Investigating the use of oligonucleotides for the treatment of muscular dystrophy

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Abstract

Antisense oligonucleotide therapy is one of the most promising strategies for treatment of myotonic dystrophy type 1 (DM1), type 2 (DM2) and Duchene muscular dystrophy (DMD). These three diseases have nuclear retained mRNA, suitable for antisense therapy targeting. The delivery of oligonucleotides to their desired target has long been an obstacle in antisense therapy with a large number of delivery reagents or methods having adverse side effects.

Promising work published detailing the successful delivery of various chemically modified oligonucleotides (CMOs) naked, *via* gymnosis, led to us investigating a number of these CMOs: deoxyribonucleic acids (DNA), Peptide nucleic acids (PNAs), 2'OMethyl (2'OMe), and Phosphorodiamidate morpholino (PMO) oligonucleotides. In DM1 expanded CUG repeat (CUGexp) mRNAs aggregate in the nucleus forming "foci". Testing the CMOs effectiveness at disrupting nuclear foci in a cell based assay, using high content imaging to visualise the number, size and intensity of foci we initially discovered that PNA and 2'OMe, were seemingly taken up *via* gymnosis by DM1 cells, and removed nuclear foci at nano-molar concentrations.

However further experimentation using live cell imaging indicated that although all CMOs could enter the cell, in all disease models tested, *via* gymnosis, the CMOs could not penetrate the nuclear membrane. In depth analysis led us to identify an artefact of the *in-situ* process used to identify these foci, explaining earlier positive results. As the target mRNA is trapped within the nuclear compartment we investigated several transfection reagents for their ability to deliver 2'OMe oligonucleotides to the nucleus using live cell fluorescent imaging and a modified northern blot based method.

It was established that polyethylenimine could successfully deliver 2'OMe oligonucleotides to the cell, with a high abundance of the oligonucleotide residing within the nuclear compartment. It was observed that PEI degrades the expanded nuclear retained repeat in the DMPK transcript of a DM1 patient cell line alone, without the addition of an antisense agent, in a concentration dependent manner.

Abbreviations

(w/v)	weight per volume
2’F	2’deoxy-2’ -fluoro
2’OEt	2’O-Ethyl
2’OMe	2’-O-Methyl
2’MOE	2’-O-Methyoxyethyl
AIDS	Acquired immune deficiency syndrome
B10K	Branched 10 000 polyethylenimine
B25K	Branched 25 000 polyethylenimine
B2K	Branched 2 000 polyethylenimine
BIR	Baculovirus inhibitor of apoptosis protein repeat
BM	Bethlem myopathy
BMD	Becker muscular dystrophy
bp	base pair
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CMOs	Chemically modified oligonucleotides
CPP	Cell-penetrating peptides
CSPD	{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl phenyl phosphate
Da	Dalton
DAPC	Dystrophin associated protein complex
DEPC	Diethyl Pyrocarbonate
DIG	Digoxigenin
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DMD	Duchene muscular dystrophy
DMPK	Myotonic dystrophy protein kinase
DMSO	Dimethyl sulfoxide
DMT	Dimethoxytrityl
DNA	Deoxyribonucleic acid

DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
GAT	Gene augmentation therapy
HEX	Hexachlorofluorescein
HGP	Human Genome Project
HIV	Human immunodeficiency virus
Hr	Hour
HSA	Human serum albumin
IAP	Inhibitors of apoptosis proteins
IFN-α	Interferon alfa
L	Litre
L10K	Linear 10 000 polyethylenimine
L25K	Linear 25 000 polyethylenimine
L2K	Linear 2 000 polyethylenimine
L40K	Linear 40 000 polyethylenimine
L5K	Linear 5 000 polyethylenimine
LHON	Leber hereditary optic neuropathy
LNA	Locked nucleic acid
LSM	Laser scanning microscope
M	Molar
MBNL	Muscleblind-like proteins
MgCl2	Magnesium chloride
Min	Minute
mL	Millilitre
Mn	Molecular number
mRNA	Messenger ribonucleic acid
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
Mw	Molecular weight
NARP	Neurogenic muscle weakness, ataxia and retinitis pigmentosa
NIH	Natonal Institutes of Health
nNOS	Neuronal nitric oxide synthase

NO	Nitric oxide
NP	Phosphoramidate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDM	Proximal myotonic dystrophy
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly(L-Lysine)
PMO	Phosphorodiamidate morpholino oligomer
PNA	Peptide nucleic acid
PROMM	Proximal myotonic myopathy
PS	Phosphorothioate
RAC	Recombinant deoxyribonucleic acid advisory committee
RISC	Ribonucleic acid -induced silencing complex
RNA-FISH	Ribonucleic acid fluorescence *in-situ* hybridisation
RNAi	Ribonucleic acid interference
Rpm	Revolutions per minute
SDW	Sterile distilled water
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SSC	Saline-Sodium Citrate
ssRNA	Single-stranded RNA
TAE	Tris base, acetic acid and EDTA
TBE	Tris/Borate/EDTA
TFN-α	Tumor necrosis factor alpha
Tm	melting temperature
UTR	Untranslated region
XIAP	X-linked inhibitor of apoptosis protein
ZNF9	Zinc finger 9
µL	Microlitre

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Chapter One: Introduction

Antisense therapy

In 1953 Francis Crick claimed to have "discovered the secret of life". This announcement was made with regarded to his and James Watson's breakthrough in their efforts to elucidate the structure of deoxyribonucleic acid (DNA), (Watson J.D. and Crick, 1953). Undeniably the most fundamental breakthrough to the events that define life: the storage, transmission, and translation of genetic information. A very important part of this discovery described the interactions involved in the bonding of the DNA double helix, in which there are complementary base pairings which bond together though hydrogen bonding. The knowledge that cytosine will only pair with guanine and adenine with thymine has allowed the development of a highly specific therapeutic approach, now known as antisense therapy, to be derived.

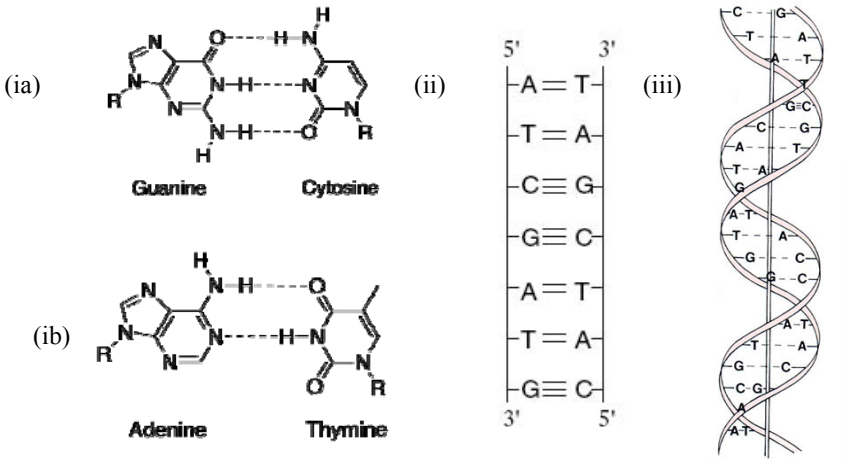


Figure 1-1: Schematic of the complementary base parings of (ia) guanine and cytosine and (ib) adenine and thymine. (ii) Schematic of base pairing between two short DNA segments. (iii) Alpha helix formation of DNA.

Since this discovery numerous advances have been made specifically in an effort to treat various diseases specifically based on their genetic cause, (Uhlmann, 1990, Bennett, 2010). The field has drawn a large degree of attention in part due to the significant advantages of antisense DNA based drugs over current pharmaceuticals, principally their potential to selectively recognise desired molecular targets and pathways, which imparts tremendous specificity of action. It is hoped that this specificity of action will reduce the number of negative side effects arising from treatment. However there are still numerous factors to be determined about the cellular uptake, distribution, toxicology and metabolism of DNA based drugs. Critical to the development of DNA based therapeutics is an understanding of the different mechanisms behind the causation of various genetic diseases. Identifying the underlying basis of genetic diseases has allowed different antisense based strategies to be hypothesised in order to meet the treatment requirements of a specific disease, (Bunnell, 1998).

The basis of genetic disease

Several important discoveries spanning centuries have powered the precise understanding of the molecular genetic basis of many human disorders: Mendel's law of heredity, the discovery of the chemical basis of inheritance, the explanation of the structure and function of DNA and the development of *in vitro* methods for DNA manipulation (Castle, 1903, Fleischman, 1970, Matveeva, 1998). There are a number of distinct types of genetic disease, categorised by their mechanistic cause, these include: chromosomal, single-gene, mitochondrial, somatic mutations and polygenic disorders.

Chromosomal disorders

Chromosomal disorders arise from either alterations in chromosome number or structure. Numerical aberrations are defined as any abnormality in chromosome number, and can be further divided into two categories: polyploidy, where the chromosome number is a multiple of the haploid number but exceeds the diploid number, and aneuploidy, where the chromosome number is not an exact multiple of the haploid number. A common syndrome caused by aneuploidy is Down syndrome, in which there are 3 copies of chromosome 21, (Lejeune, 1959). Three babies affected by the disease are born on average every day in the UK, making it one of the most common chromosome abnormalities in humans, (Malt, 2013). Structural aberrations which arise from chromosome breakage result in either DNA deletion or translocation, (Moore, 2001).

Single-gene disorders

The outcome of the Human Genome Project (HGP) has provided one of the most important tools in the successful identification of genes involved in single gene disorders, (Collins, 2001). Once specific genes are identified, progress can be made to characterise the mechanism of the causative mutation(s) for each disorder. Single-gene disorder inheritance can be classified as autosomal or X linked, (Chial, 2008), in the former the mutated gene is found on chromosomes 1-22, in the latter the mutated gene is found on the X or Y chromosome. Inheritance is also classified as either dominant, where the mutation of a single copy of the gene results in disease, or recessive, where both copies of the gene must carry the mutation for the disease to manifest. Diseases caused by the mutation of a single gene can manifest in a number of ways, these include: point mutations, deletions and insertions. Point mutations occur when a single nucleotide base is replaced with a different nucleotide base,

this can result in amino acid changes if the mutation occurs within the coding region, premature stop codons if the mutation results in the formation of a TGA sequence, and splice site mutations which occur in the splice site sequences adjacent to exon and introns. Deletions occur when fragments are deleted out of either the genomic DNA or mRNA sequences; these fragments can range in size from a single nucleotide base to an entire gene. Proteins produced from genes with this type of mutation are either absent or of a ~~of reduced~~reduced ~~in~~ size. The final type of single-gene mutations are insertions, ~~and as with deletions~~the mutation ~~there~~ can ~~range~~ involve the incorporation of a ~~from singles nucleotide~~single nucleotide base~~s~~ up to the insertion of many thousands of base ~~pairs~~s. In the cases of both insertions and deletions, even the smallest of mutations can cause in a shift in the reading frame of the mRNA, resulting in an altered protein which may not be functional (Hu, 2012).

Mitochondrial disorders

Mitochondria are organelles found in high numbers in all human cells, with the exception of red blood cells. They are responsible for creating a large amount of the energy required by the body to sustain life. The human mitochondrial genome consists of a single, circular double-stranded DNA molecule, containing 37 genes (Taanman, 1999). Due to its small size the mitochondrial genome is a small target for mutation, however the proportion of clinical diseases due to mutations in its genome is not as low as statistically expected. The bulk of the mitochondrial genome is composed of coding sequence, and as a result mitochondrial mutations are a significant contributor to human disease. Inherited mitochondrial DNA mutations have been implicated in deafness, (Guan, 2006), Leber hereditary optic neuropathy

(LHON), Leigh's syndrome and neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP), (McKenzie, 2004).

Somatic mutation disorders

Due to the complicated nature of cell division mutations can occur sporadically, the effect of these mutations depends, in part, on when in the cell cycle they occur. If a mutation is present in a zygote, then the mutation will be passed to all daughter cells, however if the mutation arises after the first division then this mutation will only be found in a proportion of cells. Individuals whose cells do not all contain the same genetic make-up are termed mosaic. The type of cells in which sporadic mutation occur play a key role in inheritance. If mutations occur in gonadal cells then there is a chance that the mutation will be passed on to successive generations, whereas if the mutation occurs only in somatic cells it cannot be inherited. Most individuals carry somatic mutations without an obvious phenotype. The somatic mutations which give rise to the most problems are those in genes linked to the cause of cancer, (Forbes, 2010), although there are non-cancerous disorders caused by somatic mutations such as Neurofibromatosis 1 and 2 and Paroxysmal nocturnal hemoglobinuria, (Erickson, 2003).

Polygenic disorders

These disorders, rather than being caused by the action of a single gene, are caused by the interaction of many genes as well as by environmental factors. Accordingly a person's genetic material does not automatically result in disease, instead makes the individual more susceptible to disease contraction , (Strachan, 2014). Identifying the genes responsible for an increase in susceptibility to disease has become feasible due to the increased efficiency of genetic mapping. One of the most challenging tasks for researchers is in estimating the

relative contributions of the environment *vs.* genetic background in causing each disorder. Often only once a significant genetic contribution to disease causation has been determined is it worth trying to identify the genes involved in causing the disease. Twin and adoption studies have historically played a major role in distinguishing the contribution of environment *vs.* genetic background for numerous disorders. Yet to be elucidated to, the identification of the genes involved in common disorders such as diabetes and obesity (Cauchi, 2008 and Scuteri, 2007) are of enormous interest to a wide range of groups, from pharmaceutical industries to ethicists.

Treatment strategies

As previously mentioned many genetic diseases are multifactorial, i.e. they are caused by mutations in several genes compounded by environmental factors. Due to the fact that the molecular basis of various diseases can differ widely, some gene therapy strategies are particularly suited to certain types of disorders ahead of others. As treatment *via* antisense therapy requires a specific genetic target by nature, only certain genetic diseases have the potential to be improved by an antisense approach. The type of treatment required heavily depends on the type of disorder; treatment strategies can be divided into four well recognised categories: targeted inhibition of gene expression, targeted killing of specific cells, gene augmentation therapy (GAT) and targeted mutation correction, all of which are detailed below.

Targeted inhibition of gene expression

Infectious diseases, cancer and inherited disorders caused by inappropriate gene activity can theoretically all be treated with targeted inhibition of gene expression. If the diseased cells

display inappropriate gene expression or produce a destructive product, then the targeted inhibition of that particular genes expression is a suitable strategy for treatment. This strategy involves introducing an agent which can interact with the faulty gene and either upregulate or downregulate gene expression or interfere with the activity of the destructive product. Using this type of treatment it is also theoretically possible to achieve allele specific inhibition in order to treat the dominant mutated gene without inhibiting the functional wild-type gene. This type of treatment strategy, to date, is used in a wide range of diseases.

Targeted killing of specific cells

There are currently numerous approaches being investigated in order to target specific apoptotic regulators in certain cell types including: organic small molecules, oligonucleotide therapy and agonistic antibodies in order to activate key receptors, (Fischer, 2005). As the major form of cell suicide, apoptosis is central to various physiological processes and therefore has been identified as a key target in various diseases, including: cancer, autoimmune disease, heart failure, stroke, AIDS and neurodegenerative disease. Several animal studies have provided proof of principle evidence to demonstrate the therapeutic potential of apoptotic drugs, employing gene therapy and antisense strategies *via* the inhibition of apoptosis proteins. There are currently known to be eight endogenous inhibitors of apoptosis proteins (IAPs) in humans which are characterised by a conserved sequence region known as the baculovirus inhibitor of apoptosis protein repeat (BIR) domain. Certain IAPs have been found to be overexpressed in several types of cancer cells, including Survivin and X-linked inhibitor of apoptosis protein (XIAP), and are currently being exploited in preclinical trials by Isis Pharmaceuticals (Carlsbad, CA)/Eli Lilly (Indianapolis, IN) and Aegera (Montreal, Quebec, Canada) using antisense approaches.

Gene augmentation therapy (GAT)

This type of therapy is appropriate for the treatment of inherited disorders caused by the loss of a functional gene product. By introducing extra copies of the normal gene back into the genome, increased expression levels of the introduced gene may be able to restore a normal phenotype. This type of therapy is targeted at clinical disorders where the pathogenic effects of the disease are reversible. GAT is significantly more successful in autosomal recessive disorders, where even modest expression levels of an introduced gene may make a substantial difference, (Ray, 2002). Autosomal dominant disorders are less amenable to this type of treatment as far higher expression levels of the introduced gene are required to reverse the disease phenotype, such protein levels can be hard to achieve. Gain of function mutations are not correctable by this strategy.

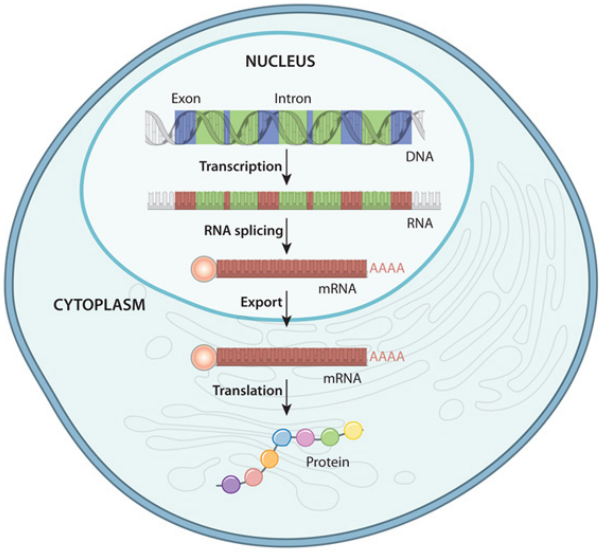
Targeted mutation correction

Significant progress has been made in the past decade towards viable therapies which correct various types of mutation. Genetic mutations can be largely categorised into four major groups: splicing, missense, nonsense, and frameshift. Splicing mutations all cause an underlying disruption in a sequence essential for the optimal site for splicing of pre mRNA. Although splicing mutations can vary, the result is an aberrant splice site which can lead to the translation of an abnormal protein. Nonsense mutations result in truncated proteins *via* the switch of a single base resulting in the introduction of a premature stop code. Missense mutations are also typically single base changes which either alter the amino acid sequence of the protein, or not, depending on the nature of the base change. Although similar to nonsense

mutations mechanistically, in practice they can be very hard to define as they often alter just one amino acid, sometimes causing issues in protein folding and lack of activity, (Needham, 2006). Finally frameshift mutations result from the insertion or deletion of one or more bases which "shifts" the reading frame, disrupting translation of the sequence downstream of the mutation, (Cartegni, 2002).

Each different mutation mechanism requires a slightly different optimised treatment strategy based on its inherent causation. Splicing abnormalities can be corrected using antisense oligonucleotides~~, (~~, (Douglas, 2011); these sequence specific molecules target pre-mRNA and redirect the cells splicing machinery away from the mutation and to a more suitable splice site *via* steric blocking. Small drug molecules such as aminoglycosides could potentially combat nonsense mutations; these typically act by inducing a local conformational change at the site of the mutation thereby allowing translation to continue through the premature stop codon, resulting in a fully functional protein, (Hu, 2009). Both frameshift and missense mutations result in a variety of effects, including proteins with folding defects and gain of function proteins. Both small molecules and antisense oligonucleotides could be employed to correct either protein activity or reduce toxic gain of function proteins. Another exciting potential strategy is the use of chimeric oligonucleotides, which contain a single base mismatch at the site of the mutation. These act by inducing the cellular mismatch repair machinery, which reverses the missense mutation. This technology has been shown to be effective in a Duchene muscular dystrophy mouse model, correcting both nonsense and frameshift mutations, (Cirak, 2011).

A large number of human diseases have been demonstrated to manifest as a direct result of malfunctioning proteins, either by the way of incorrect protein production, inappropriate levels of protein production or the misfolding of the produced protein, (Dobson, 2003). The majority of current conventional drug molecules typically are designed to interact with protein complexes, modulating their function. In contrast antisense agents act at the mRNA level, before protein translation, a schematic of transcription and translation can be seen below in Scheme 1-1.



Scheme 1-1: An overview of the flow of information from DNA to protein in a eukaryote cell, *Nature Education, 2010.* ~~Schematic of the environment and process of transcription and translation~~

As antisense drugs act earlier in the cellular information pathway, they are thought to have a number of advantages over conventional drug molecules. Firstly they have the ability to act

prophylactically, or at very early stages of disease development, it is thought they can prevent diseases as well as treat them. Antisense drugs are also designed to only affect specific genes *via* complementary based pairings, therefore theoretically, adverse side effects from off target binding should be minimised compared to traditional pharmaceuticals, which typically have less specificity.

In 1978 Zamecnik and Stephenson proposed the potential for antisense therapeutics when they discovered the possibility of inhibiting viral replication using short sequences of DNA to act as antisense agents in cell culture, (Zamecnik, 1978). Since this discovery a vast number of studies encompassing a wide range of different types of antisense agents have taken place, including studies involving plasmids, aptamers, ribozymes, small interfering RNAs (siRNAs) and oligonucleotides.

Therapeutic agents

Since the birth of antisense therapy in 1978 the understanding of critical parameters such as efficiency, cellular uptake, circulation and kinetic stability has been at the forefront of researcher's objectives. Various classes of DNA based therapeutics have emerged as promising curative agents, and although this project principally involved work with oligonucleotides, the design and mode of action of other prominent antisense agents is summarised below.

Plasmids

Identified in 1952, (Lederberg, 1952), plasmids are high molecular weight, double-stranded DNA constructs that can replicate independently of chromosomal DNA. Naturally found in bacteria, archaea and eukaryotes, their use in gene therapy was built upon the discoveries and

developments of restriction enzymes, DNA ligase and gel electrophoresis. They have been likened to traditional prodrug molecules due to their ability to utilise the DNA transcription and translation apparatus within the cell to synthesise specific proteins for which they encode. In this way they can be used to restore the levels of functioning protein, relieving the symptoms which arise from low quantities. Due to their inherent stability, ease to work with and diverse set of applications, plasmids have become an essential tool in molecular biology with a significant number of examples of plasmid drugs in clinical trials, although a large number of these are for vaccine therapy, (Xu, 2010).

Aptamers

Aptamers are comprised of short single-stranded nucleic acid segments, which fold to form a well-defined three dimensional structure which can directly interact with small molecules, proteins, nucleic acids, and even cells, tissues and organisms. It has been demonstrated that their molecular recognition properties rival that of antibodies, making them strong drug candidates due to their specificity of binding as well as in diagnostic and target validation applications, (Charlton, 1997). Clinical trials have demonstrated aptamers' use in a variety of diseases including the treatment of cancer, (Ma, 2015), and most prominently in the treatment of age-related macular degeneration, (Rask-Andersen, 2014), there have also been promising results for the treatment of cardiovasuclar disease, (Yamagishi, 2014 and Ngo, 2014). However this technology does have considerable disadvantages, primarily when compared to other anti-sense agents, the identification of an aptamer is a highly labour-intensive process. This consequently leads to the other technologies being employed for both therapeutic and diagnostic applications ahead of aptamer based molecules due to cost, competition and time scale implications.

Ribozymes

First described by Cech ~~*et al*~~*et al*. ribozymes are RNA enzymes which catalytically cleave target RNA, (Cech, 1981). Through sequence specific interactions they are able to bind and cleave suitable targets in order to supress the required genes. The main advantage over other therapeutic agents is their ability to act both catalytically and independently, therefore not relying upon the host cell's biological machinery for activation, (Doudna, 2005). Of particular interest to this study was the use of ribozymes in the treatment of myotonic dystrophy, explained in detail in section 1.3. Within the study it was demonstrated that using a *trans*-splicing ribozyme it is feasible to address expanded repeat mutations, experimentation established a reduction in the number of $(CAG)_n$ repeats from 12 to ~~five~~5, at the 3′ end of the *DMPK* transcript in mammalian cells, (Phylactou, 1998). In spite of these advantages the presence of the RNA backbone in ribozymes potentially makes them unsuitable molecules for therapeutic use *in vivo*, as they are rapidly degraded by ubiquitous RNases, (Wang, 1996). DNA analogues have also been identified, known as DNAzymes, which confer a larger degree of biological stability, (Akhta, 2000). Both DNAzymes and ribozymes have been investigated in Phase I and II clinical trials for cancer, (Schubert, 2004).

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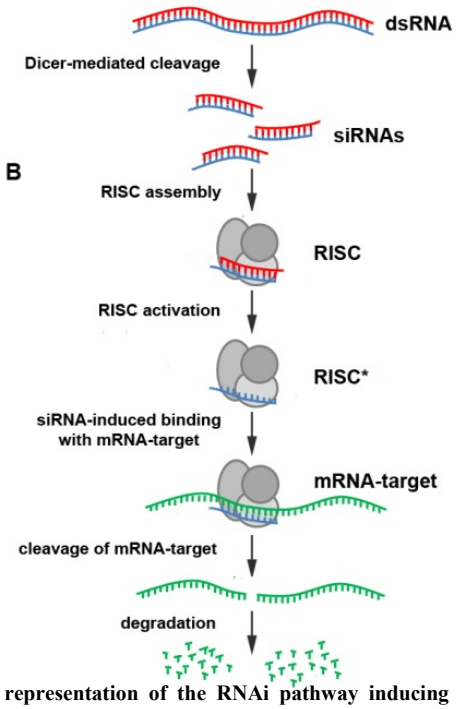
Small interfering RNAs

As recent additions to the array of antisense therapeutic tools available, small interfering RNAs (siRNAs) are typically defined as short (20 to 24 bp) double-stranded RNA molecules with a phosphorylated 5' end and a hydroxylated 3' end, often with two overhanging nucleotides. Although siRNA can play many roles, their main function is *via* a post-transcriptional gene silencing process called RNA interference (RNAi) pathway, Scheme 1-2.

Fire ~~et al~~*et al*. first described this natural phenomenon in a nematode worm model, (Fire, 1998); here they found these siRNA molecules were capable of post-transcriptional gene silencing. These therapeutic agents function in nature after processing *via* the ribonuclease protein Dicer, which processes double stranded RNA into siRNAs *via* specific cleavage. From here the siRNA molecules are incorporated into the Argonaute protein complex (Ago) which selects the guide strand and creates an active RNA-induced silencing complex (RISC). The activated RISC recognises the target mRNA *via* base pair interactions with the complexed single stranded RNA, stimulating mRNA degradation mechanisms, such as nuclease activity, resulting in a significantly decreased level of protein translation. The use of siRNA as a therapeutic agent is still in its infancy when compared to other antisense approaches, however they do offer several advantages over plasmid DNA molecules and oligonucleotides. It is thought that as they have been shown to specifically target mRNA and also have a high resistance to nucleases, siRNAs could perform more efficiently and with low cytotoxic effects. Promising results have been reported against various cancer cells, (Shi, 2014) and in combating viral diseases, (Dong, 2014), both *in vitro* and *in vivo*. Synthetic siRNA are however more expensive to produce than oligonucleotides due to being double stranded and concerns have been raised with reference to off target effects of the sense strand which is not incorporated into the RISC. It has been demonstrated that the sense strand is not required for RISC activation and that single-stranded RNAs (ssRNA) can reduce targeted mRNA level *via* and RNAi pathway. Unfortunately, undoubtedly due to their lack of stability, the effectiveness of ssRNAs were much lower when compared with their double-stranded counterparts, (Xu, 2004).



Scheme 1-2: Schematic representation of the RNAi pathway inducing mRNA cleavage using double stranded RNA (dsRNA) or single hairpin RNA (shRNA). ***Natalya S. Petrova, Marina A. Zenkova and Elena L. Chernolovskaya (2013). Structure - Functions Relations in Small Interfering RNAs, Practical Applications in Biomedical Engineering, Dr. Adriano Andrade (Ed.), ISBN: 978-953-51-0924-2, InTech, DOI: 10.5772/53945.***

Oligonucleotides

Oligonucleotides are found in nature in many organisms, including: plants, animals, and some viruses inherently, functioning in the regulation of gene expression, (Lagos-Quintana, 2001). The value of oligonucleotides has long been recognised, being used in a high number of experimental techniques including DNA microarrays, Southern blots, allele specific

oligonucleotide analysis and fluorescent *in-situ* hybridization, (Hyyro, 2005, Rychlik, 1989). Therapeutically oligonucleotides are indispensable elements for antisense applications, they are primarily utilised in targeted mutation correction where upon cellular internalisation they interact and form a duplex with complementary mRNA or pre-mRNA, (Stein C. A., 1993).

Main features

In the most basic terms oligonucleotides are short sequences of DNA or RNA. Oligonucleotide design is critical for their efficiency, with a number of factors requiring consideration, notably: length, secondary conformation, hybridisation capacity and chemical modification. The optimal oligonucleotide length for therapeutic use is considered to be between 17-28 bases, any longer than this can impair cellular uptake and chemical synthesis, any shorter results in a statistical risk of behaving non-specifically, (Patil, 2005). The formation of secondary structures is dependent on the oligonucleotide sequence, therefore care must be taken when choosing an RNA target to ensure that the complementary oligonucleotide does not form deactivating hyperstructures. It has been shown that activity and cellular uptake can be reduced when oligonucleotides form secondary structures, (Vickers, 2000). Rooted in the nature of antisense therapy is the need for an oligonucleotide to hybridise to its RNA target, in order for this to occur efficiently the secondary and tertiary spatial structure of the mRNA target needs to be considered to ensure all bases are accessible for oligonucleotide binding. Chemical modifications of oligonucleotides are often introduced to increase hybridisation, uptake and to minimise the susceptibility to degradation, (Englisch, 1991). These modifications will be discussed in further detail later in this chapter.

Chemical synthesis

Due to the multi-step nature of oligonucleotide synthesis the majority are synthesised by exploiting solid state chemistry techniques, almost exclusively using specialised automated equipment. Solid state chemistry is highly advantageous for oligonucleotide synthesis for numerous reasons: the process is very amenable to automation, can complete a large number of reactions quickly by using large excesses of liquid phase reagents and allows for simple washing and purification steps. Oligonucleotides are widely used in *in vivo* and *in vitro* studies; a practice which requires a high degree of sequence specificity. Each reaction step during the synthesis must therefore proceed in a high yield with a low level of side reactions. Oligonucleotides are synthesised using natural or chemically modified nucleotides. Although various methods have been established since the 1950s, the phosphoramidite method founded in the 1980s, (Beaucage, 1981), has since been the technique used by the majority of DNA synthesisers. This has led to an enormous increase in commercially available phosphoramidite reagents containing numerous chemical modifications. Unlike DNA replication in nature, phosphoramidite oligonucleotide synthesis proceeds in the 3'-5' direction with one nucleotide base added per synthetic cycle. The synthetic cycle can be broken down into a series of repetitive steps, outlined in more detail on page 20, Scheme 1-3, (ATDbio and Albericio, 2000).

Start: Oligonucleotides are synthesised on solid phase cartridges, which can be purchased in various sizes relative to the yield of oligonucleotide required. The first nucleotide is pre-loaded onto the cartridge and is protected at the 5' position by a dimethoxytrityl (DMT) protecting group.

Detritylation: Removes the DMT group from the terminally attached nucleoside *via* acid catalysis.

Activation and Coupling: A large excess of phosphoramidite reagent is activated using a solution of an acidic catalyst, typically tetrazole, allowing for the formation of a tetrazolyl phosphoramidite intermediate. This intermediate then proceeds to react with the hydroxyl group of the tethered nucleoside, covalently linking them together. This process is typically very rapid, however for modifications which sterically hinder the 2' position of the sugar ring, coupling times of up to 15 minutes can be required.

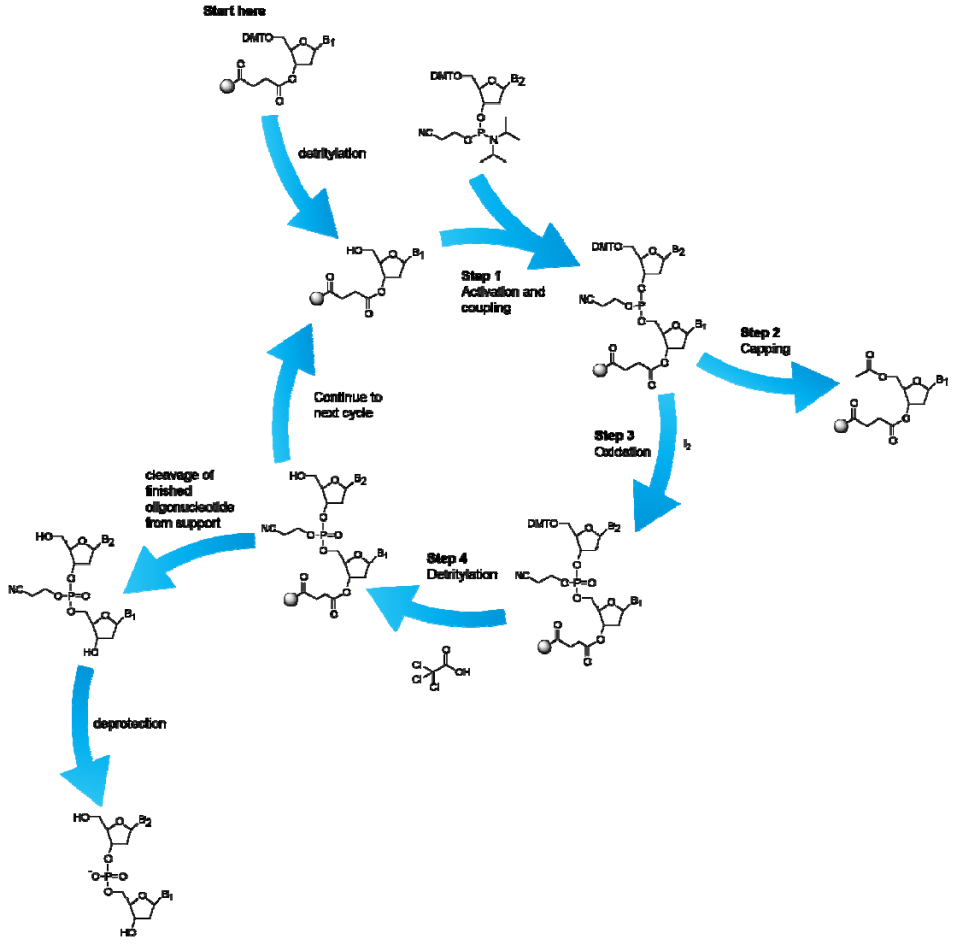
Capping: After the completion of the coupling reaction a small percentage, typically <1%, of the hydroxyl groups from the previous base remain unreacted. To ensure oligonucleotides are not formed with internal base deletions these need to be permanently blocked from further reaction. This is performed by immersing the solid support cartridge with a solution of acetic anhydride and 1-methylimidazole. For the synthesis of phosphorothioate oligonucleotides this step is sometimes performed after oxidation in order to avoid the capping mixture interfering with the sulphur transfer.

Oxidation: As a result of the coupling reaction a phosphite linkage is generated, inherently these are of limited stability and therefore require oxidation. For phosphate backbone oligonucleotides this is performed by immersing the solid support cartridge with a solution of iodine and water in the presence of a weak base, typically pyridine. For phosphorothioate oligonucleotides oxidation is replaced with sulphurisation which employs Beaucage reagent (3H-1,2-benzodithiol-3-one 1,1-dioxide) in order to sulphurise the phosphate backbone. This has been widely used as a way of increasing the stability of oligonucleotides both *in vivo* and *in vitro*.

After the synthesis has completed the oligonucleotide molecules are typically released from the solid support cartridge using aqueous ammonia alone or in a mixture with methylamine. This has a dual function of removing the oligonucleotide molecules from the solid support linker and at the same time removing the remaining protection groups from the nucleobases. Before use, many applications require the oligonucleotide to undergo a desalting procedure and some form of purification. This can be performed in a number of ways with numerous commercial desalting products available. Purification is typically conducted using high performance liquid chromatography with full length oligonucleotides often analysed *via* mass spectrometry.

Important considerations

As previously mentioned numerous different chemically modified oligonucleotides are available. Unmodified oligonucleotides are not suitable for the majority of therapeutic purposes due to their ease of degradation by nucleases. There are many conflicting reports on which chemical modifications are the most valuable for assorted applications, (Deleavey, 2012 and Lennox, 2011) with studies often now utilising mixed backbone oligonucleotides, (Agrawal, 1999). The main considerations to take into account when selecting which variety of oligonucleotide modifications to use are: affinity for its complementary target, its sequence and its stability.



Scheme 1-3: The cycle of solid phase oligonucleotide synthesis. ***ATDbio, Solid-phase oligonucleotide synthesis, 2015. < http://www.atdbio.com/content/17/Solid-phase-oligonucleotide-synthesis>***

Affinity

There are a number of different parameters to rationalise when considering the strength and stability of interactions between oligonucleotides and complementary targets. The five key factors are: Secondary structure prediction of the oligonucleotide, identification of an available, complementary RNA binding site, the binding energy prediction for increased melting temperature (T_m), an optimum GC content and the chemical modification incorporated in the oligonucleotide. Oligonucleotides with internal complementary sequences are to be avoided as they are able to form hairpin structures with themselves, significantly reducing the therapeutic use of such oligonucleotides. The secondary structure of the native RNA is less easy to predict, however it is generally recognised that effective oligonucleotide design is dependent on accurate folding pattern prediction software. Although not an exact technology, predictions of overall minimum free energy of different folding possibilities of native RNA allows scientists to identify local structures within a target transcript where there are accessible hybridisation sites such as internal loops, hairpins, bulges and joint sequences. Once an accessible site has been identified an optimal sequence of the desired oligonucleotide length has to be chosen. The reduction in expression of one or more of an organism's genes by a drug molecule is known as gene knockdown. There are number of different motifs which have been identified as advantageous in oligonucleotide knock down studies, these include: CCAC, TCCC, ACTC, GCCA and CTCT, (Chan, 2006). Conversely there are also sequence motifs which adversely affect oligonucleotide effectiveness, these include: GGGG, ACTG, AAA and TAA. Often the most important consideration for high oligonucleotide binding affinity is the binding energy at 37 ˚C, numerous iterations of software exist which calculate the optimum oligonucleotide sequence for a given mRNA sequence. Using certain databases it has been demonstrated that a 6-fold increase in potency

and a 3-fold increase in activity can be achieved simply by considering the binding affinity, (Chan, 2006). The final major consideration for binding affinity is the type of modification incorporated into the oligonucleotide. It has been demonstrated that certain modifications to the phosphate backbone, such as phosphorothioate modifications, decrease the binding affinity by as much as 0.5 ˚C per nucleotide. However numerous second and third generation modifications have significantly improved binding affinity, this will be covered in more detail later in this section.

Specificity

Crucial to antisense therapy is the specificity of the oligonucleotide for the chosen RNA target. Studies into oligonucleotide specificity are often conflicting, with some suggesting that oligonucleotides can discriminate differences in target RNA with just a single base alteration, (Lee, 2015), whereas others state oligonucleotides have various biological consequences not attributable to specific effects on the RNA target, (Fisher, 2002, Burgess, 1995). There are numerous mechanisms which can contribute to off target effects, not all of which can be combatted against. Off target effects can be caused by non-sequence specific binding of the oligonucleotide to proteins or functional cellular machinery., Undesirable effects can also occur when partial oligonucleotide binding results in the cleavage of ~~cleaving~~ other no targeted RNA transcripts~~which are partially complementary and can also be caused by reagents used to deliver the oligonucleotides~~. In order to validate antisense activity on a specific transcript, investigators must include a number of well thought out positive and negative controls in each experiment.

Stability

Like all drug molecules stability is also an essential consideration in the design of antisense oligonucleotides. Unmodified DNA and RNA are highly unstable in a biological environment, often degrading rapidly due to nuclease cleavage of the phosphodiester linkages. As well as being unstable *via* degradation, pharmacokinetics needs to be taken into account to ensure the oligonucleotide remains in a patient's blood stream long enough to reach its target and have a beneficial effect. It is known that unmodified DNA and RNA molecules have a low binding affinity to protein in plasma and are therefore filtered rapidly from the body, (Crooke, 2007). In order to prevent this chemical modifications are required, and a number of different strategies for modification have already been developed to improve all properties talked about in this section.

Chemical modifications

In order for an oligonucleotide to be active it is imperative that it reaches its target RNA intact. As previously quoted, it is well documented that unmodified DNA and RNA molecules are inherently unstable in biological systems, largely due to ubiquitously expressed nucleases degrading the susceptible phosphodiester bond, (Patil, 2005). Just like current small drug molecules, oligonucleotide molecules are amenable to medicinal chemistry to improve attributes such as binding affinity, tolerance to nucleases and bioavailability. However, careful consideration must be taken to ensure modifications made do not result in significant divergence from the DNA or RNA helical structure to ensure binding by Watson-Crick base pairing is still possible. Equally as important to feasibility, although not efficiency linked, are the synthetic routes available for synthesising the modified building blocks, A, C, G and T, which make up oligonucleotides. Modifications which require complex chemical

synthesis with no cheap source of starting materials are highly unlikely to compete on the drug market. Extensive medicinal chemistry has focused on three main areas of modification, those made to the ribose of the nucleotide, the base and also backbone modifications altering the phosphodiester group, Figure 1-2.

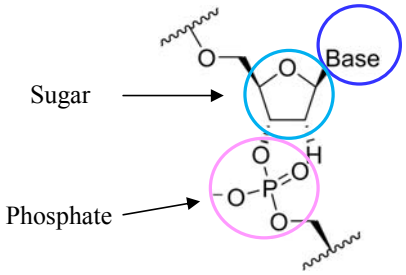


Figure 1-2:. ~~Schematic detailing the k~~Key areas typically modified in the structure of DNA.

Ribose modification

Virtually all therapeutic oligonucleotides contain some fraction of non-natural nucleotides. The majority of these are ribose modifications; those discussed in this chapter are shown in Figure 1-3, although this list is by no means exhaustive, it details the most commonly used modifications at the time of writing. As previously emphasised, in order for oligonucleotides to act as antisense agents it is fundamental that they have a high affinity for their complementary target. In order to ensure this high degree of affinity it is important that modifications do not perturb the oligonucleotide from forming a native conformation. The conformation of sugar rings can be described using phase angle values, calculated from the dihedral angles of the furanose rings. Modifications at the 2'-position of the sugar moiety have been shown to be well tolerated, resulting in increased affinity for RNA and in some cases increased nuclease resistance without adversely impacting the conformation.

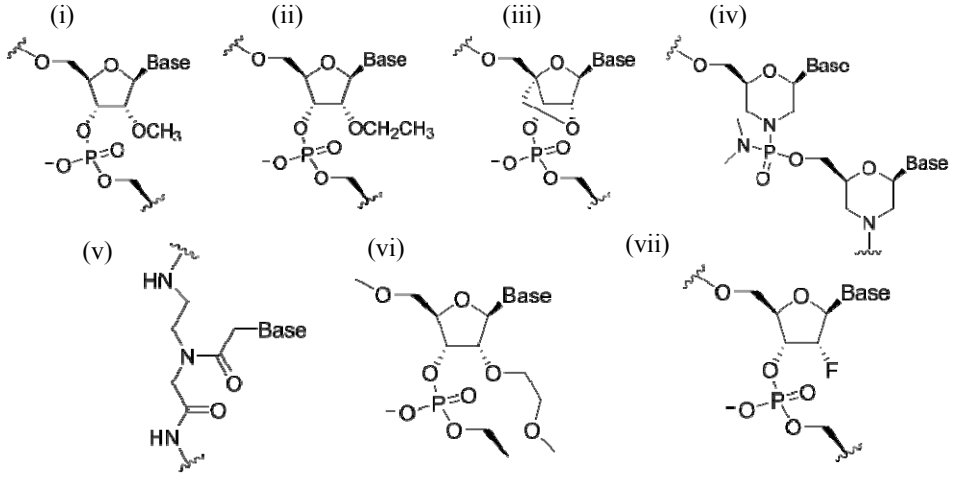


Figure 1-3. Structures of various nucleotides with ribose modifications. (i) 2'OMethyl (2'OMe), (ii) 2'OEthyl (2'OEt), (iii) Locked Nucleic Acid (LNA), (iv) Phosphorodiamidate morpholino oligomer (PMO), (v) Peptide Nucleic Acid (PNA), (vi) 2'OMethyoxyethyl (2'MOE) and (vii) 2'deoxy-2' -fluoro-β -D-arabinonucleic acid (2'F).

2'-*O*-alkyl

The 2'-O-alkyl modifications, such as the 2'-O-Me and 2'-O-Et modifications, offer several advantages over standard DNA oligonucleotides. Firstly, the binding affinity (Tm) is substantially higher for duplex formation with RNA targets. Additionally, they decrease the activity of many nucleases, resulting in better pharmokinetic properties. These advantageous properties are, however, counterbalanced by the inability of these 2'-O-alkyl modified oligonucleotides to induce RNase H cleavage of the target RNA. It has been demonstrated, through the use of mechanistic studies of the RNase H reaction, that these oligonucleotides interfere with the width of the minor groove of the oligonucleotide/RNA duplex, disrupting RNase H activity. Researchers have however used these modifications to design chimeric oligonucleotides termed "gapmers" which can activate RNase H mediated cleavage, Figure 1-6. These oligonucleotides contain a central block of unmodified DNA monomers

sufficiently long enough to induce RNase H cleavage (typically 7-12) flanked by blocks of 2'alkyl modified nucleotides that provide protection to the internal DNA block from nuclease degradation and increase the oligonucleotides affinity for its RNA target, (Cook, 1998).

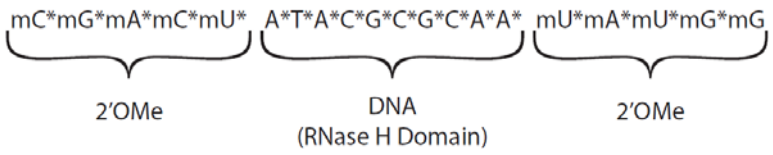


Figure 1-4: Typical structure of a gapmer oligonucleotide.

The omission of modified nucleotides from the central portion of the oligonucleotides allows the gapmer to efficiently and selectively pair with a target mRNA while preserving its ability to induce target cleavage at the centre of the duplex. Although fully modified 2'alkyl oligonucleotides cannot be used to induce RNase H cleavage, they are still very useful for non-destructive antisense strategies.

Phosphorodiamidate morpholino oligomer

Typically abbreviated to PMO, these oligonucleotides are one of the most studied third generation antisense agents. The standard ribose sugar is replaced by a six-membered morpholino ring and the phosphodiester bond is replaced by a phosphorodiamidate linkage. Chemically these changes confer excellent stability against nucleases and have been demonstrated to have similar target affinity to that of the unmodified oligonucleotides with the same sequence. Their mechanism of action is thought to be *via* steric blocking, which prohibits either translation or certain splicing events *via* complementary pairing and restricting the access of bio-molecular machinery. One of the key differences between PMO

and first and second generation oligonucleotides is their net charge. As neutral species these molecules were thought to negate any unwanted interactions with proteins, minimising off target effects which could lead to cytotoxicity. Reports detailing cellular uptake of the uncharged PMO however are conflicting, with some stating poor cellular delivery, (McClorey, 2006) and other demonstrating improved uptake, (Sazani, 2001). *In vivo* cellular uptake has been demonstrated when oligonucleotides are attached to suitable delivery moieties, administration is typically intravenously or for specific cellular tissues *via* local injections, (McClorey, 2006). PMOs have shown promising results in both *in vitro* and *in vivo*, there are currently several drug molecules based on PMO technology in clinical trials against diseases such as Duchene muscular dystrophy (DMD) and certain cancers, (Kole, 2012, Chan, 2006).

Peptide nucleic acid

Developed in the laboratory of Nielsen in 1992 PNA oligonucleotides are another third generation agent and like PMOs have a net neutral charge, (Egholm, 1992). In terms of their structure they are the most radical of the modifications widely used by researchers, the phosphodiester backbone is replaced with polymeric N-(2-aminoethyl)glycine with the nucleobases being attached to the backbone *via* a methylenecarbonyl-linkage. Although far from the typical phosphate/base/sugar configuration PNA molecules maintain the ability to Watson-Crick base pair with complementary RNA or DNA. Their production is usually *via* peptide synthesis rather than the automated process explained previously. As with the majority of neutral oligonucleotides their functional mechanism is *via* steric blocking for transcriptional or splicing interference, they also demonstrate low protein binding limiting off target effects, (Kurreck, 2003). With similar uptake disadvantages due to their net neutrality

various studies have been undertaken to conjugate a range of moieties to improve *in vivo* bioavailability, most promisingly Sazani ~~et al~~*et al*. observed that the conjugation of four lysine molecules at the carboxyl-terminus of the PNA molecule significantly improved delivery, (Sazani P. G., 2002). PNA therapeutics have been demonstrated to be well tolerated and effective for exon skipping in murine models of DMD, (Yin, 2007), however compared to other modifications there are only a limited number of PNA based agents currently in clinical trials.

