293 cells by transient gene expression (TGE) and stable gene expression (SGE). The VLP of interest is based on HIV-1 Gag polyprotein which has potential applications in therapeutic AIDS vaccine development. Three chapters present strategies based on TGE while the last chapter consist of the generation of a stable HEK293 cell line for the production of HIV-1 VLPs.

In the first chapter, the combination of extended gene expression (EGE) strategy with chemical additives supplementation was tested. EGE (based on repeated medium exchanges and retransfections) improves VLP production titers in 12-fold while chemical media supplementation leads to a 4-fold enhancement of VLP titers, despite their effect on cell viability. The combination of EGE protocol with chemical additives resulted in a 1.5-fold improvement in production compared with the EGE protocol alone ($4.45x10^{10}$ VLPs/mL compared with $2.89x10^{10}$ VLPs/mL). As an alternative, the expression of shRNAs that could provide the same effects was also tested. This novel strategy enhanced VLP production by 2.3 fold without any detrimental effect on cell viability. Finally, the combination of shRNA with EGE resulted in more than 1.3-fold improvement compared with the EGE protocol traditionally used.

In the second chapter, the scalability of EGE at bioreactor level was tested and the VLP production was compared to the results obtained in shake flasks. Cell viability was comparable between the two systems tested; however, the bioreactor enabled to reach much higher cell densities and specific growth rates than shake flasks. Due to this increased cell growth in the bioreactor, the final percentage of GFP-positive cells was considerably lower than in the shake flasks, which could be improved by performing further retransfections of the cell culture. GagGFP VLP titers were similar in both shake flasks and bioreactor. Nanoparticle tracking analysis revealed that the ratio of VLPs/total particles (VLPs and microvesicles) was higher in the shake flasks than in the bioreactor, possibly due to higher cell densities achieved in the bioreactor. EGE methodology was successfully carried out in a bioreactor system for the first time while maintaining GagGFP production titers.

In the third chapter, DNA and PEI concentration optimization was carried out for three different HEK293 cell lines (HEK293SF-3F6, T and 6E) and three different expression

plasmids (pSV40, p(-), and pOriP)). The concentration of DNA and PEI was optimized for the nine combinations and the obtained resulted are very similar in all cases (DNA: 2.34 ± 0.18 µg/mL; and PEI: 5.81 ± 0.18 µg/mL), revealing that transfection efficiency was not dependent on the cell line or vector type. Furthermore, two of the cell lines tested recognize specific origins of replication: HEK293T/SV40 and HEK293E/oriP. Origins of replication were included in the vector sequences to test their capacity to increase production titers. HEK293T/SV40 resulted in a decrease of cell density and productivity of 2.3 fold compared to a control plasmid. On the other hand, HEK293E/OriP platform enabled a 3-fold improvement in HIV-1 VLP production keeping the same cell densities and viabilities compared to a control plasmid.

In the last chapter of this work, the generation of a stable HEK293 cell line expressing Gag VLPs was attempted through a random site integration strategy. After a screening process based on GagGFP production, five clones were selected for adaptation to suspension and serum-free conditions. 10H9 clone presents similar duplication time and maximum cell density than the other clones. Furthermore, 10H9 showed highest GagGFP productivity and also highest specific productivity and was hence the selected GagGFP producer HEK293 clone.

Introduction

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and Francesc Gòdia

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1. Viral vaccines

1.1 Types of vaccine

The vast majority of preventative viral vaccines consist of an attenuated or inactivated virus, for administration to individuals to provoke a protective immune response. These types of vaccines are very effective, and generally a second administration or the use of adjuvants is not required. Nevertheless, attenuated vaccines in particular present a risk at the level of manufacture or administration since they can in principle revert to a pathogenic form (Mäkelä 2000; Ulmer et al. 2006; Plotkin 2014). Newer generation vaccines improve safety by removing whole viruses from the formulation and using alternatively protein subunits, DNA, or virus-like particles (VLPs) (Figure 1). Subunit vaccines are composed of recombinant viral proteins or purified proteins from the wild-type virus, which act as antigens (Schiller and Lowy 2015). DNA vaccination is a technique based on the direct administration to the recipient of plasmid DNA encoding an antigenic protein which is expressed by the recipient's cells, subsequently generating an immune response (Liu 2003).

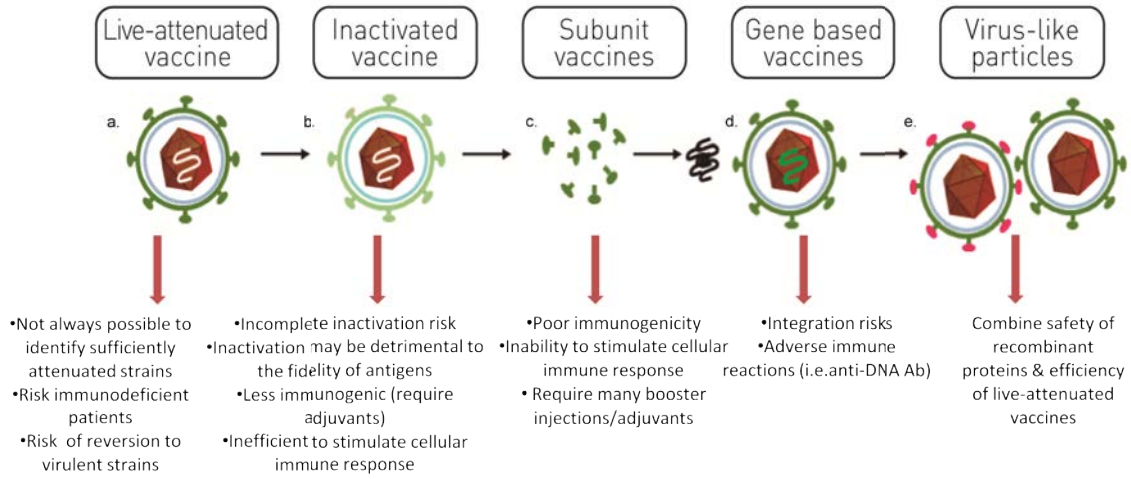

Figure 1. Types of vaccines: live-attenuated vaccine; inactivated vaccine; subunit vaccines; gene base vaccines; virus-like particles.

Virus-like particles are artificial nanostructures that resemble a virus. They are composed of all or some of the proteins that form the viral capsid but lack genomic material, precluding any possibility of reversion mutations or pathogenic infection. VLPs are

unable to replicate in the recipient but stimulate the immune system through recognition of repetitive subunits, producing a high cellular and humoral immune responses (Roldao et al. 2010). Due to the potentialities compared to other types of vaccines, the interest in VLP technology has increased in recent years (Figure 2).

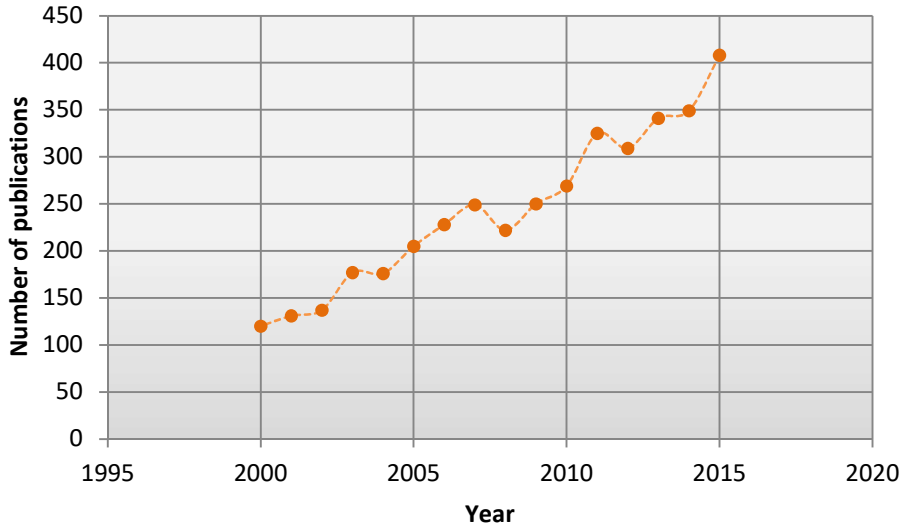

Figure 2. Increase of the interest in Virus-Like Particles. Total number of publications in PubMed about VLPs.

1.2 Types of virus-like particles

Virus-like particles are composed of one or several structural proteins that have the ability to self-assemble when recombinantly expressed. The proteins can be arranged in single, double or triple layers (Lua et al. 2014). In the case of human papillomavirus (HPV) (Abdoli et al. 2013), the VLPs are formed by a single structural protein that forms the basic capsid of the particle. Other more complex VLPs comprise several structural proteins, e.g. VLPs of the Reoviridae family are formed by 2 to 4 different proteins disposed in several layers (Lua et al. 2014). VLPs can also have an external lipid envelope. In this case, the structural protein core exits the cell through a budding process, enveloping the capsid within part of the cell membrane. This is the case for HIV-1 VLPs, which are formed by the Gag polyprotein and take part of the host cell membrane as the envelope (Cervera et al. 2013). Influenza VLPs are also formed by the protein core and the hemagglutinin spikes that are displayed on its surface (Liu et al. 2013). Hence, the choice of the producer cell line

is very important since enveloped-VLPs will contain the proteins expressed on its membrane.

The potentialities of VLPs could be further extended to the development of the concept of chimeric VLPs. Their structure is composed of a viral protein while the envelope proteins are derived from a second virus. Recently, a porcine circovirus type 2 VLP was developed displaying a porcine reproductive and respiratory syndrome virus GP5 epitope B (Hu et al. 2016). This opens the possibility of using VLPs as a delivery system. Envelope proteins can act as signals for specific tissue receptors. In this way, VLPs may be targeted to a given tissue, with capsid proteins linked to components for delivery to the targeted tissue. Thus, VLPs have high potential in drug delivery, gene therapy, and cancer treatment (Yan et al. 2015).

2. Human immunodeficiency virus (HIV)

2.1 HIV structure and infective cycle

The human immunodeficiency virus (HIV) is a lentivirus that belongs to the *Retroviridae* family. It is responsible for causing the acquired immune deficiency syndrome (AIDS) which consist of a depression of the immune system allowing the development of opportunistic infections and viral-induced cancers in the affected individuals (Sharp and Hahn 2011). It was firstly reported in the US in the 1980s and it has infected since then at least 60 million people, causing more than 25 million deaths. HIV derived from the simian immunodeficiency virus (SIV) and was firstly transmitted to humans in the 1910s (Sharp and Hahn 2011).

HIV is a complex lentiviral virus with a single-stranded RNA genome which is retrotranscribed into double-stranded DNA and integrated into the host chromosal DNA. Its genome is composed by 9 genes (gag, pol, env, tat, rev, nef, vif, vpr and vpu) (Figure3) which encode for 15 proteins (Young et al. 2006). Gag, pol and env are common in all lentiviruses and encode for essential proteins involved in the generation of the new viral particles. Gag generates the structural protein of the particle, pol encodes for the enzymes of the virus and env for the external receptor binding proteins. Tat and Rev are both regulatory proteins. Tat promotes and enhances the viral transcription while Rev is involved

in nuclear export of unspliced viral RNAs. Nef, Vpu, Vpr and Vif are accessory proteins related to the down-regulation of CD4 and MHC-I, the nuclear localization of the pre-integration complex and the increase of the viral infectivity (Young et al. 2006).

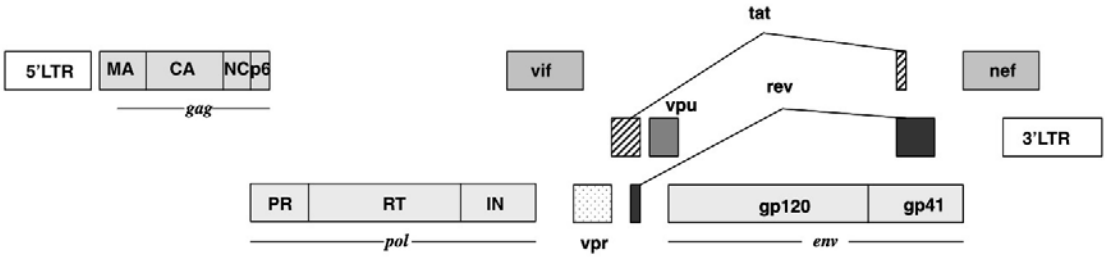

Figure 3. HIV-1 genome (Young et al. 2006)**.**

Once the host cell is infected, the viral genome is transcribed and translated into proteins. Gag protein travels to the vicinity of the cell membrane and anchors in the inner layer of the membrane for subsequent assembly. Gag protein also interacts with RNA molecules. Env is directed to the cell membrane by its transmembrane tail and it is enriched in raft-like areas where sphingomyelin and cholesterol are present. The other accessory and regulatory proteins, the genomic RNA, $tRNA^{Lys,3}$, and ribomal RNAs are also incorporated into the virions. Gag is accumulated in the cell membrane until the viral particle releases by a budding process. During or after budding, the virions undergo a maduration process (Sundquist and Kra 2015).

2.2 HIV treatment and vaccination

HIV viral infection was firstly described in 1981 and was related to the subsequent AIDS pandemics in 1983. Thanks to prevention in sexual relations, the number of new infections has been significantly reduced since then. However, in 2016 36.7 million people were living with HIV infection and 1.8 million were infected. In mid-2017, among the people living with HIV infection only a 56.9% were being treated with some of the established antiretroviral treatments (ARTs) (http://www.who.int/mediacentre/factsheets/fs360/en/).

There are more than 30 ART drugs available in the market and their use prolongs the live span of infected patients almost as in a healthy person; therefore converting AIDS in a chronic disease. Furthermore, ART are also utilized as a prophylactic treatment in high risk groups and preventive campaigns have decreased the number of new infections in the

recent years. However, ART treatment is not worldwide accessible and preventive campaigns do not have a deep impact in developing countries. Moreover, it is extremely expensive and it is not a curative treatment. The generation of an effective and affordable HIV vaccine for the eradication of the AIDS pandemic is hence crucial (Fauci et al. 2014).

HIV virus initiates the infection by breaking the mucosa barrier or infecting T-cells. In every virus replication, changes in the viral genome are generated (Jetzt et al. 2000; Zhang et al. 2003). Classical approaches for generating a HIV vaccine have been unsuccessful. Live attenuated vaccines have reported good results in monkeys; nevertheless, the high risk of vaccine reversion into the viral form has discarded this option for HIV immunization (Mäkelä 2000). On the other hand, whole inactivated or subunit vaccines have shown low capacity for generating an effective CD8+ T cell response (Girard et al. 2006). New approaches are currently being investigated based on plasmid DNA vaccines and live recombinant vector modified to express HIV antigens (Barouch 2009). As it has been previously discussed, HIV VLPs based on the protein Gag are considered a promising alternative for the generation of an effective vaccine (Roldao et al. 2010).

2.3 HIV VLPs

HIV VLPs are complex nanostructures composed by a structural internal protein, a lipidic membrane and envelop proteins. The core is composed by around 2500 Gag molecules per VLP (Chen et al. 2009). Gag release is carried out by a budding process, resulting in the incorporation of part of the cell membrane to the VLP. Membrane proteins from HIV can be incorporated to the cell membrane and hence will be disposed in the outer part of the VLP. Envelop proteins from HIV are gp41 and gp120. The first one is a transmembrane protein and the second one in an outer protein bound to gp41. Both gp41 and gp120 are forming trimers (Figure 4). A mature VLP measures around 120-200 nm and it can be visualized under electron microscopy, containing an internal electrodense region.

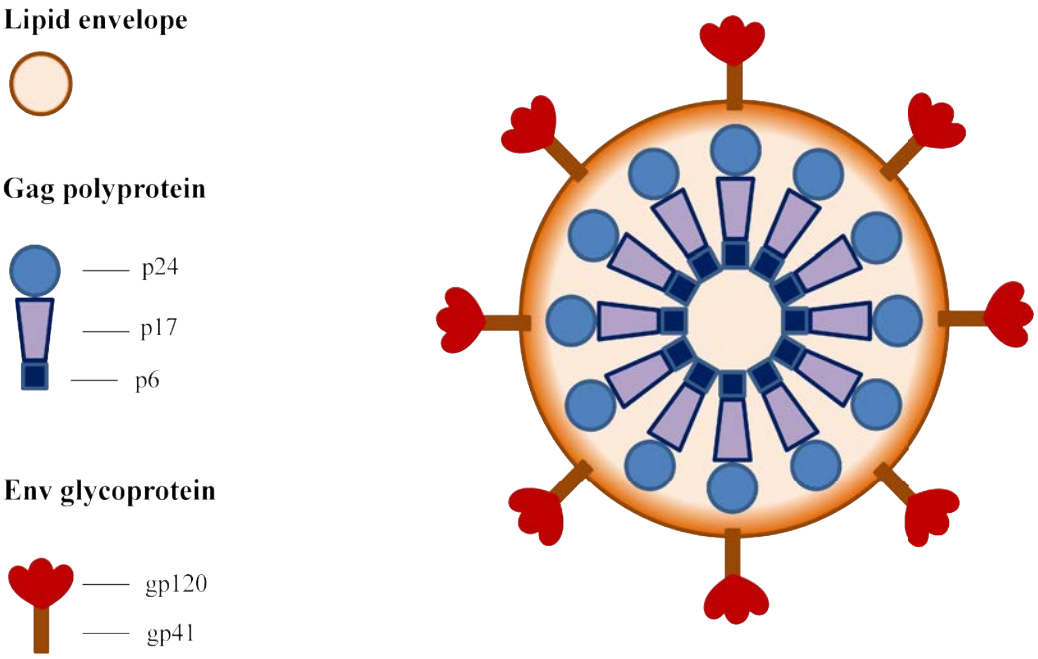

Figure 4. Schematic representation of HIV-1 Gag-based VLP.

3. Production platform for VLP production

The expression system adopted for VLP production should take into consideration the requirements for protein folding and post-translational modifications. Several expression systems are available, and the main advantages and disadvantages of each system are summarised in Table 1.

Table 1. Advantages and disadvantages of the different VLP production platforms

Production platform	Advantages	Disadvantages
E. coli	• Ease of expression • Ability to scale-up • Low production cost	• No glycosylation. • Endotoxins
Yeast	• Ease of expression • Ability to scale-up • Low production cost	• Non-appropriate protein glycosylation (i.e. high mannose glycoprotein modification). • Risk of incorrect folding and assembly.
Insect cells	• Can produce large amounts of correctly folded VLP in high density cell culture conditions • Ability to scale-up • The risk of culturing opportunistic pathogens is minimised compared to mammalian cell culture • Host-derived insect cell/baculovirus components may act as vaccine adjuvants , help trigger a more effective immune response	• Limited to high mannose glycoprotein modification. • Baculovirus contaminants may be difficult to remove • Host-derived insect cell/baculovirus components may also mask the immune response against the desired epitope
Mammalian cells	• Producer cells more closely related to the natural host • Appropriate PTMs and authentic assembly of VLPs	• Higher production cost • Lower productivities
Plants	• Ease of expression • No human-derived virus contamination	• Cannot undergo PTMs and VLP assembly • Low expression levels • Stability: antigen degradation

3.1 Bacteria and Yeast

Bacteria and yeast are cost-effective and easy to scale production systems. Bacteria are a more suitable expression system for VLPs formed with just one or two structural proteins and no envelope. The main advantage is the high yield of the proteins of interest; however, bacteria are unable to perform post-translational modifications, which can be very important for VLP immunogenicity (Roldao et al. 2010). Production of HPV Type 16 L1 VLPs has been successfully carried out using *Lactobacillus casei,* where immunofluorescence was used to confirm the presence of conformational epitopes (Aires et al. 2006). Conversely, *E. coli* bacteria were used for the production of recombinant

norovirus capsid, which was found to be useful in antigenic and also receptor-binding studies, but not as a vaccine candidate (Tan et al. 2004).

Due to the ability of yeast to perform post-translational modifications, it represents a step forward in VLP production. Recently, Chikungunya VLPs were produced using *P. pastoris* and promising results were obtained in terms of murine immunisation (Saraswat et al. 2016). Indeed, several yeast-produced VLPs have already reached approval by regulatory agencies, such as the commercialized human papillomarvirus-like particle Gardasil® produced in *Saccharomyces cerevisiae* by Merck & Co., Inc. (Luna et al. 2013). Nevertheless, their PTM pattern is not exactly the same as in humans.

3.2 Baculovirus/Insect Cell (B/IC) expression system

The B/IC system process is divided into two phases: an infection phase and a production phase. Baculovirus design is a fast and easy procedure, which makes it suitable for the production of vaccines for viruses whose surface protein can vary between each outbreak (Felberbaum 2015). The B/IC system can produce protein quantities comparable to those achieved with bacteria or yeast, and additionally its capacity to perform complex PTMs is greater (Roldao et al. 2010). Two main insect cell lines are used for recombinant protein production using B/IC expression, Sf9 (*Spodoptera frugiperda*) and High Five cells (*Trichoplusia ni*). Many VLP types have been produced using the B/IC system, including Chikungunya, HIV or porcine parvovirus-like particles (Maranga et al. 2003; Buonaguro et al. 2006; Metz et al. 2013). Indeed, the licensed human papillomarvirus-like particle vaccine Cervarix® is produces using High Five cells by GlaxoSimthKline (Roldao et al. 2010). The main disadvantage is that enveloped baculoviruses are also produced at the same time than VLPs, making purification a laborious and costly step (Liu et al. 2013).

There are currently other platforms to produce VLPs avoiding the use of baculovirus, hence simplifying purification. Stable cell lines can be generated in which the protein of interest is continuously expressed. HIV-1 VLPs were produced by stably transfected *Drosophila* S2 cells (Yang et al. 2012). If the protein produced has a cytotoxic effect, it can be regulated by an inducible promoter. Transient transfection can also be carried out in insect cells. Cellfectin has been used for the production of influenza A VLPs consisting of haemagglutinin (HA) and matrix protein (M1) in Sf9 cells (Pushko et al. 2007). Little

research has investigated the use of cheaper transfection reagents, such as polyethyleneimine (PEI), for recombinant protein production in insect cells (Shen et al. 2013).

3.3 Plants

Transgenic plants have also been used for VLP production. *Agrobacterium tumefaciens* is commonly used for infection and transformation of the cells (Ma et al. 2003). These bacteria can infect plant cells and introduce a specific gene of interest into the host genome. Several examples are available of VLP production in plants, such as for HPV type 16 or influenza (Liu et al. 2005; D'Aoust et al. 2010). The most commonly used plants for recombinant protein production are *Nicotiana tabacum* and *Arabidopsis thaliana* (Greco et al. 2007); others include potato or tomato (Huang et al. 2005) (Fruit et al. 2006).

3.4 Mammalian cells

Several mammalian cell types are suitable for VLP production. Although mammalian cells produce less protein of interest compared to other systems, they have the capacity to produce more complex and accurate PTMs (Zhu 2012). For this reason, mammalian cells are typically used to produce complex enveloped VLPs composed of multiple structural proteins. Several mammalian cell lines are available for recombinant protein production and are adapted to grow in suspension using serum-free chemically defined media (Wurm 2004). One of the most extensively utilised is the Chinese Hamster Ovary (CHO) cell line. In comparison with other mammalian cell lines, it has the advantage that it is not human-derived and therefore presents a lower risk of contamination by human viruses (Jayapal et al. 2007), maintaining similar glycosolitation patterns to the human cells. This cell line is the most widely used platform for biopharmaceutical production. CHO cells have already been used for the generation of hantavirus-like particles, which were able to induce a specific immune response in mice (Li et al. 2010). The HEK293 cell line is another widely used mammalian production platform, which has been tested for the production of many different types of VLP, such as rabies and influenza (Cervera et al. 2013; Thompson et al. 2013; Fontana et al. 2015). Other human cell lines being evaluated for the production of complex recombinant proteins include CAP-T cells, derived from human amniotic fluid, for HIV-1 VLP production (Gutiérrez-Granados et al. 2016).

In this work, HEK293 cell lines have been used for the production of Gag-based VLPs in order to maximize its production following several approaches. Even though, CHO cell is one of the most used platforms for the production of recombinant proteins, monoclonal antibodies and VLPs (Wurm 2004); this cell line do not efficiently assemble and release HIV-1 VLPs. Gag polyprotein is not able to reach the cell membrane when recombinantly expressed in CHO cells (Chen et al. 2001; Reed et al. 2002). A myristolation in Gag polyprotein is necessary for anchoring to the cell membrane which is not active in the murine CHO cell line (Lindwasser and Resh 2002). On the other hand, HEK293 cell line is able to assemble and release well formed HIV VLPs (Cervera et al., 2013;). This is a very well characterized cell line and a GMP cell bank is available. Furthermore it has been used for the production of viral vector with very promising results (Anderson et al. 2000), and it is compatible with large scale bioreactor processes (Baldi et al. 2005). One of the main drawbacks of HEK293 platform for the production of Gag-based VLPs is the inherent generation of exosomes. These are cell-derived vesicles that play a key role in intercellular signaling and waste management functions among others. Due to their physical characteristics, they are co-purified with the VLPs and may represent the major contaminant of the vaccine preparation (Venereo-Sanchez et al. 2016; Milián et al. 2017). Further purification steps must be carried out in other to remove this contamination. Other approach is the reduction of exosome production in the cellular level in other to diminish the quantity of vesicles in VLP preparations.

3.5 VLP production yields

Comparison of VLP production yields among different systems is not always straightforward, since production is dependent not only on the system but also on the complexity of the VLP. Nevertheless, a wide range of yields can be estimated. As previously discussed, bacteria and yeast are high-concentration production systems, and yields can vary from 0.75 to 700 µg of protein per ml of culture (Leavitt et al. 1985; Schädlich et al. 2009). Animal-based systems achieve lower production yields: between 0.2 and 18 µg/ml in the case of B/IC system (Mortola and Roy 2004; Aucoin et al. 2007) and between 0.018 and 10 µg/ml for mammalian cell technology (Holzer et al. 2003; Taube et al. 2005). Animal cells tend to be lower VLP-producers, but for complex enveloped VLPs, they have become the platform of choice. Transgenic plants are the most difficult to compare with the other

systems, since their production is generally calculated per mg of vegetal tissue, with yields ranging from 4 to 2380 pg/mg of leaf (Warzecha et al. 2003; Huang et al. 2006).

4. VLP production approaches in mammalian cell platforms

There are two different approaches for VLP production in animal cell-based platforms: transient gene expression (TGE) and stable gene expression (SGE). Transient gene expression is based on the episomal expression of a gene of interest (GOI) coded in a plasmid DNA. This methodology is generally used in the beginning of the development of a new biopharmaceutical when several protein candidates need to be tested. It allows obtaining small quantities of the product of interest in a short period of time for carrying out preclinical or product characterization analysis (Baldi et al. 2007; Merten et al. 2014). However, the expression of the GOI is lost over cultivation time and cell growth, since the progeny cells do not carry the plasmid of interest with them. Once the product of interest has been selected, the generation of a stable cell line is carried out for the final production process. In SGE the gene of interest is integrated in the cell genome and consequently expression is not lost over time. There are two approaches for stable cell line generation: random integration and directed integration which will be further explained. SGE is a long process which can take a year; nevertheless, the final production titers tend to be higher than in TGE (Gutiérrez-Granados et al. 2016). Table 2 summarizes the principal differences among the two technologies proposed in this work.

Table 2. Stable gene expression and transient gene expression comparison

	SGE	**TGE**
Genetic selection	Yes	No
Time for product harvesting	3-12 months	Weeks
Specific productivity (pg/(cell*day))	Up to 50	Bellow 10
Volumetric productivity (g/(d*L))	Up to 5	0.02-0.08
Scalability	Up to 20.000 L	Up to 500 L (mainly small scale)
Application	Industrial production at large scale of recombinant proteins	Generally, small scale for research protein production

4.1 Transient gene expression

For TGE, the introduction of the plasmid DNA can be carried out by several physical or chemical methods. Physical methods are based on electroporation of the cells. It has been mainly used at small scale (up to 1 mL) reporting high yields and transfection efficiencies; nevertheless, electroporation has to be carried out in very high cell concentrations and very specific buffers, which turns the overall process more complex (Gutiérrez-Granados et al., 2018). MaxCyte Company is dedicated to cell engineering using Flow Electroporation™ Technology which allows to transfect primary cells, stem cells and cell lines with transfection efficiencies over 90%. On the other hand, there are several chemical agents available for transient transfection such as cationic lipids, calcium phosphate or cationic polymers. Cationic lipids offer very high transfection efficiencies and hence production titers; nevertheless, they are mainly used at small scale, since their high cost makes them not suitable for large-scale production (Jordan et al. 1998). Calcium phosphate transfection method has been widely used over the last 30 years at both small and large scale. It is a cost-effective method that provides high transfection efficiencies (Meissner et al. 2000; Batard et al. 2001). Its main drawback though is its incompatibility with the use of serum-free medium, which is required for the production of biopharmaceuticals for clinical applications (Geisse 2009). Cationic polymers such as polyethylenimine (PEI) have also been extensively used for mammalian cell transfection. PEI was firstly discover to be a good gene transfer agent for cells in culture in experiments *in-vivo* and for this reason it gained importance in gene therapy technologies (Goula et al. 1998).

