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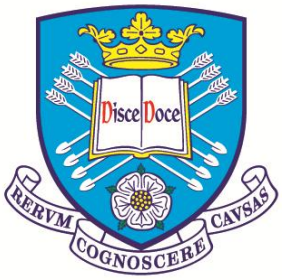
For the degree of Doctor of Philosophy

A Mechanistic Dissection of Polyethylenimine Mediated Transfection of Chinese Hamster Ovary Cells



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Declaration

I, Olivia Louise Mozley, declare that I am the sole author of this thesis and that the research presented within is a result of my own efforts and achievements. Where this is not the case, this has been clearly stated. I confirm that this work has not been submitted for any other degrees.

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LIST OF ABBREVIATIONS

ACA	anti-clumping agent
AF	auto-fluorescence
bPEI	branched PEI
BSA	bovine serum albumin
CPV	culture percentage viability
CDR	complimentarity determining region
CHO	Chinese Hamster Ovary
CS	CellScrub
CTB	cholera toxin B
DHFR	dehydrofolate reductase
DMEM	Dulbecco's modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EST	exostosin
FBS	fetal bovine serum
FC	ferric (III) citrate
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
Gal	galactose
GALT	galactosyltransferase
GalNac	n-acetylgalactosamine
GFP	green fluorescent protein
GlcA	glucuronic acid
GLCAT	glucuronyltransferase
GlcNAc	n-acetylglucosamine
GPI	glycosylphosphatidylinositol
GS	glutamine synthetase
HACA	human anti chimeric antibody
HAMA	human anti-mouse antibody
HS	heparan sulphate
HSPG	heparan sulphate proteoglycan
HSnST	2-O-sulphotransferase
IdoA	iduronic acid
lPEI	linear PEI
LDC	limiting dilution cloning
mAb	monoclonal antibody
MβCD	methyl beta cyclodextrin
MSX	methionine sulphoximine
MTX	methotrexate

NDST	n-sulphotransferase
OFAT	one factor at a time
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine
PFA	paraformaldehyde
PLL	polylysine
rDNA	recombinant DNA
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute (culture medium)
PEG	poly(ethylene glycol)
rProtein	recombinant protein
RSM	response surface model
SEAP	secreated alkaline phosphatase
SEC	size exclusion chromatography
SFM	serum free medium
siRNA	small interfering ribonucleic acid
SPION	superparamagnetic iron oxide nanoparticles
TGE	transient gene expression
VCD	viable cell density
XYLT	xylosyltransferase

Lipids use for transfection

DOPE	dioleoylphosphatidylethanolamine
DOTMA	-N,N,N-trimethyl-2,3-bis(z-octadec-9-enyloxy)-1-propanaminium chloride
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DOSPA	-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride
DDAB	Dimethyldioctadecylammonium (Bromide Salt)
DMRIE	-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide

ABSTRACT

Biopharmaceutical production through transient gene expression (TGE) is used within industry for the rapid supply of product for early stage testing. A key requirement of the process is the large scale transfection of mammalian cells, for which the cationic polymer, polyethylenimine (PEI), is widely used.

In this thesis, the mechanism of PEI mediated transfection of CHO-S cells is explored at the cell surface, a fundamental barrier to successful transgene delivery. By approaching the question from first principles, exploring the kinetics of transfection at the cell surface, bio-physical and bio-molecular interactions governing polyplex binding to the cell surface, three key findings were made.

Firstly, polyplex uptake was biphasic. Initial, rapid endocytosis of polyplex and heparan sulphate proteoglycans (HSPG) was followed by a slower phase of polyplex uptake, on depletion of cell surface HSPGs. Enzymatic depletion of cell surface HSPGs was found to reduce TGE by 25%, whereas sequestration of cholesterol using methyl-β-cyclodextrin abrogated TGE. Taken together, the data indicate that HSPGs mediate maximal TGE (via an early, rapid phase of endocytosis) but that the predominant mechanism of polyplex uptake is through the clustering of lipid rafts, occurring at depleted cell surface HSPG levels.

Secondly, the role of both electrostatic and hydrophobic interactions in polyplex binding to the cell surface was investigated. These experiments revealed that at statistically optimized conditions for TGE (with respect to PEI:DNA ratio) the net charge of the polyplex in chemically defined medium was approximately neutral. Under these conditions polyplexes bound to the cell surface, predominantly, via a hydrophobic interaction, independent of cell surface HSPGs. Accordingly polyplex binding to the cell surface was disrupted by both non-ionic surfactant and depletion of plasma membrane cholesterol by methyl-β-cyclodextrin. An increase in polyplex zeta potential at elevated polyplex PEI:DNA ratio increased polyplex binding to the cell surface, but was accompanied by increased cytotoxicity with elevated PEI internalization. A decrease in polyplex zeta potential using ferric (III) citrate resulted in decreased polyplex binding to the cell surface. Both alterations in polyplex charge reduced TGE. Taken together,

these data indicate that hydrophobic binding of polyplexes to cell surface lipid rafts (bearing passenger HSPGs) is the primary molecular interaction that promotes subsequent lipid raft clustering and polyplex micro/macropinocytosis to facilitate maximal TGE.

Lastly, in order to engineer increased binding and endocytosis of recombinant DNA, alkylated PEIs varying in alkyl chain length and degree of substitution were chemically synthesized in order to increase polyplex hydrophobicity. Compared to unmodified PEI in TGE processes, optimized by Design of Experiments Response Surface Modelling, propyl-PEI was found to mediate more efficient TGE at similar reporter gene titre via a reduction in plasmid DNA load. Propyl-PEI formed polyplexes were found to mediate enhanced polyplex uptake relative to polyplexes formed of unmodified PEI.

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CHAPTER 1

Biopharmaceuticals and their Production

This chapter provides an overview of biopharmaceuticals and production of biopharmaceuticals. Specific emphasis is given to recombinant protein production through transient gene expression. The aim of this chapter is to contextualize the research presented in this thesis, with respect to its industrial application.

1.1 Biopharmaceuticals

A “biopharmaceutical” or “biologic” is “a protein or nucleic acid based pharmaceutical substance used for therapeutic or *in vivo* diagnostic purposes, which is produced by means other than direct extraction from a native (non-engineered) biological source” (Walsh, 2002). Biologics can be broadly divided into nine classes: monoclonal antibodies (mAbs), hormones, growth factors, fusion proteins, cytokines, blood factors, therapeutic enzymes, recombinant vaccines and anticoagulants (Aggarwal, 2012). Biologics are used to treat a plethora of disease conditions, ranging from cancer to chronic autoimmune conditions to viral infections (Marasco and Sui, 2007). The first gene therapy was approved in 2012, Glybera®, for treatment of lipoprotein lipase deficiency (Yla-Herttuala, 2012).

In 2006 biopharmaceuticals made up 44% of all drugs in development (Walsh, 2006) and since then the sector has seen moderate year on year growth of approximately 5% (Aggarwal, 2012). Blockbusters drugs, defined as having >$1 billion world-wide annual sales, have contributed to this increase (Lawrence 2007).

Whilst numerous biopharmaceuticals have become hugely profitable for pharmaceutical companies, their inherent cost of production has and continues

to limit their uptake; the individual cost burden is considerable, whether it be for the individual patient, the insurance companies or the National Health Service (NHS) in the United Kingdom (Kelly and Mir, 2009). Moreover, the high cost of biopharmaceuticals limits their potential market, making their use in numerous developing countries impossible.

1.1.1 Recombinant Protein Technology

Pioneering research in the 1970s, describing the production of hybrid DNA molecules, through restriction enzyme digestion and recombination of the fragments (Jackson *et al.*, 1972; Cohen *et al.*, 1973) and production of recombinant proteins from the recombinant DNA, provided the framework for the production of therapeutic recombinant proteins/ biopharmaceuticals. The human hormone, somatostatin, was the first protein to be produced through recombinant DNA technology and chemical synthesis of the somatostatin gene sequence (Itakura *et al.*, 1977), in *Escherichia coli*. Recombinant somatostatin paved the way for production of human insulin (Humulin®), also in an *Escherichia coli* expression platform (Johnson, 1983).

1.1.2 Antibody Therapeutics

Immunotherapies, based on antibodies, make up the largest class of biologics (Aggarwal, 2012) and thus the technology (Reichert *et al.*, 2005) for their production will be discussed here and in Table 1.1.

Immunotherapies were first used in the form of serum from inoculated animals or humans, from the work of Kitasato, Behring and Ehrlich, at the turn of the 20th century (Bosch and Rosich, 2008). However, serum sickness was frequently associated with such therapies.

As part of the natural immune system, antibodies are produced by plasma cells, terminally differentiated B cells (Shaprio-Shelef and Calame, 2005; Dinnis and James, 2005). Thus, monoclonal antibodies (mAbs) were first produced by fusing a murine myeloma cell with a murine B cell from an immunised mouse, creating an immortal, mAb secreting “hybridoma” cell line (hybridoma technology) (Kohler and Milstein, 1975). The mAbs produced by this method, however, had potentially serious immunogenic side effects in the clinic, such as

the human anti-mouse antibody (HAMA) response (Tjandra *et al.*, 1990). They also had poor efficacy due to lack of synergy between with the patient's immune system, failure to activate the complement system or initiate antibody dependent cellular cytotoxicity (Waldmann, 2003).

'Chimeric antibodies' were produced by joining variable region genes from a murine myeloma cell line with known antigen specificity with human immunoglobulin constant domain genes through recombinant DNA technology (Morrison *et al.,* 1984; Boulianne *et al.*, 1984). Chimeric antibodies activated the desired responses in the human immune system, mimicking human antibodies, but also undesired immunogenic reactions, the human anti chimeric antibody (HACA) response for example (Mirick *et al.*, 2004). 'Humanized' antibodies were then developed, comprising an entirely human protein sequence, except for a murine complimentarity determining region (CDR) (Jones *et al.*, 1986).

Better success in the clinic was achieved through production of fully 'human' antibodies without associated HAMA or HACA side effects. Two technologies: phage display and transgenic animals enabled this break-through. The technique of phage display (McCafferty *et al.,* 1990; Carmen and Jermutus, 2002), involves cloning of human variable antibody genes into bacteriophage (Griffiths and Duncan, 1998). Screening the bacteriophage (expressing an array of antibody variable genes on their surface) with an array of antigens, allows an antigen to be matched to an antibody variable gene. Transgenic mice can also be used for the development of human antibodies, whereby the mice express a repertoire of human antibody gene sequences and antibody variable sequences can be isolated for a given antigen (Lonberg, 2005; Green *et al.,* 1994; Lonberg *et al.*, 1994.)

Coming full circle, polyclonal antibodies have re-emerged as immunotherapies, with a manifold of inherent benefits due to the therapeutic approach being closer to the natural immune response than treatment with a mAb (Haurum, 2006). The first method, Symplex™ technology involves sorting of antibody producing cells from immune individuals, followed by antibody heavy and light chain mRNA reverse transcription and amplification by linked Simplex PCR technology and (enabling original pairing of heavy and light chains) and finally phage display (Haurum, 2006; Waltz, 2006). Alternatively, polyclonal antibodies

Table 1.1 Immunotherapies: Development Chronology

Type	Antibody Technology	Reference	First Drug Approved *[Trade name]*
Polyclonal	Immunized human serum	-	Multiple therapies
	Immunized animal serum	-	
Monoclonal	Hybridoma (murine)	Kohler and Milstein, 1975	Muromonab-CD3 (1986) *Orthoclone OKT3*
	Chimeric recombinant antibody (66% human)	Morrison *et al.*, 1984	Abciximab (1994) *ReoPro*
	CDR grafted antibody (humanized, 90% human)	Jones *et al.*, 1986	Daclizumab (1997) *Zenapax*
	Phage Display (human)	McCafferty *et al.*, 1990	Adalimumab (2002) *Humira*
	Transgenic (human)	Lonberg *et al.*, 1994 Green *et al.*, *1*994	Panitumumab (2006) *Vectibix*
Polyclonal	Symplex technology (human)	Meijer *et al.*, 2006	Numerous in pipeline
	Transgenic (human)	Kuroiwa *et al.*, 2002	Numerous in pipeline
Fc fusion proteins	Human Fc domain	Capon *et al.*, 1989	Etranercept (1998) *Enbrel*
Bispecific monoclonal antibodies	Hybridoma (rat/ mouse)	Staerz *et al.*, 1985; Perez *et al.*, 1985	Catumaxomab (2009) *Removab*

can be developed in transgenic animals (Kuroiwa *et al.*, 2002; Kuroiwa *et al.*, 2009; Waltz, 2006).

Alongside developments in antibody technologies, recombinant fusion proteins were developed, based on the immunoglobulin Fc domain linked to a targeting peptide (Czajkowsky *et al.*, 2012). In addition, the first bispecific antibody (Kontermann, 2012), Removab®, recently came to the market, produced by hybridoma technology (Chames *et al.*, 2009).

Recombinant DNA technology and in vitro expression in mammalian cells is used for production of therapeutic antibodies and other proteins in the vast majority of cases (Wurm, 2004). Alternative methods of production include hybridoma technology and direct synthesis in transgenic animals.

1.2 Production of Biopharmaceuticals

1.2.1 Expression Systems

Marketed biopharmaceuticals have been produced in mammalian cells, bacterial, yeast and insect cells; and plant based expression systems for production of health care products (Walsh, 2010). Cell free protein expression systems, although promising, are not at the commercial stage (Kovtun, 2011).

Unlike insulin, antibodies have a complex quaternary structure and require post translational glycosylation to function in vivo (Jefferis, 2005). Whilst *E. coli* is still used as an expression system for simple proteins, such as Humulin®, for production of more complex proteins, such as antibodies, eukaryotic expression systems are utilized (Jenkins *et al.*, 1996, Walsh and Jefferis, 2006; Walsh, 2010). Yeast glycosylation is high mannose type, which leads to a short half life in vivo, reduced efficacy and in some cases immunogenicity (Gerngross, 2004; Demain and Vaishnav, 2009), so has limited suitability for therapeutic protein product at present.

The majority of biopharmaceuticals are produced in mammalian cell culture (Wurm, 2004; Birch and Racher, 2006), mainly cell lines derived from the Chinese hamster and original CHO-K1 cell line, or other rodent cell lines, such as murine lymphoid cell lines, NS0 and SP2/0. CHO cell lines possess key features amenable to biomanufacturing, such as fast growth rate in a synthetic

culture environment (Birch and Racher, 2006), susceptibility to foreign DNA integration and resistance to majority of viruses that infect humans (Berting *et al.*, 2010).

Human derived cell lines, due to their ability to confer human post translational modifications, are increasingly attractive for biomanufacturing (Durocher and Butler, 2009; Schiedner *et al.*, 2008). For example human retina derived PER-C6® cells (Crucell) and human amniocyte derived cells CAP® (Cevec) are used for production of numerous therapies in clinical trial at present. Despite the benefits of human cell line expression systems, CHO cells have the most established history of regulatory approval and thus are an attractive, safe option for biomanufacturing and remain the industry "work-horse" (Jayapal *et al.*, 2007).

1.2.2 Engineering Strategies to Improve Production

In 1986 typical recombinant protein titres from mammalian systems were approximately 50 mg L^{-1}; nearly 30 years later, productivity has increased over 100 fold and titres ranging from 10-13 g L^{-1} are commonly achieve within industry (Wurm, 2004; Kelley, 2009; Hacker *et al.*, 2009). Increased productivity has been achieved through a myriad of strategies, that can be broadly defined as 1) process design optimizations and 2) Cell line/ vector engineering strategies (O'Callaghan and James, 2008; Davies and James, 2009; Hacker *et al.*, 2009; Dietmair *et al.*, 2012; Butler and Meneses-Acosta, 2012). Optimization of chemically defined culture media, free of animal derived products (e.g. serum), has been a key factor in increased productivity (Keenan *et al.*, 2006; Jayme and Smith, 2000; Wurm, 2004; Birch and Racher, 2007).

Biopharmaceuticals can be produced through three distinct processes: stable cell line generation, stable transfectant pool technology or through transient gene expression, illustrated in Figure 1.1. The choice of process is determined by the end point requirements. Stable gene expression produces the highest titres of recombinant protein but can take many months. Often therapeutic product is needed on a shorter time scale, for example in the development stages of drug development or for toxicology testing, for example. In such

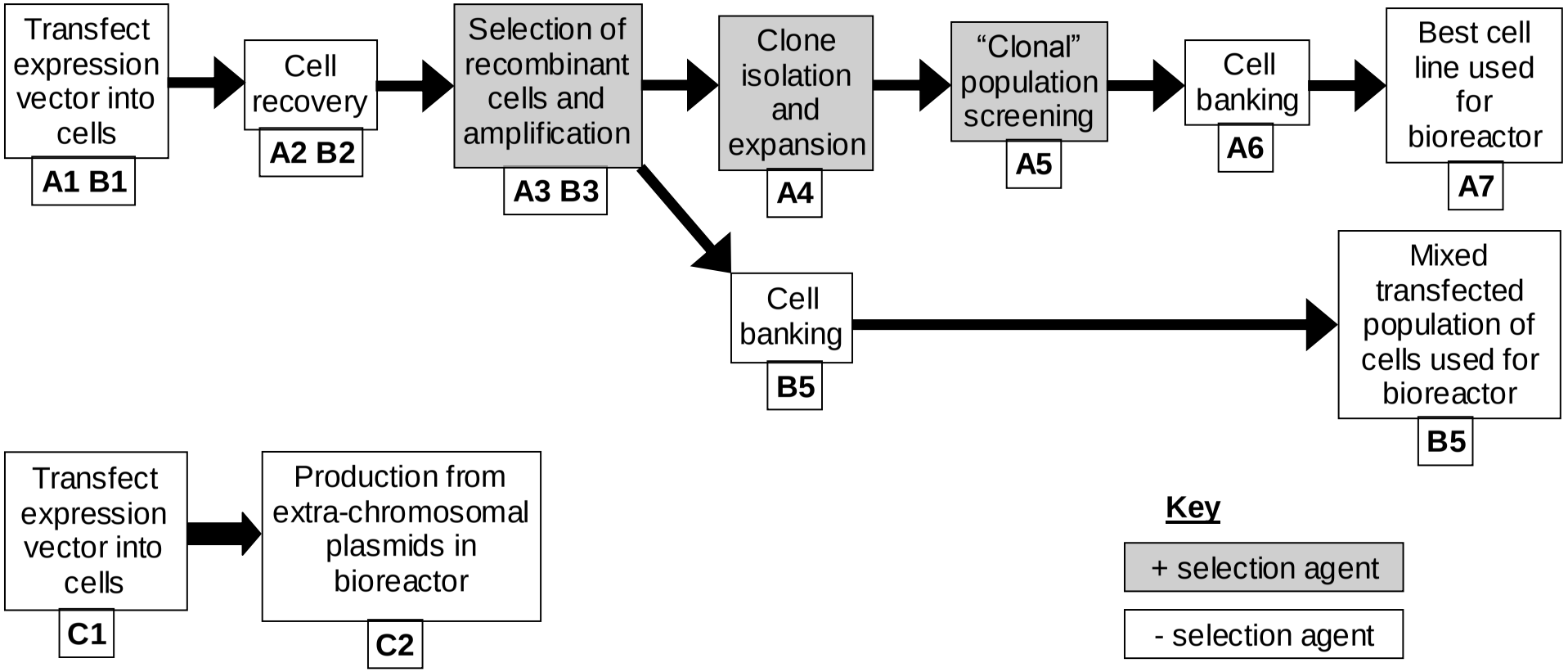


Figure 1.1 Strategies for Therapeutic Protein Production

A) Stable Cell Line Generation
B) Stable Transfectant Pool Technology
C) Transient Gene Expression

cases, a transient gene expression platform or stable transfectant pool technology may be implemented (Table 1.2).

In addition to the relatively high product titres obtained by stable gene expression, production from a “clonal” cell line is used for biomanufacturing, to aid product homogeneity and meet regulatory requirements. However, there is evidence that mAbs from whole transfected populations (i.e. transfectant pool technology) are comparable in glycosylation to mAbs produced from a stable cell line (Ye *et al.*, 2010).

1.2.3 Stable Cell Line Generation

Large scale production of therapeutic recombinant proteins involves generation of recombinant cell lines. To isolate cells that have successfully integrated the transgene/ daughter cells that have inherited the transgene following mitosis, a selection and amplification system is used. The dehydrofolate reductase (DHFR)/ methotrexate (MTX) (Figure 1.2A) and glutamine synthetase (GS)/ methionine sulphoximine (MSX) (Figure 1.2B) systems are commonly used in industry (Bebbington *et al.*, 1992; Kaufman *et al.*, 1985; Matasci *et al.*, 2008). Both systems follow the same principles: an expression vector containing the DHRF or GS gene along with the gene of interest (GOI) is transfected into the cells, after a certain length of time the only cells that can survive have successfully integrated the expression vector into a transcriptionally active region of the host cell chromosomes. Amplification can be achieved by adding a selection pressure, methotrexate or methionine sulphoximine, which inhibits the action of the selection gene (dehydrofolate reductase or glutamine synthetase respectively).

Clones are then isolated from the recombinant cells, expanded and screened for productivity and a single cell line used for bioreactor production (Birch and Racher, 2006; Browne and Al-Rubei, 2007; Porter *et al.*, 2010; Porter *et al.*, 2010b). For production of recombinant polyclonal antibodies through Sumpress™ technology, multiple vials of cell expressing an array of recombinant antibodies are used to inoculate the bioreactor (Haurum, 2006). Although lengthy, the selection and amplification process, followed by clone

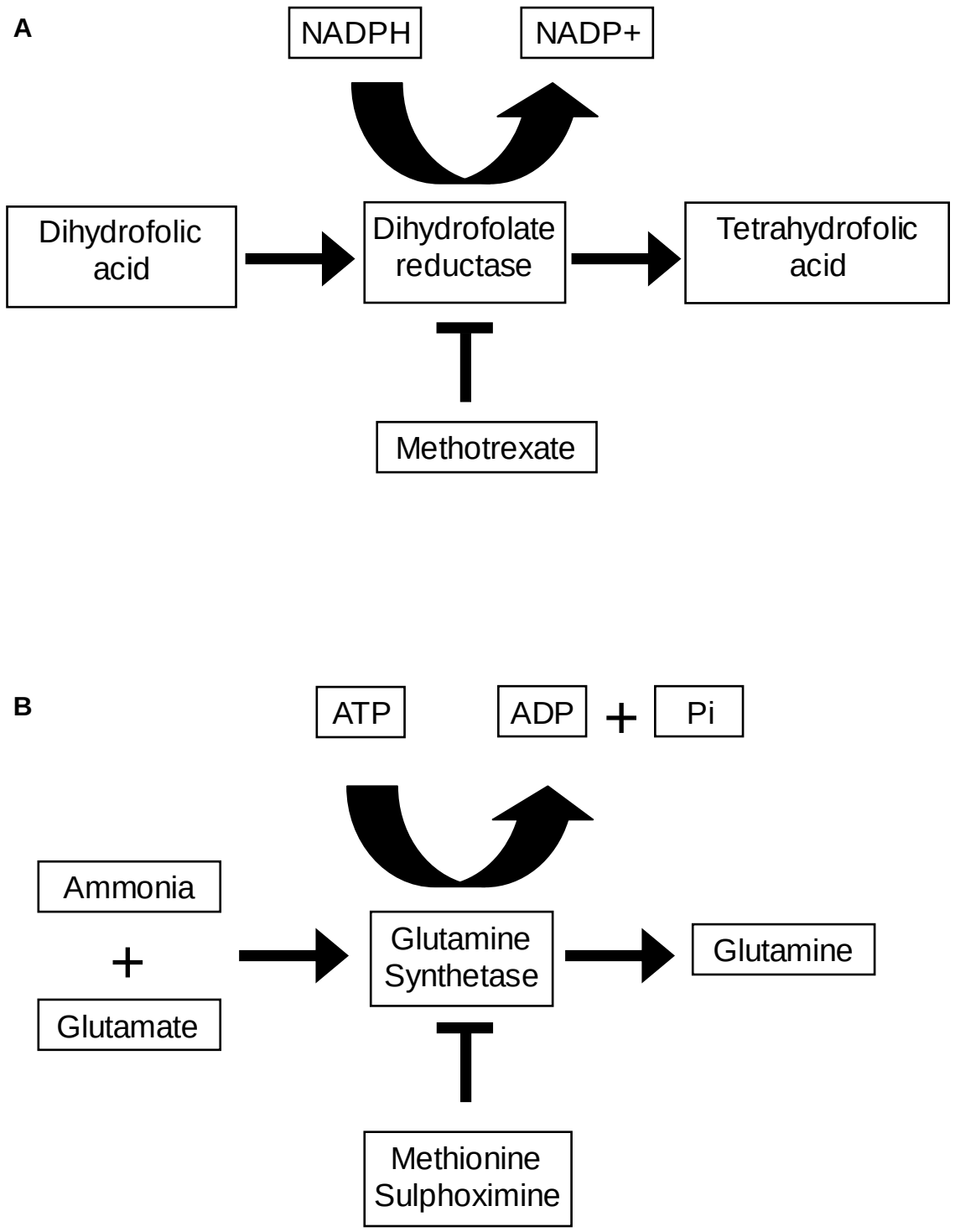


Figure 1.2 Systems used for selection and amplification of recombinant cells: DHFR and GS.
Dehydrofolate reductase (DHFR) (**A**) or glutamine synthetase (GS) (**B**) enzymes are included on the expression vector and the cells cultured in media lacking hypoxantine and thymidine (HT) or glutamine respectively. Methotrexate or methionine sulphoximine are added to amplify expression of the transgene.

isolation, expansion and screening, can result in isolation of cells with excellent specific productivities (qP) (O'Callaghan *et al.*, 2010).

One of the biggest challenges facing the process of recombinant protein production through stable gene expression is production instability. Despite the use of "clonal" cell lines for production, they remain genetically heterogeneous (Altschuler and Wu, 2010; Hyman and Simon, 2011; Davies *et al.*, 2012). Loss of productivity is a fundamental problem for biopharmaceutical production (Chusainow *et al.*, 2009), through loss of recombinant genes and epigenetic gene silencing over time (Yang *et al.*, 2010; Kim *et al.,* 2011).

Table 1.2

Biopharmaceutical Production Strategies: Development Timelines

Production Strategy	Time scale	Reference
Stable cell line generation	4-12 months	Ye *et al*., 2009
Stable transfectant pool technology	2 months	Ye *et al*., 2010
Transient gene expression	1-14 days	Hacker *et al.*, 2009

1.2.4 Stable Transfectant Pool Technology

Recombinant protein production through stable transfectant pool technology is similar to production through stable gene expression, except that the step of producing a clonal cell line is omitted, thus reducing the duration of the production process. The stably transfected parental population, following selection and amplification, is used for bioreactor production of recombinant proteins, as illustrated in Figure 1.1. Within two months post transfection, gram quantities of mAb can be generated, on a 200 L scale, with productivities ranging from 100mg L^{-1} to 1000 mg L^{-1} (Ye *et al.*, 2010). Stable pools offer an advantage over transient gene expression (TGE) platforms, in that production does not require large quantities of non-GMP plasmid DNA, potentially contaminated with endotoxin (Schmid *et al.*, 2001; Bertschinger *et al.,* 2006a). Therefore, recombinant proteins produced from stable pools rather than by TGE are more likely to be used for early stage testing in the clinic, in the future.

1.2.5 Transient Gene Expression

Transient gene expression (TGE) platforms rely on the expression of plasmid DNA in the parental cell line. TGE has been used for decades in biology laboratories for analytical and research purposes (often termed transient transfections) but only in the last fifteen years has it been scaled-up and used for rapid supply of biopharmaceuticals (Baldi *et al.*, 2007; Geisse, 2009).

HEK293 cells were originally used for scaled up TGE, but more recently, high titres have been achieved with CHO cells (>2g L^{-1} in industry data reported at conferences). HEK293 cells were used due to a system for episomal replication being well established, whereas systems for episomal replication in CHO cells came later (Geisse, 2009). Within the literature, the highest titres reported are >1 g L^{-1} in HEK293E cells, with episomal replication, co-expression of human acidic Fibroblast Growth Factor (aFGF) and cell cycle regulators, p18 and p21 (Backliwal *et al.*, 2008a). In CHO cells, the highest reported titre within the literature is 875 mg L^{-1}, described later (Cain *et al.*, 2013).

Whilst HEK293 cells are regularly used in TGE, for expression of various proteins (Swiech *et al.*, 2011), for alignment of early stage (TGE) production with manufacturing production through (SGE), use of the same cell line is desirable. Hence, TGE processes have been developed in CHO cells (the industry “work horse” for biomanufacturing (Codamo *et al.,* 2011a; Codamo *et al.,* 2011b). Similarly, for the human aminocyte derived cell line CAP®, a complimentary TGE-specific cell line, CAP-T®, has been developed (Fischer *et al.,* 2012).

Strategies to improve TGE platforms can be broadly divided into three approaches:

1) Cell line and/ or vector engineering

2) Nutritional/ non nutritional additives post transfection

3) Optimization of culture modality

1.2.5.1 Cell Line and Vector Engineering

Several episomal TGE systems have been developed that allow maintenance and replication of the plasmid within the mammalian cell (Meissner *et al.,* 2001; Durocher *et al.*, 2002; Geisse 2009) (Table 1.3). Systems include: constitutive expression of the large SV40 large T-antigen in trans with a plasmid containing the SV40 origin of replication (SV40ori) or constitutive expression of Epstein-Barr virus nuclear antigen 1 (EBNA1) protein in trans with a plasmid containing the Epstein-Barr virus origin of replication (OriP) (Yates *et al.,* 1985; Young *et al.*, 1988). Alternatively, the EBNA-1 element can be included in the expression vector for transient expression (Durocher *et al.*, 2002). Another system is the epiCHO one (Codamo *et al.*, 2011a; Codamo *et al.*, 2011b), which consists of cells constitutively expressing the Polyomavirus large T antigen (PyLT) combined with an expression vector containing the Py origin of replication (PyOri) and Epstein Barr Virus OriP for plasmid retention (Kunaparaju *et al.,* 2005). Finally, episomal replication, at low copy number, has been achieved without viral transformation of the CHO cell line, by including in the expression vector, chromosomal scaffold/ matrix attachment regions (S/MARs) in cis with the SV40 ori (Piechaczek *et al.*, 1999).

It has been reported that translation and post translational mechanisms are limiting bottlenecks in transient protein production in CHO cells (Mason *et al.*, 2012). Recently, an engineering strategy based on increasing the secretary capacity of the host cell line for TGE was employed, whereby CHO-S cells were engineered to express X box binding protein (XBP-1S) and endoplasmic reticulum oxidoreductase (ERO1-Lα) (Cain *et al.*, 2013). XBP-1 A is a regulator of protein secretion and the unfolded protein response (Lee AH *et al.*, 2003), its over-expression has been found to enhance recombinant protein production in CHO cells (Tigges and Fussenegger, 2006; Becker *et al.*, 2008). Antibody structure is dependent on disulphide bonds between heavy chains and heavy and light chains. Over expression of the protein disulphide isomerase (PDI) enzyme, which forms and isomerizes disulfide bonds, was found to increase secretion rate of recombinant monoclonal antibodies in CHO cells (Borth *et al.*, 2005). ERO1-Lα in an oxidoreductase enzyme that catalyses the re-oxidation of disulphides in the active site of the protein disulphide isomerase (PDI) enzyme;

PDI over expression has previously been shown to enhance mAb production in recombinant CHO cells (Mohan *et al.*, 2007; Mohan and Lee, 2010). The CHO-SXE cell line, expressing XBP-1 and ERO1-Lα (Cain *et al.*, 2013), increased antibody yields, 6 fold relative to CHO-S cells.

Other strategies to enhance TGE yields have included engineering CHO cells to express the anti-apoptotic protein, Bcl-xL (Majors *et al.*, 2008), which was found to enhance both mAb production and culture viabilities. The same group then over-expressed another Bcl-2 family protein, Mcl-1, resulting in, again, improved mAb yields and higher culture viabilities (Majors *et al.*, 2009). Work on TGE at Genentech has focussed on Bax and Bak, which are pro-apoptotic proteins that induce apoptosis by permeabilizing the mitochondrial membrane and activating the caspase proteolytic cascade (Wei *et al.*, 2001). A Bax Bak double knock out CHO cell line was found to have higher PEI:DNA polyplex uptake capacity than the standard CHO cells and higher transient antibody production (3-4 fold) (Macaraeg *et al.*, 2013). Higher polyplex uptake capacity in DKO cells relative to CHO-K1 was concluded to be due to the enhanced ability of the DKO cell line to resist transfection induced apoptosis (Macaraeg *et al.*, 2013). Cell line engineering strategies for TGE are summarized in Table 1.3.

Table 1.3 Cell Line Engineering for Therapeutic Protein Production through Transient Gene Expression

Engineering strategy	System	Constitutively expressed elements	Examples	Parental Cell Line	DNA Expression Vector elements (example vectors)
Episomal replication and maintenance	Epstein-Barr virus EBNA-1/ *OriP* [a]	(EBV) nuclear antigen-1 (EBNA-1)	293-EBNA [b]	HEK293 (human embryonic kidney)	EBV *oriP* *(pTT)* [e] *(pCEP4)* [b]
			293 SFE [c]	HEK293	
			CHO-3E7 [d]	CHO-K1 (Chinese hamster ovary)	
	Simian Virus 40 (SV40) large T antigen/ *Ori* [f]	SV 40 large T antigen	COS-1 and COS–7 [f]	CV-1 (monkey kidney)	SV40*ori* *(pCDNA3.1)* [b]
			HEK293 T [g]	HEK 293	
			CAP-T™ [h]	(Human aminocyte)	
	Epi-CHO [i]	Polyomavirus (Py) large T antigen	CHO-T [j]	CHO-K1	1)Polyomavirus Ori (*pyOri*) 2)EBV nuclear antigen-1 (EBNA-1) 3) EBV *OriP*
	pEPI-1[k] (vector only)	—	—	—	1)SV40*ori* 2) S/MAR
Increased protein secretion	CHOS-XE [l]	XBP-1S ERO1-Lα	CHOS-XE	CHO-K1	—
Transfection competent and suspension adapted cells	—	—	Freestyle™ CHO-S[b]	CHO-K1	—
	—	—	Freestyle™ 293-F[b]	HEK293	—
Anti-apoptotic	DG44-Bcl-x_L [m]	Bcl-x_L	—	CHO-K1	—

a) Young *et al.*, 1998. b) www.lifetechnologies.com. c)Durocher *et al.*, 2005. d) Durocher and Loignon, 2011. e) Durocher *et al.*, 2002. f) Gluzman, 1981. g) www.thermoscientificbio.com. h) Fischer *et al.*, 2012. i) Kunaparaju *et al.*, 2005. j) Codamo *et al.*, 2011. k) Piechaczek *et al.*, 1999. l) Cain *et al.*, 2013. m) Majors *et al.*, 2008.

1.2.5.2 Yield Enhancing Additives Post Transfection

Epigenetic methods of gene regulation, methylation and histone deacetylation (Nan *et al.*, 1998), as with processes based on stable cell line generation (Kim *et al.*, 2011), impact on TGE platforms (Hong *et al.*, 2001; Nan *et al.*, 2004). Chemical inhibitors of histone deacetylase have been found to improve TGE yields, as described in Table 1.4.

As described in Table 1.4, other strategies to enhance TGE have included addition of growth factors (Galbraith *et al.*, 2006) or transient co-expression of growth factors (Backliwall *et al.*, 2008a). Cell cycle attenuation, either through nocodazole supplementation (Tait *et al.*, 2004) or transient co-expression of p18 or p21 cell cycle modulators (Backliwal *et al.*, 2008a), have also been shown to enhance TGE. The process-improving effects of lithium acetate have been demonstrated on numerous occasions (Ye *et al.*, 2009), but a mechanism of action never suggested.

Nutritional additives, such as peptones, when added post transfection, have been shown to significantly enhance transient protein yields (Pham *et al.*, 2003; Pham *et al.*, 2005). Fed batch culture has also been shown to enhance transient protein yields (Sun *et al.*, 2006).

1.2.5.3 Optimization of Culture Modality

Hypothermic growth conditions were found to enhance recombinant interferon-gamma production in CHO cells (Fox *et al.*, 2005a), due to elevated recombinant mRNA levels (Fox *et al.*, 2005b). For TGE, hypothermic conditions have been shown to dramatically enhance transient protein titres on numerous occasions (Galbraith *et al.*, 2006, Wuhlfard *et al.*, 2008; Rajendra *et al.*, 2011).

Recent advances in bioreactor technology have been highly suited to large scale TGE, such as WAVE™ bioreactors (Haldanker *et al.*, 2006). Strategies, such as high density transfection (Sun *et al.*, 2008; Backliwal *et al.*, 2008b; Thompson, 2011) have also enhanced yields.

Just as consistency of host cell line between transient and stable production phases is desirable, so is culture modality (Silk *et al.*, 2010). Increasingly, automated micro-scale bioreactor systems, such as ambr™ (TAP Biosystems),

are being used for cell line development and TGE processes, to better predict the performance of a cell line in the bioreactor.

Table 1.4
Yield Enhancing Strategies for Therapeutic Protein Production through Transient Gene Expression

Engineering strategy	Method (Physical)	Method (Genetic)	Reference
Unknown	Lithium acetate	–	Ye *et al.*, 2009
	Sodium butyrate	–	Backliwal *et al.*, 2008a
Histone deacetylase inhibitor	Valproic Acid	Effects can be enhanced with co-expression of protein kinase B (AKT)*	Wulhfard *et al.*, 2010 Durocher and Loignon, 2011*
	DMSO	–	Ye *et al.*, 2009
	Trichostatin A	–	Nan *et al.*, 2004
Increased translation	LR3 IGF Growth factor	Co-expression of aFGF*	Galbraith *et al.*, 2006 Backliwal *et al.*, 2008a*
Cell cycle attenuation	Nocodazole	Co-expression of p18 or p21*	Tait *et al.*, 2004 Backliwal *et al.*, 2008a*
Enhanced recombinant mRNA transcription	Hypothermic culture temperature	–	Galbraith *et al.*, 2006 Wulhfard *et al.*, 2008
Hyper-Osmolarity	Sodium chloride	–	Zhang *et al.*, 2010

CHAPTER 2

Transfection

Chapter Overview

This chapter provides a synopsis of methods of transfection, with specific focus on the cationic polymer, polyethylenimine (PEI). Mechanistic insights into PEI:DNA polyplex cyto-delivery and cyto-trafficking are discussed. However, literature describing key steps in this process, including polyplex-cell surface binding and cyto-internalization, the focus of research presented in this thesis, are summarized in later results chapters. The aim of this chapter is to contextualize the data presented within this thesis, with respect to the body of literature describing transfection and specifically the mechanism of PEI mediated transfection.

2.1 Methods of Transfection

A fundamental process across biotechnology is transgene delivery to mammalian cells or “transfection” (Luo and Saltzman, 2000a). It is a process particularly important to therapeutic protein production through TGE, as the process relies on large scale transfection. Gene delivery strategies or methods of transfection (including viral transduction) are summarized in Table 2.1 and below.

2.1.1 Viral

Millions of years of evolution have rendered viruses preeminent gene delivery vehicles (Verma and Weitzman, 2005). Viral vectors are the most frequently used method for gene therapy applications (Warnock *et al.*, 2011), with numerous in clinical trial (Sheridan, 2011) and the first gene therapy gaining approval, Glybera®, itself an adeno-associated viral vectors expressing lipoprotein lipase (Yla-Herttuala, 2012). Viral vectors have been used on numerous occasions for TGE in cell culture (Blasey *et al.*, 1997; Blasey *et al.*, 2000). Systems use include alpha virus (including Semliki Forest virus),

adenovirus and vaccinia virus vectors (Wurm and Bernard, 1999). However, the limited DNA carrying capacity and the lengthy process of recombinant virus isolation (Pear *et al.*, 1993), combined with biosafety concerns (Pham *et al.*, 2006), have resulted in episomal vectors (Van Craenenbroeck *et al.*, 2000), combined with a delivery vehicle, being used for biomanufacturing and TGE rather than viral vectors. Nevertheless, much can be learned from viral mediated gene delivery in the design and development of non-viral delivery vehicles (Zuber *et al.*, 2001; Wagner, 2004).

2.1.2 Physical

A plethora of physical transfection methods have been reported within the literature, including: microinjection (Capecchi, 1980; Derouazi *et al.*, 2006a), particle bombardment (Sanford *et al.*, 1993), electroporation (Neumann *et al.*, 1982), pressure mediated transfection (Mann *et al.*, 1999) laser irradiation/ "optical transfection" (Tsukakoshi *et al.*, 1984; Tao *et al.,* 1987; Tirlapur and Konig, 2002) and ultrasound (Taniyama *et al.*, 2002). Until recently, none of these methods has been suitable for scale-up. However, with the advent of MaxCyte™ large scale electroporation technology, electroporation for TGE is becoming an increasingly attractive option (Cain *et al.*, 2013).

2.1.3 Chemical: Co-precipitation

The calcium phosphate co-precipitation method of transfection was first used by Graham and Van der Eb, (1973), and optimized by Jordan *et al.*, (1996) with a view to producing recombinant proteins though large scale transfection and TGE (Jordan *et al.*, 1998). Despite the widespread use of calcium phosphate for transfection and its efficacy (Girard *et al.*, 2001; Girard *et al.*, 2002; Chenuet *et al.*, 2008), the requirement of serum in the media (Jordan and Wurm, 2004) renders the method sub-optimal for biomanufacturing.

2.1.4 Cationic lipids

Cationic lipids, such as DOTMA, DOTAP, DDAB, DOSPA, have been widely used for transfection, often in proprietary formulations (Choosakoonkriang *et al.*, 2001; Midoux *et al.*, 2008). "Helper", neutral lipids, such as DOPE or

Table 2.1 Gene Delivery Strategies

Method	Examples	Features	Suitability for biopharmaceutical production through TGE	Reference (company)
Physical	Ultrasound	Designed for use *in vivo*	Not designed for scale-up.	Targeson®
	Micro-injection	• Suitable for single cell transfection • Direct nuclear delivery		–
	Electroporation	Recently, Maxcyte® technology for large scale electroporation has made the transfection method an attractive option for large scale TGE.	Large scale electroporation is being increasingly used for TGE within industry.	Maxcyte®
Co-precipitation	Calcium phosphate Calcium chloride	Inexpensive	No. Serum required for production.	Jordan et al., 1996
Viral	Semliki Forest Virus Adenovirus Vaccinia virus Baculovirus	Highly evolved transfection method	No. Immunogenicity concerns; limited DNA carrying capacity; expensive to produce.	Wurm and Bernard, 1999
Cationic lipids	Lipofectamine® 2000 Lipofectamine® LTX DMRIE-C	Highly cited within the literature and versatile	No. Expensive.	Lifetechnologies™
Cationic polymer	**Polyethylenimine (PEI)** Polylysine (PLL) Polyamidoamine dendrimer (PAMAM) DEAE-dextran	Inexpensive	Polyethylenimine is commonly used for TGE within industry.	Polyplus transfection™

cholesterol, are often added to the lipid formulation to aid transfection (Dabkowska *et al.*, 2012). Despite its widespread use for microscale research applications, lipid based transfection, is not used for large scale TGE simple due to its prohibitive cost. In addition, higher transient protein production has been reported following transfection mediated by the much less expensive alternative, PEI, compared to some lipid based formulations (Wiseman *et al.*, 2003).

2.1.5 Cationic polymers

The cationic polymer, polyethylenimine, first presented as a transfection reagent by Boussif *et al.*, (1995) has immerged as leading method of transfection for bioproduction through TGE (Geisse, 2009, Wong *et al.*, 2010; Codamo *et al.*, 2011b; Rajendra *et al.*, 2011, Thompson *et al.*, 2012, Raymond *et al.*, 2012). Combined with its efficacy as a method of transfection, it is suitable for large scale transfection due to its low cost. Other cationic polymers, such as polylysine, have shown inferior transfection properties compared to PEI (Putman *et al.*, 2001; Mannisto *et al.*, 2007).

2.1.6 PEI

Within the literature, a plethora of PEIs have been described, linear or branched (Figure 2.1), of different molecular weights and with derivatized chemical groups. The branched form is produced by cationic polymerization from aziridine monomers via a chain growth mechanism and the linear form by cationic polymerization of a 2-substituted 2-oxazoline monomer (Godbey *et al.*, 1999a). Of a range of different molecular weights available (2, 22, 25, 60, 800, 1600 kDa) (Schlaeger and Christensen, 1999; Fischer *et al.*, 1999; Godbey *et al.*, 1999b), 25 kDa linear PEI has emerged as the PEI of choice for large scale TGE in HEK 293 and CHO cell platforms (Backliwal *et al.*, 2008a; Eberhardy *et al.*, 2009; Rajendra *et al.*, 2011; Raymond *et al.*, 2012; Thompson *et al.*, 2012). Although, 22 kDa jetPEI™ (Polyplus transfection) is frequently used within industry (Wong *et al.*, 2010).

2.2 Engineering PEI mediated Transfection to improve the TGE process

At the most basic level, the process of transfection (mediated by PEI) has been engineered through optimization of transfection conditions, with respect to amount of DNA, PEI etc. (discussed in more detail in Chapter 4). Further improvements have been reported by transfecting cells cultured at high density. Sun *et al.*, (2008) reported the success of high density transfection (10^7 cells mL^{-1}) followed by perfusion culture for large scale TGE. High density transfection (~$20x10^6$ cells mL^{-1}), followed by a lower density production period (10^6 cells mL^{-1}) was also reported by Backliwal *et al.*, (2008b). One of the benefits of the high density transfection protocol is that a priori PEI:DNA complex formation is not required (Backliwal *et al.*, 2008b). It has been reported that population context determines cell to cell variability in endocytic capacity (Snider *et al.*, 2009) and it is possible that at relatively high cell densities, endocytic trafficking, for which PEI:DNA transfection is dependent, could be up-regulated.

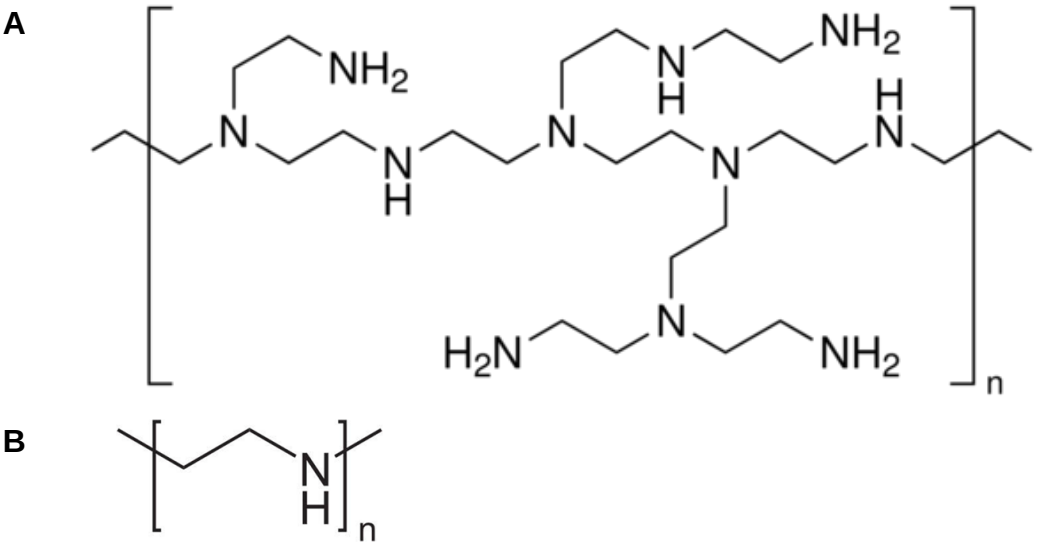


Figure 2.1
Chemical structure of polyethylenimine, branched (A) and linear (B).
Branched PEI contains primary, secondary and tertiary amines and linear PEI only secondary amines. The molecular weight of the linear and branched PEI monomer is 41.3g mol^{-1} and 489.8 g mol^{-1} respectively.

PEI was originally tested as a transfection agent, due to its predicted ability to 1) condense DNA and 2) protect DNA from degradation within lysosomes (the "proton sponge" hypothesis) (Boussif *et al.*, 1995), discussed in section 2.3. To

target other intracellular obstacles (Figure 2.2) various derivatized PEIs have been developed. For example, acetylated PEI was reported to enhance transgene expression by providing decreased affinity with DNA, relative to unmodified PEI (Gabriel son and Pack, 2006). Alkylated PEIs were found to enhance *in vivo* transgene expression, developed under the hypothesis that PEIs with enhanced hydrophobicity would display enhanced binding to the cell surface (Thomas and Klibanov, 2002; Fortune *et al.*, 2011) (discussed in Chapter 5 and 8). To avoid obstacles specific to *in vivo* delivery, such as opsonization, PEGylated PEIs were found to be efficacious (Ogris *et al.*, 1999) (discussed in Chapter 5). Biodegradable PEI was developed to reduce the cytotoxicity of PEI, and was found to provide excellent transgene expression *in vitro* (Breunig *et al.*, 2007). However, despite the plethora of examples within the literature of derivatized PEIs, for large scale transfection for industrial biopharmaceutical production by TGE, unmodified PEI continues to be utilized as the gene delivery vehicle.

To enable knowledge based engineering of PEI mediated transfection, an understanding of the process is required, from PEI:DNA polyplex formation, cyto-delivery to cyto-trafficking (Figure 2.2).

2.3 PEI:DNA Polyplexes: Cyto-Delivery and Cyto-Trafficking

Following PEI:DNA polyplex formation (1), as illustrated in Figure 2.2, the polyplex interacts with cell culture media components (2), binds to the cell surface (3), is internalized by the cell (4), escapes from endosomes (5), is trafficked to the nucleus (6), enters the nucleus (7); once inside the nucleus, the recombinant DNA is transcribed (8). Numerous reviews have described intracellular trafficking of transfection complexes (Godbey *et al.*, 1999c; Wiethoff and Middaugh, 2003; Guillem and Alino, 2004; Medina-Kauwe *et al.*, 2005; Belting *et al.*, 2005; Lungwitz *et al.*, 2005; Midoux *et al.*, 2008; Adler and Leong, 2010; Thompson, 2011).

Stages 1 and 5-8 will be discussed below and stages 2-4 within Chapters 4, 5, 6, 7 and 8.

(1) PEI:DNA Polyplex Formation

PEI was first tested as a transfection agent due to its high charge/ density ratio (the highest of any organic macromolecule) and its predicted ability to ionically condense DNA though amine-phosphate interactions, between PEI and DNA, respectively (Boussif *et al.*, 1995). Condensation of the DNA by PEI, protects it from digestion by extra and intracellular nucleases (Godbey *et al.*, 2000; Moret *et al.*, 2001).

Condensation of DNA by PEI, resulting in the formation of polyplexes (Godbey *et al.*, 1999d), is considered as fundamental for successful gene delivery. The thermodynamics of PEI:DNA binding and condensation was explored by Utsuno and Uludag (2010), who used isothermal titration calorimetry. They reported two types of binding, firstly, PEI binding to the DNA groove and secondly PEI binding to the DNA phosphate back bone, accompanied by condensation of the DNA.

