EVALUATION OF BIODEGRADABLE POLYMER MICROSPHERES AND AN ANIONIC DENDRIMER FOR THE POTENTIAL DELIVERY OF ANTISENSE OLIGONUCLEOTIDES

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SUMMARY

Antisense oligonucleotides (AODNs) can selectively inhibit individual gene expression by binding specifically to mRNA. The over-expression of the epidermal growth factor receptor (EGFR) has been observed in human breast and glioblastoma tumours and therefore AODNs designed to target the EGFR would be a logical approach to treat such tumours. However, poor pharmacokinetic/pharmacodynamic and cellular uptake properties of AODNs have limited their potential to become successful therapeutic agents. Biodegradable polymeric poly (lactide-co-glycolide) (P(LA-GA)) and dendrimer delivery systems may allow us to overcome these problems. The use of combination therapy of AODNs and cytotoxic agents such as 5-fluorouracil (5-FU) in biodegradable polymeric formulations may further improve therapeutic efficacy.

AODN and 5-FU were either co-entrapped in a single microsphere formulation or individually entrapped in two separate microsphere formulations (double emulsion method) and release profiles determined *in vitro*. The release rates (biphasic) of the two agents were significantly slower when co-entrapped as a single microsphere formulation compared to those obtained with the separate formulations. Sustained release over 35 days was observed in both types of formulation.

Naked and microsphere-loaded AODN and 5-FU (in separate formulations) were tested on an A431 vulval carcinoma cell line. Combining naked or encapsulated drugs produced a greater reduction in viable cell number as compared with either agent alone. However, controls and Western blotting indicated that non-sequence specific cytotoxic effects were responsible for the differences in viable cell number.

The uptake properties of an anionic dendrimer based on a pentaerythritol structure covalently linked to AODNs (targeting the EGFR) have been characterised. The cellular uptake of AODN linked to the dendrimer was up to 3.5-fold higher in A431 cells as compared to naked AODN. Mechanistic studies suggested that receptor-mediated and adsorptive (binding protein-mediated) endocytosis were the predominant uptake mechanisms for the dendrimer-AODN.

RNase H cleavage assay suggested that the dendrimer-AODN was able to bind and cleave the target site. A reduction of 20%, 28% and 45% in EGFR expression was observed with 0.05μM, 0.1μM and 0.5μM dendrimer-AODN treatments respectively with a reduction in viable cell number. These results indicated that the dendrimer delivery system may reduce viable cell number by an antisense specific mechanism.

Keywords:- nucleic acids, 5-fluorouracil, epidermal growth factor receptor, poly(lactide-co-glycolide), dendrimers.

DEDICATION

I dedicate this thesis to my parents as a token of my gratitude for their everlasting kind, compassionate upbringing and for instilling a profound appreciation of education in my heart. Also, to my brother Yasar and sisters Safina, Khalina, Sumyra and Naseeba for putting up with my unique way of mentally relaxing at home; my wife Shazia who was kind and patient with my late evening work. Last but by no means least to my nephews Qasim, Hasan and niece Aisha for making me smile after a long days work.

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Finally, I am especially appreciative of my dear friend Qamar Nawaz for convincing me that there really is an unlocked door just waiting for me to open, for making me laugh until I could not stop and providing me with some of the best memories a person could have.

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LIST OF ABBREVIATIONS

2'-O-Me	2'-O-methylated
5-FU	5-fluorouracil
Ab	antibody
AIDS	acquired immune dificiency syndrome
AODN	antisense oligonucleotide
AE	adsorptive endocytosis
A, G, C, T, U	adenine, guanine, cytidine, thymidine, uridine
ASGP-R	asialoylglycoprotein receptor
ATP	adenosine tri-phosphate
BCA	Bicinchoninic acid
BSA	bovine serum albumin
cm, mm, μm, nm	centimetre, millimetre, micrometre, nanometre
CMV	Cytomegalovirus
°C	degrees Celsius
CO_2	carbon dioxide
cpm	counts per minute
dendrimer-AODN	dendrimer linked to AODN
ddH_2O	double distilled water
DCM	dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra acetic acid
EGFr	epidermal growth factor receptor

EMSA	electrophoretic mobility shift assay
FBS	foetal bovine serum
FDA	food and drug agency
FITC	fluoroscein isothiocyanate
FPE	Fluid-phase endocytosis
g, mg	grams, milligram
HBSS	Hank's balanced salt solution
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)
HIV	human immunodeficiency virus
HRP	Horseradish peroxidase
im	Intra-muscular
ip	intra-peritoneal
iv	intravenous
kDa	kilo Daltons
LA	lactic acid
LSC	liquid scintillation counting
MDR1	multidrug resistance gene 1
MES	2-[N-morpholine]-ethanesulphonic acid
M, mM, μM, nM, pM	molar, millimolar, micromolar, nanomolar, picomolar
mL, μL,	millilitres, microlitres,
MP	methylphosphonates
MPG	
mRNA	messenger ribonucleic acid
Mw	molecular weight

ODN	oligodeoxynucleotide
PACA	polycyanoacrylate
PAGE	polyacrylamide gel electrophoresis
PAMAM	Polyamidoamine
PBS	phosphate buffered saline
PEI	polyethyleneimine
PGA	Poly(glycolic) acid
PLA	Poly(lactic acid)
PKC	protein kinase C
PNA	Peptide-nucleic acid
P(LA-GA)	polylactide-co-glycolide
PMSF	phenylmethylsulphonylfluoride
PO	phosphodiester
Pre-mRNA	unspliced messenger ribonucleic acid
PS	phosphorothioate
PS_2	phosphorodithioate
PVA	poly(vinylalcohol)
RES	reticulo-endothelial system
RME	receptor-mediated endocytosis
RNA	ribonucleic acid
RNase H	ribonuclease H
rpm	revolutions per minute
sc	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate

