PEI-mediated Transient Gene Expression in Cell Culture for the Rapid Production of Therapeutic Proteins

A thesis submitted to the University of London for the degree of

DOCTOR OF ENGINEERING

By

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Abstract

The aim of this study was to investigate the feasibility of using transient gene expression in mammalian cell culture for the production of recombinant therapeutic proteins. This would allow rapid production of candidate molecules for pre-clinical trial testing and characterisation. For unsuccessful candidates, this would remove the time consuming and costly requirement of producing a stable cell line. For effective candidates, the removal of the immediate requirement of producing a stable cell line could reduce development times by up to 6 months. This thesis describes the development of a simple and robust transient expression system utilizing the 25 kDa branched cationic polymer polyethylenimine (PEI) as a vehicle to deliver plasmid DNA into suspension-adapted Chinese hamster ovary cells (CHO-S) for the large-scale production of recombinant therapeutic proteins. Optimisation was done in a serum free environment, to make the process industrially applicable. However, removal of serum was found to dramatically affect the cell specific growth rate and transient transfection expression rate. This problem was negated by the addition of bovine serum albumin, and a concentration of 6 mg L⁻¹ was used in subsequent optimisations.

The PEI mediated transfection process was optimised with respect to PEI nitrogen to DNA phosphate ratio and the plasmid DNA mass to cell ratio, using a reporter construct encoding firefly luciferase. Optimal production of luciferase was observed at a PEI nitrogen to DNA phosphate molar ratio of 10:1 and 5 µg DNA per 10⁶ cells. It was found that PEI is cytotoxic to CHO-S cells, and the final concentration of PEI in the growth medium appears to be the critical factor. At high PEI concentrations, cell growth is reduced, which is coupled with a reduction in the transgene expression. Therefore at ratios greater than 10:1, or DNA concentrations higher than 5 µg DNA per 10⁶ cells, cell growth rate, and therefore transgene expression was lower. This suggested that transient gene expression is linked to cell growth, and therefore cell division. Cell cycle blocking experiments demonstrated that transition into mitosis is a pre-requisite for efficient transient gene expression.

In order to manipulate transgene expression at mitosis, cells were arrested in the G2/M phase of the cell cycle using the microtubule depolymerising agent
nocodazole. Using secreted human alkaline phosphatase (SEAP) and enhanced green fluorescent protein (eGFP) as reporters it was shown that continued inclusion of nocodazole in cell culture medium significantly increased both transfection efficiency and reporter protein production. In the presence of nocodazole, greater than 90% of cells were eGFP positive 24 h post transfection and the cell specific SEAP production rate ($q_{SEAP}$) was increased almost five-fold, doubling total SEAP production. Under optimal conditions for PEI-mediated transfection, transient expression of a recombinant chimeric IgG4 encoded on a single vector was similarly enhanced by nocodazole, with a final yield of approximately 5 μg mL$^{-1}$ achieved at an initial viable cell density of 1 x 10$^6$ cells mL$^{-1}$. The glycosylation of the recombinant antibody at Asn$^{297}$ was not significantly affected by nocodazole during transient production by this method. The mode of action of nocodazole was apparently related primarily to increased cell volume. On treatment with nocodazole for 48 h, CHO cells increased in volume by up to five-fold, with a corresponding increase in recombinant DNA content. However, the nuclear membrane of nocodazole treated CHO cells exhibited a progressive lack of integrity, which may be associated with increased nuclear trafficking of recombinant DNA.

In summary, the development of an optimised transient expression system that can utilize parental CHO cells and simple plasmid constructs "off the shelf", without the necessity for host cell or gene vector engineering has been demonstrated. The process was optimised in serum free medium and CHO-S cells for optimal industrial relevance. This generic process could therefore be used for the rapid production of any new therapeutic protein, once a plasmid construct has been produced.
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<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby Hamster Kidney</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine Spongiform Encephalitis</td>
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<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>COS</td>
<td>African green monkey kidney cell transformed with SV40</td>
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<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>DOPE</td>
<td>Dioleoylphosphatidylethanolamine</td>
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<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin, of the G subclass</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MAAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localisation Signal</td>
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<tr>
<td>NPC</td>
<td>Nuclear Pore Complex</td>
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<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Light Units</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>SEAP</td>
<td>Secreted Alkaline Phosphatase</td>
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<td>SFM</td>
<td>Serum Free Medium</td>
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<td>SV40</td>
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<td>ULB</td>
<td>Ultra Low Binding</td>
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Chapter 1. Introduction

1.1 The current status of therapeutic protein production

The aim of this project is to investigate the possibility of using transient gene expression for the production of recombinant therapeutic proteins. Since 1982, when the first recombinant therapeutic protein (human insulin) was released onto the market, there has been an explosion in research and development into other potential candidates (Lubiniecki, 1998). Therapeutic proteins were originally developed to treat diseases where patients were deficient in, or had a lower than average concentration of a particular protein. Previously, the proteins would have been extracted from human or animal donors and purified before administration. This process was limited by the supply of source material, which also carried the risk of viral contamination. However, with the advent of recombinant expression technologies, the need for this source has been removed. Recombinant expression is the insertion of foreign genes into host organisms or cells that subsequently express the protein of interest. The advantages that recombinant expression technologies gave were: the removal of risk of contamination; the ability to develop proteins previously unavailable; and the ability to produce large quantities of protein material.

There are now a number of licensed therapeutic proteins available for the treatment of a range of diseases (Buckel, 1996; Lubiniecki, 1998). The trend in their popularity is likely to continue, because they are generally more successful and have fewer side effects than traditional small-molecule drugs (Dove, 2002). Of the therapeutic proteins presently under investigation a large proportion are monoclonal antibodies. This class of therapeutic proteins have certain advantages, and therefore will be used as the model recombinant protein in this discussion.

1.1.1 Monoclonal Antibodies the ‘magic bullets’ of therapies

Antibodies are large protein structures of the immune system that recognise and eliminate foreign material. Antibodies are multimeric proteins consisting of two identical heavy chains, and two identical light chains. The light chain consists of two domains, the constant and variable regions. The heavy chain consists of four
domains, three constant and one variable (Figure 1.1). The heavy and light chains are covalently linked through disulphide bonds. The two heavy chains are similarly linked in what is known as the hinge region. Antibodies have two binding regions, the antigen and complement/Fc receptor binding regions, which together bring about an immune response. The latter binding region contains an N-glycosylation site at Asn297, which is thought to be critical for its bioactivity (Wright, et al., 1997).

When the immune system is presented with foreign material (antigens), antibodies bind to it via specific antigen binding sites. The antibody then binds to components of the complement pathway, initiating clearance of the antigens. In addition, the antibody binds to B-cells, activating them to produce more antibodies. However, because there is a plethora of potential antigens available, the antibodies of the immune system are polyclonal, i.e. different antibody molecules have slightly different antigen binding sites. However, when an immune response is elicited, some of the activate B-cells mature to produce monoclonal antibodies (i.e. the same antigen binding site). It was therefore thought that if monoclonal antibodies could be produced against protein epitopes of diseases, effective treatments could be delivered.

![Antigen Binding Site](image.png)

**Figure 1.1 A representation of the structure of an antibody molecule.** Taken from www.biology.arizona.edu/immunology/tutorials/antibody/structure.html
Monoclonal antibodies were thought to be the ‘magic bullets’ of therapeutic proteins because of their high specificity, and inherent ability to recruit the body’s natural immune system. They also potentially allow the treatment of any disease that has a protein component that can be selectively targeted. However, antibodies are large (~150 kDa) proteins that have complex carbohydrate moieties and are therefore difficult to produce recombinantly. In 1975, Kohler and Milstein (Kohler, et al., 1975) developed methods for the production of murine antibodies in mammalian cell culture. However, these were generally unsuccessful as therapeutic proteins because they had a low serum half-life, were not able to trigger human effector functions. They were also found to elicit human antimouse-antibodies (Khazaeli, et al., 1991; Kipriyanov, et al., 1999). However, since these initial trials, it was found that only a hypervariable region of the antigen-binding site was required for efficacy. Therefore, chimera antibodies were produced by recombination of human and mouse antibody genes. These antibodies can now be expressed with approximately a 90 to 95 % human content, with only the antigen specific region of the antibody being murine (Jones, et al., 1986). These ‘humanised’ antibodies were much more successful because they were able to evade the human immune system, and in 1994 the first chimaeric antibody was approved by the Food and Drug Administration (FDA). Since then a further nine antibodies have been approved for production and sale (Gura, 2002).

Research into creating entirely human antibodies continues, and is lead by the development of phage display libraries. These consist of a library of human antibody genes that are incorporated into bacteriophages. The phages can then reproduce in *Escherichia coli* and an antibody can be selected from the library using an immobilised antigen (Winter, 1994). Other groups however, have genetically engineered mice to produce human antibodies. Mouse antibody genes were knocked out and replaced with human antibody genes. Therefore, when the mice are injected with an antigen, they produce human antibodies (Choi, et al., 1993; Fishwild, et al., 1996). It is clear that there is great potential in using monoclonal antibodies to treat a wide range of indication. However, the increased activity in their development has lead to a worldwide shortage of production facilities, which has lead to the search for alternative methods for the manufacture and selection of promising candidates.
1.1.2 Alternative expression systems for the production of therapeutic proteins

For any therapeutic protein to be successful there must be an easy and reliable system in which expression is possible (i.e. the protein can be cloned for expression in a particular cell line). The cell line must provide an environment that allows expression of a protein that is as close to its native form as possible. This is to ensure that its therapeutic properties are retained. In addition, the expression system should allow easy recovery of the recombinant protein using a cost effective process. There are a number of different systems available for recombinant protein expression, ranging from the use of bacterial cells through to transgenic animals. Each system has its advantages and disadvantages, which will be discussed here.

1.1.2.1 Bacterial

The expression of heterologous proteins in bacterial cell lines is a very cost-effective method for producing simple proteins or polypeptides. The most commonly used organism is *Eschericia coli* (*E.coli*), the so-called ‘work horse’ of expression systems. *E.coli* is a very well characterised organism, and is therefore considered by the regulatory authorities as an acceptable system for recombinant expression. It also has the advantages that it can be easily grown on cheap substrates; has a short doubling time (~20 minutes) and can be grown to very high cell densities (Baneyx, 1999). Proteins are most commonly expressed from plasmids that are replicated by the host organism. This means that plasmid copy number can remain high even in daughter cells. Heterologous proteins either accumulate into the cytoplasm, or can be engineered for secretion into the periplasm. The former usually leads to protein aggregates called inclusion bodies, which are more easily purified, but usually have to be refolded *in vitro.*

Although bacterial expression systems are cheap and simple to use, they are limited in their abilities to produce mature, active human proteins. The *in vitro* folding that can be required often doesn’t yield a high percentage of correctly folded protein. This problem becomes more pronounced with larger, and more complicated proteins, such as monoclonal antibodies. Another major draw back of bacterial expression systems is they lack the cellular machinery to glycosylate proteins, a factor that has
been shown to be important in their efficacy (Wright, et al., 1997). Therefore, only simple, aglycosylated proteins can be produced in bacterial systems, such as insulin or Fab and scFv fragments of antibodies (Sanchez, et al., 1999). This system therefore precludes most therapeutic antibodies under investigation, which are generally whole and glycosylated.

1.1.2.2 Yeast

Recombinant expression in yeast cells has many of the advantages of bacterial expression, in that they are easy and relatively cheap to grow, they have a short doubling time and can be grown to high cell densities. However, they have additional eukaryotic specific post-translational functions, such as certain folding mechanisms, the ability to form disulphide bonds, and post-translational glycosylation. In this respect, they produce more human like therapeutic proteins. The most commonly used strains are Saccharomyces cerevisiae and Pichia pastoris both of which have been used to produce a number of recombinant proteins (Cereghino, et al., 1999; Romanos, et al., 1992). However, although some glycosylation occurs in yeast, it is different to that found in mammals (Brethauer, et al., 1999; Cupit, et al., 1999). Yeast cells produce similar O-linked glycans to mammals; however, its N-linked glycans are different (Romanos, et al., 1992). These incorrectly glycosylated proteins are usually not active or can be immunogenic. For this reason, yeast cells are not used to produce whole monoclonal antibodies.

1.1.2.3 Transgenic Plants

An alternative expression system that has recently received a lot of interest is the production of recombinant proteins in plants, or plant cell suspensions. This is because their glycosylation pathways are very similar to those of mammals, and the post-translational modifications yield correctly folded, multimeric, active proteins. Antibodies that have been produced in plants (‘plantibodies’) have as high specificity and affinity as those produced in the parental monoclonal cell (Fischer, et al., 1999). Recombinant proteins can be expressed in plants cells in suspension, or in fully-grown plants. The obvious advantages to transgenic plants are: they are
relatively cheap to grow, only requiring water, a few nutrients and light; they are easy to scale-up, using agricultural techniques; and they could potentially be hybridised to form more complex multimeric proteins. However, because the recombinant protein expressed currently represents a very small proportion of the total protein present, typically less than 1 % (Kusnadi, et al., 1997) there may be problems with downstream processing, they also have a relatively long lead time before any protein material can be produced. In addition, there are concerns regarding public opinion about the release of genetically modified organisms into the open environment (Doran, 2000). However, transgenic plants represent a very cost effective method for the production of native like therapeutic proteins.

1.1.2.4 Insect cells

Another expression system that produces proteins with post-translational modifications similar to mammalian cells is insect cell culture. In fact, only slight differences in the glycosylation of proteins sets them apart from mammalian cells (Altmann, et al., 1999). Insect cells are therefore able to produce recombinant proteins with similar activities to those produced in mammalian cells. Most insect cell expression systems are by infection of the cells with recombinant baculovirus particles. Upon infection, virus particles begin to replicate themselves, whilst also expressing the heterologous gene. However, this has the disadvantage that virus particles must be produced for infection of the insect cells. Therefore, some groups are now looking into producing established insect cell lines (McCarroll, et al., 1997; Pfeifer, 1998). Insect cell cultures can be grown in serum free media, and sometimes protein free media, which makes the process less expensive and reduces the biosafety risk associated with serum products. However, when compared to mammalian expression systems the fermentation can be more complicated because of the high oxygen demand and shear sensitivity of insect cells (Pfeifer, 1998). Although not the preferred method, insect cells are a viable alternative for the production of therapeutic proteins.
1.1.2.5 Mammalian cells

Production of therapeutic proteins, and more specifically therapeutic antibodies, is most commonly done in mammalian cell systems. In the period from January 1996 to November 2000, 21 of the 33 biological products approved by the FDA were manufactured in mammalian cells (Chu, et al., 2001). The popularity of mammalian cells is their ability to correctly fold and glycosylate large and complex proteins (Birch, 1991). For this reason, there has been a lot of research into the optimisation of mammalian cell cultures, which can now yield high titres of recombinant antibodies (Reff, 1993). This has yielded very robust and efficient expression systems, which are now recognised by regulatory bodies. Mammalian cell culture also has the huge advantage over transgenic animals and plants that protein material can be produced approximately 6 months after cloning of the transgene. There are three main cell lines used in industrial processes, Chinese Hamster Ovary cells (CHO), Mouse myeloma cells (NS0) Baby Hamster Kidney cells (BHK) (Birch, 1991; Chu, et al., 2001). One of the disadvantages of mammalian cell culture is the requirement for serum in the growth medium, which increases costs, and has associated biosafety risks. However, recent development in serum free media has meant that a lot of mammalian cell culture can be done in a serum free environment. An additional drawback is that at present the cost of production is very high compared to other recombinant expression systems, being approximately 150 times more expensive than transgenic animals (Dove, 2002). Regardless of this, mammalian cell culture is still the preferred method for the production of therapeutic proteins.

1.1.2.6 Transgenic animals

Recombinant protein expression in transgenic animals works in a similar way to expression in transgenic plants. The transgene is inserted into one-cell embryos, from which transgenic animals are bred. This system has been used in goats, where the recombinant protein is expressed in the milk (Pollock, et al., 1999) and in chickens, where it is expressed in their eggs (Morrow, 2000). Once established, transgenic animal are a very cost effective way of producing therapeutic proteins, and scale-up is based on agricultural economics (Dove, 2002). In addition, the
expression sites are from cells that are specifically designed for the secretion of very high protein concentrations. However, although the antibodies produced so far are near human, it has yet to be shown whether this will affect their activity in humans. Transgenic animals also have the disadvantage that production of protein material for phase I clinical trials can take 18 and 24 months to produce, in chickens and goat respectively (Chadd, et al., 2001), which could be an unacceptable delay in the development process.

1.1.3 Addressing the bottleneck in mammalian cell culture

Although the previous section demonstrated that there are a number of systems available for the production of recombinant proteins, it remains that mammalian cell culture is the preferred method. However, the massive increase in the number of therapeutic proteins being developed has meant that production facilities are struggling to keep up with the demand for clinical grade material. If we just consider therapeutic monoclonal antibodies, there are currently ten on the market (Gura, 2002), which nearly consume the manufacturing capabilities presently available. In addition to this, there are as many as 500 in development (Dove, 2002), which will require manufacturing capacity to produce material for clinical trials. This is compounded by the fact that on average, biopharmaceuticals have a 40% success rate from pre-clinical trials to launch (Struck, 1994), suggesting there could be an explosion in the number of licensed biopharmaceuticals being manufactured. Although some of the alternative recombinant expression systems may alleviate some of this problem, it is clear that other strategies are required. In addition, most therapeutic treatments require repeat doses, which need to be affordable. Therefore, it would be advantageous to reduce the development and production costs of therapeutic proteins.

One of the most labour intensive and costly procedures in early-stage development of a recombinant protein is the production of stable cell lines. This is the incorporation of the recombinant gene into the host genome. This can take up to 6 months, which could ultimately be wasted if the candidate does not pass pre-clinical trials. Transient transfection is an alternative method for the rapid production of protein material (Durocher, et al., 2002; Wurm, et al., 1999), which might then be
used for pre-clinical trials. Using this technique, the recombinant protein is expressed episomally from plasmids transfected into the mammalian cells. This has the advantage that protein material can be produced in a matter of weeks, rather than months. It therefore potentially enables the: (i) initial high-throughput in vitro studies (e.g. bioassay development, biophysical characterization) implicit in a choice of therapeutic product and (ii) production of larger quantities of recombinant protein for in vivo testing. This will reduce development times, and therefore costs, and eliminate the need to make stable cell lines of unsuccessful candidates, which will also help reduce the manufacturing demand. However, the titre from transient transfection is relatively low compared to that from stable cell lines (~20 mgL⁻¹ compared with up to 1 gL⁻¹). In addition, transient transfection until recently has only been used at the laboratory scale. Hence, for transient transfection to become a useful tool in recombinant gene expression, it must be inherently scaleable and industrially applicable. The following discussion will describe the challenges presented by this problem, and how these might be overcome.

1.2 Transient transfection in mammalian cell culture

Transient transfection is the process of transporting recombinant DNA from the external cellular environment to the nucleus of cells, where expression of the transgene can occur. The whole process from isolation of the recombinant gene through to its expression represents a number of obstacles that need to be overcome. The first obstacle is the cloning of the recombinant gene into a vector that allows efficient delivery and expression of the transgene. In addition, the vector must be easy to propagate, so that large numbers of the vector can be produced. Once a suitable vector has been chosen and engineered, the issue of entry into the cell must be considered. Figure 1.2 shows a representation of the route of entry a vector must take.

There are two main barriers that a recombinant vectors must overcome before expression can be achieved. (1) Transfer from the extracellular environment into the cytoplasm. After which, the vector must then be transported through the cell and across the nuclear envelope (2). The vector must therefore be capable of overcoming
these cellular barriers, and delivering the recombinant DNA into the nucleus in a configuration compatible with translation. The following section will discuss the types of vectors available, and how they overcome these transfection challenges.

![Diagram of recombinant vector transfection](image)

**Figure 1.2 A representation of the route taken by recombinant vectors during transient transfection.** (1) Transfer from the extracellular environment into the cytoplasm. (2) Transfer from the cytoplasm to the nucleus.

### 1.2.1 DNA transfection vectors

Recombinant gene vectors fall into two broad categories, viral and non-viral. Transfection using a viral vector is done by integration of the recombinant gene into the genome of a virus. This is followed by infection of the host cell with the virus particle, with subsequent expression of the viral genome. In contrast, non-viral methods normally use plasmid DNA vectors to transport the recombinant gene to the nucleus. The plasmid contains the information required for replication (and therefore propagation) in bacterial cell lines and the transgene of interest under the
control of a mammalian promoter. It does not, therefore have an inherent ability to enter mammalian cells.

1.2.1.1 Viral transfection

A number of viral vectors for the transfection of mammalian cells already exist, including alphaviruses (Blasey, et al., 1997), adenoviruses (Nadeau, et al., 1996), and vaccinia viruses (Bleckwenn, et al., 2003). Viral vectors are normally attenuated version of viruses that naturally infect mammalian cells. This means that their ability to infect cells and express their genome is maintained. However, they are unable to make complete viral particles that can then go on to infect further cells. Viruses have naturally evolved to overcome the cellular barriers identified in the previous section, and are therefore extremely efficient at transferring recombinant genes into their nuclei. They also harbour mechanisms by which they can hijack the cellular machinery to divert energy into the expression of their own proteins. This combined with their ability to replicate results in the accumulation of very high concentrations of the recombinant protein (up to 10 – 20 % of cellular protein in adenoviruses; (Garnier, et al., 1994). However, whilst they show promise for recombinant protein production and gene therapeutic applications, they suffer the limitations of restricted targeting to specific cell types, limited DNA carrying capacity, relative complexity and high costs of production (Crystal, 1995). In addition, although viral vectors are in an attenuated form, there remains the biosafety risk that reverted, viable virus particles could appear in the final product. With this in mind, viral transfection was ruled out as a suitable tool for large-scale transient transfection in a modern industrial setting.

1.2.1.1 Non-viral transfection

Non-viral vectors, whilst less efficient than viral vectors, are safer, not cell type specific and have a much higher DNA carrying capacity. The vector, which is normally a small plasmid, does not inherently have the sequences necessary for efficient expression of the recombinant protein and these must therefore be engineered into the DNA. The minimum required sequences a plasmid must contain is: (1) a bacterial origin of replication; (2) a bacterial selectable marker; (3) a
mammalian promoter and termination sequence for expression of the transgene. The first two elements are required for the amplification of the vector in a bacterial host; the last is required for correct translation of the gene in the mammalian host. These elements are the minimum required for transient transfection, however, most vectors also have a mammalian selectable marker for cloning of a stable cell line.

Plasmid vectors are normally replicated and amplified in bacterial hosts, such as *E.coli*. For this a bacterial origin of replication with a high copy number is important. This will allow the easy production of large quantities of recombinant DNA. One such origin of replication is a mutant form of pMB1, which was found to yield copy numbers of 500-800 (Brown, 2000). In addition, the vector must also have a bacterial selectable marker. The marker imparts upon the bacteria a selectable advantage over those that do not contain the vector. Therefore, only bacteria that contain the vector will grow. The most commonly used method is the addition of an antibiotic resistance gene. Selection can then be done through the addition of the appropriate antibiotic.

Non-viral vectors also require a mammalian promoter and termination sequence, to ensure correct transcription and translation of the transgene in the cell host. The promoter and termination sequences for recombinant gene expression in mammalian cells are normally taken from viruses that infected mammals (Colosimo, *et al*., 2000). This is because they are specifically designed for the efficient expression of proteins in hijacked mammalian cells. Promoters direct the translation of genes through the recruitment of transcription factors to the start of the gene. The most commonly used promoters in mammalian cell expression systems are the human cytomegalovirus (hCMV) immediate early promoter, and the SV40 virus promoter (Makrides, 1999). This is because they have been shown to have a high affinity for mammalian transcription factors, and therefore direct efficient expression. Most eukaryotic mRNAs posses a polyadenylate tail (n ~ 200) at the 3' end, which is important in mRNA stability and translation (Jackson, *et al*., 1990). This is added during cleavage of the primary mRNA molecule, which involves a coupled polyadenylation reaction (Proudfoot, 1996). The signals for polyadenylation are well documented, and the most commonly used sequence in vectors is that from the SV40 early transcription unit (Van der Hoff, *et al*., 1993).
Although certain qualities can be engineered into non-viral vectors to make them more viral-like in their expression, they still lack the ability to efficiently ‘infect’ cells and transport the recombinant DNA into the cells nucleus. For this they need a transfection agent to overcome the barriers outlined in diagram 1.1. These will be discussed in the following section.

1.3 Transport of vectors into mammalian cells

Viral vectors have the advantage that they are highly efficient at infecting cells, however, non-viral vectors require transfection agents to aid transfer of the recombinant DNA to the nucleus. As previously discussed, the main barriers to DNA vectors are the cell membrane, transfer through the cytoplasm, and transport across the nuclear envelope. Most transfection methods were originally designed to overcome the plasma membrane, although since then modifications have been made to tackle the additional barriers within the cell. The transfection methods available can be split into two categories, mechanical/electrical and chemical. The former rely on physical disruption of the cell membrane, which allows transfer of DNA vectors into the cell. The latter rely on adjusting the DNA vector chemically, so that cells internalise the newly formed complex through its endocytotic pathway.

