TRANSIENT GENE EXPRESSION FOR RAPID PROTEIN PRODUCTION: STUDIES & OPTIMIZATIONS UNDER SERUM-FREE CONDITIONS

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PAR

Natalie MULLER

B.Sc. in Chemical Engineering, Wayne State University, Detroit, Michigan, Etats-Unis
et de nationalité américaine

acceptée sur proposition du jury:

Prof. F. Wurm, directeur de thèse
Dr S. Geisse, rapporteur
Prof. K. Johnsson, rapporteur
Dr I. Luescher, rapporteur

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This work is dedicated to the memory of Vincent Frank Meli

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Summary

Recombinant proteins are important for biomedical research and for the treatment of human disease. Therefore it is necessary to develop reproducible bioprocesses to rapidly produce proteins of adequate quality and quantity. Expression in mammalian cells is preferred if the proteins are to be properly folded and post-translationally modified. For the rapid production of milligram to gram quantities of a protein in mammalian cells, large-scale transient gene expression is an attractive option. The two most important components of this technology are the cell cultivation system and the gene delivery method. For this reason the goal of this thesis was to develop novel cost-efficient cell cultivation systems and gene delivery methods for the large-scale transfection of mammalian cells in chemically defined media free of any animal-derived components including serum.

Cultivation of mammalian cells in suspension is essential for large-scale transient gene expression. A superior system for the cultivation of mammalian cells based on the agitation of cells in “square-shaped” glass bottles (square bottles) was developed. It is an inexpensive but efficient means to grow cells to a high density without instrumentation. The growth and viability of cultures of both human embryo kidney (HEK) 293E and Chinese hamster ovary (CHO) DG44 cells in agitated one-liter square bottles exceeded that in spinner flasks, reaching cell densities 1.5 times higher on average. Furthermore, an efficient and reproducible method to transfect cells in agitated square bottles was developed for both HEK 293E and CHO DG44 cells.

A novel gene delivery method termed calfection that relies on the addition of DNA and $CaCl_2$ directly to a suspension culture was developed. Calfection has a number of advantages over existing gene delivery methods such as calcium phosphate-DNA coprecipitation in that there are no time-dependent steps. The DNA/CaCl2 solution can be stored for long periods and is filterable. It is suitable for both large-scale transfections in bioreactors and for high-throughput transfections in microtiter plates. One drawback, however, is its dependence on the presence of serum in the culture medium.

A second gene delivery method, serum-free transfection with polyethylenimine (polyfection), proved to be highly efficient for the transfection of both HEK 293E and CHO DG44 cells. A reproducible method for polyfection in microtiter plates, 50 ml centrifuge tubes, agitated square and round bottles, spinner flasks, and bioreactors was optimized for these two cell lines. Polyfection proved to be a simple and successful gene transfer method in both instrumented and non-instrumented cultivation systems. Importantly, it could be performed in serum-free chemically defined media. With HEK 293E cells, 80 mg/l of antibody was produced in agitated square bottles and with CHO DG44 cells, more than 2 g of antibody were produced in one week in a 150-liter bioreactor.

Résumé

Les protéines recombinants sont devenues incontournables pour la recherche bio-médicale ainsi que la prévention et le traitement de nombreuses maladies. C'est pourquoi, il est nécessaire de développer des procédés permettant la production quantitative de ces protéines à qualité constante. L'utilisation de systèmes de production dérivant de cellules de mammifères permet de reproduire les modifications post-translationnelles et d'obtenir un repliement adéquat des protéines. L'expression transitoire d'un transgène à grande échelle permet la production rapide de quantité de protéines allant du milligramme au gramme. Les deux paramètres fondamentaux de cette technologie sont le système de culture cellulaire et le transfert de gènes dans les cellules. Ce travail a pour but d'améliorer ces deux paramètres, d'une part en développant un nouveau système de culture de faible coût, et d'autre part en optimisant le système de transfert de gènes dans les cellules de mammifères en milieu chimiquement défini.

La culture de cellules mammifères en suspension est indispensable pour l'expression transitoire de gènes à grande échelle. Pour répondre à cette exigence, nous avons développé un système performant pour la culture de cellules mammifères sur support mobile et dans des bouteilles en verre dont la forme carrée assure une agitation optimale. Il s'agit d'un moyen peu coûteux permettant la culture de cellules à forte densité dans un bio-réacteur non instrumenté. La croissance et la viabilité de cellules de reins d'embryons humains (HEK) 293E et des cellules d'ovaires de hamster chinois (CHO) DG44 cultivées en bouteille carrée de un litre dépassent celles d'une culture en réacteur de type spinner, atteignant une densité en moyenne 1.5 fois supérieure.

Deux méthodes de transfection ont été développées pour assurer un transfert efficace de gènes dans des cellules HEK 293E et CHO DG44 cultivées en bouteilles carrées sous agitation.

La première, dénommée calfection, repose sur l'addition d'ADN et de $CaCl_2$ directement dans la suspension cellulaire. Contrairement à la méthode classique de coprécipitation calcium phosphate-ADN, la calfection se fait sans précipitation de l'ADN permettant d'éviter l'étape critique du temps de maturation du complexe ADN-agent de transfection. De plus, la solution de chlorure de calcium contenant l'ADN peut-être filtrée et stockée sur de longues périodes. La calfection est particulièrement adaptée pour les transfections à grande échelle en bio-réacteurs ou pour les boîtes de culture de faible contenant. L'inconvénient de cette technique est qu'elle nécessite un milieu sans sérum.

La seconde méthode de transfection, dénommée polyfection, s'appuie sur le polyéthylénimine comme agent de transfection. Cette méthode s'avère particulièrement efficace pour le transfert de gène dans les cellules HEK 293E et les CHO DG44 cultivées sans sérum en systèmes instrumentés ou non-instrumentés. Une méthode reproductible pour la polyfection en boîte de culture de faible contenant, en tube de centrifugation de 50 mL, en bouteilles agitées carrées ou rondes, en spinner et en bio-réacteur, a été optimisée pour ces deux lignées. La polyfection des cellules

HEK 293E en bouteilles carrées agitées a permis de produire jusqu'à 80 mg/l d'anticorps. La polyfection des cellules CHO DG44 en bioréacteur de 150 litres a conduit à la production de 2 g d'anticorps en une semaine.

Zusammenfassung

Rekombinante Proteine sind wichtig für die biomedizinische Forschung und für die Behandlung von Krankheiten. Es ist deswegen wichtig Fermentationsprozesse zu entwickeln, die Proteine in angemessener Qualität und Quantität reproduzierbar und schnell bereitstellen können. Die Expression von Proteinen in Säugetierzellen ist bevorzugt, wenn die Proteine korrekt gefaltet und post-translational modifiziert werden müssen. Für die schnelle Produktion von milligram- bis gram- Mengen eines rekombinanten Proteins ist die transiente Gen Expression im grossen Masstab eine sehr attraktive Möglichkeit.

Die zwei entscheidenden Parameter der transienten Gen Expression sind das Zellkultivierungssystem und der Transfer von Plasmid DNA in die Zelle (Gene Delivery) . Aus diesem Grund war das Ziel dieser Doktorarbeit die Entwicklung von neuen Methoden der kostengünstigen Zellkultivierung als auch des Gene Delivery für die Transfektion von Säugetierzellen im grossen Massstab in chemisch definierten Medien ohne Zusatz von aus Tieren gewonnenen Bestandteilen (einschliesslich Serum).

Die Kultivierung von Säugetierzellen in Suspension ist essentiel für die transiente Transfektion im grossen Massstab. Für die Kultivierung von Säugetierzellen wurde eine neue Technik entwickelt, die auf dem orbitalen Schütteln von Zellen in Flaschen mit quadratischem Querschnitt (Square bottles) basiert. Dies ist eine billige, aber effiziente Möglichkeit Zellen in hoher Dichte ohne aufwendige Technik zu kultivieren. Das Wachstum und die Viabilität von Human Embryo Kidney (HEK) und Chinese Hamster Ovary (CHO) DG44 Zellen in geschüttelten Einliter Square Bottles waren besser als in vergleichbaren Spinnern. Die dabei erreichten Zelldichten lagen bis zu 1.5 fach höher.

Eine neue Transfektionsmethode wurde entwickelt, um HEK 293E and CHO DG44 Zellen in geschüttelten Square bottles zu transfizieren, die auf der direkten Zugabe von DNA und CaCl2 zu der Suspensionskultur beruht. Diese Methode wurde Calfection genannt. Sie hat zahlreiche Vorteile im Vergleich zu den bereits existierenden Gene Delivery Methoden, wie zum Beispiel der Kalzium Phosphat Transfektion. Bei der Calfection gibt keine zeitabhängigen Schritte in der Prozedur, die DNA/CaCl2 Lösung kann für lange Zeit aufbewahrt werden und ist filterbar. Die Transfektionsmethode ist einsetzbar sowohl für Transfektionen im grossen Massstab im Bioreaktor, als auch für Transfektion im Mikrotiter Platten Massstab im hohen Durchsatz. Ein Nachteil jedoch ist die Notwendigkeit von Serum im Zellmedium.

Eine zweite Transfektionsmethode ist die serumfreie Transfektion mit Polyethylenimine (Polyfection). Diese Methode ist sehr effizient für die Transfektion von HEK 293E und von CHO DG44 Zellen. Die Polyfektion von Mikrotiter Platten, 50 ml Zentrifugationsröhrchen, geschüttelten Flaschen mit rundem oder quadratischem Querschnitt, Spinnerflaschen und Bioreaktoren wurde optimisiert für diese beiden Zelllinien. Dabei zeigte die Polyfection äusserst reproduzierbare Ergebnisse. Polyfection ist eine einfache und erfolgreiche Transfektionsmethode in

sowohl geregelten als auch ungeregelten Kultivierungssystemen. Ein äusserst wichtiger Vorteil ist, dass die Transfektion in serumfreien, chemisch definierten Medium durchgeführt werden konnte. Mit Hek 293E Zellen wurden 80 mg/L eines Antikörpers in geschüttelten Square bottles produziert und mit CHO DG44 Zellen in einem 150 Liter Bioreaktor in einer Woche mehr als 2 g eines Antikörpers produziert.

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1. Abbreviations

CaPi	calcium phosphate-DNA-coprecipitation
CHO	Chinese hamster ovary cells
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
GFP	green fluorescent protein
HEK 293E	human embryo kidney cells 293 EBNA
IgG	immunoglobin G
PEI	polyethylenimine

Introduction

2. Introduction

The need for recombinant proteins (r-proteins) in fundamental research and for clinical applications is continually increasing. R-protein expression requires the introduction of foreign DNA into a host cell, a process called transformation when applied to prokaryotes and transfection when applied to eukaryotic cells. Although many hosts are available for r-protein expression, cultivated mammalian cells are often the primary choice when functional protein folding and post-translational modifications are required.
The first recombinant protein product from mammalian cells to achieve market approval was human tissue plasminogen activator (tPA, Activase; Genentech, S. San Francisco, CA, USA) in 1986. For six of the past seven years, an average of 7-9 r-proteins have been approved in the U.S. per year (Pavlou and Reichert 2004). Approximately 300 monoclonal antibodies (mAbs) and 440 r-protein products entered clinical trials between January 1980 and May 2004. Of these, 130 mAbs and 140 r-proteins are currently under study in humans (Reichert 2004). These recombinant proteins are produced in a variety of hosts including: *Escherichia coli, Sacchoromyces cerevisiae*, plants, insect cell lines, and mammalian cell lines. The most widely used cell lines are mouse NSO, baby hamster kidney (BHK), Chinese hamster ovary (CHO), mouse SP2/0, and human embryonic kidney (HEK 293) cells. To date, all r-proteins expressed in mammalian cells are from stable cell lines in which the recombinant gene is integrated into the host's genome. Although this is the standard approach for market-scale production, r-protein expression from transiently transfected mammalian cells allows the production of milligram to gram quantities of protein in a short timeframe (days to weeks) at scales up to 100s of liters. In the future, r-proteins produced by large-scale transient transfection may gain regulatory approval. Improving this technology to achieve higher protein yields is the goal of this thesis.

2.1 Production of recombinant proteins

E. coli is a very attractive host because of its ability to grow rapidly in inexpensive media. In addition, *E. coli* is a well-characterized microorganism both genetically and physiologically (Baneyx 1999). Due to the ease of genetic manipulation, short doubling time, capacity for high-level protein expression, and low cost, *E. coli* is the most widely used host for r-protein production (Dai, Chen et al. 2005). R-proteins expressed in *E. coli*, however, usually lack correct post-translational modifications, are often misfolded, and form insoluble aggregates (Clark 2001). Unlike prokaryotes, eukaryotes are able to perform a number of post-translational modifications including disulfide bridge formation, proteolytic cleavage, and glycosylation, yielding stable and functional mature proteins (Glick and Pasternak 1998).
R-protein expression in yeast is usually performed in either *Pichia pastoris* or baker's yeast *S. cerevisiae* (Cereghino and Cregg 1999). Fungi offer fast, economical growth and are easy to genetically manipulate compared to other eukaryotic hosts. Fungi possess the ability to perform many post–translational modifications including simple glycosylation (Eckart and Bussineau 1996). Their inability to perform certain complex post-transcriptional modifications, such as prolyl hydroxylation and

amidation as well as some types of phosphorylation and glycosylation also limit their utility (Cregg and Higgins 1995).
Cultivated insect cell lines are a prominent host for r-protein expression, usually via infection with a recombinant baculovirus vector. One advantage is that they can easily be cultivated at very high cell densities. The drawbacks of insect cell expression systems are the limited capacity to properly process proteins that are synthesized as larger inactive precursor proteins due to the absence of pro-protein convertases. Protein yield may also be limited due to accumulation of insoluble protein within the cells (Kost and Condreay 1999). Differences in glycosylation patterns in comparison to mammalian cells are also problematic (James, Freedman et al. 1995).
Plant cell suspension cultures especially those from tobacco and genetically modified plants are being used for r-protein expression (Doran 2000). The major advantages associated with *in vitro* plant systems include the rapidity of protein production and the ability to manipulate environmental conditions for better control over protein production and quality. The drawbacks are the relatively slow growth of plant cells and differences in glycosylation compared to mammalian cells (Doran 2000). Most efforts to genetically modify plants were done to improve resistance against pest and extreme environmental conditions or to enhance flavor and appearance (Herbers and Sonnewald 1999). The same genetic engineering tools were used to generate plants expressing foreign proteins (Hood and Jilka 1999). The major advantage of transgenic plants is their ease of cultivation and cost-effectiveness. However, protein purification is more difficult than for cultivated plant cells (Hood and Jilka 1999).
Mammalian cells can produce the most therapeutically effective r-proteins because they can perform the necessary post-translational modifications most accurately among the various hosts mentioned here. Theoretically, any cell which can be maintained in culture can also be manipulated to express foreign genes. Unfortunately, mammalian cells are also expensive to maintain in culture and grow more slowly in comparison to the other hosts mentioned above. Finally it has to be mentioned that mammalian cells are susceptible to infection with mammalian viruses, potentially leading to a contamination of the protein product.

2.2 Transient gene expression

As noted above, the main approach to the expression of r-proteins in mammalian cells remains the establishment of a cell line in which the recombinant gene(s) is integrated into the host genome and stably expressed over time, but the identification and characterization of stable cell lines is a costly and time consuming process. This labor intensive process for producing an r-protein is based on the isolation, characterization, and expansion of a highly productive clonal cell line from a pool of transfected cells. In all, this is a very lengthy process that can take up to 18 months to complete.
It may frequently be necessary to rapidly evaluate many candidate proteins or several variants of a single candidate protein for their potential as therapeutics. Furthermore, for some therapeutic proteins a small quantity may be sufficient to meet the desired needs. This is foreseeable for applications in individualized medicine (Pereira, Botelho et al. 2004; Muller-Zellenberg and Muller 2005). For these examples rapid approaches to the expression of milligram to gram quantities of a recombinant protein are needed. One promising method is transient gene expression (TGE) in transfected

mammalian cells (Wurm and Bernard 1999). Using low cost DNA delivery agents such as calcium phosphate (Jordan, Schallhorn et al. 1996) and polyethylenimine (PEI) (Boussif, Lezoualc'h et al. 1995; Akinc, Thomas et al. 2004), suspension cultures of mammalian cells at volumes ranging from 1 ml to 100 L have been transiently transfected to produce recombinant proteins in the mg/L range in 5-10 days after transfection (Schlaeger and Christensen 1999; Meissner, Pick et al. 2001; Durocher, Perret et al. 2002; Girard, Derouazi et al. 2002; Pham, Perret et al. 2003; Schlaeger, Kitas et al. 2003; Derouazi, Girard et al. 2004; Baldi, Muller et al. 2005). Transient expression in mammalian cells does not require stable DNA integration (Paborsky, Fendly et al. 1990). After the DNA enters the cell's nucleus, transcription of the transgene starts, and r-protein synthesis begins. Usually the protein can be detected within a few hours after transfection.
Although TGE was originally used for adherent cultures, the demand for larger quantities of r-proteins led to the development of large-scale transient expression in suspension cultures (Wurm and Bernard 1999). Several transfection techniques with both viral and non-viral vectors have been optimized for suspension cells. Only the latter are discussed here, however.

2.3 Non-viral gene delivery

Non-viral gene transfer methods can be divided into two areas: mechanical and non-mechanical. Mechanical methods include intracellular microinjection of DNA and continuous flow electroporation (Parham, Iannone et al. 1998). Some examples of non-mechanical methods include calcium phosphate-DNA coprecipitation (CaPi), lipofection, and polyfection. All three methods rely on the formation of positively charged nanoparticles which can enter cells after binding to the negatively charged cell surface. CaPi transfection is based on the co-precipitation of DNA with calcium phosphate (Jordan, Schallhorn et al. 1996) while lipofection and polyfection are based on the compaction of DNA with liposomes (lipoplexes) and cationic polymers (polyplexes), respectively(Graham and Eb 1973; Jordan, Schallhorn et al. 1996; Thomas and Klibanov 2003). In general, the transfection method must be chosen with regard to the cell line and the application.
Non-viral transfection methods profit from a greater flexibility in the size and number of genes to be transferred as well as the general absence of biosafety issues. The low efficiency of non-viral gene delivery methods is due to the existence of several physical and chemical barriers in cells (Lechardeur, Sohn et al. 1999; Lukacs, Haggie et al. 2000; Hong, Sherley et al. 2001; Chan and Jans 2002; Wiethoff and Middaugh 2003). Contact between the DNA and the cell membrane, transit of the DNA across the cell membrane, escape from endosomal/lysosomal compartments, transport across the cytoplasm and entry into the nucleus are some of these barriers. Different synthetic transfection reagents e.g cationic lipids, cationic polymers (Kircheis, Wightman et al. 2001; Thomas and Klibanov 2003), and calcium phosphate-DNA coprecipitates (Graham and van der Eb 1973; Jordan, Schallhorn et al. 1996) are used to improve the efficiency with which these barriers are overcome. The reason these transfection agents are believed to be successful is that they condense DNA into compact particles, which facilitates the interaction between transfection particles and the cellular surface, stimulating the internalization of the carrier-DNA complex. The

condensing agent may also provide protection from nucleases once the DNA is internalized. A high percentage of the DNA that enters the cell, however, is degraded before it reaches the nucleus (Godbey, Barry et al. 2000; Pollard, Toumaniantz et al. 2001).
Following uptake by endocytosis, the DNA must escape the endosome to be released into the cytosol and enter the nucleus. For each transfection method, the mechanisms involved in these events may differ. Another factor is cell cycle dependency. The efficiency of gene delivery by CaPi, lipofection and polyfection has been shown to be cell cycle-dependent (Lechardeur, Sohn et al. 1999; Brunner, Furtbauer et al. 2002; Mannisto, Ronkko et al. 2005). Higher recombinant protein expression is observed after CaPi transfection of adherent CHO cells during the S phase of the cell cycle (Brunner, Sauer et al. 2000). Transfection with liposomes and branched PEI is more efficient when added to cells in late S phase (Grosjean, Batard et al. 2002). These observations indicate that for all of these transfection methods plasmid DNA probably enters the nucleus after the breakdown of the nuclear membrane during mitosis. The exact mechanisms involved in these events remain to be investigated.

2.3.1 CaPi

CaPi transfection is well established for many different cell lines at many scales of operation up to 100 liters (Brunner, Sauer et al. 2000). Until recently when small modifications that improved the efficiency of CaPi were introduced, the protocol was very similar to the original method, using the same concentrations of calcium and phosphate (Graham and van der Eb 1973; Jordan, Schallhorn et al. 1996; Mannisto, Ronkko et al. 2005). The DNA/CaPi is produced by first mixing $CaCl_2$ and DNA and then adding phosphate to form the precipitate. The DNA is immediately adsorbed to or entrapped in the CaPi precipitate (Jordan, Schallhorn et al. 1996; Wurm and Jordan 1996; Jordan, Kohne et al. 1998; Urabe, Kume et al. 2000). After reaching the desired size the DNA/CaPi complexes are added to the cell culture. It has been suggested that CaPi/DNA particles are taken up by energy-dependent phagocytosis (Loyter, Scangos et al. 1982). Within the cell the precipitates are contained in lysosome-like vesicles within the cytoplasm. The DNA is then released into the cytoplasm, either as the CaPi particles dissolve due to pH reduction in the vesicles, or directly in the cytoplasm, after rupture of the vesicle due to the calcium concentration. The DNA probably enters the nucleus at the time of nuclear membrane breakdown during mitosis (Loyter, Scangos et al. 1982; Jordan, Schallhorn et al. 1996).
Although the CaPi transfection is a simple method, several critical parameters need to be carefully optimized in order to obtain reproducible transfection efficiencies. These include cell density, pH of the phosphate solution, pH of the medium, time of precipitate formation, calcium and phosphate concentrations, and time of medium dilution. Of these, the most critical one is the time of precipitate formation. Reduction of the DNA/CaPi complex formation time from 20-30 min to 1 min and addition of the phosphate rapidly instead of drop-wise resulted in a small and effective precipitate (Loyter, Scangos et al. 1982). One group suggested forming the precipitate at 50°C instead of at room temperature for the transfection of insect cells (Loyter, Scangos et al. 1982). It was also proposed to replace the calcium with the more soluble strontium (Loyter, Scangos et al. 1982). The most widely optimized parameters for CaPi/DNA precipitate formation are the pH of the phosphate buffer and the DNA

concentration (Loyter, Scangos et al. 1982; Brash, Reddel et al. 1987; Kjer and Fallon 1991; Jordan, Schallhorn et al. 1996). Adjustments in the Ca^{2+} concentration also affect the transfection efficiency (Graham and van der Eb 1973). The major drawback of this method, however, is its reliance on serum in the culture medium. There have been attempts to remove the serum but transfection efficiencies were greatly compromised (Chu and Sharp 1981).

2.3.2 Calfection

For most transfection methods there are usually one or more procedural steps which is time-dependent (Chen and Okayama 1987; O'Mahoney and Adams 1994). Additionally, the complexes usually have to be added to cells while freshly prepared, since storage of active vehicle complexes appears to be reduce their activity (Cohen, Kahn et al. 2000; Girard, Porte et al. 2001). Therefore a new method was developed to eliminate time critical steps. Calfection involves the simple addition of a mixture of DNA and $CaCl_2$ directly to the culture medium. This method was shown to be simple, reproducible, and scaleable in suspension cultures (Kircheis, Wightman et al. 2001). It does not involve any time-dependent steps, making it convenient for serial transfections and large-scale transfections. Other advantages include the one-step preparation of the active transfection solution and the feasibility of its storage for long periods without any negative effects on transfection efficiency. Calfection was shown to be effective for transient gene expression in HEK 293, BHK 21, and CHO DG44 cells with reporter protein expression at levels comparable to those observed with calcium phosphate-DNA coprecipitation. The major drawback is its requirement for serum in the medium. Calfection is described in more detail in Chapter 4.

2.3.3 PEI

It is generally believed that transfection with PEI involves the formation of positively charged nanoparticles containing DNA which enter cells after binding to the negatively charged cell surface and endocytosis (Jordan, Schallhorn et al. 1996; Seelos 1997; Brown, Schatzlein et al. 2001). Once inside the cell, the cationic PEI is thought to promote escape of DNA from the endosome by binding protons following endosome acidification, promoting an influx of chloride ions. This “proton sponge effect” results in osmotic swelling of the endosome and subsequent disruption of the endosome, allowing the release of the DNA into the cytoplasm (Lindell, Girard et al. 2004). It is believed that PEI can help promote DNA delivery to the nucleus (Akinc, Thomas et al. 2004). Much has been published on the formation of the complex and its intracellular transport (Boussif, Lezoualc'h et al. 1995; Dunlap, Maggi et al. 1997; Pollard, Remy et al. 1998; Choosakoonkriang, Lobo et al. 2003). Many different cell types have been successfully transfected with PEI in the presence and absence of serum at many scales of operation (Boussif, Lezoualc'h et al. 1995; Godbey, Wu et al. 1999; Godbey, Wu et al. 1999; Godbey, Wu et al. 1999; Godbey, Wu et al. 1999; Durocher, Perret et al. 2002; Derouazi, Girard et al. 2004).

2.4 Culture vessels and cell lines for transient gene expression.

The classical cultivation apparatus at moderate scales of operation for transient transfection is the glass spinner flask with a magnetically-driven impeller for mixing. Spinner flasks are less expensive and easier to handle than glass or stainless steel bioreactors. Nevertheless, there is a considerable amount of work associated with their cleaning and sterilization. In addition, the magnetic stirrers and the glass spinners themselves are expensive and require a large amount of incubator space.
The major alternatives to spinner flasks and bioreactors for cultivating mammalian cells in suspension are culture vessels (Erlenmeyer flasks or multiwell plates) agitated on an orbital shaker. This method has mainly been developed for bacterial and fungal cultures, but applications for animal cells have also been reported (Schlaeger and Christensen 1999; Pham, Perret et al. 2003). For bacterial and fungal cultures grown in shaken systems, an important parameter for maximum cell growth is oxygen transfer. In Erlenmeyer flasks under conditions of high cell density, oxygen may become limiting. In spinner flasks, oxygen limitations have been observed in the growth for insect cells (Schlaeger, Kitas et al. 2003). Operating parameters that have a considerable impact on oxygen transfer rate and thus on cell growth and viability include the flask size and shape, the orbital shaking speed and diameter, the filling volume, and the surface properties of the flask material (Girard, Jordan et al. 2001; Liu and Hong 2001; Annathur, Pierce et al. 2003; Thomas and Klibanov 2003).
In this thesis a new cultivation system was developed for the maintenance of suspension cultures of mammalian cells in commercially available square-shaped bottles (square bottles). These were mounted and agitated on an orbital shaker within a temperature-controlled chamber. Due to their geometry, square bottles have negative baffles which are expected to increase the surface renewal of the medium, resulting in improved mixing and gas exchange within the cultivation system. Effects of filling volume, agitation speed, and container geometry, size, and composition on cell growth and viability have been studied (Buchs 2001). Cultures of HEK 293 EBNA (HEK 293E) or CHO DG44 cells maintained in square bottles outperformed cultures grown in spinner flasks (see Chapter 5 for details).
The most widely used host cell lines for transient recombinant protein production are HEK 293 and Chinese hamster ovary (CHO) both of which are approved by regulatory agencies as hosts for recombinant protein expression (Duetz, Ruedi et al. 2000; Maier and Buchs 2001; Meissner, Pick et al. 2001; Girard, Derouazi et al. 2002; Gupta and Rao 2003; Pham, Perret et al. 2003; Baldi, Muller et al. 2005; Muller, Girard et al. 2005). TGE has been performed in several different non-instrumented systems including agitated microtiter plates (Derouazi, Girard et al. 2004; Tait, Brown et al. 2004), spinner flasks up to a volume of 3 L (Schlaeger and Christensen 1999; Girard, Jordan et al. 2001; Schlaeger, Kitas et al. 2003), and shake flasks up to a volume of 250 mL (Derouazi, Girard et al. 2004), and in instrumented stirred-tank bioreactors at volumes from 1 to 100 L (Schlaeger and Christensen 1999; Meissner, Pick et al. 2001; Tait, Brown et al. 2004; Baldi, Muller et al. 2005). The diversity of cultivation systems and experimental approaches demonstrates the adaptability and potential of the transient gene expression method.

2.5 Serum-free, chemically defined media for transient gene expression.

In addition to choosing a high performing cultivation system and host cell line (section 1.3), the medium plays a very important role in the growth and transfection of mammalian cells in suspension. Serum-containing media are often complex, undefined, and include various mixtures of hormones, nutrients, binding proteins, growth factors and other constituents that may tend to vary from batch to batch (Girard, Derouazi et al. 2002). In order to facilitate downstream processing, serum-free transfection is preferred. It is also important to eliminate serum and other animal-derived components from the medium to reduce the risk of contamination of the r-protein with animal viruses and/or prions and to reduce the cost of the process. Therefore, one of the major areas of potential improvement in transient gene expression in mammalian cells involves medium optimization (Barnes and Sato 1980). For this reason calcium phosphate-mediated transfection and calfection are less attractive since their efficiency as DNA delivery agents are dependent on the presence of serum (Pham, Perret et al. 2003; Derouazi, Girard et al. 2004). The role of serum in transfection is not clear. It is thought to function as a protective coating for the cells or for the DNA/CaPi complex, but it may also be involved in buffering the effects of an elevated Ca^{2+} concentration on cells by chelating excess Ca^{2+} (Meissner, Pick et al. 2001). Ca^{2+} is known to influence many cellular processes (Barnes and Sato 1980). Attempts to remove serum from both processes by optimizing the Ca^{2+} concentration, cell density, DNA concentration, and pH and by adding supplements such as albumin have not been successful (Jordan, Schallhorn et al. 1996; Lindell, Girard et al. 2004). The elimination of serum may result in the increased uptake of Ca^{2+} by cells. Intracellular Ca^{2+} appears to effect transient recombinant protein production indirectly through second messenger systems (Girard, Porte et al. 2001).
Interactions between Ca^{2+} and serum appear to be complex, making it difficult to find serum substitutes. Peptones of plant and animal origin have been used as medium additives to serve in part as a serum replacement (Haberland, Knaus et al. 1999; Girard, Porte et al. 2001). Recombinant insulin or growth factors may also be added to media to enhance cell growth and viability in the absence of serum (Preuss, Connor et al. 2000; Lindell, Girard et al. 2004; Pham, Perret et al. 2005).

