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Variables affecting HEK293 cells PEI-mediated transient transfection for Gag-GFP VLP production

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1 RIASSUNTO

Le particelle simil-virali (*virus-like particles*, VLPs) sono candidati molto promettenti per lo sviluppo di nuovi vaccini. Gli approcci per la produzione in larga scala di HIV-1 Gag VLPs si sono focalizzati principalmente nel sistema di espressione basato sui baculovirus. In questo lavoro, le HIV-1 Gag VLPs sono state prodotte utilizzando un protocollo di espressione transiente, precedentemente sviluppato e ottimizzato dal gruppo di ricerca, in coltura in sospensione di cellule di mammifero. Lo scopo di questo lavoro è di studiare le principali variabili presenti nel processo per capire quale effetto possano avere sulla trasfezione transiente delle cellule e sulla produzione stessa delle VLPs.

Per facilitare la quantificazione delle VLPs, la proteina Gag viene espressa in fusione con la eGFP (*enhanced Green Fluorescence Protein*), generando così VLPs fluorescenti che possono essere quantificate per fluorimetria. Da studi precedenti eseguiti dal gruppo di ricerca, la grande maggioranza delle Gag-eGFP prodotte e presenti nel sopranatante della coltura sono correttamente assemblate in VLPs le quali mostrano la morfologia e le dimensioni previste, simili a quelle delle particelle virali immature dell'HIV-1.

Il medium di coltura utilizzato è stato precedentemente ottimizzato per lo scopo, utilizzando il disegno di esperimenti (*Design of Experiments*, DoE). Il risultato è un medium di coltura supplementato con componenti di derivazione non-animale i quali comprendono proteine ricombinanti e lipidi sintetici in grado di aumentare la crescita cellulare e la produzione delle VLPs. Le cellule utilizzate sono una linea di cellule di rene di embrione umano (*Human Embryonic Kidney 293*, HEK293) che, in queste condizioni, crescono fino a una concentrazione di 4,8 milioni di cellule per ml se coltivate in *batch*.

La migliore *performance* di produzione si ottiene quando le cellule sono trasfettate durante la loro fase di crescita esponenziale, con ricambio del medium di coltura con medium fresco al momento della trasfezione ed utilizzando concentrazioni standard di DNA plasmidico e polietilienimina (PEI).
Utilizzando questo metodo, mediamente, la metà delle cellule in coltura viene trasfettata positivamente e vengono prodotti circa 2,7 miliardi di VLPs per ml. Il problema intrinseco del metodo consiste nella variabilità dell'efficienza di trasfezione che porta anche ad una variabilità nella quantità di produzione delle VLPs. In questo lavoro, i risultati ottenuti mantengono questa tendenza. Grazie a una migliore comprensione delle variabili presenti, è possibile ridurre la variabilità osservata ed aumentare la produttività.

Le variabili testate sono state diverse: variabili che influenzano la formazione dei poliplessi di DNA e PEI, altre che influenzano la procedura di trasfezione ed anche variabili che non dipendono dal protocollo di trasfezione. Per quanto riguarda la

formazione dei poliplessi, è stato studiato l'effetto dell'utilizzo di medium supplementato come solvente per la soluzione di DNA e PEI comparato con l'utilizzo di medium non supplementato; l'effetto della temperatura di tale medium e l'effetto del "*vortex-step*". Riguardo la procedura di trasfezione sono stati studiati l'effetto dello stress cellulare dovuto al ricambio di medium e l'effetto dell'età delle beute utilizzate per il coltivo in *batch*. Da ultimo si è studiato l'effetto del passo cellulare, della concentrazione e della fase del ciclo cellulare al momento della trasfezione.

Per studiare l'effetto della concentrazione cellulare sono stati adattati metodi di concentrazione e metodi di trasfezione alternativi. Mentre, per studiare l'effetto del ciclo cellulare sono state ottenute colture cellulari sincronizzate. Per raggiungere questo scopo due composti antineoplastici sono stati utilizzati. Di questi è stata studiata la tossicità, con saggi MTT, e il potere antineoplastico, grazie alla citometria di flusso.

Per conoscere i tempi di trasfezione è stata effettuata una ricerca bibliografica i cui risultati hanno permesso di trasfettare con le tempistiche corrette le colture sincronizzate ottenute.

Grazie agli studi effettuati è stato possibile concludere che il protocollo di trasfezione è robusto e non influenzato dai fattori operativi. Inoltre, è stato possibile definire un effetto positivo del medium supplementato usato per la preparazione dei poliplessi sulla vitalità cellulare ed è stata sottolineata l'importanza dello stato fisiologico delle cellule per poter ottenere buoni risultati di trasfezione e produzione. L'effetto da densità cellulare (*cell density effect*) è stato incontrato quando sono stati svolti esperimenti di trasfezione con concentrazione della coltura, benché, finora questo effetto sia stato descritto solamente in colture di baculovirus, tale effetto è stato evitato adattando un metodo di trasfezione alternativo. Lo studio della fase del ciclo cellulare ha posto in relazione una particolare fase del ciclo con la trasfezione, rivelando che cellule che si trovano nella fase G2/M sono più recettive alla trasfezione. Questi ultimi studi hanno evidenziato un forte effetto sulla trasfezione e sulla produzione del ricambio del medium precedente alla trasfezione, al riguardo del quale saranno necessari ulteriori approfondimenti.

In chiusura vengono proposti esperimenti per risolvere i dubbi evidenziati dagli studi effettuati. Si propone nel dettaglio uno studio di microscopia confocale per ricavare i tempi precisi di trasfezione nel sistema utilizzato. Viene illustrato un metodo per eliminare l'effetto del cambio di medium negli studi sulle colture sincronizzate ed da ultimo metodi alternativi per lo studio dell'effetto della fase del ciclo cellulare.

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3 ABBREVIATIONS

μ_{max}	maximum growth rate
Ad-5	Type 5 Adenovirus
AIDS	Acquired Immunodeficiency Syndrome
BRI	Biotechnology Research Institute
cGMP	current Good Manufacturing Practice
CMV	Cytomegalovirus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DoE	Design of Experiments
eGFP	Enhanced Green-Fluorescence Protein
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FU	Fluorescence Unit
G0	Gap 0 phase
G1	Gap 1 phase
G2	Gap 2 phase
Gag	Group specific Antigen
GECIT	Grup d'Enginyeria Cel.lular i Tissular
GMP	Good Manufacturing Practice
HBV	Hepatitis B Virus
HDAC1	Histone Deacetylase 1
HEK293	Human Embryonic Kidney 293 cell
HIV	Human Immunodeficiency Virus
HIV-1	Type 1 Human Immunodeficiency Virus
hpmc	hours post medium exchange
hpp	hours post drug addition
hpt	hours post transfection
HPV	Human Papilloma Virus
M	Mitosis phase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NRC	National Research Council
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PI	Propidium Iodide
Rev	Regulator of Expression of Virion Proteins
RFU	Relative Fluorescence Unit
S	Synthesis phase
UAB	Universitat Autónoma de Barcelona
VLP	Virus-Like Particle
WHO	World Health Organization

4 ABSTRACT

The virus-like particles (VLPs) are very promising candidates for the development of new vaccines. The approaches for the large scale production of HIV-1 Gag VLPs have focused mainly on the expression system based on baculovirus. In this work, the HIV-1 Gag VLPs were produced using a protocol of transient expression, previously developed and optimized by the research group, in suspension culture of mammalian cells. The purpose of this work is to study the main variables in the process to understand what effect they can have on the transient transfection of the cells and on the same VLP production.

To facilitate the quantification of VLPs , the Gag protein is expressed in fusion with the eGFP (enhanced Green Fluorescence Protein), thus generating fluorescent VLPs that can be quantified by fluorimetry. From previous studies performed by the research group, the great majority of the Gag-eGFP produced and present in the supernatant of the culture are properly assembled into VLPs, which show the provided morphology and dimensions, similar to those of immature viral particles of HIV-1.

The culture medium used was previously optimized for the purpose, using the design of experiments (DoE). The result is a culture medium supplemented with non-animal derived components, which include recombinant proteins and synthetic lipids, capable of increasing cell growth and VLP production. The cells used are of a GMP-compliant line of human embryo kidney cells (Human Embryonic Kidney 293, HEK293) that, in these conditions, grow up to a concentration of 4.8 million cells per ml, when grown in batch.

The best performance of production is obtained when the cells are transfected during their exponential growth phase, with replacement of the culture medium with fresh medium at the time of transfection and using standard concentrations of plasmid DNA and polietilienimina (PEI).
Using this method, on average, half of the cells in culture is positively transfected and produces about 2.7 billion VLPs per ml. The inherent problem of the method lies to the variability of transfection which also leads to a variability in the amount of VLP production. In this work, the results obtained maintain this trend. Thanks to a better understanding of the variables involved, it is possible to reduce the observed variability and increase productivity.

It has been concluded that the transfection protocol is robust and not influenced by operational factors. Moreover, it was possible to define a positive effect of the supplemented medium used for the preparation of polyplexes on cell viability and

underline the importance of the physiological state of the cells in order to obtain good results of transfection and production. The cell density effect was met when transfection experiments were carried out with the concentration of the culture, although, so far, this effect has been described only in cultures of baculovirus , and, it was avoided by adapting an alternative transfection method. The study of the phase of the cell cycle has placed in connection a particular phase of the cycle with transfection, revealing that the cells that are located in the G2/M phase are more susceptible to transfection. These studies have shown a strong effect on transfection and production of the replacement of the medium prior to transfection, the matter of which will require further study.

5 INTRODUCTION

5.1 TRADITIONAL VACCINES AND THIRD GENERATION VACCINES

A vaccine is a biological preparation that improves immunity to a particular disease; it typically contains an agent that resembles a disease-causing microorganism and that stimulates the body's immune system to recognize such agent as foreign, destroy it and "remember" it, so that to easily recognize it in later encounters.

The major types of vaccines licensed for use in humans can be divided into two main categories: traditional ones and modern ones (1). Traditional vaccines are classified in three kinds and there are many kinds of modern vaccines.
Traditional vaccine kinds are:

- Live attenuated vaccines: they consist in the same pathogen but with its virulent proprieties attenuated; the success of this approach depends on decoupling virulence from induction of protective immunity. Those were the firsts vaccines to be developed and work really well as they resemble the natural conditions and provide a lasting immunity. There are many examples of highly successful vaccines that have been developed like the current vaccine for tuberculosis. Often, multiple rounds of growth under conditions that weaken the organism such as in tissue culture or harsh physical conditions obtain the attenuated form of the pathogen. In these cases, the molecular basis of attenuation is unknown. New attenuated forms are likely to include the introduction of one or more specific mutations that interfere with synthesis of an amino acid or of a nucleic-acid essential for the growth of the organism (2). There are many disadvantages related with the use of live attenuated vaccines, as it multiplies in the patient and there is the possibility of reversion to virulence, due to a secondary mutation. This implies that live attenuated vaccines are suitable only for the vaccination of healthy adults (3).

- Inactivated or killed organisms: these vaccines are safer in use as the living pathogen is inactivated in its virulence or killed. The inactivation is obtained through the action of chemicals, heat, radioactivity or antibodies, destroying the ability of the organism to replicate, making it impossible to retro-mutate. But, they tend to provide a shorter term immunity compared to live vaccines. For example, the Polio and the Hepatitis A vaccines are among this kind.

- Inactivated toxins: this kind of vaccine form the toxoid vaccines category and consists of compounds of the pathogen that cause illness rather than the pathogen itself. Because of their inability to replicate in vivo, these vaccines are generally less immunogenic than live vaccines and usually do not induce immune cellular response. One example for the development of inactivated toxins is tetanus, where the symptoms are caused by the neurotoxin produced by the bacteria and not by the bacteria itself.

There have been many successes with traditional vaccines as it has been possible to improve immunity to Smallpox, Tuberculosis, Influenza, and Typhoid fever (only to name some of the most important ones), in fact, 90% of all recommended vaccines by the World Health Organization (WHO) belong to this category (4). However, an important limitation is safety: it is not always possible to identify sufficiently attenuated strains and there is an underlying risk of reversion to virulence, not to speak about the safety for who has to manipulate the vaccine. Moreover, traditional approaches have not been effective for many vaccine challenges, such as preventing or treating chronic infections and cancer, which may require induction of potent cellular immune responses. Some pathogens cannot be grown in vitro or, like in the case of Human Immunodeficiency Virus (HIV), some mutation rates are so high that make it impossible to develop a vaccine (5).

With the term "new generation vaccines" or "modern vaccines" the following vaccine types are included:

- Peptide vaccines: are based on chemical synthesis of a peptide that structurally resembles an antigen of the pathogen and which, when introduced into the host, the immune system recognizes as foreign antigens. These vaccines have the drawback that they are very quickly removed, therefore, the response that produce is much lower than conventional vaccines. On the other hand, they are more difficult to produce as the number of amino acids to form the peptide is the limiting step, with 50 being the maximum number of amino acids that can be bound in a chemical way. This type of vaccine is also valued as cancer treatment (6).

- Live recombinant or genetically attenuated vaccines are the case in which the microorganism is genetically modified and may lead to total loss of pathogenicity of the organism (7). However, the immune response that occurs has to provide resistance, when the virulent pathogen will be present.

One of the major disadvantages of these vaccines is the probability to reverse.

- DNA vaccines: this kind of vaccines is still in the experimental stages. They consist of a strand of DNA that codes for the antigens of the selected pathogen which will be introduced in the host cells, making them able to secrete such antigen and display it on their surface. In this manner, the host immune system will be stimulated to create a response. These vaccines would be relatively easy and inexpensive to design and produce. Naked-DNA vaccines against influenza and herpes are being tested.

- Recombinant vector vaccines are experimental vaccines that use an attenuated virus or bacterium to introduce part of the pathogen DNA into host cells. Thus, they work like DNA vaccines, but, a vector carries the genetic information. These vaccines closely mimic a natural infection, therefore are able to stimulate the immune system. By now, research focuses onto development of vaccines for HIV, rabies and measles.

- Recombinant protein vaccines: this type of vaccine provides the advantage of not presenting a risk for the vaccinated nor for manipulators since it consists in the production by a particular cell type, either prokaryotic or eukaryotic, of recombinant proteins able to induce a protective immune response in the vaccinated. These proteins are generally pathogen antigens. The DNA encoding these antigens is introduced stably or transiently into cells, which synthesize the recombinant proteins. Later purification processes are performed, in which the vaccine is obtained. Within this type of vaccine, Virus Like Particles (VLPs) are found. These are particles that are structurally very similar to the wild-type virus, however, lack the necessary genetic information to replicate therein.

5.2 VIRUS-LIKE PARTICLES

Virus-like particles are supra-molecular assemblages, usually icosahedral or rod-shaped structures with diameters in range of 25-100 nm (8). They incorporate key immunogenic features of viruses like highly repetitive and ordered structures, particulate nature, ability to induce innate immunity for appropriate conditioning of adaptive immune responses, while lack genetic information with replicative capacity, just like recombinant subunits vaccines; thus represent an advanced vaccine technology platform (9) (10).

Their recombinant nature means they can usually be manufactured economically in large-scale following current GMP. They display antigens in an ordered and repetitive way and thus induce rapid immune responses. Their particulate nature and dimensions means they are efficiently taken up by dendritic cells and transported to lymph-nodes. The ability of VLP polypeptide subunits to spontaneously assemble allows them to be disassembled and resembled in vitro, a process that permits stimulators to be packaged within their interiors. Nonetheless, antigens can be incorporated onto or into VLPs' surface. Those are called VLP chimeras and have been extensively explored as vaccine-candidates since the mid-1980s (11): the introduced epitopes are presented in a repetitive and ordered fashion, at relatively high density and ideally with optimal accessibility and conformation, making them able to activate even B-cells (12).

Vaccines in clinical development include those where the VLP itself represent the target epitope and those where the VLP is used to present a non-VLP derived epitope, i.e. an epitope that does not come from the proteins forming the VLP but from a third party. Two VLP-based antiviral vaccines have reached market: one against Hepatitis B Virus (HBV) and one against Human Papilloma Virus (HPV); and others are undergoing a number of clinical development programs such as VLP vaccines for influenza A, Norwalk virus, nicotine dependence, hypertension, Alzheimer's disease (12). Several attempts have been made to induce an effective anti-HIV immune response (13) (10). Retroviral VLPs have also been successfully generated through the inoculation of Gag-Pol-expressing vectors in mice (14).

5.2.1 HIV-1 PR55^{GAG} VLPS

The VLP expressed in this work is the HIV-1 Pr55^{Gag} fused with the enhanced Green Fluorescent Protein (eGFP) to enable quantification of the final product (15).

The use of such VLPs has a number of advantages: the particle has the same size as the HIV and is easily identified by the immune system and the proteins that form the VLP can be engineered to exhibit at the surface of the particle one or more HIV antigens or other immunostimulatory molecules. HIV pseudovirion immunization has been shown to generate promising cell-mediated immune response in animal models and $Pr55^{Gag}$-based VLPs have elicited strong cytotoxic T lymphocyte responses in mice (16). Intramuscular administration in macaques resulted in long-lived cytotoxic T lymphocyte responses (17). Given such promising potential, it seems remarkable that no HIV pseudovirion approaches have advanced beyond early preclinical testing, maybe because of the complexities of production, envelope protein incorporation and stability, and purification.

HIV is a lentivirus (slowly replicating retrovirus) that causes AIDS (**a**cquired **i**mmuno**d**eficiency **s**yndrome), a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive (18). HIV infects vital cells in the human immune system such as helper T cells, macrophages, and dendritic cells (5) leading to low levels of CD4+ T cells meaning loss of cell-mediated immunity (19). The HIV does not consist only of the Gag protein as its fundamental genes are *gag*, *pol* and *env*, which code for structural as well as enzymatic proteins, but, besides *gag* genes, these are not used for the expression of VLPs. The *pol* gene encodes for the virus polymerase and the *env* gene encodes for the envelope protein (Env, gp160), which is processed by the cellular protease to produce the membrane lipoprotein gp120 (SU), able to recognize and bind to the CD4 receptors. Apart from these, HIV also encodes proteins that have certain regulatory and auxiliary functions. It comprises two key regulatory elements: Tat and Rev and some other accessory proteins such as Nef, Vpr, Vif and Vpu related with the entrance in the host cell and the replication enhancing. (20)

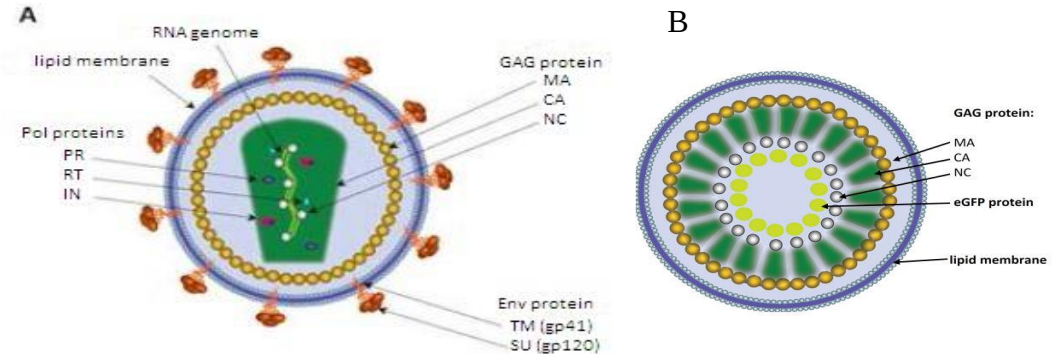


FIGURE 1– (A) STRUCTURE OF THE MATURE HIV-1. (B) STRUCTURE OF THE PRODUCED VLP.

