Evaluating the use of cross-linked PVA nanoparticles for gene and drug delivery

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Abstract

Due to the safety concerns surrounding viral vectors, non-viral alternatives are desirable for fulfilling the aim of gene therapy. In this project gel mobility shift assays demonstrated how cross-linked PVA nanoparticles successfully form complexes with plasmid DNA and are of a size and charge that should, theoretically, permit endocytosis by eukaryotic cells. However, during *in vitro* transfection studies no reporter (GFP) gene expression was noted. The collective evidence from electroporation, fluorescent-DNA-tagging, Lipofectin® or calcium phosphate chimeric and chloroquine experiments suggest that a lack of cell uptake is responsible. Nevertheless, the same cross-linked PVA nanoparticles have been shown to exhibit much promise in the field of drug delivery during *in vitro* experiments, even when used to target the same cell types as those used during transfection studies. Nanagel®, a cross-linked PVA nanoparticle containing budesonide, achieved higher levels of drug delivery than a commercially available form of the same drug (Pulmicort®) after 1 or 24 hours drug exposure. Furthermore, by measuring superoxide production during a stimulated respiratory burst, the budesonide delivered to cells appears fully functional and significantly more effective than Pulmicort® in preventing the formation

of reactive oxygen species, following a 24-hour pre-treatment period with the formulation. These findings have exciting possibilities for the use of hard-to-dissolve corticosteroids in the treatment of respiratory disease.

Keywords: *transfection, gene therapy, drug delivery, budesonide, COPD, asthma, PVA*

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1.1 Introduction

The aim of this project was to investigate whether cross-linked PVA hydrogels were able to successfully transfect eukaryotic cells. It was postulated that, owing to their positive charge, cross-linked PVA nanoparticles would form complexes with DNA through charge interaction and that a net positive charge on hydrogel/DNA complexes could facilitate cell uptake. During the project PVA-based nanoparticle transfection was compared against established transfection technologies in an attempt to validate the hypothesis being tested.

Transfection can be simply defined as the transfer of foreign DNA into cells and can be either transient or stable. During transient transfection exogenous DNA is not integrated into the host cell genome but is instead maintained extra-chromosomally within the nucleus. In contrast, during stable transfection, exogenous DNA becomes integrated into the host cell genome (Reese, 2004).

If naked DNA is applied to cells, however, DNA uptake in those cells is poor as naked DNA is unable to penetrate the plasma membrane of most cells (Feng *et al.* 2006). The size and charge of the DNA also prevents efficient

uptake (Felgner *et al.* 1987). As a consequence, carriers or vectors are required to deliver exogenous DNA into target cells (Feng *et al.* 2006).

Workers have developed various means of breaching the plasma membrane to deliver DNA into cells, with the ultimate goal of 'gene therapy' in mind. Gene therapy has been described as "the introduction of healthy copies of mutated or absent genes into target cells so as to promote the expression of a normal protein and to restore correct cellular function" (Mehier-Humbert & Guy, 2005). However, despite the fact that gene therapy concepts are well established, with the first approved protocol of human gene therapy dating back to 1989 (Journal of Gene Medicine, 2007), Gendicine, approved for use in China by the Chinese State Food and Drug Administration in 2003 for the treatment of head and neck squamous cell carcinoma, remains the only gene therapy treatment that has been approved for clinical use (Biopharm International, 2004). Although statistics from the Journal of Gene Medicine show in excess of 1,000 gene therapy clinical trials have been approved worldwide between 1989 and 2007 (Journal of Gene Medicine, 2007), there are, at present, no gene therapy treatments clinically available in the USA or Europe.

The failure of gene therapy treatments to reach the clinic is a consequence of the many problems encountered in both the lab and during clinical trials.

1.1.1 Viral Vectors

Genetically modified viruses were the vectors of choice for much of the early gene therapy research due to their inherent ability to efficiently transfect cells, delivering their DNA to the nucleus to subsequently direct gene expression (Maitra, 2005). Viruses have been genetically engineered by deleting genes permitting replication, assembly or infection and replacing them with therapeutic genes of interest (Gardlik *et al.*, 2005). Recent statistics from the Journal of Gene Medicine show that adenoviruses and retroviruses are the most popular vectors used during gene therapy research – amounting to approximately 45 % of vectors used in clinical trials - whilst poxvirus, vaccinia virus, adeno-associated virus and herpes simplex virus have also been used to a lesser extent (Journal of Gene Medicine, 2007).

Workers have persisted with the development of viral vectors because, broadly speaking, they introduce their DNA into cells with high efficiency (Gardlik *et al*., 2005). However, their clinical use is limited because of immunogenicity, cytotoxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems and high cost (Maitra, 2005). Furthermore, there is always the underlying fear that, through recombination, viruses may retrieve genes for assembly and replication that were originally removed, rendering them virulent once more (Gardlik *et al.* 2005). The cytotoxic and immunogenic responses elicited in patients during

clinical trials have even led to the death of several patients and for the US Food and Drug Administration to temporarily halt gene therapy trials using viral vectors in the past (Weiss, 2005).

1.1.2 Non-viral vectors

In view of the limitations of utilizing a viral approach for gene delivery, the field of gene therapy has moved towards developing non-viral transfection techniques which are considered much safer alternatives (Gardlik *et al.* 2005). Non-viral vectors have a virtually unlimited capacity for transgenes, demonstrate low toxicity allowing repeated application and are much simpler to construct (Gardlik *et al.* 2005). Non-viral approaches developed to date include both physical and chemical transfection methods

1.1.2.1 Physical Methods

The benefit of using physical methods for gene delivery is that transfection efficiency is only weakly dependent upon or even independent upon cell type – as direct transfer across the plasma membrane is possible (Mehier-Humbert & Guy, 2005). This means gene transfer to a wide range of cell types is thus possible.

Electroporation

A well established physical method of efficiently transfecting a wide variety of cell types *in vitro* is electroporation, or electropermeabilisation, a technique first used in 1982 by Wong and Neuman to introduce the thymidine kinase (tk) gene into tk deficient mouse cells (Wong & Neuman, 1982). Electroporation uses brief, high intensity electrical field pulses generated by an electroporator to disrupt the lipid matrix in the cell membrane to create tiny pores through which DNA can enter the cell from surrounding medium (Harrison *et al.*, 1998). However, problems associated with this technique are that DNA internalized via electroporation is not delivered directly to the nucleus it is still subjected to DNase degradation in the cytoplasm (Mehier-Humbert & Guy, 2005). An additional problem is that during the electroporation procedure the electrical currents to which cells are subjected can damage protein channels, which in some cases are permanently denatured (Tsong, 1991).

Particle Bombardment

Another physical means of attaining mammalian transfection is to use particle bombardment, a modified biolistics technique originally developed as a means of transfecting plant cells (Heiser, 1994). The basis of the technique is to transport naked DNA into target cells on an accelerated particle carrier (Mehier-Humbert & Guy, 2005). Heiser (1994) has used DNA-coated gold microparticles, fired though a gas cylinder at high pressure, to show greater

levels of transfection in COS-7 and CHO cells compared to electroporation and lipofection (lipofection is described in further detail below). After the DNA is physically delivered inside the cell it is gradually released inside the cell post-bombardment (Mehier-Humbert & Guy, 2005). The technique has been used to propel DNA coated beads into the epidermal layer for the genetic immunization of skin, however, the depth of penetration is shallow, limiting the region to which DNA is delivered (Mehier-Humbert & Guy 2005).

1.1.2.2 Chemical Methods

Calcium-phosphate

One of the simplest chemical transfection methods devised to date is to form complexes of DNA co-precipitated with calcium phosphate (Song & Lahiri, 1995), which is both simple and inexpensive and utilizes the basic procedure of Graham and van der Eb (Wilson *et al.*, 1995). Calcium phosphate-DNA particles are formed electrostatically (Mozafari & Omri 2007) by mixing DNA and calcium phosphate in buffer and then incubating the resulting complexes with target cells (Song & Lahiri, 1995). The calcium phosphate is thought to provide a stabilizing function to the nucleic acids (Mozafari & Omri 2007), which are uptaken into cells by endocytosis. It has been suggested that the presence of calcium phosphate results in an osmotic imbalance inside the endosome, causing complex release into the cytoplasm (Mozafari & Omri, 2007).

However, despite optimization of several of the parameters involved in precipitate formation, including the pH of the precipitate forming solution, the concentrations of calcium and phosphate and the incubation time of cells with the precipitate (Wilson & Smith, 1997), transfection rates achieved are still much lower than those attained using viral vectors, even when additional agents such as glycerol are included in the medium to improve rates of transfection (Wilson *et al.*, 1995). An additional problem with the calcium phosphate technique is that the precipitates formed are unstable and must be made fresh to avoid nano-sized crystals aggregating to form micro-sized crystals, which renders them too large to be endocytosed (Mozafari & Omri, 2007) and although this problem can be circumvented by coating the particles with a surrounding layer of poly(ethylene glycol) (PEG) or even another coat of calcium phosphate (Sokolova *et al.*, 2006), the technique as a whole remains limited for *in vivo* applications because of DNase I degradation of the complexes and because the complexes interact unfavourably with serum proteins, causing aggregation and increases in particle sizes (Mozafari & Omri 2007).

Liposomes

Complexing cationic lipids, or liposomes, with DNA is simple because positively charged lipids and negatively charged DNA spontaneously form complexes (lipoplexes) through ionic interactions (Felgner *et al.* 1987).

Following an initial rapid interaction between liposomes and DNA through electrostatic interaction, a slower lipid rearrangement process occurs (Pedroso de Lima *et al.* 2001).

The simplest way to prepare lipoplexes is to mix diluted solutions of preformed liposomes with plasmid DNA, which yields heterogeneously sized lipoplexes with a similar morphology (Gao *et al.* 2007). However, an alternative approach is to mix a DNA-containing solution directly onto a dried film of cationic lipid and DOPE (Hofland *et al.* 1996). The latter approach entraps DNA within multilamellar liposomes rather than sandwiching DNA between liposomes (Hofland *et al.* 1996). Thus, liposomes can form one of two structures with DNA – either a spherical, smooth surface structure, in which DNA is entirely wrapped within the liposome (Wasungu & Hoekstra, 2006), or a flat structure in which DNA strands are intercalated between lipid bilayers into a multilamellar structure (Rädler *et al.* 1997).

Following DNA binding, complexes that maintain a net positive charge are then able to interact with negatively charged cell membranes (Ourlin *et al.* 1997) to be uptaken through either endocytosis (Li & Huang, 2000; Mozafari & Omri, 2007, Wasungu & Hoekstra, 2006) or alternatively by membrane fusion (Mozafari & Omri, 2007; Ourlin *et al.* 1997) (see Fig. 1 below). It has been suggested that DNA is released from liposomes once inside the cell because of an interaction between cationic liposomes and anionic cell lipids,

leading to charge neutralization and the release of DNA from the complex (Gardlik *et al.,* 2005; Mozafari & Omri, 2007). In liposome-based transfection, DNA is released from the liposome prior to nuclear entry (Xu *et al.* 1996) and for successful transfection, must then be trafficked to the nucleus, the ultimate site of action for subsequent gene expression (Shenoy & Amiji, 2007). It is this transport of DNA to the nucleus that has proven the critical barrier to success using liposomal transfection (Dunlap *et al.*, 1997).

A number of liposome formulations have shown enough promise during *in vitro* studies to warrant evaluation in clinical trials for the treatment of cystic fibrosis. DOTAP [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane], first described by Leventis & Silvius (1990), demonstrated reduced toxicities because the ester bonds it possessed, linking the cationic head group to the lipid anchor, facilitated degradation in eukaryotic cells. However, DC-Chol{3b[*N*-(*N*´,*N*´-dimethylaminoethane)-carbamoyl]cholesterol} mixed with DOPE, first described by Gao & Huang (1991), remains the most widely used lipid in cystic fibrosis clinical trials as it shows even lower toxicities still. The use of cholesterol, which stabilizes lipid bilayers, rather than hydroxyalkyl chains as the lipid anchor, meant not only were toxicities reduced with DC-Chol, liposome stability was improved and effectiveness was enhanced. Lipid GL-67^{TM} has a similar structure to DC-Chol but has a triple amine head-group rather than a single amine head. However, despite its increased transfection efficiencies, Lipid GL-67^{TM} appeared to cause greater pulmonary toxicity than

DC-Chol (Lee *et al.* 2005). Summarising the effects of polymer structure on transfection efficiency, Lee *et al.* (2005) conclude that trivalent cationic head-groups were more effective than equivalent monovalent, bivalent or quadrivalent lipids. Also, carbamate linkers and cholesterol anchors appeared most effective when compared with alternative structures.

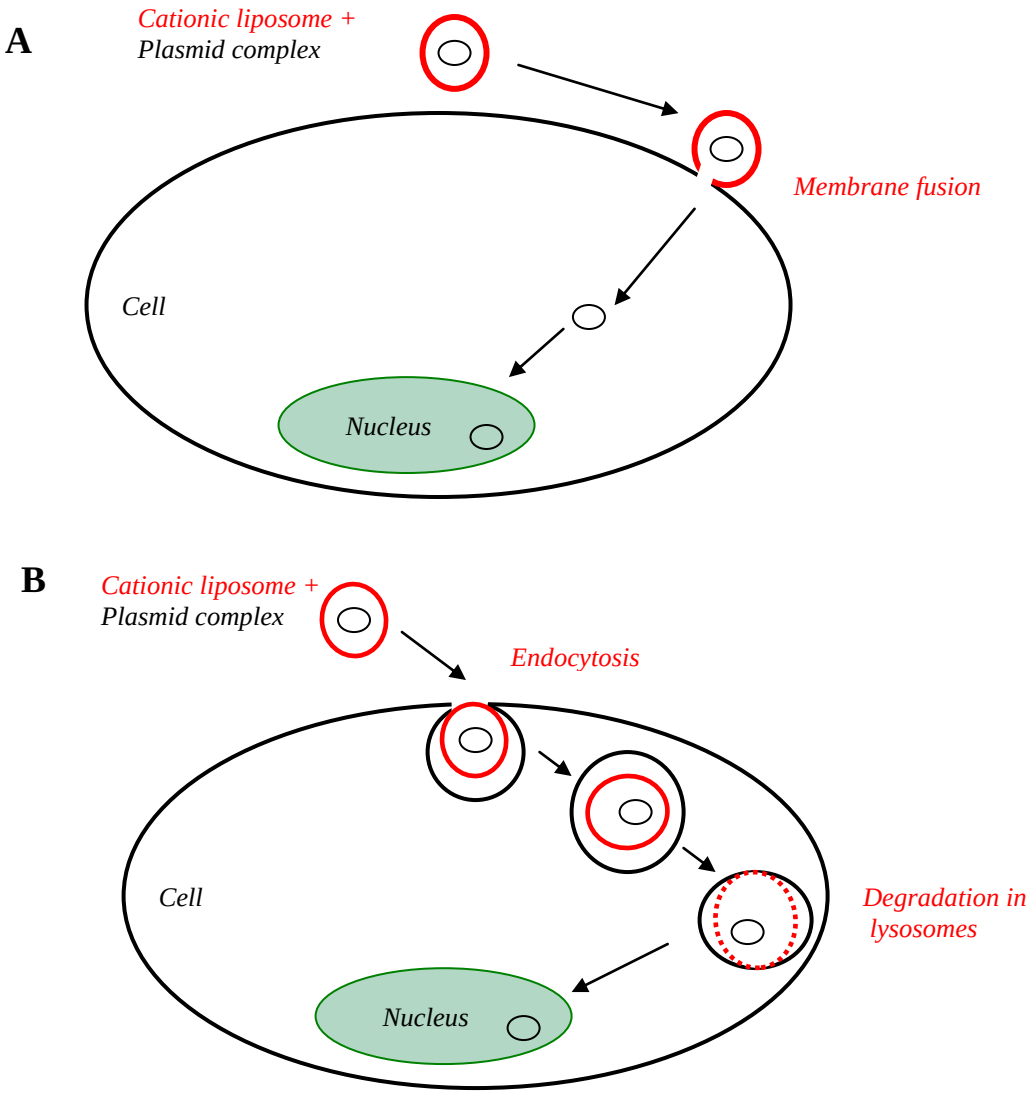

Figure 1: A: uptake of liposomes through membrane fusion – liposomes fuse with the plasma membrane and deliver their DNA payload directly into the cytoplasm. DNA must then be trafficked to the nucleus for transcription to take place. B: uptake of liposomes through endocytosis. Cells ingest the liposome/DNA complex within the plasma membrane, which encircles the lipoplex before pinching off to entrap the lipoplex within an endosome. The

lipoplex must then escape the endosomal compartment to avoid lysosomal degradation, and must then be trafficked to the nucleus for transcription to take place. Redrawn from Lee et al. (2005).

One of the reasons why many liposome formulations are more effective *in vitro* than *in vivo* is because there is plethora of negatively charged and often amphipathic proteins in polysaccharides present in the blood, mucus, epithelial lining fluid or tissue matrices to which a candidate transfection vector would not be exposed to *in vitro* (Gao *et al.* 2007). In the presence of such components, dramatic changes in size, surface charge and lipid composition of liposomes are observed (Gao *et al.* 2007). Additionally, when administered *in vivo*, many lipoplexes never even reach their intended target as they interact with negatively charged blood components, forming large aggregates which are absorbed onto the surface of red blood cells or become trapped in a thick mucus layer for example (Gao *et al.* 2007).

Polyplexes

The potential of polymers as an alternative to liposomes for gene delivery has long been known. Wu & Wu (1988) used poly-L-lysine DNA vectors to deliver SV2 CAT plasmid DNA to rat liver cells and a whole host of other polymers have since been used to achieve transfection.

Polymers used in the past to achieve gene delivery include cationic polymers such as chitosan (Strand *et al.* 2005; Zhao *et al.* 2006), gelatin (Kaul & Amiji,

2004; Leong *et al.* 1998), poly(ethyleneimine) (PEI) (Bertschinger *et al.* 2006; Feng *et al.* 2006; Nimesh *et al.* 2006) and poly(ethylene glycol) (PEG) (Jang *et al.* 2006) but also non-ionic polymers such as poly(lactic-co-glycolic acid) (PLGA) (Tinsley-Bown *et al.* 2000;) and non-condensing polymers such as poly(vinyl pyrollidone) (PVP) (Jang *et al.* 2006; Mumper *et al.* 1996; Mumper *et al.* 1998) and poly(vinyl alcohol) (PVA) (Mumper *et al.* 1996; Mumper *et al.* 1998; Wittmar *et al.* 2005).

Polymers designed for gene delivery must fulfill several biological functions including encapsulating DNA and stabilizing it by providing a protective hydrophilic polymer coating (Shenoy & Amiji, 2007). Once uptaken by cells, vectors must release the genetic material and facilitate its transport to the nucleus – the ultimate site of action for genetic expression (Shenoy & Amiji, 2007).

Although cationic polymers remain the most commonly used as they have a higher DNA encapsulation efficiency than neutral polymers (the low encapsulation efficiency of DNA in non-ionic polymers is due to the difficulty in incorporating hydrophilic DNA into a hydrophobic polymer), and despite the fact encapsulation of DNA with non-ionic polymers requires higher energy which denatures some of the DNA (Jang *et al.* 2006), Mumper *et al.* (1996) have shown that neutral, non-condensing polymers such as PVA can still bind to DNA and afford it a level of protection from nucleases, achieving improved

distribution in rat tibialis muscle compared to plasmid administered in a saline solution.

PVA is an amphiphilic molecule with a hydrophobic backbone and hydrophilic side chains and acts as a hydrogen bond donor. As it is charge neutral it stabilizes DNA through hydrogen bonding and/or van der Waals forces rather than charge neutralization - as is the case with cationic polymers (Holtorf & Mikos, 2002), which form complexes with DNA spontaneously in solution through electrostatic interactions between negatively charged phosphate groups on the DNA and the positively charged groups of the polycation (Jang *et al.* 2006).

The number of authors reporting successful transfection with PVA nanoparticles, relative to commonly used polymers such as 25 kDa PEI, remains small, with only a handful of papers reporting successful transgene expression following transfection with PVA nanoparticles. In addition to papers by Mumper *et al.* (1996) and Mumper *et al.* (1998) - which described *in vivo* transfection in rat skeletal muscle - Wittmar *et al.* (2005) have reported successful transfection with amine-modified PVA vectors. However, transgene expression was only noted when transfections were performed in the presence of an endosome disruptor, chloroquine (Wittmar *et al.* 2005), despite the fact cellular uptake of nanoparticles had been reported,

suggesting that an inability to escape the endosomal compartment may have been responsible for the lack of reporter gene expression observed.

Those findings were consistent with those previously described by Kimura *et al.* (2004), who fluorescently labelled pDNA with rhodamine and formed subsequent complexes through ultra high pressure technology with four different PVA's which differed in their degree of polymerization and saponification. Those PVA's with higher degrees of saponification (98.5 %, 99.3 % and 99.8 %) were better internalized into the Raw264 cell line, whilst the PVA with an 88 % degree of saponification did not appear to have been internalized into cells.

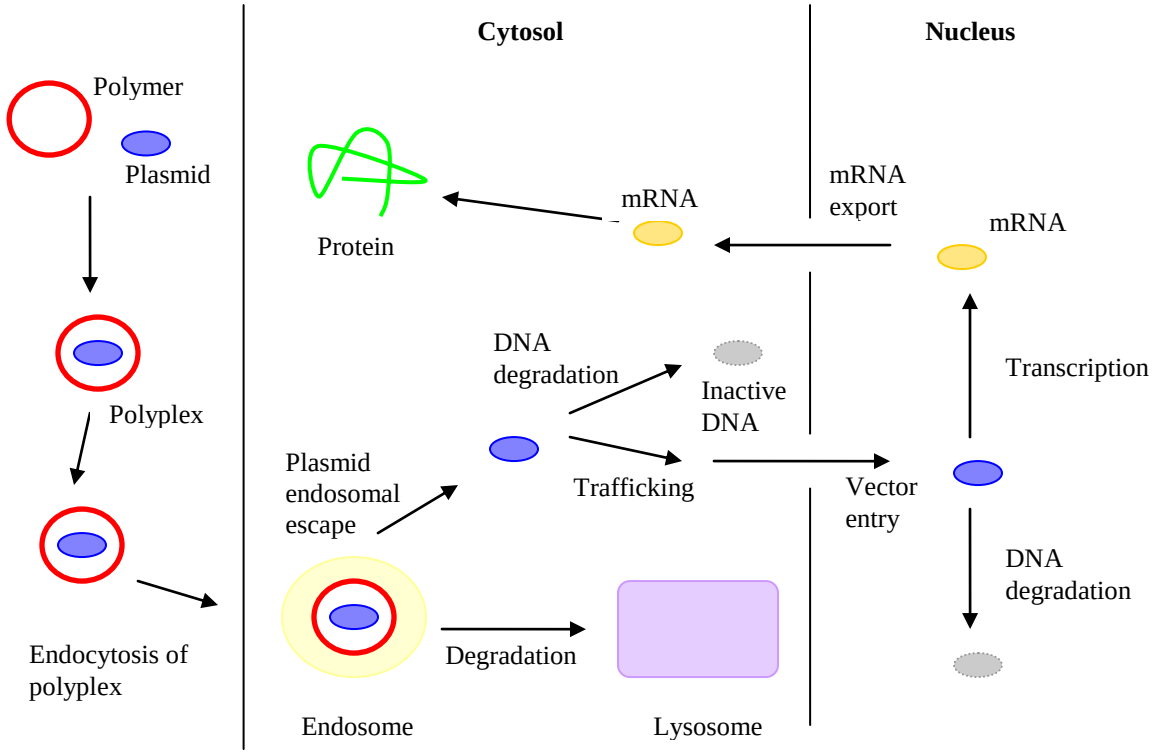

Fig 2. – schematic illustration of the process involved in gene expression. Redrawn from Maitra (2005).

Nevertheless, in all cases, no reporter gene expression was reported from the study because it would appear, as in one of the possible fates for an internalized vector in Figure 2 above, that the plasmid encapsulated in the nanoparticles in question had failed to escape the endosome and was degraded by the lysosome rather than being trafficked to the nucleus.

An intracellular trafficking study undertaken by Godbey *et al.* (1999) yielded important results for workers in the field as it identified the pathway fluorescently labelled polyplexes complexes take during transfection. Doubly-labeled PEI/DNA complexes attached to the cell membrane at discrete locations, suggesting endocytosis as the route of entry in cells, after 30 minutes. Nuclear localization was noted 3.5 - 4.5 hours after polyplexes had been administered to cells, however, transgene expression was not noted until a further hour later (Godbey *et al.* 1999). In some instances, large aggregations of fluoresecence were noted, indicating polyplexes had become trapped inside intracellular vesicles, whilst in other cases, polyplexes could freely disperse in the cytoplasm after endosomes or endolysosomes had been disrupted (Godbey *et al.* 1999). As both Kimura *et al.* (2004) and Wittmar *et al.* (2005) reported cellular uptake of PVA nanoparticles but failed to report transgene expression, it would appear that in both instances the lack of gene expression was a result of inefficient endosomal escape. The fact Wittmar *et al.* (2005) reported gene expression only when transfections were performed in the presence of chloroquine further reinforces this conclusion.

Perhaps the most active and most studied polymer for gene delivery system is poly(ethyleneimine) (PEI) (Gao *et al.* 2007). The success of PEI in comparison to other polymers stems from the fact that it possesses an inbuilt mechanism for endosomal escape and thus is avoids lysosomal trafficking (Akinc *et al.* 2005). PEI is only partially protonated at physiological pH (only one of six amino nitrogens) and thus, upon acidification within the endosome, PEI presumably acts as a proton sponge, with an influx of protons into the endosome occurring. To ensure charge neutrality, chloride ions are pumped into the endosome at the same time which increases the ionic strength of the medium, and this leads to osmotic swelling and destabilization of the endosomal vesicle (Akinc *et al.* 2005).

Akinc *et al.* (2005) have used proton pump inhibitors and N-quaternized forms of PEI and shown that transfection efficiency is reduced by two orders of magnitude, and can only be increased if chloroquine, an endosome disruptor, is included in the transfection medium. Chloroquine is a weak base, FDA approved for the treatment of malaria (Pack *et al.* 1999), and is often used at a final concentration of 100 µM in transfection media (Akinc *et al.* 2005; Leong *et al.* 1998; Reschel *et al.* 2002) to buffer the endosome to maintain a neutral endosomal pH and is able to improve rates of transfection in two ways. Firstly, it reduces the activity of the lysosomal nucleases and secondly, the buffering it provides may produce expansion of the internalized vector, leading to subsequent destabilization of the endosome (Wiethoff and

Midaugh, 2002). However, it is impractical for *in vivo* gene therapy because it is toxic to many cell types *in vitro* and, in high doses, may lead to a variety of undesirable side effects (Pack *et al.*, 1999). As a consequence, developing a vector that does not rely on chloroquine for endosomal escape is desirable. Although glycerol, which presumably increases transfection efficiency by interfering with lysosomal degradation and increasing DNA released into the cytoplasm (Wagner, 1998) and is typically used at a concentration of 1 – 1.8 M (Zauner *et al.*, 1996), and inactivated adenoviruses have also been used for the same purpose (Wagner *et al.*, 1992), both are also equally undesirable for *in vivo* applications - high local concentrations of glycerol that would be required to bring about improvements in transfection efficiency whilst inactivated adenovirus particles, although they appear non-toxic *in vitro*, would be expected to be immunogenic and add an extra step to the vector synthesis procedure (Pack *et al.* 1999).

Although Oster *et al.* (2004) have shown that chemically modifying PVA can lead to successful transfection - grafting PLGA onto the PVA backbone produced nanoparticles that transfected L929 mouse fibroblast cells eight times more effectively than PEI – PVA has also been used during transfection studies when used as an emulsifier (Panyam *et al.* 2002; Prabha & Labhasetwar, 2004) or a stabilizer (Li *et al.* 2003; Perez *et al.* 2001) in the production of polyplexes between DNA and another polymer, rather than as the main constituent of a nanoparticle.

For example, Li *et al.* (2003) have reported that adding PVA to a plasmid DNA-containing solution during encapsulation with PEGylated polycyanoacrylate nanoparticles can reduce pDNA damage, although Prabha & Labhasetwar (2004) note that the levels of reporter gene expression observed are dependent upon the molecular weight and degree of hydrolysation of the PVA used as an emulsifier.

Perhaps the most successful examples of gene delivery using PVA have been achieved when the polymer has been used to coat superparamagnetic iron oxide nanoparticles (SPIONSs) with which cells are incubated with in the presence of a magnetic field during transfections. Petri-Fink *et al.* (2005) have synthesized PVA-coated SPIONs with a mean diameter of 9 mm, with various ratios to iron oxide of either PVA, carboxylate-functionalised PVA, thio-functionalised PVA and amino-functionalised PVA. Their results showed only amino-PVA coated SPIONS were uptaken in an interesting amount of the four SPION preparations tested.

Kamau *et al.* (2006) have also demonstrated high levels of gene expression using PVA or coated SPIONs, further functionalized with amino groups and fluorochromes, to transfect synovial cells in the presence of a magnetic field. PVA nanoparticles were uptaken by more then 80 % of synovial cells, whilst GFP expression was detected in 17.7 % of 293 T cells. In comparison, PEI coated SPIONS produced transfection in 43.5 % of 293 T cells and produced

a GFP signal of higher intensity which was visible after 24 hours, rather than 48 hours as in the case of PVA. PVA nanoparticles were less toxic, however. Thus, Kamau *et al*.'s (2006) results suggest either a difference in particle uptake by the cells for the two different polymers or different release in DNA release from these polymers.

Lastly, DNA release from PVA nanoparticles can be controlled by the nanoparticle production method, as shown by Kimura *et al.* (2007). Complexes between PVA and DNA were formed either through UHP technology or freeze thaw methods and their DNA release profiles were compared, with less DNA released by the pressurized hydrogels. The cumulative amount of DNA released decreased as the PVA content of the hydrogels increased, whilst varying the amount and duration of pressure used to form hydrogels can also influence DNA release (Kimura *et al.* 2007).

