Universitat Autònoma de Barcelona

HIV VIRUS-LIKE PARTICLE PRODUCTION IN CAP CELLS

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Memòria presentada per Sonia Gutiérrez Granados per optar al grau de Doctora per la Universitat Autònoma de Barcelona

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Resum

Les *virus-like particles* (VLPs) derivades de l'HIV presenten un gran potencial com a vacunes de nova generació. La proteïna Gag s'ensambla de forma espontània a la membrana cel·lular, donant lloc a VLPs amb embolcall. Per a generar VLPs en suficient quantitat i amb la qualitat requerida per a estudis clínics, es necessiten processos de producció robustos. A més, la disponibilitat de mètodes analítics senzills i confiables és crítica per al desenvolupament, optimització i monitorització d'aquests processos. Les cèl·lules de mamífer són la plataforma preferida per a produir VLPs, ja que proporcionen l'ambient necessari per a la seva correcta formació i ensamblatge. En aquest treball, es va desenvolupar un procés de producció per a VLPs de Gag-GFP mitjançant la línia cel·lular CAP, que destaca per la seva novetat.

El primer pas va ser el desenvolupament d'una tècnica de quantificació basada en fluorescència, per a poder-la utilitzar de forma rutinària en el desenvolupament del bioprocés. A més, el mètode es va validar d'acord a l'ICH. El mètode era específic per a Gag-GFP i les mesures de fluorescència correlacionaven amb les mesures d'ELISA de referència. El mètode era adequat ja que mostrava poca variabilitat, un rang de treball de 3 ordres de magnitud i un límit de detecció suficientment petit com per a poder quantificar mostres sense purificar.

La producció de VLPs es va dur a terme mitjançant transfecció transitòria amb PEI a línia cel·lular CAP-T. El procés a petita escalar es va optimitzar de forma sistemàtica. Primer, algunes variables es van estudiar per separat per a establir les condicions inicials. La falta d'un medi de cultiu compatible amb el creixement cel·lular i la transfecció forçava la realització d'un recanvi de medi abans de la transfecció. La densitat cel·lular en el moment de la transfecció, i les concentracions de DNA i de PEI es van optimitzar mitjançant una metodologia de Disseny d'Experiments. El títol aconseguit va ser de 6×10^{10} VLP/mL, amb les característiques desitjades.

A continuació, es va dur a terme l'escalat del procés de transfecció a bioreactor d'1L, centrat en dos aspectes principals: el creixement cel·lular amb un estat fisiològic òptim i evitar el recanvi de medi per centrifugació abans de la transfecció, ja que no és desitjable a escales grans. Les cèl·lules CAP-T es van cultivar en bioreactor controlant el pH amb bicarbonat de sodi i amb aeració superficial. El recanvi de medi es va evitar cultivant les cèl·lules en una combinació de medis (FreeStyleF17 + 1% de PEM) compatible amb el creixement cel·lular i la transfecció transitòria. Aquesta innovadora estratègia va permetre simplificar significativament la transfecció transitòria a gran escala, mentre que

es van aconseguir un bon creixement cel·lular, bona eficiència de transfecció i una producció de VLPs d'alta qualitat comparable al sistema a petita escala.

El següent pas va ser la generació d'una línia cel·lular per a produir de forma estable les VLPs. La metodologia que es va utilitzar va ser la *limiting dilution*. Es van analitzar ~80 clons per a seleccionar el més productiu. A més, es va millorar la productivitat del clon seleccionat mitjançant *sorting* de les cèl·lules amb major intensitat de GFP. El cultiu d'aquest clon en bioreactor en *fed-batch* va donar lloc a un títol de 2×10^9 VLP/mL.

Finalment, es va utilitzar l'espectroscòpia dielèctrica per a monitoritzar *in-line* els dos processos de producció de VLPs, basats en expressió estable i transient. La mesura de la permitivitat va permetre monitoritzar la densitat cel·lular en temps real. A més, els paràmetres de la corba de dispersió β mostraven perfils específics que es relacionaven amb events crítics durant el bioprocés, com ara canvis metabòlics o mort cel·lular.

Summary

HIV-1 virus-like particles (VLPs) have great potential as new generation vaccines. Upon expression, the Gag polyprotein of HIV-1 assembles spontaneously in the vicinity of the plasma membrane giving rise to enveloped VLPs. Robust production processes are required to generate VLPs of sufficient quality and quantity for pre-clinical and clinical evaluation. In addition, the availability of simple, fast and reliable analytical tools is critical to develop, optimize and monitor such processes. Mammalian cells are the preferred platform to produce VLPs, since they provide the required environment for their correct formation and assembly. In this work, a production process for Gag-GFP VLPs was developed using the novel CAP cell line.

The first step was the development of a simple and cost-effective fluorescence-based quantitation technique that could be used routinely for bioprocess development. In addition, the method was validated according to ICH guidelines. The method showed to be specific for Gag-GFP and the fluorescence signal correlated well with the measurements of a reference ELISA assay. Little variability compared to ELISA, linearity over a 3-log range and a sufficiently low limit of detection to quantify crude samples demonstrated the suitability of the technique.

The CAP-T cell line was used to produce Gag-GFP VLPs by PEI-mediated transient transfection. A systematic optimization of the process at small scale was performed. First, some variables were studied separately to set the conditions. The lack of a culture medium that was compatible with suitable cell growth and PEI-mediated transient transfection, forced the performance of a medium replacement step prior to transfection. Then, the cell density at the time of transfection, and the DNA and PEI concentrations were optimized by means of a Design of Experiments approach. The final titer obtained was 6×10^{10} VLP/mL, with the desired quality attributes.

The scale-up of the transient transfection protocol to 1L bioreactor followed, focusing in two main aspects: cell culture with optimal cell physiological conditions and avoiding the medium replacement step by centrifugation prior to transfection, which is not desirable at large-scales. CAP-T cells were cultured in bioreactor by addition of carbonate to control pH and surface aeration. Remarkably, the medium replacement step was avoided by culturing the cells in a combination of media (FreeStyleF17 + 1% of PEM) compatible with both cell growth and PEI-mediated transient transfection. This novel strategy significantly simplified large-scale transient transfection, while suitable cell

growth, transfection efficiency and high quality VLP production comparable to small scale were achieved.

The generation of a stable CAP cell line producing correctly assembled Gag-GFP VLPs was the next step. The methodology employed was the classic limiting dilution, which has the advantages of being straightforward and easily implementable at any cell culture lab. A screening of ~80 clones was performed to select the most productive one. Moreover, the specific productivity of the selected clone was further improved by sorting the most intense GFP expressing cells. The culture of this clone at bioreactor level in fed-batch mode was successful, obtaining a VLP titer of around 2×10^9 VLP/mL.

Finally, dielectric spectroscopy was employed for the in-line monitoring of the two VLP production processes set in this work, based on transient and stable expression. The measurement of the permittivity during the culture allowed to monitor the cell density in real time. Moreover, specific profiles of the β-dispersion parameters were identified, and related to critical events in the bioprocess, such as metabolic shifts or cell death.

Introduction

HIV virus-like particles

Introduction, part 1

1. Virus-like particles as promising vaccine candidates

1.1. History of vaccines

Vaccination is one of the major successes of human medicine. Over the last 300 years vaccines have had a great impact on human health and longevity. The concept of vaccination was first introduced by Edward Jenner in the 18th century. He inoculated cowpox-derived pus to healthy individuals and challenged them with smallpox, demonstrating the protection provided by the cowpox inoculated material. Later, Pasteur introduced some novel vaccines by attenuating viruses and bacteria with oxygen treatment or heat. But the real breakthrough in attenuation of pathogens arrived in the middle of the 20th century with cell culture. Viruses submitted to a high number of passages would mutate to be adapted to *in vitro* culture and this would make them incompetent to replicate in a human host, while they were still generating protective immune responses [1]. Inactivated vaccines were first developed not long after pathogen attenuation in the 19th century. At first whole organisms were inactivated and as technology was advancing, purified subunits from the pathogens (such as toxoids and polysaccharides) were used as vaccines [2]. Later in the 1980's, recombinant DNA technology enabled the development of DNA-based and recombinant vaccines. Such was the success of vaccines that specific laws were created to vaccinate the population since the beginning of the 19th century in the United States. The impact of vaccines is reflected in the eradication of smallpox (1977) and in the number of diseases that have been controlled with the use of vaccines such as polio, measles, mumps, rubella, influenza, hepatitis A, diphtheria or tetanus [2]. The different types of vaccines are represented in Figure 1.

1.2. Types of licensed viral vaccines

The most classical vaccines are live attenuated and inactivated viruses, and nowadays represent the majority of the licensed viral vaccines. Only three of the recommended vaccines are of recombinant nature, and still no DNA-based vaccine has been licensed for human use (Table 1).

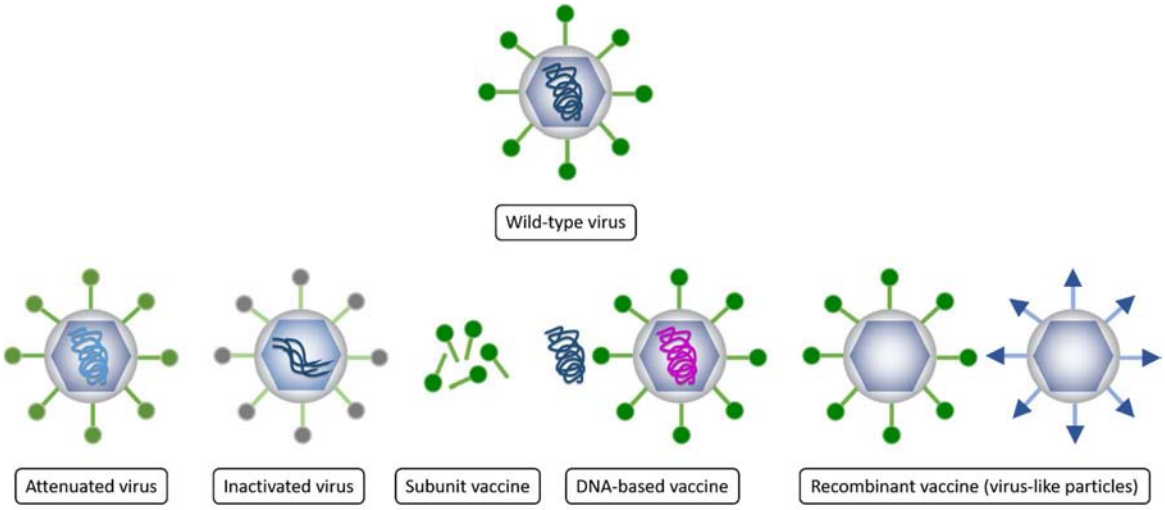

Figure 1. Schematic representation of the different types of vaccines.

Table 1
Viral vaccines recommended by WHO

Etiological agent	**Type of vaccine available**
Hepatitis B virus	Recombinant
Polio virus	Inactivated and live-attenuated
Rotavirus	Live-attenuated
Measles virus	Live-attenuated
Rubella virus	Live-attenuated
Human papilloma virus	Recombinant
Japanese encephalitis virus	Inactivated and live-attenuated
Yellow fever virus	Live-attenuated
Tick-borne encephalitis virus	Inactivated
Hepatitis A virus	Inactivated
Rabies virus	Inactivated
Mumps virus	Live-attenuated
Influenza virus	Recombinant, live-attenuated and inactivated

Adapted from [3]

Live attenuated vaccines are microorganisms able to generate a protective immune response against a certain pathogen, but their ability to infect a human host and cause the disease has been hampered. Attenuation can be achieved by several strategies, including the use of related animal viruses, chemical attenuation, passage in broth or cell culture or the use of RNA reassortants. Efficient humoral and cellular immune responses can be achieved with relatively low doses of the vaccine. Moreover, if the attenuated strains can be properly cultured *in vitro*, high viral yields can be obtained, which reduces

production costs. On the other hand, it may be not always possible to find a sufficiently attenuated strain for human administration. Moreover, the use of live viruses involve an underlying risk of reversion from attenuated to virulent strains [4].

Inactivated vaccines are based on the administration of killed pathogenic microorganisms. They are able to present the antigens, but their ability to replicate and generate the disease is suppressed by the inactivation process. The production process can be straightforward if the virus efficiently replicates *in vitro.* Inactivation is usually performed chemically with formalin or β-propiolactone treatments. Since the chemical inactivation can cause some structural modifications, immune responses are not so efficiently elicited, and higher doses of vaccine and adjuvants are often required to increase their potency. Moreover, cellular immune responses are not so always efficiently elicited [4,5].

DNA-based vaccines have not been licensed yet for human use, although two products have been licensed for animals. They consist in plasmid DNA molecules coding for the immunogens of interest. The administration of DNA into the individuals leads to the expression of the antigens, which can elicit effective cellular and humoral immune responses. DNA can be delivered “naked” or within a vector (viral or non-viral). The technologies for DNA production and purification are well-established, so production would not be a bottleneck in case of approval [4].

Recombinant vaccines are based on the heterologous expression of proteins in host cell platforms (prokaryotic or eukaryotic) that can be used as immunogens. Recombinant vaccines are a safer option, since only the immunogenic parts are present, avoiding the whole pathogen and hence the possibility of developing the disease. Production and purification processes of recombinant vaccines are more demanding than production of viruses by infection, although biosafety is not a concern at manufacturing level [3,4].

Although classical vaccine approaches are still the most widely administered, the trend in new vaccine development is moving towards recombinant vaccines. The fact that the whole pathogen is absent poses a great advantage both at manufacturing and administration levels. Moreover, there are some viruses that scientists have not been able to culture yet, which is a limitation to generate attenuated or inactivated vaccines to prevent the diseases they cause. However, if the immunogenic antigens are known, they can be expressed in a host and the vaccine can be obtained without the need to culture the whole virus.

1.3. Virus-like particles

Virus-like particles (VLPs) are one type of recombinant vaccines. They are multiprotein biological entities that mimick the structure of their corresponding wild-type virus. Upon heterologous expression in a host system, the proteins have the ability to self-assemble and form VLPs, which can be then purified from the cells or the cell culture supernatants. VLPs do not contain the virus genetic material, hence they are non-infectious and no safety concerns are associated at the administration level. The recombinant nature of VLPs exempts them of biological hazard at manufacturing level as well. The fact that they present the antigens in a particulate form and with repetitive surface patterns makes them efficient immunogens. While other recombinant vaccines based on viral subunits fail to provide both humoral and cellular immune responses, VLPs are able to elicit both, which makes them suitable as prophylactic and therapeutic vaccines. In general, large-scale production of VLPs can be achieved by different host systems, although the more complex types of VLPs may present some challenges at manufacturing level [6,7].

VLPs are structurally diverse. They are classified in two categories, non-enveloped or naked and enveloped VLPs. Non-enveloped VLPs are formed by the assembly of one or more structural proteins. If they are formed by a single protein, they can be produced in prokaryotic or eukaryotic platforms, purified and later assembled in a cell-free environment. However, the more complex entities formed by two or more proteins, usually need to be assembled upon co-expression within the cell environment. For this reason, they need to be produced in higher eukaryotic systems such as yeast, insect cells or plants. Papilloma virus (HPV) VLPs are non-enveloped and formed by a single protein. Rotavirus (RV) and Bluetongue virus (BTV) VLPs are examples of naked VLPs formed by more than one protein. Enveloped VLPs harbor a lipid envelope outside the core protein/s. Assembly takes place inside the cell and VLPs are released by budding taking a piece of the host cell membrane. They display glycoprotein spikes on the lipid envelope surface from the host cells or of recombinant nature if they are co-expressed with the main core protein/s. They are more complex than naked VLPs, for this reason eukaryotic cell platforms are required for their production. Animal cells are preferred since the cell membrane is required for the correct VLP formation. Influenza virus, human immunodeficiency virus (HIV), Ebola virus (EBV), and Chikungunya virus (CHIKV) are examples of enveloped VLPs [8].

The first VLPs were discovered in the blood of Hepatitis B (HBV) infected individuals. They were subviral particles formed basically by one of the envelope proteins (small antigen HBsAg). Their immunogenic properties were acknowledged in the early 1980's by the fact that individuals who recovered from Hepatitis B, presented antibodies against these subviral particles. The first VLP-based vaccine was based on HBsAg particles purified from human blood, but some safety concerns arose from the use of blood-derived products. To overcome this limitation, the Hepatitis B antigens were produced recombinantly (in yeast and CHO), leading to the first approved VLP-based vaccine in 1986. This paved the way for the next VLP vaccines in the market. HPV VLPs were approved as vaccines in 2006. There are also two different versions available, Gardasil from Merck (produced in yeast) and Cervarix from GSK (produced in insect cells). In 2011 Hecolin from Xiamen Innovax Biotech Co. was approved as a vaccine to prevent Hepatitis E virus infection. This vaccine is produced in bacteria and assembled *in vitro* [8,9]. The latest VLP approved for human use is FluBlok from Protein Sciences to prevent Influenza infcetion, which is produced by baculovirus-infected insect cells.

The number of advantages provided by VLPs as vaccines is reflected in the great number of VLPs being tested in clinical trials [9]. One of the most promising examples of new VLPs is the so-called RTS,S malaria vaccine. This chimeric VLP is formed by the fusion of the small antigen from HBV and *Plasmodium falciparum* circumsporozoite protein antigen. The vaccine reached Phase III clinical trials with 30-50% of protection, which is a significant advance compared to other malaria vaccine approaches [7,10]. With the promising results and the positive opinion from EMA, it is pending for market approval.

1.4. Immunogenicity of VLPs

The complexity of the human immune system is huge. There are many pathways and mechanisms that vaccines undergo to generate immune protection against pathogens. A brief and simplified description of the main events taking place upon the administration of a VLP-based vaccine follows.

The most important feature of VLPs as immunogens is their capacity to elicit both humoral and cellular immune responses, similarly to whole viruses, but without the possibility of causing the disease. VLPs are taken up by professional antigen presenting cells (APC), mainly dendritic cells (DC), which process them and present the epitopes both

in the Major Histocompatibility Complex (MHC)-I and MHC-II. MHC-I usually present intracellular epitopes and these are recognized by CD8+ T-lymphocytes, that are responsible of generating cellular immune response (clearance of infected cells). MHC-II presented epitopes are usually extracellular molecules that are internalized via Fc receptors by APCs, and are recognized by naïve CD4+ T-lymphocytes. The latter are also known as T-helper cells, and activate B-lymphocytes, which secrete antibodies that will help neutralizing the circulating virus (humoral immune response) [11–13].

The immunogenic properties of VLPs rely mainly in their size and morphology. VLPs present the antigens in a particulate form and with repetitive patterns, mimicking the wild-type viruses. For these reasons, they are able to be internalized in APCs and be presented both in MHC-I and -II (cross-presentation) [11,14]. Moreover, unlike whole viruses, they do not contain proteins with immune system evasion functions that can preclude the activation of the immune response [15]. In contrast, subunit vaccines are mainly presented by MHC-II, so they elicit more efficiently humoral responses rather than cellular responses. For this reason, subunit vaccines need to be accompanied by adjuvants that help elicit the cellular response [14].

Innate immunity is a relevant part of the protective response. It has been described that VLPs elicit innate responses due to the presence of “danger signals” that come from the host system in which they have been produced (yeast, insect cells, etc.). These danger signals are also known as pathogen-associated molecular patterns (PAMPs) and can be nucleic acids, unmethylated CpG sequences, lipopolysaccharides, etc. PAMPs are recognized by Toll-like receptors and other pattern recognition receptors (PRRs) inducing the maturation of DCs and macrophages. Mature APCs travel to the lymph nodes where can interact with T-cells and produce cytokines (IL-6, IL-12, TNF-α, INF-γ, etc.) that are necessary to completely activate T-cells [6,11].

1.5. Other potential applications of VLPs

VLPs have other applications in the biomedicine field. The versatility offered by their structures make them useful not only as immunogens *per se*, but also as platforms to carry antigens against other diseases and as drug delivery vehicles.

Chimeric VLPs can be constructed either by molecular fusion or chemical conjugation of molecules. Enveloped VLPs are especially suited to present surface antigens of various viruses that can be incorporated in the VLP surfaces upon expression in a host cell. The malaria vaccine candidate RTS,S is an example of the recombinant fusion of the small antigen from HBV and *Plasmodium falciparum* circumsporozoite protein antigen. VLPs have been also engineered to generate antibodies against disease-associated self-molecules, such as allergies, Alzheimer's, diabetes type II, hypertension or cancer. For example, the glycoprotein Trop2 was fused to the Gag protein so a chimeric SIV VLP was formed. Since this glycoprotein is extensively expressed in tumor cells, immunization with chimeric Trop2 VLPs can break tolerance to this self-protein and generate a specific cellular and humoral immune response significantly reducing tumor growth [16].

VLPs are able to encapsulate or attach proteins, nucleic acids and small molecules. The desirable characteristics of a biological vector are met by VLPs: biocompatibility, solubility, uptake efficiency, target delivery and high drug loading capacity [6]. Anticancer drugs such as adriamycin and aleomycin are bound to VLP surfaces with hydrazone bonds created by amino acid residues [17]. In addition, anticancer drug molecules can also be encapsulated into VLPs by the reversible process of self-assembly with changes of the external conditions, such as specific ionic strength [18]. DNA loaded VLPs are also being developed for gene therapy applications [8].

Finally, VLPs can be used in some cases as research surrogates. Some viruses are catalogued as biosafety level 4, which complicates the experimentation due to biosafety concerns. Since VLPs present the same structure, ligands, bioavailability, etc. they can be used for some studies, evading the biosafety issues of their wild-type viruses [6].

2. Human immunodeficiency virus (HIV)

2.1. HIV structure and infective cycle

Human immunodeficiency virus (HIV) is a lentivirus member of the *Retroviridae* family targeting human lymphocytes T CD4+. HIV is the etiological agent causing acquired immune deficiency syndrome (AIDS) which consists in the increased susceptibility to develop opportunistic infections and viral-induced cancers. HIV was first described in the

1980s, however, phylogenetic analyses have dated the first HIV virus around 1910 in west central Africa, originated from simian immunodeficiency virus (SIV) [19].

HIV contains two copies of a single stranded RNA (ssRNA) genome. The viral genome contains 9 open reading frames (Figure 2) that encode for 15 proteins whose functions are summarized in Table 2. There are 3 major genes (Gag, Pol and Env), 2 regulatory proteins (tat and rev) and 4 accessory genes (nef, vif, vpu and vpr). 2 long terminal repeats (LTRs) are flanking the genes 5' and 3'. The 5' LTR contains the promoter and enhancer for gene transcription [13]. Gag-Pol is transcribed as one mRNA at a ratio of 20 Gag to 1 Gag-Pol molecules to ensure that the viral components are produced at the correct stoichiometry [20].

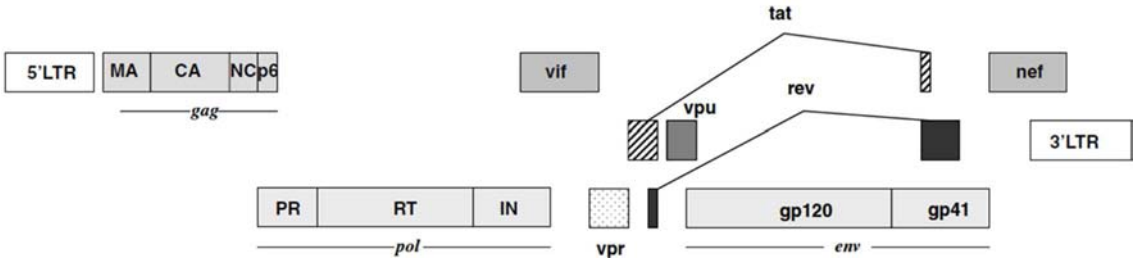

Figure 2. Schematic representation of HIV-1 proviral genome (from [13]).

A complete viral particle measures 80-120 nm and contains all the necessary elements for viral replication (Figure 3). Approximately, 50% of the particle is formed by Gag (the main structural protein), ~30% is the lipid envelope (taken from the host cell membrane) and the other viral proteins are the ~20%. The viral RNA only accounts for the 2.5% of the particle. In addition to the proviral RNA, the viruses also contain $tRNA^{Lys,3}$ to prime cDNA synthesis inside the host cell [20]. The three domains of the Gag polypotein form the capsid of the virion. Specifically, the matrix subdomain is bound to the inner face of the lipid envelope that surrounds the particle. The capsid domain forms a conical and more condensed inner capsid (core), that contains the viral RNA bound to the nucleocapsid domain and the viral enzymes. The envelope proteins are placed in the lipid bilayer, displayed to the viral surface [20].

The viral infectious cycle starts with binding of the envelope proteins to target cell receptors (CCR5 and CXCR4 from lymphocytes T $CD4^+$). The viral membrane fuses with the cell membrane and the core of the capsid is released to the cell cytoplasm. Then, the viral RNA genome is retro-transcribed to DNA by the viral retrotranscriptase. This dsDNA forms

the pre-integration complex (PIC) with the viral integrase, capsid and some cell proteins, to enter the host cell nucleus by an active transport pathway. The HIV genome integrates in the host cell genome. Integration takes place at preferential sites, for example in areas where there is a high gene density or high transcription activity. However, other factors can influence the integration site such as the nuclear entry route, the cell cycle phase or the chromatin structure. The integrated viral genome can undergo transcriptional silencing, leading to the generation of a latent viral reservoir [21].

Table 2
Summary of HIV proteins

Protein	Function
Gag	Structural protein Cleaved into capsid (CA or p24), matrix (MA or p17), nucleocapsid (NC or p7) and p6 Viral core and encapsidation of RNA
Pol	Enzymatic protein Cleaved into protease (PR), reverse transcriptase (RT) and integrase (IN) PR cleaves Gag-Pol and Gag into seven mature proteins RT converts single stranded genomic RNA into double stranded proviral DNA IN directs proviral integration into host chromosome
Env	Structural protein Cleaved into transmembrane (TM or gp41) and surface (SU or gp120) domains Virus binding and entry into susceptible cells
Tat	Regulatory protein Promoting and enhancing viral transcription
Rev	Regulatory protein Nuclear export of unspliced viral RNAs
Nef	Accessory protein Down-regulation of CD4 and MHC-I; increases infectivity
Vpu	Accessory protein Down-regulation of CD4; enhances virus release
Vpr	Accessory protein Nuclear localization of the pre-integration comples; cell cycle arrest (G2)
Vif	Accessory protein Enhances infectivity

Adapted from [13]

The viral genome is transcribed and the viral proteins translated. Upon expression, Gag travels to the cell membrane where it polymerizes. It has been described that a myristoylation in MA domain is required for Gag anchoring in the inner layer of the membrane and subsequent assembly. In addition, Gag interacts with RNA molecules. Env proteins reach the membrane independently of Gag, and are enriched in the raft-like

areas, directed by the TM tail of Env. Consequently, VLP membrane is enriched with sphingomyelin and cholesterol, the lipids present on these raft-like areas. Vpr, Vif and Nef are also incorporated into the virions, as well as, the genomic RNA, $tRNA^{Lys,3}$, and ribosomal RNAs [20].

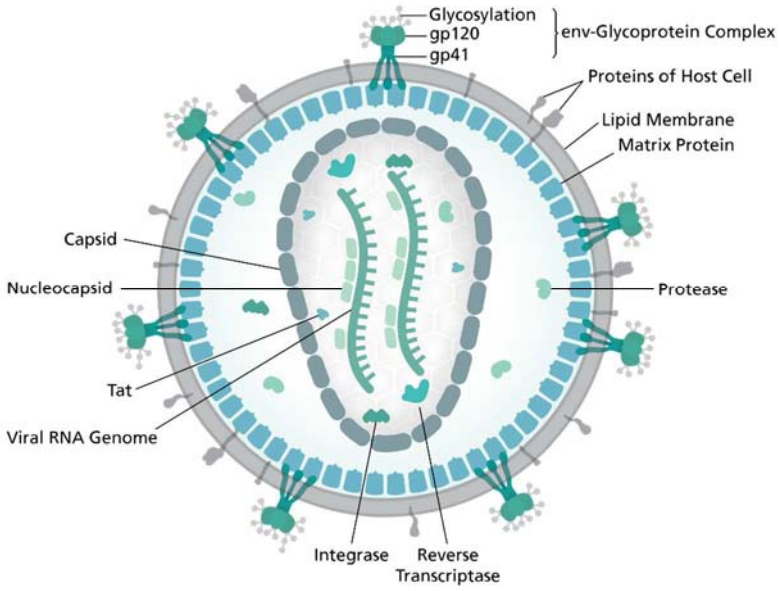

Figure 3. Schematic representation of an HIV mature virion (from www.scistyle.com).

Gag is accumulated in the membrane until a plateau is reached and viral release by budding takes place. p6 recruits the endosomal sorting complexes required for transport (ESCRT) machinery to terminate Gag polymerization and catalyze particle release. After (or during) budding, the virions undergo a maturation process. The viral PR is inactive in the immature virion, but it is believed that two Gag-Pol molecules can interact, become catalytic and trigger particle maturation. PR cleaves Gag and Gag-Pol translation product into their domains (MA, CA, NC, p6, PR, RT and IN). This leads to very significant conformational changes that include the rearrangement of CA into the core capsid, giving rise to mature virions [20].

2.2. Relevance of an HIV vaccine

HIV infection was first clinically diagnosed in 1981 in the United States, and the virus was identified as the cause of AIDS in 1983. Ever since, 70 million people have been infected with the HIV virus and 35 million died due to HIV

(http://www.who.int/gho/hiv/en/). The efforts in prevention and the successes in treatment of the last 30 years have led to a reduction of HIV incidence and mortality [22], even in the absence of an effective vaccine. However, the HIV challenge persists: 2 million people were newly infected in 2015, accounting for 36.7 million people living globally with HIV in 2015, of which only 17 million are currently receiving antiretroviral treatment (ART) [23].

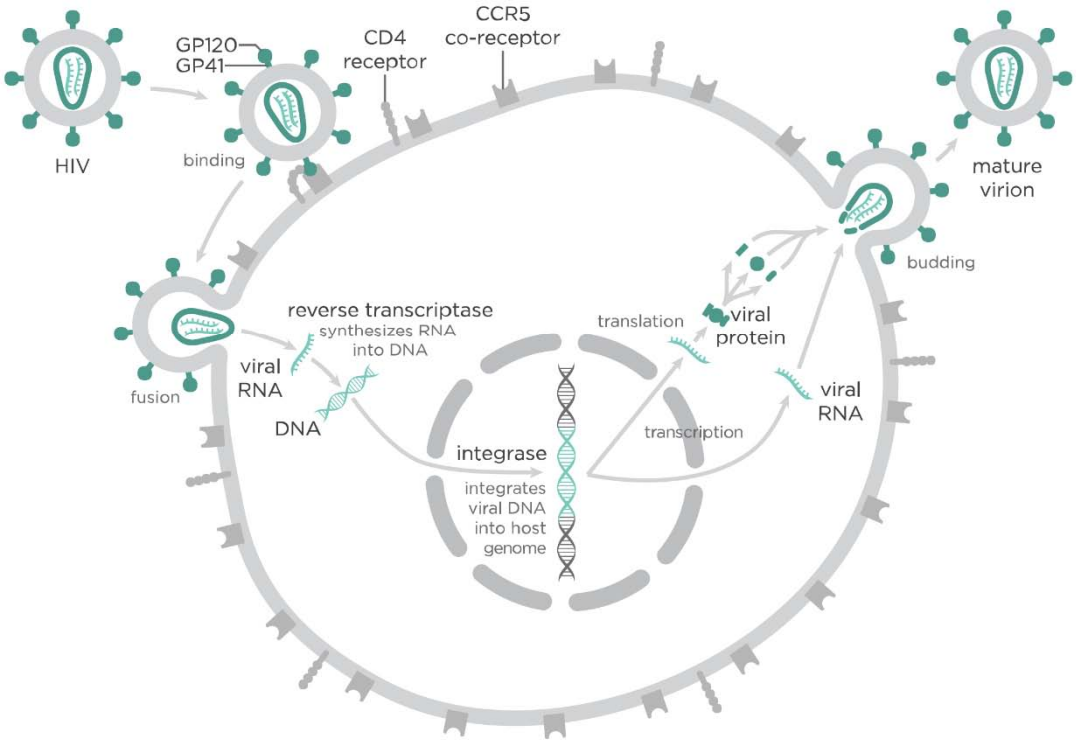

Figure 4. Schematic representation of an HIV infection cycle (from www.scistyle.com).

More than 30 ART drugs are licensed. Provided that early ART upon diagnosis is guaranteed, HIV infected individuals can have a life expectancy almost as long as a healthy person (~70 years). In addition, ART are being used as prophylactics in high risk groups (to prevent mother-to-child transmission, in non-infected sexual partners of infected individuals, etc.) and other prevention campaigns are being successfully applied globally [22]. However, prevention and treatment of HIV have some limitations that become especially important in developing countries. First, economic limitations preclude the availability of treatments or application of prevention campaigns. Second, sometimes human resources are insufficient. Third, some people are reluctant to accept some

prevention methods, or do not seek for health care due to social and cultural barriers [22,24]. For these reasons, a safe and effective vaccine able to prevent HIV infection upon a single administration is essential for the durable control of the global HIV pandemic.

Ever since the discovery of the virus, more than 200 clinical trials have been conducted with over 30 vaccine candidates. However, limited success has been found up to date. Inactivated viruses showed poor immunogenicity, while attenuated vaccines were too risky, since they could generate the disease [25]. The first candidates were mainly focused on recombinant subunit envelope proteins, eliciting humoral responses. After realizing that neutralizing antibodies were not enough to achieve a robust protection, the candidates were based on immunogens that elicited cellular responses. The first candidate to show some efficacy was RV144 (2003-2009). The approach was based in the combination of Gag-Pol and Env proteins, delivered by a canarypox vector. Although the efficacy was limited (30% of protection at month 42), the results shed light on the importance of eliciting broadly neutralizing antibodies and, at the same time, having structural-based vaccine design for developing an effective prophylactic and therapeutic HIV-1 vaccine [24,25].

2.3. HIV-based VLPs

HIV VLPs are mainly formed by the structural Gag protein, that is able to self-assemble in the absence of any other viral protein or gene. Upon expression in a host cell, Gag-VLPs are formed and released from the cells by a budding process, like the wild-type virus. The main difference with the native virus is that maturation does not take place, since the viral protease is not present to cleave Gag. Therefore, Gag-VLPs resemble immature HIV virions. If Env is co-expressed with Gag, the VLPs will present the glycoproteins in their surface, along with other host cell membrane proteins (Figure 5a) [26].

VLPs meet the characteristics required as a vaccine for HIV. They have the capacity to present native Env spikes on their surface that elicit broadly neutralizing antibodies. In addition, the fact that they are particulated facilitates VLP internalization by DCs and presentation in MHC-I and -II to elicit cellular responses [25,27]. For these reasons, different VLPs have been constructed and tested pre-clinically with success. For example, Gag-RT fusion particles [28,29], Env epitopes fused to the Gag sequence [30,31] or gp120 expressed on the surface of Gag VLPs [32–34]. Furthermore, the Gag core forms very

stable structures that can serve as scaffold to present surface antigens from a variety of sources. Gag VLPs presenting influenza surface antigens [35] and equine herpes virus glycoprotein [36] are examples of chimeric VLPs used for immunization purposes. Cancer epitopes have also been presented in the surface of Gag VLPs to elicit immune responses against tumors [16].

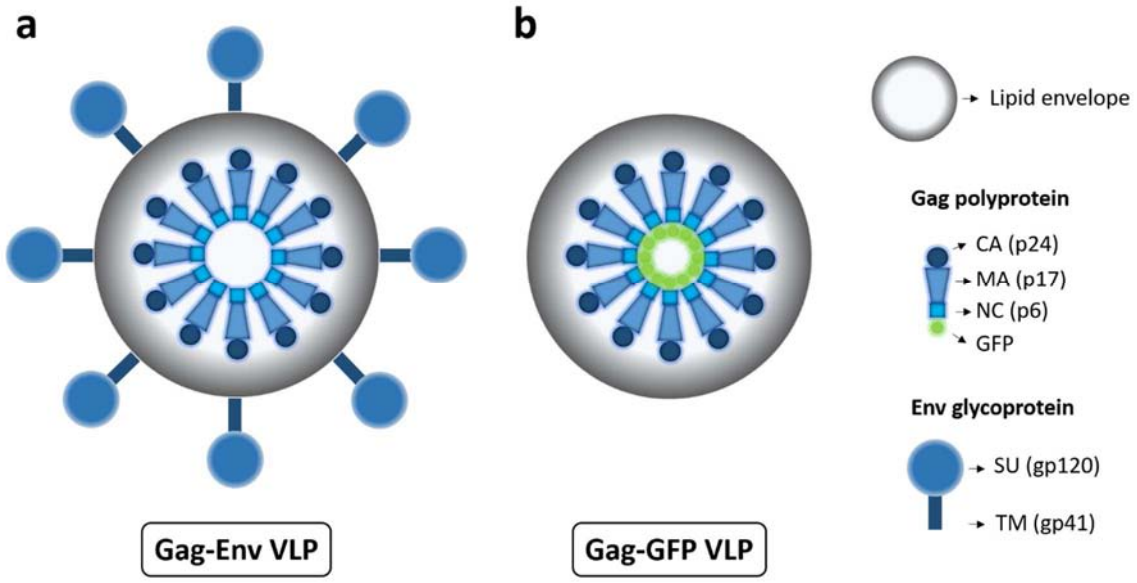

Figure 4. Schematic representation of Gag-based HIV virus-like particles.

The model candidate used in this work is a VLP formed by the HIV-1 Gag polyprotein fused in frame with a GFP protein at C-terminus. The expression of this protein in a host cell, specifically in the human-derived CAP cell line, give rise to fluorescent Gag-GFP VLPs (Figure 5b).

3. VLP quantitation and characterization methodologies

VLP quantitation and characterization are essential for vaccine bioprocess development and clinical studies. Poorly characterized vaccines may lead to misinterpretation of immunological results which can impact vaccine design. In the same manner, wrong vaccine titers can impact bioprocess development and can also lead to errors in the doses administered. Characterization and quantitation of the final product is

as relevant as doing it in every step of the bioprocess. The quality and titer in every step should determine the design of the upstream and downstream processes. Furthermore, the quality of the vaccines should be also determined by the evaluation of critical process parameters (CPP) that influence critical quality attributes (CQA), to meet the requirements of Quality by Design (QbD), demanded by the regulatory agencies. Process Analytical Technologies (PAT) are used to this end.

There are many tools that can be used for vaccine characterization and quantification. VLPs are generally complex entities, and the combination of several methodologies is required to have a more complete characterization and a more reliable titer.

3.1. Characterization methods applicable to VLPs

VLP quality assessment is of major importance since VLP size, polydispersity, purity and composition contribute to their potency and safety. VLP characterization techniques can be classified into biochemical, biophysical and biological (Table 3) [8].

The more classical biochemical techniques are useful to identify the protein/s of interest or analyze the VLP composition. However, it is common to have monomeric proteins that are not forming VLPs [37], and these techniques often fail to differentiate assembled and non-assembled proteins. Electron microscopy techniques can be complementary used to visualize the morphology of the VLPs, although it is laborious and some technical expertise is required to correctly interpret the images.

In the last years, novel techniques able to identify nanoparticles have been developed. These methods can often count the particles and analyze the size distribution of a nanoparticle population in a few minutes. Some examples are Nanoparticle Tracking Analysis, Tunable Resistive Pulse Sensor or Dynamic Light Scattering (Table 3) [38]. These methods can substitute electron microscopy for the identification of correctly assembled particles. However, the presence of cellular vesicles and exosomes, with similar properties to VLPs, is quite common in cell-based virus preparations. This can lead to misinterpretation of the results when using techniques based on particle identification. Therefore, the combination of specific protein identification methods along with particle-based techniques will ensure more reliable VLP characterization [39].

Table 3
Characterization tools for VLPs

Type of analytical method	Analytical method	VLP features analyzed
Biochemical	Total protein	Identity (antigen)
	MS	Composition
	SDS-PAGE + WB	Impurities (HCP, HCDNA)
	Agarose gel	
	Protein sequencing	
Biophysical	TEM	Identity (particles)
	Cryo-EM	Size
	DG-UC	Morphology
	NTA	Polydispersity
	TRPS	Aggregation
	DLS	
	ES-DMA	
	AF4-MALS	
	HPLC-MALS	
Biological	ELISA	Identity (antigen)
	Immunoblot	Functionality
	Hemagglutination	Potency
	In vivo tests	

Adapted from [8,37]. MS: mass spectrometry. SDS-PAGE: Sodium docecyl sulfate polyacrylamide gel electrophoresis. WB: western-blot. TEM: transmission electron microscopy. Cryo-EM: cryo-electron microscopy. DG-UC: density gradient ultracentrifugation. NTA: Nanoparticle tracking analysis. TRPS: Tunable Resistive Pulse Sensing. DLS: Dynamic light scattering. ES-DMA: Electrospray differential mobility analysis. AF4-MALS: asymmetric flow field flow fractionation-Multiangle light scattering. HPLC: High pressure liquid chromatography. ELISA: Enzyme-linked immunosorbent assay. HCP: Host cell proteins. HCDNA: Host cell DNA.

The VLP stage of development also need to be considered when choosing analytical tools. In this way, it is preferred to use particle identification methods when VLPs are non-purified, and antibody-based techniques for purified VLP preparations [39]. In addition, there are some methodologies that are not so easily implementable at any regular lab, since they require highly specialized equipment and specifically trained personnel, and this limits their widespread use. Some examples are, mass spectrometry (MS), electrospray differential mobility analysis (ES-DMA) or asymmetric flow field flow fractionation (AFFF) [38,39].

3.2. Quantitation methods applicable to VLPs

VLPs are non-infectious since they do not contain the viral genome. In addition, they are usually formed by structural virus proteins. For these reasons, the quantitation

methodologies based on virus infectivity (plaque assay, TCID50) cannot be used for VLP quantitation. In the same manner, those methods based on viral genome quantitation (qPCR) or viral enzymatic activity are discarded for VLP applications. Therefore, the methodologies that remain useful for VLP quantitation are those based on physical particle counting or protein quantitation [40,41]. Table 4 summarizes the most widely used VLP quantitation techniques, including the most classical methodologies and some novel techniques that have risen from the accelerated growth of the nanotechnology field [41].

An ideal quantitation method should be simple, fast, cost-effective, robust, sensitive and specific. Simplicity, speed and cost-effectiveness are critical to perform quantitation routinely in all the steps of the process, which is key to improve bioprocess development. Robustness and reproducibility are with no doubt necessary for reliable results. Low limit of detection is especially beneficial in the steps in which the products may be diluted. Last, specificity not only refers to unequivocally identify the protein of interest, but in the case of VLPs refers also to the specific identification of correctly assembled, instead of monomeric proteins or defective VLPs. Moreover, vesicles and exosomes produced by the cells with similar characteristics to enveloped VLPs may interfere in quantitation, especially in physical particle-counting techniques [37,39,41]. Due to the diversity and complexity of VLPs and the different purification degrees in which can be quantified, all techniques have some limitations. The most reasonable approach is, then, the combination of several methodologies to ensure reliable titers.

Between the different choices (Table 4), a fluorescence-based assay was developed in this work, taking advantage of the GFP tag fused to Gag polyprotein, which generated fluorescent VLPs. Spectrofluorometry consists in reading the fluorescence signal of a sample with a spectrofluorometer. It is a simple methodology that can be implemented at any regular molecular biology lab and it does not require highly specialized equipment that sometimes may be unaffordable. It is cost-effective, and a high number of samples can be analyzed in hours (~80 samples in 1h). In addition, it is specific for GFP expressing molecules, which is beneficial since VLP crude samples can be reliably analyzed. In general, the limit of detection is low enough to quantify crude supernatants and the working range is wide enough to perform quantification of highly concentrated VLP samples. Since it is not specific to particulate VLPs, alternative characterization methodologies are required to confirm the presence of correctly assembled Gag-GFP VLPs, however this is the general trend for complex VLPs.

Table 4
Most commonly used methods for VLP quantitation

	Detection principle	Specificity	Requirement of standard material	Require purification	Ref.
Classic virus quantitation techniques					
Transmission electron microscopy (TEM)	Direct visualization of the particles (negative staining required)	Specific to viral particles, however in the case of enveloped VLPs, difficult to differentiate from cellular vesicles	Direct method, does not require standard material	No	[42]
Enzyme-linked immunosorbent assay (ELISA)	Antibody binding assay	Specific to viral antigens, but not in a particulate form. Risk of overestimation of VLPs by the presence of protein monomers	Indirect method, requires quantified standard material	No	[43]
Spectrophotometry	Detection of protein and nucleic acid	Specific to protein and nucleic acids	Indirect method	Yes	[44]
High performance liquid chromatography (HPLC)	Separation of VLPs and detection by spectrophotometry or fluorometry	Specific to particles. Risk of overestimation of VLPs by the presence of cell-derived vesicles	Indirect method	Yes, HPLC provides separation of VLPs from other components.	[45]
Tag-based	Detection of protein by fluorometry or luminescence (label required)	Specific to viral antigens, but not in a particulate form	Indirect method	No	[46]
Modern virus counting techniques					
Flow-field flow fractionation and Multi-angle light scattering (FFF-MALS)	Light scattering of particles (label free)	Specific to particles	Direct method	No	[47]
Nanoparticle tracking analysis (NTA)	Light scattering of particles (label free)	Specific to particles	Direct method	No	[48]
Tunable-Resistive Pulse Sensor (TRPS)	Coulter principle (label free)	Specific to particles	Direct method	No	[49]
Flow virometry	Fluorescence in-flow detection (dual fluorescence label)	Specific to labeled particles	Direct method	No	[50]
Bio-layer interferometry (BLI)	Based on antibody binding	Specific to viral antigens, but not in a particulate form?	Direct method	No	[51]

3.3. Process Analytical Technologies (PAT) for VLP bioprocess characterization

The PAT initiative for biopharmaceutical products was proposed by the FDA in 2004 and it was internationally supported [52]. It was also described in ICH guideline Q8 [53]. It consists in the set of tools used to design, analyze and control the manufacturing of a biopharmaceutical product with the desired characteristics (usually functionality and safety). PAT includes multivariate data analysis, Design of Experiments (DoE), bioprocess modelling and new sensor technologies [54–56].

The characterization of CQA needs to be performed not only by the study of the final product, but also by the study of the CPP that determine these attributes. The establishment of relationships between CPP and CQA is key to this end. Monitoring of CPP allow to establish these relationships, and in addition enables increasing the knowledge of the system, which is useful to have more robust processes and improved productivities. In-line non-invasive monitoring is recommended to obtain a large number of data without interfering with the process itself [55].

In-line and on-line monitoring technologies are in its infancy [57]. In general, very few parameters are usually monitored in bioprocesses [55]. The most common variables monitored in-line in a widespread manner are the physical (temperature, level, pressure, foam, weight, power input) and some chemical (pH, pO_2, pCO_2). However, there are other key variables within a bioprocess, such as metabolite concentration, product characteristics and cell density and state. There are many off-line techniques commonly used to monitor them, and in the last years, the efforts have been focused in reducing sampling and analysis time and increase automatization in order to comply with the PAT initiative [57]. In addition, spectroscopy-based techniques have been developed in the last years to monitor these parameters in real-time, avoiding the necessity of sampling [54,56]. The most common technologies recommended by the FDA are near-infrared spectroscopy (NIR), mid-infrared spectroscopy (MIR), far-infrared spectroscopy (FIR), ultraviolet-visual spectroscopy (UV-VIS), fluorescence spectroscopy, and nuclear magnetic resonance spectroscopy (NMR). The applications of these spectroscopy techniques are summarized in Figure 5.

Dielectric spectroscopy (DS) is emerging as a PAT tool. DS measures the permittivity (the ability to store electrical charge) of a biological solution exposed to an alternating

electrical field. This measurement is performed in real time and correlates with the biovolume or cell density [58]. It has been successfully used to monitor bacterial, yeast, plants and animal cell processes [55]. Remarkably, the dielectric data also provides information about the culture state and relevant events during the process. For instance, data from DS measurements was correlated to glutamine consumption rates in an hybridoma culture [59]. CHO batch and fed-batch cultures were monitored using DS. In these cultures, nutrient availability and depletion were correlated with changes in the cell dielectric properties of the cells, which could be observed in real time [60,61]. Virus production processes were monitored by DS. Since viral formation affects the cell membrane and consequently the properties of the cells, the time of viral release could be identified [62,63]. Finally, the onset of the death phase can also be monitored by DS [64].

4. VLP manufacturing

VLPs have been expressed in bacteria, yeast, plants, insect cells and mammalian cells. The ease of expression, the ability of scale-up, cost-effectiveness, yields, glycosylation capabilities, and the correct folding and assembly of VLPs dictate the most suitable expression system for each type of VLP. Hence, non-enveloped VLPs are usually produced in the simplest systems (bacteria, yeast) since usually they do not require post-translational modifications (PTMs) or the cell environment to be assembled. On the other hand, more complex or enveloped VLPs that require complex PTMs and the cell environment for assembly and taking the lipid envelope, are usually produced in more complex systems (yeast, animal cells and plants) [8,15,65]. Table 5 summarizes the main features of each system.

Table 5
Comparison of VLP production host systems

	Bacteria	**Yeast**	**BEVS-Insect cells**	**Mammalian cells**	**Plants**
Speed	++++	+++	++	++	++
Scalability	++++	+++	++	++	++
Yield	++++	+++	++	+	+
VLP complexity	+	++	++++	++	+++
Regulatory	++++	+++	++	++++	+

From [8]. BEVS: Baculovirus Expression Vector System

HIV VLPs are complex enveloped particles, and the Gag polyprotein needs a myristoilation to be able to travel to the cell membrane and assemble. Therefore, they need to be produced in more complex systems. Production in yeast spheroplasts [66], plants [67], insect [29,68,69] and mammalian cells [70] has been reported. However, mammalian cells are one of the most suitable platforms for Gag VLP production because they provide PTMs very similar to those of the wild-type virus.

Overexpression of proteins in mammalian cells is achieved upon transfection of an expression vector coding for the protein of interest. Expression can be transient if the product is harvested a few days later and expression is lost over time. Otherwise, if the vector is inserted into the cell genome, expression can be stable [71]. Gag VLPs can be produced either by Stable Gene Expression (SGE) or Transient Gene Expression (TGE). While SGE is the most widely used approach for industrial biopharmaceutical production, TGE is moving from being used in research phases to GMP clinical lot production. Due to the relevance of Transient Gene Expression in this thesis, it has been addressed with detail in a review paper, presented in *Introduction, part 2*.

4.1. CAP cells

Among the different mammalian cell lines available for production of biological products, the most popular are CHO, for industrial applications, and HEK293 more limited to research (although there are some HEK293-derived products approved by FDA and EMA) [72,73]. The increasing market of biological therapeutic products and also their increasing complexity have led to the development of new cell lines as substrates to produce them [72,74]. In this regard, CAP is one of this novel *designer* cell lines first developed for the production of adenoviral vectors [75].

CAP cells are property of CEVEC Pharmaceuticals (Cologne, Germany). They are derived from human amniotic fluid, which can be obtained after a routine amniocentesis. Therefore, they are not of tumor origin and no ethical concerns are associated to their use. Primary amniocytes were immortalized by transformation with E1A, E1B and pIX from Adenovirus 5 [75,76]. Therefore, they provide the E1 functions in *trans* for the production of first and second generation recombinant adenoviral vectors lacking this gene [75,77].

CAP cells represent a suitable cell line for biopharmaceutical production since, besides meeting the regulatory requirements (non-tumor origin, accepted source, fully-documented history, GMP bank available), they accomplish the industrial standards: growth in suspension in serum-free media to high cell densities, performance of human PTMs, permittivity to human virus replication, high transfectability, and high yields [78,79]. It was hypothesized that due to the stem cell-like genetic background of CAP cells, they express a larger repertoire of enzymes and chaperones to successfully produce a broader variety of proteins with good secretion rates [80]. In any case, their suitability has been demonstrated in previous reports for the production of influenza virus [78,81], human cytomegalovirus [82], difficult-to-express recombinant proteins [76,80], and more recently, adeno-associated and lentiviral vectors for gene therapy applications. VLP production in CAP cells has not been yet explored.

Interestingly, CEVEC also developed the CAP-T cell line, which has the same genetic background than CAP cells but they additionally express the SV40 large T antigen. This feature is especially interesting for TGE applications, where the plasmids containing the SV40 origin of replication can replicate episomally and enhance protein production yields [80]. (More details on TGE are addressed in *Introduction, part 2*). Therefore, a platform is available comprising CAP-T cells for transient expression of different protein candidates, and CAP cells for stable expression of the selected protein. This system can be used from screening phases to commercial production guaranteeing a constant genetic background.

4.2. Stable Gene Expression

Generation of recombinant cell lines stably producing the protein of interest is currently the workhorse in industrial bioprocesses [83]. Figure 6 summarizes the steps for cell line development. Briefly, it consists in the delivery of an expression vector containing the gene of interest (GOI) and a selectable marker into the host cells, usually by transfection. The cells that have integrated the vector in the genome are further selected by the selectable marker. The isolation of clones to obtain genetically homogeneous populations follows and intensive screening of a large number of clones is required in order to finally select the highest producing cells. Finally, the stability and performance of the selected clones at larger-scales is usually tested [83,84]. The development of cell lines, from transfection to cell banking is a laborious process usually taking 3-12 months [85].

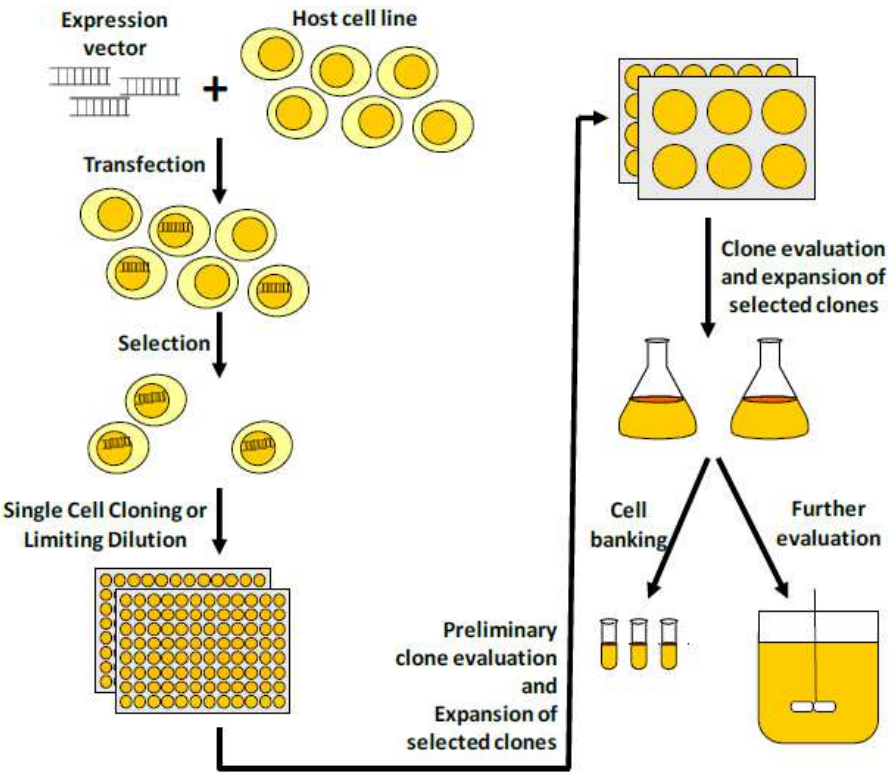

Figure 6. Flow chart of the development of stable cell lines.

Historically, tPA was the first approved drug (in 1986) produced by stable expression in CHO cells. Since then, many others have followed, from insulin and other simple peptides to complex virus structures and monoclonal antibodies. Although CHO is the preferred platform to produce biotherapeutics, other cell lines have been also used, such as HEK293, HKB11, BHK21, PerC6, EB66 and CAP [84]. For the last 30 years, genetic engineering, media development, and process optimization have been performed to improve protein titers and quality. These strategies have proven successful, leading to a 100-fold improvement in productivities from a few mg/L to 5-10 g/L [84].

The productivity of a cell line depends on the modulation of the co-expression of the product and the selection marker genes, the stringency of the selection marker, the expression vector set-up, and the integration site [83].

Antibiotics are the most common selectable markers. Among the most widely used are G418/Neomycine, Puromycine, Hygromycine, Zeocine, and Blasticin. Alternatively, metabolic genes can be used for selection, such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS) genes. The generation of engineered strains lacking these

genes are required to use them as selectable markers when co-expressed with the gene of interest. The expression of the selectable marker can be linked to the gene of interest by an IRES (Internal ribosome entry site) or they can be independently expressed.

Regarding the set-up of the expression vector, several features need consideration. The combination of strong promoters and enhancers to drive expression in mammalian cells is key to achieve high productivities. Examples are CMV and those of translation elongation factors (EFs). Susceptibility to silencing by histone modification is also a criterion for the selection of promoters. Inclusion of an intron in the expressed RNA is believed to be beneficial for RNA stabilization. Use of chromatin modulating motifs such as S/MARs (scaffold/matrix attachment regions) or UCOEs (ubiquitous chromatin opening elements) to flank the GOI has been described to increase the ratio of high producing clones after random integration of the vector and to support long term stability of transgene expression [84].

Integration site determines productivity since there are genome areas more transcriptionally active than others. Moreover, gene silencing usually takes place upon integration, and this is also determined by the chromatin area where the gene is located [71]. The most widely used strategy for the generation of stable cell lines is random integration, but there are alternative technologies that enable targeted integration (summarized in Table 6). The main drawback of targeted integration is that only one copy is usually inserted, and the titer also depends on the copy number. In random integration, several copies can be integrated, increasing the final protein titers. On the other hand, targeted integration simplifies screening because the pool generated is more homogenous, expression is more stable and gene silencing may be overcome. Some of these technologies are bound to IP property and may not be universally accessible [71,84,86].

On top of all the elements mentioned above, the factor influencing the most the process of cell line development is probably the screening technology. The higher the throughput, the more labor and time-consuming and the more chances of success there will be. An equilibrium between labor/time required and success probability needs to be found. The technologies have evolved towards higher throughput systems. Table 7 summarizes the technologies available. The choice will depend on the urge for very high producers and the economic possibilities of the research group or company. LCD is simple and straightforward, can be implemented in any lab. Results can be obtained in 3 months.

Importantly, it does not depend on reporter tags, but on selectable markers instead. However, is the lowest throughput strategy. FACS-based technologies depend on reporter proteins that are not always available. On top of that, the capture-based technologies are technically challenging. Finally, automated screening requires very specialized and expensive equipment that may not be available [83,84,86].

Table 6
Integration methods for the generation of stable cell lines

	Advantages	Disadvantages	Ref.
Random integration	Simple and straightforward Multiple copies	Non-predictable integration Gene silencing Heterogeneous expression levels Higher screening efforts	[87]
Targeted integration	Predictable site Silencing can be avoided Homogenous expression levels Less screening efforts	Single copy (lower expression levels)	
Strategies for targeted integration	Description		Ref.
RMCE	Master cell line with a reporter gene integrated in a *hot spot* flanked by recombination sites (e.g. FRT). Donor plasmid contains GOI flanked by recombination sites and a recombinase (eg. Flp).		[88]
Lentivirus	Transduction with a recombinant lentivirus containing the GOI. Integration of the lentivirus genome is a frequent event and usually takes place in transcriptionally active chromatin sites.		[89]
Phage ΦC31 integrase	Donor vector contains attB sites from *Streptomyces* flanking the GOI, and the integrase. Recombination with pseudo-attP sites takes place in the host cell genome.		[90]
Transposons	The transposon contains inverted terminal repeats and a recombinase. The inverted terminal repeats flanking the GOI are recognized by the transposon's integrase and it is inserted in TTAA sites in the cell genome.		[91]
CRISPR-Cas9	Donor vector contains GOI, Cas9 nuclease and guideRNA to direct the double stranded DNA break performed by the nuclease. The donor plasmid is integrated upon DNA break by homology directed repair or non-homologous end joining repair of the DNA.		[92]

Adapted from [71,84,86]. RMCE: Recombinase-mediated cassette exchange. GOI: Gene of interest. CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats.

