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Establishing a High Titer Transient Gene Expression Process in Conditioned Media for CHO-DG44 Cells

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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“Muitos anos depois, diante do pelotão de fuzilamento, o Coronel Aureliano Buendía havia de recordar aquela tarde remota em que seu pai o levou para conhecer o gelo.

(...)

Desconcertado, sabendo que os meninos esperavam uma expllicação imediata, José Arcadio Buendía atreveu-se a murmurar:

- É o maior diamante do mundo.

- Não – corrigiu o cigano – É gelo!”

Gabriel García Marquez “Cem anos de Solidão”

“Many years later, as he faced the firing squad, Colonel Aureliano Buendía was to remember that distant afternoon when his father took him to discover ice.

(...)

Disconcerted, knowing the children were waiting for an immediate explanation, José Arcadio Buendía ventured a murmur:

- It’s the largest diamond in the world.

- No – the gipsy countered – It’s ice!”

Gabriel García Marquez “One Hundred Years of Solitude”

Abstract

Transient gene expression (TGE) allows for fast protein production in mammalian cells and has become a very important technology in the product development pipeline of biopharmaceuticals. Polyethylenimine (PEI) mediated, high-density transfections have allowed for transient processes exceeding ~300mg/L in CHO-DG44 cells. As such, the bottleneck of TGE is no more in the titers, but in the scale-up to volumes higher than 1L, because of the need for a medium exchange before transfection. It is known that if the transfection is done in a running culture, without a medium exchange (i.e in conditioned medium), the yields obtained are very low (~5 mg/L). In CHO-DG44 cells, this problem was explored from the point of view of transfection efficiency, gene delivery and transcription. A new insight is presented in this work: The low productivities are not due to a deficient gene delivery, but instead, to lower mRNA levels that we hypothesize to be related to a lower gene accessibility of the transfected plasmid. Further, the yields were improved from ~5mg/L to ~90mg/L (18-fold) by optimizing the conditions for transfecting in conditioned medium and utilizing sodium butyrate as a transcription enhancer. These results are expected to open paths for the successful scale-up of TGE.

Keywords: Transient gene expression; Recombinant protein production; CHO cells: Conditioned medium; Scale-up

Resumo

A expressão transiente de genes (ETG) permite a produção de proteínas recombinantes em células de mamífero num curto espaço de tempo e tornou-se uma tecnologia muito importante no *pipeline* de desenvolvimento de biofármacos. Transfecções de grande densidade celular mediadas por polietileno-amina (PEI) têm permitido processos transientes excendendo os 300mg/L em células CHO-DG44. Desta forma, a limitação da ETG actualmente não está nos títulos, mas sim no *scale-up* para volumes maiores que 1L, devido à necessidade de efectuar uma mudança de meio de cultura antes da transfecção. É sabido que se a transfecção é feita numa cultura corrente, sem uma mudança de meio de cultura (em meio condicionado), a productividade é muito baixa (~5mg/L). Em células CHO-DG44, este problema foi explorado do ponto de vista da efficiência da transfecção, da transferência genética e da transcripção. Neste revelou-se que as baixas productividades não se devem a uma transferência genética deficiente, mas sim a uma limitação na transcripção. Discute-se também a hipótese de que esta deficiência está relacionada com uma baixa acessibilidade do plasmídeo aos mecanismos de transcripção. A productividade também foi melhorada de 5mg/L para 90mg/L (18 vezes) através da optimização das condições de transfecção e utilizando butirato de sódio como um agente promotor da transcripção. Espera-se que estes resultados abram caminhos para o sucesso do *scale-up* da ETG**.**

Palavras Chave: Expressão transiente de genes; Produção de proteínas recombinantes; Células CHO; Meio condicionado; Scale-up

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List of Abreviations

CaPi	-	Calcium phosphate precipitation
cDNA	-	Complementary DNA
CHO	-	Chinese Hamster Ovary cells
DHFR	-	Dihydrofolate reductase
DMSO	-	Dimethyl sulfoxide
EBNA1	-	Epstein-Barr nuclear antigen 1
EBV	-	Epstein - Barr virus
EF1-α	-	Elongation Factor 1 alfa
ELISA	-	Enzyme Linked Immunosorbent assay
GFP	-	Green Fluorescent Protein
HCMV	-	Human Cytomegalovirus
HEK293	-	Human Embryo Kidney 293
IgG	-	Imunnoglobulin G
mRNA	-	Messenger RNA
NaBut	-	Sodium butyrate
PBS	-	Phosphate Saline Buffer
PCV	-	Packed Cell volume
pDNA	-	Plasmid DNA
PEI	-	Polyethylenimine
PTFE	-	Polytetrafluoroethylene
SGE	-	Stable Gene Expression
STR	-	Stirred Tank Reactor
SV40	-	Simian Vacuolating virus 40
TGE	-	Transient Gene Epxression
WPRE	-	Woodchuck hepatitis virus Posttranscriptional Regulatory Element
μ	-	Growth rate (h^{-1})

1. Introduction

1.1. Therapeutic proteins produced from mammalian cells

Mammalian cells have become the dominant system for the production of therapeutic recombinant proteins for clinical applications, due to their capacity for proper protein folding, assembly and post-translational modification (Wurm, 2004). Since 1986, the date of the approval of the first therapeutic protein produced from mammalian cells, human tissue plasminogen activator (tPA, Activase; Genentech, S. San Francisco, CA, USA), the number of biopharmaceuticals in the market has risen to over 200 products (Walsh, 2010). Nowadays, the top ten biopharmaceutical products account for sales of almost 50 $billion (Table 1.1).

Table 1.1: Top 10 biopharmaceutical sales 2010 (adapted from **(Walsh, 2010)**)

Product	Sales ($billions)	Company
Enbrel (etanercept)	6.58	Amgen, Wyeth, Takeda Pharmaceuticals
Remicade (infliximab)	5.93	Centocor (Johnson & Johnson), Schering-Plough, Mitsubishi Tanabe Pharma
Avastin (bevacizumab)	5.77	Genentech, Roche, Chugai
Rituxan/MabThera (rituximab)	5.65	Genentech, Biogen-IDEC, Roche
Humira (adalimumab)	5.48	Abbot, Eisai
Epogen/Procrit/Eprex/ESPO (epoetin alfa)	5.03	Amgen, Ortho, Janssen-Cilag, Kyowa Hakko Kirin
Herceptin (trastuzumab)	4.89	Genentech, Chugai, Roche
Lantus (insulin glargine)	4.18	Sanofi-Aventis
Neulasta (pegfilgrastim)	3.35	Amgen
Aranesp/Nespo (darbepoetin alfa)	2.65	Agmen, Kyowa Hakko Kirin
Total Sales	49.51	

The drug development pipeline is a process that can take up to 15-years, and on average, for each 250 molecules that enter this pipeline, one will become an approved product (Figure 1.1). Thus, when designing a pharmaceutical product to enter the pipeline it is important to test different variants of the same molecule for efficacy, and also safety. Although currently, all the marketed recombinant protein therapeutics are produced using stable gene expression, in the pre-clinical phase, transient gene expression presents itself as a faster production platform (Baldi et al., 2007).

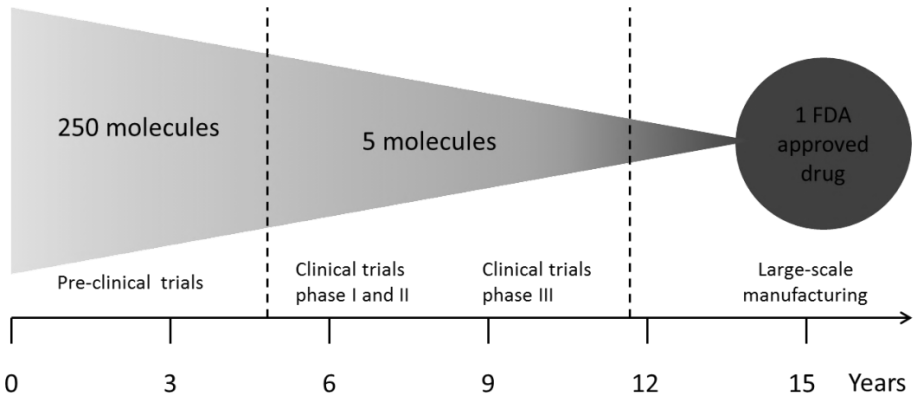

Figure 1.1: The drug development pipeline. Source: (http://www.phrma.org/research-development)

1.2. Transient gene expression

Transient gene expression (TGE) is defined as the introduction of foreign genetic material in a cell host, without selection for stable integration of the transfected plasmid in the host genome. This approach has been used for a long time (Graham e van der Eb, 1973) and offers many advantages to research: it is rapid, simple and can be applied to a large number of cell lines (Wurm e Bernard, 1999)(Geisse, 2009).

1.2.1. Transient gene expression for the production of recombinant proteins

The expansion of the biopharmaceutical industry pipeline depends on the identification of suitable candidate proteins for clinical trials (Figure 1.1). Establishing a stable cell line - the "classical" approach" - for each of the candidate molecules is a time consuming process (months) (Figure 1.2). TGE allows shortening of this early phase in a sensible way: following DNA transfection, the proteins are produced by an unselected pool of cells in a 2-10 days long production. Therefore, the candidate molecules produced transiently can enter the drug development pipeline and screening process in a very short time – weeks, instead of months (Baldi et al., 2007). Transient gene expression has established itself as an important technology for the production of recombinant proteins from mammalian cells, for the early phase clinical studies in the biopharmaceutical industry.

The key features of most transient expression systems are (i) simplicity, (ii) extremely short time-frame for the generation of product, (iii) intrinsic genetic stability, due to the short time-frame between generation of the vector and product recovery (iv) the applicability to a wide range of host cell lines, and the suitability to multiple processing, as it allows the study of different genes and mutations in parallel (Wurm e Bernard, 1999).

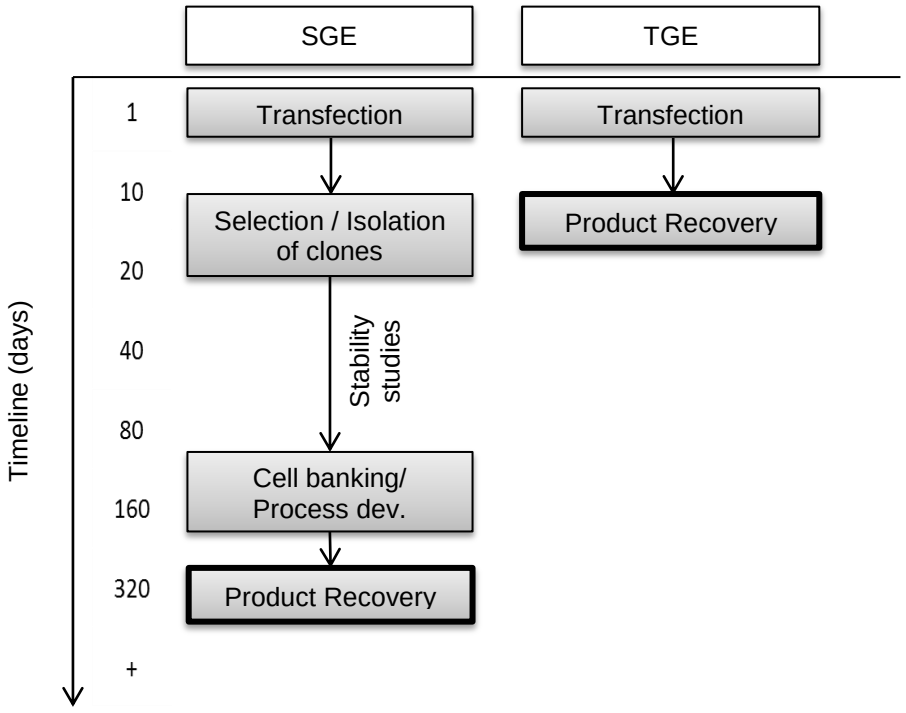

Figure 1.2: Comparison of the process time-line for stable gene expression (SGE) and transient gene expression (TGE).

