
Department für Biotechnologie
Institute für angewandte Mikrobiologie (IAM)
Universität für Bodenkultur Wien

Institutsvorstand: O. Univ. Prof. Dipl.-Ing. Dr. nat. tech. Hermann Katinger
Betreuerin: Ao. Univ. Prof. Dipl.-Ing. Dr. nat. tech. Renate Kunert

# The influence of gene copy number and mRNA level on the mAb secretion of stable recombinant mammalian cell lines and the application of two chemically defined transfection systems

Dissertation
zur Erlangung des akademischen Doktorgrades rerum naturalium technicarium an der Universität für Bodenkultur Wien

Eingereicht von Mag. rer. nat. Hannes Reisinger
Wien, im September 2008

*"If we knew what it was we were doing, it would not be called research, would it?"*

**- Albert Einstein -**

# Acknowledgements

Ich möchte mich hier herzlich bei O. Univ. Prof. DI. Dr. Katinger bedanken, der mir die Möglichkeit gab meine Dissertation an seinem Institut durchzuführen.

Besonderer Dank gilt natürlich meiner Betreuerin Ao. Univ. Prof. DI. Dr. Kunert die mich während meiner Dissertation betreute, stets ein offenes Ohr für mich hatte, mich durch so manche Höhen und Tiefen begleitete und mir mit Rat und Tat zur Seite stand. Vielen Dank liebe Renate für deine umfassende Betreuung während dieser drei Jahre.

Besonders möchte ich mich auch bei Johannes für die wundervolle und unbeschwerte Zeit bedanken, mir immer zur Seite stand und ein offenes Ohr für mich hatte.

Spezieller Dank gilt auch Willi, der mir alle Tricks und Kniffe rund um die Zellkultur beibrachte und mich in meiner Arbeit immer Unterstützte.

Auch dem Rest der Arbeitsgruppe, Helga, Roman, Alex und Martina sei hier für die schöne Zeit uns die anregenden Diskussionen gedankt.

Nicht zuletzt gilt mein Dank meinen lieben Kollegen aus dem 2, 3, 4 Stock des und all jenen Leuten, die mich stets in meiner Arbeit unterstützt haben und derer es leider zu viele sind, um ihnen an dieser Stelle einzeln zu danken.

Weiters gilt mein Dank meiner Familie, die mich während meines gesamten Werdeganges unterstütz hat und somit diese Arbeit erst ermöglicht hat.

# Abstract

Recombinant human antibody production in Chinese hamster ovary (CHO) cells represents a growing field within the last decades. In the present study we developed and improved two transfection systems based on cationic liposomes and polycationic carriers, to introduce exogenous DNA into CHO cells and generate transgenic cell lines.

Cationic liposomes consisting of DOTAP and DOPE form lipoplexes with the DNA via electrostatic interaction. These lipoplexes are taken up at the cell membrane via endosomes and facilitate the escape of the DNA into the cytosol thus reaching the nucleus. In a second approach the polycation PEI served as shuttle, again the DNA is bound and complexed via electrostatic interaction and actively supports the DNA transfer directly into the nucleus thereby preventing the degradation of the DNA.

After determining the cellular toxicity, the ideal ratio between the DNA and the two systems using EGFP as a reporter gene were determined. These systems were compared with several commercial systems showing that our systems are highly competitive during transient transfection using EGFP. The next step was to investigate the potential of these systems for transient and stable transfection using a more complex molecule, the HIV neutralizing human mAb 2F5 antibody. These experiments demonstrated that our systems worked during stable transfection resulting in 99.2 and 98.3% transfected cells for DD and PEI, respectively after selection and subcloning.

Thus, our systems served to generate different recombinant CHO cell lines, which were analyzed regarding genetic modifications to enhance protein production. Still the limitations for recombinant protein production in the cellular machinery and how amplification of gene copy number by MTX affects the protein production are unclear, although several previous studies dealt with these issues. We tried to answer some of these questions by using real-time qPCR, Northern-Blot and flow cytometry. All characterized CHO production clones expressed the neutralizing mAb 2F5. Different expression plasmids were used to create these stable mAb 2F5 expressing cell lines. One system contained signal peptide derived for the naturally secreted luciferase of the copepod *Gaussia princeps* which was shown to enhance recombinant protein production. Additionally, in one approaches the 2F5 cDNA was optimized with respect to AT and GC rich sequence stretches, repeat sequences and RNA secondary structures to increase the mRNA half-life and to shorten its turnover time. The best producing cell clones were selected, sub-cloned, amplified with MTX and cultivated for some weeks to confer long-term stability.

The gene copy number and promoters seemed to have no influence on the transcript amount suggesting a position effect of the transgene, whereas codon optimization resulted in elevated mRNA levels, without any expression increase. Translation and post-translational modifications seem responsible for decreasing antibody expression.

# Zusammenfassung

Durch den biotechnologischen Fortschritt während der letzten Jahre wurden rekombinante Antikörper zu einem der meistgefragten biopharmazeutischen Produkte. In dieser Arbeit haben wir zwei alternative Transfektionsmethoden, basierend auf kationischen Liposomen und auf dem kationischem Polymer PEI weiterentwickelt um rekombinante Ziellinien herzustellen.

Kationischen Liposomen wurden durch mischen von DOTAP und DOPE hergestellt. Die positiv geladenen Liposomen binden durch elektrostatische Wechselwirkung an die negativ geladene DNS und bilden Lipoplexe. Diese werden durch Endozytose aufgenommen und in Endosomen in die Zelle transportiert. Durch Fusion der Lipide mit der Endosomenmembran wird die DNS in das Zytoplasma entlassen von wo sie in den Zellkern gelangt. Beim zweiten Ansatz bindet das kationische PEI die DNS und komplexiert diese. PEI unterstützt den Transport der DNS in den Zellkern und verhindert den Abbau der DNS.

Zunächst wurde die für Zellen toxische Konzentration beider Systeme und die optimale Verhältnis mit dem Reporterprotein EGFP bestimmt. Der Vergleich unserer Transfektionssysteme mit diversen kommerziellen Systemen zeigte, dass unsere Systeme bei der transienten Transfektion mit EGFP vergleichbare Ergebnisse brachten.

Anschließend untersuchten wir das Potential unserer Systeme zur transienten und stabilen Expression eines komplexeren Moleküls, des HIV neutralisierenden humanen Antikörper 2F5. Diese Experimente zeigten dass unsere Systeme gut für die stabile Transfektion geeignet sind, da nach Selektion/ Subklonierung, DD 99,2% und PEI 98,3% der Zellen den Antikörper produzierten.

Diese Systeme wurden verwendet, um Antikörper produzierende Zelllinien herzustellen. Die limitierenden Faktoren der zellulären Sekretionsmaschinerie und der Einfluss der MTX-Amplifikation auf die Proteinproduktion sind noch unklar, obwohl sich schon mehrere Studien mit diesem Thema befasst haben. In der vorliegenden Arbeit wurden real-time qPCR, Northern-Blot und Durchflußzytometrie verwendet um diese Fragen zu klären.

Die Zelllinien wurden mit unterschiedlichen Plasmiden hergestellt und die besten Produzenten wurden durch Selektion, Subklonierung, MTX Amplifikation selektiert und anschließend über mehrere Wochen kultiviert um Stabilität zu gewährleisten. Ein Klon wurde mit dem Signalpeptid von *Gaussia princeps* vor der LC und HC versehen, was zu höherer Proteinexpression führen soll. Weiters wurde auch kodon-optimierte komplementäre DNS für die Herstellung eines Klons verwendet, welche einen höheren GC Gehalt und keine repetitiven Sequenzen hatte und keine

Sekundärstrukturen bilden. Diese Veränderungen bewirken eine längere mRNS Halbwertzeit und eine kürzere Turnover-Zeit.

Die Ergebnisse zeigten, dass die Anzahl der Genkopien und die unterschiedlichen Promotoren keinen Einfluss auf die Transkriptmenge hatten, was einen Positionseffekt impliziert. Die Optimierung der Codons hatte den Effekt, erhöhter mRNS Stabilität ohne Zunahme der Expression. Deshalb werden Translation und post-translationale Modifikation als Ursache für die verminderte Antikörperproduktion gesehen werden.

## Keywords

Cationic liposomes

PEI

Serum-free transfection

Monoclonal antibody 2F5

Stable and transient transfection of CHO cells

# Table of contents

# 1 Recombinant Protein Expression in Mammalian Cells

Nowadays, CHO cells are used to produce the majority of recombinant protein therapeutics manufactured by mammalian cell culture (Birch and Onakunle 2005). This cell line is preferred because of its ability to confer appropriate post-translational modifications, a variety of selection/ amplification systems (e.g. glutamine synthetase (GS) - and dihydrofolate reductase (DHFR)-system), and it can be adapted to serum-free conditions (Galbraith et al. 2006).

Since the demand for recombinant proteins in fundamental and clinical application escalates, they gain increasing importance (Muller et al. 2005) and a broad variety of methods have been developed to facilitate the uptake and integration of DNA (Deoxyribonucleic acid), because the spontaneous entry of DNA is a highly unlikely and inefficient mechanism (Felgner et al. 1987). These methods involve several approaches like polycations (Boussif et al. 1995; Farber et al. 1975; Kawai and Nishizawa 1984; McCutchan and Pagano 1968), calcium phosphate (Graham and van der Eb 1973; Loyter et al. 1982), liposome fusion (Cudd et al. 1984), micro-injection (Graessmann and Graessmann 1983), electroporation (Neumann et al. 1982) and protoplast fusion (Schaffner 1980) which will be explained later in more detail. Nowadays the majority of non-viral transfection systems rely either on cationic liposomes or cationic polymers (Khalil et al. 2006). Compared to viral DNA delivery methods, non-viral delivery systems confer the advantage of lower costs, easier and safer production of stable cell lines and long-term storage of transfectants without special conditions (Godbey et al. 1999a). Furthermore cationic liposomes and polymers enable the delivery of much larger DNA fragments than most viral vectors (Abdallah et al. 1995). In general cells can internalize macromolecules by a variety of mechanisms passing the cellular and nuclear membranes which will be explained in the following sections.

## 1.1 Membrane Structures

Biological molecules that are insoluble in aqueous solutions and soluble in organic solvents are classified as lipids. The lipids of physiological importance for humans have four major functions. They serve as structural components of biological membranes; provide energy reserves, predominantly in the form of triacylglycerols; both lipids and lipid derivatives serve as vitamins and hormones and finally lipophilic bile acids aid in lipid solubilization. Membrane lipids consist of four major groups including phospholipids, steroids, glycolipids and other lipids.

### 1.1.1 Phospholipids

Phospholipids represent one of the major compounds of cell membranes and lipoproteins. In general they consist of a polar head group and two hydrocarbon tails (Fig 1). The top region consists with the amino-group representing the charged group connected by glycerol to two fatty acid tails, one tail is a straight fatty acid chain (saturated) and the other contains a kink in the tail due to a cis double bond (unsaturated), which influences packing and movement in the lateral plane of the membrane. A common model for membranes is the fluid mosaic membrane model by Singer and Nicholson (1963). As shown in figure 2 the phospholipids form a bilayer with their hydrophobic tails facing to the inner site. Proteins associate to membranes via different ways, forming peripheral proteins, integral membrane proteins or transmembrane proteins.

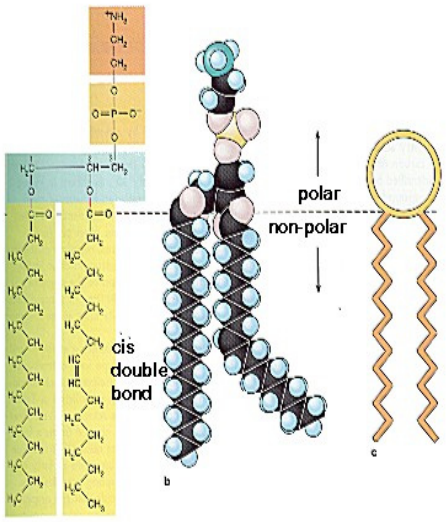


Fig 1 General composition of phospholipids (Molekularbiologie der Zelle/ 4.Auflage).

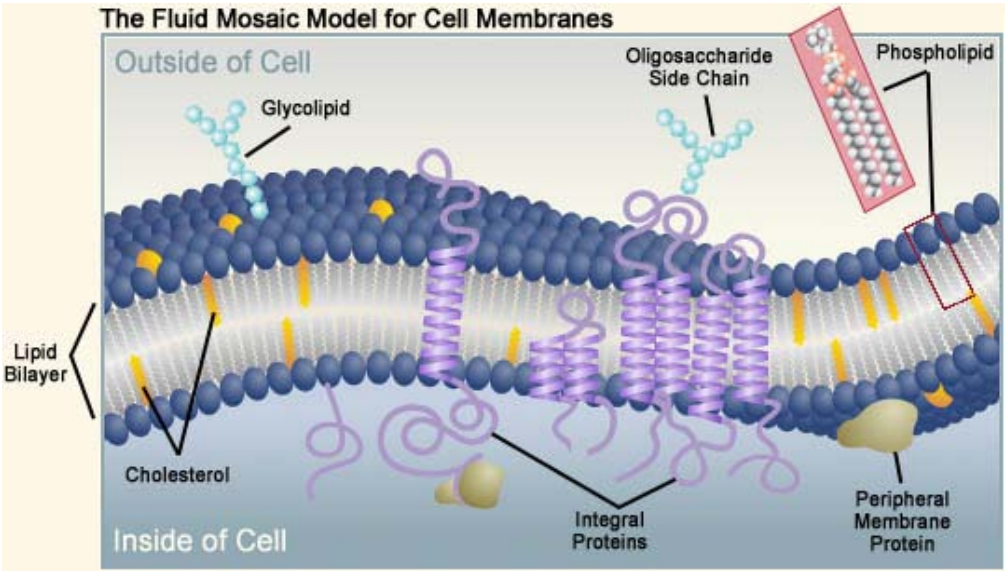


Fig 2 Fluid mosaic model for cell membranes according to Singer and Nicholson (http://student.biology.arizona.edu/honors2002/group02/images/fluid_mosaic_model.jpg)

The phospholipids can be sub-divided into four major classes appearing in mammalian cells that differ in their polar groups such as choline, ethanolamine, serine or sphingomyelin (Fig 3) attached to their phosphate.

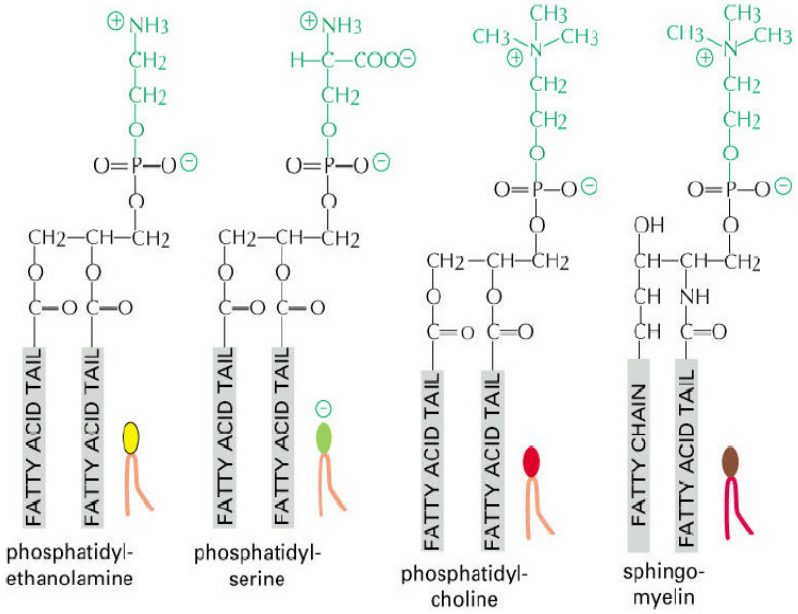


Fig 3 Major classes of phospholipids, differing in the polar groups attached to their phosphate (Molekularbiologie der Zelle/ 4.Auflage).

### 1.1.2 Steroids

Steroids increase membrane rigidity, make the lipid bilayer less deformable and decrease the permeability to small water-soluble molecules. Additionally cholesterol prevents crystallization of hydrocarbons and phase shifts in the membrane. They include cholesterols and cholesterol hemisuccinates (CHEMS).

### 1.1.3 Glycolipids and Other Lipids

Several animal membranes contain glycolipids. Sugars form their polar head group and they are common in nerve cells. Strange lipids include cardiolipin and isoprenoids such as vitamin A & E.

## 1.2 Cellular Uptake

For successful and efficient gene delivery to the nucleus, several biological barriers must be overcome (Bally et al. 1999). These steps include binding to the cell surface, traversing the plasma membrane, avoiding lysosomal degradation and overcoming the nuclear envelope (Khalil et al. 2006). Polyethylenimine (PEI) and Dioleoyltrimethylammoniumpropane/ Dioleylphosphatidylethanolamine (DOTAP/ DOPE) have the ability to enhance and mediate the cytosolic release through different mechanisms. To explain the uptake several internalization mechanisms have been proposed (Felgner et al. 1987; Felgner et al. 1995; Friend et al. 1996; Labat-Moleur et al. 1996; Matsui et al. 1997; Zuhorn and Hoekstra 2002).

Endocytosis has been described as the major mechanism for the internalization of non-viral vectors into the cells (Friend et al. 1996; Labat-Moleur et al. 1996; Zuhorn and Hoekstra 2002). It refers to the cellular uptake of macromolecules and solutes into membrane bound vesicles formed by the invagination and pinching off the plasma membrane (Khalil et al. 2006). Endocytosis can be subdivided in pinocytosis and phagocytosis (Conner and Schmid 2003). Furthermore, pinocytosis can be sub-divided into clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and clathrin/caveolae-independent endocytosis (Lamaze and Schmid 1995). These uptake mechanisms should be seen as models that are not static. The pathways are not strictly divided and the transition between them is smooth. Thus, nobody can exactly predict which pathway is used and why it is used.