SEM	scanning electron microscopy
SFM	serum-free media
$t_{1/2}$	half-life
TBE	tris-borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TETD	tetraethyldisulphide
Tris	tris(hydroxymethyl)amino methane
Triton x-100	octyl phenoxy polyethoxyethanol
UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
w/o/w	Water- in- oil- in –water

CHAPTER ONE

INTRODUCTION

1.1 THE ANTISENSE APPROACH

The ability to control gene expression with a high degree of selectivity has provided a new route for researchers to address questions about gene function and allow them to design therapies for many human diseases including cancer, cardiovascular disorders, autoimmune diseases and HIV. Such an exciting prospect has become possible due to the antisense approach. This approach utilises several types of nucleic acids such as antisense oligonucleotides (AODNs), ribozymes (RNA enzymes) and more recently DNAzymes (DNA enzymes). The aim of the antisense approach is to down-regulate the expression of disease-causing proteins by inhibiting gene expression at the mRNA level. Conventional therapies utilise compounds which act upon the protein itself and often require the non-rational approach of screening thousands of compounds to find an active molecule which even then may result in a compound which lacks specificity. In contrast the antisense strategy, at least in principal, allows for the rational design of highly sequence-specific nucleic acid drugs that can target and even result in degradation of a given mRNA. The fidelity of antisense molecules arises from their ability to form Watson-Crick-type hydrogen bonds with their target mRNA in hybridisation-accessible sites (for reviews see: Sharma *et al.*, 1995; Akhtar *et al.*, 2000; Hughes *et al.*, 2001). Successful *in vitro* and *in vivo* studies have paved the way for several clinical trials and the approval of fomivirisen (Vitravene^{TM}) in USA and Europe as the first antisense drug (see section 1.6).

1.1.1 Antisense Oligonucleotides

AODNs are short synthetic nucleotide sequences (DNA) designed to bind to the mRNA of a gene. By binding to these sequences AODNs may inhibit the expression of that gene (Zamecnik *et al.*, 1978) by several different possible mechanisms. These include direct blockage of ribosomal read-through due to the presence of the AODN (Crooke 1992) and

activation of RNase H, an enzyme found in the nucleus and cytoplasm (Crum *et al.*, 1988). However, the concentration of RNase H in the nucleus is thought to be greater and some of the enzymes found in the cytoplasmic preparations may be due to nuclear leakage (Crum *et al.*, 1988). RNase H recognises and attaches to the RNA-DNA hybrid, leading to the hydrolytic cleavage of the RNA strand whilst leaving the DNA strand untouched (Chiang *et al.*, 1991; Ho *et al.*, 1999). The RNA may be further degraded by cellular exonucleases. Activation of RNase H is strongly dependent upon the ODN chemistry *i.e.* charged ODNs such as phosphorothioates activate RNase H whereas uncharged methylphosphonate ODNs are unsuccessful in such a process (Miller 1991). The extent to which one or the other mechanism predominates depends largely on the chemistry of the ODNs used (see section 1.3.2). In either case, the end result is the same, decreased levels of the protein product (figure 1.1).

Alteration of the secondary RNA structure by AODNs is another theory for their mechanism of action. RNA adopts a variety of three-dimensional structures defined by intramolecular hybridisation such as the "stem-loop" structure which provides additional stability for RNA and acts as recognition motif for a number of proteins, nucleic acids and ribonucleoproteins that participate in RNA metabolism and translation. For example, in HIV a number of ODNs that bind to the "stem-loop" of the RNA structure known as trans-activating response (TAR) sequence have been shown to inhibit HIV replication in both acute and chronically infected viral assays (Vickers *et al.*, 1991).

An important step for the stabilisation of the pre-mRNA and consequently the mature mRNA is 5' methylguanylate capping. It is also important in binding to the nuclear matrix and transport of mRNA out of the nucleus. Several ODNs that bind near the cap site of the mRNA have been shown to have an antisense effect, presumably by inhibiting the binding of proteins to the 5'cap of the RNA (Leonetti *et al.*, 1993).

The ability of ODNs to bind within the major groove of double helical DNA and form a localised triple helix is referred to as the antigene strategy. The triple helix-forming ODNs can be used to control gene expression at the transcriptional level. Inhibition of binding of transcription factors by triplex formation modulates the level of transcription of the target gene (Helene, 1991b). Binding of a triplex-forming ODN immediately downstream of the RNA polymerase binding site can inhibit transcription initiation as shown with the *E. coli* β-

lactamase gene (Helene, 1991a). The formation of a triple helix structure requires that the targeted sense sequence within the DNA molecule contain a pyrimidine- or purine-rich motif (Cooney *et al.*, 1988). The binding of an ODN to such a motif follows the Hoogsteen's binding principle where thymidine binds to adenine-thymidine pairs, creating a triplet (TAT) and protonated cytosine recognises guanine-cytosine pairs, creating another triplet (C^+GC) (Sun *et al.*, 1994). The effectiveness of the antigene strategy to directly inhibit transcription is limited by the scarcity of sufficiently long runs of pyrimidine- or purine-rich motifs in the genomic sequence of interest to allow the formation of stable triple helices.