1.3.1 Mechanical/Electrical transfection of mammalian cells

Non-viral vectors are too big to be transported across the plasma membrane of cells through transport channels. Therefore, the basis behind mechanical transfection is the physical tearing of the plasma membrane to create pores, which allow vectors to enter the cell. This ‘opening’ of the plasma membrane must be a transient process and therefore the conditions used should be chosen carefully. If the conditions are too harsh, the cell will lyse and die. However, if the conditions are too mild, pores will not form for long enough, and transfer of the vector will not be efficient. There are currently two main mechanical transfection methods that have been described, electroporation (Neumann, et al., 1982), and_filtroporation (Williams, et al., 1999).
1.3.1.1 Transfection using electroporation

Electroporation uses the principle that an electrical pulse can cause polarisation in a cell, which allows the plasma membrane to become permeable to charged macromolecules (Mir, et al., 1988). Since its invention, it has probably become the most commonly used transfection method at the lab scale. The efficiency of transfection by electroporation is dependent upon several factors including electrical field, pulse duration and pulse number (Rols, et al., 1992), which is usually cell type specific. Electroporation has the advantage that it gives a relatively high transfection efficiency of about 80% (Parham, et al., 1998) of viable cells. However, at least 30% of the cells do not survive the treatment, giving an overall transfection efficiency of approximately 55%. Recent attempts to scale-up electroporation by using a flow chamber, have reported flow rates of 7ml/min and 18.5 ml min$^{-1}$, and transfection efficiencies of between 25 and 35% (Parham, et al., 1998; Rols, et al., 1992). However, the technique seems to be inherently incompatible with large-scale transient transfection. Not only would transfection take a long time (using the flow rates described above), it would require a lot of energy.

1.3.1.2 Transfection using filthroporation

Filtroporation, and a similar technique, syringe loading (Clarke, et al., 1992; Waldman, et al., 1998), are relatively new techniques. They work using the principle that if cells in a vector containing solution, are forced through an orifice smaller than their diameter, shear forces will create transient pores, allowing transfer of the DNA vectors into the cell. This is a relatively new technique and has therefore only been used for the transfer of macromolecules (Clarke, et al., 1992; Williams, et al., 1999), although its use for the transfer of plasmid DNA is implied. Although it might seem feasible to use filthroporation at a large-scale, the technique is also very harsh upon the cells. There is typically a 30% loss in cell viability after transfection with only 40-60% of viable cells being transfected (Williams, et al., 1999).

Mechanical transfection methods are by their nature very naïve methods for the introduction of DNA material into cells. Although the plasmid DNA is introduced
directly into the cytoplasm, there is no provision for the transport through the cell, and into the nucleus. Although attempts have been made to scale-up mechanical transfection methods, the harsh shear environments required for efficient transfection are still a major drawback

1.3.2 Chemical transfection of mammalian cells

The transfection of naked plasmid DNA into mammalian cells is a very inefficient process (Pollard, et al., 1998). Therefore, different chemicals and macromolecules have been developed to aid this process. One of the main barriers identified in plasmid-based transfection is the cell membrane (Nishikawa, et al., 2001; Pouton, et al., 1998). This is because the extracellular face of mammalian cell membranes are negatively charge, and therefore represent a repelling force to the anionic plasmid DNA vectors. Therefore, most chemical transfection methods are designed to overcome these repulsion forces, to allow an interaction between the plasmid vectors and the cell membrane. It is thought that once a plasmid vector can become associated with the cell membrane, it will then be internalised through the cells endocytotic pathways (Colosimo, et al., 2000; Luo, et al., 2000).

Endocytosis is a continual process by which mammalian cells internalise macromolecules from their surrounding environment (Figure 1.3). The process begins with formation of a small indentation of the plasma membrane, which eventually buds off into the cell to form an endosome (1). The endosome now contains any material that was bound to the external face of the plasma membrane. The endosome is transported through the cytoplasm, during which time useful nutrients and molecules are removed. The pH in the endosome is slowly reduced, via a H⁺ pump mechanism, which allows fusion with the lysosome (2). Degradation enzymes then break down any remaining matter to their basis units, which are either used within the cell, or targeted for removal (3).
Figure 1.3 A diagram to represent the endocytosis pathway in mammalian cells (1-3) and the desired route of plasmid-based recombinant gene vectors (1, 4, and 5). (1) The vesicles which have budded of the plasma membrane fuse with endosomes (~pH 7). (2) Hydrogen pumps reduce the pH of the endosome to ~pH 5, allowing fusion with the lysosome. (3) After fusion with the lysosome, macromolecules are degraded to the basic units. (4) Transfer of recombinant vectors to the cytoplasm before endosomal fusion with the lysosome. (5) Transport to, and across the nuclear envelope for eventual expression.

It is clear that if chemically mediated transfection does utilise this pathway a large proportion of the transfected plasmids will be degraded in the lysosome (Akinc, et al., 2002). However, some plasmids must avoid this fate because transgene expression does occur. It is not clear how plasmid vectors avoid trafficking to the lysosome, but early release from the endosome would obviously be an advantage (El Ouahabi, et al., 1997; Luo, et al., 2000; Luo, et al., 2000). Once plasmids have escaped from the endosome into the cytoplasm (4), it must then be transported through the cell and across the nuclear envelope (5). Transfer through the cytoplasm is a critical stage, and it has been estimated that due to the presence of nucleases, the half-life of plasmids in the cytoplasm is approximately 50-90 minutes (Lechardeur, et al., 1999). Once at the nucleus, the vector must then overcome the next major barrier, the nuclear envelope. This is now considered the major challenge of transfection technologies today (James, et al., 2000; Pollard, et al., 1998). Again, the mechanism behind this process is unknown, and various strategies to overcome
this have been investigated. The following discussion will describe the basic transfection agents presently available, and how these may be enhanced to tackle the problems identified above.

1.3.2.1 Calcium Phosphate precipitation

Calcium phosphate precipitation was one of the first non-viral transfection techniques described (Graham, et al., 1973), and has been used to transfect a number of different cell lines (Chen, et al., 1987; Chen, et al., 1988; Meissner, et al., 2001). Transfection is achieved through the mixing of calcium and phosphate ions with vector DNA. Theses bind together through electrostatic attractions, forming co-precipitates. The resulting precipitate can then adsorb to cell membranes, and be internalised through endocytosis (Jordan, et al., 1996). It is also possible to form the co-precipitate in the culture medium (Chen, et al., 1987), which is thought to increase transfection efficiency, because the particles are able to form directly on the cell surface. This method has been shown to be effective in a number of cell types. Although this is a simple method to use, it has the disadvantage that transfection efficiency is typically only between 40-60%, depending on the cell line (Jordan, et al., 1996). In addition, optimal transfection conditions are seldom the same in different cell types (Urabe, et al., 2000). It can also become cytotoxic if left in the cell culture too long, and must therefore be removed a few hours after transfection. It also has the drawback that its optimal conditions fall into a narrow range of a number of physiological parameters, including the temperature and pH of the medium; time for precipitate to form; and DNA concentration, and the transfection efficiency drops dramatically if precipitates are left for too long before transfection (Jordan, et al., 1996). However, with all these drawbacks, calcium phosphate precipitation is a very cost effective method, which makes it appealing for large-scale transient transfection.

1.3.2.2 Cationic polymers

Cationic polymers are macromolecules of repeating subunits that contain ammogroups. They included synthetic and natural polymers that vary in shape, size and cationic species (Ramsay, et al., 2000) However, all have the ability to condense
DNA through electrostatic interaction (Bloomfield, 1996; Mahato, et al., 1999). This reaction allows the targeting, and subsequent transfection of DNA particles in mammalian cells. Three cationic polymers commonly used for transient transfection are poly-L-lysine (PLL)(Ramsay, et al., 2000), polyethylenimine (PEI)(Boussif, et al., 1995) and chitosan (Leong, et al., 1998). Poly-L-lysine is a linear macromolecule of repeating lysine residues, commercially available at molecular weights of between 1 to 300 kDa. However, these preparations are heterogeneous, which can complicate complex formation. In addition, PLL is cytotoxic, which may limit its application (Putnam, et al., 2001). Polyethylenimine (PEI) is a water-soluble polymer with either a branched or linear structure and a variable molecular weight (Horn, 1980). Noted for its ability to precipitate DNA in the early 1970’s (Atkinson, et al., 1973), PEI has also been demonstrated to effectively mediate the efficient transfection of a variety of mammalian cell types (Boussif, et al., 1995; Ramsay, et al., 2000). It has the same problems associated with PLL, although its cytotoxicity appears to be lower (Mahato, et al., 1999). Chitosan is a biodegradable polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine (Özbastur, et al., 2003). Chitosan is thought to condense DNA through the interaction with its phosphate groups. Chitosan molecules are also available in different molecular weights ranging from 2 to 540 kDa.

The ability of cationic polymers to promote transfection is dependant on the molecular weight, and the specific ratio of polymer to DNA. This ratio must normally be optimised for different cell types before efficient transfection can be achieved. The advantage that cationic polymers have over other transfection agents is their ability to produce a highly condensed particle. Of the cationic polymers available, PEI is probably the most promising candidate for transient gene expression, and is therefore probably the most described in the literature. This is because as well as efficient condensation of DNA, PEI is also thought to prevent lysosomal degradation of the recombinant DNA. After condensation, the PEI-DNA complexes are internalised by through endocytosis and directed towards the lysosome. However, unlike other transfection methods, it is thought that release from the endosome into the cytoplasm is much more efficient. It has been hypothesised that PEI acts as a buffer, and therefore prevents lowering of the endosomal pH. This buffering capacity is thought to prevent fusion of the endosome
and lysosome, resulting in eventual rupture of the endosome (Abdullah, et al., 1996; Dunlap, et al., 1997). The PEI-DNA complexes are therefore released into the cytoplasm before being exposed to the degradation enzymes of the lysosome. In addition, it is thought that cationic polymers remain tightly bound with the DNA in the cytoplasm, and therefore protect it from nucleases present. It is also thought that the basic nature of these molecules act like and nuclear localisation signal, targeting the complex to the nucleus. The localised complex then enters the nucleus allowing expression of the recombinant gene. Although cationic polymers are cytotoxic to some cells, they have all the advantages mention above and they are relatively simple and cost effective to produce. This makes them a very viable candidate for large-scale transient gene expression.

1.3.2.3 Lipid-mediated transfection

Cationic lipids have become a popular method for the introduction of plasmid DNA into mammalian cells (Huang, et al., 1997; Nishikawa, et al., 2001). They are very efficient systems because their charged heads are able to bind to DNA, whilst their hydrophobic tails interact with the cell membrane. The complexes formed between cationic lipids and DNA are termed lipoplexes, and are thought to enter the cell via direct fusion with the cell membrane (Matsui, et al., 1997) or through the endocytosis pathway (Furth, et al., 1991; Zabner, et al., 1995). Since their inception, several hundred cationic gene delivery systems have been described (Lasic, et al., 1996), such as quaternary ammonium detergents, cationic derivatives of diacylglycerol and lipid derivatives of polyamines. Most cationic lipid systems include neutral lipids, such as DIOleoylphosphatidylethanolamine (DOPE) or cholesterol, in their complex, which act to increase release of the complex from the endosomal compartment (Farhood, et al., 1995; Hafez, et al., 2001). However, the amount of DNA uptake into cells does not necessarily correlate with transfection efficiency (Farhood, et al., 1992), suggesting that intracellular trafficking, or transport into the nucleus is a limiting factor. The structure function relationship of lipoplexes has been extensively investigated to determine the characteristics that give optimal expression (Audouy, et al., 2001; Birchall, et al., 1999; Braun, et al., 2003; Ferrari, et al., 2002). Although the zeta potential of the complex is clearly important (Son, et al., 2000), there has been no single characteristic that determines efficient
transient gene expression, and is generally dependent on the liposome used, the lipid:DNA ratio and the cell type.

Although lipoplexes are apparently very efficient at introducing DNA into mammalian cells, their multi-component nature makes their construction and optimisation inherently complicated. This is further complicated when neutral helper lipids are added. Lipoplexes are also relatively expensive to produce, as the lipid components can be costly to synthesis. In addition, there is some evidence that lipoplex become cytotoxic at higher doses (Templeton, et al., 1997). However, because cationic liposome transfection is commonly used in gene therapy, they have already been used in clinical trials (Caplan, et al., 1995; Porteous, et al., 1997; Sorscher, et al., 1994). Therefore, the technology has already been accepted by the regulatory authorities, which could be a consideration in the context of this thesis.

1.3.2.4 Enhancement of chemically mediated transient transfection

There are a number of techniques available for the enhancement of the basic transfection agents mentioned above. These are all designed to overcome the intracellular barriers to transient gene expression. Therefore, they are mainly concerned with avoidance or early release from the endosome pathway, and targeting to, and translocation across the nuclear envelope. The endosomal barrier is normally overcome using peptides derived from common viruses, whereas the nuclear barrier is tackled with short nuclear localisation signals (NLS).

Virus particles achieve early release from the endosome using fusogenic peptide sequences. These are normally active membrane sequences, located at the amino-terminus of viral protein, which mediate membrane translocation upon acidification. These contain hydrophobic and hydrophilic repeats that are able to form an amphipathic α-helical structure, which is thought to either cause membrane disruption, or pore formation (Plank, et al., 1998; Wagner, 1998). The most commonly used fusogenic peptide has been the N-terminal sequence of the influenza virus haemagglutinin subunit HA-2 (Wagner, et al., 1992), however, others are available (Kichler, et al., 1999; Morris, et al., 2000; Wolfert, et al., 1998). These
fusogenic peptides have been shown to increase transfection efficiency when associated with PLL, cationic lipids, PEI or polyamidoamine cascade polymers (Morris, et al., 2000). An alternative approach to avoiding lysosomal degradation of recombinant vectors is to bypass the endosomal pathway all together. Morris, et al, have achieved this through the utilisation of a combined peptide that contains an HIV-1 fusion peptide and the SV40 nuclear localisation signal (Morris, et al., 1997). This mediates the translocation of the complex directly into the cytoplasm, and its trafficking to the nucleus.

Although most transfection agents are able to mediate recombinant vectors to the cytoplasm, to a greater or lesser extent, there still remains the issue of nuclear targeting. This has been identified as a major barrier to transfection, and therefore strategies to improve this stage of transfection have been investigated. The most commonly used technique is the addition of nuclear localisation signals (NLS) to the transfection agent. NLS are normally short basic peptides that interact with the nuclear pore complex (NPC), and ultimately direct transport into the nucleus (Chan, et al., 2002). A number of different NLS have been show to increase transfection efficiency in a range of cell types (Branden, et al., 1999; Cartier, et al., 2002; Chan, et al., 1998; Colin, et al., 2001; Ludtke, et al., 1999; Petrulis, et al., 2001; Tachibana, et al., 1999), however the mechanism by which this occurs remains unclear. During active transport, the NPC is known to increase in diameter to 26 nm (Dworetzky, et al., 1988). However, a fully condensed single plasmid molecule has a diameter of approximately 25 nm (Blessing, et al., 1998). It is therefore difficult to believe that transfection complexes are able to enter the nucleus by active transport and must enter during division. It seems likely that NLS merely increase the transport to, and association with the NPC, thereby increasing DNA transfer to the nucleus during division. They are however, an effective way to increase transient gene expression. An alternative method to the direct addition of NLS to recombinant DNA complexes is to recruit proteins that naturally occur in the cytoplasm, which are being targeted to the nucleus. It has been shown that DNA containing specific sequences bind to cytoplasmic proteins that are targeted to the nucleus. Addition of these sequences increase nuclear uptake of the recombinant genes. However, this method has the drawback that the DNA sequence required is dependent upon the DNA binding
protein being available in the cytoplasm. This will therefore require the identification of nuclear-targeted DNA binding proteins in new cell lines.

1.4 Large-scale transient gene expression and its industrial use

The use of large-scale transient gene expression in an industrial process limits some of options available for the different aspects of the process. This includes the cell line, the production medium, the recombinant vector and the transfection agent. Not only must the combination of these elements result in a system that is applicable to large scale, it must also be simple to use and implement in an industrial setting. This section describes the choices available and suggests a model for an ideal system.

1.4.1 Choice of cell line

Traditionally, transient gene expression in mammalian cells was only considered in those transformed to enable plasmid replication (Makrides, 1999). The most commonly used of these are COS and HEK293(EBNA). COS cells were generate by the transfection of African green monkey kidney cells with an origin defective SV40 (Gluzman, 1981). COS cells are therefore able to replicate plasmids containing the SV40 origin of replication. HEK293 cells were engineered to express the Epstein-Barr nuclear antigen-1, which directs replication of plasmid vectors harbouring the oriP origin of replication. This system enables both nuclear retention and autonomous replication of episomal plasmid DNA (Langle-Rouault, et al., 1998), (Sclimenti, et al., 1998; Van Craenenbroeck, et al., 2000). However, only HEK293(EBNA) cells have been investigated for their use in large-scale transient gene expression, and have been employed in a batch production processes for a variety of recombinant proteins deriving from plasmid DNA delivered to the cell via the endosomal pathway, yielding up to 20 mg L$^{-1}$ (Durocher, et al., 2002; Girard, et al., 2002; Meissner, et al., 2001)

However, at present, the most commonly used cell lines for the production of recombinant therapeutic proteins are Chinese Hamster Ovary cells (CHO), mouse myeloma cells (e.g. NS0) and Baby Hamster Kidney cells (BHK) (Birch, 1991;
Chadd, et al., 2001; Chu, et al., 2001; Lubiniecki, 1998). This is an undesirable situation, as it is well known that different host cell types confer different post-translational modifications, which may significantly affect the properties of therapeutic recombinant proteins. That is, the ability to transiently produce recombinant proteins at an early stage in product screening/development in the same host cell type that would likely be employed for the final bioprocess would clearly be advantageous. For example, HEK293 cells are known to differ to CHO cells with respect to N-glycan processing, where HEK293 cells confer specific N-glycan modifications (e.g. laciDiNAc) not associated with recombinant proteins produced by CHO cells (Van den Nieuwenhof, et al., 2000; Yan, et al., 1993). Related to this, the same recombinant protein produced by both HEK293 and CHO cells may have a different bioactivity in vitro (Haack A, et al., 1999). It would therefore be beneficial to develop a transient gene expression system that is applicable to these industrially used cell lines. The disadvantage that CHO, NS0 and BHK cells have to the HEK293(EBNA) cells is that the plasmid DNA is not replicated and would therefore be diluted after cell division. This will gradually lead to a reduction in cell specific production and therefore reduce the efficiency of the production process. Therefore, this issue must be considered during development of the process.

1.4.2 Choice of cell culture medium

Another consideration regarding the large-scale production of recombinant therapeautic proteins is the cell culture medium used. Mammalian cell culture was traditionally performed in foetal calf serum containing media. This was because the serum contains growth factors and hormones required for the efficient cultivation of mammalian cells. In addition, serum has been shown to protect cells against the apoptosis induced by nutrient deprivation and the shear forces experienced in culture (Zanghi, et al., 1999). There is also some evidence that serum increases transient gene expression (Durocher, et al., 2002). However, the emergence of Bovine Spongiform Encephalalitus (BSE) has resulted in regulatory concerns regarding the transmission of prions, along with the risk of viral contamination (Chu, et al., 2001; Lubiniecki, 1998). This has lead to a move towards serum free, and even protein free media for the production of human therapeutic proteins. The term protein free, however, can be misleading, and sometimes refers to media that only
contains proteins produced recombinantly. However, at least 50% of recombinant proteins are now produced in serum free medium (Chu, et al., 2001). It would therefore be advantageous to develop a transient gene expression that is effective in such an environment.

1.4.3 Choice of recombinant DNA vector

Although viral transient gene expression is very efficient in mammalian cells, the limitations mentioned above preclude it as a viable option for large-scale production. It is therefore evident that a plasmid-based transfection vector should be used. The vector should ideally have all the elements previously discussed. In this study, most of the plasmids used were developed in house by Lonza Biologics. However, they have been adapted to have a high copy number in bacteria, contain the gene for resistance to the β-lactam, ampicillin, and have the recombinant genes under the control of an hCMV promoter and polyA termination sequence, and are therefore ideal. A further consideration for large-scale transient transfection will be the production of the plasmid vector. However, the emergence of gene therapy has meant that there are a number of techniques available for this (Kendall, et al., 2002; Lander, et al., 2002; Ribeiro, et al., 2002).

1.4.4 Choice of transfection agent

The previous discussion has shown that there is a plethora of transfection agents available for transient gene expression. There are also a number of ways in which these basic agents can be enhanced to further increase different stages of the transfection process. However, not all the methods described would be applicable at scale. The main criteria that need to be met for this are: (1) readily available to commercial companies; (2) cost effective at scale; (3) high transfection efficiency; (4) easy to use at scale; (5) low cellular cytotoxicity; (6) does not complicate downstream processing. Of the methods described, only CaPO₄ precipitation and electroporation have been used at scale, the latter of which seems inherently impractical. The synthesis of lipid-based complexes would probably be too costly at scale, as would the use of commercial transfection agents. Although peptide based
systems appear to be promising, there seems to be little evidence that they are universally effective in a range of cell types. This leaves a choice between CaPO₄ and cationic polymers. Although CaPO₄ precipitation is an established technique, which is readily available, cost effective and has relatively high transfection efficiency, it has the drawback that cytotoxicity is an issue. Most of the protocols described therefore have a medium replacement step. This, although possible at scale, would dramatically complicate, and increase the costs, of fermentation. Cationic polymers do have an associated cytotoxicity, however, it has been shown that PEI can be left in the cell culture medium without adverse effects (Schlaeger, et al., 1999). Although PEI has been shown to complicate downstream processing (Bulmer, 1992), it was chosen as the best candidate for large-scale transient gene expression, as it meets five of the six criteria outlined above. It also has the added advantage that is can be derivatised with other elements that enhance transfer of DNA through the cell.

1.4.5 A model for large-scale transient gene expression in mammalian cells

This discussion has identified they key parameters that must be considered in the development of a large-scale transient gene expression process for the production of recombinant therapeutic proteins. These are represented in figure 1.2 and are: (1) the host cell; (2) the recombinant gene vector; (3) the vector transfection agent; (4) the cellular environment. In this discussion, the best combination for this was identified as being a suspension adapted cell line (CHO-S, NS0 or BHK) grown in a serum free medium being transfected with a PEI-DNA complex. However, to date, no such system has been described. The following thesis will therefore investigate the optimisation of such a process and describe strategies for its scale-up.
Figure 1.4 A representational diagram of the critical components of large-scale transient gene expression. Four critical components of large-scale transient gene expression have been identified, the cell line, the transfection agent, the plasmid DNA and the medium choice.
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2.1 Plasmid DNA vectors

Purified vector encoding secreted human embryonic alkaline phosphatase (SEAP), gWIZ\textsuperscript{TM} SEAP, in sterile water was obtained directly from Aldevron (Fargo, USA). Plasmid DNA covalently labeled with rhodamine via a peptide-nucleic acid linker (pGeneGrip\textsuperscript{TM} Rhodamine/Luciferase), for fluorescence microscopy analysis was obtained from Gene Therapy Systems (San Diego, U.S.A.). Enhanced green fluorescent protein (pEE6.4 eGFP) and firefly luciferase (pEE6.4 FLuc) reporter vectors were derivatives of those described previously (Stephens, et al., 1989; Underhill, et al., 2003). The recombinant chimeric IgG\textsubscript{4} monoclonal antibody (b72.3) in a glutamine synthetase expression vector (Bebbington, et al., 1992) was supplied by Lonza Biologics. The vectors not bought commercially were transformed into \textit{E.coli} (DH5-\textalpha) and amplified, and purified as described in the following sections.

2.1.1 Preparation of competent cells

At all stages in the preparation of competent cells, aseptic techniques were used. A stock of competent \textit{E.coli} (DH5-\textalpha) cells were prepared using the rubidium chloride protocol. A single colony of \textit{E.coli} (DH5-\textalpha) from an LB plate (10 g L\textsuperscript{-1} tryptone, 5 g L\textsuperscript{-1} yeast extract, 10 g L\textsuperscript{-1} NaCl, 15 g L\textsuperscript{-1} Agar) was inoculated into 2.5 mL LB medium (10 g L\textsuperscript{-1} tryptone, 5 g L\textsuperscript{-1} yeast extract, 10 g L\textsuperscript{-1} NaCl) and incubated overnight at 37 °C, 200 rpm. The overnight culture was used to inoculate 250 mL of LB medium, containing 20 mM MgSO\textsubscript{4} and grown at 37 °C, 200 rpm, until the \(A\textsubscript{600}\) reached 0.4-0.6. The culture was centrifuged at 5,000 g for 5 minutes, and the supernatant was decanted. The cells were re-suspended in 100 mL (0.4 x original culture volume) of ice-cold Transformation Buffer 1(TFB1, 30 mM potassium acetate, 10 mM CaCl\textsubscript{2}, 50 mM MnCl\textsubscript{2}, 100 mM RbCl, 15% glycerol. Adjusted to pH 5.8 with 1M acetic acid and filter sterilised) and incubated on ice for 5 minutes. The suspension was centrifuged at 5,000 g for 5 minutes, and the supernatant was decanted. The cells were carefully re-suspended in 10 mL (\(1/_{25}\text{th}\) original culture
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volume) of ice-cold Transformation Buffer 2 (TFB2, 10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15% Glycerol. Adjusted to pH 6.5 with 1M KOH and filter sterilised) and incubated on ice for 15-60 minutes. Aliquots of 200 µL were quickly frozen and stored at −80 °C for up to 3 months.

2.1.2 Transformation of competent cells

Competent cells were thawed on ice and 10 ng of purified plasmid DNA were added. Cells were incubated on ice for 30 minutes, heated shocked at 42 °C for 60 seconds and placed on ice for a further 2 minutes. After addition of 3 mL LB medium, cells were incubated for 45 minutes at 37 °C, 150 rpm. 100 µL of transformation mix was put on ampicillin selection plates (LB plates with 100 µg mL⁻¹ ampicillin added) and incubated overnight at 37 °C. Plates were stored for up to 1 week.

2.1.3 Amplification of plasmid DNA

At all stages in the amplification of plasmid DNA, aseptic techniques were used. A colony of transformed E.coli (DH5-α) was placed in 10 mL of LB medium and incubated for 8 hours at 37 °C, 200 rpm. 2.5 mL of the culture were used to inoculate 500 mL LB medium, and cells were incubated overnight (14-16 hrs) at 37 °C, 200 rpm.