### 1.2.1 Clathrin-Mediated Endocytosis

The best characterized and major uptake mechanism is the clathrin-mediated endocytosis (Fig 4)(Lamaze and Schmid 1995; Takei and Haucke 2001). Generally, the internalization includes first the binding of a ligand to cell surface. Afterwards, the clathrin coated pits assemble to form a polygonal lattice on the membrane surface (Conner and Schmid 2003; Takei and Haucke 2001). This is followed by invagination and pinching off the plasma membrane, thus forming clathrin coated vesicles (Fig 4). The clathrin coat depolymerizes, resulting in an early endosome which matures to a late endosome. This maturation is connected with a pH drop to 5 in the late endosome and further reduction during fusion with lysosomes (Maxfield and McGraw 2004).

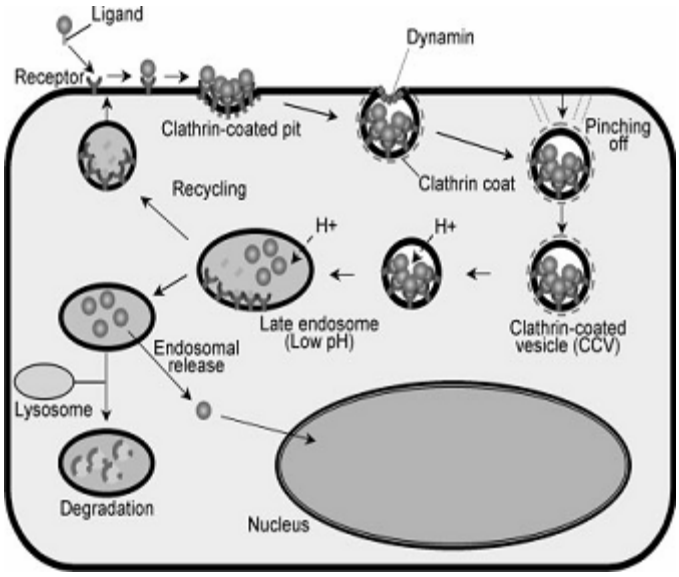


Fig 4 Clathrin mediated endocytosis pathway (Khalil et al. 2006).

### 1.2.2 Caveolae-Mediated Endocytosis

The caveolae-mediated endocytosis uptake (Fig 5) is a non-acid and non-digestive internalization route (Ferrari et al. 2003). Caveolae are small, hydrophobic membrane micro domains, rich in glycosphingolipids and cholesterol (Harris et al. 2002; Matveev et al. 2001). They were defined as flask-shaped invaginations of the plasma membrane (Harris et al. 2002; Pelkmans et al. 2001) and appear in many cell types (Conner and Schmid 2003). Caveolin containing lipid rafts are referred to as caveolae (Matveev et al. 2001), characterized through its association to caveolins a group of cholesterol-binding proteins, which function to generate and

mediate these structures (Lamaze and Schmid 1995). The macromolecules internalized by the caveolae pathway can be directly transported into the cell, thus avoiding lysosomal degradation (Ferrari et al. 2003; Harris et al. 2002) making it an interesting target for DNA delivery.

### 1.2.3 Macropinocytosis

Macropinocytosis (Fig 5) refers to the formation of large endocytic vesicles by actin-driven invagination of the plasma membrane (Amyere et al. 2002; Swanson and Watts 1995), which normally is accompanied by membrane ruffling (Conner and Schmid 2003; Swanson and Watts 1995). The ruffles become longer and broader forming large macropinosomes (Fig 5) (Swanson and Watts 1995) providing an efficient non-selective transport route (Conner and Schmid 2003). The intracellular fate of the vesicles differs depending on the cell type (Meier and Greber 2003; Swanson and Watts 1995).

This pathway provides the advantage of increased uptake of macromolecules, avoidance of lysosomal degradation and easier escape due to their leaky nature (Khalil et al. 2006).

### 1.2.4 Phagocytosis

Merely specialized cells conduct phagocytosis (Fig 5)(Allen and Aderem 1996) and internalization is mediated via interaction of specific receptors on the phagocytes with ligands on the surface of the particles (Khalil et al. 2006). After internalization the phagosomes mature via several fusion steps resulting in the formation of mature phagosomes degrading the internalized particles (Allen and Aderem 1996). This mechanism plays no significant role in gene delivery, whereas a similar mechanism is proposed for liposome/DNA and PEI/DNA complexes (Kopatz et al. 2004; Matsui et al. 1997).

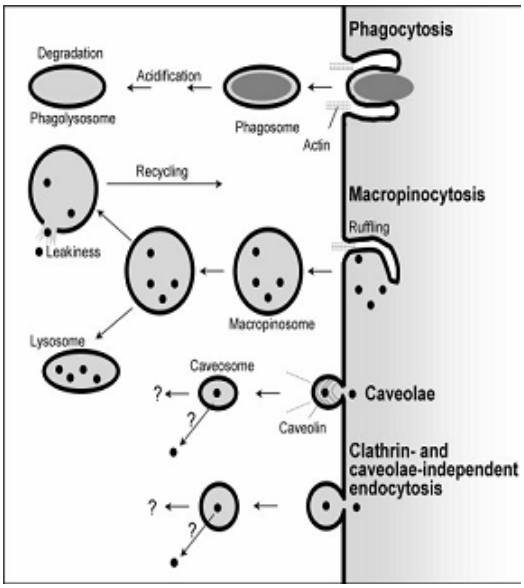


Fig 5 Phagocytosis and clathrin-independent endocytosis (Khalil et al. 2006).

### 1.2.5 Clathrin- and Caveolae-Independent Endocytosis

Finally there exists another endocytosis pathway. Despite the fact that most receptors are internalized via the clathrin-mediated endocytosis, other pinocytosis pathways are capable of selective receptor-mediated endocytosis (Parton et al. 1994; Subtil et al. 1994) via clathrin- and caveolae-independent endocytosis. The receptor-mediated endocytosis is a possible approach to introduce foreign DNA into cells.

### 1.2.6 Cellular Uptake of Lipoplexes and Polyplexes

PEI and cationic liposomes form complexes upon mixing them with DNA called polyplexes and lipoplexes respectively. Both systems have to pass cellular binding, cellular uptake, endosomal escape and nuclear delivery to enable the successful targeting of a gene. Both systems use their excess of positive charges to interact with the cell surface electrostatically (Khalil et al. 2006). The internalization mechanism of lipoplexes is yet not fully understood. Early reports suggested a fusion between the lipids and the plasma membrane for DNA delivery into the cytosol (Felgner et al. 1987; Felgner et al. 1995). However, recent experiments support evidence that endocytosis is involved in the entrance route. Currently, it is assumed that the membrane fusion is important for transfection, but that most uptake occurs via endocytosis solely by clathrin-mediated endocytosis (Khalil et al. 2006).

The polyplex uptake occurs through endocytosis, but without fusion with the cell membrane. Their uptake can be mediated via clathrin-dependent or -independent pathways (Goncalves et al. 2004) the latter involving the caveolae pathway (Rejman et al. 2005).

In general endosomal escape is the crucial step for lipoplex- and polyplex-mediated transfection. Lipoplexes contain the pH sensitive fusogenic lipid DOPE that mediates DNA release into the cytosol (Farhood et al. 1995b) and upon acidic pH by forming inverted hexagonal phases (Cullis et al. 1986). PEI shows a similar ability although with a different mechanism (Behr et al. 1989). For endosomal escape a proto sponge effect is proposed (Boussif et al. 1995), which suggests that PEI becomes more protonated upon pH decrease, triggering an influx of $Cl^-$ ions thereby causing a water influx finally disrupting the endosome (Khalil et al. 2006).

The nuclear envelope is another major barrier upon successful plasmid DNA delivery (Khalil et al. 2006). Basically three routes exist that enable DNA to enter the nucleus, the DNA can pass a nuclear pore, physically associate with chromatin during cell division or the DNA crosses of the nuclear envelope (Wiethoff and Middaugh 2003). The latter was never proved experimentally. The nuclear envelope contains pores with a diameter of 10nm (Melchior and Gerace 1995), which is too small for the delivered DNA to pass. Generally the cell division model is the most accepted suggesting that cell division is an important step enabling the foreign DNA to enter the nucleus (Wiethoff and Middaugh 2003). Concerning lipoplexes the generally accepted model by Xu and Szoka proposes the release of DNA for endosomal escape. Thus, merely naked DNA reaches the nuclear membrane (Xu and Szoka 1996). In contrast, PEI supports the entrance of DNA into the nucleus and even though it is associated with the DNA it does not hamper expression (Zabner et al. 1995).

## 1.3 Lipid Structures

Upon hydration lipids form several structures to protect their hydrophobic moieties. The shape of the formed aggregates is determined by their lipid composition. Lipids with large head group areas have a cone like geometry, self-assemble into micelles and are said to have positive membrane curvature (Fig 6A). Lipids with cylindrical shape, having equal head group to hydrocarbon area form bilayers (Fig 6B), while lipids with small head group areas build inverted hexagonal phases and exhibit negative membrane curvature (Fig 6C) (Hafez and Cullis 2001).

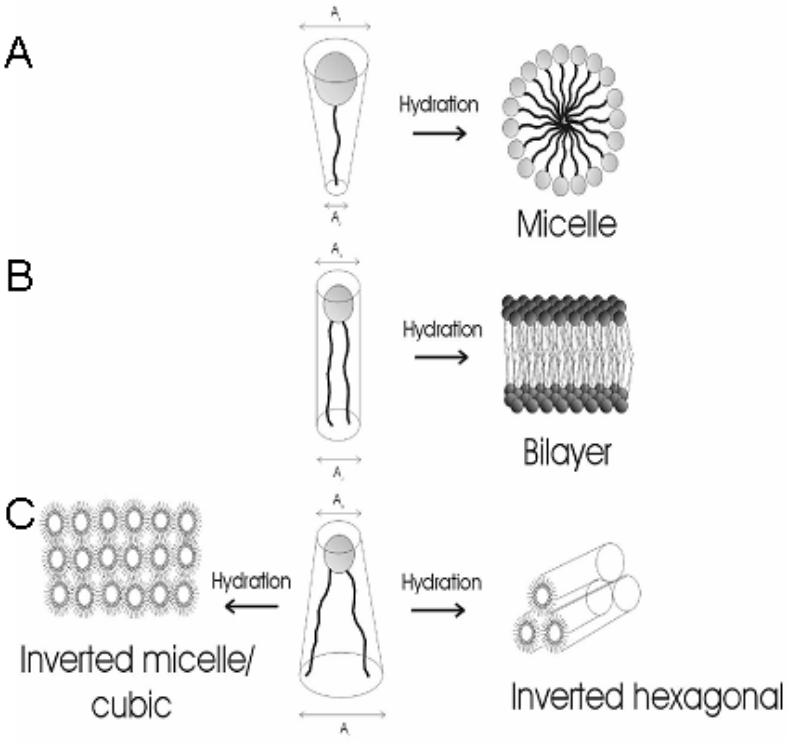


Fig 6 Molecular geometry of lipids and the predicted self-assembly of morphologically distinct structures (Hafez and Cullis 2001).

## 1.4 Liposomes

The membrane model of Singer and Nicholson has to be extended, because of the ability of phospholipids to form other structures than double layers playing an important part in membrane fusion and pore formation.

Lipids minimize the contact between the water and the hydrophobic part by transforming the bilayer into a closed compartment called liposome (Fig 7). Liposomes were discovered about 30 years ago by Bangham (Bangham and Horne 1964; Bangham et al. 1965) and since then they became very versatile tools in biology, biochemistry and medicine. Liposomes are the smallest artificial vesicles of spherical or elliptical shape, consisting of lipid double layers. The hydrophobic effect determines their structure. Amphiphilic molecules arrange so as to minimize the interaction between the water molecules and the hydrophobic part of the lipid. The two-tailed lipids tend to form bilayers due to their cylindrical shape (Fig 8).

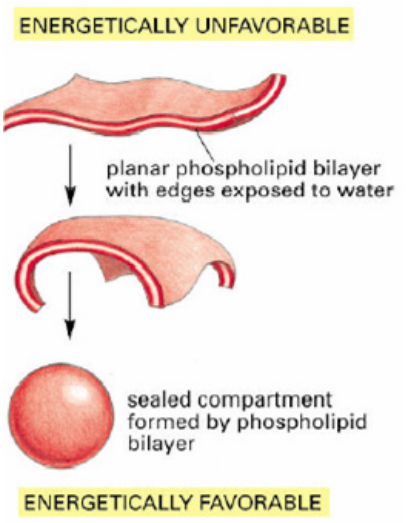


Fig 7 Liposomes formation (Molekularbiologie der Zelle/ 4.Auflage).

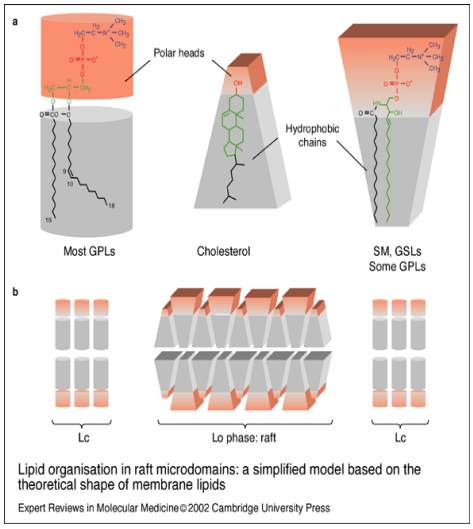


Fig 8 Lipid organization based on the theoretical shape of membrane proteins. Expert Reviews in molecular Medicine © 2002 Cambridge University press.

Liposomes are classified into multilamellar (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV). Upon hydration and agitation of dry phospholipid MLV consist of bilayers are formed. Once these particles have formed, their size can be reduced via sonication or extrusion to receive homogenous unilamellar liposomes (Fig 9). The membrane extrusion is likely to produce liposomes of more uniform size compared to sonication (Altin and Parish 2006). Liposomes offer a broad field of application, ranging from treatment of infectious diseases to gene delivery (Voinea and Simionescu 2002). Liposomes include proteoliposomes containing

fusogenic proteins (Kaneda et al. 1987; Mannino and Gould-Fogerite 1988), pH-sensitive liposomes (Slepushkin et al. 1997; Yatvin et al. 1980), cationic liposomes (Farhood et al. 1995a; Felgner et al. 1987), target sensitive liposomes (Ho et al. 1986) and immunoliposomes (Bendas et al. 1998; Bloemen et al. 1995).

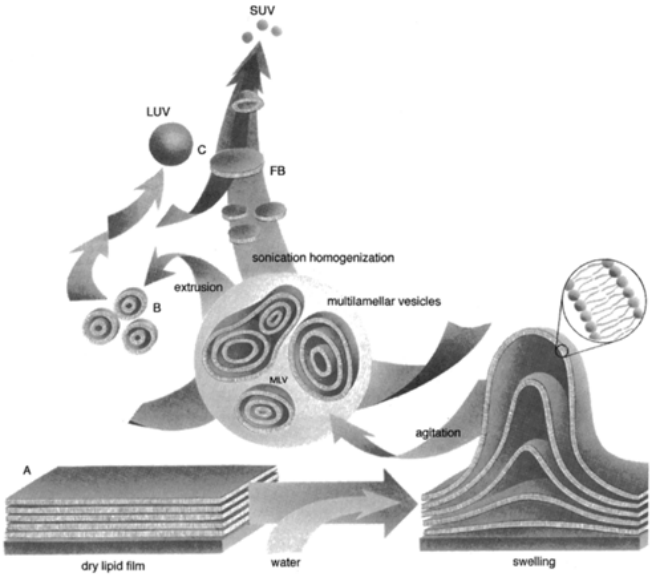


Fig 9 Production of liposome (http://www.avantilipids.com/PreparationOfLiposomes1Big.html).

For gene delivery a good transfection system provides several abilities that include protection of the DNA from degradation, facilitation of the uptake, support of the escape from the endosome and release the plasmid, which has to pass the nuclear membrane before transgene expression can occur (Gao and Huang 1995; Zabner et al. 1995). Nowadays advances in the field of non-viral vectors are made in cationic polymers and cationic lipids (Martin et al. 2005). Generally complexes are generated with a positive charge excess to permit the interaction with the negatively charged cellular membrane (Cullis et al. 1986; Farhood et al. 1995b).

### 1.4.1 Cationic Liposomes

Cationic liposomes consist of cationic lipids containing a hydrophobic domain and a positively charged head group (Karmali et al. 2006). Generally liposomes are not thermodynamically stable, they are stably trapped systems (Lasic 1991; Lasic et al. 1999). Their properties depend on the preparation conditions and physical instabilities provide the ability to aggregate and fuse

(Walde and Ichikawa 2001). The hydrophobic moieties and head groups cause the cationic vesicles to assemble into bilayer vesicles upon dispersion to aqueous solutions (Khalil et al. 2006). Frequently, cationic liposomes are formulated by combining neutral “helper” lipids like DOPE (Fig 10) with a cationic lipid like DOTAP (Fig 11). DOTAP is a widespread transfection reagent consisting of a monocationic head group and two unsaturated hydrocarbon chains (Regelin et al. 2000), while DOPE has a small lipid head group with neutral net charge and bulky tails. DOPE destabilizes lipoplex structures and the endosomal membrane upon exposure to low pH thereby facilitating the membrane fusion. Additionally it is supposed to form inverted hexagonal phases thus releasing the plasmid DNA into the cytoplasm (Rejman et al. 2005; Simberg et al. 2001; Smisterova et al. 2001). The inverted hexagonal conformations of lipoplexes appear to promote more efficient DNA delivery (Koltover 1998; Smisterova et al. 2001) than lamellar their counterparts (Smisterova et al. 2001).

