Tesis doctoral

Departament de Química

Strategies for improving production levels of HIV-1 VLPs by transient transfection of HEK 293 suspension cultures

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Abstract

Virus-like particles (VLPs) offer great potential as candidates for new vaccine production. In this work, the development and optimization of an HIV-1 Gag VLP production protocol by transient gene expression in HEK 293 suspension cultures is presented. Transient transfection enables a rapid generation of recombinant proteins of sufficient quantity and quality to perform pre-clinical trials, and it is particularly interesting in the early development phases. This work is divided in four main chapters.

In the first chapter, the serum-free commercial medium Freestyle 293 is optimized using non-animal derived components as supplements. The use of chemically defined animal derived component free media and supplements is a basic requirement for any further use of a vaccine for humans. The maximum cell density attained using the optimized medium (supplemented with 0.9*X* of Lipid mixture, 19.8 mg/L of r-insulin and 1.6 mg/L of r- transferrin) was 5.4×10^6 cells/mL in batch mode, almost double of that observed using the unsupplemented medium (2.9×10^6 cells/mL). Moreover, after the medium optimization, the transfection protocol is also improved. Best production performance is attained when cells were transfected at mid-log phase ($2–3 \times 10^6$ cells/mL) with medium exchange at the time of transfection using 1 μg/(mL of culture) of plasmid DNA and 2 μg/(mL of culture) of polyethylenimine. By using this protocol, VLP titers are increased 2.4-fold, obtaining 2.7×10^9 VLPs/mL. The optimized medium and transfection protocol defined in this chapter are used in the rest of the work.

In chapter two, the kinetics of the transient transfection process is studied with the aim to characterize and understand the complete process at intracellular level leading to the VLP production, and to determine important time points to drive process improvement. Polyplexes start to interact with the cell membrane just after addition to the culture.

After 1.5 hpt complexes are detected in the cytoplasm of the cells and reach the nucleus around 4 hours post transfection. After 10 hours post transfection GFP fluorescence is detected inside the cells, but generalized budding of VLPs from the cells is not observed until 48 hours post transfection. The optimal harvest time is determined as 72 hpt as VLP production is highest while high viability of the culture is maintained.

In chapter three, the enhancement of VLP production using specific compounds is studied. Two main groups of transfection enhancers are tested, selected on the basis that they can either facilitate the entry of PEI/DNA transfection complexes into the cell or nucleus or they can increase the levels of gene expression. Among the eight transfection-enhancers tested (Trichostatin A, Valproic acid, Sodium Butyrate, DMSO, Lithium Acetate, Caffeine, Hydroxiurea and Nocodazole) an optimal combination of compounds exhibiting the greatest effect on gene expression is subsequently identified. The addition of 20 mM Lithium Acetate, 3.36 mM of Valproic Acid and 5.04 mM of Caffeine increases production levels by 4 fold, while maintaining cell culture viability at 94 %.

As transient gene expression (TGE) is based on episomal plasmid DNA expression, conventional TGE is limited to a short production period of usually about 96 h, therefore limiting productivity. In chapter four, a novel gene expression approach termed extended gene expression (EGE) is proposed. The aim of EGE is to prolong the production period by the combination of medium exchange and repeated transfection of cell culture with plasmid DNA to improve overall protein production. The benefit of this methodology is evaluated for the production of three model recombinant products: intracellular GFP, secreted GFP, and a Gag-GFP virus-like particle (VLP). Using this

novel EGE strategy, the production period is prolonged between 192 and 240 h with a 4–12-fold increase in production levels, depending on the product type considered.

Introduction

1. Vaccines

1.1 Vaccine history and the impact of vaccination

Vaccination has been without a doubt one of the greatest medical interventions in human history. The origins of vaccination date back to 1796, when Edward Jenner observed that milkmaids who were exposed to cowpox developed a mild form of variola but were immune to smallpox. Dr. Jenner decided to inoculate an 8-year old boy with the fluid of a cowpox blister from a milkmaid and later challenged him with smallpox demonstrating that this treatment which was called vaccination provided the boy protection against deadly variola virus (Hilleman, 2000). In the nineteenth century, vaccination became a cause of national prestige and the first vaccination laws were passed. The development of vaccines reached its golden age during the twentieth century with the implementation and widespread use of many successful vaccines. As a result, smallpox has been eradicated and many other infectious diseases that have threatened humanity for centuries have virtually disappeared (Ulmer et al., 2006) (Table 1). Today vaccines are used for nearly thirty out of more than seventy known human infectious diseases.

Table 1. The impact of vaccination. Comparison of 20th century annual morbidity and current morbidity in United States

Disease	20th century annual morbidity*	2010 reported cases**	% Decrease
Smallpox	29,500	0	100%
Dipheteria	21,053	0	100%
Pertussis	200,752	21,291	89%
Tetanus	580	8	99%
Polio (paralytic)	16,316	0	100%
Measles	530,217	61	<99%
Mumps	162,344	2,528	98%
Rubella	47,745	6	<99%
CRS	152	0	100%
H.Influenzae b	20,000	270	99%

*(Roush and Murphy, 2007) ** CDC. MMWR January 7, , 2011;59(52);1704-1716

1.2 Types of vaccines

Vaccination is the administration of a biological preparation that helps the body fighting infectious pathogens. A vaccine typically contains an agent (generally known as antigen) that resembles the structure of the pathogen the vaccine is intended for. The agent acts as bait that is recognized as foreign by the recipient´s immune system and attacked. This first encounter between the agent and the immune system generates "memory" and allows, upon a second encounter, a fast and effective immune response that quickly eliminates the pathogen. The better the vaccine mimics the natural infection the more effective it will be.

In addition to preventive vaccines, there are also therapeutic vaccines. These are vaccines that are designed to treat people who already have a disease. Some scientists prefer to refer to therapeutic vaccines as "therapeutic immunogens." Currently, there is only one FDA-approved therapeutic vaccine for advanced prostate cancer in men (Cheever and Higano, 2011).

The great majority of vaccines available today are based on laboratory grown microorganisms (Table 2). Live-attenuated vaccines are obtained by selecting a weakened mutated version of a wild-type microorganism (Ulmer et al., 2006). These vaccines are very efficient at inducing a potent immune response (both humoral and cellular) since they mimic very well the natural infection and typically require only a single dose. However, due to their ability to replicate in the host (although limited) they may induce adverse reactions particularly in immunosuppressed hosts. In addition, it is not always possible to identify a sufficiently attenuated strain and there is a minor but realistic risk of reversion to a virulent strain, which precludes the use of these vaccines for life-threatening infections (Mäkelä, 2000). Inactivated vaccines represent a step forward towards safety since the microorganism is killed, typically by a chemical

treatment, at the end of the production process (Plotkin, 2014; Ulmer et al., 2006). Unfortunately, the latter may result in the loss of fidelity of the antigens. Besides, due to the nature of the vaccine, cellular immunity is not effectively induced (Mäkelä, 2000). Overall, these vaccines are less immunogenic than the live-attenuated vaccines and need to be administered in several doses along with adjuvants. A third type of vaccine that is widely used consists in purified subunits of the pathogen such as toxoids, polysaccharides or proteins (Mäkelä, 2000; Plotkin, 2014; Ulmer et al., 2006). These protective antigens are purified from the whole organisms after their amplification. Even though this type of vaccine is very safe, it is usually the least immunogenic requiring higher amounts of antigen and a greater number of doses to attain the same level of protection.

There is a continuous need for new vaccines, not only to prevent diseases for which vaccines are still not available, but also to improve the potency, quality and safety of the existing ones. As opposed to the conventional vaccines described above, nowadays the trend is to evolve towards recombinant vaccines benefiting from advances in recombinant DNA technology. This avoids the risks related to the manipulation of infectious pathogens in the laboratory making them a safer and more attractive option for vaccine manufacturers. Furthermore, the vaccine is generated under more controlled conditions and thus, better characterized.

Among the most promising new generation vaccines are recombinant subunit vaccines, DNA vaccines, viral vectors and virus-like particles (Table 2). Recombinant subunit vaccines, even though produced under safer and more controlled conditions, present the same limitations as already described for purified subunits in terms of immunogenicity

(Ulmer et al., 2006). Other modern but still highly experimental approaches are DNA vaccines and viral vectors. Both of these strategies, also termed gene-based strategies, rely on the patient's own cellular machinery to produce the antigens *in vivo* following the introduction of foreign genes coding for such antigens into the cells. The main difference resides in the way DNA is delivered. Plasmid DNA delivery needs to be forced by physical methods to give rise to acceptable antigen expression levels; whereas, viral vectors use their own sophisticated delivery machinery that has improved throughout the course of evolution to efficiently introduce their genetic payload into cells. As a result, viral vector vaccines tend to induce a stronger immune response than do DNA vaccines (Donnelly et al., 1997). From the manufacturing point of view, DNA vaccines are very simple to produce using bacterial cultures, and since DNA is stable, the transport and storage of the final product is easy and low cost (Donnelly et al., 1997). On the other hand, viral vector manufacturing is far more challenging as producer cell lines, vector design, stability, production, purification and characterization techniques need to be established on a per case basis (Bråve et al., 2007; Draper et al., 2008).

The concept of VLPs derives from the finding of subviral particles in the blood of patients with Hepatitis B (HBV) in the 1980's. When administered to healthy individuals, these plasma-derived subviral particles provided protection against HBV giving rise to the first VLP-based vaccine (Lua et al., 2014; Roldão et al., 2010). This vaccine was later replaced by a safer recombinant version. The success of this vaccine motivated further research on VLP vaccines over the past twenty years. As a result, a vaccine for Human Papilloma virus is licensed today for human use and many more preventive VLP vaccines are in advanced clinical trials (Roldão et al., 2010). Virus-like

particles are self-assembled particles that mimic the virus structure. However, they do not contain the virus genome and are thus non-infective virus particles. Due to their repetitive organized structure and particulate nature, they are very efficiently uptaken by antigen-presenting cells giving rise to a potent immune response with stimulation of both arms of the immune system: humoral and cellular. The latter is often not achieved using conventional vaccines and is essential to combat chronic infections and diseases (Berzofsky et al., 2004). These unique VLP properties make them appealing in many aspects as an alternative to the existing vaccines and are therefore the subject of intensive research and represent an advanced vaccine technology platform (Grgacic and Anderson, 2006; Ludwig and Wagner, 2007; Noad and Roy, 2003).

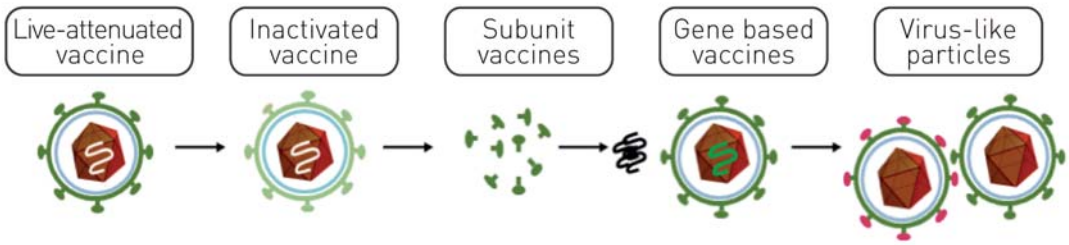

Figure 1 Evolution of vaccines, addapted from(Ulmer et al., 2006)

Table 2 Vaccines available today

Vaccine type	Selected disease target
Live attenuated viruses	Smallpox Yellow fever Tuberculosis Polio Chickenpox Rotavirus Influenza Measles Mumps Rubella Varicella
Inactivated viruses	Polio Influenza Typhoid fever Plague Rabies Whooping cough Hepatitis A
Purified subunit	Diphteria (toxoid) Pneumococcus (toxoid) Meningococcus Tetanus Haemophilus influenzae B Pertussis Anthrax
Recombinant subunit	Borrelia burgdorferi (recombinant Osp A)
Viral vectors	Under evaluation in clinical trials
DNA Vaccine	Under evaluation in clinical trials (some veterinary vaccines licenced)
Virus-like particles	Hepatitis B HPV

1.3 Types of virus-like particles

VLPs are composed of one or more recombinantly produced structural viral proteins, which upon expression self-assembles into particles. The simplest VLP structure is represented by a non-enveloped single protein particle such as the HPV. In this case, the expression of a single major capsid protein (L1) is sufficient to generate the VLP (Kirnbauer et al., 1992). However, in other cases production of VLP is not so straightforward. For instance, members of the Reoviridae family (i.e. rotavirus) are

formed by a viral capsid made up of concentric interacting layers of different capsid proteins (O'Neal et al., 1997). Assembly of multilayered, multiprotein VLPs can be achieved by co-expression of 2 to 4 capsid proteins (depending on the virus) either from multiple genes or a single gene coding for a polyprotein. VLPs for viruses with a lipid envelope such as Influenza and HIV-1 represent a more complex structure to produce (Roy and Noad, 2008). In this case the choice of the producer cell line is crucial. Indeed, enveloped VLPs bud from host cells incorporating in their structure part of the host cell membrane including anchored cellular components. The budding efficiency may vary depending on the producer cell system used (mammalian, insect, yeast, etc) and this will determine the quantity of VLPs produced, if any (Doan et al., 2005). Aside from the simple VLPs described above, that imitate the structure of the corresponding virus the vaccine is intended for, VLPs can be used as carriers to present foreign epitopes to the immune system to either vaccinate against completely unrelated pathogens or treat chronic diseases such as cancer (Deml et al., 2005). Carrier VLPs are generated through modification of the VLP capsid gene sequence to form a fusion protein or by chemically conjugating foreign epitopes to pre-formed VLPs. Of note, the generation of carrier chimeric VLP based on non-enveloped viruses may be problematic as the fused peptide may interfere with particle self-assembly, the length of the fused antigen is typically restricted and folding of the introduced antigen inside the capsid structure may render it inaccessible to the immune system. Although little has been published, the generation of carrier VLPs based on an enveloped virus model may be a more interesting vaccine platform.

2. HIV

The human immunodeficiency virus (HIV) infects cells of the immune system such as helper T-cells (CD4+), macrophages and dendritic cells. The viral infection leads to a severe reduction of CD4+ T-cells provoking that the cell-mediated immune response is compromised in the infected individuals, leading to the development of the acquired immunodeficiency syndrome (AIDS) (Douek et al., 2009).

HIV is a lentivirus, member of the family of Retroviridae. Two types of HIV virus have been differentiated: HIV-1 and HIV-2. HIV-1 is highly infective and is globally spread while HIV-2 is less infective, virulent, transmissible and restricted to West Africa (Girard et al., 2006). HIV-1 can be divided into three groups: major (M), outlier (O) and non-M non-O (N). The majority of HIV-1 belongs to group M. This group has evolved into 10 genetic subtypes and 13 circulating recombinant forms (CRF). HIV is an enveloped virus with a positive stranded RNA genome that is converted to double-stranded DNA and integrates into the host genome. The viral RNA consists of 9 genes (gag, pol, env, tat, rev, nef, vif, vpr and vpu) encoding 15 proteins (Young et al., 2006). Gag, pol and env encode essential proteins for the generation of new viral particles (Leitner et al., 2008). Gag generates the structural proteins, pol encodes the enzymes for the replication of the virus and env encode the receptor binding proteins (Leitner et al., 2008). Tat and rev are regulatory gens and nef, vif, vpr and vpu are accessory genes required for the correct replication and maturation of the virus. The structural composition of the mature virion consists of a conical capsid that is formed by the single protein p24. The RNA interacts with the protein p7 and the capsid is surrounded by the protein p17. The capsid encloses two strands of viral RNA, the replication enzymes, proteases, ribonuclease, integrase and cellular proteins (Young et al., 2006).

The envelope encloses the matrix and is composed by a host cell lipidic bilayer and the Env protein that in turn is formed by the external subunit gp120 and the transmembrane subunit gp41. Gp120 binds to CD4 receptors of the surface of the cells and gp41 is involved in the fusion between the cell membrane and the virus. A detailed review of the insights of the replication cycle can be found in (Engelman and Cherepanov, 2012).

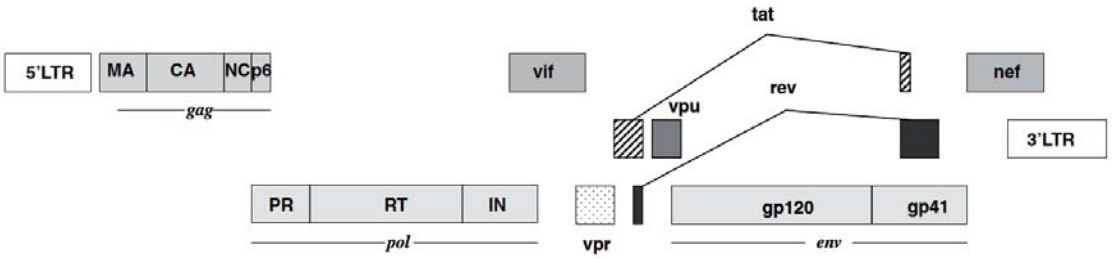

Figure 2. HIV-1 genome (Young et al., 2006)**.**

HIV-1 has infected over 60 million people since 1981 with a rate of annually new infections of 5 million people. Therefore, HIV/AIDS has become a global epidemic with 35.3 million people infected globally and 1.8 million deaths. Most of the HIV cases occurred in sub-Saharan Africa with a prevalence of 5% of the population (www.unaids.org).

The current treatment for AIDS is restricted to highly active antiretroviral therapy (HAART). Administration of HAART to HIV patients prolongs their lifespan, however, these treatments are not curative and extremely expensive. Therefore, developing an effective and affordable vaccine against HIV-1 is a major health issue (Chhatbar et al., 2011).

HIV-1 has several special characteristics that must be considered in any potential vaccine development. HIV-1 can initiate the infection breaking the mucosa barrier or infecting T-cells and the virus generates changes in the genome in ever replication (Letvin, 2006). A successful vaccine must elicit both humoral and cellular responses (Ross et al., 2010). Classical approaches have become unsuccessful for HIV-1. Live attenuated HIV-1 approach, although showed good protective effects in monkeys, has been discarded due to safety reasons, as it might be an option for the virus to recover the pathogenic activity. On the other hand, whole inactivated viruses and recombinant proteins showed low capacity to elicit CD8+ T cell response. Therefore, novel approaches have to be considered in developing an HIV vaccine (Barouch, 2008). Those new strategies include plasmid DNA vaccines and live recombinant vectors that are modified to express HIV-1 antigens. Plasmid DNA vaccines are expected to decrease the virus burden by reducing the virus replication. Recombinant vectors consist of a live viral non-replicative vector that encodes viral antigens. Different vectors have been studied such as adenovirus and poxvirus vector (Girard et al., 2006).

An alternative strategy is the use of lentiviral viral-like particles (VLPs) containing Gag and Env to form a pseudovirus that mimics the complete virus. The administration of VLP vaccine may be good candidates to elicit immune response against HIV-1 (Young et al., 2006).

3. Gag virus-like particles

Gag is known as “the particle-forming machine” as, in the absence of any other viral protein or genome, it is able to self-assemble in the vicinity of the plasma membrane and bud forming VLPs (Deml et al., 2005; Young et al., 2006). During the budding process, Gag virus particles acquire their lipid envelope from the producer cell.

Gag virus-like particles are virtually identical to the wild-type HIV-1 particles but carry an immature protein core and lack the Env-protein spikes on the surface and the genome inside the core (Figure 3).

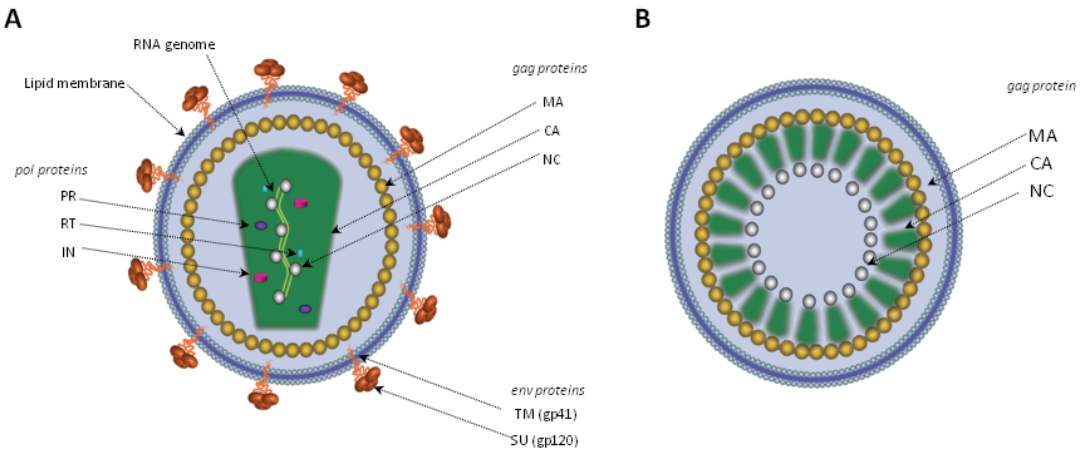

Figure 3 Structure of wild type HIV-1 and Gag-based VLP.(A) Wild type HIV-1 is composed of the cleavage products of 3 major viral polyproteins: Gag, Pol and Env. (B) The sole expression of Gag polyprotein gives rise to the generation of Gag virus-like particles which are basically immature HIV-1 particles carrying uncleaved Gag capsids surrounded by a host cell lipid layer.

Gag-based VLPs could consist an ideal platform for the generation of vaccine candidates. First, Gag-based VLPs are based on the HIV-1 virion, one of the best characterized virus particles of all times. Much is known about the biology, structure and immunogenicity of HIV-1. This scientific knowledge could be exploited for the generation of stable and immunogenic Gag-based VLPs vaccines. In fact, this knowledge has already been used for the development of HIV-1 based viral vectors (lentiviral vectors), which are among the most used gene transfer tools in gene therapy. Second, co-expression of Gag and the HIV-1 own Env-protein would generate a VLP for HIV-1 against which there is no effective vaccine yet available. Third, Gag-based VLPs could be used as carriers for the presentation of foreign antigens. The Gag particle is particularly well-suited for the incorporation of foreign antigens on the surface

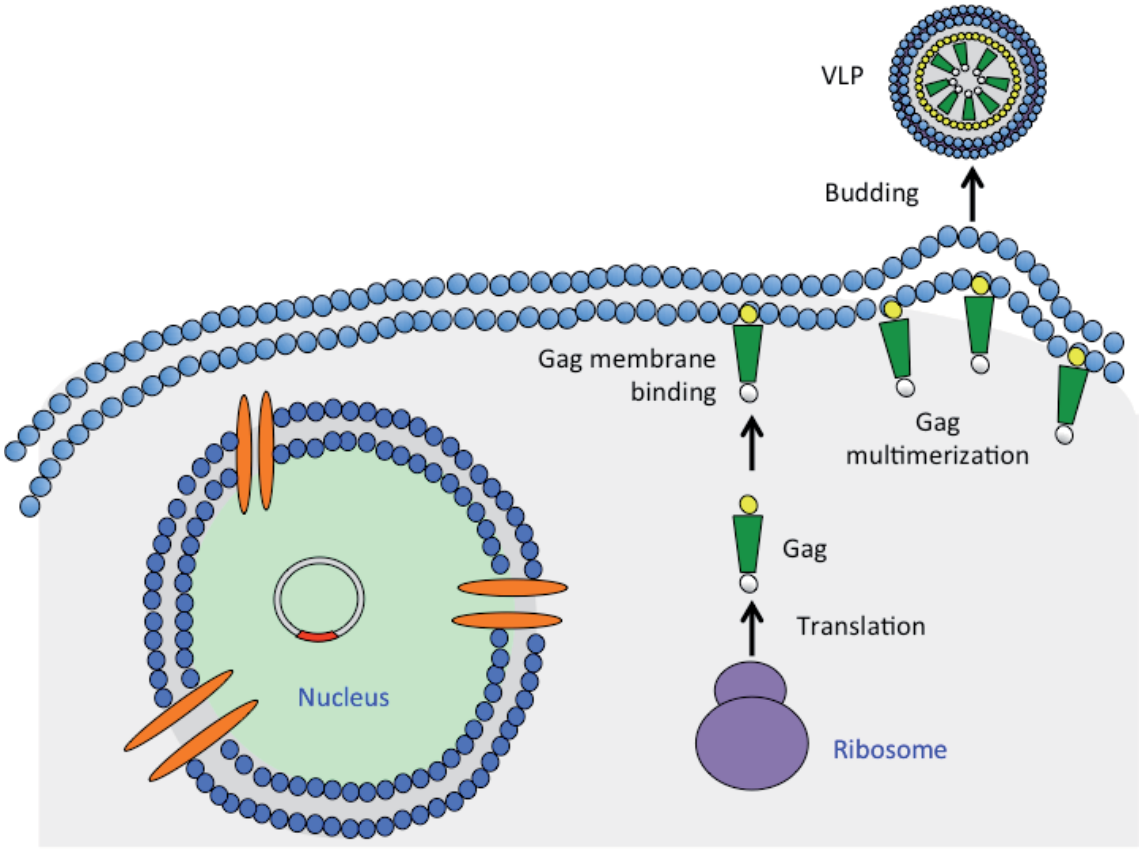

Figure 4 VLP production procés. Gag polyprotein self-assembles in the vicinity of plasma membrane and bud formin VLPs.

4. Production methods

4.1 Expression systems

A wide range of expression systems is available to produce recombinant proteins or viral vectors. These systems include bacteria, yeast, baculovirus mediated insect cell expression systems and mammalian cells. Each expression system offers advantages and disadvantages referred in relation to cost, easy of use, and post-translational modification profiles.

4.1.1 Bacteria

Bacteria although being the most widely used expression system for the production of recombinant protein is not the first choice in VLP production due to numerous factors

such as the absence of mammalian-like post-translational modifications. Nevertheless, bacteria are used to produce non-enveloped VLPs based on components of a pathogen with the ability to self-assemble into the host or as fusions of vaccine target antigens to bacteriophage surface proteins (Kushnir et al., 2012).

4.1.2 Yeast

Yeast is a well-established, robust, easily scalable and cost-efective platform for recombinant protein expression and continues to be used for VLP production, since two licensed VLP-based vaccines: Recombivax HB® and Gardasil® (Merk, USA) are using this platform. Despite these successful results, yeast differ from mammalian cells in post-translational modification patterns, concretely in protein glycosylation, so this system is generally limited to the production of non-enveloped VLPs (Kushnir et al., 2012). Nevertheless, production of enveloped HIV- Pr55Gag VLPs in yeast, using *Saccharomyces cerevisiae* spheroplasts, has been reported (Sakuragi et al., 2002). In this case, despite it has been demonstrated to activate dendritic cells in vitro and CD8 + memory cell response, some specific Gag-specific T cell populations remained unresponsive (Tsunetsugu-Yokota et al., 2003).

4.1.3 Baculovirus-Insect cell expression system

Another system widely used in VLP production is the baculovirus-insect cell expression system. Insect cells have been used to produce a number of VLP-based vaccines. In particular, High Five™ is the cell line used to produce one of the current HPV vaccines, Cervarix ® (Douglas R. Lowy and John T. Schiller, 2006). Insect cell system possesses eukaryotic–type post-translational modifications including glycosylation, accumulates high-level of foreign proteins and lacks mammalian pathogens. Large amounts of correctly folded VLPs are easily attained in high-density culture conditions. Concerning

the immunogenicity of the insect cell derived VLP, host-derived or baculovirus-derived components may act as vaccine adjuvants. On the other hand, this extra immunogenicity may mask the immune response against the desired epitope and the contamination of the product with co-produced enveloped baculovirus particles is the main limitation in this system. Insect cell expression system has been also studied for the generation of HIV vaccine, concretely expressing Pr55 Gag VLP (Kushnir et al., 2012).

4.1.4 Mammalian cells

As compared to insect cells, glycosylation in mammalian cells has the most similar pattern to human cells, which is an important aspect in terms of immunogenicity and safety. Mammalian cells are a widely extended expression platform for the production of recombinant proteins but also viruses since these cells are closely related to their natural hosts and thus typically perform appropriate post-translational modifications and authentic assembly of viral particles. The only caveat of this system is that the cost of production is higher than non-mammalian systems.

A number of mammalian cell lines have been used for protein expression with the most common one being HEK 293 (Human embryonic kidney) and CHO (Chinese hamster ovary).

4.1.4.1 CHO cell line

The first mammalian cell line approved for the production of recombinant protein was the CHO-derived cells for the production of tissue plasminogen activator (tPA) in 1986 which revolutionized medicine and opened the field of mammalian cell lines as expression systems (Lai *et al.* 2013). Nowadays, CHO cell line remains the first choice for the production of biopharmaceuticals owing to the capability to grow in suspension, low risk of contamination by human viruses, ability to grow in serum-free and

chemically defined media and finally their capacity to perform post-translational modifications to the recombinant proteins which make them compatible and bioactive in humans (Lai *et al.* 2013).

4.1.4.2 HEK 293

HEK 293 cells are also a widely and currently used mammalian-based expression system. This cell line is easy to grow, relatively easy to transfect, and it is also widely used in both research and production scale for the production of recombinant proteins and is the first choice for the production of viral vectors. HEK 293 is advantageous when human post-translation modifications, as opposed to those observed in hamster cells, confer beneficial properties to the product (Dietmair et al., 2012). This proved to be the case for human activated protein C, marketed as Xigris®, which required certain human post-translational modifications to maintain its biological activity (Durocher and Butler, 2009).

This cell line was generated in the early '70s from the co-culture of human embryonic kidney cells and adenovirus in the laboratory of Alex Van der Eb in Leiden, the Netherlands. Human cells were obtained from a single healthy fetus legally aborted. The original culture of kidney cells was carried out by van der Eb, while transformation with the adenovirus was performed in the laboratory Fran Graham van der Eb (Graham et al., 1977). Since the human kidney contains many kinds of cell types, it could not be determined the exact cell type from which HEK 293 were derived.

Of the available mammalian cells, the cell of choice for this work is HEK 293, as murine cells do not support efficient assembly and release of HIV-1 virions. This assembly defect may result from a failure of the Gag polyprotein to target to the cell membrane. This preclude the use of CHO cell line to produce Gag virus-like particles (Chen et al., 2001; Reed et al., 2002).

4.2. Stable cell lines and transient transfection

The production of recombinant proteins is usually achieved by two main approaches: stable gene expression (SGE) and transient gene expression (TGE).

SGE is characterized by the generation of a cell line which has the codified DNA of the recombinant protein integrated in its genome. The process to establish a stable cell line can be costing and time consuming, and it is normally difficult to obtain a high-titter production in the case of toxic products, as retrovirus or lentivirus (Pluta and Kacprzak, 2009).

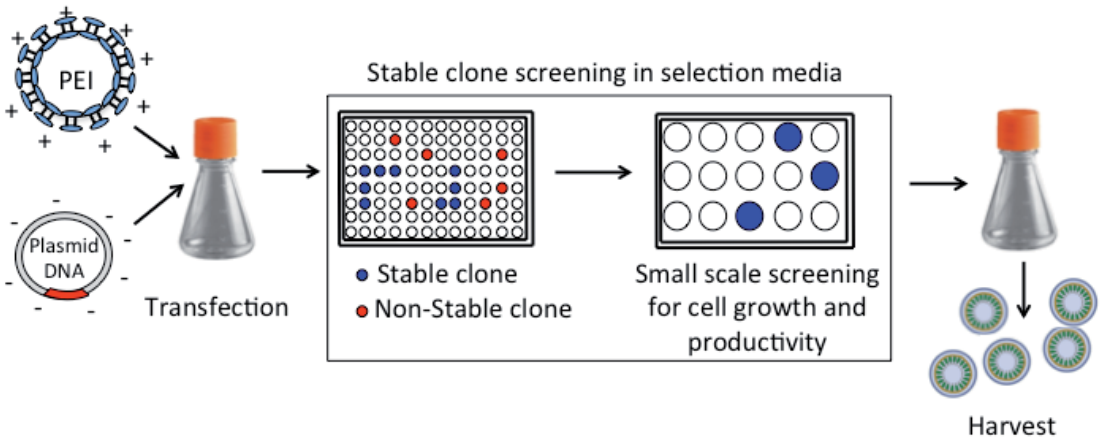

Figure 5. Proces to generate an stable cell line for cell culture processes for the generation of recombinant proteins of interst (VLPs in this exemple)

On the other hand, TGE is based on episomal plasmid DNA expression. The plasmid is introduced into the cells by using a transfection reagent and the product is harvested normally after 48-72 hours post transfection (hpt).

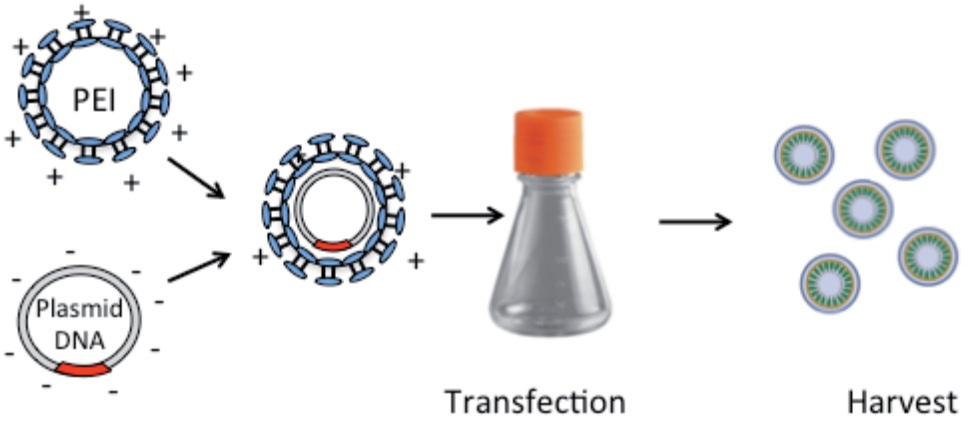

Figure 6. Transient gene expression proces.

Stable cell lines are used, therefore, in industrial processes, while transient transfection is used in the development stages, where it is necessary to have a minimum amount of product faster and easier, although this production is not applied in large quantities. For that reason, TGE is especially suitable for pre-clinical or structural studies of the obtained product. In many cases, both expression systems are combined, using the TGE in the development phase to generate prove of concept of the activity of the molecule under study, and the SGE in establishing the industrial production process.

Table 3. Comparison of SGE and TGE. Adapted from (Wulhfard et al., 2010)**.**

	SGE	**TGE**
Genetic selection	Yes	No
Time from DNA to product	6-12 months	Weeks
Specific productivity (pg/cell/day)	Up to 50	Below 10
Volumetric productivity (g/L)	Up to 5	0.02-0.08
Scalabillity	Up to 20.000 L	Small-scale production
Application	Large scale production of therapeutic recombinant protein	Small-scale production of protein for research

4.2.1. Methods of transfection

There are several complexing agents of DNA used to perform transient transfection, such as cationic lipids, calcium phosphate or cationic polymers.

4.2.1.1 Cationic lipids

There are several commercially available cationic lipids such as Lipofectamine 2000™ (Invitrogen) in adherent cultures and 293fectin™ (Invitrogen) and Fugene HD™ (Roche Diagnostics) for suspension cultures, all of them very effective in terms of transfection efficiency but very expensive which precludes its use in large-scale processes (Geisse, 2009).

4.2.1.2 Calcium phosphate

Calcium phosphate has been the most widely used method for transient transfection during the last 30 years (Batard et al., 2001; Jordan et al., 1996; Meissner et al., 2001). The method is easy and consist in mixing the DNA with a calcium chloride solution and then add a phosphate solution to form the Ca-Pi complexes. After incubation, when the complexes have reached an optimal size, they are added to the culture (Pham et al., 2006). This method is cost-effective and has been used for large-scale transfections. Its only drawback is its incompatibility when serum-free media, with low-calcium content used to prevent cell aggregation, are used (Geisse, 2009; Jordan and Wurm, 2004).