Polyethylenimine can be used in linear or branched forms and also in different molecular weights and with derivatized chemical groups. 25KDa linear PEI is the most widely used for transfection of HEK293 and CHO cultures (Derouazi et al. 2004; Carpentier et al. 2007) (Figure 5). It is also a cost-effective agent for transient transfection (Tait et al. 2004). PEI cost is around 0.5-1 Euro/mL of transfection solution, which is 650 times lower than the commercial cationic lipid Lipofectamine™ 2000 transfection reagent. PEI can condense DNA through amino-phosphate interactions and form positively charged complexes. The positively charged complexes interact with the negatively charged glycoporteins and proteoglycans in the cell membrane. These complexes protect the DNA from nuclease digestion when it is introduced into the cell (Bieber et al. 2002; Cervera et al.

2017). The cell internalizes the complexes through an endocytosis mechanism and they are incorporated in endosomes in the cytoplasm (Remy 2004). PEI complexes escape from the vesicles afterwards and the DNA can be released; nevertheless, the exact mechanism of this phenomenon is not totally understood. The most highly extended hypothesis is the so-called "proton sponge effect". PEI promotes the endosome osmotic swelling which eventually leads to the disruption of the endosome membrane and intracellular release of the DNA (Schermant et al. 1995).

This DNA needs to enter in the nucleus to be transcribed and further translated into protein. There are two extended theories describing the mechanism of entrance. The first one postulates that the complexes enter into the cell nucleus in the G2 phase of the cell cycle when no nuclear membrane is present (Tait et al. 2004; Monsigny and Fajac 2006). The other proposed hypothesis is that the complexes are transported to the cell nucleus by active transport through the nuclear envelope (Elouahabi and Ruysschaert 2005). DNA staining for complex tracking inside the cell has allowed the detection of the limiting step of the process. All DNA complexes interact with the cell membrane immediately after addition to the cell culture; they are internalized into the cell cytoplasm at 1.5 hours post-transfection (hpt) and arrive to the cell nucleus at 4 hpt. Despite the fact that all the cells uptake the complexes, not all of them arrive to the cell nucleus and hence not all the cells are able to express the GOI. The DNA delivery to the cell nucleus is hence the limiting step of the tranfection process (Cervera et al. 2017).

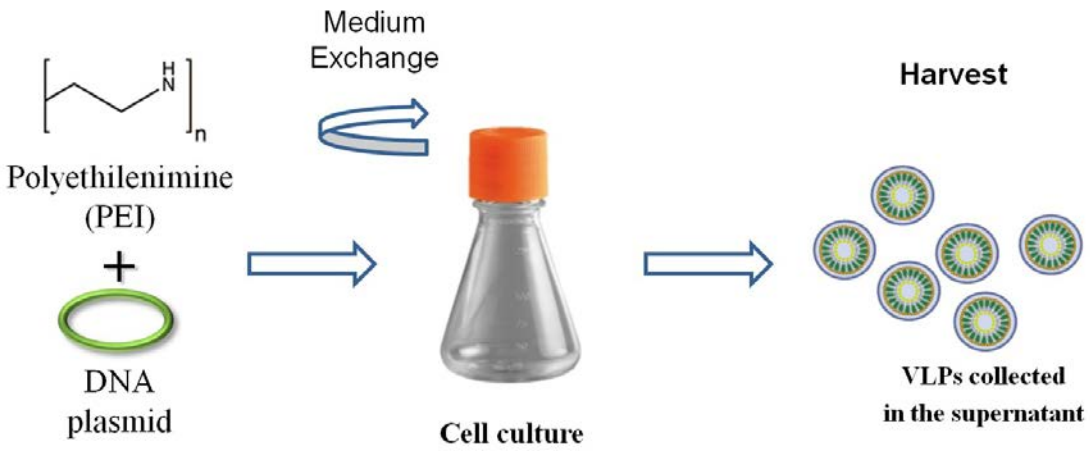

Figure 5. Transient gene expression process.

4.2 Methods for enhancing transient tranfection

Transient transfection is a highly extended method for obtaining sufficient quantities of the product of interest in a short period of time, and its optimization for obtaining higher product titers is therefore of high importance (Baldi et al. 2007; Merten et al. 2014). In this work, several strategies have been tested in order to accomplish this objective.

4.2.1 Origins of replication

One of the main drawbacks of transient transfection is that the DNA is not integrated in the cell genome and there is an episomal expression of the GOI. The plasmid is diluted in every cell division and the expression is lost over time (Middleton and Sugden 1994; Wade-Martins et al. 1999). In order to overcome this problem, two HEK293 cell lines have been developed: HEK293T and HEK293-EBNA1. HEK293T stably expresses the large T antigen from Simian Vacuolating virus 40 (SV40 virus) (Dean et al. 1987). This protein is involved in the transcription and replication of the viral genome. It is able to recognize the SV40 ori and carry out the replication of the DNA. If SV40 origin of replication is present in the plasmid DNA used for HEK293T transfection, the cell will carry out the sustained replication of the DNA (Heinzel et al. 1988). Increasing the amount of gene copies in the cell might lead to an increase in the product titers. In the same way, HEK293-EBNA1 cell line stably expresses the Epstein-Barr virus Nuclear Antigen 1 (EBNA-1) protein from Epstein-Barr virus (EBV) (Baldi et al. 2007; Segura et al. 2007). This protein is related with the gene expression and DNA replication of the viral genome. It binds to the origin of replication OriP and enables the replication of the DNA. Tranfection of HEK293 with a plasmid containing OriP enables the sustained replication of the DNA (Gahn and Schildkraut 1989; Middleton and Sugden 1992). In this work these two cell lines have been compared in terms of specific VLP production rates and growth and viability after transfection. The HEK293 cell line (HEK293SF-3F6) that has been widely used in the research group and which has a GMP cell bank was used as a control. This cell line does not stably expressing any viral protein has been used to compare with the other two.

4.2.2 PEI:DNA concentration optimization

PEI:DNA complexes have specific size characteristics that affect their intrinsic capacity for cell transfection (Choosakoonkriang et al. 2003). Several factors affect the physical properties of the complexes: the time of DNA and PEI interaction, the medium where the complexes are formed, PEI and DNA concentrations, and ratio among others. PEI and DNA concentration optimization have been widely studied for improving HEK293 transfection (Cervera et al. 2015a).

Transfection conditions, such as DNA and PEI concentrations, may vary for every HEK293; complexes physical characteristics may not be the most appropriate for each cell line. PEI:DNA complexes are nanostructures of 300 nm that quickly undergo aggregation. They are compacted and highly concentrated and their intrinsic characteristics directly affect transfetion efficiency of HEK293 cell cultures (unpublished results).

The DNA employed in each transfection can vary in the GOI or even in the plasmid backbone. When several recombinant proteins need to be produced, the plasmid DNA used for cell transfection need to be changed. DNA and PEI optimal concentrations might vary if the plasmid DNA differs in sequence, in CG content or even in size.

4.2.3 Extended gene expression

The extended gene expression (EGE) protocol was previously developed for prolonging the production phase of TGE (Cervera et al. 2015b). EGE consists on the transfection of a cell culture followed by several medium exchanges and retransfections. Every 48 hours, complete medium exchange is performed so the cells are provided with fresh nutrients and the product of interest can be removed from the culture broth. This enables to increase the duration of the cell culture, and can be considered a pseudo-perfusion system. Medium exchanges are combined with the retransfection of the cell culture. After the initial transfection, there are two different cell populations. The first one is composed of the transfected cells, which are the ones expressing the GOI. The second one is formed by the non-transfected cells. In EGE protocol, retransfections are carried out at 48 and 96 hpt in order to provide the non-transfected cells with plasmid copies so that they can start expressing the GOI. The extended gene expression leads to a 12-fold

improvement in Gag-based VLP production. It has also reported good results with intracellular and secretory reporter proteins (Cervera et al. 2015b).

4.2.4 Chemical additives

Mammalian cells produce relatively low protein titers compared with other biological systems (Roldao et al. 2010). Transient transfection production titers can be increased by the use of chemical additives. Chemical additives have been widely used for enhancing TGE in several animal cell lines and for the production of different proteins, viruses, and vaccines (Suzuki and Ollis 1990; Tait et al. 2004; Backliwal et al. 2008d; Ye et al. 2009). The production of Gag-based VLPs by TGE has been optimized with the supplementation of the cell medium with lithium acetate, caffeine and valproic acid (Cervera et al. 2015a).

Lithium acetate has shown to improve transfection efficiency due to its capacity for creating porous in the cell membrane which is meant to favor the entrance of the DNA:PEI complexes into the cell (Schiestl and Gietz 1989). Since it is a transfection enhancer, it has to be added prior to transfection in order to have an effect. Valproic acid has a histone deacetylase inhibiting effect (Backliwal et al. 2008d). Histone deacetylases (HDAC) are a class of enzymes that eliminate the acetyl groups from the lysine residues present in the histones providing them with a positive charge (Khochbin et al. 2001). This positive charge helps the DNA to join to the histones. When the DNA is packed with histones, transcription is prevented and also gene expression. Valproic acid forces the detachment of the DNA from the histones. DNA is hence free to be transcribed and the expression of the transgene is enhanced. Caffeine has a phosphodiesterase (PDE) inhibition effect (Choi et al. 1988). This class of enzymes is involved in the inactivation of intracellular cAMP and cGMP. This inactivation leads to the enhancement of recombinant protein production (Ellis et al. 2011). Caffeine and valproic acid are added after transfection in order to not interfere with the transfection process.

Previous work has demonstrated that the combination of lithium acetate (20 mM), valproic acid (3.36 mM) and caffeine (5.04 mM) leads to a 4-fold improvement in VLP production in HEK293 cell line. Nevertheless, the use of these chemical additives provokes the cell arrest in phase G1, inhibiting cell proliferation and decreasing cell viability (Roy et al. 2005).

4.2.5 Gene inhibitors

Valproic acid and caffeine interfere the catalytic action of specific groups of enzymes. Furthermore, they also alter other cellular processes such as cell proliferation; they have hence a wide action over several cellular routes. These processes can be also disrupted at a genetic level. The use of interference RNA (iRNA) against specific enzymes can provide the same biologic effect but with a higher specificity; not altering other important cellular mechanisms.

Transgene expression is regulated by many different cellular proteins which have a function at different levels: transcription, translation, protein folding, secretion… etc. The control of the genes encoding for these regulatory proteins can lead to transgene expression regulation. It has been previously discussed that valproic acid inhibits HDAC group of enzymes while caffeine inhibits PDE group of enzymes. It has been demonstrated that the use of miR-2861, which is responsible for HDAC5 inhibition, led to an increase in the expression of the recombinant protein SEAP and IgG by 1.5-fold in stable CHO producer cell lines (Fischer et al. 2015). shRNA are small pieces of DNA that, upon transcription, form a harping. This harping is processed firstly by the enzyme DICER resulting in ssRNA which is complementary to the gene to interfere. These ssRNAs bind the mRNA and the RNA-induced silencing complex (RISC) cuts them. mRNA translation is then inhibited and the protein is down-regulated (Lowe 2014).

4.3 Stable gene expression

Stable gene expression (SGE) consists on the generation of a cell line in which the GOI is constitutively expressed (Figure 6). It is widely used at industrial scale when the product of interest has been already selected and characterized (Wurm 2004; Lai et al. 2013). The process starts in the same way as TGE. A cell culture is transfected using a DNA plasmid (which can be circular or linearlized) and a transfection agent. The plasmid DNA codes for the GOI and also for a selection marker and it is integrated in the cell genome by a low probability event. This incorporation can be random or directed. In the random integration, the transgene is incorporated randomly in the cell genome and the number of copies integrated and the locus of integration can vary from cell to cell, so the cell population tends to be more heterogeneous. On the other hand, in the directed

integration, the transgene is incorporated in a specific region of the genome and generally only one copy is integrated. The locus of integration selected is generally a region of the genome of high transcription, where it is known that the transgene will not be silenced. There are three methodologies available for the directed gene integration: zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats associated with Cas9 system (CRISPR/Cas9) (Hsu et al. 2014b). All of them rely on a restriction enzyme for introducing double stranded breaks in the genome at specific locations.

The selection gene provides an advantageous phenotype against the non-expressing cells. It is generally based on an antibiotic resistance, but there are also some other systems based on a specific compound consumption. After transfection, the selection marker is added to the cell culture in order to increase the selective pressure among the cells that are expressing the resistance gene. The population is hence enriched in producer cells and a stable cell pool is generated. The specific production rates of each cell might be very different and it is highly relevant to generate and select a stable monoclonal cell line based on cell growth specific production titers. Finally, the stability of the expression of GOI in genome of the selected clone is tested (Jostock 2011; Lai et al. 2013).

The generation of a clonal cell line starts by the separation of the cells one by one. Traditionally, cells can be sorted by dilution into 96-well plates; then the wells where only one cell has been incorporated are selected. This is a very labour intensive process which can limit the number of clones to be isolated, and hence reduce the probabilities of identifying the more productive ones. More recently, the sorting technology has simplified the process. This separation platform automatically sorts the cells one by one. Furthermore, it can be coupled with a fluorescent laser which can recognize and sort the cells by their intrinsic fluorescence. This is particularly interesting when the GOI codes for a reporter protein or the protein of interest has been fused to a fluorescence protein (Priola et al. 2016).

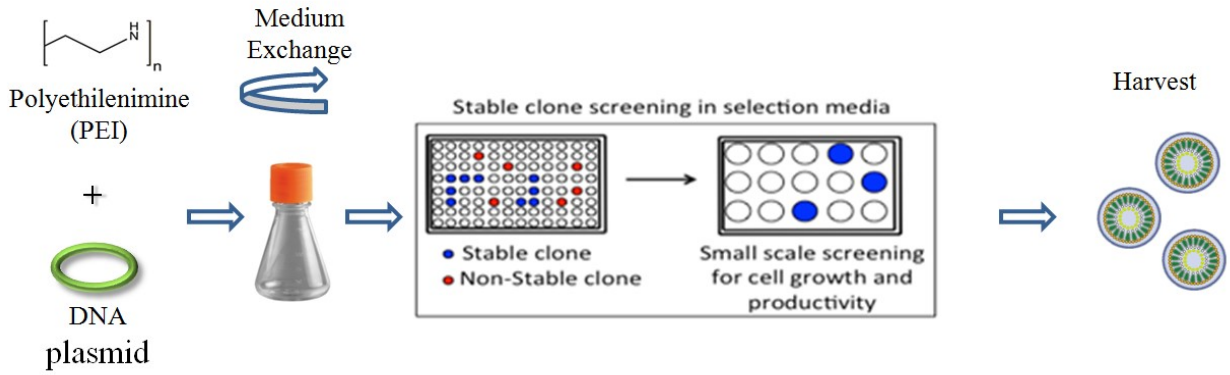

Figure 6. Stable cell line generation process.

After the clonal selection, cells are grown and screened. The clonal cell lines that present higher growth rates and higher production titers are selected and further characterized. The success in finding a high producer cell line depends on the number of clones analyzed. In industrial cell line generation, this is an automated process which allows the screening of a large amount of clones and hence the probabilities of finding a high producer cell are increased. Despite the whole clonal population comes from a single cell, regular mutations in cell division provoke the increase in heterogeneity.

TGE at bioreactor scale

TGE is generally utilized at small laboratory scale; nevertheless, the application of TGE to large industrial bioreactor scale is gaining importance in the recent years. In this context it is relevant to take into consideration that many of the growth media that are available for mammalian cell growth are not compatible with transfection (Gutiérrez-Granados et al., 2018). Many of the ones that are compatible require a complete medium exchange prior to transfection for improving transfection efficiency (Backliwal et al. 2008c; Ye et al. 2009; Rajendra et al. 2011; Rajendra et al. 2015). At small scale, the complete medium exchange is carried out by centrifugation; with larger volumes, this procedure tends to be tedious, involves high contamination risk and detrimental for cell growth. The use of other media that do not require a medium exchange before transfection has been previously explored (Gutiérrez-Granados et al. 2017). Furthermore, there are several intrinsic challenges of TGE scale-up to bioreactor with large volumes such as medium

replacement, large-scale high-quality plasmid production, DNA:PEI complexes formation, inoculums preparation and cell density for transfection (Gutiérrez-Granados et al., 2018).

Extended gene expression is based on repeated transfections and medium exchanges, becoming a pseudo-perfusion system. In order to scale up EGE to higher volumes in bioreactor, centrifugations for medium exchange cannot be considered. As EGE allows for obtaining a high amount of vaccine in a short period of time and the product of interest can be stored at adequate temperature conditions (which is very important for VLP generation), it is of great interest to study this novel methodology at the bioreactor scale, which will certainly be a step forward for its potential application in VLP manufacturing.

There are many technologies available for perfusion such as sedimentation (inclined settler, acoustic settler), filtration (filtration by tangential flow or cross-flow filer (i.e. hollow fiber filter (HF)), spin-filter, and crossflow micro filtration (Shirgaonkar et al. 2004). Among them, tangential flow filtration (TFF) is very utilized and well-established technology that has been used for the production of recombinant proteins using several cell types and at different production scales. Opposite to conventional filtration, in which the culture flows in orthogonal direction to the filter surface, in TFF the cell suspension flows tangentially to the membrane, which potentially reduces the membrane fouling. Alternating flow filtration (ATF) uses the same technology as TFF; nevertheless, the cell broth flow direction is constantly alternated using a diaphragm pump, which reduces the shear stress applied over the cells. Furthermore, the back flash performed in ATF further reduces the probability of membrane clogging (Carin et al. 2013).

The acoustic filter is based on sedimentation technology and has been widely used for perfusion at laboratory and industrial scale. It is composed of a chamber with a transducer and a reflector. The transducer is able to generate a standing wave which is then reflected in the opposite direction by the reflector. These two waves generate nodes in which the cells are trapped and sediment and the clear medium can easily flow through (Shirgaonkar et al. 2004). The medium is constantly removed and the product of interest can be continuously stored under the appropriate conditions, especially when the product of interest is thermolabile (Clincke et al. 2013; Carin et al. 2013). This perfusion system has been selected for the bioreactor scale-up of the extended gene expression for the production of Gag-based VLPs in HEK293 cells.

In this work, several approaches have been tested for TGE optimization for the production of Gag-based VLPs in HEK293 cell cultures. Gag was previously fused with the fluorescent protein GFP in order to simplify detection and quantification. First, the extended-gene expression strategy has been combined with the use of chemical additives for maximizing VLP production. Furthermore, the use of specific gene inhibitors has been found to be a good substitutive for chemical additive supplementation. Second, extended gene expression has also been scaled-up to a bioreactor carrying out perfusion mode and using the acoustic filtration technology. Third, HEK293 cell lines (EBNA-1 and T) have been compared with the standard HEK293SF-3F6 for the production of VLPs in terms of transfection efficiency, cell growth, viability, and production titers. Finally, a HEK293 stable cell line has been developed by random gene integration for the production of GagGFP VLPs.

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Objectives

The main objective of this work was the enhancement of HIV-1 virus-like particles in HEK293 cell systems using transient and stable gene expression. In more detail, the following specific objectives can be outlined:

1. Combine the previously developed extended gene expression protocol with the supplementation with chemical additives for maximizing HIV-1 VLP production and select shRNAs for substituting the chemical additives used and obtain similar HIV-1 VLP titers.

2. Perform the extended gene expression protocol at bioreactor scale using the acoustic filtration technology for perfusion.

3. Optimize the DNA and PEI concentration in TGE using three different HEK293 cell lines and three different plasmids. Using the optimized TGE protocol, compare the nine systems presented in terms of cell growth, viability, transfection efficiency and HIV-1 VLP production.

4. Generate a HEK293 stable cell line for HIV-1 VLP production using the random integration method.

Results

Enhancement of HIV-1 VLP production using gene inhibition strategies

Results, chapter 1

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Abstract

Gag polyprotein from HIV-1 is able to generate virus-like particles (VLPs) when recombinantly expressed in animal cell platforms. HIV-1 VLP production in HEK293 cells can be improved by the use of different strategies for increasing product titers. One of them is the so-called extended gene expression (EGE), based on repeated medium exchanges and retransfections of the cell culture to prolong the production phase. Another approach is the media supplementation with gene expression enhancers such as valproic acid and caffeine, despite their detrimental effect on cell viability. Valproic acid is a histone deacetylase inhibitor while caffeine has a phosphodiesterase inhibition effect. Here the combination of the EGE protocol with additive supplementation to maximize VLP production is first tested. As an alternative to the direct additive supplementation, the replacement of these chemical additives by iRNA for obtaining the same inhibition action is also tested. The combination of the EGE protocol with caffeine and valproic acid supplementation resulted in a 1.5-fold improvement in HIV-1 VLP production compared with the EGE protocol alone, representing an overall 18-fold improvement over conventional batch cultivation. shRNAs encoded in the expression vector were tested to substitute valproic acid and caffeine. This novel strategy enhanced VLP production by 2.3 fold without any detrimental effect on cell viability (91.7%) compared with the batch cultivation (92.0%). Finally, the combination of shRNA with EGE resulted in more than 15.6-fold improvement compared with the batch standard protocol traditionally used. The methodology developed enables the production of high titers of HIV-1 VLPs avoiding the toxic effects of additives.

Keywords: Virus-like particles, transient transfection, gene inhibition, extended gene expression, vaccines

Introduction

Biopharmaceutical production in mammalian cell platforms has been gaining relevance over the last years (Roldao et al. 2010). Mammalian cells are able to perform accurate post-translational modifications (PTMs) which are very important in the function and the effect of the biopharmaceuticals (Zhu 2012). Mammalian cell technology has the main disadvantage that product titers are not as high as other biological production platforms, such as baculovirus-insect cell (B/IC) technology or yeast cells (Roldao et al. 2010). The improvement of these product titers is hence of big interest. Virus-like particles (VLPs) are nanostructures that resemble the conformation of a virus. These particles are a promising platform for the development of new vaccines due to the similarity of structure to the wild virus but not containing any viral DNA which makes them non-infective and then safer for both operators and vaccine recipients (Fuenmayor et al. 2017b). The gene of interest in this work is the Gag polyprotein from HIV-1. This polyprotein has the ability to travel to the vicinity of the cell membrane when recombinantly expressed and bud from the cell leading to enveloped VLPs production (Cervera et al. 2013).

Transient gene expression (TGE) is a cost-effective and fast process for recombinant protein production in mammalian cells. TGE is a very useful tool especially in early stages of the development when many different variants need to be tested. TGE allows the rapid production of sufficient product quantities to carry out these tests and also structural and functional assays (Baldi et al. 2007). Nevertheless, the transgene does not integrate into the cell genome and hence the expression is lost over time due to dilution associated with cell division (Middleton and Sugden 1994; Wade-Martins et al. 1999).

In order to overcome this limitation, the so-called extended gene expression (EGE) protocol was developed (Cervera et al. 2015b). This protocol consists in the retransfection of the cell culture at 48 and 96 hours post transfection (hpt). By doing this, non-transfected cells are likely to receive the plasmid DNA and start producing the recombinant protein and so the production phase can be prolonged. Complete medium exchanges are also carried out every 48 hours in order to provide the cell culture with nutrients and also remove both the product of interest and waste products.

As previously discussed, mammalian cell production platforms have the main drawback of lower production titers when compared to other systems. Many additives have been used to either improve transfection efficiencies (lithium acetate, DMSO, nocodazole) (Tait et al. 2004; Ye et al. 2009) or production (valproic acid, butyric acid, caffeine, trichostatin A) (Suzuki and Ollis 1990; Tait et al. 2004; Backliwal et al. 2008a; Backliwal et al. 2008b; Ellis et al. 2011). In previous work, a screening of several transfection and production enhancers was done for the production of Gag-based VLPs in HEK293 cell (Cervera et al. 2015a). The optimal condition found was based on a combination of lithium acetate, caffeine, and valproic acid, providing a 4-fold improvement in VLP production.

Lithium acetate has been shown to improve transfection efficiency due to its capacity for creating porous in the cell membrane which is meant to help the entrance of the DNA:PEI complexes to the cell nucleus (Schiestl and Gietz 1989). Valproic acid has a histone deacetylase inhibiting effect on the cell (Backliwal et al. 2008d). Histone deacetylases (HDAC) are a class of enzymes that eliminate the acetyl groups from the lysine residues present in the histones which give them a positive charge (Khochbin et al. 2001). This positive charge helps histones in the attachment to the DNA. As a result, chromatin becomes more compact and hence DNA transcription is prevented. The use of HDAC inhibitors prevents the deacetylation of the histones which reduces the interaction between histones and DNA. As a consequence, chromatin remains open and transcription can be enhanced. HDAC inhibitors also arrest cell culture in the production phase (G1) inhibiting cell division (Roy et al. 2005). Caffeine has a phosphodiesterase (PDE) inhibition effect (Choi et al. 1988). PDE are a class of enzymes involved in the inactivation of the intracellular cAMP and cGMP. PDE inactivation has been demonstrated to have a positive effect on recombinant protein production (Ellis et al. 2011). Specific compound addition to the cell culture enables an increase in recombinant protein production in TGE. However it also increases the production costs not only because of the cost of supplements but also for the additional purification steps.

This work has two main objectives. First, the combination of EGE protocol with additive supplementation in order to maximize HIV-1 VLP production. Second, the use of small interfering RNA (siRNA) is proposed as an alternative to the use of additives for enhancing production of VLPs in HEK293 cells using TGE or EGE. siRNA can be directly introduced to the cell culture and provoke a direct effect on gene silencing, or they can be

encoded in a plasmid DNA, in this case, they are called short harping RNA (shRNA). shRNAs are transcribed and then processed by the enzyme DICER resulting in a ssRNA which is complementary to the gene to interfere. These ssRNAs bind the mRNA and the RNA-induced silencing complex (RISC) cuts them preventing their translation. At the end, a knock-down for the specific protein is achieved (Lowe 2014).

In this work plasmid DNA containing the gene of interest has been modified to incorporate specific shRNA against HDAC5 and PDE8A. Both shRNAs have been cloned in the same plasmid as the GagGFP gene in order to make the process simple and comparable to the usual transient transfection protocol.

Materials and Methods

Cell line, media and culture conditions

The cell line used in this work was a serum-free suspension-adapted HEK293 cell line called HEK293SF-3F6 (kindly provided by Dr. Amine Kamen from the BRI of National Research Council of Canada and McGill University (Montreal, Canada)). The cell line was derived from a cGMP master cell bank that is available for manufacturing of clinical material. Cells were cultured in Freestyle 293 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% Pluronic® (Invitrogen, Carlsbad, CA, USA). Medium was also supplemented with 1.6 mg/L of r-transferrin (Merck Millipore, Kankakee, IL, USA), 19.8 mg/L of r-insulin (FeF Chemicals/Novo Nordisk, Køge, Denmark.) and 0.9X of an in-house lipid mixture to maximize cell growth and productivity (Cervera et al. 2013). Cells were routinely maintained in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL of culture medium. Flasks were shaken at 110 rpm using an orbital shaker (Stuart, Stone, UK) placed in an incubator maintained at 37ºC in a humidified atmosphere of 5% CO_2 in air. Cell count and viability were determined using Nucleocounter NC-3000 (Chemometec, Allerod, Denmark).

Plasmids and shRNAs

The pControl plasmid encodes for a Rev-independent HIV-1 Gag protein which was previously fused in frame to the enhanced GFP (Hermida-Matsumoto and Resh 2000) to

allow easy quantification of the produced VLPs. pControl was cloned, prepared and purified as previously described (Cervera et al. 2015a). The pControl plasmid was modified in order to obtain pshRNA. U6 promoter plus each of the shRNAs were obtained from GenScript (Nanjing, China) and then cloned into the pControl before the CMV promoter (Figure 1). The specific shRNA sequences are summarized in Table 1.

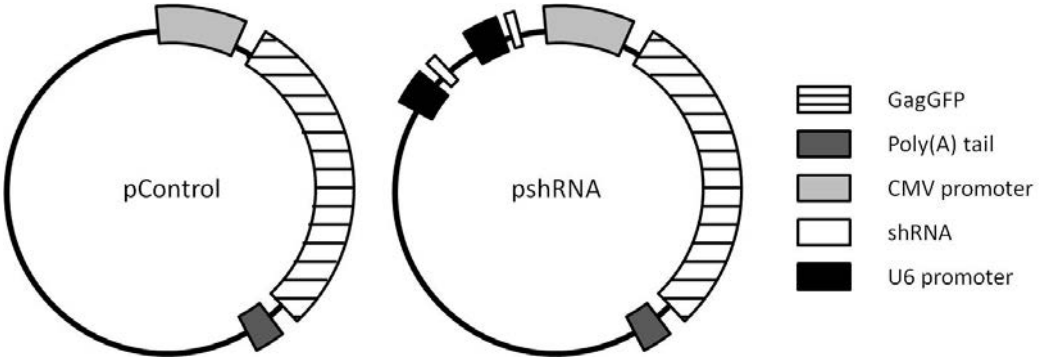

Figure 1. Expression vectors. pControl is formed by the GagGFP gene; in the pshRNA two U6 promoters plus two shRNA have been added.

Table 1. HDAC5 and PDE8A shRNA sequences.