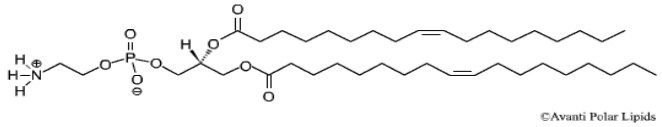


Fig 10 Structure of neutral 1, 2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine (DOPE/ http://www.avantilipids.com/ProductStructures.asp?n=850725).

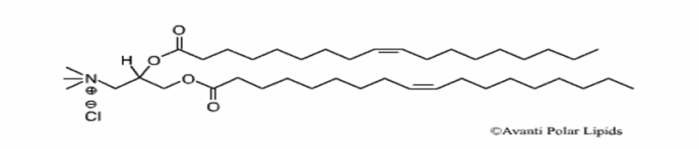


Fig 11 Structure of cationic 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP/ http://www.avantilipids.com/ProductStructures.asp?n=890890)

Perhaps upon understanding the uptake mechanism used by a broad variety of viruses, this will allow us to adapt some of their advantages and features and avoid their disadvantages by designing superior non-viral transfection methods (Zabner et al. 1995) by copying their mechanism of entrance.

## 1.5 Cationic Polymers

Generally cationic polymers include polybrene (Kawai and Nishizawa 1984), dendrimers (Haensler and Szoka 1993; Kukowska-Latallo et al. 1996) and PEI (Boussif et al. 1995) whereas PEI is the most efficient and versatile polymer for non-viral gene delivery (Doyle and Chan 2007). It binds and condenses the DNA via electrostatic interaction (Godbey et al. 2000) and avoids lysosomal degradation (Kichler et al. 2001; Sonawane et al. 2003), by a yet unknown mechanism. Additionally, the mechanism of nuclear translocation and entry of polyplexes is not clear (Doyle and Chan 2007). Despite these transports several possibilities were proposed including the interaction with microtubules (Kulkarni et al. 2005; Suh et al. 2003), but the active uptake is questionable (Lungwitz et al. 2005) and the hypothesis exists suggesting interaction with the nuclear pore complex or the nuclear membrane itself (Godbey et al. 1999b).

PEI is available either highly branched or linear (Fig 12) and at different molecular weights; However, the linear 25 kDa PEI exhibits highest transfection results (Derouazi et al. 2004). In contrast to liposomes, polymers are in its infancy which seems paradox since it was used long before liposomes (Fraley et al. 1980; Henner et al. 1973). Transfection progress was minimal until the introduction of PEI for transfection (Boussif et al. 1995). In presence of sufficient PEI, DNA will be bound and condensed (Dunlap et al. 1997). The extend of condensation depends on the ratio between PEI and DNA (Minagawa et al. 1991). Additionally polymers promote gene delivery from the cytoplasm to the nucleus and allow transgene expression in the nucleus, while complexation with cationic lipids prevents this (Pollard et al. 1998).

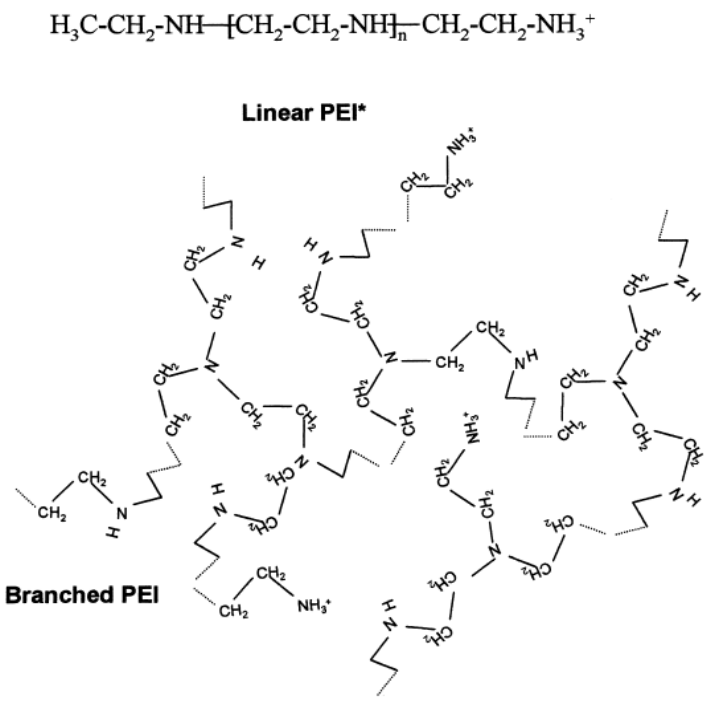


Fig 12 Structure of linear and branched PEI (Godbey et al. 2000).

## 1.6 Transfection

Transfection is the process to introduce foreign DNA into a cell by non-viral methods. Modern mammalian cell culture gains increasing importance since new transfection and clonal selection methods enable the generation of recombinant proteins, stably or transiently expressing cell lines (Meissner et al. 2001).

The pioneer work of Vaheri and Pagano (Vaheri and Pagano 1965) and Graham and van der Eb (Graham and van der Eb 1973) with diethylaminoethyl cellulose (DEAE) and calcium phosphate-mediated transfection was a milestone in the creation of recombinant protein producing eukaryotic cell lines. In the beginning the progress was rather slow until the purification and modification of DNA became easier. Another milestone was the use of chloramphenicol acetyltransferase (CAT) as reporter gene system in 1982 by Gorman et al. together with a detection assay (Gorman et al. 1982) and selection systems which expanded the applications of gene transfer technology. The combination of reporter gene and assay system allowed expression studies of promoter/ enhancer sequences, transcription factors, protein-protein interaction, messenger ribonucleic acid (mRNA) processing, translation and recombinant effects

(Groskreutz and Schenborn 1997). Since the introduction of CAT several other systems appeared such as luciferase, ß-galatosidase, alkaline phosphatase and green fluorescence protein (Groskreutz and Schenborn 1997).

The development of selection systems like neomycin (Southern and Berg 1982) were important due to low frequency of chromosomal integration of the plasmid DNA. Cells which survive the selection pressure expand into clones that can by propagated, analyzed and used for recombinant protein expression. Nowadays a broad variety of transfection methods and reporter gene systems exist to generate and investigate transgenic organisms. These methods can be divided into chemical and physical methods.

### 1.6.1 Chemical Reagents

**DEAE-dextran** was developed as transfection reagent by Vaheri and Pagano (Vaheri and Pagano 1965), which is a cationic polymer and interacts with the negatively charged DNA. An overall positive charge of the DNA/polymer complex allows the interaction with the negatively charged cell membrane followed by uptake via endocytosis. Other synthetic cationic polymers using the similar principle include polybrene (Kawai and Nishizawa 1984), PEI (Boussif et al. 1995) and dendrimers (Haensler and Szoka 1993; Kukowska-Latallo et al. 1996).

**Calcium phosphate** co-precipitation (Graham and van der Eb 1973) is a widely used method characterized by components that are easy by accessible at moderate costs combined with protocols applicable to a broad variety of cell types. The precipitate is taken up by cells via endocytosis and also appears to provide protection against nucleases (Loyter et al. 1982).

**Synthetic liposomes** were introduced by 1980 (Fraley et al. 1980) for DNA delivery and Felgner was the first who used cationic liposomes for transfection (Felgner et al. 1987). Liposomes offer high transfection efficiency and can be employed to a broad range of cells. They successfully deliver all sizes of load ranging from oligonucleotides to yeast artificial chromosomes (Felgner et al. 1987; Lamb and Gearhart 1995; Lee and Jaenisch 1996), RNA (Malone et al. 1989) and proteins (Debs et al. 1990). Additionally this technique can be utilized for transient as well as stable transfections and the application for *in vivo* RNA or DNA transfer into animals or human is possible (Felgner et al. 1995).

### 1.6.2 Physical Methods

In 1982 **electroporation** was first reported for the introduction of genes (Wong and Neumann 1982). This method is based on perturbation of the cell and nuclear membrane by an electrical

pulse, which forms pores that enable the DNA to enter the nucleus (Shigekawa and Dower 1988). Important for this technique is the fine-tuning and optimization of the pulse for each cell type, to find an equilibrium between efficient delivery and conditions that do not kill the cells.

Direct **microinjection** into the cells or the nucleus is laborious but effective to deliver DNA into the cells. This technique originates from embryonic stem cell research where it was used to produce transgenic organisms (Cappechi 1980). This method is not recommendable for large numbers of transfection events.

The **biolistic particle** delivery is an additional physical method, relying on a high velocity delivery of DNA on microprojectiles to target cells (Ye et al. 1990). This approach has been successfully applied *in vitro* and *in vivo* (Klein 1987).

## 1.7 Transient Protein Expression

Nowadays nearly 70% of all recombinant protein pharmaceuticals are produced in mammalian cells (Wurm 2004). Transient transfection is an emerging field, with the ability to speed up the screening process for new therapeutically active proteins and offers increased versatility (Meissner et al. 2001). Thereby, the new product becomes available in advance of stable manufacturing cell lines (Birch and Racher 2006). For *in vitro* experiments and preliminary clinical studies, it is beneficial to use transiently expressed material to gain approval from regulatory authorities (Derouazi et al. 2004), furthermore it is cheaper and less time consuming than generating a stable cell line (Meissner et al. 2001) and the investments for stable cell line creation might be lost if the recombinant protein is not approved for further clinical trails (Derouazi et al. 2004). Large-scale (more than 100L) transient expression systems are being developed to meet demands for preclinical studies (Derouazi et al. 2004). During transient transfection the introduced DNA can be lost by the cell at any time depending on environmental conditions. To date, mammalian cells have been successfully transfected with DNA-containing complexes generated with calcium phosphate, polycations and liposomes (Derouazi et al. 2004).

## 1.8 Stable Protein Expression

Major advances have been made over the past two decades in the use of mammalian cells for recombinant protein production (Barnes and Dickson 2006), to maximize the protein yield using gene expression in mammalian cells. Currently, 18 monoclonal antibodies are approved for therapeutic use. Ten of them are produced in CHO cell lines and 8 in murine lympoid cell lines (NS0 and Sp2/0-Ag 14) (Birch and Racher 2006). Several approaches exist to optimize stable

protein expression interfering at different stages of recombinant protein production (Fig 13), including gene delivery, vector design, plasmid integration (chromosomal environment), mRNA stability and processing, translational, secretory pathways and screening of medium components to optimize recombinant protein production (Barnes and Dickson 2006). The specific productivity and stability of production processes are important features to be addressed (Barnes et al. 2003; Butler 2005; Wurm 2004).

Stable transfection requires the genomic integration of genetic material into a cell to be maintained during reproduction. Only these clones allow replication of the exogenous DNA during cell division. Stable transfection bears the advantage of long-term reproducible production. The major applications include large scale protein production (Wurm 2004), *in vitro* analysis of gene function and regulation analysis of primary cells (Grimm 2004), as well as gene therapy (Glover et al. 2005). Additionally during stable transfection the site of integration is very important for the expression, know as position effect (Wurm 2004), but still the exact plasmid integration mechanism is not fully understood in heterologous transfection. While viral systems integrate the foreign DNA into the host genome via viral integration mechanisms, non-viral delivery systems integrate the DNA by the cell machinery, possibly via DNA repair and recombination enzymes (Haber 1999). While the integration into inactive heterochromatin results in low transgene expression, integration into active euchromatin enables efficient transcription (Wurm 2004). Several strategies have been developed to avoid negative position effects due to random integration including site-specific, homologous and transposon-mediated integration strategies (Murnane et al. 1990). Those require the expression of integration enzymes or additional sequences on the plasmid (Ivics et al. 1997; Keng et al. 2005).

## 1.9 Improvement of Recombinant Protein Expression

### 1.9.1 Transcription Efficiency, Integration and Chromosomal Environment

Developments in mammalian gene expression technology to increase recombinant gene copy number (GCN) or transcriptional activity include the use of highly active promoters (Running Deer and Allison 2004). In general, they are derived from animal viruses or highly expressed mammalian genes, including cytomegalovirus (CMV)(Boshart et al. 1985), rous sarcoma virus (RSV) (Luciw et al. 1983; Norton and Coffin 1987) and simian vacuolating virus 40 (SV40) (Benoist and Chambon 1981) as the most prominent.

Additionally the initiation of transcription depends on the rearrangement and opening of the chromatin structure and the generation of an accessible DNA conformation by chromatin

remodeling factors so the transcription factors (TF) and RNA polymerase II (RNAPII) can bind at the promoter of the gene to be transcribed (Darzacq et al. 2005). Besides the core promoter, regulatory elements up- and downstream of the promoter affect the association of the transcription, including activators, enhancers, repressors and which silencers play an important role (Fiering et al. 2000; Lee and Young 2000; Verrijzer et al. 1995).

In general, transcription starts with the association and binding of core promoter elements. All eukaryotic expression cassettes contain a TATA box (consensus TATAWAWR, W is A or T, while R is a purine)(Faiger et al. 2006) and/or an insulator element (consensus $YYA_{+1}NWYY$, Y is a pyrimidine, W is A or T and N any nucleotide)(Verrijzer et al. 1995). The TATA box is situated 28 to 33bp upstream of the transcription initiation site and associates to the TATA binding protein (TBP)(Faiger et al. 2006). The pre-initiation complex is formed by association of TFs and RNAP II (Muller and Tora 2004).

Another limiting step is RNA elongation which is a property of several promoters (Darzacq et al. 2005), as well as the interaction between RNAP II and the initially transcribed sequences or templates (Carninci et al. 2006; Pal et al. 2001).

A key factor for high transcription rates is the site of integration, know as position effect (Barnes et al. 2003). In general, the integration of transgenes is a random process, but to overcome negative position effect several possibilities exist including insulators, boundary elements, scaffold attachment regions and matrix attachment regions (Girod et al. 2005), ubiquitous chromatin opening elements (Antoniou et al. 2003) and conserved antirepressor (Kwaks et al. 2003).

Either these approaches physically prevent the influence of transgene transcription or alter the overall epigenetic environment of the surrounding chromosomal DNA. To minimize the epigenetic effect of surrounding chromatin on integrated transgenes, various cis-acting DNA elements have been incorporated into vectors (Kwaks and Otte 2006). For vector engineering, these elements should be small, confer universal function and depend on the copy number. Elements such as locus control regions (Dean 2006; Li et al. 2002) and insulators (Emery et al. 2000) have been known for many years, but a wide range of other elements are now available. Antirepressor or STAR (stabilizing and antirepressor) elements, which flank transgenes in mammalian expression vectors, prevent spreading of methylation and histone deacetylation patterns from the surrounding genome into the recombinant DNA (Kwaks et al. 2003). Scaffold/ matrix-associated regions (S/MARs), which bind to the nuclear matrix, are thought to affect the arrangement of chromatin into loops which contain genes with coordinated regulation (Hancock 2000; Heng et al. 2004). The MAR element is thought to insulate the genes of the loop from the surrounding chromatin, thus providing independent transcription activity. Integration of these

elements into expression vectors normally results in higher transcription of the gene of interest, because the insulation effect reduces a negative position effect due to their ability to inflict topological changes of chromatin (Banan et al. 1997; Bode et al. 2003; Phi-Van and Stratling 1996; Van Leeuwen et al. 2001). Reports about the effectiveness of these methods demonstrate controversial results and their precise mechanism is largely unknown (Kwaks and Otte 2006).

Another approach involves ubiquitous chromatin opening elements (UCOEs). They are derived from the promoters of housekeeping genes, which are normally transcriptionally active owing to a significant extent of histone acetylating. Recently, reports have demonstrated that the integration of UCOEs in expression vectors increased production and stability of transgene expression in CHO cells (Antoniou et al. 2003; Williams et al. 2005).

Alternatively to the integration of regulatory elements into expression vectors, the general epigenetic environment of the chromatin surrounding the sites of transgene insertion can be altered by components influencing the acetylation of histones. Acetylation is generally associated with enhanced transcription. A positive effect arises from the balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities (Barnes and Dickson 2006). Other researchers prevent deacetylation using HDAC inhibitors (e.g. sodium butyrate, NaBu) to inhibit silencing thereby increasing the protein expression. Unfortunately these agents do not act specifically and, in addition to its role as an HDAC inhibitor, NaBu has been reported to have a number of adverse general cytotoxic effects on mammalian cells (Davie 2003; Kim and Lee 2002a).

Finally to increase the recombinant protein production of a cell line the GCN can be increased by systems including the methotrexate (MTX) system which will be explained later in detail.

### 1.9.2 mRNA Procession and Stability

Strong consecutive promoters lead to high levels of primary transcript (pre-mRNA), and a larger population of processed transcripts (mRNA) and ensure higher levels of template for translation. Another important issue concerns RNA stability and refers to the incorporation of introns into the expression vectors. The DNA is often isolated as cDNA without introns, whereas it is known that efficient cytoplasmic transport and translation of mRNA depends on splicing (Le Hir et al. 2004), thus most expression vectors nowadays contain introns (Wurm 2004). Splicing can promote elongation close to the promoter thereby supporting the polymerase binding and transcription of a gene (Bentley 2005; Orphanides and Reinberg 2002). One study demonstrated an average 6.3 fold expression increase (Cullen 2003).As an example a specific RNA processing event has been shown to have a detrimental influence on productivity based on alternative splicing of the

X-box binding protein transcript (XBP-1). A single XBP-1 transcript (containing a 26 nucleotide intron) can be translated to generate either a 30 kDa protein (XBP-1(U)) or a 54 kDa protein (XBP-1(S) via alternative splicing to remove the intron and change the reading frame. XBP-1(S) leads to the expansion of the secretory pathway and enables higher protein expression. The alternatively generated XBP-1(U) is responsible for the activation of apoptosis (Brewer and Hendershot 2005; Shaffer et al. 2004; Sriburi et al. 2004; Yoshida et al. 2006). Another important point to consider is the addition of a poly-A tail. Kim and colleagues (Kim et al. 2003) quadruplicates the expression by changing the sequence downstream of the polyA-sequence. Upon disruption of cleavage and polyadenylation processes by cis- or trans-acting mutations, the transcripts accumulate in the nucleus, indicating that polyadenylation is an important point for the mRNA export (Lei and Silver 2002).