1.2.2. Expression vectors for transient gene expression

For transient gene expression, the expression vector can be non-viral and viral and has to be designed to transfer the foreign gene into mammalian cells. The choice of the vector depends on the application, the host cell, the time limitation and the safety (Baldi et al., 2007).

There are three essential elements for a non-viral TGE vector: A constitutive promoter, a transcription terminator and a prokaryotic cassette with a replication origin and a selection marker for vector production in bacteria. The most commonly used promoters are derived from viral genomes and include the human cytomegalovirus immediate early promoter (HCVM), the simian virus (SV40) early promoter and the Rous sarcoma virus long-terminal repeat promoter. Constitutive cellular promoters like the human elongation factor-1-alpha (EF-1α) have also been shown to be efficient (Baldi et al., 2007). Other elements can be used to enhance transient protein expression. The insertion of an intron between the promoter and the 5'-end of the cDNA, or the inclusion of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the 3'UTR region of the transgene mRNA (Baldi et al., 2007)(Wulhfard et al., 2008). Mammalian cells expressing the Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1), like the HEK293-EBNA1 cell line, are capable of episomal amplification of plasmid DNA containing the EBV or the SV40 origin of replication. This strategy has also been used for improving the recombinant protein production by increasing the plasmid copy number in transiently transfected cells (Van Craenenbroeck et al., 2000).

Viral expression vectors are also used for TGE. The Semliki Forest Virus (SFV) is the most widely used viral expression vector for TGE. The baculovirus system has also been used for transient gene expression in both mammalian and insect cell lines (Kost e Condreay, 2002). For mammalian cells, the baculovirus system has several advantages, including: The absence of viral replication, the lack of cytotoxicity and the simplicity of vector production. Vaccinia virus vectors have also been used for transient gene expression, although they raise more regulatory concerns, as they require containment in bio-safety level 2 laboratories (Baldi et al., 2007)(Sutter e Moss, 1992).

1.2.3. Non-viral gene delivery vehicles

Several DNA carriers for gene delivery into mammalian cells have been used. However, only a few are applicable to large-scale TGE, due to their cost-efficiency. The non-viral DNA vehicles can be categorized in (i) cationic lips, (ii) inorganic compounds and (ii) cationic polymers. Calcium phosphate (CaPi) precipitation was the first transfection method, described by Graham in 1973 (Graham e van der Eb, 1973), and still the most widely used method employing inorganic compounds (Jordan et al., 1996)(Baldi et al., 2007).

Cationic lipids are very effective transfection agents, however, they are too expensive for large-scale transfections, and are not considered for transient gene expression for manufacturing purposes (Baldi et al., 2007).

CaPi is one of the most efficient DNA delivery methods, allowing transfection efficiencies of 80-90% in HEK293 cells (Meissner et al., 2001). The requirement for serum during the transfection, however, is a critical constraint. Furthermore, CHO cells require an osmotic shock to be transfected with this method (Baldi et al., 2007). Currently, the most widely used DNA delivery agent for TGE is 25kDa linear

polyethylenimine, which was demonstrated as an efficient gene carrier in 1995 (Boussif et al., 1995).Transfections with 25kDa PEI have been the most productive to date with reported antibody yields up to 1g/L in HEK293 cells and 300mg/L in CHO cells (Rajendra et al., 2011).

1.2.4. Cell lines for transient gene expression

HEK293 cells are the most commonly used cell lines in TGE, due to the ease of maintenance and the high transfection efficiencies observed (Baldi et al., 2007)(Geisse, 2009). Several genetically modified or clonally selected HEK293 variants have been developed. HEK293 cell lines grow well in adherent mode in medium supplemented with serum or in suspension in serum free media (Geisse, 2009).

However, when TGE is used as a screening tool for drug development, it is preferable to obtain CHO-derived material for pre-clinical trials (Baldi et al., 2007). The pharmaceutical industry prefers to use CHO cell lines for production because of the established track record of this cell line in manufacturing (Wurm, 2004)(Derouazi et al., 2006a).

Only recently major attempts to use CHO cells as hosts for TGE have emerged. The first large scale transient transfection of CHO cells in a bioreactor was reported in 2004 (Derouazi et al., 2004). The interest in CHO cells for transient gene expression is high. However, the overall productivity of CHO cells for the manufacturing of recombinant proteins is lower than HEK239 cells (Geisse, 2009).

Other cell lines like COS cells and Vero cells (both derivative of CV-1 cells of Simian origin), various carcinoma cells, and recently human embryonic stem cells have also been used for transient transfections, although with no proven success for protein manufacturing (Geisse, 2009).

1.2.5. Transient gene expression in CHO cells

Until recently, the yields of transiently produced proteins from CHO cells have been far behind the yields obtained in HEK293 cells, which have achieved 1 g/L (Backliwal et al., 2008). Before 2008, the yields of TGE processes in CHO cells were in the 1-25mg/L range. Since then, several improvements in the process were made, and yields of 80-100mg/L were reported (Wulhfard et al., 2008)(Ye et al., 2009)(Wulhfard et al., 2010). Very recently, a TGE process in CHO cells achieving antibody yields of 300mg/L was reported (Rajendra et al., 2011).

Several factors are responsible for these improvements: Mild hypothermia culture conditions, vector design, high density transfections and the optimization of PEI and DNA concentrations for transient gene expression (Wulhfard et al., 2008)(Rajendra et al., 2011)

The highest yields reported so far in CHO cells have been obtained by transfecting the cells with linear 25kDa PEI following incubation of the culture at 30-30ºC (Wulhfard et al., 2008)(Rajendra et al., 2011). The enhancement of TGE in mild hypothermia conditions (31ºC) correlates with an accumulation of cells in the G1 phase of the cell cycle, reduced cellular metabolism, greater cell viability, increased steady state levels of transgene mRNAs, and increased cell size as compared to cells kept at 37ºC(Wulhfard et al., 2008). The vector design, as for example, the inclusion of the Woodchuck hepatitis virus post-transcriptional element (Wulhfard et al., 2008)

or the inclusion of an intron between the promoter and the 5'-end of the transgene cDNA have shown to improve recombinant protein production(Baldi et al., 2007).

1.2.6. Scale-up of transient gene expression

Some private companies have reported high yields in transient gene expression at large-scale. However, they do not publish the methods used for these breakthrough achievements, as they are of great commercial value.

The first review on large scale TGE was published in 1999 (Wurm e Bernard, 1999). Regarding the DNA delivery method, at that time polyethylenimine was already seen as a major technology for large-scale operations, as it is cost-efficient and it can be applied in big quantities. However, the state of the art delivery method for large scale TGE was still Calcium phosphate. The cell lines dominating the field were HEK-293, COS cells (Simian origin) and baby hamster kidney (BHK) cells. Only later CHO cells started to give satisfying levels of protein expression when transfected transiently (Derouazi et al., 2004).

For large-scale processes, mostly standard stirred-tank (STR) bioreactors have been used, but in recent years, the trend to use disposable bioreactors has increased. For large-scale TGE processes, orbital shaking bioreactors have been shown to be efficient (Zhang et al., 2010a), but also the Wave™ bioreactor has proven to be well suited for TGE processes (Geisse e Henke, 2005).

For transient gene expression in CHO cell lines, the largest scales reported were of 5-100 L in orbital shaking bioreactors, with obtained antibody titers in the range of 20-60mg/L (Muller et al., 2007)(Stettler et al., 2007) and in Wave™ bioreactors with yields of 9 mg/L(Geisse, 2009). More recently, one transient 14-day fed-batch process with CHO cells yielding 80mg/L at the 20 L scale was reported, being the highest titer every reported for large scale TGE process in CHO cells (Ye et al., 2009). These processes, although being at large-scale, include (i) a centrifugation step prior to transfection to eliminate the spent medium and (ii) a low cell density at the time of transfection.

Transfections at high cell density for TGE processes have proved to be the most high-yielding, in both CHO and HEK-293 cell lines (Backliwal et al., 2008)(Rajendra et al., 2011). The high density transfection procedure described for CHO cells is performed at a cell density of 4-5 million cells/mL (Rajendra et al., 2011), whereas for HEK-293 cells, it is performed at a cell density of 20 million cells/mL (Backliwal et al., 2008). The high-density HEK-293 transfections, while very efficient, are only feasible at small scales, as these cells are not known to achieve such densities (20 million cells/mL) in batch cultures. Thus, this high cell densities at the time of transfection can only be achieve by concentrating the cells before a medium exchange, i. e with a centrifugation step. From this point of view, the high density process in CHO cells is more likely to be successfully scaled-up, as CHO cells, grow easily to densities of 4-5 million cells/mL in serum-free media. However, for high-density transfections in CHO cells, there is still a need for a medium exchange by centrifugation to remove the spent medium (Rajendra et al., 2011).

While a centrifugation step for a medium exchange is very easy to perform at smaller scales of operation, it is not a viable alternative for large scale processes (over 2 L) and it is a limiting step on scaling-up high-yielding transient gene expression processes. Therefore, the alternative is to transfect a large scale culture without a

medium exchange (i.e in conditioned medium). However, this results in lower transfection efficiencies and loss of productivity (Schlaeger e Christensen, 1999).

1.2.7. Transient gene expression in conditioned medium

For the purpose of transient gene expression, conditioned medium is defined has the spent medium where cells have been cultivated for more than 2 days. This medium is lower in nutrients due to cell growth, it contains cell derived compounds (proteins, glycans, etc.) and other molecules that are the product of cell metabolism.

Transfecting cells in conditioned medium results in lower transfection efficiencies. This has been shown for cationic lipid mediated gene transfer (Belting e Petersson, 1999) and for PEI-mediated gene transfer (Schlaeger e Christensen, 1999). The contrary has also been claimed (but not shown) at least in one publication on PEI-mediated TGE in HEK293 cells (Raymond et al., 2011). However, the titers of these transient processes in HEK293-cells are still 40-fold lower than those obtained when a medium exchange is performed: 4000 mg/L vs 100mg/L (Divor Kiseljak, unpublished data) (Raymond et al., 2011)).

Processes where the transfection is done into a running culture have been reported for HEK293 cells (Raymond et al., 2011). For CHO cells, a transient gene expression procedure where the transfection is performed without a medium exchange has not been published yet.

It has been shown that the negative effect of the conditioned medium on the transfection efficiency is mainly due to the presence of cell-secreted high molecular weight compounds. It is thought that these compounds interact with the PEI-DNA complex, inhibiting the transfection procedure, although the mechanisms and the molecular entity of these compounds are not known.

Cell secreted high molecular weight compounds have been shown to interfere with cation-mediated gene delivery, and although they are not studied in this work, they are worth to mention. Positively and negatively charged molecules interact with DNA and the cationic delivery vehicle, and interfere with the transfection process. These include proteoglycans and glycosaminoglycans, like heparin or dextran sulphate. (Mislick e Baldeschwieler, 1996)(Ruponen et al., 2001)(Ruponen et al., 2004).

1.3. The CHO-DG44 cell line

Most of the therapeutic proteins in the market are produced in Chinese Hamster Ovary (CHO) cells, but also other cell lines such as NS0 (mouse myeloma), BHK (Baby Hamster Kidney), HEK-293 (Human embryo kidney) and PerC 6 (Human retinal cells). All these cell lines, among others, have gained regulatory approval for the production of recombinant proteins (Wurm, 2004).