4.2.1.3 Cationic polymers

Polyethylenimine (PEI) was discovered as an efficient non-viral gene transfer in cultured and in-vivo cells (Boussif et al., 1995) and firstly gained attention in the context of gene therapy approaches (Lungwitz et al., 2005).

There are several types of PEI, linear or branched, with different molecular weights and with derivatized chemical groups. In TGE approaches the most widely used is the 25kD linear PEI, both in CHO and HEK 293 cultures (Carpentier et al., 2007; Derouazi et al., 2004; Durocher et al., 2002a). Furthermore, it is an economical method for transient transfection (Tait et al., 2004).

PEI ionically condenses DNA through amino-phosphate interactions between PEI and DNA forming positively charged polyplexes that protect the DNA from digestion by nucleases. Then the negatively charged glycoproteins and proteoglycans in the cell membrane interact with the polyplexes and finally the cell internalizes the polyplexes through endocytosis, which are later found in endosomes inside the cytoplasm (Bieber et al., 2002; Godbey et al., 1999).

The exact mechanism by which the PEI is able to escape from the vesicles has not yet being well defined nonetheless it is suggested that the presence of protonable amino nitrogen in PEI structure may act as buffer (proton sponge effect) of the endosomal environment that may delay the fusion with the lysosomes (Boussif et al., 1995) (Akinc et al., 2005), and ultimately lead to an osmotic swelling and rupture of some vesicles, releasing the polyplexes into the cytoplasm. Once the polyplexes are released into the cytosol they have to be internalized to the nucleus by a process not yet elucidated. One proposed mechanism suggests that the polyplexes may enter into the nucleus during the break down of the nuclear membrane during cell division (Grosse et al., 2006; Tait et al., 2004). Alternatively, a second mechanism postulates that the polyplexes are transferred to the nucleus by active transport through the nuclear envelope, independently of cell division (Han et al., 2009). The transfer of PEI–DNA complexes to the nucleus is a limiting step in cell transfection, as only a very small fraction od plasmid delivered into the cell is

finally translocated into the nucleus and transcribed (Bieber et al., 2002; Carpentier et al., 2007; Cohen et al., 2009).

For the purpose of this study, the transfection agent of choice is polyethylenimine (PEI) since calcium phosphate is not effective in serum-free media and lipofectamine is expensive, what makes it inadequate for scale up approaches.

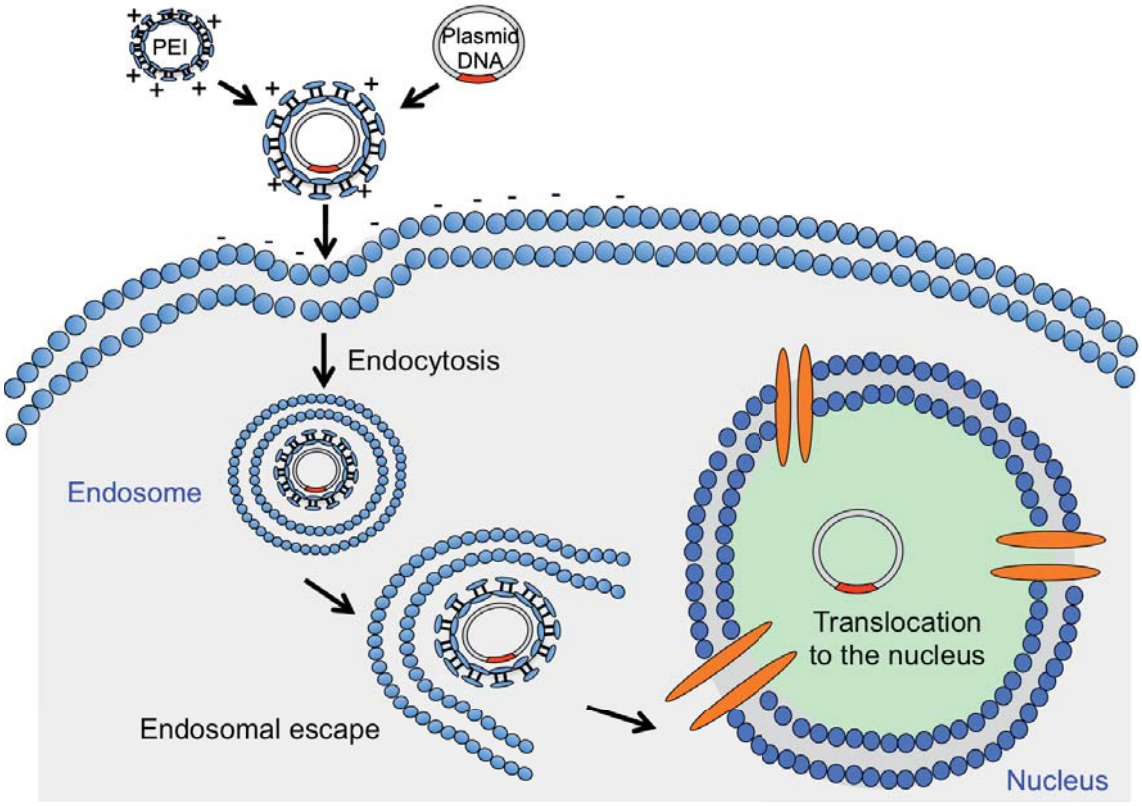

Figure 7. DNA entrance to the nucleus. DNA is complexed using PEI and enters the cell by endocytosis. Polyplexes are released to the cytoplasm by endosomalscape and enter into the nucleus

5. Methods to improve the process

5.1 Serum free media

The need of serum removal from bioprocess was recognized decades ago. First attempts in serum free media (SFM) development included components of animal origin that tried to mimic the components supplied by the serum, such as insulin, transferrin and

lipids of animal origin as well as other poorly defined mixtures (extracts, hydrolysates) (Keenan et al., 2006). Currently, the overall trend in SFM formulation is to avoid completely any animal-derived components to avoid any possible contact with new viruses or prion strains. The increasing number of animal-derived component free (ADCF) and chemically defined (CD) media formulations available in the market and the relatively recent commercial availability of recombinant versions of key serum proteins produced in *Escherichia coli* or yeast (e.g. albumin and transferrin), as well as supplements of plant origin or synthetic nature should facilitate the switch to efficient animal-derived component-free production processes.

5.2 Strategies to increase production

5.2.1 Cell lines/plasmids

A key point in the recombinant protein expression is the stability of the plasmid within the cell. To increase plasmid persistence a successful strategy developed has been the constitutive expression of the large T antigen of simian virus 40 (SV40) in 293-T, CHO-T or CAP-T cell lines, increasing therefore the replication of vectors containing the SV40 origin of replication (Baldi et al., 2007; Berntzen et al., 2005; Van Craenenbroeck et al., 2000; Durocher et al., 2002b; Geisse, 2009).

Another genetic element engineered for the optimization in mammalian cells, is the addition of EBNA-1 gene of the Epstein –Barr virus in conjunction with its replication origin, oriP, when provided in trans are claimed to boost protein expression plasmids. Furthermore, EBNA-1 appears to act as a transcriptional enhancer in human as well as rodent cells (Van Craenenbroeck et al., 2000; Durocher et al., 2002b; Geisse, 2009; Meissner et al., 2001; Young et al., 1988).

5.2.2 Additives to increase transient transfection and protein production

Several additives have been tested to enhance transient transfection efficiency. Lithium acetate and DMSO are used to increase cell membrane porosity to increase the capacity of the polyplexes to enter the cell (Ye et al., 2009). One of them is Nocodazole that acts in terms of cell cycle arrest in the G2/M phase which is thought to enhance nuclear uptake of the DNA/PEI complexes when the nuclear membrane dissolves during mitosis (Tait et al., 2004).

Once the DNA is in the nucleus another group of additives used to increase production has also proven to be efficient in several cases. Sodium butyrate (Ansorge et al., 2009; Backliwal et al., 2008c; Damiani et al., 2013; Jiang and Sharfstein, 2008; Mimura et al., 2001; Palermo et al., 1991; Rodrigues Goulart et al., 2010; Sung et al., 2004), Valproic acid (Backliwal et al., 2008a; Backliwal et al., 2008b; Backliwal et al., 2008c; Fan et al., 2005; Wulhfard et al., 2010) and Trichostatin A (Backliwal et al., 2008c; Fan et al., 2005; Spenger et al., 2004) are used to inhibit histone (HDAC), resulting in hyperacetylation of histones and consequently, alterations in DNA transcription (Rodrigues Goulart et al., 2010).

Hydroxyurea is used to block cell cycle in the G1 phase of the cell cycle which also leads to an increase in production (Fussenegger et al., 1999; Suzuki and Ollis, Tait et al., 2004).

Caffeine is a well established inhibitor of several kinases, including ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which are important signaling proteins involved in the repair of DNA double-stranded breaks (Block et al., 2004; Hall-Jackson et al., 1999; Sarkaria et al., 1999). This feature is able to increase lentivirus titer in HEK 293 cells (Ellis et al., 2011).

5.2.3 Cell culture modes

The system used for production of recombinant proteins by transient transfection play a central role to increase the yield of the protein of interest. Optimization processes carried out in fed-batch and perfusion systems have been reported. Perfusion was successfully used to increase titers of adherent H6–18 cells (derived from HEK 293 T) immobilized on microcarriers to produce Drosophila cytokine Spätzle (Cheeks et al., 2009) and for producing lentiviral particles using a suspensión adapted HEK 293 and an acoustic filter to retain cells in the bioreactor (Ansorge et al., 2009). Productivity has been also enhanced by medium feeding post transfection of HEK 293 EBNA for the production of GFP and secreted alkaline phosphatase (SEAP) (Pham et al., 2005) or GFP and erythropoietin (EPO) (Sun et al., 2006).

5.2.4 Use of anti-apoptotic genes

Another strategy to increase transient transfection yields include engineering cells to express anti-apoptotic proteins such as Bcl-xL (Majors et al., 2008) or another Bcl-2 family protein, Mcl-1 (Majors et al., 2009), resulting in higher yields and culture viabilities. A different approach has focused on knock-down of the pro-apoptotic genes Bax and Bak involved in the permeabilization of the mithocondrial membrane and trigger of caspase cascade activation (Macaraeg et al., 2013).

6. Scalability of the transient transfection process

Although TGE has been typically used at low and medium scale, during the past decade significant effort has been made towards developing large-scale TGE protocols. However since the average amount of DNA per one million transfected cells is 1 μg, the DNA cost is a factor that should be taken into consideration when selecting TGE for large-scale process. Large-scale production using both PEI and CaPi transfection

reagents has been demonstrated at production scales ranging from 1 to 110 L (Baldi et al., 2005). For instance, successful generation of IgG antibody by TGE at 10 L scale in Wave bioreactors and 50 and 100 L scale in stirred tank bioreactors has been reported (Tuvesson et al., 2008). Titers exceeding 1 g/L have been attained by TGE (Backliwal et al., 2008b).

The manufacturing of clinical grade complex biopharmaceuticals such as viral gene therapy vectors by TGE for human clinical trials has also been described (e.g., retroviral, lentiviral, and adeno-associated viral vectors) (Ausubel et al., 2012; Ayuso et al., 2010; Cornetta et al., 2008; Merten et al., 2011; Wright, 2008; Wright, 2009).

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Objectives

The main objective of this work is to develop a process for Gag-GFP VLPs production using HEK 293 cells by transient transfection. In more detail, the following specific objectives can be outlined:

1. Select and optimize a cell culture medium for cell growth and transient transfection, based on the use of commercial serum-free and chemically defined medium with further supplementation of non-animal derived components as additives to maximize cell growth, transient transfection and protein production.
2. Establish a transient transfection method for the production of Gag-GFP VLPs using PEI as transfection reagent, by evaluating the key variables affecting transient transfection efficiency such as cell concentration, DNA concentration and cell cycle phase of the culture.
3. Characterize the transient transfection process at intracellular level in order to first understand the process and second, determine relevant times related to the kinetics of transfection that can help in process optimization.
4. Optimize the production of Gag-GFP VLPs using additives to enhance transfection efficiency and production.
5. Develop a novel gene expression approach to prolong the production period by the combination of medium exchange and repeated transfection of cell cultures with plasmid DNA to improve overall protein production.

Results

Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium

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Journal of Biotechnology 166 (2013) 152–165

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium

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ARTICLE INFO

Article history:
Received 3 January 2013
Received in revised form 29 April 2013
Accepted 2 May 2013
Available online xxx

Keywords:
Animal cell culture
Media supplementation
Design of experiments (DoE)
Virus-like particles (VLP)
HIV-1 vaccine
Transient gene expression (TGE)

ABSTRACT

Virus-like particles (VLPs) offer great promise as candidates for new vaccine strategies. Large-scale approaches for the manufacturing of HIV-1 Gag VLPs have mainly focused on the use of the baculovirus expression system. In this work, the development and optimization of an HIV-1 Gag VLP production protocol by transient gene expression in mammalian cell suspension cultures is reported. To facilitate process optimization, a Gag-GFP fusion construct enabling the generation of fluorescent VLPs was used. The great majority of Gag-GFP present in cell culture supernatants was shown to be correctly assembled into virus-like particles of the expected size and morphology consistent with immature HIV-1 particles. Medium optimization was performed using design of experiments (DoE). Culture medium supplementation with non-animal derived components including recombinant proteins and lipids of synthetic or non-animal-derived origin resulted in improved HEK 293 cell growth and VLP production. The maximum cell density attained using the optimized Freestyle culture medium was 5.4×10^6 cells/mL in batch mode, almost double of that observed using the unsupplemented medium (2.9×10^6 cells/mL). Best production performance was attained when cells were transfected at mid-log phase ($2–3 \times 10^6$ cells/mL) with medium exchange at the time of transfection using standard amounts of plasmid DNA and polyethylenimine. By using an optimized production protocol, VLP titers were increased 2.4-fold obtaining 2.8 μg of Gag-GFP/mL or 2.7×10^9 VLPs/mL according to ELISA and nanoparticle tracking quantification analyses, respectively.

1. Introduction

Upon expression in heterologous systems, the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein is able to self-assemble giving rise to non-infectious VLPs in the absence of any other viral protein or virus RNA (Buonaguro et al., 2001; Rovinski et al., 1995; Sakuragi et al., 2002). HIV-1 Gag VLPs have shown great promise as platforms for the presentation of envelope antigens (Deml et al., 2005). However, the complexities associated with their manufacturing have hindered their evaluation beyond early-pre-clinical testing (Hammonds et al., 2007). Large-scale approaches for the manufacturing of HIV-1 Gag VLPs have mainly focused on the use of the baculovirus-expression system (Cruz et al., 1998; Pillay et al., 2009; Visciano et al., 2011). The generation of HIV-1 Gag VLPs using mammalian cells is less reported in the literature (Hammonds et al., 2007; Jalaguier et al., 2011), with no reports describing their production in suspension cell cultures.

The development of a scalable HIV-1 Gag VLP production strategy in human embryonic kidney 293 (HEK 293) suspension cell cultures is reported in this work. An improved culture medium supplemented with non-animal derived components was developed. The need for removal of serum from industrial manufacturing processes was recognized decades ago. In order to ensure cell line growth and productivity, early developments in serum-free media (SFM) resulted in formulations containing components normally supplied by serum such as insulin, transferrin and lipids of animal origin as well as other poorly defined mixtures (extracts, hydrolysates) (Keenan et al., 2006). However, during the past few decades there has been increasing safety concerns associated to the emergence of new viruses and prion strains prompting regulatory authorities to recommend the use of not only SFM but also animal-derived component free (ADCF) media formulations for the manufacture of biopharmaceutical products. The increasing

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http://dx.doi.org/10.1016/j.jbiotec.2013.05.001

number of ADCF and chemically defined (CD) media formulations available in the market and the relatively recent commercial availability of recombinant versions of key serum proteins produced in *Escherichia coli* or yeast (e.g. albumin and transferrin), as well as supplements of plant origin or synthetic nature should facilitate the switch to efficient animal-derived component-free production processes.

Design of experiments (DoE) has been chosen as a valuable tool for medium optimization. Statistically relevant information can be extracted from experimental designs with a minimum number of experiments. Non-animal derived additives evaluated in this work include three recombinant proteins (r-albumin, r-transferrin and r-insulin) and an *in-house* designed animal-component free lipid mixture containing synthetic cholesterol and fatty acids. A two-phase experimental design was used. Screening of components was performed in a first phase using a Plackett–Burman experimental design (Plackett and Burman, 1946). In a second phase, a response surface methodology (Box–Behnken design) was used to determine optimal concentration levels for each component showing a significant effect on HEK 293 cell growth (Box and Behnken, 1960). The performance of the optimized cell culture medium for the production of HIV-1 Gag VLPs was evaluated.

The selected production method was transient gene expression (TGE) as it offers a convenient means for the generation of recombinant products for pre-clinical and early clinical phases (Baldi et al., 2007; Geisse, 2009; Pham et al., 2006). TGE becomes particularly attractive when a large number of product variants needs to be tested and/or in cases where the expression of cytotoxic genes complicate the generation of stable cell clones, which is the case for HIV-1 VLPs. Considerable progress has been made in the past several years toward establishing large-scale transient transfection protocols (Backliwal et al., 2008a; Durocher et al., 2002; Geisse and Henke, 2005; Pham et al., 2003; Tuvesson et al., 2008). HEK 293 is the preferred host system due to the many industrially relevant features this cell line offers including ease of genetic manipulation, ability to grow in suspension culture, ability to grow to high cellular densities and adaptation to serum-free culture conditions. In addition, the HEK 293 cell line and its variants (e.g. HEK 293T, HEK 293E) are used for the production of many virus-based products including viral vaccines and most viral vectors (Durocher et al., 2007; Ghani et al., 2006; Kamen and Henry, 2004; Le Ru et al., 2010; Segura et al., 2007). Moreover, HEK 293 cells are rapidly gaining industry acceptance as they have been approved for the production of the first adenovirus-based gene therapy product (Gendicine®) in China and a therapeutic recombinant protein (Xigris®) by FDA and EMA.

2. Materials and methods

2.1. Cell line, media and culture conditions

The cell line used in this work is a serum-free suspension-adapted HEK 293 cell line (HEK293SF-3F6) kindly provided by Dr. Amine Kamen at the National Research Council of Canada (Montreal, Canada). It was derived from a cGMP master cell bank that is available for manufacturing of clinical material. Five commercial serum-free media formulations for HEK 293 were tested for cell growth, transfection efficiency and VLP production. These include HyQ SFM4 Transfx293 from HyClone Thermo Scientific (Logan, UT, USA), ExCell 293 from SAFC Biosciences (Hampshire, UK), Freestyle 293, CD 293 and 293 SFM II from Invitrogen (Carlsbad, CA, USA). All formulations were supplemented with GlutaMAX™ (4–6 mM) (Invitrogen, Paisley, UK) with the exception of Freestyle 293 media that already contains GlutaMAX™ in its formulation. Freestyle 293 medium and 293 SFM II medium were also supplemented with 0.1% Pluronic® (Invitrogen). Cell cultures were pre-adapted to each formulation prior to experimentation. An YSI 7100 MBS glucose/lactate/glutamine analyzer (YSI, Yellow Springs, OH, USA) was used to measure the concentrations of the major nutrients and by-products in cell culture supernatants. 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 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell count and viability were determined using trypan blue and a microscope counting chamber.

2.2. Transient transfection

The pGag-EGFP plasmid used in this work codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP (Hermida-Matsumoto and Resh, 2000). The plasmid from the 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, Palo Alto, CA, USA). The plasmid was prepared and purified as previously described (Segura et al., 2007). HEK 293 suspension cells were transiently transfected using 25-kDa linear polyethylenimine (PEI) (PolySciences, Warrington, PA, USA). Transfections were performed using a final DNA concentration of 1 μg/mL of media unless otherwise stated and a DNA to PEI mass ratio of 1:2. 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. The percentage of GFP positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) at different hours post-transfection (hpt).

2.3. Fluorescence confocal microscopy

The visualization of VLP producer cells was achieved using a Fluoview® FV1000 confocal microscope (Olympus, Tokyo, Japan). Transfected cells were mixed with 0.1% of Hoechst (Invitrogen, Eugene, OR, USA) and 0.1% of CellMask™ (Invitrogen) in order to stain the cell nucleus and lipid membrane, respectively. Two washes were performed by centrifuging the cells at 300 × *g* for 5 min and resuspending the pellets in PBS. Samples were placed in 35 mm glass bottom petri dishes with 14 mm microwell (MatTek Corporation, Ashland, MA, USA) for visualization.

2.4. Transmission electron microscopy

Gag-GFP VLP samples were prepared by air-dried negative staining method at the Servei de Microscòpia (UAB, Spain). Briefly, 5 μL of the mixture were placed on carbon-coated copper grids and incubated at room temperature for 5 min. Excess sample was carefully drained off the grid with the aid of filter paper. Samples were negatively stained with 5 μL of uranyl acetate (2%) by incubation for 1 min at room temperature. Excess stain was drained off as before and grids were dried for a minimum of 50 min at room temperature before the examination in a Jeol JEM-1400 transmission electron microscope equipped with a Gatan ES1000W Erlangshen CCD Camera (Model 785).

2.5. VLP quantitation

The concentration of Gag-GFP VLPs was assessed by fluorimetry using an *in-house* developed and validated 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

Table 1
HEK 293 growth kinetics and PEI compatibility in various commercially available serum-free media formulations.

Medium	Chemical definition	Max cell density (cells/mL)	Viability at max cell density (%)	$t_{1/2}$ (h)	PEI compatibility
HyQ SFM4 Transfx 293	ADCF	2.9×10^6	90	33.6	Yes
Excell 293	ADCF	4.5×10^6	91	36.3	No
Freestyle 293	CD	2.9×10^6	92	33.1	Yes
SFM II 293	ADCF	2.8×10^6	99	52.0	No
CD 293[a]	CD	–	–	–	n.a.

Abbreviations: Max: maximum; $t_{1/2:}$ duplication time; PEI: polyethilenimine; ADCF: animal-derived component free; CD: chemically defined; n.a.: not applicable.
[a] The HEK 293 suspension cell line used in this work could not be grown in CD293 medium.

Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) set as follows: λ_{ex} = 488 nm (slit 5 nm), λ_{em} = 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:

$$\text{Gag} - \text{GFP} \quad (\text{ng/mL}) = (3.245 \times \text{RFU} - 1.6833) \times 36 \tag{1}$$

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.

2.6. VLP recovery from cell culture supernatants

VLPs were isolated from cell culture supernatants following a standard procedure used for retrovirus particle separation consisting on virus pelleting by ultracentrifugation followed by size-exclusion chromatography (SEC) (Transfiguracion et al., 2003). Briefly, clarified VLP supernatants were obtained by low speed centrifugation ($500 \times g$ for 15 min at 4 °C) of cell cultures harvested 48 hpt. Viral pellets were obtained by ultracentrifugation through a 30% sucrose cushion (3 mL) at 26,000 rpm ($115{,}254 \times g$) for 3 hours at 4 °C using a SW32 rotor and a Optima L100XP Beckman ultracentrifuge. The pellets were resuspended in pre-chilled PBS (250 μL/tube) and incubated at 4 °C for 2 h. For residual nucleic acid removal, samples were subjected to Benzonase® (Merck Millipore, Germany) treatment for 1 h at room temperature using an enzyme concentration of 200 U/mL. The concentrated VLPs were analyzed by SEC using an ÄKTA Explorer™ 100 low-pressure liquid chromatography system controlled by UNICORN software (GE Healthcare, Sweden). For this purpose, Sepharose 6FF resin (GE Healthcare) was packed into a XK 16/70 column (GE Healthcare) to a final bed volume of 130 mL. The void volume of the column was determined using Blue Dextran 2000 (HMW calibration kit, GE Healthcare). Prior to VLP separation, the column was washed with 3 column volumes of degassed Milli-Q water and equilibrated with 1 CV of PBS pH 7.4 at 2 mL/min (60 cm/h). Concentrated VLPs obtained by ultracentrifugation (3 mL) were filtered through 0.45 μm pore-size syringe-mounted filters and loaded into the column. Isocratic elution in PBS (pH 7.4) was carried out at a flow rate of 2 mL/min (60 cm/h). Absorbance at 280 nm and 260 nm was measured on-line. Fractions (2.5 mL) were collected using a Frac 950 fraction collector (GE Healthcare) throughout the chromatography run and further analyzed by fluorometry.

2.7. Cell cycle analysis

Freshly harvested cells were pelleted by centrifugation, rinsed, re-suspended in PBS and fixed with cold ethanol (70%) for a minimum of 2 h at −20 °C. For cell cycle analysis, cells were stained with propidium iodide (PI) (Sigma) for 30 min at room temperature prior to data acquisition using a FACSCalibur (BD Biosciences, San Jose, CA, USA). The Modfit software (Verity Software House, Topsham, ME, USA) was used for data analysis.

2.8. Medium optimization using statistically designed experiments

Non-animal derived media components used for medium supplementation (DoE variables) included 3 recombinant proteins (albumin, transferrin and insulin) (Merck Millipore, Kankakee, IL, USA) and an *in-house* lipid mixture. The lipid mixture was prepared in a 100-fold concentrated stock containing synthetic cholesterol (100× Synthecol, Sigma), fatty acids (100× F7050, Sigma), tocopherol acetate (0.2 mg/mL T1157, Sigma) and the emulsifying agent polysorbate 80 (2.5 mg/mL P4780, Sigma). In order to determine maximum cell density attainable (DoE response) under each experimental condition analyzed, HEK 293 cells were seeded at a density of 0.3×10^6 cells/mL and their growth kinetics were analyzed during 10 days as the maximum cell density time differed for each condition.

2.8.1. Plackett–Burman design

A fractional factorial Plackett–Burman design was used to identify supplements with a significant effect on HEK 293 cell growth and screen out irrelevant variables. The four selected variables were screened at two levels: a low level (no additive) coded as −1 and a high level coded as +1 as indicated in Table 2. High levels for each variable were defined based on pre-existing knowledge extracted from the literature (Keenan et al., 2006).

The main effects of independent variables on HEK 293 cell growth were calculated using the following equation:

$$E_{ij} = \frac{\Sigma Y_{j(+)}}{n/2} - \frac{\Sigma Y_{j(-)}}{n/2} \tag{2}$$

where E_{ij} is the effect of the variable i on the response Y_j when the variable is used at its high level $Y_{j(+)}$ or low level $Y_{j(-)}$, and n is the number of experimental runs.

Plackett–Burman experimental results were fitted to a first-order polynomial function described below by regression analysis:

$$Y = \beta_0 + \sum \beta_i X_i \tag{3}$$

where Y is the response (in million cells/mL), β_0 is the model intercept and β_i is the linear coefficient for the independent variable X_i.

2.8.2. Box–Behnken design

In order to define the optimal concentration for each supplement selected in the previous step, a Box–Behnken design was used.

The three significant variables were screened at three levels: a low level coded as −1, a medium level coded as 0 and a high level coded as +1 as indicated in Table 3. Box–Behnken experimental results were fitted to a second-order polynomial equation described below by non-linear regression analysis:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (4)$$

where Y is the response (in million cells/mL), β_0 is the offset term, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient and X_i and X_j are the independent variables. This equation was used to predict the optimum values of the independent variables using the solver feature of Microsoft Excel 2007. Three-dimensional surface plots were generated to facilitate model interpretation.

2.8.3. Statistical analyses

Statistical analyses of the models were performed using Sigma Plot 11.0 (Systat Software Inc.) or Design-Expert® 8 Software (Stat-Ease, Inc.). The quality of the fit of the model equation is expressed by the coefficient R^2 obtained by regression analysis. Additionally, a lack of fit test was performed in order to compare the experimental error to the prediction error. The overall significance of the model was determined by analysis of variance (ANOVA) F-test, whereas the significance of each coefficient was determined by the corresponding t-test.

2.9. Quantification of Gag-GFP VLPs by nanoparticle tracking analysis (NTA)

Quantification of VLPs was performed using a NanoSight® LM20 device (NanoSight Ltd., Amesbury, UK) at the Institut de Ciència de Materials de Barcelona (ICMAB, CSIC, Campus UAB). NanoSight® NTA 2.2 software was employed for analyses. Samples were diluted in 0.22 μm-filtered PBS prior to injection in the device chamber in order to obtain samples with a concentration around 10^8 particles/mL. Three injections of the sample and three independent analyses were carried out. Videos of 60 s were recorded and subsequently, particles were identified and tracked by their Brownian motion at room temperature. NTA software allowed determining the number of VLPs/mL from the tracking data. As the average statistical mode obtained was 154 nm, only nanoparticles in the range of 100 and 200 nm were quantified.

3. Results

3.1. Identification of basal serum-free medium for supplementation

Five serum-free commercially available formulations specific for HEK 293 suspension growth were selected with the aid of the "good cell culture" interactive online database (Brunner et al., 2010). All formulations tested are also free of supplements of animal origin. According to the degree of chemical definition they can be classified either as ADCF or CD (Table 1). The latter do not contain hydrolysates or components of unknown composition and therefore are completely defined regarding their chemical composition. Nonetheless, the exact composition of the media is proprietary to the manufacturers. In preliminary experiments, HEK 293 growth kinetics in batch culture in the different formulations was evaluated (Fig. 1A). HEK 293 cells were subcultured directly into HyQ SFM4 Transfx293 (HyQ), Excell 293 (Excell), Freestyle 293 (Freestyle) and 293 SFM II (SFM II) media formulations requiring little or no adaptation. Cells maintained a high viability (>90%) and showed a doubling time of 33–52 h during the exponential growth phase (Table 1 and Fig. 1A). The maximum cell density

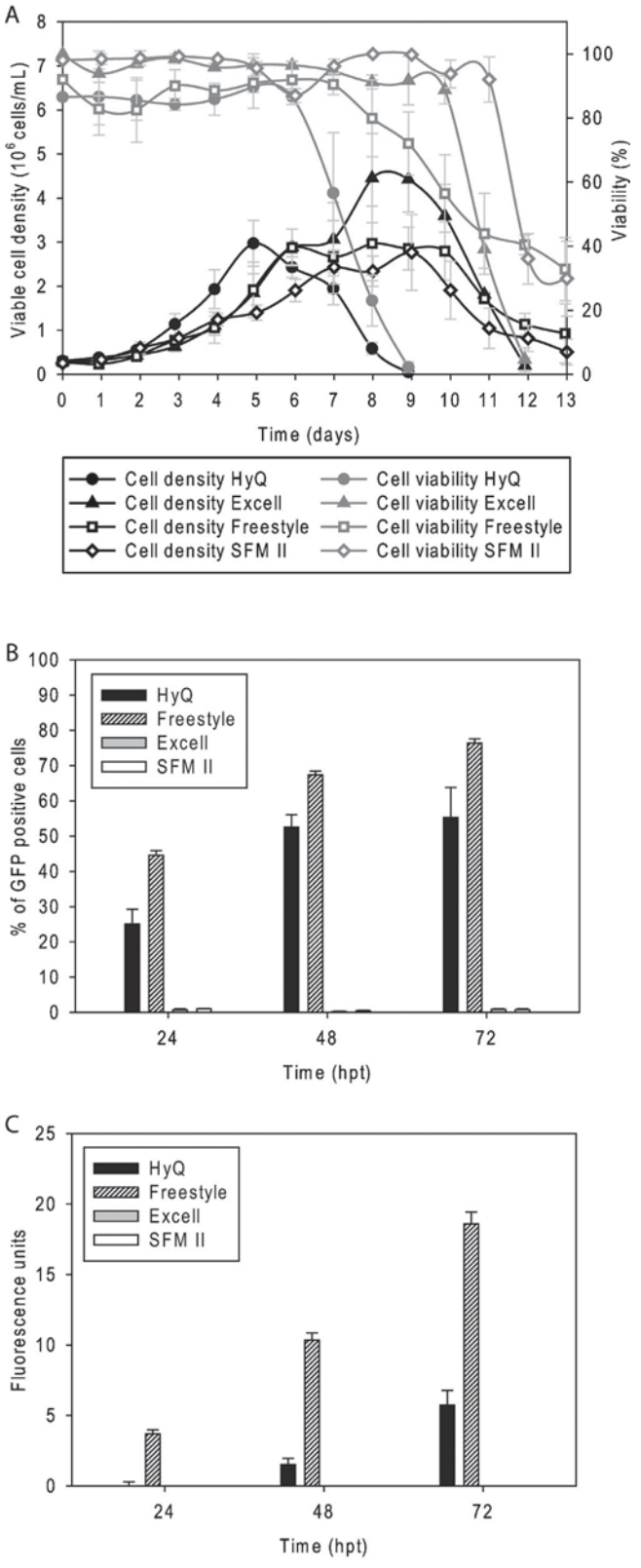

Fig. 1. Growth kinetics of HEK 293 in batch culture in different culture media. (A) Exponentially growing cells were seeded at 0.3×10^6 cells/mL in 125-mL flasks. Cell density and viability in each culture media were determined every 24 h. Mean values ± standard deviation of triplicate experiments are represented. (B) and (C) Generation of VLPs by transient transfection in different culture media. Exponentially growing HEK 293 cells were seeded at 0.5×10^6 cells/mL in 125-mL flasks the day before transfection. HEK 293 transfection efficiency (B) and VLP fluorescence intensity (C) in cell culture supernatants were measured. Mean values ± standard deviation of triplicate experiments are represented.