2.1.4 Purification of plasmid DNA

The OD₆₀₀ of overnight cultures was determined and plasmids were purified using a commercially available kit (Aurum™ maxiprep kit; BioRad, Hercules, U.S.A.) according to the manufacturer’s protocol. For quantification, plasmids were diluted in the elution solvent provided with the kit and the absorbance at 260 nm and 280 nm was measured spectrophotometrically. Plasmid preparations with A₂₆₀/A₂₈₀ ratios between 1.8 and 2.0 were used. Purified plasmids were analysed on agarose gels (0.7% (w/v), 50V constant voltage) to ensure their purity.
2.2 Mammalian Cell Lines

Cell lines used in this study were: CHO-S suspension-adapted cells (Invitrogen/Gibco, La Jolla, USA); NS0 cells (ECACC); adherent CHO-K1 cells (Underhill, 2003); and adherent CHO-L761 (Original Paper, Underhill, 2003). All cells were stored in liquid nitrogen in their respective media, with 10% DMSO added. For experimental studies, cells were thawed rapidly and centrifuged at 150 g for 3 minutes. After removal of the supernatant, cells were re-suspended in 10 mL of growth medium and incubated in a Heraeus HeraCell™ incubator (Kendro Instruments, Lane Cove, NSW, Australia) set at standard conditions (37 °C, 5% CO₂).

2.2.1 Maintenance of suspension adapted CHO-S cells

After recovery from cryopreservation, cultures were maintained in CHO-S Serum Free Medium II (CHO-SFM II, Invitrogen/Gibco, La Jolla, USA), at viable cell densities between 2 x 10⁵ – 3 x 10⁶ viable cells per mL. Cells were cultured in 250 mL glass Erlenmeyer flasks (50 - 100 mL working volume) at 100 rpm (Bioline orbital shaker) in a Heraeus HeraCell™ incubator (Kendro Instruments, Lane Cove, NSW, Australia) set at standard conditions (37 °C, 5% CO₂). Cells were maintained as described, and used for experiments up to 25 passages.

2.2.2 Maintenance of NS0 cells

After recovery from cryopreservation, cultures were maintained in growth medium at viable cell densities between 2 x 10⁵ – 2 x 10⁶ viable cells per mL. The growth medium used was Dulbecco’s Modified Eagles Medium (DMEM) supplemented with: 30 μM Adenosine, cytidine, uridine, guanosine, and thymidine; 4.5 μM L-Glutamic acid and L-Asparagine; 8mM L-Glutamine and 10 Foetal Calf Serum. Cells were cultured in 250 mL glass Erlenmeyer flasks (50 - 100 mL working volume) at 100 rpm (Bioline orbital shaker) in a Heraeus HeraCell™ incubator (Kendro Instruments, Lane Cove, NSW, Australia) set at standard conditions (37 °C,
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5 % CO₂). Cells were maintained as described, and used for experiments up to 25 passages.

2.2.3 Maintenance of CHO-K1 and CHO-L761 cells

After recovery from cryopreservation, cultures were maintained in growth medium at between 10 and 100 percent confluence in cell treated T-flasks. CHO-K1 cells were grown in DMEM supplemented with 10 % foetal calf serum and 4 mM L-glutamine. CHO-L761 cells were grown in DMEM supplemented with 10 % glutamine free foetal calf serum. Cells were cultured in either T-25 (10 mL) or T-75 (20 mL) flasks, in a Heraeus HeraCell™ incubator (Kendro Instruments, Lane Cove, NSW, Australia) set at standard conditions (37 °C, 5 % CO₂). Transfections were done in the cell lines respective media.

2.2.4 Cell Physiology Analysis

2.2.4.1 Determination of viable cell density, percentage viability and apoptosis

Viable cell density and cell viability were determined by either haemocytometer cell counting (trypan blue exclusion) or the ViaCount™ assay using a GUAVA™ personal cell analyzer (PCA, Guava Technologies, California, USA). Cell samples were manually mixed with Guava Viacount reagent, which consists of two proprietary dyes. One of the dyes is permeable to all cells while the other is only permeable to dead cells. Therefore, after excitation with the laser, the guava can differentiate between dead cells (containing both dyes) and live cells (only containing one of the dyes). The guava arrives at a total cell count determination by waiting until 1000 events have been measured and then measuring the volume of liquid that passed through the machine to arrive at 1000 counts. The theoretical total cell density is then multiplied by the known dilution factor to arrive at the final total cellular density. The percent of apoptotic cells was determined using the PCA and a Nexin reagent (Guava Technologies) according to manufacturers instructions. The Nexin reagent also uses two dyes that indicate early and late apoptosis. The Annexin dye conjugate Annexin 5-PE is used to determine the presence of phosphatidylserine
on the membrane surface of cells, which is an early indicator of apoptosis. The membrane impermeant, DNA binding dye 7-ADD is used to determine the integrity of the cell membrane and indicates late apoptotic cells. Together these dyes are sued to determine the apoptotic state of cell cultures.

2.2.4.2 Analysis of cell size

Cell size was determined using a fluorescence microscope (BX61, Olympus, Mount Waverely, Vic, Australia) to take digital images of cells. The Image-Pro® PLUS (Media Cybernetics) analysis tool was used to manually measure the circumference of cells from which the diameter, and volume were calculated. Approximately 30-50 cells were measured for each condition.

2.2.5 Cell Cycle Arrest

Cell cycle arresting agents (hydroxyurea and nocodazole) were obtained from Sigma. Hydroxyurea was dissolved in water while nocodazole was dissolved in dimethyl sulfoxide (DMSO) to create working stocks of 2 mM. To synchronize CHO-S populations for kinetic studies, cells in mid-exponential phase were treated with hydroxyurea and nocodazole (2 μM) for 16 h prior to transfection. Cells were released from the cell cycle arrest by transfer into fresh medium without arresting agents and transfected with pEE6.4 FLuc as described in section 2.3.1. Otherwise, the effect of anti-microtubule agents on PEI-mediated transient transfection was determined by addition of agents (2 μM final concentration) at the time of transfection. The effect of nocodazole on the stable production of b72.3 by GS-CHO cells was examined by addition of nocodazole (2 μM final concentration) to cells in mid-exponential phase cultures (4 x 10^6 cells mL^-1).
2.3 Transfection of Mammalian Cells

2.3.1 Transfection with PEI-DNA complexes

Polyethylenimine (PEI 25 kDa; branched) was obtained from Sigma (Castle Hill, NSW, Australia). A stock solution (0.9 mg mL$^{-1}$) was prepared in deionised water, neutralized to pH 7.0 with HCl and filter sterilized. Stock solutions were stored at RT. Purified plasmid DNA was added to an aliquot of CHO-S SFM II culture media equivalent to 5% of the final culture volume and left at RT for 2 minutes. PEI was then added and the complex in an appropriate ratio and was incubated at RT for 10 min (Schlaeger, et al., 1999). This solution containing PEI/DNA complexes was then added to the cell culture and left in for the duration of the experiment.

2.3.1.1 PEI-DNA transfection of CHO-S cells

Small-scale transfection experiments were performed in ultra low-binding (ULB) 24-well plates (Corning, Lindfield, NSW, Australia) to ensure that cells remained in suspension. Cells taken from mid-exponential growth phase cultures were seeded into fresh medium supplemented with 6 mg mL$^{-1}$ BSA (Sigma, Castle Hill, Australia) at 2 x 10$^5$ cells mL$^{-1}$ and 1 mL per well, then transfected with 50 μL of PEI/DNA transfection complex, and incubated at 37 °C, 5 % CO$_2$. Breathe Easy™ membranes (Diversified Biotech, Boston, MA, U.S.A.) were applied to plates to ensure even gaseous exchange and evaporation from all wells. The total well volume was taken when sampling. Larger scale transfection experiments employed 100 mL shake flasks (20-40 mL working volume) or 250 mL flasks (50-100 mL). Unless otherwise stated cells were seeded at a viable cell density of 2 x 10$^5$ cells mL$^{-1}$ in fresh medium, transfected and incubated at 37 °C, 5 % CO$_2$. All transfections were carried out in triplicate.

2.3.1.2 PEI-DNA transfection of CHO-K1 and CHO-L761

Transfection experiments were performed in cell culture treated 24-well plates to allow attachment of cells. Cells taken from sub-confluent culture in T-flasks were
washed with warm PBS and treated with Trypsin (Sigma-Aldrich) for 2-3 minutes. Once cells were detached from the flask surface, growth medium was added and viable cell density was determined by haemocytometer counting. Cells were then seeded into 24-well plates at $5 \times 10^5$ cells mL$^{-1}$ and 1 mL per well, transfected with 50 μL of PEI/DNA transfection complex, and incubated at 37 °C, 5 % CO$_2$. All transfections were carried out in triplicate.

2.3.2 Transfection of CHO-S cells with Lipofectamine™ and Lipofectamine 2000™

Transfections with Lipofectamine™ and Lipofectamine 2000™ were done using the manufacturers recommended protocols, with slight adjustments for better comparisons between the techniques. The media used and the DNA to cell ratio were the same as those described in section 2.3.1.1.

2.3.2.1 Transfection of CHO-S cells with Lipofectamine™

To 1.5 mL CHO-S SFM II, 90 μL of Lipofectamine™ (Invitrogen) was added. To a further 1.5 mL CHO-S SFM II, 15 μg of plasmid DNA (fluc pEE6.4) was added. The solutions were mixed and incubated at RT for 15-45 minutes, after which 12 mL of CHO-S SFM II, supplement with 6 mg mL$^{-1}$ BSA, was added. CHO-S cells taken from mid-exponential growth were re-suspended in the solution to a final concentration of $2 \times 10^5$ cells mL$^{-1}$, aliquoted into ULB 24-well plates (1 mL per well) and incubated at 37 °C, 5 % CO$_2$. All transfections were carried out in triplicate.

2.3.2.2 Transfection of CHO-S cells with Lipofectamine 2000™

To 0.5 mL CHO-S SFM II, 37.5 μL of Lipofectamine 2000™ was added. To a further 0.5 mL CHO-S SFM II, 15 μg of plasmid DNA (fluc pEE6.4) was added. Both solutions were incubated at RT for 5 minutes, and then mixed and incubated at RT for a further 20 minutes. CHO-S cells taken from mid-exponential growth were re-suspended in 5 mL of CHO-S SFM II, supplement with 6 mg mL$^{-1}$ BSA, to a final
concentration of $3 \times 10^6$ cells mL$^{-1}$. This was mixed with the DNA-Lipofectamine 2000 complex solution, aliquoted into ULB 24-well plates (400 μL per well) and incubated at 37 °C, 5 % CO₂. After 4 hours, 600 μL of fresh medium was added to each well, and the cultures re-incubated. All transfections were carried out in triplicate.

### 2.4 PEI-DNA complex particle size analysis

PEI-DNA transfection complex size measurements were carried out using a Zetasizer 3000Hsa (Malvern Instruments, Worcestershire, UK) calibrated with nanosphere size standards (Duke Scientific Corporation, CA, USA) of 199 ± 6 nm, in water. Measurements were taken using the settings: ten measurements per sample; 150 μs duration between samples; viscosity 1.078 cP; refractive index of pure water 1.333; temperature 25 °C. Transfection complexes were formed as described in section 2.3.1 and 2.5 mL added to a Sarstedt acrylic cuvette (Technology Park, SA, Australia). When studying the affects of BSA on complex size, a concentrated solution was added to give a final volume of 6 mg mL$^{-1}$ in the complex mixture.

### 2.5 Reporter Gene Assays

#### 2.5.1 Assay of Luciferase activity

Firefly luciferase expression was determined using Luclite™ luciferase assay (PerkinElmer, Boston, MA, U.S.A.). Cells were recovered by centrifugation (1000 rpm, 3 min) and washed twice in phosphate buffered saline (PBS), and then re-suspended in PBS containing 1 mM Ca$^{2+}$ (CaCl$_2$) and Mg$^{2+}$ (MgSO$_4$). Luciferase was measured according to the Luclite™ protocol using a Fluoroskan FL microplate reader (Thermo Labsystems, Vantaa, Finland) set to an integration time of 100 milliseconds. Each sample was assayed in triplicate.
2.5.2 Assay of Secreted Alkaline Phosphatase activity

SEAP activity in cell-free supernatant was measured using a chemiluminescent SEAP reporter gene assay (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. SEAP activity was measured 10 min after addition of substrate using the Fluoroskan FL (100 ms integration time). Each sample was assayed in triplicate.

2.5.3 Flow cytometric analysis

The proportion of cells in discrete phases of the cell cycle, and transfection efficiency were determined by flow cytometry using a Partec-PASIII flow cytometer (Munster, Germany) equipped with Flomax™ software.

2.5.3.1 Flow cytometric analysis of cell cycle phases

Cells were harvested by centrifugation (1000 rpm, 3 min), washed once with 5 mL of PBS, fixed in 5 mL of 4% (v/v) paraformaldehyde (PFA) and incubated at -20°C for 15 min. Cells were then washed twice with PBS and stored in PBS at 4°C. Nuclear DNA was stained in a final concentration of 1 μM 4',6-diamidino-2-phenylindole, dilactate (DAPI; Sigma) in 100 mM Tris-HCl pH 7.6 and incubated at 4°C overnight. Prior to analysis, cells were passed through a 25-gauge needle to disperse aggregates. DAPI fluorescence was measured by excitation with a UV mercury laser and emission monitoring at 435-465 nm. At least 50,000 events were collected per sample.

2.5.3.2 Flow cytometric analysis of Green Fluorescent Protein

CHO-S cells were transfected with eGFP pEE6.4 plasmid DNA as described in section 2.3.1.1. Cells were harvested by centrifugation (1000 rpm, 3 min), washed once with 5 mL of PBS, fixed in 5 mL of 4% (v/v) paraformaldehyde (PFA) and incubated at -20°C for 15 min. Cells were then washed twice with PBS and stored in PBS at 4°C. Transfection efficiencies (defined as the percentage of eGFP positive cells in a transfected population) were determined by excitation of eGFP with an
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Argon laser at 488 nm, with emission measured at 510-530 nm. The eGFP positive cells were defined as those with a higher fluorescence intensity than 99% of control untransfected cells.

2.6 Fluorescence microscopy of transiently transfected CHO-S cells

CHO-S cells were transfected with pGeneGrip™ rhodamine/luciferase plasmid DNA in ultra-low cell binding 24-well plates as described in section 2.3.1.1 using a PEI N: DNA P ratio of 10:1 and DNA concentration of 1 μg mL⁻¹. Cells were harvested by centrifugation (1000 rpm, 5 min), washed with PBS, fixed in 1 mL of 4% PFA as described above and incubated at -20 °C for 5 min. DAPI (6 μM final concentration) was added to each sample and incubated at RT for a further 10 min. Fixed cells were then washed twice with 1 mL PBS and resuspended in a further 10 μL PBS. Fluorescent mounting medium (5 μL, DAKO) and fixed, DAPI-stained cells (5 μL) were placed onto a poly-l-lysine coated microscope slide (Menzel-Glaser), mixed with a pipette tip and incubated at RT for 3 min. A cover slip was carefully placed over the sample, sealed and stored at 4 °C. Cells were examined with a fluorescence microscope (BX61, Olympus, Mount Waverely, Vic, Australia) using the 60x objective and images were captured with a charge-coupled device camera (CoolSNAP-Proef monochrome, Media Cybernetics, Singapore). Images of cells were taken in brightfield and with 2 different fluorescent filter pairs to show gross cell structure, DAPI-stained nuclei (excitation 330-385 nm, broadband emission filter max. 420 nm) and rhodamine-labeled DNA particles (excitation 510-550 nm, broadband emission filter max. 590 nm) respectively. Exposure times of 0.5 s for brightfield and DAPI stained images and 5 s for the rhodamine images were used. Composite images were produced by overlaying single colour images. Images were analyzed using Image-Pro® PLUS (Media Cybernetics) software to determine DNA particle size and the distribution of fluorescence intensity. Particle size was determined by placing a circle by hand around the visible particle and the software was used to report the diameter.
2.7 Antibody Analysis

2.7.1 ELISA of recombinant chimeric IgG4 monoclonal antibody

Monoclonal antibody b72.3 was quantified by enzyme linked immunosorbent assay (ELISA). Microplates (96-well Maxisorp™, Nalge Nunc, Rochester, NY, U.S.A.) were coated with a human Fc specific capture antibody (Sigma, I2136) (1.25 ng μL⁻¹ in coating buffer, 15 mM Na₂CO₃, 35 mM NaHCO₃ pH 9.6; 100 μL well⁻¹) and incubated at 4°C overnight. Coating solution was removed and the wells washed 3 times with 100 μL of washing solution (PBS containing 0.1% (w/v) BSA and 0.005 % (v/v) Tween 20). Wells were then blocked in PBS containing 1% (w/v) BSA and 0.5% (v/v) Tween 20 for 30 min at RT. Following 3 rinses with washing solution, cell free supernatant containing b72.3 Mab was added and incubated for 1 h at 37 °C. Sample was discarded and the wells were washed 3 times as above. A Fab specific detection antibody conjugated to horseradish peroxidase (Sigma, A0293) was then added (in blocking buffer, 100 μL well⁻¹) and incubated for 30 min at 37 °C. Wells were washed 3 times. Substrate solution (100 μL well⁻¹, SIGMA FAST™, o-phenylenediamine dihydrochloride tablet set reconstituted according to manufacturer’s instructions) was added and the reaction proceeded at RT in the dark for 30 min. The reaction was stopped by the addition of 50 μL of 3 M HCl and the OD₄₉₀ was measured using a Spectromax 250 (Molecular Devices) microplate reader. On each assay plate, the assay was calibrated using purified b72.3 Mab spiked into cell culture supernatant over the concentration range 0-500 ng mL⁻¹. Each sample was assayed in duplicate.

2.7.2 Analysis of recombinant IgG₄ N-glycosylation at Asn³⁹⁷

Recombinant IgG₄ was purified from CHO-S cell culture medium by affinity chromatography using Protein L Agarose (Pierce, Rockford, IL, USA). Briefly, 100 μL bed volume matrix was added to 20 mL cell free culture supernatant and an equal volume of PBS. After 24 h incubation at RT on a rotary mixer, the matrix was recovered by centrifugation, transferred to a 0.2 μm spin-filter (Alltech, Baulkham Hills, NSW, Australia), washed three times in 0.5 mL PBS and bound IgG₄ was
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eluted in 200 μL 50 mM Gly-HCl pH 2.0. Purified Mab was desalted immediately by direct adsorption onto a microplate C8 reverse-phase matrix (Empore™, 3M, St. Paul, MN, USA) equilibrated in 0.1% TFA (binding capacity ~50 μg IgG well⁻¹). Mab was eluted in 80 % (v/v) CH₃CN, 0.1 % (v/v) TFA directly into a UV-transparent microplate and quantified by determination of absorption at 280 nm with a UV microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Solvent was then removed by centrifugal evaporation using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY, USA). Mab was then digested with modified, sequencing grade trypsin (Sigma) at an substrate:protease ratio of 20:1 (w/w) in 50 mM ammonium bicarbonate containing 0.5 % (w/v) Rapigest™ (Waters Corporation, Milford, MA, USA) for 12 h at 37°C in a final reconstituted volume of 25 μL. Asn²⁹⁷ containing glycopeptides were selectively recovered and desalted by reverse-phase chromatography using C18 ZipTips™ (Millipore, Billerica, MA, USA) pre-equilibrated in 0.1 % (v/v) TFA with elution at 12 % (v/v) CH₃CN. The glycopeptide-containing fraction was then subjected to MALDI-MS using α-cyano 4-hydroxycinnamic acid (Sigma) as matrix (15 mg mL⁻¹ in 60 % (v/v) CH₃CN, 0.1 % (v/v) TFA) with an Applied Biosystems Voyager DE-STR instrument (Foster City, CA, USA) operating in positive ion mode at an accelerating voltage of 20 kV. Spectra are the average of 200 laser shots at a laser repetition rate of 20 Hz.

3.1 Introduction

In 1995, the ability of polyethylenimine to condense plasmid DNA to form a positively charged complex that can be internalisation by mammalian cells was described (Boussif, et al., 1995). Since then, its use in the transfection of a variety of in vivo and in vitro host cells has been demonstrated (Godbey, et al., 2001; Goula, et al., 1998a; Goula, et al., 1998b; Pollard, et al., 1998; Schlaeger, et al., 1999). To date, the most successful example of in vitro PEI-mediated transient transfection for the production of recombinant proteins at scale has employed suspension adapted human embryonic kidney (HEK293) cells, most commonly those engineered to express the Epstein-Barr nuclear antigen-1 (EBNA-1)(Durocher, et al., 2002; Girard, et al., 2002). In combination with plasmid vectors harbouring the oriP origin of replication, this system enables both nuclear retention and autonomous replication of episomal plasmid DNA. However, unlike rodent derived cell lines (e.g. CHO, BHK, NS0), HEK293 cells are not commonly employed in industry to produce recombinant therapeutic proteins at scale.

It would therefore be advantageous to develop a transient system in industrially relevant cells lines. At present, such cell lines have not been developed to enable continuous replication of episomal plasmids and therefore depend upon the efficient transfection of DNA for transient gene expression to occur. This chapter describes the comparison of cell lines more commonly used in industry, i.e. suspension adapted Chinese hamster ovary cells (CHO-S) and mouse myeloma cells (NS0) and their respective ability to transiently express genes encoded on plasmid vectors. Another important consideration in developing a process that can be used in industry is the medium composition in which the cells are cultured and is discussed here.
3.2 Comparison of Transient Transfection in CHO-S and NS0 Cell Lines

The industrial cell lines NS0 (Lonza Biologics, Slough) and CHO-S were compared for their ability to transiently express the Firefly Luciferase gene driven by the strong human cytomegalovirus (hCMV) promoter, encoded on a minimal plasmid vector (FLuc pEE 6.4). When using PEI-mediated transfection, an important consideration is the PEI to DNA ratio used (Schlaeger, et al., 1999). A previous study showed that the optimal PEI to DNA molar ratio for transient transfection of NS0 cells was 30:1 (Nitrogen to Phosphate, data not shown). Therefore, plasmid DNA was complexed with PEI at this optimal ratio, and added to cell cultures at final concentrations of 0.1, 0.5 and 1.0 µg mL⁻¹ DNA. Control, untransfected cells and cells transfected with free PEI (4.05 µg mL⁻¹, equivalent to that used to complex 1 µg mL⁻¹ DNA) or naked DNA (1 µg mL⁻¹) were also cultured. For rapid comparison and evaluation, cells were transfected and cultured in ULB 24-well plates (1 mL per well) and incubated at 37 °C, 5 % CO₂. The NS0 cell line used has not been adapted to growth in serum free medium and therefore, as serum has been demonstrated to aid transfection, CHO-S cells (although adapted to growth in SFM) were also grown in medium with 10 % serum. To demonstrate the effect serum has on transient gene expression, CHO-S cells were also grown in SFM and transfected with the PEI-DNA complexes described above.

3.2.1 Comparison of transient gene expression in NS0 and CHO-S cell lines

Luciferase production observed in NS0 cells and CHO-S cells (Figure 3.1) clearly demonstrates that transient gene expression is affected by host cell line. Although these data are from single samples, it is clear that expression in CHO-S cells (in serum containing medium) is much higher (approximately 600 times higher at maximum expression) than that observed in NS0 cells. Even when grown in SFM, CHO-S cells show approximately 10 times higher expression.

It is also evident from the data that CHO-S cells achieve optimal expression with a higher concentration of DNA, 1.0 µg mL⁻¹ compared to 0.5 µg mL⁻¹ for NS0. This
Optimisation of PEI-mediated transient gene expression

suggests that CHO-S cells are more permissive to the transfection process than NS0 cells, a premise born out by the growth data recorded. If we consider the viable cell density (Figure 3.2) and percentage viability (Figure 3.3) of NS0 cells and CHO-S cells transfected with the different concentrations of DNA (dashed lines) we see a clear pattern. NS0 cells show an immediate reduction in percentage of viable cells (which gradually recovers) and sometimes a reduction in the viable cell density (compared to control, untransfected cells), upon transfection. This phenomenon is more exaggerated at higher concentrations of DNA. However, CHO-S cells do not demonstrate this susceptibility, when grown in serum containing medium, and follow control cell growth. Upon removal of serum from the CHO-S medium, growth is similar to that observed in NS0 cells. The effects of serum are further discussed later in this chapter.

It is evident that PEI-DNA complexes are cytotoxic to cells, however we must consider whether the DNA or PEI component causes this. Comparing the conditions where cells are transfected with either naked DNA or free PEI we see that with naked DNA in all cells (with or without serum) follow control growth characteristics. However, upon transfection with free PEI both NS0 cells and CHO-S cells in SFM manifest the cytotoxicity previously observed. It can therefore be concluded that the PEI component of the complex is the cytotoxic agent. This issue will be discussed in more detail in chapter 4.
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Figure 3.1 Luciferase expression from transient transfection of NS0 and CHO-S cells. NS0 and CHO-S cells taken from mid-exponential growth were transfected with 0.1, 0.5 or 1.0 μg mL⁻¹ Fluc pEE6.4 DNA. Cells were grown in 100 mL shake flasks and the luciferase activity was measure over 48 hrs. NS0 cells were grown in serum containing media, CHO-S cells were grown in serum free and serum containing medium. n = 1.
Figure 3.2 Affect of transient transfection on viable cell density in NS0 and CHO-S cells. NS0 and CHO-S cells taken from mid-exponential growth were transfected with 0.1 (Δ), 0.5 (□) and 1.0 (○) μg mL⁻¹ FLuc pEE6.4 DNA, free PEI (■) and free DNA (▲). Control, untransfected cells were also cultured (●). Cells were grown in 100 mL shake flasks and the viable cell density was measured over 48 hrs. NS0 cells were grown in serum containing media, CHO-S cells were grown in serum free and serum containing medium. n = 1.
Figure 3.3 Affect of transient transfection of percentage viability in NS0 and CHO-S cells. NS0 and CHO-S cells taken from mid-exponential growth were transfected with 0.1 (△), 0.5 (□) and 1.0 (○) µg mL⁻¹ FLuc pEE6.4 DNA, free PEI (■) and free DNA (▲). Control, untransfected cells were also cultured (●). Cells were grown in 100 mL shake flasks and cell viability was measure over 48 hrs. NS0 cells were grown in serum containing media, CHO-S cells were grown in serum free and serum containing medium. n = 1.
3.2.2 Effect of Removing Serum from Culture Medium

The previous section showed that CHO-S cells when grown in the presence of serum give much higher transient gene expression than those grown without. However, because of regulatory standards and the downstream processing complications associated with serum, it would be advantageous to use serum free medium in our process. The previous section indicates that serum acts ‘protect’ cells from the cytotoxic effects of PEI, indirectly increasing transient gene expression. To determine whether there is a direct correlation between serum concentration, cell growth and transgene expression, CHO-S cells were transfected in medium containing no serum; 0.5 %; 2 %; 5 % and 10 % serum. In addition, to test whether bulk protein has the same effect, cells were transfected in SFM supplemented with 6 mg mL\(^{-1}\) Bovine Serum Albumin (BSA, approximately equivalent to the concentration of protein in a 10 % v/v serum solution). Control, untransfected cells were also cultured.