1.2 Materials and Methods

1.2.1 Abbreviations, reagents and materials

Abbreviations		**Supplier/code (if applicable)**
PBS	Phosphate Buffered Saline	Sigma, UK P3813
HBSS	Hanks Balanced Salt Solution	Promocell, Germany C-40390
RPMI 1640	Roswell Park Memorial Insititute (cell culture medium)	Promocell, Germany C-76015
Advanced DMEM	Advanced Dulbecco's Modified Essential Medium	GIBCO, UK 12491
DAPI	4',6-diamidino-2-phenylindole	A constituent of Vectashield mounting medium
PVA	poly(vinyl alcohol)	
PEI	poly(ethyleneimine)	
PEG	poly(ethylene glycol)	
DOTMA	{*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium chloride}	
DOPE	(dioleoyl phosphatidylethanolamine)	
DOSPA	{2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-l-propanaminium trifluoroacetate}	
DOTAP	[1,2-bis(oleoyloxy)-3-(trimethylammonio)propane]	
DC-Chol	{3b[*N*-(*N´*,*N´*-dimethylaminoethane)-carbamoyl]cholesterol}	
PLGA	poly(lactic-co-glycolic acid)	
PVP	poly(vinyl pyrollidone)	
PD2000	Patented cross linker used by AGT Sciences Ltd in nanoparticle production	
pEGFP-N1	Plasmid DNA used in the project, coding for green fluorescent protein, under the control of a cytomegalovirus promoter	
DH5-α™	Strain of *Escherichia coli* that was	Kindly provided by Dr.

	transformed with the plasmid	Steven Picksley, University of Bradford, UK.
NIH 3T3	Mouse embryonic fibroblast cells used during transfection studies	Kindly provided by Debbie Fischer University of Bradford, UK.
HaCaT	Keratinocyte cells used during transfection studies	Kindly provided by Dr. Annie Smith, University of Bradford, UK.
POVAL	Hydrogel formulation used to produce nanoparticles, 99 % hydrolysed	
KP-08	Hydrogel formulation used to produce nanoparticles, 80 % hydrolysed	
EtBr	Ethidium Bromide	Fluka, UK 46067
TAE	Tris-acetate EDTA buffer solution	Invitrogen, UK
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma, UK H0887
FCS	Foetal calf serum	Promocell, Germany C-37360
NCS	Newborn calf serum	Sigma UK N4762
LB broth/agar	Luria-Bertani broth/agar	
GFP	Green fluorescent protein	
	Chloroquine	Sigma, UK C6628
	Kanamycin	Sigma, UK K1377
	Ampicillin	Sigma, UK A0166
	Tween 20	Sigma UK, P-7949
	Amphotericin B (fungizone)	Sigma UK, A2942
	Optimix solution (electroporation buffer)	Equibio, UK
	Lipofectin®	Invitrogen 18292-011
	LabelIT® Tracker Intracellular Nucleic Acid Localisation Kit	Mirus Bio Corp, USA MIR 7013
TAE	tris-acetate EDTA buffer solution	Invitrogen, UK 15558

	Electrophoresis Grade Agarose	Invitrogen, UK 15510
	Trypsin-EDTA	Sigma, UK T 4049

Table 1 – Abbreviations, reagents and materials and source of supply

All hydrogel nanoparticles used during the project were synthesised and provided by AGT Sciences Ltd, Bradford, UK in distilled water as a 10 mg/ml colloidal suspension. Nanoparticles were synthesised from PVA and were crosslinked with AGT Sciences' patented cross-linker, PD2000, giving the nanoparticles a positive charge. The main hydrogel formulation evaluated during the project was 'KP-08', which contains 30 % residual acetate and is thus more hydrophobic than the 'POVAL' formulation which was later evaluated. POVAL contains less than 1 % residual acetate and thus its surface is less hydrophobic compared to KP-08.

1.2.2 General Methods

In order to provide a source of cells for transfection experiments, cell culture work was first undertaken to establish and maintain cell lines that could readily provide cells when required. Microbiological work was also undertaken to transform competent DH5-α^{TM} *Escherichia coli* (*E. coli*) to uptake reporter plasmid DNA encoding enhanced green fluorescence protein (EGFP-N1). The transformed *E. coli* were then cultured on a large scale to yield large amounts of the EGFP-N1 plasmid which was subsequently purified on a large

scale using an endotoxin-free gig-prep kit (Qiagen, UK) for use in hydrogel binding and transfection experiments.

1.2.2.1 Cell Culture

Three types of cells were used on the project – HaCaTs (spontaneously transformed human keratinocytes), NIH 3T3's (mouse embryonic fibroblasts) and neonatal articular chondrocytes (isolated from mice). Experiments switched to using different cell types for transfection at various points during the project as certain cell types have proven more difficult to transfect than others in the literature.

HaCaT cell culture

HaCaT cells were cultured in RPMI media, fully supplemented with 10 % (v/v) FCS, 2 mM U/ml L-Glutamine, 100 U/ml penicillin-streptomycin and 1 µg/ml amphotericin B. Cells were cultured in either T-25 or T-75 cell culture flasks and incubated at 37ºC in a 5 % CO_2 atmosphere and split once they had reached 70 % confluence.

NIH 3T3 cell culture

NIH 3T3 cells were grown in advanced DMEM fully supplemented with 5 % (v/v) NCS, 20 mM U/ml L-Glutamine, 100 U/ml penicillin-streptomycin, 1

µg/ml amphotericin B and 20 nM HEPES. Cells were cultured in either T-25 or T-75 cell culture flasks and incubated at 37ºC in a 5 % CO_2 atmosphere until they had reached 70 % confluence.

Chondrocyte cell culture

Articular chondrocytes obtained from neonatal mice (passage one) were kindly provided by Dr. Abbas Din (AGT Sciences Ltd, Bradford, UK) and cultured in RPMI media, fully supplemented with 10 % (v/v) FCS, 2 mM U/ml L-Glutamine, 100 U/ml penicillin-streptomycin and 1 µg/ml amphotericin B. Cells were cultured in either T-25 or T-75 cell culture flasks and incubated at 37ºC in a 5 % CO_2 atmosphere and split once they had reached 70 % confluence.

Upon reaching confluence cells were briefly washed with HBSS and then trypsinised using 4ml (T-25) or 8ml (T-75) trypsin-EDTA. The enzymatic activity of trypsin was neutralised using 0.125 volumes of fully supplemented cell culture media and cells were then centrifuged in a Hettich Rotofix 32 centrifuge at 2000 rpm for 5 minutes. Cell pellets were then re-suspended in an appropriate volume of the appropriate cell culture media (fully supplemented) and were plated back out into T-25 or T-75 cell culture flasks containing appropriate cell culture media.

1.2.2.2 Plasmid Purification

A stock of pDNA was obtained through four steps which are subsequently described;

1. Preparing competent DH5-α^{TM} *E. coli* cells
2. Purifying small (µg) quantities of pEGFP-N1 from previously transformed *E. coli*
3. Transforming the *E. coli* from step 1 with the pEGFP-N1 obtained in step 2
4. Culturing the transformed *E. coli* from step 3 on a large-scale sufficient to yield (mg) quantities of pEGFP-N1 from plasmid purification.

Preparation of competent cells

Using a sterile inoculating loop, DH5-α^{TM} *E. coli* were transferred from a frozen glycerol stock and streaked out onto a Luria-Bertani (LB) agar plate lacking antibiotics. A control plate was also prepared by streaking out *E. coli* onto a kanamycin selective (50 µg/ml) LB agar plate. Both plates were then incubated at 37 ºC for 24 hours to promote bacterial growth, after which time they were stored, inverted, at 4ºC in the fridge.

Preparation of pEGFP-N1 DNA

Plasmid DNA was purified from previously transformed DH5-α *E. coli* using a mini-prep plasmid purification kit (Qiagen, UK) to yield 20 µg of pDNA that could be incorporated into *E. coli* during transformation.

Previously transformed *E. coli* containing the pEGFP-N1 plasmid were streaked out from a frozen glycerol stock onto a kanamycin (50 µg/ml) selective agar plate. Control plates were also prepared by streaking out *E. coli* onto ampicillin selective (100 µg /ml) and plain agar plates. All plates were then incubated at 37ºC for 24 hours to promote bacterial growth.

The following day a single colony of the transformed bacteria was inoculated into 5 ml of LB broth supplemented with 50 µg/ml of kanamycin in a 15 ml falcon tube. A falcon tube containing LB broth only was used as a negative control and for subsequent spectrophotometric readings. Both cultures were grown overnight at 37 ºC with moderate shaking at 180 rpm in an orbital shaker.

The following morning, 1.5 ml of the overnight culture was transferred into two separate 1.5 ml eppendorf tubes and centrifuged at 13,000 rpm for 1 minute in an MSE Micro Centaur table top centrifuge to obtain a bacterial pellet. The bacterial pellets were combined into one tube by re-suspension in 250 µl of

buffer P1 after supernatants had been discarded, and the plasmid purification procedure was followed according to manufacturer's instructions. In the final step of the procedure, plasmid DNA was eluted into 50 µl of EB buffer and the DNA concentration was determined spectrophotometrically by diluting 5 µl of the DNA stock 1:200 in EB buffer to a final volume of 1 ml in a quartz cuvette and measuring the absorbance reading at 260 nm. Purified DNA was then stored at -20°C until required.

Transformation of cells

An isolate from a streaked plate of untransformed bacteria was then inoculated into 5 ml of LB broth in a 15 ml falcon tube and also into 5 ml of LB broth containing 50 µg/ml kanamycin (positive control). A negative control was also prepared by transferring 5 ml of LB broth into a separate falcon tube but adding no bacteria. Both tubes were then incubated at 37ºC in an orbital shaker with moderate shaking at 180 rpm overnight.

100 µl of overnight culture was then inoculated into 10 ml of LB medium (1:100 dilution) in 50 ml falcon tubes and then incubated in an orbital shaker at 37ºC with shaking of 180 rpm until an OD_{650nm} of 0.3-0.4 (mid-log phase) had been reached.

1 ml of the bacterial culture was then transferred into two separate 1.5 ml eppendorf tubes (one to act as a negative control), which were then

centrifuged at 13,000 rpm for 1 minute at room temperature in a MSE Micro Centaur table-top centrifuge.

The supernatant was decanted from each tube and each pellet was re-suspended in 800 µl of ice cold 0.1 M $CaCl_2$ solution. Both tubes were held on ice for 20 minutes and then centrifuged as before. Supernatants were discarded and each pellet was re-suspended in 400 µl of ice cold 0.1 M $CaCl_2$ solution and held on ice for 10 minutes.

Both solutions were then centrifuged again, as above, and re-suspended in 200 µl of 0.1 M $CaCl_2$ solution. 200 µl of each solution was then transferred into two new 1.5 ml eppendorfs. 5 µl of pEGFP-N1 pDNA was then added to one of the tubes, which was tapped gently to mix the solution, and then both tubes were held on ice for 30 minutes.

The bacteria were then heat shocked by submerging them into a 42°C water bath for 45 seconds followed by a further 5 minutes of incubation on the bench top on ice. 1 ml of LB broth was then added to each tube, mixed by inverting the tube, and then incubated at 37°C for 45 minutes in an orbital shaker set to 200 rpm to allow expression of the kanamycin resistance protein. 200 µl of the bacterial suspension was then transferred onto agar plates containing either LB agar alone, kanamycin (50 µg/ml) or ampicillin (100 µg/ml) using sterile spreaders. 200 µl of the control suspension lacking

pDNA was also transferred onto plates containing either LB agar alone or kanamycin (50 µg/ml). Plates were left to dry on the bench top for a few minutes and then inverted and incubated overnight at 37°C.

The following day a single colony was isolated from the LB-selective plate, inoculated into 5 ml of LB broth containing 50 µg/ml kanamycin in a 15 ml falcon tube, and grown overnight. The following morning, 800 µl of the overnight culture was then mixed with 200 µl of sterile 80% glycerol and frozen down in screw top vials at -20ºC. This provided a long-term stock of transformed bacteria that could be defrosted, cultured and harvested for the plasmid during the project whenever required.

Large – Scale Plasmid Purification

Plasmid DNA was purified from previously transformed DH5-α *E. coli* on a large scale using an EndoFree Giga-prep plasmid purification kit (Qiagen, UK).

Using a sterile inoculating loop, transformed *E. coli* were streaked out onto agar plates containing either kanamycin 50 µg/ml, ampicillin 100 µg/ml or LB agar alone. The plates were left on the bench top for a few minutes to dry and were then incubated at 37ºC for 24 hours.

The following day a single colony of bacteria from the kanamycin selective plate was inoculated into a starter culture of 10 ml of LB broth containing 50 µg/ml of kanamycin and grown in an orbital shaker at 37ºC with 200 rpm, for 8 hours.

1 ml of the starter culture was then transferred into each of four 2 litre conical flasks, each containing 500 ml of LB broth supplemented with 50 µg/ml of kanamycin. All flasks were then transferred to an orbital shaker set to 200 rpm for 16 hours growth at 37ºC.

The following day the cultures were centrifuged using a Beckman Coulter Avanti J-25 centrifuge equipped with a JLA 10.500 rotor pre-cooled to 4ºC. Cultures were spun at 6000 x g for 15 minutes at 4ºC to avoid overheating of the samples. All supernatant was re-collected for autoclaving, whilst the wet pellet weight of the culture was determined to ascertain bacterial density.

The bacterial pellet was then re-suspended in 125 ml of buffer P1 and the plasmid purification procedure was followed according to manufacturer's instructions. Centrifugation during the latter stages of the procedure was conducted on a Beckman Coulter Avanti J-25 centrifuge equipped with a JA 25.50 rotor. Again, centrifugation was performed at 4ºC to avoid overheating of the samples and was performed at 18,000 x g for the necessary time intervals.

In the final purification step, pDNA was dissolved in endotoxin free TE buffer or sterile distilled water, and the pDNA concentration was determined spectrophotometrically on three spectrophotometers by reading the absorbance at 260 nm. pDNA was adjusted to an appropriate concentration and then aliquotted into eppendorf tubes for long term storage at -20ºC. Lastly, DNA quality was determined via electrophoresis by running a 1 µg sample of the purified plasmid on an agarose gel.

1.2.2.3 Rhodamine-labelling of pDNA

For intracellular tracking of pDNA during transfection, µg quantities of pDNA were labelled with rhodamine using a LabelIT® tracker TM-rhodamine labelling kit according to manufacturer's instructions and using a Heraeus bench-top mini centrifuge pre-cooled to 4 ºC.

After rhodamine-labelled plasmid had been pelleted in the final step following ethanol precipitation, the stock of labelled plasmid was re-suspended in sterile distilled water in 2 µg aliquots ready for transfection when required.

1.2.2.4 Polyplex formation

To synthesise polyplexes the required amount of hydrogel (typically 40 µl of a 10 mg/ml stock: 400 µg of hydrogel) was mixed with a 2 µg aliquot of pDNA (re-suspended in 25 µl of sterile distilled water) in 1.5 ml eppendorf tubes,

mixed by tapping the bottom of the tube, and incubated at room temperature for 15 minutes.

For electrophoresis experiments the polyplexes formed were typically diluted into HBSS, PBS or cell culture media such as RPMI 1640, advanced DMEM or Opti-MEM to test the stability of the complexes in buffered solutions.

For transfection experiments, polyplexes (typically of volume 65 µl, with 2 µg of pDNA in each sample) were diluted in a final volume of 2 ml of Opti-MEM media and incubated at room temperature for at 5 minutes before being added to cells.

1.2.2.5 Agarose Gel Electrophoresis

Samples were prepared by mixing 20 µl of solution with 5 µl of gel loading buffer (a 50:50 mix of sterile glycerol and distilled water) before being loaded into the agarose gel. The presence of the loading buffer added density to the sample to prevent samples floating out of the wells. However, bromophenol blue, which is typically included in loading buffers so that DNA fragments can be visually tracked during electrophoresis, was omitted from the loading buffer to prevent any possible interaction the dye may have had with the hydrogel nanoparticles in each sample.

Electrophoresis was performed on 0.8 % (w/v) agarose gels made up using molecular biology grade agarose dissolved in 1 X TAE buffer using a microwave oven. For some experiments 0.5 µg/ml ethidium bromide (EtBr) was incorporated into the cast gel whilst in others, gels were cast without EtBr and then subsequently stained in a small plastic box filled with 400 ml of 1 X TAE containing 0.5 µg/ml EtBr. In the latter case, gels were soaked for 30 minutes and agitated periodically before being briefly rinsed under a cold water tap to remove excess dye. Omitting EtBr from the gels and staining them after the samples had been run in the electrophoresis tank improved the resolution of the subsequent image produced; however, it lengthened the time taken for each experiment.

For electrophoresis, agarose gels were placed into Bio Rad sub-cell tank linked to a Bio-Rad Power Pac 200 power supply and run at 90 V for 90 minutes. After this time gels were stained in an ethidium bromide solution if required and then transferred under a UV light box to be photographed using UVI photo imaging software.

For each experiment a sample of free pDNA, which was re-suspended in distilled water but unattached to any vector, and a DNA ladder (Hyperladder I DNA, Bioline, USA) were included in separate lanes on each agarose gel. The inclusion of free DNA allowed a comparison to be made with DNA mixed with hydrogel in polyplex samples. The presence of the DNA ladder, which

contained DNA fragments of known length, allowed an estimate of the size of the DNA fragments in each sample to be made. By mixing the DNA ladder with a loading buffer containing bromophenol blue it was possible to visually track the movement of the DNA fragments across the gel.

1.2.2.6 Transfection

On the day preceding transfection, cells were seeded onto glass coverslips (Chance Propper, UK), placed inside 35 x 10 mm Petri dishes, typically at a density of 1 x 10^5 cells per dish (cell counts were determined using a Neubauer haemocytometer). Cells were incubated for 15 mins at 37 ºC in a 5 % CO_2 atmosphere to allow cell attachment and then an additional 2 ml of the appropriate cell culture media was added to each dish. Cells were then grown overnight at 37 ºC in a 5 % CO_2 atmosphere, giving rise to a cell layer that was around 70 % confluent on the day of polyplex addition.

During transfection, cells were transfected with polyplexes diluted into Opti-MEM cell culture medium, typically in the absence of serum, over a 5 hour period. A negative control of free pDNA diluted into Opti-MEM media was also included into each transfection experiment, as was a positive control comprised of Lipofectin® complexed with 2 µg of pDNA according to manufacturer's instructions.

When the transfection period had elapsed, polyplex containing solutions were aspirated off cells and a media change, with fully supplemented media, was performed. Cells were typically grown for a further 43 hours post-transfection before being washed three times with PBS-T for five minutes for each wash at 37ºC. After the third wash, cells were fixed with cell fixing solution (4 % paraformaldehyde (w/v) in PBS) for 30 minutes at room temperature and then washed with PBS-T a further three times, again with 5 minutes for each wash at 37ºC.

Coverslips were then inversely mounted onto clean glass slides using Vectashield Mounting Medium (containing DAPI) and kept dark in tinfoil until analysis to prevent bleaching of the fluorescence signal. Slides were analysed on one of two microscopes – a Leitz Dialux 22 microscope connected to a Kodak DC290 zoom digital camera for imaging of GFP or rhodamine detection or a Nikon Eclipse 80i microscope for GFP, rhodamine, or DAPI detection.

Images taken using the Leitz microscope were taken using Adobe Photoshop imaging (version 6) software. Images taken using the Nikon microscope were taken using ACT-2U imaging software and were subsequently edited in Adobe Photoshop if necessary.

1.2.3 Examining the properties of KP-08 Nanoparticles for gene delivery

1.2.3.1 Gel mobility shift assay to determine the optimal KP-08/pDNA binding ratio

Complexes of KP-08 and pDNA were formed at varying ratios; 6:1, 8:1, 12:1, 16:1, 24:1, 31:1, 48:1, 63:1, 94:1, 126:1 and 189:1 (µg of hydrogel: µg of pDNA) and an agarose gel mobility shift assay for DNA binding was performed. This revealed the minimum amount of hydrogel needed to bind all DNA in a given sample. 0.53 µg of DNA was used in all samples.

When KP-08 had been mixed with pDNA the final volume of each solution was 22.5 µl. 5 µl of loading buffer was then added to each solution before 25 µl was loaded into an 0.8 % agarose gel, prepared as previously outlined. A DNA ladder and a sample of free pDNA (0.53 µg) were also included in control lanes.

1.2.3.2 Determining minimum charge needed to bind pDNA and complex stability at differing pH through agarose electrophoresis

Five KP-08 solutions with different levels of positive charge were provided; ‘1/.01’, ‘1/.02’, ‘1/.03’, ‘1/.04’ and ‘1/.05.’ 40 µl of each formulation was mixed

with 2 µg of DNA and incubated at room temperature for 15 minutes to form polyplexes and then diluted into 135 µl Opti-MEM media of differing pH (7.65, 7.00, 6.42, 6.15, 5.15 or 4.04) for further incubated for 30 minutes at room temperature. 20 µl of the diluted polyplex solutions were then mixed with 5 µl of loading solution before loading into an 0.8 % agarose gel along with control lanes of free DNA and a DNA ladder.

1.2.3.3 Evaluating KP-08 nanoparticles during transfection

NIH 3T3 cells were cultured and prepared for transfection as outlined previously. KP-08 polyplexes were formed by mixing 40 µl of 10 mg/ml KP-08 with 2 µg of pDNA and incubated at room temperature for 10 minutes. Polyplexes were produced in duplicate so that transfections could take place over either 5 hours or 24 hours and either in the presence of absence of 100 µM chloroquine. When transfections were performed over 24 hours 5 % NCS was included in the transfection media to maintain cell growth. Naked DNA controls were also prepared for transfection periods of either 5 hours or 24 hours (with 10 5 % serum), as were positive controls of Lipofectin®. Cells were prepared for analysis post-transfection as described previously.

1.2.3.4 Examining DNA release from KP-08 nanoparticles at different pH

Complexes of KP-08 and pDNA were formed at a ratio of 200:1 in duplicate by mixing 80 µl of 10 mg/ml KP-08 with 4 µg of pDNA (total volume 130 µl)

and incubating at room temperature for 10 minutes. Eight different solutions of Opti-MEM media were then prepared by adjusting the pH to either 7.8, 7.15, 6.54, 5.93. 5.15, 4.68 or 4.01 and then the KP-08/pDNA complexes were diluted with 270 µl of one of the Opti-MEM solutions. Diluted complexes were then incubated at room temperature for 30 minutes and then 20 µl of the diluted complexes (containing 0.4 µg of pDNA) was mixed with 5 µl of gel loading solution before being loaded into an agarose gel.

1.2.3.5 Transfection of KP-08/Lipofectin® chimeric complexes

NIH 3T3 cells were seeded at a starting density of 2.1 x 10^5 cells per coverslip. Cells were grown overnight and then transfected the following day.

Transfection solutions were made up as follows;

1. KP-08/pDNA nanoparticles - 2 µg of pDNA and 40 µl of 10 mg/ml KP-08 were mixed together in an eppendorf tube and incubated at room temperature for 60 minutes.
2. 'Chimeric' nanoparticles - 2 µg of pDNA and 40 µl of KP-08 were mixed together in an eppendorf tube and incubated at room temperature for 45 minutes. Simultaneously, 5 µl of Lipofectin® was diluted into 100 µl of Opti-MEM cell culture medium and also incubated at room temperature for 45 minutes. 35 µl of Opti-MEM media was added to the KP-08/pDNA containing solution to bring

the volume of the solution to 100 µl and then both solutions were then mixed together and incubated at room temperature for a further 15 minutes.

Lipofectin® and free DNA controls were also prepared as described previously, using 2 µg of pDNA for each control. Transfection solutions were made in double quantities - half was used for agarose electrophoresis (to determine whether DNA had bound to the hydrogel before the addition of Lipofectin®), the other half was used in transfection. In all cases the final volume of each transfection solution was adjusted to 2 ml using serum-free Opti-MEM media and all cells were transfected over a 5 hour period at 37ºC. Cells were prepared for analysis post-transfection as outlined previously.

1.2.3.6 Using rhodamine-labelled pDNA to detect successful vector internalisation

KP-08 polyplexes were prepared by mixing 40 µl of KP-08 with a 2 µg aliquot of rhodamine-labelled plasmid and incubating at room temperature for 15 minutes.

A chimeric transfection solution was prepared by mixing a 2 µg aliquot of rhodamine-labelled plasmid with 40 µl of KP-08 nanoparticles and incubating at room temperature for 45 minutes and simultaneously diluting 5 µl of

Lipofectin® into 100 µl of Opti-MEM media. The diluted Lipofectin® was then mixed with polyplexes and incubated at room temperature for a further 15 minutes.

A positive control was made up using Lipofectin® as described previously (except this time the stock of DNA was rhodamine-labelled) whilst a free DNA control (rhodamine labelled) was also prepared as in previous experiments. All transfection solutions were prepared in duplicate to a final volume of 2 ml in Opti-MEM media. One set of solutions was analysed immediately after transfection, the other was analysed 43 hours post-transfection. When cells were ready for analysis they were prepared as outlined previously, and were analysed on a Nikon Eclipse 80i microscope.

1.2.3.7 Investigating primary cells as an alternative target cell type

A flask of articular chondrocytes, obtained from neonatal rats, was provided by Dr. Din. Chondrocytes (passage 2) cultured in fully supplemented RPMI 1640 media were seeded out onto glass coverslips at a density of 1×10^5 cells per coverslip and grown overnight before transfection.

Transfection solutions were produced by mixing 40 µl of the KP-08 nanoparticles with either 2 µg of unlabelled pDNA or 2 µg of rhodamine-labelled pDNA. Control solutions of naked pDNA (unlabelled and rhodamine-

labelled) were also prepared, as were positive controls of Lipofectin® complexed with either unlabelled or rhodamine-labelled pDNA. All transfection solutions were brought to a final volume of 2 ml in serum-free Opti-MEM media. Cells were transfected over 5 hours at 37ºC and were prepared for analysis as outlined previously.

1.2.3.8 Electroporation of HaCaT cells with KP-08/pDNA polyplexes

HaCaT cells cultured in RPMI 1640 media were grown in T-75 tissue culture flasks and split upon reaching confluence. Trypsin-EDTA was used to strip the cells from the flasks and after the trypsin had been neutralised, cells were centrifuged to obtain a cell pellet and then re-suspended in 2.150 ml of Opti-Mix washing solution A electroporation buffer (Equibio). 50 µl of cell suspension was used for calculating cell density using a Neubauer haemocytometer and the remaining cell suspension was split into three 700 µl volumes in 1.5 ml eppendorf tubes.

A sample of polyplexes was prepared by mixing 40 µl of KP-08 with 2 µg (25 µl) of pDNA and incubated at room temperature for 10 minutes. The 65 µl sample of polyplexes was then added into one of the tubes containing 700 µl of cell suspension and was brought to a final volume of 800 µl using Opti-Mix electroporation buffer.

Control solutions of KP-08 (without DNA) and naked DNA (without KP-08) were also prepared. For the KP-08 control, 40 µl of hydrogel nanoparticles was added to 700 µl of cell suspension and the final volume of the solution was brought to 800 µl using Opti-Mix electroporation buffer. The naked DNA control was prepared by adding 2 µg (25 µl) of pDNA to a sample of cell suspension, and the final volume was brought to 800 µl using Opti-Mix electroporation buffer.

Each 800 µl sample was then separately pipetted into a 0.4 cm electroporation cuvette ready for electroporation. Cells were electroporated using a Gene Pulser II electroporator (Bio-Rad, USA), with 1.8 million cells electroporated in each treatment. Voltage was set to 0.250 kV on the Gene Pulser unit, with capacitance set at 1,000 µF for each electroporation.

Once cells had been electroporated the cell suspension was pipetted back out of the electroporation cuvette and placed into a Petri dish containing a glass coverslip and 2 ml of pre-warmed cell culture media. Cells were then grown for 4 hours to allow ample time for attachment to the coverslip, and the media was then aspirated off each set of cells and replaced with 2 ml of fresh RPMI media. Cells were then grown until the following morning and were analysed 20 hours post-transfection. Cells were prepared for analysis by washing in PBS-T multiple times and fixing with 4 % paraformaldehyde as in previous protocols.

1.2.3.9 Electrophoresis of KP-08/pDNA complexes re-suspended in electroporation buffer

A sample of polyplexes was prepared by mixing 40 µl of KP-08 with 25 µl of pDNA and incubating at room temperature for 15 minutes. This was done in duplicate and then one sample of polyplexes was diluted into 735 µl of electroporation buffer (thus bringing the polyplexes to the same final concentration as in an electroporation run) whilst the second sample of polyplexes was diluted into 335 µl of electroporation buffer. The rationale behind the second dilution was that DNA may not have been detectable during electrophoresis when a 20 µl sample of polyplexes was subsequently taken from the 800 µl electroporation sample for analysis on the agarose gel.

20 µl of each sample was then separately mixed with 5 µl of loading solution and loaded into the wells of an 0.8 % agarose gel containing 0.5 µg/ml ethidium bromide.

A sample of free DNA (2 µg) and a DNA ladder were also run on the agarose gel, with which the banding patterns of DNA in the hydrogel sample could be compared.

Samples were run for 45 minutes at 95 V and the gel was then transferred to a UV light box for analysis.

1.2.3.10 Electrophoresis of CaP and CaP/KP-08 precipitates

Agarose electrophoresis was undertaken on calcium phosphate precipitates formed either with or without KP-08 to compare the banding patterns of the DNA in each solution. Past electrophoresis results showed that KP-08 binds DNA and forms a distinct DNA band in the top of the lane of each well during electrophoresis and that there is no free DNA present in each lane.

Calcium phosphate/KP-08 precipitates were formed as follows – 100 µl of KP-08 was mixed with 5 µg of pDNA in a 1.5 ml eppendorf tube and incubated at room temperature for 10 minutes. 285 µl of dd$H_2$0 and 61 µl of 2 M $CaCl_2$ was then added to the hydrogel bound DNA and the tube was tapped to mix the solution. Separately, 500 µl of 2X HBS was pipetted into a 15 ml centrifuge tube. The DNA/KP-08/$CaCl_2$ solution was then added dropwise into the HBS solution, with gentle tapping on the side of the centrifuge tube after each drop. 20 µl of the resulting precipitation was immediately mixed with 5 µl of loading solution (without tracking dye) and loaded into the agarose gel.

Calcium phosphate (control) precipitates were formed in the same manner as above except no KP-08 was added to the DNA at the first step and 385 µl rather than 285 µl of ddH20 was used to bring the volume of the solution up to 500 µl prior to mixing with the 2X HBS. As trial experiments at the beginning of the month had shown that incubating the precipitates prior to addition to

cells could affect transfection efficiency, two sets of CaP control precipitates were analysed through electrophoresis – one set that had been formed and then immediately loaded into the agarose gel, and another in which complexes were incubated for 30 minutes prior to being mixed with gel loading solution and being added to cells.

Samples of free DNA and DNA mixed with calcium chloride (but not HBS in the second precipitate formation step) were also included in lanes of the gel along with a DNA ladder.

1.2.3.11 Transfection of KP-08/Calcium Phosphate precipitates

Precipitates were formed between KP-08-bound-pDNA and calcium phosphate, firstly by mixing 100 µl of KP-08 (10 mg/ml stock) with 5 µg of pDNA in an 1.5 ml eppendorf tube and incubating at room temperature for 10 minutes. 61 µl of 2 M $CaCl_2$ and ddH_20 to a final volume of 500 µl were then added into the KP-08 solution and gently tapped before being added dropwise into 500 µl of 2X HBS (a HEPES buffered solution containing 1.5 mM dibasic sodium phosphate) in a 15 ml centrifuge tube. The side of the tube was gently finger tapped after the addition of each drop. 100 µl of the precipitate was then diluted into a final volume of 2 ml of fully supplemented advanced DMEM media and then added onto cells (HaCaT cells pre-seeded

on the day before transfection onto 18 x 18 mm glass coverslips in 35 mm Petri dishes at a density of 5 x 10^5 cells/slide).

The complexes were incubated with the cells for 20 hours at 37ºC (media change performed with fully supplemented RPMI 1640 the following morning) after which time the transfection solutions were aspirated off cells and replaced with 2 ml of fully supplemented advanced DMEM. The cells were then grown for a further 24 hours before being washed, fixed and washed again for analysis. Control treatments of CaP with pDNA (no KP-08) and pDNA alone were also run simultaneously.

1.2.3.12 Particle Sizing of KP-08/pDNA/CaP complexes

KP-08/pDNA/CaP complexes were formed and incubated for either 0 or 30 minutes to determined whether the particles grew in size over time.

KP-08/Calcium phosphate precipitates were formed by mixing 100 µl of KP-08 with 5 µg of pDNA in a 1.5 ml eppendorf tube and incubating at room temperature for 10 minutes. 61 µl of 2 M $CaCl_2$ and 340 µl ddH_20 were then added into the KP-08 solution and gently tapped before being added dropwise into 500 µl of 2X HBS (a HEPES buffered solution containing 1.5 mM dibasic sodium phosphate) in a 15 ml centrifuge tube. The side of the tube was gently finger tapped after the addition of each drop.

Standard calcium phosphate precipiates were formed as above except KP-08 was omitted from the solution and 385 µl of ddH_20 rather than 285 µl of ddH_20 was used when forming precipitates.

Particle size analysis was carried out on dynamic light scattering particle sizing equipment (Brookhaven Instruments) by Dr Nicholas Crowther of AGT Sciences Ltd. The particle sizing instrument used photon correlation spectroscopy to generate values for particle diameters in each sample (nm). Samples were run for 30 minutes and results were generated using ZetaPALS particle sizing software version 3.48.