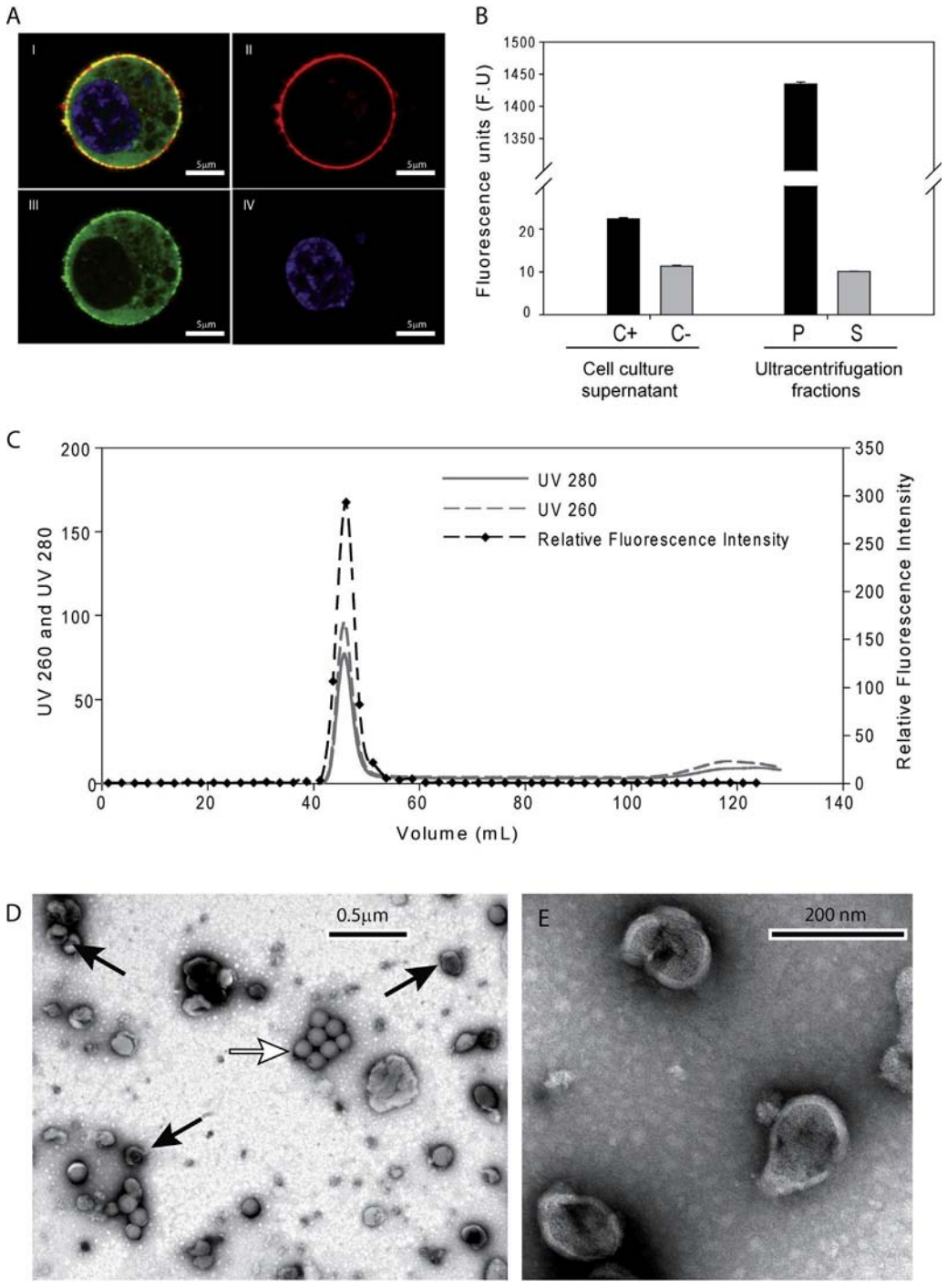

Fig. 2. Transient expression and assembly of Gag-GFP into VLPs. (A) Confocal image of HEK 293 cells transfected in Freestyle medium (72 hpt). Cell nucleus was stained with Hoechst (blue) (IV) and the lipid membrane with CellMask™ (red) (II). Gag-GFP is observed in green. The Gag-GFP was found accumulated in close proximity to the plasma membrane and in specific points inside the cell (III). Co-localization of Gag-GFP (green) and lipid membrane (red) is observed in yellow in the merged image (I). (B) Fluorescence intensity of cell culture supernatants from untransfected (C−) and transfected (C+) cultures 48 hpt and ultracentrifugation fractions. P is the pellet and S is the supernatant resulting from ultracentrifugation. Mean values ± standard deviation of triplicate experiments are represented. (C) SEC profile of purified VLP samples obtained by ultracentrifugation. Samples were loaded onto a 130-mL Sepharose 6FF packed column. Viral particles were eluted from the column by isocratic elution in PBS buffer. Optical density at 260 and 280 nm and green fluorescence intensity were monitored throughout the run. (D) and (E) Negative stain electron microscopy image of Gag-GFP VLPs. (D) Gag-GFP VLPs (full arrows) and latex beds used for quantitation purposes (empty arrows) are indicated. (E) High magnification images showing roughly spherical virus particles of ~140 nm in diameter surrounded by a lipid envelope and containing electrodense material. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

reached varied with the different formulations, being highest in Excell medium (4.5×10^6 cells/mL) but also high in the other three media ($\sim 3 \times 10^6$ cells/mL). When cell viability started to decrease, the availability of glucose and glutamine was not limiting (glucose > 1.2 g/L, glutamine > 1.16 mM) and lactate production did not exceed 1.8 g/L in any media tested (data not shown). Curiously, the cells did not grow in CD 293 and this medium was not included in further experiments. While HyQ and Freestyle medium supported efficient transfection (50 and 70%, respectively, 48 hpt), and consequently Gag-GFP production, no transfection was achieved in Excell

and SFM II culture media under the conditions tested (Fig. 1B and C). As Freestyle medium has shown to be superior to HyQ in terms of transfection efficiency and Gag-GFP production (as assessed by GFP intensity in cell culture supernatants), it was selected as basal medium for further optimization by DoE.

3.2. Transient expression and assembly of Gag-GFP into VLPs

A confocal image of HEK 293 producer cells in Freestyle base medium 72 hpt is shown (Fig. 2A). Upon transfection, Gag-GFP concentrates in close proximity to the plasma membrane of the producer cell, site at which the budding process takes place. Additionally, specific sites of Gag-GFP accumulation inside the cell can be observed (Fig. 2AIII). This result is consistent with the punctiform distribution of Gag described by Jalaguier et al. (2011) and with the observation that in epithelial-like 293 cells, HIV-1 assembles and buds both at the plasma membrane and in endosomes (Grigorov et al., 2006). Correct assembly of Gag-GFP into VLPs was confirmed by isolation of Gag-GFP VLPs from cell culture supernatants. The isolation procedure consisted in VLP pelleting by ultracentrifugation followed by further isolation and analysis by SEC. Fluorescence analyses of the fractions obtained upon ultracentrifugation is shown in Fig. 2B. Following ultracentrifugation, green fluorescence intensity in the pellet fraction significantly increases. In contrast, the fluorescence intensity in the supernatant fraction obtained after centrifugation is reduced to levels comparable to negative controls. These results provide a first indication that Gag-GFP in the cell culture supernatant is correctly assembled into virus particles of the expected size and density as they can be pelleted using ultracentrifugation procedures commonly used for the separation of retrovirus particles. The composition of the viral pellet was further analyzed by SEC. The chromatography profile is shown in Fig. 2C. VLPs eluted in a single peak at the SEC column void volume (44 mL) as expected due to their large size. The peak showed a characteristic OD_{260}/OD_{280} ratio >1 observed with retrovirus particles. Offline measurement of fluorescence intensity in the eluted fractions allowed detection of a fluorescent peak overlapping with that observed by UV online monitoring (Fig. 2C). This result indicated that the concentrated pellet obtained by ultracentrifugation contained fluorescent nanoparticles and was essentially free of significant protein or nucleic acid contamination. Examination of the ultra concentrated material by electron microscopy revealed the presence of multiple roughly spherical particles of 141 ± 22 nm ($n = 100$) in diameter consistent with Gag VLPs (Fig. 2D). In higher magnification micrographs it can be observed that the VLPs are surrounded by a lipid envelope (Fig. 2E).

3.3. Medium optimization using design of experiments

3.3.1. Screening of non-animal derived supplements using Plackett–Burman design

The potential beneficial effect of non-animal derived supplements on HEK 293 cell growth was investigated using a Plackett–Burman design of experiments. Non-animal derived additives evaluated included three recombinant proteins (r-albumin, r-transferrin and r-insulin) and an *in-house* designed animal-component free lipid mixture composed of synthetic cholesterol, fatty acids, tocopherol acetate and tween 80. The experimental design matrix in coded values, response and statistical analysis are shown in Table 2. Estimation of the main effect for each variable according to Eq. (2) indicated that the *in-house* developed lipid mixture had the greatest positive effect on cell growth (Fig. 3A). Recombinant insulin and transferrin also showed a pronounced positive effect on cell growth, whereas r-albumin had no effect. The ANOVA F test associated *p*-value for each regression coefficient can also be used as an indicator of the statistical significance of the

Table 2
Matrix design, response and ANOVA analysis for Plackett–Burman design.

Exp no.	X	X_1	X_2	X_3	Response a	Response b
1	+1	+1	+1	+1	4.84	5.04
2	−1	+1	−1	+1	4.84	4.16
3	−1	−1	+1	−1	3.72	3.84
4	+1	−1	−1	+1	3.84	3.72
5	−1	+1	−1	−1	4.00	3.24
6	−1	−1	+1	−1	2.76	3.04
7	−1	−1	−1	+1	4.00	4.35
8	+1	−1	−1	−1	3.00	2.88
9	+1	+1	−1	−1	3.24	3.06
10	+1	+1	+1	−1	4.40	4.45
11	−1	+1	+1	+1	4.48	5.20
12	+1	−1	+1	+1	4.65	4.40

	Coefficient	t-Value	p-Value
Constant	3.96500	52.0840	<0.001
X	−0.00458	−0.0602	0.953
X_1	0.28100	3.6950	0.002
X_2	0.27000	3.5530	0.002
X_3	0.49500	6.5080	<0.001

All variables were studied at two levels: a low level (no additive) coded as −1 and a high level coded as +1 being 1 g/L for r-albumin (X), 10 mg/L for r-insulin (X_1), 10 mg/L for r-transferrin (X_2) and 1X for the lipid mixture (X_3). Responses a and b are maximum cell density values from duplicate experiments in millions of cells/mL. *Abbreviations*: Exp. no.: experiment number; r-: recombinant.

factors. Variables with a *p*-value lower than 0.05 were accepted as significant factors affecting HEK 293 growth. These include r-insulin and r-transferrin with $p < 0.005$ and the lipid mixture with a $p < 0.001$. The effect of r-albumin was not statistically significant ($p > 0.10$). The three components showing a significant effect on HEK 293 cell growth were selected for further optimization experiments.

3.3.2. Optimizing the concentration of non-animal derived supplements using Box–Behnken design

A three-factor, three-level Box–Behnken experimental design was used to further optimize the concentrations of r-insulin, r-transferrin and lipid mixture in the culture medium. Table 3 outlines the experimental design matrix in coded values, response and statistical analysis of the method. These data were fitted to the second-order polynomial Eq. (4) by non-linear regression analysis. The generated model equation is:

$$Y = 4.53 + 0.63X_1 - 0.17X_2 - 0.41X_3 - 0.68X_1X_2 + 0.02X_1X_3 - 0.07X_2X_3 - 0.18X_1^2 - 0.45X_2^2 - 1.02X_3^2$$

where Y is the maximum cell density (in million cells/mL), X_1 is the coded value for r-insulin, X_2 the coded value for r-transferrin and X_3 the coded value for lipid mixture.

Regression analysis showed that the model was adequate with a coefficient R^2 of 0.8408. This indicates that the model is consistent with 84% of the variability in the data. The correlation between the experimental and predicted responses is observed in Fig. 3B. The lack of fit parameter is $p = 0.0893$, as it is not significant at 0.05 level, the prediction error is less than the experimental error and the model is consistent with the data. The statistical significance of the model was confirmed by ANOVA analysis. The Fisher's *F*-test associated *p*-value of <0.0001 indicated the model was significant. The second-order polynomial model was used to calculate optimal factor levels and construct response surface graphs. By solving the model equation, optimum concentrations of r-insulin, r-transferrin and lipid mixture in Freestyle medium were calculated as 19.8 mg/L, 1.6 mg/L and 0.9X, respectively. Under these conditions the predicted response was 5.4×10^6 cells/mL, almost double the cell density attained using unsupplemented Freestyle medium. Three-dimensional plots were constructed for visual observation

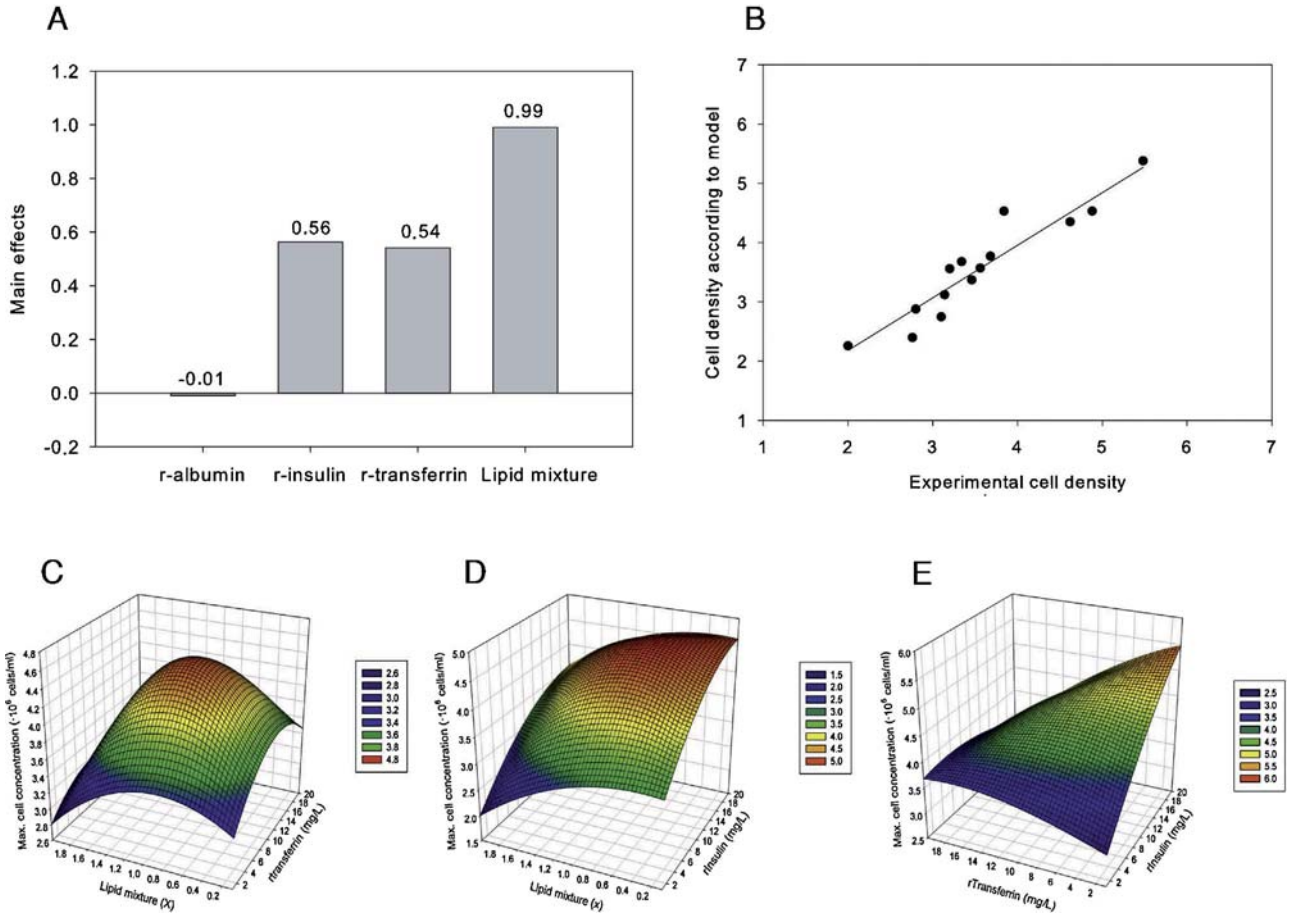

Fig. 3. Medium optimization by design of experiments. (A) Main effect of non-animal derived supplements on HEK 293 cells growth in Freestyle medium according to Plackett–Burman DoE Eq. (2). (B) Correlation between the experimental and Box–Behnken model predicted cell densities in million cells/mL. Response surface graphs based on Box–Behnken experimental results. These 3D graphs were constructed by depicting two variables at a time while keeping the third one at its middle level. HEK 293 maximum cell density as a function of the concentrations of (C) lipid mixture (*X*) vs. r-transferrin (mg/L), (D) lipid mixture (*X*) vs. r-insulin (mg/L) and (E) r-transferrin (mg/L) vs. r-insulin (mg/L). Cell density values presented are in millions of cells/mL.

of the trend of maximum responses and the interactive effects of the significant variables on the response (Fig. 3C–E). Evaluation of the response over the experimental region indicates that the optimum level of lipid mixture is in the middle of the evaluated concentration range, whereas optimal concentrations of r-transferrin and r-insulin are toward the edge of the range of concentrations tested. This was in agreement with the calculations obtained with the model.

3.3.3. Validation of the model

In order to validate the model, a verification experiment was carried out. Under the optimal culture medium conditions predicted by the model, a maximum cell density of $5.4 \pm 0.3 \times 10^6$ cells/mL was reached ($n = 3$) (Fig. 4), just as predicted, confirming the model adequacy. An additional experiment was carried out to ensure that the optimal concentration of r-insulin was well established. HEK 293 growth kinetics in the presence of 20, 40 or 60 mg/L of r-insulin was evaluated ($n = 3$). The concentration of r-transferrin and lipid mixture was kept at optimal levels in all experiments. All growth curves were similar to the one shown in Fig. 4, regardless of the concentration of insulin used (data not shown). This indicated that increasing the concentration of r-insulin would not result in further media improvement. It is worth mentioning that even though formation of small cell clumps (4–6 cells/clump) was typically observed at high cellular densities ($2–3 \times 10^6$ cells/mL) when growing cells in unsupplemented Freestyle medium, no cell aggregation was observed in the presence of the supplement mixture, even at high cellular densities.

3.4. Evaluating the performance of the supplemented medium

3.4.1. Effect of cell density on transfection efficiency and VLP production

The production of VLPs by PEI-mediated transient transfection in the supplemented cell culture medium was evaluated. According to TEM, the additives had no obvious effect on VLP structure as the size and morphology were preserved (data not shown). Fig. 5 shows the % of GFP transfected cells and the fluorescence intensity of supernatants from cultures transfected at different cell densities in unsupplemented Freestyle medium (Fig. 5A and B) and supplemented Freestyle medium (Fig. 5C and D). In general terms, it was observed that both the percentage of GFP positive cells and fluorescence intensity in supernatants increased over time. In addition, the percentage of GFP positive cells declined as the cell density at the time of transfection increased. Compared to the unsupplemented medium, transfection efficiency was usually lower in the supplemented medium, although only a minor difference (~10%) was observed (Fig. 5A and C). However, in terms of VLP production, the supplemented Freestyle medium showed to be superior. Best results in unsupplemented Freestyle were obtained when transfecting cells at 1×10^6 cells/mL (10.3 ± 0.5 F.U., 48 hpt), whereas the optimal concentration in supplemented Freestyle

Table 3
Matrix design, response and ANOVA analysis for Box–Behnken experimental design.

Exp. no.	X_1	X_2	X_3	Response a	Response b
1	−1	−1	0	3.08	3.12
2	+1	−1	0	5.92	5.04
3	−1	+1	0	3.68	3.68
4	+1	+1	0	3.00	3.68
5	−1	0	−1	3.20	3.08
6	+1	0	−1	4.32	4.92
7	−1	0	+1	2.12	1.88
8	+1	0	+1	3.32	3.80
9	0	−1	−1	3.52	2.88
10	0	+1	−1	3.76	3.16
11	0	−1	+1	2.64	2.96
12	0	+1	+1	3.12	2.40
13	0	0	0	4.88	
13	0	0	0	4.88	
13	0	0	0	3.84	

	Coefficient	*t*-Value	*p*-Value
Constant	4.5333	16.8051	<0.0001
X_1	0.6350	5.4362	<0.0001
X_2	−0.1675	−1.4340	0.1697
X_3	−0.4125	−3.5314	0.0026
$X_1 \cdot X_2$	−0.6800	−4.1164	0.0007
$X_1 \cdot X_3$	0.0200	0.1211	0.9051
X_3^2	−1.0242	−5.2089	<0.0001
$X_2 \cdot X_3$	−0.0750	−0.4540	0.6556
X_1^2	−0.1792	−0.9112	0.3749
X_2^2	−0.4542	−2.3099	0.0337

All variables were studied at 3 levels: a low level coded as −1, a medium level coded as 0 and a high level coded as +1 being 1, 10 and 20 mg/L for r-insulin (X_1), 1, 10 and 20 mg/L for r-transferrin (X_2) and 0.1, 1 and 2*X* for the lipid mixture (X_3). Responses a and b are maximum cell density values from duplicate experiments (runs 1–12) in millions of cells/mL. Run 13 was performed in triplicate because it was the center point.
Abbreviations: Exp. no.: experiment number; r-: recombinant.

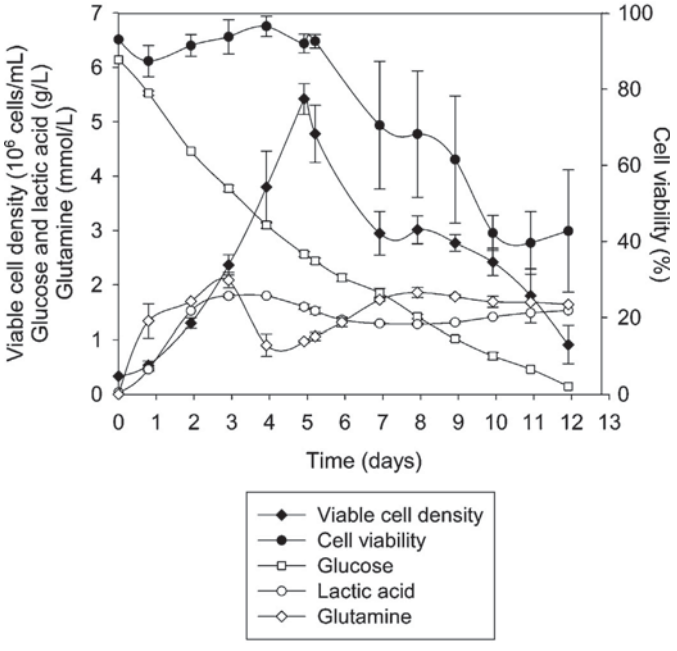

Fig. 4. Model validation. HEK 293 cell growth in Freestyle medium supplemented with the optimal levels of non-animal derived supplements were seeded at 0.3×10^6 cells/mL in 125 mL flasks. Cell density, viability, glucose, glutamine and lactic acid concentrations were determined daily. Mean values ± standard deviation of triplicate experiments are represented.

was 2×10^6 cells/mL (16.8 ± 0.4 F.U., 48 hpt) (Fig. 5B and D) representing an improvement of 1.6-fold. The best producing cell density coincides with the mid-log phase density in each culture medium since cells grow up to 2.9×10^6 cells/mL in Freestyle and 5.4×10^6 cells/mL in supplemented Freestyle medium. Therefore, production in supplemented Freestyle medium was increased proportionally to the improvement in cell growth (1.9-fold). At high cell densities (beyond the mid-log phase point), VLP production decreases drastically. This decrease in production efficiency at higher cell densities is accompanied with a decrease in transfection efficiency observed at higher cell concentrations. In an attempt to overcome this problem, a medium exchange just prior to transfection was performed (Fig. 5E and F). VLP production was significantly improved with medium exchange at all cell densities. However, in terms of transfection efficiency a significant increase was observed at high cell densities ($3–5 \times 10^6$ cells/mL) whereas transfection efficiency was negatively affected by medium exchange at low cell densities ($0.5–2 \times 10^6$ cells/mL). The best production performance in supplemented Freestyle media with medium exchange was also achieved at mid-log phase between 2 and 3×10^6 cells/mL (23.8 ± 1.0 F.U. and 25.0 ± 0.7 F.U. respectively, 48 hpt). This equals to 2.8 μg of Gag-GFP/mL according to the in-house validated Gag-GFP VLP quantitation method (Eq. (1)). VLP concentrations obtained under optimal conditions were also determined using nanoparticle tracking analysis (NTA) giving a total of 2.7×10^9 VLPs/mL. Considering both values and the molecular weight of Gag-GFP, a rough estimation of ~7700 Gag-GFP copies/VLP was obtained, which is within the expected range according to published literature (Briggs et al., 2004; Carlson et al., 2008; Chen et al., 2009). This production is 1.5-fold higher than that observed without medium exchange. Overall, by using supplemented Freestyle medium with medium exchange, production of VLPs was increased 2.4-fold.

3.4.2. *Effect of DNA/PEI complex concentration on transfection efficiency and VLP production*

Since the experiments described above were carried out using standard transient transfection conditions (1 μg of DNA/mL of culture and a DNA to PEI ratio of 1:2) optimized for transfecting 1×10^6 cells/mL (Geisse et al., 2005; Tom et al., 2008) the possibility that the DNA/PEI complex concentration was insufficient to efficiently transfect higher cell densities was investigated. As both PEI and HIV-1 Gag have known cytotoxic effects at high concentrations (Ansorge et al., 2009; Aravindan et al., 2009; Gebhart and Kabanov, 2001; Hammonds et al., 2007), the effect of DNA/PEI complex concentrations on cell density and viability was evaluated 48 hpt (Fig. 6A). Cells were transfected at a concentration of 1×10^6 cells/mL. In the absence of DNA/PEI complex or the presence of very low amounts (0.1 μg of DNA/mL, data not shown), cells grew from 1 to almost 3×10^6 cells/mL while maintaining high viability (Fig. 6A). However, cell density and viability drop in a dose dependent manner when the complex concentration is increased. Over 3 μg of DNA/mL, cells present a viable density lower than at the time of transfection, indicating a high DNA/PEI toxicity. Transfection efficiency is maintained around 50–60% regardless of the concentration of complex used (Fig. 6B). However, the concentration of VLPs in harvested supernatants gradually declines with increasing DNA/PEI concentrations (Fig. 6B), in agreement with the cytotoxicity observed. To test whether transfection efficiency, particularly at cell densities over 1×10^6 cells/mL, could be improved by increasing the amount of DNA/PEI used for transfection, cells were seeded from 0.5×10^6 to 4×10^6 cells/mL and transfected with increasing complex concentrations (1–3 μg of DNA/mL). No significant improvement in the transfection efficiency nor the VLP production was observed with higher concentrations of DNA/PEI complex (Fig. 6C and D) at any cell density tested. As previously observed

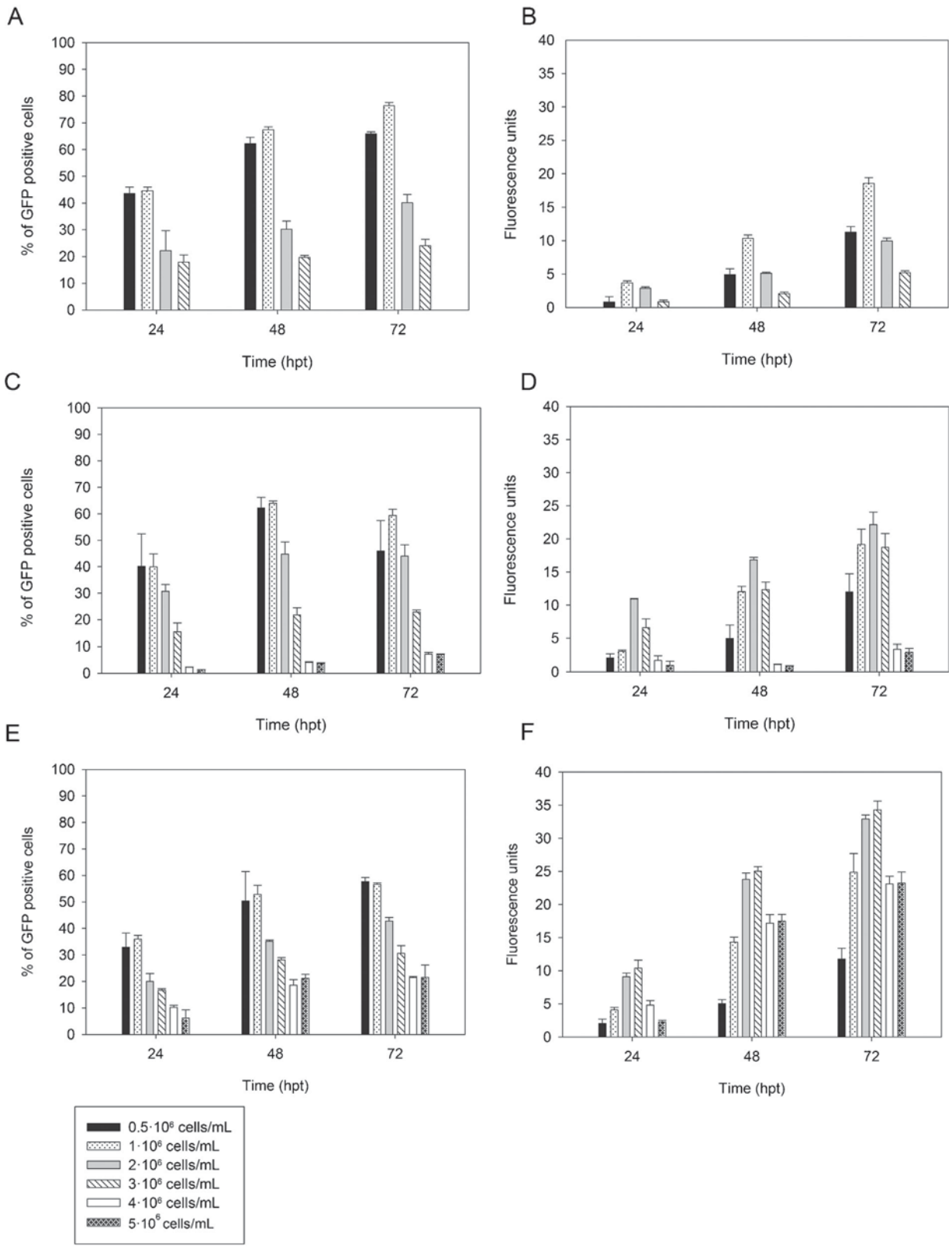

Fig. 5. Effect of the cell density on transfection efficiency and VLP production. HEK 293 cells were seeded at 0.3×10^6 cells/mL in 125-mL flasks and allowed to grow to the desired cell concentration. Cell densities ranged between 0.5 and 3×10^6 cells/mL for Freestyle medium (A and B) and between 0.5 and 5×10^6 cells/mL for supplemented Freestyle medium either without (C and D) or with (E and F) medium exchange performed before transfection. Cells were transfected with 1 μg of DNA/mL of culture and a DNA to PEI mass ratio of 1:2. HEK 293 transfection efficiency (A, C and D) and VLP fluorescence intensity (B, D and F) in cell culture supernatants were measured. Mean values ± standard deviation of triplicate experiments are represented.

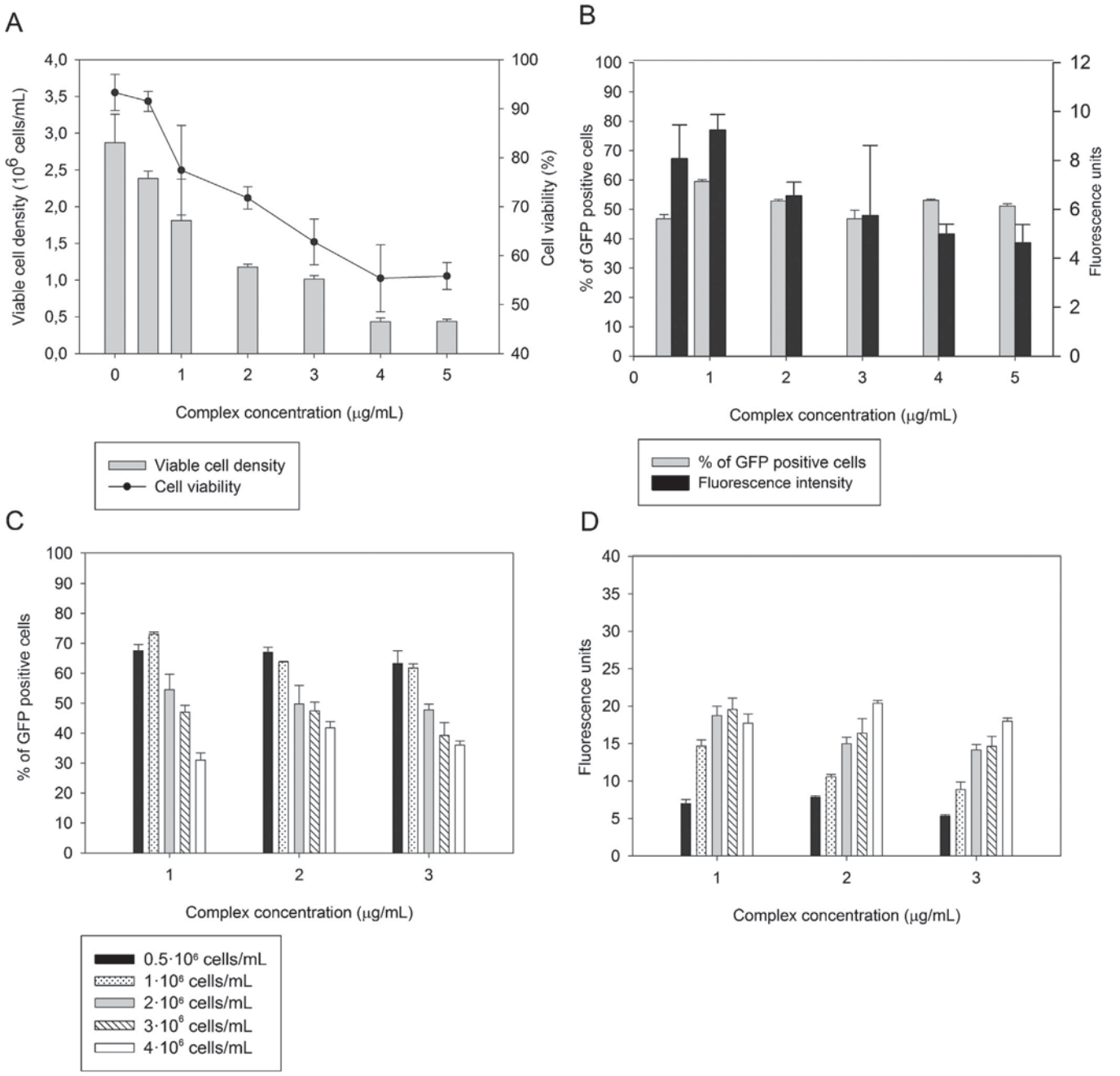

Fig. 6. Effect of DNA/PEI complex concentration on transfection efficiency and VLP production. The toxicity of Gag-GFP plasmid DNA/PEI polyplexes upon transfection of HEK 293 cells growing in supplemented Freestyle medium was evaluated (A and B). Cells were seeded at 0.5×10^6 cells/mL in 125-mL flasks the day before transfection. On the day of transfection, 2 mL of cell suspension typically at 1×10^6 cells/mL with >90% viability were transferred into six-well plates and transfected with 1–5 μg of DNA/mL of culture and a DNA to PEI mass ratio of 1:2. HEK 293 cell density and viability (A) and transfection efficiency and VLP fluorescence intensity (B) in cell culture supernatants were measured at 48 hpt. The effect of DNA/PEI complex concentration on transfection efficiency (C) and VLP production (D) 48 hpt in supplemented Freestyle medium was evaluated at different cell densities ($0.5–4 \times 10^6$ cells/mL). Mean values ± standard deviation of triplicate experiments are represented.

(Section 3.4.1), the best production performance was achieved by transfecting cells at $2–3 \times 10^6$ cells/mL with 1 μg of DNA/mL. Therefore, it can be concluded that the use of 1 μg of DNA/mL at these cell densities is not limiting transfection efficiency.