The sulphated glycosaminoglycan, heparin, has been shown to displace DNA from PEI:DNA complexes (Moret *et al.*, 2001; Clamme *et al.*, 2003; Bertschinger *et al.*, 2006b). Dextran sulphate has also been shown to relax PEI:DNA polyplexes (Ikonen *et al.*, 2008). Osmolarity was found to affect PEI:DNA complex stability (Bertschinger *et al.*, 2006b); at high salt concentrations (≥0.5M) DNA disassociated from PEI. Bovine serum albumin (BSA) was also found to cause PEI:DNA polyplex disassembly (Bertschinger *et al.*, 2006b).

(5) Endosomal Release

Endocytosed cargo in mammalian cells is degraded within the acidic environment within lysosomes (Asokan and CHO, 2002; Luzio *et al.*, 2009). A key feature of PEI as a transfection reagent, is believed to be its ability to accept protons or act as a “proton sponge” within endosomes/ lysosomes (Boussif *et al.*, 1995; Behr, 1997). There are two complimentary theories for the function of PEI within endosomally contained PEI:DNA polyplexes: the buffering properties of PEI leads to 1) an influx of protons and chloride ions, subsequent osmotic swelling and rupture, allowing release of polyplexes into the cytoplasm, and 2) neutralization of the lysosomal compartment, thus inhibiting the action of

lysosomal nucleases (active at low pH) and protecting DNA from degradation (Behr, 1997). Convincing evidence supporting the theory was provided by

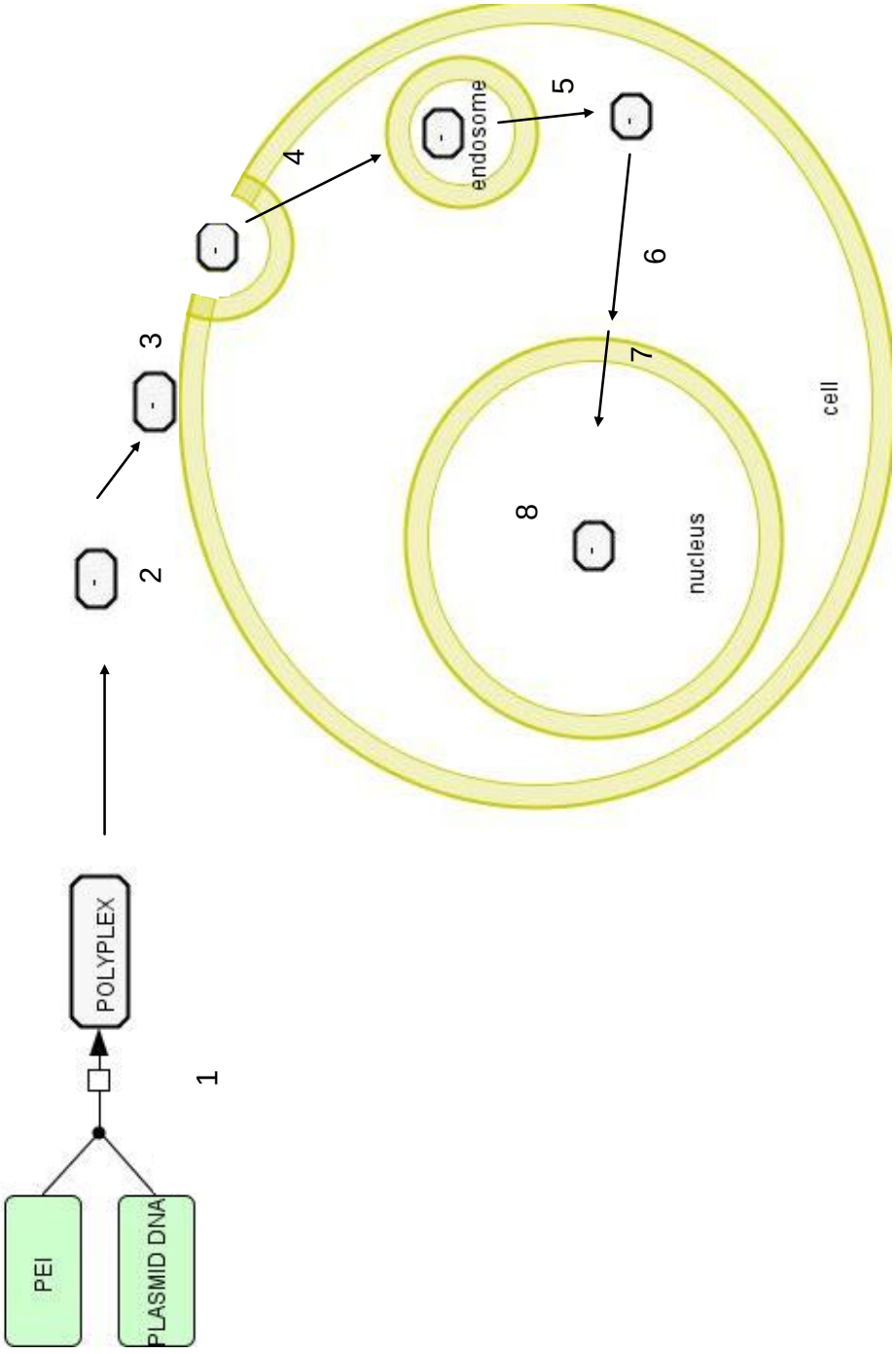


Figure 2.2 Polyplex Formation, Cyto-Delivery and Cyto-Trafficking
1) PEI-DNA polyplex formation; 2) polyplex interacts with cell culture media components; 3) polyplex binds to the cell surface; 4) polyplex is internalized by the cell; 5) polyplex escapes from endosomes; 6) polyplex is trafficked to the nucleus; 7) polyplex translocates into the nucleus; 8) transcription of recombinant DNA.

Akinc *et al.*, (2005), who reported that N-quaternization of PEI (reducing its buffering capacity) and addition of bafilomycin A1 (a proton pump inhibitor) reduced TGE by approximately two orders of magnitude (Kichler *et al.*, 2001; Thomas and Klibanov, 2002).

(6) Nuclear Trafficking

PEI:DNA polyplexes have been shown to be trafficked to the nucleus via active motor protein driven transport on microtubules (Suh *et al.*, 2003). Nuclear localization signals (Kalderon *et al.*, 1984), have been covalently attached to plasmid DNA for transfection (Sebestyen *et al.*, 1998; Zanta *et al.*, 1999). NLS have also been used with polyethylenimine-DNA complexes (Matschke *et al.*, 2012; Zhang *et al.*, 2013).

(7) Nuclear Uptake

There are two hypothesise for polyplex nuclear entry; 1) polyplexes enter the nucleus during the break down of the nuclear membrane during cell division and 2) polyplexes enter the nucleus by active transport through the nuclear envelope, independently of cell division.

Cell cycle control has been exploited to enhance transgene expression following PEI mediated transfection (Tait *et al.*, 2004; Backliwal *et al.*, 2008a), putatively enhancing transgene accumulation in the nucleus. However, it is likely polyplexes are taken into the nucleus through both mechanisms 1 and 2. Cell cycle was found to affect gene expression following transfection mediated by linear 22 kDa PEI (6 fold increase in gene expression recorded in G1 synchronized cells compared to cell synchronized in the late S phase), but to a much lesser extent than it did for gene expression mediated by lipofectamine (Brunner *et al.*, 2002), branched PEI or adenovirus (Brunner *et al.*, 2000). In addition, Grosse *et al.*, (2006) demonstrated that whilst mitosis lead to a higher proportion of GFP expressing cells, cell division was not necessary for gene expression. Han *et al.*, (2009) further demonstrated nuclear uptake of polyplexes in non dividing cells (Horbinski *et al.*, 2001).

Only a small fraction of plasmid delivered to the cell is translocated to the nucleus (~10%) (Carpentier *et al.*, 2007; Cohen *et al.*, 2009). Plasmid copy

number per nucleus was found to approach saturation with respect to gene expression (Cohen *et al.*, 2009; Glover *et al.*, 2010) or to increase linearly with gene expression (Cohen *et al.*, 2009) for different cell lines. The relationship between plasmid copy number per nucleus and gene expression is likely to be transfection protocol dependent (i.e. nuclear plasmid DNA copy number may increase beyond the level required for maximum gene expression if the cell is transfected with excess DNA and *vice versa* if too little DNA is transfected).

(8)Transcription of plasmid DNA

Once in the nucleus, transcription of plasmid DNA is thought to occur 1) whilst complexed with PEI or 2) following dissociation from PEI. Either way, transcription factors and polymerases must be able to access the plasmid DNA.

Schaffer *et al.*, (2000) and Bieber *et al.*, (2002) present conflicting data on the effect of PEI on plasmid DNA, measured using in-vitro transcription studies; Schaffer *et al.*, (2000) reported (without showing data) that PEI complexation inhibited plasmid DNA transcription whereas Bieber *et al.*, (2002) reported no difference in transcription between PEI-complexed and uncomplexed plasmid DNA. Following microinjection into the nucleus, transgene expression was similar for polyethylenimine condensed- and naked plasmid DNA (Pollard *et al.*, 1998). Similar results were obtained by Honore *et al.*, (2005), who reported that at N/P ratios between 5 and 15, transgene expression was similar for PEI condensed- and naked plasmid DNA following intranuclear microinjection. One hypothesis is that plasmid DNA is displaced from the PEI polyplex by nuclear RNA or DNA (Bertschinger *et al.*, 2006b). It should be noted that within the nucleus this displacement reaction could take place, following PEI:DNA polyplex microinjection.

2.4 Thesis Overview

The cell surface is the initial barrier that that transgene complex must pass to gain entry into the mammalian cell factory (Luo and Saltzman, 2000b). PEI was first tested as a transfection regent due to its ability to accept protons and act as a “proton sponge” within lysosomes, both protecting DNA from degradation and allowing release into the cytoplasm (Boussif *et al.*, 1995). Thus PEI was not utilized for its ability to facilitate DNA delivery across the plasma membrane, but for its ability to overcome a subsequent intracellular obstacle for DNA trafficking to the nucleus. whilst there are numerous reports within the literature of PEI derivatives for transfection (Neu *et al.*, 2005), none have been used for CHO based bioprocessing. Thus, in this thesis, the mechanism of PEI mediated transfection of suspension CHO-S cells is explored at the cell surface, approaching the question from first principles (Chapters 4-7). In the final chapter of this thesis, novel, knowledge based engineering strategies are explored, based on mechanistic insights to PEI mediated transfection at the cell surface provided by published literature and the data presented in Chapters 4-7.

Transfection kinetics, with respect to total polyplex cellular association and cyto-internalization are characterized, along with basic system-specific transfection parameters (Chapter 4). Bio-physical interactions between polyplexes and the cell surface are explored in Chapter 5; specifically, by manipulation of polyplex charge and use of non-ionic surfactants to explore the role of electrostatic and hydrophobic interactions. In Chapter 6, bio-molecular polyplex-cell surface interactions are investigated, in particular, focusing on the role of lipid rafts and heparan sulphate proteoglycans in PEI mediated transgene delivery. The efficacy of canonical chemical inhibitors of endocytosis in CHO cell platforms are described in Chapter 7. Based on data presented in Chapter 4-6, engineering strategies to improve PEI mediated transfection are explored in Chapter 8, namely by utilizing PEIs with enhanced hydrophobicity and cell line selection through clone screening. In Chapter 9, work within previous chapters is discussed as a whole.

CHAPTER 3

Materials and Methods

The materials and methods used for experiments described throughout this thesis are described in this chapter.

Mammalian cell culture and microbial work were conducted in separate laboratories. Cell culture sterility was maintained by conducting work in a laminar flow hood and by using a solution of 70% industrial methylated spirits (IMS) on all surfaces and plastic-wear. All materials used were of the highest purity available, unless otherwise stated. Deionised Milli-Q water (Millipore) was used, unless otherwise state.

3.1 Mammalian Cell Culture

The suspension adapted CHO-S cell line, derived from the CHO-K1 cell line, was cultured in CD-CHO medium (Life Technologies, Paisley, UK), supplemented with 8 mM L-glutamine (Life Technologies). Cells were routinely cultured in vented, flat bottomed Ehrlenmeyer shake flasks (Corning) (Fisher Scientific, Loughborough, UK), of 125 mL, 250 mL, 500 mL or 1 L total volume. Culture volume was 20-25% of total flask capacity. Every 3-4 days cells were passaged, seeding at a density of $2x10^5$ cells mL^{-1}. Cells were incubated at 37°C, 5% CO_2 140 rpm incubators (Infors, Derby, UK), without humidification.

Cell counts, cell viability and cell diameter was measured using a ViCell™ Cell Viability Analyser (Beckman Coulter, High Wycombe, UK), which uses trypan blue exclusion to indicate cell viability. A Nikon Eclipse TS100 inverted light microscope, with a 40x objective lens, was used to check for bacterial contamination. Cultures were also routinely tested for mycoplasma infection.

To constrain genetic diversity, master- and working cell banks were created and cells were maintained for 20 passages only. Five passages separated the master and working cell banks. For creation of cell banks, cells were pelleted and resuspended in 9 parts CD-CHO medium, 1 part dimethyl sulphoximide

(DMSO) (Sigma-Aldrich, Dorset, UK) at a density of 1x10^7 viable cells mL^{-1} and aliquoted into cryovials (Nunclon) (Sigma-Aldrich, Dorset, UK). Cryovials were gradually frozen to -80°C using a Nalgene® Mr Frosty container filled with isopropanol, before transferring to cryostats containing liquid nitrogen (-196°C) for long term storage. Cells were brought up from liquid nitrogen storage by incubating the cryovial in water at 37°C, until the culture had melted, and resuspending in warmed CD-CHO media. To remove DMSO, the cells were pelleted and resuspended in CD-CHO medium supplemented with 8 mM L-glutamine and maintained as described above. The entire vial (1x10^7 cells) was used to inoculate the flask following cryo-storage.

Cell doubling time was calculated as described by equation 3.1. Generation number was calculated as described in equation 3.2. Cell specific growth rate (μ) was calculated as described in equation 3.3. Where t is time, f is final, VCD is viable cell density.

$$\text{Cell doubling time} = \frac{(t_f - t_0)\, ln2}{\ln\,(VCD)t_f - \ln\,(VCD)t_0} \qquad 3.1$$

$$\text{Generation number} = \frac{t_f \cdot \ln\,(VCD)t_f - \ln\,(VCD)\,t_0}{(t_f - t_0)\, ln2} \qquad 3.2$$

$$\mu = \frac{\ln\,(VCD)\,t_f - \ln\,(VCD)t_0}{(t_f - t_0)} \qquad 3.3$$

3.2 Cell Fixing

Cells were fixed in a 4% (w/v) paraformaldehyde (PFA) (Sigma-Aldrich) phosphate buffered saline (PBS) solution. Cells were washed in PBS prior to incubating for 15 min at a concentration of 1x10^7 cells mL^{-1} in PFA PBS solution at 4°C. Cells were resuspended at a concentration of 1x10^6 cell mL^{-1} in PBS and stored at 4°C.

3.3 Plasmid DNA Preparation

Plasmid gWIZ™ SEAP (Genlantis) (AMS-Biotechnology, Abingdon, UK) was transformed into Library Efficiency® DH5α™ *Escherichia coli* competent cells (Life Technologies). Cells (DH5α™) were thawed on wet ice and mixed gently

with plasmid DNA at a ratio of 1:50, incubated on ice for 30 min, incubated in water at 42°C for 20 s, incubated on ice for 2 min and finally added to 0.9 mL Luria broth and incubated at 37°C for 1 h. The inoculum was then used to streak Luria broth (Fisher Scientific) agar plates (100 µg mL^{-1} kanamycin), which were incubated for 12-16 hours at 37°C. A colony was picked and used to inoculate 5 mL Luria broth (100 µg mL^{-1} kanamycin), which was incubated for 12-16 h at 37°C. Glycerol solution (50:50 glycerol to water) was added to the starter culture at a 0.3:1 ratio of glycerol solution to culture, for long term storage at -80°C of the transformed DH5α™ cells. For bulk amplification of plasmid DNA, starter culture or glycerol stocks were used to inoculate Luria broth (100 µg mL^{-1} kanamycin), which was incubated at 37°C, 170 rpm for ~20 h.

Plasmid DNA was purified using Maxi- or Mega-Prep Kits (Qiagen, Manchester, UK), following the manufacturer's protocol (2005). Plasmid DNA was stored in Tris-HCl buffer, pH 8.5 in aliquots at -20°C. The concentration of DNA was calculated according to the Beer Lambert Law (equation 3.4), whereby the extinction co-efficient of double stranded DNA is 0.02 µg mL^{-1} cm^{-1} at a wavelength of 260 nm (equation 3.5). Absorbance at 260 and 280 nm was measured using a Biomate™ UV spectrophotometer. The purity of the DNA solution was determined by using the 260/280 ratio and DNA solutions with 260/280 ratios between 1.8 and 1.9 were used for transfections.

$$A = \varepsilon bc \qquad 3.4$$

Where A is absorbance, ε is molar absorptivity (L mol^{-1} cm^{-1}), b is path length (cm), c is concentration (mol L^{-1}).

$$\text{DNA concentration (mg L}^{-1}) = \frac{A_{260}}{0.02} \qquad 3.5$$

If subsequent purification of plasmid DNA was required, nucleic acid was precipitated using ethanol. A solution of 0.1 volume 3 M sodium acetate (pH 5.2) and 2-3 vol. 100% ethanol were added to the DNA solution, which was then incubated at -20°C for several hours. The DNA was pelleted by centrifuging at 13 000g for 30 min and resuspended in Tris-HCl buffer, pH 8.5.

3.4 Fluorescent labelling of DNA

Plasmid DNA was labelled with fluoroscein or cy5 using Mirus *Label* IT® nucleic acid labelling kit (Cambridge Biosciences, Cambridge, UK), at a ratio of 1:4 (w/v) plasmid DNA to labelling reagent. Following incubation of the plasmid DNA and *Label* IT reagent solution, DNA was purified by ethanol precipitation, as described above.

3.5 Transfections

Linear 25kDa polyethylenimine (PEI) (Polysciences) (Park Scientific, Northampton, UK) was dissolved in deionised water (heated to 50°C and vortexed) at a concentration of 1 mg mL^{-1}, the pH of the solution adjusted to 7.2 using hydrochloric acid and 0.22 µm filter sterilized. Solutions of PEI were stored in single use aliquots at -80°C. The solution of PEI, DNA and 150 mM sodium chloride (NaCl), of total volume 66.6 µL per mL transfection volume, was mixed by pipetting and incubated at room temperature for exactly 1 min prior to addition to cells (CHO-S). Unless otherwise stated, CultiFlask 50 disposable bioreactors (Sartorius-Stedim, Epsom, UK) were used for transfection in shaking modality, with incubation at 37°C, 5% CO_2 170 rpm rotation and without humidification (Infors). For static micro-scale culture, Corning® Costar® Ultra-Low attachment 24 well plates (Sigma-Aldrich), with a neutral hydrophilic hydrogel surface, were used and cells cultures at 37°C, 5% CO_2 with humidification (Heraeus, Fisher Scientific). The discrete and continuous parameters used for transfections are summarized in Table 3.1 and Table 3.2 respectively.

Table 3.1 Discrete Transfection Parameters

Protocol	Modality	PEI	Plasmid	PEI:DNA incubation solution	Cell line	Media and supplements
A	Static 24 well plate	25 kDa linear	pgWiz™ SEAP	150 mM NaCl sol.	CHO-S	CD CHO, 8 mM L-glutamine.

Table 3.2 Continuous Transfection Parameters

Protocol	[PEI] µg/ 10^6 cells	[DNA] µg/ 10^6 cells	PEI:DNA ratio w/w *molar N/P*	PEI:DNA incubation time	PEI:DNA incubation solution volume	[Cells] (cells mL^{-1})	Culture Volume (mL)
A	4.7	4	*1.2* ***9***	1 min	66.6 µL per mL culture volume	$1x10^6$	0.5

The molar ratio of PEI to DNA was calculated as described by equation 3.6.

$$\text{PEI/DNA ratio} = \frac{[PEI]\cdot 327}{[DNA]\cdot 43.1} \qquad 3.6$$

Where the molecular weight of the ethylenimine monomer (C_2H_5N) is 43.1 grams $mole^{-1}$ and the average molecular weight of deoxyribonucleotide monosphosphate is 327 grams $mole^{-1}$.

3.6 Transfections with fluorescently labelled plasmid DNA

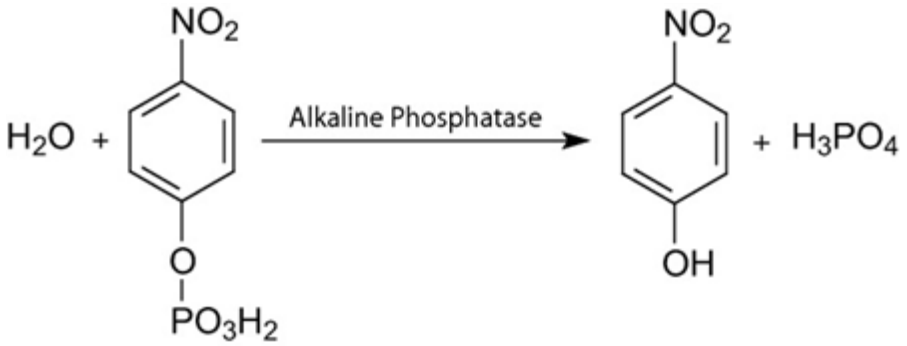
Transfections were performed as described above and at the indicated times post transfection, cells were either resuspended in CellScrub™ (AMS Biotechnology) or DPBS (to distinguish between internalized polyplex and total cell associated polyplex respectively) and incubated for 10 min at 4°C, washed a second time with PBS and stored on ice before flow cytometric analysis or confocal microscopy.

For polyplex-cell surface binding experiments, transfections were incubated at 4°C for 4 h, washed twice with PBS and incubated on ice before flow cytometric or analysis or microscopy.

3.7 Secreted Alkaline Phosphatase (SEAP) reporter protein assay

Relative secreted alkaline phosphatase (SEAP) reporter protein (Berger *et al.*, 1998) was measured using a colorimetric Anaspec SensoLyte® *pNNP* Secreted Alkaline Phosphatase Reporter Gene Assay Kit (Cambridge Bioscience). Alkaline phosphatase catalyses the hydrolysis of paranitrophenyl phosphate to paranitrophenol (equation 3.6), which has a yellow colour,

measured spectrophotometrically at 405 nm. Thus absorption is a measure of SEAP activity and concentration. Supernatant was stored at -20°C prior to relative SEAP quantification. In a 96 well plate, 50 µL paranitrophenyl phosphate (pNPP) was added to 50 µL of (diluted) supernatant. The absorbance was measured at 405 nm using a PowerWave™ spectrophotometer plate reader (BioTek, Potton, Bedfordshire, UK). Kinetic readings were taken, to ensure absorbance readings were within linear range. Where normalized values are given, maximum absorbance at 405 nm was given a value of 1. For quantification of reporter output, a human placental SEAP standard curve was prepared by serial dilutions in CD CHO media (Figure 3.1).



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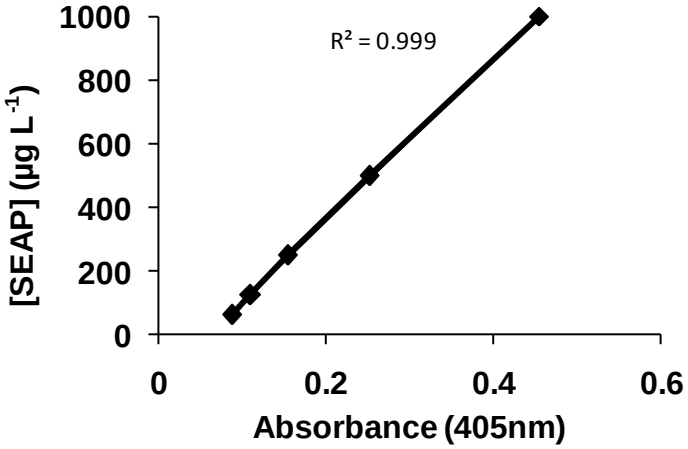

Figure 3.1 Example SEAP standard, concentration (µg L^{-1}) against absorbance (405 nm).

3.8 Flow Cytometry

A BD FACSCalibur™ (Oxford, UK) was used for flow cytometry. The cell population was gated according to side and forward scatter, Figure 3.2. Fluoroscein was excited using a 488 nm laser and detected using a 530/30 band pass filter. Cells without a fluorescent label were measured to determine cellular auto-fluorescence. Unless otherwise stated, a sample of 5000 cells was measured. Data was analyzed using FloJo™ software.

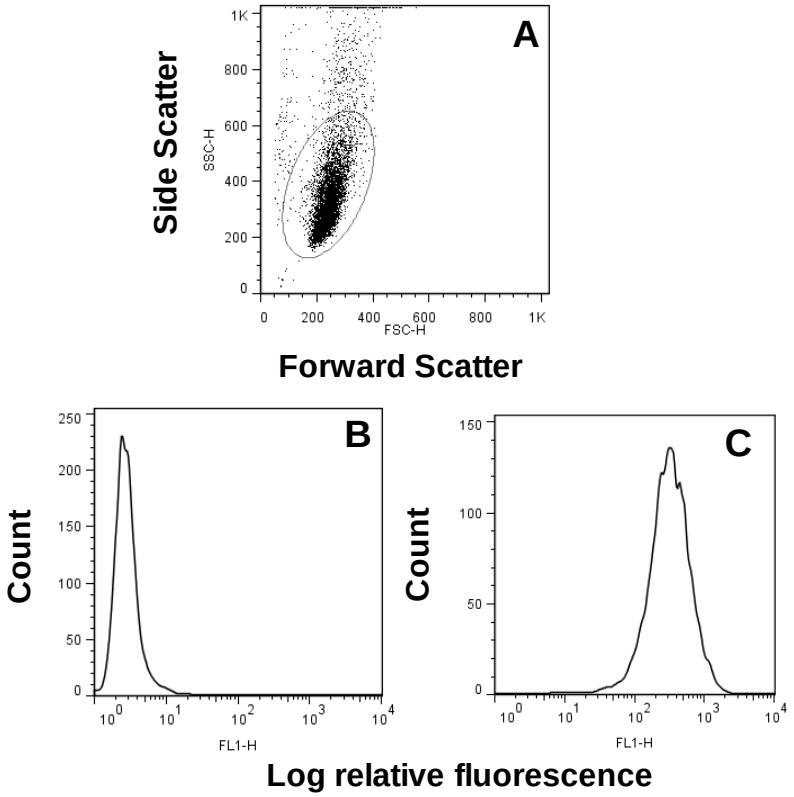

Figure 3.2 Example flow cytometry plots
Cell population gated for granularity and size (**A**). Example histogram of fluorescence for cells without a fluorescent stain/ label (auto-fluorescence) (**B**). Example histogram of cells with fluorescent label (**C**).

3.9 Microscopy

A Nikon Eclipse TS100 microscope was used for light microscopy. Images were captured using a Nikon Digital Sight camera. For confocal microscopy, upright and inverted Zeiss LSM 510 microscopes were used and images analysed using Zen 2009 light edition software.

3.10 Zeta potential measurements

PEI:DNA polyplexes were formed in 150 mM sodium chloride solution, as described above. PEI:DNA polyplexes were added to solutions at the same concentration as for transfections and incubated for 5 min at room temperature prior to measurement. Particle electrophoretic mobility was measured using a Brookhaven ZetaPALS (Cheadle, Cheshire, UK) system, using phase analysis light scattering. Zeta potential was obtained by using the Smoluchowsky model.

3.11 Statistics

Unless otherwise stated, the mean of triplicate experiments is shown and the error bars represent one standard deviation about the mean. To test for significance, an unpaired, two tailed, student's t-test was used.

CHAPTER 4

Characterization of Transfection Parameters and Kinetics

Acknowledgements

DoE-RSM transfection optimization was designed and conducted by Dr Ben Thompson, data presented in Figure 4.2.

Dr Markus Ariaans assisted with confocal microscopy.

Chapter Overview

This chapter describes a DoE-RSM optimized protocol for the micro-scale transfection of CHO-S cells. Key parameters surrounding the transfection process are explored, such as the kinetics of polyplex cell-association and cyto-internalization and the cytotoxicity of PEI and PEI mediated transfection. The aims of this chapter are to:

- Describe the DoE-RSM optimized PEI mediated transfection protocol.
- Characterize the host cell line (CHO-S) for parameters relevant to PEI mediated transfection and transient gene expression.
- Establish the kinetics of transfection, with respect to polyplex-cell association and cyto-internalization.
- Investigate the toxicity of PEI and PEI:DNA polyplexes.

In summary, a DoE optimized PEI mediated transfection protocol is described, justified and explored at the molecular level.

4.1 Introduction

4.1.1 Cell line specific transfection optimization

As described in section 1.2.1, amongst other mammalian cell lines, cell lines derived from a Chinese Hamster Ovary (CHO) are the most frequently used for the production of complex, glycosylated biopharmaceuticals. First taken from the Chinese Hamster Ovary in 1956 (Puck *et al.*, 1956), the cell line has diverged and numerous variant, parental CHO cell lines now exist (Derouazi *et al.*, 2005). Frequently used CHO variant cell lines for biomanufacturing include the CHOk1sv and CHO DG44 cell lines.

For the study presented in this thesis, the CHO-S parental cell line was used throughout. The CHO-S cell line in a suspension adapted CHO cell line variant, adapted to grow in serum free culture and is freely available to purchase from Life Technologies, unlike several other variant CHO cell lines, which are propriety cell lines. A suspension adapted, rather than adherent, cell line was utilised, due to its relevance to biomanufacturing.

Heterogeneity exists between cell lines, between variant CHO cell lines and between clones of the same cell line (Wurm and Hacker, 2011). It is likely that the the results presented in this thesis are cell line specific. However, the CHO-S cell line is a suitable “model” cell line for biomanufacturing, rendering the data presented in this thesis applicable to other cell lines with similar characterisitics.

Optimization of transfection involves the fine tuning of numerous discrete and continuous parameters (Thompson *et al.*, 2012). Discrete parameters include host cell line, vector or system (Table 1.3). Recently, the variability in CHO cell line specific TGE capacity has been demonstrated. CHO cell lines were found to vary in their reporter protein output, following transfection (CHO-S cells were found to secrete >4 fold the level of SEAP secreted by CHO-L and CHO-M cells) (Thompson *et al.*, 2012). In addition, CHO-K1SV derived clones have been found vary dramatically in their clone specific transient mAb or GFP production capacity following transfection by lipofection or electroporation (Davies *et al.*, 2012).

Continuous parameters for optimization include the cell density at time of transfection and PEI and plasmid DNA concentration. Optimization of these

variables can be achieved via a one factor at a time approach (OFAT) (Rajendra *et al.*, 2011; Raymond *et al.*, 2012), or via design of experiments (DoE) methodology (Bollin *et al.*, 2011; Thompson *et al.*, 2012). As described by Mandenius and Brundin (2008), optimization using DoE methodology is superior to OFAT in that, often, fewer experiments are required and a true optimum is more likely to be achieved. Using OFAT, if one variable is kept constant, a quasi optimum may result due to the inter-dependency of the variables (Mandenius and Brundin, 2008).

Optimization of continuous parameters is often specific to discrete parameters, for example plasmid vector, culture modality, media and cell line. Thus, transfection optimization is an unavoidable step in PEI mediated TGE.

4.1.2 Transfection kinetics: polyplex-cell-surface association and cyto-internalization

The kinetics of polyplex nuclear delivery have been explored (Glover *et al.*, 2010), but not the kinetics of polyplex-cell association or cyto-internalization.

Transfection kinetics were explored, indirectly, by Bertschinger *et al.,* (2008), by adding excess competitor DNA to the culture at set times post transfection, which was taken to inhibit subsequent uptake of PEI:DNA polyplexes. At 60 min post transfection with PEI, addition of excess stuffer DNA resulted in TGE (GFP reporter protein) at 80% of control levels (without addition of stuffer DNA).

Polyplex-cell association and cyto-internalization was explored by Thompson *et al.,* (2012) for three variant CHO cell lines. Cell-line specific polyplex internalization capacity was found to be >4 fold higher in CHO-S cells, relative to CHO-L and CHO-M. Cyto-internalization, but not total cell-association, of fluorescently labelled polyplexes, across three cell-lines, was linked to SEAP reporter protein production at cell line specific optimized conditions.

The role of cell surface constituents (e.g. heparan sulphate proteoglycans) in polyplex-cell surface interactions will be discussed and explored in Chapter 6.

4.1.3 Cyto-toxicity of PEI and PEI mediated transfection

Two outputs are normally required for TGE processes: high transgene expression and high culture viability post transfection (Lv *et al.*, 2006). Low cell viabilities, due to stress at transfection or during the TGE process, leading to release of intracellular proteases or glycosidases, can adversely affect product quality and/or titre (Cruz *et al.*, 2002). Of a range of cationic polymer/ macromolecular transfection agent, polyethylenimine was found to be relatively toxic (Fischer *et al.*, 2003). Thus an understanding of toxicity caused by PEI mediated transfection may help improve TGE platforms.

Cyto-toxicity of PEI and PEI:DNA polyplexes has been reported to occur in two phases (Moghimi *et al.*, 2005). The first stage of toxicity was found to occur within the first hour post addition of PEI/ PEI:DNA polyplexes, attributed to necrotic damage to the plasma membrane (assessed by lactate dehydrogenase release and phosphatidylserine translocation to the cell surface). The second stage of toxicity was reported at 24 hours post addition of PEI/ PEI:DNA polyplexes and was attributed to mitochondrially mediated apoptosis (assessed by release of proapoptotic cytochrome c, activation of caspase c and alteration of mitochondrial membrane potential) (Moghimi *et al.*, 2005).

Necrotic changes at the plasma membrane have also been reported at 1-2 hours following PEI:DNA polyplex addition (phosphatidyl serine exposure and plasma membrane permeability measured through propidium iodide staining) by Grandinetti *et al.* (2011). The authors also reported apoptotic markers at early time points post transfection (1-2 h), by measuring caspase 9 activity and a decrease in mitochondrial membrane potential. PEI:DNA polyplexes were also shown to co-localize with mitochondria, within two hours post transfection. Additionally, Grandinetti *et al.*, (2011) demonstrated that the effect of PEI on mitochondrial membrane potential was potentiated by addition of cyclosporin A (an inhibitor of the mitochondrial membrane permeability transition pore), rotenone and oligomycin (respiratory chain inhibitors).

Mitochondria are a source of reactive oxygen species (ROS) in mammalian cells (Murphy, 2009), which can in turn lead to the induction of apoptosis (Anderson *et al.*, 1999; Green and Reed, 1998; Simon *et al.*, 2000) (Figure 4.1).

Recently, efforts to reduce ROS following PEI mediated transfection have been reported (Lee *et al.*, 2013). Transfection with PEI lead to significant production of ROS in HepG-2 cells, which was abated by addition of the anti-oxidant, tocopherol. An amphiphilic copolymer (PEI-PLGA) was synthesized and used to co-transport the anti-oxidant alpha-tocopherol alongside plasmid DNA (PEI-PLGA/Toco/pDNA polyplex); it was found to enhance transfection efficiency dramatically in HepG2, MCF-7 and HCT-116 cells (Lee *et al.*, 2013).

Acylated PEIs have been reported as efficacious for *in vitro* and *in vivo* gene delivery (Forrest *et al.*, 2004; Nimesh *et al.*, 2007; Aravindan *et al.*, 2009). Recently, acylated PEI nano-particles have been shown to result in lower levels of ROS production relative to non-acylated PEI based nano-particles (Calarco *et al.*, 2013).

The toxicity of charged medical nano-particles is well reported (Baek *et al.*, 2011; Frohlich, 2012). Substantial work has been directed towards reducing the toxicity of PEI:DNA polyplexes by shielding the net positive charge on their surface through PEGylation and lipid coating (Luo *et al.*, 2012). PEGylation and lipid coating of polyplexes successfully reduced their zeta potential and numerous PEG-PEI:DNA polyplexes displayed lower toxicity and higher transfection efficiency relative to PEI:DNA polyplexes, especially at higher N/P ratios (Luo *et al.*, 2012). Acylated- and PEGylated-PEIs are lipo-polyplexes are discussed in more detail in Chapters 5 and 8.

The relative toxicity and transfection efficacy of PEI:DNA polyplexes, PEI:DNA polyplexes purified by size exclusion chromatography (SEC) or PEI:DNA polyplexes purified by SEC with additional free PEI added was explored (Boeckle *et al.*, 2004). Purified polyplexes were found to be severely detrimental to gene expression in the case of branched 25 kDa PEI. However, for linear 22 kDa PEI, purified polyplexes were found to have no effect or enhance gene expression, for a selection of the cell lines tested (CT26, B16-F10, HeLa). Furthermore, transfection with purified polyplexes dramatically improved the toxicity profile and, for both branched and linear PEI, increased levels of cell associated DNA (Boeckle *et al.*, 2004). In addition, Godbey *et al.*, (1999d) reported the detrimental effects of free PEI on transfection.

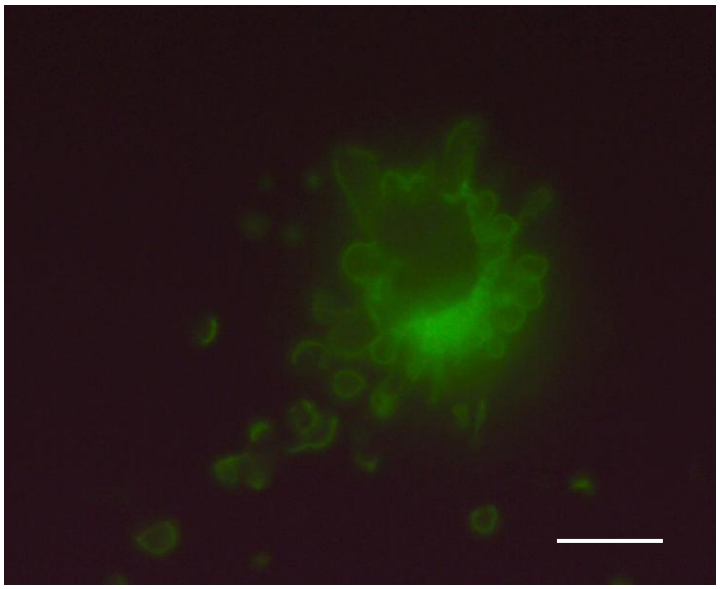

Figure 4.1 Apoptotic CHO-S cell.
Cells (CHO-S) were biotinylated and stained with streptavidin FITC and viewed using a Cytoviva. Membrane blebbing (Coleman et al., 2001) is a morphological change characteristic to apoptosis. Cells were imaged using a 100x objective lens. Bar=10µm.

4.2 Materials and Methods

For all experiments described in this chapter, CHO-S cells were used, using the culture conditions described in Chapter 3. Cells were transfected with 25 kDa linear polyethylenimine according to protocol A, as described in Chapter 3, Tables 3.1 and 3.2.

4.2.1 Transfection Optimization

Transfection was optimized using design of experiments software (Design Expert 7, Statease (PRISM-TC, Cambridge, UK), specifically using a central composite design. Cell density at time of transfection was kept constant, at 1 $x10^6$ cells mL^{-1}. Cells were cultured in a total volume of 500 µL in 24 micro-well plates. DNA concentration was varied between 1 and 5 µg mL^{-1} and PEI was varied, as a ratio of DNA mass, between 1 and 3.

4.2.2 Biotinylating and staining with streptavidin-FITC

Sulpho-NHS-SS Biotin was purchased from Fisher Scientific. A 10 mM solution was prepared immediately before use and added to 10^6 live cells at a concentration of 250 µM. Cells were incubated for 30 min on ice, washed once

with PBS and fixed with PFA. Biotinylated, fixed cells (10^6 in 1 mL volume) were incubated at 4°C, in darkness for 20 min with 2.5 µg streptavidin FITC (BD Biosciences) and washed and resuspended in PBS.

4.2.3 Analysis of reactive oxygen/nitrogen species

The Oxiselect™ *In vitro* ROS/RNS Assay Kit (Cambridge Bioscience) was used to measure free radical levels in the culture supernatant. The method employs the fluorogenic probe, 2, 7-dichlorodihydrofluorescein (DCFH). In the presence of ROS and RNS, DCFH is oxidized to the fluorescent 2,7-dichlorofluorescein (DCF) ($\lambda_{excitation}$ =498nm; $\lambda_{emission}$ =522nm). Fluorescence intensity is proportional to the total ROS/RNS level within the sample. Fluorescence was measured using a Fluoroskan-Ascent plate reader (Fisher Scientific).

4.2.4 Fluorescent microscopy

Fluorescent cells were visualised using an Olympus BX51 microscope, a Cytoviva Dual Mode Fluorescence Module (Aetos Technologies), an Exfo X-cite 120 Fluorescent light source and a Progress C5 camera and software (Jenoptik).

4.3 Results

4.3.1 Optimization of micro-scale PEI mediated transfection of CHO-S cells using design of experiments methodology

Using design of experiments, a micro-scale protocol was developed in static 24 well plate format, using 25 kDa linear PEI, CHO-S cells in CD CHO media and a pgWiz™ SEAP expression vector (Figure 4.2). SEAP activity increased with increasing DNA concentration and decreasing PEI:DNA ratio (Figure 4.2A). However, by reducing DNA concentration, culture percentage viability increased significantly (Figure 4.2B). Balancing SEAP production and culture viability, a DNA concentration of 4 mg L^{-1} and PEI concentration of 4.7 mg L^{-1} (PEI:DNA ratio of 1.2 w/w or molar N/P ratio of 9) were predicted by the model and validated experimentally.

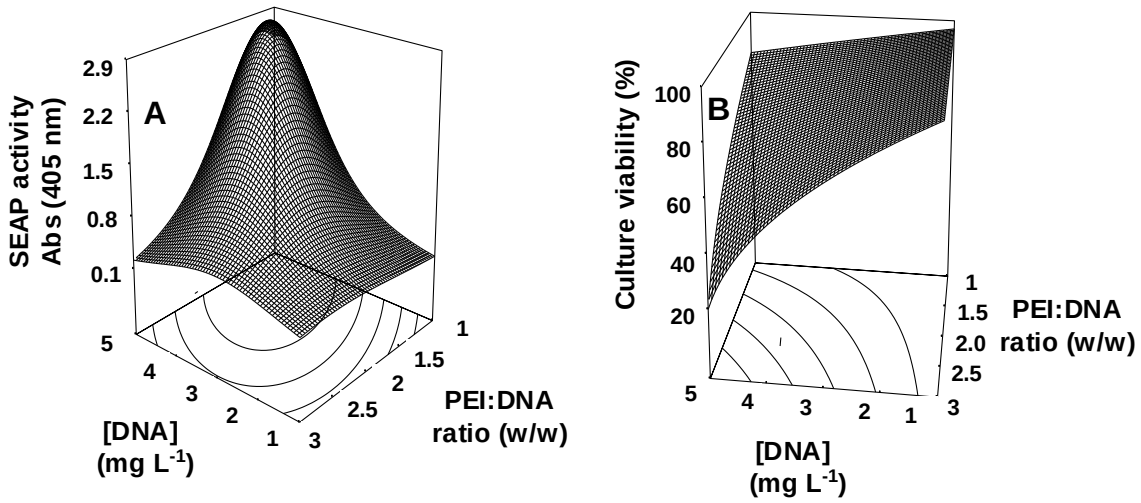

Figure 4.2 Design of experiments response surface models (DoE-RSM) for micro-scale PEI mediated transfection of CHO-S cells with pgWiz™SEAP expression vector.
Transfection of CHO-S, suspension adapted cells cultured in a static 24 well micro-plate modality was optimized using design of experiments soft-ware (Design Expert 7) (a central composite design). Optimized conditions were based on a sweet point between maximum SEAP production and high culture viabilities. **A:** SEAP response surface and **B:** culture % viability response surface.

4.3.2 Characterization of host cell line for TGE mediated by PEI

The CHO-S cell-line was used as a host cell line due to its ability to grow to high density in suspension culture and its superior susceptibility to transfection and TGE (Thompson *et al.*, 2012). The cell growth and the culture percentage viability were measured from cultures over a 200 h period, with readings taken at approximately 24 h intervals (Figure 4.3A). Cell specific growth rate, μ, was calculated for the period that the cells were in exponential growth phase (up to 96 h), and found to be 0.04 h^{-1}. It was determined that cells would be passaged and transfected at approximately 72 h post sub-culture (in the mid-exponential phase). Up to approximately 100 h, the cells doubled at an approximately constant rate and between 100 and 125 h, the doubling rate increased substantially.

To constrain the genetic diversity of the CHO-S host cell line, cell banks were created and cells were maintained for 20 passages only. Cells at passage 1 and

21 were transfected using standard conditions (Figure 4.4). Limited difference in SEAP production, viable cell density or culture percentage viability was seen between transfections at passages 1 and 21 (Figure 4.4 A, B). In addition, there was no significant difference between viable cell density and culture percentage viability of un-transfected cells (Figure 4.4 C).

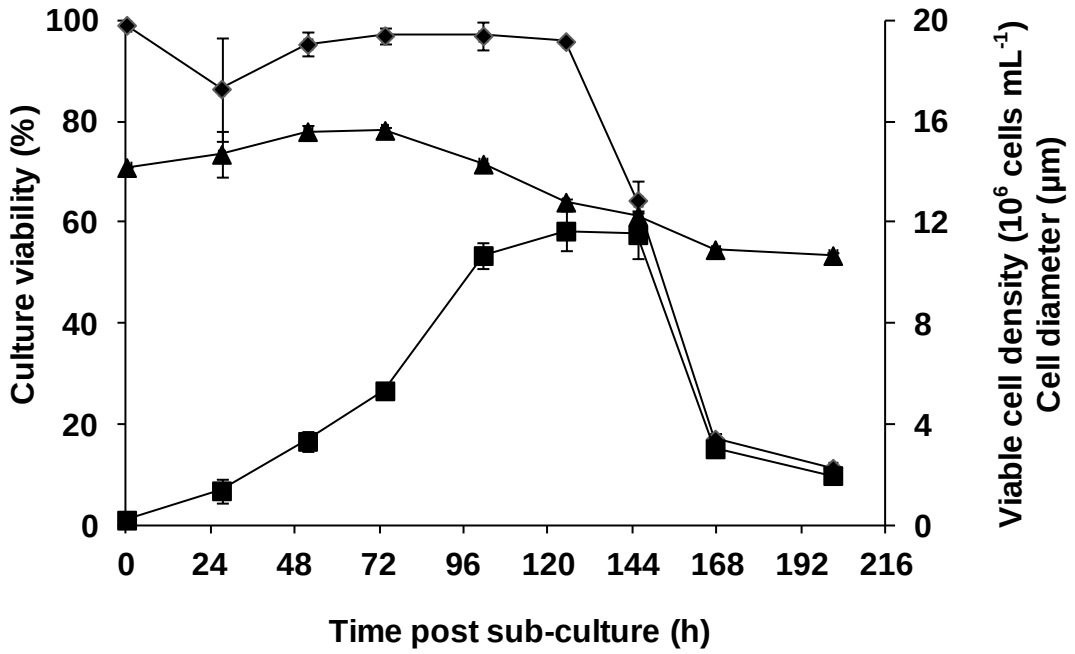

Figure 4.3 Growth of CHO-S Cells in batch culture.
CHO-S cells were grown in CD CHO media with 8mM L-glutamine in a total volume of 50 mL in 250 mL Erlenmeyer flasks, at 37°C, 5% CO_2 and 170 rpm. At the indicated times post sub-culture, viable cell density (squares, right axis), culture %viability (diamonds, left axis) and average cell diameter (triangles, right axis) were measured. The mean value ± standard deviation from triplicate shake-flasks is shown.

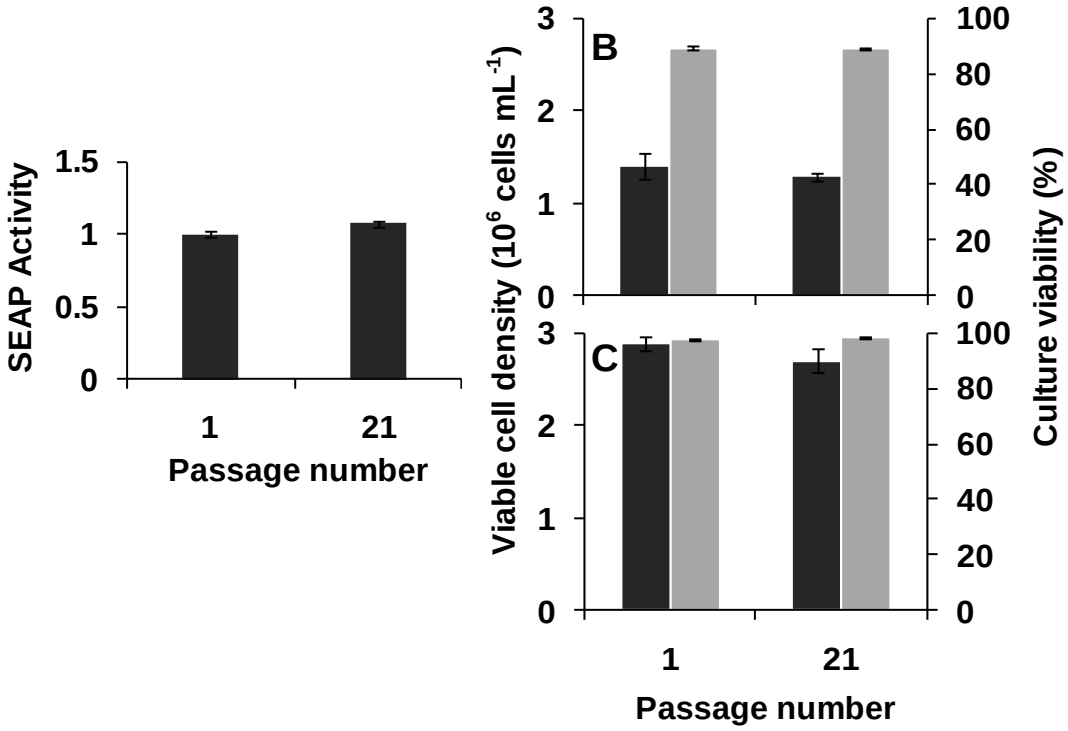

Figure 4.4
Transfection at passage 1 and passage 21 (Δ generations ~100).
Cells at passage 1 and 21 were transfected according to protocol A. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured at 24 h post transfection (**B**) or at 24 h post sub-culture (**C**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

4.3.3 Transfection kinetics: polyplex-cell surface association and cyto-internalization

Given the dynamic nature of the cell surface and the transfection process, kinetic measurements were taken to characterize the experimental platform (Figure 4.5). Polyplex cell surface attachment and internalization were assessed by transfecting cells using PEI and fluoro-labelled plasmid DNA. By washing cells with CellScrub, cell surface attached polyplex was removed (Zuhorn *et al.*, 2002; Mckeon and Khaledi, 2003; Moriguchi *et al.*, 2005; Thompson *et al.*, 2012). A sample of cells was washed with PBS, to obtain a value for total cell associated fluorescence

Confocal micrographs (Figure 4.6) illustrate cyto-internalized polyplexes (CellScrub washed) and cell associated polyplexes (PBS washes). Images were taken through the cell to establish whether polyplex was located intracellularly or on the surface of the cell. The images are similar to those obtained by Godbey *et al.*, (1999c) using confocal microscopy and Mishra *et al.*, (2004) using electron microscopy, showing polyplexes to enter the cells as relatively large complexes.