1.2.3.13 Transfection of KP-08/pDNA/CaP complexes prepared with and without an incubation period

Previous particle sizing experiments had highlighted how KP-08/pDNA/CaP complexes grew in size over time so it was decided that complexes formed either with or without an incubation period should be compared in a transfection experiment.

NIH 3T3 cells cultured in fully supplemented advanced DMEM culture media were seeded out onto glass coverslips at a density of 5×10^4 cells per coverslip and grown overnight before transfection. Cell culture media was

replaced two hours prior to transfection with fresh media, which was then replaced with the appropriate transfection solution at the start of transfection. Transfection solutions were prepared as follows:

KP-08/Calcium phosphate precipitates – 100 µl of KP-08 was mixed with 5 µg of pDNA in a 1.5 ml eppendorf tube and incubated at room temperature for 10 minutes. 61 µl of 2 M $CaCl_2$ and 340 µl ddH_20 were then added into the KP-08 solution and gently tapped before being added dropwise into 500 µl of 2X HBS (a HEPES buffered solution containing 1.5 mM dibasic sodium phosphate) in a 15 ml centrifuge tube. The side of the tube was gently finger tapped after the addition of each drop. 200 µl of the precipitate was then either immediately diluted into a final volume of 2 ml of fully supplemented advanced DMEM media and then added onto cells. Alternatively, precipitates were incubated at room temperature for 30 minutes before being diluted into cell culture media and being added to cells.

Standard calcium phosphate precipitates – formed as above except KP-08 was omitted from the solution and 385 µl of ddH_20 rather than 285 µl of ddH_20 was used when forming precipitates. Complexes were added to cells either immediately or after 30 minutes of incubation.

The complexes were incubated with the cells for 20 hours, after which time the transfection solutions were aspirated off cells and replaced with 2 ml of

fully supplemented advanced DMEM. The cells were then grown for a further 24 hours before being washed, fixed and washed again for analysis.

1.2.3.14 Nebulised delivery of KP-08/pDNA/CaP precipitates to cells

NIH 3T3 cells were grown to confluence and then split 1:2 for transfection the following day. KP-08/CaP precipitates were prepared for transfection as follows:

KP-08/Calcium phosphate precipitates – 100 µl of KP-08 was mixed with 5 µg of pDNA in a 1.5 ml eppendorf tube and incubated at room temperature for 10 minutes. 61 µl of 2 M $CaCl_2$ and 340 µl ddH_20 were then added into the KP-08 solution and gently tapped before being added dropwise into 500 µl of 2X HBS (a HEPES buffered solution containing 1.5 mM dibasic sodium phosphate) in a 15 ml centrifuge tube. The side of the tube was gently finger tapped after the addition of each drop. The 1 ml of precipitate prepared was then added to the well at the top of the nebuliser, which was sealed to the neck of the culture flask with parafilm to maximise the amount of mist entering the flask. When the nebuliser (Aeroneb go® nebuliser, Aerogen, Ireland) was connected to the mains supply the formulation immediately formed a mist, which filled the culture flask. After a few minutes when all mist had been produced by the nebuliser the top was screwed back onto the culture flask and the flask was laid down in the flow hood for 5 minutes to allow the mist to settle on the cells. This procedure was performed in duplicate with or without

8 ml of fully supplemented advanced DMEM present in the culture flask at the point of exposure to the nebulised formation. In the latter scenario, cells were exposed to the mist for 5 minutes (incubated at room temperature) before the addition of 8 ml of media. Cells were then returned for overnight incubation at 37ºC.

Standard CaP precipitates were formed in the same way (except KP-08 was omitted during precipitate formation) and nebulised as a control treatment. In the control, cells were exposed to the mist in the presence of 8 ml of culture media.

After 24 hours flasks of cells were then trypsinised using 8 ml of trypsin-EDTA and then centrifuged to obtain a cell pellet. Supernatant was discarded off the cells and the pellet was re-suspended in 4 ml of fully supplemented advanced DMEM cell culture medium. 500 µl of the re-suspended cells was then seeded out onto 18 x 18 mm glass coverslips placed inside 35 mm Petri dishes containing 2 ml of media. Cells were grown for a further 2 hours before being washed, fixed and mounted for analysis.

1.2.3.15 Optimiaztion of KP-08/pDNA/CaP complexes for transfection

NIH 3T3 cells cultured in fully supplemented advanced DMEM media were seeded out onto glass coverslips at a density of 2.5 x 10^4 cells per coverslip

and grown in 35 mm Petri dishes overnight at 37º before transfection. When the cells were ready for transfection overnight growth media was aspirated off cells and replaced with the appropriate transfection solution. Transfection solutions were made up to investigate three parameters that may affect the efficiency of complex formation and thus transfection:

1) The time for which precipitate was incubated for prior to being added to the cells
2) The time for which KP-08/DNA was incubated with $CaCl_2$ for before the KP-08/DNA/$CaCl_2$ solution was added dropwise into a 2 X HBS solution.
3) The incubation period of KP-08 with DNA prior to the addition of $CaCl_2$ to the polyplexes.

All transfection solutions were made up with 5 µg of pDNA and 100 µl of KP-08. A media change (2 ml fully supplemented advanced DMEM) was performed on all cells 1 hour prior to the onset of the transfection solutions.

To investigate parameter 1, a 1 ml transfection solution was made up by mixing DNA with KP-08 and incubating the polyplexes for 10 minutes at room temperature. 285 µl of dH_20 and 61 µl of 2 M $CaC1_2$ was then added to complexes and the solution was mixed by tapping the side of the eppendorf tube in which complexes were formed. The 500 µl of KP-08/DNA/$CaCl_2$

solution was then added dropwise into 500 µl of 2 X HBS. The resulting precipitate was then incubated at room temperature for as long as necessary and added to cells either immediately after formation, 10 minutes or 40 minutes after formation. In each case 200 µl of the transfection solution was pipetted dropwise into each Petri dish.

To investigate parameter 2, KP-08/DNA were mixed and incubated at room temperature for ten minutes. 285 µl of dH_20 and 61 µl of 2 M $CaC1_2$ was then added to complexes and the $CaC1_2$ was incubated with the polyplexes for either 0, 5 or 20 minutes before the KP-08/DNA/CaP solution was added dropwise into 2 X HBS. Once the two solutions had been mixed, 200 µl of each transfection solution was immediately pipetted into the appropriate Petri dish by dropwise addition.

To investigate parameter 3, KP-08 and DNA were mixed and incubated at room temperature for either 0, 5 or 20 minutes before dH_20 and $CaCl_2$ were added into the polyplex solution. After the $CaCl_2$ had been added, the KP-08/DNA/$CaCl_2$ solution was immediately added dropwise into 500 µl of 2 X HBS and once the two solutions had been mixed, 200 µl of each transfection solution was immediately pipetted into the appropriate Petri dish by dropwise addition.

A control treatment of standard CaP precipitates was also made up. 5 µg of pDNA, 385 µl of dH_20 and 61 µl of 2 M $CaCl_2$ were mixed together and immediately added dropwise into 500 µl of 2 X HBS. 200 µl of the resulting precipitate was then added to cells.

All cells were grown at 37ºC in the presence of the respective transfection solution for 24 hours. After 24 hours a media change was performed using 2 ml fully supplemented advanced DMEM media and cells were grown for a further 24 hours at 37ºC.

All growth media was then aspirated off cells, which were washed for 10 seconds at room temperature with PBS, fixed for 30 minutes at room temperature with 4 % paraformaldehyde and washed a further time for 10 seconds at room temperature, again with PBS.

1.2.3.16 Particle Sizing of Nebulised KP-08/pDNA/CaP precipitates

1 ml of transfection solution was made up as in transfection experiments – 5 µg of pDNA and 100 µl of 2 % KP-08 were mixed together and immediately mixed with 285 µl of dH_20 and 61 µl of 2 M $CaCl_2$. The KP-08/DNA/CaP solution was then added dropwise into 500 µl of 2 X HBS and immediately nebulised. The nebuliser outlet was sealed to the neck of a 20 ml sample tube using parafilm and the nebulised mist was collected into 4 ml of dH_20. The

solution of dH_20 containing nebulised formulation was then taken for particle sizing.

Particle size analysis was carried out on dynamic light scattering particle sizing equipment (Brookhaven Instruments). The particle sizing instrument used photon correlation spectroscopy to generate values for particle diameters in each sample (nm). Samples were run for 30 minutes and results were generated using ZetaPALS particle sizing software version 3.48.

1.2.3.17 Filtration of Nebulised KP-08/pDNA/CaP precipitates prior to transfection

NIH 3T3 cells cultured in fully supplemented advanced DMEM media were seeded out onto glass coverslips at a density of 2.5×10^4 cells per coverslip and grown in 35 mm Petri dishes overnight at 37º before transfection. When the cells were ready for transfection overnight growth media was aspirated off cells and replaced with the appropriate transfection solution.

A Transfection solution was made up as in previous experiments - 100 µl of KP-08 was mixed with 5 µg of pDNA in a 1.5 ml eppendorf tube and 285 µl ddH_20 and then 61 µl of 2 M $CaCl_2$ were immediately added to the polyplex solution. The KP-08/DNA/$CaCl_2$ solution was then immediately pipetted dropwise into 500 µl of 2X HBS (a HEPES buffered solution containing 1.5

mM dibasic sodium phosphate) in a 15 ml centrifuge tube. The side of the tube was gently finger tapped after the addition of each drop.

200 µl of the transfection solution was pipetted into cells in one Petri-dish containing 2 ml of fully supplemented advanced DMEM. The remaining 800 µl of the same solution was then quickly filter sterilised though a 450 nm filter and then 200 µl of the filtered transfection solution was then pipetted onto cells in a different Petri dish.

This enabled a direct comparison to be made between filtered and non-filtered transfection solutions.

1.2.3.18 Evaluating the use of a different nebuliser

NIH 3T3 cells were grown to confluence in T-75 culture flasks and then split 1:2 for transfection the following day. The hydrogel stock used was 2 % KP-08 (30/6/08) and had a pH adjusted to 2.75 with acetic acid.

3 flasks of cells were transfected:

1. Side Stream Nebuliser – A transfection solution was made up as follows: 100 µl of KP-08 was mixed with 10 µg of pDNA and then 285 µl of dH_20 and 61 µl 2 M $CaCl_2$ were immediately added. The DNA/hydrogel/$CaCl_2$ solution was then added dropwise into 500 µl 2 X

HBS. The 1 ml of transfection solution was then nebulised into a flask of cells containing 8 ml of advanced DMEM. The neck of the culture flask was sealed to the outlet on the nebuliser using parafilm during nebulisation.

2. Transfection solution made up as in 1. but the solution was nebulised using the E-flow nebuliser.
3. Transfection solution made up as in 1. but the solution was pipetted dropwise into a flask of cells rather than being nebulised.

After nebulisation flasks were left in a horizontal position for five minutes to allow the mist time to settle on the media. Culture flasks were then returned to the incubator for incubation at 37ºC for 24 hours.

At the end of the transfection period transfection media was aspirated off cells and cells were then trypsinised using 8 ml of trypsin-EDTA and then centrifuged to obtain a cell pellet. After the supernatant had been discarded the cell pellet was re-suspended in 4 ml of fully supplemented advanced DMEM cell culture medium. 500 µl of the cell suspension was then seeded out onto 18 x 18 mm glass coverslips placed inside 35 mm Petri dishes. Cells were given 15 minutes to allow attachment to the coverslips and then 2 ml of media was added to each well.

Cells were grown for a further 2 hours at 37 ºC before being washed, fixed and mounted for analysis.

1.2.3.19 Agarose electrophoresis to detect pDNA in the nebulised solution

An 0.8 % agarose gel containing 0.5 µg/ml ethidium bromide was made up using electrophoresis grade agarose (Invitrogen) and 1 X TAE buffer (Invitrogen).

Samples for analysis were made up as follows:

Naked DNA: 50 µg of pDNA was mixed with 950 µl of dH_20 and nebulised with the E-flow. The neck of the nebuliser was sealed to a 20 ml specimen bottle containing 500 µl dH_20. As much of the mist was recovered into the water as possible by swilling the collection tube and then the 500 µl was divided into two; one half of which was filtered through a 0.45 micron filter.

A normal transfection solution was made up by adding 200 µl KP-08 (31/7/08) to 50 µl of pDNA and adding 190 µl water. 61 µl of 2 M $CaCl_2$ was then added dropwise into 500 µl 2 X HBS. The resulting precipitate was nebulised in the E-flow and collected in 500 µl dH_20. Half of the solution was passed through a 0.45 micron filter.

20 µl of each of the four solutions was taken and mixed with 5 µl of loading buffer prior to loading into wells on the gel. A DNA ladder and a sample of free DNA were also included in separate lanes as reference samples.

1.2.4 Examining the properties of POVAL Nanoparticles for gene delivery

1.2.4.1 Examining stability of POVAL/pDNA complexes through electrophoresis

Polyplexes were formed by mixing 2.12 µg of pDNA with 25 µl of a 10 mg/ml POVAL stock in 1.5 ml eppendorf tubes and incubating at room temperature for 10 minutes to produce sample a). 25 µl of sample a) was then pipetted into a new eppendorf and was diluted with 25 µl of sterile PBS to prepare sample b). 25 µl of sample b) was then pipetted into a new eppendorf and, after dilution with a further 25 µl of sterile PBS, formed sample c). 25 µl of sample c) was then pipetted into a new eppendorf and, after dilution with a further 25 µl of sterile PBS, formed sample d). In all cases, samples were incubated at room temperature for 10 minutes after being diluted with PBS before the next dilution was performed.

The procedure was then repeated after another set of POVAL/pDNA polyplexes had been synthesised. However, all dilutions took place in Opti-MEM cull culture media to yield samples e) to h).

Diluting samples in this way meant the following amounts of DNA were loaded into the gel after the 25 µl of the final samples had been supplemented with 5µl loading solution;

a) 0.88 µg – samples A and E (lanes 4 and 8 respectively)
b) 0.44 µg – samples B and F (lanes 5 and 9 respectively)
c) 0.22 µg – samples C and G (lanes 6 and 10 respectively)
d) 0.11 µg – samples D and H (lanes 7 and 11 respectively)

Samples of free DNA and a DNA ladder (Bioline Hyperladder I) were also run on the gel and prepared as outlined in 2.2.9, in which electrophoresis parameters are also described.

1.2.4.2 Evaluating POVAL nanoparticles during transfection

NIH 3T3 cells were cultured as outlined in 2.2.1 and were seeded at a density of 5 x 10^4 cells per coverslip on the day preceding transfection as described in 2.2.10.

POVAL polyplexes were prepared by mixing 2.12 µg pDNA with 25 µl of 10 mg/ml POVAL and incubating at room temperature for 15 minutes. Polyplexes were synthesised in duplicate and transfected over a period or either 5 or 24 hours. In the case of the 5 hour transfection solution, the final volume of the solution was adjusted to 2 ml with Opti-MEM media. For the 24 hour transfection solution, Opti-MEM was added to the polyplexes to a final volume of 1.9 ml and then 100 µl NCS was added to the solution prior to addition to cells.

Negative naked DNA controls were included using 2µg DNA suspended in either TE buffer or ddH_20. Positive Lipofectin® controls were also included forming lipoplexes according to manufacturers instructions with 2µg pDNA suspended in either TE buffer or ddH_20.

1.2.5 Miscellaneous transfection-related experiments

1.2.5.1 Transfection of HAE primary cells with POVAL/CaP precipitates

Primary HAE cells purchased from Lonza were maintained in BEBM cell culture media and grown at 37ºC in a 5 % CO_2 atmosphere. Prior to transfection cells were split from the culture flasks using trypsin-EDTA and seeded out onto 18 x 18 mm class coverslips placed inside 35 mm Petri dishes at a density of 2.5 x 10^4 cells per coverslip. As the primary cells were slow growing it was necessary to grow the cells for a further week prior to transfection, with a media change (2 ml fresh BEBM) performed after three days.

On the day of transfection growth media was aspirated off cultures and replaced with 2 ml of transfection solution. Transfection solutions were made up as follows.

1. Calcium phosphate control – 10 µg of pDNA was mixed with 390 µl dH_20 and then 61 µl of 2 M $CaCl_2$. The DNA/$CaCl_2$ solution was then added dropwise into 500 µl of 2 X HBS and incubated at room temperature for 5 minutes. 50 µl of the resulting CaP precipitate was then added to the cells instead of 200 µl as is typically done during transfection, because at the latter volume the precipitate had proven toxic to cells in a preliminary transfection experiment.

2. POVAL/CaP precipitate – transfection solution formed as in 1) except 200 µl of 0.5 % POVAL (1/8/08) was added to the pDNA before the addition of 190 µl of dH_20. 50 µl of the final solution was pipetted onto cells.
3. Lipofectin control – A Lipofectin control was made up according to manufacturers instructions, as in previous experiments. Cells were exposed to transfection solution containing a total of 2 µg of pDNA in the lipoplex solution.
4. Lipofectin/POVAL transfection solution. Transfection solution was made up as in 3 except 40 µl of 0.5 % POVAL (1/8/08) was mixed with DNA during the first step.
5. POVAL transfection solution - 40 µl of 0.5 % POVAL (1/8/08) was mixed with 2 µg of DNA and incubated for 15 minutes prior to addition to cells.
6. Naked DNA control – cells were exposed to 2 µg of DNA diluted to a final volume of 2 ml in BEBM cell culture media.

The final volume of all cultures was adjusted to 2 ml using BEBM cell culture media and cells were transfected over a 24 hour period. After this time a media change was performed (2 ml fresh BEBM) and cells were grown for a further 24 hours to permit the expression of any GFP.

Growth media was then aspirated off cells, which were washed, fixed and mounted for analysis as in previous protocols.

1.2.5.2 Electrophoresis of PEI/pDNA complexes

In order to verify the efficacy of the plasmid pDNA and confirm the cell types used were transfectable an additional control technique was evaluated – PEI, polymer-based, transfection.

Firstly, polymer and pDNA were mixed at various nitrogen: phosphate ratios to determine the minimum amount needed to bind all pDNA in a given sample. 0.0675 g of PEI (Acros Organics, approx MW 60,000, 50 % wt a.q. solution, branched) was diluted into 75 ml of sterile filtered (0.2 micron) ddH_20 to yield a stock of 10 mM PEI. The solution was stirred with a spatula for 5 minutes until all PEI had dissolved. The pH was adjusted to 7.3 using HCl and the solution was then sterile filtered through a 0.2 micron filter and stored at 4ºC until use.

Various quantities of the stock solution were then taken and mixed with pDNA to yield final ratio's of 16:1, 32:1, 64:1 and 128:1 N:P (5.2 ug of pDNA in each sample).

pDNA and PEI were separately diluted into 50 ul of 150 mM NaCl and vortexed on a low speed for ten seconds. The solution were incubated at room temperature for ten minutes before being mixed together and being subjected to another 10 min incubation period. 20 µl of the resulting solution was mixed with 5 µl of loading buffer and loaded into a 0.8 % agarose gel containing 0.5 µg/ml ethidium bromide. Samples were ran in a Bio Rad electrophoresis tank for 1 hour at 80 V, after which time the gel was photographed under UV light box.

1.2.5.3 Transfection of PEI-pDNA polyplexes

HaCaT cells were seeded out onto glass coverslips at a starting cell density of 5 x 10^4 cells per well and grown overnight in fully supplemented RPMI media.

The following morning transfection solutions were prepared as in the 2.3.21 experiment at a ratio of 4:1 (5ul of PEI stock solution diluted into 50 ul of 150 mM NaCl solution, and 20.5 ul pDNA separately diluted into 50 ul of 150 mM NaCl solution and incubated for ten minutes prior to mixing).

200 ul was then pipetted onto the cells, which were incubated for 5 hours at 37 degrees C in serum-free media.. After that time a 2 ml media change was performed with fully supplemented RPMI and the cells were grown overnight.

The following morning another media change was performed and the cells were grown for a final 24 hours before being washed, fixed and mounted for analysis.

1.2.5.4 Examining complex formation through particle size analysis

A sample of free DNA was prepared by diluting DNA to a concentration of 62.5 µg/ml to a total volume of 5 ml. Samples of POVAL nanoparticles were prepared by diluting the stock solution 1:10 into either PBS or distilled water to yield working concentrations of 1 mg/ml. Samples of polyplexes were synthesised by mixing 675 µl of hydrogel nanoparticles from the working stock (diluted in PBS) straight with 4.32 ml of pDNA (62.5 µg/ml) in TE buffer. A second sample of polyplexes were prepared by diluting the nanoparticles and DNA prior to mixing by mixing 96 µl of pDNA (62.5 µg/ml) into 300 µl of Opti-MEM and incubating at room temperature for 45 minutes. Simultaneously, 15 µl of 1 mg/ml POVAL (diluted in PBS) was diluted into 300 µl of Opti-MEM and left for incubation at room temperature for 30 minutes. Both pDNA and hydrogel solutions were then combined, mixed gently, and incubated for a further 10 minutes at room temperature before a further 5.4 ml of Opti-MEM was added. 5 ml of the final sample was taken for particle size analysis using dynamic light scattering particle sizing equipment (Brookhaven Instruments). The particle sizing instrument used photon correlation spectroscopy to generate values for particle diameters in each sample (nm).

Samples were run for between 24 and 52 minutes and results were generated using ZetaPALS particle sizing software version 3.48.

1.2.5.5 Conductimetric titrations

The lack of nanoparticle uptake observed in transfection experiments could possibly have been attributable to a charge effect. Thus it was decided that conductimetric titrations would be performed, first of PD2000 against calf thymus DNA, and then with KP-08 against calf thymus DNA, to determine whether KP-08 and DNA were being mixed at appropriate amounts during polyplex formation for transfection experiments.

PD2000 vs calf thymus DNA

25 ml of a 1 % (w/w) PD2000 was made up using distilled water and pipetted into a beaker. A solution of 1 % (w/w) calf thymus DNA was similarly prepared with distilled water and was titrated into the beaker at 0.5 ml intervals, with conductivity readings recorded using a handheld conductivity meter (Hanna Instruments).

KP-08 vs calf thymus DNA

25 ml of a 0.5 % KP-08 formulation was pipetted into a beaker. A solution of 1 % (w/w) calf thymus DNA was similarly prepared with distilled water and was titrated into the beaker at 0.25 ml intervals, with conductivity readings recorded using a handheld conductivity meter (Hanna Instruments).

1.3. Results

1.3.1 Examining the properties of KP-08 nanoparticles for gene delivery

1.3.1.1 Gel mobility shift assay to determine the optimal KP-08/pDNA binding ratio

Figure 3 shows at low ratios of KP-08:DNA, such as 6:1 or 8:1, there is an insufficient amount of KP-08 present to bind pDNA in a 0.53 µg DNA sample. As the amount of KP-08 used in polyplex formation increases (moving towards the right across Figure 3) less and less DNA appears in the lanes of each lane as it has been bound to the hydrogel and remains in the wells at the top of each lane.

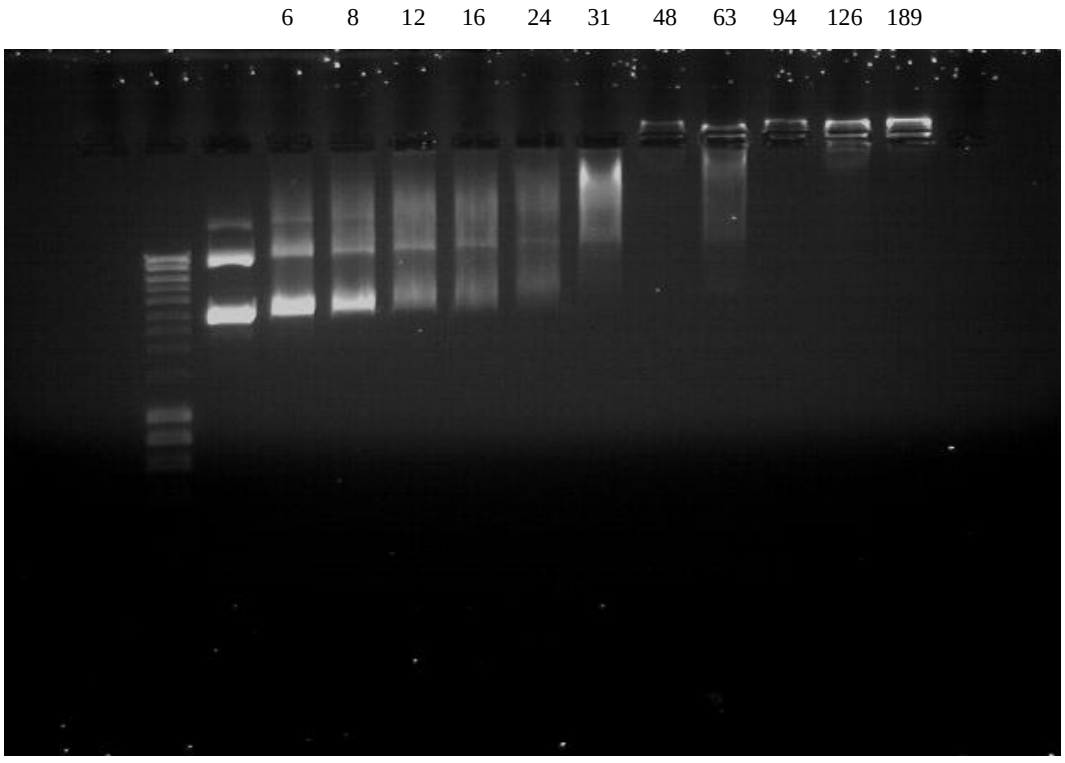

Fig. 3 – agarose gel mobility shift assay of KP-08(18/7/07)/pDNA complexes. Complexes formed at hydrogel to pDNA ratios in indicated at the top of each lane per µg of pDNA . Total

amount of pDNA in each lane was 0.53 µg. The two samples in the far left hand lanes are a DNA ladder and a sample of free pDNA (0.53 µg)

At a ratio of 48:1 there appears to be little, if any, pDNA in the lane. However, it is only at a ratio of 189:1 that a thick DNA band, indicating complete polyplex formation (i.e. no residiual, unbound DNA in the lane), is really visible in the well at the top of a lane.

It was decided that for future experiments polyplexes would be formed at a ratio of 200:1 µg KP-08: µg DNA as a surplus of KP-08 would at least ensure that all DNA in a given sample was bound to the hydrogel. Preliminary experiments had shown KP-08 to have no adverse affects on cell viability, so an excess of KP-08 had not proven to be detrimental to cell cultures.

1.3.1.2 Determining minimum charge needed to bind pDNA and complex stability through agarose electrophoresis

Figure 4 a) shows that with the 1/0.01 nanoparticles (least positively charged), complexes between KP-08 and DNA formed, as indicated by the band of DNA in the well at the top of lane 5. When the polyplexes were diluted into Opti-MEM (lanes 6 – 12), however, DNA release was observed in all samples, regardless of the pH of the media. As a result, the 1/.01 solution is of no use for transfection as any DNA that is coupled to the hydrogel would be released upon dilution into buffered media (with an identical pH to that in lane 6), which is part of the transfection procedure.

Similarly, Figure 4 b) shows that with the 1/0.02 KP-08, complexes initially formed between the KP-08 and DNA (lane 4) but that DNA was immediately released upon dilution into Opti-MEM media (lanes 5 – 11). Again, DNA dissociation was observed at all pH's, including at physiological pH (lane 5), indicating that 1/.02 complexes would also be of no use for transfection studies.

However, Figure 4 b) shows with the 1/.03 KP-08 that DNA dissociation was not apparent when KP-08/pDNA complexes were diluted into Opti-MEM suggesting that nanoparticles with this PD2000 concentration were able to bind DNA (lane 12) and maintain the interaction when diluted into media (lanes 13-19).

There does appear to be a smeared pattern in some of the lanes in both Figures 4 b) and c), but this may have been produced by the contrast settings on the picture. What is clear in Fig. 4 b) is that bands of DNA that are visible in the 1/.02 solutions, level with the arrow to the left of the image, are not present in the 1/.03 solutions, as DNA has remained bound to the 1.03 KP-08 (represented by faint bands in the wells at the top of lanes 13-19), rather than dissociating and appearing as free pDNA further down the lanes as is the case with the 1/.02 solution (lanes 5-11 Fig. 4 b).

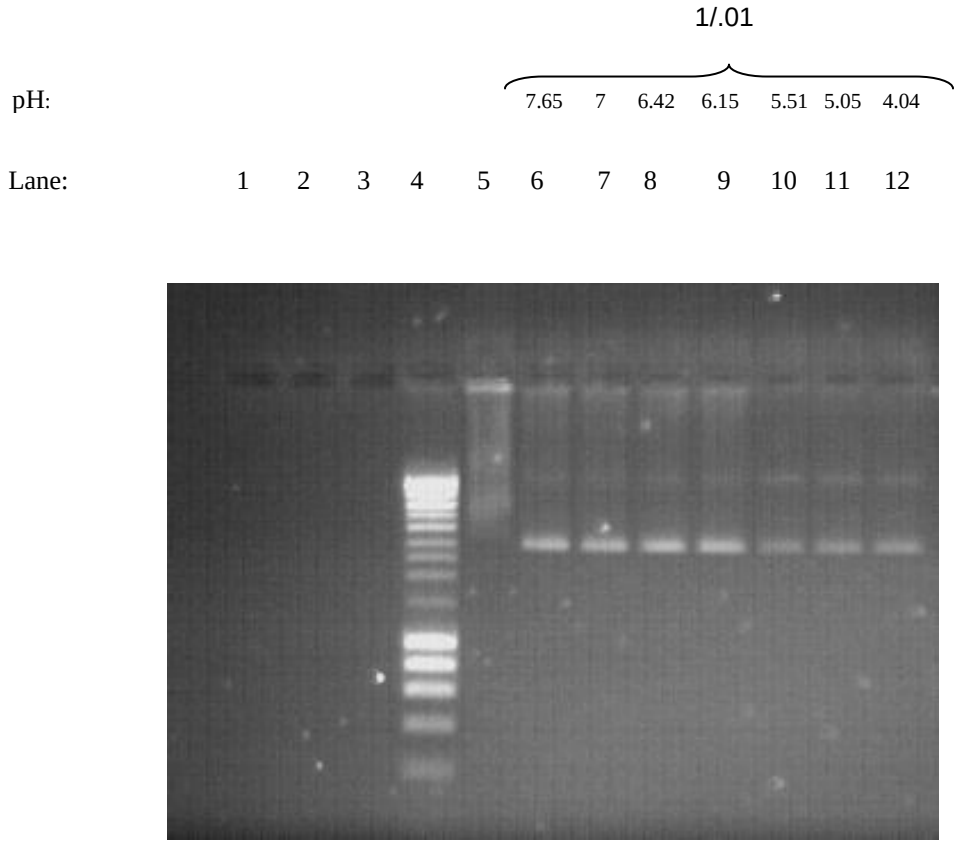

Fig. 4 a) – electrophoresis of KP-08 (1/.01)/pDNA complexes diluted into Opti-MEM media of differing pH. A DNA ladder was included in lane 4. In lane 5 was a sample of KP-08/pDNA which had not been diluted into Opti-MEM (to check complexes had formed before dilution into Opti-MEM). Lane numbers and the pH of the buffer used in sample preparation for each lane are indicated above the image. Lanes 1-3 were blank.

Figure 4 c) shows that with the 1/.03 and 1/.04 solutions DNA bound to the KP-08 (lanes 4 and 12 respectively) and that in both cases, the resulting complexes that formed remained stable when diluted into Opti-MEM. For both the 1/.03 solution (lane 5-11) and the 1/.04 solution (lanes 13-19) DNA bands are visible in the wells at the top of each lane as the DNA was bound in a complex with a net positive charge that did not migrate towards the positive electrode as unattached, negatively charged DNA would have done.