The HIV type I (HIV-1) *gag* (**g**roup specific **a**nti**g**en) gene encodes for the highly conserved structural polyprotein $Pr55^{gag}$, which directs the viral assembly process and is present at about 5000 copies per virion (21). Accurate crystal structures of $Gag_{1\text{-}278}$ (i.e., the fused MA and CA_N domains) at 2.2 Å resolution and the free $CA^N{}_{133\text{-}278}$ domain at 1.9 Å resolution have been obtained (22). The Gag protein comprises the major structural proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC) and p6 linker (LI), that are processed by the viral protease as virions bud from the host cell, thereby releasing discrete new proteins, as the small spacers peptides p2 and p1, required for maturation (23).

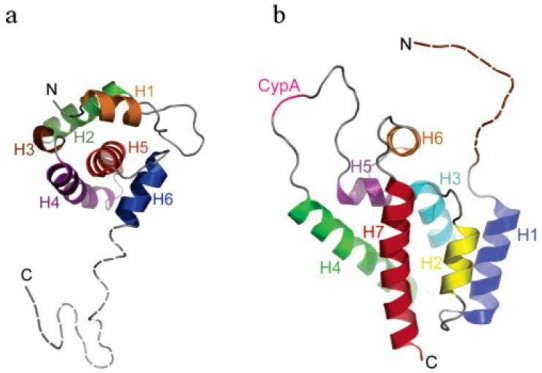


FIGURE 2 - (A) CARTOON REPRESENTATION OF THE GAG1-278 MA DOMAIN WITH SECONDARY STRUCTURES LABELED AND DISORDERED RESIDUES SHOWN AS DASHED LINES. (B) SAME AS (A) BUT FOR THE GAG1-278 CAN 133-278 DOMAIN (22)

The N-terminal MA domain confers membrane-binding activity to Gag, and the MA protein remains associated with the viral membrane after processing. Membrane binding is bipartite, with electrostatic interactions made by positively charged MA side chains and hydrophobic interactions provided by the insertion of MA's N-terminal myristate moiety into the lipid bilayer (24).

The CA region immediately follows MA in the HIV-1 Gag polyprotein and is comprised of two domains (25). The N-terminal domain mediates hexamer formation in the viral capsid, and the C-terminal domain mediates CA association of adjacent CA hexamers in the core (26). The extended Gag molecules adopt a radial distribution in the spherical immature particle, and the CA layer is located ~100 Å from the membrane (27). During the subsequent process of maturation, ~1000-1500 CA protein molecules rearrange to form the distinctive conical capsid of infectious virions.

The p7 (NC) domain has the role to encapsulate and protect viral RNA and is important for efficient targeting and binding Gag to the plasma membrane (28) (29).

The role of RNA in virion assembly is so vital that HIV recruits other cellular RNAs if viral RNA is not available (30). (22)

When the *gag* gene is expressed in appropriate cells, the Gag polyprotein is synthesized in the cytoplasm and then is targeted to the plasma membrane, where it acquires a lipid envelope and buds in form of VLP. During the cytosolic transport, it forms assembly intermediates, recruits single-strand RNA and aggregates at the inner face of plasma membrane as dense patches (10).

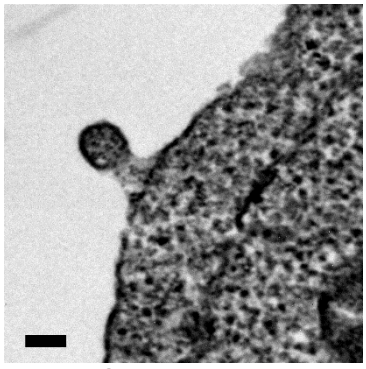


FIGURE 3 – TEM IMAGE OF ULTRATHIN SECTION SHOWING VLP BUDDING FROM HEK293 PRODUCER CELL. BAR 100μM (60)

Depending on the specific target of the VLP vaccine, different expression systems should be employed for expression of the required subunits: bacteria, yeast, plant cells, insect cells and mammalian cells can be used with pros and cons related (31). In fact, considerations such as appropriate protein glycosylation, correct folding and assembly favored mammalian cells as expression system, although they are more expensive (32).

Bacteria are a wild used expression system in which the production is easy and the cost is low. Even the scaling process in easy, but bacteria are unable to introduce post-transcriptional modifications. The efficiency of the expression of viral components is variable and, usually, VLPs do no assemble if this system is used.

The yeast expression system is similar to the bacterial expression system, with the pro that it is a eukaryotic system. Post-transcriptional modification are introduced but not with the same path as in mammalian cells. The main problem in the VLPs production is that, when produced, they are of low consistency and are separated from the cell cytoplasm into spheroplasts; the disruption of the cellular wall will be needed as well.

Insect cells are a well studied expression system, the production cost is intermediate, scalability is easy and post-transcriptional modification are quite similar to mammalian cells. Producing VLPs in this system is hard, due to the use of baculovirus in this expression system, which has to be removed during downstream process (33). Moreover, the incorporation of insect proteins or baculovirus-derived proteins in the lipid envelope of HIV-1 Gag VLPs is known to provoke a strong immune response that can potentially mask the response against the desired envelope antigen (10) (33).

If a mammalian cell is used as expression system, the production system will be very similar to the native pathogen-host cell, post-transcriptional modification will

be adequate and the final product will have a good quality. Although, their cost is high and the productivity is low. (34)

5.3 HUMAN EMBRYONIC KIDNEY CELL LINE

The expression system used in this work is a mammalian cell line, precisely the Human Embryonic Kidney cell line HEK293 SF-3F6. HEK293 cells are easy to grow and transfect, they are an excellent cell line to use in transfection experiments, or to produce recombinant DNA or gene products. The introduction of plasmid vectors such as those under the control of the CMV promoter very effectively hijacked the HEK cell's synthetic protein machinery and forced the translation of gene products artificially incorporated into the plasmid (35). This has made them a popular research tool in cell biology studies, and an ideal cell line for therapeutic protein and virus production by the biotechnology industry.

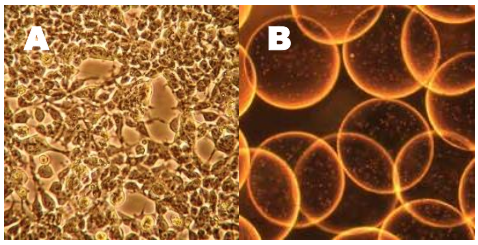


FIGURE 4 – HEK293 OPTICAL MICROSCOPE IMAGE. (A) CULTURE IN ADHESION, ALMOST CONFLUENT. (B) CULTURE IN SUSPENSION.

HEK293 cells were generated over 40 years ago by transformation of human embryonic kidney cell culture with sheared fragments of adenovirus type 5 (Ad5) DNA in the laboratory of Alex Van Der Eb in Leiden, Holland. The original culture was created by Van Der Eb using some kidney cells from an aborted fetus and were transformed by Graham (36) as he was working on DNA-Calcium Phosphate transfection of rat embryo and kidney cells since a couple of years (37), hence, the number 293 is used because that was his 293rd transfection experiment. Since many kinds of cell belong to the kidney tissue, it is impossible to know exactly from which the HEK293 cell line comes from, but, their genomic characteristics have been studied (38). The transformation permanently incorporated around 4.5 kb of the left arm of the Ad5 genome into chromosome 19 of the host genome; such modification was subsequently used for the generation of transcription incompetent human adenoviral vectors. This cell line presents a mode of 64 chromosomes per cell (i.e. two or more copies of each chromosome), including three copies of the X chromosome and four of the chromosomes 17 and 22.

When cultivated, these cells have to be grown in adhesion until they are ~ 70% confluent; after some passages in adhesion, they can be adapted for growth in suspension. In order to make them adhere, incubation at 37 °C is necessary,

meaning also that they can be transported, without any mechanical damage, in suspension.

The cell line used in this work grows in suspension in the FreeStyle culture medium optimized with the addition of animal-free supplements, that permits to reach high cell densities (39). Cell morphology is shown in Figure 4: suspension cells have a round shape and do not interact physically with each others.

5.4 TRANSIENT TRANSFECTION

Transfection is the process of deliberately introducing foreign nucleic acids, such as supercoiled plasmid DNA, into eukaryotic cells to get them expressed. There are different methods to obtain transfection: some rely on physical treatment, such as electroporation, others on chemical materials, such as the use of calcium phosphate or cationic polymers, like polyethylenimine (PEI), or biological particles, such as viruses as carriers. Transfection can result in the generation of a stable line expressing the introduced trans-genes. Stable transfection in more difficult to obtain because the transfected DNA has to remain in cell genome and to be passed to the daughter cells through generations. Transient transfection is easier, compared to stable transfection, as the transgenic material is not integrated into the nuclear genome, although it enters the cell nucleus, allowing expression of the transgene, which will be diluted through mitosis or degraded. This is why stable transfection is usually preferred by industries, as they want a product to be expresses in a stable manner without warring about the production rates. Nonetheless, transient transfection is a suitable technique for academics or early expression studies that could be done before and to test the feasibility of a stable line. In fact, this method is very flexible, as it is possible to generate different candidate products in a fast and easy way, in order to test them for the searched characteristics.

The technique used in this work for the production of VLPs is the transient transfection mediated by PEI. Since its introduction in 1995, PEI has become one of the most efficient non-viral gene-transfection agent. It is a cost-effective and efficient method (21) based on the use of cationic polymers. Cationic polymers have a positive charge and thus are able to complex DNA, which has a negative charge, and to cross the cellular membrane, which has a global negative charge too and an inner hydro-carbonic appearance, even reaching the nucleus through diffusion.

Despite the high efficiency of the method there is a high variability in the positive cell transfection percentage and the recombinant protein production.

Many points of the methods have been widely investigated to find ways to reduce its variability. Now it is known that branched and linear PEI/DNA complexes differ in their ability to transfect cells. The greater efficiency of linear PEI might be due to an inherent kinetic instability under salt solution (40). The structure PEI/DNA complexes has been quantitatively analyzed as well.

The role of the cellular state has been studied as well. It has been shown that beyond the mid-log phase point of the growth of cell that have to be transfected, VLP production is limited by a significant loss in transfection efficiency. These observations are also in agreement with those reported by Carpentier et al. (41) who concluded that successful transgene expression is more likely to depend on a "cellular competent state" than on the quantity of plasmid DNA delivered per cell. In this sense, Brunner and collaborators have indicated that transfection efficiency is strongly dependent on the cell cycle stage at the time of transfection, with cells in the G2/M phase giving greater levels of transfection than G1 cells (42). The authors have hypothesized that transport of DNA into the nucleus is a very inefficient process and that transfection of cells shortly before their next cell division (close to M phase) is facilitated by nuclear membrane breakdown. In support of these studies, Tait et al. (43) have shown that both transfection efficiency and protein expression can be enhanced by arresting cells in G2/M phase using Nocodazole.

5.5 CELL CYCLE

Through the cell division process, known as cytokinesis, a mother cell divides into two daughter cells, transmitting them its whole genetic information in equal quantity. Each new cell division corresponds to a new cell cycle. Cells going through the actual division process are in the mitosis (M) phase and non-dividing cells are in interphase. Interphase may last for different periods of time (hours, days, weeks, or longer) depending on the cell type and it is divided into three phases: gap one (G1), synthesis (S) and gap two (G2).

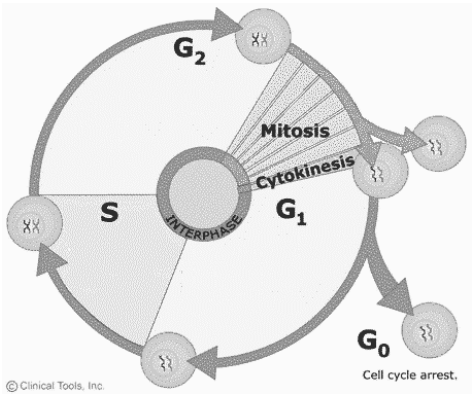


FIGURE 5 - CELL CYCLE SCHEME

To provide a complete set of genetic material for each of its two daughter cells, the genetic information of the mother cell has to be duplicated, resulting in a cell with twice the normal amount of DNA. This duplication of the genome is performed during the S phase. Afterwards, mitosis occurs and the two daughter cells each have a full copy of the DNA. The S phase and mitosis are separated by the G2 phase where a checkpoint for completed DNA synthesis occurs. Mitosis and the S phase are separated by the G1 phase in which a checkpoint for DNA damage occurs. Cells can reversibly enter a resting phase (G0) during G1 phase, if environmental conditions are not sufficient for growth and division.

5.5.1 STUDY OF THE CELL CYCLE

Different approaches exist to study the cell cycle such as monitoring the cell concentration with time as well as the use of specific stains, combined with flow cytometry; this provides determination of percentage of cells present in the different phases of the cell cycle.

A normal cell culture appear asynchronous, as each cell proceeds independently through cell cycle, so it results that the whole culture is randomly distributed throughout the different cell cycle phases. In order to study the effect of a specific cell cycle phase it is necessary to have a synchronized culture, or at least a culture enriched with a good amount of cells in the same cycle phase.

5.5.1.1 CELL SYNCHRONIZATION

Many different approaches have been published to achieve cell synchronization among a given population in culture. The two main approaches are by using a drug that inhibits the progression in the cell cycle and by physiological means such as contact inhibition or nutrient deprivation. Reversible inhibitors of cellular DNA synthesis are useful tools for synchronization as cycling resumes upon drug removal. However, when an asynchronous cell population is treated, only cells reaching the point in the cell cycle inhibited by the drug will get affected by the synchronization agent and accumulate at that point.

Synchronization over more than one or two cell cycles is difficult to achieve due to the fact that as soon as the synchronization factor is removed, each cell will again cycle on an individual basis, independently of the state of the cells surrounding it. The cause of de-synchronization is not understood but has been attributed to intracellular fluctuations in the number of key molecules necessary for the initiation of DNA synthesis (44).

CHEMICAL SYNCHRONIZATION

In this work, two chemical reagents have been used with the aim to obtain two different synchronized cell cultures: Hydroxyurea and Nocodazole.

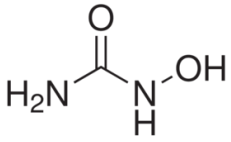


FIGURE 6 - HYDROXYUREA

Hydroxyurea: it is an anti-neoplastic drug used against chronic myeloproliferative syndrome, besides being used for pain in sickle cell disease. It also has antiretroviral capabilities. Advantage was token from its propriety as DNA replication inhibitor

since it inhibits conversion of ribonucleotides to deoxyribonucleotides (45). The effect is cell line dependent: HEK293 resulted blocked in G1 phase.

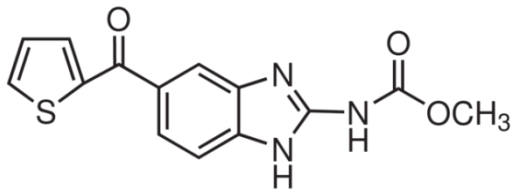


FIGURE 7 - NOCODAZOLE

Nocodazole: it is an anti-neoplastic agent that arrests the cell cycle interfering with microtubule polymerization thus blocking cells in G2 or M cell cycle phase. Cells are able to enter mitosis but are unable to form the spindle apparatus as microtubules cannot be polymerized, thus activating the mitosis checkpoint (46). Long exposure to Nocodazole can lead to apoptosis. Furthermore, as organizing the perinuclear Golgi structure is microtubule polymerization dependent, Golgi structure is inhibited by Nocodazole too. Blocking cells in G2-M phase could lead to high transfection percentages, due to lack of cellular membrane; however, Nocodazole does not synchronize cells, as, when removed, a synchronized culture is not obtained (47).

6 AIM OF THIS WORK

This work aims to optimize the production of Gag-eGFP VLPs in HEK293 cells through a better understanding of some important variables that have to be taken into account during the process.

The independent study of different variables provides ways to understand better the mechanisms implied in the formation of the PEI/DNA polyplexes used for the transient transfection of cells and the process of PEI mediated transient transfection itself.

Various methods exist to transfect mammalian cells in culture. It is generally accepted that individual methods have to be optimized for each of the cell lines or cell types used. Despite the fact that PEI transfection is widely used, many questions about its mode of action remain. Repetitive transfections in apparently identical conditions may results in significant day-to-day variation efficiency, despite the use of optimized protocols. Parameters as cell density, cell physiological state, cell stress state, cell cycle phase but also as modes of preparation of the PEI/DNA polyplexes play an important role in efficient transfer of plasmid DNA into nuclei of host cells.

A better understanding of the PEI transfection is expected to result in a more efficient ratio between plasmid DNA input and recombinant protein output.

7 MATERIALS AND METHODS

7.1 PLASMID PREPARATION

The pGag-eGFP plasmid used for generation of fluorescent VLPs was kindly provided by Dr. Julia Blanco at Fundació IRSI Caixa, Hospital Germans Trias i Pujol in Badalona, Spain. This plasmid codes for a Rev-independent HIV-1 Gag protein fused in frame to the enhanced GFP (48). The plasmid from the NIH AIDS Reagent Program (Cat 11468) was constructed by cloning the Gag sequence from pCMV55M1-10 (49) into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA).

The plasmid was amplified in competent *Escherichia Coli* DH5α strain stored in 100 µl samples at -80 °C, transfected by thermal shock and grown in LB medium (Conda, Madrid, Spain) supplemented with Kanamicin (10 µg/ml, Sigma, Saint Louis, MO, USA). Plasmid purification was carried out using the Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, as previously described (50).