3.4.3. *Effect of cell cycle on transfection efficiency and VLP production*

A plot showing the relation between fluorescence intensity and number of GFP positive producer cells is shown in Fig. 7A. A good correlation is observed ($R^2 = 0.8490$). This observation confirmed that the higher the number of transfected producer cells, the higher the amount of VLPs produced. However, as shown in previous experiments it is not sufficient to transfect higher density cultures as, in high density cultures, a low percentage of cells actually became transfected producer cells. Cell cycle analysis of HEK 293 cell cultures throughout exponential growth was performed (Fig. 7B). Interestingly, the percentage of cells in G2/M phase gradually decreased as cell density increased. Based on these observations we hypothesized that the decrease in transfection efficiency observed with increasing cell densities was related with the cell cycle state. To investigate this further, an additional experiment was carried out. HEK 293 cell cultures were grown

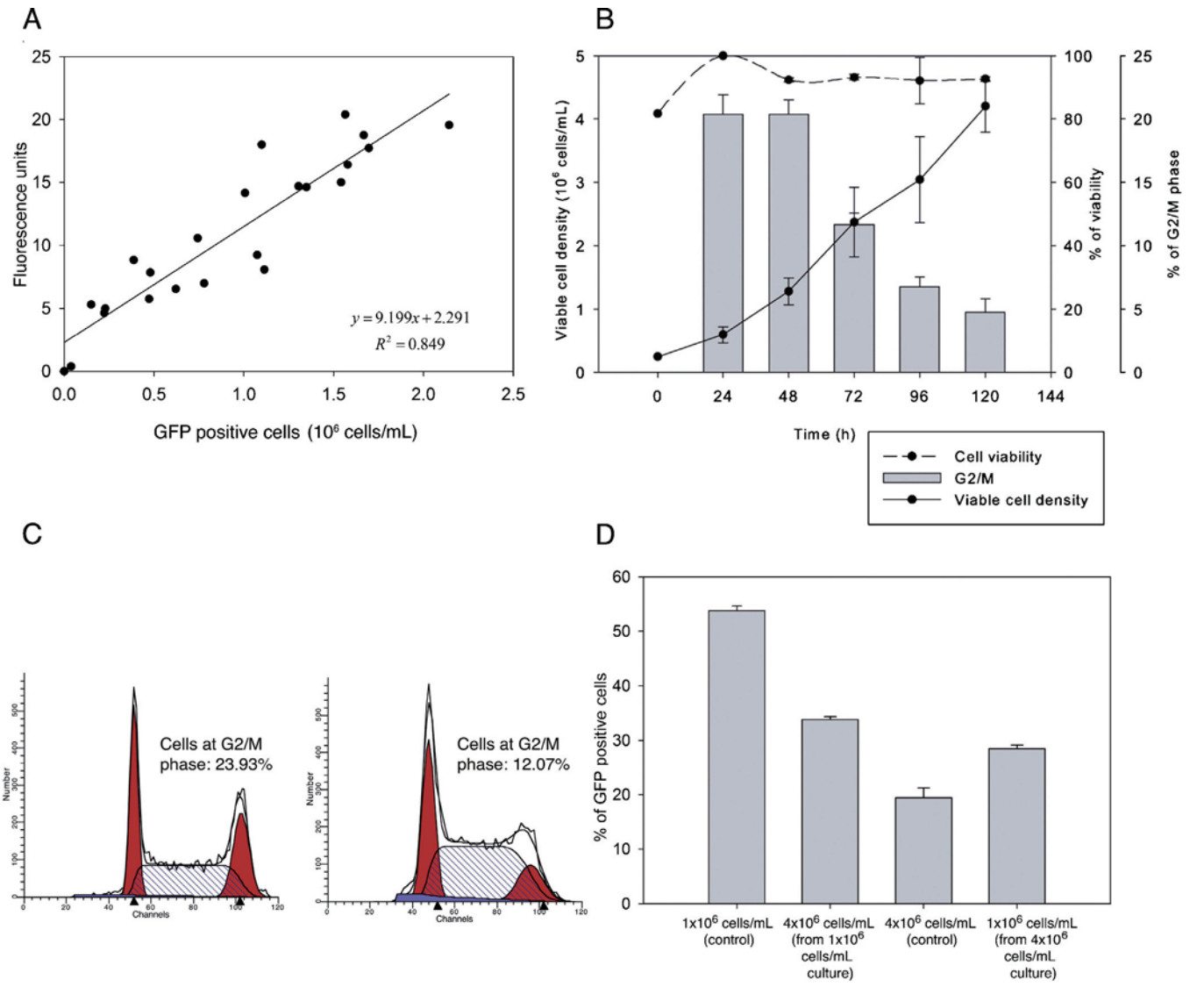

Fig. 7. Effect of cell cycle on transfection efficiency and VLP production. Correlation between the number of GFP positive producer cells and VLP fluorescence 48 hpt as obtained from data presented in Fig. 6C and D. The number of GFP positive cells was calculated as viable cell density times the % of GFP positive cells (A). Percentage of cells in G2/M phase throughout cell culture growth (B). Cells were seeded at 0.25×10^6 cells/mL in 125-mL shake flasks. Cell density, cell viability and percentage of cells in G2/M phase were monitored until the culture reached 4×10^6 cells/mL. Cell cycle analysis of the cells grown up to 1×10^6 cells/mL or 4×10^6 cells/mL (C). Percentage GFP positive cells 24 hpt of cultures grown to 1×10^6 cells/mL (control early log phase) and the same cells concentrated to 4×10^6 cells/m and cultures grown to 4×10^6 cells/mL (control late log phase) and the same cells diluted to 1×10^6 (D). In all cases a complete medium exchange prior to transfection was performed.

to 1×10^6 cells/mL (early log-phase) or 4×10^6 cells/mL (late log-phase). Following medium exchange, cells were re-suspended in fresh medium to either 1 or 4×10^6 cells/mL and transfected with Gag-GFP plasmid. As expected, the percentage of cells in G2/M phase at the time of transfection was lower for cells derived from cultures in late log-phase (grown to 4×10^6 cells/mL) (12.1%) than cells in early log-phase (grown to 1×10^6 cells/mL) (23.9%) (Fig. 7C). Transfection efficiencies were analyzed 24 hpt and results are shown in Fig. 7D. As previously observed, transfection efficiency 24 hpt was around 50% for cells in early log-phase transfected at 1×10^6 cells/mL but low (~18%) for cells in late log-phase transfected at 4×10^6 cells/mL. Unexpectedly, transfection efficiency of cells in early log-phase, concentrated and transfected at 4×10^6 cells/mL was significantly lower than 50% (~30%). Additionally, transfection efficiency of cells in late log-phase, diluted and transfected at 1×10^6 cells/mL was significantly higher than 18% (~30%). These results suggest that besides the percentage of cells in G2/M phase, other factors related to the cellular density influence cell transfection efficiencies. These aspects will be further investigated in future work.

4. Discussion

HIV-1 Gag VLPs represent an attractive platform for the generation of particulate vaccine candidates. Although there are a number of reports describing the generation of HIV-1 Gag VLPs using the baculovirus expression system, to the best of our knowledge the production of these VLPs using an industrially relevant suspension adapted mammalian cell line, such as the GMP-compliant HEK 293 used in this work, has not yet been reported. The use of mammalian as opposed to insect cell systems may be preferred for a number of reasons. For instance, the incorporation of insect proteins or baculovirus-derived proteins, such as the gp64, in the lipid envelope of HIV-1 Gag VLPs is known to provoke a strong immune response that can potentially mask the response against the desired envelope antigen (Deml et al., 2005; Hammonds et al.,

2007). In addition, removal of contaminating baculovirus particles during downstream processing is challenging as these viruses share similar physicochemical properties with VLPs (Deml et al., 2005; Hammonds et al., 2007). Moreover, the post-translational and proteolytic capabilities of insect cells are not identical to those of mammalian cells, which results in VLP structures that do not accurately mimic authentic HIV-1 particles (Deml et al., 2005; Hammonds et al., 2007).

The use of a culture media devoid of any animal-derived compound is critical for the acceptability of vaccine products. Several ADCF and CD media formulations are commercially available for culture of HEK 293 suspension cells. Our results showed that most of these media can efficiently support the growth of the HEK 293 clone used in this work (HEK293SF-3F6). Similar growth properties were observed by other authors working with HEK 293 EBNA cells (Geisse et al., 2005), although they were able to grow this cell line in CD 293 medium. The inability for HEK 293SF-3F6 cells to grow in CD293 (despite repeated attempts) was not investigated, yet, it may be related with highly specific nutritional requirements that can be encountered with different clones of the same cell line and even different sources of the same clone (Butler, 2005). Importantly, not all culture media can be used for PEI-mediated transient transfection as shown in this work and previously by others (Geisse et al., 2005; Pham et al., 2003; Tom et al., 2008). It has been speculated that the presence of anti-clumping agents such as heparin or dextran sulfate polyanions (frequently added to suspension culture media formulations to prevent cells from aggregating) interfere with DNA/PEI complex entry. More recently, Geng et al. (2007) have shown that, at the concentrations used in Excell medium, dextran sulfate is able to completely inhibit transfection. It has also been reported that this inhibition can be overcome by using higher amounts of complex than usual (Backliwal et al., 2008b).

During process development, the use of reporter proteins and marker genes is extremely common to facilitate quantitation and monitoring of a product of interest. In this work, we have made use of this approach by expressing a Gag-GFP fusion protein that enabled the generation of fluorescent VLPs that were easily tracked and quantified in cell culture supernatants. The great majority of Gag-GFP present in cell culture supernatants have shown to be correctly assembled into virus-like particles as verified in this work by isolation of assembled fluorescent Gag-GFP VLPs by ultracentrifugation followed by subsequent analyses of the pellet and supernatant fractions by fluorometry and SEC. The fluorescence intensity in supernatants obtained after ultracentrifugation was under the limit of detection confirming the absence or low contamination of harvested cell culture supernatants with Gag-GFP monomers. It should be noted that in the absence of viral protease, HIV-1 maturation (process by which the Gag polyprotein is cleaved into its distinct subunits and the virus particle structure is reorganized) does not take place. Viral particles generated were of the expected size (~140 nm) and morphology as determined by electron microscopy and NTA, consistent with immature HIV-1 particles (Briggs et al., 2004; Valley-Omar et al., 2011).

The design of a supplemented culture medium for improved HEK 293 cell growth is reported here. A well established and proven statistical method for medium optimization was employed. Both HEK 293 cell growth and VLP production were increased in a proportional manner. Further VLP production improvement was achieved by complete medium replacement at the time of transfection. A similar positive effect of medium exchange on production has previously been reported (Backliwal et al., 2008b). The supply of nutrients in fresh culture medium that might be exhausted in the conditioned medium may contribute to this effect. Notably, the production improvement is more remarkable at higher cell densities and it is accompanied by higher transfection efficiencies suggesting potential removal of metabolism by-products present in the conditioned medium that could interfere with transfection as previously reported (Durocher et al., 2002; Schlaeger and Christensen, 1999; Tom et al., 2008). Metabolism by-products are expected to be more concentrated in conditioned medium of high rather than low cell density cultures. A negative effect of medium exchange on transfection efficiency at low cell densities ($0.5–2 \times 10^6$ cells/mL) was even observed, which may be attributed to centrifugation (Tom et al., 2008). The overall production improvement was 2.4-fold. This should result in a considerable reduction of the manufacturing costs considering that the supplements added to the base serum-free medium represent only a small fraction of the medium cost (approximately 25%), not to mention other savings in manufacturing expenses associated with the production scale. Although it is relatively easy to perform discontinuous medium exchange (batch replacement) at scales of a few liters by centrifugation in the laboratory, this operation is not desirable at scales of hundreds to thousands of liters in the industrial setting. At these large scales it is more convenient to use a perfusion process (Altaras et al., 2005). For instance, the medium can be continuously exchanged during the cell growth phase prior to transfection by introducing perfusion elements as previously shown (Ansorge et al., 2009).

Further VLP production improvement was limited by a significant loss in transfection efficiency observed at cellular densities beyond the mid-log phase point ($2–3 \times 10^6$ cells/mL). As transfection efficiency seemed to be critical for any additional process improvement, the role of several parameters on transfection efficiency and VLP production were analyzed in this work. No significant improvement in transfection efficiency or VLP production was observed by increasing the amount of DNA/PEI used for transfection indicating that DNA/PEI complex concentrations of 0.3–0.5 μg of DNA/million cells are sufficient to efficiently transfect cells. This is in line with the concentrations recommended by others working with high cell density cultures (0.4–0.6 μg of DNA/million cells) (Ansorge et al., 2009; Rajendra et al., 2011; Sun et al., 2008; Swiech et al., 2011). These results are also in agreement with those reported by Carpentier et al. (2007) who concluded that successful transgene expression is more likely to depend on a "cellular competent state" than on the quantity of plasmid DNA delivered per cell. In this sense, Brunner and collaborators have indicated that transfection efficiency is strongly dependent on the cell cycle stage at the time of transfection, with cells in the G2/M phase giving greater levels of transfection than G1 cells (Brunner et al., 2000). The authors have hypothesized that transport of DNA into the nucleus is a very inefficient process and that transfection of cells shortly before their next cell division (close to M phase) is facilitated by nuclear membrane breakdown. In support of these studies, Tait et al. (2004) have shown that both transfection efficiency and protein expression can be enhanced by arresting cells in G2/M phase using nocodazole. In light of these reports, a further attempt to understand the loss in transfection efficiency observed at high cell densities was carried out in this work. In agreement with reported results, it was observed that cells grown beyond the mid-log point had a lower percentage of cells in G2/M phase. However, this did not seem to be the only factor controlling transfection efficiency.

To conclude, an optimized HIV Gag VLP production strategy is outlined in this work. The strategy consists in growth of HEK 293 cells up to $2–3 \times 10^6$ cells/mL (mid-log phase point) in supplemented Freestyle medium and subsequent transfection with 1 μg of DNA/mL of culture and a DNA to PEI mass ratio of 1:2 after a complete medium exchange. The strategy should facilitate the generation of sufficient VLP material for pre-clinical studies. By using a GMP-compliant suspension adapted cell line and a culture medium devoid of animal-derived components, a fast translation into clinical trials is envisioned. It is expected that the ability to transfect an even higher number of HEK 293 cells should result in further VLP production improvement. This may be accomplished by growing

cells in fed-batch or perfusion as opposed to batch mode (Ansorge et al., 2009; Sun et al., 2008) or by arresting cells in G2/M (e.g. using nocodazole) (Tait et al., 2004) in order to attain a higher number of VLP producer cells.

Conflict of interest

No conflict of interest to declare.

Acknowledgements

The authors wish to thank Dr. Amine Kamen (NRC, Montreal, Canada) for valuable discussions and for providing the HEK 293 cell line used in this study. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pGag-EGFP (Cat#11468) from Dr. Marilyn Resh. We would also like to thank Dr. Jorge Carrillo at IrsiCaixa (Badalona, Spain) and Gavin Whissell for helpful advice and comments. The help of Manuela Costa from Institut de Biotecnologia i Biomedicina with FACS analyses and Dr. Salvador Bartolomé from Departament de Bioquímica i de Biologia Molecular is appreciated. The recombinant albumin was a generous gift from Merck Millipore. This work is supported by a grant of Ministerio de Innovación y Ciencia of Spain (MICIN BIO2011-2330). Laura Cervera is a recipient of a PIF scholarship from UAB.

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Intracellular characterization of VLP production by transient transfection in HEK 293 cells.

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Abstract

Transient Gene Expression is a widely used technique to introduce foreign DNA into mammalian cells. It is a very suitable technique to produce enough quantities of biomaterial to perform pre-clinical studies and the scalability of the process has been demonstrated in recent years. Even though it is a very widespread technique, less is known about the transfection process at its cellular level. The aim of this work is to study the process by which DNA crosses the cell membrane and reaches the nucleus after transfection using PEI as transfection reagent in a process in which Gag-GFP VLPs are produced. Flow cytometry assays show that DNA:PEI polyplexes start to interact with the cells from the very beginning and a linear increase in transfection efficiency is observed until 60 minutes of contact between the cells and the complexes. No change in transfection efficiency is obtained afterwards. The same profile is observed when fluorescence in the supernatant is analyzed. When cells are observed under confocal microscopy, it can be observed the DNA/PEI complexes seem to be attached to the plasma membrane since the moment that the culture is transfected. After 1.5 hpt complexes are detected in the cytoplasm of the cells and reach the nucleus around 4 hours post transfection. After 10 hours post transfection GFP fluorescence is detected inside the cells, but generalized budding of VLPs from the cells is not observed until 48 hours post transfection. The optimal harvest time is determined as 72 hpt as VLP production is highest while high viability of the culture is maintained.

1. Introduction

Transient gene expression (TGE) is a methodology to introduce foreign DNA to cells. TGE has been routinely used in laboratory for mechanistic studies. Nowadays, the interest of the industry has focused on TGE as it facilitates rapid production of biopharmaceuticals in early development phases for characterization assays and pre-clinical studies. Apart from the electroporation methods used to introduce DNA into the cells, several transfection agents have been proven effective to complex with the plasmid DNA to transfect the cells. Cationic lipids, such as Lipofectamine 2000™ (Invitrogen), have shown high efficiency of transfection, but its high price precludes its use in large-scale processes (Geisse, 2009). One of the most extended complexing agents is Calcium phosphate as it has been used at large-scale, is easy to use and cost-effective (Batard et al., 2001; Jordan et al., 1996; Meissner et al., 2001). The only inconvenient of Calcium phosphate is that cannot be used with serum-free media, as the low-calcium concentration of these media used to avoid cell clumping, interfere with calcium-phosphate transfection (Geisse, 2009; Jordan and Wurm, 2004). A third group of complexing agents are the cationic polymers. Polyethylenimine (PEI) has gained interest in recent years due to its easy use, low price and good performance in suspension CHO and HEK 293 cells when serum-free media is used. PEI was discovered to efficiently introduce DNA into the cells by (Boussif et al., 1995) showing good transfection efficiencies both *in vitro* and *in vivo*. PEI is known to complex the DNA resulting in positive charged polyplexes that interact with the negative charged parts of the cell membrane such as heparin sulfate proteoglycans (Kopatz et al., 2004). After interaction, polyplexes are up-taken by the cells through endocytosis into acidified endosomal compartments (Godbey et al., 1999). After the introduction to the cells PEI

acts as a buffer and through its known “proton sponge” effect is able to escape from the endosomes and liberate the polyplexes to the cytoplasm, preventing them of enzymatic decradation of DNA (Boussif et al., 1995). Then polyplexes have to reach the nucleus and enter, which is thought to be the limiting step in cell transfection, as only a very small fraction od plasmid delivered into the cell is finally translocated into the nucleus and transcribed (Bieber et al., 2002; Carpentier et al., 2007; Cohen et al., 2009). The way polyplexes enter the nucleus is the less characterized step of gene delivery. One proposed mechanism suggests that the polyplexes may enter into the nucleus during the break down of the nuclear membrane during cell division (Grosse et al., 2006; Tait et al., 2004). Alternatively, a second mechanism postulates that the polyplexes are transferred to the nucleus by active transport through the nuclear envelope, independently of cell division (Han et al., 2009).

Transient transfection can be carried out to produce viral vectors, recombinant proteins and antibodies. In the present work, the product of interest is HIV-1 virus-like particles. Virus-like particles are promising as new generation vaccines, because while they mimic the native virus structure, they lack of genetic material, which makes them very safe both for patients and manufacturers involved in their production (Deml et al., 2005; Young et al., 2006). One of the intrinsic characteristics of HIV-1 VLPs is that, like the virus itself, they are enveloped VLPs. During the budding process, Gag virus particles acquire their lipid envelope from the producer cell.

In this work, the focus is on the kinetics of the different steps involved in this process, which has been divided in the following steps: a) Polyplexes/cell interaction, b) Polyplexes penetration into the cytoplasm, c) Polyplexes penetration into the nucleus, d) Initiation of gene expression, e) VLP budding from the cells and f) Optimal harvest time. This time points are considered of high importance in process optimization as its

knowledge can lead to better understanding and identification of checkpoints to make the transient transfection process more efficient.

2. Materials and Methods

2.1 Cell line, media and culture conditions

The cell line used in this work is a serum-free suspension-adapted HEK 293 cell line (HEK293SF-3F6) kindly provided by Dr. Amine Kamen from the BRI of National Research Council of Canada (Montreal, Canada). It 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). 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 (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).

2.2 Plasmids

The pGag-EGFP plasmid used in this work codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP (Hermida-Matsumoto and Resh, 2000). The plasmid from the 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).

The plasmids were prepared and purified as previously described (Segura et al., 2007).

2.3 Transient transfection

HEK 293 suspension cells were transiently transfected using 25-kDa linear polyethilenimine (PEI) (PolySciences, Warrington, PA, USA). Transfections were performed using a final DNA concentration of 1 µg/mL of 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 incubated for 15 min at room temperature to allow complex formation prior to its addition to the cell culture.

2.4 DNA labeling

To study the kinetics of the PEI-DNA complexes entrance into the cell, the plasmid Gag-GFP was labeled with the fluorochrome Cys3 ™. This marker absorbs at 649 nm and emits at 670 nm so it has a red fluorescent color. The labeled DNA was generated using the Label IT® Traker ™ Kit (Mirus Bio Technology) following manufacturer's instructions. The plasmid was quantified using NanoDrop 1000 (Thermo Scientific, Wilmington, Delaware, USA) and diluted to a concentration of 100 µg/mL using MiliQ water.

2.5 VLP quantitation

The concentration of Gag-GFP VLPs was assessed by fluorometry using an *in-house* developed and validated quantification assay (Gutiérrez-Granados et al., 2013).VLP containing supernatants were recovered by cell culture centrifugation at 1000×g for 5 min. On the other hand, Gag-GFP containing cell lysates were prepared by subjecting cell pellets to 3 freeze-thaw cycles followed by centrifugation at 13.800 × g for 20 min to eliminate cell debris.

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:

$$Gag-GFP\left(ng/mL\right)=(3.245\times RFU-1.6833)\times 36 \quad (1)$$

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.

2.6 Confocal microscopy

In order to study the kinetics of the transient transfection system, the visualization of VLP producer cells was performed by Flouview® FV100 confocal microscope (Olympus, Tokyo, Japan). Firstly, HEK 293 cells were dyed with CellMask™ 10 mg/mL (Life Technologies, Masachussets, USA), which stains the plasma membrane in a deep red color and with Hoechst 33342, Trihydrochloride, Trihydrate 10 mg/mL (Life Technologies, Masachussets, USA), which stains the nuclear DNA in blue cyan color. The excitation/emission parameters of each dye used for Confocal Microscopy were 649nm/ 666nm for Cell Mask™, 649nm/670nm for Cys 3 ™, 350nm/461nm for Hoescht and 488nm/510nm for Gag-GFP VLPs.

1 µL of each dye was added to 1000 µL of cell culture, after 10 minutes of incubation at room temperature and protected from light, the mixture was centrifuged at 300g for 5 minuts and cell pellet was resuspended with fresh medium. Then, the transient transfection with Cys3™ labeled DNA was performed. Samples were placed in glass bottom dishes (MatTek, Massachusetts, USA) for visualization under the microscope. Final images were processed using LAS AF™ software (Leica Microsystems,Weztlar, Germany).

2.7 Flow citometry

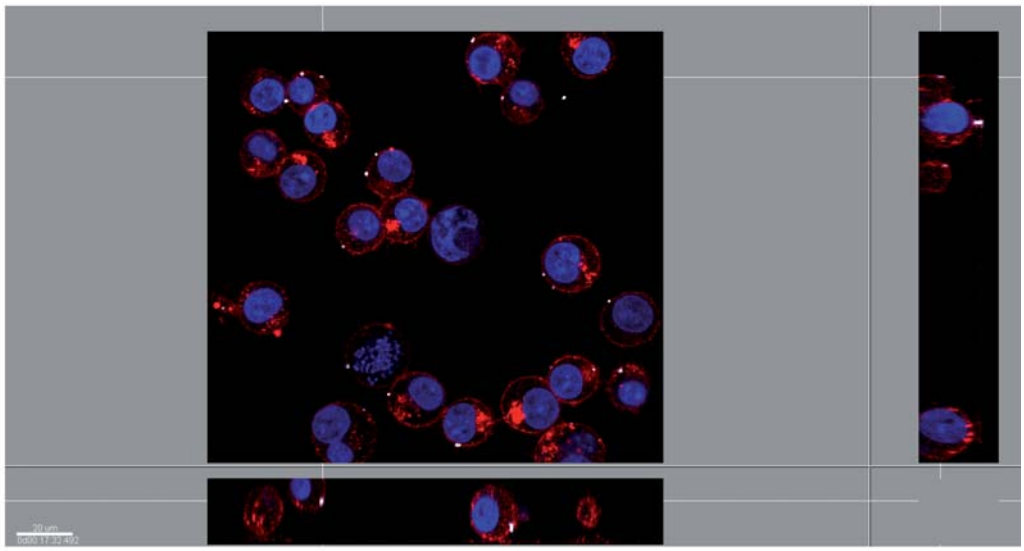
The percentage of GFP positive cells and the percentage of cells united to DNA/PEI polyplexes was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA).

3. Results and discussion

3.1 Study of the kinetics of DNA/PEI polyplexes penetration into the cells.

As it can be observed in figure 1A, polyplexes can be found localized on the cell membrane since the first moment after addition to the cell culture. At 30 minutes post transfection the polyplexes are still attached to the external surface of the cell membrane, while after 90 minutes post transfection, polyplexes are detected inside the cell (figure 2B).

A



B

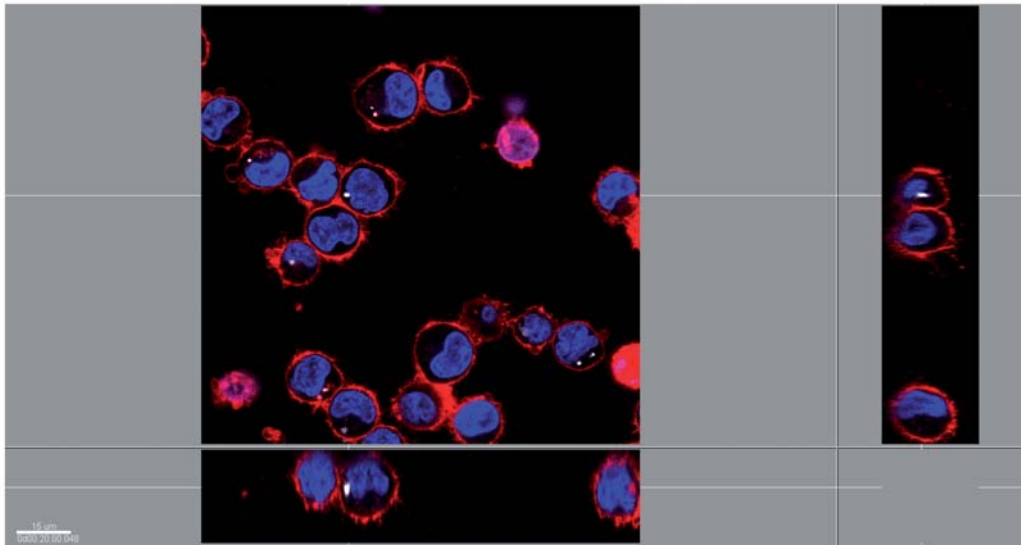


Figure 1 Confocal images of HEK 293 cells taken at 30 minutes post addition (A) and 90 minutes post addition (B).

In order to establish the exact moment when DNA/PEI polyplexes enter into the cells, an experiment was performed to study the effect of different contact times between polyplexes and cells. Cells were transfected using the standard transfection protocol and after 0 minutes post adition of the polyplexes, samples were taken every 5 minutes and centrifuged at 300xg and 5 minutes. After centrifugation, cells were seeded again in 6 well plates with fresh media. During the period from 45 minutes post transfection to 2 hours post transfection, samples were taken every 15/30 minutes. The percentage of transfection efficiency was analyzed at 24 and 48 hours post transfection and fluorescence in the supernatant (concentration of VLPs) was analyzed at 48 hours post transfection.

Figure 2 A and B shows the results of the kinetics of the penetration of the PEI/DNA polyplexes into the cells as obtained in this experiment, and reflected in the percentage of GFP positive cells. It can be observed a clear saturation type kinetics. The interaction between PEI/DNA polyplexes starts just when the complexes are added to the culture, at almost a constant rate, that further decreases with time. After 60 minutes, the percentage of transfected cells (both measured at 24 and 48 hours post transfection) as well as the fluorescence in the supernatant, reaches a plateau. These kinetics shows that 1 hour of contact is enough to transfect the maximum number of cells, or from another point of view, that the entrance of the DNA/PEI polyplexes into the cells takes 1 hour. Taking into consideration this kinetics and the previous observations with confocal microscopy, it can be deduced, that during the first hour post transfection, the interaction of the cells with the polyplexes becomes stronger until the complex enters into the cell, which happens around 1 hour post transfection.

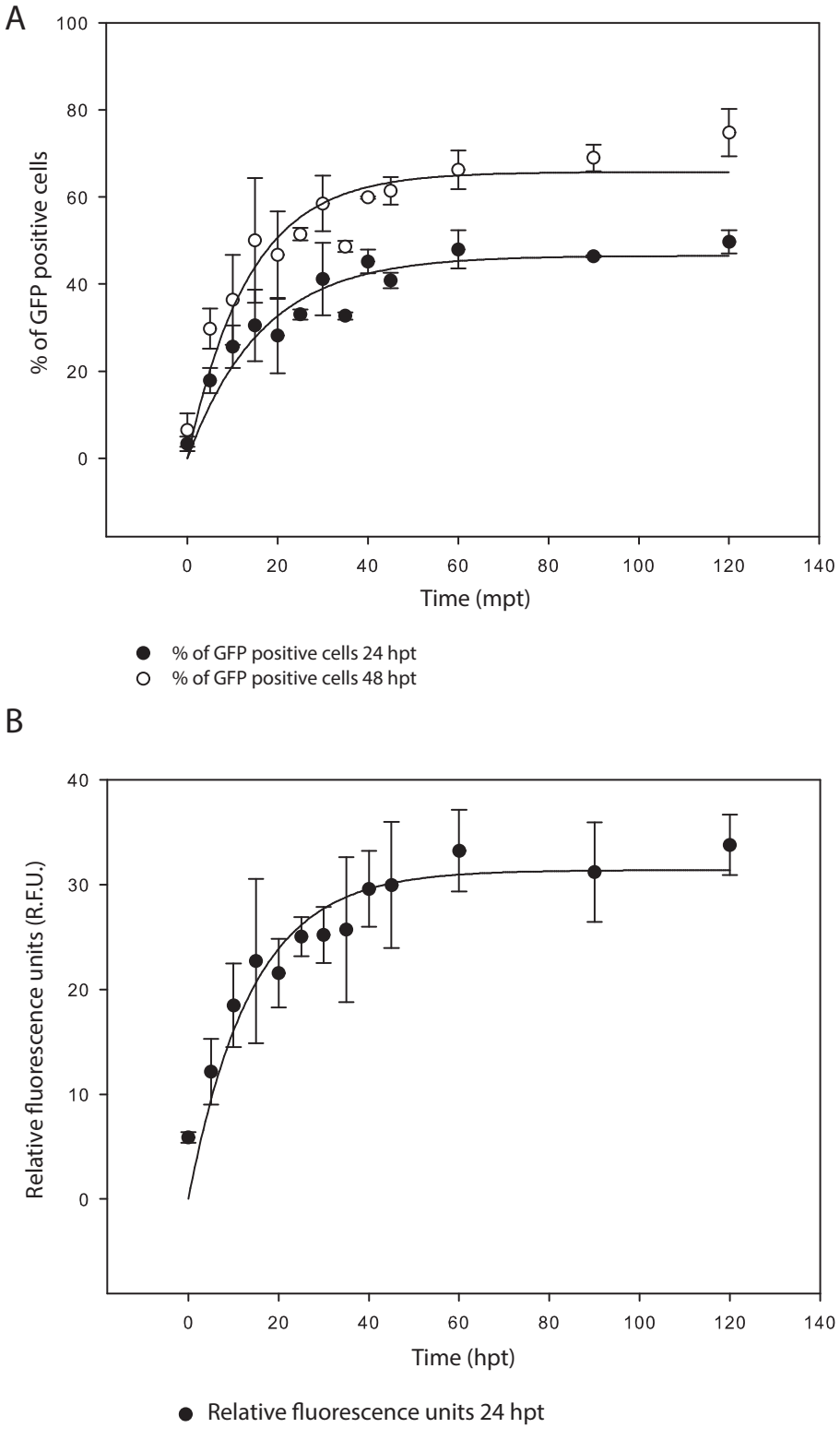


Figure 2 A) Percentage of GFP positive cells after 24 and 48 hpt after different times of contact between cells and DNA/PEI complexes. B) Relative fluorescence in the supernatant of the culture, corresponding to Gag-GFP VLPs produced 48 hpt.

The next step pursued to observe the kinetics of transfection and also the kinetics of production and the release of the VLPs from the cell by the budding process. To this respect, cells were transfected with a mixture of labeled and not-labeled DNA. Cells were transfected using the standard protocol, but with 0.5 µg/mL of labeled DNA and 0.5 µg/mL non-labeled DNA, mixed together and then complexed with the PEI to form the polyplexes. By this approach, the transfection process can be followed by flow cytometry. In figure 3 A it can be observed that after 20 minutes post transfection more than 95% of the cells have a complex either inside or interacting with the cell membrane. The comparison between this experiment, in which the interaction of polyplexes with the cells is observed, and the experiment in which the transfection efficiency was evaluated, can lead to the following conclusion. In relation to the observation that the difference between cell interaction with a complex at one hour post transfection was 98%, while the percentage of GFP positive cells that have been in contact with the polyplexes for the same time was 60 % at 48 hpt. These results suggest that in the 38% of the cells the polyplexes that have interacted with the cell are not able to express the protein, even because of the DNA did not reach the nucleus or once in the nucleus the protein was not expressed.

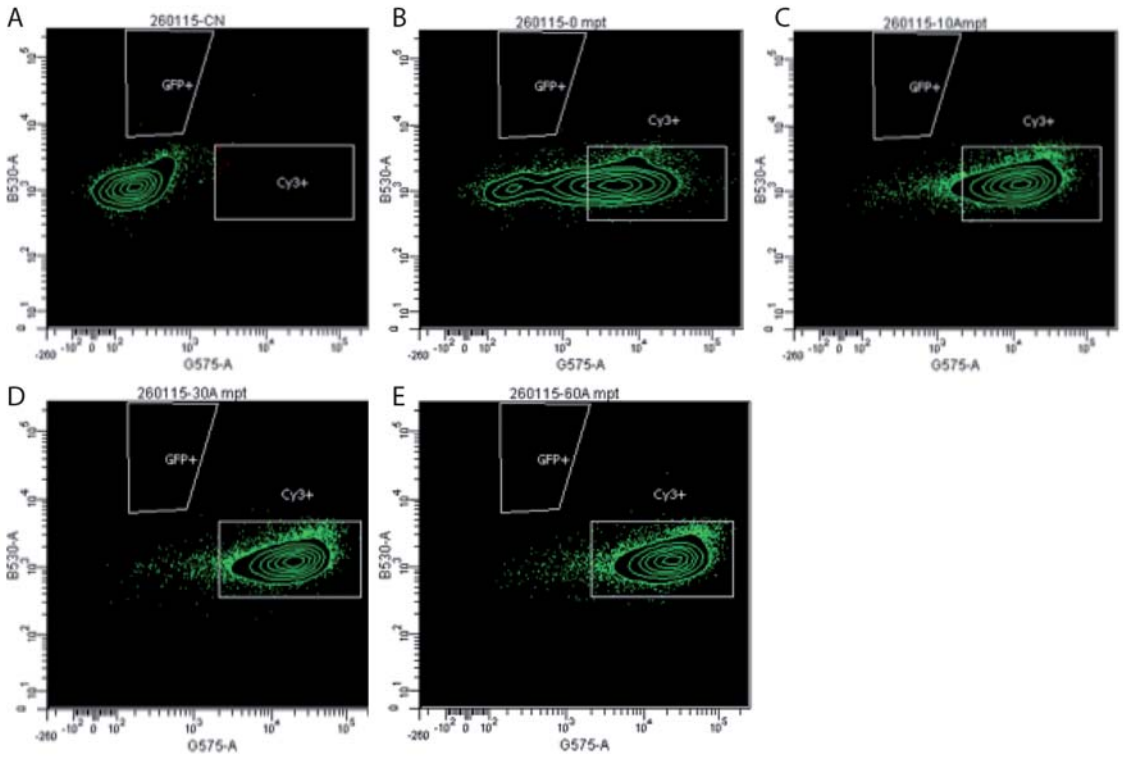


Figure 3 Flow cytometry analysis of the % of cells interacting with polyplexes. A) Non tranfected cells. B) 1 minute post addition, C) 10 minutes post addition, D) 30 minutes post addition, E) 60 minutes post addition.

In addition to the previous observations, an additional aspect to be considered is the observation, in figure 2A of an increase in transection efficiency between 24 and 48 hours post transfection. One possible hypothesis could relate this increase in the percentage of GFP positive cells to produced VLPs entering into other cells. To elaborate on this hypothesis, an experiment consisting in using the supernatant of a culture 24 hpt and 72 hpt to resuspend a cell pellet of non transfected cells. Cells were observed by flow cytometry 24 hours post resuspension with VLP containing medium.