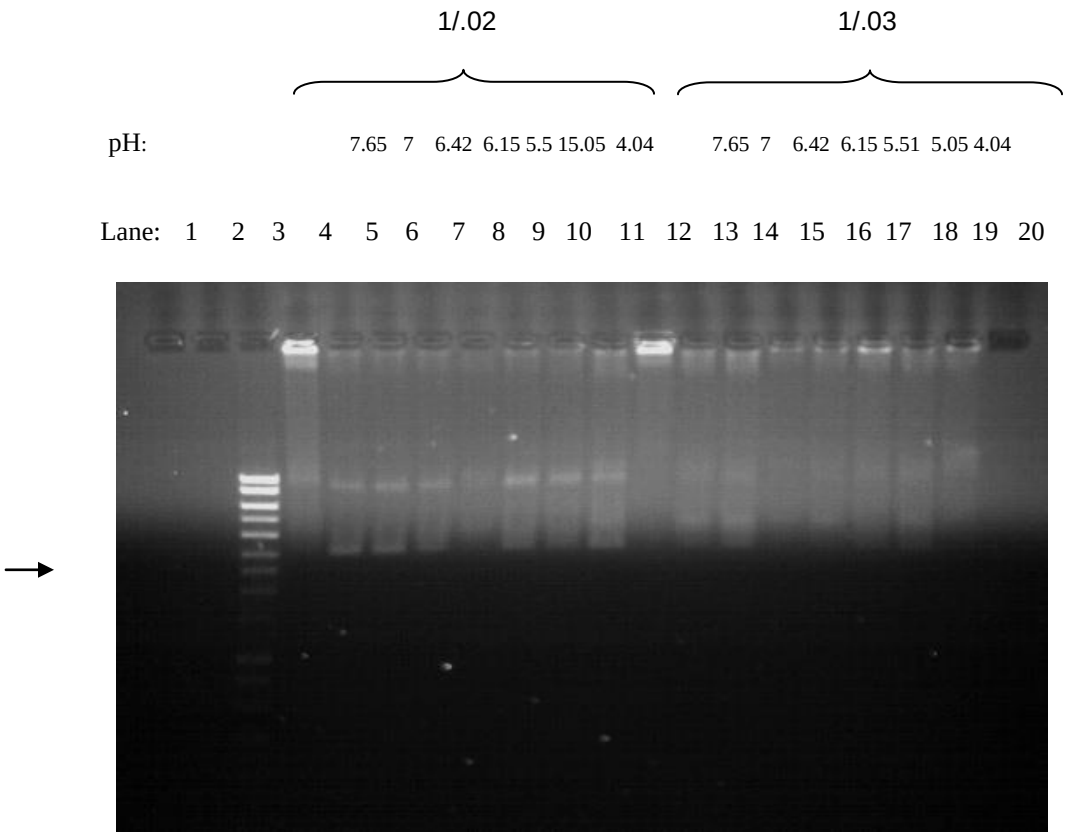

Fig. 4 b) – electrophoresis of KP-08 (1/.02 and 1/.03)/pDNA complexes diluted into Opti-MEM media of differing pH. A DNA ladder was included in lane 3. In lane 4 was a sample of KP-08 (1/0.02)/pDNA which had not been diluted into Opti-MEM (to check complexes had formed before dilution into Opti-MEM). Lane numbers and the pH of the buffer used in sample preparation for each lane are indicated above the image. Lanes 1-2 and 20 (at each end of the gel) were blank.

In summary, these results suggest that somewhere between the 1/.02 and 1/.03 solutions is a critical level of PD2000 required to bind all DNA in a sample and maintain the interaction when diluted into cell culture media. With the KP-08 formulation at least, if the concentration of PD2000 is below this level, any DNA bound to the hydrogel will simply dissociate from the hydrogel upon dilution into cell culture media. It was decided that in order to ensure complete binding, a 0.05 % concentration of PD2000 would be used thereon in.

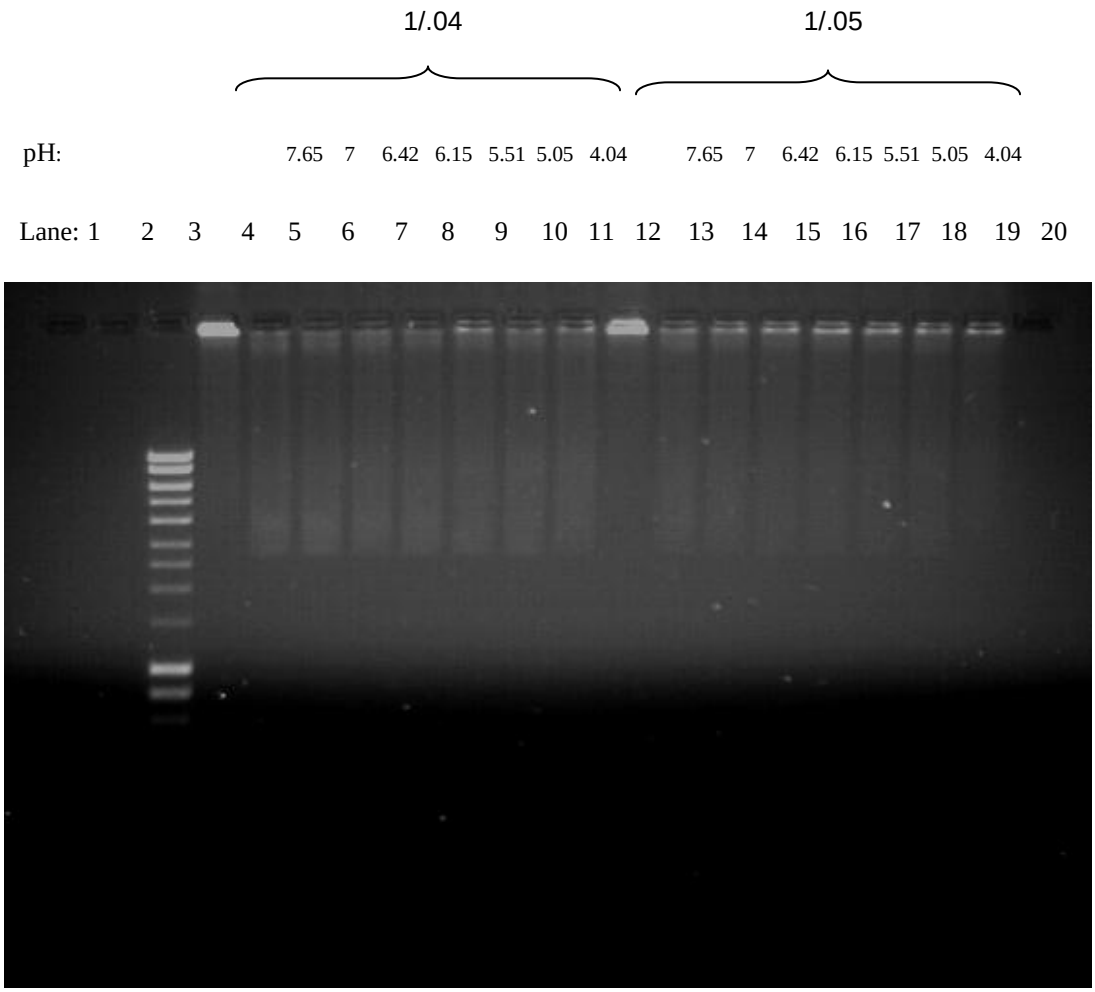

Fig. 4 c) – electrophoresis of KP-08 (1/.04 and 1/.05)/pDNA complexes diluted into Opti-MEM media of differing pH. A DNA ladder was included in lane 3. In lane 4 was a sample of KP-08/pDNA which had not been diluted into Opti-MEM (to check complexes had formed before dilution into Opti-MEM). Lane numbers and the pH of the buffer used in sample preparation for each lane are indicated above the image. Lanes 1-2 and 20 (at each end of the gel) were blank.

1.3.1.3 Evaluating KP-08 nanoparticles during transfection

At the point of analysis following transfection, GFP expression was noted in cells transfected with the hydrogel. Fig. 5 below shows two GFP-expressing 3T3 cells, which were transfected over a period of 5 hours in the absence of

serum and chloroquine. In total, only a small number of NIH 3T3 cells were transfected, with 5 counted in total. In comparison, Lipofectin® controls have demonstrated much higher levels of transfection with over 2,000 GFP-expressing NIH 3T3 cells counted in previous experiments.

However, no GFP-expressing cells were observed in any of the other hydrogel treatments, regardless of whether transfection had taken place over 5 or 24 hours or had been performed in the presence or absence of chloroquine.

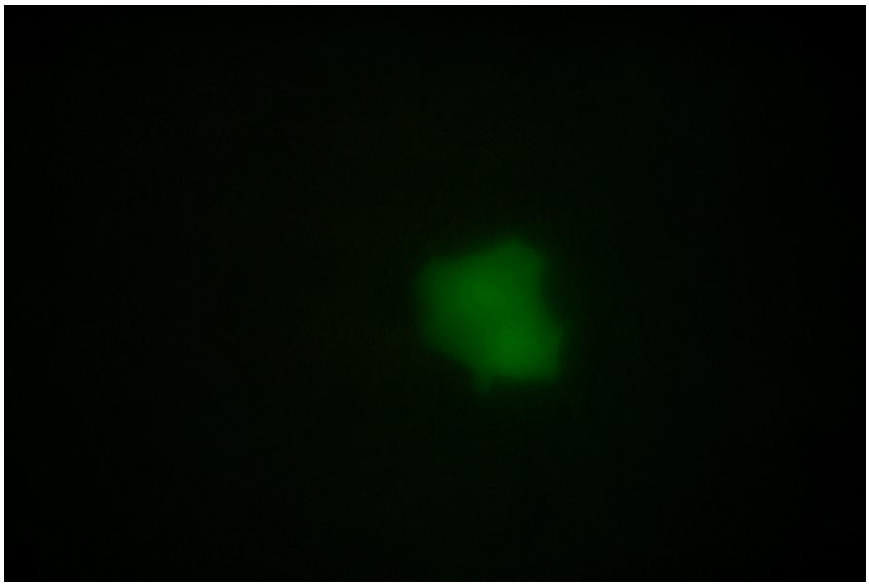

Fig. 5 – GFP expression detected in NIH 3T3 cells exposed to KP-08/pDNA complexes. Cells transfected over 5 hours in the absence of serum and chloroquine.

As no GFP expression was detected in cells exposed to naked DNA over either 5 or 24 hours it would appear that the observed GFP expression in Fig.

5 resulted from DNA carried into the cells as part of the KP-08 vector rather than in a free form.

1.3.1.4 Examining DNA release from KP-08 nanoparticles at different pH

Figure 6 shows that complexes of KP-08 and pDNA had successfully formed (a sample of complexes which were not diluted into Opti-MEM media was included in lane 13) and that when the complexes were subsequently diluted into Opti-MEM media of differing pH, the KP-08 maintained an interaction with pDNA in all cases except when complexes had been diluted into media of pH 5.15 (lane 10). The result in lane 10 show that, at pH 5.15, pDNA was released from the KP-08, a result not witnessed at the higher or lower pH's tested in this experiment.

pH:					7.8	7.15	6.54	5.93	5.57	5.15	4.68	4.01			
Lane:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

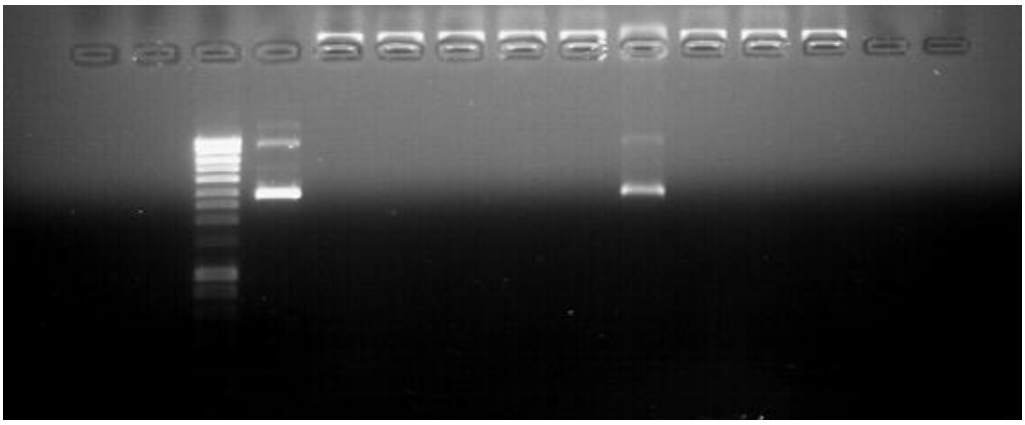

Figure 6 – electrophoresis of KP-08/pDNA complexes diluted into Opti-MEM media of differing pH. In lane 3 is a DNA ladder and in lane 4 is a sample of free pDNA (equivalent amount to that in lanes 5-13). In lane 13 was a sample of KP-08/pDNA which had not been

diluted into Opti-MEM (to check complex formation was still occurring prior to dilution into media). Lane numbers and the pH of the buffer used in sample preparation for each lane are indicated above the image. Lanes 1-2 and 14-15 (at each end of the gel) were blank.

The fact that DNA appeared to be released at pH 5.15 suggested there was the possibility DNA may be released from the hydrogel in the endosome (which has a pH of ~5) if complexes were internalised by endocytosis.

1.3.1.5 Transfection of KP-08/Lipofectin® chimeric complexes

The result in figure 7 suggested that all of the plasmid DNA in the sample had been bound to the hydrogel as there was no evidence of free, unattached DNA in lane 4. Thus, it can be assumed that any GFP expression that may be detected in cells exposed to chimeric nanoparticles in the subsequent experiment was produced by transfecting with DNA simultaneously bound to both KP-08 and Lipofectin®.

Successful transfection was noted in cells exposed to the chimeric complexes – 134 GFP expressing cells were counted in total (see Fig. 8), compared to 0 GFP expressing cells in the KP-08/pDNA treatment. 1,360 GFP expressing cells were counted in the Lipofectin® control but none were counted in the cells exposed to naked DNA which had not been bound to any vector.

Lane: 1 2 3 4

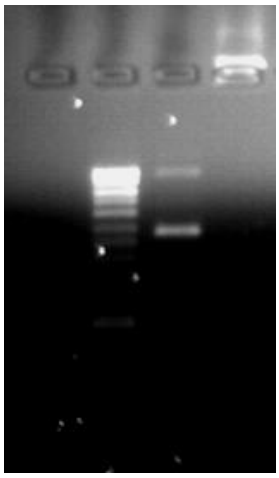

Figure 7. – electrophoresis of chimeric complexes. In lane 2 is a DNA ladder, lane 3 is naked pDNA whilst in lane 4 is a sample of chimeric nanoparticles.

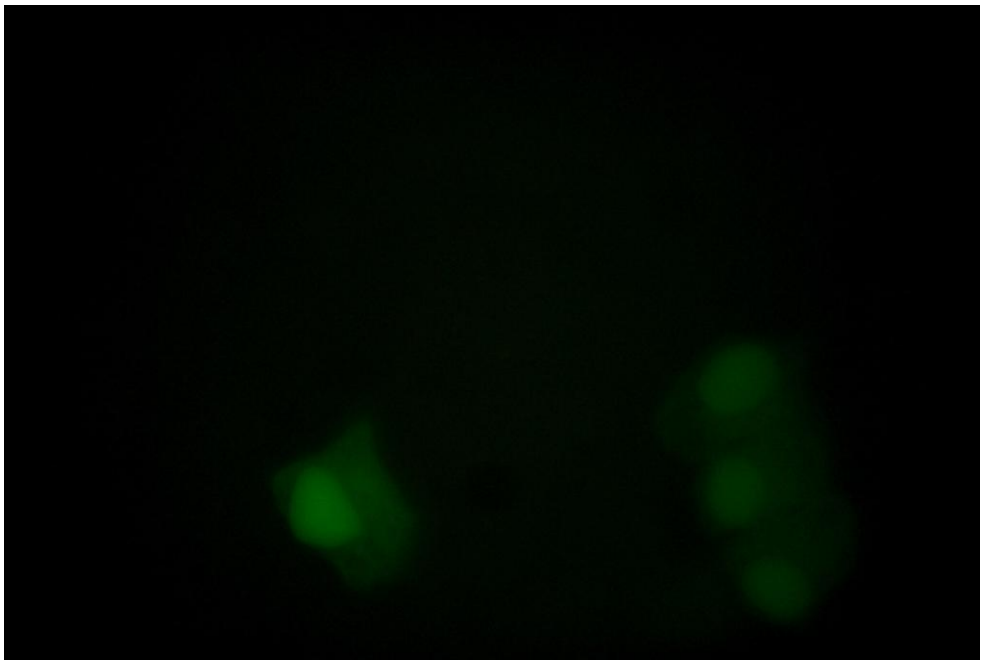

Figure 8. – 3T3 cells transfected with chimeric KP-08/Lipofectin® nanoparticles. Transfection was performed over 6 hours in the absence of serum, with a total of 350 GFP expressing cells counted in total. x 50 magnification.

If it is assumed that all pDNA in the sample was simultaneously bound to both KP-08 and Lipofectin® during transfection then the results from this experiment suggest that pDNA is able to dissociate from the hydrogel inside the cell – otherwise GFP expression would not have been noted in cells transfected with the chimeric nanoparticles. A hypothetical mechanism for cellular entry for the chimeric nanoparticles could be membrane fusion with the plasma membrane, as opposed to endocytosis (thus circumventing the problem of escape from the endosome for any complexes that are endocytosed), which would lead to localisation of the KP-08/pDNA complex in the cytoplasm. If the pDNA was then able to dissociate from the KP-08 and traffic itself to the nucleus then GFP expression could subsequently be induced.

During liposomal based transfections, opinion is divided as to how lipoplexes are internalised into cells. Authors such as Li & Huang (2000) and Leng and Mixson (2005) state lipoplexes are endocytosed whilst Kamata *et al.* (1994), Ourlin *et al.* (1997) and Mozafari & Omri (2007) suggest membrane fusion is the route of entry. It may be that a combination of the two processes permits internalisation of lipoplexes. Evidence from this project has shown liposomal based reagents to be effectively internalised into the cell.

However, the results from this experiment, like the majority of those previously recorded during the project, showed no GFP expression in cells

transfected with KP-08/pDNA complexes. It was postulated that this may because of a lack of nanoparticle uptake.

1.3.1.6 Using rhodamine-labelled pDNA to detect successful vector internalisation

Cells analysed immediately after transfection

There was evidence of rhodamine-labelled plasmid in cells exposed to KP-08 nanoparticles, chimeric nanoparticles and in the Lipofectin® control. However, there was no evidence of rhodamine in free DNA control.

However, there were large differences between the amount of rhodamine-labelled plasmid detected in cells exposed to the different transfection vectors. In cells exposed to Lipofectin®, which has repeatedly been shown to successfully transfect cells in previous experiments, there was a widespread abundance of tiny red speckles distributed all across the coverslip (see Fig. 9 a). Such fluorescent patterns were absent from the free DNA control, in which no speckles of rhodamine were noted.

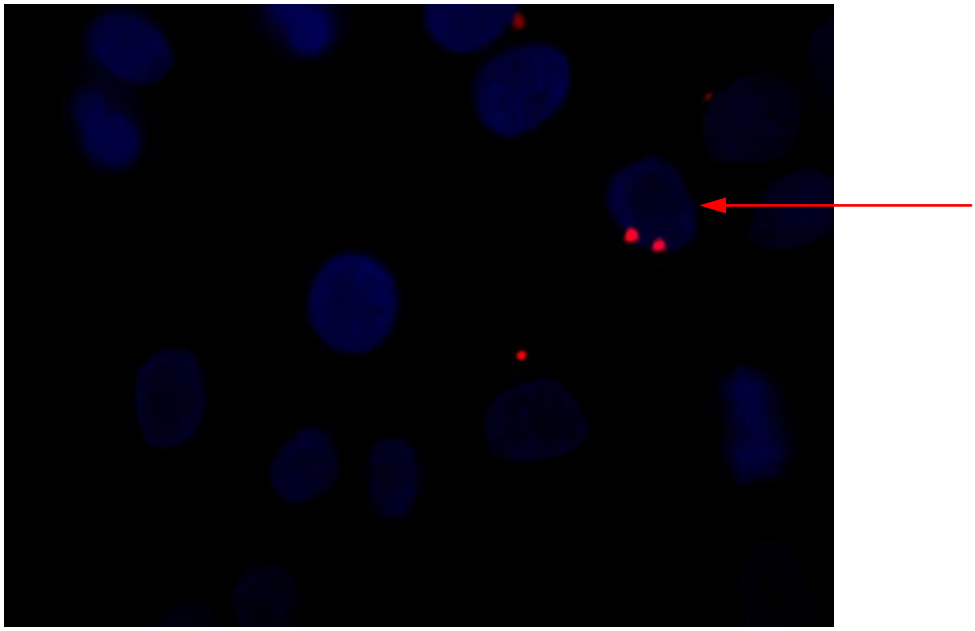

Figure 9 a) – cells exposed to Lipofectin®, analysed immediately after the 5 hour transfection period. Evidence of rhodamine-labelled plasmid around and inside the nucleus (indicated by arrow) was noted. The number of rhodamine speckles noted was extremely large (not all visible on the photo), with rhodamine distributed across all of the coverslip. The point of analysis was too early for GFP detection, however. Images were taken using ACT-2U software as in the previous experiment, through x 100 magnification.

In cells exposed to the chimeric nanoparticles (synthesised by mixing polyplexes of KP-08 and DNA with Lipofectin®), there was widespread evidence of internalised plasmid (see Fig. 9 b), although not to the same extent as in the Lipofectin® control. Lastly, in the cells exposed to KP-08 nanoparticles there was evidence of rhodamine labelled plasmid in cells (only faintly visible to the left of the arrow in Fig. 9 c), but it was very sparse and abundance of rhodamine was largely reduced in comparison to the chimeric particles and Lipofectin® control.

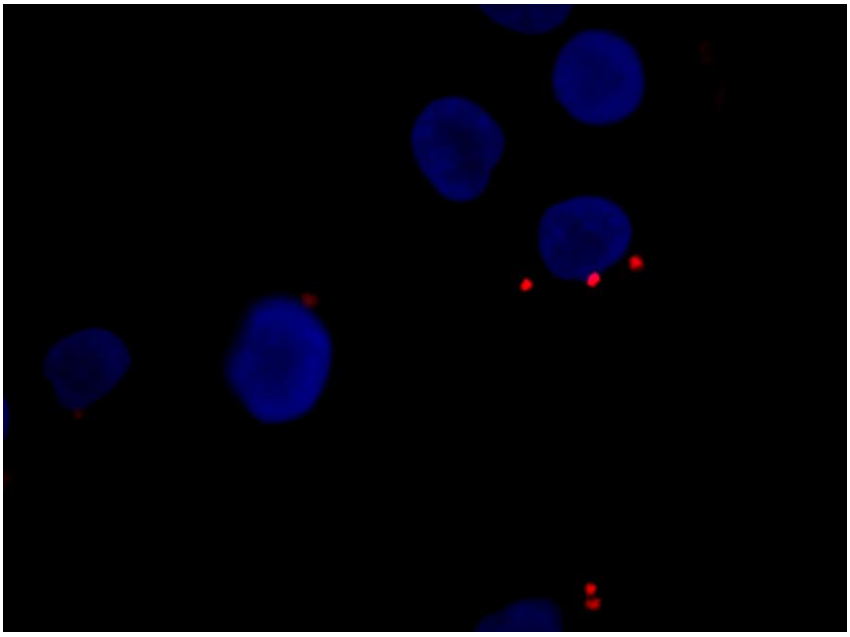

Figure 9 b) – Cells exposed to chimeric nanoparticles, analysed immediately after the 5 hour transfection period. Evidence of rhodamine-labelled plasmid inside cells was noted although in the cells captured in this image the plasmid had not entered the nucleus by the point of analysis.

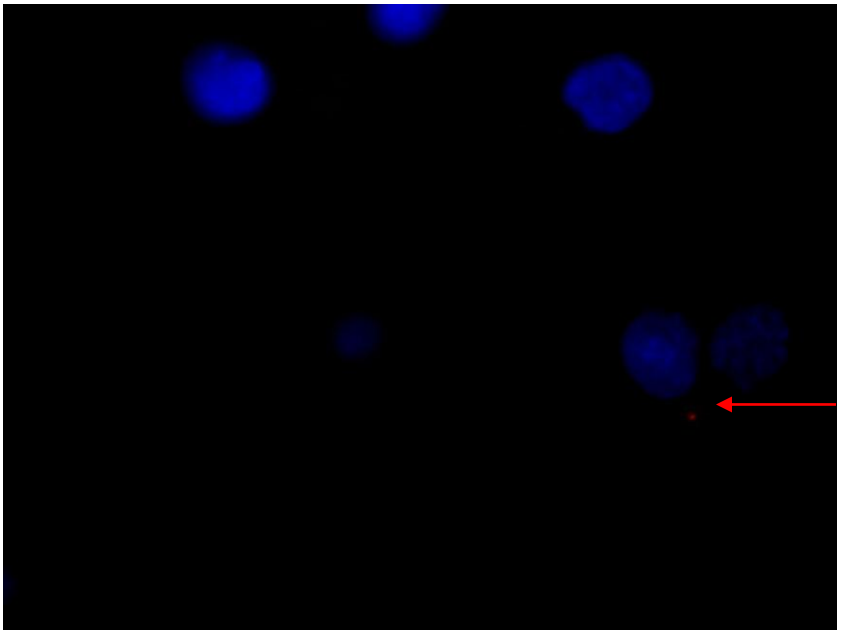

Figure 9 c) – cells exposed to KP-08/pDNA nanoparticles, analysed immediately after the 6 hour transfection period. One speckle of rhodamine is present towards the bottom right hand corner of the image, and although was not located in the nucleus, appeared to have localised in the cell cytoplasm. The number of rhodamine speckles noted on this coverslip overall was very low, however.

Cells analysed 43 hours post-transfection:

In the cells analysed 43 hours post transfection the same patterns of rhodamine fluorescence were noted compared to when cells were analysed immediately after transfection – the greatest distribution of rhodamine was in the Lipofectin® control with the least amount of plasmid in the cells exposed to KP-08. No rhodamine was detected in cells exposed to free DNA, whilst a good amount of rhodamine was detected in the chimeric treatment, although not to the same levels as in the Lipofectin® control.

As cells had been grown for longer prior to analysis the cell density was increased compared to those analysed straight after transfection (greater number of DAPI-stained nuclei visible).

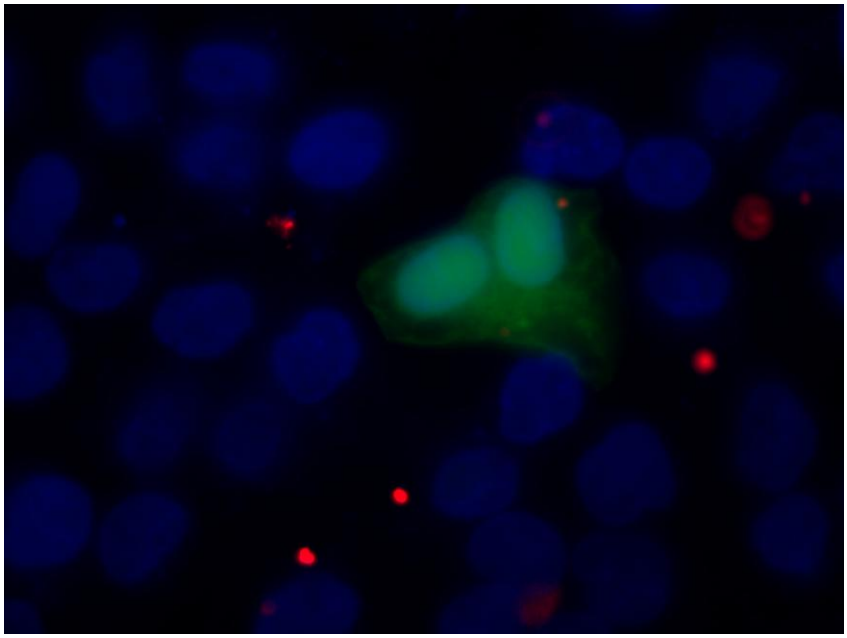

Figure 9 d) – cells exposed to Lipofectin®, analysed 42 hours post transfection. Delaying the onset of analysis provided transfected cells sufficient time to express the GFP protein, as indicated in the above photo. There are around ten visible rhodamine dots on the photo indicating the presence of internalised plasmid. In all cases, internalised rhodamine is located in close proximity to the cell nuclei, but only in two of the above cells has GFP been expressed. It may have been that one of the two transfected cells was stably transfected (i.e. the plasmid became integrated into the host cell chromosome) and that the second transfected cell is a 'clone' daughter cell. Alternatively, both cells may have been separately transfected. To determine whether the cells had been stably or transiently transfected, antibiotic selection of the culture would be required as the plasmid encodes neomycin resistance to successfully transfected cells.

However, lengthening the time prior to analysis meant GFP expressing cells were now visible at the point of analysis in both the Lipofectin® (see Fig. 9 d) and chimeric treatments (Figure 9 e). There were approximately 400 GFP expressing cells counted in cells exposed to Lipofectin® compared to 200 with the chimeric nanoparticles. However, no GFP expression was detected in cells exposed to free DNA or KP-08 nanoparticles, despite the fact rhodamine-labelled plasmid was located inside cells transfected with KP-08 nanoparticles (albeit in very small numbers).

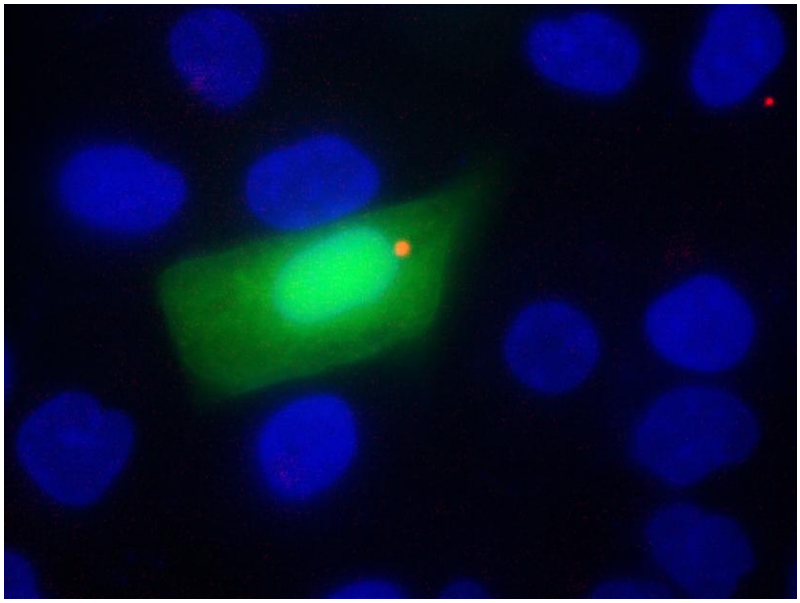

Figure 9 e) – cells exposed to chimeric nanoparticles, analysed 42 hours post transfection. Delaying the onset of analysis provided transfected cells sufficient time to express the GFP protein, as indicated in the above photo. GFP expression is noted in both the nucleus – where the DAPI and GFP images overlap and in the cell cytoplasm, where the green area of fluorescence marks the boundaries of the cell. There is evidence of rhodamine in the cytoplasm of the transfected cell, as indicated by the red dot, but also in the cell in the top right of the photo. In the latter case, however, transfection has not been successful as no GFP expression was observed.