Table 7
Summary of selection methods for the isolation of high producing clones

Method	Summary	Advantages	Disadvantages
Traditional methods			
Limiting dilution cloning	Dilute transfected stable pool, plate in 96-well plates at 1–10 cells/well	Inexpensive, simple, does not require complex equipment	Long timeline, LTP, additional analysis required to characterize each clone
Selection via antibiotic resistance	Select cells using an antibiotic for each GOI-containing plasmid that contains a resistance gene	Inexpensive, simple, does not require complex equipment	Selection is indirect, LTP, non-producing clones may survive
DHFR selection systems	Transfect GOI and DHFR gene into DHFR-deficient cells, add MTX at increasing concentrations, select transfectants that have "amplified" DHFR (and hopefully GOI) copy number and expression	Widely-used technique, particularly for CHO. More HTP than LDC	Long development time, amplified gene can be unstable, selection pressure is indirect
GS selection system	Transfect GOI and DHFR gene cells, add MSX at increasing concentrations, select transfectants that have "amplified" GS (and hopefully GOI) copy number and expression	More HTP than LDC, CHO cells GS gene knocked out have been recently developed	Long development time, amplified gene can be unstable (more than DHFR), selection pressure is indirect
FACS-based methods			
GOI-IRES-reporter gene	Express GOI and reporter on same plasmid, separated by an IRES	Flow cytometry and FACS are HTP, GOI and reporter are expressed to the same relative level	Expression of the gene downstream of IRES is lower than gene upstream of IRES, does not work well with secreted proteins.
GOI-reporter fusion	Directly fuse reported gene to GOI	Flow cytometry and FACS are HTP, expression level of GOI and reporter should be identical	Reporter can influence structure and function of protein of interest
Link expression level of secreted protein to the cell that secreted it	Trap selected protein near cell that secreted it via gel microdrop, affinity capture surface display or cold capture; anchor protein to cell membrane via a transmembrane peptide, transiently anchor secreted protein to extracellular transmembrane cytosolic domain using a furin cleavage peptide	Facilitates flow cytometry of secreted proteins, flow cytometry and FACS are HTP	More time consuming and expensive than methods used for non-secreted proteins, trapping of secreted protein can be inefficient
Automated methods			
Fluorescence-based systems	Automate cell selection and picking based in fluorescence. Systems include ClonePix, CellCelector, LEAP, and GenePix	Very HTP, selection of high producing clones in 4 weeks, minimal labor required	Cost, more suited for secreted proteins than membrane proteins

Robotic systems	Automated cell culture, clone scale-up and analytical analysis, such as ELISA, via systems such as Cello and CellSpot	Very HTP. Decreased labor and increased sensitivity compared to manual ELISA	Cost, clone productivity is typically not much better than clones isolated via manual methods
Complete automation	Automate clone selection, analysis, and cell culture via FACS, Clone Select Imager, and TECAN Liquid Handling System, respectively	Very HTP, minimal manual labor required, stringent selection process based in gene expression and specific productivity	Very expensive, cost makes complete automation impractical for academic labs

Adapted from [86]. LTP: Low-throughput. GOI: Gene of interest. DHFR: Dihydropholate reductase. MTX: Methotrexate. HTTP: High-throughput. GS: Glutamine synthetase. MSX: methionine sulphoximine, FACS: Fluorescence-activated cell sorter. IRES: Internal ribosome entry site. LEAP: Laser-Enabled Analysis and Processing.

References

[1] Plotkin S. History of vaccination. Proc Natl Acad Sci U S A. 2014;111:12283–12287.

[2] Plotkin S. Vaccines : past , present and future Early successes. Nat. Med. 2005;11:5–11.

[3] Cox MMJ. Recombinant protein vaccines produced in insect cells. Vaccine. 2012;30:1759–1766.

[4] Ulmer JB, Valley U, Rappuoli R. Vaccine manufacturing: challenges and solutions. Nat. Biotechnol. 2006;24:1377–1383.

[5] Nunnally BK, Turula VE, Sitrin RD. Vaccine analysis: Strategies, principles, and control. Vaccine Anal. Strateg. Princ. Control. 2015.

[6] Yan D, Wei YQ, Guo HC, et al. The application of virus-like particles as vaccines and biological vehicles. Appl. Microbiol. Biotechnol. 2015;99:10415–10432.

[7] Pitoiset F, Vazquez T, Bellier B. Enveloped virus-like particle platforms: vaccines of the future? Expert Rev. Vaccines. 2015;14.

[8] Lua LHL, Connors NK, Sainsbury F, et al. Bioengineering virus-like particles as vaccines. Biotechnol. Bioeng. 2014;111:425–440.

[9] Roldão A, Mellado MCM, Castilho LR, et al. Virus-like particles in vaccine development. Expert Rev. Vaccines. 2010;9:1149–1176.

[10] Outcomes AP. New England Journal. 2011;1991–2002.

[11] Deml L, Speth C, Dierich MP, et al. Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. Mol. Immunol. 2005;42:259–277.

[12] Stern d. HLA-DR : Molecular insights and vaccine design. 2013;15:3249–3261.

[13] Young KR, McBurney SP, Karkhanis LU, et al. Virus-like particles: Designing an effective AIDS vaccine. Methods. 2006;40:98–117.

[14] Vartak A, Sucheck SJ. Recent Advances in Subunit Vaccine Carriers. Vaccines [Internet]. 2016 [cited 2017 Feb 19];4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27104575.

[15] Grgacic EVL, Anderson DA. Virus-like particles: Passport to immune recognition. Methods. 2006;40:60–65.

[16] Cubas R, Zhang S, Li M, et al. Chimeric Trop2 Virus-like Particles. J. Immunother. [Internet]. 2011 [cited 2017 Feb 28];34:251–263. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21389873.

[17] Zochowska M, Paca A, Schoehn G, et al. Adenovirus Dodecahedron, as a Drug Delivery Vector. Jagetia GC, editor. PLoS One [Internet]. 2009 [cited 2017 Feb 28];4:e5569. Available from: http://dx.plos.org/10.1371/journal.pone.0005569.

[18] Huhti L, Tamminen K, Vesikari T, et al. Characterization and immunogenicity of norovirus capsid-derived virus-like particles purified by anion exchange chromatography. Arch. Virol. [Internet]. 2013 [cited 2017 Feb 28];158:933–942. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23229011.

[19] Sharp PM, Hahn BH. Origins of HIV and the AIDS pandemic. Cold Spring Harb. Perspect.

Med. 2011;1:1–22.

[20] Sundquist WI, Kräusslich HG. HIV-1 assembly, budding, and maturation. Cold Spring Harb. Perspect. Med. 2012;2:1–24.

[21] Lusic M, Siliciano RF. Nuclear landscape of HIV-1 infection and integration. Nat. Rev. Microbiol. [Internet]. 2016;69–82. Available from: http://www.nature.com/doifinder/10.1038/nrmicro.2016.162.

[22] Fauci AS, Folkers GK, Marston HD. Ending the global HIV/AIDS Pandemic: The critical role of an HIV vaccine. Clin. Infect. Dis. 2014;59:S80–S84.

[23] UNAIDS. Global AIDS Update 2016. World Heal. Organ. [Internet]. 2016;422. Available from: http://www.unaids.org/sites/default/files/media_asset/UNAIDS_Gap_report_en.pdf.

[24] Shin SY. Recent update in HIV vaccine development. Clin. Exp. Vaccine Res. 2016;5:6–11.

[25] Zhao C, Ao Z, Yao X. Current Advances in Virus-Like Particles as a Vaccination Approach against HIV Infection. Vaccines [Internet]. 2016;4:2. Available from: http://www.mdpi.com/2076-393X/4/1/2.

[26] Buonaguro L, Tagliamonte M, Visciano ML, et al. Developments in virus-like particle-based vaccines for HIV. Expert Rev. Vaccines. 2013;12:119–127.

[27] Williamson A-L, Rybicki EP. Justification for the inclusion of Gag in HIV vaccine candidates. Expert Rev. Vaccines [Internet]. 2016 [cited 2017 Mar 9];15:585–598. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26645951.

[28] Halsey RJ, Tanzer FL, Meyers A, et al. Chimaeric HIV-1 subtype C Gag molecules with large in-frame C-terminal polypeptide fusions form virus-like particles. Virus Res. 2008;133:259–268.

[29] Pillay S, Meyers A, Williamson AL, et al. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. Biotechnol. Prog. 2009;25:1153–1160.

[30] Wagner R, Deml L, Schirmbeck R, et al. Construction, expression, and immunogenicity of chimeric HIV-1 virus-like particles. Virology. 1996;220:128–140.

[31] Griffiths JC, Harris SJ, Layton GT, et al. Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. J. Virol. [Internet]. 1993 [cited 2017 Mar 9];67:3191–3198. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8497047.

[32] Tagliamonte M, Visciano ML, Tornesello ML, et al. HIV-Gag VLPs presenting trimeric HIV-1 gp140 spikes constitutively expressed in stable double transfected insect cell line. Vaccine. 2011;29:4913–4922.

[33] Visciano ML, Diomede L, Tagliamonte M, et al. Generation of HIV-1 Virus-Like Particles expressing different HIV-1 glycoproteins. Vaccine [Internet]. 2011 [cited 2017 Mar 9];29:4903–4912. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21596074.

[34] Hammonds J, Chen X, Fouts T, et al. Induction of Neutralizing Antibodies against Human Immunodeficiency Virus Type 1 Primary Isolates by Gag-Env Pseudovirion Immunization. J. Virol. 2005;79:14804–14814.

[35] Venereo-Sanchez A, Gilbert R, Simoneau M, et al. Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate. Vaccine. 2016;34:3371–3380.

[36] Osterrieder N, Wagner R, Brandmüller C, et al. Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). Virology. 1995;208:500–510.

[37] Vicente T, Roldão A, Peixoto C, et al. Large-scale production and purification of VLP-based vaccines. J. Invertebr. Pathol. [Internet]. 2011;107:S42–S48. Available from: http://dx.doi.org/10.1016/j.jip.2011.05.004.

[38] Thompson CM, Petiot E, Lennaertz A, et al. Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches. Virol. J. [Internet]. 2013;10:141. Available from: http://www.virologyj.com/content/10/1/141.

[39] Manceur AP, Kamen AA. Critical review of current and emerging quantification methods for the development of influenza vaccine candidates. Vaccine [Internet]. 2015;33:5913–5919. Available from: http://dx.doi.org/10.1016/j.vaccine.2015.07.104.

[40] Steppert P, Burgstaller D, Klausberger M, et al. Quantification and characterization of virus-like particles by size-exclusion chromatography and nanoparticle tracking analysis. J. Chromatogr. A. 2016;1487:89–99.

[41] Heider S, Metzner C. Quantitative real-time single particle analysis of virions. Virology [Internet]. 2014 [cited 2017 Mar 13];462–463:199–206. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24999044.

[42] Malenovska H. Virus quantitation by transmission electron microscopy, TCID50, and the role of timing virus harvesting: A case study of three animal viruses. J. Virol. Methods [Internet]. 2013 [cited 2017 Mar 15];191:136–140. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23603437.

[43] Pecora A, Aguirreburualde MSP, Rodriguez D, et al. Development and validation of an ELISA for quantitation of Bovine Viral Diarrhea Virus antigen in the critical stages of vaccine production. J. Virol. Methods. 2009;162:170–178.

[44] Sommer JM, Smith PH, Parthasarathy S, et al. Quantification of adeno-associated virus particles and empty capsids by optical density measurement. Mol. Ther. 2003;7:122–128.

[45] Transfiguracion J, Manceur AP, Petiot E, et al. Particle quantification of influenza viruses by high performance liquid chromatography. Vaccine [Internet]. 2015 [cited 2017 Mar 15];33:78–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25448111.

[46] Sakuragi S, Sakuragi J ichi, Morikawa Y, et al. Development of a rapid and convenient method for the quantification of HIV-1 budding. Microbes Infect. 2006;8:1875–1881.

[47] Bousse T, Shore DA, Goldsmith CS, et al. Quantitation of influenza virus using field flow fractionation and multi-angle light scattering for quantifying influenza A particles. J. Virol. Methods [Internet]. 2013 [cited 2017 Mar 15];193:589–596. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23916678.

[48] Kramberger P, Ciringer M, Štrancar A, et al. Evaluation of nanoparticle tracking analysis for total virus particle determination. Virol. J. [Internet]. 2012 [cited 2017 Mar 9];9:265. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23140220.

[49] Yang L, Yamamoto T. Quantification of virus particles using nanopore-based resistive-pulse sensing techniques. Front. Microbiol. 2016;7.

[50] Rossi CA, Kearney BJ, Olschner SP, et al. Evaluation of ViroCyt® Virus Counter for rapid filovirus quantitation. Viruses [Internet]. 2015 [cited 2017 Mar 15];7:857–872. Available from: http://www.mdpi.com/1999-4915/7/3/857/.

[51] Dong X, Broglie JJ. Evaluation of Bio-Layer Interferometric Biosensors for Label-Free Rapid Detection of Norovirus Using Virus Like Particles. J. Anal. Bioanal. Tech. [Internet]. 2016;7:329. Available from: http://www.omicsonline.org/open-access/evaluation-of-biolayer-interferometric-biosensors-for-labelfree-rapid-detection-of-norovirus-using-virus-like-particles-2155-9872-1000329.php?aid=76693.

[52] FDA. Guidance for Industry PAT: A Framework for Innovative Pharmaceutical Development, Manufacuring, and Quality Assurance. FDA Off. Doc. 2004.

[53] ICH. ICH Harmonised tripartite guideline: Pharmaceutical Development (Q8). 2009.

[54] Teixeira AP, Oliveira R, Alves PM, et al. Advances in on-line monitoring and control of mammalian cell cultures : Supporting the PAT initiative. Biotechnol. Adv. [Internet]. 2009;27:726–732. Available from: http://dx.doi.org/10.1016/j.biotechadv.2009.05.003.

[55] Justice C, Brix A, Freimark D, et al. Process control in cell culture technology using dielectric spectroscopy. Biotechnol. Adv. 2011;29:391–401.

[56] Claßen J, Aupert F, Reardon KF, et al. Spectroscopic sensors for in-line bioprocess monitoring in research and pharmaceutical industrial application. Anal. Bioanal. Chem. [Internet]. 2017;651–666. Available from: http://dx.doi.org/10.1007/s00216-016-0068-x.

[57] Pais DAM, Carrondo MJT, Alves PM, et al. Towards real-time monitoring of therapeutic protein quality in mammalian cell processes. Curr. Opin. Biotechnol. 2014;30:161–167.

[58] Davey CL, Davey HM, Kell DB, et al. Introduction to the dielectric estimation of cellular biomass in real time, with special emphasis on measurements at high volume fractions. Anal. Chim. Acta. 1993;279:155–161.

[59] Noll T, Biselli M. Dielectric spectroscopy in the cultivation of suspended and immobilized hybridoma cells. J. Biotechnol. 1998;63:187–198.

[60] Ansorge S, Esteban G, Schmid G. Multifrequency permittivity measurements enable on-line monitoring of changes in intracellular conductivity due to nutrient limitations during batch cultivations of CHO cells. Biotechnol. Prog. 2010;26:272–283.

[61] Opel CF, Li J, Amanullah A. Quantitative modeling of viable cell density, cell size, intracellular conductivity, and membrane capacitance in batch and fed-batch CHO processes using dielectric spectroscopy. Biotechnol. Prog. 2010;26:1187–1199.

[62] Zeiser A, Bédard C, Voyer R, et al. On-line monitoring of the progress of infection in Sf-9 insect cell cultures using relative permittivity measurements. Biotechnol. Bioeng. 1999;63:122–126.

[63] Emma P, Kamen A. Real-time monitoring of influenza virus production kinetics in HEK293 cell cultures. Biotechnol. Prog. 2013;29:275–284.

[64] Patel P, Markx GH. Dielectric measurement of cell death. Enzyme Microb. Technol. 2008;43:463–470.

[65] Noad R, Roy P. Virus-like particles as immunogens. Trends Microbiol. 2003;11:438–444.

[66] Sakuragi S, Goto T, Sano K, et al. HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 2002;99:7956–7961.

[67] Scotti N, Alagna F, Ferraiolo E, et al. High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. Planta. 2009;229:1109–1122.

[68] Tagliamonte M, Visciano ML, Tornesello ML, et al. Constitutive expression of HIV-VLPs in stably transfected insect cell line for efficient delivery system. Vaccine. 2010;28:6417–6424.

[69] Yang L, Song Y, Li X, et al. HIV-1 virus-like particles produced by stably transfected Drosophila S2 cells: a desirable vaccine component. J. Virol. [Internet]. 2012;86:7662–7676. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22553333%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3416305.

[70] Cervera L, Gutiérrez-Granados S, Martínez M, et al. Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J. Biotechnol. 2013;166:152–165.

[71] Büssow K. Stable mammalian producer cell lines for structural biology. Curr. Opin. Struct. Biol. 2015;32:81–90.

[72] Genzel Y. Designing cell lines for viral vaccine production: Where do we stand? Biotechnol. J. 2015;10:728–740.

[73] Dumont J, Euwart D, Mei B, et al. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. Crit. Rev. Biotechnol. 2015;1:1–13.

[74] Aggarwal SR. What ' s fueling the biotech engine — 2012 to 2013. Nat. Biotechnol. 2014;32:32–39.

[75] Schiedner G, Hertel S, Kochanek S. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. Hum. Gene Ther. [Internet]. 2000 [cited 2015 Mar 3];11:2105–2116. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11044912.

[76] Schiedner G, Hertel S, Bialek C, et al. Efficient and reproducible generation of high-expressing, stable human cell lines without need for antibiotic selection. BMC Biotechnol. 2008;8:13.

[77] Silva AC, Simão D, Küppers C, et al. Human amniocyte-derived cells are a promising cell host for adenoviral vector production under serum-free conditions. Biotechnol. J. 2015;10:760–771.

[78] Genzel Y, Behrendt I, Rödig J, et al. CAP, a new human suspension cell line for influenza virus production. Appl. Microbiol. Biotechnol. 2013;97:111–122.

[79] Essers R, Kewes H, Schiedner G. Improving volumetric productivity of a stable human CAP cell line by bioprocess optimization. BMC Proc. 2011;5 Suppl 8:P66.

[80] Fischer S, Charara N, Gerber A, et al. Transient recombinant protein expression in a human amniocyte cell line: The CAP-T® cell system. Biotechnol. Bioeng. 2012;109:2250–2261.

[81] Genzel Y, Vogel T, Buck J, et al. High cell density cultivations by alternating tangential flow (ATF) perfusion for influenza A virus production using suspension cells. Vaccine [Internet]. 2014;32:2770–2781. Available from: http://dx.doi.org/10.1016/j.vaccine.2014.02.016.

[82] Krömmelbein N, Wiebusch L, Schiedner G, et al. Adenovirus E1A/E1B transformed amniotic fluid cells support human cytomegalovirus replication. Viruses. 2016;8.

[83] Lai T, Yang Y, Ng SK. Advances in mammalian cell line development technologies for recombinant protein production. Pharmaceuticals. 2013;6:579–603.

[84] Jostock T. Expression of Antibody in Mammalian Cells. Springer Netherlands; 2011 [cited 2017 Mar 25]. p. 1–24. Available from: http://www.springerlink.com/index/10.1007/978-94-007-1257-7_1.

[85] Bandaranayake AD, Almo SC. Recent advances in mammalian protein production. FEBS Lett. 2014;588:253–260.

[86] Priola JJ, Calzadilla N, Baumann M, et al. High-throughput screening and selection of mammalian cells for enhanced protein production. Biotechnol. J. 2016;11:853–865.

[87] Rouiller Y, Bielser JM, Brühlmann D, et al. Screening and assessment of performance and molecule quality attributes of industrial cell lines across different fed-batch systems. Biotechnol. Prog. 2016;32:160–170.

[88] Zhou H, Liu Z, Sun Z, et al. Generation of stable cell lines by site-specific integration of transgenes into engineered Chinese hamster ovary strains using an FLP-FRT system. J. Biotechnol. [Internet]. 2010 [cited 2017 Mar 26];147:122–129. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20371256.

[89] Oberbek A, Matasci M, Hacker DL, et al. Generation of stable, high-producing cho cell lines by lentiviral vector-mediated gene transfer in serum-free suspension culture. Biotechnol. Bioeng. 2011;108:600–610.

[90] Chalberg TW, Portlock JL, Olivares EC, et al. Integration Specificity of Phage φC31 Integrase in the Human Genome. J. Mol. Biol. [Internet]. 2006 [cited 2017 Mar 26];357:28–48. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16414067.

[91] Matasci M, Baldi L, Hacker DL, et al. The PiggyBac transposon enhances the frequency of CHO stable cell line generation and yields recombinant lines with superior productivity and stability. Biotechnol. Bioeng. 2011;108:2141–2150.

[92] Bachu R, Bergareche I, Chasin L a. CRISPR-Cas targeted plasmid integration into mammalian cells via non-homologous end joining. Biotechnol. Bioeng. 2015;112:2154–2162.

Advancements in mammalian cell transient gene expression technology for accelerated production of biologics

Introduction, part 2

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Abstract

Transient Gene Expression (TGE) in animal cell cultures has been used for almost 30 years to produce milligrams and grams of recombinant proteins, virus-like particles and viral vectors, mainly for research purposes. The need to increase the amount of product has led to scale-up of TGE protocols in bioreactors. Moreover, product quality and process reproducibility are also of major importance, especially when TGE is employed for the preparation of clinical lots. This work gives an overview of the different technologies available for TGE, and how they can be combined depending on each application. Then, a critical assessment of the challenges of large-scale transient transfection follows, focusing on suspension cell cultures transfected with polyethyleneimine, which is the most widely used methodology for transfection. Finally, emerging opportunities for transient transfection arising from the gene therapy, personalized medicine and vaccine development are reviewed.

Keywords: Transient transfection, large-scale, bioreactors, polyethylenimine (PEI), animal cell culture, protein production, viral vector production.

1. Introduction

Transient Gene Expression (TGE) is a technology consisting in the introduction of foreign genes into host cells (transfection), to be expressed in a limited time span. Currently, several transfection technologies allow an efficient introduction of DNA plasmids containing the gene of interest into eukaryotic cells [1]. After transfection, plasmid DNA molecules remain in the cells without integrating into the genome (episomally), and are eventually lost over time and cell division. For this reason, gene expression lasts approximately between 48 and 200 hours post-transfection.

TGE allows obtaining milligrams to grams of protein within 2-4 weeks, from gene cloning to protein. This represents a major advantage compared to the time and resource-consuming process of generating stable cell lines. Establishment of stable clones usually takes 3-12 months with the requirement of either a labor-intensive clonal selection process, or complex and expensive laboratory equipment [2]. In contrast, TGE can be set up in almost every cell culture laboratory, since it does not require highly specialized equipment. Another remarkable advantage is the flexibility of TGE. Several protein candidates can be expressed only by changing the gene of interest contained in the plasmid DNA. This makes TGE especially useful for high throughput screening campaigns of biomolecules at early development phases. TGE is also a suitable alternative when the protein of interest cannot be constitutively expressed by the host cell line due to toxicity issues [3,4]. In addition, TGE can be performed from very small scale (96-well plates) [5] to large-volume cultures (hundreds of liters in bioreactors) [6].

The first ever reported transfection was performed by Graham and van der Eb in 1973 [7] using calcium phosphate (CaPi) and DNA co-precipitation in KB cells, aiming to analyze the infectivity of the adenovirus 5 genome. In fact, the broadly known and commonly used HEK293 (Human Embryonic Kidney) cell line was established using this technique [8]. Ever since, a number of transfection approaches have been reported with different purposes such as study of molecular pathways or recombinant protein production [9–14]. Remarkably, the breakthrough of transient transfection was introduced by Boussif et al. [15] in 1995 with the use of polyethylenimine (PEI). PEI allowed high transfection efficiencies of HEK293 suspension cell lines in serum-free media in a cost-effective manner. Since then, transfection became a tool for the rapid expression of recombinant proteins, mainly dedicated to physico-chemical characterization or bioactivity studies. The rapid testing of several proteins spared

the necessity to generate a stable cell line for each candidate, which economically benefited process development. Up-scaling PEI-mediated transfection was required to produce enough material for testing before one candidate was selected for stable expression in a host cell line. Schlaeger and Christiensen [16] were the first to scaleup the PEI-mediated transfection protocol to 23L stirred tank bioreactor using HEK293 cells. Thenceforth, more than 50 successful attempts of large-scale transient transfection have been reported [17], even at a scale of 500L. Initially, HEK293 was the preferred cell line at early development phases to produce recombinant proteins in weeks. However, since CHO (Chinese Hamster Ovary) is the mostly used cell line for commercial manufacturing of recombinant proteins, the set-up of transfection protocols in CHO cells was soon introduced in the field [18]. By maintaining the same cell line from early development stages to manufacturing, constant product characteristics would be guaranteed. However, HEK293 cells are still preferred for viral-based products. Currently, the trend is moving towards viral vector production for clinical testing, being adeno-associated viruses (AAVs) and lentiviruses (LVs) the most widely produced type of vectors [19,20]. New opportunities are raising for large-scale transfection, especially with the accelerated development of gene therapy products and personalized medicine and the increasing number of clinical trials involving biotherapeutics manufactured by TGE technology [21,22].

Some disadvantages or concerns have been associated to TGE. For instance, there is a generalized perception that titers are low, that TGE is not reproducible enough, or that the amount of DNA or transfection reagent needed are limiting for large-scale applications. These concerns and others will be extensively discussed in this review. The main focus will be large-scale TGE. Its implementation still involves some challenges that have been addressed through several approaches. A special emphasis will be put on the description of suspension-growing mammalian cell lines transfected with PEI because of the well-demonstrated scalability of this approach.

2. Available technologies for Transient Gene Expression

Transfection of mammalian cell lines has been performed for the last 40 years with many different purposes. The flexibility to change the candidate to be expressed by only changing the plasmid or the gene of interest, makes transient gene expression a versatile technology

for different applications. Gene silencing, cell engineering, gene therapy, stem cell reprogramming and differentiation, recombinant protein production and virus production are the main fields where TGE has been exploited [1]. In this regard, a wide variety of cell types, genes, culture vessels, culture volumes and transfection reagents have been combined to achieve the best outcome for every objective. Focusing on recombinant protein and virus production purposes, a brief overview of the current technologies available for TGE follows, addressing (1) cell platforms and applications, (2) transfection reagents and (3) culture systems. Considering all these elements forming part of a transfection process, there is not one universal strategy for setting up a transfection protocol. There is a wide number of possibilities depending on the specific needs, and the interaction of many variables will define the technologies and conditions eventually selected (Figure 1).

2.1 Cell platforms and applications

Biopharmaceutical compounds produced by TGE include monoclonal antibodies, soluble proteins, recombinant vaccines, and viral vectors. A variety of cell platforms have been used to produce them.

The most widely used cell platform for transient transfection is HEK293 cells, since they are highly transfectable and grow to high cell densities. Moreover, many suspension and serum-free media adapted HEK293 subtypes are available, which facilitates scale-up [23]. Noticeably, different modified HEK293 cell lines have been developed in order to improve episomal plasmid sustainability after transfection and consequently, protein yields. For instance, HEK293-EBNA (expressing Epstein Barr Nuclear antigen) and HEK293-T (expressing SV40 large T antigen) allow the episomal replication of plasmids containing OriP and SV40ori origins of replication respectively. This aspect has been recently reviewed in detail by Jäger et al. [23]. Many proteins have been produced in this platform, including small peptides and monoclonal antibodies. Moreover, as a human cell line, it represents a highly suitable platform for the production of complex entities such as viral vectors and virus-like particles (VLPs). Glycosylation patterns of viral proteins are highly dependent on the cell line in which they are produced, which highly influences their immunogenicity and potency [24,25]. Since these virus-based products are naturally optimized to replicate in human cells, they are usually produced in HEK293 cells [4,26–30]. On top of that, the availability of GMP Master Cell Banks, the large number of ongoing clinical trials with viral vectors produced in HEK293,

and the fact that there are already some products on the market produced by HEK293 cells, are indicators that regulatory clearance will not be a problem for biopharmaceutical manufacturing in these cells [25].

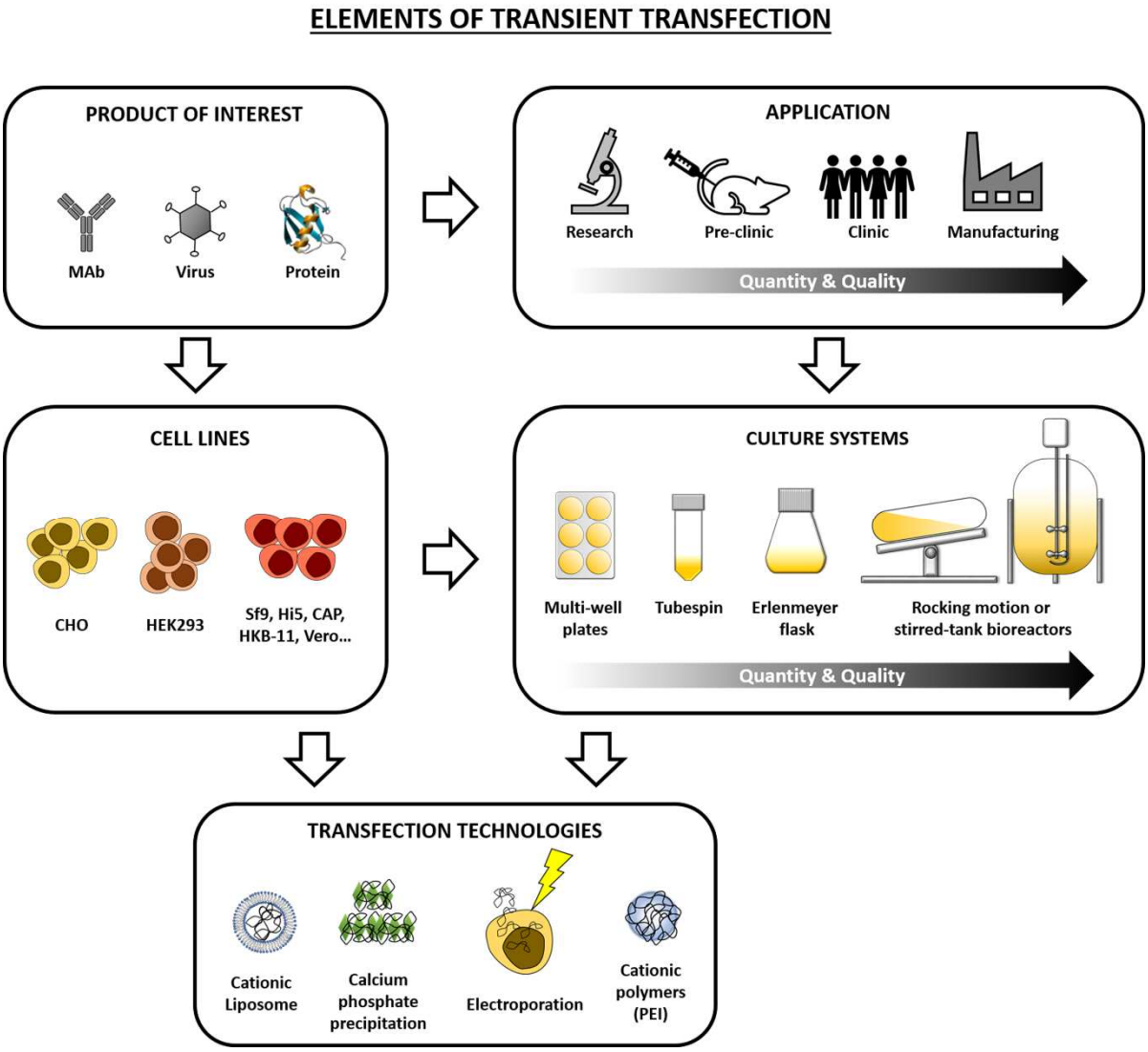

Figure 1. Elements of transient transfection. The type of product required usually influences the selection of the cell line, as for instance, CHO are often preferred for monoclonal antibodies, whereas HEK293 are normally chosen for production of viruses. The selection of the cell line will also depend on its availability, the existence of a GMP bank, or the licensing opportunities. The application and amount of product required will determine the culture systems (from multi-well plates to large-scale bioreactors). Finally, the transfection methodology will highly depend on the cell line, the working scale, and the final application of the product.

CHO cell lines are the workhorse for the production of many of the commercially available pharmaceutical molecules, most of which are monoclonal antibodies [18,31–34]. Maintaining the same cell line at early research stages and manufacturing level guarantees consistency in product quality from development to clinic [35,36]. Consequently, CHO has

become a platform of choice for transient expression of proteins in the early screening phases. CHO cells grow in suspension in serum-free media to high concentrations, they are able to perform human-like post-translational modifications, and have a well-known regulatory track record [25]. Thus, they represent a suitable candidate to transiently express proteins at small and large scales.

Insect cells have been extensively used for expression of proteins, VLPs and gene therapy vectors by baculovirus expression vector system (BEVS) [37]. The first step before baculovirus infection is transfection of the insect cells with the modified virus genome to generate the viral stock. In general, liposome-mediated transfection is used in this step due to its high efficiency [38]. However, some efforts have been made to avoid the use of baculovirus in protein expression as it has some important limitations, regarding baculovirus stock generation and downstream processing. To that end, PEI-mediated transfection protocols have been set [39–42]. Upon optimization of critical variables influencing transfection, high transfection efficiencies (~90%) were achieved in Sf9 and Hi5 cells. However, the protein yields achieved by TGE were not superior to those attained by BEVS (100-150 µg/mL vs. 100 mg/mL). In general, TGE in insect cells has not been extensively pursued to date.

Novel cell lines have been developed in the last years, which have also been used for transient expression purposes. That is the case of CAP-T cell line, property of Cevec Pharmaceuticals (Cologne, Germany), PER.C6 from Crucell (Leiden, The Netherlands), EB66 from Valneva (Lyon, France) or AGE1.CR from ProBioGen (Berlin, Germany) (reviewed in [43]). These cell lines were initially generated to allow the replication of viruses, however, they may also offer an alternative to the classical HEK293 or CHO for other specific applications. For example, CAP-T cells are able to produce difficult-to-express proteins that could not be successfully expressed in HEK293 [44]. On the other hand, the use of PER.C6 and avian-derived cell lines have not been yet fully explored for transient transfection. Nonetheless, their potential to express proteins is reflected in the successful transfection of adherent PER.C6 with calcium phosphate for the production of adenoviruses [45], and the successful expression of MAbs in stable avian cell lines [46]. HKB11 is a human cell line fruit of the fusion between HEK293S and Burkitt's lymphoma derivative 2B8 cells. It was first established by Cho et al. [47] and was demonstrated to be superior to HEK293S and CHO for transient protein production mediated by electroporation and a cationic lipid reagent. Adherent Vero cells cultured on microcarriers were also successfully transfected with PEI, although transfection efficiency was low (~10%) because the transfection conditions were not specifically optimized for this cell line [48].

Notably, protein yields have improved since the first transient transfections in HEK293 and CHO. Some examples from the early 2000's range between 1-20 mg/L of protein [16,18,49–52], and more recently titers of 1 g/L for HEK293 [53] and 2 g/L for CHO [33] have been reported. The efforts made in the development and optimization of new protocols have resulted in yield improvement of 1000-fold. However, it should not be neglected that yields reached by stable cell lines are still much higher (5-10 g/L and up to >20 g/L, [54]), even though they harbor only one to a few copies of the gene of interest, in contrast to thousands of gene copies in the cells after transient transfection. Therefore, more efforts are required towards understanding the intracellular molecular events after transfection, which should enable to further improve the yields of TGE in a rational fashion. Some examples of these studies are already reported [55–57].

2.2 Transfection technologies

DNA delivery into cells for recombinant protein expression has been achieved by different methodologies, which will be addressed briefly since they have been recently reviewed by Jäger *et al*. [23].

Currently, the most widely used method and the main focus of this review is PEI-mediated transfection, described in 1995 by Boussif *et al*. [15]. Its many advantages (non-expensive, good transfection efficiencies) make PEI a suitable choice to achieve high yields of recombinant protein production, especially when the goal is to scale-up the process. On top of that, PEI was first produced as a bulk chemical (mainly for applications in chemical processes [58]), but now GMP compliant versions of PEI are available (for instance, PEIpro®-HQ from Polyplus transfection, Illkirch, France). This represents a significant step towards GMP clinical material production by transient transfection (see Discussion). However, a number of commercially available culture media are not compatible with PEI transfection and therefore medium replacement is often required before transfection. This issue is discussed in more detail below (see section 3.1), since it represents a challenge at large-scale.

Calcium phosphate (CaPi)-mediated transfection was the first reported methodology for large scale transient transfection of suspension cells [59]. Similarly to PEI, CaPi is a non-expensive bulk chemical that enables high transfection efficiencies (40-60%) [50]. However, CaPi transfection has also some medium compatibility issues. Since a critical concentration of calcium is required for transfection, serum-free low-calcium medium formulations are not

suitable for CaPi-mediated transfection, and addition of serum is sometimes required [60]. On top of that, CaPi is especially efficient to transfect adherent cell lines, whereas it promotes aggregation in suspension cell lines [50,61]. In addition, CHO cells are not efficiently transfected with CaPi [18]. Finally, very short incubation times (1-2 minutes) are required for the optimal formation of the CaPi precipitates. This is a limitation for large-scale applications, since delays can often occur in the handling of large volumes [50]. Altogether, these issues have caused PEI-mediated transfection to replace CaPi in the last years.

The use of cationic lipids for gene delivery was first reported by Felgner *et al*. [10] in 1987. Ever since, they have shown great success in transfection of diverse cell lines [62]. This is also reflected in the amount of cationic lipids that are commercially available (reviewed in [23]). The main disadvantage of this type of reagents is their relatively high prices. While lipofection represents an appropriate candidate for low-scale transfection, scale-up is limited by the price of the cationic lipids.

Electroporation is also a successful strategy for low scale transfection (volumes up to 1mL), yielding very high efficiencies. This method has been used to deliver DNA into cell lines as well as primary cells for ex vivo gene therapy with low toxicity profiles [63]. Large-scale electroporation was successfully performed some years ago in COS cells [14]. More recently, flow electroporation has been developed by MaxCyte (Gaithersburg, MD, USA) and it has been scaled-up to 1×1010 cells [32]. However, electroporation needs to be performed in a very specific buffer where cells need to be highly concentrated. Thus, a medium replacement and concentration step before electroporation is required. Sometimes, this step can be seen as a drawback for large scale applications, as it makes the transfection process more complex (see section 3.1).

Other alternative DNA carriers have been used (reviewed in [64]). Some examples of natural polymers used for transfection are chitosans, collagen and gelatin [65]. Others have developed new synthetic polymers as is the case of INVect [66], developed by InVivo Biotech (Hennigsdorf, Germany) which demonstrated to be superior to PEI in CHO, HEK293 and CAP-T cells both in transfection efficiency and protein yields. Chemical modifications of PEI have also been reported. For instance, Thomas *et al*. [67] were able to improve PEI25 and PEI2 transfection efficiencies in COS-7 cells by derivatizing the polycation with different molecules such as alanine or dodecyl groups. In general, these alternative reagents have been tested in small-scale applications to date.

To sum up, PEI is the most widely used transfection reagent for recombinant protein or virus production, especially for large scale applications. Literature reports on the use of PEI for this purpose clearly outnumber the reports of any other methodology. The limitations of the other reagents or techniques are overcome in a great measure by the use of PEI, offering at the same time good transfection efficiencies and production levels all at a very low cost. PEI has become so popular that novel reagents will need to prove themselves highly superior in many aspects to surpass PEI in TGE applications.

2.3 Types of bioreactor

Development and optimization of transient transfection protocols are usually performed at small scale in non-instrumented flasks with non-controlled conditions. The high number of variables influencing transfection processes make small-scale screening the best approach to optimize them [68,69]. Thus, systems that allow high throughput analysis are preferred in early optimization stages of TGE. These types of flasks are usually placed in orbital shakers in incubators with control of temperature, CO2 and humidity, but not pH or dissolved oxygen (DO) control. In this regard, shaken polycarbonate flasks are the preferred system for mammalian cell lines at small scale [29,35,44,70]. Tubespin bioreactors have also been used for screening purposes at low scale. These bioreactors allow to perform medium replacement very easily as they are conical centrifugation tubes [56,69,71]. Transfection optimization using multi-well plates and spinner flasks can be found in literature as well [18,35,72,73].

Once the optimal conditions are set, scale-up of the process usually follows to obtain the desired production. Many examples of successful large-scale transfections have been reported involving a wide variety of bioreactor systems and working volumes. Examples can be found from non-instrumented shaking bottles to more sophisticated stirred tank bioreactors, from 0.1 to 100L.

Among the non-instrumented systems, large shake flasks are a suitable option for straightforward scale-up. Working volumes from 200mL to 1L have been reported [4,40,44,74,75]. The protocols set in small scale do not need to be changed, just scaled-up proportionally, as the system is basically the same. Squared-shaped or cylindrical bottles are orbitally shaken bioreactors that have also been used for transfection purposes with direct scale-up protocols [69,76,77]. Wave bioreactors have been used for transient transfection in

its simplest configuration, controlling temperature but not pH or DO, mimicking the conditions of a shake flask. Haldankar *et al*. [78] used a Wave bioreactor (1 and 10 L) to transfect CHO-S cells, only controlling temperature and DO, but not pH. Abbot *et al*. [79] used a 25L Wave bioreactor to transfect CHO-EBNA-GS cells with no pH or DO control, and many other examples have been reported in the literature [5,20,33,79,80]. In the same way, other authors have used Cell Factories for adherent cells to produce proteins or viral vectors [19,81,82], as a means to increase surface for cell culture and direct scale-up from T-flaks.

Stirred tank bioreactors have also been successfully used for TGE applications, both for CHO and HEK293 cell lines. In some cases the yields could be maintained from the small scale system to the bioreactor, even at such a high volume as 80L [18,30,49,69]. In other cases, lower productivities in bioreactor were reported compared to small scale systems, due in some cases to lower transfection efficiencies [6,48,83–85]. Unfortunately, very often the causes of the reduced transfection efficiencies were not further investigated.

3. Challenges in large scale transfection

The need to produce higher amounts of proteins for research, pre-clinical or even clinical testing forced the development of many scale-up operational approaches for TGE protocols (summarized in Figure 2). However, the implementation of TGE at large scale in bioreactors, achieving at least the same productivities than at small-scale still poses some challenges. A critical review of some of these challenges and solutions follows, focusing mainly in PEI-mediated transfection of suspension cell lines. Table 2 gathers some relevant examples of large-scale transient transfection processes from the literature.

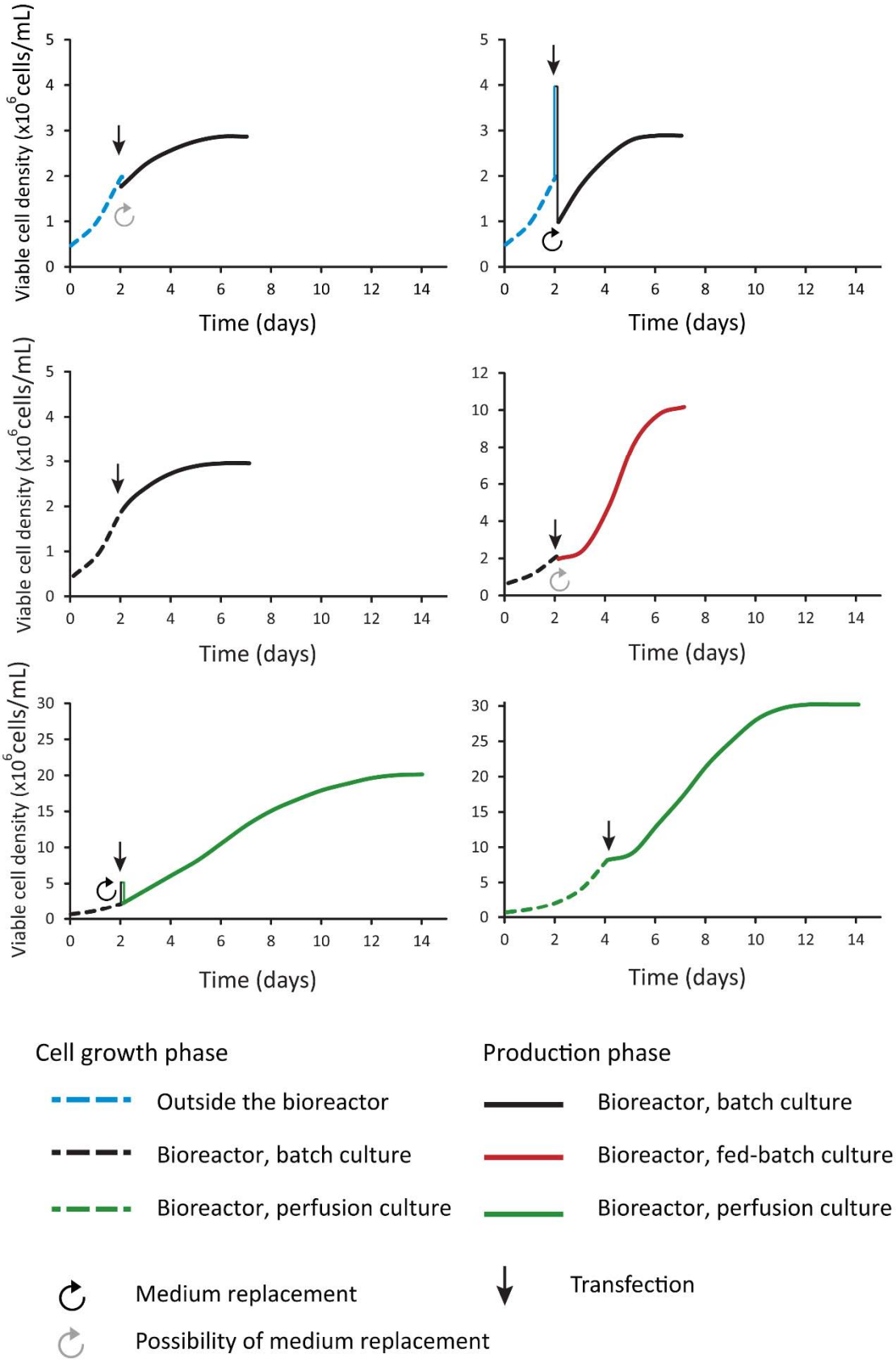

Figure 2. Operational approaches for large-scale transient transfection in bioreactor. Batch, fed-batch and perfusion strategies can be combined before and after transfection, including or not a medium replacement step, to achieve the highest yield for every system.

Table 1
Examples of large scale transient transfection

Cell line	Reactor type	Working volume	Agitation	DO control	pH control	Seed train in the BR	Medium replacement	Cell density	DNA concentration	DNA:PEI ratio	Complexation	Product	Titer	Titer vs small scale	Ref.
HEK293	Wave	10L	ns	ns	ns	Yes (48h)	Dilution	1×10^6	1.5 µg/10^6 cells	1:2	Yes	Adeno-associated vectors	>1×10^{13} vg/mL	~100%	[20]
293SF-3F6	Stirred-tank	3L	80 rpm	40%	7.15	Yes	No	nd	nd	nd	nd	Influenza VLPs	0.5 µg/mL of HA	>100%	[30]
CHO K1SV GS KO	Shake flask	2L	200 rpm	none	6-8% CO_2	No	Yes	4×10^6	3.2 µg/mL	1:1.5	No	Antibodies	1 g/L	~100%	[75]
CHO-EBNA-GS	Wave	13L	25 rpm, 7°	none	5% CO_2	Yes	No	1×10^6	0.5 µg/mL	1:9	No	RON (ECD) his tagged	nd	>100%	[79]
CHO-EBNA-GS	Wave	6L	ns	ns	ns	Yes	No	1×10^6	1 µg/mL	1:7	Yes	MAbs	1.8 g/L	~90%	[33]
HEK EBNA SF and Vero	Stirred-tank	1.5L	30 rpm	30%	7	Yes	Yes	0.8-1.2×10^6	30 µg/mL	1:10	Yes	GFP	(17% GFP+ cells)	~20%	[48]
HEK293-6E	Wave	5L	25 rpm, 8°	ns	ns	Yes (48h)	No	1.5-2×10^6	0.75 µg/mL	1:3	Both, direct and indirect with similar results	MAb	120 mg/L	ns	[5]
CHO-DG44	Squared shaped bottle	3L	110 rpm	none	5% CO_2	No	Yes	12×10^6	2 µg/mL	1:4	Yes	MAb	50 mg/L	~100%	[86]
HEK293SF-3F6	Stirred-tank	2.7L	85 rpm	40%	7.1-7.2	Yes (48h)	Yes	4.5×10^6	1 µg/10^6 cells	1:2	Yes	Lentiviral vector	8×10^7 tu/mL	~80%	[83]
CHOK1SV	Wave	10L	20 rpm	ns	5% CO_2	No	Yes	0.5×10^6	1 µg/mL	1:3	Yes	MAb	66 mg/L	~80%	[84]
HEK293E	Glass round bottle	2L	110 rpm	none	5% CO_2	No	Yes	20×10^6	50 µg/mL	1:2	No	IgG	860 mg/L	~78%	[77]

Table 1 continued

Cell line	Reactor type	Working volume	Agitation	DO control	pH control	Seed train in the BR	Medium replacement before transfection	Cell density at transfection	DNA concentration	DNA:PEI ratio	Complexation	Product	Titer	Titer vs small scale	Ref.
HEK293 EBNA 1	Stirred-tank	1.6L	100 rpm	50%	7.2	Yes (5 days)	Yes	10×10^6	0.6 µg/10^6 cells	1:3	Yes	EPO	6×10^3 IU/mL	ns	[87]
HEK293-EBNA	Stirred-tank	100L	40 rpm	50%	7.2	No	Yes	1×10^6	0.8 µg/mL	1:2.5	Yes	IgG	7 mg/L	ns	[6]
CHO DG44	Stirred-tank	80L	150 rpm	20%	7.1	No	Yes	2×10^6	2.5 µg/mL	1:4	Yes	IgG	22 mg/L	~100%	[69]
CHO DG44	Orbital shaker bioreactor	30L	90 rpm	none	6.8-7	No	Yes	2×10^6	2.5 µg/mL	1:4	Yes	IgG	30 mg/L	~100%	[76]
HEK293-6E	Stirred-tank	3L	73 rpm	40%	7.2	Yes (48h)	No	0.9×10^6	1 µg/mL	1:3	Yes	Lentiviral vector	1.2×10^6 IVP/mL	~100%	[88]
CHO-S	Wave	10L	Set by DO	40-50%	5% CO_2	No	Yes	1×10^6	1 µg/mL	1:3	Yes	IgG	7.2 mg/L	~100%	[78]
HEK293 and HEK293T	Spinferm bioreactor	2L	60 rpm	30-50%	<6.9	Yes (24-48h)	Yes	0.5×10^6	3 µg/mL	1:0.9	Yes	Adeno-associated vector (serotype 2)	10×10^{11} infectious particles	>100%	[61]
HEK293.EBNA	Wave	10L	18 rpm, 7°	ns	ns	No	No	1.4×10^6	1 µg/mL	1:2	Yes	r-protein	9 mg/L	75%	[89]
CHO DG44	Stirred-tank	13L	200 rpm	20%	7.1	No	Yes	2×10^6	2.5 µg/mL	1:2	Yes	IgG	8 mg/L	125%	[18]
HEK293SFE	Stirred-tank	10L	70rpm	40%	7.1	Yes (24h)	No	0.5×10^6	1 µg/mL	1:2	Yes	Tie-2 and Neuropilin-1	ns	ns	[51]

Table 1 continued

Cell line	Reactor type	Working volume	Agitation	DO control	pH control	Seed train in the BR	Medium replacement before transfection	Cell density at transfection	DNA concentration	DNA:PEI ratio	Complexation	Product	Titer	Titer vs small scale	Ref.
HEK293E	Stirred-tank	3L	70 rpm	40%	7.2	Yes	No	0.5×10^6	1 µg/mL	1:2	Yes	SEAP	20 mg/L	200%	[49]
HEK293 EBNA	Stirred-tank	23L	ns	ns	Ns	Yes (24h)	No	0.5×10^6	0.2 µg/mL	1:4.5	Yes	TNFRp55	1.2 µg/mL	Lower (ns)	[16]

rpm: revolutions per minute; ns: not specified; vg/mL: vector genomes/mL; HA: hemagluttinin; tu: transducing units; IU: infectious units; IVP: infectious viral particles

3.1 Medium replacement steps

A complete medium replacement is often performed before transfection regardless of the cell type or product. When performed at low scale (6-well plates, Erlenmeyer or spinner flask, Tubespin, etc.), this step is as easy as a low speed centrifugation and pellet resuspension in fresh medium. There are three main reasons to perform the transfection in fresh instead of conditioned medium. First, cells perform better with fresh medium, show better viabilities and productivities, basically because they have more nutrients available. Second, conditioned medium contains cell by-products that may partially or totally inhibit the transfection process. Cervera *et al*. [90] demonstrated that medium exchange before transfection of HEK293SF-3F6 cells increased HIV-1 VLP production by 1.5-fold, compared to transfection in conditioned FreeStyle293 medium. Rajendra *et al*. [56] performed a complete medium replacement before transfection of CHO cells to avoid the use of conditioned ProCHO medium. Other examples can be found in the literature [34,77,84]. Third, there is a recurrent problematic of incompatibility of media suitable for cell growth and PEI transfection at the same time. A wide variety of commercial chemically-defined serum-free media for animal cell culture have been developed in the last years. Although their composition remains non-disclosed, they are GMP compliant and thus, accepted for production of biotherapeutics. However, very few of these media are compatible with PEI transfection. Media that support good cell growth (high cell densities, high viabilities, growth as single cells) usually contain compounds that hinder transfection. On the other hand, media that allow transient transfection usually promote cell aggregation and do not support high cell densities. 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 cell entry [90]. Other examples of these incompatibilities can be found in the literature [55,68,75,91].

Upscaling this medium exchange step by centrifugation is labor-intensive and it is associated with potential contamination risks. Alternative strategies have been proposed, aiming either to avoid the medium exchange, or to reduce contamination risk by retaining the cells inside the bioreactor.

First of all, in order to avoid medium replacement, media formulations compatible with both cell growth and transfection have been pursued (see Table 1). For instance, FreeStyleF17 and FreeStyle293 media (ThermoFisher Scientific, Whaltam, MA, USA) were used to transfect HEK293 cells in bioreactor without previous medium replacement

[5,20,88,92]. In the case of CHO cells, CD-CHO medium (ThermoFisher Scientific) is a formulation compatible with growth and transfection [79]. However, it is not trivial to find a compatible medium, and therefore alternative solutions are required. Backliwal *et al.* could overcome ExCell medium inhibition by transfecting HEK293 cells at 20×10^6 cells/mL and using very high amounts of DNA and PEI (25 and 50 µg/mL, respectively) [77]. Alternatively, diluting the conditioned medium with fresh medium before transfection helps reducing the concentration of cell by-products and at the same time the nutrient availability for transfection is enhanced. In addition, feeding the culture with rich medium formulations after transfection is a common practice to increase the productivity, since media compatible with transfection may not be rich enough to support high cell densities and protein production. Both strategies are operationally simple at large-scale [20]. A compatible medium was developed for CAP-T cells by the supplementation of FreeStyleF17 (compatible with transfection, but not with cell growth) with low percentages of PEM (necessary for cell growth, but incompatible with transfection), achieving high transfection efficiencies and VLP titers upon transfection, without the need of a medium replacement (Gutiérrez-Granados *et al*., in preparation).

The use of adherent cell lines for transfection facilitates the medium exchange step. Ausubel *et al*. [19] performed 10L transient transfection of adherent HEK293T cells in 10-layer trays for the production of GMP-grade lentiviral particles, with medium replacement steps before and after transfection. Since cells are adhered to the tray surface, the medium can easily be harvested. Zhao *et al*. [93] proposed to perform large-scale transient transfection of adherent HEK293T cells using a CompacTSelecT automatic system. In this case, a robot performed medium replacement by pouring media into a waste receptacle while cells kept attached to the culture flasks. Nevertheless, the use of adherent cell lines is not desirable in large scale applications. Increasing the surface needed to attach higher quantities of cells can be considered more as a scale-out than a scale-up process. Manipulation becomes highly time-consuming and requires more equipment, which translates into larger time investment (or more human resources), and larger footprint [21]. Alternatively, adherent cells can be cultured in microcarriers or solid beds in bioreactors. This allows scale-up of the culture and at the same time, easy retention of the cells for medium replacement [48,80], although working with microcarriers can be to some extent cumbersome. Alternatively, the novel iCeLLis® fixed-bed bioreactor from PALL, has been successfully used for the production of AAV viral vectors by transient transfection in adherent HEK293T/17 cells. The authors demonstrated high and homogenous transfection efficiencies

throughout the bed and competitive AAV yields [94]. This system is scalable up to 500 m2 (equivalent to 3000 roller bottles).

When medium replacement cannot be avoided in bioreactor cultures of cells in suspension, several alternatives may be used. Some authors grow the cells outside (1L bottles, shake flasks, spinner flaks, etc) and perform a complete medium replacement before transferring the cells into the bioreactor. In this case, the process is simplified since the seed train is performed outside the bioreactor [50,69,78]. Tuvesson *et al*. [6] used a Centritech Lab II continuous centrifuge to replace the medium and transfer the cells from the seed train 20L bioreactor to the 100L bioreactor prior to transfection. Ansorge *et al*. [83] performed HEK293 cell growth up to 4.5×10^6 cells/mL in a 3L stirred tank bioreactor in perfusion mode, using an acoustic filter to retain cells inside the bioreactor. Thus, medium was fresh before transfection while centrifugation was avoided. Sun *et al*. [87] also used a perfusion strategy with a spin filter device to grow cells up to 1×10^7 cells/mL before transfection, performing high cell density transfection with fresh medium. Warner [95] observed that the presence of 50% of conditioned and 50% of fresh medium yielded similar productivities than 100% of fresh medium. For this reason, a replacement of half the medium was performed in a 3L stirred tank bioreactor before transfection of CHO cells, using a tangential flow filtration system.

In the author's opinion, technology has to move towards achieving media compatible with high cell density growth and transient transfection at the same time. This would solve the need of medium replacement before transfection, remarkably simplifying operation in bioreactors. On the other hand, media replacement after transfection provides some advantages. The removal of excess PEI would be a clear benefit, as cell toxicity would be decreased [48] (Kadlevova *et al*. [55] showed that around 70% of the PEI molecules remain in the supernatant after transfection). The addition of nutrients would also be beneficial, as higher productivities would be achieved. In addition, it would allow for a more continued product harvest, rather than at final point, which is often beneficial for product quality and stability [20]. In this regard, focusing on the development and optimization of methodologies that allow a relatively fast medium replacement in the bioreactor with minimum invasion of the culture will be key to success.

Table 2
Cell culture media compatible with transient transfection

Cell culture medium	Composition	Additives	Cell line	Fresh or conditioned?	Cell density at the time of transfection (cells/mL)	Feeding post-transfection	Product	Ref.
FreeStyle293 (ThermoFisher Scientific)	AOF, CD, PF, SF (Non-disclosed)	Pluronic F-68 (0.1%), r-Insulin (19.8 mg/L), r-Transferrin (1.6 mg/L), In-house developed lipid mixture (0.9X)	HEK293SF-3F6	Conditioned (24-48h) or fresh	2×10^6	-	HIV-1 Gag-GFP Virus-like particles	[90]
		Pluronic F68 (0.1%), Glutamine (4mM) and Geneticin G148 (25 ug/mL)	HEK293-6E	Conditioned (24h)	1×10^6	Peptone TN-1 (0.5%) (24hpt)	Secreted interleukins	[92]
		-	HEK293-EBNA1	Fresh	10×10^6	Perfusion with SFMII supplemented with aminoacids, glutamine and glucose	EPO	[87]
		Pluronic F68 (0.1%), Glutamine (4mM) and Geneticin G148 (25 ug/mL)	HEK293-6E	Conditioned (48h)	1×10^6	Total medium replacement 3, 4 and 5 days post-transfection	Lentiviral vectors	[88]
FreeStyle F17 (ThermoFisher Scientific)	AOF, CD, PF, SF (Non-disclosed)	Pluronic F68 (0.1%), Glutamine (4mM) and Geneticin G148 (25 ug/mL)	HEK293-6E	Conditioned (48h)	$1.5\text{-}2\times10^6$	Peptone TN-1 (0.5%) (24-48hpt)	SEAP, GFP, chimeric B72.3 antibody	[5]
		Pluronic F68 (1g/L), Glutamine (4mM) and Geneticin G148 (25 ug/mL)	HEK293-6E	Conditioned (48h)	$\sim2\times10^6$	Peptone TN-1 (0.5%) and 1 volume of fresh culture medium (48hpt)	scFV-Fc antibody	[29]
		Pluronic F68 (0.2%)	HEK293	Conditioned (48h) and diluted with 1.125 volumes of fresh medium	2×10^6	10% of CDM4HEK293 medium (3hpt)	AAV vectors	[20]

Table 2 continued

Cell culture medium	Composition	Additives	Cell line	Fresh or conditioned?	Cell density at the time of transfection (cells/mL)	Feeding post-transfection	Product	Ref.
SFM4Transfx293 (HyClone)	AOF, SF (Non-disclosed)	-	HEK293SF-3F6	Fresh, perfusion rate before transfection 1 reactor volume per day	4.5×10^6	Perfusion with medium and single addition of sodium butyrate 16 hpt (5 mM)	Lentiviral vectors	[83]
		Glutamine (4-6 mM)	HEK293SF-3F6	ns	ns	ns	Influenza VLPs	[30]
Ex-Cell293 (Sigma-Aldrich)	APF, SF (Non-disclosed)	Glutamine (4mM)	HEK293E	Fresh	20×10^6	Dilution in ExCell medium 4hpt to 1×106	IgG	[77]
In house developed media blend CD-CHO/DMEM/F12	DMEM and F12: CD, SF	L-Glutamine (8 mM)	CHO-S	Fresh	1×10^6	Unespecified feed 2, 4 and 6 dpt	IgG1	[78]
CD-CHO (ThermoFisher Scientific)	AOF, CD, PF, SF (Non-disclosed)	-	CHO-EBNA-GS	Conditioned (24h)	1×10^6	Peptone TN-1 (0.5%) (24hpt)	RON(ECD)-His tag	[79]
CHO-TNX	Propietary blend of media	N,Ndimethylacetamide (0.125%)	CHO K1SV GS KO	Fresh	4×10^6	Propietary feed solutions (2-4 hpt)	54 antibodies	[75]
Low-calcium hybridoma serum-free medium (ThermoFisher Scientific)	LP, SF (Non-disclosed)	Pluronic F-68 (0,1%), HEPES (10mM) and BCS (1%)	HEK293E	Conditioned (24h)	0.5×10^6	ns	SEAP	[49]
RPMI 1640	Defined and public	Pluronic F-68 (1%), HEPES (25mM) and L-Glutamine (4mM)	HEK293E	Fresh	2×10^6	Dilution in ExCell medium 4hpt to 1×10^6	IgG	[53]

Table 2 continued

Cell culture medium	Composition	Additives	Cell line	Fresh or conditioned?	Cell density at the time of transfection (cells/mL)	Feeding post-transfection	Product	Ref.
Ham's F12-based medium	Defined and public	-	CHO-K1 and CHO-DKO	Fresh	1.2×10^6	Proprietary solution containing hydrolysates, amino acids, salts, and glucose (25%, 24 hpt).	IgG	[96]
DMEM	Defined and public	ITS-X (4mL/L), FBS (1%)	HEK293	Fresh	2X final concentration after dilution	FreeStyle293 +ITS-X (4mL/L) (1.5hpt)	IgG1	[91]
		ITS-X (4mL/L)	CHO	Fresh	2X final concentration after dilution	ProCHO 5+ITS-X (4mL/L)+ Glutamine (4mM) (3hpt)	IgG1	
SF-medium (Bayer corporation)	Non-disclosed	-	HKB11	ns	1×10^6	Dillution with SF-medium 3 and 7 dpt	Tissue factor and Factor VIII	[47]

AOF: Animal origin-free; CD: chemically-defined; PF: Protein-free; SF: serum-free; LP: low-protein; APF: animal protein free; ns:non-specified; hpt: hours post-transfection; dpt: days post-transfection

3.2 Large scale high quality plasmid production

Plasmid DNA is one of the major limiting factors for large scale transient transfection, since obtaining milligrams of high quality plasmid has a relevant economic impact in the process. Production of plasmid DNA is non-expensive itself, as it consists in transforming bacteria (usually E. coli) with the desired plasmid and culturing them using standard lab equipment and methodologies. Importantly, the plasmid needs to be optimized for replication in bacteria, in order to obtain high copy numbers. However, limitations usually arise in plasmid purification, since such processes can be to some extent expensive and will determine plasmid quality. Plasmid DNA quality is an important factor influencing transfection efficiency. A260/A280 ratio (~1.8-2) and the percentage of supercoiled plasmid (~70-90 %) are the most usual parameters that determine plasmid quality. Endotoxin removal is desirable when a final human application is pursued, but it seems not to affect transfection efficiency [97–99]. For low scale transfection, micrograms of plasmid DNA are needed, which can be easily obtained with commercial purification kits based on anion-exchange chromatography. These kits guarantee that the final product will meet the required quality characteristics. However, for large-scale transfection milligrams to grams of DNA are required and scale-up of such purification methods is limited and costly.