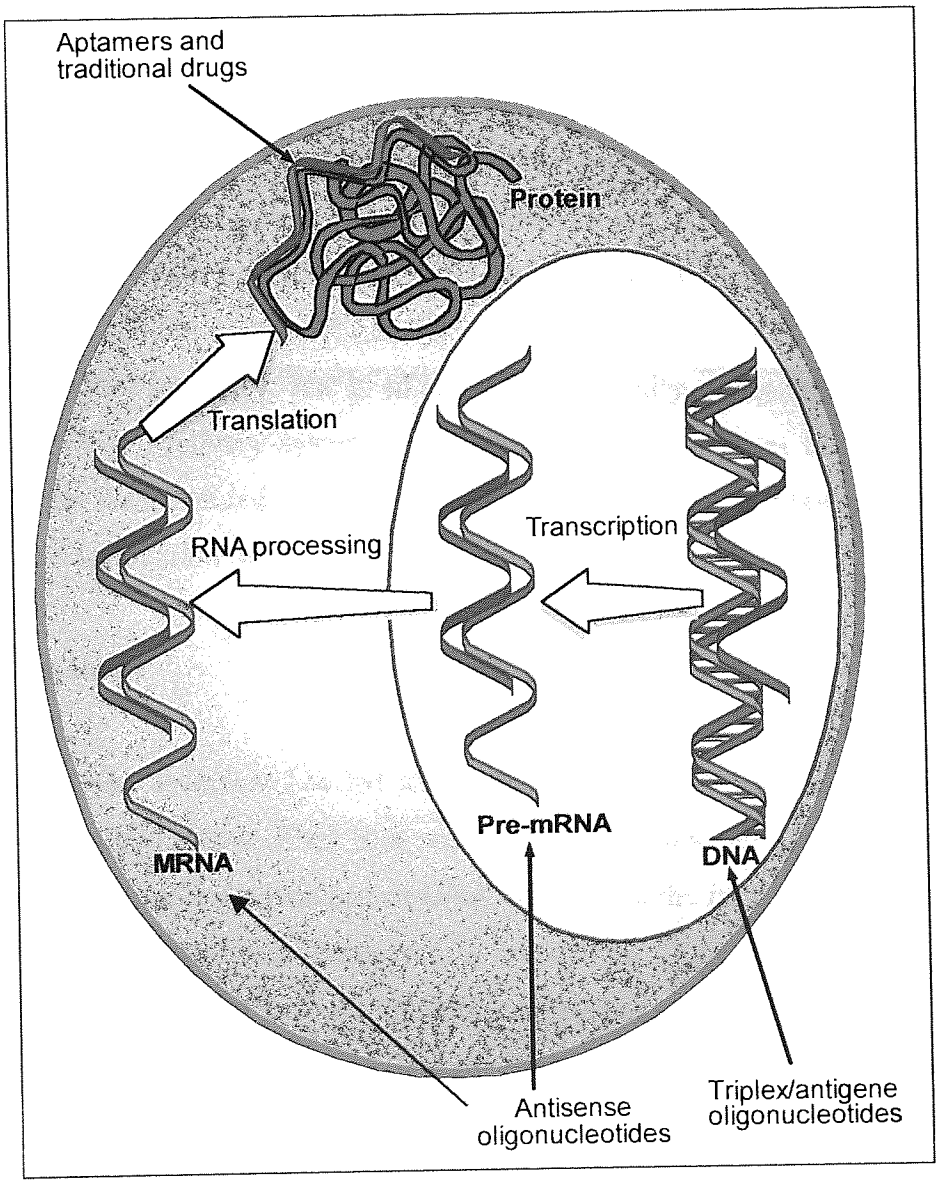


Figure 1.1 A schematic representation of the basis of AODN-mediated inhibition of gene expression (adapted from Sharma and Narayanan, 1995).

1.1.2 Ribozymes

RNA transcription from a gene involves the splicing of the intron sequences (non-coding regions) and consequently the ligation of the exons to form the mature mRNA. Certain RNA splicing reactions are catalysed by RNA of intervening sequences. Such RNA molecules possessing enzymatic activity are called ribozymes (for review see: Lewin *et al.*, 2001). These catalytic RNAs or ribozymes have been found in a variety of biological systems. Ribozymes can be engineered to bind specifically to the desired RNA targets and cleave them resulting in the inhibition of gene function in the absence of proteins or enzymes. A ribozyme can be regarded as essentially an ODN that contains a catalytic region of conserved nucleotides, which in the presence of divalent cations such as magnesium, can exhibit multiple turnover of substrate mRNA molecules (Hudson *et al.*, 1996). The entry of ribozymes in clinical trials confirms their potential as therapeutic agents (see section 1.6). However the full potential of ribozymes is hampered by their extremely poor nuclease stability in biological environments. Although chemical modifications to ribozymes can enhance nuclease stability, catalytic activity is often compromised, therefore repeated administration is often necessary but is likely to be clinically undesirable in many cases (Ruffner *et al.*, 1990). Delivery systems which can protect ribozymes from nucleases and simultaneously provide sustained delivery over extended time periods may be useful for the biopharmaceutical application of ribozymes.

1.1.3 DNA Enzymes

In recent years *in vitro* selection has led to the development of DNA enzymes composed entirely of DNA that can cleave RNA in a sequence-specific manner (Santoro *et al.*, 1997; Sioud *et al.*, 2000). The added advantage of DNA enzymes is the fact that they are relatively easy and inexpensive to synthesise compared to ribozymes. Rapid catalytic turnover and stability to nucleases make DNA enzymes an important part of antisense technology.