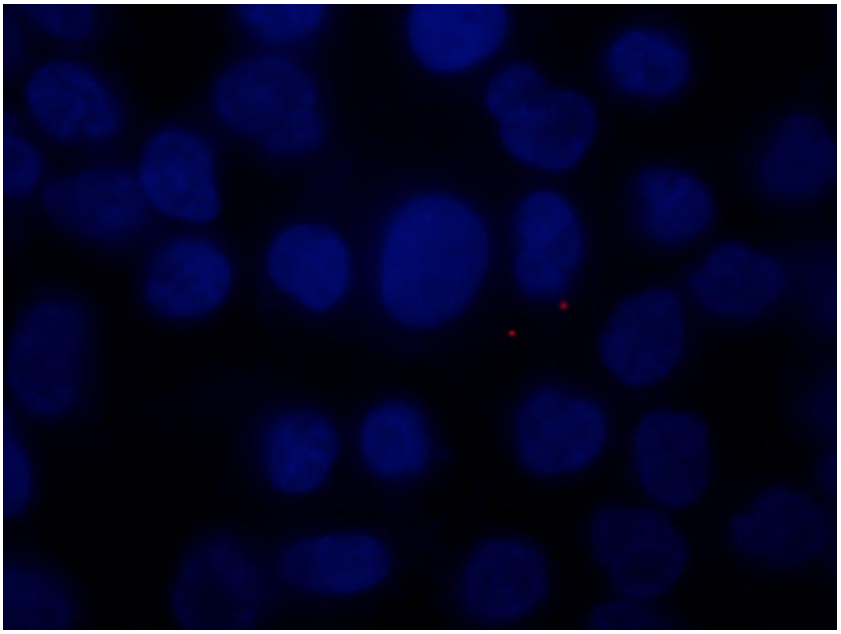

Figure 9 f) – cells exposed to KP-08 nanoparticles. Viewing cells exposed to KP-08 nanoparticles through DAPI and rhodamine filters showed that cells were present on the coverslip and that there was rare evidence of rhodamine speckles on the slide, indicating internalisation of the polyplex. However, no GFP expression was detected in any cells on the slide. The large difference in the number of rhodamine speckles present on this slide compared to the Lipofectin and chimeric nanoparticles suggested that the vast majority of KP-08 was not being internalised into cells because of inefficient endocytosis.

1.3.1.7 Investigating primary cells as an alternative target cell type

Evidence of rhodamine uptake and GFP expression was only noted in cells transfected with Lipofectin, not in cells exposed to KP-08 nanoparticles or in the naked DNA control (regardless of whether DNA had been labelled or not).

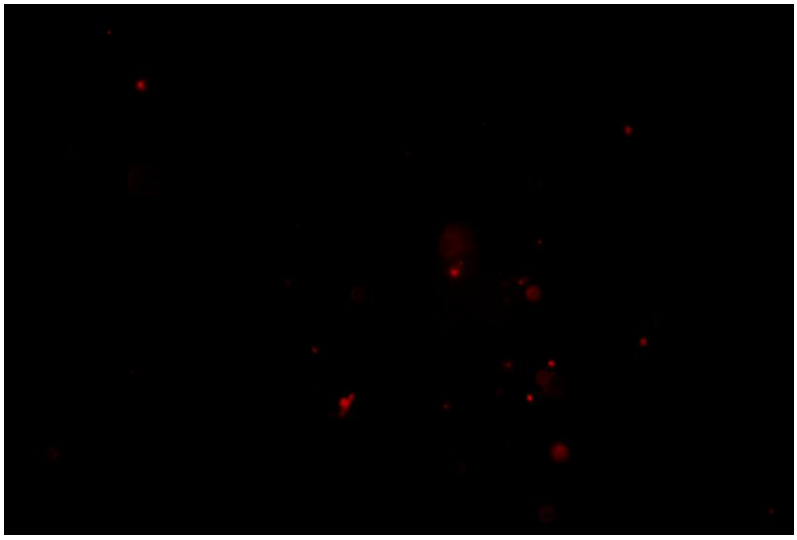

Fig. 10a – articular chondrocytes transfected with Lipofectin® complexed to rhodamine-labelled plasmid DNA. GFP expression was also noted in the same cells, although levels of GFP expression were much higher when transfections were performed using unlabelled pDNA.

Chondrocytes were shown to be successfully transfected by the Lipofectin® complexed with unlabelled DNA with around 700 GFP-expressing cells counted in total (see Figure 10b below)

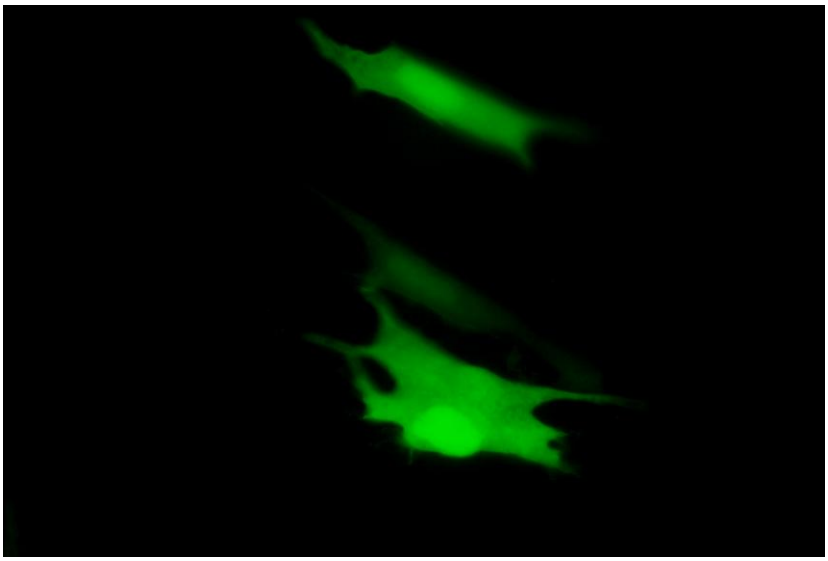

Fig. 10b) – GFP expression noted in articular chondrocytes transfected with Lipofectin® and unlabelled plasmid.

1.3.1.8 Electroporation of HaCaT cells with KP-08/pDNA complexes

Evidence of GFP expression in the naked DNA control (see Fig. 11a) below) confirmed the efficacy of the plasmid DNA stock used during the experiment and that electroporation parameters were appropriate for transfection. Secondly, a lack of GFP expression in the KP-08 control (in which no DNA was added) suggested that KP-08 alone could not achieve transfection.

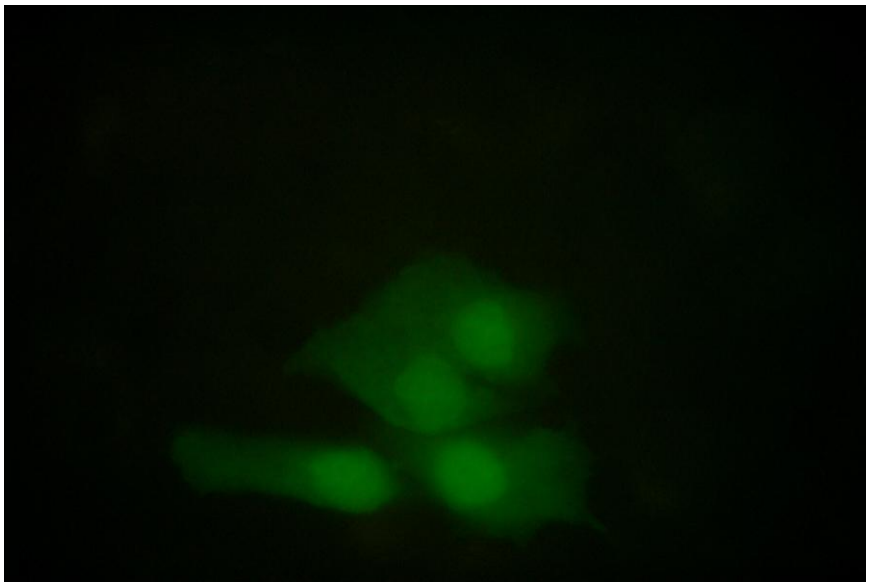

Fig 11a) – HaCaT cells electroporated with naked DNA, analysed 24 hours post-electroporation. x 50 magnification

In the experimental treatment in which cells had been electroporated in the presence of KP-08/pDNA complexes, GFP expression was noted, indicating successful transfection in at least 250 cells. This finding, assuming that all DNA was bound to the hydrogel during the electroporation procedure, suggested that the KP-08 formulation did not prevent DNA release into the

cytoplasm and thus did not prevent GFP expression from taking place if complexes were located into the cytoplasm.

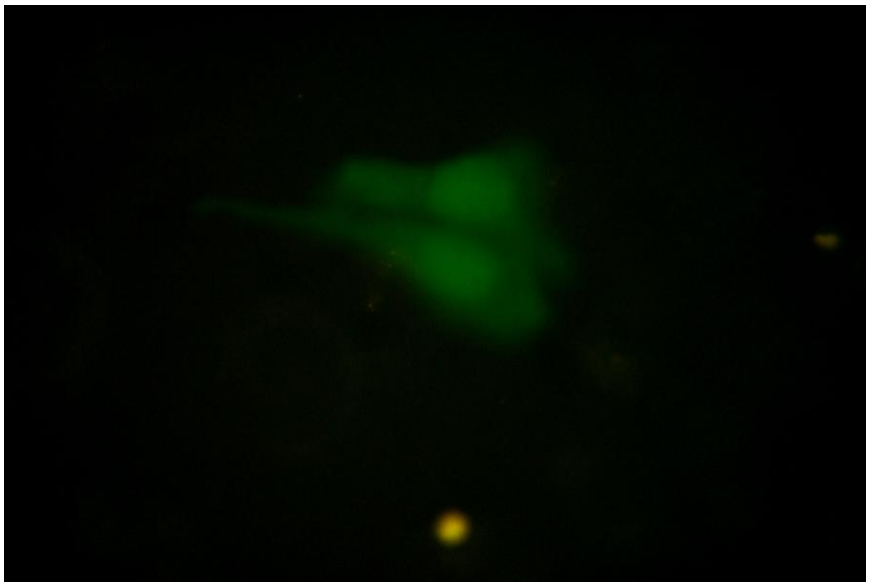

Fig 11b) – HaCaT cells electroporated with KP-08/pDNA complexes, analysed 24 hours post transfection. x 50 magnification

1.3.1.9 Electrophoresis of KP-08/pDNA complexes re-suspended in electroporation buffer

Figure 12 suggested that KP-08 had successfully bound DNA into polyplexes (lane 6) which remained intact when diluted into electroporation buffer at the two concentrations selected (lanes 7 and 8). In lanes 7 and 8 a band of DNA was faintly visible in the well at the top of each lane, however, there was no evidence of unbound DNA migrating down the lane towards the positive electrode as in lane 5 (control lane).

Thus, these results suggested that during the previous electroporation experiment, the DNA that was successfully uptaken into the cells during the electroporation procedure was likely to have been bound to KP-08 at the point of entry into cells.

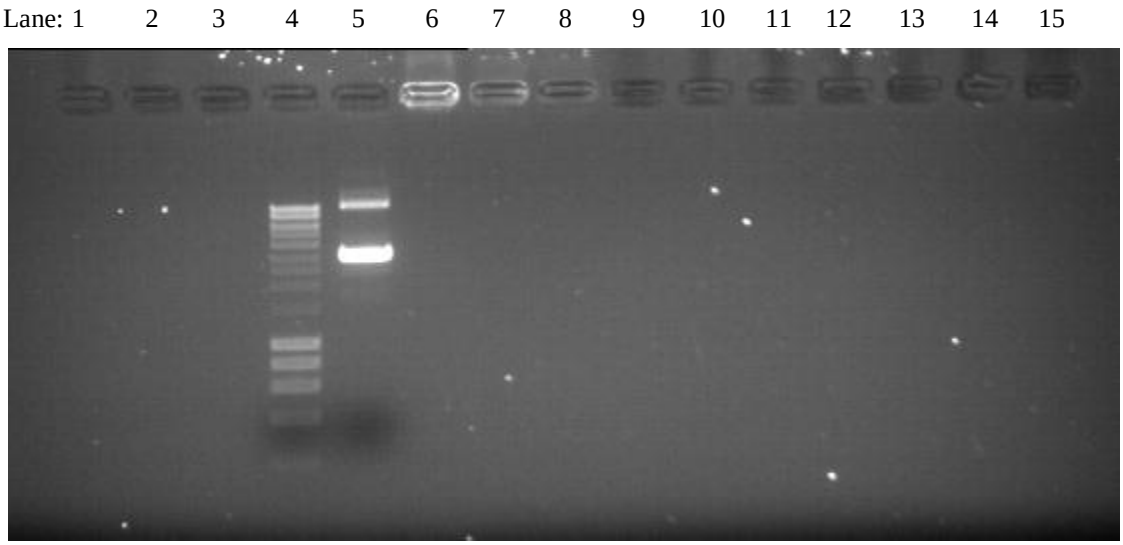

Fig 12 – Electrophoresis of the following samples: lanes 1-3: blank, 4: DNA ladder, 5: sample of free DNA, 6: sample of KP-08/DNA complexes undiluted into buffer, 7: KP-08/DNA complexes diluted into electroporation buffer (400 µl final volume), 8: KP-08/DNA complexes diluted into electroporation buffer (800 µl final volume). All other lanes are blank.

1.3.1.10 Electrophoresis of CaP and CaP/KP-08 precipitates

The results showed that DNA present in standard calcium phosphate precipitates migrated down the lane (lanes 4 and 5) in the same manner as free DNA (control lane 3). When DNA was mixed with calcium chloride but not mixed with HBS thereafter (as would be the case in precipitate formation) the result remained identical, as the DNA fragment migrated the same distance down lane 7.

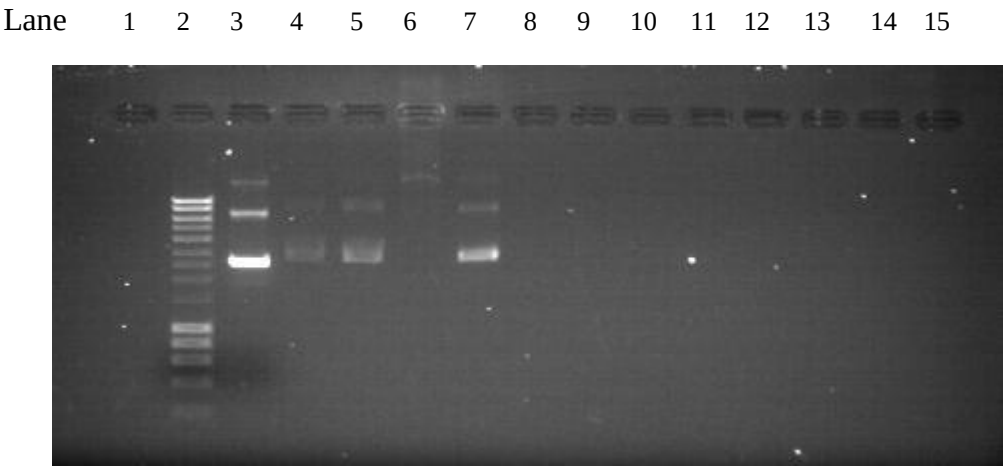

Fig. 13 – Agarose electrophoresis of KP-08/CaP and CaP precipitates. Lanes 1 and 8-15 are blank. In lane 2 is a DNA ladder whilst lane 3 contains a sample of free DNA. Lanes 4 and 5 are samples of CaP formed with or without a pre-incubation period. Lane 6 is a sample of KP-08/CaP and lane 7 is a sample of DNA mixed with calcium chloride but which was not taken through mixing with sodium phosphate in the second precipitate formation step.

In contrast, when KP-08 is added to the DNA during precipitate formation the majority of DNA remains in the well at the top of lane (lane 6). A band of nicked DNA is present around a third of the way down lane 6, which is also present in the other lanes, however, the thick band of plasmid DNA present in lanes 3,4,5 and 7 is not observable, suggesting that the DNA is bound to the hydrogel. The fact that transfection has been achieved with such precipitates would suggest that the hydrogel-gel-bound DNA is still precipitating with calcium phosphate thereafter, just that it fails to produce the same banding pattern on an agarose gel.

1.3.1.11 Transfection of KP-08/Calcium Phosphate precipitates

When cells were observed at the point of analysis, GFP expression was noted in cells exposed to the hydrogel/CaP precipiates (see Figs. 14 a-c))

with around two hundred GFP expressing cells counted in total. This was a notable increase from previous experiments when either hydrogel nanoparticles alone or hydrogel/liposome ‘chimeric’ vectors had been used.

GFP expression was also noted in the positive CaP control, in which around 500 GFP-expressing cells were counted. However, there was no evidence of transfection in the naked DNA negative control.

It was interesting to note that GFP expression had been detected in 3T3 cells because HaCaT cells exposed to the same precipitates (both the KP-08/CaP precipitates or standard CaP precipitates) had previously displayed no evidence of GFP expression.

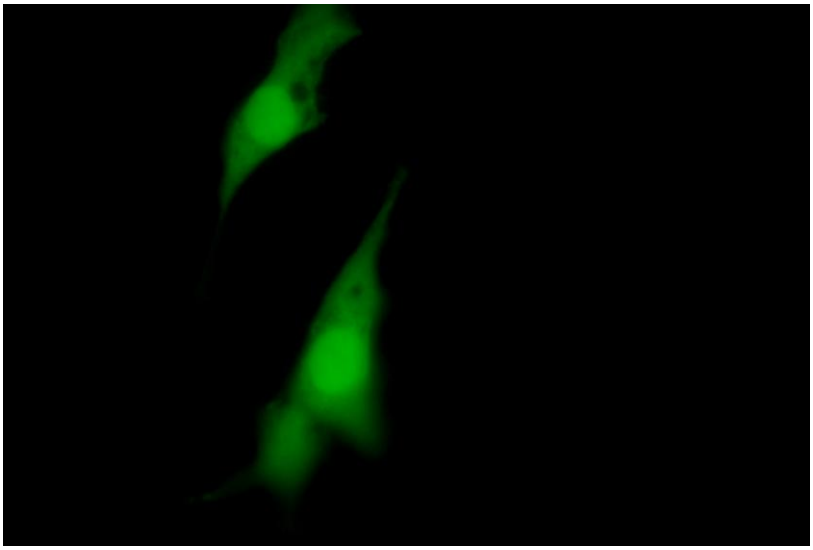

Fig 14a) - NIH 3T3 cells transfected with KP-08/CaP precipitates, a few hundred GFP expressing cells were counted in total

Although there were a greater amount of GFP-expressing cells in the CaP control, suggesting that the presence of the hydrogel may, in some way, hinder gene expression, it may be that adding hydrogel to the formulation confers some other benefit, such as the ability to nebulise the formulation.

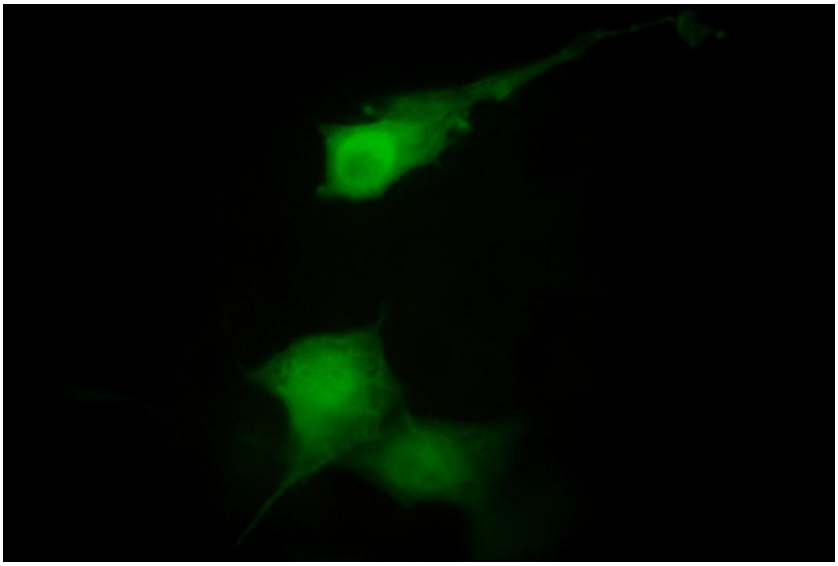

Fig 14b) - NIH 3T3 cells transfected with KP-08/CaP precipitates, a few hundred GFP expressing cells were counted in total

The results of this experiment (see Figs. 14a) and b)) show a marked improvement in the transfection efficiency of the hydrogel, albeit in the presence of CaP. It appears that mixing hydrogel-bound DNA with calcium chloride and sodium phosphate does not prevent precipitation, and thus the complexes are still endocytosed in the same manner as standard CaP precipitates. It must be acknowledged, however, that an assumption has been made during the experiment – that all of the DNA present in the KP-08/CaP precipitates was bound to the KP-08 prior to the addition of the calcium phosphate. Previous electrophoresis results would suggest that this is the

case, however, if DNA and KP-08 were not associated with one another then the gene expression observed could have been a result of transfection achieved with residual DNA forming precipitates with CaP.

1.3.3.12 Particle Sizing of KP-08/pDNA/CaP complexes

Particle sizing measurements showed both in the presence and absence of KP-08, calcium phosphate-pDNA precipitates grew in size over time. When KP-08 was present, the complexes were larger (started off around 1 micron in diameter in the presence of KP-08 as opposed to around 850 µm without KP-08) and they grew at a faster rate over time.

In conclusion it appears that because the KP-08/CaP precipitates start off much larger than CaP precipitates, they can only be incubated for a short time before addition to cells. Prolonging the incubation period of such complexes for too long means the precipitate grows excessively, and may be too large to be endocytosed by cells. Thus, in future experiments Kp-08/CaP precipitates will be added immediately to cells in an attempt to maximise cell uptake.

1.3.3.13 Transfection of KP-08/pDNA/CaP complexes prepared with and without an incubation period

With standard CaP-pDNA precipitates formed in the absence of KP-08, only 98 GFP-expressing cells were counted when complexes were immediately added to cells, however, after a 30 min incubation period with precipitates, this number was increased to 486.

In contrast, however, when KP-08 was present in the precipitate the opposite result was noted, with 915-GFP expressing cells counter when the precipitates were immediately added to cells, with this figure dropping to only 49 in a 30 min incubation step was added before the complexes were added to cells. Together with the particle sizing experiments, these results indicate that because the complexes grow in size over time there is a small window of opportunity for them to become exposed to cells before they grow in size too big to be uptake by the target cells. When KP-08 is excluded in the control precipitates, transfection efficiency increases in time over time, although it is expected to only increase up to a point and is worth noting that such complexes start at a smaller size to those containing KP-08 anyway. Studies in the literature have revealed that taking absorbance readings of precipitates at an optical density of 320 nm can reveal whether precipitates are likely to be efficient during transfection. A reading of 0.2/0.3 is preferable in the absence/presence of DNA. When transfection solutions were formed and

analysed 1 hour post preparation the standard CaP precipitates produced a reading of 0.117, or 0.187 if the precipitates had been incubated prior to addition to cells. When KP-08 is added to the solution the starting reading is larger, although increases in size over time are also noted.

1.3.3.14 Nebulised delivery of KP-08/pDNA/CaP precipitates to cells

On the coverslip of cells exposed to the nebulised KP-08/DNA/CaP precipitate (transfected in the presence of media) a total of 977 GFP-expressing cells were counted (see Figure 15), however, no GFP expression was observed in the control treatment in which cells had been exposed to a nebulised formulation of the standard CaP precipitate or when transfection of the KP-08/DNA/CaP precipitate was performed with media omitted from the flask.

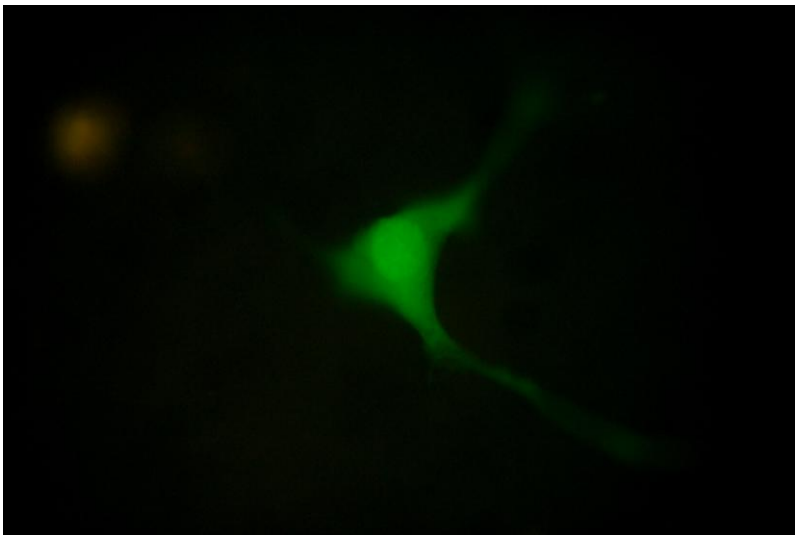

Fig 15): NIH 3T3 cells transfected with the nebulised KP-08/DNA/CaP precipitate

Thus, it would appear that including cell culture media in the flask during transfection improves the rate of transfection as a drastic increase in GFP expression was observed compared to when media was omitted from the flask.

Additionally, the absence of GFP expression in the control suggests that the presence of the hydrogel in the precipitate confers some advantage over the standard CaP precipitate as a transfection solution.

1.3.3.15 Optimiaztion of KP-08/pDNA/CaP complexes for transfection

Parameter 1

The aim of investigating parameter 1 was to ascertain whether incubating the KP-08/CaP precipitates prior to addition to cells could increase levels of gene expression. Although 249 GFP-expression cells were counted when the precipitate was immediately added to cells (see Fig. 16) below), levels of gene expression dropped when the incubation period of the precipitate was extended, with 104 GFP expressing cells visible after 5 minutes incubation, and only 50 noted after 20 minutes of incubation.

Particle sizing measurements taken last month showed that the KP-08/CaP precipitates grow over time and thus the reduced levels of gene expression

observed after an extended incubation period are probably attributable to the precipitates growing to a size when they become too big to be endocytosed.

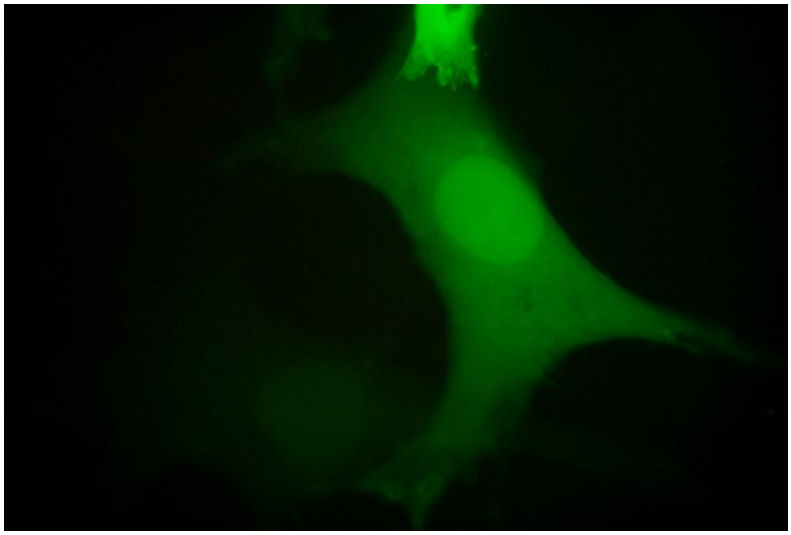

Figure 16 - KP-08/CaP precipitate formed and added straight to cells without any prior incubation at room temperature.

Parameter 2

The aim of investigating parameter 2 was to determine whether there was an optimal time with which KP-08/DNA polyplexes should be incubated with $CaCl_2$ for prior to dropwise addition into the HEPES buffered solution containing the sodium phosphate (2X HBS).

Results showed that if the KP-08/DNA/$CaCl_2$ solution was immediately added onto the 2 X HBS that 302 GFP-expressing cells were observed (see Fig 17) below). However, if the polyplexes were incubated with $CaCl_2$ for 5 or 20 minutes prior to addition into the HBS then the number of GFP expressing cells observed decreased to 213 and 144 respectively.

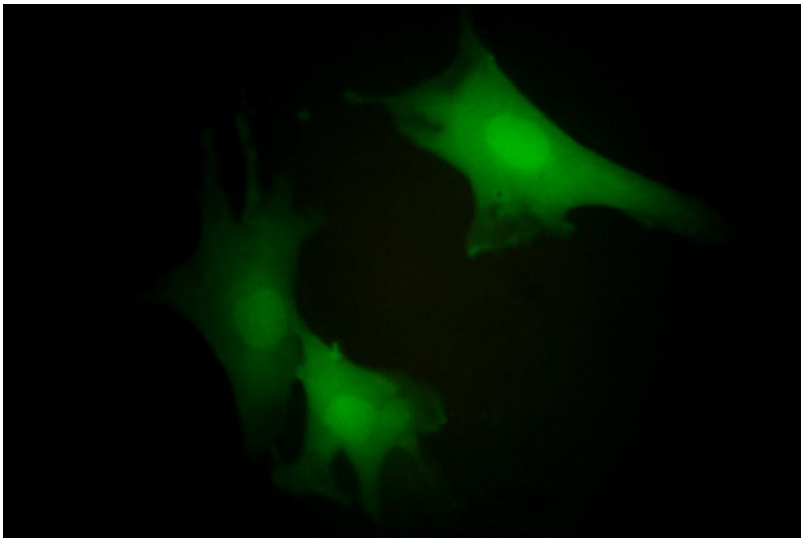

Figure 17) - KP-08/DNA/$CaCl_2$ solution immediately added to 2 X HBS during precipitate formation, with no incubation period of $CaCl_2$ with the polyplexes

Again, this highlighted the benefit of making up the precipitate as quickly as possible, shortening incubation periods as much as possible in between each step of the precipitate forming procedure.

Parameter 3

The aim of investigating parameter 3 was to determine whether there was an optimal time for which KP-08 and DNA should be incubated together for prior to the addition of $CaCl_2$.

When KP-08 and DNA were mixed and $CaCl_2$ was added immediately afterwards, a total of 485 GFP-expressing cells were counted. However, if the polyplexes were incubated for either 5 or 20 minutes before $CaCl_2$ was added then the number of transfected cells observed decreased to 268 and 123 respectively.

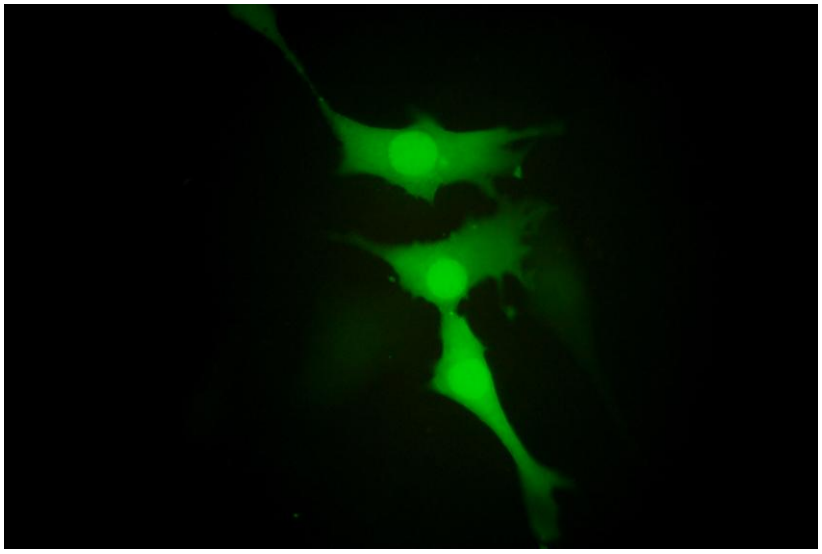

Figure 18) CaCl2 immediately added to cells after KP-08 and DNA initially mixed together during the polyplex formation step

Finally, GFP expression was noted in the standard CaP control, with a total of 188 GFP-expressing cells counted. However, this transfection efficiency remained lower than the most efficient transfection achieved with the hydrogel.