Using flow cytometry, polyplex–cell association was found to occur rapidly, within the first 15 min post transfection and then increase until saturation at approximately 240 min post transfection (Figure 4.4, squares). At 14 min post transfection, total cell associated polyplex reached 41% its maximum level (measured at 240 min post transfection).

Internalized polyplex was also measured at 14 min post transfection and saturated at approximately 240 min post transfection (Figure 4.5, circles). At 14 min post transfection, internalized polyplex reached 24% its maximum level (measured at 480 min post transfection). Thus between 14 and 480 min post transfection, the remaining 76% of polyplex was internalized.

As a percentage of total associated polyplex, internalized polyplex increased from 47% at 14 min post transfection to 81% at 480 min post transfection. Thus polyplex gradually translocated from the cell surface to within the plasma membrane at a rate faster than the rate of extracellular polyplexes binding to the cell surface.

Polyplex attached to the cell surface was calculated by subtracting internalized polyplex from total cell associated polyplex (Figure 4.5 dotted line, triangles). Cell-surface-attached polyplex increased rapidly immediately post tranfection and then gradually, between 14 and 240 min post transfection.

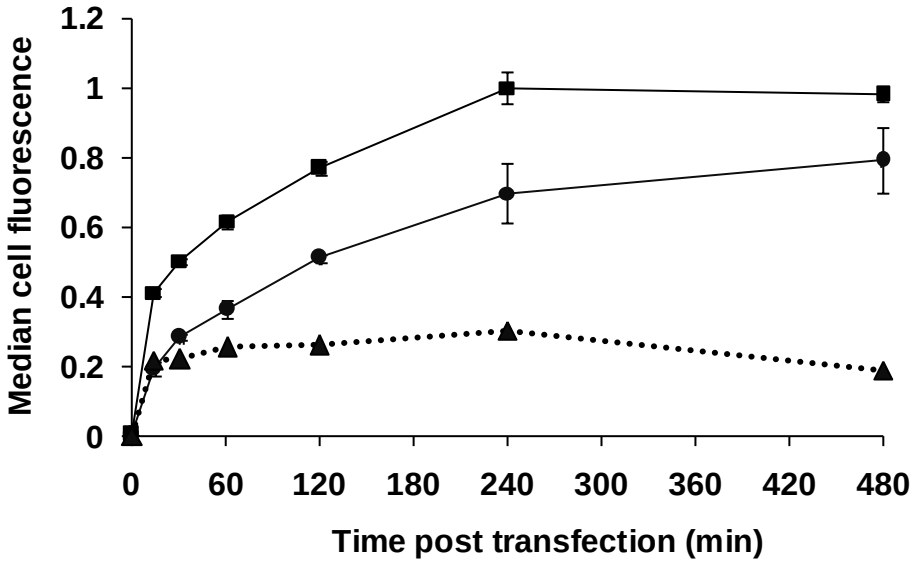

Figure 4.5 Kinetics of cell-association and cyto-internalization of PEI:DNA polyplexes by CHO-S cells.
Cells were transfected with PEI and fluorescently labelled plasmid DNA according to protocol A. Total cell associated (squares) and cyto-internalized polyplex (circles) was measured by flow cytometry, at the indicated times post transfection, following washing with PBS or CellScrub, respectively. Levels of cell surface-attached polyplex (triangle) were obtained by subtracting cyto-internalized (CellScrub washed) from total cell associated (PBS washed) fluorescence levels. The mean value ± standard deviation from triplicate transfections is shown.

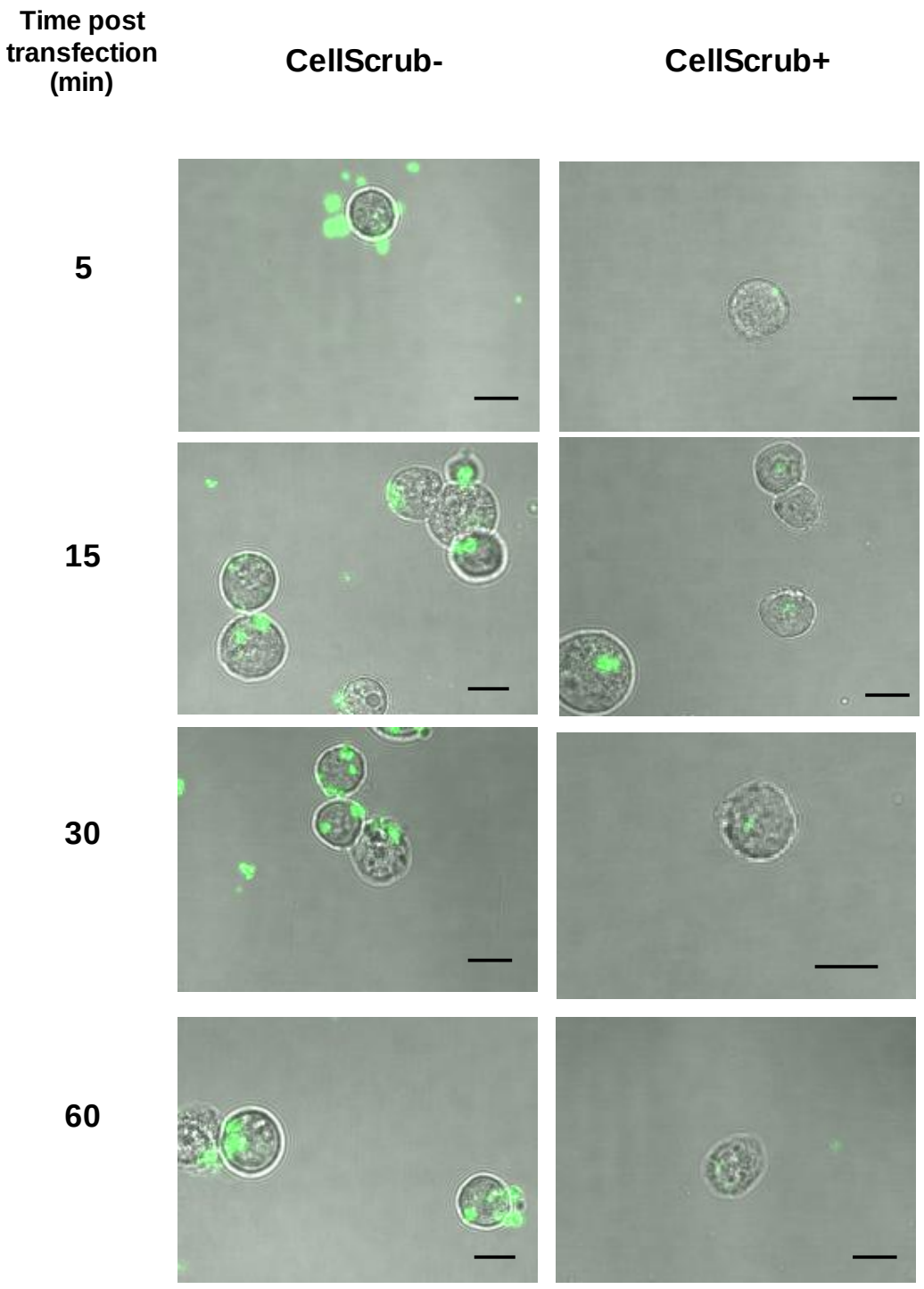

Figure 4.6 Cyto-associated and cyto-internalized polyplexes.
CHO-S cells were transfected with PEI and fluoroscein labelled DNA. At the indicated times post transfection cells were removed from the transfection, treated with or without CellScrub and incubated on ice. Cells were imaged with an upright Zeiss LSM microscope with a 40x/0.8 dipping lens and 488 nm laser. Bar = 10µm.

The rate of polyplex-cell association, cyto-internalization and cell-attachment were also calculated according to equations 4.1, 4.2 and 4.3. The rate of polyplex-cell association, calculated according to equation 4.1, decreased from 15.8 FU $cell^{-1}min^{-1}$ between 0 and 14 min post transfection, to 1.0 FU $cell^{-1}min^{-1}$ between 120 and 240 min post transfection (Figure 4.7, squares). The rate of polyplex internalization, calculated according to equation 4.2, decreased from 7.4 FU $cell^{-1}min^{-1}$ between 0 and 14 min post transfection, to 0.9 FU $cell^{-1}min^{-1}$ between 120 and 240 min post transfection (Figure 4.7, circles). The rate of polyplex cell-surface attachment, calculated according to equation 4.3, decreased from 8.4 FU $cell^{-1}min^{-1}$ between 0 and 14 min post transfection, to 0.2 FU $cell^{-1}min^{-1}$ between 120 and 240 min post transfection (Figure 4.5, dotted line, triangles). A biphasic process was measured, whereby the rate of polyplex-cell association and cyto-internalization was >5 fold and >2 fold greater during the first 14 min post transfection, then at subsequent time points, respectively. The rate of polyplex-cell surface attachment was >10 fold greater during the first 14 min post transfection than at subsequent time points.

The data presented in Figures 4.5, 4.7 and 4.8, combined with data presented in Figures 6.4, 6.5 and 6.6, is used to form the biphasic model of polyplex uptake, described in Chapter 9 and Figure 9.1.

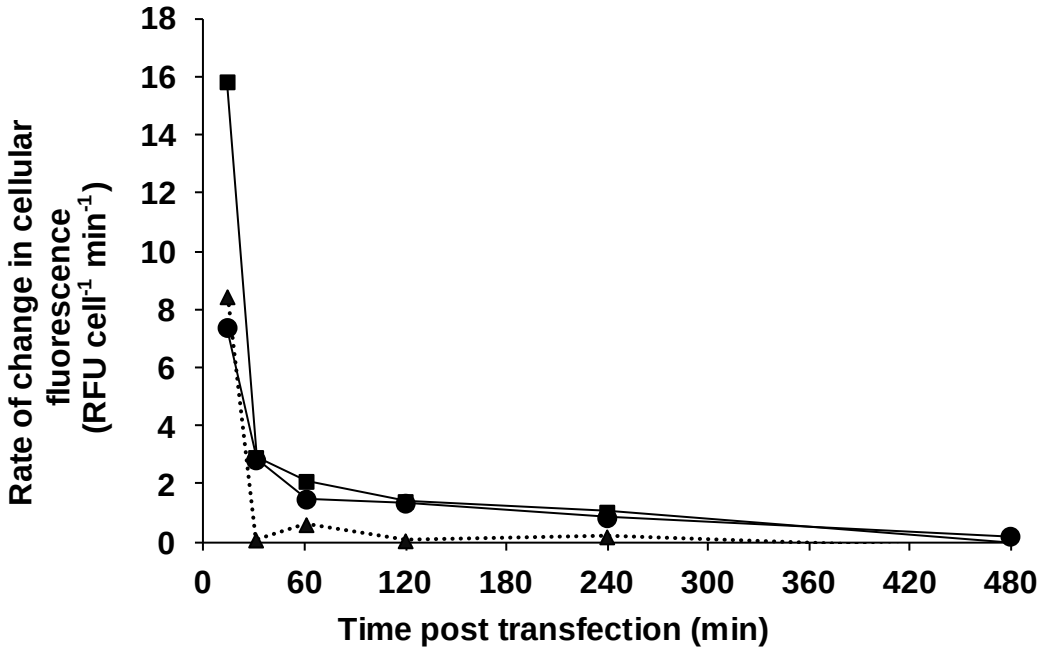

Figure 4.7 Rate of polyplex cell-association and cyto-internalization. Rate of change of total cell-associated polyplex at time *t* (squares), rate of change of cyto-internalized polyplex at time *t* (circles), rate of change of cell-surface attached polyplex at time *t* (triangles). Rates were calculated using equations 4.1, 4.2 and 4.3 and data presented in Figure 4.5.

$$\frac{F(as)t_n - F(as)t_{(n-1)}}{(mt_n - mt_{(n-1)})} \qquad 4.1$$

$$\frac{F(int)t_n - F(int)t_{(n-1)}}{(mt_n - mt_{(n-1)})} \qquad 4.2$$

$$\frac{F(as-int)t_n - F(as-int)t_{(n-1)}}{(mt_n - mt_{(n-1)})} \qquad 4.3$$

Equation 4.1 describes the rate of change of polyplex-cell association, equation 4.2, the rate of change of polyplex-cyto-internalization and equation 4.3, the rate of change of polyplex-cell attachment, where $F(as)$ represents median cell fluorescence attributed to total cell associated polyplex (following a PBS wash), $F(int)$ represents median cell fluorescence attributed to cyto-internalized polyplex (following a CellScrub wash) and m is minutes.

Polyplex cyto-internalization data was transformed and lines of best fit added (Figure 4.8). Interestingly, a single equation could not be fitted to the entire series accurately, but a power law function provided good fit to the data between 14 and 480 min post transfection. An exponential function was added between 0 and 14 min post transfection (Figure 4.8). The two lines of best fit were solved using WolframAlpha software; at 16.8 min post transfection, polyplex cyto-internalization began to be best described by a power law function.

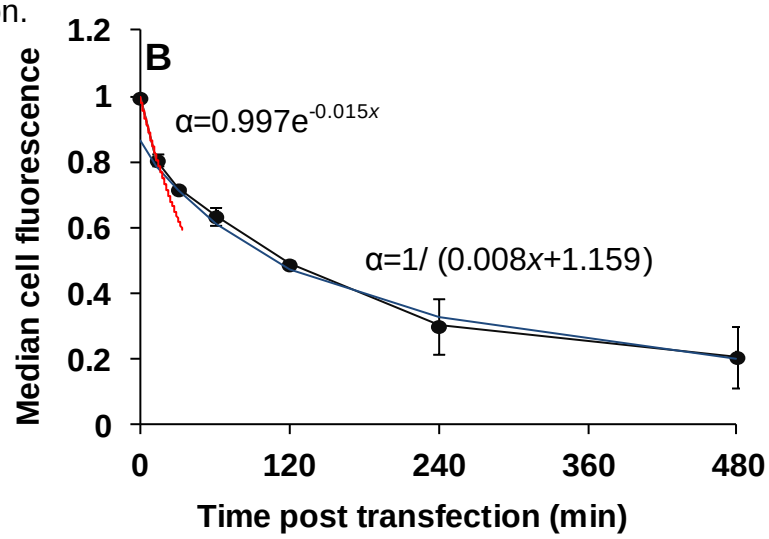

Figure 4.8
Equations fitted to kinetic data for cyto-internalization of polyplexes.
Polyplex cyto-internalization kinetic was transformed according to 1-y. Between t=14 and t=240 polyplex cyto-internalization was found to follow a polynomial function. Between t=0 and t=14, an exponential function was fitted to the data set.

4.3.4 Cyto-toxicity of PEI and PEI mediated transfection

To investigate the toxicity of free PEI relative to PEI:DNA polyplexes, cells were cultured with a range of PEI concentrations, both free and complexed with DNA. Furthermore, to investigate the cause of PEI or PEI:DNA polyplex mediated toxicity, cells were cultured at hypothermic conditions (4°C) to establish whether toxicity was attributed to physical damage to the cells. In addition, ROS/ RNS levels in the supernatant were assayed.

At hypothermic incubation temperature (4°C), PEI:DNA polyplexes had minimal effect on viable cell density and culture percentage viability at 4 h post addition of polyplexes (Figure 4.9). At a PEI:DNA ratio of 3 (12 µg :4 µg) there was a reduction in viable cell density to $0.9x10^6$ cells mL^{-1} relative to $1x10^6$ cells mL^{-1} in control conditions. At a PEI:DNA ratio of 3 there was also a decrease in culture viability from 97% in control conditions to 86%.

In its free form, however, PEI dramatically reduced viable cell density and culture percentage viability at higher concentrations, at hypothermic culture temperatures (Figure 4.9). At a PEI concentration of 8 µg 10^6 $cells^{-1}$ and 12 µg 10^6 $cells^{-1}$, viable cell density reduced to $0.7x10^6$ cells mL^{-1} and $0.5x10^6$ cells mL^{-1} relative to $1x10^6$ $cellsmL^{-1}$ in control conditions; cell culture viability also decreased to 67% and 47% relative to 96% in control conditions, respectively.

At hypothermic incubation temperature, as PEI concentration increased, PEI was relatively more toxic to cells in its free form, compared to when complexed with plasmid DNA, as shown in Table 4.1. At a concentration of 8 µg 10^6 $cells^{-1}$, following addition of the free form of PEI to cells, culture viability reduced to 67%, whereas following addition of PEI:DNA polyplexes, culture viability reduced to only 96%. At 12 µg 10^6 $cells^{-1}$ PEI, following addition of the free form of PEI to cells, culture viability reduced to 45%, whereas following addition of PEI:DNA polyplexes, culture viability reduced to only 86%.

Reactive oxygen and nitrogen species (ROS and RNS) were measured in the culture supernatant. At hypothermic culture temperatures (4°C), addition of free PEI and PEI:DNA polyplexes, at difference concentrations, had no effect on ROS/RNS levels in the supernatant at 4 hours post sub-culture (Figure 4.9B).

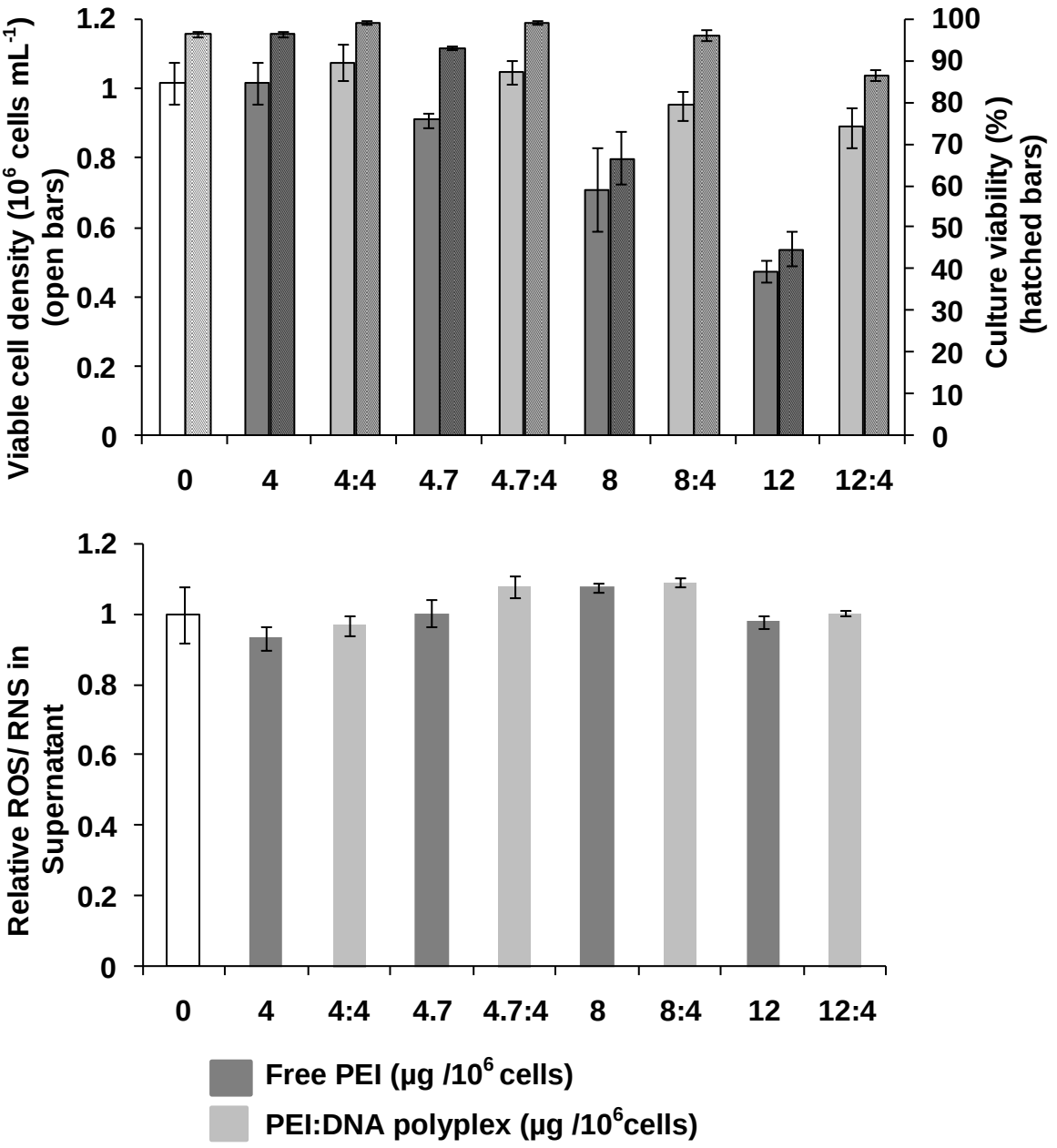

Figure 4.9 Cytotoxicity of polyethylenimine and PEI:DNA polyplexes at hypothermic culture temperature.
The indicated amount of free PEI (dark grey bars) or PEI:DNA polyplexes (light grey bars) were added to cells, and following incubation at 4°C for 4 h, the viable cell density (open bars) and culture % viability (hatched bars) measured (**A**). Total reactive oxygen species or reactive nitrogen species level in the culture supernatant was also measured (**B**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

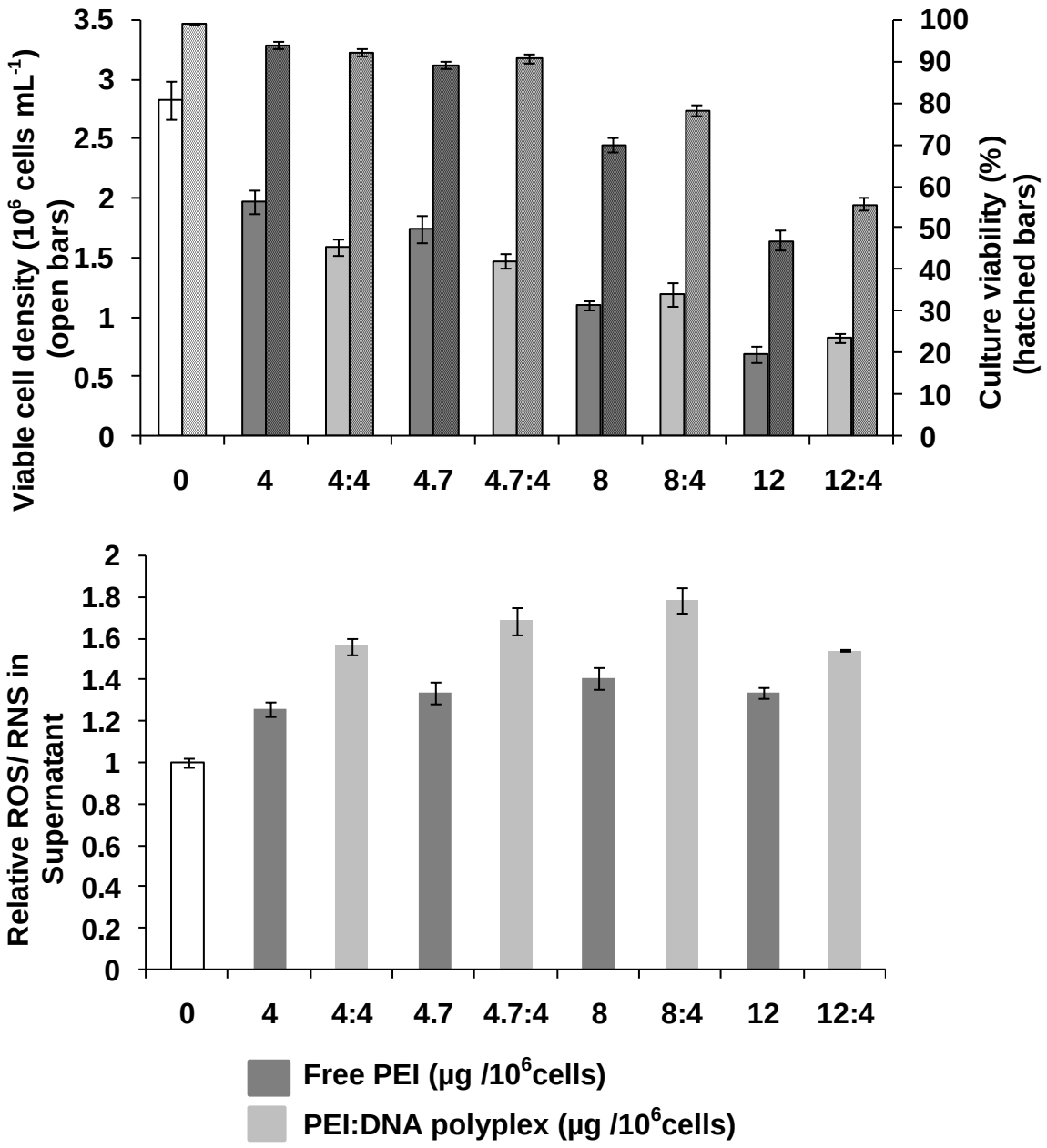

Figure 4.10 Cytotoxicity of polyethylenimine and PEI:DNA polyplexes at physiological culture conditions.
The indicated amount of free PEI (dark grey bars) or PEI:DNA polyplexes (light grey bars) were added to cells, and following incubation at 37°C for 24 h, the viable cell density (open bars) and culture % viability (hatched bars) measured (**A**). Total reactive oxygen species or reactive nitrogen species level in the culture supernatant was also measured (**B**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

At physiological incubation temperature (37°C, 5% CO_2, with humidification), addition of PEI:DNA polyplexes significantly reduced viable cell density (Figure 4.10A). At 24 h post transfection, using standard conditions (protocol A), viable cell density was $1.5x10^6$ cells mL^{-1} relative to $2.8x10^6$ cells mL^{-1} for un-transfected cells. Higher PEI:DNA ratios lead to a further decrease in viable cell density at 24 h post transfection. At a PEI:DNA ratio of 2 (8:4 µg 10^6 $cells^{-1}$), viable cell density was $1.2x10^6$ cells mL^{-1} and at a PEI:DNA ratio of 3 (w/w) (12:4 µg 10^6 $cells^{-1}$) viable cell density was $0.8x10^6$ cells mL^{-1}. Culture percentage viability reduced from 99% in un-transfected conditions, to 91, 78 and 56% at 24 h post transfection at PEI:DNA ratios of 1.2 (protocol A), 2 and 3 respectively.

Viable cell density and culture viability was also reduced by addition of free PEI (Figure 4.10A). With increasing concentrations of PEI culture percentage viability decreased. Culture percentage viability decreased as free PEI concentration increased, falling to 47% at a concentration of 12 µg 10^6 $cells^{-1}$ relative to 99% in control conditions. Similarly viable cell density decreased with increasing concentrations of free PEI, falling to $0.7x10^6$ cells mL^{-1} at a concentration of 12 µg 10^6 $cells^{-1}$ free PEI relative to $2.8 x10^6$ cells mL^{-1} in control conditions.

There were significant differences between the viable cell density following addition of free PEI and an equivalent amount of PEI complexed with plasmid DNA, as shown in Table 4.1. For example, viable cell density following addition of 4 µg 10^6 $cells^{-1}$ free PEI was $2x10^6$ cells mL^{-1}, significantly higher than following addition of 4 µg 10^6 $cells^{-1}$ PEI complexed with plasmid DNA, which was $1.6x10^6$ cellsmL^{-1}. Addition of 4.7 µg 10^6 $cells^{-1}$ free PEI resulted in a viable cell density of $1.7x10^6$ cells $mL^{-1,}$ whereas the equivalent PEI:DNA polyplex resulted in a viable cell density of $1.5x10^6$ cells mL^{-1}. At a concentration of 8 µg 10^6 $cells^{-1}$, there was no significant difference between the viable cell density following addition of free PEI or the equivalent PEI:DNA polyplex. At a concentration of 12 µg 10^6 $cells^{-1}$, opposite to the pattern observed at lower concentrations, viable cell density was significantly lower following addition of free PEI than PEI:DNA polyplexes, with viable cell densities of 0.7 and $0.8x10^6$ cells mL^{-1}, respectively. At higher PEI concentrations, culture percentage

viability was lower following addition of equivalent amounts of free PEI. At concentrations of 8 and 12 µg 10^6 cells^{-1} free PEI, culture percentage viability was 70% and 47% following addition of free PEI, compared to 78% and 56% following addition of PEI:DNA polyplexes, respectively.

Reactive oxygen and nitrogen species were measured at 24 hours post incubation at 37°C (Figure 4.10B). ROS/ RNS levels, following addition of free PEI, were between approximately 25% and 40% higher relative to control levels (cells incubated without additional free or DNA-complexed PEI). Addition of PEI:DNA polyplexes to the culture, increased ROS/RNS to between approximately 50% and 80% above control levels. At each concentration of PEI tested, higher ROS/RNS levels were measured for cultures with added PEI:DNA polyplexes relative to the respective concentration of free PEI.

Table 4.1 Index of PEI:DNA polyplex toxicity relative to free PEI mediated cytotoxicity

	4°C 4 hr incubation		37°C 24 hr incubation	
X	VCD[1]	CPV[2]	VCD[1]	CPV[2]
1	106	103*	81*	98
1.175	115*	107*	84*	102
2	134*	144*	108	112*
3	187*	193*	120*	118*

1) $\frac{100 \cdot \mathrm{VCD}_{(xPEI:DNA)}}{\mathrm{VCD}_{(xPEI)}}$

2) $\frac{100 \cdot \mathrm{CPV}_{(xPEI:DNA)}}{\mathrm{CPV}_{(xPEI)}}$

Free PEI or PEI complexed with DNA (polyplexes) were added to CHO-S cells and incubated at 4°C for 4 h or 37°C for 24 h. Viable cell density and culture percentage viability were measured following the incubation period. Plasmid DNA concentration was kept constant at 4 µgmL^{-1} and thus PEI concentration is a multiple of 4, i.e. for x=2, PEI concentration = 8 µgmL^{-1}. An index value of >100 indicates that PEI is relatively more toxic in its free form (in decreasing VCD or CPV) than in its complexed form with DNA (as a polyplex).

***** Delta VCD_{xPEI} and $VCD_{xPEI:DNA}$ and delta CPV_{xPEI} and $CPV_{xPEI:DNA}$ significant to the 95% confidence level.

4.4 Discussion

4.4.1 DoE-RSM optimization

Response surface models for the DoE optimization of PEI mediated transfection, with respect to DNA amount and PEI:DNA ratio were presented in Figure 4.2. For industrial TGE, utilizing PEI mediated transfection, numerous additional factors would be optimized, such as temperature shift, use of yield enhancing additives etc. (as detailed in Chapter 1). Furthermore, for maximum production, a cell line/ vector system enabling plasmid maintenance would be used. However, for the purpose of the research presented in this thesis, a simple, micro-scale, optimized method was required for transfection of CHO-S cells and subsequent TGE, with high post-transfection culture viabilities.

We have previously used DoE to optimize transfection conditions in three variant CHO cell lines (Thompson *et al.*, 2012) and CHO-S cells. For CHO-S cells, the DOE-RSM predicted optimum was 9 mg L^{-1} DNA and 16.3 mg L^{-1} PEI, however this was accompanied by a cell density of $2.5x10^6$ cells mL^{-1}. A wide range of optimized PEI and DNA amounts have been reported within the literature, using a range of media types, additives, cell lines, vectors etc. Thus, whilst reported transfection optima can be used to build a design space for transfection optimization, optimization, in most cases, cannot be avoided

Work within the group has explored the heritability of TGE across a panel of 199 CHO-K1Sv clones (Davies *et al.*, 2012). For the majority of clones, transient mAb production was not heritable (Davies *et al.*, 2012). However, clones were isolated which displayed heritable traits with respect to transient mAb production. In this chapter, transient SEAP production by parental CHO-S cells, following PEI mediated transfection, was compared at culture points approximately 100 generations apart (20 passages) (Figure 4.4). There was found to be no significant difference in SEAP production at these two points, and thus the parental CHO-S cell line, used throughout this thesis, was found to display heritability in TGE. Interestingly, in the Davies *et al.* study, clones that displayed heritable traits in transient mAb production, did so close to the parental average, and a parental cell line was used in the work presented here.

4.4.2 Transfection Kinetics

An understanding of transfection kinetics, with respect to polyplex-cell association and cyto-internalization, is relevant to industrial TGE platforms as follows. Typical industrial TGE platforms employ a biphasic strategy, separating transfection and production. Data describing transfection kinetics, enables knowledge based decision making regarding the cut-off point for the biphasic production process, i.e. when to shift to mild-hypothermic culture temperatures (Galbraith *et al.*, 2006).

Previously, polyplex-cell surface binding and uptake was measured by Godbey *et al.* (1999c), using confocal microscopy, who reported fluorescent polyplexes attached to the cell surface to appear at 30 min post transfection. Data presented in this chapter has expanded on this knowledge. The data presented in Figure 4.5 and Figure 4.7 show polyplex-cell association and cyto-internalization to be a biphasic, process. Within the first ~15 min post transfection, the rate of cyto-association and cyto-internalization of polyplex was significantly higher than at later time points, indicating a relatively fast phase of binding/ uptake followed by a slower phase. The biphasic model for polyplex uptake is described in Chapter 9.

Polyplex-cell association and uptake saturated at approximately 240 min post transfection (Figure 4.5). It is possible that by this point, polyplexes had aggregated (Sharma *et al.*, 2005) to a degree, whereby they were too large to be endocytosed. The presence of cell surface-attached polyplex throughout the measured period is indicative of a trafficking process in which endosomal recycling is a bottleneck for gene delivery (Nichols *et al.*, 2001; Maxfield and McGraw, 2004).

The fast polyplex cyto-internalization kinetics in the first 15 min post transfection, are consistent with time-scales reported for endocytosis (Fujimoto *et al.*, 2000). Slower internalization of polyplex is well described as bulk, non-specific uptake (Simons and Gerl, 2010).

4.4.3 Cytotoxicity of free PEI and PEI mediated transfection

At hypothermic culture conditions, free PEI displayed significant cyto-toxicity, especially at higher concentrations (Figure 4.9A). At hypothermic conditions, reduction in cell viability is more likely to be attributed to necrosis than apoptosis. It is unlikely that PEI would be trafficked into the cell at hypothermic culture temperatures. Thus, the data is consistent with the early, “necrotic like changes at the plasma membrane” reported by Moghimi *et al.* (2005). Moreover, at hypothermic culture temperatures, toxicity could be attributed to physical damage of the plasma membrane.

Interestingly, the same concentrations of PEI, when complexed with plasmid DNA, lead to much lower reductions in cell viabilities (less toxicity) (at 4°C) (Figure 4.9A, Table 4.1). When complexed with DNA, PEI has a different physical entity (explored in Chapter 5). At hypothermic culture temperatures, it is unlikely that PEI:DNA polyplexes would be internalized by the cells, through an endocytic mechanism. Thus, the data indicate that PEI:DNA polyplexes, in CD CHO media at 4°C, confer relatively low level physical toxicity at the plasma membrane (Frohlich, 2012), whereas, in its free form, PEI causes significant physical damage to the cell. The increase in toxicity at higher amounts and PEI:DNA ratios could be attributed to free, uncomplexed PEI or the positive charge of the polyplexes at higher PEI:DNA ratios (Boeckle *et al.*, 2004; Frohlich *et al.*, 2012). At hypothermic culture temperatures, no ROS in the supernatant could be measured (Figure 4.9B). This is indicative of mitochondrially mediated ROS (Murphy, 2009), following internalization of PEI or PEI:DNA polyplexes, which was inhibited by hypothermic culture conditions.

At physiological culture conditions, reduction in cell growth, following addition of PEI:DNA polyplexes (transfection) could be attributed to the stress on the cell caused by transgene expression (Figure 4.10A). Toxicity could be caused by the PEI:DNA polyplex particles, or by free PEI following dissociation from DNA, intracellularly. At higher concentrations of PEI (8 and 12 µg mL^{-1}) free PEI was significantly more toxic to cells than PEI:DNA polyplexes at hypothermic culture conditions than at physiological culture conditions (Table 4.1); it is possible that at physiological conditions, PEI had dissociated from PEI:DNA polyplexes,

intracellularly, thus PEI:DNA polyplexes provided significant free PEI and free PEI mediated toxicity.

At physiological culture conditions, at optimal PEI:DNA ratio/ PEI amount (4 and 4.7 µg mL^{-1} PEI), PEI:DNA polyplexes reduced cell growth relatively more than respective quantities of free PEI (Table 4.1); gene expression caused cell growth inhibition more than the undesirable 'side effect' of toxicity from the transfection vehicle itself. However, at high, sub-optimal, PEI:DNA ratios/ PEI amount (12 µg mL^{-1}), free PEI lead to higher toxicity than PEI:DNA polyplexes (Figure 4.9A, Table 4.1). The data indicate that the sweet spot for PEI:DNA ratio and PEI amount lie where PEI:DNA polyplexes and gene expression are relatively less toxic to cells than the transfection vehicle itself, PEI.

At 37°C, free PEI and PEI:DNA polyplexes lead to ROS production (Figure 4.10B). However, at all concentrations tested, ROS levels were higher following addition of PEI:DNA polyplexes, rather than free PEI. It could be that gene expression lead to enhanced levels of ROS production. Alternatively, the physical properties of PEI:DNA polyplexes, within the cytosolic environment, lead to mitochondrial stress and ROS generation (Grandinetti *et al.*, 2011; Lee *et al.*, 2013).

The data presented here, combined with that presented by other authors, (Lee *et al.*, 2013; Calarco *et al.*, 2013, Grandinetti *et al.*, 2011, Moghimi *et al.*, 2005), provides a basis for further work regarding oxidative stress caused by PEI mediated transfection. Anti-oxidant supplements, such as ascorbic acid, uric acid, tocopherols, tocotrienols or small molecule iron chelators (Yun *et al.*, 2003) could be added to cultures to reduce intracellular oxidative damage. A DoE-RSM approach could be applied to the relative quantities of anti-oxidants added to culture. Alternatively, expression vectors containing superoxide dismutase or catalase genes could be co-transfected into the cells.

4.5 Chapter Conclusions

Data presented in this chapter show that polyplex binding to the cell surface and uptake follows a biphasic, saturable kinetic pathway. Approximately 25% of total polyplex was internalized within the first ~15 min post transfection, via a rapid uptake pathway, and the remaining 75% of polyplex internalized between ~15 and 240 min post transfection, via a slower internalization mechanism. Over a period of 480 min post transfection, polyplex was found localized to the cell surface, showing the cell surface to be a bottleneck for transgene delivery.

Experiments conducted at hypothermic culture temperatures highlighted the different mechanisms of toxicity attributed to free PEI as opposed to PEI complexed with DNA (PEI:DNA polyplexes). Free PEI was found to mediate significant physical damage to cells at higher concentrations (≥8 μg mL^{-1}). At physiological culture conditions, PEI and PEI:DNA polyplexes mediated significant levels of ROS/RNS within culture supernatant; these data indicates that measures to ameliorate levels of cellular oxidative stress may improve the TGE process.

CHAPTER 5

Bio-Physical Interactions Governing Polyplex Binding to the Cell Surface

Chapter Overview

Biophysical interactions surrounding the PEI mediated transfection process, such as those governing polyplex-cell surface binding, are explored in this chapter. The mechanism behind the inhibitory effect of ferric (III) citrate on PEI mediated TGE is investigated. The aims of this chapter are to:

- Investigate the effect of PEI:DNA polyplex ratio on polyplex cell surface binding.
- Investigate the effect of PEI:DNA ratio and culture media on the zeta potential of PEI:DNA polyplexes.
- Test the hypothesis that hydrophobic interactions contribute to polyplex binding to the cell surface.
- Explore the inhibitory effect of media additives, ferric (III) citrate and anti-clumping agent on PEI mediated transfection.

5.1 Introduction

5.1.1 Polyplex surface properties

Given that it is the surface of the PEI:DNA polyplex that interacts with the surface of the cell, its properties are fundamental for transfection and endocytosis (Adler and Leong, 2010). The zeta potential of polyplexes has been measured previously by several groups (Godbey *et al.*, 1999d; Choosakoonkriang *et al.*, 2003; Ikonen *et al.*, 2008, Wu, 2009; Rajendra *et al.*, 2012; Fortier *et al.*, 2013). PEI:DNA polyplexes have been reported to have a positive zeta potential at N/P ratios used for transfection. At low NP ratios, N/P≤4 (Choosakoonkriang *et al.*, 2003) and N/P<1.5 (Ikonen *et al.*, 2008)

PEI:DNA polyplexes with a negative zeta potential have been reported. However, Pollard *et al.*, (1998) reported PEI:DNA polyplexes, at an N/P ratio of 3.5, to have a neutral zeta potential.

In the context of exploring the aggregation of polyplexes, Sharma *et al.*, (2005) studied the zeta potential of polyplexes. At an N/P ratio of 10, in a PBS 5% glucose solution, polyplexes were measured to have a zeta potential of +5.1 mV and were described as having patches of neutral, positively and negatively charged regions. Aggregation of polyplexes was found to occur through hydrophobic interactions and ameliorated by the use of non-ionic surfactants; whereby the hydrophobic tail of the non-ionic surfactant binds to the surfaces of the polyplexes and the hydrophilic head group of the non-ionic surfactant provides steric hindrance of polyplex-polyplex interactions (Sharma *et al.*, 2005). Polyplex aggregation was not characterized in this study and is discussed in more detail in section 9.5.

For particles with diameters between 1 nm and 1 µm (colloids), adsorbed ions determine the surface properties of the particle (Shaw, 1993). An electric double layer exists, consisting of ions directly adsorbed onto the surface of the particle (stern layer) and a second, consisting of ions loosely associated with the particle (ion diffuse layer) (Figure 5.10). The diffuse layer can move upon tangential stress, thus the zeta (ζ) potential is measured at the point which separates the particle-associated fluid from mobile fluid (the slipping/shear plane). Thus, whether the polyplex is used *in vitro* or *in vivo* transfection, immersion solution will determine the zeta potential of the polyplex.

The use of serum for *in vitro* cultivation of mammalian cells provides a growth environment with similarities to that *in vivo*. However, for biomanufacturing, as described in Chapter 1, serum is not used.

5.1.2 Polyplex surface properties, *in vitro*

Little work has focussed on the effect of culture medium on the zeta potential of PEI:DNA polyplexes. However, Wu (2009), reported that the zeta potential of PEI:DNA polyplexes was dramatically influenced by immersion in culture media. Polyplexes formed in 150 mM NaCl solution, DMEM or CHO-S-SFM II had a zeta potential of +30 mV, -20 mV and +2 mV, respectively. Following

subsequent dilution in CHO-S-SFM II culture media, however, the surface charge of the polyplexes, in all cases, was approximately -5 mV.

Petri-Fink *et al.*, (2008) measured the effect of medium and serum on the colloidal stability, cytotoxity and cyto-uptake of a range of polymer coated super-paramagnetic iron oxide nanoparticles (SPIONs), including PEI:DNA:SPIONs. Viability of HeLa cells following exposure to PEI:DNA:SPIONs was dramatically affected by culture media (RPMI, DMEM, with or without 10% foetal calf serum). In addition Ye *et al.*, (2009) reported dramatically different TGE following transfection of CHO cells with PEI, in a range of commercially available culture media. In both studies, it is difficult to distinguish between the superior properties of certain media types in supporting production post transfection as opposed to the transfection step itself.

5.1.3 Polyplex surface properties, *in vivo*

For *in vivo* applications, polyplexes must avoid opsonization and clearance by the immune system and thus, covalently coupled PEI and poly(ethylene glycol) (PEG) complexed with DNA (PEGylated PEI:DNA complexes) are widely used. PEGylation of polyplexes was found to provide resistance to binding of human or murine serum proteins, including IgM, fibronectin, fibrinogen, albumin and complement C3 (Ogris *et al.*, 1999). PEGylated transferrin PEI:DNA complexes displayed longevity in blood and plasma compared to non PEGylated transferrin PEI:DNA polyplexes. In addition, PEGylation was found to reduce the zeta potential of the polyplexes and reduce plasma mediated aggregation of polyplexes (Ogris *et al.*, 1999).

PEG shielding of the PEI:DNA polyplex positive charge was also reported by Merdan *et al.*, (2005), who reported, at an N/P ratio of 6, PEI:DNA polyplexes to have a zeta potential of +26 mV (±2 mV) and PEG-PEI:DNA polyplexes to have a zeta potential of 0.5 mV (±0.9 mV).

5.1.4 Bio-physical interactions governing polyplex cell-surface binding: the electrostatic theory

The electrostatic theory of polyplex binding to the cell surface is based on the following logic: that positively charged regions of polyplexes bind to anionic

heparan sulphate proteoglycans (Elouahabi and Ruysschaert, 2005; Biri *et al.*, 2007; Tros de Ilarduya *et al.*, 2010). There is evidence within the literature, that depletion of HSPGs is detrimental to PEI mediated transfection and TGE (discussed in Chapter 6). The theory of polyplex cell surface binding is wide-spread and a generally accepted paradigm.

5.1.5 Implications for bioprocessing: inhibitory media components of PEI mediated TGE

Iron is an essential nutrient, required for basic cellular function. Under normal physiology and in the majority of cases, it is delivered to cells via the plasma protein iron carrier, transferrin (Anderson and Vulpe, 2009). For biomanufacturing, given the requirement for chemically defined culture medium, free of animal-derived products, small molecule iron delivery systems are used (Bai *et al.*, 2010). Small molecule iron carriers, such as selenite tropolone (2-hydroxy-2, 4, 6-cycloheptarin-1-one), ferric ammonium citrate and ferric citrate are frequently used in biomanufacturing (Zhang *et al.*, 2006; Bai *et al.*, 2010). In fact, culture supplementation with iron and citrate, when added in concert, has been found to significantly enhance recombinant mAb production (Bai *et al.*, 2010).

However, ferric (III) citrate is known to inhibit TGE following transfection mediated by PEI (Eberhardy *et al.*, 2009). Culture supplementation with EDTA or serum was found to have a protective effect against the reduced titres reported in the presence of ferric citrate, explainable by the higher affinity that both EDTA and transferrin have for iron, relative to citrate. Ferric (III) citrate was found to inhibit TGE at an early stage in the transfection process and its mechanism of inhibition was hypothesized to be at the stage of PEI:DNA polyplex formation, polyplex binding to the cell surface or intracellular trafficking (endosomal release or nuclear entry) (Eberhardy *et al.*, 2009).

5.2 Materials and Methods

5.2.1 Chemical treatments prior to transfection

Ferric (III) citrate, polyoxyethylene40 stearate (POES 40) and Pluronic F-68, were purchased from Sigma-Aldrich (Dorset, UK) and Anti-Clumping Agent from Life Technologies (Paisley, UK). Ferric (III) citrate were dissolved in de-ionised water and polyoxyethylene (40) was dissolved in PBS, before 0.22 µm filter sterilization. Pluronic F68 was dissolved in CD CHO medium. For ferric (III) citrate, POES40, final concentrations were obtained by diluting 1:100 to the cells in medium. An equal volume of the reconstitution solution was added to control wells. At 1 or 2 h prior to transfection and at 4°C or 37°C, as indicated, chemicals were added to cells in 24 well plates.

5.2.2 Micro-beads

Mono-disperse micro-beads based on polystyrene, in aqueous suspension, of diameter 15 µm, were purchased from Sigma-Aldrich. Micro-beads ($5x10^5$) in a total volume of 500 µL in a 24 multi well plate were immersed in CD CHO medium supplemented with 8 mM L-glutamine or phosphate buffered saline solution. Fluorescently labelled PEI:DNA polyplexes were formed according to protocol A, added to micro-beads in suspension and incubated for 4 h at rt. Microbeads were washed with either CD CHO or PBS, prior to flow cytometric analysis.

5.3 Results

5.3.1 Effect of PEI:DNA ratio on polyplex-cell surface binding

It was hypothesized that PEI:DNA ratio would affect the binding to the cell surface and the zeta potential of polyplexes in CD CHO medium. PEI:DNA ratio was altered, by changing PEI concentration and keeping the DNA concentration constant. At a PEI:DNA ratio of 2 and 3, polyplex-cell surface binding was 8% and 20% above binding using standard PEI:DNA ratios (protocol A) (Figure 5.1A and B). However, the percentage of intact cells (in gate 1) reduced from 92% in standard conditions, to 84% and 66% of cells at PEI:DNA ratios of 2 and 3 respectively (Figure 5.1 C, E and E).

5.3.2 Effect of immersion solution and PEI:DNA ratio on polyplex zeta-potential

It was hypothesized that immersion solution (CD CHO medium or deionized water) would affect the zeta-potential of PEI:DNA polyplexes, in addition to the PEI:DNA ratio. Across a range of PEI:DNA ratios, the zeta potential of the polyplexes was approximately +40 mV in deionized water, with no significant difference in the zeta potential between polyplexes formed at different PEI:DNA ratios (Fig 5.2), similar to data reported by Godbey *et al.*, (1999d) and Rajendra *et al.*, (2012). In CD CHO medium, PEI:DNA polyplexes had a dramatically different zeta potential compared to the zeta potential in deionized water. At PEI:DNA ratios of 1, 1.2, 2 and 3, the average zeta potential was -0.7, 0.7, 3.4 and 11.5 respectively (Figure 5.2). Thus, at DoE-RSM optimized PEI:DNA ratio (section 4.3.1), the polyplex zeta-potential in CD CHO medium was approximately neutral.