For manufacturing purposes, cell culture is done in adherent or suspension culture, being suspension, by large, the most common. Suspension adapted CHO cell lines are the dominant host for the mass production of therapeutic products (Wurm, 2004).

The CHO cells used in protein manufacturing originated in 1957 by immortalization of a cell from a primary culture of ovarian cells of a Chinese Hamster (Hacker et al., 2007).

The CHO-K1, a glycine dependent strain derived from the original CHO cell line, was mutagenized to generate CHO-DXB11, a cell line lacking DHFR activity (Urlaub et al., 1983). Subsequently, a CHO-pro3 strain was mutagenized to generate the CHO-DG44, which also lacks DHFR activity. Because of that, these two DHFR-minus strains require glycine, hypoxanthine and thymidine (GTH) for cell growth (Kaufman et al., 1985)

The DHFR-minus CHO cells were not originally intended for manufacturing of recombinant proteins, but several pioneering experiments were done on a stable transfection with an exogenous *dhfr* gene via selection in a GTH-minus medium (Hacker et al., 2007). The CHO-DG44 cell line has thus become a dominant, and well characterized, mammalian cell host for recombinant protein manufacturing.

1.4. TubeSpin® bioreactor 50 and orbitally shaken bioreactors

The TubeSpin® Bioreactor 50 (TPP, Switzerland) is, an orbitally shaken scale-down system, successfully used in the screening and optimization processes of suspension cells(De Jesus et al., 2004). It has a working volume from 1 to 35mL and 5 openings of different sizes above the gas permeable sterile PTFE filter of the screw cap which allow for custom optimization of the gas transfer. The sterile gas exchange is done through the 0.22 µm filter membrane. High cell densities can be achieve with only passive aeration through the opening cap (Muller et al., 2005). With passive aeration, the mass transfer coefficient of oxygen for the TubeSpin® Bioreactors 50 (kLa) is above $15h^{-1}$ and from the 50mL scale up to the 2000L scale the kLa is in the range of $4\text{-}30h^{-1}$(Zhang et al., 2009)(Tissot et al., 2011). This disposable bioreactor system allows bioprocesses to be run without probes or controllers (Tissot et al., 2011). For these reasons, the TubeSpin® bioreactor 50 is very advantageous for the high-throughput study of culture conditions or optimization of transfection processes.

Furthermore, the orbitally shaken culture system has been scaled-up to volumes higher than 1000L in a very straightforward approach(Zhang et al., 2010b) which leaves the door open to further volumetric scale-up. Transient gene expression has been performed in these bioreactors at the 100 L scale (Zhang et al., 2010b).

Figure 1.3 – Left to Right: Disposable TubeSpin® bioreactors 50 and 600 (TPP, Switzerland; *www.tpp.ch*)

2. Goal of this thesis

Currently, one of the key problems for TGE in CHO cells is its volumetric scale-up with high yields. This can be approached by developing a high-density transfection procedure without a medium exchange (i.e in conditioned medium). However, it is known that if the transfection is done in a running culture, without a medium exchange, the yields obtained are very low (Schlaeger e Christensen, 1999).

This work examines this problem from two different perspectives:

1. Analysis of gene delivery in fresh medium *vs* conditioned medium.
2. A process development approach for the establishment of a high-titer TGE process for CHO-DG44 cells in conditioned medium.

By comparing the gene delivery in the TGE process in fresh medium (high yielding) and in conditioned medium (low yielding) it was expected to gain better insights about what is happening to the transfected DNA. This was done by quantitative analysis of the transfected plasmid DNA and comparison with the respective mRNA levels and protein production. Studies were done in order to unravel the factors that are limiting the protein production when the transfections are done in conditioned medium. These insights were applied in the design of strategies for the improvement of yields.

With a step-by-step approach of process development, using the TubeSpin® bioreactor 50 system, several parameters known to have an influence of transient gene expression process productivities were studied: (i) The study of different cell culture media formulations (ii) The optimization of the PEI-DNA ratios for high density transfections in conditioned medium and (iii) the study of chemicals known to have a positive effect on the mRNA levels and protein productivities in cell culture processes.

This work was aimed at the identification of important parameters for conditioned medium transfections and strategies that can be used to improve TGE processes in CHO cells for larger scales. A process development approach was also followed in order to develop a fully scalable TGE process for CHO cells that does not involve a medium exchange prior to transfection.

3. Materials and Methods

3.1. Cell culture

Suspension-adapted CHO DG44 cells (Urlaub chasin) were routinely grown in 250-mL squared shaped glass bottles (Schott Glass, Mainz, Germany) as previously described (Whulfard 2008) at 37ºC in 85% humidity and 5% CO_2. The cells were cultivated in ProCHO5 medium (Lonza, Verviers, Belgium) (Henceforth refered to as "ProCHO5") supplemented with 14µg/L hypoxanthine, 4µg/L thymidine, and 4mM glutamine (SAFC Biosciences, St. Louis, MO). The cells were passaged 2 times per week and maintained in exponential growth between 0.3 – 6 million cells/mL.

The suspension cell culture done was done at a working volume of at most 40% of the nominal volume of the vessel. The bottles were fixed to a horizontal model ES-W orbital shaker with a rotational diameter of 2.5 cm (Kühner AG, Birsfelden, Switzerland) using double sided adhesive transfer tape (3M Corp, Minneapolis, MN) and agitated at 110 rpm.

Before transfection and at the time of transfection the cell density was always determined by the packed cell volume method (PCV) (Stettler 2006) with the VoluPAC system (Sartorius AG , Göttingen, Germany). For CHO-DG44 cells, a PCV of 0.18-0.2 correlates to a cell density of 1 million cells/mL. Manual cell counting after transfection was performed, when necessary, using the Trypan blue exclusion method.

3.2. Transfections

3.2.1. Plasmid DNA preparation

Plasmid DNA was produced by transformation of *E.Coli* DH5α and growth of the bacteria in LB broth with ampicillin. The plasmid DNA purification was performed with a Nucleobond AX anion exchange column (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Plasmid DNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The quality ratios 260/280 and 260/230 were measured and the accepted values were of at least 1.8 and 2.0, respectively.

3.2.2. Plasmids used

3.2.2.1. Plasmid pXLG^{CHO}-A3 (pA3)

The dual expression vector pXLG^{CHO}-A3 with the human anti-Rhesus D IgG1 heavy and light chain cDNAs cloned in separate expression cassettes in a head-to-head orientation was kindly provided by Excellgene SA. It was constructed by removing the IgG light chain expression cassette from pXLG^{HEK}-RhLC and cloning it into the unique AflII site of pXLG^{HEK-}RhHC. Each expression cassette included the human CMV immediate early promoter/enhancer, an artificial intron in the 5′-untranslated region, the cDNA for IgG light or heavy chain with the translational start codon present within the Kozak consensus sequence, the woodchuck hepatitis virus post-transciptional regulatory element (WPRE) in the 3′-untranslated region, and the bovine growth hormone polyadenylation signal (Rajendra et al., 2011).

3.2.2.2. Plasmid pMYKEF1-EGFP (pEGFP)

The plasmid pMYKEF1-EGFP has been previously described (Derouazi et al., 2006b). It was generated by the enhanced GFP gene from pEGFP-N1 (Clontech, Palo Alto, CA) cloned as an EcoRI/NotI fragment into EcoRI/NotI-digested pMYKEF-1 (Kim et al. 2002) to produce pMYKEF1-EGFP.

3.2.3. Preparation of the stock solution of 25kDa linear polyethylenimine

For the preparation of the 1mg/mL stock solution of 25kDa linear polyethylenimine (PEI), 1 g of PEI is dissolved in 800mL dH_2O by adding 1N HCl (PEI will not dissolve until the pH is lower than 7). The pH is adjusted to 7.0 with 1N NaOH and dH_2O is added until the volume is 1L. If the pH is lower than 7, 1N NaOH is added. The solution is sterile filtered trought a 0.22µm filter and stored at -80ºC in 40 mL aliquots in 50mL tubes.

3.2.4. Transfection with a medium exchange (fresh medium)

All transfections were done in duplicate at a final volume of 5 mL in TubeSpin® Bioreactors 50, unless specified otherwise. Cells from the seed culture were spin down and re-suspended in 5 mL of ProCHO5 medium at a density of 5 million cells/mL (PCV = 1 - 1.1). The cells were transfected with 15ug (at a concentration of ~1mg/mL) of DNA (95% $pXLG^{CHO}$-A3 and 5% pMYKEF1-EGFP) and 75 ug of of 25kDa linear polyethylenimine (PEI) (at a concentration of ~1mg/mL). The DNA was added directly to the cells followed by addition of PEI, and the culture was incubated at 31ºC with 5% CO_2 and 85% humidity at a shaking speed of 180 rpm.

3.2.5. Transfection without a medium exchange (conditioned medium)

For transfecting without a medium exchange, cells from the seed train, in exponential growth phase, were sub-cultivated when at a density of ~5million cells/mL into fresh medium at a density of 0.4-0.5 million cells/mL. The cell culture was incubated at 37ºC as previously described, and on day 3 (72h) the transfection was performed. On the day of transfection cell density was controlled with the packed cell volume (PCV) method. When the PCV of the culture reached 1.05-1.1, cells were transfected with different concentrations of DNA and PEI (as described in the results and discussion section) by direct addition of DNA and PEI, and incubated at 31ºC with 5% CO_2 and 85% humidity at a shaking speed of 180 rpm. Sodium butyrate and Dimethyl sulfoxide were added to the culture at the time of transfection, when specified.

3.2.6. Transfection for the media screening experiment

Thirty-eight different commercially available cell growth media were inoculated with 0.5 million cells/mL, and cells were allowed to grow in this media in the previously described cell culture conditions. After 3 days, the medium supernatant (i.e the conditioned medium) was harvested by centrifugation. Cells from the seed culture were then transfected as described in 3.2.4, using the harvested conditioned medium and also fresh medium out of the bottle.

3.3. Reporter protein assays

3.3.1. Transfection efficiency: %GFP positive cells

Cells transfected with the pEGFP plasmid were diluted 20x in PBS to a density of ~0.3 million cells/mL in a 96-Well plate and the percentage of GPF positive cells was measured with a GUAVA Easy-Cyte™ multi-well system (GUAVA Technologies, Hayward, CA).

3.3.2. ELISA: Recombinant antibody concentration

After transfection with the pA3 plasmid, the IgG1 (human anti-Rhesus-D IgG) concentration in the medium was determined by sandwhich ELISA using an anti-human kappa light chain antibody (coating IgG) (Biosource International, Camarillo, CA) for the IgG capture and alkaline phosphatase conjugated anti-human IgG for detection (detection IgG) (Invitrogen AG). Microtest™ 96-well ELISA plates (BD Biosciences, Bedford, MA) were incubated for 3-4 hours at 37ºC with 100 µL of 1000x diluted (in PBS with 0.1% of tween-20) coating IgG. For the assay, the samples were diluted in a blocking buffer and added to each well. A standard of known concentration was also added to the plate. The incubation time was 45 min at 37ºC. After this incubation time, the plate was washed 3 times and incubated again at 37ºC with 100 uL of 1000x diluted (in blocking buffer) detection IgG. After 45 min of incubation, the plate was washed and the substrate solution was added. Absorbance was measured at 405 nm against 490 nm using a microplate reader (SPECTRAmax™ 340, Molecular devices, Palo Alto, CA).