In summary, these results highlighted several parameters during the precipitate formation procedure that influence transfection efficiency, presumably by optimising precipitate formation. The highest rates of

transfection were achieved when the precipitate formation procedure was performed without incubation periods in between each step.

1.3.3.16 Particle Sizing of Nebulised KP-08/pDNA/CaP precipitates

After the nebulised solution had been collected it was transferred into a cuvette for particle sizing measurements. However, the solution was not producing a clear signal and it was thus necessary to filter the solution through a 0.45 micron filter and take particle sizing measurements from the filtered solution. This acted to remove any background from the solution and showed that the transfection particles were approximately 190 nm in diameter prior to nebulisation but that after nebulisation particle size increased to approximately 300 nm. Particle sizing measurements were taken every two minutes for twenty minutes, which showed that the particles grew by no more than 10 % of their original size by the end of the sampling period.

Past particle sizing experiments sized KP-08/DNA/CaP precipitates at around 1 micron at the start of the sampling period and 1.7 microns at the end. This was larger than DNA/CaP precipitates which were around 850 nm to start with and grew to around 1.3 microns by the end of the sampling period. These findings tied in with spectophotometric readings of DNA CaP precipitates from earlier in the project, which indicated that precipitates grew in size over time.

As the solutions had been filtered the results were not going to show particles as large as before. However, even after filtration it was possible to ascertain that they had grown in size during nebulisation.

1.3.3.17 Filtration of Nebulised KP-08/pDNA/CaP precipitates prior to transfection

At the point of analysis 42 GFP expressing cells were counted in the non-filtered treatment.

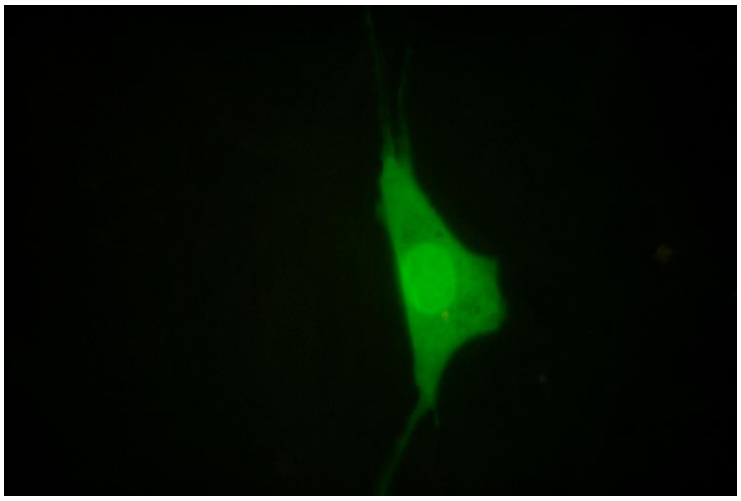

Fig. 19 – GFP-expressing cell observed after exposure to a non-filtered transfection solution.

However, 0 GFP expressing cells were counted in the filtered treatment, suggesting that it is the KP-08/DNA/CaP precipitates sized above 450 nm that are responsible for achieving transfection.

1.3.3.18 Evaluating the use of a different nebuliser

No GFP-expression was noted in the cells exposed to the precipitate nebulised with the Sidestream nebuliser, however, 6 GFP-expressing cells were noted when the E-flow nebuliser had been used. Strangely, no GFP expression was noted in the treatment in which the transfection solution had been pipetted dropwise into the culture flask, even though such treatments have demonstrated transfection in the past. It may have been that transfection did take place in this solution at a low rate and that transfected cells were not identified during analysis.

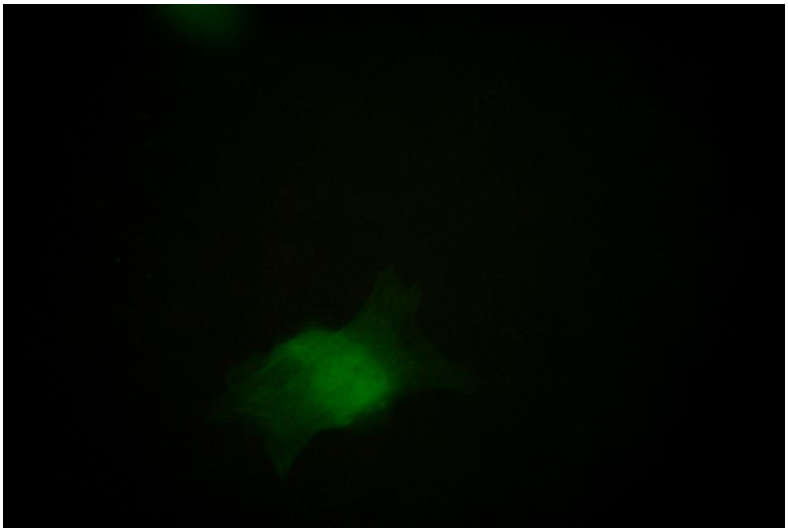

Fig. 20 – GFP expression in NIH 3T3 cells exposed to transfection solution nebulised with the E-flow nebuliser.

The fact that only 6 GFP-expressing cells were counted following E-flow nebulisation suggested that transfection remained very inefficient as there were several hundred thousand viable cells present on each coverslip at the

point of analysis. It was decided that a new hydrogel solution would be formulated and nebulisation would be repeated with the E-flow nebuliser.

1.3.3.19 Agarose electrophoresis to detect pDNA in the nebulised solution

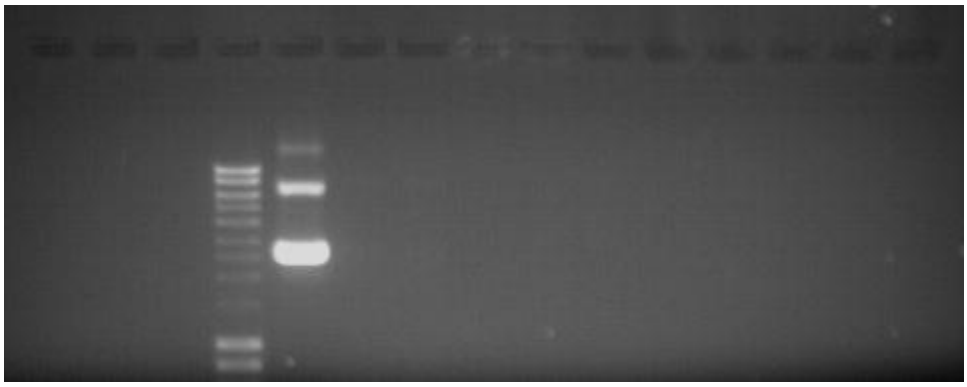

Fig 21: Agarose electrophoresis of the following samples: lanes 1-3 blank, lane 4 DNA ladder, lane 5 a sample of free DNA, lane 6 nebulised sample of free DNA, lane 7 nebulised sample of free DNA filtered, lane 8 KP-08/CaP precipitate nebulised and collected, lane 9 KP-08/CaP precipitate nebulised, collected and filter sterilised. Lanes 10-15 blank.

The results showed that in the hydrogel solution (non-filtered) DNA was present in the well at the top of lane 8, suggesting that there was DNA coming through the nebuliser and that it remained bound in a complex with a net positive charge, which did not migrate towards the negative electrode. However, when the solution was filter sterilised little if any DNA was present in the well or lane in lane 9, suggesting that the DNA is bound to a complex larger than 0.45 microns.

This has implications for transfection because it suggests that the DNA is being presented to cells during transfections and that the nebuliser isn't

blocked and preventing cells from being exposed to the transfection solution. The results also suggest that the majority, if not all of the DNA that is being presented to cells during nebulisations, is bound in a complex that is sized above 0.45 microns. The size of the complexes alone does not explain why transfection has proven inefficient because the standard calcium phosphate precipitates are similarly sized around a micron and still produce transfection.

Finally, the results in figure 21 also show that when DNA was nebulised by itself (attached to no vector), none was detected in the collected samples (non-filtered, lane 6 or filtered, in lane 7), suggesting that a simple DNA solution cannot be nebulised.

1.3.2 Examining the properties of POVAL Nanoparticles for gene delivery

1.3.2.1 Examining the stability of POVAL/pDNA complexes through electrophoresis

By forming polyplexes and performing serial dilutions in either PBS or Opti-MEM it was possible to examine the stability of the POVAL-pDNA complexes when they were diluted down to low concentrations. Such stability at low concentrations was essential during transfection, when a 50 µl volume of polyplexes would be diluted into a final volume of 2 ml of cell culture media.

The results in Figure 22 show that polyplexes remained stable upon dilution into both PBS (lanes 4-7) and Opti-MEM media (Lanes 8-11) and that all DNA had remained bound to the hydrogel in each sample. In lanes 4-11 there was an absence of free, unbound DNA, which would have migrated as in lane 12 (control lane of free DNA)

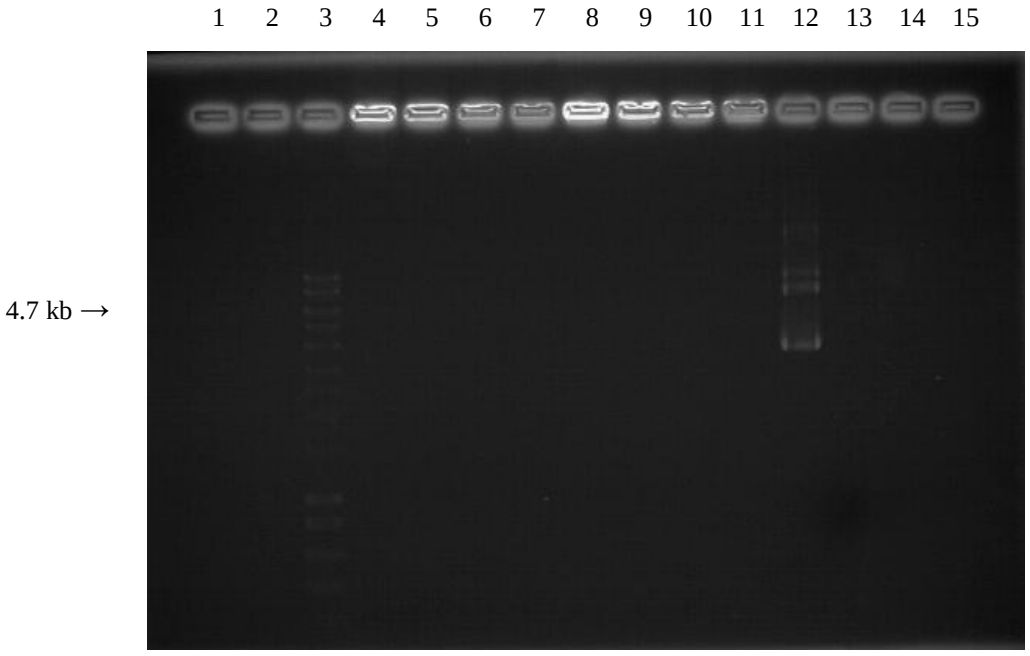

Fig. 22 – electrophoresis of the following samples;
Lane 1 – Blank
Lane 2 – Blank
Lane 3 - DNA molecular weight ladder
Lane 4 – pDNA/hydrogel complex diluted in PBS after complex formation
Lane 5 – two fold dilution of the sample loaded in lane 4
Lane 6 – two fold dilution of the sample loaded in lane 5
Lane 7 – two fold dilution of the sample loaded in lane 6
Lane 8 - pDNA/hydrogel complex diluted in Opti-MEM media after complex formation
Lane 9 – two fold dilution of the sample loaded in lane 8
Lane 10 – two fold dilution of the sample loaded in lane 9
Lane 11 – two fold dilution of the sample loaded in lane 10
Lane 12 – naked pDNA (re-suspended in distilled water)
Lane 13 – dye used to track the migration of fragments across the gel
Lanes 14 and 15 – blank

The banding intensity varied between lanes because of the varying amounts of DNA in each lane – a consequence of the serial dilutions performed in sample preparation.

As in the previous experiment it appeared that all of the DNA in the hydrogel samples had a decreased mobility on the agarose gel because of charge neutralisation of the phosphate backbone of the DNA and because the polyplexes had an increased molecular size (Akinc *et al.* 2005; Chen *et al.* 2007; Pack *et al.* 1999). As the POVAL polyplexes were shown to be stable it was decided that a transfection attempt would be undertaken.

1.3.2.2 Evaluating POVAL nanoparticles during transfection

Although NIH 3T3 cells exposed to POVAL nanoparticles appeared healthy no GFP expression was detected regardless of whether transfection period had been 5 or 24 hours. However, in the Lipofectin® controls GFP expression was observed regardless of whether the DNA stock had been re-suspended in TE buffer (see Fig. 23a) or ddH_20 (see Fig. 23b), confirming that the plasmid used was able to produce transfection if complexed with the right vector.

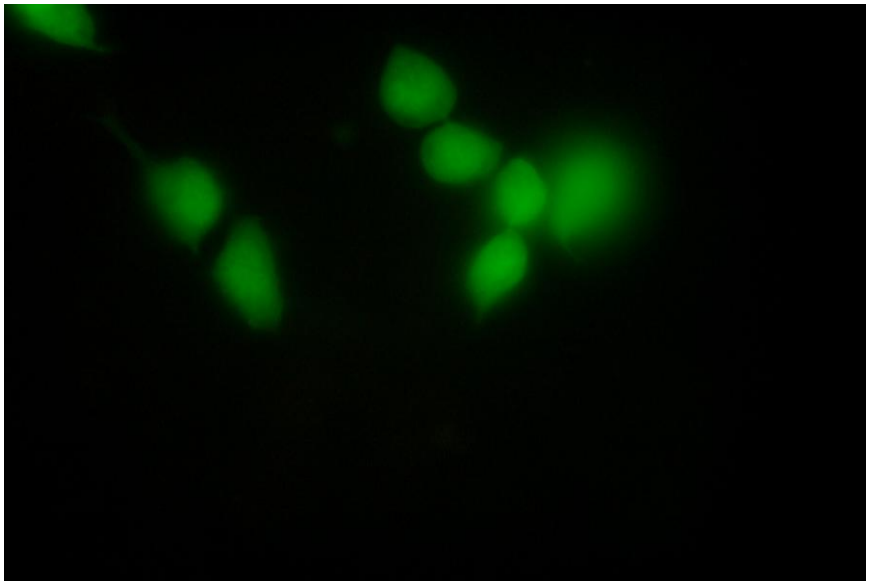

Fig. 23a) – NIH 3T3 cells transfected with Lipofectin® complexed with pDNA re-suspended in TE buffer. GFP-expression was also noted in the Lipofectin® control in which plasmid had been suspended in ddH_20, however. Image taken on a Leitz Dialux 22 microscope through x50 magnification.

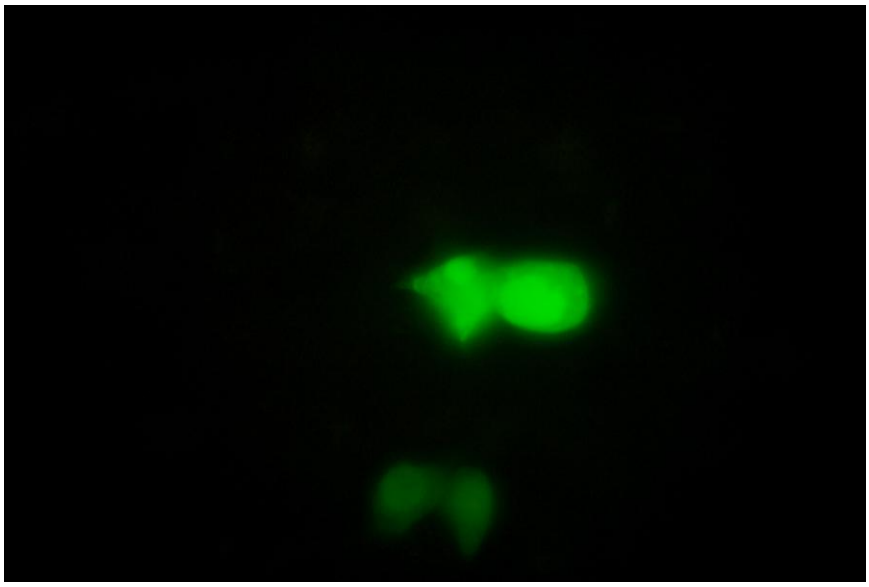

Fig. 23b) – NIH 3T3 cells transfected with Lipofectin® complexed with pDNA re-suspended in TE buffer. GFP-expression was also noted in the Lipofectin® control in which plasmid had been suspended in ddH_20, however. Image taken on a Leitz Dialux 22 microscope through x50 magnification.

The lack of GFP expression observed in the cells exposed to POVAL nanoparticles could have been observed for several reasons. Firstly, the nanoparticles may not have been uptaken by cells during transfection. Secondly, if nanoparticles had been uptaken, GFP release may have been hindered if the DNA was too tightly bound to the hydrogel and unable to escape in order for transcription factors to access the DNA (Bertshinger *et al*. 2006). Alternatively, the polyplex may have been internalised into the cell but was unable to escape the endosomal compartment and was thus degraded, which was the fate of the PVA nanoparticles administered to cells by Wittmar *et al.* (2005).

1.3.3 Miscellaneous transfection-related experiments

1.3.3.1 Transfection of HAE primary cells with POVAL/CaP precipitates

No transfection was noted when POVAL alone was used for transfection or when naked DNA had been incubated with cells. Surprisingly, no transfection was observed using the calcium phosphate precipitate, even though this same formulation has been shown to repeatedly transfect cell lines *in vitro* (but not HaCaTs). Similarly, no transfection was observed in cells exposed to the POVAL/CaP precipitate, although the results from the Lipofectin treatments confirmed that the primary cells were infact transfectable; with 114 GFP expressing cells noted in the Lipofectin control

(fig 24a) and 26 GFP expressing cells noted in the POVAL/Lipofectin treatment (fig 24b).

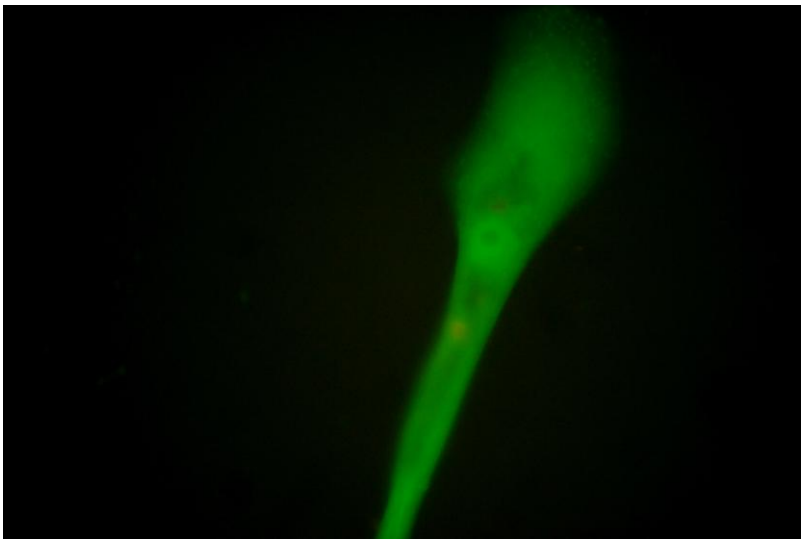

Fig 24a): Primary HAE cells transfected with Lipofectin. A total of 114 GFP-expressing cells were counted in total.

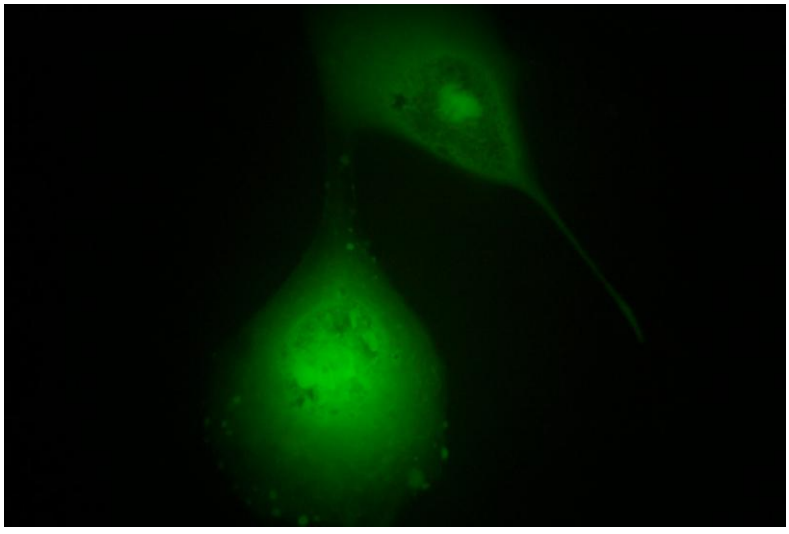

Fig 24b): Primary HAE cells transfected with 0.5 % POVAL/Lipofectin. A total of 26 GFP-expressing cells were counted in total.

Although the total number of GFP expressing cells counted in these treatments remains much smaller than that in cell lines, it is worth noting

that the overall density in these treatments was much smaller because the primary cells were slow growing.

1.3.3.2 Electrophoresis of PEI/pDNA complexes

Results showed that at low N:P ratios (1:1 or 2:1) there is an unsufficient amount of nitrogen present in the sample to bind all pDNA. However, at a ratio of 4:1 or greater DNA was clearly visible as a band in the top of the well in each lane, suggesting complex formation has taken place. There was no migration of free pDNA down the lane, suggesting the polymer has bound all pDNA.

1.3.3.3 Transfection of PEI-pDNA polyplexes

330 GFP-expressing cells were noted in the PEI polyplex treatment at the point of analysis. This was in contrast to the naked DNA control, in which no GFP expression was noted. These results show PEI to be an easy method to follow and it proved fairly effective, without any attempt at optimization.

1.3.3.4 Examining complex formation through particle size analysis

Particle sizing of POVAL polyplexes was undertaken to gain an idea of the size of the POVAL nanoparticles and plasmid DNA to determine whether

they would be of a small enough size to be endocytosed either alone, or when combined, during transfection. It has generally been suggested that smaller nanoparticles are more desirable for gene delivery as they are more effectively endocytosed (Pack *et al.* 1999) and Guy *et al.* (1995) reported efficient endocytosis of complexes sized under 150 nm in diameter. Nevertheless, authors such as Gan *et al.* (2005), Kaul & Amiji (2005), Yi *et al.* (2006) and Zhao *et al.* (2006), amongst others, have produced nanoparticles with size ranges in excess of this and have still reported successful transfection. For example, Kaul & Amiji (2005) synthesised gelatin nanoparticles within a 100-500 nm range, with an average particle diameter of 200 nm.

Particle sizing results showed that POVAL nanoparticles had a diameter of 37 nm when diluted in water, but that particle diameter increased to 76.3 nm when nanoparticles were diluted into PBS, most probably due to flocculation in the phosphate containing buffer. As pDNA alone was sized at 118.9 nm the diameter of the polyplexes may have been expected to be approximately the sum of the POVAL and DNA values. However, this was not the case as the diameter of the polyplexes was 114.7 nm, a reduction on the diameter of pDNA alone.

Sample	Particle Diameter (nm)
pDNA alone	118.9

Hydrogel nanoparticles diluted in water	37
Hydrogel nanoparticles diluted in PBS	76.3
Hydrogel nanoparticles complexed straight with pDNA (polyplexes) without prior dilution	114.7
Hydrogel/pDNA polyplexes with prior dilution in media	17894.4

Table 6 – particle sizes of hydrogel nanoparticles, pDNA and hydrogel/pDNA complexes

The reduced value of 114.7 nm is probably a mean value of two discrete particle populations in the sample if DNA and POVAL had not formed complexes, skewed towards the value for free DNA as there was a greater proportion of pDNA in the sample relative to the hydrogel nanoparticles. In order to investigate this matter further agarose electrophoresis was subsequently performed on a sample of polyplexes.

When the POVAL and DNA were diluted in Opti-MEM prior to mixing and the resulting polyplexes were sized a particle diameter of 17894.4 nm was recorded. This would appear not be a true value, and rather could be attributable to background in the sample instead as the POVAL and DNA were diluted down to such a low concentration in the Opti-MEM media.

1.3.3.5 Conductimetric titrations

PD2000 vs calf thymus DNA

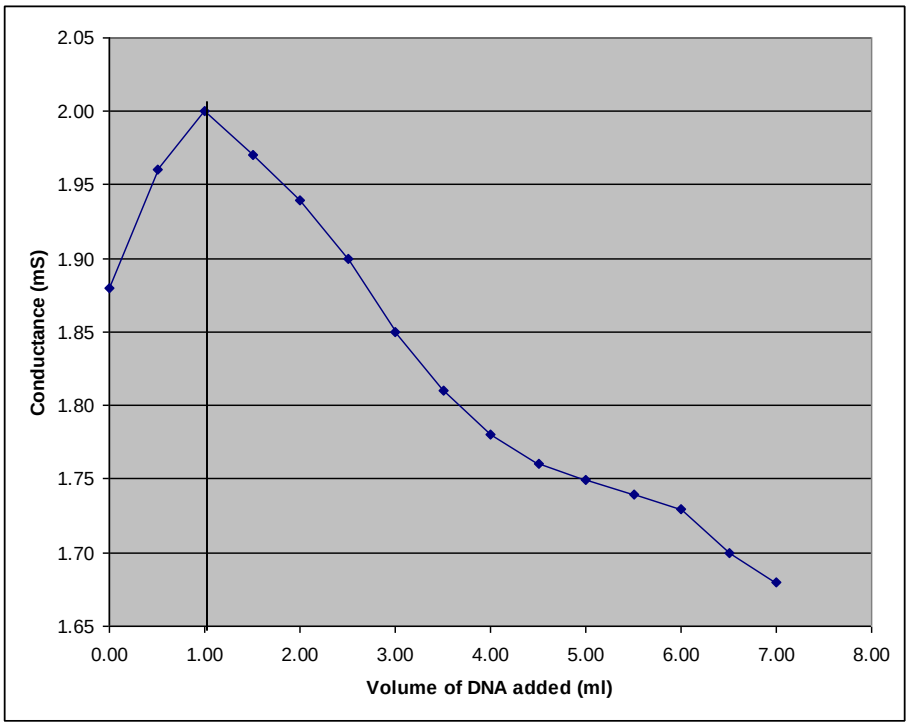

Fig 25a) – Conductimetric titration of calf thymus DNA vs PD2000. The endpoint appeared to be reach after 1 ml of DNA had been added to the solution of PD2000

Figure 25a) above shows that the endpoint of the experimental run appeared to be reached after 1 ml of DNA had been added to the solution of PD2000. As both the PD2000 and DNA solutions had been made up to identical concentrations (1% w/w), the results suggested that for every 1 volume of DNA, 25 volumes of PD2000 were required to ensure the resulting complexes possessed a positive charge. It was decided that the procedure would be repeated except DNA would be titrated against KP-08 rather than PD2000 alone.

KP-08 vs calf thymus DNA

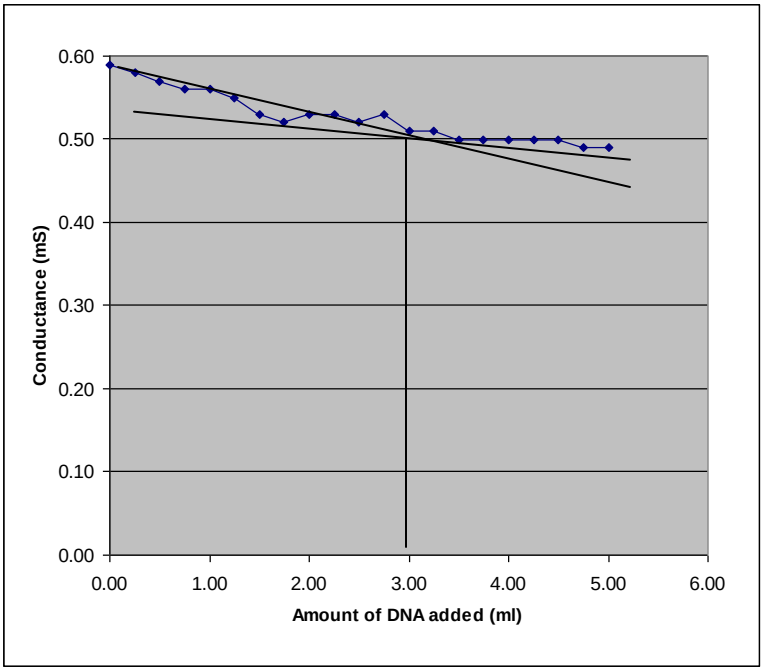

Fig 25b) – Conductimetric titration of calf thymus DNA vs 0.5 % KP-08 formulation. The endpoint appeared to be reach after 3 ml of DNA had been added to the solution of KP-08.

With reference to figure 25b), in terms of the relative amounts of PD2000 (present in the KP-08 formulation) mixed together with calf thymus DNA at the end point – after 3 ml of DNA had been added - there was, on a weight by weight basis, 0.03 g of DNA mixed with 0.00625 g of PD2000. However, taking into account the fact that DNA has a much large molecular weight compared to PD2000 (1,000,000 compared to 3,000 for PD2000), in terms of a molar basis, one mole of DNA was mixed with 69.44 moles of PD2000 at the titration endpoint.

In comparison, during transfection experiments, when polyplexes are formed, 40 µl of KP-08 is mixed with 25 µl of pDNA. In terms of a weight/weight ratio that involves mixing 400 µg of KP-08 with 2 µg of DNA. On a molar basis, that equates to mixing 3333 moles of PD2000 for every 1 mole of DNA used during complex formation. As a result, this calculation suggests that the amount of PD2000 present in complex formation is in fact in surplus and that the lack of a positive charge is unlikely to be hampering uptake of the nanoparticles.

Furthermore, these results - suggesting a net positive charge of the complex - confirm what the electrophoresis results have repeatedly showed previously – that PD2000 has neutralised the negative charge on the DNA and prevented movement towards the positive electrode.

2. Evaluating the use of cross-linked PVA nanoparticles for drug delivery

2.1 Introduction

The aim of the second part of the project was to determine whether cross-linked PVA particles would be able to enhance drug delivery of budesonide to eukaryotic cells. Previous work within AGT Sciences Ltd (unpublished) showed Nanagel® (a cross-linked PVA nanoparticle) to improve the solubility of hard to dissolve drugs such as budesonide and tobramycin and previous transfection work conducted as part of this project revealed Nanagel® to be non-toxic to cell cultures when combined with pDNA.

Asthma and chronic obstructive pulmonary disease (COPD) are common respiratory conditions characterized by episodic and reversible airway obstruction (Huynh *et al.* 2010). The worldwide prevalence of both diseases is increasing, with societal implications in terms of morbidity, mortality and costs (Thorsson & Geller, 2005).

Inhaled corticosteroids (ICS) are considered the cornerstone of therapy for patients with asthma and reduce exacerbations in COPD (Thorsson & Geller, 2005). Their use is justified by their efficacy as they are very effective in controlling symptoms, reducing exacerbations, improving the health status of patients and reducing asthma mortality (Barnes, 2006). The benefits of their

use outweigh the unwanted side effects, such as reduced growth velocity and decreases in bone mineralization (Allen *et al.* 2003).