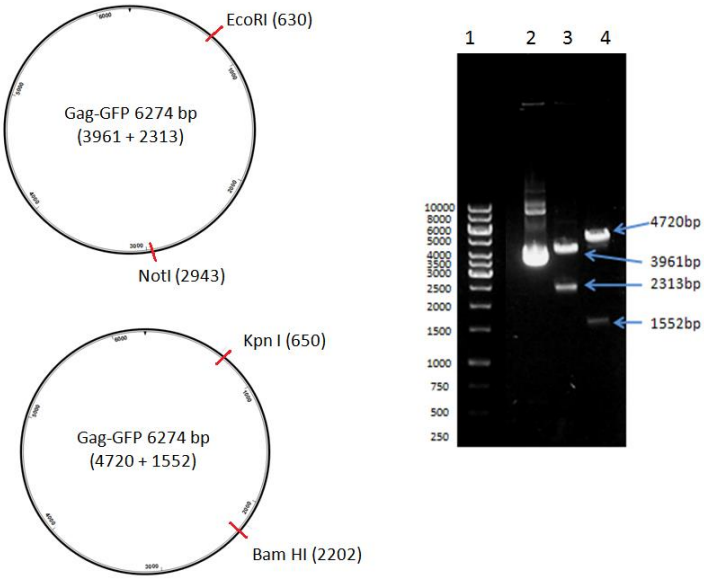


FIGURE 8 – PGAG-EGFP DIAGRAM AND AGAROSE GEL IN WHICH THE PLASMID WAS RUN AFTER SEVERAL ENZYME DIGESTIONS. RUN 1: MOLECULAR WEIGHT MARKER; 2: CONCENTRATED PLASMID; 3: PLASMID DIGESTION WITH ECO-NOT; 4: PLASMID DIGESTION WITH BAM-KPN.

DNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA integrity was evaluated by agarose gel electrophoresis and its identity confirmed by restriction mapping. Purified plasmids were typically in supercoiled form, free of RNA contamination and showed an A260/280 ratio of approximately 1.85. They were

diluted in mQ sterile water to achieve a final concentration of 100 ng/µl in the stock solution.

7.2 CELL LINE

The cell line used for VLP production is the HEK 293SF-3F6, kindly provided by Dr. Amine Kamen from the Biotechnology Research Institute (BRI), National Research Council (NRC) in Montreal, Canada. It was derived from a cGMP master cell bank that is available for manufacturing of clinical material. These cells were adapted to grow in suspension and serum-free conditions. Cells were cultured in FreeStyle 293 medium (Invitrogen, Carlsbad, CA, USA) optimized with the addition of non-animal derived components (39). The supplements used are r-insulin and r-transferrin from Merk Millipore (Kankakee, IL, USA) and an *in-house* developed animal component free lipid mix composed of synthetic cholesterol (SyntheChol®, Sigma-Aldrich, Steinheim, Germany), fatty acids (F7050, SAFC Biosciences), tocopherol (T1 157, Sigma) and emulsifying agents (PS80, Sigma), whose optimal concentrations were defined by DoE (39). Cells were routinely maintained in 125 ml disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, New York, USA) shaken at 110 rpm using an orbital shaker (Stuart, Stone, UK) containing a working volume of 20 ml. HEK 293 cells were maintained at a concentration between 0.3 and 2×10^6 cells/ml and viability usually between 85 and 95% in a humidified incubator at 37°C and 5% CO_2 in air. Cell density and viability were determined using a haemocytometer chamber (Neubauer) under an inverted phase contrast microscope (Zeiss, Germany) and trypan blue exclusion dye (Sigma, Steinheim, Germany) at a final concentration of 2% v/v. The average density of cells in the diluted sample for cell density and viability determination was around 25 cells per field. Equation [1] was used for cell density determination both of living and death, while equation [2] was used for viability assessment; *n* factors are the number of cells counted per field, discarding the most and the less populated four fields among the total eight.

$$Cellular\ concentration\ \left(\frac{10^6 cells}{ml}\right) = \frac{n_1+n_2+n_3+n_4}{2} * \frac{final\ volume}{initial\ volume} \qquad [1]$$

$$Viability\ (\%) = \frac{Living\ cells\ concentration}{Living\ cells\ concentration+Dead\ cells\ concentration} * 100 \qquad [2]$$

Characterization of the exponential growth curve was done determining the kinetic parameters of the phase. The specific growth rate (μ_{max}) is constant and maximum

in this phase and describes the duplication rate; it can be calculated through regression of the living cells concentration. The doubling time was calculated through the equation [3]:

$$t_d(h) = \frac{\ln(2)}{\mu_{max}} \quad [3]$$

7.3 PRODUCTION AND HARVEST OF GAG-EGFP VLPS

VLPs were produced by transient transfection of HEK 293 cells with Gag-eGFP plasmid DNA using 25 kDa linear PEI (PolySciences, Warrington, PA) as transfection reagent. Cells were typically transfected at 2×10^6 cells/ml (unless stated otherwise). The final DNA concentration was 1 µg/ml and the PEI:DNA mass ratio used for transfection was 2:1. DNA-PEI polyplexes were prepared in a volume of culture medium that corresponded to 10% of the final volume to be transfected. PEI was added to plasmid DNA diluted in culture medium. The mixture was vigorously vortexed three times and incubated at room temperature for 15 min before being added to the cell culture. Cell suspensions were harvested at the indicated time post-transfection. Cells were centrifuged at 1000×g for 5 min to separate cells and cell debris from cell culture supernatants containing VLPs.

7.4 QUANTIFICATION OF GAG-EGFP VLPS BY SPECTROFLUOROMETRY

Green fluorescence intensity in Gag-eGFP VLPs 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 values (RFU) were calculated by subtracting fluorescence intensity (FU) of negative control samples that were systematically included in every assay (usually, supernatant from untransfected cells or culture media) from the fluorescence given by the sample. RFU values can be converted to Gag-eGFP VLPs concentration values using equation [4], as previously described by the research group (15). There is a linear correlation between fluorescence intensity and p24 values determined using the INNOTEST ELISA HIV antigen mAb (Innogenetics NV, Gent, Belgium).

$$Gag - eGFP\ [\frac{ng}{ml}] = (3.6126 \times RFU - 14.935) \times 36 \quad [4]$$

Where Gag-eGFP is the estimated concentration of polyprotein and RFU is the measured eGFP fluorescence intensity of the samples. The first term is the correlation equation between fluorescence values and p24 concentrations determined by ELISA (Enzime-Lynked ImmunoSorbent Assay) and 36 is a correction factor that takes into account the difference in molecular weight between p24 and Gag-eGFP and an underestimation arising from using the p24 ELISA to estimate p55 Gag concentrations.

7.5 QUANTIFICATION OF TRANSFECTED CELLS

The percentage of positively transfected cells at the indicated time post-transfection was quantified by flow cytometry. Cells were fixed in order to enable later subsequent analysis. A 500 µl sample was harvested and centrifuged at 300×g for 5 min and consequently fixed through resuspension in formaldehyde (2% in PBS) for 10 min; afterwards the sample was centrifuged at 500×g to remove the fixation agent and resuspended in PBS. Fixed cells were analyzed in a FACSCanto Flow Cytometer (BDBiociencies, San Jose, CA) before five days after fixation.

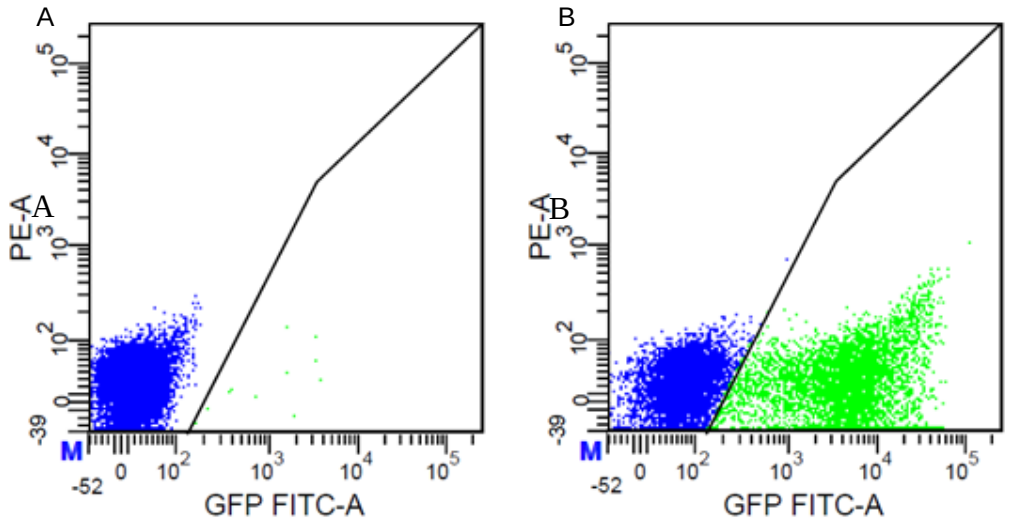


FIGURE 9 – TYPICAL TRANSFECTION RESULTS. GRAPH A SHOWS THE TYPICAL ANALYSIS RESULT OF A CONTROL (I.E. NOT TRANSFECTED) CULTURE. GRAPH B SHOWS THE TYPICAL ANALYSIS RESULT OF A TRANSFECTED CULTURE; TWO POPULATIONS ARE VISIBLE: BLUE ONE (NOT TRANSFECTED CELLS) AND GREEN ONE (POSITIVELY TRANSFECTED CELLS).

7.6 DETERMINATION OF CELL CYCLE PHASE

At the indicated time-point a 1 ml sample was taken from the cell culture and fixed for cell cycle analysis.

7.6.1 CELL FIXATION AND STAINING

The sample was centrifuged at 200×g for 6 min to harvest cells, rinsed and re-suspended in 500 µl PBS, than fixed with 4.5 ml cold 70% v/v ethanol for a minimum of 2 h at -20 °C to fix cells. Subsequently, fixed cells were centrifuged at 200×g for 5 min, rinsed and stained with 1 ml of staining solution for at least 1 h at room temperature prior data acquisition. The staining solution consist of 20 µl of Propidium Iodide 1 mg/ml (PI) (Sigma) solution, 20 µl RNase DNase-free 10 mg/ml solution and 1 µl 0.1% v/v TritonX-100 solution in PBS per each ml of final volume.

PI is an intercalating agent with excitation wavelength at 535 nm and emission at 617 nm, when it is bound to nucleic acids; nucleic acids bounding also enhances around 30-fold its fluorescence. Thus, PI does not interfere with the excitation wavelength of the eGFP. PI has no sequence preference but binds to RNA too, thus necessitating a previous sample treatment with RNases (51).

7.6.2 FLOW CYTOMETRY ANALYSIS

The DNA content of a cell doubles during S phase and maintains its maximum quantity during G2 and M cell cycle phase before cytokinesis. It is possible, using specific fluorescent DNA, stains such as PI to quantify the relative amount of DNA in each cell of a population using flow cytometry. The result is a cell cycle profile of the analyzed population, a histogram reflecting the distribution of cells according to their DNA content. Cells with double DNA amount will have twice the fluorescent signal than cells with normal DNA quantity. Cells actively duplicating their DNA will have a fluorescent signal that falls in-between.

Forward scatter, side scatter and UV-fluorescence (FL4) were acquired with a FACSCalibur Flow Cytometer (BD, San Jose, CA, USA) for each sample. Cell cycle analysis was performed on the acquisition of minimum 20’000 events in order to have well defined profiles. Determination of the percentage of cells in the different phases of the cell cycle was done through software analysis (Mod Fit LT,

Variety Software House, Tropsham, ME, USA) when well distinguishable G0/G1 and G2/M peaks were present. For blocked or synchronized populations, when G0/G1 or G2/M peaks were not present (or not significantly enough to be recognized as such by software analysis), gating of regions of interest was performed to calculate the percentage of cells in the different phases. Regions gated manually were compared with software analysis for well-defined profiles to ensure that the estimation was correct.

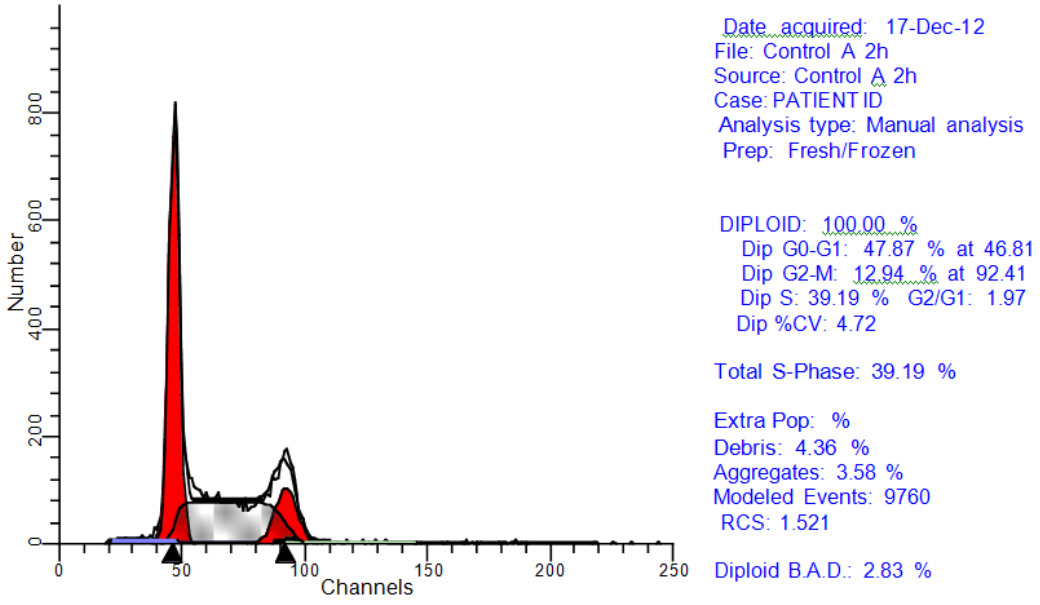


FIGURE 10 – TYPICAL RESULT OF A CELL CYCLE ANALYSIS. Y-AXIS INDICATES THE NUMBER OF CELLS WITH SUCH FLUORESCENCE, X-AXIS IS DIVIDED INTO CHANNELS CORRESPONDING TO AN IDENTICAL AMOUNT OF DETECTION WINDOW FOR INCREASING FLUORESCENCE INTENSITY. RED PEAK AT CHANNEL 46.81 INDICATES G0/G1 PHASE CELLS; RED PEAK AT CHANNEL 92.41 INDICATES G2/M PHASE CELLS; GREY AREA INDICATES S PHASE CELLS.

7.7 MTT ASSAY

MTT assay is a colorimetric assay for measuring activity of cellular enzymes which reduce the tetrazolium dye (MTT) to its insoluble form (formazan), giving a purple sample. In cell biology, this test is used to assess cell viability. The MTT is reduced to formazan in the presence of living cells.

In this work, the MTT assay was used for toxicity testing. In a 96-well plate were cultivated 100 µl HEK293 at the concentration of 0.5×10^6 cells/ml together with 10 µl of different concentrations of the compound under toxicity testing. The plate was grown under standard HEK293 cell culture conditions. After three days, 20 µl of MTT reagent were added to each well and the plate was again incubated for one

hour at 37 °C with agitation of 110 rpm. The day of the test was conducted using a 12 wells as calibration line. In each of these 12 wells, 100 µl of HEK293 cells at various concentrations, ranging from 0 up to a maximum concentration 2×10^6 cells/ml were placed. Such curve was performed in duplicate.

Absorbance was measured in a spectrophotometer at a wavelength of 490 nm. Using data obtained from the calibration curve, it was possible to extrapolate the absorbance values at the cell concentration, which indicates the toxicity of the various compounds tested.

7.8 CELL CYCLE CHEMICAL BLOCKING AND CELL SYNCHRONIZATION

The cell cycle phase profile of a normal, i.e. non-synchronized, inoculum was followed. To draw this profile the growth in supplemented FreeStyle medium of an inoculum of 20 ml of cells at the concentration of 0.3×10^6 cells/ml was followed for eight days and cell cycle analysis done every 24 h. Exact percentages highly depend on several variables such as cell state, exact time of analysis and more. In order to be used as control, the cell cycle phase profile of cells at the concentration of 2×10^6 cells/ml was also followed for 24 h and analyzed every hour.

Cell cycle blocking was performed using two different reagents in separated experiments to control that no side effects arose from the use of the drug. Nocodazole was used to arrest the cells in the prometaphase and thus enrich the culture with G2/M phase cells, as it is known that Nocodazole-treated cells arrest with a G2- or M-phase DNA content when analyzed by flow cytometry. Hydroxyurea was used to arrest the cells in the G0/G1 phase. Drug blocking effect was assessed by following cell cycle phase kinetics.

7.8.1 CELL CULTURE CHEMICAL SYNCHRONIZATION

Drug removal was done by medium exchange and flask change: the whole culture volume was centrifuged at 200×g for 6 min to harvest cells which were then immediately and gently resuspended in the same volume of fresh pre-heated medium in a new Erlenmeyer flask.
Cell cycle phase deregulation kinetics were followed by cell cycle analysis, cell concentration and viability.

8 RESULTS AND DISCUSSION

8.1 GROWTH PROFILE

The growth of the HEK293 cell line in supplemented FreeStyle medium has been followed starting with an inoculum of 0.3×10^6 cells/ml. Cell concentration and viability have been assessed every twenty-four hours (Figure 11).

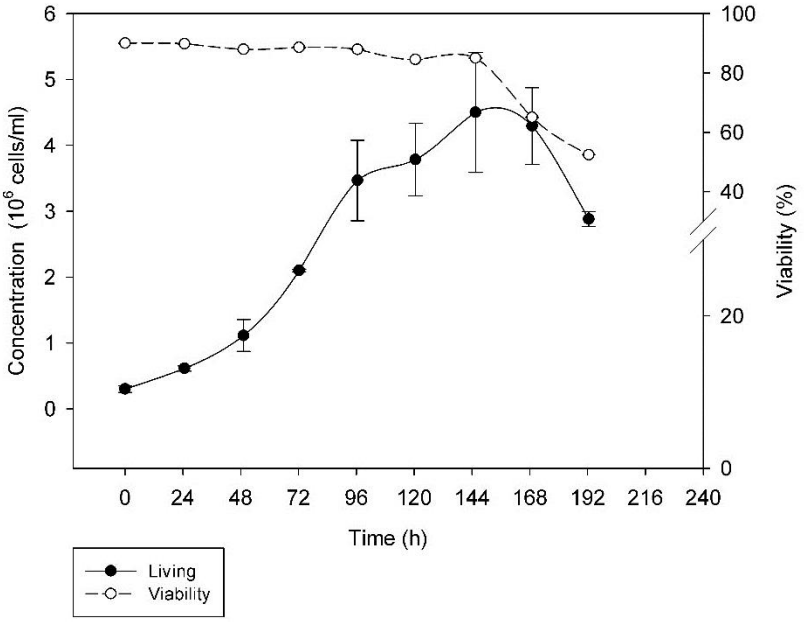


FIGURE 11 - HEK293 GROWTH CURVE IN SUPPLEMENTED FREESTYLE CULTURE MEDIUM.

The cell inoculum is adequate as there is no lag phase and cells directly enter log phase; exponential growth lasts four days, consequently cells enter stationary phase and, at seventh day, start to die. The culture grows to a maximum concentration of 4.80×10^6 cells/ml with over 85% viability and shows an average doubling time of 33.2 h, the calculated maximum growth rate (μ_{max}) is 0.021 h^{-1}; these data are in agreement with previous work (39).