Locked nucleic acid

Another of the most promising oligonucleotide modifications are locked nucleic acids (LNA), which belongs to the group of 2',4'-bicyclic nucleosides. These oligonucleotides contain a methylene bridge that connects the 2'oxygen of ribose with the 4'carbon, resulting in the formation of a conformationally restricted bicyclic nucleoside. This linkage enforces a Northern sugar conformation that closely mimics A-form RNA, resulting in preorganisation of the LNA oligonucleotide for target binding, increasing the Tm. Numerous studies have demonstrated the potential of LNA modifications to significantly increase the Tm of the oligonucleotide/RNA complex when compared to an unmodified DNA oligonucleotide, (Deleavey, 2012). The study found that the substitution of 10 DNA nucleotides for LNA monomers resulted in a 24 °C Tm increase when compared with a full length unmodified DNA control, (Lennox K. A., 2006). Whilst LNA modifications increase the binding affinity towards an RNA target, LNA:LNA hybrids show an even higher Tm; as such, oligonucleotides which are fully LNA modified often form stable hairpin and self-dimer structures that can interfere with function. In order to avoid the formation of these secondary

structures researchers often design chimeric oligonucleotides with interspersed LNA and DNA monomers. The relative potency of a variety of chimeric oligonucleotides have been tested, with independent research groups finding LNA mixmers were among the most potent designs, (Kurreck, 2002), although significant toxicity was observed in one study, (Swayze, 2007).

2'-*O*-Methyoxyethyl

Another 2'-O-alkyl modification described is 2'-O-methoxyethyl (2'-MOE), shown to provide remarkable protection from nuclease degradation, reduce nonspecific protein binding and to increase T_m by about +2° C per modification when compared to RNA, (Manoharan, 1999). It is also reported that 2'MOE modifications also improve nuclease resistance above that of the 2'OMe modification due to the larger alkyl chain sterically hindering attack, (Altmann, 1996). The modification has also been successfully used in gapmer, (Manoharan, 1999) and siRNA, (Prakash, 2005) technologies. Unfortunately, due to existence of a current patent, this modification is not commercially available and as such fewer studies have been conducted using CMOs containing this modification. Even so, with numerous 2'MOE therapeutics currently in clinical trials, (Disterer, 2014 and Watts, 2012), there is no doubt it will feature prominently in antisense medicine over the coming years. Already featured in a licensed drug, Kynamro (a 2'-MOE containing gapmer), was the second antisense FDA approved drug, legalised for the treatment of homozygous familial hypercholesterolemia in order to reduce low density lipo-protein-cholesterol, apolipoprotein-B, total cholesterol and non-high density lipoprotein–cholesterol in patients with the condition. There were however found to be some serious side effects including liver toxicity, with the packaging citing the

risk of hepatic toxicity. All together 2'MOE drugs show great potential, with the outcome of current clinical trials eagerly anticipated.

2'-Deoxy-2'-fluoro

First discovered over 20 years ago 2'F-RNA oligonucleotides have a vastly increased hybridisation affinity compared with unmodified agents, with various studies demonstrating an increase in Tm as high as +2 °C per modification when compared to DNA, (Bennett, 2010 and Kawasaki, 1993). One of the largest advantages of this modification is the adoption of the sugar ring into a C3'-*endo* conformation, which consequently ensures the RNA:Oligonucleotide duplex adopts the more thermodynamically stable A-form helix upon hybridization. This allows 2'F-RNA oligonucleotides to act *via* the RNAi pathway as recognition occurs with the necessary bio-molecular machinery, (Watts, 2008). One disadvantage of 2'F-RNA modification is its inability to activate RNase H, however its epimer, 2'-deoxy-2'-fluoro-β-D-arabino nucleic acid (2'F-ANA) is a structural mimic of DNA rather than RNA and therefore activates RNase H, (Trempe, 2001). The two fluorine modified nucleotides are structurally identical, only the stereochemistry of the fluorine alters. In 2'F-ANA the 2' F-substituent projects into the major groove of the helix, therefore not obstructing the binding of RNase H allowing for degradation to take place, (Denisov, 2001). It has been demonstrated that DNA, 2'F-ANA chimers can outperform first generation phosphorothioate DNA oligonucleotides as RNase H mediated cleavage is accelerated, resulting in efficient gene silencing in the low nano molar range, (Kalota, 2006).

Backbone modifications

The inter-nucleotide phosphodiester linkages which make up the backbone structure of DNA and RNA sequences lend themselves to modification. The most-studied of the backbone modifications to date are the phosphorothioate (PS) analogs, in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulphur atom, Figure 1-5.

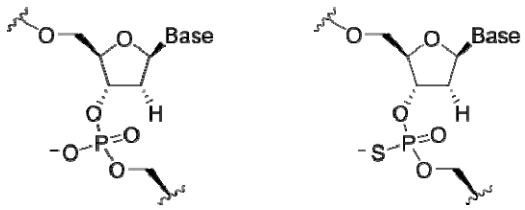


Figure 1-5: Structures of nucleotides with different phosphate backbones. (i) unmodified phosphate backbone (PO) and (ii) phosphorothioate backbone (PS).

PS oligonucleotides have a negatively charged backbone and are capable of supporting RNase-H activity similar to phosphodiester oligonucleotides, crucial for the activity of a number of antisense mechanisms. PS oligonucleotides also have a significantly greater resistance to nuclease degradation than unmodified phosphodiester analogs, (Agrawal, 1999), and have an enhanced affinity for serum albumin, important for improved pharmacokinetics and prolonged circulation time. PS oligonucleotides have been tested in a number of different models. Safety studies of PS oligonucleotides in mice and rats demonstrated sequence-dependent side effects, the severities of which were dependent on the dose and the frequency of administration. The observed toxicity profile of these oligonucleotides differed in monkeys and humans, when administered by intravenous infusion side effects were sequence independent, however did still depend on the dose, duration of the treatment and the frequency of administration, (August, 1997). Another type of backbone modification involves the replacement of both non-bridging phosphodiester oxygen atoms with sulphur

atoms, Figure 1-6; these are known as phosphorodithioates oligonucleotides, (Cummins, 1996).

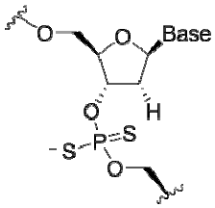


Figure 1-6: Structure of phosphorodithioate backbone modification.

Compared with the corresponding phosphorothioate modification, the duplex stability of phosphorodithioate modified oligonucleotides with DNA is decreased, resulting in a lower binding affinity, (Ghosh, 1993). They have also been found difficult to synthesis on scale, therefore although these modifications do have advantages over phosphorothioate modified oligonucleotides, (they have been shown to exhibit less non-specific protein binding and more resistance to nucleases) these disadvantages have resulted in far fewer studies based on phosphorodithioate-modified oligonucleotides, (Mesmaeker, 1995).

As with base modifications there are an ever growing number of backbone modifications. Figure 1-7 displays other analogues which have been less widely studied.

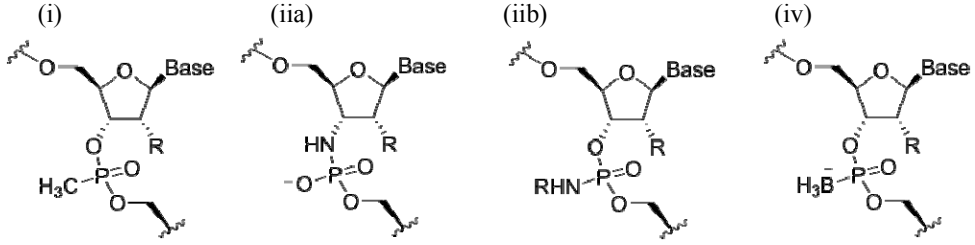


Figure 1-7: Structures of various nucleotides with less widely studied backbone modifications. (i) methylphosphonates, (ii) phosphoramidate and (iii) boranophosphate.

Figure 1-7(i) depicts the most studied group of non-ionic backbone modification, methylphosphonates. These have been shown to have melting temperatures with complementary RNA/DNA strands similar to those observed with wild-type backbones. It is thought that the neutral backbone could help facilitate uptake, (Miller, 1981). These modifications are thought to be as stable, if not more so than PS backbone modification. Two types of phosphoramidate (NP) modifications can be seen in Figure 1-7(iia/b), here either the 3'-amino group is substituted for 3'-oxygen in the deoxyribose ring (iia) or one of the non-bridging oxygen atoms in the phosphate backbone is replaced by an amine group (iib). NP containing oligonucleotides do not activate RNase H, but have good binding affinity for targeted sequences and high nuclease resistance; they can therefore be used to down regulate gene expression, (Gryaznov, 1995 and (Mesmaeker, 1995). Finally boranophosphate internucleotide linkages also have been shown to demonstrate increased nuclease stability compared to their unmodified counterpart, Figure 1-7(iii). Although these molecules have been shown to function in gene silencing assays, (Hall, 2006) few are in clinical trials due to the lack of availability of the starting monomers from commercial sources.

Base modifications

Although less common than sugar and backbone modifications, nucleobase modifications have also found purpose in improving stability and specificity of antisense agents. The major considerations to this type of modification are the steric implications as well as the necessity to maintain the integrity of hydrogen bonds which are critical for base pair matching. Although a large number of base modifications exist there are only a handful which show promise for therapeutic use, typically by increasing specific base pair recognition. The 5-bromo uracil and 5-iodo uracil substitutes, Figure 1-8 (ia/ib), as well as the 2,6-

diaminopurine in place of Adenosine, Figure 1-8 (ii), have been shown to improve the stability of the A-U base pairing (Peacock, 2011).

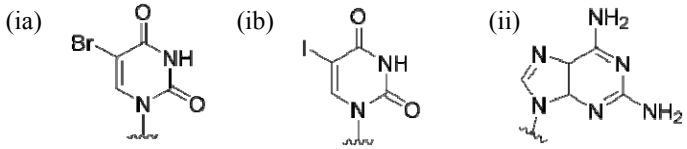


Figure 1-8: Structures of (ia) 5-bromo Uracil, (ib) 5-iodo Uracil and (ii) 2,6-diamino Adenosine.

It has also been demonstrated *in vitro* that a propynyl group can increase the stability of DNA when linked to the 5' position of a pyrimidine or to the 7' position of a purine whilst retaining RNase H functionality. Two specific examples published by He ~~et al~~*et al*, (He, 2002), are depicted in Figure 1-9 below. These improvements however were only seen in oligonucleotides containing certain ribose modifications, with others showing no enhancement. When tested *in vivo* these oligonucleotides exhibited acute toxicity characterised by mortality in the animals tested, negating their therapeutic use, (Shen, 2003).

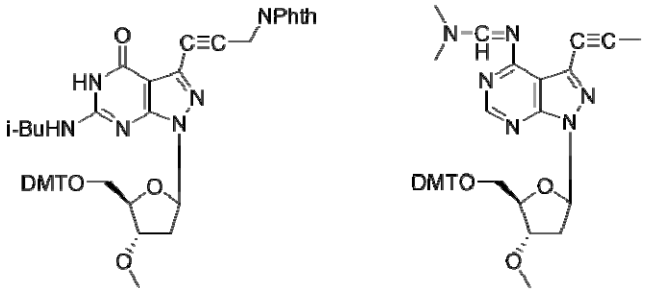


Figure 1-9: Structures of propynyl modified nucleotides which are thought to increase the stability of DNA oligonucleotides.

A contrasting strategy by Addepalli ~~et al~~*et al*. established that local destabilisation of the central region in an oligonucleotide:RNA duplex could be induced by the placement of nucleobase modifications removing or decreasing the ability of hydrogen bonding,

(Addepalli, 2010). This can be achieved in various ways by removing the hydrogen bond accepting and donating atoms from the relevant bases, Figure 1-10.

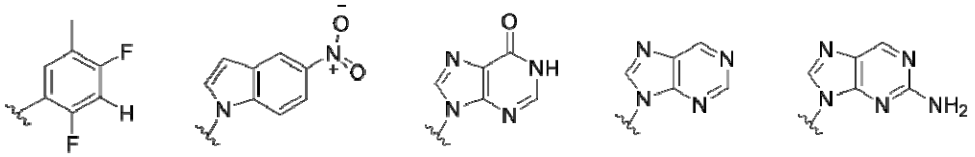


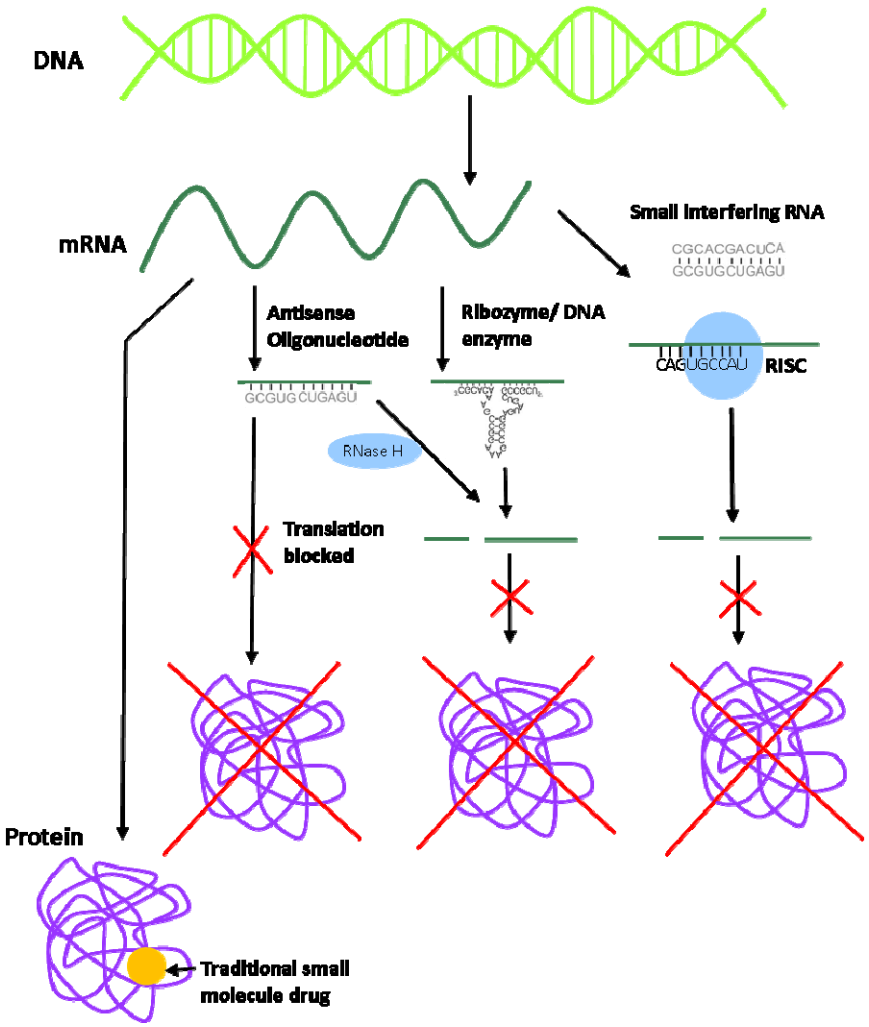
Figure 1-10: Nucleobase modifications where atoms capable of hydrogen bond donation or acceptance are removed.

Silencing activity was found to be highly dependent on the position and type of residue modified. The destabilisation could significantly improve silencing activity if the passenger and guide strand dissociate prior to cleavage, an alternative mechanism which has been proposed for RNA-induced silencing complex (RISC) assembly, (Addepalli, 2010). This type of modification would only be useful for oligonucleotides which act *via* a specific mechanism, explained in more detail in the next section.

Modes of action

A further major consideration for the selection of a therapeutic oligonucleotide is the mechanism by which different oligonucleotides function. Although not fully understood, it has been established that certain oligonucleotide modifications influence their mode of action, (Crooke, 1999 and Deleavey, 2012). These insights suggest it may be possible in future to select the modifications incorporated within an oligonucleotide to match to the desired mechanism. The current proposed mechanisms can broadly be categorised as either occupancy only mediated, occupancy activated, or oligonucleotide induced cleavage

mechanism. Scheme 1-4 compares the use of oligonucleotides with current small drug molecules, including their mode of action as understood at present.



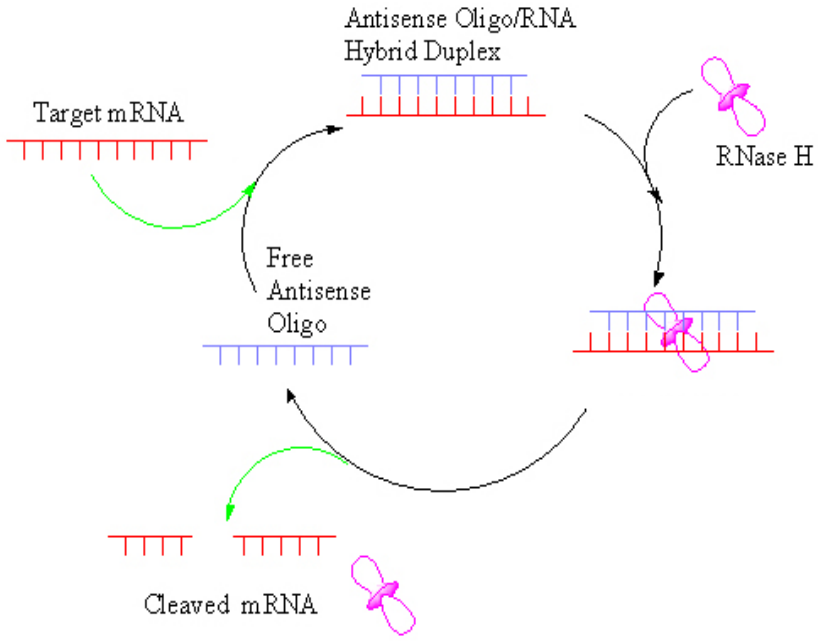
Scheme 1-4: Mechanism and stage at which different antisense agents are able to act during protein synthesis, modifed from Kurreck, 2003.

Occupancy only

Oligonucleotides which act in this way alter biological activity by preventing the interaction between the target RNA and proteins or other factors, therefore interfering with the RNAs function without promoting RNA degradation. These oligonucleotides are usually targeted at specific parts of the RNA sequence, most commonly the initiation codon, which results in translational arrest. These oligonucleotides can also be used to inhibit splicing by targeting splice junction sequences thus preventing any biological machinery necessary for the process from binding and inhibiting the production of mature mRNA. Finally these oligonucleotides can be used to disrupt necessary RNA structures, such as stem loops, destabilizing the RNA and preventing proteins, nucleic acids, and ribonucleoproteins from participating in intermediary metabolism and activities, (Crooke, 1999 and Bennett, 2010).

Target degradation

A number of oligonucleotides which act by degrading their DNA or RNA targets have been used successfully in various studies, (Lee, 2011, Vickers, 2003 and Giles, 1992). These oligonucleotides are thought to degrade RNA through endogenous enzymes, such as RNase H or Argonaute 2, (Sharma, 2014). Oligonucleotides that work through an RNA cleavage mechanism are currently the best understood group of oligonucleotides with a number in advanced clinical trials, (Bennett, 2010), examples of which can be found in Table 1-1. RNase H is a family of enzymes, thought to be present both in the nucleus and the cytoplasm, which recognises RNA-DNA hetroduplexes, cleaving the RNA strand, Scheme 1-5. Although proven to be a robust antisense mechanism, oligonucleotides must be carefully designed to ensure any modifications do not interfere with the enzymes recognition of a DNA/RNA heteroduplex.



Scheme 1-5. Scheme representation of how RNase H causes enzymatic degradation of oligonucleotide/RNA duplexes, taken from "Kinetic analysis of the RNA cleavage of the conformationally-constrainedoxetane-modified antisense-RNA hybrid duplex by RNase H" (Amirkhanov, 2002).

Drug	Indication	Target	Chemistry[a]	Status
Fomiversen	Cytomegalovirus	IE2 gene	PS ODN	Approved
Oblimersen	Oncology	Bcl-2	PS ODN	Phase 3
Mipomersen	Cardiovascular	Apolipoprotein B	2′-MOE chimera	Phase 3
Trabedersen	Oncology-glioblastoma	Transforming growth factor-β	PS ODN	Phase 3
GS-101	Corneal neovascularization	Insulin receptor substrate-1	PS ODN	Phase 3
LOR-2040	Oncology	Ribonucleotide reductase	PS ODN	Phase 2
Archexin	Oncology	AKT-1	PS ODN	Phase 2
TPI ASM8	Asthma	CCR3 and IL-5 receptors (two oligos)	PS ODN	Phase 2
Alicaforsen	Colitis	Intercellular adhesion molecule-1	PS ODN	Phase 2
Custirsen	Oncology	Clusterin	2′-MOE chimera	Phase 2
LY2181308	Oncology	Survivin	2′-MOE chimera	Phase 2
AEG35156	Oncology	X-IAP	2′-*O*-Me chimera	Phase 2
TV/ATL1102	Multiple sclerosis	CD49D	2′-MOE chimera	Phase 2
ISIS 1113715	Diabetes, type 2	Protein tyrosine phosphatase-1B	2′-MOE chimera	Phase 2
Monarsen	Myasthenia gravis	Acetylcholine esterase	2′-*O*-Me chimera	Phase 2

Table 1-1: A selection of modified oligonucleotides currently in clinical trials which degrade the complementary target, (Bennett, 2010).

Oligonucleotides which induce cleavage of the target RNA by recruiting endogenous Ago 2 are said to work through and RNA interference (RNAi) mechanism. It is proposed that these oligonucleotides mimic naturally occurring siRNAs which regulate gene expression, whose mechanism has been previously described. When oligonucleotides enter the RNAi effector complex, RISC, it is possible to select silencing targets based on sequence complementarity. Mechanistic studies have clearly identified parallels between RNase H and Ago2 mediated cleavage mechanisms, however the key difference in their mode of action is RNase H mediated cleavage the oligonucleotide binds to the target RNA prior to enzyme interaction, (Bennett, 2010), whereas in Ago2 mediated cleavage the oligonucleotide is bound to the enzyme prior to target recognition.

Oligonucleotide induced cleavage

The selective destruction of target RNA by complementary oligonucleotides conjugated to a chemical cleaving moiety has long been a goal of chemistry/biology collaborations. Numerous analogues have been synthesised incorporating a wide range of chemical groups including conjugating oligonucleotides to reactive metal complexes and photon activated moieties. Sequence specific cleave has been demonstrated with the conjugation of reactive metal species which generate free radicals that can cleave nucleic acids. Europium (III) complexes, (Hall, 1994) and EDTA.Ce(IV) are two examples, Figure 1-11, which have been found to improve the antisense activity in cells, (Crooke, 1999 and Moser, 1987).

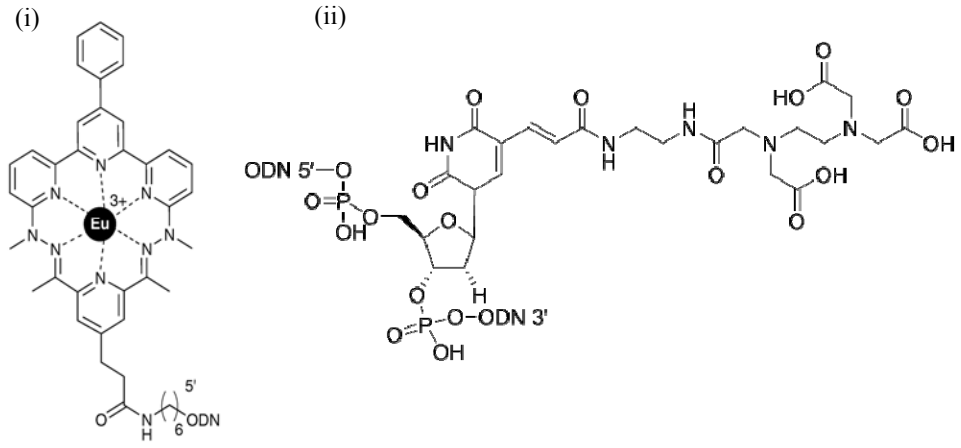


Figure 1-11. Reactive metal conjugation to induce sequence specific cleavage. (i) Europium (III) complex and (ii) EDTA.Ce(IV)

Dervan ~~et al~~*et al*. have employed EDTA-oligonucleotide conjugates to demonstrate cleavage of single-stranded DNA. Their mechanism proposes that EDTA-chelated iron generates hydroxyl radicals *via* a Fenton-like reaction that cleaves the DNA. However, the cleavage occurs at several bases near the nucleotide at which EDTA is attached rather than with absolute specificity, (Moser, 1987). The potential disadvantages are obvious, with oligonucleotide degradation resulting in off target effects, almost certainly resulting in high levels of toxicity. Preliminary data has however suggested that covalently modified oligonucleotides are feasible and possibly enhance the potency, increasing target degradation. Although there are to date no example of this type of chemistry in clinical trials, it seems likely that studies in progress will address this in the coming years. Photo-conjugates have also been developed which demonstrate the potential for specific activation *in vivo via* UV light activation, giving rise to DNA/RNA damage by photo-crosslinking, (Singh, 2010). This allows specific sequences to be cleaved at desired times and sites of interest without changing chemical or physical conditions. Tanaka ~~et al~~*et al*. synthesised acridine modified

oligonucleotides which contain a photo responsive azobenzene, Figure 1-12, by combining it with Mn(II) ions they showed site-selective RNA scission which was photo-controlled *in vitro*, (Tanaka, 2008). The major disadvantages to both photo cleavable and the conjugation of so called metallonucleases are they are difficult to synthesise and currently do not appear to offer an advantage over simpler approaches which employ endogenous nucleases present in cells.

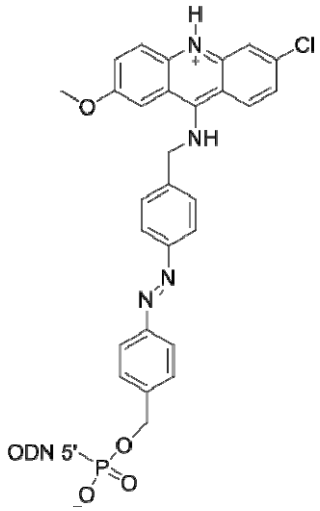


Figure 1-12. Structure of acridine modified phosphate group which can be incorporated into oligonucleotides to induce photo cleavage, (Tanaka, 2008).

Another intrinsically more attractive concept for therapeutic application is the conjugation of ribozymes and DNAzymes to oligonucleotides. In theory this would allow the destruction of RNA/oligonucleotide complexes by the cells' own machinery. A number of reports have demonstrated the potential of ribozymes conjugates to cleave RNA targets in cell culture, however both ribozymes and DNAzymes have proven not to be as effective *in vivo* as some other less complicated approaches therefore have attracted little attention recently as a therapeutic strategy, (Bennett, 2010).

Delivery technology

Successful intercellular delivery of oligonucleotides is possibly the most significant impediment to the clinical success of oligonucleotide therapy. There are a number of delivery strategies available, some chemical and others physical, however efficacy has often come at the price of increased toxicity. There are two essential components when considering suitable delivery reagents, firstly the reagent must protect its cargo from degradation, and secondly it must deliver its contents to the required part of the cell. Historically there are three well defined subsets of delivery technology methods, i) physical methods, ii) viral vectors and iii) polymer based delivery. Each method has its own advantages and disadvantages with varying reagents as outlined below.

Physical methods

Physical methods are relatively new in the field of antisense therapy however they have demonstrated great promise to deliver antisense agents *in vitro*. Here I discuss the basic principles behind the physical methods which have shown the most promise for therapy. Although not all of the methods described have been investigated in this project it is important to briefly note their positive and negative features, both in terms of efficiency and practicality.

Gymnosis

Unarguably the most simplistic delivery method is that of oligonucleotides alone without any additional reagents. In 2009 Stein ~~et al~~*et al*. described a method called gymnosis, derived

from the Greek “gymnos” meaning naked. Here it is thought that oligonucleotide uptake is attributed to the natural growth properties of the cells and seems to be a general phenomenon and not cell type dependent. Although naked oligonucleotide injection is a safe and simple method, its efficiency for delivery is low and significantly slower than when compared to other transfection protocols. A considerable advantage of gymnotic delivery is the reduction in off-target effects when compared to the use of chemical transfection reagents which have been shown to interact with cell membranes and consequently their receptors and signalling processes altering other downstream functions. (Stein, 2010, Souleimanian, 2012 and Macron, 2011b).

Hydrodynamic

Initially established for gene delivery in mice, hydrodynamic delivery has been demonstrated to be effective for the transfer of various water soluble biomolecules into cells. The technique relies upon the physical force generated by the rapid injection of fluid into a blood vessel, increasing the cells permeability. The key components of this technique include: large volume, high injection speed and vasculature for needle placement. The lack of toxicity, and large delivery efficiency have driven this technique to be used in multiple animal experiments, however difficulties have been identified in the translation of this method into an effective procedure applicable for human therapy. It has been proposed that if animal dosing were extrapolated to human scale an injection volume of up to 7.5 L would be required for therapeutic doses, (Bonamassa, 2011). Even in light of this considerable hurdle hydrodynamic delivery for human therapy is still a desirable goal due to advantages such as reduction of cost of delivery, high efficiency and simplistic equipment needs. Recent studies using rabbit and pig models have encouragingly demonstrated liver transfection using

adapted procedures with lower volumes, indicating that with modification, hydrodynamic delivery could have potential in clinical therapy, (Bonamassa, 2011).

Magnetofection

Whilst strictly not a purely physical method, magnetofection combines the advantages of physical and non-viral delivery methods together into one system. It employs superparamagnetic nanoparticles to deliver biocompatible molecules using an external magnetic field to preferentially concentrate the particles into the target cells (Scherer, 2002). The current understanding suggests that the magnetic field may draw the magnetic particles across the plasma membrane and into the cell, improving cellular uptake. Rather than a standalone method of oligonucleotide delivery, magnetofection is an enhancement of non-viral delivery methods as it is ineffective unless the oligonucleotide is complexed with a carrier reagent and a magnetic particle. Encouragingly good transfection efficiencies have been reported with fast delivery times. *In vitro* transfection efficiencies were demonstrated to be as high as 84% within 15 minutes, including nuclear localisation within 2 hr, (Hrotz, 2003). The key advantages of this technique include: drastically lowered vector dose; considerably reduced incubation time required to achieve high transfection efficiency; and the ability of oligonucleotide delivery to a wide range of cell types. As one of the most recently established techniques, development of focused strong magnetic fields will be a challenge for physical and medicinal sciences in order for this delivery method to reach its full therapeutic potential, (Prosen, 2013).

Sonoporation

Sonoporation relies upon the application of ultrasound technology in order to generate the transient formation of small pores in the cell membrane, allowing for an increase in the permeabilisation of surrounding molecules into the cell, (Mehier-Humbert, 2005). The transfection efficiency of ultrasound mediated uptake is determined by the frequency, output strength and treatment time and has been shown to be enhanced by using contrast agents. Ultrasound is thought to be a promising delivery technique due to its non-invasive nature and ability to penetrate soft tissues making it ideal for oligonucleotide delivery into internal organs. The major disadvantage facing ultrasound-facilitated delivery is low delivery efficiency. Even so for certain applications this lack of efficacy can be tolerated with Tezel ~~*et al*~~*et al*. demonstrating that low frequency ultrasound could heterogeneously deliver therapeutically significant quantities of oligonucleotides into skin without skin damage, with estimated delivery concentrations ranging from 0.5 ~ 5% of the total administered dose detectable in the skin, (Tezel, 2004).

Electroporation

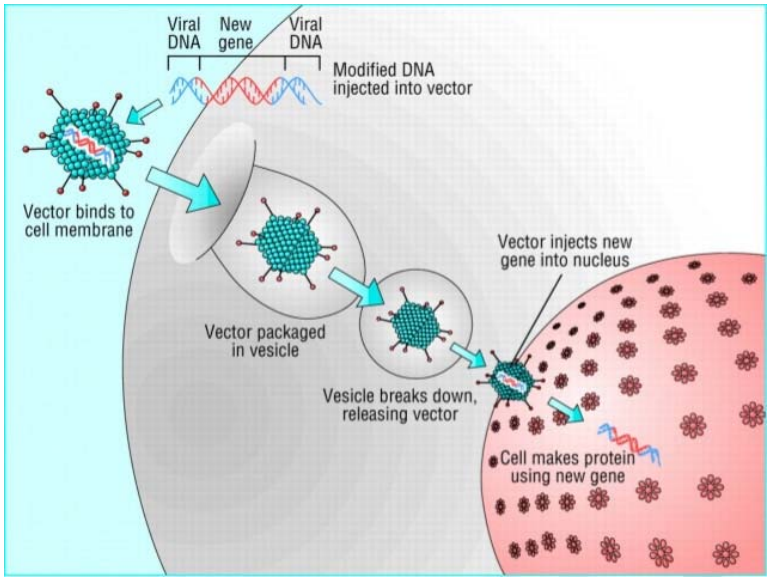
Possibly the least clinically relevant of the physical methods described here, electroporation is arguably also the best understood. By exposing the cellular membrane to high intensity electric pulses the membrane integrity is perturbed and molecules localised in the area of the destabilised membrane can penetrate into the cell. The temporary permeability is thought to arise from the creation of electric field-induced pores, (Mehier-Humbert, 2005). Efficacy of delivery *via* electroporation can be influenced by several factors, both physical (such as duration and strength of the electric pulse) and biological (such as cell type and oligonucleotide concentration), (Gehl, 2003, Andreason, 1988). As previously alluded to

however electroporation comes with a number of significant drawbacks. Firstly it is difficult to transfect larger tissues due to the limited effective range of just 1 cm between the electrodes, secondly high voltages can cause irreversible damage to the treated tissue, third the method requires unfavourable surgical procedures and finally there are concerns over the lack of selectivity over the position of insert of the antisense agent into genomic DNA, (Bolhassani, 2014). Although some of these concerns could potentially be resolved; the high number of disadvantages has led to a decline of *in vivo* experimentation. The technique is still widely used in *in vitro* cell culture experiments, although again cell death is notably high in comparison to other transfection methods.

Viral vectors

As previously mentioned, in order for gene therapy to be successful, enough therapeutic agent must reach its target to have a beneficial affect without high levels of toxicity. Viral vectors were a part of one of the first strategies applied to successfully deliver therapeutic antisense agents into living cells, (Nayerossadat, 2012). By applying what was known about viral replication, researchers saw the potential for persistent gene correction in differentiated, less accessible tissues. In brief, viruses attack host cells and introduce their genetic material which contains replication instructions. Using the host's replication machinery, multiple copies of the virus are produced, infecting a high number of cells. Certain virus types even have the ability to physically incorporate their genes into the host's genome, so that upon replication these genes are passed onto daughter cells. As natural viruses have evolved to deliver their infecting genes to a cell, reengineering these viruses to contain unmutated copies of non-functional genes or oligonucleotides could potentially correct a range of genetic

diseases. The exact way in which viruses are reengineered is beyond the scope of this introduction; however Scheme 1-6 illustrates a basic schematic representation.



Scheme 1-6: The basic concept of viral gene delivery. Figure taken from "*Gene Ther.* science fiction or science fact?" (Soliman, 2013).

One of the largest hurdles to overcome when using this technology was the body's own immune response against these modified viruses. The primary function of the human immune system is to detect, limit and hopefully eliminate an infection. Activation of the immune system by viral vectors has been widely studied, and a thorough understanding of the mechanism by which these viral vectors initiate an immune response has allowed for modifications which reduce the immunotoxicity of these agents. These undesired immune responses not only impede the delivery of genes to target cells but can cause severe complications for the patient.

There are a number of different viruses which lend themselves to modification, with decisions of which to use dependent on target cell type and on the requirement for either transient or long-term gene expression. The modification of viruses has evolved over many years, the most studied of which are adenovirus, retrovirus and lentivirus, (McTaggart, 2002, Galimi, 2002). Adenovirus vectors are probably the most frequently used viral vectors, as they have been found to accommodate large transgenes, be highly efficient vectors in a range of tissues and code for proteins without integrating into the host cell genome, (Nayerossadat, 2012). They are medium-sized, non-enveloped viruses containing a double stranded DNA genome. Early versions of adenovirus vectors showed unacceptably high levels of toxicity, with the first reported fatality from gene therapy occurring in 1999, (Patil, 2005). Since then second and third generation vectors have shown reduced toxicity upon removal of certain non-essential viral genes from the vector, however these deletions are complex and can have a negative effect on replication, (Alba, 2005). The production process for these vectors is difficult to scale to pharmaceutical levels and it has been demonstrated that adeovirus formulations may lose their potency when stored in commonly used pharmaceutical vials, (Nyberg-Hoffman, 1999).

Retroviruses are another common vector used in a range of pharmacological studies, including tissue repair and engineering, and gene therapy. Rather than DNA they are composed of RNA and rely upon reverse transcriptase to transcribe their RNA into DNA after entering a cell, (Difford, 2003). Unlike adenoviral and lentiviral vectors they are able to pass through the nuclear pores of mitotic cells allowing them to transfect cells which are dividing. Retroviral vectors for therapeutic purposes are created by the removal of the

inherent retroviral gag, pol, and env genes replacing them with the necessary therapeutic gene of interest.

Unfortunately there are a number of significant disadvantages to the use of retroviruses in a therapeutic capacity. Firstly they have relatively small carrying capacity, although this would not limit their capacity to carry oligonucleotides, rather larger gene constructs. Secondly, and highly problematic, is the reported incidence of leukaemia in some patients, this arises due to the insertion of the genetic material in an unfavourable position, (Hacein-Bey-Abina, 2003). The integrase enzyme, which inserts the genetic material from the virus, can insert the sequence in any arbitrary position in the genome of the host, if this happens to be in a gene regulating cell division uncontrolled activation can occur leading to cancer. Insertions in other genes can also lead to insertional mutagenesis, disrupting the function of potentially important genes. Historically, after adverse findings which occurred almost three years after the use of a retrovirus, gene therapy trials were halted or restricted in the USA, (Fox, 2003). This was as a direct result of a clinical trial for the treatment of severe combined immunodeficiency where three children developed T-cell acute lymphoblastic leukaemia. In 2005 the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) found that although the majority of children treated had significant clinical improvement, "The occurrence of leukaemia in this protocol is not a random event and constitutes a serious inherent risk in this study". This led to the NIH RAC making the following recommendation: "Retroviral gene transfer studies for X-linked severe combined immunodeficiency should be reviewed, on a case-by-case basis, and limited, pending further data, to patients who have failed identical or haploidentical stem-cell transplantation or for whom no suitable stem cell donor can be identified. Case-by-case review would include appropriate risk:benefit analysis

accompanied by implementation of appropriate informed consent and monitoring plans". As a result of this, limited clinical trials have taken place using retroviruses to insert antisense material.

Lentiviruses are a subclass of retroviruses, and similarly rely upon reverse transcriptase to transcribe the inherent viral RNA sequence into the complementary DNA format. Unlike typical retroviruses they are able to naturally integrate with non- dividing cells as well as dividing cells. This unique feature has resulted in their extensive use in *ex vivo* gene transfer with no significant immune responses and no unwanted side effects. To date lentiviruses have been successfully used for transfection of several mouse models, including studying the treatment of diabetes and haemophilia. The use of lentiviral vectors has increasingly become more common as studies reveal novel insights into safe integration preferences of the various vector types, (Cockrell, 2007). The first clinical trial to use a lentiviral vector was conducted in 2005 using a HIV-1 based vector for anti-HIV therapy after significant regulatory considerations, (Liechtenstein, 2013).

Although certain viral vectors are now proving safe and efficient in a variety of clinical studies, their use is often to insert large gene constructs rather than short length oligonucleotides. In light of the safety concerns and bad publicity a large number of non-viral delivery reagents have emerged as promising alternatives to deliver smaller antisense drugs such as oligonucleotides.

Non-viral vectors

To address the toxicity associated with viral vector delivery many synthetic alternatives have been devised. To date the two most scientifically important categories of these are liposomes and polymers, (Patil, 2005), with numerous functional analogues of each category having been synthesised and tested for efficacy and toxicity. Both methods address critical parameters for *in vivo* use such as ease of production, not oncogenic and non-immunogenic, however toxicity is still proving to be a significant obstacle in the use of antisense therapy.

Liposomes

Although liposomes were not utilised in this project they are an important type of non-viral delivery agents and are therefore worth mentioning briefly. Liposomes are composed of self-assembled amphiphilic molecules which accumulate *via* interacting with one another in an energetically favourable manner to form spheres. Lipid molecules containing a hydrophilic head group and hydrophobic tails Figure 1-13(i) associate in aqueous solutions to form leaflets, Figure 1-13(ii), in order to minimise unfavourable interactions of the hydrophobic tails with the aqueous environment. Self-assembly continues as the bilayer formation is still energetically unfavourable due to the outer lipid molecules tails still being exposed to the aqueous environment. This disadvantageous energy state is overcome through curvature of the bilayer, resulting in the sphere like structure seen in Figure 1-13(iv).

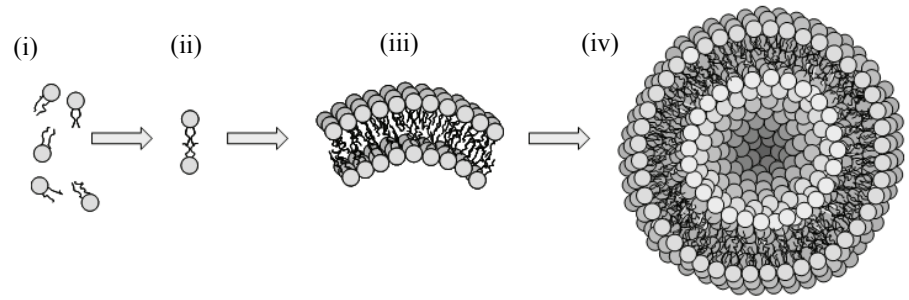


Figure 1-13: Lipid molecules (i) in aqueous solution spontaneously form leaflets (ii), then bilayer membranes (iii), and eventually liposomes. ***(Balazs, 2010).***

Liposome technology has a number of unique advantages, including: their diverse range of morphologies, compositions, ability to transport many types of therapeutic biomolecules, lack of immunogenic response, low cost, and their differential release characteristics, (Balazs, 2010). These characteristics have led to liposomes finding application in both industry and scientific research, including: food technologies, cosmetics, analytics and drug delivery, (Gregoriadis, 2006), however, this report will focus on their use as delivery reagents for oligonucleotides. Liposomes found potential as delivery reagents over 35 years ago, and since numerous developments have been made allowing their use in a broad range of pharmaceutical applications. Liposomes, like the previous delivery strategies, address the two key issues of oligonucleotide delivery: access across the cellular membrane and protecting the oligonucleotide from nuclease degradation.

There are two distinct ways in which liposomes bind oligonucleotides, either *via* encapsulation in the liposome interior, or bound to the liposome surface, (Torchilin, 2003). Encapsulation has been found to be the most utilised approach as this protects the

oligonucleotide from degradation and also leaves scope for the engineering of specialised delivery opportunities *via* pH mediated release, (Simoes, 2001).

The functional properties of liposomes can also be controlled by altering their chemical properties, selecting cationic, neutral or anionic lipid formulations. Anionic liposome formulations have been found to have limited use in oligonucleotide delivery due to poor delivery and encapsulation ability. This is due to the repulsive electrostatic forces present between the negative phosphate groups of the oligonucleotide and the anionic lipid head groups, as well as the repulsion between the anionic head groups and the negatively charged cellular components. However reports of lipid-polymer hybrid systems have been made in which anionic lipids result in a reduction of cytotoxicity thought in part to be due to the overcoming of the surface charge issue associated with cationic lipid-polymer-DNA complexes. Here the oligonucleotide is complexed typically with a cationic polymer and then coated with an anionic lipid layer.

More successful in this field has been the use of cationic lipid compounds, which have been formulated and well characterised, with a variety of different structural aspects such as head group size and tail length. The mechanism of delivery for these cationic particles is widely accepted to be *via* an endocytic pathway, with oligonucleotides being delivered predominantly to the nuclear compartment. There are a number of commercially available transfection reagents which utilise cationic lipid technology, including: Lipofectin® and Transfectam®. As demonstrated by these commercial reagents, liposomes for use in oligonucleotide delivery are often formulated using cationic and neutral lipids, with the neutral species being referred to as “helper” lipids. These formulations improve stabilisation

at low pH in order to improve endolysosomal escape. Cationic lipids are favoured as the positive charge results in spontaneous electrostatic interactions between the liposome and the negative phosphate groups of the oligonucleotide, forming the resultant lipoplex *via* condensation of the oligonucleotide molecules into a tightly packed structure. The overall positive charge of the lipoplex also facilitates its binding to the negatively charged components which make up the cellular membrane, important for cellular uptake. However the use of cationic lipids also has its disadvantages and they must be used with caution and with appropriate controls as cell growth is inhibited by N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), the cationic detergent in Lipofectin, in a linear dose-dependent fashion in some cell lines, (Yeoman, 1992). Cationic detergents may not be useful in promoting oligonucleotide uptake in all cell types. In one study, keratinocytes were found to take up phosphorothioate oligonucleotides equally well both with and without Lipofectin, (Nestle, 1994).

Irrespective of which lipid technology is utilised, generally for both *in vitro* and *in vivo* applications, smaller particles are preferred for oligonucleotide delivery. Complex size has been shown to be dependent on factors such as the individual lipid size, the positive:negative charge ratio, the ionic strength of the growth medium, and the concentration of all reagents. Doxil, the first approved liposomal product, has stimulated the investigation of new liposome formulations and brought about various products as well as numerous clinical trials, (Sanna, 2014). New successful technologies are constantly emerging with the majority of new discoveries consisting of hybrid technologies, with formulations consisting of both polymeric and lipid components. Many exciting studies have shown the potential for controlled and

targeted release of oligonucleotides using pH sensitive, stealth and immunoliposome systems which could drastically reduce off target affects and improve efficiency, (Immordino, 2006).

Polymers

One highly efficient and widely used subset of transfection reagents are synthetic cationic macromolecules, unlike liposomes they do not contain hydrophobic components and are therefore completely soluble in an aqueous environment. These have been shown to condense genetic material into nanoconstructs for transfection, primarily *via* electrostatic interactions, (Brown, 2001). The condensation of oligonucleotides is extremely important as high molecular weight molecules can be restricted from internalisation due to their size because of the plasma membrane. Recent years have seen a large progression in the development of polymeric delivery reagents with many different derivatives and architectures being investigated with much being learnt about their structure–function relationships. Numerous studies have demonstrated that polymeric reagents have: improved cellular uptake, biodegradability and biocompatibility. A large advantage for polymeric delivery platforms is the possibility to incorporate components such as ligands in order to introduce a more targeted delivery strategy *via* receptor-mediated endocytosis, (Xu, 2014). It has also been demonstrated that it is possible to incorporate molecules to facilitate endosomal escape. Although many derivatives have been tested the main focus of polymeric delivery reagents has been based on a two different chemistries: polyethylenimine (PEI) and poly($_{L}$-Lysine) (PLL), (Nayerossadat, 2012). With the growing understanding of polymer gene-delivery mechanisms and continued efforts of creative polymer chemists, it is likely that polymer-based gene-delivery systems will become an important tool for human gene therapy.

Poly($_L$-Lysine)

Poly($_L$-Lysine) (PLL) polymers were one of the first poly cationic polymers to be employed in gene transfer, (Satchi-Fainaro, 2006). Their cellular delivery capability was first realised in studies evaluating the delivery of traditional small drug molecules, with Hugues ~~et al~~*et al*. demonstrating that cellular uptake of PPL conjugate drug molecules was far in excess of the uptake of free drug. PLL polymers consist of repeating units of amino acid lysine (Figure 1-14), forming linear polymers. The repeating units bond with DNA based molecules *via* electrostatic interaction between the negatively charged phosphate group of DNA and the primary amine group of lysine, which are protonated in a physiological environment. As polypeptide structures, PPL polymers are biodegradable by nature, a positive trait for carrier molecules, however they are also rapidly bound by plasma proteins resulting in a fast clearance from the circulatory system, affecting transfection efficiency. Studies evaluating the transfection efficacy and toxicity of various PLL polymers of different molecular weights found a relationship between the number of repeating units and efficiency, as PLL polymers below 3000 Da could not form stable complexes, (Mintzer, 2009). It has also been shown that increased toxicity is linked with increasing molecular weight of PLL therefore a balance of delivery efficiency and toxicity must be considered, (Wolfert, 1999). In order to address adverse cytotoxicity various derivatives of PPL have been tested, including dendritic PPL, amphiphilic PLL and PLL conjugates with targeting moieties.

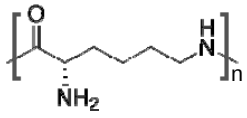


Figure 1-14: Structure of the repeating unit in Poly(L-Lysine).