AU-rich elements (ARE) cause a faster degradation of the poly(A)-tail, maybe through mediation of the exosome, thereby influencing translational efficiency (Mignone et al. 2002; Mukherjee et al. 2002; Wilusz and Wilusz 2004). Besides the ARE, other destabilizing elements have been found in the 5' and 3' untranslated regions (UTR) of transcripts (Day and Tuite 1998). In contrast, stabilizing elements have been found on mRNAs encoding proteins required at constantly high levels, including histones, β-globulin and cytokines (Jiang et al. 2006a).

Deeper understanding of the influence of the 5' and 3' UTR of the transcripts regarding mRNA, targeting, stability and translatability (Mazumder et al. 2003; Meijer and Thomas 2002; Mignone et al. 2002; Ringner and Krogh 2005), will result in further possibilities to improve expression plasmids (van der Velden et al. 2001).

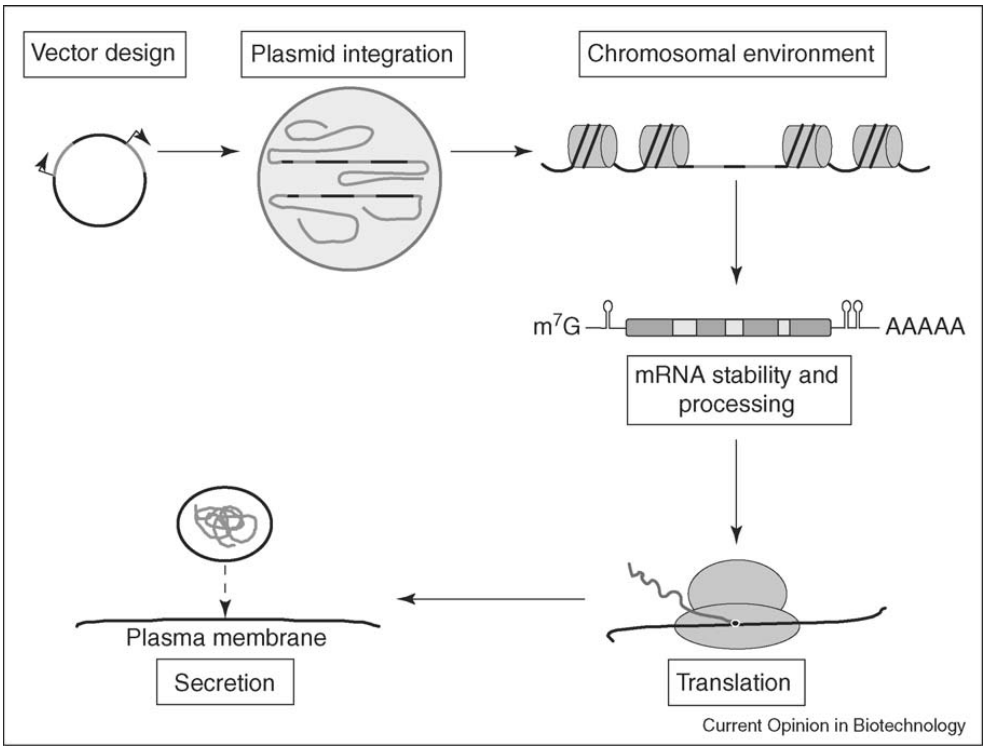


Fig 13 Typical recombinant protein production pathway. The protein production pathway is highly complex with multiple steps that influence the efficiency and stability of recombinant protein production. The pathway can be divided into three distinct phases: vector design, plasmid integration and chromosomal environment; mRNA stability and processing; and translation and secretory events (Barnes and Dickson 2006).

## 1.9.3 Translation, Protein Processing and Secretion

Mammalian cells provide correct folding, assembly, glycosylation, full functionality and safety of the recombinant products, therefore it is often mandatory to generate therapeutic proteins in mammalian cells (Stern et al. 2007).

The initiation of translation is one of the key determining factors for efficient mRNA translation. The majority of control elements for initiation is located within the UTR of the transcript, but also other features along the mRNA may influence translation efficiency (Mignone et al. 2002).

Most commonly mRNAs are translated by a cap-dependent translation mechanism. Thereby the eukaryotic initiation factor 4F associates with cap, followed by binding of the 43S pre-initiation complex, consisting of 40S ribosomal subunit, eIF2 and Met tRNA. Thus the complex scans the 5’ UTR for the start codon and upon binding to it the 60S ribosomal subunit joins and translation starts (Preiss and M 2003).

The start codon surrounding sequence is of huge importance for translation, since it confers binding site for ribosomal subunits. It is known as **Kozak sequence**, occurs on eukaryotic mRNA and has the consensus sequence GCCRCCaugG. The purine R, usually A, at position -3 and the G at +4 seem to be the most crucial bases (Kozak 1987; Kozak 1999), because mutations within these positions cause severe disease (Kozak 2002).

Furthermore, secondary structures close to the cap may block or hamper the accessibility of eIF4F and is therefore an inhibitory feature, resulting in lower translation levels if hairpin structures close to 5' end appear (Kozak 2005; Roberts et al. 1997). Additionally, the presence of AUGs upstream of the start site can interfere with translation in a negative way (Meijer and Thomas 2002).

In eukaryotic cells the nascent polypeptides first enter the ER before its transport into the Golgi complex. A signal peptide attached to the N-terminal end of the nascent polypeptide directs the molecule into the ER lumen and after targeting the protein to or into the ER, a peptidase cleaves it off (Stern et al. 2007). The size of these signals varies between 15 to 30 amino acids, but can be up to 50 (Johnson and van Waes 1999; Martoglio and Dobberstein 1998). Beside variations in the amino acid sequence signal sequences share some common features. Through increased basicity and hydrophobicity in different parts of the interleukins (IL)-2 leader, Zhang et al (Zhang et al. 2005) observed a 3.5 fold expression increase. Since the different signal peptides show such sequence diversity, it is expected that they also show different affinities to the signal recognition particle (SRP), which dictates the efficiency with which a nascent polypeptide enters the secretory pathway (Zhang et al. 2005; Zheng and Gierasch 1996). Based on this observations a novel approach is to use specific signal peptides from *Gaussia* luciferase mediating high level protein production and to increase protein expression (Knappskog et al. 2007).

Furthermore, rarely used codons might be a rate limiting step causing a translation pause, due to the low concentration of tRNA corresponding to a particular codon (Stern B 2007). Basically the idea is to increase protein yield by using codons that frequently occur in the expression organism (Hu et al. 2006; Narum et al. 2001). The potential to increase protein synthesis of a recombinant gene utilizing codon optimization is a common tool (Gustafsson et al. 2004), although the beneficial effect on mRNA stability and transport into the cytoplasm has not been investigated properly (Narum et al. 2001). High mRNA concentration also might be connected to codon optimization as demonstrated by GenArt, since higher GC content and shorter turnover prolong m-RNA half-life (Reisinger et al. submitted).

Further, recombinant antibody production is limited at a post-transcriptional level. *In vitro* studies have shown that the rate of antibody folding in the ER is relatively slow (Goto and Hamaguchi

1981; Lilie et al. 1994). For antibody expression, equimolar expression, folding and assembly of both heavy (HC) and light chains (LC) are necessary and at low levels of antibody mRNA, antibody expression is limited by the amount of transcript and mRNA expression are positively correlated (Barnes et al. 2004; Dorai et al. 2006). In case of high levels of mRNA the expression rate can be restricted by other factors like saturation of translational and/or secretory events (Barnes et al. 2004). The accumulation of unfolded proteins in the endoplasmatic reticulum (ER), results in an acute stress state of the cell which triggers the unfolded protein response (UPR) resulting in the competition between apoptosis for cell survival (Forman et al. 2003; Paschen and Frandsen 2001).

Several studies using clonally-derived mammalian cell lines or hybridomas demonstrate that at higher production rates, no correlation between mAb and the corresponding cellular availability of HC or LC mRNA was observed (Barnes et al. 2004; Flickinger et al. 1992; Kim et al. 1998a), although some studies observed a correlation of antibody expression with LC mRNA content (Borth et al. 1999; Merten et al. 1994; Strutzenberger et al. 1999). Intensively studied are synergistic interactions of the immunoglobulin binding protein (BiP) chaperone GRP78 and protein disulfide isomerase (PDI) catalyzing the formation of intra- and intermolecular disulfide bonds (Dinnis and James 2005). Unfortunately, genetic up regulation of these chaperones in mammalian cells to improve expression has not been particularly successful (Dinnis and James 2005). In general, it is difficult to draw firm conclusions from these conflicting reports, despite the fact that production of mammalian recombinant proteins in yeast or insect cells can be enhanced by the co-expression of mammalian folding chaperones, foldases and their cofactors.

The production of recombinant proteins is highly complex, especially if the proteins are multimeric (e.g. antibodies), thus defining the influencing factors for recombinant protein production is difficult (Barnes and Dickson 2006). Smales et al. (Smales et al. 2004) found that expression of certain endoplasmic chaperones BiP and PDI correlated with high levels of specific productivity. Proteomic techniques and clonal analysis can also be applied to dissect the sites and mechanisms by which environmental regulators modulate production. Recently a number of reports outlined the positive effect of low culture temperatures or biphasic temperature processes on volumetric production (Bollati-Fogolin et al. 2005; Fogolin et al. 2004; Fox et al. 2004; Rodriguez et al. 2005; Yoon et al. 2004).

Another major bottleneck following the proper assembly of antibody chains is the ER to cis-Golgi transport which might limit the rate of secretion (Hooker et al. 1999). It had been shown recently that active X-box binding protein (XBP1) induced synthesis of ER membrane phospholipids and could increased the surface area and volume of rough ER to direct secretion of expressed proteins (Shaffer et al. 2004).

In summary, it is clearly possible to overload the ‘‘capacity’’ of engineered mammalian cells to synthesize and secrete recombinant proteins (Schroder and Friedl 1997), and different proteins will impose different demands on the cell. Protein folding reactions can be considered to constitute a sequence of potential bottlenecks where fractional alleviation of one bottleneck may improve $q_P$. However, even with a relatively rapid folding pathway, the next major bottleneck involving multiple protein components, ER to cis-Golgi transport (Sollner and Rothman 1996), may subsequently limit $q_P$ (Hooker et al. 1999). Despite identifying and isolating the machinery mediating the transport between intracellular compartments, still we are far from understanding the whole mechanism in detail. Manipulation of cellular secretion by altering the intracellular content of chaperone/ foldase proteins leave some questions and seem to be product and cell-line specific. For example the over expression of GRP78 (BiP) in CHO cells can selectively retain proteins that do require GRP78 for folding, including Factor VIII, but has no effect on the secretion rate of proteins that do not require GRP78 for folding (Dorner and Kaufman 1994). Whereas, the expression of a heterologous BiP protein in insect cells, raised the secretion of recombinant IgG by 90% (Hsu and Betenbaugh 1997).

### 1.9.4 Host Cell Engineering and Medium Optimization

Upon genetic engineering, the growth, lifespan and productivity of host cell lines can be improved (Wurm 2004). Several approaches have been tested (Arden et al. 2004; Vives et al. 2003) including the integration of, proto-onkogenes, cell cycle control genes, growth factor genes and anti-apoptotic genes, most commonly by expression of basal cell lymphoma-2 (bcl-2) or bcl-xL (Fussenegger and Bailey 1998; Itoh et al. 1995; Mastrangelo et al. 2000; Meents et al. 2002), but also caspase inhibition (Kim and Lee 2002b; Sauerwald et al. 2002), and expression of the molecular chaperone heat shock proteins (HSP) 70 (Lasunskaia et al. 2003), to cell lines to generate superior production hosts (Arden et al. 2004). Although most studies report an improvement in cellular ‘‘robustness,’’ measured as resistance to an imposed stress, just a few report an actual increase in product yield.

Metabolic engineering has been employed to increase viable cell concentration and volumetric production indirectly by inhibition of the accumulation of toxic byproducts of metabolism such as lactate and ammonium (Bell et al. 1995; Chen et al. 2001; Park et al. 2000) or by an engineered improvement in the efficiency of primary metabolic pathways. Whereas the latter may be achieved by nutrient limitation (Cruz et al. 2000; Haggstrom et al. 1996), a recent genetic engineering approach based on pyruvate carboxylase expression to increase glucose flux into the tricarboxylic acid cycle has proven successful. Engineered cells showed reduced glucose

consumption, less accumulation of lactate and ammonium as well as a prolonged production phase (Elias et al. 2003; Irani et al. 2002; Irani et al. 1999).

Volumetric product yield is basically a function of cell specific production rate and the integral of viable cell concentration. The ideal combination for typical fed-batch production processes is a rapid accumulation of cellular biomass and maintenance at high viable cell concentration for as long as possible (Bibila and Robinson 1995; Sauer et al. 2000). Mainly this has been achieved by systematic optimization of media formulation and rational design of feeding regimes.

The inverse relationship between cell proliferation rate and cell specific recombinant protein production has been reported in a variety of circumstances including the use of specific metabolites or nutrient limitations (Altamirano et al. 2001; Carvalhal et al. 2003), the reduction of culture pH (Miller et al. 1988; Muthing et al. 2003), reduced culture temperature (Fox et al. 2004; Kaufmann et al. 1999), or hyperosmotic pressure (Kim and Lee 2002b; Lee and Lee 2000). To increase volumetric concentration of recombinant product a ‘‘biphasic’’ cell culture process strategy was proposed (Fussenegger and Bailey 1998). During the first phase, cell proliferation must be supported to accumulate viable cell concentration, whereas during the second phase cell division is arrested to enable high cell volumetric titers (Dinnis and James 2005).

A crucial point is the medium, since it provides the growth environment for the cells and is responsible for the maximal reachable cell density which correlates with the expression of recombinant protein. According to Wurm, several media formulations are necessary for a single production process, designed specifically for the manufacturing phase (Wurm 2004). Often these positive effects are achieved by using fetal calf serum (FCS), which is no option because due to regulatory reasons the expression of recombinant therapeutic proteins in chemically defined conditions without animal derived components is mandatory (Muller et al. 2007). In general, medium design is of high importance and has to be performed, for each cell line individually (Wurm 2004). The main supplements of basal medium include vitamins, trace elements, glucose and nucleotides (Hammond et al. 1984).

### 1.9.5 Selection, Gene Amplification and Cell Line Screening

Selection systems are co-transfected and confer some advantage for cell growth under selection conditions. Several selection systems exist, including resistance to antibiotics such as neomycin phosphotransferase, conferring resistance to G418, DHFR, or GS (Southern and Berg 1982; Wurm 2004). The basic antibody gene expression systems used including GS, DHFR selection/amplification systems have not been changed significantly since their introduction,

indicating specific productivity of 20–50 pg/cell/day (Bebbington et al. 1992; Bianchi and McGrew 2003; Kaufman and Sharp 1982).

Selection with DHFR works merely in DHFR deficient cells like CHO-DUXB11 (Urlaub and Chasin 1980) or CHO-DG44 (Urlaub et al. 1986), while the GS system works with a broad variety of cells.

In case of the DHFR system, the recombinant protein expression can be increased by exposing the cells to MTX, which blocks DHFR activity (Gandor et al. 1995; Pallavicini et al. 1990). After the amplification surviving cells frequently contain several hundred gene copies (Wurm et al. 1986), of the plasmid integrated into their chromosome (Pallavicini et al. 1990). Cells with low transgene copy number are more stable in terms of production, because it is assumed that cell clones containing multiple copies might be more susceptible to copy number loss or tandem-repeat-induced gene silencing caused by methylation (Derouazi et al. 2006). Whereas, the integration of several copies in tandem repeats raises the probability that the DNA is located away from repressive elements in the genome thus improving transcription (Jiang et al. 2006b).

Upon amplification of cells with MTX they show 10- to 20-fold expression improvements compared to unamplified cells (Wirth et al. 1988). A recent investigation of MTX amplified CHO cells have shown that amplification not merely caused an increase in the recombinant gene copy number, but also enhanced transcriptional efficiency per copy until a maximum of ~250 copies. At higher copy numbers the specific productivity per copy decreased (Guarna et al. 1995).

An alternative provides the GS system. GS catalyzes the production of glutamine from glutamate and ammonium, the latter being undesirable waste product of cells. Thus, this system provides three main advantages, the reduction of ammonium, providing the essential and unstable amino acid glutamine to the cells and it can be used with a broad variety of cell lines. Whereas the DHFR-system is limited to cell lines where the DHFR gene is mutated or deleted. The transgene complex containing the GS gene and the gene of interest is amplified, by using the specific and irreversible inhibitor Methionine sulphoimine (MSX) for this system (Bebbington et al. 1992).

Due to the fact that protein expression level varies up to two orders of magnitude between cell clones, including those derived from amplification (Barnes et al. 2004; Jones et al. 2003), the identification of high producers is indispensable and numerous cell lines have to be screened. Additionally during screening the cell growth must be taken into account, since producing cell lines often show reduced growth rates, but it is the combination of fast growth and high $q_P$ that makes a good production cell line (Wurm 2004).