3.4. Plasmid DNA extraction and quantification

At different times after transfection, 1 million cells were sampled and centrifuged, followed by two consecutive washes with PBS buffer. Total DNA was isolated using DNeasy blood and tissue kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed used Absolute qPCR SYBR green ROX mix (ABgene UK, Epsom, UK) in Ligthcycler® 480 optical 96-well reaction plates (Roche Diagnostics, Mannheim, Germany). DNA isolated from cells was diluted 100 x with high pure water before use for qPCR. A standard curve was made by sequential dilution of the plasmid of interest in water. The primers used for the quantification of the pA3 plasmid amplify a region of the gene of the IgG light-chain and were purchase from SAFC Biosciences (St. Louis, MO, USA (Table 3.1). All the experiments were performed on a Lightcycler® 480 (Roche Diagnostics, Mannheim, Germany). The following thermal cycling program was used: preheating at 95ºC for 5 min, 35 cycles of heating at 95ºC for 15s, 59ºC for 30s and 72ºC for 45s. For each sample, 4 measurements were performed.

Table 3.1 – Primers used for the quantification of DNA by qPCR

Gene of Interest	Forward primer (5' – 3')	Reverse primer (5' – 3')
IgG light chain	TGTCTTCATCTTCCCGCC	GCGTTATCCACCTTCCACTGT
B-Actin	GCTCTTTTCCAGCCTTCCTT	GAGCCAGAGCAGTGATCTC

3.5. mRNA extraction and quantification

At different times after transfection, 1 million cells were collected and centrifuged, followed by two consecutive washes with PBS buffer. Total RNA was extracted using the GenElute mRNA kit (Sigma-Aldrich GbmH, Buchs, Switzerland) according to the

manufacturer's protocol. Samples were treated with 1 U of DNAse I (Invitrogen AG, Basel, Switzerland) for 15' at room temperature, the enzyme was then inhibited by addition of EDTA and by heating.

First-strand cDNA synthesis was performed from 1µg of RNA with the M-MLV reverse transcriptase (Invitrogen AG) using oligo dT (New England Biolabs, Ipswich, MA) as the primer. Each reaction was diluted 10-fold in RNAse-free water for quantitative PCR. The oligolucleotide primers for the amplification (Table 3.1) were purchased from SAFC Biosciences (St. Louis, MO, USA).

The samples from the first-strand cDNA synthesis were mixed with the appropriate primer pair and the reaction mix from the Absolute qPCR SYBR Green ROX mix (ABgene UK, Epsom, UK), and amplified according to the manufacturer's protocol. All experiments were performed on a Lightcycler® 480 (Roche Diagnostics, Mannheim, Germany). The comparative Ct method was used to calculate the relative quantity of IgG light chain mRNAs relative to the quantity of β-actin mRNA (Housekeeping gene).

3.6. Detection of DNA in the medium with PicoGreen ®

The PicoGreen® dye from the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen AG) was diluted in TE Buffer at a 200-fold dilution according to the manufacturer's instructions to make a working solution. Then, at different times post-transfection, cells were centrifuged and medium supernatant removed. Afterwards, 100µL of the medium samples were sampled into a black 96-well plate, followed by the addition of 50µL of the PicoGreen® working solution. The plate was incubated in the dark for 5 minutes and Fluorescence (excitation 480 nm, emission 520 nm) was measured in a TECAN Safire^2 ™ Spectrofluorometer (Tecan Trading, Männedorf, Switzerland).

3.7. Nuclear extraction and nuclear pDNA quantification

1 million viable cells were collected, centrifuged (4ºC, 400g, 5min), and washed twice with PBS buffer. The cell pellet was resuspended in 1mL nuclear extraction buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, and 0.5% Nonidet P-40 (NP40)), and a 10 min incubation in ice followed. Nuclei were then collected by centrifugation (4ºC, 400g, 5min) and re-suspended in PBS buffer. The nuclei extraction method used was based on a publish protocol (Wang et al., 2007) and validated in house by microscopy, to be sure that only intact nuclei were isolated, and not cells. Nuclear DNA extraction and quantification was executed as previously described for the total DNA extraction (3.4).

4. Results and Discussion

4.1. Transfection without a medium exchange results in lower recombinant protein yields

4.1.1. Introduction

Transiente gene expression (TGE) processes are known to be less efficient if at the time of transfection a medium exchange to remove the spent medium is not performed (Schlaeger e Christensen, 1999). In this section, a transient gene expression process that was developed involving a medium exchange by centrifugation is characterized. Two production runs, one with and one without a medium exchange (i.e. in conditioned medium) are compared side by side in terms of protein titers.

Further, the negative influence of the conditioned medium on the transfection efficiency is analyzed in a time-dependent way and the influence of the conditioned medium on transfection efficiency and protein production is studied.

4.1.2. Comparison between a TGE process in fresh media and in conditioned media

In order to study this TGE process in conditioned medium and in fresh medium, two transient production runs were done in parallel. In one process, a medium exchange was performed before transfection, whereas in the other process, the transfection was done without a medium exchange (i.e in conditioned medium) (Figure 4.1).

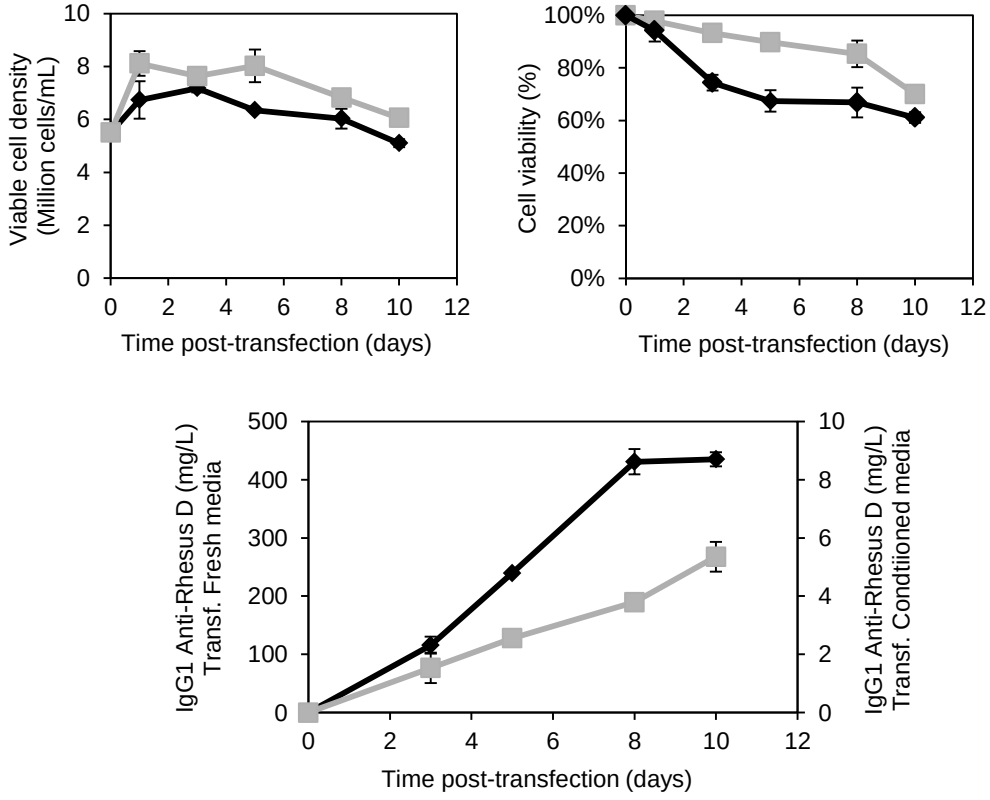

Figure 4.1 – Comparison between a TGE process in fresh medium (■) and in conditioned medium (■). *n=2*

The titers obtained on a 10 day batch process are very low when the transfection is performed without a medium exchange, compared to when a medium exchange is done to eliminate the conditioned medium: ~450 mg/L *vs* ~6 mg/L. Although the titer obtained is low, the cell viability and cell density are high throughout the culture and it is worth noticing that in the process in conditioned medium, the viable cell density and the cell viability are higher (this was observed throughout all the experiments performed). If the transfection is performed in spent medium, the cell viability, and consequently the cell density are always higher than when the same transfection procedure is done in fresh medium. This makes sense, as the spent medium contains many factors secreted by cells, which might act with a *serum-like* effect by protecting the cells, but making the transfection less efficient. The low transfection efficiency can be seen by analyzing the %GFP positive cells 24h post transfection by flow cytometry (Figure 4.2). This data correlates with the low productivity of the process – the negative effect of the conditioned medium on transfection is already observed on the percentage of cells that express GFP at 24 hours post-transfection.

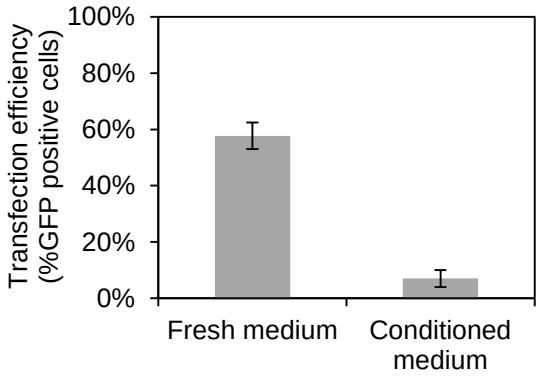

Figure 4.2 – Transfection efficiency (% of GFP positive cells 24-post transfection) in conditioned medium and in fresh medium. *n=2*

4.1.3. The transfection efficiency decreases with the time of conditioning of the media

An experiment was setup to analyze the impact of the time of conditioning of the medium on transfection efficiency. Time of conditioning of the medium is defined as the time cells are allowed to grow on the culture medium before being transfected.

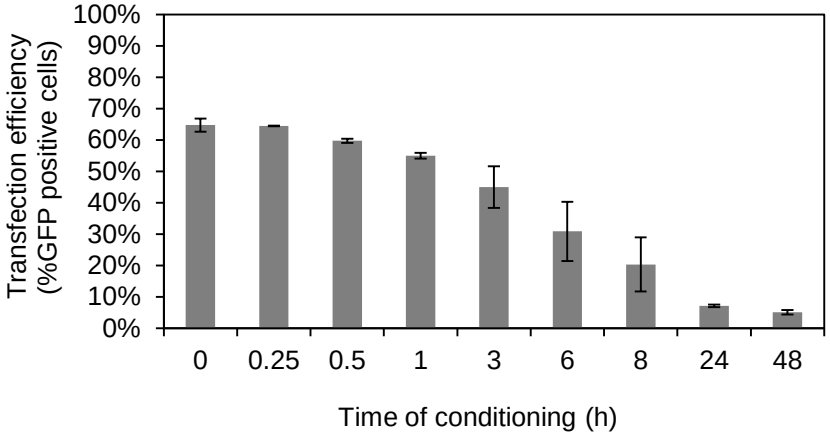

Figure 4.3 – The effect of the time of conditioning of the medium on the transfection efficiency (% of GFP positive cells 24-post transfection. *n=2*

It is possible to observe that the transfection efficiency decreases with the time of conditioning of the medium (Figure 4.3), i.e. if the cells are transfected in the same medium where they were grown, the transfection efficiency decreases. This effect can already be seen at 3-6h of conditioning time and limits the possibility of growing the cells and transfecting without a medium exchange prior to transfection. It had been previously suggested that this is due to cell derived components that interfere with the transfection process (Ralph Duhr, EPFL) – The longer the time of culture, more secreted compounds will be in the medium, the more conditioned the medium will be resulting in a lower transfection efficiency.