8.2 TRANSFECTION PROTOCOL STRENGTH TESTING

The main problem of the transient transfection is its efficiency variability, which makes industries discard this technique when they want to prepare a product for the market. In the results obtained in this work, such variability could also be observed, as it can be noticed in Figure 12. Each replicate was performed by following always the standard protocol, but, even so, variability on positively transfected cells and quantity of VLPs produced was high. Usually, the average transfection percentage is 45% 48 hpt and 55% 72 hpt, while the average VLPs production is quantified around 22 RFU 48 hpt and 35 RFU 72 hpt.

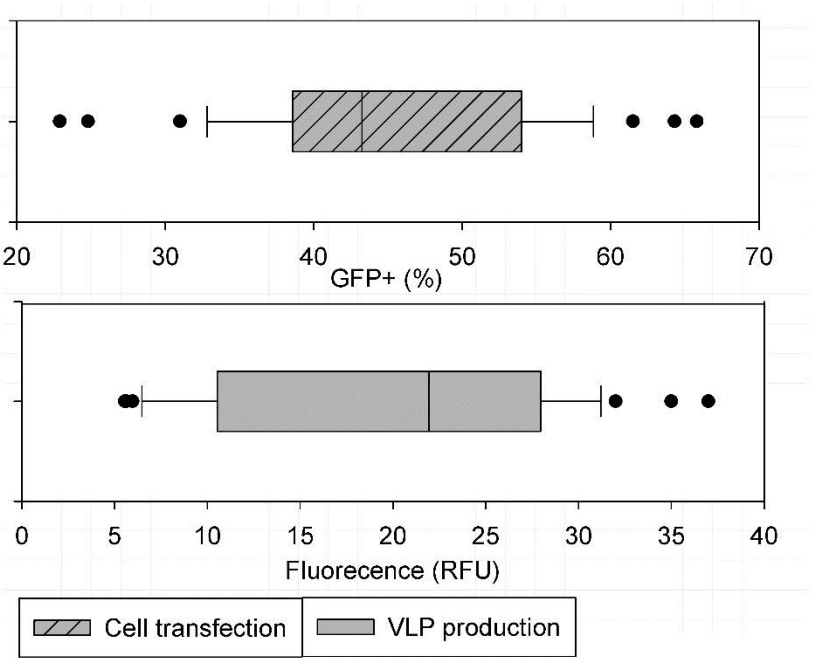


FIGURE 12 – POSITIVELY TRANSFECTED CELLS PERCENTAGES AND THE RELATIVE FLUORESCENCE UNITS OF THE CULTURE SUPERNATANTS 48 HPT IN THIRTY-THREE INDEPENDENT EXPERIMENTS.

In order to reduce this variability a wide range of variables in the transfection protocol was taken into account; after compiling a list of those convided more relevant, a large number of experiments was done to evaluate each variable effect independently. Each experiment has been done in triplicate to evaluate standard deviation and repeated more times to assess repeatability.

The tested variables were the following:

- Variables affecting the polyplexes formation:
    - Supplemented vs. non-supplemented FreeStyle culture medium as solvent for the DNA and PEI mixture;

  - Pre-heated vs. not pre-heated culture medium as solvent for the DNA and PEI mixture;
  - Vortexed vs. not-vortexed mixture for the preparation of polyplexes;

- Variables affecting the transfection procedure:

  - Age of used flasks;
  - Cell stress due to medium exchange;

- Not-depending on the protocol variables:

  - Cell passage at transfection time-point;
  - Cell concentration at transfection time-point;
  - Cell cycle phase at transfection time-point.

For each, the routine transfection procedure was strictly followed a part of the tested variable; cell growth, viability and positively transfected cells percentage was quantified 24, 48 and 72 hpt, as well as VLPs production; p-values were calculated with the null hypothesis that the results are significantly different.

8.2.1 SUPPLEMENTED VS. NON-SUPPLEMENTED FREESTYLE CULTURE MEDIUM AS SOLVENT FOR THE DNA AND PEI MIXTURE

Supplemented FreeStyle medium was used as solvent for the DNA and PEI mixture to analyze if it has any effect over transfection and VLP production. As control, the solvent used to prepare the mixture was non-supplemented FreeStyle medium. Results are shown
Figure 13.

There is no significant difference in transfection efficiency and VLP production between using one or the other medium, although it could be sad that cells are positively affected by a second addition of a 10% in culture volume of fresh medium, leading to a higher viability of cells in the off 72 hours. Nonetheless, the supplemented components added to the FreeStyle medium do not interact with DNA and PEI prejudicing polyplexes formation. Based on these data, supplemented FreeStyle medium was from now on used as solvent for the preparation of polyplexes.

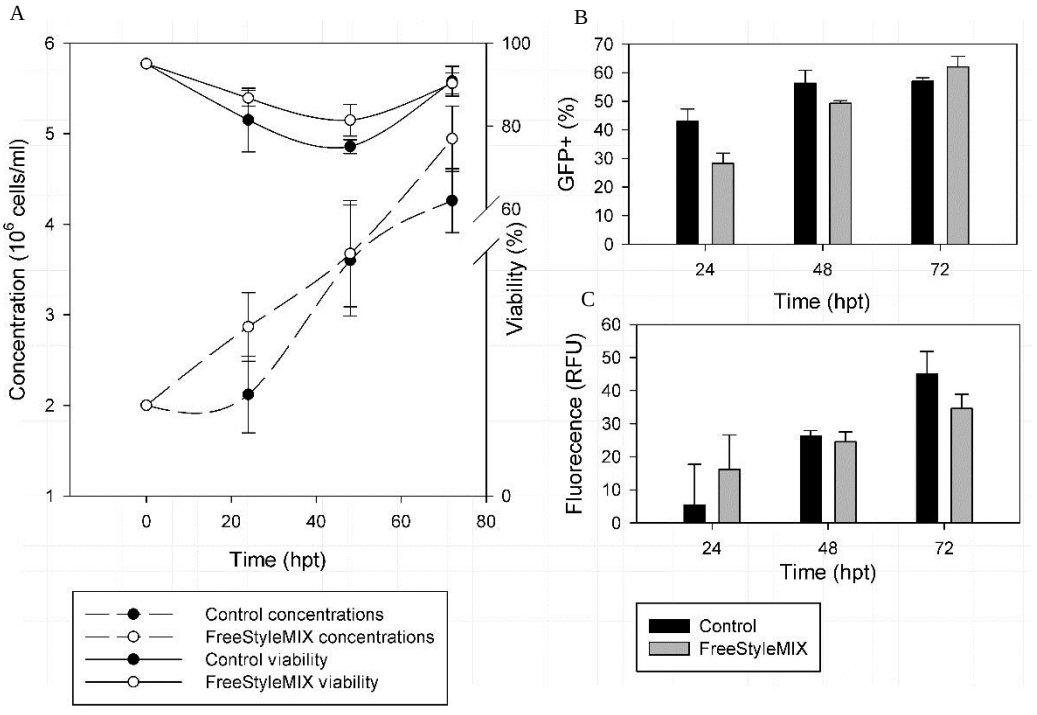


FIGURE 13 – SUPPLEMENTED FREESTYLE CULTURE MEDIUM AS SOLVENT FOR THE DNA AND PEI MIXTURE RESULTS. (A) CELL CONCENTRATION AND VIABILITY; (B) TRANSFECTION PERCENTAGES; (C) VLP PRODUCTION.

8.2.2 PRE-HEATED VS. NOT PRE-HEATED CULTURE MEDIUM AS SOLVENT FOR THE DNA AND PEI MIXTURE

Cell growth, transfection efficiency and production were compared between cells transfected using a not-pre-heated solvent per mixture and a control mixture where the solvent was pre-heated. Following the routine procedure, the solvent used to prepare the mixture has to be pre-heated at 37 °C (the same temperature as the cultivated cells) before the reagents addition. But, as it is stored at 4 °C, this could lead to loss of time and/or to inhomogeneous proper heating of the culture medium. Results are shown in
Figure 14.

The addition of such cold volume affects cells viability for the firsts hours post transfection but has no effect on the percentage of transfected cells and quantity of VLPs produced at the hours post transfection (hpt) of interest.

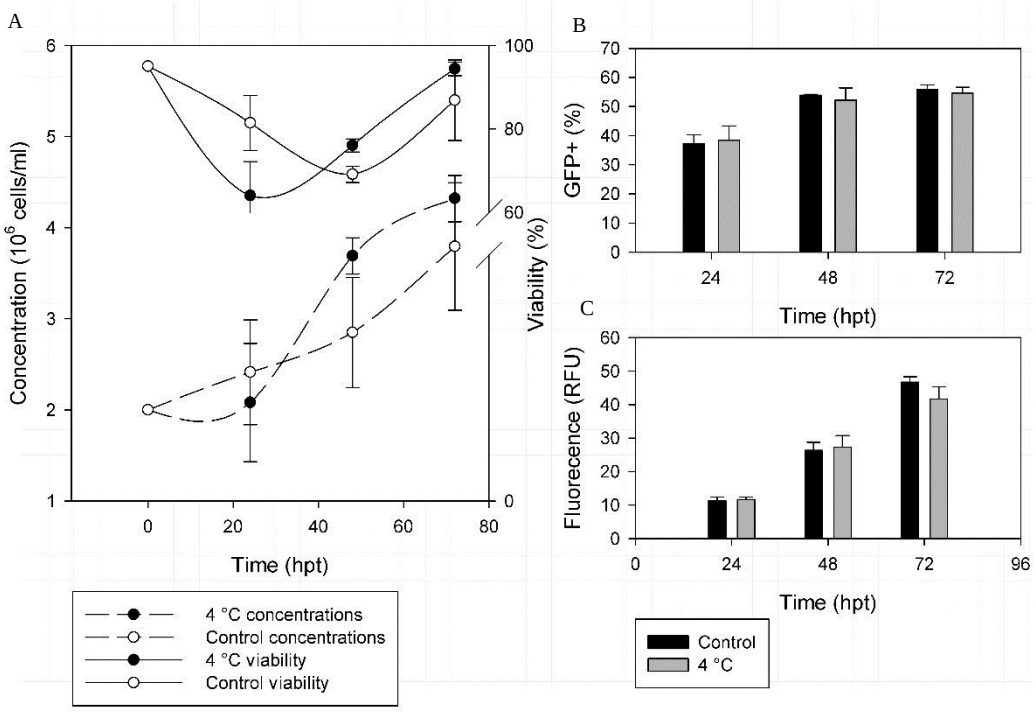


FIGURE 14 – NOT PRE-HEATED SOLVENT FOR THE DNA AND PEI MIXTURE RESULTS. (A) CELL CONCENTRATION AND VIABILITY; (B) TRANSFECTION PERCENTAGES; (C) VLP PRODUCTION

8.2.3 VORTEXED VS. NOT-VORTEXED MIXTURE FOR THE PREPARATION OF POLYPLEXES

One of the lasts steps in the mixture preparation is the vortexing step: the mixture has to be vortexed 10 sec to dissolve DNA and 3 times per 2 sec to solubilize PEI. Cell concentration and viability after transfection, positively transfection percentages and VLP production data of a cell culture transfected using a not-vortexed mixture were compared with a control culture transfected using a vortexed mixture. The not-vortexed mixture was gently inverted one time and the control mixture was vortexed as stated by the standard protocol. Results are shown in Figure 15.

Cells are transfected even in the not-vortexed case, meaning that the polyplexes are however able to form. Transfection percentages are comparable with the control at the indicated hpt. Cell concentration and viability are not affected by the vortexing step as these data are comparable between the not-vortexed case and the control.

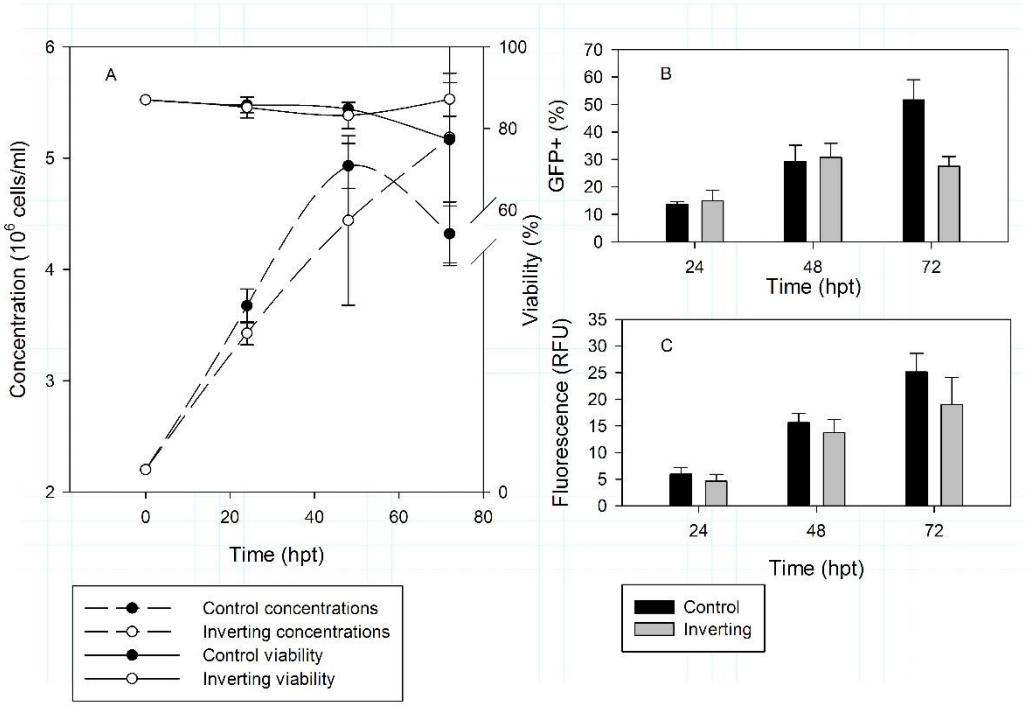


FIGURE 15 - NON-VORTEXED DNA AND PEI MIXTURE RESULTS. (A) CELL CONCENTRATION AND VIABILITY; (B) TRANSFECTION PERCENTAGES; (C) VLP PRODUCTION.

8.2.4 AGE OF FLASKS

The polycarbonate Erlenmeyer flasks used in this work are designed for single use, but, due to their expensive cost, they were re-used. The flasks are routinely cleaned at the end of an experiment, but the cleaning and sterilization procedure could in some way affect their integrity and thus affect the final results of the experiments. In fact, the cleaning and sterilization procedure includes 10 min sonication at room temperature while washing with 1 M sodium hydroxide, abundant water and mP water rinsing, and irradiation with gamma ray. Old flaks, i.e. flasks that have undergone a large number of cleaning and sterilization cycles, appear matter than new ones.

In order to evaluate if the age of the flask used to culture cells may have some effect over cell growth, transfection and production, cells were re-suspended after medium exchange (and before transfection) in a completely new Erlenmeyer flask. Results were compared with a parallel experiment done using some visibly very opaque flasks. Results are shown in
Figure 16. There is no considerable effect on cell growth post-transfection, transfection efficiency and VLPs production so, this variable has no influence.

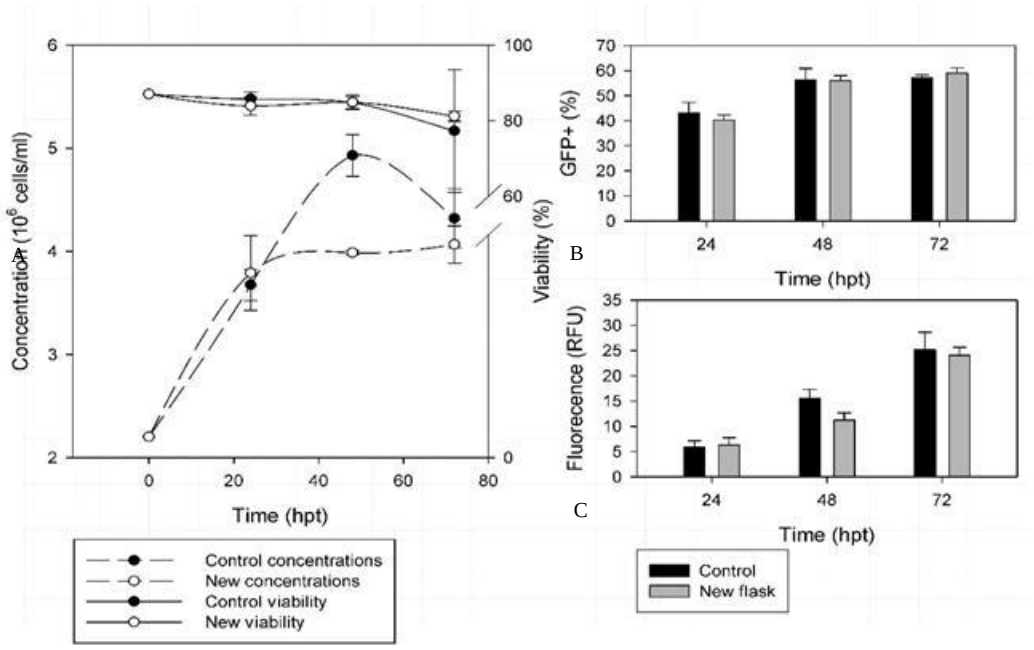


FIGURE 16 – NEW FLASK AS CULTURE FLASK AFTER TRANSFECTION. (A) CELL CONCENTRATION AND VIABILITY; (B) TRANSFECTION PERCENTAGES; (C) VLPS PRODUCTION.

8.2.5 CELL STRESS DUE TO MEDIUM EXCHANGE

The procedure used for transfection states to exchange culture medium with fresh medium before transfecting, as this influences cells making them more active and permits to eliminate possible sub-products secreted by cells during their culture, that could interfere with transfection. As it involves a centrifugation step and some re-suspensions trough pipetting, it could also stress cells. To test if this variable has some effect, a cell culture was incubated four hours after medium exchange and before transfection, to let cells escape such "stress state".

Cell growth, transfection efficiency and VLP production were quantified comparing with a control experiment where cells were not subjected to the extra incubation time. Results are shown in
Figure 17. No significant differences between the two conditions were observed, thus, this variable has no effect.

