Optimization of transient protein production by chemically transfected CHO suspension cells

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Dedication

To my family

Declaration

This work described here in the current thesis was carried out at the Institute of Technical Chemistry, Leibniz University of Hannover under the guidance of Prof. Dr. Thomas Scheper. I hereby declare that the present work in my own and to the best of my knowledge and belief, it contains no material previously neither published or written by another person or submitted by another person for the award of any other university degree, except where acknowledgment has been made in the text.

Hannover, September 2017

Abdalla Adel SabryAbdelarzik Mohamed Elshereef.

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Abstract

During the last decade, transient mammalian cell transfection technology has become wide spread and accepted for the fast expression (a matter of weeks) of different recombinant proteins, for both pharmaceutical application and research development. The development of a robust, scalable, transient protein production platform based on fundamental knowledge of this complex process is an essential task. Strategies to optimise and develop transient transfection aim to improve the scalability and to extend production time via repeated transfection. Although polyethylenimine (PEI) is one of the most studied transient transfection mediating agents, many open questions still exist concerning its mechanism, toxicity and transfection efficiency; this lack of knowledges hinders the production of large amounts of proteins in comparsion to stable cell lines.

In the present work, cell viability and transfection efficiency are the most important response parameters evaluated to study such fundamental processes. Transfection efficiency has been investigated by transfecting a plasmid-expressing green fluorescent protein (GFP) into the CHO-K1 cell which can easily be detected and quantitatively measured. CHOMACS CD medium supports cultivation of a high transfected cell density of about 10-18 million cells per mL with a viability of over 80%, which elucidates a transfection culture volume of up to 12.5 mL in a Tubespin®50 bioreactor and in small shake flasks. Several conditions for transfection efficiency (TE) have been explored and guided by a design of experiment approach (DoE). A high TE of 70% combined with a high cell viability of over 95%, and an extended expression time of 120 hours post-transfection, (hpt) have been achieved through a modified repeated transient transfection strategy utilizing a low amount of 6 µg pDNA:30 µg PEI for 10 million cells per mL.

Through this optimization, transforming growth factor (TGF-β1) tagged with a tryptophan label and a Strep-tag was successfully expressed in CHO-K1 cells. The total purified volumetric yield of TGF-β1 from culture supernatant was about 27 µg/mL. The bioactivity of this mature TGF-β1 exhibited the same extent of inhibition effect on A549 epithelial cell growth as the commercial one. Fluorescence spectra reveal a direct increase in fluorescence intensity correlated with purified protein concentration. This would represent an ideal application of 2D-fluorescence spectroscopy for monitoring the protein productivity process.

Keywords: PEI-transient transfection, GFP-transfection efficiency, viability, TGF-β1 expression.

Zusammenfassung

Technologien zur transienten Transfektion von Säugetierzellen zur Expression rekombinanter Proteine haben im vergangenen Jahrzehnt sowohl für die pharmazeutische Anwendung als auch für Forschung und Entwicklung an großer Bedeutung gewonnen. Die Entwicklung einer robusten, skalierbaren Transfektionsplatform ist dabei die Grundlage für qualitativ hochwertige Produkte und reproduzierbare Bioprozesse. Die Transfektion mit Polyethylenimin (PEI) ist die bisher am besten untersuchte Methode. Trotz des großen Aufwands, der in den vergangenen Jahren betrieben wurde, um die Zusammenhänge der chemischen Transfektion zu verstehen, blieben bisher viele Fragen offen, die sich beispielsweise mit Toxizität, Wirkmechanismen und Transfektionseffizienz beschäftigen. Dies ist einer der Gründe, warum pharmazeutische Wirkstoffe derzeit ausschließlich mit stabilen Zelllinien produziert werden.

Der Fokus dieser Arbeit liegt darin zu untersuchen, welche Versuchsparameter sich auf die Zellviabilität nach Transfektion und die Transfektionseffizienz (TE) auswirken und die Wirkzusammenhänge zu verstehen. Zur Untersuchung der TE wurde eine CHO-K1 Zelllinie mit einem Plasmid zur Expression von grün fluoreszierendem Protein (GFP) transfiziert und die TE über das Fluoreszenzsignal verfolgt. Die Versuche wurden in Tubespin®50 Bioreaktoren mit 12,5 mL Arbeitsvolumen sowie in 125 mL Schüttelkolben mit einem Arbeitsvolumen von 25 mL durchgeführt. Es wurden sukzessive DoE Experimente durchgeführt um die Einflüsse verschiedener Versuchsparameter quantitativ zu bestimmen. Bei einer Zellviabilität von über 95% wurden Expressionszeiten von über 120 h und TE von über 70% erreicht.

Außerdem wurde eine bereits bestehende Methode zur mehrfachen, wiederholten Transfektion einer CHO-K1 Zelllinie zur Expression von TGF-β1 (transforming growth factor) in der CHO-K1 Zelllinie optimiert. Dabei wurden wesentlich geringere Mengen an PEI und Plasmid-DNA verwendet. Der Inhibierungseffekt auf das Zellwachstum ist dabei ebenso hoch wie bei einem kommerziellen Vergleichsprodukt. Die Ausbeute an aufgereinigtem Produkt lag dabei bei 27 µg/mL. Mithilfe eines Tryptophan-Tags konnte die Produktbildung mithilfe von Fluoreszenzspektroskopie online verfolgt werden. Dabei korrelierte die Fluoreszenzintensität direkt mit der Konzentration an aufgereinigtem TGF-β1.

Keywords: PEI-transiente Transfektion, GFP-Transfektionseffizienz, Viabilität, TGF- β1 Expression.

List of Abbreviations

°C	Celsius
2D	Two-dimensional
A549	Human Lung Carcinoma epithelial adherent cell line
aa	Amino acid
ANOVA	Analysis of variance
BM40	Secretion signal peptide (from basement membrane protein 40)
bp	base pairs
CCC	a central composite circumscribed design
CHO-K1	Chinese hamster ovary cell line (suspension)
Conc.	Concentration
CTB	CellTiter Blue (Assay)
ddH_2O	double distilled water
DLS	Dynamic light scattering
DNA	deoxyribonucleic acid
DoE	Design of Experiment
E	Elution buffer fraction
E.coli	Escherichia coli
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
GFP	Green Fluorescence Protein
GFP-TE	Green fluorescent protein transfection efficiency
GMP	Good manufacturing practices
hpt	hours post-transfection time
hpt_1	Hour post-transfection time step one
hpt_2	Hour post-transfection time step two
hr/hrs	hour/s
IgG	Immunoglobulin G
kV	Kilovolt
LAP	latency associated peptide
LB	Luria-Bertani medium
L-PEI	Linear Polyethylenimine (PEI)
LTBP	latent TGF-β binding protein
M	Mole

min	minutes
mOsmol kg^{-1}	milliosmols (one-thousandth of an osmole) per kilogram of water
N:P	Nitrogen to phosphate
O.D	Optical Density
PBS	phosphate buffered saline
pDNA	plasmid DNA
pEGFP-N1	plasmid enhanced fluorescent protein N1
PEI	Polyethylenimine
PVDF	Polyvinylidene difluoride membrane
rpm	revolutions per minute
RPMI F68	RPMI media with pluronic F68
SD	Standard Deviation
SDS	sodium dodecyl sulphate
SLC	small latent complex
TCC	Total cell count
TE%	Transfection efficiency percent
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TGF-β1	transforming growth factor beta 1
T-test	T-test tiales test.
Twin-Strep-tag®	Twin-Strep-tag Two moieties of Strep-tag®II
UV	Ultraviolet light
V	Volume
v/v	volume by volume
VCC	Viable cell count
W	Weight
w/w	weight by weight
xg	Earth's gravitational G-force

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1. Introduction

The growing worldwide demand for therapeutic recombinant proteins has created intense interest in the establishment of several mammalian protein production platforms. A fast tool to meet this growing need is necessary for these platforms and an important development goal. Global sales in these market segments exceeds US $120 billion per year [1]. In addition, more than 50% of all biopharmaceutical products on the market are produced through mammalian cell protein expression systems [2].

Normally, recombinant protein expression requires the introduction of foreign DNA into a host cell, a process known as transformation when applied to prokaryotes, and transfection when applied to eukaryotic cells. The biotechnology and pharmaceutical industry sectors rely heavily on mammalian cell culture as the bioproduction system to produce such proteins with glycosylation patterns similar to those of human proteins. The most widely used host mammalian cells for producing various kinds of protein are Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK 293) cells, baby hamster kidney (BHK) cells and mouse myeloma cells, including NS0 and Sp2/0 cells [2–4]. CHO cells are one of the most prominent mammalian cells used for expression of biopharmaceutical proteins since 1957 [5]. The pharmaceutical industry prefers to use CHO cell lines because of its well-known cell system [6].

To date, the standard approach to supply the market with recombinant proteins is a genetically engineered stable transfection cell line. The recombinant gene is integrated into the host's genome followed by screening and selection of high producing clones [3]. The establishment of highly stable producer cells takes a long time – i.e. between 6 to 12 months. Recombinant protein expression by transient transfection of a mammalian cell line is called transient gene expression (TGE), a system that allows the rapid production of milligram to multigram quantities of protein, up to 100s of liters, in a short time frame (days to weeks) [7–9].

This TGE system is a promising means of producing of a wide range of proteins, including kinases, receptors, and enzymes in as short a time as one week. Polyethylenimine (PEI) is considered the most common transfection reagent to achieve transient transfection flexibly and economically [10]. Transfection criteria, for both transfection reagent and cell line are necessary not only for transient transfection in protein expression, but also for establishing scalable transient production processes. In addition, several critical parameters need to be carefully optimized in order to obtain reproducible and highly efficient transfections. These factors include media type and vector design, as well as continuous basal variables such as cells, plasmid DNA and PEI. Also, process design variables such as scale, temperature, osmolality are important parameters for scalable TGE [11]. In the future, establishment and development of

an efficient and rapid protein expression platform are crucial to support early drug development or novel recombinant protein expression which is required for structural biology and therapy.

1.1. Aim and objectives

In this thesis, I study transient protein expression technology, with the aim to achieve higher and reproducible protein yields. This goal requires optimization of the gene delivery method to be more efficient, simpler, higher yielding and scalable at an affordable cost. Plasmid Enhanced Green Fluorescence Protein (pEGFP-N1) is used as a model. This plasmid, expressing green fluorescence protein (GFP), and the linear polyethylenimine (PEI) polymer as the transfection reagent to deliver the plasmid for the CHO-K1 host cell line, are the focus of this research work. The results of this model can be applied to other recombinant proteins for the evaluation of established transient transfection systems. The goal of this study was extended to include production of transforming growth factor beta-1 (TGF-ß1) on a lab scale using the TGF-ß1 plasmid. This plasmid encodes a fusion protein with a Strep-tag and fluorescent tag, which provides an easier way to purify protein and monitor it offline, respectively.

There are three major objectives in achieving this aim. They are as follows:

- **Optimization of GFP transient transfection.**
  - A. Independent study of transfection parameters
    1) Media-related factors, such as media type, media exchange, and media osmolality.
    2) Cell culture-related factors, like pre-culture age, pre-culture cell density, and cell density at transfection time.
  - B. Interaction study of transfection parameters using DoE approach
    1) Cell density, PEI and pDNA amount
    2) PEI and pDNA amount
  - C. Repeated transient transfection process one more time to improve transfection efficiency
  - D. Physicochemical properties of pDNA/PEI polyplex nano-particles
    1) Nanotracking particle size formation dynamics analysis
    2) Imaging analysis of pDNA/PEI polyplex
- **Scalability of transient transfection protocol.** From 1 ml to 12.5 mL in Tubespin bioreactor®50 and small shake flasks.
- **Transient expression of TGF-ß1 in shake flasks**
  1) TGF-ß1 purification, detection and quantification
  2) TGF-ß1 bioactivity test

2. Theoretical Background

There is no doubt that the production of recombinant protein is an important issue for biotechnology research, bioprocess development and industry. The traditional methods for down-streaming amounts of functional protein from large amounts of animal or plant tissue are ineffective from an economic point of view. With advances in molecular biology and the science of gene-to-protein, it becomes easier to construct a plasmid with the desired gene and express it through an appropriate cell host mechanism. The following theoretical background discusses the issues involved in obtaining recombinant proteins.

2.1. Recombinant protein production host systems

Escherichia coli (E.coli) is the most widely used host for commercial recombinant protein production. It has the capacity for high-level protein expression and grows rapidly on inexpensive media. Therefore *E.coli* would be the first choice as a host due to its relatively simple, well-established cultivation requirements. The bacterial production is however defined by the limited capability of *E.coli* to form a recombinant protein with accurate quaternary protein folding structures and has a limited capacity with regard to post-translational modifications such as glycosylation, amidation, acetylation and phosphorylation. In addition, it often forms insoluble aggregates (inclusion bodies) or aggregates with low solubility [12–14].

Another promising host for recombinant protein production is yeast, including *Saccharomyces cerevisiae*, *Pichia pastoris* and *Hansenula polymorpha.* Many eukaryotic post-translational modifications can be performed with yeasts, such as glycosylation, disulfide bond formation and proteolytic processing. However, other modifications, such as prolyl-hydroxylation and amidation as well as some types of phosphorylation and glycosylation are not possible with yeasts [15–17]. In contrast, mammalian cells can be used to perform complex post-translational modifications of recombinant proteins [18]. Furthermore, the chaperone system of mammalian cells ensures that proteins are secreted in a correctly folded manner [19]. However, differences in glycosylation and post-translation patterns between different mammalian cell lines are also problematic [20]. The process is very expensive with poor secretion in comparison to the other hosts mentioned here.

The filamentous fungi expression host system, particularly with *Aspergillus spp.,* and *Trichoderma reesei,* provide a high level secretion of heterologous proteins at low cost, though our limited understanding (compared to bacteria) of the complex physiology of filamentous fungi, and significant differences in glycosylation compared to mammalian cells, have hindered this technology up to now [21–23]. Recombinant baculovirus-insect cell expression systems are able to produce recombinant proteins with more complex post-translational modifications

associated with the eukaryotic system. Because in insect cells, signal peptides are cleaved as in mammalian cells, disulfide bonds are formed in the endoplasm reticulum and pro-protein-converting enzymes are available for proteolytic processing. Glycosylation in insect cells is not identical to that in mammalian cells. This can influence protein solubility, half-life, activity and interactions with other molecules [20,24,25].

While one single perfect host for all proteins does not exist, bacteria remain attractive hosts for producing simple proteins. For proteins that require glycosylation, mammalian cells, fungi, or the baculovirus system are used.

In general, the mammalian cell host has the advantage in mimicking human glycosylation with direct application of the produced protein. Therefore, it represents a good host for functional recombinant protein production. In particular, Chinese hamster ovary (CHO) cells are the most commonly used mammalian cell host as they provide about 50% of the therapeutic protein market [26].

2.2. Transfection methods mediate recombinant protein expression

After selection of the suitable protein expression host for the target protein expression plasmid, the transfection method to deliver this plasmid to the host cell is an important issue. Many transfection methods have been developed to transfer genetic material into a host cell. These include virally, physically, and chemically mediated transfection methods. Each method should be compatible with the cell type and purpose. Method types, application, and advantages and disadvantages have been reviewed by Jin and Al-Dosari [27,28]. Some methods are preferred to make stable transfections while others for transient transfection. Techniques and main difference between the two transfection systems are described in Figure 1.

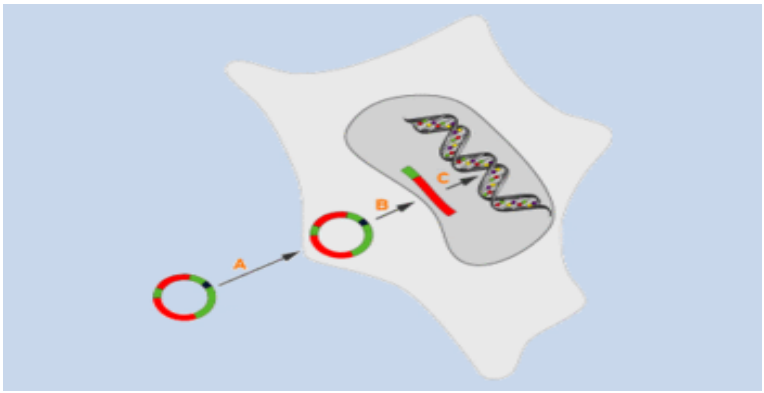

Figure 1: Difference between transient and stable transfection systems [29].
With transient transfection, the genetic material transfers until step B and is not integrated to the cell chromosome (remains in episomal form). Thus, the transfected cells lose the genetic material within a short time. It is chemical- or electroporation-based. With stable transfection, the genetic material transfers until step C and the gene is chromosomally integrated. Thus, all cells have transfected DNA. It is viral- or microinjection-based.

Viral vector transfection involves modification of the viral genomes by deleting some areas of their genomes so that their replication becomes deranged; but it has to overcome genotoxicity and immunogenicity challenges [30]. Physical methods introduce isolated DNA into the nucleus of single or multiple target cells. These methods are less dependent on cell type and condition [31] and are therefore preferred for single cell stable transfection. These physical methods are based on achieving transient penetration into the cell membrane by mechanical, electrical, ultrasonic, hydrodynamic, or laser-based energy so that "naked DNA" in media enters into the targeted cells. Chemical systems are more common than physical methods. They are easy to use and effective for transfecting suspension cells and adherent cell tissues, but have to overcome the variability in transfection efficiency by cell type or condition. These chemical methods include compaction of negatively charged nucleic acid by cationic liposome/micelle or cationic polymers constitute nano-particles called lipoplex and polyplex, respectively.

While all these viral and non-viral gene delivery systems have been developed in the last three decades, no delivery system has been designed that can be applied in gene delivery of all kinds of cell types *in vitro* and *in vivo* with no limitation and side effects [32].

Chemical transfection methods enable research and development efforts to combine low toxicity with high transfection efficiencies, particularly with linear polyethylenimine (L-PEI) 25 kDa molecular weight [33]. The polyethylenimine cationic polymer introduced by Boussif [34] has become the gold standard of non-viral gene delivery in mediating transient transfection. Recently, application of this polyethylenimine for various protein productions in HEK-293 and CHO suspension cells has been reviewed by Hacker [35]. The more recent study suggests that other higher molecular weights of linear PEI, such as 40 kDa PEI, are better for obtaining optimal transient protein production in both cell lines than 25 kDa PEI [36], while the lower molecular weight of PEI 1.8 kDa has low transfection efficiency compare to L-PEI 25 KDa [37]. In this study linear 25 kDa PEI was used to mediate transient transfection in CHO-K1 suspension cell line because of its successful track record in transient gene expression approaches [38,39].

2.2.1. Transfection efficiency

Transfection efficiency (TE) is a very efficient response parameter to evaluate the effectiveness of a transfection method for the transfer of genetic material into the host cells with minimal toxicity. TE determines the percentage of transfected cells in the population, either visually by fluorescence microscopy or quantitatively by flow cytometry. Transfection efficiency is usually monitored either by green fluorescence proteins (GFP) which express cytosolic fluorescent protein and can easily detect positively expressed cells or by luciferase reporter genes which

produce bioluminescence in the presence of luciferin. Protein yield is directly dependent on the transfection efficiency [40] and post-transfection time. Improvement in the transfection efficiency is one of the most important research objectives. Therefore, all considered transfection methods should have a high transfection efficiency, low cell toxicity and be easily reproducible [41].

The linear 25 kDa polyethylenimine chemical transfection method was found to produce cost effective transfection efficiency with other transfection reagents of Lipofectamine® 2000, TransIT-PRO® ExGen 500 and Effectene in CHO cells [42,43].

An optimal level of GFP transfection efficiency has been reported for CHO cells between 50-72% [35,39,44] depending on cell type suspension or adherent and media condition. The cell cycle as described in Figure 2 [45] at S phase increases the transfection efficiency level, and, protein productivity increases by arresting the cells at G2/M phase [46].

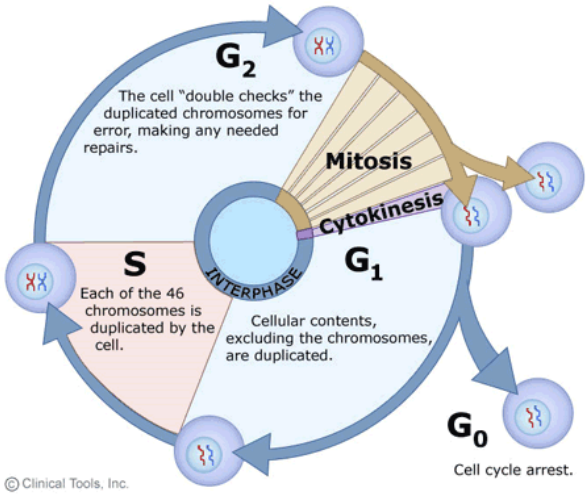

Figure 2: Cell cycle diagram [47].

2.3. Character of the transfection host cell and media

Host cell properties and cultivation media that are used in transient gene expression (TGE) systems are another important factor for efficient transfection. Screening host cell processes reveals that the transient productivity of secreted protein varies 10-fold among host CHO cells [11]. The transfectable cells should have the ability to grow in serum-free media at high density 5 x10^6 cells/mL. In general, younger, actively dividing cells of less than 50 passages take up DNA better than quiescent cells [48]. HEK-293 and CHO cells are the most used transfectable cell host in TGE systems utilizing polyethylenimine (PEI) for DNA delivery [35]. Host cell engineering dealing with the relation between genomics/transcriptomics and protein production can increase the protein glycosyaltion capability of the cells. Regulating genes during exponential growth conditions can also increase protein productivity [49].

Culture state characterized by working cell clone explores narrow phenotype diversity corresponding to metabolic and physiological activities increase the productivity potential [50].

Hence, it is important to use a medium that supports PEI-mediated transfection. The fresh medium must be used if it contains chemically unstable components, such as thiamine [48]. Some commercially available media inhibit PEI-mediated transfection due to the presence of known components such as dextran sulfate, heparin sulfate, ferric ammonium citrate, and certain hydrolysates or other unknown components [51]. The presence of more salt ions in media, PEI amount improves the aggregation of pDNA/PEI particle size complexes and transfection efficiency [52]. Polyplex formation was found to be sensitive to changes in the media ionic strength which could affect the ability of PEI to compact pDNA, protect, binding of pDNA extracellular and release the pDNA intracellular. Combining polyethylenimine with Fe (III) ions was found as a good supporting condensing agent enhances pDNA incorporation in the complex [53]. The presence of dextran sulfate likewise iron (III) citrate components in the media composition can inhibit PEI transfection [54,55].

2.4. Mechanism of polyethylenimine (PEI) mediated transient transfection

Linear PEI consists of repeated units of aliphatic carbon groups and secondary amines [56]. The amine group is responsible for the protonation state of the polymer, making it highly positively charged and condensing the pDNA to nano-polyplex size. This dual capability enables it to bind electrostatically with the negatively charged phosphate group of plasmid DNA as described in Figure 3, to compact it to nano-particle size and protect the pDNA from intracellular enzymatic digestion [57–59]. Polyethylenimine condensation ability is based on its molecular weight. With branched 25 kDa PEI, the surface charge increased while polyplex size decreased compared to other low molecular weight PEI [60].

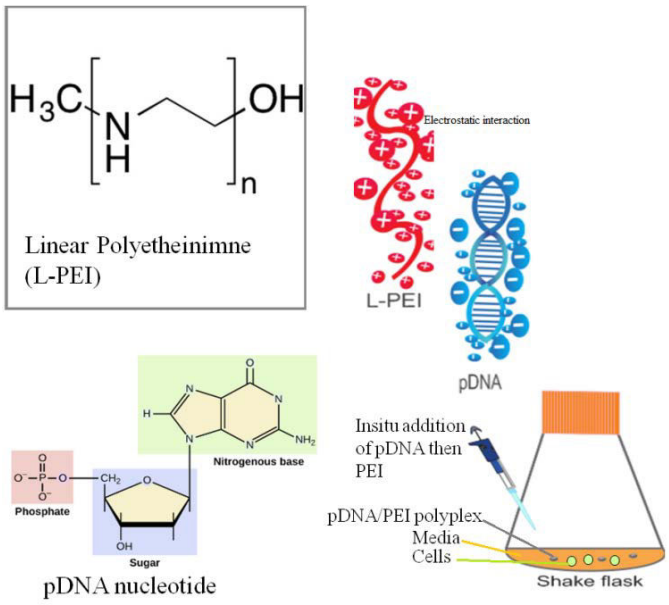

Figure 3: Structure of linear PEI, pDNA nucleotide and pDNA/PEI polyplex formation.

Plasmid DNA/PEI polyplex spontaneously forms *in situ* by addition of pDNA followed by PEI through electrostatic interaction. This interaction occurs between the phosphate group's negative charge of pDNA nucleotide and the amine group's positive charge of polyethylenimine polymer.

Recently, it has been reported that this interaction stabilizes not only due to electrostatic interaction but also because of its interacting with groove atoms (minor and major grooves are shown in Figure 4) of the DNA nucleic acid [61].

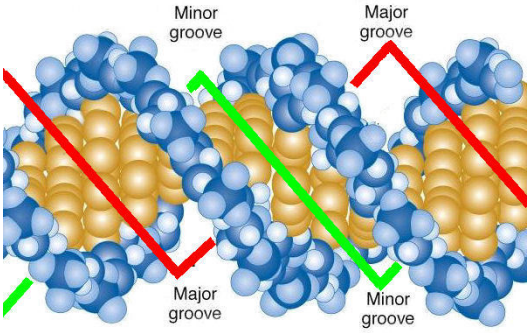

Figure 4: The major and minor grooves in a space-filling model of DNA [62].
Both grooves arise from the anti-parallel arrangement of the two backbone strands. They are important in the attachment of the DNA-binding proteins involved in replication and transcription.

Once the polyplex has reached the target cell, it binds to the plasma membrane proteins that are negatively charged, like heparin sulfate proteoglycans and integrins. The plasma membrane represents the first barrier which regulates the entrance of nano-particle polyplex (as illustrated in Figure 5). The interaction of cationic polymers with cells and nano-particle uptake have been reviewed [63,64]. There are several mechanisms that allow small polyplexes to internalize the cells, including clathrin-mediated endocytosis (for endocytic vesicles with a size of ~100–150 nm), caveolae-mediated endocytosis (~50–80 nm), micropinocytosis (~90 nm) and macropinocytosis (~500–2000 nm) [42,65–67]. The polyplexes pass into the cells through endocytosis, macropinocytosis, or phagocytosis [68]. Additionally, both clathrin and caveolae appear to be major internalization routes for PEI polyplexes [69]. Generally, the polyplexes have to be positively charged and their size should be in the range <100 nm for efficient endocytosis [36].

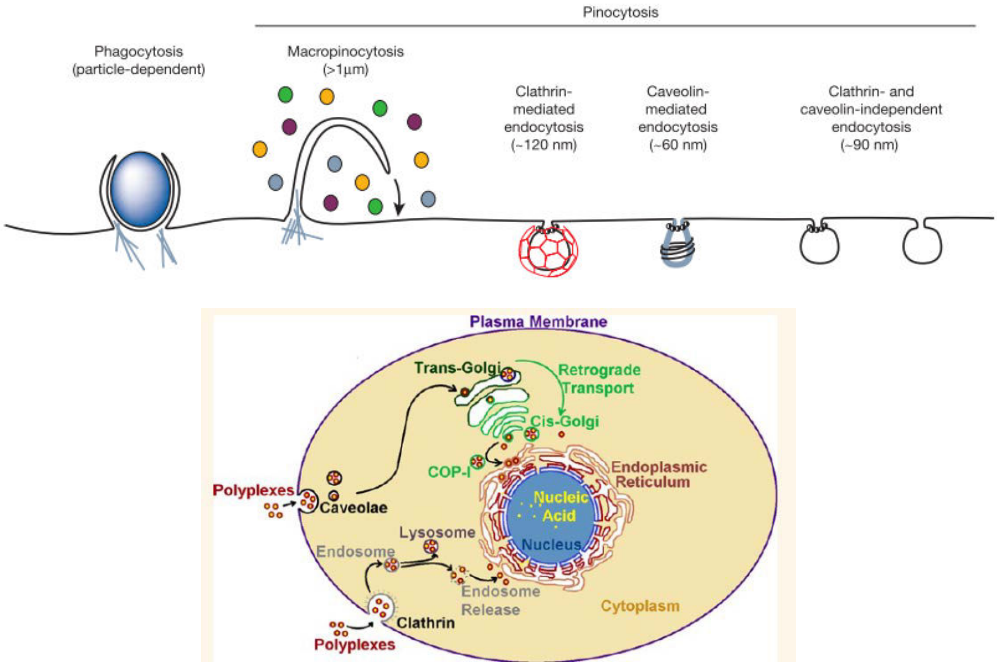

Figure 5: Pinocytosis pathways and tracking pDNA/PEI polyplex [65,69].

The protonation state of the polymer depends on pH values and salt concentration and enables the polyplex to escape from endo-lysosomes lysis and deliver the pDNA to the nucleus [70]. Recent studies in atomic force microscopy (AFM) showed that sub-50-nm PEI/pDNA polyplex binds with nuclear pore complex without affecting the nuclear membrane [71]. To date, it is still unknown whether the PEI dissociates from pDNA before transfection or not, and what happens to the PEI the fate of PEI inside the cells [72].

2.4.1. Physico-chemical properties of pDNA/PEI polyplex

As previously mentioned, polyplex formation occurs as a result of the ionic interaction. Therefore, the shape, size, stability and overall surface charge of the resulting polyplex particles from that interaction depends on the preparation conditions. There are two ways of pDNA/PEI polyplex particle preparation. The first is called the conventional method (two steps), where the pDNA and PEI solutions are dispersed separately in 150 mM NaCl or 278 mM glucose, then the PEI solution is added to the DNA and the mixture is incubated for 10 min [73] before added it to the cell suspension. The second is called *in situ* (one step), and is quite simple: the pDNA solution and PEI are both added to the culture without the prior preparation step [74]. For both methods of polyplex preparation, the size and surface charge of the polyplex particle are important transfection parameters. The polyplex size is determined by measuring the hydrodynamic radius of pDNA/PEI polyplex based on dynamic light scattering (DLS) in which changes in the polyplex surface charge are found by measuring the zeta potential [75]. Recently, nano-particle tracking analysis (NTA) has begun to be used to determine polyplex size,

distribution, concentration, and for imaging [71]. Also characterization of L-PEI/pDNA polyplexes by transmission electron microscope (TEM) is performed as described in Figure 6 [76]. The PEI-pDNA nano-particles are almost spherical with a diameter of ~20-100 nm.

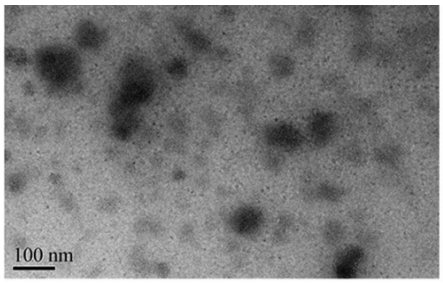

Figure 6: TEM image of pDNA/25kDa L-PEI [76].
Polyplex formation between interactions of 2 µg/14 µg, respectively.

The particle size in pDNA/PEI formation as monitored by DLS increases and then decreases with the PEI/DNA (N:P) molar ratio in the 2.2-15840 ratio range. At the same time, the zeta potential of PEI/DNA polyplex changes from negative to positive with an increase in the PEI/DNA ratio [75] , as represented in Figure 7. This study does not include a transfection efficiency correlation.

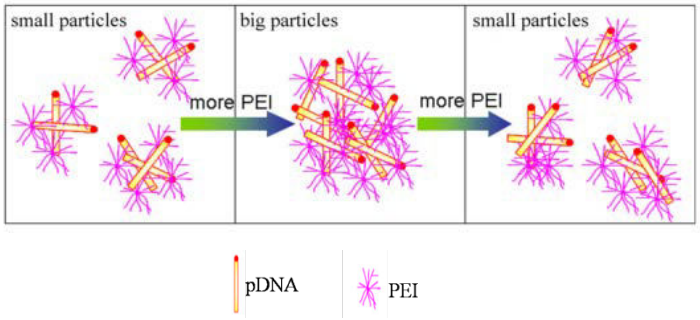

Figure 7: Schematic image showing polyplex particle size with the ratio.
Showing the trend towards the size of PEI/DNA polyplex being related to the PEI/DNA ratio [75].

Simulation studies investigated the stability of polyplex, finding that it depends on the charge distribution and transfection facilitating by free of PEI (unbound PEI particles with pDNA) [77]. To date, the positive charge comes from the interaction between PEI amines' positive and the phosphate group's negative nucleic acid charge; this binding is facilitated by the release of water from the nucleic acid to form stable polyplex [61]. This interaction is reversible and allows PEI molecules to replace each other, as described in a previously reported simulation study [78]
One of the important properties required for a transfectable polyplex molecule is the surface charge, which is usually determined by measurement of the zeta potential. It is well known that only a high +/- charge ratio of polyethylenimine polyplexes improves transfection where free uncomplexed PEI is present. An excess of PEI gives polyplexes a positive surface charge which

enhances binding to the anionic cell membrane [79]. On the other hand, it was reported that free PEI can mediate toxicities before uptake by cells through membrane destabilization and after entering into the cells, thus inducing apoptosis [80]. At the same time, to overcome the problem that high transfection efficiency is accompanied by high cytotoxicity, it may be possible to optimize the concentration of pDNA, PEI and host cells.

2.5. Optimization of PEI transient transfection conditions

In addition to choosing a high-performance host cell line and medium for cultivation, the ratio of pDNA:PEI plays a very important role in the transfection of mammalian cells. Screening of the transfection parameters, including media, cell type, and the ratio of PEI to DNA in response to protein expression time are crucial steps in determining the optimum conditions for maximizing protein expression [57,81]. Dilution of transient transfection culture with hyperosmolar media of 490 mOsm kg^{-1} can increase productivity 4-fold and also reduce cell growth [82]. Even the method of PEI/pDNA polyplex formation, either through *in situ* or conventional methods, affects gene expression. Higher transfection efficiency was obtained with the one-step transfection procedure with higher cell densities and higher concentrations of PEI and DNA. In addition, *in situ* polyplex formation is more amenable to up-caling and GMP compliance because of the lower contamination risk and easier handling involved [44,74]. *In situ* transfection under hypothermic conditions increases volumetric recombinant protein yield. This ca. two-fold increase coincides with a reduction in the amount of both PEI and plasmid DNA used. This study was performed with 4 $x10^6$ cells/mL cell density and without a media dilution step after transfection [83]. Polyplex cellular uptake is dependent on the PEI/pDNA ratio, the cell density at the time of transfection, and the extent of particle aggregation. The rate of particle uptake was constant within the first 60 min post-transfection at a DNA:PEI ratio of 1:2 (w/w) and at a cell density of 2 $x10^6$ cells/mL. Aggregates become larger over time [84].

Another Design of Experiment (DoE) study explored how productivity titer increased with a parallel increase in cell density and PEI concentration while fixing the pDNA concentration [85].

Normally transient transfection introduces foreign DNA without integrating it into the genome, but the genes are expressed for a limited time (24–96 hr), thus transient transfection is hindered by a short expression period and low productivity in comparison to constant gene expression. Repeated transient gene expression is applied to prolong the productivity time and improve productivity 4- to 12-fold based on harvest time [86]. The repeated transient transfection approach is described in Figure 8.