Gene	shRNA
HDAC5	5'CCGGGCCGGGTTTGATGCTGTTGAACTCGAGTTCAACAGCATCAAACCCGGCTTTTT 3'
PDE8A	5'TGCTGTTGACAGTGAGCGAGCTAAGATCATGGTTACAAATTAGTGAAGCCACAGATGTAATTTGTA ACCATGATCTTAGCGTGCCTACTGCCTCGGATTTTT 3'

Transient transfection

HEK293 suspension cells were usually grown to reach a cell concentration of $2x10^6$ cells/mL and were transiently transfected using 25-kDa linear polyethyleneimine (PEI) (PolySciences, Warrington, PA, USA). Prior to transfection, a complete medium exchange was performed by centrifuging the cell culture at 300xg for 5 minutes. Cells were resuspended in fresh supplemented FreeStyle medium. Transfections were performed using 1 µg/mL of plasmid DNA and 2 µg/mL of PEI. PEI:DNA complexes were formed by adding PEI to plasmid DNA diluted in fresh culture media (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 prior to its addition to the cell culture. When production

enhancers were added to cell culture, lithium acetate (20 mM) was added 3 hours prior transfection and valproic acid (3.36 mM) and caffeine (5.04 mM) were added 4 hpt (Cervera et al. 2015a). The percentage of GFP-positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) at different hpt.

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×g for 5 min. Green fluorescence was measured at room temperature using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) set as follows: λex= 488 nm (slit 5 nm), λem= 510 nm (slit 10 nm). Relative fluorescence units 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 GagGFP concentration is divided by the molecular weight of a GagGFP monomer (84 kDa) and then divided by the number of monomers that a VLP contains (2500 monomers/VLP) (Gutiérrez-Granados et al. 2013).

Cell cycle analysis

Freshly harvested cells were centrifuged at 300g for 5 minutes, re-suspended in PBS and fixed with cold ethanol (70%) for at least 2 h at -20°C. In order to carry out cell cycle

analysis, cells were stained with propidium iodide (PI) (Sigma, Saint Louis, MO, USA) for 30 min at room temperature before data acquisition using FACSCalibur (BD Biosciences, San Jose, CA, USA). The ModFit software (Verity Software House, Topsham, ME, USA) was used for data analysis.

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, Biosciences, San Jose, CA, USA). 75000 cells were sorted for each population and subsequently centrifuged at 300g for 5 minutes, re-suspended in PBS and stored at -20ºC. 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). 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.

Statistical analyses

All the protocols developed where 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.

Results

Extended gene expression with additive supplementation

EGE procedure, based on periodic medium exchanges and retransfections of the cell culture, provided a 12-fold improvement in HIV-1 VLP production in HEK293 cells (Cervera et al. 2015b). On the other hand, the addition of valproic acid, caffeine and lithium acetate

to the cell culture provided a 4-fold improvement in VLP production (Cervera et al. 2015a). In this section, the possibility of combining both protocols was explored in order to maximize VLP production.

To do so, HEK293 cells were transfected following the standard EGE procedure with pControl plasmid, but in every retransfection, the medium was supplemented. Lithium acetate was added to the cell culture 3 hours prior transfection in order to give enough time to have an effect over the cells. Valproic acid and caffeine were added 4 hours post transfection to let the DNA complexes get into the cells. In the steps where no retransfection was required, only valproic acid and caffeine were added at the same time as the medium replacement. This procedure is named EGE+Add in the rest of the manuscript. For clarity, all the protocols used in this work are summarized in Tables 2 and 3.

In order to make a comprehensive comparison of both systems, accumulated VLPs in the supernatant were calculated (this is the sum of VLPs quantified in the supernatant from 0hpt to 240hpt). As it is shown in Figure 2A, EGE+Add ends up with a 1.5-fold improvement compared with the standard EGE. This means, that in comparison with the most conventional process of batch cultivation for VLP production, an 18-fold improvement can be achieved by the use of a combination of EGE protocol with additive supplementation. Figure 2B shows VLP concentration in the supernatant for the EGE+Add protocol compared with the standard EGE. VLP production remains higher for EGE+Add compared with the control for more than half of the cultivation. Nevertheless, after 144 hpt EGE control protocol produced higher amounts of VLPs than EGE+Add. The improvement was considerably lower at the end of the experiment compared with the initial phase. Additive supplementation is known to have cell cycle arrest consequences. Retransfection efficiency could be influenced by the presence of additives in the culture media. Additive supplementation has hence a detrimental effect over cell density. In the EGE condition, cell density reached $7.5x10^6$ cells/mL compared with $1.1x10^6$ cells/mL reached in the EGE+Add condition. Furthermore, in EGE+Add the cell density tended to diminished trough all the cultivation which means a loss in number of cells in every medium exchange. Cell viability was 60% at the end of the cultivation in both protocols. The effect of additives on cell viability was not clearly observable when medium exchanges were carried out since cell debris and dead cells can be removed and not accumulated during the cultivation.

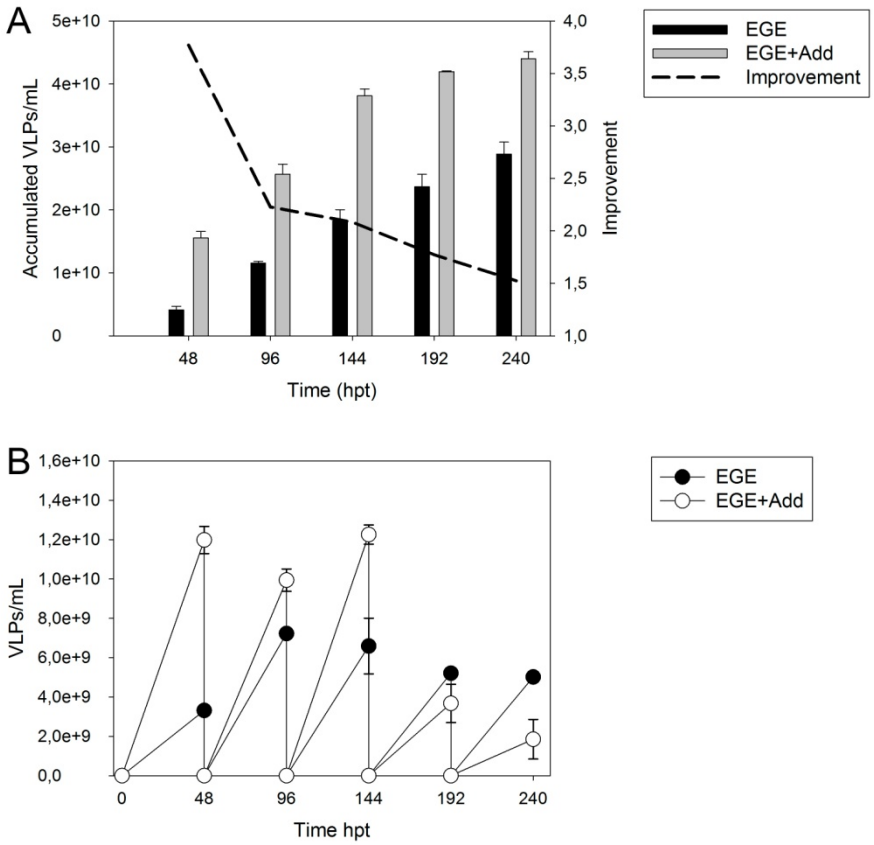

Figure 2. A GagGFP VLP accumulated production of the EGE Control and the EGE+Add protocols and the improvement of the EGE+Add over the EGE Control. B GagGFP VLP production of the EGE Control and the EGE+Add protocols. All the transfection were carried out with pControl plasmid.

Valproic acid enhances recombinant protein production since it produces the detachment between histones and DNA, provoking chromatin decondensation. The cell cycle is stopped and cell culture is arrested in G1 phase. In EGE+Add experiment, cell growth diminished from $2x10^6$ to $0.8x10^6$ cells/mL during the cultivation while in the standard EGE protocol cell density increased from $2x10^6$ to $5.3x10^6$ cells/mL. It has been reported that the best cell cycle phase for transfection success is G2/M (Tait et al. 2004; Liu et al. 2008) since there is no nuclear membrane and the DNA:PEI complexes can reach easier the condensed chromatin. In EGE+Add protocol, cell cycle could be arrested in phase G1 which is the best for DNA transcription but not for transfection. Therefore, the relevance of retransfecting the cell culture at 48 and 96 hpt should to be tested.

Retransfection requirement in EGE+Add protocol

A cell culture triplicate was transfected with pControl plasmid following EGE+Add procedure. In parallel, a triplicate was transfected using the ME+Add protocol. This protocol consists of the transfection of a cell culture and the supplementation of medium. Medium exchanges are carried out in the same way as in the EGE+Add protocol, but in this case, no retransfections are performed (Table 2).

Table 2. Summary of the EGE-based protocols tested and their specific VLP production and the improvement obtained by each of them compared with the standard EGE. The improvement given by all the protocols developed is statistically significant (*p*-value <0.05)

Protocol name	Description	VLPs/mL	Improvement
Standard EGE	• Extended gene expression. After the initial transfection, medium is exchanged every 48hpt. Retransfections are carried out at 48 and 96 hpt	2.89E10	1
EGE+Add	• Extended gene expression. After the initial transfection, medium is exchanged every 48hpt. Retransfections are carried out at 48 and 96 hpt. Lithium acetate is added 3 hours prior each transfection and caffeine and valproic acid are added 4 hours after each transfection	4.45E10 (*p*-value<0.01)	1.5
ME+Add	• After the initial transfection, medium is exchanged every 48 hpt. Lithium acetate is added 3 hours prior transfection and caffeine and valproic acid are added 4 hpt. In every medium exchange, caffeine and valproic acid are added to the culture medium.	3.97E10 (*p*-value<0.02)	1.4
EGE+shRNA	• Standard extended gene expression where two shRNA have been included into the DNA plasmid sequence	3.88E10 (*p*-value<0.02)	1.3

Figure 3A shows that there were no remarkable differences in terms of VLP production between EGE+Add and ME+Add protocols. This means that retransfections are not crucial for enhancing the production of VLPs when the media has been supplemented with lithium acetate, valproic acid and caffeine. Cell cycle was analyzed in order to corroborate the cell cycle arrest. As it is shown in Figure 3B, the use of additives provoked an increment in the percentage of cells in G1/G0 phase of the cell cycle, which affects directly transfection efficiency of the cell culture. ME+Add protocol performed similar VLP productions but no retransfections were performed and was hence selected over the

EGE+Add protocol, which could be discarded in further experiments. In terms of cell density and viability, both protocols followed the same trends.

As it was mentioned before, valproic acid provokes an inhibition of HDAC group of enzymes; while caffeine has the same effect on PDE enzymes. The additives had the detrimental effect of the cell cycle arrest and consequently diminished the transfection capacity of the cells in EGE+Add procedure. shRNA coded in the transfection vector could have the same effect as the additives and act only among transfected cells, leaving the non-transfected population available for transfection. shRNA could be a good alternative for additive supplementation in EGE procedure, since no cell cycle arrest would happen and non-transfected cell could acquire the plasmid in the further retransfections.

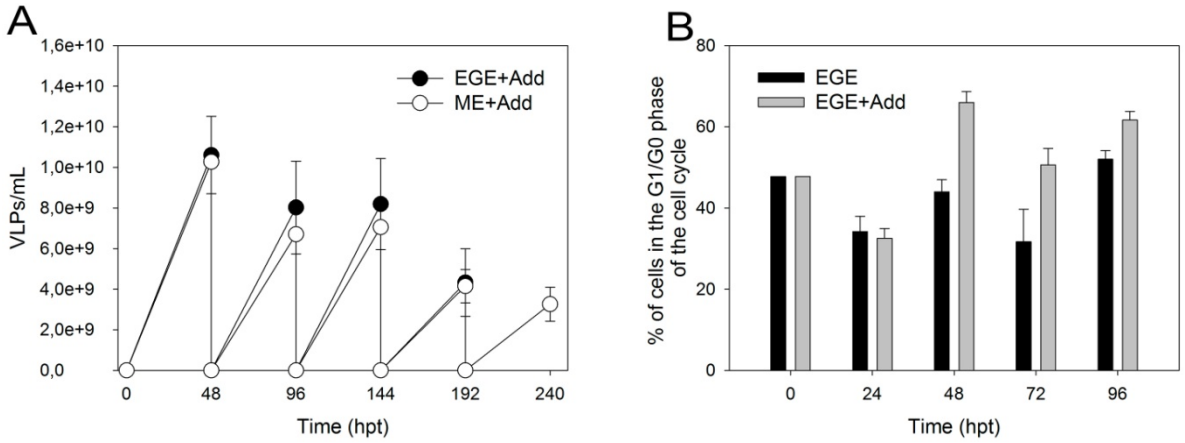

Figure 3. A GagGFP VLP production of the EGE+Add protocol compared with the ME+Add protocol. Cell cycle analysis (G1/G0 phase) of the EGE and the EGE+Add protocols. B All the transfection were carried out with pControl plasmid.

Utilization of shRNAs for the substitution of media additives in batch culture

As it was previously discussed, shRNA could be used in EGE procedure as an alternative to additive supplementation. First of all, shRNA coded in the transfection vector were tested in batch cultivation in order to analyze if a considerable increase in VLP production could be achieved. pGagGFP was the reference plasmid were GagGFP polyprotein had been cloned (Hermida-Matsumoto and Resh 2000). In order to obtain the silencing of the mentioned genes, U6 promoters followed by HDAC5 and PDE8A shRNA sequences were added in the plasmid construction. Figure 1 shows the plasmid generated to evaluate gene silencing as well as the control plasmid. HEK293 cell cultures were

transfected using each one of the plasmids, as well as a transfected culture supplemented with additives (Table 3).

Table 3. Summary of the TGE-based protocols tested and their specific VLP production and the improvement obtained by each of them compared with the standard transfection. The improvement given by all the protocols developed is statistically significant (*p*-value <0.05).

Protocol name	Description	VLPs/mL	Improvement
Standard transfection	• Batch standard PEI-based transfection	3.52E9	1
Add	• Batch transfection where lithium acetate is added to the cell culture 3 hours prior transfection and caffeine and valproic acid are added 4 hpt	1.57E10 (*p*.value<0.01)	4.5
shRNA	• Batch standard protocol where two shRNA have been included into the DNA plasmid sequence	9.27E9 (*p*.value<0.02)	2.6

Figure 4A shows the production of VLPs over time for the three conditions tested. Figure 4B shows the improvement under the different conditions in respect to control condition. Additive supplementation of the medium resulted in 4.5-fold improvement, confirming results from previous work (Cervera et al. 2015a). Cell cultures transfected with pshRNA resulted in more than a 2.6-fold improvement in respect to the control condition, with an obvious advantage of not requiring additive supplementation.

Control culture experienced some growth after transfection reaching $6.5x10^6$ cells/mL and a viability of 92% at 72 hpt. Comparable results were obtained with the shRNA condition where cell viability remained high and cell growth was not stopped. Conversely, when media was supplemented with the additives, cell growth was stopped and viability dropped faster than in the other conditions. At 72 hpt, the viability of the cell culture was 79.5% and cell density $2.9x10^6$ cells/mL.

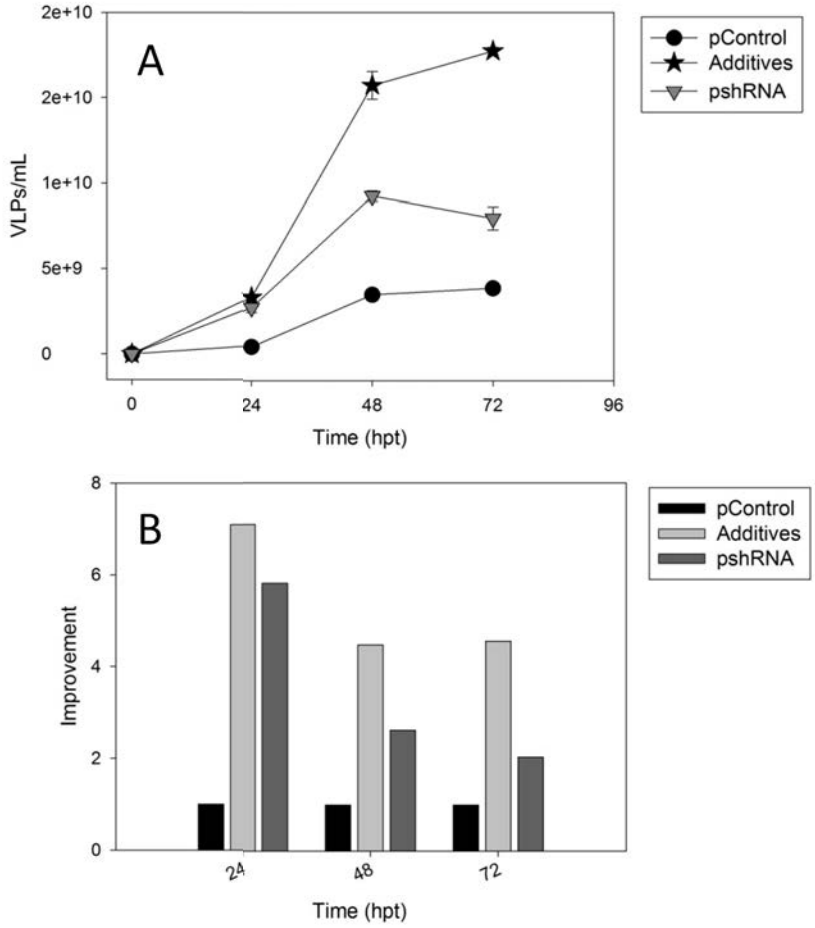

Figure 4. A GagGFP VLP production using pControl plasmid, the additives protocol and the pshRNA plasmid. B Improvement of the pshRNA and the additives protocol over the pControl expression vector.

Gene expression analyses were carried out to corroborate the shRNA effect on HDAC5 and PDE8A genes. Samples were taken from shRNA and control conditions at 48 hpt. Cells were sorted into transfected and non-transfected populations, in order to compare GFP positive and GFP negative cells from the two conditions studied. There were not significant differences between the two GFP negative populations in terms of relative HDAC5 and PDE8A mRNA levels. In the case of GFP positive cells, significant 67% inhibition was found in HDAC5 gene when comparing the standard transfection condition with the shRNA condition (p-value<0.01); nevertheless, non-significant inhibition was found in the case of the PDE8A gene. Consequently, HDAC5 shRNA inhibition is the main responsible in the increase observed in GagGFP production. Interfering HDAC5 gene has a big impact in recombinant protein production. The fact that PDE8A was not inhibited may have caused that the increase of the VLP production was not as high as when the culture was

supplemented with the additives. Caffeine specific effect over VLP production was analyzed in order to corroborate this hypothesis.

Caffeine addition evaluation in pshRNA

A cell culture triplicate was transfected with the pshRNA and caffeine was added to the medium 4 hours post transfection. This condition was compared with a standard additive protocol condition and also with a cell culture triplicate

transfected with pshRNA. Figure 5 shows the VLP production of the three protocols tested. pshRNA plus caffeine reaches comparable VLP concentrations than the additive condition, demonstrating that the fact that PDE8A was not inhibited was the cause for not reaching higher VLP production titers. Other shRNAs against PDE8A should be found and tested for further enhancing VLP production.

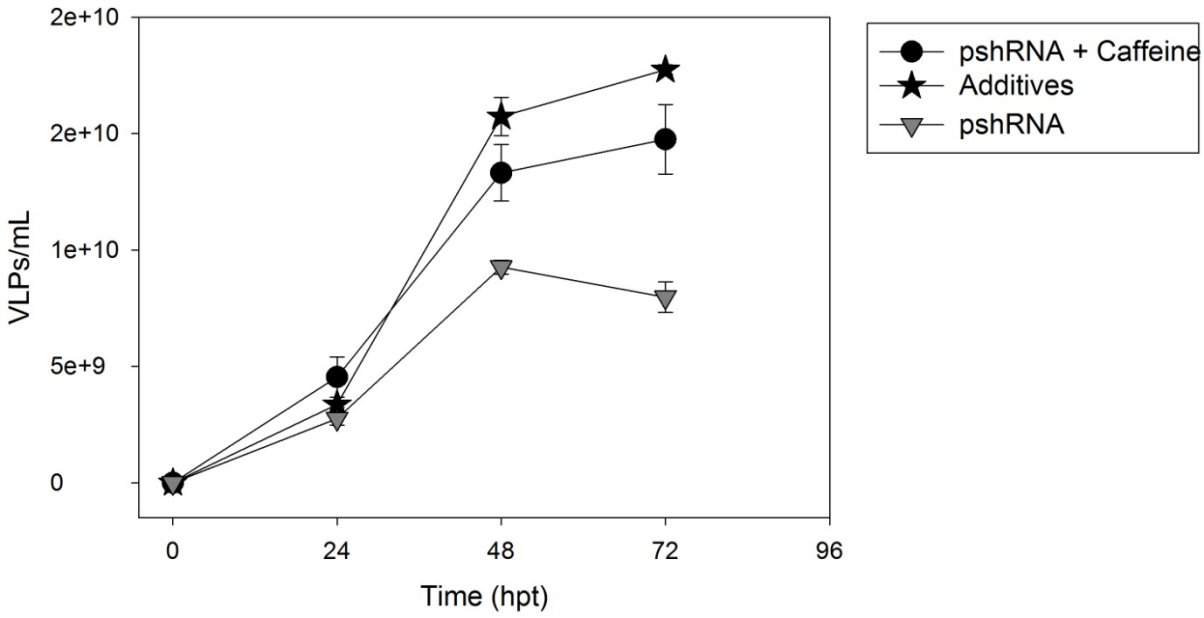

Figure 5. Caffeine relevance in GagGFP VLP production when pshRNA is transfected compared with the standard pshRNA transfection and also the Additive condition (pControl transfection supplemented with lithium acetate, caffeine and valproic acid)

Lithium acetate addition evaluation in pshRNA

Lithium acetate has been also used for improving VLP production in HEK293 cells since it has the capacity of forming pores in the membrane which it is considered that facilitates the DNA:PEI complex entrance into the cell (Schiestl and Gietz 1989; Ye et al. 2009). Here, in addition to the fact that the pshRNA construct has been proven to be an effective substitute of valproic acid, the effect of lithium acetate was also analyzed in order to corroborate if its addition was useful even when using the pshRNA plasmid for transfection. To do so, HEK293 cell culture was transfected as previously described with the pshRNA plasmid. In one condition, lithium acetate was added 3 hours before transfection and in the other one no additive was used. Figure 6 shows that there was no improvement when lithium acetate was used when using pshRNA plasmid. In order to simplify the process, lithium acetate was discarded for further experiments.

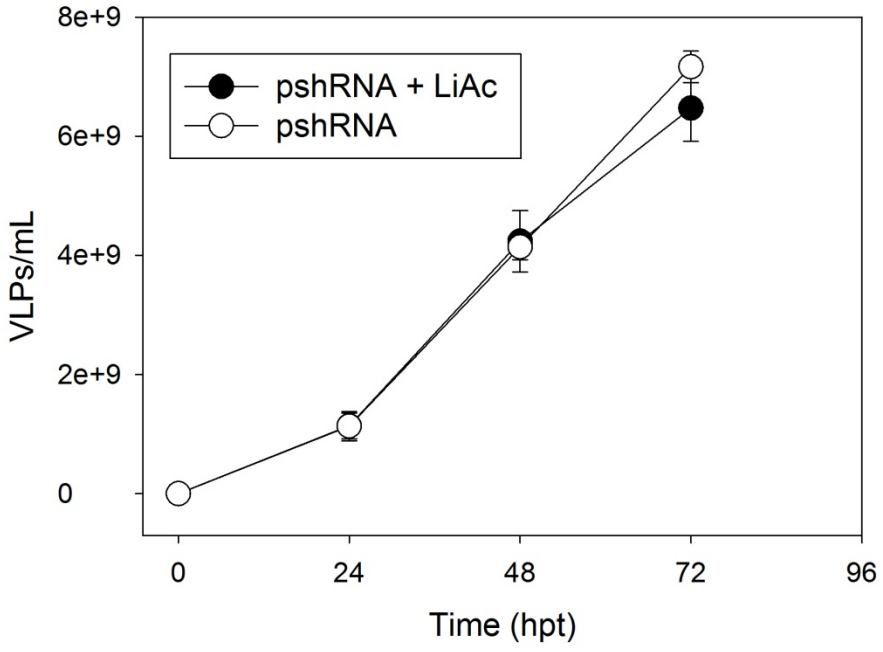

Figure 6. Lithium acetate relevance in GagGFP VLP production when pshRNA is transfected.

Extended gene expression with pshRNA combination

In EGE protocol, the cells that have not been transfected in the first transfection are suspectible of being transfected in the second or the third retransfection. When media was supplemented with valproic acid and caffeine, non-transfected cells were not able to

incorporate the DNA and thus the potential of EGE+Add was limited, since cell were arrested in G1 phase. As previously discussed, EGE with additive supplementation was simplified and the retransfections were avoided, resulting in the ME+Add protocol. Transfection with pshRNA enhanced recombinant protein production in transfected cells. This means that it represents a good substitutive for the use of media additive supplementation. Furthermore, the effect provoked by the shRNA is present only in the transfected cells and thus the non-transfected ones would be susceptible of incorporating the plasmid DNA in the EGE protocol, since no cell cycle arrest is produced. For this reason, the use of pshRNA was tested in combination with EGE protocol (retransfecting cell culture at 48 and 96 hpt), to investigate the potential for higher VLP titers (Table 2). EGE and ME+Add protocol were carried out with pControl plasmid, while EGE+shRNA protocol was carried out with pshRNA plasmid.

Figure 7A shows VLP production titers of the EGE, the ME+Add and the EGE+shRNA protocols. As previously observed, for ME+Add protocol reported higher VLP production from the beginning of the cultivation until 144 hours post transfection. After that point, VLP production was reduced, achieving lower values than the EGE control. In a similar way, the use of pshRNA resulted in an increment in VLP production at the beginning of the cultivation and this was maitained during the first half of the cultivation. Then, in the last part of the culture, VLP production was reduced to similar values than EGE protocol.

Figure 7B shows the accumulated VLP production as well as the improvement provided by the two improved procols. ME+Add protocol promoted more than 1.4-fold improvement compared with the standard EGE as previously observed. In the case of EGE+shRNA, it provided a 1.3-fold improvement. shRNA inhibits the expression of concrete proteins by interfering with the mRNA. The cells are able to find alternative pathways to bypass the interfered routes. Due to this reason, the effect of the shRNA was much more pronunced at the beginning of the cultivation that at the end, when the cells were already adapted to this inhibition. Furthermore, only HDAC5 gene was inhibited; which made that the overal VLP production was not as high as in the ME+Add protocol.

Figure 7C shows both cell growth and viability of the three protocols tested. In terms of cell growth, in both EGE and EGE+shRNA cell were able to grow, reaching about 6 milion cells/mL. In ME+Add protocol, cell growth was inhibited and viability started to fall after 96 hpt, due to the effect of additives. Viabilities were comparable in all cases. The

effect of additives on cell viability was not clearly observable when medium exchanges were carried out since cell debris and dead cells can be removed and not accumulated during the cultivation.