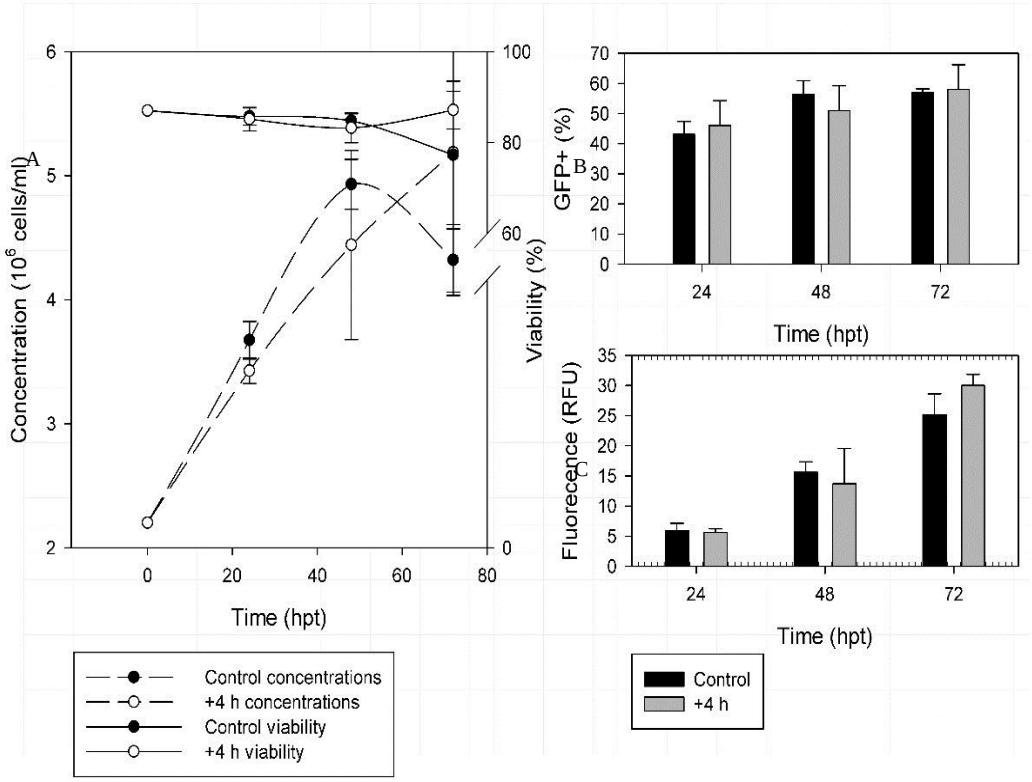


FIGURE 17 – ADDITIONAL INCUBATION TIME RESULTS.. (A) CELL CONCENTRATION AND VIABILITY; (B) TRANSFECTION PERCENTAGES; (C) VLPS PRODUCTION.

8.2.6 CELL PASSAGE AT TRANSFECTION TIME-POINT

For maintenance, every two or three days cells are reseeded at a final concentration of 0.3×10^6 cells/ml, resulting in a new passage. Cells available in the master cell stock are around passage 40 and can typically be maintained until passage 100. To know if cells react differently to transfection due to their passage, recently de-frozen cells (passage 47), mid-age cells (passage 77) and old cells (passage 97) were transfected. Results are shown in
Figure 18.

Thus, the passage number does not influence transfection, neither VLP production, but, as the technique has a toxic effect over cells, it is important not to use too old cells. Many cells are killed by transfection and those who survive decrease their growth rate, as VLP production requires a strong metabolic effort, even due to the CMV promoter. This is why the use of use of old cells must be avoided.

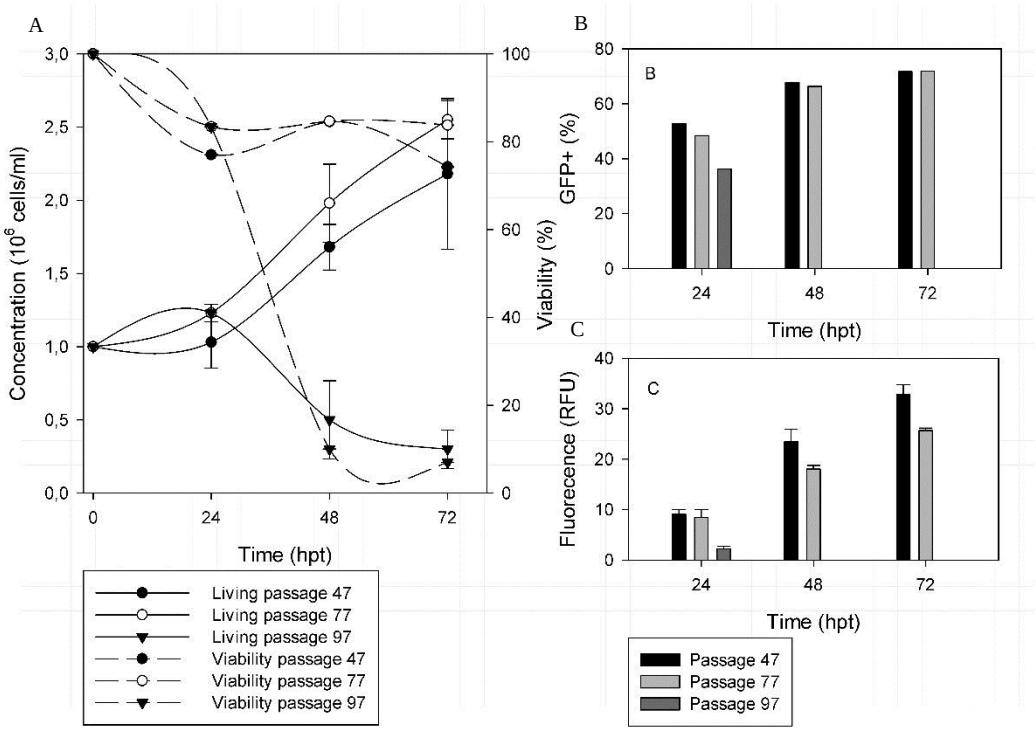


FIGURE 18 - CELL PASSAGE EFFECT RESULTS. (A) CELL CONCENTRATION AND VIABILITY; (B) TRANSFECTION PERCENTAGES; (C) VLPS PRODUCTION.

8.3 CELL CONCENTRATION AND CELL CYCLE PHASE EFFECT

In order to understand better the correlation between the healthy state of cells in the culture, their concentration and the cycle phase of the culture related to transfection and VLP production, data coming from independent replicates were deeply analyzed.

Moreover, with the same aim, a further experiment was done. The growth curve of an inoculum of cells at the concentration of 0.3×10^6 cells/ml was followed by analyzing every 24 hours its cell concentration, viability and cell cycle phase. Every 24 h a volume of 20 ml was taken from the culture and separated into a new flask to be transfected. The experiment scheme is explained in Figure 19. Transfection was carried out without medium exchange. Cell growth, transfection percentages and VLP production of the separated and transfected volumes were quantified at 48 and 72 hpt.

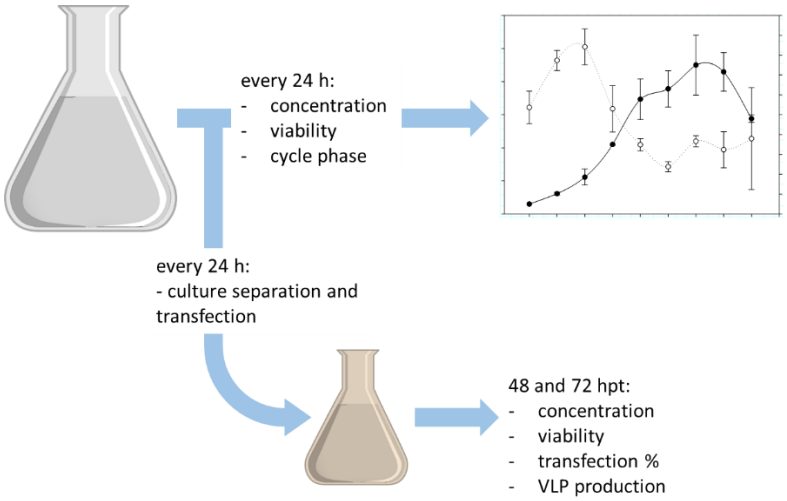


FIGURE 19 – SCHEMATIC REPRESENTATION OF THE TRANSFECTION OF A VOLUME COMING FROM A GROWTH CURVE.

8.3.1 DEEPER DISCUSSION OVER CELL CONCENTRATION AT THE TIME-POINT OF TRANSFECTION

Analyzing the data shown in Figure 12, as it was expected, it can be noticed how a major quantity of positively transfected cells leads to higher concentrations of VLP in the supernatant, as it is described by Figure 20.

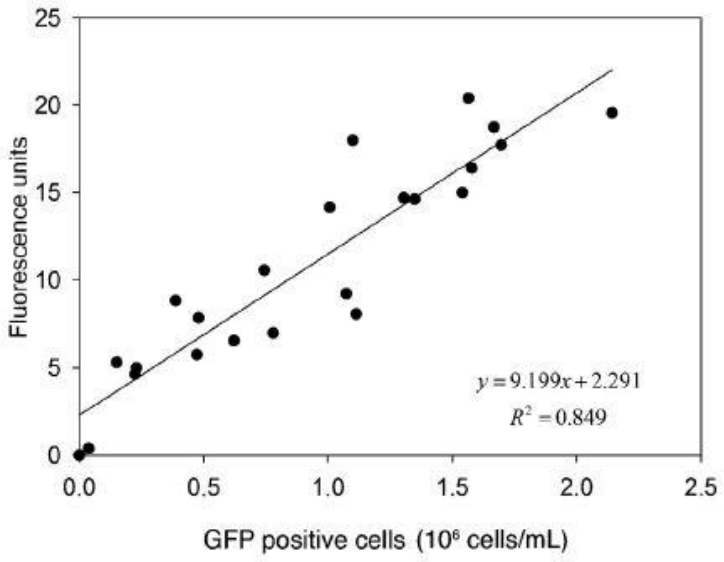


FIGURE 20 - DIRECT CORRELATION BETWEEN POSITIVELY TRANSFECTED CELLS AND VLP PRODUCTION 48 HPT.

What could be thought, is that transfecting a higher concentration of cells should give as result a higher protein production; but this is not the case, as stated by the *cell density effect, i.e. the drop in the specific productivity in the baculovirus-insect cells expression system when cells are infected at high cell densities* which, actually, can be extended even to mammalian and in suspension cell cultures. *So, if specific productivity can be maintained at its maximum level, increasing cell density improves volumetric production; unfortunately, it is well documented that insect cell lines currently used for protein production undergo a severe loss in specific productivity when infected at high cell densities* (52) (53) (54). This effect is not so well described for mammalian cells and suspension cultures, while there are studies that relate the initial cell density and the specific growth in batch culture, where kinetics studies have been done to quantitatively analyze the reduced growth rates (55). The results of the experiment described in paragraph 8.3 can be traced to some sort of cell density effect and will be presented and described in the following paragraph.

Transfected volumes were separated from a growing culture started with an inoculum, thus, every 24 h, i.e. the time the volume was separated to be immediately

transfected and cell concentration assessed, the cell concentration in the separated volume was higher. This means that every 24 h a denser culture was transfected. Positive transfected cell percentages were analyzed 48 and 72 hpt (which is also 48 and 72 hours post volume separation). This allowed to relate transfection percentages and cell concentration. Results are shown in Figure 21.

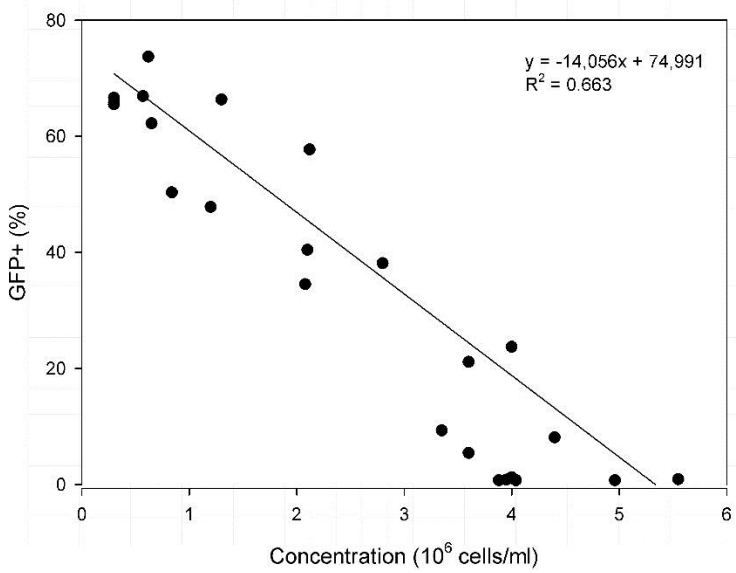


FIGURE 21 - PERCENTAGE OF GFP+ CELLS 48 HPT RELATED TO CELL CONCENTRATION AT TRANSFECTION TIME-POINT IN MILLION CELLS PER ML.

As it can be noticed, the higher the cell concentration at the transfection time-point the lowest the percentage of positively transfected cells 48 and 72 hpt (72 hpt data are not shown, but the trend is maintained). Over ~ 4×10^6 cells/ml transfection percentages reach zero, thus there is no transfection. On the other hand, the highest transfection percentages are reached at very low cell concentrations, for example at ~ 0.6×10^6 cells/ml around ¾ of cells in the culture are transfected. But, it is necessary to refer to the parameters of transfection and to the physiological state of the culture. The subtracted volume was always transfected with the same quantity of polyplexes (1µg/ml of DNA, cf. Materials and Methods), which can justify the high and the low transfection rates respectively. When cells are at a very low concentration, when the polyplexes are added into the culture, they are very available for cells, which can easily get in contact with them; as cells become more concentrated, the meeting between the two is always more difficult. But, this is not the only reason. High cells concentrations are achieved after days of incubation (typically the highest cell concentration is reached at the seventh day, cf. Figure 11); this means that, as time passes, the culture gets older, accumulating waste products and housing cells entering a worst physiological state.

Due to the toxic effect of transfection and the metabolic effort necessary for VLP production, it appears not to be appropriate to transfect cells that do not show a good physiological state. This assumption is confirmed by the growth rates after transfection. In spite of the effort cells have to do after transfection, the growth rates are positive until the fourth day of transfection (cell concentration: ~ 2×10^6 cells/ml) and from the fifth are negative (data not shown).

8.3.1.1 CELL PHYSIOLOGICAL CONDITIONS-INDEPENDENT CELL DENSITY EFFECT

In order to analyze deeper the effect of polyplexes availability and the physiological condition of cells, the following experiments were designed.

An appropriate volume of cells was expanded to the concentration of 2×10^6 cells/ml, than cells were concentrated at 2, 3 and 4 $\times10^6$ cells/ml and transfected with standard and higher final DNA concentration (respectively 1 and 3 µg/ml). An alternative transfection protocol was followed too. This protocol has been proposed by Bollin F. (56) and adapted to the conditions used in this work: an appropriate culture volume was centrifuged, keeping in mind that the final volume of transfection was 20 ml, and the cell pellet was gently re-suspended in the transfection mix, transferred in a flask containing 50% of pre-warmed fresh culture medium. Cell culture was than incubated under agitation for 1 h and the other half of medium was added. Using this second transfection protocol, cells were concentrated at 4×10^6 cells/ml and separately transfected with a final DNA concentration of 1 and 3 µg/ml. These two experiments are schematically explained in Figure 22.

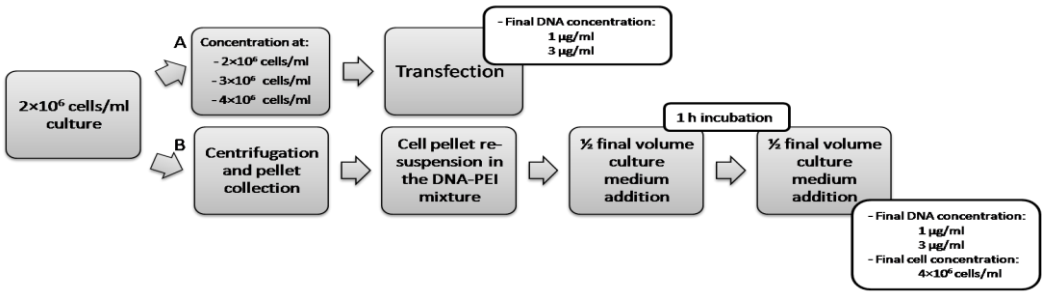


FIGURE 22 – SCHEMATIC REPRESENTATION OF THE EXPERIMENTS DONE TO ASSESS THE CELL DENSITY EFFECT INDEPENDENTLY FROM THE PHYSIOLOGICAL CELL STATE. WAY (A), CONCENTRATION AND TRANSFECTION EXPERIMENTS. WAY (B) "BOLLIN METHOD" EXPERIMENTS.

A 2×10^6 cells/ml culture was concentrated in order to reach higher concentration levels but with cells which physiological conditions were the same as in the

standard protocol and thus separate the effects of cell concentration and physiological state of the culture. The higher quantities of DNA used for the polyplexes formation permitted to be sure that polyplexes concentration was enough to transfect even really high cell concentrations. The expected effect of the so called "Bollin method" was to force contact between cells and polyplexes, thus increasing transfection rates. Results are shown in Figure 23.

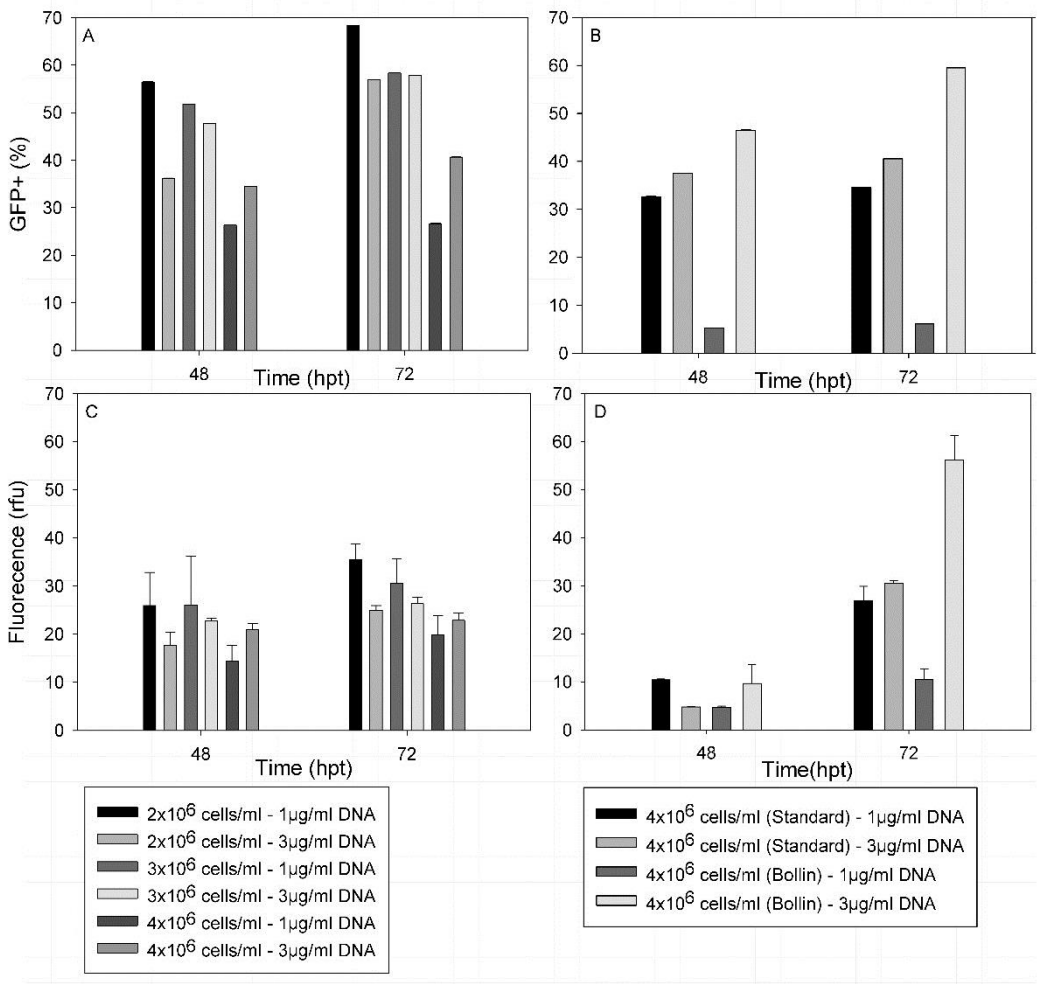


FIGURE 23 - POSITIVELY TRANSFECTED CELL PERCENTAGES ARE SHOWN 48 AND 72 HPT. (A) SHOWS THE RESULTS OF THE TRANSFECTION DONE FOLLOWING THE STANDARD PROTOCOL, CONCENTRATING CELLS AND USING TWO DIFFERENT FINAL CONCENTRATIONS OF DNA FOR THE PREPARATION OF THE POLYPLEXES. (B) SHOWS THE TRANSFECTION RESULTS OBTAINED WITH THE "BOLLIN METHOD". (C) AND (D) SHOW THE VLP PRODUCTION RESPECTIVELY OBTAINED THROUGH THE STANDARD AND THE BOLLIN PROTOCOL.