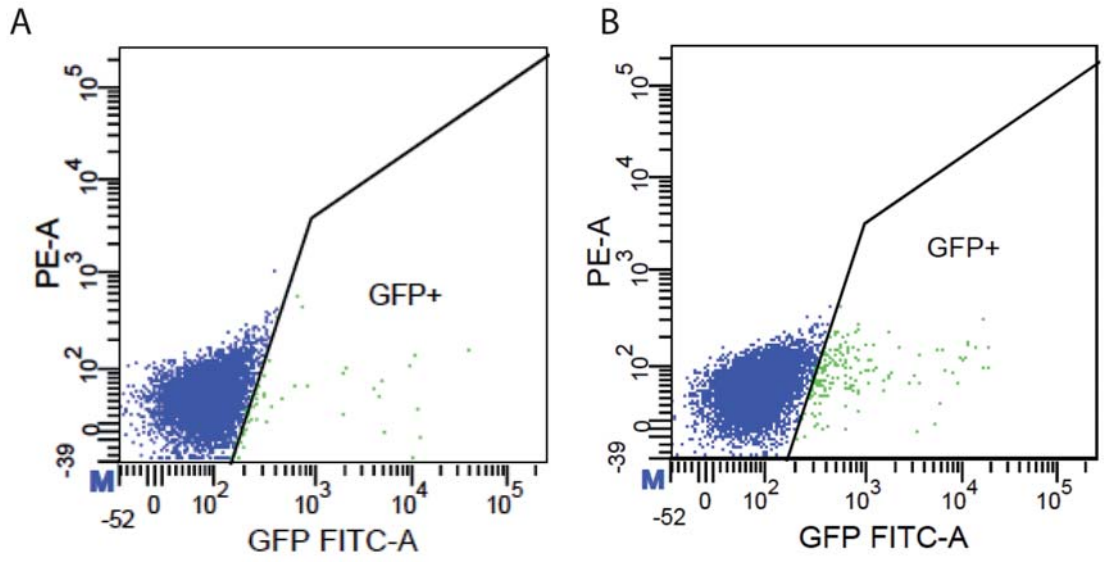


Figure 4 Percentage of GFP positive cells after 48 hours of resuspension with medium containing supernatant with VLPs collected at 24hpt (A) and 72 hpt (B)

As it is shown in figure 4 A and B no retransfection with produced VLPs can be observed, and for this reason the observed increase in transfection efficiency between 24 and 48 hpt might be related to additional polyplexes nuclear uptake, due to the fact that not all the cells pass mitosis at the same time, or due to late expression.

3.2. Gag-GFP production kinetics

An experiment following the expression of Gag-GFP was performed by taking samples to analyze the fluorescence in the supernatant, the fluorescence in the lysate (cell pellet plus supernatant), transfection efficiency and cell culture viability (Figure 5). It can be observed that the Gag-GFP VLPs assembly and budding process is a limiting step in Gag-GFP VLP production as there is fluorescence inside the cells that is excreted out the cell. On the other hand, it can be observed that once the cell viability of the culture starts decreasing, there is a degradation of the protein inside the cell. From this observation it can be determined that the time of harvest should be 72hpt since the

fluorescence in the supernatant does not further increase and the culture has a viability of 89%.

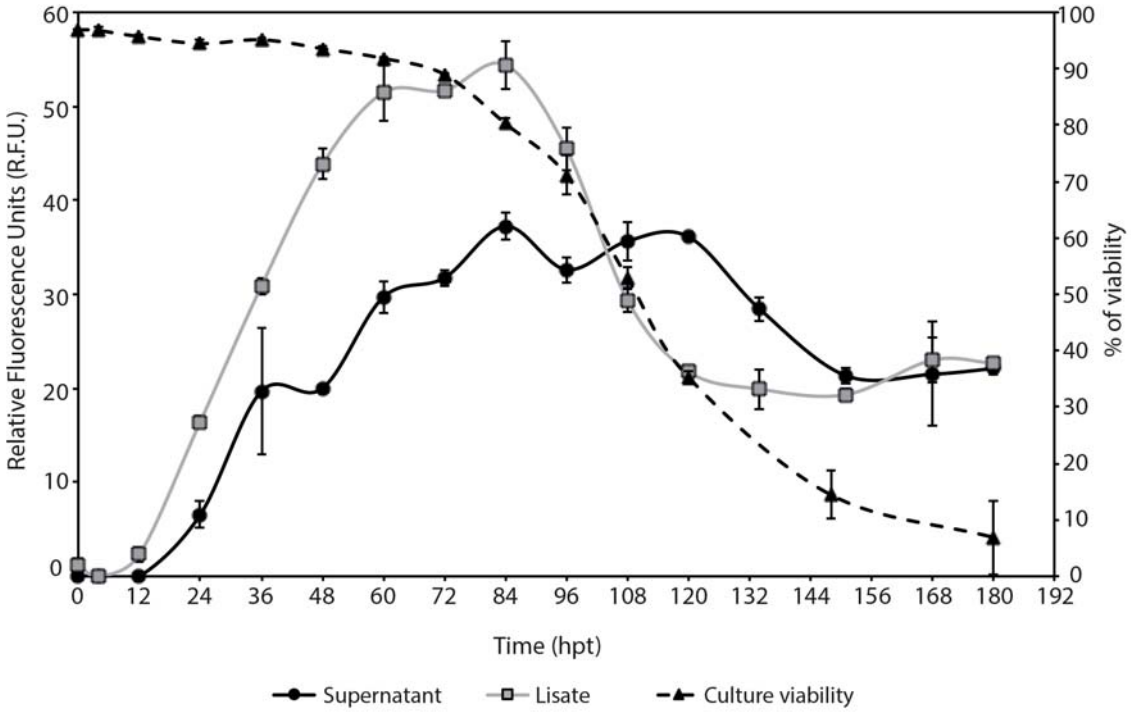
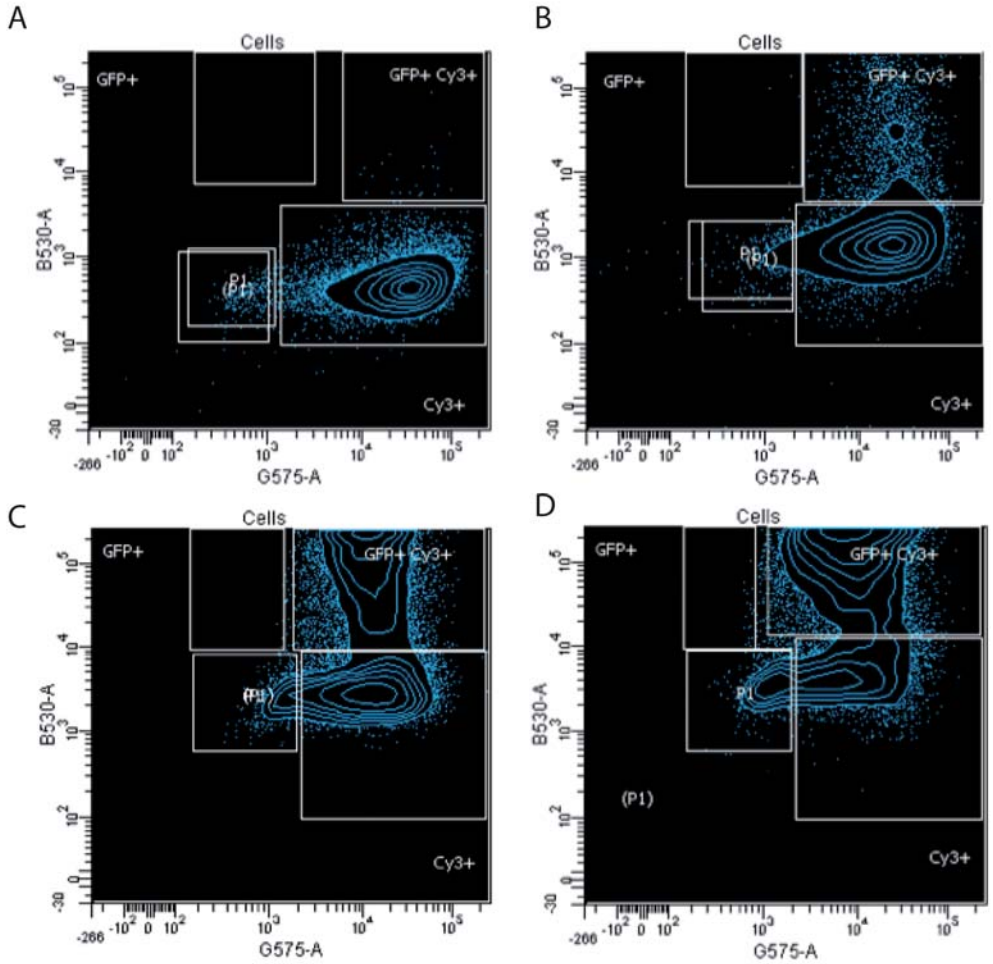


Figure 5 Gag-GFP production kinetics

The kinetics of Gag-GFP expression was also studied using flow cytometry (Figure 6). Samples taken at different time points after transfection were analyzed and as can be observed in figure 6, at 4 hpt no expression of Gag-GFP can be detected. At 10 hours post transfection 11% of the cells are expressing the protein and the % increases at 24 hpt (43.5%) and at 48 hpt (61.7%). Interstingly the percentage of Cy3 positive population decreases over time, suggesting a lost of the plasmid in dividing cells. On the other hand there can not be observed cells expressing Gag-GFP, without having a complex inside, wich is in agreement with the results presented above were no retransfection of cells with released VLPs was observed.


A
Cells
GFP+
GFP+ Cy3+
P1
Cy3+
B530-A
G575-A
B
Cells
GFP+
GFP+ Cy3+
P1
Cy3+
B530-A
G575-A
C
Cells
GFP+
GFP+ Cy3+
P1
Cy3+
B530-A
G575-A
D
Cells
GFP+
GFP+ Cy3+
P1
Cy3+
B530-A
G575-A

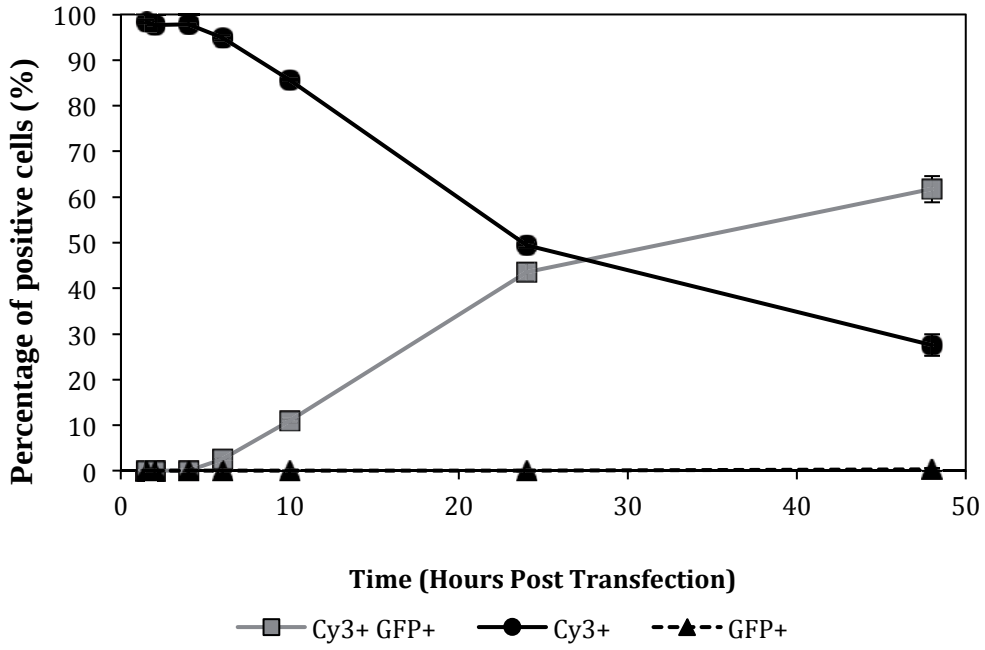


Figure 6 Kinetics of Gag-GFP expression by flow citometry. Images of flow cytometry analysis a different time points. A) 4 hpt, B) 10 hpt, C) 24 hpt, D) 48hpt and E) Graph representing the percentages of cells Cy3 positive, expressing Gag-GFP and Cy3 positive and only expressing Gag-GFP.

In confocal images taken 24 hours post transfection (Figure 6A), it can be observed that almost all the cells have a complex inside even though not all the cells express the Gag-GFP protein. In the majority of the cells no colocalization of the VLPs with the cell membrane can be observed, suggesting that the budding process starts after this time, which is in agree with figure 5, were the fluorescence in the supernatant was only 6.5 R.F.U. at 24hpt. On the other hand, colocalization of Gag-GFP VLPs budding from the cells can be observed in the cells at 48 hpt. Colocalization was confirmed representing the intensity of the different channels. No overlapping of the red channel (cell membrane) and green chanel (Gag-GFP) was observed at 24 hpt, but clear overlapping can be observed at 48 hpt.

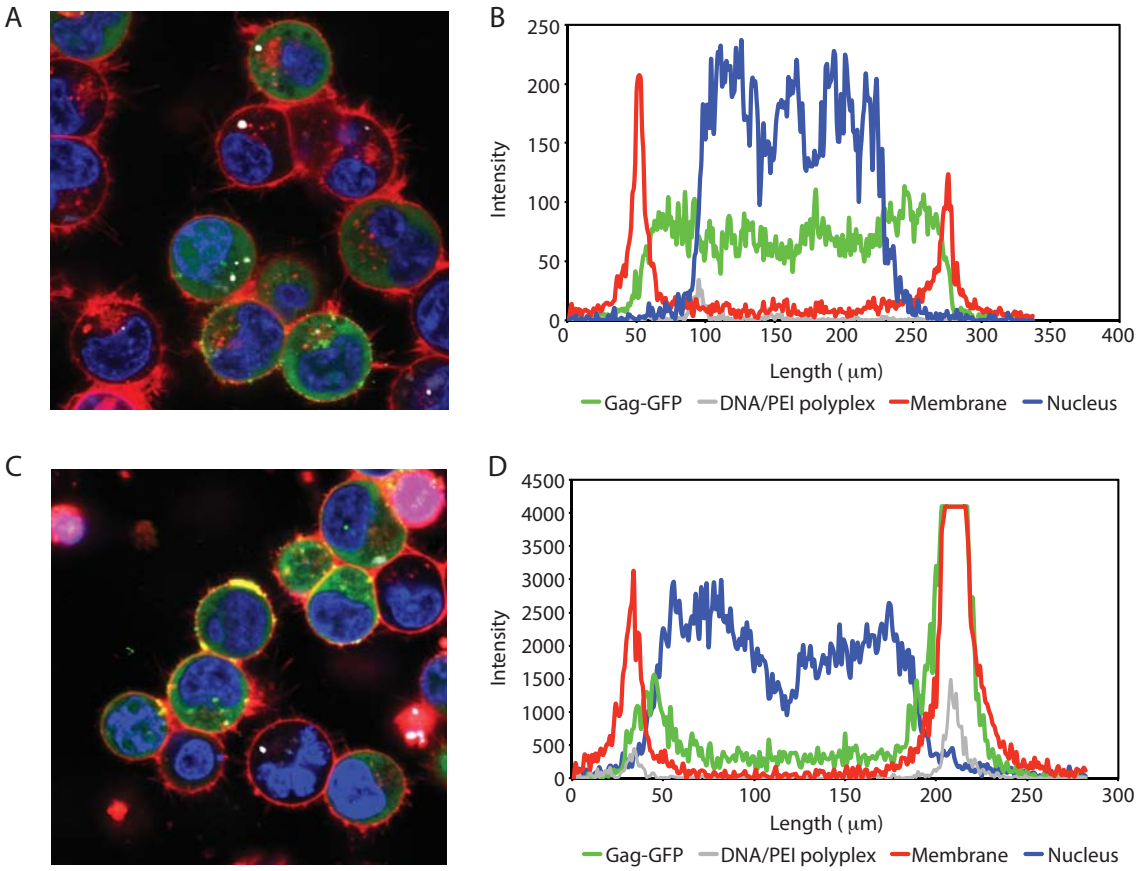


Figure 7 Confocal images (A,C) and graphs of intensity on the diameter of the cell. A,B correspond to 24hpt images and C,D to 72 hpt images were colocalization can be observed.

4. Discussion

The work performed first focused on the polyplexes uptake kinetics. It was observed that 5 minutes after the addition of DNA-PEI complexes, the interaction between cells and polyplexes is already evidenced. The maximum interaction was found at 30 minutes post transfection (as observed by confocal microscopy). The complexes were located in the cell membrane (Figure 1A), and cannot be detached by centrifugation as shown in Figure 2), this meaning that the DNA/PEI polyplexes are truly internalized in the cell membrane after this period of time. This result was corroborated using flow cytometry as all the cells interacted with polyplexes at 30 minutes post addition (Cy3 positive in figure 3D). Confocal microscopy images showed that at 90 minutes post transfection almost all observed cells have a complex inside (Figure 1B).

Several authors have already observed that the vast majority of the cells internalize polyplexes (Carpentier et al., 2007; Doyle and Chan, 2007; Han et al., 2009). For example, internalization of polyplexes was observed at 4 hpt independently of the cell cycle while only 1.8% of cells were expressing GFP at this time (Han et al., 2009). However, it is noteworthy that approximately half of the cells with complexes inside failed to express the protein at 20hpt (Han et al., 2009). These results are in agreement with the observations done in this work, indicating that all the cells internalize polyplexes, but only 60% of them are GFP-positive after 24 hpt (Figure 2).In the same direction, Doyle and Chan (Doyle and Chan, 2007) observed that polyplexes could be found in the cytoplasmatic periphery after 1 hpt, with evidence of polyplex aggregation in specific areas of the cell surface. They also noted that 92.23% of the cells internalized polyplexes after 2 hours, and, within 4 hours, significant localization within the nuclear proximity was observed. Carpentier (Carpentier et al., 2007), also found that 24 hours post transfection the fluorescence derived from DNA was homogenously distributed in expressing and non-expressing cells. These results suggested that polyplex uptake by the cells is a very efficient mechanism, and one or several of the next steps that proceed or preceded protein expression is the limiting step.

Godbey (Godbey et al., 2000) showed that complexation with PEI provided DNA protection from DNases and that endosomes, after endocytosis, did not fuse with lysosomes. They also showed that complexes appeared at the cell surface at 30 minutes post transfection. Endosome is formed between 2-3hpt, at 3.5 hpt endocytotic vesicles are larger and more numerous, and that complex starts to enter inside the nucleus by 3.5-4.5 hpt (Godbey et al., 1999). Although some DNA/PEI polyplexes are dissociated in the cytoplasm, it has been reported that, when DNA is complexed with PEI it mostly

enters the nucleus as a complex (Godbey et al., 1999; Godbey et al., 2000; Grosse et al., 2006).

The increase in transfection efficiencies observed between 24 ad 48 hours post transfection is related to cells that between this two-time points have the chance to pass through mitosis and incorporate the plasmid DNA into the nucleus. This phenomenon has been already suggested by Brunner (Brunner et al., 2000), who also proposed that this increase was low due to the inactivation or degradation of plasmid DNA. In this direction, (Lechardeur et al., 2005) showed that after 4h, 25% of the complexed DNA was degraded in the cytosol. This hypothesis is also reinforced by the fact that the increase in the percentage of transfected cells is not due to previously produced VLP entrance to the cells as shown in figure 4.

The expression of Gag-GFP protein starts between 4 and 10 hpt and there is an accumulation of intracellular protein, as it can be observed in Figure 5. In confocal images it is observed that at 24 hpt the fluorescence is homogenously distributed in the cytoplasm of the cell (Figure 6 A, B), but at 48 hpt Gag-GFP is accumulated at the vicinity of the cell membrane where colocalization of the cell membrane and VLPs during the budding process can be observed.

The kinetics of the transient transfection process was studied with the aim to characterize and understand the complete process at intracellular level leading to the VLP production, and to determine important time points to drive process improvement. Polyplexes start to interact with the cell membrane just after addition to the culture. After 1.5 hpt complexes are detected in the cytoplasm of the cells and reach the nucleus around 4 hours post transfection. After 10 hours post transfection GFP fluorescence is

detected inside the cells, but generalized budding of VLPs from the cells is not observed until 48 hours post transfection. The optimal harvest time is determined as 72 hpt as VLP production is highest while high viability of the culture is maintained.

Two limiting steps in VLP production could be identified in this work, as polyplexes entry into the nucleus and VLP budding.

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Selection and optimization of transfection enhancer additives for increased virus-like particle production in HEK293 suspension cell cultures

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Abstract

The manufacturing of biopharmaceuticals in mammalian cells typically relies on the use of stable producer cell lines. However, in recent years transient gene expression has emerged as a suitable technology for rapid production of biopharmaceuticals. Transient gene expression is particularly well suited for early developmental phases, where several therapeutic targets need to be produced and tested *in vivo*. As a relatively new bioprocessing modality, a number of opportunities exist for improving cell culture productivity upon transient transfection. For instance, several compounds have shown positive effects on transient gene expression. These transfection enhancers either facilitate entry of PEI/DNA transfection complexes to the cell or nucleus or increase levels of gene expression. In this work, we evaluated the potential of combining transfection enhancers to increase Gag-based virus-like particle production levels upon transfection of suspension-growing HEK293 cells. Using Plackett-Burman design of experiments, we first tested the effect of eight transfection enhancers: Trichostatin A, Valproic acid, Sodium Butyrate, DMSO, Lithium Acetate, Caffeine, Hydroxyurea and Nocodazole. An optimal combination of compounds exhibiting the greatest effect on gene expression levels were subsequently identified using a surface response experimental design. The optimal consisted on the addition of 20 mM Lithium Acetate, 3.36 mM Valproic Acid and 5.04 mM Caffeine which increased production levels 3.8 fold, while maintaining cell culture viability at 94%.

1. Introduction

The number of approved biopharmaceuticals produced in mammalian cell cultures continues to increase over time. The interest in mammalian cell culture bioprocesses is associated with the need to generate complex biopharmaceuticals that require the post-translational machinery exclusively available in eukaryotic cells. The manufacturing of

biopharmaceuticals in mammalian cells typically relies on the use of stable producer cell lines. However, in recent years transient gene expression (TGE) has emerged as a suitable technology for rapid production of biopharmaceuticals. TGE is particularly suited for early stages of development, where several therapeutic targets are produced at small to medium scale for structural characterization and *in vivo* studies.

As an early bioprocessing modality, a number of opportunities exist for improving TGE in order to increase productivity of transfected suspension mammalian cells. Optimization of media composition [1] and process parameters [2–5] is one way to attain higher titers. In addition, a number of compounds have shown positive effect in cell productivity upon TGE. These compounds, also known as transfection enhancers, can be classified into those that facilitate the transfection complex entry into the cell or nucleus including Lithium Acetate (LiAc), Dimethyl sulfoxide (DMSO) and Nocodazole and those that increase the level of transient gene expression such as Valproic acid (VPA) Sodium Butyrate (NaBut), Caffeine (Caf.), Hydroxyurea (HU) and Trichostatin A (Tricho) (Table 1).

Among those compounds described in the first group, LiAc and DMSO are used to improve the porosity of the cell membrane facilitating the entry of DNA/PEI complexes inside the cell. DMSO improves electroporation capacity of different mammalian cell lines probably due to its capacity for making the cell membrane more porous [6]. LiAc has been widely used in yeast [7–9] and bacterium transformation [10]. LiAc and DMSO used in combination have shown to significantly increase transient expression levels in mammalian CHO cell cultures[11]. Nocodazole is known to arrest cell cycle in G2/M phase, which facilitates DNA entry into the nucleus as the nuclear membrane

brakes down during mitosis. It has shown to increase transfection efficiency to 94% and correspondingly, the production of the reporter protein SEAP and IgG_4 by 2-fold [12]. Among the additives described to improve protein expression levels, hydroxyurea acts by blocking the cell cycle in G1 which increases cell productivity [12–14]. Caffeine has shown to enhance the production of lentiviral vectors in HEK 293 suspension cultures. The effect of caffeine was improved even further by combining it with 1mM of Butyric acid, yielding an 8-fold increase of functional Lentivirus titer [15].The mechanism of action of caffeine has been described as inhibition of several kinases, including ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which are important signaling proteins involved in the repair of DNA double-stranded breaks [16–18]. Another group of additives that can increase transient expression levels includes the inhibitors of histone deacetylases (HDAC), which includes NaBut, VPA and Trichostatin A. Hyperacetylation of histones is thought to open up the usually highly compacted nucleosome, allowing the transcription machinery to come into contact with the DNA template enabling gene transcription.[19]. The ability of NaBut to improve transient gene expression has been demonstrated in a wide range of cell lines and products such as Human Prolactin [19], Human Thyrotropin [20], Human Thrombopoietin [21], IgG [22,23], t-Pa [24] and lentiviral vectors [3]. Similarly, VPA has been reported to increase recombinant protein production levels in several cell lines and products as IgG [23,26–28] and LacZ [29]. The increase in protein production has shown to correlate with an increase in the relative steady-state levels of mRNAs and a decrease in cell growth and viability. VPA does not measurably affect pDNA uptake and/or stability following PEI-mediated transfection [26]. Additional HDAC inhibitors tested for transient transfection improvement include Trichostatin A which has shown

to enhance LacZ [29], IgG [23] and GFP and Luciferase expression using both plasmid DNA and a BacMam virus DNA templates. Responses observed were different for different promoters and cell lines tested [30].

Table 1 Additives used to improve gene expression levels

Additive	Mechanism of action	References
Trichostatin A (TSA)	Inhibition of histone deacetylases	[23,29,30]
Valproic acid (VPA)	Inhibition of histone deacetylases	[23,26–29]
Sodium Butyrate (NaBut)	Inhibition of histone deacetylases	[3,19–25]
DMSO	Increases cell membrane porosity	[6,11]
Lithium Acetate (LiAc)	Increases cell membrane porosity	[7–11]
Caffeine	Inhibition of DNA-PKcs (and probably ATM, ATR)	[15]
Hydroxyurea	Arrest cells in the G1 phase of the cell cycle	[12–14]
Nocodazole	Arrest cells in the G2/M phase of the cell cycle	[12]

The effect of several transfection enhancers on the production of Gag-based virus-like particles (VLPs) in HEK 293 cultures is investigated in this work using Design of experiments methodology. The VLPs are purposely modified and include a GFP marker to facilitate their quantitation by fluorescence measurements. The proposed approach focuses first on the identification of the compounds with the highest impact on production levels and second on the identification of the most effective combination of compounds and the optimization of their relative concentrations to achieve the highest level of gene expression possible.

2. Materials and Methods

2.1 Cell line, media and culture conditions

The cell line used in this work is a serum-free suspension-adapted HEK 293 cell line (HEK293SF-3F6) kindly provided by Dr. Amine Kamen from the BRI of National Research Council of Canada (Montreal, Canada). It 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). 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 [1].

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).

2.2 Plasmids

The pGag-EGFP plasmid used in this work codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP [31]. The plasmid from the NIH AIDS Reagent Program (Cat 11468) was constructed by cloning the Gag sequence from pCMV55M1-10 [32] into the pEGFP-N1 plasmid (Clontech). The plasmids were prepared and purified as previously described [33].

2.2 Cell Cycle analysis

Freshly harvested cells were pelleted by centrifugation, rinsed, re-suspended in PBS and fixed with cold ethanol (70%) for a minimum of 2 h at −20°C. For cell cycle analysis, cells were stained with propidium iodide (PI) (Sigma) for 30 min at room temperature prior to data acquisition using a FACSCalibur (BD Biosciences, San Jose,CA, USA). The Modfit software (Verity Software House, Topsham,ME, USA) was used for data analysis.

2.3 MTT assay

MTT assay is based on colorimetric assay, which detect the viability of cell culture. MTT (tetrazolium) is reduced in presence of viable cells in the sample. MTT precipitate and generate the formazan, which is responsible for the purple color. In this work, MTT assay is used to study the toxicity of the different additives. 96-well plates were used to analyze the toxicity of each component. Each well was filled with 100µL of HEK293 cells at $0.5 \cdot 10^6$ cells/mL with 10 µL of different concentrations of the component to be tested. The 96-well plate was incubated at 37ºC and 110 rpm for 48 hours. A calibration curve was generated at the time of analysis with 100 µL of HEK 293 with concentrations ranging from 0 to $2 \cdot 10^6$cells/mL. 20 µL of the MTT reagent was added to every well and incubated at 37ºC at 110 rpm during 1 hour. Absorbance at 490 nm was measured with an spectrophotometer Victor3 Plate Reader (PerkElmer, Massachusetts, USA).

2.4 Transient transfection

HEK 293 suspension cells were transiently transfected using 25-kDa linear polyethilenimine (PEI) (PolySciences, Warrington, PA, USA). Transfections were performed using different concentrations of DNA and PEI to find the optimal concentration and ratio. 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. The percentage of GFP positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) at different hours post-transfection (hpt).

2.5 VLP quantitation

The concentration of Gag-GFP VLPs was assessed by fluorimetry using an *in-house* developed and qualified quantification assay [34]. 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.

2.6 Additive concentration optimization using statistically designed experiments

The additives used in this work to optimize VLP production were Nocodazole (M1404, Sigma, Saint Louis, MO, USA), Lithium Acetate (62393, Sigma), DMSO (D2438, Sigma), Valproic acid (P4543, Sigma), Sodium Butyrate (B103500, Sigma), Caffeine (C0750, Sigma), Hydroxyurea (H8627, Sigma) and Trichostatin A (T1952, Sigma).

2.6.1 Plackett-Burman design

A fractional factorial Plackett-Burman design was used to identify supplements with a significant effect on transfection efficiency and VLP production and screen out irrelevant compounds. The eight selected compounds were screened at two levels: a low level (no additive) coded as -1 and a high level coded as +1 as indicated in Table 2. High levels for each compound were defined based on pre-existing knowledge extracted from the literature and toxicity assays.

The main effects of independent variables (in this case, compounds tested) were calculated using the following equation:

$$E_{ij} = \frac{\sum Y_{j_{(+)}}}{n/2} - \frac{\sum Y_{j(-)}}{n/2} \qquad (2)$$

where E_{ij} is the effect of the variable i on the response Y_j when the variable is used at its high level $Y_{j_{(+)}}$ or low level $Y_{j(-)}$, and n is the number of experimental runs.

Plackett-Burman experimental results were fitted to a first-order polynomial function described below by regression analysis:

$$Y = \beta_0 + \sum \beta_i \, X_i \qquad (3)$$

where Y is the response (in percentage of GFP positive cells), β_0 is the model intercept and β_i is the linear coefficient for the independent variable X_i.

2.6.2 Box-Behnken design

In order to define the optimal combination and concentration for each additive selected in the previous step, a Box-Behnken design was used. Compounds with significant effect on transient transfection and VLP production were screened at three levels: a low level coded as -1, a medium level coded as 0 and a high level coded as +1 as indicated in Table 4 and 5. Box-Behnken experimental results were fitted to a second-order polynomial equation described below by non-linear regression analysis:

$$Y = \beta_0 + \sum \beta_i \, X_i + \sum \beta_{ii} \, {X_i}^2 + \sum \beta_{ij} \, X_i X_j \qquad (4)$$

where Y is the response (in relative fluorescence units, R.F.U. at 48 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 (i.e. tested compounds). This equation was used to predict the optimum concentration values of the independent variables using R project for statistical computing. Three-dimensional surface plots were generated to facilitate model interpretation.

2.6.3 Statistical analyses

Statistical analyses of the models were performed using R project for statistical computing. The quality of the fit of the model equation is 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.

3 Results and discussion

3.1 Preliminary tests to define key experimental conditions

3.1.1 PEI/DNA ratio optimization

The DNA/PEI ratio used for transient transfection was first optimized using Central Composite Design of experiments (CCD). Five levels for each variable were established as shown in Table 2, taking the standard concentration of DNA and PEI used as central point [1]. The resulting matrix consisted in 13 experiments and the percentage of GFP positive cells was analyzed 48 hours post-transfection (hpt) (Table 2). The results obtained were fitted to a second order polynomial (equation 5).

$$\%GFP = 66.15 + 2.13\times DNA - 11.76\times DNA^2 + 20.64\times PEI - 16.13\times PEI^2 + 5.5\times DNA\times PEI \qquad (5)$$

where %GFP is the percentage of GFP positive cells at 48 hpt, DNA is the concentration of plasmid Gag-GFP and PEI is the concentration of PEI both in µg/ml of culture.

Regression analysis showed that the model was adequate with a coefficient R^2 of 0.753, indicating that the model is consistent with 75.3% of the variability in the data. The statistical significance of the model was confirmed by ANOVA analysis (Table 2). The Fisher's *F*-test associated *p*-value of 0.0421 indicated that the model had a level of confidence of 95.8%. The response surface graph generated from the obtained results (Figure 1) allows observing the effect of each variable on the transfection efficiency in terms of percentage of GFP positive cells.

The second-order polynomial model was used to calculate optimum concentrations of DNA and PEI, being 1 µg of DNA/ml of culture and 3 µg of PEI/mL of culture. Under these conditions the predicted response was 73% of GFP positive cells at 48 hpt.

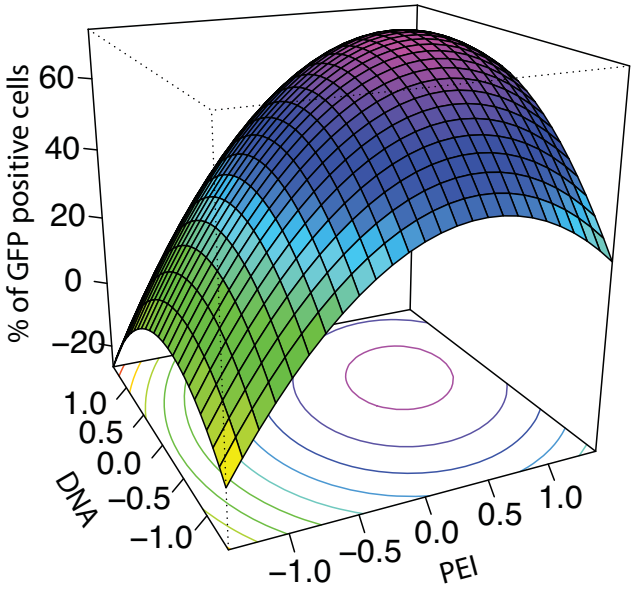

Figure 1. Transfection efficiency optimization by design of experiments. Response surface graphs based on central composite design experimental results show the percentage of GFP positive cells as a function of the concentrations of DNA (µg/mL) and PEI (µg/mL).

Table 2 Code levels, matrix design, response and regression coefficients for central composite experimental design

Independent variables	Coding levels				
	-√2	-1	0	1	√2
DNA (ug/mL)	0.1	0.4	1	1.6	1.9
PEI (ug/mL)	0.1	0.7	2	3.3	3.9

N°	DNA	PEI	Response[a]
1	√2	0	56.7
2	1	-1	0.1
3	1	0	60.3
4	1	1	64.4
5	0	-√2	0.1
6	0	-1	45.9
7	0	0	57.1
8	0	1	57.9
9	0	√2	62.2
10	-1	-1	26.7
11	-1	0	62.8
12	-1	1	69
13	-√2	0	17.8

	Coefficient	t-value	*p*-value
Constant	-66.15	5.6458	0.0008
DNA	2.13	0.4186	0.6881
PEI	20.64	4.0539	0.0048
DNA²	-11.76	0.6831	0.5165
PEI²	-16.13	-1.4872	0.1806
DNA × PEI	5.5	-2.0398	0.0807

[a] Responses are percentage of GFP positive cells at 48 hpt. Abbreviations: N°, experiment number

3.1.2 Time of additive addition

The time of additive addition to the cell culture relative to the time of culture transfection with PEI/DNA complex addition was established prior to conducting the design of experiments. LiAc and DMSO were added to cell cultures 3 hours prior to

transfection as described in the literature [11]. The best time for addition of Nocodazole is less well defined in the current literature and can be cell line dependent. Therefore, a kinetic study was performed to ensure that the maximum number of HEK293 cells would be arrested at G2/M phase at the time of transfection (section 3.1.2.1. Determination of Nocodazole action kinetics). The rest of the additives evaluated in this work were added 4 hours post-transfection in accordance to the published literature [23,28].