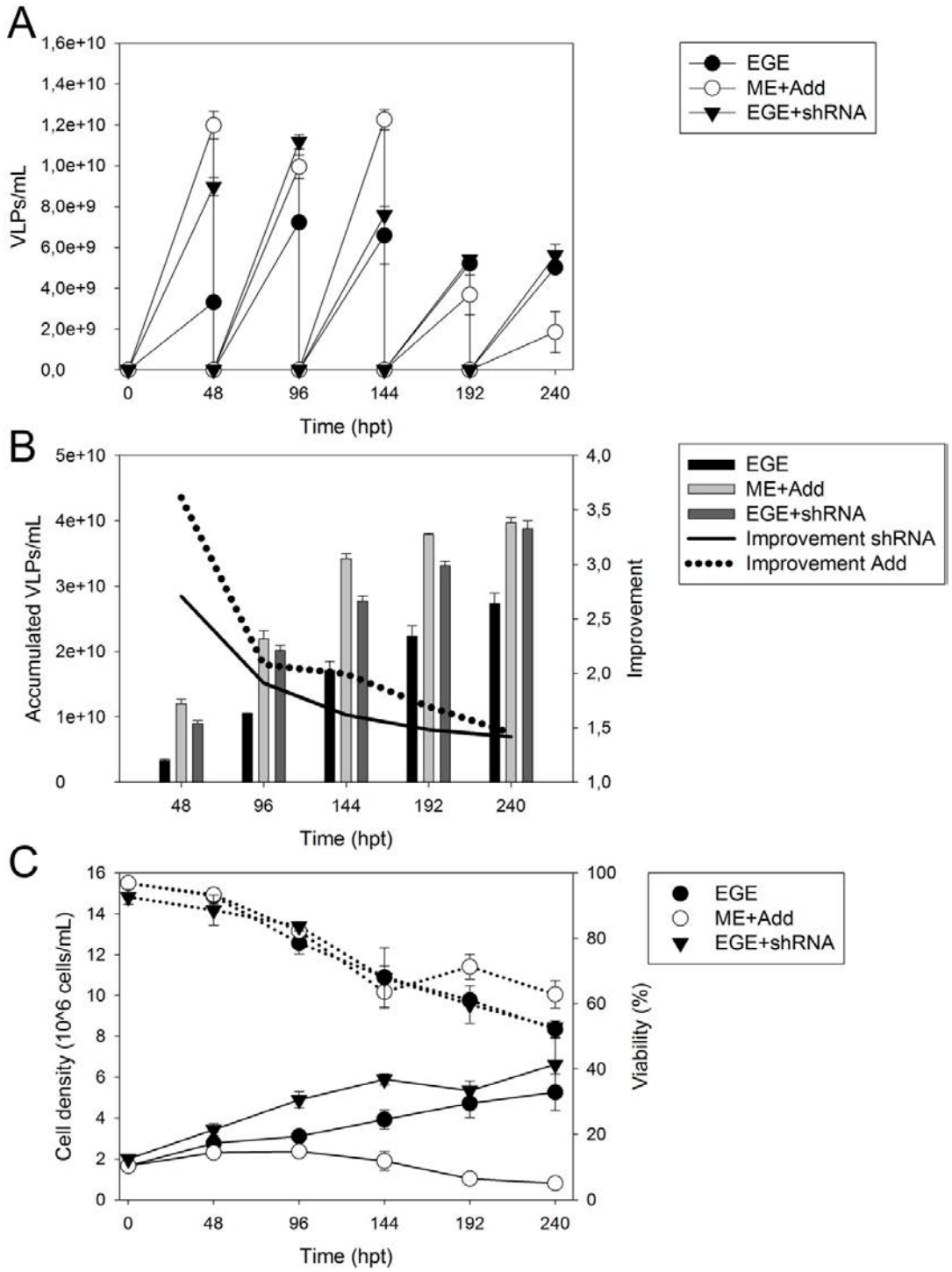

Figure 7. A GagGFP VLP production of the EGE Control, the ME+Add and the EGE+shRNA protocols. B GagGFP VLP accumulated production for the three protocols and the improvement of the shRNA and the ME+Add

protocols over the EGE Control. C Cell density (straight line) and viability (dotted line) of the EGE Control, the ME+Add and the EGE+shRNA cultivations.

pshRNA has been demonstrated to be an efficient additive substitutive. It provides a 2.6-fold improvement in conventional batch cultivation, avoiding the use of additive supplementation which has a detrimental effect over cell density and cell viability. In the case of EGE protocol, it provided a 1.3-fold improvement compared with the standard EGE procol. ME+Add protocol, in which the retransfections were avoided but the medium was supplemented, a 1.4-fold improvement has been achieved over the EGE control.

Two enhanced EGE protocols have been proposed in this work. The first one is based on the supplementation of the cell medium with additive and avoinding the cell retransfections. The second one is based on shRNA which have an inhibition effect over HDAC5 enzyme. Both protocols produce a similar improvement over the standard EGE procedure which represents an important progress towards increasing recombinant protein titers in mammalian cell platforms.

Discussion

Transient gene expression using mammalian cell platforms has been widely utilized for the production of virus-like particles. TGE in mammalian cells has the main disadvantage of relative low production titers (Roldao et al. 2010). Some approaches have been developed for enhancing recombinant protein production in mammalian cells. There are described in the literature several chemical additives that lead to an increase in either transfection efficiency or production phase. Cervera et al (2015), described that a combination of three chemical additives (lithium acetate, valproic acid and caffeine) led to a 4-fold increase in VLP production in batch cultivation. In transient transfection, the plasmid is not integrated into the cell genome and the gene expression is carried out episomally. The plasmid DNA containing the transgene is lost over time due to dilution and hence the expression is lost with cell division (Middleton and Sugden 1994; Wade-Martins et al. 1999; Fuenmayor et al. 2017a). In order to overcome this problem, the extended gene expression protocol was previously developed (Cervera et al. 2015b). By carrying out several medium exchanges and retransfections, the production phase was elongated and VLP titers increased by 12-fold.

The combination of the extended gene expression and additive supplementation protocols previously explained for the maximization of VLP production has been tested in this work. EGE was supplemented with the three proposed additives, obtaining an increase in VLP production of 1.5-fold compared with the standard EGE approach. However, retransfections carried out in EGE when additives were used were found to be non-efficient since valproic acid arrests cells in G1/G0 cell cycle phase and then blocking retransfection (Tait et al. 2004). By eliminating the retransfections, the improvement was maintained and the protocol was simplified. The use of addivities for medium supplementation had a detrimental effect on cell density which provoked a decrease in cell density over the cultivation.

Additive supplementation has several disadvantages such as the increment in the process costs or the increase of downstream steps for VLP purification. Valproic acid inhibits HDAC group of enzymes (Backliwal et al. 2008d) while caffeine inhibits PDE group of enzymes (Choi et al. 1988). The transfection of miR-2861 has been previously reported to inhibit HDAC5, enhancing SEAP and IgG productivity by 1.5-fold in stable CHO producer cell lines (Fischer et al. 2015). In this work, a plasmid containing two shRNA against HDAC5 and PDE8A was developed in order to obtain an alternative for additive supplementation. In batch culture, a significant gene inhibition was found for HDAC5 gene but not for PDE8A gene. shRNA plasmid led to a VLP production increment of 2.3-fold while additive supplementation led to a 4-fold improvement in batch cultivation. Cell viability was maintained high (over 90%) in all the cultivation when using pshRNA compared with the additive protocol (>80%). qPCR analyses showed an inhibition of HDAC5 gene; nevertheless, the PDE8A gene was not inhibited, since PDE8A shRNA sequence did not have an inhibition effect over PDE8A mRNA. shRNA plasmid led hence to a lower improvement due to the non inhibition of PDE8A as observed by qPCR results. In order to demonstrate this hypothesis, a shRNA plasmid transfection was supplemented with caffeine, obtaining comparable VLP production titers to those obtained with the additive supplementation protocol. In this work, by incorporating to the transgene plasmid specific shRNAs, the productivity of VLPs could be increased by 2.3-fold.

Finally, pshRNA was also tested in EGE strategy providing a 1.3-fold improvement in total VLP production compared with the standard EGE protocol, avoiding additive supplementation throughout the whole process. The use of shRNAs has been therefore demonstrated to be a good alternative for medium supplementation. The pshRNA

expression vector tested showed positive results for both standard batch cultivations and also perfusion-based strategies such as extended gene expression protocol enhancing VLP productivities without requirements for additive supplementation. Furthermore, pshRNA did not prevent cell growth and had no detrimental effect over cell viability.

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Extended gene expression for Gag VLP production achieved at bioreactor scale

Results, chapter 2

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Abstract

Transient gene expression has been widely used for VLP production in both small- and large-scale systems. The extended gene expression technique is based on repeated medium exchanges and retransfections of cell culture to prolong production phase. The aim of this study was to demonstrate scalability of the approach by operating EGE continuously in a controlled bioreactor to obtain similar results to those achieved with shake flask EGE for VLP production. Cell viability was comparable between the two systems tested; however, the bioreactor allowed for much higher cell densities and specific growth rates than did shake flasks. Due to this increased cell growth in the bioreactor, the percentage of GFP-positive cells was considerably lower at the end than in the shake flasks. GagGFP VLP titers were similar in both shake flasks and bioreactor. Nanoparticle tracking analysis revealed that the ratio of VLPs/total particles (VLPs and cell vesicles) was higher in the shake flasks than in the bioreactor, possibly due to higher cell densities achieved in the bioreactor. In this study, EGE methodology was carried out in a bioreactor system for the first time while maintaining GagGFP production titers. Overall, our findings call for further optimization and implementation of common downstream processing steps to improve yield and VLP quality.

Keywords: Acoustic filter, Bioreactor, Perfusion, Transient transfection, Virus-Like Particles.

Introduction

Mammalian cell platforms are one of the most commonly used systems for the production of biopharmaceuticals (Bandaranayake and Almo 2014). Among the several cell lines that are currently available for recombinant protein production, the HEK293 cell line is one of the most utilised owing to its capacity for growth in serum-free media and suspension conditions (Meissner et al. 2000; Thomas and Smart 2005; Baldi et al. 2007). HEK293 has been widely employed for the production of several pharmaceuticals in both laboratory and industrial scale systems (Thompson et al. 2013; Fontana et al. 2015; Grieger et al. 2016). Virus-like particles (VLPs) are nanostructures that resemble the configuration of a native virus (Fuenmayor et al. 2017b). VLPs are composed of one or several viral proteins but lack the genetic material and hence cannot revert into the virulent form, which makes them safe for both vaccine production operators and vaccine recipients (Abdoli et al. 2013; Liu et al. 2013; Lua et al. 2014). Furthermore, the repetitive subunits of VLPs provoke high cellular and humoral responses by the immune system. Several VLPs are already available on the market and many more are in preclinical or clinical development stages (Roldao et al. 2010). Post-translational modifications (PTMs) provided by mammalian cell production platforms are critical in vaccine production since they enhance the immune response. The Gag polyprotein from HIV-1 can travel to the vicinity of the cell membrane and bud generating VLPs when recombinantly expressed. These VLPs can be functionalised with other envelop proteins from HIV or other viruses by co-expression of the Gag polyprotein and other viral membrane proteins (Weber et al. 1995; Doan et al. 2005; Zhang et al. 2011).

Transient gene expression (TGE) is one of the most commonly used systems for recombinant protein production in mammalian cell platforms. TGE consists of delivering a plasmid encoding the gene of interest to the cell nucleus. DNA delivery can be performed by physical or chemical methods (Baldi et al. 2007; Merten et al. 2014). Polyethylenimine (PEI) is a positively charged polymer that can form complexes with plasmid DNA; these complexes are internalised by the cell and eventually disassembled, after which the DNA is transported into the cell nucleus where it can be transcribed for protein expression (Cervera et al. 2017). PEI is a very cost-effective reagent widely used in HEK293 transfection from the small-scale of a few millilitres (Baldi et al. 2005; Cervera et al. 2013; Gutiérrez-Granados et al. 2016) to bioreactor-scale until 500 L (Gutiérrez-Granados et al. 2018). TGE

offers the possibility of obtaining sufficient quantities of the product of interest in a very short period of time (in the order of weeks). This is very important during initial stages of development when several candidates need to be tested on a small scale and when the product of interest varies often and rapidly. This is the case of several viruses such as the influenza virus, where a new vaccine has to be generated on a yearly basis (Petiot and Kamen 2013; Milián et al. 2017).

One of the main drawbacks of TGE is that plasmid DNA is not integrated into the cell genome and thus the gene of interest is lost as cells divide (Middleton and Sugden 1994; Wade-Martins et al. 1999). Some experimental approaches have been developed to overcome this problem. One approach is the so-called extended gene expression (EGE) technique. EGE relies on cell culture transfection followed by several medium exchanges and retransfections. The medium is exchanged by centrifugation so that cell waste and the product of interest are removed and collected; thus, the cells are fed with fresh nutrients every 48 hours. Two retransfections are carried out 48 and 96 hours post transfection (hpt) to transfect cells that are not expressing the gene of interest. EGE has been tested in Erlenmeyer flask experiments with a working volume of 20 mL reaching 12-fold improvement in Gag-based VLP production compared with the standard batch transfection protocol (Cervera et al. 2015b). Considering that EGE allows for obtaining a high amount of vaccine in a short period of time, it is of great interest to study this novel methodology at the bioreactor scale, which will certainly be a step forward for its potential application in VLP manufacturing. The original EGE methodology relies on centrifugation; in every medium exchange, cells are removed from the flask, pelleted, and resuspended back into the Erlenmeyer flask, providing a pseudo-perfusion mode of operation with a exchange rate of 0.5 reactor volume per day (vvd). Centrifugation is a convenient approach for a small working volume but it becomes more difficult with larger production scales. Furthermore, the contamination risk for the culture is high. Perfusion allows for prolonging the production phase due to removal of waste products as well as the continuous feeding of nutrients to the cells. Perfusion is interesting in TGE processes where production phases must be extended to maximise product titers. If the product is secreted, it has a short residence time which is important in unstable glycoprotein production and also allows for lower bioreactor working volumes. On the large scale, perfusion main disadvantage is the handling of large volumes generated and the relatively low product concentration obtained in such volumes (Carin et al. 2013; Clincke et al. 2013). There are many devices available for

perfusion, such as sedimentation (inclined settler or acoustic settler), filtration (by tangential flow or cross-flow filter, i.e. hollow fibre filter [HF]), spin-filter, and crossflow microfiltration (Shirgaonkar et al., 2004). An acoustic filter is composed of a chamber with a transducer and a reflector; the transducer generates a standing wave that is reflected in the opposite direction. Cells are trapped in the nodes generated by these two waves while the conditioned medium can flow freely through. Cells start forming aggregates and eventually sediment by gravity. VLPs are not aggregated and can thus flow through the harvest as well as cell waste products. The main disadvantage of acoustic filtration is that oxygen and other nutrients can be rapidly consumed when cells remain inside the acoustic chamber. Shorter times inside the chamber imply higher cell viabilities (Shirgaonkar et al. 2004).

In this study, bioreactor scale-up of EGE is described for the first time in producing Gag-based VLPs; furthermore, analyses of both bioprocess operations (bioreactor and shake flasks) and product quality are compared.

Materials and Methods

Cell lines, media, and culture conditions

The cell line used in this study was the serum-free suspension-adapted HEK293 cell line HEK293SF-3F6. The cell line was derived from a cGMP master cell bank that manufactures clinical material (National Research Council, Montreal, Canada). Cells were cultured in FreeStyle 293 media (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% F68 Pluronic® (Invitrogen, Carlsbad, CA, USA). The medium was also supplemented with 1.6 mg/L r-transferrin (Merck Millipore, Burlington, MA, USA), 19.8 mg/L r-insulin (Nordisk Pharmatek, Køge, Denmark), and 0.9×of an in-house developed lipid mixture to maximise cell growth and productivity as previously described (Cervera et al. 2013). Cells were routinely maintained in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL culture medium. Flasks were shaken at 110 rpm using an orbital shaker (Stuart, Stone, UK) and placed in an incubator maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell count and viability were determined using the Vi-Cell XR counter (Beckman Coulter, Pasadena, CA, USA).

Plasmids and transient transfection

The pGagGFP plasmid encodes a Rev-independent HIV-1 Gag protein fused in-frame to enhanced GFP. The plasmid from the National Institute of Health AIDS Reagent Program (Cat 11468) was constructed by cloning the Gag sequence from pCMV55M1-10 into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA). HEK293 suspension cells were transiently transfected using 25-kDa linear PEI (PolySciences, Warrington, PA, USA) at a cell density of 2×10^6 cells/mL. Prior to transfection, a complete medium exchange was performed by centrifuging the cell culture at 300 × *g* for 5 min. Cells were resuspended in fresh supplemented FreeStyle medium. PEI:DNA complexes were formed by adding PEI to plasmid DNA diluted in fresh culture media (10% of the total volume of cell culture to be transfected). The mixture was incubated for 15 min at room temperature to allow complex formation prior to its addition to the cell culture. The percentage of GFP-positive cells was assessed using a BD Accuri flow cytometer (BD Biosciences, San Jose, CA, USA) at different hpt.

Bioreactor operation, acoustic filter, and cell transfection

Cells were cultured in a 3-L glass autoclavable bioreactor (Applikon Biotechnology B.V., Delft, the Netherlands) at 37 °C, 135 rpm, and in a total working volume of 1.35 L. N_2 was constantly aerated through the sparger at 100 mL/min, while the pO_2 set point was established at 40% of air saturation and controlled by O_2 pulses. The pH was set to 7.1 ± 0.2 and controlled by CO_2 pulses.

A total of 1.35 L of HEK293 cells were grown in the incubator following standard procedures previously explained in Section 2.1 to reach a cell density of 2×10^6 cells/mL. Prior to inoculation, the medium was completely exchanged by centrifuging the cell culture at 300 × *g* for 5 min. Immediately after inoculation, cells were transfected using the standard PEI:DNA complex concentration (2 µg/mL of PEI and 1 µg/mL of DNA). At 4 hpt, perfusion was achieved at a perfusion rate of 0.5 vvd using a BioSep acoustic filter (Applikon Biotechnology B.V.) to separate cells from the supernatant, as illustrated in figure 1. Masterflex L/S peristaltic pumps (model 7521-40; Cole-Palmer, Vernon Hills, IL, USA) were used were used for feeding the culture with fresh medium, harvesting, and pumping culture through the recirculation loop. At 48 and 96 hpt, perfusion was stopped and cells

were retransfected using half of the DNA than in the first transfection (as optimized at small scale); perfusion was restored 4 hours post retransfection; in order to let the DNA:PEI complexes enter the cells.

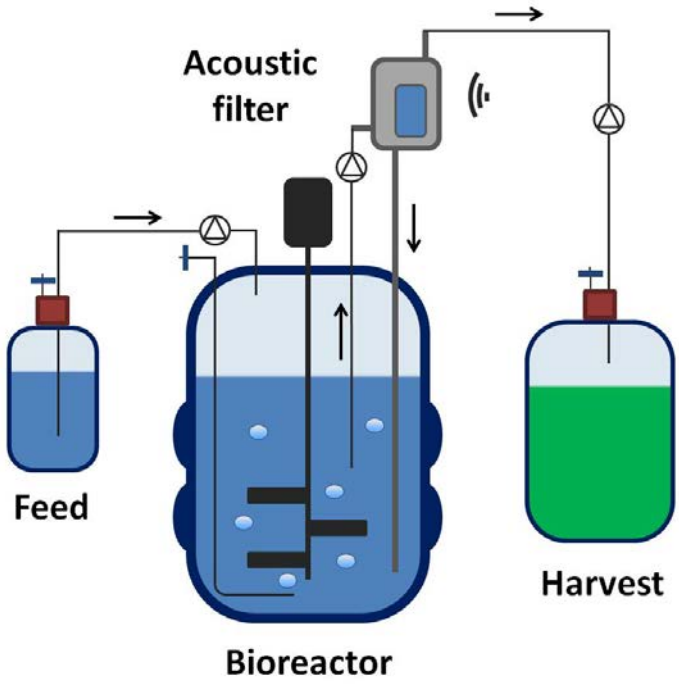

Figure 1. Bioreactor configuration for extended gene expression using acoustic filter perfusion system.

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 10000 × *g* for 5 min. Green fluorescence was measured at room temperature using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) with the following parameters, λex = 488 nm (slit 5 nm) and λem = 510 nm (slit 10 nm). Fluorescence units (FU) of an untransfected negative control were subtracted from each experimental sample to calculate the relative fluorescence units (RFU).) There is a linear correlation between fluorescence intensity and p24 antigen values as determined by the INNOTEST HIV antigen mAb immunoassay (Innogenetics NV, Gent, Belgium). RFU values were 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 of the samples. The first term (3.245xRFU – 1.6833) is the correlation equation between fluorescence values and p24 antigen levels determined by enzyme-linked immunosorbent assay (ELISA), while 36 is a correction factor that takes into account differences in molecular weight between p24 and Gag-GFP and the underestimation arising from using the p24 ELISA to estimate p55 Gag concentrations. In order to obtain the number of VLPs per mL, the GagGFP concentration (ng/mL) has to be divided by its molecular weight (1.39^{-10} kDa) and the results divided by the number of GagGFP monomers present in a VLP (2500).

VLP purification

Harvest supernatants were centrifuged at 10000 × *g* for 5 min to remove cell debris as well as dead and living cells. Supernatants containing the Gag-GFP VLPs were purified by ultracentrifugation (UC) through a sucrose cushion as previously described (Gutiérrez-Granados et al. 2013). VLP pellets were resuspended in PBS and kept at -80 °C for further quality analysis.

VLP characterization by nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was employed for VLP characterization using a NanoSight®NS300 device (Nanosight Ltd., Amesbury, UK) by the Preparation and Characterization of Soft Material services at the Institut de Ciència de Materials de Barcelona (ICMAB-CSIC, Bellaterra, Spain). Sample analysis was performed as previously described (Gutiérrez-Granados et al. 2013).

VLP characterization by transmission electron microscopy

VLP samples were prepared by negative staining of air-dried specimens with uranyl acetate at the Servei de Microscòpia (Universitat Autònoma de Barcelona, Bellaterra, Spain) as previously described (Gutiérrez-Granados et al. 2013). Samples were then imaged with a Jeol JEM-1400 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Gatan ES1000W Erlangshen CCD Camera model 785 (Imaging & Microscopy, Weinheim, Germany).

Determination of host cell protein and host cell DNA

The HEK293 host cell protein (HCP) ELISA kit (Cygnus Technologies, Southport, NC, USA) was employed to determine the concentration of HCPs in the samples. Host cell DNA was evaluated using the Quant-iT™ PicoGreen® dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Both kits were used according to manufacturer's instructions.

Results

In the present study, the extended gene expression methodology was adapted to the bioreactor scale and compared with standard batch cultivation and EGE using Erlenmeyer shake flasks. The following results compare the bioprocess and quality of VLPs obtained by the three different cultivation modes.

Cell density and viability

First, cell density and viability were compared for the three methodologies tested. Figure 2 shows the differences in terms of cell growth and viability between the three modes of operation. Batch cultivation reached 4.8×10^6 cells/mL by 96 hpt. After this point, cell viability rapidly dropped under 50%. EGE at the 20-mL scale exhibited higher growth after transfection, reaching a plateau of 8×10^6 cells/mL by 216 hpt with viability remaining over 50% for more than 288 hpt. Cell growth in the bioreactor exhibited a lag phase (between 48 and 72 hpt) where cells did not grow and cell viability dropped to 70%. Changes to the cultivation system from shake flasks to a bioreactor may have provoked this adaptation phase. Furthermore, cells were subjected to transfection and subsequent acoustic filtration after a short period of time, which could also explain the decreased viability. After 72 hpt, cell viability recovered and the values obtained were comparable to those achieved with flask-scale EGE. Cell density reached a plateau at 192 hpt and was maintained at a constant 14×10^6 cells/mL until the end of the cultivation (288 hpt). Taking into consideration the exponential growth phase reported for shake flasks and bioreactor EGE, the specific growth rate was 0.0109 h^{-1} in Erlenmeyer flasks and 0.0320 h^{-1} in the bioreactor, demonstrating that cell growth is significantly promoted in the bioreactor system by perfusion.

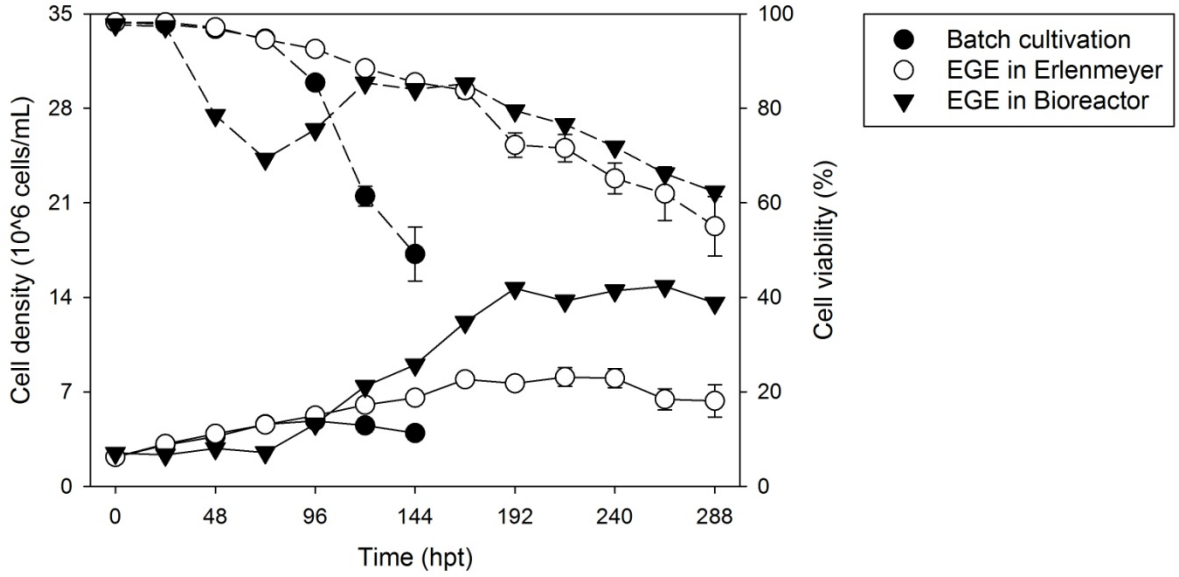

Figure 2. Cell density and cell viability over time for the batch cultivation, EGE in Erlenmeyer flaks and bioreactor scale.

Pseudo-perfusion carried out in shake flasks provided cells with nutrients that allowed for growth and maintenance of high cell viability for more than 288 hpt. In contrast, cells in the bioreactor reached cell concentrations 2-fold higher than those in the Erlenmeyer flask. Perfusion has the advantage of nutrients being constantly fed to the cell culture with concurrent waste product removal. Cells can grow under these conditions more efficiently than in a pseudo-perfusion mode, where nutrients are added at a given time point. Centrifugation steps carried out for the shake flasks may also slow down cellular growth.

Percentage of GFP-positive cells and VLP production

The percentage of GFP-positive cells was analysed every 24 h by flow cytometry (Figure 3a). Transfection efficiency at 48 hpt was comparable between the three systems tested (around 60%); this percentage was relatively stable during the first 96 hpt. Transfection was thus successfully carried out in the bioreactor and no observable differences could be detected between the different systems tested. After 96 hpt, the percentage of GFP-positive cells in the bioreactor started to drop to 25%. There was also a reduction in the percentage of GFP-positive cells for shake flask EGE; nevertheless, the

percentage of GFP-positive cells stabilised to approximately 50%, 2-folds more than GFP-positive cells found in the bioreactor.

As previously mentioned, bioreactor perfusion allows for improved growth and hence higher cell densities. After 96 hpt, cells in the bioreactor started to grow faster, corresponding with the observed drop in the percentage of GFP-positive cells. The large amounts of non-transfected cells also suggest the possibility of performing more retransfections of the culture to increase production titers. Non-transfected cells are susceptible to transfection during the second and the third rounds of retransfection. In the bioreactor, the number of non-transfected cells was higher and thus the number of retransfections performed can be increased. By augmenting the number of retransfections, the total amount of GFP-positive cells can be increased. If there are a higher number of producer cells, the amount of VLPs that can be produced would also be higher. Extended gene expression at the bioreactor scale allows for higher cell densities which leaves room for improving the process and obtaining higher production titers.

For GagGFP quantification, samples were taken every 24 hpt from EGE in shake flask, EGE in bioreactor, and batch cultivation in shake flaks. Fluorescence in the supernatant was used for GagGFP quantification. Figure 3b shows GagGFP production in ng/mL over time for the three different systems tested. A total amount of 29767.4 µg of GagGFP protein was produced in the bioreactor compared with 429.0 µg in the shake flask following EGE. To equally compare both systems, the total amount of protein produced was divided by the working volume of the system (19 mL for the shake flask and 1350 mL for the bioreactor). After this normalization, 22.6 µg/mL ($6.50x10^{10}$ VLPs/mL) was found produced in the shake flask compared with 22.1 µg/mL ($6.36x10^{10}$ VLPs/mL) of GagGFP produced in the bioreactor (Figure 3c). Therefore, GagGFP production following EGE was demonstrated to be scalable to bioreactor operations. Percentage of GFP-positive cells in the bioreactor was much lower than in the Erlenmeyer flask, meaning that if higher percentages of GFP-positive cells were achieved in the bioreactor, higher VLP titers could be obtained. This could be achieved by further retransfections or increased DNA concentration at the retransfections.

Absolute GagGFP protein quantification does not necessarily indicate efficient assembly into mature VLPs. To further analyse VLP quality, several assessments were

carried out to characterise the product and compare the performances of the systems evaluated.