## 1.10 Therapeutic Antibody Expression

During the last decades the technological advances made antibodies to one of the most prominent and growing class of biotherapeutics (Chadd and Chamow 2001). The number of monoclonal antibodies currently developed is higher than any other therapeutic category (Davis et al. 1999).

In recombinant expression technologies the major challenges are the correct processing of the product and to meet manufacturing regulatories (Chadd and Chamow 2001).

Köhler and Milstein (Kohler and Milstein 1975) established methods to generate mouse clonal antibodies in 1975. The use of murine antibodies in human therapy is limited by their immunogenicity (Kipriyanov and Little 1999; Richards et al. 1999). In 1984, chimeric antibodies consisting of 66% human and 34% mouse sequences were generated for the first time (Morrison et al. 1984). Between 1988 and 1991 humanization attempts of murine antibodies reduced the mouse portion to 5-10% (Padlan 1991; Riechmann et al. 1988). Alternatively fully human antibodies can be produced by phage display (Huls et al. 1999) and afterwards the variable regions are grafted to constant regions in cell culture. During the 1990s transgenic mice emerged which were genetically engineered to contain a human antibody repertoire (Green et al. 1994; Mendez et al. 1997; Taylor et al. 1994). To generate monoclonal antibodies B-cells are isolated from the spleen after antigen challenge. Thus, the B-cells are fused with myeloma cells to provide indefinite growth in culture. The hybridomas grow rapidly and produce the desired antibodies.

For antibody production several systems exist including yeast, mammalian cells or transgenic animals and plants, they show the best capacity for full-length recombinant human antibody expression, while bacteria seem favorable for the expression of antibody fragments, as bacteria lack the machinery for post-translational modifications (Chadd and Chamow 2001). In contrast to bacteria there is discussion about the ability of yeast cells to express functional antibodies, since their glycosylation pattern is differs in its complexity in mammalian systems (Cupit et al. 1999; Freyre et al. 2000). Glycosylation is crucial, for recombinant antibodies to confer full biological activities including antibody-dependent cell cytotoxicity and complement mediated lysis *in vivo*. Recently, human-like glycosylation of recombinant glycoprotein produced by genetically engineered tobacco, moss, and yeast has been accomplished (Bakker et al. 2001; Decker and Reski 2004; Hamilton et al. 2003), although as production vehicles, these systems are still very much in their infancy. Concerning mammalian production system their potential increases due to improvement of recombinant expression vectors, identification of expression hotspots, medium

optimization and enhancement of biomass accumulation (Chadd and Chamow 2001). An alternative method still under development for large-scale production of antibody is the generation of transgenic organism. Besides regulatory difficulties, these approaches have to cope with social rejection and ethic concerns.

### 1.10.1 Anti-HIV-1 Monoclonal Antibody 2F5

The human monoclonal antibody (mAb) 2F5 IgG1 isotype was established as hybridoma cell line by fusion of peripheral blood lymphocytes with CB-F7 myeloma cells (Buchacher et al. 1994; Grunow et al. 1988). 2F5 neutralizes the human immunodeficiency virus (HIV)-1 (Purtscher et al. 1994) and recognizes and binds the highly conserved sequence ELDKWA on the virus envelope gp41 (Muster et al. 1994). Furthermore this antibody efficiently inhibits infection of laboratory strains and primary HIV-1 isolates (Li et al. 1998; Mascola et al. 1997). Unfortunately the primary structure of this antibody is highly complex especially concerning its long complementary determining region 3 (CDR3) loop (Kunert et al. 1998) which might be responsible for low secretion rate (Kunert et al. 2000) even in stable cell lines notably under serum-free conditions (Reisinger et al. submitted). In general, the low $q_P$ of the antibody 2F5 might be referred to the fact that, each antibody has a characteristic expression efficiency, affected by a combination of several factors including the primary sequence of light- and heavy chain (Bentley et al. 1998).

# 2 Constructs

In case of our experiments we used several versions of expression plasmids, differing in their backbone, their promoters, their leaders and their cDNA (Figure 14) to express the mAb 2F5. The human monoclonal antibody (mAb) 2F5 IgG1 isotype neutralizes the human immunodeficiency virus (HIV)-1 (Purtscher et al. 1994) and recognizes and binds the highly conserved sequence ELDKWA on the virus envelope gp41 (Muster et al. 1994). If the plasmids contained no dhfr cassette, an additional plasmid was co-transfected, containing the mouse dhfr cistron, to allow amplification with methotrexate (MTX)(Alt et al. 1978).

For the first plasmid generation, pRC/RSV (Invitrogen) was used as a backbone for LC and HC coding region under control of a RSV promoter in clones 2F5/MCB, 2F5/WCB and 2F5/NF. The cDNA of LC and HC was amplified from the original hybridoma, the signal peptides were from the highly germline related antibody 3D6 (Kohl et al. 1991).

The next plasmid generation, used for 2F5/CO, the vector pRoM410 was used as expression backbone for expression of LC behind the CMV promoter. The genes for HC and dihydrofolate-reductase (dhfr) on pRoM423 were controlled by two independent SV40 promoters/terminators (Fig 14).

In another approach the first generation plasmid backbones as well as LC and HC cDNA (pRC/RSV/GPSP) were combined with the *Gaussia* luciferase signal peptide (Fig 14), thereby replacing the highly antibody 3D6 signal peptides (Kohl et al. 1991) to generate 2F5/LG (Knappskog et al. 2007).

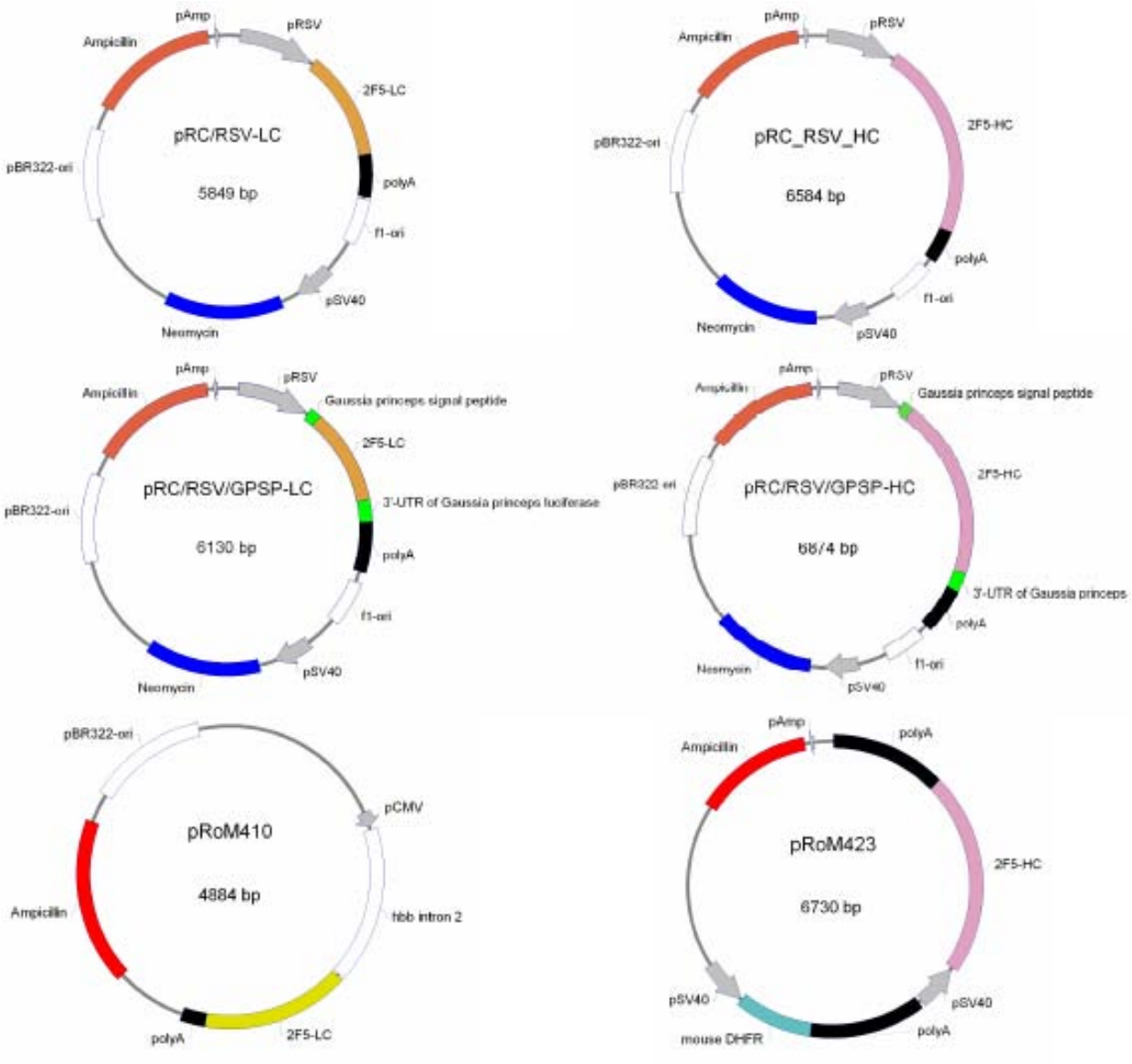


Fig 14: Shows the detailed maps of the plasmids used for the generation of stable cell lines, carrying either the HC or LC of mAb 2F5.

# 3 Conclusion of Experiments

At the beginning of this study, our lab had no experience with self-developed transfection systems. We merely used commercially available systems which bear the disadvantage of high costs and limited knowledge about the compounds for the customer which is important when developing a cell-line. Thus it was important for us to develop our own chemically defined transfection systems. Therefore we employed two different strategies, one dealing with liposomes and the other dealing with cationic polymers. During our work we investigated the usability of these systems for the transient expression of enhanced green fluorescent protein (EGFP), for the transient expression of the mAb 2F5 and the generation of stable cell lines expressing 2F5.

## 3.1 Transient EGFP Experiments

After defining the lipid composition we investigated DOTAP/DOPE (DD) and CHEMS/DOPE (CD) liposomes via SAXS analysis. During transfection, the formation of a hexagonal phase is associated with high transfection rates. DD formed cubic phases which are a transition state between lamellar and inverse hexagonal phase (Seddon 1990), but no hexagonal phases were observed. In combination with DNA the CHEMS/DOPE neither formed cubic phases nor lamellar stacks. This might be ascribed to the uptake mechanism of DD and CD. CD are a pH sensitive liposome, the general assumption is that they destabilize endosomal membranes at acidic pH, but the detailed mechanism is yet not well defined (Straubinger 1993), while cationic DD interact with the negatively charged cellular membrane and initiate the membrane transfer process (Scarzello et al. 2005).

In parallel the fate of both liposome/DNA complexes within the cell was observed. Therefore the DNA and the liposomes were stained and analyzed via confocal microscopy revealing that the DNA enters the nucleus while the liposomes remain in the endosomes (Reisinger et al. 2007). These observations confirm that the degradation of the liposome/DNA complex and the nuclear uptake of the DNA is the crucial step for an optimal transcription (Xu and Szoka 1996). Due to poor transfection results, the approach using the CD was abandoned although they worked in other studies (Slepushkin et al. 1997).

The increase of liposome concentration resulted in a reduced viability of the cells. Positively charged lipoplexes enable efficient binding to the cell surface, but an overload of positive charge enhances the toxicity of the complex (Templeton 1997), because the excess of positive charges surrounding the cell destabilizes the membrane and leads to its disruption.

As for PEI, a cationic polymer was used for DNA transfer. PEI condenses DNA and serves as a non viral gene delivery system (van der Aa et al. 2007). First, we addressed to several parameters to develop and improved the PEI transfection protocol. Thus, the toxicity of the PEI complexes and the ideal ratio between DNA and PEI were determined since the ratio between PEI nitrogen and DNA phosphates is important for transfection efficiency and cell toxicity (Reisinger et al. submitted) and has to be adapted to each medium (Muller et al. 2007). In our experiments the cultivation medium was Dulbecco's modified Eagle's medium (DMEM/Biochrom KG, Berlin, Germany) containing 4mM L-glutamine (Life Technologies, Grand Island, NY, USA), 0.25% Soya-peptone/UF, 0.1% Pluronic-F68, in-house developed protein-free supplement (Polymun Scientific Immunbiologische Forschung GmbH, Vienna, Austria) and HT (Hypoxanthine, Thymidine: Sigma-Aldrich Handels GmbH, Vienna, Austria). A DNA to PEI ratio of 1:36 (w/w) resulted in a maximum transfection rate in combination with 30 minutes incubation at 22°C for the complex formation.

The comparison of our transfection techniques, with Lipofectin, DMRIE-C, Lipofectamine and Nucleofector during transient transfection of EGFP, showed that DD and PEI can compete with the commercially available systems.

## 3.2 Transient 2F5 Expression

During transient transfection using LC and HC of mAb 2F5 cDNA, both systems showed poor transfection rates below 1% and their volumetric antibody titer was below 50ng $ml^{-1}$. Therefore we decided to move on to a stable transfection. The mAb 2F5 has a complicated primary structure especially concerning the long CDR3 loop (Kunert et al. 1998) which is referred to be responsible for low secretion rate (Kunert et al. 2000) even in stable cell lines under serum-free conditions (Reisinger et al. submitted). Despite those poor transient transfection results, viability was kept high, providing a better starting position for the generation of stable recombinant cell lines.

## 3.3 Stable 2F5 Expression

When comparing the transfection abilities of DD and PEI with EGFP, PEI proved to be the better method for transient transfection but both systems performed equal during stable transfection. This might be referred to the transfection mechanism, since different endocytic pathways exist and it should be taken into account that each pathway has its own characteristics (Khalil et al.

2006). The cellular uptake of lipoplexes is performed mainly by endocytosis via clathrin coated pits, which occur after non-specific charge mediated interaction with the cellular surface (Zuhorn and Hoekstra 2002). After internalization, the complex travels into an endosomal compartment and escapes before reaching the lysosome. However, the exact mechanism of DNA transfer across the nuclear membrane is still unsolved (Escriou et al. 2001).

It is postulated that PEI can either be taken up via the clathrin mediated or the caveolae pathway. During the clathrin-dependent pathway the polyplexes are transported in early and late endosomes that ultimately fuse with lysosomes and the trans-Golgi network (Takei and Haucke 2001). PEI is taken up either by the clathrin mediated or the caveolae pathway, thus bypassing degradation in endosomes. In contrast to DD, PEI seems to support the entrance of DNA into the nucleus actively and even though it is associated with the DNA it does not hamper expression (Zabner et al. 1995).

## 3.4 Generation and Comparison of Stable Cell Lines

Five recombinant cell lines, were generated and investigated, each expressing the HIV neutralizing human monoclonal antibody 2F5 (Kunert et al. 1998).

The results of genetic characterization indicate that, despite the usage of similar promoters, the cell lines differed in their transcription rate. The GCN did not reflect the mRNA content determined by Northern-Blot. Responsible for this effect might be a position effect of the target gene on the chromosome (Jiang et al. 2006b; Wurm 2004). Besides several studies which confirmed that the GCN is proportional to the level of MTX amplification (Pendse et al. 1992), the GCN is not sufficient for high $q_P$ as described previously (Lattenmayer et al. 2007).

The cell lines generated with codon-optimized cDNA showed increased mRNA amounts. This increase might result from prolonged mRNA half-life related to higher GC-content and shorter turnover (Stern et al. 2007). Thus, the detected intracellular content of HC and LC were high which is referred to the optimized cDNA, but the bottleneck seems to be the secretion of the antibodies, because the volumetric titer is similar to the other protein-free cultivated cell lines.

Furthermore the signal peptide of the *Gaussia princeps* luciferase was used with LC and HC cDNA on plasmids. It was shown that they mediate increased synthesis and more efficient secretion of a model protein (Knappskog et al. 2007). These plasmids were used to generate a cell line, which showed low HC mRNA amounts but similar $q_P$ compared to the other generated cell lines.

Flow cytometry analysis confirmed that the varying protein secretion rates are often not reflected by the intracellular content of the antibody as already described by Kim et al (Kim et al. 1998b).

Others working in that field propose increase volumetric concentration of recombinant product via a ‘‘biphasic’’ cell culture process strategy (Fussenegger and Bailey 1998), and consequently suggested that cell division should be arrested to obtain a high $q_P$ since cell division drains resources required for recombinant protein production (Dinnis and James 2005). During batch cultivation under nutrient starvation, elevated levels of intracellular HC and LC in clones cultivated without FCS were observed.

Several *in vitro* studies concluded that the folding and assembly of LC and HC to obtain a functional antibody is the rate-limiting step in recombinant mAb production (Barnes and Dickson 2006; Dinnis and James 2005). Furthermore, no correlation between intracellular LC and HC mRNA content and antibody expression seems to exist (Barnes et al. 2004; Flickinger et al. 1992; Kim et al. 1998a), although the strong correlation between recombinant mAb production and LC mRNA had been postulated in several studies (Borth et al. 1999; Strutzenberger et al. 1999).

Another major bottleneck following the proper assembly of antibody chains is the ER to cis-Golgi transport which might limit the rate of secretion (Hooker et al. 1999). It had been shown recently that active X-box binding protein (XBP1) induced synthesis of ER membrane phospholipids and thereby the surface area and volume of rough ER are increased, thus raising the secretion of expressed proteins (Shaffer et al. 2004).

Our two chemically defined systems are suitable for stable serum-free transfection of CHO cells and they can compete with commercially available transfection systems (Reisinger et al. 2007), they are cost efficient and provide easy scalability and are also usable for larger scales. They provide a fast and easy method for stable transfection and are highly competitive with common transfection techniques used in our lab (Reisinger et al. submitted).