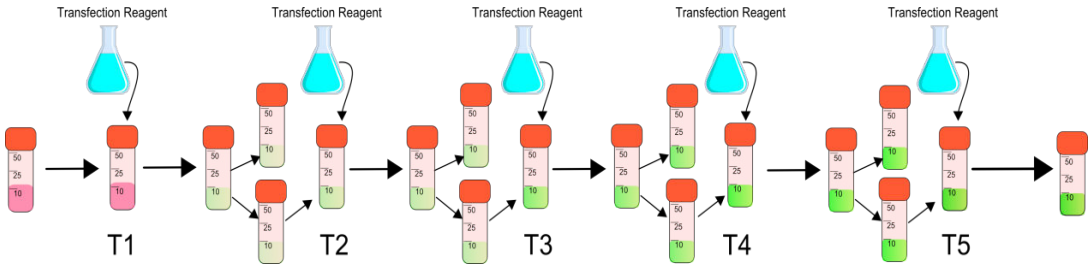

Figure 8: Flow diagram explaining the repeated transient transfection process.
T1 to T5 stand for transfection number.

Generally, it is quite difficult to compare all these findings due to the difference in PEI:pDNA ratio, mixing order, incubation time, different polyplexes concentrations, medium composition, volume, cultivation vessel, and cell type. All of these variables reflect the need to optimize the method of transfection in CHO-K1 cells.

2.5.1. Design of Experiment (DoE) approach for transfection optimization

Gene delivery into cells by a process of transient transfection is influenced by a number of interrelated factors as presented in Figure 9. Optimizing this complex process requires the study of these factors in isolation and in combination. The DoE method is an essential modeling tool for this purpose.

A wide range of optimized PEI and DNA amounts have been reported in the literature, using a range of media types, cell lines, vectors. The most important factors to consider in optimizing transfection include the cell density, PEI and plasmid DNA concentration at the time of transfection. The easiest way to handle problems with multiple factors is to start by fixing one factor and then bring in other factors one after another, a procedure called ‘‘One Factor At a Time’’ (OFAT) [39] and referred to as the “independent” way.

The DoE approach is superior to OFAT in that it more quickly investigates the interrelation (“dependent” way) amongst a number of factors and requires fewer experiments for all factors at the same time. The DoE methodology is particularly preferable when the optimal condition discovered from the interaction of several factors rather than from the addition of just one factor.

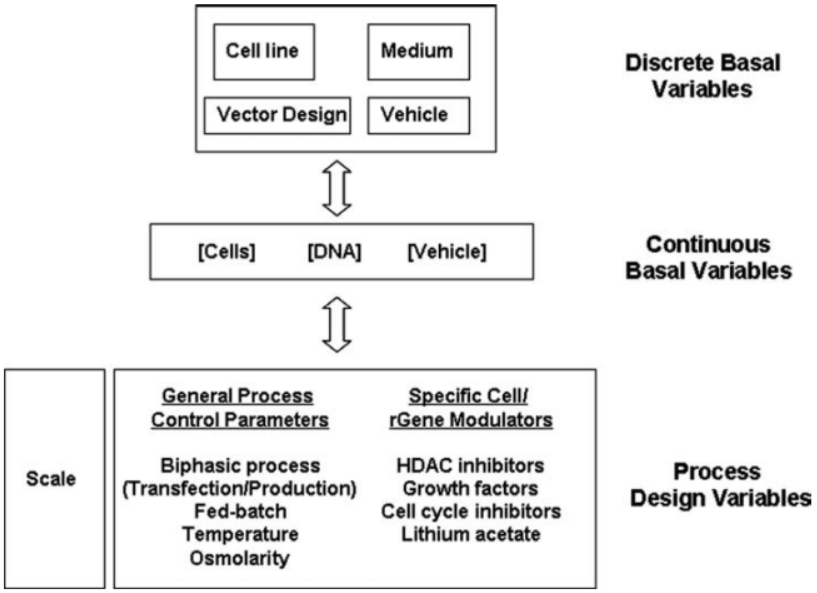

Figure 9: Interacting variables underpinning transient transfection process. Mammalian host cell based transient production process design [11].

DoE screening is used at the beginning of the experimental procedure to explore how many factors could affect the process and to identify their appropriate ranges. This is followed by the optimization step, which predicts the response value for all possible combination factors within the experimental region and identifies the optimal experimental point. The DoE methodology can design a series of experiments to be conducted in parallel. This helps in monitoring the process which is influenced by many factors, in estimating the interaction of factors, and in distinguishing between the noise and real effects.

DoE findings represented by a contour plot display the relationship between input factors and responses. When the objective is screening, full factorial designs can easily be run, but the number of experiments would increase dramatically by a number of factors. Therefore fractional factorial designs are used to reduce the number of experiments. This design is based on linear or polynomial interaction. Hence, optimization of the already identified factor is required, a quadratic model is flexible and closely the relationship between factors and response named response surface modeling (RSM). One of the commonly used RSM designs which is applied in this study is a central composite circumscribed (CCC) [87,88].

2.6. Transforming growth factor-β (TGF-ß) superfamily

After achieving the desired GFP-transfection efficiency with host cells in specific media and appropriate transfection reagent, it is important to evaluate that transfection system with other target recombinant proteins, like transforming growth factor beta 1 (TGF-β1) within this study. TGF-β1 is one of three closely related mammalian members of the large TGF-β superfamily [89]. TGF-ß1, 2, 3 growth factors display a shared identity of about 64-82% [90]. Most cell lines and

tissues encode human TGF-β1 cDNA, a 390 amino acid (aa) precursor that contains a 29 aa signal peptide and a 361 aa pro-protein. The endopeptidase furin enzyme convert pro-protein precursor to mature moiety residues which consist of an N-terminal 249 aa latency-associated peptide (LAP) and a C-terminal 112 aa mature TGF-β1 [91,92]. Due to the absence of latent TGF-beta binding proteins (LTBP) in CHO cells, the recombinant human TGF-β1 is generally produced as a small latent complex (SLC) as seen in Figure 10, where LAP binds to mature TGF-β1 through a non-covalent bond and an intramolecular disulfide bond [93,94]. The SLC can be visualized on the SDS page, non-reducing as a dimer at 75 kDa [94,95] and the mature active TGF-ß1 dimer at 25 kDa.

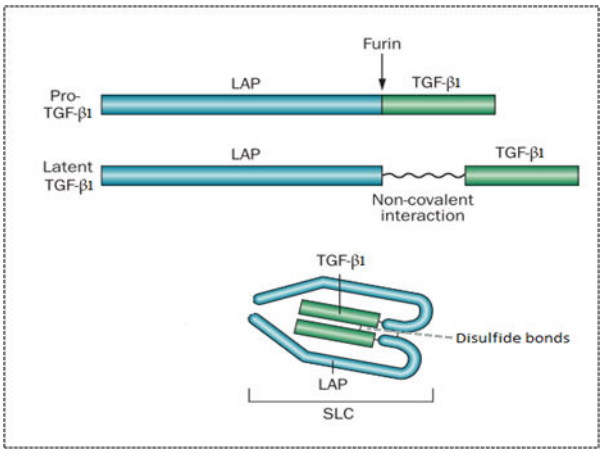

Figure 10: Cleavage of TGF-ß1 precursor.
This graph shows the process of a dimeric recombinant TGF-ß1 protein production in CHO cells [96].

The TGF-β family plays an important role in cell proliferation and embryo implantation through its biologically active form (monomer 12.5 kDa to ~17 kDa or dimer) [97,98]. *In vitro*, the active (mature) form is liberated from the large latent TGF-β complex and small TGF-β complex under extreme pH, heating to high temperature, or in the presence of SDS and urea [92].
Previously, the recombinant production of mature TGF-ß1 was achieved by stable transfection of CHO cells with improvement in the production concentration from 7 mg/L to 30 mg/L [94,99]. More recently, Abdulkerim et al., was able to produce TGF-ß1 through lentiviral transduction of CHO cell [100].
The maximum reported recombinant antibody titer in transient transfection platform was 160 mg/L in CHO- K1 cells, estimated by enzyme-linked immunosorbent assay (ELISA) [101].
TGF-ß1 is known to inhibit or stimulate cell growth as the bifunctional regulator, to regulate the immune system, and to enhance wound healing through deposition of extracellular matrix [102]. It can inhibit the proliferation of most cell lines by inducing cell cycle G1 arrest [103]. To regulate cell function, mature TGF-ß1 requires liberation from SLC (inactive unit) through different

activation mechanisms (mentioned above) in order to act on its receptors (TGF-ß Rs), which in turn activate SMAD proteins [95]. More details about the regulation of TGF-ß signaling through TGF-ß receptors in control of cell proliferation have been reported by many scientists [104,105] and are described in Figure 11.

The activity of transforming growth factor beta (TGF-ß) can be determined by assessing the inhibition of the growth of lung epithelial cell lines [106]. Bioassays for quantification of active and total transforming growth factor-ß are more sensitive than ELISA [107], and provide insight into the activation of latent TGF-ß1 to mature TGF-ß1.

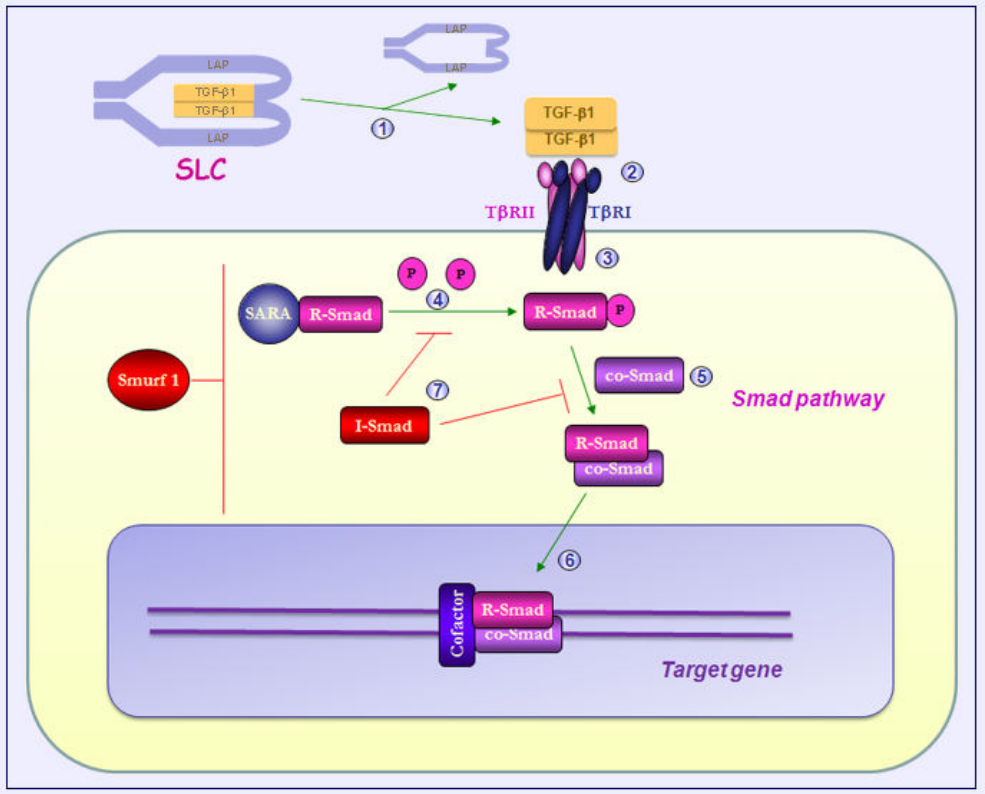

Figure 11: TGF-β1 signaling through the Smad-dependent pathway.
Mature TGF-β1 is released by different mechanisms such as degradation of LAP by proteases, induction of conformational change in LAP by interaction with thrombospondin or by rupture of noncovalent bonds between LAP and TGF-β1. 2) Active TGFβ-1 binds to receptor type II (TβRII) which is constitutively phosphorylated and active. 3) The TGF-β1-TβRII complex recruits and activates TβRI by transphosphorylation of the GS domain. 4) The heterotetrameric receptor complex phosphorylates R-SMAD at the C-terminal SSXS domain. SARA protein promotes the binding of R-SMAD with TβRI. 5) The phosphorylation of R-SMAD allows the interaction with Co-SMADs. 6) This complex can translocate to the nucleus, joining the DNA and inducing or modulating the transcription of different target genes. 7) I-SMAD can inhibit signaling through the blockade of the access of the receptor complex to R-SMAD by mechanical interaction or induce TβRI degradation by ubiquitination [108].

2.6.1. Fusion TGF-ß1 recombinant protein

After optimizing the transfection factors with GFP plasmid, there are two ways to transiently transfect the desired expression plasmid. The first way is the co-transfection of the dual plasmid system, one fluorescence GFP for monitoring transfection efficiency and the other for target protein [35]. The second way is to replace the GFP plasmid with a target fused plasmid like pGag-EGFP plasmid [86]. This enhanced GFP (fluorescent) fused to the target protein (not fluorescent) allows the target protein to be monitored via fluorescence detection. Recombinant fusion proteins have become an important category of biopharmaceuticals. Fusion proteins were developed by genetically fusing two or more protein domains together to add multi-functional properties, such as protein purification and on-line monitoring [109,110]. Florescent fusion tags are commonly used for detecting recombinant proteins during cultivation. Short chain tryptophan tag is comes superior for GFP tag family. It is compromise only 5-11% of its molecular size. Proteins of large molecular size may stress the host cell metabolism during production [111]. The binding specificity of antibody recombinant protein is not affected by this tag, while an increasing amount of tryptophan residue slightly decreases the binding activity.

Tryptophan has maximum excitation at 280 nm and maximum emission at 350 nm with a relatively high quantum yield and a larger Stokes shift than the other aromatic amino acids (~70 nm). Its fluorescence signal is highly sensitive to the properties of the surrounding environment and neighboring amino acids [112]. This system has been used to monitor on-line the production of antibody fragments in *E.coli* cultivated on a microtiter plate using an adapted BioLector fiber optic monitoring device [113,114].

For protein purification directly from culture supernatant, short peptide affinity tags have become indispensable in protein downstream research. His-tag fused to recombinant protein provides a powerful application for purification, detection and assay of recombinant proteins[100]. Twin-Strep-tag® is particularly popular for providing recombinant proteins at high purity and functionality by using physiological conditions within a rapid one-step protocol. The affinity receptor for Twin-Strep-tag® is the engineered streptavidin, known as Strep-Tactin resin column chromatography. The presence of biotin in culturing media competes with Twin-Strep-tag® binding and inactivates Strep-Tactin® columns. Therefore it is important when purifying Twin-Strep-tag® fusion proteins, that pH be adjusted to 8 and to remove biotin, blocking it using BioLock solution 70 U/ml (1 U blocks 1 µg biotin) [115].

Implementation of short chain tryptophan (3 residues) tags for off-line protein monitoring by 2D-florescence spectroscopy and Twin-Strep-tag® for protein purification technology was developed in this thesis.

3. Material and Methods

3.1. Materials

3.1.1. Plasmid DNA

Two plasmids were used throughout this study, namely pEGFP-N1 plasmid (Figure 12) purchased from Clontech (Clontech Laboratories Inc., USA) and TGF-ß1 plasmid (Figure 15). The former was directed to express green fluorescence protein (GFP) intercellular for transfection efficiency evaluation. The other plasmid was targeted to extracellular expression of TGF-ß1 protein. TGF-ß1 plasmid orginated from integration of acceptor plasmid (Figure 13) with entry plasmid (Figure 14), which were purchased from IBA (IBA GmbH, Göttingen, Germany) using the Stargate protocol [116].

- **pEGFP-N1 plasmid map:**

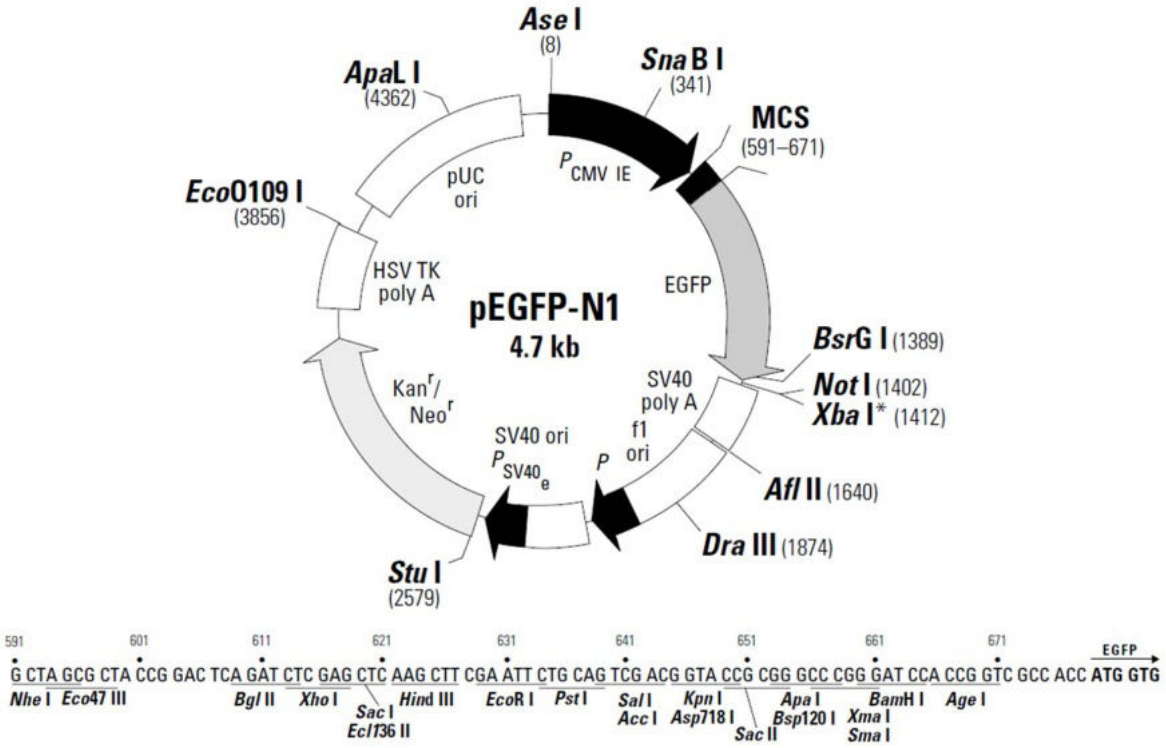

Figure 12: Map of pEGFP-N1 plasmid encoded green fluorescence protein (GFP).
Restriction map is represented in the plasmid map.

Generation of TGF-ß1 production plasmid:

TGF-ß1 production plasmid Figure 15 originated from integration of acceptor plasmid Figure 13 with entry plasmid Figure 14 using Stargate protocol [116].

- **Acceptor plasmid:**

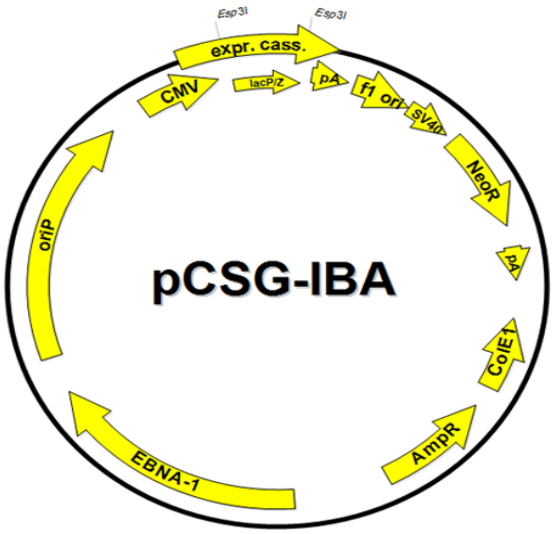

Figure 13: Map of pCSG-IBA-102 acceptor vector.
The vector carries the BM40 signal sequence for secretion of the recombinant protein into the medium and the Twin-Strep-tag® for C-terminal fusion to the recombinant protein. The episomal replication is mediated through the Epstein Barr Virus replication origin (oriP). For selection and propagation in *E.coli*, the vector carries an ampicillin resistance.

- **Entry plasmid:**

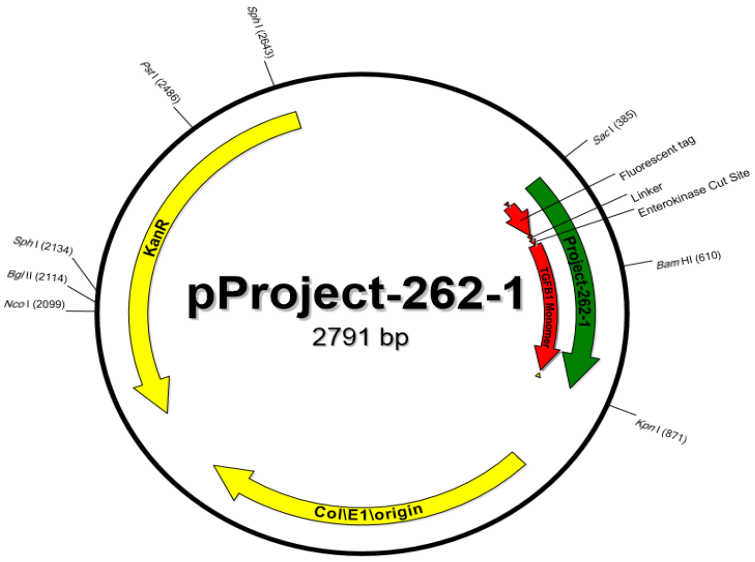

Figure 14: Map of entry vector with target TGF-ß1 sequence and a fluorescent tag.
This vector was integrated with the previously mentioned acceptor vector to produce the target expression plasmid.

- **TGF-ß1 production plasmid:**

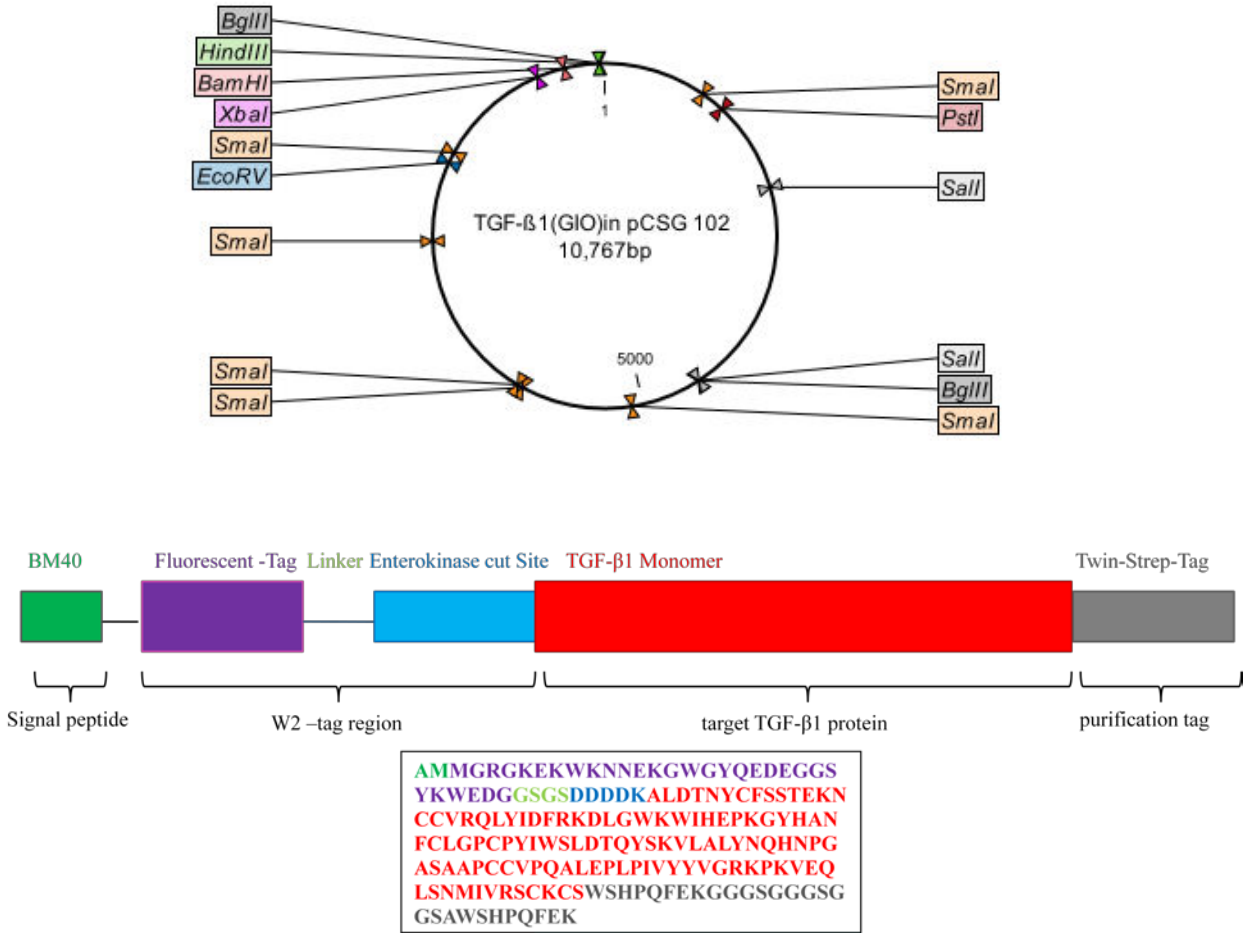

Figure 15: Map of TGF-ß1 fusion protein destination expression plasmid.
This represents the final TGF-ß1 production plasmid used in this study, which was prepared according to the manufacturer's (IBA) instructions [117]. The plasmid backbone contains ampicillin resistance gene (AmpR), cytomegalovirus (CMV) enhancer promoter and Epstein Barr Virus origin of replication (oriP). The TGF-ß1 sequence is genetically linked to the W2-tag accession number [Gen Bank: JN120907] with three tryptophan residues. The W2-tag is linked to the cleavable enterokinase site by a GS-linker. The TGF-ß1 segment is linked to Twin-Strep-Tag® for further purification as shown in the schematic diagram. The amino acid sequence consists of 39 amino acids (aa) for the fluorescent chain (W2-tag region), 112 aa for mature TGF-ß1 (monomer form) and 28 aa for Twin Strep-tag®.

3.1.2. Sequencing primer

pCSG forward 5′- GAGAACCCACTGCTTACTGGC -3′

pCSG reverse 5′- TAGAAGGCACAGTCGAGG -3′

3.1.3. Cells

1) One Shot® TOP10 chemically competent E.coli was obtained from IBA (IBA GmbH, Göttingen, Germany); cells used for plasmid propagation.
2) Chinese hamster ovary (CHO-K1) suspension cells were obtained from Bielefeld University, Germany; cells used for cultivation and protein expression by transient transfection.

3) Adenocarcinoma human alveolar basal epithelial cells (A549-cells), ACC107, were purchased from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). These adherent cells were used for a further TGF-ß1 bioactivity test.

3.1.4. Media and supplements

- **For *E.coli* cultivation:**
  1) LB-Agar consists of 5 g yeast extract, 1 g NaCl, 10 g Trypton, 15 g Agar added to 1 L ddH2O at pH 7.6
  2) LB consists of 5 g yeast extract, 1 g NaCl, 10 g Trypton added to 1 L ddH2O at pH 7.6
- **For CHO-K1 cell cultivation and transfection:**

Table 1: List of media and supplement.

Media type	Suppliers
CHOMACS CD	Miltenyi Biotec, Germany
CD CHO	Thermo Fisher Scientific, Germany
ProCHO-5	Lonza, Sartorius AG, Germany
Ex-cell CD CHO	Sigma-Aldrich, Germany
Opti-MEM	Gibco, Thermo Fisher Scientific, Germany
DMEM/F12	Thermo Fisher Scientific, Germany
L-Glutamine added at conc. of 8 mM/L	Biochrom, Germany

- **For osmotic pressure:**

CHOMACS CD media with different osmolality was prepared by addition of NaCl directly to the media, and osmolality was measured with the Osmomat 3000 (Gonotec GmbH, Germany).

- **For A549-cell cultivation:**
  1) DMEM media for A549-cell cultivation obtained from Life Technologies, Germany.
  2) Fetal calf serum (FCS) purchased from PAA Laboratories GmbH, Austria.

3.1.5. Transfection reagent polyethylenimine (PEI)

Polyethylenimine, Linear, MW 25 kDa was purchased from Polysciences GmbH, Germany. A stock solution of 1 mg/mL was prepared according to the Cold Spring Harbor protocol [118].

3.1.6. Kit and accessory reagent

1) Plasmid purification Gega Kit (Qiagen, Netherlands).
2) Twin-Strep-tag® protein purification buffer set 10x (IBA GmbH, Göttingen, Germany).
3) WET FRED System (IBA GmbH, Göttingen, Germany).
4) CellTiter Blue Assay reagent purchased from (Promega GmbH, Germany).

3.1.7. Antibodies and commercial TGF-ß1

Table 2: List of antibodies.

Antibody used in ELISA and Westen blot	Manufacturer, Country
Primary antibody mouse-anti-human-TGF-β1	Dianova GmbH, Germany
Second goat anti-mouse IgG AP-conjugate	BD Biosciences Pharmingen, USA
Second goat anti-mouse IgG HR-conjugate	BD Biosciences Pharmingen, USA
Commercial human TGF-β1 recombinant protein	Peprotech GmbH, Germany

3.1.8. Concentration of protein sample

Vivaspin membrane (MWCO: 10,000 kDa), Sartorius Stedim Biotech, Göttingen

Vivaspin 2 centrifugal concentrators (MWCO: 3,000 kDa), Sartorius Stedim Biotech, Göttingen

3.1.9. Statistical analysis

The GraphPad Prism 7 software was used for the ANOVA, T-test and P-value calculations.

3.2. Methods

3.2.1. Plasmid production, purification, quantification, and identification

E.coli aliquots were stored at -80 °C. For transformation, 50 µL of thawed *E.coli* was mixed with 10 µL of 0.5 µg/µL plasmid DNA (pDNA). This starting transformation mixture was incubated on ice for 30 min then heat-shocked for 5 min at 37 °C; afterward, the bacteria were cooled on ice for 2 min, taken up in pre-heated 900 µL LB medium and incubated at 37 °C for 45 min as a heat-shock. A prepared LB agar plate with 50 µg/mL kanamycin was inoculated by 100 µL of previously transformed bacteria suspension. In the case of TGF-ß1 plasmid, the LB agar plate was prepared with 100 µg/mL ampicillin. The plates were incubated overnight at 37 °C to allow only the transformed *E.coli,* which contains the plasmid, to grow under antibiotic selection pressure. The colony of bacteria carrying a specific plasmid was isolated for further propagation. The same antibiotic selection marker was used in LB liquid medium to scale up transformed cell growth and to obtain a large amount of cells.

After scaling up, the transformed *E.coli*, containing the target plasmid at a total volumetric scale of 10 L, came from several Erlenmeyer baffled shake flasks (propagation step). The cells were centrifuged at 4000 rpm for 30 min, collected as a pellet and stored at -20 °C. This harvested bacteria pellet was used for the next plasmid extraction step which was performed using a Qiagen's plasmid Giga kit.

On the day the plasmid was purified, the transformed *E.coli* pellet was thawed and resuspended in 125 mL of P1 buffer followed by an addition of 125 mL buffer P2, mixed well and incubated for 5 min at room temperature. Then 125 mL pre-cooled buffer P3 was added, mixed well and incubated for 30 min on ice. Afterward, the supernatant of cell lysate containing the plasmid was separated by centrifugation twice for 30 min at 14,000 xg and 4 °C.

For pDNA purification from previous cell lysate supernatant, the activated QIAGEN-tip10000 with QBT buffers was loaded by the cell lysate supernatant which carries the target plasmid. The solutions pass by gravity flow through the column. The column was washed with 600 mL QC buffer. pDNA was eluted with 100 mL of 65 °C pre-warmed QF buffer. The pDNA was precipitated by adding 70 mL of isopropanol at room temperature followed by centrifugation for 30 min at 14,000 xg and at 4 °C. The pellet was washed with 10 mL of 70% ethanol and transferred to a new 1.5 mL Eppendorf tube with ethanol for centrifugation, again at 12,000 xg for 10 min. The ethanol was discarded and the pDNA left to dry under a laminar flow for 15 min then dissolved in water for further quantification with the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Germany). All these steps were done according to the QIAGEN ® plasmid purification manual [119] . After the pDNA concentration was

determined, the plasmid and target gene were identified by agarose gel electrophoresis, which separates DNA fragments by size. The pDNA fragments were generated by restriction enzymes. Procedure: 1.5% agarose gel is used. For this purpose, 1.5 g of agarose is dissolved in 100 mL of 1x TAE buffer (preparation was described in Appendix section page 85) and then heated in the microwave for 120 s (900 watt). Subsequently, 5 µL of a Roti®Safe GelStainer is added and the solution is poured into a gel chamber. Stencils are then used for the sample pockets. After the gel has been fixed, the gel is placed in the electrophoresis chamber and superimposed with 1x TAE buffer. A comb was withdrawn and 20 µL of the DNA samples with restriction enzymes (prepared according to instructions of Thermo scientific, Germany) were pipetted into the pockets, together with 6 µL of the 10000 bp marker (3 µL marker + 3 µL of 10x FastDigest Green buffer, Thermo scientific, Germany). The samples were separated at a voltage of 100 V for 45 min. The gels were photographed under UV excitation.

Before getting into the mammalian cell transfection, it is important to validate the plasmid components. There should be a gene of interest included. The validation is achieved either by pDNA gel electrophoresis or sequencing and protein expression. On the one hand, the GFP validation was done by gel electrophoresis and expression as shown in Figure 16. On the other hand, TGF-β1 plasmid validation performed by gel electrophoresis, sequencing as shown in Figure 16 & 18 and its expression afterward in Chapter 4.4.

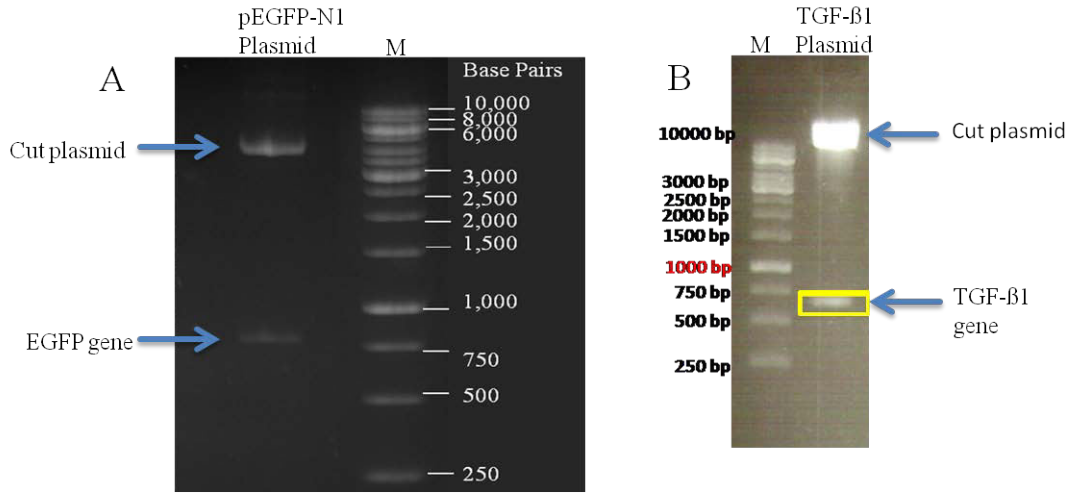

Figure 16: Agarose gel in the target gene identification.
A) EGFP gene with approximately 761 base pairs (bp). B) TGF-ß1 gene fused by BM40 + Tryptophane Tag + Twin-Strep-tag®. M: Marker.