2.6 Goals of the thesis

The major goal of this work was to develop a transient transfection system for suspension-adapted cells applicable to both instrumented and non-instrumented cultivation systems at a broad range of scales in serum-free, chemically defined medium. It was anticipated that the achievement of this goal would enhance transient recombinant protein expression in mammalian cells from current yields in the 5-20 mg/l range to yields in the 100-500 mg/l range.
One of the main challenges in transient gene expression is the maintenance of large cell populations. Spinner flasks have been the culture vessel of choice for many laboratories. Here the development of an alternative vessel, the agitated square bottle, is described. The objective of this part of the thesis was to demonstrate that HEK

293E and CHO DG44 cells, two of the most commonly used cell lines for large-scale transient gene expression, could be cultivated and transfected in this vessel.
Delivery of DNA into cells in serum-free chemically defined medium is the second major challenge in transient gene expression. The three methods that have shown promise to date for scalable operations are calcium phosphate-DNA coprecipitation (Pham, Perret et al. 2003), polyfection (Fitzsimmons, Sanyal et al. 2004), and electroporation (Fukumoto, Sperling et al. 2003). The experiments described here focus on two methods of gene delivery, calfection and polyfection. The objective of this part of the thesis was to demonstrate efficient gene delivery to HEK 293E and CHO DG44 cells in a serum-free, chemically defined environment using a process that was scaleable to at least 100 liters.

2.7 References

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Materials and Methods

3. Materials and Methods

3.1 Cell culture

Suspension-adapted HEK 293E and CHO DG44 (a dihydrofolate reductase-deficient mutant) cells were routinely grown in 250 ml square-shaped glass bottles (Schott Glass, Mainz, Germany). The former were cultivated in serum-free, chemically defined Pro293s CDM (Cambrex, East Rutherford, NJ) or Ex-Cell 293 medium (JRH Biosciences; Lenexa, KS). CHO DG44 cells were cultivated in ProCHO5 CDM medium (Cambrex, East Rutherford, NJ) supplemented with 0.68 mg/l hypoxanthine, 0.194 mg/l thymidine (HT), and 4 mM glutamine (Muller et al. 2005). The cells were seeded at a density of $3.5x10^5$ cells/ml in 100 ml of medium and subcultivated every 3-4 days. The cultures were incubated at 37°C in a roll-in incubator (Bellco Glass Inc., Vineyard, NY) that was outfitted with a horizontal Model ES-W orbital shaker (Kühner AG, Birsfelden, Switzerland) having a rotational diameter of 2.5 cm unless otherwise stated. The bottles were fixed to the shaker with double-sided adhesive transfer tape (3M Corp., Minneapolis, MN). The caps were tightly closed after inoculation and opened by one quarter of a turn when the culture reached an approximate pH of 7.0 (usually after 24 h of incubation) as judged by visual inspection of the medium containing phenol red. Suspension-adapted BHK21 cells were maintained in 500 ml spinner flasks (Bellco Glass Inc., Vineland, NJ) in serum-free ProCHO5 CDM medium (Cambrex) and subcultivated every 3-4 days at a seeding density of $2\text{-}3x10^5$ cells/ml.

For some experiments, cells were grown in one-liter square-shaped glass bottles (Schott Glass, Mainz, Germany). For comparative purposes, one-liter square-shaped bottles composed of polycarbonate (PC), polyethylene terephthalate copolyester (PETG), or polypropylene (PP) (Nalge Nunc Int., Rochester, NY), one-liter glass Erlenmeyer flasks with screw caps (Corning, Acton, MA), and 500-ml and one-liter spinner flasks (Bellco) were also used.

The cell number was determined using three different methods. Automated counting was performed with either a CASY1 Counter (Scharfe System, Reutlingen, Germany) or a CEDEX Cell Counter (Innovatis AG, Bielefeld, Germany). Manual counting was performed with a Neubauer hemocytometer. Cell viability was assessed using the Trypan blue exclusion method. When necessary, a BioProfile 200 bioanalyser (NOVA Biomedical Corp., Waltham, MA) was used to measure pH and the concentrations of dissolved oxygen, glucose, sodium bicarbonate, glutamine, ammonium, and lactic acid.

3.2 Plasmid DNA

pEAK8-LH39 and pEAK8-LH41 with the full-length cDNAs of the light and heavy chain IgG genes, respectively, under the control of the human EF-1α promoter were described previously (Pick et al., 2002). pEAK8 was purchased from Edge Biosystems (Gaithersburg, MD). pEGFP-N1 with the enhanced green fluorescence

protein (GFP) gene under the control of the human cytomegalovirus major immediate early promoter was purchased from Clontech (Palo Alto, CA).

The NY-ESO-1 cDNA clone in pcDNA3.1(-), designated pNYESO1 in this study, was kindly provided by Dr. Y.-T. Chen and has been described previously (Chen et al. 1997). pLH-NYESO1 was generated by first amplifying by PCR the Ig κ-chain leader sequence and the six histidine tag sequence from pSecTagA (Invitrogen AG, Basel, Switzerland) with the forward and reverse primers 5'-GCTGG**CTCTAGA**CCATGGAGACAGACACAC-3' (the sequence in bold is an XbaI restriction site) and 5'-GGTAC**GGATCC**ATGATGATGATGATGATGGTCACCAGTGGAACCTGG-3' (the sequence in bold is an EcoRI restriction site), respectively. The PCR product was digested with XbaI and EcoRI and cloned into pNYESO1 digested with the same two enzymes. pcH-NYESO1 was constructed by first amplifying the six histidine codons and the NY-ESO-1 coding sequence from pLH-NYESO1 using the forward and reverse primers 5'-GCACC**TCTAGA**ATGGAGCATCATCATCATCATCATGGATCC-3' (the sequence in bold is an XbaI restriction site) and 5'-GACTCAGC**AAGCTT**AGCGCCTCTGCCCTGAGG-3' (the sequence in bold is a HindIII restriction site), respectively. The PCR product was digested with XbaI and HindIII and cloned into pcDNA3.1(-) that had been digested with the same two enzymes.

Plasmid DNA was purified on a Nucleobond AX anion exchange column (Macherey-Nagel, Düren, Germany) and stored at -20°C at a concentration of 1 mg/ml in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

3.3 DNA transfection

3.3.1 Calcium phosphate-DNA coprecipitation

250 mM $CaCl_2$ was prepared in ultra high purity (UHP) water, filtered (0.22 m pore size), and autoclaved. For preparation of the phosphate buffer, Hepes and NaCl were dissolved in UHP water at concentrations of 50 mM 140 mM, respectively. The pH was adjusted to 7.05 with 1 M NaOH and then 300 mM Na_2HPO_4 was added to a final concentration of 1.4 mM.

Prior to transfection the cells were suspended in modified DMEM/F12 medium (Applichem, Darmstadt, Germany). The standard DMEM/F12 medium was supplemented with 10 mM Hepes (Applichem), 2.5 mg/l insulin (Sigma), 2.5 mg/l holo-transferrin (Sigma), 0.1 mM diethanolamine (Fluka Chemie AG, Buchs, Switzerland), 0.1 mM L-proline (Sigma), 2.5 mM L-glutamine (Applichem), and 1.85 g/l L-glucose (Applichem). The medium was sterilized by filtration, and fetal bovine serum (FCS) (JRH Biosciences, Lenexa, KS) was added to a concentration of 2% unless otherwise stated.

For each ml of culture medium, 2.5 µg of plasmid DNA was mixed with 50 µl of 250 mM $CaCl_2$. Just prior to transfection, 50 µl of phosphate buffer was added to the DNA (Jordan et al. 1996). Particle formation was allowed to proceed for 60 sec at room temperature. Thereafter, 100 µl of precipitate was added per ml of culture at a cell density of $5x10^5$ cells/ml. At 4 h post-transfection one volume of modified DMEM/F12 medium with 2% FCS was added to the culture.

3.3.2 Calfection

Various amounts of 250 mM $CaCl_2$ were mixed with plasmid DNA as indicated in the text and added directly to suspension cultures in modified DMEM/F12 medium with 1% FCS. This solution, representing 5% of the volume of the culture, was added rapidly to the culture and then briefly mixed. Unless otherwise noted, one volume of modified DMEM/F12 medium with 1% FCS was added to the cell culture 4 h post-transfection. (Lindell et al. 2004).

Besides $CaCl_2$ several other salts including NaCl, LiCl, KCl, $MgCl_2$, $BaCl_2$, and $SrCl_2$ were used as transfection reagents. The solutions were prepared with UHP water, filtered (0.22 pore size), and autoclaved.

3.3.3 Polyfection

Linear 25 kDa PEI (pH 7.0) (Polysciences, Eppenheim, Germany) was prepared in water at a final concentration of 1 mg/ml and sterilized by filtration (0.22 µM pore size). For the transfection of 1 ml of cells, 2.5 µg of DNA was added to 50 µl of 150 mM NaCl. Varying amounts of PEI were used for transfection depending on the cell line and the transfection medium. CHO DG44 cells were transfected in either RPMI 1640 (Applichem, Darmstadt, Germany) with 25 mM Hepes (pH 7.1) (Derouazi et al. 2004) or ProCHO5 CDM supplemented with HT and glutamine. The DNA:PEI ratios for transfection in these two media were 1:2 and 1:4 (w/w), respectively. HEK 293E cells were transfected in RPMI 1640 containing 25 mM Hepes (pH 7.1) or Ex-Cell 293 medium at a DNA:PEI ratio of 1:3 or 1:11 (w/w), respectively. In all cases, the PEI was added to 50 µl of 150 mM NaCl for each ml of medium to be transfected. Prior to transfection, the PEI/NaCl solution was added to the DNA/NaCl solution and allowed to stand at room temperature for 10 min. For each ml of culture, 100 µl of this mixture was added to the cells.

3.4 Transfection in different culture vessels

3.4.1 12-well microtiter plates

Cells were centrifuged and resuspended in the appropriate medium at cell densities ranging from $0.5\text{-}2x10^6$ cells/ml. The cells were then distributed into 12-well microtiter plates (TPP, Wohlen, Switzerland) using 1 ml aliquots. After addition of the DNA by calcium phosphate-DNA coprecipitation, calfection, or polyfection the plates were incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity on an orbital shaker at 180 rpm as previously described (Girard et al. 2001) (Fig. 1). At 4 h post-transfection, 1 ml of the appropriate medium was added to each well.

Figure 1. 12-well microtiter plates were fixed on a horizontal orbital shaker with double-sided adhesive transfer tape.

3.4.2 50 ml centrifuge tubes

Cells were centrifuged and resuspended in the appropriate medium at a cell density of 0.5-2x10^6 cells/ml. A 5 ml aliquot of cells was added to each 50 ml centrifugation tube fitted with a filter cap (TPP, Wohlen, Switzerland) (De Jesus et al. 2004). Following the addition of DNA by polyfection or calfection, the tubes were agitated at 140 (for CHO DG44 cells following polyfection) or 160 rpm (for HEK 293 cells following polyfection or for any cell type following calfection) in an ISF-4-W shaker incubator (Kühner AG, Birsfelden, Switzerland) at 37°C in a 5% CO_2 atmosphere with 95% humidity (Fig. 2). After 4 h, one volume of the appropriate medium was added and the agitation speed was increased to 160 (for CHO DG44 cells following polyfection) or 180 rpm (for HEK 293 cells following polyfection or for any cell type following calfection).

Figure 2. 50 ml centrifuge tubes fitted with a filter cap. The tubes were maintained in racks fastened to an orbital shaker in an incubator.

3.4.3 Square bottles

Cells were centrifuged and resuspended in one-liter square bottles at a density of 2x10^6 cells/ml in 200 ml of the appropriate medium. For cultivation in square bottles the RPMI 1640 medium was supplemented with 25 mM Hepes (pH 7.1) and 0.1% (w/v) Pluronic F-68 (Applichem, Darmstadt, Germany). Following transfection by calcium phosphate-DNA coprecipitation or polyfection, the bottles were agitated at

100 rpm in an ISF-4-W shaker (Kühner) at 37°C in a 5% CO_2 atmosphere with 95% humidity with the caps open one quarter of a turn. After 4 h one volume of the appropriate medium was added. The bottle caps remained open, but the agitation speed was increased to 110 rpm. At 24 h post-transfection, the bottles were transferred to an incubator without CO_2 and humidity control (Fig. 3). The bottles were agitated on an ES-W orbital shaker (Kühner) at a speed of 110 rpm. During the cultivation period the cells were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate unless otherwise stated. For smaller or larger square-shaped bottles, the same procedure was followed. In all cases, the volume of the culture at the time of transfection was 20% of the nominal volume of the bottle.

Figure 3. One-liter square bottles mounted on a horizontal orbital shaker with double-sided adhesive transfer tape.

3.4.4 Spinner flasks

Cells were centrifuged and resuspended in one-liter spinner flasks (Fig. 4) at a density of $2x10^6$ cells/ml in 200 ml of the appropriate medium. If RPMI 1640 was used, it was supplemented with 25 mM Hepes (pH 7.1) and 0.1% (w/v) Pluronic F-68. Following the addition of DNA by calcium phosphate-DNA coprecipitation, calfection, or polyfection, the flasks were stirred at 80 rpm in an incubator at 37°C in a 5% CO_2 atmosphere with 95% humidity with the caps open one quarter of a turn. After 4 h the cultures were diluted with one volume of the appropriate medium. At 24 h post-transfection, the bottles were transferred to an incubator at 37°C without CO_2 and humidity control. The cultures were fed upon need with glucose, glutamine, and sodium bicarbonate as indicated above. For smaller or larger spinner flasks, the same protocol was followed. The volume of the culture at the time of transfection was always 20% of the nominal volume of the spinner flask.

Figure 4. One liter spinner flasks on a magnetic stirrer.

3.4.5 Bioreactors

Cells were centrifuged and resuspended in the appropriate medium. When RPMI 1640 was used it was supplemented with 25 mM Hepes (pH 7.1) and 0.1% (w/v) Pluronic F-68. The cells were seeded at $2x10^6$ cells/ml into either a 3-liter (Applikon, The Netherlands) (Fig. 5), a 20-liter (Bioengineering, Wald, Switzerland) (Fig. 6), or 150-liter bioreactor (Bioengineering, Wald, Switzerland) (Fig. 7) containing the same prewarmed medium as for the resuspension of the cells. The final volume of each culture is indicated in the text. DNA addition by calcium phosphate-DNA coprecipitation or polyfection was performed immediately after seeding, and the culture was stirred at 150 rpm. After 4 h the culture was diluted with one volume of the appropriate medium, and the agitation speed was increased to 200 rpm. The pH was maintained at 7.1 with 1 M NaOH and CO_2, and the level of dissolved oxygen was maintained at 20% by sparging air into the culture. The cells were fed upon need with glucose, glutamine, and sodium bicarbonate as indicated above.

For calfection, HEK 293E cells were centrifuged and resuspended in modified DMEM/F12 supplemented with 1% FCS in a 150-L bioreactor. The medium was supplemented with 10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 μg/ml amphotericin B, and 0.1% Pluronic F-68. The cells were seeded at $5x10^5$ cells/ml in a total volume of 30 L. After seeding, 150 mg DNA (29% pEAK39-LH1, 69% pEAK41-LH2, and 2% pEGFP-N1) was added to 1.5 L of 250 mM $CaCl_2$, filtered (0.22 pore size), and added to the culture. The pO_2 was controlled by surface aeration, and the pH was maintained at 7.6. At 4 h post-transfection, 30 L of modified DMEM/F12 medium with 1% FCS was added and the pH was maintained at 7.0.

Figure 5. 3-liter bioreactor.

Figure 6. 20-liter bioreactor.

Figure 7. 150-liter bioreactor.

3.5 Reporter protein quantification

3.5.1 Quantification of GFP

Cells (1-2 ml) in a 12-well microtiter plate were lysed by addition of 0.5 volumes of PBS containing 1% Triton X-100 (AxonLab, Baden-Dättwil, Switzerland). After 1 h of agitation at 37°C, the fluorescence was measured with a Cytoflour^{TM} 4000 plate reading fluorometer (PerSeptive Biosystems, Inc., Farmington, MA). The excitation wavelength was 485 nm with a bandwith of 20 nm and the emission wavelength wavelength was 530 nm with a bandwidth of 25 nm. The background fluorescence from mock transfected cells was subtracted from each value to give relative fluorescence units (RFU). Error bars on the graphs in this work represent the standard deviation of three samples.

3.5.2 ELISA

The IgG concentration in culture medium was determined by sandwich ELISA using unconjugated goat anti-human kappa light chain antibody for IgG capture and alkaline phosphatase-conjugated goat anti-human IgG for detection as previously described (Meissner et al. 2001). Error bars on the graphs in this work represent the standard deviation of three samples.

3.5.3 Western Blot

For the transfections with pcH-NYESO1in 12-well microtiter plates, the cultures from three wells were pooled and centrifuged at 2,000 rpm for 5 min. The cell pellet was

washed once in PBS and lysed in 0.5 ml Mammalian Protein Extraction Reagent (M-PER) (Perbio Science, Lausanne, Switzerland) according to the manufacturer's recommendations. After centrifugation to remove the cellular debris, 5 µl of the soluble fraction was analyzed by Western blot following denaturing 12.5% SDS-PAGE. The blot was probed with mouse monoclonal antibody ES121 raised against recombinant NY-ESO-1 produced in *E. coli*. Antibody binding was detected with horseradish peroxidase-conjugated anti-mouse IgG (Sigma Chemical, St. Louis, MO). For the transfections in one-liter square-shaped bottles, 2 ml aliquots of the culture were collected. The cells were harvested by centrifugation, lysed in 0.5 ml M-PER, and analyzed by SDS-PAGE and western blot as described above.

3.6 References

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Results and Discussion

4. Calfection

A new method termed calfection was developed for gene delivery into mammalian cells in suspension (Lindell et al. 2004). This work was carried out to simplify the calcium phosphate-DNA coprecipitation method of gene transfer because it includes a time-dependent step which is inconvenient for large-scale transfections. Calfection (Fig. 1) eliminates the need to form the calcium phosphate-DNA coprecipitate before its addition to the cells (Fig. 2). The calfection method is simple and reproducible and has been shown to be applicable from the milliliter to the bioreactor scale without alterations. The method does not involve any time-dependent steps, making it a convenient method for both serial and large-scale transfections. Other advantages include the one-step preparation of transfection solutions and the possibility to store them for long periods without any negative effects on transfection efficiency. Plasmid DNA was simply diluted into a $CaCl_2$ solution and then added directly to the cell culture for transfection. We evaluated and optimized this approach using suspension-adapted HEK 293E cells. The highest reporter protein expression levels were obtained when cells were transfected in the presence of 12.5 mM calcium and 5 µg/ml plasmid DNA while maintaining the pH of the culture medium at 7.6 at the time of transfection. Suspension-adapted BHK 21 and CHO DG44 cells were also transfected with this method. Calfection differs from calcium phosphate-DNA coprecipitation in several ways. For calfection, the transfection mix (DNA in a $CaCl_2$ solution) remained highly efficient during long-term storage at temperatures ranging from 20°C to –20ºC. In contrast calcium phosphate-DNA co-precipitates were only efficient for gene transfer when prepared fresh. Furthermore, passing the calfection mixture through a 0.2 µm filter did not compromise transfection efficiency, whereas calcium phosphate-DNA coprecipitates were retained by the filter. High protein expression levels, a limited number of manipulations, and the possibility of filtration prior to transfection make calfection suitable for both large-scale transfection in bioreactors and for high-throughput transfections in microtiter plates.

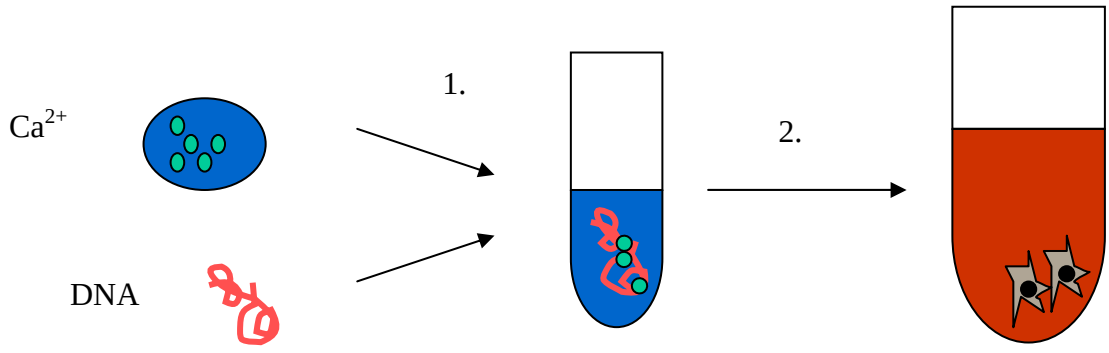

Figure 1. Schematic diagram of calfection protocol. Solutions of plasmid DNA and calcium chloride were mixed (1) and added to a suspension culture of mammalian cells (2). At 4 h post-transfection, the culture can be diluted with one volume of medium but this step is not necessary.

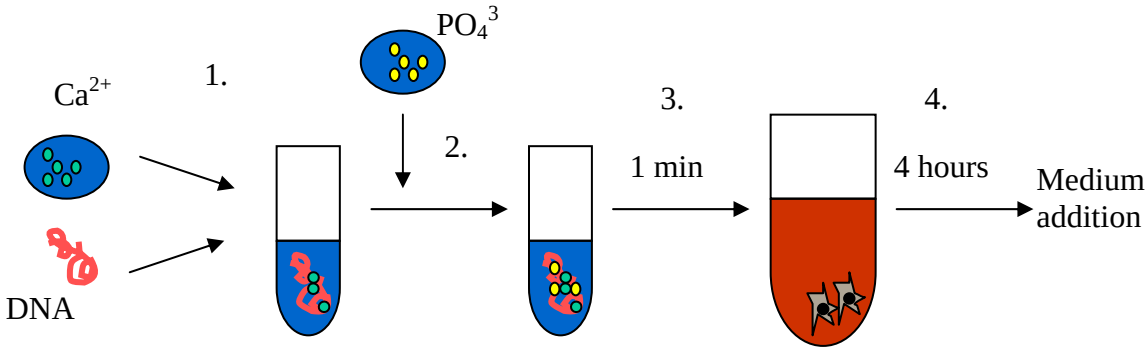

Figure 2. Schematic diagram of calcium phosphate-DNA coprecipitation protocol. Solutions of plasmid DNA and calcium chloride were mixed (1). and one volume of a phosphate buffer was added (2). After 1 min the calcium phosphate-DNA coprecipitate was added to a suspension culture of mammalian cells in the presence of 1-2% FCS (3). Four hours after addition of the DNA, the culture was diluted with one volume of medium (4).

4.1 Results

4.1.1 Parameters for calfection.

Initially, several mono- and divalent cations including Li^{+}, Na^{+}, K^{+}, Cs^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} were tested for their potential to support the transfer of plasmid DNA into suspension-adapted mammalian cells. The chloride salts of these cations at a range of concentrations were mixed individually with plasmid DNA and added directly to 1 ml cultures of HEK 293E in 12-well microtiter plates at a density of $5x10^{5}$ cells/ml in modified DMEM/F12 medium with 1% FCS. While transfection in the presence of most of these cations yielded little or no GFP expression (data not shown), high levels of expression were observed in the presence of some concentrations of Ca^{2+} (Fig. 3) when transfected with a a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41. For all DNA concentrations tested the highest GFP expression occurred at a Ca^{2+} concentration in the medium of 12.5 mM (Fig. 3). For Ca^{2+} concentrations less than 8 mM the GFP level was below or near the limit of detection whereas Ca^{2+} concentrations up to 62.5 mM yielded measurable amounts of GFP depending on the DNA concentration (Fig. 3). Overall, the highest level of GFP expression was seen at a DNA concentration of 5 µg/ml and a Ca^{2+} concentration of 12.5 mM (Fig. 3). While the presence of elevated concentrations of Ca^{2+} for a few hours was neither toxic nor prevented cell growth, it did induce the formation of cell aggregates (data not shown).

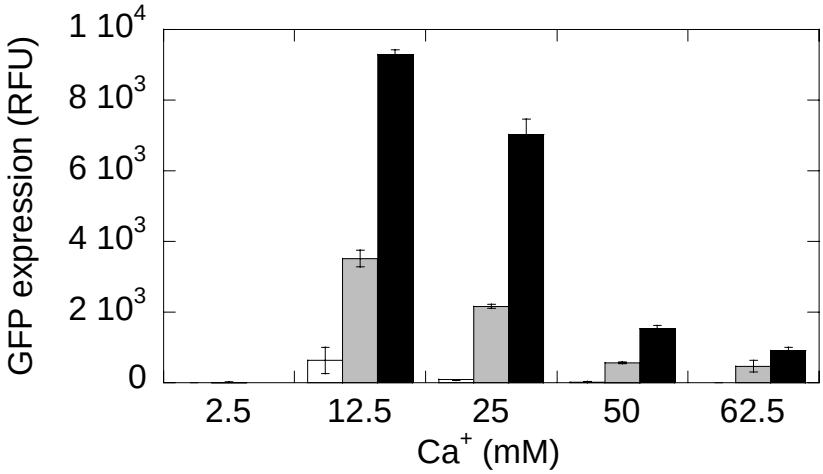

Figure 3. Calfection of HEK 293E cells with various Ca^{2+} and DNA concentrations. Cells were seeded into 12-well microtiter plates at a density of $5x10^5$ cells/ml in modified DMEM/F12 medium with 1% FCS and transfected at various final DNA and Ca^{2+} concentrations as indicated. The DNA was a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41, with a final concentration in the medium of either 0.5 g/ml (white bars), 2.5 g/ml (gray bars), or 5.0 g/ml (black bars). The cells were diluted with one volume of medium at 4 h post-transfection. GFP expression was measured at 3 days post-transfection after cell lysis.

For the experiment described above the optimum DNA concentration for the range of Ca^{2+} concentrations tested was 5 μg/ml. To determine if this was indeed the optimum level of plasmid DNA for calfection, HEK 293E cells were exposed to various concentrations of pEGFP-N1 in the presence of 13 mM Ca^2. GFP expression increased in a dose-dependent manner when the DNA concentration in the medium was increased from 1 to 5 μg/ml (Fig. 4). A plateau of GFP expression appeared at DNA concentrations equal to or higher than 5 μg/ml. Based on these results, a final DNA concentration of 5 μg/ml was used for all subsequent experiments.

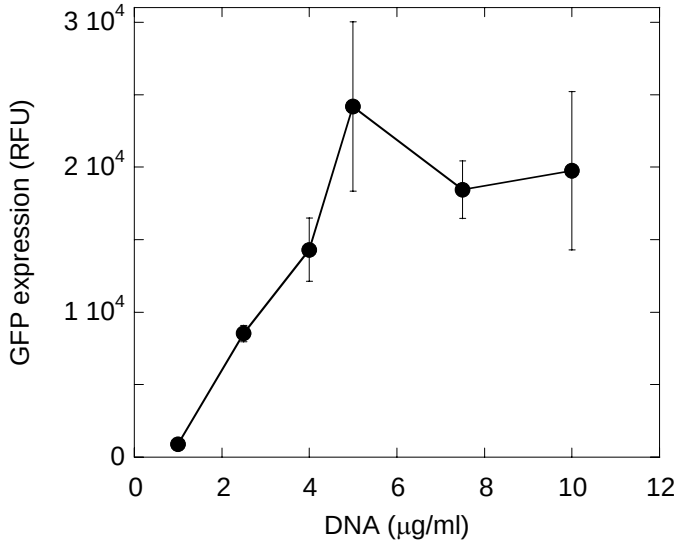

Figure 4. Calfection of HEK 293E cells at various DNA concentrations. The cells were seeded into 12-well microtiter plates at a density of $5x10^5$ cells/ml in modified DMEM/F12 medium with 1% FCS. pEGFP-N1 was added to the cells at various final concentrations as indicated. $CaCl_2$ was also added to a final concentration in the medium of 13 mM. The cells were diluted with one volume of fresh medium at 4 h post-transfection. GFP expression was measured at 3 days post-transfection following cell lysis. The data for this figure was kindly provided by Jeanette Lindell.

The cell density at the time of calfection was also expected to be an important parameter for high-level reporter protein expression. Thus the cell density at the time of DNA addition was varied from $2\text{-}10x10^5$ cells/ml. In addition, the cells were transfected at two different DNA (2.5 and 5.0 µg/ml) and Ca^{2+} concentrations (12.5 and 25 mM) to determine if there was any interdependence of these three parameters on the efficiency of calfection. A DNA mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 was used for the transfection. The highest GFP expression was observed at a DNA concentration of 5 µg/ml, a Ca^{2+} concentration of 12.5 mM, and a cell density of $5x10^5$ cells/ml (Fig. 5). Therefore, additional experiments to characterize the calfection method were performed under these conditions.

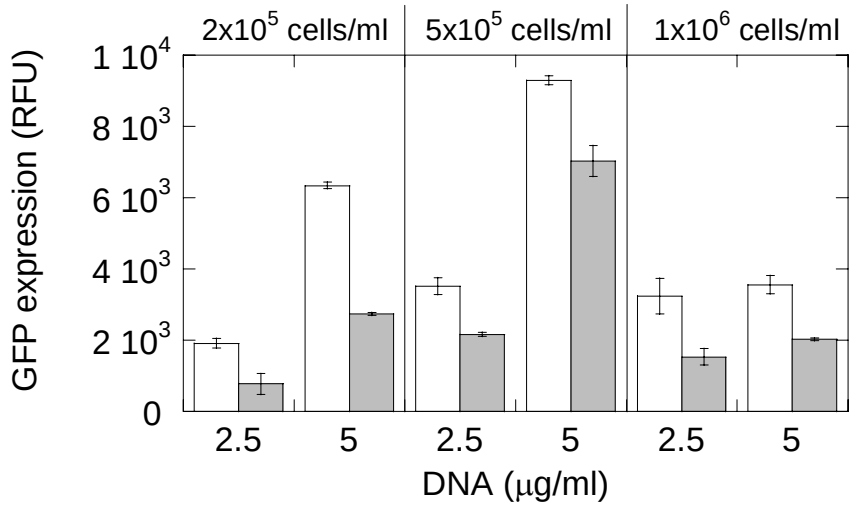

Figure 5. Calfection of HEK 293E cells at various conditions of cell density, DNA concentration, and Ca^{2+} concentration. The cells were seeded into 12-well microtiter plates at different cell densities and treated with various final DNA and Ca^{2+} concentrations as indicated. The DNA was a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41. GFP expression was measured at 3 days post-transfection following cell lysis. The white bars indicate a final concentration of 12.5 mM Ca^{+} in the medium, and the gray bars indicate a final concentration of 25 mM Ca^{+} in the medium.