As it is shown in Figure 23A, the highest transfection percentages are reached only strictly following the standard protocol, where there is no actual cell concentration and the final DNA concentration is 1 µg/ml. Using higher DNA concentrations doesn't mean to transfect more cells, as long as cell concentrations are not really high (2 and 3×10^6 cells/ml). In fact, comparing transfection percentages, those are lower as DNA quantity is increased but cell density is maintained constant,

probably due to a toxic effect of DNA on cells, as, if viability of cells after transfection is compared (data not shown), it can be noticed a higher decreasing rate of viability when cells are transfected with 3 µg/ml DNA. This means that, as DNA quantity is increased, the living cells concentration after time-point post-transfection decreases, resulting in a lower living cell transfected population.

This is not true when cells concentrated at 4×10^6 cells/ml are transfected. Using higher DNA concentration a larger percentage of cells is transfect, compared to cells transfected with 1 µg/ml DNA. However, transfection percentages are generally lower than those reached transfecting cells at lower concentrations. This could be because the quantity of polyplexes used to transfect cell at these high concentrations is not sufficient to reach higher transfection percentages or because the polyplexes are not available for cells in the culture medium.

The results obtained using the "Bollin method" are shown in Figure 23B. The percentage of GFP+ cells transfected using the "Bollin method" is comparable to the percentage of GFP+ cells reached when following the standard protocol. Cells transfected using the "Bollin method" and a DNA final concentration of 1 µg/ml almost weren't transfected (transfection percentages are around 5% at 48 as well as at 72 hpt), but, by increasing DNA concentration, higher transfection percentages are reached. So, if cells are concentrated to 4×10^6 cells/ml and transfected with a final DNA concentration of 3 µg/ml following the "Bollin method", transfection percentages reach 60% 72 hpt and are comparable to the results obtained using the standard protocol.

The quantity of VLP production is shown in Figure 23C and D. As production is directly related to transfection percentages, as, assuming that each cell has got more or less the same specific productivity, the more cells are transfected the higher will be the production; these data mirror the result just described. It has to be noticed that the relative fluorescence of the supernatant of the culture of cells concentrated and transfected with the "Bollin method" and with the higher DNA concentration is the highest among all. This is because volumetric productivity depends on cell density that here is the highest, so, even if the transfection percentage is comparable with the one reached following the very standard protocol, VLP production reached is higher.

Thus, it has been demonstrated that cell concentration has a big effect on transfection and production and that, by transfecting higher cell concentrated cultures, it is possible to reach a higher production. As some cell density effect

appears, it is possible to avoid it by concentrating cells that are in an active and healthy state and by forcing polyplexes of transfection entering cells. VLP production was also improved, increasing the fluorescence of the culture supernatant harvested 72 hpt of around twenty-five arbitrary units, which means an increment of production of ~ 2.5 µg/ml.

8.3.2 DEEPER DISCUSSION OVER CELL CYCLE PHASE

The effect of cell cycle phase over transfection and VLP production was studied too. As already introduced, cell cycle phase may influence the transfection efficiency. The cell cycle analysis of the data presented in Figure 12 was therefore performed. As observed in Figure 24 a direct correlation between the cell cycle phase and the transfection percentage could be observed, so, the higher the percentage of cells in G2/M phase at the time-point of transfection, the higher the percentage of transfected cell.

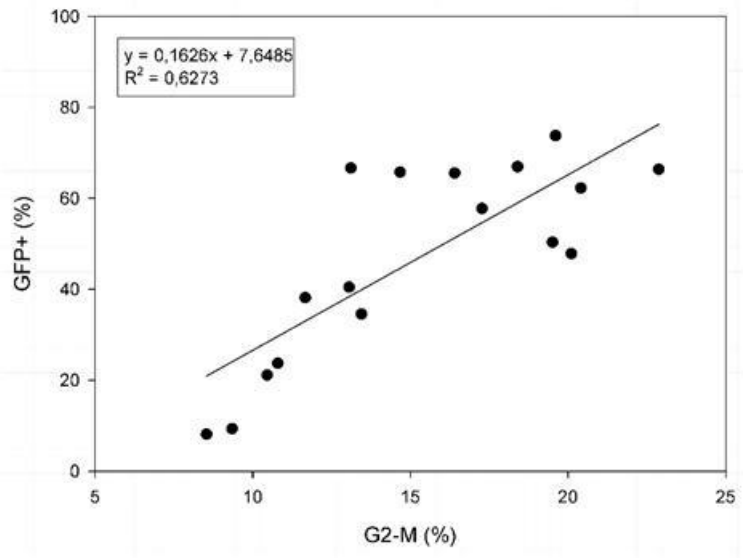


FIGURE 24 - CORRELATION BETWEEN CELLS IN G2/M PHASE PERCENTAGE OF CELLS IN THE CULTURE AND TRANSFECTION PERCENTAGE 48 HPT.

These data are also in agreement with those obtained in the experiment described in paragraph 8.3 and shown in Figure 25, but here, as already described, some cell density effect has to be taken into account.

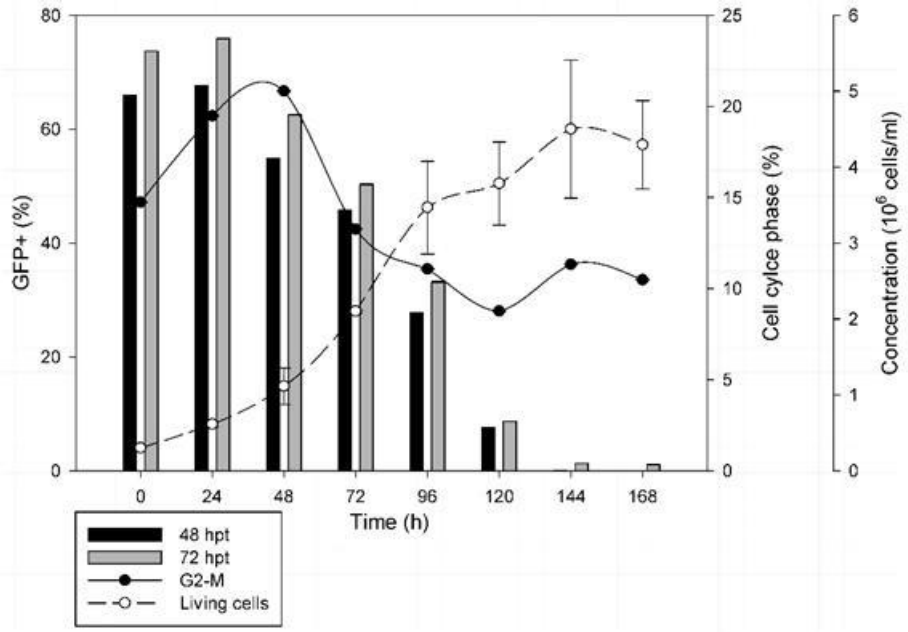
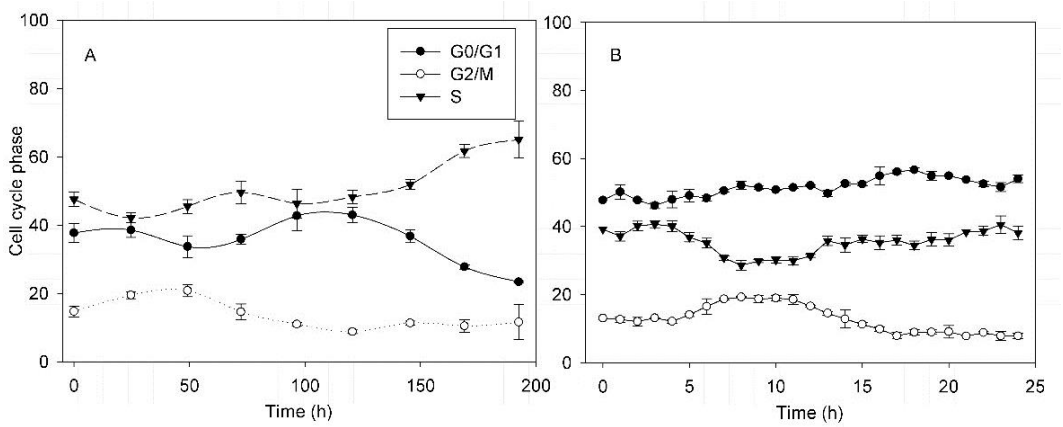


FIGURE 25 - RESULTS IN TRANSFECTION PERCENTAGES RELATED BOTH TO CELL CYCLE PHASE OF THE CULTURE AND TO CELL CONCENTRATIONS.

In order to analyze the cell cycle phase effect, two different cell cultures were blocked in two specific cycle phases using two different reagents. As control, the cell cycle phase of a normal, i.e. non-blocked and asynchronous, cell culture was analyzed starting from a culture inoculum.

In a normal inoculum, cells are usually divided into phases as the followings: ~ 35% G0/G1 phase, ~ 15% G2/M phase and ~ 50% S phase. As cell culture grows, G2/M phase cell percentages reach a peak around 20% at the third day, than low down and S phase percentages increase as cell culture moves to death phase.



GRAPH 1- (A) SHOWS THE TYPICAL CYCLING PROFILE OF A CULTURE THROUGH ALL ITS GROWTH, STARTING WITH AN INOCULUM AT THE CONCENTRATION OF 0.3×10^6 CELLS/ML. (B) SHOWS THE PHASES PROFILE FOR 24 H OF A 2×10^6 CELLS/ML CULTURE

Nocodazole and Hydroxyurea were use to block cell division and to obtain two different blocked cell cultures. Previously cytotoxicity was determined and the kinetics of cell cycle blocking was followed. As last, after-drug-removal kinetics were followed.

Results showed that:

- Nocodazole has to be added at a final concentration of 2 µg/ml and cells have to be incubated with for 26 h to reach the maximum blocking effect and have a culture were ~ 86% of cells are in G2/M phase;
- Hydroxyurea final concentration has to be 1.25 mM and incubation time 24 h as to have a ~ 75% G0/G1 cell culture;
- In both cases, drug removal is simply possible by complete medium exchange and flask change; after that, cells recover from the effect and start to divide again, but, only in the case of Hydroxyurea a synchronized cell culture is obtained (in the case of Nocodazole cells slowly leave G2-M phase).

8.3.2.1 NOCODAZOLE CYTOTOXICITY, EFFECT AND WHOLE CULTURE BLOCKING

Nocodazole cytotoxicity was studied by incubating cells in 96 well plates at different drug concentrations performing an MTT assay. Results are illustrated in Figure 26. Calculated ranges for Nocodazole were 0-5 µg/ml as final concentration and are in agreement with bibliography data (57).

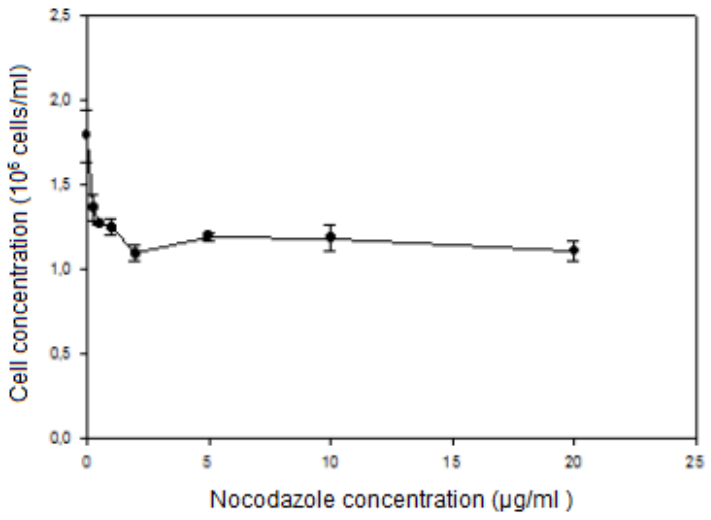


FIGURE 26 - NOCODAZOLE TOXICITY EXPERIMENT RESULT.

Four Nocodazole concentrations among the range were tested to know which has the major effect. So, cells were amplified at a final concentration of 2×10^6 cells/ml and incubated with 0 (as control), 0.2, 2 and 5 µg/ml Nocodazole for 12 h. After the incubation time, cell cycle phase analysis was performed. Results are shown in Figure 27. The major blocking effect is reached when 2 µg/ml Nocodazole are added to the culture, so that the major percentage of cells is blocked in G2/M phase.

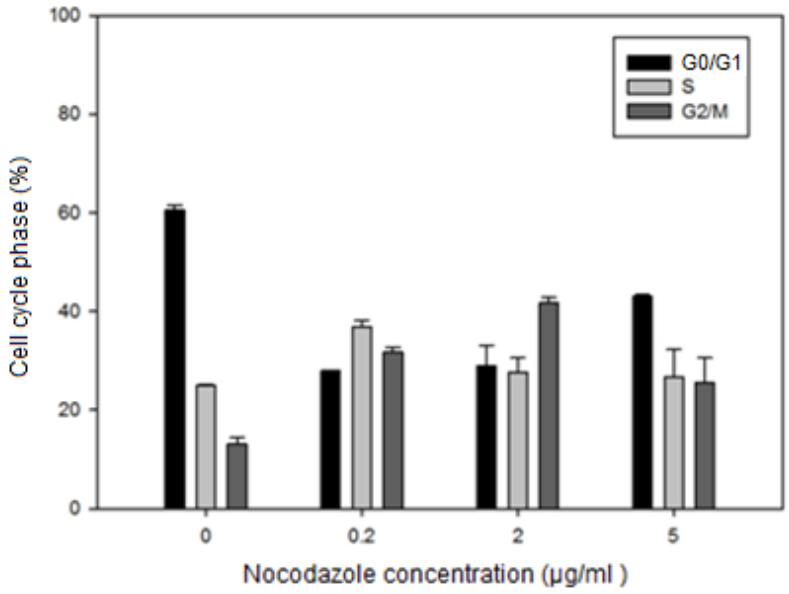


FIGURE 27 - DIFFERENT NOCODAZOLE CONCENTRATIONS RELATED TO THEIR EFFECT OVER CELL CYCLE PHASE

To know the blocking profile, a culture of 2×10^6 cells/ml was blocked using 2 µg/ml Nocodazole and cell cycle was analyzed every hour for 24 h. The blocking profile curve is shown in Figure 28.

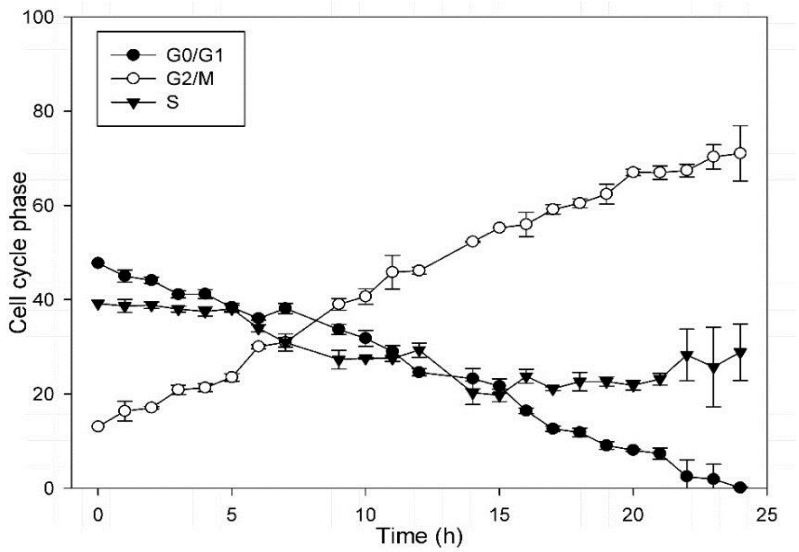


FIGURE 28 - CYCLE PHASE PROFILE OF CELLS BLOCKED WITH NOCODAZOLE.

It can be noticed that Nocodazole blocks cells in G2/M phase but at 24 h the percentage is not stabilized into a constant value. Thus, a second experiment was done, incubating cells for a longer time and analyzing the cell cycle every hour but between 24 and 33 h post Nocodazole addition. Results show that after 26 h the percentages stabilize as G0/G1 and S percentages reach zero and G2/M percentage is now stable (data not shown).

8.3.2.2 CELL CYCLE DEREGULATION AFTER NOCODAZOLE CYCLE BLOCKING

In order to know the profile of cells cycling after having been blocked with Nocodazole, cell cycle phase of an enriched culture has been analyzed after drug removal. An appropriate volume of cell culture was expanded to the concentration of ~ 2×10^6 cells/ml and incubated for 26 h with 2 µg/ml Nocodazole, medium exchange and flask change were than performed and culture deregulation followed by cycle phase analysis every hour until 57 hpmc (hours post medium exchange). Figure 29 illustrates the cycling profile of the culture that does not appear to be synchronized. After 9 hpmc cells recover from the blocking effect and start to cycle by slowly leaving G2/M phase and entering G1/G0 phase and thus S phase. Around 33 hpmc the culture stabilizes and start to have the normal cycle phase profile.