Five corticosteroids are available as inhaled formulations for the treatment of asthma in the UK: beclometasone dipropionate, fluticasone propionate, mometasone furoate, ciclesonide and budesonide (NICE 2008) and a range of inhalation devices have been developed as a means of targeting ICS to airways. Ultimately, the choice of drug and inhalation device varies from patient to patient and is determined on an individual basis, however, budesonide offers flexibility to clinicians as it is available in metered dose inhalers in both proprietary (Pulmicort®, AstraZeneca) and non-proprietary (Novalizer, Meda) formulations, dry powder inhalers (Pulmicort Turbohaler, AstraZeneca), hard capsule powder inhalers (Cyclocaps, APS) (Nice, 2008) and as a nebulisable solution, Pulmicort Respules® (Thorsson & Geller, 2005).

Despite their widespread use, research into corticosteroid delivery has continued as it is estimated that only 10 – 40 % of ICS are deposited in the lung, with the remainder swallowed and passed through the GI tract (Allen *et al.* 2003), wastage that is increased in pediatric patients (Huynh *et al.* 2010). As a consequence, any means of improving drug delivery would be desirable. Additionally, there is a need to improve drug dissolution, especially important in the case of budesonide as the drug has a low solubility in water of < 1 mg/100 ml (Jacobs & Muller, 2002).

In view of the need for improved drug dissolution and enhanced drug delivery AGT Sciences Ltd, Bradford, have recently developed cross-linked poly(vinyl alcohol) based Nanagel® nanoparticles that have been shown to efficiently entrap budesonide and are fully nebulisable (D. Eagland, pers. comm). The purpose of this study was to demonstrate that the budesonide contained within the nanosuspension (BUDAS) was readily uptaken by cells *in vitro* and effective in suppressing the production of reactive oxygen species (ROS) during a stimulated respiratory burst.

In previously described work cellular respiratory bursts have been produced and measured *in vitro* using stimulants including formyl-methionyl-leucyl-phenylalanine (fMLP) (Braga *et al,* 2005, Dal Sasso *et al*. 2005, Pruett & Loftis, 1990) and phorbol-12-myristate-13-acetate (PMA) (Braga et al, 2005, Black *et al,* 1994, Elferink 1984, Tan & Berridge, 2000, Theron 1994) and measuring the reduction of cytochrome c (Black *et al* 1991, Bjorquist *et al.* 1994, Pick & Mizel, 1981) MTT (Burdon *et al.* 1993, Pruett & Loftis, 1990), WST-1 (Tan & Berridge, 2000) or using luminol-amplified (Dal Sasso *et al.* 2005) or lucigenin (Theron *et al.* 1994) chemiluminescence. Typically neutrophils have been extensively used for such studies, but here we present an alternative but simple and inexpensive means of stimulating SOD-inhibitable ROS production in HaCaTs, measurable by the turnover of WST-1.

HaCaTs are an ideal cell to study in an inflammatory model as they are deficient in the intracellular redox mediator, NAD. This interferes with their ability to use glycolysis as their main energy source and thus they circumvent the problem by consuming glutamine, which inadvertently leads to the accumulation of NADPH oxidase, the ROS products of which are used for energy (Benaventre & Jacobsen, 2008). The readily accessible supply of latent NOX allows the possibility of an enzyme being mobilized by the application of external stimuli.

2.2 Methods

2.2.1 TNF-α/fMLP stimulation of HaCaTs

TNFα in cojunction with fMLP was used in an attempt to stimulate an oxidative burst in HaCaT cells.

HaCaT cells grown in RPMI media were seeded into tissue-culture-treated 96-well plates at a concentration of 1 x 10^4 cells per well and grown overnight at 37ºC to permit cell attachment and spreading. Media totalling a volume of 250 µl was added to each well for the duration of this period. The following morning overnight media was aspirated off cells which were rinsed with 200 µl HBSS which was then discarded.

Cells were stressed with 150 µl solution per well for 45 minutes, primed with 135 µl of 10 ng/ml TNF (the batch of TNF used had already been pre-aliquotted in RPMI media in the lab and thus it was not possible to eliminate all traces of media from the solution. Subsequent dilutions were prepared in HBSS however) mixed with 10 % (v/v) WST-1. After that point 50 µl of FMLP was added, diluting down the FMLP to a final concentration of 10^{-6} M. Absorbance readings (450 nm) were taken every 15 minutes.

2.2.2 Comparison of media vs TNF-α as a stress mediator

HaCaT cells grown in RPMI media were seeded into tissue-culture-treated 96-well plates at a concentration of 1 x 10^4 cells per well and grown overnight at 37ºC to permit cell attachment and spreading. Media totalling a volume of 250 µl was added to each well for the duration of this period. The following morning overnight media was aspirated off cells which were rinsed with 200 µl HBSS which was then discarded.

Stressor solutions were made up as in the previous experiment, to a final volume of 150 µl per well. TNF (aliquotted in RPMI media) was diluted 1:10 in HBSS in the experimental treatment whilst in the controls RPMI media was diluted 1:10 (v/v) in HBSS ("diluted media") or mixed straight with WST-1 without any traces of RPMI present in the well ("control"). Readings were taken every 15 minutes.

2.2.3 Cell Penetration studies

Primary human airway epithelial cells (NHBE) cells (Lonza, UK) were grown at 37ºC in a 5 % CO_2 atmosphere in BEGM® cell culture media, supplemented with BulletKit® growth supplements, as recommended by the supplier. Cells were grown in ventilated T-75 culture flasks (Corning, UK), sub-cultured upon reaching 80 % confluence, and seeded into tissue-culture-treated 12-well plates when required for experimental purposes.

NHBE cells were grown to confluence, growth media was aspirated off cells, which were rinsed with 1 ml of HBSS (Promocell, Germany) which was subsequently discarded. BUDAS and Pulmicort® were diluted to a final budesonide concentration of 0.5 µg/ml in BEGM media and 1 ml added to cells for either 1 or 24 hours, after which time drug-containing medium was aspirated off cells. Cells were rinsed with 1.5 ml HBSS (subsequently discarded) and then lysed by the addition of 1ml ddH_20 with incubation at 4ºC overnight. Any remaining cells attached to the bottom of the culture flask were removed with a cell scraper and the cell lysate soution was extracted using solid phase extraction techniques. Samples were then analysed using HPLC by Dr Dinesh Nadarassan at the Institute of Pharmaceutical Innovation, Bradford.

2.2.4 Cell Retention Studies

NHBE cells were grown as previously described. Cells were seeded into tissue-culture-treated 12 well-plates and grown to confluence. All growth media was then aspirated off cells which were rinsed with 1 ml of HBSS which was subsequently discarded. BUDAS and Pulmicort® were diluted to a final budesonide concentration of 0.5 µg/ml and 1 ml added to cells for a period of 24 hours. After this time cells were either prepared for analysis, or washed, and in the latter case a media change was performed with 1 ml fresh BEGM for a further 23 hours incubation at 37ºC. When ready for analysis, growth media was aspirated off cells, which were rinsed with HBSS. 1 ml of

ddH_20 was then used to lyse cells by incubation at 4ºC overnight. Any remaining cells attached to the bottom of the culture flask were removed with a cell scraper and the cell lysate soution was extracted using solid phase extraction techniques. Samples were then analysed using HPLC by Dr Dinesh Nadarassan at the Institute for Pharmaceutical Innovation, Bradford.

2.2.5 Measuring background absorbance of MTT/WST-1 in the absence of cells

The background turnover of MTT (Sigma-Aldrich, UK) and WST-1 (Roche, France) in media solutions was determined by diluting 5 mg/ml MTT (aliquotted in PBS, sterile filtered) or neat WST-1, 1:10 into either HBSS (Promocell, Germany) or RPMI 1640 (Lonza, UK) medium, the latter of which was pre-supplemented with HEPES and L-Glutamine and was further supplemented with 10 % (v/v) foetal calf serum (Promocell, Germany), 100 U/ml penicillin-streptomycin (Sigma, UK) and 1 µg/ml amphotericin B (Sigma, UK) (fully supplemented RPMI),

200 µl of each solution was loaded into 96-well plates and the resulting absorbance (450 nm for WST-1 or 595 nm for MTT) read at ten minute intervals on a MRX microplate reader (Dynex Technologies, UK).

2.2.6 Identifying the media component responsible for ROS production

HaCaTs were maintained in fully supplemented RPMI 1640 medium, supplemented as described previously. Cultures were incubated at 37ºC with 5 % CO_2 in a humidified atmosphere, and grown to 80 % confluence in T-75 tissue culture flasks, with sub-culturing taking place three times per week.

When required for experimental purposes, HaCaTs were seeded into tissue-culture-treated 96 well plates (Corning, UK) at a density of 1 x 10^4 cells per well and incubated overnight in a 100 µl volume of fully supplemented RPMI to permit cell attachment and spreading. The following morning overnight media was aspirated off cells, which were rinsed with 200 µl HBSS which was subsequently discarded.

Media solutions were prepared by diluting 200 µl of either fully supplemented or unsupplemented RPMI or 20 µl foetal calf serum into a final volume of 2 ml HBSS. HBSS was used as a negative control. 20 µl of all these solutions was then discarded and replaced with 20 µl of WST-1 per treatment. 200 µl of each media solution containing WST-1 was then added to each well and the resulting absorbance read at 450 nm on a MRX microplate reader at hourly intervals.

2.2.7 SOD inclusion during cell stressing

HaCaT cells were cultured as previously described until required for experimental use, upon which they were seeded into tissue-culture-treated 96-well plates at a concentration of 2 x 10^4 cells per well and grown overnight at 37ºC in a 100 µl volume of fully supplemented RPMI to permit cell attachment and spreading.

The following morning overnight media was aspirated off cells which were rinsed with 200 µl HBSS which was then discarded. Media solutions were prepared by diluting fully supplemented RPMI 1:10 in HBSS, alternatively a solution comprised of HBSS was used as a negative control. WST-1 (aliquotted neat) was added to each solution to a final concentration of 10 % (v/v) and 150 µl was added per well. Absorbance measurements were taken at 450 nm every 30 minutes for a total of four hours.

2.2.8 Attenuation of OB after pre-treatment with corticosteroids

HaCaT cells grown in RPMI media were seeded into tissue-culture-treated 96-well plates at a concentration of 1 x 10^4 cells per well and grown overnight at 37ºC to permit cell attachment and spreading. Media totalling a volume of 100 µl was added to each well for the duration of this period. The following morning overnight media was aspirated off cells which were rinsed with 200 µl HBSS which was then discarded.

50 µl of BUDAS was diluted into a final volume of 5 ml fully supplemented RPMI to yield a working concentration that contained 5 µg/ml budesonside. 200 µl of the resulting solution was then loaded into wells (200 µl RPMI added into control wells). Pulmicort®, at an equivalent budesonide concentration, was used as control. Cells were grown in the presence of the BUDAS for 24 hours at 37ºC, after which time the BUDAS solution was aspirated off cells, which were rinsed with 250 µl HBSS that was subsequently discarded.

Cells were then stressed with 10 % fully supplemented RPMI medium diluted in HBSS (10 % WST-1) (as in the positive control in which cells were exposed to the same solution but had not been pre-incubated with either BUDAS or Pulmicort® previously) with a total volume of 150 µl per well. A negative control (HBSS and WST-1) was made up to indicate the background level of absorbance on the HaCaT cells. Absorbance readings (450 nm) were taken at an end point of 2 hours.

2.2.9 Stressing Cells in the presence of corticosteroids

HaCaT cells grown in RPMI media were seeded into tissue-culture-treated 96-well plates at a concentration of 2 x 10^4 cells per well and grown overnight at 37ºC to permit cell attachment and spreading. Media totalling a volume of 100 µl was added to each well for the duration of this period. The following

morning overnight media was aspirated off cells, which were rinsed with HBSS (wash solution subsequently discarded).

BUDAS was diluted into 10 % RPMI (diluted in HBSS) and WST-1 was added to a final concentration of 10 % (v/v). The final concentration of budesonide in this BUDAS solution was 5 µg/ml, with 200 µl loaded per well. Pulmicort® was prepared in the same manner and used for comparison at an identical concentration. Positive controls of 10 % RPMI containing WST-1 and negative controls of WST-1 diluted in HBSS were also prepared. Cells were stressed for six hours, with absorbance readings recorded every hour (A450 nm).

2.3 Results

2.3.1 TNF-α/fMLP stimulation of HaCaTs

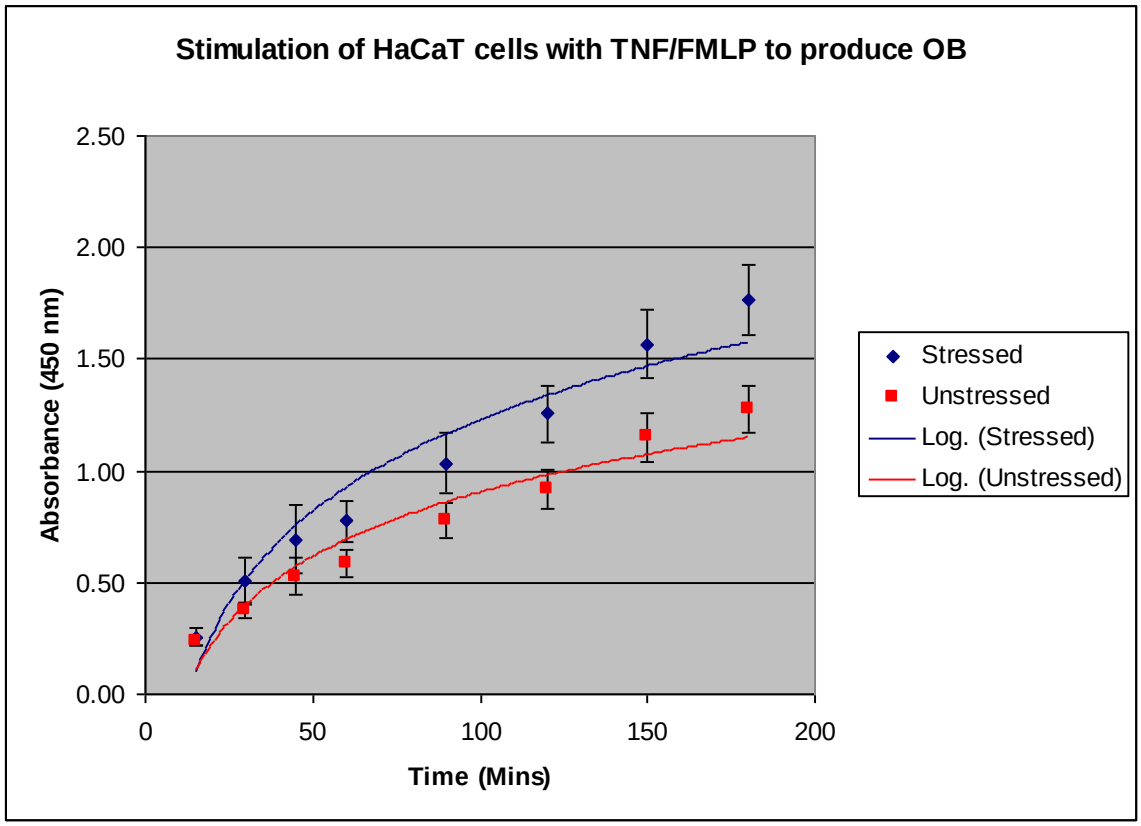

Figure 26 – stimulation of HaCaTs with stressors (in media.) Error bars represent standard deviation.

The result appeared to indicate successful stressing had been produced by the TNF however there remained the possibility that it was the media component in which the TNF was diluted that was causing stressing. As suggested by the manufacturer of the TNF, the initial batch had been diluted into RPMI media and it was not possible to extract the TNF out of this media

to remove all traces of media from the experiment. As a result, a subsequent experiment attempted to stimulate an inflammatory response simply by incubating cells with RPMI diluted down in HBSS to the same concentration as in this experiment (10 % v/v) to ascertain whether such low levels of media would stimulate HaCaTs in the absence of any TNF. Readings from such a treatment could then be compared against cells exposed to TNF.

2.3.2 Comparison of media vs TNF-α as a stress mediator

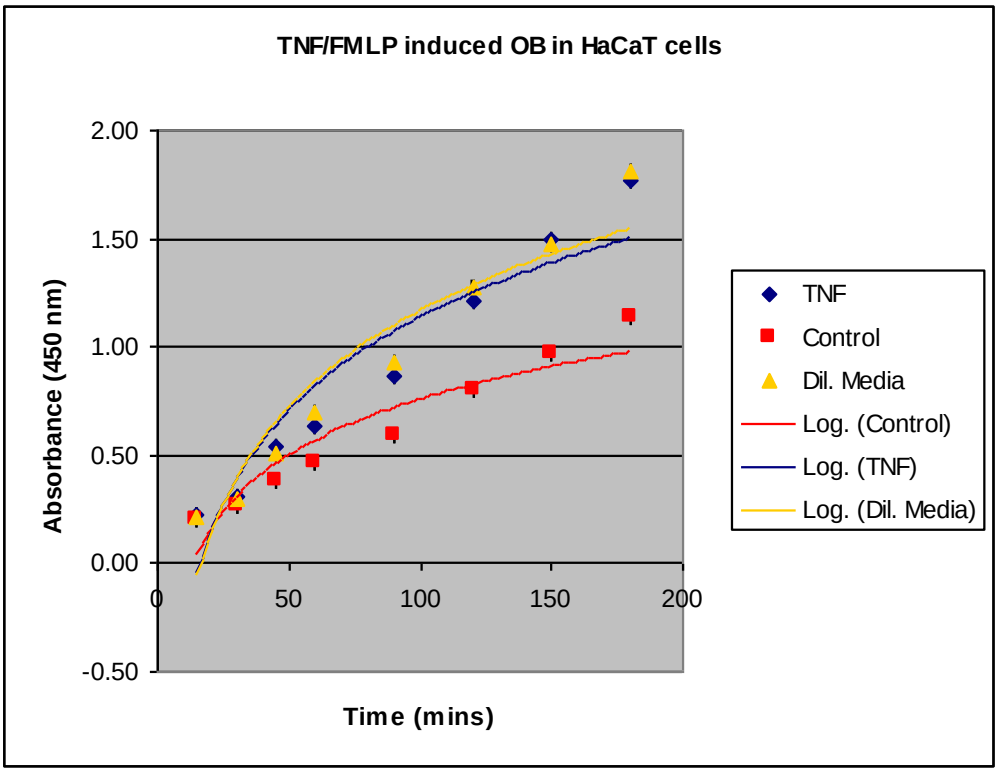

Figure 27 – illustrates that if RPMI media is diluted to 10 % in HBSS that the same level of 'stress' is produced as in cells exposed to TNF. Both sets of absorbances are almost identical and both are significantly higher than that of the negative control (cells exposed to WST-1 diluted into HBSS). Error bars represent standard deviation.

RPMI media diluted 1:10 in HBSS produced almost identical readings to that of the TNF suggesting that it was the media itself and not the TNF producing the apparent inflammatory response relative to the negative control. Given that the unstressed cells in the negative control yielded a much lower baseline absorbance it was now possible to establish two benchmarks for fully stressed and unstressed cells even though it was RPMI media and not a combination of TNF and FMLP that was producing the higher benchmark, as originally hoped. Nevertheless, it was now possible to pre-treat HaCaT cells with BUDAS to look for potential anti-inflammatory effects on those cells.

2.3.3 Cell penetration studies

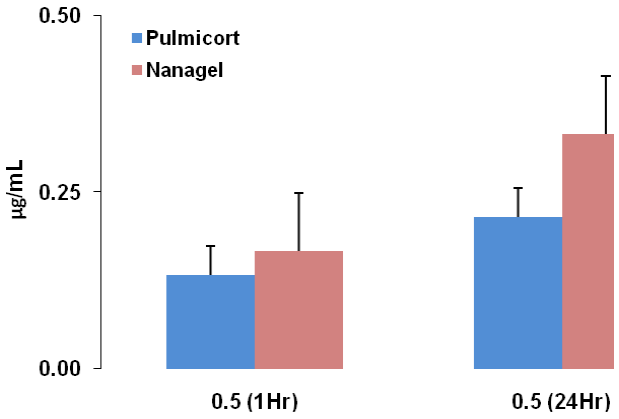

Fig. 27 Concentration of budesonside in human airway epithelial cell lysate after either 1 or 24 hours exposure to 0.5 µg/ml BUDAS or Pulmicort. Assayed by HPLC analysis of the cell lysate. Errors bars represent standard deviation, n=6

The concentration of budesonide in NHBE cells after exposure to BUDAS or Pulmicort® at an equivalent concentration is similar after 1 hour, but greater in cells exposed to BUDAS after 24 hours.

2.3.4 Cell Retention Studies

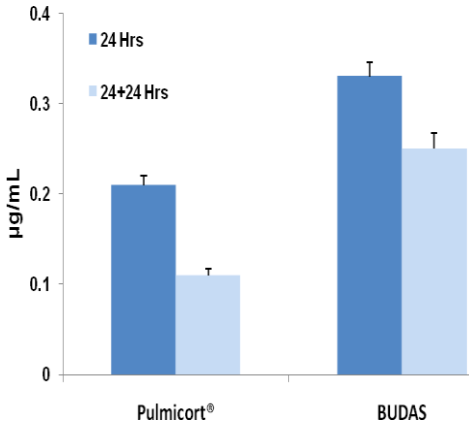

Fig. 28 Concentration of budesonside in human airway epithelial cell lysate after either 24 or 48 hours incubation. In the latter treatment, cells were subjected to a media change after 24 hours. Assayed by HPLC analysis of the cell lysate. Error bars represent standard deviation. N=6

A greater proportion of internalized budesonide is retained in NHBE cells after exposure to BUDAS than with Pulmicort® at an equivalent dose (Figure 28).

2.3.5 Measuring background absorbance of MTT/WST-1 in the absence of cells

When MTT or WST-1 were diluted into HBSS the resulting levels of background absorbance were lower than when the reagents had been diluted into RPMI cell culture media (Figure 29). Furthermore, the magnitude of absorbance change at the end of the sampling period was also reduced when WST-1 dilutions were performed with HBSS. In both cases, however, background turnover remained at acceptably low levels.

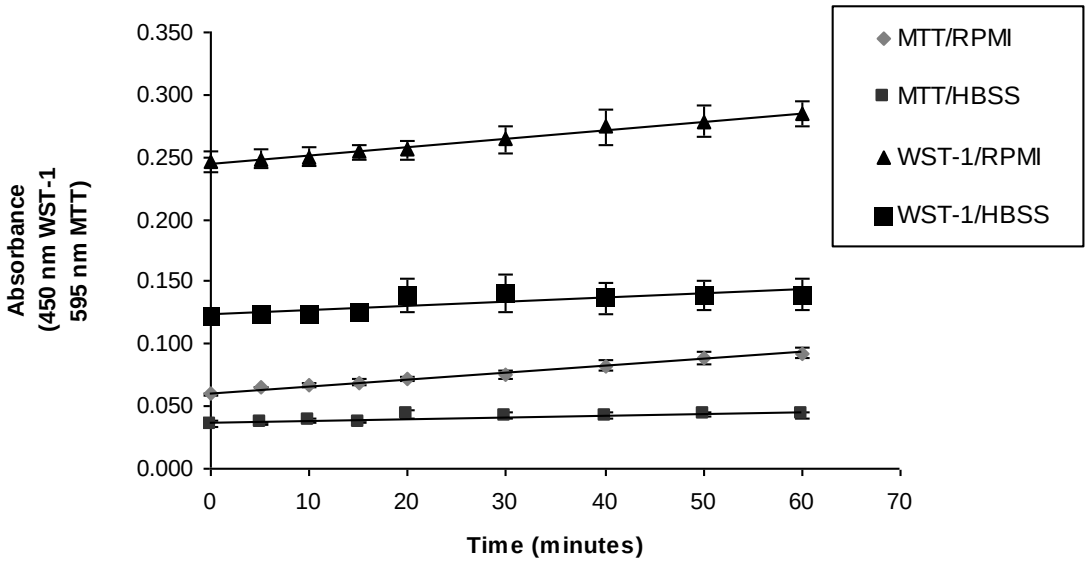

Fig. 29. Changes in background absorbance of WST-1 (450 nm) and MTT (595 nm) over time after dilution into HBSS or RPMI 1640 cell culture medium. Error bars represent standard deviation (n = 3).

2.3.6 Identifying the media component responsible for ROS production

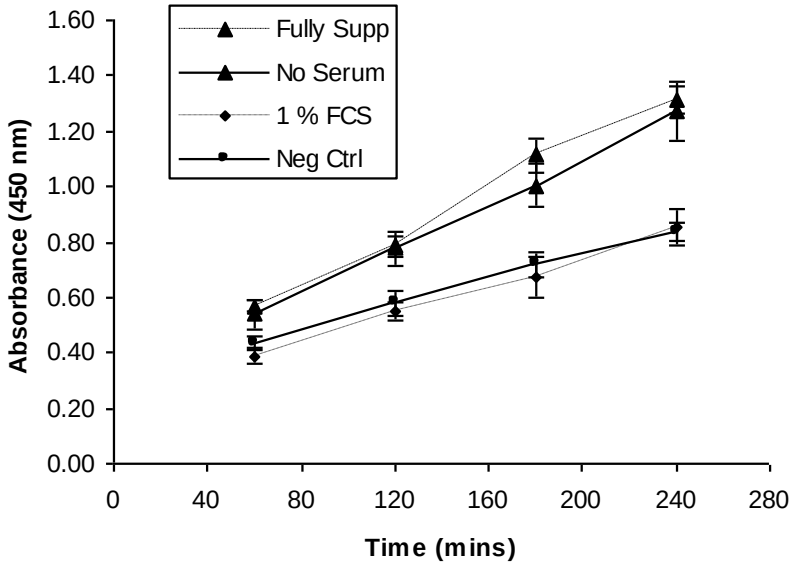

Fig. 30. Turnover of WST-1 in HaCaT cells exposed to one of four media solutions; 1) fully supplemented RPMI 1640 cell culture medium diluted 10 % in HBSS, 2) unsupplemented RPMI 1640 cell culture medium diluted 10 % in HBSS, 3) FCS diluted to 1 % in HBSS or 4) HBSS. All dilutions were v/v. Error bars represent standard deviation (n=6).

The levels of absorbance observed in the negative control represent that of background turnover of WST-1 diluted in HBSS and of the three solutions tested 1 % FCS diluted in HBSS produced readings most similar to the negative control. This suggests that, at a final concentration of 1 % (v/v), FCS does not induce ROS production in HaCaT cells (Figure 30).

In contrast, the level of absorbance observed when cells are exposed to fully supplemented RPMI cell culture media (diluted in HBSS) represent that of the positive control – fully 'stressed' cells in which ROS production had been stimulated. That RPMI medium lacking serum produced a pattern of absorbance most similar to the positive control for the duration of the experiment suggests a component of the RPMI and not FCS is responsible for inducing the inflammatory response in HaCaT cells.

2.3.7 SOD inclusion during cell stressing

The inclusion of superoxide dismutase (SOD) in experimental solutions drastically reduced the turnover of the 10 % RPMI stress solution (a 59 % reduction compared to when SOD was omitted) (Figure 31). SOD inclusion reduced absorbance levels nearer to the baseline level of the negative control (HBSS –SOD) which represented the levels of absorbance in unstressed cells.

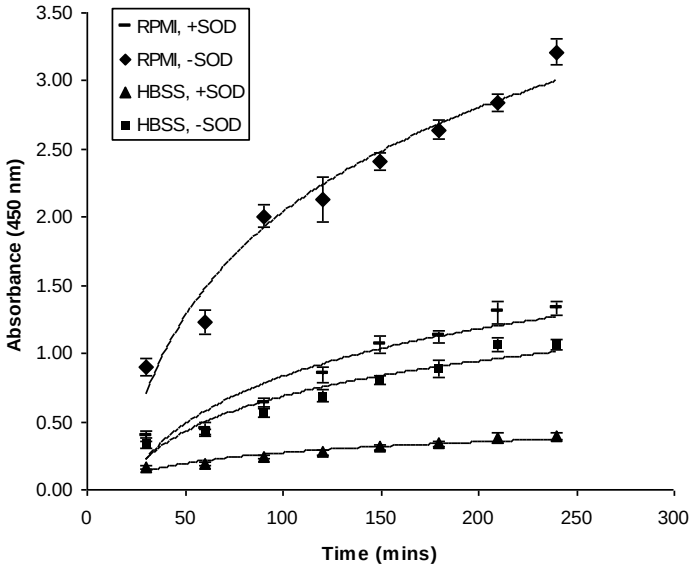

Fig. 31. Turnover of WST-1 in HaCaT cells exposed to either a 'stress' solution of 10 % (v/v) RPMI 1640 diluted into HBSS or a negative control solution of HBSS, both either in the presence or absence of the anti-oxidant, superoxide dismutase. Error bars represent standard deviation (n=6).

2.3.8 Attenuation of OB after pre-treatment with corticosteroids

Pre-treatment of HaCaT cells with BUDAS (which contained 5 µg/ml budesonide) significantly lowered the level of stressing compared to the positive control of fully stressed cells (Figure 32). Unpaired student's t-test results show there to be a significant difference between BUDAS and the positive control ($P < 0.001$) but not between Pulmicort® and the positive control ($P > 0.05$) suggesting Pulmicort® failed to inhibit ROS production to the same extent as BUDAS.

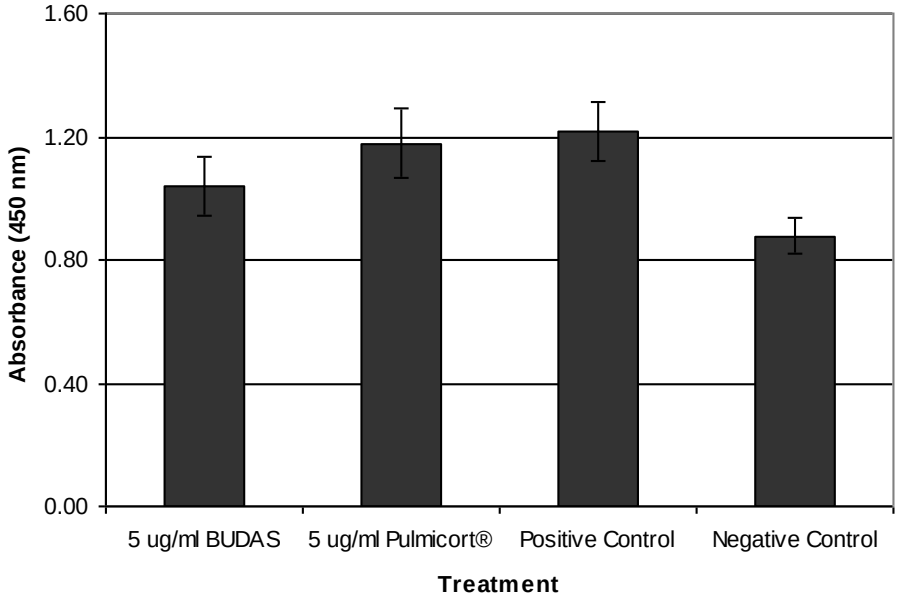

Fig. 32. Pre-incubation of HaCaT cells with either a) BUDAS or b) Pulmicort® over a 24 hour period prior to stressing. Error bars represent standard deviation (n =12).

2.3.9 Stressing Cells in the presence of corticosteroids

If there is no-treatment period with corticosteroids, and cells are instead exposed to budesonide whilst being stressed, suppression of the inflammatory response is immediately noted (Figure 33) with both BUDAS and Pulmicort®. However, with both formulations, the ability to suppress the inflammatory response decreases over time, to the point where, at six hours, there is no apparent therapeutic benefit. At time intervals of 1,2,3,4 or 5 hours all observed readings for BUDAS or Pulmicort are significantly lower than in the positive control ($P < 0.001$ in all cases) but at the end point of 6 hours, no significant difference was observed ($P > 0.05$).