4.1.2 Effects of pH and FCS on calfection.

In the experiments described above, variability in reporter protein expression was often observed in independent experiments. One possible cause was the pH of the medium at the time of transfection. To determine if this parameter was important for calfection the sodium bicarbonate buffer in the transfection medium (modified DMEM/F12) was replaced with 25 mM Hepes at a pH range of 6.5 to 7.8. HEK 293E cells were resuspended in each of the different media and inoculated into 12-well microtiter plates. Following calfection with pEGFP-N1, the cultures were incubated in a 37°C chamber with humidified air but without CO_2. By 4 h post-transfection the pH of the different media had not changed more than 0.1 units. At 4 h post-transfection, one volume of modified DMEM/F12 medium containing 48 mM sodium bicarbonate but without Hepes was added to each culture to reduce the calcium concentration and to restore the sodium bicarbonate buffer system. A dramatic dependency of reporter gene expression on the pH of the medium at the time of calfection was found (Fig. 6). For pH values below 7.4 little or no GFP expression was observed, but calfection in the pH range from 7.4 to 7.8 yielded high levels of GFP expression (Fig. 6). Based on these results further experiments to characterize calfection were performed in medium with a pH of 7.6.

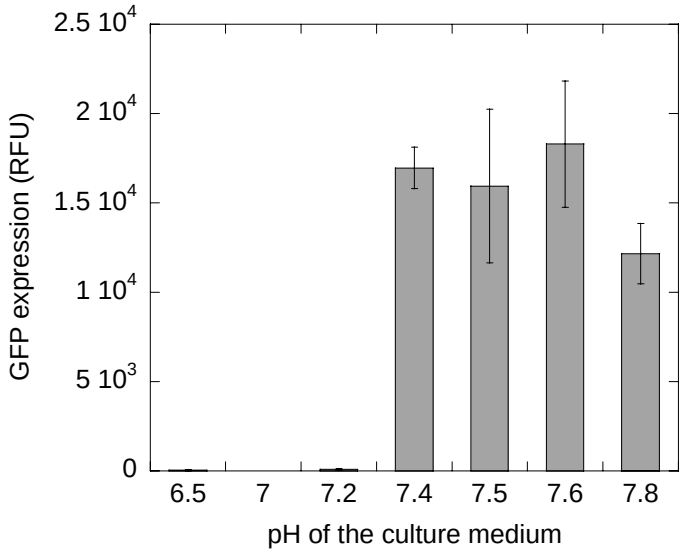

Figure 6. The effect of pH on calfection. HEK 293E cells were seeded in 12-well microtiter plates at a density of $5x10^5$ cells/ml in modified DMEM/F12 with 1% FCS at the pH values indicated. Calcium and pEGFP-N1 were added to final concentrations of 13 mM and 5 μg/ml, respectively. GFP expression was measured at 2 days post-transfection following cell lysis.

The presence of FCS in the medium at the time of transfection was also thought to be a critical parameter for obtaining a high level of reporter protein expression following calfection. To investigate the effect of FCS on calfection HEK 293E cells were seeded in 12-well microtiter plates in modified DMEM/F12 medium with or without FCS and then transfected with a combination of pEAK8-LH39 and pEAK8-LH41. Under serum-free conditions the cells formed exceptionally large aggregates after addition of the calcium/DNA mixture and antibody expression was low (Fig. 7). Addition of 1–5% FCS to the medium improved antibody expression up to 10-fold relative to its expression in the absence of serum (Fig. 7). The highest antibody expression were seen at 2-3% FCS (Fig. 7).

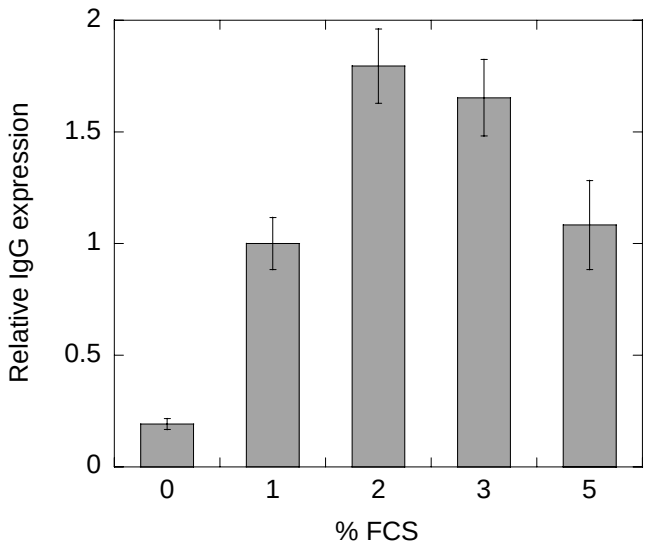

Figure 7. Effect of FCS on calfection. HEK 293E cells were seeded at a density of $5x10^5$ cells/ml in 12-well microtiter plates in modified DMEM/F12 medium (pH 7.6) with or without FCS as indicated. Calfection was then performed by addition of calcium and DNA to final concentrations of 13 mM and 5 μg/ml, respectively. The DNA was a mixture of pEAK8-LH39 and pEAK8-LH41 at a 3:7 (w:w) ratio. At 4 h post-transfection the cells were diluted with one volume of medium without serum. The antibody concentration was measured by ELISA at 3 days post-transfection, and the results were normalized to the antibody level observed in the transfection with 1% serum.

4.1.3 Calfection with other mammalian cell lines.

To determine if the protocol established for calfection with HEK 293E cells was applicable to other mammalian cell lines, suspension-grown BHK 21 and CHO DG44 cells were seeded in 50 ml centrifugation tubes at a density of $5x10^5$ cells/ml in modified DMEM/F12 medium with 1% FCS, and then pEGFP-N1, in the presence of or absence of $CaCl_2$, was added to the cells. GFP expression was observed in both CHO DG44 and BHK 21 cells after plasmid addition in the presence but not in the absence of Ca^{2+} (Fig. 8). The calfection protocol, however, was not further optimized for these two cell lines or for this cultivation system. It is likely that the efficiency of calfection of these cells can be improved with a more thorough analysis of different transfection parameters.

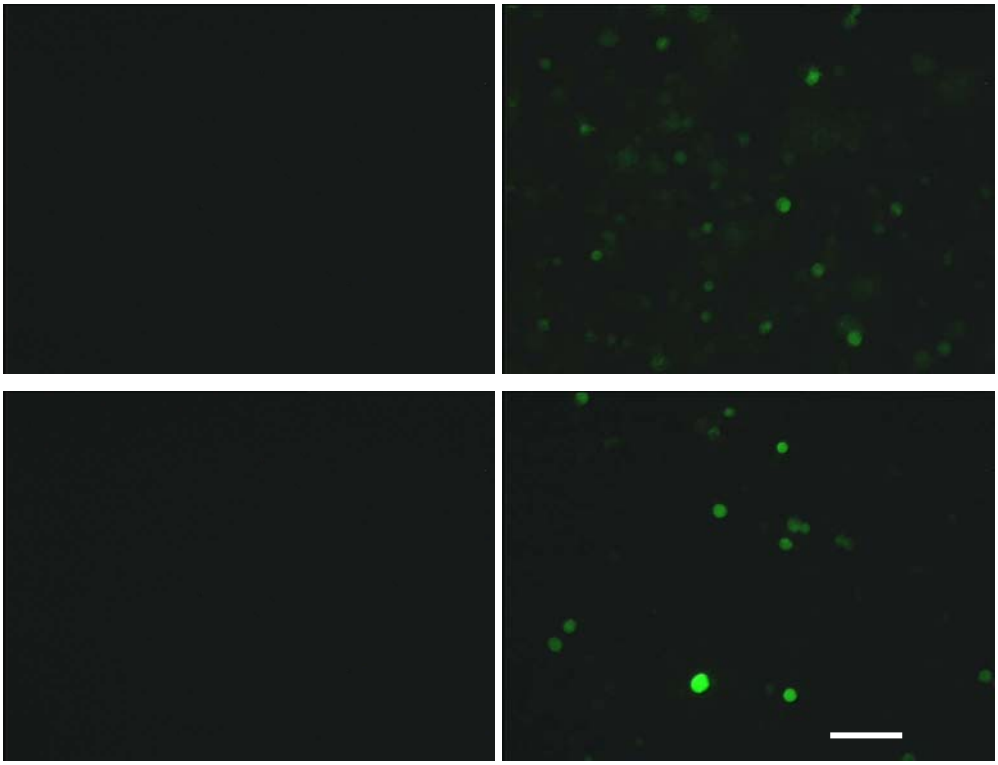

Figure 8. Calfection of BHK 21 (upper panels) and CHO DG44 cells (lower panels). pEGFP-N1 in the presence (right panels) or absence (left panels) of $CaCl_2$ was added to the 10 ml cultures in 50 ml centrifuge tubes to a final concentration in the medium of 5 μg/ml. The images were produced at 3 days post-transfection. The bar represents 50 μm and is the same for all images. The images were kindly provided by Jeanette Lindell.

4.1.4 Stability of the calfection mixture.

In the experiments described above $CaCl_2$ was mixed with plasmid DNA and then added to the culture. The addition of each of these components to the culture sequentially in either order resulted in the same level of GFP expression as seen when the two components were added at the same time (data not shown). These results demonstrated that the two components are essential for successful DNA transfer into cells, but unlike calcium phosphate-DNA co-precipitation, pre-mixing of the components for a defined time prior to addition to the cells is not necessary. The results suggested that the mixture of $CaCl_2$ and plasmid DNA may be physically and chemically stable and thus amenable to long-term storage. A single calfection mixture containing 50 μg/ml pEGFP-N1 and 250 mM $CaCl_2$ was prepared and stored at different temperatures for up to 9 weeks. At different times HEK 293E cells in 12-well microtiter plates were transfected with aliquots of the stored mixtures. No significant variation in GFP expression was observed between a freshly prepared mixture and the stored mixtures (Fig. 9).

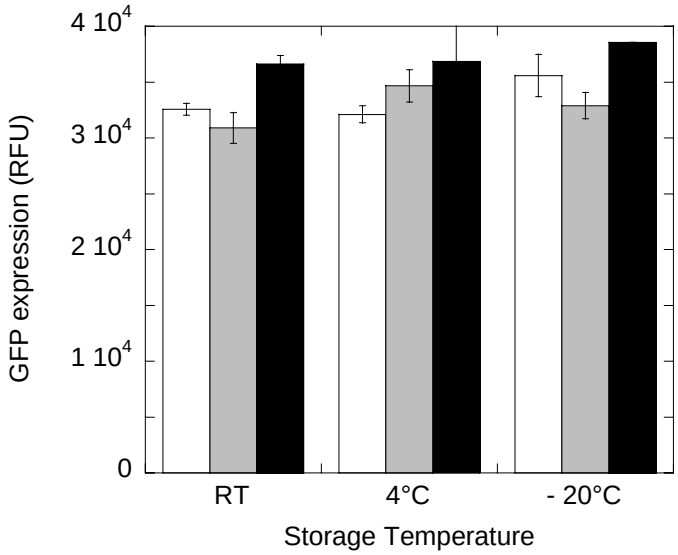

Figure 9. Stability of the calfection solution at different temperatures. A solution containing 50 µg/ml pEGFP-N1 and 250 mM $CaCl_2$ was divided into aliquots and stored at temperatures ranging from room temperature (RT) to -20°C for up to 9 weeks. After either 4 (gray bars) or 9 weeks (black bars) of storage, aliquots of the calfection solution were used to transfect HEK 293E cells in 12-well microtiter plates. Transfection with a freshly prepared calfection solution at the same DNA and $CaCl_2$ concentrations was used as a control (white bars). GFP expression was measured at 3 days post-transfection following cell lysis. The data for this figure was kindly provided by Jeanette Lindell.

4.1.5 Effect of filtration of the calfection solution.

To evaluate the feasibility of filtering the calfection solution prior to its addition to the cells, a mixture of 50 µg/ml plasmid DNA pEGFP-N1 and 250 mM $CaCl_2$ was passed through a 0.22 µm low protein-binding filter and then used to transfect cultures of HEK 293E cells in 12-well microtiter plates. At 3 days post-transfection the level of GFP expression was about the same for the filtered and non-filtered calfection solutions (data not shown). Furthermore, filtration did not result in a measurable loss of DNA.

4.1.6 Effect of medium addition after calfection.

To evaluate the necessity of the addition of one volume of medium at 4 h after calfection, HEK 293E cells were seeded in 12-well microtiter plates with three different conditions: (1) an initial volume of 2 ml with no medium addition after calfection, (2) an initial volume of 1 ml with no medium addition after calfection, and (3) an initial volume of 1 ml with the addition of one volume of medium after calfection. The cells were seeded in modified DMEM/F12 with 1% FCS at a density of $5x10^5$ cells/ml and transfected with pEGFP-N1. For condition 3 the addition of one volume of medium was carried out at 4 h post transfection. At 3 days post-transfection, GFP expression was measured following cell lysis. Suprisingly, GFP

expression was about similar for all three conditions tested (Fig. 10). These results demonstrated that it was not necessary to dilute the cultures following calfection.

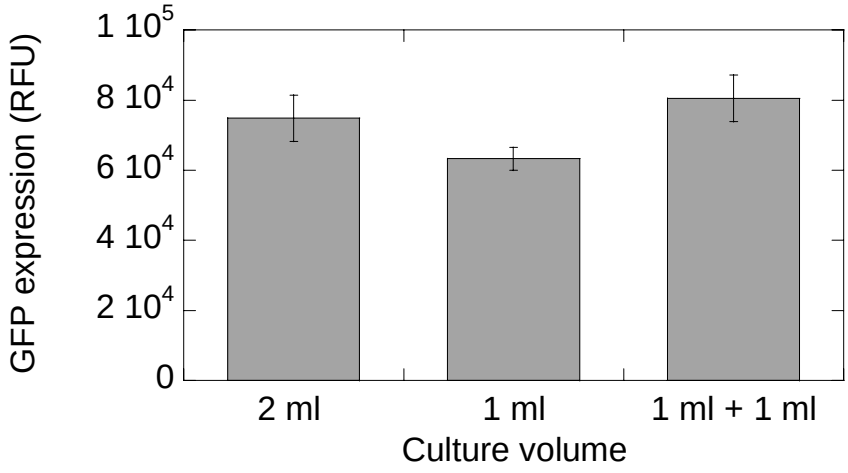

Figure 10. Effect of medium addition after calfection. HEK 293 cells were inoculated into 12-well microtiter plates at a density of $5x10^5$ cells/ml in modified DMEM/F12 medium with 1% FCS. The volume at the time of transfection was either 1 or 2 ml. The final concentrations of pEGFP-N1 and $CaCl_2$ were 5.0 µg/ml and 12.5 mM, respectively. At 4 h post-transfection some of the wells containing 1 ml of medium were diluted with one volume of medium. GFP was measured after cell lysis at 3 days post-transfection.

4.1.7 Effect of calcium exposure on cells.

The effect of an elevated Ca^{2+} concentration on cultivated cells was investigated by the calfection of HEK 293E cells with pEGFP-N1. As in other experiments the final Ca^{2+} concentration was 12.5 mM. The cells were then observed microscopically. Both cell viability and morphology remained normal during the first 4 h after calfection. In this experiment, the culture was not diluted with one volume of medium. By 3 days post-transfection, the cells remained healthy and the level of GFP expression was equivalent to that of control cultures which had been diluted with medium at 4 h after calfection (data not shown). The growth rate of the cells, however, was reduced in the non-diluted cultures as compared to the control cultures (data not shown).

4.1.8 Scalability of calfection.

To determine if calfection was applicable at larger scales than those described above, 100 ml of HEK 293E cells in 250 ml spinner flasks at a density of $5x10^5$ cells/ml in modified DMEM/F12 with 1% FCS were transfected with a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 by the calfection method. The culture was not diluted following DNA addition. As a control 50 ml of HEK 293E cells at the same cell density in a 250 ml spinner flask were transfected with the same mixture of DNAs using the calcium phosphate-DNA coprecipitation method. The culture was diluted to a final volume of 100 ml by the addition of one volume of

medium at 4 h post transfection. Following calfection, the antibody concentration reached a level of 25 mg/L by day 6 post-transfection (Fig. 11). By comparison the antibody level was only about 6 mg/L in the culture transfected by calcium phosphate-DNA coprecipitation (Fig. 11). For this culture, the antibody titer was about two-fold lower than normal. Similar results were also observed for the calfection of HEK 293E cells in a final volume of 500 ml in one-liter spinner flasks (data not shown).

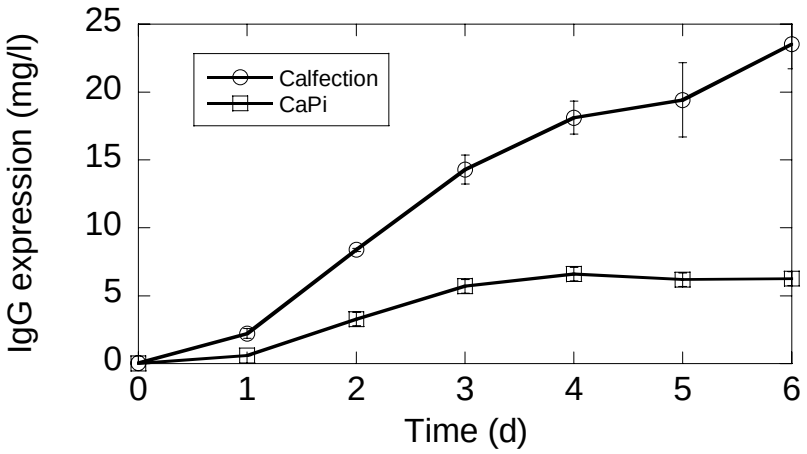

Figure 11. Comparison of calfection and calcium phosphate-DNA coprecipitation in 250 ml spinner flasks. HEK 293E cells in 250 ml spinner flasks were transfected with pEAK8-L41, pEAK8-LH39, and pEGFP-N1 by either calfection or calcium phosphate-DNA coprecipitation. The final volumes in each case were 100 ml. The antibody concentration in the medium was determined by ELISA at the times indicated.

Subsequently, the calfection of a 30 l culture of HEK 293E cells in a 150-liter bioreactor was attempted. The culture in modified DMEM/F12 medium with 1% FCS at a density of $5x10^5$ cells/ml was treated with a calfection solution containing a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 (150 mg DNA in 1.5 L of 250 mM $CaCl_2$). At 4 h after calfection, one volume of medium was added to the cells. Antibody was initially detected about 24 h post-transfection (Fig. 12). The antibody level increased over the next 5 days and attained a maximum concentration of about 3 mg/l (Fig. 12).

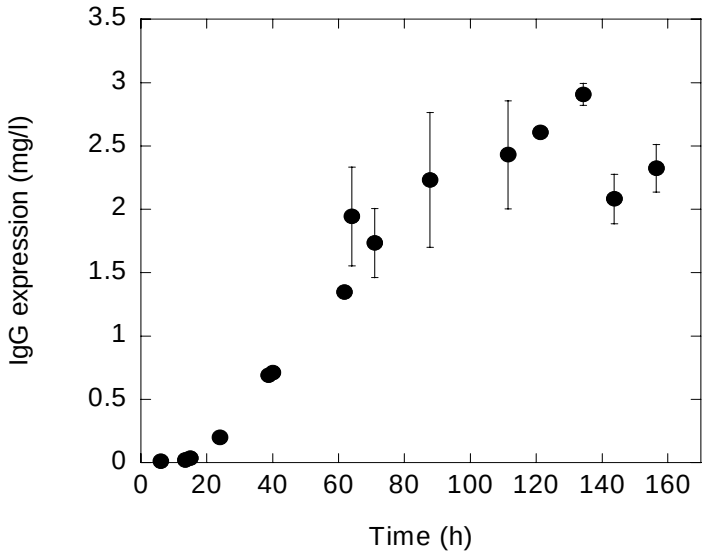

Figure 12. Large-scale calfection of HEK 293E cells in a 150-liter bioreactor. The 30 L culture of HEK 293E cells was transfected with 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 by calfection. At 4 h post-transfection the cells were diluted with an additional 30 L of medium. The antibody concentration in the medium was determined by ELISA at the times indicated.

4.1.9 Effect of the cell density of the seed train on calfection.

To determine if the cell density of the culture that was prepared one day prior to calfection played an important role in the efficiency of DNA transfer, four different cultures of HEK 293E cells in Ex-Cell 293 medium were prepared in 250 ml square bottles 24 h prior to calfection. The cell densities in the bottles were approximately $5x10^5$, $1x10^6$, $1.5x10^6$, and $2.5x10^6$ cells/ml. On the day of calfection, the bottles had cell densities of $9x10^5$, $2x10^6$, $3x10^6$, and $4.7x10^6$ cells/ml. Cells from each of the bottles were transferred to 12-well microtiter plates at a density of $5x10^5$ cells/ml in 2 ml of modified DMEM/F12 medium (pH 7.6) with 1% FCS, and pEGFP-N1 and $CaCl_2$ were added to final concentrations of 5 µg/ml and 12.5 mM, respectivley. The cultures were not diluted with medium following DNA addition. At 3 days post-transfection the highest level of GFP expression was observed in cells originating from the bottle with a density of $3x10^6$ cells/ml on the day of calfection (Fig. 13). The GFP level in this case was 2-fold higher than that in cells originating from the bottle with the lowest cell density on the day of calfection (Fig. 13). Similar results were observed when the cells were inoculated into 12-well plates at a density of $1x10^6$ cells/ml (data now shown). The results demonstrated that the density of the culture on the day of calfection is an important factor for efficient gene transfer by this method.

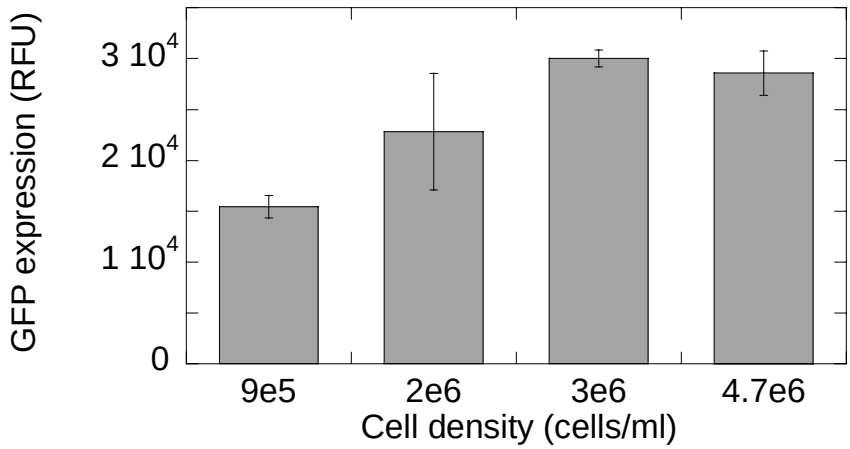

Figure 13. Effect of different seed culture densities on the efficiency of calfection. HEK 293E cells were grown to different cells densities as indicated after subcultivation the day before calfection. The cells were transferred to 12-well microtiter plates at a density of $5x10^5$ cells/ml in modified DMEM/F12 with 1% FCS and then transfected by addition of pEGFPN-1 and $CaCl_2$. GFP expression was measured on day 3 post-transfection after cell lysis.

4.1.10 Effect of culture age on calfection.

In all of the experiments described above, the cells were subcultivated one day prior to calfection by centrifugation and then resuspension in fresh medium. To determine if this step is necessary, HEK 293E cells were subcultivated either 24 h (designated N-1), 48 h (N-2), or 96 h (N-4) prior to calfection. On the day of calfection, the density of each culture was approximately $3x10^6$ cells/ml. Cells from the three cultures were inoculated into 12-well microtiter plates at a density of $5x10^5$ cells/ml in modified DMEM/F12 medium (pH 7.6) with 1% FCS and then transfected by the addition of EGFP-N1 and $CaCl_2$. As a control the cells were also transfected with pEGFP-N1 by calcium phosphate-DNA coprecipitation after resuspension in modified DMEM/F12 medium (pH 7.2) with 1% FCS. The age of the culture prior to transfection had a significant effect on calfection but not on calcium phosphate-DNA coprecipitation (Fig. 14). For calfection, the older the culture prior to transfection, the higher the reporter gene expression (Fig. 14). It was also surprising to see that transfection by calcium phosphate-DNA coprecipitation was just as efficient with cells subcultivated 4 days before transfection as with cells subcultivated the day before transfection (Fig. 14).

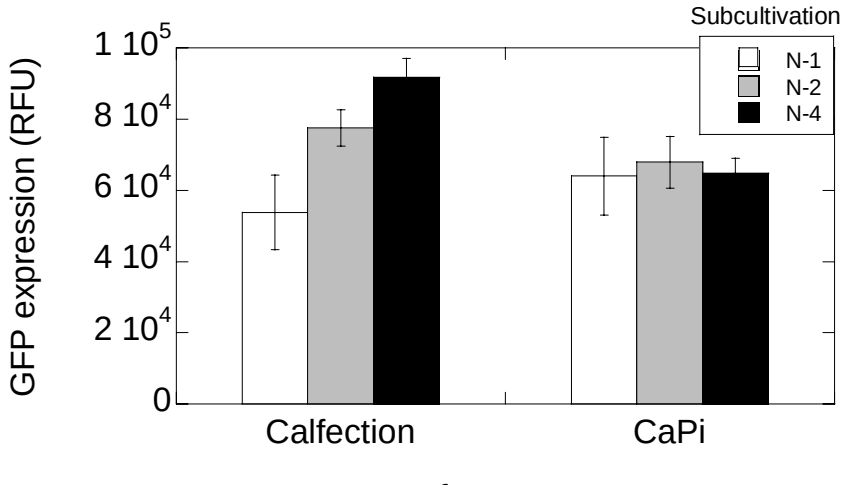

Figure 14. Effect of culture age on calfection. HEK 293E cells were subcultivated 24 h (N-1), 48 h (N-2), or 96 h (N-4) prior to calfection. The cells were inoculated into 12-well microtiter plates at a density of 5x105 cells/ml in modified DMEM/F12 with 1% FCS and then transfected with pEGFP-N1 by either calfection or calcium phosphate-DNA coprecipitation. GFP expression was measured on day 3 post-transfection following cell lysis.

4.1.11 Effect of medium on calfection.

To determine if media other than modified DMEM/F12 support gene transfer by calfection, HEK 293E cells in three different media with and without FCS were transfected with pEGFP-N1 by calfection and by calcium phosphate-DNA coprecipitation. The media chosen for this experiment were modified DMEM/F12, Pro293s CDM, and Ex-Cell VPro. The latter two are chemically defined media that support growth of cells to a high density in suspension culture. HEK 293E cells after 4 days of cultivation were inoculated into 12-well microtiter plates at $5x10^5$ cells/ml in the three different media with or without FCS. The cells were then transfected with pEGFP-N1 either by calfection or calcium phosphate-DNA coprecipitation. For the latter the cells were diluted at 4 h post-transfection with one volume of the same medium as that used for transfection. The cells were not diluted after calfection. Following calfection in modified DMEM/F12 and in Pro293s CDM in the presence of FCS, significant levels of GFP expression were observed, but only low levels of GFP expression were seen following calfection in Ex-Cell VPro or in any media in the absence of FCS (Fig. 15A). In contrast, transfection with calcium phosphate-DNA coprecipitation only yielded a high level of GFP expression in modified DMEM/F12 in the presence of FCS (Fig. 15B).

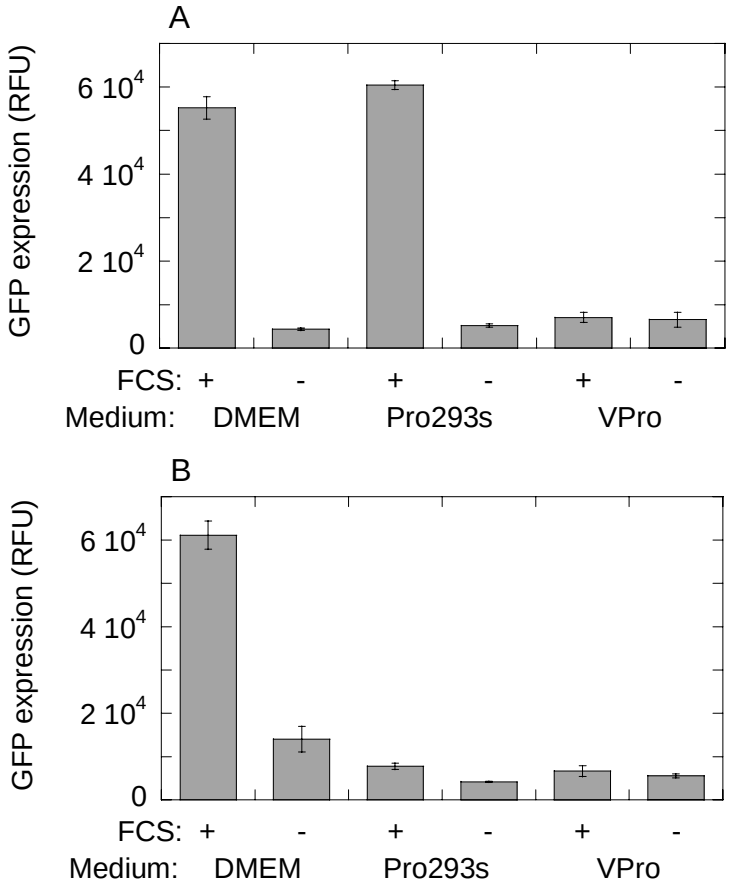

Figure 15. Calfection in different media with or without FCS. HEK 293E cells that were subcultivated 4 days before transfection were inoculated into 12-well microtiter plates at a density of $5x10^5$ cells/ml in either modified DMEM/F12 (DMEM), Pro293s CDM (Pro293s), or Ex-Cell VPro (VPro) with or without 1% FCS. The cells were transfected with pEGFP-N1 by calfection (A) or by calcium phosphate-DNA coprecipitation (B). GFP expression was measured at 3 days post-transfection following cell lysis.