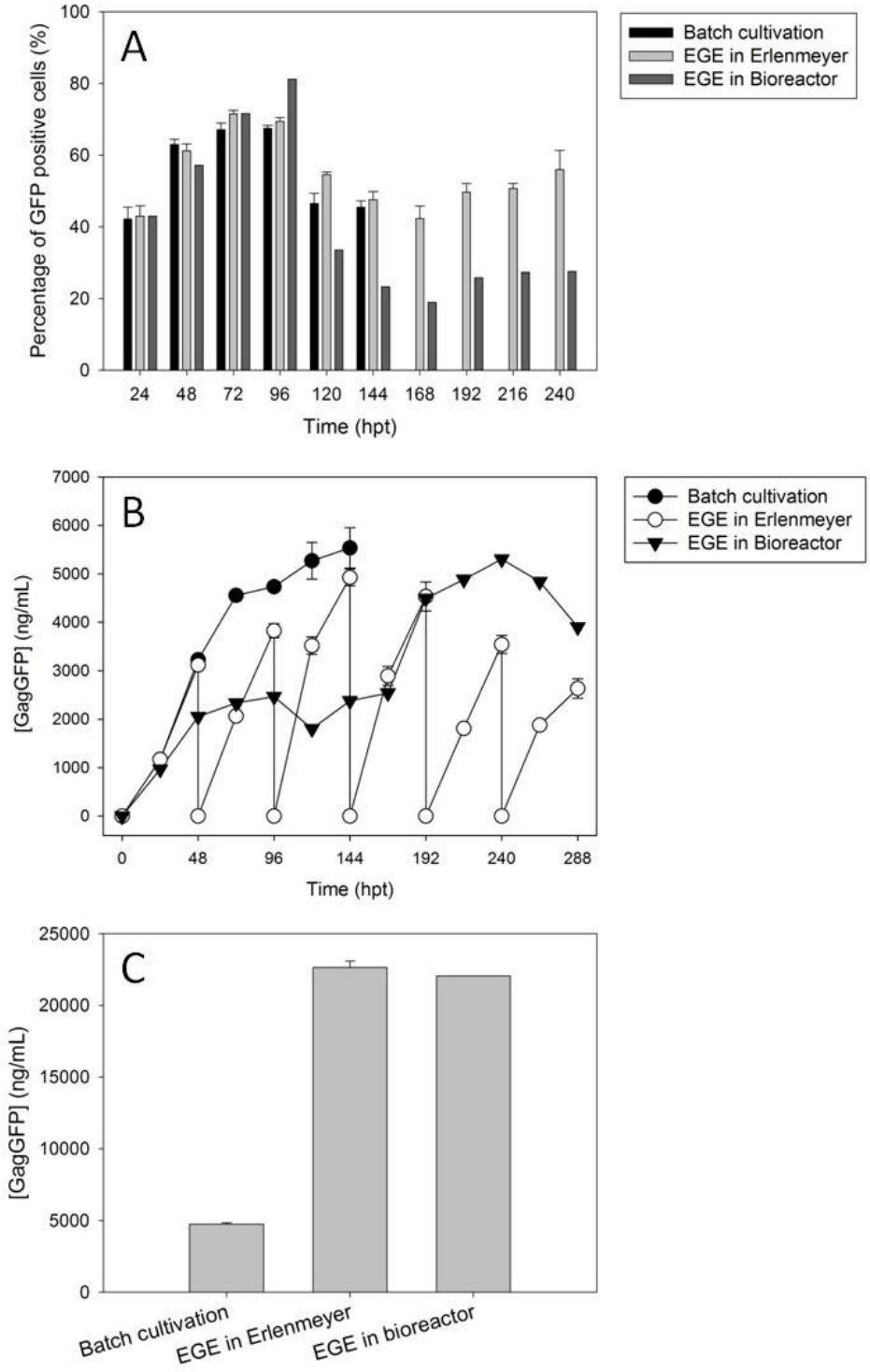

Figure 3. A Percentage of GFP positive cells at different time points. B GagGFP production in ng/mL over time. C Accumulated GagGFP production in ng/mL for the three cultivation systems tested.

VLP quality assessment

VLPs were purified by ultracentrifugation from batch cultivation as well as from the harvests of shake flask- and bioreactor-scale EGE. Harvests were purified at 96, 192, and 288 hpt and then VLPs were resuspended in PBS to perform quality determination analyses of VLPs at different time points.

Nanoparticle tracking analysis and transmission electron microscopy

NTA was carried out using the purified harvest samples from different time points as well as from batch cultivation. Figure 4 shows the fluorescent particle distribution of different time points and systems analysed. Fluorescent particles between 100 and 200 nm correspond to self-assembled GagGFP VLPs. VLPs produced by the EGE protocol in both systems exhibited similar sizes. Surprisingly, VLP size decreased over time. VLPs showed a mean diameter of 195 nm, 145-155 nm, and 125-135 nm at 96 hpt, 192 hpt, and 288hpt, respectively. Transfected dead cells release non-assembled GagGFP that may form small agglomerates which can perturb the experimental means observed (Gutiérrez-Granados et al. 2013). Fluorescent particles may also correspond to GagGFP aggregates that may not be forming VLPs. Both fluorescent and non-fluorescent particles were quantified to establish a correlation between the two particle populations. Figure 5 shows the percentage of fluorescent particles (corresponding to VLPs) with respect to total particles (VLPs and cellular vesicles); this percentage serves as a quantitative indicator of sample purity. Both shake flask and bioreactor EGE exhibited a similar trend for this indicator, having the highest percentage at 192 hpt. Nevertheless, bioreactor sample quality remained lower than the shake flask. The number of non-transfected cells present in the bioreactor was much higher than in the shake flasks, as previously mentioned. Cells generally produce exosomes that are co-purified with VLPs due to their similar characteristics and represent the main contamination of VLP preparations. Exosomes are produced by both transfected and non-transfected cells, whereas VLPs are only produced by transfected cells. At the beginning of the cultivation, the percentage of non-transfected cells was the same in both system; nevertheless, it decreased much rapidly over time in the bioreactor than in the shake flasks due to higher cell growth, and thus the ratio between VLPs produced and total particles was lower. In shake flasks, the percentage of transfected cells (50%) was higher than in the bioreactor (25%), which leads to a higher percentage of VLPs/total particles.

Bioreactor VLP preparation had a higher presence of exosomes due to the higher cell densities reached by this system. Regular downstream processing can be implemented to increase concentration and quality of VLP preparations. Figure 4c shows the polydispersity of the sample, demonstrating that the lowest polydispersity corresponds to the 192 hpt time point in both bioreactor and shake flasks.

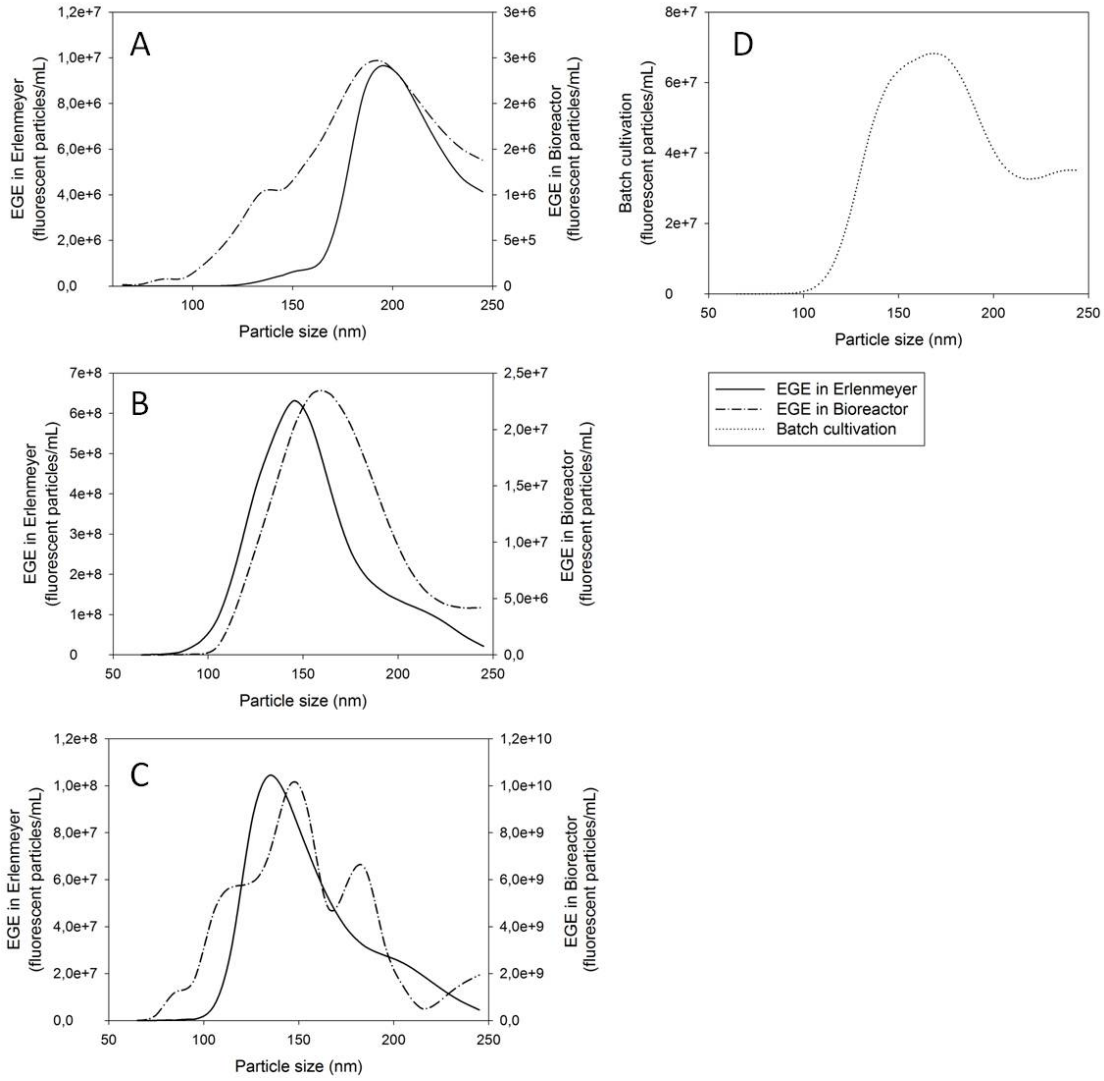

Figure 4. A-C Fluorescence particle size distribution for three different time points (96, 192 and 288 hpt, respectively) and for EGE in Erlenmeyer flask and bioreactor. D Fluorescence particle size distribution for batch cultivation.

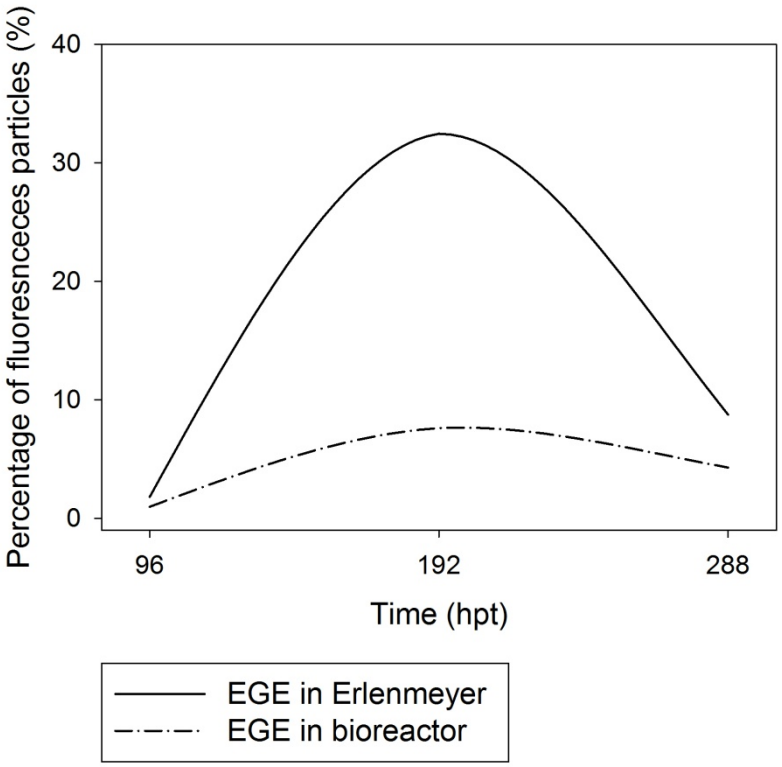

Figure 5. Percentage of fluorescence particles (florescence particles/total particles) over time for the EGE in Erlenmeyer flask and bioreactor.

VLP presence in the samples was corroborated by observation under transmission electron microscopy where electrodense, circular-shaped structures of approximately 100–200 nm were visualised (Figure 6).

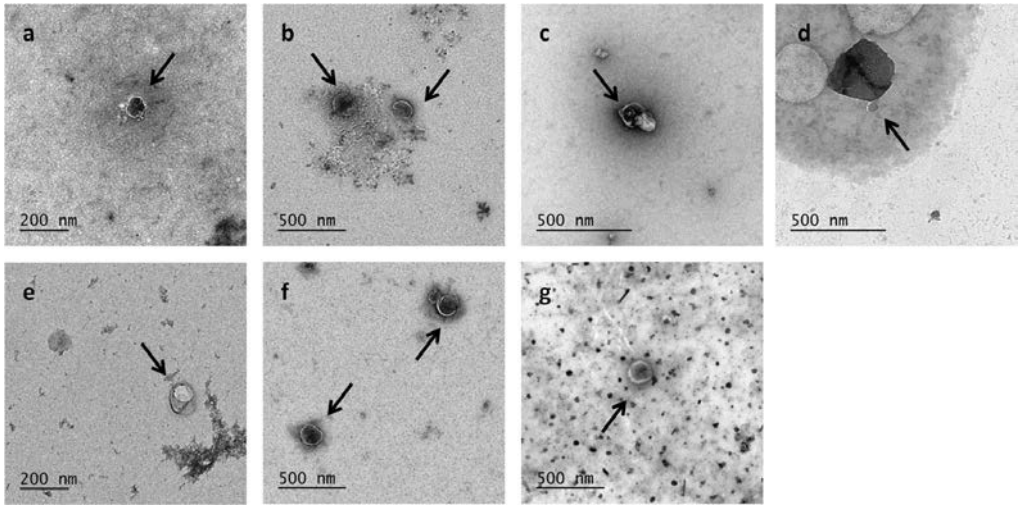

Figure 6. Transmission electron microscopy VLP observation. A-C EGE in Erlenmeyer flaks (96, 192 and 288 hpt respectively). E-G EGE in bioreactor (96, 192 and 288 hpt respectively). D Batch cultivation.

Host cell protein and host cell DNA assays

HEK293 cell DNA and protein residues were analysed in the purified samples to investigate significant differences in the purity of different samples. Table 1 lists all values of HCP and HCD obtained for all samples analysed. Bioreactor and Erlenmeyer flask samples were highly comparable in HCP and HCD residues at 96 hpt. Nevertheless, bioreactor samples showed higher impurity concentrations during later time points of the process. Since bioreactor perfusion systems allow for higher cell densities, this may result in the production of more HCP and HCD for the same amount of VLPs. Downstream processing implementation can reduce levels of HCP and HCD present in VLP preparations.

Table 3. Host cell DNA and host cell protein quantification (μg/mL)

Cultivation system	Time (hpt)		
	96	192	288
EGE in Erlenmeyer	4.84/6.70	14.52/61.71	8.11/69.57
EGE in Bioreactor	3.62/6.47	22.46/14.98	169.83/129.83
Batch cultivation	7.66/29.40		

Conclusions

Extended gene expression was successfully scaled up to a bioreactor level. Perfusion cultivation allowed for the constant addition of fresh medium to the cell culture which led to an increase in cell density and favoured specific growth rate, compared with the 20-mL shake flask. In terms of transfection efficiency and GagGFP production titers, there were no remarkable differences between the two systems tested. The percentage of GFP-positive cells rapidly diminished in the bioreactor after 96 hpt which may be associated with the high cell growth recorded. VLP quality analyses were also carried out to further compare the two production scales. Round shaped, electrodense structures 200 nm in size were observed under transmission electron microscopy corresponding to mature VLPs. Nanoparticle tracking analysis was performed to analyse the percentage of fluorescence particles (VLPs) among total particles (VLPs and other cellular vesicles). Maximum

percentage was obtained at 192 hpt for the two systems, the percentage being higher in shake flasks, a consequence of the higher cell densities achieved in the bioreactor. Similarly, levels of host cell protein and host cell DNA residues were increased in the bioreactor preparations.

The results obtained show that bioreactor EGE allowed for much higher cell densities which had a detrimental effect on the purity of the preparation and a decrease in the percentage of GFP positive cells. In terms of VLPs, $6.50x10^{10}$ VLPs/mL were produced following EGE in shake flasks compared with $6.36x10^{10}$ VLPs/mL in the bioreactor. Further retransfections could be performed to augment the number of GFP-positive cells throughout the cultivation system and thus increase bioreactor production titers. This infers that EGE at bioreactor scale has a huge room for improvement optimizing the concentration of DNA used in each retransfection and the number of retransfections. This optimization would lead to an increase in production titers

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Transient gene expression optimization and expression vector comparison to improve HIV-1 VLP production in HEK293 cell lines

Results, chapter 3

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Abstract

Transient gene expression (TGE) has been used at small and medium scale for the production of biologicals in sufficient quantities to perform pre-clinical and characterization studies. PEI-mediated transfection offers a low toxicity and non-expensive method for cell transfection. DNA and PEI concentration for transient gene expression has been extensively optimized in order to increase product titers. However, the possibility to extrapolate the optimal concentrations found for a specific bioprocess when expression vectors or cell lines need to be changed has not been investigated. In this work, the combination of three different HEK293 cell lines with three different vectors was studied for the production of HIV-1 virus-like particles (VLPs). The concentration of DNA and PEI was optimized for the nine combinations. The obtained results were very similar in all cases (DNA: 2.34 ± 0.18 μg/mL; and PEI: 5.81 ± 0.18 μg/mL), revealing that transfection efficiency is not dependent on the cell line or vector type, but on DNA and PEI quantities. Furthermore, two of the cell lines tested stably expressed a protein able to recognize specific origins of replication: HEK293T/SV40 and HEK293E/oriP. Origins of replication were included in the vector sequences in order to test their capacity to increase production titers. HEK293T/SV40 resulted in a decrease of cell density and productivity of 2.3 fold compared to a control plasmid. On the other hand, HEK293E/OriP platform enabled a 3-fold improvement in HIV-1 VLP production keeping the same cell densities and viabilities compared to a control plasmid.

Keywords: Transient gene expression, virus-like particles, HEK293, origin of replication, expression vectors

Introduction

Biopharmaceutical production using mammalian cell technology has been gaining importance in the industry over the last years (Bandaranayake and Almo 2014). Transient gene expression (TGE) is one of the most used technologies for the production of recombinant proteins, especially in early development phases, but it is also gaining importance in the development of large scale processes for novel products such as viral vectors for gene therapy (Baldi et al. 2007; Merten et al. 2014). It is based on the transfection of a cell culture where a heterogeneous population of transfected and non-transfected cells is generated. After 48 to 72 hours post transfection (hpt) the product of interest is harvested (Cervera et al. 2013). This is an easy, cost-effective and fast process which allows obtaining sufficient quantities of the product of interest in a short period of time. TGE is very convenient in the initial phases of the development of a new biopharmaceutical when several candidates need to be tested. It is also very useful when the product of interest has a toxic effect on the cell line. Furthermore, it has been widely used in both laboratory and industrial scale for the production of different proteins and viruses (Baldi et al. 2007). One of the main drawbacks of TGE is that the expression of the gene of interest is lost over time. Since the gene of interest is not integrated into the cell genome, it is expressed episomally and lost due to dilution in each cell division (Middleton and Sugden 1994; Wade-Martins et al. 1999).

In this work, TGE was used for the generation of HIV-1 virus like particles (VLPs). Mammalian cell technology allows incorporating accurate post-translational modifications (PTMs) which are very important for the efficacy of the VLPs (Zhu 2012). These are nanostructures that resemble the configuration of a virus (Roldao et al. 2010). They are composed of one or multiple structural proteins from the virus which can be membrane or core proteins (Abdoli et al. 2013; Lua et al. 2014). VLPs can also have a lipid envelope (Liu et al. 2013). Their repetitive structure provides efficient humoral and cellular immune responses which are advantageous in front of other recombinant vaccines. They are not able to pack viral genome which makes them safer for both patient and vaccine operators (Roldao et al. 2010). HIV-1 VLPs are generally formed by the polyprotein Gag. This protein has the ability to anchor to the cell membrane and assemble into VLPs that are released by a budding process. Part of the cell membrane is incorporated to the VLPs and therefore

membrane proteins can be integrated to the external envelop (Weber et al. 1995; Doan et al. 2005; Zhang et al. 2011).

HEK293 has been used for the production of recombinant proteins in TGE, since it is able to grow in suspension and under serum free media conditions (Meissner et al. 2000; Thomas and Smart 2005; Baldi et al. 2007). Furthermore, it has been widely used for the production of viruses (Le et al. 2010; Petiot and Kamen 2013), vectors (AAVs (Grieger et al. 2016), lentiviral vectors (Merten et al. 2016)) and vaccines candidates (D'Aoust et al. 2010; Cervera et al. 2013; Fontana et al. 2015).

HEK293SF-3F6 cell line has been previously used for the production of HIV-1 VLPs at laboratory scale (Cervera et al. 2013). Nevertheless, the expression is lost over time due to the plasmid dilution (Cervera et al. 2017). To overcome this problem, there are two available HEK293 cell lines. First, HEK293T stably expresses the SV40 large T antigen from Simian Vacuolating virus 40 (SV40 virus). Its genome encodes for the large T antigen which is related with transcription and also replication of the DNA (Dean et al. 1987). This protein can recognize a piece of the viral genome: the SV40ori origin of replication which can also interact with the DNA polymerase from the host cell. This leads to the episomal replication of the plasmid when SV40ori is present in its sequence (Heinzel et al. 1988). Gene copy number increases considerably, enabling high expression levels (Van Craenenbroeck et al. 2000). Nevertheless, this high plasmid amplification can also have a detrimental effect on cell growth and viability (Durocher et al. 2001). An additional drawback of the system is the high number of mutations incorporated to the DNA during the episomal replication (Van Craenenbroeck et al. 2000). Second, HEK293-EBNA1 expresses Epstein-Barr virus Nuclear Antigen 1 (EBNA-1) protein from Epstein-Barr virus (EBV) (Baldi et al. 2007; Segura et al. 2007). Its genome encodes for the protein EBNA-1 which has several functions in gene regulation and viral genome replication. It binds to the origin of replication OriP enabling the episomal replication of the plasmid (Gahn and Schildkraut 1989; Middleton and Sugden 1992). This replication is though not as high as in HEK293T/SV40ori system. Indeed, the episomal replication occurs at the same rate as cell division (Kumar et al. 1990). Consequently, it has no detrimental effects on cell density and production. Furthermore, OriP has a transactivation capacity which increases transcription of the gene of interest encoded in the plasmid (Van Craenenbroeck et al. 2000).

The gene of interest of this work was the Gag polyprotein that generates HIV-1 virus-like particles (VLPs). In order to simplify detection and quantification, a GFP fused to Gag was used (Gutiérrez-Granados et al. 2013). This method allows an easy follow-up of the percentage of transfected cells and the production of VLPs. Three different HEK293 cell lines: T, 6E (EBNA-1) and SF-3F6 (not expressing any recognition protein) were compared in terms of production, transfection efficiency, cell growth, and viability. These cell lines were studied in combination with 3 plasmids, one containing the SV40ori origin of replication, another one containing the OriP and another one without any origin of replication. By combining the three cell lines with the three types of plasmid, the influence of different expression systems in the performance of TGE was studied in this work.

Materials and Methods

Cell line, media and culture conditions

The cell lines used in this work are serum-free suspension-adapted HEK293 cell lines: HEK293SF-3F6 (kindly provided by Dr. Amine Kamen, BRI, National Research Council of Canada (Montreal, Canada)), HEK293T (kindly provided by Dr. Claudio Prieto from the Laboratory of Cell Culture, Universidad Nacional del Litoral (Santa Fe, Argentina)), and HEK293-EBNA1 (acquired from the National Research Council of Canada (Montreal (Canada)). Cells were cultured in FreeStyle 293 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% Pluronic® (Invitrogen, Carlsbad, CA, USA). The medium was also supplemented with 1.6 mg/L of r-transferrin (Merck Millipore, Kankakee, IL, USA), 19.8 mg/L of r-insulin (FeF Chemicals/Novo Nordisk, Køge, Denmark.) and 0.9X of an *in-house* developed lipid mixture to maximize cell growth and productivity, following previous results on medium supplementation enhancement (Cervera et al. 2013). Cells were routinely maintained in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL of culture medium. Flasks were shaken at 110 rpm using an orbital shaker (Stuart, Stone, UK) placed in an incubator maintained at 37ºC in a humidified atmosphere of 5% CO_2 in air. Cell count and viability were determined using Nucleocounter NC-3000 (Chemometec, Allerod, Denmark).

Plasmids

The pSV40 plasmid codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP (Hermida-Matsumoto and Resh 2000). This plasmid (NIH AIDS Reagent Program (Cat 11468)) was constructed by cloning the Gag sequence from pCMV55M1-10 (Schwartz et al. 1992) into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA). The Gag-EGFP gene was cloned from pSV40 into a pCEP4 plasmid (Invitrogen, Carlsbad, CA, USA) using *Kpn*I and *Not*I restriction enzymes. EBNA1 gene was removed from pCEP4:GagGFP using *Csi*I and *Xag*I restriction enzymes, resulting in pOriP. EBNA-1 gene and OriP origin of replication were also removed from the pCEP4:GagGFP plasmid using *Psc*I and *Xag*I restriction enzymes, resulting in p(-). The plasmids were prepared and purified as previously described (Segura et al. 2007).

Transient transfection

A comparison of three different HEK293 cell lines and three different transfection vectors was carried out in this work. A general transfection protocol had hence to be established in order to equally compare the performance of the cell lines used. HEK293 suspension cells were grown as usual to reach a cell concentration of $2x10^6$ cells/mL and were transiently transfected using 25-kDa linear polyethyleneimine (PEI) (PolySciences, Warrington, PA, USA). Prior to transfection, a complete medium exchange was performed by centrifuging the cell culture at 300xg for 5 minutes. Cells were resuspended in fresh tempered supplemented FreeStyle 293 medium. Transfections were performed using different concentrations of DNA and PEI to find the optimal concentrations and ratios. PEI:DNA complexes were formed by adding PEI to plasmid DNA diluted in fresh culture media (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 prior to its addition to the cell culture (Cervera et al. 2013). 4 hours post-transfection (hpt) a complete medium exchange was performed by centrifuging the cell broth at 300xg for 5 minutes and replaced by Ex-Cell® 293 medium (Sigma-Aldrich, San Luis, MO, USA). The percentage of GFP-positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) at 24 hpt.

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 10000×g for 5 min. Green fluorescence was measured at room temperature using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) set as follows: λex= 488 nm (slit 5 nm), λem= 510 nm (slit 10 nm). Relative fluorescence units 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.

PEI and DNA concentration optimization using design of experiments

Central composite design (CCD)

In order to find the optimal concentration of PEI and the three different DNA plasmids for each cell line, a 10-experiment CCD design was used. 5 different concentrations of DNA and PEI were used to perform the experiments and were coded as: -√2, -1, 0, 1, √2 as indicated in Table 1. CCD experimental results were fitted to a second-order polynomial equation described below by non-linear regression analysis:

$$Y = \beta_0 + \sum \beta_i \, X_i + \sum \beta_{ii} \, {X_i}^2 + \sum \beta_{ij} \, X_i X_j \qquad (2)$$

where Y is the response (in percentage of GFP positive cells at 24 hpt), β_0 is the offset term, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient and *Xi* and *Xj* are the independent variables (in this case, PEI and DNA concentrations). This equation was used to predict the optimum concentration values of the independent variables using R software for statistical computing. Three-dimensional surface plots were generated to facilitate model interpretation.

Table 1. Coded experimental concentrations used in the Central composite design.

Experiment number	DNA	PEI
1	-√2	√2
2	0	1
3	√2	√2
4	-1	0
5	0	0
6	0	0
7	1	0
8	-√2	-√2
9	0	-1
10	√2	-√2

Statistical analyses

Statistical analyses of the models were performed using R software for statistical computing. The quality of the fit of the model equation was expressed by the coefficient R^2 obtained by regression analysis. The overall significance of the model and coefficients was determined by analysis of variance (ANOVA) *F*-test.

Results

Preliminary tests to define experimental space and toxicity

DNA and PEI concentration ranges were selected in order to find an appropriate experimental space to perform the tests without toxicity effects on the cells. DNA

concentration range was: 0.1, 0.67, 2.05, 3.43 and 4 µg/mL and PEI concentration range was: 0.5, 1.38, 3.5, 5.62 and 6.5 µg/mL. HEK293T cells were transfected using p(-) plasmid to be able to extrapolate conclusions to the other cell lines and plasmid types. 24 hpt flow cytometry and viability data were used to establish the concentration ranges for each variable.

Both maximum DNA and PEI concentrations did not have a detrimental effect on cell culture viability, since viability remained over 85% at 24 hpt in all the experiments. Low PEI concentrations coded as -√2 and -1 corresponding to 0.5 and 1.38 µg/mL led to very low transfection efficiencies, for this reason, PEI concentration range was modified in further experiments. On the other hand, since low DNA concentrations did not appear to have a negative effect on transfection the minimum DNA concentration was maintained.

The percentage of GFP positive cells at 24 hpt was represented in a contour plot (Figure 1). As it can be observed, coded PEI concentrations below -1 led to very low transfection efficiencies (lower than 0.5%). In contrast, low DNA concentration did not lead to low percentage of GFP positive cells (over 15%). Experimental space was, therefore, redesigned and higher PEI concentration range was established as follows: 2.5, 3.23, 5, 6.77 and 7.5 µg/mL.