1.2 THERAPEUTIC APPLICATIONS OF ANTISENSE ODNs

Therapeutically, AODNs have been applied to situations involving the inhibition of over-expressed genes. The gene target may be foreign, as in viral infection, or may be a normal gene which has undergone mutation such as an activated proto-oncogene. The most obvious

areas of antisense therapy to date are cancer and HIV. AODNs are also potential agents for various other disease states such as cardiomyopathy, cardiac hypertrophy and other cardiovascular disorders, transplant rejection and several autoimmune diseases (for review see Reddy 1996). Some of the more common therapeutic applications of AODNs are described below.

1.2.1 Cancer

Changes in specific genes controlling cell growth and apoptosis is a crucial difference between normal cells and cancer cells. These genes are mutations of proto-oncogenes which are normally found in cells and are activated during embryogenesis, specific tissue regeneration or cell growth. Induction of uncontrolled growth occurs due to a mutation which leads to the alteration of the corresponding protein expression. Since these genes are over-expressed and produce RNA which is distinguishable from the proto-oncogene, oncogenes are potentially excellent targets for AODNs. AODNs have been tested against many types of tumours in different experimental models; below is a brief overview of some of the studies conducted.

The *c-myc* proto-oncogene is active in well-characterised human xenograft models of leukaemia and melanoma. The efficacy of a phosphorothioate *c-myc* AODN in a microencapsulated delivery system was tested in human melanoma xenografts in immunocompromised mice (Putney *et al.*, 1999). Reduced *c-myc* expression (51%) resulted in decreased tumour weight (2-fold) and increased survival times. Exposure of human leukemia K562 cells in mice to a phosphorothioate (PS) AODN, complementary to a *c-myb*-encoded mRNA led to 3.5-fold longer survival times as compared to no treatment. This study utilised an infusion pump (100μg/day for 4 or 14 day treatment) to deliver the AODN (Nieborowska-Skorska *et al.*, 1994). Antisense-mediated inhibition of *bcr-abl* gene expression which is involved in chronic myelogeneous leukaemia has also been demonstrated (Mahon *et al.*, 1995). In this study, a concentration of 5μM of a 56-mer phosphodiester (PO) AODN demonstrated an antisense-specific 50% reduction in viable cell number for K562 cell line (CML erythroblastic).

In glioblastoma multiforme, a highly malignant brain cancer for which there is no effective therapy, the most commonly overexpressed oncogene is the *c-erbB1* gene, which encodes the epidermal growth factor receptor (EGFR). In addition to glioblastomas abnormal EGFR expression has been reported in squamous epidermoid cancers and breast cancer (for review see: Woodburn 1999). Therefore strategies that down-regulate EGFR expression such as AODN may be therapeutically useful (see section 1.7).

1.2.2 HIV

AODNs have been shown to inhibit HIV-1 by three different mechanisms:-

1. PS AODNs have been shown to interfere with virus-mediated syncytia formation. The phosphorothioates strongly bind to the viral receptor CD4 and to the viral envelope protein gp 120 (Stein *et al.*, 1993).
2. Once inside the cytoplasm ODNs have the potential to inhibit reverse transcription of the viral RNA in a sequence-independent manner, probably by binding directly to the reverse transcriptase (Stein *et al.*, 1993).
3. PO AODNs may inhibit reverse transcription in a sequence-specific manner by annealing to their target sequence on the viral RNA (Anazodo *et al.*, 1995).

Cell-culture experiments have been used to study the effects of a 25-mer PS ODN complementary to the *gag* initiation site of HIV-1 in combination with the nucleoside analog drug zalcitabine (Veal *et al.*, 1998). The PS ODN exhibited synergism with zalcitabine (2', 3'-dideoxycytidine) with an approximate 5-fold decrease in the zalcitabine IC_{50} value. This interaction was not seen with any of the mismatched ODNs suggesting an involvement of a sequence-specific mechanism of action.

A B4.14 cell line derived from the CMT3 monkey kidney was stably transfected with a plasmid containing the *rev* gene of HIV. This was used as a model to assess the effectiveness of a 20-mer PS ODN. Densitometric analysis of data from Western blot analysis showed sequence-specific and concentration-dependent ODN inhibition of HIV *rev* protein synthesis. When lipofectin was used as a delivery vehicle, a markedly increased potentiation of the

AODN activity of the sequence was observed at a lower concentration (0.1μM) following a 24-hr preincubation (Anazodo *et al.*, 1995).

A series of PO and PS AODNs which specifically bind to the HIV TAR sequence have been evaluated. The TAR sequence forms a stable RNA stem-loop structure which binds the HIV *tat* (trans-activator) protein and mediates increased viral gene expression. Specific binding to the TAR sequence was demonstrated *in vitro* with enzymatically synthesised TAR RNA resulting in the inhibition of HIV replication in both acute and chronically infected viral assays (Vickers *et al.*, 1991). These studies provide encouragement regarding the potential use of AODNs in the management of HIV infection.