Alternatively, plasmid purification can be addressed by precipitation, which is less expensive and more scalable. Moreover, it has been demonstrated to be efficient in the generation of high quality plasmid for transient transfection [97,100]. Another alternative was proposed by Sun *et al*. [87], which consisted in the re-use of commercial single-use chromatography columns. By using one column more than 10 times for two months, they were able to obtain 8.1 mg of plasmid DNA (compared to 0.9 mg of a single use), and they did not observe any loss of plasmid quality or transfection efficiency. Similarly, Püngel *et al*. [98] proposed the use of an anion exchange chromatography column that can be re-equilibrated and re-used several times.

Several authors have tried to overcome this limitation focusing on the reduction of DNA quantities instead. Abbot *et al*. [79] transfected CHO at low cell densities, allowing them to use low DNA concentrations. After three days post-transfection the culture temperature was decreased to 30°C and the same yield as transfecting high cell density cultures with higher amounts of DNA was achieved. With the aim of reducing the amount of plasmid DNA used for complex formation, it is common to substitute it with filler or stuffer DNA. This is unspecific DNA usually derived from salmon or herring sperm that is able to form complexes

with PEI, and it is commercially available at low cost. Rajendra *et al*. [74] observed that reducing the plasmid DNA by 67% resulted in only a reduction of 25% of product titer. They even observed that complexes containing stuffer DNA yielded better transfection efficiencies, although the mechanisms of this phenomenon were not further investigated. Therefore, a reduction in the amount of coding plasmid does not affect transfection efficiency nor product titers as long as total DNA amount is optimal for complex formation [44,55,56].

Finally, other authors have proposed optimizing the upstream step of bioprocessing for the cost-effective large-scale production of plasmid DNA. Cheng *et al*. [97] proposed a fed-batch E. coli fermentation followed by a low-cost precipitation-based purification with high yields of high quality DNA. The DNA was efficiently transfected in HEK293 cells with better transfection efficiencies than DNA purified with commercial kits. Ramirez *et al*. [101] proposed the use of an engineered E. coli strain that utilized glucose more efficiently than the parental strain, and a slow glucose release strategy to enhance plasmid DNA production by 89%.

In summary, minimizing the DNA quantity in transfection as well as improving the production yields and purification efficiency are key strategies to reduce the economic impact of the DNA in transfection, especially at larger scales.

3.3 DNA-PEI polyplex formation

As mentioned earlier, DNA molecules require vehicles, either of viral or non-viral nature, to be efficiently introduced into cells. Among the different vectors available, PEI is the most popular DNA carrier for transient expression of proteins in host cell lines. For this reason, this section focuses on PEI-DNA complex formation.

PEI is a cationic polymer able to condense DNA molecules by electrostatic interactions and form complexes (polyplexes) with a net positive charge. The degree of condensation depends on the ratio of the two molecules. Polyplexes are able to interact with heparan sulfate proteoglycans at the cell membrane (negatively charged), and enter into the cells by endocytosis [57,102]. Once inside the cells, complexes are able to escape from the late endosomes by the so-called “proton-sponge effect”, and travel to the nucleus [15]. Complex entry into the cells occurs within about 1 hour after transfection, is highly efficient since most

of the cells have complexes inside after transfection, and seems to be independent of cell physiological state or cell cycle phase [57,103].

The next step is the entry of the polyplexes into the nucleus. Diverse hypotheses are found in the literature regarding this step. Some authors consider that complexes enter the nucleus by passive mechanisms, for example, when nuclear membrane breakdown occurs during cell division. For this reason, these authors claim that transfection is dependent on the cell cycle, and report strategies of cell synchronization at S or G2/M phase before transfection to enhance protein yields [104–106]. Other authors claim that the transport into the nucleus is active, and that only complexes of a certain size are able to enter and deliver DNA for transcription [103]. Finally, some believe that complexes enter the nucleus both by active and passive transport [107,108]. Most of these authors agree in the fact that complex entry into the nucleus is a bottleneck in the transfection process.

Several authors have studied complex formation kinetics using different conditions and techniques such as atomic force microscopy (AFM), transmission electron microscopy (TEM) or dynamic light scattering (DLS). Complex size increases over time between 20 and 1800 nm, while the typical time of incubation of 10-20 minutes yields complexes of around 300-400 nm [55,58,103]. Complex size and polydispersity seem to play a role in transient transfection [55,109]. Moreover, incubation time influences the degree of association between DNA and PEI. Since DNA needs to be released from the polyplex to be transcribed, the stronger the association the poorest the protein expression [103]. Hence, polyplex incubation time highly influences transfection efficiency and transcription, and therefore it is a critical parameter for optimizing transient gene expression.

The great majority of the published protocols perform a pre-complexation step before transfection [30,34,78,90,92,95]. Briefly, DNA and PEI are dissolved separately in the transfection media (culture media, NaCl solution, etc.). Next, the PEI solution is added to the DNA solution and they are thoroughly mixed (usually vortexed), previous to an incubation period of 10-20 minutes. After this incubation time, the complexes are added to the cells. This is a fairly simple protocol for small scales, since mixing small volumes is not problematic, incubation time can be easily controlled, and complex addition is just a pipetting step. On the contrary, large volume manipulation can cause delays in complex addition to the bioreactor, having an impact in complex size distribution and consequently, in transfection efficiency. Additionally, mixing DNA and PEI solutions at larger volumes is performed by manually swirling the bottles, which could also lead to undesired variability. To overcome these time

limitations, Daramola *et al*. [33] observed that the size of the complexes was more stable over time when performed in a NaCl solution, instead of culture medium. Therefore, NaCl allowed having reproducible complex characteristics at larger scales, when complex incubation time is not so easily controlled.

Some protocols based on direct transfection have been reported in the last years. They consist in direct addition of a DNA solution and a PEI solution to the culture, without the previous pre-complexation step [41,68,71,77,79,87]. The great advantage of this strategy would be the simplification of the protocol, which could have a relevant impact at larger scales. Addition of DNA and PEI separately could reduce the variability associated to large volume manipulation in bioreactors. For instance, Rajendra *et al*. [75] demonstrated that addition of PEI from 0 to 60 minutes after addition of DNA resulted in reproducible production yields, providing a very flexible protocol for large-scale transfections. On the other hand, when complexes are formed in situ, many new variables may play a role: agitation, the presence of cells, pH, osmolality, etc. Such number of variables could lead to non-reproducible complex characteristics from small shake flasks to bioreactors or even batch-to-batch differences. Therefore, a deep characterization of the influence of the environment on complex formation and characteristics would be necessary in order to achieve high reproducibility.

To sum up, the nature of the plasmid, gene, product, host cell, scale, etc. will heavily influence the molecular mechanisms of the transfection process. This may explain that both approaches (pre-complex formation and direct addition) have generated satisfactory results in different systems, although with high case-to-case variability. Therefore, the best option will need to be experimentally tested for every platform. For both strategies, it is critical to determine the parameters that influence the characteristics of the complexes in order to keep them constant. This would be especially interesting for applications of TGE to industrial GMP-compliant processes where QbD (Quality by Design) is key (see Discussion).

3.4 Inoculum preparation and cell density for transfection

Cell inoculum preparation has a great influence on transfection efficiency and productivity. Inoculum must be prepared always in the same way to obtain reproducible results. Commonly, cells are seeded at low cell densities one or two days before transfection,

either with or without medium replacement. This will allow the culture to be in the mid exponential growth phase at the time of transfection [61,79,88,90]. The relevance of cell seeding before transfection is acknowledged in the field and therefore, it is usually well described in the methods section along with the transfection protocols. Moreover, some authors have analyzed the effect of seeding the cells different days before transfection. Rajendra *et al*. [75] observed that seeding CHO cells 1, 2 or 3 days before transfection yielded similar results. Similarly, production was maximized when CAP-T cells were seeded two or three days before transfection, whereas lower yields were obtained when cells were seeded one day before transfection [68]. Müller *et al*. observed that CHO cells needed to be cultured for no more than 1 day prior transfection to obtain optimal protein expression levels [69].

The fact that cells are in the mid-exponential growth phase is closely related to cell physiological state, which is a critical parameter for transfection [57]. The most obvious quantitative parameter indicating cell health is viability, which is often recommended to be over 90-95% at the time of transfection. Other parameters like intracellular nucleotide ratios [72], or cell vitality [68] have been reported to indicate cell population health. Relying on several variables to quantify cell physiological state would help to have more reproducible transfections. This is especially interesting when working with bioreactors, as although cells are able to grow with good viabilities, transfection could be hindered due to cell stress caused by the operational conditions. In such case, the seed train could be also performed in shake flasks, promoting better cell physiological state with milder culture conditions, as proposed by some authors [18,61,78].

Cell age also influences transfection outcomes. Some authors have compared cells at high and low passage numbers and have observed a loss in transfection efficiency and productivity over passages [20,29,72,91]. For this reason, determining the passage range in which the cells have the desired behavior is essential for reproducibility purposes. In this regard, De Los Milagros-Bassani *et al*. [72] observed that the nucleotide ratio was dependent on cell age, and therefore this parameter could become a quantitative criterion to discard aged cells.

Regarding cell density at the time of transfection, a common procedure is to transfect cells at 1-2×10^6 cells/mL. Since the usual maximum cell density attained is 3-5×10^6 cells/mL in batch cultures, and transfection needs to be performed optimally at mid-exponential growth phase, cell density for transfection is often limited to these low values [73,90]. However, high cell density cultures have also been successfully transfected, demonstrating in some cases higher yields than low cell density transfections. High cell densities are usually

achieved by a centrifugation step prior to transfection. Backliwal *et al*. [77] demonstrated that by transfection of HEK293 cells at 20×10^6 cells/mL, antibody yields were improved compared to their standard protocol at 2×10^6 cells/mL. Shen *et al*. [40] concentrated Sf9 cells to 30×10^6 cells/mL before PEI transfection as well. Cells are usually diluted afterwards with richer media formulations that support high cell densities and protein titers (not necessarily compatible with transfection). Alternatively, perfusion strategies have been used to increase maximum cell density (10 and 18×10^6 cells/mL [83,87]) which allowed transfection at higher cell densities (4.5 and 10×10^6 cells/mL) while cells were still in mid-exponential growth phase, increasing the productivities. Implications of applying the mentioned strategies to large-scale bioreactors has been discussed in section 3.1.

In conclusion, cell density at the time of transfection, cell physiological state and inoculum preparation are key parameters influencing transient transfection, and need to be carefully considered in order to achieve maximum yield and reproducibility. In addition, there seems to be a limitation in the cell densities to transfect, having to perform concentration steps when seeking for higher cell densities. Again, studies of the molecular events may allow to determine the drivers of those limitations and design rational strategies to overcome them and improve productivities at higher cell densities. Maybe similar strategies to those employed for infection (cell growth until maximum cell density before infection) could be applied to transfection in the future in order to intensify processes.

3.5 Maximizing productivity

Tremendous efforts have been conducted over the years to increase TGE titers. Maximizing productivity has a major influence in scaling-up. Importantly, the strategies for increasing productivity are often set at small-scale since it is more cost-effective, sparing raw materials and time, and simpler to operate. For this reason, a careful evaluation of cost-benefit should be performed for every strategy set at small scale before scaling it up. Jäger *et al*. [23] reviewed some of the reported methodologies to increase titers in TGE approaches. Here, a critical assessment of different approaches regarding their application to large-scale follows.

The most immediate strategy to maximize productivity upon transient transfection is the study of the variables influencing TGE. The most relevant ones have been already covered:

the type and concentration of transfection reagent, DNA concentration, presence of stuffer DNA, performing or not the complexes before transfection, time of complex incubation, cell culture medium for cell growth, transfection and protein production, influence of the passage number, and cell density at the time of transfection. This high number of variables need to be considered together, since it is likely to miss interactions between them if studied separately. Therefore, the most common practice to optimize the factors influencing TGE is Design of Experiments (DoE) as it allows obtaining statistically significant results with a reduced number of experimental runs [35,68,70,91]. Optimization of cell density at the time of transfection, along with DNA and PEI concentrations (or DNA to PEI ratio) is often performed in TGE, since the optimal levels of each variable are cell line and product dependent.

Cell line engineering has contributed to the improvement of productivities. The most classical example is the generation of cell lines expressing viral antigens that allow the episomal replication of plasmids (see Section 2.1 and [110]). Another more novel approach was recently reported by Daramola *et al*. [33]. They generated a CHO cell line expressing EBNA-1 antigen along with glutamine synthetase (GS) gene. The isolated clones presented an enhanced antibody yield after PEI-mediated transient transfection compared to the wild-type CHO. In fact, they reported the highest yields for transient transfection up to date (2 g/L) with the CHO-EBNA1-GS cell line. Pichler *et al*. [111] reported a strategy for the selection of CHO subclones presenting a phenotype of higher antibody production rates after electroporation. Briefly, after three rounds of transfection and selection of the most productive subpopulations by cell sorting, CHO clones with specific productivities 3-fold higher than the parental cell line were isolated. This strategy opens new directions to investigate which are the molecular mechanisms driving the increase of productivity after transfection. By identifying specific genes responsible for that, targeted mutagenesis could be used to generate new-engineered cell lines with improved characteristics. Other examples of cell line engineering can be found in the literature, especially for CHO cell lines [96,112]. Regarding large-scale production, ensuring the capability of these modified cell lines to grow to high cell densities, as well as maintaining their transfectability and specific productivity in bioreactor would be of much relevance. Stability of the cell line is also a feature to consider for scaling-up, since seed train processes may be long (depending on the final volume needed) and the cell line characteristics need to be constant.

Expression vector optimization improved production yields by 10-fold in HEK293E cells [53]. The combination of a strong promoter (CMV), an artificial intron and the woodchuck

post-transcriptional regulatory element (WPRE) was the optimal approach in this work to enhance antibody titers. However, the optimal vector configuration is specific to cell line and product [29,113]. Modification of expression vectors has minimal implications in large-scale transfection. Nonetheless, the ability of replicating to high copy numbers in bacteria must be at least maintained, if not enhanced, to make the DNA production step as cost-effective as possible.

Temperature shift from the physiological temperature (37°C) down to 30-32°C is a common practice to increase protein titers in CHO cell cultures [75,79,114–116]. Decreasing the culture temperature causes an arrest of the cell cycle in G1 phase, as well as reduced metabolic rates. At the same time, it increases protein productivity. Bioreactors harbor very precise temperature control systems and therefore, the translation of this strategy to large-scale becomes straightforward. Successful scale up (50L) has been reported [76].

Addition of chemical compounds is often employed to increase transfection efficiency and productivity. The most commonly used are inhibitors of the histone deacetylases (HDACi), which upon addition to the cell culture promote histone hyperacetylation which translates in higher DNA transcription. They are usually added 4 hpt, when the DNA is inside the nucleus and transcription starts. Among the most common HDACi are valproic acid and sodium butyrate [44,53,70,83]. Some compounds are reported to increase membrane porosity and, therefore increase transfection efficiency, like DMSO or lithium acetate [70,84]. These are usually added before transfection, followed by a medium replacement after their function is accomplished. Other compounds can be added to arrest cells in a specific cell cycle phase. For instance, nocodazole to arrest cells in G2/M phase or hydroxyurea to arrest them in G1 [104]. Insulin growth factor (IGF) or polar solvents like DMA (N,N-dimethylacetamide) have also been reported to increase productivities [75,115]. Finally, product-specific compounds can be also added to increase the yields, for example Cervera *et al*. [70] supplemented a transfected HEK293 culture for the production of HIV virus-like particles with caffeine, which is known to increase lentivirus yields. These compounds are usually cost-effective, even at large-scales. For this reason, the only implication to consider in this case for large-scale productions is the necessity to remove the compounds out of the final product formulation if a potential commercial application is envisaged.

Regulatory proteins have been co-expressed with the gene of interest to increase protein titers. For instance, Backliwal *et al*. [53] co-transfected HEK293 cells with three plasmids coding for the regulatory proteins p18, and p21, and the acidic Fibroblast Growth Factor

(αFGF). Higher cell viability was observed, as well as an increase in specific antibody productivity. Zustiak *et al*. [35] co-transfected a plasmid coding for the bcl-xL antiapoptotic gene, and a reduction in apoptosis was observed along with increased protein titers. This co-transfection strategy has a minimal impact at large-scale as long as the final amount of DNA is not increased compared to standard transfection. To maintain the same amount of total DNA, Backliwal *et al*. reduced the amount of plasmid coding for the protein of interest to include the plasmids coding for p18, p21 and aFGF. In the case of Zustiak *et al*., the plasmid coding for the bcl-xL gene was added, but it only represented a 10% of the plasmid of interest.

Feeding strategies after transfection have been extensively reported. Rich media formulations supporting high cell densities and high protein productivities are added usually 3-24 hpt, since they often inhibit the transfection process itself. Single medium additions are required 4hpt when the transfection medium does not support proper cell growth and production [5,68]. Other approaches consist in performing several additions of peptones or concentrate feeds during the culture to extend the production time [33,78,115–118]. Addition of medium in a bioreactor is an easy operation that does not add difficulty to the process. Other approaches consisting in medium replacement steps during the production phase have been reported, either using perfusion strategies [83] or batch centrifugation [20,115]. Medium replacement increases product titers as well as expand production time. However, as already discussed in Section 3.1, medium replacement at large-scale can be to some extent cumbersome.

A novel strategy based on repeated transfection was recently reported by Cervera *et al*. [119]. A 12-fold increase of the production of virus-like particles in HEK293 was achieved by repeated medium replacement and re-transfection steps. Work is ongoing to scale-up this approach to bioreactor (Fuenmayor, unpublished work). A careful evaluation of the cost-benefit of this strategy should be carried out, since the amount of DNA and culture medium used are 2-fold and 5-fold increased, respectively compared to the standard batch transfection.

4. New applications and emerging opportunities

Transient Gene Expression has been for many years focused on the production of research-grade material for screening phases of biopharmaceutical product development.

Milligrams to grams of different protein candidates can be obtained within weeks in a very rapid, simple and flexible manner. The classical generation of the stable cell line producing the product of interest has been up to now the workhorse for the production of proteins by mammalian cell hosts. It is reasonable to spend time and resources in the generation of such cell lines when the product of interest is well-defined and high amounts are needed. However, new applications are being pursued in the biopharma industry that will probably change the industrial paradigm. The success of gene therapy, the growing field of personalized medicine or the new challenges faced by vaccine development are some examples of these new applications, characterized by smaller target populations, broader number of therapeutic products and reduced times from genetic sequence to the clinic.

Gene therapy introduced a breakthrough in medicine 30 years ago. Unfortunately, some safety concerns related to the use of viral vectors stopped abruptly the clinical trials. However, gene therapy research continued and around 2008, a new wave of clinical trials started along with the generation of many start-up companies. The success of the field is reflected in the approval in the EU of two gene therapy treatments: Glybera from UniQure (Amsterdam, The Netherlands) in 2012 and Strimvelis from GSK (Brendfort, UK) in 2016. The keys to these successes have been the development of safer vectors along with greater knowledge of the molecular aspects of the diseases [120]. The most widely used vectors for gene therapy are currently adeno-associated vectors (AAV). Moreover, the majority of the vector preparations for clinical trials are produced by transient transfection [21]. TGE is a flexible methodology allowing to change the therapeutic gene or the virus serotype only by changing the plasmid. Moreover, high AAV yields can be obtained and the ratio of infectious vs. total particles are within the accepted standard. In addition, TGE does not require the generation (and validation) of viral stocks (baculovirus, adenovirus or herpes virus) or stable cell lines expressing proteins (packaging or producer cell lines), instead, they require high quality DNA preparation which is a much simpler technology. Finally, downstream processing is also simplified due to the absence of other helper viruses. Considering this, the versatility and the relative simplicity of the protocols make TGE a very suitable methodology to generate a variety of AAV serotypes targeting a variety of diseases in a very rapid manner.

Personalized medicine is a potentially growing field. The knowledge of the human genome allows to diagnose personally and design treatments tailored to every person needs. This mean that instead of having limited number of drugs produced by kg, drug batches will be smaller and more diverse [121,122]. As the variety of drugs grow, and processes are scaled-down, the less effort will probably be dedicated to the generation of stable cell lines.

Production facilities will need to be more versatile and faster to respond to the need of producing small quantities of very different drugs every year. Although it may be still decades until personalized medicine is a reality, Transient Gene Expression will play an essential role in this field, since its main characteristics (versatility and speed) comply with the main requirements of personalized medicine.

In this regard, vaccine development is a field potentially benefiting from TGE features. Although vaccination has been one of the most successful medical applications, the new emerging diseases and recent viral outbreaks (Ebola, Zika, Influenza, Chikungunya, etc.) make necessary some changes in the paradigm of vaccine production. Precisely, faster and more flexible production systems are needed to overcome the challenges arising from these new diseases [123], and TGE meets these requirements.

As the number of drugs produced in one facility will increase, single use bioprocess technologies (up and downstream) will enable the implementation of more versatile facilities to comply with this new paradigm. The need to produce many different drugs in the same facility enhances the need of more reliable and robust cleaning and validation processes to avoid cross-contamination events, which require high resource and time investment without totally eliminating the risk of contamination. Therefore, the most reasonable alternative in this case is to bypass cleaning and validation steps, by the use of single-use equipment. In addition, single-use equipment is generally ready to use and allow to rapidly and safely move from one process to another [124].

Envisioning these potential applications, the next challenge is the approval by regulatory agencies of TGE-derived drugs. The establishment of robust and competitive TGE processes will pave the way to accomplish this goal. The best response to this need will be the rational optimization of these processes, which should be addressed from both intracellular and operational points of view.

There is already a lot of ongoing work to fully elucidate the intracellular events occurring after transfection. Despite the wide variety of protocols used, the development of new techniques for characterization of the product, the DNA-PEI complexes or the monitoring of intracellular events will allow gathering deeper knowledge of transfection processes. The efforts dedicated to the optimization and systematization of operational aspects in large-scale TGE protocols have already retributed better reproducibility and yields. Nevertheless, as discussed over this review there are still some challenges to be faced, such as the development of new media formulations enabling both robust cell growth and transfection,

better understanding of the limitations posed by some bioreactor systems or the cost-efficient generation of large amounts of DNA. The development and implementation of new Process Analytical Technologies (PAT) will enable to establish Quality by Design (QbD) strategies in which the identity of the product will not solely rely on its physico-chemical properties, but also on the reproducibility of all steps of the process [125]. A relevant impact is therefore, expected from all this knowledge regarding yields and reproducibility. Eventually, TGE will become an industrial drug production technology competing with stable and infection-mediated expression in animal cell lines.

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Declaration of interest

The authors report no declaration of interest.

References

[1] Kim TK, Eberwine JH. Mammalian cell transfection: The present and the future. Anal. Bioanal. Chem. 2010;397:3173–3178.

[2] Bandaranayake AD, Almo SC. Recent advances in mammalian protein production. FEBS Lett. 2014;588:253–260.

[3] Nallet S, Amacker M, Westerfeld N, et al. Respiratory syncytial virus subunit vaccine based on a recombinant fusion protein expressed transiently in mammalian cells. Vaccine. 2009;27:6415–6419.

[4] Mignaqui AC, Ruiz V, Perret S, et al. Transient Gene Expression in Serum-Free Suspension-Growing Mammalian Cells for the Production of Foot-and-Mouth Disease Virus Empty Capsids. PLoS One. 2013;8:1–9.

[5] Raymond C, Tom R, Perret S, et al. A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications. Methods. 2011;55:44–51.

[6] Tuvesson O, Uhe C, Rozkov A, et al. Development of a generic transient transfection process at 100 L scale. Cytotechnology. 2008;56:123–136.

[7] Graham FL, van der Eb AJ. A New Technique for the Assay of Infectivity of Human Adenovirus 5 DNA. Virology. 1973;52:456–467.

[8] Graham FL, Smiley J, Russell WC, et al. Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. J. Gen. Virol. 36:59–72.

[9] Lau JT, Pittenger MF, Cleveland DW. Reconstruction of appropriate tubulin and actin gene regulation after transient transfection of cloned beta-tubulin and beta-actin genes. Mol. Cell. Biol. 1985;5:1611–1620.

[10] Felgner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U. S. A. 1987;84:7413–7417.

[11] Pear WS, Nolan GP, Scott ML, et al. Production of high-titer helper-free retroviruses by transient transfection (retroviral packaing cells/gene therapy). Cell Biol. 1993;90:8392–8396.

[12] Wick PF, Senter RA, Parsels LA, et al. Transient transfection studies of secretion in bovine chromaffin cells and PC12 cells: Generation of kainate-sensitive chromaffin cells. J. Biol. Chem. 1993;268:10983–10989.

[13] Pahl HL, Burn TC, Tenen DG. Optimization of transient transfection into human myeloid cell lines using a luciferase reporter gene. Exp. Hematol. 1991;19:1038–1041.

[14] Blasey HD, Aubry J-P, Mazzei GJ, et al. Large scale transient expression with COS cells. Cytotechnology. 1996;18:183–192.

[15] Boussif O, Lezoualc'h F, Zanta MA, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U. S. A. 1995;92:7297–7301.

[16] Schlaeger EJ, Christensen K. Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotechnology. 1999;30:71–83.

[17] Almo SC, Love JD. Better and faster: Improvements and optimization for mammalian

recombinant protein production. Curr. Opin. Struct. Biol. 2014;26:39–43.

[18] Derouazi M, Girard P, Van Tilborgh F, et al. Serum-free large-scale transient transfection of CHO cells. Biotechnol. Bioeng. 2004;87:537–545.

[19] Ausubel LJ, Hall C, Sharma A, et al. Production of CGMP-Grade Lentiviral Vectors. Bioprocess Int. 2012;10:32–43.

[20] Grieger JC, Soltys SM, Samulski RJ. Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. Mol. Ther. 2016;24:287–297.

[21] Clément N, Grieger JC. Manufacturing of recombinant adeno-associated viral vectors for clinical trials. Mol. Ther. Methods Clin. Dev. 2016;3:16002.

[22] Merten OW, Gaillet B. Viral vectors for gene therapy and gene modification approaches. Biochem. Eng. J. 2016;108:98–115.

[23] Jäger V, Büssow K, Schirrmann T. Transient Recombinant Protein Expression in Mammalian Cells. Anim. Cell Cult. Springer International Publishing; 2015. p. 27–64.

[24] Schwarzer J, Rapp E, Hennig R, et al. Glycan analysis in cell culture-based influenza vaccine production: Influence of host cell line and virus strain on the glycosylation pattern of viral hemagglutinin. Vaccine. 2009;27:4325–4336.

[25] Dumont J, Euwart D, Mei B, et al. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. Crit. Rev. Biotechnol. 2015;1:1–13.

[26] Berntzen G, Lunde E, Flobakk M, et al. Prolonged and increased expression of soluble Fc receptors, IgG and a TCR-Ig fusion protein by transiently transfected adherent 293E cells. J. Immunol. Methods. 2005;298:93–104.

[27] Sena-Esteves M, Tebbets JC, Steffens S, et al. Optimized large-scale production of high titer lentivirus vector pseudotypes. J. Virol. Methods. 2004;122:131–139.

[28] Shen CF, Lanthier S, Jacob D, et al. Process optimization and scale-up for production of rabies vaccine live adenovirus vector (AdRG1.3). Vaccine. 2012;30:300–306.

[29] Jäger V, Bussow K, Wagner A, et al. High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. BMC Biotechnol. 2013;13:52.

[30] Venereo-Sanchez A, Gilbert R, Simoneau M, et al. Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate. Vaccine. 2016;34:3371–3380.

[31] Walsh G. Biopharmaceutical benchmarks 2014. Nat. Biotechnol. 2014;32:992–1000.

[32] Steger K, Brady J, Wang W, et al. CHO-S Antibody Titers >1 Gram/Liter Using Flow Electroporation-Mediated Transient Gene Expression followed by Rapid Migration to High-Yield Stable Cell Lines. J. Biomol. Screen. 2015;20:545–551.

[33] Daramola O, Stevenson J, Dean G, et al. A high-yielding CHO transient system: Coexpression of genes encoding EBNA-1 and GS enhances transient protein expression. Biotechnol. Prog. 2014;30:132–141.

[34] Rajendra Y, Kiseljak D, Baldi L, et al. A simple high-yielding process for transient gene expression in CHO cells. J. Biotechnol. 2011;153:22–26.

[35] Zustiak MP, Jose L, Xie Y, et al. Enhanced transient recombinant protein production in CHO cells through the co-transfection of the product gene with Bcl-xL. Biotechnol. J. 2014;9:1164–1174.

[36] Baldi L, Hacker DL, Adam M, et al. Recombinant protein production by large-scale transient gene expression in mammalian cells: State of the art and future perspectives. Biotechnol. Lett. 2007;29:677–684.

[37] Felberbaum RS. The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol. J. 2015;10:702–714.

[38] Grady LM, Bai P, Weller SK. HSV-1 protein expression using recombinant baculoviruses. Methods Mol. Biol. 2014;1144:293–304.

[39] Roest S, Kapps-Fouthier S, Klopp J, et al. Transfection of insect cell in suspension for efficient baculovirus generation. MethodsX. 2016;3:371–377.

[40] Shen X, Hacker DL, Baldi L, et al. Virus-free transient protein production in Sf9 cells. J. Biotechnol. 2013;171:61–70.

[41] Shen X, Pitol AK, Bachmann V, et al. A simple plasmid-based transient gene expression method using High Five cells. J. Biotechnol. 2015;216:67–75.

[42] Buchs M, Kim E, Pouliquen Y, et al. High-throughput insect cell protein expression applications. Methods Mol. Biol. 2009;498:199–227.

[43] Genzel Y. Designing cell lines for viral vaccine production: Where do we stand? Biotechnol. J. 2015;10:728–740.

[44] Fischer S, Charara N, Gerber A, et al. Transient recombinant protein expression in a human amniocyte cell line: The CAP-T® cell system. Biotechnol. Bioeng. 2012;109:2250–2261.

[45] Subramanian S, Kim JJ, Harding F, et al. Scaleable production of adenoviral vectors by transfection of adherent PER.C6 cells. Biotechnol. Prog. 2007;23:1210–1217.

[46] Olivier S, Jacoby M, Brillon C, et al. EB66 cell line, a duck embryonic stem cell-derived substrate for the industrial production of therapeutic monoclonal antibodies with enhanced ADCC activity. MAbs. 2010;2:405–415.

[47] Cho MS, Yee H, Chan S. Establishment of a human somatic hybrid cell line for recombinant protein production. J. Biomed. Sci. 2002;1986:631–638.

[48] Fliedl L, Kaisermayer C. Transient gene expression in HEK293 and vero cells immobilised on microcarriers. J. Biotechnol. 2011;153:15–21.

[49] Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 2002;30:E9.

[50] Girard P, Derouazi M, Baumgartner G, et al. 100-Liter transient transfection. Cytotechnology. 2002;38:15–21.

[51] Pham PL, Perret S, Doan HC, et al. Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: Peptone additives improve cell growth and transfection efficiency. Biotechnol. Bioeng. 2003;84:332–342.

[52] Wright JL, Jordan M, Wurm FM. Transfection of partially purified plasmid DNA for high level

transient protein expression in HEK293-EBNA cells. J. Biotechnol. 2003;102:211–221.

[53] Backliwal G, Hildinger M, Chenuet S, et al. Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res. 2008;36:e96.

[54] Kuystermans D, Al-rubeai M. Antibody Expression and Production. Cell Eng. 2011;7:25–52.

[55] Kadlecova Z, Nallet S, Hacker DL, et al. Poly(ethyleneimine)-Mediated Large-Scale Transient Gene Expression: Influence of Molecular Weight, Polydispersity and N-Propionyl Groups. Macromol. Biosci. 2012;12:628–636.

[56] Rajendra Y, Kiseljak D, Baldi L, et al. Transcriptional and post-transcriptional limitations of high-yielding, PEI-mediated transient transfection with CHO and HEK-293E cells. Biotechnol. Prog. 2015;31:541–549.

[57] Carpentier E, Paris S, Kamen A a., et al. Limiting factors governing protein expression following polyethylenimine-mediated gene transfer in HEK293-EBNA1 cells. J. Biotechnol. 2007;128:268–280.

[58] Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J. Control. Release. 1999;60:149–160.

[59] Jordan M, Köhne C, Wurm FM. Calcium-phosphate mediated DNA transfer into HEK-293 cells in suspension: Control of physicochemical parameters allows transfection in stirred media: Transfection and protein expression in mammalian cells. Cytotechnology. 1998;26:39–47.

[60] Geisse S. Reflections on more than 10 years of TGE approaches. Protein Expr. Purif. 2009;64:99–107.

[61] Park JY, Lim B-P, Lee K, et al. Scalable production of adeno-associated virus type 2 vectors via suspension transfection. Biotechnol. Bioeng. 2006;94:416–430.

[62] Majzoub RN, Ewert KK, Safinya CR. Cationic liposome-nucleic acid nanoparticle assemblies with applications in gene delivery and gene silencing. Philos. Trans. A. Math. Phys. Eng. Sci. 2016;374.

[63] Li L, Shivakumar R, Feller S, et al. Highly Efficient , Large Volume Flow Electroporation. Technol. Cancer Res. Treat. 2002;1:341–349.

[64] Mintzer MA, Simanek EE. Nonviral vectors for gene delivery. Chem. Rev. 2009;109:259–302.

[65] Dang JM, Leong KW. Natural polymers for gene delivery and tissue engineering B. Adv. Drug Deliv. Rev. 2006;58:487–499.

[66] Püngel S, Veiczi M, Welsink T, et al. INVect - a novel polycationic reagent for transient transfection of mammalian cells. BMC Proc. 2013;7:P26.

[67] Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci U S A. 2002;99:14640–14645.

[68] Gutiérrez-Granados S, Cervera L, Segura MM, et al. Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells. Appl. Microbiol. Biotechnol. 2015;100:3935–3947.

[69] Muller N, Derouazi M, Van Tilborgh F, et al. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol.

Lett. 2007;29:703–711.

[70] Cervera L, Fuenmayor J, González-Domínguez I, et al. Selection and optimization of transfection enhancer additives for increased virus-like particle production in HEK293 suspension cell cultures. Appl. Microbiol. Biotechnol. 2015;99:9935–9949.

[71] Xie Q, Xinyong G, Xianjin C, et al. PEI/DNA formation affects transient gene expression in suspension Chinese hamster ovary cells via a one-step transfection process. Cytotechnology. 2013;65:263–271.

[72] De Los Milagros Bassani Molinas M, Beer C, Hesse F, et al. Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring. Cytotechnology. 2014;66:493–514.

[73] Baldi L, Muller N, Picasso S, et al. Transient gene expression in suspension HEK-293 cells: Application to large -scale protein production. Biotechnol. Prog. 2005;21:148–153.

[74] Rajendra Y, Kiseljak D, Manoli S, et al. Role of non-specific DNA in reducing coding DNA requirement for transient gene expression with CHO and HEK-293E cells. Biotechnol. Bioeng. 2012;109:2271–2278.

[75] Rajendra Y, Hougland MD, Alam R, et al. A high cell density transient transfection system for therapeutic protein expression based on a CHO GS-knockout cell line: Process development and product quality assessment. Biotechnol. Bioeng. 2015;112:977–986.

[76] Stettler M, Zhang X, Hacker DL, et al. Novel orbital shake bioreactors for transient production of CHO derived IgGs. Biotechnol. Prog. 2007;23:1340–1346.

[77] Backliwal G, Hildinger M, Hasija V, et al. High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. Biotechnol. Bioeng. 2008;99:721–727.

[78] Haldankar R, Li D, Saremi Z, et al. Serum-free suspension large-scale transient transfection of CHO cells in WAVE bioreactors. Mol. Biotechnol. 2006;34:191–199.

[79] Abbott WM, Middleton B, Kartberg F, et al. Optimisation of a simple method to transiently transfect a CHO cell line in high-throughput and at large scale. Protein Expr. Purif. 2015;116:113–119.

[80] van der Loo JCM, Swaney WP, Grassman E, et al. Scale-up and manufacturing of clinical-grade self-inactivating γ-retroviral vectors by transient transfection. Gene Ther. 2012;19:246–254.

[81] Aydin H, Azimi FC, Cook JD, et al. A Convenient and General Expression Platform for the Production of Secreted Proteins from Human Cells. J. Vis. Exp. 2012;1–7.

[82] Merten O-W, Charrier S, Laroudie N, et al. Large-scale manufacture and characterization of a lentiviral vector produced for clinical ex vivo gene therapy application. Hum. Gene Ther. 2011;22:343–356.

[83] Ansorge S, Lanthier S, Transfiguracion J, et al. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. J. Gene Med. 2009;11:868–876.

[84] Ye J, Kober V, Tellers M, et al. High-level protein expression in scalable CHO transient transfection. Biotechnol. Bioeng. 2009;103:542–551.

[85] Rosser MP, Xia W, Hartsell S, et al. Transient transfection of CHO-K1-S using serum-free

medium in suspension: a rapid mammalian protein expression system. 2005;40:237–243.

[86] Zhang X, Fernandez I, Wurm FM. Hyperosmolarity enhances transient recombinant protein yield in Chinese hamster ovary cells. Biotechnol. Lett. 2010;32:1587–1592.

[87] Sun X, Hia HC, Goh PE, et al. High-density transient gene expression in suspension-adapted 293 EBNA1 cells. Biotechnol. Bioeng. 2008;99:108–116.

[88] Segura MM, Garnier A, Durocher Y, et al. Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification. Biotechnol. Bioeng. 2007;98:789–799.

[89] Geisse S, Henke M. Large-scale transient transfection of mammalian cells: A newly emerging attractive option for recombinant protein production. J. Struct. Funct. Genomics. 2005;6:165–170.

[90] Cervera L, Gutiérrez-Granados S, Martínez M, et al. Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J. Biotechnol. 2013;166:152–165.

[91] Bollin F, Dechavanne V, Chevalet L. Design of experiment in CHO and HEK transient transfection condition optimization. Protein Expr. Purif. 2011;78:61–68.

[92] Carter J, Zhang J, Dang TL, et al. Fusion partners can increase the expression of recombinant interleukins via transient transfection in 2936E cells. Protein Sci. 2010;19:357–362.

[93] Zhao Y, Bishop B, Clay JE, et al. Automation of large scale transient protein expression in mammalian cells. J. Struct. Biol. 2011;175:209–215.

[94] Powers AD, Piras BA, Clark RK, et al. Development and optimization of AAV hFIX particles by transient transfection in an iCELLis® fixed bed bioreactor. Hum. Gene Ther. Methods. 2016;27:112–121.

[95] Warner CM. Rapid, large-scale production of full-length, human-like monoclonal antibodies. (Doctoral Thesis). Keck Graduate Institute of Applied Life Sciences; 2012.

[96] Macaraeg NF, Reilly DE, Wong AW. Use of an Anti-Apoptotic CHO Cell Line for Transient Gene Expression. Biotechnol. Prog. 2013;29:1050–1058.

[97] Cheng L, Sun X, Yi X, et al. Large-scale plasmid preparation for transient gene expression. Biotechnol. Lett. 2011;33:1559–1564.

[98] Püngel S, Veiczi M, Beckmann TF, et al. Reconciling pillars of transient gene expression: From DNA prep via media, reagent and cell line development to holistic process optimization. BMC Proc. 2015;9:P18.

[99] Schmid G, Schlaeger E, Wipf B. Non-GMP plasmid production for transient transfection in bioreactors. Cytotechnology. 2001;35:157–164.

[100] Wright JL, Jordan M, Wurm FM. Extraction of plasmid DNA using reactor scale alkaline lysis and selective precipitation for scalable transient transfection. 2001;165–173.

[101] Ramírez EA, Velázquez D, Lara AR. Enhanced plasmid DNA production by enzyme-controlled glucose release and an engineered Escherichia coli. Biotechnol. Lett. 2016;38:651–657.

[102] Kopatz I, Remy JS, Behr JP. A model for non-viral gene delivery: Through syndecan adhesion molecules and powered by actin. J. Gene Med. 2004;6:769–776.

[103] Han X, Fang Q, Yao F, et al. The heterogeneous nature of polyethylenimine-DNA complex formation affects transient gene expression. Cytotechnology. 2009;60:63–75.

[104] Tait AS, Brown CJ, Galbraith DJ, et al. Transient production of recombinant proteins by Chinese hamster ovary cells using polyethyleneimine/DNA complexes in combination with microtubule disrupting anti-mitotic agents. Biotechnol. Bioeng. 2004;88:707–721.

[105] Grosjean F, Batard P, Jordan M, et al. S-phase synchronized CHO cells show elevated transfection efficiency and expression using CaPi. Cytotechnology. 2002;38:57–62.

[106] Brunner S, Sauer T, Carotta S, et al. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther. 2000;7:401–407.

[107] Grosse S, Thévenot G, Monsigny M, et al. Which mechanism for nuclear import of plasmid DNA complexed with polyethylenimine derivatives? J. Gene Med. 2006;8:845–851.

[108] Tseng WC, Haselton FR, Giorgio TD. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. Biochim. Biophys. Acta - Gene Struct. Expr. 1999;1445:53–64.

[109] Dunlap D, Maggi A, Soria MR, et al. Nanoscopic structure of DNA condensed for gene delivery. Nucleic Acids Res. 1997;25:3095–3101.

[110] Kunaparaju R, Liao M, Sunstrom N-A. Epi -CHO, an episomal expression system for recombinant protein production in CHO cells. Biotechnol. Bioeng. 2005;91:670–677.

[111] Pichler J, Galosy S, Mott J, et al. Selection of CHO Host Cell Subclones With Increased Specific Antibody Production Rates by Repeated Cycles of Transient Transfection and Cell Sorting. 2011;108:386–394.

[112] Cain K, Peters S, Hailu H, et al. A CHO Cell Line Engineered to Express XBP1 and ERO1-L a Has Increased Levels of Transient Protein Expression. Biotechnol. Prog. 2013;29:697–706.

[113] Vink T, Oudshoorn-Dickmann M, Roza M, et al. A simple, robust and highly efficient transient expression system for producing antibodies. Methods. 2014;65:5–10.

[114] Wulhfard S, Tissot S, Bouchet S, et al. Mild hypothermia improves transient gene expression yields several fold in Chinese hamster ovary cells. Biotechnol. Prog. 2008;24:458–465.

[115] Galbraith DJ, Tait AS, Racher AJ, et al. Control of culture environment for improved polyethylenimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. Biotechnol. Prog. 2006;22:753–762.

[116] Davami F, Baldi L, Rajendra Y, et al. Peptone Supplementation of Culture Medium Has Variable Effects on the Productivity of CHO Cells. Int. J. Mol. Cell. Med. 2014;3:146–156.

[117] Pham PL, Perret S, Cass B, et al. Transient gene expression in HEK293 cells: Peptone addition posttransfection improves recombinant protein synthesis. Biotechnol. Bioeng. 2005;90:332–344.

[118] Sun X, Goh PE, Wong KTK, et al. Enhancement of transient gene expression by fed-batch culture of HEK 293 EBNA1 cells in suspension. Biotechnol. Lett. 2006;28:843–848.

[119] Cervera L, Gutiérrez-Granados S, Berrow NS, et al. Extended gene expression by medium exchange and repeated transient transfection for recombinant protein production enhancement. Biotechnol. Bioeng. 2015;112:934–946.

[120] Brender E. Gene Therapy Industrial Strength. Nature. 2016;537:S57–S59.

[121] Brescia BA. Personalized Medicine and Public Health, A Clinical Testing Perspective. Bioprocess Int. 2006;24–29.

[122] Robinson J. Precision Biomanufacturing. Med. Mak. 2015;504.

[123] Grabherr R, Reichl U. Editorial: Can modern vaccine technology pursue the success of traditional vaccine manufacturing? Biotechnol. J. 2015;10:657–658.

[124] Whitford WG. Single-use systems as principal components in bioproduction. Bioprocess Int. 2010;8:34–42.

[125] Pais DAM, Carrondo MJT, Alves PM, et al. Towards real-time monitoring of therapeutic protein quality in mammalian cell processes. Curr. Opin. Biotechnol. 2014;30:161–167.

Objectives

Gag-based VLPs are a promising tool as HIV vaccine candidates and as scaffolds for the presentation of other viral antigens. In addition, VLPs have the ability to encapsulate and deliver small molecules, proteins or nucleic acids, which widens their potentiality in biomedicine. The establishment of robust bioprocesses, with high production titers and reproducible results is key for the success of VLPs in the clinic. Moreover, VLP quality is also essential to ensure a correct functionality. In this regard, mammalian cells are the preferred production system for the correct formation of complex viral particles, such as Gag-based enveloped VLPs. CAP is a novel cell line whose superiority compared to others has already been demonstrated, yet they remain unexplored for VLP production.

The general objective of this thesis was the development of a bioprocess platform for the production of HIV VLP-based vaccines in CAP cells. To achieve this goal, several specific objectives were planned:

1. The development and validation of a quantitation assay for Gag-GFP based VLPs, that could be used in a routine basis.

2. The set up and optimization of the transient gene expression technology in CAP-T cells to produce Gag-GFP VLPs.

3. The scale-up of the transient gene expression technology in CAP-T cells to 1L stirred-tank bioreactor.

4. The generation of a CAP stable cell line to produce Gag-GFP VLPs and its culture up to 1L stirred-tank bioreactor.

5. The implementation of dielectric spectroscopy as a technology to monitor in-line the VLP production processes (transient and stable), to gain deeper knowledge about them that could lead to further titer and quality improvements.

Results

Development and validation of a quantitation assay for fluorescently tagged HIV-1 virus-like particles

Results, chapter 1

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Development and validation of a quantitation assay for fluorescently tagged HIV-1 virus-like particles

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Upon expression, the Gag polyprotein of HIV-1 assembles spontaneously in the vicinity of the plasma membrane giving rise to enveloped virus-like particles (VLPs). These particulate immunogens offer great promise as HIV-1 vaccines. Robust VLP production and purification processes are required to generate VLPs of sufficient quality and quantity for both pre-clinical and clinical evaluation. The availability of simple, fast and reliable quantitation tools is critical to develop, optimize and monitor such processes. Traditionally, enzyme-linked immunosorbent assays (ELISA) are used to quantify p24 antigen concentrations, which reflect tightly virus particle concentrations. However, this quantitation technique is not only time-consuming, laborious and costly but it is also prone to methodological variability. As an alternative, the development and validation of a fluorescence-based quantitation assay for Gag VLPs is presented here. A Gag polyprotein fused to the enhanced green fluorescent protein was used for generation of fluorescent VLPs. A purified standard reference Gag-GFP VLP material was prepared and characterized *in house*. The method was validated according to ICH guidelines. The validation characteristics evaluated included accuracy, precision, specificity, linearity, range and limit of detection. The method showed to be specific for Gag-GFP. The fluorescence signal correlated well with p24 concentrations measured using a reference p24 ELISA assay. The method showed little variability compared to ELISA and was linear over a 3-log range. The limit of detection was ~10 ng of p24/mL. Finally, fluorescence-based titers were in good agreement with those obtained using transmission electron microscopy and nanoparticle tracking analysis. This simple, rapid and cost-effective quantitation assay should facilitate development and optimization of bioprocessing strategies for Gag-based VLPs.

1. Introduction

In the past few years, there has been interest in moving toward recombinantly produced vaccines, which currently represent a minor fraction of the licensed vaccines (10%) (Cox, 2012). Recombinant protein vaccines provide a safer option for vaccination than conventional vaccines, as they do not require administration and/or manipulation of infectious agents (Cox, 2012; Ulmer et al., 2006). Virus-like particles (VLPs) are formed typically by multiple copies of a viral structural antigen that spontaneously assembles yielding particles that mimic the viral structure. Due to their particulate nature, repetitive surface structure and the fact that they can present viral antigens in a more authentic conformation, they are typically more immunogenic and stable than soluble antigen recombinant vaccines (Ludwig and Wagner, 2007). VLPs do not contain a viral genome and thus, cannot cause the disease minimizing safety concerns (Ulmer et al., 2006). At present, there are VLP-based vaccines for human papillomavirus (HPV) and hepatitis B virus (HBV) commercially available. Many other VLPs are being tested in clinical trials (Roldao et al., 2010).

Currently there is no effective vaccine against the human immunodeficiency virus (HIV), the etiologic agent of AIDS (acquired immunodeficiency syndrome). HIV is an enveloped single-stranded RNA virus. Its genome contains three major coding regions (gag, pol and env) that give rise to three precursor polyproteins: Gag, Gag–Pol and Env (Frankel and Young, 1998; Joshi and Joshi, 1996). 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). During the budding process, Gag virus particles acquire their lipid envelope from the producer cell. Embedded in this lipid membrane is the viral Env protein (when co-expressed with Gag) composed by its two subunits (gp41 and gp120) generated by proteolytic cleavage by cellular proteases. In addition, host cellular proteins present at the site of virus budding are acquired by

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the virus (Ott, 1997; Tremblay et al., 1998). After budding, immature HIV virions undergo a maturation process during which Gag is cleaved into its four domains: matrix ($p17^{MA}$), capsid ($p24^{CA}$), nucleocapsid ($p7^{NC}$) and linker (p6). This gives place to conformational changes and leads to the formation of mature HIV particles (Freed, 1998).

Successful generation of several types of Gag-based VLPs has been reported. Gag VLPs can be generated by sole expression of Gag polyprotein (Lynch et al., 2010; Sakuragi et al., 2002), Gag–Pol fusion protein (Adamson et al., 2003), Gag fused to specific viral enzymes or accessory proteins (Pillay et al., 2009) or by co-expression of Gag along with Env (Deml et al., 2005; Hammonds et al., 2007; Tagliamonte et al., 2011; Yao et al., 2003). The latter type is the most attractive for the development of a prophylactic HIV vaccine since Env is the major target for neutralizing antibodies (Phogat et al., 2007). Gag has also been co-expressed with antigens from other viruses to form chimeric or pseudotyped VLPs (Garnier et al., 1995; Haynes et al., 2009; Osterrieder et al., 1995), making Gag-based VLPs an interesting platform for antigen carrying in a particulate form.

Robust VLP production and purification processes are essential to generate VLPs of sufficient quantity and quality for pre-clinical and eventually clinical testing. Simple, rapid and inexpensive analytical methods for quantitation of VLPs are required to develop and monitor these bioprocesses. There are a number of methods for viral particle quantitation reported in the literature (Altaras et al., 2005; Aucoin et al., 2008). Among the most common assays are $TCID_{50}$ and plaque assays (Graham and van der Eb, 1973), transgene expression assays (Chang and Zaiss, 2002), real-time PCR (Segura et al., 2010), transmission electron microscopy (TEM) (Alain, 1997), densitometry (Sutjipto et al., 2005), spectrophotometry (Maizel et al., 1968), high performance liquid chromatography (HPLC) (Transfiguracion et al., 2011), enzyme-linked immunosorbent assay (ELISA) (Pecora et al., 2009) and those based on viral enzymatic activity (Clark et al., 1996). However, not all these techniques can be used for VLP quantitation. As VLPs are non-infectious particles, infectivity-based assays are not applicable. Similarly, assays that are based on the quantitation of nucleic acids or transgene expression are not useful, as VLPs typically do not carry the viral genome. In addition, enzymatic activity-based assays can often not be used for VLP quantitation as the proteins required for VLP generation are usually structural antigens whereas viral enzymes are dispensable.

Among the methods that are applicable to VLP quantitation are TEM, spectrophotometry, densitometry, HPLC and ELISA. The latter is currently the most commonly used technique for lentiviral particle quantitation. This is reflected clearly by the large number of commercially available p24 ELISA kits. However, this technique is expensive and labor-intensive. Moreover, it consists in a multi-step protocol which leads typically to experimental error and variability. New technologies for virus particle quantitation and characterization are emerging rapidly along with the growing field of nanotechnology. Examples of novel methods that are applied to virus particle quantitation are nanoparticle tracking analysis (NTA) (Filipe et al., 2010), nanospectroscopy (Block et al., 2012), nanopore-based virus analysis (Roberts et al., 2012), flow field flow fractionation (Pease et al., 2009) or biosensor-based technologies (Caygill et al., 2010). However, these techniques require specialized equipment not usually available in cell culture laboratories.

Even though the final vaccine candidate will likely not contain a tag, the availability of a reporter protein allows for rapid and easy quantitation of the product of interest and comparison of different conditions studied throughout the developmental phases of the bioprocess. Tagged virus particles carrying fluorescent or luminescence reporters have been quantified previously using fluorescence-based (Hermle et al., 2010) and luminescence-based assays (Sakuragi et al., 2006) for HIV budding studies.

The development and validation of a quantitation technique for fluorescent Gag VLPs using a standard laboratory spectrofluorometer is reported here. A Gag polyprotein fused to the enhanced green fluorescent protein (GFP) was used for generation of fluorescent VLPs. Validation was carried out according to the International Conference Harmonisation (ICH) guideline about validation of analytical procedures: text and methodology (ICH, 2005). p24 ELISA was used as the reference quantitation technique. The reliability of the method was studied by comparison with other quantitation techniques.

2. Materials and methods

2.1. Cell line and plasmid DNA

The cell line used for production of VLPs was the human embryonic kidney 293SF-3F6 (HEK 293), kindly provided by Dr. Amine Kamen from the Biotechnology Research Institute of the National Research Council of Montreal, Canada. Cells were cultured in FreeStyle™ 293 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% Pluronic® (Invitrogen, Paisley, UK). Cells were maintained routinely in exponential growth phase in 125 mL disposable polycarbonate erlenmeyer flasks (Corning, NY, USA) shaken at 110 rpm in a humidified incubator at 37 °C and 5% CO_2 in air. The pGag-EGFP plasmid used in this work codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP. The plasmid from the NIH AIDS Reagent Program (Cat #11468) (Hermida-Matsumoto and Resh, 2000) was constructed by cloning the Gag sequence from pCMV55M1-10 (Schwartz et al., 1992) into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). Plasmid DNA was amplified in *Escherichia coli* DH5α strain grown in LB medium (Conda, Madrid, Spain) supplemented with kanamycin (10 μg/mL, Sigma, Saint Louis, MO, USA). Plasmid purification was carried out using the Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Generation of Gag-GFP VLPs

VLPs were produced by transient transfection of HEK 293 cells with pGag-EGFP plasmid DNA using 25 kDa linear polyethylenimine (PEI, PolySciences, Warrington, PA, USA) as transfection reagent. Cells were transfected at 1×10^6 cells/mL with 1 μg/mL of DNA and a PEI:DNA mass ratio of 2:1. DNA–PEI polyplexes were prepared by adding PEI to plasmid DNA diluted in a volume equal to 10% of the final culture volume. The mixture was incubated for 15 min at room temperature before being added to the cell culture. Cell suspensions were harvested at the indicated time-point post-transfection and supernatants were recovered by centrifugation at $500 \times g$ during 10 min. Lactate dehydrogenase (LDH) activity in harvested supernatants was determined using the cytotoxicity detection kit (Roche, Mannheim, Germany). 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. Separation of free Gag-GFP protein and assembled Gag-GFP VLPs was carried out by centrifugation of cell culture supernatants at $11{,}200 \times g$ using Vivaspin500 centrifugal filter units (300 kDa MWCO; Sartorius Stedim Biotech, Goettingen, Germany) according to manufacturer's instructions.

2.3. Flow cytometry analysis and confocal fluorescence microscopy

The percentage of GFP expressing cells was assessed by flow cytometry using a fluorescence-activated cell sorter (FACS) (BD

FACS Canto, BD BioSciences, San Jose, CA, USA). 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, Oregon, USA) and 0.1% of CellMask™ (Invitrogen) in order to stain cell nuclei and lipid membranes, respectively. Two washes were performed by centrifuging cells at 300 × *g* for 5 min and resuspending cell pellets in PBS. Samples were placed in 35 mm glass bottom Petri dishes with 14 mm microwell (MatTek Corporation, Ashland, MA, USA) for visualization under the microscope.

2.4. Purification of Gag-GFP VLPs

A standard reference Gag-GFP VLP material was prepared using a common retrovirus purification protocol consisting of virus particle pelleting by ultracentrifugation through a sucrose cushion (Transfiguracion et al., 2003). Briefly, a volume of 35.5 mL of clarified supernatant harvested 48 h post-transfection (hpt) was layered on top of a 30% sucrose cushion (3 mL) and centrifuged at 26,000 rpm (115,254 × *g*) for 3 h at 4 °C using a SW32 rotor in a Beckman Optima L100XP centrifuge. Pellets were resuspended in pre-chilled PBS (250 μL/tube) and incubated at 4 °C for 2 h. The concentrated and purified standard reference material obtained by ultracentrifugation was aliquoted and stored at −80 °C for future studies. For residual nucleic acid removal prior to size-exclusion chromatography (SEC) analysis, samples were subjected to Benzonase® (Merck-Millipore, Germany) treatment for 1 h at room temperature using a final enzyme concentration of 200 U/mL. SEC was performed using an ÄKTA Explorer™ 100 low-pressure liquid chromatography system controlled by UNICORN software (GE Healthcare, Sweden). For this purpose, Sepharose 6FF size exclusion chromatography resin (code 17-0159-01, GE Healthcare) was packed into a XK 16/70 column (GE Healthcare) to a final volume of 130 mL. An average pore size of 24.7 nm has been reported for this resin (DePhillips and Lenhoff, 2000; Schroder et al., 2006). The void volume of the column was determined using Blue Dextran 2000 (HMW calibration kit, GE Healthcare). The standard reference Gag-GFP VLP material (3 mL) was filtered through 0.45 μm pore-size membrane filters and loaded onto 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 monitored on-line. Fractions (2.5 mL) were collected throughout the run and analyzed by p24 ELISA and spectrofluorometry.

2.5. Quantitation of Gag-GFP by spectrofluorometry

Green fluorescence intensity in Gag-GFP samples was measured by spectrofluorometry using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The instrument parameters were set as follows: λ_{ex} = 488 nm (slit 5 nm), λ_{em} = 510 nm (slit 10 nm). Readings were carried out at room temperature. Relative fluorescence units (RFU) values were calculated by subtracting fluorescence units (FU) of negative control samples from that given by the sample. Negative controls were included systematically in every assay (supernatant from untransfected cells, culture media or PBS). As part of this study, the correlation between RFU values and p24 concentration has been established, as further detailed in Section 3.2.2. The conversion to Gag-GFP concentrations is addressed in Section 3.3.

2.6. Quantitation of Gag-GFP by p24 ELISA

Gag-GFP polyprotein concentrations were determined by p24 ELISA using the commercially available kit Innotest® HIV antigen mAb (Innogenetics NV, Gent, Belgium). The assay was performed according to manufacturer's instructions.