3.1.2.1 Determination of Nocodazole action kinetics

Nocodazole is known to arrest cells in the G2/M phase of the cell cycle resulting in enhanced transfection efficiency, as cells are more receptive to the uptake of foreign DNA into the cell nucleus. An experiment was carried out to determine at which time nocodazole would cause the maximum effect. In a first experiment, different concentrations of nocodazole were tested. Best results were obtained with a concentration of 2 μg of Nocodazol/mL of culture, which resulted in 50% of cells arrested at G2/M phase at 12 hours after Nocodazol addition (figure 2A). This concentration was used in a second experiment consisting of a kinetics study in which the percent of cells at different cell cycle phases was monitored during 24 hours to determine the time necessary to reach the maximum percentage of cells in G2/M phase. Figure 2B shows that in the absence of nocodazole, the percentage of cells in G2/M ranges between 7 to 16.6% during the 24 hours. When nocodazole is added to the culture (Figure 2C), the percent of cells arrested in the G2/M phase of the cell cycle continuously increases over time from 13% at time of nocodazol addition until they reach a plateau 23 hours after, with a percentage of cells at G2/M phase of 71%. It is also remarkable that the percentage of cells in G0/G1 phase was zero after 23 hours of

nocodazole exposure. It can be concluded from these experiments that cells should be exposed to nocodazole 24 hours before transient transfection in order to observe the maximum effect of nocodazole.

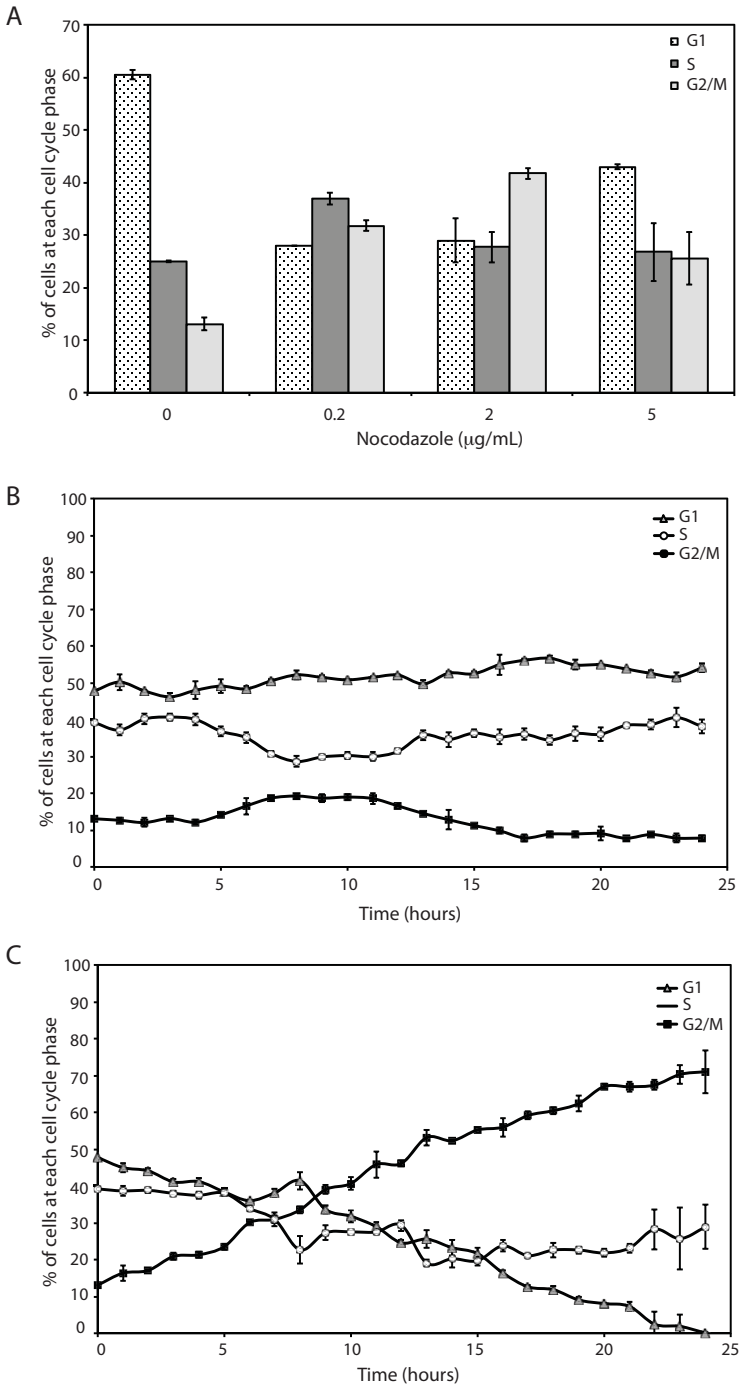

Figure 2. Toxicity assays. Viable cell density (10^6 cells/mL) attained after 72 hours of seeding analyzed using MTT assay in response to different concentrations of Nocodazole (μg/mL) (A), DMSO (V/V) (B), Lithium Acetate (mM) (C), Valproic acid

(mM) (D), Sodium butyrate (mM) (E), Trichostatin A (mM) (F), Caffeine (mM) (G) and Hydroxyurea (μM) (H).

3.1.3 Toxicity assays

To avoid cytotoxic effect elicited by compounds being tested in the design of experiments, a maximum and a minimum concentration for the different tested compounds were established. In order to define the maximum level of each additive while maintaining a high viable cell count, a toxicity assay using the MTT assay was carried out. The results of the toxicity assays are shown in Figure 3. Experimental ranges for the Plackett-Burman design were determined based on these results (Table 3). The maximum additive concentration to be used in the experiments was that resulting in no less than 50% cell density compared to that reached by the negative control cell culture in which no additive was added.

Table 3. Code levels, matrix design and regression coefficients for Plackett-Burman experimental design.

Independent variables	**Coding levels**	
	-1	1
Nocodazole (μg/mL)	0	2
DMSO (V/V%)	0	1
Sodium Butyrate (mM)	0	10
Butiric acid (mM)	0	4
Valproic acid (mM)	0	2
Trichostatin A (μM)	0	0.6
Caffeine (mM)	0	4
Hydroxyurea (mM)	0	1.25

Nº	Noco	DMSO	LiAc	NaBut	VPA	Trico	Caf	Hydroxyurea	R.F.U.	Transfection efficiency	% of viability
1	+1	+1	+1	+1	+1	+1	+1	+1	7.3	20.3	71.5
2	−1	+1	−1	+1	+1	+1	-1	-1	22.5	66.5	65.8
3	−1	−1	+1	−1	+1	+1	+1	-1	28.2	71.3	75.1
4	+1	−1	−1	+1	-1	+1	+1	+1	7.8	25.7	70.0
5	−1	+1	−1	−1	+1	-1	+1	+1	10.1	19.8	81.8
6	−1	−1	+1	−1	-1	+1	-1	+1	10.7	28.4	77.5
7	−1	−1	−1	+1	-1	-1	+1	-1	26.3	62.5	65.8
8	+1	−1	−1	−1	+1	-1	-1	+1	8.0	21.1	80.1
9	+1	+1	−1	−1	-1	+1	-1	-1	11.6	56.4	75.0
10	+1	+1	+1	−1	-1	-1	+1	-1	13.4	60.5	76.6
11	−1	+1	+1	+1	-1	-1	-1	+1	11.2	39.8	71.9
12	+1	−1	+1	+1	+1	-1	-1	-1	16.9	67.0	76.6

Coefficients:

	Coefficient	*t*-value	*p*-value
Constant	22.0978	10.862	0.00167
Nocodazole	-7.3291	-5.404	0.01242
DMSO	-3.6211	-2.670	0.07569
Lithium acetate	0.2597	0.191	0.86039
Sodium Butyrate	1.6908	1.247	0.30098
Valproic acid	1.9908	1.468	0.23843
Trichostatin A	0.3795	0.280	0.79781
Caffeine	2.0323	1.499	0.23094
Hydroxyurea	-10.6168	-7.828	0.00434

Abbreviations: EXP N°, experiment number

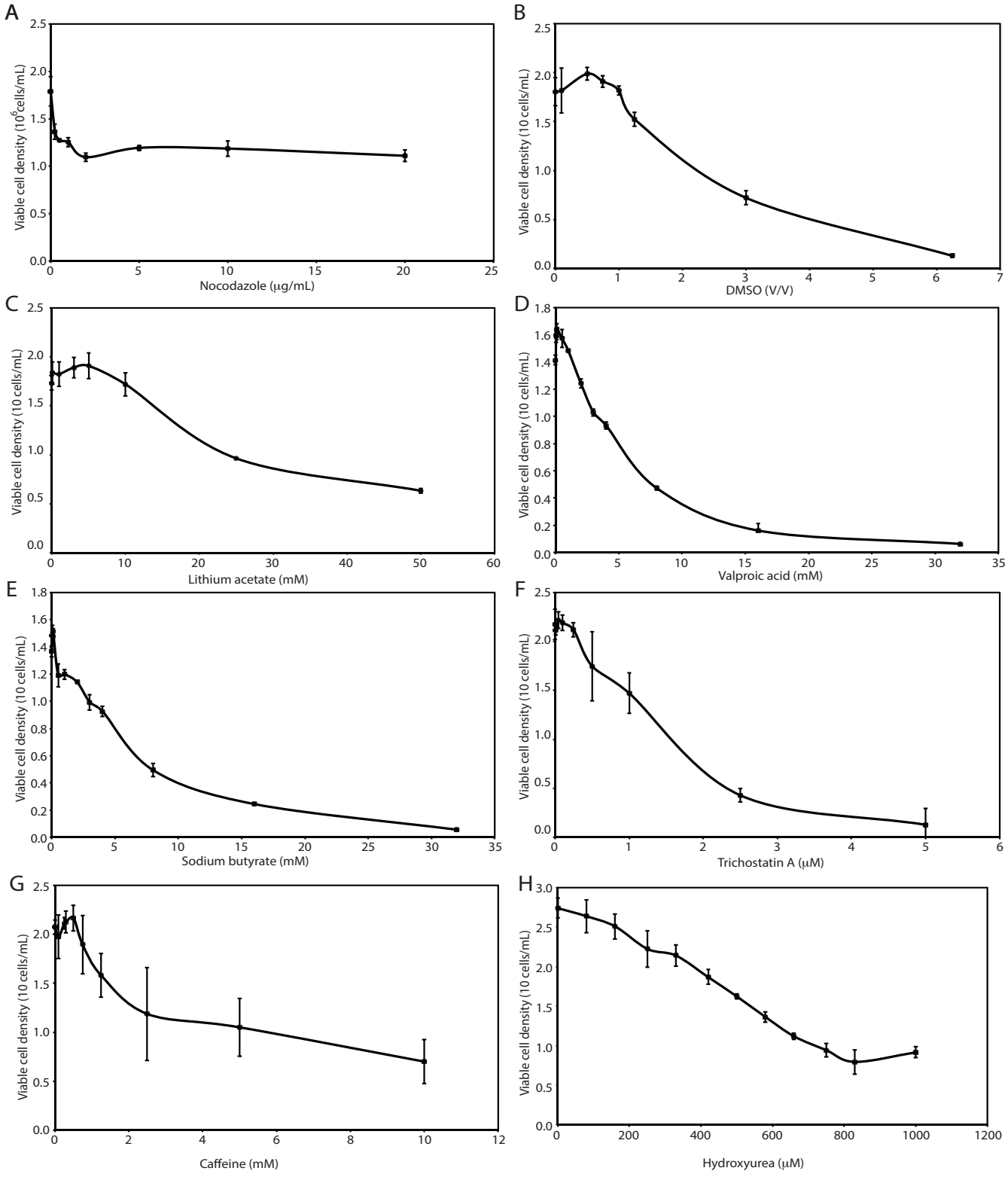

Figure 3. Cell growth arrest kinetics. Percentage of cells at each phase of the cell cycle depending on Nocodazole concentration (A). Percentage of cells at each phase of the

cell cycle followed during 24 hours when no additive is added to the culture (B) and when exposed to 2 (μg/mL) of Nocodazole (C).

3.2 Optimization of Gag-GFP VLP production using additives.

The optimization of the production was performed using a two-step design of experiments methodology. In the first step, a Plackett-Burman design was performed to evaluate the main effect of each additive and discard those not having any effect of gene expression or even having a negative effect. Subsequently, a Box-Behnken experiment was performed with the selected additives to find the optimal concentration of each of them to reach maximum levels of gene expression.

3.2.1 Screening additives using Plackett-Burman design of experiments

A statistically designed experiment arranged according to the Plackett-Burman design was carried out to decipher which additives had a significant effect on VLP production as described in the materials and methods section. The experimental design matrix in coded values, response and statistical analysis are shown in Table 3.

For the performance of the Plackett-Burman design, 24 hours in advance cells were split in 12 shake flasks and Nocodazole was only added to those flasks coded as +1 (high level of the additive) as indicated in Table 3. Three hours before the experiment, DMSO and LiAc were also added to the flasks with high level of these compounds. Just before transfection, a complete medium exchange was performed to all the flasks and finally, four hours post transfection, the remaining additives were added as indicated in Table 3.

Samples were taken at 48 and 72 hpt and cell density, viability, % of GFP positive cells and fluorescence in the supernatant was analyzed. Fluorescence in the supernatant and

transfection efficiency at 48 hpt were used as DoE responses. Fluorescence was adjusted to a first order polynomi (Equation 2).

$$Fluorescence = 22.10 - 7.33\times Noco. - 3.62\times DMSO - 0.26\times LiAc + 1.69\times NaBut + 1.99\times VPA + 0.38\times Trich. + 2{,}03\times Caf. - 10.62\times HU \quad (2)$$

The regression analysis showed that the model was adequate for the experimental data with a coefficient R^2=0.972 indicating that the model is consistent with 97.2% of the variability of the data. The statistical significance of the model was confirmed by ANOVA analysis (Table 3). The Fisher's *F*-test associated *p*-value of 0.029 indicated that the model had a level of confidence of 97,1%.

Observing the main effects of each variable (Figure 4B) it can be concluded that the components with clear positive effect on fluorescence are Sodium butyrate, Valproic acid and Caffeine. Therefore, these supplements were selected for further optimization. Lithium acetate was also selected, as it showed a great effect in transfection efficiency and there is a possibility of interaction with the other components that leads to an overall improvement in Gag-GFP VLP production levels.

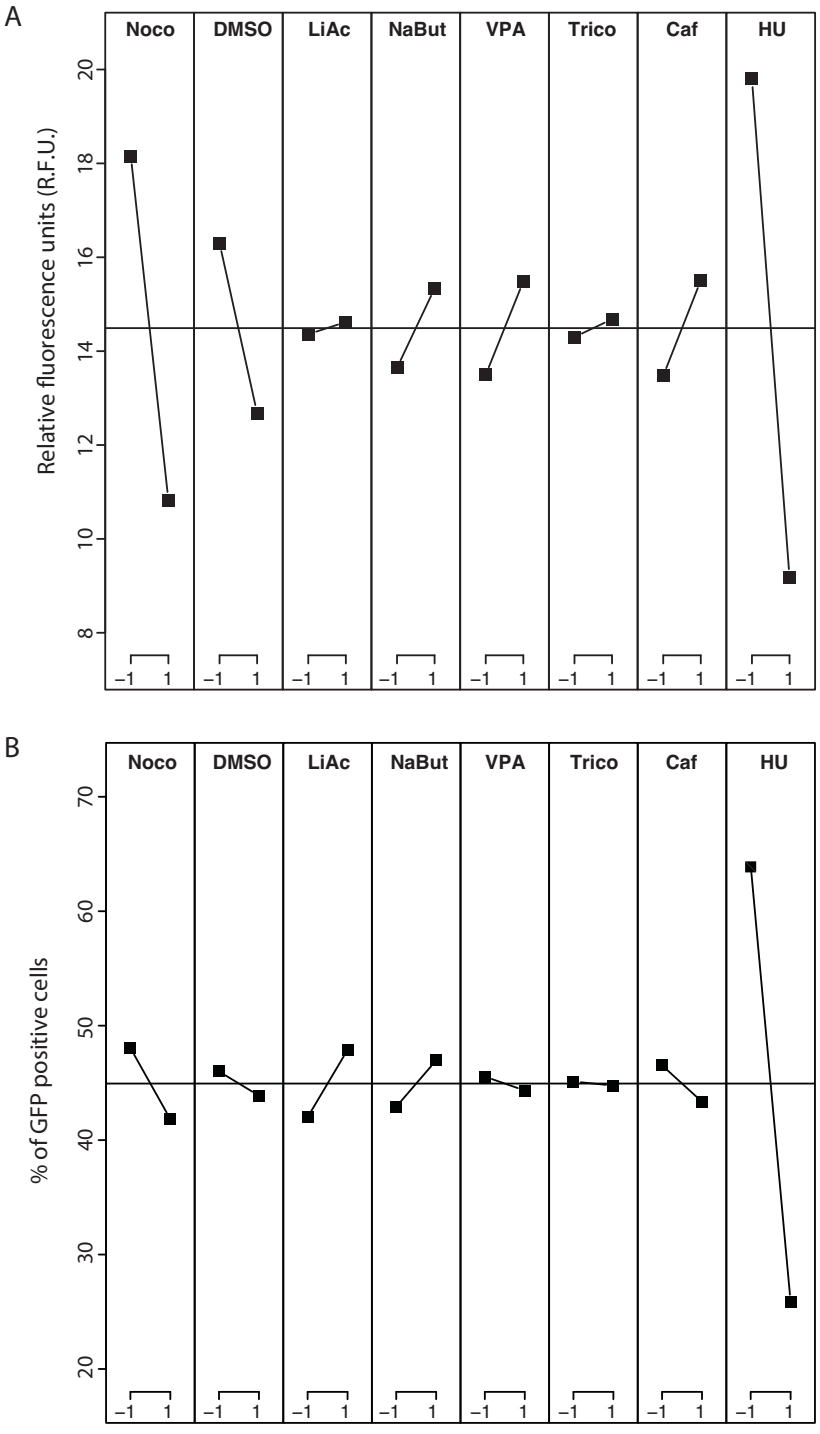

Figure 4. Main effect of non-animal derived supplements on HEK 293 cells growth in Freestyle medium according to Plackett–Burman DoE. Taking relative fluorescence (A) and transfection efficiency (B) as a response

3.2.2 Optimization of the concentration of the additives used in combination using Box-Behnken design of experiments

For the Box-Behnken design of experiments, three levels of concentrations of each additive were needed, a minimum, a medium and a maximum level (Table 4), all within the limits of minimal toxicity for each component. The experimental design matrix in coded values, response and statistical analysis of the method are outlined in Table 4. A total of 27 experiments were required to perform the Box-Behnken design (BB1). Samples were taken 48 and 72 hpt and cell density, viability, percentage of GFP positive cells and fluorescence in the supernatant were analyzed. Three shake-flasks without any component addition were used as control and included to the design of experiments matrix.

Fluorescence in the supernatant at 48 hpt was measured and the response and was adjusted to a second order polynomial (Equation 3).

$$\begin{aligned} Fluorescence = {} & 48.09 - 0.11\times LiAc + 2.19\times VPA + 2.58\times NaBut - 5.30\times Caf \\ & + 0.26\times LiAc\times VPA - 3.00\times LiAc\times NaBut + 2.65\times LiAc\times Caf \\ & - 10.85\times VPA\times NaBut + 0.89\times VPA\times Caf - 6.32\times NaBut\times Caf \\ & - 6.12\times LiAc^2 - 5.36\times VPA^2 + 0.92\times NaBut^2 - 1.44\times Caf^2 \quad (3) \end{aligned}$$

Regression analysis showed that the model was adequate with a coefficient R^2 of 0.956. This indicates that the model is consistent with 95.6% of the variability in the data. The statistical significance of the model was confirmed by the Fisher´s *F*-test. An associated *p*-value of <0.0001 indicated the model is significant. The significance of each regression coefficient was analyzed by student´s *t*-test (Table 4).

The general trend of the observed experimental responses and the interactive effects on the response can be observed in the three-dimensional plots presented in Figure 5. In

general it can be observed that the level of lithium acetate is optimal at its center value independently of the other compounds used and that production increases as the concentration of sodium butyrate does. In Figure 5A, B, C, it can be observed that as the concentration of sodium butyrate increases, less valproic acid has to be used, possibly because they complement each other as they have a similar mechanism of action. In Figure 5 D, E and F, it can be observed that caffeine optimum corresponds to its highest level when butyric acid is at its lowest level, but once butyric acid concentration increases, the optimal level of caffeine drops to its lowest level, which would also suggest some kind of complementation between both compounds. In Figures 5 G, H, I, it can be observed that as the concentration of butyric acid increases, the effect of caffeine and valproic acid is negative.

Table 4. Code levels, matrix design, response and regression coefficients for Box-Behnken experimental design (BB1).

Independent variables	**Coding levels**		
	-1	0	1
Lithium Acetate (mM)	0	10	20
Sodium Butyrate (mM)	0	2.5	5
Valproic acid (mM)	0	2.5	5
Caffeine (mM)	0	3	6

Nº	**Lithium Acetate**	**Valproic acid**	**Sodium Butyrate**	**Caffeine**	**R.F.U. [a]**	**%viability**
1	1	0	-1	0	41.9	80.7
2	0	0	0	0	46.7	69.5
3	0	0	-1	1	48.2	83.2
4	1	0	0	-1	41.7	80.6
5	1	0	0	1	35.7	80.7

6	-1	0	0	-1	52.8	72
7	1	0	1	0	44.1	69
8	0	1	-1	0	56.0	80.1
9	-1	0	1	0	46.5	75.1
10	-1	-1	0	0	34.4	75.2
11	0	0	1	1	39.8	79.3
12	0	0	0	0	47.1	73.7
13	1	-1	0	0	35.7	72.6
14	0	0	-1	-1	47.4	76.3
15	0	1	0	-1	45.7	75.7
16	0	-1	0	-1	45.8	63.8
17	1	1	0	0	40.3	74.5
18	0	0	0	0	46.9	70.4
19	0	-1	1	0	51.6	45.6
20	-1	0	0	1	38.7	78.7
21	-1	0	-1	0	35.9	77.2
22	0	0	1	-1	60.8	67.5
23	0	1	1	0	40.9	72
24	-1	1	0	0	38.2	73.7
25	0	1	0	1	35.2	82.7
26	0	-1	0	1	35.4	76.7
27	0	-1	-1	0	26.9	68.8
28	-1	-1	-1	-1	18.4	70.8
29	-1	-1	-1	-1	15.4	70.7
30	-1	-1	-1	-1	18.4	76.2

	Coefficient	*t*	*p*-value
Constant	48.09	29.08	<0.0001
LiAc	-0.11	-0.12	0.9058
VPA	2.18	2.56	0.0221
NaBut	2.58	2.96	0.0098
Caf	-5.30	-5.65	<0.0001
LiAc × VPA	0.26	0.19	0.8536
LiAc × NaBut	-3.00	-2.06	0.0573
LiAc × Caf	2.65	1.47	0.1617
VPA × NaBut	-10.84	-7.44	<0.0001
VPA × Caf	-0.89	-0.61	0.5498
NaBut × Caf	-6.32	-4.34	0.0006
$LiAc^2$	-6.12	-4.59	0.0004
VPA^2	-5.36	-4.20	0.0008
$NaBut^2$	0.92	0.73	0.4757
Caf^2	-1.44	-1.07	0.2998

[a] Responses are percentage fluorescence at 48 hpt. Abbreviations: N°, experiment number

By solving the model equation, optimum concentrations of Lithium Acetate, Valproic Acid, Butyric Acid and Caffeine were calculated as 5.14 mM, 0.66 mM, 5 mM and 0 mM, respectively. One relevant aspect to be considered in the previous series of experiments is the negative interactions observed between Sodium butyrate with the rest of compounds studied and the low cell viability observed when butyric acid is used. Indeed, the negative effects of Sodium butyrate on cells viability have also been observed by other authors, reporting that the positive effect of Sodium butyrate in terms of production is accompanied by an evident deleterious effect on cell growth triggering cell arrest and apoptosis [19,25]. Consequently, a second Box-Behnken design (BB2) is proposed, in which butyric acid is excluded, with the aim to investigate if the effects on cell viability could be mitigated while preserving the obtained production enhancement. Therefore, this second optimization experiment (BB2) is performed with Lithium Acetate, Valproic Acid and Caffeine. The experimental design matrix in coded values, response and statistical analysis of the method are outlined in Table 5.

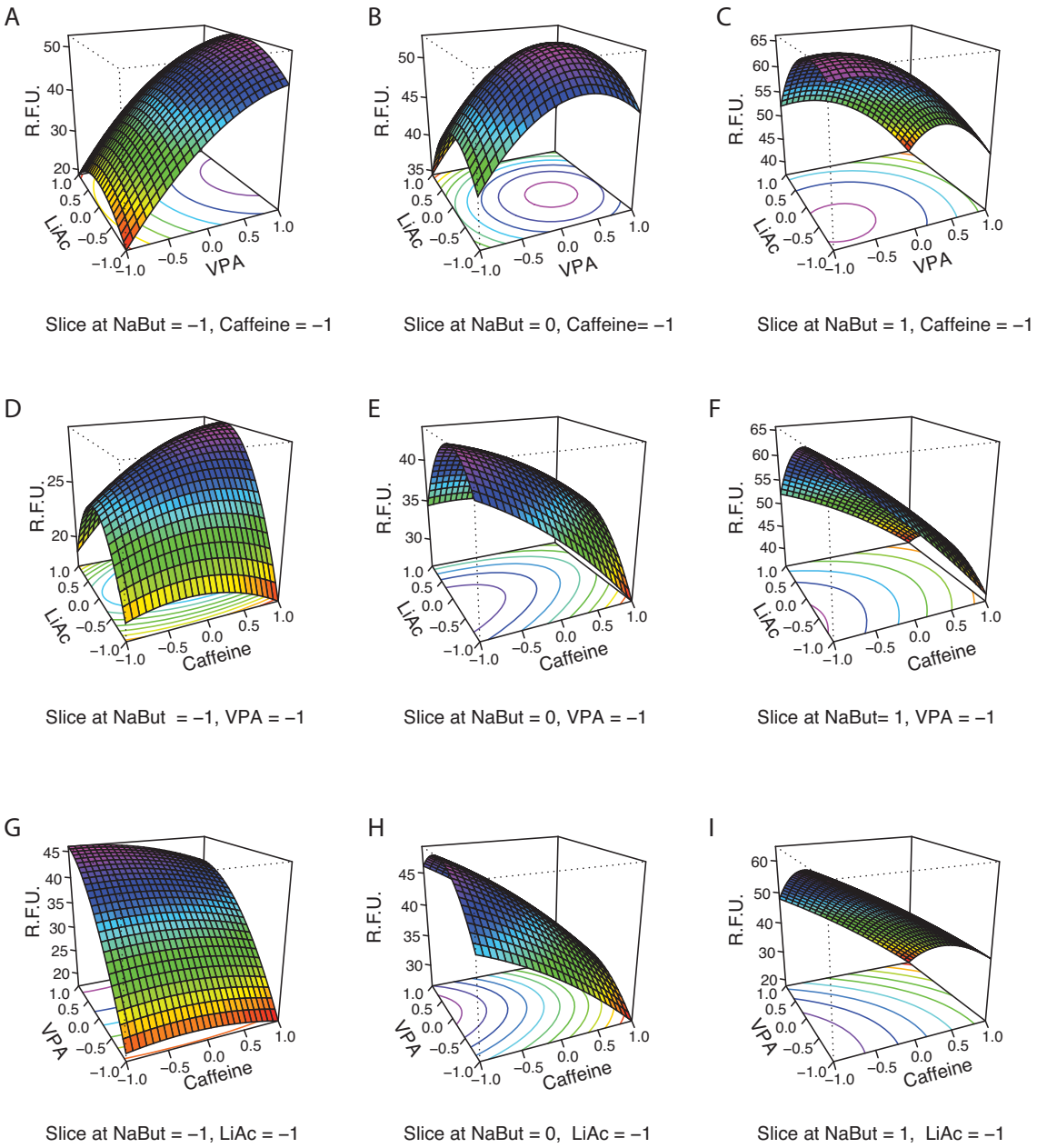

Figure 5. Response surface graphs based on Box–Behnken (BB1) experimental results. These 3D graphs were constructed by depicting two variables at a time while keeping the third one at its lowest level. The increase in the concentration of Sodium Butyrate is studied for each combination. Relative fluorescence units as a function of the concentrations of Lithium acetate (mM) vs. Valproic acid (mM) (A, B, C), of Lithium acetate (mM) vs. Caffeine (mM) (D, E, F) and Valproic acid (mM) vs. Caffeine (mM) (G, H, I).

A total of 15 experiments were carried out, samples were obtained at 48 and 72 hpt and cell density, viability, percentage of GFP positive cells and fluorescence in the supernatant were analyzed (Table 5). Fluorescence in the supernatant at 48 hpt was adjusted to a second order polynomial (Equation 4).

$$Fluorescence = 50.00 + 3.46\times LiAc + 8.01\times VPA + 5.90\times Caf - 1{,}80\times LiAc\times VPA - 1.43\times LiAc\times Caf - 4.23\times VPA\times Caf + 15.54\times LiAc^2 - 17.61\times VPA^2 - 4.54\times Caf^2 \quad (4)$$

Regression analysis showed that the model was adequate with a coefficient R^2 of 0.960. This indicates that the model is consistent with 96.0% of the variability in the data. The Fisher´s F-test confirmed the statistical significance of the model. An associated *p*-value of 0.0004 indicated the model is significant. The significance of each regression coefficient was analyzed by student´s *t*-test (Table 5).

Table 5. Code levels, matrix design, response and regression coefficients for Box-Behnken experimental design (BB2).

Independent variables	**Coding levels**		
	-1	0	1
Lithium Acetate (mM)	0	10	20
Valproic acid (mM)	0	3	6
Caffeine (mM)	0	3.5	7

Nº	Lithium acetate	Valproic acid	Caffeine	R.F.U.[a]	% viability
1	1	0	-1	60.8	94.4
2	-1	0	-1	56.3	92.2
3	1	0	1	65.0	93.9
4	0	-1	-1	12.5	89.1
5	0	1	-1	27.4	89.6
6	0	1	1	38.0	93,2
7	-1	1	0	54.8	88.3
8	1	-1	0	41.7	93.5
9	0	0	0	49.1	91.7
10	0	0	0	49.0	91.3
11	0	-1	1	34.5	93.5
12	1	1	0	60.5	91.6
13	-1	0	1	60.7	93.3
14	-1	-1	0	34.3	90.2
15	0	0	0	46.9	92.4
16	-1	-1	-1	16.7	92.5
17	-1	-1	-1	14.5	91.5

	Coefficient	*t*	*p-value*
Constant	49.23	16.79	<0.0001
LiAc	3.40	1.91	0.0972
VPA	7.90	4.45	0.0030
Caf	5.82	3.28	0.0135
LiAc × VPA	-1.77	-0.73	0.4886
LiAc × Caf	-1.38	-0.57	0.5855
VPA × Caf	-4.17	-1.72	0.1283
$LiAc^2$	15.28	5.77	0.0007
VPA^2	-17.35	-6.55	0.0003
Caf^2	-4.47	-1.69	0.1344

[a] Responses are relative fluorescence units at 48 hpt. Abbreviations: N°, experiment numbers

Three-dimensional plots were constructed for visual observation of the trend of maximum responses and the interactive effects of the significant variables on the response (Figure 6). Upon close evaluation of the behavior of the response over the

experimental region it can be observed that the optimum level of Valproic acid and Caffeine is in the middle of the evaluated concentration range, whereas optimal concentration of lithium acetate is towards the edge of the range of concentrations tested.

By solving the model equation, optimum concentrations of Lithium Acetate, Valproic Acid and Caffeine, were calculated as 20 mM, 3.36 mM and 5.04 mM, respectively.

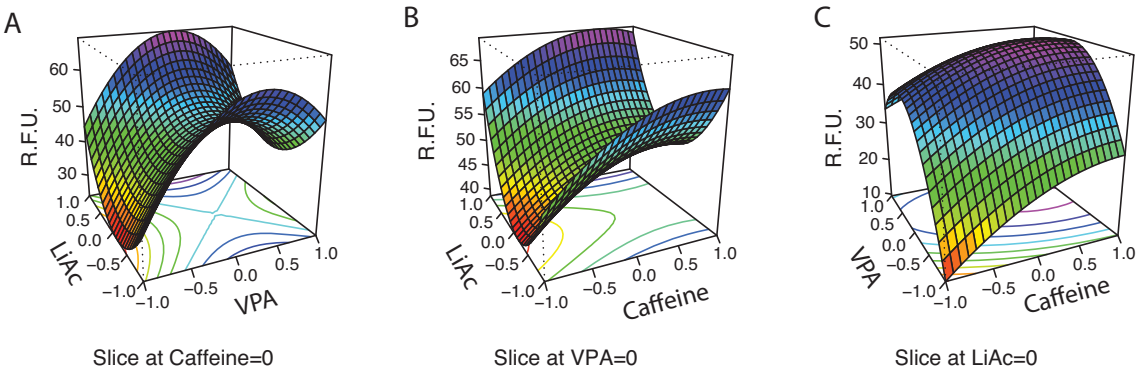

Figure 6. Response surface graphs based on Box–Behnken (BB6) experimental results. These 3D graphs were constructed by depicting two variables at a time while keeping the third one at its middle level. Relative fluorescence units as a function of the concentrations of Lithium acetate (mM) vs. Valproic acid (mM) (A), of Lithium acetate (mM) vs. Caffeine (mM) (B) and Valproic acid (mM) vs. Caffeine (mM) (C).

3.2.3 Validation of the model.

In order to validate the results, an experiment using the optimal concentrations of the additives was performed. The results obtained in BB1 (LiAc: 5.14 mM, VPA: 0.66 mM, NaBut: 5 mM) and in BB2 (LiAc: 20 mM, VPA: 3.36 mM and Caffeine: 5.04 mM) were validated. Other two conditions were also tested, one using the optimal

concentration of NaBut founded in a lineal experiment (data not shown) and a control using no additives (Figure 7),

It can be observed that results obtained in terms of fluorescence by using the optimal concentration found in BB1 and the results obtained using only Sodium Butyrate at 48 hpt are equivalent and 88.8. R.F.U. (2.94×10^{10}VLPs/mL), with viabilities of 81% and 71% respectively. These results suggest that by adding Lithium Acetate and Valproic Acid to Butyric Acid the viability of the culture increases 10% higher. The results using BB2 were slightly lower than when butyric acid is used, 69.1 R.F.U. (2.3×10^{10}VLPs/mL), but the viability of the culture is the highest of all the conditions tested (94%), even higher than when no additives are added to the culture. When fluorescence is compared with the negative control the increase when BB1 or butyric acid by its own is 4.9 fold, and the increase when the combination when BB2 is used is 3.8 fold.