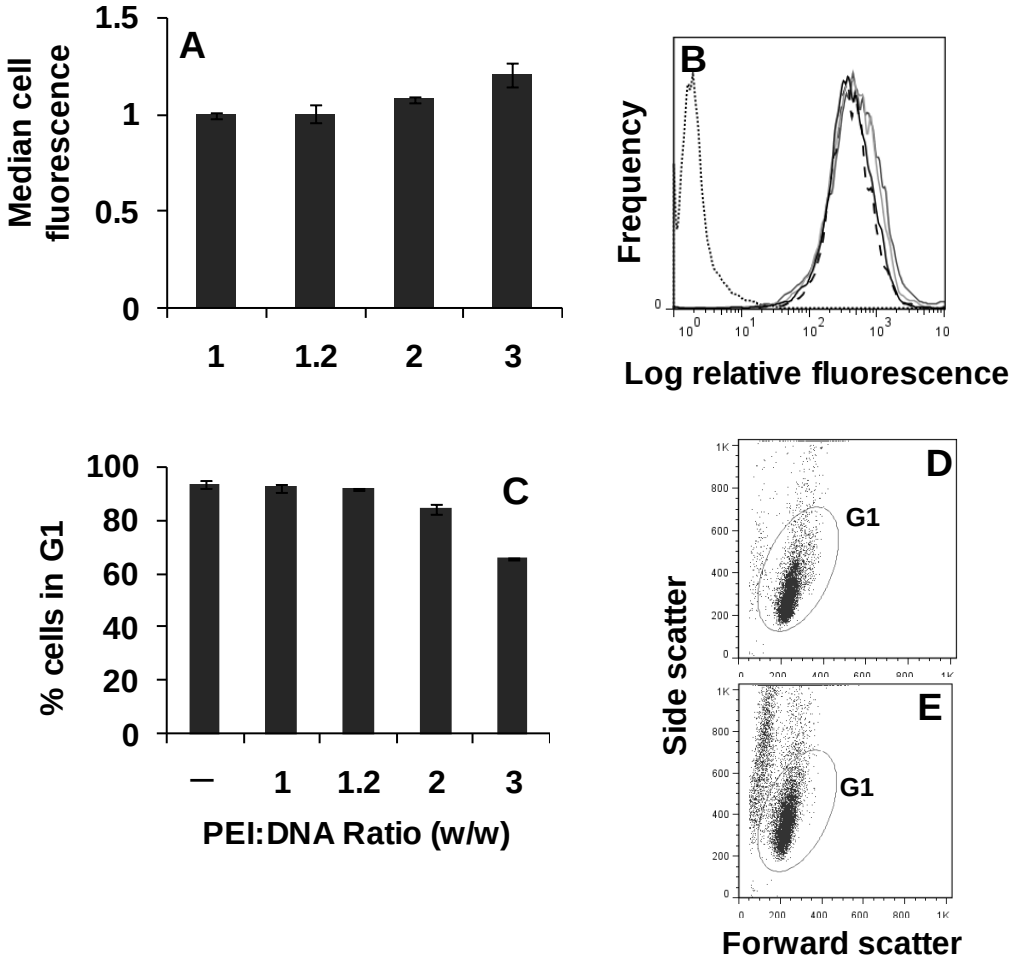

Figure 5.1
PEI:DNA polyplex cell-surface binding at varying PEI:DNA ratios
PEI:DNA polyplexes were formed at the indicated PEI:DNA ratios. The quantity of plasmid DNA was kept constant, varying only PEI quantity. Polyplex-cell surface binding (**A** and **B**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. (—) indicates control cells, without added polyplex.
Cytotoxicity at different PEI:DNA ratios. The percentage of intact cells, following transfection with polyplexes, at the indicated PEI:DNA ratios, and incubation at 4°C for 4 h (**C**). Side scatter/ forward scatter plots for cells treated with polyplexes at a 1.2 PEI:DNA ratio **(D**) and a 3 PEI:DNA ratio (**E**). The mean value ± standard deviation from triplicate cultures is shown.

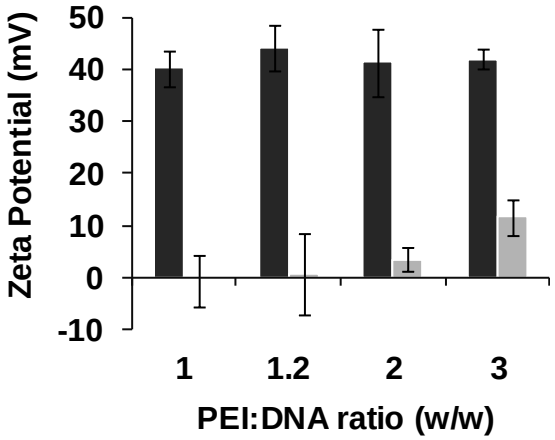

Figure 5.2 Zeta potential of polyplexes, at different PEI:DNA ratios, in deionised water and CD-CHO culture medium.
PEI:DNA polyplexes were formed at the indicated PEI:DNA ratios. The quantity of plasmid DNA was kept constant, varying only PEI quantity. PEI:DNA polyplexes were added to deionised water (black bars) or CD CHO medium (grey bars) and incubated for 5 min prior to analysis of electrophoretic mobility. The mean value ± standard deviation from five technical replicates, of 20 cycles each, is shown.

5.3.3 Effect of immersion solution on polyplex binding to micro-beads

The effect of immersion solution on binding of polyplexes to micro-beads was assessed. Mono-disperse polystyrene beads with a sulphate derivatized surface, 15 µm in diameter, were used due to being of a similar size to CHO-S cells and immersed in either CD CHO medium or PBS solution. Polyplex binding to microparticles was only observed when they were incubated in PBS and not CD CHO medium (Figure 5.3A and B). When incubated in PBS, polyplex-microparticle binding was measured for 38% of the microbeads, with a geometric mean fluorescence of 344, dramatically higher than basal fluorescence. Thus, immersion solution had a dramatic effect on binding of PEI:DNA polyplexes to micro-beads.

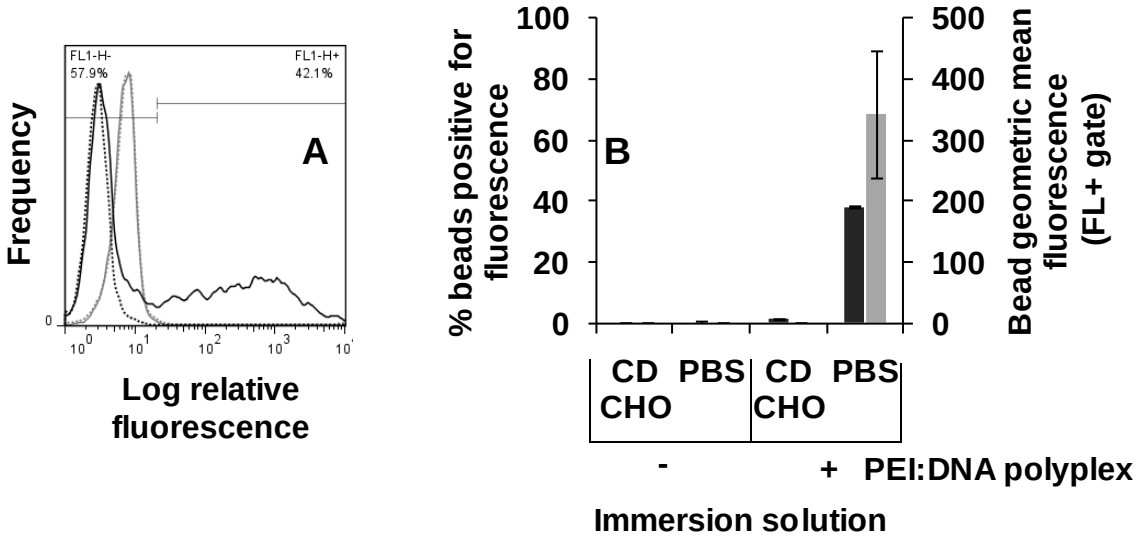

Figure 5.3
Effect of immersion solution on polyplex binding to micro-beads
Micro-beads, based on polystyrene, with a sulphate derivatized surface, were incubated in CD CHO or phosphate buffered saline solution (PBS) and incubated with FITC labelled PEI:DNA polyplexes (formed according to protocol A) for 4 h at room temperature, prior to flow cytometric analysis. **A:** flow cytometric analysis of PEI:DNA polyplex-micro-bead binding; black lines: micro-beads immersed in PBS; grey lines: micro-beads immersed in CD CHO; dotted lines: micro-bead auto-fluorescence (no FITC labelled polyplex added); solid lines: FITC labelled polyplexes added to micro-bead suspension. **B:** % of beads in gate FL1-H+ (black bars) and median geometric fluorescence (grey bars). The mean value ± standard deviation from triplicate samples of beads is shown.

5.3.4 Inhibition of polyplex-cell surface hydrophobic interactions using non-ionic surfactant

The hypothesis was tested that hydrophobic interactions contribute to polyplex binding to the cell surface. In order to inhibit hydrophobic interactions between polyplexes and the cell surface, the non-ionic surfactant, polyoxyethylene 40 stearate (POES40), was added to the culture, as previously used to inhibit polyplex-polyplex hydrophobic interactions (Sharma *et al.*, 2005).

Addition of POES40 reduced SEAP activity (Figure 5.4A) at 24 h post transfection to 27% and polyplex binding to the cell surface (Figure 5.4D and E) to 11% of control levels, at a concentration of 0.1% w/v POES40. At 24 h post transfection, viable cell density and culture percentage viability reduced somewhat, from $1.4x10^6$ cells mL^{-1} and 88% in control conditions to $1.1x10^6$ cells mL^{-1} and 80% at 0.1% (w/v) POES40, respectively (Figure 5.4B).

For untransfected cells (Figure 5.4C), at 24 h post sub-culture, viable cell density decreased to $1.7x10^6$ cells mL^{-1} at 0.1% (w/v) POES40 relative to $2.9x10^6$ cells mL^{-1} in control conditions. The presence of POES40 reduced culture percentage viability incrementally in untransfected cells.

The inhibitory effect of the non-ionic surfactant, POES40 on PEI mediated tranfection of CHO cells, is similar to that reported by Thompson, 2011.

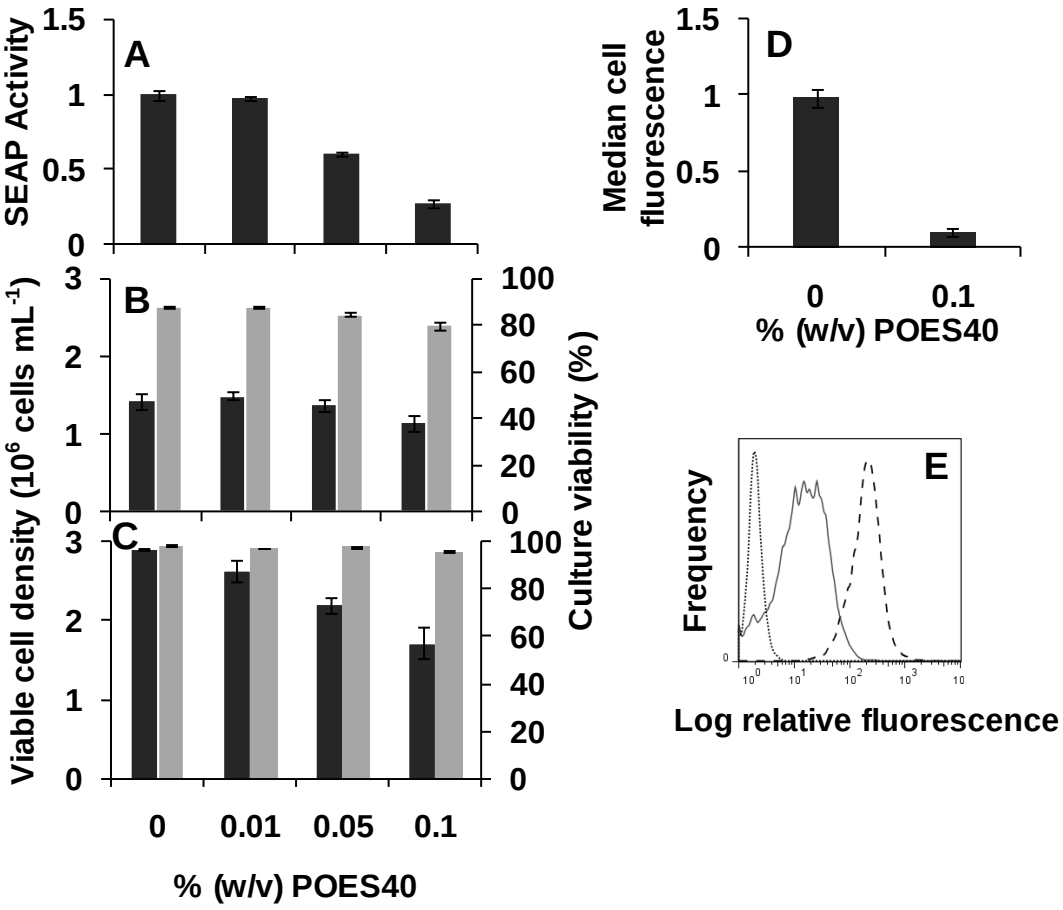

Figure 5.4 Non-ionic surfactant, polyoxyethylene (40) stearate (POES40), reduces polyplex-cell surface binding and SEAP production.
One hour prior to transfection with PEI, the indicated concentration of POES40 was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and cell culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). Polyplex-cell surface binding (**D** and **E**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection and measuring cellular fluorescence using flow cytometry. **E**: black dashed line: 0% POES40, grey line: 0.1 % POES40, dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or cultures is shown.

5.3.5 Effect of Pluronic F68 on PEI mediated TGE

Box 5.1 Pluronic F68 and bioprocessing

Cell surface hydrophobicity leads to cell clumping and cell-bubble interactions during bioreactor sparging, both detrimental to biomanufacturing processes (Ghebeh *et al.*, 2002).

To alleviate the effect of sheer stress on cells in bioreactors, caused by sparging and stirring, non ionic polymers, such as Pluronic F-68, are frequently added (Tharmalingam *et al.*, 2008). Pluronic is a tri-block copolymer based on hydrophilic ethylene oxide and hydrophobic propylene oxide (Batrakova and Kabanov, 2008; Hillmyer *et al.*, 1996). Pluronic F-68 (and other similar molecules) are thought to alleviated sheer stress by 1) stabilizing the foam layer on the surface of sparged cultures and/or 2) increasing the resistance of cells to sheer stress through physical incorporation into the plasma membrane (Gigout *et al.*, 2008; Clincke *et al.*, 2011).

Given that the non-ionic surfactant, POES40, was found to inhibit polyplex binding to the cell surface and SEAP levels, the effect of Pluronic F-68 on TGE following PEI mediated transfection was tested, due to its amphiphilic structure.

At concentrations of approximately ten-fold higher than would be used in bioreactors, at 1%, 2% and 4% w/v, a reduction in SEAP activity was observed (Figure 5.5A). At 4% w/v Pluronic F68, SEAP activity reduced to 74% of control levels. In transfected and untransfected conditions, Pluronic F68 had an incremental effect on viable cell density and culture percentage viability at 24 h post transfection or sub-culture. At 24 hours post transfection, at 4% w/v Pluronic F-68, viable cell density reduced to $1.3x10^6$ cells mL^{-1} compared to $1.5x10^6$ cells mL^{-1} without Pluronic and culture percentage viability was 79% compared to 89% without Pluronic (Figure 5.5B).

In untransfected conditions, at 24 h post sub-culture, at 4% w/v Pluronic F-68, viable cell density reduced to $2.6x10^6$cells mL^{-1} compared to $3.3x10^6$ cells mL^{-1} in control conditions but Pluronic had little effect on culture percentage viability (Figure 5.5C).

It is possible that due to the higher molecular weight of Pluronic F68 compared to polyoxyethylene 40 stearate (8350 compared to 328.5, respectively), that a higher concentration of Pluronic F68 (w/v) compared to POES40 was required to inhibit polyplex binding to the cell surface and subsequent SEAP production. Alternately, the different chemical properties of the non-ionic surfactant and tri-block co-polymer (Box 5.1) may have different physico-chemical interactions with polyplexes and the cell surface.

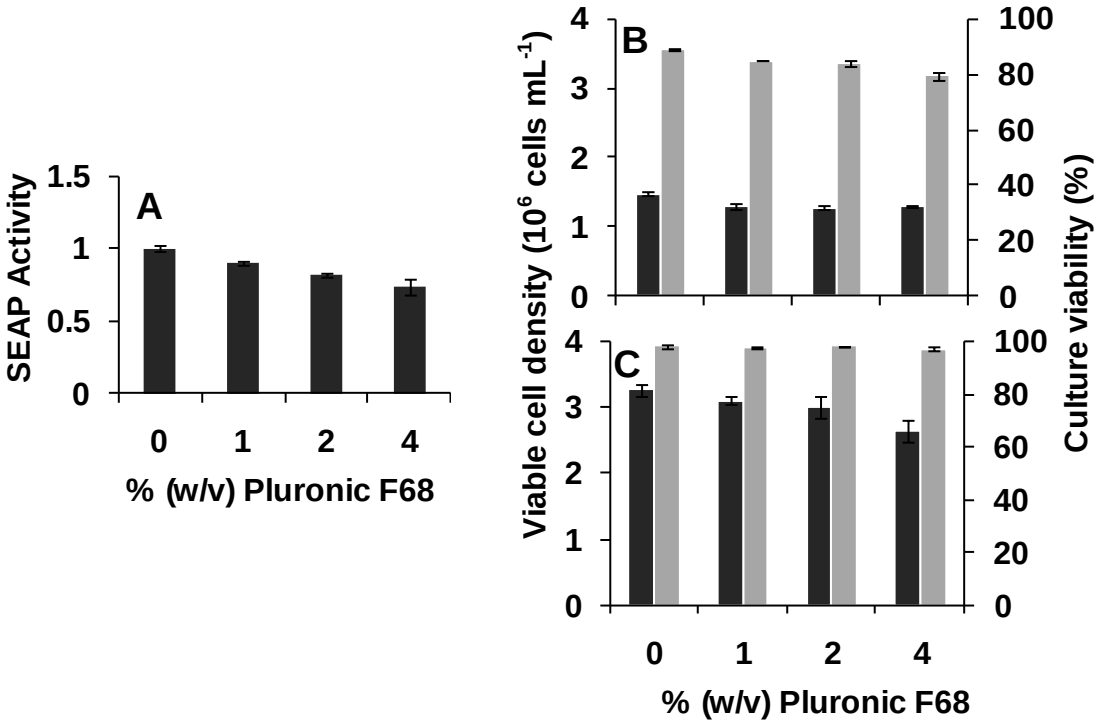

Figure 5.5 Effect of non-ionic block copolymer, Pluronic F68, on transient SEAP production

One hour prior to transfection with PEI, the indicated concentration of Pluronic F68 was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % percentage viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). The mean value ± standard deviation from duplicate transfections or cultures is shown.

5.3.6 On the mechanism of the inhibitory effect of media additives, ferric (III) citrate and anti-clumping agent, on PEI mediated TGE

Expanding on work presented by Eberhardy *et al.*, (2009), it was hypothesized that addition of ferric (III) citrate (FC) to media would reduce binding of polyplexes to the cell surface and alter the zeta potential of polyplexes in CD CHO medium. As expected, FC inhibited transient SEAP production in a concentration dependent manner, abrogating SEAP activity at a concentration of 50 µM (Figure 5.6A). At the same concentration (50 µM) of FC, polyplex-cell surface binding was reduced to 20% of control levels (Figure 5.6D and E).

At 24 h post transfection, viable cell density and culture percentage viability increased with increasing concentrations of FC (Figure 5.6B). At a concentration of 50 µM FC, viable cell density was $2.5x10^6$ cells mL^{-1} relative to $1.3x10^6$ cellsmL^{-1} following transfection in the absence of FC. Culture percentage viability was 98% at a concentration of 50 µM FC, relative to 87% in the absence of FC (Figure 5.6B).

In untransfected conditions, FC had no effect on culture percentage viability and slightly enhanced viable cell density (Figure 5.6C).

It was hypothesized that Gibco Anti-Clumping Agent (ACA), known to inhibit PEI mediated transfection, similarly to FC, would inhibit polyplex binding to the cell surface and effect the zeta potential of polyplexes in CD CHO medium. As expected, transient SEAP production was abrogated by the presence of ACA (Figure 5.7A). In addition, polyplex-cell surface binding decreased by >98% in the presence of 0.1% (v/v) ACA (Figure 5.7D and E). In the presence of ACA, viable cell density and culture percentage viability increased to $2.8x10^6$ cells mL^{-1} and 98% relative to $1.7x10^6$ viable cells mL^{-1} and 90.7% following transfection in the absence of ACA (Figure 5.7B). In untransfected conditions, ACA had no significant effect on viable cell density and culture percentage viability at 24 h post sub-culture (Figure 5.7C).

When FC and ACA were added to CD CHO, culture viabilities were equivalent to those in untransfected conditions. These data, combined with binding data, suggest that PEI:DNA interactions with the cell surface, were significantly minimized in the presence of FC and ACA.

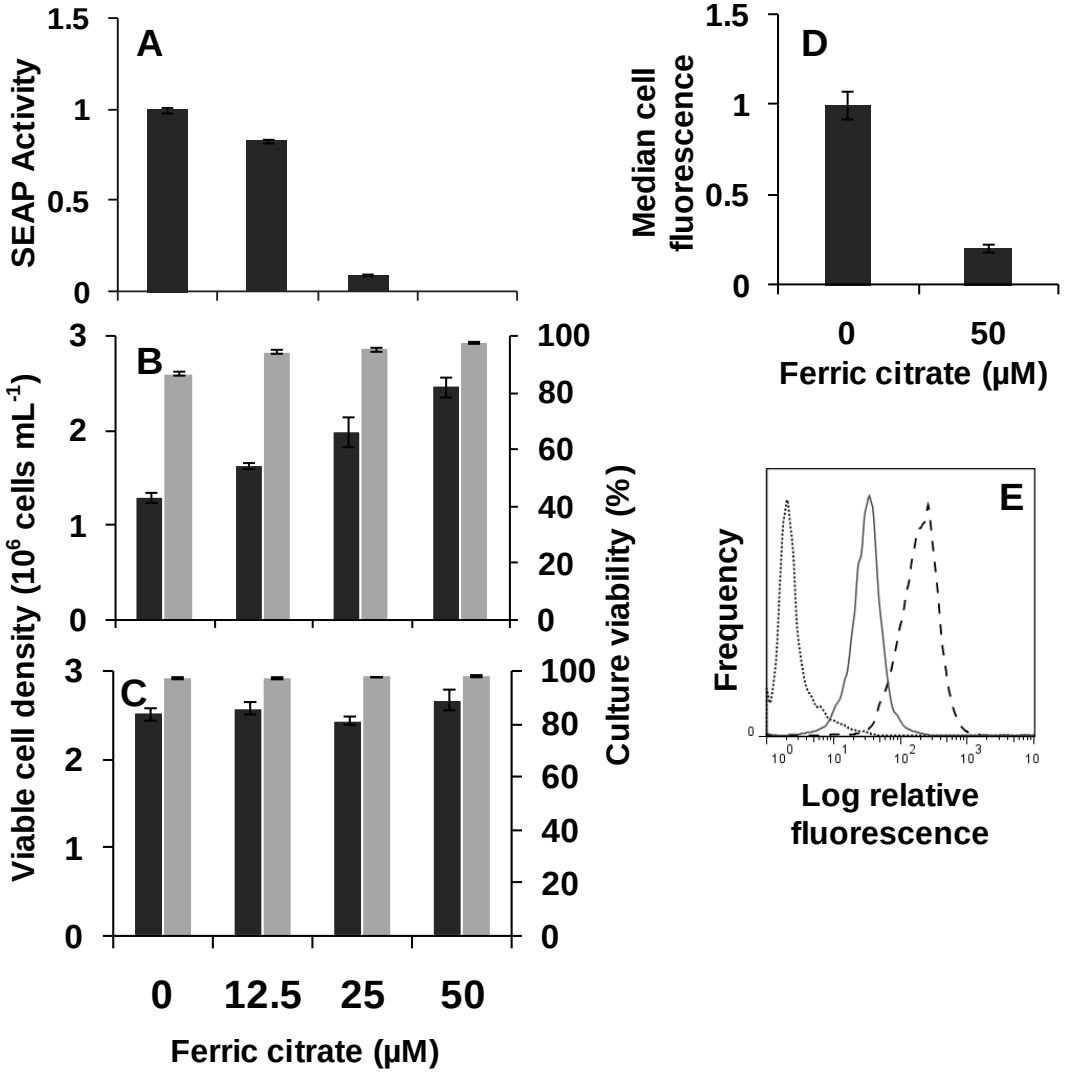

Figure 5.6 Ferric (III) citrate reduces polyplex-cell surface binding and transient SEAP production.

One hour prior to transfection with PEI, the indicated concentration of ferric (III) citrate was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). Polyplex-cell surface binding (**D** and **E**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. **E**: dashed line: 0 μM ferric citrate; solid line: 50 μM ferric citrate; dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or cultures is shown.

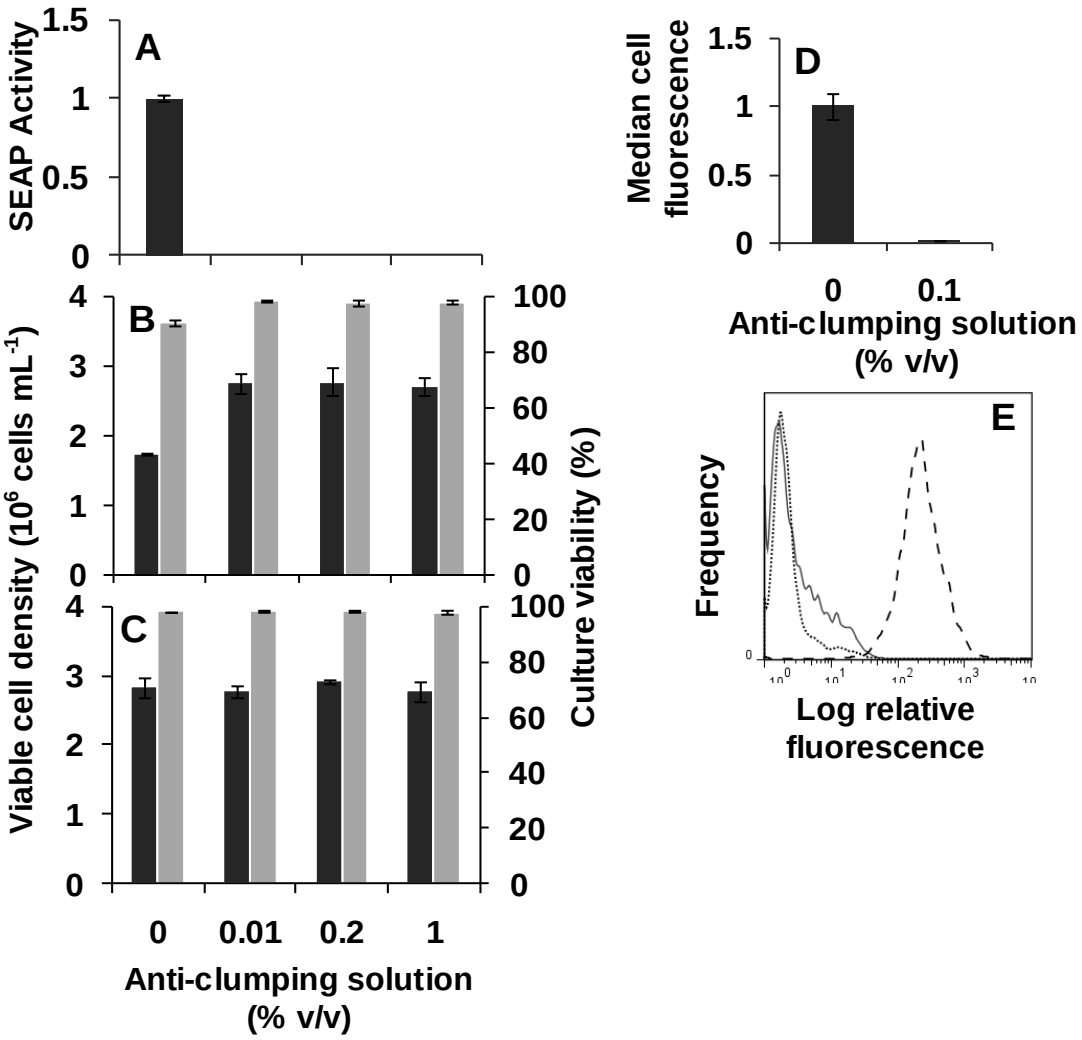

Figure 5.7 Anti-clumping agent reduces polyplex-cell surface binding and transient SEAP production.

One hour prior to transfection with PEI, the indicated concentration of anti-clumping agent was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). Polyplex-cell surface binding (**B** and **D**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. **E**: dashed line: 0 anti-clumping agent; solid line: 0.1 %(v/v) anti-clumping agent; dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or cultures is shown.

Expanding on data presented in Figure 5.2, immersion solution was found to have a dramatic effect on the zeta potential of polyplexes (formed at standard PEI:DNA ratio) (5.8). The zeta potential of polyplexes immersed in CD CHO and 50 µM ferric citrate or 0.1% (v/v) anti clumping agent were found to have a negative zeta potential, of -17 mV and -28 mV respectively.

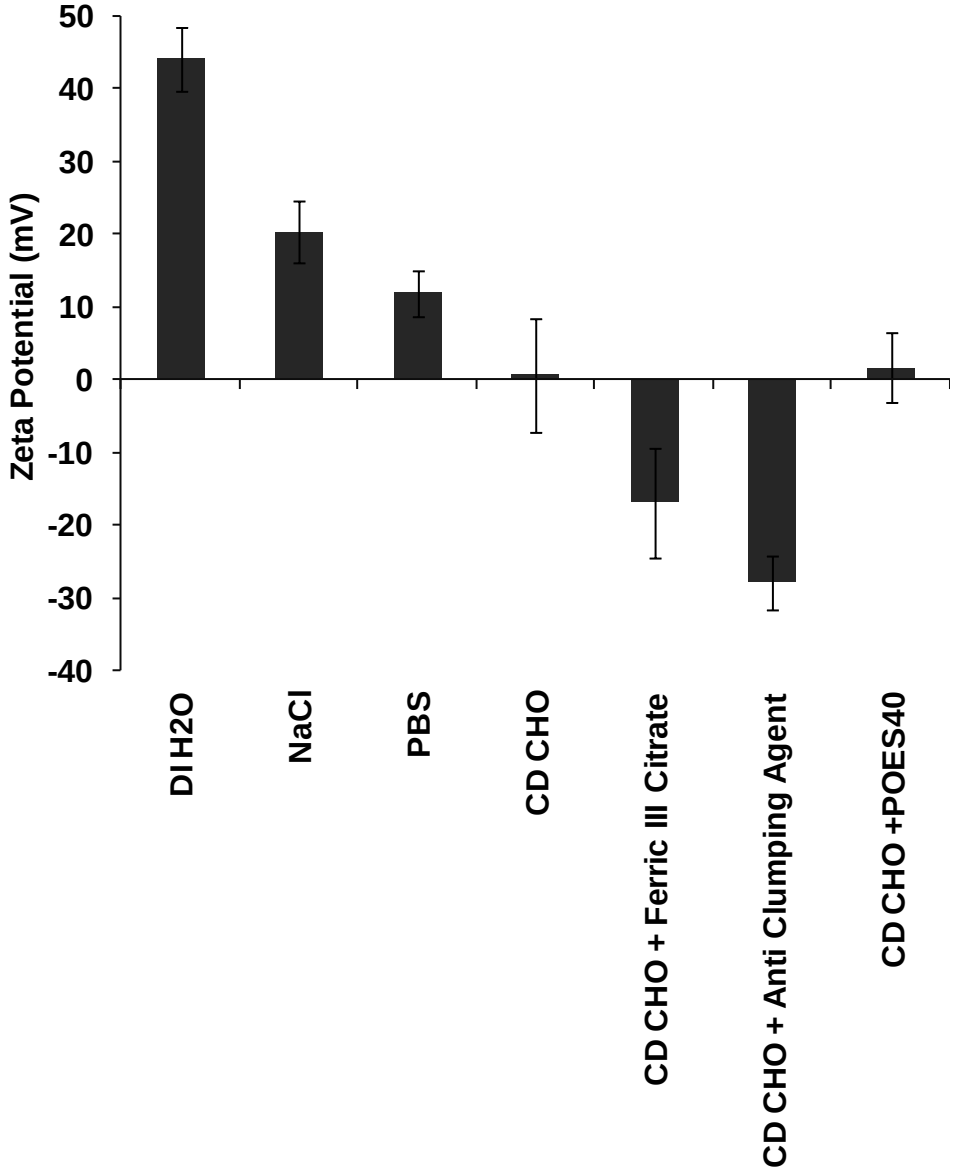

Figure 5.8 Immersion solution determines the cell surface charge of polyplexes

PEI:DNA polyplexes were formed, according to protocol A, and added to the indicated aqueous solutions and incubated for 5 min at rt prior to measurement. Polyplexes were immersed, from left to right, de-ionised water, 150mM sodium chloride solution, phosphate buffered saline solution, CD CHO medium, CD CHO medium supplemented with 50 µM ferric III citrate, CD CHO medium supplemented with 0.1% (v/v) anti-clumping agent and CD CHO medium supplemented with (0.1% w/v) POES 40. Electrophoretic mobility was measured on a ZetaPals. The mean value ± standard deviation from five technical replicates, of 20 cycles each, is shown.

5.3.7 The effect of PEI:DNA complex solution on TGE

It was hypothesized that the solution used to mix PEI and DNA could impact on the formation of polyplexes, with respect to condensation of DNA, and thus gene expression post transfection. However, none of the aqueous solutions tested, deionised water, 150 mM sodium chloride solution, phosphate buffered saline solution and CD-CHO medium, for PEI:DNA complexation, had any effect on subsequent TGE, at 24 or 48 h post transfection (Figure 5.9). Thus immersion solution affected the surface of polyplexes and interactions dependent on the polyplex surface, but not PEI:DNA polyplex formation.

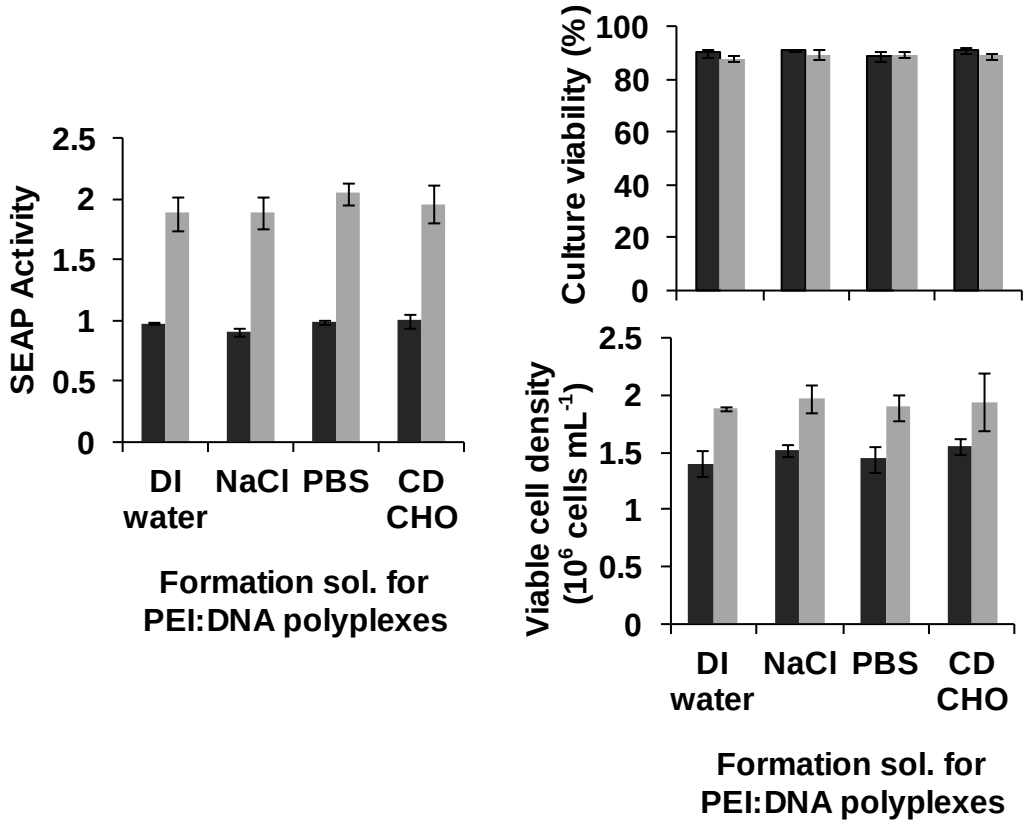

Figure 5.9 Effect of PEI:DNA complex solution on transient SEAP production and culture viabilities post transfection.
Polyethylenimine and plasmid DNA solutions, according to standard transfection conditions, were mixed in a total volume of 33.4ul made up with the indicated solutions (90% total volume), de-ionized water, 150mM sodium chloride solution, phosphate buffered saline solution or CD CHO medium. Samples were taken at 24 (black bars) and 48 (grey bars) h post transfection. SEAP (**A**), viable cell density (**B**) and culture percentage viability (**C**) were measured. The mean value ± standard deviation from triplicate transfections or cultures is shown.

5.4 Discussion

The mammalian cell surface or plasma membrane consists of a lipid bilayer, containing numerous lipid moieties (Van Meer, 2005) and interspersed membrane proteins. Glycerophospholipids of the plasma membrane are amphiphatic, possessing a hydrophilic head (phosphate and choline) and hydrophobic fatty acid tail (Cooper, 2000). The glycerophospholipids align, in an energetically favourable order, whereby the hydrophilic head group faces towards the cytosol and extracellular space and the fatty acid chains are protected within the leaflets. In addition, the extracellular leaflet possesses negatively charged molecules, for example sialic acid and sulphate groups, on membrane gangliosides and heparan sulphate proteoglycans, respectively (Byrne *et al.*, 2007; Taube *et al.*, 2009, Bishop *et al.*, 2007). Nonetheless, unlike the surrounding media, the surface of the cell is not an aqueous solution and to minimize disruption of hydrogen bonds between H_2O molecules, cells aggregate and particles bind to the cell surface through hydrophobic interactions, improving the entropy of the system (Chandler, 2005).

PEI:DNA polyplexes are positively charged entities (Choosokoonkriang *et al.*, 2003; Rajendra *et al.*, 2012). However, as reported by Sharma *et al.*, (2005) they possess charge-neutral regions (in addition to positively and negatively charged regions) and aggregate through hydrophobic interactions.

Given that polyplexes have a diameter in the 1-1000 nm range, they are classic colloids (Shaw, 1992), whose surface properties are determined by the adsorbed ions (Shaw, 1993; Petri-Fink *et al.*, 2008). When immersed in an ionic solution, it is relevant to consider the surface properties of the electrokinetic unit (Figure 5.10), rather than PEI:DNA polyplex, denuded of adsorbed ions, similar to the approach taken by Ogris *et al.* (1999) for studying the effect of serum on the PEI:DNA polyplex electrokinetic unit. At DoE-RSM optimized PEI:DNA ratio, in CD CHO medium, polyplexes were found to have a net charge of approximately zero, a result similar to one obtained by Wu (2009).

The net charge of the PEI:DNA polyplex electrokinetic unit (in CD CHO) was altered by increasing PEI:DNA ratio or using media additives (e.g. ferric (III) citrate) (Figure 5.2 and 5.8 respectively). A net positive charge (achieved

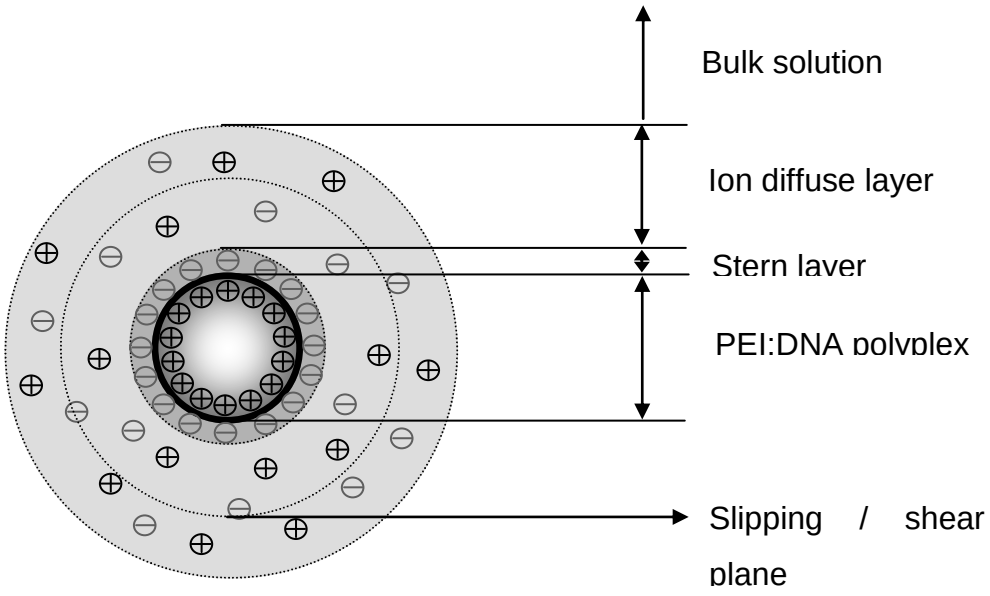

Figure 5.10
Schematic representation of the zeta potential of a PEI:DNA polyplex
The zeta-potential is measured at the slipping plane. Thus, the zeta potential and surface charge of the polyplex is dependent on the dispersion solution i.e. culture medium.

through increasing PEI:DNA ratio) was found to increase polyplex-cell surface binding (Figure 5.1A) and a net negative charge (achieved through supplementation with FC), was found to decrease polyplex-cell surface binding (Figure 5.6A). The non-ionic surfactant POES40 was used to physically inhibit polyplex-cell surface binding (Figure 5.4D and E); through binding of the hydrophobic tail to the cell surface and hydrophobic patches on the polyplex and the hydrophilic head group providing steric hindrance between polyplexes and the cell surface. (Incidentally, the effect of an ionic surfactant on polyplex-cell surface binding was not tested, because it was predicted to disrupt the PEI:DNA complex.) Taken together, it is likely that both electrostatic and hydrophobic interactions contribute to polyplex binding to the cell surface.

The involvement of hydrophobicity in polyplex binding to the cell surface, is consistent with the wide-spread reported efficacy of PEGylated PEI:DNA polyplexes for gene delivery *in vivo*. If polyplex binding to the cell surface was solely mediated by electrostatic interactions, it would follow that a decrease in zeta potential, as reported for PEGylated polyplexes (Ogris *et al.*, 1999; Merdan *et al.*, 2005), would substantially decrease transgene expression, by decreased

association of polyplexes with the cell surface. Despite the benefits of PEGylation by reducing polyplex clearance, if polyplex-binding to the cell surface was significantly inhibited, so too would transgene expression.

Furthermore, the influence of hydrophobicity in polyplex binding to the cell surface is in accordance with the reported efficacy of alkylated PEIs for transfection (Thomas and Klibanov, 2002; Fortune *et al.*, 2011). Another note worthy point, is that the inclusion of neutral lipids (e.g. cholesterol) in cationic lipid transfection formulations increased lipoplex binding to the cell surface (Crook *et al.,* 1998), indicating the role of hydrophobicity in transfection complex binding to the surface of mammalian cells.

The application of the work presented in this chapter, is that it is not necessary or even desirable for transfection to increase the zeta potential of the polyplex through increasing PEI:DNA ratio. Whilst electrostatic interactions are involved in polyplex-binding to the cell surface, they are not the sole force mediating the process, hydrophobia plays a role also. Polyplexes formed at higher PEI:DNA ratios were found to result in increased toxicity and reduced reporter protein titre (section 4.3.1) and whilst polyplex binding to the cell surface increased at higher PEI:DNA ratios (Figure 5.1A) it did so by only 20%. Thus, it seems that for optimal transfection, a sweet-spot exists between polyplex-cell surface binding and PEI / polyplex mediated toxicity to achieve maximum transgene expression.

The data indicate the importance of medium in transfection optimization and that transfection optimization, in addition to being cell-line specific, is possibly also medium-type specific. The data presented in this chapter also offer a facile method for predicting the effect of medium additives on transfection and of screening additives prior to bioreactor production. For example, a culture additive that confers a negative charge on the polyplex, such as ferric (III) citrate, is likely to be detrimental, or indeed abrogate, PEI mediated TGE.

5.5 Chapter Conclusions

In this chapter data is presented supporting the hypothesis that hydrophobic, in addition to electrostatic interactions, contribute to polyplex binding to the cell surface. Data is presented demonstrating the fundamental role of culture medium in the properties of the PEI:DNA polyplex electro-kinetic unit. Data elucidating the inhibitory effect of ferric (III) citrate on PEI mediated transfection is also presented; showing that ferric (III) citrate, not only dramatically reduces polyplex binding to the cell surface, but also confers a negative charge on the electrokinetic unit of the PEI:DNA polyplex in CD CHO medium.

CHAPTER 6

Polyplex-cell surface bio-molecular interactions

Acknowledgements

Dr Nicola Green assisted with confocal microscopy.

Chapter Overview

In this chapter, the role of bio-molecular plasma membrane moieties in PEI mediated transfection are explored through biochemical manipulations. The aims of the chapter are to:

- Explore the role of lipid rafts in PEI mediated transfection.
- Explore the role of lipid raft constituents, such as heparan sulphate proteoglycans, in PEI mediated transfection.

6.1 Introduction: Putative bio-molecular plasma membrane targets for polyplex binding

6.1.1 Lipid Rafts

The concept of lipid rafts was first presented by Simons and Ikonen, (1997), expanding on the fluid mosaic model of the plasma membrane, presented by Singer and Nicolson in 1972 (Vereb *et al.*, 2003). Before the lipid raft model, membrane functionality was completely attributed to membrane proteins, whereby membrane lipids solely provided a fluid mosaic for the proteins to reside in. Although controversial (Munro, 2003), the functional role of lipids in membrane function is well established.

Lipid rafts are nano-scale assemblies of sphingolipids, cholesterol and certain membrane proteins, which coalesce to form up to micron scale assemblies upon activation by a ligand, which function in plasma membrane signaling,

trafficking and pathogen entry (Simons and Ikonen, 1997; Harder *et al.*, 1998; Del Pozo *et al.*, 2004; Lingwood and Simons, 2010). Originally, defined by insolubility in detergent (Triton X-100 etc.), the definition is now considered inaccurate (Brown, 2006). Notable lipid rafts associated proteins, glycolipids and glycoproteins include, GPI anchored proteins (Brown and Rose, 1992; Friedrichson and Kurzchalia, 1998), gangliosides (Fujinaga *et al.*, 2003) and heparan sulphate proteoglycans (Chu *et al.*, 2004).

Raft biochemistry is attributed to the properties of sphingolipids alone and in concert with cholesterol. Saturated hydrocarbon chains of sphingolipids results in reduced fluidity compared to the rest of the lipid bilayer, which consists of un-saturated phospholipids (Simons and Ehehalt, 2002). In addition, the "condensing" effect of cholesterol has been reported, whereby a cholesterol-phospholipid mixture constitutes a smaller surface area than the sum of its parts (McConnell and Radharkrishnan, 2003; Xu *et al.*, 2005; Hancock, 2006). Additionally, a model has been proposed whereby hydrophobic cholesterol is shielded from the extra-cellular milieu by polar sphingolipid head groups: the "umbrella effect" (Huang and Feigenson, 1999). Cholesterol is so fundamental to lipid raft functionality that its sequestration from the cell surface, using methyl beta cyclodextrin (MβCD) is a well established method for disturbing and studying lipid raft functionality (Christian *et al.*, 1997; Ilangumaran and Hoessli, 1998; Simons and Toomre, 2000; Zidovetzki and Levitan., 2007).

Coalescence of nano-scale raft components is dependent on lipid-lipid, lipid-protein and protein-protein oligomerizing reactions (Simons and Gerl, 2010). Following coalescence, Simons and Ehehalt (2002) present three, non mutually exclusive models for signal induction by lipid rafts. The first is that lipid raft proteins are activated by ligand binding. The second, that receptors with weak affinity for lipid rafts, on ligand activation, oligomerize and remain resident in rafts for an increased length of time. Third, that activated receptors recruit cross-linking proteins that bind to proteins in other rafts, resulting in coalescence of rafts. Raft clustering exposes proteins to a new membrane environment, enriched in specific enzymes, such as kinases, phosphatises, palmitoylases and depalmitoylases: crucial for signal transduction pathways (Simons and Toomre, 2000).

Numerous pathogenic agents have been shown to gain entry into the cell through binding to lipid rafts constituents and subsequent lipid raft clustering (Manes *et al.*, 2003; Rajendran *et al.*, 2010). Plasmodium uses lipid rafts domains of erythrocytes for their entry (Murphy *et al.*, 2006). Lipid raft associated GPI anchored proteins are used by the protective antigen of anthrax toxin to gain entry into the cell (Gruenberg and van der Goot, 2006). Gangliosides are used by cholera and Shiga toxins (Kovbasnjuk *et al.*, 2001; Fujinaga *et al.*, 2003). The T cell receptor, CD4, which locate in lipid rafts, is used by HIV to gain entry into the cell and lipid rafts are also used by the virus for subsequent assembly and release steps (Manes *et al.*, 2000; Popik et al, 2002).

The role of lipid rafts functionality in ligand-HSPG interactions, for example, fibroblast growth factor (FGF) and HIV-1 Tat protein transduction domain (TatP) ligands, has been reported (Chu *et al.*, 2004; Imamura *et al.*, 2011). Perturbation of lipid rafts with MβCD and filipin reduced fibroblast growth factor-2 (FGF-2) binding to HSPGs by 2-5 fold, although cell surface HSPG level was not affected (Chu *et al.*, 2004). Imamura *et al.*, (2011), described multi valent (≥8) TatP induced HSPG clustering at the cell surface, recruitment of activated Rac1 to adjacent lipid rafts and TatP/HSPG localization in actin associated micro-domains and subsequent internalization.

Lipid rafts have previously been implicated in PEI mediated transfection (Kopatz *et al.*, 2004; Paris *et al.*, 2008). Previously, sequestration of cholesterol using MβCD has been shown to reduce TGE following PEI mediated transfection (Kopatz *et al.*, 2004) and inhibit polyplex induced clustering of syndecans (Paris *et al.*, 2008).

6.1.2 Heparan sulphate proteoglycans

Heparan sulphate belongs to the family of glycosaminoglycans (GAGs), also home to heparin, chondroitin sulphate and hyaluronic acid (and tissue type specific GAGs, dermatan sulphate and keratan sulphate) (Jackson *et al.*, 1991; Hardingham and Fosang, 1992). The proteoglycan chains of the GAG family are either carboxylated or sulphated (or both), and thus GAGs are anionic species (although this depends on the cell line specific expression of enzymes such as sulphotransferases).

GAGs have a repeating disaccharide structure. Heparan sulphate consists of alternating *N*-acetyl-glucosamine (GlcNAc) or *N*-acetyl-galactosamine (GalNAc) and uronic acids (glucuronic acid (GlcA) or iduronic acid (IdoA)) (Hardingham and Fosang, 1992). Heparan sulphate is assembled in the Golgi and due to its enzyme-catalyzed assembly (Bishop *et al.*, 2007), the product is highly diverse and also cell type specific (Perrimon and Bernfield, 2000; Park *et al.*, 2000; Esko and Selleck, 2002; Belting, 2003).

Key enzymes that mediate HSPG biosynthesis (Bishop, 2007) within the Golgi body include xylosyltransferase (XYLT1, XYLT2), which catalyses attachment of the GAG chain to specific serine residues of the core protein domain (Schon *et al.*, 2006; Cuellar *et al.*, 2007). The tetrasaccharide primer (GlcA-Gal-Gal-Xylose) is assembled by galactosyltransferases (GALT1, GALT2) and glucuronyltransferase (GLCAT1) (Bishop, 2007). Exostosin glycosyltransferases (EXT1, EXT2) catalyses GlcA–GlcNAc polymerization/ chain elongation within the Golgi (Wei *et al.*, 2000; Busse *et al.*, 2007).

N-sulphation of *N*-acetyl-glucosamine is catalyzed by *N*-sulphotransferases (NDST1-4) whereas the sulphation of glucuronic acid and iduronic acid is catalyzed by heparan sulphate *n*-O-sulphotransferases (HS*n*ST) (*n*=1-6) (Bishop *et al.*, 2007). Interestingly, although CHO-K1 cells possess homologues to the majority of human sulphotransferases, the majority are not expressed (Xu *et al.*, 2011). HS6ST and HS2ST expression was reported in CHO-K1 cells, but not that of HS3ST (Xu *et al.*, 2011).