2.7. Quantitation of Gag-GFP by gel electrophoresis and densitometry

The content of Gag-GFP polyprotein in purified Gag-GFP VLP stocks was analyzed by quantitative densitometry. For this purpose, the purified samples were fractionated by electrophoresis in NuPAGE® Novex 4–12% Bis–Tris polyacrylamide gels (Invitrogen) run under reducing conditions (SDS-PAGE) using a XCell SureLock™ Mini-Cell electrophoresis system. Known amounts of purified bovine serum albumin (BSA; Micro BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA) were also loaded in the gel as mass standards. The molecular weight standard used was SeeBlue® Plus2 Pre-Stained (Invitrogen). Protein bands were visualized by Coomassie Blue staining (Coomassie G250, Bio-Rad, Hercules, CA, USA). Densitometry analysis was performed using ImageLab 4.0 (Bio-Rad) software. By plotting BSA mass (*y*-axis) vs. band density (*x*-axis), a linear regression curve was obtained (R^2 = 0.9929). The equation was as follows:

$$y = 1.26 \cdot 10^{-6} \cdot x - 0.0295 \quad (1)$$

The amount of Gag-GFP protein loaded in the gel was calculated using this equation. The identity of Gag-GFP polyprotein band was confirmed by Western blot analysis. For this purpose, SDS-PAGE fractionated proteins in a second gel were transferred to a nitrocellulose membrane using iBlot® Gel Transfer Device (Invitrogen) and iBLot Transfer Stack Mini (Invitrogen) according to manufacturer's instructions. Immunodetection of Gag-GFP protein was carried out using a primary Anti-HIV-1 p24 mouse monoclonal antibody (Mab) [39/5.4A] (Abcam, Cambridge, UK) and a peroxidase-conjugated Goat Anti-Mouse AffiniPure F(ab')2 Fragment IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). The primary antibody was incubated overnight at 4 °C whereas the secondary was incubated at room temperature for 1 h. Finally, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 min at room temperature and visualized using a ChemiDoc-It® Imaging System (UVP, Upland, CA, USA) device and VisionWorks®LS software (UVP).

2.8. Quantitation of Gag-GFP VLPs by TEM

For VLP particle quantitation by TEM, purified reference Gag-GFP VLP material was diluted 1:2 with a solution of known concentration of latex spheres (1×10^{11} particles/mL, 100 nm). The mixture was then 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 drained carefully off the grid with the aid of filter paper. Samples were stained negatively 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). The ratio between viral particles and latex spheres was estimated by counting both types of particles in 50 fields containing between 10 and 30 particles. The mean ratio was used to calculate the Gag-GFP VLP concentration using the following equation:

$$\left[\frac{VLPs}{\text{mL}}\right] = \frac{n_{VLPs}}{n_{spheres}} \cdot \left[\frac{spheres}{\text{mL}}\right] .DF \quad (2)$$

where [*VLPs*/mL] is the concentration of the VLP sample, $n_{VLPs}/n_{spheres}$ is the mean ratio between the number of VLPs and

latex spheres, [*spheres*/mL] is the concentration of the latex spheres (considering dilution with the sample) and *DF* is the dilution factor.

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

Quantitation of VLPs in purified reference material was performed using a NanoSight® LM20 device (NanoSight Ltd., Amesbury, UK) at the Service of Preparation and Characterization of Soft Materials located at Institut de Ciència de Materials de Barcelona (ICMAB, CSIC, Campus UAB). The data were analyzed with the NanoSight® NTA 2.2 software. Samples were diluted in 0.22 μm-filtered PBS prior to injection in the device chamber in order to obtain 1 mL of sample 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 determination of particle size and number from the tracking data.

3. Results

3.1. Gag-GFP expression kinetics and evaluation of assembled vs. free Gag-GFP in cell culture supernatants

Gag-GFP VLPs were generated by transient transfection of HEK 293 suspension cells using PEI as transfection reagent. The percentage of GFP positive cells 48 hpt was typically between 60% and 70%. Green fluorescent cells were visualized by confocal fluorescence microscopy 72 hpt. The green fluorescence was observed in the cytoplasm of transfected cells (Fig. 1A I). Gag-GFP molecules were particularly enriched in the vicinity of the plasma membrane (Fig. 1A II). By staining the lipid membrane with CellMask™ (red), strong co-localization of green and red fluorescence was visualized as yellow regions on the membrane (Fig. 1A III), location of virus particle budding.

The kinetics of Gag-GFP expression upon transient transfection of HEK 293 cells was evaluated by monitoring green fluorescence intensity in harvested supernatants at different times post-transfection (Fig. 1B). Gag-GFP fluorescence in cell culture supernatants increased continuously after transfection. Cell viability was maintained relatively high (over 70%) until 96 hpt. As cell viability decreases, disrupted cells release their intracellular content into the culture media including free Gag-GFP monomers. In order to determine the percentage of free vs. assembled Gag-GFP, cell culture supernatants were subjected to filtration through 300 kDa MWCO membranes using Vivaspin500 centrifugal filters. This allows separation between free and assembled Gag-GFP due to their significant difference in size. The content of p24 in permeate and retentate fractions was assessed by ELISA. The analysis was performed with supernatant samples harvested at different times post-transfection. This experiment indicated that the percentage of free Gag-GFP molecules in the cell culture supernatant increases continuously with time (Fig. 1C). A parallel increase in LDH activity in harvested supernatants was observed (data not shown). The percentage of contaminating free Gag-GFP in cell culture supernatants early after transfection (48 hpt) was low (25.6 ± 2.0%). However, the percentage of contaminating free Gag-GFP nearly doubles at 120 hpt (49.7 ± 9.6%).

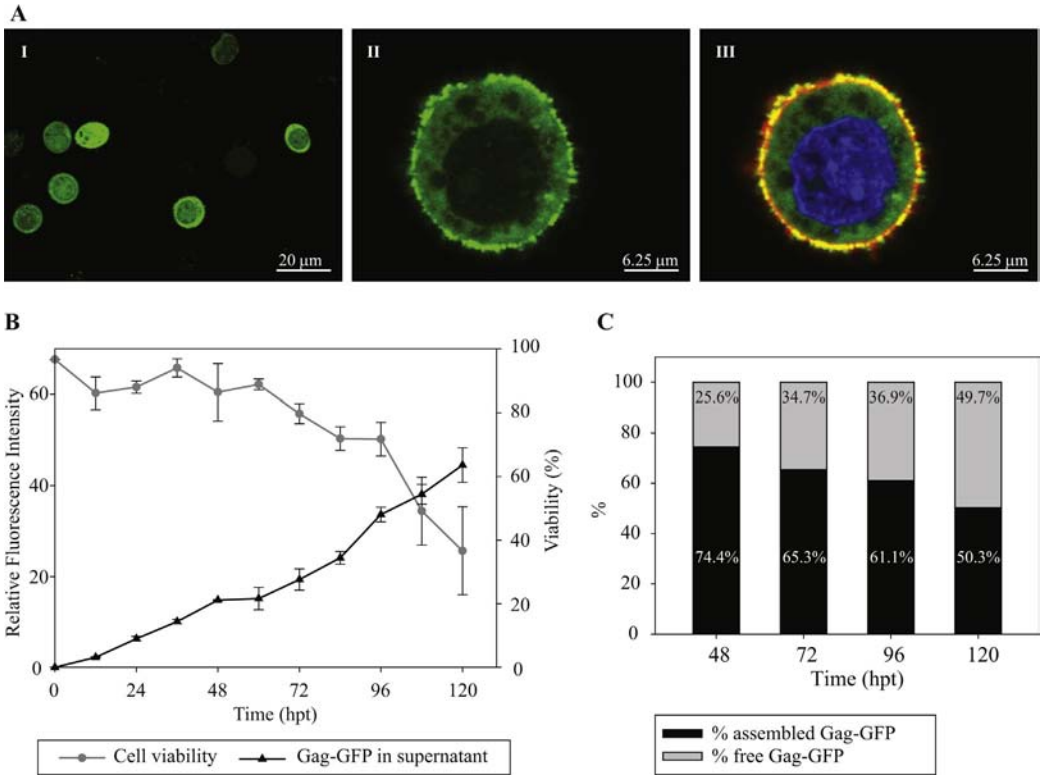

Fig. 1. (A) Confocal fluorescence microscopy images of HEK 293 producer cells at 72 hpt. Gag-GFP transfected cells at low (I) and high magnification (II). (III) High magnification merged image of producer cells with double staining: the cell nucleus was stained with Hoechst (blue) and membrane was stained with CellMask™ (red). The green fluorescence indicates the presence of Gag-GFP molecules. Overlay of green and red fluorescence (yellow) indicated co-localization of Gag-GFP molecules and lipid membrane. (B) Kinetics of Gag-GFP production in HEK 293 cells and cell viability. Error bars indicate standard deviation ($n = 3$). (C) Analysis of the percentage of free and assembled Gag-GFP molecules in cell culture supernatants over time post-transfection ($n = 3$) using Vivaspin500 filtration units. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

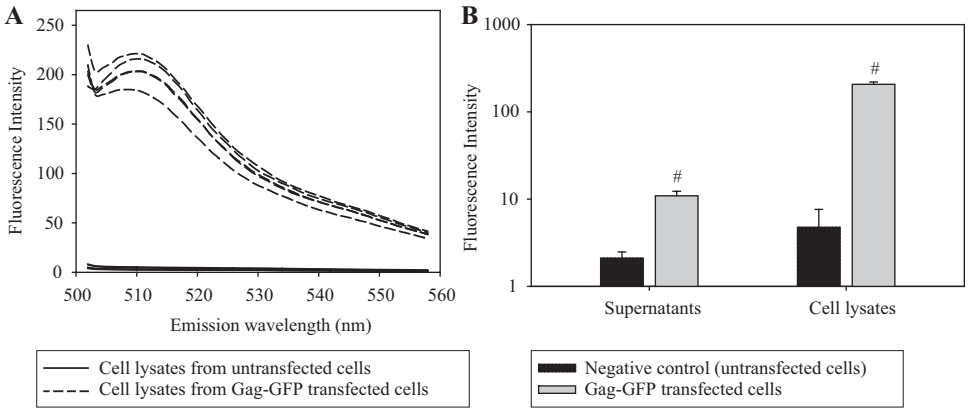

Fig. 2. (A) Fluorescence emission spectra of cell lysates from Gag-GFP transfected cells showed a maximum emission at 510 nm upon excitation at 488 nm. Untransfected cell lysates were used as negative control. (B) Mean fluorescence intensity from cell culture supernatants and cell lysates obtained 48 hpt and untransfected negative controls. Error bars indicate standard deviation ($n = 6$). A significant fluorescence signal was detected both in cell culture supernatants and cell lysates (# indicates $p < 0.001$, t-test).

3.2. Development and validation of a fluorescence-based quantitation method

A fluorescence-based method for the quantitation of Gag-GFP VLPs was developed and validated. Validation was carried out according to the ICH guideline about validation of analytical procedures: text and methodology (ICH, 2005). The validation parameters evaluated included specificity, linearity, quantitation range, limit of detection, precision, and accuracy. For validation purposes, a standard reference material containing concentrated and purified Gag-GFP VLPs was prepared by ultracentrifugation through a sucrose cushion and fully characterized *in house*. The VLP concentration in such reference material was determined by several methods (see Section 3.4). The sample showed to be highly pure. A first indication of VLP purity was observed by elution of a single peak in the void volume of a SEC column as assessed by OD280 and OD260 online monitoring. The peak displayed an expected OD260/OD280 ratio (1.24) consistent with purified retrovirus particles (McGrath et al., 1978). In addition, p24 ELISA and fluorescence measurements of all eluted fractions confirmed the presence of Gag-GFP only in the peak eluting at the column's void volume, but not in the rest of the eluted fractions, which indicates absence of free Gag-GFP monomers in the purified reference preparation obtained by ultracentrifugation. SDS-PAGE and Western blot analyses were used to confirm the purity of the standard reference material (see Section 3.3).

3.2.1. Specificity

Specificity is the ability to assess unequivocally the product of interest in the presence of components which may be expected to be present (ICH, 2005). As a proof of concept, cell lysates from untransfected cells (negative controls) and transfected cells containing concentrated Gag-GFP polyprotein were analyzed initially by spectrofluorometry. A strong fluorescence signal was detected in transfected cell lysates but not in negative control lysates. The fluorescence emission spectra showed a maximum peak at 510 nm when excited at 488 nm, corresponding to the typical GFP emission pattern (Fig. 2A). Likewise, a statistically significant fluorescence signal was detected in cell culture supernatants from transfected cultures only (Fig. 2B). These preliminary results suggested that spectrofluorometry was specific and sensitive enough to detect fluorescent VLPs directly from cell culture supernatants with no need for previous sample processing steps (e.g. purification and/or concentration). The specificity of fluorometry was confirmed by spiking a known concentration of VLPs from the purified standard reference material in cell culture supernatants from untransfected cell cultures. The latter contained representative impurities expected to be found in problem samples. Recovery was calculated to be $96.6 \pm 4.5\%$ ($n = 3$). This experiment demonstrated specific detection of Gag-GFP by spectrofluorometry.

3.2.2. Linear correlation with p24 ELISA reference assay

The correlation between spectrofluorometry and p24 ELISA measurements was assessed. For this purpose, two concentrated samples were prepared, one representing free Gag-GFP monomers (a cellular lysate from transfected cells) and the other one representing highly purified assembled Gag-GFP VLPs (the standard reference material). Serial dilutions of both samples were analyzed by spectrofluorometry and p24 ELISA. Correlation plots were constructed and are shown in Fig. 3A (free p24) and B (assembled p24). A linear correlation between p24 concentration values and green fluorescence intensity was observed in both cases with an R^2 of 0.9971 and 0.9996, respectively. A statistical analysis was carried out in order to compare the regression equations obtained for both samples and a new plot was constructed (Fig. 3C). The plot shows a new regression line resulting from all the points from Fig. 3A and B and the 95% limits of agreement. As shown in Fig. 3C, all measurements fall within the confidence limits. This study indicated that no significant difference exists between both correlation equations. This observation implies that the VLP lipid envelope does not interfere with the fluorescence reading.

The correlation equation for enveloped Gag-GFP VLPs is as follows:

$$\text{p24(ng/mL)} = 3.245 \cdot \text{RFU} - 1.6833 \quad (3)$$

This equation can be used to convert relative fluorescent units (RFU) values into ng/mL of p24.

To confirm the validity of the equation, the p24 content and fluorescence intensity of a battery of 15 randomly selected problem samples, including cell culture supernatants and concentrated supernatants obtained by ultrafiltration, were measured (Fig. 3D). All problem sample double measurements fell in close proximity of the correlation curve (Eq. (3)). In order to assess quantitatively the proximity of the data to the correlation curve, a Bland–Altman plot (Bland and Altman, 1995) was constructed (Fig. 3E). The Bland–Altman plot shows the difference of the measured and the predicted p24 values (y-axis) vs. the mean of the measured and predicted p24 values (x-axis). All measurements fell within the 95% confidence interval confirming the good performance of the fluorescence quantitation technique.

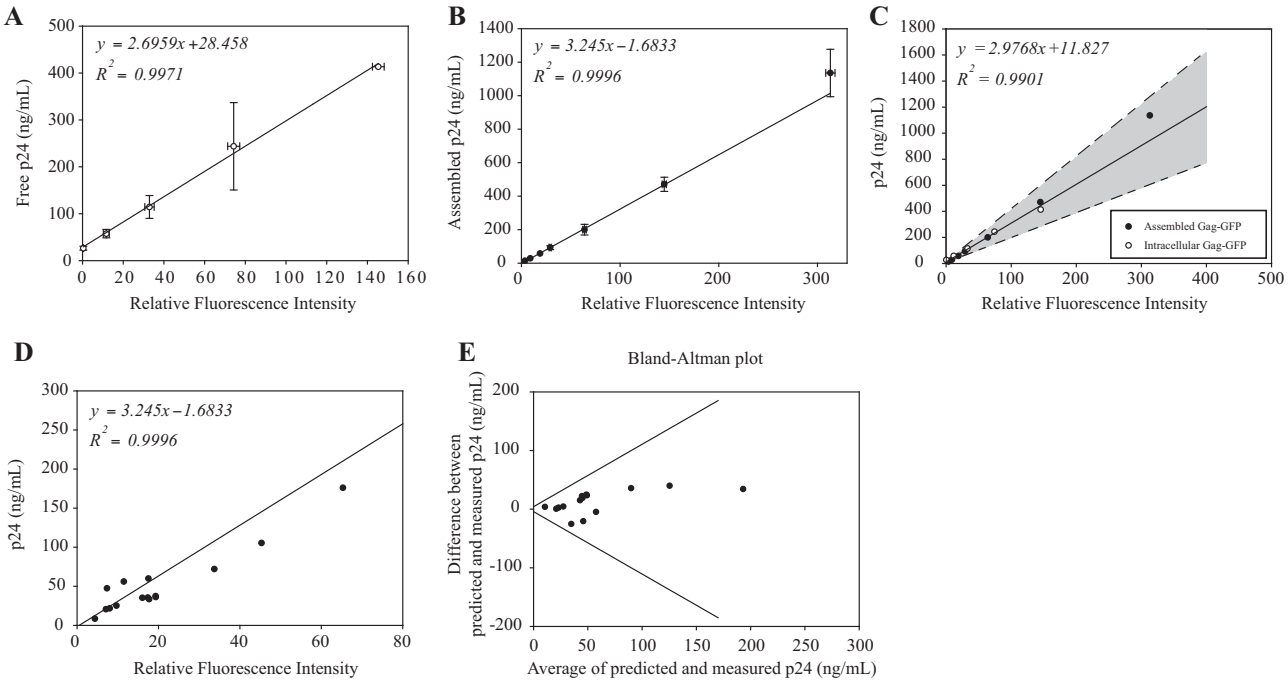

Fig. 3. (A) Linear correlation between ng of p24/mL determined by ELISA and relative fluorescence intensity (RFU) determined by spectrofluorometry using cell lysate serial dilutions. Error bars indicate standard deviation ($n = 3$). (B) Linear correlation between ng of p24/mL determined by ELISA and relative fluorescence intensity (RFU) determined by spectrofluorometry in the purified reference VLP sample. Error bars indicate standard deviation ($n = 3$). (C) Comparison of regression equations for free and assembled p24. The plot shows a new regression line resulting from all the points from Fig. 3A and B and the limits of agreement. The bands indicating the limits of agreement have been calculated as $x \pm x \cdot \%CV \cdot 1.96$, where %CV is the mean coefficient of variation of all data and 1.96 indicates a confidence level of 95%. (D) Gag-GFP VLP concentrations in randomly selected samples determined by ELISA and spectrofluorometry. All problem sample double measurements (dots) fell in close proximity of the correlation curve (Eq. (3)). (E) Bland–Altman plot using the data set from D. All points fell within the upper and lower limits of agreement, calculated as $\pm x \cdot \%CV \cdot 1.96$, with a confidence interval of 95%.

3.2.3. Linear range and limit of detection

The standard reference VLP material was used to determine the linear range and limit of detection (LOD) of the method. A linear response between p24 concentration values measured by ELISA and green fluorescence intensity is observed up to 1000 RFU, after which the signal is saturated (Fig. 4A). Throughout this 3-log linear range, the ratio between fluorescence intensity and p24 concentration was constant (0.28 ± 0.03) (Fig. 4B).

The detection limit is the lowest amount of product of interest that can be detected (ICH, 2005). The LOD was established based on signal-to-noise ratios according to ICH guidelines. The noise is the signal given by the matrix in which VLPs are suspended (fluorescence intensity of negative control samples). A signal-to-noise ratio of 2:1 was considered acceptable based on inspection of fluorescence emission scans as samples with a lower ratio did not show an adequate fluorescence emission scan with the characteristic maximum emission peak at 510 nm. In the case of supernatant samples, in which VLPs are suspended in culture medium, the LOD was approximately 14 FU as the noise in the culture medium was approximately 7 FU. This is equivalent to 20 ng/mL of p24 according to Eq. (3) shown in Section 3.2.2. When VLPs were suspended in PBS, as is the case of the purified samples, the LOD was lower (6 FU) as the noise of PBS was only 3 FU. This LOD equals to 8 ng/mL of p24.

3.2.4. Precision

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample (ICH, 2005). The precision of the fluorescence-based quantitation method was investigated by analysis of the purified Gag-GFP VLP sample. Precision was considered at two levels: repeatability and intermediate precision. Results are summarized in Table 1. Repeatability was assessed at 3 different

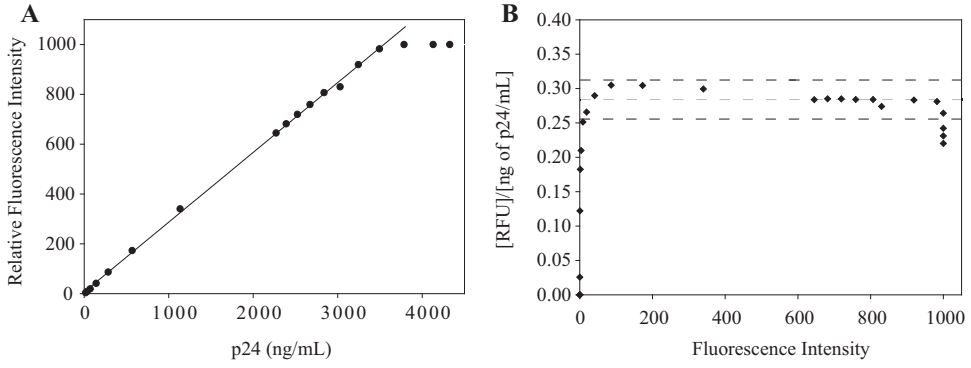

Fig. 4. (A) Linear relation between RFU and p24 measures. (B) The ratio of the fluorescence intensity and the concentration of p24 protein determined by ELISA remains constant (0.28 ± 0.03) within this linear range. The gray dashed line indicates the mean ratio and the black dashed lines indicate the mean ±10% ratio.

Table 1
Spectrofluorometry precision.

Standard reference VLP material dilution	Fluorescence intensity (RFU)			
	Repeatability mean ($n = 3$) (%CV)[a,b]			Intermediate precision mean ($n = 9$) (%CV)[a,b]
	Day 1	Day 2	Day 3	
1/2	143.59 (2.63)	145.01 (0.49)	145.72 (0.47)	144.77 (0.75)
1/4	64.27 (1.02)	63.26 (2.19)	64.30 (4.88)	63.94 (0.92)
1/8	28.65 (5.74)	29.92 (0.80)	28.96 (0.79)	29.18 (2.26)

[a] Coefficient of variation was calculated as %CV = 100·SD/<x>.
[b] %CV are presented in parenthesis.

Table 2
p24 ELISA precision.

Standard reference VLP material dilution	p24 concentration (ng/mL)			
	Repeatability mean ($n = 3$) (%CV)[a,b]			Intermediate precision mean ($n = 9$) (%CV)[a,b]
	Day 1	Day 2	Day 3	
1/2	435.00 (7.70)	506.03 (1.46)	471.15 (9.90)	470.73 (7.55)
1/4	175.27 (5.97)	219.99 (19.47)	205.08 (11.47)	200.12 (11.38)
1/8	99.88 (13.02)	93.90 (14.78)	85.08 (3.54)	92.95 (8.01)

[a] Coefficient of variation was calculated as %CV = 100·SD/<x>.
[b] %CV are presented in parenthesis.

concentrations with 3 replicates of each. A coefficient of variation (%CV) was calculated for each concentration and was found to be consistently low (below 6%). Additionally, intermediate precision that considered inter-day variations (9 replicates for each concentration in three different days) showed low %CV values (below 3%). According to established criteria, a %CV lower than 15% is considered acceptable. For comparison purposes, p24 ELISA precision was also studied in the same manner using the same set of samples. As shown in Table 2, %CV estimated for ELISA were considerably higher than those determined for spectrofluorometry.

3.2.5. Accuracy

Accuracy expresses the closeness of agreement between an accepted reference value and the value found (ICH, 2005). Accuracy was analyzed at three different concentrations with three replicates each. Results are summarized in Table 3. p24 concentrations were estimated from fluorescence values obtained using the linear correlation equation (Eq. (3)) and compared with p24 concentrations measured by ELISA. Accuracy values were between 0.05% and 2.85%. Accuracy is acceptable when % error values are under 15% according to FDA Industry Guidance on Bioanalytical Method Validation (FDA, 2001), thus the method shows appropriate accuracy. A *t*-test showed no significant differences (95% confidence interval) between p24 concentrations measured by ELISA and those estimated from fluorescence measurements. This indicates a good concordance between reference values and experimental values obtained by spectrofluorometry.

Table 3
Accuracy.

Measured p24 concentrations (ng/mL ± SD)	Estimated p24 values based on fluorescence (ng/mL ± SD)[a]	Accuracy[b] (%)
470.73 ± 35.52	468.10 ± 3.51	0.56
200.12 ± 22.77	205.81 ± 1.92	2.85
92.95 ± 7.44	93.00 ± 2.14	0.05

[a] Fluorescence values were converted to p24 concentrations using Eq. (3).
[b] Accuracy was calculated as Accuracy (%) = |(Estimated<x> − Measured<x>)|/Measured<x>·100.

3.3. p24 ELISA underestimates Gag-GFP protein content

Commercial p24 ELISA kits are usually developed to recognize epitopes present in mature HIV particles. Some of these kits fail to recognize with the same efficiency p24 epitopes present in the uncleaved protein, which is the Pr55 Gag polyprotein present in immature HIV particles as well as most VLPs (Sakuragi et al., 2006), as they do in the cleaved form of p24. This was the case for the Innotest® ELISA HIV antigen mAb kit used in this work (personal communication Dr. J. Blanco, IrsiCaixa, Badalona, Spain). In order to determine the underestimation factor resulting from using this particular ELISA kit, the amount of Gag-GFP protein in purified reference material VLP sample was quantified by densitometry and the result compared with that obtained using p24 ELISA.

The protein profile of the standard reference material and this material after SEC is shown in Fig. 5A (lanes 2 and 3, respectively). An 84 kDa band consistent with the expected mass of Gag-GFP fusion protein (88 kDa) is clearly observed in both samples. The identity of Gag-GFP polyprotein was confirmed by Western blot analysis using a p24 Mab (Fig. 5B). Curiously, a lower molecular weight band (66 kDa) was also recognized by the p24 Mab (Fig. 5B). This band could also be observed in the SDS-PAGE gel (Fig. 5A) but represented only a minor fraction of Gag in the sample (less than 5%). Densitometry analysis of the standard reference material protein bands confirmed the high purity of the sample as the Gag-GFP polyprotein alone accounted for over 65% of the total protein content in the sample. The rest of the proteins observed in the lane should correspond to cellular proteins that are typically found associated with retroviral particles (Ott, 1997, 2002; Segura et al., 2006, 2008; Tremblay et al., 1998).

For quantitative densitometry, a calibration curve was generated by loading known amounts of purified BSA (66 kDa) into the gel (Fig. 5A) ranging from 3000 ng (lane 4) to 47 ng (lane 10) of protein. The calibration curve equation (Eq. (1)) was used to convert

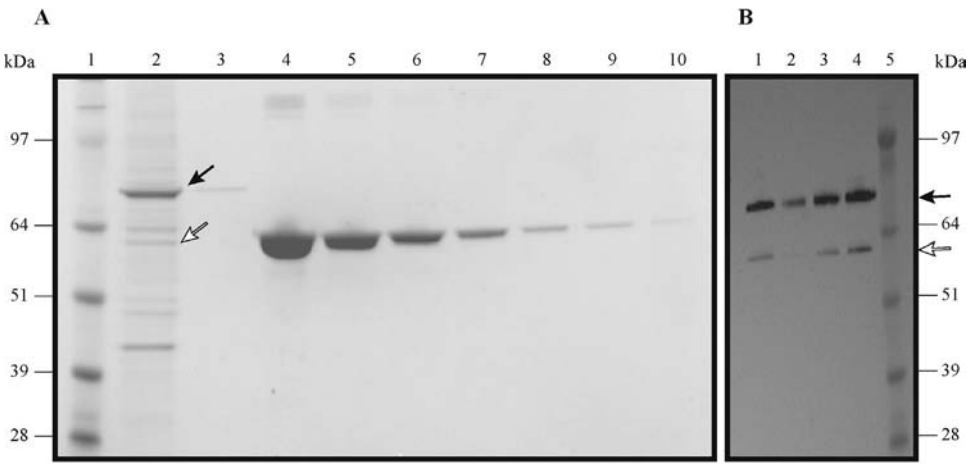

Fig. 5. (A) SDS-PAGE stained with Coomassie Blue. Lane 1: MW ladder. Lane 2: purified standard reference VLP sample. Lane 3: purified standard reference VLP sample after SEC. Lanes 4–10: BSA standards (3, 1.5, 0.75, 0.38, 0.19, 0.09, 0.05 μg/lane). Full arrow indicates Gag-GFP band (84 kDa). Empty arrow indicates Gag-GFP fragment band (66 kDa). (B) Western-blot analysis of highly purified VLP samples using an anti-p24 antibody. Lane 1: purified standard reference VLP sample. Lanes 2–4: purified standard reference VLP sample after SEC diluted 1/2 (lane 2), 1/4 (lane 3) and 1/8 (lane 4). Lane 5: MW ladder. Full arrow indicates Gag-GFP band (84 kDa). Empty arrow indicates Gag-GFP fragment band (66 kDa).

band density values to protein mass units. The Gag-GFP polyprotein band is indicated in the gel with a black arrow. The amount of Gag-GFP protein loaded in the gel (lane 2) was estimated as 555 ng of protein corresponding to a concentration of 37.0 μg/mL of Gag-GFP polyprotein in the original reference material sample.

According to p24 ELISA, the concentration of p24 in the standard reference material was 1101 ng of p24/mL. This is equivalent to 3.85 μg of Gag-GFP/mL considering the difference in molecular weight between p24 and Gag-GFP. Thus, it was determined that Innotest® p24 ELISA kit underestimated Gag-GFP concentrations by a factor of ~10 (3.85 vs. 37 μg of Gag-GFP/mL). Taking into account both the difference in molecular weight between p24 and Gag-GFP and the calculated underestimation factor, p24 concentrations obtained by ELISA need to be corrected by a factor of 36 in order to estimate Gag-GFP concentration values. Thus, the corrected concentration of Gag-GFP in the purified VLP sample according to p24 ELISA was 39.6 μg/mL.

Considering these results, RFU values should be converted into ng/mL of Gag-GFP using the following equation:

$$\text{Gag} - \text{GFP}(\text{ng/mL}) = (3.245 \cdot \text{RFU} - 1.6833) \cdot 36 \quad (4)$$

where the correlation equation between fluorescence values determined by spectrofluorometry and p24 concentrations determined by ELISA is shown between parentheses and 36 is the correction factor.

3.4. Comparison with other virus particle quantitation methods

Besides p24 ELISA and densitometry, the fluorescence-based quantitation method was compared with two other quantitation techniques: TEM and NTA. TEM is a gold standard technique for virus quantitation whereas NTA represents a new technology for nanoparticle counting developed by NanoSight Ltd. In addition to VLP quantitation, both TEM and NTA also provide information on particle size. Representative VLP micrographs of the standard reference material obtained by TEM are shown in Fig. 6A and B. Roughly spherical virus particles surrounded by a lipid envelope can be distinguished in a homogeneous background (Fig. 6C and D). The mean particle diameter was determined to be 141 ± 22 nm (n = 100) with a statistical mode of 146 nm. In good agreement, NTA allowed for detection of a main peak with an average statistical mode of 147 nm.

The concentration of Gag-GFP VLPs in the standard reference VLP material was evaluated using the different titration methods. Quantitation results obtained are summarized in Table 4. The concentration of Gag-GFP VLPs in protein mass/mL was estimated by densitometry (37.0 μg of Gag-GFP/mL) and p24 ELISA (39.6 μg of Gag-GFP/mL), showing comparable results (see Section 3.3). In terms of particles/mL, the concentration in the standard reference VLP material was 1.30×10^{11} particles/mL according to TEM analysis and 1.24×10^{11} particles/mL by NTA. In order to compare quantitation results obtained by TEM and NTA with those determined by densitometry and p24 ELISA, the concentrations expressed as mass of Gag-GFP/mL need to be converted into number of particles/mL. Assuming that a single VLP contains 2500 Gag-GFP molecules (Chen et al., 2009), the concentration of VLPs in the purified sample was 1.07×10^{11} particles/mL according to densitometry and 1.14×10^{11} particles/mL according to p24 ELISA. These estimated titers are in agreement with those obtained by TEM and NTA.

To confirm the validity of the fluorescence-based quantitation method developed, the fluorescence intensity of the standard reference VLP material was determined. The sample showed a fluorescence of 451.6 RFU. This is equivalent to 52.7 μg of Gag-GFP/mL considering Eq. (4) and 1.52×10^{11} particles/mL considering Gag stoichiometry as presented above. These results are in close agreement with those obtained by TEM and NTA quantitation methods confirming the reliability of spectrofluorometry for VLP quantitation.

4. Discussion

Due to the flexibility of the retrovirus particle assembly process, fluorescently tagged Gag virions of the expected morphology can be easily generated when expressing Gag as a fusion construct with the green fluorescent protein (GFP) or its variants (e.g. Gag-YFP, Gag-RFP, Gag-CFP) (Hermle et al., 2010; Muller et al., 2004; Schwartz et al., 1992; Sherer et al., 2003). Gag-GFP forms virus-like particles with an efficiency equivalent to that of Gag (Hermida-Matsumoto and Resh, 2000). Gag fluorescent labeling has mainly been used so far to study retrovirus replication in living cells (Dale et al., 2011; Hermida-Matsumoto and Resh, 2000; Lindwasser and Resh, 2002; Muller et al., 2004; Perlman and Resh, 2006). This attractive feature is exploited in our laboratory for the development of VLP bioprocessing strategies as fluorescently

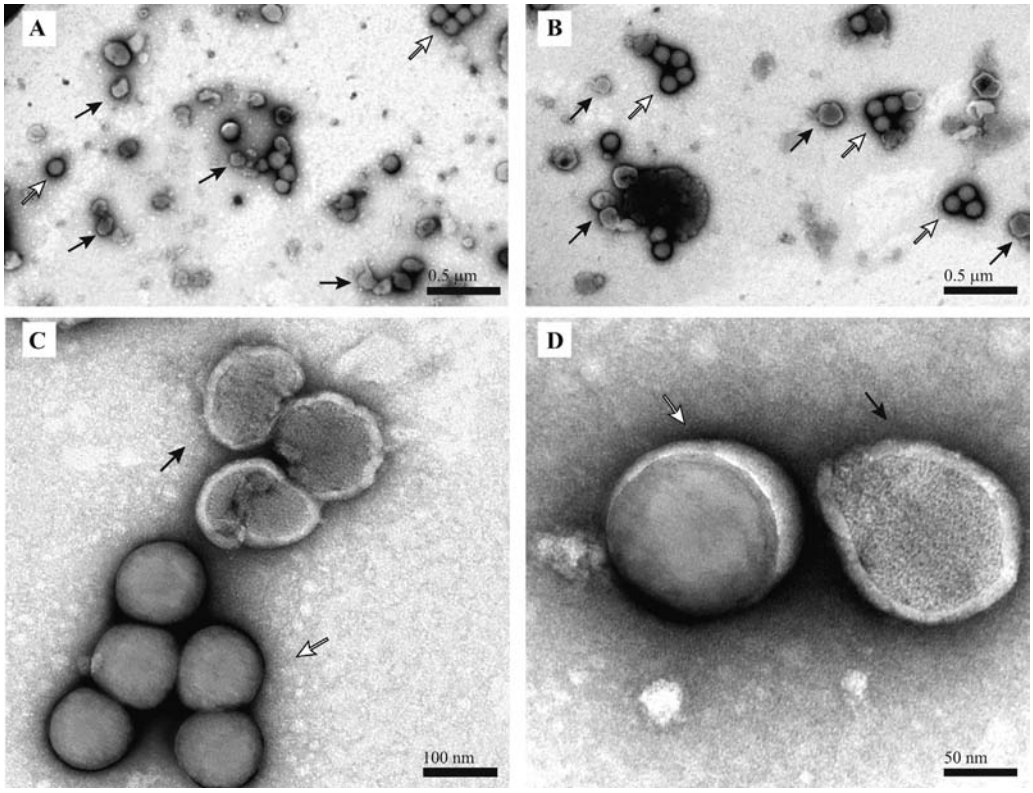

Fig. 6. (A and B) Negatively stain electron microscopy image of Gag-GFP VLPs. Gag-GFP VLPs (full arrows) and latex beads used for quantitation purposes (empty arrows) are indicated. (C and D) High magnification images showing roughly spherical virus-like particles surrounded by a lipid envelope (full arrows), and latex beads (empty arrows).

tagged VLPs can be easily detected and quantified. Although keeping the fluorescent tag is likely not of interest in the final VLP vaccine product, the use of this label during product development phases is very convenient, allowing fast and simple quantitation of the various process alternatives. Comparatively, viral gene therapy vectors carrying reporter genes that allow identification of transduced cells and quantitation of functional virus particles are widely used in process development.

It is well described in the literature that Gag VLPs as well as other immature HIV-1 forms lacking the viral-encoded protease have a significantly larger diameter than mature HIV-1 particles (Valley-Omar et al., 2011; Wilk et al., 2001). Indeed, Wilk et al. (2001) stated that Gag VLPs resembled immature HIV particles with a mean size of 169 ± 29 nm and a statistical mode of 133 ± 17 nm and Valley-Omar et al. (2011) showed that Gag VLPs show a diameter between 120 and 150 nm by TEM. This contrasts with the size of wild-type mature HIV-1 particles, typically between 100 and 120 nm in diameter. To no surprise, the Gag VLPs analyzed in this study have shown a large diameter (140 nm) based on TEM and NTA, consistent with immature HIV-1 particles.

A simple, rapid and cost-effective fluorescence-based method for the quantitation of GFP-tagged Gag VLPs has been developed. This technique is being applied routinely for the development and optimization of production and purification processes for HIV-1 Gag VLPs in our laboratory. Compared to p24 ELISA, the standard technique used for Gag VLP quantitation, the fluorescent-based assay presents numerous advantages. These advantages are associated largely to the fact that, as opposed to a multi-step p24 ELISA quantitation, the fluorescence-based assay consists in a single-step protocol. This is an important requirement for routine virus particle quantitation as it reduces the time associated with the procedure. In addition, it makes the technique less prone to experimental error. Moreover, the use of this alternative fluorometric assay should result in significant cost savings as commercial p24 ELISA kits are quite expensive and the proposed methodology only requires standard laboratory equipment.

Method reliability is essential and can be achieved through method validation according to pre-established criteria. Validation of the fluorescence-based method was carried out according to ICH guidelines. p24 ELISA was selected as a reference method for validation purposes. All parameters met the acceptable criteria for analytical method validation. The spectrofluorometry quantitation method showed to be specific for Gag-GFP. The method was linear over a 3-log range from 7 to 1000 RFU corresponding to 10 to 3600 ng of p24/mL. This represents a clear advantage over ELISA that has a narrower linear range (2-log from 10 to 300 pg/mL) that

Table 4
Comparison of the quantitation results of VLP purified sample by five different methods.

	Spectrofluorometry[a]	ELISA[b]	Densitometry	TEM[c]	NTA Nanosight®[d]
μg of Gag-GFP/mL (±SD)	52.7 (±0.49)	39.6 (±12.5)	37.0	n.a.	n.a.
Particles/mL (±SD)	1.52×10^{11} ($\pm 1.40 \times 10^{9}$)	1.14×10^{11} ($\pm 3.58 \times 10^{10}$)	1.07×10^{11}	1.30×10^{11} ($\pm 4.49 \times 10^{10}$)	1.24×10^{11} ($\pm 3.21 \times 10^{10}$)

[a] Upon conversion using Eq. (4), considering that RFU was 451.6 (±4.69).
[b] Concentrations in mass of p24/mL were converted to μg of Gag-GFP/mL according to the correction factor calculated in Section 3.3.
[c] Only nanoparticles from 90 to 160 nm and consistent with VLP morphology were counted.
[d] Only nanoparticles from 90 to 160 nm were counted.

makes it troublesome to find the correct dilutions for sample quantitation. In addition, the method showed to be more precise and less prone to experimental error than p24 ELISA with a mean %CV of 2 vs. 10. The method has shown adequate accuracy as demonstrated by concordance between reference p24 concentration values and those obtained by spectrofluorometry. The developed quantitation method was found to be significantly less sensitive than p24 ELISA (LOD ~10 ng vs. 10 pg of p24/mL, respectively). Thus, the latter may still be required in specific cases in which VLP samples contain less than 10 ng of p24/mL. However, the sensitivity of spectrofluorometry is high enough to quantify Gag-GFP VLPs in most samples as shown by significant fluorescence intensities in multiple cell culture supernatant samples analyzed throughout this work.

A common limitation of ELISA and spectrofluorometry quantitation methods is that they cannot distinguish between free Gag-GFP monomers and correctly assembled Gag-GFP VLPs. Although Gag-GFP monomers would be relatively easy to remove during downstream processing operations, they can still interfere with both ELISA and spectrofluorometry quantitation methods. This highlights the importance of maintaining cell viability high throughout production to minimize contamination with free Gag-GFP species. Harvest of VLPs can be performed at early stages when the percentage of assembled Gag-GFP is still high. In any case, the level of contamination with free Gag-GFP monomers in harvested VLP supernatants should be assessed in order to avoid overestimation of Gag-GFP VLP titers. To assess the content of these two types of species in VLP samples, a separation step prior to analyses is required. This can be accomplished, by using centrifugal filter units as shown in this work or by viral particle pelleting prior to quantitation (QuickTiter™ Lentivirus Titer Kit), for instance.

A calibration curve was constructed to convert fluorescence values into p24 titers. In order to convert p24 to Gag-GFP concentration values a correction factor was estimated using densitometry. This correction factor takes into account the difference in molecular weight of both proteins and an underestimation factor arising from the use of p24 ELISA to estimate Gag polyprotein concentrations. In order to compare the titers obtained by spectrofluorometry with TEM and NTA results it was assumed that each Gag VLP contains 2500 Gag-GFP molecules based on reported values (Chen et al., 2009). The good agreement between VLP concentration values obtained by spectrofluorometry with all other quantitation techniques evaluated in this work confirmed the value of the proposed method.

In conclusion, a simple, reliable and cost-effective technique for Gag VLP quantitation is reported here. This method should facilitate the development and optimization of bioprocessing strategies for Gag-based VLPs.

Conflict of interest

No conflict of interest to declare.

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References

Adamson, C.S., Nermut, M., Jones, I.M., 2003. Control of human immunodeficiency virus type-1 protease activity in insect cells expressing Gag–Pol rescues assembly of immature but not mature virus-like particles. Virology 308, 157–165.
Alain, R., 1997. Quantitation of virus particles by negative stain electron microscopy. Microsc. Today 97, 20.
Altaras, N.E., Aunins, J.G., Evans, R.K., Kamen, A., Konz, J.O., Wolf, J.J., 2005. Production and formulation of adenovirus vectors. Adv. Biochem. Eng. Biotechnol. 99, 193–260.
Aucoin, M.G., Perrier, M., Kamen, A.A., 2008. Critical assessment of current adeno-associated viral vector production and quantification methods. Biotechnol. Adv. 26, 73–88.
Bland, J.M., Altman, D.G., 1995. Comparing two methods of clinical measurement: a personal history. Int. J. Epidemiol. 24 (Suppl. 1), S7–S14.
Block, O., Mitra, A., Novotny, L., Dykes, C., 2012. A rapid label-free method for quantitation of human immunodeficiency virus type-1 particles by nanospectroscopy. J. Virol. Methods 182, 70–75.
Caygill, R.L., Blair, G.E., Millner, P.A., 2010. A review on viral biosensors to detect human pathogens. Anal. Chim. Acta 681, 8–15.
Chang, L.J., Zaiss, A.K., 2002. Lentiviral vectors. Preparation and use. Methods Mol. Med. 69, 303–318.
Chen, Y., Wu, B., Musier-Forsyth, K., Mansky, L.M., Mueller, J.D., 2009. Fluorescence fluctuation spectroscopy on viral-like particles reveals variable gag stoichiometry. Biophys. J. 96, 1961–1969.
Clark, K.R., Voulgaropoulou, F., Johnson, P.R., 1996. A stable cell line carrying adenovirus-inducible rep and cap genes allows for infectivity titration of adeno-associated virus vectors. Gene Ther. 3, 1124–1132.
Cox, M.M., 2012. Recombinant protein vaccines produced in insect cells. Vaccine 30, 1759–1766.
Dale, B.M., McNerney, G.P., Hubner, W., Huser, T.R., Chen, B.K., 2011. Tracking and quantitation of fluorescent HIV during cell-to-cell transmission. Methods 53, 20–26.
Deml, L., Speth, C., Dierich, M.P., Wolf, H., Wagner, R., 2005. Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. Mol. Immunol. 42, 259–277.
DePhillips, P., Lenhoff, A.M., 2000. Pore size distributions of cation-exchange adsorbents determined by inverse size-exclusion chromatography. J. Chromatogr. A 883, 39–54.
FDA, 2001. Guidance for Industry, Bioanalytical Method Validation.
Filipe, V., Hawe, A., Jiskoot, W., 2010. Critical evaluation of nanoparticle tracking analysis (NTA) by nanosight for the measurement of nanoparticles and protein aggregates. Pharm. Res. 27, 796–810.
Frankel, A.D., Young, J.A., 1998. HIV-1: fifteen proteins and an RNA. Annu. Rev. Biochem. 67, 1–25.
Freed, E.O., 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology 251, 1–15.
Garnier, L., Ravallec, M., Blanchard, P., Chaabihi, H., Bossy, J.P., Devauchelle, G., Jestin, A., Cerutti, M., 1995. Incorporation of pseudorabies virus gD into human immunodeficiency virus type 1 Gag particles produced in baculovirus-infected cells. J. Virol. 69, 4060–4068.
Graham, F.L., van der Eb, A.J., 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 12.
Hammonds, J., Chen, X., Zhang, X., Lee, F., Spearman, P., 2007. Advances in methods for the production, purification, and characterization of HIV-1 Gag-Env pseudovirion vaccines. Vaccine 25, 8036–8048.
Haynes, J.R., Dokken, L., Wiley, J.A., Cawthon, A.G., Bigger, J., Harmsen, A.G., Richardson, C., 2009. Influenza-pseudotyped Gag virus-like particle vaccines provide broad protection against highly pathogenic avian influenza challenge. Vaccine 27, 530–541.
Hermida-Matsumoto, L., Resh, M.D., 2000. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. J. Virol. 74, 8670–8679.
Hermle, J., Anders, M., Heuser, A.M., Muller, B., 2010. A simple fluorescence based assay for quantification of human immunodeficiency virus particle release. BMC Biotechnol. 10, 32.
ICH, 2005. Validation of Analytical Procedures: Text and Methodology Q2(R1).
Joshi, S., Joshi, R.L., 1996. Molecular biology of human immunodeficiency virus type-1. Transfus. Sci. 17, 351–378.
Lindwasser, O.W., Resh, M.D., 2002. Myristoylation as a target for inhibiting HIV assembly: unsaturated fatty acids block viral budding. Proc. Natl. Acad. Sci. U.S.A. 99, 13037–13042.
Ludwig, C., Wagner, R., 2007. Virus-like particles-universal molecular toolboxes. Curr. Opin. Biotechnol. 18, 537–545.

Lynch, A.G., Tanzer, F., Fraser, M.J., Shephard, E.G., Williamson, A.L., Rybicki, E.P., 2010. Use of the piggyBac transposon to create HIV-1 gag transgenic insect cell lines for continuous VLP production. BMC Biotechnol. 10, 30.
Maizel Jr., J.V., White, D.O., Scharff, M.D., 1968. The polypeptides of adenovirus, I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology 36, 115–125.
McGrath, M., Witte, O., Pincus, T., Weissman, I.L., 1978. Retrovirus purification: method that conserves envelope glycoprotein and maximizes infectivity. J. Virol. 25, 923–927.
Muller, B., Daecke, J., Fackler, O.T., Dittmar, M.T., Zentgraf, H., Krausslich, H.G., 2004. Construction and characterization of a fluorescently labeled infectious human immunodeficiency virus type 1 derivative. J. Virol. 78, 10803–10813.
Osterrieder, N., Wagner, R., Brandmuller, C., Schmidt, P., Wolf, H., Kaaden, O.R., 1995. Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). Virology 208, 500–510.
Ott, D.E., 1997. Cellular proteins in HIV virions. Rev. Med. Virol. 7, 167–180.
Ott, D.E., 2002. Potential roles of cellular proteins in HIV-1. Rev. Med. Virol. 12, 359–374.
Pease 3rd, L.F., Lipin, D.I., Tsai, D.H., Zachariah, M.R., Lua, L.H.L., Tarlov, M.J., Middelberg, A.P.J., 2009. Quantitative characterization of virus-like particles by asymmetrical flow field flow fractionation, electrospray differential mobility analysis, and transmission electron microscopy. Biotechnol. Bioeng. 102, 11.
Pecora, A., Perez Aguirreburualde, M.S., Rodriguez, D., Seki, C., Levy, M.S., Bochoeyer, D., Dus Santos, M.J., Wigdorovitz, A., 2009. Development and validation of an ELISA for quantitation of bovine viral diarrhea virus antigen in the critical stages of vaccine production. J. Virol. Methods 162, 170–178.
Perlman, M., Resh, M.D., 2006. Identification of an intracellular trafficking and assembly pathway for HIV-1 gag. Traffic 7, 731–745.
Phogat, S., Wyatt, R.T., Karlsson Hedestam, G.B., 2007. Inhibition of HIV-1 entry by antibodies: potential viral and cellular targets. J. Intern. Med. 262, 26–43.
Pillay, S., Meyers, A., Williamson, A.L., Rybicki, E.P., 2009. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. Biotechnol. Prog. 25, 1153–1160.
Roberts, G.S., Yu, S., Zeng, Q., Chan, L.C., Anderson, W., Colby, A.H., Grinstaff, M.W., Reid, S., Vogel, R., 2012. Tunable pores for measuring concentrations of synthetic and biological nanoparticle dispersions. Biosens. Bioelectron. 31, 17–25.
Roldao, A., Mellado, M.C., Castilho, L.R., Carrondo, M.J., Alves, P.M., 2010. Virus-like particles in vaccine development. Expert Rev. Vaccines 9, 1149–1176.
Sakuragi, S., Goto, T., Sano, K., Morikawa, Y., 2002. HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 99, 7956–7961.
Sakuragi, S., Sakuragi, J., Morikawa, Y., Shioda, T., 2006. Development of a rapid and convenient method for the quantification of HIV-1 budding. Microbes Infect. 8, 1875–1881.
Schroder, M., von Lieres, E., Hubbuch, J., 2006. Direct quantification of intraparticle protein diffusion in chromatographic media. J. Phys. Chem. B 110, 1429–1436.
Schwartz, S., Campbell, M., Nasioulas, G., Harrison, J., Felber, B.K., Pavlakis, G.N., 1992. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. J. Virol. 66, 7176–7182.
Segura, M.M., Garnier, A., Di Falco, M.R., Whissell, G., Meneses-Acosta, A., Arcand, N., Kamen, A., 2008. Identification of host proteins associated with retroviral vector particles by proteomic analysis of highly purified vector preparations. J. Virol. 82, 1107–1117.
Segura, M.M., Garnier, A., Kamen, A., 2006. Purification and characterization of retrovirus vector particles by rate zonal ultracentrifugation. J. Virol. Methods 133, 82–91.
Segura, M.M., Monfar, M., Puig, M., Mennechet, F., Ibanes, S., Chillon, M., 2010. A real-time PCR assay for quantification of canine adenoviral vectors. J. Virol. Methods 163, 129–136.
Sherer, N.M., Lehmann, M.J., Jimenez-Soto, L.F., Ingmundson, A., Horner, S.M., Cicchetti, G., Allen, P.G., Pypaert, M., Cunningham, J.M., Mothes, W., 2003. Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. Traffic 4, 785–801.
Sutjipto, S., Ravindran, S., Cornell, D., Liu, Y.H., Horn, M., Schluep, T., Hutchins, B., Vellekamp, G., 2005. Characterization of empty capsids from a conditionally replicating adenovirus for gene therapy. Hum. Gene Ther. 16, 109–125.
Tagliamonte, M., Visciano, M.L., Tornesello, M.L., De Stradis, A., Buonaguro, F.M., Buonaguro, L., 2011. HIV-Gag VLPs presenting trimeric HIV-1 gp140 spikes constitutively expressed in stable double transfected insect cell line. Vaccine 29, 4913–4922.
Transfiguracion, J., Jaalouk, D.E., Ghani, K., Galipeau, J., Kamen, A., 2003. Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications. Hum. Gene Ther. 14, 1139–1153.
Transfiguracion, J., Mena, J.A., Aucoin, M.G., Kamen, A.A., 2011. Development and validation of a HPLC method for the quantification of baculovirus particles. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 879, 61–68.
Tremblay, M.J., Fortin, J.F., Cantin, R., 1998. The acquisition of host-encoded proteins by nascent HIV-1. Immunol. Today 19, 346–351.
Ulmer, J.B., Valley, U., Rappuoli, R., 2006. Vaccine manufacturing: challenges and solutions. Nat. Biotechnol. 24, 1377–1383.
Valley-Omar, Z., Meyers, A.E., Shephard, E.G., Williamson, A.L., Rybicki, E.P., 2011. Abrogation of contaminating RNA activity in HIV-1 Gag VLPs. Virol. J. 8, 462.
Wilk, T., Gross, I., Gowen, B.E., Rutten, T., de Haas, F., Welker, R., Krausslich, H.G., Boulanger, P., Fuller, S.D., 2001. Organization of immature human immunodeficiency virus type 1. J. Virol. 75, 759–771.
Yao, Q., Bu, Z., Vzorov, A., Yang, C., Compans, R.W., 2003. Virus-like particle and DNA-based candidate AIDS vaccines. Vaccine 21, 638–643.

Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells

Results, chapter 2

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Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells

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Abstract HIV-1 virus-like particles (VLPs) have great potential as new-generation vaccines. The novel CAP-T cell line is used for the first time to produce Gag-GFP HIV-1 VLPs by means of polyethylenimine (PEI)-mediated transient transfection. CAP-T cells are adapted to grow to high cell densities in serum-free medium, and are able to express complex recombinant proteins with human post-translational modifications. Furthermore, this cell line is easily transfected with PEI, which offers the flexibility to rapidly generate and screen a number of candidates in preclinical studies. Transient transfection optimization of CAP-T cells has been performed systematically in this work. It is determined that for optimal production, cells need to be growing at mid-exponential phase, Protein Expression Medium (PEM) medium has to be added post-transfection, and cells can be transfected by independent addition of DNA and PEI with no prior complexation. A Box-Behnken experimental design is used to optimize cell density at time of transfection, DNA/cell and PEI/cell ratios. The optimal conditions determined are transfection at a density of 3.3E + 06 cells/mL with 0.5 pg of DNA/cell and 3 pg of PEI/cell. Using the optimized protocol, 6×10^{10} VLP/mL are obtained, demonstrating that CAP-T is a highly efficient cell line for the production of HIV-1 VLPs and potentially other complex viral-based biotherapeutics.

Keywords Transient gene expression · HIV-1 virus-like particles · CAP-T · Design of experiments

Introduction

The majority of vaccines commercially available today are based on inactivated or live-attenuated viruses. Although they are immunologically potent, these classical vaccines often present side effects on vaccinated individuals and they represent a risk at manufacturing level before being inactivated or attenuated. Recombinant vaccines are a safer alternative; however, they show less immunogenicity and higher doses may be required (Kushnir et al. 2012). Virus-like particles (VLPs) represent a novel approach in the generation of recombinant vaccines as they combine some advantages of classical and new-generation vaccines. VLPs are formed by the spontaneous assembly of the main virus structural protein(s), but they do not contain the viral genetic information and they are non-infectious. Therefore, no safety concerns are associated to their manufacture, manipulation, and administration. On the other hand, they have shown to be immunologically more potent than recombinant subunit vaccines, as they present the antigens in a repetitive form and in a very similar conformation than in the wild virus. For these reasons, VLPs represent high potential as novel vaccine candidates (Roldao et al. 2010; Buonaguro et al. 2013).

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One of the viruses for which VLPs have been studied as potential vaccines is HIV. The main structural protein of the virus (Gag polyprotein) is able to self-assemble upon expression in a heterologous system, without the need of any other viral component, giving rise to VLPs that resemble the structure of immature HIV virions. VLP assembly takes place in the vicinity of the plasma membrane, and the particles are released from cells by a budding process taking part of the membrane as a lipid envelope (Buonaguro et al. 2013). Gag-based VLPs can expose on their surface membrane proteins from the host cells, HIV surface antigens (when co-expressed with Gag) (Hammonds et al. 2007; Tagliamonte et al. 2011), or proteins from other viruses (Garnier et al. 1995; Haynes et al. 2009). Therefore, they can be exploited as platforms for displaying a variety of antigens.

HIV-1 VLPs have been generated using a variety of expression systems (Pillay et al. 2009; Sakuragi et al. 2002; Scotti et al. 2009; Tagliamonte et al. 2010); however, mammalian cells are one of the most suitable platforms for Gag VLP production because they are able to perform post-translational modifications (PTMs) very similar to those of the wild-type virus (Cervera et al. 2013). Protein production in mammalian cells can be achieved by generation of stable cell lines (SGE) or by transient gene expression (TGE). While the former has been up to now the preferred approach in industry (high titers and lot-to-lot consistency are its main advantages), TGE has been relegated to early development stages of drugs and vaccines, or as the system to obtain research-grade material. However, in the last years, extensive efforts have been made toward the improvement of production titers and scale-up of TGE strategies (Bandaranayake and Almo 2014; Geisse 2009). As a result, titers of 2 g/L of protein using a TGE approach at 6 L scale (Daramola et al. 2014) and a TGE-based protein production at more than 100 L scale (Girard et al. 2002; Tuvesson et al. 2008) have been reported. Moreover, TGE offers the flexibility to easily change the protein to be expressed and milligram to gram quantities of protein can be obtained within 2–4 weeks (Backliwal et al. 2008a).

TGE has been extensively used in HEK293 and CHO cell lines. However, new emerging cell lines suitable for transient protein expression have been developed (Geisse 2009). CAP (Cevec Pharmaceuticals) is a novel cell line derived from human amniocytes and transformed by the E1 and pIX functions of Ad5 (Schiedner et al. 2000). CAP-T cells are based on CAP, but they express the large T antigen of simian virus 40 (SV40LT) which supports episomal replication of plasmids containing SV40 ori (Wolfel et al. 2011). This advantageous feature allows working both at early development stages (using TGE on CAP-T cells) and production levels (using SGE on CAP cells) without the need of changing the cell line (Geisse 2009). Moreover, CAP-T cells are adapted to grow in suspension and in serum-free media to high cell densities, which make them suitable for large-scale TGE, and are highly transfectable. There are limited reports on this cell line because of its novelty; however, it has been previously described to support expression of difficult-to-express proteins (Fischer et al. 2012) and virus production (Genzel et al. 2013).

The aim of this work is to establish an optimized TGE approach to produce HIV-1 derived Gag-GFP VLPs using the CAP-T cell platform. The plasmid DNA used in this work codes for a HIV-1 Gag polyprotein fused to GFP that allows easy quantification of the fluorescently labeled VLPs and transfection efficiency. Polyethylenimine (PEI) Max is used as a transfection reagent for CAP-T cells. Besides its many advantages (easy to use, efficient with suspension cells, compatible with some serum-free media, and cost-effective) (Baldi et al. 2007), it has been previously reported as the most efficient reagent for this cell line (Fischer et al. 2012). Production of correctly assembled VLPs is initially demonstrated. Then, the variables affecting TGE are discriminated between discrete and continuous factors. The former are addressed one by one in a stepwise optimization, and the latter are optimized by a Design of Experiments (DoE) approach. High production levels are reported, demonstrating the suitability of the CAP-T system for this specific production of HIV-1 VLPs. To the best of our knowledge, this is the first reported case of VLP expression in CAP-T cells by means of TGE.

Materials and methods

Cell line and culture conditions

CAP-T cells were kindly provided by Cevec Pharmaceuticals (Cologne, Germany). Cells were cultured in suspension in Protein Expression Medium (PEM, Invitrogen, Grand Island, NY, USA) supplemented with 4 mM of GlutaMAX (Invitrogen, Paisley, UK). Cells were routinely maintained at exponential growth phase with viabilities over 95 % in 125 mL disposable polycarbonate Erlenmeyer flasks (Corning, New York, NY, USA). Erlenmeyer flasks were placed in an orbital shaker (orbit diameter 16 mm, Stuart, Stone, UK) at 130 rpm in a humidified incubator at 37 °C and 5 % CO_2 in air. Cell counts, viability, and vitality assay were performed using the NucleoCounter®NC-3000 automatic cell counter (Chemometec, Allerod, Denmark) according to manufacturer's instructions.

Plasmid DNA

The pGag-EGFP plasmid used for transfection codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP. The plasmid from the NIH AIDS Reagent Program (Cat #11468) (Hermida-Matsumoto and Resh 2000) was constructed by cloning the Gag sequence from

pCMV55M1-10 (Schwartz et al. 1992) into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). Plasmid DNA amplification and purification was carried out as previously reported (Gutierrez-Granados et al. 2013).

Standard transient transfection

VLPs were generated by transient transfection of CAP-T cells with pGag-EGFP plasmid DNA using 40 KDa linear polyethylenimine (PEI Max, PolySciences, Warrington, PA, USA) as transfection reagent. The standard transfection protocol consisted of the steps explained here. However, as part of the optimization study, changes are introduced in the final protocol as detailed in the "Results" section. Prior to transfection, cells exponentially growing in PEM were subjected to a medium exchange to FreeStyle™ 293 (Invitrogen, Grand Island, NY, USA) supplemented with 0.1 % Pluronic (Invitrogen, Paisley, UK) by centrifugation at 200×*g* during 5 min. In this step, cells were concentrated to 5×10^6 cells/mL in 4 mL of medium. DNA at 1 pg/cell and PEI at 3 pg/cell (DNA:PEI mass ratio of 1:3) were diluted separately in FreeStyle™ 293 and vortexed for 10 s then the DNA solution was added to the PEI solution and the mixture was vortexed three times for 3 s. After 15 min of incubation at room temperature, 2 mL of complexes were added to the concentrated culture. Culture volume was completed with PEM (24 mL) 5 h post-transfection (hpt). The percentage of GFP expressing cells was assessed by flow cytometry using a fluorescence-activated cell sorter (FACS) (BDFACS Canto, BD BioSciences, San Jose, CA, USA).