4.1.12 Mechanism of calfection.

The mechanism of DNA transfer by calfection is not completely understood. One possibility is that the mechanism is similar to that of calcium phosphate-DNA co-precipitation. The calcium in the calfection solution may react with phosphate in the culture medium to form a precipitate which includes the plasmid DNA. To address this possibility an attempt was made to visualize a precipitate after addition of DNA and $CaCl_2$ to modified DMEM/F12 (pH 7.6) in the presence of 1% FCS but in the absence of cells. As a control, a calcium phosphate-DNA co-precipitate was also formed and added to the same medium at pH 7.2. By 30 min after DNA addition a precipitate was visible in the plate with the calcium phosphate-DNA coprecipitate but not in the calfection plate (Fig. 16). By 24 h after DNA addition no precipitate was visible in the calfection plate even though large particles were present in the plate with the calcium phosphate-DNA coprecipitate (Fig. 16).

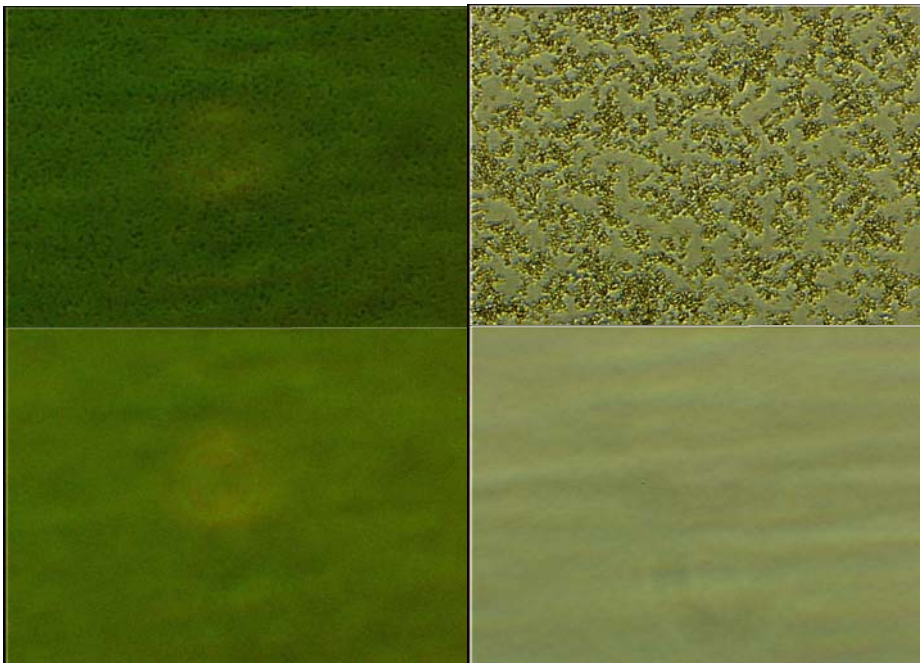

Figure 16. Microscopic analysis of calcium phosphate-DNA coprecipitate formation. A calcium phosphate-DNA co-precipitate (top panels) or a calfection solution (bottom panels) was added to modified DMEM/F12 medium in the absence of cells. Images were taken at a magnification of 320X either 30 min (left panels) or 24 h (right panels) after addition of the DNA to the medium. The images were kindly provided by Jeanette Lindell.

4.2 Discussion

We have found Ca^{2+} to act as an enhancer of gene delivery to suspension-adapted mammalian cells. Previously, several authors observed synergistic effects of Ca^{2+} on gene transfer efficiency using different delivery vehicles. Ca^{2+} increases the transfection potency of plasmid DNA-cationic liposome complexes up to 20-fold with the greatest improvement for Ca^{2+} concentrations between 5 and 25 mM (Lam et al. 2000). Similar levels of Ca^{2+} were shown to enhance polycation-mediated transfection (Haberland et al. 1999) and adenovirus-mediated gene delivery (Walters et al. 1999). The same range of optimal calcium concentrations were observed for calfection in the studies described here (Fig. 3). While previous reports investigated Ca^{2+} as an enhancer of different transfection agents, here it is demonstrated that excellent gene transfer rates can be promoted by Ca^{2+} alone. At this stage the mechanism by which Ca^{2+} promotes gene transfer into cultured cells is not known. The calfection procedure can be divided into two stages: (1) the pre-mixing of Ca^{2+} and plasmid DNA and (2) the period after the addition of this solution to the culture medium, and both of them need to be considered when discussing the mechanism of gene delivery by calfection.

As far as the pre-mixture of DNA and Ca^{2+} is concerned, questions may arise as to whether or not a direct interaction occurs between Ca^{2+} and plasmid DNA resulting in DNA condensation. The results have demonstrated that ready-to-use transfection solutions are stable and maintain their efficiency for at least 9 weeks even at room

temperature. If Ca^{2+} does condense DNA, the results from the storage experiment indicate that the kinetics of such a condensation are almost instantaneous (within seconds) or slow enough to prohibit any significant change in the complex within 60 days at room temperature. The observation that the Ca^{2+} and DNA concentrations in the transfection solution are much less critical than their final concentrations in the culture medium does not support the idea of a direct interaction between the two components. Neither does the fact that separate additions of Ca^{2+} and plasmid DNA to the culture medium (both added within minutes) were as efficient in terms of recombinant protein expression as the addition of the pre-mixed solution. This is in agreement with another report concluding that divalent cations do not provoke condensation of DNA in aqueous solution at room temperature (Knoll et al. 1998).

The most crucial step of the calcium phosphate transfection, the addition of phosphate to the calcium-DNA solution to form the precipitate, is absent during calfection. Jordan and co-workers did not detect any recombinant protein expression when using transfection solutions lacking appropriate concentrations of phosphate (Libby 1984). The results reported here, however, show that high levels of reporter gene expression can be obtained without phosphate in the transfection solution.

Once the two transfection components have been added to the culture medium many questions concerning their fate remain. It is not clear whether a transfection-active species is formed, i.e. a particle or a complex containing plasmid DNA, or what the composition of such a species might be. From the data in Fig. 6 it is clear that the difference between an unsuccessful and an efficient transfection occurs within a narrow range of pH. This observation supports the idea of the appearance of a microprecipitate whose solubility strongly depends on pH. In such a case, phosphate is the most likely candidate anion (Kejnovsky et al. 1998). However, the participation of other medium components in the formation of microprecipitate cannot be excluded. It should be noted that the final concentrations of Ca^{2+} and phosphate in the transfection medium during calfection and calcium phosphate transfection are similar (Girard, Derouazi et al. 2002). Thus, in both cases a *de novo* formation of a microprecipitate is possible. A slightly opalescent appearance of the medium after calcium and DNA addition supports the formation of a microprecipitate that may be the relevant transfer vehicle for calfection. In contrast to the calcium phosphate method, however, it was not possible to visualize particles under a light microscope at 200× magnification. The small size of the hypothetical precipitate formed within the medium may be an explanation for the failure of calfection to transfect adherent cells which is dependant on the sedimentation of a DNA complex onto the cells (data not shown). As can be observed under the microscope, particles created by calcium phosphate precipitation settle onto the cells and within a few hours every cell is covered with a large number of particles. This phenomenon was not observed following calfection of adherent cells.

Calfection at the current stage of development requires the presence of serum during transfection for efficient gene expression (Fig. 7, 15). This might be a drawback for certain applications. Downstream processing is frequently hampered by the presence of serum in the medium, leading to the demand for serum-free processes. Another reason to omit serum in bioprocesses is its non-defined chemical composition. The

role of serum in calfection remains unclear, although albumin, present in serum at a high concentration, may play a key function. Acting as a chelator, albumin may shield, to some extent, cells from the high concentration of extracellular calcium. Furthermore, in the case of a transfection-active species, a direct interaction of serum components with the transfection complex cannot be excluded.

In our opinion, calfection is one of the easiest methods for gene transfer to suspension-grown cells. With a simple mixture of Ca^{2+} and plasmid DNA, genes can be delivered to cell populations with efficiencies comparable to those observed with current well-established methods such as calcium phosphate-DNA coprecipitation. Gene transfer by calfection depends on only a few parameters. These include the concentrations of the plasmid DNA and Ca^{2+}, the pH of the medium, and the presence of serum in the medium. Importantly, it does not require cells to be subcultivated the day prior to DNA transfer. It can be easily and rapidly optimized for expression in a new system and has been shown to be applicable in the common suspension-grown cell lines CHO DG44 and BHK 21 (Fig. 8) as well as at large scale in HEK 293E cells (Fig. 12). Finally, calfection combines simplicity with reproducibility. One drawback is that it may not be feasible in all media (Fig. 15). Taking into account these considerations, we believe that calfection will become a valuable tool for transient gene expression.

4.3 References

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5. Cultivation of animal cells in agitated square bottles

For large-scale applications in biotechnology, cultivation of mammalian cells in suspension is an essential prerequisite. In this chapter a new cultivation system for suspension cultures of mammalian cells is described. Typically, suspension cultures are grown in glass spinner flasks filled to less than 50% of the nominal volume. Here a superior system based on orbital shaker technology is proposed. It was found that square-shaped bottles (square bottles) provide an inexpensive but efficient means to grow HEK 293E and CHO DG44 cells to high density. Cultures in agitated one-liter square bottles exceeded the performance of cultures in spinner flasks, reaching densities up to $7x10^6$ cells/ml for HEK 293E cells and $5x10^6$ cells/ml for CHO DG44 cells in comparison to $2.5\text{-}4x10^6$ cells/ml for cultures of the same cells grown in spinner flasks. Transient reporter gene expression following gene delivery by calcium phosphate-DNA co-precipitation was the same or slightly better for HEK 293E cells grown in square bottles as compared to those grown in spinner flasks. Cost reduction, simplified handling, and better performance in cell growth and viability make the agitated square bottle a very promising tool for the cultivation of mammalian cells in suspension.

5.1 Results

5.1.1 Vessel comparison for HEK 293E cells grown in agitated systems.

To determine the growth characteristics in square bottles of suspension-adapted HEK 293E cells, one-liter square bottles made of glass, PC, PP, or PETC were inoculated at a density of $3.5x10^5$ cells/ml in Pro293s CDM medium. For comparison, cells were also cultivated in one-liter Erlenmeyer flasks and in one-liter spinner flasks. The filling volumes were set to 30% of the nominal volume in each case. The agitation speed of the orbital shaker for the square bottles and Erlenmeyer flasks was set to 120 rpm based on observations that this condition prevented both cell settling and medium foaming (data now shown). These conditions were also successful in avoiding out-of-phase operating conditions (Durocher, Perret et al. 2002). The spinner flasks were stirred at 80 rpm. Parallel cultures in the three different vessels were initiated with a single homogeneous cell population maintained in Pro293s CDM medium in a spinner flask. Cultures grown for 150 h in square bottles achieved higher cell densities ($4.5\text{–}5.0x10^6$ cells/ml) than those grown in Erlenmeyer flasks ($\sim 2x10^6$ cells/ml) or spinner flasks ($\sim 3.5x10^6$ cells/ml) (Fig. 1).

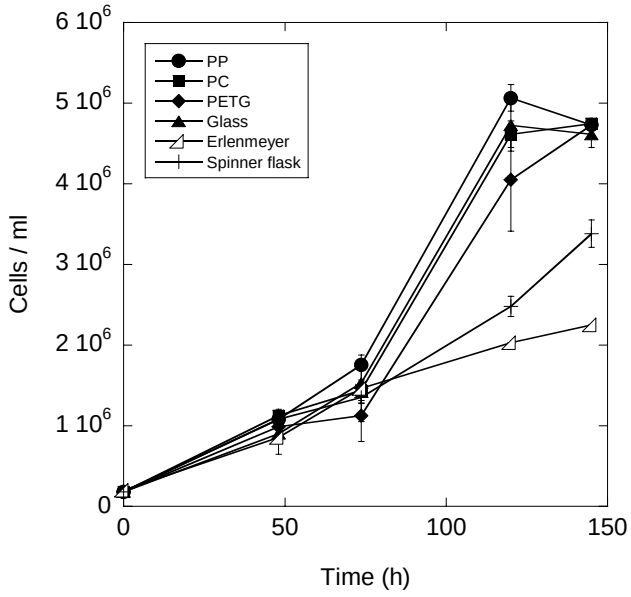

Figure 1. Growth comparison HEK 293E cells in different cultivation vessels. One-liter square bottles of glass, polycarbonate (PC), polypropylene (PP), or polyethylene terephthalate copolyester (PETG) and one-liter Erlenmeyer flasks and spinner flasks were inoculated in duplicate with HEK 293E cells at a density of $3.5x10^5$ cells/ml in 300 ml of Pro293s CDM medium. The viable cell number was determined with a CASY1 cell counter at the times indicated.

The composition of the square bottles used for the cultivation of HEK 293E cells had little effect on cell growth (Fig. 1). Therefore, glass bottles were used in all subsequent experiments because of their lower cost and durability. The glass bottles were also easier to clean and allowed visual monitoring of the culture. As noted in Methods and Materials, the screw cap of the glass bottle was opened one quarter of a turn after approximately 24 h of incubation. However, it was observed that if the cap was opened one quarter of a turn, a half a turn, or one or two full turns, the gas exchange was the same (Gleich and Wurm, unpublished data).

5.1.2 Effect of filling volume on growth of HEK 293E cells in agitated square bottles.

For mammalian cells cultivated in spinner flasks and square bottles, filling volume is critical because the headspace of the vessel functions as a reservoir for O_2 and CO_2. It also affects foam formation caused by the agitation of the medium. In square bottles, foaming resulted if the filling volume was too low. To determine the effect of filling volume on cell growth in square bottles, HEK 293E cells were inoculated at a density of $3.5x10^5$ cells/ml in square bottles containing 300 to 700 ml of Pro293s CDM medium, and cell growth was monitored for three consecutive passages. Culture volumes between 300 and 500 ml yielded the best cell growth with the maximum cell densities ranging from $3.5\text{-}5x10^6$ cells/ml, while poor growth was observed with a

filling volume of 700 ml (Fig. 2). Culture volumes of less than 300 ml yielded improved growth when the agitation speed was decreased from 120 rpm to 105 rpm (data now shown).

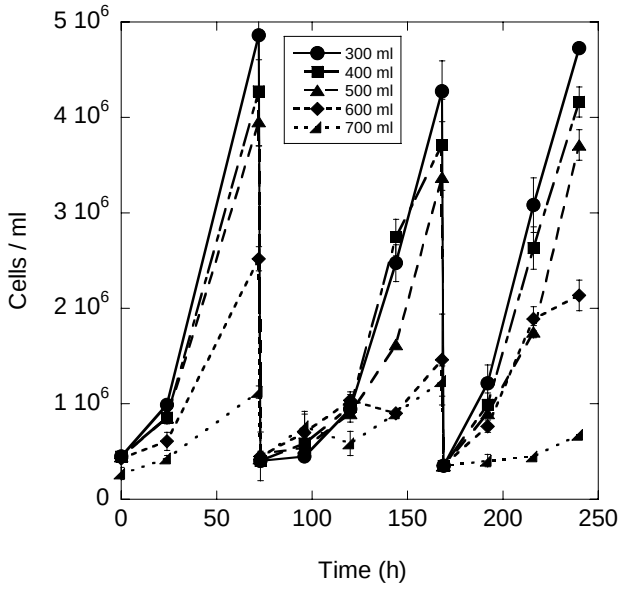

Figure 2. Effect of filling volume on growth of HEK 293E cells in square bottles. One-liter square bottles were inoculated in duplicate with HEK 293E cells at a density of $3.5x10^5$ cells/ml in different volumes of Pro293s CDM medium as indicated and agitated at 120 rpm. Cells were subcultivated at both 72 h and 168 h post-inoculation. The viable cell number was determined using the Trypan blue exclusion method at the times indicated.

5.1.3 The effect of square bottle size on the growth of HEK 293E cells in suspension.

The effect of bottle size on cell growth was investigated by inoculating square bottles of 250 ml, 500 ml, or 1 l to 40% of the nominal volume with HEK 293E cells in Pro293s CDM medium. The cell density and agitation speed were the same as described above. The growth of the cultures was monitored for three passages. Bottle size did not affect cell growth as all the cultures routinely resulted in cell densities ranging from $4\text{-}5x10^6$ cells/ml (Fig. 3).

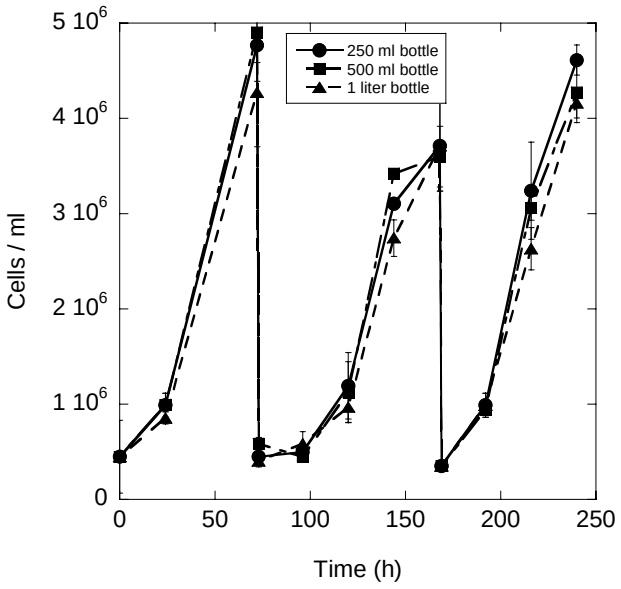

Figure 3. Effect of square bottle size on cell growth. Square bottles of three different sizes were inoculated in duplicate with HEK 293E cells at a density of $3.5x10^5$ cells/ml at 40% of the nominal volume and agitated at 120 rpm. Each culture was subcultivated at 72 h and 168 h post-inoculation. Viable cell number was determined using the Trypan blue exclusion method at the times indicated.

The growth of animal cells in square-shaped bottles with volumes greater than 1 liter was also investigated by inoculating two- and ten-liter square bottles to 40% of the nominal volume with HEK 293E cells in Pro293s CDM medium. The cell density and agitation speed were the same as described above. These containers were mounted on an orbital shaker using either double-sided tape (two-liter bottle) or storage boxes fastened to the shaker with belts (ten-liter bottle). The double-sided tape was not effective for retention of the 10-liter bottles. The two-liter bottle was square-shaped but had a built-in handle which altered the geometry of one face. The ten-liter bottle was slightly rectangular. It was necessary to use these vessels since the one-liter square bottles described above were not available in larger sizes. In two experiments in two-liter square bottles the cell density at day 4 post-inoculation reached $2.5x10^6$ cells/ml (Fig. 4). The cell density was lower than what was routinely achieved in one-liter square bottles. This may have been due to the different geometries of the two types of bottles. For the two-liter bottle, out-of-phase mixing was observed when agitation speed was increased from 120 rpm to 140 rpm or higher.

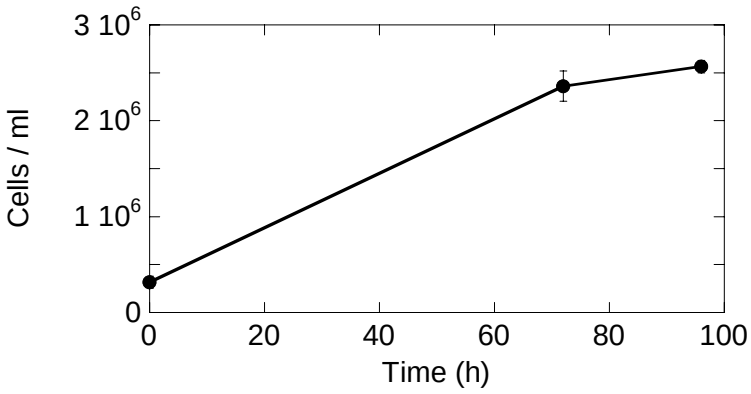

Figure 4. Cell growth in two-liter square bottles. The bottles were inoculated in duplicate with HEK 293E cells at a density of $3.5x10^5$ cells/ml in 800 ml of Pro293s CDM medium and agitated at 120 rpm. No subcultivation was performed during the course of the experiment. Viable cell number was determined using the Trypan blue exclusion method at the times indicated.

When using the ten-liter bottles it was difficult to maintain a sterile culture. This was perhaps due to a larger opening in this bottle as compared to those in the one- and two-liter square bottles. As a consequence, sampling was difficult. Three experiments in ten-liter bottles were started, but only one was successfully completed, and it yielded almost $4.5x10^6$ cells/ml after 8 days of cultivation (Fig. 5). These results suggest that the growth of suspension cultures is feasible in ten-liter square bottles and that this approach may be an alternative for producing cells for the inoculation of large-scale bioreactors. However, improvements in the bottle need to be made to facilitate sampling.

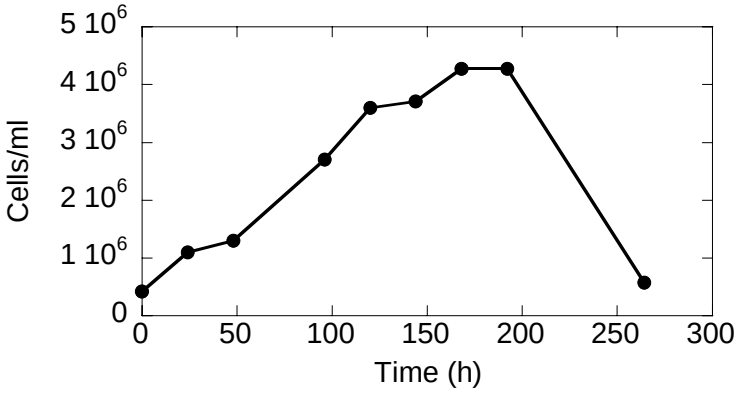

Figure 5. Cell growth in ten-liter square bottles. A single bottle was inoculated with HEK 293E cells at a density of $3.5x10^5$ cells/ml in 4 l of medium and agitated at 120 rpm. No subcultivation was performed during the course of the experiment. Viable cell number was determined using the Trypan blue exclusion method at the times indicated.

5.1.4 Effect of both shaking speed and diameter on growth of HEK 293E cells in square bottles.

The effect of shaking speed on cell growth for a given culture volume was determined by inoculating one-liter square bottles with HEK 293E cells at a density of $3.5x10^5$ cells/ml in 300, 400, or 500 ml of Pro293s CDM. The cultures were agitated at speeds ranging from 100 to 140 rpm, but only the results for the cultures agitated at 120 and 130 rpm are shown (Fig. 6). Overall, better cell growth was observed with agitation at 130 rpm as compared to 120 rpm for all of the volumes tested (Fig 6). An increase in agitation speed can improve the rate of oxygen transfer in a suspension culture (Parham, Iannone et al. 1998), but in this experiment agitation at speeds higher than 130 rpm resulted in foam formation that was accompanied by a decline in cell viability (data not shown). Agitation at speeds below 120 rpm did not affect the viability of the culture, but the maximal cell density was reduced as compared to agitation at 120 or 130 rpm (data not shown). At agitation speeds below 100 rpm, cells settled on the bottom of the bottle. Although increasing the agitation speed from 120 to 130 rpm yielded a higher cell number in all volumes tested, only a small increase was observed for the 500 ml culture. This may have been due to the combination of large culture volume and small headspace limiting the gas exchange. However, if the speed was increased to try to achieve better mixing, the liquid reached the nonsterile barrier (the cap).

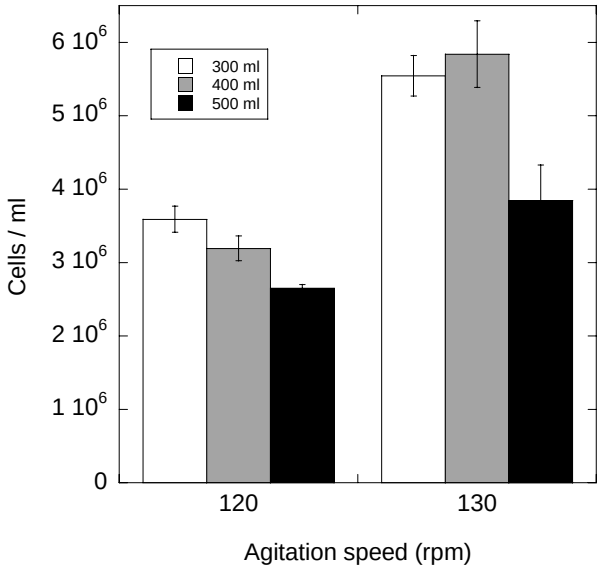

Figure 6. Comparison of agitation speed on cell growth in square bottles. One-liter bottles were inoculated in duplicate with HEK 293E cells at a density of $3.5x10^5$ cells/ml in different volumes of Pro293s CDM medium as indicated and agitated at 120 or 130 rpm. Viable cell number was determined at 3 days post-inoculation using the Trypan blue exclusion method.

The experiments described above were performed with an orbital shaker having a rotational diameter of 2.5 cm. However, most commercially available shakers have an adjustable rotational diameter. To determine the effect of this parameter on cell

growth in square bottles, cultures of HEK 293E were agitated on an orbital shaker with a rotational diameter of 5 cm. With filling volumes of 300-400 ml, cell growth at an agitation speed of 110 rpm was the same as that at an agitation speed of 130 rpm on a shaker with a rotational diameter of 2.5 cm (data not shown).

5.1.5 Maximum growth of HEK 293E cells in square bottles.

Based on the results from the experiments described above, the maximum growth of HEK 293E cells in square bottles and in spinner flasks was compared. One-liter square bottles and spinner flasks were each inoculated in duplicate at a density of $3.5x10^5$ cells/ml in Ex-Cell 293 medium. Exponential growth was observed for the first 120 h of cultivation in square bottles but only for the first 75 h in spinner flasks (Fig. 7A). In square bottles, the maximum cell density reached $6\text{-}7x10^6$ cells/ml whereas the cultures in spinner flasks only achieved a maximum cell density of $4x10^6$ cells/ml by 150 h post-inoculation (Fig. 7A). The cell viability of the cultures grown in square bottles remained rather constant for 150 h post-inoculation at a level greater than 95% (Fig. 7B). In contrast, the viability of cultures in spinner flasks began to decrease around 50 h post-inoculation and eventually fell below 85% by 150 h post-inoculation (Fig 7B). Despite growth to a high cell density, not many cell aggregates were observed in the cultures in square bottles. The oxygen level of each culture was analyzed at various times after inoculation. At 72 h post-inoculation when the cell density of the two cultures was approximately the same, the pO_2 levels in square bottles and spinner flasks were 11 kPa and 6 kPa, respectively. Therefore, oxygen limitation did not appear to be a problem for either culture under the conditions tested. The pH was also determined throughout the cultivation period. From an initial pH of 7.2, the pH stabilized at approximately 6.8 in spinner flasks and 6.5-6.7 in square bottles by 72 h post-inoculation.

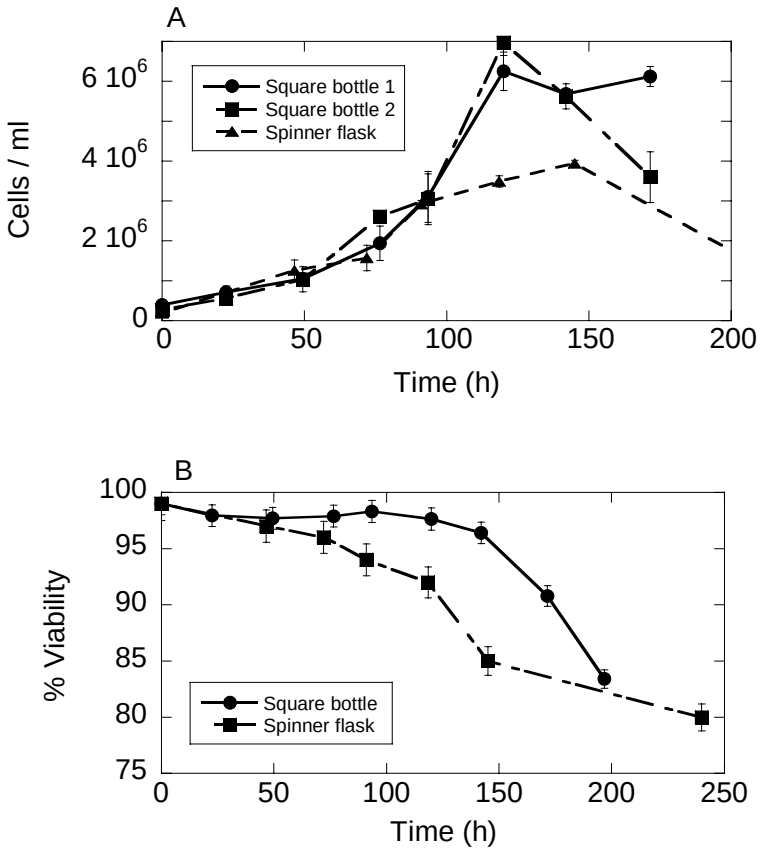

Figure 7. Maximum growth of HEK-293E cultures in square bottles and spinner flasks. Four one-liter square bottles and two one-liter spinner flasks were inoculated with HEK 293E cells at a density of $3.5x10^5$ cells/ml in 300 ml of Ex-Cell 293 medium. The square bottles were agitated at 130 rpm, and the spinner flasks were stirred at 80 rpm. (A) The cell number in the square bottles labeled 1 was determined with a CEDEX cell counter. For the square bottles labeled 2 and for the spinner flasks, the cell number was determined manually using a Neubauer hemocytometer. (B) The viability of each culture was determined with the Trypan blue exclusion method at the times indicated.

5.1.6 Gene transfer into HEK 293E cells grown in square bottles.

The major objective of the cultivation of mammalian cells in square bottles was to provide large numbers of cells with a high viability for large-scale transient gene expression. Therefore it was important to determine if cells grown in square bottles were transfected with the same efficiency as those grown in spinner flasks since it has been shown that some operational changes or media additives appear to alter specific productivity by impacting gene transcription, cell cycle progression, or cellular metabolic activity (Jordan, Kohne et al. 1998). HEK 293E cells grown in either square bottles or spinner flasks were used to seed 12-well microtiter plates. The cells were then transfected with a mixture of pEAK8-LH41, pEAK8-LH39, and pEGFP-N1 in a ratio of 69:39:2 (w/w/w). At 3 days post-transfection the levels of the two reporter proteins were about the same regardless of the source of the cells (Table 1). These results demonstrated that HEK 293E cells grown in an agitated square bottle were efficiently transfected.