Okuda ~~et al~~*et al*. reported the synthesis of several generations of dendritic PLL molecules, Figure 1-15, and evaluated their binding and transfection abilities into cells, (Ohsaki, 2002, Okuda, 2004). It was found that low generations, by definition lower molecular weight dendrimers, could not bind DNA whereas generations 5 and 6 had good binding ability even at 1:1 ratio of PLL:DNA. Dendrimers of generations 5 and 6 also demonstrated high transfection abilities into a variety of cell lines with cell *via*bility over 80% with optimised conditions.

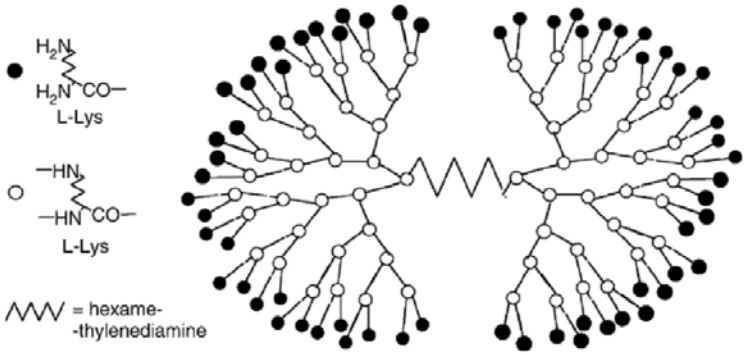


Figure 1-15: Structure of a sixth generation PLL dendrimer. ***(Lv, 2006)***

As mentioned, copolymers of PLL and polyethylene glycol (PEG), Figure 1-16, have also been shown to reduce toxicity without compromising on delivery efficiency, (Brown, 2000). It is thought that the major cause of toxicity of pure PLL polymers is the cationic nature of the complexes surface. In order to mediate this observed toxicity copolymers can be synthesised to reduce the overall charge, reducing the cytotoxicity resulting from the high cationic nature. The addition of PEG results in improved complex stability and reduced inter particle aggregation, prolonged time *in vivo* circulation time and transfection efficiency.

(i) (ii)

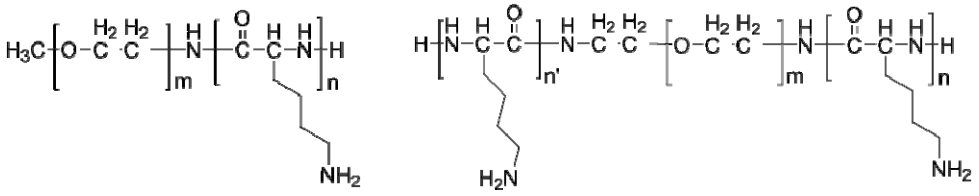


Figure 1-16: (i) Block copolymers or (ii) comb copolymers can be synthesised with a varying ratio of PPL:PEG in order to control the desired properties of the copolymers. (Vanderkerken, 2000).

Finally in order to address the lack of inherent selectively of cell surface binding inherent in non-viral delivery reagents various ligands have been covalently bonded to PLL containing polymers and copolymers, (Vanderkerken, 2000). The conjugation of arterial wall binding peptide, a peptide containing the arterial wall binding domain, resulted in a dramatic increase in transfection efficiency of the aorta cells and smooth muscle cells in a bovine model. Antibody conjugation takes advantage of one of the most specific biological interactions, potentially allowing for directed treatment of cancer cells containing a target surface antigen. Various other conjugates are also being investigated including sugar conjugation and folate conjugation, Figure 1-17, for selective transfection.

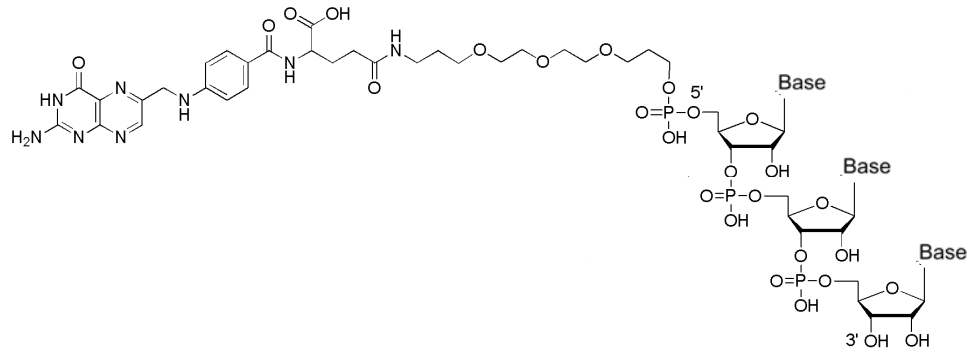


Figure 1-17: 5' folate conjugation is thought to target tumour cells due to the upregulated expression of receptors in tumour tissues, (Puri, 2009).

One of the largest disadvantages of delivery with PPL based polymer is the requirement for endosomolytic or lysosomotropic agents in order to facilitate uptake, (Suhorutsenko, 2011, Shoji-Moskowitz, 2009). These reagents themselves have been shown to demonstrate various levels of toxicity, a significant downside to the use of this type of polymer.

Polyethylenimine

Another well-defined polymeric delivery reagent is polyethylenimine (PEI), a cationic polymer which can form various polymeric architectures, Figure 1-18. PEI has been used for many years in various industries such as paper production, shampoo manufacture and water purification, (Godby, 1999). However, several groups demonstrated the ability of both branched and linear PEI molecules to act as gene delivery reagents, (Boussif, 1995, Boletta, 1997, Ferrari, 1997). Several structural derivitives have been shown to promote efficent cellular delivery of oligonucleotides at various molecular weights without the requirement of additional agents to facilitate uptake, required when using PLL polymers, (Kadlecova, 2013).

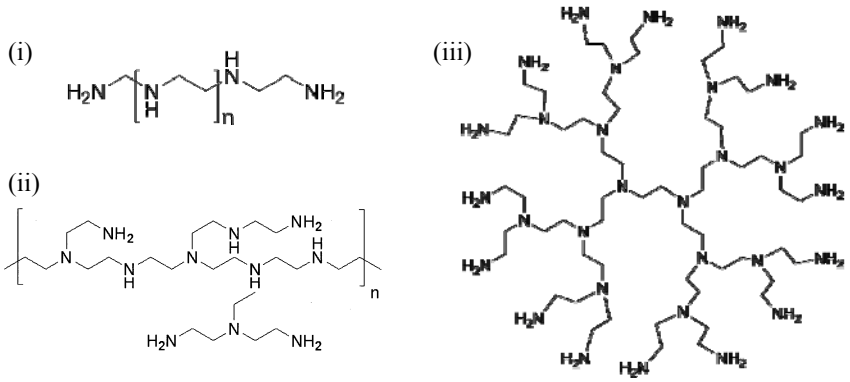


Figure 1-18: Chemical structures of (i) Linear, (ii) Branched and (iii) Dendritic PEI molecules.

As with PLL polymers, PEI molecules have the ability to protect oligonucleotides from degradation upon complexation. The electrostatic interactions between the negatively charged phosphate group of the oligonucleotide and the positively charged amine groups of the PEI monomers again form the basis of this complexation. Polyplexes formed by the complexation of oligonucleotides with PEI molecules retain their overall net positive charge, and it is thought that this promotes interactions between the polyplex and the negatively charged polysaccharides on the cell surface. PEI has been shown to have significantly higher transfection efficiency over PLL owed to a higher buffer capacity over a very broad pH range, (Kircheis, 2001). This buffer capacity is as a result high number of amines present within the molecule, with every third atom on the polymer backbone being a nitrogen atom capable of protonation. Mechanistically the buffering capacity allows for the acceptance of protons, acting as a "proton sponge" within the endosomes or lysosomes, (Boussif, 1995). It has been hypothesised that once electrostatic interactions form between the polyplex and cell surface the polyplexes enter the cell *via* endocytosis. Here the "proton sponge" effect causes an influx of protons and water into the endosome causing swelling and eventual rupture releasing the polyplex into the cell, (Boussif, 1995, Behr, 1997). Although there is a wealth of evidence to support the "proton sponge" hypothesis, a number of publications have challenged its accuracy, (Thomas, 2002, Funhoff, 2004). Seemingly more work is required in order to fully understand how PEI molecules interact with cellular membranes and the endosomal environment in order to design more efficient and less toxic PEI derivatives, (Freemann, 2012).

Previous studies using PEI mediated delivery of oligonucleotides have demonstrated that both cytotoxicity and transfection efficacy were dependent on many factors including

molecular weight and the degree of branching of the PEI carrier, (Wightman, 2001). Of the larger number of PEI reagents available the linear 25 kDa PEI, (Backliwal, 2008, Godbey, 2001, Eberhardy, 2009, Raymond, 2012), and the branched 25 kDa PEI, are the most widely studied. There are conflicting reports in the literature as to which PEI reagents are the most effective for *in vivo* applications, (Goyal, 2012, Kircheis, 2001). One report proposed that linear PEI molecules outperform their branched counterparts *in vivo* due to the higher presence of primary amines reducing complexation strength which allows for more efficient dissociation of complexes, (Wightman, 2001). More recently a study that hyper branched PEI molecules outperformed their linear analogues in transfection tests with COS-7 cells, (Martelloa, 2012). It is feasible that different PEI molecules have a differing delivery capacity based on cell type and delivery environment/method, (Goyal, 2012, Martelloa, 2012).

Although the high degree of cationic charge is thought to be what makes PEI molecules such useful carrier regents, the downfall of such a high charge density is thought to be an increase in toxicity. Godby ~~et al~~*et al*. proposed two distinct mechanisms of PEI mediated toxicity, one immediate and one delayed, (Godby, 2001). Immediate toxicity was reportedly caused by the free cationic PEI molecules interaction with anionic serum proteins resulting in the precipitation of large structures which then adhere to cell surfaces destabilising the plasma-membrane. This was supported by evidence that complexed PEI in polyplexes does not demonstrate the same immediate toxicity, rather a delayed effect related to the release of the oligonucleotides from the polyplex resulting once again in free PEI molecules. Seemingly toxicity can be attributed to free PEI molecules interacting in a biological system however removal of free PEI from polyplexes resulted in a decrease in transfection efficiency,

suggesting that free PEI may be required for successful endosomal release. This theory was supported by evidence that transfection efficiency was restored upon the addition of free PEI to the polyplexes before transfection, (Dai, 2011). Other studies have observed increasing molecular weight of the PEI structures results in higher observed toxicities, (Godby, 1999). It has also been demonstrated that the toxicity of PEI can be reduced by chemical modifications such as PEGylation, (Ouji, 2002).

Numerous research groups are currently trying to combine the effectiveness of PEI delivery whilst lowering the toxicity *via* copolymer synthesis or coated nanoparticle technology as mentioned previously. Over the last decade PEI has seen a large degree of development as a non-viral delivery reagent. Delivery of a plasmid for the treatment of bladder cancer *via* PEI transfect has demonstrated a good safety profile, with no serious adverse event and no disease progression reported, (Matouk, 2008). Zhang ~~et al~~*et al*. also demonstrated the promise of PEI mediated delivery *in vivo*, evaluating the toxicity and production of pro-inflammatory cytokines after siRNA-based therapy *via* PEI transfection, identifying no change serum TNF-α or IFN-α levels compared with glucose-treated mice, indicating a lack of severe immune response, (Zhang, 2013).

Conjugated delivery

As previously eluded to it is seemingly becoming more relevant to talk about successfully delivery constructs rather than single element delivery reagents. Although not used in the present study, due their increasing presence in the literature, details of the most promising are included for completeness. The synthetic work required in order to develop such tools has progressed rapidly thanks to focused collaborative efforts between organic chemists and cell

biologists. The conjugation of various ligand classes to PEI has been studied including: lipids, peptides, polyamines and carbohydrates, (Gao, 2007, Järver, 2012, Morille, 2008). It has been demonstrated that both the stability and bio-distribution of certain oligonucleotides can be enhanced by the attachment of small lipophilic molecules, potentially due to the introduction of hydrophobic character, (Avino, 2014). Conjugation of ligands to antisense agents has also been investigated. A construct containing a 2'OMe phosphorothioate oligonucleotide tethered to cholesterol was found to silence the endogenous human gene encoding apolipoprotein B in a transgenic mouse model, (Soutschek, 2004). Similar findings have been demonstrated using bile acids, docosanyl and stearoyl ligands, (Winkler, 2013). It has also been established that antisense down regulation *in vivo* is dependent on fatty acid chain length, with shorter compounds (less than 18 carbons) unable to reduce mRNA levels compared to their longer counterparts (18-22 carbons), (Winkler, 2013). Peptide–oligonucleotide conjugates have been investigated extensively and show great promise *in vitro*. Widely known as cell-penetrating peptides (CPP) they have been shown to carry both proteins and nucleic acid constructs into cells, (Lee, 2013, Lindberg, 2011). Although the mechanism of uptake is not fully understood, it is thought that CPP's internalisation involves the interaction of the construct with anionic cell surface markers, (Winkler, 2013). Various studies have demonstrated that this technology functions best with neutral oligonucleotides, namely PNA and PMO chemistries, (Winkler, 2013). This observation is thought to be due to the neutral species inability to neutralise the CPPs positive charge, known to be crucial for its activity. *In vivo* studies demonstrated the exon skipping potential of CPP-PMO oligonucleotides when injected intravenously, leading to a significant enhancement of the expression of dystrophin in mdx mice, (Yin, 2007). Polyamines have also found use as delivery conjugates, like peptides they also have the ability to alleviate the negative

consequences of the poly-anionic charge of the phosphate backbone. A publication by Noir ~~et al~~*et al*. demonstrated that covalently linking serval spermine molecules to an LNA oligonucleotides resulted in an increased binding affinity target due to minimising the electrostatic repulsion between the two strands. Again, as shown with peptide conjugation, both binding affinity and target silencing have been shown to be length dependent, (Winkler, 2013), with shorter polyamines lacking silencing capacity *in vitro*. Intuitively, as a large number of pathways for cellular recognition are based on carbohydrate-protein interactions, various studies have been undertaken covalently linking carbohydrate moieties to oligonucleotides, (Davis, 2002). There are currently three siRNA:carbohydrate conjugates developed by Alnylam Pharmaceuticals in Phase I/II clinical trials for the treatments of transthyretin amyloidosis, hypercholesterolemia and haemophilia. Also as this involved siRNA-based drugs and not single stranded oligonucleotides, it demonstrated promise for carbohydrate conjugated oligonucleotides being compatible with RNA interference machinery.

Specific genetic diseases amenable to antisense therapy

There are an extensive number of genetic diseases potentially responsive to antisense therapy, however recent clinical trials have involved a subset of these, namely muscular dystrophies. Muscular dystrophies are a heterogeneous group of disorders which can be caused by various mutations in a wide range of genes. The term “dystrophy” is derived from “dys”, meaning difficult and “trophe”, relating to nourishment, due to such disorders affect skeletal muscles and are accompanied by muscle wasting and weakness of variable distribution and severity. The majority of muscular dystrophies are caused by single gene mutations, making them promising targets for antisense therapy using oligonucleotides.

Myotonic dystrophy types 1 (DM1) and 2 (DM2), and Duchenne muscular dystrophy (DMD) are all caused by single gene mutations, although all in separate genes, and the type of mutation is well understood.

Myotonic Dystrophy type 1

History and nomenclature

Myotonic dystrophy type 1 (DM1) is the most common adult form of muscular dystrophy with an estimated incidence of 1 per 8000 people worldwide, (Emery, 1991). Two independent groups, Steinert and Batten and Gibbs, marked the delineation of DM1 as a disorder in its own right in 1909. Over the course of their careers Steinert, Batten and Gibbs authored numerous reports detailing involvement of non-muscular symptoms. Although DM1 was initially characterised as a muscular disease, subsequent clinical studies have revealed other abnormalities such as insulin resistance, cataracts and cardiac disease, resulting in the disease being re-categorised as a multisystemic disorder, (Harper, 2001).

Even though the clinical picture of DM1 had been well established, with a wide range of symptoms and a highly variable phenotype, the molecular mechanism underlying the disease remained unknown until 1992. Three independent groups identified a trinucleotide CTG expansion as the mutation responsible for DM1 in 1992, the repeat lying in the 3' UTR region of the *DMPK* gene, located on chromosome 19q13.3, (Brook, 1992, Fu, 1992 and Mahadevan, 1992). There was subsequently found to be a high variability in repeat length, with unaffected individuals having 5-37 CTG repeats and individuals affect with DM1 having between 50 and several thousand.

Clinical symptoms

As referenced earlier DM1, is a multisystemic disorder affecting many different functions. The most characteristic features of DM1 and the level of severity are included in Table 1-2. The most characteristic feature of symptomatic myotonic dystrophy is myonia, especially of the grip. Patients are able to form a tight fist when asked but show significant delay upon relaxation, (Harper, 2001). Muscular weakness is another common feature, commonly affecting distal limbs, neck muscles and sometimes proximal limbs. Cardiac defects of varying degrees of severity are observed, with patients commonly presenting with cardiac conduction defects, although SCG abnormalities in most patients are asymptomatic. Cataracts are another common symptom with patients showing obvious abnormalities in old age.

Feature	Severity/Stage
Muscle	
Weakness	
Face	+++
Ptosis	+++
Sternomastoid	+++
Proximal legs	Late
Distal	+++
Any location	+
Muscle pain	+
Myotonia	+
Calf hypertrophy	–
Systemic	
Cataracts	+++
Balding	+++
Cardiac arrhythmias	+++
Gonadal failure	+++
Hypersomnia	+++
Hyperhidrosis	+
Cognitive disorder	+ to +++

Table 1-2. Features of DM1 with their severity, (Turner, 2010).

Variable penetrance and anticipation

Symptoms of DM1 can vary greatly among family members and the disease can be divided into three distinct clinical groups: minimally affected or late onset, classical adult onset, and congenitally affected individuals. These groups are related to the clinical symptoms and age at onset of the disorder. Many patients remain undiagnosed or misdiagnosed due to the symptomatic variability, often only identified upon the birth of a child affected with DM1.

Phenotype	Clinical signs	CTG repeat size‡	Age of onset (years)	Age of death (years)
Pre-mutation	None	38–49	N/A	N/A
Mild/late onset/ asymptomatic	Cataracts mild myotonia	50–100	20–70	60 to normal life span
Classic	Weakness Myotonia Cataracts Conduction defects Insulin insensitivity Balding Respiratory failure	50–1000	10–30	48–60
Childhood onset	Facial weakness, myotonia, psychosocial problems, low IQ, conduction defects	50–1000	1–10	N/A
Congenital	Infantile hypotonia Respiratory failure Learning disability Cardiorespiratory complications in third and fourth decade	>1000*	Birth	45†

Tabel 1-3. Summary of the repeat length and phenotypes associated to each distinct group of DM1 patients.
***typically above 1000 however Redman ~~et al~~ *et al* reported several individuals with a congenital phenotype but with between 730-1000 repeats. ł Data does not include neonatal deaths. ǂ Correlation of CTG length and phenotype can vary.**

The division into the three clinical groups is a result of an important hallmark of DM1, the genetic phenomena known as anticipation, as through successive generations patients demonstrate an increase in disease severity and a decrease in the age of onset, (Harley, 1993, Tsilfidis, 1992). Figure 1-19 demonstrates the disease progression through the generations of two families affected with DM1. Minimally affected individuals are often difficult to diagnose, with onset occurring in middle to old age and being characterised by cataracts with

little muscle involvement. Minimally affected patients often lead fully active lives and have a normal or minimally shortened life span. Classical adult onset occurs in early adult life or adolescence, with patients displaying the classical wide ranging symptoms of DM1 including: cardiac conduction defects, diabetes, grip myotonia and mild muscle weakness. Patients affected with this form of DM1 often find myotonia, (the delayed relaxation of skeletal muscles after voluntary contraction) can interfere with daily life, with many patients becoming severely disabled later in life. A reduced life expectancy has been reported in this group of patients, (Bird, 1999).

Figure 1-19: comparison of clinical groups. A: Grandmother, son and mother (left to right). B: Mother, son and grandmother (left to right). (Left - Engel, 1986) (Right - Jorde, 2000).

Molecular mechanism for pathogenesis

Three models for the mechanism of pathogenesis of DM1 have been proposed; the haploinsufficiency model, the chromatin structure model and the RNA dominance model. Whilst the haploinsufficiency and chromatin structure can both account for certain symptoms of DM1, several studies strongly suggest the model of RNA dominance to be the most

probable mechanism of pathogenesis of DM1. I have briefly explained the theory behind each model below and the evidence supporting or contradicting each model.

Haploinsufficiency model

In this model it is proposed that the CTG repeat expansion in the 3'-UTR of the *DMPK* gene alters *DMPK* expression, reducing the level of *DMPK* protein. The decrease of *DMPK* protein results in a reduction in function, thought to cause the multisystemic symptoms seen in DM patients. This theory was initially supported by a number of expression studies, (Fu, 1992, Hoffmann-Radvanyi, 1993) which showed reduced *DMPK* mRNA and protein levels in patient muscle and cell culture. However a study by Reddy ~~et al~~*et al*, (Reddy, 1996), involving *DMPK* knockout mice to test the hypothesis found that haploinsufficiency of *DMPK* alone cannot account for DM pathogenesis as the mice did not show typical multisystemic features as observed in DM patients only mild, late onset myopathy and cardiac conduction abnormalities. Subsequent studies were carried out using *DMPK* null mice, again these mice failed to show characteristic multisystemic features common in DM1, (Jansen, 1996). Although this model could account for certain observed features in DM1 many of the major characteristics are absent.

Chromatin Structure

It has been demonstrated that DNA with $[CTG]_n$ repeat expansions can alter the chromatin structure, (Liu, 2012) which in turn can alter the expression of not only the gene containing the mutant expansion but also the neighbouring genes. The *DMPK* gene lies within an extremely gene rich region of the DM locus, (Eriksson, 1999). It has been suggested that the

presence of the repeat expansion results in the formation of a more condense chromatin structure, (Otten, 1995 and Wang, 1995) resulting in a hindrance to transcription of *DMPK* and the neighbouring genes, Figure 1-20.

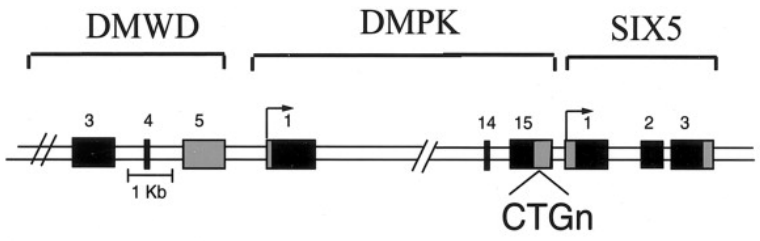


Figure 1-20: *DMPK* and neighbouring genes.

Haploinsufficiency of upstream DMWD, found to be strongly expressed in adult mice brain and testis, and SIX5, involved in photoreceptor development, could explain the multi-systemic nature of features such as cataracts, mental retardation and male infertility, (Jansen, 1995).

The major contradicting insufficiency of this model is its inability to account for the unusual multi-systemic clinical parallels between DM1 and another type of muscular dystrophy, myotonic dystrophy type 2 (DM2) described in detail later in this chapter. The significant number of overlapping symptoms heavily suggests a similar pathogenic mechanism; however as the DM2 locus is mapped to chromosome 3 and not the DM1 region of chromosome 19, it makes it highly unlikely that regional effects of gene expression play a major role in causing the common clinical features of the diseases, (Liquori, 2001). Therefore the chromatin structure model is unable to account for both DM1 and DM2 phenotypes.

RNA gain of function

The RNA gain-of-function hypothesis proposes that the mutant RNA transcribed from the expanded allele is sufficient to induce symptoms of the disease, Figure 1-21.

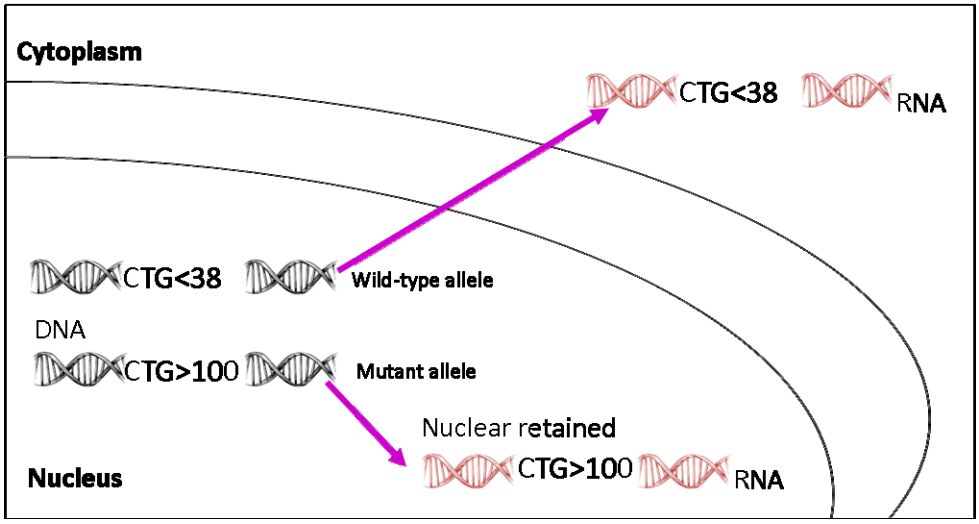


Figure 1-21: RNA containing expanded repeat CUG transcripts are retained in the nucleus of DM1 patient cells.

Taneja ~~et al~~*et al*. first proposed that a gain of function at the RNA level disrupts splicing and reduces nucleo-cytoplasmic mRNA export. Although CTG repeats are located in an untranslated region of the *DMPK* gene, and thus not translated to protein, the mutant repeats are still transcribed to RNA. Studies using fluorescent *in-situ* hybridisation techniques demonstrated that the transcribed transcripts aggregate in the nuclei, forming distinct structures – termed foci, Figure 1-22. (Liquori, 2001 and Taneja, 1995). No foci are seen in the nuclei of unaffected individuals.

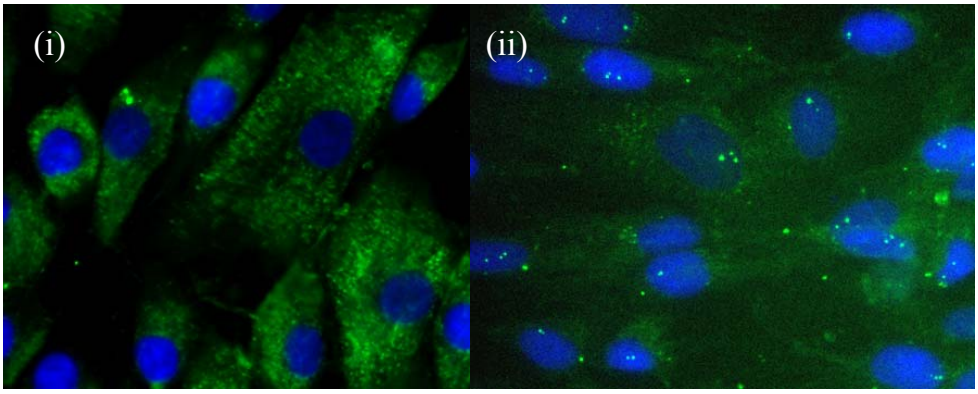
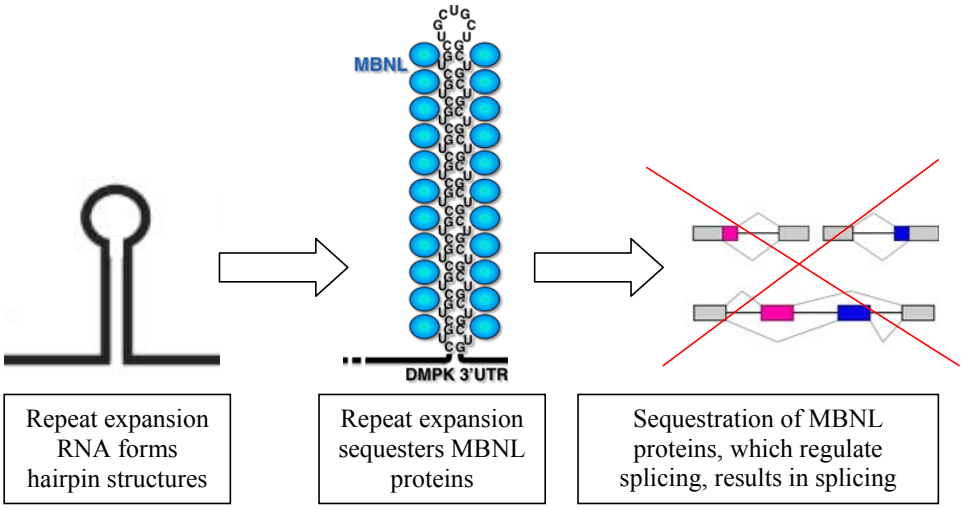


Figure 1-22: Fluorescent microscopy images taken in the Brook lab after in-situ hybridisation with $(CAG)_{10}$ DNA probe of (i) control and (ii) DM1 cells. Blue areas represent Hoechst nuclear stain. Green represents the fluorescence of the oligonucleotide probe.

It was suggested that the concentration of mutant RNA transcripts retained in the nuclei led to abnormal RNA processing which could explain the disease phenotype. In support of this observation Davis ~~*et al*~~*et al* found that mutant *DMPK* transcripts were found exclusively in the nucleus and not in the cytoplasm, (Davis, 1997). Further experimentation demonstrated the nuclear retention of the expanded repeats occurs above a critical threshold between 80 and 400 CTG repeats, (Hamshere, 1997). Studies have shown that RNA containing expanded repeats of pathogenic length fold to form hairpin structures which interact with RNA binding proteins thus preventing these proteins from carrying out their normal cellular functions, Scheme 1-7. Studies have shown that muscleblind-like proteins (MBNL) colocalise with expanded CUG repeats to form these nuclear foci, (Miller, 2000). All three MBNL isoforms (MBNL1, MBNL2 and MBNL3) are sequestered, (Fardaei, 2002), diverting the proteins from the appropriate function. Support for protein sequestration being a feasible mechanism for the diverse symptoms of the disease was presented by Kanadia ~~*et al*~~*et al*. who demonstrated that mice homozygous for Mbnl1 deficiency ($Mbnl1^{\Delta 3/\Delta 3}$) developed myotonia, cataracts and splicing irregularities in ClC-1, TNNT2, and skeletal muscle troponin T

(TNNT3) all of which are phenotypic and molecular changes characterised in DM1 patients, (Kanadia, 2003).

CUG BP1, another RNA binding protein, has also been shown to be sequestered in the nucleus. It has been demonstrated that increased CUG-BP1 levels in DM1 myoblasts are involved in the aberrant splicing of additional genes, (Timchenko, 2001), leading to the multi-systemic phenotype observed. Alteration of activity of these RNA binding proteins can result in a range of splicing abnormalities which could explain the multi-systemic nature of the disease, (Cho, 2007).



Scheme 1-7: Repeat expansion RNA sequesters MBNL proteins resulting in splicing defects.

Current treatment

To date only supportive care is available for DM1 patients, with no curative treatment having been identified. The use of ankle-foot orthoses, wheelchairs, or other assistive devices is typically used to help when muscle weakness gets worse. It is also possible to treat other specific symptoms of DM1 such as myotonia, heart problems, and eye problems with

medication. Although no restorative therapy has yet been approved a number of different strategies are being employed by researchers in order to identify a suitable treatment. For example, overexpression of MBNL1 in HSA^{LR} mice reversed the myotonia and mis-splicing events, thought to be a significant cause of the DM1 pathology (Kanadia, 2006 and Chamberlain, 2012). This was demonstrated using a recombinant adeno-associated viral vector transduced into the skeletal muscle of the mouse model, with Chamberlain ~~et al~~*et al*. establishing that overexpression was well tolerated, premature shifts in alternative splicing of MBNL1-regulated genes in multiple organ systems did not cause embryonic lethality, and MBNL1 overexpression prevented myotonia, myopathy and splicing abnormalities associated with DM1.

Small molecule inhibitors have also been identified which are capable of inhibiting the interaction between MBNL1 and the expanded $(CUG)_n$ repeat, (Gareiss, 2008). Initial work identified three compounds capable of inhibiting the interaction *in vitro*, with further work identifying an additional two compounds which were shown to partially restore missplicing in a DM1 cell model as well as disperse ribonuclear foci, (Jahromi, 2013). Pentamidine, Figure 1-23, another small molecule, was also found to inhibit the interaction between the expanded repeat and MBNL1 *in vitro*, (Coonrod, 2013), and was shown to improve splicing defects associated with DM1.

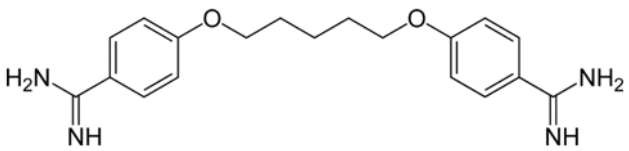


Figure 1-23: Chemical structure of pentamidine.

Antisense therapy using oligonucleotides has also been widely investigated, (Mulders, 2009, Pandey, 2015, Sobczak, 2013, Wheeler, 2012). A 2'OMe oligonucleotide targetted at the $(CUG)_n$ expanded repeat was shown to be capable of degrading the *DMPK* transcript in an RNase independent mechanism *in vitro*, (Mulders, 2009). Wheeler ~~et al~~*et al*. demonstrated the use of gapmer technology in the treatment of DM1, demonstrating rapid knockdown of the expanded *DMPK* RNA in skeletal muscle of the HSA^{LR} mouse model. Physiological, histopathologic and transcriptomic features of the disease were shown to be corrected with therapeutic effects being sustained for up to a year after the discontinuation of treatment, (Wheeler, 2012). More recently an Isis Pharmaceuticals oligonucleotide (ISIS 486178) demonstrated potent activity *in vitro* and *in vivo* against mouse, monkey and human *DMPK*, (Pandey, 2015). It was reported that the oligonucleotide, which utilises gapmer technology, when delivered systematically to wild-type mice decreased *DMPK* mRNA levels by up to 90% in liver and skeletal muscle. Systemic delivery to either human *DMPK* transgenic mice or cynomolgus monkeys also led to up to 70% inhibition of *DMPK* in multiple skeletal muscles and ~50% in cardiac muscle in both species, (Pandey, 2015). The first clinical trial involving a drug specifically designed for the treatment of myotonic dystrophy was announced by Isis Pharmaceuticals in collaboration with Biogen Idec in late 2014. The Phase I/II trial is said to contain 36 adults who will be randomly assigned to receive subcutaneous injections of the antisense drug or a placebo for six weeks.

All of the methods described demonstrate potential for the treatment of DM1 with important findings continually providing promising therapeutic strategies. Undoubtedly more drugs for the treatment of DM1 will soon be being assessed in clinical trials.

Myotonic Dystrophy type 2

History and nomenclature

Before the identification of the mutation responsible for DM1, (Brook, 1992), genetic heterogeneity in myotonic dystrophy (DM) was unsuspected. Shortly after genetic testing became available however another dominantly inherited form of DM, which lacked the CTG expansion at 19q.13.3, was described by various research groups. These patients presented with similar symptoms, but demonstrated significantly less muscle weakness which occurred in proximal muscles. Initially known as proximal myotonic myopathy (PROMM) or proximal myotonic dystrophy (PDM) due to the muscle types involved, the substantial clinical overlap of the new syndrome with DM1 led to a revision of the nomenclature and the disease is now identified as myotonic dystrophy type 2 (DM2). On a molecular level the mutation responsible for DM2 was mapped to a 3 cM region at 3q21, with further experimentation later identifying a tetra nucleotide expansion as the causative event, (Liquori, 2001). Using positional cloning an unstable CCTG repeat expansion was identified in the first intron of zinc finger protein 9 (ZNF9). Further investigations using genetic testing has defined the expansion threshold in unaffected individuals to be approximately 70 repeats. Within the affected population the range of expanded allele sizes is extremely broad, reaching up to ~11 000, with a reported average of ~5000 repeats, (Margolis, 2006). Unlike the pattern observed in DM1, there is a distinct lack of correlation between repeat size and disease severity, with larger repeats not resulting in progressively severe pathogenic effects.

Clinical symptoms

The phenotypic manifestations of DM1 and DM2 are very similar and due to the multi systemic nature of myotonic dystrophy affected individuals often show varied symptoms. At the time of diagnosis most patients, affected by DM1 or DM2, have clinically identifiable muscle involvement. The significant clinical difference involves the type of muscle affected. DM1 patients show distal muscle involvement, severe muscle atrophy and symptomatic finger flexor weakness, whereas DM2 patients show more proximal muscle involvement. A comparison of the clinical features in DM1 and DM2 is detailed in Figure 1-24. Patients are often clinically diagnosed with DM2 after presenting with hip girdle muscle weakness, where climbing stairs or arising from the floor becomes problematic, although the majority of patients also demonstrate weakness at neck flexors and deep flexors of the thumb and lateral digits, similar to DM1, upon testing, (Day, 2004). In DM2 patients manual skills remain largely intact and there is no prominent late weakness of the bulbar, facial or respiratory muscles. There are however reports of very severe variants of the disease in which fatal cardiac complications have occurred coupled with serious muscle weakness, (Schoser, 2004). A key variant between the two types of DM is the presence of a congenital form of the disease. To date no congenital cases have been reported in DM2, (Cardani, 2012), though it is possible that very large CTGG expansions in the DM2 gene have lethal effects, therefore terminating pregnancy.

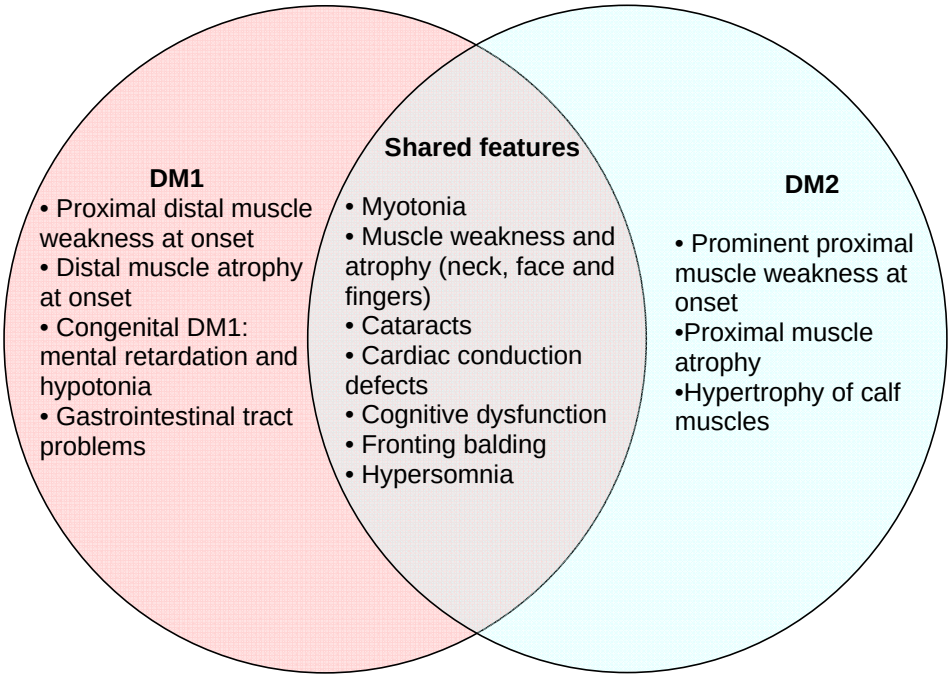


Figure 1-24: Venn diagram demonstrating the shared clinical symptoms of DM1 and DM2.

Another marked symptom characteristic of DM2 is muscle pain, presenting prior to any apparent muscle weakness or wasting. Overall it is currently considered that the clinical course of DM2 is more favourable than that of DM1 as in general, it is much less severe, the onset is later and the progression is slower than in adult-onset. DM1. Except for families with severe cardiac conduction abnormalities, the life span of a DM2 patient is normal. As with DM1, the large repeat $[CCUG]_n$ expansions were demonstrated to form multiple discrete foci under fluorescence in-situ hybridization conditions when a $(CAGG)_{10}$ probe was used, Figure 1-25. It was also observed that MBNL1 co-localised with the expanded RNA, (Cardani, 2012).

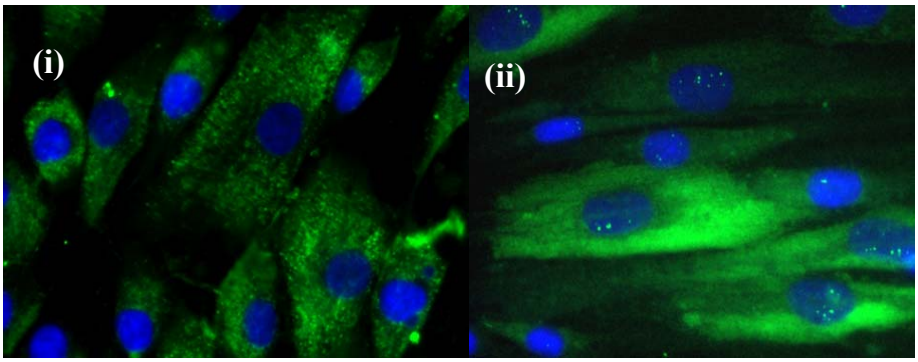


Figure 1-25: Fluorescent microscopy images taken in the Brook lab after in-situ hybridisation with (CAGG)10 DNA probe of (i) control and (ii) DM2 cells. Blue areas represent Hoechst nuclear stain. Green represents the fluorescence of the oligonucleotide probe.

Penetrance and anticipation

Ascertainment of the frequency of DM2 within the global population has proved difficult due to the large variation in clinical symptoms and severity. Initially it was proposed that fewer than 2% of patients with DM had DM2, (Fardaei, 2002). Recently however, although generally still considered much more infrequent than DM1, preliminary data on DM2 suggests that both types share a similar prevalence within certain populations, (Thornton, 2014). As a genetic disorder, prevalence can vary significantly in different populations, especially in genetic isolates; Suominen ~~et al~~*et al*. found a remarkably high frequency 1/1830 of the DM2 mutation within the Finnish population, (Suominen, 2011). Unlike DM1, anticipation in DM2 is the exception and not the rule, an as of yet unexplained phenomenon.

Molecular mechanism for pathogenesis

The molecular and clinical parallels between DM1 and DM2 strongly indicate that the clinical features common to both diseases including myotonia, muscular dystrophy, cataracts, cardiac arrhythmias, insulin insensitivity and diabetes, and testicular failure are caused by the

pathogenic effects of RNA containing the CUG and CCUG expansions rather than any haploinsuffiency mechanism. The fact that two repeat sequences located in entirely different genes can cause such similar disease features strongly implies a common pathogenic mechanism by RNA gain-of-function. It was expected that due to the presence of expanded RNA foci that the splicing pattern of ZNF9 pre-mRNA would be affected, however two independent studies failed to detect this, (Margolis, 2006 and Botta, 2006). It has been propsed that the foci observed may be pure CCUG repeated, formed after excision of the mutant intron 1 of ZNF9, (Margolis, 2006). Defining the downstream differences between the CUG and CCUG expansions will be important for fully understanding any mechanistic variances between DM1 and DM2, potentially explaining the milder phenotype shown in DM2.

Current treatment

As with DM1 there is currently no cure and clinicians can only treat the symptoms of the disease as they manifest. A correct diagnosis is just as important in DM2 as DM1, again to ensure patients are appropriately treated, ensuring correct management of the disease in order to avoid possible complications of DM2 such as heart conduction defects. Historically, more is known about DM1 than DM2, (Ulane, 2014), therefore it is comprehensible that progress towards the treatment of DM1 is more advanced. It is hoped any breakthrough in the treatment of DM1 will be applicable to DM2 due to their shared mechanism of action.

Duchenne muscular dystrophy

History and nomenclature

Duchenne muscular dystrophy (DMD) is one of the most common and severe forms of muscular dystrophy, affecting approximately 1 in 3500 males born each year, (Engel, 1986). The disease caused by mutations, most often deletions in the dystrophin gene, resulting in DMD patients producing a severely truncated protein, (Hoffman, 1987). As an X-linked recessive disorder it rarely affects females and female carriers are either asymptomatic or minimally affected, (Juan-Mateu, 2012). This is thought to be due to the ability of the functional dystrophin copy on their second X chromosome compensating for the defect. The disease drastically influences affected individuals' life span, with men inheriting the condition usually only living into their 20s or 30s. Although first described in the mid-1800s by Meryon ~~et al~~*et al*, (Laing, 2011), it was not until much later that the genetic basis of the disease was known with identification of the molecular mechanism of pathogenesis being initiated by the isolation of the wild-type DMD gene. Important prerequisites for this discovery were ascertaining the localization of the genetic locus within the X chromosome, accomplished *via* the analysis of rare female DMD patients who demonstrated X-autosome translocations, the breakpoint of which was thought to be near the DMD gene, (Boyd, 1986), and using random cDNA fragments to undertake linkage analysis (Davies, 1983). These experiments indicated in agreement that the DMD gene was likely to be located in the Xp21 region on the short arm of the X chromosome. There is currently no effective long-term treatment available, with supportive therapies such as artificial ventilation being able to moderately prolonged patients' survival, (Scheuerbrandt, 2009).

Clinical symptoms

DMD is one of the most devastating muscular dystrophies, with affected individuals exhibiting no obvious clinical symptoms until the age of 3 to 5 years. The first symptoms present as proximal muscle weakness, often identified when patients have difficulty rising from the floor or climbing the stairs. Diagnosis is often clinically confirmed by largely elevated serum creatine kinase levels, a muscle biopsy which confirms fibrosis and an electromyogram. The prognosis is bleak, with muscle weakness being progressive in a high number of muscle groups, affected individuals are often unable to walk past the age of 12, (Whitehead, 2006). As muscles are replaced with fibrous/fatty tissue patients succumb to cardiac or respiratory failure by the mid to late 20s, although with adequate supportive treatment life expectancy can be increased into the early 30s. Although early reports are conflicting, (Sherwin, 1961 and Lincoln, 1977), there is now an overwhelming amount of evidence in support of significant cognitive observations. The average boy with DMD has an I.Q of just 85. Deficits in verbal expression and memory have also been reported alongside underperformance in reading, mathematics and spelling, (Anderson, 2002).

Molecular mechanism for pathogenesis

As previously cited DMD is caused by a defect in a single gene located at Xp21, the dystrophin gene. When transcribed correctly dystrophin strengths muscle cells by anchoring elements of the internal cytoskeleton to the surface of the membrane, Figure 1-26. Without dystrophin muscles are susceptible to mechanical injury and undergo repeated cycles of necrosis and regeneration until their regenerative capabilities are exhausted or inactivated, (De Palma, 2014).

The expression of dystrophin is regulated by three individual promoters which splice unique first exons with the other remaining 78 exons, for activity in the brain, muscle and Pukinje neurones, Figure 1-27. Further internal promoters also express other isoforms, producing smaller dystrophin proteins, however it is the complete absence of the 427 kDa isoform which is attributed to causing DMD, as patients who transcribe shorter or mutated dystrophin often have a milder phenotype which is recognised as a different type of muscular dystrophy known as Becker muscular dystrophy (BMD), discussed later, (Koenig, 1989).

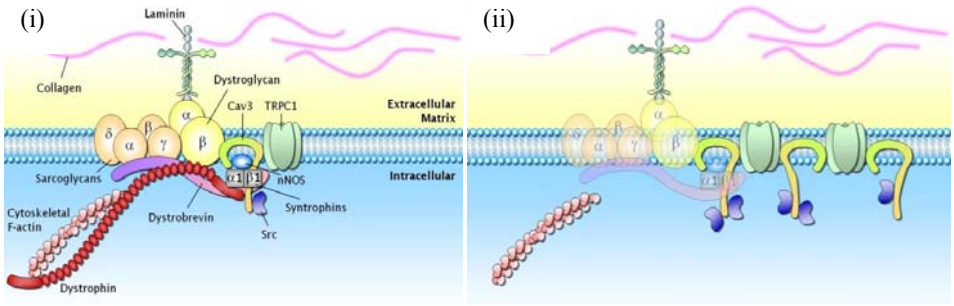


Figure 1-26: Schematic representation of (i) dystrophin linking the internal cytoskeleton to the surface membrane in unaffected individuals, (ii) the result on the membrane when there is a lack of functioning dystrophin. Figure taken from "Duchenne muscular dystrophy--what causes the increased membrane permeability in skeletal muscle?" Allen, 2011.

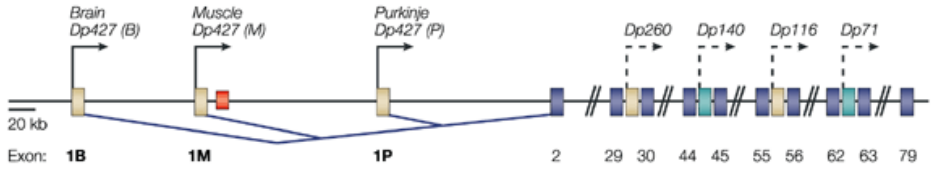


Figure 1-27: The organization of the exons within the gene encoding dystrophin, Arrows indicate transcription start sites. Figure taken from "Pharmacological strategies for muscular dystrophy" Khurana, 2003.