Heparan sulphate proteoglycans consist of a heparan sulphate GAG chain and a core protein, which is either transmembrane (the syndecans) or glycosylphosphatidylinositol (GPI) linked (the glypicans) (Couchman, 2003; Lander and Selleck, 2000; Sarrazin *et al.*, 2011). The core protein, either syndecan (the family consisting of four gene products) or glypican (six gene products) (Park *et al.*, 2000), determines the biological function of the proteoglycan (Belting, 2003). The GAG chain is anchored through the attachment of xylose to serine residues in the core protein (for syndecans and glypicans) (Sarrazin *et al.*, 2011).

HSPGs have been associated with the binding and cyto-internalization of multiple ligands (Park *et al.*, 2000; Esko and Selleck, 2002, Sarrazin *et al.*, 2011). HSPGs are involved in ligand interactions in normal cell functioning and in infection (bacterial and viral). Two well characterized interactions are those of FGF and the HIV-1 TatP with cell surface HSPGs (Chu *et al.*, 2004; Bishop, 2007; Imamura *et al.*, 2011). Interestingly, cells of the CHO lineage are relatively resistant to viral infection (a desirable trait for biomanufacturing). For example, CHOK1 cells are resistant to Herpes Simplex Virus 1 (HSV-1) infection (Shukla *et al.*, 1999), a virus which relies on HSPGs for entry into the cell (Shieh *et al.*, 1992). Data obtained through the CHO genome project (Xu *et al.*, 2011), highlighting the lack of expression of enzymes involved with HSPG biosynthesis (see above) is consistent CHO-K1 resistance to HSV-1.

The role of cell surface proteoglycans in polylysine (cationic polymer) and cationic lipid mediated transfection has been demonstrated (Mislick and Baldeschwieer, 1996). Treatment of HeLa cells with chlorate (Baeuerle and Huttner, 1986) resulted in reduced PLL:DNA complex binding to the cell surface and TGE. Addition of exogenous heparin and HSPG resulted in reduced gene expression following PLL mediated transfection. Enzymatic cleavage of cell surface HSPGs using heparitinase resulted in ~80% lower gene expression following PLL mediated transfection (Mislick and Baldeschwieer, 1996). Similarly transfection of proteoglycan deficient CHO cell line (CHO-pgs745) resulted in dramatically lower TGE compared to transfection of wild type CHO cells with PLL and a range of cationic lipids. Although, interestingly, the lipid based transfection complexes with the highest dependence on cell surface proteoglycans, were those consisting of two cationic lipids; TGE following transfection with lipid formulations consisting of a cationic lipid/ neutral lipid combination or single cationic lipid showed relatively less dependence on cell surface glycosaminoglycans (Mislick and Baldeschweiler, 1996). The role of proteoglycans in cationic lipid mediated gene expression was further demonstrated by Mounkes *et al.*, (1998) and Belting and Petersson, (1999).

The role of proteoglycans and specifically HSPG in transfection mediated by polyethylenimine is contentious, with conflicting reports of the role of cell surface HSPGs in PEI mediated transfection provided. The literature is

summarized in Table 6.1. From the same group, several papers have demonstrated that cell surface GAGs inhibit TGE, with higher transfection efficiencies reported in GAG deficient CHO relative to wild type CHO cell lines (Ruponen *et al.*, 2004; Lampela *et al.*, 2004; Hanzlikova *et al.*, 2011). However, given that the GAG deficient cell lines used in these papers were obtained by clonal isolation, an explanation for the results could be the inherent variability in transfectability/ TGE capacity exhibited by clones of the same cell line (Davies *et al.*, 2012).

Numerous other groups have provided evidence that HSPGs are necessary for successful TGE and PEI:DNA polyplex cyto-internalization, both directly and indirectly (Payne *et al.*, 2007; Paris *et al.*, 2008; Wong *et al.*, 2010, Thompson *et al.*, 2012). Payne *et al.*, (2007) demonstrated co-localization of PEI:DNA polyplexes with fluorescently labeled anti-HSPG antibody, and reduced transfection efficiency in proteoglycan deficient CHO cells and following chlorate treatment of BS-C-1 cells. Wong *et al.*, (2010) reported significantly reduced PEI:DNA polyplex uptake and subsequent TGE following enzymatic removal of cell surface HSPGs. Paris *et al.*, (2008) demonstrated the opposing roles of syndecan 1 and syndecan 2 in transfection efficiency with PEI of HEK-EBNA1 cells. The inhibitory role of extracellular glycosaminoglycans on TGE mediated by PEI has been demonstrated (Ruponen *et al.*, 2001). However, it was not possible to find within the literature, data describing the role of HSPGs in PEI:DNA polyplex cell surface binding (as opposed to cyto-internalization or TGE).

The surface of CHO cells has been previously engineered to aid transfection mediated by PEI (Wong *et al.*, 2010). Human IgG1 antibodies, deficient in fucose of the Asn^{297} linked carbohydrate, were found to improve binding to the receptor, human FcγRIII and improve antibody dependent cellular toxicity (ADCC) (Shields *et al.*, 2002). However, production of afucosylated antibodies posed challenges to biomanufacturing, especially through PEI mediated TGE. Afucosylated cell lines also displayed low levels of cell surface HSPGs, relative to wild type CHO cells (Wong *et al.*, 2010). Exostosin 1 (EXT1), an enzyme that catalyses HS GAG polymerization/ chain elongation (Busse *et al.*, 2003; Busse *et al.*, 2007; Okada *et al.*, 2010); generation of a stably expressing FUT-8 KO

cell line stably expressing EXT1 was found to have equivalent levels of cell surface HSPGs and to yield equivalent protein expression relative to CHO WT cells following PEI mediated transfection (Wong *et al.*, 2010). Interestingly, EXT1 and EXT2 enzymes were also found to affect NDST1 expression and thus HSPG sulphation (Presto *et al.*, 2008).

Table 6.1 part 1 Heparan sulphate proteoglycans and transfection mediated by polyethylenimine: literature summary

PEI:DNA polyplex cell surface interactions	Experimental Approach	Reference
• Cholesterol sequestration reduces TGE. • Protein kinase C inhibition reduces TGE and polyplex uptake.	• MβCD to sequester cholesterol prior to transfection. • Staurosporine treatment. • 25 kDa bPEI; HeLa (adherent, 10% serum).	Kopatz *et al.*, 2004
• Polyplexes induce syndecan (SDC1 and SDC2) clustering. • Cholesterol sequestration inhibits clustering. • Polyplex and SDC1 and SDC2 co-localize on the cell surface. • SDC1 over-expression increases transfection efficiency by 12%. • SDC2 over-expression decreases transfection efficiency by 80%. • The ectodomain of SDC2 has an inhibitory effect on transfection efficiency. • Co-expression of SDC1 and SDC2 reduces transfection efficiency relative to SDC1 expression only.	• Cells transfected transiently using 293fectin™ with GFP tagged SDC1, SDC2, truncated SDC1 and SDC2, chimeric SDC1 and SDC2 and CD4 control 24 h prior to transfection mediated by PEI. • bPEI and 25kDa lPEI; HEK293-EBNA1 cells.	Paris *et al.*, 2008
• HSPGs mediate polyplex internalization and TGE.	• Sodium chlorate reduces transfection efficiency (BS-C-1 cells). • Reduced transfection efficiency in proteoglycan deficient CHO cells. • Polyplex and anti HSPG antibody co-localize • Jet PEI; BS-C-1 cells.	Payne *et al.*, 2008
• HSPGs mediate PEI:DNA polyplex internalization and TGE.	• Treatment with heparitinase decreases polyplex internalization and TGE. • Enhanced cell surface HSPG complement (through exotosin overexpression), on low HSPG-expressing FUT8KO cell line, increases polyplex uptake and TGE.	Wong *et al.*, 2010

Table 6.1 part 2 Heparan sulphate proteoglycans and transfection mediated by polyethylenimine: literature summary

PEI:DNA polyplex cell surface interactions and outcome	Experimental Details	Reference
• Extra-cellular GAGs inhibit PEI:DNA polyplex formation and condensation of plasmid DNA. • Extra-cellular GAGs reduce TGE.	• 25 kDa PEI and RAA SMC (rabbit aortic smooth muscle cells).	Ruponen *et al*., 1999
• Extra-cellular GAGs reduce polyplex uptake and TGE.	• Extracellular HSPGs and hyaluronic acid reduce polyplex uptake and TGE. • 25 kDa PEI; RAA SM cells. • NB, apparently very low proportion of cells GFP positive.	Ruponen *et al*., 2001
• Cell surface GAGs reduce transfection efficiency. • Extra-cellular GAGs reduce transfection efficiency.	• Enhanced transfection efficiency in pgsB-618 (galactosyltransferase I -), pgsA-745 (xylosyltransferase-) and pgsD-677 (heparan sulphate polymerase-) cell lines relative to wild type CHO. • Enhanced transfection efficiency following treatment with chlorate, xyloside, chondroitinase ABC and *Streptomyces* hyaluronidase to remove cell surface GAGs of SM cells. • Higher luciferase expression in pgsB-618 and pgsA-745 cell lines relative to wild type CHO. • Extra-cellular HSPG and hyaluronic acid reduce transfection efficiency. • 25kDa PEI; SMC and CHO cell lines.	Ruponen *et al*., 2004
• Cell surface GAGs reduce TGE.	• Lower beta-galactosidase activity in pgsB-618 cells relative to wild type CHO. • 25 kDa PEI.	Lampela *et al*., 2004
• Cell surface GAGs reduce TGE.	• Lower beta galactosidase activity in pgsB-618 and pgsD-677 cells relative to wild type CHO. • 25 kDa bPEI.	Hanzlikova *et al*., 2011

6.1.3 Gangliosides

Gangliosides are glycolipids, which contain an oligosaccharide with one or more sialic acid residue, giving the molecule an anionic charge. The sialic acids (Byrne *et al.* 2007) are a diverse family of 9-carbon carboxylated sugars, of which *N*-acetyl neuraminic acid (Neu5Ac) is the most commonly occurring and is believed to be the biosynthetic precursor of all the other sialic acid family members (Varki, 1992).

The cell surface gangliosides play crucial roles in disease pathogenesis. The GM1 ganglioside is the cell surface receptor for cholera toxin B sub-unit, the primary enterotoxin produced by the bacterium *Vibrio cholerae* (Holmgren *et al.*, 1993). Gangliosides have also been identified as the cell surface receptors for the Human Influenza A Virus (Suzuki *et al.,* 1986) and sialic acid was identified as the cell surface receptor for Adenovirus Type 37 (Arnberg *et al.* 2000).

Cholera toxin subunit B is widely reported to bind to lipid rafts (Harder *et al.*, 1998, Janes *et al.*, 1999) although their ganglioside-specific binding has been disputed (Cuatrecasas, 1973; Fujinaga *et al.*, 2003; Harder *et al.*, 1998; Rusnati *et al.*, 2002; Blank *et al.*, 2007). Blank *et al.* (2007) specifically reported CTB binding to cells treated with a chemical inhibitor of ganglioside synthesis and the enzyme glucosidase. Nonetheless, fluorescently labelled recombinant cholera toxin B is a canonical ganglioside/ lipid raft marker (Palmer *et al.*, 2007; Zhu *et al.*, 2008; Simons and Gerl, 2010).

6.2 Materials and Methods

For all experiments described in this chapter, CHO-S cells were used, using the culture conditions described in Chapter 3. Cells were transfected with 25 kDa linear polyethylenimine according to protocol A, described in Chapter 3, Tables 3.1 and 3.2.

6.2.1 Chemical and enzymatic treatments prior to transfection

Methyl-beta-cyclodextrin (MβCD), water soluble cholesterol and phospholipase-C, were purchased from Sigma-Aldrich (Dorset, UK). MβCD and phospholipase-C were dissolved in de-ionized water. The HSPG lyase, heparitinase, was purchased from AMS Biotechnology (Abingdon, UK) and resuspended in 0.1 % BSA in PBS. For phospholipase-C and heparitinase, final concentrations were obtained by diluting 1:100 to the cells in media and 1:50 for MβCD. Chemical solutions were filter sterilized (0.22 µm). An equal volume of the reconstitution solution was added to control wells. At 1 or 2 h prior to transfection and at 4°C or 37°C, as indicated, chemicals or enzymes were added to cells.

Trypsin (0.05%) in EDTA and dialyzed fetal bovine serum (FBS) were purchased from Life Technologies (Paisley, UK). For trypsinization of cell surface proteins, 85 µl 0.05% trypsin in EDTA was added to $1x10^6$ cells at a concentrations of $2.5x10^6$ cells mL^{-1} in CD CHO media. Cells were incubated with trypsin (or control cells with EDTA) for 10 min, 37°C and 170 rpm. Cells were then pelletted and then incubated with CD CHO 10% dialyzed FBS for 2 min, 37°C and 170 rpm, washed with CD CHO and resuspended at a concentration of $1x10^6$ cells mL^{-1} in CD CHO media.

Cells were enriched with monosialoganglioside (GM1) from bovine brain (lyophilized gamma irradiated) (Life Technologies) by seeding cells at $2x10^5$ cells mL^{-1} in culti-flaks, with 100 µM ganglioside. At three days post sub-culture, cells were washed with media and transfected according to protocol A.

6.2.2 Heparan sulphate proteoglycan immunostaining

For anti-heparan sulphate immunostaining, $5x10^6$ cells mL^{-1} were fixed in 4% PFA (w/v) solution and stained as previously described (Wong *et al.*, 2010). Fixed cells were washed with PBS 1% BSA and incubated with anti-HS

antibodies 10E4 or HepSS1 (Seikagaku, AMS Biotechnology) at 1:100 for 30 min at 4°C. Cells were washed twice in PBS 1% BSA and stained with anti-mouse IgM FITC antibody (Life Technologies, Paisley, UK) or anti-mouse IgM Alexa 633 antibody (Life Technologies) at 1:500 for 30 min at 4°C. After thee washes in PBS 1% BSA, cells were analyzed by flow cytometry (FACSCalibur™) or confocal microscopy. For HSPG micrographs, Vectashield HardSet™ (Vector Laboratories, Peterborough, UK) mounting solution containing DAPI was used on the coverslips and glass slides.

6.2.3 Staining with Nile red

Microscopy grade Nile red was purchased from Sigma-Aldrich and diluted in acetone to 20 µg mL^{-1} and added to $1x10^6$ mL^{-1} live cells at a concentration 100 ng mL^{-1}. Cells were incubated at 4°C for 20 min and washed once with ice cold PBS. For single Nile red staining, cells were kept on ice before fixing. For polyplex staining, $5x10^5$ Nile red treated cells in 500 µl chilled CD CHO media, were added to a 24 well plate (Corning®, ultra low bind) and transfected, according to protocol A, with Mirus Label IT® Cy5 (Cambridge Bioscience, Cambridge, UK) plasmid DNA . Cells were incubated for 30 min at 4°C and washed once with cold PBS. Cells were fixed with 4% (w/v) PFA in PBS for 15 min and resuspended in PBS at a concentration of $1x10^6$ cells mL^{-1}. Slides were prepared by centrifuging cells and resuspending in ProLong® Gold Antifade Reagent (Life Technologies), pipetting gently onto slides, adding coverslip and leaving to cure for 24 h at rt in darkness, before sealing the edges with nail varnish.

Images were obtained using a Zeiss LSM 510Meta inverted confocal microscope and 63x/1.4 Oil DIC with a pixel dwell time of 2.4 µs. Nile red was excited using a 543 nm laser (47% transmission) and emission detected between 560 and 615nm. Cy5 was excited using a 633 nm laser (31% transmission) and emission detected between 650 and 710 nm. All image analysis was performed using LSM Image Examiner software.

6.2.4 Staining with fluorescent aerolysin (FLAER)

Cells ($4x10^5$) were fixed in 4% PFA (w/v) and then resuspended in 2.5% fluorescent aerolysin (FLAER) (Pinewood Scientific Services, Victoria, BC, Canada) in PBS at a concentration of $8x10^6$ mL^{-1}. Cells were incubated on ice for 1 h in the dark, washed twice in PBS and the cell-specific fluorescence measured by flow cytometry.

6.2.5 Staining with alexa555 cholera toxin B

Recombinant alexa555 cholera toxin B (Life Technologies) was added to live cells at a 1:500 ratio ($5x10^5$ cells in 500 µL PBS), which were incubated for 10 min at 4°C in darkness and washed twice in PBS before fixing in PFA.

6.3 Results

6.3.1 Reduction in cell surface cholesterol, using methyl-beta cyclodextrin (MβCD), reduces polyplex binding to the cell surface and TGE

It was hypothesized that sequestration of membrane cholesterol with MβCD, thus reducing membrane fluidity, would reduce TGE following transfection mediated by PEI and polyplex binding to the surface.

Sequestration of cholesterol with MβCD was found to reduce SEAP activity, at 24 h post transfection with PEI in a concentration dependent manner (Figure 6.1A). At a concentration of 2.5 mM, MβCD abrogated SEAP production (Figure 6.1A). At a concentration of 1.9 mM MβCD, SEAP activity decreased to 10% of control levels and polyplex-cell surface binding decreased to 3% of control levels (Figure 6.1A and D).

Treatment with MβCD reduced viable cell density of PEI transfected cells in a concentration dependent manner (Figure 6.1B). At 1.9 mM MβCD, viable cell denisty reduced to $1.2x10^6$ cells mL^{-1} and culture viability to 78% relative to $1.5x10^6$ cells mL^{-1} and 89% in control conditions.

Treatment with MβCD also reduced the viable cell density and culture viability of untransfected cells (Figure 6.1C). Viable cell density reduced by over 50% at 2.5 mM for untransfected cells but culture percentage viability to only 87%, relative to 96% in control conditions.

To test whether reduced polyplex-cell surface binding in the presence of MβCD was due to polyplexes binding to extracellular MβCD-cholesterol clumps, following MβCD treatment and prior to transfection, media was replaced and polyplex-cell surface binding measured as normal (Figure 6.1E). Polyplex-cell surface binding was found to decrease with increasing concentrations of MβCD. At a concentraion of 2.5 mM MβCD, polyplex-cell surface binding reduced to 13% of control levels. The pattern observed was the same as without a media replacement prior to transfection, but a higher concentration of MβCD was required to achieve a similar reduction in polyplex-cell surface binding. The data indicates that depletion of plasma membrane cholesterol, reducing membrane

fluidity, reduces polyplex-binding to the cell surface. The data also indicates, that polyplexes may bind to extracellular MβCD-cholesterol clumps.

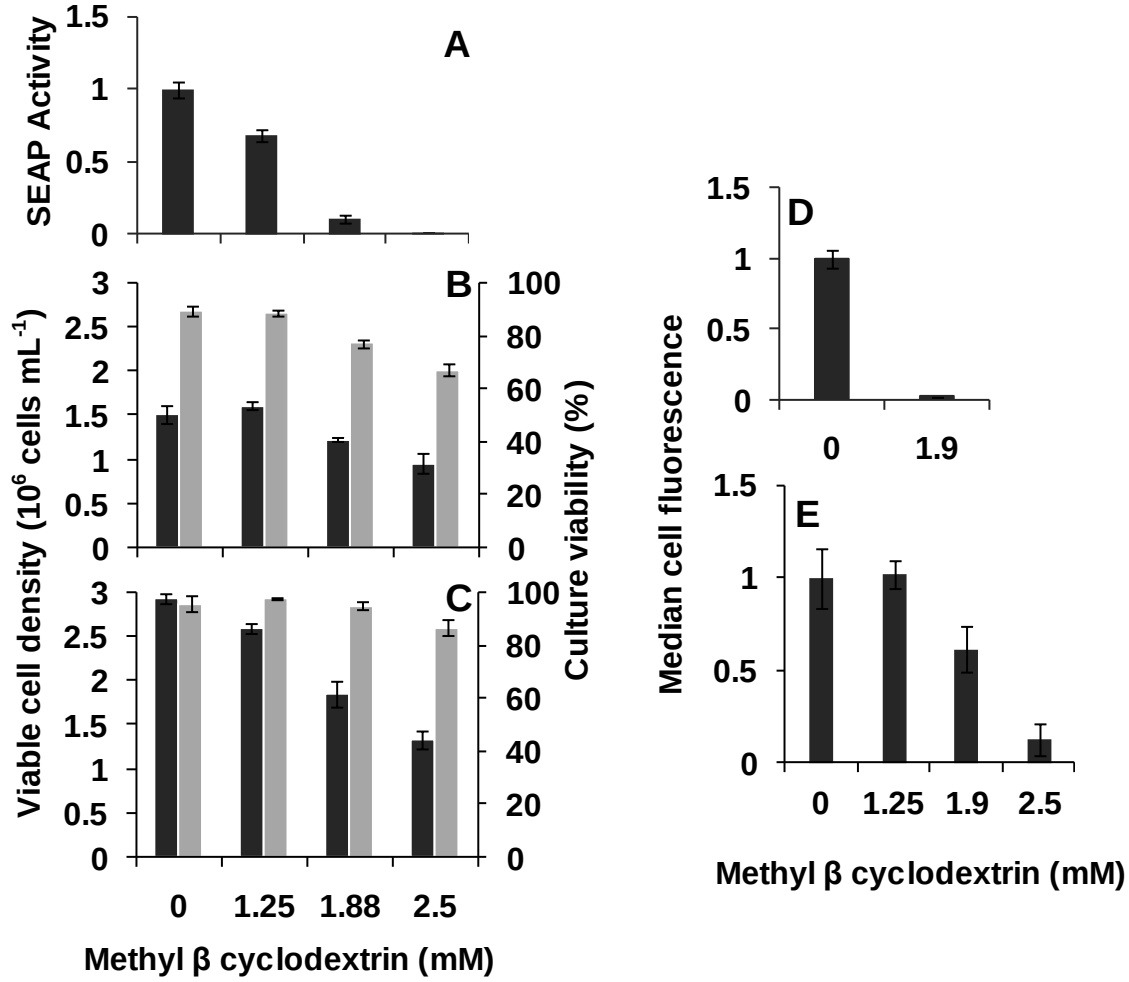

Figure 6.1 Sequestration of cholesterol by methyl beta cyclodextrin reduces polyplex-cell surface binding and SEAP production.
One hour prior to transfection with PEI, the indicated concentration of MβCD was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) (**B**) and cell culture percentage viability (grey bars) (**C**) were measured at 24 h post transfection. Viable cell density (black bars) and culture % viability (grey bars) (**E**) were measured at 24 h post sub-culture. Polyplex-cell surface binding (**D** and **E**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. **E**: Media was replaced post treatment with MβCD and pre-transfection: polyplex-cell surface binding was measured as above. The mean value ± standard deviation from triplicate transfections or cultures is shown.

Box 6.1 Methyl β-cyclodextrin

Methyl β-cyclodextrin is a cyclic heptasaccharide, belonging to the cyclodextrin family of cyclic oligosaccharides. It is used to sequester cholesterol from natural and model cell membrane. Of all the cyclodextrins, it has the highest affinity for cholesterol (Zidovetzki and Levitan, 2007).

In addition to the role of MβCD in disrupting lipid raft functionality, described in section 6.1, it has also been used as an inhibitor of numerous endocytic pathways (Ivanov, 2008). However, the role of cholesterol in clathrin and caveolin dependent endocytosis and macropinocytosis has been demonstrated (Grimmer *et al.*, 2002), thus its use at physiological conditions is likely to disrupt numerous endocytic pathways. Specifically, MβCD has been shown to cause flattening of caveolae, mislocalization of caveolin-1, to inhibit uptake of cholera toxin B (a marker of caveolae mediated endocytosis) and at high concentrations, to inhibit uptake of transferrin (a marker of clathrin mediated endocytosis) (Ivanov, 2008).

6.3.2 Effect of addition of water soluble cholesterol to TGE and culture viabilities

Converse to addition of MβCD, water soluble cholesterol (carried by MβCD) was added to cells. Addition of water soluble cholesterol to cells has previously been provided as a method for enhancing membrane cholesterol content (Christian *et al.*, 1997) and it was hypothesized that by increasing membrane cholesterol content, PEI mediated TGE would increase (by increased polyplex binding to the cell surface).

Water soluble cholesterol was added to cells prior to transfection with PEI, with and without medium replacement following cholesterol treatment and prior to transfection. Without media replacement pre-transfection, addition of cholesterol at 25 µM resulted in SEAP activity at 95% of control levels and at concentrations of 50 and 100 µM, SEAP activity was abrogated (Figure 6.2A). However, at 50 and 100 µM cholesterol, a signiciant reduction in viable cell density and culture percentage viability was observed (Figure 6.2B). For example, at 50 µM cholesterol, viable cell density was 0.9×10^6 cells mL^{-1}

relative to $1.5x10^6$ cells mL^{-1} in control conditions and culture percentage viability reduced to 69%. Addition of cholesterol to untransfected cells also resulted in a significant reduction in cell growth and culture viabilty (Figure 6.2C).

With media replacement prior to transfection and following incubation of cells supplemented with water soluble cholesterol, at 24 h post transfection, cholesterol treatment at a concentration of 50 µM and 100 µM resulted in SEAP activity at 34% and 10% of control levels (Figure 6.2D). In the presence of cholesterol the viable cell density of transfected cells also decreased, but only at a concentration of 100 µM cholesterol (Figure 6.2E). Similarly, a significant reduction in culture percentage viability to 74% relative to 88% for control, was observed at 100 µM cholesterol. For untransfected cells, treatment with one hour prior to media replacement, resulted in a concentration dependent reduction in viable cell density but had minimal effect on culture percentage viability, at 100 µM cholesterol, decreasing viability to 92% (Figure 6.2F).

Membrane cholesterol trafficking is thought to be a highly sensitive process (Leyt *et al.*, 2007) and it is possible that addition of water soluble cholesterol disrupted membrane cholesterol in a similar fashion to that achieved by MβCD. Furthermore, as indicated by the difference in SEAP output at a concentration of 50 µM cholesterol, with and without media replacement prior to transfection, it is possible that polyplexes bound to extracellular cholesterol dispersed in the media, reducing polyplex binding to the cell surface.

6.3.3 Localization of cellular hydrophobic regions and polyplexes

It was hypothesized that PEI:DNA polyplexes would alter the distribution of hydrophobic lipids at the cell surface and in the cytoplasm. Nile red (9-diethylamino-5-benzo(α)phenoxazinone) is selectively fluorescent in a hydrophobic environment (i.e. intracellular lipid droplets) (Greenspan *et al.*, 1985). Thus, cells were stained with Nile red and subsequently, fluorescently labelled PEI:DNA polyplexes were added to cells on ice (Figure 6.3). Patches stained with Nile red were seen in the peri-nuclear area, consistent with previously published micrographs of Nile red stained CHO cells (Listenberger *et al.*, 2003). Polyplexes were seen in distinct patches on the cell membrane,

consistent with data presented in Figure 4.6 and in Godbey *et al.*, (1999c). However, comparing micrographs Figure 6.3B and C, no difference in the distribution of lipid droplets was observed between transfected and untransfected cells and furthermore, no co-localization between cy5 (polyplexes) and Nile red (lipid droplets) was observed.

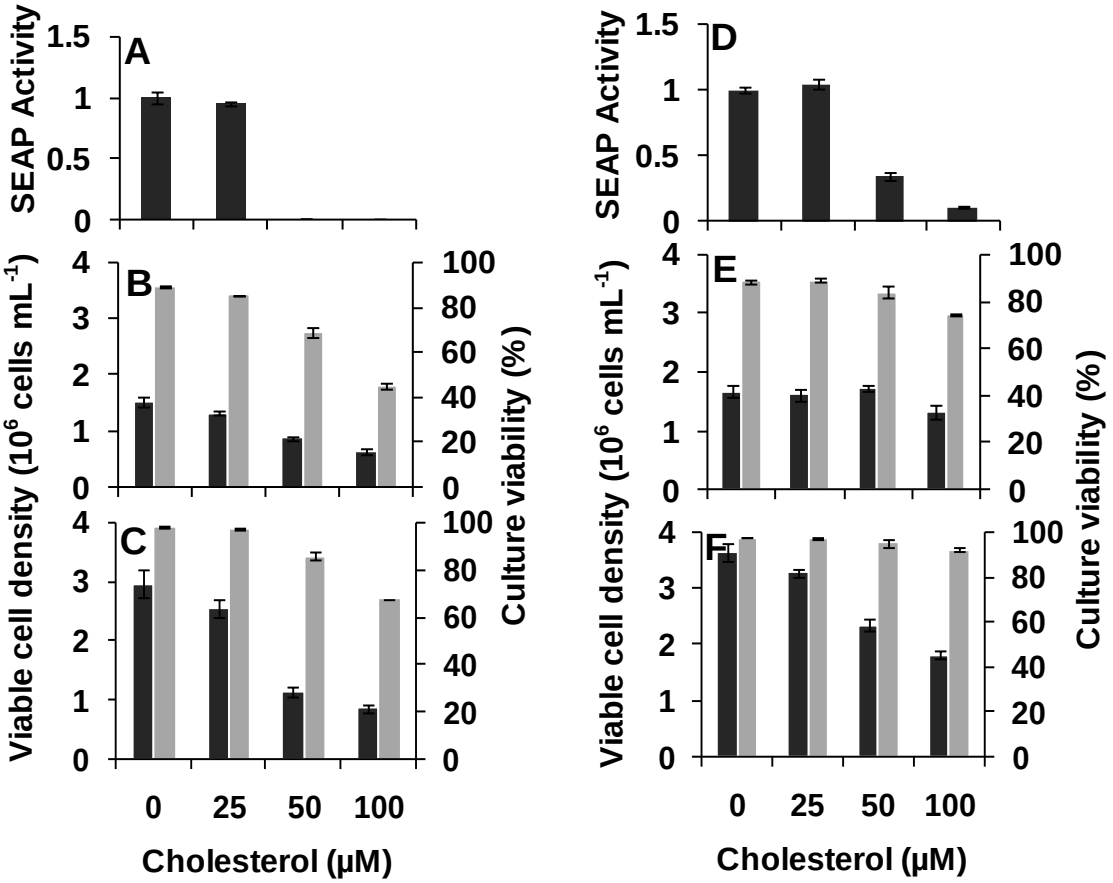

Figure 6.2 Addition of water soluble cholesterol to cells prior to transfection with PEI.
CHO-S cells, in CD CHO medium, were incubated with the indicated concentration of cholesterol (dissolved in water) at 37°C, 5% CO_2, 180rpm for 1 h. Cells were then directly transfected (**A, B, C**) or pelleted and resuspended in fresh CD CHO prior to transfection (**D, E, F**). Transfections were performed according to protocol A. At 24 h post transfection SEAP was measured (**A** and **D**). Viable cell density (black bars) and culture % viability (grey bars) was measured at 24 hours post transfection (**B** and **E**) or 24 h post sub-culture (**C** and **F**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

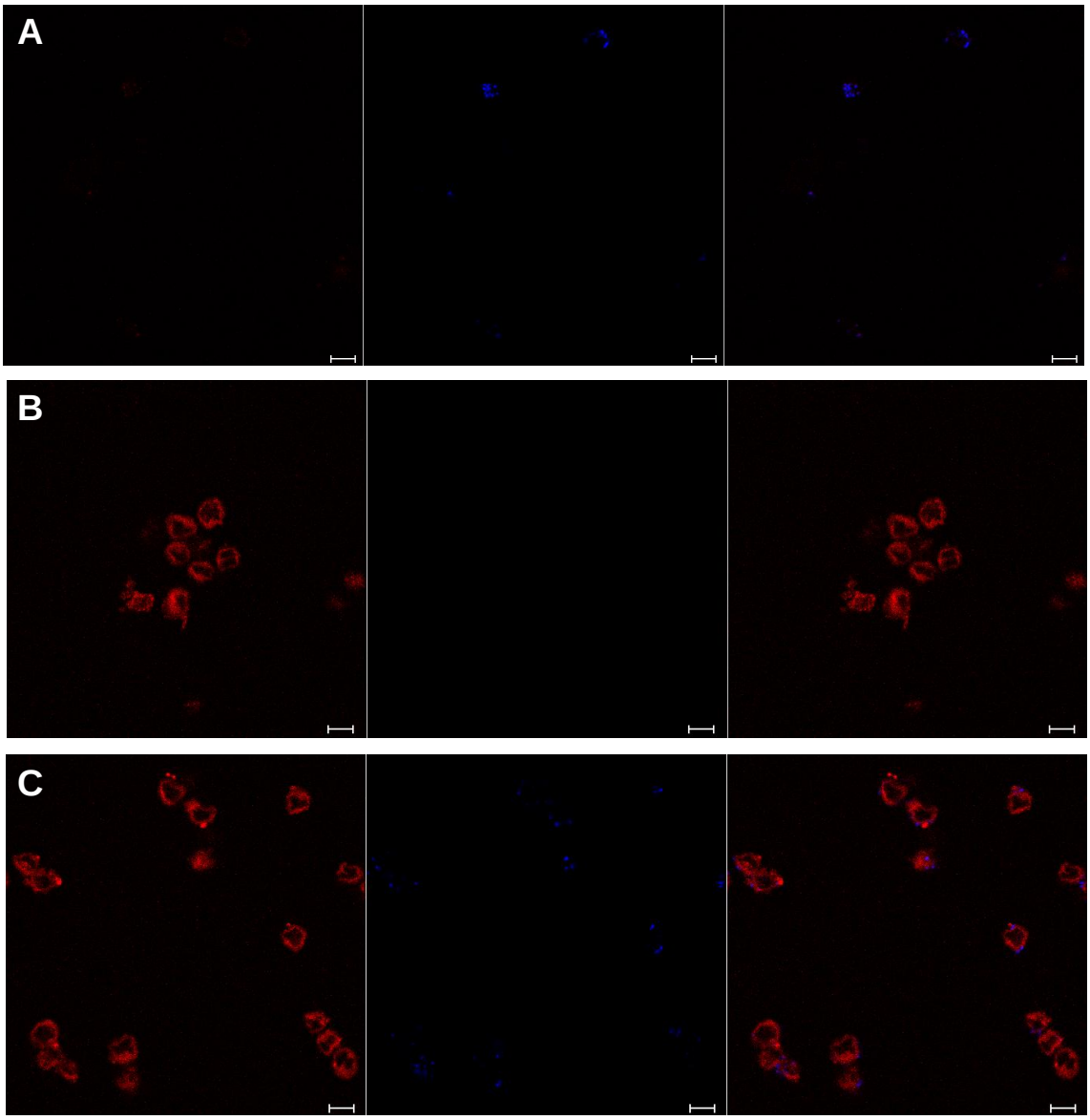

Figure 6.3 Cells labelled with Nile red and cy5 polyplexes.
Live cells (CHO-S) were incubated at a concentration of 10^6 mL^{-1} in PBS and Nile red (20 µg mL^{-1}) at 4°C for 20 min in the dark. For single Nile red staining, cells were kept on ice before fixing in 4% (w/v) PFA. Cy5 labelled PEI:DNA polyplexes were formed according to protocol A and incubated with cells at 4° C for 30 min, prior to fixing. Cells were resuspended in ProLong® Gold Antifade reagent mounting agent. **A**-cells with cy5 labelled polyplex only; **B**-cells stained with Nile red only; **C**-cells stained/ labelled with Nile red and cy5 polyplexes. Column one (560—615 nm filter). Column two (650-710 nm filter). Column three-combined images. Bar = 10µm.

6.3.4 Cell surface heparan sulphate proteoglycans (HSPGs) deplete from the cell surface following transfection but regenerate rapidly following enzymatic cleavage.

Given the plethora of data demonstrating HSPG involvement in PEI mediated transfection (Table 6.1) and specifically that PEI:DNA polyplexes and anti-HSPG antibodies co-localize intra-cellularly (Payne *et al.*, 2007) the hypothesis was tested that cell surface HSPGs would deplete following transfection with PEI. Cells were fixed at set times pre and post transfection or trypsinization and immunostained using anti-HSPG mAbs (Figure 6.4). At 7.5 min post transfection, cell surface HSPGs reduced to 25% of the level pre transfection. At 30 min post transfection, the level dropped further to 14% and remained at this level at 8 h post transfection. In contrast, in un-transfected conditions, following enzymatic cleavage of HSPGs using trypsin, cell surface HSPGs fell to 16% of pre trypsinization levels but gradually regenerated on the cell surface. At two and eight hours post trypsinization, HSPGs had regenerated to 47% and 73% of pre-trypsin levels, respectively (Figure 6.4).

The data describing HSPG depletion post transfection was transformed according to $y_{(t,\ 480)}=0$, and an exponential function fitted to the data (Figure 6.5A). Using calculus, the point where the time point where the rate of change in fluorescence was less than or equal to 1% was calculated:

$$\alpha = c + 0.8691e^{-0.266x}$$

Find time at which change in fluorescence is <1%

$$\frac{dy}{dx} \geq -1\%$$

$$-1\% \leq -0.2311806\ e^{-0.266x}$$

$$X = 11.8\ \text{min}$$

The time point where HSPG depletion was ≤1% was calculated to occur at 11.8 min post transfection.

A linear function was found to provide good fit to HSPG regeneration post trypsinization between 1 and 240 min post trypsinization (Figure 6.5B)

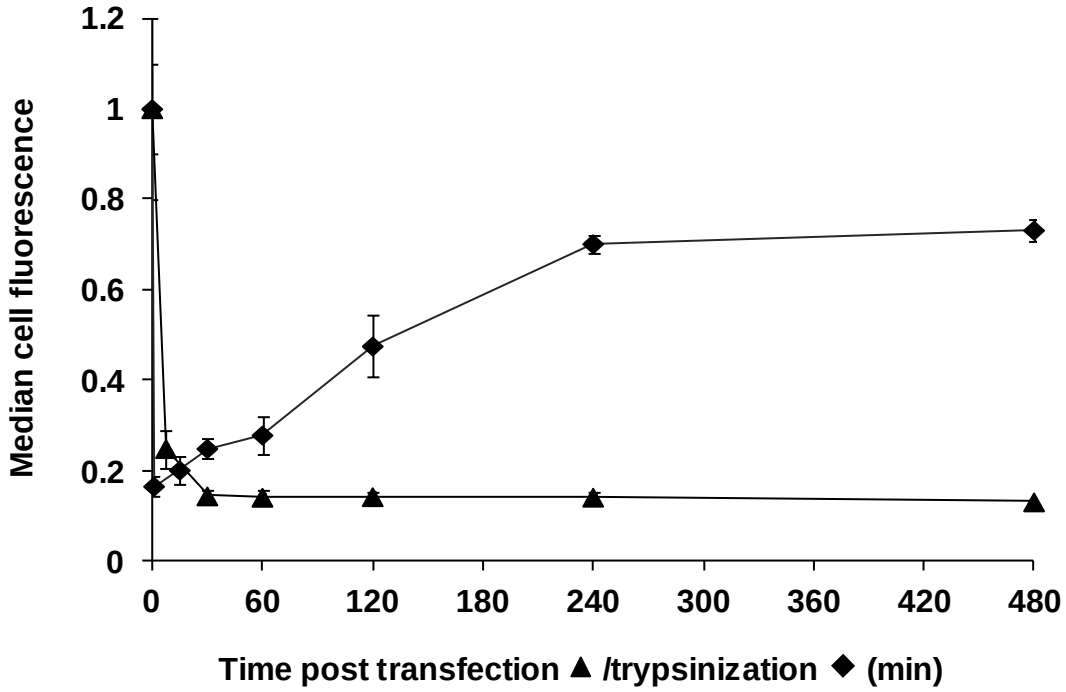

Figure 6.4 Heparan sulphate proteoglycans deplete from the cell surface following transfection but regenerate rapidly following enzymatic cleavage.

CHO-S cells were transfected with PEI or trypsinized and at the indicated times post or prior to transfection/ trypsinization, washed with PBS and fixed. Fixed cells were then immunostained for HSPGs and the fluorescence measured by flow cytometry or confocal microscopy. Diamonds: HSPGs regenerate over time following trypsinization. Triangles: HSPGs deplete from the cell surface following transfection. The mean value ± standard deviation from triplicate transfections or cultures is shown.

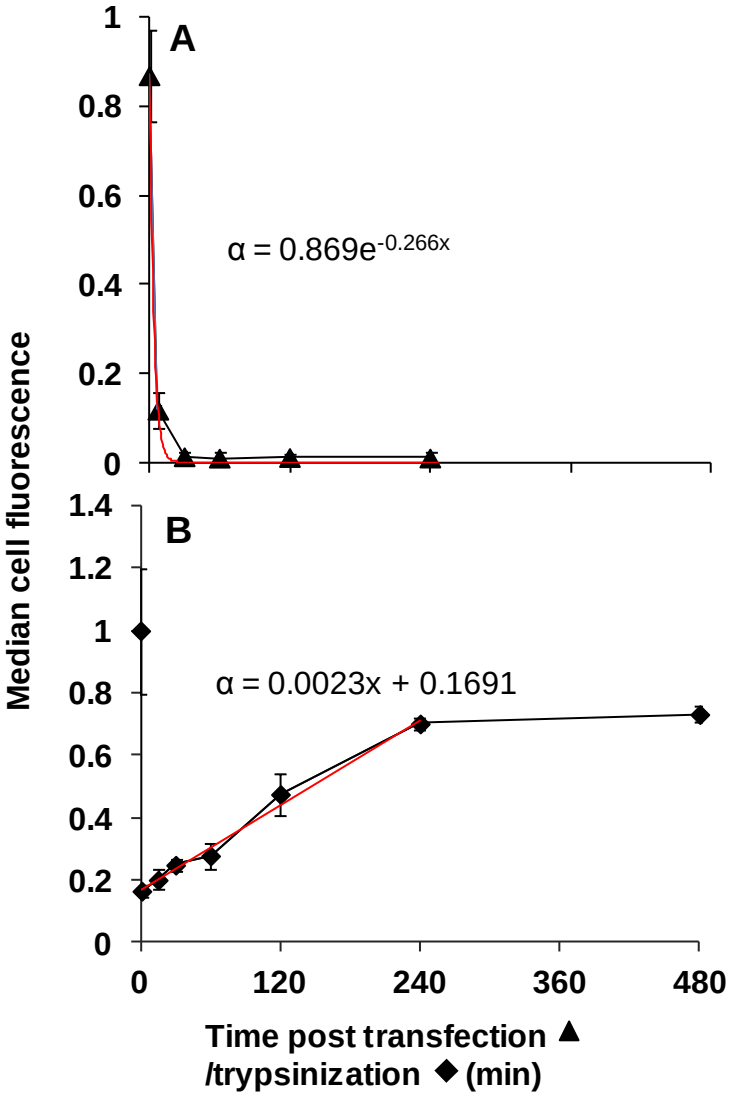

Figure 6.5 Equations fitted to kinetic data for HSPG depletion post transfection or trypsinization.
A: HSPGs post transfection. Data was transformed whereby $y_{(t,480)}=0$. The exponential function was found to provide good fit to the data.
B: HSPGs post trypsinization. A linear function provided good fit to the data between 0 and 240 min post transfection

Rate of change of cell surface HSPGs was also calculated, according to equation 6.1. Following trypsinization, the rate of regeneration of cell surface HSPGs was calculated to be approximately constant, over 8 h post transfection (Figure 6.6). Following transfection, the rate of change in cell surface HSPGs fell >10 fold between 7.5 and 30 min post transfection. Between 60 and 480 min post transfection, the rate of change of cell surface HSPGs was approximately constant (Figure 6.6).

$$\text{Rate of change in cell surface HSPGs} = \frac{FUt_n - FUt_{(n-1)}}{(mt_n - mt_{(n-1)})} \qquad 6.1$$

Where FU is fluorescence unit, t is time and m is minutes.

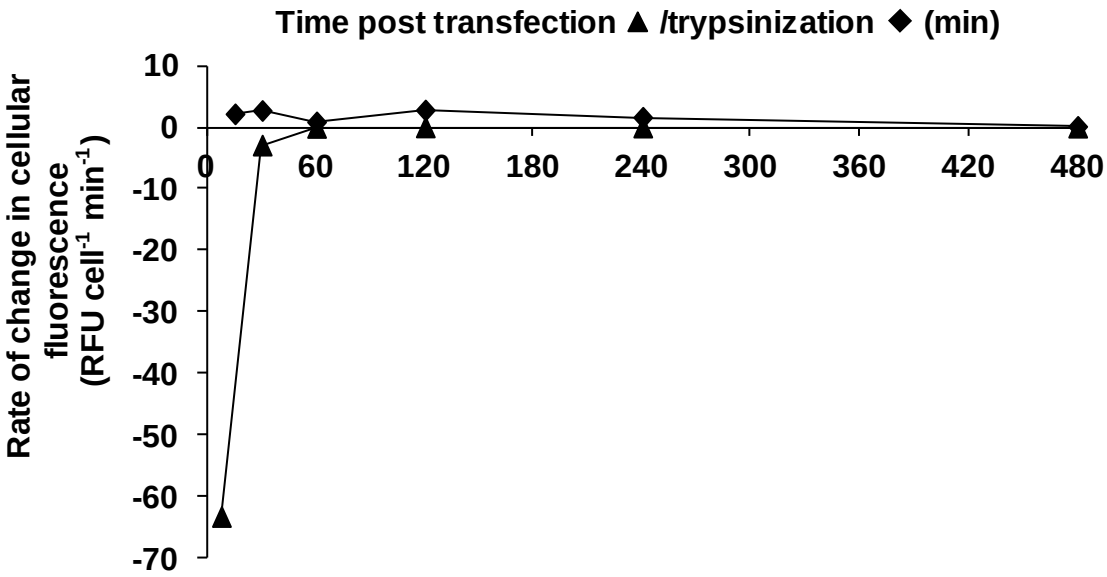

Figure 6.6 Rate of change in cell surface HSPG complement, post transfection or trypsinization.
Diamonds: rate of cell surface HSPG regeneration following trypsinization. Triangles: rate of cell surface HSPG depletion following transfection. Rates of change of cell surface HSPG complement were calculated using equation 6.1 and data presented in Figure 6.4.

The data presented in Figures 6.4, 6.5 and 6.6, combined with data presented in Figures 4.4, 4.5 and 4.6, is used to form the biphasic model of polyplex uptake, described in Chapter 9 and Figure 9.1.

6.3.5 Immunostaining and confocal microscopy shows cell surface HSPGs located in distinct rafts on the cell surface

To illustrate the data on HSPG depletion post transfection, confocal micrographs were taken. In addition, it was hypothesized that HSPGs would appear at distinct patches around the plasma membrane, indicative of lipid-raft localization. Confocal micrographs of cell surface HSPG complement, pre and post transfection with PEI, supported the data obtained by flow cytometry, illustrating a substantial reduction in cell surface HSPGs post transfection (Figure 6.7). Furthermore, confocal micrographs showed HSPGs in distinct, localized patches on the cell surface, both pre and post transfection with PEI.

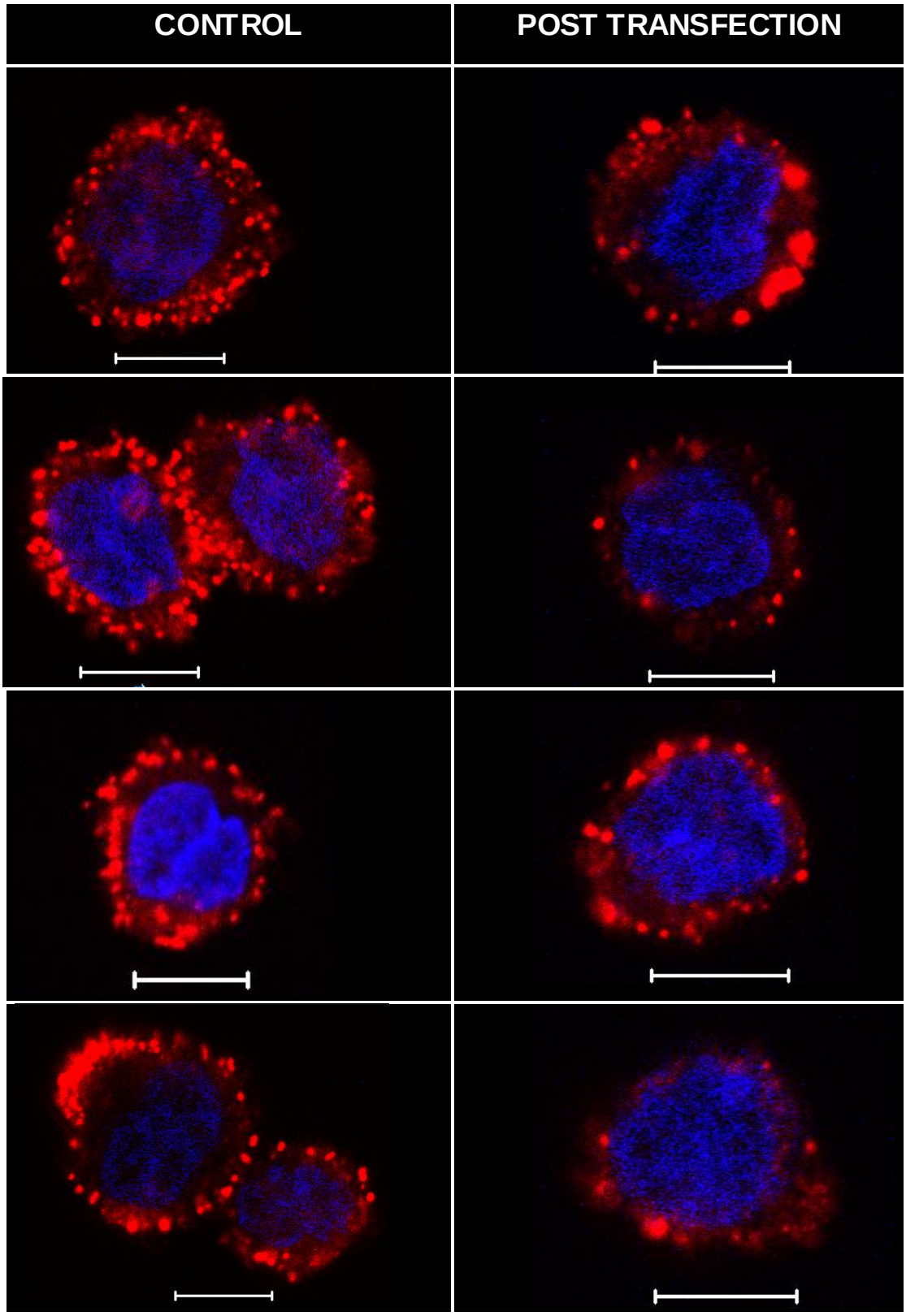

Figure 6.7 HSPG clusters pre- and post transfection with PEI.
Cell were fixed, without prior treatment or 10 min post transfection with PEI. Cells were immunostained for HSPG with the primary murine IgM monoclonal antibody (10E4) and the secondary alexa-633 anti mouse IgM antibody. Cells were mounted on cover-slips with DAPI containing medium. An inverted LSM510 meta confocal microscope with a 63x/1.4 oil DIC and 633 and 800 nm lasers was used. Bar = 10µm.

6.3.6 Cell surface HSPGs are not absolutely required for polyplex binding to the cell surface but HSPG depletion reduces TGE by 25 percent

To test the hypothesis that HSPGs mediate polyplex-cell surface binding and PEI mediated TGE, HSPGs were enzymatically cleaved using heparitinase and the HSPG reduction validated by anti-HSPG immunostaining. Treatment with heparitinase resulted in a 90% reduction in cell surface HSPGs, validated by immunostaining at the point of transfection (and a validated >85% reduction at 24 h post transfection, data not shown) (Figure 6.8F). However, there was no significant difference in polyplex-cell surface binding (Figure 6.8D), between heparitinase treated or control cells, measured at 4 h post transfection. Although heparitinase treatment resulted in a ~25% reduction in SEAP activity at 24 h post transfection (Figure 6.8A). Treatment with heparitinase had no significant effect on viable cell density or culture % viability at 24 h post transfection (Figure 6.8B).