As shown in Figure 16A, the pEGFP-N1 plasmid was treated with the restriction enzymes Sal1 and Not1 and the cutting was successfully performed. The EGFP gene fragment corresponded to the expected size of 761 bp according to the plasmid design. The delivery of the EGFP gene to the CHO-K1 cell was analyzed through its fluorescent protein expression. The transfection efficiency was always monitored in this study through measurements of the GFP+ cells

percentage which are transfected by pEGFP-N1 plasmid at different hour's post-transfection (hpt). This analysis measurement was done quantitatively with flow cytometry and qualitatively by observing fluorescence under a fluorescence microscope as shown in Figure 17.

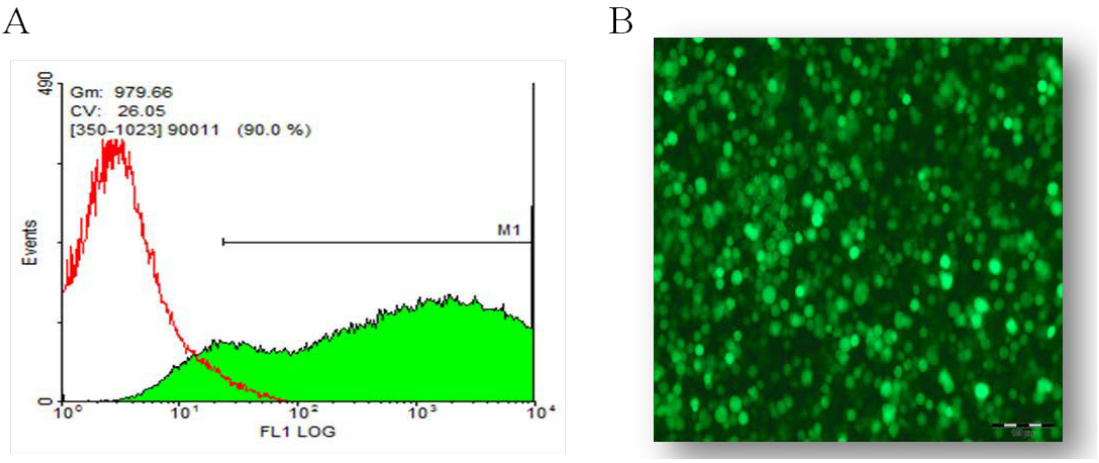

Figure 17: EGFP level determination by flow cytometry and a fluorescence microscope.
Panel (A) shows comparison histograms of GFP-transfected cells and non-transfected cells (red line). The data was obtained with flow cytometer EPICS XL-MCL, Beckmann-Counter, USA. Panel (B) shows a photograph of GFP transfected sample obtained with a fluorescence contrast microscope. The image was obtained from submitted work using media combination Opti-MEM + CHOMACS CD 50:50 (v/v). This protocol was not used here in this study.

- **Flow cytometric analysis:**

For flow cytometric analysis, one million cells are collected from pEGFP-N1 transfected culture and from non-transfected culture then centrifuged for 5 min at 200 g and resuspended in 1 mL of 1x PBS. The sample is then analyzed on the flow cytometer BD Accuri™ C6. Generally, the data obtained show different histograms and dot plots produced from FL1-H of non-transfected samples and transfected ones, which represents the transfection efficiency percent as shown in Figure 34.

- **Microscopical analysis:**

Cells were photographed by fluorescence microscope (Olympus 1X 50) equipped with a digital camera and an image analysis system with an N.B filter.

Also, validation of TGf-ß1 plasmid design encoding mature TGF-ß1 protein tagged with tryptophan and Twin-Strep-tag® was performed either by digesting the plasmid with Xba1 and Hind III restriction enzymes. The expected size of the DNA fragment was 667 bp according to the plasmid design. Gel electrophoresis analysis shown in Figure 16B confirms that the produced DNA fragment of digestion was this size. Furthermore, to confirm that the fragment had a TGF-ß1 gene sequence with fusion tags of about 453 bp according to the plasmid design in Figure 15, DNA sequencing results in Figure 18 show that TGF-β1 was successfully integrated into the acceptor vector and was 100% identical to the TGF-β1 sequence.

- **DNA sequencing analysis:**

For the sequencing of the pDNA, 15 µL of the purified plasmid DNA solution to be examined were sent to the company EurofinsGenomics with 5 µL of the primer. The solution has a pDNA concentration of 70 ng/µL. The results of the sequencing were evaluated with the CLC Sequence Viewer as shown in Figure 18.

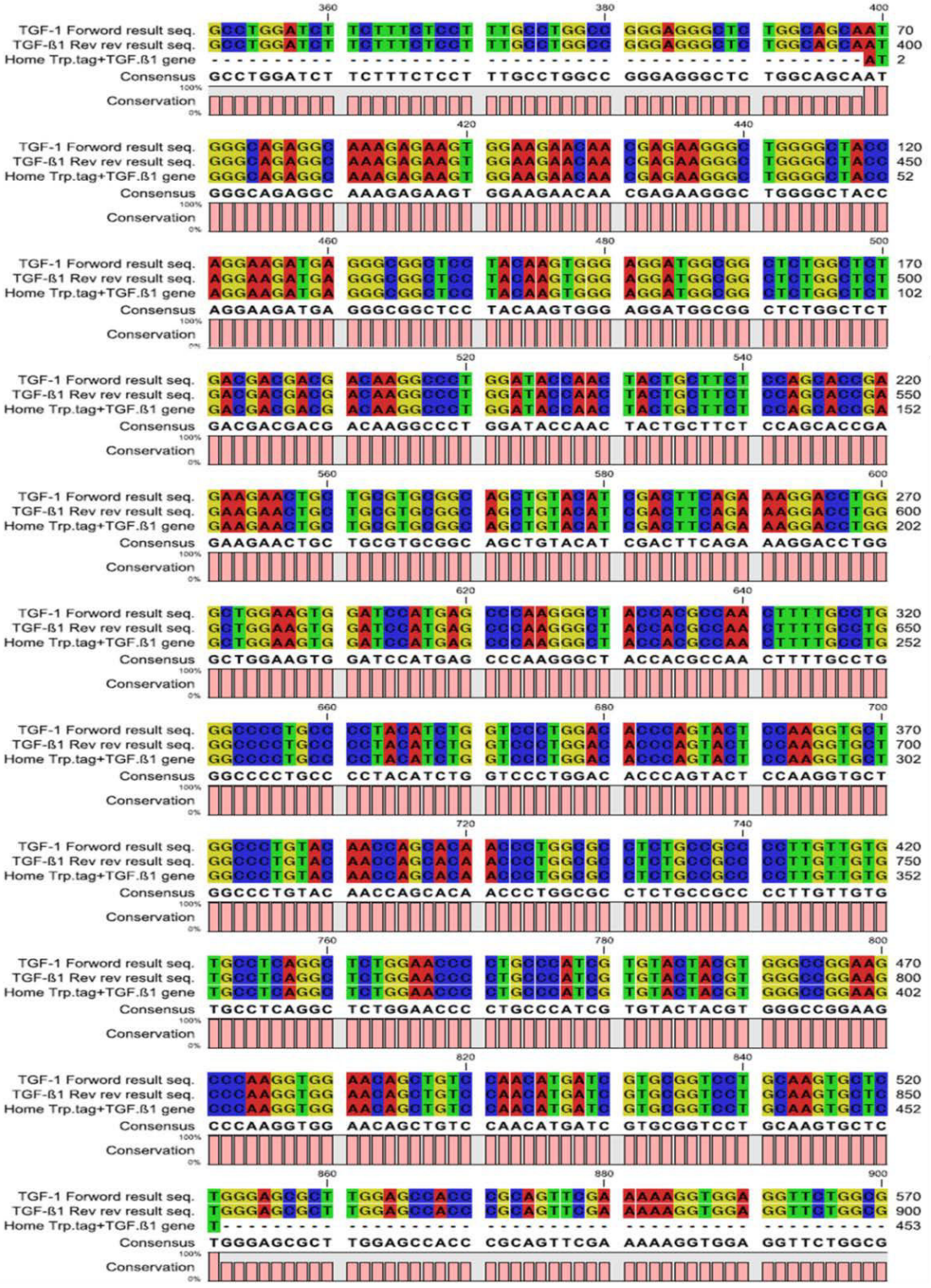

Figure 18: Alignment of pDNA sequences with the TGF-β1 gene.
The forward and reverse primer sequence was used to sequence target plasmid. Listed is the TGF-β1 sequence (453 bp) from whole TGF-β1 expression plasmid 10767 bp.

3.2.2. CHO-K1 transient transfection and further analysis

For routine cell cultivation, the CHO cell suspension is grown in shake flasks at 150 rpm of 20 mm orbital shaker and in Tubespin bioreactor®50 at 270 rpm of 10 mm orbital shaker as shown in Appendix section Figure 44. The cultures were kept at 37 °C., 5% CO_2 and 95% humidity in the incubator and continuously shaken.

The culture was taken twice a week in fresh CHOMACS CD medium to obtain highly active cell growth with a viability of 99%. Automated counting of the cells and cell viability using the Trypan blue exclusion method were performed with the Cedex analyzer (Cedex Roche Innovates, Germany). The cell suspension was centrifuged at 200 xg for 5 min and the supernatant discarded. The cell pellet was resuspended in as much fresh medium necessary to reach an optimal cell density of 0.5 x10^6 cells/mL.

Cultivation in the Tubespin bioreactor®50 was found to be a good alternative to shake flasks. It has the advantage of needing only a small culture cultivation volume of 1 mL. Furthermore, this kind of cultivation vessel is suitable to obtain higher cell densities of up to 16 x10^6 cells/ml and for high throughput cell cultivation [120,121].

Pre-culture is the last culture prepared before transfection. Usually, the pre-culture is prepared one day before transfection. Cells with a viability of more than 99% were obtained from the routine cultivation shake flask or Tubespin bioreactor®50 to inoculate new fresh medium at a cell density of 1.3-1.5 x10^6 cells/mL.

For media screening, the transfection was carried out according to procedure (A) or (B) as following:

- **Procedure A (transfection without dilution):**

  Media volume of 5 mL was treated with 10 million cells (pre-culture viability > 99%) and kept in the incubator for 1 hr to obtain single dispersed cells in solution and to guarantee homogenous cell dispersion. At transfection time, 6 µg or 8 µg pDNA (3.6 µg/µL stock solution), and 30 µg PEI (1 mg/mL) were added. Here transfection culture volume and final culture volume are the same. The experiments were done once for each condition.

- **Procedure B (transfection with dilution):**

  Based on procedure (A), procedure (B) was developed by modifying transfection media volume to 1 mL, which was treated at transfection time with the same amount of cells, pDNA, and PEI as for procedure (A). After 5 hours post-transfection (hpt), the final volumes of all transfected cultures were adjusted to 5 mL (equivalent to procedure A).

Here the screening transfection procedure was carried out in a reduced culture volume followed by expansion in additional media. The experiments performed in duplicate.

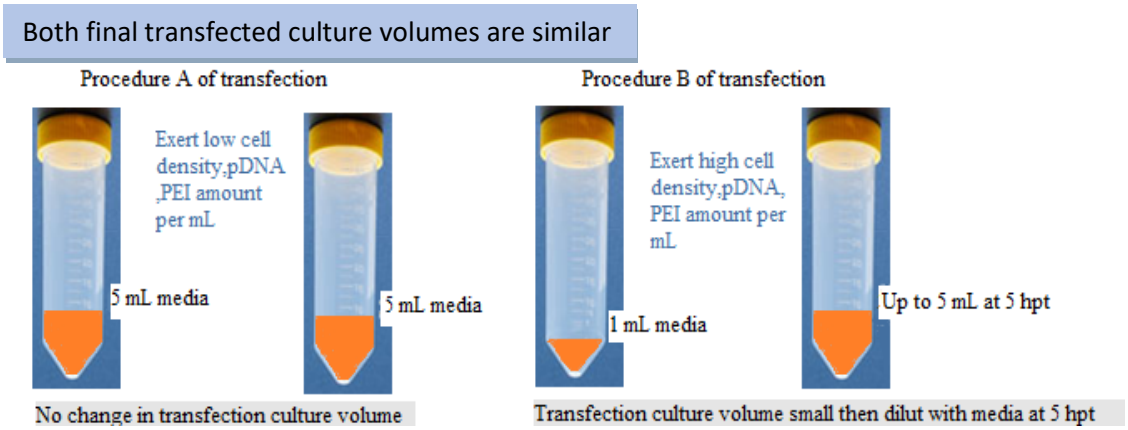

Figure 19: Describing media screening for transfection in two stages Procedures (A & B).
Both procedure (A & B) were transfected with the same amount of cells, pDNA, and PEI but in media volume 5 mL or 1 mL respectively.

Further transient transfection protocols were conducted in this study based on procedure (B) as summarized in Table 3.

To standardize the experimental setup, all the following transfection protocols were performed unless otherwise stated in the Tubespin bioreactor®50 at 1 mL transfection culture volume. Pre-culture cells 24 hrs of age was resusepnded in fresh CHOMACS CD medium. Cells were dispersed through shaking in a CO_2 incubator for 1 hour. This was followed by the addition of pDNA and polyethylenimine linear PEI 25 kDa transfection reagent simultaneously with the cell culture, known as the *in situ* transient transfection process. After 5 hpt the transfection culture was diluted by an equal volume of fresh media. All cultures were harvested at 48 hpt for further cell and protein analysis.

Table 3: Summary of transfection protocol conditions.

Methods of transfection	Media volume at transfection	Amount of viable cells	Amount of pDNA [µg]	Amount of PEI [µg]	Media addition volume	Culture vessel
Screen procedure (A)	5 mL	10 x10^6	6 or 8	30	0 mL	Tubespin
Screen procedure (B)	1 mL	10 x10^6	6 or 8	30	4 mL	Tubespin
Routine transfection	1 mL	10 x10^6	24	45	1 mL	Tubespin.
Very high density transf.	1 mL	14-50 x10^6	24	45	1 mL	Tubespin
DoE transf. conditions	1 mL	10 x10^6	appendix table 8, 9, 10 and 11		1 mL	Tubespin
Scale Up transfection	12.5 mL	12.5 x10 x10^6	12.5x24	12.5x45	12.5 mL	Tubespin - shake fla.
volumetric scalability	1 mL	10 x10^6	6	30	0,1,3,7	Tubespin.
Repeated transfection (spaced time 24 hr)	4 mL	4 x10 x10^6	1st transf. 24 µg 2nd transf. 6 µg	1st transf. 45 µg 2nd transf. 30 µg	4 mL	Tubespin
TGF-β1 transfection protocol	12.5 mL	12.5 x10 x10^6	12.5x17	12.5x38	12.5 mL	shake fla
	12.5 mL	12.5 x10 x10^6	12.5x6	12.5x30	12.5 mL	shake fla
	12.5 mL	12.5 x10 x10^6	12.5x24	12.5x45	12.5 mL	shake fla

transf. =transfection, Tubespin. =Tubespin bioreactor®50 and shake fla= shake flask

- **Design of Experiments (DoE):**

Several experiments were conducted based on the design of experiment (DoE) approach to obtain optimal results. The experiments were done firstly by full factorial analysis design: three factors involving cell density, pDNA amount, and PEI amount in two levels from the central point. The central point was repeated four times to validate the model design shown in Table 8. This was followed by a series of experiments under fixed cell density conditions using central composite circumscribed (CCC) designs with the two factors, PEI and pDNA, in two level ranges from the central point, as described in Table 9-Table 11. All designs were created by the

MODDE software program (modde 9 umetrics, Sweden). The list of factors and variation ranges are shown in the Appendices section, pages 89-91.

- **Cell cycle analysis:**

One million cells were collected from each pre-culture age, ranging from 0 hr to 120 hr before transfection, then centrifuged for 5 min at 200 xg and resuspended in 1 mL of 1x PBS. The cells were fixed by taking up a cell pellet in 300 µL of PBS and diluting this slowly by directly adding 700 µL of 100% ethanol drop by drop. The solution was left on ice for at least 1 hour before storing it at -20 °C.

On the day of analysis, the previously fixed cells were collected again in pellets and resuspended in 425 µL of PBS buffer treated with 50 µL of RNAse solution (1 mg/mL, DNAse free) to ensure only DNA rather than RNA would be stained. This was followed by incubation for 20 min at 37 °C in a water bath. Before measuring with flow cytometer at fluorescence (FL3) channel, 25 µL of propidium iodide stain solution (1 mg/mL) was added to distinguish the cell population according to the changes in cellular DNA content.

- **Polyplex characterization analysis:**

Nano-particle tracking analysis (NTA, Malvern Instruments Ltd.) allows sizing, counting and visualizing of the pDNA/PEI particle polyplex formation between the electrostatic interaction of oppositely charged pDNA and PEI. The exact number of particles per mL is measured by nano-tracking analysis. The device is equipped with a sample chamber and a 405 nm blue laser. All measurements were made at room temperature and within a tracking time of 80 minutes (min). An EMCCD camera and NTA software were used for size measurement. Freshly prepared polyplexes at a concentration of 24 µg pDNA:24µg PEI and 24µg pDNA:45µg PEI per 1 mL CHOMACS CD medium were analyzed. The analysis was done under static (non-shaking) condition.

As described above, 5 kV analyses were carried out using the scanning electron microscope (SEM) (phenom desktop scanning electron microscope G1) with a backscatter electron detector thermionic source. A droplet from each sample was deposited on clean coverslips and kept overnight at 4 °C to allow the material to attach to the glass coverslips. The coverslips were carefully rinsed several times with distilled H_2O then dehydrated through immersion in ethanol and dried in the oven at 60 °C for 6 hrs. After sputter coating, the samples were examined under microscope.

3.2.3. Transforming growth factor (TGF-ß1) protein analytics

Fused recombinant protein was purified with short peptide affinity tags such as the Twin-Strep-tag® directly from the culture supernatant using Strep-Tactin® affinity resins. For efficient purification of Strep-tag-fused recombinant protein, the pH has to be adjusted to 8. However, some culture media supplemented with biotin competes with the Twin-Strep-tag® binding site and inactivates Strep-Tactin® columns. Seven mL/L of the BioLock® solution was used to complete the biotin blocking task [115].

For purification, all supernatants of TGF-β1 transfected cultures were centrifuged for 30 min at 4000 rpm at 4 °C and the pellets were discarded. The pH of the supernatant was adjusted to pH 8.0 with 1x wash buffer (W1): 1 M Tris-Cl, 1.5 M NaCl, 10 mM EDTA at pH 10. At the same time, the column was equilibrated with 1X W1 buffer at pH 8.0 while being cooled. The supernatant was loaded onto the column using the WetFred System (Figure 20). The column was washed with 1x W1 buffer 5 times then once with elution buffer (E): 1 M Tris-Cl, 1.5 M NaCl, 10 mM EDTA, 25 mM desthiobiotin at pH 8.0 was added three times to extract the target protein in fractions E1, E2 and E3. To reuse the column, regenerated buffer was loaded followed by a 1x W1 buffer. All these steps were done according to the Twin-Strep-tag® protocol [122].

Figure 20: Structure of the WetFred purification system
This system was used for Twin-Strep-tag® protein purification.

The concentration of purified protein was determined based on the spectrum measurement of Nanodrop ND-1000 UV-Vis Spectrophotometer at 280 nm. After that several samples were used to perform Western blots.

- **Protein quantification by ELISA**

10 µL of protein extracted in elution buffer was taken up in 90 µL Na_2CO_3/$NaHCO_3$ buffer and incubated in 96-well polystyrene plates overnight at 4 °C. Thereafter, the protein sample was stacked with the plate. On the next day, the plate was washed three times with 200 µL of 1x TBS buffer 1 L: 50 mM Tris-Cl, 150 mM NaCl at pH 7.6. The plate was then blocked with 200 µL of TBS (5% skimmed milk) at room temperature for 2 hrs and afterwards washed three times with TBS buffer. All samples on the plate were then incubated with 100 µL of the primary antibody mouse-anti-human-TGF-β1 solution at a final concentration of 8 µg/mL of diluent solution for 1 hr at room temperature. After this, the samples were washed three times with 200 µL of 1x TBS buffer and then incubated for 1 hr at room temperature with 100 µL of the second antibody goat Anti-Mouse IgG HR-conjugate solution at a final concentration of 4 µg/mL of diluent solution. Subsequently, it was washed again three times with 1x TBS. Of course with each washing step, the solution was discarded completely by inverting the plate several times onto tissue paper.

The color development was started by adding the staining solutions peroxidase substrate tetramethylbenzidine (TMB) 100 µL to each well. The reaction was stopped after 30 min with the addition of 100 µL 1 M H_2SO_4, and the color changed from blue to yellow. The O.D. was detected using a 96-well plate reader Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Germany) at 450 nm absorbance. The concentration of purified active TGF-ß1 in the elution was calculated by correlating each sample to the standard calibration curve of commercial TGF-ß1 presented in Appendix (Figure 47).

- **Protein qualification by SDS-PAGE and Western blot**

SDS-PAGE was applied for separating protein bands to detect a specific TGF-ß1 protein band. After SDS-PAGE and electro-transfer of the protein to a membrane, the Western blot protocol was applied.

A polyacrylamide gel is formed by crosslinking acrylamide with N, N-methylene bisacrylamide. Ammonium persulfate (APS) is an oxidizing agent used with tetramethylethylenediamine (TEMED) to catalyze the polymerization of acrylamide and bisacrylamide to prepare polyacrylamide gels for electrophoresis.

Procedure: The 15% separating gel is poured between two glass plates. After the polymerization, the 6% stacking gel is poured over the separating gel. This is left standing with a comb until it is solid. The protein samples are mixed in equal portions with Laemmli buffer: 800 µl of 2x SDS buffer (20 mg of bromophenol blue, 58 mg of EDTA sodium salt, 5 g of SDS, 0.315 g of tris-HCl, and 80 mL of ddH_2O), 100µl glycerin at 55%, 100µl ß-mercaptoethanol,

and shaken for 10 min at 95 °C. The electrophoresis chamber is then filled with 1x SDS running buffer: 0.3 g tris, 14.0 g glycine, and 1.0 g SDS are added to 1 L ddH_2O at pH 8.3 and the polymerized gel is placed in the chamber. The comb is carefully removed from the gel. In the case of gels with ten pockets, 17 µL of the prepared samples are applied to the gel bags as well as to a gel bag with 4 µL of the molecular weight marker. The electrophoretic separation is carried out for about 2 hrs at a voltage of 100 V until the running front (bromophenol blue) reaches the end of the gel.

By means of the Western blot, the protein band from the SDS-page gels is transferred to the PVDF membranes; the target protein band can then be detected using specific antibodies.

Procedure: First, the PVDF membranes are stored in methanol for 20 min. The membrane is then transferred to the transfer buffer and incubated for at least 5 min. A Whatman-paper immersed in transfer buffer is then placed onto the anode of the blotting apparatus. Now the PVDF membrane and SDS-PAGE gel are placed over it. This is followed by another wet Whatman-paper. The blots are moistened with transfer buffer: 0.3 g tris, 1.5 g glycine, 20 mL methanol, and 0.1% SDS and the apparatus is sealed. The blotting is carried out for 45 min at 15 V. The membrane is then swirled briefly in ddH_2O. To saturate free binding sites, the membrane is incubated overnight in the block solution: 5 g of skimmed milk powder, 500 µL of Tween 20, to 100 mL of 1x PBS at 4 °C. The membrane is then washed three times for 5 min in PBST (500 mL PBS buffer + 0.5% Tween 20) 1x PBS buffer: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.27 g KH_2PO_4, add 100 mL ddH_2O at pH 7. This is followed by membrane incubation for one hour in 20 mL of the primary antibody solution (3 µg/mL of diluent solution as shown in Appendix). The membrane is then washed three times again with PBST for 5 min. This is followed by its incubation for one hour in the secondary antibody solution (0.2 µg/mL of diluent solution). Again, the membrane is washed 3 times for 5 min with PBST and once for 10 min in alkaline phosphatase buffer (AP buffer: 100 mM tris HCl, 100 mM NaCl, 5 mM $MgCl_2$ $7H_2O$, added to 25 mL ddH_2O at pH 9.5). The membrane is developed with 25 mL of the AP staining solution: 250 µL color reagent A, 250 µL color reagent B, added to 25 mL AP buffer. The staining is stopped by transferring the membrane to ddH_2O.

- **2D-Fluorescence spectroscopy**

Two-dimensional fluorescence (2D-fluorescence spectroscopy, Hitachi model F 7000, Japan) intensity scans analyzed the fluorescence intensity of 1 mL purified TGF-ß1 in the elution buffer E2 fraction with different concentrations as estimated with the nanodrop method. Fluorescence spectra were measured using a fluorescence spectrophotometer with horizontal beam geometry in a standard cuvette. The parameters had an excitation wavelength of 200–550

nm and an emission wavelength of 250–550 nm (20 nm steps). The whole spectrum was obtained within 400 V (scan speed: 2400 nm/min).

3.2.4. Protein bioactivity determination (cell-based assay)

- **A549 cell cultivation**

A549 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin and subcultured until required for assay treatment. Cultures were incubated at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% (v/v) CO_2 in air. The cell number in the trypsinized cell suspension was determined using a haemocytometer.

The cells were washed with warm PBS buffer and then incubated with a Trypsin/EDTA buffered solution at 37 °C for 5 min in an incubator. After that, the equivalent amount of serum-enriched medium was added to stop trypsinization and to prevent cell damage. Detached cells were collected and washed twice with PBS through centrifugation at 300 xg for 5 min. The supernatant was discarded and the harvested cells were counted and seeded in 96-well plates in fresh DMEM media with 10% FCS. The cell suspension was diluted to give a final concentration of $4x10^4$ cell/ml, and 200 µl was pipetted into each well of the 96-well plate (i.e. 8000 cells/well). After a 24h adhesion time, the incubation medium was removed and DMEM supplemented with 1% FCS and various TGF-ß1 concentrations ranging from 44 ng/mL to 176 ng/ml and controls (0 ng/ml) were added. Each concentration was tested in four independent wells. The inoculated plates were incubated in a CO_2-incubator at 37 °C, 5% (v/v) CO_2 and at 90% relative humidity for five days.

- **CellTiter-Blue Assay**

After visual assessment, the TGF-ß1-containing media and negative controls were removed and an indirect estimation of cell proliferation was made using a CellTiter-Blue® Cell Viability Assay (Promega, Cat. Number G8080). A working solution of CellTiter-Blue reagent in DMEM or alpha-MEM without antibiotics and serum was prepared: 1 mL CellTiter-Blue reagent + 9 mL DMEM (10 mL of total volume). After the medium was removed, 100 µL of the working solution was added to each well with the help of a repeating pipette. Additionally, 4 side wells without cells were filled with CellTiter Blue working solution for the blank measurement. The plates were incubated at 37 ± 1 °C in a humidified atmosphere of 5 ± 1% (v/v) CO_2 in air for approximately 4 hours. The fluorescence was determined at 560 nm (excitation) and 590 nm (emission). The dye used for this assay is the blue, non-fluorescent resazurin (7-hydroxy-3h-phenoxazin-3-one-10-oxide). In the mitochondria of living cells, it is reduced to the pink fluorescent resorufin. Thus a measure of the metabolic activity of the living cells is based on the degree of fluorescence intensity.

4. Results

Production of recombinant proteins is important for therapeutic and research development purposes. Establishing efficient transient protein expression is a prerequisite for the rapid delivery and production of these recombinant proteins.

Methods of transient transfection depend on a number of different factors, including media type, cell density, physiological state and age of the culture, and the amount of plasmid and transfection reagent. Therefore, it is necessary to develop a reproducible transfection protocol to rapidly produce proteins of adequate quality and quantity. For this reason, the goal of this thesis has been to develop a novel means of cost-efficient transfection by optimizing the parameters of the transfection process that have an impact on transfection efficiency and cell viability. To do so we used linear polyethylenimine (PEI) polymer as a transfection reagent and green fluorescence protein (GFP) expression plasmid as a model for protein expression.

After optimizing the transfection process, we applied the method for producing transforming growth factor TGF-ß1 protein, which was then purified. The next step was detection by Western blot and 2D-fluorescence spectroscopy. In the same time, the concentration was determined with a Nanodrop spectrophotometer and ELISA methods. Finally, the bioactivity was tested through a cell growth inhibition assay.

The major objectives of this work are divided into four chapters. The first chapter focusses on establishing a suitable transient transfection procedure in CHOMACS CD serum-free media. This helps in the development of an efficient routine transfection protocol that combines high transfection efficiency with high cell viability. The next chapter takes into account a wide range of variables relating to the cell culture in the routine transfection protocol. These variables include pre-culture cell density, cell density at the time of transfection , and pre-culture age in relation to the cell cycle. The third chapter focusses on a wide range of pDNA and PEI amounts in the routine transfection protocol. Various transfection conditions were tested using the design of experiment (DoE) method to understand the interaction between these variables and transfection efficiency. Repeated transient transfection was implemented to further improve transfection efficiency by 60%; this extended protein expression and reduced the amount of PEI and pDNA per transfection step. The scalability of the routine transfection protocol from 1 mL to 12.5 mL was evaluated using the Tubespin bioreactor®50 and shake flasks. The last chapter focuses on the transient expression of recombinant transforming growth factor TGF-ß1 protein in shake flasks. This chapter provides preliminary information about using 2D-fluorescence spectroscopy to detect purified TGF-ß1 protein regarding their concentrations. Finally, the TGF-ß1 activity corresponding to the inhibitory growth of A549 cells was evaluated.

4.1. Transient transfection in CHOMACS CD media

Cell culture media represent the first barrier that faces the PEI/pDNA transfection polyplex before it interacts with cells. Six different commercially available media were screened for their influence on cell growth (indirectly indicated by cell viability) and transfection efficiency. The screening was done under fixed transfection parameters concerning the number of cells, i.e. the amount of pDNA and PEI, but involving two different volumes of transfection culture. The first of these is the large culture volume (5 mL) called here “procedure (A)”. The second is the small culture volume (1 mL) called here “procedure (B)”. In an indirect way procedure (A) exerts the transfection parameters in a lower concentration than procedure (B), as described in ‘Materials and Methods’ section Figure 19. Furthermore, this study used procedure (B) to evaluate the effect of adding media at different post-transfection time points, from 1 hpt up to 20 hpt. We extended the study to also investigate the necessity of adding media at 5 hpt, which coincides with the up-scaling of the transfected culture volume. The need to exchange the medium prior to transfection was evaluated. Finally, we study the effects of media osmolality on transfection efficiency.

4.1.1. Screening of transfection medium for CHO-K1 cells

Six different media named (ProCHO-5, CD CHO, CHOMACS CD, Ex-cell CD CHO, Opti-MEM and DMEM/F12) were tested for transfection as shown in Figure 21.

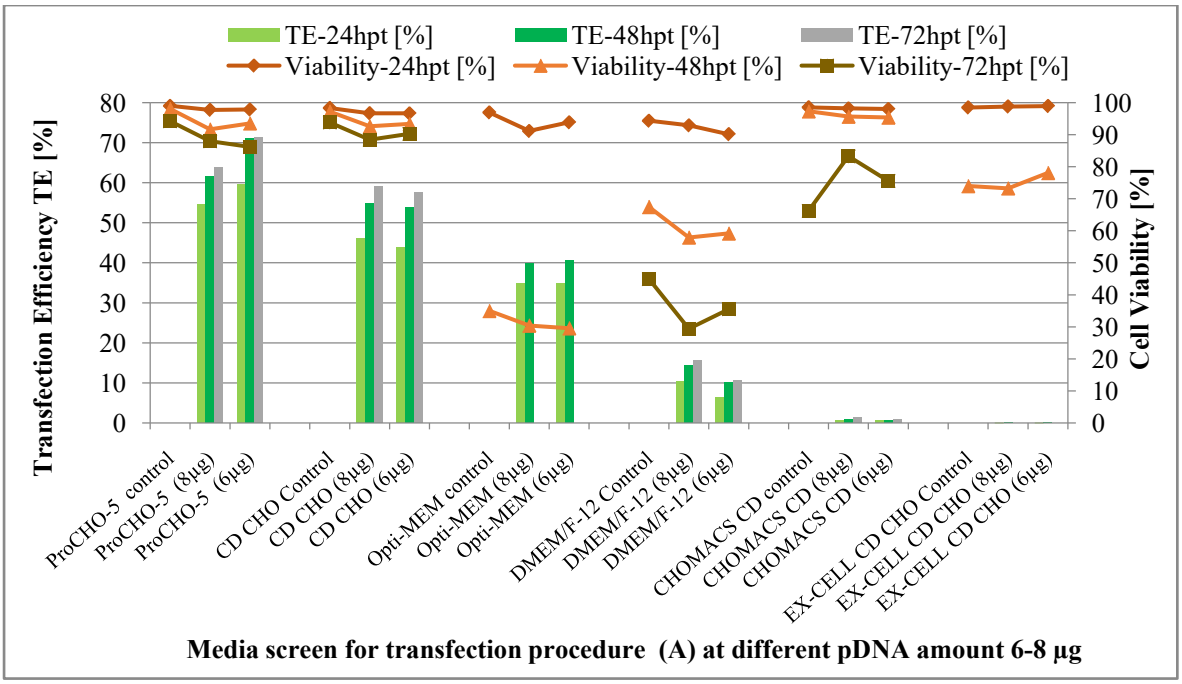

Figure 21: Media screen for transfection and cultivation Procedure (A).
Screening of the transfection media was done according to the procedure (A). Transfection efficiency and cell viability in different transfection media (ProCHO-5, CD CHO, CHOMACS CD, Ex-Cell CD CHO, Opti-MEM and DMEM/F12) were determined at 24, 48, and 72 hours post-transfection (hpt).

Cell growth and transfection efficiency are the main targets of establishing a transient transfection protocol. Importantly, high cell growth is indicated indirectly here by high viability, and high transfection is related to high transfection efficiency (TE). The media screening procedure (A) (Figure 21) reveals the different effects of various media on cell growth and transfection efficiency, the two targets of the protocol. ProCHO-5 and CD CHO media help support both cell growth and transfection, while Opti-MEM media enhances only transfection. CHOMACS CD showed better results only with regard to cell growth, and DMEM/F12 slightly assisted transfection, unlike Ex-Cell CD CHO.

This result clearly shows that ProCHO-5 and CD CHO media are the best choices for achieving the targets of supporting transfection and growth and that the other media are not suitable for this purpose. However, the media which support either transfection or growth can also be made to support both targets (transfection and growth), and hence were tested by using the alternative method of transfection and adapting CHO cells to grow in such media. For this reason, rather than neglecting CHOMACS CD the second part of this experiment was conducted with this media with the aim of achieving this target.