1.2.3 Other Applications

Most work to date regarding potential AODN therapy has been focused on diseases for which only inadequate conventional therapy exists such as cancer and HIV (see sections above). However, AODNs have been used as therapeutic agents for various other disease states. Below is a brief summary of some of the studies conducted:-

Genetic variants of angiotensinogen are implicated in the development of hypertension. Liposome-encapsulated AODNs have been utilised in rats to target angiotensinogen mRNA to test whether peripheral angiotensinogen reduction would lower their hypertensive blood pressure (Wielbo *et al.*, 1996; Wielbo *et al.*, 1997a). Mean arterial pressure was significantly reduced in rats administered intra-arterially with liposome encapsulated AODN (-24.66±2.43 mm Hg). No significant blood pressure changes were observed with the scrambled AODN.

AODNs have been used against myotonic dystrophy, an inherited neuromuscular disease. The molecular basis of this disease is due to expansion of an unstable poly-CTG repeat in the 3' transcribed and untranslated region of a gene encoding for a putative cAMP-dependent serine-threonine protein kinase (DMPK), named myotonin. It has been demonstrated that DMPK gene expression can be reduced by addition of PS ODNs in K562 cell lines (Galderisi *et al.*, 1996). The DMPK mRNA level showed a reduction of about 50-55% in cells treated with 1μM AODN compared to the sense and scrambled controls. A total reduction of about 75% was observed in cells treated with 5μM AODN for 6-hrs.

The above studies have been selected to give the reader an appreciation for the varied therapeutic applications of AODNs. The list of diseases in which the antisense strategy may be applied clinically is growing rapidly along with further developments of the strategy itself.

1.3 CHALLENGES TO ANTISENSE DRUG DEVELOPMENT

For AODNs to have a therapeutic effect various hurdles need to be overcome. Firstly, the ODNs must be delivered to target cells, where they must then penetrate the plasma membrane to reach their target site in the cytoplasm or nucleus. Secondly, once inside the cell the ODN must be able to withstand enzymatic degradation presented by various endogenous nucleases. Thirdly, the ODN must be able to find and then bind specifically to its intended target site in order to inhibit expression of the disease-causing gene. In addition, the ODN must avoid binding to non-target sites as this may produce undesirable side effects. It is important to realise that the studies described so far are amongst the most successful and that poor pharmacokinetic/pharmacodynamic and cellular uptake properties are probably the most important limiting factors for AODNs to become successful therapeutic agents. To date, there have been many studies which have attempted to overcome some of the barriers for effective AODN technology. Below is a brief overview of the main challenges to AODN technology and also steps which have been undertaken to overcome these problems.

1.3.1 Target Accessibility

One of the hurdles for the consistent and effective use of AODNs is the identification of active antisense sequences *i.e.* accessible sites (*e.g.* single stranded regions) for ODN hybridisation to the target mRNA. One would think that these regions could be selected from computer-predicted folding of RNA, however this approach has proven unsuccessful and it is clear that currently available computer algorithms do not readily predict the complex secondary and tertiary structure of RNA molecules due to the fact that the bases required for Watson-Crick base pairing are involved in intramolecular pairing (Akhtar, 1998). The problem of target accessibility has triggered development of a number of alternative strategies to try to overcome this problem. These are described below:-

1.3.1.1 'Gene-walking'

The strategy of 'gene-walking' has been employed whereby a series of ODNs has been generated against the target mRNA and each one evaluated in cell culture or *in vivo*. This strategy has been successful in identifying active sequences but is far from exhaustive. To obtain a complete picture of RNA accessibility using this method would be time consuming and expensive (for review see: Hughes *et al.*, 2001).

1.3.1.2 RNase H Mapping of Accessible Sites

The ability of the enzyme RNase H to recognise and selectively cleave mRNA at sites within the hybridised ODN:mRNA duplex forms the basis of the RNase H mapping technique. The target mRNA transcript is combined with a random, or semi-random library of chemically synthesised ODNs in the presence of RNaseH. This leads to the production of mRNA cleavage fragments *i.e.* sites accessible for hybridisation. Subsequent sequencing of the RNase H-cleaved fragments identifies the 'open' mRNA sites for the targeting of AODNs. This strategy has been successful in identifying regions of the angiotensin type-1 receptor (AT_1) mRNA that were accessible to PS ODNs. PS ODNs targeted against accessible sites reduced AT_1 receptor levels by at least 50% in cell culture and 65% when injected in to rat brains (Ho *et al.*, 1998). Due to numerous RNase H cleavage sites within the ODN:mRNA duplex, the precise selection of antisense sequence is not possible with this strategy (Monia *et al.*, 1996), however it is possible to combine this strategy with other technologies to precisely identify active antisense sequences.

1.3.1.3 Combinatorial ODN Arrays

A series of overlapping ODNs are synthesised by standard phosphoramidite chemistry on a modified polymer membrane or glass surface. This array of ODNs cover every possible sequence within the target RNA (Sohail *et al.*, 1999). Precise antisense sequences can then be identified by hybridising the radiolabelled target RNA transcript to the immobilised antisense ODNs *in vitro*. Although this approach can be used to cover the full length of the RNA in practice only short regions are used because the exhaustive walking of the RNA can be tedious and unnecessary. RNase H mapping and combinatorial ODN array technology has

been used in combination to select a RNA region with good accessibility and to define target antisense sequences respectively (Milner *et al.*, 1997).