Confocal microscopy

The visualization of VLP producer cells was performed using a FluoView®FV1000 confocal microscope (Olympus, Tokyo, Japan). Transfected cells were dyed with Hoechst and CellMask™ and subsequently observed under the microscope as previously reported (Gutierrez-Granados et al. 2013).

Quantitation of Gag-GFP VLPs by spectrofluorometry

The concentration of Gag-GFP VLPs was assessed by spectrofluorometry using an in-house developed and validated quantification technique (Gutierrez-Granados et al. 2013). The equation used to convert RFU values to Gag-GFP concentration values is the following:

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

where Gag-GFP is the estimated concentration of polyprotein and RFU is the measured GFP fluorescence intensity in the samples. To further convert Gag-GFP concentration to VLP concentration, it was assumed that one VLP contains 2500 Gag-GFP monomers (Chen et al. 2009) with a molecular weight of 84 kDa per monomer. Percentage of assembled Gag-GFP molecules was assessed using Vivaspin500 centrifugal filter units (300 kDa MWCO; Sartorius Stedim Biotech, Goettingen, Germany) to separate Gag-GFP monomers from VLPs and then quantifying both species by p24 ELISA as described in Gutierrez-Granados et al. (2013). The percentage of assembled Gag-GFP was over 80 %, so monomeric Gag-GFP molecules were not taken into account for further quantification.

VLP purification

VLPs from CAP-T transfected cell culture supernatants were further purified using a common retrovirus purification protocol consisting of virus particle pelleting by ultracentrifugation through a sucrose cushion (Transfiguracion et al. 2003). Briefly, a volume of 35.5 mL of clarified supernatant harvested 96 hpt was layered on top of a 30 % sucrose cushion (3 mL) and centrifuged at 26,000 rpm (115,254×*g*) for 3 h at 4 °C using a SW32 rotor in a Beckman Optima L100XP centrifuge. Pellets were resuspended in pre-chilled PBS and incubated overnight at 4 °C. Size exclusion chromatography (SEC) was performed using PD-10 Desalting Columns (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions. Elution was carried out with PBS.

VLP characterization by nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was employed for VLP characterization in a NanoSight®LM20 device (NanoSight Ltd., Amesbury, UK) at the Service of Preparation and Characterization of Soft Materials (Institut de Ciència de Materials de Barcelona, ICMAB, CSIC, Bellaterra, Spain). Sample analyses were performed as previously reported (Gutierrez-Granados et al. 2013).

VLP characterization by transmission electron microscopy

VLP samples were prepared by air-dried negative staining method at the Servei de Microscòpia (UAB, Bellaterra, Spain) as previously reported (Gutierrez-Granados et al. 2013) before the examination in a Jeol JEM-1400 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Gatan ES1000W Erlangshen CCD Camera (Model 785).

VLP characterization by tunable resistive pulse sensing

Purified VLP samples were measured using an Izon qNano (Izon Science Europe Ltd., Magdalen Centre, The Oxford Science Park, Oxford, UK). Polystyrene calibration particles with a concentration of 1.2×10^{13} particles/mL and mode size

of 110 nm were purchased from Thermo Fischer Laboratories (Waltham, MA, USA). All samples were dispersed in PBS for analysis. Particle concentration and size were calculated using Izon Control Suite Software V3.2 on a minimum of 500 particle events.

Optimization of transient transfection using design of experiments

Transient transfection was optimized in order to maximize VLP production (DoE response). The three variables chosen for optimization were cell density at the time of transfection, DNA/cell ratio, and PEI/cell ratio. A Box-Behnken design was selected to define the optimal value for each variable. The three 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 1. Box-Behnken experimental results were fitted to a second-order polynomial equation described below (Eq. 2) by non-linear regression analysis:

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

where Y is the response in RFU, β_0 is the offset term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is 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 2010 and R software (RStudio, Inc., Boston, MA, USA).

Statistical analysis

Statistical analysis of the models was performed using SigmaPlot 11.0 (Systat Software Inc.) and R Studio software (RStudio, Inc., Boston, MA, USA). 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 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.

Results

Initial characterization of cell growth and transfection

As starting point of the study, some aspects related to the characterization of cell growth and transfection were assessed. CAP-T cell line is adapted to grow in suspension in PEM, and FreeStyle™ 293 has been previously reported as the transfection medium for these cells (Fischer et al. 2012). Particularly, the need to use two different media, one for cell growth and one for cell transfection, was first investigated with the aim to avoid medium exchange, which may add complexity to the process. First, transfection in PEM medium was performed and no GFP expressing cells were obtained (data not shown). In fact, PEM has been previously reported as incompatible with PEI transfection (Fischer et al. 2012). Second, cell growth in FreeStyle™ 293 was tested and compared with growth in PEM. CAP-T cells grew up to 7×10^6 cells/mL with a duplication time of 25 h in PEM medium. However, they only grew up to 3.6×10^6 cells/mL in FreeStyle™ 293 with a duplication time of 49 h, with lower viabilities (Fig. 1a). Transfection in FreeStyle™ 293 was required, while cell growth in PEM allowed obtaining higher cell densities with higher viabilities. Thus, medium exchange could not be avoided due to the lack of a culture medium that simultaneously supports high cell densities and transfection of CAP-T cells.

Gag-GFP VLP production upon transfection with the standard protocol in CAP-T cells was studied. The percentage of GFP-positive cells was 30 % at 24 hpt and almost 60 % at 48 hpt. Then, the percentage of GFP-positive cells decreased (Fig. 1b), although the concentration of GFP+ cells was maintained over time up to 120 hpt (data not shown). Cells were able to grow up to 6×10^6 cells/mL after transfection, while the untransfected culture grew up to 7.9×10^6 cells/mL with higher viabilities (data not shown).

Green fluorescent cells were visualized by confocal fluorescence microscopy 48 hpt. Green fluorescence was observed in the cytoplasm of transfected cells (Fig. 1c), but Gag-GFP molecules were particularly enriched in the vicinity of the plasma membrane. By staining the lipid membrane with CellMask™ (red), co-localization of green and red fluorescence was visualized as yellow regions on the membrane, where the virus particle budding occurs. Then, the kinetics of Gag-GFP expression upon transient transfection of CAP-T cells was evaluated by monitoring green fluorescence intensity in harvested supernatants at different times post-transfection (Fig. 1b). Gag-GFP fluorescence in cell culture supernatants increased continuously after transfection and stabilized at 120 hpt. Maximum fluorescence was 120 RFU, which corresponds to 4×10^{10} VLPs/mL according to Eq. (1). Cell viability was maintained over 80 % until 120 hpt.

The Gag-GFP VLPs produced in CAP-T cells upon transfection were further studied in order to demonstrate that Gag-GFP was correctly assembled into VLPs with the expected characteristics. Representative micrographs from VLP purified material were obtained by transmission electron microscopy (TEM) (Fig. 1d). Roughly spherical virus particles surrounded by a lipid envelope can be distinguished with a particle diameter around 138 ± 25 nm. The same sample was analyzed by NTA and tunable resistive pulse sensing (TRPS). NTA results showed a main

Table 1 Box-Behnken design, results, and ANOVA analyses

	−1		0		1
Cell density (E + 06 cells/mL)	2		5		8
DNA/cell ratio (pg/cell)	0.5		1.25		2
PEI/cell ratio (pg/cell)	3		5		8
Experimental run	Cell density	DNA/cell ratio	PEI/cell ratio	Max fluorescence in SN (RFU[a])	Viability (%)
1	0	1	1	80.96	64.70
2	1	0	−1	117.20	55.30
3	0	1	−1	102.17	85.60
4	0	0	0	130.15	53.10
5	1	0	1	81.67	77.00
6	1	1	0	78.01	49.50
7	0	−1	1	134.40	88.30
8	−1	1	0	103.07	64.00
9	1	−1	0	162.63	68.30
10	0	0	0	138.17	67.60
11	−1	0	−1	116.42	84.20
12	−1	−1	0	171.33	80.80
13	0	−1	−1	174.26	83.40
14	−1	0	1	110.56	79.90
15	0	0	0	131.93	67.60
Model	*F* test, *p* value[b]		Lack of fit test, *p* value[c]	R^2	
A (fluorescence-based)	0.0003		0.279		0.988
B (viability-based)	0.0948		0.598		0.860
Parameters (model A)	Coefficient		*t*		*p* value
Constant	129.856		40.265		<0.0001
[Cells]	−7.734		−3.916		0.0112
[Cells][b]	−8.789		−3.023		0.0293
[DNA]	−34.802		−17.622		<0.0001
[DNA][b]	7.694		2.647		0.0456
[PEI]	−12.806		−6.484		0.0013
[PEI][b]	−14.603		−5.023		0.004
[Cells] × [DNA]	−4.091		−1.465		0.2029
[Cells] × [PEI]	−7.418		−2.656		0.0451
[DNA] × [PEI]	4663		1670		0.1558

[a] RFU: relative fluorescence units

[b] *p* values under 0.05 are considered statistically significant with 95 % confidence, and *p* values under 0.1 are considered statistically significant with 90 % confidence

[c] *p* values associated to lack of fit test above 0.05 mean that the hypothesis arguing that the model is suitable cannot be rejected

peak size of (average statistical mode) 169.5 ± 60 nm (Fig. 1e), and 46 % of the particles in the supernatants were determined to be between 100 and 200 nm. Accordingly, when particle size analysis was performed by TRPS technique, a mean size of 154 nm was determined.

Stepwise optimization of the transient transfection protocol for CAP-T cells

Several features of the transfection process were further studied in order to improve the standard production protocol

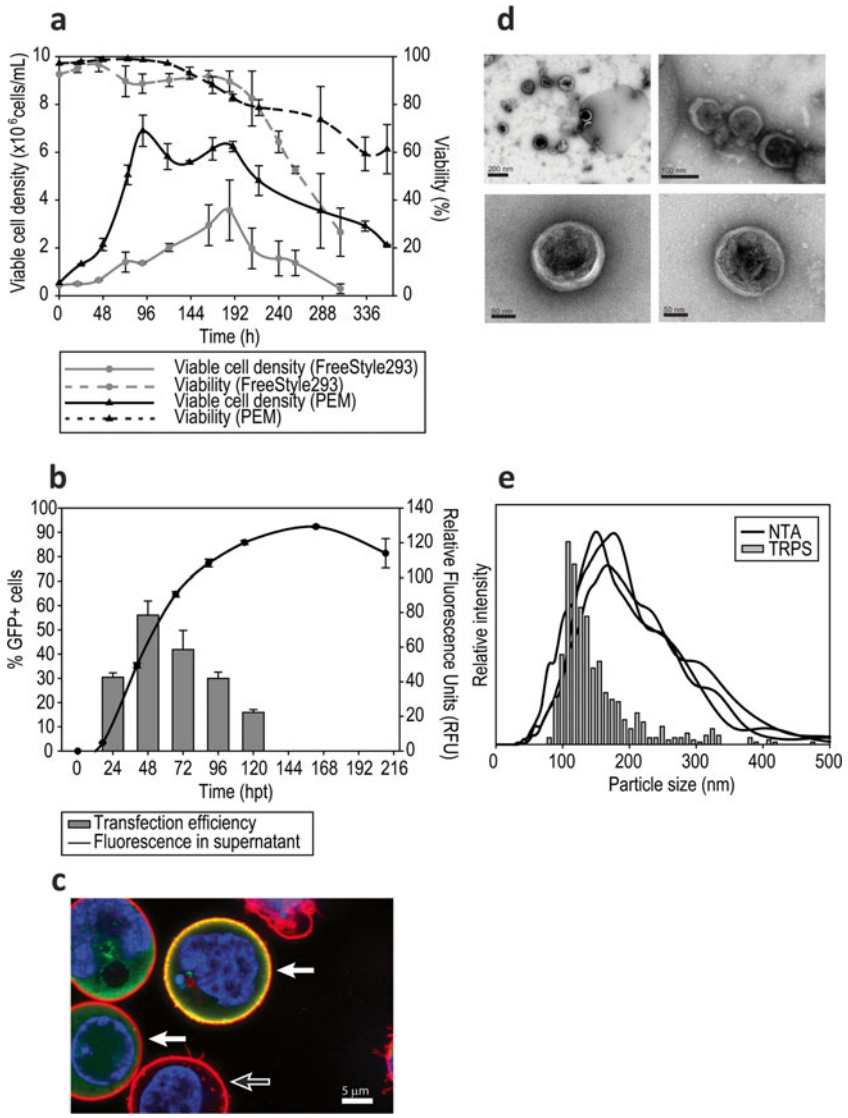

Fig. 1 **a** Cell growth in 125-mL disposable Erlenmeyer flasks of CAP-T cells in batch. **b** Percentage of GFP+ cells and fluorescence signal in cell culture supernatants at different times post-transfection of CAP-T cells transfected with the standard protocol. **c** Confocal fluorescence microscopy images of CAP-T producer cells 48 hpt. Cells were subjected to double staining: cell nucleus was stained with Hoechst (*blue*) and membrane was stained with CellMask™ (*red*). The *green fluorescence* indicates the presence of Gag-GFP molecules. *Solid arrows* indicate transfected cells, and the *empty arrow* indicates a non-transfected cell. **d** Electron microscopy image of negatively stained Gag-GFP VLPs. **e** NTA and TRPS analysis of purified Gag-GFP VLP samples. All experiments were performed in triplicate. *Error bars* represent standard deviation in all cases

described before. These include cell state at the time of transfection, DNA-PEI complexation, and PEM addition after transfection. The mentioned factors are considered as discrete variables affecting transient transfection that need to be addressed individually (Thompson et al. 2012).

Study of cell state at the time of transfection

A growth curve in PEM medium was performed in order to study if cell state has an influence on the productivity of CAP-T cells, and cell density, viability and vitality were analyzed every 24 h (Fig. 2a). Cells grew up to 8.7×10^6 cells/mL with viabilities over 80 % and with a duplication time of 25 h. The vitality assay indicates the percentage of cells with high concentrations of thiol-containing molecules. These molecules are easily oxidized; thus, they are able to protect cells from the reactive oxygen species. That is the reason why thiol-containing molecules have an essential role on maintaining the redox state of cells (Circu and Aw 2010). The percentage of healthy cells was maintained high (over 70 %) during the exponential growth phase. Viability and vitality of cells decreased after reaching the maximum cell concentration.

Additionally, cells obtained at various times of the growth curve in PEM (indicated with gray arrows in Fig. 2a) were transfected using the standard protocol (as described in "Materials and methods" section). The percentage of GFP positive cells (24 hpt) and fluorescence in supernatant (120 hpt) of each transfected cells were analyzed (Fig. 2b). Transfection efficiency was higher at days 2, 3, and 4 after cell seeding, which coincides with the mid-exponential phase than when cells were transfected at 1, 5, and 7 days after seeding. VLP production was significantly (p value <0.001) increased when transfected cells were derived from mid-exponential phase (days 2–4), obtaining almost threefold fluorescence than when transfected cells were derived from days 1 and 7 after seeding. Thus, the best performance coincided with mid-

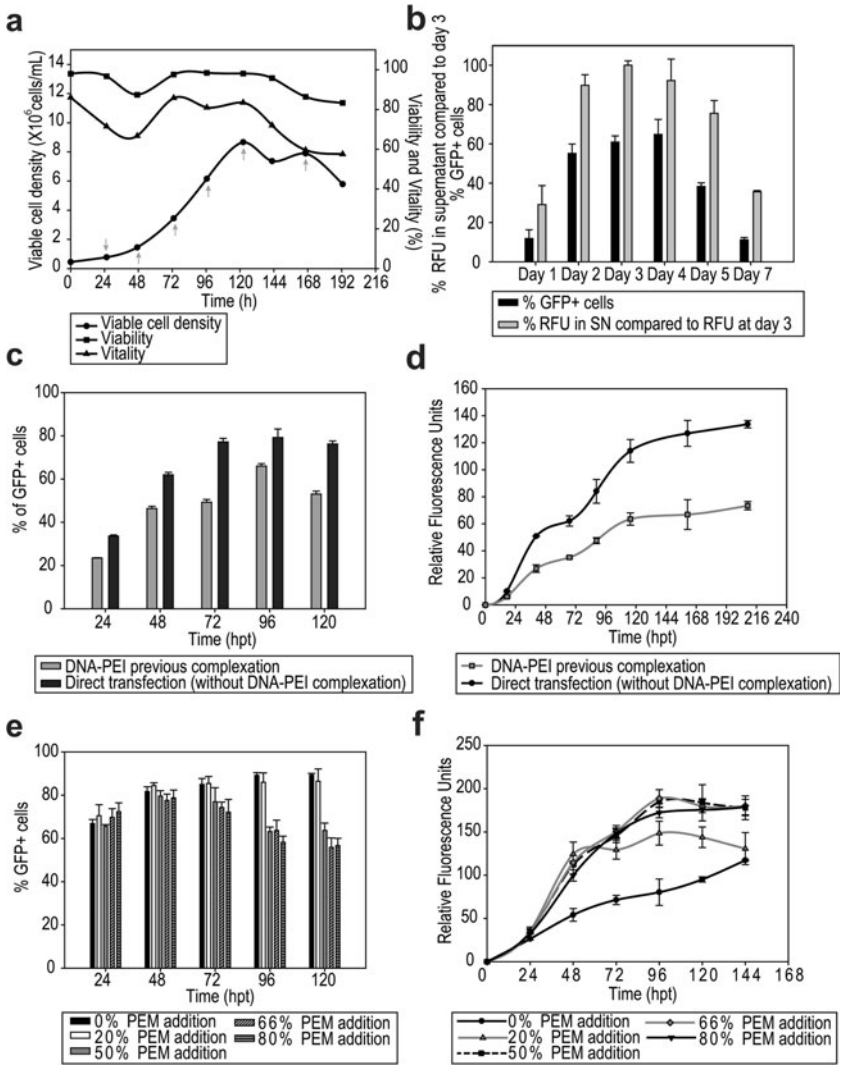

Fig. 2 Stepwise optimization of the standard transfection protocol. **a** Cell growth in 1 L disposable Erlenmeyer flasks of CAP-T cells in PEM. *Arrows* indicate the times at which transfections were performed. **b** Percentage of GFP+ cells 24 hpt of the cells transfected on different times of the curve. Fluorescence in cell culture supernatants 120 hpt expressed as percentage respect fluorescence obtained at day 3. **c**, **d** Percentage of GFP+ cells and fluorescence in cell culture supernatants after transfection when CAP-T cells are transfected with and without prior DNA-PEI complex formation. **e**, **f** Percentage of GFP+ cells and fluorescence in cell culture supernatants after transfection when different percentages of PEM were added 5 hpt. All experiments (except **a**) were performed in triplicate. *Error bars* represent standard deviation in all cases

exponential phase, when cells are healthier and highly dividing. All subsequent transfections were performed 2 days after seeding cells at 0.5×10^6 cells/mL in an attempt to maximize production and gain reproducibility of the process.

Study of direct addition of DNA and PEI avoiding pre-complexation step

Transfection with prior DNA-PEI complexation was studied in comparison with the direct transfection approach, consisting in adding DNA and PEI solutions separately to the culture. This approach has been successfully used in HEK293 and CHO cell lines (Backliwal et al. 2008b; Schlaeger and Christensen 1999) and also in CAP-T cells (Fischer et al. 2012), yielding good transfection efficiencies and product levels. The standard protocol was compared to a transfection performed by adding 1 mL of DNA solution and 1 mL of PEI solution, both at the same final concentration than in the standard protocol but in this case without previous complexation. The percentage of GFP-positive cells and the fluorescence in cell culture supernatants were monitored every 24 hpt. Both transfection efficiency and fluorescence in supernatants (Fig. 2c, 2d) were significantly higher (p value <0.001) when DNA and PEI were added separately in contrast to DNA-PEI complex formation prior to transfection. Since complexation of DNA and PEI did not contribute to an improvement in VLP production, all subsequent transfections were performed using the direct transfection method.

Study of PEM addition after transfection

Finally, the addition of PEM medium after transfection was studied in order to determine the effect of different percentages of PEM in VLP production medium. To this end, five transfections were performed using the standard protocol, this time without DNA-PEI complexation. After 5 hpt, the culture was completed with 80 % of the final volume with different percentages of PEM (0, 20, 50, 66, and 80 %) and FreeStyle™

293 (80, 60, 30, 14, and 0 %). Transfection efficiency and fluorescence in supernatants were analyzed every 24 hpt. The percentage of GFP-positive cells was higher in the cultures that contained 0 and 20 % of PEM (Fig. 2e), probably due to the absence of cell growth after transfection (data not shown). Conversely, when 50 % or more PEM was added after transfection, the percentage of GFP-positive cells decreased over time (from 48 to 120 hpt), probably due to the fact that cells were able to grow after transfection up to 2×10^6 cells/mL (data not shown). Regarding VLP production, Fig. 2f shows that addition of PEM after transfection allowed increasing VLP titers in comparison to the transfection where no PEM was added. A minimum of 50 % of PEM was required to maximize VLP titers.

Summarizing, in order to achieve better performance of CAP-T cells producing Gag-GFP VLPs by transient transfection, cells should be transfected in the mid-exponential phase, DNA and PEI should be added separately, avoiding complex formation before transfection and the final transfected cells should be cultured in a medium containing at least 50 % of PEM.

Transient transfection optimization by design of experiments

Besides the discrete variables studied in the previous section, there are some parameters influencing transient transfection that should be considered as continuous. These are DNA to cell ratio, PEI to cell ratio, and cell concentration at the time of transfection. Continuous variables were studied by means of a design of experiments (DoE). A response surface methodology (RSM), specifically a Box-Behnken design, was selected as it is an efficient methodology in terms of the number of experimental runs to be performed related to the statistically relevant information that can be obtained (Montgomery 1997).

It should be highlighted that the design space must be carefully considered when using DoE approaches since the results will depend on it. Regarding cell density and DNA to cell ratio, the working ranges were set based on the literature (Backliwal et al. 2008a; Cervera et al. 2013; Fischer et al. 2012; Thompson et al. 2012). In the case of PEI to cell ratio, an empirical identification of the working range was performed. Since PEI has a toxicity effect on cells, the upper limit was set at 8 pg/cell based on a toxicity assay (data not shown). 3 pg/cell was set as the minimum PEI necessary to obtain transfected cells. Working ranges for each variable are presented in Table 1.

A three-factor three-level Box-Behnken design was constructed using the selected concentration ranges for each variable as the design space boundaries. A 15-experiment matrix was defined (Table 1) in which the central point was triplicated to assess the pure experimental error. Transfections were performed with a 2 day-old culture previously seeded at 0.5×10^6 cells/mL, without pre-complexation and adding in all cases 50 % of PEM after transfection, according to the conditions defined as optimal in the previous section.

The response variables considered in this study were the maximum fluorescence in cell culture supernatants after transfection and the viability at the time of maximum production (Table 1). The data were fitted to a second-order model by non-linear regression analysis obtaining a model for each one of the response variables. The statistical significance of the non-linear regressions was confirmed by ANOVA analysis (Table 1).

The data from model A were used to construct the response surface plots where the interactions between the factors can be depicted (Fig. 3a–i). Noticeably, cell density does not have a strong influence on fluorescence outcome as can be seen in Fig. 3a–c, where cell density increases from 2 to 8×10^6 cells/mL and the maximum fluorescence does not change. Otherwise, DNA and PEI have a stronger influence on VLP production with smaller associated p values than the p value for cell density (Table 1). The optima for DNA and PEI are located in the lower boundaries of the design space, as can be seen in Fig. 3d–f, g–i, respectively, the increase in DNA/cell ratio or PEI/cell ratio was translated into a decrease of fluorescence in supernatant. A statistically significant interaction between cell concentration and PEI/cell ratio was identified (p value 0.0451, see Table 1). As cells are more concentrated at the time of transfection, the amount of PEI/cell required to achieve maximum fluorescence decreases (Fig. 3a–c).

The effect of cell density, DNA and PEI on viability of the cultures was explored by analyzing a third model (C) whose response variable was $Y = \text{Fluorescence} \cdot (\text{Viability}/100)^n$ (3), where n is between 0 and 1. When $n = 0$, the viability has no importance in the model; thus, the weight of viability in this particular model increases with n. Figure 3j shows the value of each factor studied as a function of the different n values tested. As can be observed, DNA concentration is not influencing the viability of the cultures, as it remains constant regardless of the weight of the viability in the model. Otherwise, PEI and cell density have an influence on viability: the more important viability is in the model (n increases), the lower PEI and cell concentration need to be to achieve the optimum.

Model A was used to predict an optimal combination of the three factors to maximize fluorescence signal in supernatants, but a restriction was applied to this optimization: the viability predicted by model B with the conditions found should be at least 88 % which is the maximum viability attained experimentally. With these conditions, a predicted maximum fluorescence in supernatant of 170.31 ± 10.95 RFU was obtained, with a viability of 88 ± 15.34 % predicted by model B. The optimization of model A without restricting the viability predicted a fluorescence of 177.64 RFU (only a 4 % increase) while the predicted viability was dropped to 74 %. Thus, the optimal concentration of each variable was 0.5 pg of DNA/cell, 3 pg of PEI/cell (PEI:DNA mass ratio of 6:1) and 3.3×10^6 cells/mL at the time of transfection.

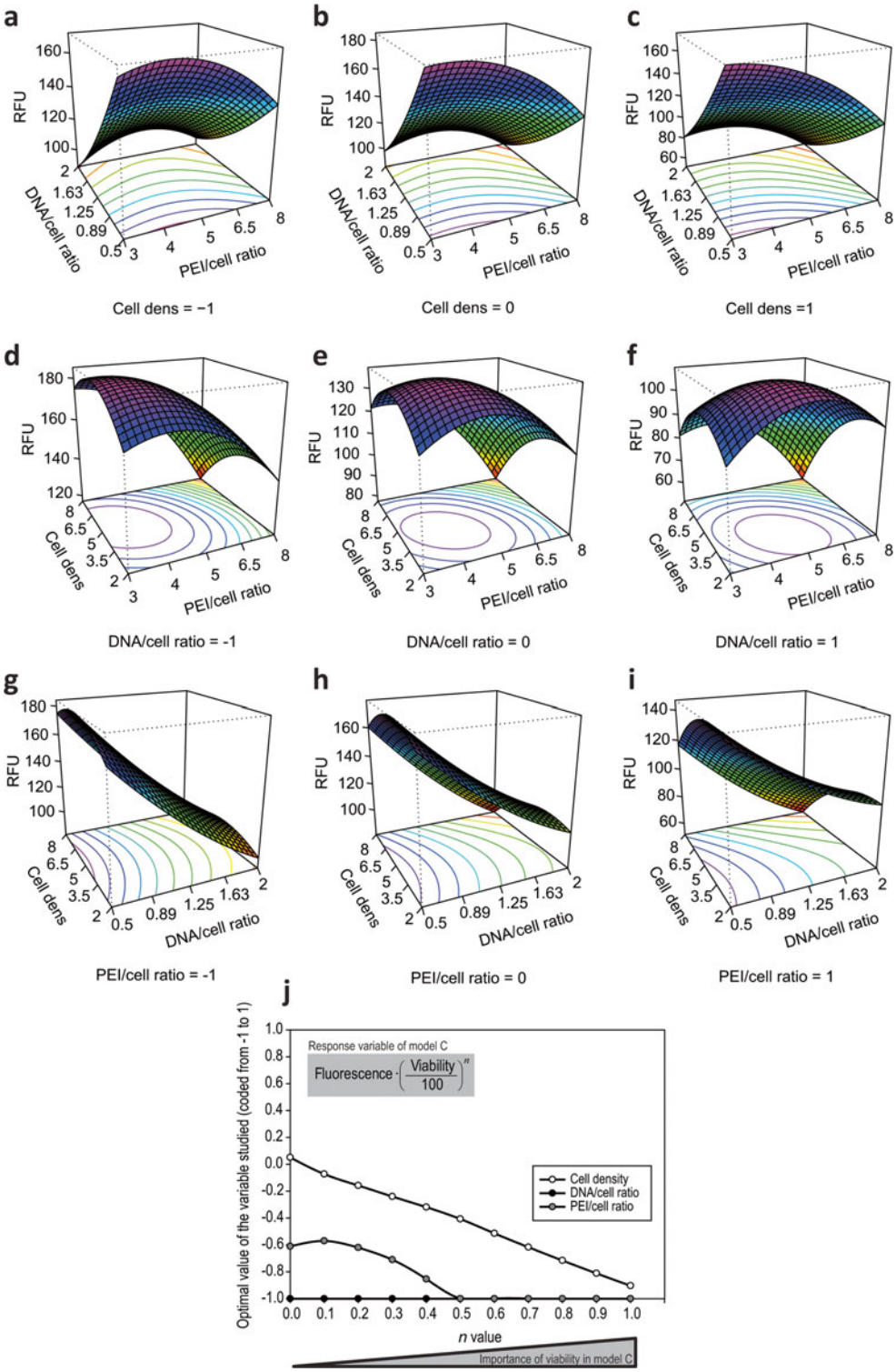

Fig. 3 Response surface graphs based on Box-Behnken experimental results. Maximum fluorescence in cell culture supernatants as a function of **a**, **b**, **c** cell density vs. DNA/cell ratio; **d**, **e**, **f** cell density vs. PEI/cell ratio; and **g**, **h**, **i** DNA/cell ratio vs. PEI/cell ratio. The graphs were constructed by depicting two variables at a time and maintaining the third one at a fixed level. **j** Analysis of the influence of cell density, DNA, and PEI on cell culture viability. The optimal values of each of the factors studied are represented as a function of the *n* value. The value of *n* is an indication of the importance of cell culture viability in the model; thus, the influence of each factor in viability can be depicted

Validation of the model

A confirmation experiment was carried out with the aim of validating the model. To this end, the optimal cell density, DNA and PEI to cell ratios predicted by the model were tested experimentally. With these transfection conditions, the experimental output was a maximum fluorescence in supernatant of 172.83 ± 19.61 RFU (Fig. 4a), which was within the range of the predicted response (170.31 ± 10.95 RFU), confirming model adequacy (Fig. 4b). Cell culture viability at the time of maximum fluorescence (96 hpt) was 90.43 ± 0.86 % (Fig. 4a), in good agreement with the predicted 88 % viability.

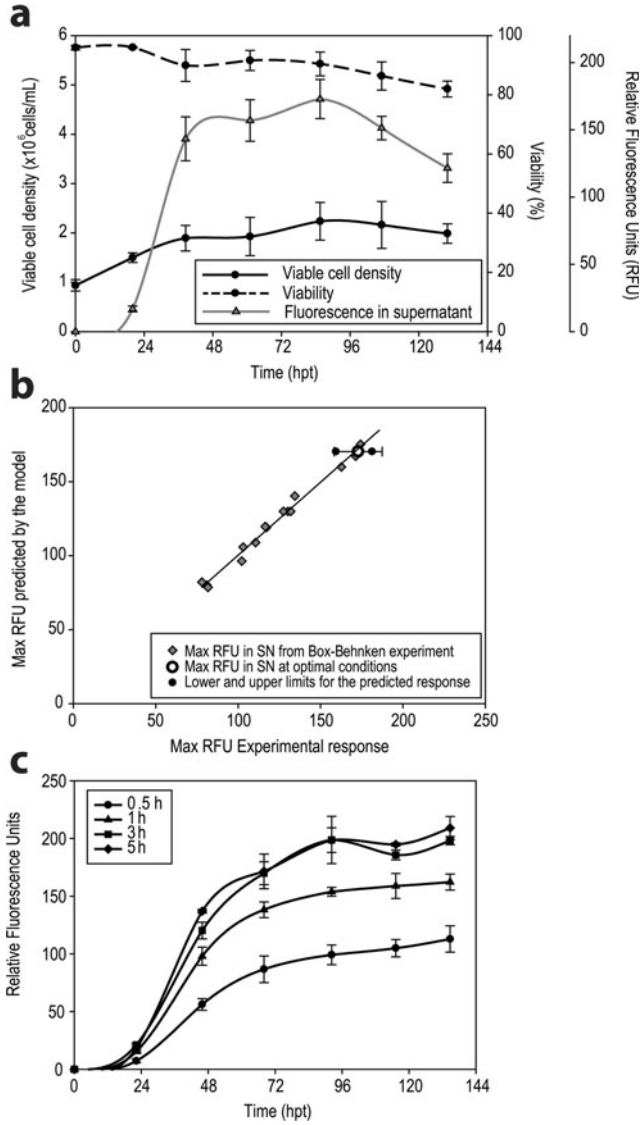

Fig. 4 Model validation. **a** Cell growth, viability, and fluorescence in supernatant after transfection with the optimal conditions. **b** Correlation between the experimental and Box-Behnken model predicted fluorescence values in RFU. The *white dot* represents the experimental vs. predicted value when the optimal conditions for transfection are used, and *solid dots* represent the upper and lower bounds for the predicted value, calculated as predicted value $\pm 1.96 \times \sqrt{\mathrm{MS}}$, with a confidence interval of 95 %. **c** Fluorescence in cell culture supernatants over time after transfection with the optimal conditions, but with different times of PEM addition after transfection. All experiments were performed in triplicate. *Error bars* represent standard deviation in all cases (*MS* mean squared error)

Finally, to further refine the protocol, different times of PEM addition were tested. These included 0.5, 1, 3, and 5 hpt. Figure 4c shows that a minimum time of 3 h after transfection is necessary before adding PEM to the culture in order to obtain maximum fluorescence signal in supernatants. When PEM addition was performed earlier, transfection process was altered likely because of the interference that PEM medium has on PEI-mediated transfection, and consequently, fluorescence intensities were lower.

Discussion

The systematic optimization of a transient transfection protocol allowed to develop a simple, robust, and high yielding method for the production of HIV-1-based VLPs in CAP-T cells. A concentration of 5.8×10^{10} VLPs/mL was achieved, which represents a 44 % of improvement compared to the initial conditions. CAP-T cells were proven to be a suitable platform for Gag-based VLP production. Generated VLPs presented the expected size (~120–150 nm) and morphology consistent with immature HIV-1 particles and similar to VLPs produced in Sf9 or HEK293 (Cervera et al. 2013; Valley-Omar et al. 2011). CAP-T cells have been used for influenza virus production before (Genzel et al. 2013); nonetheless, to the best of our knowledge, this is the first evidence of HIV-1-based VLP production in this cell line.

Despite the recent novelty of CAP-T cells, it has already been demonstrated that they represent a powerful system for expression of heterologous proteins (Fischer et al. 2012). A comparison of Gag-based VLP production levels between CAP-T and other reported systems is summarized in Table 2. These results support that CAP-T cells are a suitable and promising alternative to classical systems because they are able to produce higher titers of complex VLPs in the correct conformation. Noticeably, the high expression levels of VLPs upon transfection would allow obtaining, in 1 L of culture, enough material to immunize 500 mice (Table 2).

Even though CAP-T cells represent a strong platform, there are many variables influencing TGE yields that can be optimized. These variables are the medium chosen for transfection, cell density at the time of transfection and the cell state, DNA concentration, transfection reagent, its mass ratio to DNA, and the DNA-PEI complexation conditions. After study of the latter, the final transfection protocol is as follows: cells are seeded in PEM at 0.5×10^6 cells/mL, after 2 days, thus in mid-exponential phase, they are subjected to a medium exchange to FreeStyle™ 293 prior to transfection. In this step, cells are concentrated to 3.3×10^6 cells/mL in 30 % of the final volume. DNA at 0.5 pg/cell and PEI at 3 pg/cell (DNA:PEI mass ratio of 1:6) are diluted in FreeStyle™ 293 and vortexed for 10 s then both solutions are added separately to the concentrated culture. Culture volume is completed with PEM 3 hpt.

Although PEM is not compatible with PEI transfection, it better supports cell growth and protein production compared to FreeStyle™ 293. Addition of PEM after transfection allows improvement of VLP production, which is in good agreement with many reported TGE protocols that also include a dilution step with fresh medium after transfection to improve cell

Table 2 Comparison of production levels of HIV-1 Gag-based VLPs in different systems

VLP	Cell line	Production approach	Gag/L (μg)	Fold-improvement of CAP-T compared to alternative system	Number of mice that could be immunized with 1 L of culture[b]	Reference
Gag-GFP	CAP-T	TGE	20130[a]	1	503	This work
Gag-GFP	HEK293SF-3F6	TGE	4170[a]	5	104	(Cervera et al. 2015)
Gag-GFP	HEK293SF-3F6	TGE	950[a]	21	24	(Cervera et al. 2013)
Gag	High Five	SGE	655	31	16	(Tagliamonte et al. 2010)
GagRT and GagTN	T.ni ProTM cells and Sf9	Baculovirus infection	504	40	13	(Pillay et al. 2009)
Gag	*Saccaromyces cerevisiae* spheroplasts	Yeast transformation	196	102	5	(Sakuragi et al. 2002)

[a] Converted from relative fluorescence units according to (Gutierrez-Granados et al. 2013)

[b] Assuming a mouse is immunized with two doses of 20 μg of VLPs according to (Tagliamonte et al. 2010) and not considering purification losses

growth and increase mRNA levels and consequently, protein concentration (Backliwal et al. 2008b; Fischer et al. 2012; Tuvesson et al. 2008). On the other hand, the availability of new chemically defined and serum-free media that can support both high cell densities and transient transfection is crucial to overcome the potential limitations associated with medium replacement steps, especially in bioreactors (Geisse 2009; Tuvesson et al. 2008). In this regard, media development projects are ongoing by the authors to address this issue. The lack of such culture media makes that many authors end up using medium replacement strategies in bioreactor, even at scales of 100 L because of the associated benefits (addition of nutrients to improve cell performance and removal of metabolism by-products that may interfere with cell transfection) (Girard et al. 2002; Haldankar et al. 2006). Alternatively, large-scale TGE involving medium exchange could be performed by perfusion mode (Ansorge et al. 2009) or by continuous centrifugation (Tuvesson et al. 2008).

Optimization of the discrete variables affecting TGE have led to reduction of steps and increased simplicity of the protocol, which make it very suitable for routinely producing recombinant proteins. Moreover, efforts towards variability minimization are also relevant in TGE processes. Namely, the health of the cells at the time of transfection may be a source of variability if it is not appropriately considered (Carpentier et al. 2007; Grosjean et al. 2002; Liu et al. 2008); thus, transfection of cells 2 days after seeding in PEM enables to avoid this specific source of variability. Furthermore, direct transfection strategy enables to circumvent variability arising from the complex formation, thus making the protocol more reproducible and the number of steps is minimized, which simplifies the process. Valuably, the direct transfection approach is advantageous for process scale-up and automation. Since DNA-PEI complexation depends on many variables such as the medium composition, mixing, or incubation time, accurate control of all variables involved in this step is not operationally trivial when working with large-scale bioreactors and also it hinders process automation (Raymond et al. 2011).

Optimization of cell density, DNA, and PEI to cell ratios was carried out by a DoE approach which is a powerful tool to rapidly identify the optimal combination of the main process variables and has been used in other works to improve the yields of transiently expressed proteins (Backliwal et al. 2008a; Pillay et al. 2009; Thompson et al. 2012). The main advantages of this approach are that statistically relevant results are obtained with fewer experiments compared to the "one factor at a time" strategy and that interactions between variables can be depicted. The optimal PEI to DNA ratio found for CAP-T cells is in good agreement with previously reported results (Fischer et al. 2012). Noticeably, the DNA concentration was kept relatively low, what can be regarded as an advantage, since DNA is one of the major-cost factors of TGE, and producing high quantities of high-quality DNA for transfection is a bottleneck in TGE scale-up (Pham et al. 2006).

In the specific case of Gag-based VLP production, cell culture viability becomes very relevant. Considering that dead cells would theoretically release their intracellular content into the supernatant, contaminating the VLP preparations with intracellular proteins as well as free Gag-GFP monomers, a higher viability at the time of harvest is preferred. That is why optimal conditions are determined by restricting a minimum cell culture viability value, even with the associated decrease in fluorescence compared to the optimization without restricting the viability value. Not surprisingly, PEI is the variable with the highest effect on viability, so it needs to be minimized in order to avoid toxicity effect and gain viability of the culture. On top of the relevance of PEI/cell ratio by itself, its statistically significant interaction with cell density in the second-order model suggests that volumetric concentration of PEI is also important since the more concentrated cells

are at the time of transfection, the lower volume is used to transfect and PEI is more concentrated, thus more toxic to the cells.

In conclusion, although there is room for further improvement of TGE in this cell line by applying a number of strategies previously reported (Backliwal et al. 2008a; Cervera et al. 2015; Vink et al. 2014), high expression of HIV-1-based VLPs was achieved in this work upon variables optimization. Future efforts toward scale-up of this process should allow the rapid and efficient production of enough material for pre-clinical studies.

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Compliance with ethical standards This article does not contain any studies with animals or human participants performed by any of the authors.

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References

Ansorge S, Lanthier S, Transfiguracion J, Durocher Y, Henry O, Kamen A (2009) Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. J Gene Med 11(10):868–876. doi:10.1002/jgm.1370

Backliwal G, Hildinger M, Chenuet S, Wulhfard S, De Jesus M, Wurm FM (2008a) Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res 36(15):e96. doi:10.1093/nar/gkn423

Backliwal G, Hildinger M, Hasija V, Wurm FM (2008b) High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. Biotechnol Bioeng 99(3):721–727. doi:10.1002/bit.21596

Baldi L, Hacker DL, Adam M, Wurm FM (2007) Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol Lett 29(5):677–684. doi:10.1007/s10529-006-9297-y

Bandaranayake AD, Almo SC (2014) Recent advances in mammalian protein production. FEBS Lett 588(2):253–260. doi:10.1016/j.febslet.2013.11.035

Buonaguro L, Tagliamonte M, Visciano ML, Tornesello ML, Buonaguro FM (2013) Developments in virus-like particle-based vaccines for HIV. Expert Rev Vaccines 12(2):119–127. doi:10.1586/erv.12.152

Carpentier E, Paris S, Kamen AA, Durocher Y (2007) Limiting factors governing protein expression following polyethylenimine-mediated gene transfer in HEK293-EBNA1 cells. J Biotechnol 128(2):268–280. doi:10.1016/j.jbiotec.2006.10.014

Cervera L, Fuenmayor J, Gonzalez-Dominguez I, Gutierrez-Granados S, Segura MM, Godia F (2015) Selection and optimization of transfection enhancer additives for increased virus-like particle production in HEK293 suspension cell cultures. Appl Microbiol Biotechnol. doi:10.1007/s00253-015-6842-4

Cervera L, Gutierrez-Granados S, Martinez M, Blanco J, Godia F, Segura MM (2013) Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J Biotechnol 166(4):152–165

Circu ML, Aw TY (2010) Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 48(6):749–762. doi:10.1016/j.freeradbiomed.2009.12.022

Chen Y, Wu B, Musier-Forsyth K, Mansky LM, Mueller JD (2009) Fluorescence fluctuation spectroscopy on viral-like particles reveals variable gag stoichiometry. Biophys J 96(5):1961–1969. doi:10.1016/j.bpj.2008.10.067

Daramola O, Stevenson J, Dean G, Hatton D, Pettman G, Holmes W, Field R (2014) A high-yielding CHO transient system: coexpression of genes encoding EBNA-1 and GS enhances transient protein expression. Biotechnol Prog 30(1):132–141. doi:10.1002/btpr.1809

Fischer S, Charara N, Gerber A, Wolfel J, Schiedner G, Voedisch B, Geisse S (2012) Transient recombinant protein expression in a human amniocyte cell line: the CAP-T(R) cell system. Biotechnol Bioeng 109(9):2250–2261. doi:10.1002/bit.24514

Garnier L, Ravallec M, Blanchard P, Chaabihi H, Bossy JP, Devauchelle G, Jestin A, Cerutti M (1995) Incorporation of pseudorabies virus gD into human immunodeficiency virus type 1 Gag particles produced in baculovirus-infected cells. J Virol 69(7):4060–4068

Geisse S (2009) Reflections on more than 10 years of TGE approaches. Protein Expr Purif 64(2):99–107. doi:10.1016/j.pep.2008.10.017

Genzel Y, Behrendt I, Rodig J, Rapp E, Kueppers C, Kochanek S, Schiedner G, Reichl U (2013) CAP, a new human suspension cell line for influenza virus production. Appl Microbiol Biotechnol 97(1):111–122. doi:10.1007/s00253-012-4238-2

Girard P, Derouazi M, Baumgartner G, Bourgeois M, Jordan M, Jacko B, Wurm FM (2002) 100-liter transient transfection. Cytotechnology 38(1–3):15–21. doi:10.1023/A:1021173124640

Grosjean F, Batard P, Jordan M, Wurm FM (2002) S-phase synchronized CHO cells show elevated transfection efficiency and expression using CaPi. Cytotechnology 38(1–3):57–62. doi:10.1023/A:1021197830091

Gutierrez-Granados S, Cervera L, Godia F, Carrillo J, Segura MM (2013) Development and validation of a quantitation assay for fluorescently tagged HIV-1 virus-like particles. J Virol Methods 193(1):85–95

Haldankar R, Li D, Saremi Z, Baikalov C, Deshpande R (2006) Serum-free suspension large-scale transient transfection of CHO cells in WAVE bioreactors. Mol Biotechnol 34(2):191–199

Hammonds J, Chen X, Zhang X, Lee F, Spearman P (2007) Advances in methods for the production, purification, and characterization of HIV-1 Gag-Env pseudovirion vaccines. Vaccine 25(47):8036–8048. doi:10.1016/j.vaccine.2007.09.016

Haynes JR, Dokken L, Wiley JA, Cawthon AG, Bigger J, Harmsen AG, Richardson C (2009) Influenza-pseudotyped Gag virus-like particle

vaccines provide broad protection against highly pathogenic avian influenza challenge. Vaccine 27(4):530–541. doi:10.1016/j.vaccine.2008.11.011

Hermida-Matsumoto L, Resh MD (2000) Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. J Virol 74(18):8670–8679

Kushnir N, Streatfield SJ, Yusibov V (2012) Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. Vaccine 31(1):58–83

Liu C, Dalby B, Chen W, Kilzer JM, Chiou HC (2008) Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. Mol Biotechnol 39(2):141–153. doi:10.1007/s12033-008-9051-x

Montgomery DC (1997) Design and analysis of experiments. Wiley, Fourth edn

Pham PL, Kamen A, Durocher Y (2006) Large-scale transfection of mammalian cells for the fast production of recombinant protein. Mol Biotechnol 34(2):225–237. doi:10.1385/MB:34:2:225

Pillay S, Meyers A, Williamson AL, Rybicki EP (2009) Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. Biotechnol Prog 25(4):1153–1160. doi:10.1002/btpr.187

Raymond C, Tom R, Perret S, Moussouami P, L'Abbe D, St-Laurent G, Durocher Y (2011) A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications. Methods 55(1):44–51. doi:10.1016/j.ymeth.2011.04.002

Roldao A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM (2010) Virus-like particles in vaccine development. Expert Rev Vaccines 9(10):1149–1176. doi:10.1586/erv.10.115

Sakuragi S, Goto T, Sano K, Morikawa Y (2002) HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 99(12):7956–7961. doi:10.1073/pnas.082281199

Scotti N, Alagna F, Ferraiolo E, Formisano G, Sannino L, Buonaguro L, De Stradis A, Vitale A, Monti L, Grillo S, Buonaguro FM, Cardi T (2009) High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. Planta 229(5):1109–1122. doi:10.1007/s00425-009-0898-2

Schiedner G, Hertel S, Kochanek S (2000) Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. Hum Gene Ther 11(15):2105–2116. doi:10.1089/104303400750001417

Schlaeger EJ, Christensen K (1999) Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotechnology 30(1–3):71–83. doi:10.1023/A:1008000327766

Schwartz S, Campbell M, Nasioulas G, Harrison J, Felber BK, Pavlakis GN (1992) Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. J Virol 66(12):7176–7182

Tagliamonte M, Visciano ML, Tornesello ML, De Stradis A, Buonaguro FM, Buonaguro L (2010) Constitutive expression of HIV-VLPs in stably transfected insect cell line for efficient delivery system. Vaccine 28(39):6417–6424. doi:10.1016/j.vaccine.2010.07.054

Tagliamonte M, Visciano ML, Tornesello ML, De Stradis A, Buonaguro FM, Buonaguro L (2011) HIV-Gag VLPs presenting trimeric HIV-1 gp140 spikes constitutively expressed in stable double transfected insect cell line. Vaccine 29(31):4913–4922. doi:10.1016/j.vaccine.2011.05.004

Thompson BC, Segarra CR, Mozley OL, Daramola O, Field R, Levison PR, James DC (2012) Cell line specific control of polyethylenimine-mediated transient transfection optimized with "Design of experiments" methodology. Biotechnol Prog 28(1):179–187. doi:10.1002/btpr.715

Transfiguracion J, Jaalouk DE, Ghani K, Galipeau J, Kamen A (2003) Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications. Hum Gene Ther 14(12):1139–1153. doi:10.1089/104303403322167984

Tuvesson O, Uhe C, Rozkov A, Lullau E (2008) Development of a generic transient transfection process at 100 L scale. Cytotechnology 56(2):123–136. doi:10.1007/s10616-008-9135-2

Valley-Omar Z, Meyers AE, Shephard EG, Williamson AL, Rybicki EP (2011) Abrogation of contaminating RNA activity in HIV-1 Gag VLPs. Virol J 8:462. doi:10.1186/1743-422X-8-462

Vink T, Oudshoorn-Dickmann M, Roza M, Reitsma JJ, de Jong RN (2014) A simple, robust and highly efficient transient expression system for producing antibodies. Methods 65(1):5–10. doi:10.1016/j.ymeth.2013.07.018

Wolfel J, Essers R, Bialek C, Hertel S, Scholz-Neumann N, Schiedner G (2011) CAP-T cell expression system: a novel rapid and versatile human cell expression system for fast and high yield transient protein expression. BMC Proc 5 Suppl 8:P133. doi:10.1186/1753–6561-5-S8-P133

Production of HIV virus-like particles by transient transfection of CAP-T cells at bioreactor scale avoiding medium replacement

Results, chapter 3

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Abstract

Human-derived CAP-T cell line has been demonstrated to be a powerful platform for high-titer production of HIV virus-like particles (VLPs) by PEI-mediated transient transfection. Scale-up of transfection processes is key to ensure the necessary quantities for pre-clinical and clinical testing. One of the major operational challenges of large-scale transient transfection is the medium replacement step that is often required before transfection. In this work, CAP-T cells were cultured in 1L bioreactor with addition of sodium bicarbonate and surface aeration, which were observed to improve cell physiological state for transfection. Remarkably, the medium replacement step was avoided by culturing the cells in a combination of media (FreeStyleF17 + 1% of PEM) compatible with cell growth and PEI-mediated transient transfection. In the conditions developed in this work, 0.5×10^6 cells/mL were seeded in 1L bioreactor. Two days later, ~2×10^6 cells/mL were transfected without medium exchange, using 0.5 pg of DNA/cell and 3 pg of PEI/cell. Transfection efficiency and VLP production comparable to shake flasks were obtained with a production of 4×10^{10} VLPs/mL. This novel strategy significantly simplifies large-scale transient transfection, while suitable cell growth, transfection efficiency, and high quality VLP production are achieved.

1. Introduction

Virus-like particles (VLPs) are promising vaccine candidates. They are formed by expression of the main virus structural protein/s, and mimic the native virus structure which enables efficient immune responses. However, VLPs are non-infectious since they do not contain the viral genome, which reduces biosafety concerns during manufacturing and upon administration to the patients (Liu et al., 2016; Noad and Roy, 2003; Rodríguez-Limas et al., 2013). VLPs can be engineered to present different surface antigens (chimeric VLPs), which makes them highly versatile biological entities (Lua et al., 2014). Their success is reflected in the already approved VLPs in the market and the number of VLPs being developed for a great variety of viruses (Roldão et al., 2010; Saraswat et al., 2016; Warfield and Aman, 2011). Moreover, they also have potential application in the gene therapy and nanobiotechnology fields, since they have the ability to encapsulate and deliver molecules, proteins and nucleic acids (Lua et al., 2014; Yan et al., 2015). Altogether, VLPs are interesting biological entities with great potential in biomedicine.

HIV-1 VLPs are formed upon expression of the Gag polyprotein in the host cells. Gag is able to self-assemble in the vicinity of the plasma membrane forming the VLPs, which will be then released by a budding process, taking a part of the cell membrane as their lipid envelope (Buonaguro et al., 2013). This rather complex entities are preferably produced in animal cell platforms, which provide the necessary environment (post-translational modifications and the cell membrane) to correctly form the enveloped VLPs. Among different alternatives available, HEK293 and insect cell lines have been the most widely used (Cervera et al., 2013; Pillay et al., 2009; Tagliamonte et al., 2010). Previous studies have shown that the novel human CAP-T cell line produces high titers of HIV-1 Gag-based VLPs upon transient transfection with PEI (Gutiérrez-Granados et al., 2015). CAP-T cells show many advantages for protein and virus manufacturing: they are adapted to serum-free media, they grow in suspension to high cell densities, and they are highly transfectable. They have also been successfully used for the production of viruses (Genzel et al., 2013) and expression of difficult-to-express proteins (Fischer et al., 2012).

Transient Gene Expression (TGE) is a technology consisting in the introduction of foreign genes into host cells (transfection), to be expressed in a limited time span. After transfection, plasmid DNA molecules remain in the cells without integrating into the genome (episomally), and are eventually lost over time and cell division. Milligrams to grams of proteins can be obtained within weeks using this technology (Geisse, 2009).

Scale-up of transfection protocols is necessary to cover the needs for high amounts of protein that may arise from structural, bioactivity or pre-clinical testing. Large-scale transfection approaches have already been reported mainly to obtain research-grade material (Jäger et al., 2015). However, new applications are being pursued in the biopharma industry that will potentially embrace TGE as the production strategy. The success of gene therapy and the growing field of personalized medicine are two examples of these new applications. Large-scale transfection has been already used to produce viral vectors for gene therapy clinical trials (Ausubel et al., 2012; Grieger et al., 2016).

Large-scale transfection in stirred-tank bioreactors has been explored mainly in HEK293 and CHO cell lines. Some of the large-scale productions have been successful (Muller et al., 2007; Venereo-Sanchez et al., 2016), while others showed lower performances compared to small-scale productions (Ansorge et al., 2009; Fliedl and Kaisermayer, 2011). Unfortunately, the specific reason for the loss of transfection efficiency or product titers upon scale-up in bioreactors is not well understood. The multiple operational parameters that could impact the process make rather difficult to depict the causes of these phenomena. In addition, there are no previous reports of CAP-T cell transfection in stirred-tank bioreactors.

One of the major operational challenges for transfection at large-scale is the need to perform a medium replacement before transfection. Many of the protocols available today incorporate this step, including the protocol developed for HIV VLP production in CAP-T cells (Gutiérrez-Granados et al., 2015). However, scaling-up medium replacement operations is not straightforward. One of the reasons to perform medium replacement is that cells perform better in fresh than in conditioned medium: more nutrients are available and cell by-products that may partially or totally inhibit the transfection process are removed (Backliwal et al., 2008; Cervera et al., 2013; Rajendra et al., 2012; Ye et al., 2009). Another reason of great relevance to perform medium replacement prior to transient transfection is the lack of media formulations compatible with cell growth (high cell densities, high viabilities, lack of cell aggregation) and PEI-mediated transfection. Media that support suitable cell growth usually contain compounds that hinder transfection. On the other hand, media allowing transient transfection can promote cell aggregation and typically, do not support high cell densities. 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 cell entry (Schlaeger and Christensen,

1999). As a result, medium replacement in bioreactor by centrifugation steps or using cell-retention devices (Ansorge et al., 2009; Sun et al., 2008; Tuvesson et al., 2008) is needed. With the aim of simplifying the process, some have performed seed trains in shake flask before inoculating the bioreactor for transfection (Haldankar et al., 2006; Muller et al., 2007), but this is only possible in lab-scale bioreactors. The simplest (and therefore most desirable) strategy would be to use a medium that is compatible with good growth and transfection and does not require a replacement. Yet the development of new media formulations is quite complex and there has been increasing interest in the last years with limited success regarding compatibility with transfection. There are some media compatible like FreeStyle293 for HEK293 or CD-CHO for CHO cells (Abbott et al., 2015; Cervera et al., 2013). However, due to their novelty, there is still not a formulation specifically designed for CAP-T cells enabling growth to high cell densities and transfection without medium replacement.

The aim of this work was to scale-up transient transfection of CAP-T cells to 1 L stirred-tank bioreactor. First, growth conditions in bioreactor to achieve a suitable cell physiological state for transfection were optimized. Then, a strategy based on the combination of two media for CAP-T cell growth and transfection was developed. Importantly, this strategy allowed to perform transient transfection with no medium replacement, significantly simplifying the process at bioreactor level.

2. Materials and methods

2.1. Cell line and culture conditions

CAP-T cells were kindly provided by Cevec Pharmaceuticals (Cologne, Germany). Cells were cultured in suspension in Protein Expression Medium (PEM, Gibco, Grand Island, NY, USA) supplemented with 4 mM of GlutaMAX (Gibco, Paisley, UK). Cells were routinely maintained at exponential growth phase with viabilities over 95% in 125 mL disposable polycarbonate shake flasks (Corning, New York, NY, USA). Shake flasks were placed in an orbital shaker (orbit diameter 16 mm, Stuart, Stone, UK) at 130 rpm in a humidified incubator at 37 °C and 5% CO_2 in air. Cell counts and viability were performed using the

NucleoCounter®NC-3000 automated cell counter (Chemometec, Allerod, Denmark) according to manufacturer's instructions. For the growth experiments, cultures were inoculated at 0.5×10^6 cells/mL and samples were taken every 24 h for cell counting and viability determination.

2.2. Cell culture in 1L bioreactor

A BioStat B Plus bioreactor (Sartorius AG, Goettingen, Germany) equipped with a 3-blade segment impeller was used for CAP-T cell cultivation. The agitation was set at 160 rpm. Temperature was set at 37°C. The pH was set at 7 and controlled with CO_2 and $NaHCO_3$ (7.5% w/v). The dissolved oxygen was controlled at 40% of air saturation by supplementing air or oxygen by headspace or sparger, as detailed in the results section. For the growth experiments, 500-600 mL of cell culture medium were introduced in the bioreactor and acclimated to the culture conditions before addition of cell inoculum (growing exponentially in disposable polycarbonate 1L shake flasks, Corning). Then, the tubing was rinsed with additional medium up to 1L containing AntiFOAM C (Sigma, St. Louis, MO, USA) to a final concentration of 100 mg/L. Inoculum concentration was 0.5×10^6 cells/mL. Samples were taken every 24 h for cell counting and viability determination.

2.3. Plasmid DNA

The pGag-EGFP plasmid used for transfection codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP. The plasmid from the NIH AIDS Reagent Program (Cat #11468) (Hermida-Matsumoto and Resh, 2000) was constructed by cloning the Gag sequence from pCMV55M1-10 (Schwartz et al., 1992) into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). Plasmid DNA amplification and purification was carried out as previously reported (Gutiérrez-Granados et al., 2013).

2.4. Transient transfection at small-scale with medium replacement (standard protocol)

VLPs were generated by transient transfection of CAP-T cells with pGag-EGFP plasmid DNA using 40 KDa linear polyethylenimine (PEI Max, PolySciences, Warrington, PA, USA) as transfection reagent. A previously optimized protocol was used (Gutiérrez-Granados et al., 2015). Briefly, prior to transfection, cells exponentially growing in PEM were subjected to a medium exchange to FreeStyle293 (Gibco, Grand Island, NY, USA) supplemented with

0.1 % Pluronic (Gibco, Paisley, UK). In this step, cells were concentrated to 3.3 × 10^6 cells/mL. DNA at 0.5 pg/cell and PEI at 3 pg/cell (DNA:PEI mass ratio of 1:6) were diluted in FreeStyle293, vortexed for 10 seconds, and added separately to the cells. Culture volume was completed with PEM 3h post-transfection (hpt). The percentage of GFP expressing cells was assessed by flow cytometry using a fluorescence activated cell sorter (FACS) (BDFACS Canto, BD BioSciences, San Jose, CA, USA).

2.5. Transient transfection at small-scale without medium replacement

Alternatively, transfection was performed without medium replacement in different culture media. Cells were allowed to adapt to each new medium for a few passages before starting the experiments. The conditions used were:

a) CAP-T Express (Cevec Pharmaceuticals, Cologne, Germany). Cells were transfected 72h after seeding at 0.5×10^6 cells/mL. Prior to transfection, 1 volume of fresh medium was added to dilute the conditioned medium.

b) FreeStyle293 supplemented with 3% of PEM. Cells were transfected 48h after seeding at 0.5×10^6 cells/mL. One volume of PEM medium was added to the transfected cultures 3hpt.

c) FreeStyleF17 (Gibco, Grand Island, NY, USA) supplemented with 0.1% of Kolliphor (Sigma, St. Louis, MO, USA), 4 mM of GlutaMAX, 50 µL/L of Insulin Growth Factor (IGF, Sigma, Lenexa, KS, USA) and 1% of PEM. The same protocol as in b was used.

2.6. Transient transfection in bioreactor

The transfection protocol was lineally scaled-up from Erlenmeyer flask (30mL) to 1 L stirred-tank bioreactor. Cells were cultured in PEM medium in the bioreactor for two days before transfection. Prior to transfection, cells were taken out of the bioreactor and subjected to a medium replacement by centrifugation. Once resuspended in the appropriate amount of FreeStyle293, they were introduced back in the bioreactor. Temperature was controlled at 37°C, agitation at 160 rpm and aeration was set through headspace. DNA and PEI solutions were sequentially added to the bioreactor, and the tubing was rinsed with more FreeStyle293. 4 hpt, PEM medium supplemented with Antifoam C was added to a final volume of 1L. Aeration was then set through sparger for

the production phase. A simplified protocol without medium replacement was developed as indicated in the results section.