# 4 Perspectives

For the generation of high producing cell lines, that generate high $q_P$, the next step could be to combine the transfection techniques described in this work. Thereby the advantages of cationic liposomes and the cationic polymers could be used to get high transfection rates and highly recombinant protein expressing cells. Several studies reported the successful combination of the cationic liposomes and polymers in one system for transfection (Kamiya et al. 2003; Kogure et al. 2004; Lee and Huang 1996). It is possible to complex the PEI with the DNA and additionally load the liposomes with these polyplexes. Thereby the liposomes would serve to cross the cellular membrane, while the PEI would support the transport via the cytoplasm into the nucleus providing high transfection rates.

In a first step, the modification of the expression plasmids should include the integration of at least one intron, codon-optimized cDNA of the antibody HC and LC under control of CMV promoters on different vectors. Additionally the plasmids should be as small as possible to simplify the integration into the genome. Thus, it might be better not to overload the plasmids with proposed genetic elements for the increase of antibody production. After investigating the influence of these changes one can proceed to the addition of leader sequences or other genetically engineering approaches.

Another possible approach might be the identification of the amino acids in the primary sequence that are not necessary for biologic activity of the antibody and switch to other amino acids, thereby increasing the expression rate of the antibody.

Additionally the “omics” tools which became available during recent years might play a major role in providing knowledge upon understanding the biology of the cell. It can show which regulatory elements are up regulated and how the secretion via ER and Golgi is influenced.

The highest titers published so far were 4.7 g/l claiming without mentioning the probable variations (Wurm 2004), while it is believed that understanding of cell biology using the omics tool in combination with progress in media composition and feed development might lead to antibody concentrations of at least 10g/l (Birch and Racher 2006).

The design and development of new expression vectors can be beneficial as well as modifying the growth conditions for the host cells reducing stress and enabling the cells to grow to higher densities thereby producing higher volumetric antibody titer. Often the $q_P$ is limited by events downstream of transcription and translation, which can be partly addressed by vector engineering. The antibody production is limited at folding, assembly and secretion reactions, thus the secretory pathways must be modified. Since foldases and chaperones exist as large

protein complexes, the global expansion of all components of the secretory pathway is required for genetic improvement of antibody secretion rather than over-expression of selected proteins. The processing and the secretion of the recombinant proteins can be increased upon understanding the detail mechanism to obtain higher $q_P$ of the recombinant protein.

# References


Abdallah B, Sachs L, Demeneix BA. 1995. Non-viral gene transfer: applications in developmental biology and gene therapy. Biol Cell 85(1):1-7.

Allen LA, Aderem A. 1996. Mechanisms of phagocytosis. Curr Opin Immunol 8(1):36-40.

Alt FW, Kellems RE, Bertino JR, Schimke RT. 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J Biol Chem 253(5):1357-70.

Altamirano C, Cairo JJ, Godia F. 2001. Decoupling cell growth and product formation in Chinese hamster ovary cells through metabolic control. Biotechnol Bioeng 76(4):351-60.

Altin JG, Parish CR. 2006. Liposomal vaccines--targeting the delivery of antigen. Methods 40(1):39-52.

Amyere M, Mettlen M, Van Der Smissen P, Platek A, Payrastre B, Veithen A, Courtoy PJ. 2002. Origin, originality, functions, subversions and molecular signalling of macropinocytosis. Int J Med Microbiol 291(6-7):487-94.

Antoniou M, Harland L, Mustoe T, Williams S, Holdstock J, Yague E, Mulcahy T, Griffiths M, Edwards S, Ioannou PA and others. 2003. Transgenes encompassing dual-promoter CpG islands from the human TBP and HNRPA2B1 loci are resistant to heterochromatin-mediated silencing. Genomics 82(3):269-79.

Arden N, Nivtchanyong T, Betenbaugh M. 2004. Cell engineerimg blocks stress and improves biotherapeutic production. Bioprocessing 3:23-28.

Bakker H, Bardor M, Molthoff JW, Gomord V, Elbers I, Stevens LH, Jordi W, Lommen A, Faye L, Lerouge P and others. 2001. Galactose-extended glycans of antibodies produced by transgenic plants. Proc Natl Acad Sci U S A 98(5):2899-904.

Bally MB, Harvie P, Wong FM, Kong S, Wasan EK, Reimer DL. 1999. Biological barriers to cellular delivery of lipid-based DNA carriers. Adv Drug Deliv Rev 38(3):291-315.

Banan M, Rojas IC, Lee WH, King HL, Harriss JV, Kobayashi R, Webb CF, Gottlieb PD. 1997. Interaction of the nuclear matrix-associated region (MAR)-binding proteins, SATB1 and CDP/Cux, with a MAR element (L2a) in an upstream regulatory region of the mouse CD8a gene. J Biol Chem 272(29):18440-52.

Bangham AD, Horne RW. 1964. Negative Staining of Phospholipids and Their Structural Modification by Surface-Active Agents as Observed in the Electron Microscope. J Mol Biol 8:660-8.

Bangham AD, Standish MM, Miller N. 1965. Cation permeability of phospholipid model membranes: effect of narcotics. Nature 208(5017):1295-7.

Barnes LM, Bentley CM, Dickson AJ. 2003. Stability of protein production from recombinant mammalian cells. Biotechnol Bioeng 81(6):631-9.

Barnes LM, Bentley CM, Dickson AJ. 2004. Molecular definition of predictive indicators of stable protein expression in recombinant NS0 myeloma cells. Biotechnol Bioeng 85(2):115-21.

Barnes LM, Dickson AJ. 2006. Mammalian cell factories for efficient and stable protein expression. Curr Opin Biotechnol 17(4):381-6.

Bebbington CR, Renner G, Thomson S, King D, Abrams D, Yarranton GT. 1992. High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. Biotechnology (N Y) 10(2):169-75.

Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. 1989. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. Proc Natl Acad Sci U S A 86(18):6982-6.

Bell SL, Bebbington C, Scott MF, Wardell JN, Spier RE, Bushell ME, Sanders PG. 1995. Genetic engineering of hybridoma glutamine metabolism. Enzyme Microb Technol 17(2):98-106.

Bendas G, Krause A, Schmidt R, Vogel J, Rothe U. 1998. Selectins as new targets for immunoliposome-mediated drug delivery. A potential way of anti-inflammatory therapy. Pharm Acta Helv 73(1):19-26.

Benoist C, Chambon P. 1981. In vivo sequence requirements of the SV40 early promotor region. Nature 290(5804):304-10.

Bentley DL. 2005. Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr Opin Cell Biol 17(3):251-6.

Bentley KJ, Gewert R, Harris WJ. 1998. Differential efficiency of expression of humanized antibodies in transient transfected mammalian cells. Hybridoma 17(6):559-67.

Bianchi AA, McGrew JT. 2003. High-level expression of full-length antibodies using trans-complementing expression vectors. Biotechnol Bioeng 84(4):439-44.

Bibila TA, Robinson DK. 1995. In pursuit of the optimal fed-batch process for monoclonal antibody production. Biotechnol Prog 11(1):1-13.

Birch JR, Onakunle Y. 2005. Biopharmaceutical proteins: opportunities and challenges. Methods Mol Biol 308:1-16.

Birch JR, Racher AJ. 2006. Antibody production. Adv Drug Deliv Rev 58(5-6):671-85.

Bloemen PG, Henricks PA, van Bloois L, van den Tweel MC, Bloem AC, Nijkamp FP, Crommelin DJ, Storm G. 1995. Adhesion molecules: a new target for immunoliposome-mediated drug delivery. FEBS Lett 357(2):140-4.

Bode J, Goetze S, Heng H, Krawetz SA, Benham C. 2003. From DNA structure to gene expression: mediators of nuclear compartmentalization and dynamics. Chromosome Res 11(5):435-45.

Bollati-Fogolin M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R. 2005. Temperature reduction in cultures of hGM-CSF-expressing CHO cells: effect on productivity and product quality. Biotechnol Prog 21(1):17-21.

Borth N, Strutzenberger K, Kunert R, Steinfellner W, Katinger H. 1999. Analysis of changes during subclone development and ageing of human antibody-producing heterohybridoma cells by northern blot and flow cytometry. J Biotechnol 67(1):57-66.

Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41(2):521-30.

Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 92(16):7297-301.

Brewer JW, Hendershot LM. 2005. Building an antibody factory: a job for the unfolded protein response. Nat Immunol 6(1):23-9.

Buchacher A, Predl R, Strutzenberger K, Steinfellner W, Trkola A, Purtscher M, Gruber G, Tauer C, Steindl F, Jungbauer A and others. 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res Hum Retroviruses 10(4):359-69.

Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol 68(3):283-91.

Cappechi MR. 1980. Cell 22:479.

Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, Ponjavic J, Semple CA, Taylor MS, Engstrom PG, Frith MC and others. 2006. Genome-wide analysis of mammalian promoter architecture and evolution. Nat Genet 38(6):626-35.

Carvalhal AV, Santos SS, Calado J, Haury M, Carrondo MJ. 2003. Cell growth arrest by nucleotides, nucleosides and bases as a tool for improved production of recombinant proteins. Biotechnol Prog 19(1):69-83.

Chadd HE, Chamow SM. 2001. Therapeutic antibody expression technology. Curr Opin Biotechnol 12(2):188-94.

Chen K, Liu Q, Xie L, Sharp PA, Wang DI. 2001. Engineering of a mammalian cell line for reduction of lactate formation and high monoclonal antibody production. Biotechnol Bioeng 72(1):55-61.

Conner SD, Schmid SL. 2003. Regulated portals of entry into the cell. Nature 422(6927):37-44.

Cruz HJ, Moreira JL, Carrondo MJ. 2000. Metabolically optimised BHK cell fed-batch cultures. J Biotechnol 80(2):109-18.

Cudd A, Labbe H, Gervais M, Nicolau C. 1984. Liposomes injected intravenously into mice associate with liver mitochondria. Biochim Biophys Acta 774(2):169-80.

Cullen BR. 2003. Nuclear RNA export. J Cell Sci 116(Pt 4):587-97.

Cullis PR, Hope MJ, Tilcock CP. 1986. Lipid polymorphism and the roles of lipids in membranes. Chem Phys Lipids 40(2-4):127-44.

Cupit PM, Whyte JA, Porter AJ, Browne MJ, Holmes SD, Harris WJ, Cunningham C. 1999. Cloning and expression of single chain antibody fragments in Escherichia coli and Pichia pastoris. Lett Appl Microbiol 29(5):273-7.

Darzacq X, Singer RH, Shav-Tal Y. 2005. Dynamics of transcription and mRNA export. Curr Opin Cell Biol 17(3):332-9.

Davie JR. 2003. Inhibition of histone deacetylase activity by butyrate. J Nutr 133(7 Suppl):2485S-2493S.

Davis CG, Gallo ML, Corvalan JR. 1999. Transgenic mice as a source of fully human antibodies for the treatment of cancer. Cancer Metastasis Rev 18(4):421-5.

Day DA, Tuite MF. 1998. Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview. J Endocrinol 157(3):361-71.

Dean A. 2006. On a chromosome far, far away: LCRs and gene expression. Trends Genet 22(1):38-45.

Debs RJ, Freedman LP, Edmunds S, Gaensler KL, Duzgunes N, Yamamoto KR. 1990. Regulation of gene expression in vivo by liposome-mediated delivery of a purified transcription factor. J Biol Chem 265(18):10189-92.

Decker EL, Reski R. 2004. The moss bioreactor. Curr Opin Plant Biol 7(2):166-70.

Derouazi M, Girard P, Van Tilborgh F, Iglesias K, Muller N, Bertschinger M, Wurm FM. 2004. Serum-free large-scale transient transfection of CHO cells. Biotechnol Bioeng 87(4):537-45.

Derouazi M, Martinet D, Besuchet Schmutz N, Flaction R, Wicht M, Bertschinger M, Hacker DL, Beckmann JS, Wurm FM. 2006. Genetic characterization of CHO production host DG44 and derivative recombinant cell lines. Biochem Biophys Res Commun 340(4):1069-77.

Dinnis DM, James DC. 2005. Engineering mammalian cell factories for improved recombinant monoclonal antibody production: lessons from nature? Biotechnol Bioeng 91(2):180-9.

Dorai H, Csirke B, Scallon B, Ganguly S. 2006. Correlation of heavy and light chain mRNA copy numbers to antibody productivity in mouse myeloma production cell lines. Hybridoma (Larchmt) 25(1):1-9.

Dorner AJ, Kaufman RJ. 1994. The levels of endoplasmic reticulum proteins and ATP affect folding and secretion of selective proteins. Biologicals 22(2):103-12.

Doyle SR, Chan CK. 2007. Differential intracellular distribution of DNA complexed with polyethylenimine (PEI) and PEI-polyarginine PTD influences exogenous gene expression within live COS-7 cells. Genet Vaccines Ther 5:11.

Dunlap DD, Maggi A, Soria MR, Monaco L. 1997. Nanoscopic structure of DNA condensed for gene delivery. Nucleic Acids Res 25(15):3095-101.

Elias CB, Carpentier E, Durocher Y, Bisson L, Wagner R, Kamen A. 2003. Improving glucose and glutamine metabolism of human HEK 293 and Trichoplusia ni insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnol Prog 19(1):90-7.

Emery DW, Yannaki E, Tubb J, Stamatoyannopoulos G. 2000. A chromatin insulator protects retrovirus vectors from chromosomal position effects. Proc Natl Acad Sci U S A 97(16):9150-5.

Escriou V, Carriere M, Bussone F, Wils P, Scherman D. 2001. Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. J Gene Med 3(2):179-87.

Faiger H, Ivanchenko M, Cohen I, Haran TE. 2006. TBP flanking sequences: asymmetry of binding, long-range effects and consensus sequences. Nucleic Acids Res 34(1):104-19.

Farber FE, Melnick JL, Butel JS. 1975. Optimal conditions for uptake of exogenous DNA by Chinese hamster lung cells deficient in hypoxanthine-guanine phosphoribosyltransferase. Biochim Biophys Acta 390(3):298-311.

Farhood H, Gao X, Barsoum J, Huang L. 1995a. Codelivery to mammalian cells of a transcriptional factor with cis-acting element using cationic liposomes. Anal Biochem 225(1):89-93.

Farhood H, Serbina N, Huang L. 1995b. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim Biophys Acta 1235(2):289-95.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A 84(21):7413-7.

Felgner PL, Tsai YJ, Sukhu L, Wheeler CJ, Manthorpe M, Marshall J, Cheng SH. 1995. Improved cationic lipid formulations for in vivo gene therapy. Ann N Y Acad Sci 772:126-39.

Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. 2003. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Mol Ther 8(2):284-94.

Fiering S, Whitelaw E, Martin DI. 2000. To be or not to be active: the stochastic nature of enhancer action. Bioessays 22(4):381-7.

Flickinger MC, Goebel NK, Bibila T, Boyce-Jacino S. 1992. Evidence for posttranscriptional stimulation of monoclonal antibody secretion by L-glutamine during slow hybridoma growth. J Biotechnol 22(3):201-26.

Fogolin MB, Wagner R, Etcheverrigaray M, Kratje R. 2004. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J Biotechnol 109(1-2):179-91.

Forman MS, Lee VM, Trojanowski JQ. 2003. 'Unfolding' pathways in neurodegenerative disease. Trends Neurosci 26(8):407-10.

Fox SR, Patel UA, Yap MG, Wang DI. 2004. Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling. Biotechnol Bioeng 85(2):177-84.

Fraley R, Subramani S, Berg P, Papahadjopoulos D. 1980. Introduction of liposome-encapsulated SV40 DNA into cells. J Biol Chem 255(21):10431-5.

Freyre FM, Vazquez JE, Ayala M, Canaan-Haden L, Bell H, Rodriguez I, Gonzalez A, Cintado A, Gavilondo JV. 2000. Very high expression of an anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast Pichia pastoris. J Biotechnol 76(2-3):157-63.

Friend DS, Papahadjopoulos D, Debs RJ. 1996. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim Biophys Acta 1278(1):41-50.

Fussenegger M, Bailey JE. 1998. Molecular regulation of cell-cycle progression and apoptosis in mammalian cells: implications for biotechnology. Biotechnol Prog 14(6):807-33.

Galbraith DJ, Tait AS, Racher AJ, Birch JR, James DC. 2006. Control of culture environment for improved polyethylenimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. Biotechnol Prog 22(3):753-62.

Gandor C, Leist C, Fiechter A, Asselbergs FA. 1995. Amplification and expression of recombinant genes in serum-independent Chinese hamster ovary cells. FEBS Lett 377(3):290-4.

Gao X, Huang L. 1995. Cationic liposome-mediated gene transfer. Gene Ther 2(10):710-22.

Girod PA, Zahn-Zabal M, Mermod N. 2005. Use of the chicken lysozyme 5' matrix attachment region to generate high producer CHO cell lines. Biotechnol Bioeng 91(1):1-11.

Glover DJ, Lipps HJ, Jans DA. 2005. Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet 6(4):299-310.

Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. 2000. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J Biomed Mater Res 51(3):321-8.

Godbey WT, Wu KK, Mikos AG. 1999a. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 45(3):268-75.

Godbey WT, Wu KK, Mikos AG. 1999b. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci U S A 96(9):5177-81.

Goncalves C, Mennesson E, Fuchs R, Gorvel JP, Midoux P, Pichon C. 2004. Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells. Mol Ther 10(2):373-85.

Gorman CM, Moffat LF, Howard BH. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2(9):1044-51.

Goto Y, Hamaguchi K. 1981. Formation of the intrachain disulfide bond in the constant fragment of the immunoglobulin light chain. J Mol Biol 146(3):321-40.

Graessmann M, Graessmann A. 1983. Microinjection of tissue culture cells. Methods Enzymol 101:482-92.

Graham FL, van der Eb AJ. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52(2):456-67.

Green LL, Hardy MC, Maynard-Currie CE, Tsuda H, Louie DM, Mendez MJ, Abderrahim H, Noguchi M, Smith DH, Zeng Y and others. 1994. Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. Nat Genet 7(1):13-21.