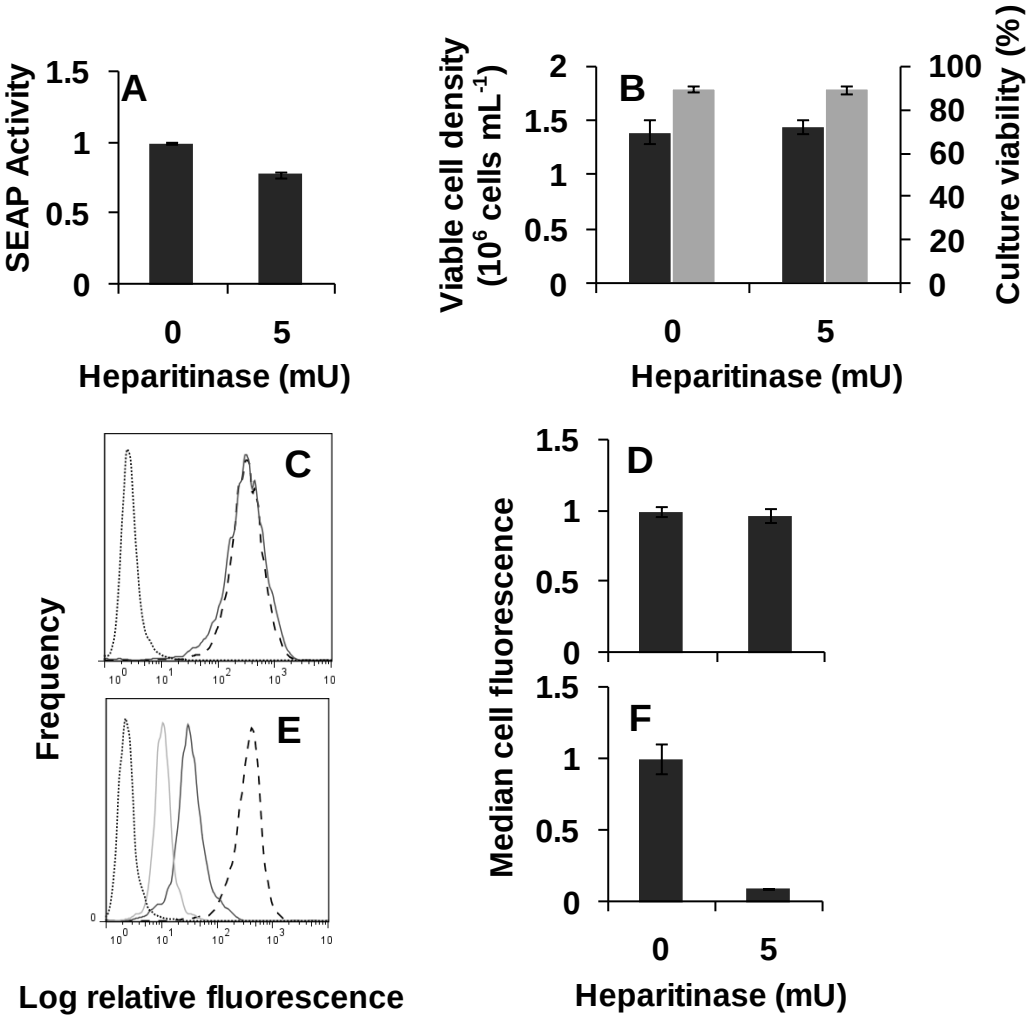

Figure 6.8 Depletion of HSPGs has no effect on polyplex-cell surface binding at 4 h post transfection, but reduces SEAP activity by 25% at 24 h post transfection.

Prior to transfection with PEI (2 h), heparitinase was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured at 24 h post transfection. Polyplex-cell surface binding (**C** and **D**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. **C**: dashed line: 0 mU heparitinase; solid line: 5 mU heparitinase; dotted line: auto-fluorescence. At the point of transfection, cells were fixed and immunostained for cell surface HSPGs (**E** and **F**) and the fluorescence measured by flow cytometry. **E**: dashed line: 0 mU heparitinase, solid line: 5 mU heparitinase, light grey line: secondary antibody control, dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or samples is shown.

6.3.7 Trypsinization, removing cell surface proteins, reduces PEI mediated TGE by 23 percent

The role of the cell surface proteome in PEI mediated transfection was tested, by enzymatically depleting cell surface proteins, using trypsin, which cuts at arginine and lysine (Olsen *et al.*, 2004) and sometimes before proline (Rodriguez *et al.*, 2008). In addition, the regeneration of cell surface proteins was considered, by transfecting cells at set times post trypsinization. At set times post trypsinization, cells were transfected with PEI and at exactly 24 h post transfection, samples were taken for SEAP and cell viability analysis (Figure 6.9). For cells transfected at 10 min post trypsinization, SEAP activity was at 77% that of control levels (Figure 6.9A). Cells transfected at 60, 120 and 240 min post trypsinization had SEAP activity at 85, 88 and 85% of control levels, respectively. It is possible that regeneration of cell surface proteins/ glycoproteins facilitated the incremental increase in TGE when cells were transfected at 60/ 120/ 240 min post trpsinization, rather than at 10 min post trypsinization, when cell surface protein levels would be expected to be minimal.

The process of trypsinization had no significant effect on culture viability. Neither viable cell density or culture percentage viability at 24 h post sub-culture or transfection was significantly different for cells with or without prior trypsinization (Figure 6.9 B and C).

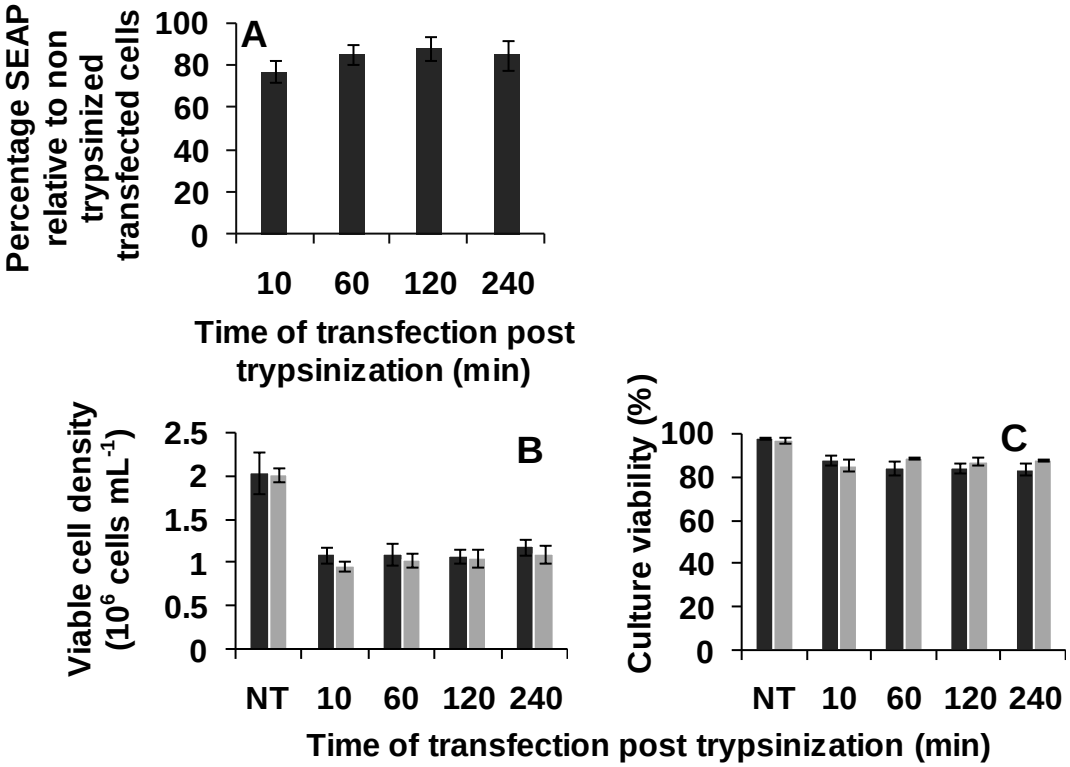

Figure 6.9 Transfection of cells at set times post trypsinization.
Cells were transfected, at the indicated times post trypsinization, with PEI. At 24 h post transfection, SEAP (**A**), viable cell density (**B**), and culture % viability (**C**) of trypsinized (dark grey bars) and non trypsinized (light grey bars) were measured. The mean value ± standard deviation from triplicate transfections or cultures is shown.

Box 6.2 Anti-heparan sulphate proteoglycan mAbs, HepSS1 and 10E4: Epitope Specificity

Anti-HSPG murine mono-clonal antibodies 10E4 and HepSS1 were produced through hybridoma technology (David *et al.*, 1992; Kure and Yoshie, 1986). The saccharide epitopes of the mAbs was identified by inhibition ELISA, using heparin like polysaccharides (Van den Born *et al.*, 2005). The epitope of the 10E4 mAb was found to occur commonly in heparan sulphate and is distinguished by a *N*-acetylated and *N*-sulphated glucosamine sequence (GlcNAc and GlcNS). In contrast, the epitope of HepSS1 was found to be rare in heparan sulphate, characterized sequences rich in *N*-sulphated glucuronic acid (Van den Born *et al.*, 2005).

6.3.8 Total cell surface HSPG level varies across CHO cell lines.

As presented by Thompson *et al.*, (2012) three CHO cell lines, CHO-L, CHO-M and CHO-S, following transfection optimization, were found to vary in SEAP output, polyplex binding and polyplex cyto-internalization capacity. It was hypothesized that the cell surface HSPG level would also vary across the three variant CHO cell lines. Thus, CHO-S, CHO-L and CHO-M cells, were stained with anti-HSPG murine monoclonal antibodies (10E4 and HepSS1) and a secondary anti-mouse IgM FITC-labelled mAb. The CHO-S cells were more heavily stained with both 10E4 and HepSS1 mAbs, relative to CHO-L and CHO-M (Thompson *et al.*, 2012). With anti-HSPG 10E4, staining of CHO-L and CHO-M was 24% and 19% the level for CHO-S, respectively (Figure 6.10). With HepSS1, staining of CHO L and CHO M was 35% and 24% the level for CHO-S, respectively (Figure 6.10).

For each cell line, the level of staining with the HepSS1 was lower compared to staining with 10E4, as expected (Van den Born *et al.*, 2005). Staining of CHO-S, CHO-L and CHO-M using HepSS1 was 24%, 37% and 31% the level of 10E4 staining, for each respective cell line (Figure 6.10).

Interestingly, CHO cell lines stained with the same mAbs, HepSS1 and 10E4, DG44, DG44 fut8 -/-, CHO WT and FUT 8 KO (Wong *et al.*, 2010), were stained by HepSS1 at >70% the level of 10E4 staining (estimated), much higher than

the ratio of HepSS1/10E4 staining reported here. These data indicate the diversity in HSPGs micro-structure amongst variant CHO cell lines.

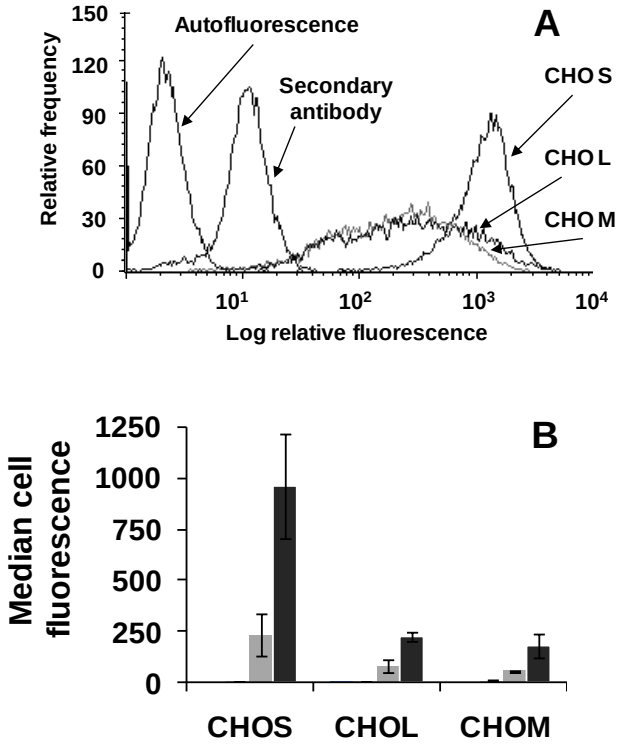

Figure 6.10 Variable heparan sulphate proteoglycan level across three CHO cell lines.
Host CHO cells ($1x10^6$ cell mL^{-1}) were fixed in 4% (w/v) paraformaldehye then labelled with murine anti-HSPG monoclonal antibodies (HepSS1-grey bars and 10E4-black bars) and a secondary FITC labelled goat anti-mouse secondary antibody, prior to flow cytometric analysis (**A** and **B)**. The mean value ± standard deviation from triplicate biological samples is shown.

6.3.9 GPI anchored cell surface proteins do not mediate polyplex-cell surface binding or PEI mediated TGE

The involvement of GPI anchored proteins, in polyplex cell surface binding and TGE were investigated. The core protein domain of HSPGs is either a transmembrane, syndecan, or GPI anchored, glypican. The involvement of syndecans in PEI:DNA polyplex cyto-internalization and TGE has been previously reported (Paris *et al.*, 2008). However, it was not possible to find data describing the role of GPI anchored proteins in PEI mediated transfection. It was hypothesized that enzymatic depletion of GPI anchored proteins would affect PEI mediated TGE and polyplex binding to the cell surface.

GPI anchored proteins were enzymatically depleted using phospholipase-C, which has been used previously to cleave GPI anchored proteins from the surface of CHO cells (Jarousse and Coscoy, 2008). Depletion of GPI anchored proteins was validated using and fluorescent aerolysin (FLAER), which has been used to stain for GPI anchored proteins (Brodsky *et al.*, 2000).

Treatment of CHO-S cells with phospholipase-C, resulted in a 78% reduction cell surface GPI anchored proteins, measured by staining with fluorescent aerolysin (Figure 6.11 E and F). However, treatment with phospholipase-C did not affect SEAP production (Figure 6.11A) or polyplex-cell surface binding (Figure 6.11 C and D). Viable cell density and culture percentage viability at 24 h post transfection was not affected by treatment with phospholipase-C (Figure 6.11B).

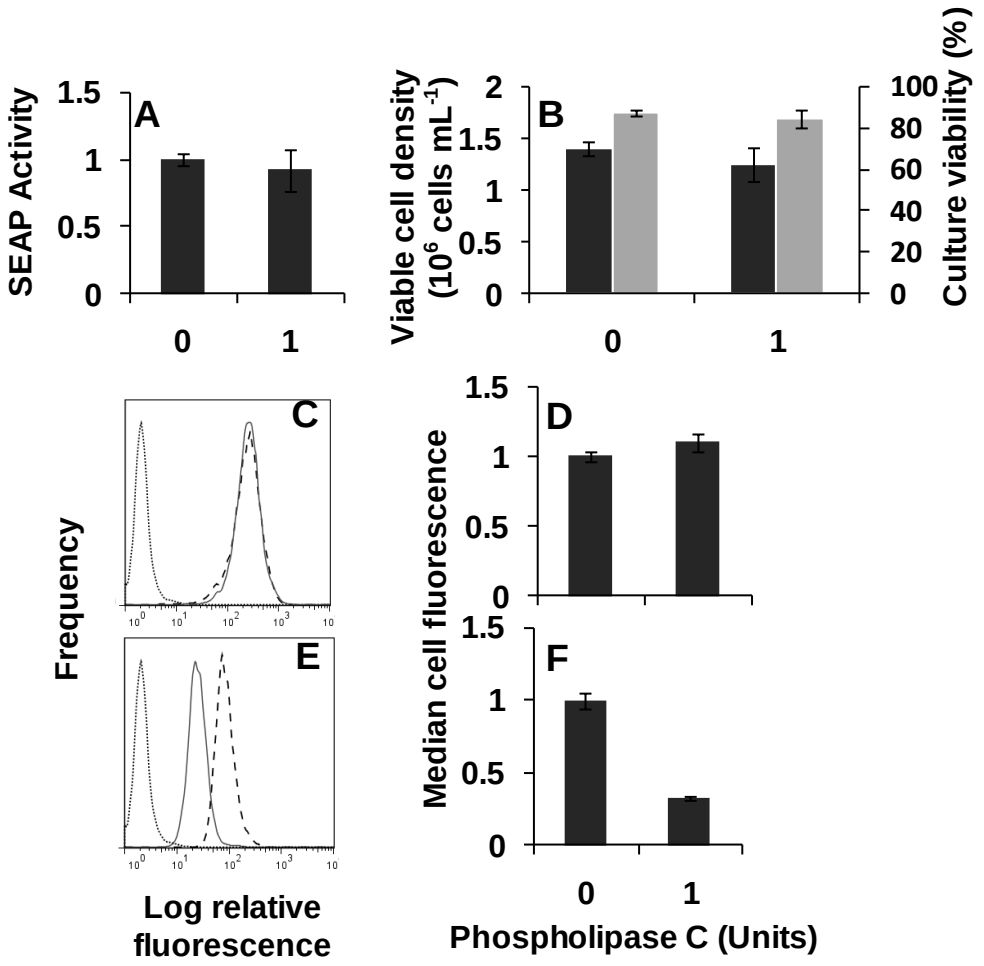

Figure 6.11 Depletion of GPI anchored proteins on the cell surface using phospholipase c does not significantly reduce polyplex-cell surface binding or SEAP production after transfection with PEI.
One hour prior to transfection with PEI, phospholipase C was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured at 24 h post transfection. Polyplex-cell surface binding (**C** and **D**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. At the point of transfection, cells were fixed and stained for cells surface GPI anchored proteins using fluorescent aerolysin (FLAER) (**E** and **F**). **C and E**: dashed line: 0 mU phospholipase C; solid line: 1 U phospholipase C; dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or samples is shown.

6.3.10 Gangliosides are not exogenously expressed by CHO-S cells. Enrichment of the cell surface with ganglioside (GM1) does not affect polyplex-cell surface binding or PEI mediated TGE

The CHO-K1 cell line has been shown to be deficient in gangliosides (Rusnati *et al.*, 2002). It was hypothesized that increasing levels of cell surface gangliosides would enhance polyplex binding to the cell surface.

Lipid raft associated gangliosides have previously been enriched in ganglioside-deficient CHO-K1-pgsA745 cells, through addition of exogenous bovine ganglioside (GM1) (Rusnati *et al.*, 2002). Thus, bovine ganglioside was added to CHO-S cells, three days prior to transfection. Since the CHO-S cell line is derived from CHO-K1, it was not surprising that CHO-S stained with cholera toxin B were not fluorescent above back-ground levels. Following enrichment with gangliosides, however, staining with fluorescently labelled CTB was dramatically above background fluorescence (Figure 6.12 E and F). However, enrichment with gangliosides did not affect polyplex cell-surface binding (Figure 6.12 C and D). SEAP production was 21% lower in ganglioside enriched cells compared to control (Figure 6.12A). Viable cell density, at 24 h post transfection, was slightly higher for ganglioside enriched cells, 1.8 $x10^6$ cells mL^{-1} compared to 1.2 $1.8x10^6$ cells mL^{-1} in control conditions (Figure 6.12B). Culture percentage viability was 89% for ganglioside enriched cells compared to 84% for control cells at 24 h post transfection (Figure 6.12B).

It is possible that the process of ganglioside enrichment, as described above, caused stress to the cells (e.g. through the trafficking of gangliosides) that was detrimental to PEI mediated transfection and SEAP production. Thus a cell line engineering approach to increase cell surface ganglioside complement might be a more lengthy, but unavoidable step to accurately test whether gangliosides improve PEI mediated transfection and TGE.

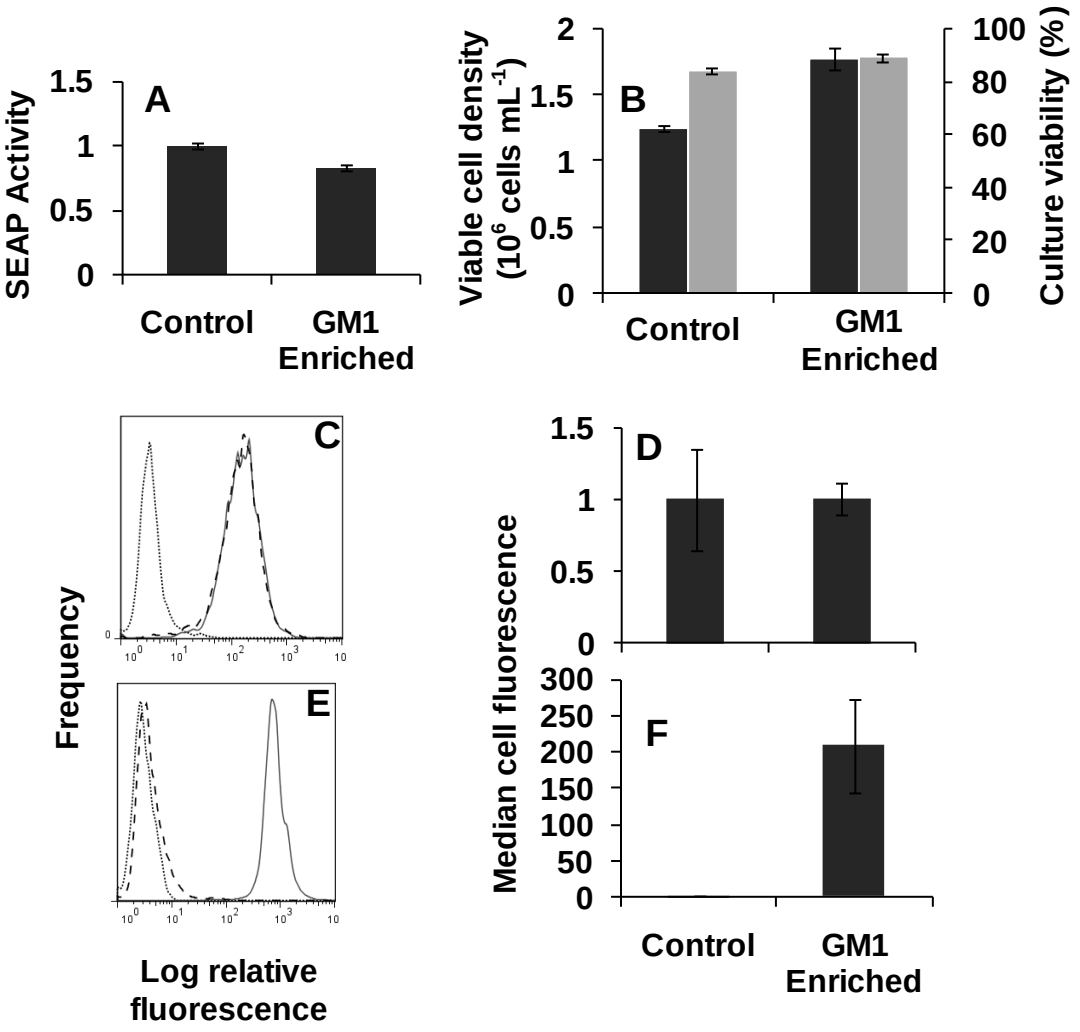

Figure 6.12 Enrichment of CHO cells with ganglioside (GM1) has no significant effect on polyplex-cell surface binding.
Cells (CHO-S) were cultured with or without 100 µM GM1 for 72 h prior to transfection with PEI. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured at 24 h post transfection. Polyplex-cell surface binding (**C** and **D**) was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C and at 4 h post transfection measuring cellular fluorescence using flow cytometry. Enrichment with ganglioside GM1 was validated by fixing cells with PFA and staining using alexa-555 labelled cholera toxin B (CTB) (**E** and **F**). **C** and **E**: dashed line: control cells; solid line: ganglioside enriched cells; dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or samples is shown.

6.4 Discussion

Several groups have demonstrated the role of HSPGs in polyplex internalization and subsequent TGE (Payne *et al.*, 2007; Paris *et al.*, 2008; Wong *et al.*, 2010), as described in detail in section 6.1 and Table 6.1. Thus, it was not surprising that heparitinase treatment was found to reduce TGE (Figure 6.7). However, it was expected that heparitinase treatment would completely oblate TGE, rather than the observed 25% reduction in TGE, in accordance with the theory that HSPGs are the cell surface receptors for polyplexes and that HSPGs are absolutely necessary for polyplex binding to the cell surface. In contrast to the limited reduction in TGE following heparitinase treatment, MβCD ablated TGE at the highest concentration tested, similar to data presented by Kopatz *et al.*, (2004). These data can be explained by using the biphasic model of polyplex uptake, described in Chapter 9 and Figure 9.1. According to the model, heparitinase treatment would oblate phase 1 uptake (HSPG endocytosis, Payne *et al.*, 2007), but have no effect on phase 2 uptake. MβCD treatment would reduce polyplex internalization via both pathways, membrane fluidity being fundamental for almost all forms of endocytosis reported (Grimmer *et al.,* 2002) (Box 6.1) (Table 6.2). Approximately 25% of polyplex was found to be internalized in phase 1 (within approximately 15 min post transfection) (Figure 4.5), equal to the reduction in reporter protein expression caused by heparitinase treatment.

Table 6.2 Putative effects of biochemical manipulation on transfection using the biphasic model for the uptake of PEI:DNA polyplexes

	Uptake	
Treatment	Phase 1	Phase 2
Heparitinase	x	—
MβCD	↓	↓

Heparitinase treatment abrogates uptake via phase 1 (HSPG-endocytosis) but has no effect on phase 2 uptake. Treatment with MβCD reduces phase 1 and phase 2 uptake.

In addition to heparitinase treatment, trypsinization of the cell surface was found to only reduce PEI mediate TGE by ~25% (Figure 6.9). Since HSPGs consist of

a protein core domain, it is possible that the reduction in TGE could be explained by reduced cell surface HSPGs, as described above. However, the trypsinization data indicate that other protein based membrane moieties are not absolutely necessary for transfection, but as with HSPGs, are necessary for TGE at maximum levels.

Interestingly, MβCD, but not heparitinase treatment, reduced polyplex binding to the cell surface, at 4 h post transfection (Figures 6.1 and 6.8). The data indicate that membrane fluidity/ lipid raft functionality is necessary for polyplex-cell surface binding. It is possible that had polyplex-cell surface binding been measured at earlier time points (i.e. <10 min post transfection), heparitinase treated cells may have displayed lower polyplex binding than control cells and that by 4 h post transfection, cell-surface binding may have saturated. Nonetheless, the data indicate that polyplexes bind to the cell surface in the absence of HSPGs.

The data presented in Figures 6.1 and 6.7 fit with data presented in Chapter 5 and support the theory that hydrophobic interactions contribute to polyplex-cell surface binding as follows: hydrophobic cholesterol assemblies are absolutely necessary for polyplex binding to the cell surface whereas HSPGs (generally anionic species) are not absolutely required for polyplex-cell surface binding. Furthermore, since there was no change of medium following heparitinase treatment, the data also demonstrate that extracellular HSPGs do not inhibit polyplex cell surface binding, opposite to the findings reported by Ruponen *et a l.*, (2001) (Table 6.1).

HSPGs have been demonstrated as lipid raft associated (Fuki *et al.*, 2000; Chu *et al.*, 2004) and confocal micrographs (Figure 6.7) indicate HSPG localization in distinct regions on the cell membrane, with and without addition of polyplexes, as described in section 6.1 (Chu *et al.*, 2004; Imamura *et al.*, 2011). Putatively, HSPGs provide a secondary layer of order, in addition to the PEI:DNA polyplex ligand, i.e. HSPGs aid the clustering of lipid rafts, following addition of PEI:DNA polyplexes, and subsequent lipid raft endocytosis. Alternatively, HSPGs, on co-internalization with polyplexes, may act as buffers within endosomes/ lysosomes, supporting the proton sponge capacity of PEI.

In summary, the reduction in PEI mediated TGE following cell surface HSPG depletion could stem from numerous bio-molecular scenarios, the most likely listed below:

1) Cell surface HSPGs mediate polyplex-cell surface binding immediately post transfection.
2) Polyplexes are endocytosed via HSPG-dependent endocytosis.
3) HSPGs aid polyplex induced lipid raft clustering and subsequent endocytosis.
4) HSPGs co-internalized with polyplexes act as an additional buffer to PEI, aiding lysosomal protection and escape, according to the “proton sponge hypothesis”.

Data presented in this chapters indicates that cell surface HSPGs do not mediate polyplex-cell surface binding, absolutely i.e. that HSPGS are not the sole cell surface receptor for polyplexes.

6.5 Chapter Conclusions

Following transfection, HSPGs were found to rapidly deplete from the cell surface, residing at 15% pre-transfection levels, between 30 min and 480 min post transfection; the data are indicative rapid HSPG endocytosis of polyplexes. Cell surface HSPGs are not absolutely required for PEI mediated TGE, HSPG lyase treatment reducing SEAP by ~25 % and having no effect on polyplex binding to the cell surface, at 4 hours post transfection. On the other hand, membrane fluidity and lipid raft functionality, assessed by MβCD treatment, was found to be fundamental for polyplex binding to the cell surface and subsequent TGE. These data support the data presented in Figure 5.4, demonstrating the role of hydrophobicity in polyplex-cell surface binding.

CHAPTER 7

Impact of canonical chemical inhibitors of endocytosis on PEI mediated TGE

Chapter Overview

The effect of canonical chemical inhibitors of endocytosis on PEI mediated TGE are explored in this chapter. The efficacy of the chemical inhibitors is explored through assessment of their toxicity to transfected and untransfected cells. The aims of this chapter are to:

- Determine the effect of a selection of canonical endocytic inhibitors on PEI mediated TGE.
- Assess the cytotoxicity of the same endocytic inhibitors on CHO cells.

7.1 Introduction. Endocytosis.

Doherty and McMahon (2009) describe endocytosis as "*the de novo production of internal membranes from the plasma membrane lipid bilayer*" by which "plasma membrane lipids and integral proteins and extracellular fluid become fully internalized into the cell" (Doherty and McMahon 2009:858).

Endocytosis is required for the uptake of polar molecules into the cell, that cannot pass through the hydrophobic cell membrane. It can be divided into phagocytosis (cell eating) or pinocytosis or (cell drinking). Phagocytosis only occurs in specialised macrophage cells and describes the process by which cells take up large particles, such as a bacterium. Pinocytosis (also known as fluid phase endocytosis) describes the process by which cells take up fluids and solutes. Efficiency of pinocytosis is largely dependent on the concentration of solutes in the medium. The efficiency of endocytosis is increased by non-specific binding of solutes to the cell membrane (adsorptive pinocytosis). Solutes are most efficiently endocytosed by cells through uptake by high affinity

receptors (receptor mediated endocytosis) which are themselves co-transported into endocytic vesicles (Conner and Schmid, 2003).

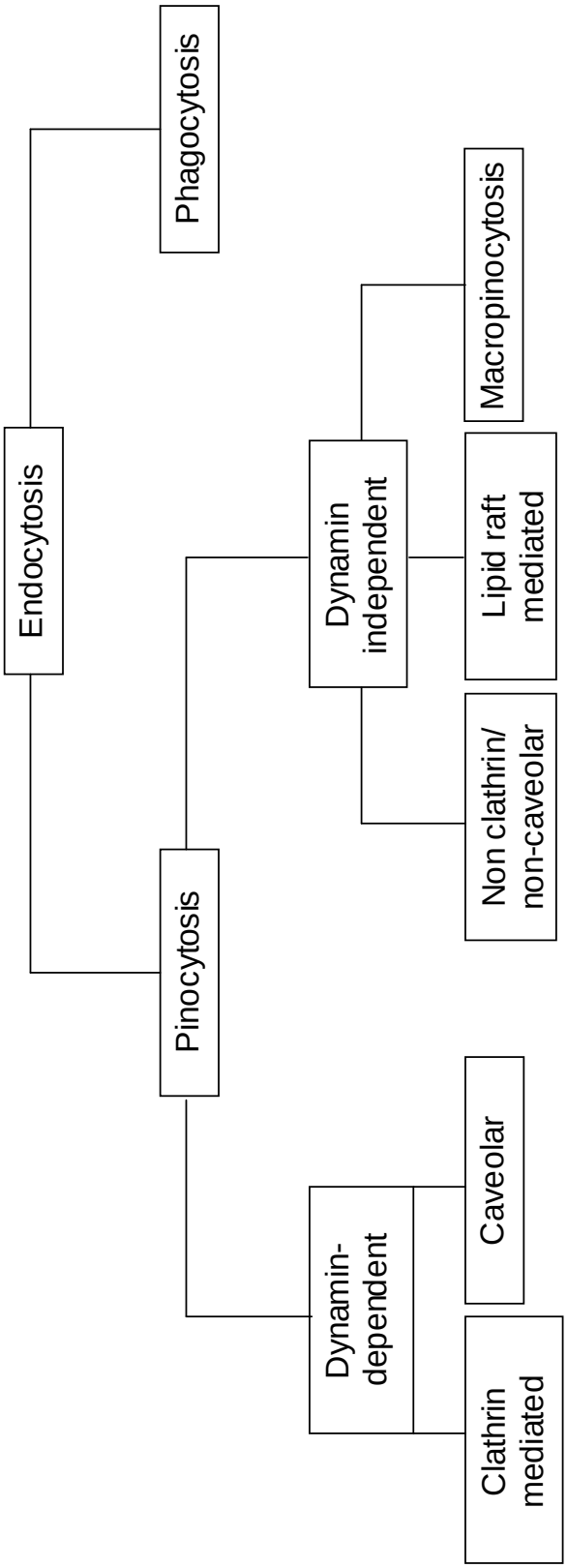

Figure 7.1 Schematic of major endocytic pathways (from Mercer and Helenius, 2009)

Pinocytic pathways can be further divided into those which require the GTPase, dynamin, for vesicle fission (Macia *et al.*, 2006; Ferguson and Camilli, 2012). As illustrated in Figure 7.1, caveolar and clathrin mediated pathways are dynamin dependent. Macropinocytosis, lipid raft mediated endocytosis and numerous other pathways (non clathrin/ non caveolar) are dynamin independent (Mercer and Helenius, 2009). However, Payne *et al.* (2007) reported a clathrin and caveolin independent but dynamin dependent pathway for internalization of proteoglycan bound ligands, so the definition used here is not universally applied. The level of endocytosis by a specific pathway is likely to be cell line or cell type specific, although clathrin and caveolae mediated endocytosis are thought to be equi-prevalent in fibroblasts (Doherty and McMahon, 2009). Recently, systems levels surveys of endocytosis have been undertaken through measurement of multiple key parameters (Collinet *et al.*, 2010), adding greatly to the detailed but fragmented knowledge of endocytosis.

7.1.1 Dynamin dependent: Clathrin

The clathrin protein has a three legged, triskelion structure, with three clathrin heavy chains each with an associated clathrin light chain (Conner and Schmid, 2003). The formation of clathrin polygonal cages, by the self-association of clathrin proteins, requires additional coat assembly proteins (APs) in physiological conditions (under non physiological conditions, of low salt and high calcium concentrations, formation of clathrin cages requires no additional proteins) (Conner and Schmid, 2003). The AP2 complexes are targeted to the plasma membrane by the α-adaptin subunit, its β2 subunit mediates clathrin assembly and the μ2 subunit interacts directly with motifs on the cargo molecule (Motley *et al.*, 2003). Dynamin, a multidomain GTPase acts at the neck of the clathrin coated pit and acts as a collar, to control membrane fission and the release of the clathrin coated pits (Conner and Schmid, 2003).

7.1.2 Dynamin dependent: Caveolar

Caveolae are flask shaped invaginations, present in cholesterol and sphingolipid rich micro-domains of the plasma membrane (Conner and Schmid, 2003; Midoux *et al.*, 2008). There are approximately 100-200 molecules of the dimeric protein, caveolin-1, in caveolae; they bind to cholesterol and confer a

loop like formation into the plasma membrane and self-associate to form a striated caveolin coat on the surface of the invaginations (Conner and Schmid, 2003). Mundy *et al.*, (2002) reported three types of caveolin compartments in CHO cells: caveolae at the cell surface kept in place by cortical actin filaments, intracellular caveosomes and caveolar vesicles, which move bi-directionally along microtubules between the cell surface and caveosomes.

Caveolae are slowly internalized, with a half time >20 min and the process is unlikely to significantly contribute to bulk endocytosis into the cell (Conner and Schmid, 2003). The SV40, Ebola Zaire, Marburg and echoviruses have been found to gain entry into cells through caveolae mediated endocytosis (Midoux *et al.,* 2008).

7.1.3 Dynamin independent: macropinocytosis

Macropinocytosis is non-selective and allows relatively large volumes to be taken up by the cell. A signalling cascade, involving Rho-family GTPases, as in phagocytosis, trigger actin driven membrane protrusions that then collapse and fuse with the plasma membrane, encapturing a volume of extracellular milieu; a process which accompanies membrane ruffling stimulated by growth factors amongst other things (Conner and Schmid, 2003; Doherty and McMahon, 2009). The role of macropinocytosis in amino acid supply to oncogenic Ras transformed cells has recently been reported (Commisso *et al.*, 2013).

7.1.4 Dynamin independent: lipid raft mediated

As described in section 6.1, the role of lipid rafts in the cyto-internalization of numerous agents (viruses etc.) has been reported (Simons and Gerl, 2010). The process relies on ligand-induced clustering of lipid rafts and subsequent formation of invaginations, which are cyto-internalized. A steric specific model for lipid raft endocytosis has been proposed (Bhagatji *et al.*, 2009), describing a bulk, non specific mechanism for lipid raft mediated endocytosis (Simons and Gerl, 2010).

The pentavalent Shiga toxin B gains entry into the cell via lipid raft endocytosis, following binding to their lipid raft glycosphingolipid receptor (Roemer *et al.*, 2007).

A noteworthy point is that caveolar caveosomes are a subset of membrane assemblies, defined by the presence of caveolin 1, and not synonymous with lipid rafts (Nabi and Le, 2003; Parton and Simons, 2007).

7.1.5 Dynamin independent: non-clathrin/ non-caveolar

In recent years other pinocytic pathways have been described; these include the CLIC/ GEEC, IL2Rβ, Arf6 dependent, flotillin dependent and circular dorsal ruffle pathways (Glebov *et al.,* 2005; Doherty and McMahon, 2009; Hansen and Nichols, 2009; Sandvig *et al.*, 2008)

Box 7.1 Chemical Inhibitors of Endocytosis

Genistein is a tyrosine kinase inhibitor that causes disruption of the actin cytoskeleton (Nabi *et al.,* 2003; Parton *et al.* 1994). Genistein has been widely used as inhibitor of caveolae mediated endocytosis (Rejman *et al.*, 2005; Van der Aa *et al.*, 2007), but given that it disrupts actin, it is likely to inhibit additional endocytic pathways, such as clathrin mediated and macropinocytosis (Robertson *et al.,* 2009).

Filipin belongs to the class of polyene antibiotics. Filipin has been found to cause aberrations in caveolar shape, dispersion of GPI anchored proteins, inhibition of lipid raft ligand internalization and to interact with membrane phospholipids (leading to membrane permeabilization at concentrations of 10 μM or above). In addition, it has been found to disrupt linkages between cortical actin and the plasma membrane (Ivanov, 2008).

Rottlerin was originally described as an inhibitor of protein kinase c (Gschwendt *et al.* 1994) but has since been described as ineffective for this use (Soltoff, 2007). Its non specific effects include mitochondrial uncoupling and protein phosphorylation (Soltoff, 2007). Rottlerin has also been described as an inhibitor of macropinocytosis by Sarkar *et al.* (2005), in monocyte derived dendritic cells. The tentative inhibitory properties of rottlerin against protein kinase c were not attributed to its inhibitory effect on macropinocytosis, since other PKC inhibitors did not have the same effect on macropinocytosis. Rottlerin's given IC_{50} value of 400 nM for fluid phase endocytosis inhibitor was approximately 7 fold lower than any value for PKC

inhibition and a 10 fold lower concentration than that required to inhibit uptake of transferrin by clathrin mediated endocytosis (Sarkar *et al.* 2005). Hufnagel *et al.* (2009) used rottlerin to inhibit fluid phase endocytosis.

Chlorpromazine hydrochloride, a cationic amphiphile, is a canonical inhibitor of clathrin mediated endocytosis. In the micro molar range, its inhibitory affect on clathrin mediated endocytosis is thought to be due to the loss of clathrin and adaptor protein (AP2) complexes from the cell surface (Ivanov, 2008). The chemical can be used to discriminate between clathrin and caveolae mediated endocytosis but it has been shown to block the uptake of fluid phase markers and to inhibit phagocytosis in cells of the immune system (Ivanov, 2008). Other associated non-specific effects include, reorganization of the cortical actin cytoskeleton and fluidization of the plasma membrane (due to its amphiphatic structure).

Phorbol 12-myristate 13-acetate (PMA) or Phorbol ester 12-O-tetradecanoylphorbol 13 acetate (TPA). Macropinocytosis was induced in A431 cells by PMA (Grimmer et al, 2002). PMA was shown to markedly increase syndecan shedding in SVEC4-10 endothelial cell by Subramanian *et al.* (1997) and the finding exploited by Jarousse and Coscoy (2008) to study the cell surface attachment of the murine gammaherpesvirus 68 to CHO cells. PMA is also used to stimulate cell proliferation (Rusnati *et al.*, 2002).

DMSO has been shown to enhance recombinant protein production in stably transfected CHO cells and to reduce growth rate (Liu *et al.*, 2001). In addition, DMSO was found to enhance transient protein production in CHO cells following transfection by PEI and to reduce viable cell density (Thompson, 2011).

7.1.7 Endocytosis of polyplexes

Rejman *et al.*, (2005) reported that treatment with filipin, genistein and chlorpromazine resulted in reduced polyplex uptake, but only filipin and genistein reduced transfection efficiency, in A459 and HeLa cells. Similarly, von

Gersdorff *et al.*, (2006) reported a reduction in transfection efficiency following treatment with chlorpromazine hydrochloride or fillipin, but that the effects were highly dependent on the cell line used and PEI type (e.g. branched or linear) used for polyplex formation. Hufnagel *et al.* (2009) reported that rottlerin (an inhibitor of fluid phase endocytosis) reduced polyplex uptake and gene expression following transfection with PEI in CHO cells. Conversely, Payne *et al.* (2007) reported that neither chlorpromazine or filipin reduced polyplex internalization by BS-C-1 cells, indicating clathrin and caveolin independent uptake. However, dynasore and Dyn-2 siRNA treatment indicated dynamin dependent uptake. In addition, internalization was found to be flotillin-1 dependent, since Flot-1 siRNA treatment reduced polyplex uptake (Payne *et al.*, 2007). Van der Aa *et al.* (2007) used a range of chemical inhibitors: nocodazol, wortmannin, LY239004, MβCD, chlorpromazine hydrochloride and genistein, in COS-7 cells prior to transfection with PEI. Only nocodazol, MβCD and genistein reduced polyplex uptake and gene expression, indicative of a caveolin mediated endocytic pathway.

The endocytosis of PEI:DNA polyplexes in CHO-K1 and CHO-DKO cells (Bax and Bak double knock out) was investigated using canonical chemical inhibitors of endocytosis (Macaraeg *et al.*, 2013). In both cell lines, clathrin, caveolae and macropinocytosis were found to mediate polyplex cyto-internalization, as a reduction in polyplex associated fluorescence was reported following incubation with chlorpromazine hydrochloride, genistein, MβCD, filipin and amiloride.

Internalization of PEI:DNA polyplexes is a fundamental step in TGE. It has been hypothesized that polyplexes are internalized via a specific endocytic pathway, clathrin, caveolin or via macropinocytosis (or fluid phase endocytosis). Much of the evidence surrounding PEI:DNA polyplex endocytic internalization pathways is rested on the use of chemical inhibitors of endocytosis.

7.2 Materials and Methods

For all experiments described in this chapter, CHO-S cells were used, using the culture conditions described in Chapter 3. Cells were transfected with 25 kDa linear polyethylenimine according to protocol A, in Nunc 24 well plates (Sigma-Aldrich, Dorset, UK) as described in Chapter 3, Tables 3.1 and 3.2.

7.2.1 Chemical treatmeents prior to transfection

Chlorpromazine hydrochloride, filipin, genistein, rottlerin, PMA and DMSO were all purchased from Sigma Aldrich. Genistein and rottlerin were dissolved in DMSO (Sigma Aldrich) and the other chemicals in water. Cells were transfected one hour post addition of chemical inhibitor.

7.2.2 Confocal microscopy

Alexa-555 dextran (Life Technologies, Paisley, UK) and PEI:DNA polyplexes were added to cells simultaneously. Cells were incubated at 37°C, 5% CO_2 for 90 min, prior to washing with CellScrub and resuspension in ice cold PBS, prior to live confocal microscopy analysis. Cells were transfected and dextran added at the same time. At 90 min following transfection, the cells were washed with CellScrub, to removed cell surface associated molecules, and resuspended in ice cold PBS. Cells were imaged using a Zeiss upright LSM510 confocal microscope using a 40x/ 0.8 objective, dipping lens and 488 and 543 nm lasers.

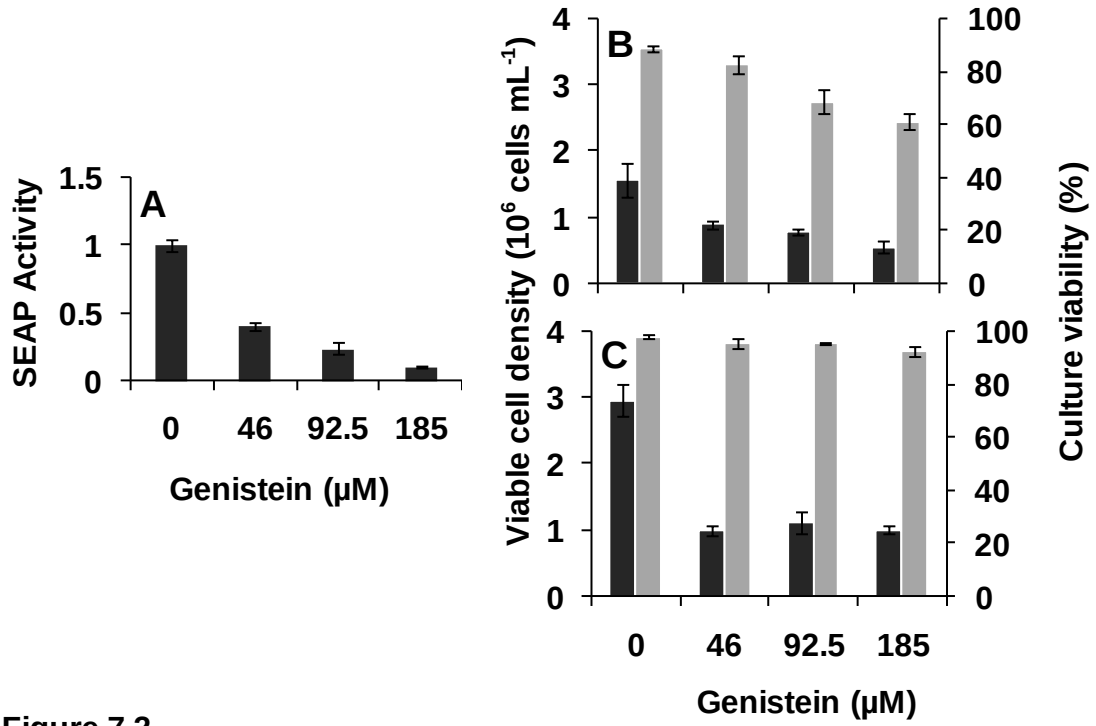

Figure 7.2
Effect of genistein on transient SEAP production and culture viability.
One hour prior to transfection with PEI, the indicated concentration of genistein was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

7.3 Results

7.3.1 Genistein

Addition of genistein resulted in a concentration dependent reduction in SEAP activity, at 24 h post transfection (Figure 7.2). At concentrations of 46.3, 92.5 and 185 µM genistein, SEAP activity was 41%, 24% and 10% relative to control, respectively (Figure 7.2A).

In transfected conditions, viable cell density decreased from $1.6x10^6$ cells mL^{-1} to 0.9, 0.8 and 0.6 $x10^6$ cells mL^{-1} at concentrations of 0, 46.3, 92.5 and 185 µM genistein (Figure 7.2B). Culture percentage viability reduced from 89% in control conditions to 83%, 69% and 62% at concentrations of 46.3, 92.5 and 185 µM respectively (Figure 7.2B).

Genistein significantly inhibited cell division in untransfected cells; reducing viable cell density from $3x10^6$ cells mL^{-1} in control conditions to approximately $1x10^6$ cells mL^{-1} in the presence of genistein (Figure 7.2C). Similarly, culture viability reduced from 98% in control conditions to 93% in the presence of 185 µM genistein (Figure 7.2C). The drop in viable cell density between genistein treated and control cells was greater for untransfected than transfected cells.

Given the loss in viable cell density due to genistein treatment, SEAP activity per cell was calculated. Based on this calculation, a step-wise, significant reduction in SEAP per cell relative to the control was seen at each tested concentration.

7.3.2 Filipin

Treatment with filipin resulted in a concentration dependent reduction in SEAP activity, at 24 h post transfection (Figure 7.3). Higher concentrations of filipin, 1.8 µM and above, abrogated SEAP production (Figure 7.3A). At a concentration of 0.9 µM filipin, SEAP activity was at 75% of control levels.