As expected, the component "media" is an important factor for compatibility with PEI transfection, acting as either an inhibitor or supporter of the process. The transfection condition was modified in procedure (B) as shown in Figure 22. The idea behind this is to produce a high density of cells with a smaller amount of inhibitor. Transfection in Procedure (B) was carried out with only three media (ProCHO-5, CD CHO, and CHOMACS CD), based on the preliminary results (Figure 21) which showed they had the best viability record.

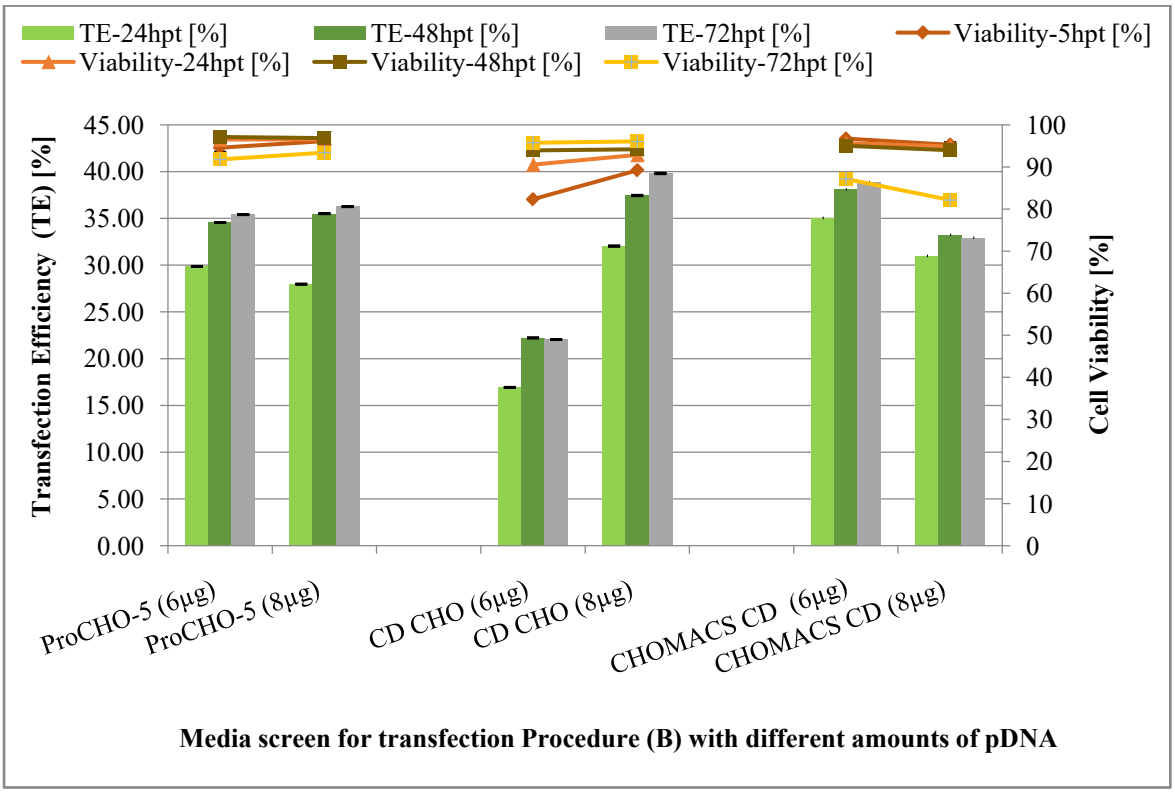

Figure 22: Media screen for transfection according to Procedure (B).
Transfection efficiency and cell viability were determined in the three different media of CHOMACS CD, ProCHO-5 and CD CHO using transfection Procedure (B). All experiments were done in duplicate.

Transfection in low amounts of media under Procedure (B) affected the transfection efficiency differently, depending on the type of media (Figure 22). Transfection efficiency with CHOMACS CD media increased >35 fold compared to Procedure (A). Since an equal cell amount at high cell density was used in 1 mL CHOMACS CD (Figure 22), this increases the cell polyplex (CHO/PEI/pDNA) interaction. Thus the (CHO/PEI/pDNA) interaction was better in low media volume (1 mL) but not the best with high media volume (Figure 21). The better efficiency can be explained by the presence of a number of media components that interacted negatively with the pDNA/PEI polyplex. This interaction could occur through blocking or neutralizing the charge of pDNA/PEI polyplex. Accordingly, with regard to the pDNA/PEI polyplex aspect, the presence of such negative components in CHOMACS CD is greater in the 5-mL-volume with Procedure (A) than in the 1-mL-volume with Procedure (B). Therefore the CHO/PEI polyplex interaction could be diminished.

The highest transfection efficiency was achieved with 5 mL ProCHO-5 in Procedure (A), where high media volume was used for a transfection of nearly 70% at 72 hpt (Figure 21), but with a culture volume of 1 mL and Procedure (B) the transfection efficiency of the same media was decreased to 35%. Interestingly, the CD CHO media showed the same tendency, with around 60% in the 5-mL volume and 30% in the 1-mL volume.

This transfection behavior is contrary to that of the CHOMACS CD media, where the influence of inhibited media components on PEI-mediated transfection was clear. This influence had a less negative impact in the 5-mL transfection volume for both ProCHO-5 and CD CHO compared to CHOMACS CD. Apparently, the different methods of transfection in the two procedures (A & B) can determine whether the media supports high cell density transfection or low density transfection. The high cell density transfection with Procedure (B) was used as a starting point towards improving transfection efficiency in the CHOMACS CD media used in this study. To explore the robustness and flexibility of Procedure (B) for transfection, we studied the effect on transfection efficiency of extending or shortening the post-transfection time of 5 hpt before the stage of transfected culture dilution.

4.1.2. Determination of post-transfection time required before media dilution

With the aim of determining whether increasing or decreasing the post-transfection time could affect transfection efficiency, different numbers of hours post-transfection (hpt) , i.e. 1, 5, 10 and 20 hpt were evaluated. According to the hypothesis that the media has an inhibitory effect (comes from media dilution) on pDNA/PEI polyplex mediated transfection, the expectation would be that dilution occurring after only a few hours post-transfection (hpt) would have a more negative impact on transfection than dilution at a later hpt. This is because the effect is greater after subsequent additions of media 1:1 (v/v), which interact with the polyplex and exert an inhibitory effect.

CHO-K1 cells were transfected on an initial volume of 1 mL with the addition of one volume of media (media volume addition was reduced from 4 to 1 mL), modified from the previously mentioned Procedure (B). From now and hereafter (unless otherwise stated) pDNA and PEI amounts were modified to 24 µg pDNA and 45 µg PEI rather than 6, 8 µg for pDNA and 30 µg for the PEI as described in the previous experiments. This change was based on DoE studies described in Chapter 4.3 to obtain high transfection efficiency.

Interestingly, the findings confirm previous expectations concerning the ability of media components to inhibit PEI transfection. The transfection efficiency was lower in cultures transfected for 1 hpt, while other cultures transfected in the 5-20 hpt range have a similarly higher transfection efficiency, as shown in Figure 23. With regard to the cell viability, cultures

with 1 hpt, showed relatively higher cell viability compared to those with 5, 10, 20 hpt. This reflects the inverse relationship trend between transfection efficiency and viability percentage.

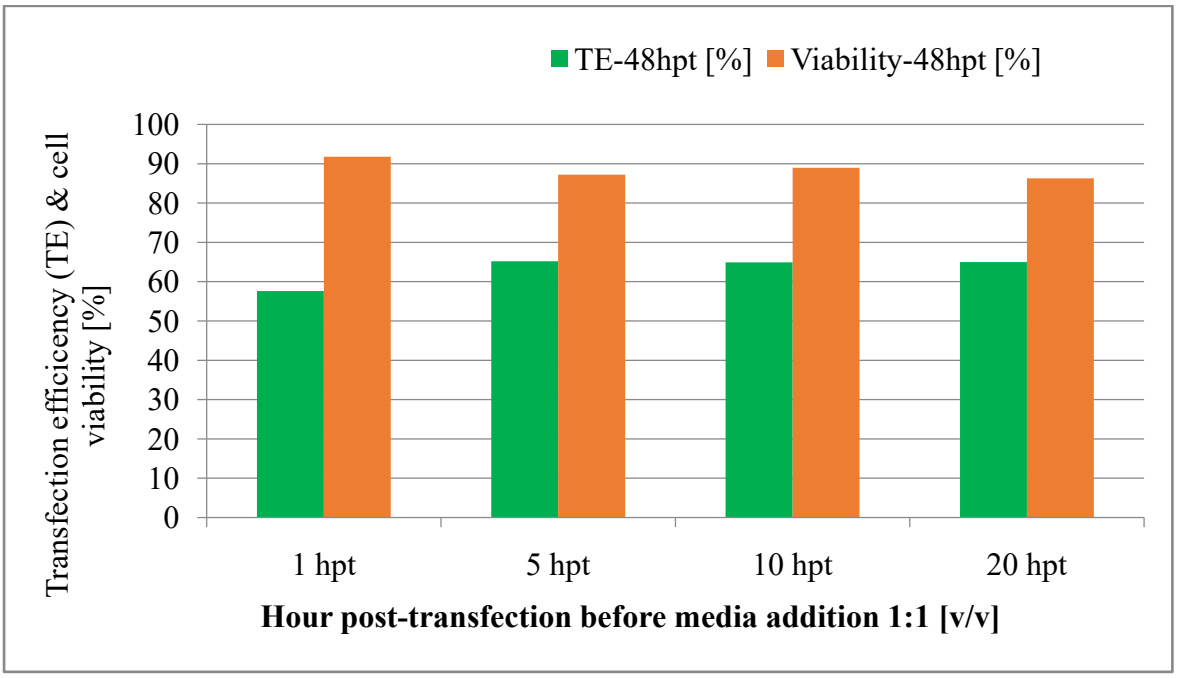

Figure 23: Effect of post-transfection time prior to dilution.
CHO-K1 cells transfected in a constant culture volume of 1 mL as in Procedure (B) followed by an equal volume addition of media. The additions were made at the indicated hours post-transfection (hpt). The cultures were harvested to determine transfection efficiency and measure viability at only 48 hpt to avoid further starvation stress.

The data in Figure 23 shows that extending post-transfection times from 5 to 20 hours before transfected culture dilution provides no further increase in transfection efficiency, and also that reducing this time to 1 hpt resulted in relatively low transfection efficiency. The expectation was that increasing transfection time would allow such delayed cells that were not in mitotic stage (cell cycle) to enter it and maintain the pDNA/PEI polyplex. Unfortunately, no improvement in transfection efficiency occurred. This could be due to two limitations. First, the PEI/pDNA polyplex aggregate over time and become bigger in size, which means they cannot pass through the cell membrane via pinocytosis. Second, the cells were saturated by the amount of PEI/pDNA polyplex which had already passed through by the 5 hpt period.

These results indicate that 5 hpt is enough time to allow the entrance of transfection polyplex and that a further increase in transfection time before culture dilution has no effect on transfection efficiency and viability. To confirm this finding, the transfected culture was diluted with a different volume of media as described in the experiment below. Generally, this result also indicates that during scale-up transient transfection, there is no need to dilute the culture at a fixed time after 5 hpt. Of course, this flexibility in media addition time, gives us more chance to observe contamination within the 20 hpt with a large-scale bioreactor.

4.1.3. Effect of additions to media volume on transfected culture at 5 hpt

As discussed above, the pDNA/PEI polyplex has enough time to enter the cells within 5 hpt. Now the study evaluate the necessity of adding one volume of medium at the 5-hpt time point, and test the effect of volumetric scale-up (addition of 1-7 volumes of culture media) on transfection efficiency (further confirmation for the previously mentioned media component inhibitory hypothesis). The expectation was that additions of different volumes of media at the 5-hpt time point should not have a negative impact on transfection efficiency. Cultures of CHO-K1 cells were resuspended and transfected with an initial volume of 1 mL according to Procedure (B) in a Tubespin bioreactor®50 with four different dilution volumes at 5 hpt: (1) no media addition, (2) addition of one volume (1 mL) of media, (3) addition of 3 volumes (3 mL) of media and (4) addition of 7 volumes (7 mL) of media.

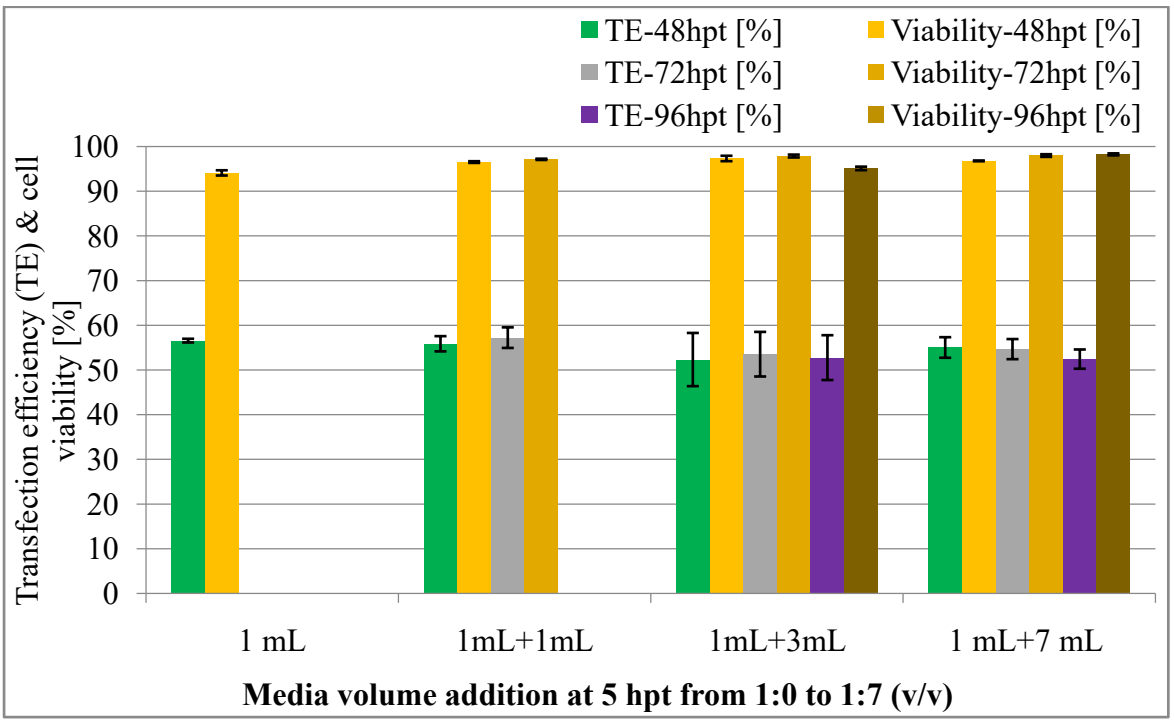

Figure 24: Different volumes of media added at 5 hpt.
Effect of media additions after transfection 5 hours post-transfection time (hpt) on transfection efficiency and cell viability% were described. All experiments were done in triplicate.

The results in Figure 24 demonstrates that as expected transfection efficiency was about similar for all four tested conditions, and that it is not necessary to dilute the culture after transfection. In addition, transfection efficiency remained stable in all cultures, regardless of media addition at 5 hpt in the tested ranges of 1:1 to 1:7 (v/v). The data confirm the feasibility of volumetric scale-up for the transfected culture by the strategy of adding media. This will increase the period of recombinant protein expression. Overall, the transfection efficiency obtained was about 52% ± 5% for the different post-transfection time ranging from 48-96 hpt.

Generally, transfection efficiency level was high at 48 hpt and increased by increasing post-transfection time to 72 hpt, but further increasing it decreased to the same level of 48 hpt. This behavior may be explained by the increasing growth of a non-transfected cell (GFP-cell) population compared to a transfected one (GFP+ cell) within the same culture during the time period 72 to 96 hpt. Another explanation is that this is due to the loss of plasmid DNA (pDNA) through extended cultivation time because it's episomal and there is loss by cell division. According to this experiment, 72 hpt represents the maximum point of reporter GFP expression and 48 hpt is an optimal point for harvesting the culture. However, viability remained higher above 95% in all transfected cultures, which is a good indicator for cell growth activity. In order to avoid deterioration of viability in the cultures with no media addition or with a low volume of added media, the culture harvested earlier.

It is worth comparing the efficiency of transfection within the individual experiments carried out. The efficiency of transfection with a low amount of pDNA 6 µg combined with 30 µg PEI ranged from 52% (Figure 24) to 38% (Figure 22). This variability in the percent values of transfection efficiency could be related to the physiological intra-variability of the cultures or the stability of the formed pDNA/PEI polyplex. This observation endorses the use of a high amount of 24 µg pDNA:45 µg PEI (mentioned previously) as a routine transfection protocol. This is recommended for repeated assessment within and between the conducted experiments. Perhaps the high amount of pDNA and PEI forming stable polyplex with less variability coincides with stability in transfection efficiency.

4.1.4. Medium exchange at transfection time point

According to routine the transfection protocol described in 'Materials and Methods', cells should be prepared one day before transfection (pre-culture), centrifuged and then resuspended in fresh medium at transfection time. To determine if these steps are required, CHO-K1 cells were pre-cultivated either for one or two days (pre-culture duration) followed by media exchanges either in fresh or consumed media (Figure 25) at transfection time.

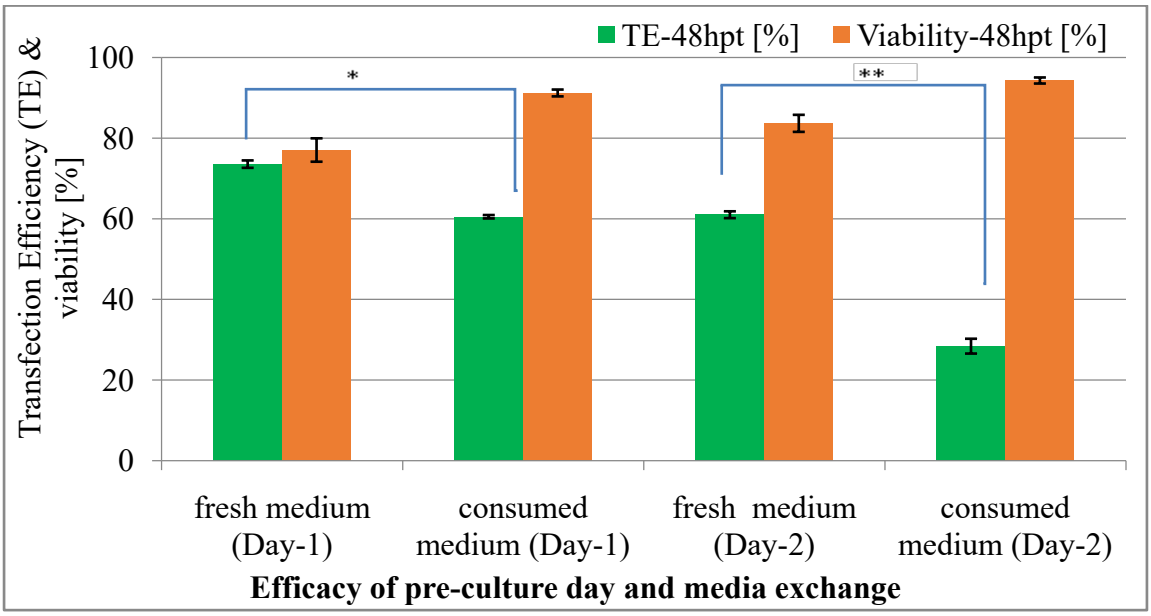

Figure 25: Efficacy of pre-culture day and media exchange.
Media exchange for transfection either by fresh or consumed media, 1 or 2 days pre-culture. All experiments were done in duplicate.

The results demonstrate that higher transfection efficiency (TE) was obtained for cells resuspended in fresh media within one day of pre-culture, like in the routine transfection protocol. Increasing the pre-culture time to two days and resuspending the cells in fresh media decreased transfection efficiency by more than 10% compared to one-day pre-culture. The decrease in transfection efficiency occurred for those cells resuspended in consumed media either one or two days pre-culture compared to those cells resuspended in fresh media. This underlines the necessity of resuspending cells in fresh medium within one day of pre-culture. Higher viability is related to lower transfection efficiency. This reflects the inverse relationship trend between transfection efficiency and viability, which is consistent with the previous results. These results suggest that transfection is possible in consumed media after one day pre-culture, which facilitates easier scale-up of transient gene expression and avoids media exchange. Obtaining transfection efficiency of around 60% is acceptable for afterward production process.

Also, it is worth observing here and regarding the previous results, that transfection efficiency under the same transfection conditions varied from 74% (Figure 25) to 65% (Figure 23).

4.1.5. Effect of media osmolality on cell growth and transfection efficiency

To determine if osmolality can improve transfection efficiency without affecting cell growth, different media with various applied osmotic pressures ranging from 288 to 334 mOsm Kg^{-1} were tested, firstly for cell cultivation as indicated in Appendix Figure 45. The result showed no big difference in the maximal cell number achieved or in cell viability; these ranged from 25 to 24 $x10^6$/mL and 98 to 99% respectively for 48 hrs of cultivation in this osmotic stress range.
The CHOMACS CD media with the same osmotic pressure as noted previously were used for transfection of the same amount of cells, pDNA and PEI as in the routine transfection protocol. At 5 hpt, one volume of normal medium 288 mOsm Kg^{-1} was added to all transfected cultures.

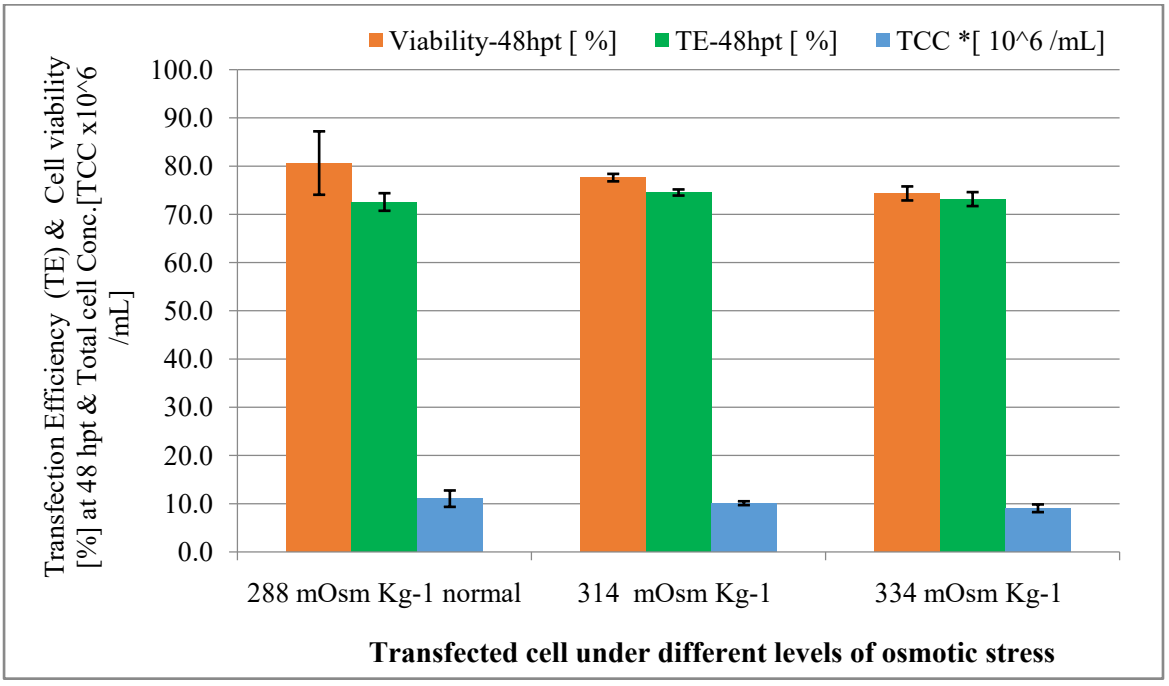

Figure 26: Effect of media osmolality on transfection.
Normal media without NaCl addition has an osmotic pressure of around 288 mOsm Kg^{-1}. All experiments were done in triplicate.

Transfection efficiency and viability at 48 hpt as shown in Figure 26 exhibited no big differences in percentage. Under various applied osmolality conditions, transfection efficiency averaged 72-74% for all media and viability, 74-80%. Increasing osmolality up to 314 mOsm kg^{-1} slightly improved transient transfection efficiency, but with a reduction in viability to 77% compared to normal culture media with 288 mOsm kg^{-1} and 80% viability. Generally, there is no strong effect (less than 10%) on either viability or on transfection with this osmotic range.

4.1.6. Discussion of transient transfection in CHOMACS CD media

The medium is one of the most critical factors in the production of recombinant protein from high-density transfected suspension cultures [35]. Transient transfection depends on the cultivation media, which is necessary for cell growth and protein production. But there are some media components that inhibit or diminish transfection efficiency. Performing transfection in deficient media (transfection media), which comprises 50% of the final production medium, followed by feeding of complete media, is the most-used method for the transfection [6,123]. This strategy has been described in submitted work. In this study, a transfection method in CHOMACS CD production media was developed, consistent with a recently reported transfection method in production ProCHO-5 media [35]. CHOMACS CD serum-free medium is better for providing high cell density growth of up to 18 x10^6 cells/mL [124]. But, what about its transfection capability? Optimal medium composition is necessary for transfection and production since only one media type is used. This has the advantage of avoiding the need for cell adaptation to newly introduced transfection media or medium exchange after transfection, thus maximizing protein productivity.

Screening media transfection according to Procedure (A) provides an overview on which kinds of media are compatible with PEI transfection. Evaluation the same media with Procedure (B) exhibits a different level of transfection efficiency, the best of which is for CHOMACS CD media. This type of screening strategy, which is carried out in two stages (A & B), has not, to our knowledge, so far been used. The most applied method have been published describes how a pre-adapted cell culture is used in each media, then transfection is conducted under specific transfection condition one time, after which it is decided which media is suitable or not suitable for transfection [125]. With this method, some types of media will be rejected for PEI transfection, although they could indeed be used for transfection by modifying the transfection procedure and testing them under different transfection conditions. The transfection screening procedures (A&B) in this study make it possible to distinguish the media that support high or low density cell growth and transfection and to quickly evaluate them. In addition, conducting screening under two transfection conditions is an excellent tool for confirming whether the media support transfection or not, particularly for establishing a starting transfection protocol.

Since the composition of CHOMACS CD and ProCHO-5, like most commercial media, is confidential, some complex media are not amenable to high-density transfection due to the presence of a high concentration of inhibitory factors [74]. Concerning CHOMACS CD, it is likely to provide high transfection efficiency and high cell density with few inhibitory factors under Procedure (B). The basic principle behind components in the media that inhibit

pDNA/PEI transfection is presumably related to the ability to neutralize the positive charge in pDNA/PEI polyplex, which limits the arrival of polyplex to the cell surface [54]. Efficient transfection occurs when the anionic DNA phosphate backbone is neutralized with an excess positive charge of PEI to bind with the anionic cell surface charge [79].

This study found that the post-transfection dilution step does not affect transfection efficiency for the time periods 5-20 hpt, consistent with literature, which states that most transfection events occur within the first post-transfection period, when 80% of the pDNA/PEI polyplex is uptaken [44,84]. At 4.5 hpt the pDNA enters the cell nuclei [126]. Therefore volumetric upscaling of the transfected culture through media dilution by adding up to 1:7 (v/v) in media volume at 5 hpt is a promising and feasible way to scale up the volume of transfection culture after transfection.

Raising the amount of pDNA from 6 µg to 24 µg and PEI from 30 µg to 45 µg increases transfection efficiency from 55% to 65% but also reduces the viability from 96% to 87%. However, an inverse relation between transfection efficiency and toxicity has been previously reported [127]. Thus the balance between viability and transfection efficiency needs to be maintained, since high cell viability and growth rate, as well as high transfection efficiency, are prerequisites for obtaining high recombinant protein yields [40,52].

The most crucial step in PEI transfection concentrates the pre-culture cells and resuspends them in fresh medium. Wurm and co-workers found that fresh media produces higher transfection efficiency than consumed media, the latter which severely reduces transfection efficiency [128]. Consistent with this the use of consumed medium in transfection is significantly linked to the age of the culture. These observations also agree with those reported by Schlaeger et al [129]. The logically low transfection efficiency derived with consumed media suggests the presence of a component that inhibits PEI transfection or the absence of one that is necessary for it. As the pre-culture time becomes longer, the presence of this component in the consumed media also is longer, thus reducing efficiency of the transfection.

The addition of sodium chloride (NaCl) increases the culture osmolality, which aids DNA delivery [130], especially with conventional transfection methods. The results reported here, however, show that addition of NaCl increases media osmolality from 288 to 334 mOsm Kg^{-1} , but neither had a significant effect on transfection efficiency or on total cell number and viability.

Collectively, these findings outline the initial routine *in situ* transient PEI transfection protocol originating from the Procedure (B), including, 1) transfection at a high cell density of 10

million cells per mL in CHOMACS CD production media. 2) Dilution of transfection culture with 1:1 (v/v) of fresh medium at 5 hpt. This initial transfection protocol provides flexibility for culture dilution after transfection, either for dilution up to 1:7 (v/v) with fresh media (volumetric scale up) or without dilution (which would be better for fed-batch transient transfection). Overall, this study observes a transfection efficiency of about 60-70% for CHO-K1 cell in CHOMACS CD media at 24 µg and 45 µg amount of pDNA and PEI respectively. The same PEI transfection reagent solution mediates about 50–60% transfection efficiency for CHO-DG44 cells in ProCHO-5 production media [35]. A high level of reproducibility in performance was also seen; the cell density, transfection efficiency and viability of parallel cultures usually differed by less than 10%.

4.2. Cell parameters for efficient transfection

For transient recombinant protein expression, it is important to identify the critical parameters needed to obtain a reproducible transfection process with constant transfection efficiency. In this chapter, several critical parameters were carefully optimized to obtain reproducible and high transfection efficiency. These parameters are related to the host cell (which is CHO-K1 itself) and the conditions of the culture. The variables include the pre-culture cell density, pre-culture age and cell cycle and cell density at transfection time, which will be analyzed to assess the strength of the previously mentioned high-density routine transfection protocol. Each variable was tested independently. Pre-culture is designed to prepare cells for transfection.

4.2.1. Pre-culture cell density

A culture which is prepared one day (if not specified otherwise) prior to PEI transfection is usually called a pre-culture. To determine if the cell density of the pre-culture plays an important role for transfection efficiency, three different pre-cultures of CHO-K1 cells in CHOMACS CD medium were prepared in shake flasks. During the pre-culture, the cell densities were doubled till transfection time as indicated in Figure 27 (1.2, 2.6, 4.0 and 5.3 x10^6 cells/mL). The differences in pre-culture cell density were results from variations in initial cell inoculum. For transfection, equal cell densities from each pre-culture were prepared and transfected according to the routine transfection protocol mentioned in 'Materials and Methods' in order to investigate the effects of pre-culture cell density.

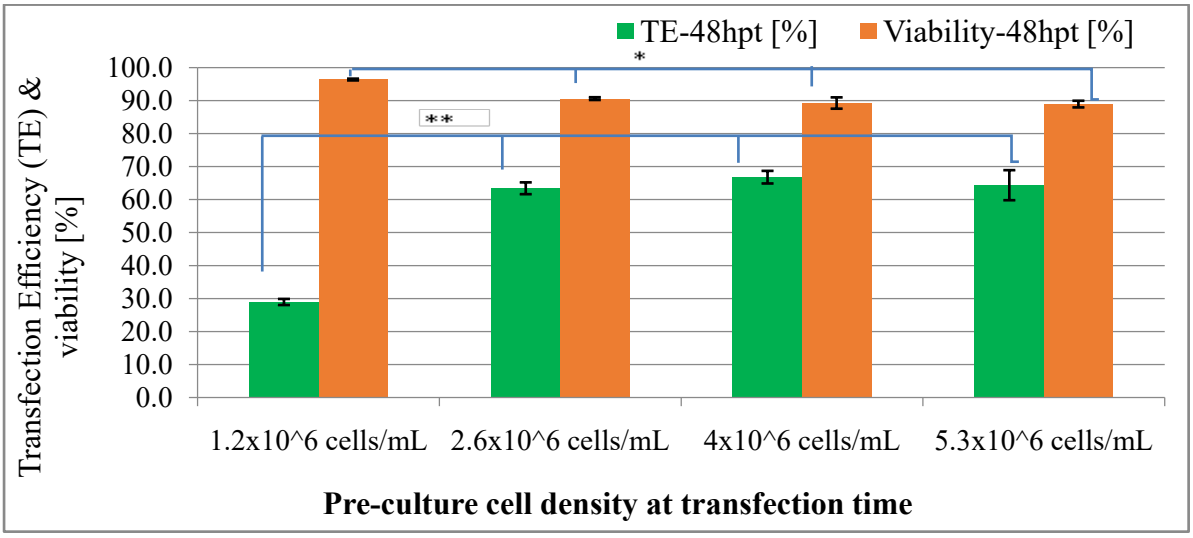

Figure 27: Effect of pre-culture cell density at transfection time.
Transfection efficiency and viability depending on different pre-culture cell densities and constant transfected cell density. Comparison of transfection efficiency and cell viability were done using one-way ANOVA test n=3. Summary of P value; *p<0.05, ** P<0.01 was considered significant and very significant, respectively.

Figure 27 demonstrates that the highest level of transfection efficiency was observed for a pre-culture density of 2.6, 4.0 or 5.3 x10^6 cells/mL. These conditions give a two-fold higher

transfection efficiency (TE%) than for a pre-culture density of 1.2 x10^6 cells/mL with a highly significant difference. In contrast, the viability was significantly higher for the low cell density pre-culture of 1.2 x10^6 cells/mL compared to other condition.

This increase in viability is related to the low transfection efficiency, while PEI toxicity decreased consistent with the previously mentioned results shown in Figure 23. This directly indicated cell density of the pre-culture at transfection time is a very important factor for efficient gene transfection, particularly in the range tested. From these results, it can be concluded that pre-culture cell density over 1.2 x10^6 cells/mL is necessary for efficient transfection. This can be related to the physiological state of the cells. Cell-cell communication can trigger transfection.

4.2.2. Pre-culture age and cell cycle

In order to evaluate more thoroughly the effect of pre-culture age and cell cycle dependency on transfection efficiency and viability, an equal number of CHO-K1 cells were transfected in fresh media from the different pre-cultures aging 15, 40 and 63 hrs. The applied pre-culture age in routine transfection protocol is 24 hr.