Mutation in the dystrophin gene can be caused via a number of different mechanisms, detailed in Table 1-3. Deletions represent the largest subgroup of DMD mutations and

although there are a number of different sites where deletions can take place the major DMD deletion "hot spot" which has been found in a large cohort of DMD patients has been identified between exons 45 and 53.

Mutation Class	Total	%
Deletion	284	44
Stop	176	27
Subexonic	69	11
Exonic duplication	87	14
Splice	22	3
Missense	2	<0.1
Other	2	<0.1

Table 1-3. Summary of mutations detected in DMD patients, (Flanigan, 2009).

The 427 kDa isoform of dystrophin consists of four structural domains: the amino-terminal actin binding domain, the large rod domain, the cysteine-rich domain and the carboxy-terminal domain, (Campbell, 1995). The previously mentioned mutations lead to the production of dystrophin without the cysteine-rich domain and the carboxy-terminal domain meaning that dystrophin can no longer link together the necessary compartments of the muscle, Figure 1-28.

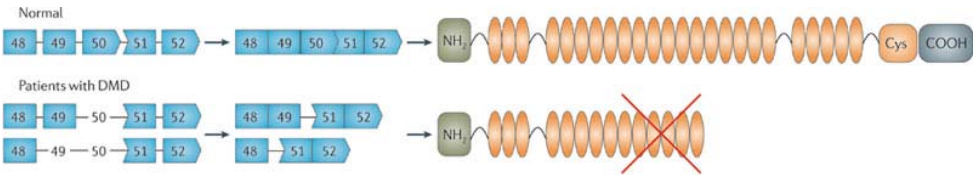


Figure 1-28: Schematic representation of the most prominent exon mutations involved in the causation of DMD, resulting in the formation of dystrophin protein without the cysteine-rich domain and the carboxy-terminal domain. Figure taken from "Targeting RNA to treat neuromuscular disease" Muntoni, 2011.

Evidence established over the course of a decade identified dystrophin to be a membrane cytoskeletal protein, with biochemical experiments identifying the tight association of dystrophin with sarcolemmal glycoproteins, (Campbell, 1989, Ervastic, 1991). One of the most important discoveries on the path to understanding DMD was in the deduction that dystrophin complexes with several novel sarcolemmal protein and glycoprotein components, forming a dystrophin associated protein complex (DAPC), (Ervasti, 1993). It is now understood that dystrophin links with the DAPC in various ways, firstly dystrophin connects the subsacolemmal cytoskeleton to the sarcolemmal through interactions between F-actin and its amino-terminal domain. Secondly dystrophin links the glycoprotein complex through its carboxy-terminal domain. Dystrophin also directly associates *via* interactions through its carboxy-terminal domain with the syntrophin triplet. Together the associated DAPC and dystrophin provide a bridge between the extracellular matrix and the F-actin cytoskeleton. Based on current understanding it is thought that the lack of dystrophin results in the disruption to DAPC assembly, as it has been demonstrated that in the absence of dystrophin mRNA levels of the components of DAPC are normal but the complex is not correctly formed, (DUNANT, 2003). Although these finding are well documented the exact role of dystrophin and the mechanisms by which its absence leads to the observed phenotype are still ambiguous. Various hypotheses have been proposed, and the current accepted theory is that a combination of different mechanisms may contribute to the observed phenotype, including: reduced membrane stability, reduced nitric oxide levels and Ca^{2+} dysregulation.

Membrane stability

Based on the current understanding, in the muscle of non-affected individuals, the DAPC, by linking the cytoskeleton and extracellular matrix, confers mechanical stability to the plasma

membrane. In patients afflicted with DMD the lack of dystrophin results in the muscles becoming susceptible to mechanical stress leading to tearing of the plasma membrane due to the lack of stabilisation. Evidence to support this has come from a number of sources, including: raised levels of muscle-specific proteins in blood, (Blake, 2002), entry of membrane impermeable dyes when muscles are subjected to repeated contractions (Wallace, 2009) and dystrophin deficient muscle fibres of mdx mice (a well-established DMD mouse model) show an escalated vulnerability to exercise induced membrane rupture, (Wallace, 2009).

Nitric oxide levels

As previously cited, the lack of dystrophin protein results in misassembly of the DAPC components leading to instability of the plasma membrane. Besides providing mechanical stabilisation, the DAPC is also responsible for recruiting neuronal nitric oxide synthase (nNOS) to the sarcolemma to produce the signalling molecule nitric oxide (NO), through the conversion of L-arginine (L-Arg). In the absence of dystrophin nNOS detaches from the sarcolemma reducing NO production leading to a restriction in blood flow due to compromised vascular relaxation. Although decreased NO production is known to be linked to cardiomyopathy, an important cause of death in DMD patients, little is known about the NO signalling pathway in the cardiac muscle. Recently Ramachandran ~~et al~~*et al*. reported that dystrophin and nNOS do not co-localize at the membrane of WT mouse cardiomyocytes (Ramachandran, 2013), concluding that the physical proximity of dystrophin and nNOS is not a factor in nNOS regulation, suggesting an indirect although efficient role for dystrophin in modulating nNOS activity. Interestingly, transgenic nNOS expression and sildenafil treatment, which enhances NO signalling, led to multiple benefits, including reduced fibrosis and inflammation, and the subsequent improvement of electrocardiography data. Even

though the direct mechanisms of correction are not understood, the findings suggest a biochemical compensatory mechanism of the NO pathway and a potential entry site for therapeutics.

Ca^{2+} ion dysregulation

The exact involvement of calcium and its role in the pathogenesis of DMD is not fully understood, it is proposed that an imbalanced in Ca^{2+} ions cycling through the sarcolemma and the sarcoplasmic reticulum may contribute to enhanced degradation of muscle proteins leading to necrosis. The involvement of Ca^{2+} ions in the development of DMD assumes a connection exists between the degeneration of sarcolemmal integrity and Ca2+ ion dependent proteolysis, Figure 1-29 outlines the main mechanistic roles played by Ca^{2+} ions.

In brief, the rupturing of the plasma membrane results in the activation of a Ca^{2+} dependent membrane resealing process at the sites of tearing. Ca^{2+} leak channels present during the resealing process causing an increased influx of Ca^{2+} ions into the cytoplasm of dystrophic fibres, rendering the dystrophin deficient muscle fibres more susceptible to necrosis. Ca^{2+} influx gives rise to a pathophysiological cycle as Ca^{2+} influx causes increased protein hydrolysis, and further protein breakdown causes increased Ca^{2+} influx. Studies using dysferlin-null mice support the theory of involvement of Ca^{2+} ion influx. These mice maintain a functional DAPC, resulting in a fully supported plasma membrane, however lack dysferlin protein, involved in surface membrane repair. It was found that dysferlin deficient muscle fibres were defective in Ca^{2+} dependent sarcolmma resealing, and, even though they maintain a functional DAPC, they developed progressive muscular dystrophy upon the disruption of their muscle membrane. This study clearly demonstrated the importance of an efficient Ca^{2+} dependent sarcolemma resealing process to counter-act injuries to the muscle

plasma membrane. It has been proposed that preceding this cycle further Ca^{2+} handling proteins, such as nicotinic acetylcholine receptors and transient receptor potential channels, are involved, although this is yet to be fully understood. The involvement of other proteins does not challenge the overall concept that intracellular Ca2+ imbalance contributes to DMD development.

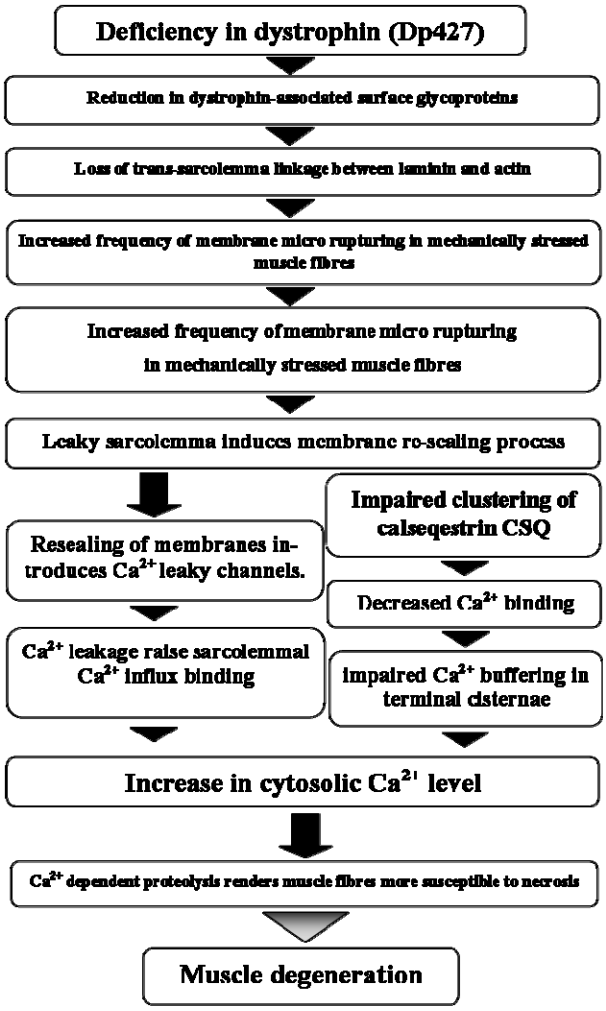


Figure 1-29: Calcium's role in inflammation leading to phenotypic symptoms of DMD.

Current treatment

The current clinical treatment for DMD is limited to managing the disease symptoms, including corticosteroids to improve muscle strength and function, mechanical respiratory support due to deterioration in respiratory muscles and cardiac assessment as a result of the high incidence of cardiac complications. Although there have been major advances into the pathogenic mechanisms of DMD there is still currently no cure. However thanks to multidisciplinary medicine including input from numerous scientific research organisations, the emergence of encouraging novel therapeutic from the antisense field is bringing hope to many. Although there are numerous strategies for treating DMD being investigated by researchers the five seemingly most promising are detailed below.

Viral gene therapy

As previously described viral vectors can be engineered to carry genetic information of interest in order to produce required protein. As the lack of dystrophin is seen as the major cause of DMD researchers have attempted to defined the critical components of the dystrophin protein for its function in order to produce a truncated version of the gene which can be inserted in the viral capsid, (Odom, 2011). The adeno-associated virus has shown promise in a number of *in vivo* murine model studies including induction of a truncated dystrophin into the tibialis anterior, diaphragm and heart in young mdx mice (Bowles, 2012). The treatment also showed positive effects in old mice, where muscle function was severely deteriorated, suggesting the possibly of improving muscle pathology even after extensive damage. However the treatment failed to restore heart function, a major disadvantage when cardiac involvement is linked to a high number of deaths in DMD patients, (Verhaert, 2010). Although positive results have been seen in smaller animal studies a higher level of toxicity

has been observed in larger animal studies leading to questions being raised of the safety of this course of therapeutic action regarding immunogenicity and problematic repeat administration.

Cell based therapy

In the late 1980s experiments in the mdx mouse model demonstrated the potential of cell transplantation in order to produce dystrophin expression in muscle cells. It was demonstrated that by injecting dystrophin producing cells into pre-existing mdx muscle fibres it was possible to render many fibres dystrophin positive and partially or wholly rescue the mice from biochemical defect, (Partridge, 1989). Clinical trials based on this technology were completed in the early 1990s, however no clear functional benefit was observed in the injected muscles, (Cossu, 2007). Adult derived stem cells have also been invesitgated in pre-clinical animal model studies, potentially opening up new treatment avenues, although early trials were again documented as largely inefficient, (Péault, 2007).

Stop codon read though

One of the major causes of DMD is nonsense mutations which result in the insertion of a premature stop codon resulting in the premature translational termination of the dystrophin protein. Ataluren, formally known as PTC124, was conditionally approved by the EU in 2014 to treat patients with this type of mutation. The orally bioavailable drug is currently in Phase (III) clinical trials, (Therapeutics, 2014). Mechanistically it works by selectively inducing a ribosomal read-through of premature but not normal termination codons, (Welch, 2007). It is thought that if successful the drug will have broad clinical potential for the treatment of various diseases caused by mutations which insert premature stop codons.

Utrophin up-regulation

Utrophin is a large protein expressed in high abundance during muscle development and, like dystrophin, is a large protein. In DMD patients, where dystrophin is absent, utrophin is seen to be upregulated, however not enough to compensate for the lack of dystrophin. It has been demonstrated in mdx mice that the up-regulation of utrophin with small drug molecules can dramatically reduce the observed muscular dystrophy pathology, (Manzur, 2008). Currently Summit Therapeutics has a lead compound (SMT C1100) in Phase (Ib) clinical trials.

Exon Skipping

One of the most common causes of DMD is the deletion of exons from the dystrophin gene. Using antisense oligonucleotides it has been demonstrated that it is possible to skip mutated exons in order to restore the reading frame, (Tidball, 2003). Although this would result in the formation of a truncated dystrophin protein, studies have shown that it is functional. It has been estimated that over 70% of DMD suffers could be treated by skipping just 12 exons, (Cossu, 2007). The skipping of exon 51 is thought to be the most abundant mutation in DMD sufferers and therefore initial exon skipping therapies have targeted this sequence. Preclinical trials in mdx mouse models were encouraging, (Lu, 2003), using a variety of chemically modified oligonucleotides including 2'OMe phosphorothioate backbone agents and PMO molecules, (Tidball, 2003, Alter, 2006). More recently two drugs based on this technology have been in clinical trials, (McClorey, 2014). Drisaperse, (GSK and Prosensa's 2'OMe based oligonucleotide) demonstrated great promise in pre-clinical and earlier Phase (I/II) trials, (Goemans, 2011). Despite this encouraging early results the outcome of Phase (III) trials reported a failure to meet its primary endpoint of a significant improvement in the six-minute walk distance test, typically used as a diagnostic and output measure in such

studies. Earlier clinical outputs of etiplirsen (Sarepta Therapeutics Inc's PMO based oligonucleotide) demonstrated a marginal in dystrophin positive fibres however as before no significant difference in the six minute walk test was observed between treatment and placebo groups, (McClorey, 2014).

Aim of the study

As discussed there are currently no globally clinically approved drugs for the treatment of DM1, DM2 and DMD. Numerous studies by various research groups have demonstrated the potential of antisense oligonucleotides in the treatment of these diseases. It has also been discussed that different chemical modifications afford different mechanisms of action. Therefore the aim of this study was to understand the mechanism by which different chemically modified oligonucleotides (CMOs) act and to identify suitable reagents for the treatment of diseases with nuclear retained transcripts.

The aim of the first part of this thesis (chapter three) was to test four different CMOs in order to identify any difference in the gymnotic delivery potential to cells from patients with DM1, DM2 and DMD. The aim of the second part of my thesis (chapter four) was to investigate the use of PEI molecules as delivery reagents as it had previously been established that oligonucleotides do not reach the cell nucleus when delivered gymnotically. The aim of the final part of this thesis (chapter five) was to initially investigate what effect the delivery of various CMOs by PEI transfection had on the *DMPK* transcript. However findings resulted in the investigation of the effects of PEI on CUG containing transcripts.

Chapter two: Materials and Methods

All experiments were carried out under standard conditions: room temperature, typically 18-22 °C (unless stated) and atmospheric pressure.

Cell Culture

All cell culture work was carried out inside a laminar flow cabinet in a tissue culture room.

Cell lines

Cell lines used in this study include human fibroblast cells from DM1 (KB-Telo MYOD), DM2 (KAGO-Telo), and a wild type cell line (SB-Telo MYOD), and have been reported previously, (Ketley, 2013). One human Duchenne muscular dystrophy fibroblast culture was a gift from Denis Furling, (UPMC University of Paris). Becker muscular dystrophy and Bethlem myopathy cells were obtained *via* a material transfer agreement from the University of Newcastle commercial cell bank.

Media and solutions

Cells were cultured in Dulbecco's minimum essential media (DMEM, Gibco life technologies), supplemented with 10% (v/v) foetal bovine serum (FBS) EU approved origin (Gibco BRL) and 1% (v/v) penicillin-streptomycin (Gibco BRL).

Additional solutions: Phosphate buffered saline (PBS) (Gibco BRL), was used cell washing during sub-cultures. 0.25% trypsin-EDTA solution (Sigma), was used in the passaging

process to removed adherent cells from the surface of tissue culture treated plastics. Dimethyl sulphoxide (DMSO) (Sigma) was used in cell cryopreservation.

Passaging of cells

Cells were visualised under the microscope in order to ascertain their degree of confluence. Once cell confluence reached 80%-100% cells were split at a ratio appropriate for the speed of growth of the cell line. All solutions used in the sub-culture process were incubated at 37 °C before use. Media was removed and cells were washed once with 10 mL of PBS. PBS was replaced with 2 mL of trypsin-EDTA. Flasks were then incubated at 37 °C for 2-5 min in order to detach cells from the bottom of the flask. Cells were then re-suspended in 12 mL of new media and mixed thoroughly to give a homogenous solution. Cells were then split into new sterile 75 cm^3 flasks at an appropriate density, and new media added to make the total volume up to 20 mL.

Cryopreservation of cell lines

Adherent cells were dissociated using trypsin and re-suspended in 10 mL of media and pelleted by centrifugation at 1000 rpm for 5 min. Cell pellets were then re-suspended in 1 mL of media containing 10% DMSO (v/v), transferred to a cryogenic vial (Nalgene) and immediately stored at -80 °C for 24 hr. Finally the cryogenic vial was transferred to liquid nitrogen for long term storage.

Cell defrosting

Cryogenic vials containing cells were removed from liquid nitrogen and thawed quickly by immediate incubation in a water bath (37 °C). Once thawed the cell solution was immediately

transferred into a 20 mL universal tube containing 10 mL of pre-incubated media (37 °C). Cells were pelleted by centrifugation at 1000 rpm for 5 min, the media replaced by 10 mL of new media and pelleted again. This media was replaced once again with new media and the cell solution was transferred into a sterile 75 cm^3 flask. The cell solution was made up to 20 mL and the flask incubated at 37 °C to allow growth.

Chemically modified oligonucleotides

Purchased oligonucleotides

Fluorescently labelled and unlabelled oligonucleotides were purchased from various sources as listed below. Morpholino oligonucleotides were purchased from GeneTools. PNA and LNA oligonucleotides were purchased from Eurogentec. DNA oligonucleotides were purchased from Sigma Aldrich. 2'OMe oligonucleotides were either purchased from Eurogentec or synthesised as described below.

Oligonucleotide synthesis

Equipment

Oligonucleotides were synthesised on an ABI 394 DNA/RNA synthesiser.

Reagents

Solid phase support columns (SynbaseTM CPG 1000 Å, 0.2 M), standard DNA and 2'OMe phosphoramidites and reagents for the synthesiser were purchased from Link Technologies Ltd. Ammonium hydroxide (33% in ethanol) was purchased from Sigma Aldrich. Oligonucleotide purification cartridges (OPCTM) were purchased from Applied Biosystems UK. Acetonitrile was distilled over CaH_2 and stored over 4Å molecular sieves under argon.

Deblock mix (3% trichloroacetic acid in dichloromethane), BTT activator (0.3M 5-benzylthio-1-*H*-tetrazole in acetonitrile, anhydrous), Cap Mix A (THF/pyridine/acetic anhydride = 8:1:1), Cap Mix B (10% methylimidazole in THF) and sulphurising reagent 0.05 M were also purchased from Link Technologies Ltd.

Purchased Phophoramidites

2'OMe reagents used: 5'-(4,4'-Dimethoxytrityl)-N-benzoyl-adenosine,2'-O-methyl, 3'-[(2-cyanoethyl)-(N,N-diisopropyl))]–phosphoramidite. 5'-(4,4'-Dimethoxytrityl)-N-acetyl-cytidine,2'-O-methyl, 3'-[(2-cyanoethyl)-(N,N-diisopropyl))]–phosphoramidite, 5'-(4,4'-Dimethoxytrityl)-N-dimethylformamidine-guanosine,2'-O-Methyl, 3'-[(2-cyanoethyl)-(N,N-diisopropyl))]-phosphoramidite. DNA reagents used: 5'-(4,4'-Dimethoxytrityl)-N-benzoyl-2'-deoxyadenosine,3'-[(2-cyanoethyl)-(N,N-diisopropyl))]–phosphoramidite, 5'-(4,4'-Dimethoxytrityl)-N-acetyl-2'-deoxycytidine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl))]–phosphoramidite, 5'-(4,4'-Dimethoxytrityl)-N-dimethylformamidine-2'-deoxyguanosine,3'-[(2-cyanoethyl)-(N,N-diisopropyl))] phosphoramidite. All were purchased from Link Technologies Ltd.

Cell treatment

Cells were treated with a range of chemically modified oligonucleotides in 96 well plates, 25 cm^3 or 75 cm^3 flask format *via* various methods.

Gymnosis

Cells were plated 24 hr before treatment. In a centrifuge tube the required volume of stock oligonucleotide (100 μM) was added to the fresh medium, and mixed well to form a homogenous solution. Growth medium was removed and cells were washed once with 10 mL of PBS. The treatment medium was then added directly to the cells and the cells returned to the incubator for the required time period before analysis.

PEI transfection

Cells were plated 24 hours before treatment. The required volume of stock oligonucleotide (100 μM) was added to the require volume of stock PEI reagent (10 μM) in a suitably sized centrifuge tube and homogenised. Complexation between the oligonucleotide and PEI reagent occurred appropriately at room temperature in 15 min. After this time the required volume of fresh medium was added to the centrifuge tube and again the solution homogenised. After medium removal cells were washed once with PBS and then the treatment medium was then added directly to the flask/plate and the cells returned to the incubator for the required amount of time before analysis.

MTT assay

The MTT assays were performed as first described by Mosmann, (Mosmann, 1983) with any modifications detailed below. In brief, cells were seeded in Corning® Costar® 96-well cell culture plates at 6000 cells per well. After 24 hours incubation at 37 °C, cells were treated with the required reagents for the experiment. After the required time period for the experiment the treatment medium was removed. Cells were washed once with PBS. A stock solution of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was added

to fresh culture medium at a final concentration, 0.5 mg/mL and 100 µL of this solution added to each well. The plate was incubated for 4 hr at 37 °C (the time period was reduced to 2 hr for very dense cells) to allow for crystal formation. After the required time period the MTT containing solution was removed carefully, so not to disturb the crystals, and 150 µL of DMSO added. After 5 min incubation at room temperature, samples were homogenised by pipetting in order to fully dissolve all crystals. The optical density was measured at a wavelength of 560 nm. A well with DMSO alone was measured and this blank reading was subtracted from all values.

RNA fluorescence *in-situ* Hybridisation

RNA-FISH analysis was performed to identify RNA transcripts with expanded repeat units that appear as nuclear foci.

Seeding of cells

Cells were seeded in Corning® Costar® 96-well cell culture plates at 6000 cells per well. After 24 hours incubation at 37 °C cells were ready for treatment.

Fixation

Treatment media was removed and cells washed three times with PBS (5 min intervals between washes) before the fixation solution was added. Cells were fixed with 4% paraformaldehyde (w/v) in PBS. 50 µL was added to each well of the plate and incubated at room temperature for 30 min. Cells were subsequently washed three times with PBS to remove the fixing agent.

Permeabilization and hybridisation

Cells were permeabilised using 50 µL per well of 80% ethanol (v/v) in water for 15 min at room temperature. Cells were subsequently washed three times with PBS to remove the ethanol. Cells were then prepared for hybridisation by incubation in pre-hybridisation solution (50 µL per well, Table 2-1) for 15 min at room temperature. The cells were then exposed to hybridisation solution (50 µL per well) containing the correct fluorescent DNA oligonucleotide probe for the RNA target ($[CAG]_{10}$ for the DM1 cell line, $[CAGG]_{10}$ for the DM2 cell line) for 16-24 hr at 37 °C.

Formulation for 10 mL		
Prehybridisation solution	Chemical	Hybridisation solution
4 mL	Formamide	4 mL
1 mL	20X Saline Sodium Citrate	1 mL
5 mL	DEPC H_20	5 mL
	Bovine Serum Albumin	20 µL
	ssDNA	100 µL
	Oligonucleotide probe	5 µL

Table 2-1: Formulation of prehybridisation and hybridisation solutions for *in-situ* hybridisation protocol.

After the hybridisation period cells were washed three times with 5mM $MgCl_2$ in PBS to remove the hybridisation solution. This was followed by Hoechst staining using 0.1 µg/mL Hoechst in 5mM $MgCl_2$ in PBS for 15 min. The Hoechst solution was then replaced with 5mM $MgCl_2$ in PBS and the plate imaged using a molecular devices micro high content imaging system under two distinct wavelengths for Hoescht and the fluorescent probe.

Nucleic Acid Amplification

RNA extraction

RNA samples were obtained from cultured fibroblast cells. Cells from 75 cm^3 flasks were trypsinised and cell pellets obtained by centrifugation. All equipment was sterilised with a solution of 70% ethanol and DEPC water to avoid RNase activity. Cell pellets were washed twice with PBS before being re-suspended in 400 µL DEPC-treated water in a 1.5 mL micro-centrifuge tube. 100 µL of lysis solution was added, followed by 800 µL of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 solution (Sigma Aldrich) and the solution vortexed for 20 seconds. The solution was then centrifuged at 12 000 rpm for 10 min and the aqueous phase transferred into a fresh 1.5 mL micro-centrifuge tube. To the aqueous phase was added another 800 µL of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 solution, and the same vortex and centrifuge steps followed. For a second time the aqueous phase was transferred into a fresh 1.5 mL micro-centrifuge tube followed by the addition of 800 µL of isopropanol and 100 µL of 3M sodium acetate. The resulting solution was then homogenised and incubated at -20 °C for an hour before being centrifuged at 4 °C for 30 min at 12 000 rpm. The supernatant was carefully removed from the RNA pellet, and 1 mL of 80% ethanol added. After inverting the tube several times it was again centrifuged at 12 000 rpm for 30 min before finally drying the pellet and re-suspending it in the required volume of DEPC water.

Reverse transcription

In order to produce cDNA for downstream experiments, total RNA was reverse transcribed using the following protocol and reagent: 2 µg of total RNA, 2 µL random hexamers, 8 µL dNTPs were combinded in a 200 µL tube and the total volume made to 20 µL with SWD. The solution was incubated at 70 °C for 5 min. 2 µL of RNase inhibitor and 4 µL of the reverse transcription buffer were added and the solution homogenised. Half the sample was then aliquoted into a separate 0.2 µL tube for use as a negative control. 1 µL of reverse transcriptase was then added to the positive sample. Both tubes were incubated at 42 °C for 90 min.

Primer design

The design of oligonucleotides to be used as primers was assisted by Primer 3 software, Whitehead Institute for Biomedical Research (Rozen).

Polymerase Chain Reaction (PCR)

PCR reactions were performed in volumes of 10 and 20 µL, Table 2-2, dependent on the amount of material required for downstream applications. All reactions were carried out in autoclaved 200 µL tubes. Thermal cyclers equipped with heated lids were used. Amplification parameters were manipulated for individual primer sets. Standard cycling conditions were typically: initial denaturation step at 95 °C for 3-5 min, denaturation step at 95 °C for 0.5 min, annealing step at individualised T_a for 0.5 min, extension time of 0.5-2 min at 72 °C, product specific cycle number and a final extension step lasting 3 min at 72 °C.

Each PCR reaction contained:

	10 µL reaction	20 µL reaction
Forward primer 10 µM (Sigma)	1 µL	2 µL
Reverse primer 10 µM (Sigma)	1 µL	2 µL
Megamix (Microzone)	7.5 µL	15 µL
cDNA template	0.5 µL	1 µL

Table 2-2: Reagents and the volumes of which make up the PCR reaction.

Northern blotting analysis

A modified northern blotting protocol has been used to identify the subcellular presence of oligonucleotides.

Sub cellular fractionation

In order to separate nuclear and cytoplasmic cellular compartments sub-cellular fractionation was carried out. Briefly, after the required treatment time, cells were washed three times with ice cold PBS, and a minimal amount of 10% Nodient p-40 was added in order to lyse the cytoplasmic membrane. After 3 minutes the supernatant was transferred to a microcentifuge tube, this being the cytoplasmic fraction. A further 1 mL of Nodient p-40 solution was then added to the flask and the nuclei detached from the flask. The nuclear fraction was transferred to a 15 mL centrifuge tube with the flask washed twice with ice cold PBS and the washings added to the tube. The nuclear solution was then centrifuged for 10 min at 3500 g in order to pellet the nuclei. Once pelleted, 400 µL of DEPC water was added along with 100 µL of lysis buffer and the solution transferred to a microcentifuge tube. 1 µL of proteinase K

was added to each of the nuclear or cytoplasmic fractions. RNA/DNA was then extracted using the same protocol as previously mentioned.

Sample preparation

Gel Loading Buffer, Novex® Tris/Borate/EDTA(TBE)-Urea Sample Buffer (2X), 8 µL was added to RNA/DNA samples (~50 ng) and the total solution made up to 14 µL. Samples were denatured (95 °C, 10 min) then chilled on ice for 5 min.

Denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis was performed using commercial reagents supplied by Invitrogen. 200 mL of 5X Novex® TBE Running Buffer was added to 800 mL of SDW to give a final concentration of 1X. Pre-cast 15% Novex® TBE-Urea Gels were removed from packaging, the comb removed and all wells rinsed with 1X running buffer. The gel was assembled in a Biorad cell surelock mini cell system. The upper and lower chambers were filled with 1X running buffer to a level which covered all wells. RNA/DNA samples were removed from ice and loaded. The gel was run at a constant voltage of 200 V for 1 hr.

Transfer to positive nitrogen membrane

After separation by electrophoresis, RNA/DNA samples were transferred to a positive nylon membrane. Casting plastic was removed from the gel and the gel washed with TBE buffer for 5 min. Meanwhile the required size membrane and 6 sheets of 3MM Whatman paper were cut to the size of the gel and equilibrated in TBE for 5 min. Starting from the side of the positive electrode of the Biorad transfer station, three sheets of Whatman paper, the nylon

membrane, the gel, and an additional three sheets of Whatman papers were layered to form a paper–membrane–gel–paper sandwich. Once assembled, using a glass sterile pipette, any bubbles between the gel, membrane and papers were rolled out to ensure reliable transfer of the entire membrane. Sample transfer was carried out at 25 V for 30 min. After transfer the membrane was exposed to UV light in order to cross link the DNA/RNA samples to the positive nylon membrane. The membrane underwent UV cross-linking on both sides at 254 nm with an exposure of 250 milliJoules/cm^2.

Hybridisation

After completion of cross-linking, the membrane was rolled with RNA/DNA side facing inwards and inserted into a hybridization bottle. 10 mL of ULTRAhyb hybridization buffer (Ambion®) was added to the bottle and the membrane underwent pre-hybridisation at 37 °C for 30 min in a rotating hybridization oven. To probe the membrane for the presence of the treatment oligonucleotide, an LNA/DNA oligonucleotide complementary in sequence was synthesised by Exiqon to be used as a probe. The probe oligonucleotide was digoxigenin (DIG) labelled using a DIG labelling kit (Roche) following the manufacturer's instructions. Once labelled the probe was denatured at 95 °C for 1 min and added to the hybridization buffer already in the bottle to yield a final concentration of 0.5 nM. The membrane was hybridised at 37 °C for 4-12 hr with slow rotation.

Washing, blocking and detection

All solutions were prepared as instructed by Kim ~~et al~~*et al*, (Kim, 2009). After hybridisation was complete, the hybridisation solution was discarded and membrane washed twice with low stringent buffer (10mL) at 37 °C for 15 min. The membrane was then washed twice with

high stringent buffer (10mL) at 37 °C for 5 min and finally rinsed with washing buffer (10mL) at 37 °C for 10 min. After washing the membrane was incubated in blocking buffer (10mL) overnight at 4 °C. The next day the blocking buffer was discarded and replaced with a solution consisting of DIG antibody and blocking buffer at a ratio of 1:15,000. The membrane was incubated at room temperature for 30 min, before being washed four times in DIG washing buffer (10mL) for 15 min (1 hr total). Next, the membrane was incubated in development buffer (10mL) for 5 min before being removed from bottle with clean tweezers and placed on saran wrap. Substrate solution was prepared by adding disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD, Roche) to development buffer at a ratio of 1:100. The substrate solution was then carefully applied to the cover the entire membrane (2.5 mL) and incubated at room temperature for 5 min. After this time excess substrate solution was removed from the membrane by blotting with Whatman paper. The membrane was then sealed in saran wrap and incubated at 37 °C for 10 min. The membrane was finally exposed to X-ray film (Fuji) and digital images were captured using a ChemiDoc-IT Imaging System which detects photoemissions from the membrane.

DNA manipulations

Agarose gel electrophoresis

Gels were formed at a range of agarose percentages (1-3%) depending on the size of the DNA product(s) to be analysed. The gels were prepared using analytical grade agarose in 1X TAE buffer (40 mM Tris-HCl, 50 mM EDTA, pH 8.0, 0.1142% (v/v) glacial acetic acid) followed by microwave heating. Once slightly cooled 3 µL of ethidium bromide solution (10 mg/mL) was added and uniformly dispersed through the gel. 10X DNA loading dye (10X

TAE, 2% (w/v) glycerol and bromophenol blue) was added to each DNA sample for a final concentration of 1X, prior to loading. Gels were run at a constant voltage of 80-110 V (depending on the gel percentage and fragment size of the DNA) and the DNA products visualised using a BioRad GelDoc system.

Gel extraction

In order to extract DNA from agarose gel for sequencing, the QIAGAN Gel Extraction Kit was used in accordance with the instructions provided by the manufacturer.

In brief, DNA bands were visualised on a UV transilluminator, excised from the gel with a sterile scalpel and placed into a pre weighed micro-centrifuge tube. The excised gel fragment was dissolved in the required amount of buffer (weight dependent) containing guanidine thiocyanate. Once entirely dissolved isopropanol was added and the entire solution applied to a QIAquick silica column and centrifuged. Salts were washed away by application of an ethanol containing buffer to the column followed again by centrifugation. The DNA was eluted from the column using the elution buffer provided into a sterile micro-centrifuge tube.

Sequencing

DNA samples were sent to The University of Nottingham's sequencing lab. Sequencing was performed on an ABI Prism 377 automated sequencer using both forward and reverse primers for each sample.

DNA digestion

BPMI restriction enzyme was used according to the manufacturer's specification. For complete digestion to take place, samples were left at 37 °C overnight.

Measurement of nucleic acid concentration

All extracted RNA, DNA and synthesised oligonucleotide samples were quantified using a Nanodrop spectrophotometer. Each sample was measured 3 times and an average taken.

Microscopy

Control microscopy was carried out using a Zeiss LSM Exciter microscope. Wildfield microscopy was carried out using a Zeiss Molecular Devices Micro High Content Imaging system.

Chapter Three: The delivery of various chemically modified oligonucleotides *via* gymnosis.

Introduction

A number of different chemically modified oligonucleotides (CMOs) have been introduced in chapter one alongside selected methods for the transfection of cultured cells. As discussed, it has previously been concluded that passive diffusion of oligonucleotides across the cellular membrane was an unlikely mechanism of uptake (Akhta, 1991). This was in part due to the large size and polar nature of the oligonucleotide species, and the polarity of the phospholipid bilayer which makes up the cellular membrane. Therefore, for antisense technology to be utilised, it was assumed that the intracellular delivery of oligonucleotides required some form of transfection method whether it be electroporation, chemical transfection or *via* viral vector.

There are a number of serious drawbacks associated with the use of transfection methods. The use of viral vectors have had a problematic past, the disastrous ornithine transcarbamylase trial in 1999 made it apparent that non-toxic delivery *via* vector based methods was highly patient specific due to the degree in variability between immune responses in different individuals, (Thomas, 2003). Overall the use of viral vectors has shown promise, however the variability of immune response is still a large hurdle to overcome.

In response to the observed problems with viral vectors, work on non-viral vector development has created a number of different delivery methods and reagents, (Akhta, 2000). However even though delivery *via* non-viral based methods are seemingly a feature of a large

number of publications in the fields of genetics, biochemistry, molecular biology and developmental biology, optimisation of such methods is lacking when concerned with an understanding of toxicity.

Contrary to wide held scientific beliefs it has recently been shown that oligonucleotides have the potential to cross cellular membranes using the normal growth properties of the cells to promote intercellular uptake, (Stein, 2010). The establishment that delivery of oligonucleotides can occur without the need for any transfection additives was of significant interest, in particular to the drug discovery and development community, as it removes the risk of delivery systems resulting in undesirable side effects. The group did note that very little nuclear delivery was observed in comparison to good cytoplasmic uptake. For a large number of disease types cytoplasmic delivery sufficient for treatment, however efficient nuclear internalisation of oligonucleotides is paramount in diseases where the target RNA resides within the nuclei.

In this chapter, I study the gymnotic delivery of a variety of CMOs to human fibroblast cells from DMD, DM1 and DM2 patients, as well as a control cell line. Although the complete picture is still unknown as to the exact cause of DM1 and DM2, the molecular basis of pathogenesis for both disease types is thought to involve a dominant RNA gain-of-function. This model developed from the observation that large RNA transcripts, (containing CUG or CCUG repeats, for DM1 and DM2 respectively) aggregate within the nuclei of cells forming structures know as foci. It has previously been observed within the Brook lab that 2'OMe and PNA oligonucleotides, delivered gymnotically, can remove nuclear foci in DM1 cells when targeted at the repeat sequence. Interestingly it was also observed that morpholino and DNA

oligonucleotides with the same sequence had no effect on foci, Figure 3-1. This work was carried out by Ami Ketley and is unpublished at the time of writing.

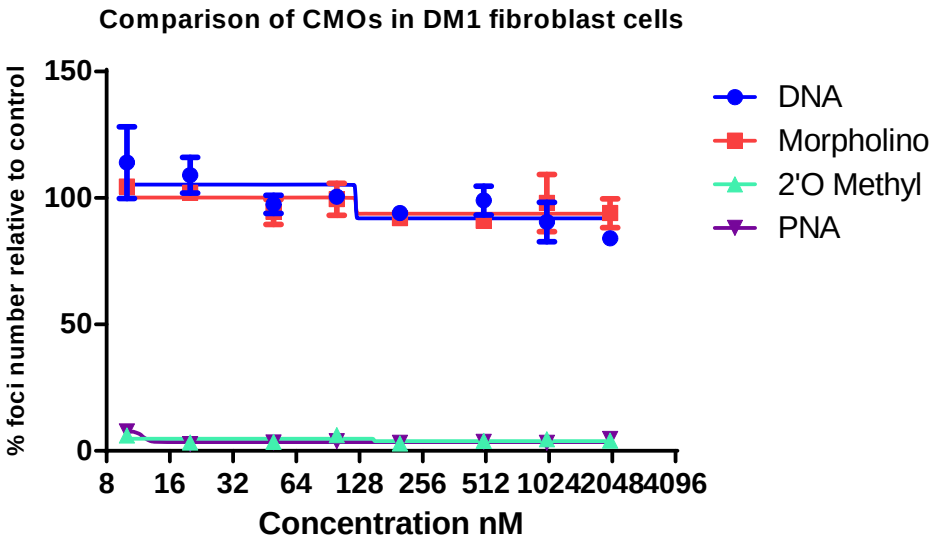


Figure 1-3: Comparison of the removal of nuclear foci in DM1 fibroblast cells after treatment with various chemically modified oligonucleotides, with the sequence $(CAG)_7$, at a range of concentrations. X axis represents the concentration of the oligonucleotide in nM. Y axis represents the percentage number of nuclear foci remaining compared to untreated control cells. All oligonucleotides used were purchased, see supplementary information for table. All oligonucleotides were 21 nucleotides in length.

Although unsure of the mechanism, in order for these oligonucleotides to be acting upon the nuclear foci they must be penetrating the nuclear membrane without the requirement of transfection reagent. Additionally 2'OMe oligonucleotides of the same sequence have also been shown to degrade *DMPK* transcripts which contain the repeat, (Mulders, 2009), although these experiments were carried out with the use of a transfection reagent.

The use of oligonucleotides to treat DMD is also well established. It has long been inferred that DMD cells are more permeable to oligonucleotides, potentially as a result of leaky membranes. Early literature precedent used electron microscopy studies to demonstrate focal disruptions of the surface membranes of muscle cells, (Mokri, 1975). Experimentally the increased membrane permeability has been repeatedly confirmed by studies in which markers which are normally membrane impermeant, such as albumin and Evans Blue dye, can be found inside muscle fibres, (Allena, 2011). It has been hypothesised that this increased permeability of the muscle membrane may allow for easier access of antisense agents, increasing the efficacy of treatment.

Aim of study

Oligonucleotides have long been hailed as an ideal therapeutic strategy for a number of genetic disorders due to their sequence specificity arising from complementary base pairings formed though hydrogen bonding. Fundamental in working towards a human therapy containing antisense technology is the understanding of the delivery of these molecules across cellular membranes.

Certain chemistries of oligonucleotides have previously been shown to successfully cross the cell membrane without the aid of transfection reagent, with 2'OMe oligonucleotides specifically demonstrating the ability to remove nuclear foci gymnotically, Figure 3-1. It is therefore assumed that 2'OMe oligonucleotides cross both the outer cellular and the nuclear membrane *via* gymnosis.

The initial aims of this work will be to identify any difference in cellular uptake between the range of CMOs in the foci assay to establish whether the variance in foci disruption in linked to different delivery profiles. An increased general understanding of oligonucleotide delivery *via* gymnosis in our disease models will come as a result of testing a variety CMOs in a range of cell lines: DM1, DM2, DMD and a control line. I hope to identify suitable conditions for nuclear penetration, an important requirement in these disease types, and identify whether the increased membrane permeability of DMD cells supports increased oligonucleotide uptake.

Results

2'OMethyl oligonucleotide delivery

Initial experiments were carried out using a synthesised 2'OMe phosphorothioate (PS) oligonucleotide labelled with a hexachlorofluorescein (HEX) fluorophore. This oligonucleotide has the sequence $(CAG)_7$ and was targeted at the DM1 repeat sequence. As previously mentioned 2'OMe oligonucleotides have been shown to degrade mutant *DMPK* transcripts in various DM1 models when combined with a transfection reagent, (Mulders, 2009). 2'OMe oligonucleotides have also been shown to penetrate the cell *via* gymnosis and remove RNA foci at low concentrations. It is therefore feasible that 2'OMe oligonucleotides could be used therapeutically without the need of transfection reagents, making them a more promising choice for treatment.

Comparison of oligonucleotide delivery in diseased and unaffected cell lines

In order to investigate whether 2'OMe oligonucleotide delivery varies between the different disease models selected, live cell imaging was performed using confocal microscopy in DM1, DM2, DMD and a control cell line. As shown in Figure 3-3, after treatment with the fluorescently labelled hexachlorofluorescein (HEX) 2'OMe PS oligonucleotide (lab synthesised), fluorescent signal from the oligonucleotide (green) appeared predominantly cytoplasmic in all of the disease models tested, no over lapping with the nuclear staining of Hoechst (blue).

Although the images evidently suggest the absence of oligonucleotide in the nuclear compartment, in order to gain a quantitative measure of fluorescent intensity the images were analysed using MetaExpress software which allowed for the nuclear and cytoplasmic areas to be defined independently. After imaging using the plate reader the high-throughput software used the presence of Hoechst staining (represented in blue) above a set intensity threshold to create an oval mask of the nuclear area and gave the average fluorescent intensity from the oligonucleotide (represented in green) within this area. The cytoplasmic intensity was defined as an area a set distance outside the nuclear mask dependent on nuclear size. An example of this concept is demonstrated in Figure 3-2. Using this technique allowed for the measurement of hundreds of cells per treatment, ensuring a robust data set.

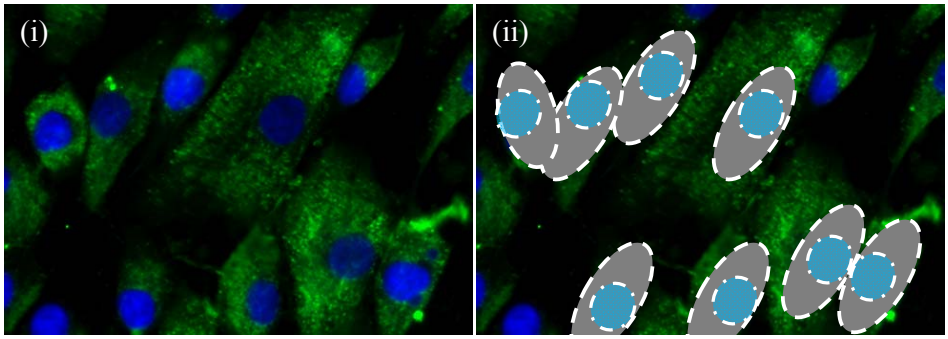


Figure 3-1: i) Images captured using high throughput microscopy, ii) Illustration of how the software creates masks identifying the nuclear (seen in blue in ii) and cytoplasmic areas (seen in grey in ii) of the cell. The fluorescent intensity within each area was recorded and analysed.

There was mp statistically significant difference in fluorescent intensity arising from the fluorescently labelled oligonucleotide in the nuclear compartment when treated cells were compared to untreated controls, Figure 3-4. This quantified data supported the evidence from the confocal images, demonstrating that *via* gymnosis HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotides do not permeate the nuclear membrane.

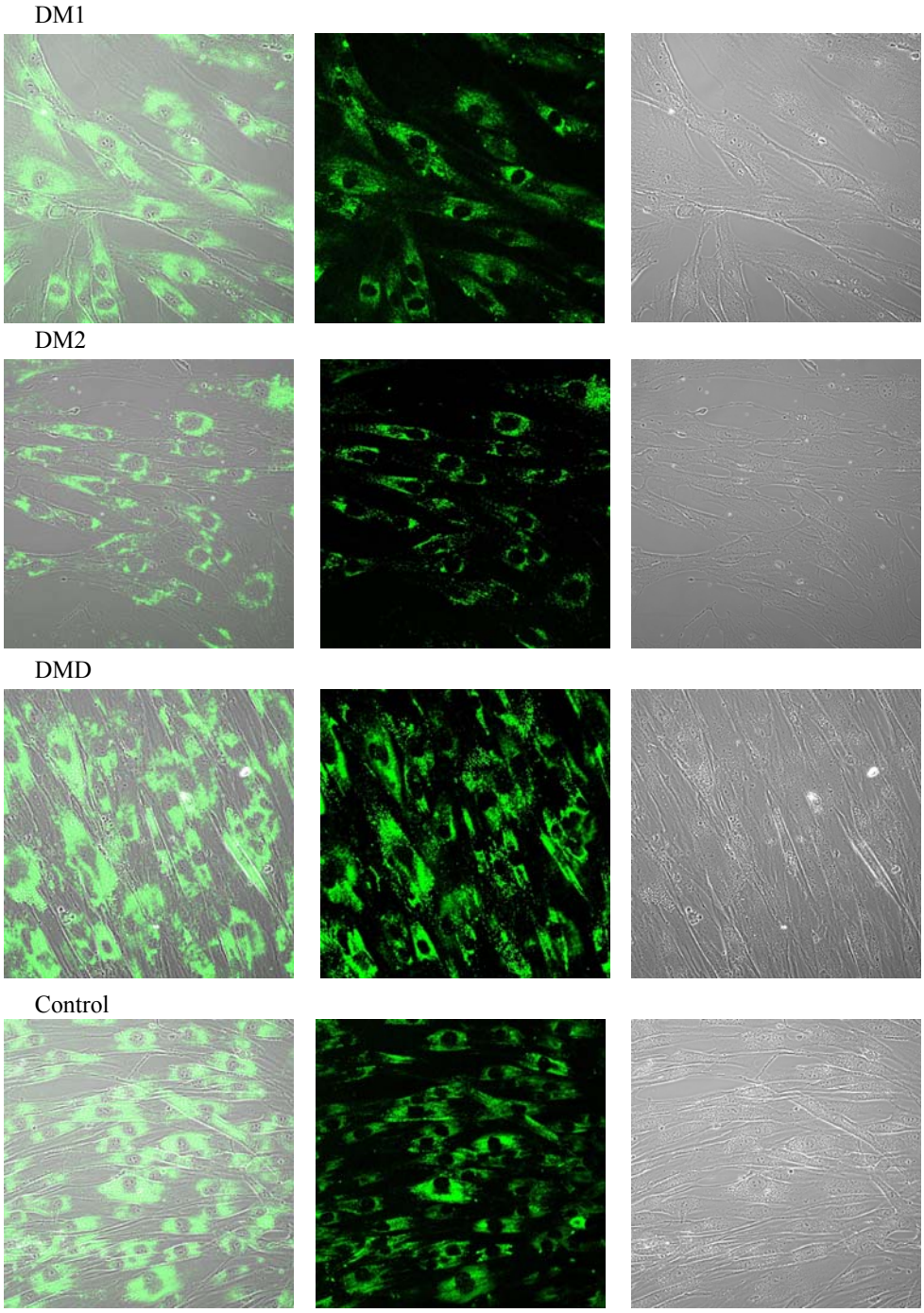
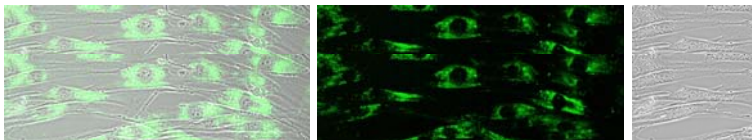


Figure 3-2. Combined, fluorescent and phase images of live cells from various disease models after treatment with HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotide, 500 nM, *via* gymnotic transfection for 48 hrs. Oligonucleotide fluorescence is identified as the green signal.