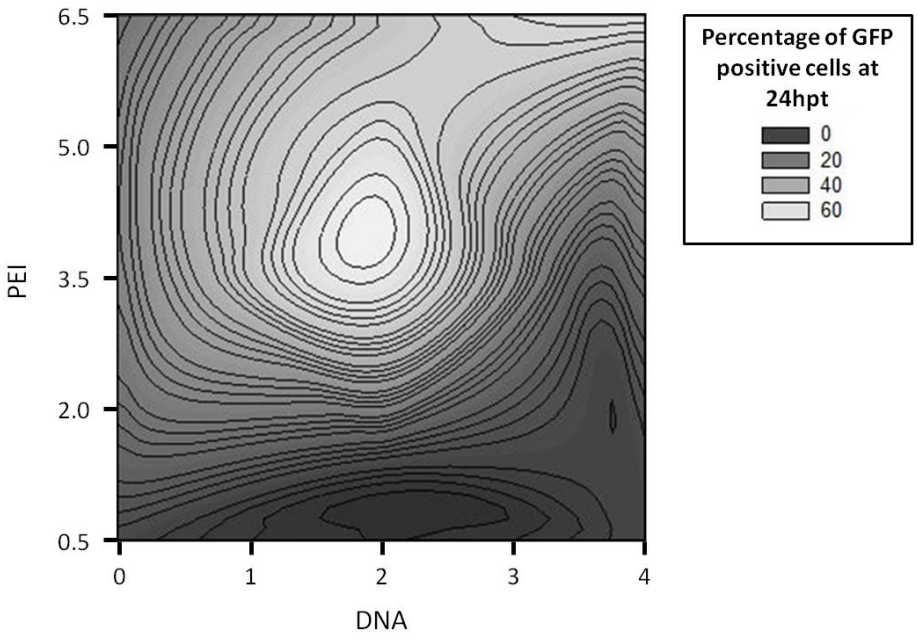

Figure 1. PEI (µg/mL) and DNA (µg/mL) variable contour plot. Effect of DNA and PEI preliminar concentrations on transfection efficiency. Colour intensity corresponds to the transfection efficiency as shown in the legend.

Optimization of DNA and PEI concentrations

A central composite design was used to find optimal PEI and DNA concentrations for every cell line/plasmid type combination. As previously mentioned, three different HEK293 cell lines (T, 6E, SF-3F6) and three different plasmids (pSV40, p(-), pOriP) were used. Using the percentage of GFP+ cells at 24 hpt as the response, 9 quadratic regression models were adjusted (Table 2) and 9 optimal concentrations were found (Table 3).

Table 2. Regression coefficients for the 9 Central composite designs adjusted and their specific p-values in brackets.

Model parameters	**293SF-3F6**			**293T**			**293E**		
	pSV40	**p(-)**	**pOriP**	**pSV40**	**p(-)**	**pOriP**	**pSV40**	**p(-)**	**pOriP**
Constant	35.72 (<0.01)	36.26 (<0.01)	32.65 (<0.01)	50.16 (<0.01)	56.84 (<0.01)	48.53 (<0.01)	34.15 (<0.01)	29.65 (<0.01)	36.9 (<0.01)
DNA	-1.91 (0.32)	0.67 (0.78)	0.75 (0.74)	0.56 (0.78)	4.05 (0.13)	2.31 (0.49)	0.3 (0.79)	0.67 (0.71)	0.49 (0.66)
DNA^2	-3.03 (0.35)	-5.07 (0.25)	-3.08 (0.44)	-9.35 (0.05)	-11.43 (0.04)	-10.39 (0.11)	-3.47 (0.13)	-2.86 (0.38)	-5.44 (0.04)
PEI	6.1 (0.02)	4.46 (0.12)	4.78 (0.09)	7.34 (0.02)	6.82 (0.04)	7.29 (0.07)	3.21 (0.04)	2.98 (0.15)	4.70 (0.01)
PEI^2	-6.14 (0.01)	-7.23 (0.13)	-7.45 (0.11)	-7.75 (0.08)	-7.89 (0.10)	-6.68 (0.26)	-5.8 (0.03)	-5.83 (0.11)	-5.48 (0.04)
DNA*PEI	2.81 (0.10)	3.28 (0.14)	3.06 (0.14)	3.5 (0.08)	5.19 (0.04)	5.01 (0.10)	4.33 (0.01)	3.35 (0.07)	3.73 (0.01)
R^2	90.74	87.48	86.45	94.95	95.07	89.42	95.51	87.54	96.68
p-value	0.03	0.06	0.07	0.01	0.01	0.04	<0.01	0.06	<0.01

Table 3. Optimal DNA and PEI concentrations generated by the models (ng/µL).

	293SF-3F6	**293T**	**293E**
pSV40	1.92 / 5.84	2.22 / 5.87	2.44 / 5.68
p(-)	2.30 / 5.62	2.46 / 5.94	2.49 / 5.61
pOriP	2.48 / 5.68	2.42 / 6.14	2.35 / 5.88

Regression analysis showed that the models were adequate with a coefficient R^2 higher than 86% in the 9 models. This indicates that the models were consistent with more than 86% of the variability of the data. The consistence of the models was confirmed by the Fisher's *F*-test. An associated *p*-value of <0.07 indicated the models were significant.

The general trend of the experimental responses and the interactive effects on the response can be observed in the three-dimensional plots presented in Figure 2. It is observed that the response varies with the same trend in all 9 combinations. For all of them, the highest responses are located in a similar DNA and PEI range of concentration (between 1.92-2.49 ng/µL for DNA and 5.61-6.14 ng/µL for PEI). As it is shown in Table 3, the optimal concentrations found for all the combinations were very similar. This means that for transfection efficiency at 24 hpt, neither the plasmid type nor the cell lines appear to have a strong influence on optimal concentrations. DNA and PEI optimal concentration means and standard deviation (SD) were found at 2.34 ± 0.18 µg/mL for DNA and 5.81 ± 0.18 µg/mL for PEI. SD only represents a 7.7% of the mean in the case of DNA and 3.1% in the case of PEI. This result shows that regardless which HEK293 cell line and which vector are being used in TGE, DNA and PEI concentrations must remain constant to obtain the highest transfection efficiencies with the protocol used in this work.

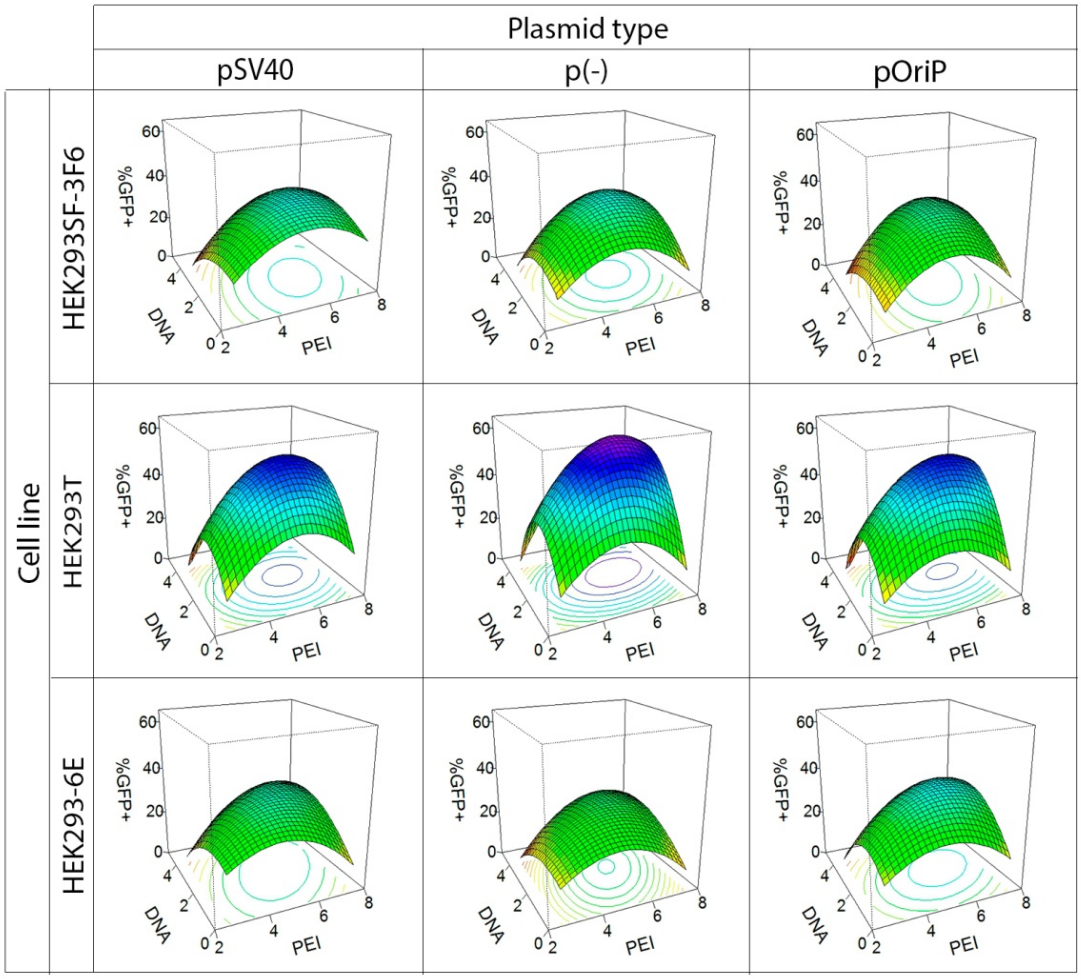

Figure 2. Three dimentional models obtained for each cell line/vector combination. The percentage of GFP positive cells is plotted in respect to PEI (µg/mL) and DNA (µg/mL) variables.

In order to further analyze cell line and plasmid type dependency on the optimal concentrations found, the mean DNA and PEI concentrations were calculated for each cell line and for each vector. Figure 3 shows that there is not a significant effect of the cell line or the vector type on the optimal DNA and PEI concentration used. Thus, DNA and PEI concentrations found here could be extrapolated to other HEK293 cell lines or other vectors since transfection efficiency would remain optimal with this particular transfection protocol.

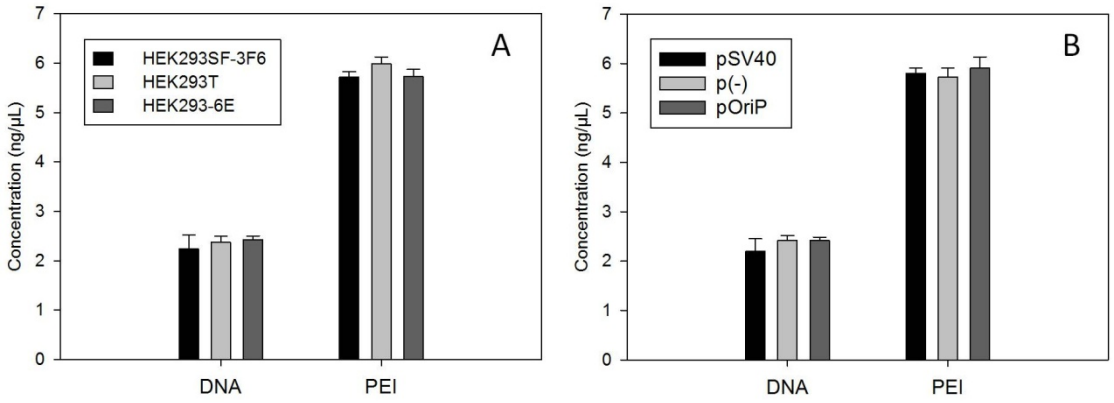

Figure 3. ACell line dependency over DNA and PEI optimal concentrations found. B Vector dependency over DNA and PEI optimal concentrations found.

Taking into consideration that the plasmids differ in base pairs, optimal DNA concentrations correspond to different plasmid gene copy number. pSV40 is 6.4kb, p(-) is 8 kb and pOrip is 10kb. For the overall DNA optimal concentration (2.34 ng/µL), $3.39 \cdot 10^8$ gene copies/mL were found to be optimal for pSV40, $2.71 \cdot 10^8$ for p(-), and $2.17 \cdot 10^8$ for pOriP. This proves that gene copy number is not a strong variable when optimizing transfection efficiency. Indeed, it has been previously reported that reducing the copy number but maintaining DNA concentration by the addition of stuffer DNA provides comparable transfection efficiencies (Kichler et al. 2005). Other physical features such as PEI:DNA complex characteristics could be highly influencing the process instead (Choosakoonkriang et al. 2003).

Validation of the models

The optimal DNA and PEI concentrations obtained by the 9 models were validated experimentally to corroborate the results predicted by the statistical models. All the three HEK293 cell lines were transfected with the three different plasmids using the optimal DNA and PEI concentrations (2.34 ng/µL and 5.81 ng/µL, respectively). Transfection efficiency at 24 hpt was used to validate the models, but samples were also taken at 48, 72 and 96 hpt and cell density, viability, and VLP concentration were analyzed.

The percentage of GFP positive cells at 24 hpt found for every cell line/plasmid type combination is shown in Table 4. These values corroborate the results predicted by the models. Figure 4 shows the predicted data versus the experimental data for each of the 9

models. It can be observed that all the model predicted values are in agreement with the observed experimental values. The distance between the dots and the line in Figure 4 reflects the accuracy of each model in predicting the transfection efficiency. HEK293T presented the highest transfection efficiency while HEK293-EBNA1 presented the lowest. Furthermore, highest transfection percentage was reached at 24 hpt in HEK293T, but in the other two cell lines at 48hpt. HEK293T cell line reached at 24 hpt around 50% of transfection efficiency while HEK293SF-3F6 and HEK293-EBNA1 reached this percentage at 48 hpt. HEK293T is able to faster start the expression of the recombinant protein while it takes longer to the other two to initiate protein expression.

Table 4. Percentage of GFP+ cell at 24 hpt predicted by the model versus the obtained experimentally.

	293SF-3F6		**293T**		**293E**	
	Predicted	**Experimental**	**Predicted**	**Experimental**	**Predicted**	**Experimental**
pSV40	37.0 ± 7.3	40.0 ± 0.6	52.0 ± 8.0	54.0 ± 1.0	34.7 ± 5.4	32.6 ± 1.5
p(-)	37.0 ± 9.1	31.0 ± 0.3	59.2 ± 8.8	53.5 ± 0.9	30.1 ± 7.3	29.6 ±1.2
pOriP	33.6 ± 8.5	31.7 ± 0.8	51.0 ± 11.4	50.2 ± 0.8	38.1 ± 5.3	36.1 ± 1.6

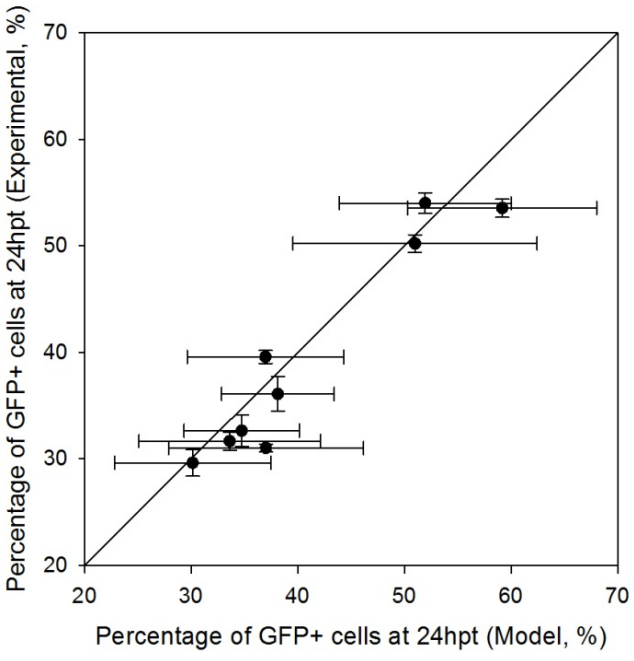

Figure 4. Experimental versus predicted data for the optimal DNA and PEI concentrations in the models generated. The straight line is driven by the equation y=x (vertical error bars represent SD of three replicates while horizontal error bars represents the upper and lower bounds for the predicted values, calculated as predicted value ± 1.96 x √MS, with a confidence interval of 95%).

Comparison of HIV-1 VLP production with the presence of origins of replication

After transfection, HEK293T showed cell growth, reaching cell densities of $7.5x10^6$ cells/mL at 96 hpt and viabilities higher than 85% during the whole cultivation when transfected with p(-) and pOriP. Conversely, HEK293T transfected with pSV40 plasmid did not grow as fast as when transfected with the other two plasmids, reaching a cell concentration of $4x10^6$ cells/mL at 96 hpt (Figure 5B). pSV40 had hence a detrimental effect on cell growth in HEK293T. In terms of GagGFP polyprotein production, less than 52 R.F.U. (6 µg/mL) were produced using the pSV40 compared to more than 120 R.F.U. (14 µg/mL) with the other two plasmids (Figure 5A). By including the SV40ori in the expression vector, GagGFP production was decreased by more than 2.3-fold. Therefore, the use of this origin of replication was detrimental for HIV-1 VLP production in HEK293T cells. This result is in agreement with observations previously published (Van Craenenbroeck et al. 2000; Durocher et al. 2001). The authors observed that the amplification of plasmids containing the SV40ori is a fast and demanding process in cell nutrients and energy which ends up in a detrimental effect over cell division and protein production. Furthermore, Van Craenenbroeck noted that mutation incorporation to the gene of interest coded in the plasmid provokes a decrease in recombinant protein expression (Van Craenenbroeck et al. 2000).

In the case of HEK293-EBNA1, cell growth was stopped after transfection and the concentration remained constant for all the plasmids tested. Cell viability dropped significantly between 48 and 72 hpt. There were no observable differences in terms of cell density and viability among the three plasmids in HEK293-EBNA1 (Figure 5D). Highest GagGFP production was reached by the use of pOriP, obtaining more than 78 R.F.U. (9 µg/mL) compared to 27.7 R.F.U. (3.2 µg/mL) and 33.5 R.F.U. (3.9 µg/mL) of pSV40 and p(-) respectively (Figure 5C). The presence of the oriP origin of replication in the plasmid caused a 2.8 fold improvement over the other two plasmids. Plasmid amplification is, in this case, not as fast as in the HEK293T/SV40 system. It has been reported that plasmid replication occurs along with the cell cycle (Kumar et al. 1990). Additionally, OriP origin of replication has a transactivation effect over DNA transcription which entailed an increase in recombinant protein production (Van Craenenbroeck et al. 2000).

Finally, HEK293SF-3F6 presented cell growth after transfection, like HEK293T. Nevertheless, in this case, there was no detrimental effect on cell density when the pSV40 was used (Figure 5F). HEK293SF-3F6 produced 28.2 R.F.U. (3.2 µg/mL) of GagGFP when transfected with pSV40 followed by the production obtained using p(-) and pOriP which was 49.7 R.F.U. (5.7 µg/mL) and 81.1 R.F.U. (9.4 µg/mL) respectively (Figure 5E). Since HEK293SF-3F6 does not recognize any origin of replication, in this case, differences in global GagGFP production can be due to specific interactions with each vector.

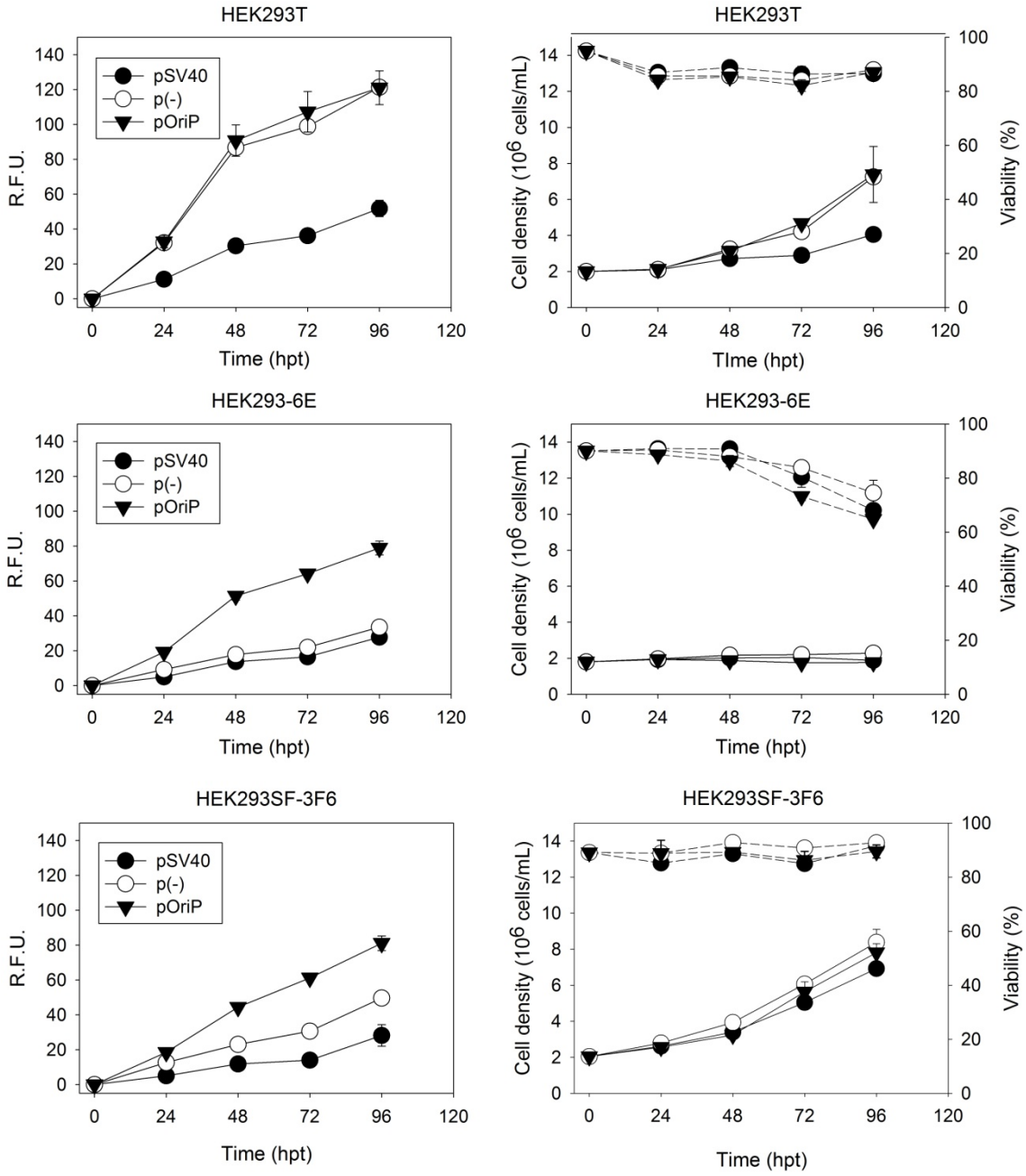

Figure 5. A, C and E show VLP production over time. B, D and F show cell density and viability over time for each vector and each cell line.

In order to make an overall comparison of the 9 systems tested, the specific production in terms of pg of GagGFP per cell was calculated (Figure 6). HEK293-EBNA1/oriP system generated highest quantities of GagGFP protein per cell, reaching 5.2 pg/cell, which is 3 times higher than the other two HEK293-EBNA1-based systems. HEK293T had, using all three plasmids, a high volumetric production; nevertheless, when it comes to specific production it remained 2.7-fold below the HEK293-EBNA1/oriP system. Indeed, HEK293T/SV40 combination had the lowest productivity for HEK293T cell line, corroborating the detrimental effect of SV40ori in HEK293T for HIV-1VLP production. Finally, HEK293SF-3F6 presented the lowest specific production rates compared to any of the other systems tested.

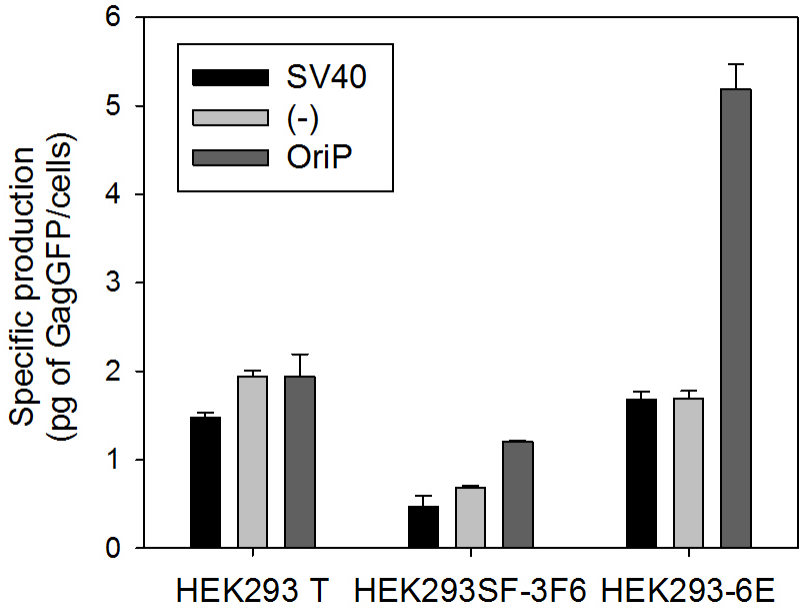

Figure 6. Comparison of the 9 specific production of GagGFP per million of cells for each combination.

Remarkable differences in growth were also observed among the three cell lines tested. HEK293T and HEK293SF-3F6 were able to grow after transfection in Ex-Cell medium. This enabled a high overall VLP production mainly due to the high cell densities reached more than to the cell specific capacity for VLP production. On the other hand, no cell growth of HEK293-EBNA1 cells was observed in the culture medium used (ExCell) after transfection. Low cell densities and hence production were reached and so overall production. Nevertheless, the highest specific production rates were reached with this cell

line, suggesting that OriP origin of replication had a very significant influence on VLP production.

HEK293-EBNA1/oriP system has been confirmed to be a very suitable platform for VLP production. Promising results have been obtained with this system, considering the high cell specific productivity observed. However, the volumetric productivity has been limited by the low growth observed with the specific medium used. Further improvements, on this direction should lead to even better performances.

Discussion

PEI-mediated TGE is a fast and cost-effective method for obtaining small quantities of the product of interest in a short period of time. It is very convenient when several candidates need to be tested in structural or preclinical studies. It has been widely used at laboratory scale for the production of recombinant proteins (Meissner et al. 2000), viruses (Grieger et al. 2016), and vaccines candidates (Cervera et al. 2013; Fontana et al. 2015). Recently, it has also been gaining importance at industrial scale (Baldi et al. 2005). DNA and PEI concentrations optimization has been deeply studied over several cell lines and recombinant proteins, such as HEK293 (Cervera et al. 2015a; Yang et al. 2017), CHO (Derouazi et al. 2004), or CAP-T (Gutiérrez-Granados et al. 2016), among others. Nevertheless, the possibility of extrapolating the found optimal concentrations to other cell lines or expression vectors has not been previously studied. In this work, DNA and PEI concentrations were optimized for three different HEK293 cell lines and using three different expression vectors. Optimal concentrations of DNA and PEI for the TGE protocol used were obtained for the nine different combinations were the same: 2.34 μg/mL for DNA and 5.81 μg/mL for PEI. These concentrations were found to be independent on the HEK293 cell line or the expression vector, demonstrating that DNA and PEI optimal concentrations do not need to be modified when changing the HEK293 cell line or the expression vector. This fact may lead to money and time saving in bioprocess development at both laboratory and industrial scale. Furthermore, differences in plasmid size and hence gene copy number were not influencing transfection efficiency.

Optimal DNA and PEI concentrations were validated with each of the cell line/plasmid type combination. HEK293SF-3F6 was the reference cell line which did not

recognize any origin of replication and was maintained as the control cell line. HEK293SF-3F6 presented lowest specific production rates compared with the other two cell lines and for all the three plasmids tested. HEK293T stably expresses the large T antigen which is able to recognize the origin of replication SV40ori (Dean et al. 1987). HEK293T when transfected with pSV40 presented lowest specific production and growth rates compared with the other two expression vectors, which is in agreement with other studies previously reported (Van Craenenbroeck et al. 2000; Durocher et al. 2001; Pham et al. 2006). The episomal replication of the DNA is a very fast process which can end up in detrimental effects over recombinant protein production. It provokes a stop in cell growth and it incorporates a high number of mutations in the DNA sequence leading to a production decrease (Van Craenenbroeck et al. 2000; Durocher et al. 2001; Pham et al. 2006). HEK293-EBNA1 stably expresses EBNA-1 protein which can recognize OriP origin of replication and carry out the episomal replication of the DNA (Baldi et al. 2007; Segura et al. 2007). For GagGFP VLP production, HEK293-EBNA1 when transfected with pOriP presented the highest specific productivity compared with the other two vectors and cell lines. Episomal DNA replication in this case occurs at the same time as cell division (Kumar et al. 1990). Furthermore, it has been reported that OriP origin of replication has a transactivation effect over the gene codified in the vector sequence (Van Craenenbroeck et al. 2000; Durocher et al. 2001; Pham et al. 2006). These two features have a beneficial effect in recombinant gene expression that is observed in an increase of GagGFP VLP production. HEK293-EBNA1/OriP combination demonstrated to be a very good choice for HIV-1 VLP production among the other systems tested and production conditions should be optimized to further enhance GagGFP VLP production.