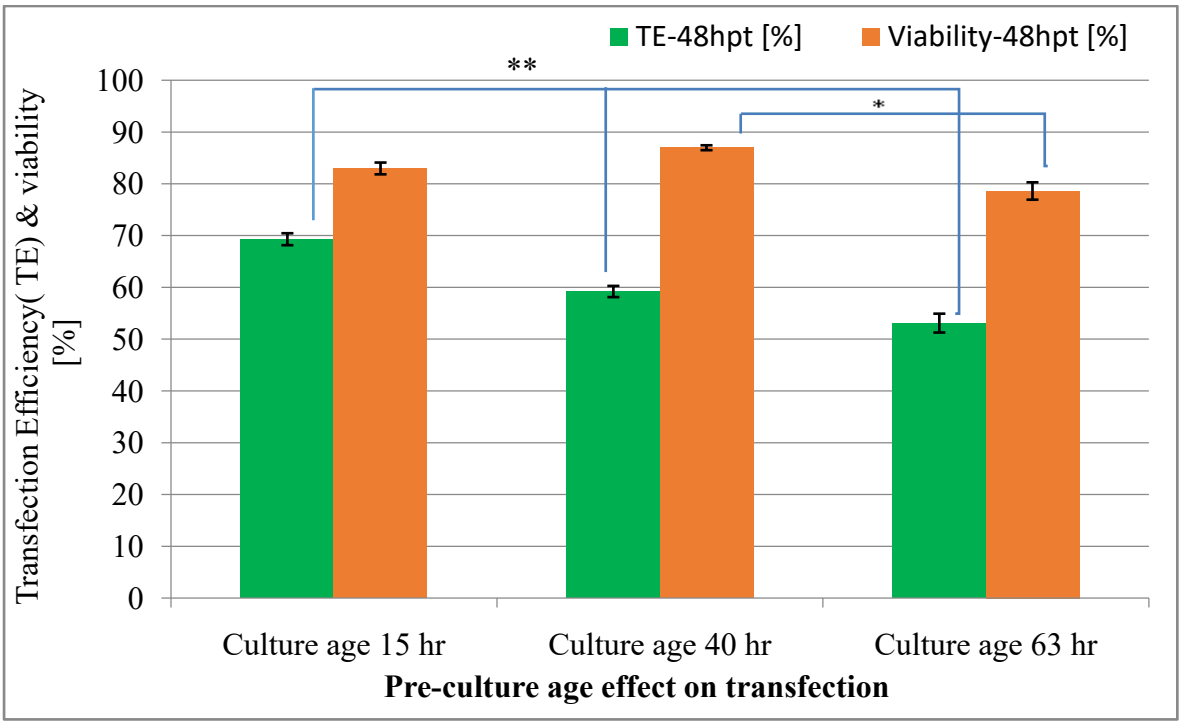

Figure 28: Pre-culture age effect on transfection.
The graph represents the transfection efficiency and viability at 48 hpt, depending on different pre-culture ages. All experiments were done in triplicate.

Analyzing the data from the Figure 28, it is obvious that the highest transfection efficiency was observed for a pre-culture age of 15 hrs, after which efficiency decreased proportionally with increasing pre-culture age. Our observations strongly suggest that the age of the pre-culture has

a significant effect on transfection efficiency. The younger the pre-culture, the higher is the transfection efficiency, consistent with the previously obtained results as shown in Figure 25.

To guarantee that this transfection efficiency effect depends on pre-culture age and not on pre-culture cell density belonging to longer cultivation times, the cell density of the pre-culture was found to be in the range of over 1.2 to 5 x10^6 cells/mL (compare to Figure 27), while the cell density for a 63-hr-old pre-culture was over this tested range. Therefore, the possible reason for why a reduction was observed in transfection efficiency of pre-culture 63 hr age. This would interrelate not only to culture age but also to cell density effect.

Further transfection conditions over 63-hrs-old were not conducted with regard to the viability issue. Because as the culture gets older, accumulated waste products and housing cells begin to deteriorate physiologically, cell viability starts to decrease to less than 99%. It is worth noting that in all conducted transfection experiments, the viability of pre-culture at transfection time was always over 99%.

A decrease in viability after transfection not only depends on transfection toxicity but also on the physiological state of pre-culture. Further analysis was carried out to explore the physiological state (cell viability) as well as cell cycle distribution pre-culture age from 0 to109 hrs cultivation time.

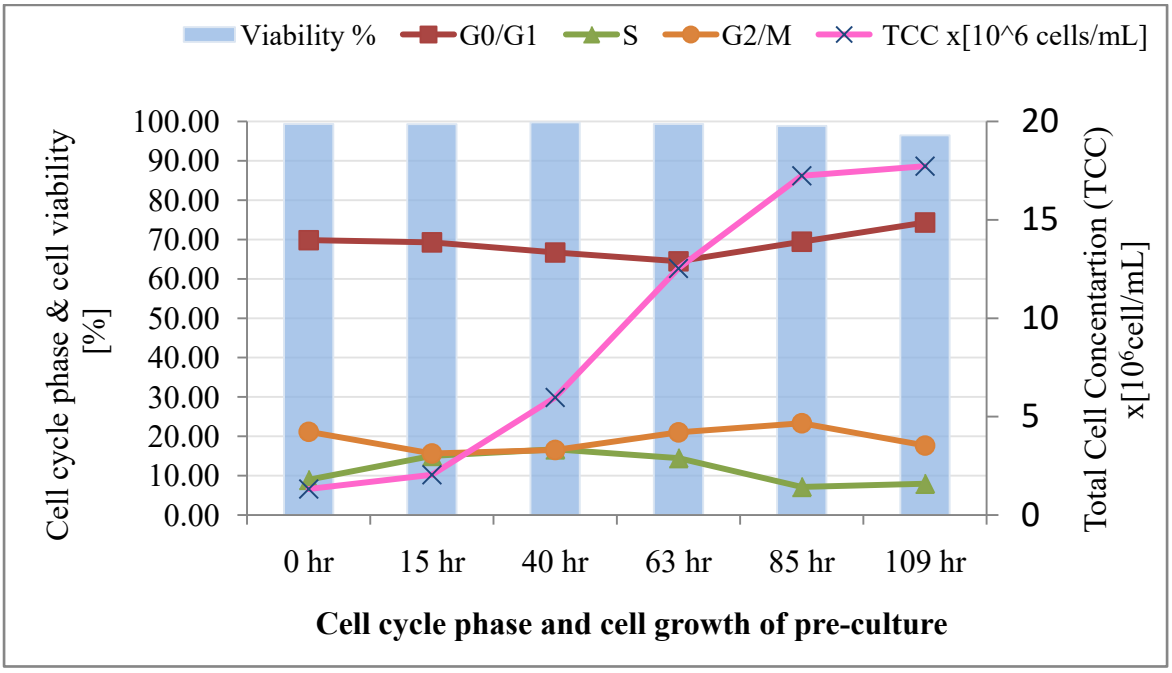

Figure 29: Pre-culture cell cycle phase and cell growth.
The graph represents the typical CHO-K1 cell cycle profile and viability of pre-culture within 109 hr cultivation time.

As observed in Figure 29, cells are usually divided into phases as follows; 70% G0/G1 phase, 21% G2/M phase and 9% S phase. As the cell culture grows, the S phase starts to increase opposite in order to the G0/G1 phase until 40 hrs of cultivation. After that, the G2/M phase

starts to increase inversely to the S phase until 85 hrs. Before the cultivation ends at 109 hrs, the G2/M phase decreases as the cell culture moves into its death phase.

Correlation between the cell cycle phase and transfection efficiency can be observed by combining the data in Figure 28 and Figure 29 as follows: the transfection efficiency is inversely proportional to the increase in the pre-culture age, from 15 hr to 63 hr, coinciding with a decrease in percentage of the cell population in the S phase after a cultivation period of 15-40 hrs to 63 hrs. Although these results reveal a minor dependency of transfection on cell cycle in the range of 15 to 40 hrs of age, conducting transfection after only one day pre-culture age is recommended, consistent with the previously reported results from experiments in media exchange described in Chapter 4.1. This finding needs further assessment and confirmation, and suggests that cell cycle synchronization be linked to the underlying cell cycle phase effect.

4.2.3. Cell density at the transfection time point

We also expect cell density at the time of transfection to be an important parameter in high transfection efficiency. Thus cell density at the time of pDNA addition was varied from 4-18 x10^6 cells/mL. The response of this parameter corresponds to transfection efficiency and viability at 48 hpt, as well as in the statistical analysis illustrated in Figure 30. The amount of pDNA and PEI is constant in relation to cell density.

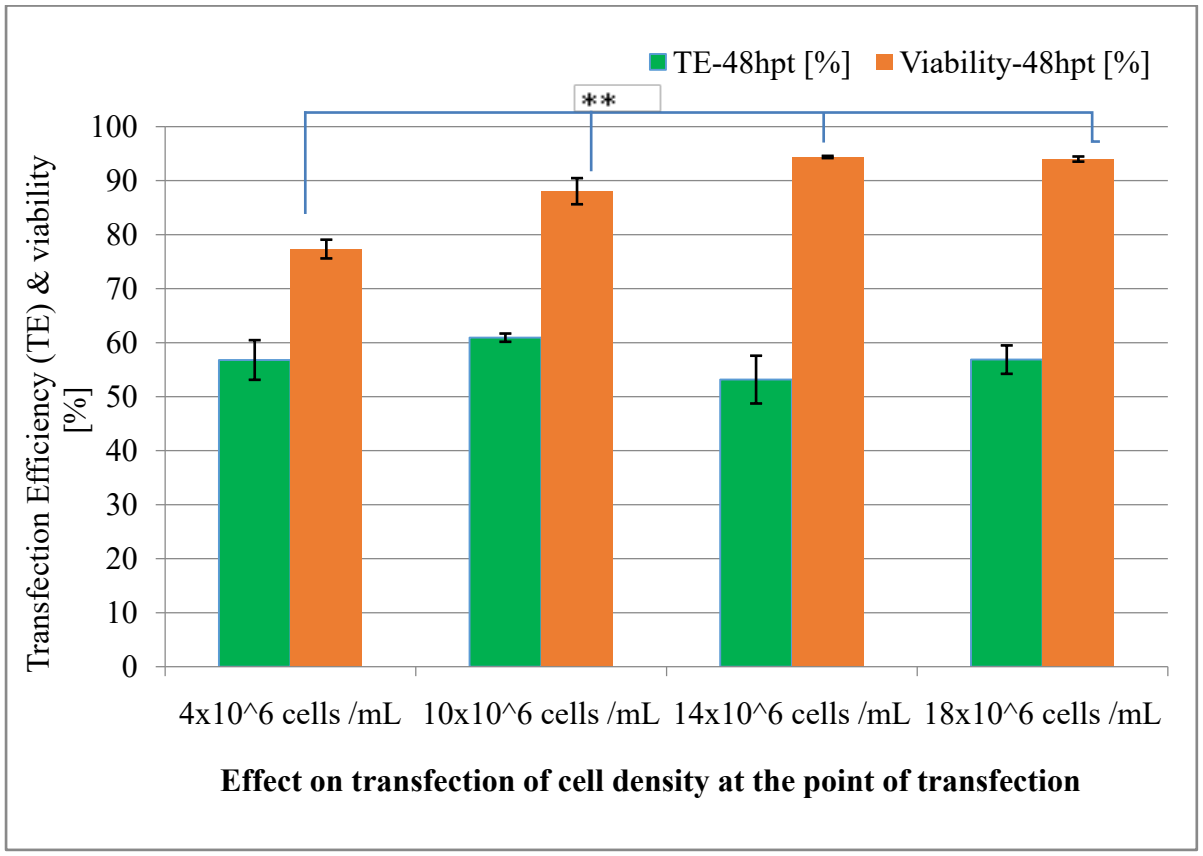

Figure 30: Cell density at transfection point.
Study on the effect of different cell densities at the time of transfection on transfection efficiency and cell viability -48 hpt.

Data in Figure 30 demonstrate that the highest transfection efficiency can be observed when transfection is carried out at a cell density of 10 x10^6 cells/mL, like in the routine transfection protocol. According to the ANOVA test, the difference in transfection efficiency was not significant for any of tested cell densities. These results were surprising because the same amount of pDNA and PEI did not appear to be a problem for transfected cultures with either a low density of 4 million cells or a higher density of 18 million cells per mL.

The cell viability of all cultures was over 80% except at the lowest cell density transfected. This difference was very significantly corresponding to other culture viability variance. Therefore, the participation of 4 x10^6 cells/mL cell density in the routine transfection condition can be excluded. The negative effect of free PEI (unbound PEI particles with pDNA) seems to be

responsible for reducing cell viability. Since the cells present at relatively low cell density at transfection time disperse more singly than those at high cell density, they come in contact with more toxic-free PEI than those at high cell density. Free PEI contributes to 85% of the total PEI in the PEI/DNA polyplexes used for transfection and are responsible for cell membrane destabilization [131]. Reasons for PEI toxicity are not completely clear, while increasing PEI concentrations have a deleterious effect on viability [44].

Although these results suggest that transfection is efficient in the range of 10-18 x10^6 cells/mL, transfection of 10 x10^6 cells/mL was considered to be the best condition due to a transfection efficiency higher than 50% and with viability above 80%, as well as experiment reproducibility with low variation. However, also achieving a high level of transfection efficiency with minimum toxicity is necessary. Transfection efficiency of approximately 60% with viability of 80% is a very promising basis from which to start target recombinant protein production. These findings also suggest that performing transfection with a higher cell density of up to 18 x10^6 cells/mL is the much more feasible approach, as well as being cost-efficient. The cost of production can be further reduced by using the same amount of pDNA and PEI with high cell density so that the amount of pDNA per cell is also reduced. In addition, this approach will extend the range of scale-up transfection culture volume after transfection by more than 1:7 (v/v) as indicated in Figure 24.

4.2.4. Discussion of cell parameters for efficient transfection

The most important parameters for optimal cell transfection are pre-culture cell density and a pre-culture age of one day. The initial cell density affects the initial growth rate and monoclonal antibody productivity within hybridoma cells [132], and the physiological status of a cell line has an impact on PEI transfection [133]. Therefore, the communication state would be better for a high pre-culture density of up to 5.2 x10^6 cells/mL than for a lower cell density of 1.2 x10^6 cells/mL. The maximum cell density for high transfection achieved in literature is 5 x10^6 cells/mL for CHO and 20 x10^6 cells/mL for HEK-293 cells [35,74]. This study reports a maximum cell number of up to 18 x0^6 cells/mL for CHO cell transfection. However, very high cell density of up to 50 x10^6 cells/mL with high viability can be transfected if the culture disperses well as a single suspension cell (data not shown). In contrast to the literature, this study found no effect of cell density during transfection for cell densities between 4-18 x10^6 cells/mL. Transfection of that cell range with an equal amount of pDNA and PEI had the same impact on transfection efficiency over 50%. Conversely, an increase in cell density can be compensated by increasing the PEI:DNA ratio for efficient transfection, because the extent of particle uptake decreases with an increase in cell density [74,84]. A possible explanation for transfection efficiency,

regardless of cell density at that range, is the applied amount of pDNA and PEI. These constitute transfectable polyplex pDNA/PEI particles sufficient for this cell density. This creates an additional important factor called polyplex particle formation [44].

The transfection efficiency increased with younger pre-culture age and the presence of a high cell population in the S cell cycle phase, as indicated for the 15-40-hr age. In agreement with this, PEI transfection would be more efficient when performed with cells in the S phase with a minimal efficiency at the G1 phase, because of the cellular uptake of polyplexes (80–90%) recorded at in the S phase and (5–30%) in the G1 phase [134]. Conversely, the transfection efficiency of linear PEI polyplexes and electroporation is insensitive to the cell cycle [135].

Collectively, all these findings demonstrate that transfection efficiency is limited by pre-culture cell density, age, and the necessity to concentrate the cells in fresh media. Cell density at transfection time provides a deeper understanding of the PEI transfection efficiency. Nowadays many conditions affecting efficiency are being identified, in addition to the already-known conditions of the routine transfection protocol. These include 1) for efficient transfection, a pre-culture cell density in the range of 2.6-5.3 $x10^6$ cells/mL is required; a further increase in cell density was not tested here; 2) a one-day pre-culture age and concentration of the cells in fresh media are a crucial step for transfection; 3) A cell density of 10 $x10^6$ cells/mL at transfection time is optimal for efficient transfection, and a higher cell density of up to 18 $x10^6$ cells/mL is possible. The reasons why the transfection efficiency achieved in the routine protocol still does not exceed 60-70% remain unclear. This study therefore sought further optimization of the protocol by applying a design of experiment DoE approach, to explore the interaction between the three basic factors (cell, pDNA, and PEI amount) in the transfection process in an effort to improve on the previously achieved transfection efficiency percentage.

Variability in transfection efficiency (TE%) was often observed in independent experiments, probably due to the difference in the percentage of the S phase cell population and passages effect which were not tested.

4.3. Design of experiment for optimizing transfection performance

This optimization study investigates the relationship between pDNA, PEI and cell density, and their single or combined effects on transfection efficiency. This optimization step is an essential prerequisite before proceeding to the target of optimizing TGF-β1 recombinant protein expression. A full factorial design of experiment (DoE) approach was applied and investigates systematically the impact of these parameters on transfection efficiency. In doing so, a central composite circumscribed (CCC) design was used, in which the cell density factor was fixed and tested against a wide range of the other parameters in order to improve the efficiency of transfection in the routine transfection protocol.

The study used a nano-tracking analysis (NTA) method to explore the role of particle size pDNA/PEI polyplex formation dynamics in the transfection process. These polyplexes result from the electrostatic interaction of negatively charged pDNA and positively charged PEI. This NTA analysis aims to combine the advantages of these dynamics with transfection efficiency.

The study implements another recent strategy, namely repeated transient transfection which uses a lower amount of pDNA and PEI. This strategy aims to improve transfection efficiency and extend the post-transfection period of transient gene expression.

Finally, the feasibility of scaling-up the transfection volume in the routine transfection protocol from 1 mL (as always applied) to 12.5 mL either in a Tubespin bioreactor®50 and shake flasks were evaluated. This step will be useful for further TGF-β1 protein production.

4.3.1. Interaction of main factors influencing transfection efficiency

In the first DoE setting dealing with viable cell density, the amount of pDNA and PEI was studied, these ranging between 7–13 x10^6 cells/mL, 10–26 µg and 40–60 µg, respectively. The full factorial design was adjusted at two levels for each factor, and has four center points in the middle of the design space to assess the significance of each effect, as shown in Appendix Figure 48. The data for 10 x10^6 cells/mL (the cell density of interest in routine transfection protocol) is presented in a contour plot as shown in Figure 31. This contour represents effect of the main factors on transfection efficiency. In these experiments, pDNA, PEI and cell density were considered as the main factors affecting transfection efficiency.

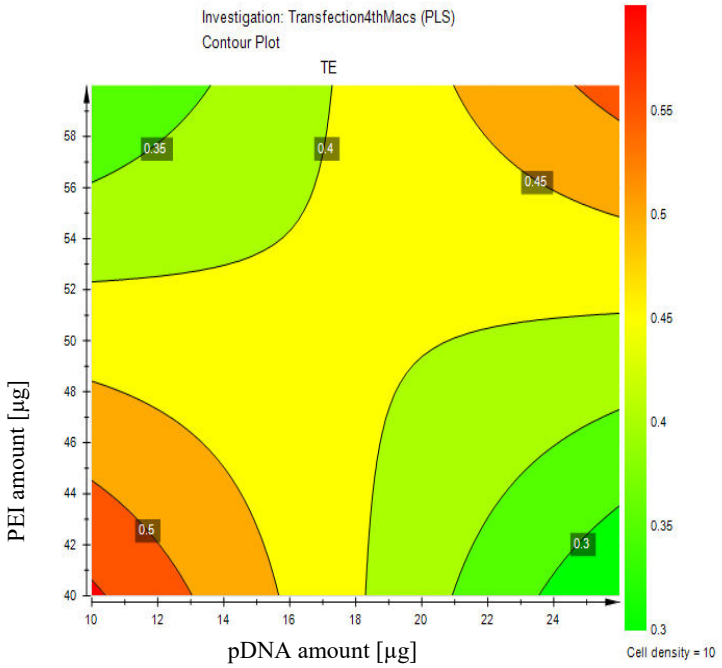

Figure 31: Contour plots of TE with pDNA and PEI amount.
Contour plots of transfection efficiency against amounts of PEI and pDNA by µg at 1 mL transfection volume. Cell density at transfection time was around 10 x10^6 cells/mL. Transfection efficiency (TE) was determined at 48 hpt and represented by a number.

Figure 31 shows that high transfection efficiency is quite closely linked to a low amount of pDNA at 10 µg and PEI at 40 µg, as well as to a high amount of 26 µg and 60 µg for pDNA and PEI, respectively. An unexpected result was that the correlation between pDNA, amount of PEI, and transfection efficiency was hard to establish through this study.

An explanation for this might be that the polyplex of pDNA/PEI particles were generated from a different combination of pDNA and PEI amount. Although these complexes originated either from interactions between the high and low amounts of pDNA-PEI, they could somehow have the same biophysical character, which would enable them to exert a similar influence on transfection efficiency, ~50%.

The ployplex biophysical character involves surface charge on the one hand. Particle size, on the other hand, is responsible for the formation of transfectable polyplexes. These polyplexes interact with the cells electrostatically and non-specifically. Thus, transfection efficiency can be strongly influenced by the polyplex particle size formation and concentration. With reference to the optimization of transfection efficiency, these results suggest that evaluating the other experimental spaces in the upper right-hand corner and lower left-hand corner of contour Figure 31 might provide more scope for optimal transfection efficiency. In general, since no direct relation between pDNA, amount of PEI and transfection efficiency was found here, we performed further investigation for an optimal set of conditions.

4.3.2. pDNA/PEI polyplex mediates a wide variety of transfection conditions

As the above contour plots suggest, two transfection conditions called region A and B were evaluated as indicated in Figure 32. The experiment conditions with a new range of PEI and pDNA amounts were determined using a central composite circumstance design (CCC) as described in Appendix Table 9 and 10. Afterwards, using the same DoE approach as described in Table 11, area C of Figure 32 was investigated. All experiments were carried out with a fixed amount of cells (10 x10^6 cells/mL) in 1 mL transfection volume diluted with fresh media at 1:1 (v/v) and 5 hpt.

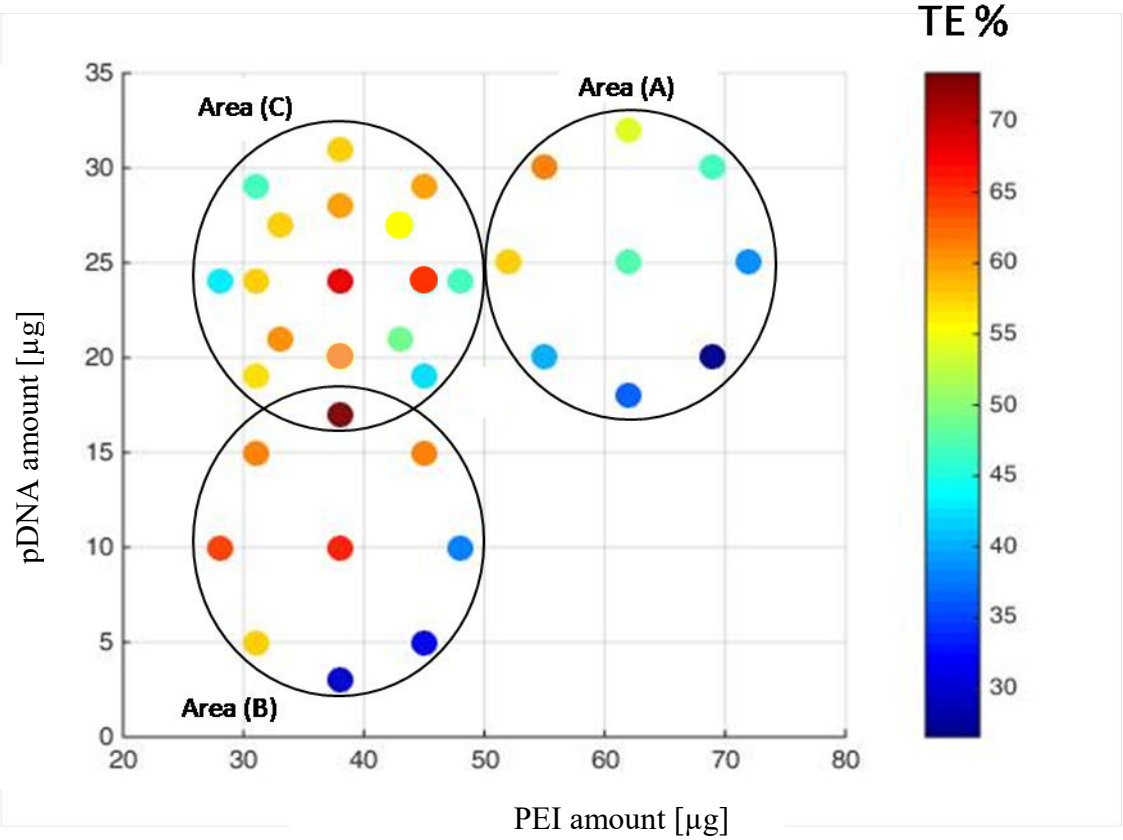

Figure 32: Identificatin of optimal transfection area by DoE method.
Different amounts of pDNA interacting with PEI to affect transfection efficiency. Design spaces area A and B represent the interaction of large and small amounts of pDNA and PEI respectively and their effect on transfection efficiency (TE%). Area C represents the optimal region. The middle point was represented by the average of three experiments to validate the DoE design.

In Figure 32, area A and B reveal that a transfection efficiency (TE) of over 50% was obtained when a higher amount of pDNA interacted with a lower amount of PEI. In the same context, a transfection efficiency of less than 50% was also exhibited in both areas (A&B) of the design space when influenced by a large amount of PEI with a small amount of DNA. Low transfection efficiency could be related to a low amount of pDNA, when this amount was too small to transfect 10 million cells per mL as in the case of area B, or related to un-transfectable pDNA/PEI polyplexes in the case of area A.

To investigate how a pDNA:PEI ratio (amount of PEI/amount of pDNA) is related to transfection efficiency, we visualize the relationship in Figure 33 .

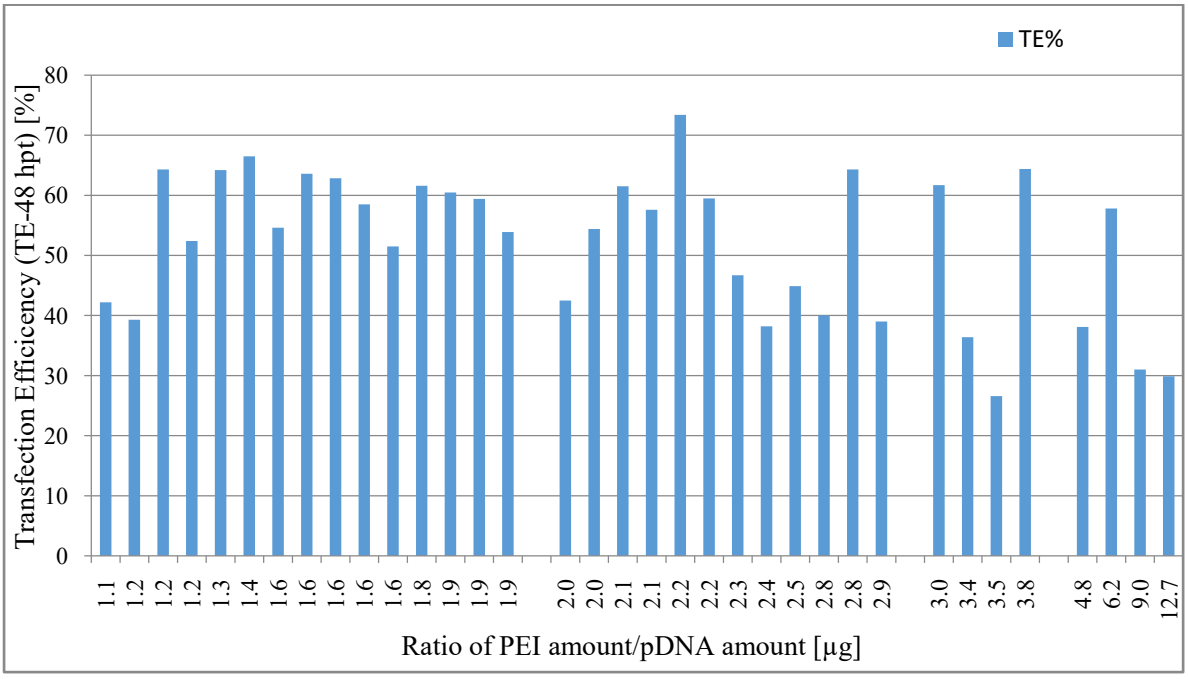

Figure 33: Polyplex profile between pDNA:PEI ratio evaluated by GFP-TE.
The data represented here contains the same information as Figure 32, twisted by the ratio (w/w) pDNA:PEI.

The threshold of 50% was chosen as the limit of acceptable transfection efficiency (TE%) for recombinant protein production. The data in Figure 33 corresponds to that threshold and is related to a pDNA:PEI (w/w) a ratio between 1:1.3-1.9. These ratios are interrelated with the amounts of pDNA and PEI, i.e. 19-32 µg and 31-62 µg respectively.

Based on these results and previous findings, this study concludes that the size of polyplex pDNA/PEI particle formation needs to be considered in a future study. The variance in transfection efficiency can be correlated to the diversity in particle size formation resulting from the interplay between different amounts of pDNA and PEI.

Regarding cell viability, these results indicate that transfection efficiency is over 50% with a high percent of viability in area B but not in area A (data not shown). Because of this positive aspect combined with the use of less pDNA during transfection compared to the area A, it was decided to conduct subsequent experiments in area C Figure 32 with the expectation of identifying a means of achieving perhaps even 70% transfection efficiency.

Unexpectedly, the results clearly show there are no large increases in transfection efficiency. The data of area C helps to close the gap between the amount of pDNA-PEI interaction with cells and high transfection efficiency. This area provides several choices for acceptable transfection efficiency from 50%-67% with viability over 80% and disappearance of low transfection condition >38%.

These variations in acceptable transfection efficiency originate from the differences in amounts of PEI and pDNA interacting with the same amounts of cell. a various interaction amount of PEI and pDNA with the same amount of cells. This offers the possibility of selecting any combination for further recombinant TGF-β1 protein production later on, since the PEI polymer has a property which allows optimal transfection conditions governed by different amounts of pDNA and PEI in the same media. This could explain why PEI exerts such a different influence, depending on the type of media, as stated in Chapter 4.1. Bearing this aspect in mind, no absolute value can be used to achieve optimal transfection conditions mediated by PEI regardless of the cell density and media type.

This DoE study suggested that a threshold exists for a transfection efficiency of between 50-73%. The power to increase transfection efficiency over 73% seems to be limited to the CHOMACS CD medium.

Further improvements to increase efficiency without deterioration and loss of viability could rely on a better understanding of the influence of the polyplex particle size parameter on plasmid uptake. Therefore another study based on a nano-particle tracking analysis (NTA) for monitoring the particle size of pDNA/PEI polyplex formation was performed, in an attempt to understand the mechanism behind how polyplex formation relates to transfection efficiency.

4.3.3. Transfection efficiency reproducibility and nano-tracking analysis (NTA)

Cells at 48 hpt from two transfection conditions were investigated in a flow cytometer to determine transfection efficiency and determine the reproducibility of a routine transfection assay as shown in Figure 34. These two transfection conditions were chosen because one was based on the DoE result at a ratio 1:1.9 with an expectation of high transfection efficiency and the other one at 1:1 pDNA:PEI (w/w) with the expectation of low transfection efficiency. A tracking particle formation analysis was then performed for both conditions to find the difference in dynamic behavior between low and high TE as indicated Figure 35.

To achieve this, a fixed amount of pDNA was allowed to interact with different amounts of PEI to track the particle size of pDNA/PEI polyplex formation. Once PEI was added to the pDNA, this polyplex particle formation began and was monitored at different time intervals up to 80 min, as shown Figure 35. As expected and previously mentioned in Chapter 4.1, most of the transfection particle entered the cells within 60 min. Based on this, an 80-min tracking time was thought to be enough to monitor the major differences in the dynamics of polyplex particle size formation.

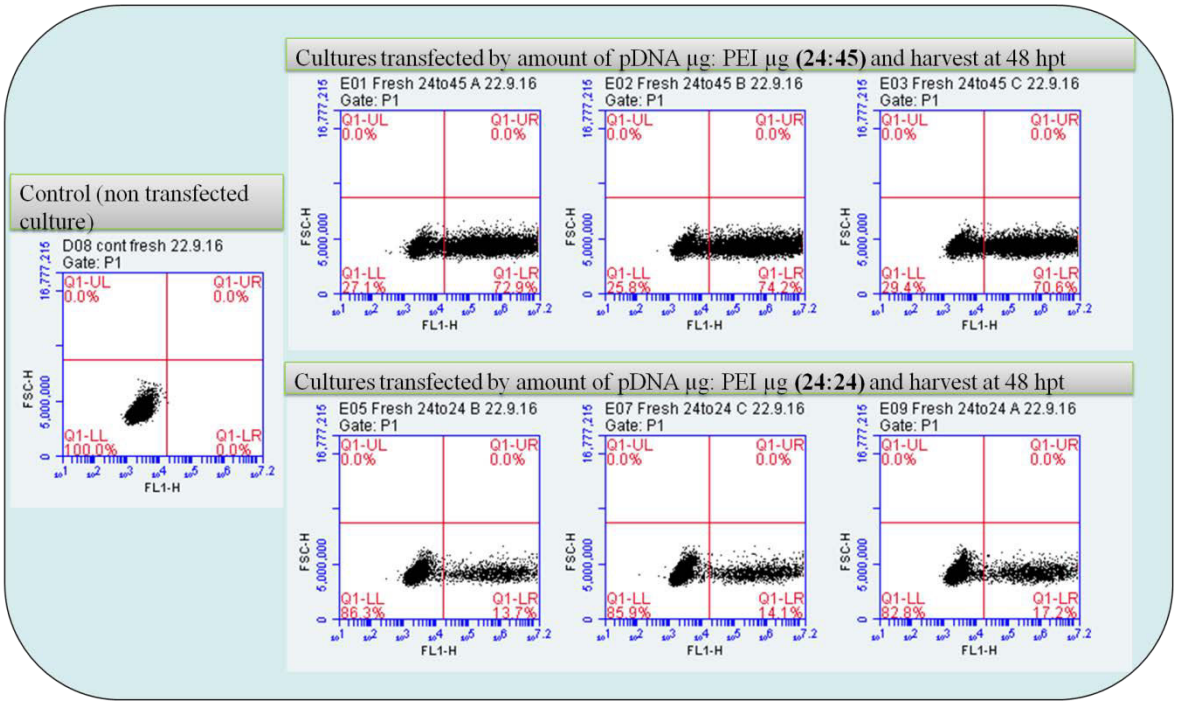

Figure 34: Reproducibility of transfection results obtained with a flow cytometer.
Ten million CHO-K1 cells per mL were transfected with pEGFP-N1 using the amount of pDNA and PEI as indicated in the graph. Each condition was tested three times. A representative dot plot with the flow cytometer (Accuri 6 model) of normal CHO-K1 cells and GFP-transfected cells is shown.

As shown in Figure 34 (upper plot), there was an increase in the number of cells that express green fluorescence protein (GFP) compared to those in the lower plot. The only difference in GFP expression must be regarded as a difference in the amount of PEI between both transfection conditions.

The combination of a high amount of PEI at about 45 µg and the amount of pDNA 24 µg yielded a higher transfection efficiency of about 72% (upper plot). This is in comparison to 14% from the combination of a low amount of PEI 24 µg (lower plot) with the same amount of pDNA. The flow cytometric analysis was done referring to the non-transfected culture.