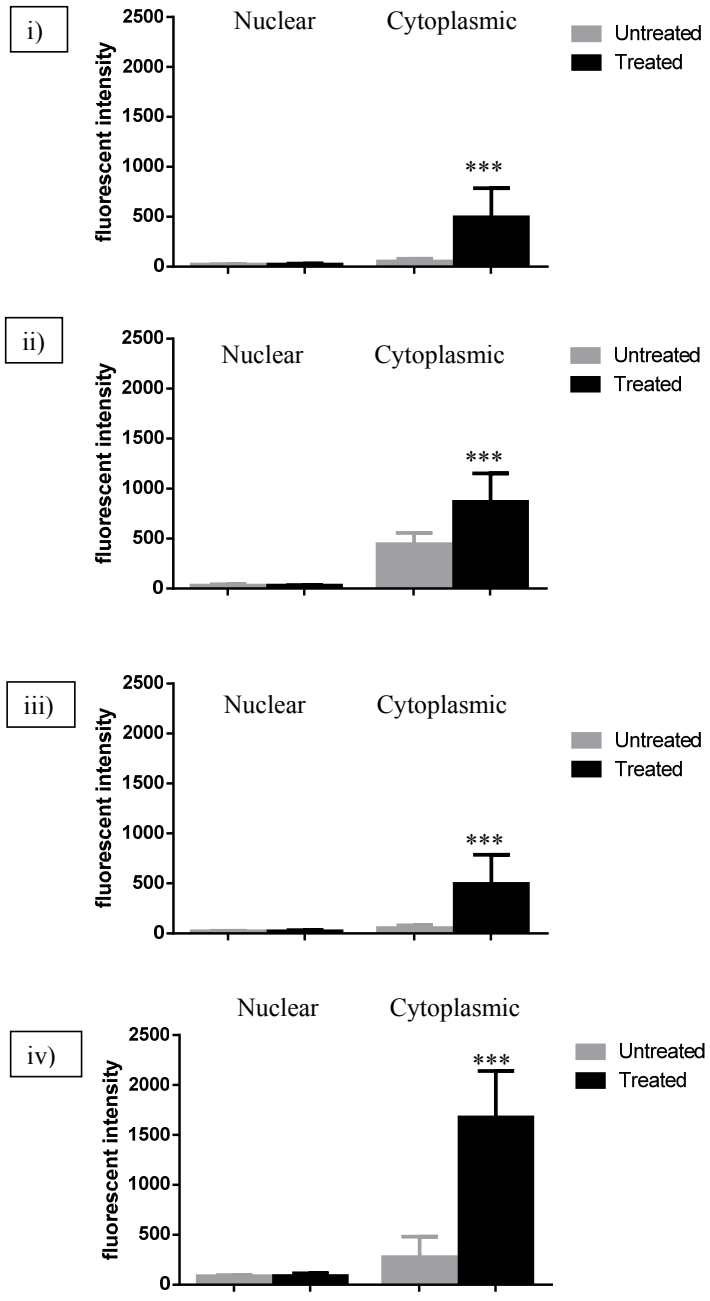


Figure 3-4. Quantification of the compartmental fluorescent intensity from live cells treated gymnotically for 48 hr with HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotide separated by location in live cells. i) DM1, ii) DM2, iii) DMD, iv) Control cells. ($P < 0.001$, $n > 100$).

Identification of oligonucleotide localisation *via* northern blot analysis

The uptake of oligonucleotides gymnotically is thought to be *via* endocytosis. Although the exact proteins and recognition sites are unknown it is highly likely that chemical structure influences membrane recognition, which is paramount for successful delivery. Live cell imaging requires the addition of a fluorophore molecule to the oligonucleotide in order to visualise its location within the cell. The Northern blotting method allows the intracellular location of the oligonucleotide to be identified without the use of microscopy and therefore does not require the treatment oligonucleotide to carry a fluorophore. For these experiments cells were treated with an oligonucleotide which did not have a fluorescent molecule attached in order to confirm that the fluorophore had not impeded nuclear delivery. This would experimentally give the best view of the delivery potential *in vivo* as fluorescent molecules are only required for diagnostic purposes and not for treatment.

To validate the observations made by live cell imaging, the distribution of 2'OMe oligonucleotide between the cytoplasm and nuclear compartments was examined after treatment and cell fractionation. The experiment was carried out in DM1, DM2, DMD and control cells. Approximately 2^6 cells were treated with 2'OMe PS oligonucleotide at 200 nM for 48 hours. After this time cytoplasmic and nuclear extracts were obtained in biological triplate for each cell type. An equal amount of extracted DNA was loaded into each well of a Novex TBA-Urea 15% gel and northern blotting carried out using an LNA probe designed to hybridise to the treatment oligonucleotide. In order to demonstrate that nuclear samples did contain nucleic acid and the nuclear exaction had been successful, samples were also loaded onto 1% agarose gels containing ethidium bromide, run at 100V for 1 hr and imaged. This was carried out to ensure the lack of oligonucleotide in the nuclear compartment was due to

the oligonucleotide failing to cross the nuclear membrane and not observed due to an artefact of nucleic acidic extraction. Northern blotting confirmed that the unlabelled $(CAG)_7$ 2'OMe PS oligonucleotide did not penetrate the cell nuclei *via* gymnosis in all four cell types tested, Figure 3-5.

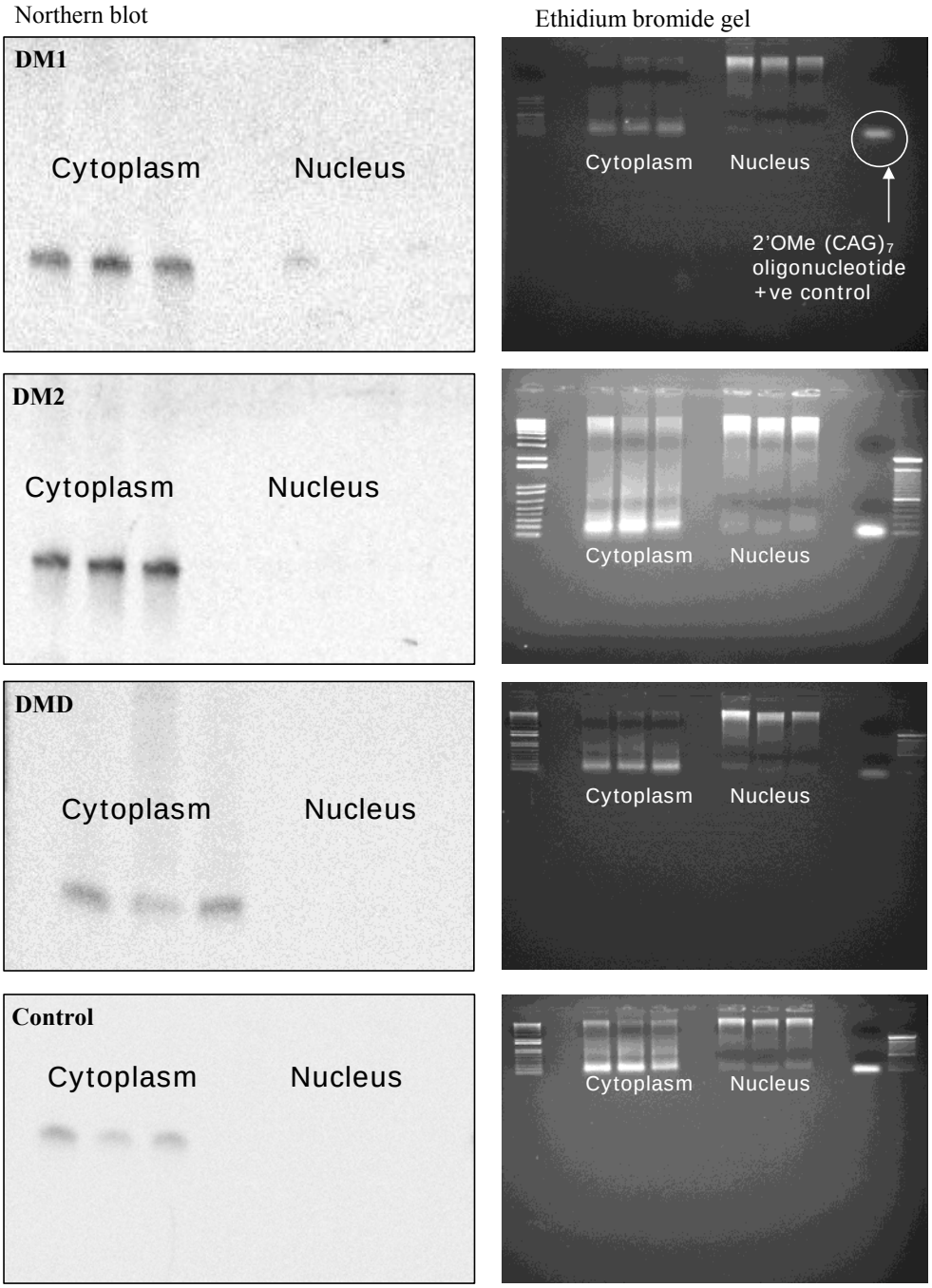


Figure 3-5: Northern blot analysis after sub fractionation of the cellular compartments. Cells were treated for 48 hr with a synthesised 2'OMe PS $(CAG)_7$ oligonucleotide Northern blots demonstrate the presence of the oligonucleotide in the cytoplasm but not the nucleus. Ethidium bromide gels demonstrate the presence of nucleic acid in the nuclear lanes, confirming successful extraction of nucleic acid.

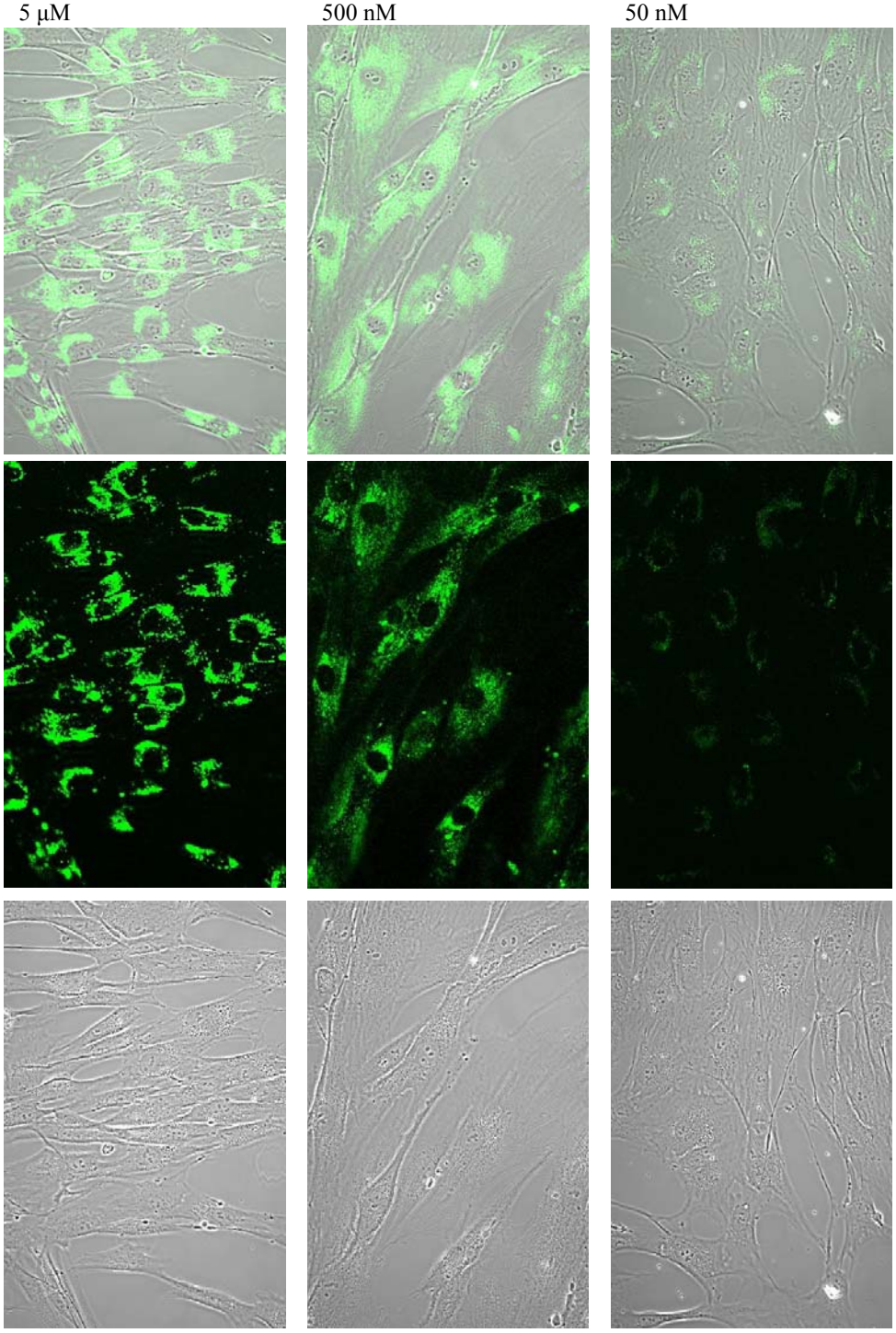
Concentration effects on delivery

As mentioned in the introduction of this chapter, 2'OMe PS oligonucleotides have been shown to remove nuclear RNA foci at a range of concentrations. Concentration dependent delivery is fundamentally important when developing any potential therapeutic agent as delivery must occur within a window of tolerated toxicity in order for any treatment to be considered effective as a therapy.

The preceding results suggest that 2'OMe oligonucleotides do not permeate the nuclear membrane at a concentration of 500 nM. In order to determine the extent at which concentration affects subcellular delivery *via* gymnosis, live cell imaging was performed using a range of HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotide concentrations in DM1, DM2, DMD and control cells. Figure 3-6 shows that even at concentrations significantly higher than those required to remove RNA foci all fluorescent signal resides in the cytoplasm.

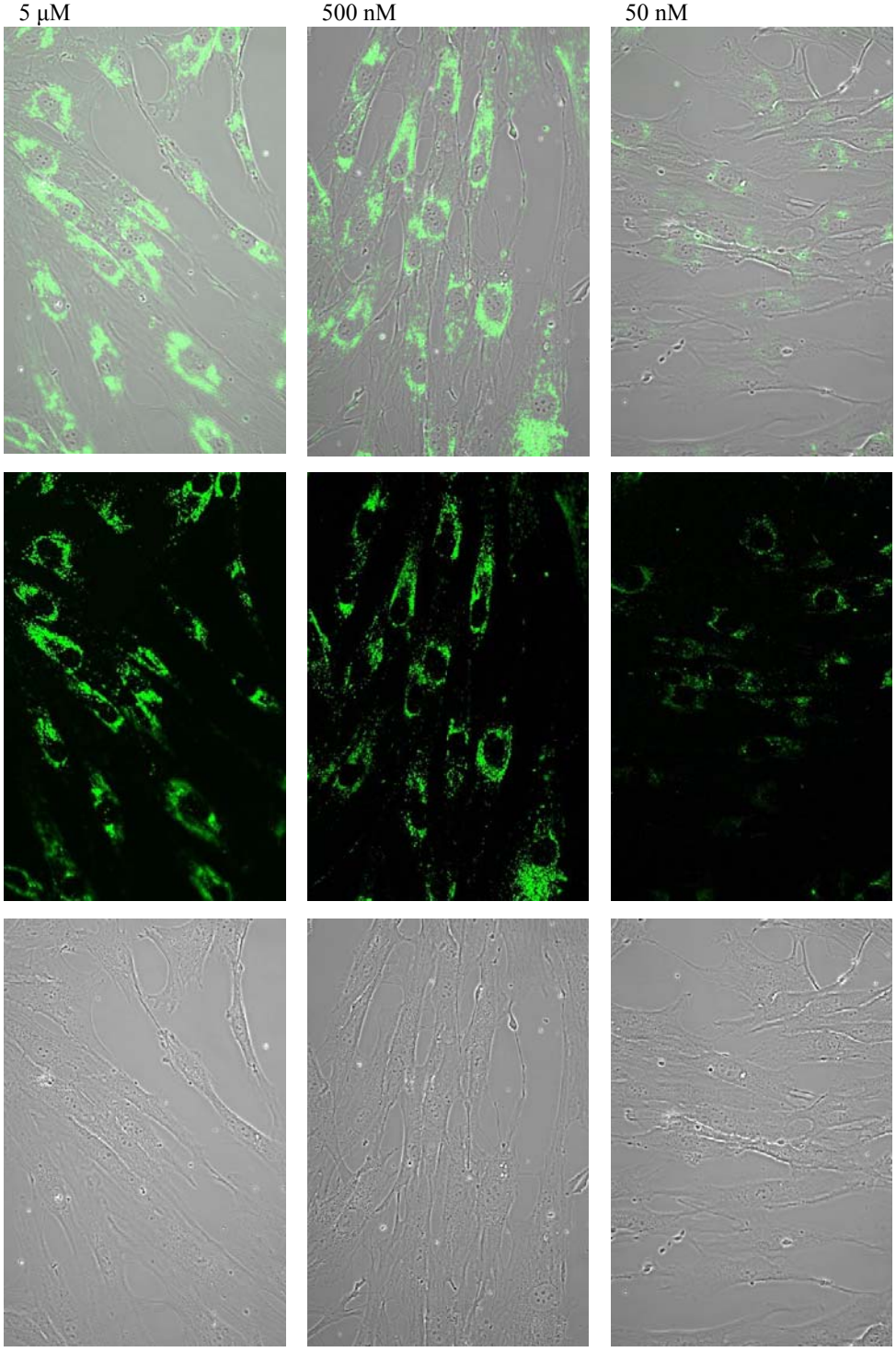
DM1

5 µM 500 nM 50 nM



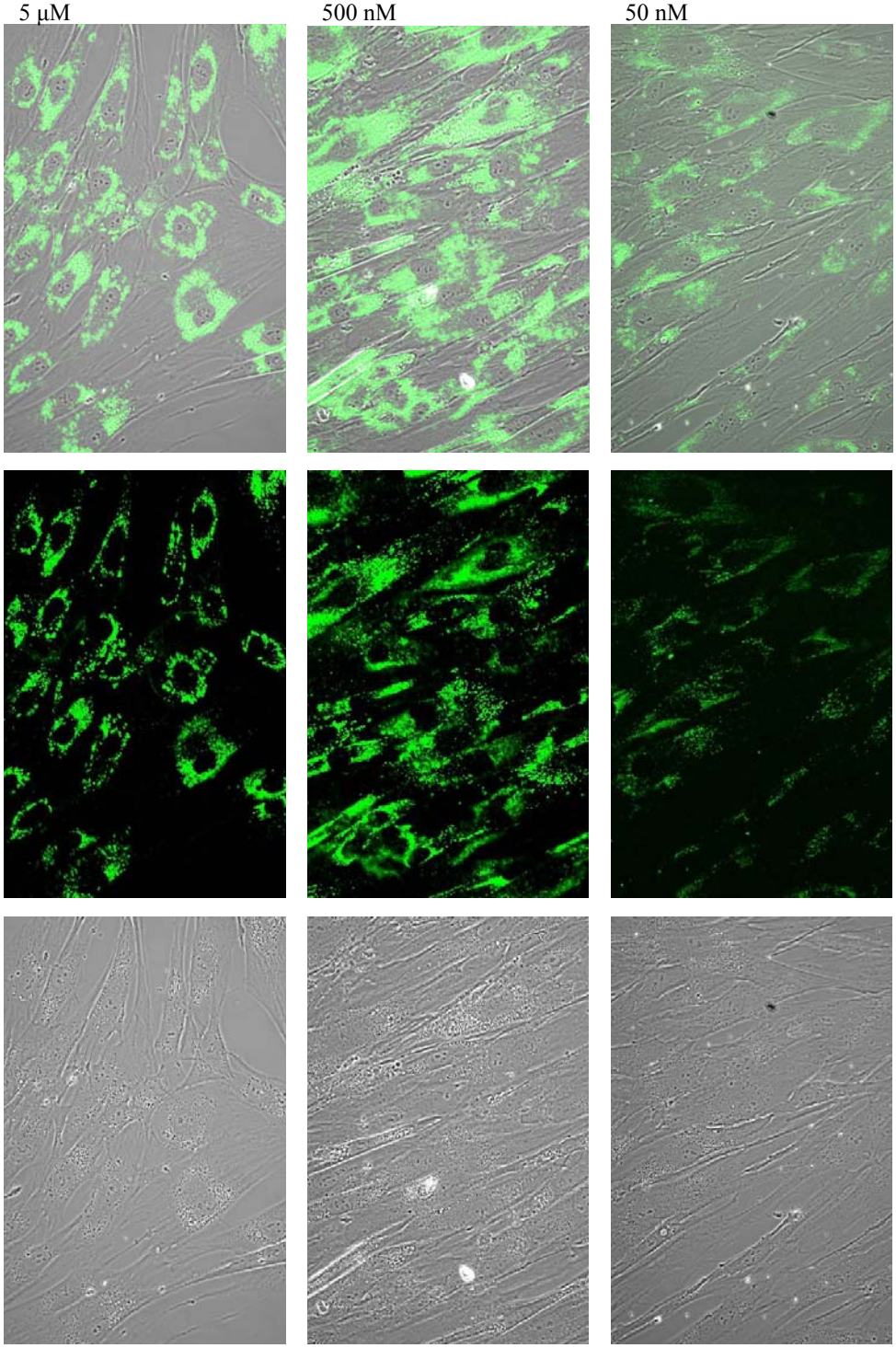
DM2

5 µM | 500 nM | 50 nM



DMD

5 µM 500 nM 50 nM



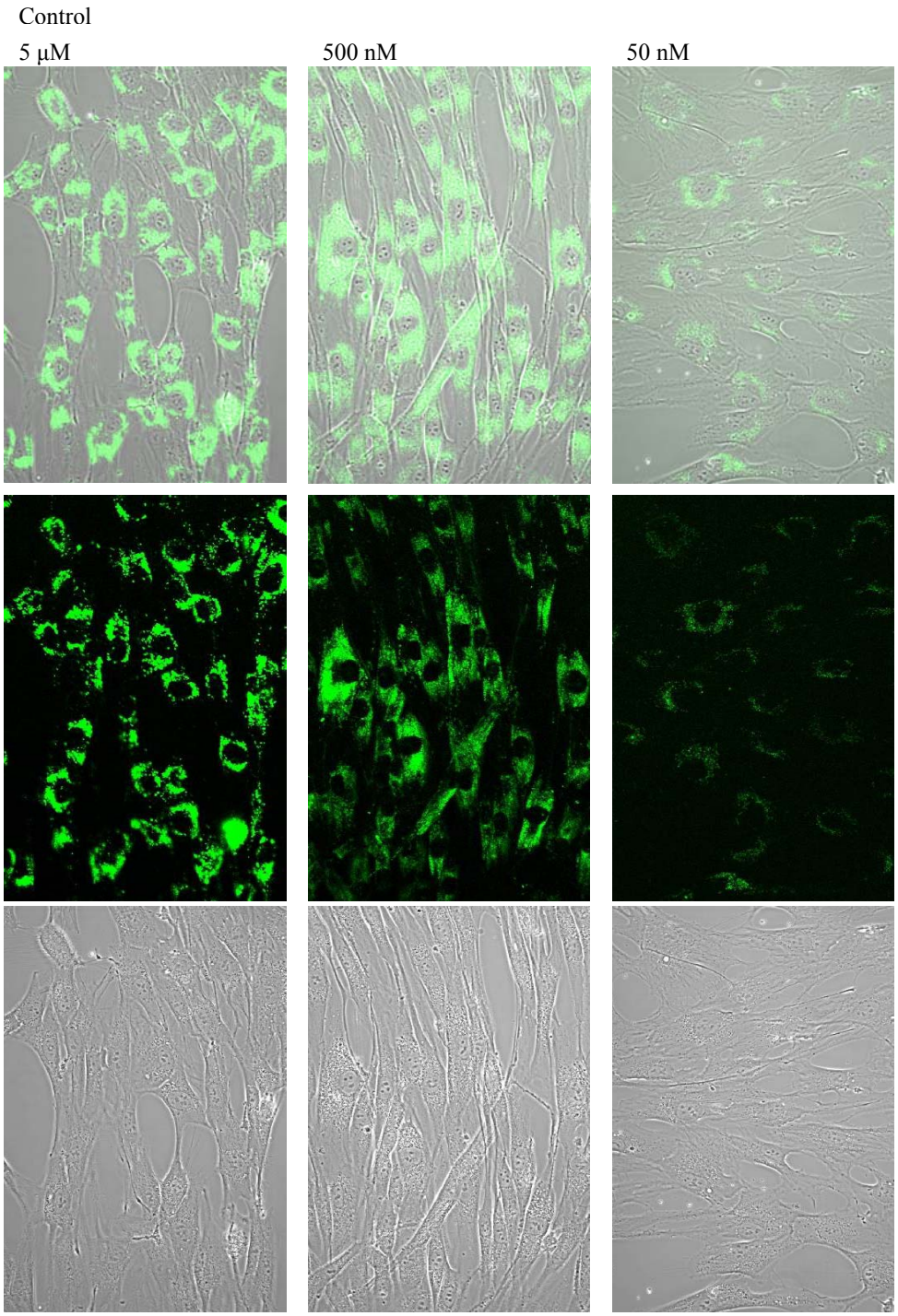


Figure 3-3: Combined, fluorescent and phase images of live cells from various disease models after treatment with HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotide at a range of concentrations *via* gymnotic transfection for 48 hrs. Oligonucleotide fluorescence is identified as the green signal.

Toxicity of 2'OMe oligonucleotides *via* gymnotic delivery

Although only a relatively small number of studies using oligonucleotides gymnotically have been carried out, it has been shown by others that oligonucleotides delivered this way can significantly silence gene activity, (Souleimanian, 2012), with minimal toxicity in cell culture. Stein ~~et al~~*et al*. found that silencing could be continuously maintained with little or no toxicity for up to 240 day with the correct dosing strategy.

In order to establish the toxicity profile of the $(CAG)_7$ 2'OMe PS oligonucleotide when delivered gymnotically, cells from each of the disease models were treated and an MTT assay performed after 48 hrs of treatment. Oligonucleotides were tested at a range of concentrations, Figure 3-7. It was observed that even at the highest concentration of 1 μM $(CAG)_7$ 2'OMe PS oligonucleotides delivered *via* gymnosis confer no toxicity in our disease models.

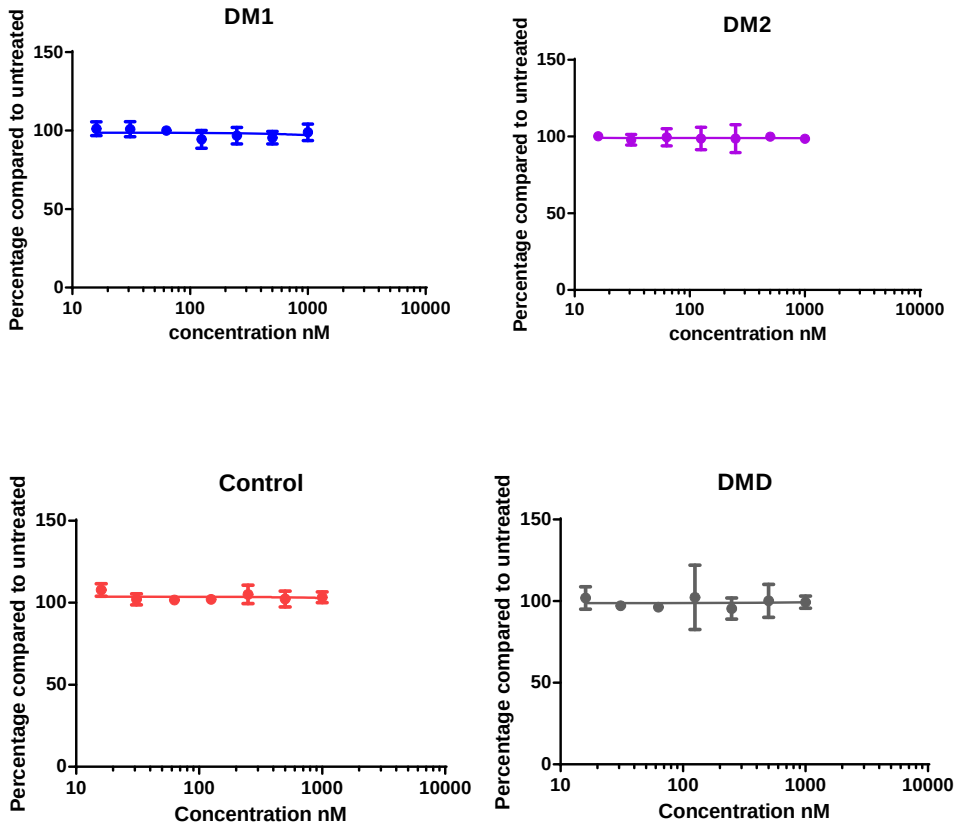


Figure 3-7: Assessment of the viability of cells from different disease models after treatment with varying concentrations of a $(CAG)_7$ 2'OMe PS oligonucleotide *via* gymnosis for 48 hr. X axis represent the concentration of the oligonucleotide in nM. Y axis displays the percentage viability of the cells compared to untreated controls of each cell type.

Delivery of various other chemically modified oligonucleotides *via* gymnosis

As reported in chapter one there are a wide range of CMOs which are commercially available. Previous work within the Brook lab demonstrated that PNA oligonucleotides, as with 2'OMe oligonucleotides, were also capable of removing nuclear foci, whereas DNA and PMO oligonucleotides of the same sequence were not. In order to further understand whether the lack of potency was due to a differential in delivery or another effect (i.e. structural relationship or melting temperature of the duplex) I again carried out live cell imaging to track the cellular location of fluorescently labelled analogue of each of the CMOs.

Deoxyribonucleic acid oligonucleotide delivery

Unmodified DNA oligonucleotides were among the first to be tested as antisense agents. Although these compounds showed successful, sequence-specific antisense activity, their use is limited due to poor biological stability (Uhlmann, 1990). It was demonstrated by Uhlmann ~~et al~~*et al*. that the enzymatic cleavage of an unmodified DNA 20mer oligonucleotide can take place in just 15 minutes, indicated by the increasing ladder like banding as time goes on in the blot shown in Figure 3-8.

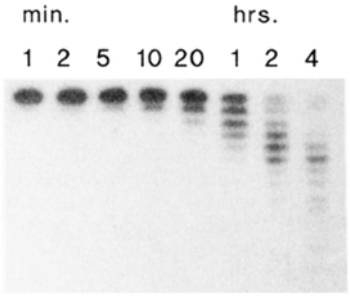


Figure 3-4. Kinetics of exonucleolytic cleavage of a radiolabeled DNA 20-mer oligonucleotide in fetal calf serum. Taken from Uhlmann, 1990.

In order to combat the degredation by endogenous enzymes an obvious approach to stabilisation was to modify the phosphate center, the target cleavage site of these enzymes. As a result one of the earliest modifications in oligonucleotide chemistry came in the form of phosphorothioate analogs, which enhance nuclease stability (Agrawal, 1999). Phosphorothioate DNA oligonucleotides were shown to significantly inhibited ICAM-1 expression, a glycoprotein expressed on the surface of both hematopoietic and non-hematopoietic cells, as early as 1991 by Chiang ~~et al~~*et al*.

To further the understanding of cellular uptake of DNA oligonucleotides a synthesised HEX labelled $(CAG)_7$ PS DNA oligonucleotide was tested using live cell imaging to identify whether this type of CMO was dispersed throughout the cell or whether, as with the 2'OMe oligonucleotide, delivery was exclusively cytoplasmic. The fluorescence from the oligonucleotide was once again found to be exclusively cytoplasmic, Figure 3-9.

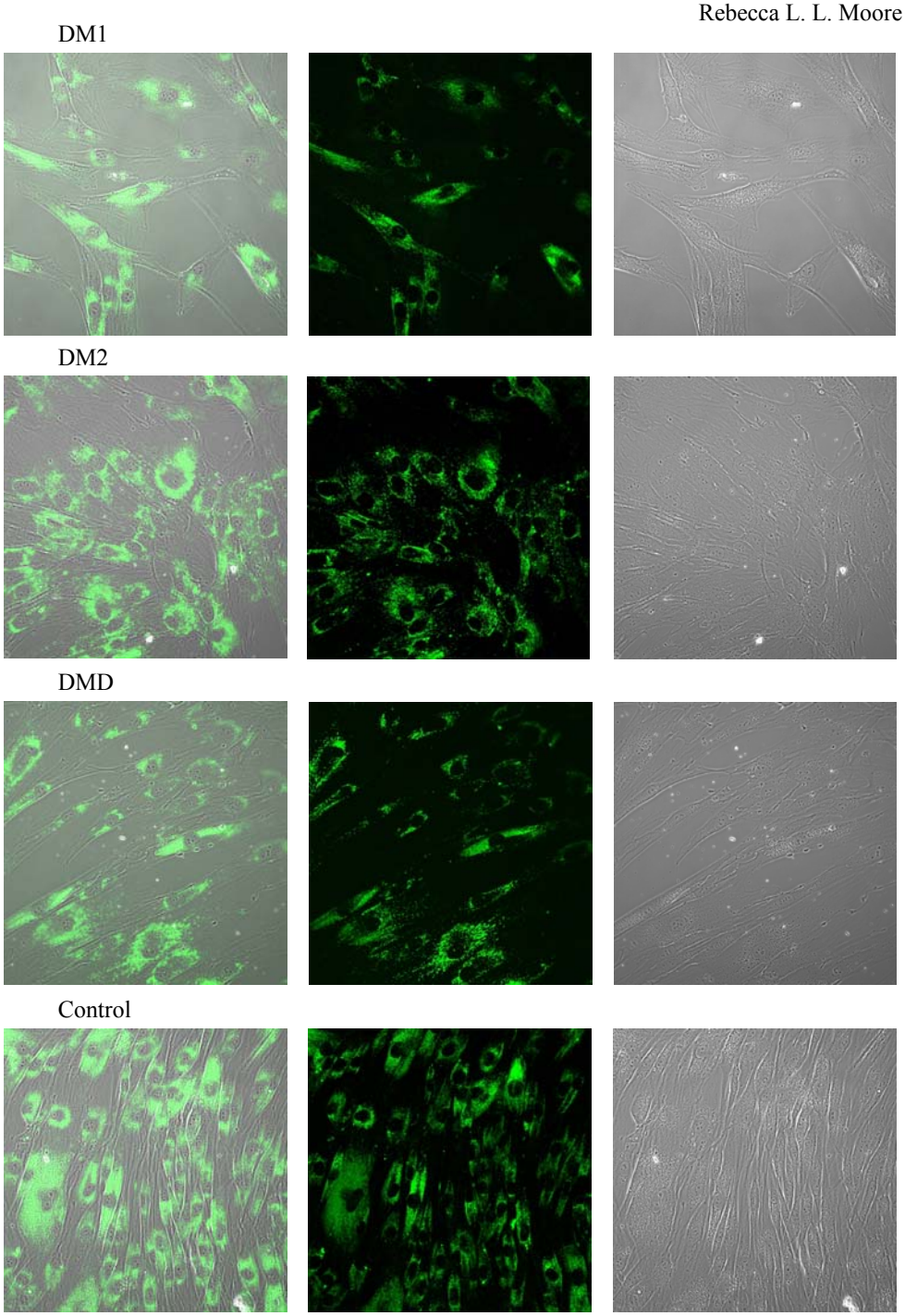


Figure 3-9: Combined, fluorescent and phase images of live cells from various disease models after treatment with HEX $(CAG)_7$ labelled DNA PS oligonucleotide at 500 nM *via* gymnotic transfection for 48 hrs. Oligonucleotide fluorescence is identified as the green signal.

Peptide nucleic acid oligonucleotide delivery

PNA oligonucleotides are structurally distinctive as they are formed by replacing the sugar phosphate backbone of the native nucleic acid with a synthetic peptide backbone. Surprisingly, even with the replacement of the entire sugar phosphate backbone, they are able to hybridize through Watson–Crick binding, (Egholm, 1993). As one of the later generations of oligonucleotide chemistries they are known to be stable and highly resistant to ubiquitous enzymes whilst demonstrating a high-binding affinity and sequence specificity, (Larsen, 1999). Their largest disadvantages as antisense agents are their poor cellular uptake and water insolubility, however it has been demonstrated that these can be improved with the conjugation of peptide conjugates, (Turner, 2009). It has also been shown that they are cleared more rapidly from the body compared to other well used oligonucleotides, potentially explaining why they have not been investigated *in vivo* or in clinical trials as often as other technologies (Rayburn, 2008).

PNA oligonucleotides have been shown to be effective at gene siliencing and the induction of exon skipping making them potential candiates for therapeutic use in both DM1/2 and DMD. Yin ~~et al~~*et al*. demonstrated a significant length-dependent systemic restoration of dystrophin expression by 25mer PNA oligonucleotides in mdx mice, a Duchene specific mouse model. This work established that PNA oligonucleotides have potential as systemic splice correcting agents for DMD and related disorders where the modification of target gene splicing is likely to be of high efficacy as a therapy. Fabani ~~et al~~*et al*. were the first to show knock down of microRNA function in a cell line using a PNA oligonucleotide, however the mechanism of knock down was not clear.

In order to identify whether this chemical modification had a different delivery potential *via* gymnosis I used a CY3 labelled $(CAG)_7$ PNA oligonucleotide to treat cells from the each of the disease models previously tested. Once again it was observed that the fluorescent signal was exclusively cytoplasmic, Figure 3-10. This was somewhat paradoxical as PNA oligonucleotides, as with 2'OMe oligonucleotide, had been identified previously in the group to be capable of foci removal at a range of concentration. At this point I could propose no mechanism by which these agents could infer the therapeutic activity observed when not present in the nucleus.

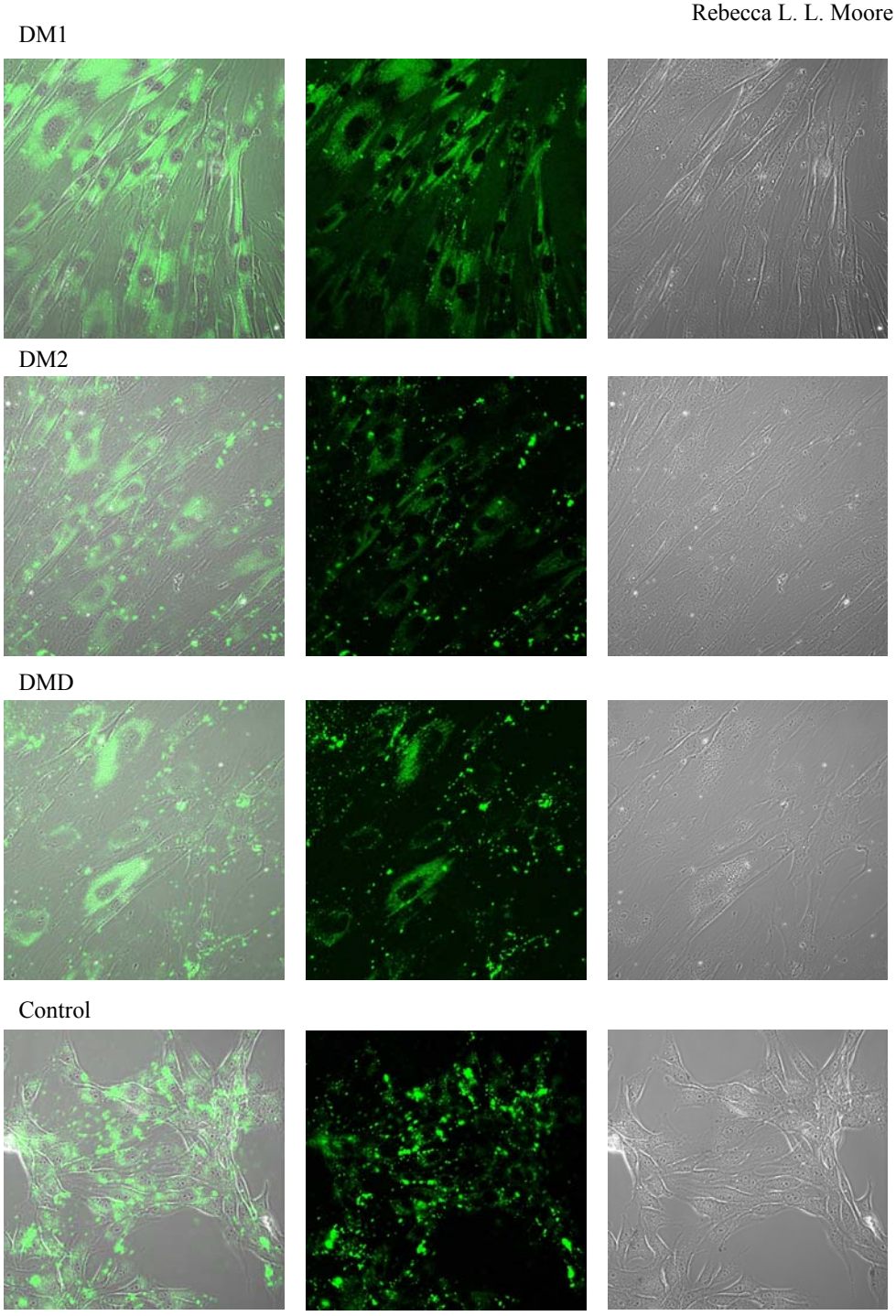


Figure 3-10: Combined, fluorescent and phase images of live cells from various disease models after treatment with Cy3 labelled $(CAG)_7$ PNA oligonucleotides at 500 nM *via* gymnotic transfection for 48 hrs. Oligonucleotide fluorescence is identified as the green signal.

Phosphorodiamidate morpholino oligonucleotide delivery

PMO oligonucleotides were first introduced into developmental biology by Heasman ~~*et al*~~*et al*. in the year 2000. They are widely used agents employed to further the understanding of the molecular basis of early development as they allow researchers to specifically inhibit the functions of particular gene at specific points in embryonic models. Although the bulk of the earlier literature was concerned with developmental pathways in models such as zebra fish, frogs, chick and xeonpus embryos, therapeutic uses of PMOs are coming to light. As therapeutic agents they belong to the small subset of non-ionic oligonuceotides which are thought decrease non-specific interactions with cellular proteins, lowering off target effects. Their binding efficiencies have been shown to be similar to unmodified DNA and RNA, necessitating the use of slightly longer sequences to ensure good hybridisation, typically ~25bp. The key features of PMO oligonucleotides is the presence of morpholine rings instead of deoxyribose rings and that nucleosides are linked through phosphorodiamidate groups instead of phosphates. These modifications allow for Watson-Crick base pairing but also ensure PMOs are resistant to digestion from nucleases, inferring a greater stability in a biological system.

PMO oligonucleotides efficiency has been demonstrated in cell cultures and in animal models. Their specific use in one of our diseases of interest, DMD, is widely documented, with on-going clinical trials. In 2011 AVI-4658, a PMO based antisense agent was found to be safe and biochemically efficient as a disease-modifying drug for DMD, (Cirak, 2011). When the same live cell imaging tests were carried out using PMO oligonucleotides it was observed that these agents, as befre with the other CMOs tested, were unable to permeate the nucleus *via* gymnotic delivery, Figure 3-11.

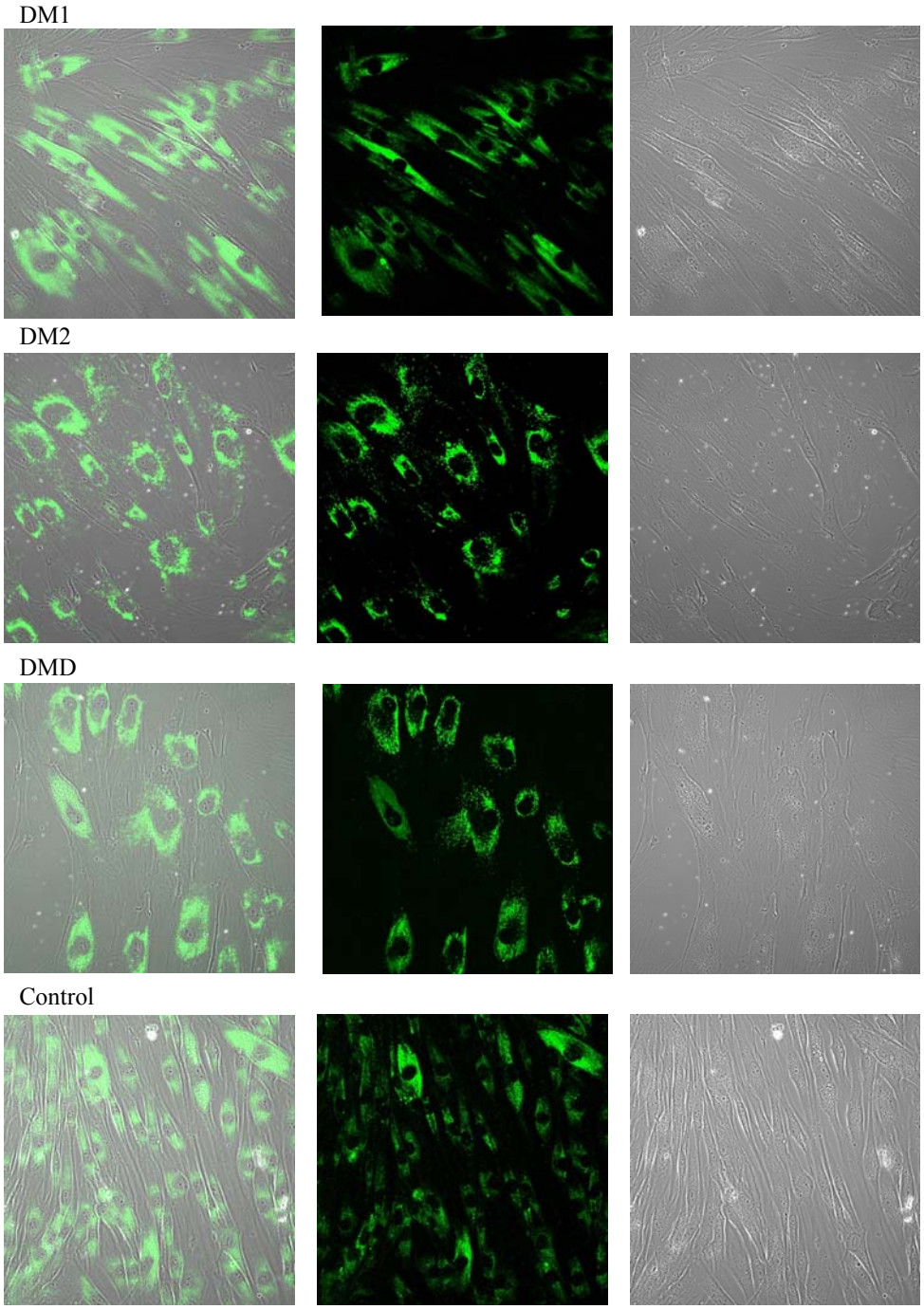


Figure 3-11: Combined, fluorescent and phase images of live cells from various disease models after treatment with HEX labelled $(CAG)_7$ PMO oligonucleotides at 500 nM *via* gymnotic transfection for 48 hrs. Oligonucleotide fluorescence is identified as the green signal.

Toxicity of different CMOs *via* gymnotic delivery

As previously mentioned, in order for oligonucleotides to be of any therapeutic value they must be functional in a non-toxic range. Although none of the CMOs tests appeared to result in nuclear penetrance of the oligonucleotide they may have use against cytoplasmic targets.

As with the 2'OMe oligonucleotide, the toxicity of each CMO was tested at a range of concentrations using cells from DM1, DM2 and DMD patients and a control cell line. An MTT assay was performed after 48 hours of continuous gymnotic treatment. It was observed at high concentrations the oligonucleotides tested may result in a small decrease in viability although this was not statistically significant. Overall none of the oligonucleotides demonstrated a high level of toxicity, Figure 3-12.