4.1.4. Transfection efficiency in conditioned media *vs* fresh media

In the previous section (4.1.3) it was shown that the protein expression is lower when the transfection is performed in conditioned medium, and this effect is higher when the medium is conditioned for longer time. However, it is necessary to study whether the lower protein expression in conditioned medium is due to a deficient transfection, or to a negative effect of the spent medium on protein production. Transfections were performed in conditioned medium and in fresh medium and a medium exchange into fresh medium and conditioned medium was performed 4h post-transfection.

If the transfection is performed in conditioned medium and after 4h the cells are changed to fresh medium, the percentage of GFP expression is very low (Figure 4.4). On the other hand, if the transfection is performed in fresh medium and cells are changed to conditioned medium 4h post transfection, the GFP expression is at the level of the cells transfected in fresh medium and changed into fresh medium (Figure 4.4). It is possible to conclude that the lower GFP expression is due to an effect of the conditioned medium on the transfection, and not an influence of the conditioned medium on protein expression.

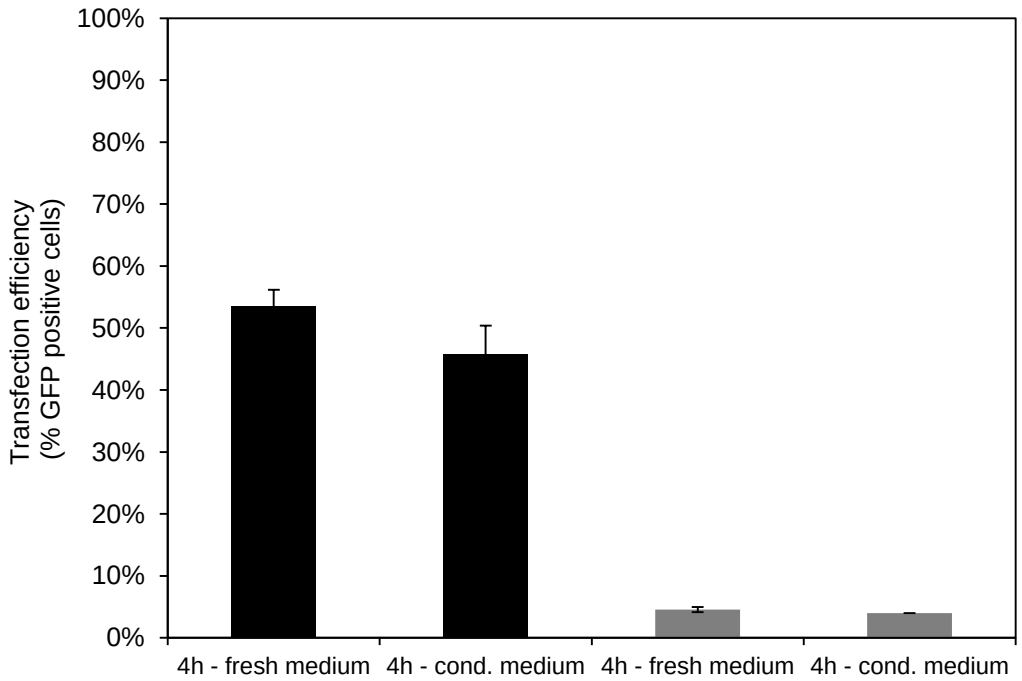

Figure 4.4 – Transfection efficiency (% of GFP positive cells 24-post transfection) of cells transfected in fresh media (■) and in conditioned media (■). A medium exchange to fresh medium or conditioned medium was performed 4h post-transfection. *n=2*

4.1.5. Conclusion

Transient gene expression processes, with and without a medium exchange, were studied and compared. Furthermore, the reasons for the low protein production observed in TGE processes in conditioned medium were assessed: (i) The low protein production is not due to a low cell viability, as the cellular viability post-transfection is even higher when cells are transfected in conditioned medium, in comparison to fresh medium (ii) A higher conditioning time of the medium leads to a lower transfection efficiency, suggesting that compounds that accumulate in the medium could interfere with the transfection procedure (iii) The lower protein expression is due to a negative effect of the conditioned medium on the transfection.

One might think that the lower transfection efficiency (the percentage of GFP positive cells 24-h post transfection) is due to a reduced DNA delivery into the cell due to cell derived components in the conditioned medium that interfere with the PEI-DNA complex (Mislick e Baldeschwieler, 1996)(Ruponen et al., 2001). This hypothesis is explored in the following section.

4.2. Plasmid DNA delivery in conditioned media

4.2.1. Introduction

The results presented in the previous section lead to the conclusion that the transfection in conditioned medium is less efficient that in fresh medium and that this is the reason for the lower protein production. However, the observed transfection efficiency is representative of the percentage of GFP expressing cells. The expression of the reporter protein GFP, although being an indicator, does not give any information about whether or not the transfected DNA is internalized by the cell.

In this chapter, DNA delivery into the cells is studied. This is done first from the point of view of DNA disappearing from the culture medium after transfection, and afterwards by quantifying the copy number of transfected plasmid DNA inside the cells. Further, exogenous mRNA levels are studied and the isolation of transfected plasmid DNA from the cell nuclei is also done.

4.2.2. The transfected plasmid DNA is not detectable in the cell culture medium after transfection in conditioned and in fresh medium

A first approach to study the disappearance of DNA from the culture medium was to collect medium supernatant samples at different time points after transfection, and analyzing them on a 1% agarose gel (Figure 4.5).

When the transfection is performed in conditioned medium the GFP expression is lower. It was expected that DNA would not be internalized by the cells and would stay in the cell culture medium after transfection. Surprisingly, it was observed that 1h after transfection, no DNA was detected in the medium, both when the transfection is performed in conditioned medium, or in fresh medium.

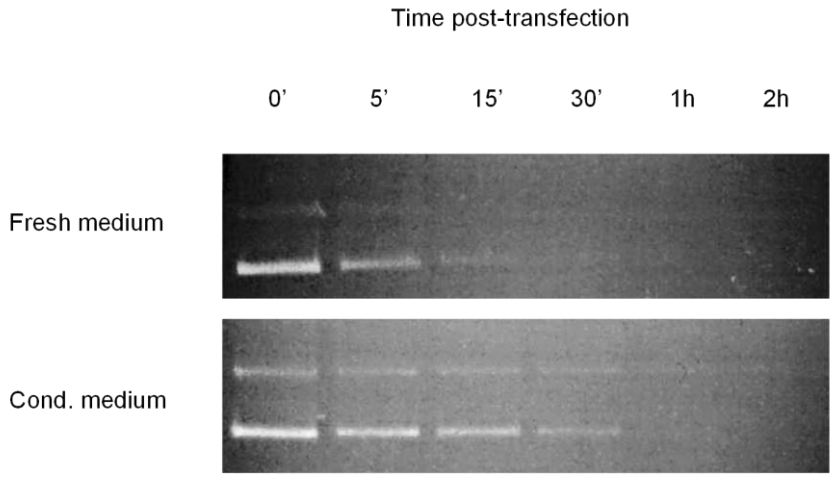

Figure 4.5 – Agarose gel electrophoresis (1%) of medium supernatant after transfection in conditioned medium and in fresh medium. The bands show the plasmid used for transfection, in supercoiled and relaxed form. Both sets (fresh medium and conditioned medium) were run on the same gel, with the same volume of supernatant loaded in every well.

In order to confirm the results of the medium analysis on the agarose gel, a different methodology was used. As before, supernatant samples were collected at different time-points after transfection, and analyzed for their fluorescence when treated with the DNA intercalating dye PicoGreen® (Figure 4.6). The decrease in fluorescence of the medium samples collected after transfection shows the same trend as the agarose gel analysis - One hour after transfection, DNA is not detectable in the cell culture medium. A control experiment (not show) was done, where DNA was incubated with medium to assess whether or not its disappearance from the medium could be due to degradation, and this hypothesis was excluded.

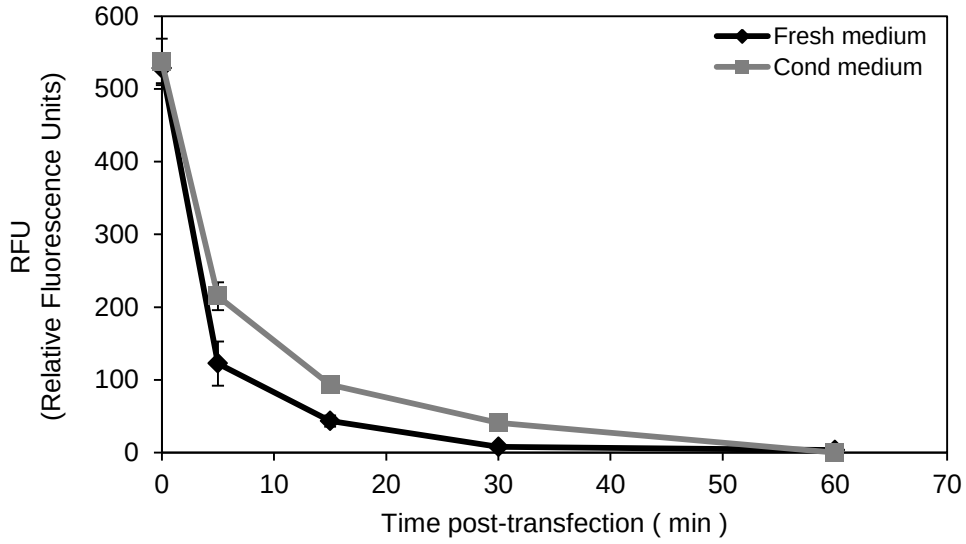

Figure 4.6 – Detection of DNA in the culture medium after transfection using the PicoGreen® dye. *n=2*

If the DNA is not detectable in the culture medium after transfection, there is a strong indication that it is being internalized by the cell. The evidence of internalization of DNA by the cells when the transfection is done in conditioned medium is surprising, because the protein expression is reduced.

4.2.3. Plasmid DNA is being delivered to the cells in conditioned media, but mRNA levels are lower

The information obtained by analyzing the medium supernatant is strongly indicative that the plasmid DNA is being taken up by the cell, but is not conclusive. In order to further study the DNA delivery into the cell, total intracellular DNA was extracted after transfection, and quantitative real-time PCR was used to assess the pDNA copy number per cell. Transfections were performed in fresh medium and in conditioned medium, and at different time-points post-transfection, samples were taken and the plasmid copy number per cell was quantified (Figure 4.7).

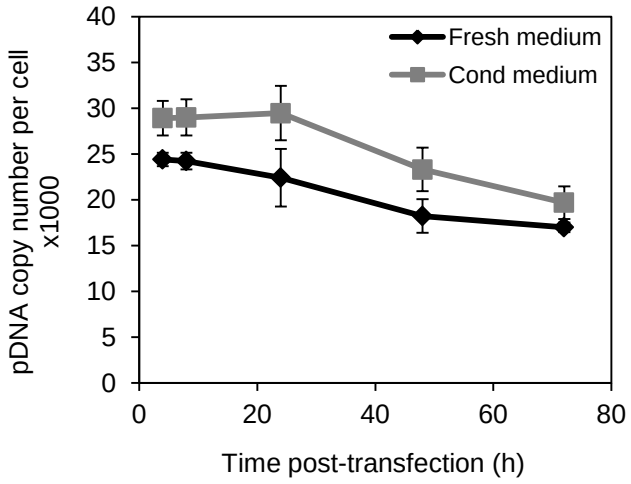

Figure 4.7 – Plasmid DNA copy number per cell (x1000) at different time-points after transfection in fresh medium and in conditioned medium. *n=2*

It was expected to detect pDNA inside the cells, even if the transfection is done in conditioned medium, as the plasmid DNA was disappearing from the cell culture medium. However, it was thought that when the transfection was performed in conditioned medium, a decrease in the plasmid copy number per cell would be seen over time and this could explain the lower protein production. The hypothesis was that in conditioned medium, cell derived compounds in the medium would interfere with the PEI-DNA complex, and a weaker complex would enter the cell (Ruponen et al., 2004). A weaker PEI-DNA complex makes DNA less protected inside the cell and more subject to degradation by nucleases. A big decrease in the pDNA copy number per cell after transfection could explain the low protein expression observed and would confirm this hypothesis, however, this was not observed.