On the one hand, this indicates that the amount of PEI has a reliable effect on transfection. On the other hand, it indirectly indicates that both amounts of applied PEI are able to condense the pDNA molecule to transfectable pDNA/PEI polyplex particles, which in turn influenced transfection efficiency. Put another way, a higher ratio of pDNA:PEI 1:1.9 (w/w) would condense pDNA better than a lower ratio 1:1 (w/w). This strongly suggests that a high ratio could induce a higher concentration of transfectable PEI/pDNA polyplex particles that a low ratio. An analysis of the dynamics of particle size formation cannot not prove this, however, as long as identification of these transfectable polyplex particles are still unknown.

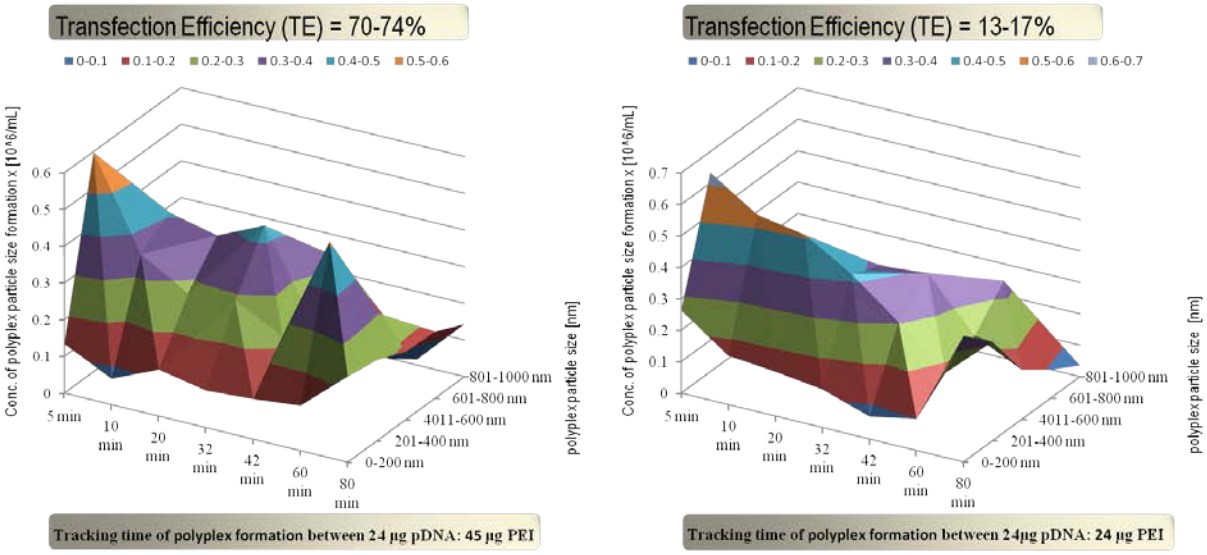

Figure 35: The polyplex particle concentration by selectable size class.
The left-hand side shows the dynamics of particle size concentration by time at a high ratio of pDNA:PEI (w/w). The right-hand side shows these dynamics under a low ratio (w/w).

For correlating dynamical polyplex particle size with GFP transfection efficiency, the graphs in Figure 35 demonstrate the concentration of pDNA/PEI polyplex particle size formation by time. The interaction of the same pDNA amount with different amounts of PEI produced polyplex aggregates of variable sizes >1000 nm within 80 min. On the one hand, the distribution of particle size concentration for both conditions (PEI=24 and PEI=45 µg) is nearly the same. On the other, the formation dynamics of this particle size relating to the concentration appears slightly different. In both conditions, the interaction starts by forming all types of polyplex particle sizes >1000 nm, but with high PEI, polyplex tends to form smaller-sized particles at

high concentrations compared to low PEI. This aspect was investigated in NTA and with a scanning electron microscope (SEM) images.

This data exhibits a homogeniety in particle size formation order and dissimilarity in the dynamics of this particle formation during tracking over 80 min between both conditions. The fluctuation in the dynamics of particle formation coincides with size and concentration. This fluctuation was higher up and downlinked with a high amount of PEI in the transfection condition, as well as high transfection efficiency − and vice versa. This inconsistency with regard to particle size formation and concentration results from electrostatic interaction (binding and dissociation of polyplex dynamics) and could indicate high transfection efficiency.

Particle shape is also an interesting parameter that could provide some reason for investigating properties of highly transfectable polyplex. NTA analysis gives information about the shape of these particles resulting from light scattering, as shown in Figure 36. Regarding the formation of polyplex, both previously mentioned conditions show similarity with a spherical structure which started smaller after 5 min of interaction then underwent dynamic changes with time, ending with spherical shapes either singularized or aggregated.

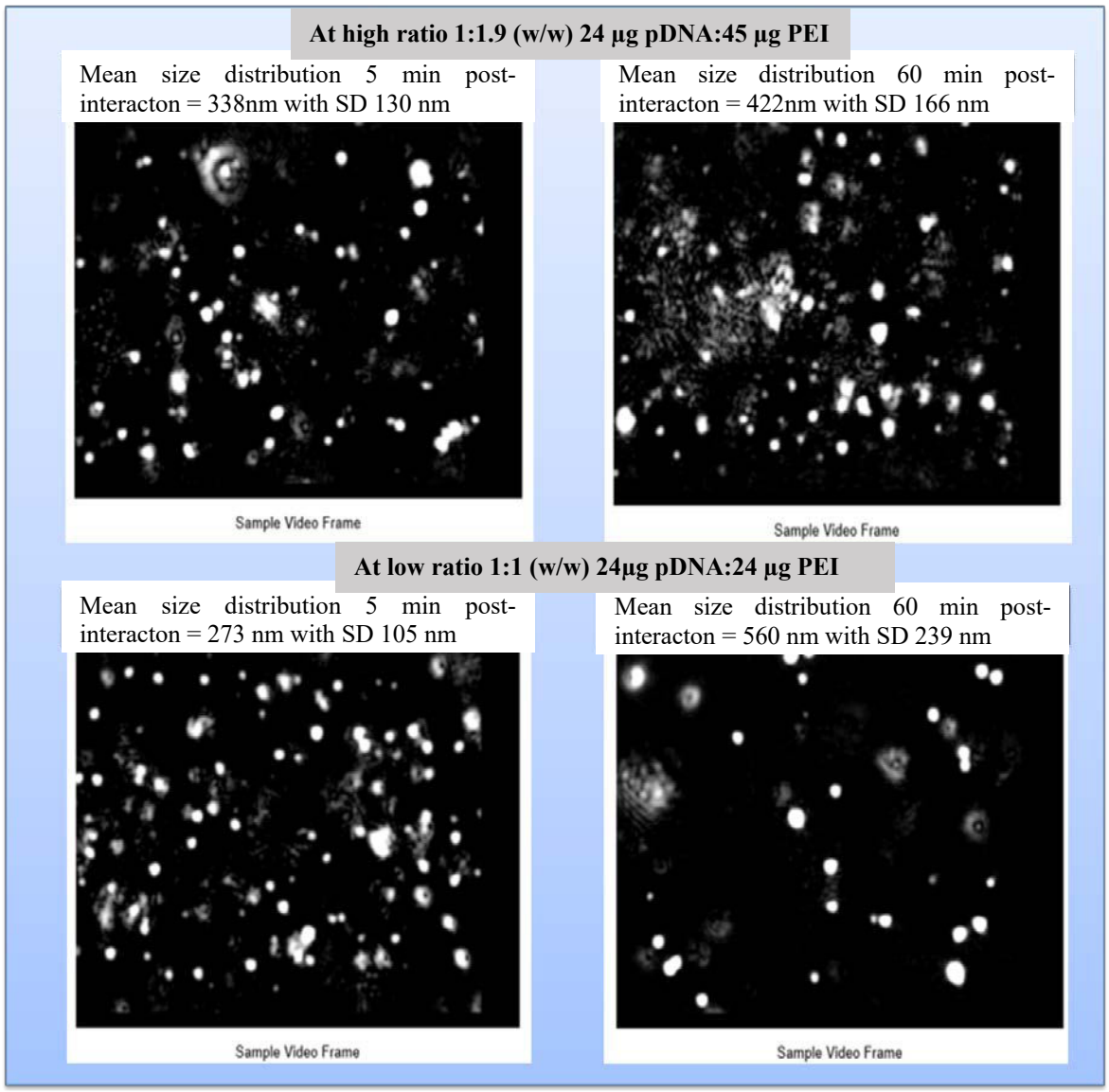

Figure 36: Nano-particles pDNA/PEI polyplex visualized by their light scattering.
The images were obtained from video images taken by a microscope.

The only difference that can be slightly noticed at 60 min post-interaction is that the mean particle size of these polyplex spheroids was about 422 ± 166 nm in the high transfection efficiency condition, while it was 560 ± 239 nm for the low transfection condition. Thus transfection condition characterized by smaller-sized spheroid polyplex formation would positively influence transfection efficiency. The same difference was confirmed in an independent experiment by SEM.

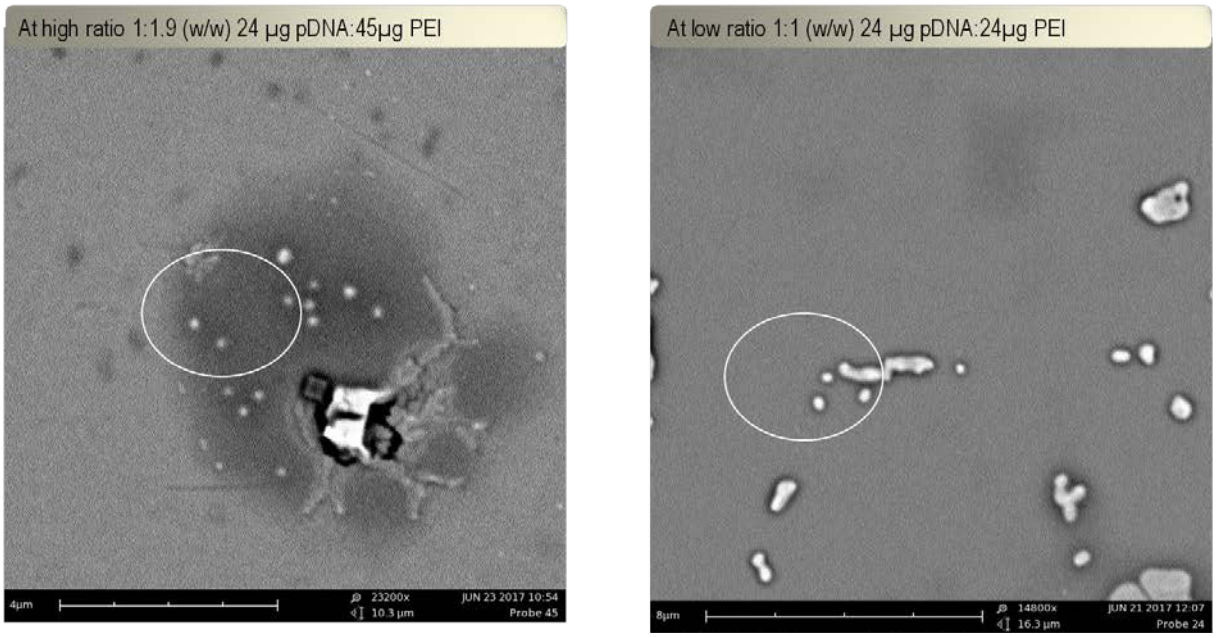

Figure 37: Scanning electron photograph of pDNA/PEI polyplexes.
These polyplexes result from the interaction between different amounts of pDNA and PEI as indicated in above the image. On the left side are scanning electron micrographs of pDNA:PEI (w/w) with a high ratio scale bar, 4µm. On the right side are scanning electron micrographs of pDNA:PEI (w/w) at a low ratio scale bar, 8µm.

Micrographs of SEM Figure 37 reveal that an increase in the amount of PEI to the pDNA (related to high transfection efficiency) is characterized by smaller single spherical polyplexes of between 170 and 230 nm. In the case of an equal amount of pDNA and PEI, most single particle sizes are in the range of 260-300 nm. Larger polyplexes were formed due to re-aggregation of single spheroids within the PEI polymer, creating a branched spherical shape. While still unclear whether the presence of polyplex in dispersed single form or aggregated in chain form would be more effective for transfection, the spherical morphology of polyplex formation seems to have no effect on transfection efficiency. The spherical polyplex was found in high and low transfection efficiency.

4.3.4. Repeated transient transfection

In order to overcome the transfection efficiency limitation above 73% and prolong the production period afterwards, a repeated transient transfection protocol was implemented by media exchange and the low amount of pDNA was used only one time (round). The first (1^{st}) transfection step was performed under the routine transfection protocol. The transfection efficiency at 48 hpt_1 was determined. The cells were passaged like a pre-culture and incubated for 24 hrs for the second (2^{nd}) transfection. The second transfection was performed like the first but with a smaller amount of pDNA and PEI. Using a smaller amount of pDNA and PEI avoids more toxicity. The experiment scheme is explained in Figure 38.

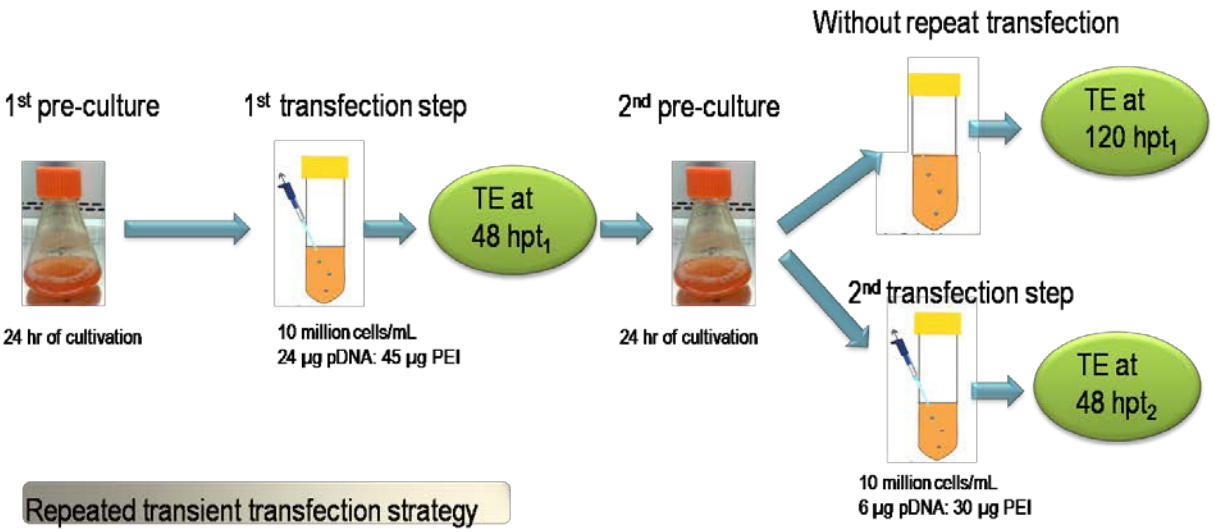

Figure 38: Schematic representation of the repeat transient transfection strategy.
The diagram shows the first (1st) and second (2nd) transfection steps.

The effect of the repeated transfection strategy on transfection efficiency was investigated as shown in Figure 39.

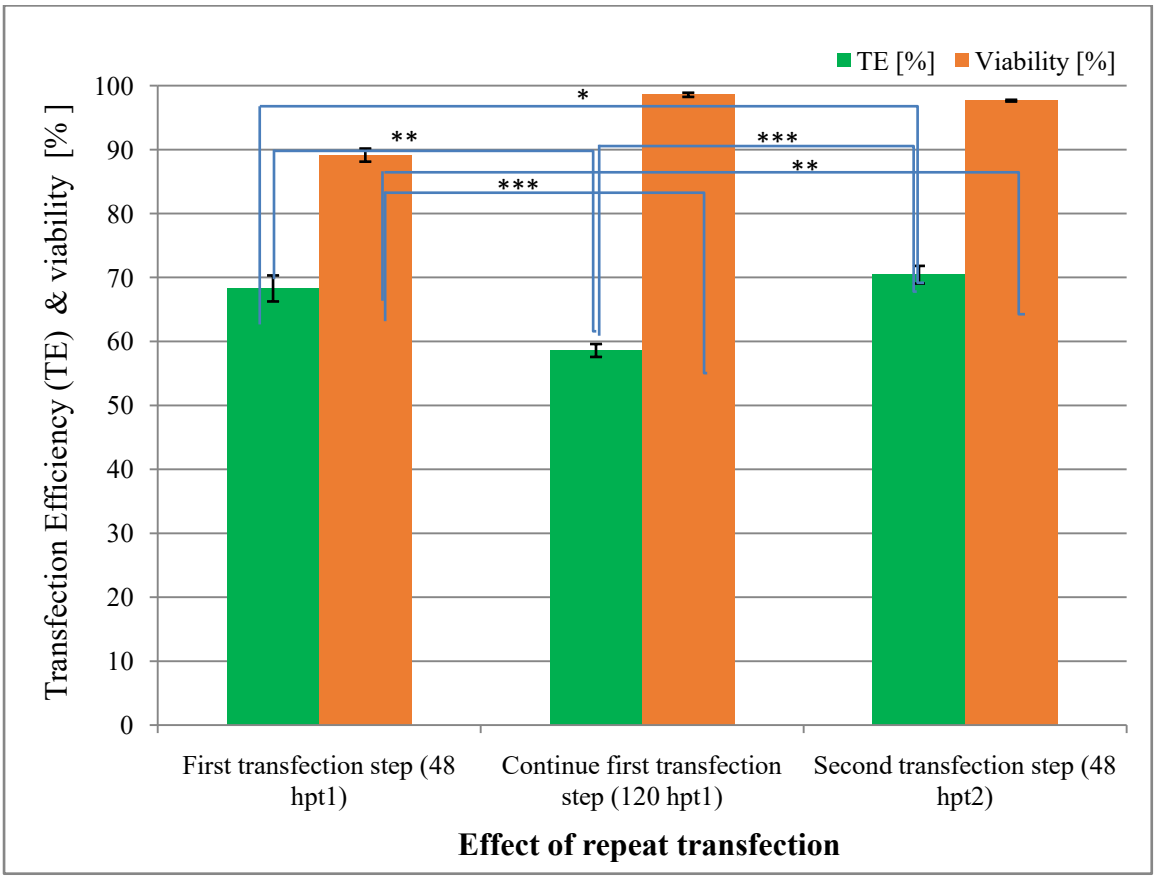

Figure 39: Repeated transient transfection strategy.
Effects of second transfection step on transfection efficiency and viability. Error bars represent the standard deviation from the mean (N=4). Data were analyzed using T-test, and *p<0.05, ** P<0.01 *** P<0.001 was considered significant, very significant and highly significant, respectively.

In comparing the first transfection step and the second, repeated transfection step, transfection efficiency at the 48-hour post-transfection point in step one (hpt_1) and the further 48-hour post-

transfection point in step two (hpt_2) seems to be a similar at about 68% and 70% respectively as shown in Figure 39. However, this result contradicts the intention of the second transfection step, which was to increase transfection efficiency up to 100%. As expected, the amount of pDNA and PEI used produces 38-50% transfection efficiency as tested in a previous procedure (B) Chapter 4.1. However, this transfection level does not occur with repeated transfection. This could be associated with a ceiling limiting improved transfection above 73%. But the second transfection step improves transfection by about 12% compared to the first transfection after 120 hpt_1.This difference is quite significant and keeps the transfection efficiency high by using a smaller amount of pDNA and PEI.

Importantly, these results demonstrate that the transfection efficiency of the first transfection step is not lost completely after 120 hpt_1. With a further increase in cultivation to 144 hpt_1, transfection efficiency starts to decrease for both conditions (data not shown) reflecting the need to apply another repeat transfection round.

On the other hand, the second transfection step was accompanied by a significant increase in viability at 48 hpt_2 compared to the first transfection step, although the initial pre-culture viability at transfection time was 95% and 99%, respectively. This is probably due to the toxicity of the higher amount of pDNA and PEI which applied in the first transfection step compared to that used in the second transfection step.

The possible reasons for increases in viability are the time intervals of about 24 hr in the pre-second transfection culture, which was established between the first transfection 48 hpt cultures and the start of second transfection step. The time interval for the culture in fresh media allows viability to increase from 89% to 95%. The second reason are smaller amounts of pDNA and PEI of about 6 and 30 µg, respectively, that were applied during the second transfection step, and which differed from the amounts in first transfection step.

The repeat transfection strategy requires a smaller amount of pDNA and PEI for a high transfection efficiency (70%) combined with high viability (97%) at 48 hpt_2; this allows better performance in cell growth and protein expression. These features make this repeat transfection approach especially attractive for extended protein expression and more suitable for continuous transfection culture. The intermediate pre-culture between the first and second transfection step helps to enhance cell growth and viability from 89% to 95%. Further work is required on the feasibility of performing repeat transfection strategy through direct addition of pDNA and PEI without the need for cell centrifugation and pre-culture preparation step prior transfection.

4.3.5. Scale-up of transient transfection

The scalability of transient transfection was investigated using the Tubespin bioreactor®50 and shake flasks. A CHOMACS CD media volume of 12.5 mL was suspended with 10 x10^6 cells/mL and transfected with 24 µg of pDNA/mL and 45 µg of PEI/mL. At 5 hpt the cultures were diluted with an equivalent volume of media. With a 1-mL transfection volume in the Tubespin bioreactor®50, another fresh media at 48 hpt was added to allow measurement at 72 hpt.

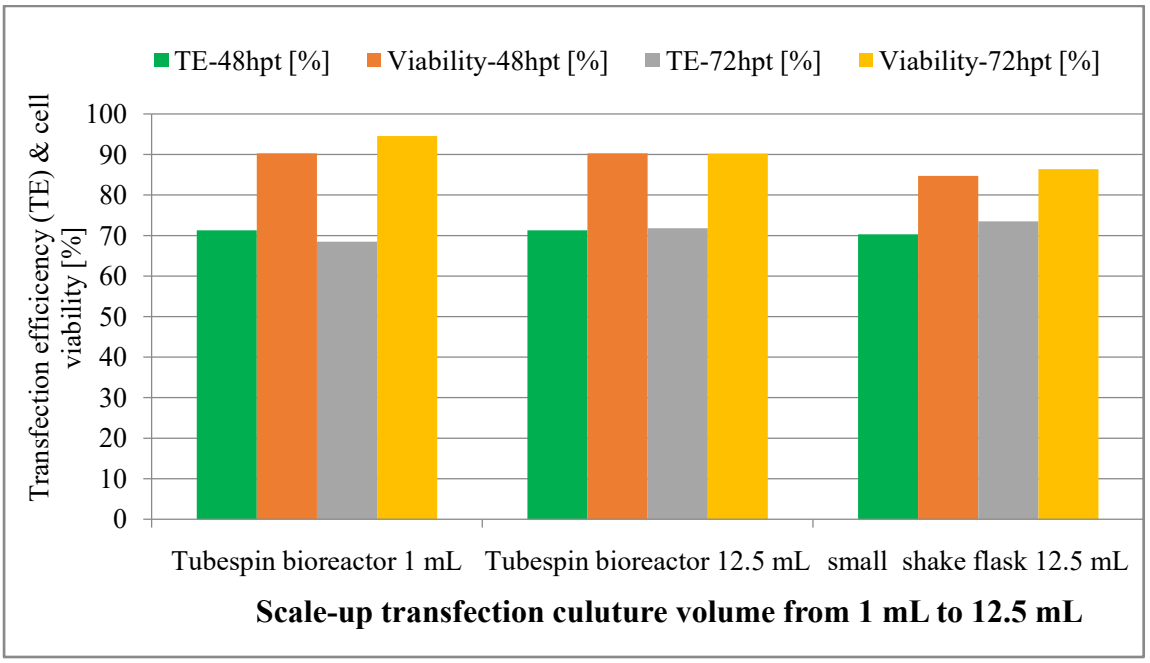

Figure 40: Transient transfection scale-up in shake flasks and Tubespin bioreactor®50.
Comparison of transient transfection in a Tubespin bioreactor®50 and shake flasks with 12.5 mL of transfected CHO-K1. Transfection efficiency and viability were measured at 48 and 72 hpt. A transfection volume of 1 mL was used here as a reference.

As shown in Figure 40, the transfection efficiency for both cultures at 48 hpt was about 70%, similar to that obtained with a volume of 1 mL transfected culture using the routine transfection protocol. The reason that viability increased in 1 mL in the Tubespin bioreactor®50 at 72 hpt is related to the addition of fresh media. Generally, this experiment successfully evaluated the transient transfection scale-up protocol to 12.5 mL in different culture vessels, i.e, the Tubespin bioreactor®50 versus to shake flasks. This step is quite necessary for TGF-β1 production and will facilitate the production of recombinant protein in shake flasks.

4.3.6. Discussion on the DoE to optimize transfection performance

In the first DoE, the surprising finding was that the highest transfection efficiency was achieved by combining the highest amount of pDNA with the highest amount of PEI and vice versa. These data suggest that the amount of PEI and pDNA are more important factors than cell density, which is consistent with previously findings concerning the tested range of 4-18 million cell density per mL discussed in Chapter 4.2. Contrary to our observation, Xie et al [44] report that higher cell densities and higher amounts of PEI and pDNA are important for *in situ* transfection. Higher concentrations of pDNA and cells with lower ratios of PEI:pDNA have been shown to have the absolute highest transfection efficiency [136].

Each media such as Ex-Cell medium or RPMI F68 has only one optimal transfection efficiency point achieved by increasing cell density, PEI and pDNA concentrations [137]. But our design spaces, called areas A, B, and C, assign more-than acceptable transfection efficiency over 50% to CHOMACS CD. There is indeed an advantage to using this as the high transfection efficiency is also accompanied by increased viability and less pDNA is required to obtain optimal transfection efficiency.

One major concern with the pDNA:PEI (w/w) ratio as related to transfection efficiency is the independently on concentrations. However, most with transfection efficiencies over 50% have ratios of around 1:1.3-1.9 (w/w), which are related to the combination of the pDNA and PEI amounts of 19-32 µg and 31-62 µg, respectively, in 1 mL transfection volume. Thus the precise ratio actually depends on the DNA concentration [129].

Interestingly, transfecting cells using a wide range of pDNA:PEI ratios (w/w) − 1:1.6, 1:1.8, 1:2.1 and 1:3 − did not significantly affect the expressing cell population of 61%. In agreement with this study, other one reported that the highest transfection efficiency of 72% was the same for the DNA:PEI ratio of 1:5 and 1:7 [44]. However, the diversity of PEI transfectability properties is discussed in greater detail in the literature. The property which enables PEI polymer to display these numerous optimal features originates from the different magnitudes of pDNA and PEI ratios (w/w) of *in situ* interactions, which could explain the diversity of optimal PEI transfection conditions among all reported studies. This property could depend on polyplex particle size formation between opposite charged pDNA and PEI electrostatic interaction.

The dissimilarity in the dynamics of these particle formations during tracking over 80 min results from the interaction between equal amount of pDNA and different amounts of PEI, which is related to a dissimilarity in transfection efficiency of both conditions. Therefore, polyplex particles were generated in low ratios, transfecting only 14 ± 2% of the cells and 72 ± 1.8% in a high ratio.

In addition, NTA and SEM analysis revealed spherically shaped polyplexes with sizes smaller than 422 nm and 230 nm, respectively, at 60 min post-polyplex interaction, which would be recommended for PEI mediated transfection.

Zetasizer analysis studies over 60 min have illustrated that 277 to 950 nm small particle size formation seems to be responsible for a high transfection efficiency of 72% [44]. Recently, a study reported that generating positively charged polyplexes with a size range of <100 nm is optimal for PEI-mediated transfection [36].

However, this study highlights an important aspect of the dynamics of small-sized spherical-shaped polyplex particle formation, which is may be combined with high transfection efficiency. A further polyplex dynamic analysis study over all previously mentioned DoE experiments is required to prove this concept.

Transfection efficiency level changes over time post-transfection. After 48 hpt, the level peaks at 72 hpt; at 96 hpt, the efficiency returns to a value equal to that recorded at 48 hpt with no significant difference as shown in Chapter 4.1. This could be explained by the fact that transfected culture consists of 60% transfected cells (GFP+ cells) and 40% non-transfected cells (GFP- cells). By the time post-transfection is running, this GFP-cell population will have grown faster than the GFP+ cells. Moreover, GFP+ cells lose their episomal pDNA through cell division [138,139]. Since transfection efficiency depends on the ratio between transfected and non-transfected cells, this tends to decrease with prolonged post-transfection time.

Recently repeated transfections had been introduced by Cervera [86] to tackle the problem of losing episomal pDNA in the short period after transient transfection of HEK 293 suspension cells. They were able to keep viability of HEK-293 cells over 50% and to extend protein expression between 192 and 240 hr by several transfection rounds.

Here the proposed repeat transfection strategy for only one round in CHO-K1 cells was able to increase viability from 89 to 95% and transfection efficiency to 70% in comparison to 98% viability and 58.6% transfection efficiency in the first transfection step at 120 hpt_1. A pre-culture 24-hr time interval before conducting the second transfection step enables the culture to recover viability again.

Interestingly, this is the first time in this study that a high transfection efficiency of 70% with a high viability of 97% was obtained; in contrast, higher transfection efficiency has been shown in the past to be associated with a decrease in cell viability [133].

Scalability of the routine transient transfection was achieved successfully up to 12.5 mL transfection volume and revealed a similarly high transfection efficiency of about 70% with a pDNA:PEI amount of 24 µg:45 µg, consistent with previous small-scale 1 mL transfection

volumes. This finding is in agreement with the reported reproducible and scalable method with 300 mL reported by Shen [140]. Nevertheless, the scalability of our high transfection efficiency protocol is restricted by the relatively high pDNA requirement.

Altogether, this work shows no further improvement with regard to the level of transfection efficiency of over 73% that can be achieved for the routine transfection protocol. The same transfection efficiency level can be obtained with other combinations of pDNA and PEI amounts. This reflects the significance of particle size formation between the pDNA and PEI interaction. This factor should be the key element in any transfection process. Moreover, choosing a transfection condition to optimize recombinant protein production is another important factor that needs to be considered later on.

4.4. Transient TGF-ß1 recombinant protein expression

This study has already described a simple transient transfection procedure in CHOMACS CD medium; the pEGFP-N1 plasmid was utilized to evaluate the transfection efficiency. In this chapter, the TGF-β1 plasmid is used instead of the pEGFP-N1 plasmid. The objective of the experiments and analysis described in this chapter is to express TGF-ß1 in culture supernatant for further purification with Twin-Strep-tag® technology. To do so, the purified protein was determined and detected using Nanodrop, Western blot assay, and 2D-fluorescence spectroscopy. This was followed by the specific quantification of the active TGF-ß1 by enzyme-linked immunosorbent assay (ELISA) in order to determine the bioactivity of the purified TGF-ß1 in comparison to the commercial one. This was done through a lung A549 cell growth inhibition assay.

4.4.1. Transient production of TGF- β1 in shake flasks

Based on the efficiency achieved with GFP-transfection mentioned above in the DoE experiments, the transfection conditions that achieved the highest transfection efficiency (of about 73%) were used here for TGF-β1 production. The pEGFP-N1 plasmid was replaced by TGF-β1 plasmid for this purpose. Although there is a difference in size between pEGFP-N1 plasmid (4.7 kb) shown in Figure 12 and TGF-β1 plasmid (10.7 kb) shown in Figure 15, the delivery limitation due to plasmid size was not considered as a discrete factor in this study.

For transfection, the contents of three shake flasks each with 12.5 mL of CHOMACS CD media were mixed with 12.5 x10 million cells based on the above-mentioned scale-up protocol as described in Chapter 4.3. TGF-β1 proteins were successfully produced using 17 µg TGF-β1 plasmid and 38 µg PEI per mL.

At 72 hpt all cultures were harvested for cell counting, protein purification, and further analysis as shown in Appendix Figure 46. This harvest time was determined based on a previous GFP transfection study which focussed on finding the best transfection efficiency time. This is in agreement with that reported by Cervera [125]. Further study to determine the optimal time for harvest is required.

The viability post-transfection was decreased due to PEI toxicity. In general, a transfected cell culture with a viability of above 80% is a good indication of the success of the transfection process, similar to what occurred with the pEGFP-N1 plasmid.

The TGF-ß1 purified protein from culture supernatant was detected by applying the reducing conditions of the SDS-page protocol, followed by a Western blot of that protein with a TGF-β1 specific antibody. The result is shown in Figure 41. Commercial TGF-β1 served as a positive control.

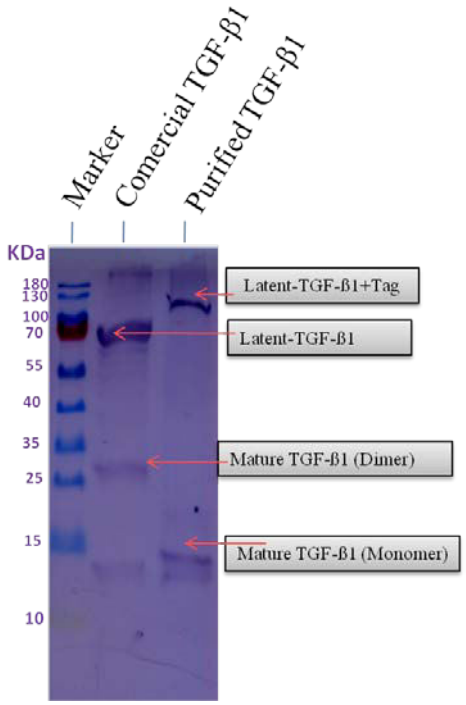

Figure 41: Evaluation of purified TGF-β1 protein detection by Western blotting.
Lane 1: shows a protein-size marker. Lane 2: shows commercial TGF-β1. Lane 3: shows the purified TGF-β1 from transfected culture supernatant.

Western blot analysis of the commercial TGF-β1 shown in Lane 2 of Figure 41 shows that the upper band is about 75 kDa, which can be assigned to the latent form of TGF-β1. The other bands of about 25 kDa and 14 kDa were assigned to the active (mature) TGF-β1-dimer and monomer form, respectively. Regarding TGF-β1 purified from culture supernatant, the upper band is also assigned to latent-TGF-ß1 fused tag protein, which increased in size at 100 kDa compared to the commercial one of about 75 kDa of latent TGF-β1. The second band illustrates the mature form at 14 kDa monomer structure, as shown in Figure 41.

Based on this, the commercial and purified TGF-β1 proteins show a similarity in band size with regard to the mature TGF-ß1 monomer form. This data is consistent with the notion that mature TGF-β1 can be visualized under non-reducing conditions as a dimer form of 25 kDa and under reducing conditions in a monomer form of 12-15 kDa [94,141]. However, the difference in band size between the latent purified TGF-β1 protein and commercial TGF-β1 were caused by the presence of tags in the purified one and the fact that the two proteins originate from different CHO cell clones. Latent TGF-β1 can be visualized on the non-reducing Western blot analysis at 75, 80, 84, 90, or 100 kDa [93,97,142].

However, the TGF-ß1 plasmid design was intended to produce the monomer form of the mature recombinant human TGF-β1 112 amino acid polypeptide chain (12.7 kDa). It is well known

that the cells secrete TGF-ß1 in a latent form (homodimer of latency-associated peptide and mature TGF- ß1) and the mature form releases this under acidic conditions [143]. This explains why both forms are revealed in the membrane of the Western blot analysis in Figure 41.