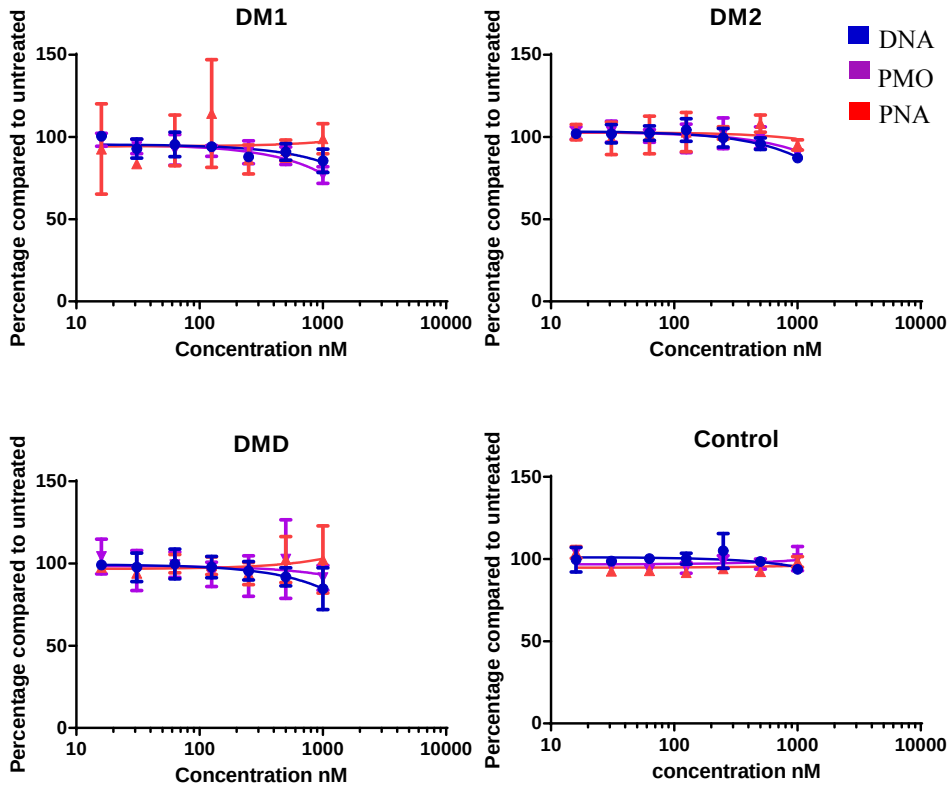


Figure 3-12: Assessment of the viability of cells from different disease models after treatment with varying concentrations of a $(CAG)_7$ oligonucleotide of various chemistries *via* gymnosis for 48 hr. X axis represent the concentration of the oligonucleotide in nM. Y axis displays the percentage viability of the cells compared to untreated controls of each cell type.

Effect of fluorescent *in-situ* hybridisation (FISH) processes on cellular localisation of oligonucleotides

Once established that oligonucleotide uptake *via* gymnosis gave entirely cytoplasmic delivery for all the CMOs tested, with little evidence of nuclear penetration, the removal of nuclear foci by a subset of these oligonucleotide seems paradoxical. In order to test whether the observed foci removal was an artefact of the FISH process, fluorescent images were taken after cells had been through each stage of the experimental process. This allowed a comparison of the subcellular oligonucleotide location before and after the FISH processes, in order to rule out any misleading results.

As Figure 3-13 confirms, fluorescent $(CAG)_7$ PNA DNA and 2'OMe oligonucleotides were all heavily redistributed across the cell after FISH processes had been carried out, whereas the PMO oligonucleotide appeared to remain cytoplasmic.

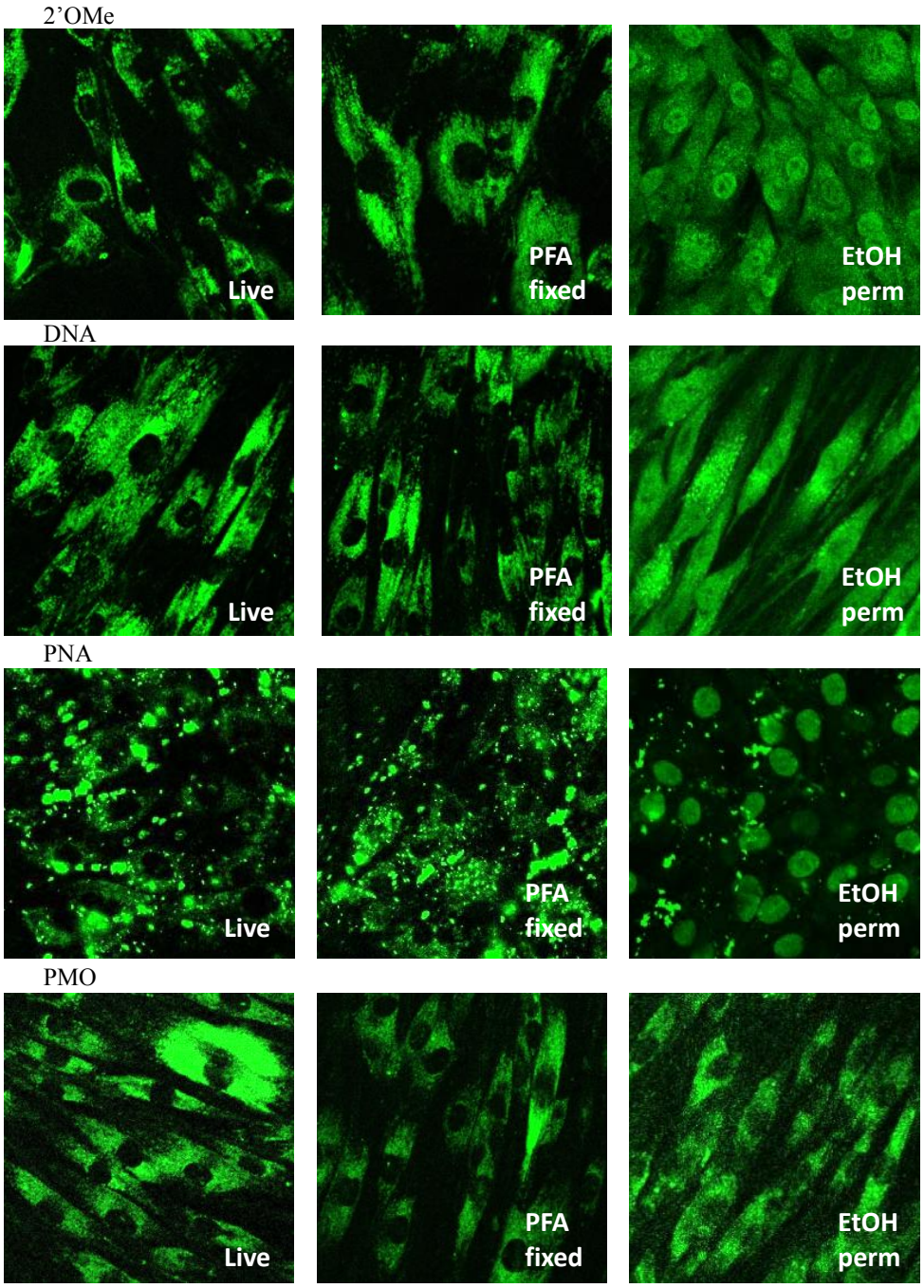


Figure 3-5. Confocal microscopy images of live, fixed and permeabilised control cells after treatment with each of the fluorescently labelled $(CAG)_7$ oligonucleotides at 500 nM *via* gymnotic transfection for 48 hrs. Oligonucleotide fluorescence is identified as the green signal.

Discussion

The direction for this project came as a result of the observed diverse potential of different CMOs to remove foci in DM1 cells. Taneja ~~et al~~*et al*. first reported the consistent appearance of nuclear foci in DM1 fibroblasts and their absence in normal fibroblasts, commenting that it could provide a simple analytical approach for disease diagnosis, (Taneja, 1995). Since this finding nuclear foci have been utilised as an important diagnostic tool for potential treatments, with the high through-put screening of numerous small molecules relying upon an observed effect on foci to identify hit compounds, (Ketley, 2013). Using foci as a diagnostic tool it was shown by previous lab members that both 2'OMe and PNA oligonucleotides could completely remove foci at concentrations as low as 5 nM, whereas PMO and DNA oligonucleotides failed at even the highest concentrations tested. In order to establish an explanation for this differential activity observed between the various CMOs, live cell images were taken to ascertain whether the lack of biological activity was as a direct result of different delivery capabilities.

To date the majority of the work referencing the delivery of various CMOs concerns their delivery using transfection reagents and not *via* gymnosis. Therefore I attempted to determine whether the observed results from the previous foci assay could be explained by varying levels of gymnotic uptake of the CMOs. As I am also concerned with the treatment of DM2 and DMD *via* oligonucleotide antisense therapy I endeavoured to establish whether cells from a range of disease models could uptake the various CMOs to the same extent. To this end 2'OMe oligonucleotides were first tested in cells from DM1, DM2 and DMD patients as well as an unaffected individual. Fluorescent images indicated that 2'OMe oligonucleotides were unable to penetrate the nuclear membrane in all 4 cell lines tested. To ensure the

fluorophore moiety was not impeding nuclear delivery, the fluorescent imaging data was then validated using a modified northern blotting method. Northern blotting data did suggest that 2'OMe oligonucleotides remain full length and are not degraded *in vitro*, although this observation could be subject to the length of the probe used in detecting the oligonucleotide. These findings were in direct conflict with the previous foci data, as 2'OMe oligonucleotides were able to remove foci, which are retained in the nucleus. As mentioned, this was seemingly paradoxical as I could not propose a mechanism by which the oligonucleotide could act upon foci whilst located in the cytoplasm. Although nuclear delivery of oligonucleotides is paramount for the disease models I am investigating, I briefly investigated the toxicity profile of 2'OMe oligonucleotides in all 4 cell lines. No decrease in viability was observed as assessed by MTT assays even at the highest concentrations tested, 1 μM.

Various studies have demonstrated the ability of both locked nucleic acid (LNA) and 2′-fluoro-arabino nucleic acid (2'FANA) gapmers produce sequence-specific gene silencing *via* gymnotic delivery, (Souleimanian, 2012, Stein, 2010). Although these findings were encoraging other CMOs have not been tested in the same way. Therefore in order to investigate whether the other CMOs examined in the foci assay behaved similarly to the previously tested 2'OMe oligonucleotide, further live cell experimentation and imaging was carried out using a fluorescently labelled analogue of each CMO of interest. As before it was observed that all fluorescent signal appeared cytoplasmic, demonstrating again a lack of nuclear delivery of each of these agents. This data does not parallel with the observations from the previous foci assays, as PNA oligonucleotides are again located solely in the cytoplasm. It is therefore apparent that differences in the membrane composition between the nuclear and cytoplasmic membranes deny entry of these agents to the nuclear compartment .

It was also observed that the PNA, DNA and PMO CMOs only resulted in a slight decrease in viability at the highest concentrations tested, with cells treated at concentrations below 500 nM not presenting any toxicity in any of the four cell lines tested.

In order to attempt to decipher the conflicting data from the foci assay and the live cell imaging each step of the FISH protocol, used to visual the nuclear foci, was examined. The results obtained clearly confirmed an inbuilt artefact of the FISH process, where upon permeabilisation the treatment oligonucleotide is heavily redistributed in the case of PNA, 2'OMe and DNA oligonucleotides. It was noted that PMO oligonucleotides do not seem to redistribute to the same extent, potentially explaining why they did not remove nuclear foci. The reasons for this are unclear, as they are not considerably larger than the other CMOs, and they have the same neutrality as the PNA oligonucleotides, which do heavily redistribute. However it must be taken into account that the observations here concerning PMO oligonucleotides could be as a result of a decrease in overall delivery, as it is difficult to quantify absolutely the level of delivery by the observed fluorescence. This redistribution result in the nuclear delivery of the oligonucleotide, which is not representative of the live biological situation. In the case of the 2'OMe and PNA oligonucleotides this redistribution resulted in the oligonucleotides being able to bind to their target, sterically blocking the observation probe from binding, consequentially showing an artificial clearance of foci. Although redistributed upon permeabilisation, the lack of foci reduction by the DNA oligonucleotide can be explained by the enthalpic benefit of the probe binding compared to the treatment oligonucleotide. The foci observation probe is a 30bp labelled DNA oligonucleotide whereas the treatment oligonucleotide is just 21bp, therefore thermodynamically the energy provided from the probe binding is far higher and therefore

foci are still observed. The melting temperature between the RNA and PNA or 2'OMe duplexes are higher than that between the RNA and DNA duplex therefore the PNA and 2'OMe treatment oligonucleotides can overcome the thermodynamic disadvantage of being shorter than the DNA foci observation probe because of their increased binding affinity, afforded by their modifications.

Chapter four: The delivery of 2'-O-Methyl oligonucleotides *via* polyethylenimine based transfection

Introduction

The findings from the previous chapter suggest that in order to successfully treat diseases *via* antisense therapy a delivery reagent may be required in diseases where the target is retained in the nucleus. As previously discussed in chapter one there are a number of different reagents and methods available to transfect oligonucleotide species into cells. The Wansink laboratory have had great success at treating various DM1 models *in vitro* using a subset of transfection reagents known as polyethylenimine (PEI), (Mulders, 2009), which have been shown to deliver oligonucleotides to the nucleus (Godby, 1999). PEI molecules can exist in various forms including branched, linear and dendritic, Figure 4-1, each with different delivery and toxicity properties, (Kadlecova, 2013).

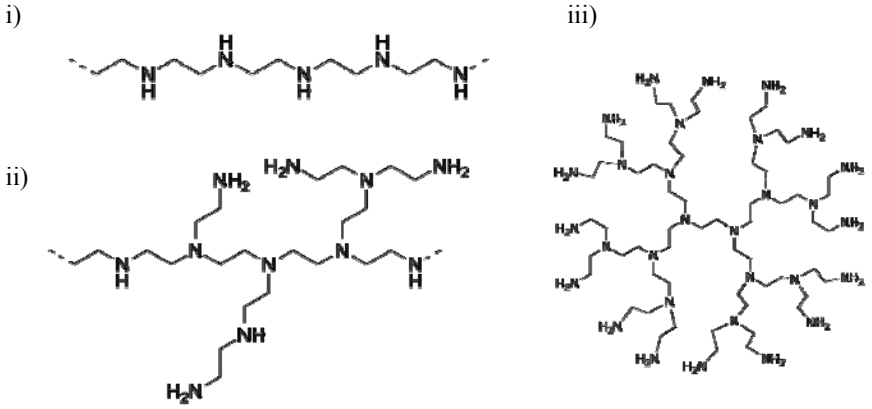


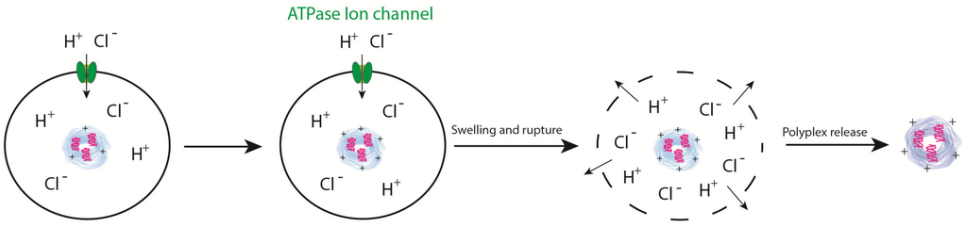
Figure 4-1: Different PEI molecular structures, (i) linear, (ii) branched and (iii) dendritic.

Chemically each variant of PEI contains different amino functionality, branched PEI molecules contain primary, secondary and tertiary amine groups, dendritic PEI molecules contain primary and tertiary groups. Finally linear PEI molecules contain predominantly secondary amines. Before being studied as therapeutic agents PEI molecules have found a wide range of applications including water purification, detergents, adhesives, shampoos and cosmetics, establishing them as an innocuous agent, (Boussif, 1995).

PEI was first demonstrated to be a successful delivery reagent in 1995, (Boussif, 1995), with reports of up to 100% of cells exhibiting bright fluorescent nuclei after treatment with 1.25 μM DNA based rhodamine labelled 18-mer oligonucleotide. Initially considered non-toxic reagents, the group's studies revealed a somewhat less clear-cut picture of PEI induced toxicity. Experimentation showed no perturbation of cell metabolism, even at concentrations up to twice the optimal required for gene delivery, whereas higher concentrations did result in toxicity. It seems there is an apparent inconsistency between the reported innocuous nature of PEI (Bikales, 1985) and the toxic response in cell culture to PEI/Oligonucleotide complexes, which has raised a number of questions in the literature.

Since its discovery as a successful delivery agent, PEI molecules have been shown to transfect a wide range of cell types including lung, cerebrum, neuron and kidney, (Boussif, 1995). As detailed in chapter one, PEIs great transfection ability is attributed to its high charge density as a result of the large number of primary, secondary and tertiary amino groups (structure dependent). The molecules have a significant buffering capacity over a wide pH range, as the pKas of the unprotonated amines differ with their environment. This buffering capacity allows for endosomal escape *via* a mechanism coined the 'proto-

sponge'effect, Scheme 4-1, (Behr, 1997). It is hypothesised that ATPase transports protons across the endosome in order to reach the desired pH, proton accumulation results in an influx of counter ions causing osmotic swelling and endosomal rupture releasing the oligonucleotide (Yamashiro, 1983, Maxfield, 1987).



Scheme 4-1: Schematic of the 'proto-sponge' effect. Scheme taken from "Low Molecular Weight Chitosan (LMWC)-based Polyplexes for pDNA Delivery: From Bench to Bedside" (Agirre, 2014).

Another advantage of the high charge density of PEI molecules is the strong ionic interactions it affords between the oligonucleotide and the PEI macromolecule. This results in the condensation of the DNA based therapeutic into a polyplex, leading to stable spherical particles. Studies have shown that linear PEI molecules with lower molecular weights were the most efficient for the transfection of shorter antisense agents such as oligonucleotides, (Breunig, 2005) although there are conflicting reports within the literature. However, although a high cationic charge confers the favourable attributes mentioned above, it is also thought to be responsible for the observed toxicity, (Eliyaha, 2005) as detailed in chapter one.

To date one of the most important considerations in order to obtain successful delivery is the ratio of positive and negative charges, which allow the electrostatic interactions to occur. As the positive charge is contributed by the amine (NR_4^+) of the PEI molecule and the negative charge is contributed by the phosphate (PO_6^-) of the oligonucleotide, the ratio of these

components is widely known as the N/P ratio. Numerous reports have addressed the required N/P ratio as it is apparent that adequate amounts of PEI are necessary for complete condensation of nucleic acids, (Eliyaha, 2005, Godbey, 2001, Boussif, 1995). Therefore PEI molecules are often used in excess in order to ensure full condensation takes place as it has been reported that polyplexes prepared at N/P ratios are particularly efficient transfection agents (Boeckle, 2004). However, a high N/P ratio is also thought to cause increased toxicity as a result of the large amount of un-complexed PEI reagent, (Zou, 2000, Boeckle, 2004).

As PEI reagents condense oligonucleotides *via* electrostatic interactions they have primarily been used to investigate the delivery of positively charged oligonucleotide, such as 2'OMe, 2'MOE and DNA chemistries (Zou, 2000, Boussif, 1995). As there are many shortcomings of unmodified DNA oligonucleotides, detailed previously, this chapter will focus on the deliveryof 2'OMe oligonucleotides *via* PEI transfection which has been shown to be therapeutically effective in various DM1 and DMD animal models.

Aim of study

It has been demonstrated that molecular weight heavily influences the capacity of PEI molecules to deliver oligonucleotides. It is also known that different structures of PEI may generate different complex formulations with oligonucleotides, thus demonstrating a range of transfection efficiencies and biocompatibility. In this chapter I aim to investigate the nuclear delivery efficiency of each PEI molecule tested in our specific cell based models from DM1, DM2 and DMD patients. This will allow me to identify the most suitable PEI molecule with which to test the various CMOs in our models with a hope of identifying their ability to affect their nuclear RNA targets.

Results

Delivery of 2'OMe oligonucleotides *via* various PEI molecules

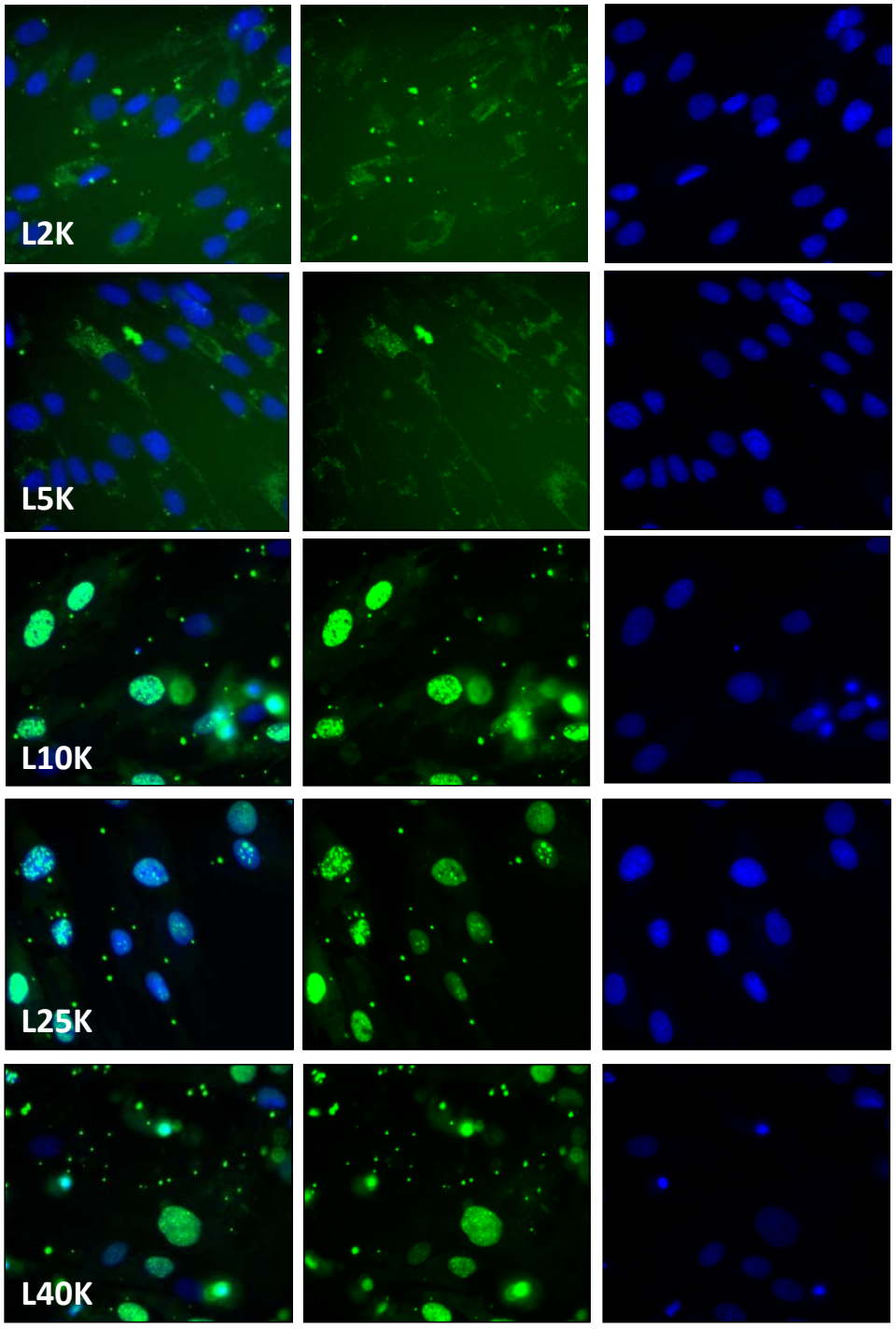
Exgen, a commercially formulated PEI based transfection reagent, and eight other available PEI molecules were assessed in order to establish their effectiveness to deliver 2'OMe oligonucleotides in cell culture.

Assessment *via* live cell fluorescent imaging

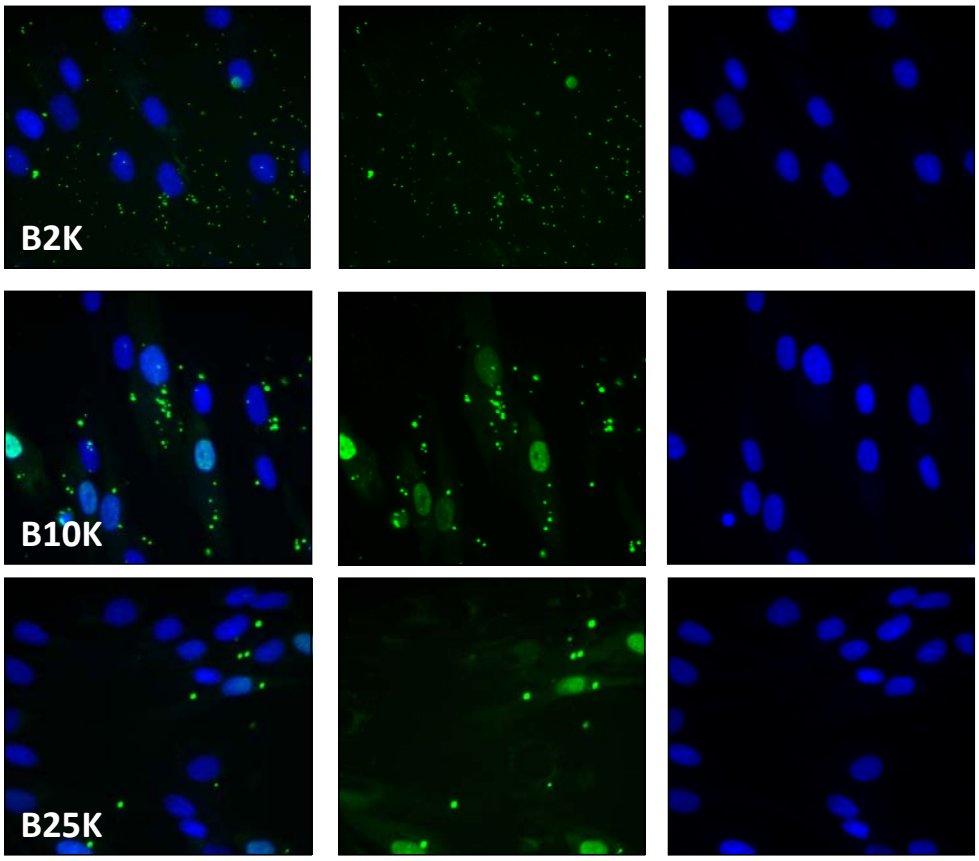
In order to identify the delivery capacity of each PEI molecule, live cell images were taken after treatment with a $(CAG)_7$ 2'OMe PS oligonucleotide labelled with a hexachlorofluorescein (HEX) fluorophore. The results are shown in Figure 4-2. Oligonucleotide and PEI were left to complex for 15 min before the addition of 2 mL of culture media. Solutions were mixed well and added directly to adherent cells for 48 hrs.

The concentration of each reagent upon complexation were found to be important as any further fold dilution of either the oligonucleotide or PEI solution led to unsuccessful complexation and complete lack of delivery, (data not shown). The results obtained identified that linear PEI molecules with a molecular number above 10 000 gave good nuclear delivery. Linear PEI molecules with a molecular number 5000 and below gave poor delivery, both nuclear and cytoplasmic, showing an observed minimum molecular weight for successful delivery. Branched PEI molecules also demonstrated a molecular weight differential, with the 2000 Mn PEI showing poor capacity to deliver oligonucleotide to both the cytoplasm and the nucleus, whereas the 10 000 Mn and the 25 000 Mn PEI molecules delivered successfully. As predicted, due to their similarity in structure, Exgen gave similar delivery performance to the linear higher Mn PEIs, Figure 4-2.

(i) Linear PEI molecules



(ii) Branched PEI molecules



(iii) Exgen 500 (commercial reagent)

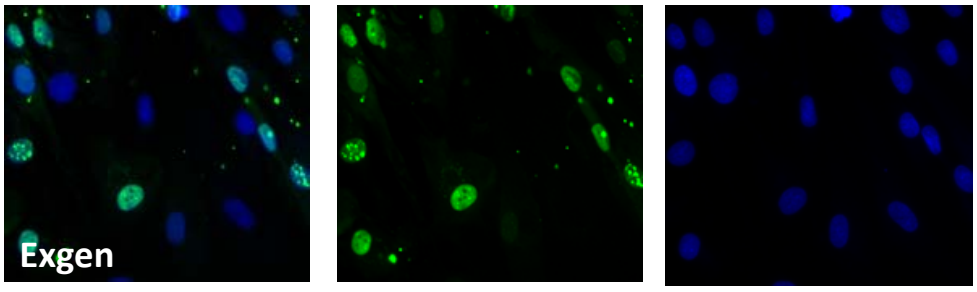


Figure 4-2: Fluorescent images from cells treated with HEX labelled 2'OMe $(CAG)_7$ PS oligonucleotide and various PEI reagents, either (i) Linear or (ii) Branched in structure and (iii) Exgen 500, a commercially sold transfection reagent. Far left column is an overlay of Hoechst nuclear stain (blue) and fluorescence from the oligonucleotide (green). Middle column is oligonucleotide fluorescence only (green). Far right column is Hoechst nuclear stain only, (blue).

Assessment *via* northern blot analysis

To validate the observations made by live cell imaging, northern blotting was carried out, using a similar approach to that outlined in chapter 3 when assessing delivery *via* gymnosis. A selection of the most successful PEI reagents were chosen, including: Linear 25 kDa Mn (L25K), Linear 10 kDa Mn (L10K), Linear 5 kDa Mn (L5K) PEI and Exgen.

As previously described, the distribution of 2'OMe oligonucleotide between the cytoplasmic and nuclear compartments was examined after cell fractionation in DM1 cells. The required volume of 2'OMe oligonucleotide stock (100 μM) was aliquoted into a 15 mL centrifuge tube, the required volume PEI stock solution (10 μM) was added and the resulting mixture left to complex for 15 min. After the addition of 8 mL of culture medium the solution was mixed well and added directly to cells seeded in a 75 cm^3 culture flask, and left for 48 hrs. Each treatment was carried out in triplicate, after 48 hours the cells were washed with PBS and fractionated before extracting nucleic acid. The nucleic acid concentration was quantified using a Nanodrop 2000 and Northern blot analysis was carried out as described in chapter 2. The results are shown in Figure 4-3.

L25K and L10K PEI molecules as well as Exgen 500 all demonstrated good nuclear delivery of the 2'OMe oligonucleotide, whereas the L5K PEI molecule gave significantly less delivery, both nuclear and cytoplasmic, corroborating the results from live cell imaging.

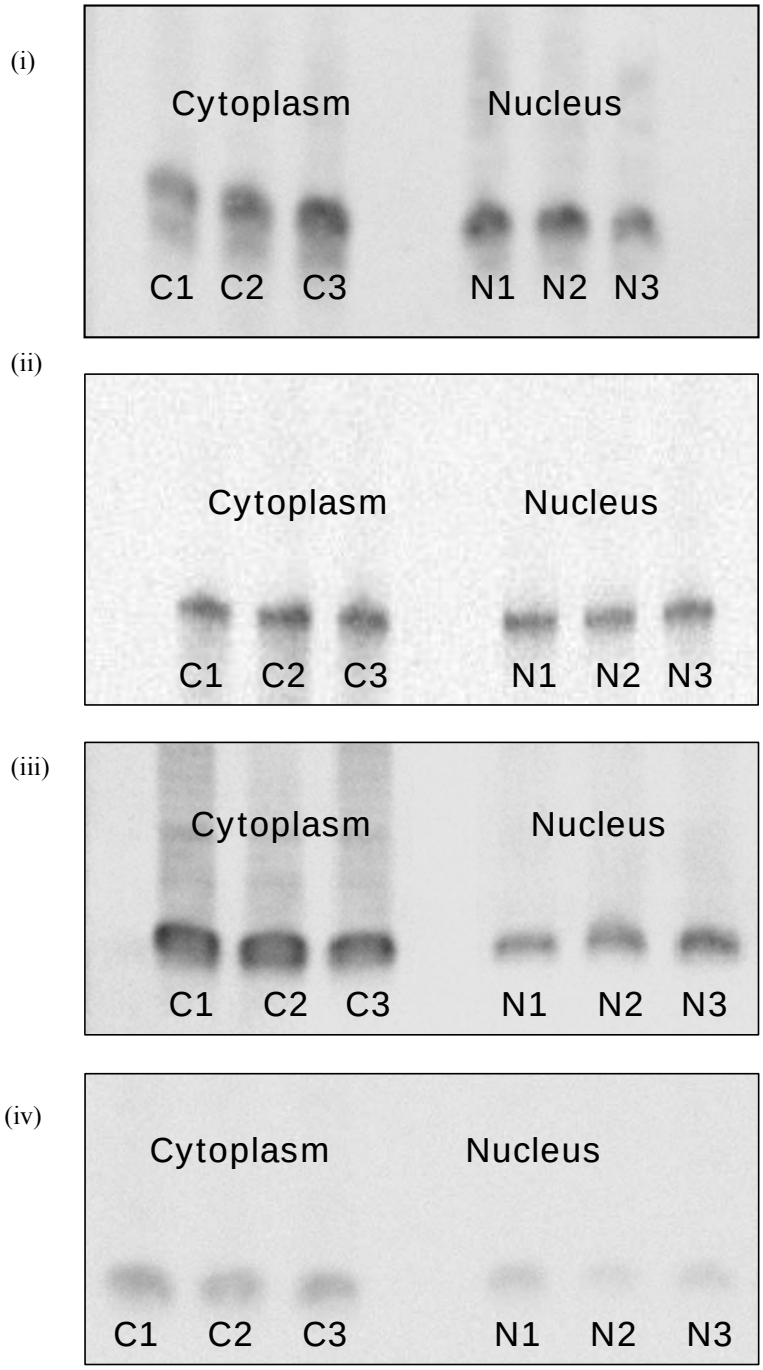


Figure 4-3: Northern blot analysis of 2'OMe $(CAG)_7$ PS oligonucleotide delivery to sub-cellular compartments *via* PEI transfection with (i) Exgen, (ii) L10K, (iii) L25K and (iv) L5K. Blot were probed with a complementary probe to the oligonucleotide - $(CUG)_7$.

Assessing toxicity

Unlike gymnotic delivery, there is a wealth of data dating back to 1995 which details the toxicity of various PEI molecules when used as transfection reagents. Unfortunately as previously mentioned a large amount of this data is conflicting. In order to try and further understand the toxicity profile of the various PEI molecules in our cell models, MTT tests were carried out on all four cell lines: DM1, DM2, DMD and a control line, to ascertain the extent of toxicity in the relevant disease models.

Toxicity of PEI molecules alone

Numerous articles propose that cytotoxic effects observed upon transfection with PEI molecules are caused by free PEI molecules, unincorporated into PEI polyplexes. The majority of evidence for this however is based upon experimental evidence where upon removal of the PEI molecules the oligonucleotides themselves are not toxic. As preceding experiments have shown, delivery *via* gymnosis results in far less oligonucleotide uptake than when oligonucleotides are combined with PEI reagents. I therefore set out to investigate whether PEI molecules alone are toxic in a variety of cell lines.

As Figure 4-4 illustrates, different PEI molecules have different toxicity profiles. It is observed that PEI molecules with a linear structure appear more toxic than their branch counterparts of the same molecular number. The results also establish a trend pertaining to size, when analysing the data from linear or branched PEI molecules, toxicity appears to be increased the higher the molecular number of the PEI reagent.

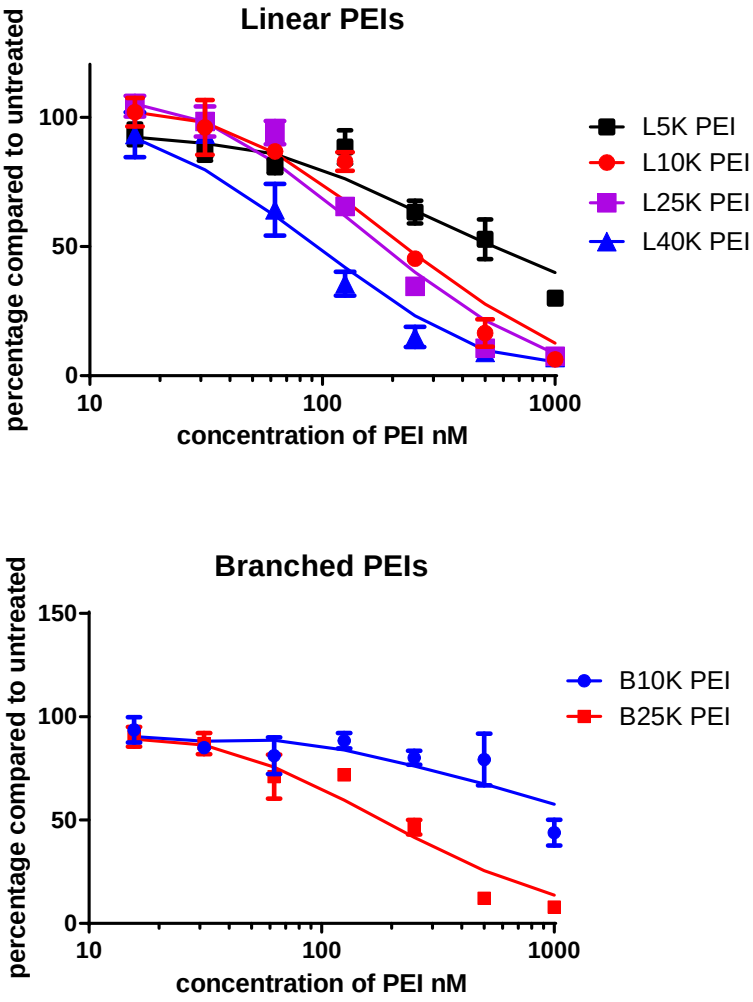
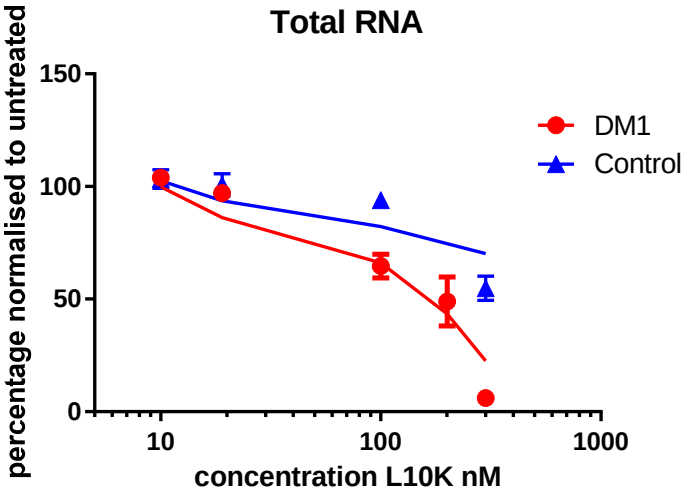


Figure 4-4: Toxicity profiles of various PEI molecules without oligonucleotides as measured by an MTT assay. X axis reprents the concentration of the PEI molecule being tested, nM. Y axis represents the percentage viability compared to untreated control cells.

MTT assays have long be used to establish cell viability, however in order to validate the results at a more basic level I extracted total RNA from control and DM1 cells treated with linear L10K PEI at a range concentrations. L10K was chosen as it demonstrated the highest degree of nuclear delivery compared to observed toxicity and therefore appeared to be the most promising candidate to optimise. Cells from the required patient models were seeded in 25 cm^3 flasks. After 24 hrs cells were treated with the necessary concentration of L10K in new media and left for 48 hrs. Each treatment was carried out in triplicate in order to obtain an average. RNA extraction was carried out as described in chapter two followed by quantification *via* Nanodrop. Cell counts were obtained using a Haemocytometer. This allowed any global effect on cell division or RNA concentration to be detected in both DM1 and control cells. Both RNA levels and cell number appeared stable at concentration of up to 19 nM, with a significant reduction at 100 nM. This data supported the MTT assay findings. Figure 4-5.

(i)



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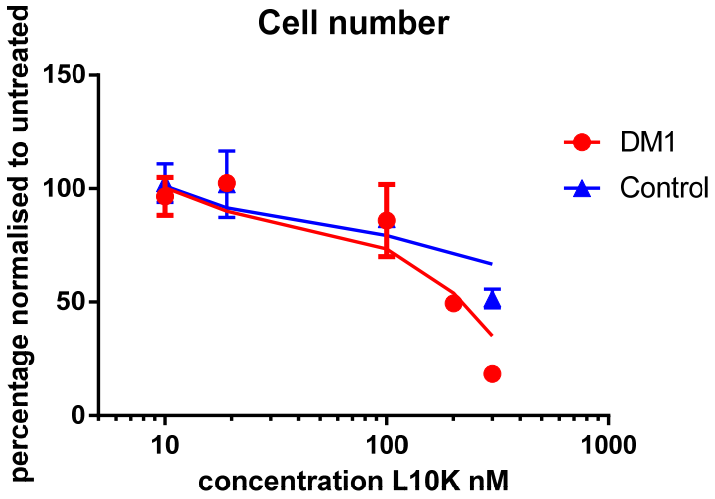


Figure 4-5: (i) Level of total global RNA after treatment with various concentrations of L10K, measured as a percentage of untreated RNA level. (ii) Cell number after treatment with various concentrations of L10K, measured as a percentage of untreated cell number.

Toxicity of combined PEI and oligonucleotide treatment.

In order to determine whether PEI molecules and polyplexes conferred different toxicity levels, DM1 and control cells were treated with various concentrations of the formed polyplex. PEI and oligonucleotide condensation was carried out as described in chapter two using an N/P ratio of 7 regardless of the oligonucleotide concentration.

Cells were plated in 96 well plates at 6000 cells per well and left to settle for 24 hrs. After this time cells were treated for 48 hrs with the required concentration of polyplex in fresh media. MTT assays were then carried out in the plate as described in chapter two. As shown in Figure 4-6, L10K alone required higher concentrations to demonstrate the same level of toxicity as the polyplexes. It appears possible that the entry of oligonucleotides into the nuclei of cells could decrease cell viability to a higher extent than PEI molecules alone. Another potential theory is that the polyplexes c could do a greater degree of damage to the cellular membranes compared to the PEI molecules alone due to their size, therefore also resulting in a decrease in viability. This theory is support by evidence that polyplexes particle size correlates with increased toxicity (Flore, 2002). Further work will be required to gain a deeper understanding of what is causing the increased toxicity observed upon oligonucleotide complexation.

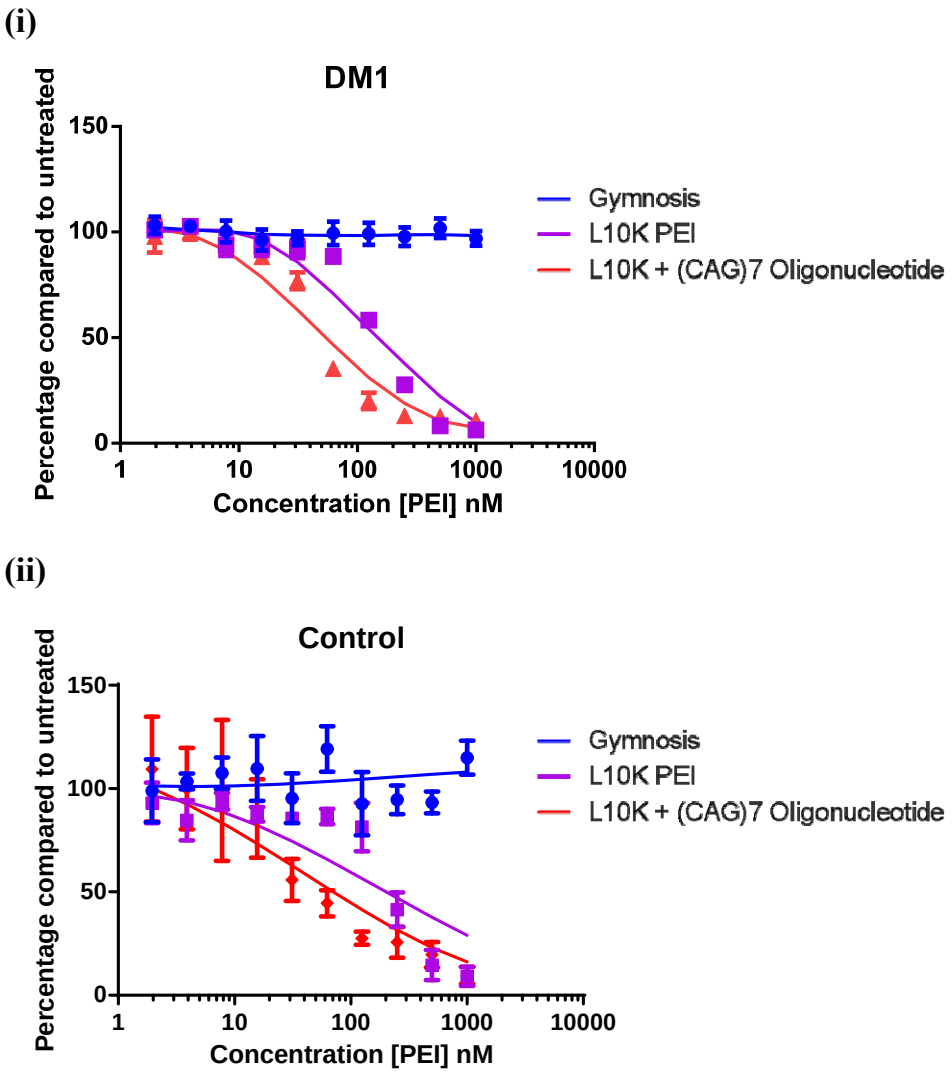


Figure 4-6. Comparable toxicity profiles of cells treated *via* gymnosis, PEI alone and polyplexes in (i) DM1 and (ii) control cell lines. Polyplexes were created using unlabelled 2'OMe $(CAG)_7$ PS oligonucleotides. X axis represents the concentration of the PEI reagent either alone or in the polyplex. For cells treated *via* gymnosis the X axis represents the concentration of the oligonucleotide. The Y axis represents the percentage of viable cells after treatment compared to untreated cells.

Additive toxicity of nuclear delivery

As commented upon by Boeckle, effective intracellular delivery of oligonucleotides is dependent on the molar ratio of PEI:Oligonucleotide, (Boeckle, 2004). Once the excess of PEI is not significant enough, the delivery efficiency of the oligonucleotide to the nucleus falls. Preceding data suggests that treatments with linear L10K PEI causes less toxicity alone than when combined with an oligonucleotide. Increased delivery has been reported to be associated with increasing N/P ratio. With the purpose of identifying a possible association between intracellular oligonucleotide delivery and toxicity, cells were treated with 2'OMe oligonucleotides delivered with complexes formed at various N/P ratios. The oligonucleotide concentration was kept consistent at 75 nM, chosen as it was previously observed to be within the non-toxic range. As before cells were plated in 96 well plates at 6000 cells per well and left to settle for 24 hrs. After this time cells were treated for 48 hrs with the required polyplexes in fresh media. MTT assays were then carried out in the plate as described in chapter two. It was observed that N/P ratios between 10 and 6 led to a high decrease in cell viability. Figures 4-7 and 4-8 show a decreasing impact on cell viability was observed as the N/P ratio approached 1. This data however does not lead to a robust conclusion as this viability profile could be as a result of the combination of decreasing delivery, rather than excess free PEI, as the PEI excess becomes limiting at the lower N/P ratios.