What is shown here is that the pDNA copy number per cell when the transfection is performed in conditioned medium is even slightly higher than when the transfection is performed in fresh medium (this phenomenon was always observed in different qPCR experiments). More importantly, the pDNA copy number is stable up to 3 days after transfection. Longer duration studies cannot be made because DNA extraction and quantification is not reliable when cells have a viability lower than 70%. The slight decrease observed in the pDNA copy number is due to cell division, as cells coming from cell division will not contain the transiently transfected plasmid.

The copy number per cell and the stability of the plasmid DNA copy number is contradictory to the low protein productivity when the transfection is performed in conditioned medium. However, lower mRNA levels can explain the low protein production when the transfection is performed in conditioned medium (Figure 4.8).

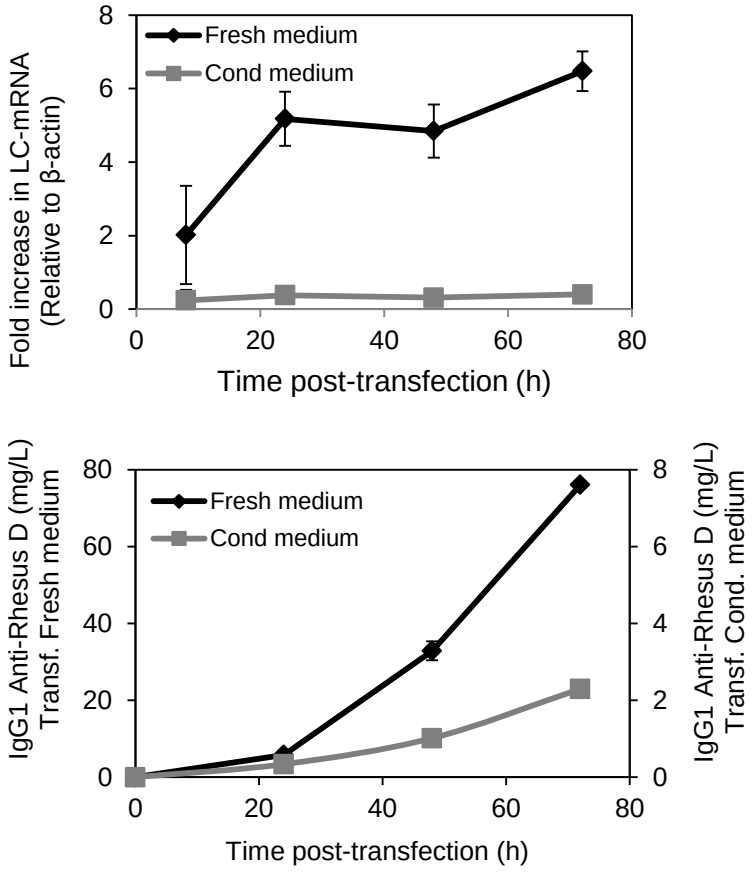

Figure 4.8 – Top: LC-mRNA fold increase relative to β-actin after transfection in fresh medium and conditioned medium. Bottom: Protein titers correlating to the mRNA levels when transfections are performed in fresh medium and in conditioned medium. *n=2*

Three days post-transfection, the mRNA levels when the transfection is performed in conditioned medium, are about 12-fold lower compared to when the transfection is done in fresh medium. The lower mRNA levels correlate to the lower protein production.

The fact that the transfected pDNA is inside the cell, but the mRNA levels are lower, suggests that the limitation in transfecting in conditioned medium is not on the gene delivery into the cell, as it was thought before (4.1.5). The lower mRNA levels could be explained by: (i) The intracellular trafficking of the PEI-DNA complex - if the DNA does not get to the cell nuclei it is not transcribed (ii) The accessibility of the gene for transcription or/and (iii) The state of degradation of the plasmid DNA.

It is known that free chains of PEI (i.e. not in complex with DNA) are important for an efficient intracellular trafficking of the PEI-DNA complexes to the cell nucleus (Yue et al., In Press). PEI is a positively charged molecule that can bind negatively charged molecules present in the conditioned medium. One could imagine that due to this binding, less free PEI chains are available when the transfection is done in conditioned medium. With less free molecules of PEI available, the intracellular trafficking to the nucleus could be affected and the DNA would not be transcribed.

Polyethylenimine, a positively charged molecule, and DNA, a negatively charge molecule, bind to each other making a PEI-DNA complex. It is also possible that other cell-derived components that are present in conditioned cell culture medium (glycans,

proteins, etc.) can bind to this complex making it tighter. A tighter PEI-DNA complex might not be as accessible to transcription as a normal PEI-DNA complex. For example, if histone proteins would be present in the cell culture medium, they could bind to DNA making it less accessible for transcription. A tighter PEI-DNA complex would also make the DNA less susceptible to degradation by nucleases and would explain the higher pDNA copy numbers observed when the transfection is performed in conditioned medium (Figure 4.7).

It is not likely that the DNA is degraded, as extracellular degradation does not occur (4.2.2) and intracellular degradation by exo-nucleases would destroy the whole plasmid making it undetectable by qPCR. However, the hypothesis that the DNA is degraded can only be fully excluded by a southern hybridization, which was not done in this work.

The intracellular localization of the transfected pDNA is studied on the next section. The gene accessibility for transcription was not studied by molecular biology means, but this hypothesis was taken into consideration on the studies on process development for the improvement of productivity and it will be discussed further in this work.

4.2.4. Quantification of pDNA in the cell nuclei

Quantification of intracellular pDNA copy numbers has shown that the DNA is being delivered to the cell when cells are transfected in either fresh medium or conditioned medium. However, the mRNA levels and the protein production are lower in conditioned medium, which means that the delivered DNA is not being utilized by the cell.

For a plasmid to be transcribed it has to reach the cellular nucleus. Cell nuclei from cells transfected in fresh and conditioned media were isolated 24 hours post-transfection and the pDNA copy number was quantified. It was observed that when cells are transfected in conditioned medium, and in fresh medium, an equivalent plasmid copy number is detected inside the cell nucleus. (Figure 4.9).

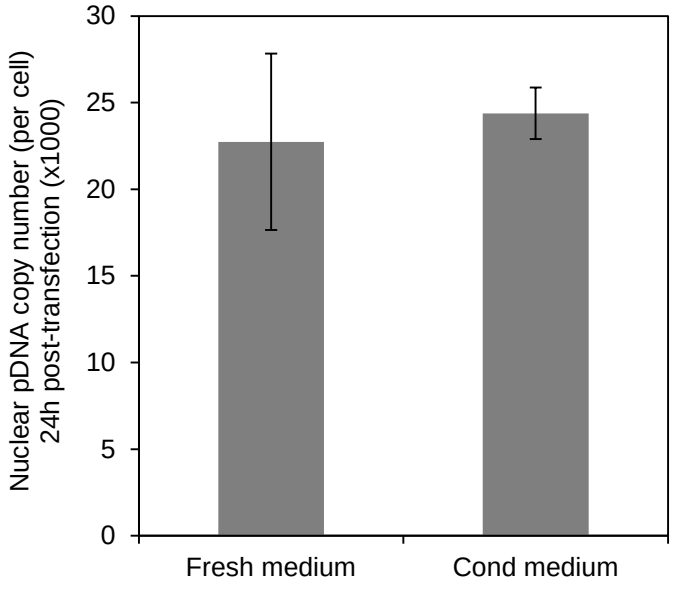

Figure 4.9 – Plasmid DNA copy number (x1000) per nuclei 24h post-transfection when transfections are performed in fresh medium and in conditioned medium . *n=2*

The fact that the same pDNA copy number is found in the nucleus 24h post-transfection, whether cells are transfected in fresh medium of in conditioned medium is a new insight on the understanding of what happens in the transfection when cells are transfected in conditioned medium and in fresh medium.

4.2.5. Conclusion

The systematic study of the fate of the transfected pDNA when transfections are performed conditioned medium and in fresh medium revealed that there are no apparent differences in the number of pDNA copies that are delivered into the cells between these two conditions (Figure 4.7). Further, it revealed that, although the transfected pDNA is present in the cell nuclei, it is not being transcribed into mRNA, which results in a lower protein expression (Figure 4.8; Figure 4.9).

If the transfected DNA is present in the cell nuclei, the possibility of an incorrect cellular trafficking of the PEI-DNA complex when transfections are performed in conditioned medium can be put aside. However, questions are raised on why the pDNA is not being transcribed into mRNA. As previously discussed, there are two main hypotheses that could explain the lower mRNA levels: (i) The transfected DNA is not in a condition which allows it to be correctly transcribed (i.e degraded), (ii) The DNA is not accessible to the RNA polymerase.

In mammalian cells, the enzymes responsible for DNA degradation, the exo-nucleases, are not sequence specific. It is then reasonable to assume that if there would be intracellular DNA degradation due to an inefficient transfection, this would affect all the plasmid, and a lower pDNA copy number would be detected, which is not the case (Figure 4.7).

The reason behind the lower mRNA levels could be a lower accessibility of the plasmid to transcription by the RNA polymerase. As discussed before (4.2.3), it is possible that charged proteins (for example, histones) or other components of the conditioned medium bind to the PEI-DNA complex making it tighter and less accessible to the RNA polymerase transcription complex.

4.3. Improving a TGE process without a medium exchange by process development at small scale

Several strategies were studied in order to improve the TGE process without a medium exchange for CHO-DG44 cells transfected with polyethylenimine (PEI). In order to improve the process, the impact of several parameters was studied: (i) The cell culture medium, (ii) The increase of the concentrations of PEI and DNA (iii) The study of Sodium butyrate (NaBut) and Dimethyl sulfoxide (DMSO) as enhancers of mRNA levels and protein production.

4.3.1. Media screening

Thirty-eight different commercially available media formulations were tested for TGE in conditioned medium. The aim was to find out if the problem of transfecting in conditioned medium can be solved by changing the cell culture media. The 38 media formulations were tested for cell growth and for transfections in fresh medium and in conditioned medium. Before the transfection, cell growth was accessed with the PCV method, and 7 days post-transfection the protein titer was measured by ELISA (Figure 4.10).