2.7. VLP quantification

The concentration of Gag-GFP VLPs was assessed by spectrofluorometry using an in-house developed and validated quantification technique (Gutiérrez-Granados et al., 2013). The equation used to convert RFU values to Gag-GFP concentration values is the following:

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

where Gag-GFP is the estimated concentration of polyprotein and RFU is the measured GFP fluorescence intensity in the samples. To further convert Gag-GFP concentration to VLP concentration, it was assumed that one VLP contains 2500 Gag-GFP monomers (Chen et al., 2009) with a molecular weight of 84 kDa per monomer.

2.8. VLP characterization by nanoparticle tracking analysis

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

2.9. VLP characterization by transmission electron microscopy

VLP samples were prepared by air-dried negative staining method with uranyl acetate at the Servei de Microscòpia (UAB, Bellaterra, Spain) as previously reported (Gutiérrez-Granados et al., 2013) before the examination in a Jeol JEM-1400 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Gatan ES1000W Erlangshen CCD Camera (Model 785).

3. Results and discussion

3.1. CAP-T cell growth in 1L bioreactor

The first step towards the scale-up of the transient transfection protocol was the optimization of cell growth in 1L bioreactor. The goal was to implement the protocol as it had been optimized previously (Gutiérrez-Granados et al., 2015), consisting in culturing the cells for 48 h in the bioreactor before being transfected. This becomes relevant when envisioning scale-up to higher volumes, where the seed train cannot be performed in shake flasks, but instead must be performed in bioreactors.

CAP-T cells were previously adapted to grow in suspension in PEM medium at small scale. Since there are not previous reports regarding growth or transfection of CAP-T cells at bioreactor scale, the conditions used by other authors for CAP cell growth were initially adopted (Genzel et al., 2014). The cultures were performed at pH 7.2 controlled with CO_2 and NaOH. Aeration was controlled at 40% DO with oxygen pulses through the sparger. Under these first bioreactor culture conditions tested, CAP-T cells grew up to 3.9×10^6 cells/mL with viabilities over 90% up to 110 h and a doubling time of 37.9 h. However, in shake flasks CAP-T cells grew up to 7×10^6 cells/mL in 96 h, with a doubling time of 24.5 h (Figure 1a). Even though 4×10^6 cells/mL could be considered an acceptable cell density to perform transfection, a lower growth rate indicates that the physiological state of the cells is suboptimal, which could negatively impact transient transfection efficiency (Gutiérrez-Granados et al., 2015). For this reason, the culture conditions were further refined to further improve growth rates in bioreactor cultures.

Carbonate addition was considered, as it is a key nutrient for cells (Gray et al., 1996) and there is a difference in availability from shake flasks and bioreactor. Shake flasks are placed in an incubator with 5% of CO_2 in the atmosphere, so the carbonate equilibrium is constantly fed by CO_2, and the carbonate concentration in the medium remains constant. On the other hand, pH is controlled with CO_2 and NaOH in bioreactor, but CO_2 addition stops when pH drops, causing limited availability of carbonate. Therefore, the growth curve was reproduced using in this case CO_2 and $NaHCO_3$ to control pH and ensure a constant carbonate availability. As can be seen in Figure 1b, cell density in bioreactor was comparable to shake flasks (6.72×10^6 cells/mL, td=27.1 h). In conclusion, CAP-T cell batch culture in bioreactor was optimal using the following settings: pH 7 controlled with CO_2

and $NaHCO_3$ (7.5% w/v), 160 rpm and DO controlled at 40% with constant air and oxygen enrichment through sparger.

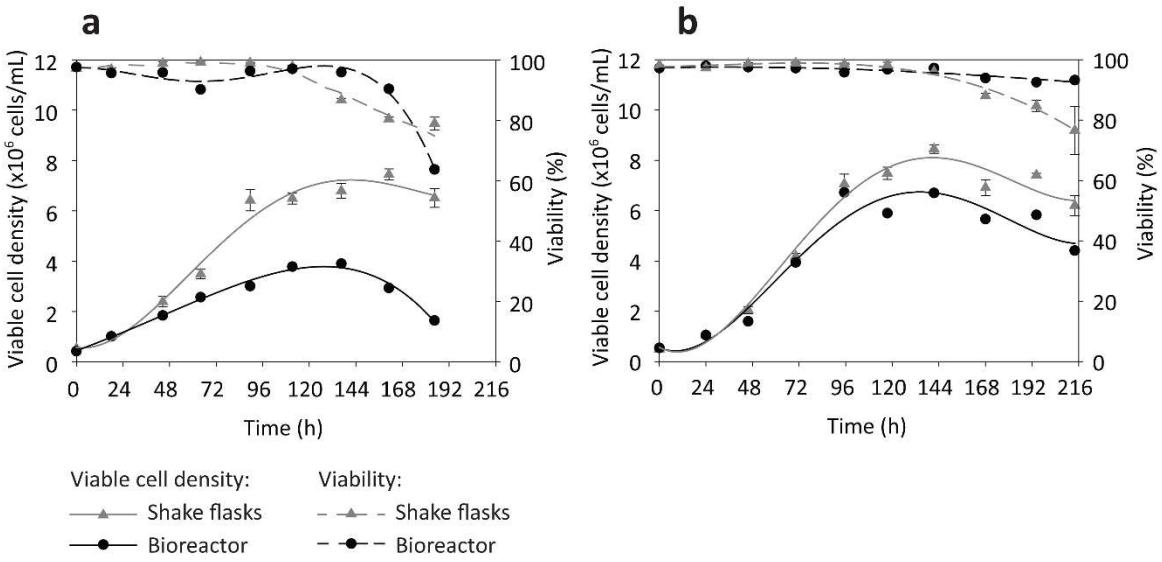

Figure 1. CAP-T cell growth in bioreactor. Batch cultures in PEM medium are shown along with culture in shake flasks as controls. Viable cell density and viability are represented over time. Shake flask data are the mean of triplicate experiments and the error bars represent standard deviation. (a) Preliminary conditions, pH was controlled with CO_2 and NaOH in bioreactor. (b) Optimized conditions, pH was controlled with CO_2 and $NaHCO_3$.

3.2. Definition of operating conditions for transfection in bioreactor

Before performing transient transfection in bioreactor, some aspects have to be considered: the influence of antifoam on transient transfection and Gag-GFP VLP production and the minimal working volume available in the bioreactor.

The use of antifoam was not necessary in shake flasks, but it was in bioreactor to avoid the foaming caused by continued air sparging (Handa-Corrigan et al., 1989). Therefore, the impact of antifoam on transfection and VLP production at shake flask scale was evaluated. To this purpose, antifoam was added to CAP-T cultures before transfection (in the medium replacement step) or 3h after transfection (along with PEM medium addition). As presented in Figure 2a, the presence of antifoam did not affect transfection efficiency or VLP production in supernatant compared to control transfection without antifoam.

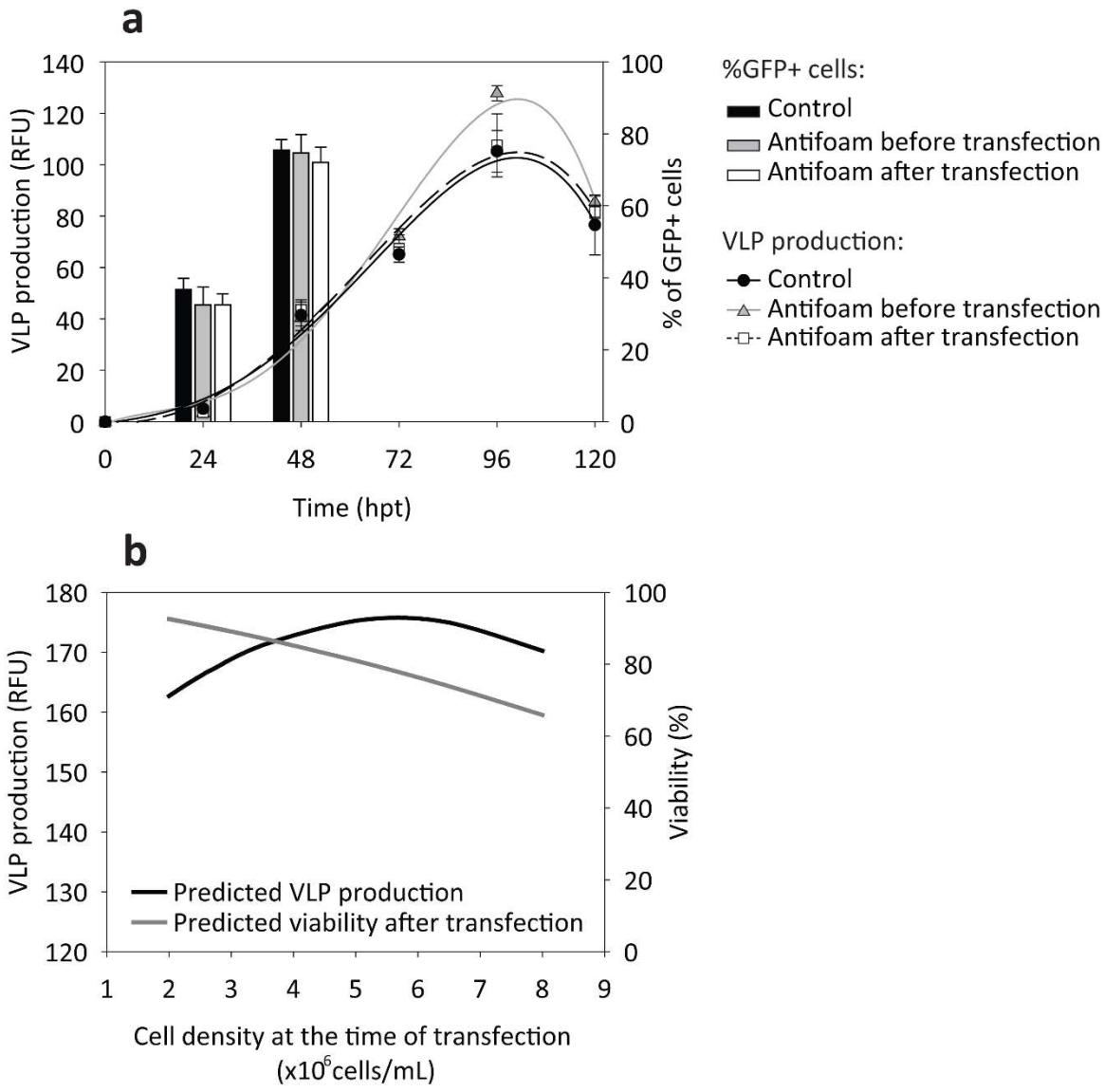

Figure 2. Preliminary considerations for transient transfection in bioreactor. (a) Effect of antifoam addition in transfection and VLP production. Antifoam (100 mg/L) was added either before transfection (in the medium replacement step) or after transfection (along with PEM addition). Transfection efficiency and VLP production were analyzed. The data are the mean of triplicate experiments and the error bars represent standard deviation. (b) Predicted VLP production and cell culture viability are represented vs. the cell density at the time of transfection according to the correlations defined previously (Gutiérrez-Granados et al., 2015). DNA and PEI were fixed at their optimal levels (0.5 and 3 pg/mL).

Regarding the physical limitations in bioreactor, a minimum volume of 500 mL was required in order to maintain correctly the agitation, pH, DO, and temperature control. However, as described in section 2.4, the standard transfection protocol included a medium replacement and cell concentration step to 3.3×10^6 cells/mL which required reducing the volume down to a 30% of the final working volume. A working volume of 1L

was fixed. The final cell density after PEM addition was also fixed at 1×10^6 cells/mL, as in the small-scale protocol. Therefore, in order to perform transfection in 500 instead of 300 mL, the cell density at the time of transfection was changed to 2×10^6 cells/mL. The influence of transfecting CAP-T cells at 2×10^6 cells/mL was studied based on correlations that predict the fluorescence in supernatant and viability after transfection as a function of cell density, DNA and PEI concentrations (original data from (Gutiérrez-Granados et al., 2015)). The fluorescence in supernatant and cell culture viability predicted by the models were plotted against cell density at the time of transfection (from 2 to 8×10^6 cells/mL) while fixing DNA and PEI at their optimal concentrations (0.5 and 3 pg/cell, respectively). As it can be observed in Figure 2b, there was not a significant difference (less than 5%) between the fluorescence predicted by the model when the cells are transfected at 2 or 3.3×10^6 cells/mL. Regarding viability, the model predicted lower viabilities after transfection with higher densities, while a viability of 93% was predicted for transfection at 2×10^6 cells/mL. Maintaining higher viabilities is relevant in this case because Gag-GFP monomers assemble inside the cells and are budded to the supernatant in the form of VLPs. If cells die, the intracellular content is released, and monomeric Gag-GFP contaminates the VLP preparation. Thus, transfection at 2×10^6 cells/mL should not negatively impact the levels of VLPs compared to transfection at 3.3×10^6 cells/mL and, as an additional advantage, higher cell culture viabilities should be obtained.

Transient transfection was performed in bioreactor scaling-up the standard protocol previously set for transfection of CAP-T cells in shake flask (see section 2.4). Cells grew in the bioreactor for 48 h in PEM medium (with the optimal conditions set in section 3.1) and a medium replacement before transfection to FreeStyle293 was performed by centrifugation. Shake flask controls (consisting in performing cell growth and transfection in shake flasks) and satellite controls (consisting in transfecting bioreactor-grown cells in shake flasks) were analyzed in parallel. Transfection efficiency in bioreactor was 17% lower than in shake flask controls (Figure 3). Regarding the satellite controls, there was no significant difference with the Erlenmeyer controls. However, VLP production was significantly hampered in bioreactor (Figure 3). Gag-GFP VLPs in bioreactor, measured in fluorescence units, were 60% lower than in shake flask controls (96hpt). Remarkably, the fluorescence values achieved in the satellite controls were 44% lower than in the shake flask controls. It was hypothesized that this loss in VLP production observed in satellite controls was due to the fact that the cells cultured in bioreactor were at suboptimal conditions to be transfected. In the case of the bioreactor, an additional 16% of VLP

production was lost compared to satellite controls. This correlated with the lower transfection efficiency obtained.

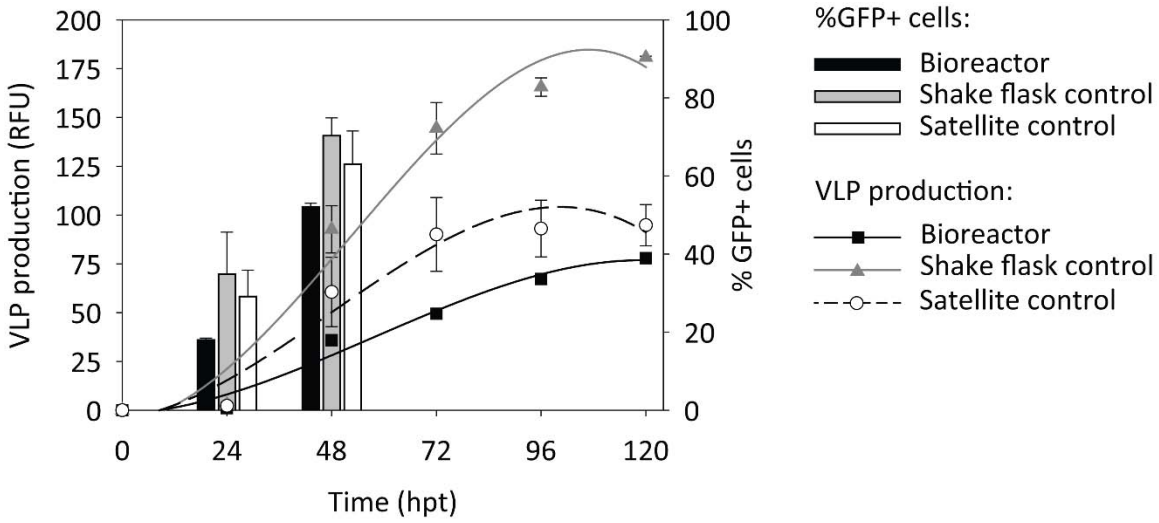

Figure 3. CAP-T transient transfection in 1L bioreactor. CAP-T cells were transfected by scaling-up the standard protocol. The percentage of GFP positive cells and the VLP concentration in cell culture supernatants are represented. Two controls were run in parallel: cells grown and transfected in shake flasks (Shake flask control) and cells grown in bioreactor and transfected in shake flasks (Satellite control). Shake flask data are the mean of triplicate experiments and the error bars represent standard deviation.

Obtaining lower performance in bioreactor was not a totally unexpected result. Other authors reported large-scale transfections in stirred-tank bioreactor obtaining lower transfection efficiencies and protein titers than in small-scale cultures with HEK293 and Vero cell lines (Ansorge et al., 2009; Fliedl and Kaisermayer, 2011; Tuvesson et al., 2008). Interestingly, in all cases cells grew in the bioreactor and were subjected to medium replacement before transfection. However, the causes for lower performances were not further investigated.

3.3. Surface aeration improves CAP-T cell state for transfection

The deleterious effect of gas sparging in mammalian cell lines has been described before (Chisti, 2000; Handa-Corrigan et al., 1989). Therefore, aeration was set by the headspace for the bioreactor cell growth phase 48 h before transfection, to study if the state of the cells improved by avoiding sparging. Then, transfection was performed in

shake flasks (satellite transfection). A control consisting in transfection of cells grown in shake flask was also performed in parallel (shake flask control).

The doubling time of the cells growing in the bioreactor was comparable to that achieved in shake flasks (Figure 4a). More importantly, bioreactor-grown cells were able to produce VLPs to the same levels than Erlenmeyer control cells. Transfection efficiency was independent of the system where the cells had been previously grown (Figure 4b).

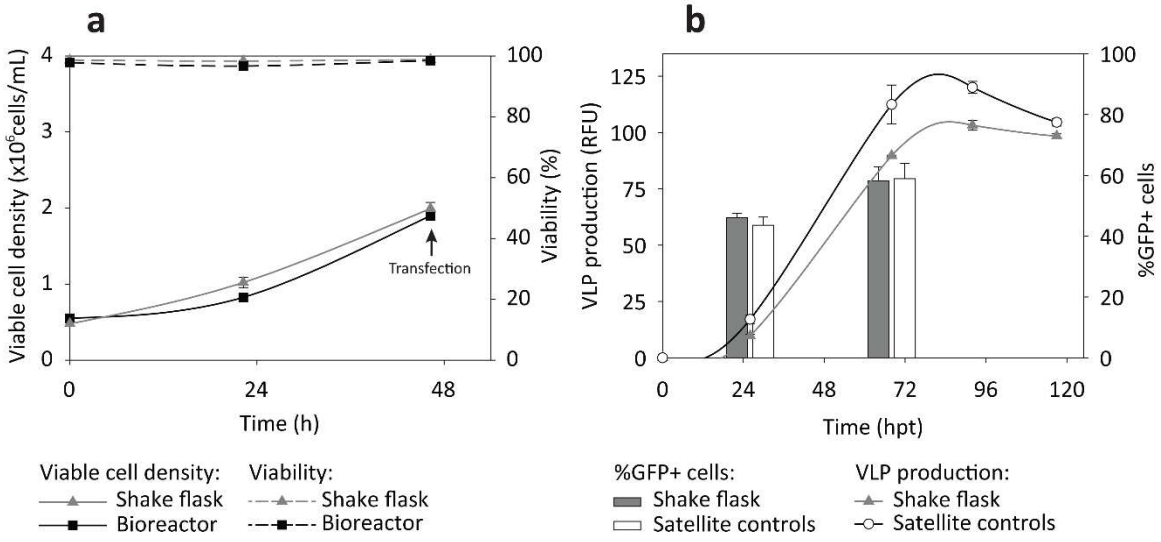

Figure 4. Effect of headspace aeration on transient transfection in bioreactor. (a) CAP-T cell growth in 1L bioreactor with aeration through headspace and control growth in shake flasks. CAP-T cells were seeded at 0.5×10^6 cells/mL in PEM medium in shake flask or 1L bioreactor. Viable cell density and viability are represented. (b) Transfection of CAP-T cells previously grown in shake flasks (control) or in 1L bioreactor (Satellite control). Transfections were performed in shake flasks using the standard transfection protocol detailed in Section 2.4. The percentage of GFP positive cells and the VLP concentration in cell culture supernatants were analyzed. The data are the mean of triplicate experiments and the error bars represent standard deviation.

Therefore, avoiding bubbling in the bioreactor is a solution to improve the ability of the cells to produce the protein of interest upon transfection. Considering the relatively low cell densities needed in this protocol for transfection (~2×10^6 cells/mL), headspace aeration is a rather simple strategy to implement in bioreactor. However, headspace aeration may not be sufficient if higher cell densities are pursued for transfection. Alternatively, the use of cell-protective additives, such as Pluronic or albumin, or the use of bubble-free oxygenation through microporous or diffusion tubing made of silicone rubber could be tested (Chisti, 2000). Cells could also be cultured outside the bioreactor before transfection, in a system offering milder culture conditions. Shake or spinner flaks

are the preferred systems to this end (Girard et al., 2002; Haldankar et al., 2006; Muller et al., 2007). However, as mentioned before, when the volumes to transfect are high, performing the seed train in shake flasks may become cumbersome.

3.4. Combining different media to avoid medium replacement before transfection

Medium replacement in bioreactor by centrifugation is a time-consuming operation and it involves risks of contamination, since it requires massive manipulation of the culture. In addition, centrifugation is not an easily scalable technology. Albeit at 1L it is still manageable, it is certainly not at larger volume scales. Medium replacement by centrifugation has been circumvented by different approaches, for instance, by performing the seed train outside the bioreactor (Haldankar et al., 2006) or by using cell retention devices (Ansorge et al., 2009; Sun et al., 2008). However, the simplest strategy would be to avoid a medium replacement altogether. To that end, media formulations compatible with high cell densities, appropriate cell physiological state and PEI-mediated transient transfection are required. In this way, cells can grow for 1 or 2 days in the bioreactor and be transfected without taking them out (Carter et al., 2010; Grieger et al., 2016; Segura et al., 2007).

With the aim of avoiding medium replacement before transfection, different cell culture media were screened at small scale (shake flasks) for cell growth and transfection compatibility. Table 1 summarizes the main results obtained. PEM medium was the reference for cell growth. CAP-T cells were previously adapted to this medium and, when inoculated at 0.5×10^6 cells/mL, they attained a cell density of around 2×10^6 cells/mL in 48 h (corresponding to their typical growth pattern, allowing to reach 7×10^6 cells/mL in 96-120 h). However, as mentioned before, PEM is not compatible with transient transfection. On the other hand, FreeStyle293 is compatible with transfection, but it does not support suitable cell growth. The alternative formulations tested that were compatible with cell growth and transfection without the need of a medium replacement step were FreeStyle293 supplemented with 3% of PEM and FreeStyleF17 supplemented with 1% of PEM. FreeStyle293 + 3% of PEM and FreeStyleF17 + 1% of PEM yielded a 44% and 60% of the VLPs produced with the standard control conditions, respectively. Hence, the use of these two media was further studied to improve the VLP yields.

Table 1
Alternative cell culture media for cell growth and transfection without medium replacement.

Culture medium	Viable cell density after seeding at 0.5×10^6 cells/mL ($\times10^6$ cells/mL)	Duplication time (h)	Medium replacement required?	Transfection efficiency at 48hpt (%GFP+ cells)	Fluorescence in supernatant 96hpt (RFU)
PEM	2.05 (48h)	24.5	Yes	~70	~170
FreeStyle293	1.03 (72h)	66.7	No	50.1	77.9
FreeStyle293 + 10% PEM	2.2 (72h)	26.3	Yes	nd	nd
FreeStyle293 + 3% PEM	2.15 (48h)	29.6	No	63.7	59.2
FreeStyleF17	2.25 (72h) Aggregation in 3-4 days	35	No[1]	nd	nd
FreeStyleF17 + 2%PEM	2.67 (48h)	19.9	Yes	nd	nd
FreeStyleF17 + 1%PEM	2.32 (48h) Aggregation in 6-8 days	21.7	No	49.7	58.5

1. Transfection could not be performed due to massive aggregation and cell death. RFU: Relative Fluorescence Units. nd: non-determined.

Regarding FreeStyle293 + 3% of PEM, an experiment was performed to elucidate the causes of the reduced VLP production compared to the control. Briefly, CAP-T cells were cultured in FreeStyle293 + 3% of PEM and transfected without medium replacement, with a medium replacement to fresh FreeStyle293 + 3% of PEM or with a medium replacement to fresh FreeStyle293. A control of cells cultured in PEM and transfected in fresh FreeStyle293 with a medium replacement was included. As can be observed in Figure 5a, the fact that the cells were cultured in FreeStyle293 + 3% of PEM reduced VLP production by 24% compared to the control. Then, the presence of 3% of PEM in the transfection medium further reduced VLP yields by an extra 17%. Finally, the presence of conditioned medium instead of fresh, reduced VLP titers by a total of 55% compared to the control. With the aim of increasing VLP titers, the percentage of PEM and conditioned medium at

the time of transfection were reduced to one half by diluting the culture with 1 volume of fresh FreeStyle293 before transfection. This strategy did not result in an improvement of VLP titers, meaning that the remaining PEM and conditioned medium still hampered transfection (Figure 5b). The effect of conditioned FreeStyle293 medium on VLP production has also been reported for HEK293 cells. In this case, medium replacement allowed obtaining an increase of 30% of VLP titers (Cervera et al., 2013). In conclusion, using FreeStyle293 medium in combination with PEM is not the best alternative to perform transient transfection without medium replacement, mainly due to the adverse effect of conditioned medium.

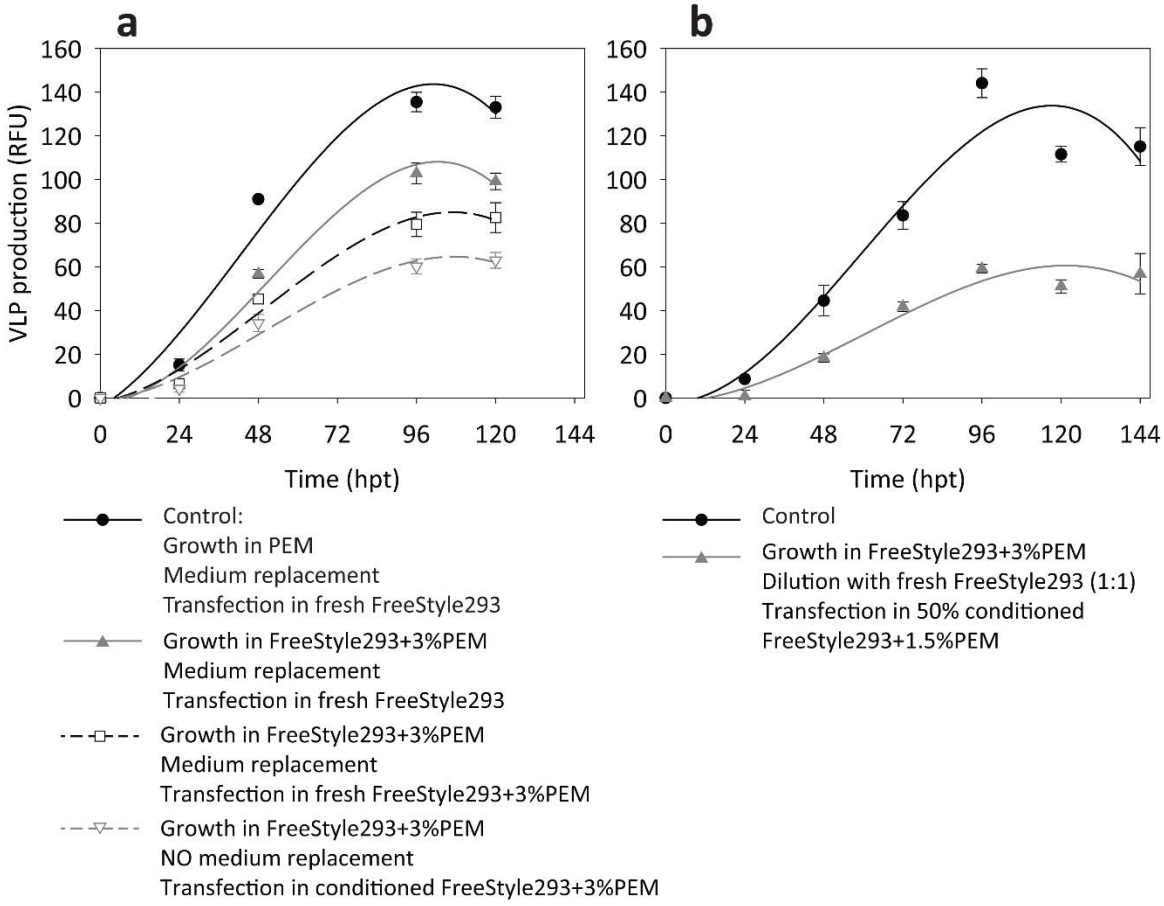

Figure 5. VLP production after transfection without medium replacement using FreeSyle293 medium supplemented with PEM in shake flasks. Control transfections were performed following the standard protocol detailed in section 2.4. (a) Transfection in FreeStyle293 + 3%PEM and different control conditions. (b) Transfection in conditioned FreeStyle293 + 3%PEM diluted 1:1 with fresh FreeStyle293 before transfection. The percentage of GFP positive cells and the VLP concentration in cell culture supernatants were analyzed. The data are the mean of triplicate experiments and the error bars represent standard deviation.

In the case of FreeStyleF17 + 1% of PEM, reduced VLP production was caused by massive cell aggregation after 3-4 passages when it was supplemented with 1% of PEM (Table 1). However, Figure 6a shows that VLP production reached similar levels than controls in shake flasks when CAP-T cells were transfected in FreeStyleF17 + 1% of PEM before aggregation occurred. Taking this into consideration, a window of operation can exist, where cells are first grown with high PEM percentages and no aggregation, and they are diluted 48 h before transfection (1 passage) in order to have them in 1% of PEM, non-aggregated and ready for transfection. To test this hypothesis, CAP-T cells were cultured for 4 passages in FreeStyleF17 + 5% or + 1% of PEM. 48 h before transfection they were both subcultured in 1% of PEM. Cells cultured in 1% of PEM were aggregated at the time of transfection (macroscopic aggregates were observed) while the ones in 5% of PEM were not. Figure 6b shows how VLP production was comparable to the control transfection levels for cells grown in 5% of PEM and transfected in 1% of PEM, and it was significantly reduced for cells cultured always in 1% of PEM. Therefore, culture in FreeStleF17 + 5% of PEM and dilution to 1% of PEM 48 h before transfection was selected as the condition to scale-up transfection in bioreactor.

Although PEM was not compatible with transfection and needed to be used at very low concentrations in the pre-transfection cultures, it was previously shown that addition of 1 volume of PEM 3 hpt was required for optimal VLP production (Gutiérrez-Granados et al., 2015). On top of that, combining media to achieve the desired cell growth and, at the same time, maintaining the ability of transfection without medium replacement is a rather novel strategy. It represents a highly desirable approach to adopt for large-scale transfection, because it simplifies the protocol by avoiding the medium replacement step.

3.5. Optimized transfection protocol for bioreactor

The culture conditions tested before that proved to be beneficial for optimal growth, transfection and VLP production in bioreactor were eventually applied together in a 1 L process. Cells were cultured in FreeStyleF17 supplemented with 5% of PEM to prepare the seed train in shake flasks. The bioreactor was inoculated with 0.5×10^6 cells/mL in FreeStyleF17 so the PEM concentration was diluted to 1%. Aeration was set through headspace for this growth phase. 48 h after inoculation, half of the culture was removed and cells were transfected without performing a medium replacement step. 4 hpt, one

volume of PEM was added, so the final production volume was restored to 1 L. The aeration for the production phase was set through the sparger. Cells grown in bioreactor and transfected in shake flasks were analyzed in parallel (satellite controls) and cells grown and transfected in shake flasks were also analyzed as the positive control.

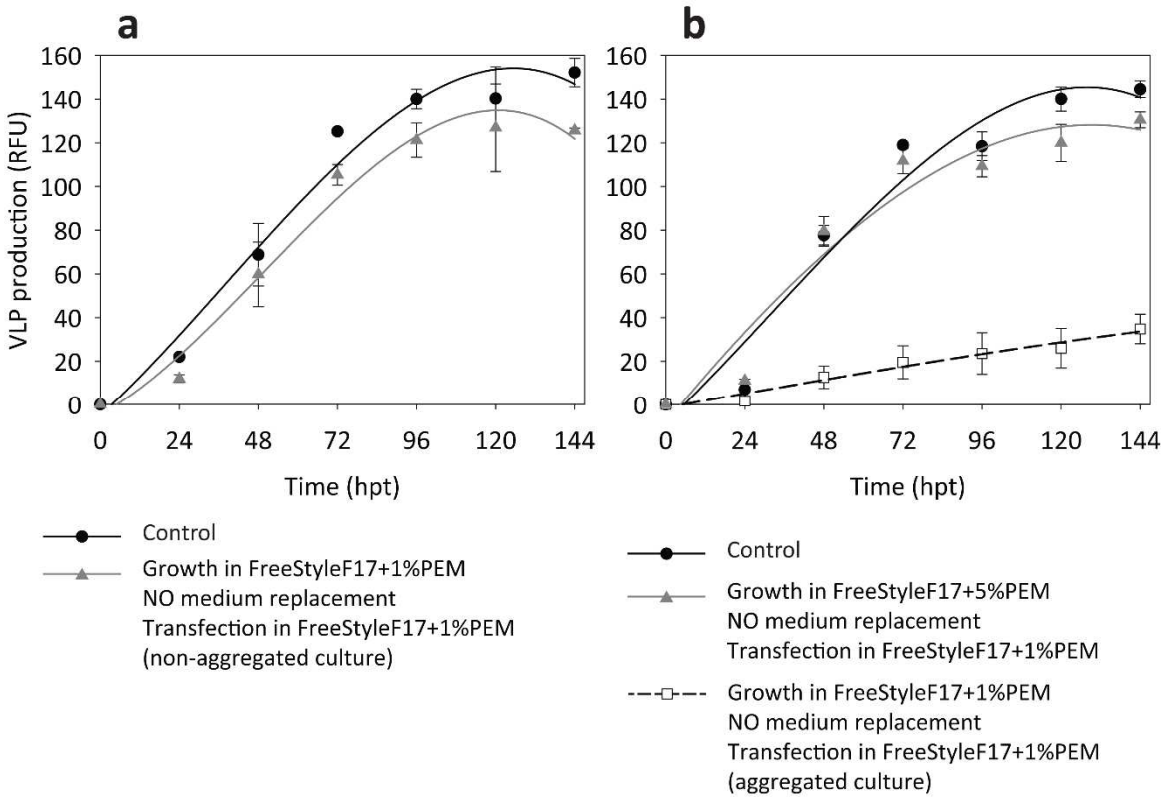

Figure 6. VLP production after transfection without medium replacement using FreeSyleF17 medium supplemented with PEM in shake flasks. Control transfections were performed following the standard protocol detailed in section 2.4. (a) Transfection in FreeStyleF17 + 1%PEM in a non-aggregated culture. (b) Transfection in FreeStyleF17 + 1%PEM aggregated culture and in FreeStyleF17 + 1%PEM non-aggregated culture that was cultured in FreeStyleF17 + 5%PEM until 48 h before transfection. The percentage of GFP positive cells and the VLP concentration in cell culture supernatants were analyzed. The data are the mean of triplicate experiments and the error bars represent standard deviation.

Figure 7a shows that the transfection efficiency in bioreactor was comparable to the controls. This was translated in equivalent levels of VLP concentration in cell culture supernatants. In terms of cell growth and cell culture viabilities there were not significant differences in the behavior between shake flaks and bioreactor (Figure 7b). As expected, cells grew up to 4×10^6 cells/mL after transfection in 5 days, and viability was maintained over 80% up to day 4 after transfection. This experiment was repeated several times with similar results. These results corroborate that superficial aeration in bioreactor allows for a suitable cell physiological state for optimal transfection efficiency. Importantly, the

medium replacement step by centrifugation was avoided, which highly simplified the process operationally. The transfection protocol is less time-consuming, and the risk of contamination due to massive manipulation of the culture is minimized. In addition, the cost of the process is significantly reduced, since the required volume of culture medium is lower and no specific equipment and material for medium exchange is required.

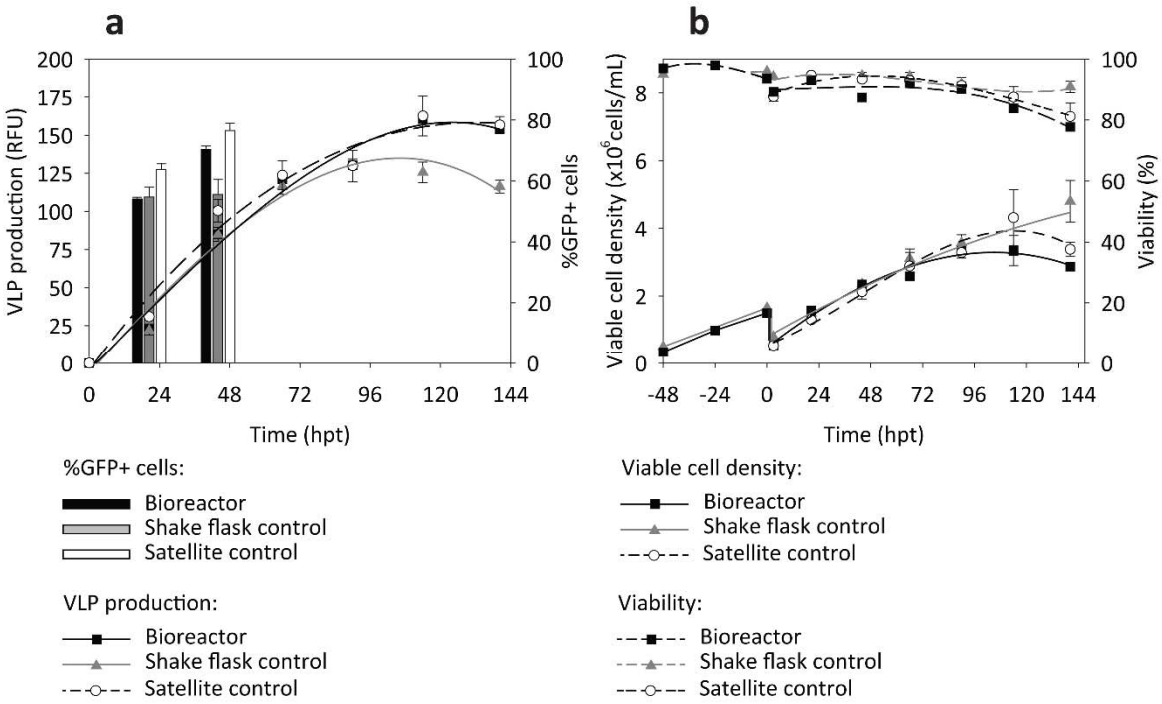

Figure 7. CAP-T transient transfection in 1L bioreactor using the optimized protocol. Briefly, cells grew in FreeStyleF17 + 1%PEM for 48 h before transfection. Transfection was performed without medium replacement, and 1 volume of PEM was added 4 hpt. Cells grown and transfected in shake flask and cells grown in bioreactor and transfected in shake flasks (Satellite control) were analyzed as controls. (a) Percentage of GFP positive cells and VLP concentration in cell culture supernatants were analyzed. (b) Viable cell density and cell culture viability. Shake flask control and satellite control data are the mean of triplicate experiments and the error bars represent standard deviation.

Finally, the quality of bioreactor-produced VLPs was assessed. The fact that Gag-GFP protein is correctly assembled into particles is relevant since the immunogenic properties of VLPs heavily rely on their particulate form (Ludwig and Wagner, 2007). If the cells were lysed during the culture, monomeric Gag-GFP would be released to the medium, causing an overestimation of the VLP content measured by fluorescence readings (Gutiérrez-Granados et al., 2013). The viability profile of the bioreactor culture did not indicate massive cell lysis that could lead to monomeric Gag-GFP release (Figure 7b). Nevertheless,

the product was characterized to determine the nature of the Gag-GFP detected in the supernatant. Nanoparticle Tracking Analysis (NTA) allows following fluorescent nanoparticles in suspension and determining their size distribution. Fluorescent particles were successfully identified by NTA in the supernatants from the bioreactor and shake flasks controls (96hpt) (Figure 8a). The size distribution observed in these populations was the expected for Gag-GFP VLPs with the mode around 130-150 nm (Gutiérrez-Granados et al., 2015). Representative VLP micrographs are shown in Figure 8b (from bioreactor) and c (from shake flasks). Roughly spherical virus particles surrounded by a lipid envelope can be distinguished. In accordance with NTA, the particle diameter observed by TEM was around 150 nm. These results indicated that fluorescence signal detected in the supernatant corresponded to correctly assembled VLPs rather than monomeric Gag-GFP.

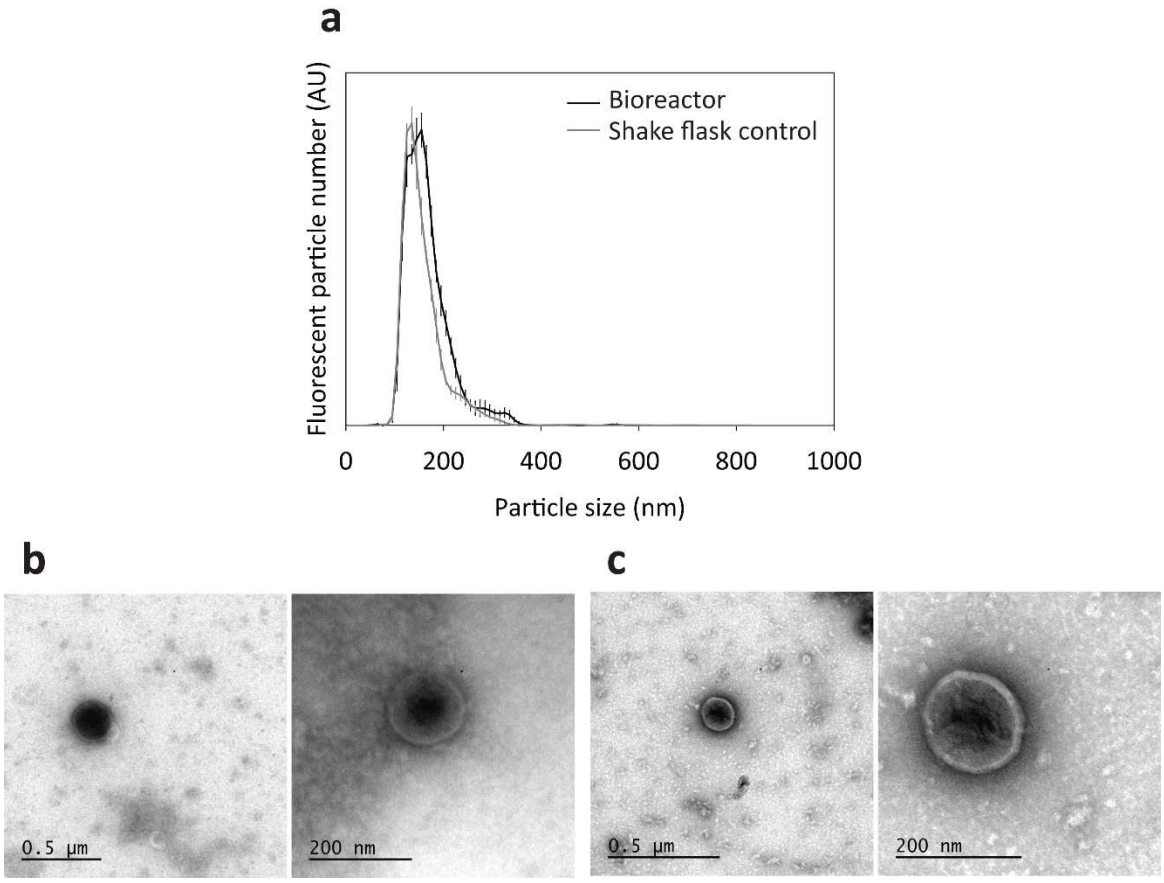

Figure 8. VLP characterization. (a) VLPs from culture supernatants were analyzed by fluorescence Nanoparticle Tracking Analysis (NTA). A fluorescent particle population of the expected size (130-150 nm) was identified in bioreactor and shake flask controls (96 hpt). Error bars indicate the standard error of triplicate quantifications of the same sample. (b and c) Transmission electron microscopy micrographs obtained by negative staining of cell culture supernatants containing VLPs. (b) corresponds to bioreactor culture and (c) to shake flask controls both at 96hpt. AU: arbitrary units.

4. Conclusions

Here we describe the successful scale-up of CAP-T transient transfection to 1L stirred-tank bioreactor. The cell growth conditions in bioreactor were fine-tuned in order to achieve the suitable cell state for optimal transfection performance. Addition of carbonate and aeration through headspace were found to be critical variables to this purpose. Moreover, by culturing CAP-T cells in FreeStyleF17 medium supplemented with 5% of PEM and then diluting the PEM to 1% of PEM 48 h before transfection, the cumbersome medium replacement step could be avoided. This novel media combination strategy significantly simplified the large-scale process, while VLP production levels achieved were comparable to small-scale controls. Remarkably, VLP quality was maintained in bioreactor production. The strategy proposed here enables to perform cell growth and transient transfection of CAP-T cells in bioreactor without medium exchange and can contribute to the application of transient gene expression in larger volumes.

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Conflict of interest

Kerstin Hein is an employee of CEVEC Pharmaceuticals, the company that has developed the CAP-T cell line and has contributed with scientific advice to this work.

References

Abbott, W.M., Middleton, B., Kartberg, F., Claesson, J., Roth, R., Fisher, D., 2015. Optimisation of a simple method to transiently transfect a CHO cell line in high-throughput and at large scale. Protein Expr. Purif. 116, 113–119.

Ansorge, S., Lanthier, S., Transfiguracion, J., Durocher, Y., Henry, O., Kamen, A., 2009. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. J. Gene Med. 11, 868–76.

Ausubel, L.J., Hall, C., Sharma, A., Shakeley, R., Lopez, P., Quezada, V., Couture, S., Laderman, K., McMahon, R., Huang, P., Hsu, D., Couture, L., 2012. Production of CGMP-Grade Lentiviral Vectors. Bioprocess Int. 10, 32–43.

Backliwal, G., Hildinger, M., Chenuet, S., Wulhfard, S., De Jesus, M., Wurm, F.M., 2008. Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res. 36, e96.

Buonaguro, L., Tagliamonte, M., Visciano, M.L., Tornesello, M.L., Buonaguro, F.M., 2013. Developments in virus-like particle-based vaccines for HIV. Expert Rev. Vaccines 12, 119–127.

Carter, J., Zhang, J., Dang, T.L., Hasegawa, H., Cheng, J.D., Gianan, I., O'Neill, J.W., Wolfson, M., Siu, S., Qu, S., Meininger, D., Kim, H., Delaney, J., Mehlin, C., 2010. Fusion partners can increase the expression of recombinant interleukins via transient transfection in 2936E cells. Protein Sci. 19, 357–362.

Cervera, L., Gutiérrez-Granados, S., Martínez, M., Blanco, J., Gòdia, F., Segura, M.M., 2013. Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J. Biotechnol. 166, 152–65.

Chen, Y., Wu, B., Musier-Forsyth, K., Mansky, L.M., Mueller, J.D., 2009. Fluorescence fluctuation spectroscopy on viral-like particles reveals variable Gag stoichiometry. Biophys. J. 96, 1961–1969.

Chisti, Y., 2000. Animal-cell damage in sparged bioreactors. Trends Biotechnol. 18, 420–432.

Fischer, S., Charara, N., Gerber, A., Wölfel, J., Schiedner, G., Voedisch, B., Geisse, S., 2012. Transient recombinant protein expression in a human amniocyte cell line: The CAP-T® cell system. Biotechnol. Bioeng. 109, 2250–2261.

Fliedl, L., Kaisermayer, C., 2011. Transient gene expression in HEK293 and vero cells immobilised on microcarriers. J. Biotechnol. 153, 15–21.

Geisse, S., 2009. Reflections on more than 10 years of TGE approaches. Protein Expr. Purif. 64, 99–107.

Genzel, Y., Behrendt, I., Rödig, J., Rapp, E., Kueppers, C., Kochanek, S., Schiedner, G., Reichl, U., 2013. CAP, a new human suspension cell line for influenza virus production. Appl. Microbiol. Biotechnol. 97, 111–122.

Genzel, Y., Vogel, T., Buck, J., Behrendt, I., Ramirez, D.V., Schiedner, G., Jordan, I., Reichl, U., 2014. High cell density cultivations by alternating tangential flow (ATF) perfusion for influenza A virus production using suspension cells. Vaccine 32, 2770–2781.

Girard, P., Derouazi, M., Baumgartner, G., Bourgeois, M., Jordan, M., Jacko, B., Wurm, F.M., 2002. 100-Liter transient transfection. Cytotechnology 38, 15–21.

Gray, D.R., Chen, S., Howarth, W., Inlow, D., Maiorella, B.L., 1996. CO2 in large-scale and high-density CHO cell perfusion culture. Cytotechnology 22, 65–78.

Grieger, J.C., Soltys, S.M., Samulski, R.J., 2016. Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. Mol. Ther. 24, 287–297.

Gutiérrez-Granados, S., Cervera, L., Gòdia, F., Carrillo, J., Segura, M.M., 2013. Development and validation of a quantitation assay for fluorescently tagged HIV-1 virus-like particles. J. Virol. Methods 193, 85–95.

Gutiérrez-Granados, S., Cervera, L., Segura, M.M., Wölfel, J., Gòdia, F., 2015. Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells. Appl. Microbiol. Biotechnol. 100, 3935–3947.

Haldankar, R., Li, D., Saremi, Z., Baikalov, C., Deshpande, R., 2006. Serum-free suspension large-scale transient transfection of CHO cells in WAVE bioreactors. Mol. Biotechnol. 34, 191–199.

Handa-Corrigan, A., Emery, A.N., Spier, R.E., 1989. Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles. Enzyme Microb. Technol. 11, 230–235.

Hermida-Matsumoto, L., Resh, M.D., 2000. Localization of Human Immunodeficiency Virus Type 1 Gag and Env at the Plasma Membrane by Confocal Imaging Localization of Human Immunodeficiency Virus Type 1 Gag and Env at the Plasma Membrane by Confocal Imaging. J. Virol. 74, 8670–9.

Jäger, V., Büssow, K., Schirrmann, T., 2015. Transient Recombinant Protein Expression in Mammalian Cells, in: Animal Cell Culture. Springer International Publishing, pp. 27–64.

Liu, J., Dai, S., Wang, M., Hu, Z., Wang, H., Deng, F., 2016. Virus like particle-based vaccines against emerging infectious disease viruses. Virol. Sin. 31, 279–287.

Lua, L.H.L., Connors, N.K., Sainsbury, F., Chuan, Y.P., Wibowo, N., Middelberg, A.P.J., 2014. Bioengineering virus-like particles as vaccines. Biotechnol. Bioeng. 111, 425–440.

Ludwig, C., Wagner, R., 2007. Virus-like particles-universal molecular toolboxes. Curr. Opin. Biotechnol. 18, 537–545.

Muller, N., Derouazi, M., Van Tilborgh, F., Wulhfard, S., Hacker, D.L., Jordan, M., Wurm, F.M., 2007. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol. Lett. 29, 703–711.

Noad, R., Roy, P., 2003. Virus-like particles as immunogens. Trends Microbiol. 11, 438–444.

Pillay, S., Meyers, A., Williamson, A.L., Rybicki, E.P., 2009. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. Biotechnol. Prog. 25, 1153–1160.

Rajendra, Y., Kiseljak, D., Manoli, S., Baldi, L., Hacker, D.L., Wurm, F.M., 2012. Role of non-specific DNA in reducing coding DNA requirement for transient gene expression with CHO and HEK-293E cells. Biotechnol. Bioeng. 109, 2271–8.

Rodríguez-Limas, W.A., Sekar, K., Tyo, K.E.J., 2013. Virus-like particles: The future of microbial

factories and cell-free systems as platforms for vaccine development. Curr. Opin. Biotechnol. 24, 1089–1093.

Roldão, A., Mellado, M.C.M., Castilho, L.R., Carrondo, M.J.T., Alves, P.M., 2010. Virus-like particles in vaccine development. Expert Rev. Vaccines 9, 1149–1176.

Saraswat, S., Athmaram, T.N., Parida, M., Agarwal, A., Saha, A., Dash, P.K., 2016. Expression and Characterization of Yeast Derived Chikungunya Virus Like Particles (CHIK-VLPs) and Its Evaluation as a Potential Vaccine Candidate. PLoS Negl. Trop. Dis. 10, 1–19.

Schlaeger, E.J., Christensen, K., 1999. Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotechnology 30, 71–83.

Schwartz, S., Campbell, M., Nasioulas, G., Harrison, J., Felber, B.K., Pavlakis, G.N., 1992. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. J. Virol. 66, 7176–7182.

Segura, M.M., Garnier, A., Durocher, Y., Coelho, H., Kamen, A., 2007. Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification. Biotechnol. Bioeng. 98, 789–99.

Sun, X., Hia, H.C., Goh, P.E., Yap, M.G.S., 2008. High-density transient gene expression in suspension-adapted 293 EBNA1 cells. Biotechnol. Bioeng. 99, 108–16.

Tagliamonte, M., Visciano, M.L., Tornesello, M.L., De Stradis, A., Buonaguro, F.M., Buonaguro, L., 2010. Constitutive expression of HIV-VLPs in stably transfected insect cell line for efficient delivery system. Vaccine 28, 6417–6424.

Tuvesson, O., Uhe, C., Rozkov, A., Lüllau, E., 2008. Development of a generic transient transfection process at 100 L scale. Cytotechnology 56, 123–136.

Venereo-Sanchez, A., Gilbert, R., Simoneau, M., Caron, A., Chahal, P., Chen, W., Ansorge, S., Li, X., Henry, O., Kamen, A., 2016. Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate. Vaccine 34, 3371–3380.

Warfield, K.L., Aman, M.J., 2011. Advances in Virus-Like Particle Vaccines for Filoviruses 204, 1053–1059.

Yan, D., Wei, Y.Q., Guo, H.C., Sun, S.Q., 2015. The application of virus-like particles as vaccines and biological vehicles. Appl. Microbiol. Biotechnol. 99, 10415–10432.

Ye, J., Kober, V., Tellers, M., Naji, Z., Salmon, P., Markusen, J.F., 2009. High-level protein expression in scalable CHO transient transfection. Biotechnol. Bioeng. 103, 542–551.

Generation of a CAP stable cell line to produce HIV virus-like particles

Results, chapter 4

1. Introduction

Virus-like particles (VLPs) are formed by the main structural protein of a virus, mimicking its natural structure. Since the viral antigens are presented with the native conformation, efficient immune responses can be elicited, thus VLPs are promising vaccine candidates [1]. The main structural protein of HIV is Gag. This polyprotein has the ability to self-assemble in the cell membrane upon expression in a eukaryotic host cell line to form VLPs. The HIV VLPs are released from the cells by budding, taking part of the membrane as a lipid envelope. Therefore, the envelope of VLPs contains membrane-associated host cell proteins [2]. The presence of other viral proteins or genes is not required for VLP formation. However, if the surface antigens of the HIV (Env) are co-expressed with Gag in the same host cell, they will be expressed in the cell membrane and eventually incorporated in the VLP envelope [3,4]. Moreover, not only HIV antigens can be incorporated into VLPs, but also membrane antigens from other viruses if they are co-expressed with Gag. The presence of envelope antigens in the VLP surfaces is interesting since these are usually the molecules eliciting humoral immune responses [5], so VLPs can act as scaffolds to present a variety of immunogens.

Co-expression of Gag along with other viral antigens can be achieved either by transient or stable expression of the proteins of interest. Transient expression is achieved by the co-transfection of the plasmids coding for the proteins of interest. This is the case of the production of lentiviral vectors by co-transfection of two plasmids coding for Gag and Env or VSV-G glycoprotein [6,7]. For the generation of stable cell lines, co-transfection of the plasmids of interest need to be followed by antibiotic selection. This was the case of the generation of a HEK293 stable cell line expressing Gag-Pol-Env VLPs [8]. Alternatively, stable expression of Gag-Env VLPs was achieved in two steps by Tagliamonte *et al*. They first generated a cell line constitutively expressing Gag, to which stable expression of Env was added afterwards [4,9]. The combination of transient and stable expression is also a possibility. In this regard, Venereo-Sánchez *et al*. generated a HEK293 cell line expressing influenza surface antigens (HA and NA) in a stable manner. Expression of Gag in this cell line was then performed by transient transfection, which resulted in Gag VLPs displaying the influenza surface antigens [10]. This last approach is particularly interesting when the VLPs are formed by more than one protein, and while one of them is fixed, the second need to be tested among several candidates before selection of the most appropriate one.

The aim of this work was to generate a stable CAP cell line expressing Gag-GFP VLPs, that could be used in the future to produce VLPs with surface antigens from diverse viruses. CAP cells were developed by Cevec Pharmaceuticals (Cologne, Germany) [11] and, since then, extensive experience has been accumulated on the generation of CAP recombinant cell lines. For this reason, the generation of a VLP-expressing CAP clone was carried out at Cevec. The methodology employed was the one already implemented at the company for the generation of other stable cell lines consisting in random integration of the gene of interest followed by limiting dilution and manual screening of the clones.

The random integration of the gene of interest has the advantage that many copies can be inserted into the genome, and the number of copies usually correlates with the productivity [12,13]. On the other hand, limiting dilution is the most classic methodology for the generation of stable cell lines, and interestingly it is also the most widely used in industry, because of its simplicity and straightforwardness. Additionally, it does not require any specific and expensive equipment and it is not subjected to IP restrictions [13,14].

2. Materials and methods

2.1. Cell line and culture conditions

CAP cells were kindly provided by Cevec Pharmaceuticals (Cologne, Germany). Cells were cultured in suspension in Protein Expression Medium (PEM, Gibco, Grand Island, NY, USA) supplemented with 4 mM L-alanyl-L-glutamine (Biochrom, Berlin, Germany). Cells were routinely maintained at exponential growth phase with viabilities over 95% in 125 mL disposable polycarbonate Erlenmeyer flasks (Corning, New York, NY, USA). Erlenmeyer flasks were placed in an orbital shaker (orbit diameter 16 mm, Stuart, Stone, UK) at 130 rpm in a humidified incubator at 37 °C and 5% CO2 in air or in a ISF4-X shaker (Kühner shaker, Birsfelden, Switzerland) at 185 rpm (orbit diameter 5 cm).

2.2. Plasmid construction

The gene coding for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP was cloned in the pStbl-bsd-CMV-MCS(-) expression vector. The plasmid was linearized with Eco32I, which generated blunt ends. The Gag-GFP insert was extracted from the original plasmid (pGag-EGFP, NIH AIDS Reagent Program, Cat #11468 [15]) using NotI and KpnI cloning sites followed by a treatment with Klenow Fragment to generate blunt ends. The subsequent amplified ligation products were sequenced to corroborate the correct orientation of the insert into the new vector pStbl_GagGFP (Figure 1). Plasmid DNA amplification and purification was carried out as previously reported [16]. Finally, the pStbl_GagGFP plasmid was linearized with ScaI and further purified with a standard phenol/chloroform protocol.

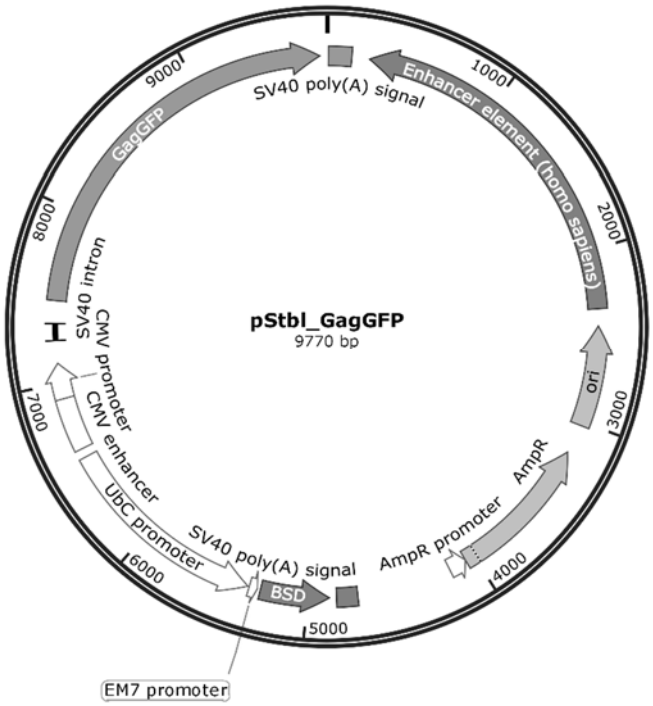

Figure 1. pStbl_GagGFP vector map. Gag-GFP expression is driven by the CMV promoter and blasticidin expression is under the ubiquitin C promoter. The SV40 intron upstream of the gene of interest promotes mRNA stability and transport to the cytoplasm. The enhancer element increases expression levels. Ampicillin is the antibiotic resistance for bacteria.

2.3. Analysis of cell culture main parameters and product quantification

Viable cell density and viability were determined using either the Cedex automated cell counter (Innovatis, Bielefeld, Germany) or the NucleoCounter®NC-3000 automatic cell

counter (Chemometec, Allerod, Denmark) according to manufacturer's instructions. The percentage of GFP expressing cells was determined by the NucleoCounter®NC-3000 automatic cell counter (Chemometec) according to manufacturer's instructions or by flow cytometry using a fluorescence-activated cell sorter (FACS) (BDFACS Canto, BD BioSciences, San Jose, CA, USA). Gag-GFP polyprotein concentrations were determined using the commercially available kit Innotest® HIV antigen mAb (Innogenetics NV, Gent, Belgium) according to the manufacturer's instructions.