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Stable HEK293 cell lin generation for the production of GagGFP VLPs

Results, chapter 4

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Abstract

Virus-like particles are nanostructures that resemble the configuration of a virus and induce a strong immune response. They do not contain genetic material which makes them safe for both vaccine manufacturers and recipients. HIV-1 VLPs are based on the polyprotein Gag which can form spherical structures when recombinantly expressed. Mammalian cell platforms are the selected systems for such complex and enveloped VLPs. This approach allows the incorporation of accurate post translational modifications into the VLP which is important for the vaccine efficacy. Recombinant protein production in mammalian cells can be achieved through two traditional methods: transient gene expression (TGE) or stable gene expression (SGE). In this work, the SGE of a HEK293 cell line for the production of Gag VLPs has been achieved through a random site integration strategy. After a screening process based on GagGFP production, five clones were selected for adaptation to suspension and serum-free conditions. Four clones were successfully adapted (10H9, 9A7, 4A7, 3A1). 10H9 presented similar duplication time and maximum cell density than the other clones. Furthermore, 10H9 showed highest GagGFP productivity (67.58 ng/(mL·d)) and also highest specific productivity ($1.74x10^{-2}$ pg of GagGFP/(cell·d) and was hence the selected GagGFP producer HEK293 clone.

Keywords: Stable cell line, virus-like particles, HEK293, bioprocess

Introduction

Recombinant protein production in mammalian cell systems is usually performed by two methodologies: transient gene expression (TGE) and stable gene expression (SGE). Transient gene expression consists on the introduction of a plasmid DNA into a cell. The DNA can be introduced by physical or chemical methods. The DNA arrives into the nucleus where it is transcribed and then translated into the protein of interest. This is a cost-effective and fast process in which the product can be obtained in a short period of time (in the order of weeks) (Baldi et al. 2007; Cervera et al. 2013; Merten et al. 2014). TGE is very suitable in the early stages of the development of a new pharmaceutical when several product variants need to tested; it is also convenient when the product of interest is toxic for the cell. TGE has the main disadvantage that the expression is lost over time. Since the plasmid is not integrated in the cell genome it is not duplicated and the DNA is lost in each cell division (Middleton and Sugden 1994; Wade-Martins et al. 1999). TGE at large scale requires high amounts of plasmid DNA which makes the process more expensive and increases the variability from batch to batch. Transient transfection generally improves by performing a partial or complete medium exchange before DNA addition. This is a feasible process at small scale but it turns more complicated at bioreactor scale (Gutiérrez-Granados et al. 2018). In more advanced phases of the biopharmaceutical development, the stable gene expression (SGE) is the selected approach. SGE consists in the integration of a piece of DNA into the cell genome. This integration can be done randomly or directed (Wurm 2004). There are several methodologies available for the directed DNA integration (Lafountaine et al. 2015) and all of them rely on a restriction enzyme for introducing double stranded breaks in the genome DNA at specific locations. Random integration relies on the ability of naked DNA to be integrated in the cell genome. A portion of DNA is delivered to the cells which can eventually integrate in a random locus of the genome (Jostock 2011). Random integration allows the integration of several copies of the gene of interest through the genome; while the site specific integration targets the gene of interest to the selected site. Desirably, this locus of the genome generally corresponds to a hot spot, a region of the genome described for high protein expression and low gene silencing. Both random and directed DNA integration are followed by a selection and screening process. The introduced DNA encodes for the gene of interest but also for a selection marker (generally an antibiotic resistance). The cells are afterward subjected to a selective pressure and the population is enriched with producer cells (Fontana et al. 2015; Venereo-Sanchez et al. 2016). A

heterogeneous population of cells is obtained which is called polyclonal cell line. This population is composed of many different cells that produce the product of interest at different titers. In order to obtain higher titers a clonal selection is normally carried out and to do so a single cell separation process is performed. Cell sorter can be used to separate the cells one by one in a well plate (Pichler et al. 2011). These clonal cells are then expanded and the best producers and the ones exhibiting better growth are selected. If the product of interest has been fused to a fluorescence protein, the cell sorter can also differentiate between fluorescent and non-fluorescent cells and seed them separately (Priola et al. 2016). The single clones are expanded and growth curves are carried out in order to select the best producers and the ones with best specific growth rates. Random integration relies very much on the selection process. The probability of finding a high producer clone relies directly on the number of single clones analyzed (Priola et al. 2016). If an exhaustive selection process can be carried out in the random integration, very high producer cell lines can be found and also new integration sites. At industrial scale an exhaustive process for selecting the best clone is carried out in order to achieve high specific productivities. The use of inducible promoters should also be taken into consideration when cytotoxic products need to be produced. Constitutive expression of a cytotoxic protein in the cell leads to low cell growth and viability; and eventually to cell death. Inducible promoters allow the expression of the protein at the point when they are activated according to the production process; not affecting cell physiological state in the rest of it (Venereo-Sanchez et al. 2016).

Virus-like particles are nanostructures that resemble the natural conformation of a virus. They are formed by one or several viral proteins that can assemble into particles. They do not contain viral genetic material which makes them safer for both recipients and operators in vaccine manufacturing sites. They generate a potent immune response due to their repetitive subunits (Roldao et al. 2010; Fuenmayor et al. 2017b). Post translational modifications (PTMs) play a very important role in the antigen-immune system recognition. Mammalian cell platforms are able to incorporate accurate PTMs to the recombinant proteins and hence are very good candidates for VLP production (Zhu 2012). Gag polyprotein from HIV-1 has the ability to self-assemble into VLPs when recombinantly expressed. It can travel to the vicinity of the cell membrane and bud from it creating enveloped vaccine candidates (Cervera et al. 2017). Since part of the cell membrane is taken by the VLP, membrane proteins can be co-expressed with Gag and the VLP

incorporates them in its surface. These membrane proteins can be HIV viral proteins but also proteins from other viruses, enabling to develop chimeric virus-like particles (Holzer et al. 2003; Doan et al. 2005; Zhang et al. 2011). Gag-based VLPs can also be used as nanocarriers for the transportation of different pharmaceuticals to target specific cell types. Gag protein has been widely expressed in HEK293 cell lines both stably and transiently. HEK293 is a cell-derived human cell line which has been deeply characterized. It is able to grow under suspension and serum-free conditions which makes it a very good production platform candidate for the production of VLPs and other pharmaceuticals (Meissner et al. 2000; Thomas and Smart 2005; Baldi et al. 2007). Gag protein is a very promising platform for the development of many different VLPs or nanocarriers. It is hence of great interest the development and characterization of a stable cell line for the production of this polyprotein. This stable cell line can be used to introduce other membrane proteins candidates by TGE allowing to produce functionalized VLPs. In this work, a Gag fused with GFP has been used in order to facilitate the cell clone screening as well as the follow-up of the protein production. The objective of this work is the development of a stable HEK293 cell line for the expression of GagGFP able to grow under serum-free and suspension conditions by random integration.

Materials and Methods

Cell line, culture media and conditions

The serum-free suspension-adapted HEK293 cell line HEK293SF-3F6 (kindly provided by Dr. Amine Kamen from the BRI at the National Research Council of Canada and McGill University (Montreal, Canada)) has been used to perform this work. The cell line was derived from a cGMP master cell bank that is available for manufacturing of clinical material. Cells were cultured in Freestyle 293 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% Pluronic® (Invitrogen, Carlsbad, CA, USA). Medium was also supplemented with 1.6 mg/L of r-transferrin (Merck Millipore, Kankakee, IL, USA), 19.8 mg/L of r-insulin (Novo Nordisk Pharmatek, Køge, Denmark.) and 0.9X of an *in-house* developed lipid mixture to maximize cell growth and productivity (Cervera et al. 2013). For cell selection, the medium was also supplemented with the antibiotic G418 (ThermoFisher, MA, USA). After cell sorting, 10% FBS was added to the cell medium, as well as gentamicin (ThermoFisher, MA, USA) and antibiotic-antimycotic (ThermoFisher, MA, USA)

Suspension cell cultures were routinely maintained in 125-mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL of culture medium. Flasks were shaken at 110 rpm using an orbital shaker (Stuart, Stone, UK) placed in an incubator maintained at 37ºC in a humidified atmosphere of 5% CO_2 in air. After cell sorting, the cells were seeded individually in 96-well plates and grown under adherent conditions in a humidified incubator. At higher cell densities, they were transferred to 25 mm^2 T-flasks, 75 mm^2 T-flask (Sigma-Aldrich, Saint Louis, MO, USA), 96-well, 24- well and 6-well plates (Sigma-Aldrich, Saint Louis, MO, USA) depending on the moment of the cell culture expansion. Cells growing in adherence in monolayers were re-suspended by gently shaking the T-flask or the well plate and pipetting, avoiding the use of trypsin.

Cell count and viability were determined using the automatic cell counter NucleoCounter NC-300 (Chemometec, Allerod, Denmark). Cells were observed under the Olympus CKX41 optical inverted microscope (Olympus, Tokyo, Japan) to assess cell morphological states. Nikon Eclipse TE2000E optical fluorescence inverted microscope (Nikon, Tokyo, Japan) was used for qualitative analysis of GagGFP expression.

Plasmids and cell transfection

The pGagGFP plasmid codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP and for the KanR resistance gene which confers resistance to G418. The plasmid from the NIH AIDS Reagent Program (Cat 11468) was constructed by cloning the Gag sequence from pCMV55M1-10 into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA). HEK293 suspension cells were transfected using 25-kDa linear polyethilenimine (PEI) (PolySciences, Warrington, PA, USA) at a cell density of $2x10^6$ cells/mL. The DNA used for transfection was either circular or linearized. To obtain the linearized DNA, the circular plasmid was digested with FastDigest *Vsp*I (ThermoFisher, Maltham, MA, USA) following manufacturer's instructions. Prior to transfection, a complete medium exchange was performed by centrifuging the cell culture at 300xg for 5 minutes. Cells were resuspended in fresh tempered supplemented FreeStyle medium. PEI:DNA complexes were formed by adding PEI to plasmid DNA diluted in fresh culture media (10% of the total volume of the culture to be transfected). The mixture was vortexed three times for three seconds and was incubated for 15 min at room temperature to allow complex formation prior to its addition to the cell culture.

The percentage of GFP positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA). Cell sorting was carried out using a fluorescence activated BD FACSJazz Cell Sorter (BD, Biosciences, MA, USA).

Toxicity assays

G418 antibiotic was used for selecting GagGFP expressing cells. In order to select the G418 concentration for cell producer population enrichment, a toxicity assay was first carried out. 100 µL of HEK293 cell were seeded at $0.5x10^6$ cells/mL in a 96-well plate with 10 µL of antibiotic at increasing concentrations in triplicate. The plate was cultured under suspension following the standard HEK293 culture conditions. After 48 hours, 20 µL of MTT reagent (Promega, Fitchburg, WI, USA) was added to the wells. The plate was then incubated at 130 rpm and 37ºC for 1 hour. A standard curve with cell concentrations from 0 to $4x10^6$ cells/mL was also added in duplicate and the same MTT assay was performed. The plate was read on a Multilabel Plate Reader VICTOR3 (Perkin Elmer, MA, USA) at 490 nm.

VLP quantitation

The concentration of GagGFP VLPs was assessed by fluorimetry using an *in-house* developed and qualified quantification assay. VLP-containing supernatants were recovered by cell culture centrifugation at 10000×g for 5 min. Green fluorescence was measured at room temperature using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) set as follows: λex= 488 nm (slit 5 nm), λem= 510 nm (slit 10 nm) (Gutiérrez-Granados et al. 2013).

GagGFP polyprotein concentrations were also quantified by p24 ELISA using the Innotest HIV antigen mAb (Innogenetics NV, Gent, Belgium). Analysis was performed according to manufacturer's instructions.

Results and discussion

Toxicity assays

The plasmid used for cell transfection encodes for the GagGFP polyprotein which generates VLPs. In order to carry out the selection of the producer cells, the plasmid also encodes for G418 resistance gene. This gene confers the cells with resistance to G418 antibiotic. The antibiotic concentration used for cell selection was selected based on antibiotic toxicity assays. A wide range of antibiotic concentrations ranging from 25 to 1125 µg/mL was tested. G418 is able to bind to the 30S subunit ribosome and inhibits protein translation, stopping cell growth. As it is shown in Figure 1, only the 25 µg/mL concentration allowed growth during the 48 hours of the test; 125 µg/mL was the minimum G418 inhibitory concentration. Nevertheless, in order to incorporate higher selective pressure to the transfected culture, 225 µg/mL was the chosen concentration for cell selection.

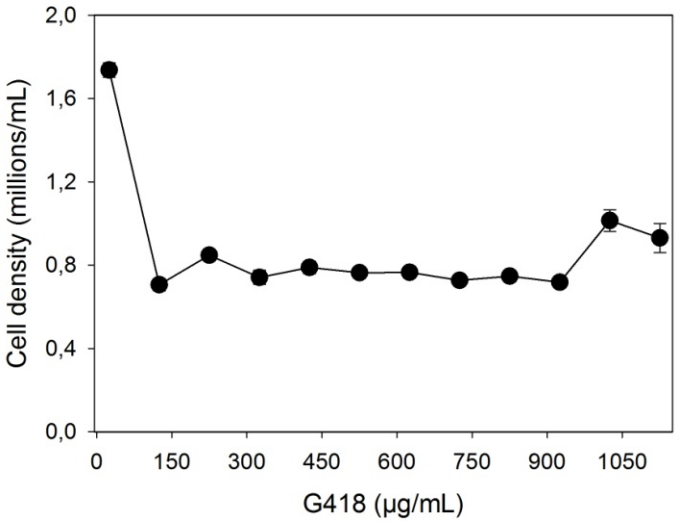

Figure 1. G418 toxicity assay. Cell concentration after 48 h of culture at different G418 concentrations.

Cell pool generation

pGagGFP plasmid was used for transfecting HEK293 cell cultures. Cells were transfected as previously described in suspension in 6-well plates. Both circular and linearized plasmids were used for transfecting the cells in order to know if there were any

differences in the generation of the stable cell line. Cells were transfected at $2x10^6$ cells/mL without G418 antibiotic to let the cells adapt and start producing the resistance. At 48 hpt, transfection efficiency in percentage of GFP positive cells was 30.7% in the circular-transfected culture and 24.4% in the linear-transfected culture. The culture was diluted to $0.5x10^6$ cells/mL in order to provide fresh nutrients and remove waste products and G418 was added to the cell culture for cell selection at a concentration of 225 µg/mL. When cell density was higher than $2x10^6$ cells/mL, the culture was diluted; if no cell growth was observed, a complete medium exchange was carried out. Figure 2 shows the cell growth and viabilities of the two cultures during the whole process of the generation of the stable cell pool. After supplementing the medium with G418 at 2 dpt, cell growth was not prevented and decrease cell viability was still high. The antibiotic concentration was thus duplicated to make a stronger selection and eliminate the cells that were not stably transfected as indicated in Figure 2. After that point a deep decrease in cell viability was observed and cell growth was stopped. After day 9 post-transfection, cell viability started to recover. Nevertheless, a regular cell growth was not observed in the culture. Once the cell viability was totally recovered (16 dpt), cell sorting was performed to obtain VLP producer single cells. Cell density was still not fully recovered, but in order to be conservative, cell sorting was in any case performed.

There were no observable differences in terms of cell growth or viability between the cell culture that was transfected with circular plasmid and the one transfected with linearized plasmid. Using a circular plasmid was as efficient as a linearized one for stable cell line generation.

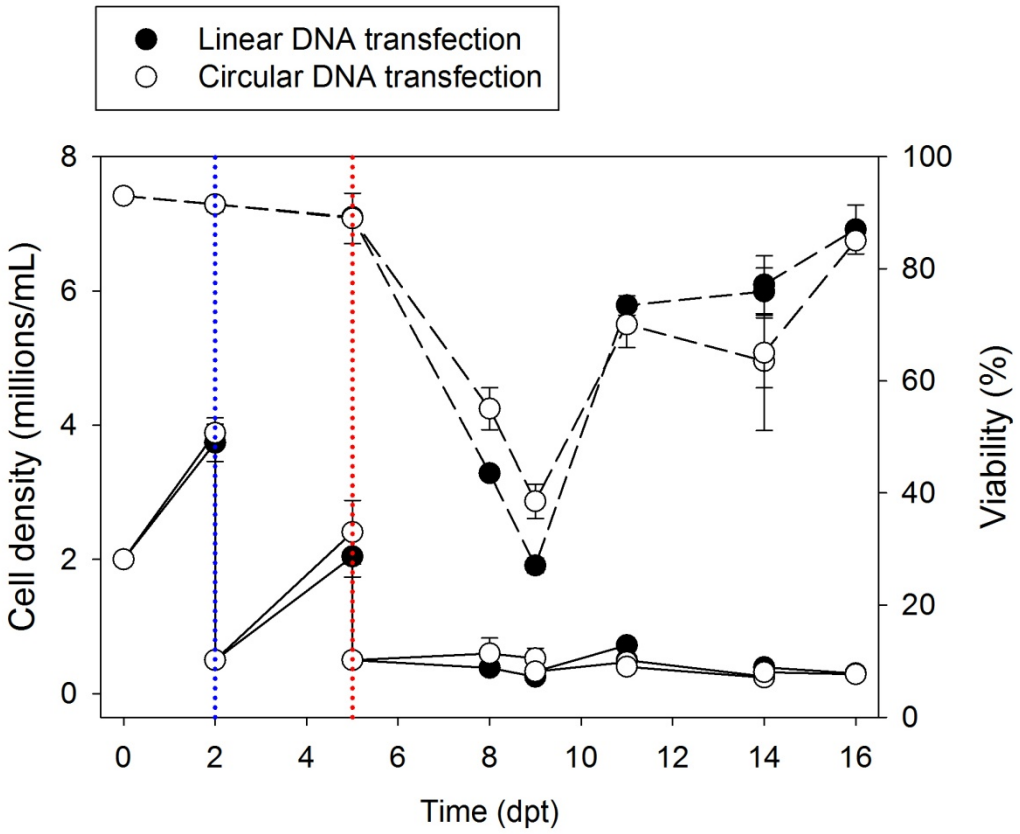

Figure 2. Stable cell pool generation. G418 at a final concentration of 225 µg/mL was added at 2 dpt (blue dotted line) and duplicated at 5 dpt (red dotted line).

Cell sorting

At day 16 after transfection, cell sorting was carried out. The cell culture was a mix composed of several different cell types: some of the cells might have acquired only the G418 resistance gene but not the GagGFP gene; others might have incorporated both. Among the ones that have incorporated both resistance and GagGFP genes, the number of copies acquired will vary cell to cell. Furthermore, the specific locus of the genome where the genes have been integrated can be different from cell to cell. In order to select only the cells that are both resistant and GagGFP-producer and selecting individual cells leading to have monoclonal cell lines, a fluorescence-based cell sorting was performed. Seven 96-well plates were filled up with single GFP positive cells. Two different sorting criteria were used. The first one was to sort GFP positive cells with higher fluorescein isotiocyanate (FITC) values than 70-80; and the second was to sort GFP positive cells with higher FITC values than 200-250. This discrimination was done in order to establish a relation between the fluorescence intensity of the clones at that point and the final specific GagGFP production.

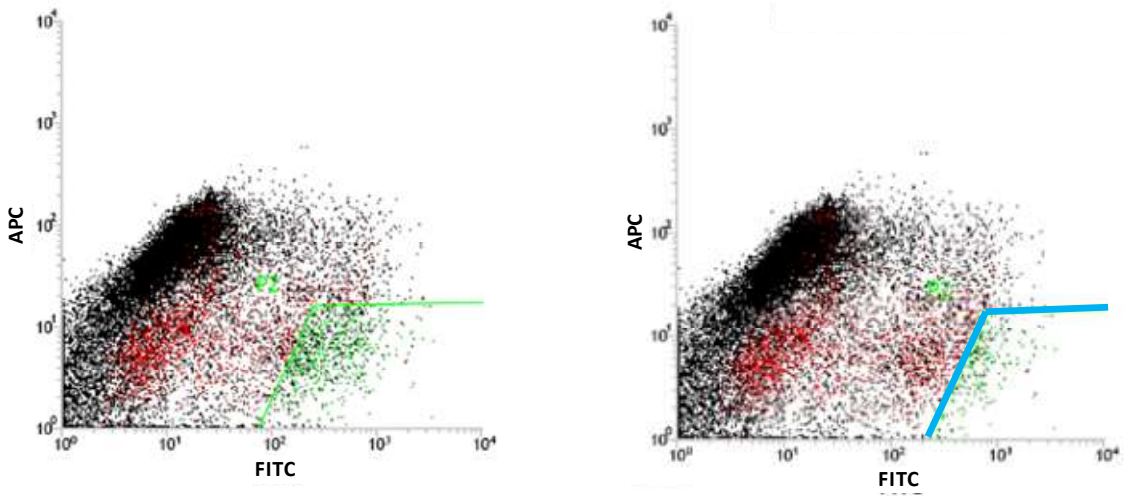

Figure 3. Flow citometry plots. The green line indicates the less restrictive selection criteria (FITC>70-80) and the blue line indicates the more restrictive criteria (FITC>200-250).

Clone amplification

Previous experiments were carried out with non-transfected HEK293SF-3F6 in order to determine if cell amplification in adherent 96-well plates from a single cell was possible. Single cell growth was only achievable with the supplementation of 10% FBS. Sorted GFP positive cells were seeded one per well and 10% FBS. Throughout visual observation under the inverted microscope, cell growth was detected in about 102 wells out of 672 that were seeded. Medium replacement was done in a weekly basis to all the plates until cell confluency was observed. At 18 days post cell sorting, the clones were transferred to 24-well plates and at day 21 post cell sorting to 6-well plates. At that point, cell culture supernatant from each single well was recovered to analyze GagGFP production. A final number of 102 clones could be amplified and at that point cryopreserved at -80 ºC.

Under the whole amplification process, clones were eventually observed under fluorescence inverted microscope to ensure that GagGFP production was not lost. Fluorescence intensity was different throughout the different clones. Figure 4 shows some examples of evident GagGFP producer clones.

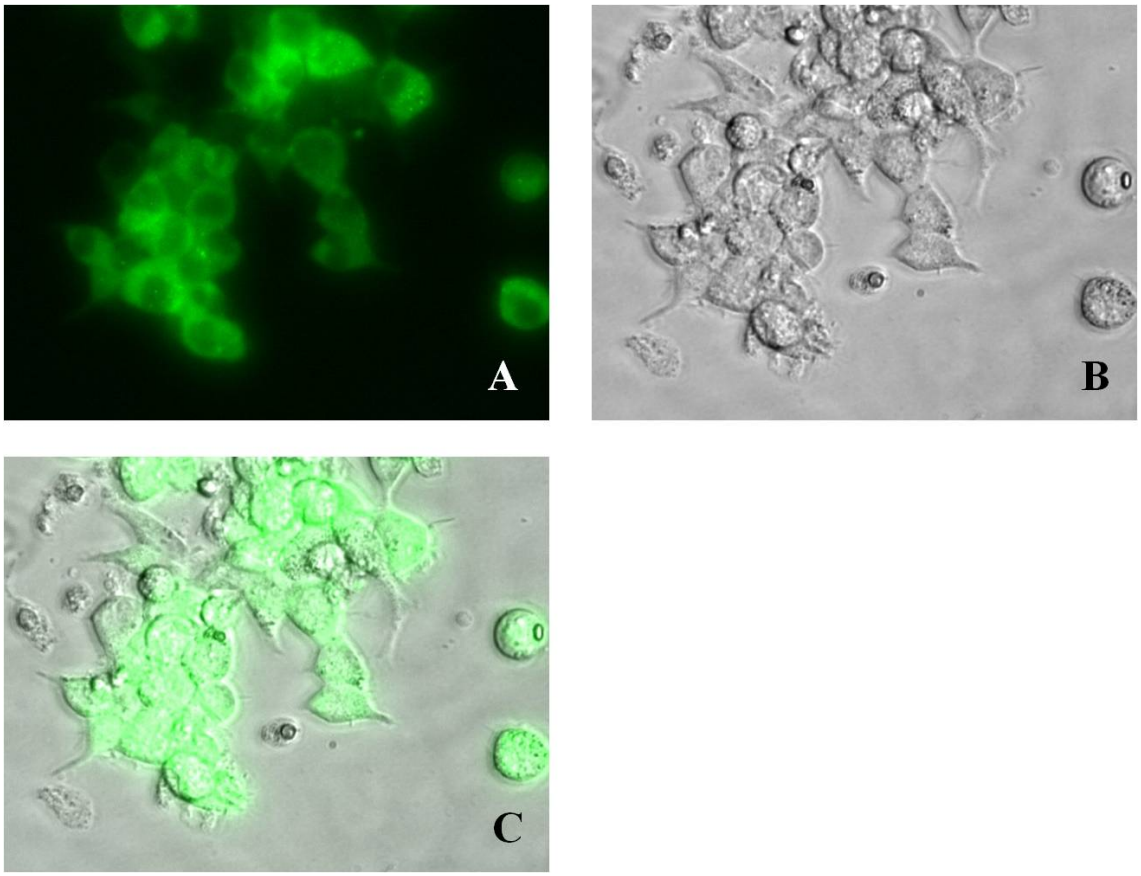

Figure 4. 24E9 clone observation under the inverted microscope (x40). A fluorescence flied. B Clear field. C Fluorescence and clear field images overlapping.

GagGFP production quantification

In order to make an initial comparison among the clones, fluorescence in the supernatant was analyzed in days 21 and 22 after sorting. The 40 clones exhibiting highest fluorescence were selected and p24 ELISA analysis was also carried out for a more exhaustive comparison. Figure 5 shows the different clones analyzed ordered by GagGFP concentration in the supernatant at day 22 after cell sorting.

The five HEK293 selected clones according to higher GagGFP production were 4A7, 10H9, 9A7, 9C1 and 3A1. These clones were thawed and amplified in adherence for creating a bigger cell bank. All the clones analyzed had similar cell densities and hence the best producers were the ones exhibiting higher GagGFP concentration in the supernatant.

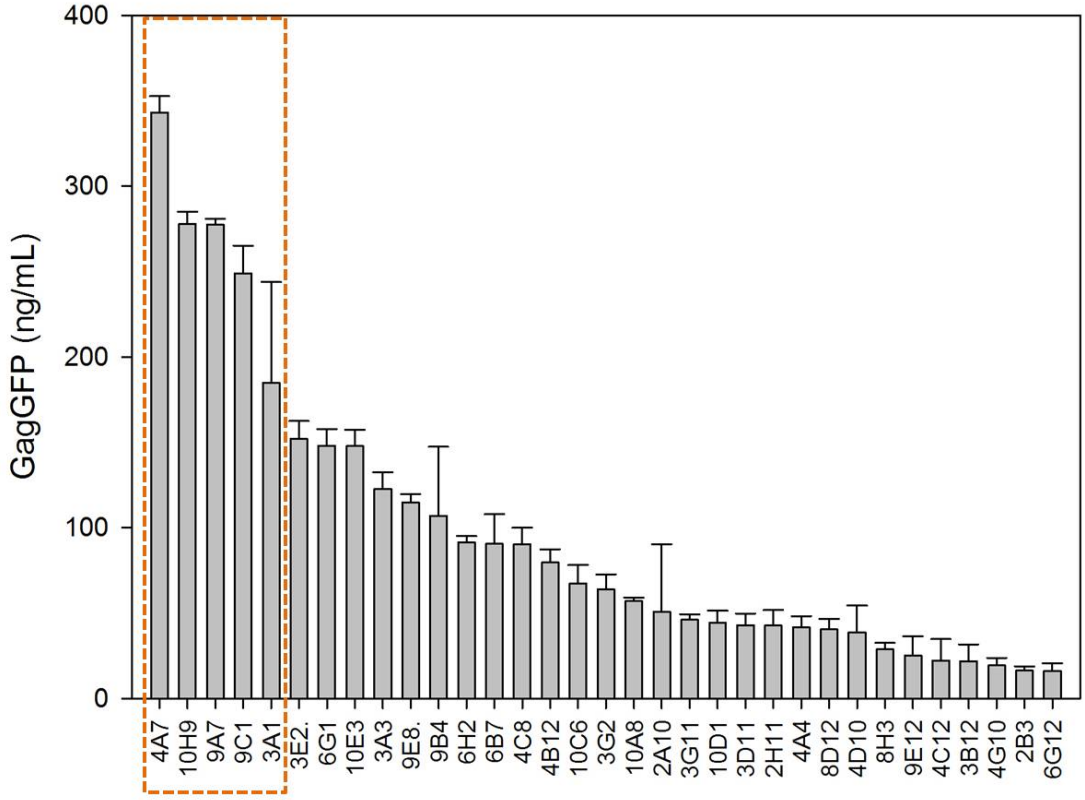

Figure 5. Fluorescence clones ordered by GagGFP production.

Suspension and serum-free culture adaptation

As pointed out, the selected clones were growing in adherence and in the presence of serum conditions to facilitate clonal cell growth in the well plates. Serum supplementation is not recommended due to its variable composition, its non-compatibility with GMP procedures, the possibility of containing viruses, prions, or growth inhibitors, its cost and also the interferences that it causes in further downstream processes. Re-adaptation of the selected clones to suspension and serum-free conditions was then a necessary step to do next, since the ultimate goal is to have a stable clone that can be used in high volumes and cell densities for production processes.

The adherent clones exhibiting more than 90% viability and 80-95% of confluence were seeded at $0.5x10^6$ cells/mL in 125-mL polycarbonate Erlemenyer flasks with 10% FBS. Cells were diluted to $0.5x10^6$ cells/mL when cell concentration was higher than $1x10^6$ cells/mL. If no cell growth was observed, cell medium was exchanged every 4 days. Clones

were considered adapted when cell growth was observed longer than 3 passages with more than 95% viability.