Table 1. Reporter protein expression in transfected HEK 293E cells.

Cultivation system	**IgG expression**[a]	**GFP expression**[b]
Spinner flask	3.5 +/- 0.6 mg/l	4900 +/- 300 RFU
Square bottle	4.0 +/- 0.7 mg/l	4500 +/- 200 RFU

[a]Determined by ELISA at 3 days post-transfection. Each value is the average of 3 transfections.
[b]Determined by fluorometry at 3 days post-transfection. Each value is the average of 3 transfections.

5.1.7 Growth of CHO DG44 cells in square bottles.

To determine if cell lines other than HEK 293E could be grown in square bottles, one-liter bottles and spinner flasks were inoculated in duplicate with CHO DG44 cells at a density of $3.5x10^5$ cells/ml in ProCHO5 CDM medium at a filling volume of 300 ml. As observed for HEK 293E cells, growth of CHO DG44 cells in square bottles over several passages was superior to that in spinner flasks (Fig. 8). The cultures in the square bottles routinely reached a cell density of about $5x10^6$ cells/ml, while that in spinner flasks was $2.5\text{-}4x10^6$ cells/ml (Fig. 8).

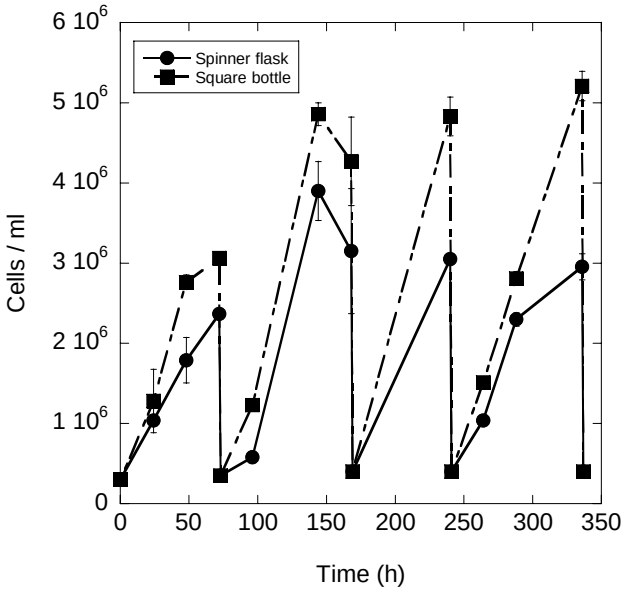

Figure 8. Cultivation of CHO DG44 cells in square bottles. One-liter square bottles and spinner flasks were inoculated in duplicate with CHO DG44 cells at a density of 3.5x105 cells/ml in 300 ml ProCHO5 CDM medium. The cultures in the square bottles were agitated at 130 rpm and those in spinner flasks were stirred at 80 rpm. The cells were subcultivated every 3-4 days as indicated. Viable cell number was determined using the Trypan blue exclusion method at the times indicated.

As for HEK 293E cells, a study for determining the maximum cell density in square bottles was performed with CHO DG44 cells. Duplicate cultures in one-liter square bottles and spinner flasks were inoculated at $2.5x10^5$ cells/ml. As before, the cultures in square bottles reached a higher cell density than those in spinner flasks (Fig. 9). The highest cell density for the cultures in spinner flasks was about $2.5x10^6$ cells/ml, whereas the cultures in the square bottles reached nearly $5x10^6$ cells/ml (Fig. 9). Importantly, for the cultures in square bottles, the viability remained above 95% for a four-day period and above 90% for a one-week cultivation (data not shown). The results demonstrated that the square bottle cultivation system described here is applicable to cell lines other than HEK 293E.

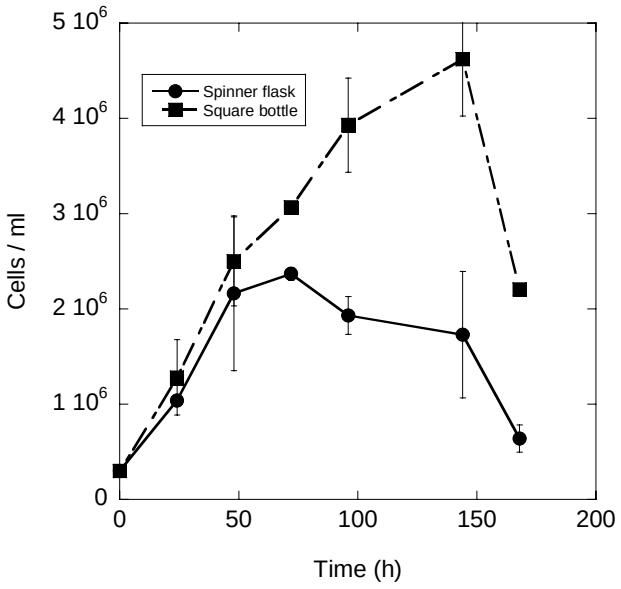

Figure 9. Maximum growth of CHO DG44 cells in square bottles and spinner flasks. One-liter square bottles and spinner flasks were inoculated in duplicate at $2.5x10^5$ cells/ml in 300 ml of Pro CHO5 CDM medium. The square bottles were agitated at 130 rpm, and the spinner flasks were stirred at 80 rpm. The viable cell number was determined at the times indicated using the Trypan blue exclusion method.

5.1.8 Comparison of cell growth in square bottles and in a bioreactor.

The maximum growth of cells in square bottles was compared to that in a 3-liter bioreactor. HEK 293E cells were used to inoculate duplicate cultures in one-liter square bottles and in a single bioreactor at a density of $3.5x10^5$ cells/ml in Ex-Cell 293 medium. The final volumes were 300 ml for the square bottles and 1 l for the bioreactor. Square bottles were agitated at 130 rpm and the bioreactor was stirred at 200 rpm. All cultures were fed upon need with glucose (to 4 g/L), glutamine (to 4 mM), and sodium bicarbonate (to 10 mM). Exponential cell growth was observed in both cultivation systems for the first 160 h after inoculation (Fig. 10). In square bottles the maximum cell density reached $5.5x10^6$ cells/ml whereas that in the bioreactors was nearly $7x10^6$ cells/ml (Fig. 8). The cell viability in the square bottles and in the bioreactor remained nearly constant at more than 90% for the first 170 h of incubation (Fig. 10). Despite the high cell densities, not many cell aggregates were observed. For each culture the oxygen level was analyzed at various times throughout the experiment. At 72 h post-inoculation when the cell density of the two cultures was approximately the same, the pO_2 levels in square bottles and in the bioreactor were about 6 kPa. Therefore, oxygen limitation did not appear to be a problem for either cultivation system under the conditions tested. The pH, glucose, glutamine, ammonium, and lactate levels were similar for both culture systems throughout the course of the experiment (data not shown).

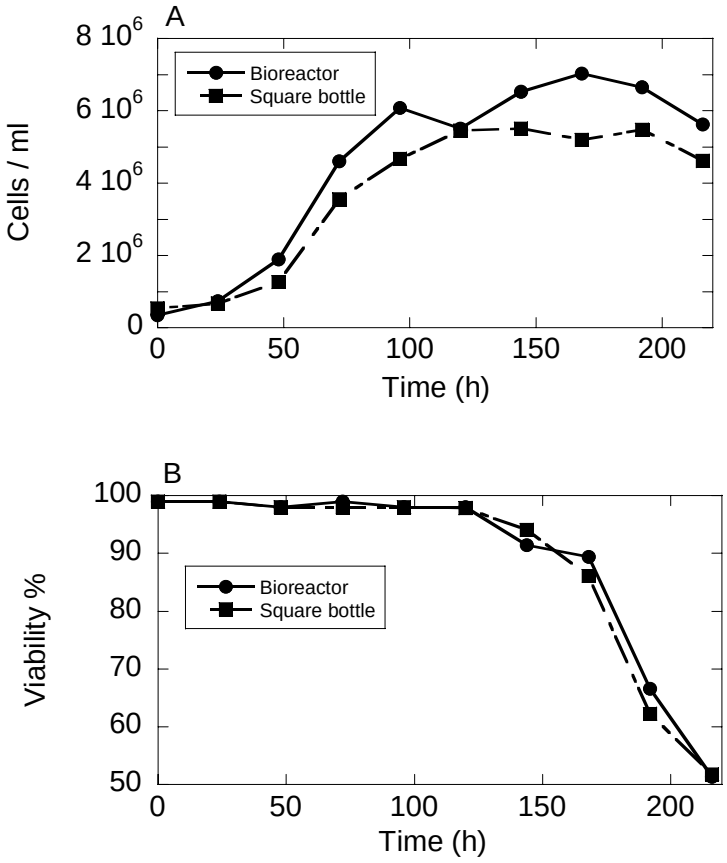

Figure 10. Growth of HEK 293E cultures in square bottles and in a three-liter bioreactor. Two one-liter square bottles and one three-liter bioreactor were inoculated with HEK 293E cells at a density of $3.5x10^5$ cells/ml in Ex-Cell 293 medium. (A) The viable cell number and (B) the viability were determined with the Trypan blue exclusion method at the times indicated.

5.1.9 Application of the square bottle cultivation system to medium optimization.

A comparative study of the growth of HEK 293E cells in different cell culture media was performed with Pro293s CDM, CD 293, 293 SFM II, G-0791 Gene-Therapy II, Ex-Cell 293 and Ex-Cell 520 media. For each medium, duplicate cultures inoculated in one-liter square bottles at a cell density of $3.5x10^5$ cells/ml were evaluated. The cultures were agitated at 130 rpm, and the cells were routinely passaged every three or four days. The study was not directed towards determining which of these media supported the maximum cell density. Therefore the densities were slightly lower than these in the optimization studies described above. With Ex-Cell 293 medium, the average cell density for the five passages ranged from $2\text{-}2.5x10^6$ cells/ml (Fig. 11), and the viability of the cultures remained above 98% (data now shown). Lower cell densities were observed for the cultures grown in Pro293s CDM and 293 SFM II media (Fig. 11). Throughout these studies, the pH and metabolite levels of each

culture were determined but no significant differences were observed (data not shown).

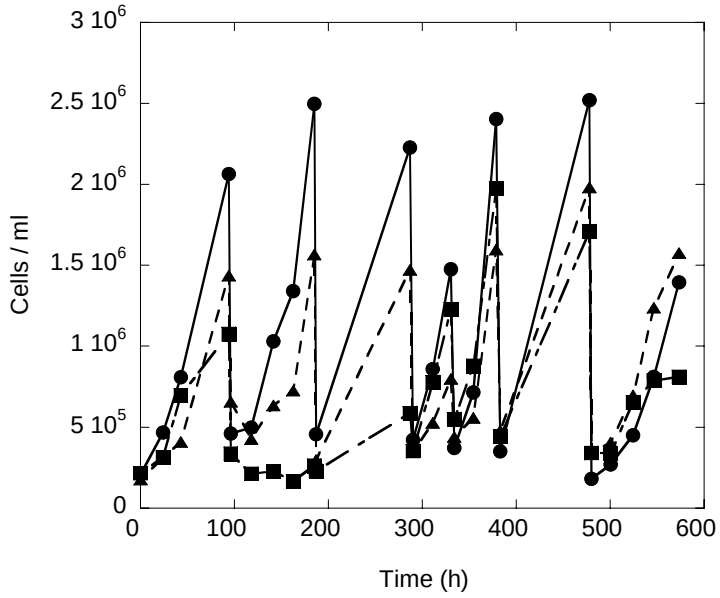

Figure 11. Comparison of cell growth in different media. One-liter square bottles were inoculated in duplicate with HEK 293E cells at a density of $3.5x10^5$ cells/ml in 300 ml of Ex-Cell 293 (filled circles), Pro293s CDM (filled triangles), or 293 SFM II medium (filled squares). The viable cell number was determined at the times indicated using the Trypan blue exclusion method.

5.2 Discussion

Square bottles mounted on a horizontal orbital shaker were evaluated with respect to their potential for the propagation of suspension cultures of HEK 293E and CHO DG44 cells. For these two widely used cell lines, very good cell growth, which depended on specific conditions for the filling volume and the agitation speed, was observed. In fact, maximal cell densities following regular subcultivations exceeded the densities observed with cultures grown in spinner flasks, the standard system to cultivate cells using a non-instrumental approach. A high level of reproducibility in performance was also seen; the cell density and viability of parallel cultures usually differed by less than 10%.

Filling volume and shaking speed, each highly dependent on the other, were found to be among the most important parameters for optimal cell growth in square bottles. For a one-liter square bottle, a filling volume of 400 ml and a shaking speed of 130 rpm were shown to be optimal when using a rotational diameter of 2.5 cm. However, other combinations of filling volume and shaking speed also resulted in good cell growth. For applications requiring expansion to a large-scale culture, a filling volume of 500

ml in a one-liter bottle is possible, but the maximum cell density may not be as high as observed for the optimal conditions described here. It is also possible to increase the volume of the culture by using larger bottles. Here the cultivation of HEK 293E cells in two- and ten-liter square bottles was demonstrated. Growth of suspension cultures of animal cells to a high cell density with high viability has also been achieved in small volumes (0.125 to 2.0 ml) using microtiter plates (McDaniel and Bailey 1969; Jordan, Kohne et al. 1998; Buchs, Lotter et al. 2001; Brorson, De Wit et al. 2002) and in larger volumes (up to 56 l) in various containers (Duetz and Witholt 2004) when the mixing parameters were optimally adjusted.

The agitation of suspension cultures by orbital shaking can improve gas exchange into and out of the medium (Girard 2001; Büchs 2001b), and the geometry of square bottles may further enhance gas exchange. The corners in these bottles may act similarly to baffles in bioreactors, increasing shear stress, speed distribution, and surface renewal, resulting in a more turbulent flow and in a significant increase in the oxygen transfer rate (OTR). An optimal surface to volume ratio in square-shaped vessels may also lead to turbulent flow (Duetz and Witholt 2001). For shaken vessels, the ratio of the volume of the liquid to the volume of the headspace in combination with the speed of agitation may limit the OTR or result in out-of-phase mixing conditions which in turn influence the efficiency of agitation (Büchs et al. 2001a; Duetz and Witholt 2001). This may explain why drastically reduced cell growth was observed for culture volumes between 600 and 800 ml in a one-liter square bottle despite the partial opening of the bottle and the absence of out-of-phase operating conditions. Although it has been shown in the past that the type of closure can inhibit or reduce growth rates in *E. coli* (Gupta and Rao 2003; McDaniel and Bailey 1969), this was not the case when different sized caps and bottle openings were tested under our optimal conditions.

There are many benefits to using glass rather than plastic bottles. They are economical, easy to clean, and allow for visibility of the culture. Additionally, these bottles were the easiest to fit onto the orbital shaker with double-sided tape. It was also possible to agitate the square bottles within racks attached to the shaker, but the double-sided tape was easier to handle and allowed for the optimization of incubator space. As for bottle reuse, the PC and PP bottles did not always survive a second or third round of autoclaving and were therefore not as durable as the glass bottles. The single-use PETG bottles were not cost efficient.

The main purpose of this research was to evaluate a cultivation system for producing large quantities of mammalian cells in suspension for large-scale transient gene expression. We have shown that cells cultivated in square bottles were transfected with the same efficiency as cells grown in spinner flasks. Cultures in square bottles not only yielded a higher number of cells per unit volume than cultures in spinner flasks, but the quality of the cells as measured by viability was also better. Cultures grown in square bottles had viabilities higher than 90% for up to a week after inoculation. We have found that this parameter of culture quality can be predictive of success for large-scale transient gene expression in suspension cultures. Experiments on the transfection of cells in square bottles are described in Chapters 6 and 7.

5.3 References

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6. Polyfection of HEK 293E cells in chemically defined medium

The large-scale transfection of HEK 293E cells has been reported using either calcium phosphate-DNA coprecipitation or polyfection for DNA delivery (Amstutz, Miescher et al. 1999; Girard, Jordan et al. 2001; Liu and Hong 2001; Meissner, Pick et al. 2001; Duetz and Witholt 2004). None of these experiments were performed, however, in a chemically defined medium. For the transfections using calcium phosphate-DNA coprecipitation it was necessary to include serum in the medium. Although polyfection does not require serum, but it has not been accomplished in a chemically defined medium (Durocher, Perret et al. 2002; Girard, Derouazi et al. 2002). The objective of the experiments described in this chapter was to develop a method for the large-scale transfection of HEK 293E cells in serum-free, chemically defined medium. Using PEI as a DNA delivery it was possible to achieve high level recombinant protein expression in chemically defined Ex-Cell 293 CDM medium in both instrumented (3-L bioreactors) and non-instrumented (microtiter plates, round and square glass bottles, and 50 ml centrifuge tubes) cultivation systems at scales from 1 ml to 1 liter.

6.1 Results

6.1.1 Polyfection of HEK 293E cells in serum-free RPMI 1640 medium.

The initial studies to determine the conditions for the transfection of suspension-adapted HEK 293E cells with PEI focused on the DNA:PEI ratio. The transfections were performed in RPMI 1640 since the polyfection of CHO DG44 cells was successful in this medium (Derouazi et al. 2004). At the time of these experiments, our HEK 293E cells were routinely grown in chemically defined Pro293s CDM medium. On the day before transfection, the cells were seeded in fresh Pro293s CDM, and on the day of transfection the cells were washed once in RPMI 1640 and then seeded in this medium at a density of $2x10^6$ cells/ml in 12-well microtiter plates. The cells were transfected with a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 to give a final DNA concentration of 2.5 µg/ml in the medium. The DNA:PEI ratio was varied from 1:1 to 1:5 (w/w). At 4 h post-transfection, the cells were diluted with one volume of Pro293s CDM. AT 3 days post-transfection, GFP expression was the highest at DNA:PEI ratios of 1:3 to 1:5 (Fig. 1A) and IgG expression was the highest at a ratio of 1:3 but other DNA:PEI ratios gave similar results (Fig. 1B). When the transfections were performed at a cell density of $1x10^6$ cells/ml, reporter protein expression was about 50% lower than in Fig. 1 (data not shown). Subsequent transfections of HEK 293E cells with PEI were performed at a density of $2x10^6$ cells/ml and a DNA:PEI ratio of 1:3 (w/w).

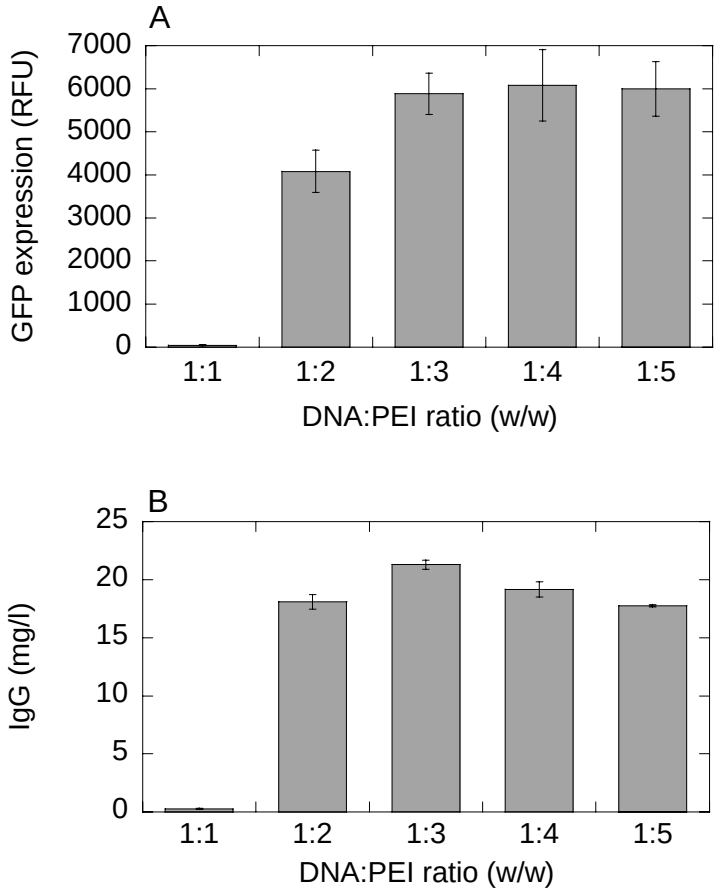


Figure 1. Effect of DNA:PEI ratio on the polyfection of HEK 293E cells in RPMI 1640 medium. Cells were seeded into 12-well microtiter plates at a density of $2x10^6$ cells/ml in RPMI 1640. The cells were transfected with a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 using various DNA:PEI ratios. The cells were diluted with one volume of Pro293s CDM at 4 h post-transfection. At 3 days post-transfection, (A) GFP expression was determined by fluorometry after cell lysis and (B) the IgG concentration was determined by ELISA.

6.1.2 Polyfection in chemically defined medium.

Since our HEK 293E cells were being routinely cultivated in chemically defined Ex-Cell 293 CDM, it was decided to attempt to transfect them in this medium rather than in RPMI 1640. Ex-Cell 293 CDM is a medium optimized for high density cell growth in suspension culture. Therefore, it may provide better support for cell growth and viability after transfection than RPMI 1640, a minimal medium. Initially, transfections at DNA:PEI ratios of 1:1 to 1:5 (w/w) were attempted, but under these conditions no reporter protein expression was detected (data not shown). For this reason, transfections at higher DNA:PEI ratios were performed. HEK 293E cells were seeded at either $0.5x10^6$, $1x10^6$, or $2x10^6$ cells/ml in Ex-Cell 293 CDM in 12-well microtiter plates. The cells were transfected with pEGFP-N1 at DNA:PEI ratios ranging from 1:8 to 1:13 (w/w). As a control, cells seeded at a density of $2x10^6$ cells/ml in RPMI 1640 were transfected with pEGFPN-1 at a DNA:PEI ratio of 1:3 (w/w), the optimal conditions for this medium. All cultures were diluted with one volume of Ex-Cell 293 media at 4 h post-transfection. For the transfections in Ex-Cell

293 CDM, GFP expression was detected at DNA:PEI ratios of 1:10 or higher for all the cell densities tested (Fig. 2). The highest GFP expression was seen at a cell density of $2x10^6$ cells/ml and a DNA:PEI ratio of 1:11 (w/w) (Fig. 2). Under these conditions GFP expression was about 4-fold higher than for the control transfection in RPMI 1640 (Fig. 2). It was also observed that transfection in Ex-Cell 293 at a DNA:PEI ratio of 1:11 (w/w) resulted in more single cells and less cell aggregates than transfection in RPMI 1640 (Fig. 3).

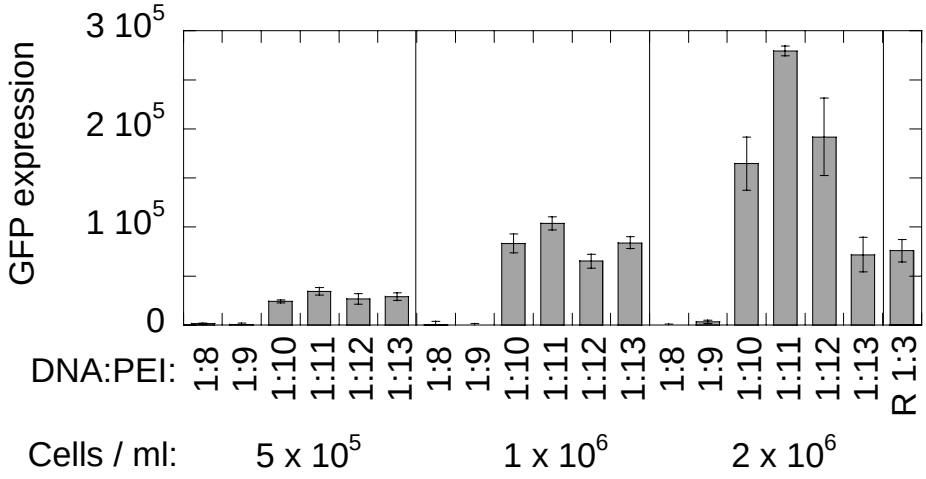


Figure 2. Polyfection of HEK 293E cells in Ex-Cell 293 CDM medium. The cells were seeded in 12-well microtiter plates at different cell densities as indicated in Ex-Cell 293. The cells were transfected with pEGFPN-1 at various DNA:PEI ratios as indicated. Cells were also seeded in RPMI 1640 at a density of $2x10^6$ cells/ml and transfected with pEGFP-N1 at a DNA:PEI ratio of 1:3 (denoted R 1:3). The cells were diluted with one volume of Ex-Cell 293 at 4 h post-transfection. GFP expression was measured at 3 days post transfection after cell lysis.

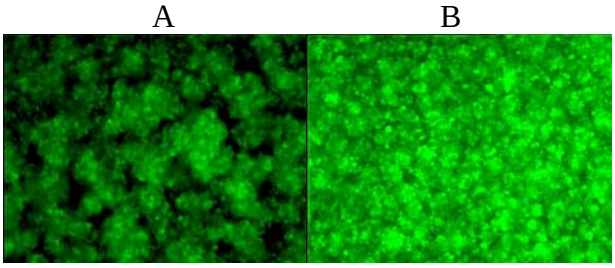


Figure 3. HEK 293E cells polyfected with pEGFP-N1. (A) Polyfection in RPMI 1640 at a density of $2x10^6$ cells/ml and a DNA:PEI ratio of 1:3 (w/w). (B) Polyfection in Ex-Cell 293 CDM at a density of $2x10^6$ cells/ml and a DNA:PEI ratio of 1:11 (w/w). Images were taken at a magnification of 100X.

6.1.3 Effect of culture conditions on polyfection of HEK 293E cells.

Normally, HEK 293E cells were subcultivated in Ex-Cell 293 CDM the day before transfection, washed once in fresh medium, and then centrifuged and resuspended in fresh medium prior to transfection. To determine if all of these steps were necessary for efficient gene transfer by polyfection, two separate experiments were performed. In the first, the necessity of the wash step was investigated, and in the second, the

necessity of two steps, the subcultivation of cells 1 day before transfection and the seeding of cells in fresh medium at the time of transfection, was addressed.

The importance of the wash step was determined using two different cultivation conditions: (1) cells grown in Ex-Cell 293 CDM medium for 1 day were centrifuged and resuspended in fresh medium (either Ex-Cell 293 CDM or RPMI 1640) without a wash step (termed N-1) and (2) cells were grown as in (1) except that they were washed once with fresh medium (either Ex-Cell 293 CDM or RPMI 1640) prior to resuspension in the appropriate medium for transfection (N-1 washed). After transfer into 12-well microtiter plates at a density of $2x10^6$ cells/ml, the cells were transfected with pEGFP-N1 at a DNA:PEI ratio of either 1:11 (w/w) for cells in Ex-Cell 293 CDM or 1:3 (w/w) for cells in RPMI 1640. At 4 h post-transfection the cultures were diluted with one volume of Ex-Cell 293 CDM. Regardless if the cells were transfected in Ex-Cell 293 CDM or RPMI 1640, the wash step prior to transfection was not necessary (Table 1). In fact, it may have been detrimental for polyfection in Ex-Cell 293 CDM medium (Table 1).

Table 1. Effect of washing step on the efficiency of polyfection of HEK 293E cells.

Culture condition[a]	**Normalized GFP expression in Ex-Cell 293 CDM**[b]	**Normalized GFP expression in RPMI 1640**[c]
(N-1)	1.00+/-0.12	1.00+/-0.15
(N-1 washed)	1.21+/-0.09	0.99+/-0.16

[a]Cells grown in Ex-Cell 293 CDM medium for 1 day were centrifuged and resuspended in fresh medium (either Ex-Cell 293 CDM or RPMI 1640) without a wash step (N-1). Cells were grown as above except that they were washed once with fresh medium (either Ex-Cell 293 CDM or RPMI 1640) prior to resuspension in the appropriate medium for transfection (N-1 washed).

[b]Polyfection of HEK 293E cells with pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w) in Ex-Cell 293 CDM. GFP expression was determined at day 3 post-transfection after cell lysis and normalized to the value obtained for the N-1 culture in Ex-Cell 293 CDM.

[c]Polyfection of HEK 293E cells with pEGFP-N1 at a DNA:PEI ratio of 1:3 (w/w) in RPMI 1640. GFP expression was determined at day 3 post-transfection after cell lysis and normalized to the value obtained for the N-1 culture in RPMI 1640.

To investigate the requirement for both the subcultivation of cells 1 day before transfection and the seeding of cells in fresh medium at the time of transfection, HEK 293E cells were subcultivated in Ex-Cell 293 CDM either 1 day (N-1) or 4 days (N-4) before the day of transfection. On the day of transfection the cells were treated in two different ways, either they were centrifuged and resuspended in fresh Ex- Cell 293 CDM or they were left in their conditioned medium. In either case, the cells were seeded into 12-well microtiter at a density of $2x10^6$ cells/ml and transfected with a mixture of 90% pEAK8 and 10% pEGFPN-1 at DNA:PEI ratios ranging from 1:11 to 1:13 (w/w) unless otherwise stated. At 4 h post-transfection the cells were diluted with one volume of fresh Ex-Cell 293 CDM. The highest GFP expression was observed following transfection of the N-1 cells that were resuspended in fresh

medium at the time of transfection and then transfected at a DNA:PEI ratio of 1:11 (w/w) (Fig. 4). At this DNA:PEI ratio, GFP expression was about 30% lower for the N-1 cells that were maintained in conditioned medium as compared to the N-1 cells that were resuspended in fresh medium (Fig. 4). For the other DNA:PEI ratios tested, GFP expression was the same for the N-1 cells transfected in conditioned or in fresh medium (Fig. 4). For the transfections of N-4 cells in fresh or conditioned medium, GFP expression was always lower than with the N-1 cells treated under the same conditions (Fig. 4). These results suggest that HEK 293E cells seeded in fresh medium 1 day before transfection can be transfected in either conditioned or fresh medium. However, higher recombinant protein expression is expected if the cells are transfected in fresh medium. The results also demonstrated that the seeding of cells in fresh medium 1 day before transfection is necessary to achieve the highest possible recombinant protein expression.