2.4. Nucleofection and cell pool maintenance

Exponentially growing CAP cells were electroporated with the pStbl_GagGFP plasmid using Nucleofection technology (Lonza, Basel, Switzerland). Briefly, 1×10^7 cells were resuspended in nucleofector solution V (Lonza) and electroporated with 1 µg of the linearized plasmid in the Nucleofector device (Lonza) following the manufacturer's instructions. Then, the cells were cultured in agitation in 15 mL of PEM. After 3 days, blasticidin (ThermoFisher Scientific) was added to a final concentration of 5 µg/mL to select the cells that had incorporated the vector in the genome. The cell pool was maintained at exponential growth phase by reseeding twice a week to a concentration of 1×10^6 cells/mL. Cell density, percentage of GFP expressing cells and Gag-GFP concentration in supernatants were analyzed until the pool was frozen 3 weeks after transfection.

2.5. Limiting dilution and further amplification of the CAP selected clones

Cells from the pool were seeded in 96-well plates (TPP, Trasadingen, Switzerland) at a concentration of 0.25 cells/well in cell culture medium. Single clones were cultured in static mode for 5 weeks, with a PEM medium replacement (supplemented with 4 mM L-alanyl-L-glutamine), twice a week, starting after week 2. The selected clones were seeded in 24-well plates (TPP) in PEM supplemented with blasticidin (5 µg/mL) and cultured for 10 days with a medium replacement every 3-4 days. The selected clones were further amplified in static cultures in 6 and 10 cm dishes (TPP) until an appropriate number of cells was achieved to freeze four vials of each clone.

2.6. Fed-batch experiments in 24-deep well plates, shake flasks and 1L bioreactor

CAP cells were subjected to a medium replacement by centrifugation at 200×g, 5 min to CAP-CDM medium (Cevec Pharmaceuticals, Cologne, Germany) supplemented with 6 mM of L-alanyl-L-glutamine, LONG® R3 IGF-I (50 µL/L, Sigma, Lenexa, KS, USA) and blasticidin (5 µg/mL), and they were allowed to adapt for 2-3 passages before starting the experiments. For the fed-batch experiments, cells were seeded at 1×10^6 cells/mL in CAP-CDM without antibiotic in 24-deep well plates (Qiagen, Hilden, Germany) and at 0.5×10^6 cells/mL in shake flasks and 1L bioreactor. At determined time points (indicated in the results section) CAP-CDM Feed (10% of the initial culture volume, Cevec Pharmaceuticals) was added along with GlutaMAX (2% of the initial culture volume). Samples were taken to analyze the viable cell density, viability and p24 concentration in supernatants. The BioStat B Plus bioreactor (Sartorius AG, Goettingen, Germany) was equipped with a 3-blade segment impeller. The agitation was set at 160 rpm. Temperature was set at 37°C. The pH was set at 7.1, controlled with CO_2 and $NaHCO_3$ (7.5% w/v). The dissolved oxygen was controlled at 40% of air saturation by supplementing air and oxygen through the sparger. The medium was additionally supplemented with AntiFOAM C (Sigma, St Louis, MO, USA) to a final concentration of 100 mg/L.

2.7. Analysis of gene copy number by qPCR

Total DNA was extracted from 1×10^7 cells from the three higher-ranked and two negative clones, using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Real time quantitative PCR (qRT-PCR) was carried out in a Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. 2X Brilliant Multiplex QPCR Master Mix (Agilent Technologies) was used. Since the number of chromosomes of immortalized cells can increase over cell passage, the copy number per genome was calculated. The genome of a CAP cell line previously determined to have only one genome set was used as a reference. A multiplex reaction was carried out, in which the CMV promoter was detected to determine the gene copy number and PrP and E1a sequences were detected as controls, since there are two and one copies of these genes per genome, respectively.

Table 1 summarizes the sequences of the primers and probes used to amplify and detect CMV, PrP and E1a.

Table1
Primers and probes used in qPCR for copy number analysis

Primers and probes	Sequence
Primer E1a "Forward"	5'-GACTGTGGTTGCTTCATGCT-3'
Primer E1a "Backward"	5'-ACGTGAATGGTCTTCAGCAG-3'
Primer PrP "Forward"	5'-CAGTGGAACAAGCCGAGTAA-3'
Primer PrP "Backward"	5'-CCTCATAGTCACTGCCGAAA-3'
Primer CMV "Forward"	5´-AAATGGCCCGCCTGGCATTATG-3'
Primer CMV "Backward"	5´-AAACCGCTATCCACGCCCATTG-3'
Probe E1a-HEX-BH1	5'-TGAGAGGCCCTGTCCTCGCA-3'
Probe PrP-FAM-BH1	5'-AGCATGTAGCCGCCAAGGCC-3'
Probe CMV-ROX-BHQ2	5'-ACATGACCTTATGGGACTTTCCTACTTGGCAGTACATC-3'

2.8. Cell sorting

Cell sorting was performed in a BD FACSJazz cell sorter (BD Biosciences, San Jose, CA, USA) equipped with a blue (488 nm) and a red laser (640 nm) at Servei de Cultius Cel·lulars, Anticossos i Citometria (SCAC, MRB, UAB). Briefly, exponentially growing cells were concentrated at 4×10^6 cells/mL in PEM medium before sorting. The gating strategy was as follows: first, viable and single cells were selected by forward and side scatter and forward scatter and trigger pulse width plots, respectively. Then, GFP expressing cells were detected by FL1 detector (530/40 band pass filter). The sheath fluid was DPBS (Gibco, Grand Island, NY, USA) with a constant pressure set to 27 psi. A 100 µm nozzle was used. Sorting rate was ~500 cells/s, and 1.5×10^6 cells were collected in PEM medium supplemented with penicillin-streptomycin (1X, Sigma Aldrich, MI, USA). After sorting, cells were pelleted (300xg for 5 min) and kept for two weeks in PEM medium with penicillin-streptomycin and blasticidin in static culture until freezing.

2.9. VLP characterization by NTA and TEM

Nanoparticle tracking analysis (NTA) was employed for VLP characterization in a NanoSight®NS300 device (NanoSight Ltd., Amesbury, UK) at the Service of Preparation and Characterization of Soft Materials (Institut de Ciència de Materials de Barcelona, ICMAB, CSIC, Bellaterra, Spain). Sample analyses were performed as previously reported

[16]. For VLP visualization under transmission electron microscopy (TEM), samples were prepared by air-dried negative staining method at the Servei de Microscòpia (UAB, Bellaterra, Spain) as previously reported [16] before the examination in a Jeol JEM-1400 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Gatan ES1000W Erlangshen CCD Camera (Model 785).

3. Results and discussion

3.1. Study of the stability of the cell pool

The CAP cell pool expressing Gag-GFP VLPs was generated by culturing transfected cells for three weeks under antibiotic selection. The percentage of GFP-expressing cells increased until stabilizing at around 50% (data not shown). At that time, the pool was frozen and clone screening by limiting dilution started.

In order to assess the stability of the expression under no selective pressure the CAP pool was splitted in two and cultured with and without antibiotic for six weeks. Cell growth, viability, percentage of GFP-expressing cells and VLP productivity over time were analyzed (Figure 2). Regarding cell growth and viability (Figure 2a), there was no significant difference between the pool with antibiotic (average duplication time of 34.59 ± 5.1 h) and without it (average duplication time of 34.18 ± 5.3 h). The percentage of GFP+ cells and the volumetric productivity of the pool without selective pressure were reduced by a 13.1 and 15.3%, respectively compared to the pool with the antibiotic selection. These parameters remained constant in the latter (Figure 2b).

A slight reduction of the percentage of cells expressing VLPs was observed after antibiotic removal. This result demonstrated that the majority of the cells in the pool expressing the protein of interest were stable, and therefore, antibiotic was not required to maintain high percentages of VLP expressing cells. As expected, the reduced number of expressing cells resulted in a reduced volumetric productivity. The average productivity was 84.3 ± 42.5 pg/mL per day when no selective pressure was applied while it was 99.6 ± 35.4 pg/mL per day with the blastidicin selection. Since the variability over time was significant, this decrease of about 15% in productivity was not relevant, and it can be concluded that the CAP pool was stable over time without the need of antibiotic selection.

Cells producing proteins at high titers usually grow to lower cell densities than lower-producer or non-producer cells, since they use the energy both for growth and production [17]. In this case, the reduction of the productivity observed when the antibiotic was removed did not influence the duplication time of the cells, corroborating that it was not very significant.

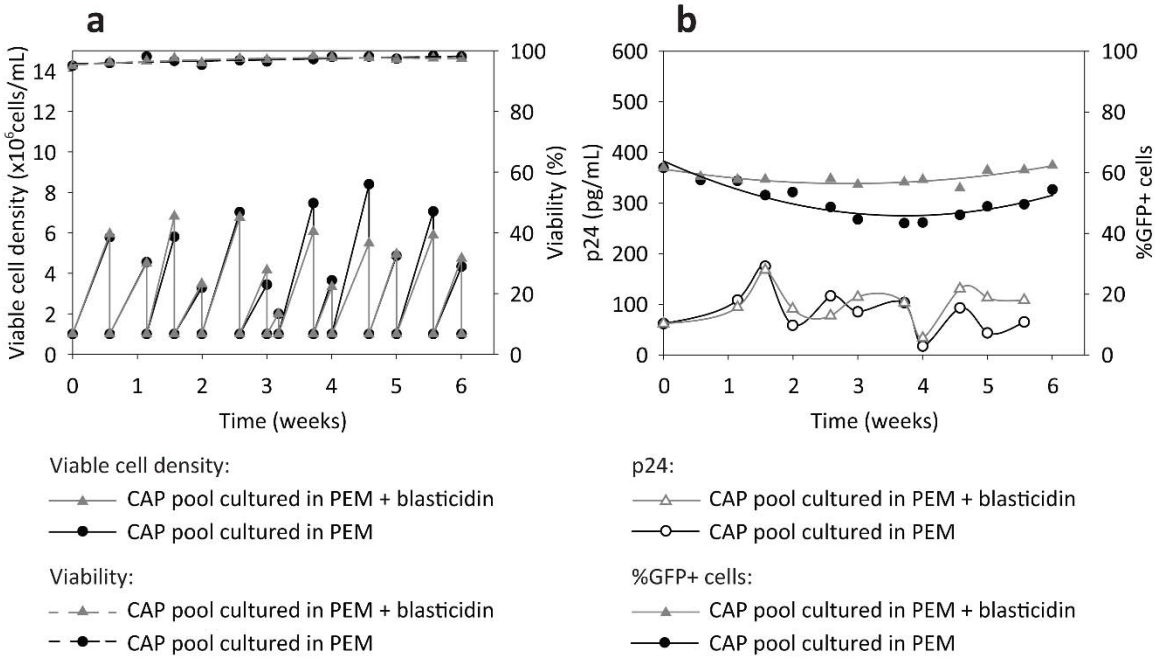

Figure 2. Study of the stability of the transfected CAP pool. The transfected CAP pool was cultured with and without antibiotic pressure for six weeks. (a) Viable cell density and viability are represented. (b) The percentage of GFP expressing cells (analyzed using the NucleoCounter NC-3000) and the VLP production in supernatants are represented.

The fact that after six weeks of blasticidin pressure, the percentage of GFP+ cells and the volumetric productivity did not increase was an indication that there was a significant number of cells expressing the antibiotic resistance but not the protein of interest. This could be expected since the expression of both cassettes is not linked as in bi-cistronic cassettes. Moreover, the CMV promoter and enhancer driving the Gag-GFP expression are known to be easily silenceable by epigenetic events [18,19] while ubiquitin C promoter, driving blasticidin expression, is not [20].

3.2. Clone screening and selection after limiting dilution

The screening methodology used was limiting dilution. Briefly, the cells from the stably transfected pool were seeded in 96-well plates at a concentration of 0.25 cells/well to ensure the presence of single cells in at least some of the wells. After 5 weeks of culture, wells with colonies originated from a single cell (determined by visual inspection of the plates after seeding) were further amplified in 24-well plates. The clone selection was then carried out in two steps. In the first round, the VLP concentration in supernatants of static cultures in 24-well plates was the selection criterion. In the second phase, fed-batch experiments in 24-DWP were carried out and VLP concentration in supernatant, percentage of GFP+ cells and maximum cell density achieved were the variables considered to rank the clones and select the best performing ones.

Figure 3 shows the concentration of VLPs (measured by p24 ELISA) of each clone cultured in static mode in 24-well plates (first screening round). Considering that 75 out 960 wells could be amplified from 96- to 24-well plates, of which 53 were positive for Gag-GFP expression, this represents a global success rate of around 6%. Considering only the analyzed clones, a success of 70% was obtained.

The best 22 clones were further amplified for a second screening round. Specifically, they were adapted to suspension culture in CAP-CDM medium and subjected to growth in 24-DWP in fed-batch mode. Additionally, two negative clones were also analyzed as controls (8F8 and 2H7). The maximum viable cell density attained varied between 2.5 and 14×10^6 cells/mL at 168 h of culture (Figure 4a) and the viability was over 85% for all the clones (not shown). Only one clone (2G11) did not grow and was not further analyzed. The percentage of GFP positive cells and VLP concentration at 168 h were analyzed for every clone. Surprisingly, some of the clones presented percentages of GFP positive cells lower than 80% (ranging between 20-70%). It was hypothesized that these populations may not be clonal, either because they came from more than one cell or because they suffered genetic drifting over cell passage. Another possibility could be that the NucleoCounter (used in this phase to analyze the percentage of GFP+ cells) was not sensitive enough to detect GFP fluorescence in the lower producer cells of every clone, considering that not all cells will produce the same amount of VLPs within a clonal population. Since the causes for these heterogeneous percentages of GFP positive cells were not further investigated, it was decided to give some priority to the clones presenting higher percentages of GFP positive cells, without totally excluding the ones

with lower percentages. Therefore, the selection in this round was based on the VLP titer (60% of the weight), the percentage of GFP expressing cells (30% of the weight) and the maximum viable cell density achieved (10% of the weight). Figure 4b shows the ranked clones by their relative scores. The best three clones (3F11, 9B10 and 8D5) were selected for further evaluation.

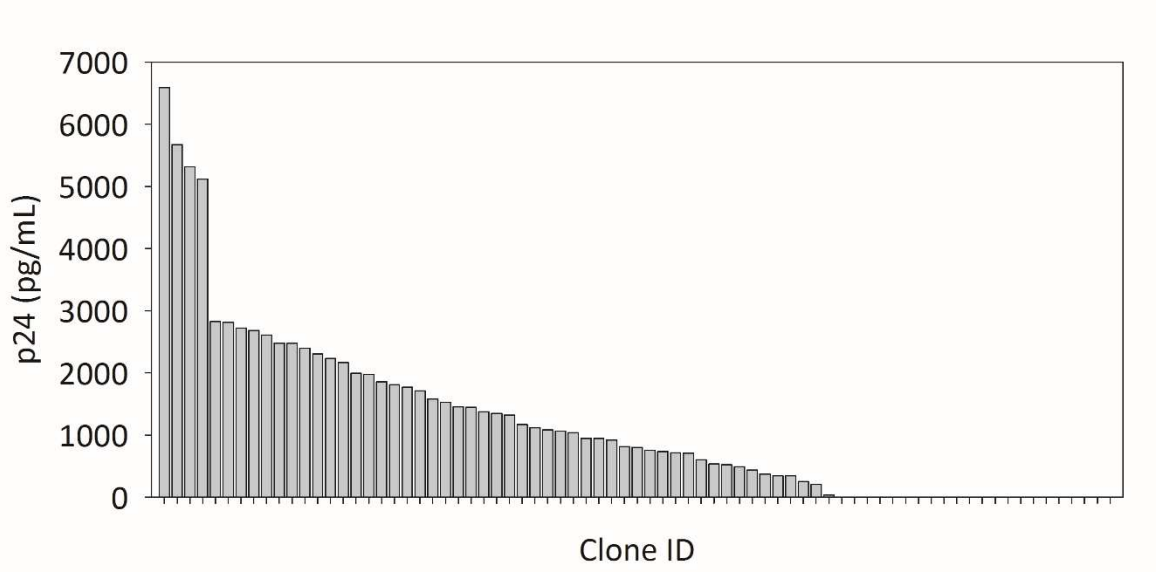

Figure 3. First screening round. After limiting dilution, the wells with colonies originated from one single cell were amplified to 24-well plates in static cultures (75 clones). VLP production in supernatants was analyzed after 4 days of culture for all the clones amplified. The 22 clones showing higher p24 titers in supernatants were selected for the second screening round.

Noticeably, it is relevant to screen the clones under the same culture conditions that will be used in larger-scales. In this case, suspension culture in fed-batch mode was the methodology used for screening, since these conditions reproduce with more reliability the large-scale culture situation [13]. This was demonstrated recently for CHO cells expressing a monoclonal antibody. Rouiller *et al.* used a robotic liquid handler that allowed them to screen 470 clones in fed-batch mode in a primary screening phase (in 96-DWP), allowing them to identify clones producing 3 g/L of antibody. The selected clones were cultured in larger-scales (shake tubes, micro-scale and lab-scale bioreactors) with highly reproducible results [21].

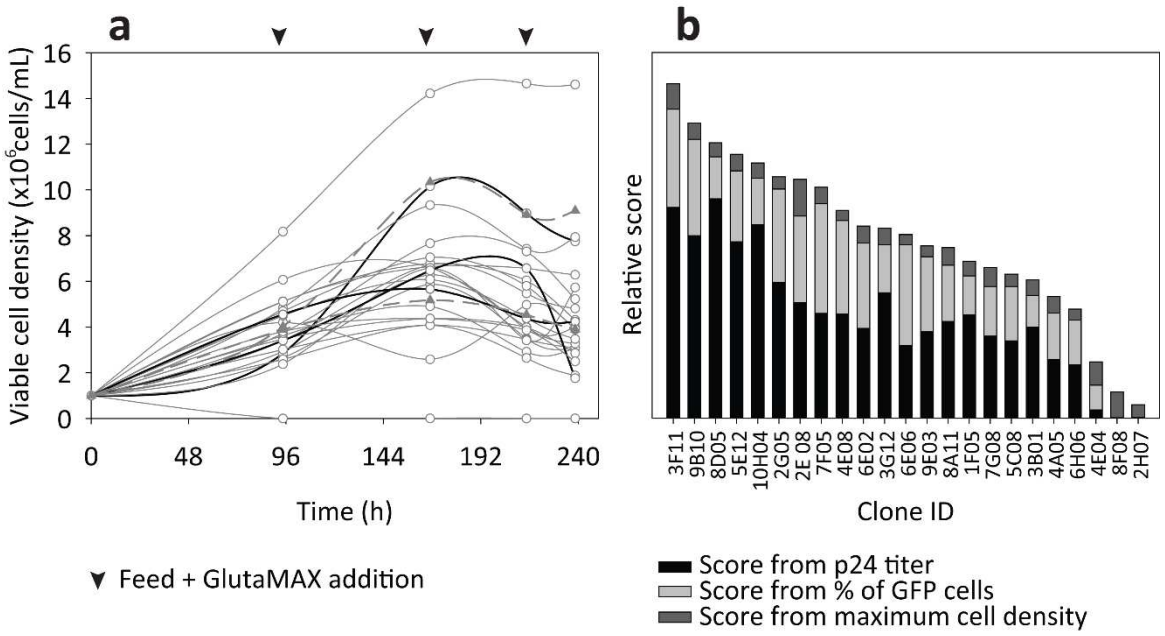

Figure 4. Second screening round. (a) The best 22 clones from the first screening round were adapted to growth in suspension in CAP-CDM medium and were subjected to fed-batch cultures in 24-DWP. Black lines correspond to the best ranked clones (shown in b), dashed lines correspond to the negative clones analyzed and the rest are represented with gray lines. (b) The clones were ranked according to the p24 titer at 168h of culture (60% of the weight), the percentage of GFP-expressing cells at 168 h (30% of the weight) and the maximum cell density achieved (10% of the weight). The three best scored clones were further analyzed, along with the two negative clones.

3.3. Evaluation of clone performance at small scale

The gene copy number of the three selected clones was analyzed by qPCR along with the two negative clones and the CAP parental cell line. As can be observed in Figure 5, 8D5 presented 10 copies of the gene, 3F11 had 5 copies and 9B10 had 1 copy. As expected, the parental cells did not harbor any copies. Surprisingly, both negative clones analyzed contained some copies of the plasmid.

One of the advantages of random compared to targeted integration is that many copies of the gene of interest can be inserted into the genome. This leads to higher transcription levels and thus enhanced protein titers [13,21]. In fact, p24 titers observed in the 24-DWP fed-batch experiments (section 3.2) correlate with the relative copy numbers determined by qPCR. For this reason, the two clones containing more copies

(8D5 and 3F11) were selected for testing in shake flasks. 9B10 was discarded at this point for being the one with less copies (Figure 5) and with lower p24 titers (Figure 4b).

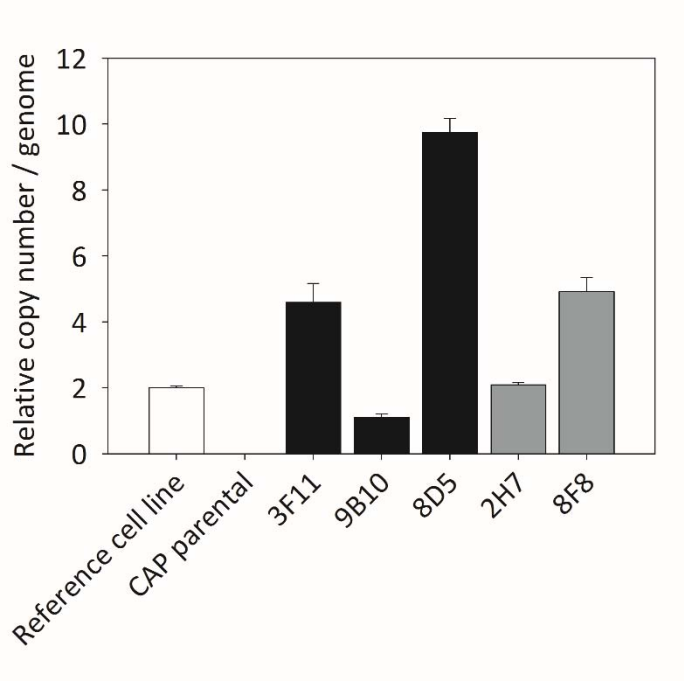

Figure 5. Analysis of gene copy number of the three clones selected in the second screening phase was determined by qPCR. The two negative clones and the CAP parental cell line were also analyzed. The reference cell line is a cell line previously characterized to contain 2 copies of PrP and 1 copy of E1a. Error bars indicate the standard deviation of triplicate reactions.

Regarding the negative clones, they were found to contain 2 and 5 copies of the gene although no VLP expression was ever detected. Silencing of the CMV promoter due to epigenetic events [19], insertion in transcriptionally inactive genome sites or mutations (deletions/insertions) hindering Gag-GFP expression and correct VLP formation could explain this phenomenon. The two last options are believed to be less probable, mainly for 8F8 since it contained 5 copies of the gene. However, this phenomenon was not further investigated since it was out of the scope of this work.

Clones 3F11 and 8D5 were cultured in fed-batch mode in CAP-CDM medium in shake flasks. Figure 6a and b show the growth profiles of each clone, cell viability and VLP concentration in supernatant. As it can be observed, 3F11 grew up to 8.06×10^6 cells/mL with a duplication time of 35 h and viabilities over 90% until day 9. Similarly, maximum cell density attained by 8D5 was 6.49×10^6 cells/mL with a duplication time of 38 h and viabilities over 90% until day 9. The maximum VLP concentration attained was different

for each clone. 8D5 produced 27 ng/mL of p24 and 3F11 only achieved 16 ng/mL. This was not surprising since 8D5 had already shown higher titers than 3F11 in screening phase 2 in 24-DWP. Moreover, 8D5 contained more copies of the gene of interest, thus better productivities were expected. The specific productivity was consistently higher for 8D5 than for 3F11 (Table 2), as it had been also in the second screening phase. Both clones were alternatively cultured in batch in PEM medium, obtaining similar cell densities but significantly lower VLP titers (data not shown). Therefore, PEM was discarded as the culture medium for VLP production.

8D5 clone was finally selected for further evaluation and optimization, cultured in CAP-CDM medium in fed-batch mode.

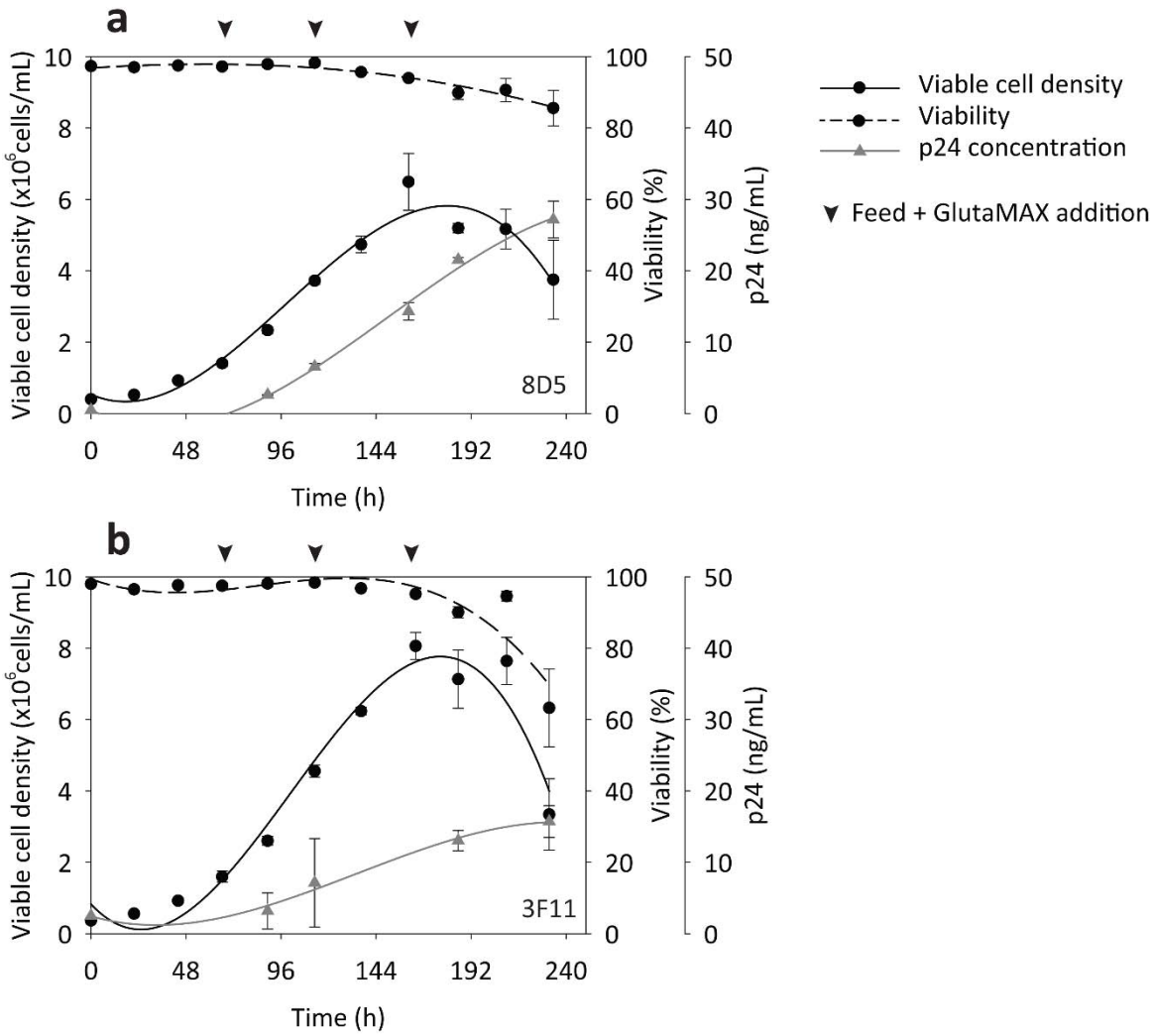

Figure 6. Clone evaluation in fed-batch mode in shake flasks. Viable cell density, viability and VLP titer (p24 concentration) are represented. The mean of triplicate experiments is represented. Error bars indicate the standard deviation. (a) 8D5 clone. (b) 3F11 clone.

Table 2
Specific productivity of the clones selected in screening phase two

Clone ID	Specific productivity (pg/cell·day)		
	24-DWP	Shake flask[1]	Stirred-tank bioreactor
3F11	3.94×10^{-4}	3.50×10^{-4} $\pm2.01\times10^{-5}$	-
8D5	2.73×10^{-4}	8.62×10^{-4} $\pm9.65\times10^{-5}$	8.17×10^{-4}
8D5+	-	1.77×10^{-3} $\pm4.44\times10^{-4}$	-
8D5+ (+VPA)[2]	-	2.17×10^{-3} $\pm4.74\times10^{-4}$	-

1. Average of three experiments ± standard deviation. 2. VPA: Valproic acid 4 mM added at 120h of culture.

3.4. 8D5 clone evaluation in lab-scale bioreactor

The selected clone was cultured in 1L stirred-tank bioreactor using the same feeding regime than in shake flaks experiments. Triplicate shake flask cultures were run in parallel as controls.

As can be observed in Figure 7a, cell viability was similar in both systems during all culture. In terms of maximum cell density achieved, it was slightly lower in bioreactor (7.5 compared to 8.5×10^6 cells/mL). Duplication time was 38.5 h in shake flask and 39.8 h in bioreactor. Despite the performance in bioreactor regarding cell growth was slightly lower than in shake flasks, the differences were not very significant and the 8D5 cell line behavior can be considered reproducible when scaled-up to stirred-tank bioreactor. Accordingly, VLP titer and specific productivity was similar in both systems (Table 2).

Upon expression in a host cell, Gag-GFP assembles into VLPs which are released from the cells by budding. If the cells are lysed during the culture, monomeric Gag-GFP will be released to the medium, causing an overestimation of the VLP content measured by ELISA or by fluorescence readings [16]. The viability profile of the bioreactor culture did not indicate massive cell lysis that could lead to monomeric Gag-GFP release (Figure 7a). Nevertheless, the product was characterized to determine the nature of the Gag-GFP detected in the supernatant by ELISA. Nanoparticle Tracking Analysis (NTA) allows following fluorescent nanoparticles in suspension and determining their size distribution. Fluorescent particles were successfully identified by NTA in the supernatants from the bioreactor and shake flask cultures (144 h) (Figure 7b). The size distribution observed in these populations was the expected for Gag-GFP VLPs with the mode around 130-150 nm

[22]. However, the sample from the bioreactor culture showed higher particle diameters, with a significant peak at 275 nm indicating some VLP aggregation may have occurred, while this was not so pronounced in shake flasks. Representative VLP micrographs are shown in Figure 7c and d. Roughly spherical virus particles surrounded by a lipid envelope can be distinguished. In accordance with NTA, the particle diameter observed by TEM was around 150 nm. These results indicated that the p24 detected in the supernatant corresponded to correctly assembled VLPs rather than monomeric Gag-GFP.

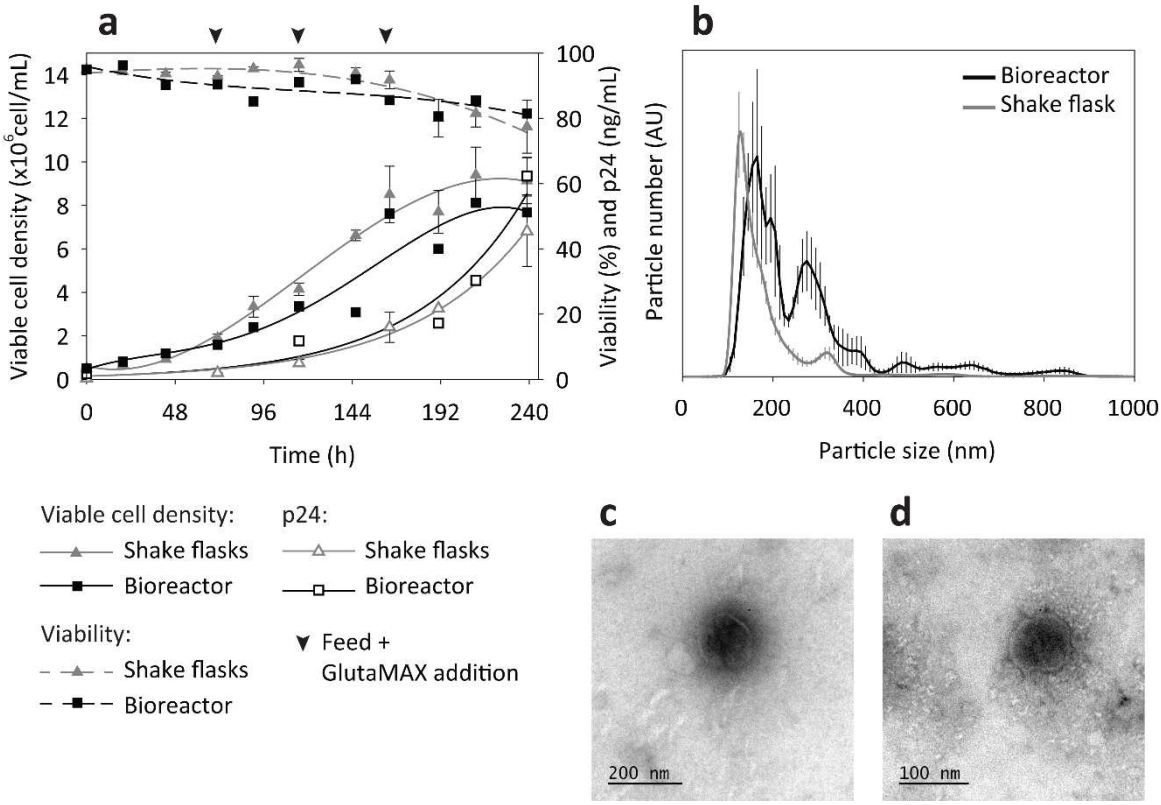

Figure 7. 8D5 evaluation in 1L stirred-tank bioreactor. (a) 8D5 clone was cultured in 1L stirred-tank bioreactor under the same feeding regime that shake flask cultures. Error bars represent the standard deviation of triplicate experiments. In the case of p24 from bioreactor, error bars indicate the standard deviation of triplicate quantifications of the same sample. (b) VLPs from culture supernatants were analyzed by fluorescence Nanoparticle Tracking Analysis (NTA) (c and d) Transmission electron microscopy micrographs obtained by negative staining of cell culture supernatants containing VLPs. (c) corresponds to bioreactor culture and (d) to shake flask controls both at 192 h. AU: arbitrary units.

3.5. Strategies to further increase productivity

By analyzing the 8D5 clone population by flow cytometry, a single population could be observed (instead of two populations as it was observed by the NucleoCounter at the

secondary screening phase). However, the population was rather heterogenous, indicating that some cells expressed Gag-GFP with higher intensities than others. In light of these results, 8D5 was subjected to one round of cell sorting in which the 50% more intense cells were selected with the aim of enriching the clone population with higher producer cells (Figure 8).

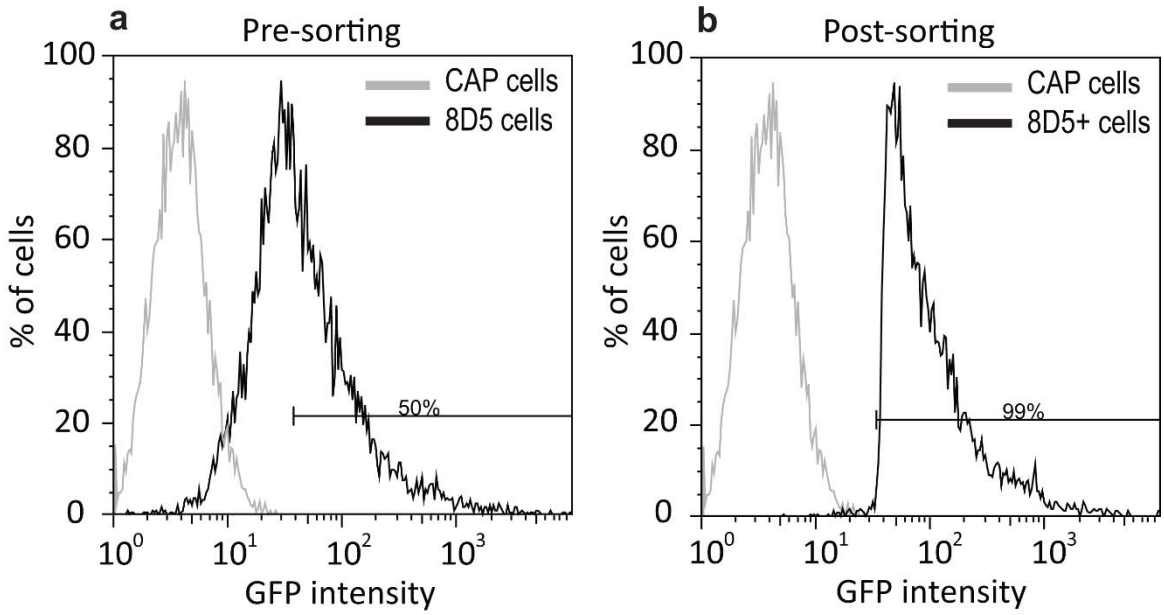

Figure 8. Sorting of the 8D5 clone population. (a) 8D5 viable single cells distribution by GFP intensity. CAP cells were used as a negative control. The 50% more intense cells from the 8D5 clone were selected. (b) Distribution of the sorted 8D5 cells (8D5+) by GFP intensity.

After amplification, the new sorted cells (8D5+) were cultured in fed-batch in shake flasks, in parallel with the unsorted 8D5 clone. Figure 9 shows the cell growth and VLP production of each population. 8D5 cells grew up to 7.9×10^6 cells/mL while the maximum cell density attained by 8D5+ was 6.5×10^6 cells/mL. 8D5+ cells produced 48.5 ± 1.6 ng of p24/mL which represents an increase of ~50% compared to the titers obtained in the same experiment with the 8D5 unsorted cells. Regarding specific productivity, a two-fold increase was observed after sorting compared to the parental clone (Table 2).

The fact that the population was enriched with high producer cells corroborated the phenotypic heterogeneity within 8D5 clone. If this heterogeneity was also genotypic remains unclear. Previous reports of cell line screening by cell sorting demonstrated that after several rounds, the sorted pools presented higher specific productivities and titers [23]. Therefore, more rounds of sorting with more stringent conditions would likely allow

to increase more significantly the specific productivity of the selected clone. Unfortunately, further sorting rounds could not be carried out due to time constraints.

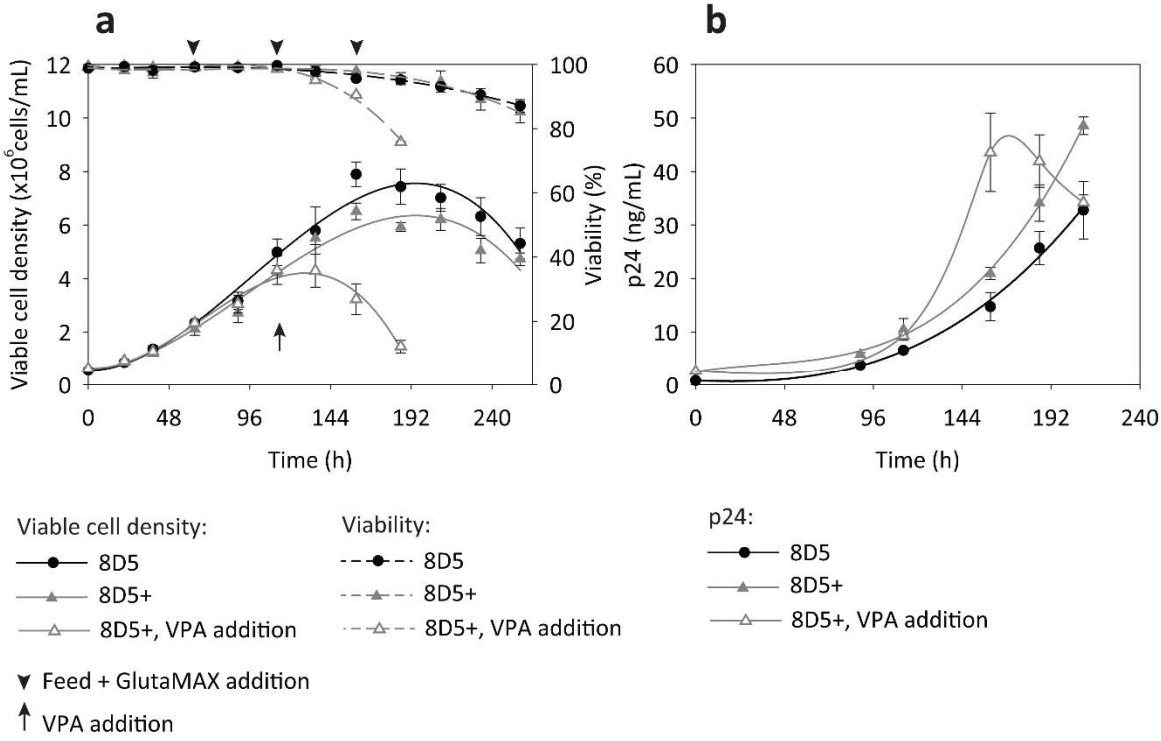

Figure 9. 8D5+ clone evaluation in shake flasks. (a) 8D5+ sorted cells were cultured in fed-batch mode. 8D5 clone was cultured in parallel as a control. Addition of 4mM of valproic acid (VPA) at 120 h of culture was tested for the 8D5+ clone. Error bars represent the standard deviation of triplicate experiments. (b) Determination of the VLP concentration in supernatants by p24 ELISA of 8D5, 8D5+ and 8D5+ with VPA addition. Error bars represent the standard deviation of triplicate experiments.

Additionally, the effect of the addition of valproic acid (VPA) was tested in this experiment. VPA is an inhibitor of histone deacetylases and its enhancing effect on protein production (stable and transient) has been extensively described [24–27]. VPA is also known to inhibit cell growth, for this reason it was added at 120 h of culture. At this time, the cells were growing exponentially, thus their physiological state for VLP production would be optimal. Moreover, the cell density at 120 h was already relatively high (~4×10^6 cells/mL), which was interesting since VLP production is a function of viable cell density, and therefore adding VPA at the beginning of the culture would have resulted in no cell growth and no VLP production. The concentration tested was 4 mM, since it is the standard used in the literature.

Interestingly, VLP titer was substantially increased 48 h after VPA addition, reaching a titer of 41.9 ± 4.9 ng of p24/mL (168 h). However, a detrimental effect of VPA on cells could also be observed. As expected, cell growth stopped upon VPA addition, and the viability dropped. The harvest time in this case should be 168 h, when the Gag-GFP concentration showed its maximum. Afterwards the viability further decreased and so did the VLP concentration.

Overall, the obtained clone expressing Gag-GFP VLPs (8D5+) showed a productivity of 2.17×10^{-3} pg/cell per day when cultured in CAP-CDM medium in fed-batch mode and with VPA addition at 120 h. This represents a 12-fold increase compared to the initial cell pool generated. Since Gag-GFP VLPs are a highly complex product, the productivity observed not only depends on Gag-GFP expression levels but also on the particle assembly and budding rates. For this reason, it is not comparable with the productivities achieved by industrial cell lines producing monoclonal antibodies (10-20 pg/cell·day) [13]. However, the productivity can be directly compared to the same VLP production by transient transfection of CAP-T cells [22]. In this case, a specific productivity of $9.94\times10^{-2} \pm 1.2\times10^{-2}$ pg/cell·day was obtained, which is 45-fold higher than the stable cell line.

If the comparison is done based on the VLP titer achieved (41.9 ng of p24/mL), the CAP stable cell line is around 14-fold below the transient production by CAP-T cells (~580 ng of p24/mL). Although this cell line did not reach the expected production levels for a stale CAP cell line, the titers were within similar levels as other reported Gag-based VLP production systems. For better comparison, the titer obtained by culture of the 8D5+ clone with VPA addition is converted to ~1400 µg of Gag/L, considering the underestimation factor of the p24 ELISA [16]. A HEK293 cell line producing the same Gag-GFP VLPs by transient transfection produced ~4200 µg of Gag/L [24]. Stable and baculovirus-mediated expression in Hi5 and Sf9 cell lines yielded around ~600 µg of Gag/L [9,28]. Finally, expression of Gag-VLPs in yeast yielded ~200 µg of Gag/L, being the least productive platform.

Other VLPs have been expressed in stable cell lines. For instance, expression of Japanese encephalitis virus-based VLPs expressed in BHK-21 cells have yielded titers of 15-20 µg/mL [29]. Expression of rabies virus VLPs in stable HEK293 cell lines was also reported [30]. However, it is difficult to compare the titers obtained for different VLPs. First because virus quantification is not always straightforward, and therefore not all reports include quantification of the product. Second, there are many different

technologies available for virus quantification, and the correct comparison of results from different methods is not always possible. Third, the productivities heavily depend on the nature of every VLP, the complexity and toxicity of the capsid protein, the assembly rates, the presence or absence of a budding release process, etc.

4. Conclusions

The successful generation of a stable CAP cell line producing correctly assembled Gag-GFP VLPs has been demonstrated in this work. The methodology employed was the classic limiting dilution, which has the advantages of being simple and straightforward and easily implementable at any cell culture lab. Moreover, it can be applied to a wide variety of proteins without the need of fluorescent reporters or cell staining.

By allowing the gene of interest to be randomly integrated in the genome, more than one copy can be integrated, which usually correlates with higher productivities. In this case, the productivity and titer obtained with the final selected clone are within the range of the titers reported in the literature for HIV VLPs. However, it was not competitive compared to the CAP cells potential capacity to express VLPs, as demonstrated by transient gene expression. Therefore, low production cannot be only attributed to the high complexity of the product. A higher-throughput screening should be performed in future attempts to obtain a clone with a more relevant performance.

On the other hand, one of the advantages of the stable cell line is that VLP production depends on the viable cell density attained. Therefore, if seeking for higher titers, intensification of the cell culture may help increasing the number of cells and consequently the VLP concentration. Perfusion mode cultures are a strategy that could be applied to this end [31]. This kind of strategies cannot be so easily applied in the case of transient transfection processes, where generally, relatively low cell densities are required for transfection (0.5-3×10^6 cells/mL) and the productivity not only depends on the cells but also on the availability of the DNA coding for the protein of interest [32]. In this case, intensification of the process must be linked to high DNA input which can become an economic limitation.

In any case, the established cell line will be the starting point for the development of new VLP-based vaccines expressing antigens in their surface from HIV or other viruses (influenza, malaria, etc.).

References

[1] Noad R, Roy P. Virus-like particles as immunogens. Trends Microbiol. 2003;11:438–444.

[2] Deml L, Speth C, Dierich MP, et al. Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. Mol. Immunol. 2005;42:259–277.

[3] Hammonds J, Chen X, Zhang X, et al. Advances in methods for the production, purification, and characterization of HIV-1 Gag-Env pseudovirion vaccines. Vaccine. 2007;25:8036–8048.

[4] Tagliamonte M, Visciano ML, Tornesello ML, et al. HIV-Gag VLPs presenting trimeric HIV-1 gp140 spikes constitutively expressed in stable double transfected insect cell line. Vaccine. 2011;29:4913–4922.

[5] Phogat S, Wyatt RT, Karlsson Hedestam GB, et al. Inhibition of HIV-1 entry by antibodies: potential viral and cellular targets. J. Intern. Med. 2007;262(1):26–43.

[6] Tang Y, Garson K, Li L, et al. Optimization of lentiviral vector production using polyethylenimine-mediated transfection. Oncol. Lett. 2015;9:55–62.

[7] Segura MM, Garnier A, Durocher Y, et al. Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification. Biotechnol. Bioeng. 2007;98:789–799.

[8] Zhang X, Wang X, Zhao D, et al. Design and immunogenicity assessment of HIV-1 virus-like particles as a candidate vaccine. Sci. China Life Sci. 2011;54:1042–1047.

[9] Tagliamonte M, Visciano ML, Tornesello ML, et al. Constitutive expression of HIV-VLPs in stably transfected insect cell line for efficient delivery system. Vaccine. 2010;28:6417–6424.

[10] Venereo-Sanchez A, Gilbert R, Simoneau M, et al. Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate. Vaccine. 2016;34:3371–3380.

[11] Schiedner G, Hertel S, Bialek C, et al. Efficient and reproducible generation of high-expressing, stable human cell lines without need for antibiotic selection. BMC Biotechnol. 2008;8:13.

[12] Obayashi H, Kawabe Y, Makitsubo H, et al. Accumulative gene integration into a pre-determined site using Cre/loxP. J. Biosci. Bioeng. 2012;113:381–388.

[13] Priola JJ, Calzadilla N, Baumann M, et al. High-throughput screening and selection of mammalian cells for enhanced protein production. Biotechnol. J. 2016;11:853–865.

[14] Matasci M, Hacker DL, Baldi L, et al. Protein therapeutics Recombinant therapeutic protein production in cultivated mammalian cells : current status and future prospects. Drug Discov. Today. 2008;5:37–42.

[15] Hermida-Matsumoto L, Resh MD. Localization of Human Immunodeficiency Virus Type 1 Gag and Env at the Plasma Membrane by Confocal Imaging Localization of Human Immunodeficiency Virus Type 1 Gag and Env at the Plasma Membrane by Confocal Imaging. J. Virol. 2000;74:8670–8679.

[16] Gutiérrez-Granados S, Cervera L, Gòdia F, et al. Development and validation of a quantitation assay for fluorescently tagged HIV-1 virus-like particles. J. Virol. Methods. 2013;193:85–95.

[17] Okumura T, Masuda K, Watanabe K, et al. Efficient enrichment of high-producing

recombinant Chinese hamster ovary cells for monoclonal antibody by flow cytometry. J. Biosci. Bioeng. 2015;120:340–346.

[18] Kim M, O'Callaghan PM, Droms KA, et al. A mechanistic understanding of production instability in CHO cell lines expressing recombinant monoclonal antibodies. Biotechnol. Bioeng. 2011;108:2434–2446.

[19] Osterlehner A, Simmeth S, Göpfert U. Promoter methylation and transgene copy numbers predict unstable protein production in recombinant chinese hamster ovary cell lines. Biotechnol. Bioeng. 2011;108:2670–2681.

[20] Hyde SC, Pringle IA, Abdullah S, et al. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. Nat. Biotechnol. 2008;26:549–551.

[21] Rouiller Y, Bielser JM, Brühlmann D, et al. Screening and assessment of performance and molecule quality attributes of industrial cell lines across different fed-batch systems. Biotechnol. Prog. 2016;32:160–170.

[22] Gutiérrez-Granados S, Cervera L, Segura MM, et al. Optimized production of HIV-1 virus-like particles by transient transfection in CAP-T cells. Appl. Microbiol. Biotechnol. 2015;100:3935–3947.

[23] Brezinsky SCG, Chiang GG, Szilvasi A, et al. A simple method for enriching populations of transfected CHO cells for cells of higher specific productivity. J. Immunol. Methods. 2003;277:141–155.

[24] Cervera L, Fuenmayor J, González-Domínguez I, et al. Selection and optimization of transfection enhancer additives for increased virus-like particle production in HEK293 suspension cell cultures. Appl. Microbiol. Biotechnol. 2015;99:9935–9949.

[25] Fischer S, Charara N, Gerber A, et al. Transient recombinant protein expression in a human amniocyte cell line: The CAP-T® cell system. Biotechnol. Bioeng. 2012;109:2250–2261.

[26] Backliwal G, Hildinger M, Kuettel I, et al. Valproic acid: A viable alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures. Biotechnol. Bioeng. 2008;101:182–189.

[27] Essers R, Kewes H, Schiedner G. Improving volumetric productivity of a stable human CAP cell line by bioprocess optimization. BMC Proc. 2011;5 Suppl 8:P66.

[28] Pillay S, Meyers A, Williamson AL, et al. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. Biotechnol. Prog. 2009;25:1153–1160.

[29] Hua R, Li Y, Chen Z, et al. Generation and characterization of a new mammalian cell line continuously expressing virus-like particles of Japanese encephalitis virus for a subunit vaccine candidate. 2014;14:1–9.

[30] Fontana D, Kratje R, Etcheverrigaray M, et al. Rabies virus-like particles expressed in HEK293 cells. Vaccine. 2014;32:2799–2804.

[31] Clincke M-F, Mölleryd C, Samani PK, et al. Very high density of Chinese hamster ovary cells in perfusion by alternating tangential flow or tangential flow filtration in WAVE Bioreactor™-part II: Applications for antibody production and cryopreservation. Biotechnol. Prog. 2013;29:768–777.

[32] Jäger V, Büssow K, Schirrmann T. Transient Recombinant Protein Expression in Mammalian Cells. Anim. Cell Cult. Springer International Publishing; 2015. p. 27–64.

On-line monitoring of CAP-T and CAP VLP production processes by dielectric spectroscopy

Results, chapter 5

1. Introduction

Viable cell density is a parameter of major importance in cell culture processes. It is typically monitored by sampling and off-line counting the cells once or twice a day. In addition, parameters that can be related to cell physiological state are of much value in cell-based processes.

In-line monitoring of the critical process variables has many advantages. First, sampling can be reduced, and consequently contamination risk. Second, sudden changes or unexpected events can be detected immediately, which may give the operator capacity to react on time. Third, the process can be deeply characterized which is a relevant part of product characterization, according to the PAT and QbD initiatives [1]. Fourth, process monitoring is more robust and reliable, since large amount of data are generated from in-line acquisition.

The most widely used technologies for in-line or on-line monitoring of cell density are the calculation of the oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) [2], dielectric spectroscopy [3], turbidity [4] and *in-situ* microscopy [5]. While the first three are related to physiological state of the cells, the last two are insensitive, thus less informative.

Dielectric spectroscopy or capacitance measures the permittivity of a biological solution. A good correlation between the biovolume and permittivity can be established. In-line monitoring in biological processes was first reported by Harris *et al*. in 1987 [6], and currently represent an established tool used in cell-based processes, as well as plants, yeast and bacterial cultures [7]. The cell concentration in the exponential growth phase is accurately monitored by the permittivity signal, while this correlation is typically weakened at the plateau and death phases. In addition, the correlation between permittivity data and cell density allows to use the signal for advanced bioreactor control, such as fed-batch and perfusion processes [8,9].

The resulting data from dielectric spectroscopy (β-dispersion parameters, addressed with detail below) can be also related to cell properties, mainly size and membrane characteristics. Therefore, its analysis gives additional information related to the state of the culture. Specific profiles of these parameters could be related to nutrient depletion and availability in CHO batch and fed-batch cultures [4,10]. Furthermore, these data have

also been used to identify some key events in cell culture processes, related to major changes in the membrane such as virus production and cell death [11–14].

The aim of this work was to use for the first time a capacitance sensor to monitor viable cell density in-line in CAP-T and CAP cultures. Specifically, HIV-based VLP production processes by transient gene expression of CAP-T and stable gene expression in CAP cells were studied. In addition, the β-dispersion parameters were analyzed throughout the cultures by comparing the VLP production processes with cultures without VLP production of both cell lines. The findings in these preliminary experiments with CAP and CAP-T cells open the possibility to use capacitance monitoring as a tool to implement advanced control strategies and improve the VLP production processes.

2. Theoretical background on dielectric spectroscopy

Dielectric spectroscopy or capacitance measures the permittivity, which is defined as the measure of a material's ability to store electric charge. When cells are in the presence of an electric field, ion migration at the poles of the cell (polarization) takes place. This happens because the cytoplasm is a conductive solution where ions can move, but ion movement is restricted by the cell membrane (non-conducting material). Consequently, the cells act as small capacitors. The permittivity is proportional to the magnitude of polarization of the sample, therefore, the higher the membrane enclosed volume with polarization capacity, the higher the permittivity signal will be. Consequently, when an alternating electric field is applied to a cell suspension, the biovolume, and therefore, the cell density, can be measured through the permittivity signal [7]. Dead cells with the membrane compromised and cell debris do not contribute to the capacitance signal [15]. Importantly, the electric field does not affect the cells.

The cell polarization rate depends on the frequency of the alternating electric field. In this way, between the radio frequency interval (0.1-10 MHz), cells do not have enough time to polarize at high frequencies and permittivity values are low, while at low frequencies, polarization is complete and permittivity signals are higher. This loss of cellular polarization is referred as β-dispersion curve. This curve can be defined by three parameters: (1) the characteristic frequency at which polarization if half complete (f_c), (2) the angle of the curve (α), and (3) the maximum difference between the permittivity signal at high and low frequencies ($\Delta\varepsilon$) (Figure 1) [7,15].

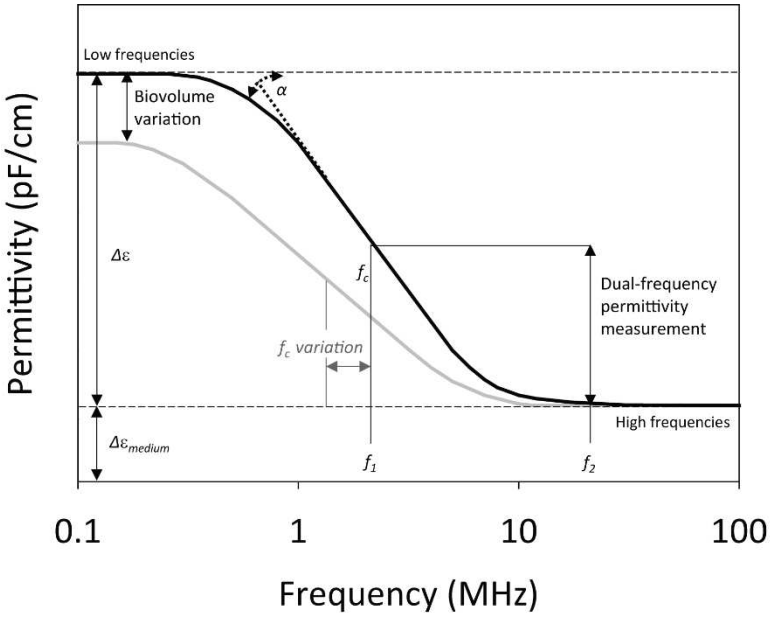

Figure 1. Schematic representation of the β-dispersion curve of the permittivity in the radio frequency range. Variations of the β-dispersion parameters (f_c, $\Delta\varepsilon$, and α) due to changes in the cell properties or the cell density are represented as well. Adapted from [16].

Dual-frequency measurement is the most common approach to measure the biovolume. It consists in subtracting the permittivity values at high frequencies (f_2, background permittivity) to the values at close to the characteristic frequency (f_1) (Figure 1) [15].

According to the work of Schwan [17], the permittivity also depends on the cell size and the state of the cell membrane, reflected in the membrane capacitance. The permittivity increment from high to low frequencies ($\Delta\varepsilon$) for spherical particles and low fraction volumes of cells ($P < 0.2$) can be described by Eq. 1 [17]:

$$\Delta\varepsilon = \frac{9 \times r \times P \times C_M}{4} \qquad \text{(Eq. 1)}$$

$$P = \frac{4 \times \pi \times r^3 \times N}{3} \qquad \text{(Eq. 2)}$$

$$\Delta\varepsilon = 3 \times \pi \times r^4 \times C_M \times N \qquad \text{(Eq. 3)}$$

where $\Delta\varepsilon$ is the permittivity (F/m), r is the cell radius (m), P is the volume fraction of cells in the medium (%), C_M is the membrane capacitance per area unit (F/m^2), and N is the cell density (cell/m^3).

The characteristic frequency (f_c) can be described by the following equation [17]:

$$f_c = \frac{1}{2\times\pi\times r\times C_M\times\left(\frac{1}{\sigma_i}+\frac{1}{2\sigma_m}\right)} \quad \text{(Eq. 4)}$$

where f_c is the characteristic frequency (Hz), σ_i is the intracellular conductivity (mS/cm), and σ_m is the medium conductivity (mS/cm). Since σ_m is significantly higher than σ_i it is usually neglected, thus Eq. 4 can be further simplified:

$$f_c = \frac{\sigma_i}{2\times\pi\times r\times C_M} \quad \text{(Eq. 5)}$$

Finally, α is an empirical parameter that is believed to increase when the properties of the cell suspension are more heterogeneous[18].

3. Materials and methods

3.1. VLP production by transient transfection of CAP-T cells

The experimental details regarding VLP production in CAP-T cells by transient transfection in 1L bioreactor are described in *Results, chapter 3*, section *2.* Briefly, a plasmid DNA coding for a Gag polyprotein fused to a GFP was used for transient transfection. The transfection protocol was as follows: CAP-T cells were cultured in FreeStyleF17 + 1% of PEM medium in the bioreactor for two days before transfection. Prior to transfection, the volume was reduced to 500mL. DNA and PEI solutions were sequentially added and 4 hours post transfection (hpt) the bioreactor volume (1L) was restored with PEM medium for the production phase. During these 4 hours, dielectric spectroscopy data was not acquired. VLP quantitation was performed by an in-house developed spectrofluorometry method.

3.2. VLP production by a CAP stable cell line

The experimental details regarding VLP production in CAP stable cell line in 1L bioreactor are described in *Results, chapter* 4. Specifically, the generation of the CAP cell

line stably expressing Gag-GFP VLPs is described throughout section *3*. The selected clone was cultured in fed-batch mode in CAP-CDM medium. Briefly, cells were seeded at 0.5×10^6 cells/mL and CAP-CDM feed and GlutaMAX were added at 72, 120, 168 and 240h. VLP quantitation was performed by ELISA.