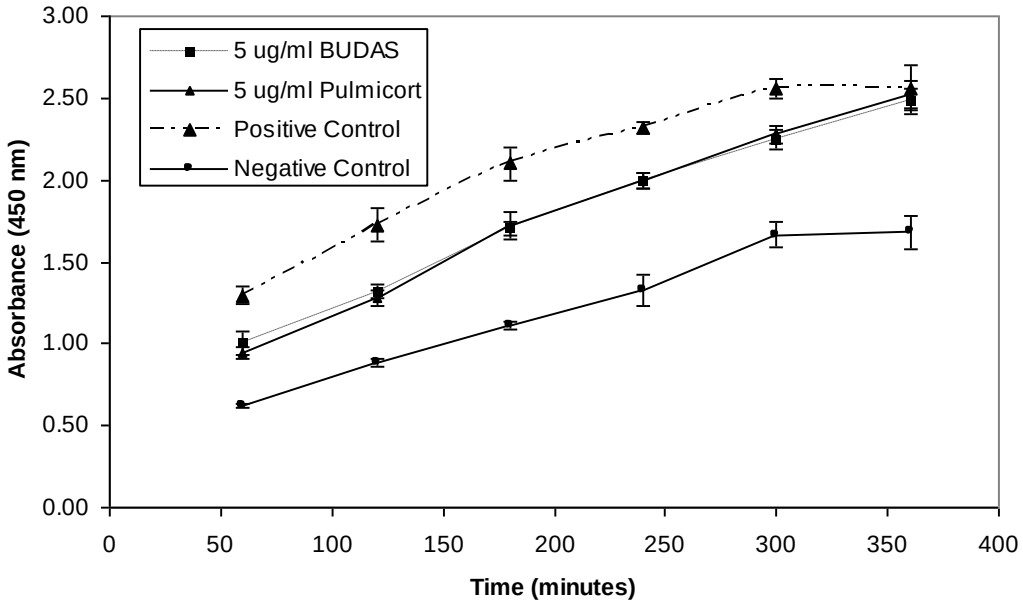

Fig. 33. HaCaTs exposed to BUDAS or Pulmicort® during stressing. Error bars represent standard deviation (n=6).

3. Discussion

Cross-linked PVA nanoparticles for DNA delivery

GFP detection was selected as a means of determining transfection efficiency as it is a widely used reporter of gene expression and provides a reliable indicator of transfection as the protein is only expressed upon successful internalization into the cell and if all subsequent barriers to gene expression are overcome.

Results in this project confirmed the findings of Felgner *et al.* (1987) – that naked DNA is unable to produce transfection unless complexed with a carrier or physically delivered into the cytoplasm.

Stocks of GFP plasmid were purified and tested for efficacy by transfecting them with a commercial transfection reagent, Lipofectin®, known to produce successful transfection in a variety of cell lines and even primary cells *in vitro*. Once culture conditions were optimized for Lipofectin® transfections, liposomal based transfection was used as a positive control technique all throughout the project to verify the efficacy of the plasmid stock and also the suitability of the culture conditions during each transfection.

As results confirmed the efficacy of the plasmid, experiments moved onto looking at hydrogel nanoparticles as DNA carriers. KP-08 nanoparticles were evaluated via a mobility shift assay to test whether DNA binding was occurring. As in studies by Akinc *et al.* (2005), Chen *et al.* (2007) and Pack *et al.* (1999), DNA had a restricted mobility on the agarose gel when it had been mixed with KP-08 prior to electrophoresis. As the amount of KP-08 (in terms of µg) relative to DNA was increased, DNA movement across the gel decreased to the point where there was no movement across the gel and all DNA remained bound to the hydrogel (hydrogel:DNA ratio of 189:1), forming a distinct band visible in the well at the top of the lane. These results suggested that DNA was successfully forming polyplexes with KP-08 nanoparticles.

The following experiment mixed DNA with KP-08 nanoparticles with varying concentrations of the crosslinker, PD2000, as nanoparticle charge is positively related to PD2000 concentration. These results suggested a minimum PD2000 concentration of 0.03 % was necessary in order to bind all DNA in a given sample when KP-08 and DNA were mixed at a ratio around 200:1. This experiment also demonstrated the stability of the KP-08/DNA polyplexes in Opti-MEM cell culture media, a pre-requisite if the polyplexes were to be incubated with cells during transfection studies.

During transfection, NIH 3T3 cells were shown to uptake KP-08 polyplexes, however, the overall rate of transfection remained negligible with only 5 GFP-

expressing cells counted on a coverslip that contained several hundred thousand cells. Furthermore, this result was not replicable under identical experiment conditions, thus questioning the validity of the result.

Kimura *et al.* (2004) showed that the hydrophobicity of PVA nanoparticles can influence whether they are uptaken by cells or not, however, even when POVAL nanoparticles (minimal level of hydropobicity) were used in transfection studies during this project (identical methodologies followed to that described for KP-08) no GFP expression was noted. This was despite the fact that POVAL nanoparticles, like KP-08 nanoparticles, were shown to effectively bind DNA during electrophoresis studies.

It was postulated that the lack of GFP expression may have been a consequence of inefficient endosomal escape of the polyplexes if they had been uptaken by cells. For that reason cells were transfected in the presence of chloroquine, which had been shown to increase the transfection efficiencies of PVA nanoparticles by Wittmar *et al.* (2005), incidentally, who also reported a lack of endosomal escape in the absence of the drug. However, even following clhoroquine addition, NIH 3T3 cells displayed no evidence of GFP expression after exposure to KP-08 or POVAL nanoparticles.

One of the most successful polymers used in transfection research to date is PEI. It has been suggested that its success (again demonstrated as an

efficient transfector during this project) stems from the fact it is only partially protonated at physiological pH. Once internalizated into cells, PEI nanoparticles are localized in the endosome, in which the environment is more acidic (Wagner, 1998), and this leads to protonation as protons and chloride ions are pumped into the cell, providing a mechanism for endosomal escape due to osmotic swelling and subsequent rupture (Akinc *et al.* 2005). With this in mind we sought to examine the stability of KP-08 nanoparticles when placed into a more acidic environment, such as in the endosome.

At pH 5.15 (similar to that of the endosome) DNA appeared to be released from the KP-08. However, when the experiment was repeated by making up fresh solutions of Opti-MEM and forming new complexes, the result was not reproducible. As a result it is not possible to determine from these results whether DNA release would occur inside or outside of the endosome in successfully internalized cells.

There was every possibility that the lack of transfection observed during the project may have been because cells had simply not uptaken nanoparticles in the first instance, and that transfection was being prevented at the first hurdle. To investigate this possibility, stocks of pDNA were labelled with a fluorescent-rhodamine tag and examined for following transfection, as in Kaul & Amiji (2005). In contrast to the Lipofectin® control in which rhodamine-labelled plasmid was conspicuous and widespread in cells following

transfection, cells exposed to KP-08 nanoparticles displayed little evidence of internalized labeled-plasmid.

However, when KP-08-bound pDNA was subsequently mixed with Lipofectin®, to form chimeric complexes, both rhodamine-labelled plasmid and GFP expression was noted in cells following transfection. There remains the possibility that the internalized plasmid may not have been bound to KP-08 and Lipofectin® simultaneously, and may have instead formed Lipofectin®-DNA complexes that transfected cells as in a positive control treatment. However, electrophoresis results suggested that all DNA presented to the Lipofectin® would have been bound to the KP-08 as polyplexes were formed in line with previous hydrogel:DNA ratios. Although particle sizing analysis did not clarify the issue – with several species sized in the sample – electroporation was undertaken to address the same question – if polyplexes are delivered into cells does GFP expression take place?

When HaCaT cells were electroporated with an electroporation buffer containing KP-08/DNA polyplexes, GFP expression was subsequently noted. Again, it is only assumed that the DNA had remained bound to the KP-08 at the point at which the cell membrane was electropermeabilised. However, as the total number of GFP-expressing cells counted was reduced two fold when cells were electroporated in the presence of the KP-08 it would appear that the KP-08 hindered, but did not totally prevent, subsequent gene expression once KP-08 nanoparticles were located inside the cell.

Particle sizing analysis of the hydrogel nanoparticles revealed that they are of a small enough size to be endocytosed. Particle sizing measured KP-08/pDNA polyplexes at 120 nm, whilst authors such as Kaul & Amiji (2005) and Zhao *et al.* (2006), amongst others, have reported particle sizes in excess of this yet still reported successful transfection. Conductimetric titration results also suggest that the particles have a net positive charge and thus charge repulsion with the negatively charged cell membrane seems an unlikely explanation for the lack of cell uptake.

It has been reported by Gao *et al.* (2007) that cell type affects transfection efficiency and for that reason transfection studies have been undertaken on four cell types – HaCaT and NIH 3T3 cell lines and primary chondrocyte and human airway epitelial cells – however, KP-08 has never transfected any of those cell types with high efficiency and even in the one instance in which GFP-expression was noted the result was not reproducible. In contrast, the Lipofectin® positive control has repeatedly produced transfection (detected by GFP expression) despite modification of experimental parameters such as target cell type, starting cell density, duration of transfection period, duration of time cells grown for prior to analysis etc.

Collectively, the results gathered throughout the duration of the project suggest that the barrier to transfection with cross-linked hydrogel nanoparticles is inefficient cell uptake. The nanoparticles used for

transfection appear of a size small enough to be endocytosed by cells and complex formation with pDNA appeared to be occurring. Nevertheless, the target cells at present seem to lack a stimulus to uptake the formed polyplexes, preventing transfection from taking place. However, although they failed to produce transfection after exposure to cells during nebulisation, the fact the hydrogel nanoparticles were nebulisable and non-toxic to cells opened up the possibility an alternative application in the field of drug (namely budesonide) delivery and the same nanoparticles were used for *in vitro* investigations.

Cross-linked PVA nanoparticles for drug delivery

Measurement of the respiratory burst is a useful *in vitro* tool for studies of respiratory disease as it enables workers to screen possible antioxidants that may have potential use as anti-inflammatory agents.

Previous studies have demonstrated using chemiluminescence (Braga *et al.* 2005 and Dal Sasso, 2005), that budesonide can yield lower chemiluminescence readings in neutrophils, after exposure to fMLP or PMA. Budesonide was functional at concentrations of either 5 or 10 µg/ml either in isolation or when used in conjunction with erdosteine (Dal Sasso, 2005). Inclusion of the glucocorticoid receptor antagonist mifepristone in respiratory burst experiments on neutrophils has inhibited the effect of budesonide on

luminol-amplified chemiluminescence, suggesting budesonide reacts with glucocorticoid receptors to exert an antioxidant activity (Braga *et al.,* 2005).

As in previous studies (Tan & Berridge, 2000) these results have demonstrated the use of WST-1 for measuring antioxidant activity. Neutrophils have been widely used for research into this area as they release large amounts of toxic molecules, such as reactive nitrogen and reactive oxygen species (Folkerts *et al.* 2001) that can be stimulated *in vitro* by exposing cells to fMLP (Braga *et al*, 2005, Dal Sasso *et al.* 2005, Pruett & Loftis, 1990) or PMA (Braga *et al*, 2005, Black *et al*, 1994, Elferink 1984, Tan & Berridge, 2000, Theron 1994). Here the present an alternative means of stimulating a SOD-inhibitable respiratory burst, using standard cell culture media (RPMI 1640) on the HaCaT cell line. Results suggest it is a component of the RPMI media itself, and not the serum supplement, that is responsible for producing the witnessed oxidative burst. Serum has been shown to increase NADPH-oxidase activity of stimulated neutrophils by preventing premature, oxidative inactivation of cellular energy metabolism (Theron *et al.* 1994) but given that HaCaTs can circumvent this problem by consuming glutamine (Benavente & Jacobsen, 2008), which inadvertently leads to the accumulation of NADPH oxidase and subsequent ROS production, serum starvation did not prevent an OB from being produced in our experiments.

Importantly, HPLC analysis shows our BUDAS formulation delivers more budesonide to NHBE cells than Pulmicort® at an equivalent concentration of 5

µg/ml and that a greater proportion of the delivered drug is retained in the cell. This is potentially a useful clinical finding as it means it may be possible to reduce the number of budesonide inhalations, improving patient compliance.

When ROS production is stimulated in HaCaTs in the presence of budesonide, both BUDAS and Pulmicort demonstrate virtually identical readings in lowering the turnover of MTT suggesting that in the immediate hours of budesonide exposure the levels of active drug available in HaCaT cells is similar for both formulations. Cellular uptake results indicated the internalization of BUDAS and Pulmicort® was similar after 1 hour, but after 24 hours, a marked difference was observable. However, studies incorporating a 24-hour pre-treatment period of budesonide prior to OB-stimulation reveal that BUDAS production at a concentration of 5 µg/ml is effective at inhibiting ROS production whereas Pulmicort® is not.

Given the budesonide can be present in cells in both functional and esterified forms (Brattsand & Miller Larsson, 2003), the enhanced drug retention and prolonged duration of action of BUDAS over Pulmicort® that was evident in respiratory burst studies is most likely a consequence of increased long-term storage of esterified-budesonide in the cells exposed to BUDAS. When the intracellular concentration of budesonide decreases, budesonide esters are hydrolysed back into their active state (Brattsand & Miller-Larsson, 2003). This appears to confer the benefits of increased drug retention and a

prolonged duration of action with BUDAS, and may also reduce the risk of systemic effects in future clinical trials.

These findings highlight the potential value in using cross-linked PVA-based Nanagel® nanoparticles to improve budesonide dissolution and delivery for the treatment of asthma and COPD.

4. References

Akinc, A., Thomas, M., Klibanov, A.M. & Langer, R. (2005) Exploring polyethyleneimine-mediated DNA transfection and the proton sponge hypothesis. *The Journal of Gene Medicine*. **7**: 657-663

Allen, D.B., Bielory, L., Derendorf, H., Dluhy, R., Colice, G.L. & Szefler, S.J. (2003) Inhaled corticosteroids: Past lessons and future issues. *The Journal of Allergy and Clinical Immunology*. **112**: S1-40

Barnes, P.J. (2006) Corticosteroids: The drugs to beat. *European Journal of Pharmacology*. **533**: 2-14

Benavente, C.A. & Jacobson, E.L. (2008) Niacin restriction upregulates NADPH oxidase and reactive oxygen species (ROS) in human keratinocytes. *Free Radical Biology and Medicine*. **44**: 527-537

Bertschinger, M., Backliwal, G., Schertenleib, A., Jordan, M., Hacker, D.L. & Wurm, F.L. (2006) Dissasembly of polyethyleneimine-DNA particles in vitro: implications for polyethyleneimine-mediated DNA delivery. *Journal of Controlled Release*. **116**: 96-104

Biopharm International (2004) The Genesis of Gendicine: The Story Behind the First Gene Therapy. *New Brunswick Scientific*. Accessed from http://www.nbsc.com/files/papers/BP5-08-04Ae.pdf on 22/11/06

Björquist, P., Palmer, M. & Ek, B (1994) Measurement of superoxide anion production using maximal rate of cytochrome (III) C reduction in

phorbol ester stimulated neutrophils, immobilized to microtiter plates. *Biochem Pharmacol*. **48**: 1967-72

Black, C.D., Cook, J.A., Russo, A. & Samuni, A. (1991) Superoxide production by stimulated neutrophils: temperature effect. *Free Radic Res Commun*. **12-13**: 27-37

Braga, P.C., Dal Sasso, M., Culici, M., Bianchi, T. & Guffanti, E.E. (2005) Budesonide reduces superoxide and peroxynitrate anion chemiluminescence during human neutrophil bursts. *Pharmacology*. **75**: 179-86

Brattsand, R. & Miller-Larsson, A. (2003) The role of intracellular esterification in budesonide once-daily dosing and airway selectivity. *Clinical Theraputics*. **25**: C28-C41

Burdon, R.H., Gill, V. & Rice-Evans, C. (1993) Reduction of a tetrazolium salt and superoxide generation in human tumor cells (HeLa). *Free Radic Res Commun*. **18**: 369-80

Chen , J., Tian, B., Yin, X., Zhang, Y., Hu, D., Hu, Z., Liu, M., Pan, Y., Zhao, J., Li, H., Hou, C., Wang, J. & Zhang, Y. (2007) Preparation, characterization and transfection efficiency of PEGylated PLA nanoparticles as gene delivery systems. *Journal of Biotechnology*. **130**(2): 107-113

Dal Sasso, M., Culici, M., Guffani, E.E., Bianchi, T., Fonti, E. & Braga, P.C. (2005) A combination of budesonide and the SH-metabolite I or erdosteine acts synergistically in reducing chemiluminescence during human neutrophil respiratory burst. *Pharmacology*. **74**: 127-34

Dunlap, D.D., Maggi, A., Soria, M.R. & Monaco, L. (1997) Nanoscopic structure of DNA condensed for gene delivery. *Nucleic Acids Research*. **25**(15): 3095-101

Elferink, J.G. (1984) Measurement of the metabolic burst in human neutrophils: a comparison between cytochrome c and NBT reduction. *Res Commum Chem Pathol Pharmacol*. **43**: 339-42

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. & Danielsen, M. (1987) Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *PNAS*. **84**: 7413-7417

Feng, M., Lee, D. & Li, P. (2006) Intracellular uptake and release of poly (ethyleneimine)-*co*-poly(methyl methacrylate) nanoparticle/pDNA complexes for gene delivery. *International Journal of Pharmaceutics*. **311**: 209-214

Folkerts, G., Kloek, J., Muijsers, R.B.R. & Nijkamp, F.P. (2001): Reactive nitrogen and oxygen species in airway inflammation. *European Journal of Pharmacology*. **429**: 251-262

Gao, X. & Huang, L. (1990) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Comm*. **179**: 280-385. *In*: Lee, T.W.R., Matthews, D.A. & Blair, G.E. (2005) Novel molecular approaches to cystic fibrosis gene therapy. *The Biochemical Journal*. **387**: 1-15

Gao, X., Kim, K-S. & Liu, D. (2007) Nonviral Gene Delivery: What we Know and What Is Next. *The AAPS Journal*. **9**(1): E92-E104

Gardlik, R., Pálffy, R., Hodosy, J., Lukács, Turňa, J, & Celec, P (2005) Vectors and delivery systems in gene therapy. *Med Sci Monit*. **11**(4): RA110-121

Godbey, W.T., Wu, K.K. & Mikos, A.G. (1999) Tracking the intracellular path of poly(ethyleneimine)/DNA complexes for gene delivery. *PNAS*. **96**: 5177-5181

Guy, J., Drabek, D. & Antoniou, M. (1995) Delivery of DNA into mammalian cells by receptor-mediated endocytosis and gene therapy. *Mol. Biotechnol*. **3**(3): 237-48

Harrison, R.L. Bryne, B.J. & Tung, L. (1998) Electroporation-mediated gene transfer in cardiac tissue. *FEBS Letters*. 435: 1-5

Heiser, W.C. (1994) Gene transfer into mammalian cells by particle bombardment. *Analytical Biochemistry*. **217**(2): 185-96

Hofland, H.E.J., Shephard, L. & Sullivan, S.M. (1996) Formation of stable cationic lipid/DNA complexes for gene transfer. *PNAS*. 93: 7305-7309

Holtorf, H.L. & Mikos, A.G. (2002) *Cationic and non-condensing polymer based gene delivery*. *In* Mahato, R.I & Kim, S.W. (eds). Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics. CRC Press, pp382

Huynh, P.N., Scott, L.G., & Kwong, K.Y.C. (2010) Long-term maintenance of pediatric asthma: focus on budesonide/formoterol inhalation aerosol. *Therapeutics and Clinical Risk Management*. **6**: 65-75

Jacobs, C. & Müller, R.H. (2002) Production and characterization of a Budesonide Nanosuspension for Pulmonary Administration. *Pharmaceutical Research*. **19**: 189-194

Jang, J.S., Kim, S.Y., Lee, S.B., Kim, K.O., Han, J.S. & Lee, Y.M. (2006) Poly(ethylene glycol)/poly(ε-caprolactone) diblock copolymeric nanoparticles for non-viral gene delivery: The role of charge group and molecular weight in particle formation, cytotoxicity and transfection. *Journal of Controlled Release*. **113**: 173-182.

Journal of Gene Medicine (2007) Accessed from http://www.wiley.co.uk/genmed/clinical/ on 3/3/07

Kamata, H., *et al.* (1994) Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection. *Nucleic Acids Research.* 22: 536-537.

Kamau, S. W., Schulze, K., Steitz, B., Petri-Fink, A., Hassa, P.O., Hottiger, M.O., Hofmann, H., Hofmann, M., von Rechenberg, B. (2005) Superparamagnetic iron oxide nanoparticles (SPIONs) as non-viral vectors for gene delivery *in vitro*. *European Cells and Materials*. **10**: supp 5: pp 10

Kaul, G. & Amiji, M. (2005) Cellular Interactions and *In Vitro* DNA Transfection Studies with Poly(ethylene glycol)-Modified Gelatin Nanoparticles. *Journal of Pharmaceutical Sciences*. **94**(1):184-198

Kimura, T., Okuno, A., Miyazaki, K., Furuzono, T., Ohya, Y., Ouchi, T., Mutsuo, S., Yoshizawa, H., Kitamura, Y., Fujisato, T. & Kishida, A. (2004) Novel PVA-DNA nanoparticles prepared by ultra high pressure technology for gene delivery. *Materials Science and Engineering C*. **24**(6-8): 797-801

Kimura, T., Iwai, S., Moritan, T., Nam, K., Mutsuo, S., Yoshizawa, H., Okada, M., Furuzono, T., Fujisato, T. & Kishida, A. (2007) Preparation of poly(vinyl alcohol)/DNA hydrogels via hydrogen bonds formed on ultra-high pressurization and controlled release of DNA from the hydrogels for gene delivery. *Journal of Artificial Organs*. **10**(2): 104-8

Lee, T.W.R., Matthews, D.A. & Blair, G.E. (2005) Novel molecular approaches to cystic fibrosis gene therapy. *The Biochemical Journal*. **387**: 1-15

Leng, Q. & Mixson, A.J. (2005) Modified branched peptides with a histidine-rich tail enhance in vitro gene transfection. *Nucleic Acids Research*. 33(4): e40

Leong, K.W., Mao, H.-Q., Truong-Le, V.L., Roy, K., Walsh, S.M. & August, J.T. (1998). DNA-polycation nanospheres as non-viral gene delivery vehicles. *Journal of Controlled Release*. **53**: 183-193.

Leventis, R. & Silvius, J.R. (1990) Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta*. **1023**: 124-132. *In*: Lee, T.W.R., Matthews, D.A. & Blair, G.E. (2005) Novel molecular approaches to cystic fibrosis gene therapy. *The Biochemical Journal*. **387**: 1-15

Li, S. & Huang, L. (2000) Nonviral gene therapy: promises and challenges. *Gene Therapy*. **7**: 31-34

Li, Y., Ogris, M., Wagner, E., Pelisek, J. & Rüffer, M. (2003) Nanoparticles bearing polyethyleneglycol-coupled transferring as gene carriers:

preparation and in vitro evaluation. *International Journal of Pharmaceutics*. **259**(1-2): 93-101

Maitra, A. (2005) Calcium phosphate nanoparticles: second-generation nonviral vectors in gene therapy. *Expert Rev. Mol. Diagn*. **5**(6): 893-905

Mehier-Humbert, S. & Guy, R.H. (2005) Physical methods of gene transfer: Improving the kinetics of gene delivery into cells. *Advanced Drug Delivery Reviews*. **57**: 733-753.

Mozafari, M.R. & Omri, A. (2007) Importance of Divalent Cations in Nanolipoplex Gene Delivery. *Journal of Pharmaceutical Sciences*. **96**(8): 1955-66

Mumper, R.J., Duguid, J.G., Anwer, K., Barron, M.K., Nitta, H. & Rolland, A.P. (1996) Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. *Pharmaceutical Research*. **13**(5): 701-9.

Mumper, R.J., Wang, J., Klakamp, S.L., Nitta, H., Anwer, K., Tagliaferri, F. & Rolland, A.P. (1998) Protective interactive noncondensing (PINC) polymers for enhanced plasmid distribution and expression in rate skeletal muscle. *Journal of Controlled Release*. **52**(1) 191-203

National Institute for Clinical Excellence (2008) Inhaled corticosteroids for the treatment of chronic asthma in adults and in children aged 12 years and over.

Nimesh, S., Goyal, A., Pawar, V., Jayaraman, S., Kumar, P., Chandra, R., Singh, S. & Gupta, K.C. (2006) Polyethylenimine nanoparticles as

efficient transfecting agents for mammalian cells. *Journal of Controlled Release*. **110**: 457-468

Oster, C.G., Wittmar, M., Unger, F., Barbu-Todoran, L., Schaper, A.K. & Kissel, T. (2004) Design of amine-modified graft polyesters for effective gene delivery using DNA-loaded nanoparticles. *Pharmaceutical Research*. **21**(6): 927-931

Ourlin, J.C., Vilarem, M.J., Daujat, M., Harricane, M.C., Domergue, J., Joyeaux, H., Baulieux, J. & Maurel, P. (1997) Lipid-mediated transfection of normal adult human hepatocytes in primary culture. *Analytical Biochemistry*. **247**(1): 34-44

Pack, D.W., Putnam, D. & Langer, R. (1999) Design of Imidazole-Containing Endosomolytic Biopolymers for Gene Delivery. *Biotechnology and Bioengineering*. **67**(2): 217-223

Panyam, J., Zhou, W-Z., Prabha, S., Sahoo, S.K. & Labhasetwar, V. (2002) Rapid endo-lysosomal escape of poly(DL-lactide-*co*-glycolide) nanoparticles: implications for drug and gene delivery. *The FASEB journal*. **16**: 1217-1226

Pedroso de Lima, M.C., Simões, S., Pires, P., Faneca, H. & Düzgüneş, N. (2001) Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Advanced Drug Delivery Reviews*. **47**(2-3): 277-94

Perez, C., Sanchez, A., Putnam, D., Ting, D., Langer, R. & Alonso, M.J. (2001) Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new

carriers for the delivery of plasmid DNA. *Journal of Controlled Release*. **75**(1-2): 211-224

Petri-Fink, A., Chastellain, M., Juillerat-Jeanneret, L., Ferrari, A. & Hofmann, H. (2005) Development of functionalised superparamagnetic iron oxide nanoparticles for interaction with human cancer cells. *Biomaterials*. **26**(15): 2685-94.

Pick, E. & Mizel, D. (1981) Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods*. **46**: 211-26

Prabha, S. & Labhasetwar, V. (2004) Critical determinants in PLGA/PLA nanoparticle-mediated gene expression. *Pharmaceutical Research*. **21**(2): 354-364

Pruett, S.B. & Loftis, A.Y. (1990) Characteristics of MTT as an indicator of viability and respiratory burst activity of human neutrophils. *Int Arch Allergy Appl Immunol*. **92**: 189-92

Rädler, J.O., Koltover, I., Salditt, T. & Safinya, C.R. (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science*. 275: 810-814

Reese (2004) *Analysis of genes and genomes*. Wiley, Chichester, UK

Reschel, T., Koňák, C., Oupický, D., Seymour, L.W. & Ulbrich, K. (2002) Physical properties and in vitro transfection efficiency of gene delivery

vectors based on complexes of DNA with synthetic polycations. *Journal of Controlled Release*. **81**: 201-217

Shenoy, D.B. & Amiji, M.M. (2007) An overview of condensing and noncondensing polymeric systems for gene delivery. *In* Friedmann, T. & Rossi, J. (Eds.) *Gene transfer: delivery and expression of DNA and RNA* (pp 395-405). Cold Spring Harbor Laboratory Press: New York, USA

Sokolova, V.V., Radtke, I., Heumann, R. & Epple, M. (2006) Effective transfection of cells with multi-shell calcium phosphate-DNA nanoparticles. *Biomaterials.* **27**(16): 3147-3153

Song, W. & Lahiri, D.K. (1995) Efficient transfection of DNA by mixing cells in suspension with calcium phosphate. *Nucleic Acids Research*. **23**(17): 3609-11

Strand, S.P., Danielsen, S., Christensen, B.E. & Vårum, K.M. (2005) Influence of chitosan structure on the formation and stability of DNA-chitosan polyelectrolyte complexes. *Biomacromolecules*. **6**(6): 3357-66

Tan, A.S. & Berridge, M.V. (2000) Superoxide produce by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. *Journal of Immunological Methods*. **238**: 59-68

Theron, A.J., Steenkamp, K.J. & Anderson, R. (1994) NADPH-oxidase activity of stimulated neutrophils is markedly increased by serum. *Inflammation*. **18**: 459-67

Thorsson, L. & Geller, D. (2005) Factors guiding the choice of delivery device for inhaled corticosteroids in the long-term management of stable asthma and COPD: Focus on budesonide. *Respiratory Medicine.* **99**: 836-849

Tinsley-Bown, A.M., Fretwell, R., Dowsett, A.B., Davis, S.L. & Farrar, G.H. (2000) Formulation of poly (D,L-lactic-co-glycolic acid) microparticles for rapid plasmid DNA delivery. *Journal of Controlled Release*. **66**: 229-241

Tsong, T.Y. (1991) Electroporation of cell membranes. *Biophysical Journal*. **60**: 297-306

Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D.T. & Birnstiel, M.L. (1992) Coupling of adenovirus to transferring-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *PNAS*. 89: 6099-6103

Wagner, E. (1998) Effects of membrane-active agents in gene delivery. *Journal of Controlled Release*. **53**: 155-158

Wasungu, L. & Hoekstra, D. (2006) Cationic lipids, lipoplexes and intracellular delivery of genes. *Journal of Controlled Release*. **116**: 255-264

Weiss, R. (2005) Boy's Cancer Prompts FDA to Halt Gene Therapy. Washington Post 4/3/05 Accessed from http://www.washingtonpost.com/ac2/wp-dyn/A3926-2005Mar3?language=printer on 10/11/06

Wiethoff, C.M. & Midaugh, C.R. (2002) Barriers to nonviral gene delivery. *Journal of Pharmaceutical Sciences*. **92**(2): 203-217

Wilson, S.P. Liu, F., Wilson, R.E. & Housley, P.R. (1995) Optimization of calcium phosphate transfection for bovine chromaffin cells: relationship to calcium phosphate precipitation formation. *Analytical Biochemistry*. **226**(2): 212-220

Wilson, S.P. & Smith, L.A. (1997) Addition of glycerol during DNA exposure enhances calcium phosphate transfection. *Analytical Biochemistry*. **246**(1): 148-150

Wittmar, M., Ellis, J.S., Morell, F., Unger, F., Schumacher, J.C., Roberts, C.J., Tendler, S.J., Davies, M.C. & Kissel, T. (2005) Biophysical and transfection studies of amine-modified poly(vinyl alcohol) for gene delivery. *Bioconjugate Chemistry*. **16**(6): 1390-8

Wong, T.-K. & Neumann, E. (1982) Electric field mediated gene transfer. *Biochemical and Biophysical Research Communications*. **107**(2): 584-587

Wu, G.R. & Wu, C.H. (1988) Receptor-mediated Gene Delivery and Expression in vivo. *The Journal of Biological Chemistry*. **263**(29): 14621-14624

Xu, Y. & Szoka, F.C. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*. **35**: 5616-5623. *In* Bertschinger, M., Backliwal, G., Schertenleib, A., Jordan, M., Hacker, D.L. & Wurm, F.L. (2006) Dissasembly of polyethyleneimine-DNA particles in vitro: implications for polyethyleneimine-mediated DNA delivery. *Journal of Controlled Release*. **116**: 96-104

Zauner, W., Kichler, A, Schmidt, W., Sinski, A. & Wagner, E. (1996) Glycerol enhancement of ligand-polylysine/DNA transfection. *Biotechniques*. **20**(5): 905-13

Zhao, X., Yu, S.-B., Wu, F.-L., Mao, Z.-B. & Yu, C.-L.(2006) Transfection of primary chondrocytes using chitosan-pEGFP nanoparticles. *Journal of Controlled Release*. **12**: 223-228.