Figure 3.4 shows that when serum is present, viable cell density is similar at all concentrations tested. However, the cell viability shows that cells are more susceptible to PEI’s cytotoxic effects in lower serum concentration. This data confirms that serum initially protects cells against PEI. The transient gene expression data (Figure 3.5) shows that cell specific expression is also related to serum concentration, i.e. serum also increases the number of high producing cells. Whether this is related to cell growth or other factors that increase transfect efficiency is, at present, unclear, but will be address later in this chapter. The growth and expression data for SFM containing BSA is similar to that of serum, indicating that it could be used as a substitute and that further investigation would be beneficial.
Figure 3.4 Serum protects cells against the cytotoxic effects of transfection. CHO-S cells were transfected with FLuc pEE 6.4 and grown in serum free medium supplemented with no serum (□), 0.5 % serum (△), 2 % serum (○), 10 % serum (◇) and 6 mg mL⁻¹ BSA (●). Control, untransfected cells were also cultured (▲). Cell density (A) and viability (B) were measure for 96 hrs. n = 1.
Figure 3.5 Serum and Bovine Serum Albumin enhance transient transfection. CHO-S cells were transfected with FLuc pEE 6.4 and grown in serum free medium supplemented with no serum, 0.5 % serum, 2 % serum, 10 % serum and 6 mg mL⁻¹ BSA. Luciferase activity was measured for 96 hrs. n = 1.
3.3 Effects of the addition of Bovine Serum Albumin (BSA) to serum free medium

Serum is rarely used in industrial mammalian cell processes because of its inherent cost, variability and risk of transfer of adventitious agents to end-users. Therefore, following from the results observed in section 3.2.2, further experiments were performed to determine the affects of BSA on transient gene expression. Bovine serum albumin is a single polypeptide chain of 583 amino acids, and has a molecular weight of 66.43 kDa. In cell culture, albumin acts to bind pyrogens, and trace elements such as vitamins and hormones. It is also thought to protect cells from shear forces. With this in mind, the affect of BSA upon cell physiology and upon the PEI-DNA complex was investigated. Further optimisation of the PEI to DNA ratio and DNA concentration in CHO-S cells showed maximum expression at 10:1 and 1 μg mL⁻¹ DNA (discussed in more detail in Chapter 4). These optimised conditions were used throughout this section.

3.3.1 Optimisation of the use of BSA in transient gene expression culture medium

In section, 3.2.2 it was shown that reducing the serum concentration in the culture medium of transfected cells lead to a decrease in cell specific transgene expression. As little is known about the exact mechanisms occurring, an informed choice of the BSA concentration required to maintain the beneficial effects observed cannot be made. Therefore, the optimal BSA concentration was investigated. It was also hypothesised that the final concentration of BSA might be reduced by adding it to the complex mixture prior to mixing with the cell culture. This would allow the BSA to interact with the PEI-DNA complexes at a high BSA concentration, but reduce the final concentration in the medium.

3.3.1.1 Effect of BSA concentration in bulk medium

To determine whether the BSA concentration in the medium can be reduced without loss of expression, CHO-S cells were transfected with the FLuc pEE 6.4 vector as
described in 2.3.1.1. Cells were transfected (PEI:DNA 10:1 and 1 \( \mu \)g mL\(^{-1}\)) and cultured in ULB 24-well plates in 1 mL of serum free medium supplemented with BSA at 0, 0.0006, 0.006, 0.06, 0.6 and 6 mg mL\(^{-1}\). Control, untransfected cells were also cultured. At BSA concentrations below 0.6 mg mL\(^{-1}\) transient gene expression was the same as cultures with no BSA. At concentrations, 0.6 and 6 mg mL\(^{-1}\) expression was higher than control levels and were dose dependent (data not shown). Therefore, concentrations between 1.5 mg mL\(^{-1}\) and 6 mg mL\(^{-1}\) BSA were tested. The experiment described above was repeated using medium supplemented with 0, 1.5, 3, and 6 mg mL\(^{-1}\) BSA. In addition, a positive control of medium with 10 % serum was used.

Figure 3.6 shows that cell specific expression increases with increased BSA concentration. If the total luciferase production over 48 hours is calculated (expressed as light hours, see appendix A) and plotted against BSA concentration (Figure 3.7), a strong linear relationship is observed. Of interest to note, from Figure 3.6, is that the kinetics of expression appears to be different between serum and BSA, although the total luciferase production at 48 hours is the same. Growth data from this experiment (Figure 3.8) shows that, in contrast to serum, cell viability is not affected by a reduction in BSA concentration. However, viable cell density is seen to increase slightly with an increase in BSA concentration (the 30 hour time point for 1.5 mg mL\(^{-1}\) appears to be anomalous). Although the data presented here suggests that a further increase in the BSA concentration could result in increased transient gene expression, a concentration of 6 mg mL\(^{-1}\) BSA was used for future work, as higher protein concentrations will inevitably complicate downstream processing.
Figure 3.6 Bovine Serum Albumin enhances transient gene expression in serum free medium. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA (1µg mL⁻¹, N:P 10:1) and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 0, 1.5, 3 and 6 mg mL⁻¹ BSA and 10 % serum. Luciferase activity was measured for 48 hrs, and the cell specific luciferase production (A) and the total luciferase production were calculated. n = 3 ± s.d.
Figure 3.7 There is a strong correlation between Bovine Serum Albumin concentration and Luciferase production. The total luciferase production at 48 hrs post transfection (from Figure 3.6) was plotted against BSA concentration. The line represents the best linear fit.
Figure 3.8 Bovine Serum Albumin enhances the growth of transfected CHO-S cells. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA (1μg mL⁻¹, N:P 10:1) and cultured in low binding 24-well plates. Transfected cells were cultured in serum free medium supplemented with 0, 1.5, 3 and 6 mg mL⁻¹ BSA. Control, untransfected cells were also culture in serum free medium and serum free medium supplemented with 6 mg mL⁻¹ BSA. Viable cell density (A) and cell viability (B) were measured 48 hrs post transfection. n = 3 ± s.d.
3.3.1.2 Effect of BSA addition to complex mixture

In order to reduce the final concentration of BSA in bulk medium, the effect of adding BSA to PEI-DNA complexes prior to mixing with the cell culture was examined. PEI-DNA complexes were formed as normal, or with the addition of BSA (6 mg mL\(^{-1}\) final concentration in complex mix) 10 minutes after PEI addition. CHO-S cells grown in SFM II were transfected with both complex types described, and cells grown in SFM II with 6 mg mL\(^{-1}\) BSA were transfected with complexes formed as normal. Control, untransfected cells were also cultured.

The cell specific expression observed (Figure 3.9a) indicates that it is the bulk medium final concentration, and not the concentration at which the BSA and PEI-DNA complexes first interact, that is critical in determining expression. When BSA is added to the complex mixture, and not the bulk medium, on mixing it is reduced to 0.6 mg mL\(^{-1}\) and reverts to expression levels obtained with control, transfected cells. The growth data (Figure 3.9b) indicates that addition of BSA to complexes, rather than bulk medium, does not affect the cell specific growth rate compared to the control, whereas using BSA in the bulk medium does give a reduction in growth rate. This is probably due to an inefficient transfection in the former case. In a separate experiment, BSA was added to complexes prior to PEI addition and was found to dramatically reduce expression levels (data not shown)
Figure 3.9 Bulk medium Bovine Serum Albumin is an important factor. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA (1 μg mL⁻¹, N:P 10:1) and cultured in low binding 24-well plates. BSA was added to either the bulk medium (6 mg mL⁻¹), the complex after formation and prior to addition to cells (6 mg mL⁻¹) or not at all. Control, untransfected cells were also cultured. Luciferase production and the viable cell density were measured 24 hrs post transfection and the cell specific luciferase (A) production and cell specific growth rate (B) calculated. n = 3 ± s.d. P values are calculated using a two-tailed T-test assuming equal variance, using the null hypothesis.
3.3.1.3 Effect of BSA on transfection efficiency

The data presented in section 3.3.1.1 shows that an increase in BSA concentration leads to an increase in cell specific luciferase expression. The increase in cell specific production could either be due to an increase in the number of plasmids entering the cells (i.e. the transfection efficiency) or an increase in expression from the plasmids present increased protein synthesis machinery). To determine whether BSA acts to increase transient gene expression by increasing the percentage of cells transfected, CHO-S were transfected with a plasmid vector encoding enhanced green fluorescent protein (eGFP). Cells were transfected as described in 2.3.1.1 and cultured in ULB 24-well plates under standard conditions. Cells were fixed and analysed for GFP expression by flow cytometry (Figure 3.10).

The presence of BSA increases transfection efficiency from 18 % to 53 % 24 hours post-transfection (3-fold increase) and from 48 % to 85 % 48 hrs post transfection (below 2-fold increase). The 6-fold increase in cell specific luciferase production cannot be entirely explained by an increase in transfection efficiency, suggesting that BSA not only increases complex uptake, but also acts to increase cellular gene expression. Comparing the distribution of GFP intensity observed with (Figure 3.10c) and without (3.10b) BSA present, it is seen that there is not a major peak shift. This indicates that expression in a small number of cells is increasing a lot, rather than expression increasing by a small amount in all cells, i.e. BSA allows high producers to occur.
Figure 3.10: Bovine Serum Albumin increases transfection efficiency. CHO-S cells taken from mid-exponential growth were transfected with GFP pEE 6.4 DNA (1μg mL⁻¹, N:P 10:1) and cultured in low binding 24-well plates. Cells were culture in serum free medium without BSA and with 6 mg mL⁻¹ BSA. Control, untransfected cells were also cultured. Cells were removed from culture at 24 and 48 hrs post transfection and fixed using para-formaldehyde. GFP intensity was measured by flow cytometry and the transfection efficiency was calculated. The profiles represent the average of three cultures.
3.3.2 Effect of BSA on PEI-DNA particle Size

Complex formation between PEI and DNA is due to the interaction between the two opposing charges of the molecules. Reports in the literature indicate that particle size of transfection complexes is an important factor in transient gene expression (Gebhart, et al., 2001; Rudolph, et al., 2002; Tang, et al., 1997). As BSA is a charged molecule, it can be assumed that it will interact with the PEI-DNA complexes in some way, altering the particle size. To study whether this is the case, and whether it has a bearing upon increasing transient gene expression, photon correlation spectroscopy was used to measure complex size before and after BSA addition. Complexes were formed in SFM II (PEI:DNA ratio 10:1 μg mL⁻¹ DNA) and particle size was measured for 3 hours, after which BSA was added to the mixture to give a final concentration of 6 mg mL⁻¹. Particle size was again measured for a further hour. It can be seen from figure 3.11 that the average diameter of PEI-DNA complexes slowly increases with time. Addition of BSA to the complex mixture results in a reduction, and stabilisation of the average particle size. It is difficult to conclude from these data whether this property of BSA is the cause of the increase in transient gene expression. This issue will be discussed further in this chapter with respect to expression from different BSA preparations, and in chapter 4 with respect to PEI : DNA ratios.
Figure 3.11 Bovine Serum Albumin reduces and stabilises the complex particle size. Polyethylenimine and FLuc pEE 6.4 DNA (1 µg mL⁻¹, N:P 10:1) were allowed to form complexes for 3 hrs, after which time BSA (final concentration 6 mg mL⁻¹) was added. Throughout this time the average dynamic diameter was measured using photo correlation spectroscopy. n = 3 ± s.d. Work done by Michael Hines.
3.3.3 BSA preparation and lot number variation

The previous sections have described how BSA improves transfection efficiency. However, this effect could be greatly effected by the preparation and quality of the BSA used. In addition, it is likely, as with serum, that lot-to-lot variation occurs. In order to determine whether the observed effects of BSA upon transfected cell growth and transient gene expression are dependent upon the BSA formulation, different BSA preparations (from Sigma-Aldrich) were tested. The following sigma BSA products and lot numbers were investigated: sigma catalogue number A 3350; sigma catalogue number A 9647; sigma catalogue number A 2153, (lot numbers 109H1073, 12H0183, 26H1013, 35H1065 and 032K1444); and Sigma catalogue number A 4161. BSA solutions were tested for: their effect on transient gene expression; their effect on average particle size; the proteins present; and nuclease activity.

3.3.3.1 Effect of different BSA preparations on transient gene expression

CHO-S cells were transfected with Fluc pEE 6.4 in the presence of different BSA preparations and cultured in ULB 24-well plates under standard conditions. Control cells were also transfected in SFM without BSA. Figure 3.12 shows the luciferase production and growth rate 24 hours post transfection. It is clear from these data that not only the method of preparation, but also the specific lot number, is important in enhancement of expression (compared with the control). Lot 26H1013 shows higher expression than other preparations, which in general do not enhance expression above control levels, and even inhibit expression in some cases (A 3350, A 2153, 032K1444). Even when compared, with cell culture tested BSA (Figure 3.13a), lot 26H1013 gives higher expression (note: this comparison was an independent experiment). Interestingly, enhancement of expression does not correspond with an enhancement in growth rate (Figures 3.12b and 3.13b). Even if we compare information from the certificates of analysis obtained (Appendix B, summarised in Table 3.1), there is no clear correlation between enhanced expression and any specific characteristic of the preparation.
Figure 3.12 The positive effects of Bovine Serum Albumin are lot dependent. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE6.4 DNA (1 μg mL\(^{-1}\), N:P 10:1) and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 6 mg mL\(^{-1}\) BSA from the lots given above. Control cells were transfected in serum free medium. The luciferase production and viable cell density were recorded 24 hrs post transfection, and the cell specific luciferase production (A) and cell specific growth rate (B) were calculated. \(n = 2\) ± determined values. P values are calculated using a one-tailed T-test assuming equal variance, using the null hypothesis, comparing with the control.
Figure 3.13 The positive effect of Bovine Serum Albumin does not correlate with its purity. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA (1μg mL⁻¹, N:P 10:1) and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 6 mg mL⁻¹ standard (A-2153, lot number 26H1013) or cell culture tested (A-4161) BSA. Control cells were transfected in serum free medium. The luciferase production and viable cell density were recorded 24 hrs post transfection, and the cell specific luciferase production (A) and cell specific growth rate (B) were calculated. n = 3 ± s.d. P values are calculated using a two-tailed T-test assuming equal variance, using the null hypothesis.
<table>
<thead>
<tr>
<th>BSA (catalogue #, lot #)</th>
<th>Solubility</th>
<th>Elemental Analysis</th>
<th>pH test</th>
<th>Purity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Luciferase&lt;sup&gt;b&lt;/sup&gt; Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 2153, 26H1013</td>
<td>Yellow solution at 10 g plus 250 mL of water</td>
<td>15.84 % Nitrogen</td>
<td>7.0 (1 % in 0.15M NaCl)</td>
<td>97 %</td>
<td>16.44&lt;sup&gt;c&lt;/sup&gt; 130.38&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>A 2153, 35H1065</td>
<td>Yellow solution at 10 g plus 250 mL of water</td>
<td>15.46 % Nitrogen</td>
<td>7.3 (1 % in water)</td>
<td>&gt; 98 %</td>
<td>6.93&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A 2153, 32K1444</td>
<td>Yellow solution at 10 g plus 250 mL of water</td>
<td>15.7 % Nitrogen</td>
<td>6.7 (1 % in 0.15M NaCl)</td>
<td>98 %</td>
<td>0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A 4161, 57H9304</td>
<td>Yellow solution at 50 mg mL&lt;sup&gt;-1&lt;/sup&gt; in water</td>
<td>15.9 % Nitrogen</td>
<td>N/A</td>
<td>&gt;99 %</td>
<td>78.20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.1 The physical and chemical characteristics of different BSA preparations. Information taken from the certificates of analysis sent by Sigma-Aldrich (Appendix B)

<sup>a</sup> determined by agarose gel electrophoresis  
<sup>b</sup> relative light units per cell x 10⁶  
<sup>c</sup> and <sup>d</sup> are the results from independent experiments and therefore levels of expression can only be compared between those from the same experiment.
3.3.3.2 Effect of different BSA preparations on PEI-DNA complex size

To show whether PEI-DNA complex size is affected by different BSA preparations, complexes were formed as normal, and BSA (final concentration 6 mg mL\(^{-1}\)) was added 1 hour after PEI addition. Table 3.2 summarises the particle sizes measure and the respective luciferase production observed. After the addition of BSA, particle sizes are observed to reduce to between 200 and 250 nm in diameter. Comparing the relative expression, there does not appear to be any correlation between particle size and transgene expression. Although broadly speaking, the preparations that yield particle sizes below 200 nm show higher expression.

<table>
<thead>
<tr>
<th>BSA (catalogue #, lot #)</th>
<th>Average dynamic diameter at 0 h (nm)</th>
<th>Average dynamic diameter at 1 h (nm)</th>
<th>Size immediately after BSA addition (nm)</th>
<th>Luciferase* Production</th>
<th>Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 2153, 26H1013</td>
<td>321 ± 13.4</td>
<td>1060 ± 19.8</td>
<td>194 ±17.0</td>
<td>16.44(^b)</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>130.38(^c)</td>
<td></td>
</tr>
<tr>
<td>A 2153, 12H0138</td>
<td>307 ± 27.8</td>
<td>1050 ± 85.3</td>
<td>242 ± 24.3</td>
<td>6.93(^b)</td>
<td>42.2 %</td>
</tr>
<tr>
<td>A-3350, 1140628</td>
<td>307 ± 6.24</td>
<td>960 ± 160.0</td>
<td>219 ± 11.7</td>
<td>0.03(^b)</td>
<td>0.2 %</td>
</tr>
<tr>
<td>A 4161, 57H9304</td>
<td>322 ± 4.55</td>
<td>1030 ± 53.0</td>
<td>196 ± 15.8</td>
<td>78.20(^c)</td>
<td>60.0%</td>
</tr>
</tbody>
</table>

Table 3.2 PEI-DNA complex particle size and luciferase expression with different BSA preparations. PEI-DNA complexes were formed as described in section 2.4 using the different BSA lots indicated. In separate experiments, CHO-S were transfected with the Fluc pEE6.4 plasmid and grown in the presence of the BSA lots indicated.

* The relative light units per cell x 10^6.

\(^{b}\) The results from independent experiments and therefore levels of expression can only be compared between those from the same experiment.

\(^{d}\) The relative expression was calculated using A2153, 26H1013 as the reference point.
3.3.3.3 Protein content and nuclease activity of different BSA preparations

The effect being observed could also be due to the impurities present in the BSA preparations. Two likely candidates for causing different expression levels are the levels of other proteins present (which might interact with the complex or the cell), and the presence of nucleases (which will degrade DNA, and reduce the final cellular content). The different BSA preparations were analysed for protein content using an SDS-PAGE gel. Comparing BSA lot 26H1013 (best enhancer of expression), with the other BSA preparations, there does not appear to be a significant difference in protein content. The intensity of the low molecular weight protein bands is slightly less, as it is in the cell culture tested BSA. However, there are no proteins uniquely present or absent. The cell culture tested sample does have a band missing at approximately 75 KDa, which corresponds to antibody heavy chains, which are removed in this product.

To test for nuclease presence, purified plasmid DNA (FLuc pEE 6.4) was incubated in SFM supplemented with different BSA preparations (50 mg mL⁻¹) at 37 °C for 1 hour. After incubation, samples were analysed by agarose gel electrophoresis. This analysis showed that incubation with BSA results in a greater proportion of the DNA being in the open circular conformation than supercoiled, when compared with untreated DNA (data not shown). This clearly shows that there are endonucleases present in the BSA preparations. However, it is difficult to establish from this whether DNA degradation has an effect on expression levels, as all BSA preparations cause DNA degradation to a certain extent.

3.4 Conclusions and Discussion

In any industrial process, the choice of cell line and medium environment used is a critical factor. This chapter has demonstrated that this is even more pertinent for transient gene expression. Until now, research into transient gene expression has usually been done using mammalian cell lines that are not commonly used in industry. It has been shown here, using industrially relevant cell lines, that transient transfection levels are cell line dependent. For this reason two widely used
industrially cell lines, NS0 and CHO-S, were compared for their ability express recombinant protein from a transiently transfected minimal plasmid.

When comparing suspension adapted Chinese Hamster Ovary (CHO-S) cells and mice myeloma (NS0) cells it was clear that CHO-S cells are much more amenable to transient transfection, demonstrating expression levels of between 10 and 600 times higher (depending on the absence or presence of serum respectively). In addition, CHO-S cells were less susceptible to the cytotoxic effects of PEI-mediated transfection, allowing higher DNA doses to be used. CHO-S cells have been described as being generally more robust than NS0 cells (personal communication, Dr David James), which is the likely reason they are less susceptible to the PEI cytotoxicity. This robustness is probably why CHO-S cells show much higher transient gene expression levels than NS0 cells.

Whilst comparing CHO-S cells and NS0 cells it became apparent that the presence of foetal calf serum greatly increases transient gene expression levels. Although serum-containing medium is a good source of nutrients and growth factors for mammalian cells, it is gradually being phased out and replaced with serum free medium. This is because regulatory authorities discourage its use in the manufacturing of biopharmaceuticals, because of the associated risk of contamination with adventitious agents (Macleod, 1991). In addition, the presence of serum in medium inherently complicates the down-stream processing of biopharmaceuticals. However, removal of serum from transiently transfected CHO-S cells leads to a reduction in growth rate, and transgene expression, suggesting the serum somehow ‘protects’ the cells and increases gene expression. It is know that transcription factors within serum increase expression from CMV promoters (Butler, 1994), however it is unlikely this is the reason there is approximately a 30-40 times increase in transient gene expression on addition of serum. It is therefore more likely that the increase is associated with the higher specific growth rate and viability observed. This sort of effect is also observed with addition of bovine serum albumin to media (Durocher, et al., 2002), which was therefore considered a good candidate to replace serum. In addition, other reports suggest that bulk protein in media increase transient gene expression (Pham, et al., 2003; Rhaese, et al., 2003).
The data presented in this chapter demonstrates that the enhancement of PEI-mediated transfection by serum is also observed when BSA is added to the medium. The enhancements observed are similar to that of serum in that the PEI cytotoxicity at high DNA concentration is reduced and that an increase in BSA concentration gives higher cell specific transient gene expression, in a dose dependent response. In addition, it was shown that addition of BSA also increase transfection efficiency. However, the mechanism by which BSA, and similarly serum, acts to enhance transient gene expression is still not fully understood. It is clear that serum and BSA protect cells against the cytotoxic effects of PEI, increase transfection efficiency and increase transient gene expression. However, whether the increase in transient gene expression is only due to the other observed effects is not evident.

A reduction in cytotoxicity is likely to be a critical factor in increasing transient gene expression, as this will both increase the number of cells available for transfection and reduce the amount of DNA degraded and lost in dying cells. This appears to be confirmed by the fact that the increase in transfection efficiency is due to the presence of more high producers, rather than an increase in expression over the whole population. Combined with the reduction in the initial loss of viability observed (more evident with serum), this indicates that cells that uptake a higher concentration of PEI-DNA complex (i.e. those that are about to divide when transfected) are not killed in the presence of BSA, but are in the absence of BSA. However, although it is clear that a reduction in cytotoxicity is important, the mechanism by which BSA achieves this is not clear. It may improve the general health of the cells, which are then more able to survive; it may act as a solvent, binding and absorbing excess PEI molecules; or it may ‘mask’ the PEI-DNA complexes from the cell and therefore reduce DNA degradation, and reduce the cellular response to naked DNA. Establishing this could be difficult; as it has been shown here that different BSA lots and preparations act on transient gene expression in different and unpredictable ways. However, a PEI affinity column has been used to purify a BSA homologue (Holderman, 2002) suggesting that BSA could interact with PEI in some way.
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Although PEI cytotoxicity is an influencing factor, the data suggests that this is not the only way in which BSA is increasing transient transfection. If we consider the cell specific luciferase production rate, there is almost a six times increase when BSA is added to the medium. Therefore, not only are more cells surviving, the productivity appears to be higher as well. This could be due to an increase in transcription and/or an increase in DNA uptake. Some BSA preparations are likely to contain the transcription factors found in serum that increase expression from CMV promoters, however, this cannot be established from the data presented here. What can be drawn from these data is that BSA directly interacts with the PEI-DNA complex, and that this is a relatively loose association. This is demonstrated by the fact that BSA consistently reduces and stabilises the average particle size of PEI-DNA complexes. However, upon dilution, the beneficial effects of BSA are lost, suggesting it has become dissociated from the PEI-DNA complex. This suggests that BSA binding to the PEI-DNA complex is probably in a dynamic equilibrium and therefore concentration dependent. It is also clear that the order of addition is important, as when added before PEI, BSA acts to reduce expression. This association of BSA with the complex could influence transfection in many ways. There are many reports that suggest that particle size is an important factor in transfection (Gebhart, et al., 2001; Rudolph, et al., 2002; Tang, et al., 1997), and therefore the simple reduction in size may increase transfection. There have also been reports that conjugation of peptides or proteins to PEI-DNA complexes act to increase cellular uptake (Kircheis, et al., 2001; Orson, et al., 2002; Rhaese, et al., 2003). However, the information presented here indicates that BSA binds to complexes, reduces their size, and perhaps forms an outer ‘shell’ around the complex, allowing greater uptake and gene expression.