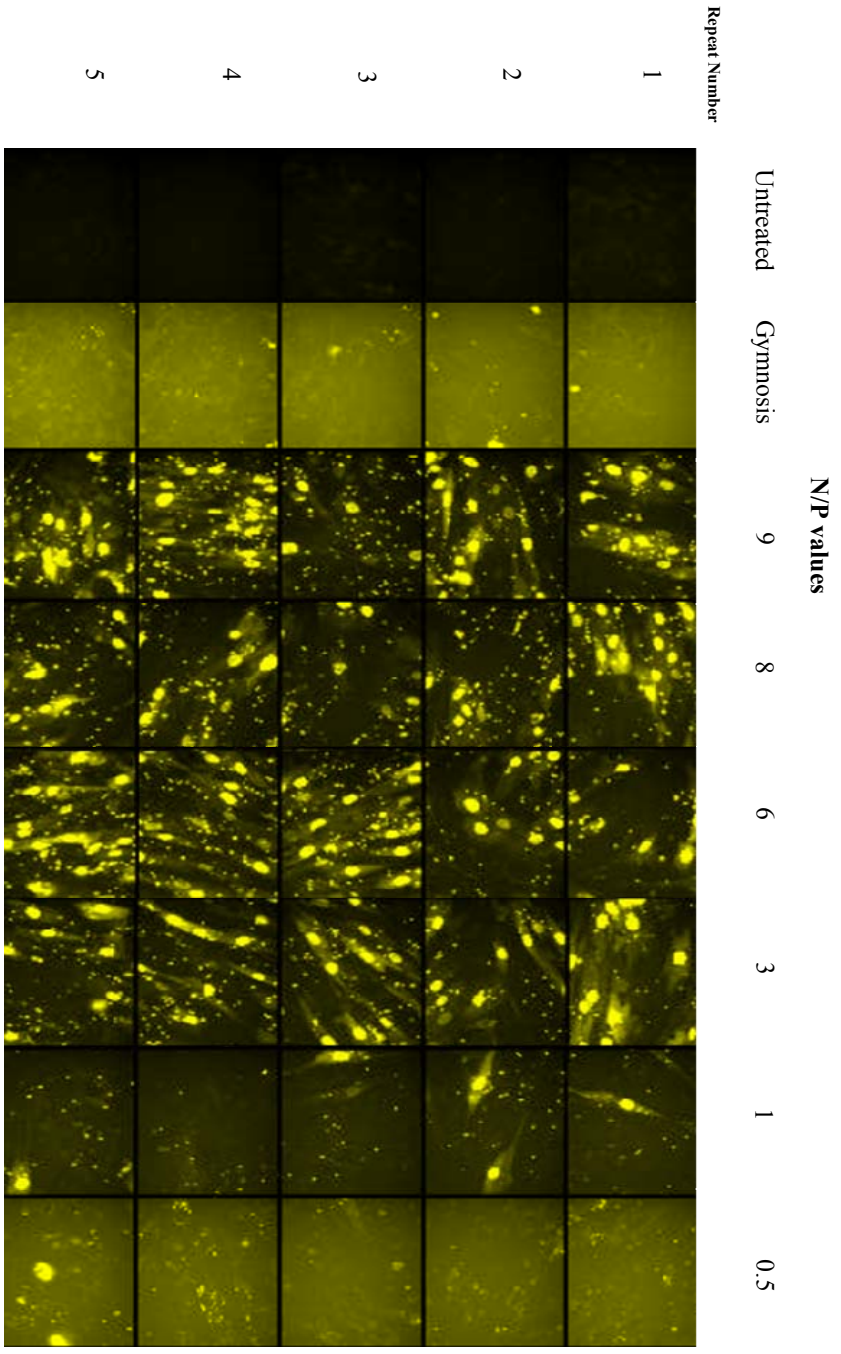


Figure 4-7: Live cell imaging demonstrating the decrease in both cellular and nuclear delivery of $(CAG)_7$ 2'OMe PS oligonucleotide at decreasing N/P ratios with L10K PEI in control cells.

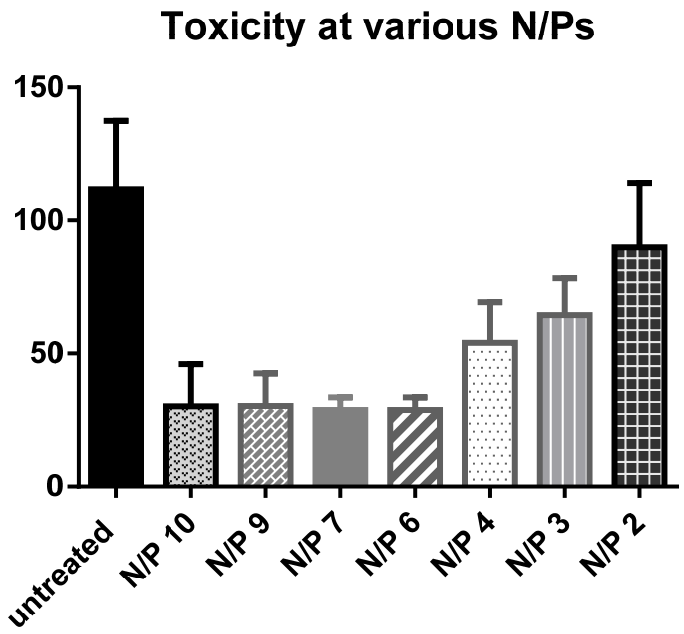


Figure 4-8: Control cell viability upon treatment with polyplexes formed at different N/P ratios using $(CAG)_7$ 2'OMe PS oligonucleotide and L10K PEI.

To further investigate this phenomenon, control cells were treated with both nuclear delivering and non-nuclear delivering PEI reagents, as identified previously, and with and without the presence of 2'OMe $(CAG)_7$ PS oligonucleotide. An N/P ratio of 7 was used as this conferred significant toxicity in control cells when an oligonucleotide concentration of 75 nM was used. As before cells were plated in 96 well plates at 6000 cells per well and left to settle for 24 hrs. After this time cells were treated for 48 hrs with the required free PEI or polyplexes in fresh media. MTT assays were then carried out in the plate as described in chapter two.

Figure 4-9 demonstrates that nuclear delivering PEI molecules led to far higher levels of toxicity when complexed with 2'OMe oligonucleotides than without. It was also observed that non-nuclear delivering PEI reagents did not show the same level of decreased viability as

their nuclear delivering counterparts. This provides evidence that nuclear delivering polyplexes attribute far more toxicity than free PEI. However this does not address whether the toxicity arises from the PEI or the oligonucleotide molecule once the polyplex has entered the cell nuclei.

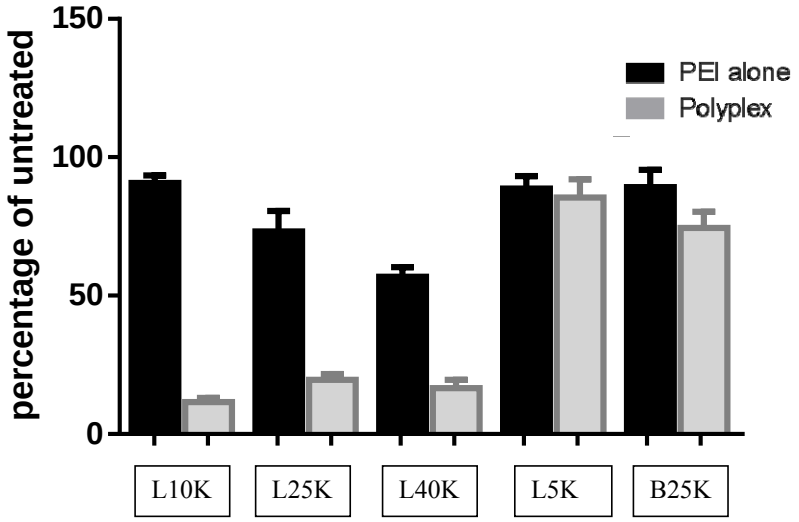


Figure 4-9: MTT assay assessing the viability of control cells upon treatment with free PEI molecules or with polyplexes formed at N/P 7 using $(CAG)_7$ 2'OMe PS oligonucleotide.

In order to validate the MTT assay results in Figure 4-9 the toxicity associated with the nuclear delivery of oligonucleotide was assessed *via* live cell imaging after 24 hrs of treatment. Nuclear delivering PEI molecules *vs.* a PEI molecule which delivers almost exclusively to the cytoplasm were compared using a HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotide, concentration of 100 nM and an N/P ratio of 7, Figure 4-10. Changes in morphology, brightness and adherence were observed in cells where nuclear delivery was high.

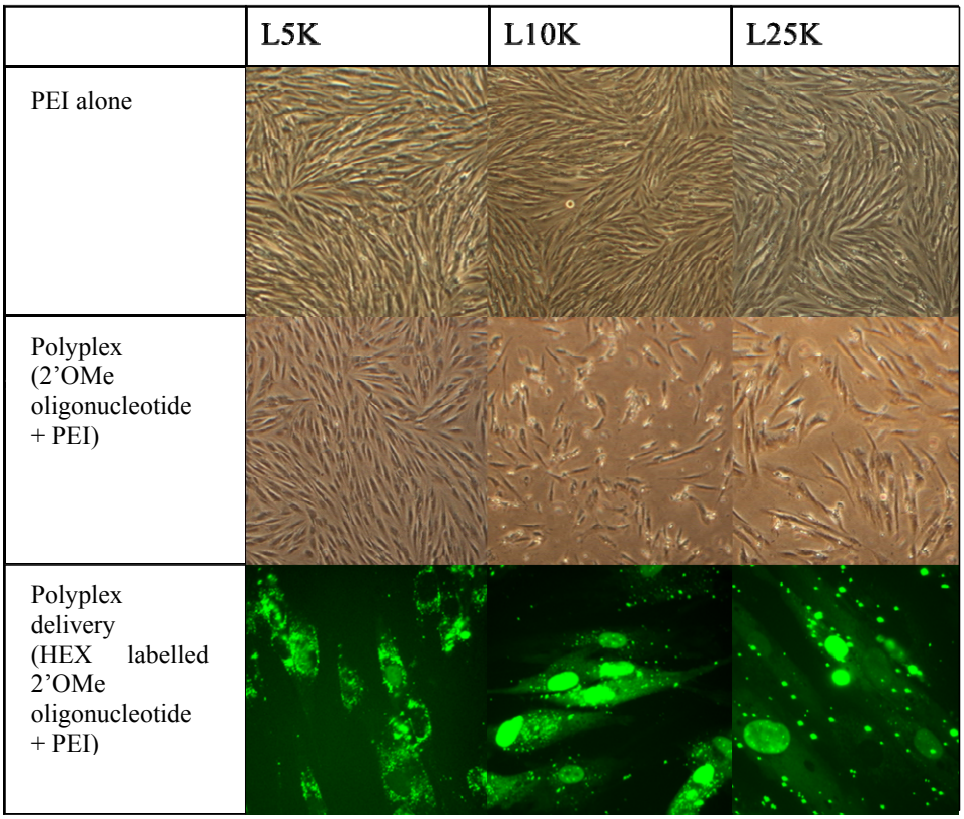


Figure 4-10: Live cell imaging 24 hr (delivery images) or 48 hr (light microscopy) after treatment under various conditions.

Thus, this data suggest that it is the nuclear delivery of these polyplexes that contributes to a significant decrease in cell viability, although it is not clear whether the two molecules synergistically cause the observed toxicity or whether toxicity is due solely to one component over the other, which cannot be accounted for without further experimentation. This observation is supported by work carried out by Godbey ~~*et al*~~*et al*. who found that transfection with certain forms of PEI did have an impact on cell viability and that the effect was parallel to the transfection efficiency, (Godbey, 2001).

Assessing factors affecting delivery

In order to determine the conditions which allow for minimal toxicity with a large uptake of oligonucleotide into the nucleus I tested the variation of five factors to identify their relationship with oligonucleotide delivery. These included PEI structure, PEI molecular weight, PEI:Oligonucleotide ratio (N/P), cell line and oligonucleotide concentration.

PEI structure and molecular weight

The molecular weight dependence on each PEI molecule's ability to deliver $(CAG)_7$ 2'OMe PS oligonucleotides has previously been eluded to, however further studies were undertaken in order to quantify this phenomenon. Five linear and three branched PEI molecules were tested in our control cell line. Delivery was evaluated *via* live cell fluorescent imaging, with the oligonucleotide used at a standard concentration of 75 nM for 48 hrs. As previously described, cells were plated in 96 well plates at a density of 6000 cells per well and left to settle for 24 hrs. After this time, cells were treated for 48 hrs with the required polyplexes and the plate imaged using a wide field microscope. Each well was imaged 9 times, with treatments being run in biological triplicates, ensuring a large number of cells were averaged per treatment. Cytoplasmic and nuclear compartments were analysed individually as previously described in chapter 3 in order to identify whether the PEI structure influenced delivery to distinct compartments to differing extents.

From the 8 commercially available PEI molecules described previously, two sets have the same molecular weight but different structure. The L10K and L25K PEIs were compared with their branched equivalents in order to determine whether PEI structure had a significant effect on delivery. It was observed that of the PEI molecules tested, the linear larger molecular weight PEI molecules were the most successful delivery reagents for their ability

to deliver 2'OMe oligonucleotides to the nucleus. As shown in Figure 4-11 the L10K PEI molecule promoted the highest level of nuclear delivery, seemingly suggesting an optimum PEI size for nuclear delivery as the L25K and the L40K demonstrate successively less potential to promote nuclear delivery. Branched PEI molecules seemed to promote uptake less well, although again the B10K did promote the highest level of uptake compared to the other branched molecules tested.

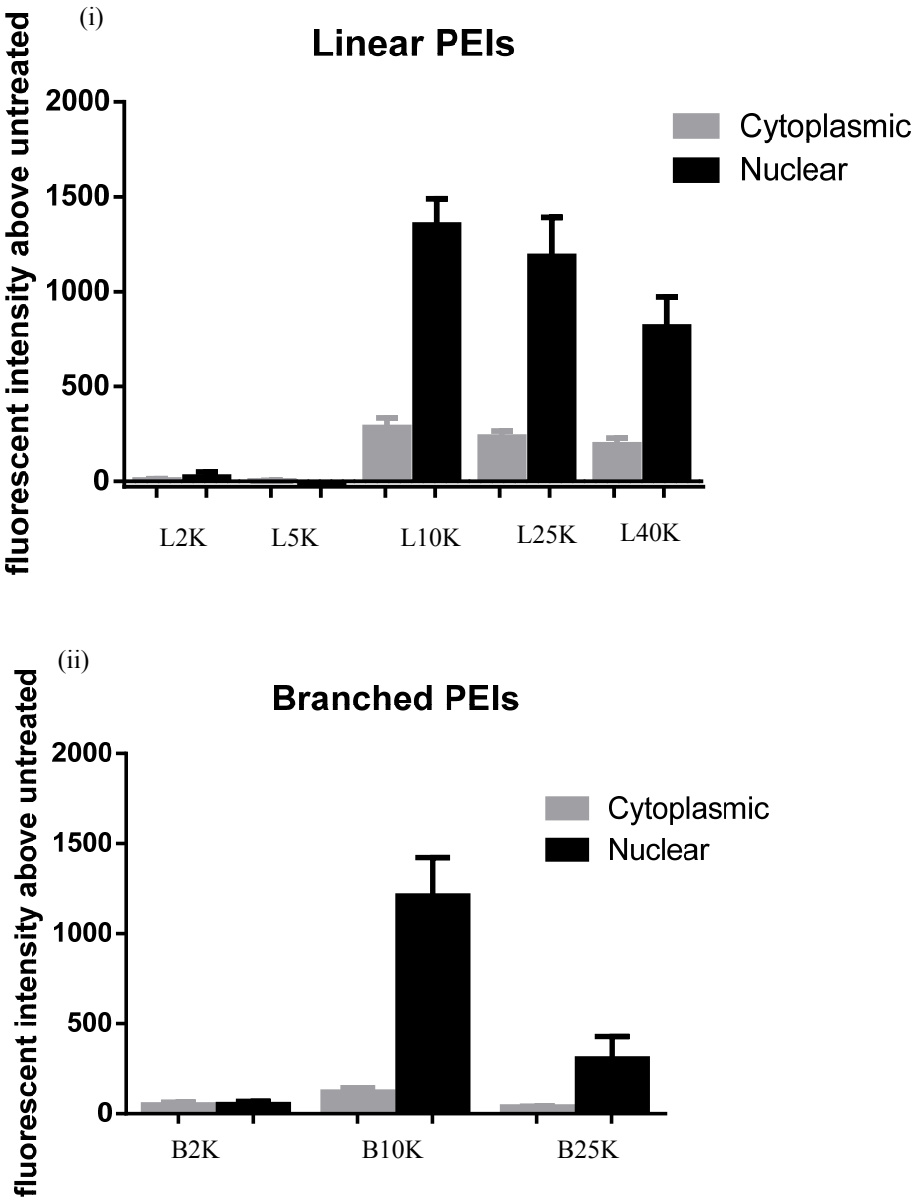


Figure 4-11: Comparison of different (i) linear and (ii) branched PEI molecules ability to deliver HEX labelled $(CAG)_7$ 2'OMe PS oligonucleotide to individual cellular compartments. X axis depicts the type of PEI reagent used. Y axis represents the increase in fluorescent intensity in treated cells above the background of untreated cells. At least 100 cells were analysed per treatment.

PEI:Oligonucleotide ratio (N/P value)

As discussed earlier, uptake *via* PEI transfection is thought to occur *via* endocytosis of nano-complexes formed as the carbocationic PEI molecules complex with the oligonucleotide species. The formation of these nano-complexes is known to be most efficient at certain PEI:Oligonucelotide ratios. This partnership is termed the N/P ratio, and relates the number of amine groups of the PEI to the number of phosphate groups of the oligonucleotide. High N/P ratios are particularly efficient for delivery of oligonucleotides, with PEI being in 5 fold excess or above; however it is proposed that this can cause cytotoxicity as a consequence of the high concentration of free PEI being delivered to the cells.

The majority of studies into the effect of N/P ratio on delivery have been conducted using DNA oligonucleotides. In order to test delivery of 2'OMe oligonucleotides at various N/P ratios live cell imagining was carried out using the wide-field microscope. Control cells were treated with a fluorescent 2'OMe oligonucleotide (75 nM) and one of three PEI molecules, (L5K, L10K, L25K), at the various concentrations needed create a range of N/P ratios.

It was observed that the L5K PEI molecule delivery was inefficient at all the N/P values tested, Figure 4-12(i). It was also observed that an N/P ratio of 6 was highly effective for delivery using L10K PEI, Figure 4-12(ii). Finally it was demonstrated that the L25K PEI molecules that an N/P ratio of 4 was sufficient to observe maximal fluorescent intensity, indicated by the plateau in the graphs shown in Figure 4-12 (iii).

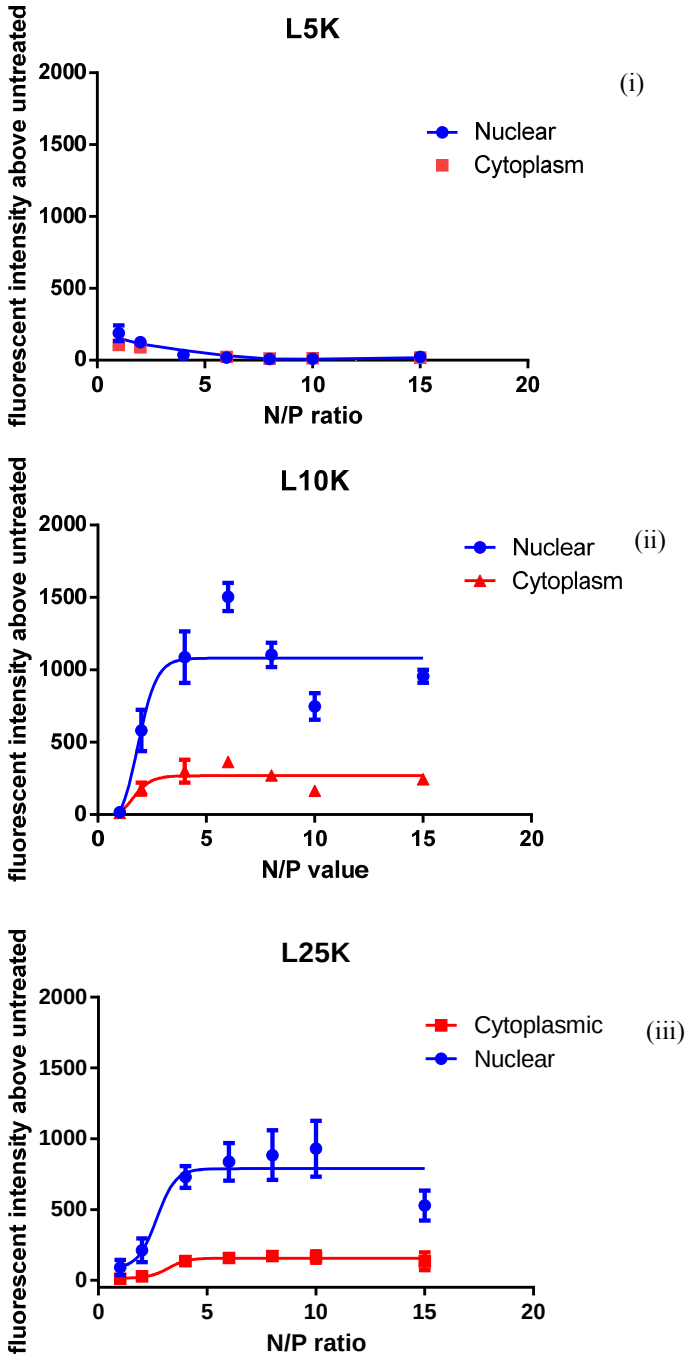


Figure 4-12: Effect of various N/P values on the delivery of $(CAG)_7$ 2'OMe PS oligonucleotide to the individual nuclear and cytoplasmic compartment of control cells using (i) L5K, (ii) L10K and (iii)L25K. X axis depicts the type of N/P ratio used upon polyplex formation. Y axis represents the increase in fluorescent intensity in treated cells above the background of untreated cells.

pH at complexation

As previously mentioned one of the prominent features of PEI molecules is their high cationic charge density due to every third atom within the polymer identifying as a nitrogen atom capable of protonation. Since the PEI polymers do not contain any quaternary amines, all cationic charges are generated *via* protonation in the complexation environment. As the level of PEI protonation varies with pH, it is reasonable to predict that varying the pH of complexation solution could alter the efficiency of complexation between the DNA and PEI molecules therefore altering cellular uptake. If this is the case it could result in inferior oligonucleotide delivery of complexes formed in basic conditions. In order to test this hypothesis, PEI and oligonucleotides were complexed at a range of pH values and tested for their efficiency to deliver to the nucleus, assessed *via* fluorescent live cell imaging. Concentration upon complexation of both reagents were kept the same to ensure this did not skew the results.

Figure 4-13 shows that delivery was successful at a range of pH values, from 3 to 13, though in the most acidic conditions, pH 1, delivery was not observed. Hypothetically this may be due to acid catalysed degradation of the oligonucleotide at such extreme conditions. The range at which delivery can take place is unsurprising as it is well documented that PEI molecules have a good buffering capacity, (Benjaminsen, 2013).

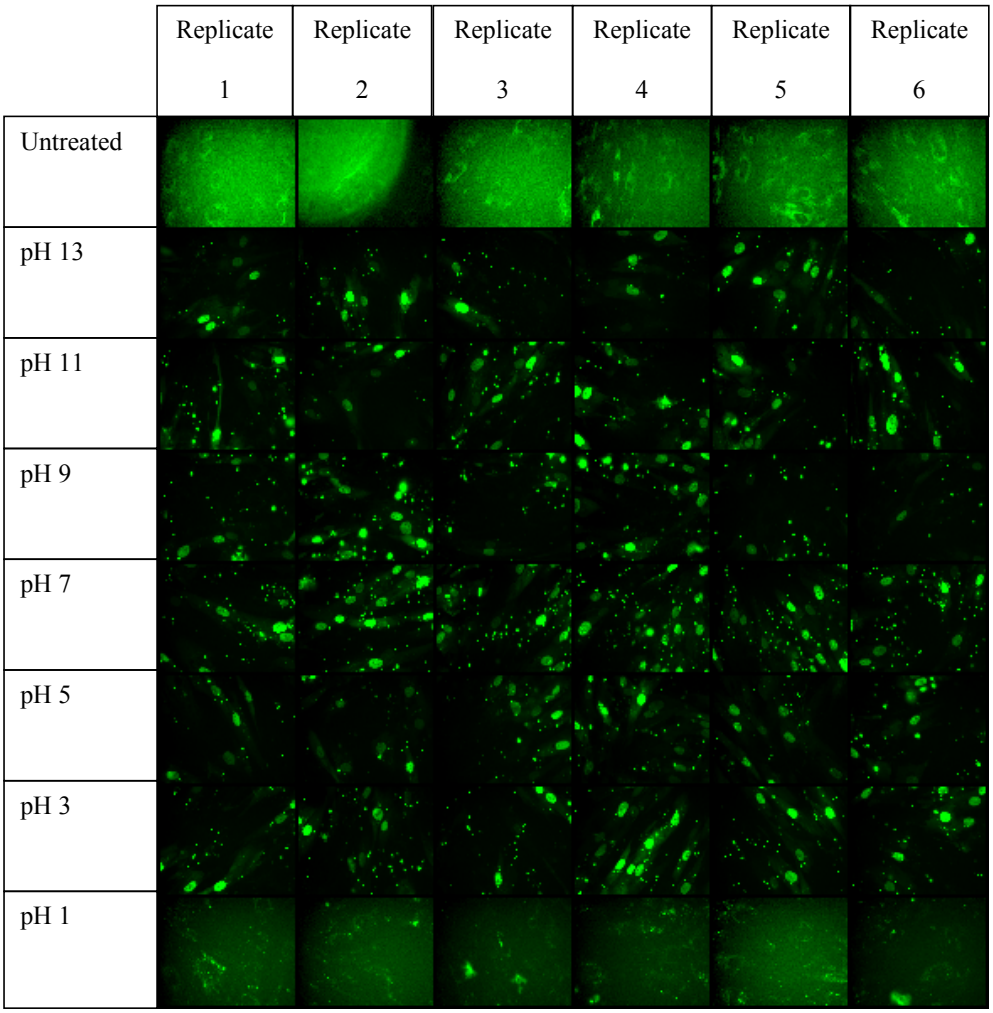


Figure 4-13. Live cell imaging demonstrating the cellular delivery of HEX labelled 2'OMe $(CAG)_7$ oligonucleotide when the pH of the polyplex solution is changed during the complexation period.

Cell line

Previous experimental work has been carried out using cells from an unaffected individual, known as a control cell line. Next I wanted to investigate whether the chosen PEI molecule, L10K, has the same delivery capacity for the 2'OMe $(CAG)_7$ PS oligonucleotide to the disease cell models investigated in this report. This is very important as, if the mutations which cause these diseases also have an impact on the nuclear deliverance *via* PEI transfection, investigations into the use of the L10K molecule would be fruitless in this study.

Delivery was investigated again using live cell fluorescent imaging using wide-field microscope. As well as the four cell lines used previously, cells were also obtained from patients with Becker Muscular Dystrophy (BMD), a milder form of DMD, and Bethlem Congenital Myopathy (BM). Cells were treated with 100 nM 2'OMe oligonucleotide and delivery *via* different transfection methods were compared. As shown in Figure 4-14 it was observed in all of the cell lines tested that transfection using L10K was significantly more efficient than using L5K and gymnosis.

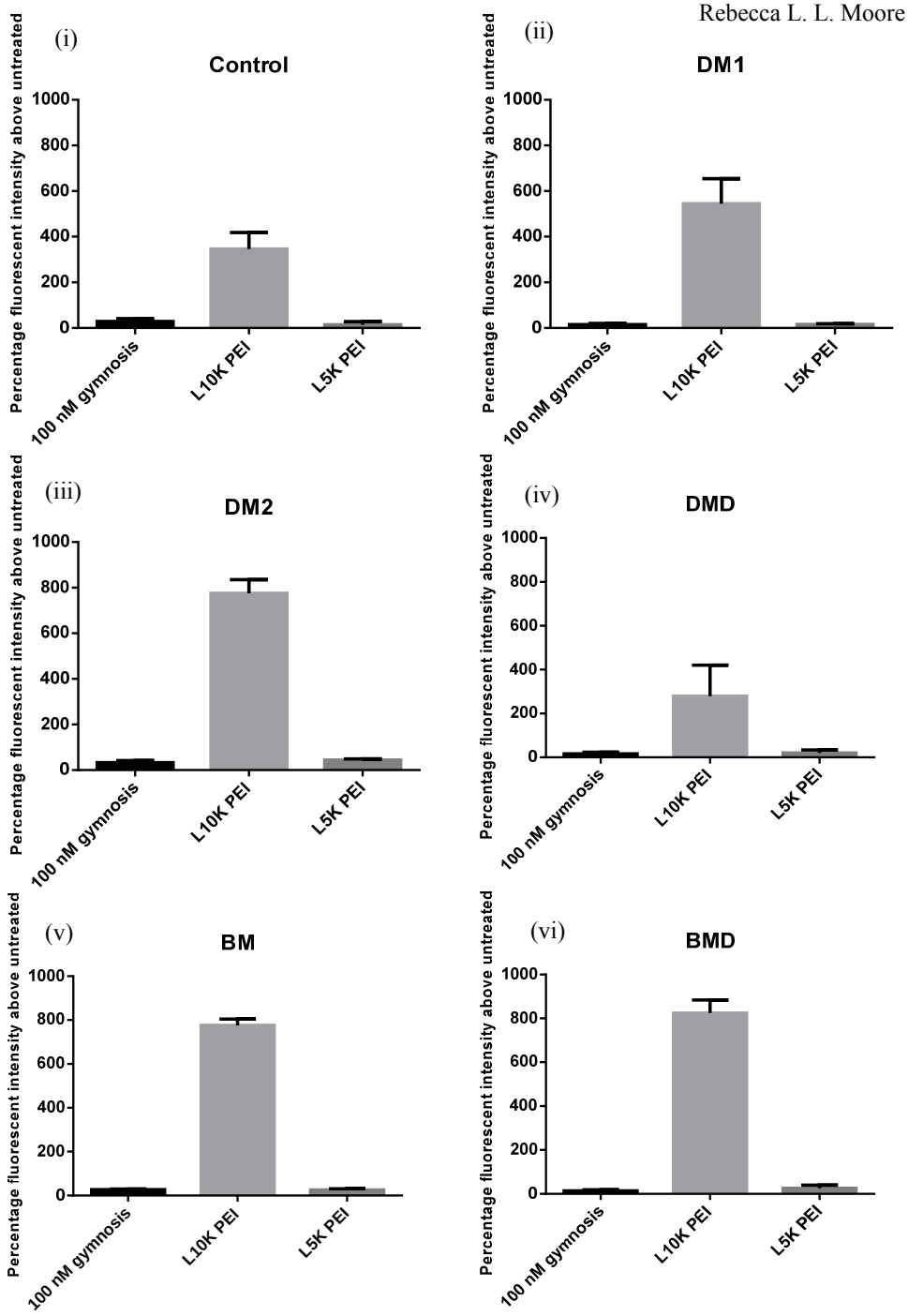


Figure 4-14. Comparative assessment of different delivery methods to assess 2'OMe oligonucleotide delivery to the nuclei in (i) control, (ii) DM1, (iii) DM2, (iv) DMD, (v) BM and (vi) BMD cells. Y axis represents the increase in fluorescent intensity in treated cells above the background of untreated cells.

Oligonucleotide concentration

Previous literature reports have used a range of oligonucleotide concentrations both *in vitro* and *in vivo,* often with concentrations depending upon numerous factors such as: chemical modifications incorporated, nature of the targeted transcript and type of delivery method used. In this set of experiments I aimed to identify whether concentration dependence exists in general for PEI based transfection when assessing nuclear delivery specifically rather than overall cellular delivery. In order to investigate this, seven of the previously tested PEI reagents were used at a range of concentrations in the control cell line.

Cells were treated with HEX labelled 2'OMe $(CAG)_7$ PS oligonucleotide at 25, 50, 100 and 200 nM for 24 hours before live cell images were obtained using a wide-field microscope. As before nuclear fluorescence was measured using software to define a mask of the nuclei based on Hoechst staining. The results obtained are shown in Figure 4-15 and indicate that concentration dependent deliverance to the nuclei occurred for all PEI molecules with a molecular weight above 5000. It was observed however that cells treated at 200 nM were beginning to change morphology, often seen as a first sign of a toxic response.

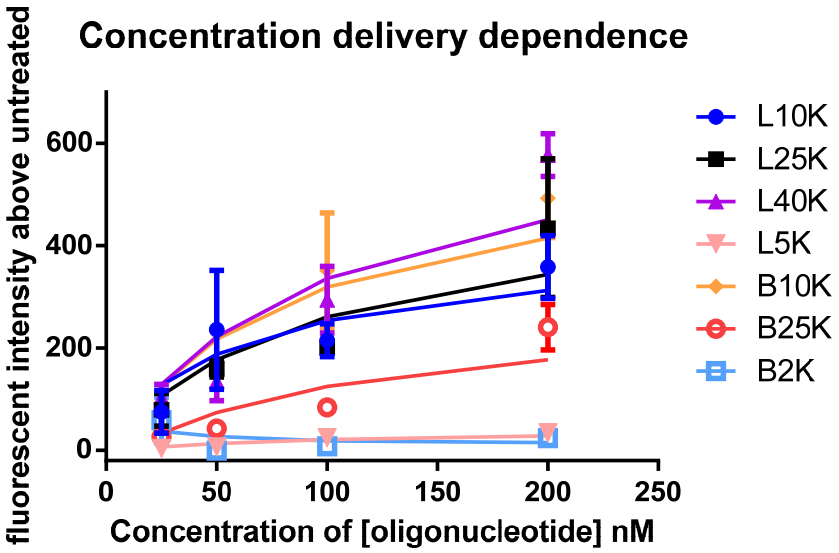


Figure 4-15: Assessment of the relationship between HEX labelled $(CAG)_7$ PS 2'OMe oligonucleotide concentration and nuclear delivery for a range of PEI molecules. Y axis represents the increase in fluorescent intensity in treated cells above the background of untreated cells.

Discussion

The focus of this study was the transfection of oligonucleotides into cell nuclei using PEI molecules. The inspiration for this was taken from the work carried out by Mulders ~~*et al*~~*et al*. published in 2009, who demonstrated that PEI transfection of oligonucleotides had an effect on the *DMPK* transcript, known to be the cause of DM1, (Brook, 1992). PEI molecules can be synthesised in a range of different ways allowing for the creation of different structures and molecular weights. Initial experiments were carried out to investigate which PEI molecules were most successful at delivering 2'OMe oligonucleotides to the nuclei. It was observed by fluorescent microscopy that both linear and branched PEI molecules were capable of nuclear delivery of the oligonucleotide as long as the PEI molecule had a molecular weight above 5 KDa. From the live cell images obtained it appeared that linear PEI molecules allowed for an increased nuclear fluorescent signal compared to the branched molecules, although this was not initially quantified. These observations were validated using a modified northern blot based method previously established in chapter three.

Once I had confirmed that PEI molecules had the capacity to transfect cell nuclei I established the concentration range at which they appear non-toxic to cells. This was primarily evaluated using MTT assays, although global RNA levels and cell number were also used to validate the MTT results. Viability was initially assessed after treatment with the PEI molecule alone, without the presence of oligonucleotide. It was observed that there appear to be a correlation between molecular weight and viability, with high molecular weight PEI molecules resulting in a higher decrease in cell viability at equivalent concentrations. It was also noted that branched PEI molecules were seemingly less toxic than their linear counterparts of the same molecular weight. As with delivery efficiency there are

contradicting reports in the literature on whether branched or linear induce the most toxicity, (Kafil, 2011, Jäger, 2012). Observations from experiments investigating global RNA levels and cell number, Figure 4-5, demonstrated the same trend in increasing concentrations leading to increased toxicity at concentrations similar to those in the MTT assay.

MTT assays were then used to assess the toxicity of the resultant polyplex once the L10K PEI molecule and 2'OMe oligonucleotide were incubated together. It was noted that the addition of an oligonucleotide to the PEI molecule resulted in a decrease in cell viability compared to the PEI molecule alone when the same concentration of PEI molecule was used. In order to assess whether the observed toxicity was a result of cellular uptake of the oligonucleotide, cells were transfected at various N/P ratios to establish whether a decrease in cellular delivery resulted in a restoration of viability. It was observed that at the higher N/P values tested cell viability fell significantly, whereas at lower N/P values cell viability improved. However, from this data set it was unclear whether the toxicity was caused by a higher cellular uptake of the polyplex or the higher amount of un-complexed PEI reagent at the higher N/P values.

In order to address the short-comings of this experimental design a range PEI reagents with differing oligonucleotide delivery capacities were tested at the same N/P ratio. It was observed that the best performing PEI reagents in terms of cellular delivery resulted in the highest decrease in cell viability when combined with oligonucleotides, whereas a PEI reagent with only a moderate capacity of delivery resulted in less observed toxicity. When used alone the PEI reagents conferred significantly less toxicity in the 24 hr time period when compared to the 48hr MTT results. This suggests a potentially different toxicity mechanism

between PEI reagents alone and PEI/oligonucleotide polyplexes, with polyplexes seemingly resulting in a decrease in viability faster than sole PEI molecules. Cells were imaged after treatment in order to demonstrate the difference in cell viability when treated with PEI reagent alone and with polyplexes, the difference in cell number and morphology were unmistakable.

Next several experimental studies were carried out in order to establish what effect various parameters had on delivery. PEI structure, N/P ratio, pH upon complexation, cell line and oligonucleotide concentration were all assessed. It has been well documented that PEI molecules of different molecular weights and structures afford varying protection from nucleases and delivery potential, (Godbey, 2001 and Brus, 2003). It was observed that for oligonucleotide delivery to be sucessful PEI moelcules must be over 5 kDa, at least for oligonucletoides of a length of 21 bp. Structurally it was evident that linear PEI moelcules were more efficient at nuclear delivery than branched molecules of the same weight.

The second factor to be considered was the N/P ratio as it is recognised that the ratio of PEI amine groups and oligonucleotide phosphate groups has an impact on complete complexation between the two reagents in the polyplex. It was observed that the L5K PEI molecule did not adequately deliver oligonucleotide at any of the N/P values tested, L10K performed best at an N/P ratio of 6 and delivery *via* the L25K PEI molecule plateaued after an N/P ratio of 4.

It is well known that complexation between PEI and oligonucleotide occurs *via* electrostatic interactions. As the cationic charge of the PEI molecule is dependent on the number of protonated amine groups it is possible that the pH of the solution at the time of complexation

could alter the overall net charge of the PEI reagent. This change in cationic charge could influence the amount of oligonucleotide condensed, consequently impacting delivery. Although I hypothesised that delivery would increase at lower pH values due to a higher overall cationic change of the PEI molecule, nuclear delivery seemingly remained constant at all the pH values tested. However this data was not quantitatively evaluated or collected from looking at a specific knockdown therefore only allows for a basic statement of delivery occurring at a wide range of pH values.

As mentioned in chapter one, there are three main disease types of interest in this study, all of which are forms of muscular dystrophy. In order to establish whether PEI transfection was suitable for a range of muscular dystrophies two other cell lines were acquired from patients with Bethlem congenital muscular dystrophy (BD) and Becker muscular dystrophy (BMD). The delivery efficiency of gymnosis, L10K and L5K were compared in each of the cell lines. It was evident that transfect *via* L10K resulted in both far higher cytoplasmic and nuclear delivery in each cell line. Although the average fluorescent intensity within the nuclear compartment varied between disease models, I am unsure whether this is as a direct result of differences in the disease models or the cell state as each cell line was tested at a different passage number with some cell lines being immortalised and other cell lines cells being primary.

Finally the last parameter tested was the concentration of the oligonucleotide. With regards to the N/P data from the L10K PEI molecule it was observed that at an N/P of 6, maximal delivery to the nuclear compartment was observed, after this point nuclear delivery as measured by fluorescent intensity plateaued. In order to identify whether such a plateau effect

existed in regards to concentration control cells were tested at a range of oligonucleotide concentrations in combination with each of the PEI reagents, up to a concentration known to be toxic. It was observed that for PEI molecules above a molecular weight of 5 kDa an increase in concentration led to an increase in nuclear fluorescent intensity, however at concentration above 100 nM morphological changes were observed in the cells, thought to be as a result of toxicity.

In conclusion the data presented in this chapter indicated that the oligonucleotide delivery potential of PEI molecules is highly dependent upon their weight and structure. PEI molecules are also seemingly more toxic when complexed to oligonucleotides although the mechanism for this is unknown. It was observed that successful PEI transfection of oligonucleotides is dependent on the ratio of PEI to oligonucleotide (N/P ratio), as well as the concentration of the oligonucleotide when the N/P was kept at a value of 7. Somewhat accidently it was also observed that the concentration of reagents at complexation is also important, as low concentrations of reagents at complexation do not lead to successful delivery hypothetically due to lack of condensation. In contrast, nuclear delivery is seemingly not dependent on the pH at complexation as well as not being dependent on the disease model tested.

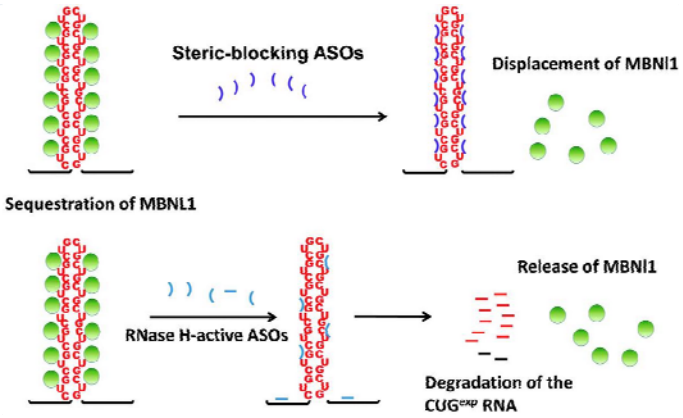
Chapter five: Effect of treatment on *DMPK* transcript

Introduction

In order to treat genetic disease the most obvious approach is to try and negate the dominating negative cause. The predominant molecular mechanism involved in the pathology of myotonic dystrophy type one (DM1) has been identified as an unstable expansion of a $(CTG)_n$ repeat sequence. Various groups have established that the features of RNA toxicity are reversible. Using a DM1 mouse model, in which $(CUG)_n$ RNA expression is inducible, Mahadevan ~~et al~~*et al*. demonstrated the first *in vivo* proof of principle for an antisense therapeutic strategy involving the degradation or silencing of the toxic repeat expansion, strongly suggesting that reducing the level of expanded *DMPK* transcripts will be beneficial to patients, (Mahadevan, 2006). Since this verification numerous studies have attempted to use various antisense agents, including oligonucleotides, small interfering RNAs, small organic molecules, ribozymes, and engineered small nuclear RNAs in order to sterically block, degrade or export the toxic expanded RNA, (Mulders, 2009, Wheeler, 2012, Ketley, 2013, Wojciechowska, 2014, Magana, 2011). Due to the nature of this project I have purposely only described those studies involving single stranded oligonucleotides as these are most applicable to the work that follows.

In 2007 Wheeler ~~et al~~*et al*. described the successful use of PMO oligonucleotides to restore normal splicing of the muscle-specific chloride channel (ClC-1) in HSA^{LR} mice by selectively repressing the inclusion of exon 7a, potential indicating the correction of ClC-1 splicing could significantly improve myotonia in DM1 patients, (Wheeler, 2007). Although this is a valid approach for targeting the symptoms of myotonia it would leave other

pathogenic aspects untreated. Unsurprisingly it seems that, from the majority of scientific literature, the most effective approach to reduce the RNA induced toxicity is to degrade or negate the effects of the toxic RNA directly, as demonstrated by Mahadevan, (Mahadevan, 2006). There are two predominant mechanisms capable of this using antisense therapy, either *via* steric hindrance to prevent protein sequestration, or by active degradation of the toxic RNA, Scheme 5-1.



Scheme 5-1: Schematic representation of how oligonucleotides interact with the expanded repeat of the *DMPK* transcript in order to produce a therapeutic response. Scheme taken from "Antisense oligonucleotides: Rising stars in eliminating RNA toxicity in myotonic dystrophy", (Gao, 2013).

Two independent studies by Wheeler, (Wheeler, 2007) and Mulders, (Mulders, 2009), demonstrated effective degradation of the $(CUG)_n$ toxic repeat using two distinct chemistries of oligonucleotide, a 25 nucleotide PMO oligonucleotide and a 21 nucleotide 2'OMe oligonucleotide respectively. It was however unanticipated that both would result in such large reductions in expanded transcripts as neither modification is thought to induce RNase H enzymatic cleavage. It is currently thought that these treatments potentially allow for nuclear export of the toxic RNA once the sequestered proteins are released, allowing for cytoplasmic degradation of the expanded repeats. The use of gapmer technology was investigated by Lee

~~et al~~*et al*. specifically to induce RNase H cleavage of the toxic RNA. Both LNA and MOE flanked gamers were capable of significantly reducing the level of toxic RNA in a cell culture model of DM1, (Lee J. E., 2011, Wheeler T. M., 2012). It has also been revealed that synergistic effects exist when combining therapeutic strategies, with the co-administration a 25mer PMO oligonucleotide with a gapmer resulting in increased degradation of the toxic RNA transcripts. Short LNA modified oligonucleotides of either eight or ten nucleotides have also been shown to be effective at reducing the expanded RNA repeat recently, (Wojtkowiak-Szlachcic, 2015), with the ability to correct the DM1- specific alternative splicing abnormalities and disrupt foci.

Although the use of oligonucleotides targeting the expanded $(CUG)_n$ repeat has been shown to have huge benefits, the off target effects of such a strategies must also be considered. It has been repeatedly stipulated that the use of any therapeutic strategy involving the degradation of the *DMPK* transcript for the treatment of DM1 should specifically target the mutant transcript whilst leaving the wild-type transcript intact. As a repeat expansion there is by definition no consistent polymorphic difference between the wild-type and mutant repeat expansions, only the repeat number differs. Statistically however due to the large size of the mutant expansion and its nuclear sequestration targeting the mutant transcript specifically should be enhanced by these characteristics, especially as PEI molecules seemingly accumulate oligonucleotides in the nucleus.

To date some of the most promising results have been those produced in the Wansink lab, who have previously demonstrated a silencing capacity of up to 80% of the expanded CUG repeat *in vivo* after treatment with a 2'OMe phosphorothioate oligonucleotide, (Gonzalez-

Barriga, 2013, Mulders, 2009). Further work by the group has progressed to test a variety of other repeat targeting oligonucleotides of different chemical identities. Initially I sought to build on this work by testing PNA and LNA oligonucleotides alongside gapmer technologies *via* gymnotic delivery; however findings in the early stages of the experimentation resulted in a separate direction for the work carried out.

Specific techniques applied

BpmI - As previously mentioned one of the critical parameters when designing an antisense therapy for the treatment of myotonic dystrophy is the specificity of the therapeutic agent for the expanded repeat. Length is typically the only differing parameter between the two *DMPK* transcripts of DM1 patients (Gonzalez-Barriga, 2013), however a small percentage of DM1 patients present a Bpm1 recognition site in one of their *DMPK* alleles. This enables the assessment of transcript levels from each *DMPK* to be determined independently based on their distinctive size after digestion, Figure 5-1.

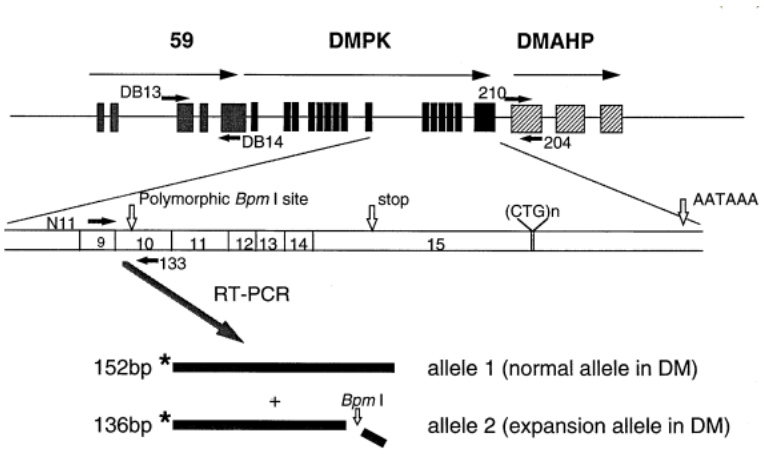


Figure 5-1: The position of the polymorphic *BpmI* recognition site in exon 10 of the *DMPK* gene in our DM1 cell line. Primer positions are indicated by short black arrows. (Hamshere, 1997).

PCR amplified products from the wild-type and mutant can be analysed independently by gel electrophoresis when a high percentage (~3%) agarose gel is used, Figure 5-2, or *via* GeneScan if a fluorescent primer is used during amplification.

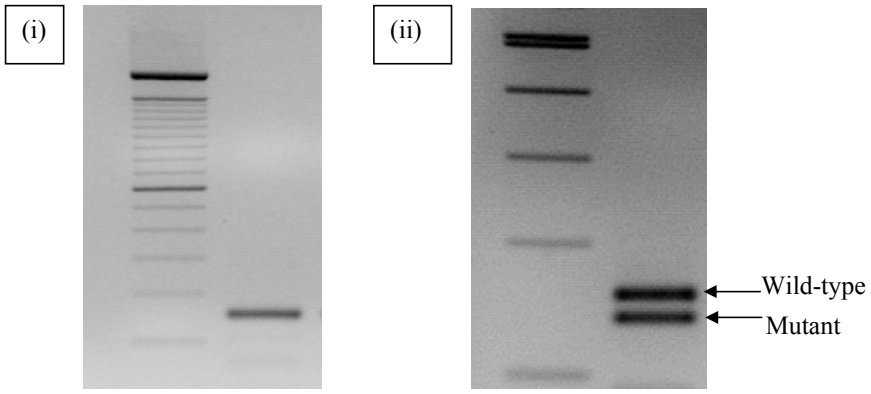


Figure 5-2. Agarose gel showing the *DMPK* amplicon (i) before and (ii) after digestion with *BpmI*.