1.3.2 Biological Stability

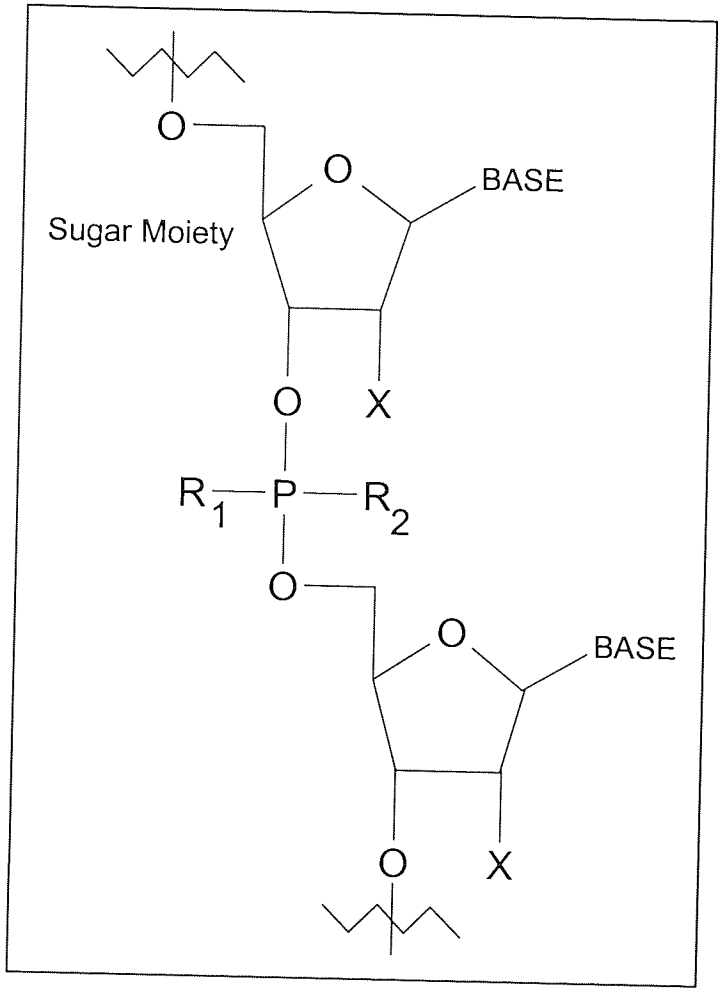
Conventionally, an ODN consists of unmodified DNA sugar phosphate backbone (figure 1.2). However, due to the unstable properties of the phosphodiester molecule in biological fluids (Akhtar *et al.*, 1992) containing cellular and extracellular nucleases various analogues have been synthesised.

Unmodified phosphodiester (PO) ODNs are rapidly degraded in biological fluids by a combination of both endo- and exo-nucleases (Walker *et al.*, 1995; Reddy 1996; Fell *et al.*, 1997; Hudson *et al.*, 1999). In many cases this degradation occurs so quickly that a PO ODN would not survive long enough to exert its pharmacological effect (Kole *et al.*, 1991). This problem has been overcome to varying extents by using chemically modified ODNs which are more resistant to nucleases (Tortora *et al.*, 1998). It should be noted that such chemical modifications do not just effect nuclease resistance as they may also modify other properties of ODNs *e.g.* alterations in the base, sugar and phosphate moieties can potentially create ODNs with enhanced affinity, more selectivity for RNA or DNA, enhanced cellular uptake, altered distribution as well as improved pharmacokinetics (for review see Reddy 1996).

1.3.2.1 Phosphodiester Internucleoside Linkage Modifications

1.3.2.1.1 Phosphorothioates/Phosphorodithoates

Replacing one of the oxygen atoms of the internucleotide phosphodiester linkage by sulphur leads to one of the most widely studied ODN modifications i.e. phosphorothioates (PS). These analogues have increased resistance to degradation both *in vitro* and *in vivo* as well as having the capability of eliciting RNase H activity, however, they have a decreased affinity for their target nucleic acid. They exhibit both sequence-specific and non-specific activity (Boiziau *et al.*, 1991; Woolf *et al.*, 1992; Dean *et al.*, 1994; Coulson *et al.*, 1996; Sommer *et al.*, 1998). Biological effects that are not related to the antisense mechanism are common to PS ODNs as a result of binding in a non-specific manner to both surface and intracellular proteins (Weidner *et al.*, 1995; Coulson *et al.*, 1996). However, studies with good controlled



R_1	*R_2*	*Name*
O=	**O-**	**Phosphodiester**
O=	**S-**	**Phosphorothioate**
S=	**S-**	**Phosphorodithioate**
O=	**CH_3-**	**Methylphosphonate**
O=	**NH_2-**	**Phosphoramidate**
O=	**BH_3-**	**Boranephosphite**

R_1	*R_2*	*X*	*Name*
O=	**O-**	**OCH_3-**	**2' O-methyl**
O=	**O-**	**OH-**	**RNA**

Figure 1.2 The chemical structure of a phosphodiester ODN with positions of structural modifications.

experiments have shown promising results. For example, a 20-mer PS ODN designed to hybridise to the AUG translation initiation codon of the mRNA encoding murine protein kinase C-α (PKC-α) reduced PKC-α mRNA expression for up to 48-hrs *in vitro* and *in vivo* with no effect when the scrambled AODN was utilised (Dean *et al.*, 1994).

PS ODNs have the potential to posses different biochemical and biophysical properties from their pure stereoregular counterparts due to the fact that they are synthesised and evaluated as diasteriomeric mixtures (Tang *et al.*, 1995). This has led to the substitution of both non-bridging oxygen atoms with sulphur atoms resulting in the formation of phosphorodithioate (PS_2) ODNs (Eckstein 2000). The phosphorodithioate linkage would in theory eliminate the chiral centre thereby removing the diastereomer problem, however the data available to date does not demonstrate that this linkage has any advantage over the simple monothioate mentioned above. Its affinity for the target and ability to activate RNase H is similar to the diastereomeric mixtures of monothioates (Eckstein 2000).