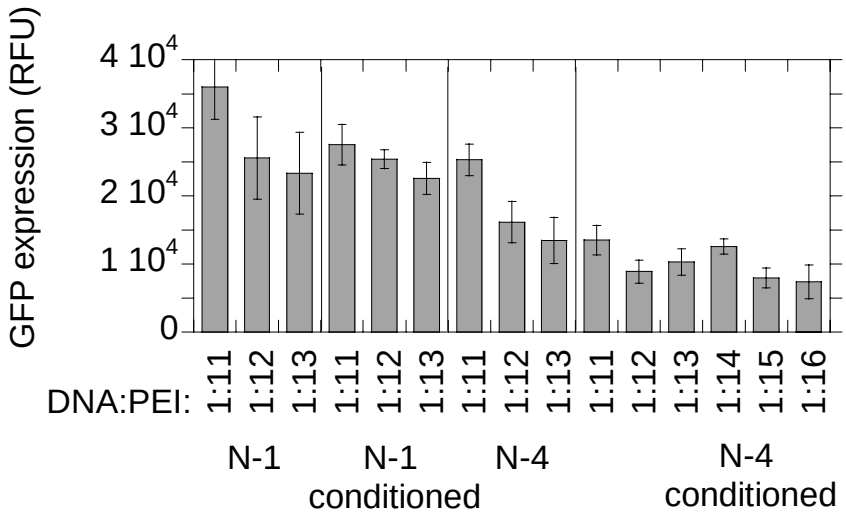


Figure 4. Effect of culture age on the polyfection HEK 293E cells. Cells were subcultivated 1 day (N-1) or 4 days (N-4) days before transfection. The cells were either centrifuged and resuspended in fresh Ex-Cell 293 CDM or retained in conditioned medium (N-1 and N-4 conditioned). The cells were seeded into 12-well plates at a density of $2x10^6$ cells/ml and transfected with a mixture of 90% pEAK8 and 10% pEGFPN-1 at various DNA:PEI ratios as indicated. The cells were diluted with one volume of fresh medium at 4 h post-transfection. GFP expression was measured at 3 days post transfection following cell lysis.

6.1.4 Polyfection of HEK 293E cells in square and round bottles.

The experiments described above were performed in 12-well microtiter plates at volumes of 1-2 ml. The next objective was to determine if it was possible to scale-up the polyfection of HEK 293E cells in Ex-Cell 293 medium in a non-instrumented cultivation system. Agitated 1-liter square bottles were chosen for this step because of the superior growth of HEK 293E cells in this vessel compared to spinner flasks. Furthermore, the eventual possibility of cultivating and transfecting cells in the same vessel was attractive. At the same time, the transfection of HEK 293E cells in agitated 1-liter "round-shaped" bottles (round bottles) was also investigated. The experiments described in this section were designed to evaluate several different cultivation

parameters for their effects on recombinant protein yield in square and round bottles: CO_2, bottle size, and agitation speed.

To determine if a CO_2 atmosphere was required for efficient gene transfer and recombinant protein expression following polyfection, HEK 293E cells were seeded in duplicate at a density of $2x10^6$ cells/ml in 200 ml of Ex-Cell 293 CDM in either 1-liter round or square bottles. The cultures were transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w). Half of the cultures were incubated at 37°C in the presence of 5% CO_2 and 95% humidity with the bottle caps opened one quarter of a turn. The other half were transferred to an incubator at 37°C without CO_2 with the bottle caps closed. All of the bottles were agitated at 100 rpm. At 4 h post-transfection, the cultures were diluted with 200 ml Ex-Cell 293 CDM, and the agitation speed was increased to 110 rpm. At 24 h post-transfection, the bottles from the CO_2 incubator were transferred to the non-CO_2 incubator. All the bottle caps were opened one quarter of a turn. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. By 3 days post-transfection there was no difference in GFP expression in cultures incubated in the presence or absence of CO_2 (Fig. 5A). GFP expression was significantly higher for the cultures in square bottles compared to those in round bottles (Fig. 5A). At 7 days post-transfection recombinant antibody expression was slightly higher in cultures incubated in the absence of CO_2 than those in the presence of CO_2 (Fig. 5B). As with GFP, antibody expression was higher for the cultures in square bottles than those in round bottles (Fig. 5B). Finally, there was no difference in either biomass (Fig. 5C) or viable cell number (data not shown) for the various cultures. Therefore, subsequent experiments in square or round bottles were incubated in a non-CO_2 incubator.

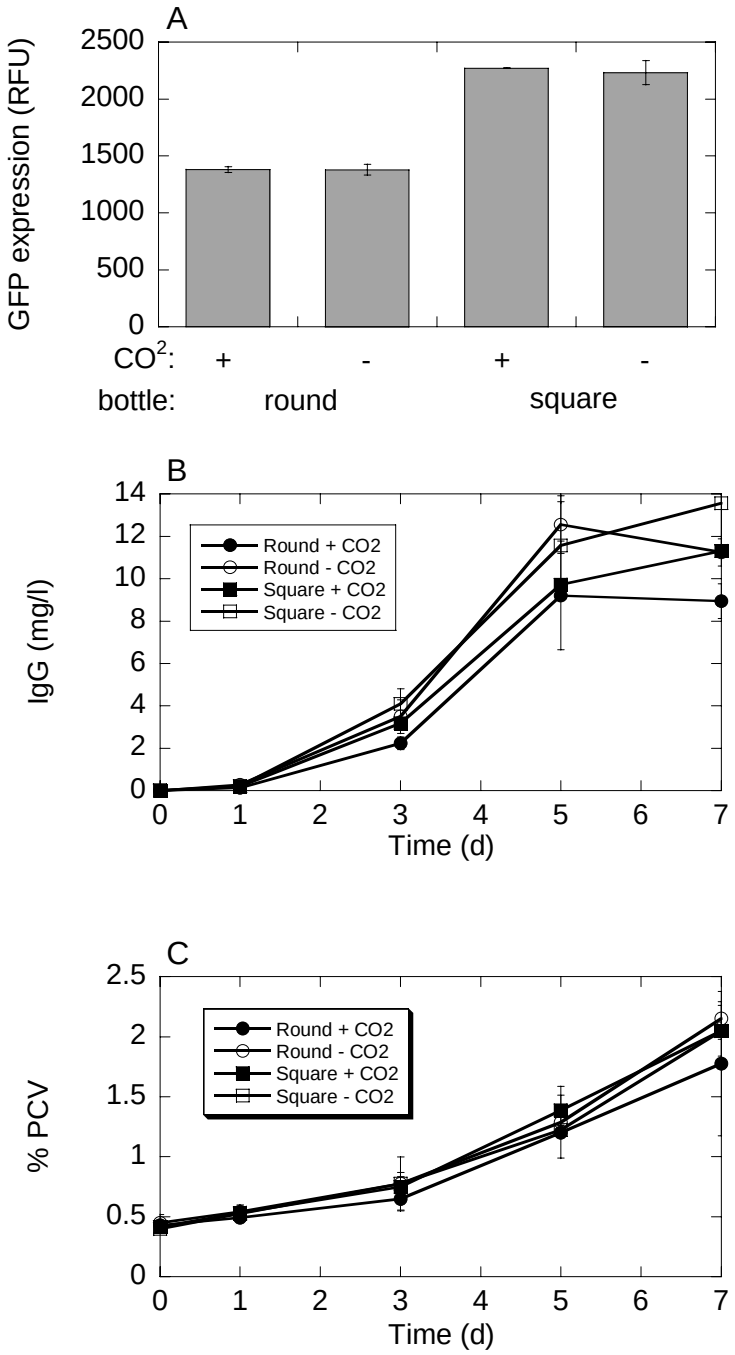


Figure 5. Polyfection of HEK 293E cells in Ex-Cell 293 CDM in the presence or absence of CO_2. HEK 293E cells seeded at a density of $2x10^6$ cells/ml in 200 ml of Ex-Cell 293 CDM in 1-liter square or round glass bottles. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-41, and pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w). The bottles were incubated in the presence or absence of CO_2 for 24 h after transfection. All the bottles were then incubated in the absence of CO_2. (A) GFP expression was determined at day 3 post-transfection following cell lysis. (B) Antibody expression was determined by ELISA at the times indicated. (C) Biomass was determined with mini PCV tubes at the times indicated.

To determine if bottle size had an effect on recombinant protein expression following polyfection, HEK 293E cells were seeded in duplicate at a density of $2x10^6$ cells/ml in either 50 ml or 200 ml of Ex-Cell 293 CDM in either 250-ml or 1-liter square bottles respectively. The cultures were transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w). At 4 h post-transfection, the cultures were diluted with one volume of Ex-Cell 293 CDM. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. Over a 5 day incubation period, GFP expression was higher for cultures in 1-liter square bottles than in 250-ml bottles (Fig. 6). However, the results did demonstrate that polyfection in Ex-Cell 293 CDM was feasible in square bottles with a volume other than 1 liter.

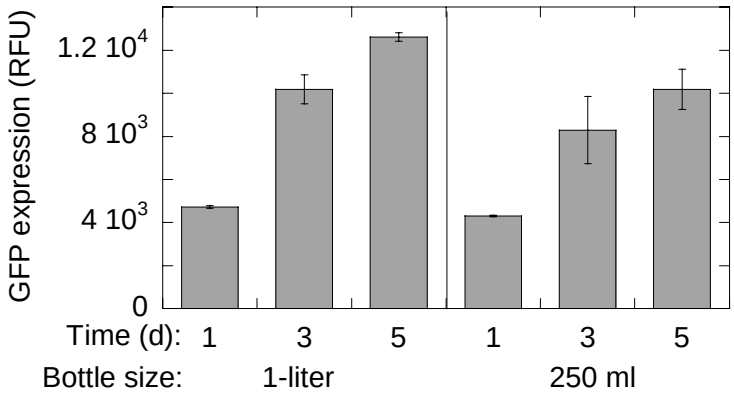


Figure 6. Polyfection of HEK 293E cells in square bottles. Cells were seeded in duplicate in 200 ml (for 1-liter bottles) or 50 ml (for 250-ml bottles) of Ex-Cell 293 CDM at a cell density of $2x10^6$ cells/ml. The cells were transfected with a mixture of 2% pEGFP-N1, 29% pEAK8-LH39, and 69% pEAK8-LH41 at a DNA:PEI ratio of 1:11 (w/w). The cells were diluted with one volume of medium at 4 h post-transfection. GFP expression was determined following cell lysis at the times indicated.

For the experiments in square and round bottles described above, the agitation speed was reduced from 110 rpm to 100 rpm for a 4 h period after DNA addition. The necessity of this step was evaluated with a polyfection of HEK 293E cells in 250-ml square bottles. The cells were seeded at a density of $2x10^6$ cells/ml in 50 ml of either Ex-Cell 293 CDM or RPMI 1640. The cultures were then transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of either 1:11 (w/w) in Ex-Cell 293 CDM or 1:3 (w/w) in RPMI 1640. The vessels were transferred to an incubator at 37°C without CO_2 and agitated at either 100 or 110 rpm. At 4 h post-transfection, the cultures were diluted with 50 ml Ex-Cell 293 CDM and the shaking speed was set at 110 rpm for all bottles. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. At 5 days post-transfection the recombinant antibody levels following transfection in Ex-Cell 293 CDM and RPMI 1640 were 80 mg/l and 70 mg/l, respectively, when the agitation speed was not decreased after DNA addition (Fig. 7A). With a decrease in agitation speed for 4 h after DNA addition, the recombinant antibody levels were about 65 mg/l for the two different cultures (Fig. 7A). Higher biomass was observed in the cultures transfected in Ex-Cell 293 CDM than in those transfected in RPMI 1640 (Fig. 7B), but

there was no difference in cell viability among the cultures (data not shown). For subsequent experiments in agitated square or round bottles the agitation speed was not decreased after DNA addition.

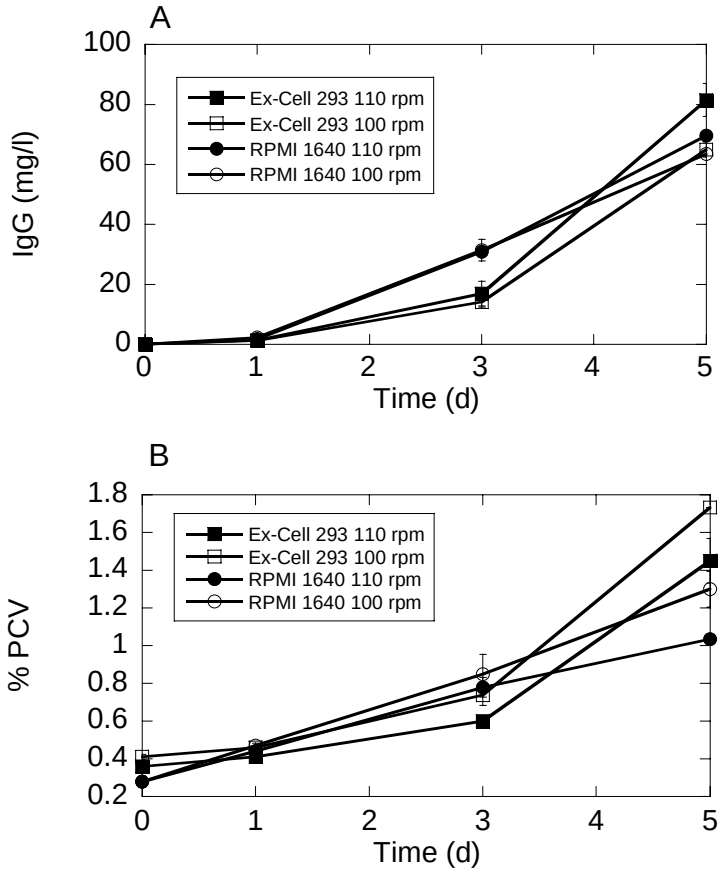
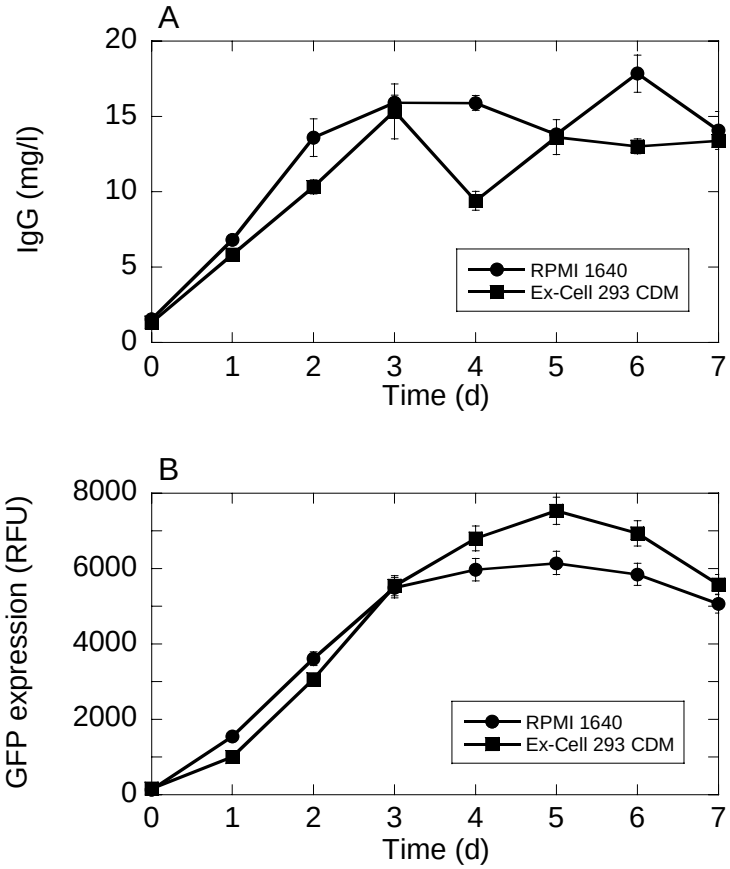


Figure 7. Effect of agitation speed on polyfection in square bottles. HEK 293E cells were seeded at a density of $2x10^6$ cells/ml in 50 ml of either Ex-Cell 293 CDM or RPMI 1640. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-41, and pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w) in Ex-Cell 293 CDM and 1:3 (w/w) in RPMI 1640. At the time of DNA addition the agitation speed was decreased from 110 to 100 rpm for half the bottles. The other bottles were agitated at 110 rpm throughout the experiment. At 4 h post-transfection one volume of Ex-Cell 293 CDM was added to each bottle, and the agitation speed was raised to 110 rpm. (A) Antibody concentration was determined by ELISA at the times indicated. (B) Biomass was determined with a mini-PCV tube at the times indicated.

6.1.5 Polyfection of HEK 293E cells in 3-liter bioreactors.

Having succeeded in scaling up the polyfection of HEK 293E cells in chemically defined medium with a non-instrumented cultivation system, the next step was to attempt this method in an instrumented cultivation system. HEK 293E cells were seeded in 3-liter bioreactors at a density of $2x10^6$ cells/ml in 500 ml of either Ex-Cell 293 CDM or RPMI 1640. Duplicate cultures were inoculated for each condition. The cells were transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w) for cells in Ex-Cell 293 CDM and 1:3 (w/w) for cells in RPMI 1640. At 4 h post-transfection the cultures were diluted

with 500 ml of Ex-Cell 293 CDM. The pH was maintained at 7.1 with 1 M NaOH and CO_2, and the level of dissolved oxygen was maintained at 20% by sparging air into the culture. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. As early as 4 h post-transfection, fluorescent cells were observed in all cultures (data not shown). By 7 days post-transfection the recombinant IgG levels reached approximately 14 mg/l for both conditions tested (Fig. 8A). GFP expression was slightly higher for the transfection in Ex-Cell 293 CDM than in RPMI 1640 (Fig. 8B). These results were surprising because the cultures transfected in Ex-Cell 293 CDM achieved a substantially higher cell density than those in RPMI 1640 (Fig. 8C). The viabilities of the two cultures, however, were not significantly different (Fig. 8D). It is not known why the recombinant proteins levels were much lower in 3-L bioreactors than in agitated square bottles. Some variables to consider, however, are the state of the cells at the time of transfection and the physical conditions in the two different cultivation systems.



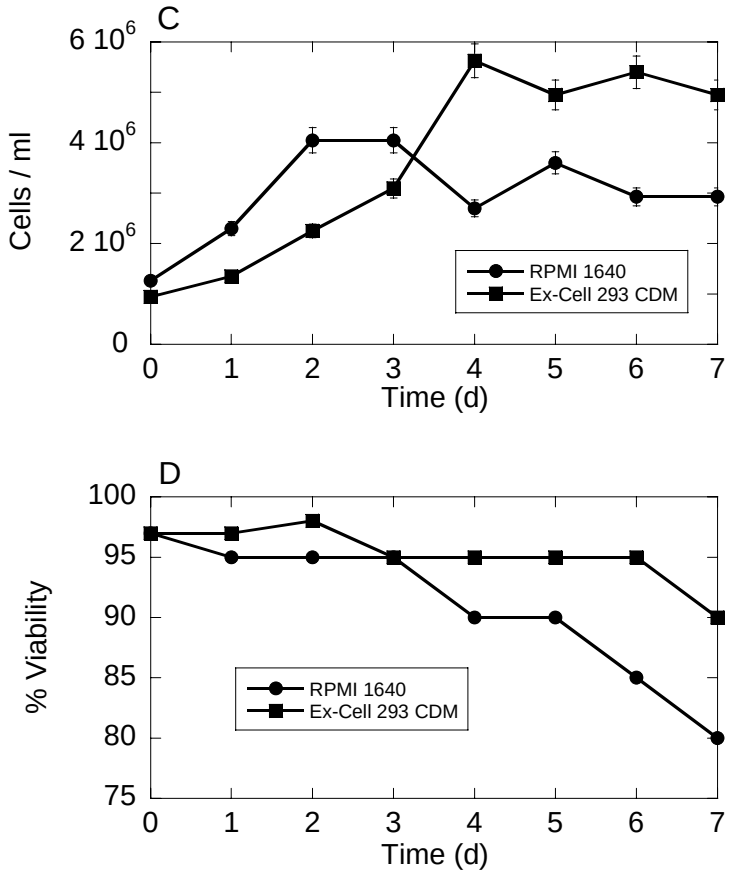


Figure 8. Polyfection of HEK 293E cells in 3-liter bioreactors. The cells were seeded in duplicate in 500 ml of either Ex-Cell 293 CDM or RPMI 1640 in 3-liter bioreactors at a density of $2x10^6$ cells/ml. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w) in Ex-Cell 293 CDM and 1:3 (w/w) in RPMI 1640. At the times indicated (A) the antibody concentration was measured by ELISA, (B) GFP expression was determined by fluorometry after cell lysis, and (C) the viable cell number and (D) the viability were determined with the Trypan blue exclusion method.

6.1.6 Effect of feeding after polyfection of HEK 293E cells.

Cell cultures that have been transiently transfected are usually fed upon need with glucose, glutamine, and sodium bicarbonate if the production phase of the culture lasts more than 1-2 days. To determine the effect of feeding on transiently transfected cultures following polyfection, HEK 293E cells were seeded in 2.5 ml of Ex-Cell 293 CDM at a density of $2x10^6$ cells/ml in 50 ml centrifugation tubes with a filter cap. These cultures of 2.5 ml were transfected with a mixture of 39% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w). The tubes were maintained in an incubator at 37°C in an atmosphere of 5% CO_2 and 95% humidity with agitation at 160 rpm. After 4 h, one volume of Ex-Cell 293 CDM was added and the agitation speed was increased to 180 rpm. The concentrations of glucose and glutamine were determined each day, and half the cultures were fed to maintain concentrations of 4 g/l glucose and 4 mM glutamine. The other cultures were not fed

during the experiment. By 7 days post-transfection, the levels of antibody expression were nearly the same for the two cultures (Fig. 9A). The biomass was also similar for the two cultures (Fig. 9B). From these results it can be concluded that the addition of glucose and glutamine following polyfection in Ex-Cell 293 CDM is not necessary. Similar results have been observed following the polyfection of CHO DG44 cells in agitated square bottles (Chapter 7).

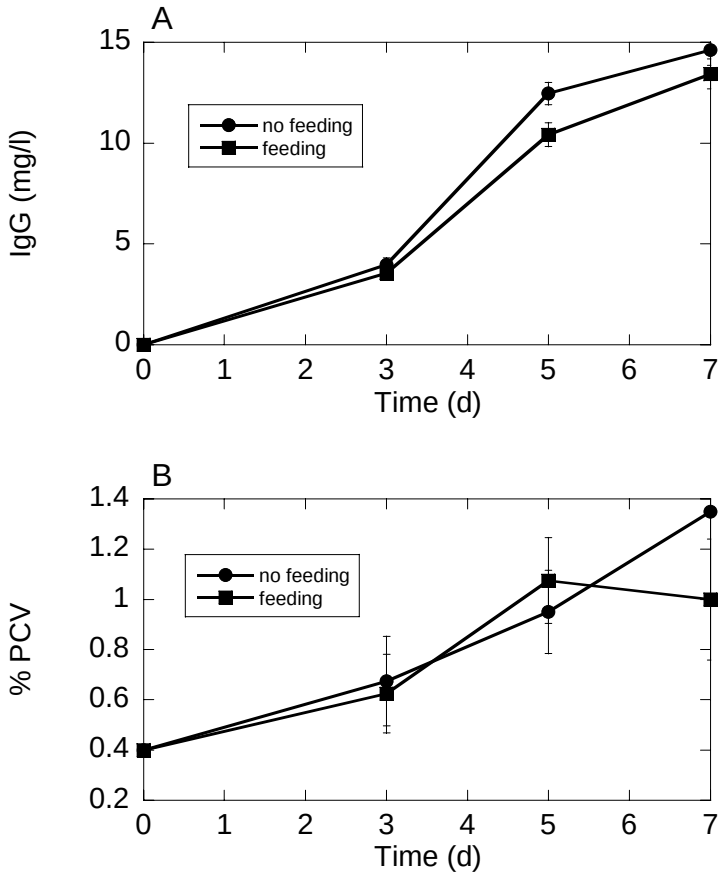


Figure 9. The effect of feeding on recombinant protein expression after polyfection. HEK 293E cells were seeded in 2.5 ml of Ex-Cell 293 CDM at a density of $2x10^6$ cells/ml in 50 ml centrifuge tubes with a filter cap. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a DNA:PEI ratio of 1:11 (w/w). The cells in half the tubes were fed with glucose and glutamine. (A) The antibody concentration was determined by ELISA at the times indicated. (B) Biomass was determined with mini-PCV tubes at the times indicated.

6.2 Discussion

Here we have demonstrated the feasibility of large-scale transfection of HEK 293E cells in serum-free, chemically defined medium (Ex-Cell 293 CDM) in both instrumented and non-instrumented cultivation systems. This was accomplished using PEI for DNA delivery since, unlike calfection and calcium phosphate-DNA coprecipitation, it efficiently transfects mammalian cells in the absence of serum. Linear 25 kDa PEI was chosen for these studies because it had previously been shown to yield high levels of reporter protein expression following the transfection of CHO

DG44 (Derouazi et al. 2004) and HEK 293E cells (Durocher et al 2002). The method developed for polyfection in chemically defined medium proved to be scaleable from 1 ml to 1 liter in microtiter plates, 50 ml centrifuge tubes, 1-L round and square glass bottles, and 3-L bioreactors.

It was determined that the optimum DNA:PEI ratio for polyfection of HEK 293E cells in Ex-Cell 293 CDM medium was 1:11 (w/w) as compared to a 1:3 (w/w) ratio for the transfection of HEK 293E cells in RPMI 1640. One possible reason for this difference is the presence of a compound(s) in Ex-Cell 293 CDM that interacts with PEI to cause its release from the DNA/PEI complex or alters the surface charge of the DNA/PEI complex to prevent binding to the cell. This may explain why more PEI is required for complex formation in the chemically defined medium as compared to a minimal medium such as RPMI 1640. In support of this hypothesis, PEI has been shown to form a precipitate with a component(s) in Ex-Cell 293 CDM and other chemically defined media (M. Bertschinger, unpublished data). The compaction of the DNA/PEI complex and its size are important for efficient polyfection (Pham, Perret et al. 2003; Baldi, Muller et al. 2005). Therefore, we initially assumed that DNA:PEI ratios resulting in a large excess of PEI would lead to less efficient transfection as the DNA/PEI complexes grew in size. However, this assumption proved to be wrong.

Several novel cultivation systems including round and square bottles and 50 ml centrifuge tubes were shown to be suitable for polyfection in a chemically defined medium. The two types of bottles are of interest for the large-scale production of recombinant proteins while the 50 ml centrifuge tubes can be used as a tool for the optimization of transfection parameters and for high-throughput approaches to recombinant protein expression. Both 250-ml and 1-L square bottles with maximum volumes of 100 and 400 ml, respectively, were used for these studies. One of the advantages of square bottles is that they are an excellent tool for the routine cultivation of mammalian cells. Based on the results shown in Fig. 4, it may eventually be possible to cultivate and transfect cells in the same vessel. The transfection of HEK 293E cells in one-day old conditioned Ex-Cell 293 CDM medium yielded only 30% less protein than transfection of the same cells following their transfer to fresh medium. With further experimentation to optimize the conditions, it should be possible to seed cells the day before transfection in a 1-L square bottle and directly transfect them the next day in the same bottle. This approach is especially attractive for large-scale transfection in the 2-L and 10-L containers described in Chapter 5.

For most of the cultivation systems described here the polyfection of HEK 293E cells with vectors carrying the light and heavy chain IgG genes resulted in antibody yields of 5-20 mg/l at 5-7 days post-transfection. This is similar to yields observed following the large-scale transfection of HEK 293E cells with calcium phosphate-DNA coprecipitation (Meissner et al. 2001; Girard et al. 2002; Baldi et al. 2005). Surprisingly, antibody yields in 250-ml square bottles were in the range of 60-80 mg/l by 5 days after polyfection in either Ex-Cell 293 CDM or RPMI 1640 (Fig. 7). This experiment was performed again two different times. For one of these two repeats, titers in the 35-40 mg/l range were achieved and for the other repeat, titers in the same range previously mentioned, 60-80 mg/l were achieved when the experiment was repeated. It is not yet clear why antibody expression was significantly higher in these

experiments than in the others. Additional experiments are needed to understand this phenomenon and to try to achieve the same yields in other cultivation systems.

In conclusion, we demonstrated transient gene expression in suspension cultures of HEK 293E cells using linear 25 kDa PEI in serum-free chemically defined medium. The optimal transfection conditions as determined here were a cell density of $2x10^6$ cells/ml in Ex-Cell 293 CDM with a DNA:PEI ratio of 1:11 (w/w) and a final DNA concentration in the culture medium of 2.5 µg/ml. The cells were diluted 4 h post-transfection with one volume of Ex-Cell 293 CDM medium. Here we have shown that the process can be simplified by incubation in the absence of CO_2, maintenance of a single agitation speed after transfection and medium addition, and elimination of a wash step after centrifugation of the overnight culture. This method is cost-efficient and suitable for serum-free operations. It offers high transfection efficiency with a simple and reproducible procedure. These features make PEI a more suitable transfection vehicle than calfection or calcium phosphate for large-scale transient gene expression with HEK 293E cells.

6.3 References

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7. Polyfection of CHO DG44 cells in chemically defined medium

For transient recombinant protein expression it is important to identify a reproducible process with controllable parameters that can be performed at a broad range of volumes. Derouazi et al. (2004) described the polyfection of suspension-adapted CHO DG44 cells with linear 25 kDa PEI. The cells were grown in ProCHO5 CDM medium and then transferred at a cell density of $2x10^6$ cells/ml to RPMI 1640 containing 25 mM Hepes at pH 7.1. Optimal reporter gene expression was achieved with a DNA:PEI ratio of 1:2 (w/w) with a final DNA concentration in the culture medium of 2.5 µg/ml. The DNA and PEI were mixed in 150 mM NaCl and allowed to incubate for 10 min prior to addition to the culture. At 5 h post-transfection, the cells are diluted with one volume of ProCHO5 CDM medium. This procedure was used as a starting point for the experiments described below. One major objective of this work was to replace RPMI 1640, a minimal medium, with a chemically defined medium that allows the high-density cultivation of suspension cells. The second objective was to develop a simple transient transfection procedure that could be utilized in both instrumented and non-instrumented cultivation systems. In this chapter the polyfection of suspension-adapted CHO DG44 cells for the transient expression of recombinant proteins at scales ranging from 1 ml to 100 l is described. These experiments were performed in both noninstrumented (microtiter plates, 50 ml centrifuge tubes, round bottles, and square bottles) and instrumented (bioreactors with capacities of 3-, 20-, and 150-l) cultivation systems. Importantly, polyfection was adapted to cells in a serum-free, chemically defined medium, eliminating the need for animal-derived components in the transient production of recombinant proteins.