Grimm S. 2004. The art and design of genetic screens: mammalian culture cells. Nat Rev Genet 5(3):179-89.

Groskreutz D, Schenborn ET. 1997. Reporter systems. Methods Mol Biol 63:11-30.

Grunow R, Jahn S, Porstmann T, Kiessig SS, Steinkellner H, Steindl F, Mattanovich D, Gurtler L, Deinhardt F, Katinger H and others. 1988. The high efficiency, human B cell immortalizing heteromyeloma CB-F7. Production of human monoclonal antibodies to human immunodeficiency virus. J Immunol Methods 106(2):257-65.

Guarna MM, Fann CH, Busby SJ, Walker KM, Kilburn DG, Piret JM. 1995. Effect of cDNA copy number on secretion rate of activated protein C. Biotechnol Bioeng 46(1):22-7.

Gustafsson C, Govindarajan S, Minshull J. 2004. Codon bias and heterologous protein expression. Trends Biotechnol 22(7):346-53.

Haber J. 1999. Hildegard Peplau. The mother of psychiatric nursing. Nurs Health Care Perspect 20(4):228.

Haensler J, Szoka FC, Jr. 1993. Synthesis and characterization of a trigalactosylated bisacridine compound to target DNA to hepatocytes. Bioconjug Chem 4(1):85-93.

Hafez IM, Cullis PR. 2001. Roles of lipid polymorphism in intracellular delivery. Adv Drug Deliv Rev 47(2-3):139-48.

Haggstrom L, Ljunggren J, Ohman L. 1996. Metabolic engineering of animal cells. Ann N Y Acad Sci 782:40-52.

Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H and others. 2003. Production of complex human glycoproteins in yeast. Science 301(5637):1244-6.

Hammond SL, Ham RG, Stampfer MR. 1984. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc Natl Acad Sci U S A 81(17):5435-9.

Hancock R. 2000. A new look at the nuclear matrix. Chromosoma 109(4):219-25.

Harris J, Werling D, Hope JC, Taylor G, Howard CJ. 2002. Caveolae and caveolin in immune cells: distribution and functions. Trends Immunol 23(3):158-64.

Heng HH, Goetze S, Ye CJ, Liu G, Stevens JB, Bremer SW, Wykes SM, Bode J, Krawetz SA. 2004. Chromatin loops are selectively anchored using scaffold/matrix-attachment regions. J Cell Sci 117(Pt 7):999-1008.

Henner WD, Kleber I, Benzinger R. 1973. Transfection of Escherichia coli spheroplasts. 3. Facilitation of transfection and stabilization of spheroplasts by different basic polymers. J Virol 12(4):741-7.

Ho RJ, Rouse BT, Huang L. 1986. Target-sensitive immunoliposomes: preparation and characterization. Biochemistry 25(19):5500-6.

Hooker AD, Green NH, Baines AJ, Bull AT, Jenkins N, Strange PG, James DC. 1999. Constraints on the transport and glycosylation of recombinant IFN-gamma in Chinese hamster ovary and insect cells. Biotechnol Bioeng 63(5):559-72.

Hsu TA, Betenbaugh MJ. 1997. Coexpression of molecular chaperone BiP improves immunoglobulin solubility and IgG secretion from Trichoplusia ni insect cells. Biotechnol Prog 13(1):96-104.

Hu S, Li L, Qiao J, Guo Y, Cheng L, Liu J. 2006. Codon optimization, expression, and characterization of an internalizing anti-ErbB2 single-chain antibody in Pichia pastoris. Protein Expr Purif 47(1):249-57.

Huls GA, Heijnen IA, Cuomo ME, Koningsberger JC, Wiegman L, Boel E, van der Vuurst de Vries AR, Loyson SA, Helfrich W, van Berge Henegouwen GP and others. 1999. A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. Nat Biotechnol 17(3):276-81.

Irani N, Beccaria AJ, Wagner R. 2002. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. J Biotechnol 93(3):269-82.

Irani N, Wirth M, van Den Heuvel J, Wagner R. 1999. Improvement of the primary metabolism of cell cultures by introducing a new cytoplasmic pyruvate carboxylase reaction. Biotechnol Bioeng 66(4):238-46.

Itoh Y, Ueda H, Suzuki E. 1995. Overexpression of bcl-2, apoptosis suppressing gene: Prolonged viable culture period of hybridoma and enhanced antibody production. Biotechnol Bioeng 48(2):118-22.

Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. 1997. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell 91(4):501-10.

Jiang Y, Xu XS, Russell JE. 2006a. A nucleolin-binding 3' untranslated region element stabilizes beta-globin mRNA in vivo. Mol Cell Biol 26(6):2419-29.

Jiang Z, Huang Y, Sharfstein ST. 2006b. Regulation of recombinant monoclonal antibody production in chinese hamster ovary cells: a comparative study of gene copy number, mRNA level, and protein expression. Biotechnol Prog 22(1):313-8.

Johnson AE, van Waes MA. 1999. The translocon: a dynamic gateway at the ER membrane. Annu Rev Cell Dev Biol 15:799-842.

Jones D, Kroos N, Anema R, van Montfort B, Vooys A, van der Kraats S, van der Helm E, Smits S, Schouten J, Brouwer K and others. 2003. High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog 19(1):163-8.

Kamiya H, Akita H, Harashima H. 2003. Pharmacokinetic and pharmacodynamic considerations in gene therapy. Drug Discov Today 8(21):990-6.

Kaneda Y, Uchida T, Kim J, Ishiura M, Okada Y. 1987. The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. Exp Cell Res 173(1):56-69.

Karmali PP, Majeti BK, Sreedhar B, Chaudhuri A. 2006. In vitro gene transfer efficacies and serum compatibility profiles of novel mono-, di-, and tri-histidinylated cationic transfection lipids: a structure-activity investigation. Bioconjug Chem 17(1):159-71.

Kaufman RJ, Sharp PA. 1982. Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary dna gene. J Mol Biol 159(4):601-21.

Kaufmann H, Mazur X, Fussenegger M, Bailey JE. 1999. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. Biotechnol Bioeng 63(5):573-82.

Kawai S, Nishizawa M. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol Cell Biol 4(6):1172-4.

Keng VW, Yae K, Hayakawa T, Mizuno S, Uno Y, Yusa K, Kokubu C, Kinoshita T, Akagi K, Jenkins NA and others. 2005. Region-specific saturation germline mutagenesis in mice using the Sleeping Beauty transposon system. Nat Methods 2(10):763-9.

Khalil IA, Kogure K, Akita H, Harashima H. 2006. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol Rev 58(1):32-45.

Kichler A, Leborgne C, Coeytaux E, Danos O. 2001. Polyethylenimine-mediated gene delivery: a mechanistic study. J Gene Med 3(2):135-44.

Kim D, Kim JD, Baek K, Yoon Y, Yoon J. 2003. Improved mammalian expression systems by manipulating transcriptional termination regions. Biotechnol Prog 19(5):1620-2.

Kim NS, Kim SJ, Lee GM. 1998a. Clonal variability within dihydrofolate reductase-mediated gene amplified Chinese hamster ovary cells: stability in the absence of selective pressure. Biotechnol Bioeng 60(6):679-88.

Kim NS, Lee GM. 2002a. Inhibition of sodium butyrate-induced apoptosis in recombinant Chinese hamster ovary cells by constitutively expressing antisense RNA of caspase-3. Biotechnol Bioeng 78(2):217-28.

Kim NS, Lee GM. 2002b. Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl-2 overexpression. J Biotechnol 95(3):237-48.

Kim SJ, Kim NS, Ryu CJ, Hong HJ, Lee GM. 1998b. Characterization of chimeric antibody producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. Biotechnol Bioeng 58(1):73-84.

Kipriyanov SM, Little M. 1999. Generation of recombinant antibodies. Mol Biotechnol 12(2):173-201.

Klein TM. 1987. Nature 327:70.

Knappskog S, Ravneberg H, Gjerdrum C, Trosse C, Stern B, Pryme IF. 2007. The level of synthesis and secretion of Gaussia princeps luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide. J Biotechnol 128(4):705-15.

Kogure K, Moriguchi R, Sasaki K, Ueno M, Futaki S, Harashima H. 2004. Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. J Control Release 98(2):317-23.

Kohl J, Ruker F, Himmler G, Razazzi E, Katinger H. 1991. Cloning and expression of an HIV-1 specific single-chain Fv region fused to Escherichia coli alkaline phosphatase. Ann N Y Acad Sci 646:106-14.

Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495-7.

Koltover I, Salditt, T., Rädler, J.O., Safinya, C.R. 1998. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 281:78-81.

Kopatz I, Remy JS, Behr JP. 2004. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. J Gene Med 6(7):769-76.

Kozak M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15(20):8125-48.

Kozak M. 1999. Initiation of translation in prokaryotes and eukaryotes. Gene 234(2):187-208.

Kozak M. 2002. Emerging links between initiation of translation and human diseases. Mamm Genome 13(8):401-10.

Kozak M. 2005. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. Gene 361:13-37.

Kukowska-Latallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA, Baker JR, Jr. 1996. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. Proc Natl Acad Sci U S A 93(10):4897-902.

Kulkarni RP, Wu DD, Davis ME, Fraser SE. 2005. Quantitating intracellular transport of polyplexes by spatio-temporal image correlation spectroscopy. Proc Natl Acad Sci U S A 102(21):7523-8.

Kunert R, Ruker F, Katinger H. 1998. Molecular characterization of five neutralizing anti-HIV type 1 antibodies: identification of nonconventional D segments in the human monoclonal antibodies 2G12 and 2F5. AIDS Res Hum Retroviruses 14(13):1115-28.

Kunert R, Steinfellner W, Purtscher M, Assadian A, Katinger H. 2000. Stable recombinant expression of the anti HIV-1 monoclonal antibody 2F5 after IgG3/IgG1 subclass switch in CHO cells. Biotechnol Bioeng 67(1):97-103.

Kwaks TH, Barnett P, Hemrika W, Siersma T, Sewalt RG, Satijn DP, Brons JF, van Blokland R, Kwakman P, Kruckeberg AL and others. 2003. Identification of anti-repressor elements that confer high and stable protein production in mammalian cells. Nat Biotechnol 21(5):553-8.

Kwaks TH, Otte AP. 2006. Employing epigenetics to augment the expression of therapeutic proteins in mammalian cells. Trends Biotechnol 24(3):137-42.

Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Furstenberger P, Oberling F, Brambilla E, Behr JP. 1996. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. Gene Ther 3(11):1010-7.

Lamaze C, Schmid SL. 1995. The emergence of clathrin-independent pinocytic pathways. Curr Opin Cell Biol 7(4):573-80.

Lamb BT, Gearhart JD. 1995. YAC transgenics and the study of genetics and human disease. Curr Opin Genet Dev 5(3):342-8.

Lasic DD. 1991. Formation of membranes. Nature 351(6328):613.

Lasic DD, Vallner JJ, Working PK. 1999. Sterically stabilized liposomes in cancer therapy and gene delivery. Curr Opin Mol Ther 1(2):177-85.

Lasunskaia EB, Fridlianskaia, II, Darieva ZA, da Silva MS, Kanashiro MM, Margulis BA. 2003. Transfection of NS0 myeloma fusion partner cells with HSP70 gene results in higher hybridoma yield by improving cellular resistance to apoptosis. Biotechnol Bioeng 81(4):496-504.

Lattenmayer C, Trummer E, Schriebl K, Vorauer-Uhl K, Mueller D, Katinger H, Kunert R. 2007. Characterisation of recombinant CHO cell lines by investigation of protein productivities and genetic parameters. J Biotechnol 128(4):716-25.

Le Hir H, Nott A, Moore M. 2004. How introns influemce and enhance eukaryotic gene expression. Trends in Biochem. Sci.:215-220.

Lee JT, Jaenisch R. 1996. A method for high efficiency YAC lipofection into murine embryonic stem cells. Nucleic Acids Res 24(24):5054-5.

Lee MS, Lee GM. 2000. Hyperosmotic pressure enhances immunoglobulin transcription rates and secretion rates of KR12H-2 transfectoma. Biotechnol Bioeng 68(3):260-8.

Lee RJ, Huang L. 1996. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J Biol Chem 271(14):8481-7.

Lee TI, Young RA. 2000. Transcription of eukaryotic protein-coding genes. Annu Rev Genet 34:77-137.

Lei EP, Silver PA. 2002. Intron status and 3'-end formation control cotranscriptional export of mRNA. Genes Dev 16(21):2761-6.

Li A, Katinger H, Posner MR, Cavacini L, Zolla-Pazner S, Gorny MK, Sodroski J, Chou TC, Baba TW, Ruprecht RM. 1998. Synergistic neutralization of simian-human immunodeficiency virus SHIV-vpu+ by triple and quadruple combinations of human monoclonal antibodies and high-titer anti-human immunodeficiency virus type 1 immunoglobulins. J Virol 72(4):3235-40.

Li Q, Peterson KR, Fang X, Stamatoyannopoulos G. 2002. Locus control regions. Blood 100(9):3077-86.

Lilie H, McLaughlin S, Freedman R, Buchner J. 1994. Influence of protein disulfide isomerase (PDI) on antibody folding in vitro. J Biol Chem 269(19):14290-6.

Loyter A, Scangos GA, Ruddle FH. 1982. Mechanisms of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. Proc Natl Acad Sci U S A 79(2):422-6.

Luciw PA, Bishop JM, Varmus HE, Capecchi MR. 1983. Location and function of retroviral and SV40 sequences that enhance biochemical transformation after microinjection of DNA. Cell 33(3):705-16.

Lungwitz U, Breunig M, Blunk T, Gopferich A. 2005. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 60(2):247-66.

Malone RW, Felgner PL, Verma IM. 1989. Cationic liposome-mediated RNA transfection. Proc Natl Acad Sci U S A 86(16):6077-81.

Mannino RJ, Gould-Fogerite S. 1988. Liposome mediated gene transfer. Biotechniques 6(7):682-90.

Martin B, Sainlos M, Aissaoui A, Oudrhiri N, Hauchecorne M, Vigneron JP, Lehn JM, Lehn P. 2005. The design of cationic lipids for gene delivery. Curr Pharm Des 11(3):375-94.

Martoglio B, Dobberstein B. 1998. Signal sequences: more than just greasy peptides. Trends Cell Biol 8(10):410-5.

Mascola JR, Louder MK, VanCott TC, Sapan CV, Lambert JS, Muenz LR, Bunow B, Birx DL, Robb ML. 1997. Potent and synergistic neutralization of human immunodeficiency virus (HIV) type 1 primary isolates by hyperimmune anti-HIV immunoglobulin combined with monoclonal antibodies 2F5 and 2G12. J Virol 71(10):7198-206.

Mastrangelo AJ, Hardwick JM, Zou S, Betenbaugh MJ. 2000. Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. Biotechnol Bioeng 67(5):555-64.

Matsui H, Johnson LG, Randell SH, Boucher RC. 1997. Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. J Biol Chem 272(2):1117-26.

Matveev S, Li X, Everson W, Smart EJ. 2001. The role of caveolae and caveolin in vesicle-dependent and vesicle-independent trafficking. Adv Drug Deliv Rev 49(3):237-50.

Maxfield FR, McGraw TE. 2004. Endocytic recycling. Nat Rev Mol Cell Biol 5(2):121-32.

Mazumder B, Seshadri V, Fox PL. 2003. Translational control by the 3'-UTR: the ends specify the means. Trends Biochem Sci 28(2):91-8.

McCutchan JH, Pagano JS. 1968. Enchancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J Natl Cancer Inst 41(2):351-7.

Meents H, Enenkel B, Eppenberger HM, Werner RG, Fussenegger M. 2002. Impact of coexpression and coamplification of sICAM and antiapoptosis determinants bcl-2/bcl-x(L) on productivity, cell survival, and mitochondria number in CHO-DG44 grown in suspension and serum-free media. Biotechnol Bioeng 80(6):706-16.

Meier O, Greber UF. 2003. Adenovirus endocytosis. J Gene Med 5(6):451-62.

Meijer HA, Thomas AA. 2002. Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. Biochem J 367(Pt 1):1-11.

Meissner P, Pick H, Kulangara A, Chatellard P, Friedrich K, Wurm FM. 2001. Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. Biotechnol Bioeng 75(2):197-203.

Melchior F, Gerace L. 1995. Mechanisms of nuclear protein import. Curr Opin Cell Biol 7(3):310-8.

Mendez MJ, Green LL, Corvalan JR, Jia XC, Maynard-Currie CE, Yang XD, Gallo ML, Louie DM, Lee DV, Erickson KL and others. 1997. Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. Nat Genet 15(2):146-56.

Merten OW, Moeurs D, Keller H, Leno M, Palfi GE, Cabanie L, Couve E. 1994. Modified monoclonal antibody production kinetics kappa/gamma mRNA levels, and metabolic activities in a murine hybridoma selected by continuous Culture. Biotechnol Bioeng 44(6):753-64.

Mignone F, Gissi C, Liuni S, Pesole G. 2002. Untranslated regions of mRNAs. Genome Biol 3(3):REVIEWS0004.

Miller WM, Blanch HW, Wilke CR. 1988. A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH. Biotechnol Bioeng 32(8):947-65.

Minagawa K, Matsuzawa Y, Yoshikawa K, Matsumoto M, Doi M. 1991. Direct observation of the biphasic conformational change of DNA induced by cationic polymers. FEBS Lett 295(1-3):67-9.

Morrison SL, Johnson MJ, Herzenberg LA, Oi VT. 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci U S A 81(21):6851-5.

Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS, Wilusz J. 2002. The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. Embo J 21(1-2):165-74.