This chapter has shown that cell and medium type are critical parameters in optimising transient gene expression. It is obvious that some cell lines are much more amenable to transient transfection than others, and it has been shown here that of the most commonly used industrial cell lines, CHO-S is clearly the most appropriate to use. It is also evident that the medium composition also dramatically effects the levels of transient gene expression observed, and given more time, could be suitably manipulated to yield high titres.
Chapter 4. Optimisation of PEI-mediated Transient Gene Expression, Part 2: The PEI-DNA complex

4.1 Introduction

In the previous chapter, it was shown that cell type and medium composition have dramatic effects upon cell growth, viability, and transfection efficiency during PEI-mediated transient transfection. It was also shown that some cell types are more amenable to transfection than others and that addition of either serum or BSA to culture medium results in an overall increase in transgene expression. However, another very important variable in PEI-mediated transfection is the composition and structure of the PEI-DNA complex itself.

To date, the most critical parameters of PEI-DNA complex formation reported have been size and overall charge (Choosakoonkriang, et al., 2003; Gebhart, et al., 2001; Rudolph, et al., 2002; Schaffer, et al., 1998; Tang, et al., 1997; Wagner, 1991). Both are primarily determined by the specific PEI nitrogen to DNA phosphate molar ratio, which has been optimised at between 6 and 10:1. The optimal ratio appears to be cell line dependent and varies between different systems. In addition, it has been shown that optimal transfection is achieved using the branched 25 kDa PEI molecule (Schlaeger, et al., 1999). However, PEI mediated transient transfection has not been optimised in CHO-S cells. This chapter describes the study of the PEI nitrogen to DNA phosphate ratio required for optimal expression in CHO-S cells. It also considers other parameters that effect complex formation, and therefore the transient gene expression.

4.2 Optimisation of the PEI nitrogen to DNA phosphate molar ratio

The optimal ratio of PEI nitrogen to DNA phosphate for PEI-mediated CHO-S cells has so far not been comprehensively studied. Therefore, an experiment was done to determine this, and the DNA concentration that gives maximum total luciferase expression. CHO-S cells were transfected with PEI-DNA complexes formed with
N:P ratios of 5 to 1, 10 to 1, 20 to 1 and 30 to 1, and with pEE6.4 Fluc DNA concentrations 0.5, 1 and 2 μg mL\(^{-1}\). Cells were cultured in ULB 24-well plates under standard conditions, and the luciferase expression was measured at 6, 24 and 48 hours post transfection. The cell specific luciferase production and, using the integral of luciferase output, the total luciferase production was calculated for the 48-hour period.

### 4.2.1 Effects of polyethylenimine to DNA ratio on transgene expression

It is evident from the cell specific data (figure 4.1) that optimal expression occurs at both an N:P ratio of 5 to 1, with 2 μg mL\(^{-1}\) DNA, or an N:P ratio of 10 to 1, with 1 μg mL\(^{-1}\) DNA. However, cell specific expression continues to increase throughout the culture using 10 to 1, with 1 μg mL\(^{-1}\) (\(p = 2.8 \times 10^{-6}\) from 6 to 24 hrs, \(p = 0.065\) from 24 to 48 hrs), whereas no increase occurs 48 hours post transfection using 5 to 1, with 2 μg mL\(^{-1}\) DNA (\(P = 0.235\)). If the integral of luciferase production (figure 4.2) over the 48 hours is considered, it is clear that overall production is similar with both conditions (\(p = 0.022\)), however an N:P ratio of 10:1 with 1 μg mL\(^{-1}\) requires half the quantity DNA to achieved this. It was therefore decided that this ratio is preferential. Interestingly, if this optimal ratio is compared with the optimal ratio at 0.5 μg mL\(^{-1}\) (N:P ratio 20:1) there is more than a doubling in the total luciferase production.

Comparing the PEI to DNA ratio that gives optimal expression at the different DNA concentrations (i.e. 5 to 1, 10 to 1, and 20 to 1 for 2, 1, and 0.5 μg mL\(^{-1}\) respectively), we find that the total PEI concentration in the medium is the same (1.35 μg mL\(^{-1}\)). This suggests that optimal expression is in some way linked to the final PEI concentration. To understand this correlation, cell growth data must be considered (Figure 4.3 and 4.4). Figure 4.3 shows the viable cell density observed using the different transfection conditions. The previously observed phenomenon of a reduction in growth when cells are transfected is seen again here. In addition the effect on cell growth becomes more pronounced as either the N:P ratio is increased or the DNA concentration is increased. If the final PEI concentration, independent of DNA concentration, is compared with cell specific growth rate (Figure 4.4), and clear correlation is observed. This correlation is also observed with free PEI (Figure
Optimisation of PEI-mediated transient gene expression

4.4, dashed line), however free DNA does not have any effect (data not shown). These data therefore indicate that an increase in PEI concentration in the culture media (presumably above a certain level) will have a direct effect upon cell specific growth rate. However, this does not explain how PEI concentration, and therefore cell specific growth rate, has an effect upon the expression levels achieved.

Further analysis of the data shows that there exists a correlation between the cell specific growth rate (and hence PEI concentration) and the rate of increase in cell specific production. These two parameters were calculated for conditions where the PEI concentration is optimal, or higher, (i.e. 1.35 \( \mu \text{g mL}^{-1} \) or above) where it was assumed that PEI concentration is the overriding influence on cell specific growth rate. The rate of increase in cell specific production was calculated on a linear basis. Figure 4.5 shows that over 24 hours post transfection and 48 hours post transfection, there is a clear relationship between these two parameters, i.e. the faster cells are growing, the more rapidly their production rate increases. If it is assumed that the cellular production rate is largely influenced by the amount of transcriptionally active plasmid DNA present in the cell, then it could be suggested that the difference in production rate could be associated with the DNA uptake rate. Therefore, those conditions that affect the cell specific growth rate also affect the DNA uptake rate. This conclusion would suggest that cell division is required for efficient transfection. To confirm this assertion, a further experiment was conducted.

If a cell population is block and synchronised in different phases of the cell cycle, and then released you would expect that population of cells to all be dividing together. If, therefore, they are transfected upon release from cell cycle blocking and cell division is required for efficient transient transfection, there would be a delay in expression equivalent to time it takes the cells to reach the mitotic stage of the cycle. Cells blocked in G1 and G2/M phases of the cell cycle, using hydroxyurea and nocodazole respectively, were placed into fresh medium and transfected with PEI-DNA (fluc pEE6.4) complexes and incubated in ULB 24-well plates under standard conditions. The luciferase production data (Figure 4.6) shows that the population of cells that were blocked in the G2/M phase of the cell cycle begin to express approximately 4-8 hours post transfection, whereas those blocked in the G1 phase do not show expression until 8-12 hours post transfection. The untreated cells also
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show expression 4-8 hours post transfection, however, the rate in increase is much less. These data clearly suggests that cell division is a pre-requirement of PEI-mediated transient transfection.
Figure 4.1 Cell specific luciferase production using different complex conditions. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 6 mg mL⁻¹ BSA. Cultures were transfected with 0.5, 1 and 2 µg mL⁻¹ DNA complexed with PEI using the molar ratios indicated. Cell viability and luciferase production were measured at the times (post transfection) indicated and the cell specific luciferase production calculated. n = 3 ± s.d.
Figure 4.2 Total luciferase production using different complex conditions. Total luciferase production 48 hrs post transfection was calculated using the figures given in Figure 4.1.
Figure 4.3 DNA and PEI concentration affects the growth of cells. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 6 mg mL\(^{-1}\). Cells were transfected with PEI-DNA complexes formed using N:P ratios of 5:1 (♦), 10:1 (■), 20:1 (▲) and 30:1 (●) and the DNA concentrations indicated. Viable cell density was recorded at the times (post transfection) indicated. \( n = 3 \pm \text{s.d.} \)
Figure 4.4 CHO-S cell specific growth rate is inversely proportional to PEI concentration in culture. From the data shown in Figure 4.3, cell specific growth rate was calculated over the 48 h period post transfection for each combination of transfection conditions. Control cells were transfected with free PEI. Specific growth rate is inversely related to PEI concentration in culture, independent of the corresponding DNA concentration. $n = 3 \pm \text{s.d.}$
Figure 4.5 DNA uptake rate is proportional to initial growth rate. From the data presented in figure 4.3 and 4.1, the cell specific growth rate and the rate of increase of cell specific luciferase production at 24 hrs post transfection were calculated for complex conditions of 1 (O) and 2 (●) μg mL⁻¹ DNA where the final PEI concentrations was higher than 1.35 μg mL⁻¹. n = 3 ± s.d.
Figure 4.6 Transition through G2/M phase is required for PEI-mediated transient gene expression in CHO cells. (A) CHO-S cells were synchronized in G2/M or G1/S phases for 16 h prior to transfection by the addition of nocodazole (2 μM) or hydroxyurea (2 μM) respectively. Control cells were not treated. In each case, the DNA content of fixed cells was analysed by flow cytometry to determine cell cycle distribution. (B) Cells were seeded into fresh medium in 24-well plates, without cell cycle inhibitors, at $2 \times 10^5$ viable cells mL$^{-1}$ and transfected with the FLuc pEE6.4 vector complexed with PEI (PEI N:DNA P = 10:1, 5 μg DNA 10$^6$ cells$^{-1}$). Post transfection, viable cell density and intracellular luciferase activity (measured as relative light units) was determined for unsynchronized control cell populations (●), or cells that had been synchronized in G2/M by nocodazole (■) or in G1 by hydroxyurea (▲). n = 3 ± s.d.
4.2.2 Effects of polyethyleneimine to DNA ratio on transfection efficiency

The experiment described above was repeated using the pEE6.4 EGFP plasmid construct to determine transfection efficiency. Transfection efficiency was measured as described in section 2.5.3.2, at 24 and 48 hours post-transfection. Figure 4.7 shows that the optimal transfection efficiency occurring at 24 and 48 hours post transfection was with a ratio of 10:1, and 1 µg mL\(^{-1}\) DNA, consistent with the luciferase data. However, the difference in transfection efficiency between different conditions at 48 hours post-transfection is not as pronounced as the difference in luciferase expression, and an increase in PEI concentration appears to have less effect. Interestingly, at 48 hours post transfection, the average GFP intensity (Table 4.1) is relatively invariant among positively expression cells with all conditions tested. The growth profiles were similar to those observed in the luciferase experiment (data not shown).

<table>
<thead>
<tr>
<th>PEI Phosphate to DNA Nitrogen Ratio</th>
<th>Final DNA Concentration (µg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>5 to 1</td>
<td>880 ± 150</td>
</tr>
<tr>
<td>10 to 1</td>
<td>960 ± 120</td>
</tr>
<tr>
<td>20 to 1</td>
<td>950 ± 180</td>
</tr>
<tr>
<td>30 to 1</td>
<td>860 ± 84</td>
</tr>
</tbody>
</table>

Table 4.1 Average GFP intensity of transiently transfected CHO-S cells using different transfection conditions. CHO-S cells were transfected with the EGFP pEE6.4 plasmid, and incubated under standard conditions. The average GFP intensity was measured by flow cytometry 48 hours post transfection. n = 3 ± s.d.
Figure 4.7 Transfection efficiency is dependent upon PEI-DNA complex conditions. CHO-S cells taken from mid-exponential growth were transfected with GFP pEE 6.4 DNA and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 6 mg mL\(^{-1}\). Cultures were transfected with 0.5, 1 and 2 \(\mu\)g mL\(^{-1}\) DNA complexed with PEI using the molar ratios indicated. Cells were fixed and the GFP intensity measured using flow cytometry. \(n = 3 \pm s.d.\)
4.2.3 Effects of polyethylenimine to DNA ratio on average complex size

Photon correlation spectroscopy was used to determine whether the size of complexes is critical in transient transfection, as has previously been suggested. PEI-DNA complexes were formed using 1 μg mL⁻¹ with PEI to DNA ratios of 5 to 1, 10 to 1, 20 to 1, and 40 to 1. Complexes were allowed to form for 3 hours, after which time, BSA was added to a final concentration of 6 mg mL⁻¹. Complexes size was measured throughout. As was shown in the previous chapter, complexes slowly aggregate over time, but are stabilised to a reduced size after the addition of BSA. Table 4.2 shows the complex sizes immediately prior and after BSA addition and the corresponding total luciferase production. If we consider the complex size after BSA addition (i.e., the presumed size they are on contact with cells) there appears to be a reduction in the complex size as the PEI to DNA concentration increases. However, this correlation is not observed in the corresponding luciferase production observed. Interestingly, there is a correlation between the particle size prior to BSA addition and the luciferase production, although it is difficult to judge whether this is significant as it is only from three data points.

<table>
<thead>
<tr>
<th>PEI Nitrogen to DNA Phosphate ratio</th>
<th>Average dynamic diameter prior to BSA addition (nm)</th>
<th>Average dynamic diameter after BSA addition (nm)</th>
<th>Luciferase Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 to 1</td>
<td>1290 ± 45.6</td>
<td>201 ± 17.5</td>
<td>753 ± 33.9</td>
</tr>
<tr>
<td>10 to 1</td>
<td>1680 ± 81.0</td>
<td>170 ± 20.2</td>
<td>1810 ± 70.1</td>
</tr>
<tr>
<td>20 to 1</td>
<td>1360 ± 63.4</td>
<td>155 ± 8.91</td>
<td>811 ± 3.43</td>
</tr>
<tr>
<td>40 to 1</td>
<td>1060 ± 9.94</td>
<td>155 ± 17.7</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 4.2 The correlation between PEI to DNA ratio, PEI-DNA complex size, and transgene expression. PEI-DNA complexes were formed using the molar ratios indicated. Complexes were allowed to form for 3 hours, after which BSA was added to a final concentration of 6 mg mL⁻¹. PCS was used to measure the average particle diameter of the complexes form. In a separate experiment, CHO-S cells were transfected with the Fluc pEE6.4 plasmid, and grown under standard conditions. n = 3 ± s.d.

a Total relative light units calculated over 48 hours.
4.3 Optimisation of other complex formation conditions

In the previous chapter, the effect BSA has upon complex formation was clearly demonstrated. It was also shown that this effect is very dependent on when BSA is added to the complex, showing very detrimental effects when added before the PEI addition, but very favourable after. Also, there are many reports of the solution in which PEI-DNA complexes are formed having dramatic effects upon transgene expression. Therefore a comparison between our system and the most commonly used solution, HEPES buffered saline, was done. The effect of complex formation time was also investigated. Cells were transfected with PEI-DNA complexes formed in either: SFM II; HEPES buffered saline (HBS); or HEPES buffered saline supplemented with BSA. Complexes were formed in each solution for either 10, 30, 60 or 180 minutes prior to transfection.

4.3.1 Effect of complex formation solution upon transient gene expression

Figure 4.8 clearly shows that complexes formed in serum free medium give much higher expression levels than those formed in HBS. In addition, the presence of BSA in HBS during complex formation leads to a dramatic decrease in transgene expression, confirming the results shown in chapter 3.

4.3.2 Effect of complex formation time upon transient gene expression

Figure 4.8 demonstrates that the length of time a complex solution is left to form for will have a large effect on the transient gene expression levels observed. The point at which maximum gene expression occurs is dependent upon the solution used for formation, however it is interesting to note that if SFM II is used as the formation solution, it appears that a plateau of maximum expression is reach at 30 minutes formation time, after which expression does not increase. However, it is worth noting that complexes that are left to form for longer than 10 minutes have a greater impact upon the cell specific growth rate (Figure 4.9) with both SFM II and HBS.
Figure 4.8 Transient gene expression is affected by complex formation conditions. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA and cultured in low binding 24-well plates. PEI-DNA complexes were formed in serum free medium (●), HBS (■) and HBS with 6 mg mL⁻¹ BSA (▲). Cells were cultured in serum free medium supplemented with 6 mg mL⁻¹ BSA and transfected with complexes allowed to form for the times indicated. The viable cell density and luciferase production were measured 48 hrs post transfection. n = 3 ± s.d.
Figure 4.9 The complex formation time affects the cytotoxic effects of PEI. CHO-S cells taken from mid-exponential growth were transfected with FLuc pEE 6.4 DNA and cultured in low binding 24-well plates. PEI-DNA complexes were formed in the solutions indicated. Cells were cultured in serum free medium supplemented with 6 mg mL\(^{-1}\) BSA and transfected with complexes that were allowed to form for 10, 30, 60 and 180 minutes. The viable cell density was measured and the cell specific growth rate over 48 hrs was calculated. \(n = 3 \pm \text{s.d.}\).
4.4 Comparison of optimised transfection protocol with commercial transfection agents

PEI-mediated transfection is inexpensive, and therefore more amenable to scale-up than commercial products. However, expression levels may be so low that this advantage becomes redundant. Therefore a comparison between PEI-mediated transfection and commercial products was done. One of the most common commercial transfection products used is Lipofectamine™ (Invitrogen) because of its high success rates and expression levels it achieves in a wide range of mammalian cell types. This product and a more recently released version, Lipofectamine2000™, was therefore taken as a benchmark for comparison with the optimised PEI-mediated transfection protocol. Cells were transfected with PEI-DNA complexes, Lipofectamine-DNA and Lipofectamine2000-DNA complexes using adapted protocols for better comparison (see section 2.3.2). Cells taken from mid-exponential growth were transfected with Fluc pEE6.4 plasmid complexed with each transfection agent and incubated under standard conditions in ULB 24-well plates for 48 hours. The SEAP production data obtained (Figure 4.10) clearly shows that transgene expression from PEI-, Lipofectamine- (p = 0.243 compared to PEI) and Lipofectamine2000- (p = 0.525 compared to PEI) mediated transfection are similar.
Figure 4.10 PEI-mediated transient gene expression is similar to commercially available transfection agents. CHO-S cells taken from mid-exponential growth were transfected with gWIZ plasmid DNA complexed with either lipofectamine™, lipofectamine 2000™ or PEI (1µg mL⁻¹ DNA, N:P 10:1) and cultured in ULB 24-well plates under standard conditions. The SEAP production was measured 24 hours post transfection. n = 3 ± s.d.
4.5 Conclusions and Discussion

In this chapter is has been shown that the characteristics of the PEI-DNA complex used to transfect CHO-S cells greatly effect the level of transient gene expression that is achievable. The ratio of PEI to DNA has previously been described as an important factor, but investigations into why are scant. In section 4.2 it was shown the optimal condition for transfection of CHO-S cells is a PEI nitrogen to DNA phosphate ratio of 10:1 with 1 μg mL⁻¹ (or 1 μg per 2 x 10⁵ cells). Further analysis of the data indicates that these conditions are arrived at through a combination of two influencing factors. Rather than the PEI to DNA ratio being an important factor, it is more the final PEI concentration in the growth medium that is important. In addition, an optimum concentration of DNA, independent of PEI to DNA ratio exists.

An inverse correlation exists between PEI concentration in culture media and cell growth rate that was independent of DNA concentration (Fig. 2). The fact that optimal transient gene expression is also influenced by the PEI concentration would therefore suggest that this is due to the change in cell specific growth rate. It has been shown that the addition of NLS sequences to PEI-DNA complexes mediates active transport of the vector DNA (Colin, et al., 2001; Dowty, et al., 1995). However, there is no conclusive evidence to indicate whether cell division is required for transient gene expression. The data in this chapter present a strong case that, in the absence of an NLS sequence, cell division is a pre-requisite to transient gene expression. Not only does the growth rate effect the rate of increase of cell specific production (which could be associated with the rate of DNA uptake and expression), it was clearly shown that cultures synchronised in particular stages of the cell cycle only begin to express the transgene at the approximate time when they would enter mitosis. The data presented so far therefore suggest that to achieve high transient gene expression, cells must be actively dividing.

The concentration of DNA will be important, because at concentrations below optimum, the amount of transcriptionally active DNA within the cell will be less. In addition, the DNA uptake rate is likely to be lower. However, at higher DNA
Optimisation of PEI-mediated transient gene expression
centrations, the rate of luciferase reporter production declined earlier than at the optimal concentration, irrespective of PEI concentration. Although not shown here, we also observed this phenomenon on transient production of b72.3 Mab at plasmid DNA concentrations of 2 μg mL\(^{-1}\) and PEI:DNA ratio of 5:1 (i.e. the same total PEI mass per mL as at the optimal PEI:DNA ratio of 10:1 and DNA concentration of 1 μg mL\(^{-1}\)), where transient production ceased at 48 hours post transfection rather than at 72 hours post transfection. The reason for this is not clear, however it is speculated that an increased dose of DNA may eventually lead to increased cytosolic DNA after PEI-mediated endosome rupture. As a consequence, and as has been shown in a previous report, heterologous plasmid DNA itself in the cytosol may function to down-regulate the global rate of protein synthesis by cellular mechanism(s) and signalling pathways that control mRNA translation (Underhill, 2003).

The transfection efficiency data presented in this chapter show that maximum expression at both 24 and 48 hours post transfection occurs at a PEI to DNA ratio of 10:1, with both 1.0 and 2.0 μg mL\(^{-1}\). It is interesting to note that, although the luciferase expression is also high at 5:1, 2.0 μg mL\(^{-1}\) the transfection efficiency is not. This would therefore suggest that although the transfection efficiency is lower, the number of high producing cells is higher. Although the average GFP intensity at 48 hours is relatively invariant (Table 4.2), it is however clear that under these conditions the average intensity is the highest. The data also suggest that a PEI to DNA ratio of 10:1 or greater is required for efficient transfection of the cell population. A reduction in transfection efficiency at higher ratios could be accounted for by the reduction in cell growth rate previous described. However, this explanation cannot be ascribed to a ratio of 5:1. It is therefore clear that there is some characteristic of particles with PEI to DNA ratios higher than 10:1 that make them inherently more transfectable.

It is acknowledged that the mean diameter of transfection complex particles has a pronounced effect on the efficiency of transient gene expression. However, it is likely that the optimal size will lie within a range at the nanometre scale. Although intuitively, smaller particles might thought to be better because they could be more easily transported, it might be that larger particles delivery more DNA efficiently. However, other groups have not satisfactorily been able to show a correlation
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between the size of transfection particles and transient gene expression (Ogris, et al., 1998; Tang, et al., 1997). In the study presented here, particle size, after BSA addition, does not correlated with expression output, although particles do become smaller as more PEI is added. This could be due to binding interactions between the PEI and DNA allowing a more densely packed particle to for after BSA addition (Clamme, et al., 2003; Remy-Kristensen, et al., 2001). Interestingly there is a correlation between particle size prior to BSA addition with larger particles giving a higher expression output. However, further investigation into the exact nature of the PEI-DNA complexes would have to be done to know whether this is relevant or not. A correlation between complex zeta potential and transfection efficiency has been identified (Son, et al., 2000), and it has been proposed that for PEI-mediated transfection, a neutral charge is optimal (Jones, 2003; Trubetskoy, 2003). It is therefore possible that a ratio of 5:1 does not contain enough PEI to create a neutrally charged particle. This would then account for why the transfection efficiency is lower at this ratio than at 10:1.

The comparison of complex formation in HEPES buffered saline and serum free medium II (supplement with BSA) showed that serum free II medium gave noticeably better results. This may be because the HBS alters the charge of the particle and therefore reduces the transfection efficiency. In addition, forming the PEI-DNA complex in a solution different to the final transfection environment means that it is likely that, upon mixing, interactions will change and therefore the particle itself will change. This will therefore make any effort to predict transfection results from characteristics of the complex particle futile. The expression results observed in serum free medium II also indicates that the level of transgene expression is linked to the length of time the complex is left to form, up to 30 minutes after which a plateau is reached. This suggests that although the complex continues to increase in size during formation, a point is reach where its characteristics cannot become any more favourable. This may be the point at which enough PEI has combined with the DNA to achieve neutrality. The cell growth data shows that complexes that are formed for only 10 minutes are not as cytotoxic as those formed for longer, which could be because less PEI has been accumulated in the complex particles prior to transfection.
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It has been hypothesised that there is a dynamic equilibrium between free PEI and PEI-DNA complexes (Tiyaboonchai, et al., 2001). It was shown in the previous chapter that BSA reduces and stabilises the PEI-DNA complex size. It might therefore be assumed that BSA prevents this dynamic equilibrium from occurring. This would therefore suggest that the optimal characteristics of the PEI-DNA complex must be achieved before addition to the transfection medium. The data presented here has demonstrated that the final PEI concentration and the final DNA concentration of the medium is a key influence in the subsequent transient gene expression. However, it has also been shown that the PEI-DNA complex characteristics can also influence the transfection efficiency and growth rate of the culture. It appears that the size of the PEI-DNA is probably only significant if it is outside a certain range. However, it appears that the composition of the particle is important. At PEI to DNA ratios below 10:1, the transfection efficiency is lower, maybe because a neutral particle is not formed. Above this, the cytotoxicity of DNA comes into play. Complexes that are left to form for less than 30 minutes show reduced transgene expression levels. This again may be because not enough PEI has complexed with the DNA to form a neutral particle before addition to the BSA containing medium. However, complexes that are left for more than 10 minutes to form have a greater cytotoxicity. It could therefore be possible that complexes that have more PEI than is required for a neutral particle (i.e. at a high N:P ratio or are left to form for longer), upon addition to BSA containing medium, have excess PEI 'trapped' in the particle. On internalisation and release of the particles into the cytoplasm of the cell, where BSA might become dissociated, the equilibrium would be towards release of free PEI into the cell, which is subsequently cytotoxic. Although there is no direct evidence for this, what is clear from the data presented in this chapter is that some characteristic of the PEI-DNA particle, possibly its charge, is responsible for efficient transfection (it may be that BSA has an influence on this as well). After the particle has entered the cell, the governing factors then become the cytotoxicity of PEI to the cell, and the response of the cell to the amount of plasmid DNA present. Achieving good PEI-mediated transfection in any cell line will therefore be a fine balance between these three factors.