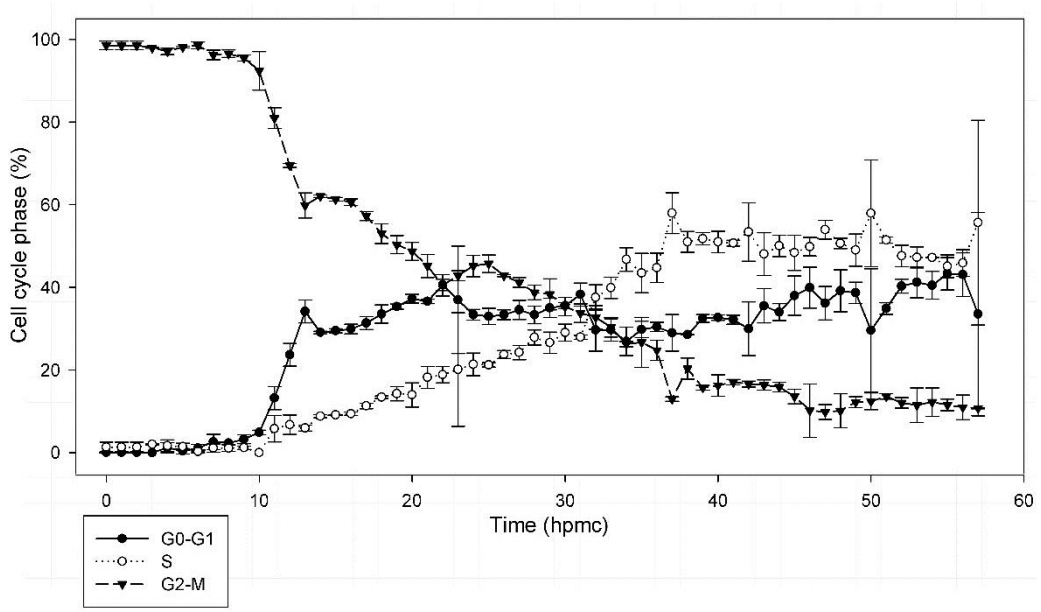


FIGURE 29 - CELL CYCLE DEREGULATION AFTER NOCODAZOLE REMOVAL.

8.3.2.3 HYDROXYUREA CYTOTOXICITY, EFFECT AND WHOLE CULTURE BLOCKING

Usually (58), Hydroxyurea is added to cell cultures in a concentration between 2 and 4.3 mM, but the effect might be different among cell types. To assess the cytotoxic effect of Hydroxyurea over HEK293 cells a wide range of concentrations has been tested. Firstly the MTT assay was performed and resulted that at very low drug concentrations (until 0.5 mM), Hydroxyurea seems not to have any effect over cells; until 110 mM no toxic effect appears. A considerable toxic effect appears over 1 M drug concentration.

As 1 M is a very high drug concentration, another set of drug concentrations was tested to assess which among the used (referring to bibliography data) has the minor cytotoxic effect at long time after addition. Thus, cells were expanded to ~ 2×10^6 cells/ml in 6 well plates and Hydroxyurea was added to the culture at the concentrations of 1.25, 2.50, 3.75 and 5 mM. After 23 h incubation, cells viability was determined. There is no considerable cytotoxic effect even after longer times of exposure.

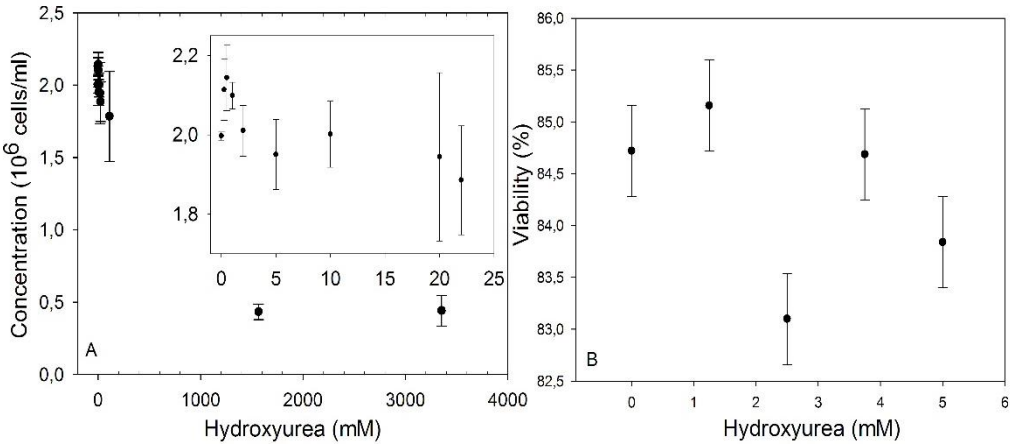


FIGURE 30 – (A) HYDROXYUREA MTT ASSAY RESULTS. INNER GRAPH ZOOMS BETWEEN 0 AND 25 MM CONCENTRATIONS OF HYDROXYUREA. (B) VIABILITY OF CELLS AFTER 23 H OF INCUBATION WITH HYDROXYUREA.

The Hydroxyurea effect over cell cycle phase of the culture has been tested. Cells were expanded to ~ 2×10^6 cells/ml in 6 well plates and Hydroxyurea was added to the culture at five chosen concentrations (1.25, 2.50, 3.75, 5.00 and 100.00 mM). Cells were than incubated and cell cycle phase of the culture was analyzed 10 and 23 hours post drug addition (hpp) to determine the blocking effect of the drug in comparison with a control culture where no Hydroxyurea was added (Figure 31).

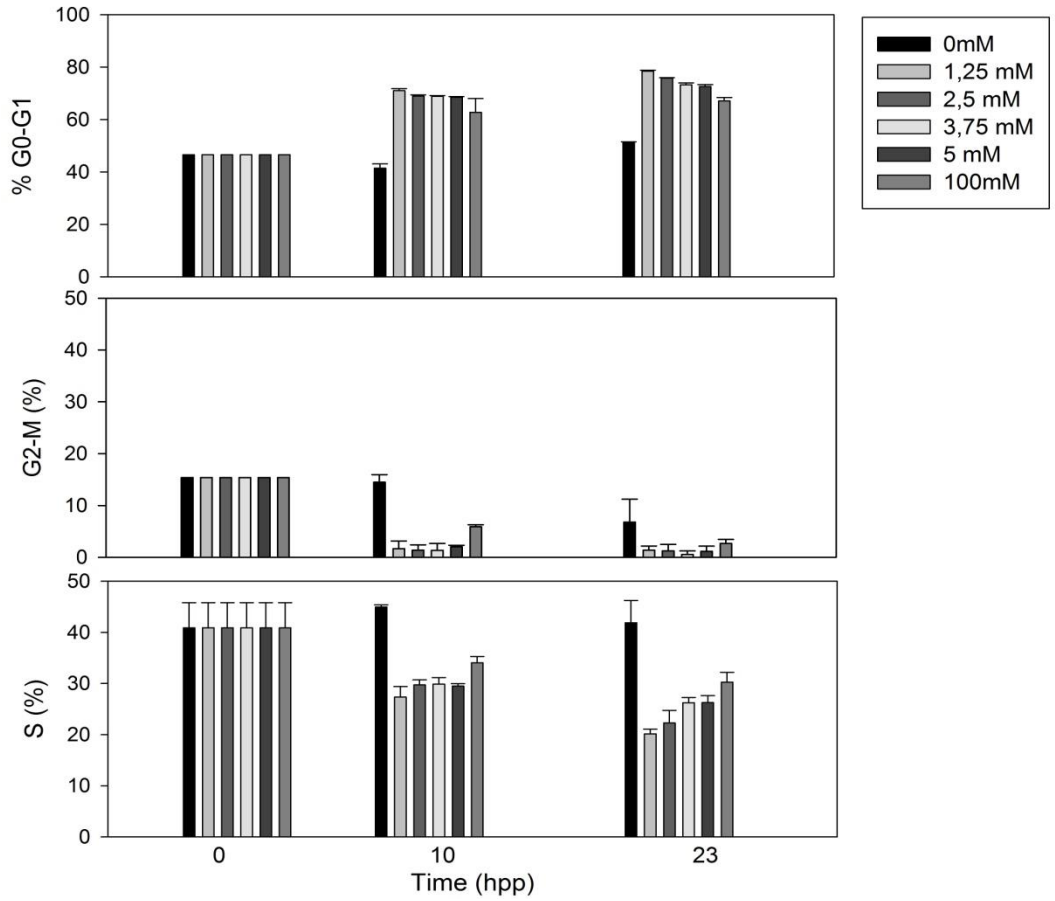


FIGURE 31 – HYDROXYUREA EFFECT OVER CYCLE PHASE.

By the addition of Hydroxyurea, cells are blocked in G0/G1 phase so the culture is enriched of this kind of cells as time passes. As there is no toxic effect at these drug concentrations, viability doesn't drop down but cell concentration does not increase, as cells are unable to divide. The major effect is reached using a concentration of 1.25 mM, so this will be the concentration used on every further experiment.

To find the incubation time necessary to have a sufficiently enriched culture, an appropriate volume of cell culture was expanded to the concentration of ~ 2×10^6 cells/ml and 1.25 mM Hydroxyurea has was added. Every hour a sample was taken to analyze cell cycle phase. After 24 h a high percentage (75%) of G0/G1 blocked cells is reached.

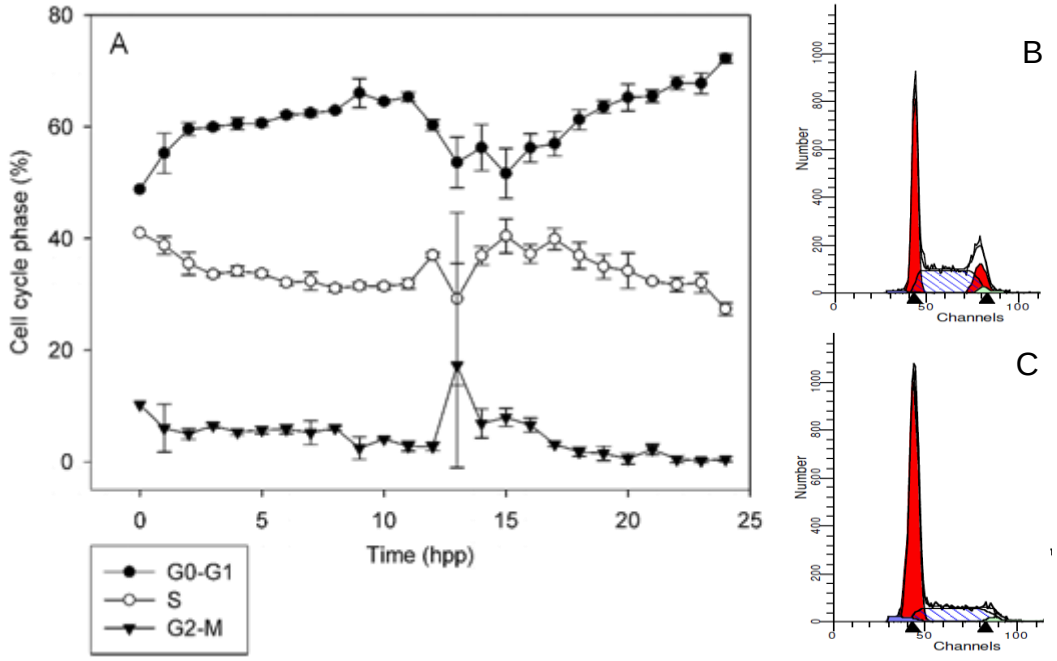


FIGURE 32 – (A) HYDROXYUREA BLOCKING CELL CYCLE PHASE PROFILE; (B) STARTING CULTURE CELL CYCLE PHASE PROFILE HISTOGRAM; (C) FINAL (AFTER 24 H INCUBATION WITH HYDROXYUREA) CELL CULTURE PHASE PROFILE HISTOGRAM.

8.3.2.4 CELL CYCLE DEREGULATION AFTER HYDROXYUREA CYCLE BLOCKING

In order to know the profile of cells cycling after having been blocked with Hydroxyurea, the cell cycle phase of an enriched culture has been analyzed after drug removal. An appropriate volume of cell culture was expanded to the concentration of ~ 2×10^6 cells/ml and incubated for 24 h with 1.25 mM Hydroxyurea, medium exchange and flask change were than performed and culture deregulation followed by cycle phase analysis every hour until 25 hpmc and every two hours between 25 and 33 hpmc. The cycling profile is described in Figure 33. After 5 hpmc cells completely recover from the blocking effect and start to cycle synchronously. As they start leaving G0/G1 phase, enter S phase until a maximum percentage; so, leave this phase too and enter G2/M phase. A full cycle is done after 21 hpmc, when the culture reaches a second maximum in G0/G1 phase, and synchronization is gradually lost, in fact, only 50% of cells is now in this phase.

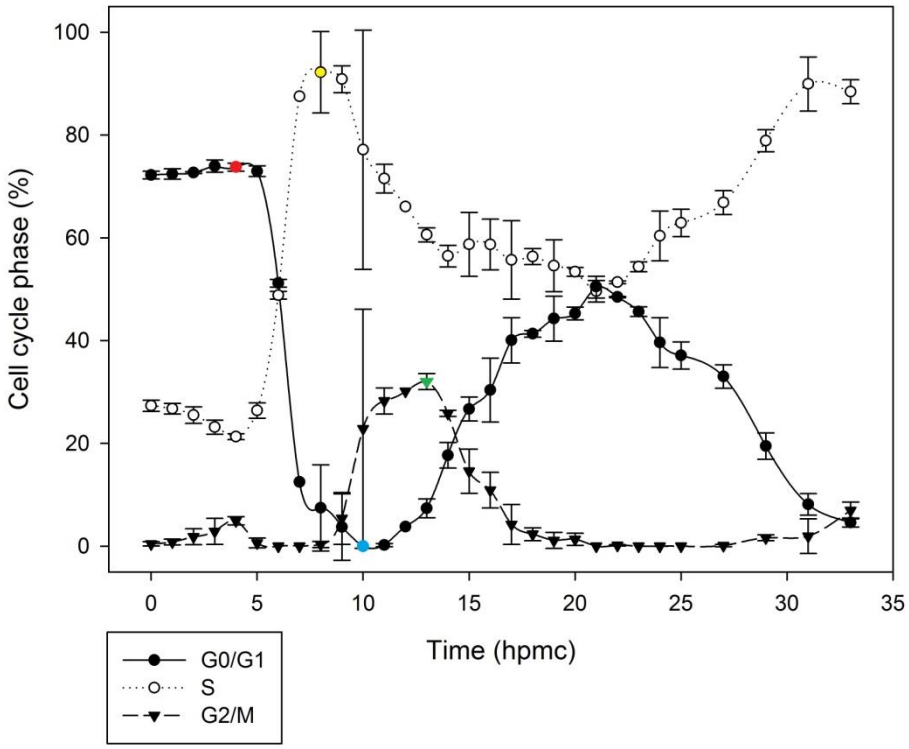


FIGURE 33 – SYNCHRONIZED CULTURE OBTAINED BY HYDROXYUREA CYCLYING PROFILE. NOTABLE TIMES ARE UNDERLINED WITH COLOURED SYMBOLS IN THE CELL CYCLE PHASE CURVES.

There are four notable times in the curves: until 5 hpmc the maximum of cells in G0/G1 phase is present and at 4 hpmc (red symbol) cells recover from the drug effect, as they start to enter S phase; at 8 hpmc (yellow symbol) the maximum percentage of cells are in S phase (usually ~ 90%); at 13 hpmc (green symbol) cells reach the maximum in G2/M phase (~ 30%). At 10 hpmc (blue symbol) there isn't any cell belonging to G0/G1 phase and this is the absolute minimum of the G0/G1 percentage curve.

8.3.2.5 TRANSFECTION TIMING

Times involved in cell transfection can be a very interesting variable that has not been well studied yet and for the aims of this work it is important to know how long do the PEI/DNA polyplexes take to enter cells in suspension. Some previous work have tracked the pathway of PEI/DNA complexes for gene delivery into different kinds of cells; this has been possible through confocal microscopy, fluorescent labeled PEI and DNA and the expression of eGFP encoded in the reporter plasmid. Thus, it has been possible to track the entrance of both PEI and DNA into cells and also estimate the time needed for the reporter gene expression. The evidence is that by 30 min post transfection PEI/DNA complexes begin to attach to cell surfaces forming aggregates. Endocytosis of the complexes begins 2-3 hours post transfection and nuclear localization is common 3.5-4.5 hpt. After one more hour, the earliest transgene expression is observed (Figure 34) Thus, in this work a time of four hours was accepted as the time polyplexes take to enter cells.

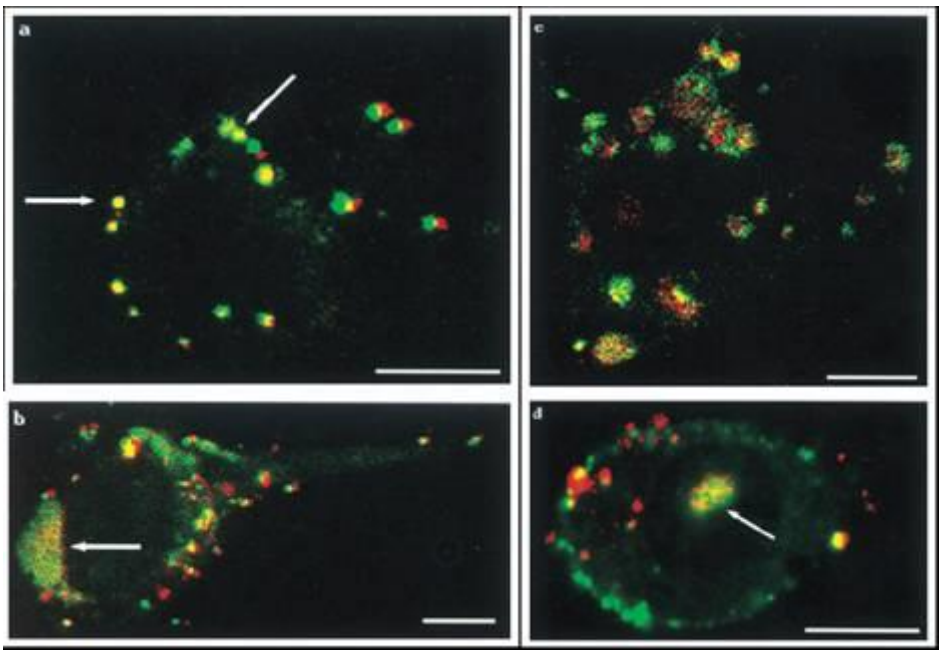


FIGURE 34 – TRACKING OF DOUBLE-LABELED PEI/DNA COMPLEXES. (A) AT 2 HPT VISIBLE COMPLEXES APPEAR AS CLUMPS ON THE CELL'S EXTERIOR, AS INDICATED BY ARROWS. (B) AT 3 HPT BOTH SURFACE AGGREGATION AND ENDOSOMES ARE VISIBLE. THE ARROW INDICATES ENDOSOMAL FORMATION. (C) AT 4 HPT ENDOSOMES ARE VISIBLE THROUGHOUT THE CELL CYTOPLASM. (D) AT 4.5 HPT FLUORESCENT STRUCTURES CONTAINING BOTH PEI AND DNA IN SIDE THE CELL NUCLEUS ARE PRESENT. (BAR = 10 µM) (61)

8.3.2.6 ENRICHED CULTURE TRANSFECTION

After having obtained two specific cycle phase enriched cultures, drug removal was performed and cycling cultures were obtained. In order to study the cell cycle phase effect over transfection, these cell cultures were transfected.