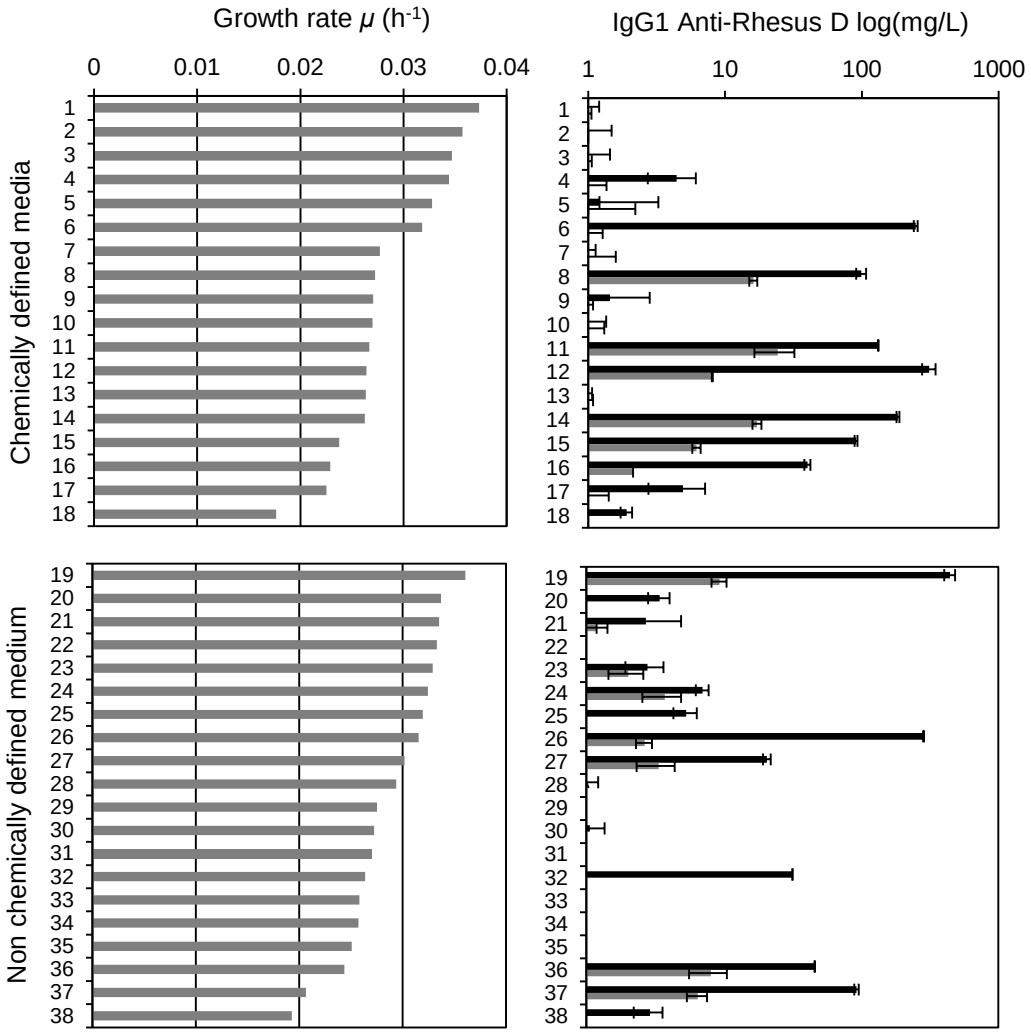

Figure 4.10 – Screening of 38 cell culture media formulations for growth (left) and productivity on a 7 day batch TGE process in fresh medium (■) and in conditioned medium (■). The cell growth calculation has an error of <5%. The 38 media samples were provided by Excellgene SA (Monthey, CH)

Through this screening approach, 15 media formulations that allow for a fast cellular growth were identified (μ>0.03 h^{-1}, doubling time < 23h). Taking into account the control transfections in fresh medium, only in 13 media formulations, transient antibody production was observed (>10 mg/L. More importantly, only in 5 media formulations high titers were achieved (>100mg/L).

In all the media formulations studied, a significant reduction in productivity is observed when the transfections are performed in conditioned medium (productivity is presented in a logarithmic scale). The highest productivity obtained in conditioned medium (24 mg/L, number 11) is still far from the maximum productivity obtained in fresh medium (450mg/L, number 19).

Changing the cell culture medium requires adaptation of the cells to the new medium and establishment of a cell bank in the new medium after adaptation. Taking these factors into account, it was considered that the slight improvement obtained with the medium formulation 11 for transfections in conditioned medium was not significant enough to consider a change from one medium to another. Other alternatives for the improvement of titers in conditioned medium were explored in the following sections.

4.3.2. Increasing the PEI and DNA concentrations improves transfection efficiency and protein production

The hypothesis of increasing the PEI and DNA concentrations in order to improve the TGE process in conditioned medium was studied. The rationale behind this approach is that some of the negative effects of the conditioned medium on the transfection could be surpassed by "overloading" the cells with DNA. Accordingly, by utilizing a higher DNA concentration, the PEI concentration also needs to be increased.

The process that had been previously established for transfecting in fresh media utilizes 3µg/million cells of PEI and 0.6 µg/million cells of DNA. Cells were transfected after being cultivated for 3 days, when the cell density reached 5-5.5 million cells/mL. At the time of transfection the concentrations of PEI and DNA were varied in a range of 3-8 µg PEI/million cells and 0.6-1.2 µg DNA/million cells. The transfection efficiency was measured 24-h post transfection (Figure 4.11) and antibody yields were measured after 7 days and are represented as a fold difference compared to the control (Figure 4.12).

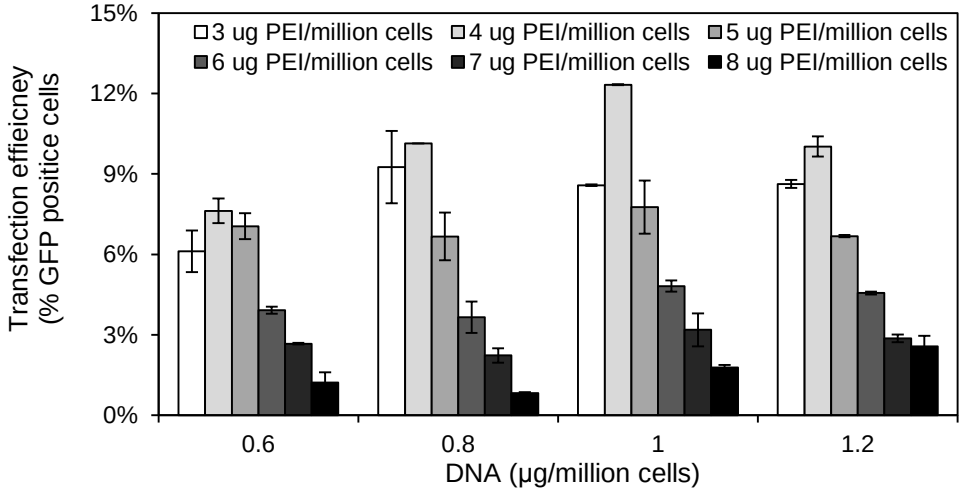

Figure 4.11 – Transfection efficiency (% of GFP positive cells 24-post transfection) in conditioned medium at different concentrations of DNA and PEI. Control (0.6µg DNA/million cells; 3µg PEI/million cells). *n=2*

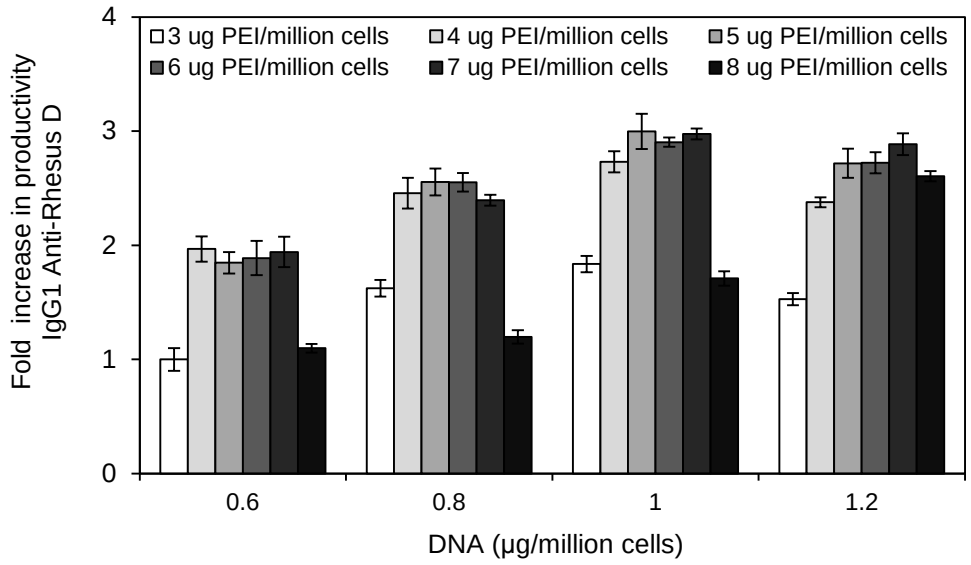

Figure 4.12 – Fold increase in productivity in conditioned medium at different concentrations of DNA and PEI. Control (0.6µg DNA/million cells; 3µg PEI/million cells)

As observed in both Figure 4.11 and Figure 4.12, increasing only the PEI concentration from 3µg/million cells to 4µg/million cells already improves the process (keeping DNA at 0.6 µg/million cells). One can imagine that the extra PEI can bind to some of the charged compounds in the conditioned medium that interfere with the transfection, and enhance this process. However, the best results are obtained when the DNA concentration is also increased, and the optimal DNA concentration for this process is 1µg/million cells. The DNA concentration of 1.2µg/million cells was not considered for further studies, as the cell viability drops very fast with this concentration of DNA (data not shown).

Considering the concentration of DNA of 1µg/million cells the optimal, further investigations were done to decide on whether to use 4 or 5 µg/million cells of PEI, as this are the two lowest concentrations of PEI that result in an improved process (Figure 4.12).

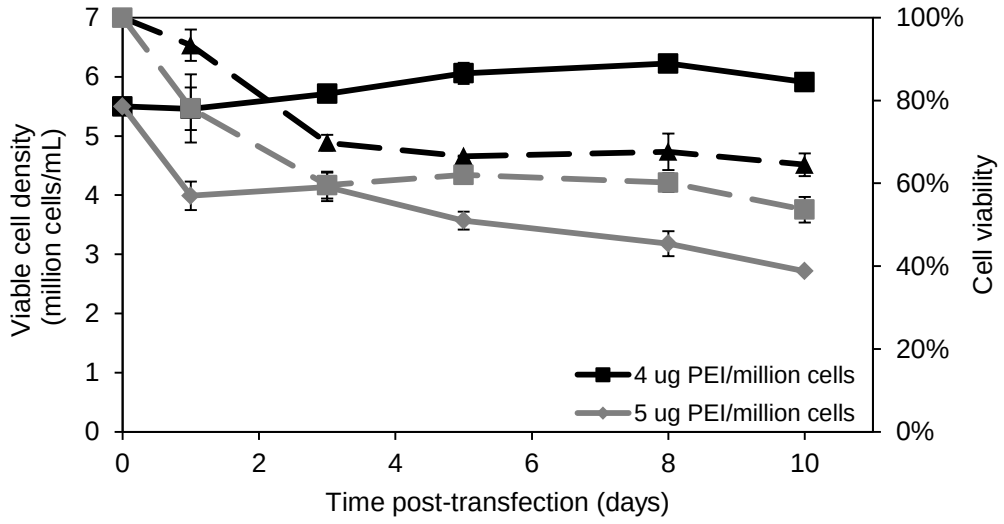

Figure 4.13 – Viable cell density (straight lines) and cell viability (dashed lines) when transfections are performed in conditioned medium with a DNA concentration of 1µg/million cells and two different concentrations of PEI.

At a concentration of 1 µg/million cells of DNA, the highest titer is obtained when using a concentration of 5 µg/million cells of PEI (Figure 4.12). However, the cell density decreases very fast after transfection, and the cell viability is also slightly lower, compared to the process with 4 µg/million cells of PEI (Figure 4.13). As such, the optimal condition for transfecting in 3 day old medium was defined as 1µg/million cells of DNA and 4 µg/million cells of PEI.

4.3.3. Strategies to improve mRNA levels and protein production in TGE processes in conditioned medium

Previous studies showed that in conditioned medium the transfected DNA reaches the cell nuclei but the mRNA levels and protein production, are reduced. As such, compounds that are known to increase the mRNA levels and recombinant protein production in mammalian cells, were tested.