This chapter has shown, through careful analysis of the data, that optimisation of PEI-mediated transient gene expression is not simply a case of finding an optimal
PEI to DNA ratio, or DNA to cell ratio, but that it involves a complicated interplay of a number of critical factors. Although it is not fully understood why particular conditions give better transfection efficiency and/or transgene expression, it has been shown that at a PEI nitrogen to DNA phosphate ratio of 10:1 and a DNA to cell ratio of 5 μg per 10⁶ cells optimum expression can be achieved. Although it was shown that the complex formation time of 30 minutes was optimal, 10 minutes was used in future experiments, with the knowledge that increasing the formation time could improve expression, but with the caveat that it will affect cell growth. It has also shown that after optimisation of the process it is possible to achieve expression levels comparable to those achieved with commercially available products. The data presented in this chapter therefore indicate that if transient gene expression is viable at large scale, then PEI-mediated transfection would be an appropriate method to use because it is easy, inexpensive, and above all, effective.
Chapter 5 Manipulation of Cell Function to Increase Transient Gene Expression in CHO-S Cells

5.1 Introduction

The previous chapters have dealt with may in which the PEI-DNA complex can be manipulated to increase transient gene expression. An alternative method to increase gene expression is to manipulate the host cell itself. Cell lines stably expressing proteins have commonly been further transformed to contain genes that augment expression, such as transactivators, translation deregulators or anti-apoptosis genes (Cockett, et al., 1990; Lasunskiaia, et al., 2003; Mercille, et al., 1998; Underhill, et al., 2003). In addition, many small molecule effectors, such as sodium butyrate, cell cycle blockers or growth hormones have been used to increase expressions levels. However, these techniques have not yet been widely applied to transient gene expression. Central to transient gene expression is the efficient transport and expression of pDNA to the cell nucleus. There are several major barriers to this process in mammalian cells including: DNA degradation by nucleases; transport to the nucleus; transport across the nuclear envelop; gene silencing through DNA modification and efficient translation of the transgene. It has been suggested that PEI acts to overcome the two former barriers, delivering pDNA efficiently to the nuclear envelop (Abdullah, et al., 1996; Dunlap, et al., 1997). However, the transport of pDNA into the nucleus and the subsequent expression has not previously been studied. Therefore, this chapter will discuss possible ways in which these barriers may be overcome, namely: cell cycle arrest in G2/M phase; inhibition of DNA methylation by 5-azacytidine; and constitutive expression of the adenovirus transactivator E1A.
5.2 Arrest in the G2/M phase of the cell cycle increase transient gene expression

In the previous chapter, it was shown that cell division is a pre-requisite to efficient transient gene expression. However, there has recently been a lot of interest in the effects of anti-mitotic drugs upon gene expression (Baru, et al., 1995; Chowdhury, et al., 1996; Nair, et al., 2002; Son, et al., 1996). Cycle arrest has been used in stable cell lines, once a high biomass concentration has been achieved, so that energy is diverted from cell growth into production formation, which increases the final titre of the culture (Ibarra, et al.; Watanabe, et al., 2002). There have also been studies into how the cell cycle stage upon transfection, effects transient gene expression (Brunner, et al., 2002; Brunner, et al., 2000). It was shown in the previous chapter that cells synchronised by nocodazole (a small molecule which inhibits microtubule polymerisation) showed a higher rate of increase in cell specific productivity than untreated cells. In addition, a control culture, where nocodazole was maintained in the medium showed that the cell specific productivity was higher throughout the culture. It was therefore hypothesised that addition of nocodazole to cell culture medium might increase PEI-mediated transient gene expression. Diagram 5.1 shows the structure of nocodazole.

![Diagram 5.1 The structure of the cell cycle blocking agent, nocodazole, molecular formula C_{14}H_{11}N_{3}O_{3}S](image-url)
5.2.1 Characterization of the effects of nocodazole upon transient gene expression

CHO-S cells were transfected with FLuc pEE6.4 DNA in the presence and absence of nocodazole. Maximum gene expression was found using a nocodazole concentration of between 1 and 8 μM, above which, it was cytotoxic. Unless otherwise stated, nocodazole (1 μM) was added at the time of transfection and maintained throughout the culture period. CHO-S cells transfected in the presence of nocodazole clearly show an increase in cell specific and overall luciferase production (Figure 5.1) with nearly a 5-fold increase in total production at the end of the culture. It is therefore clear that cells blocked in the G2/M phase of the cell cycle exhibit higher transgene expression levels than untreated cells.

In the previous experiment, nocodazole was added at the time of transfection, however it was hypothesised that expression might be increase further if cells were already synchronised in the G2/M phase. In addition, the percentage viability in nocodazole treated cells gradually reduces throughout the culture (data not shown). Therefore, cell recovery after nocodazole removal was investigated. Figure 5.2 shows the luciferase data from this experiment.

The first thing to draw from these data is that cells transfected in nocodazole free medium, whether they have been pre-incubated or not, show the same expression profile. This would suggest that nocodazole must be present at the time of transfection to increase gene expression. If we consider the cultures where nocodazole is maintained indefinitely after transfection, it can be seen that those that were pre-incubated for 12 hours actually show lower expression than those where nocodazole addition is at the time of transfection. It therefore appears that pre-incubation, and therefore synchronization, is not required for increased transient gene expression. In studying whether nocodazole can be washed out 8 hours post transfection to maintain high cell viability and therefore high expression we can see the luciferase production reduces to a level between cells that are removed from nocodazole at transfection and those that are maintain in nocodazole.
Figure 5.1 Nocodazole increases cell specific and overall transient gene expression of the luciferase protein. CHO-S cells taken from mid-exponential growth were incubated with (■) and without (●) 1 μM nocodazole for 12 hours, after which cultures were placed into fresh medium (with or without nocodazole) and transfected with the Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1). Cells were cultured in ULB 24-well plates under standard conditions for 5 days, and the viable cell density and luciferase production were monitored. The cell specific (A) and total luciferase production (B) was calculated. n = 3 ± s.d.
Figure 5.2 Pre-incubation with nocodazole is not required for increased transient gene expression. CHO-S cells taken from mid-exponential growth were either cultured under standard conditions (solid line) or pre-incubated with 1 μM nocodazole for 12 hours (dashed line). Cells were transfected with the Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1) and further cultured under standard conditions with (■) or without (♦) 1 μM nocodazole. In addition, cells pre-incubated with 1 μM nocodazole were transfected with the Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1), cultured in the presence of 1 μM nocodazole for a further 8 hours, then either spun down, and re-suspended in fresh, nocodazole free medium (▲, dashed line) or spun down, and re-suspended in its original medium (●, dashed line). The viable cell density and luciferase production were measured throughout the culture. n = 3, ± s.d.
5.2.2 Nocodazole increases transfection efficiency

Although overall protein production is an important factor of transient gene expression, it is also important to consider the transfection efficiency. This will indicate whether nocodazole is increasing the number of cells that are expressing, or the expression level of each cell. The transfection efficiency in the presence and absence of nocodazole was measured using flow cytometry. Figure 5.3 shows the flow cytometry profiles obtained, the transfection efficiency was calculated as described in the materials and methods. These data clearly show that nocodazole causes a dramatic increase from 53% to 99%, and 85% to 99%, 24 and 48 hours post transfection respectively. In other words, in the presence of nocodazole, nearly all cells are transfected. In addition, the width of the peaks suggests that, in terms of expression levels, nocodazole imparts a uniform distribution across the cell population. It is also worth noting that the average intensity in nocodazole treated cells is higher than in untreated cells. Assuming the rate of expression from individual plasmids will be at a maximum rate, this suggests that nocodazole treated cells take up more DNA over a specific period.
Figure 5.3 Nocodazole increases transfection efficiency in CHO-S cells. CHO-S cells taken from mid-exponential growth were transfected with the GFP pEE6.4 plasmid (1μg mL⁻¹, N:P 10:1) and cultured in ULB 24-well plates in the absence (B) or presence (C) of 1 μM nocodazole. Control, untransfected cells were also cultured (A). After 24 and 48 hours, cells were removed, fixed with paraformaldehyde, and analysed for GFP expression on a flow cytometer. The profiles represent the average of 3 cultures.
5.2.3 Nocodazole increases transient expression of secreted proteins

The previous experiments with nocodazole have all used luciferase as the reporter protein. Although luciferase is easy to measure by a very sensitive assay, it is not a secreted protein. Nocodazole inhibits microtubule formation, which is integral in protein secretion. Most proteins that would be produced by the process being developed would be secreted. Therefore, the effect of nocodazole upon the expression of secreted alkaline phosphatase (SEAP) was measured. Cells not pre-incubated and pre-incubated in nocodazole were transfected in nocodazole containing medium. A control transfection, without nocodazole was also done. Figure 5.4 shows the total SEAP expression observed. This clearly shows that addition of nocodazole does not prevent the expression of secreted proteins, and that again, there is an increase in expression over the control transfection. This also confirms that pre-incubation in nocodazole is not required and should in fact be avoided.

5.2.4 Nocodazole increase the average cell size

Section 5.2.2 showed that addition of nocodazole increases the average intensity of GFP expression by approximately 2-fold at 24 hours post transfection. It was assumed that this was due to an increase in the number of plasmids present per cell. In order to determine whether there is an increase in DNA plasmid numbers in nocodazole treated cells, a fluorescently labelled plasmid was used to quantify accumulation within cells. CHO-S cells were transfected with a Rhodamine labelled GFP plasmid in the presence and absence of nocodazole. At 6, 24 and 48 hours post transfection cells were fixed in para-formaldehyde and pictured using a fluorescence microscope.
Figure 5.4 Nocodazole increases transient gene expression of secreted proteins. CHO-S cells taken from mid-exponential growth were either pre-incubated with 1 μM nocodazole for 12 hours (dashed line) or cultured under standard conditions (solid line). Cells were transfected with the gWIZ™ SEAP plasmid (1μg mL⁻¹, N:P 10:1) and cultured in ULB 24-well plates in the absence (●) or presence (■) of 1 μM nocodazole. Viable cell density and SEAP production were measured throughout the culture. n = 3, ± s.d.
Figure 5.5 Treatment of CHO cells with nocodazole leads to an increase in cell size and loss of nuclear membrane integrity. CHO-S cells in suspension in 24-well plates at 2 x 10^5 cells mL\(^{-1}\) (1 mL per well) were transfected with a plasmid vector covalently labelled with rhodamine under optimal conditions for PEI-mediated transfection (PEI N:DNA P = 10:1, 5 μg DNA 10^6 cells\(^{-1}\)) in the presence or absence of 2 μM nocodazole. Cells were fixed post transfection and analysed by fluorescence microscopy. Typical false colour composite images of cells are shown. Rhodamine labeled rDNA containing particles are red, DAPI stained nuclei are blue. Bars = 20 μm. From work done by Douglas Galbraith.
<table>
<thead>
<tr>
<th>Time post transfection (h)</th>
<th>Nocodazole (2 μM)</th>
<th>Mean cell diameter (μm ± SD)</th>
<th>Mean cell volume (μm³)</th>
<th>Particles per cell (mean ± SD)</th>
<th>Particles per unit volume (particles μm⁻³)</th>
<th>Mean particle diameter (μm ±SD)</th>
</tr>
</thead>
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<tr>
<td>6</td>
<td>-</td>
<td>22 ± 4.3</td>
<td>5900</td>
<td>12.7 ± 5.7</td>
<td>2.2</td>
<td>0.8 ± 0.5</td>
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<tr>
<td></td>
<td>+</td>
<td>23 ± 3.5</td>
<td>6100</td>
<td>11.7 ± 3.8</td>
<td>1.9</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>26 ± 3.8</td>
<td>9600</td>
<td>14.2 ± 4.9</td>
<td>1.5</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>31 ± 6.7</td>
<td>15,500</td>
<td>35.0 ± 10.7</td>
<td>2.3</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>25 ± 2.8</td>
<td>7500</td>
<td>12.8 ± 4.9</td>
<td>2.2</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>41 ± 6.1</td>
<td>35,700</td>
<td>68.8 ± 21.8</td>
<td>1.9</td>
<td>1.0 ± 0.7</td>
</tr>
</tbody>
</table>

Table 5.1 Treatment of CHO cells with nocodazole leads to an increase in cell size and recombinant DNA particles per cell. As for Figure 5.5, CHO-S cells in suspension in 24-well plates at 2 x 10⁵ cells mL⁻¹ (1 mL per well) were transfected with a plasmid vector covalently labeled with rhodamine under optimal conditions for PEI-mediated transfection (PEI N:DNA P = 10:1, 5 μg DNA 10⁶ cells⁻¹) in the presence or absence of 2 μM nocodazole. Cells were fixed post transfection and quantitatively analyzed by fluorescence microscopy. The mean of 50 single cell analyses ± s.d. is shown. From work done by Douglas Galbraith.
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Figure 5.5 and table 5.1 show typical cell physiology and typical cellular parameters respectively. The first thing to observe from figure 5.5 is that nocodazole treated cells are a lot larger late in culture. This observation is borne out when we measure the average diameter of the cells, and calculate the average volume (Table 5.1). To quantify whether the amount of plasmid DNA within nocodazole treated cells is greater, the number of fluorescent particles was measured (Figure 5.5, red dots). It is unlikely that particles are made up of just one plasmid molecule, and is probably a conglomerate of a number of molecules. However, the number of particles will give a qualified estimate of whether nocodazole increases plasmid DNA abundance in cells. Table 5.1 shows that nocodazole does in fact increase the amount of DNA present within cells. However, because nocodazole treated cells are larger, the volume must be considered. If the volume specific number of DNA particles is calculated there is little difference between untreated and nocodazole treated cells. It is therefore an increase in cell volume, with a subsequent increase in plasmids per cell, which leads to the increase in cells specific expression observed in nocodazole treated cells.

5.3 Gene silencing by DNA methylation reduces transient gene expression

The only naturally occurring DNA modification in mammalian cells is cytosine methylation (Ng, et al., 1999). Methylation occurs at CpG sites in DNA, and when this is within or near the location of eukaryotic promoters, gene silencing normal occurs. Hypermethylation has been observed in cells grown in vitro (compared with those in vivo) and is thought to occur because those genes not required for growth in culture are gradually switched off. It is therefore very likely that pDNA will experience methylation to some degree, which will therefore reduce transgene expression. It has been shown in X syndrome cells that the cytosine analogue, 5-azadeoxycytidine reduces hypermethylation in the FMR1 gene (Pietrobono, et al., 2002) and that in human hepatoblastoma cells, 5-azacytidine reduces the methylation of episomal plasmids (Hong, et al., 2001). Both studies indicate that a reduction in DNA methylation leads to an increase in the genes of study. It is suggested that the cytosine analogues irreversibly inhibit the de novo DNA methyltransferase, and
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therefore prevents gene silencing by methylation. The exact mechanism by which this occurs is not fully understood although it is thought that the analogue must be incorporated into a DNA strand to inhibit the enzyme. It was therefore hypothesised that pre-incubation of cells with 5-azacytidine will give and increase in transgene expression. In addition, it was thought that plasmid DNA proliferated in *E.coli* in the presence of 5-azacytidine would also increase transgene expression. Diagram 5.2 shows a comparison of the two homologues.

![Diagram 5.2 A comparison of the naturally occurring cytidine, and the methyltransferase blocking, 5-Azacytidine.](image)

### 5.3.1 Effects of 5-azacytidine on transient gene expression in CHO-S cells

The maximum concentration at which 5-azacytidine (5-AzaC) did not effect CHO-S cell growth was found to be 0.5 μM. CHO-S cells were therefore pre-incubated in 0.5 μM 5-AzaC for 48 hours before transfection. Untreated cells were also cultured. CHO-S cells were transfected with FLuc pEE6.4 grown in the presence and absence of 5-AzaC. Figure 5.6a shows that CHO-S cells pre-incubated in 5-AzaC clearly display nearly a 100 % increase in cell specific productivity, whereas CHO-S cells that are transfected with plasmid DNA grown in the presence of 5-AzaC do not. However, when the overall luciferase production is considered for pre-incubated cells (Figure 5.6b), the increase, although significant, is approximately only 25 %. However, the viable cell density at 48 hours is reduced by approximately 25 % (data not shown).
Figure 5.6 Pre-incubation with 5-azacytidine increases cell specific transient gene expression. CHO-S cells taken from mid-exponential growth were cultured in the presence of 0.5 μM 5-azacytidine for 48 hours. Control, untreated cells were also cultured. The untreated cells were transferred to fresh medium, and transfected with either untreated Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1) or Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1) grown in the presence of 0.5 μM 5-azacytidine. The cells grown in 5-azacytidine were transferred to fresh medium and transfected with untreated Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1). The viable cell density and luciferase production were measured for 48 hours and the cell specific production (A) and total luciferase production (B) were calculated. n = 3 ± s.d. P values are calculated using a two-tailed T-test assuming equal variance, using the null hypothesis.
5.4 Constitutive expression of the E1A transactivaton increases transient gene expression

As previously mentioned, a further barrier to transient gene expression will be the efficient translation of the gene of interest. Although the hCMV promoter is very efficient in CHO cells (Cockett, et al., 1990; Foecking, et al., 1986), it contains elements that are thought to be recognised by the E1A transactivator. It has been shown that cloning of the adenovirus gene for E1A into CHO-K1 cells increases stable expression (Cockett, et al., 1990). The cloned cells, CHO-L761, constitutively express E1A, transactivating available hCMV promoters. It was therefore hypothesised that transient expression in CHO-L761 cells would by higher than in the parental CHO-K1 cells, because of the transactivation of the episomal plasmid DNA.

5.4.1 CHO-L761 cells show higher transient gene expression than parental CHO-K1 cells

Parental CHO-K1 cells and CHO-L761 cells (Lonza, Slough) were transfected with fLuc pEE6.4 DNA and cultured in 24-well plates for 72 hours under standard conditions. Control, untransfected cells were also cultured. Cell growth of parental CHO-K1 cells and CHO-L761 cells remained similar up to 48 hours, after which the growth of CHO-L761 cells slowed down (Figure 5.7a). Transfected cells showed similar growth characteristics to untransfected cells. Transgene expression in CHO-L761 cells is much higher than in the parental CHO-K1 cells, showing approximately a three-fold increase in cell specific productivity at 48 hours post transfection (Figure 5.7b). It is clear from the results that constitutive expression of E1A is leading to a dramatic increase in transient gene expression.
Figure 5.7 CHO-L761 cells show higher transient gene expression than CHO-K1 cells. Sub-confluent CHO-L761 (▲) cells and CHO-K1 (■) cell were cultured under standard conditions (solid line) or transfected with the Fluc pEE 6.4 plasmid (1μg mL⁻¹, N:P 10:1) (dashed line) in 24-well plates. The viable cell density (A) and the luciferase production (B) were monitored throughout culture. n = 3 ± s.d.
5.5 Conclusions and Discussions

In section 5.2 it was shown that addition of the anti-mitotic agent, nocodazole increases cell specific production and the total production in transiently transfected CHO-S cells. In addition, nocodazole gives almost 100% transfection efficiency 24 hours post transfection, which is maintained throughout the culture. Upon further investigation, it was found that the increase in cell specific production resulted from an increase in cell size, which subsequently gave an increase in the amount of DNA per cell. However, the DNA concentration remained constant on a per volume basis. Previous reports by Kung et al (Kung, et al., 1990; Kung A, 1993) have shown that CHO cells treated with microtubule-disrupting agents such as nocodazole continue to synthesize protein and DNA, becoming polyploid via serial doubling of DNA content on extended incubation. These data confirms that CHO-S cells arrested in mitosis by nocodazole, which is known to disrupt the formation of a functional mitotic spindle, continue to actively "cycle" without cell division.

Interestingly, an increase in cell specific production was also observed when nocodazole was added to CHO-S cells stably expressing the monoclonal antibody b72.3 (Tait, in press), which could also be attributed to an increase in the cell size. These data agree with previously published data, that although stably transfected CHO-S cells are most productive during G2/M phase, cell size is the major determinant of productivity (Lloyd, et al., 2000). It can therefore be concluded that cell size is the major determinant in cell specific productivity. However, an increase in total production was not observed in stably transfected cell lines, and must be a phenomenon specific to transient gene expression. It is known that nocodazole blocks cells in the G2/M phase of the cell cycle and that transition through mitosis is associated with a transient loss in the nuclear envelope (Aitchison, et al., 2002). This disintegration of the nuclear membrane might allow trafficking of PEI-DNA particles into the nucleus. However, there is no direct evidence that the absolute rate of PEI-DNA complex trafficking to the nucleus was itself enhanced by nocodazole, above that which occurs in untreated cells during the mitotic transition. It is inferred from our data that nocodazole (and other anti-mitotic agents) may act to maintain CHO cells in a constantly "transfectable" state when supplied to cultures.
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concurrently with PEI-mediated transfection, by causing a loss of nuclear membrane integrity. In these circumstances, nuclear trafficking of transcriptionally active PEI-DNA complexes may be a more continuous process, rather than being restricted to a short period of the cell cycle. In this way, nocodazole acts to increase the rate of DNA uptake of the population of cells, rather than the cell specific uptake rate.

Although nocodazole increases the ‘transfectability’ of the culture, the pDNA that is trafficked to the nucleus is still liable to modification by DNA methylation. Section 5.3 demonstrated that pre-incubation of cells in the cytosine analogue 5-AzaC prior to transfection (with continued use afterwards), approximately doubles cell specific production of transient gene expression. However, there is a concomitant decrease in viable cell density, which results in only a 25% increase in the total production observed. Although 5-AzaC does improve the overall expression of the culture, it is difficult to establish from the data presented the mechanism behind this increase. It is hypothesised that 5-AzaC acts to inhibit DNA methyltransferase, which prevents gene silencing. However, prolonged use of 5-AzaC is also known to block cells in the G1 phase of the cell cycle (Rodriguez, et al., 2001), and it may be this that increases the cell specific production rate. However, the increase in overall expression could not be explained by leaving cells in a more ‘transfectable’ state as with nocodazole, as the nuclear envelop would not be disintegrated in this phase of the cell cycle. It is therefore likely that 5-AzaC does act to increase transgene expression to a certain extent. This could be verified by measuring the degree of methylation of isolated pDNA (Eads, et al., 2000). Although addition of 5-AzaC does increase overall production, its effects upon the cell physiology would have to be further investigated before it could be implemented in an industrial process.

Section 5.4 showed that transient gene expression can also be increased through genetic manipulation of cells, as well as through chemical manipulation. Stable expression of the hCMV transactivator increased transient gene expression by approximately 3-fold. However, at present these cells are not suspension adapted, and are therefore less applicable to large-scale production.

The results presented in this chapter have demonstrated that the three strategies employed to manipulate cell function to increase transient gene expression are
effective to a greater or lesser extent. It was shown that arresting cells in the G2/M phase of the cell cycle, addition of 5-azacytidine to the cell culture, and the use of cells that stably expression the E1A transactiavatar all increase the overall production of the culture. It is clear that use of CHO-L761 cells would be a very easy and effective way to substantially increase expression at large scale, although it would be preferential to have them growing in suspension. Of the two chemical methods used to manipulate cell function, nocodazole appears to be the most promising candidate for use at large scale. However, it does have dramatic effects on cell physiology, which may affect product quality. This issue will be discussed further in the following chapter. What is clear from this chapter is there are probably many different strategies available to increase transient gene expression through manipulation of different cell functions and is therefore still a lot of scope for increasing expression levels from transient systems.
Chapter 6. Process Scale-up and Antibody Production

6.1 Introduction

The aim of this project was to develop a scaleable process for the rapid production of therapeutic proteins using transient gene expression. In order for this to be viable, the most appropriate cell line and transfection vehicle needed to be found. The cell line needed to be one that is commonly used in industry and was amenable to transient transfection. In chapter 1, a comparison between NS0 cells and CHO-S cells showed that CHO-S cells were more robust under the transfection conditions tested. In addition, it was found that removal of serum from growth medium decreased gene expression levels, but that levels could be recuperated by the addition of bovine serum albumin to the medium. Therefore the cell line chosen as being the most appropriate to use for this study was CHO-S cells grown in a serum free medium, supplemented with BSA.

For the process to be applicable at scale, the transfection vehicle used needed to be both inexpensive, and simple to use. Most commercially available transfection products are prohibitively expensive and were therefore immediately disregarded. The only other techniques that are available and could feasibly be used at scale were polyethylenimine (PEI) and calcium phosphate (CaPO₄) mediated transfection. The protocol used for CaPO₄ transfection involves media changes, whereas PEI transfection is an inherently more simple process. It was therefore decided that PEI was the most suitable transfection vehicle available, and in chapter 2 it was shown that after optimization, PEI-mediated transfection gave expression levels in CHO-S cells that are comparable to those achieved with commercial transfection products. It was also shown that progression of cells into mitosis is a pre-requisite for transient gene expression to occur.

In chapter 3 it was demonstrated that transient gene expression can be further increased by various manipulations of cell function. One of the most effective
methods was to block cells in the G2/M phase of the cell cycle, which both increases the transgene expression levels, and reduces the substrate requirement of the culture. However, the processes investigated in the previous chapters were all done in a 24-well plate format. This is a static format and is therefore very different to the well-mixed environment that you would expect in a bioreactor. Not only will the mixing of substrate and PEI-DNA complexes be very different, cells will experience a very different shear environment that could dramatically affect expression levels. It was therefore necessary to compare the culture characteristics observed in 24-well plates, with those found in a mixed environment. In addition, other scale-up considerations and the transient production of a monoclonal antibody were considered.