1.3.2.1.2 Methylphosphonates

The formation of methylphosphonate (MP) ODNs results from the substitution of the non-bridging oxygen of the phosphodiester bond with a methyl group. These are non ionic and relatively stable to nucleases (Miller 1991; Boiziau *et al.*, 1991). MP ODNs do not bind to proteins and demonstrate minimal toxicity (Miller 1991). Poor hybridisation properties as well as the inability to activate RNase H has led to the use of chimeric ODNs containing MP linkages in the middle of the PS ODN. These chimeric ODNs have been shown to reduce side-effects *in vivo* compared with PS-ODNs (Tortora *et al.*, 1997) (see section 1.3.2.2).

1.3.2.1.3 Phosphoramidates

The formation of phosphoramidate ODNs results from substituting the non-bridging oxygen of the phosphodiester bond with an amine group. These modified ODNs have been shown to be stable to nucleases *in vitro* and have an enhanced interaction with complementary RNA compared to phosphodiester ODNs (Schultz *et al.*, 1996). The increased lipophilicity of phosphoramidate ODNs, as result of amine substitution, enhances cellular uptake, however

phosphoramidate ODNs are unable to activate RNase H resulting in reduced antisense activity (Maher *et al.*, 1988).

1.3.2.1.4 Boranephosphite ODNs

Modification at the phosphate linkage by substitution of the phosphodiester bond with BH_3 group results in the formation of boranephosphite ODNs. This modification allows ODNs to have increased resistance to nucleases (Shaw *et al.*, 1991). The increased hydrophobicity due to the lack of lone pairs may possibly increase cellular uptake, however poor binding of this modified ODN to complementary RNA target may hamper its therapeutic efficacy (Zhang *et al.*, 1996).

1.3.2.2 Mixed-backbone ODNs

Mixed-backbone ODNs are composed of two sections, one which activates RNaseH (e.g. PS internucleotide linkages) and another which does not activate RNase H (e.g. methylphosphonates). These ODNs have been studied to further enhance the therapeutic potential of AODNs (Tortora *et al.*, 1997; Tortora *et al.*, 1998). The PS portion of the ODN would allow the activation of RNase H and the MP internucleotide linkages would reduce the overall toxicity of the ODN. Increased stability and enhanced activation of RNase H has been observed for mixed-backbone ODNs with alternating MP and PO compared to full PO ODNs (Spiller *et al.*, 1992).

1.3.2.3 Sugar-phosphate Backbone Modifications

1.3.2.3.1 The 2'-O-modified ODNs

The most common modifications of the ribose sugar have involved the 2' position (Fisher *et al.*, 1993). Substitution of all nucleosides by their 2'-O-methyl analogues stabilises the resulting ODN against various nucleases such as 2'-OH-dependent S1 nuclease, mung bean nucleases and exonuclease III. However, such ODNs are still susceptible to degradation by micrococcal nuclease and nuclease P1 (Sproat *et al.*, 1989). 2'-O-Methyl-modified ODNs have been shown not to activate RNase H upon hybridisation with target mRNA, however increased mRNA hybridisation affinity has been observed (Inoue *et al.*, 1987). To overcome

their inability to activate RNase H chimeric ODNs have been used with 5 nucleotides at the 5' end and 5 at the 3' end modified with methoxyethoxy groups on the 2' ribose position, whereas 10 interior nucleotides were phosphorothioates. This study showed that these modified ODNs were substantially more potent (10-fold) than unmodified PS ODNs in inhibiting P-glycoprotein (Alahari *et al.*, 1998).

1.3.2.3.2 Hexitol ODN

In this modification, the ribose ring has been replaced by a hexose molecule and the resulting ODN has been shown to hybridise well to RNA. This change in the ODN has been shown to enhance the affinity of the ODN to RNA as well as being stable against nucleases. However, cellular permeation is not improved by the modification (Verheggen *et al.*, 1995).

1.3.2.3.3 Peptide nucleic acids (PNA)

Replacement of the entire sugar-phosphate unit with artificial amino acids has also been accomplished and the ODN analogues produced have displayed very interesting characteristics (Nielsen *et al.*, 1991; Pooga *et al.*, 2001). PNA oligomers bind to single-stranded DNA and RNA with extraordinary affinity and high sequence specificity. These studies also showed that PNAs invade double stranded nucleic acid structures (Nielsen *et al.*, 1991; McMahon *et al.*, 2002). PNA oligomers were shown to be able to act as antisense and transcriptional inhibitors when microinjected in to cells as well being stable to nucleases and peptidases (Hanvey *et al.*, 1992). An added advantage with this modification is the ability to polymerise monomers using standard peptide coupling chemistry which is highly efficient (Hanvey *et al.*, 1992).

1.3.2.4 Base Modifications

Modifications to ODN bases have been made to enhance the stability and affinity for the target RNA (Brian *et al.*, 1992). A simple pyrimidine modification which has been shown to increase affinity for the RNA is the 5-propynyl uracil residue (figure 1.3 A). Antisense effects with propynyl pyrimidine ODNs have been seen in a number of cell culture systems when the linkage is the nuclease stable PS (Flanagan *et al.*, 1996). Purines have been investigated to a lesser degree largely because of their increased synthetic complexities. The

simple purine analogue diaminopurine improves affinities relative to adenine due to an additional hydrogen bond being formed with the complementary uracil (Gryaznov *et al.*, 1994) (figure 1.3 B).