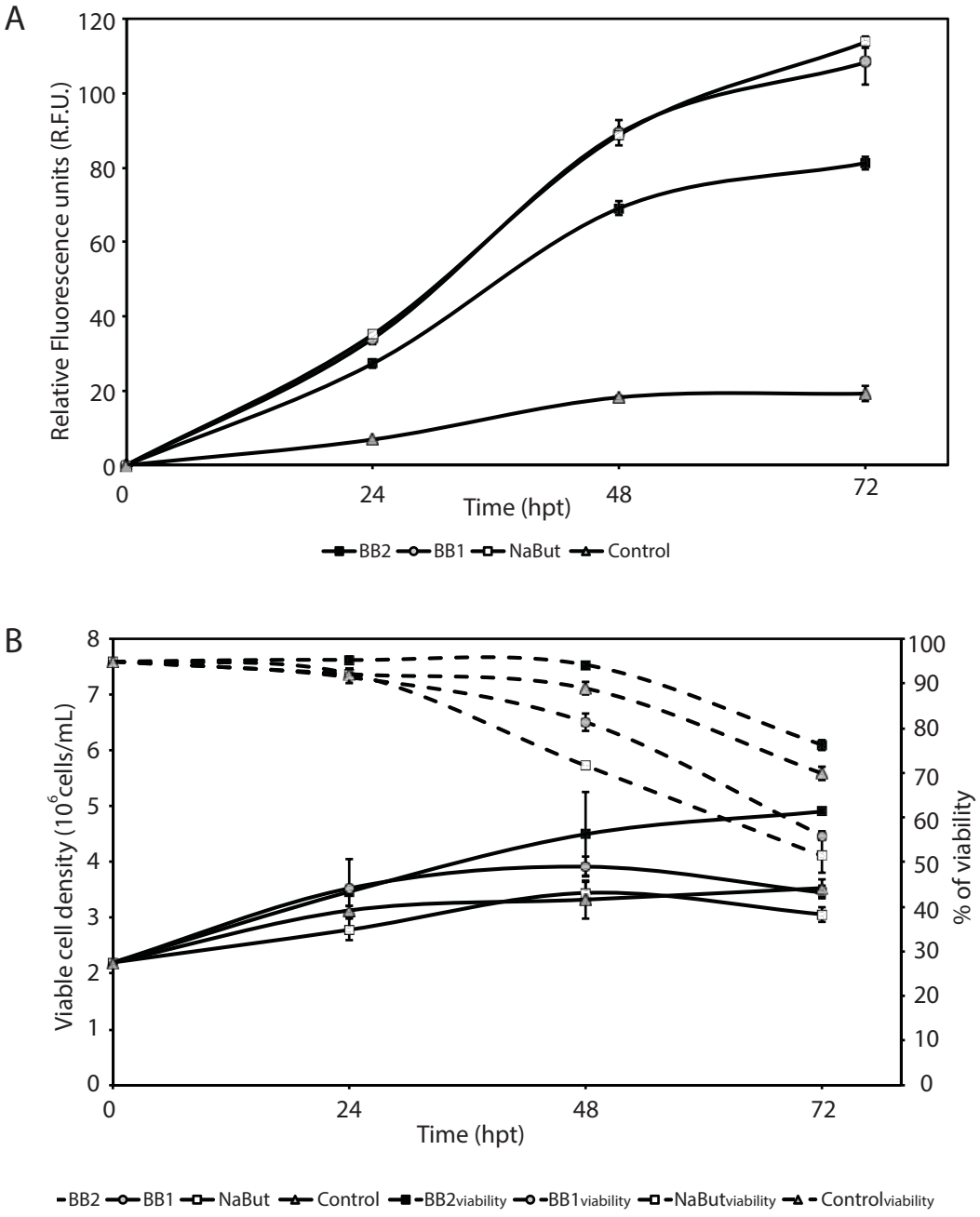

Figure 7. Model validation. Production of Gag-GFP VLPs using the optimal concentration of additives. An experiment with only Sodium butyrate and a control without additives were added for comparison. Mean values ± standard deviation of triplicate experiments are represented.

One consideration to be made is on the observed differences in final viability among the different protocols and their potential impact on the corresponding downstream process for the final purification of the VLPs, since a lower viability would imply a higher amount of cellular debris and the release of intracellular components making more difficult and expensive the production of the final VLP preparation. Additionally, considering that enveloped VLPs are exported out from the cell through a budding process, a decrease in culture viability can lead to a decrease of properly assembled and enveloped VLPs. For this reasons the optimum concentrations of additives obtained in BB2 should be considered the best option.

Conclusions

Taking into consideration the results obtained in this work, it can be concluded that a methodology has been developed for the optimization of production of HIV-1 Gag-GFP VLPs by transient transfection of HEK 293 cells using specific additives as enhancers of VLPs production. The optimal methodology proposed is: Transfect exponencially growing cells at 2×10^6 cells/mL, using 1 μg of DNA/(ml of culture) and 3 μg of PEI/(mL of culture). Add Lithium acetate to a concentration of 20mM, 3 hours prior transfection and add Valproic acid to a concentration of 3.36 mM and 5.04 mM of Caffeine, 4 and hours post transfection.

One very relevant characteristic of this methodology is that it can be performed preserving cell viability at high concentrations.

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Selection and optimization of transfection enhancer additives for increased virus-like particle production in HEK293 suspension cell cultures

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Extended Gene Expression by Medium Exchange and Repeated Transient Transfection for Recombinant Protein Production Enhancement

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ABSTRACT: Production of recombinant products in mammalian cell cultures can be achieved by stable gene expression (SGE) or transient gene expression (TGE). The former is based on the integration of a plasmid DNA into the host cell genome allowing continuous gene expression. The latter is based on episomal plasmid DNA expression. Conventional TGE is limited to a short production period of usually about 96 h, therefore limiting productivity. A novel gene expression approach termed extended gene expression (EGE) is explored in this study. The aim of EGE is to prolong the production period by the combination of medium exchange and repeated transfection of cell cultures with plasmid DNA to improve overall protein production. The benefit of this methodology was evaluated for the production of three model recombinant products: intracellular GFP, secreted GFP, and a Gag-GFP virus-like particles (VLPs). Productions were carried out in HEK 293 cell suspension cultures grown in animal-derived component free media using polyethylenimine (PEI) as transfection reagent. Transfections were repeated throughout the production process using different plasmid DNA concentrations, intervals of time, and culture feeding conditions in order to identify the best approach to achieve sustained high-level gene expression. Using this novel EGE strategy, the production period was prolonged between 192 and 240 h with a 4–12-fold increase in production levels, depending on the product type considered.

Biotechnol. Bioeng. 2014;9999: 1–13.

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Contract grant sponsor: SEIDI - Ministerio de EconomÚa y Competitividad
Contract grant number: BIO2012-31251
Contract grant sponsor: Generalitat de Catalunya
Contract grant number: 2009 SGR 1038
Received 5 September 2014; Revision received 11 November 2014; Accepted 18 November 2014
Accepted manuscript online xx Month 2014;
Article first published online in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/bit.25503

KEYWORDS: HEK 293; transient transfection; recombinant proteins; VLPs; production optimization

Introduction

Industrial production of recombinant therapeutics in mammalian cell cultures is routinely accomplished by stable gene expression (SGE). This manufacturing approach is based on the use of a cell line that, upon stable integration of a DNA sequence into the host cell genome, enables continuous gene expression. The process to establish a stable producer cell line involves transfection of cells with plasmid DNA and subsequent selection of cells that have stably integrated the plasmid using a selection marker. Cells are then clonally separated for identification of the highest producer cell clones, which are subsequently screened for optimal attributes such as cell growth capacity, specific productivity, product quality (e.g., glycosylation pattern), etc. (Wurm, 2004). This process is tedious, costly, and typically takes several months depending on the difficulty to express the protein of interest (Baldi et al., 2007). In addition, the establishment of stable producer cell lines expressing cytotoxic genes and/or multiple genes for the generation of complex biopharmaceuticals is challenging (Pluta and Kacprzak, 2009).

Transient gene expression (TGE) is based on episomal DNA expression. Plasmid DNA entry to cells is facilitated by the use of transfection reagents including inorganic compounds such as calcium phosphate (CaPi), cationic polymers such as polyethylenimine (PEI), and cationic lipids such as lipofectamine® (Geisse, 2009; Geisse and Fux, 2009; Pham et al., 2006). The product is typically harvested between 48 and 72 h post-transfection (hpt) (Cervera et al., 2013). This production approach is straightforward and allows for the generation of sufficient quantities of biotherapeutics to conduct pre-clinical and clinical studies in some cases.

The application of TGE for production of recombinant proteins has been reviewed (Geisse, 2009; Pham et al., 2006; Wurm, 2004]. TGE is particularly suitable in early phases of development when several product candidates need to be screened for clinical studies. The manufacturing of clinical grade complex biopharmaceuticals such as viral gene therapy vectors by TGE for trials in humans has also been described (e.g., retroviral, lentiviral, and adeno-associated viral vectors) (Ausubel et al., 2012; Cornetta et al., 2008; Merten et al., 2011; Wright, 2009).

Although TGE has been typically used at low and medium scale, during the past 10 years significant effort has been made towards developing large-scale TGE protocols. Large-scale production using both PEI and CaPi transfection reagents has been demonstrated at production scales ranging from 1 to 110 L (Baldi et al., 2005). For instance, successful generation of IgG antibody by TGE at 10 L scale in Wave bioreactors and 50 and 100 L scale in stirred tank bioreactors has been reported (Tuvesson et al., 2008). Titers exceeding 1 g/L have been attained by TGE (Backliwal et al., 2008).

While the most commonly used host cell for SGE is the Chinese Hamster Ovary (CHO) cell line due to its extensive characterization and well-established history of regulatory approvals, the predominant cell line for TGE is the Human Embryonic Kidney 293 (HEK 293) cell line, mainly due to its unparalleled ability to become transfected. Nonetheless, both TGE using CHO cells and SGE using HEK 293 cells have also been successfully shown (Derouazi et al., 2004; Thomas and Smart, 2005). The latter is advantageous when human post-translation modifications, as opposed to those observed in hamster cells, confer beneficial properties to the product (Dietmair et al., 2012). This proved to be the case for human activated protein C, marketed as Xigris®, which required certain human post-translational modifications to maintain its biological activity (Durocher and Butler, 2009).

Compared with SGE, TGE allows for rapid production of biopharmaceuticals, but product titer is usually lower (Dietmair et al., 2012). One of the intrinsic limits of TGE is that episomal DNA is lost as cells divide (Chiou et al., 2014; Middleton and Sugden, 1994; Wade-Martins et al., 1999). As a result, the production time is short, typically less than 96 hpt, which in turn limits the overall production capacity. Several specific cell lines have been developed in an attempt to maximize TGE production time. The most popular one is the HEK 293 T cell line that constitutively expresses the large T antigen of the simian virus 40 (SV40). The protein allows replication of vectors containing the SV40 origin of replication in mammalian cells, thus augmenting episomal persistence (Baldi et al., 2007; Berntzen et al., 2005; Durocher et al., 2002; Geisse, 2009; Van Craenenbroeck et al., 2000;). Based on the same strategy, CHO-T and recently CAP-T cells have also been developed and used for TGE (Agrawal et al., 2013; Fischer et al., 2012; Kunaparaju et al., 2005). Similarly, the HEK 293 EBNA cell line that constitutively expresses the Epstein Barr virus nuclear antigen 1 (EBNA1) supports the extra chromosomal amplification of plasmids carrying the EBV origin of replication (Durocher et al., 2002; Geisse and Fux, 2009; Meissner et al., 2001; Van Craenenbroeck et al., 2000; Young et al., 1988). For all these cell lines, improved production is attributed to an increased plasmid copy number in the transfected cell population throughout the production phase.

In order to extend TGE time, a novel gene expression approach termed extended gene expression (EGE) was evaluated in this work. The aim of this production methodology is to prolong the production period by repeatedly transfecting HEK 293 suspension cell cultures combined with repeated medium exchange. The impact of this approach on production time and overall production yield for 3 model products, including an intracellular and secretable version of GFP and complex virus-like particles (VLPs) labeled with GFP, was evaluated.

Materials and Methods

Cell Line, Media, and Culture Conditions

The cell line used in this work is a serum-free suspension-adapted HEK 293 cell line (HEK 293SF-3F6) kindly provided by Dr. Amine Kamen from the BRI of National Research Council of Canada (Montreal, Canada). It 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) supplemented with 0.1% Pluronic® (Invitrogen). Medium was also supplemented with 1.6 mg/L of r-transferrin (Merck Millipore, Kankakee, IL), 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 (Cervera et al., 2013). Cells were routinely maintained in 125-mL disposable polycarbonate erlenmeyer flasks (Corning, Steuben, NY) 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 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell count and viability were determined using trypan blue staining followed by cell counting using a hematocytometer.

Plasmids

The pGag-EGFP plasmid used in this work codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP (Hermida-Matsumoto and Resh, 2000). The plasmid from the 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 (Clonthec, Mountain View, CA).

The expression plasmids pOPINEeGFP, pOPINECherry, and pOPINGeGFP used in this study were generated in the Protein Expression Core Facility of the Institute for Research in Biomedicine (IRB) in Barcelona. Briefly, eGFP and Cherry fluorescent protein genes, originally derived from pEGFP-N1 and pmCherry-N1 (Clontech, Mountain View, CA), were amplified using the following primer pair: N-HiseGFP fwd: ataccatggcacaccatcaccaccatcacag-cagcggtatggtgagcaagggcgaggag, 3CFPrev: Cgacggtaccctgaaaca-gaacttccagaccgctgctcttgtacagctcgtccatg, the PCR products digested with NcoI and KpnI and ligated into NcoI-KpnI cut pOPINE (Berrow et al., 2007) to produce pPEU2 and pPEU3 respectively. For secreted proteins, eGFP was amplified from pEGFP-N1 using the following primer pair: pOPINGFPfwd: GCGTAGCTGAAACCGG-CATGGTGAGCAAGGGCGAGGAG pOPINE/GFP rev: GTGATGGT-GATGTTTCTTGTACAGCTCGTCCATGCC. The resulting PCR products were cloned by In-Fusion cloning (Clontech, Mountain

View, CA) into vector pOPING (Berrow et al., 2007) linearised with KpnI and PmeI to produce pOPINGeGFP. Plasmids were prepared and purified as previously described (Segura et al., 2007).

Transient Transfection

HEK 293 suspension cells were transiently transfected using 25-kDa linear polyethylenimine (PEI) (PolySciences, Warrington, PA). HEK 293 cells were seeded at 0.3×10^6 cells/mL in 125-mL disposable flasks, grown to 2×10^6 cells/mL and transfected with 1 μg of DNA/mL or 0.5 μg/mL of culture and a DNA to PEI mass ratio of 1:2 after a complete medium exchange. 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. The percentage of GFP and Cherry positive cells was assessed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA) at different hours post-transfection (hpt). Mock transfected samples were included as negative control in every experiment and served to adjust the flow cytometry gates.

Fluorescence Confocal Microscopy

The visualization of fluorescent producer cells was achieved using a Fluoview® FV1000 confocal microscope (Olympus, Tokyo, Japan). Samples were placed on 14 mm microwells from 35 mm glass bottom petri dishes (MatTek Corporation, Ashland, MA) for visualization.

Fluorescence-based product Quantitation

For intracellular GFP and Cherry, cell lysates were prepared by subjecting cell pellets to 3 freeze-thaw cycles followed by centrifugation at $13.800 \times g$ for 20 min to eliminate cell debris. Green and cherry fluorescence were measured at room temperature using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA) using the following sets for GFP protein: λex = 488 nm (slit 5 nm), λem = 510 nm (slit 10 nm) and for Cherry protein: λex = 587 nm (slit 5 nm), λem = 610 nm (slit 10 nm). In the case of secreted GFP, supernants were clarified by cell culture centrifugation at $1000 \times g$ for 5 min. Relative fluorescence units values (R.F.U.) were calculated by subtracting fluorescence units (F.U.) values of untransfected negative control samples. The concentration of Gag-GFP VLPs was assessed by fluorometry using an *in-house* developed and validated quantification assay (Gutiérrez-Granados et al., 2013). VLP containing supernatants were recovered by cell culture centrifugation at $1000 \times g$ for 5 min. There is a linear correlation between fluorescence intensity and p24 values determined using the INNOTEST p24 ELISA HIV antigen mAb (Innogenetics NV, Gent, Belgium). RFU values can be converted to Gag-GFP concentration values using the following equation:

$$Gag - GFP\left(\frac{ng}{mL}\right) = (3.245 \times R.F.U. - 1.6833) \times 36 \quad (1)$$

where Gag-GFP (ng/mL) is the estimated concentration of polyprotein and R.F.U. 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 (Gutiérrez-Granados et al., 2013). For intracellular GFP and Cherry, cell lysates were prepared by subjecting cell pellets to 3 freeze-thaw cycles followed by centrifugation at $13,800 \times g$ for 20 min to eliminate cell debris. For the quantification of Cherry protein, Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA), was set as: λex = 587 nm (slit 5 nm), λem = 610 nm (slit 10 nm).

Results and Discussion

Establishing an Extended Gene Expression Methodology

The proposed production strategy consisted in performing repeated rounds of transfection to achieve a sustained level of gene expression over time as opposed to the conventional TGE approach which entails a single transfection round. The initial transfection round was performed at 2×10^6 cells/mL using 1 μg of plasmid DNA/mL of cell culture using a standard TGE protocol described previously (Cervera et al., 2013). Retransfection rounds were then performed using various plasmid DNA concentrations, intervals of time, and culture feeding conditions in order to identify the best approach to maintain gene expression over time. Since the methodology proposed comprised a medium exchange each time a retransfection was performed, a medium exchange negative control (ME) was included in the studies. In these ME control cultures, after the first transfection round, a complete medium exchange (but no DNA addition) was performed every 48 h.

To achieve extended gene expression (EGE), a complete medium exchange followed by retransfection of cell cultures using a concentration of 1 μg of plasmid DNA/mL of cell culture every 48 h was initially carried out. In an attempt to reduce the potential toxicity caused by the addition of the PEI/DNA complexes, two alternative approaches were evaluated. The first approach was carried out as described for EGE but after the first round of transfection, cell cultures were re-transfected with only 0.5 μg of plasmid DNA/mL of cell culture (0.5 EGE). The alternative approach consisted in retransfection rounds that were spaced in time. Therefore, cell cultures were treated as described for EGE with a medium exchange every 48 h but the addition of DNA (1 μg of plasmid DNA/mL of cell culture) was only performed every 96 h (96 h EGE).

All three EGE approaches described above were evaluated for production of three recombinant products with increasing levels of expression difficulty: (1) a green fluorescent protein (GFP) expressed intracellularly; (2) a GFP secreted to the culture medium; and (3) a fluorescently labeled HIV-1 Gag Virus-Like Particle (Gag-GFP VLP). The latter is a macromolecular complex of proteins and lipids that resemble virions but are devoid of genetic material and are therefore, incapable of replication. This feature makes VLPs very promising vaccine candidates (Doan et al., 2005). The Gag-GFP polyprotein self-assembles into a particle core inside the producer cell and is released to the supernatant through a budding process.

Gag-GFP VLPs are rounded structures measuring approximately 140 nm and enveloped with the lipid membrane taken from the producer cell (Briggs et al., 2004; Gutiérrez-Granados et al., 2013; Valley-Omar et al., 2011). Of note, all three products can be monitored by detecting green fluorescence. GFP is among the most widely used reporter proteins. Its use in these studies enabled straightforward determination of the transfection efficiency by flow cytometry and protein production levels by fluorometry.

Results obtained using the five transfection experiments described above (standard TGE, ME, EGE, 0.5 EGE, and 96h EGE) are shown in Figure 1 (intracellular GFP), Figure 2 (secreted GFP), and Figure 3 (Gag-GFP VLP). Cell culture growth behavior, as assessed by cellular density and cell culture viability at different times post transfection, are shown in Figures 1A, 2A, and 3A, respectively, whereas Figures 1B, 2B, and 3B show the percentage of GFP positive cells in cell cultures and Figures 1C, 2C, and 3C show fluorescence levels over time for the three products, respectively.

Extended Gene Expression for Intracellular GFP

As expected, GFP expression using the standard TGE approach is limited to a period of 96 hpt. During this time, cell culture viability was maintained high (>80%). However, a pronounced decrease in cell density and viability was observed in the cultures beyond 96 hpt, with only 5% of the culture remaining viable 144 hpt (Fig. 1A). In contrast, both the ME control and all three EGE approaches allowed for maintenance of cell density around 6×10^6 cells/mL with viabilities over 50% for an extended time span of 240 hpt. These results are clearly due to the medium exchange performed every 48 h in all cases.

Significant differences between the various transfection protocols were observed for transfection efficiency (Fig. 1B) and protein production levels (Fig. 1C). In contrast with the traditional TGE approach in which the cell culture was unviable at 144 hpt, ME and all EGE approaches allowed for sustained and maximum levels of GFP positive cells and lysate fluorescence intensity at all times tested after 144 hpt, showing that production time can be successfully extended for a minimum of 240 hpt. Of note, the maximum percentage of GFP positive cells was clearly observed using the EGE and 0.5 EGE transfection approaches, which performed comparably (Fig. 1B). More importantly, this result correlated with GFP expression levels (Fig. 1C) further confirming that the percentage of GFP positive producer cells correlate with expression levels as shown previously (Cervera et al., 2013). Production performance for the ME and 96 h EGE procedures was similar indicating that addition of DNA needs to be performed frequently enough (every 48 h as opposed to 96 h) in order to keep plasmid DNA copy number and high expression levels.

The maximum percentage of transfected cells for the classical TGE approach reached 32% at 96 hpt with a maximum fluorescence level of 57 R.F.U. By using the proposed EGE production strategy, not only production time was extended beyond 96 hpt, but also the levels of protein expression reached were far superior with a maximum of 344 R.F.U. at 192 hpt (six-fold higher, Table I) correlating with a higher percentage of GFP positive cells (over 50%) at all times during the production process. Comparatively, the ME approach resulted in a lower percentage of GFP positive cells (maximum of 38% at 96 hpt) and a lower protein expression level (maximum 127 R.F.U., 144 hpt). This result was expected as the ME process is only benefited by medium but not DNA re-feeding. Moreover, it clearly indicates that addition of nutrients and removal of by-products as would take place in a perfusion cell culture process, is not the only factor responsible for the high protein production levels achieved by EGE. This initial set of experiments confirmed the potential of the EGE proposed strategy as it was efficient not only for maintaining cell growth and viability, but also maintaining cells transfected throughout the process, and consequently, expressing GFP with a concomitant increase in protein production yields.

Extended Gene Expression of Secreted GFP

The benefits of using the EGE strategy for the production of secreted GFP were subsequently evaluated. A general observation for this second study was that cells consistently achieved higher cell densities and viabilities for all conditions tested when compared to the cultures expressing intracellular GFP protein. This may be related with a lower toxicity or metabolic burden associated with the expression of secreted as opposed to intracellular GFP (Fig. 2A). For the standard TGE condition, cell concentration and viability was maximum at 96 hpt and sharply decreased thereafter. In contrast, cell density and viability was higher for all the other conditions tested allowing for extension of the process up to 240 hpt. These results are similar to those observed for intracellular GFP.

Significant differences were also observed in the percentage of cells transfected over time (Fig. 2B) and GFP expression kinetics (Fig. 2C) depending on the transfection protocol followed. Of note, as the GFP protein studied in this set of experiments was secreted to the cell culture supernatant, we harvested GFP at each medium exchange and results were based on calculated accumulated protein production (Fig. 2C). As observed previously, standard TGE showed a limited time span of 144 hpt compared to the rest of the transfection strategies involving periodic medium exchanges, which extended the process to 240 hpt. The EGE protocol clearly showed to be superior to any other protocol tested including the 0.5 EGE and 96 h EGE. The latter resulted in comparable percentage of transfected cells throughout production and accumulated protein levels as the negative control protocol (ME) implying that a minimum amount of DNA (besides a minimum time between DNA additions as observed before) is required to keep high plasmid copy number and attain sustained high level gene expression. The higher cell growth rate observed in cultures expressing secretable GFP as opposed to the intracellular GFP version may be related to the need for higher amounts of DNA addition.

The accumulated fluorescence obtained using the conventional TGE approach was 2707 R.F.U. at 144 hpt. Whereas using ME, 0.5 EGE, and 96 h EGE the accumulated fluorescence obtained was around 7000 R.F.U. (6694, 7697, and 7082 R.F.U., respectively) at the end of the production phase. The best performance was obtained with the EGE protocol, in which 11676 R.F.U. can be obtained at 240 h after the first transfection. The improvement obtained by using the extended gene expression when compared to the standard gene expression protocol is 4.3 fold (Table I).

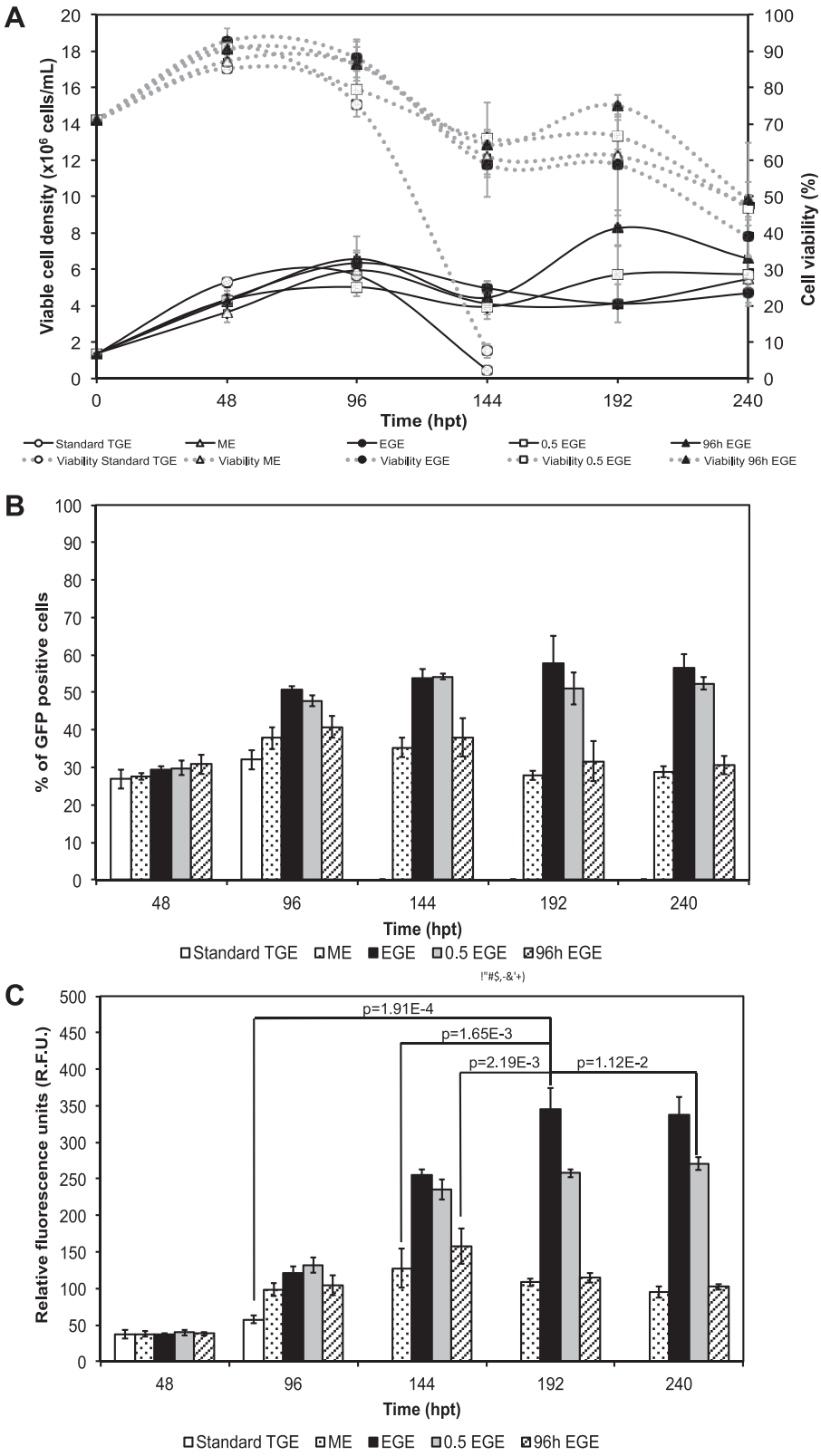

Figure 1. Performance of the different production protocols for intracellular GFP. Five different production protocols were evaluated in parallel including a standard TGE, a control medium exchange every 48 h (ME), retransfections with 1 μg/mL of DNA every 48 h (EGE), retransfections with 0.5 μg/mL of DNA every 48 h (0.5 EGE) and retransfections with 1 μg/mL if DNA every 96h (96 h EGE). HEK 293 cell density and viability (**A**), transfection efficiency (**B**), and fluorescence intensity in harvested cell suspension lysates (**C**) were measured every 48 h during 10 days. Mean values ± standard deviation of triplicate experiments are represented.

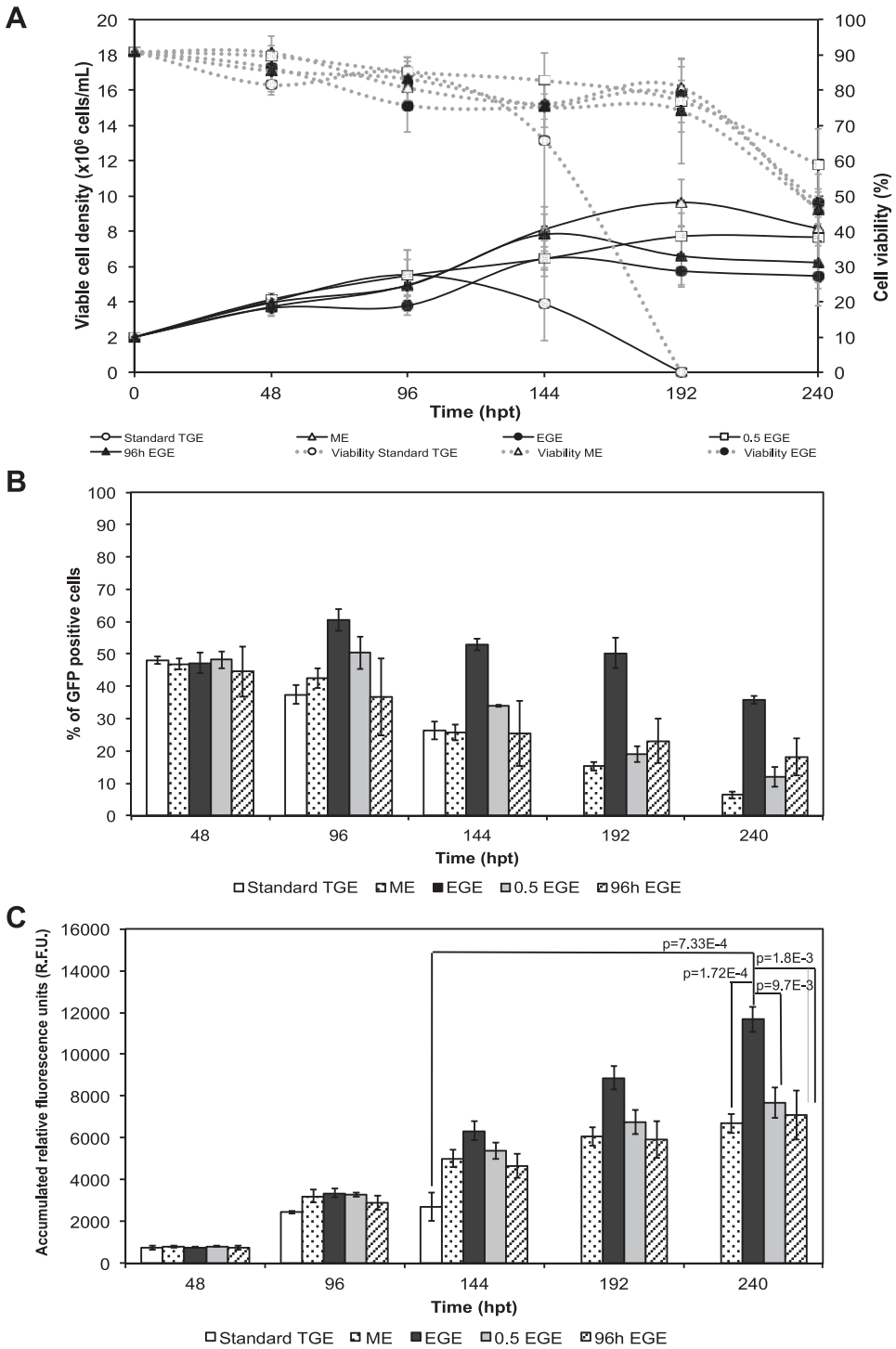

Figure 2. Performance of the different production protocols for secretable GFP. Five different production protocols were evaluated in parallel including a standard TGE, a control medium exchange every 48 h (ME), retransfections with 1 μg/mL of DNA every 48 h (EGE), retransfections with 0.5 μg/mL of DNA every 48 h (0.5 EGE) and retransfections with 1 μg/mL if DNA every 96 h (96h EGE). HEK 293 cell density and viability (**A**), transfection efficiency (**B**), and accumulated fluorescence intensity in harvested supernatants (**C**) were measured every 48 h during 10 days. Mean values ± standard deviation of triplicate experiments are represented.

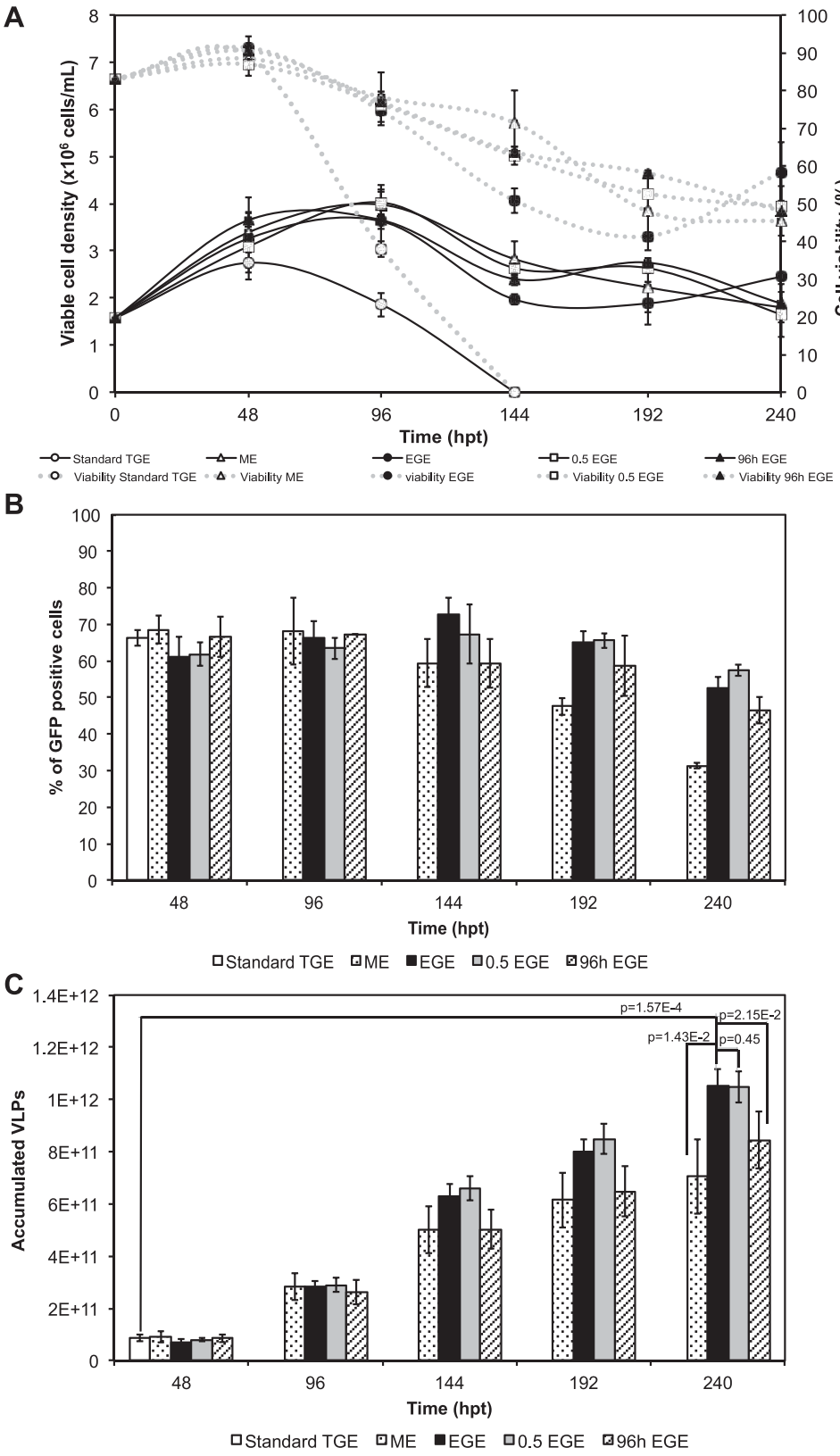

Figure 3. Performance of the different production protocols for Gag-GFP VLPs. Five different production protocols were evaluated in parallel including a standard TGE, a control medium exchange every 48 h (ME), retransfections with 1 μg/mL of DNA every 48 h (EGE), retransfections with 0.5 μg/mL of DNA every 48 h (0.5 EGE), and retransfections with 1 μg/mL if DNA every 96 h (96h EGE). HEK 293 cell density and viability (**A**), transfection efficiency (**B**), and accumulated VLPs in harvested supernatants (**C**) were measured every 48 h during 10 days. Mean values ± standard deviation of triplicate experiments are represented.