6.2 Scale-up of transient gene expression in CHO-S cells

In this section it will be demonstrated that results found using cultures grown in a static 24-well plate format can be used to predict those found in the well mixed shake flask environment. This will be done through an experiment comparing growth and expression characteristics in both formats and confirmation of the DNA concentration required for optimal gene expression. In addition, the issue of increasing the culture cell density culture will be addressed. This is because industrial processes are normally run using cell densities between 5 and 10 times those that have been used so far in this thesis.

6.2.1 Comparison of growth and expression characteristics in 24-well plates and shake flasks

CHO-S cells transfected with FLuc pEE6.4 DNA were cultured in ULB 24-well plates and 100 mL shake flasks (30 mL working volume). Control, untransfected cells were also cultured in each format. As can be seen from figure 6.1a, the viable cell density of untransfected cells (solid lines) is comparable in plates and shake flasks up to 30 hours post transfection. After 30 hours, the cell specific growth rate in shake flasks is much higher (0.040 ± 0.0003) than of cells grown in plates (0.031 ± 0.0040)(p = 0.014).
Figure 6.1 Comparison of CHO-S growth characteristics in 24-well plates and Erlenmeyer flask. CHO-S cells taken from mid-exponential growth were transfected with the Fluc pEE6.4 plasmid (N:P 10:1, 1 μg mL⁻¹) and cultured under standard conditions in either ULB 24-wells plates (dashed line, ▲) or 100 mL Erlenmeyer flasks (dashed line, ◆). Control untransfected cells were also grown in ULB 24-well plates (solid line, ▲) and Erlenmeyer flasks (solid line, ◆). Viable cell density (A), cell viability (B) and the metabolite concentration of the culture (C) were measured. n = 3 ± s.d.
Antibody production

Figure 6.2 Comparison of CHO-S expression characteristics in 24-well plates and Erlenmeyer flask. CHO-S cells taken from mid-exponential growth were transfected with the Fluc pEE6.4 plasmid (N:P 10:1, 1 µg mL⁻¹) and cultured under standard conditions in either ULB 24-wells plates (▲) or 100 mL Erlenmeyer flasks (♦). Luciferase production was measured throughout the culture and the cell specific production calculated. n = 3 ± s.d.
Comparison of untransfected and transfected cells shows that the cytotoxic effects of PEI previously described are much more pronounced in shake flasks than in 24-well plates, showing cell specific growth rate of $0.040 \pm 0.0003$ and $0.019 \pm 0.0007$ ($p = 1.11 \times 10^{-5}$) compared with $0.031 \pm 0.0040$ to $0.027 \pm 0.0015$ ($p = 0.232$) in shake flasks and plates respectively. Interestingly however, although there are observable differences in viable cell density, there are no significant differences in percentage viability (Figure 6.1b) and the glucose and lactate concentrations (Figure 6.1c) observed. The transgene expression observed (Figure 6.2) in the different formats show that the cultures are again similar up to 30 hours post transfection, after which the cell specific production is lower in a shake flask environment ($p = 0.0003$).

6.2.2 Confirmation of optimal DNA concentration for transient gene expression in shake flasks

The previous section showed that the effects of PEI-mediated transfection upon cell growth are much more pronounced in shake flasks. It might be suggested that optimal expression will therefore occur using a lower concentration of DNA, which will enable cells to grow. Therefore, CHO-S cells were transfected with Fluc pEE6.4 DNA at a concentration of 0.5, 1.0 and 2.0 $\mu$g mL$^{-1}$ and grown in shake flasks under standard conditions for 4 days. Figure 6.3a shows that the lower DNA concentration does result in a slightly high viable cell density. However, when the total luciferase is calculated (Figure 6.3b) it can be seen that this increase in viable cell density does not increase the transgene expression. As was observed in 24-well plates, 1.0 $\mu$g mL$^{-1}$ of DNA gives the optimal expression.
Figure 6.3 Comparison of transgene expression using different DNA concentrations in shake flasks. CHO-S cells taken from mid-exponential growth were transfected with the Fluc pEE6.4 plasmid (N:P 10:1) at concentrations of 0.5 (■), 1.0 (▲) and 2.0 (●) μg mL\(^{-1}\) and cultured in 100 mL Erlenmeyer flasks. Control, untransfected cells were also cultured (●). The viable cell density (A) and luciferase production (B) were measured throughout culture. \(n = 3 \pm \text{s.d.}\).
6.2.3 Effects of increasing starting cell density upon transient gene expression

An important consideration in developing a scaleable process is the cell density at which a bioreactor is operated. In the studies done so far, the cultures have been seeded at a starting cell density of $2 \times 10^5$. In comparison to most industrial mammalian cell cultures this is very low. It is therefore important to consider how increasing the cell density will effect transient gene expression. CHO-S cells were transfected with gWiz SEAP DNA and cultured in ULB 24-well plates and cultured under standard conditions. In an initial experiment the DNA concentration was kept constant, but the starting cell density was increase. As might be expected, the total expression remained the same, but the cell specific production decreased (data not shown). In a second experiment, the DNA to cell ratio (5 μg per $10^6$ cells) was maintained with an increase in the starting cell density. Figure 6.4 shows that there is a more than 2.5 increase in transgene expression when the cell density is increased from $2 \times 10^5$ to $5 \times 10^5$. However, at cell densities above $5 \times 10^5$ the increase in transgene expression is not equivalent to the increase in the number of cells, and in fact decreases at very high cell densities. If we compare the viable cell density and cell viability (Figure 6.5a and 6.5b respectively) throughout the whole culture it becomes clear why. At very high cell densities, the growth medium used is not able to support exponential growth for long, and the cell number begins to rapidly decrease. With any cells that die, through necrosis or apoptosis, there will be a concomitant loss of the plasmid DNA that was contained within them. Hence, there is a reduction of transcriptively active plasmid DNA. In addition to this problem, it can be seen from figure 6.6 that in cultures of high cell density, glucose is rapidly consumed, and cells begin to utilise the lactate which they have produced.
Figure 6.4 Increasing starting cell density increases total transgene output. CHO-S cells taken from mid-exponential growth were transfected with the gWiz SEAP plasmid (N:P 10:1, 1 µg per 2 x 10^5 cells). Cells were cultured in ULB 24-well plates with starting densities of 0.2 x 10^6 (○), 0.5 x 10^6 (▲), 1.0 x 10^6 (■) or 1.5 x 10^6 (◆) cells mL⁻¹ (maintaining the DNA to cell ratio). The SEAP activity was measured throughout culture. n = 3 ± s.d.
Figure 6.5 Viable cell density and cell viability rapidly decrease at high seed densities. CHO-S cells taken from mid-exponential growth were transfected with the gWiz SEAP plasmid (N:P 10:1, 1 μg per 2 x 10^5 cells). Cells were cultured in ULB 24-well plates with starting densities of 0.2 x 10^6 (●), 0.5 x 10^6 (▲), 1.0 x 10^6 (■) or 1.5 x 10^6 (○) cells mL^-1 (maintaining the DNA to cell ratio). Viable cell density (A) and cell viability (B) were measured throughout. n = 3 ± s.d.
Figure 6.6 High cell density cultures utilise lactate when glucose becomes deficient. CHO-S cells taken from mid-exponential growth were transfected with the gWiz SEAP plasmid (N:P 10:1, 1 μg per 2 x 10^6 cells). Cells were cultured in ULB 24-well plates with starting densities of 0.2 x 10^6 (●), 0.5 x 10^6 (▲), 1.0 x 10^6 (■) or 1.5 x 10^6 (◆) cells mL^-1 (maintaining the DNA to cell ratio). The glucose concentration (A) and lactate concentration (B) were measured throughout. n = 3 ± s.d.
6.3 Monoclonal antibody production by transient gene expression

Until now, the process optimisation has been done using reporter proteins, which are relatively small and simple molecules. However, it is important that the process developed is applicable for use with larger, more complicated molecules such as therapeutic proteins. Therefore a full study into the production of a humanised therapeutic antibody, b72.3 (Lonza Biologics, Slough) was done. The b72.3 plasmid contains the genes for the light and heavy chains of the antibody, and has the glutamine synthetase selection marker on it, so is therefore a much larger DNA molecule than has previously been used (12030 b.p. compared with 6752 b.p.). For this reason the optimal DNA to PEI ratio and DNA concentration was assessed in ULB 24-well plates before shake flask studies were conducted. Once this was established, a comprehensive study of antibody production, cell physiology and metabolism and transfection efficiency at shake flask scale was performed. In addition, the glycosylation patterns of proteins produced by transient transfection were studied.

6.3.1 PEI-DNA complex conditions for optimal monoclonal antibody expression

Changing the size of the DNA molecule used for transient transfections may change the binding characteristics of the PEI-DNA complex, and therefore the PEI to DNA ratio at which optimal expression is achieved. CHO-S cells were therefore transfected with b72.3 DNA using different ratios and DNA concentrations. Figure 6.7 shows the antibody titres achieved using the different conditions. Previous data have shown that optimal expression is achieved with a PEI to DNA ratio of 10:1 and 5 μg per 10^6 cells (section 4.2.1), however figure 6.7 clearly suggests that maximum antibody production is observed using 10 μg per 10^6 cells with either a PEI to DNA ratio of 5:1, or 10:1, although the mg of antibody per μg of DNA does decrease slightly (from 0.22 at 10:1, 1 to 0.17 at 5:1, 2). A further comparison of the conditions 5 μg per 10^6 cells, N:P 10:1 and 10 μg per 10^6 cells, N:P 5:1 (assuming the lower PEI concentration being better) at shake flask scale showed that the previously established ratio was actually better over a longer period culture (data not
Antibody production

![Graph showing antibody production vs DNA concentration for different PEI:DNA ratios.]

**Figure 6.7 Total monoclonal antibody production under different complex conditions.** CHO-S cells taken from mid-exponential growth were transfected with β72.3 DNA and cultured in low binding 24-well plates. Cells were cultured in serum free medium supplemented with 6 mg mL⁻¹ DNA. Cultures were transfected with 0.5, 1 and 2 μg mL⁻¹ DNA complexed with PEI using the molar ratios indicated. Supernatant samples were taken 48 hours post transfection and the antibody titres were determined by ELISA analysis. n = 3 ± s.d.
Antibody production

shown). The possible reasons for this will be discussed later. These results suggest that the size of the DNA molecule used does not appear to effect the PEI to DNA ratio required for optimal expression. They also highlight that there are limitations to optimisation using static 24-well plates, and that they can only be used to establish likely ranges of optimal conditions which should then be studied at a larger scale.

6.3.2 Monoclonal antibody production in shake flask cultures

In order to predict whether transient transfection would be a feasible production option at scale, an experiment was set up to study the production titres and transfection efficiency achievable using transient transfection, and how the use of nocodazole addition augments these. The effects on cell growth and death were also examined. CHO-S cells were transfected with b72.3 plasmid (N:P, 10:1, 5 µg per 10^6 cells) and cultured in 100 mL shake flasks (30 mL working volume) for 4 days. Cells were grown at a starting densities of 5 x 10^5 with and without nocodazole and 1 x 10^6 with nocodazole (assuming that glucose consumption would be less of an issue with cycle arrested cells) and the antibody titre; viable cell density; cell viability; cell apoptosis; cell size and metabolite concentration were measured. Control, untransfected cells were also cultured. In addition, an independent experiment using GFP pEE6.4 DNA was done to determine the transfection efficiency under the conditions described above.

6.3.2.1 Characteristics of transient gene expression in shake flasks

It is worth noting that in all three cultures, antibody accumulation stops 72 hours post transfection and therefore all cell specific production rates are calculated over this period. Figure 6.8a shows that transient transfection without nocodazole present yields a final antibody titre of 1.0 ± 0.12 mg L\(^{-1}\) which corresponds to a cell specific production rate of 0.37 ± 0.05 pg cell\(^{-1}\) day\(^{-1}\). When nocodazole is added the final antibody titre increases to 2.1 ± 0.22 mg L\(^{-1}\) and gives a cell specific production rate of 1.85 ± 0.213 pg cell\(^{-1}\) day\(^{-1}\). This corresponds to an approximate doubling in the antibody production from the same amount of DNA. Doubling the starting cell density (in the presence of nocodazole) and maintaining the DNA to cell ratio,
results in approximately a further doubling of the final antibody titre to $4.6 \pm 0.30$ mg L$^{-1}$ (a cell specific production rate of $2.53 \pm 0.400$ pg cell$^{-1}$ day$^{-1}$). Figure 6.8b shows that transfection efficiency achieved without nocodazole and with nocodazole at both starting cell densities. Without nocodazole, transfection efficiency gradually increases to a maximum of 89% at 48 hours post transfection, and then reduces during the rest of the culture. The presence of nocodazole, however, yields almost 100% transfection efficiency 24 hours post transfection, which is maintained throughout the culture.
Figure 6.8 Nocodazole increases transient gene expression and transfection efficiency in shake flasks. (A) CHO-S cells taken from mid-exponential growth were transfected with b72.3 DNA (N:P 10:1, 5 μg per 10^6 cells) and cultured in 100 mL Erlenmeyer flasks (30 mL working volume) under standard conditions. Cells were cultured with starting densities of 0.5 x 10^6 cells mL^-1, with (■) and without nocodazole (◆), and 1 x 10^6 cells mL^-1 with nocodazole (▲). Samples were taken throughout culture and the antibody production measured by ELISA analysis. (B) In an independent experiment, CHO-S cells were transfected with GFP pEE6.4 DNA and cultured under the conditions described above. Cells were fixed throughout the culture, and the transfection efficiency determined by flow cytometry. n = 3 ± s.d.
6.2.2.2 Physiological characteristics of CHO-S cells transiently transfected with b72.3 plasmid

Figure 6.9-6.11 shows the physiological characteristics of CHO-S cells grown under standard conditions, transfection conditions and transfection conditions with nocodazole added. Figure 6.9a demonstrates that in shake flasks, transfected cells show a characteristic reduction in viable cell density with a concomitant reduction in the cell specific growth rate (from 0.027 ± 0.0013 to 0.015 ± 0.0001, over 72 hours, p = 2.36 x 10⁻⁵). However, this is just a slowing of cell growth rather than cell death occurring, demonstrated by the similar cell viabilities observed (Figure 6.9b). Interestingly, the onset of apoptosis appears to occur earlier in transfected cells (Figure 6.10, condition B) than in untransfected cells (Figure 6.10, condition A). Although there is less cell proliferation in transfected cells, the glucose consumption and lactate production is very similar (figure 6.11a and 6.11b respectively) to untransfected cells. As cell size is not significantly effected by transfection (Table 6.1) it would appear that energy is being diverted from cell growth into product formation.

<table>
<thead>
<tr>
<th>Time post transfection (h)</th>
<th>Average cell diameter (µm ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Control Cells</td>
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<tr>
<td>24</td>
<td>27 ± 3.4</td>
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<tr>
<td>48</td>
<td>27 ± 3.2</td>
</tr>
<tr>
<td>72</td>
<td>26 ± 3.4</td>
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Table 6.1 A comparison of the average cell size of transfected, and nocodazole treated cells. CHO-S cells transfected with a b72.3 plasmid were grown in the absence and presence of nocodazole. The average diameter of cells was determined as described in section 2.2.4.2. n ~ 50 cells.
Figure 6.9 Growth characteristics of transfected CHO-S cells in shake flasks. CHO-S cells taken from mid-exponential growth were transfected with b72.3 DNA (N:P 10:1, 5 μg per 10^6 cells) and cultured in 100 mL Erlenmeyer flasks (30 mL working volume) under standard conditions. Cells were cultured with starting densities of 0.5 x 10^6 cells mL^-1, with (solid line, ■) and without nocodazole (solid line, ◆), and 1 x 10^6 cells mL^-1 with nocodazole (solid line, ◆). Control, untransfected cells were also culture at 0.5 x 10^6 cells mL^-1 (dashed line, ◆) and 1 x 10^6 cells mL^-1 (dashed line, ▲). Viable cell density (A) and cell viability (B) were measured throughout culture. n = 3 ± s.d.
Figure 6.10 Apoptotic characteristics of transfected CHO-S cells in shake flasks. CHO-S cells taken from mid-exponential growth were transfected with b72.3 DNA (N:P 10:1, 5 μg per 10^6 cells) and cultured in 100 mL Erlenmeyer flasks (30 mL working volume) under standard conditions. Cells were cultured with starting densities of 0.5 x 10^6 cells mL⁻¹, with (C) and without nocodazole (B), and 1 x 10^6 cells mL⁻¹ with nocodazole (E). Control, untransfected cells were also cultured at 0.5 x 10^6 cells mL⁻¹ (A) and 1 x 10^6 cells mL⁻¹ (D). Stages of apoptosis were determined using the Guava personal cytometer and are represented in the figure as follows: viable cells; early apoptotic cells; late apoptotic cells; dead cells and cell debris. n = 3 ± s.d.
Figure 6.11 Glucose consumption of transfected CHO-S cells in shake flasks. CHO-S cells taken from mid-exponential growth were transfected with b72.3 DNA (N:P 10:1, 5 μg per 10⁶ cells) and cultured in 100 mL Erlenmeyer flasks (30 mL working volume) under standard conditions. Cells were cultured with starting densities of 0.5 x 10⁶ cells mL⁻¹, with (solid line, ■) and without nocodazole (solid line, ◆), and 1 x 10⁶ cells mL⁻¹ with nocodazole (solid line, ▲). Control, untransfected cells were also culture at 0.5 x 10⁶ cells mL⁻¹ (dashed line, ◆) and 1 x 10⁶ cells mL⁻¹ (dashed line, ▲). Viable cell density (A) and cell viability (B) were measured throughout culture. n = 3 ± s.d.
Addition of nocodazole to cultures of transfected cells leads to a blocking of cell proliferation, and a gradual reduction in the cell viability of the population (Figure 6.9). It is also evident that nocodazole increases the amount of apoptosis observed early in the culture (figure 6.10, conditions C and D). However, nocodazole does decrease the overall substrate requirement of the culture (Figure 6.11), reducing the rate of consumption from 35 mg h\(^{-1}\) to 20 mg h\(^{-1}\). This allows a higher density of cells to be maintained, and therefore a higher final antibody titre to be achieved. As previously observed, nocodazole also leads to an increase in average cell size (Table 6.1).

6.3.3 Glycan analysis of monoclonal antibodies produced by transient gene expression

The glycosylation pattern of a therapeutic protein can have dramatic effects upon its efficacy and effect in patients. It is therefore important to establish whether correct glycosylation of proteins is occurring during transient transfection. In addition, nocodazole acts to block microtubule formation, and will therefore dramatically affect the internal cytoskeleton of the cell. This may effect the functioning of the golgi apparatus and therefore glycosylation of protein products. Figure 6.12 shows the glycosylation pattern of b72.3 antibody produced by transient transfection in the absence and presence of nocodazole. The glycosylation pattern observed from transient transfection shows that correct glycosylation of the antibody is occurring. Comparing these results with the nocodazole treated cells, we see that although glycosylation is occurring to a certain degree, the amount of fucosylation occurring is reduced and the number of high manose products (those which have not undergone complex glycosylation) present is increased.
Figure 6.12 Analysis of the Fc N-linked glycans of recombinant IgG4 Mab transiently produced by CHO cells in the presence and absence of nocodazole. MALDI-MS analysis of Asn^{297} containing tryptic glycopeptides deriving from recombinant b72.3 Mab harvested at 96 h post transfection. Mab was produced by cultures transfected at an initial viable cell density of 0.5 x 10^6 cells mL^{-1} as described in Figure 7 in the absence (A) or presence (B) of nocodazole. The observed monoisotopic masses of singly protonated molecular ions and the corresponding N-glycan structures are indicated. Note that in each case the observed mass is the sum of the N-glycan moiety plus that of a common Asn^{297} containing peptide with a monoisotopic mass of 1173.52 Da. The monosaccharide residues of N-glycan structures are represented by the following symbols: ■, N-acetylglucosamine; ●, mannose; ★, fucose; ▲, galactose.
6.4 Conclusions and Discussion

In this chapter it was shown that optimised PEI mediated transient transfection of CHO cells in suspension, in the presence of nocodazole, can yield recombinant antibody titres of approximately 5 mg L\(^{-1}\). This is broadly comparable with those obtained by PEI-mediated and calcium phosphate-mediated transfection in HEK293(EBNA) cells (Durocher, et al., 2002; Meissner, et al., 2001), where titres of 1-20 mg L\(^{-1}\) were achieved. In addition Wurm et al., have recently reported antibody titres of up to 8 mg L\(^{-1}\) using PEI-mediated transfection in CHO-S cells (Derouazi, et al., 2004). However, it is noted that comparisons of expression level using different proteins can potentially be misleading as, for example, transient expression of even different recombinant Mab’s using the same CHO host cell line and liposomal transfection reagent has been reported to yield a 200-fold difference in expression levels between Mabs (Bentley, et al., 1998).

Whereas the HEK293(EBNA) systems are based on the engineered ability of HEK cells to replicate the episomal DNA vector with each cell doubling (and thus productivity is growth associated), the simpler CHO cell transient expression system described here is based on the production principle that host cell biomass is accumulated prior to a shorter three day transient production phase. During this period, manipulation of the host cell by nocodazole or other anti-mitotic agents yields a significantly higher product titre at the expense of cell growth and ultimately, viability. In this context, this system is more analogous to recombinant protein production by virus infected mammalian cells and, for example, optimised adenovirus (Condon, et al., 2003; Cote, et al., 1998; Nadeau, et al., 1996) and Semliki Forest virus (Blasey, et al., 2000; Blasey, et al., 1997) based systems that have been described. Of course, the synthetic transfection system described here is inherently simpler to implement than viral mediated gene transfer and by comparison with data presented in the reports cited above, yields a potentially higher titre of recombinant product.

In considering potential problems with scale-up of PEI-mediated transfection, it is worth noting that although transgene expression is comparable, the cytotoxicity
associated with PEI transfection is more pronounced in shake flasks. This is probably due to the slight increase in transfection efficiency observed in the better-mixed environment of the shake flask. The better mixing will increase transfer of the PEI-DNA complexes to the cell surface, and therefore increase uptake. It is therefore clear that mixing will become a major issue for scale-up, because it will not only affect the transfection efficiency of the culture, but also the cell specific growth rate. It is noted that the maximum starting cell density employed here (in the presence of nocodazole) for Mab production was $1 \times 10^6$ cells mL$^{-1}$. Under these circumstances mean cell specific monoclonal antibody production ($q$Mab) was actually higher (albeit not significantly) than that obtained at a lower starting cell density. Therefore, we would expect that higher productivity is possible using a host CHO cell culture at higher initial viable cell density at the same optimal ratio of PEI:DNA:cells. Moreover, we strongly suspect that use of a host CHO cell line with more favourable growth characteristics in a controlled environment would further improve yield. In other words, there is ample potential for further optimization of this system.

The use of nocodazole in this system increases transient gene expression, however, another well-characterized cellular effect of nocodazole on mammalian cells is Golgi fragmentation (Cole, et al., 1996). Fragmentation and dispersal of the Golgi complex has been shown to be a necessary component of cellular re-organisation during mitosis (Sutterlin, et al., 2002), and is a microtubule directed process (Shima, et al., 1998; Shima, et al., 1997). That is, disruption of microtubule-directed Golgi partitioning by nocodazole itself contributes to G2/M arrest. We had considered Golgi fragmentation to be a potential limitation for transient production of secreted proteins, but this was not the case. In fact, despite the potential ultrastructural effects of nocodazole treatment on the Golgi apparatus, it clearly does not disrupt secretion of proteins from CHO cells; or indeed from other cell types (David James, personal communication). Furthermore, analysis of the N-glycosylation of Mab transiently produced by nocodazole treated cells indicated no significant aberrations in Golgi processing.

In summary, the work described in this thesis has shown that optimised PEI-mediated transient transfection can produce monoclonal antibody titres comparable
to those achieved by other transient systems. The system is compatible to use with
the manipulation of cell function to further increase titres and it can be scale-up for
high cell density cultures. Although the production titres are not comparable to
stable cell line production, the potential for further optimisation and scale-up so that
gram quantities can be produced is clearly there. This would enable the early
characterisation of potential new therapeutic proteins. This is an inherently simple,
cheap and scaleable process, and although in its infancy, PEI-mediated transient
transfection in mammalian cells obviously has the potential to be a very powerful
tool in the development of therapeutic biopharmaceuticals.
Chapter 7. Future Directions

7.1 Introduction

The work described in this thesis has demonstrated that large-scale transient gene expression for recombinant protein production is a feasible process. The optimal parameters for PEI-mediated transfection have been identified in Chinese hamster ovary cells, and the production of a recombinant monoclonal antibody was demonstrated. This thesis has also indicated that there are a number of potential routes of further investigation regarding this process. It is clear that, through manipulation of the cellular environment, or through manipulation of cellular function, expression levels can be expressed. It has also been shown that the process could be scaled up to bioreactors working in a high cell density format. The following chapter will discuss the approaches that could be taken to monopolise on the results presented in this thesis.

7.2 Further optimisation of PEI-mediated transient gene expression

Although the PEI-mediated transfection protocol has been optimised to a certain extent, there are still options available for further improvement. The areas that would appear to be the most amenable to manipulation are: (1) the culture medium; (2) the formulation of the PEI-DNA complex; (3) manipulation of cell function. The ways in which these might be further investigated will be discussed here.