7.1 Results

7.1.1 Polyfection in non-instrumented cultivation systems.

7.1.1.1 Polyfection in microtiter plates.

The feasibility of polyfection of CHO DG44 cells in ProCHO5 CDM was initially investigated in agitated 12-well microtiter plates. In these experiments two critical parameters, the DNA:PEI ratio and the cell seeding density, were varied. Suspension-adapted CHO DG44 cells from an overnight culture grown in ProCHO5 CDM were centrifuged, resuspended in either ProCHO5 CDM or RPMI 1640 at a density of $2x10^6$ cells/ml, and transferred to 12-well microtiter plates. The cells were transfected with a mixture of pEAK8-LH41, pEAK8-LH39, and pEGFP-N1 at a ratio of 69:39:2 (w/w/w). The final DNA concentration was kept constant at 2.5 µg/ml, and the DNA:PEI ratio was varied from 1:2 to 1:6 (w/w). For polyfection in ProCHO5 CDM, the DNA:PEI ratios of 1:4 and 1:5 (w/w) resulted in the highest levels of GFP (Fig. 1A) and IgG expression (Fig. 1B) at 3 days post-transfection. By comparison the highest levels of reporter protein expression for the transfections in RPMI 1640 were observed at a DNA:PEI ratio of 1:2 (w/w) (Fig. 1A, B; only the results for the DNA:PEI ratio of 1:2 are shown). For both IgG and GFP, slightly higher expression

levels were observed for the transfections in ProCHO5 CDM than those in RPMI 1640 (Fig. 1A,B).

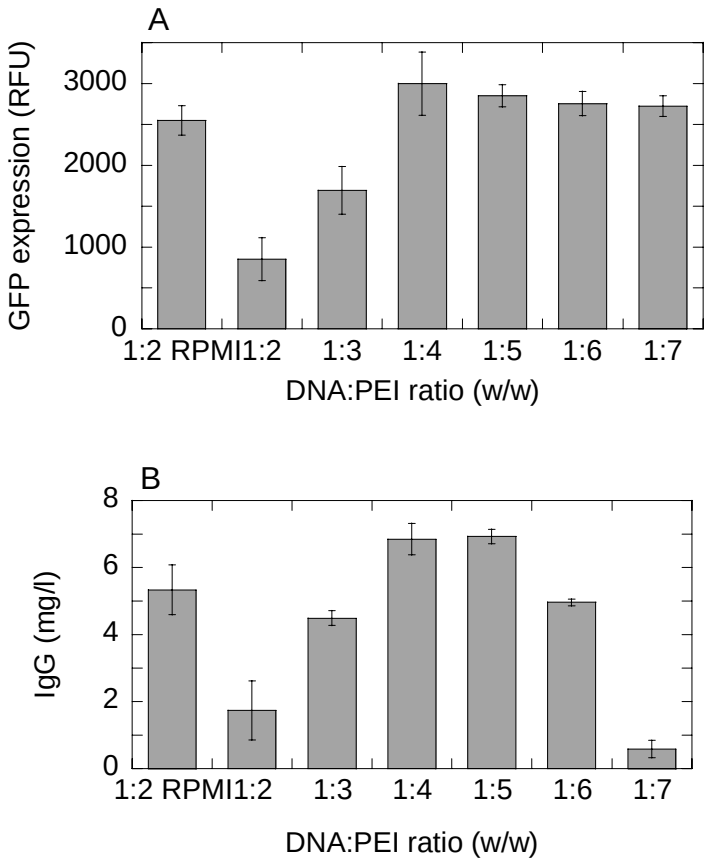


Figure 1. Polyfection in agitated 12-well microtiter plates. CHO DG44 cells were seeded in 12-well plates at $2x10^6$ cells/ml in either ProCHO5 CDM or RPMI 1640 and transfected at different DNA:PEI ratios with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1. At 3 days post transfection, GFP expression was measured by fluorometry (A) and IgG expression was determined by ELISA (B). For the transfections in RPMI 1640 only the results at a DNA:PEI ratio of 1:2 (w/w) are shown (1:2 R).

Using a DNA:PEI ratio of 1:4 (w/w), the effect of cell density on transient recombinant protein expression in ProCHO5 CDM was investigated. CHO DG44 cells at either $1x10^6$ or $2x10^6$ cells/ml were transfected with a mixture of pEAK8-LH41, pEAK8-LH39, and pEGFP-N1 as described above. At 3 days post-transfection the highest levels of GFP and IgG expression were observed at a cell density of $2x10^6$ cells/ml (Table 1). In this experiment, antibody expression was 2.5-fold higher in ProCHO5 CDM than in RPMI 1640 (Table 1). Based on these results, all subsequent transfections in this and other cultivation systems were performed at a seeding density of $2x10^6$ cells/ml.

Table 1. Effect of cell density on the polyfection of CHO DG44 cells.

Cell density (cells/ml)	GFP expression (RFU)[a]	IgG expression (mg/l)[b]
1x10^6	1200+/-300	1.8+/-0.1
2x10^6	1500+/-100	4.9+/-0.6

[a]The average GFP fluorescence of three transfections is shown. GFP expression was measured by fluorometry following cell lysis.
[b]The average IgG concentration of three transfections as determined by ELISA is shown.

As shown for calfection (Chapter 4), the cultivation conditions prior to transfection can have a significant effect on transient recombinant protein expression. In the original protocol of Derouazi et al. (2004) CHO DG44 cells were grown in ProCHO5 CDM, washed once in RPMI 1640, and finally resuspended in RPMI 1640 for transfection. To determine if the washing step was still necessary after demonstrating that polyfection in ProCHO5 CDM is possible, two different cultivation conditions were tested: (1) cells grown in ProCHO5 CDM medium for 1 day were centrifuged and resuspended in fresh medium (either ProCHO5 CDM or RPMI 1640) without a wash step (termed N-1), or (2) cells were grown as in (1) except that they were washed once with fresh medium (either ProCHO5 CDM or RPMI 1640) prior to resuspension in the appropriate medium (N-1 washed). After transfer into 12-well microtiter plates the cells were transfected with pEGFP-N1 at a DNA:PEI ratio of either 1:4 (w/w) for cells in ProCHO5 CDM or 1:2 (w/w) for cells in RPMI 1640. At 4 h post-transfection the cultures were diluted with one volume of ProCHO5 CDM, and GFP expression was measured at 3 days post-transfection. Regardless if the cells were transfected in ProCHO5 CDM or RPMI 1640, a wash step prior to transfection was not necessary (Table 2, compare N-1 to N-1 washed).

Table 2. Effect of cell cultivation conditions on the polyfection of CHO DG44 cells in ProCHO5 CDM or RPMI 1640.

Culture condition[a]	Normalized GFP expression in ProCHO5 CDM[b]	Normalized GFP expression in RPMI 1640[c]
(N-1)	1.00+/-0.13	1.00+/-0.15
(N-1 washed)	1.00+/-0.09	0.97+/-0.10

[a]Cells grown in ProCHO5 CDM medium for 1 day were centrifuged and resuspended in fresh medium (either ProCHO5 CDM or RPMI 1640) without a wash step (N-1). Cells were grown as in N-1 except that they were washed once with fresh medium (either ProCHO5 CDM or RPMI 1640) prior to resuspension in the appropriate medium (N-1 washed).
[b]Polyfection of CHO DG44 cells with pEGFP-N1 at a DNA:PEI ratio of 1:4 in ProCHO5 CDM. GFP expression was normalized to that obtained for the N-1 culture.
[c]Polyfection of CHO DG44 cells with pEGFP-N1 at a DNA:PEI ratio of 1:2 in RPMI 1640. GFP expression was normalized to that obtained for the N-1 culture.

For calfection, the number of days in cultivation prior to gene transfer is an important parameter (Chapter 4). To determine the effect of culture age on the efficiency of gene transfer by polyfection, CHO DG44 cells were cultivated in ProCHO5 CDM for one day (N-1), 4 days (N-4), or 7 days (N-7) prior to the day of transfection. On the day of transfection, the density of each culture was approximately $3x10^6$ cells/ml. The cells were either centrifuged and resuspended in fresh ProCHO5 CDM or maintained in conditioned medium. The cells were seeded at $2x10^6$ cells/ml in 12-well microtiter plates and transfected with a mixture of 90% pEAK8 and 10% pEGFPN-1 at DNA:PEI ratios ranging from 1:4 to 1:6 (w/w). At 4 h post transfection the cultures were diluted with one volume of ProCHO5 CDM. In contrast to the results with calfection, the efficiency of polyfection did not increase with the age of the culture (Fig. 2). With an increase in cultivation time prior to transfection, there was a decrease in GFP expression. In addition, the transfer of cells into fresh medium at the time of transfection was required for efficiency polyfection; little GFP expression was observed following polyfection in conditioned medium (Fig. 2). The transfection of N-7 cells in conditioned medium at DNA:PEI ratios up to 1:9 (w/w) did not result in increased GFP expression (data not shown). From this experiment it was concluded that for the polyfection of CHO DG44 cells in ProCHO5 CDM, the best approach was to use cells that were grown in ProCHO CDM for 1 day before transfection and then centrifuged and resuspended in ProCHO5 CDM at the time of transfection. Furthermore, the results from the transfections in conditioned medium suggest the presence of an inhibitor of polyfection or the absence of a required component of polyfection in conditioned ProCHO5 CDM medium.

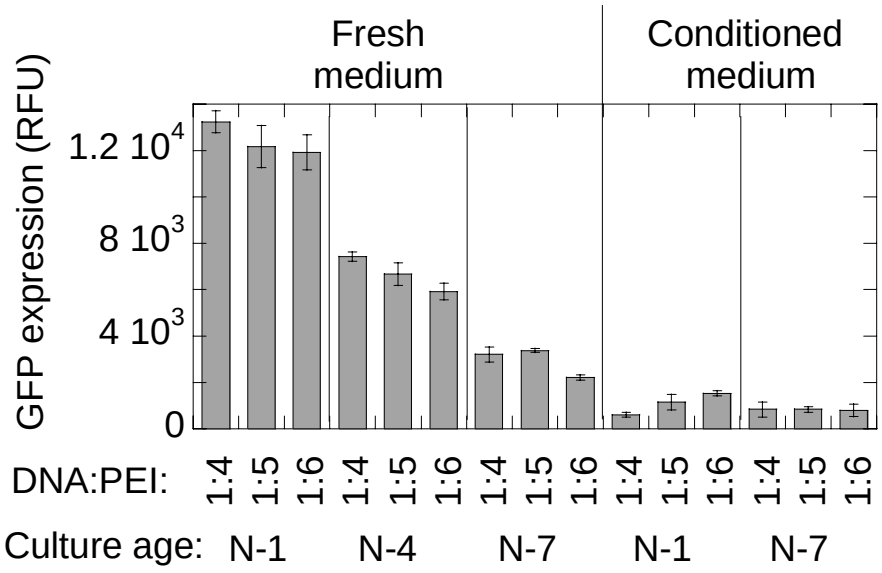


Figure 2. Effect of culture age on polyfection of CHO DG44 cells. CHO DG44 cells grown in ProCHO5 CDM for either one (N-1), four (N-4), or seven (N-7) days prior to polyfection were either transfected in fresh ProCHO5 CDM or in the conditioned medium with a mixture of 90% pEAK8 and 10% pEGFPN-1 at DNA:PEI ratios ranging from 1:4 to 1:6. Cells were seeded into 12-well microtiter plates at a density of $2x10^6$ cells/ml. The cells were diluted with one volume of ProCHO5 CDM at 4 h post-transfection. GFP expression was measured at 3 days post transfection after cell lysis.

7.1.1.2 Polyfection in 1-liter square or round bottles.

The polyfection of CHO DG44 cells in ProCHO5 CDM medium was attempted at a larger scale in agitated 1-liter square or round bottles. Using these vessels, we first investigated the requirement for atmospheric CO_2 after transfection. Eliminating the for a CO_2 incubator for non-instrumented cultivation systems such as agitated square or round bottles would significantly simplify the transient transfection process.

CHO DG44 cells were seeded in the bottles at a density of $2x10^6$ cells/ml in 200 ml of ProCHO5 CDM. The cultures were then transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w). The requirement for CO_2 was investigated by incubating the bottles at 37°C in the presence of absence of 5% CO_2. For the bottles in the CO_2 incubator the caps were opened one quarter of a turn, and in the incubator without CO_2 the bottle caps remained closed. All of the bottles were agitated at 100 rpm until 4 h post-transfection when the cultures were diluted with 200 ml ProCHO5 CDM and the agitation speed was increased to 110 rpm. At 24 h post-transfection, the bottles in the CO_2 incubator were transferred to the non-CO_2 incubator and all the bottle caps were opened one quarter of a turn. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. At 3 days post-transfection, the GFP level in the cultures was about the same (Fig. 3). There was no noticeable effect of either CO_2 or bottle geometry. At 5 days post-transfection the IgG yield was about 20% higher in square bottles as compared to round bottles (data not shown), but there was no effect of CO_2 on IgG yield (data not shown). Finally, there was no significant differences in either viable cell number or PCV among the different bottles (data not shown). Because of these results further experiments in square bottles were performed in the absence of CO_2.

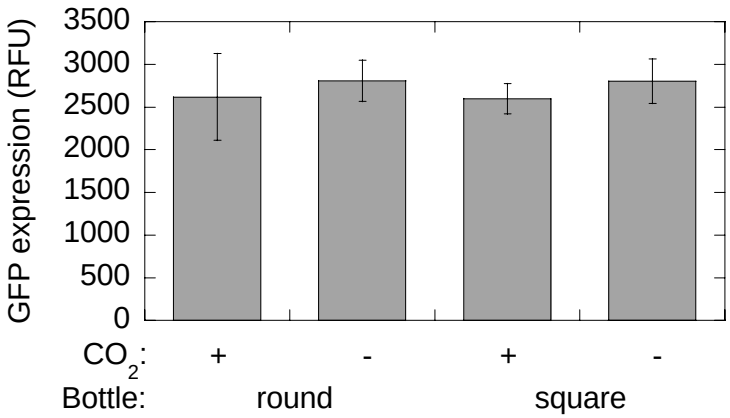


Figure 3. Effects of bottle geometry and CO_2 on efficiency of polyfection of CHO DG44 cells in ProCHO5 CDM. Cells were inoculated into 1-liter square or round bottles at a density of $2x10^6$ cells/ml in 200 ml of ProCHO5 CDM. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-41, and pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w). During the first 24 h post-transfection, half the bottles were incubated in the presence of CO_2 prior to being transferred to a non-CO_2 incubator. The other half of the bottles were located in a non-CO_2 incubator throughout the experiment. GFP expression was determined at day 3 post-transfection following cell lysis.

In the experiment described above the agitation speed was reduced from 110 rpm to 100 rpm during the first 4 h post-transfection. This step was initially implemented to correct for the lower volume (Chapter 5). There was also concern that if the mixing was too turbulent, the transfection efficiency would be decreased. To determine if the reduction in agitation speed was necessary, CHO DG44 cells were seeded at a density of $2x10^6$ cells/ml in 200 ml of either ProCHO5 CDM or RPMI 1640 supplemented with 25 mM Hepes (pH 7.1) and 0.1% Pluronic F-68. The cultures were then transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:4 or 1:2 (w/w) for transfections in ProCHO5 CDM or RPMI 1640, respectively. The vessels were incubated with agitated at either 100 rpm or 110 rpm. At 4 h post-transfection, the cultures were diluted with 200 ml ProCHO5 CDM, and all the bottles were agitated at 110 rpm. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. Neither the medium at the time of transfection nor the agitation speed during transfection had an effect on GFP expression at 3 days post-transfection (Fig. 4A). After 5 days antibody yield was higher after transfection in ProCHO5 CDM as compared to transfection in RPMI 1640 (Fig. 4B). The change in agitation speed, however, did not have an effect on antibody expression in either medium (Fig. 4B). The higher antibody expression in ProCHO5 CDM was not due to the accumulation of greater biomass in this medium since there was not a significant difference in biomasss determined by mini-PCV (Fig. 4C) or viable cell number (data not shown) for the cultures in ProCHO5 CDM or RPMI 1640. Since a reduction in agitation speed during transfection was not necessary, subsequent experiments in 1-liter square or round bottles were performed with agitation at 110 rpm during and after dilution of the transfected culture. Besides the 1-liter square or round bottles described here, it has also been possible to transfect cells using PEI in 250-ml square or round bottles with an initial volume of 50 ml and a final volume after dilution of 100 ml. Recombinant protein yields were in the range described here (data not shown).

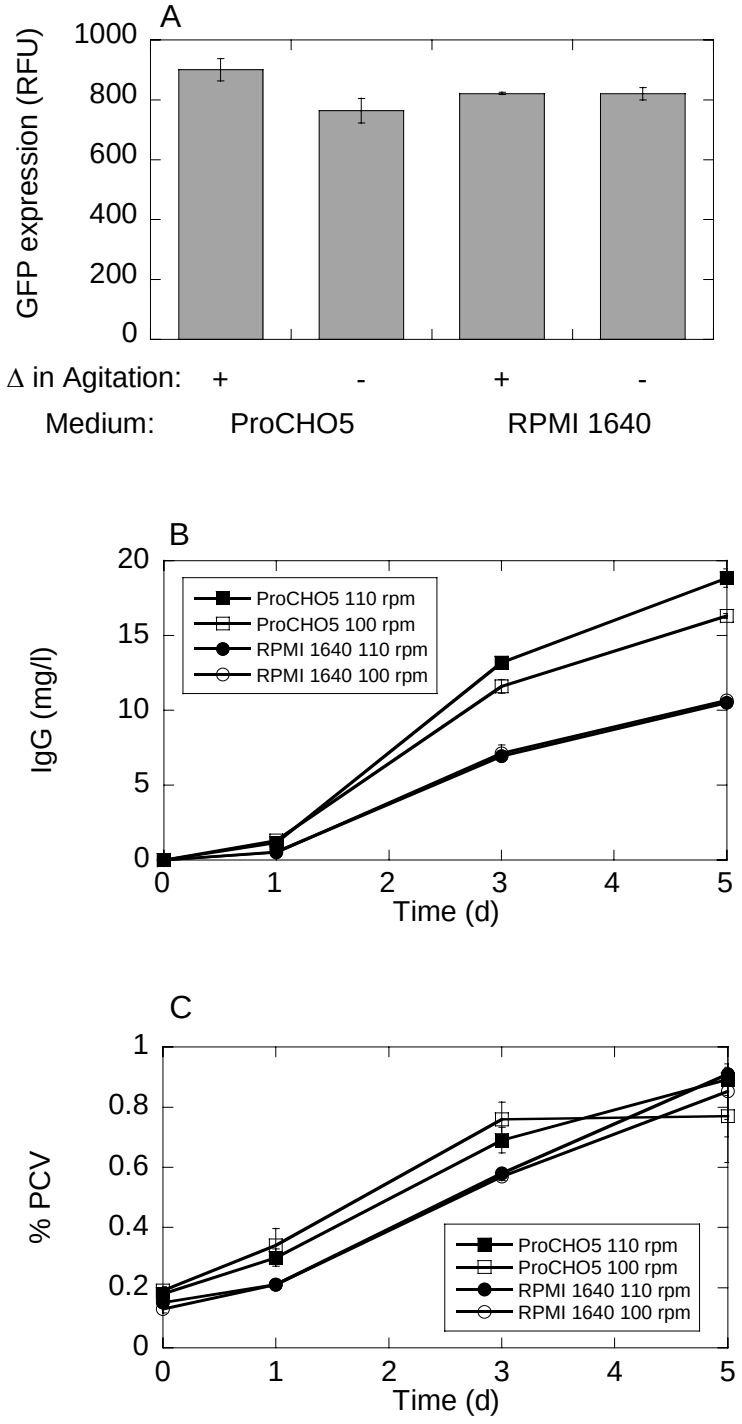
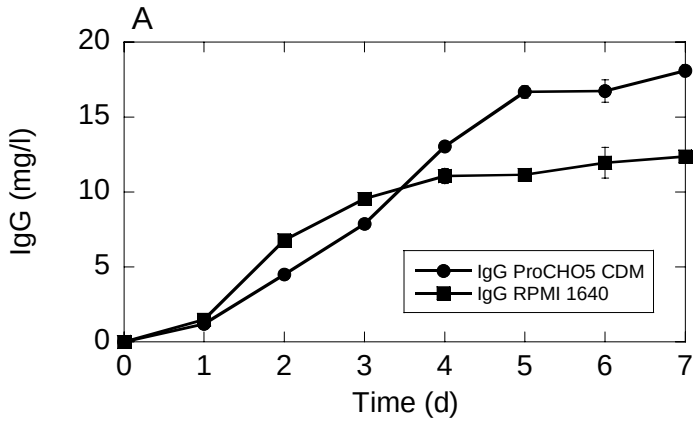


Figure 4. Effect of agitation speed on the efficiency of polyfection in 1-liter square bottles. CHO DG44 cells were seeded at a density of $2x10^6$ cells/ml in 200 ml of either ProCHO5 CDM or RPMI 1640. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-41, and pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w) in ProCHO5 CDM and 1:2 (w/w) in RPMI 1640. At the times indicated (A) GFP expression was determined by fluorometry after cell lysis, (B) antibody expression was determined by ELISA, (C) biomass was determined with a mini-PCV tube.

7.1.2 Polyfection in instrumented cultivation systems.

7.1.2.1 Polyfection in 3-liter bioreactors.

CHO DG44 cells were seeded in 3-liter bioreactors at a density of $2x10^6$ cells/ml in 500 ml of either ProCHO5 CDM or RPMI 1640. The cells were transfected with a mixture of 29% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1, and at 4 h post-transfection the cultures were diluted with 500 ml of ProCHO5 CDM. The pH was maintained at 7.1 with 1 M NaOH and CO_2, and the level of dissolved oxygen was maintained at 20% by sparging air into the culture. The cultures were fed upon need to 4 g/L glucose, 4 mM glutamine, and 10 mM sodium bicarbonate. As early as 4 h post-transfection, fluorescent cells were observed in both cultures (not shown). By 7 days post-transfection the recombinant IgG levels reached 18 and 12 mg/L for the transfections in ProCHO5 CDM and RPMI 1640, respectively (Fig. 5A). In both cultures GFP expression reached a maximum at 2 days post-transfection and then declined with time (Fig. 5B). At 7 days post-transfection, there was also a greater biomass in the culture transfected in ProCHO5 CDM (1.2% PCV) than in RPMI 1640 (0.8% PCV) (Fig. 5C). High cell viability was maintained throughout the cultivation period in both media, although the viability of the culture transfected in RPMI 1640 eventually decreased to 90% at day 7 post-transfection (Fig. 5D). For both cultures there was little increase in IgG accumulation after day 5 (Fig. 5A). This result was surprising for the transfection in ProCHO5 CDM because the biomass increased from 0.9% to 1.2% PCV during this time period (Fig. 5C). In contrast there was little change of the biomass after day 5 for the culture transfected in RPMI 1640 (Fig. 5C).



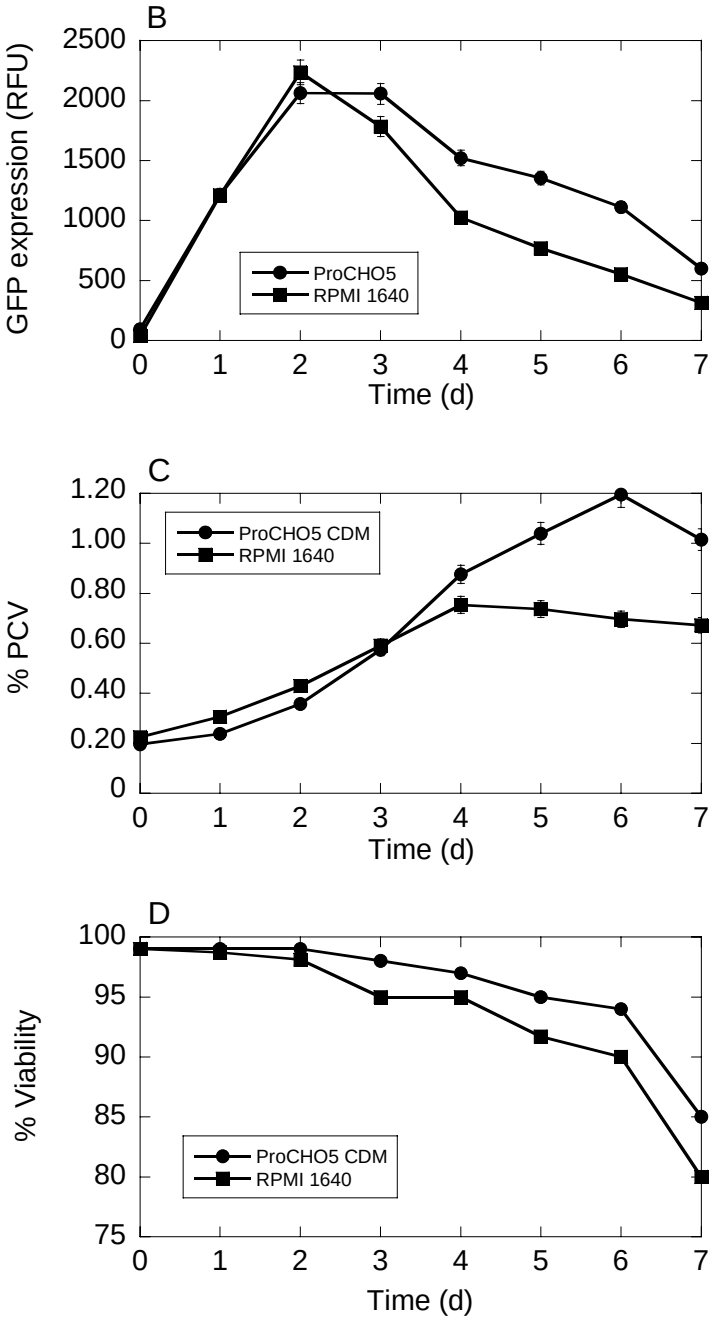


Figure 5. Polyfection in 3-liter bioreactors. CHO DG44 cells were seeded in 500 ml of either ProCHO5 CDM or RPMI 1640 in 3-liter bioreactors at a density of $2x10^6$ cells/ml. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a DNA:PEI ratio of 1:4 in ProCHO5 CDM and 1:2 in RPMI 1640. At the times indicated (A) antibody concentration was measured by ELISA, (B) GFP expression was measured by fluorometry after cell lysis, (C) biomass was determined using a mini-PCV tube, and (D) cell viability was determined by Trypan blue exclusion.

7.1.2.2 Polyfection in a 20-liter bioreactor.

CHO DG44 cells were seeded at $2x10^6$ cells/ml in 5 L of ProCHO5 CDM in a bioreactor with a capacity of 20 L. The culture was transfected with a mixture of 39% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w). At 4 h post-transfection, the culture was diluted with 5 L of ProCHO5 CDM. The pH, dO_2, glucose, glutamine, and sodium bicarbonate levels were maintained as described above, and the culture was fed upon need with glucose, glutamine, and sodium bicarbonate. In this experiment the maximum IgG yield only reached 10 mg/L (Fig. 6A). This was not due to low cell viability since it remained above 85% through day 5 and only decreased to 75% by day 6 (Fig. 6B). Instead, difficulty in the maintenance of the pH during the experiment may have been responsible for the lower yield than observed in the 3-L bioreactor (Fig. 5).

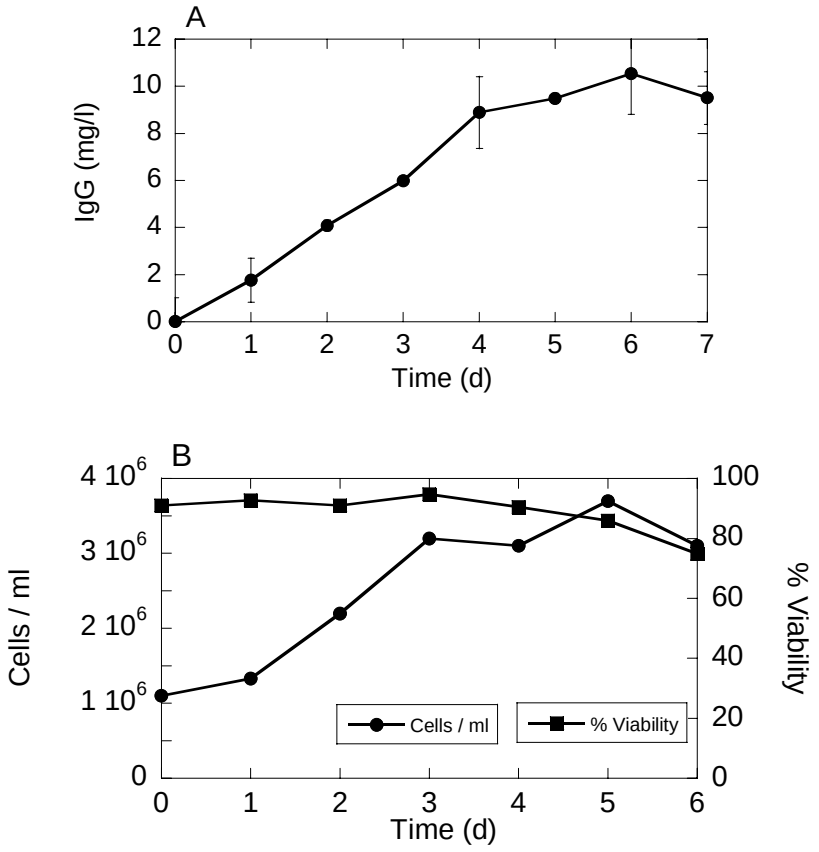


Figure 6. Polyfection in a 20-liter bioreactor. CHO DG44 cells were seeded at a density of $2x10^6$ cells/ml in 5 L of ProCHO5 CDM. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w) and diluted with one volume of ProCHO5 CDM at 4 h post-transfection. (A) The antibody concentration was determined by ELISA at the times indicated. (B) The viable cell number and percent viability were determined by Trypan blue exclusion at the times indicated.