Sodium Butyrate (NaBut), an HDAC inhibitor, has been used in mammalian cell culture for a long time to improve protein yields, and it is known to stimulate monoclonal antibody expression in CHO cells by improving gene accessibility (Jiang e Sharfstein, 2008). Dimethyl sulfoxide (DMSO), and organic solvent, has been reported to improve exogenous gene expression in CHO cells by enhancing transcription (Wang et al., 2007).

Based on the previously defined optimal transfection conditions for this process, additions of NaBut and DMSO at the time of transfection were assessed for the improvement of protein production over a 7 day period, when transfecting in conditioned media (Figure 4.14).

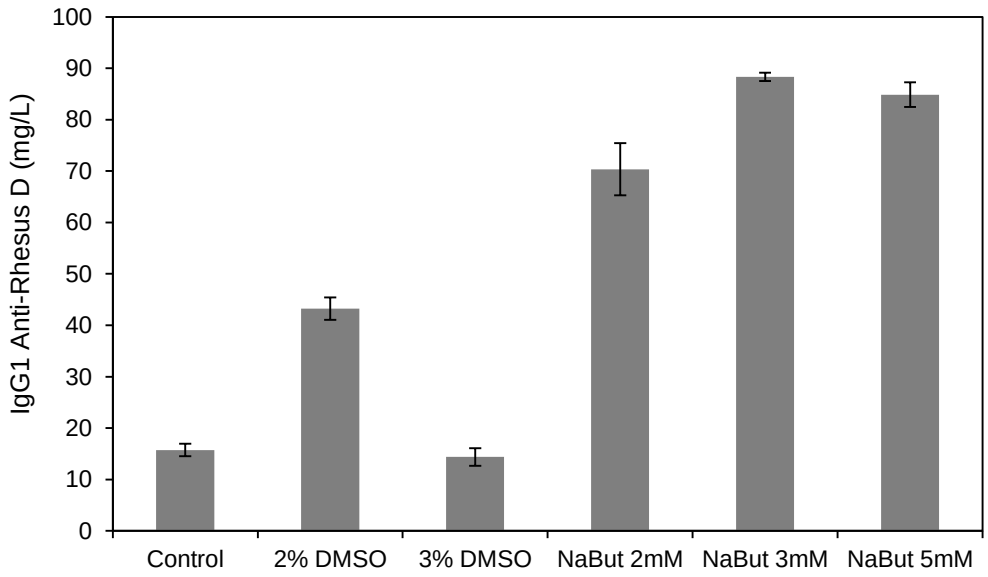

Figure 4.14 – The use of Dimethyl sulfoxide (DMSO) and Sodium Butyrate (NaBut) for the enhancement of protein production when the transfections are performed in conditioned medium. Protein concentration measured at day 7 post transfection. Control (transfection performed with 1µg of DNA and 4µg of PEI per million cells)

It was observed that both DMSO and NaBut enhance the protein productivity. This was already known for both of these compounds. In fact DMSO when used in fresh medium results in a 2-3 fold increase of transient protein titers in CHO cells and a similar effect is observed here (Yashas Ranjendra, EPFL, unpublished data). This is

consistent with the hypothesis that DMSO enhances transcription, and thus, enhances mRNA levels and protein titers by having a positive effect on the activity of the RNA polymerase (Chen e Zhang, 2005).

For transfections in conditioned medium it is seen that NaBut increases the titers more than 5-fold. However, if the transfection is performed fresh medium the addition of NaBut does not have such a big impact (data not shown).

Based on the 5-fold improvement seen when adding NaBut for transfecting in conditioned mediumThis is in accordance with the hypothesis proposed before, that components of the conditioned medium bind to the PEI-DNA complex, are internalized during the transfection, and decrease the gene accessibility, and thus, a lower protein production is observed (4.2.3;4.2.5). It could even be that cell derived histone proteins present in the conditions medium are binding to the DNA, altought this is not proved. However, the addition of NaBut helps to overcome the negative effects of the conditioned medium in TGE processes.

4.3.4. Conclusion

The advantage of process development at small scale is that it allows the study of different conditions at the same time. Here, the improvement of the transfection conditions for transfecting in conditioned medium yielded a 3-fold improvement on the protein titers. Combined with the addition of NaBut, the titers of the already improved process are increased more than 5-fold (Figure 4.15).

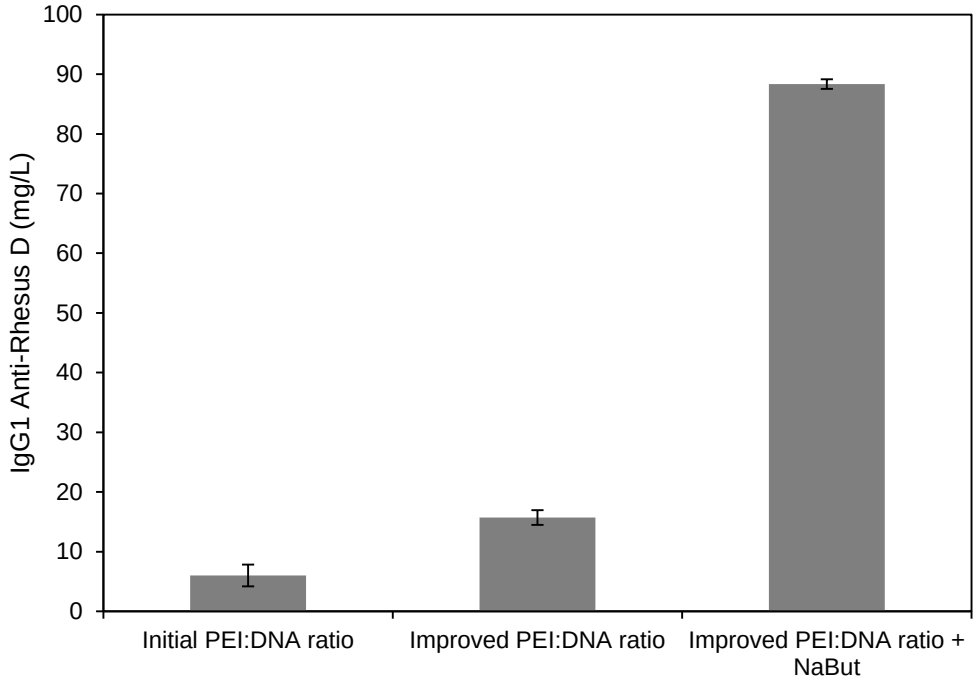

Figure 4.15 - Yield comparison between the initial process (optimized for fresh medium), the process with the improved transfection conditions for conditioned medium and the improved process with the addition of Sodium Butyrate (NaBut).

An 18-fold improvement of the process was achieved by combining the improvement of the transfection conditions with the addition of NaBut. Table 4.1 shows a summary

of what was done to improve this TGE process in CHO-DG44 cells in conditioned medium.

Table 4.1 – Summary of the improvements of the TGE process in CHO-DG44 cells in conditioned medium

	Initial process	**Improved process**
25kDa Polyethylenimine (PEI) (µg/million cells)	3	4 (33% more)
DNA (µg/million cells)	0.6	1 (66% more)
Chemical addition	None	3mM of Sodium Butyrate
Yield	~5 mg/L	~90mg/L (18-fold)

5. Future perspectives and conclusion

In this work, transient gene expression processes in conditioned medium in CHO-DG44 cells were studied. The investigations on DNA delivery provided new insights on the understanding of why these processes are less efficient. By quantifying intracellular plasmid DNA concentrations, it was unraveled that the DNA delivery into the cell is not a limitation when transfecting in conditioned medium and it is not the factor responsible for the lower mRNA levels and protein production. The hypothesis of incorrect intracellular trafficking of the plasmid was put aside by extracting the cell nuclei after transfection and quantifying the intracellular pDNA. It was observed that the transfected DNA was inside the nuclei 24h post transfection when the cells are transfected in either fresh or conditioned medium. It was hypothesized that there might be a problem of gene accessibility and that this could be the reason for the lower mRNA levels when the transfection is performed in conditioned medium.

As argued before, it is not likely that the plasmid DNA is degraded, as its detection is very stable by qPCR. However, a definitive proof can only be obtained by a southern hybridization of the DNA isolated from the cells after the transfection. This should be included in further work done on this topic.

The trafficking of the DNA to the nucleus was studied with a simple method of nuclear extraction that is based on a detergent. However, it would also be interesting to label DNA and PEI with fluorescent probes and with microscopy, study the differences of the transfection process with DNA and PEI in fresh and in conditioned medium.

The hypothesis of reduced gene accessibility due to compounds of the conditioned medium binding to PEI-DNA complexes, and making them less accessible, is not easy to prove. First, it is necessary to quantify cell-derived compounds in the conditioned medium, by measuring DNA concentrations, protein concentrations and glycan concentrations in the cell culture medium. This however, is both challenging and time consuming, as cell culture media is a highly rich mixture of components, that include proteins and glycans, and it would be difficult to analytically distinguish cell derived components in this mixture. More specifically, it would be interesting to detect the presence of DNA binding proteins in the conditioned medium. Further research should also look into *in vitro* studies to detect the possibility of a PEI-DNA complex being formed in conjugation with other charged molecules like proteins (positively charged and negatively charged) or glycans.

In the process development part of this work, the concentrations of of DNA and PEI that had been used for TGE processes involving a medium exchange before transfection, were optimized to define a TGE process without a medium exchange (i.e. in conditioned medium). An increase of the DNA concentration and PEI concentration lead to a 3-fold improvement of the yields.

Further, the possibility of enhancing mRNA levels and protein production with Sodium Butyrate (NaBut) and Dymethyl Sulfoxide (DMSO) was investigated. Although both chemicals improved the recombinant protein yields, NaBut proved the most efficient. A yield improvement of more than 5-fold was obtained by using Sodium Butyrate and this might be due to enhanced gene accessibility. This is a good example of how systematic research combined with process development tools can be used to solve otherwise complex biotechnological problems. All together, the TGE process in conditioned medium in CHO-DG44 cells was improved 18-fold, from ~5mg/L to ~90mg/L. Regarding the available literature, this was the first TGE process with significant yields in CHO cells that was performed without a medium exchange.

A remark to small scale cell culture systems should be made, as it would not have been possible to conduct this kind of process development work at larger scales. The TubeSpin® bioreactor 50 proved to be very useful, as it allows high density suspension cell culture work in volumes that can go to as low as 5 mL. This allowed, for example, 48 different bioprocesses (i.e. 48 small-scale bioreactors) to be run at the same time when optimizing the PEI and DNA concentrations.

In this approach, the use of feeding was not looked into. As the transfections are performed in conditioned media, the concentrations of medium compounds are changed, and the medium has already accumulated metabolites as lactate and ammonia and is depleted of glutamine. A feeding approach would enhance the cell metabolism and extend the process for a longer period of time, resulting in higher yields. Also, only 3 concentrations of NaBut were tested and a more refined screening of different concentrations of NaBut should be done. The timing of addition of NaBut could also be important as in this study NaBut was only added at the time of transfection. It could also be that adding NaBut before the transfection, or some hours after has a better effect on the productivity.

The development of TGE processes without a medium exchange is one of the principles that allow the scale-up of transient gene expression and these processes should continue to be developed and scaled-up. High yields obtained by transient transfections at large culture volumes would allow the manufacturing of Kg amounts of protein for the market in time-spans of as short as 1-2 weeks. This would be a very strong competitive advantage for biotechnology companies as they could enter the market faster, without having to spent months developing a stable cell line and a process for it. If high-yielding TGE is mastered at large-scale, the door is opened for a paradigm shift in the protein manufacturing industry, but that remains to be seen.

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