All the clones had a similar behavior when adapting to suspension conditions. Firstly, cell growth was stopped and a drop in cell viability was observed. Secondly, cell viability recovered but no cell growth was observed. Finally, a constant cell growth was achieved with high viabilities indicating the correct cell adaption to suspension. Clone 9C1 could not be adapted to suspension and was hence discarded (Figure 6).

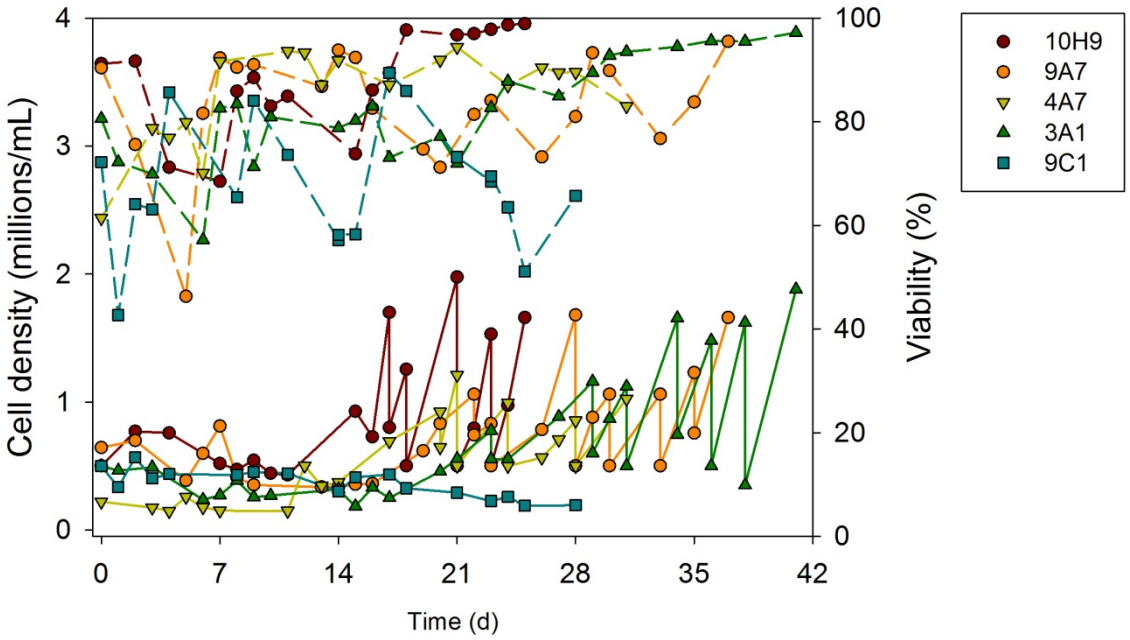

Figure 6. Suspension adaptation. All the clones were adapted to suspension but the 9C1 clone which was discarded.

The clones were also adapted to serum-free growing conditions. Once the clones were adapted to suspension, FBS was eventually removed from the cell culture. FBS was gradually removed from the cell medium from 10% to 0%. There were not observable differences in serum-free adaptation among the clones. The cell viability did not decrease during the process.

Cell clone comparison

Four out of the five selected clones could be adapted to grow in suspension and under serum-free conditions. In order to make a final selection and select the best clone, growth curves were carried out. Figure 7 shows the cell density and viability over time among the different clones. In terms of maximum cell density and specific growth rate, the four clones appear to behave very similarly (Table 1). 4A7 presented lowest cell growth, since it reached a maximum cell growth 20-30% lower than the other clones; furthermore, its duplication time was 10 hours lower than the rest.

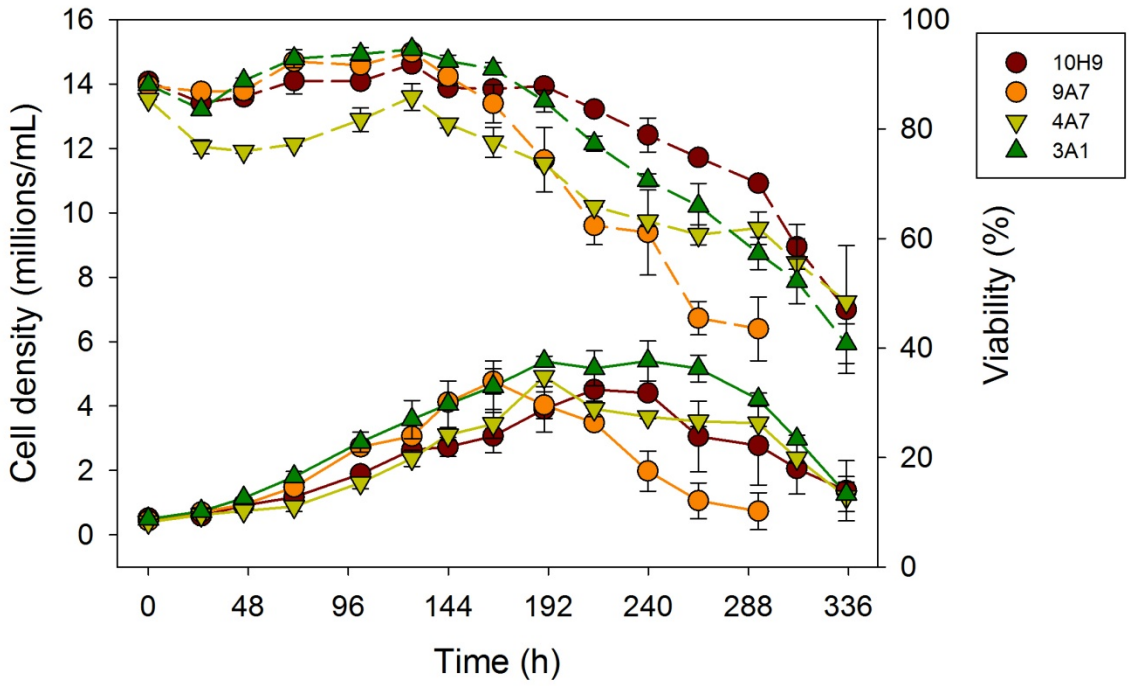

Figure 7. Cell growth kinetics for the four selected clones.

Table 1. Maximum cell density and duplication time for the four selected clones

	Duplication time	Maximum cell density
10H9	26,7 ± 1,9	4,5 ± 0,2
9A7	23,1 ± 1,2	4,8 ± 0,4
4A7	32,5 ± 3,7	3,7 ± 0,1
3A1	24,5 ± 0,9	5,2 ± 0,4

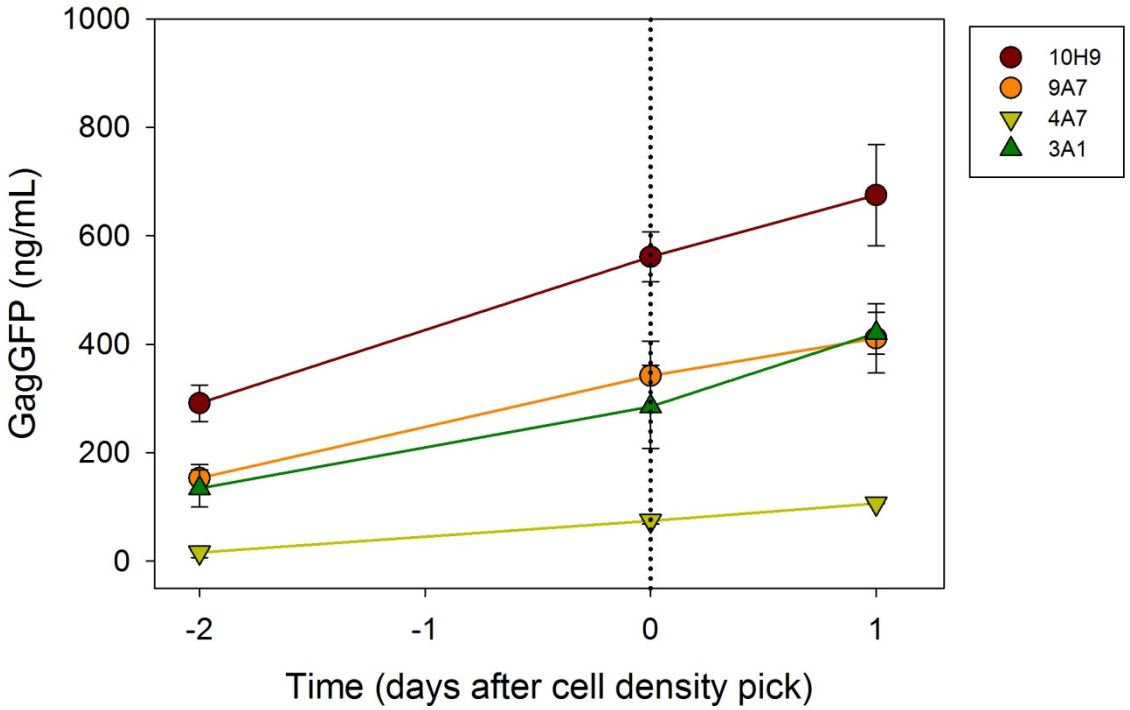

Figure 8. GagGFP production for the four selected clones. The measures were taken at the day of maximum cell growth (0), one day after (1) and 2 days before (-2).

In respect to production, Figure 8 shows the GagGFP concentration in the supernatant for the four clones at 3 different time points: the day of maximum cell growth, 2 days before and 1 after.

The clone 10H9 presented highest GagGFP production for all the time points analyzed and highest specific productivity (Table 2). Furthermore, it presented a similar maximum cell growth and duplication time than the other clones and was hence the selected clone. A cell bank with the adapted clone 10H9 to suspension and serum-free conditions was therefore generated. GagGFP VLPs are high complex structures and hence the production observed not only depends on its specific productivity. There are several cellular processing, including VLP budding, which also affect the final VLP production. For this reason, GagGFP productivity obtained in this work is not comparable with productivities achieved by industrial cell lines for the production of monoclonal antibodies (10-20 pg/(cell·day)) (Priola et al. 2016).

Table 2. Specific productivity and volumetric productivity of the adapted clones

	Specific productivity (pg/(cell·d)	Productivity (ng/(mL·d))
10H9	$1.74 \cdot 10^{-2} \pm 2.41 \cdot 10^{-3}$	67.58 ± 9.215
9A7	$1.46 \cdot 10^{-2} \pm 3.43 \cdot 10^{-3}$	11.93 ± 0.37
4A7	$3.98E\text{-}03 \pm 9.54 \cdot 10^{-4}$	51.90 ± 7.99
3A1	$1.02 \cdot 10^{-2} \pm 1.55 \cdot 10^{-3}$	47.14 ± 3.53

The performance of the selected clone was compared with the generation of GagGFP VLPs in HEK293 cells by TGE (Cervera et al. 2013; Fuenmayor et al. 2017a). HEK293 transfection reported a maximum GagGFP production of 2800 ng/mL 48 hours post transfection; on the other hand, 10H9 clone achieved 67.6 ng/(mL·d). Transient transfection of HEK293 cells produced 4 times more GagGFP than the generated stable cell line. TGE still provides higher production compared with the SGE. Nevertheless, in TGE the DNA to transfect the cells need to be produced which turn more difficult when larger production volumes have to be performed. Furthermore, in TGE the expression is lost over time, and pseudo-perfusion strategies with retransfections must be done in order to prolong the production phase (Cervera et al. 2015b). This is a first approximation for the stable cell line generation. Two parameters can be improved in order to increase production titers: cell density and specific productivity. Medium screening and optimization would lead to obtain higher specific growth rates; furthermore, other cultivation modes such as fed-batch or perfusion can be tested in order to further increase production titers. Moreover, transient transfection of membrane proteins from HIV or other viruses could be done in order to obtain functional vaccine candidates.

Conclusions

The generation of a stable HEK293 cell line for GagGFP VLP production has been successfully carried out. Both circular and linearlized plasmid have provided similar results in the process. Among the 672 clones seeded, 102 reported cellular growth in 96-well plates, which represents a 15.2% of all the seeded clones. Single cell amplification was carried out in adherence and presence of serum and GagGFP was analyzed by ELISA in the supernatant of these clones. The five clones exhibiting higher GagGFP concentration in the

supernatant were selected and adapted to suspension and serum-free conditions. 9C1 clone could not be adapted to grow under these conditions and was hence discarded. The other four clones (10H9, 9A7, 4A7 and 3A1) were successfully adapted and growth curves were carried out in order to compare them in terms of cell growth, viability and GagGFP production. 4A7 clone presented a lower maximum growth rate and also higher duplication time compared with the other ones. 10H9, 9A7 and 3A1 achieved a maximum cell density around $5x10^6$ cells/mL and a duplication time of around 25 hours in batch cultivation. In terms of GagGFP production, 10H9 showed highest specific productivity (0.73 fg of GagGFP/(cell·h)) and was hence the clone selected.

Further optimization work should be done in medium optimization and fed batch and perfusion culture modes should be optimized in order to increase VLP production in 10H9. Transient transfection could also be tested using the 10H9 clone for expressing env protein from HIV or other viral membrane proteins in order to obtain functional virus-like particles.

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Overall discussion and future directions

In this work several strategies have been followed for the enhancement of Gag VLP production titers using TGE in HEK293 cell lines.

The use of chemical additives for enhancing VLP production in HEK293 cells was previously reported (Cervera et al. 2015a). The combination of lithium acetate, valproic acid and caffeine led to an improvement of 4-fold in batch cultivation. Chemical additives have been widely used for the enhancement of recombinant proteins both in TGE and in stable cell lines. Lithium acetate is known for creating porous in the cell membrane which help the entry of DNA:PEI complexes inside the cells (Ye et al. 2009). Valproic acid is a histone deacetylase (HDAC) inhibitor and caffeine has a phosphodiesterase (PDE) inhibitory effect, which leads to an increase in the recombinant protein expression (Backliwal et al. 2008d). The use of chemical additives has some disadvantages such as the increase of costs and the complexity of downstream processing; furthermore they have a detrimental effect over cell density and viability. In order to overcome these drawbacks, the incorporation of shRNAs in the expression vector to provide the same biological effect as the additives was proposed. Two shRNAs were added to the expression plasmid for replacing the need of valproic acid and caffeine but obtaining the same effect and hence reduce the bioprocess complexity. By the use of this plasmid, HDAC5 mRNA was inhibited and the VLP production was increased by 2.5-fold; nevertheless, no PDE8A mRNA inhibition was accomplished. This explains that the improvement is not as high as when caffeine and valproic acid are added to the cell medium. The interference of HDAC5 expression by genetic control was previously reported by the transfection of miR-2861 for enhacing SEAP and IgG productivity in stable CHO cell lines (Fischer et al. 2015). The use of shRNA codified in the expression plasmid was found to be a high potential approach for increasing the recombinant protein production in TGE. Valproic acid and caffeine inhibit a large group of enzymes which are involved in the expression of recombinant proteins. Deeper proteomic analysis should be carried out to find which proteins are involved in recombinant protein expression in TGE.

In TGE, the plasmid DNA is expressed episomally and not integrated in the cell genome, and consequently it is diluted in every cell replication; which is one of the main disadvantages of TGE causing the transgene expression loss over time (Van Craenenbroeck et al. 2000). In order to prolong the production phase of the bioprocess, the extended gene expression (EGE) procedure was previously developed (Cervera et al. 2015b). This procedure is based on the combination of several medium exchanges and retransfections. The cells are provided every 48 hour post transfection (hpt) with fresh nutrients essential

for VLP production and cell duplication. Waste products are also removed and the product of interest can be stored in the appropriate conditions. Furthermore, two retransfections are carried out at 48 and 96 hpt to provide the non-transfected cells with DNA plasmid to become VLP producer cells. This procedure has increased VLP production titers by 12-fold compared with a standard batch cultivation. As it was previously discussed, chemical additive supplementation leads to an increase in the production titers. As a step forward in this bioprocess optimization, EGE was combined with the medium supplementation with lithium acetate, valproic acid and caffeine, improving the process by 1.5-fold. Additive supplementation provoked the arrest of cell division and an increase of cellular death. Cellular metabolic state is important for the quality of the production as virus-like particles require complex PTMs for providing a good immune response in the vaccine recipient (Zhu 2012). shRNA had a similar effect on VLP production but eluding the cytotoxic effect of the additives, and was hence tested in EGE as well. Similar results were obtained by combining shRNA and EGE and cell density was increased by 1.3-fold compared with the EGE with additive supplementation. The use of shRNA in TGE showed promising results in both batch and perfusion-based modes and they should be implemented in routinely TGE procedures, since they provide an increase in VLP production and present no observable drawbacks. Furthermore, other specific shRNA (especially for PDE inhibition) should be designed in order to further maximize VLP production. Other biological mechanisms involved in VLP production such as VLP budding or PTM incorporation should be also studied and shRNAs should be generated and tested for these specific biological mechanisms.

The potential of EGE has been demonstrated. EGE improvement by combining it with chemical additives or the use of shRNA leads to a remarkable increase in VLP production. Although TGE is especially used in the initial phases of the development of a new pharmaceuticals; it has also been gaining relevance in the production phases as well (Gutiérrez-Granados et al. 2018). There are some recombinant proteins that are cytotoxic for the producer cell line and hence it is more convenient to use of TGE than generating a stable cell line. Furthermore, there are some vaccines that change every year such as the influenza vaccine, and hence TGE offers an advantage in respect to the generation of a stable cell line (Venereo-Sanchez et al. 2016). Transient gene expression has several difficulties when it is carried out in bioreactor such as DNA:PEI complexes formation, inoculum preparation, high quality plasmid DNA production, among others (Gutiérrez-Granados et al. 2018). Furthermore, extended gene expression does also require medium

exchanges every 48 hpt to provide the cells with nutrients and remove the waste products. Due to all these reasons, EGE scale-up to bioreactor presents a challenge where many technical variables are involved. Acoustic filtration methodology was selected here to perform EGE at bioreactor scale in perfusion mode. The constant addition and removal of nutrients and waste products and the controlled conditions in the bioreactor led to a remarkable increase in growth rate (0.0320 h^{-1} in bioreactor compared with 0.0109 h^{-1} in the shake flasks) and also in the maximum cell density achieved in the bioreactor ($14x10^6$ cells/mL in bioreactor compared to $8x10^6$ cells/mL in the shake flasks). Since there was a high growth in the bioreactor, the percentage of transfected cells was considerably lower at the end of the cultivation than in the shake flasks. The high number of non-transfected cells indicates that further retransfections could be done in order to further increase production titers in the bioreactor. EGE at bioreactor scale achieved the same VLP production titers than the conventional shake flask scale process, showing a great potential for VLP production. Several bioprocess modifications should be done to improve the system at bioreactor scale; such as adjusting the DNA concentration to the cell density or modifying the number and the time for retransfections. These optimizations would lead to the generation of a bioprocess in which high VLP concentrations are obtained in a relatively small working volume, which is very desirable at industrial scale. The main drawback of the high cell densities achieved in the bioreactor is that the potential contaminants in the product preparations are increased. Cell exosomes and other cellular vesicles as well as host cell DNA and host cell protein residues are increased. Downstream processing steps should be implemented in the bioprocess in order to remove the contaminants produced by the high cell densities. Furthermore, cell biology modifications could also be investigated in order to force the cells to produce less cellular vesicles and hence increase the ratio VLP/total vesicles; reducing hence the downstream process steps and simplifying the overall process.

DNA and PEI concentration optimization is another key point in TGE and it has been widely studied for HEK293 and also for other mammalian cell lines such as CHO or CAP cells (Derouazi et al. 2004; Gutiérrez-Granados et al. 2016). Nevertheless, the possibility of extrapolating the optimal DNA and PEI concentration to other cell lines or vector types had not previously been investigated. In this work, DNA and PEI concentration optimization has been carried out for three different HEK293 cell lines and three vector types. The nine optimal concentrations were found to be very similar: 2.34 µg/mL for DNA and 5.81 µg/mL

for PEI. These optimal concentrations were hence independent on the HEK293 cell line and the vector type used for TGE. This fact may lead to a reduction in costs and time in biopharmaceutical development, since the same TGE protocol can be applied to different cell lines and vector types.

In TGE the gene of interest is expressed episomaly and the expression is lost over time since the plasmid is diluted in every cell division. There are some available HEK293 cell lines designed to enlarge the production phase by carrying out the episomal replication of the DNA which are HEK293-EBNA1 and HEK293T. By increasing the amount of plasmid copies in the cell nucleus, the transcription should be also increased. Both cell lines have been used for the production of recombinant proteins, viruses and vaccines using TGE at bioreactor and shake flaks scale (Meissner et al. 2000; Leopoldino et al. 2010). These two cell lines have been compared with a standard HEK293 cell line in terms of VLP production, viability and cell growth when transfected with three different plasmids: one containing the SV40ori, another one the OriP and a third one with no origin of replication. Among the nine combinations tested, the HEK293-EBNA1/OriP system presented highest specific productivity: 5.19 ng of Gag/million of cells; which is 3.5-fold higher than the one achieved with the HEK293T/SV40 system. The episomal replication of the DNA in HEK293T is a fast and energy-consuming process which provokes a decrease in cell growth having a detrimental effect over VLP production. In the case of HEK293-EBNA1 the episomal replication is carried out in a much more controlled manner. The plasmid DNA is duplicated once per cell division (Van Craenenbroeck et al. 2000). There is not a detrimental effect over cell division compared with the other plasmid types and the productivity is enhanced. Similar results were previously obtained for the production of the green fluorescence protein (GFP) and the human placental secreted alkaline phosphatase (SEAP) (Durocher et al. 2001), where HEK293-EBNA1 and HEK293T cells lines are compared in TGE using plasmids containing either OriP or SV40 origins of replication, obtaining similar conclusions. Nevertheless, in terms of cell growth, HEK2936-E presented lowest specific growth rate and cell density after transfection compared with the other cell lines. Modifications in the medium used for cell growth could be implemented in order to generated better conditions for HEK293-EBNA1 growth and hence maximize VLP production. Furthermore, other cultivation modes such as fed-batch and perfusion could be tested to increase cell density and hence obtain higher VLP titers. EGE methodology could be studied and adapted to HEK293-EBNA1/OriP system to obtained even better productivities. Considering the

obtained results and showing the potential of HEK293-EBNA1/pOriP system, this would be the selection platform for VLP production in further works.

Several different methodologies for improving or overcoming the inherent difficulties of TGE have been described and discussed. As an alternative, the generation of a stable cell line was also tested and compared with TGE in order to have a wider vision of the general possibilities for VLP production using HEK293 systems. A stable HEK293 cell line has been generated for the production of Gag VLPs. The possibility of obtaining a high producer clone is directly related to the number of clones analyzed. In this case a total number of 672 clones were analyzed and the best producer clone had a specific productivity of $1.74x10^{-2}$ pg of GagGFP/(cell·h) which is 4 times lower than those achieved by TGE. Compared with other stable cell lines expression Gag-based VLPs, Hi5 and Sf9 insect cell lines yielded around 600 ng of Gag/mL; compared to 675 ng of Gag/mL obtained with the generated clone. Comparison with other cell lines that produce other types of VLPs or even other recombinant proteins is a difficult task; since the product of interest directly affects the capacity of being produced.

In SGE by random integration, the gene of interest is inserted in one or several arbitrary loci of the cell genome. The number and the site of integration directly affect the performance of the cell line generated. The gene stability in the cell genome is also governed by the site of integration and it is also necessary to insert the gene of interest in a region where its expression will not change. Otherwise, it could lead to high variability from batch to batch. Other integration methodologies should be taken into consideration when generating a stable cell line for obtaining higher productivity and more stable clones. Directed integration methodologies such as ZFN, TALENs or CRISPR/Cas9 offer the possibility of integration of the gene of interest into a specific point of the cell genome, known for being of high transcription and low silencing (Lafountaine et al. 2015). The recently developed gene editing technology CRISPR/Cas9 has been mainly used for achieving knock-outs in human cells for identifying the function of specific genomic regions (Hsu et al. 2014a); nevertheless, it has not been so widely performed for introducing transgenes in production cells lines such as HEK293. The specific locus of integration when using directed methodologies should also be taken into deep consideration. The so called genomic safe harbors (GSHs) are regions of the human genome able to accommodate the integrated DNA with a predictable and reliable expression and with a high capacity of not silencing (Papapetrou et al. 2011). One of these GSHs is the adeno-associated virus site 1

(AAVS1) locus. The transgene is integrated in the intron 1 of the phosphatase 1 regulatory subunit 12C gene (PPP1R12C), and the resistance gene can use its promoter. The integration construct is formed by firstly the resistance gene without a promoter, followed by the gene of interest and its correspondent promoter. This construction ensures that only the cells that have been inserted the transgene in the AAVS1 locus will express the resistance gene. AAVS1 targeting for genomic modification has been previously carried out using the ZFN technology for different human cells lines and introducing different transgenes with promising results (Hockemeyer et al. 2009; Dekelver et al. 2010). This locus should be taken into consideration for generating a HEK293 stable cell line for the production of HIV-1 VLPs.

In the generation of a stable cell line at production scale it is very important to increase the number of clones for screening. It is hence of big relevance to have an automated screening method for analyzing the product quantity obtained in a fast and simple manner. Here, ELISA analyses were done over 102 clones (the ones that were able to grow after seeding in single cell) for Gag quantification which is a costly and time-consuming methodology. Furthermore, a specific medium selection should be developed after obtaining the best producer clone. The new cell line generated has specific characteristics that may not be totally covered by the medium used for selection and screening. Medium optimization must be done in order to further increase the specific productivity. In the same way, other cultivation modes should be tested such as fed-batch or perfusion for achieving higher cell densities and hence maximizing VLP production. Once a high Gag-producer HEK293 cell line is obtained, the improvements generated in this thesis regarding TGE could be used for transfecting the HEK293 stable cell line with antigenic membrane proteins (either from HIV-1 or from other viruses) for generating a complex and functionalized VLP.

Summarizing, several strategies have been followed for enhancing the production of Gag VLPs in HEK293 cell cultures with the aim to facilitate its application at larger scales. For this reason, it is important to increase production titers of TGE and also achieve TGE scale-up to bioreactor scale. EGE protocol has been combined with the use of shRNA and chemical additives for VLP production maximization. Furthermore, EGE protocol has been successfully scale-up to bioreactor. Several HEK293 cell lines have been compared and DNA and PEI concentrations have been optimized for VLP production using TGE. Finally, the development of a stable HEK293 cell line expressing VLPs has been achieved and compared

with TGE. All these investigations call for further analysis and optimization of the process which will lead to an important improvement in VLP production using mammalian cell systems at laboratory and industrial scale using both TGE and SGE.

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Conclusions

From the results obtained in this work, the following conclusions can be highlighted:

1. The supplementation of the extended gene expression protocol with lithium acetate, valproic acid and caffeine led to an increase in VLP production of 1.5-fold; generating the so-called EGE+Add protocol.

2. The EGE+Add protocol could be simplified by the elimination of the first and the second retransfections maintaining the same process performance.

3. The use of additive supplementation in EGE led to a stop in the cellular growth.

4. The inhibition of HDAC5 gene by the incorporation of a shRNA to the expression vector led to an increase in VLP production of 2.3-fold in batch cultivation; PDE8A gene could not be inhibited through genetic control.

5. The combination of the expression vector containing the HDAC5 shRNA with caffeine supplementation led to a 4-fold improvement compared to standard batch cultivation

6. EGE using the HDAC5 shRNA vector led to a 1.3-fold improvement in VLP production, maintaining cellular growth.

7. EGE was successfully performed in a 1.35 L bioreactor, obtaining similar VLP production.

8. EGE in bioreactor led to an increase in specific growth rate which turned into a higher amount of cellular vesicles and HCD and HCP in the purified samples.

9. Optimal DNA and PEI concentration for TGE were found for the combination of three different cell lines (HEK293SF3F6, T and 6E) and vector types (pSV40, p(-) and pOriP). The nine optimal concentrations were very similar between them: 2.34 µg/mL for DNA and 5.81 µg/mL for PEI.

10. HEK293-EBNA1/OriP system showed higher specific production rates; even though this cell line presented lowest specific growth rate in the conditions tested.

11. A stable HEK293 cell line expressing GagGFP VLPs was developed. The selected clone a specific production rate of 1.74×10^{-2} pg of GagGFP/cell·d, a maximum cell density of 4.5×10^{6} cells/mL and a duplication time of 26.7 hours.

From these results, it can be concluded that further optimizations could be done in order to improve the bioprocess proposed. First of all, the routine expression vector employed for TGE of HEK293 cells should include HDAC5 and PDE8A shRNAs and further proteomic analyses should be done to include other shRNAs. The selected expression platform would be HEK293-EBNA1/OriP including the new generated expression vector and the development of an expression medium for this particular system. The combination of the cell line, the expression vector and the growth medium should be scaled up to bioreactor and the EGE protocol tested over this new expression platform. Bioprocess optimization at bioreactor scale could be done to improve the system and overcome drawbacks associated with bioreactor scale. Finally the CRISPR/Cas9 methodology and high-throughput screening systems should be combined in order to provide with a high producer HEK293 cell line. This new stable platform could be combined with the transient expression of viral antigens using the TGE optimization provided in this work.