7.1.2.3 Polyfection in a 150-liter bioreactor.

CHO DG44 cells were seeded in 40 liters of ProCHO5 CDM at a density of $2x10^6$ cells/ml in a bioreactor with a capacity of 150 L. The culture was transfected with a DNA:PEI ratio of 1:4 (w/w) with a mixture of 39% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1. At 4 h post-transfection the culture was diluted with 40 L of ProCHO5 CDM. The pH, dO_2, glucose, glutamine, and sodium bicarbonate levels were maintained as described above, and the culture was fed upon need with glucose, glutamine, and sodium bicarbonate. The recombinant IgG concentration reached 22 mg/L by day 7 post-transfection (Fig. 7A). This was similar to the production observed in the 3-L bioreactor (Fig. 5) and in one-liter square bottles (Fig. 4). The cell number was as high as $4x10^6$ cells/ml, and the viability of the culture remained above 90% through day 4 and then decreased to 55% by day 6 (Figure 7B).

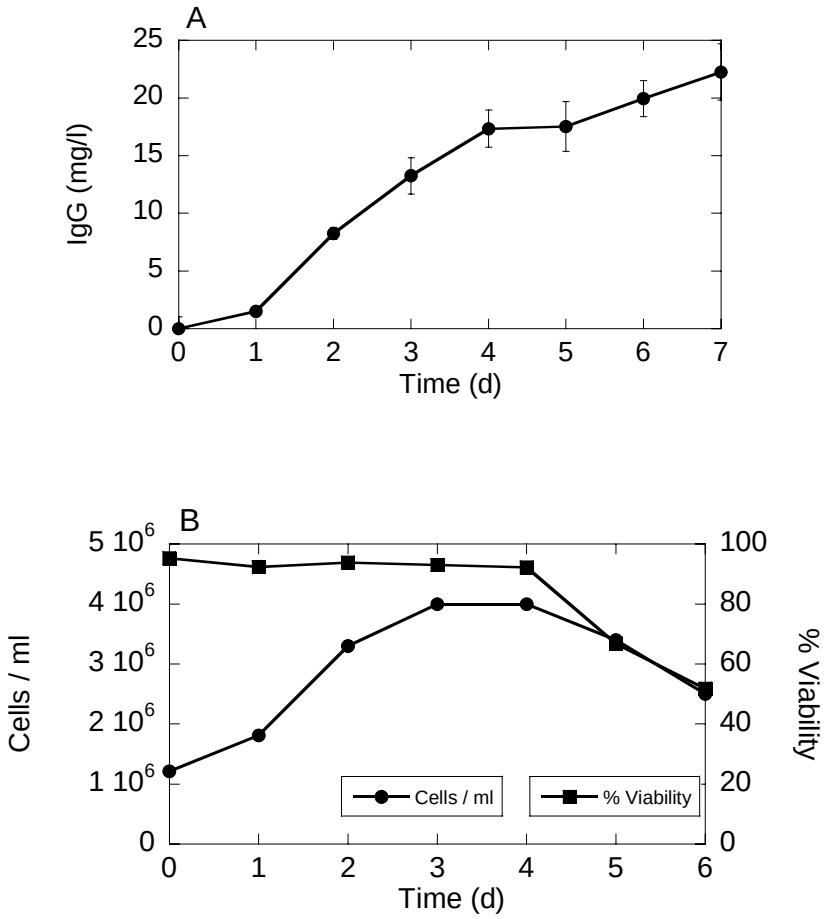


Figure 7. Polyfection in a 150-liter bioreactor. CHO DG44 cells were seeded in 40 liters of ProCHO5 CDM at a density of $2x10^6$ cells/ml. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w) and then diluted with one volume of ProCHO5 CDM at 4 h post-transfection. (A) The antibody concentration was determined by ELISA at the times indicated. (B) The viable cell number and the percent viability were determined by Trypan blue exclusion at the times indicated.

7.1.3 Effect of feeding on transient recombinant protein expression following polyfection of CHO DG44 cells.

During the large-scale transient transfections in square bottles and in bioreactors described above, the cultures were fed upon need with glucose, glutamine, and sodium bicarbonate. However, there has been contradictory evidence on whether it is necessary to feed recombinant cell lines in batch cultures (Godbey, Wu et al. 1999; Godbey, Wu et al. 1999; Derouazi, Girard et al. 2004; Tait, Brown et al. 2004). The effect of feeding on recombinant protein yield following the polyfection of CHO DG44 cells was investigated. For this experiment the cells were inoculated into 50 ml centrifuge tubes with a filter cap in 2.5 ml of ProCHO5 CDM medium at a density of $2x10^6$ cells/ml. The cells were transfected at a DNA:PEI ratio of 1:4 (w/w) with a mixture of 39% pEAK8-LH39, 69% pEAK8-LH41, and 2% pEGFP-N1. The tubes were transferred to a 37°C incubator with an atmosphere of 5% CO_2 and 95% humidity at an agitation speed of 140 rpm. After 4 h, one volume of Pro CHO5 CDM was added and the agitation speed was increased to 160 rpm. The concentrations of glucose and glutamine were determined each day, and half the cultures were fed to maintain concentrations of 4 g/l glucose and 4 mM glutamine. The other cultures were not fed during the experiment. Feeding did not result in higher recombinant antibody expression (Fig. 8A). The absence of feeding, however, did affect biomass accumulaton. By 5 days post-transfection, the biomass in the unfed cultures was significantly lower than that in the fed cultures (Fig. 8B). The results also confirm the feasibility of polyfection in another non-instrumented cultivation system, the 50-ml centrifuge tube with a filter cap.

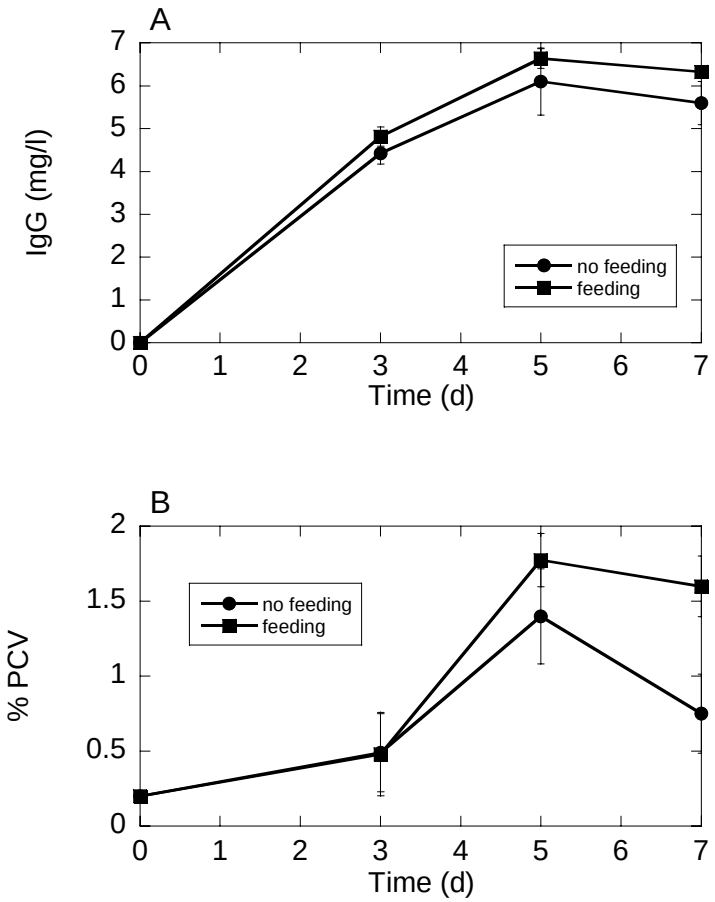


Figure 8. Effect of feeding on transient gene expression in 50 ml centrifuge tubes. CHO DG44 cells were seeded in 2.5 ml of ProCHO5 CDM at a density of $2x10^6$ cells/ml. The cells were transfected with a mixture of pEAK8-LH39, pEAK8-LH41, and pEGFP-N1 at a DNA:PEI ratio of 1:4 (w/w). When necessary, half of the cultures were fed with glucose and glutamine. (A) The antibody concentration was determined by ELISA at the times indicated. (B) The PCV was determined with a mini-PCV tube at the times indicated.

7.1.4 Polyfection of CHO DG44 cells with pcH-NYESO1.

The versatility and reproducibility of the polyfection method was tested with another model protein, the testis antigen NY-ESO-1 (Reuveny, Velez et al. 1987). CHO DG44 cells were seeded at a density of $2x10^6$ cells/ml in 200 ml of RPMI 1640 with 25 mM Hepes (pH 7.1) in ten 1-liter square bottles and transfected at a DNA:PEI ratio of 1:2 (w/w) with pcH-NYESO1, a vector which expresses histidine-tagged NY-ESO-1. Immediately following transfection, the bottles were placed in an incubator at 37°C in a 5% CO_2 atmosphere with 95% humidity with the caps open one quarter of a turn. The agitation speed was 100 rpm. At 4 h post transfection, the cells were diluted with one volume of ProCHO5 CDM and returned to the incubator at an agitation speed of 110 rpm. At 24 h post transfection, the bottles were moved into a non-CO_2 incubator at 37°C with the caps opened one quarter of a turn. The cultures were not fed during the course of the experiment. At 2 days post-transfection, the time of optimum H-NY-

ESO-1 expression, all of the bottles were harvested and cell lysates were analyzed by western blot. The steady-state level of H-NY-ESO-1 was nearly the same for most of the cultures (Fig. 9A). The biomass was also nearly the same for all of the cultures with the exception of bottle 2 (Fig. 9B). There was a slightly higher pH in this culture, which is usually indicative of a lower density.

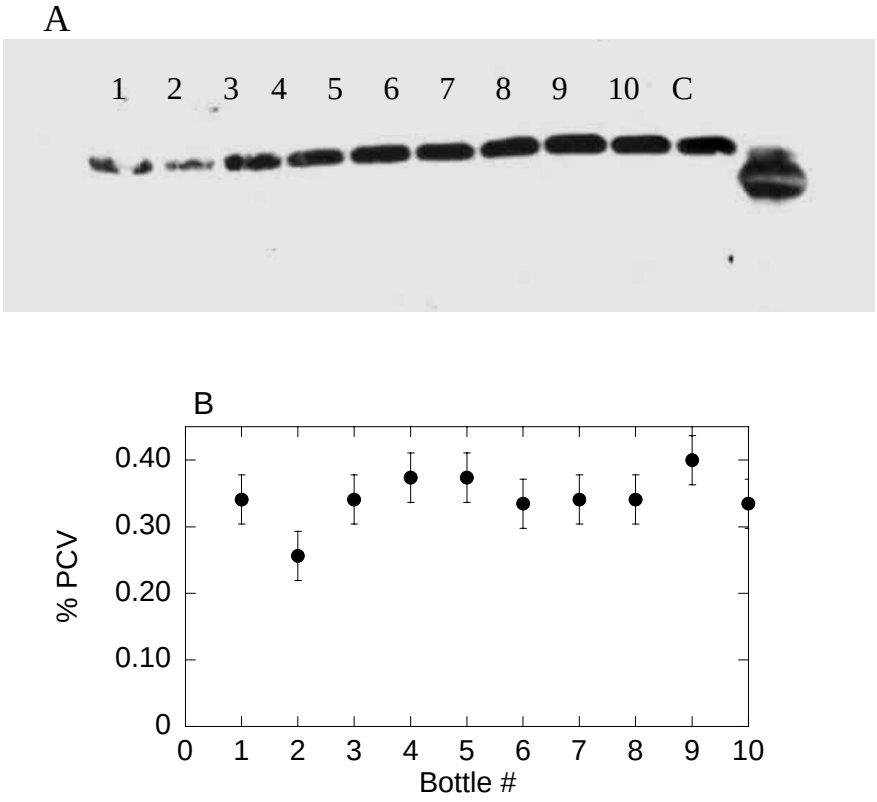


Figure 9. Polyfection of CHO DG44 cells with pcH-NYESO1. Cells were seeded at a density of $2x10^6$ cells/ml in 200 ml of RPMI 1640 supplemented with 25 mM Hepes (pH 7.1) in ten 1-liter bottles. The cells were transfected with pcH-NYESO1 at a DNA:PEI ratio of 1:2 (w/w). The cultures were diluted with one volume of ProCHO5 CDM at 4 h post-transfection. (A) At 2 days post-transfection, cell lysates were analyzed by western blot using a monoclonal antibody against NY-ESO-1. (B) At 2 days post-transfection biomass was determined with a mini-PCV tube.

7.2 Discussion

Transient gene expression in mammalian cells is a promising method for the expression of milligram to gram amounts of a recombinant protein. The objective for the research presented in this chapter was to develop a transient gene expression system that is applicable to both instrumented and non-instrumented cultivation systems at a broad range of scales in medium without any animal-derived components. This laboratory has previously demonstrated that suspension-adapted CHO DG44 cells can be transfected in a serum-free minimal medium (RPMI 1640) using PEI as a DNA delivery agent (Derouazi et al., 2004). The transfections in that case were performed in 12-well microtiter plates or in a 20-L bioreactor (Derouazi et al., 2004). More recently Tait et al. (2004) presented similar results for the polyfection of CHO cells in

agitated Erlenmeyer flasks in serum-free, but not chemically defined, growth medium (CHO-S SFM II). In this chapter the polyfection of CHO DG44 cells in a serum-free, chemically defined medium (ProCHO5 CDM) at volumes ranging from 1 ml to 80 L using both non-instrumented and instrumented cultivation systems is described. The non-instrumented systems included 12-well microtiter plates, 50 ml centrifugation tubes, and 1-liter round or square bottles (Muller et al., 2005). The instrumented cultivation systems included 3-, 20-, and 150-L bioreactors. Over this range of scales and cultivation systems, the yield of recombinant antibody ranged from 5-25 mg/L with the lowest yields observed in 12-well microtiter plates. These results demonstrated the scalability of polyfection for CHO DG44 cells in ProCHO5 CDM medium.

Transient transfection in serum-free, chemically defined medium is important for several reasons. First, the elimination of animal-derived components reduces the risk of contamination of the recombinant protein with infectious agents such as viruses or prions. Second, this approach allows the use of a single medium for both cell growth and transfection. Conceivably, with further development it may be possible to grow and transfect cells in the same culture vessel without any intervening steps involving centrifugation and resuspension of cells in fresh medium prior to transfection. Third, the elimination of serum facilitates recombinant protein purification and reduces the cost of the production process (Barnes and Sato, 1980).

In most of the cultivation systems tested here, recombinant protein expression was higher for transfections in ProCHO5 CDM as compared to those in RPMI 1640. In one-liter square bottles this difference was not due to a difference in viable cell number between the two cultures. This was not the case, however, in 3-liter bioreactors where a higher recombinant protein yield in ProCHO5 CDM was accompanied by a greater number of viable cells as compared to the culture in RPMI 1640. Additional experiments are necessary to determine more accurately the relationship between recombinant protein production and viable cell number for transient transfections in ProCHO5 CDM and RPMI 1640.

Polyfection in serum-free, chemically defined medium as described in this chapter has also been demonstrated with HEK 293E cells, another frequently used host for transient gene expression (Baldi et al., 2005; Durocher et al., 2002, Schlaeger and Christensen 1999; Schlaeger et al., 2003). As shown in Chapter 6, suspension-adapted HEK 293E cells were polyfected in serum-free, chemically defined Ex-Cell 293 medium. For HEK 293 cells transient gene expression has been successful at many scales in several different cultivation systems including spinner flasks (up to 3 L) and 3-, 20-, and 150-L bioreactors (Baldi et al., 2005; Girard et al. 2002; Meissner et al. 2001; Pham et al. 2003; Schlaeger and Christensen 1999 Schlaeger et al. 2003). In several of these examples, the cells were transfected by calcium phosphate-DNA coprecipitation in the presence of FCS (Baldi et al., 2005; Girard et al. 2002; Meissner et al. 2001). HEK 293 cells have also been polyfected in serum-free medium that was not chemically defined and that included peptones from gelatin (Pham et al., 2003).

Transfection in ProCHO5 CDM required a DNA:PEI ratio of 1:4 (w/w) as compared to a ratio of 1:2 in RPMI 1640. Similarly, the polyfection of HEK 293E cells in Ex-

Cell 293 medium required a DNA:PEI ratio of 1:11 (w/w) (Chapter 6). One possible explanation for these observations is that there are component(s) in ProCHO5 CDM and Ex-Cell 293 which interact with PEI. It has been shown that the packing of PEI/DNA complexes is important for efficient DNA delivery (Godbey et al., 1999a; Godbey et al., 1999b). If PEI has a higher affinity for a component(s) in the medium than it does for DNA, then the active transfection complex may be disrupted, resulting in a decrease in the efficiency of gene delivery to the cells. Indeed, it has been shown that PEI precipitates with unknown component(s) in both ProCHO5 and Ex-Cell 293 media (M. Bertschinger, unpublished data). Thus, transfection in these media may require an excess of PEI to preserve the integrity of PEI/DNA complexes.

The agitated square bottle is an attractive system for both cell growth and transient transfection. For CHO DG44 and HEK 293E cells, densities of $5x10^6$ and $7x10^6$ cells/ml, respectively, have been achieved in serum-free, chemically defined media (see Chapter 6). Here and in Chapter 6 we have demonstrated that cells can be transfected in square bottles. Polyfection of CHO DG44 cells in one-liter square bottles yielded about 20 mg/L of recombinant antibody (Fig. 2A) as compared to yields in a range of 10-25 mg/L following transfection in 3-, 20-, and 150-L bioreactors (Fig. 3-5). As described here, several changes in the protocol including the elimination of a wash step, maintenance of the culture in a non-CO_2 incubator, and a constant agitation speed before and after dilution of the culture have greatly simplified the polyfection protocol in square bottles. Thus, this cultivation system may eventually replace bioreactors for the transient transfection of mammalian cells. As shown in Chapter 5, it should be possible to grow and transfect cells in even larger square-shaped containers than the one-liter bottles described here.

The typical yield of secreted recombinant proteins in either CHO DG44 or HEK 293 cells following transient transfection is in the 1-75 mg/L range (Wurm, 2004). However, recombinant mammalian cell lines may yield as much as 4 g/L (Wurm, 2004). This difference suggests that further improvements in transient gene expression in mammalian cells are possible. Optimization of media, gene delivery methods, cultivation systems, and expression vectors are expected to help close this gap, making transient gene expression an even more attractive option for the rapid production of recombinant proteins.

7.3 References

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Conclusions and Perspectives

8. Conclusions

There are many different mammalian cell lines, cultivation systems, and DNA delivery methods that can be used for large-scale transient gene expression. Currently, the most popular cell lines are CHO and HEK 293, the best characterized cultivation vessels are spinner flasks, Erlenmeyer flasks, and stirred-tank bioreactors, and the most widely used non-viral DNA delivery techniques are calcium phosphate-DNA coprecipitation and polyfection. The work described in this thesis focused on the development of agitated square bottles as a vessel for the cultivation and transfection of mammalian cells in suspension and on the characterization of two gene delivery methods, calfection and polyfection, for transfecting cells in chemically defined media.

One of the first challenges in large-scale transfection is the generation of large cell populations prior to transfection. Here a new cultivation system was developed in order to produce high cell density cultures with good viability. Superior growth of suspension-adapted HEK 293E and CHO DG44 cells was observed in agitated square bottles as compared to spinner flasks. Cultures in agitated one-liter square bottles reached densities up to $7x10^6$ cells/ml for HEK 293E cells and $5x10^6$ cells/ml for CHO DG44 cells in comparison to $2.5\text{-}4x10^6$ cells/ml for cultures of the same cells grown in spinner flasks. Cell viability was maintained above 90% for as long as seven days after inoculation, making cell passage once per week a possibility. Following transfection by calcium phosphate-DNA coprecipitation, cells grown in square bottles yielded the same or slightly higher levels of recombinant protein than did cells grown in spinner flasks. Additional advantages of this cultivation system include its low cost and the ease of cleaning and handling the bottles. Thus, agitated square bottles represent an attractive alternative to other systems for the cultivation of mammalian cells in suspension.

The second objective was to develop a method for DNA delivery in serum-free, chemically defined that was applicable to both instrumented and non-instrumented culture vessels at a broad range of scales. Calfection, a transfection method based on the addition of Ca^{2+} and DNA to cultivated cells, was initiated as a simplification of calcium phosphate-DNA coprecipitation. Calfection proved to be a simple and scalable protocol without any time-dependent steps. Reporter protein expression levels similar to those achieved with calcium phosphate co-precipitation were observed. However, the efficiency of gene delivery by calfection decreased substantially in the absence of serum.

Efficient transfection of both HEK 293E and CHO DG44 cells in serum-free chemically defined medium was achieved by polyfection. The method was shown to be scaleable from 1 ml to 1 L for HEK 293E cells and from 1 ml to 80 L for CHO DG44 cells using a variety of non-instrumented (microtiter plates, 50 ml centrifuge tubes, spinner flasks, and both square and round bottles) and instrumented (3-, 20-, and 150-L stirred-tank bioreactors) cultivation systems. The highest yields of recombinant antibody were 80 mg/L in HEK 293E cells in comparison to 14-20 mg/L

reported in literature to date (Baldi et al. 2005; Durocher et al. 2002; Meissner et al. 2001) and 22 mg/L in CHO DG44 in comparison to 5-8 mg/L previously achieved (Derouazi et al. 2004; Tait et al. 2004). Importantly, recombinant protein expression was usually as high or higher in square bottles than in stirred-tank bioreactors.

9. Perspectives

Transient gene expression is a field that has many areas that still need to be studied. There are some key areas that could be addressed first:

- It would be interesting to grow and transfect cells at a higher density to determine the impact on protein yield.

- Attempts to establish a serum-free method for calfection should be made. Although serum-free polyfection is an efficient method of gene delivery, calfection is more promising because it does not require the addition of synthetic polymers.

- The characterization of cell growth and transfection in square containers larger than 1 liter and in round bottles needs to be continued. The advantage of round bottles over square ones is that there are existing glass containers up to the 5-liter size. Square bottles larger than 2-liter (other than with a slightly different geometry) are not yet commercially available.

- A more in depth study is necessary to remove one of the two medium exchange steps directly prior to transfection. Ideally, transfection could be done directly in the N-1 culture as has been shown for HEK 293E cells in Chapter 6. With the proper optimization, this should work with other cells (CHO DG44) and with other methods of DNA delivery (calfection).

- Peptones have shown promise as a serum replacement during polyfection. The screening of various peptones can be done to determine if they benefit gene delivery by polyfection, calfection, and calcium phosphate-DNA coprecipitation in chemically defined media.

- Polyfection in square bottles is a simple and reproducible method of gene delivery. Further research into calfection and calcium phosphate-DNA co-precipitation in these bottles is needed. Initial tests have given promising results, but a full optimization is necessary. Ideally, a process in chemically defined medium can be developed with these two methods.

- Semi-stable transfection to produce a genetically heterogeneous population can be established with transient gene expression. After transfection and genetic

selection, the population could be expanded without first establishing clonal populations. This would eliminate the need to establish stable cell lines.

- Polyfection as a gene delivery method for transient protein production is very promising. A better understanding of the processes during polyfection and of the fate of PEI in cells and in the medium are necessary. In depth studies of cell conditions prior to and during polyfection will probably lead to an improved transfection efficiency and higher recmbinant protein yield. The effects of various factors such as the age of the culture, the effects of cell passage number, and the viability of the cells on transfection efficiency should be carefully scrutinized.

Although we were pleased with a recombinant protein yield from transient transfection of 80 mg/l, this remains many times lower than the most productive recombinant cell lines. Titers of 4.7 g/l have been reported with specific productivities up to 90 pg/cell/day for recombinant protein production (Wurm 2004). Many reviews and publications have studied ways to improve recombinant protein production (Andersen and Krummen 2002; Chu and Robinson 2001; Wurm 2004). Research in several areas (media, cultivation system, DNA delivery, vector development, and gene expression) is expected to help narrow this gap in the coming years.

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Curriculum Vitae

10. Curriculum Vitae

Natalie Muller
58, Rte d'Annecy, 1256 Troinex, Switzerland
Natalie.Muller@epfl.ch

June 7, 1972
American
Swiss C Permit
Married

Objective

A challenging research position allowing me to apply and further my knowledge in cellular and molecular biology to the area of immunotherapy of cancer.

Education

2001-Present

Ph.D. Candidate, Life Sciences Faculty, Institute of Biological Engineering and Biotechnology, Laboratory of Cellular Biotechnology, EPFL. Thesis topic: ***Transient gene expression for rapid protein production: studies and optimizations under serum-free conditions*** under the direction of Professor Florian M. Wurm

1990-1996

Bachelor of Science in Chemical Engineering, Wayne State University, Detroit, Michigan. GPA: 3.25/4.0

Teaching responsibilities

Teaching Assistant (Cell culture, microbiology, genetic engineering, planning and implementation of new student experiments, lectures):

- Introduction to genetic engineering 2001-2003
- Optimizing extraction of a recombinant intracellular protein from mammalian cells 2001-2003
- PEI transfection of Human Embryo Kidney cells 2004

Supervision of trainees, students, and technicians

Experience

10/99-1/01

Citibank Private Bank, Geneva, Switzerland.
Assistant Manager, *Investment Finance*

3/99-4/99

Roche Molecular Systems, Pleasanton, CA, U.S.A.
Research Scientist/Engineer, *Systems Group*

11/98-2/99

America True, San Francisco, CA, U.S.A.
Marketing and Retail assistant.

5/96-9/98

Henkel Surface Technologies, Madison Heights, MI, U.S.A.
Site Engineer, *Chemical Management Division*

1/95-5/96

The Dow Chemical Company, Midland, MI, U.S.A.
Technical Service & Development, *Antimicrobials Department*
Research & Development: *Designed Thermoplastics Research*

Publications

N. Muller, M. Derouazi, F. VanTilborgh, D.L. Hacker, M. Jordan, F.M. Wurm. Reactor scale transfection of CHO cells in chemically defined medium. (Manuscript in preparation.)

N. Muller, D.L. Hacker, M. Jordan, F.M. Wurm. Shaking technology for rapid transient transfection with CHO cells. (Manuscript in preparation.)

L.Cohen, G. Ritter, D.L. Hacker, N. Muller, H-P. Thanh, S. Picasso, and F.M. Wurm. Development of a transient mammalian expression process for the production of the cancer testis antigen NY-ESO-1. (Manuscript in preparation.)

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Posters

N. Muller, J. Lindell, P. Girard, E. Derow, H. El Abridi, R. Jacquet, M. Jordan, and F.M. Wurm. *A simple and scalable gene transfer method: Calfection*.
6th Conference on Protein Expression in Animal Cells in Mont-Tremblant, Quebec, Canada 2003

L. Baldi, P. Girard, R. Jacquet, S. Picasso, H. Phan Thanh, N. Muller, M. Derouazi, F. VanTilborgh, I. Tabuas-Muller, E. Derow, D. Hacker, M. Jordan, and F.M. Wurm. *Non-viral transient gene expression at scales from milliliters to 100 liters*.
Cell Culture Engineering IX Conference, Cancun, Mexico, 2003

L. Baldi, P. Girard, R. Jacquet, S. Picasso, H. Phan Thanh, N. Muller, M. Derouazi, E. Derow and F.M. Wurm. *Recombinant proteins from mammalian cells by large-scale transient gene expression.*
NCCR Conference, Zurich, Switzerland. 2003

J. Lindell, N. Muller, P. Girard, E. Derow, R. Jacquet, M. Jordan, and F.M. Wurm. *A novel nanoparticle (?) based gene transfer method for mammalian cells*.
EPFL Nanotechnology Conference, Lausanne, Switzerland, 2003

N. Muller, P. Girard, M. Jordan, F.M. Wurm. *Shaken and not stirred: Evaluation of various small and moderate scale cultivation systems for suspension cells*.
Cell Culture Engineering VIII in Colorado, USA 2002

N. Muller, J. Lindell, P. Girard, M. Derouazi, E. Derow, P. Tromba, M. Jordan, F.M. Wurm. *Calfection II: Process refinements for a novel gene transfer method*.
Second European BioTechnology Workshop, Ittingen, Switzerland 2002

N. Muller, P. Girard, M. Jordan, F.M. Wurm *Shaking Technology: A novel system for the cultivation and transfection of mammalian cells in suspension*.
Second European BioTechnology Workshop, Ittingen, Switzerland 2002

P. Girard, R. Jacquet, N. Muller, M. Bourgeois, M. J.ordan, and F.M. Wurm. *Large scale transient gene expression*.
European BioTechnology Workshop, Ittingen, Switzerland 2002

J. Lindell, N. Muller, P. Girard, M. Jordan, and F.M. Wurm. *Calfection I: The development of a new, convenient and low-cost gene transfer method for suspension cells*.
Second European BioTechnology Workshop, Ittingen, Switzerland 2002

Oral Presentations

N. Muller and F.M. Wurm. *Calfection: A novel approach for gene transfer*.
Second European BioTechnology Workshop, Ittingen, Switzerland 2002

N. Muller and F.M. Wurm. *Growth media comparison*.
Scientific Collaboration with Serono Pharmaceutical Research Institute, Geneva, Switzerland 2001

Professional Training

2003	Flow Cytometry course in the Department of Clinical Research at the Institute of Pathology, University of Bern
2001-2002	Molecular & Cellular Biotechnology EPFL – Prof. Dr. Florian Wurm
2001-2002	Applications in Industrial Biotechnology EPFL - Prof. Dr. Ian Marison
2001	Cell and Tissue Reactor Engineering at University of Minnesota, USA
2001	Molecular Biotechnology in Champéry, Switzerland

Professional Skills

Cellular Biology

- Mammalian cell cultivation (adherent, suspension, various lines and culture systems)
- Cell growth optimization
- Transient gene expression in mammalian cells
- Bacterial cultivation

Bioengineering

- Non-viral transfection
- Large-scale transfection (up to 100L)
- Bioreactors
- Process development and optimization

Biochemistry

- ELISA assay
- Protein quantification assays

Molecular Biology

- Plasmid DNA preparation
- Gel electrophoresis
- Bacterial Transformation

Other Analytical Methods

- Bioprofile200, NOVA Biomedical (multi-parameter analysis)
- Flow cytometry
- Fluorescence microscopy

Technical Communication

Project Management

Honors & Activities

Wayne State University Presidential Merit Scholarship recipient, Michigan Competitive Scholarship recipient, Dean's List, Member of American Institute of Chemical Engineers and Society of Women Engineers.

Hobbies Skiing, swimming, music, dancing, hiking, and cooking

Languages English: mother tongue
French: good knowledge written and spoken