The plasmid-produced TGF-ß1 fusion protein is connected to the C-terminal portion with a Twin-Strep-tag® 28 amino acid (3 kDa) to enable protein purification to take place [115]. At the same time, it was connected to the N-terminal portion with a short fluorescence chain to monitor protein productivity [111]. The fluorescent chain consists of 39 amino acids (4.4 kDa), including a tryptophan fluorescent tag plus linker and enterokinase cutting sites, as shown on the plasmid map in Figure 15.

This work demonstrated that the plasmid design is able to synthesize the TGF-β1 in transfected CHO-K1. The active TGF-ß1 (mature) was secreted into the supernatant of the transfected culture as confirmed by Western blot analysis. A contradictory description appears in the literature stating that the folding and secretion of active TGF-β1 requires plasmid directed to the expression of the TGF-β1 prodomains [144,145].

4.4.2. Determining the protein concentration

Transfection efficiency is not directly measurable for TGF-ß1 protein. Because TGF-ß1 is not fluorescent and secretes extracellularly, not intracellularly like GFP, the transfection quality is instead detected by the amount of protein produced. The total protein purified by Strep-Tactin® Superflow® column in elution buffer fraction E1, E2, and E3 concentration was assessed by Nanodrop A280 measurements as shown in Table 4.

Table 4: Estimation of purified TGF-ß1 protein concentration by Nanodrop.

Elution fraction	Volume of elution fractions [mL]	Concentration of purified protein TGF [µg/mL]	Total amount of purified protein [µg]
E1	1.2 mL	510	612
E2	5.2 mL	170	884
E3	1 mL	140	140
Σ	7.4 ml		1636

Based on Nanodrop A280 analysis shown in Table 4, the calculation of the volumetric productivity of purified TGF-ß1 concentration from the whole culture supernatant (60 mL) is about 27 µg/mL (i.e. 27 mg/L). This calculation was made by dividing the total protein amount in all elution buffer fractions E1, E2, and E3 by the amount of loaded culture supernatant volume in the column.

To standardize the concentration of purified TGF-ß1 with the commercial TGF-ß1 in order to determine protein bioactivity, it is important to establish a more specified method for protein

concentration determination by ELISA. This step is required because the intensity of the active protein band is relatively much lower than the latent TGF-ß1, as appears in the Western blot analysis in Figure 41. Therefore, the concentration of E2 fraction (which represents the main fraction) was estimated according to the calibration curve of commercial TGF-β1 using the developed indirect ELISA assay as shown in Figure 47 'Appendix section'.

The total volumetric active protein concentration of fraction E2 was about 15 ± 2 ng/mL (i.e. 0.015 ± 0.002 mg/L), in relation to the total amount of culture supernatant (60 mL). This calculation was made as mentioned above. The result shows a big difference between the amount of mature and total TGF-ß1 purified from culture supernatant based on this determination method. The total TGF-ß1 estimated by Nanodrop is much higher at about 14700 ng/mL compared to the 15 ng/mL of the mature TGF-ß1, as determined by ELISA. This difference seems to be consistent with that band's intensity as indicated by Western blot analysis. The latent band shows more intestate than the mature band in the same protein fraction.

However, the literature reports that the maximum volumetric yield of TGF-ß1 produced from stable CHO cells cultivated in T-500 triple layer flasks under acid activation was about 26.6 mg/L of total protein and 10.6 mg/L of mature TGF-ß1. This is based on a determination with a bicinchoninic acid assay (BCA assay) and ELISA respectively [94].

Comparing those findings with our results above, the big difference in active protein concentration could be related to the activation process, which allows the liberation of biologically active TGF-ß1 from its latent form. This step was not applied in this experiment here.

Most cultured cells generate little, if any, active TGF-β. This may be due to the fact that some TGF-β activation occurs at the cell surface [146] and that they generate local mature TGF-β which in turn binds to the cell surface receptor and therefore were not detectable at that level.

It is also worth noting that non-transfected CHO-K1 cells produce natural TGF-β1 in picogram amounts, which contaminates the clinical products produced by these cells [147]. The ELISA determination assay shows the absolute active TGF-β1 concentration.

4.4.3. Production of TGF-β1 protein through different transfection conditions

Three experiments were conducted to estimate the optimal transfection conditions for TGF-ß1 production and to find the correlation between GFP-transfection efficiency (as determined previously) and TGF-ß1 protein productivity. Three transfection conditions with different GFP-transfection efficiency (GFP-TE) as estimated before in Chapter 4.3 are applied here with TGF-ß1 plasmid, as shown in Table 5. For each transfection condition, the contents of three flasks

with 12.5 mL of CHOMACS CD media in each were mixed with 12.5 x10 million cells followed by 1:1 (v/v) media dilution at 5 hpt as previously mentioned. At 72 hpt all cultures were harvested for direct quantification of protein by ELISA as shown in Table 5. The purification process was omitted since there was no further need to use pure TGF-ß1.

Table 5: Estimation of mature TGF-β1 from culture supernatant by ELISA.

Transfection condition per 10 x10^6 cells/mL x12.5 mL transfection volume				**Concentration of mature-TGF-β1 in culture supernatant [ng/mL]**
Condition No.	PEI [µg/mL]	pDNA-TGF-β1 [µg/mL]	GFP-TE [%]	
1	38	17	73	**26 ± 1.4**
2	45	24	62	**13 ± 2.5**
3	30	6	50	**5 ± 1.3**

Table 5 shows that different levels of TGF-β1 expression were successfully produced under different transfection conditions (1-3). The concentration of TGF-β1 varied according to the transfection conditions applied and coincide with GFP-transfection efficiency.

The volumetric productivity was calculated with 60 mL for each condition (as mentioned previously). The TGF-ß1 yields were estimated as 26, 13 and 5 ng/mL linked to respective 73, 62 and 50% GFP-transfection efficiency. This reflects the direct relation between protein production concentration and transfection efficiency, which increases hand in hand. This is consistent with the notion that protein yield is directly dependent on transfection efficiency [40]. However, there was not an equal increase in productivity and transfection efficiency.

Under the same transfection conditions (1), a difference was noticed between the mature TGF-ß1 estimated directly in culture supernatant here and that purified in fraction E2 in the previous experiment. The concentration was 26 ng/mL in the culture supernatant and about 15 ng/mL in the purified fraction E2. This reduction in the protein concentration may be due to the fact that other the fractions, E1 and E3 of the mature protein concentration, were not determined. Moreover, the purification process could negatively affect the amount of activated mature TGF-ß1.

4.4.4. Correlating protein concentration using fluorescence spectroscopy

In this analysis, different concentrations of purified TGF-β1 (E2 fractions) were obtained from different cultivation experiments. The quantification method was based on Nanodrop measurements and these samples underwent 2D-fluorescent spectroscopy. As shown in Figure 42, the absolute signals were obtained by subtracting the signal of the blank from the sample signals.

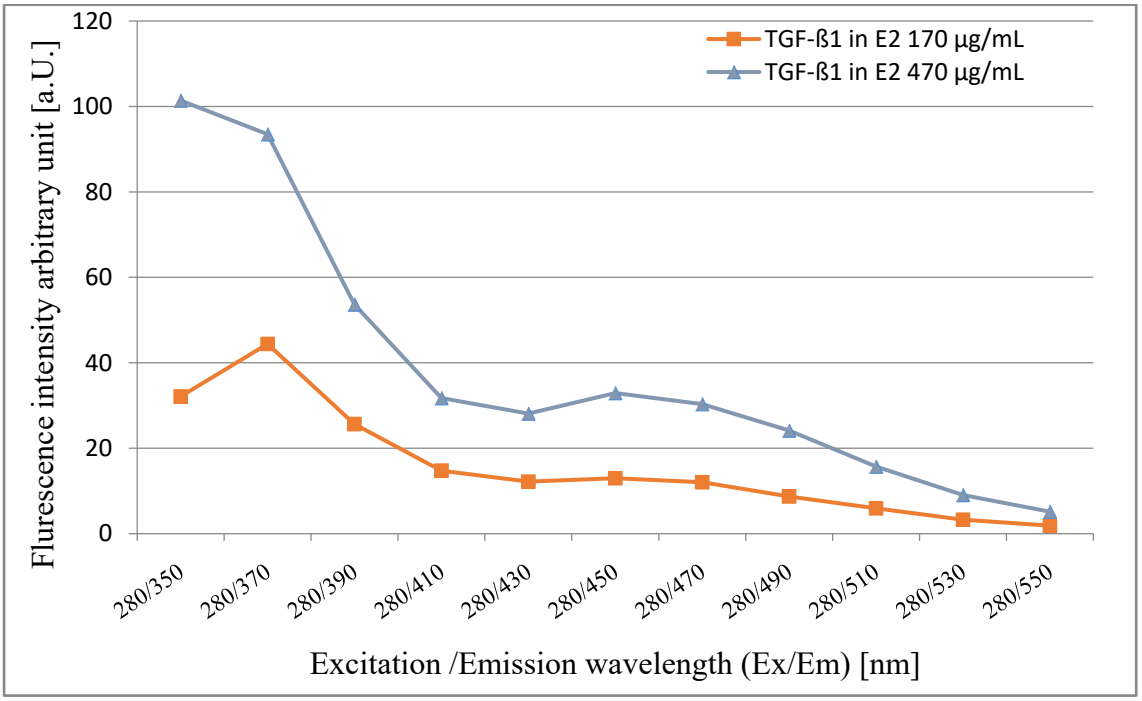

Figure 42: Excitation and emission spectra of purified TGF-β1 protein.
The total purified protein concentration in elution buffer (E2) was indicated in the graph as was the 2D-fluorescence intensity.

The result from the fluorescence spectra in Figure 42 for different concentrations of purified TGF-ß1 protein reveal that the overall fluorescence signal differed significantly. The fluorescent intensity was higher with high protein concentration and vice versa. The signal intensity was related to Ex/Em 280/350 wavelength corresponding to short-chain fluorescent tryptophan tags. Normally, tryptophan fluorescence is excited between 250-300 nm, and the resulting emission ranges from 300-400 nm [111]. In general, the result here shows that fluorescent intensity was directly related to the concentration of the protein loaded.

In this context, this difference may be related to the tryptophan tag which is able to generate stronger signals in higher protein concentrations than in lower ones. This would improve the ability of 2-D fluorescence to distinguish the target protein from other proteins in the cell culture, in particular at online measurements. Tagged antibodies with short chain tryptophan tags were used for on line detection due to the increase in the fluorescence signal, rather than

untagged protein within a maximum (Ex.nm/Em.nm) at 280 nm/350 nm [111]. However, this aspect needs further investigation, due to the absence of TGF-β1 untagged protein as a reference.

4.4.5. Bioactivity of TGF-ß1 protein test with the A549 cell line

The purified protein in elution buffer was concentrated with a vivaspin cutting membrane tube then resuspended in DEMEM media at various concentrations of 179, 88 and 44 ng/mL as determined with ELISA. The equivalent concentration of commercial TGF-β1 was tested in parallel as a positive control. For the negative control, the same experiment was performed without the addition of TGF-β1. The viable cell proliferation rate of A-549 human lung epithelial carcinoma cells (CCL-185) was measured by CellTiter Blue®-Assay. Cell viability assay (CTB-Test) as described in the 'Materials and Methods' section.

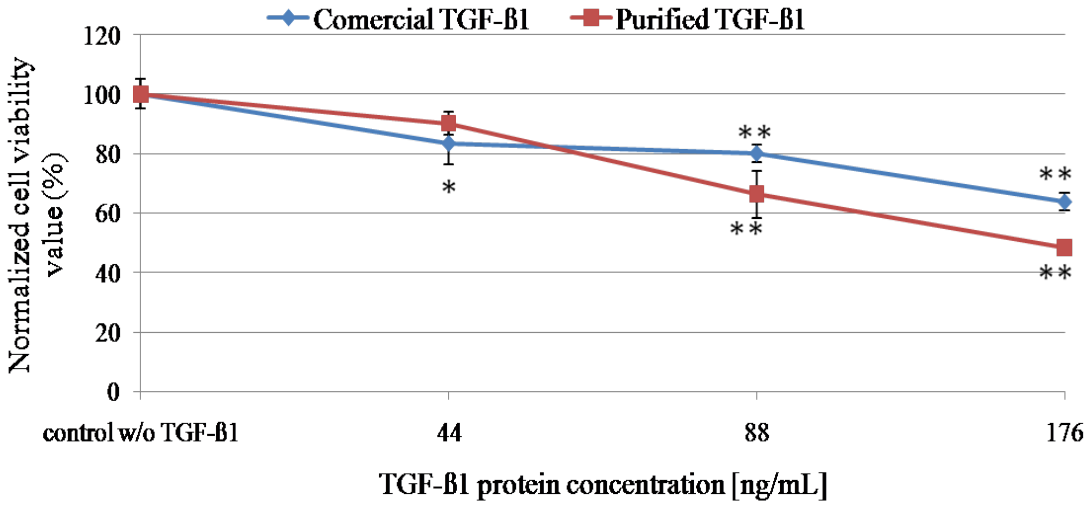

Figure 43: Bioactivity of TGF-ß1 secreted from transfected CHO-K1 cells.
Percent changes in A-549 cell proliferation based on TGF-β1 concentration. Data were analyzed using two-tailed paired t-test compared to control (N=3), and *p<0.05, ** P<0.01 t-test was considered significant and very significant respectively.

TGF-β1 is a multifunctional growth factor with various effects described in the literature. In this experiment, its capacity to inhibit A-549 cell proliferation was tested. The results in Figure 43 show that the addition of TGF-β1 inhibits the proliferation of A-549 cells compared to the negative control (0 ng/mL-TGF-β1). After 5 days of cultivation, the commercial and the purified TGF-β1 show a comparable result with regard to the effect on the A-549 cells. The increase in this effect is also evident at higher concentrations (compare 44 and 176 ng/mL TGF-β1). Cultures with concentrations of 88 and 176 ng/mL show a very significantly different effect from concentrations of 44 ng/mL. This result indicates that the TGF-ß1 expressed in transfected CHO-K1 culture is bioactive and has a similar bioactivity to the commercial one.

Measurement of TGF-β1 activity is an important step in optimizing productivity before scaling up the protein production process. TGF-β1 activity was therefore determined in this study using the A549 lung carcinoma CCL-185 cell growth inhibition bioassay via CellTiter Blue®-Assay. In agreement with the literature, we observed a decrease in cell viability (indirect indicator for cell growth) in CCL-185 in response to increasing TGF-β1 concentration [106].

The antibody used in ELIZA recognizes only the active form according to the manufacturer's instructions, and TGF-β1 exerts its effect only through the mature (active) dimer form. Therefore, it was important to measure the amount of active TGF-β1 in the total purified protein before performed the bioactivity test [143].

5. Summary

Production of recombinant proteins is important for therapeutic and research development purposes, both in the lab or on an industrial scale.

Establishing an efficient method for transient protein expression is a necessary not only for the rapid production of these recombinant proteins but also might be interesting for the fast screening and isolation of highly productive cell clones in a stable transfection process. The transient transfection method depends on a multitude of different factors that have to be adjusted to obtain an efficient and reproducible method to transfect cells in a Tubespin bioreactor®50 (small scale) or shake flasks (relatively large scale). These factors include selecting suitable transfected production media to support high density transfection. Determining the ideal post-transfection time is necessary before culture dilution with various volumes of media for further volumetric scale-up. Evaluating the scale-up of transfection culture is also a necessary step towards production of a desired protein like TGF-ß1.

Important factors in the transfection protocol include studying the state of cell culture by investigating the effect of pre-culture cell density, and resuspending cells in consumed or fresh media in parallel with culture age and cell cycle. Such knowledge will increase the protocol strength and help avoid variability noise. Testing the transfection protocol with a wide range of cell densities at transfection time point, has proved its validity for possible application in continuous culture. Plasmid and PEI transfection reagent concentration is a critical factor for choosing optimal transfection conditions for protein production. Correlation of pDNA/PEI polyplex particle size formation with the transfection efficiency is helpful for understanding the role of this physical polyplex property in the transfection process. Implementation of a repeat transfection strategy is necessary to extend the post-transfection expression time of proteins.

For all of these purposes, the goal of this thesis has been to optimize these parameters related to media, cells, and transfection polyplex with regard to their influence on transfection efficiency and cell viability. This was done either by changing factors one by one or by studying the interaction of factors using the design of experiment (DoE) approach.

The findings of this study with regard to different commercial media were screened for PEI compatibility, either at low or at high cell density. The screening study showed that the medium CHOMACS CD (well established media for bioprocess development) is suitable for high cell density transfection at 10 million cells per mL. The time of diluting this high cell density transfected culture after i.e. 5 hours post-transfection (hpt), is critical, while dilution or no dilution of the transfected culture volume has no effect on transfection efficiency. In addition, the effect on cell density of cell collection from one-day pre-culture, either in fresh or

consumed media, was also investigated here. This experiment proved that cell collection and dispersal in fresh media is a crucial step for transient transfection. Seeking to determine if the cell density of the pre-culture that is used afterward for transfection in fresh media has an impact on the transfection process, we found that pre-culture at transfection time in the range of 2.6-5.3 x10^6 cells/mL provides a good basis for further process optimization of over 60% TE and 80% viability. Increasing pre-culture age from 1 day up to 3 days causes a reduction in transfection efficiency corresponding slightly with the increase in S phase cell population. The presence of NaCl in media at transfection time leads to an increase in osmolality. This is in contrast to cell viability below 80% where transfection efficiency remains nearly unchanged.

The decrease in cell density at transfection time from 10 million cells per mL to 4 million or an increase to 18 million had no effect on transfection efficiency. However, the former transfection condition leads to a decrease in viability. A high density transfection protocol for 10-18 million cells/mL using 24 µg of pDNA and 45 µg PEI enhanced transfection efficiency ~60% with a viability of over 80% at 48 hpt, with the dual target strategies of volumetric scale-up and a smaller amount of pDNA per cell.

Using the DoE approach, we were able to identify several conditions for increasing transfection efficiency ranging from 50-70%. These conditions were derived from combining various amounts of pDNA and PEI, which interact with the same amount of cell material to improve transfection efficiency (enlarge the optimal transfection condition range). This offers the advantage of using smaller amounts of pDNA and PEI during transfection while at the same time increasing transfection efficiency to over 73% and viability to over 80%. This mainly reflects the indirect impact on transfectability of polyplex pDNA/PEI particle size formation, a factor resulting from a variation in amounts of pDNA and PEI. Tracking the dynamics of polyplex formation over 80 min via nano-tracking analysis (NTA), which utilizes a laser light scattering microscope. This study explored the dynamics of pDNA/PEI polyplex formation between a high transfection efficiency of 70% and a low transfection efficiency of 14%. Both transfection conditions resulted from a fixed amount of pDNA combined with different amounts of PEI. Although the dynamics of particle size formation, shapes and counts were different, the drawback of this analysis is the need to identify transfectable pDNA/PEI polyplex to distinguish between both conditions. Because of this, it is hard to elucidate polyplex properties and their effect on transfection efficiency. Both transfection conditions can generate nano-spherical particles, either singly or in aggregate. Higher concentrations of smaller particle size below 250 nm can be attributed to high transfection efficiency based on SEM and NTA studies.

One drawback of the previous protocol, however, is its use of relatively high amounts of pDNA and PEI. Repeat transient transfection has been shown to be a useful and reliable way to extend gene expression time for 120 hpt with a high transfection efficiency of ~70% and viability over 95%. This was achieved by using a small amount of 6 µg pDNA and 30 µg PEI for 10 million cells per mL in the second transfection step. Applying one-day pre-culture period (time interval) between the first and second transfection step improved the viability of the transfected culture.

Polyethylenimine (PEI) *in situ* transient transfection proved to be a simple and successful gene transfer method in both the Tubespin®50 bioreactor and in 125-mL shake flask cultivation. The central prerequisite for this protocol is the preparation of cells from pre-culture (less than 24 hrs of age with a cell density range of 2.6-5.3 x10^6 cells/mL). These cells are prepared at a high cell density of 10-18 x10^6 cells/mL in fresh media for transfection. *In situ* transfection by adding 24 µg pDNA then 45 µg PEI, showed 60 ± 10% green fluorescence protein (GFP) positive cells (transfection efficiency) at 48 hpt.

Scalability is achieved either volumetric through an increase in production volume after five hours of transfection (the process is linearly scalable from 1 mL to 8 mL in a Tubespin bioreactor®50) or through an increase in transfection culture volume (the process is linearly scalable up to 12.5 mL in a Tubespin bioreactor®50 and shake flask).

Efficient transfection of CHO-K1 cells with TGF-ß1 plasmid tagged by tryptophan and Twin-Strep-tag® was achieved, based on the progress of the GFP-transient transfection model. The transfection condition was obtained using the amount within a shake flask of 17 µg pDNA:38 µg PEI in CHOMACS CD. The maximum purified TGF-ß1 produced was about 27 mg/L compared to 30 mg/L for the highest yields of recombinant TGF-ß1 reported in the literature [94].

An off-line 2D-fluorescence spectroscopy assay was used to correlate fluorescence signal intensity with tryptophan TGF-ß1 tagged protein concentration, which is considered to be a reliable tool for future online technical applications. Another plasmid directed to untagged TGF-β1 will be useful for seeing the effect of tryptophan tags and to validate its fluorescence signal.

The strength of the fluorescence signal is strongly proportional to the purified TGF-β1 protein concentration.

Finally, the bioactivity of the purified mature TGF-β1 quantified by ELIZA was successfully determined with a cell-based bioactivity assay. This shows an excellent level of activity and reproducibility, consistent with commercial TGF-ß1. The increase in the TGF-ß1 concentration had a strongly inhibitory effect on cell growth in the epithelial lung carcinoma A549 cell line.

6. Conclusions and Perspectives

In conclusion, a polyetheyleneinimne (PEI) transient transfection procedure makes it easy to perform transient protein production on a lab scale using the mammalian CHO-K1 suspension cells cultivated in a CHOMACS CD medium.

The work performed in this thesis contributes to the scientific knowledge not only of transient transfection but also with regard to the development of a recombinant protein production platform. Interesting new goals were developed in connection with this subject:

- Transfection CHO-K1 suspension cell density up to 18 million cells/mL of without prior preparation of a pDNA/PEI transfection cocktail.
- The omission of a dilution step after transfection would assist in establishing very high density transient transfection for future application with perfusion culture.
- Overcoming the problem of having to use two types of media, one for transfection and the other for production (accepted protocol not published yet and not mentioned here), would involve e.g. using only one production media −CHOMACS CD − for transfection and cultivation. Transfection and production in a single medium would enhance protein productivity, cell growth, up-scaling and GMP compliance because of the lower contamination risk and easier handling involved (fewer handling steps). In this context, *in situ* transient transfection is superior to conventional transfection methods, especially when performed on a large bioreactor scale.
- Use of pre-culture one day prior to transfection with cell density between 2.6-5.3 million cells per mL at transfection time is an important step for efficient transfection, in addition to concentrating the cells to ten million cells per mL in fresh media.
- Use of a minimal amount of 6 µg pDNA:30 µg PEI to achieve 38-50% transfection efficiency with a viability of over 90% combined with high standard deviation; it was not recommended for use in this study as the first step of transfection, but would be preferable to a repeat transfection strategy.
- Use of a relatively high amount of 24 µg pDNA:45 µg PEI in the routine protocol of the first step of transfection elevates the transfection efficiency level to 60-74% with a viability of over 80% combined with less standard deviation in independent experiments (more stable condition).
- Study of DoE approach exploring various optimal transfection conditions resulting from various amounts of pDNA and PEI interaction under the same cell density, giving an opportunity to select low amounts of pDNA-PEI combined with a high level of transfection efficiency.

- Study of a nano-tracking analysis exploring the dynamics of pDNA/PEI polyplex formation and correlate this biophysical character with transfection efficiency. The most spherical shape in a small size offers the most transfection efficiency.

In general, one drawback of the transfection protocol in this work has been the need to concentrate the cells at high density for large scale transfection, especially with regard to the need for large scale culture centrifugation to collect the cells.

Additional experiments are required to further improve the process:

- Further scalability of the transient transfection protocol to larger scales such as 600 shaker bioreactor and 2-liter bioreactor.
- The omission of a concentrated pre-culture cell step required for obtaining high cell density for transfection. Extension of the pre-culture cell density range to reach 10 million cells per mL at transfection day, followed directly by *in situ* transfection would provide an attractive solution for large-scale transfection, in addition to the use of a cell retention device.
- Characterization of pDNA/PEI polyplex formation correlated to transfection efficiency.
- Expansion of the results to different mammalian cell types and a cell culture model.
- Evaluation of the purified TGF-ß1 amino acid sequences by mass spectroscopy analysis.
- Release of mature TGF-ß1 from latent TGF-ß1 through a protein activation process.
- Expansion of protein bioactivity results to different cell lines, stem cell differentiation, and cell transformation.
- Optimization TGF-ß1 protein productivity in shake flasks.
- Use of a fluorescence spectroscopy (bioview) sensor for real-time monitoring of TGF-ß1 protein productivity.

7. Appendices

7.1. Equipment

Table 6: List of required equipment.

Devices/Disposables	Manufacturer, Country
Centrifuge microfuge 3s	Thermo Fisher Scientific Inc., USA
Flow cytometer BD Accuri™ C6	BD Biosciences, USA
Flow Cytometer EPICS XL-MCL	Beckmann-Counter, Florida, USA
Laminar flow hood	Thermo Fisher Scientific Inc., USA
SDS-PAGE gel system unit	Bio-Rad Laboratories Inc., USA
Shake flasks 125, 250, 1000 mL	Corning Inc., USA
Shaker (DOS-20L)	ELMI, North America
Shaker (SHKR-03 Model DOS-10M)	ELMI, North America
Sterile pipettes 2, 5, 10, 50 mL	Corning Inc.,USA
Strep-Tactin® Superflow® column 1-mL	IBA GmbH, Germany
Tubespin bioreactor®50	TPP, Trasadingen, Switzerland
Ultra centrifuge	Thermo Fisher Scientific Inc., USA
Vortex Mixer VM-300	NeoLab Migge GmbH, Germany
Water bath	Memmert GmbH, Germany
Weight with digital display	Sartorius Stedim Biotech, Germany

7.2. Chemicals

Table 7: List of Chemicals.

Chemicals	Manufacturer, Country
Agarose	ABgene, Germany
Ammonium persulfate (APS)	Carl Roth GmbH + Co. KG, Germany
Ampicillin-sodium salt	VWR Chemicals, Germany
Ap conjugate substrate kit	Bio-Rad Laboratories Inc., USA
Bacteriological Agar-Agar	Carl Roth GmbH + Co. KG, Germany
Bromophenol blue	Merck Millipore, Germany
DNA 10000 bp Ladder	Thermo Fisher Scientific, Germany
Ethanol	Merck Millipore, Germany
Ethylenediaminetetraacetic acid (EDTA)	AppliChem GmbH, Germany
Isopropanol	Merck Millipore, Germany
kanamycin sulfate	Carl Roth GmbH + Co. KG, Germany
Methanol (MeOH)	Merck Millipore, Germany
Monosodium phosphate (NaH2PO4)	Merck Millipore, Germany
penicillin/streptomycin)	PAA Laboratories GmbH, Austria
Prestained ladder 26616	Thermo Fisher Scientific, Germany
Roti®-Safe Gel Stain	Carl Roth GmbH + Co. KG, Germany
Rotiphorese Gel 40	Carl Roth GmbH + Co. KG, Germany
Sal1, Not1, Xba1 and Hind III	Thermo Fisher Scientific Inc., USA
Sodium bicarbonate NaHCO3	Merck Millipore, Germany
sodium chloride (NaCl)	Merck Millipore, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Corporation, USA
Sodium hydroxide (NaOH)	Fluka Chemie Ag., switzerland
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific Inc., USA
Tween 20	Carl Roth GmbH + Co. KG, Germany
Yeast Extract.	Carl Roth GmbH + Co. KG, Germany
β-Mercaptoethanol	Merck Millipore, Germany

7.3. Buffer, Gel and solution preparation

Agarose gel electrophoresis 50 x TAE running buffer: 242 µL Tris, 57.1 mL acetic acid, 100 mL EDTA (0.5 M) added into 25 mL ddH_2O.

15% separation gel: 1.8825 ml of rotiphoresis gel 40, 1.4 ml of 1 M Tris (pH 8.8), 1.2175 mL of ddH2O, 10 µL of TEMED, 0.5 mL (1%) of SDS, 10 µL (25%) APS.

6% collecting gel: 0.75 mL of rotiphoresis gel 40, 0.63 ml of 1 M Tris (pH 6.8), 3.77 mL of ddH2O, 0.3 (1%) SDS, 10 µL of TEMED, 10 µL of (25%) APS.

Antibody diluents solution 40 mL: 20 mL block buffer: 20 mL PBST buffer (50:50)

Ampicillin stock solution:

100 mg Ampicillin added into 1 mL ddH_2O.

Kanamycin stock solution:

50 mg Kanamycin added into 1 mL ddH_2O.

7.4. Tubespin bioreactor®50 to an orbital shaker in an incubator

Figure 44: Tubespin bioreactor®50 fitted with a filter cap in an orbital shaker.
The tubes were maintained in racks designed in house with O-ring in the upper part to fit well on the orbital shaker in an incubator.

7.5. Cell cultivation under different osmotic stress

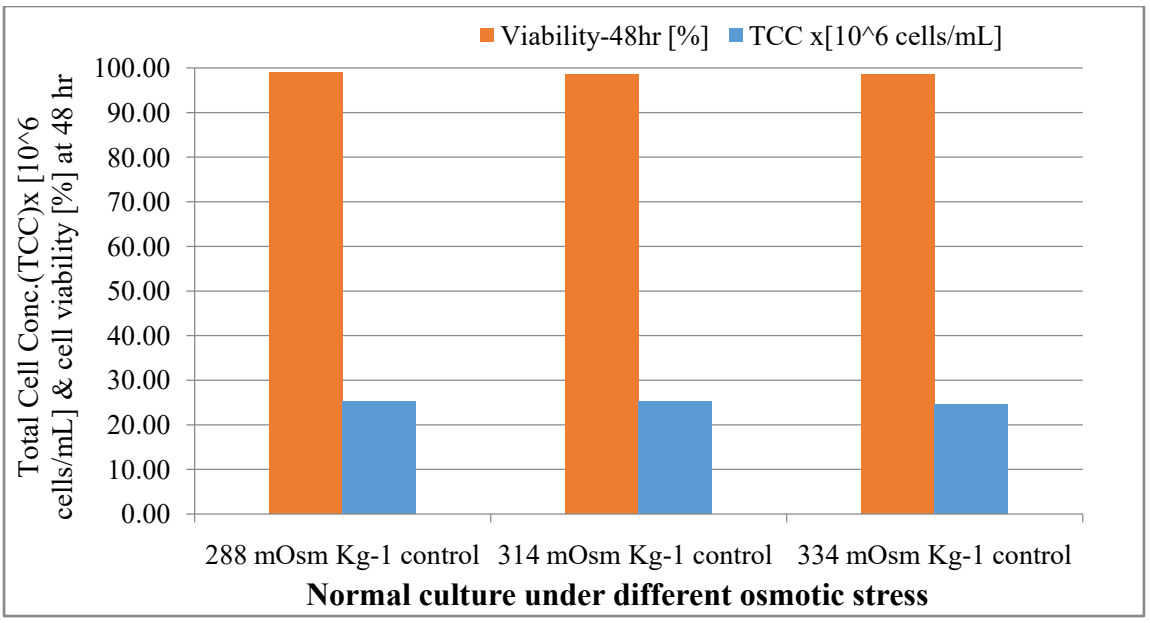

Figure 45: CHO-K1 cultivation in different osmotic stress.
The graph shows CHO-K1 cell growth and viability in different osmotic pressure media for 48 hr. The cultivation was done to mimic the transfection condition but without transfection. Cells at 10 million cells per mL were collected and seeded in 1 mL of each medium with particular osmotic pressure. The osmolality of normal media has osmotic pressure of 288 mOsm kg^{-1}. After 5 hour all culture was diluted with another 1 mL of normal media. At 48 hr of cultivation samples were obtained for viability and cell concentration determination.

7.6. Transient expression of TGF-ß1 protein

All steps which required for TGF-ß1 protein expression starting from pre-culture preparation step for 24 hr followed by cell collection at high cell density in fresh media and distributed by shaking for one hour. This ended by its transfection with pDNA and PEI amount and diluted by 1:1 (v/v) for further cultivation at 72 hpt was represented below.

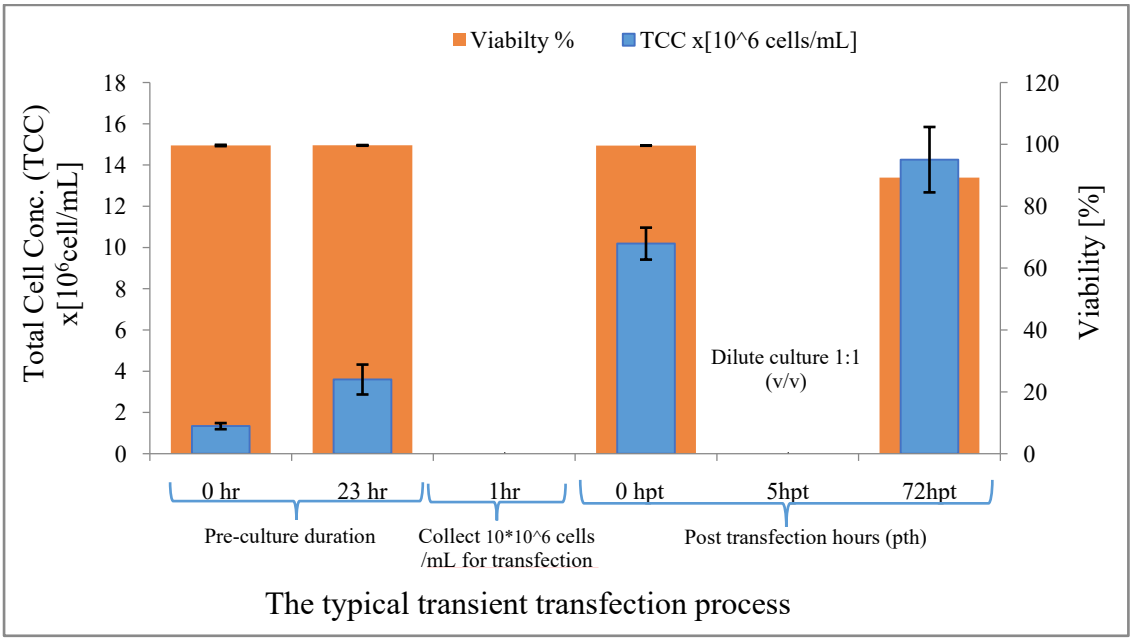

Figure 46: Transfection process for TGF-ß1 production.
The process is starting with pre-culture cells at low cell density. After that, the cells were duplicated within 24 hr. The cells were collected and resuspended in fresh media. For well cell dispersion, the cells were cultivated for 1-hour under shaken condition. The cultures were transfected *in situ* by adding the appropriate amount of pDNA and PEI respectively. The cultures were diluted after transfection i.e. 5 hpt with equal volume of fresh media. The transfected culture was left of 72 hpt prior to harvest.