In the case of Nocodazole blocked cell culture, it is possible to take advantage only of the high percentage of cells in G2/M phase. So, the enriched culture was transfected 4, 6 and 8 hours before drug removal, to take the maximum possible advantage from the cycle phase effect. Another enriched culture was transfected right after medium exchange too. Such times were selected in order to permit the complete entrance of the PEI/DNA polyplexes into the cells and to lose, through medium exchange, the minor quantity of polyplexes that still were in the culture medium.

An appropriate volume of cell was expanded to 2×10^6 cells/ml, incubated with 2 µg/ml Nocodazole for 26 h and transfected at those precise time-points, medium exchange was performed before or after transfection as designed. A control culture without any Nocodazole addition was expanded and transfected as well. It was expected to obtain very high transfection percentages as polyplexes were forced to enter cell in G2/M phase. Results are summed in Figure 35.

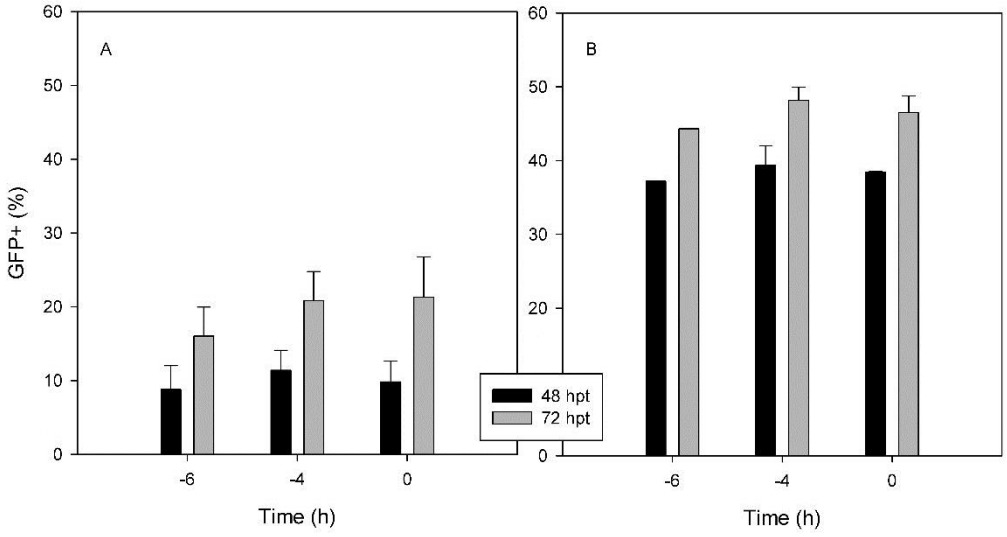


FIGURE 35 – (A) TRANSFECTION OF CELLS RELEASED FORM NOCODAZOLE EFFECT; (B) TRANSFECTION OF THE CONTROL CULTURE.

Generally, it was obtained a considerable transfection percentage even if transfection was made before medium exchange, indicating that polyplexes do enter cells in the interval time between transfection and medium exchange. There is no

difference between the samples transfected before and after medium exchange in the transfection percentages, confirming that even the shortest interval permits transfection. But, comparing these data between enriched and control culture, the transfection percentages of the enriched culture are considerably lower, which is in contrast with the expected. VLP production data reflect this trend, but also indicate that even if cells were blocked with Nocodazole, if they are transfected, they are able to produce VLPs. There must be some side effect within the cycle blocking effect of Nocodazole that does not permit cell transfection.

In the case of Hydroxyurea, a synchronized culture was obtained. The culture was transfected at the four notable times selected in the deregulation curve (Figure 33), keeping in mind the four hours gap between the transfection of the culture and the actual time polyplexes enter cells (cf. paragraph 8.3.2.5). This is because polyplexes had to enter cells at the exact maximum peak, i.e. at each of the four notable times, in order to take the maximum advantage from the cell cycle phase effect. It was expected to observe the cell cycle phase effect differentiated through phases.

Cells were expanded to 2×10^6 cells/ml, incubated with 1.25 mM Hydroxyurea for 24 h, while a control culture was only expanded to the same concentration. Medium exchange was performed and the culture was transfected 1, 4, 6 and 9 hpmc. Transfection at 1 hpmc means that the polyplexes enter cells at 5 hpmc, when they recover from drug effect, restart to cycle and the maximum percentage of cells is in G0/G1 phase. Transfecting the culture 4 hpmc means that the polyplexes enter the cells at 8 hpmc, when the S phase peak is reached; 6 hpmc is equal to transfection at 10 hpmc when there is the minimum in G0/G1 phase and culture transfection 9 hpmc is cell transfection 13 hpmc, at G2/M maximum peak.

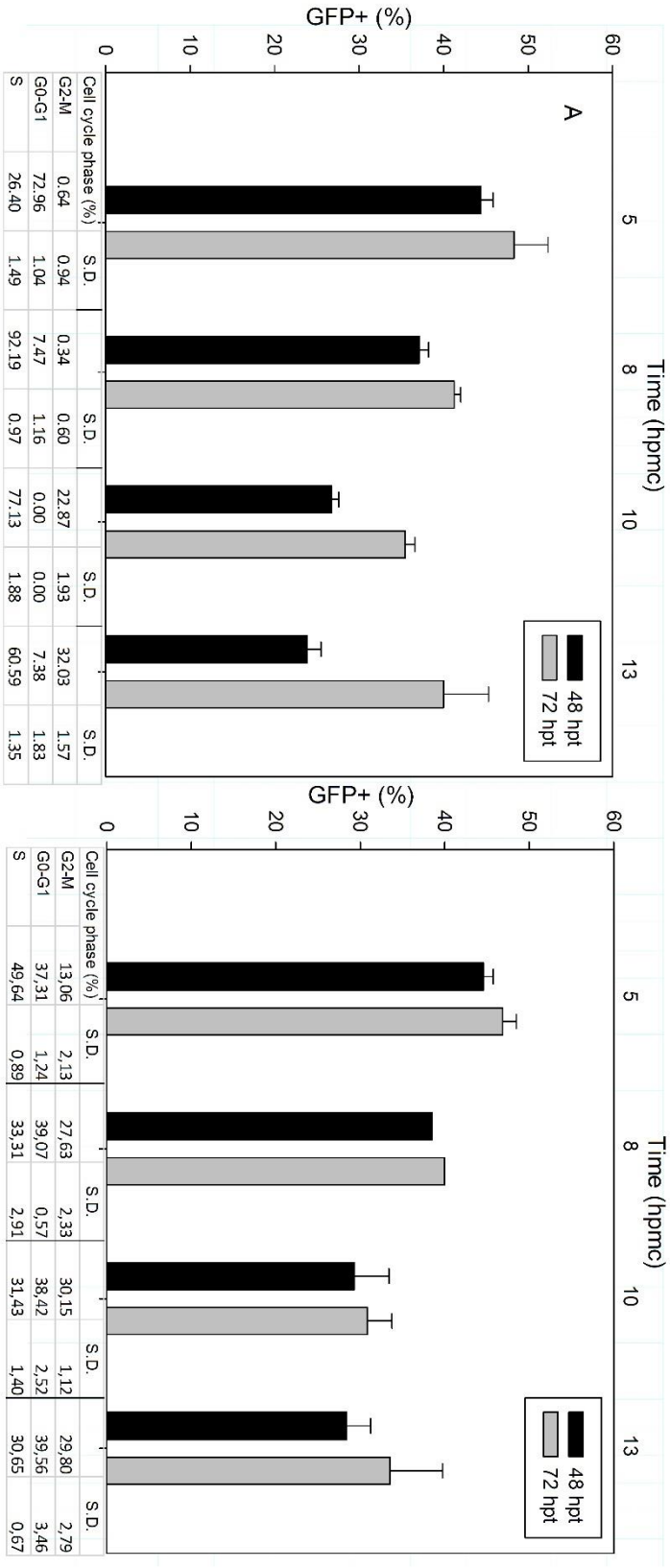


FIGURE 36 – RELATION BETWEEN POSITIVE TRANSFECTION PERCENTAGES, TIME AFTER DRUG REMOVAL AND CELL CYCLE PHASE. (A) SYNCHRONIZED CULTURE DATA. (B) CONTROL CULTURE DATA.

The observed results (Figure 36) show that the transfection percentage of the synchronized culture at the G2/M phase peak is not the highest. But there are some considerations that can be done. At the selected hpmc the cycle phase percentages are in agreement with those in the deregulation curve (Figure 33), meaning that four hours post culture transfection cell cycle phase is not affected by transfection and thus cells continue cycling as expected.

Looking at the transfection percentages 48 hpt of the synchronized culture, it can be noticed that there is transfection at every chosen hpmc, which correspond to peaks in cell cycle phases percentages curve. GFP+ cell percentage is higher when cells in G0/G1 phase peak are transfected (5 hpmc, no cell in G2/M phase) and it is the lowest when cells in G2/M phase peak are transfected (13 hpmc). Then, it could be thought that transfection is completely independent from cell cycle phase. The control culture, i.e. the non-blocked and asynchronous culture, maintains such transfection profile. It has to be noticed also that cells are transfected at increasing hpmc. It can be noticed that the transfection percentages low down as time passes from medium exchange. It is probable that transfection percentages depend much more on culture physiological state after medium exchange than they do on cycle phase, leading to the obtained results. The lower transfection percentage has been obtained 13 hpmc, which is a considerable lack of time, while the highest transfection percentage is obtained transfecting cells only 5 hpmc. Moreover, transfection percentages at 5 hpmc of the synchronized culture are comparable to the percentages usually obtained following the standard protocol, where cells are transfected right after medium exchange. A last consideration can be done observing at the percentages of transfection on the synchronized culture 10 and 13 hpmc. These two times are not so far from each other and are noticeably distant from medium exchange, and yet, the percentage of transfection 72 hpt of cells transfected 13 hpmc is significantly higher than those transfected 10 hpmc. As here we can ignore the time effect, the reason could lie in the 10% more of cells in G2/M phase in the 13 hpmc sample. Furthermore, this transfection percentage is comparable to the correspondent in the control culture, and so are the G2/M percentages.

Medium exchange has a strong effect over transfection, and it can even shadow cell cycle phase effect. If its effect can be eliminated, it is still possible to notice the phase effect and that a major G2/M phase percentage leads to higher transfection percentages, as it was shown in the upper graph (Figure 24).

9 CONCLUSIONS

The HEK293 cell line used in this work, cultured in suspension in supplemented FreeStyle at 37 °C and 5% CO_2, can grow up exponentially until day six to a maximum concentration of 4.8×10^6 cells/ml, observed around 150 h after the stating inoculum. The maximum growth rate (μ_{max}) is 0.021 h^{-1} and the doubling time is 33.2 h.

The used standard transfection protocol states to transfect a cell culture in the exponential phase at the concentration of 2×10^6 cells/ml with a mixture of PEI:DNA 2:1, with DNA final concentration of 1 µg/ml, after medium exchange. Many variables have been tested independently to analyze the strength of such protocol.

It resulted that cell positive transfection percentages and VLPs production are not affected by the mixture solvent used to prepare the PEI/DNA polyplexes (not-supplemented or supplemented FreeStyle culture medium), nor if the medium is used cold, nor if the mixture is not well vortexed. Even the age of the flasks (meant as number of times a flask has undergone a cleaning and sterilization process) used is not affecting cell transfection, VLPs production nor cell growth. Thus, it can be concluded that the transfection protocol is robust and not affected by the operating factors.

However, some of these variables have a considerable effect over cell growth and viability post transfection. Supplemented FreeStyle medium as solvent for the PEI/DNA mixture has a positive effect resulting in a slightly higher viability in the firsts hours after transfection, maybe because of the components used to supplement the medium. Cold mixture addition to the cell culture has a negative effect on viability, supposedly because it induces an adaptive response to cold in the firsts hours post transfection.

It is generally known that transfection has a toxic effect on cells, caused by exogenous DNA and PEI toxicity and even by the same protocol, resulting in a percentage of cell death post transfection and extending doubling time. The addition of a rest time after medium exchange before transfection permits to increase cell concentration days post transfection, thus having a positive effect on viability but no considerable effect over transfection and VLP production. Due to such toxic effects, health cell state has to be considered too, thus it was considered it through the cell passage. It resulted that cells at really high passage (considering the maximum reachable passage around # 100) cannot be used as it results in the death of the culture; otherwise, cell passage has no considerable effect.

Cell concentration at transfection time-point is an important variable and it affects transfection and VLPs production. It resulted that the standard protocol is already optimized in its variables, as transfecting cultures with higher cell concentrations, even with higher quantities of DNA, did not result in more cell transfection or product production. Such results are in agreement with the cell density effect; this effect can be avoided by using an alternative transfection protocol referred as "Bollin method", that forces contact between cells and polyplexes through cell re-suspension in the PEI/DNA mixture. The Bollin method permitted to transfect a higher percentage of cells and obtain more VLPs production when exponential phase cells are concentrated at 4×10^6 cells/ml and transfected with a mixture of PEI/DNA polyplexes in which the final DNA concentration is 3 µg/ml.

The cell cycle was monitored to see if it had any implication for efficient transfer of plasmid DNA molecule in the nuclear environment. Comparing many independent repetitions it is possible to show that cell transfection and G2/M phase are directly related, maybe because the lack of the lipid membrane in this cell cycle phase allows polyplexes to easily reach the nucleus. Therefore, two drugs were used (Nocodazole and Hydroxyurea) to synchronize a cell culture and monitor the cell cycle phase effect. Before using such components, their operating concentrations and times have to be assessed. So, it is shown that Nocodazole blocks cells in G2/M phase; its operation range is between 0 and 5µg/ml but has to be added to the cell culture at a final concentration of 2µg/ml to have the maximum blocking effect. After 26 hours of incubation with Nocodazole the percentage of cells in G2/M phase stabilizes reaching 100%. Hydroxyurea blocks cells in G0/G1 phase; it has no considerable toxic effect until 1 M final concentration and its maximum effect over cycle blocking is obtained at a final concentration of 1.25 mM. After 23 hours a sufficient percentage of cells (around 75%) is blocked in G0/G1 cell cycle phase. The effect over the exact percentage of cells blocked over time is dependent on the cell cycle phase profile of the starting culture, however, the results are reproducible. Drug removal is simply through medium exchange and flask change. After drug removal, Nocodazole blocked cells are not synchronized and slowly leave G2/M phase; after 33 hour post medium exchange the culture re-starts to cycle as a normal asynchronous one; while Hydroxyurea blocked cells are synchronized. Hydroxyurea synchronized cell culture shows the typical profile of a synchronized culture; after 21 hours a cycle is completed and the synchronization is gradually lost. Cell cycling profiles after drug removal are reproducible.

The locking in G2/M phase trough Nocodazole did not lead to higher transfection percentages, but to lower. Probably there is some side effect related to Nocodazole that does not permit transfection. The data obtained by the Hydroxyurea synchronized culture are not easy to explain, as a cell cycle effect is not evident. What was expected in both drug-threated cultures was a higher transfection percentage for a higher percentage in G2/M phase, but this was not the case. In the case of Hydroxyurea synchronized culture, results can be explained considering a strong effect of the medium exchange for drug removal; considerations done eliminating such effect, still directly relate G2/M phase to positive transfection.

10 FUTURE DEVELOPMENTS

Some improvements should be done looking back at results explained in this work with the aim to explain better the effects observed.

As timing in the transfection of a synchronized culture is very important, it is necessary to know how much time take the polyplexes to enter the cells. Thus, the transfection of a normal culture will be observed under a confocal microscope. The reagents suggested for this experiment are the following: Hoechst 33342 for cell nucleus staining and Cell Mask deep red membrane stain as their fluorescence does not interfere with the fluorescence of the VLPs (where the Gag protein is fused to the eGFP) and are already available in the laboratory; and Cy3 to label pDNA. All this fluorescent probes do no interfere with each others as excitation and emission wavelength are quite different. This experiment will allow to know the exact timing of transfection and could be repeated with differently treated cells. The knowledge of this variable will permit to repeat those experiments where time is an essential variable.

To eliminate the medium exchange effect from the transfection experiment of the Hydroxyurea synchronized culture, I propose to repeat the experiment exchanging the medium not only to release the culture from the drug effect but also right before transfection. As the same time will have been passed between medium exchange and transfection, the medium exchange effect could thus be eliminated, maybe allowing to see the underlying cell cycle phase effect.

As further experiment to study the cell cycle effect, cells from a normal culture could be divided for their cycle phase using a cell sorter. Once divided, the new three cultures could be transfected and the cell cycle effect revealed. As control, a normal culture could be passed through the cell sorter without dividing it and then transfected. Cell sorting stresses cells, so the same good transfection percentages as those obtained following the standard protocol might not be seen, but, as every group will undergo the same treatment, such "stress variable" will result eliminated.

As increasing DNA final concentration when transfecting cell at higher concentrations did not result in more transfection, PEI concentration for polyplexes formation should be optimized. Actually, this has been done by my work-mate Javier Fuenmayor and the results are explained it his thesis (59). Summarizing, through DoE, different DNA and PEI concentrations were elected, cell transfection was performed and data were analyzed through Sigma Plot 11.0. Results show that, for a standard transfection, PEI concentration should be increased to 3.3 leading to 6% more transfection, comparing to standard conditions.

As there is evidence that G2/M cell cycle phase has a direct effect on positive transfection and thus on VLPs production, probably because of the lack of membranes during M phase, some reagents able to solubilize the cell membrane should be tested. This work was done by my work-mate Javier too and it is explained in his thesis (59). Dimethyl-sulfoxide (DMSO) and lithium acetate gave negative results; while the use substances that could improve the production gave good results. DMSO ant lithium acetate are able to depolarize the plasmatic membrane, while, volproic acid inhibits HDAC1, thus inhibits histone deacetilation, and trichostatin A modifies the binding of transcription factors to DNA. Through DoE Plackett-Burrman the concentration of volproic acid and trichostatin A where optimized to reach higher VLPs production. Both are able to increase transcription and thus VLPs production. If they are added at a final concentration of 6 mM and 0,04 nmol/ml respectively, right after transfection, the VLPs production can rise up to five times comparing to the normal level obtained.

Once this proposed analysis will be completed, the mechanism of HEK293 transient transfection for the production of Gag-eGFP VLPs will be better understood, hopefully leading to more stable results. Thus, as partly already done, we could take advantage of most effective variable in order to obtain a more efficient transfection and a higher VLPs production.

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