GeneScan - This technique allows for the high through-put analysis of DNA fragments. The procedure uses algorithms to automatically identify and size each peak relative to an internal size standard providing size, peak area and peak height information for each sample. Internal size standards consist of fragments of known sizes, which are added to and electrophoresed with every sample being investigated. Analysis errors are minimised with the GeneScan Analysis Software which automatically analyses data collected from each sample, removing human interpretive inaccuracy. Using the peak area it is possible to determine the ratio between the mutant and wild-type transcripts.

Aim of study

Combining the knowledge of optimised delivery methods, toxicity profiles and factors affecting delivery, I aim to investigate what effect 2'OMe oligonucleotide delivery has upon

the mutant *DMPK* transcript. I will investigate oligonucleotide delivery *via* both gymnotic and PEI transfection in order to ascertain whether nuclear delivery is essential for transcript degradation. Further experimentation to test oligonucleotides of various other chemistries for their ability to silence the mutant *DMPK* transcript will identify the most potent antisense oligonucleotide for treatment.

Results

In order to investigate whether 2'OMe oligonucleotides have an effect on the degradation of the *DMPK* transcript in our hands, cells from a DM1 patient cell line were treated with oligonucleotides using various transfection methods.

Effects of oligonucleotide treatment *via* gymnosis

It is widely accepted that any antisense agents must reach the nucleus in order to reduce the level of toxicity caused by the nuclear retained, expanded RNA. Previous results in Chapter 3 clearly demonstrated that 2'OMe oligonucleotides are unable to penetrate the cell nucleus unassisted. In order to test whether antisense oligonucleotides could act from outside the nucleus, DM1 cells were exposed to naked 2'OMe $(CAG)_7$ PS oligonucleotides for 48 hr. After treatment cells were fractionated and RNA was extracted from the nuclear compartment as previously described. The resulting RNA was reverse transcribed and PCR amplified before digestion with the *BpmI* enzyme. The digestion allowed the level of mutant and wild-type *DMPK* transcripts to be distinguished separately, as illustrated above, Figure 5-2. Samples were then submitted for GeneScan analysis and the data processed using the specialised software. As stated in the introduction, GeneScan gives a separate peak area for the mutant and wild-type transcripts based on their size. The abundance of each transcript in relation to one another can be identified from their area ratios. It was found that in untreated samples a mutant:wild-type ratio of 58:42 was detected in our DM1 cell line. As shown in Figure 5-3, treatment with either a scrambled 2'OMe PS (used as a negative control) or a treatment 2'OMe PS $[CAG]_7$ oligonucleotide had no significant effect on the mutant:wild-type ratio. The observations here were made in fibroblasts; other studies on RNA levels in

untreated DM1 cells have reported conflicting results, finding varying levels of mutant RNA across tissue types. (Depardon, 2001).

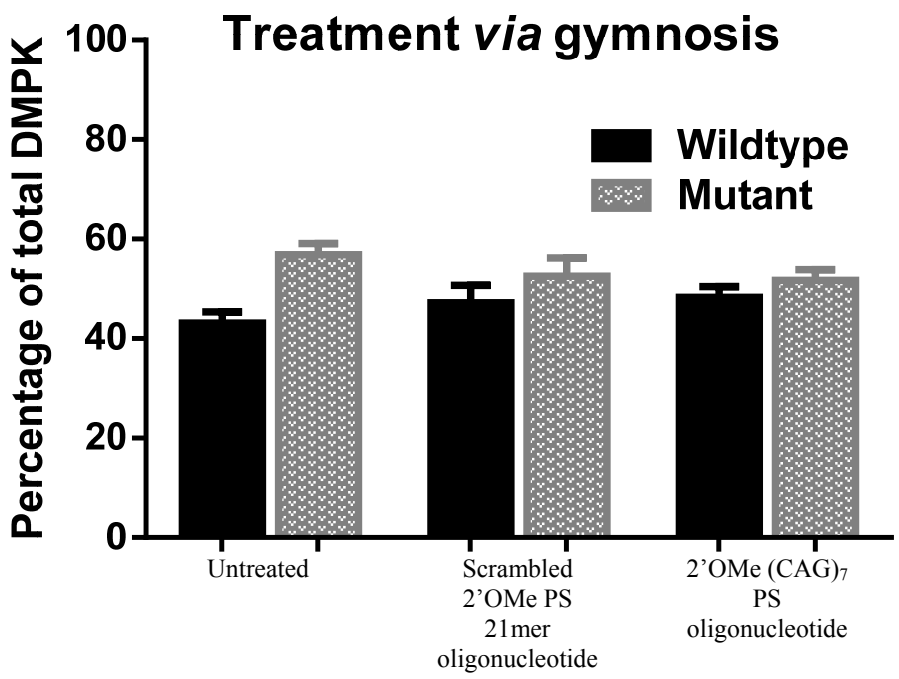


Figure 5-3: GeneScan analysis of *DMPK* transcripts from DM1 cells. Ratio of Wild-type:Mutant *DMPK* RNA transcripts. Scrambled 2'OMe PSoligonucleotide (100 nM) used as a control. 2'OMe $[CAG]_7$ (100 nM) used at the treatment agent.

Effects of oligonucleotide treatment *via* PEI transfection

Once it had been established that oligonucleotides delivered *via* gymnosis had no significant effect on the *DMPK* transcript ratio I moved on to study the effects of oligonucleotide delivery *via* polyethylenimine (PEI) transfection. As demonstrated in chapter 4 various PEI molecules have the ability to deliver oligonucleotides to the nucleus of DM1 cells. This delivery strategy holds far greater promise for the observation of an effect on the *DMPK* transcript at the drug molecule is able to access its target.

Exgen 500 transfection

As experiments performed by Mulders ~~et al~~*et al*. were carried out using a commercial PEI based transfection reagent, marketed as Exgen 500, I first studied the effects of transfection of $(CAG)_7$ 2'OMe PS oligonucleotides using this PEI based carrier.

In order to ensure the experiment was well designed, three distinct negative controls were developed utilising: untreated cells, cells treated with Exgen 500 only and cells treated with Exgen 500 and a control scrambled oligonucleotide. As above, cells were treated for 48hrs before nuclear RNA extraction, reverse transcription, PCR amplification and *BpmI* digestion were performed. Once obtained the samples were submitted for GeneScan analysis. The results were surprising, with the presence of Exgen 500 alone significantly reversing the abundance ratio of mutant:wild-type transcripts without the addition of any specific *DMPK* targeting agent, Figure 5-4. There did appear to be a further shift in favour of the wild-type transcript with the addition of the treatment 2'OMe $[CAG]_7$ PS oligonucleotide, however this was not found to be statistically significant. Although not what we set out to investigate we

felt this was an important finding as to my knowledge there are no observations within the literature of transfection reagents having the targeting effect.

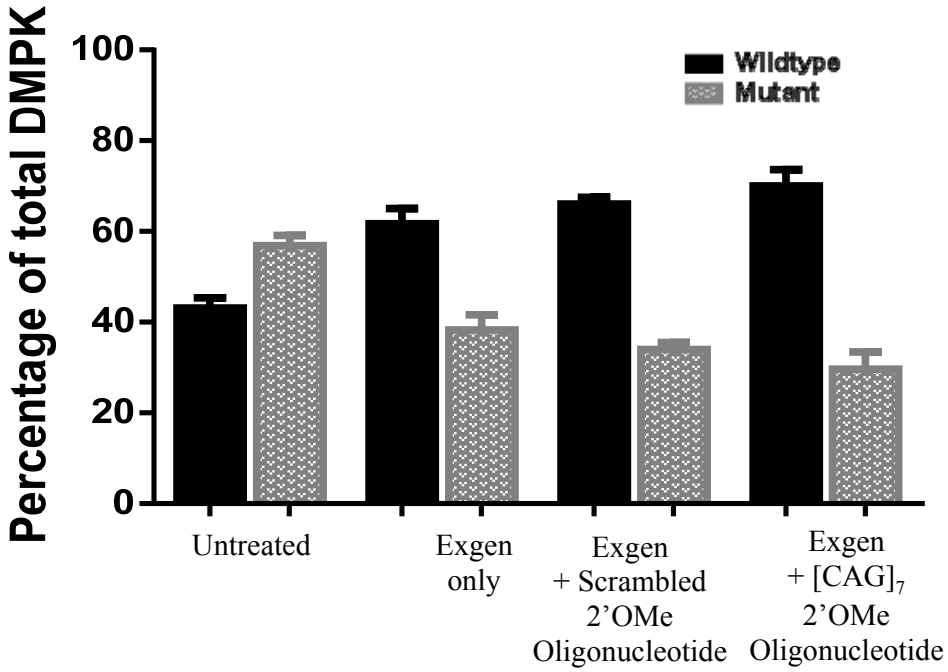


Figure 5-4: GeneScan analysis of *DMPK* transcripts from DM1 cells. Ratio of Wild-type:Mutant *DMPK* transcripts. Exgen only (75 nM) and polyplex consisting of Exgen and a scrambled 2'OMe oligonucleotide (100 nM) used as a control. 2'OMe PS $[CAG]_7$ oligonucleotide (100 nM) transfected with Exgen (75 nM).

Various other PEI molecules

As Exgen 500 is a formulated transfection reagent protected by patent restrictions it is unknown what else the reagent contains chemically. In light of the previous results I set out to identify whether the PEI element of the transfection reagent was responsible for the abundance shift of the *DMPK* transcripts. To investigate this, cells were treated with PEI molecules alone (absent of oligonucleotide) with various molecular weights and different nuclear delivering capacities tested. L10K, L25K and L5K PEI molecules were selected for treatment, both with and without 2'OMe PS $[CAG]_7$ oligonucleotide. As shown in Figure 5-5, all PEI molecules tested were able to alter the *DMPK* mutant:wild-type ratio in favour of the wild-type allele without the addition of a targeting oligonucleotide, indicating that it is the PEI chemistry resulting in an abundance shift of the DMPK transcript. Previous studies have eluded to this with it being observed that PEI particles can be delivered alone when not complexed to oligonucleotides, these were eventually localised into nuclei as ordered structures, potentially allowing them to interfere with host genes, (Chirila, 2002). This is noteworthy as the use of transfection reagent alone is rarely used as a negative control within the literature surrounding DM1. Carrier molecules are often seen as inert entities rather than the reactive chemical molecule they potentially are, seemingly studies are therefore being carried out without proper scientific controls or a full appreciation of the effects of the carrier reagents themselves.

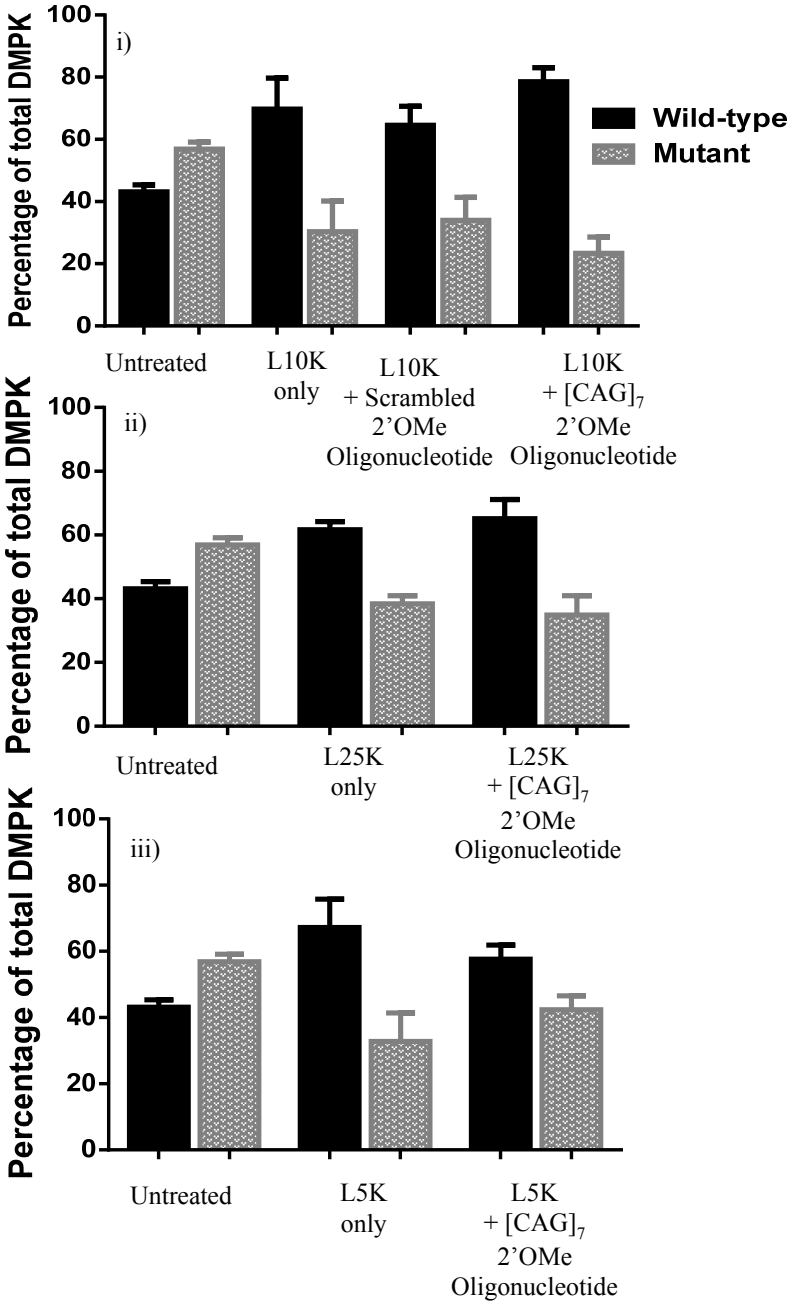
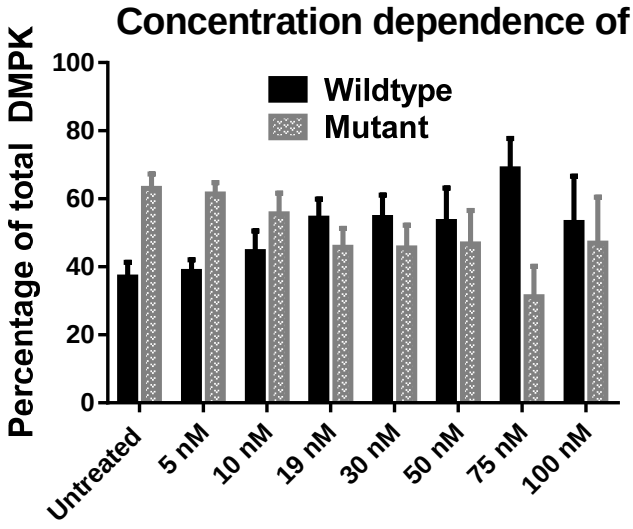


Figure 5-5: GeneScan analysis of *DMPK* transcripts from DM1 cells. Ratio of Wild-type:Mutant *DMPK* transcripts. PEI only (75 nM) (i) L10K, (ii) L25K, (iii) L5K, scrambled 2'OMe oligonucleotide where used (100 nM). 2'OMe $[CAG]_7$ PS oligonucleotides were used at the treatment agent. (100 nM).

Further investigations using L10K PEI

In order to further investigate the observed phenomenon of PEI resulting in a shift in the ratio of the DMPK transcripts, cells were treated with varying concentrations of L10K PEI to identify whether there was a concentration dependence effect on degradation. It was hypothesised that if a concentration dependent relationship did exist, it was more credible that the abundance shift of the *DMPK* transcripts was a result of a specific effect caused by the PEI reagent on the *DMPK* transcript. DM1 cells were treated for 48 hr followed by the usual protocol. Upon analysis a concentration dependence effect was clearly identified with increasing concentration of L10K leading to a greater shift in favour of the wild-type transcript. The pattern was only interrupted at the highest concentration, which may be due to extensive toxicity effects starting to manifest, Figure 5-6.



Treatment	Ratio WT : MT
Untreated	1 : 1.70
5 nM	1 : 1.58
10 nM	1 : 1.25
19 nM	1 : 0.84
30 nM	1 : 0.86
50 nM	1 : 0.88
75 nM	1 : 0.45
100 nM	1 : 0.88

Figure 5-6: GeneScan analysis of *DMPK* transcripts from DM1 cells. Concentration dependence of L10K PEI alone on *DMPK* transcript ratio.

Although appreciably an important observation there is an inherent inadequacy in the GeneScan analysis technique in its current form. Currently DMPK transcripts are only quantitatively compared to one and other, mutant to wild-type. It is therefore impossible to ascertain whether the PEI molecules are selectively degrading the mutant transcript or whether they are degrading both transcripts disproportionately, potentially due to the mutant transcripts increased size. In order to address this shortfall work was undertaken to optimise a duplex PCR amplification in order to amplify both the DMPK gene and the HPRT gene, used as a housekeeping standard, Figure 5-7.

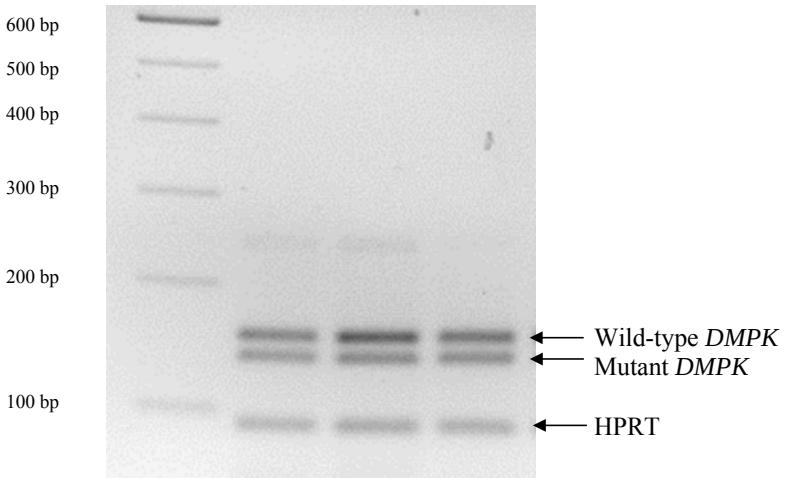


Figure 5-7: Agarose gel showing the duplex PCR amplicons of *DMPK* and HPRT after digestion with *BpmI*.

Specificity of *DMPK* transcript degradation

The importance for any drug targeting the *DMPK* transcript to selectively degrade the mutant transcript in DM1 patients has long been acknowledged, (Mulders, 2009). As mentioned above the current technique does allow for the standardised quantification in relation to an internal standard. With a duplex PCR amplification protocol in hand, it enables the separate analysis of mutant and wild-type transcripts in comparison to the housekeeping gene HPRT. There was found to be no HPRT degradation by L10K PEI, assessed *via* concentration dependent studies on gel electrophoresis. Figure 5-8 depicts a typical plot output from the GeneScan software after the duplex PCR has taken place.

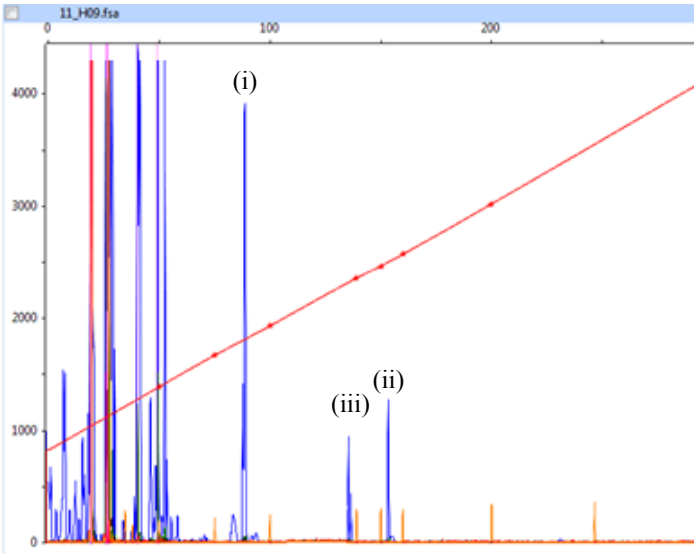


Figure 5-8: Schematic of a typical GeneScan plot observed after duplex PCR (*DMPK* and HPRT) followed by digestion with *BpmI* using RNA samples from DM1 cells. (i) HPRT, (ii) wild-type *DMPK* transcript, (iii) mutant *DMPK* transcript.

The three separate peaks can clearly be identified and integrated to obtain the area in order to assess the degradation of both *DMPK* transcripts respective to HPRT. To determine whether L10K PEI can discriminate between expanded and non-expanded RNA transcripts cells were

treated with various concentrations established from previous experimentation to alter the abundant ratio of mutant:wild-type *DMPK* products. After treatment and analysis it can clearly be observed from Figure 5-9 that there is a concentration dependent degradation of both *DMPK* transcripts, wild-type and mutant, by L10K PEI in our DM1 cell model.

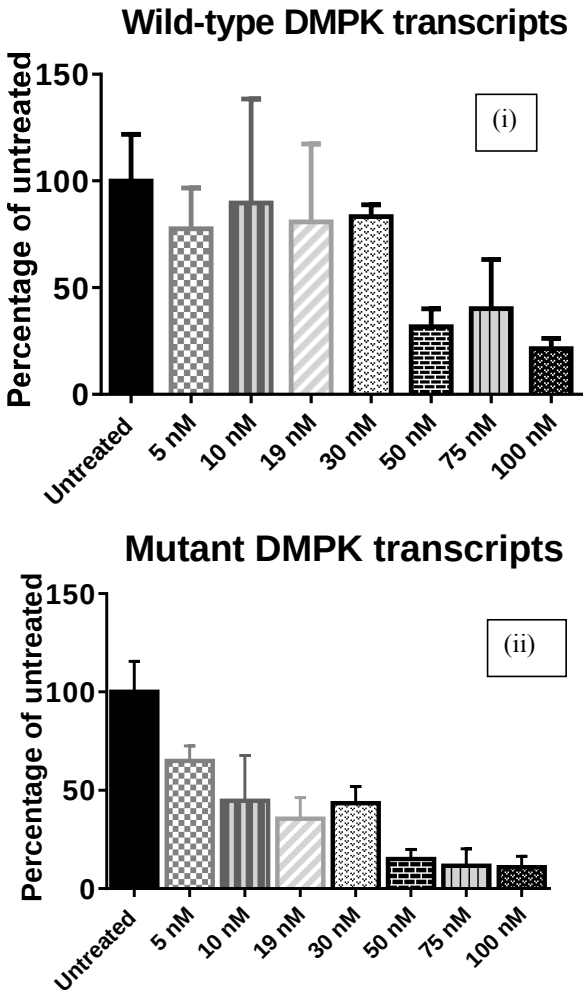


Figure 5-9. GeneScan analysis of duplex *DMPK*, HPRT transcripts from DM1 cells after treatment with various concentration of L10K PEI alone. Graphs indicate the level of either (i) Wild-type or (ii) Mutant *DMPK* transcripts normalised to HPRT. The X axis represent the L10K PEI concentration in nM. The Y axis represents the percentage of DMPK transcript remaining normalised to HPRT.

It is notable that lower concentrations of PEI can result in a significant degradation of the mutant transcript in comparison to the wild-type. As the $[CUG]_n$ repeat size is the key difference between the two DMPK transcripts it is not unreasonable to assume that the hairpin structures formed by the mutant RNA allow a larger target for degradation. Another theory takes into account the aggregate nature of the mutant transcript, which could allow for easier access of degradation due to the concentrated nature of the transcripts.

Degradation of other transcripts containing CTG repeats

As mentioned earlier, DM1 is caused by an expanded CTG repeat found in only one allele of the *DMPK* gene, with the other allele contain 5-38 repeats known to be non-pathogenic. Numerous publications stress the importance of specifically targeting the expanded CUG toxic RNA, leaving the wild-type intact to produce functioning *DMPK* protein. To determine whether L10K PEI also targets transcripts unrelated to DM1 containing $(CTG)_n$ repeats, I identified two other genes containing different sized CTG tracts. The first gene chosen, Cathepsin A (CTSA), is a gene encoding a glycoprotein which associates with lysosomal enzymes beta-galactosidase and neuraminidase to form a complex of high molecular weight multimers. Deficiencies in this gene are associated with galactosialidosis and ring chromosome 20, however for this purpose we are not interested in the biological function, rather the number of CTG repeat units, which can differ from individual to individual. Identification of suitable primers and PCR amplification conditions to amplify a repeat containing stretch of DNA were experimentally found. After amplification the DNA fragment was purified and sent for sequencing, Figure 5-10. It was found that CTSA has 9 CTG repeats in the forward direction and 8 CTG repeats in the reverse in our DM1 cell line.

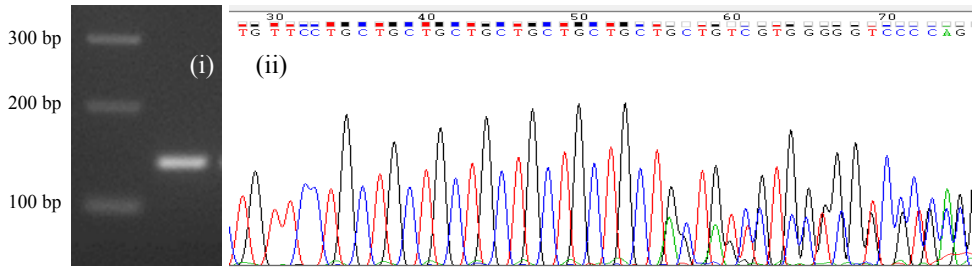


Figure 5-10: (i) PCR amplification of a DNA fragment from the CSTA gene containing the $(CTG)_n$ repeat. (ii) Sequencing plot of a fragment of the CTSA gene containing the $(CTG)_n$ repeat.

The second gene chosen was Ribosomal Protein L14 (RPL14), a protein coding gene encoding a ribosomal protein that is a component of the 60S subunit of ribosomes. It is well known that RPL14 contains a highly polymorphic CTG tract. In order to quantify the $(CUG)_n$ repeat length in the DM1 cell line being used, as before, suitable primers and PCR amplification conditions were identified, DNA amplification and product purification undertaken, and the sample sent for sequencing. As shown in Figure 5-11 sequencing demonstrated that our DM1 cell line has 15 and 16 CTG repeats within the RPL14 gene.

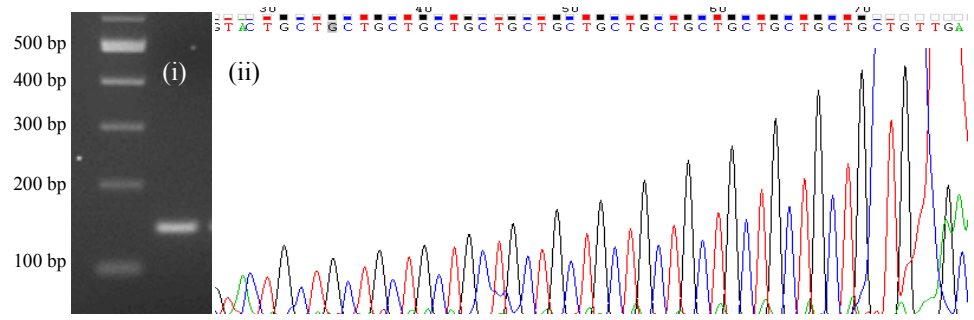
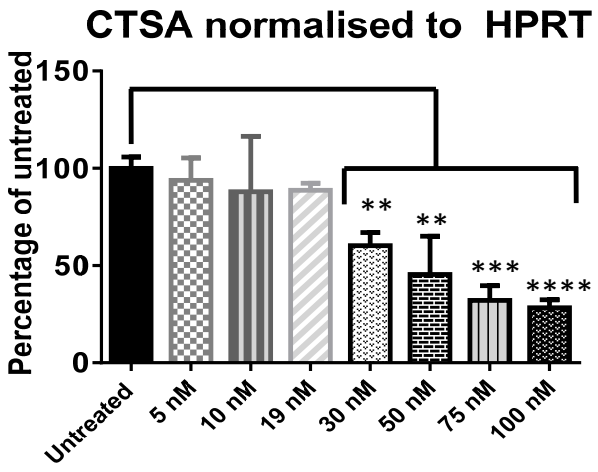


Figure 5-11: (i) PCR amplification of a DNA fragment from the RPL14 gene containing the $(CTG)_n$ repeat. (ii) Sequencing plot of a fragment of the RPL14 gene containing the $(CTG)_n$ repeat.

With the ability to amplify $(CTG)_n$ containing products from two separate genes with two different repeat sizes it is now possible to attempt to identify whether there is a link between repeat length and ability of PEI to degrade the $(CUG)_n$ transcript. It has previously been demonstrated that L10K PEI degrades the mutant DM1 DMPK gene to a higher extent at lower concentrations than the wild-type. I previously hypothesised that this could potentially be due to the size of the CUG tract. Data from the CTSA transcripts demonstrated a similar pattern of degradation to the wild-type DMPK transcripts. As these have the same number of CUG repeat units this is supportive of degradation correlating to CUG repeat length, Figure

5-12(i). Data from the RPL14 transcripts follows the same concentration dependent degradation trend, Figure 5-12(ii), however the data is not statically significant. This may be due to the high abundance of the RPL14 transcript potentially requiring a lower number of amplification cycles during PCR in order to reduce the variability between repeats.

(i)



(ii)

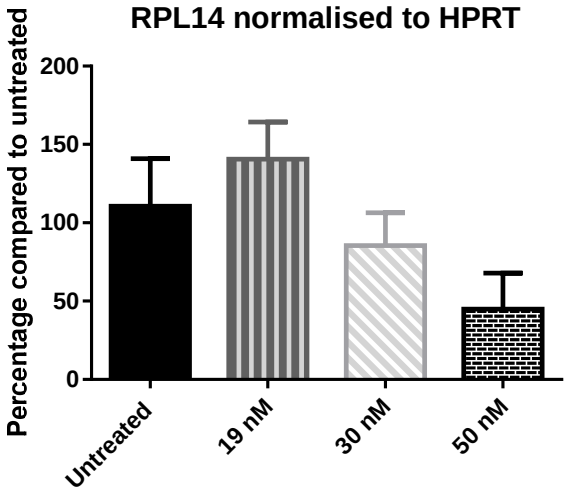


Figure 5-12: GeneScan analysis of duplex (i) CTSA/HPRT and (ii) RPL14/HPRT transcripts from DM1 cells. Concentration dependent degradation of the repeat containing RNA fragment using L10K PEI on (i) CTSA or (ii) RPL14 normalised to HPRT.

Discussion

Cationic polymers such as PEI are widely used as delivery reagents by researchers striving to improve the understanding and therapeutic value of antisense agents, (Amin, 2013, Dai, 2011, Zhang, 2013). Although the toxicity profiles of such reagents are thoroughly screened, little consideration appears to be given to their off target effects. My original intentions were to screen a range of CMOs delivered by PEI transfection to identify whether certain chemical modifications outperformed others in regards to degrading the DMPK transcript. Initial studies confirmed that 2'OMe modified oligonucleotides had no significant effect on the DMPK transcript ratio when delivered *via* gymnosis. Observations that oligonucleotides are unable to penetrate the nuclei when delivered unassisted from experimentation completed earlier in the study (chapter three), predicted this. Once confirmed that gymnotic delivery did not have an effect, my efforts concentrated on delivery using optimised delivery conditions identified in previous studies (chapter four). Experimentation into the use of PEI deliverance however detected an unexpected biological result. The data established that PEI molecules alone were able to alter the ratio of mutant:wild-type transcript in DM1 cells without the aid of sequence specific oligonucleotides, Figure 5-4 & Figure 5-5, result was repeated with a range of PEI molecules of various molecular weights, all demonstrating the ability to alter the mutant:wild-type DMPK transcript ratio when compared to untreated cells. Of the molecules tested the L10K PEI was found to result in the highest shift in favour of the wild-type DMPK transcript. Further studies were undertaken to demonstrate that the effect was concentration dependent. Numerous literature publications cited the adverse toxicity levels of PEI on cells (Godbey, 2001, Ouji Y, 2002), however, as demonstrated through earlier experimentation, the levels required to ensure nuclear delivery can be below the toxicity threshold. As expected the transcript ratio was undoubtedly dependent on the PEI concentration the cells

had been exposed to, with concentrations as low as 19 nM increasing the wild-type percentage over the mutant. A major limitation of the data to this point was it only allows for the comparison of one transcript in relation to the other, so it is not possible to define whether the mutant transcript is being degraded alone or whether both levels are dropping with the mutant transcript being most susceptible. In order to address this a duplex PCR protocol was optimised, allowing the DMPK transcripts to be separately normalised to a house-keeping gene, HPRT. Data from the next set of experimentation demonstrated that the treatment of DM1 cells with L10K PEI resulted in the degradation of both mutant and wild-type transcripts, although mutant transcript degradation became significant at lower concentrations of the L10K PEI reagent. This was promising as it revealed that PEI could selectively target the mutant transcript at certain concentrations, ensuring the wild-type transcript was left intact to function. To identify whether L10K PEI had any effect on other $(CUG)_n$ containing transcripts two genes were identified with a different number of CUG repeats. Primer design and PCR amplification conditions were optimised and the resultant amplicon sequenced. CTSA was found to have 7 or 8 CUG repeats within its RNA sequence, whereas RPL14 was found to have 15 or 16, Figure 5-12. It was observed that L10K PEI degraded both transcripts containing CUG repeats, although degradation of the RPL14 gene was found not to be significant.

Chapter six: General discussion and future work

In this chapter I will summarise the significance of my findings from the previous three chapters and discuss further work which could be undertaken in order to progress the understanding of the action and extent of degradation caused by PEI molecules.

Summary of findings

Oligonucleotide delivery *via* gymnosis

The work presented in the first part of this thesis (chapter 3) was conducted with the aim of understanding whether DM1, DM2 and DMD cells could be treated with various CMOs without the aid of a transfection reagent. The Brook lab had previously established that nuclear foci, an important diagnostic feature of DM1 and DM2, could be disrupted using 2'OMe and PNA oligonucleotides following treatment *via* gymnosis for just 2 days. Interestingly neither DNA nor PMO oligonucleotides resulted in the same reduction in foci even when similar repeat lengths and concentrations were used. In order further understand this observation 2'OMe, DNA, PMO and PNA fluorescently labelled oligonucleotides were used to treat both diseased and control cell lines followed by the image capture of live cells using confocal microscopy to identify the extent of uptake *via* gymnotic delivery. It was observed that oligonucleotide uptake to the nucleus was not significant *via* gymnosis for any of the CMOs tested in any cell type. This result was validated using an adapted northern blotting based technique which confirmed the lack of nuclear oligonucleotide delivery. This was an important observation as it directly contridicted the previous findings of nuclear foci disruption. In an attempt to resolve this conflict, images were taken at various stages of the *in-situ* hybridisation process. I detected during this investigation that the permeabilisation

step of the protocol allowed for a significant redistribution of the 2'OMe, PNA and DNA oligonucleotides from the cytoplasm into the nuclear compartment. It was this relocation which gave such promising foci disruption results, highlighting an important artefact of the *in situ* process when examining cells after oligonucleotide treatment. Viability studies demonstrated no observed toxicity of any of the CMOs tested at concentrations as high as 1 μM, therefore the use of these CMOs to treat diseases with cytoplasmic targets could be promising.

2'OMe delivery *via* PEI transfection

Once it had been established that gymnotic uptake would not allow for delivery of oligonucleotides to their desired nuclear RNA target in the disease models I had selected, I decided to employ a popular polymer-based transfection agent, polyethylenimine, PEI. To date a number of labs have successfully used oligonucleotides to target various RNA transcripts for the treatments of DM1, DM2 and DMD, however one study of great consequence was that by Mulders ~~*et al*~~*et al*. (Mulders, 2009). Here the group demonstrated successful degradation of the DMPK transcript using a 2'OMe $(CAG)_7$ oligonucleotide transfected using a PEI based formulation known as Exgen 500. This study was the motivation behind my choice of transfection reagent. Initial experimentation (chapter four) investigated the use of different PEI molecules for their ability to transport 2'OMe oligonucleotides into the nuclei of control cells. Experimentation *via* live cell fluorescent imaging demonstrated that some of the PEI molecules tested performed better than others; this was then validated using my modified northern blot based technique. It was observed that PEI reagents with a molecular weight of 10 kDa were highly efficient at delivering

oligonucleotides to the cell nuclei, with other linear PEI molecules of higher molecular weight (25 and 40 kDa) also demonstrating good delivery potential.

In an attempt to understand whether PEI molecules could be successfully used in human therapy I examined the toxicity profiles of the reagents which showed a delivery capacity for the oligonucleotide. The majority of scientific opinion suggests that higher molecular weight correlates to increased toxicity when concerned with PEI molecules, (Feng, 2010). PEI molecules were tested alone initially, without the presence of oligonucleotides. My results were found to be in agreement with these previous observations with a direct relationship between molecular weight and increased toxicity. I also observed that branched PEI molecules appeared less toxic than their linear counterparts.

My studies concentrated on the linear 10 kDa PEI molecule (L10K) as this showed the most favourable delivery *vs.* toxicity profile for the 2'OMe oligonucleotide. My results demonstrated a decrease in viability of cells treated with polyplexes compared to PEI molecules alone. Further experimentation demonstrated that nuclear delivery of oligonucleotides resulted in a significant decrease in viability, however it was not possible to identify whether this was caused by the oligonucleotide alone or a synergistic effect of the polyplex. To date there is very little literature available to explain this trend as the majority of studies have not dissected the toxicity of the individual components.

In order to optimise delivery conditions of 2'OMe oligonucleotides *via* L10K transfection, various parameters such as PEI structure, PEI molecular weight, PEI:Oligonucleotide ratio (N/P), complexation pH, cell line and oligonucleotide concentration were assessed. Results

identified that low molecular weight PEI molecules either branched or linear in nature were inefficient at oligonucleotide delivery. It was also observed that of the higher molecular weight PEIs tested, linear structures out-performed branched species. Studies investigating the importance of the N/P ratio established that an excess of 4 amines were required to allow for effective nuclear and cytoplasmic delivery. These results were in agreement with previous studies which demonstrated an N/P ratio between 4 and 10 were optimal for the transfection of various antisense agents, (Gaoa, 2005 and Sirsi, 2005). My results regarding the ability of PEI to condense oligonucleotides at various pH environments supported studies by Amin ~~*et al*~~*et al*. who indicated that the condensation ability of PEI at acidic, neutral and basic environments was similar, (Amin, 2013). Fluorescent live cell imaging data showed that at environments ranging from pH 3-12, L10K PEI could condense and successfully delivery 2'OMe oligonucleotides, although this data was not analysed quantitatively. PEI is known to allow for the transfection of various tissues, (Boeckle, 2004), and further experimentation demonstrated L10K PEI could deliver 2'OMe oligonucleotides to cells from a range of disease models. It is worth noting that L10K transfection to each of the 6 cell models tested demonstrated an increase in nuclear fluorescent intensity of several orders of magnitude above untreated, gymnotically treated and L5K PEI transfected cells. Finally it was observed that for each of the higher molecular weight PEI molecules tested, an increase in nuclear fluorescence was observed with increasing concentration of the oligonucleotide. The use of higher concentrations however is associated with a significant increase in toxicity; therefore it is imperative that a balance of delivery *vs.* viability is considered. It was observed that upon quantification of the nuclear fluorescence the B10K PEI molecule gave comparable oligonucleotide delivery at concentrations which are tolerable by the cells (<50 nM),

therefore further studies could also include this molecule as it provides the added benefit of a reduced toxicity response.

Degradation of CUG containing transcripts by PEI molecules.

Assessment of the DMPK levels after treatment *via* gymnosis confirmed that gymnotic delivery of $(CAG)_7$ 2'OMe PS oligonucleotides was not sufficient to significantly alter the mutant:wildtype ratio of the transcripts. As gymnosis does not afford nuclear delivery, and therefore contact of the oligonucleotide with the RNA target, this was supportive of our hypothesis that in order to treat diseases with nuclear retained target it is essential that oligonucleotides are able to enter the nuclear compartment.

Work by other groups has previously demonstrated significant degradation of the mutant DMPK transcript in a DM1 model when treated with 2'OMe CMOs targeted at the mutant repeat expansion, (Mulders, 2009).This work was carried out using a PEI based transfection reagent known as Exgen 500. Repeating the experiment with Exgen 500 however resulted in an interesting finding as the Exgen 500 alone, minus oligonucleotide, was able to shift the ratio of mutant:wild-type transcripts. In order to investigate this observation the experiment was repeated with three different PEI molecules, L10K, L25K and L5K. Again it was observed that the PEI molecules had the capacity to alter the mutant:wild-type transcript ratio. Further experimentation using the L10K PEI molecule found a concentration-dependent shift of the mutant:wild-type DMPK transcripts, the trend broke down when cell viability began to significantly decrease at high PEI concentrations. A duplex PCR assay was designed to allow the quantification of the DMPK transcripts independently. It was observed that significant degradation of the mutant transcript required a lower concentration of the

L10K PEI than the wild-type. It is worth noting that at a concentration of 19 nM the L10K can significantly decrease the mutant transcript whilst having no significant effect on the wild-type, this concentration has also been shown to cause very little, if any, decrease in cell viability.

Finally it was observed the L10K PEI also degrades other $(CUG)_n$ containing transcripts at comparable concentrations. This indicates that PEI molecules can potentially target $(CUG)_n$ containing transcripts *via* an unknown mechanism resulting in their degradation. Although during this study no work has been carried out to identify the mechanism of action, there is literature precedent for the degradation of RNA by poly amines.

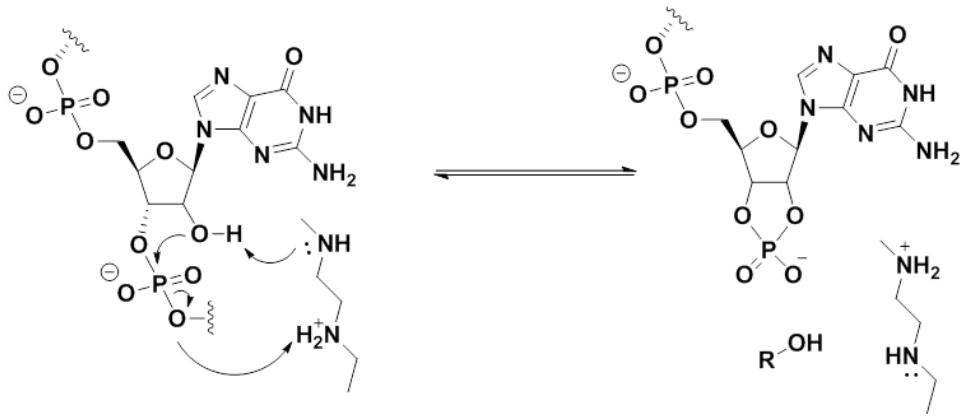


Figure 6-1: Schematic of the potential mechanism by which PEI molecules could degrade *DMPK* transcripts.

Figure 6-1 depicts a schematic taken from work carried out to identify the mechanism of enzymatic degradation of dinucleotides, (Breslow, 1996). It is theorised that an intra-molecular acid base cooperation between amine and ammonium cations promotes catalytic degradation of the RNA. Although mechanistically this would not be selective, there

hypothetically could be an increased affinity for CUG repeats by the PEI reagent, although this would need to be verified through rigorous experimentation. Prosensa Technologies are currently optimising antisense technology including the conjugation of amine containing peptides, stating in the patent that "invention in particular relates to the use of antisense oligonucleotides with improved characteristics enhancing clinical applicability as further defined herein", (De Visser, 2015).

Further work

The treatment of DM1 by the use of small organic molecules has shown good promise, (Ketley, 2013 and Wojciechowska, 2014). However, although transfection reagents are often organic compounds, numerous reaseach groups continually regard then as inert species, incapable of causing a biological effect other than decreased viability. Here I have identified a transfection reagent capable of causing a significant biology effect in our disease model which, to date, has not been recognised within the literature. The implication of such biological activity could be further assessed by micro-array techniques in order to identify whether PEI based molecules conclusively target $(CUG)_n$ containing genes specifically or whether they target hairpin forming repeats in general. Whether it were found that the extent of degradation or dis-regulation correlated with repeat length or not, it would be beneficial to further understand the mode of action of the effect of PEI. However, this could potentially be very challenging if its effects were not correlated to repeat length.

Within this investigation a non-toxic, biologically effective PEI concentration has been identified. Although unsure of its mechanism of action there is demonstrable reduction of the toxic expanded RNA transcripts, which is theoretically as a result of degredation. All work

done here has been *in vitro* therefore, in order to further our understanding, testing *in vivo* would be of great importance. One well-defined DM1 model is that of the HSA^{LR} mouse of Mankodi ~~*et al*~~*et al.*, which exhibits nuclear foci, myotonia and many of the splicing defects that are characteristic of the human disease, (Mankodi, 2000). Testing the PEI reagent in the DM1 HSA^{LR} mouse model could be used as a proof of principle for mitigation of phenotypic effects in muscle by PEI molecules alone. However this would have limitations as the model lacks some of the multisystem phenotypic representation of the human condition.

If PEI molecules do target hairpin structures they would also affect the $(CCUG)_n$ repeats of ZNF9 in DM2 patients. At the time of writing no polymorphism has been identified in our specific DM2 cell line, the same procedures as used here were applied to the DM2 cell model. Although there was an observed reduction in the ZNF9 PCR product (as assessed by gel electrophoresis) from cells treated with 75 nM PEI compared to the untreated controls, I was unable to provide statistically significant evidence from the gel images obtained. Therefore, with the identification of a SNP within the ZNF9 gene in a DM2 cell model it would be possible to carry out the same analysis and ascertain whether PEI molecules could selectively degrade the mutant transcript of the ZNF9 gene.

Finally the identification of a nuclear delivering transfection reagent which does not result in degradation of the *DMPK* transcript alone would allow for the comparison of effectiveness of 2'OMe oligonucleotides with and without the PEI reagent in order to identify any synergistic effects from the sequence specificity of the oligonucleotide. This could be carried out using the same techniques as currently employed.

Conclusion

The work performed during this study has demonstrated the importance of understanding the significance of each step within a protocol and the need for rigorous biological controls.

To conclude; gymnotic delivery of four different CMOs resulted in cytoplasmic but not nuclear uptake of the antisense agents at various concentrations, time points and in a range of disease models. The previous disappearance of DM1 and DM2 nuclear foci after treatment with PNA and 2'OMe oligonucleotides gymnotically was in fact an artefact of the *in-situ* protocol and not a live cell biological effect.

PEI molecules of various structures and molecular weights are capable of delivering 2'OMe oligonucleotides to the nucleus with great efficiency. Although very effective at delivering these reagents there is a further decrease in cell viability when polyplexes are administered to cells rather than PEI reagents alone. This therefore implicates either a synergistic toxicity or toxicity as a direct result of nuclear deliverance of oligonucleotides.

2'OMe oligonucleotides targeted at the $(CUG)_n$ expanded repeat have no effect on the ratio of mutant:wild-type *DMPK* transcripts when delivered gymnotically. When delivered *via* PEI transfection various PEI molecules are able to significantly alter the mutant:wild-type ratio without the presence of the oligonucleotide. My results suggest that transfection reagents are not inert biological entities and care must be taken to ensure any biological effects observed are as a result of the treatment molecule and other not due to co-administered agents. Further studies concluded that the L10K PEI reagent could alter the mutant:wild-type ratio in a concentration dependent manner. I have shown that upon L10K

treatment at various concentrations not only the ratio of transcripts is altered but also the total amount of each transcripts in respect to a house keeping gene. By analysing other $(CUG)_n$ containing transcript I have been able to identify a concentration range which seemingly degrades the mutant *DMPK* transcript, $(CUG)_n$, whilst leaving other $(CUG)_{<n}$ transcripts intact.

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Supplementary Information

Chemical modification	Source	Sequence	Fluorphore
DNA, PS	Synthesised or Sigma-Aldrich	$(CAG)_7$	-
DNA, PS	Synthesised	$(CAG)_7$	Hexachlorofluorescein (HEX)
2'OMe, PS	Synthesised or Eurogentec	$(CAG)_7$	-
2'OMe, PS	Synthesised	$(CAG)_7$	Hexachlorofluorescein (HEX)
PNA	Eurogentec	$(CAG)_7$	-
PNA	Eurogentec	$(CAG)_7$	$Cy3^{TM}$
PMO	GeneTools	$(CAG)_7$	-
PMO	GeneTools	$(CAG)_7$	Hexachlorofluorescein (HEX)

Table S-1. List of oligonucleotides used during experimental work