Table I. Comparison of production level attained by applying different transfection protocols relative to standard TGE production levels for various recombinant products.

	Fold production level increase relative to levels obtained by Standard TGE (best EGE strategy in Bold)				
	ME	EGE	0.5 EGE	96h EGE	Additional gain from retransfection with respect to ME
Intracellular GFP	2.2	**6**	4.7	2.7	2.7
Secreted GFP	2.5	**4.3**	2.8	2.6	1.7
Gag-GFP VLPs	8.1	12	**12**	9.7	1.5

Extended Gene Expression of HIV-1 Gag-GFP Virus-like Particles

Finally, the EGE approach was tested for the production of an enveloped virus-like-particle (Gag-GFP VLPs) representing a complex product candidate. Interestingly, cell culture viability for all conditions tested in the case of Gag-GFP VLP (Fig. 3A) was lower than for the intracellular and secretable GFP proteins. The reason for this effect is unclear but may be related to cytotoxic effects of Gag gene expression and/or the budding process. Using the conventional TGE approach, cell density and viability reached a maximum at 48 hpt and then suffered a sharp decrease forcing Gag-GFP VLP harvest at an earlier time point (48–72 hpt) compared with the other products evaluated in order to avoid massive supernatant contamination with intracellular components and cell debris. Cell concentration and viability profiles for all other protocols tested, showed to be comparable. The cell density roughly doubled that observed for TGE reaching approximately 4×10^6 cells 96 hpt and was maintained high until the end of the production phase. In addition, as opposed to the TGE approach in which the cell culture was unviable at 144 hpt, cell viability was maintained for a longer period up to 240 hpt with viabilities over 50%. This is in agreement with the medium exchange benefits observed with the other two products evaluated (intracellular and secreted GFP).

The percentage of transfected cells (Fig. 3B) is maintained high (above 50%) until the end of the process (240 hpt) for all protocols tested with the exception of ME, for which there is no retransfection and therefore the percentage of transfected cells decreases with time, as expected. It has to be highlighted that the performance of EGE and 0.5 EGE protocols in terms of transfection efficiency is very similar.

The accumulated production of VLPs with all the EGE protocols is remarkably higher than that of standard TGE (Fig. 3C). The best production performance is achieved by EGE and 0.5 EGE, with no statistically significant difference between these two protocols. In both cases, the accumulated VLP production is in the order of 1×10^{12}, a marked improvement with respect to the data obtained using the conventional TGE approach for Gag VLP production that resulted in 8.7×10^{10} VLPs representing a 12-fold increase in production.

As in the 0.5 EGE approach, the amount of DNA added during retransfections is half of the DNA added for the EGE, this is the protocol proposed for the production of VLPs. The generation of Gag-GFP VLPs by EGE was further investigated in this work with the scope of further optimizing the production protocol by gaining additional understanding on the retransfection steps.

Dynamics of Cell Culture Retransfection

To elucidate the dynamics of cell culture retransfection, an experiment consisting of two sequential transfection rounds using plasmids coding for two different fluorescent proteins was performed. The first transfection was performed with Cherry protein coding plasmid DNA at 0 h, and the second was performed with GFP protein coding plasmid DNA at 48 h. Flow cytometry analysis of the cell culture was conducted 96 h after the first round of transfection. Results indicated that the majority of cells (76.9%) expressed only the product coded by the plasmid transfected in the first round (Cherry), while 16.8% of the cells expressed only the plasmid transfected in the second round (GFP). Only a small fraction of the cells expressed both plasmids (6.3%). This experiment confirmed that the vast majority of the cells initially transfected with the first plasmid (Cherry) maintained the

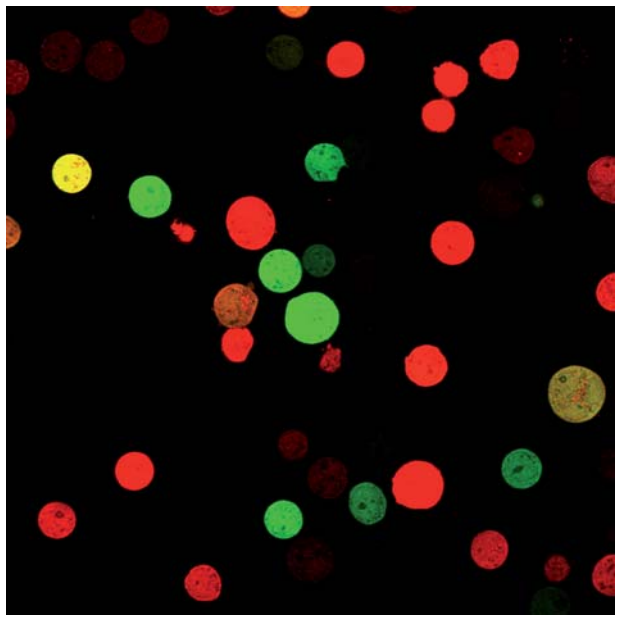

Figure 4. Analysis of the dynamics of retransfection by sequential introduction of plasmids coding for two different fluorescent proteins. Exponentially growing HEK 293 cells at 2×10^6 cells/mL resuspended in fresh cell culture medium were transfected with 1 μg of pCherry DNA/mL of culture in a first round and 0.5 μg of pGFP DNA /mL of culture in a second round that took place 48 h after the initial transfection. Confocal image showing green fluorescent cells (expressing GFP), red fluorescent cells (expressing Cherry), and cells overlaying green and red fluorescence signal (expressing both GFP and Cherry).

expression of the transgene. In addition, the experiment indicated that primarily non-transfected cells were taking up the second plasmid (GFP) as only a fraction of cells already transfected with the first plasmid showed double positivity. Therefore, retransfections seem to preferentially affect non-transfected cells, while those cells that are already transfected seem more refractory to be transfected a second time.

These results were verified by microscopic observation of the cell culture 96 hpt using confocal microscopy. Figure 4 shows a large population of Cherry positive cells, an intermediate population of GFP positive cells, and a small population of double positive (yellow) cells expressing both Cherry and GFP proteins (Fig. 4). These results support the utility of the EGE approach by confirming it is possible to increase the number of producer cells by implementing retransfection rounds targeting new populations of cells that were not transfected in the first round.

Previous work demonstrated higher transfection efficiency in cells undergoing G2/M phase rather G1, showing a close dependency between transfection and cell cycle phase (Brunner et al., 2000; Cervera et al., 2013). Brunner et al. also suggest that transfection of cells shortly before the next cell division (close to M phase) is facilitated by nuclear membrane breakdown. We hypothesize that the capacity of transfecting a new subpopulation of cells that were not transfected in the first round can be due to a change in the cell cycle phase or another cellular parameter that changes with time after the first transfection.

It is widely accepted that plasmid DNA copies are lost upon cell division following transient transfection (Middleton and Sugden,

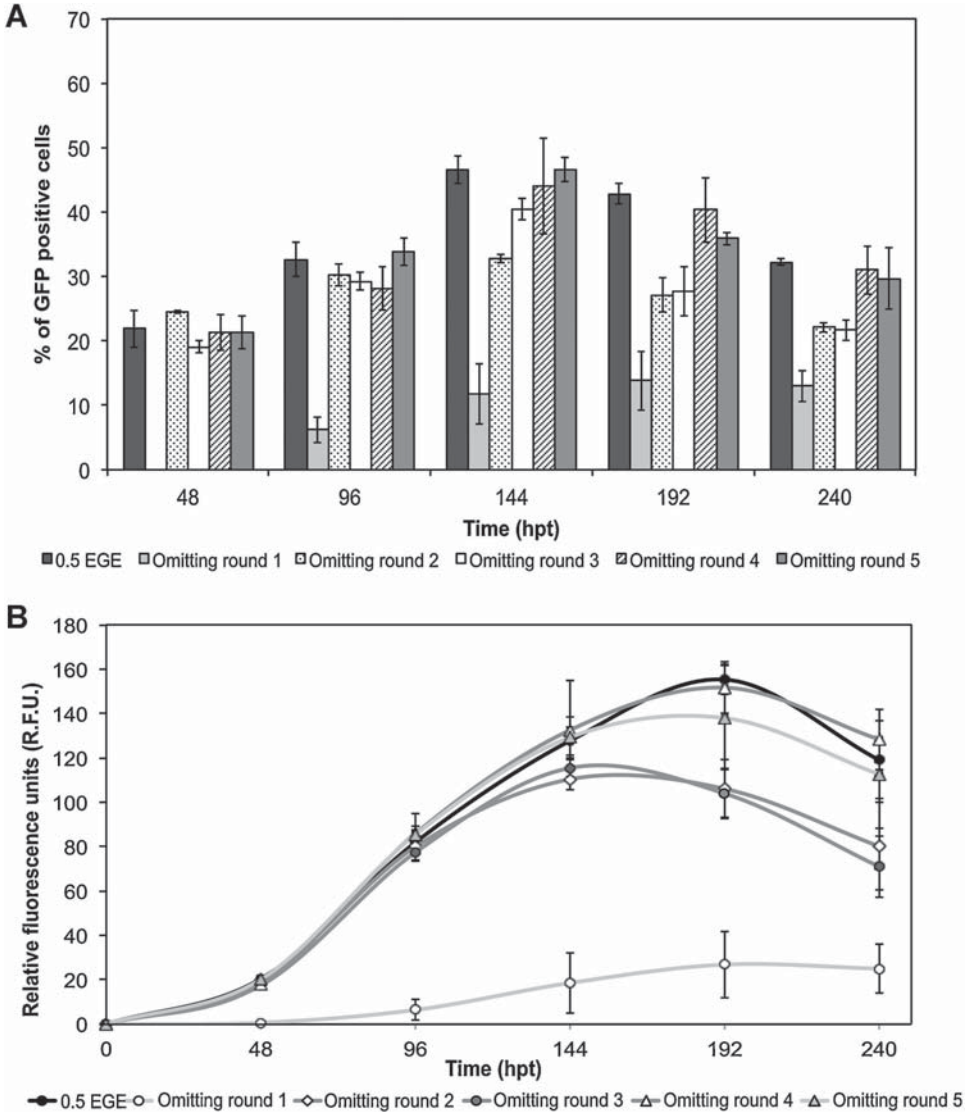

Figure 5. Evaluation of the effect of plasmid DNA addition in different EGE transfection rounds on transfection efficiency and protein production. Exponentially growing HEK 293 cells at 2×10^6 cells/mL resuspended in fresh cell culture medium were transfected with 1 μg of DNA /mL of culture in a first round and 0.5 μg of DNA /mL of culture in subsequent rounds of transfection every 48 h using the pGFP or the pCherry when indicated. The bar plots show the percentage of GFP positive cells (**A**) and green fluorescence intensity in cell culture lysates (**B**) attained at different times post-transfection. Mean values ± standard deviation of three experiments are presented.

1994; Wade-Martins et al., 1999). In fact, several authors make use of genetically modified cells containing viral elements that allow plasmid episomal persistance to improve production of biotechnological products by transient transfection (i.e., HEK 293 T and HEK 293 EBNA cell lines). An advantage of the proposed approach over the use of HEK 293 T or HEK 293 EBNA cell lines for production is that the need for demonstrating clearance of undesired genomic sequences (i.e., SV40 large T antigen) from the final product is circumvented. The retransfection of cells that may have lost the plasmid DNA during cell division is proposed here as a potential additional mechanism by which the EGE approach is achieving higher gene expression levels over time besides the transfection of cell populations originally not transfected.

Evaluation of the Effect of Plasmid DNA Addition in Different Rounds of Transfection on Transfection Efficiency and Protein Production

To complete the evaluation of the EGE protocol, the effect of each one of the multiple transfection rounds was analyzed, with respect to their contribution to the percentage of transfected cells and overall production. The objective of this experiment was to determine if any round of retransfection was not significantly contributing to the overall productivity and therefore could be eliminated with the consequent simplification of the protocol. To this end, the 0.5 EGE protocol was followed in five experiments. In each of these experiments, a given step of retransfection, normally performed using the GFP coding plasmid, was substituted by addition of the Cherry coding plasmid. Using this approach, analysis of the percentage of GFP positive cells and GFP expression levels in each one of these experiments could be compared to those obtained in a control experiment performed using the pGFP for all rounds of retransfection.

Figure 5A shows how the percentage of GFP positive cells is affected by the omission of pGFP DNA addition at different rounds in the EGE protocol. When comparing the percentage of transfected GFP positive cells at any given point, for example at 240 hpt, it can be observed that when all transfections are performed using the pGFP (complete 0.5 EGE), the percentage of transfected cells is maximum at 32%. When the first transfection was performed using the pCherry instead of pGFP (but all the following with pGFP) the percentage of transfected cells drops by 59%, further emphasizing that the first round of transfection is key to achieve a high percentage of transfected cells that is sustained throughout the process. When the second and the third retransfections were performed using pCherry instead of pGFP, the percentage of transfected cells decreased by 31%, which indicated that, the second and the third retransfection rounds also contribute to increasing the population of transfected cells. When the pGFP of the fourth and the fifth retransfections were substituted with pCherry, the percentage of GFP positive cells was equivalent to that of the complete 0.5 EGE, thus suggesting that the fourth and fifth retransfections did not significantly impact the percent of GFP positive cells.

As expected, results for the GFP production levels (Fig. 5B) parallel that of percent GFP positive cells (Fig. 5A). When the first transfection is not performed with the pGFP, GFP expression levels were very low (29 R.F.U.). When the second and the third retransfections were performed with pCherry instead of pGFP, only 110 and 115 R.F.U. were attained at 144 hpt. When the pGFP of the fourth and the fifth retransfections were substituted with the pCherry, the GFP production levels were nearly unchanged. These results further ascertain that the last two retransfections do not add any substantial improvement to the process and thus could be eliminated with no detrimental effect on production in the final optimized protocol.

Validation of the Optimized EGE Protocol

In order to validate the previous results, production of VLPs was carried out using the complete 0.5 EGE strategy and the 0.5 EGE

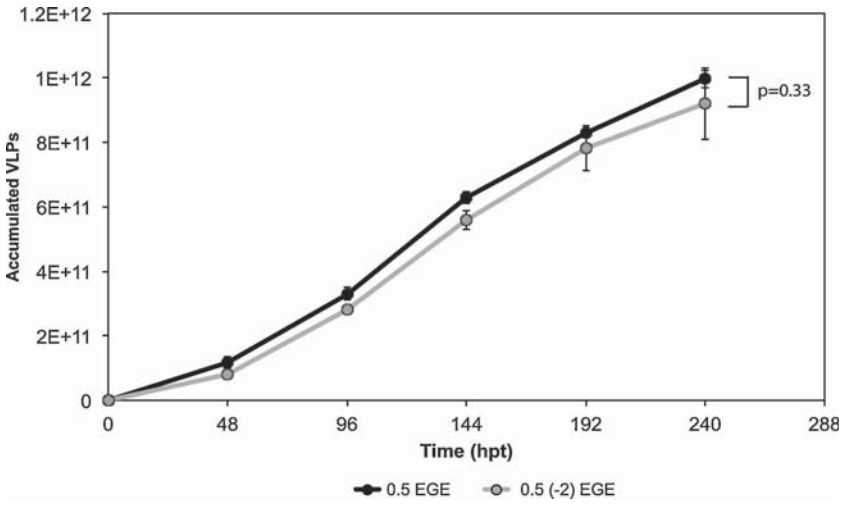

Figure 6. Validation of the optimized 0.5 EGE production protocol for VLPs. To validate the performance of the proposed optimized EGE protocol, the normal EGE, and optimized EGE protocols lacking the last two retransfections were run in parallel. Mean values of accumulated VLPs ± standard deviation of triplicate experiments are represented. A *P*-value of 0.33 confirms that there is no statistically significant difference between them.

Table II. Comparison of standard TGE and optimized EGE transfection process performance

Accumulated VLPs	Standard TGE	8.7E10
	Optimized EGE	9.2E11
VLP concentration (VLPs/mL)	Standard TGE	4.3E09
	Optimized EGE	9.2E09
Accumulated production (VLPs/h)	Standard TGE	1.8E09
	Optimized EGE	3.8E09
Volumetric productivity (VLPs/h*Vreactor)	Standard TGE	9.0E07
	Optimized EGE	1.9E08

excluding the last two rounds of retransfection. As it can be observed in Figure 6, production performance in both cases is very similar with total accumulated number of 1×10^{12} and 9.2×10^{11} VLPs, respectively. The calculated *P*-value of 0.33 indicates that there is no statistically significant difference between both results.

Table II summarizes the results obtained using the standard TGE VLP production approach and the proposed optimized EGE. It can be concluded that the optimized EGE protocol offers clear advantages in terms of total accumulated VLPs, final concentration, accumulated VLPs production, and volumetric productivity compared to standard TGE. The concentration of VLPs obtained with every medium exchange is equal or higher than that obtained by standard transient transfection (Fig. 7). This is relevant for subsequent downstream processing operations as no additional predict concentration steps would be required using the optimized approach. During the time in which one EGE is performed only three TGE can be performed considering a two-day stop for cleaning, sterilization, and cell amplification are required. If one were to use the standard TGE approach to attain the same amount of VLPs generated by the proposed EGE approach, it would require three-fold the time (30 instead of 10 days), two-fold the media (10 instead of 5 volumes) and five-fold the amount of plasmid DNA (200 instead of 40 μg). These parameters have a significant influence in the process cost and highlight the relevance of the remarkable improvements associated to the EGE approach presented here.

The results obtained in the proposed methodology are in agreement with previously reported literature. For instance, an increase in transfection efficiency was observed in adherent CMT cells (a derivative of COS cell line) when cells were transfected two times (Ishikawa and Homcy, 1992). Also, in attempt to increase transfection efficiency, HEK 293 T adherent cells were transfected up to five times every 6 h (Yamamoto et al., 1999). Best results were achieved by transfecting adherent cell cultures three times, leading to an increase in transfection efficiency from 40 to 74%. The positive effect of medium supplementation on transient transfection is well documented. Improvements in protein production made by fed-batch or perfusion after transient transfection have been reported. Perfusion was successfully used to increase titers of adherent H6–18 cells (derived from HEK 293 T) immobilized in microcarriers to produce Drosophila cytokine Spätzle (Cheeks et al., 2009) and for producing lentiviral particles using a suspension adapted HEK 293 and an acoustic filter to retain cells in the bioreactor (Ansorge et al., 2009). Productivity has been also enhanced by medium feeding post-transfection of HEK 293 EBNA for the production of GFP and secreted alkaline phosphatase (SEAP) (Pham et al., 2005) or GFP and erythropoietin (EPO) (Sun et al., 2006).

Conclusion

In this work, we present a transient transfection methodology that allows extended gene expression at higher levels for a variety of

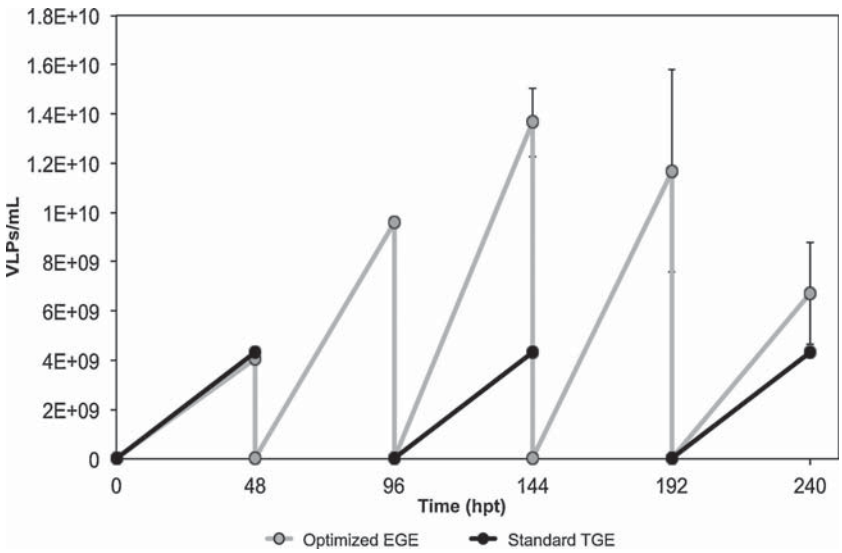

Figure 7. Comparison between the standard TGE and optimized EGE production strategies. A plot comparing VLP titers achieved by TGE and EGE is presented. The concentration of VLPs reached throughout the production phase using the EGE protocol is equivalent or higher than that reached using the TGE protocol. Two TGE runs can be carried out during the time an EGE run is performed.

bioproducts. Depending on the protein produced, the improvement made by the EGE protocol is mainly due to medium exchange or by repeated transfection of the cell culture (Table I). An optimized EGE protocol for generation of Gag-GFP VLP has shown to extend the production phase from 48 to 240 h resulting in a 12-fold increase in the amount of VLPs generated compared to the classical TGE production approach (Table I). This EGE manufacturing approach has also shown to extend the time of secreted (144–240 h) and intracellular (96–192 h) GFP expression that resulted in a four to six-fold increase in the recombinant protein production levels compared to the classical TGE approach. To conclude, the developed extended gene expression protocol provides a novel means for improving transient transfection process yields.

The authors wish to thank Dr. Amine Kamen (NRC, Montreal, Canada) for valuable discussions and for providing the HEK 293 cell line used in this study. We would also like to thank Dr. Julià Blanco at IRSI Caixa (Badalona, Spain) for providing the plasmid construct for Gag-GFP and for helpful comments. The contribution of Manuela Costa (Institut de Biotecnología i Biomedicina UAB) with FACS analyses is deeply appreciated. The support of Dr. Salvador Bartolomé (Departament de Bioquímica i de Biología Molecular UAB) in fluorometry analysis and Meritxell Vendrell (Servei de Microscòpia UAB) in confocal microscopy is recognized. Gavin Whissell provided generous help in the revision of the manuscript. This work is supported by a grant of SEIDI - Ministerio de Economía y Competitividad of Spain (BIO2012-31251) and Generalitat de Catalunya (2009 SGR 1038). Laura Cervera is a recipient of a PIF scholarship from UAB. Sònia Gutiérrez-Granados is a recipient of a FPU grant from the Ministerio de Educación y Deportes of Spain.

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General discussion and further directions

HIV-1 Gag VLPs represent an attractive platform for the generation of new generation vaccine candidates. Virus-like particles are self-assembled particles that mimic the virus structure. Due to their repetitive organized structure and particulate nature, they are very efficiently uptaken by antigen-presenting cells giving rise to a potent immune response. The use of VLPs for vaccination confers an ideal candidate, as they are very safe both for the vaccinated individual and for the manufacturers, due to the lack virus genome making them non-infective virus particles.

The production of HIV-1 Gag VLPs was previously reported using the baculovirus-insect cell expression system. The use of insect cell production platform has been widely used, even though it has some drawbacks when compared to the cell line used in this work, HEK 293-3F6 GMP compliant cell line. Since HIV-1 Gag VLPs are enveloped VLPs, they take part of the cell membrane during the budding process from the host cell. Consequently, when they are produced using insect cells, they incorporate insect or baculovirus-derived proteins, such as the gp64, in the lipid envelope, which is known to provoke a strong immune response that can potentially mask the response against the desired envelope antigen (Deml et al., 2005; Hammonds et al., 2007). In addition, removal of contaminating baculovirus particles during downstream processing is challenging as these viruses share similar physicochemical properties with VLPs (Deml et al., 2005; Hammonds et al., 2007). Moreover, the post-translational and proteolytic capabilities of insect cells are not identical to those of mammalian cells, which results in VLP structures that do not accurately mimic authentic HIV-1 particles (Deml et al., 2005; Hammonds et al., 2007).

The use of animal derived component free and chemically defined media is essential for rapid translation from the small-scale laboratory work to large-scale industry manufacture and also for human vaccine approval. For this reason, all the serum-free

and chemically defined media existing in the market at the time of performing this work for HEK 293 suspension cells, were tested to screen for better cell culture growth and productivity. The selected medium (Freestyle 293, Invitrogen, Carlsbad, CA) allows both cell growth and PEI-mediated transient transfection. The medium was further optimized using non-animal derived components as supplements, and cell growth was increased by 2.4 fold. As the best moment to transfect the culture is before the mid-log phase, the increase in maximum cell density attained by medium optimization led to a proportional increase in HIV-1 Gag VLPs production. The production was further optimized by complete medium exchange just before transfection. This medium exchange allows the supply of nutrients that might be exhausted in the conditioned medium (Backliwal et al., 2008b) and the removal of by-products in the conditioned medium that could interfere with the PEI mediated transfection (Durocher et al., 2002; Schlaeger and Christensen, 1999; Tom et al., 2008). This optimal transfection protocol lead to a final concentration of 2.7×10^9 VLPs/mL.

No significant improvement in transfection efficiency or VLP production was observed by increasing the amount of DNA/PEI used for transfection indicating that DNA/PEI complex concentrations of 0.3–0.5 μg of DNA/million cells are sufficient to efficiently transfect HEK 293 cells.

A further attempt to understand the loss in transfection efficiency observed at high cell densities was carried out. In agreement with reported results (Brunner et al., 2000; Carpentier et al., 2007) it was observed that cells grown beyond the mid-log point had a lower percentage of cells in G2/M phase. However, this did not seem to be the only factor controlling transfection efficiency, and the understanding of this situation would

require further investigation.

The kinetics of the transient transfection process has been studied with the aim to characterize the different intracellular steps and determine important time points that can guide process optimization conditions. In this direction it has been demonstrated that one hour of contact between PEI/DNA polyplexes and cells is enough to ensure efficient transient transfection. The time at which the protein starts to be produced has been determined to be between 4 and 10 hpt. This is very valuable information to determine the most appropriate moment to add additives to enhance protein production.

The optimal harvest time after transfection in batch mode of operation has been determined as 72 hours post transfection, as it gives maximum production and the culture still has high viability. In general, it is considered a desirable trend in the process that viability is high at the harvest, since this will certainly favor to have a final product with higher purity to start the corresponding downstream processing. It has also been observed that between 48 and 72 hpt, the number of cells that do not have any interaction with polyplexes increases, what could be read as a sign of plasmid loss during cell division.

On the other hand it was observed a 20% of increase in the percentage of cells expressing Gag-GFP between 24 and 48 hours post transfection. We demonstrated by adding VLP containing medium to an untransfected culture that this increase was not related to entrance of already produced VLPs inside non-transfected cells. This increase in the percentage of transfected cells could be related to that between this two time points, when the cells pass the G2/M phase of the cell cycle, they have the chance to incorporate complexes through mitosis. For this reason further investigation on the

relation of cell cycle phase and transfection efficiency could lead to better and more reproducible transfections.

Three alternatives are proposed in this work on the use of supplements to enhance VLP production. Positive results have been obtained in all case, with relative differences in terms of the level of production improvement and final viability of the culture. The observed differences in final viability among the different protocols could have a significant impact on the corresponding downstream process for the final purification of the VLPs, since a lower viability would imply a higher amount of cellular debris and the release of intracellular components making more difficult and expensive the purification of the final VLP preparation. Additionally, considering that enveloped VLPs are exported out from the cell through a budding process, a decrease in culture viability can lead to a decrease of properly assembled and enveloped VLPs. For this reasons, among the three best combinations obtained, it is considered that the optimum concentrations of enhancers are 20 mM of LiAc, 3.36 mM of VPA and 5.04 mM Caffeine, since they imply a significant improvement of the final VLP production (3.8 fold when compared with the negative control) while maintaining cell culture viability as high as 94%.

Design of experiments (DoE) has been used systematically as a valuable tool for medium supplementation and transfection/production enhancers optimization. This tool enables to obtain statistically relevant information from experimental designs with a minimum number of experiments. It also allows identifying interactive effects of many factors that could affect the response. The traditional one-factor-at-a-time approach for optimization is time-consuming and assumes that the different variables studied do not interact, which could induce to error when defining optima. In the results presented in this work, it has been very useful to first screen the variables with significant and

positive effect using the Plackett-Burman design and optimize the concentration using the Box-Behnken design.

As transient transfection is based on episomal DNA expression it has a limited time span, normally of 96 hours. With the aim to prolong the production period while mainaining high gene expression, a novel process strategy named extended gene expression (EGE) has been developed by the combination of medium exchange and repeated transfection of cell cultures with plasmid DNA to improve overall protein production. Taking as starting point the observation that between 24 and 48 hours post transfection appears a population of cells that has not interaction with DNA/PEI polyplexes, and that after this point no further increase in transfection efficiency is observed and the viability starts to slightly decrease, the first attempt for EGE was medium exchange and retransfections performed every 48 hours post transfection. In order to avoid possible toxicity from PEI/DNA polyplexes, one attempt with half of the DNA at each retransfection and another one retransfectiong only every 96 hours were also tried to find the best approach to achieve sustained high-level gene expression. The benefits of EGE were demosntrated for the production of three recombinant proteins, intracellular GFP, secreted GFP and Gag-GFP VLPs. Best results for VLP production were found with medium exchange every 48 hours and retransfection with half of the standard DNA concentration at 48 and 96 hours post transfection. For the production of intracellular GFP and for secreted GFP, best results were obtained using all DNA at each retransfection. In the case of secreted GFP, the results could be even improved as it can be observed that transfecting every 48 hours with 1ug/mL is not enough to maintain the percentage of transfected cells as the cell growth and viability is very high when this protein is produced. Using this novel EGE strategy, the production period was

prolonged between 192 and 240 h with a 4–12-fold increase in production levels, depending on the product type considered.

Taking into consideration the remarkable results obtained with the extended gene expression approach at laboratory scale, it would be important to study the scale-up to bioreactors as future work within this research. In this respect, the data obtained about the time of contact required for complexes to transfect the cells is a very relevant variable to consider. Using this data it can be determined that perfusion will need to be stopped during one hour after each retransfection to allow its success. Better results in extended gene expression using perfusion in bioreactors could be expected in comparison to the results obtained by discontinuous medium exchange, as in the last case medium had to be exchanged by centrifugation which can damage the cells, and the physiological state of the cells is crucial for good transfection efficiencies. On the other hand the perfusion rate will be a very important variable to optimize as one have to take in account that conditioned medium can interfere with PEI mediated transfection.

It could also be very interesting to analyze the combined benefits of extended gene expression and the use of the optimal levels of production enhancers determined in this work. As the viability of the culture after the addition of the optimal concentration of additives is even higher than the viability of the culture with no additives, this is the ideal combination to be used for extended gene expression, where keeping high viability of the culture as long as possible is a crucial aspect.

In addition to quantity, VLP quality is even as relevant for the final application of vaccine products, especially when intended for human applications. In those terms, one interesting aspect to further study would be the possible RNA content in VLPs. As published by (Rulli et al., 2007), assembly of retrovirus particles normally entails the selective encapsidation of viral genomic RNA. In absence of packageable viral RNA, assembly is still effective, as it takes cellular mRNAs. A second relevant aspect regarding quality is potential contamination with cellular exosomes due to their similarities (Sokolova et al., 2011). This is a key factor in VLP production using HEK 293, as exosomes elute at the same fraction when ultracentrifugation and size exclusion chromatography is performed to purify VLPs. An efficient method to separate exosomes from VLPs should be investigated to have better purified VLPs to increase the percentage of VLPs in respect to total protein in order to have more reliable results in pre-clinical studies.

In this work, a quantification method developed and validated within the research group, based on fluorescence (Gutiérrez-Granados et al., 2013) has been used to substitute the very expensive and time consuming standard method to quantify HIV-1 VLPs by ELISA. The only drawback of this method, that have in common with ELISA determinations, is that it cannot differentiate between proper assembled and enveloped VLPs and free Gag-GFP polyprotein. This highlights the importance of maintaining high percentage of viability throughout production to minimize contamination with free Gag-GFP species. For this reason it would be interesting to deeply study particle quantification, as one of the techniques used to quantify and characterize the VLP concentration, Nanoparticle tracking analysis gives high variability when measuring virus-like particles, and further efforts in using new nanobiotechnology methodologies

would certainly provide new insights in this critical part for proper characterization of VLPs as products

It would be also interesting to increase the basic understanding of the governing factors of transient transfection variability, probably one of its main drawbacks. The predominant cell cycle phase in the culture, appears to be a crucial factor to have a successful transfection. As the cell cycle arrest did not gave good results using Nocodazole, Thymidine could be used as alternative or also cell sorting could be tried to separate the cells in the different phases, and analyze deeply the relationship between cell cycle and transfection efficiency.

Finally, the use of antiapoptotic additives could help to increase cell viability in the culture which could improve even further the results obtained in a single batch, but more importantly the results obtained by extended gene expression strategiy. This could be done engineering the cells by introducing anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL (Majors et al., 2008), Mcl-1 (Majors et al., 2009)) or alternatively downregulating pro-apoptotic members of the same family (such as Bax, Bak) (Macaraeg et al., 2013). Both approaches are focused on delaying mitocondrial permeabilizaiton and activation of caspase dependant apoptosis. Co-transfecting the cells with p18 and p21 (Backliwal et al., 2008a)., also has given good results improving culture viability, length and production.

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Conclusions

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

1. The production of HIV-1 Gag-GFP VLPs has been reported, for the first time, using an industrially relevant suspension adapted mammalian cell line, such as the GMP-compliant HEK 293 used in this work.

2. Culture medium was optimized using non-animal derived media supplemented with recombinant compounds, which ensures safety and easies rapid transfer to potential clinical studies. The optimal conditions obtained for cell growth were supplementation with 0.9*X* Lipid mixture, 19.8 mg/L r-insulin and 1.6 mg/L r-transferrin leading to a maximum cell concentration of 5.4×10^6 cells/mL in batch mode, almost double of that observed using the unsupplemented medium (2.9×10^6 cells/mL).

3. A protocol for transient transfection was defined. Cells were transfected at mid-log phase ($2–3 \times 10^6$ cells/mL) with medium exchange at the time of transfection using 1 μg/(mL of culture) of plasmid DNA and 2 μg/(mL of culture) of polyethylenimine. Leading to a production of 2.7×10^9 VLPs/mL.

3. The study performed using several techniques to characterize the process of internalization of the DNA-PEI complexes into the cells and its further processing to produce VLPs through the membrane budding process, has enable to identify that the time required for the complexes to penetrate into the cells is 90 minutes post addition, and after 10 hours post transfection GFP fluorescence is detected inside the cells. Generalized budding of VLPs from the cells is not observed until 48 hours post

transfection and the optimal harvest time is determined as 72 hpt as VLP production is highest while high viability of the culture is maintained.

4. Further improvement has been achieved by the use of transfection and production enhancers as additional supplements to the culture medium at transfection. Using the optimal concentrations of the tested additives (20 mM Lithium Acetate, 3.36 mM Valproic Acid and 5.04 mM Caffeine) the production could be enhanced 3.8 fold with a viability of 94%

5. A novel process approach named as extended gene expression has been developed and proven for the production of three recombinant proteins of increasing difficulty, intracellular GFP, secreted GFP and Gag-GFP VLPs. Using this novel EGE strategy, the production period was prolonged between 192 and 240 h with a 4–12-fold increase in production levels, depending on the product considered. This approach consists in performing the standard transient transfection process as developed initially for a first step, and after perform a complete medium exchange every 48 hours followed by re-transfection of cell cultures using a concentration of 0.5 µg of plasmid DNA/mL of cell culture at 48 hpt and 96 hpt.

6. Several further work lines of interest to pursue the work presented have been identified, and will serve as a basis of future work within the research group. This approaches are: characterization of the VLP quality, combination of EGE methodology and production enhancers addition, and use of antiapoptotic genes to improve culture viability.