More detailed related to transient transfection process which applied in this study was explained in the standard operating procedure (SOP) available in house.

7.7. Purification of TGF-ß1 protein

For purifying Twin-Strep-tag® fusion proteins directly from cell culture supernatant, Iba protocol was applied by loading the Strep-Tactin® Superflow® column with the total 60 mL of transfected culture supernatant at flow rate 1 mL/min. The purification was carried out on the basis of the Twin-Strep-tag® fusion protein coupled to Strep-Tactin® affinity chromatography. In elution step, the destination protein was collected in the elution buffer. Elution buffer fraction with purified protein was quantified by Nanodrop. More detail was explained in SOP Protein Analytics available in house.

7.8. ELISA calibration

Various concentration of commercial TGF-ß1 was used to establish the calibration curve below through serial dilution method.

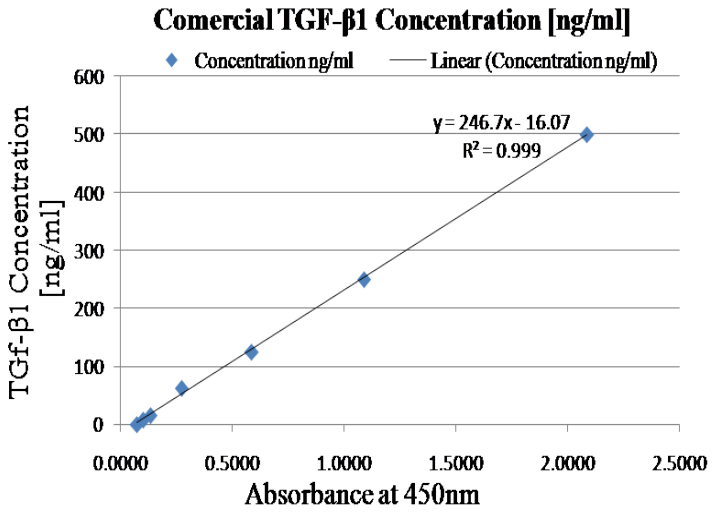

Figure 47: Calibration curve of commercial TGF-β1.
Various concentrations of commercial TGF-β1 were incubated with monoclonal antibody anti TGF-β1. After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 450 nm.

7.9. Designs regions of DoE

Table 8: Full factorial factor settings (2 levels) design of screening contour.

Exp No	Screening experiment	Run Order	DNA Conc.[µg/mL]	PEI Conc.[µg/mL]	Cell density x10^6 [cells/mL]
1	N1	12	10	40	7
2	N2	3	26	40	7
3	N3	1	26	40	13
4	N4	6	26	60	13
5	N5	7	26	60	7
6	N6	11	10	60	7
7	N7	9	10	60	13
8	N8	2	10	40	13
9	N9	8	18	50	10
10	N10	4	18	50	10
11	N11	10	18	50	10
12	N12	5	18	50	10

DoE is designed with 4 central points and 8 run distributed as shown in design region Figure 48.

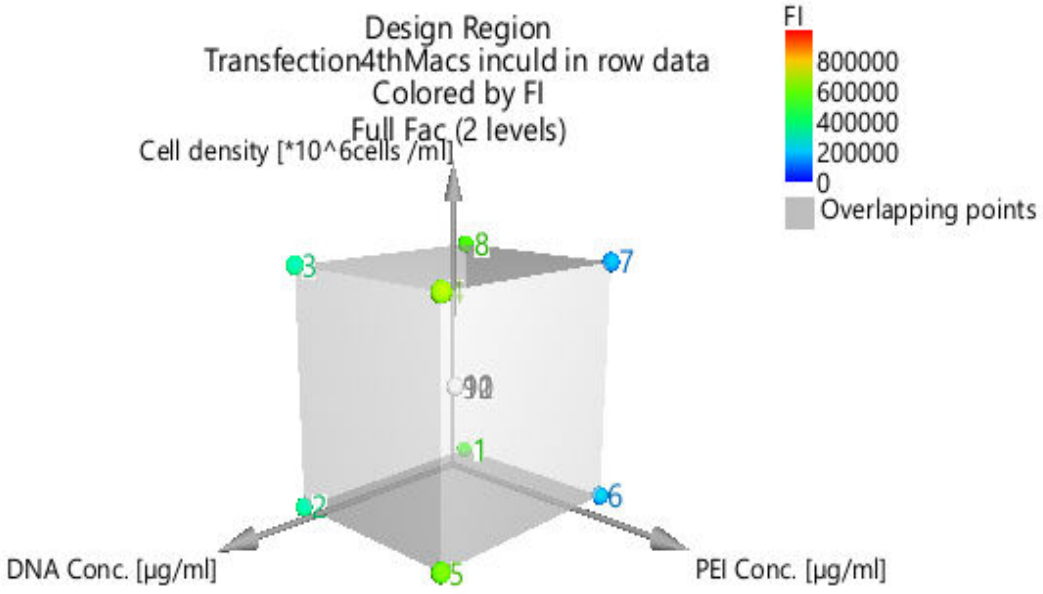

Figure 48: Full factorial 2^3 design region.

Table 9: A central composite circumscribed (CCC) DoE design of area B (low-PEI-Conc.).

Exp No	Exp Name (low Conc.)	Run Order	PEI [µg/mL]	GFP-pDNA [[µg/mL]
1	N1	5	31	5
2	N2	8	45	5
3	N3	7	31	15
4	N4	6	45	15
5	N5	11	28	10
6	N6	10	48	10
7	N7	1	38	3
8	N8	9	38	17
9	N9	2	38	10
10	N10	4	38	10
11	N11	3	38	10

Transfection condition: 10 million cells per mL. At 5 hpt the culture diluted 1:1 (v/v) then at 48 hpt TE% and viability were determined. The table illustrates the factor settings required.

Table 10: The factor settings required for (CCC) DoE design of area A (high-PEI-Conc.).

Exp No	Exp Name (high Conc.)	Run Order	PEI [µg/mL]	GFP-pDNA [[µg/mL]
1	N1	11	55	20
2	N2	7	69	20
3	N3	8	55	30
4	N4	10	69	30
5	N5	4	52	25
6	N6	5	72	25
7	N7	2	62	18
8	N8	3	62	32
9	N9	6	62	25
10	N10	1	62	25
11	N11	9	62	25

Transfection condition: 10 million cells per mL. At 5 hpt the culture diluted 1:1 (v/v) then at 48 hpt TE% and viability were determined.

Table 11: Illustrates the factor settings required for (CCC) DoE design of area C.

Exp No	Exp Name: Inner-ring	Run Order	PEI [µg/mL]	GFP-pDNA [µg/mL]
1	N1	9	33	21
2	N2	2	43	21
3	N3	1	33	27
4	N4	11	43	27
5	N5	8	31	24
6	N6	10	45	24
7	N7	6	38	20
8	N8	3	38	28
9	N9	5	38	24
10	N10	4	38	24
11	N11	7	38	24
Exp No	**Exp Name: Outer-ring**	**Run Order**	**PEI [µg/mL]**	**GFP-pDNA [[µg/mL]**
1	N1	9	31	19
2	N2	2	45	19
3	N3	1	31	29
4	N4	11	45	29
5	N5	8	28	24
6	N6	10	48	24
7	N7	6	38	17
8	N8	3	38	31
9	N9	5	38	24
10	N10	4	38	24
11	N11	7	38	24

Transfection condition: 10 million cells per mL. At 5 hpt the culture diluted 1:1 (v/v) then at 48 hpt TE% and viability were determined.

8. Bibliography

1. Ashok D., B. & Steven C., A. Recent advances in mammalian protein production. *NIH Public Access FEBS Lett. Author manuscript; PMC* **588,** 253–260 (2015).
2. Khan, K. H. Gene Expression in Mammalian Cells and its Applications. *Adv. Pharm. Bull.* **3,** 257–263 (2013).
3. Griffin, T. J., Seth, G., Xie, H., Bandhakavi, S. & Hu, W. Advancing mammalian cell culture engineering using genome-scale technologies. **25,** (2007).
4. ZHANG, J. in *Mammalian Cell Culture for Biopharmaceutical Production, p* (ed. Baltz R, Demain A, Davies J, Bull A, Junker B, Katz L, Lynd L, Masurekar P, Reeves C, Z. H.) 157–178. (Manual of Industrial Microbiology and Biotechnology, Third Edition. ASM Press, Washington, DC., 2010). doi:10.1128/9781555816827.ch12
5. Kunert, R. & Vorauer-Uhl, K. in *Straegies for efficient transfection of CHO-Cells With Plasmid DNA* (ed. Hartley, J. L.) 213–226 (Springer Science + Business Media, 2012).
6. Baldi, L., Hacker, D. L., Adam, M. & Wurm, F. M. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnol. Lett.* **29,** 677–84 (2007).
7. Shen, X. *et al.* A simple plasmid-based transient gene expression method using High Five cells. *J. Biotechnol.* **216,** 67–75 (2015).
8. Girard, P. *et al.* 100 Liter Transient Transfection. (2002). doi:10.1023/A
9. Lai, T., Yang, Y. & Ng, S. Advances in Mammalian Cell Line Development Technologies for Recombinant Protein Production. *Pharmaceuticals* **6,** 579–603 (2013).
10. Pham, P. L., Kamen, A. & Durocher, Y. Large-scale transfection of mammalian cells for the fast production of recombinant protein. *Mol. Biotechnol.* **34,** 225–37 (2006).
11. Thompson, B. C. *et al.* Cell line specific control of polyethylenimine-mediated transient transfection optimized with 'Design of experiments' methodology. *Biotechnol. Prog.* **28,** 179–87 (2011).
12. Overton, T. W. Recombinant protein production in bacterial hosts. *Drug Discov. Today* **19,** 590–601 (2014).
13. Rosano, G. L. & Ceccarelli, E. A. Recombinant protein expression in Escherichia coli: Advances and challenges. *Front. Microbiol.* **5,** 1–17 (2014).
14. Walsh, G. Post-translational modifications of protein biopharmaceuticals. *Drug Discov. Today* **15,** 773–780 (2010).
15. Cereghino, J. L. & Cregg, J. M. Heterologous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiol. Rev.* **24,** 45–66 (2000).
16. Palomares, L. A., Estrada-Moncada, S. & Ramírez, O. T. Production of recombinant proteins. *Recomb. Gene Expr.* **4,** 15–51 (2004).
17. Mattanovich, D. *et al.* Recombinant protein production in yeasts. *Methods Mol Biol* **824,** 329–58 (2012).
18. Hauser H. in *Mammalian Cell Biotechnology in Protein Production.* (eds. Hauser, H. & Wagner, R.) 3–32. (Berlin: Walter de Gruyter, 1997).
19. Sandig, V., Rose, T., Winkler, K. & R., B. in *Mammalian cells* (ed. Gellissen, G.) 233–252 (Weinheim: Wiley- VCH Verlag, 2005).
20. Hossler, P., Khattak, S. F. & Li, Z. J. Optimal and consistent protein glycosylation in mammalian cell culture. *Glycobiology* **19,** 936–949 (2009).

21. Landowski, C. P. *et al.* Enabling low cost biopharmaceuticals: high level interferon alpha-2b production in Trichoderma reesei. *Microb. Cell Fact.* **15,** 104 (2016).
22. Su, X., Schmitz, G., Zhang, M., Mackie, R. & Cann, I. Heterologous gene expression in filamentous fungi. *Adv. Appl. Microbiol* **81,** 1–61 (2012).
23. Ward, O. P. Production of recombinant proteins by filamentous fungi. *Biotechnol. Adv.* **30,** 1119–1139 (2012).
24. Katoh, T. & Tiemeyer, M. The N's and O's of Drosophila glycoprotein glycobiology. *Glycoconj. J.* **30,** 57–66 (2013).
25. Kollewe, C. & Vilcinskas, A. Production of recombinant proteins in insect cells. *Am. J. Biochem. Biotechnol.* **9,** 255–271 (2013).
26. Demain, A. L. & Vaishnav, P. Production of recombinant proteins by microbes and higher organisms. *Biotechnol. Adv.* **27,** 297–306 (2009).
27. Jin, L., Zeng, X., Liu, M., Deng, Y. & He, N. Current progress in gene delivery technology based on chemical methods and nano-carriers. *Theranostics* **4,** 240–55 (2014).
28. Al-Dosari, M. S. & Gao, X. Nonviral Gene Delivery: Principle, Limitations, and Recent Progress. *AAPS J.* **11,** 671–681 (2009).
29. bio.lonza. Stable transfection. *© by Lonza Cologne GmbH - 2012-07-30* (2012). Available at: http://bio.lonza.com/resources/applications/stable-transfection/%0A.
30. Kotterman, M. A., Chalberg, T. W. & Schaffer, D. V. Viral Vectors for Gene Therapy: Translational and Clinical Outlook. *Annu. Rev. Biomed. Eng.* **17,** 63–89 (2015).
31. Das, A. K., Gupta, P. & Chakraborty, D. Physical methods of gene transfer: Kinetics of gene delivery into cells: A Review. *Agric. Rev.* **36,** 61 (2015).
32. Nayerossadat, N., Maedeh, T. & Ali, P. A. Viral and nonviral delivery systems for gene delivery. **1,** 1–11 (2012).
33. Dai, Z., Gjetting, T., Mattebjerg, M. A., Wu, C. & Andresen, T. L. Elucidating the interplay between DNA-condensing and free polycations in gene transfection through a mechanistic study of linear and branched PEI. *Biomaterials* **32,** 8626–8634 (2011).
34. Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc Natl Acad Sci USA* **92,** 7297–7301 (1995).
35. Hacker, D. L. *et al.* Polyethyleneimine-based transient gene expression processes for suspension-adapted HEK-293E and CHO-DG44 cells. *Protein Expr. Purif.* **92,** 67–76 (2013).
36. Delafosse, L., Xu, P. & Durocher, Y. Comparative study of polyethylenimines for transient gene expression in mammalian HEK293 and CHO cells. *J. Biotechnol.* **227,** 103–111 (2016).
37. Liu, S. *et al.* Bioreducible Zinc(II)-Coordinative Polyethylenimine with Low Molecular Weight for Robust Gene Delivery of Primary and Stem Cells. *J. Am. Chem. Soc.* jacs.6b13337 (2017). doi:10.1021/jacs.6b13337
38. Geisse, S., Jordan, M. & Wurm, F. M. in *Therapeutic proteins: methods and protocols* (eds. Smales, C. & James, D.) 87–91 (2005).
39. Liu, C., Dalby, B., Chen, W., Kilzer, J. M. & Chiou, H. C. Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. *Mol. Biotechnol.* **39,** 141–53 (2008).
40. Dalton, A. C. & Barton, W. A. Over-expression of secreted proteins from mammalian

cell lines. *Protein Sci.* **23,** 517–525 (2014).
41. Kim, T. K. & Eberwine, J. H. Mammalian cell transfection: the present and the future. *Anal. Bioanal. Chem.* **397,** 3173–8 (2010).
42. Huh, S.-H. *et al.* Optimization of 25 kDa linear polyethylenimine for efficient gene delivery. *Biologicals* **35,** 165–71 (2007).
43. Sou, S., Polizzi, K. & Kontoravdi, C. Evaluation of transfection methods for transient gene expression in Chinese hamster ovary cells. *Adv. Biosci.* **4,** 1013–1019 (2013).
44. Xie *et al.* PEI/DNA formation affects transeint gene expression in suspension Chinese hamster ovary cells via a one-step transfection process. *Cytotechnology* **65,** 263–271 (2013).
45. Wang, W. *et al.* Polyethylenimine-mediated gene delivery into human bone marrow mesenchymal stem cells from patients. *J. Cell. Mol. Med.* **15,** 1989–1998 (2011).
46. Vink, T., Oudshoorn-Dickmann, M., Roza, M., Reitsma, J. J. & de Jong, R. N. A simple, robust and highly efficient transient expression system for producing antibodies. *Methods* **65,** 5–10 (2014).
47. Leicester, U. of & Genie@le.ac.uk. Virtual Genetics Education Centre Home / Projects / VGEC / Genetics for Schools and Colleges / Topics for Schools and Colleges / The cell cycle,mitosis and meiosis. Available at: http://www2.le.ac.uk/projects/vgec/highereducation/topics/cellcycle-mitosis-meiosis.
48. Ruedel, A. & Bosserhoff, A. K. *Transfection Methods Overview*. *Methods in Cell Biology* **112,** (2012).
49. Xu, X. *et al.* The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat. Biotechnol.* **29,** 735–41 (2011).
50. Wurm, F. CHO Quasispecies—Implications for Manufacturing Processes. *Processes* **1,** 296–311 (2013).
51. Meleady, P. *Heterologous Protein Production in CHO Cells*. **1603,** (2017).
52. Sang, Y. *et al.* Salt ions and related parameters affect PEI–DNA particle size and transfection efficiency in Chinese hamster ovary cells. *Cytotechnology* **67,** 67–74 (2013).
53. Jorge, A. F. *et al.* Combining polyethylenimine and Fe(III) for mediating pDNA transfection. *Biochim. Biophys. Acta - Gen. Subj.* **1850,** 1325–1335 (2015).
54. Eberhardy, S. R., Radzniak, L. & Liu, Z. Iron (III) citrate inhibits polyethylenimine-mediated transient transfection of Chinese hamster ovary cells in serum-free medium. *Cytotechnology* **60,** 1–9 (2009).
55. Tom, R., Bisson, L. & Durocher, Y. Transfection of HEK293-EBNA1 cells in suspension with linear PEI for production of recombinant proteins. *Cold Spring Harb. Protoc.* **3,** 1–5 (2008).
56. Intra, J. & Salem, A. K. Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles in vitro and after intraperitoneal injection in vivo. *J. Control. Release* **130,** 129–138 (2008).
57. Cheraghi, R., Alipour, M., Nazari, M. & Hosseinkhani, S. Optimization of conditions for gene delivery system based on PEI. **4,** 8–16 (2017).
58. Kou, X., Zhang, W. & Zhang, W. Quantifying the Interactions between PEI and Double-Stranded DNA: Toward the Understanding of the Role of PEI in Gene Delivery. *ACS Appl. Mater. Interfaces* **8,** 21055–21062 (2016).
59. Ziebarth, J. D. & Wang, Y. Understanding the protonation behavior of linear

polyethylenimine in solutions through Monte Carlo simulations. **11,** 1–29 (2011).
60. Mady, M. M., Mohammed, W. a, El-Guendy, N. M. & Elsayed, a a. Effect of polymer molecular weight on the DNA/PEI polyplexes properties. *Rom. J. Biophys.* **21,** 151–165 (2011).
61. Ziebarth, J. D., Kennetz, D. R., Walker, N. J. & Wang, Y. Structural Comparisons of PEI / DNA and PEI / siRNA Complexes Revealed with Molecular Dynamics Simulations. (2017). doi:10.1021/acs.jpcb.6b10775
62. Carr, S. M. The major and minor grooves. (2011). Available at: https://www.mun.ca/biology/scarr/MGA2_02-07.html.
63. Ware, M. J., Summers, H. U. W. D. & Godin, B. in *Cationic Polymers in Regenerative Medicine Methods and Applications* (ed. Dubruel, S. K. S. and P.) 479–502 (2015).
64. Yameen, B. *et al.* Insight into nanoparticle cellular uptake and intracellular targeting. *J. Control. Release* **190,** 485–499 (2014).
65. Conner, S. D. & Schmid, S. L. Regulated portals of entry into the cell. *Nature* **422,** 37–44 (2003).
66. Hansen, C. G. & Nichols, B. J. Molecular mechanisms of clathrin-independent endocytosis. *J. Cell Sci.* **122,** 1713–1721 (2009).
67. Pelkmans, L. & Helenius, A. Endocytosis Via Caveolae. *Traffic* **3,** 311–320 (2002).
68. El-Aneed, A. An overview of current delivery systems in cancer gene therapy. *J. Control. Release* **94,** 1–14 (2004).
69. Fichter, K. M., Ingle, N. P., Mclendon, P. M. & Reineke, T. M. Exploit Active Interorganelle Trafficking Mechanisms. *Am. Chem. Soc.* A-R (2012).
70. Choudhury, C. K. & Roy, S. Structural and dynamical properties of polyethylenimine in explicit water at different protonation states: a molecular dynamics study. *Soft Matter* **9,** 2269 (2013).
71. Andersen, H., Parhamifar, L., Hunter, A. C., Shahin, V. & Moghimi, S. M. AFM visualization of sub-50 nm polyplex disposition to the nuclear pore complex without compromising the integrity of the nuclear envelope. *J. Control. Release* **244,** 24–29 (2016).
72. Hall, A., Lächelt, U., Bartek, J., Wagner, E. & Moghimi, S. M. Polyplex Evolution: Understanding Biology, Optimizing Performance. *Mol. Ther.* **25,** 1–15 (2017).
73. Derouazi, M. *et al.* Serum-free large-scale transient transfection of CHO cells. *Biotechnol. Bioeng.* **87,** 537–45 (2004).
74. Backliwal, G., Hildinger, M., Hasija, V. & Wurm, F. M. High density transfection with HEK 293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. *Biotechnol. Bioeng.* **99,** 721–727 (2008).
75. Hou, S. *et al.* Formation and structure of PEI/DNA complexes: quantitative analysis. *Soft Matter* **7,** 6967 (2011).
76. Zhou, H. *et al.* Linear polyethylenimine-plasmid DNA nanoparticles are ototoxic to the cultured sensory epithelium of neonatal mice. *Mol. Med. Rep.* **11,** 4381–4388 (2015).
77. Antila, H. S. & Ha, M. Chemistry specificity of DNA – polycation complex salt response : a simulation study of DNA , polylysine and polyethyleneimine †. *Phys. Chem. Chem. Phys.* **17,** 5279–5289 (2015).
78. Sun, C., Tang, T. & Uludag, H. Molecular Dynamics Simulations of PEI Mediated DNA Aggregation. (2011).

79. Hanzlíková, M., Ruponen, M., Galli, E. & Tenhu, H. Mechanisms of polyethylenimine-mediated DNA delivery : free carrier helps to overcome the barrier of cell-surface glycosaminoglycans. 402–409 (2011). doi:10.1002/jgm
80. Zhang, X. Z., Zeng, X., Sun, Y. X. & R. X. Zhuo. *Bioactive Materials in Medicine: Design and Applications*. (Woodhead, Cambridge, 2011).
81. Longo, P. A., Kavran, J. M., Ming-Sung, K. & Leahy, D. J. Transient mammalian cell transfection with polyethylenimine (PEI). *Methods Enzymol.* 227–240 (2013).
82. Zhang, X., Fernandez, I. & Wurm, F. M. Hyperosmolarity enhances transient recombinant protein yield in Chinese hamster ovary cells. 1587–1592 (2010). doi:10.1007/s10529-010-0331-8
83. Rajendra, Y., Kiseljak, D., Baldi, L., Hacker, D. L. & Wurm, F. M. A simple high-yielding process for transient gene expression in CHO cells. *J. Biotechnol.* **153,** 22–6 (2011).
84. Bertschinger, M., Schertenleib, A., Cevey, J., Hacker, D. L. & Wurm, F. M. The Kinetics of Polyethylenimine-Mediated Transfection in Suspension Cultures of Chinese Hamster Ovary Cells. *Mol. Biotechnol.* **40,** 136–143 (2008).
85. Bollin, F., Dechavanne, V. & Chevalet, L. Design of Experiment in CHO and HEK transient transfection condition optimization. *Protein Expr. Purif.* **78,** 61–8 (2011).
86. Cervera, L., Gutiérrez-Granados, S., Berrow, N. S., Segura, M. M. & Gòdia, F. Extended gene expression by medium exchange and repeated transient transfection for recombinant protein production enhancement. *Biotechnol. Bioeng.* **112,** 934–946 (2015).
87. Eriksson, L., Johansson, E., Kettaneh-Wold, N., Wikström, C. & Wold, S. *Design of Experiments: Principles and Applications*. (2008).
88. Otto, M. *Chemometrics: Statistics and Computer Application in Analytical Chemistry, 3rd Edition*. (2016).
89. Derynck, R. & Miyazono, K. in *Cold Spring Harbor Monograph Series 50* 29 (2008).
90. Govindena, R. & Bhoola, K. D. Genealogy, expression, and cellular function of transforming growth factor-β. *Pharmacol. Ther.* **2,** 257–265 (2003).
91. Derynck, R. *et al.* Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **22–28,** 701–5 (1985).
92. Miyazono, K., Hellman, U., Wernstedt, C. & Heldin, C. H. Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J. Biol. Chem.* **263,** 6407–6415 (1988).
93. Gentry, L. E. *et al.* Type 1 transforming growth factor beta: amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary cells. *Mol. Cell. Biol.* **7,** 3418–3427 (1987).
94. Zou, Z. & Sun, P. D. Overexpression of human transforming growth factor-β1 using a recombinant CHO cell expression system. *Protein Expr. Purif.* **37,** 265–272 (2004).
95. Maurya, V. K. *et al.* Transforming Growth Factor-Beta 1 (TGF-B1) Liberation from Its Latent Complex During Embryo Implantation and Its Regulation by Estradiol in Mouse. *Biol. Reprod.* **89,** 84–84 (2013).
96. Lafyatis, R. Transforming growth factor β—at the centre of systemic sclerosis. *Nat. Rev. Rheumatol.* **10,** 706–719 (2014).
97. Maurya, V. K. *et al.* Transforming Growth Factor-Beta 1 (TGF-B1) Liberation from Its Latent Complex During Embryo Implantation and Its Regulation by Estradiol in Mouse.

Biol. Reprod. **89,** 84–84 (2013).
98. Barnard, J. a, Lyons, R. M. & Moses, H. L. The cell biology of transforming growth factor β. *Biochim. Biophys. Acta - Rev. Cancer* **1032,** 79–87 (1990).
99. Ramani, B. & Kondaiah, P. Recombinant expression of human transforming growth factor-beta isoforms in Chinese hamster ovary cells. *J. Biosci.* **23,** 577–583 (1998).
100. Abdulkerim, E., Baganz, S., Schambach, A., Kasper, C. & Scheper, T. Production and purification of TGFb-1 in CHO-Cells. *BMC Proc.* **5,** P134 (2011).
101. Agrawal, V. *et al.* A High-Yielding, CHO-K1–Based Transient Transfection System Rapid Production for Therapeutic Protein Development. *BioProcess Int. BioProcess Tech.* **11,** (2013).
102. Wakefield, L. M., Smith, D. M., Flanders, K. C. & Sporn, M. B. Latent Transforming Growth Factor-beta from Human Platelets. *J. Biol. Chem.* **263,** 7646–7654 (1988).
103. Li, C. Y., Suardet, L. & Little, J. B. Potential role of WAF1/Cip1/p21 as a mediator of TGF-ß cytoinhibitory effect. *Journal of Biological Chemistry* **270,** 4971–4974 (1995).
104. Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-. *Nature* **425,** 577–584 (2003).
105. Zhang, Y., Alexander, P. B. & Wang, X. TGF- b Family Signaling in the Control of Cell Proliferation and Survival. *Cold Spring Harb. Lab. Press* 1–23 (2017).
106. Zheng, X. *et al.* Evaluation of the transforming growth factor-beta activity in normal and dry eye human tears by CCL-185 cell bioassay. *Cornea* **29,** 1048–54 (2010).
107. Khan, S. A., Joyce, J. & Tsuda, T. Quantification of active and total transforming growth factor-β levels in serum and solid organ tissues by bioassay. *BMC Res. Notes* **5,** 636 (2012).
108. Isabel Fuentes, C. & Carlos Martínez, S. TGFB1 (transforming growth factor, beta 1). *Atlas Genet. Cytogenet. Oncol. Haematol.* **1,** 543–549 (2013).
109. Manuscript, A. Fusion Protein Linkers: Property, Design and Functionality. *NIH Public Access* **65,** 1357–1369 (2014).
110. Terpe, K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* **60,** 523–533 (2003).
111. Siepert, E.-M. *et al.* Short-chain fluorescent tryptophan tags for on-line detection of functional recombinant proteins. *BMC Biotechnol.* **12,** 65 (2012).
112. Vivian, J. T. & Callis, P. R. Mechanisms of tryptophan fluorescence shifts in proteins. *Biophys. J.* **80,** 2093–2109 (2001).
113. Kensy, F., Zang, E., Faulhammer, C., Tan, R.-K. & Büchs, J. Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. *Microb. Cell Fact.* **8,** 31 (2009).
114. Samorski, M., Müller-Newen, G. & Büchs, J. Quasi-continuous combined scattered light and fluorescence measurements: A novel measurement technique for shaken microtiter plates. *Biotechnol. Bioeng.* **92,** 61–68 (2005).
115. Schmidt, T. G. M. *et al.* Development of the Twin-Strep-tag® and its application for purification of recombinant proteins from cell culture supernatants. *Protein Expr. Purif.* **92,** 54–61 (2013).
116. Www.stargate.com. *StarGate ® The new dimension of combinatorial cloning Instruction manual*. (2013).
117. Manual, I. *The new dimension of combinatorial cloning*. (2011).

118. Polyethylenimine (PEI), linear (1 mg/mL). *Cold Spring Harb. Protoc.* **2008,** pdb.rec11323-rec11323 (2008).
119. Qiagen. QIAGEN ® Plasmid Purification Handbook Sample & Assay Technologies QIAGEN Sample and Assay Technologies. (2012).
120. Xie, Q. *et al.* TubeSpin bioreactor 50 for the high-density cultivation of Sf-9 insect cells in suspension. *Biotechnol. Lett.* **33,** 897–902 (2011).
121. De Jesus, M. J. *et al.* TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology. *Biochem. Eng. J.* **17,** 217–223 (2004).
122. Protocol, S. (Twin-) Strep -tag ® Purification Short Protocol (Twin-) Strep -tag ® Purification Short Protocol. 1–3 (2012).
123. Geng, Z.-H., Nudson, W., Davis, L., Luo, S. & Etchberger, K. *Optimizing Medium Components for Polyethylenimine Mediated Transient Transfection*. (JRH Biosciences, Inc., 2005).
124. Sandor, M. *et al.* Comparative study of non-invasive monitoring via infrared spectroscopy for mammalian cell cultivations. *J. Biotechnol.* **168,** 636–645 (2013).
125. Cervera, L. *et al.* Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. *J. Biotechnol.* **166,** 152–65 (2013).
126. Godbey, W. T., Barry, M. a., Saggau, P., Wu, K. K. & Mikos, A. G. Poly(ethylenimine)-mediated transfection: A new paradigm for gene delivery. *J. Biomed. Mater. Res.* **51,** 321–328 (2000).
127. Florea, B. I., Meaney, C., Junginger, H. E. & Borchard, G. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. *AAPS PharmSci* **4,** E12 (2002).
128. Pereira, J., Rajendra, Y., Baldi, L., Hacker, D. L. & Wurm, F. M. Transient gene expression with CHO cells in conditioned medium: a study using TubeSpin(®) bioreactors. *BMC Proc.* **5 Suppl 8,** P38 (2011).
129. Schlaeger, E. J. & Christensen, K. Transient gene expression in mammalian cells grown in serum-free suspension culture. *Cytotechnology* **30,** 71–83 (1999).
130. Zaric, V. *et al.* Effective polyethylenimine-mediated gene transfer into human endothelial cells. *J. Gene Med.* **6,** 176–184 (2004).
131. Clamme, J. P., Azoulay, J. & Me, Y. Monitoring of the Formation and Dissociation of Polyethylenimine / DNA Complexes by Two Photon Fluorescence Correlation Spectroscopy. **84,** 1960–1968 (2003).
132. Ozturk, S. S. & Palsson, B. O. Effect of initial cell density on hybridoma growth, metabolism, and monoclonal antibody production. *J. Biotechnol.* **16,** 259–78 (1990).
133. De Los Milagros Bassani Molinas, M., Beer, C., Hesse, F., Wirth, M. & Wagner, R. Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring. *Cytotechnology* **66,** 493–514 (2014).
134. Männistö, M. *et al.* The role of cell cycle on polyplex-mediated gene transfer into a retinal pigment epithelial cell line. *J. Gene Med.* **7,** 466–476 (2005).
135. Brunner, S., Fürtbauer, E., Sauer, T., Kursa, M. & Wagner, E. Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation. *Mol. Ther.* **5,** 80–86 (2002).
136. Abbott, W. M. *et al.* Optimisation of a simple method to transiently transfect a CHO cell

line in high-throughput and at large scale. *Protein Expr. Purif.* **116,** 113–119 (2015).
137. Bollin, F., Dechavanne, V. & Chevalet, L. Design of Experiment in CHO and HEK transient transfection condition optimization. *Protein Expr. Purif.* **78,** 61–8 (2011).
138. Chiou, H. *et al.* in *Scalable transient protein Expression.* 1104:35-55. (2014). doi:10.1007/978-1-62703-733-4_4.
139. Kunaparaju, R., Liao, M. & Sunstrom, N.-A. Epi-CHO, an episomal expression system for recombinant protein production in CHO cells. *Biotechnol. Bioeng.* **91,** 670–7 (2005).
140. Shen, X. *et al.* A simple plasmid-based transient gene expression method using High Five cells. *J. Biotechnol.* **216,** 67–75 (2015).
141. McMahon, G. a, Dignam, J. D. & Gentry, L. E. Structural characterization of the latent complex between transforming growth factor beta 1 and beta 1-latency-associated peptide. *Biochem. J.* **313 (Pt 1,** 343–51 (1996).
142. Olofsson, A. *et al.* cells contains the multifunctional cysteine-rich fibroblast growth factor. **434,** 427–434 (1997).
143. Janssens, K., Ten Dijke, P., Ralston, S. H., Bergmann, C. & Van Hul, W. Transforming growth factor-β1 mutations in Camurati-Engelmann disease lead to increased signaling by altering either activation or secretion of the mutant protein. *J. Biol. Chem.* **278,** 7718–7724 (2003).
144. Shi, M. *et al.* Latent TGF-β structure and activation. *Nature* **474,** 343–349 (2011).
145. Gray, A. M. & Mason, A. J. Requirement for activin A and transforming growth factor--beta 1 pro-regions in homodimer assembly. *Science* **247,** 1328–1330 (1990).
146. Munger, J. S. *et al.* The integrin alpha v beta 6 binds and activates latent TGF?1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96,** 319–328 (1999).
147. Beatson, R. *et al.* Transforming growth factor-ß1 is constitutively secreted by chinese hamster ovary cells and is functional in human cells. *Biotechnol. Bioeng.* **108,** 2759–764 (2011).

List of Puplication

Papers and Proceedings

- Abdalla Elshereef*, André Jochums, Antonina Lavrentieva, Janina Bahnemann, Dörte Solle, Thomas Scheper: conference paper for BMC Proceedings 2017 “Optimized transfection efficiency for CHO-K1 suspension cells through combination of transfection and culture media”. (submitted)
- Abdalla Elshereef*, Antonina Lavrentieva, Dörte Solle, Thomas Scheper: A High Cell Density Transient Transfection CHO cell System as a tool for TGF-β1 protein expression, Electronic Journal of Biotechnology. (on preparation)

Poster presentations

- Proceeding of the 25th Annual Meeting of the European Society for Animal Cell Technology (ESACT) May,14-17, 2017, Swiss Tech Convention Center, Lausanne, Switzerland.(Published)

Curriculum Vitae

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