In transfected conditions, viable cell density decreased from $1.5x10^6$ cells mL^{-1} to 1.4 $x10^6$ cells mL^{-1} and $1x10^6$ cells mL^{-1} at concentrations of 0, 0.9 and 1.8 µM filipin, respectively (Figure 7.2B). Culture percentage viability was 87, 87 and 74% at concentrations of 0, 0.9 and 1.8 µM filipin, respectively (Figure

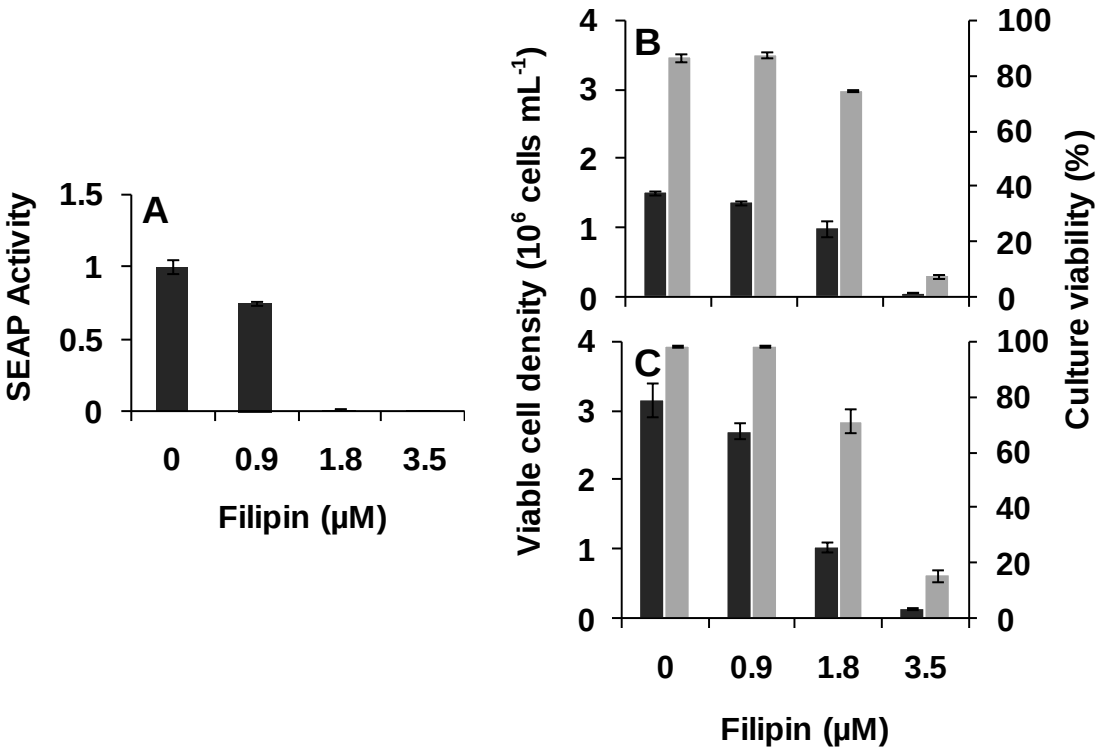

Figure 7.3 Effect of filipin on transient SEAP production and culture Viability.
One hour prior to transfection with PEI, the indicated concentration of filipin was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

7.2B). At a concentration of 3.5 µM filipin, with and without transfection, the cells were almost completely non-viable.

In untransfected cells, addition of filipin, at a concentration of 1.8 µM, reduced viable cell density to $1x10^6$ cells mL^{-1} compared to 3.2 10^6 cells mL^{-1} in control conditions (Figure 7.2C). For untransfected cells, culture percentage viability reduced from 98% to 71% at a concentration of 1.8 µM filipin (Figure 7.2C).

Given the loss in viable cell density due to filipin treatment, SEAP per cell was calculated. Based on this calculation, a step-wise, significant reduction in SEAP per cell relative to the control was seen up to a concentration of 1.8 µM filipin.

7.3.3 Rottlerin

Rottlerin significantly reduced transient SEAP production at 24 h post transfection, reducing SEAP activity to 16% relative to the control at a

concentration of 100 nM and obliterating SEAP activity at a 500 nM and 1 µM concentrations, respectively (Figure 7.4A).

Treatment with rottlerin reduced the division of transfected cells and culture percentage viability (Figure 7.4B). At a concentration of 100 nM rottlerin, viable cell density reduced to $1.1x10^6$ cells mL^{-1} relative to $1.4x10^6$ cells mL^{-1} but there was little change in culture percentage viability.

Rottlerin significantly inhibited division of untransfected cells and culture percentage viability (Figure 7.4C). The drop in viable cell density between rottlerin treated and control cells was greater for untransfected than transfected cells.

Given some loss in viable cell density due to rottlerin treatment, SEAP per cell was calculated. Based on this calculation, 100 nM rottlerin reduced SEAP activity to 20% of the control level and higher concentrations almost completely obliterated SEAP activity.

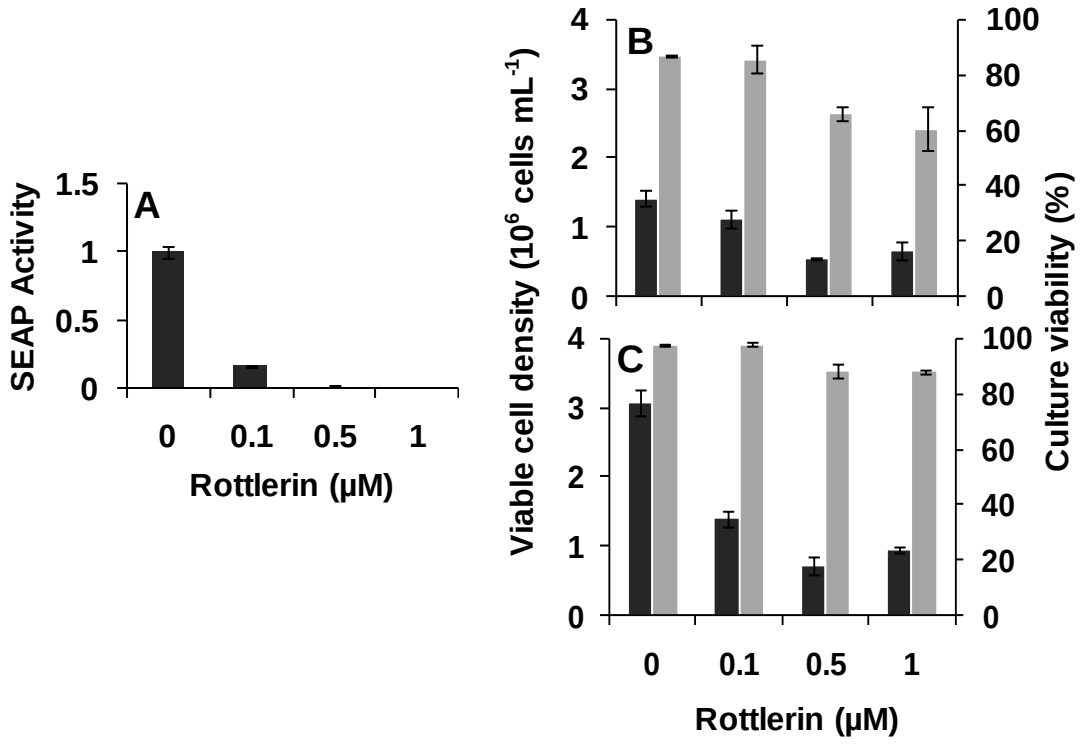

Figure 7.4
Effect of rottlerin on transient SEAP production and culture viability.
One hour prior to transfection with PEI, the indicated concentration of rottlerin was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

7.3.4 Chlorpromazine hydrochloride

Treatment with chlorpromazine significantly reduced transient SEAP production at 24 h post transfection, halving SEAP activity at a concentration 12.5 µM and abrogating SEAP activity at a concentration of 25 µM (Figure 7.5A).

Treatment with chlorpromazine, at a concentration of 12.5 µM had no significant effect on viable cell density, however, at a concentration of 25 µM, viable cell density reduced to $0.5x10^6$ cells mL^{-1} compared to $1.3x10^6$ cells mL^{-1} in control conditions (Figure 7.5B). At a concentration of 25 µM chlorpromazine, culture percentage viability also reduced to 74% relative to 88% in the absence of drug treatment.

Chlorpromazine significantly inhibited division of untransfected cells, at a concentration of 12.5 µM and 25 µM, reducing viable cell density to $2x10^6$ cells mL^{-1} and $1x10^6$ cells mL^{-1}, respectively, relative to $2.6x10^6$ cells mL^{-1} in the absence of chlorpromazine (Figure 7.5C). Culture percentage viability decreased to 89% at a concentration of 25 µM chlorpromazine, relative to 98% in control conditions.

Given some loss of viable cell density due to chlorpromazine treatment, SEAP per cell was calculated. Based on this calculation, 12.5 µM chlorpromazine almost halved SEAP activity relative to control and at a concentration of 25 µM chlorpromazine, SEAP activity was abrogated.

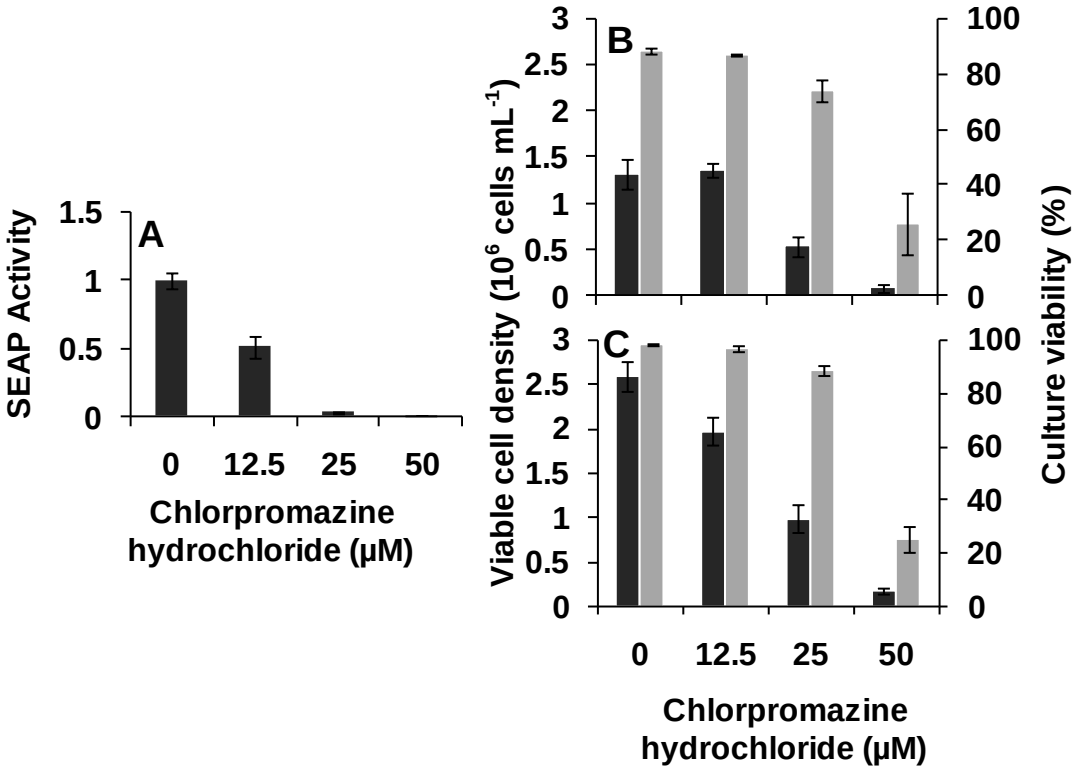

Figure 7.5
Effect of chlorpromazine hydrochloride on transient SEAP production and culture viability.
One hour prior to transfection with PEI, the indicated concentration of chlorpromazine hydrochloride was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

7.3.5 Phorbol 12-myristate 13

Phorbol 12-myristate 13 (PMA), at all concentrations tested, resulted in an incremental but significant reduction in transient SEAP production, of ~10% (Figure 7.6A). PMA had no effect on viable cell density or culture percentage viability of transfected cells, at any of the concentrations tested (Figure 7.6 B). Since PMA has also been found to trigger syndecan shedding, cells were stained for HSPGs, using anti-HSPG mAb HepSS1, but there was found to be no difference in cell surface HSPG level between PMA treated and control cells (Figure 7.6D). In addition, PMA had no effect on the culture percentage viability of untransfected cells but viable cell density reduced from 3.1×10^6 cells mL^{-1} to 2.1×10^6 cells mL^{-1} at a concentration of 2 µM PMA (Figure 7.6C).

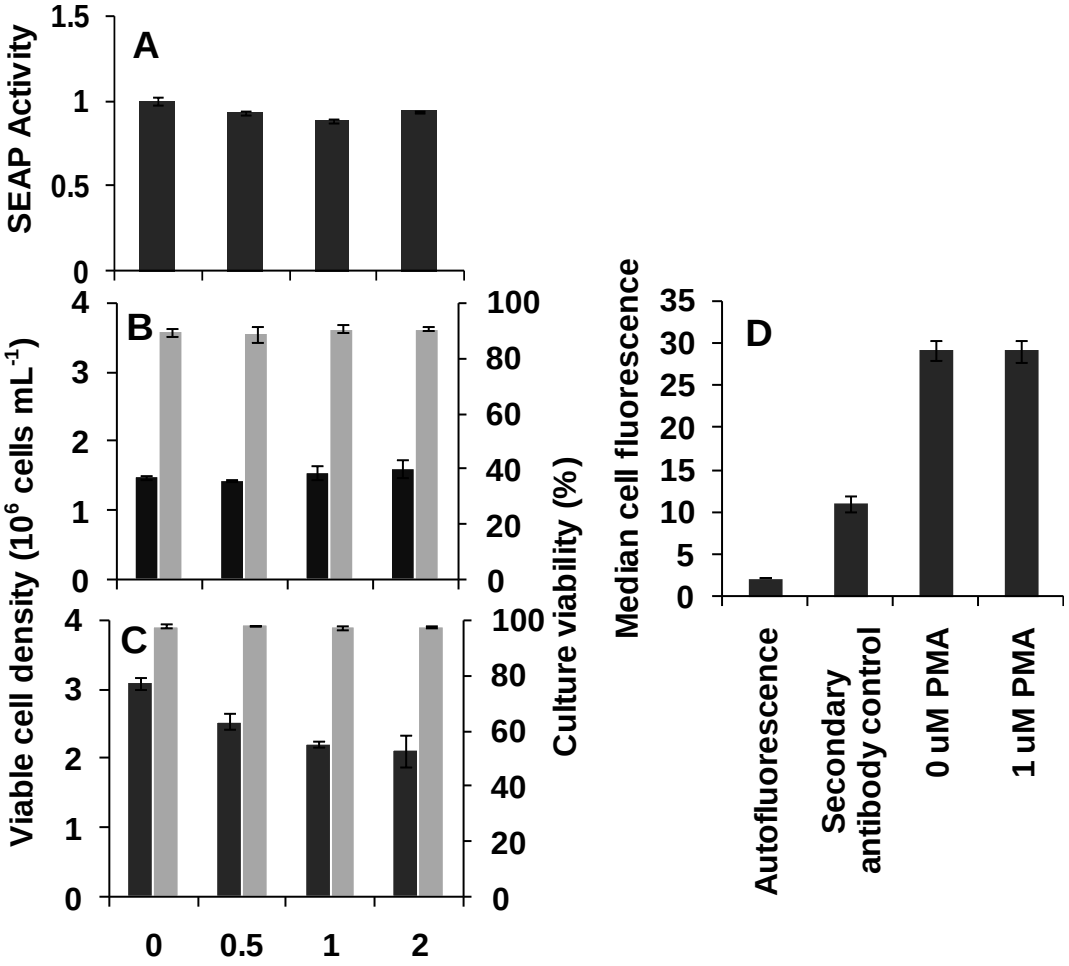

Figure 7.6
Effect of Phorbol 12-myristate 13-acetate (PMA) on transient SEAP production and culture viability.
One hour prior to transfection with PEI, the indicated concentration of PMA was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). Cells fixed were stained with anti-HSPG antibody, HepSS1 and secondary anti-mouse IgM FITC secondary antibody and cellular fluorescence measured by flow cytometry. The mean value ± standard deviation from triplicate transfections or cultures is shown.

7.3.6 DMSO

The positive effect of DMSO on TGE has been previously reported (Thompson, 2011; Ye *et al.*, 2009). Since it was used to dissolve genistein and rottlerin, the effect of the vehicle was tested at a range of concentrations (Figure 7.7). At the concentrations tested, below those reported to have an effect on TGE, it was indeed found to have no effect on transient SEAP production (Figure 7.7A) or the viable cell density or culture percentage viability of transfected or untransfected cells (Figure 7.7B and C).

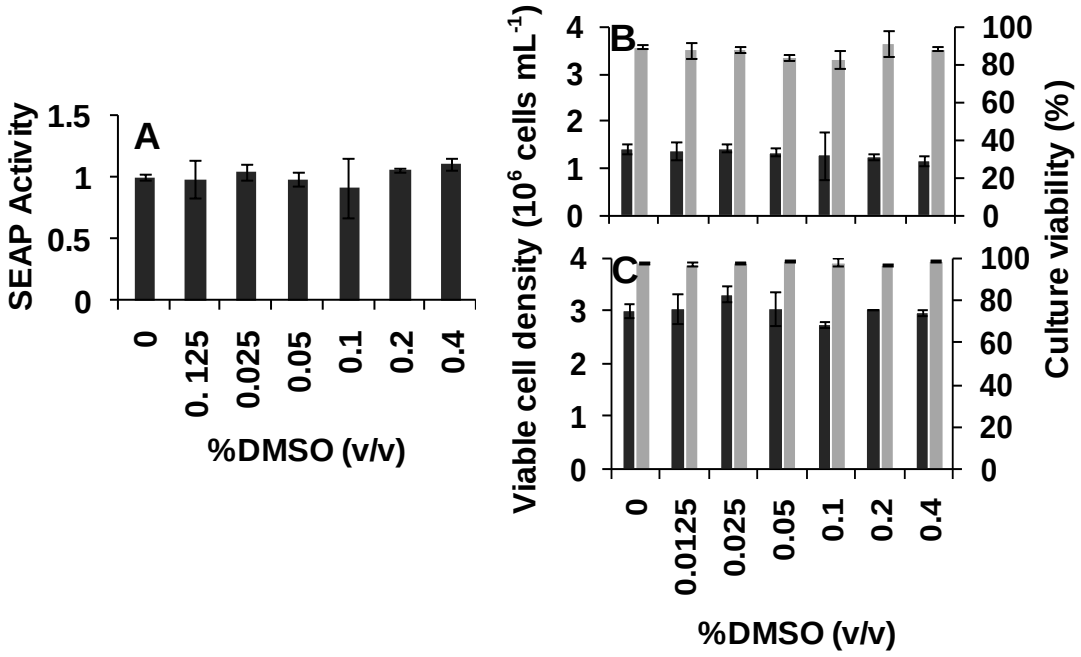

Figure 7.7
Effect of DMSO on transient SEAP production and culture viability.
One hour prior to transfection with PEI, the indicated concentration of DMSO was added to CHO-S cells. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) were measured at 24 h post transfection (**B**) or 24 h post sub-culture (**C**). The mean value ± standard deviation from triplicate transfections or cultures is shown.

7.3.7 Effect of chemical inhibitors on polyplex internalization

It was found that CellScrub wash reagent could not be used alongside chemical inhibitors. Cells were treated with chemical inhibitors of endocytosis, rottlerin, genistein and chlorpromazine hydrochloride and transfected with PEI and fluoro labelled plasmid DNA. At one hour post transfection, the cells were pelleted and resuspended in CellScrub or PBS solution. However, following flow cytometric analysis, CellScrub treated cells had higher fluorescent readings than phosphate buffered saline treated cells. The antagonistic chemical interactions between CellScrub and the endocytic chemical inhibitors made it impossible to distinguish between cell surface-attached and polyplex internalized by the cell. Within the literature, uptake of fluoro-labelled lipo-/ poly-plexes has been used only following washing cells with PBS solution, not CellScrub (Van der Aa *et al.*, 2007, Payne *et al.*, 2007, Rejman *et al.*, 2005).

7.3.8 PEI:DNA polylexes and dextran to not co-localize

Dextran is a canonical marker of macropinocytosis (Ivanov, 2008). Confocal microscopy was used to test the localization of fluorescently labelled dextran and polyplexes. It was hypothesized that PEI:DNA polyplexes are internalized via a macropinocytic pathway (or one defined by the uptake of dextran) and that co-localization between dextran and polyplexes would be observed upon examination by confocal microscopy. Using confocal microscopy, alexa-555-dextran and fluorescein labelled polyplexes were found not to colocalize and dextran and polyplexes were found to localize in distinct regions (Figure 7.8).

7.4 Discussion

The chemical inhibitors, chlorpromazine hydrochloride, genistein, rottlerin and filipin were all found to reduce TGE in a concentration dependent manner. Traditionally, the chemicals have been described as inhibitors of clathrin, caveolin, macropinocytosis or caveolin mediated endocytosis respectively. By this definition, the data suggest that all three endocytic pathways contribute to PEI:DNA polyplex delivery in CHO-S cells and subsequent TGE. However, as described in Box 7.1, non-specific inhibitory effects of chemical inhibitors of endocytosis have been reported (Ivanov, 2008).

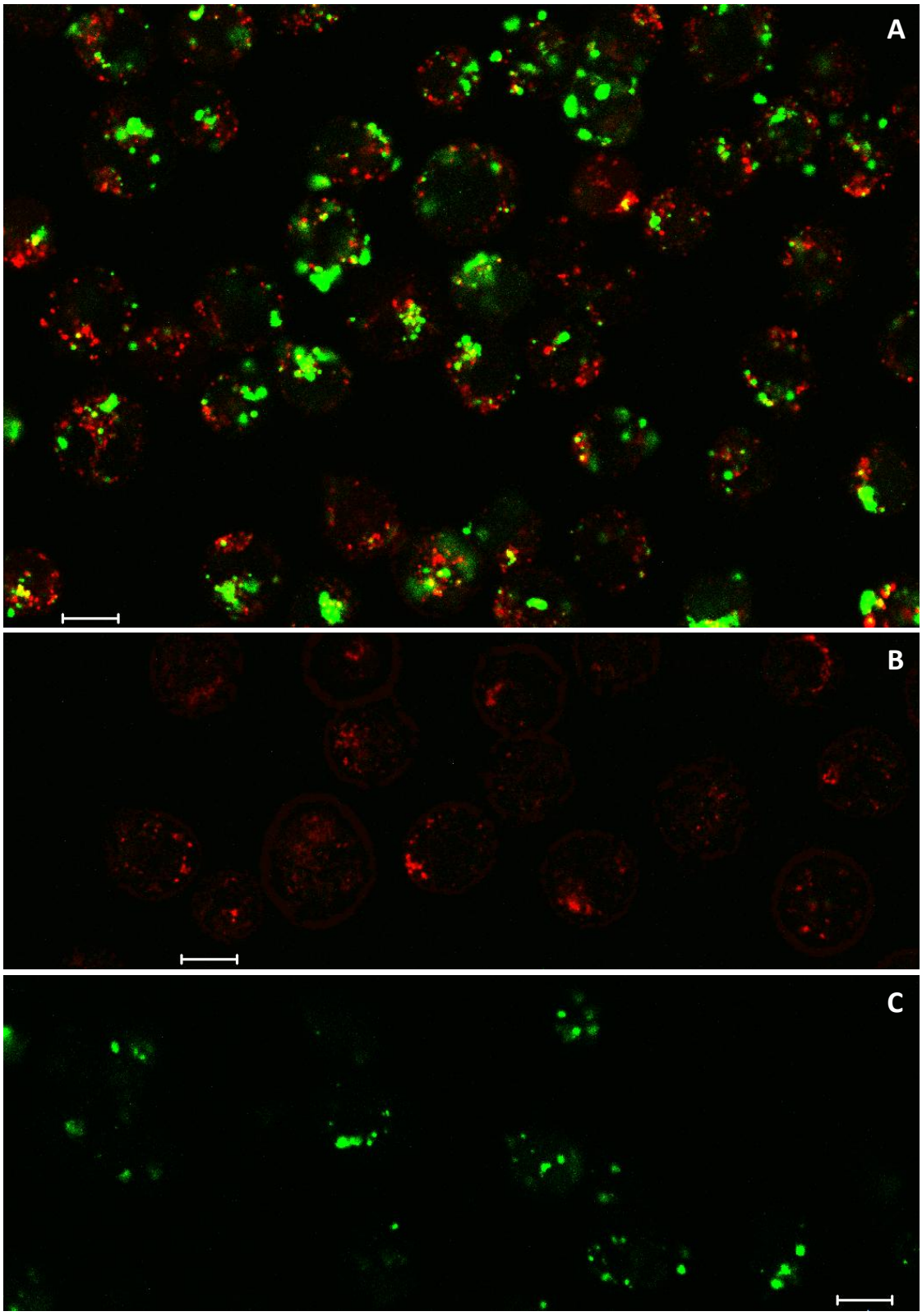

Figure 7.8 Dextran and polyplex do not co-localize.
A: Fluoroscein labelled polyplexes and alexa555 dextran do not colocalize. Single stain controls: **B**: Alexa555 dextran, **C**: fluoroscein labelled polyplex. Cells were transfected with PEI and FITC-labelled plasmid DNA and 50 µg mL^{-1} alexa 555 dextran and incubated for 1.5 h at 37°C and 5% CO_2. Cells were washed with CellScrub™ and immersed in PBS before live cell imaging using an upright LSM510 meta confocal microscope with a achroplan 40x/0.8 objective and 488 and 543nm lasers. Bar = 10 µm.

The data presented here is similar to that obtained at Genentech on CHO-K1 and their engineered CHO-DKO cell line (Macaraeg *et al.*, 2013), who reported that chlorpromazine, filipin and genistein reduced polyplex uptake and further concluded that caveolae and clathrin mediated endocytosis were utilized for polyplex uptake.

For untransfected cells, chlorpromazine hydrochloride, genistein, rottlerin and filipin all caused significant cytotoxicity, in terms of reduced cell doubling time. Although, for all inhibitors tested, reduction in viable cell density, did not account for reduced reporter protein output, the observation is an indicator of numerous non-specific drug side-effects. Thus, as described by Ivanov, (2008) and Vercauteren *et al.*, (2010), linking data obtained from use of a chemical inhibitor of endocytosis to a specifically defined endocytic pathway should be approached with caution.

Alternative methods for measuring endocytic pathways include the use of CHO knock-out cell lines (lacking caveolin 1 or clathrin for example) (Vercauteren *et al.*, 2010) or silencing RNAs against caveolin 1 or clathrin proteins (Payne 2007).

7.5 Chapter Conclusions

From the data presented in this chapter, measuring the effect of canonical chemical inhibitors on PEI mediated TGE, it can be concluded that several endocytic pathways mediate efficient transgene delivery for maximal TGE. However, this is a tentative conclusion, given the observed reduction in cell division caused by each of the inhibitors, on untrasfected and transfected cells alike.

CHAPTER 8

Engineering Strategies to enhance TGE

Clone screening and derivatized PEIs with enhanced hydrophobicity

Acknowledgements

Cloning of the CHO-S parental cell line and maintenance of clones was performed by Alejandro Fernandez-Martell.

Chapter Overview

Built on data presented in previous chapters and within the literature, explored within this chapter are engineering strategies to improve the TGE process, with respect to total recombinant protein titre, cytotoxicity and efficiency of production with respect to plasmid DNA. Engineering strategies focus on a) the gene delivery vehicle and b) the host cell line. The aims of this chapter are to:

- Explore the effectiveness of alkylated PEIs for transfection and gene expression in CHO-S cells.
- Explore the use of clonal derivatives of the parental CHO-S cell line for TGE mediated by PEI.

8.1Introduction

8.1.1 Polyethylenimines with enhanced hydrophobicity

The hydrophobicity of PEI:DNA polyplexes has been enhanced through chemical derivatization of the PEI polymer itself or though inclusion of a secondary hydrophobic (often lipid based) moiety (Incani *et al.*, 2010; Liu *et al.*, 2010).

PEIs have been chemically derivatized within the Klibanov group, with the aim of enhancing PEI mediated gene delivery, by modifying the proton sponge capacity, hydrophobicity and lipophilicity of the polymers (Thomas and Klibanov, 2002). Moderate enhancement of hydrophobicity, by *N*-acylation with alanine, increased transgene expression in COS-7 cells, relative to unmodified 25 kDa branched PEI, in the absence of serum. In the presence of serum, *N*-acylation with alanine and dodecylation of PEI tertiary amines, lead to enhanced transgene expression, relative to unmodified 25 kDa linear PEI. Hydrophobic modifications to 2 kDa PEI, through derivatization with dodecyl- or hexadecyl-groups, increased transgene expression by 400 and 550 fold respectively in the presence of serum, exceeding the level mediated by 25 kDa branched PEI (Thomas and Klibanov, 2002).

More recently, Fortune *et al.*, (2011) increased the hydrophobicity of linear 22 kDa PEI by synthesising methyl-, ethyl-, propyl-, butyl- and octyl- PEIs and explored gene delivery *in vivo*. Increased hydrocarbon chain length increased gene expression in the kidney, stepwise from methyl- to octyl- PEIs. In the liver and heart, increased chain length increased gene expression, although in both cases propyl-PEI lead to the highest gene expression, with butyl- and octyl-PEIs leading to relatively lower gene expression. In the lung, ethyl-PEI lead to a 26 fold enhanced expression relative to unmodified PEI. Using radio-active tracing, alkylated PEIs were found to localize in organs to a similar extent as unmodified PEI, indicating that alkylation of PEIs did not enhance cell-surface binding.

Primary and secondary amines of branched PEI were acylated to form primary and secondary amides respectively (Forrest *et al.*, 2004). Partial acetylation of branched PEI was found to enhance transgene expression in C2C12 and MDA-MB-231 cells, with and without serum, with negligible effects on cytotoxicity. Acylation was found to reduce the buffering capacity of the polymer at physiological conditions, reduce the zeta potential of polyplexes (14 mV to 8-11 mV), increase the particle diameter (2-3 fold) (Forrest *et al.*, 2004) and reduce binding affinity with DNA (Garbrielson and Pack, 2006). Aravindan *et al.*, (2009) increased the hydrophobicity of acyl-PEI, by increasing carbon chain length,

and found PEIs acylated with propionic anhydride to result in better transgene expression than PEIs acylated with acetic anhydride.

Lee CH *et al.*, (2003) reported PEI:DNA–DOTAP-Cholesterol complexes to result in increased transgene expression in a range of human cancer cell lines (HeLa, HepG2, 2.2.15 cells) relative to PEI or cationic lipid mediated gene delivery. Similarly, Garcia *et al.*, (2007) reported enhanced transgene expression using PEI:DNA-DOTAP-Cholesterol complexes, in HepG2 cells, at high serum concentrations, relative to PEI or DOTAP-Cholesterol mediated gene delivery. Matsumoto *et al.*, (2008) found PEI-DOTMA mediated transfection to result in higher transgene expression *in vivo* following intravenous injection in mice, relative to transfection with just PEI. Recently, the efficacy of cationic lipid coated PEI:DNA polyplexes for the transfection of mesenchymal stem cells has been reported (Song *et al.*, 2012). Putatively, lipid shells provide enhanced targeting to cell surface lipid rafts (Anderson and Jacobson, 2002).

Cyclodextrin (CD), which can encase small hydrophobic molecules, was covalently coupled to PEI (Forrest *et al.*, 2005). CD-PEI:DNA complexes were found to have an increased particle diameter than PEI:DNA complexes and cyclodextrin-PEI was found to mediated higher transgene expression in HEK293 cells than unmodified PEI. In addition, CD-PEI was found to be less toxic (to decrease metabolic activity to a lesser extent) than unmodified PEI, in HEK-293 cells.

8.1.2 Cell line selection from clonal derivatives of the parental

Chinese hamster ovary cell lines display functional heterogeneity (Yoon *et al.*, 2004; Barnes *et al.*, 2006; Davies *et al.*, 2012). The heterogeneity stems from genomic, at the level of single bases to the entire karyotype (Derouazi *et al.*, 2006b; Wurm and Hacker, 2011) and epigenetic instability (Yang *et al.*, 2010; Kim *et al.*, 2011). Derived from normal (non-cancerous) tissue from the ovary of a Chinese hamster (Tjio *et al.*, 1958; Puck *et al.*, 1958) , the karyotype of CHO cell lines differs considerably from that of the animal (Deaven and Petersen, 1973; Derouazi *et al.*, 2006b). Thus, whilst the recently published sequence of the CHO-K1 cell line (Xu *et al.*, 2011) provides a plethora of tools for cell line

engineering and development, its use has limitations due to the heterogeneity between the thousands of CHO cell lines, both “parents” and therapeutic protein-producing (Wurm and Hacker, 2011).

Measured functional heterogeneity between cell lines is based on population averages (Altschuler and Wu, 2010). Within clonal cell lines heterogeneity also exists (Pilbrough *et al.*, 2009; Davies *et al.*, 2012). In this case, the heterogeneity has also been attributed to chromosomal instability (Deaven and Petersen, 1973) and to stochastic gene expression (Elowitz *et al.*, 2002; Sigal *et al.*, 2006; Raj and Oudenaarden, 2008).

The exploitation of cell line clonal heterogeneity is well established for the generation of stable recombinant cell lines (Chapter 1) (Wurm, 2004), not just with respect to r-protein production (O’Callaghan *et al.*, 2010; Porter *et al.*, 2010), but also with respect to secondary traits required for biomanufacturing, such as ability to grow in bioreactors/ synthetic culture environments (Sinacore *et al.*, 2000).

Recently, cell heterogeneity has been exploited for biomanufacturing, to yield host cells lines with superior phenotypes for given biomanufacturing processes (Pichler *et al.*, 2010; Davies *et al.*, 2012). Pichler *et al.*, (2010) transiently transfected CHO-K1 and CHO-S cells by electroporation with an IgG coding vector and FACS sorted the cells according to anti-human IgG R-phycoerythrin staining. The highest 1% of producers were sorted three times, each at 2 days post transient transfection. The sorted population, of both CHO-S and CHO-K1SV, displayed a threefold improvement in cell specific production rate (*qP*) relative to the respective original parental cell lines, immediately post sorting and at three months post sorting, displaying the heritability of the process.

The heterogeneity within the CHOK1SV cell line was explored in detail by Davies *et al.*, (2012), who isolated (by limited dilution cloning) and maintained 199 clones from the parental CHOK1SV population. The clones were found to vary significantly in specific proliferation rate during micro-plate suspension culture and transient mAb and GFP production, following electroporation or lipofection. Transient mAb and GFP expression, following gene delivery by electroporation, varied 3.7 and 7.6 fold between clones, respectively. Following

gene delivery by lipofection (lipofectamine 2000) clones exhibited a 42.5 and 139 fold variation in mAb and GFP production, respectively. A subset of 23 clones were subjected to extended sub-culture and freeze-thawing regimes and although transient mAb production was not heritable per se, clones were identified with heritable properties, with respect to specific proliferation rate, endocytic transfectability and *N*-glycan processing.

8.2 Materials and Methods

8.2.1 Limited dilution cloning of parental CHO-S cell line

The suspension-adapted parental cell line CHO-S (Life Technologies, Paisley, UK) and cultivated in CD-CHO medium (Life Technologies) supplemented with 8 mM L-glutamine (Life Technologies). Clonally derived CHO-S cell lines were isolated by two rounds of limiting dilution cloning (LCD). Cells were plated at a cell density of 0.5 cells well^{-1} in 96-well plates and incubated at 37^{o}C, 5% (v/v) CO_2 for 21 days in a static incubator (Thermo Scientific Heraeus). At day 2 post-seeding, wells containing single colonies were identified by microscopic examination. After 21 days, cultures were subject to a second round of LCD, as described above. Clones were expanded to 24-well plates, 6-well plates, T-25 static flasks and finally 125 mL Erlenmeyer shake flasks (Corning) (Sigma-Aldrich, Dorset, UK). Clones were maintained and banked in liquid nitrogen as described in Chapter 3.

8.2.2 Alkylation of polyethylenimine

Polyethylenimine (25 kDa linear) (Polysciences – Park Scientific, Northampton, UK) (1 g) in absolute ethanol (10 mL) was treated with the appropriate alkylating agent (ethyl, propyl, octyl 10 mol%) (Figure 8.1) (Farapack Polymers, Sheffield, UK). The reaction mixture was heated overnight at 50°C, concentrated in vacuo, dissolved in water, treated with a drop of 10 M NaOH, dialyzed and then lyophilized to yield the various alkylated PEIs. The percentage alkylation was 10%, 7% and 10% for ethyl-, propyl- and octyl-PEI, respectively.

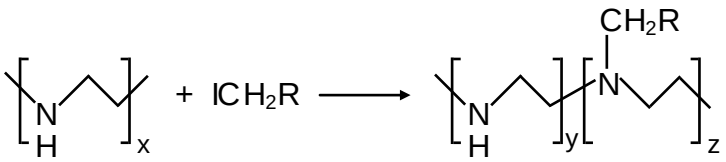

Figure 8.1 Schematic of polyethylenimine alkylation
PEI (25 kDa) linear (Polysciences) was alkylated with ethyl-, propyl and octyl iodoalkanes (Farapack polymers, Sheffield, UK).

R=CH_3, CH_2CH_3, $(CH_2)_6CH_3$

$$x = \frac{25000}{43.1} = 580$$

$$y = 580 - z$$

$$z = (\%\ alkylation) \cdot \frac{580}{100}$$

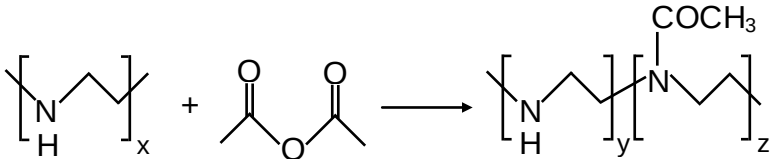

Figure 8.2 Schematic of polyethylenimine acetylation
PEI (25 kDa) linear (Polysciences) was acylated with ethanoic anhydride (Farapack polymers, Sheffield, UK).

$$x = \frac{25000}{43.1} = 580$$

$$y = 580 - z$$

$$z = (\%\ acetylation) \cdot \frac{580}{100}$$

8.2.3 Acetylation of polyethylenimine

Polyethylenimine (25kDa linear) (Polysciences) (1 g) in methanol (10 mL) was acetylated with ethanoic anhydride (10 mol%) (Figure 8.2) (Farapack Polymers, Sheffield, UK). The reaction mixture was heated overnight at 50°C, concentrated in vacuo, dissolved in water, treated with a drop of 10 M NaOH, dialyzed and then lyophilized to yield the amidated PEI.

8.3 Results

8.3.1 Screening of derivatized polyethylenimines

The hypothesis was tested that PEIs chemically derivatized for enhanced hydrophobicity (e.g. ethyl-, propyl- and octyl-PEI) would enhance TGE, through increased binding to the cell surface. Acetylated PEI, having a polar acetyl group, was included as a control. Ethyl-, propyl-, octyl- and acetyl- PEIs were screened by transfecting according to protocol A (Figure 8.3). At 48 h post transfection, TGE following transfection with ethyl PEI was <50% the level obtained following transfection with unmodified PEI. Both octyl-PEI and acetyl-PEI resulted in gene expression at <2% of levels obtained using unmodified PEI. TGE following transfection with propyl PEI was at 89% the level obtained following transfection with unmodified PEI (Figure 8.3A).

Transfection with unmodified and propyl-PEI resulted in similar cell growth and culture viability (Figure 8.3B). Viable cell density was slightly higher following transfection with ethyl-PEI, relative to unmodified or propyl-PEI. Transfection with octyl-PEI resulted in significantly lower viable cell density and culture viability, relative to unmodified PEI, at $1.3x10^6$ cells mL^{-1} and 68% compared to $2.1x10^6$cells mL^{-1} and 91% respectively. Transfection with acetyl-PEI resulted in higher growth and culture viability, relative to transfection with unmodified PEI, at $2.9\ x10^6$ cells mL^{-1} and 95%, respectively (Figure 8.3B).

Formation of PEI:DNA polyplexes with unmodified-, ethyl- or propyl- PEI had no significant effect on polyplex cell surface binding (Figure 8.3C). However, octyl-PEI:DNA polyplex binding to the cell surface was significantly lower than for polyplexes formed with unmodified PEI. No cellular fluorescence attributed to

polyplex-cell surface binding could be measured for polyplexes formed with acetyl-PEI (Figure 8.3C). Given that acetyl-PEI polyplexes did not bind to the cell surface, it was not surprising that they displayed low cyto-toxicity.

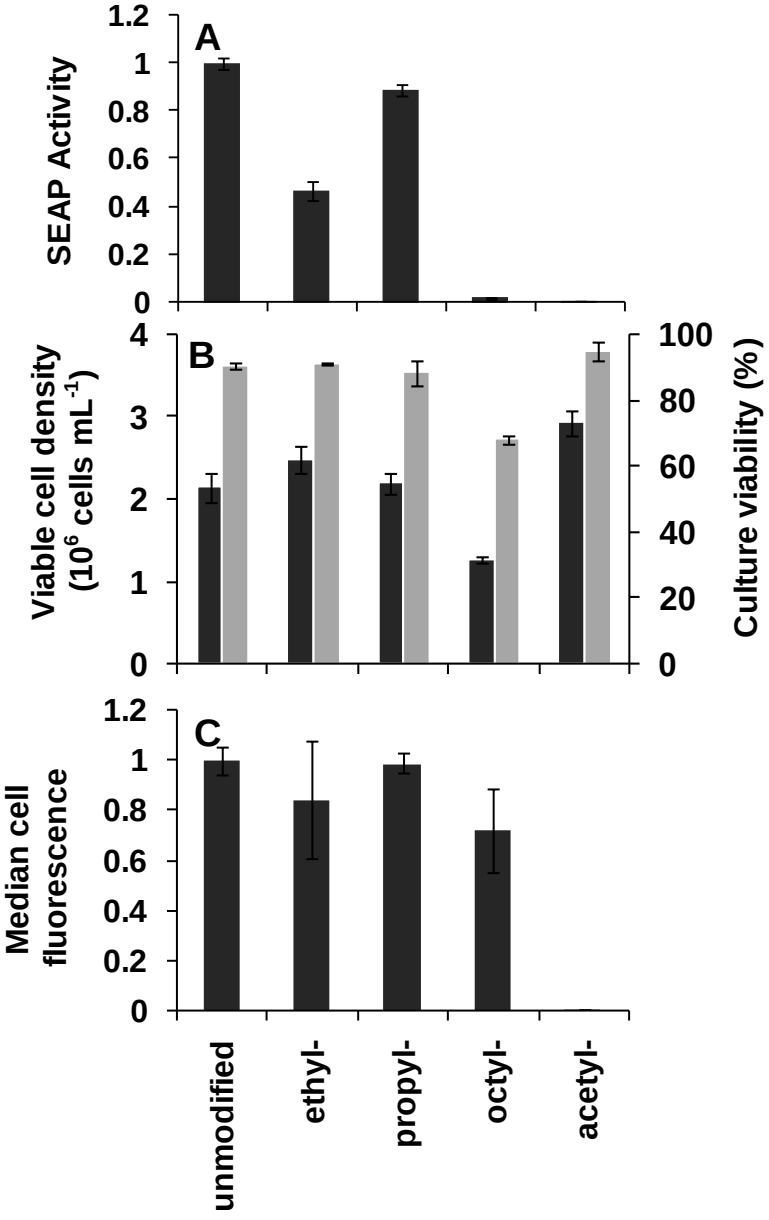

Figure 8.3 Alkylated and Acetylated PEI screening
CHO-S cells were transfected according to standard conditions (protocol A), using the indicated PEI based polymer. SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured at 48 h post transfection. Polyplex-cell surface binding was measured by using fluoroscein labelled plasmid DNA, incubating the transfection at 4°C for 4 h and measuring cellular fluorescence using flow cytometry (**C**). The mean value ± standard deviation from triplicate transfections or biological samples is shown.

The cyto-toxicity of ethyl-, propyl-, octyl- and acetyl- PEI was compared to unmodified 25 kDa linear PEI (Figure 8.4). Cells (CHO-S) were sub-cultured at a density of $1x10^6$ cells mL^{-1} in a total volume of 5 mL in culti-flasks. At a concentration of 3.75 µg 10^6 $cells^{-1}$, acetyl PEI had no significant effect on viable cell density, compared to control, untreated cells. Other than acetyl-PEI, unmodified PEI was the least cytotoxic, measured by reduced cell division (viable cell density), followed by propyl-, ethyl- and octyl- PEI (Figure 8.4A).

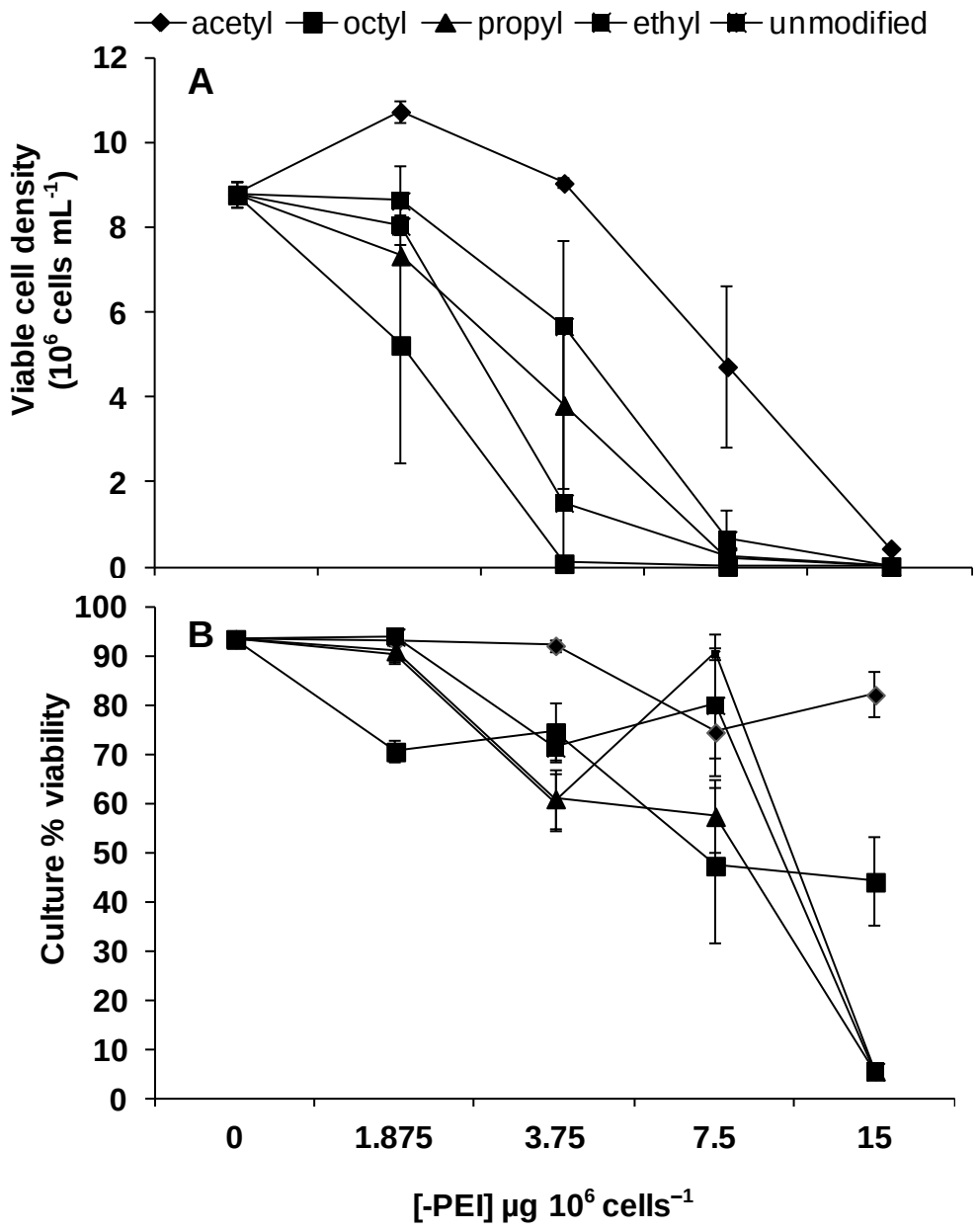

Figure 8.4 Cyto-toxicity profile of derivatized 25 kDa PEI: ethyl, propyl, octyl, acetyl (and unmodified).

Cells (CHO S) were cultured in a TubeSpin® bioreactor 50 at a density of $1x10^6$ cells mL^{-1} in CD CHO, 8mM L-glutamine, in a total volume of 5 mL. The indicated concentration of PEI (dissolved to 1mg mL^{-1} in deionised water) was added to cells, at a ratio of 3:100. Cells were in incubated at 37°C, 170 rpm for 5 days prior to measurement of culture % viability and viable cell density. The mean value ± standard deviation from triplicate cultures is shown.

8.3.2 DoE-RSM optimization of propyl-PEI and unmodified-PEI mediated transfection

Given that transfection mediated by propyl-PEI yielded TGE similar to that obtained by unmodified PEI, both unmodified PEI and propyl-PEI were optimized using DoE-RSM, varying DNA concentration and PEI:DNA ratio (Figure 8.5). Transfections were conducted in cultiflasks, in a total volume of 5 mL and incubated for a 5 day period, at 37°C, 170 rpm, 5% CO_2. Cell seeding density was kept constant at 10^6 cells mL^{-1}. Propyl-PEI and unmodified PEI were optimized for two host cell lines, the parental CHO-S and Clone 4 of CHO-S. SEAP, viable cell density and culture percentage viability were measured at 5 days post transfection. The model was used to predict optimal transfection conditions. Interestingly, although maximum SEAP activity was not significantly higher following transfection mediated by propyl-PEI relative to unmodified PEI, as observed via facile screening (Figure 8.3), propyl-PEI had a lower optimal carrying capacity for DNA, relative to unmodified PEI (Figure 8.5).

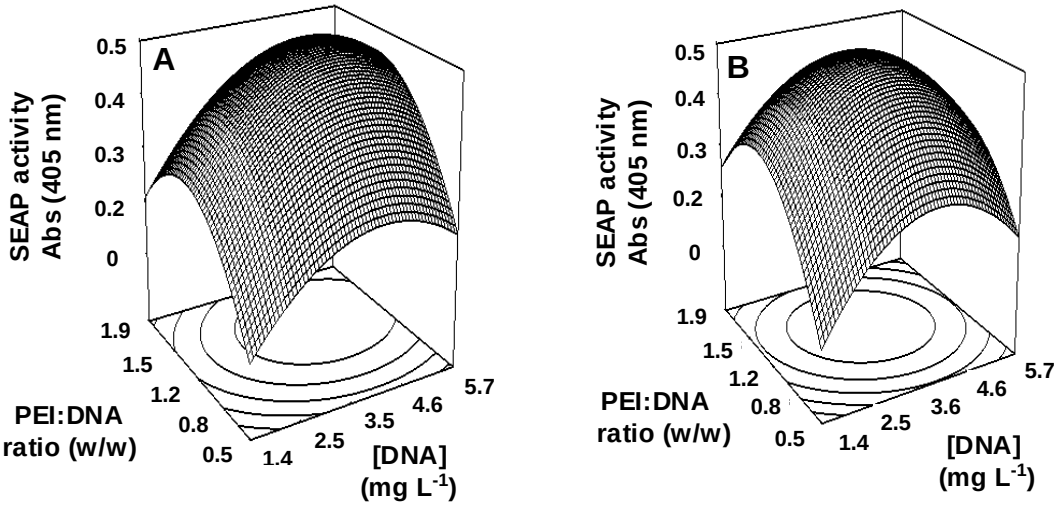

Figure 8.5 DoE RSM for transient SEAP production following transfection of parental CHO-S cells with unmodified PEI (A) and propyl PEI (B)
Transfection was optimized using response surface modelling, a central composite design. DNA quantity was varied between 1.4 and 5.7 mgL^{-1} and PEI:DNA ratio, between 0.5 and 1.9. Cell seeding density was kept constant at 10^6 cells mL^{-1}. Transfections were conducted in cultiflasks, in a volume of 5mL and cultured at 37°C, 170 rpm. SEAP was measured at 5 days post transfection.