Muller F, Tora L. 2004. The multicoloured world of promoter recognition complexes. Embo J 23(1):2-8.

Muller N, Derouazi M, Van Tilborgh F, Wulhfard S, Hacker DL, Jordan M, Wurm FM. 2007. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol Lett 29(5):703-11.

Muller N, Girard P, Hacker DL, Jordan M, Wurm FM. 2005. Orbital shaker technology for the cultivation of mammalian cells in suspension. Biotechnol Bioeng 89(4):400-6.

Murnane JP, Yezzi MJ, Young BR. 1990. Recombination events during integration of transfected DNA into normal human cells. Nucleic Acids Res 18(9):2733-8.

Muster T, Guinea R, Trkola A, Purtscher M, Klima A, Steindl F, Palese P, Katinger H. 1994. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. J Virol 68(6):4031-4.

Muthing J, Kemminer SE, Conradt HS, Sagi D, Nimtz M, Karst U, Peter-Katalinic J. 2003. Effects of buffering conditions and culture pH on production rates and glycosylation of clinical phase I anti-melanoma mouse IgG3 monoclonal antibody R24. Biotechnol Bioeng 83(3):321-34.

Narum DL, Kumar S, Rogers WO, Fuhrmann SR, Liang H, Oakley M, Taye A, Sim BK, Hoffman SL. 2001. Codon optimization of gene fragments encoding Plasmodium falciparum merzoite proteins enhances DNA vaccine protein expression and immunogenicity in mice. Infect Immun 69(12):7250-3.

Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. Embo J 1(7):841-5.

Norton PA, Coffin JM. 1987. Characterization of Rous sarcoma virus sequences essential for viral gene expression. J Virol 61(4):1171-9.

Orphanides G, Reinberg D. 2002. A unified theory of gene expression. Cell 108(4):439-51.

Padlan EA. 1991. A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. Mol Immunol 28(4-5):489-98.

Pal M, McKean D, Luse DS. 2001. Promoter clearance by RNA polymerase II is an extended, multistep process strongly affected by sequence. Mol Cell Biol 21(17):5815-25.

Pallavicini MG, DeTeresa PS, Rosette C, Gray JW, Wurm FM. 1990. Effects of methotrexate on transfected DNA stability in mammalian cells. Mol Cell Biol 10(1):401-4.

Park H, Kim IH, Kim IY, Kim KH, Kim HJ. 2000. Expression of carbamoyl phosphate synthetase I and ornithine transcarbamoylase genes in Chinese hamster ovary dhfr-cells decreases accumulation of ammonium ion in culture media. J Biotechnol 81(2-3):129-40.

Parton RG, Joggerst B, Simons K. 1994. Regulated internalization of caveolae. J Cell Biol 127(5):1199-215.

Paschen W, Frandsen A. 2001. Endoplasmic reticulum dysfunction--a common denominator for cell injury in acute and degenerative diseases of the brain? J Neurochem 79(4):719-25.

Pelkmans L, Kartenbeck J, Helenius A. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol 3(5):473-83.

Pendse GJ, Karkare S, Bailey JE. 1992. Effect of cloned gene dosage on cell growth and hepatitis B surface antigen synthesis and secretion in recombinant CHO cells. Biotech. Bioeng. 40(1):119-129.

Phi-Van L, Stratling WH. 1996. Dissection of the ability of the chicken lysozyme gene 5' matrix attachment region to stimulate transgene expression and to dampen position effects. Biochemistry 35(33):10735-42.

Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D. 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J Biol Chem 273(13):7507-11.

Preiss T, M WH. 2003. Starting the protein synthesis machine: eukaryotic translation initiation. Bioessays 25(12):1201-11.

Purtscher M, Trkola A, Gruber G, Buchacher A, Predl R, Steindl F, Tauer C, Berger R, Barrett N, Jungbauer A and others. 1994. A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. AIDS Res Hum Retroviruses 10(12):1651-8.

Regelin AE, Fankhaenel S, Gurtesch L, Prinz C, von Kiedrowski G, Massing U. 2000. Biophysical and lipofection studies of DOTAP analogs. Biochim Biophys Acta 1464(1):151-64.

Reisinger H, Sevcsik E, Vorauer-Uhl K, Lohner K, Katinger H, Kunert R. 2007. Serum-free transfection of CHO-cells with tailor-made unilamellar vesicles. Cytotechnology(54):157-168.

Reisinger H, Steinfellner W, Stern B, Katinger H, Kunert R. submitted. The effect of gene copy number and mRNA level on the amount of mAb secretion from mammalian cells. Applied Microbiology and Biotechnology(submitted).

Rejman J, Bragonzi A, Conese M. 2005. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther 12(3):468-74.

Richards J, Auger J, Peace D, Gale D, Michel J, Koons A, Haverty T, Zivin R, Jolliffe L, Bluestone JA. 1999. Phase I evaluation of humanized OKT3: toxicity and immunomodulatory effects of hOKT3gamma4. Cancer Res 59(9):2096-101.

Riechmann L, Clark M, Waldmann H, Winter G. 1988. Reshaping human antibodies for therapy. Nature 332(6162):323-7.

Ringner M, Krogh M. 2005. Folding free energies of 5'-UTRs impact post-transcriptional regulation on a genomic scale in yeast. PLoS Comput Biol 1(7):e72.

Roberts SJ, Chung KN, Nachmanoff K, Elwood PC. 1997. Tissue-specific promoters of the alpha human folate receptor gene yield transcripts with divergent 5' leader sequences and different translational efficiencies. Biochem J 326 ( Pt 2):439-47.

Rodriguez J, Spearman M, Huzel N, Butler M. 2005. Enhanced production of monomeric interferon-beta by CHO cells through the control of culture conditions. Biotechnol Prog 21(1):22-30.

Running Deer J, Allison DS. 2004. High-level expression of proteins in mammalian cells using transcription regulatory sequences from the Chinese hamster EF-1alpha gene. Biotechnol Prog 20(3):880-9.

Sauer PW, Burky JE, Wesson MC, Sternard HD, Qu L. 2000. A high-yielding, generic fed-batch cell culture process for production of recombinant antibodies. Biotechnol Bioeng 67(5):585-97.

Sauerwald TM, Betenbaugh MJ, Oyler GA. 2002. Inhibiting apoptosis in mammalian cell culture using the caspase inhibitor XIAP and deletion mutants. Biotechnol Bioeng 77(6):704-16.

Scarzello M, Smisterova J, Wagenaar A, Stuart MC, Hoekstra D, Engberts JB, Hulst R. 2005. Sunfish cationic amphiphiles: toward an adaptative lipoplex morphology. J Am Chem Soc 127(29):10420-9.

Schaffner W. 1980. Direct transfer of cloned genes from bacteria to mammalian cells. Proc Natl Acad Sci U S A 77(4):2163-7.

Schroder M, Friedl P. 1997. Overexpression of recombinant human antithrombin III in Chinese hamster ovary cells results in malformation and decreased secretion of recombinant protein. Biotechnol Bioeng 53(6):547-59.

Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A and others. 2004. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21(1):81-93.

Shigekawa K, Dower WJ. 1988. Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. Biotechniques 6(8):742-51.

Simberg D, Danino D, Talmon Y, Minsky A, Ferrari ME, Wheeler CJ, Barenholz Y. 2001. Phase behavior, DNA ordering, and size instability of cationic lipoplexes. Relevance to optimal transfection activity. J Biol Chem 276(50):47453-9.

Slepushkin VA, Simoes S, Dazin P, Newman MS, Guo LS, Pedroso de Lima MC, Duzgunes N. 1997. Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. J Biol Chem 272(4):2382-8.

Smales CM, Dinnis DM, Stansfield SH, Alete D, Sage EA, Birch JR, Racher AJ, Marshall CT, James DC. 2004. Comparative proteomic analysis of GS-NS0 murine myeloma cell lines with varying recombinant monoclonal antibody production rate. Biotechnol Bioeng 88(4):474-88.

Smisterova J, Wagenaar A, Stuart MC, Polushkin E, ten Brinke G, Hulst R, Engberts JB, Hoekstra D. 2001. Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine-DNA complexes and the efficiency of gene delivery. J Biol Chem 276(50):47615-22.

Sollner TH, Rothman JE. 1996. Molecular machinery mediating vesicle budding, docking and fusion. Cell Struct Funct 21(5):407-12.

Sonawane ND, Szoka FC, Jr., Verkman AS. 2003. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 278(45):44826-31.

Southern PJ, Berg P. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J Mol Appl Genet 1(4):327-41.

Sriburi R, Jackowski S, Mori K, Brewer JW. 2004. XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. J Cell Biol 167(1):35-41.

Stern B OL, Trösße C, Ravneberg H and Pryme IF. 2007. Improving mammalian cell factories: The selection of signal peptide has a major impact on recombinant protein synthesis and secretion in mammalian cells.

Stern B, Olsen LC, Trösße C, Ravneberg H, Pryme IF. 2007. Improving mammalian cell factories: The selection of signal peptide has a major impact on recombinant protein synthesis and secretion in mammalian cells. Trends in cell & molecular biology.

Straubinger RM. 1993. pH-sensitive liposomes for delivery of macromolecules into cytoplasm of cultured cells. Methods Enzymol. 221:361-76.

Strutzenberger K, Borth N, Kunert R, Steinfellner W, Katinger H. 1999. Changes during subclone development and ageing of human antibody-producing recombinant CHO cells. J Biotechnol 69(2-3):215-26.

Subtil A, Hemar A, Dautry-Varsat A. 1994. Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited. J Cell Sci 107 ( Pt 12):3461-8.

Suh J, Wirtz D, Hanes J. 2003. Efficient active transport of gene nanocarriers to the cell nucleus. Proc Natl Acad Sci U S A 100(7):3878-82.

Swanson JA, Watts C. 1995. Macropinocytosis. Trends Cell Biol 5(11):424-8.

Takei K, Haucke V. 2001. Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol 11(9):385-91.

Taylor LD, Carmack CE, Huszar D, Higgins KM, Mashayekh R, Sequar G, Schramm SR, Kuo CC, O'Donnell SL, Kay RM and others. 1994. Human immunoglobulin transgenes undergo rearrangement, somatic mutation and class switching in mice that lack endogenous IgM. Int Immunol 6(4):579-91.

Templeton NS, Lasic, D.D., Frederik, P.M., Strey, H.H., Roberts, D.D., Pavlakis, G.N. 1997. Improved DNA: liposome complexes for increased systemic delivery and gene expression. Nat Biotechnol. 15(7):647-52.

Urlaub G, Chasin LA. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc Natl Acad Sci U S A 77(7):4216-20.

Urlaub G, Mitchell PJ, Kas E, Chasin LA, Funanage VL, Myoda TT, Hamlin J. 1986. Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. Somat Cell Mol Genet 12(6):555-66.

Vaheri A, Pagano JS. 1965. Infectious poliovirus RNA: a sensitive method of assay. Virology 27(3):434-6.

van der Aa MA, Huth US, Hafele SY, Schubert R, Oosting RS, Mastrobattista E, Hennink WE, Peschka-Suss R, Koning GA, Crommelin DJ. 2007. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. Pharm Res 24(8):1590-8.

van der Velden AW, Voorma HO, Thomas AA. 2001. Vector design for optimal protein expression. Biotechniques 31(3):572, 574, 576-80, passim.

Van Leeuwen W, Mlynarova L, Nap JP, van der Plas LH, van der Krol AR. 2001. The effect of MAR elements on variation in spatial and temporal regulation of transgene expression. Plant Mol Biol 47(4):543-54.

Verrijzer CP, Chen JL, Yokomori K, Tjian R. 1995. Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. Cell 81(7):1115-25.

Vives J, Juanola S, Cairo JJ, Godia F. 2003. Metabolic engineering of apoptosis in cultured animal cells: implications for the biotechnology industry. Metab Eng 5(2):124-32.

Voinea M, Simionescu M. 2002. Designing of 'intelligent' liposomes for efficient delivery of drugs. J Cell Mol Med 6(4):465-74.

Walde P, Ichikawa S. 2001. Enzymes inside lipid vesicles: preparation, reactivity and applications. Biomol Eng 18(4):143-77.

Wiethoff CM, Middaugh CR. 2003. Barriers to nonviral gene delivery. J Pharm Sci 92(2):203-17.

Williams S, Mustoe T, Mulcahy T, Griffiths M, Simpson D, Antoniou M, Irvine A, Mountain A, Crombie R. 2005. CpG-island fragments from the HNRPA2B1/CBX3 genomic locus reduce silencing and enhance transgene expression from the hCMV promoter/enhancer in mammalian cells. BMC Biotechnol 5:17.

Wilusz CJ, Wilusz J. 2004. Bringing the role of mRNA decay in the control of gene expression into focus. Trends Genet 20(10):491-7.

Wirth M, Bode J, Zettlmeissl G, Hauser H. 1988. Isolation of overproducing recombinant mammalian cell lines by a fast and simple selection procedure. Gene 73(2):419-26.

Wong TK, Neumann E. 1982. Electric field mediated gene transfer. Biochem Biophys Res Commun 107(2):584-7.

Wurm FM. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22(11):1393-8.

Wurm FM, Gwinn KA, Kingston RE. 1986. Inducible overproduction of the mouse c-myc protein in mammalian cells. Proc Natl Acad Sci U S A 83(15):5414-8.

Xu Y, Szoka FC, Jr. 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry 35(18):5616-23.

Yatvin MB, Kreutz W, Horwitz BA, Shinitzky M. 1980. pH-sensitive liposomes: possible clinical implications. Science 210(4475):1253-5.

Ye GN, Daniell H, Sanford JC. 1990. Optimization of delivery of foreign DNA into higher-plant chloroplasts. Plant Mol Biol 15(6):809-19.

Yoon SK, Hong JK, Lee GM. 2004. Effect of simultaneous application of stressful culture conditions on specific productivity and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Prog 20(4):1293-6.

Yoshida H, Oku M, Suzuki M, Mori K. 2006. pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. J Cell Biol 172(4):565-75.

Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 270(32):18997-9007.

Zhang L, Leng Q, Mixson AJ. 2005. Alteration in the IL-2 signal peptide affects secretion of proteins in vitro and in vivo. J Gene Med 7(3):354-65.

Zheng N, Gierasch LM. 1996. Signal sequences: the same yet different. Cell 86(6):849-52.

Zuhorn IS, Hoekstra D. 2002. On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? J Membr Biol 189(3):167-79.

## Selected Publications

Reisinger H, Sevcsik E; Vorauer-Uhl K, Lohner K, Katinger H, Kunert R. 2007. Serum-free transfection of CHO cells with tailor-made unilamellar vesicles. Cytotechnology 54(3):157-68

Reisinger H, Steinfellner W, Stern B, Katinger H, Kunert R. 2008. The effect of absence of gene copy number and mRNA level on the amount of mAb secretion from mammalian cells. Applied Microbiology and Biotechnology. accepted.

Reisinger H, Steinfellner W, Katinger H, Kunert R. submitted. Serum-free transfection of CHO cells with chemically defined transfection systems and investigation of their potential for transient and stable transfection. Cytotechnology submitted

# Abbreviations

| | |
|---|---|
| ARE | AU-rich elements |
| bcl | Basal cell lymphoma |
| BiP | Immunoglobulin binding protein |
| CAT | Chloramphenicol transferase |
| CDR | complement determining region |
| CHEMS | Cholesterol hemisuccinate |
| CHO | Chinese hamster ovary cells |
| CMV | Cytomegalovirus |
| DD | DOTAP/DOPE |
| DNA | Deoxyribonucleic acid |
| DEAE | Diethylaminoethyl cellulose |
| DHFR | Dihydrofolate reductase |
| DOPE | Dioleylphosphatidylethanolamine |
| DOTAP | 1,2-Dioleoyl-3-Trimethylammonium-Propane |
| EGFP | Enhanced green fluorescent protein |
| ER | Endoplasmatic reticulum |
| FCS | Fetal calf serum |
| GCN | Gene copy number |
| GS | Glutamine synthetase |
| HAT | Histone acetyltransferases |
| HC | Heavy chain |
| HDAC | histone deacetylases |
| HIV | Human immunodeficiency virus |
| HSP | Heat shock proteins |
| IL | Interleukins |
| LC | Light chain |
| LUV | Large unilamellar vesicles |
| MAR | Matrix attachment regions |
| mRNA | messenger RNA |
| MSX | Methionine sulphoimine |
| MTX | Methotrexate |
| PDI | Protein disulfide isomerase |
| PEI | Polyethylenimine |
| RNA | Ribonucleic acid |
| RNAPII | RNA polymerase II |
| RSV | Rous sarcoma virus |
| SAR | Scaffold attachment region |
| SRP | Signal recognition particle |
| STAR | Stabilizing antirepressor |
| SUV | Small unilamellar vesicles |
| SV40 | Simian vacuolating virus 40 |
| TBP | TATA binding protein |
| TF | Transcription factor |
| UCOE | Ubiquitons chromatin opening elements |
| UPR | Untranslated protein response |
| UTR | Untranslated regions |
| XBP | X-box binding protein |

**From:**

steinbu@uni-muenster.de

**Subject:**

Applied Microbiology and Biotechnology - Decision on Manuscript ID AMB-08-16650.R1

**Body:**

02-Sep-2008

Dear Mr. Reisinger:

It is a pleasure to accept your manuscript entitled "The absence of effect of gene copy number and mRNA level on the amount of mAb secretion from mammalian cells" in its current form for publication in "Applied Microbiology and Biotechnology".

Thank you for your fine contribution. On behalf of the Editors of the "Applied Microbiology and Biotechnology", we look forward to your continued contributions to the Journal.

Sincerely,
Alexander Steinbuchel
Editor in Chief
Applied Microbiology and Biotechnology
steinbu@uni-muenster.de

**Date Sent:**

02-Sep-2008