3.3. Off-line determination of viable cell density, viability, cell size and glucose concentration.

Cell density, viability and cell size were determined by sampling the bioreactor every 24h and counting the cells using the NucleoCounter NC-3000 (Chemometec, Allerod, Denmark) according to the manufacturer's instructions.

Glucose concentration in cell culture supernatants was analyzed by and YSI 7100 MBS (YSI, Yellow Springs, OH, USA), according to the manufacturer's instructions.

3.4. Dielectric spectroscopy equipment

Permittivity measurements were performed using an Incyte sensor unit (Hamilton, Bonaduz, Switzerland) according to the manufacturer's instructions. Permittivity was calculated by dual-frequency capacitance measurements. The system also performed a Frequency Scan, measuring the capacitance at 17 different frequencies between 0.3 and 10 MHz, which provided information on the β-dispersion parameters.

4. Results and discussion

4.1. On-line monitoring of viable cell density in CAP-T transient transfection process

HIV VLPs were produced by transient transfection of CAP-T cells, using PEI as transfection reagent. Briefly, cells were seeded in the bioreactor 48h before transfection at 0.5×10^6 cells/mL and grew up to 2×10^6 cells/mL (Figure 2a). 4h after transfection, the

culture was diluted with 1 volume of cell culture medium for the VLP production phase. Doubling time after transfection was typically 39-40 h, compared to the 21-27 h duplication time of non-producing cells. This was expected since it is generally accepted that cells producing a recombinant protein present slower growth rates. Cell culture viability was over 90% during all the process. The first 24hpt, VLPs were hardly released from the cells, from that time on, VLPs were constantly released to the medium until 96h, when production achieved a plateau. VLP titer was ~6×10^{10} VLPs/mL (Figure 2b). For comparison purposes, CAP-T cells were cultured in batch (no transfection was performed). Briefly, cells were seeded in the bioreactor at 0.5×10^{6} cells/mL and grew up to 5.5×10^{6} cells/mL in 5 days (Figure 3a). Duplication time was 21h, and cell culture viability was over 90% during all the process. A capacitance sensor was used to monitor on-line the viable cell density during both cultures.

The permittivity signal matched the off-line viable cell density measurements both after and before transfection. This can be observed in Figure 2a corresponding to the transfection process, and in Figure 3a corresponding to CAP-T cell growth in batch. Doubling times calculated with on-line data were 35 h for transfected cells producing VLPs and 21 h for non-transfected cells (in agreement with the off-line calculated values). Figure 4 shows the linear correlations of the in-line and off-line cell density data for the previous experiments in the exponential growth phase. Both correlations were proper with R^2 values higher than 0.97. The slopes for the cultures expressing and not expressing VLPs were similar, indicating that VLP production was not influencing viable cell density monitoring.

In both cases, the correlation between the on-line and off-line data was not so strong after 72 h of culture (Figure 4, triangles), coinciding with the time of glucose depletion, and the end of the exponential growth phase (Figure 2b and 3b). The weakening of the linear relationship in the stationary phase was observed before [19]. In other applications, the permittivity signal followed the decrease of the viable cell density almost as accurately as the exponential growth phase [4]. The determination of the number of dead cells is still a subjective matter, since it depends heavily on the analysis technique and on the death mechanism [14]. The hypothesis in this case was that viable cells and viability could be overestimated by the off-line counts using the NucleoCounter NC-3000, but more data is needed to confirm it.

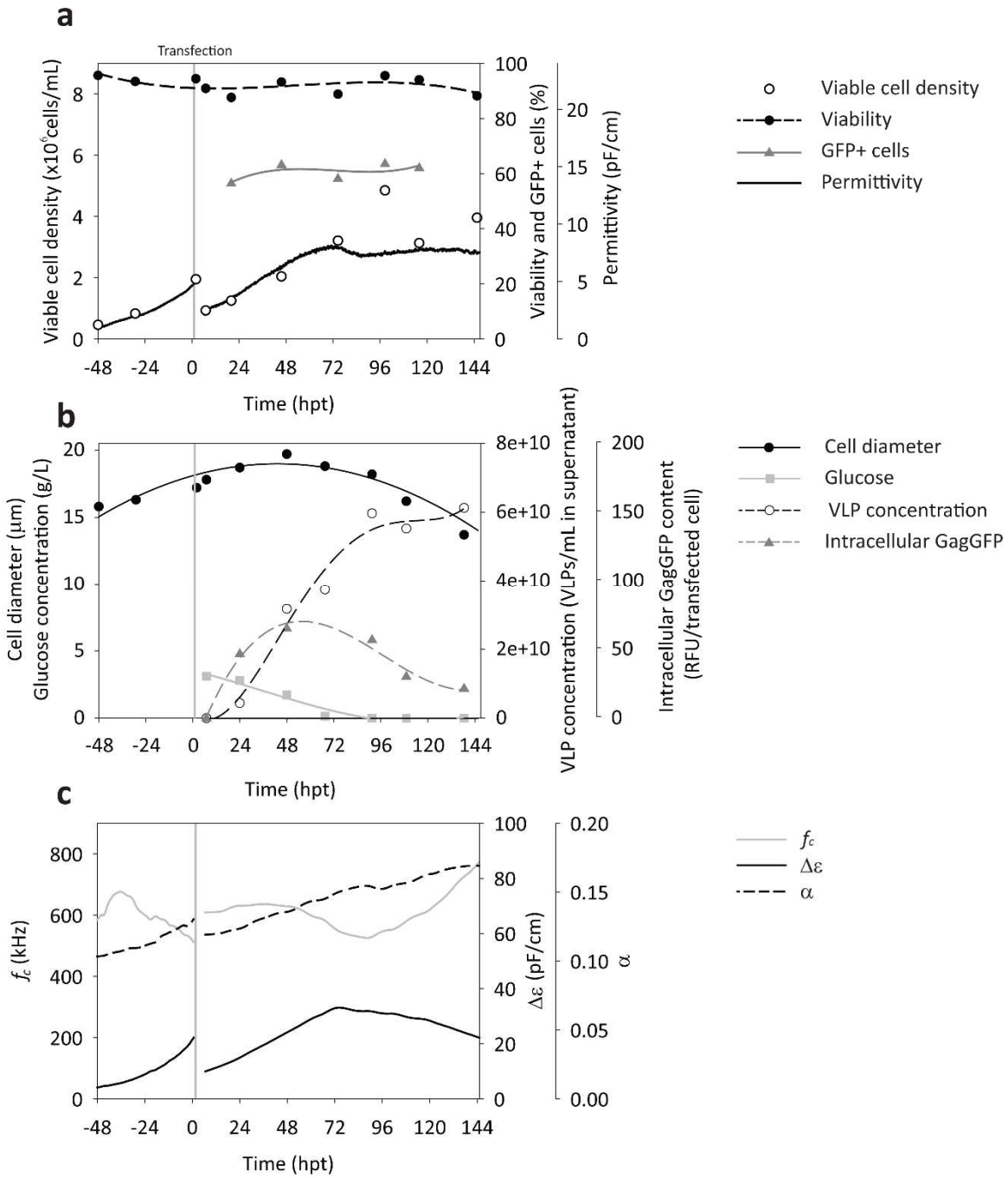

Figure 2. Dielectric spectroscopy measurements in the CAP-T transient transfection process. (a) The off-line viable cell counts and viability are represented before and after transfection in the bioreactor. The percentage of GFP expressing cells upon transfection was analyzed as well. (b) Cell size, glucose concentration in supernatant, VLP concentration in supernatant and intracellular Gag-GFP are represented. RFU: Relative fluorescence units. (c) In-line monitoring of β-dispersion parameters. Vertical grey lines indicate time of transfection.

A proper correlation between viable cell density and permittivity data was observed for the first time for CAP-T cells. This correlation would allow not only to monitor the

process, but also to establish advanced control strategies. For instance, a perfusion process was established for CHO cells in which the viable cell density was maintained constant by adjusting the purge according to the permittivity signal [9].

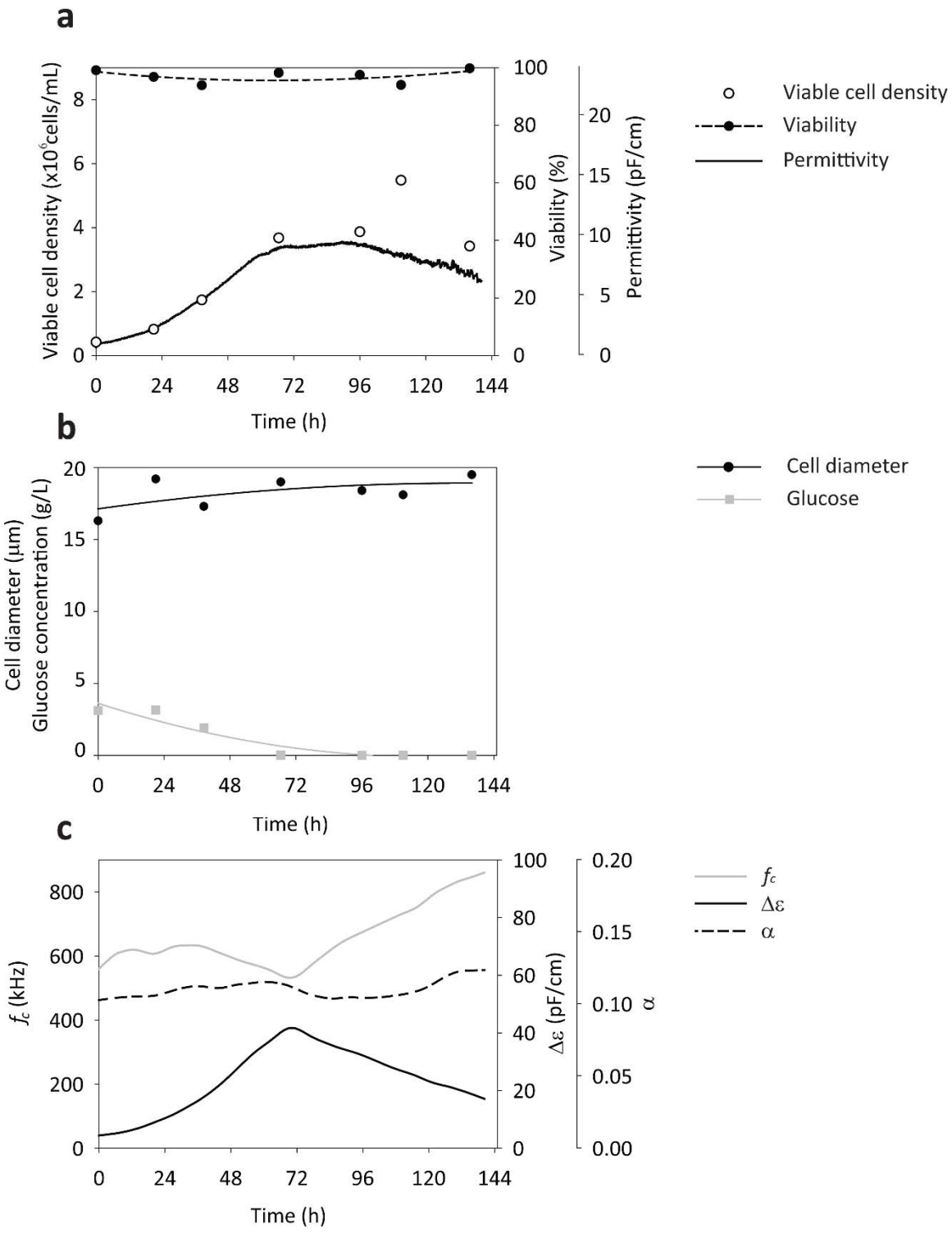

Figure 3. Dielectric spectroscopy measurements in the CAP-T batch culture. (a) The off-line viable cell counts and viability are represented before and after transfection in the bioreactor. (b) Cell size and glucose concentration in supernatant. (c) In-line monitoring of β-dispersion parameters.

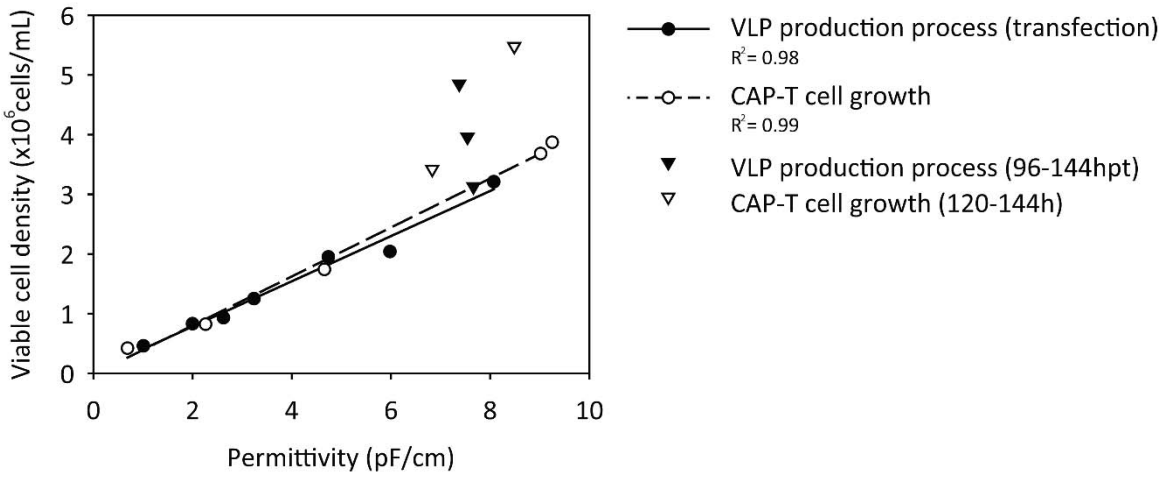

Figure 4. Correlation between the off-line viable cell density and permittivity measurements for CAP-T cells with and without transfection. The triangles represent the data of the final phases of the cultures, when the correlation was weakened.

4.2. Analysis of β-dispersion parameters in the transient transfection process

The β-dispersion parameters (f_c, $\Delta\varepsilon$, and α) were analyzed in the transient transfection process and compared to the profiles observed for CAP-T cell growth. According to Eq. 1 and 5, $\Delta\varepsilon$ depends on *P, r*, and C_M, and f_c is influenced by *r*, σ_i and C_M. Of these variables, only cell concentration and size could be experimentally determined, therefore, some assumptions were made to interpret the results, based on previously published data.

The characteristic frequency (f_c) showed a specific profile. In the first 48 hpt, f_c was steady or slightly increasing (~10%). The cell diameter slightly increased (~10%) in this phase, probably due to intracellular accumulation of Gag-GFP protein (Figure 2b). However, the increase of f_c cannot be explained by the increase of cell size, in fact it should be the opposite. This lead to the hypothesis that σ_i or C_M should be changing as well during this phase. After that, f_c declined, and again it could not be explained by the decrease observed in cell size, but instead by changes in the cell properties leading to changes in σ_i or C_M. This specific behavior of f_c was observed before for CHO batch and fed-batch cultures. The increase of f_c profiles was related by the authors with a situation of nutrient availability and good physiological state of the cells, while the following decrease was associated to nutrient limitations (glucose and glutamine depletions) [4,10]. In the CAP-T cultures, glucose was depleted 72h after transfection and after seeding (Figure 2b and 3b), therefore the decrease in f_c could be associated to nutrient limitations as well.

While off-line measurements indicated that cells were still growing after glucose depletion, $\Delta\varepsilon$ signal showed an extreme and started to decrease from this point (29%, Figure 2c). Since cell size decreased as well by a 27%, it is difficult in this case to depict the causes of this decline. However, a more pronounced decline was observed for $\Delta\varepsilon$ signal in the batch culture (Figure 3c) with no significant changes in the cell size. This suggests that changes in C_M were probably taking place in this phase. At the time of glucose depletion, the cells started the consumption of lactate. Although the lactate concentration was not available for these experiments, this phenomenon has been observed before systematically for CAP-T (non-published data) and HEK293 cells in bioreactor cultures with constant pH control [20]. Moreover, the pH in the bioreactor increased at that time, indicating lactate consumption (data not shown). Remarkably, the shift in cell metabolic behavior could be anticipated by the $\Delta\varepsilon$ signal.

This metabolic change could also be observed in the permittivity signal. Upon glucose depletion, the permittivity decreased slightly for 24h, and then it was recovered (Figure 2a). This phenomenon was observed as well in the CAP-T batch culture, although it was less pronounced (Figure 3a).

After glucose depletion, there was a significant increase of f_c until the end of the culture (72-144h), that could be observed both in the transfection process and the batch growth. Regarding the transfection process, a 26% increase in f_c was observed, which could be directly attributed to the cell diameter decline. However, in the case of the CAP-T batch culture, an increase of 37% in f_c was observed while the cell size was apparently not changing significantly (Figure 3b). This significant f_c increase at the end, was associated before with cell death and apoptosis [16]. In this case, cell viability was still high (>85%) at the time of f_c increase in both processes. As mentioned before, it is possible that off-line viability measurements were overestimated. However, deeper studies of cell death will be necessary to clarify the causes of the sharp increase of f_c. Independently of the cause of this increase, it is undeniable that this parameter is indicating a relevant event in the culture, related to nutrient depletion.

The fact that the on-line data anticipates in a systematic way the metabolic changes (that cannot be observed through off-line viable cell density and viability determinations) has important implications for bioprocess development. First, this kind of shifts can be determining to decide product harvest time. A decrease in cell performance and viability may lead to the release of intracellular content, which can have a detrimental impact in

product quality. Being able to anticipate this moment of the culture may alleviate this phenomenon. Second, on-line monitoring of this event, gives the capacity to react immediately, for instance, by adding a feed that restores the nutrient limitation. This last application could also be benefited from the f_c profile, since nutrient limitations are observed by a decrease of these parameter. This was already proven for CHO fed-batch cultures, where a close loop based on on-line permittivity measurements, allowed to control the feedings to maintain asparagine concentration at a constant level [9]. Another successful fed-batch example was reported for hybridoma cells. The authors found that permittivity correlated to glutamine consumption rate, so the permittivity signal was used to automatically add feeds and maintain constant the glutamine concentration [3].

Regarding α, it is an indicator of the heterogenicity of the cell culture. In this case, the signal increased steadily both before and after transfection (approximately by a 30%, Figure 2c). Although in the batch culture it was not increasing so significantly (Figure 3c), there was not enough evidence of this α increase being related to transient transfection or VLP production, since the same increase rate was observed first 48h before transfection. Moreover, other independent experiments of CAP-T batch culture also showed a ~30% steady increase of α.

One of the initial goals of this work was to relate the β-dispersion parameters to VLP production events. This hypothesis was based on several literature reports that were able to identify different events or phases in virus infection processes (influenza, baculovirus, herpes) and lentiviral vectors produced by transient transfection [11–13,21]. Since HIV VLP formation takes place in the cell membrane, this lead to the thought that the membrane characteristics would change, and these changes would be reflected in dielectric cell properties. However, the patterns of the different parameters analyzed were very similar to those observed for CAP-T batch growth without transfection, so there was not enough evidence to confirm the initial hypothesis. This could be due to the fact that in the transient transfection process analyzed, only 50-60% of the cells were expressing VLPs (Figure 2a), and the signal from the producer cells was mixed with the non-producers. In contrast, Petiot *et al.* [13] analyzed a population with 100% of influenza infected cells, finding significant correlations with virus production events. The other possibility is that VLP formation and budding from the cells was not altering the membrane in such significant way that is reflected in the signal. If this was the case, a more sensitive signal would be required to detect VLP budding, or more cells producing higher levels of VLPs would be needed to have higher signals. Future work will follow to

try to relate on-line signals to VLP production, since the impact on process automatization and QbD strategies would be significant.

4.3. On-line monitoring of viable cell density in CAP stable expression process

CAP cells were cultured in bioreactor in CAP-CDM medium in fed-batch mode, adding CAP-CDM Feed at 72, 120, 168 and 240 h of culture. Cells were seeded at 0.5×10^6 cells/mL and grew up to 12×10^6 cells/mL with a doubling time of 30h (Figure 5a). Cell viability was maintained high over the culture, although it started to decrease at 168 h. The CAP clone stably producing Gag-GFP VLPs (named as 8D5 clone) was cultured under the same conditions (Figure 6a). These cells grew up to 7×10^6 cells/mL with a doubling time of 33.3 h. The VLP titer obtained was 1×10^9 VLPs/mL, considering a harvest time of 144 h (end of the exponential growth phase).

The on-line permittivity signal matched the off-line viable cell density measurements in both cultures with and without VLP production. This can be observed in Figures 5a and 6a corresponding to the CAP and 8D5 clone cultures, respectively. Dilutions of the cell density caused by feed additions could be observed in real-time in the permittivity signal. Doubling times calculated with on-line data were 32 h for CAP cells and 28 h for 8D5 cells, close to the off-line calculated values. Figure 7 shows the linear correlations of the on-line and off-line cell density data for the previous experiments in the exponential growth phase. Both correlations were proper with R^2 values higher than 0.98. The slopes of the cultures expressing and not expressing VLPs slightly differed in this case (0.75 for CAP cells and 0.47 for 8D5 cells).

Again, the correlation between the on-line and off-line data was not so strong after the exponential growth phase (Figure 7, triangles). When glucose was depleted (216 h for CAP cells, Figure 5b, and 144 h for 8D5 cells, Figure 6b), the permittivity signal decreased significantly, while the viable cell density was constant. This was another indication that the viability and the cell density could have been overestimated in this phase of the culture by the off-line method used for cell counting.

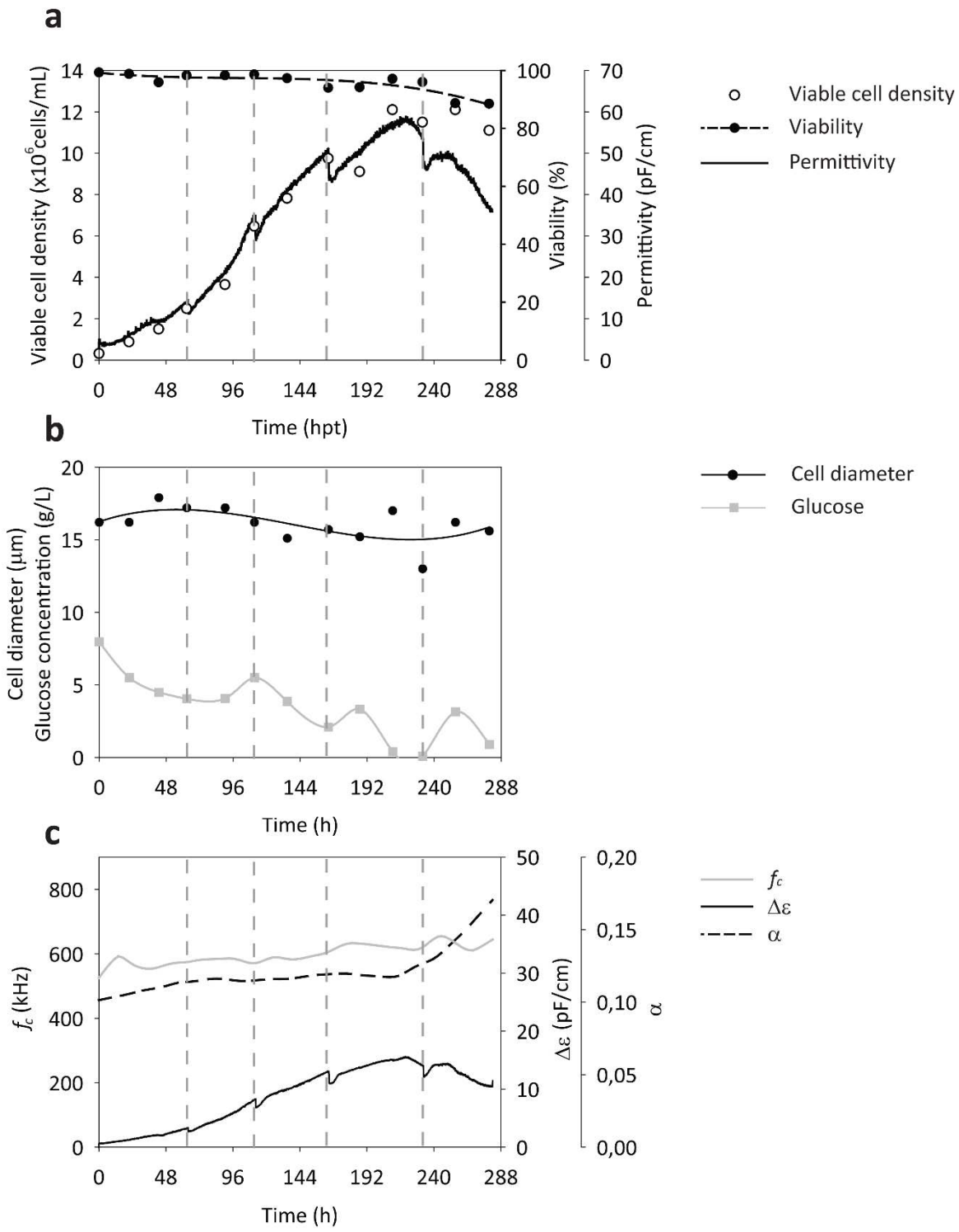

Figure 5. Dielectric spectroscopy measurements in the CAP fed-batch culture. (a) The off-line viable cell counts and viability are represented. (b) Cell size and glucose concentration in supernatant. (c) On-line monitoring of β-dispersion parameters. Dashed vertical lines indicate the times of feed addition.

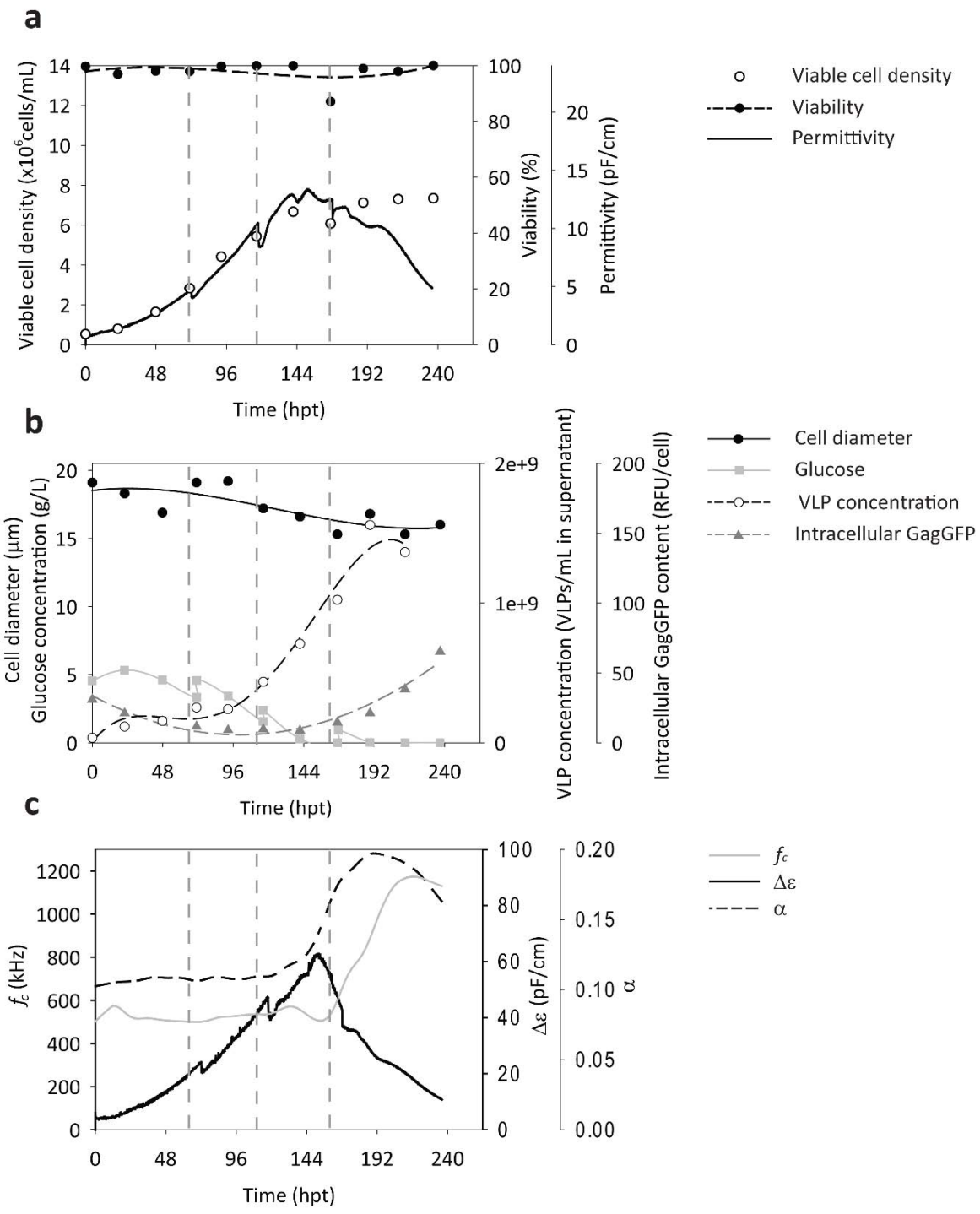

Figure 6. Dielectric spectroscopy measurements in the CAP stable cell line (8D5) process. (a) The off-line viable cell counts and viability are represented. (b) Cell size, glucose concentration in supernatant, VLP concentration in supernatant and intracellular Gag-GFP are represented. RFU: Relative fluorescence units. (c) On-line monitoring of β-dispersion parameters. Dashed vertical lines indicate the times of feed addition.

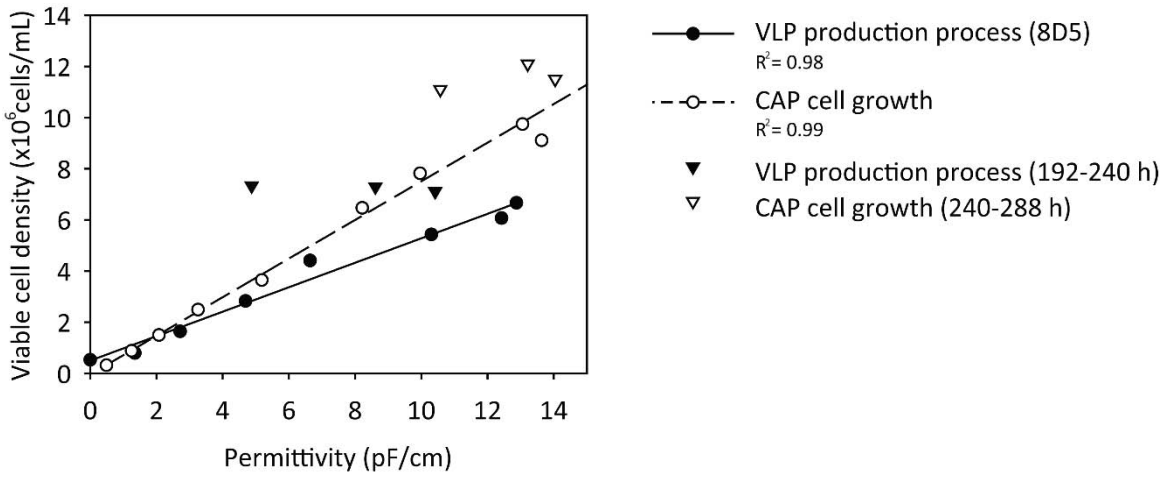

Figure 7. Correlation between the off-line viable cell density and permittivity measurements for CAP and 8D5 cells. The triangles represent the data of the final phases of the cultures, when the correlation was weakened.

4.4. Analysis of β-dispersion parameters in the stable expression process

The β-dispersion parameters were analyzed for both producing and non-producing VLP cultures. The profiles of f_c, $\Delta\varepsilon$ and α were similar for the CAP and 8D5 cultures, therefore, the profiles observed in the 8D5 culture could not be related to VLP production, similarly to the transient transfection results. In this case, even lower VLP titers were produced, and it was hypothesized that they were not affecting cell membranes so significantly so as to observe changes in the dielectric properties of the cells.

The f_c profile was similar to that observed before in CHO fed-batch cultures, where increases in f_c were observed upon feed additions [10]. Specifically, the first addition at 72 h did not result in a very significant change (Figures 5c and 6c), since at this point, nutrients were probably not limiting. Similarly, Ansorge *et al.* observed that the first feed additions did not result in a very significant change in f_c, since the main nutrients were not depleted at that point [10]. However, the following additions did result in significant f_c increases (Figures 5c and 6c). Cell diameter did not change significantly in the CAP culture (Figure 5b), thus the changes in f_c could not be attributed to changes in cell size. In the case of 8D5, the cell size was decreasing progressively during the culture (Figure 6b), thus an influence of cell size in f_c could not be discarded. The changes observed in the *fc* profiles due to nutrient depletion and availability could be exploited in the future to develop automated fed-batch cultures in which feed additions would be subjected, for instance, to *fc* decreases.

In the final phase of the 8D5 culture f_c increased sharply (from 168 h). The inflexion point coincided with an inflexion point in the $\Delta\varepsilon$ profile, which started to decrease, and with the time of glucose depletion. For these reasons, it is likely that this was an indication of the onset of the death phase of the culture, even though the viability was still high and the off-line cell density was constant. Another indication of the start of cell death is the change in VLP concentration slope observed after 120 h of culture (Figure 6b). It could be possible that monomeric Gag-GFP release could be taking place after this inflexion point. Furthermore, the intracellular Gag-GFP content was apparently increasing after that point (Figure 6b), which could be an artifact, since more fluorescence was observed in the supernatants, but the number of cells to divide it was constant. Therefore, this artificial increase, observed after 144 h, could be indicating a release of Gag-GFP monomers. Future studies will follow to observe a drop of the viability with off-line measurements.

In the case of CAP cells, an increase in f_c was observed at 264 h of culture, coinciding as well with the inflexion in $\Delta\varepsilon$ and glucose depletion. In this case, it was not so evident as in the 8D5 culture, however if the culture would have been extended, the same effect could have probably been observed also for CAP. In this case, the presence of VLPs may be influencing the differences observed in the time of cell culture death.

α had a different behavior in CAP cells compared to CAP-T. It was maintained constant until the time when the inflexion was observed in $\Delta\varepsilon$ and f_c, which at the same time coincided with glucose depletion. At that time, a sharp increase of α was observed in both producing and non-producing cultures (Figure 5c and 6c). This indicated an increase of the heterogenicity of the culture, which also supports the hypothesis that it was at that time when cell death started.

If these hypotheses are confirmed, and cell death is taking place at that inflexion time points, the dielectric spectroscopy data will proof a very powerful tool to observe the end of the culture in real-time and decide the most suited harvest time for VLPs, before release of intracellular content takes place.

5. Conclusions

Dielectric spectroscopy was used to monitor on-line the transient and stable Gag-GFP VLP production processes by CAP-T and CAP cells, respectively. The permittivity signal correlated correctly with viable cell density during the exponential growth phase. This correlation was weakened after exponential growth, which evidenced the need to recheck the off-line viability and cell density measurement technique. By comparing the β-dispersion data with negative control cultures not producing VLPs, it was concluded that VLP production could not be assessed by dielectric spectroscopy. However, other relevant events in the culture processes could be identified. The onset of the end of the culture could be identified by an increase of f_c, coinciding with a decrease of $\Delta\varepsilon$. This is very relevant to decide the time of VLP harvest, in a manner that the release of intracellular content to the supernatant is avoided. Nutrient availability and depletion could also be monitored by the f_c profile. Nutrient depletion was observed by f_c decreases and feed addition resulted in f_c increases. This feature has the potential to be used to develop automated fed-batch processes based on these data. Perfusion strategies could also be established using the permittivity data correlating with viable cell density. Altogether, these preliminary results demonstrate the potential of dielectric spectroscopy to monitor vaccine production bioprocesses and also open the possibility to use this technology to develop advanced control strategies.

References

[1] Pais DAM, Carrondo MJT, Alves PM, et al. Towards real-time monitoring of therapeutic protein quality in mammalian cell processes. Curr. Opin. Biotechnol. 2014;30:161–167.

[2] Kussow CM, Zhou W, Gryte DM, et al. Monitoring of mammalian cell growth and virus production process using on-line oxygen uptake rate measurement. Enzyme Microb. Technol. 1995;17:779–783.

[3] Noll T, Biselli M. Dielectric spectroscopy in the cultivation of suspended and immobilized hybridoma cells. J. Biotechnol. 1998;63:187–198.

[4] Ansorge S, Esteban G, Schmid G. Multifrequency permittivity measurements enable on-line monitoring of changes in intracellular conductivity due to nutrient limitations during batch cultivations of CHO cells. Biotechnol. Prog. 2010;26:272–283.

[5] Guez JS, Cassar JP, Wartelle F, et al. Real time in situ microscopy for animal cell-concentration monitoring during high density culture in bioreactor. J. Biotechnol. 2004;111:335–343.

[6] Harris CM, Todd RW, Bungard SJ, et al. Dielectric permittivity of microbial suspensions at radio frequencies: a novel method for the real-time estimation of microbial biomass. Enzyme Microb. Technol. 1987;9:181–186.

[7] Justice C, Brix A, Freimark D, et al. Process control in cell culture technology using dielectric spectroscopy. Biotechnol. Adv. 2011;29:391–401.

[8] Dowd JE, Jubb A, Kwok KE, et al. Optimization and control of perfusion cultures using a viable cell probe and cell specific perfusion rates. Cytotechnology. 2003;42:35–45.

[9] Heinrich C, Beckmann T, Büntemeyer H, et al. Utilization of multifrequency permittivity measurements in addition to biomass monitoring. BMC Proc. 2011;5:P30.

[10] Ansorge S, Esteban G, Schmid G. On-line monitoring of responses to nutrient feed additions by multi-frequency permittivity measurements in fed-batch cultivations of CHO cells. Cytotechnology. 2010;62:121–132.

[11] Zeiser A, Bédard C, Voyer R, et al. On-line monitoring of the progress of infection in Sf-9 insect cell cultures using relative permittivity measurements. Biotechnol. Bioeng. 1999;63:122–126.

[12] Ansorge S, Lanthier S, Transfiguracion J, et al. Monitoring lentiviral vector production kinetics using online permittivity measurements. Biochem. Eng. J. 2011;54:16–25.

[13] Emma P, Kamen A. Real-time monitoring of influenza virus production kinetics in HEK293 cell cultures. Biotechnol. Prog. 2013;29:275–284.

[14] Patel P, Markx GH. Dielectric measurement of cell death. Enzyme Microb. Technol. 2008;43:463–470.

[15] Davey CL, Davey HM, Kell DB, et al. Introduction to the dielectric estimation of cellular biomass in real time, with special emphasis on measurements at high volume fractions. Anal. Chim. Acta. 1993;279:155–161.

[16] Petiot E, El-Wajgali A, Esteban G, et al. Real-time monitoring of adherent Vero cell density and apoptosis in bioreactor processes. Cytotechnology. 2012;64:429–441.

[17] Schwan HP. Electrical properties of tissue and cell suspensions. Adv. Biol. Med. Phys. 1957;5:147–209.

[18] Cole KS, Cole RH. Dispersion and absorption in dielectrics: I. Alternating current characteristics. J. Chem. Phys. 1941;9:341–351.

[19] Opel CF, Li J, Amanullah A. Quantitative modeling of viable cell density, cell size, intracellular conductivity, and membrane capacitance in batch and fed-batch CHO processes using dielectric spectroscopy. Biotechnol. Prog. 2010;26:1187–1199.

[20] Liste-Calleja L, Lecina M, Lopez-Repullo J, et al. Lactate and glucose concomitant consumption as a self-regulated pH detoxification mechanism in HEK293 cell cultures. Appl. Microbiol. Biotechnol. 2015;99:9951–9960.

[21] Archer S, Morgan H, Rixon FJ. Electrorotation studies of baby hamster kidney fibroblasts infected with herpes simplex virus type 1. Biophys. J. 1999;76:2833–2842.

Discussion and future directions

A production platform for HIV VLP-based vaccines was successfully developed in this work using the human-derived CAP cell line. Previous reports indicated the suitability of this cell line for the production of recombinant proteins [1,2] and viruses [3–5]. However, this was the first proof of concept of the production of VLPs in this cell line. Importantly, Gag-GFP protein was correctly assembled in the cell environment and budded to the cell culture supernatant as VLPs upon expression in CAP-T and CAP cells. Despite CAP cells already displayed acceptable productivities, systematic process development and optimization performed in this work lead to further VLP titer improvements.

The integration of upstream (USP) and downstream processing (DSP) is of major importance in bioprocess development. The decisions taken in USP will have an impact on DSP at performance and economic level. In an industrial context, USP and DSP would need to be addressed in a more integrated manner since the beginning of process design, to avoid having to go back to change decisions already taken in USP that may have a negative impact in DSP. In the same way, detailed VLP characterization is critical to ensure the desired product quality, especially for pre-clinic and clinic applications. In this thesis, the focus was put on USP, thus DSP and characterization could not be addressed with detail. They were only used as tools to corroborate the presence of correctly assembled VLPs. However, their importance is recognized by the research group, and DSP and VLP characterization are being specifically addressed in other theses.

Before starting the development of the bioprocess, a quantitation technique was set to routinely quantify VLP samples in a reliable and cost-effective manner. The GFP tag was used to develop a fluorescence-based method, that allowed to specifically detect and quantify Gag-GFP molecules within crude and purified samples. Validation of analytical tools is essential to obtain reliable and, more importantly, comparable results between different experiments. Moreover, the validation of all analytical methods used within a bioprocess is a requirement from regulatory agencies.

The main limitation of the developed spectrofluorometry-based method is that it is not specific for assembled VLPs. The presence of monomeric Gag-GFP that may be released from dead cells would cause the overestimation of VLP titers, especially in crude samples. Before starting, when VLPs were produced in HEK293 (chapter 1), it was determined that the majority of the Gag-GFP was assembled into VLPs (70-80%), so the quantification method was developed neglecting this low percentage of non-assembled Gag-GFP. The percentage of assembled Gag-GFP was also determined for CAP-T

production by transient transfection with Vivaspin Filter separation. Although it was not shown in the results, 80-90% of the Gag-GFP was assembled at 168 hpt. In earlier time points (72 and 120 hpt) monomeric Gag-GFP content in the permeate of Vivaspin filtered samples was not detected. Therefore, the quantitation method could be used with research purposes, assuming that the percentages of assembled VLPs would not change over bioprocess optimization.

Despite that, it would be necessary to develop and validate a second (and maybe a third) quantification method that could specifically quantify assembled particles. This would be essential in three situations. First: it could be possible that some conditions tested during bioprocess development changed the fact that the majority of the Gag-GFP molecules were assembled. For instance, addition of components that reduce the viability, such as sodium butyrate could lead to that problem [6]. Second, in pre-clinical or even clinical preparations where the product would not have a fluorescent reporter, and fluorescence-based method could not be used. Third, in clinical preparations, it would be important not only to assume but also to experimentally corroborate that the titer is not being overestimated by the presence of monomeric protein, to have the best approximation of the dose to be injected.

Novel quantification techniques, based on particle counting such as NTA or flow virometry could be useful for this purpose. However, they have another problematic. The presence of cell-derived exosomes, with very similar properties to HIV VLPs may cause the overestimation of VLP titers. To overcome this problem, efficient VLP separation from these vesicles should be achieved, although it is not trivial. In this regard, the problematic of the exosomes was not fully understood at the time of the development of the quantitation method. For this reason, even though quantification of total particles with NTA resulted in the same result that spectrofluorometry, it was not surprising at that time. However, after having access to fluorescence NTA, it was acknowledged that this result could not be correct. The reasons for this error could not be further investigated.

Future work will follow to have a more reliable quantitation, based on particles, but being careful not to quantify vesicles and exosomes, and without having to rely only on a reporter label.

The availability of several production platforms widens the possibilities of success in bioprocess development. HEK293SF-3F6 cell line was already in use in the lab for VLP production by TGE, and it was the platform used to produce the VLPs for the development

of the quantitation technique. However, other platforms were explored to produce VLPs. First, CHO-S cells were tested. CHO cells were not so amenable to PEI-mediated transient transfection as HEK293. Importantly, when they were successfully transfected under very specific conditions, it was found that Gag-GFP was not correctly assembled into VLPs. Literature reports on Gag production in CHO cells revealed that human-derived MA subunit from Gag was not correctly processed in CHO (not myristoylated) and that a chimeric human Gag with a murine MA subunit would be needed to be produced in CHO cells [7]. For this reason, a human platform was preferred and at this point it was decided to explore CAP cells as a novel platform for the production of VLPs.

CAP-T cell line was first used for transient expression of Gag-GFP. It was a very robust cell line in terms of cell growth and VLP production. The titers obtained with a non-optimized protocol were already competitive compared to other reported HIV VLP production systems. Moreover, the optimization of the main variables influencing transfection (seed age, PEM addition, complexation of DNA and PEI, cell density at the time of transfection, DNA and PEI concentration) led to an improvement of 1.4-fold in VLP titer.

There are many other strategies that have been reported to improve TGE titers, mainly for CHO and HEK293 cell lines. The application of these strategies to the CAP-T TGE established protocol will enable to further increase the titers. For example the optimization of the expression vector with sequences that enhance protein yields has been reported [8]. Temperature shift from 37 to 30-32 °C in the production phase has proven useful in CHO cells [9]. The addition of chemical compounds enhanced transfection and production on HEK293 and CAP-T cells [1,6]. Co-transfection of regulatory proteins, such as antiapoptotic genes [10], feeding strategies after transfection [11], and multiple product harvesting by medium replacements [12], also increased process yields. Extended Gene Expression was a novel strategy developed for HEK293, consisting in the re-transfection of the cultures, to prolong the production phase [13]. This strategy was performed with CAP-T cells, but the preliminary tests were not successful. Despite that more DNA was added to the cells every 48h after the first transfection, this did not result in better yields compared to a batch transfection. The causes remain to be clarified, since it could be that the new DNA was not entering into the cells or that the cell machinery was already saturated and more plasmid DNA did not result in more protein. Future studies will follow on this topic to try to develop a rational Extended Gene Expression protocol suitable for CAP-T cells.

One of the main drawbacks of the transient transfection protocol established for CAP-T cells was the medium replacement step. Due to the novelty of the cell line, and the fact that is not a public cell line, so its use has not been generalized, there are not many specific culture media options developed for CAP-T. There are only two culture media specifically design for CAP and CAP-T cells. CAP-CDM is used for stable expression and is not compatible with transfection, and CAP-T Express medium, for transfection. The latter is compatible with transfection when it is fresh, but not when is already conditioned, so it had to be diluted with fresh medium before transfection. Anyway, the results obtained with this formulation were not competitive compared to the PEM + FreeStyle293 optimized protocol, so it was discarded. The lack of a culture medium compatible with cell growth and transfection is an important limitation, especially for large-scale applications. Medium replacement by centrifugation was not desirable, since it could be performed at 1L scale, but larger volumes would have not been manageable. In addition, massive manipulation of the cultures may lead to contamination events. For these reasons, a very attractive and simple alternative was developed in this work to avoid medium replacement. It was observed that supplementing cell culture media compatible with transfection with enough PEM, allowed suitable cell growth. On top of that, if the percentage of PEM was sufficiently small, it did not inhibit transfection. So, the window of operation was small, but a solution was found to avoid medium replacement. Supplementation of FreeStyleF17 medium (compatible with transfection) with 5% of PEM allowed suitable cell growth without massive cell aggregation. Then, dilution to 1% of PEM 48h before transfection allowed to perform transfection without medium replacement. The fact that FreeStyleF17 conditioned medium did not inhibit transfection was also important for the success of this strategy. The same type of strategy was tested for FreeStyle293 medium, but the results were lower due to inhibition by spent medium. Remarkably, successful application of this strategy in bioreactor transfection was demonstrated in this work. The use of media combinations to achieve a final formulation compatible with growth and transfection represent a novel strategy that can be used with multiple cell lines and products, and it is easily scalable.

Future work to optimize the conditions with this new media combination will follow, since the conditions were first optimized for growth in PEM and transfection in FreeStyle293. In a more integrated process, this media screening should have been performed at the very beginning, predicting already the necessity to avoid the medium replacement upon scale-up. In this way, the conditions would have been already

optimized for this medium. However further attempts for other cell lines or products will be performed having this into consideration, to avoid the troubles seen in this work.

As discussed in *Introduction, part 2*, TGE is being used for production of clinical lots of gene therapy products, and eventually, a commercial production application is envisaged. The broader application of TGE will potentiate the development of new media formulations compatible with transfection, to avoid medium replacement at higher scales. However, medium replacement in bioreactor has other applications, such as to provide nutrients to achieve higher cell densities, to remove spent medium during the production phase, and continued product harvest [12,14]. This can be achieved by cell retention devices, such as ATF (alternate flow filtration) or spin filters. Future work in the group will be focus on setting the ATF for medium replacement and perfusion applications, for HEK293 and CAP-T transfection processes with the aim of increasing the product titers by reaching higher cell densities before transfection and providing fresh medium in the production phase.

The scale-up of the transfection protocol to 1L stirred-tank bioreactor was successful, since the same titers than in small scale were obtained. It was the first reported transient transfection in stirred-tank bioreactor, since the other reports were based in large shake flasks [1]. The conditions of the bioreactor needed to be adjusted to achieve optimal physiological state of the cells. Even though the cells could grow, they could not produce VLPs efficiently when they were aerated through the sparger. For this reason, surface aeration was used for the first growth phase in bioreactor before transfection. The VLP production capacity was restored to the control levels with this strategy. The relevance of the state of the cells for transfection was evidenced in this work. First, it was demonstrated that seed age was important, since cells that had been in culture for more than 3 days lost productivity. Second, gas sparging had a negative effect on the cells before transfection (although not after, this should be further studied). Third, massive aggregation occurring in determined cell culture media lacking some essential components hindered transfection efficiency and VLP production. It would be very interesting to find a universal parameter that could be easily measured and could define the physiological state of the cells. Therefore, it could be used to decide whether or not cells are ready for transfection, and to define in a very precise manner the culture conditions used before transfection. Vitality assay was useful in a growth curve to observe how the percentage of healthy cells was decreasing over time, but it was not useful to differentiate cells aerated by gas sparging and surface aeration. Therefore, a more

univsersal tool should be explored. The nucleotide triphosphate/uridine ratios were proposed as a variable to distinguish between early and late passage cells [15]. This variable and others will be explored in the future for CAP-T and HEK293 cell lines. On the other hand, dielectric spectroscopy (DS) could be useful to this end, since the β-dispersion parameters give information about the state of the cells. Deeper studies using different conditions will be explored to see if DS can give information about the cell health and help to define optimal growth conditions.

The use of DS allowed to obtain real-time information of the process, and observe relevant moments of the cultures. It was useful for both transient and stable production of Gag-GFP VLPs. This was the first step towards the implementation of PAT. With the use of more in-line monitoring technologies, the processes are closer to the standards demanded by regulatory agencies, making easier the transition from research to GMP production. Moreover, product characterization can rely on these technologies as well, according to the QbD initiative. In transient production, DS allowed the identification of the time when the glucose was depleted and there was a metabolic shift. In addition, the onset of the death phase could also be identified. Therefore, a harvest time for VLPs was identified using the in-line data, just before the death phase.

Unfortunately, VLP release could not be observed through changes in β-dispersion parameters. This could be due to the fact that only 50% of the cells were expressing VLPs or in the stable production where a majority of the cells were producing VLPs, because the membrane was not sufficiently affected by the amount of VLPs released. Future work will follow to try to correlate DS signal with time of transfection and VLP production.

Regarding stable production of VLPs in bioreactor, competitive titers were achieved compared to other systems reported in the literature [16–18]. The titers could be further improved with optimized fed-batch or perfusion processes, where the feeds or culture medium are added by demand of the cells, for instance maintaining the growth rate constant. DS data could also enable the implementation of sophisticated control loops for fed-batch and perfusion processes. One advantage of having a stable cell line producing VLPs is that productivity depends on the number of cells, and not on the addition of DNA. This provides a wide window of operation to improve the yields, by increasing cell density.

The titers obtained with the stable clone were lower compared to the TGE titers. This means that the potential of CAP cells to produce VLPs was not fully exploited. This is probably due to the low throughput screening carried out in this work. But limiting dilution

cloning was chosen since it was a straightforward technology, giving results in a short period of time. It was very easily implemented, not only for Gag-GFP as the model protein of this thesis, but also for Gag (not bearing the GFP reporter). Due to time constraints, it was not possible to perform more rounds of LDC. Future attempts to generate a cell line expressing VLPs will be performed by FACS, since it is a high-throughput technology. However, this would only be possible if the VLP contained a GFP reporter. If not, the most suitable technology would be LDC. Technologies based on capturing the proteins in the surroundings of the cell (gel microdrop, cold-capture, affinity matrix) may not be useful for VLPs, since the lipid envelope would preclude antibodies to attach. Automated technologies are out of the lead of academic research groups.

Regarding integration, targeted strategies have not been tested in CAP cells. Therefore, it is not possible to know what strategy would result in better yields. Although targeted integration could result in a more homogenous population, it would only allow for one copy, in contrast to the multiple copies that could be integrated by a random procedure, and usually the copy number correlated with productivity [19]. CRISPR-Cas is an emerging technology mainly used to generate knock-out cell lines, however, it has been also used to integrate genes [20,21]. Since it is a very simple technology, that does not require pre-modified cell lines (like the RMCE or DHFR) it is likely going to be used more extensively in the future for the generation of cell lines, in combination with high-throughput screening technologies.

Gene silencing was a problem observed in some of the clones in this work. Although some clones contained more than 1 copy of the gene, no Gag-GFP expression was ever detected. To overcome this limitation, the construction of the expression vector could be improved by linking the expression of the gene of interest and the selectable marker, for instance with an IRES. On the other hand, CMV promoter is precisely prone to be silenced [22], therefore it could be substituted by alternative strong promoters without this problem associated.

Having a clonal population is a regulatory requirement. In addition, the homogeneity of the population is beneficial for bioreactor performance and product quality [23]. However, cell lines change upon passaging and even with a clonal population a 50-70% of difference in expression has been found [19]. Enrichment of the 8D5 clones by cell sorting was demonstrated in this work, meaning that there were some higher producer cells within the clonal cell lines. Since clones seem to end up being heterogeneous pools, then

it is not so clear if the screening and cloning efforts are sufficiently justified. In the future, working with cell pools rather than clonal cell lines may become a topic to debate.

The process developed in this thesis including the transient transfection technology and the stable cell line producing VLPs up to 1L bioreactor allows to have a ready-to-use platform for the production of Gag-based vaccines. On one hand, having a stable cell line producing Gag VLPs is a useful tool since they have a value as immunogens and drug carriers *per se*. In addition, transient transfection can be performed on this cell line to add many envelope antigens in the Gag scaffolds. And not only VLPs, but many different protein candidates can be tested with the optimized transfection protocol at 1L scale. The platform will continue to be studied and optimized, focusing in the implementation of in-line monitoring tools and automation of the process, to work towards a QbD and GMP-like compliant platform. The lessons learnt, will be also useful for the development of production systems based on other cell lines.

References

[1] Fischer S, Charara N, Gerber A, et al. Transient recombinant protein expression in a human amniocyte cell line: The CAP-T® cell system. Biotechnol. Bioeng. 2012;109:2250–2261.

[2] Schiedner G, Hertel S, Bialek C, et al. Efficient and reproducible generation of high-expressing, stable human cell lines without need for antibiotic selection. BMC Biotechnol. 2008;8:13.

[3] Genzel Y, Behrendt I, Rödig J, et al. CAP, a new human suspension cell line for influenza virus production. Appl. Microbiol. Biotechnol. 2013;97:111–122.

[4] Silva AC, Simão D, Küppers C, et al. Human amniocyte-derived cells are a promising cell host for adenoviral vector production under serum-free conditions. Biotechnol. J. 2015;10:760–771.

[5] Krömmelbein N, Wiebusch L, Schiedner G, et al. Adenovirus E1A/E1B transformed amniotic fluid cells support human cytomegalovirus replication. Viruses. 2016;8.

[6] Cervera L, Fuenmayor J, González-Domínguez I, et al. Selection and optimization of transfection enhancer additives for increased virus-like particle production in HEK293 suspension cell cultures. Appl. Microbiol. Biotechnol. 2015;99:9935–9949.

[7] Reed M, Mariani R, Sheppard L, et al. Chimeric Human Immunodeficiency Virus Type 1 Containing Murine Leukemia Virus Matrix Assembles in Murine Cells. Society. 2002;76:436–443.

[8] Backliwal G, Hildinger M, Chenuet S, et al. Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res. 2008;36:e96.

[9] Wulhfard S, Tissot S, Bouchet S, et al. Mild hypothermia improves transient gene expression yields several fold in Chinese hamster ovary cells. Biotechnol. Prog. 2008;24:458–465.

[10] Zustiak MP, Jose L, Xie Y, et al. Enhanced transient recombinant protein production in CHO cells through the co-transfection of the product gene with Bcl-xL. Biotechnol. J. 2014;9:1164–1174.

[11] Pham PL, Perret S, Cass B, et al. Transient gene expression in HEK293 cells: Peptone addition posttransfection improves recombinant protein synthesis. Biotechnol. Bioeng. 2005;90:332–344.

[12] Grieger JC, Soltys SM, Samulski RJ. Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. Mol. Ther. 2016;24:287–297.

[13] Cervera L, Gutiérrez-Granados S, Berrow NS, et al. Extended gene expression by medium exchange and repeated transient transfection for recombinant protein production enhancement. Biotechnol. Bioeng. 2015;112:934–946.

[14] Ansorge S, Lanthier S, Transfiguracion J, et al. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. J. Gene Med. 2009;11:868–876.

[15] De Los Milagros Bassani Molinas M, Beer C, Hesse F, et al. Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring. Cytotechnology. 2014;66:493–514.

[16] Cervera L, Gutiérrez-Granados S, Martínez M, et al. Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J. Biotechnol. 2013;166:152–165.

[17] Sakuragi S, Goto T, Sano K, et al. HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 2002;99:7956–7961.

[18] Tagliamonte M, Visciano ML, Tornesello ML, et al. Constitutive expression of HIV-VLPs in stably transfected insect cell line for efficient delivery system. Vaccine. 2010;28:6417–6424.

[19] Priola JJ, Calzadilla N, Baumann M, et al. High-throughput screening and selection of mammalian cells for enhanced protein production. Biotechnol. J. 2016;11:853–865.

[20] Wang X, Huang X, Fang X, et al. CRISPR-Cas9 System as a Versatile Tool for Genome Engineering in Human Cells. Mol. Ther. - Nucleic Acids. 2016;5:e388.

[21] Bachu R, Bergareche I, Chasin L a. CRISPR-Cas targeted plasmid integration into mammalian cells via non-homologous end joining. Biotechnol. Bioeng. 2015;112:2154–2162.

[22] Osterlehner A, Simmeth S, Göpfert U. Promoter methylation and transgene copy numbers predict unstable protein production in recombinant chinese hamster ovary cell lines. Biotechnol. Bioeng. 2011;108:2670–2681.

[23] Lai T, Yang Y, Ng SK. Advances in mammalian cell line development technologies for recombinant protein production. Pharmaceuticals. 2013;6:579–603.

Conclusions

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

1. A fluorescence-based technique was developed for the routine quantitation of Gag-GFP VLPs. Fluorescence measures correlated well with p24 ELISA concentrations. Therefore, a standard curve could be generated using purified VLPs to convert relative fluorescence values to Gag-GFP concentrations in ng/mL.

2. The quantitation method was validated according to ICH guidelines. It was demonstrated to be specific, linear over a wide range, precise, accurate and sensitive enough to quantify crude VLP samples. Moreover, quantitation by Spectrofluorometry was in agreement with other methods.

3. CAP-T cells produced correctly assembled Gag-GFP VLPs, with the expected size and morphology upon PEI-mediated transfection at small scale.

4. Systematic optimization of the transfection protocol, first by studying some discrete variables (seed age, PEM addition and separate addition of PEI and DNA), and second by studying cell density, DNA and PEI concentrations combined in a Design of Experiments approach, led to VLP titers of 6×10^{10} VLPs/mL (1.44-fold improvement).

5. CAP-T cells were cultured in 1L bioreactor in batch mode in PEM medium to high cell densities comparable to small scale shake flasks.

6. VLP production upon transfection of cells grown in bioreactor was comparable to small scale shake flasks when gas sparging was avoided during the growth phase in bioreactor.

7. A novel combination of media was used to grow and transfect CAP-T cells without the need to perform medium replacement in bioreactor. Cells were cultured in FreeStyleF17+5% of PEM and 48h before transfection, they were diluted in FreeStyleF17+1% of PEM, allowing direct transfection in bioreactor. This strategy significantly simplified the transfection protocol at bioreactor scale.

8. VLP titers obtained by TGE in 1L bioreactor were comparable to the titers obtained in small scale shake flasks.

9. A stable CAP cell line expressing Gag-GFP VLPs was developed. The selected clone produced correctly assembled VLPs with titers of 2×10^9 VLPs/mL at small scale and 1L bioreactor in fed-batch mode.

10. In-line monitoring of dielectric spectroscopy (DS) was implemented in the VLP production processes (transient and stable) at bioreactor scale. A correlation between permittivity signal and viable cell density was observed during the exponential growth phase, although this correlation was weakened at the end of the culture. Fed-batch and perfusion processes could be implemented based on the permittivity data.

11. The β-dispersion data obtained by DS allowed to identify key moments in the cultures, such as nutrient depletion, metabolic shift and the onset of the death phase. This information will be used to determine the time of VLP harvest, before massive cell death occurs.