8.3.3 Mechanistic exploration of propyl-PEI mediated transfection

The mechanistic differences between propyl-PEI and unmodified PEI, were explored. At the optimal transfection conditions for propyl-PEI, 2.8 mgL^{-1} DNA, PEI:DNA ratio 1.2, propyl PEI was found to deliver increased levels of plasmid DNA to the cells than unmodified PEI (Figure 8.6A and B). Cells were transfected with fluorescently labelled plasmid DNA and at 4 hours post transfection, cells were washed with CellScrub and PBS, and cellular fluorescence measured by flow cytometry. At a DNA concentration of 2.8 mgL^{-1} and PEI:DNA ratio 1.2, cyto-internalized DNA transfected with propyl-PEI was 1.7 fold that of cyto-internalized DNA transfected with unmodified PEI (Figure

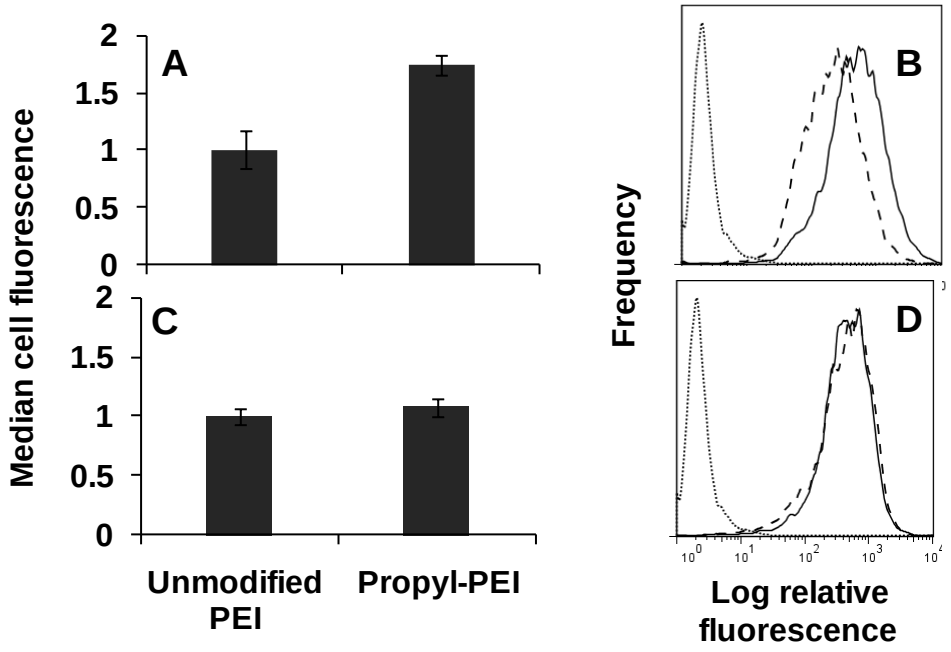

Figure 8.6 Transfection with propyl-PEI mediates increased uptake of plasmid DNA relative to unmodified PEI, but has no effect on polyplex binding to the cell surface

Cells were transfected with 2.8 mgL^{-1} FITC labelled plasmid DNA and unmodified or propyl-PEI (as indicated) at a ratio of 1.2 with respect to DNA. All other transfection conditions are as protocol A. Polyplex cyto-internalization was measured by incubating cells for 4 h post transfection at 37°C prior to washing with CellScrub to remove cell surface attached polyplex; cellular fluorescence was measured by flow cytometry (**A** and **B**). Polyplex-cell surface binding was measured by incubating cells for 4 h post transfection at 4°C prior to washing with PBS; cellular fluorescence was measured by flow cytometry (**C** and **D**). **B** and **D**: dashed line: unmodified PEI; solid line: propyl-PEI; dotted line: auto-fluorescence. The mean value ± standard deviation from triplicate transfections or biological samples is shown.

8.6A and B). At a concentration of 4 $mgL^{-1,}$ PEI:DNA ratio 1.3, cyto internalized DNA was 1.2 fold the level of cyto-internalized DNA following transfection with unmodified PEI ($p<0.05$) (data not shown). Polyplex-binding to the cell surface was again assayed, for propyl-PEI formed polyplexes and unmodified PEI formed polyplexes. As observed via facile screening (Figure 8.3C), under optimized DoE-RSM conditions, again no significant difference was observed between propyl-PEI:DNA and unmodified PEI:DNA polyplex binding to the cell surface (Figure 8.6C and D).

The DoE-RSM predicted optima for parental CHO-S cells and Clone 4, transfecting with unmodified and propyl-PEI, were compared directly (Table 8.1). At optimized conditions for the parental CHO-S cell line, SEAP production was significantly lower following transfection with propyl-PEI relative to transfection with unmodified PEI. However, a higher production efficiency (SEAP/DNA) was achieved through propyl-PEI mediated transfection (6.2 compared to 5.1 for unmodified PEI). SEAP production was higher for CHO-S Clone 4 using propyl-PEI relative to unmodified PEI for gene delivery (29.6 mgL^{-1} and 27.5 mgL^{-1} respectively). As with the parental CHO-S cell line, transfection with propyl-PEI provided a more efficient TGE platform with respect to plasmid DNA, relative to transfection mediated by unmodified PEI. For both cell lines, CHO-S parental and CHO-S Clone 4, transfection with propyl-PEI resulted in decreased culture viabilities compared to transfection mediated by unmodified PEI. For the parental cell line, average culture viability at 5 days post transfection with unmodified PEI was 66% and 61% following transfection mediated by propyl PEI. For CHO-S clone 4, culture viability at 5 days post transfection mediated by unmodified PEI was 85% relative to 78% following transfection mediated by propyl-PEI (Table 8.1).

Table 8.1 DoE RSM predicted optima, for parental CHO-S and CHO-S clone 4 cell lines and measured output at 5 days post transfection. Transfections were performed in duplicate.

Cell line	PEI	DNA (mgL^{-1})	PEI:DNA Ratio (w/w)	SEAP (mgL^{-1})	Culture viability (%)	Efficiency (SEAP/ DNA)
Parental CHO-S	Unmodified-PEI	4.0	1.3	20.2 (±1.1)	66 (±3.3)	5.1
	Propyl-PEI (7%)	2.8	1.2	17.3(±0.0)	61 (±1.5)	6.2
CHO-S Clone 4	Unmodified-PEI	3.6	1.2	27.5(±0.5)	85 (±0.3)	7.7
	Propyl-PEI (7%)	3.1	1.3	29.6(±0.7)	78 (±0.)	9.7

8.3.4 LDC to generate "parental" cell lines with superior phenotypes for PEI mediated TGE

Eleven clones of the parental CHO-S cell line were produced by limited dilution cloning, as described in section 8.2. At approximate generation number 1, 80 and 120, clones were transfected with PEI and transient SEAP production measured at 24 hours post transfection (Figures 8.7, 8.8 and 8.9). As reported by Davies *et al.*, (2013), although transient, clone-specific, SEAP expression was not heritable across the panel, a clone was identified (Clone 4) which was the highest producer at each generation measured, a clone with putatively heritable superior characteristics with respect to transient SEAP production following transfection with PEI. At generation 80 (Figure 8.9A) and 120 (Figure 8.8A), SEAP activity was 1.4 and 2 fold that of the parental, respectively. At generation 1, there was no significant difference in SEAP activity for the parental cell line or clone 4 (Figure 8.7A).

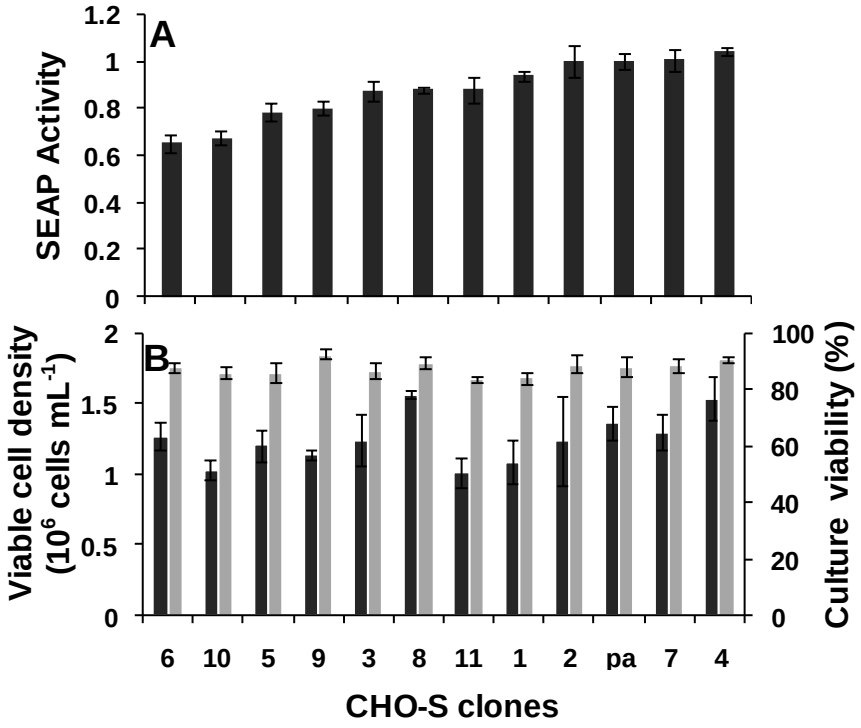

Figure 8.7 CHO-S clones display variation in transient SEAP production following transfection with PEI at generation~1
Cells were transfected with PEI in 24 well plates, at a seeding density of 10^6 cells mL^{-1}. At 24 h post transfection, SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured. The mean value ± standard deviation from triplicate transfections is shown.

With respect to SEAP activity, the order of clone specific productivity did not correlate between 1 and 120 generations (r^2=0.188) (Figures 8.7A and 8.8A). A sub-set of four clones, 4, 6, 7 and the parental CHO-S cell line, were used for uptake, binding and staining experiments at generation 80, due to the practical necessity of a small sample size for experimentation (Figure 8.10). The four cell lines were chosen because they displayed a range of SEAP productivities across the panel of 11. Using the four cell line sub set, the correlation was still weak with respect to SEAP production between generation 1 and 120, r^2=0.48. Between generations 80 and 120 there was excellent correlation in the order of clone specific productivity (r^2=0.99, $p<0.0001$). It is possibly, that once adapted to the culture environment, i.e. in 125mL shake Erlenmeyers, achieved by

generation 80 and 120, the functional phenotypes of the cell lines (with respect to SEAP production etc.) has stabilized.

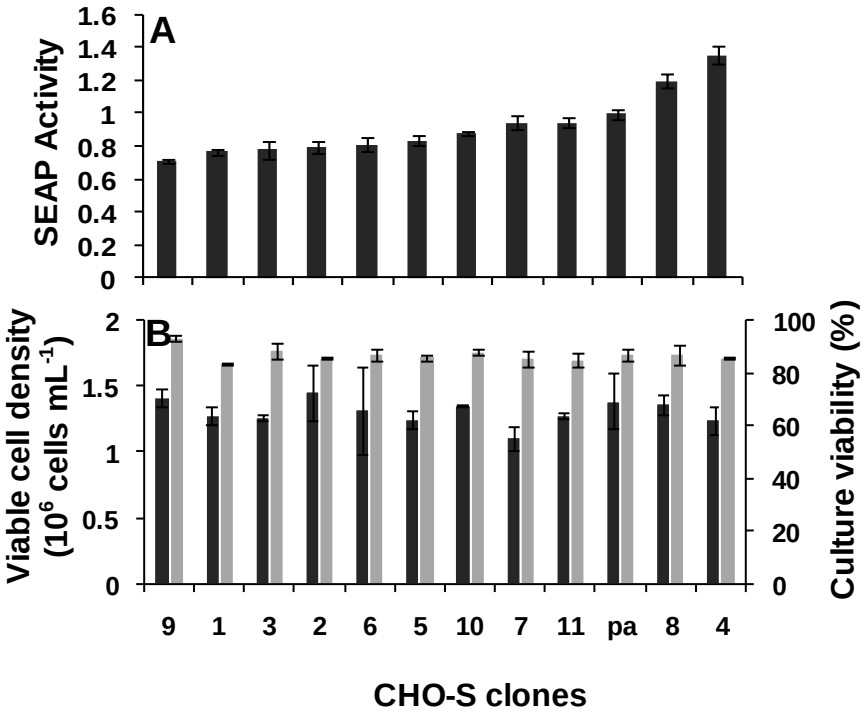

Figure 8.8 CHO-S clones display variation in transient SEAP production following transfection with PEI at generation ~120
Cells were transfected with PEI in 24 well plates, at a seeding density of $1x10^6$ cells ml^{-1}). At 24 h post transfection, SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured. The mean value ± standard deviation from triplicate transfections is shown.

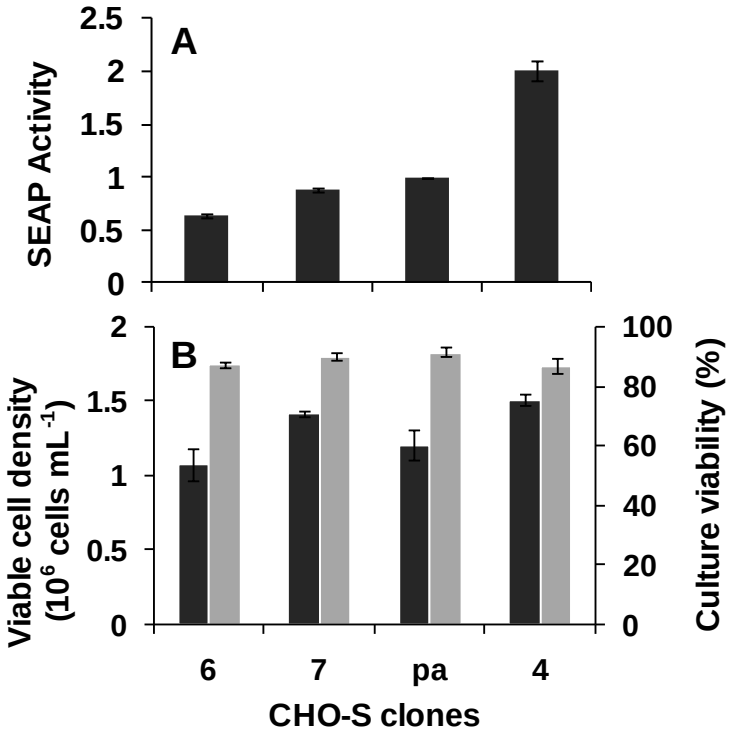

Figure 8.9 CHO-S clones display variation in transient SEAP production following transfection with PEI at generation ~80
Cells were transfected with PEI in 24 well plates, at a seeding density of 10^6 cells mL^{-1}). At 24 h post transfection, SEAP (**A**), viable cell density (black bars) and culture % viability (grey bars) (**B**) were measured. The mean value ± standard deviation from triplicate transfections is shown.

We previously observed that across three variant CHO cell lines, transient SEAP production co-varied with polyplex uptake, whereby the cell line that transiently produced the highest level of SEAP, also displayed the highest polyplex internalization capacity (CHO-S) (Thompson *et al.*, 2012). In addition, cell surface HSPG level was found to co-vary with transient SEAP production and polyplex cyto-internalization (Thompson *et al.*, 2012). It was therefore hypothesized that across four cell lines, varying in transient SEAP production, cell surface HSPGs and polyplex uptake would co-vary.

Polyplex cyto-association (Figure 8.10A) and uptake (Figure 8.10B) was higher for clone 4 than clone 7 and the parental, but polyplex cyto-association and uptake was also significantly higher for clone 6 than the parental. A similar pattern was observed for polyplex binding to the cell surface (Figure 8.10C);

clones 6 and 4 had higher binding than clone 7 and the parental cell line. There was no significant difference between the anti-HSPG staining observed for clones 6, clone 4 and the parental cell line (Figure 8.10D). Only clone 7 was stained more lightly for HSPGs, at approximately 70% the level of the parental.

Interestingly, for clone 6, in addition to SEAP activity being 64% that of the parental cell line, viable cell density at 24 h post transfection was only $1.1x10^6$ cells mL^{-1} compared to $1.2x10^6$ cells mL^{-1} for the parental cell line and $1.5x10^6$ cells mL^{-1} for high producing clone 4. The data indicate that the highest producer cell line, clone 4, displayed the highest polyplex internalization capacity combined with high, post transfection, culture viabilities.

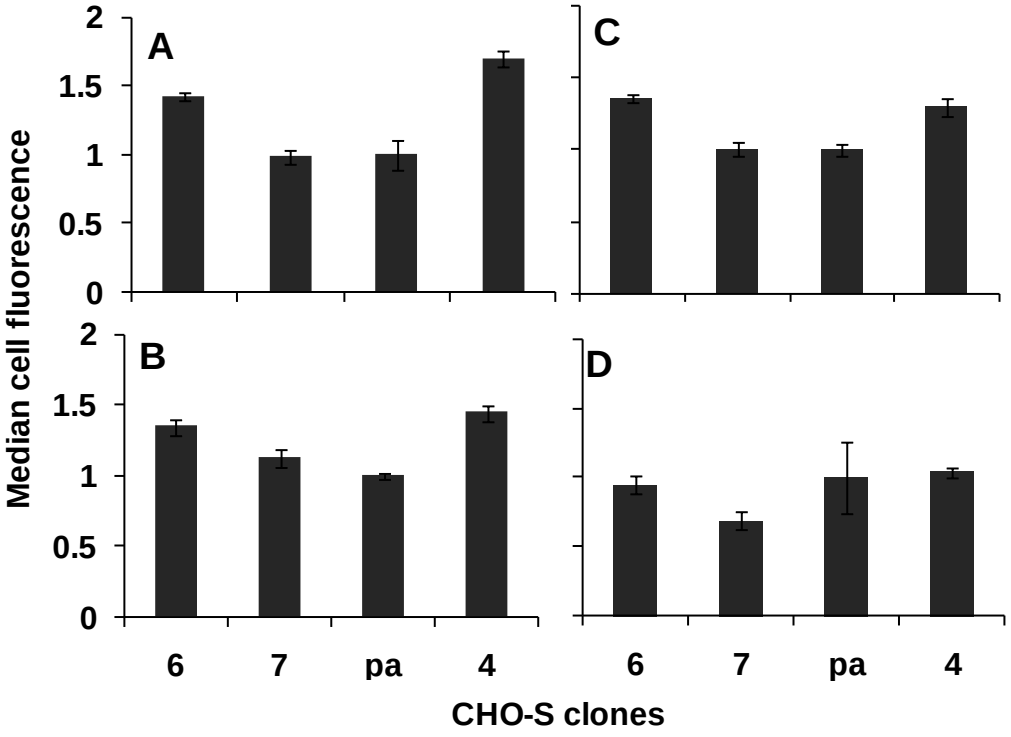

Figure 8.10 CHO-S clones exhibit variation in polyplex cyto-internalization and binding following transfection with PEI and cell surface HSPG content

Cells were transfected with PEI according to protocol A. At 4 h post transfection with fluorescently labelled plasmid DNA and PEI, polyplex cyto-association (PBS washed) (**A**), cyto-internalization (CellScrub washed) (**B**) were measured by flow cytometry. Polyplex-cell surface binding was measured using flow cytometry, at 4 h post transfection with fluorescently labelled plasmid DNA and PEI and following incubation at 4°C (**C**). Cells were fixed and immunostained with mouse anti-HSPG mAbs and secondary FITC conjugated anti-mouse IgM mAbs and fluorescence measured by flow cytometry (**D**). The mean value ± standard deviation from triplicate transfections or biological samples is shown.

8.4 Discussion

Data presented in this chapter display the promise that derivatized PEIs hold for TGE using CHO cell platforms. Using a relatively small panel of derivatized PEIs, propyl-PEI was found to display superior properties relative to unmodified PEI. In light of data presented in previous chapters, it was hypothesized alkylated PEIs (with enhanced hydrophobicity relative to unmodified PEI) would display superior binding to the cell surface, through hydrophobic interactions. However, this was not the case (Figure 8.3C and 8.6C) and is similar to the *in vivo* radio-active trace data obtained by Fortune *et al.*, (2011), indicating alkylated PEIs did not enhance cell-surface binding. It may be that the hydrophobic binding between the cell surface and unmodified PEI:DNA polyplexes are at a maximum/ saturated level. It is possible that if cell surface binding of polyplexes had been measured at an earlier time point, propyl-PEI:DNA and unmodified PEI:DNA polyplexes may have displayed different properties.

Interestingly, acetylated-PEI:DNA polyplexes showed no binding to the cell surface (Figure 8.3C). There are two explanations for this; firstly, that the acetyl group (due to its polarity perhaps) was detrimental to polyplex-cell surface binding or simply that DNA was not condensed by the acetyl-PEI, which could be tested by gel electrophoresis of polyplexes. Transfection with PEI is a multi-step process, involving numerous cell trafficking steps (Chapter 2). Two fundamental steps are DNA condensation and polyplex-binding to the cell surface. Useful data in screening derivatized PEIs could be obtained by gel electrophoresis, to obtain DNA condensation capacity, in addition to polyplex-cell surface binding capacity, TGE and culture viability post transfection etc.

As demonstrated in Chapter 4, cyto-toxicity caused by free PEI is not identical to that caused by PEI:DNA polyplexes and transgene expression, but can be used as an approximate guide for toxicity. At a concentration of 4 mg L^{-1} PEI, alkylated PEIs did not improved culture densities or viabilities relative to unmodified PEI (Figure 8.4). This could be explained several ways. Putatively, at a given concentration, alkylated PEIs are taken up by the cells more efficiently and thus display higher toxicity. Alternatively, the alkyl chain moiety might be itself cytotoxic. Alternatively, alkylated PEIs might enhance cell-cell

clumping, thus artificially reducing the cell density count by the Vi-Cell. CHO-S cells are known to be a “clumpy” cell line and PEI and PEI:DNA polyplexes increase cell-cell clumping. An extension of this experiment would be to use anti-clumping agent prior to running samples on the vi-cell.

The enhanced cyto-internalization displayed following transfection by propyl-PEI relative to unmodified PEI (Figure 8.6A), could be attributed to uptake of propyl-PEI:DNA polyplexes by a more efficient endocytic pathways compared to the pathway facilitating PEI:DNA polyplex uptake. Given that propyl-PEI was found to facilitate higher plasmid uptake relative to unmodified PEI, it was not surprising that its optimized DNA load was found be lower than that of unmodified PEI (Figure 8.5), with respect to reporter SEAP output. Taken together, propyl-PEI may facilitate more efficient uptake into the cell and thus require a lower plasmid DNA load for TGE relative to unmodified PEI.

Propyl-PEI was found to mediate higher TGE relative to unmodified PEI using clone 4 (not the case in the parental cell line (Table 8.1). Post transfection culture viabilities were also significantly higher for clone 4 relative to the parental cell line (Table 8.1). Clone 4 may have a phenotype with superior resistance to PEI/ PEI:DNA polyplex induced toxicity; it may have reduced sensitivity for apoptotic induction for example. To test this hypothesis, the expression of a selection of apoptotic genes could be tested through analysis of respective transcript levels, e.g. caspases.

For engineering targets such as transfection, multi-gene modulation is likely to be necessary, rather than a gene by gene approach. Unlike typical cell line engineering strategies, whereby a single or a hand-full of target genes are up/down regulated, selection of cell lines through clone screening is likely to result in a multi-gene up/down regulation. However, the approach relies on chance, that a cell line will be isolated with a superior phenotype for the required biomanufacturing process. Selective evolution strategies could be employed to render a clone more suitable to transfection. Alternatively, the larger the panel of clones screened, the higher the chance of isolating a cell line with superior properties for transfection and/ or TGE.

Future work could focus on screening a larger panel of alkylated PEIs, at a range of percentage alkylation. In addition, it would be interesting to test the efficacy of reversibly PEGylated PEI for CHO based TGE (Walker *et al.*, 2005). Walker *et al.*, (2005) addressed the hypothesis that PEG shielding of PEI:DNA polyplexes could hinder acceptance of protons by PEI and endosomal escape. PEIs linked to PEG via acyl-hydrazides or pyridyl-hydrazines were synthesized, which would hydrolyse at pH 5, within lysosomes, thus separating PEG from PEI. The reversibly PEGylated polyplexes provided a 100x increase in transgene expression *in vivo* and a 10x increase *in vitro*, relative to stably PEGylated polyplexes. Such reversibly PEGylated PEI:DNA polyplexes might provide superior properties for cell surface binding (Chapter 5), whilst maintaining maximum capacity for lysosomal escape.

8.5 Chapter Conclusions

In this chapter, for the first time, a PEI derivative (propyl-PEI) has been demonstrated to have superior properties *in vitro* to unmodified PEI, with respect to the efficiency of TGE with respect to plasmid DNA utilization (SEAP/ DNA). Furthermore, propyl-PEI was found to mediate enhanced uptake of plasmid DNA relative to unmodified PEI. Clone screening was found to be a successful strategy for cell line development for TGE mediated by PEI.

CHAPTER 9

Final Discussion

9.1 A biphasic model for the uptake of PEI:DNA polyplexes by CHO-S cells

"...dynamical problems lie behind the morphological problems of the cell."

Thompson, DA. 1917.

The model is based on data presented in Figures 4.5, 4.7, 4.8, 6.4,6.5,6.6 and key data represented below in Figure 9.1.

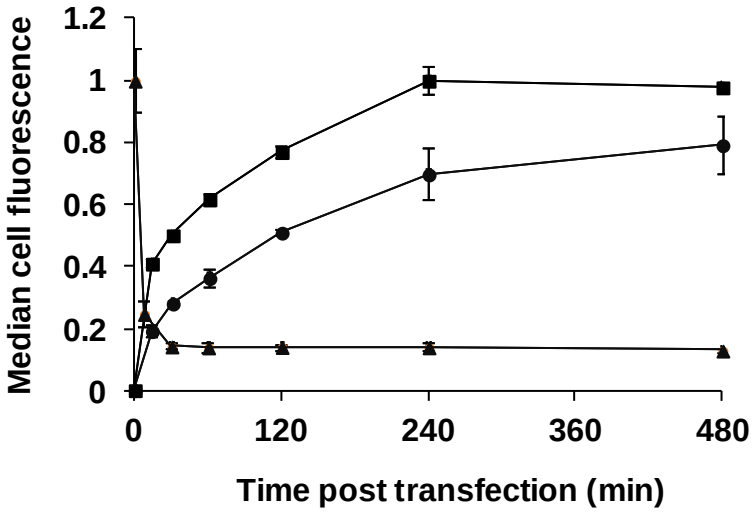

Figure 9.1
Rapid polyplex-cell association and uptake is mirrored by the decrease in cell surface HSPGs. Polyplex-cell association and uptake continues after HSPGs have depleted from the cell surface.
Squares: polyplex-cell association. Circles: polyplex cyto-internalization. Triangles: cell surface HSPGs deplete post transfection.

Two phases for polyplex uptake exist (Figure 9.2). Phase 1 uptake is relatively rapid, mediated by HSPGs (Payne *et al.*, 2007), occurring within, approximately,

the first 15 min post transfection. Phase 2 (between -15 and 240 min post transfection) is slower and occurs at diminished levels of cell surface HSPGs. Both pathways are dependent on membrane fluidity/ lipid raft clustering. It is possible that Phase 2 uptake is dependent on lipid raft regeneration and limited by the rate of regeneration.

Approximately 25% of polyplex was taken up in phase 1, in the transfection system used throughout this thesis, the remaining 75% taken up by phase 2. Similarly, 25% of TGE was mediated by phase 1 uptake and 75% via phase 2 uptake, based on data presented in Figure 6.8, where enzymatic depletion of HSPGs reduced SEAP by 25%.

In other systems, utilising different cell lines, derivatized PEIs or culture modality, it is likely that the proportion of polyplex taken up by each pathway may vary. The PEI:DNA ratio of polyplexes may also impact on pathway utilization.

The model shares some similarities with the conclusions drawn by von Gersdorff *et al.*, (2006) who reported the involvement of several endocytic pathways in polyplex uptake. Maximal transgene expression was reported to occur only if the final endocytic pathway (for polyplexes ≥1 μm in diameter) was accomanied by endocytic pathways that mediated the uptake of smaller polyplexes (Rejman *et al.*, 2004), such as clathrin mediated endocytosis.

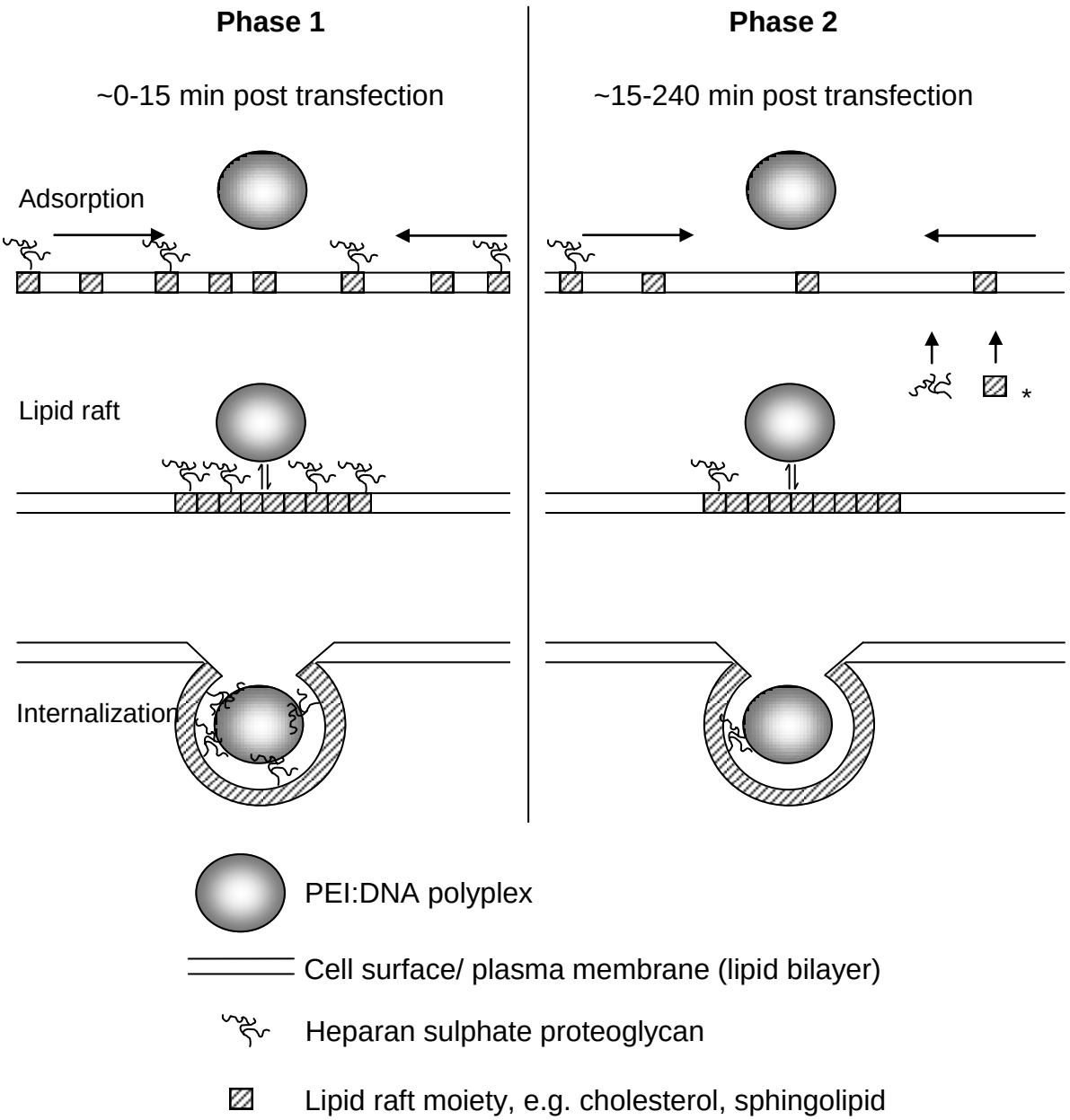

Figure 9.2
A biphasic model for the uptake of PEI:DNA polyplexes by CHO-S cells
Polyplex uptake occurs rapidly via HSPG-endocytosis (Payne *et al.*, 2007), within approximately 15 min post transfection (Phase 1). Subsequent uptake of polyplexes occurs at depleted HSPG levels (Phase 2).
*Regeneration of lipid raft moieties and HSPGs.

Phase 3

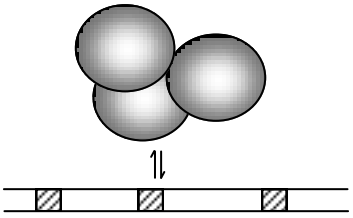

Figure 9.3 Extra-cellular attrition of transgene: PEI:DNA polyplex aggregation
PEI:DNA polyplexes aggregate (Sharma *et al.*, 2005) to an extent whereby they cannot be internalized by the cell.

Aggregation of polyplexes is a well reported phenomenon (Sharma *et al.*, 2005) (Figure 9.3). At a certain level of aggregation it is likely that polyplexes would be too large to be internalized by the cell (Rejman *et al.*, 2004). On the other hand, aggregated polyplexes bound to the cell surface, could maintain an equilibrium with extra-cellular polyplexes, suspended in the culture milieu. Thus, the kinetics of transfection may determine total cyto-internalization of polyplexes, in that polyplexes must be taken up by the cell before they have aggregated to a point where they are too large to be endocytosed.

In our virgin system, using unmodified PEI for polyplex formation and untreated CHO-S cells, cyto-internalization saturated at approximately 240 min post transfection and it is possible that polyplexes had aggregated to an extent, by this point, whereby further cyto-internalization was impossible. A working theory to improve TGE, with respect to the efficiency of DNA utilization, is that increased rate of binding/ internalization reduces plasmid DNA attrition by extracellular aggregation of polyplexes.

9.2 Polyplex binding to the cell surface

"the things which we see in the cell are less important than the actions which we recognise in the cell..." Thompson, DA. 1917.

Binding experiments were performed by incubating transfections at 4°C, thus inhibiting cell trafficking processes, including polyplex internalization and HSPG depletion from the cell surface. Thus, binding experiments were likely to maintain cell surface HSPGs at an undepleted level, representative of an untransfected cell but artificial for a transfected cell, at approximately ≥7.5 min post transfection. Under normal transfection conditions (i.e. incubation at 37°C rather than 4°C), polyplexes in contact with the cell, at time points approximately greater than or equal to 7.5 min post transfection, would bind to a plasma membrane with a reduced complement of cell surface HSPGs (≤25% untransfected levels) relative to that of a de novo transfected cell.

Hydrophobia has been described as an entropic process, due to the increase in hydrophobic interactions at higher temperatures (Chandler 2005); thus hydrophobic interactions at 4°C would be less strong than those occurring under normal physiological conditions. Also, as temperature decreases hydrogen bonds become shorter and the viscosity of water increases. Therefore, whilst, incubation at hypothermic culture temperatures, allows an almost physical approach to the biological question of polyplex-cell surface binding, this, by definition, has its limitations.

Data presented in Chapter 5 support the hypothesis that hydrophobia is a mediatory force in polyplex binding to the cell surface. Both PEI:DNA polyplexes in medium and CHO-S cells in suspension are dynamic and heterogeneous entities. The dynamic cell is well documented as is heterogeneity within individual cells (Altschuler and Wu, 2010); intra-cellular heterogeneity within "clonal" cell populations has been recently described (Davies *et al.*, 2012). PEI:DNA polyplex heterogeneity has also been described (Han *et al.*, 2009). It is likely that both electrostatic and hydrophobic interactions contribute to polyplex binding to the cell surface. Attribution of the binding mechanism solely to polar or apolar interactions is simplistic. It seems more likely that both would contribute. At the molecular level, a multitude of physical interactions take

place, at different patches of the same polyplex and at different locations of a lipid raft molecule.

On a systems level, it is possible that the relative proportion of electrostatic and hydrophobic interactions changes over time. That is, within the first few min post transfection, the cell surface possessing a full complement of HSPGs, electrostatic interactions might be relatively more predominant. As HSPGs deplete from the cell surface, hydrophobic interactions may then predominate. However, binding data presented in Chapter 5 using non-ionic surfactant was performed at hypothermic culture temperature; a condition (as described above) that would maintain cell surface HSPGs at an undepleted level on the cell surface. Thus, even possessing a full complement of cell surface HSPGs, unsurprisingly, the CHO-S cells is a hydrophobic entity.

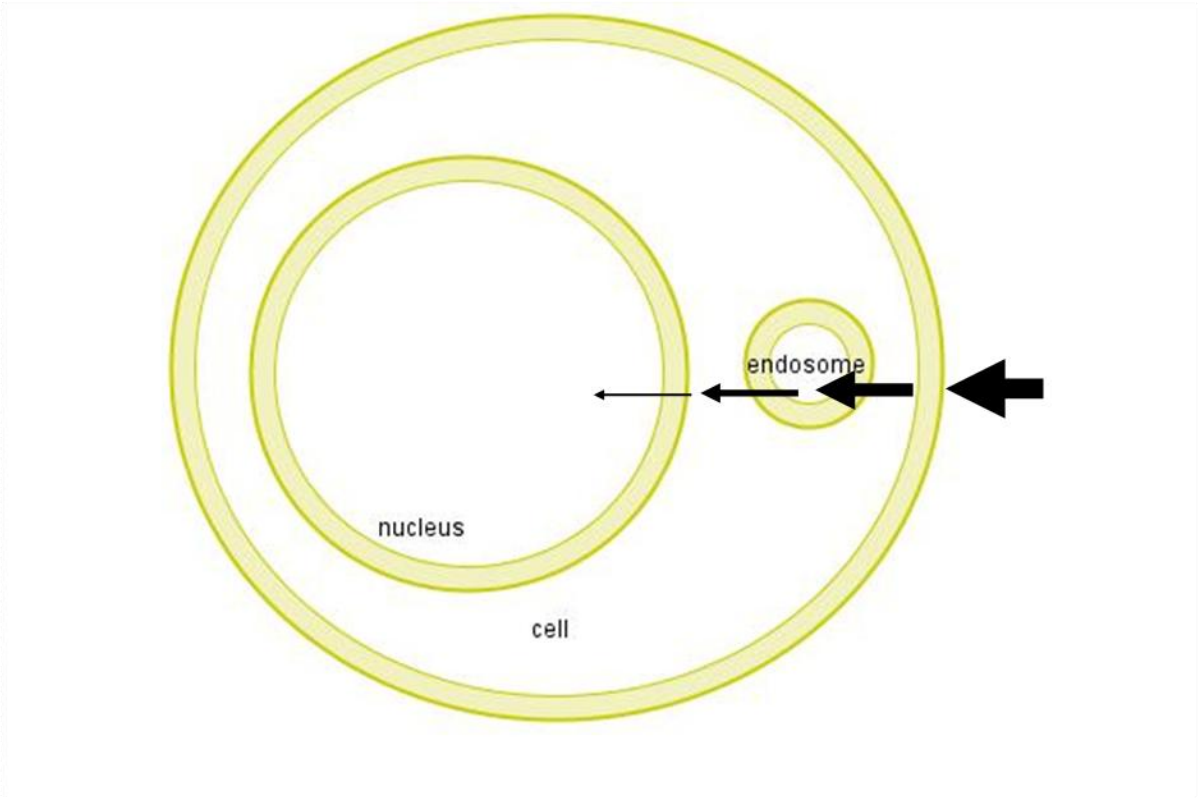

Figure 9.4 Intracellular attrition of transgene
Transgene is lost at cellular defences, such as the plasma membrane, endosomes or the nuclear membrane. The arrows (←) indicate transgene and the relative size of the arrow is an indication of transgene quantity.

9.3 The attrition-based paradigm for intracellular transgene trafficking

Within the literature, and presented in this thesis, are examples of the inefficient trafficking of plasmid DNA (complexed with a transfection reagent) within the mammalian cell (Figure 9.4). Cellular structure and biochemistry, in addition to providing the framework and functioning of the cell, provides an intrinsic barrier against foreign DNA. Bottlenecks for efficient gene delivery exist at the cell surface plasma membrane, endosomes/ lysosomes and nuclear membrane. One of the key (all be it accidental) design features of PEI, is its ability to accept protons within lysosomes, leading to osmotic swelling and subsequent rupture of lysosomes, allowing polyplexes to escape into the cytosol, avoiding lysosomal degradation (Akinc *et al.*, 2005).

In all cases, transfection is optimized (e.g. DNA amount, and transfection reagent to DNA ratio) with respect to TGE output, culture viabilities and sometimes transfection efficiency (% cells GFP positive). A system is achieved whereby the cell is flooded with transgene, so that despite attrition of transgene at various cellular bottlenecks (plasma membrane, nuclear membrane etc.) sufficient transgene is available for transcription to enable maximum TGE.

However, barriers may exist preventing transcriptionally available transgene reaching saturation level. For example, by media additives such as ferric (III) citrate preventing polyplex binding to the cell surface or HSPG depletion, preventing HSPG endocytosis of polyplexes into the cell.

With regard to HSPG endocytosis, it appears to be a rapidly saturable process. Where a cell line is HSPG deficient, HSPG over-expression may enable a further endocytic trafficking pathway for polyplex uptake into the cell (Wong *et al.*, 2010). However, given the saturability of the process, HSPG over-expression per se, in a cell line possessing a full complement of HSPGs, seems an unlikely method to increase r-protein titres. A more logical strategy would be to increase the regeneration/ cycling of HSPGs or lipid raft constituents to the cell surface, following polyplex induced endocytosis; however, this would require a genomics scale engineering approach, with limited feasibility (Maxfield and McGraw, 2004).

Engineering strategies, used to increase r-protein through TGE (described in more detail Chapter 1) have focussed on enhanced protein secretion or reduced cellular susceptibility to apoptosis or choice of cell line/ system. Given that PEI delivered nuclear-localized plasmid DNA can be close to the level required for gene expression saturation (Cohen *et al.*, 2009), mechanistic engineering of transfection (i.e. gene delivery and trafficking to the nucleus) is likely to yield improvements in efficiency of plasmid DNA utilization, rather than improvements in r-protein titres.

9.4 Engineering Transfection

Two methods could be employed to enhance the efficiency of transgene delivery; the first; by engineering the cell itself and the second by engineering the transgene delivery complex.

Regardless of engineering strategy, optimization of TGE with respect to the efficiency of plasmid DNA utilization, as illustrated in Chapter 8, requires varying DNA concentration and PEI:DNA ratio; side by side comparison at one set of conditions (DNA concentration and PEI:DNA ratio) is inadequate. Depending on process requirements, algorithms designed to maximise efficiency of TGE production with respect to plasmid DNA utilization or to maximise overall cost efficiency, could be employed when optimizing transfection conditions (DNA load etc.), rather than simply using recombinant protein titre or culture viability as a process output.

It seems that cell line engineering for transfection is fundamentally influenced by whether the original/ parental cell line is deficient in cellular machinery that facilitates a trafficking step of the polyplex or whether it is a relatively “fit-for-purpose”, transfection competent host cell line. For example, HPSG over-expression, enhanced PEI mediated TGE, in a CHO-K1 derived cell line, deficient in cell surface HSPGs (a sub-standard cell line for PEI mediated TGE) (Wong *et al.*, 2010). On the other hand, cell cycle control (using nocodazole or p18 or p21 regulators) was effective in fit-for purpose cell lines (Tait *et al.*, 2004; Backliwal *et al.*, 2008a).

Cell line engineering for enhanced transfection is likely to require multi-gene modulation. Hence, clone generation (Chapter 8), successive rounds of cell

sorting following transfection (Pichler *et al.*, 2010) or selective evolution strategies might be more appropriate to achieve the required goal, at this stage, than gene by gene cell line engineering methods. Transcriptomics of isolated (evolved) clones with superior transfection phenotypes (reverse engineering), would enable future employment of direct cell line engineering strategies.

As described in the introduction to Chapter 8, PEIs with enhanced hydrophobicity, developed by various methods, have been demonstrated as efficacious for transgene delivery and expression, in a range of platforms (*in vivo* and *in vitro*), by several groups. We tested the efficacy of a selection of N-alkylated PEIs, for two reasons: to build on data indicative of the role of hydrophobia in polyplex-cell surface binding (Chapter 5) and to build on data showing polyplex cell-surface binding to occur following cell surface HSPG depletion (Chapter 6). We hypothesized that alkyl PEIs would enhance the efficiency of TGE with respect to plasmid DNA by enhancing polyplex-binding to the cell surface. In fact, propyl-PEI was found to provide better uptake properties and more efficient TGE with respect to plasmid DNA, relative to unmodified PEI.

9.5 Limitations of the Study

In this study of PEI:DNA polyplexes, only DNA was fluorescently labelled, not PEI. In a study by Derouazi *et al.*, (2004) free DNA was found to be completely removed from solution following addition of 25 kDa linear PEI, at PEI:DNA ratios (w/w) of 0.4:1 and above. It was thus assumed that fluorescent readings associated with the cell (intracellular or cell surface attached), obtained by flow cytometry or confocal microscopy, were attributed to PEI:DNA polyplexes and not DNA alone. An alternate method would be to fluorescently label both PEI and DNA (Godbey *et al.*, 1999; Hufnagel *et al.*, 2009).

Transient gene expression or SEAP reporter production was used as an indirect measure of polyplex uptake, under the assumption that polyplex uptake and transient gene expression correlate (Bertschinger *et al.*, 2008). It was not always possible to measure polyplex uptake, as described in section 7.3.7.

DNA topology has previously been shown to have a significant effect on polyplex uptake (polylysine formed polyplexes) in CHO cells (Dhanoya *et al.*,

2011) and dendritic cells (Dhanoya *et al.*, 2012). Whether the DNA was supercoiled, open circular or linear affected the uptake kinetics of DNA delivered as polylysine polyplexes. For example, at 1 h post transfection of dendritic cells, 61% of supercoiled pDNA polyplexes were localized to the nucleus whereas only 24.3% and 3.5% of open circular or linear pDNA polyplexes, respectively, were localized to the nucleus of CHO cells (Dhanoya *et al.*, 2012). Supercoiled pDNA-polylysine polyplexes also displayed the highest transfection efficiency, compared to open circular and linear pDNA-polylysine polyplexes, putatively due to their increased resistance to nucleases. In this thesis, the topology of the DNA was not addressed but it would be an interesting area of future research.

The aggregation of polyplexes in solution has previously been explored by Shama *et al.,* (2005), who reported polyplexes to rapidly aggregate in solution and within a 4 h time period, <10% of total DNA to remain in non-aggregated polyplexes. Polyplex aggregation was not characterized in this study but an interesting area of future investigaion would be to investigate any relationship between polyplex size and endocytic uptake pathways. Previously, the effect of chemical treatments, including MβCD and chlorpromazine, on the uptake of microsphere of different sizes by murine myeloma B16-F10 cells was characterized (Rejman *et al.*, 2004). interestingly, cholesterol depletion using MβCD and chemical treatments with genistein and filipin were found to inhibit uptake of microsphere of 500 nm in diameter to a greater extent than smaller microspheres; inhibition of clathrin mediated endocytosis using chlorpromazine hydrochloride was found to have the opposite effect, inhibiting the uptake of smaller microspheres (50 nm diameter) more than larger microspheres (500 nm diameter) (Rejman *et al.*, 2004). The topology of DNA has been shown to strongly influence polylysine-DNA polyplex size and therefore the topology of the DNA may affect the uptake of polyplexes (Dhanoya *et al.*, 2011; Dhanyoya *et al.*, 2012). Future work could focus on characterizing the topology of the DNA and its effect on PEI mediated transfection of CHO cells.

9.6 Future Work

Future work, building on work presented in this thesis, could follow several branches. One avenue to explore would be the mechanism of propyl-PEI mediated transfection, e.g. the transfection kinetics or dependence of cell surface HSPGs of propyl-PEI polyplexes relative to unmodified PEI-polyplexes. Given that propyl-PEI was found to facilitate increased uptake of polyplexes, relative to unmodified PEI, the mechanisms of endocytosis could be explored, using siRNAs. Alternatively, fluorescent labelling of the respective polymers could be employed for live microscopy imaging, combined with fluorescent labelling of endocytic vesicles.

Building on data presented in Chapter 5 indicating the role of hydrophobia in polyplex-cell surface interactions, an increased panel of hydrophobically derivatized PEIs could be screened (Lynn *et al.*, 2001). The efficacy of methyl-butyl-, pentyl-, hexyl- or heptyl- PEIs could be tested, at varying alkylation percentages (Fortune *et al.*, 2011). In addition, reversibly PEGylated PEIs (Walker *et al.*, 2005) or cyclodextrin PEIs could be tested for bioprocessing (Forrest *et al.*, 2005).

Lipid encasement has been employed to enhance the hydrophobicity of polyplexes (Liu *et al.*, 2010). For large scale gene delivery TGE applications, lipid coated polyplexes are less likely to be suitable for scaled-up production than chemically derivatized PEIs (as described above), due to the cost of manufacture.

It would be desirable to screen derivatized PEIs for the respective rate of polyplex uptake by the cell, working on the hypothesis that PEIs which enable faster polyplex uptake offer superior transfection properties, due to the increased level of cellular transgene uptake prior to extracellular attrition of transgene by polyplex aggregation. Equally, it would be relevant to screen the aggregation (Sharma *et al.*, 2005) rate of polyplexes formed from a range of PEIs.

As described in Chapter 1 and 2, a plethora of strategies are employed to achieve maximum transient recombinant protein titres. Derivatized PEIs could be tested on an industry standard TGE platform, for example, a system

enabling episomal maintenance and replication with a shift to mild-hypothermic culture temperatures. The effectiveness of derivatized PEIs on such as system could be screened in micro-scale bioreactors.

Transient recombinant protein production, following PEI mediated transfection, was found to be strongly influenced by culture media (Ye *et al.*, 2009). A future line of experimentation could test for correlation between the zeta potential of polyplexes in media and the level of polyplex-binding to the cell surface in the same media. A hypothesis being that culture medium conferring a negative zeta potential on the polyplex would lead to relatively low polyplex-cell surface binding levels.

As described in Chapter 4, to manage the oxidative stress of the system, multiple strategies could be employed. For example, the parental cell line could be engineered with enhanced anti-oxidant defences, such as superoxide dismutases or catalases. Alternatively, genes for such enzymes could be included on the transient expression vector. Alternatively anti-oxidants could be added to the culture, such as ascorbic acid or alpha-toco-pherols or the redox potential of the medium itself could be engineered. The relative proportion of anti-oxidant supplements/ media additives could be optimized by DoE-RSM methodology (Grainger and James, 2013).

Other than direct engineering of the system, the mechanism of PEI and PEI:DNA mediated oxidative stress could be mechanistically explored. Future work could include comparison of cellular ROS levels following addition of PEI or PEI:DNA polyplexes, as opposed to ROS in the supernatant (Figure 4.9 and 4.10), to characterize the relationship between PEI or PEI:DNA polyplex addition, cellular ROS, cell viability and recombinant protein output.

9.7 Other applications: gene therapy, DNA vaccines and RNA interference.

Other applications for PEI mediated DNA delivery include gene therapy, DNA vaccines and RNA interference (Thomas and Klibanov, 2003; Aigner, 2006; Schaffert and Wagner, 2008; Grant *et al.*, 2012; Wegmann *et al.*, 2012). *In vivo* experimental systems are commonly used for exploring transgene delivery for such applications (Fortune, 2010) due, in no small part, to the differing colloidal properties of the transgene complexes in synthetic culture media or blood/ tissue. However, cell culture based experimentation offers numerous advantages over *in vivo* systems, for one, by allowing a higher degree of biochemical manipulation. For example, comparing cell surface-attached polyplex versus cyto-internalized polyplex *in vivo* would be extremely difficult. Thus, whilst the data presented in this thesis, has limits in its applicability to *in vivo* systems, it may also provide insight in areas that *in vivo* experimentation does not allow.

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