7.2.1 Optimisation of the culture medium

The removal of serum from the culture medium was seen to have a dramatic effect upon transient gene expression levels, however, the addition of bovine serum albumin resorted expression. It is therefore evident that the culture medium has a direct influence upon the success of transient gene expression, and could therefore be further optimised. Although the addition of bovine serum albumin is clearly beneficial to transient gene expression, it is still an animal derived product, which are becoming less accepted by the regulatory authorities. Therefore, unless
recombinant BSA is used, it would be beneficial to find an alternative. Addition of other peptide mixes has been shown to also increase transient gene expression (Geisse, 2003; Pham, et al., 2003; Rhaese, et al., 2003). However, of these, only plant-derived hydrolysates are not animal derived. Therefore, investigation into the availability of alternatives and their affect upon transient gene expression should be done. In addition to macromolecules, other medium components have been shown to increase transient gene expression, such as folic acid (Guo, et al., 2001; Wightman, et al., 2001) and calcium and zinc ions (Pichon, 2002), which could also be investigated. There is obviously a lot of potential to increase transient gene expression through medium optimisation.

7.2.2 Manipulation of the PEI-DNA complex

The work presented here has shown that although PEI-mediated transfection is a relatively efficient process, the mechanism by which PEI-DNA complexes enter and are translocated through the cell are still not well understood. What is known is that PEI is very efficient at condensing DNA (Bloomfield, 1996), which target it to the cell membrane. However, the characteristics of the PEI-DNA complex that makes this optimal are still not fully understood. From here, it is endocytosed, with eventual release into the cytoplasm. The complexes are then transported to the nucleus, where gene expression can occur (Godbey, et al., 1999b). However, although PEI is thought to buffer the endosome, increasing the release of complexes into the cytoplasm, a large proportion is still degraded in the lysosome (Bieber, et al., 2002). In addition, the transferred of plasmid DNA into the nucleus is only thought to occur via the nuclear pore complex if the DNA is conjugated to a NLS containing peptide (Munkonge, et al., 2003). There are clear a number of strategies that still remain to increase PEI-mediated transient gene expression through manipulation of the complex. The areas that could be tackled to achieve this are: (1) understanding of the interactions of the PEI-DNA complex; (2) increasing the release of PEI-DNA complexes from the endosome; (3) increasing transport to, and translocation across the nuclear envelope.
7.2.2.1 Understanding the PEI-DNA complex

There is still little known about what properties of a PEI-DNA complex make it optimal for transient transfection. It has been shown that their zetas potential (Son, et al., 2000) and size (Gebhart, et al., 2001; Rudolph, et al., 2002; Tang, et al., 1997) are important, however, this is often cell line specific, and therefore no definitive specifications can be set. In addition, it is clear that the cytotoxicity of PEI is an issue. There is clearly potential for increasing PEI-mediated transient gene expression through further optimisation of these characteristics.

Although the charge of the PEI-DNA molecule is important in its transfection, the design of an appropriately charged molecule may not be as easy as it sounds. It was shown here that variation in the PEI to DNA ratio can alter transient gene expression, however, it is difficult to quantify the affect salts and other medium components will have upon the transfection particle. It is thought that the PEI-DNA complex is relative loosely formed (Clamme, et al., 2003), and there will therefore be many charged groups available to make electrostatic interactions. This has been shown to be the case here with BSA. Therefore, in order to ‘design’ an optimal PEI-DNA complex, all the salt, and pH environments that the complex might encounter needs to be considered. Therefore, a model of the various stages of transfection would need to be established.

The issue of the cytotoxicity of PEI has already been tackled using a number of approaches. These aim either to make the PEI molecule less cytotoxic, or to hide the PEI-DNA complex from the cell. One way in which the cytotoxicity of PEI is being tackled is the production of biodegradable PEI molecules (Ahn, et al., 2002; Forrest, et al., 2003). Another way in which the cytotoxicity has been reduced is by ‘hiding’ the PEI-DNA complexes from the cell using large macromolecules, similar to the phenomena shown here with BSA. This has so far been done using transferrin and human serum albumin (Kircheis, et al., 2001; Rhaese, et al., 2003). These show promise, but are multi-component transfection agents, and therefore increase the complexity of transfection.
7.2.2.2 Increasing release of PEI-DNA complexes from the endosome

PEI is thought to increase release of plasmid vectors from the endosome, however, a proportion of molecules are still targeted to the lysosome. Virus particles achieve early release from the endosome using fusogenic peptide sequences. These are normally active membrane sequences, located at the amino-terminus of viral protein, which mediate membrane translocation upon acidification. The most commonly used fusogenic peptide has been the N-terminal sequence of the influenza virus haemagglutinin subunit HA-2 (Wagner, et al., 1992), however, others are available (Kichler, et al., 1999; Morris, et al., 2000; Wolfert, et al., 1998). These fusogenic peptides have been shown to increase transfection efficiency when associated with PLL, cationic lipids, PEI or polyamidoamine cascade polymers (Morris, et al., 2000). However, these techniques have not been used for the production of recombinant antibodies at large-scale. In addition, the optimal ratio of peptide to PEI-DNA complex has not been comprehensively investigated. Therefore, the potential for further increases in transient gene expression using these methods should be investigated. Another molecule commonly used to increase transfection is chloroquine. This buffers the lysosome, and therefore has the same affect as PEI, in that more DNA is released into the cytoplasm. This could therefore be used in conjunction with PEI. However, a recent report suggests that an additive affect is not observed, and that chloroquine actually inhibits PEI-mediated transfection (Akinc, et al., 2005). This could however be a case specific phenomena, and should therefore be tested.

7.2.2.3 Increasing the transport of PEI-DNA complexes to the nucleus

Although most transfection agents are able to mediate recombinant vectors to the cytoplasm, to a greater or lesser extent, there still remains the issue of nuclear targeting. This has been identified as a major barrier to transfection, and therefore strategies to improve this stage of transfection have been investigated. The most commonly used technique is the addition of nuclear localisation signals (NLS) to the transfection agent. NLS are normally short basic peptides that interact with the nuclear pore complex (NPC), and ultimately direct transport into the nucleus (Chan, et al., 2002). A number of different NLS have been show to increase transfection
efficiency in a range of cell types (Branden, et al., 1999; Cartier, et al., 2002; Chan, et al., 2002; Colin, et al., 2001; Ludtke, et al., 1999; Petrulis, et al., 2001; Tachibana, et al., 1999), however, the mechanism by which this occurs remains unclear. Due to the nature of PEI, conjugation of an NLS to the molecule would be simple. However, although this may increase transient gene expression, all PEI molecule used for future transfection would have to be conjugated to the NLS. This could become a costly process at large scale.

An alternative method to the direct addition of NLS to recombinant DNA complexes is to recruit proteins that naturally occur in the cytoplasm, which are being targeted to the nucleus. It has been shown that DNA containing specific sequences bind to cytoplasmic proteins that are targeted to the nucleus. Addition of these sequences increase nuclear uptake of recombinant genes (Dean, 1997; Dean et al., 1999). This would be a simple way of increasing transient gene expression if a generic plasmid and a generic cell line were used. Implementation of the technique would require the identification of DNA binding proteins in the host cell line that are nuclear targeted. The binding sequence could then be engineered into the plasmid, with the potential for increased nuclear transport. This would be a complicated procedure to undertake, but once accomplished would results in a DNA vector more suited to transfection of the chosen cell line.

7.2.3 Manipulation of cell function to increase transient gene expression

It was shown here that the simple manipulation of the cell cycle using nocodazole resulted in a doubling of production from transient gene expression in mammalian cells. In addition, the use of the E1A transformed cell line (CHO-L761) also resulted in the increase of transient gene expression. It is therefore evident that simple manipulations of the cells function can increase transient gene expression. It was found that other microtubule disrupting agents such as, paclitaxel, colchicine and vinblastine increased transient transfection (Tait, in press). However, these drugs are expensive and would not be practical for use at large scale.

The use of transformed cells, however, does have the potential for further optimisation. Other than the manipulation of protein translation, cell lines can be
engineered for better recombinant protein production in other ways. One of the most commonly used methods is addition of the gene Bcl-2 (Ibarra, et al., 2003; Mercille, et al., 1998), which expresses the Bcl-2 anti-apoptosis protein. It is clear from the results presented in this thesis that inhibition of apoptosis would be an advantage in transient gene expression. Therefore, the affect of Bcl-2 upon transient gene expression should be investigated.

7.3 Scale-up of PEI-mediated transient gene expression

It was demonstrated here that transient gene expression could be used to produce recombinant monoclonal antibodies in mammalian cell culture. However, for this to be a useful tool, the process must be able to provide larger quantities of protein material, for which scale-up would be required. It was shown here that the cell concentration and DNA concentration could be increase, with a concomitant increase in production. However, using the batch system demonstrated here, nutrient limitation became a problem. The following section will discuss the important issues regarding the scale-up of PEI-mediated transient gene expression, and the possible bioreactor designs that could be used for this purpose.

7.3.1 Critical scale-up parameters

The characteristic parameters of PEI-mediated transient gene expression that were identified in chapter 6 are: (1) plasmid DNA uptake is maximal up to 48 hours (2) in the absence of nocodazole, plasmid uptake is dependent on cell division (3) recombinant protein production occurs up to 72 hours post transfection (4) nutrient limitation becomes a problem at high cell densities. In addition mixing of the PEI-DNA complexes might become an issue at scale. However, the shear sensitivity of PEI-DNA complexes is not known and disruption of the complexes might result in a reduction in transient gene expression. Therefore, consideration of these parameters must be taken during the scale-up process.

The requirement of cell division for maximum plasmid uptake means that cell division in the first 48 should be optimal. This also suggests that it might be possible
to feed the cells with more PEI-DNA complexes, once the culture medium concentration has dropped below that required to create an efficient concentration gradient. However, feeding of more PEI-DNA complexes might result in greater cytotoxicity. It is clear that because plasmid DNA is not replicated in this system, but is also cytotoxic in its complexed form, the starting cell density should be as high as possible. This will allow the use of the maximum amount of DNA, whilst keeping the DNA to cell ratio the same. However, at high cell density, nutrient deprivation becomes a problem. It would therefore be useful to implement a feeding strategy. Ideally, full HPLC analysis of the culture medium would be done to establish why recombinant protein production ends at 72 hours post transfection. This could then be used to implement a feeding strategy to combat nutrient deprivation.

7.3.2 Choice of bioreactor for scale-up

The previous section identified that to scale-up PEI-mediated transfection in CHO-S cells, the bioreactor would have to be able to manage: (1) high cell density; (2) good mixing will low shear; (3) a feeding strategy. The standard bioreactor format for the production of recombinant proteins is the fed-batch (Sauer, et al., 2000), which extends the production time of the batch process by feeding nutrients to late exponential cultures. However, the development of perfusion cultures has meant that mammalian cell culture can be further enhanced by the continuous removal of toxic waste products, including the recombinant protein product (Yabannavar, et al., 1994; Yang, et al., 2000). Perfusion bioreactors have a retention membrane across which small molecules can pass, but cells cannot. Nutrients can therefore be fed into a reservoir, allowing diffusion across the membrane into the main bioreactor. In addition, the continual removal of liquid from the reservoir clears away waste products. This could also be potentially used for the feeding of PEI-DNA complexes, for increased transient gene expression. However, perfusion cultures often have spinning membranes, to avoid the blocking of pores. This may therefore create shear forces that cause an unfavourable disruption to PEI-DNA complexes. An alternative method is the use of disposable bioreactor bags on wave bioreactors (Singh, 1999). These bioreactors have good mixing, with low shear forces. However, these are presently not used in industry, and would therefore have some
validation implications. A comparison of a fed-batch stirred tank reactor, and a wave bioreactor would indicate the best route for further scale-up of transient gene expression.
Chapter 8. The Impact of Large-scale Transient Gene Expression upon the Management of Developing a New Recombinantal Therapeutic Protein

8.1 Introduction

This thesis has described the optimisation of transient gene expression in CHO-S cells for the production of recombinant therapeutic proteins. Although not used for large-scale production here, its potential has been demonstrated. With further development, the process could feasibly be used in the development of therapeutic proteins. Most therapeutic antibody treatments require large doses, which are usually repeated over a long-term period. Therefore, the annual production often needs to be in the kg quantities (Chadd, et al., 2001). It is therefore clear, that the optimised process described here could not be used for the large-scale manufacturing of licensed therapeutics, as a production capacity of 200 m³ would be required. However, if the process was further optimised and developed so that expression levels were similar to stable expression, it might be feasible to use it at large-scale. It is therefore clear that depending on its further optimisation; transient gene expression could be used in two different ways:

- Rapid production of protein material for pre-clinical trial testing
- Large-scale manufacture of therapeutic drugs.

The impact of transient gene expression on these two different situations will be very different, and they will therefore be discussed separately here.

For any new therapeutic protein under investigation, there are various stages of development before full-scale manufacture and release onto the marker. These can be broadly classed into: (1) pre-clinical testing and characterisation; (2) clinical trials; (3) licensing and manufacture. The pre-clinical testing involves the production and characterisation of the new therapeutic proteins. Clinical trials are required to demonstrate the therapeutic properties, and establish the safety and efficacy of the new drug. This also highlights the dosage requirements, and any side effects experienced. The licensing and manufacture involves clearance by the
regulatory authorities, with subsequent large-scale manufacture. The main issues regarding management of the development line are speed and the smooth transfer between the different stages. In addition, it is important to ensure that process decisions are made early, because latter changes are very expensive. The impact that the two uses of transient gene expression previously discussed would have upon this development process is described here. In addition, the validation issues associated with a transient gene expression system will be considered.

8.2 The impact of using transient gene expression for pre-clinical trial testing only

The transient gene expression described here is most likely to be used for pre-clinical trial testing. This obviously, will most dramatically affect the pre-clinical trial stage of development. However, there may be some impact upon the latter stages, which will be discussed here.

8.2.1 The impact of pre-clinical transient gene expression on pre-clinical testing

Figure 8.1 shows the main activities involved in pre-clinical testing and characterisation. It is clear from this diagram that the creation of a stable line is the limiting stage of this process. The early production of protein material using transient gene expression would therefore allow most activities in this development stage to be started earlier, including purification development, formulation and production stability, and toxicology studies.

Purification of new antibodies is normally done using pre-defined generic processes. However, these processes still need to be tested with any new candidates to ensure that recovery is optimal. The use of transient gene expression to rapidly produce protein material however, would allow purification testing to begin earlier. This would give more time in the development process to cope with any changes that might need to be addressed for any new or different protein molecules used. In addition, because the purification process is faster, the formulation and product stability studies can be started earlier.
Figure 8.1: The sequence of events required for pre-clinical studies in the development of therapeutic proteins.

Product formulation and stability studies must be done to ensure that the purified product can be effectively produced in a format that can be easily administered to patients. This is normally in either liquid form, or lyophilised powder. Once the appropriate formulation has been established, its stability over time must be tested, so that its shelf life can be determined. This is a time consuming process, which can be on the critical path of development. Therefore, if this were started earlier, the pre-clinical development time could be reduced. Another time-consuming stage of pre-clinical development is the toxicology studies. Again, if protein material that has been purified and had its formulation established is produced more quickly, toxicology studies can be started earlier.

The potential for transient gene expression for decreasing the time required for pre-clinical trials is clear. This will obviously have a knock-on effect for the whole development process, therefore reducing the time to market for any new therapeutic proteins. In addition, the success rate of biopharmaceuticals from pre-clinical to phase I clinical trials is 57% (Struck, 1994). Therefore, the rapid identification of ineffective drugs before pre-clinical trials may reduce the amount of time and money spend on the development of stable cell lines that are ultimately not required.
8.2.2 The impact pre-clinical transient gene expression on clinical trials

The main development issue associated with clinical trials is the management of changes required during the trials. Toxicology studies should give indications of any adverse reactions that might occur; however, these may only become apparent in clinical trials. For example, if an indication gave an adverse reaction that could be reduced by a slight structural change. The structural change would mean that this is classed as a new indication. However, with transient gene expression it would be possible to quickly check whether the new indication has the same purification profile and whether its formulation and stability is the same as the first indication. This would then indicate whether clinical trials should be continued with the first indication, accepting the adverse affects, or whether the new indication should enter the development process.

In addition, it might be possible to use material produced with transient gene expression to give an indication of how the material produced from a stable cell line will react. This could identify poor candidates, and therefore reduce the number of candidates that make it through to clinical trials, which could potentially save a lot of money.

8.2.3 The impact pre-clinical transient gene expression on licensing and manufacture

Although pre-clinical use of transient gene expression will not directly influence the manufacture of the product, it will mean the development time will have been reduced. Therefore, manufacturing will begin earlier, allowing earlier release onto the market. This will in turn, extend the length of time the released product is under patent, and therefore increase its potential earnings. There are some licensing issues regarding the use of different processes in pre-clinical trials, however, these will be discussed in section 8.4.
8.3 The impact of using transient gene expression for large-scale manufacturing of recombinant proteins

If the transient gene expression process was optimised to a point where it rivalled stable cell line expression levels, it could feasibly be used for large-scale manufacture of therapeutic proteins. This will clearly influence the pre-clinical trials in the ways discussed in section 8.2.1. However, it will also dramatically affect the licensing and manufacturing stage of development.

8.3.1 The impact of using large-scale transient gene expression on the pre-clinical and clinical stages of development

Using transient gene expression throughout the whole development process of a therapeutic protein would affect the pre-clinical and clinical trial stages as previously described. In addition to this, however, the need for producing, banking and testing of stable cell lines could be delayed until the indication has been successful in late clinical trials. This is because a standard cell line in which all transfection are done could be used. Therefore, the cell banking and characterisation will have already been done, and would only be required for the stable cells line of successful drugs. The fermentation optimisation will also have been done, and therefore only the effects of the new product on fermentation parameters will have to be done. These combined will also ease the pressure on the volume of documentation that needs to be produced. Therefore, the time required for the pre-clinical and clinical stages of development will be greatly reduced.

8.3.2 The impact of using large-scale transient gene expression on licensing and manufacture

The advantage of using large-scale transient gene expression for the manufacture of recombinant therapeutic products is the process is very generic. With the development of new proteins, the only change in the process that would occur is the vector, and therefore the product itself. However, a standard vector and cell line could be used, reducing the amount of development required. By using a standard
cell line, the fermentation parameters will be well characterised and documented. This will reduce the development times required for fermentation processes, and the scale-up of fermentation will be very easy to predict. Therefore, the only additional development that would be required is the purification stream. However, this could be made generic, further reducing development times. In addition to reducing the development times, the need for validation of the process will be reduced, making licensing of the product easier. This is because the generic nature of the process would allow historical data to be used for many stages of the validation process. These advantages gained from transient gene expression would ultimately save a lot of time in development, and would therefore results in a quick release onto the market. This will extend the time the therapeutic is covered by patent, and therefore increase the earning potential of the drug.

8.4 Validation issues associated with transient gene expression of recombinant therapeutic proteins.

The validation of production of a monoclonal antibody includes process and product validation. The process validation must show that the equipment used for the production of the protein operates within predefined set parameters, such as pH temperature of the fermenter. The product validation must show that the protein characteristics are consistent, i.e. the structure, biological activity. These together must show that the manufacturing process consistently and robustly produces a product of the appropriate quality. This ensures that the product will have the expected efficacy, and be safe to use in the patient (Moran, et al., 2000). The validation issues associated with transient gene expression would be very different if the technique is used for just pre-clinical trial testing, and if it were used for full-scale manufacture. As mention, the process described here could not be used for the latter at present, but further optimisation could allow this. The validation implications will therefore be considered for both cases.
8.4.1 Validation of transient gene expression used for pre-clinical testing only

If transient gene expression is used to produce material for the initial characterisation and toxicology studies for a new therapeutic protein, then the main validation issues will be regarding the use of two different expression systems in development. This is because transient production and stable production would not have the same process parameters. It was shown here that transient transfection affects the growth rate of cells, and the addition of DNA to the medium is quite a large process change. In addition, the change in process and the different nature of the expression systems is likely to result in different products. This would become a problem because the regulatory authorities like the pre-clinical trial and clinical trial material to be produced using the same system as the manufactured product.

To overcome this problem, a company would have to prove that although the systems are evidently very different, they essentially have the same process control parameters. They would also have to be able to prove that the transient and stable expression systems yield a product with the same characteristics. Proving that transient and stable expression can be achieved using the same process controls could be possible. The cell line will be the same, and therefore the substrate concentration, temperature and pH ranges that occur throughout the culture are likely to be similar. Therefore, if these fall within the predefined process control parameters, they might be considered the same. However, proving that the cultures behave in the same way, and produce the same crude drug product from fermentation could be a problem. Not least because there will be residual DNA in the culture media, which will need extra purification steps. In addition, the purified product may exhibit different glycoforms to that produced in a stable cell line. However, with the advent of post-fermentation manipulation of the glycosylation patterns of therapeutics proteins, this may become less of an issue.

It may be possible to validate the use of transient gene expression for the production of pre-clinical trial material. However, because it goes against the present recommended practices of the regulatory authorities, it could be an expensive process to initiate. The first company to implement the technology would have to go through the trouble of extensive validation trials and documentation, without the
guarantee that the product will be licensed at the end of its development. Although the use of transient gene expression at the stage of development would clearly be an advantage, the risk for a company to use it is presently too great. However, with further development and characterisation, it might one day be adopted.

8.4.2 Validation of transient gene expression used for large-scale manufacture

If the process were optimised to a point where transient gene expression could be used for full-scale manufacture of recombinant protein products, it is my opinion that product variation would be the main validatory issues. The reason for this is that during the research for this thesis, there appeared to be some batch-to-batch variation in the expression level achieved. There are a number of reasons this might occur, from variations in: the DNA preparations; the PEI formulation or the cell passage number. Therefore, one of the main validation problems might be the robustness of the crude product. This could easily be solved by having robust and validated methods for the large-scale production of recombinant plasmids and PEI solution. In addition, the passage number of the cells used can be set within certain predetermined limits. These together should reduce the batch-to-batch variation, and therefore allow validation of the fermentation process. A second issue that might arise from large-scale PEI-mediated transient transfection is the removal of the DNA and PEI components during purification. However, it is likely that the purification protocols already in place for recombinant proteins would be sufficient.
References


References


References


Delivery to the Nucleus in Mammalian Cells. The Journal of Biological Chemistry 273: 7507-7511.


References


References


References


Appendix A

Calculation of total luciferase production (light hours) was done using the trapezium rule:

\[ \text{Light Hours} = \sum \left( \frac{(L_1 + L_0)}{2} \right) \times (T_1 - T_0) \]

- \(T_0\) = Time of first measurement (h)
- \(T_1\) = Time of second measurement (h)
- \(L_0\) = Relative light units of first measurement
- \(L_1\) = Relative light units of second measurement
Appendix B

The following pages are reproductions of the certificates of analysis for the bovine serum albumin products tested. They are as follows:

Albumin Bovine, Serum product number A2153

Lot number 026H1013 168
Lot number 035H1065 169
Lot number 032K1444 170

Bovine Albumin, Gamma-irradiated, Sigma product number A3156
Lot number 109H83001 171

Bovine Albumin, Cell culture tested, Sigma product number A4161
Lot number 57H9304 172
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<td>CAS number</td>
<td>9048488</td>
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<td>Appearance</td>
<td>White to tan powder</td>
<td>Faint yellow powder with a faint tan cast</td>
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<td>Solubility</td>
<td>Clear to slightly hazy yellow solution at 10 g plus 250 ml of water</td>
<td>Very slightly hazy faint yellow solution at 10 g plus 250 ml of water</td>
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<td>Loss on Drying</td>
<td>Not more than 10%</td>
<td>1.3%*</td>
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<td>Nitrogen 14.5 to 16.5%</td>
<td>15.84% Nitrogen</td>
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<td>Identity by Infrared Electrophoresis</td>
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<tr>
<td>pH Test</td>
<td>6.5 to 7.5 (% in 0.15 M Sodium Chloride)</td>
<td>7.0 (1% in 0.15M NaCl)</td>
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<td>Ash assay</td>
<td>NLT 95% Albumin</td>
<td>87% Albumin</td>
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* Supplier information.
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<td>SOLUBILITY</td>
<td>CLEAR TO SLIGHTLY HAZY YELLOW SOLUTION AT 10 GM PLUS 250 ML OF WATER</td>
<td>15.45% NITROGEN OF BOVINE ORIGIN</td>
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<td>LOSS ON DRYING</td>
<td>NMT 0%</td>
<td>7.3 (1% IN WATER)</td>
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<td>WATER</td>
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<td>CHLORIDE)</td>
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<td>SHELF LIFE SOP 10-30-2007</td>
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ATTN ANDREW TAIT  
QLD UNI-CHEMICAL ENGINEERING  
FAX: 07 3365 4199

SIGMA-ALDRICH

DATE: 11/04/02

--- CERTIFICATE OF ANALYSIS ---

PRODUCT NAME: ALBUMIN, BOVINE GAMMA-IRRADIATED  
CELL CULTURE TESTED
PRODUCT NO: A3156  
LOT NO: 1094193001
CAS NO: 9048-46-8

STORE IN A COOLER

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1 OF 1
Certificate of Analysis

Albumin, Bovine Essentially Globulin Free Cell Culture Tested
Product No. A4181
Lot No: S798304
CAS No: 9048-46-0
Assay Results: July 1996

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Biological performance is assessed using appropriate cell lines. Data are counted, and growth is plotted as a logarithmic function of time in culture. Seeding efficiencies, doubling time, and final cell densities are determined. During the testing period, cultures are examined microscopically for any morphological abnormalities that may indicate toxic components.

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Publications

Conference Attendance and Presentations

- European Society for Animal Cell Technology UK Conference, Jan 2001
- Oral presentation at European Society for Animal Cell Technology UK Conference, Jan 2002
- Fermentation and Bioprocess Interest Group, April 2002
- Oral presentation at Fermentation and Bioprocess Interest Group, April 2003
- Poster presentation at European Society for Animal Cell Technology, May 2003
- Oral presentation at Asia Pacific Biochemical Engineering Conference, Dec 2003
- Poster presentation at Cell Engineering Conference, March 2004

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Tait, AS; Hoare, M; Birch, J; Galbraith, DJ; Hines, MJ; Brown, CJ; James DC (2003) Optimisation of PEI-Mediated Transient Expression in Chinese Hamster Ovary Cells. Presented at ESACT conference, Granada,