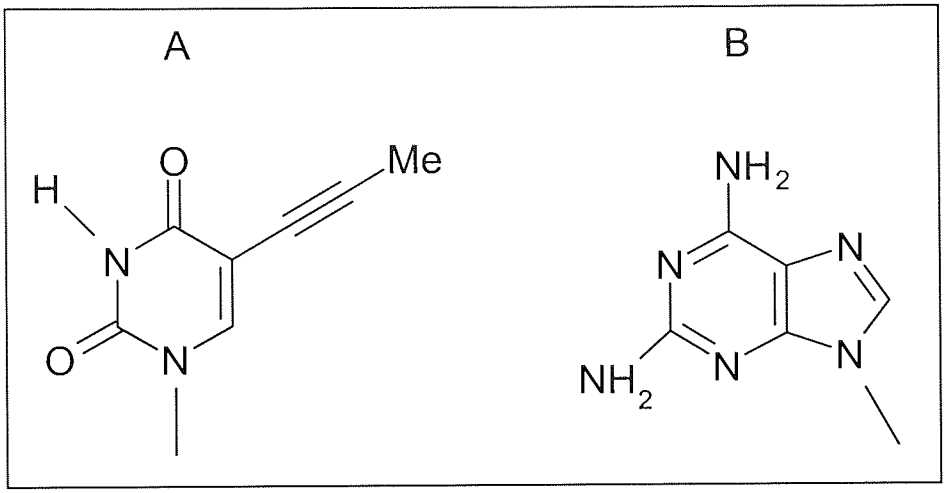


Figure 1.3 Base modifications of AODNs, (A) pyrimidine analogue, (B) purine analogue (adapted from Gryaznov and Ronald, 1994).

Evaluating the role of RNase H activity in biological efficacy is essential when developing possible new backbone analogues. ODNs are able to recruit this ribonuclease only when they contain both a charged phosphorus backbone and a deoxyribose sugar (Szymkowski 1996). Modifications that eliminate this charge or alter the sugar to improve drug stability may therefore reduce overall biological efficacy.

1.3.3 Cellular Uptake and Distribution

The entry of AODNs into cells and the subsequent interaction with pre-mRNA or mRNA in the nucleus and cytoplasm is vital for AODNS to successfully inhibit gene expression. Cellular uptake refers to the combination of both ODN membrane-binding and internalisation. AODNs are generally 15-30 nucleotides in length, highly charged hydrophilic molecules with molecular weights up to 9000 Da which cannot freely cross the plasma membrane. Permeability of the cell membrane is therefore a limiting factor for successful efficacy. Uptake of ODNs is an inefficient process; only a small fraction of the input ODN becomes bound to the cell membrane and internalised (Akhtar *et al.*, 1996; Beck *et al.*, 1996).

Studies suggest that a combination of fluid-phase (pinocytosis) (FPE), adsorptive (AE) and receptor-mediated endocytosis (RME) are responsible for the uptake of exogenously delivered AODNs (Akhtar *et al.*, 1992). FPE involves unbound ODN being internalised non-specifically. The non-specific binding of ODN to the cell membrane is classified as AE and ODNs binding to a specific cell surface receptor involves RME (figure 1.4). It appears that the major endocytic pathway for ODNs is *via* receptors on the cell membrane *i.e.* RME (Loke *et al.*, 1989; Beck *et al.*, 1996). These studies have shown that the uptake process of ODNs depends on temperature, energy and concentration and is also saturable. An approximately 80 kDa cell surface protein (in HL60 and mouse fibroblast L929 cells) and a smaller 34 kDa (in a variety of human carcinoma cells) membrane protein which function at pH 4.5 have been proposed as receptor proteins for ODN cellular association and/or uptake (Yakubov *et al.*, 1989; Loke *et al.*, 1989; Vlassov *et al.*, 1994).

ODN chemistry, concentration and conformation as well as cell type, cell cycle and cell culture conditions are important factors in the precise uptake mechanism involved for ODNs (Akhtar *et al.*, 1992). The uptake of methylphosphonate ODNs is *via* a pathway distinct from that of charged nucleotides and may involve either passive diffusion and/or adsorptive endocytosis (Dagle *et al.*, 1991), however the precise mechanisms of uptake are poorly understood.

The intracellular localisation of AODNs have been studied to find out their possible fate following cellular uptake (Shoji *et al.*, 1998). Fluorescently labelled ODNs accumulate in vacuoles within the cells in tissue culture media, forming a punctate perinuclear pattern which are presumably endosomes. A significant amount of the ODN is also compartmentalised within other organelles, such as the Golgi complex and the endoplasmic reticulum (Islam *et al.*, 2000). Following microinjection of fluorescently labelled ODNs into cells, the ODN has been shown to rapidly accumulate within the nucleus. This suggests that after endosomal exit ODNs are able to migrate to the nucleus (Leonetti *et al.*, 1991). Endosomal compartments are acidic (pH range 5-7) and their membranes usually have a lipid composition distinct from that of the plasma membrane. Diffusion of ODNs across model lipid membranes of similar composition to those of endosomes is very slow and is thus an unlikely mechanism of ODN release from endosomal compartments. It may be likely that the leakage of ODNs during the fusion process or through simple rupturing of endosomes is an important factor for the release of ODNs from the endosomes into the cytosol (Akhtar *et al.*, 1992).

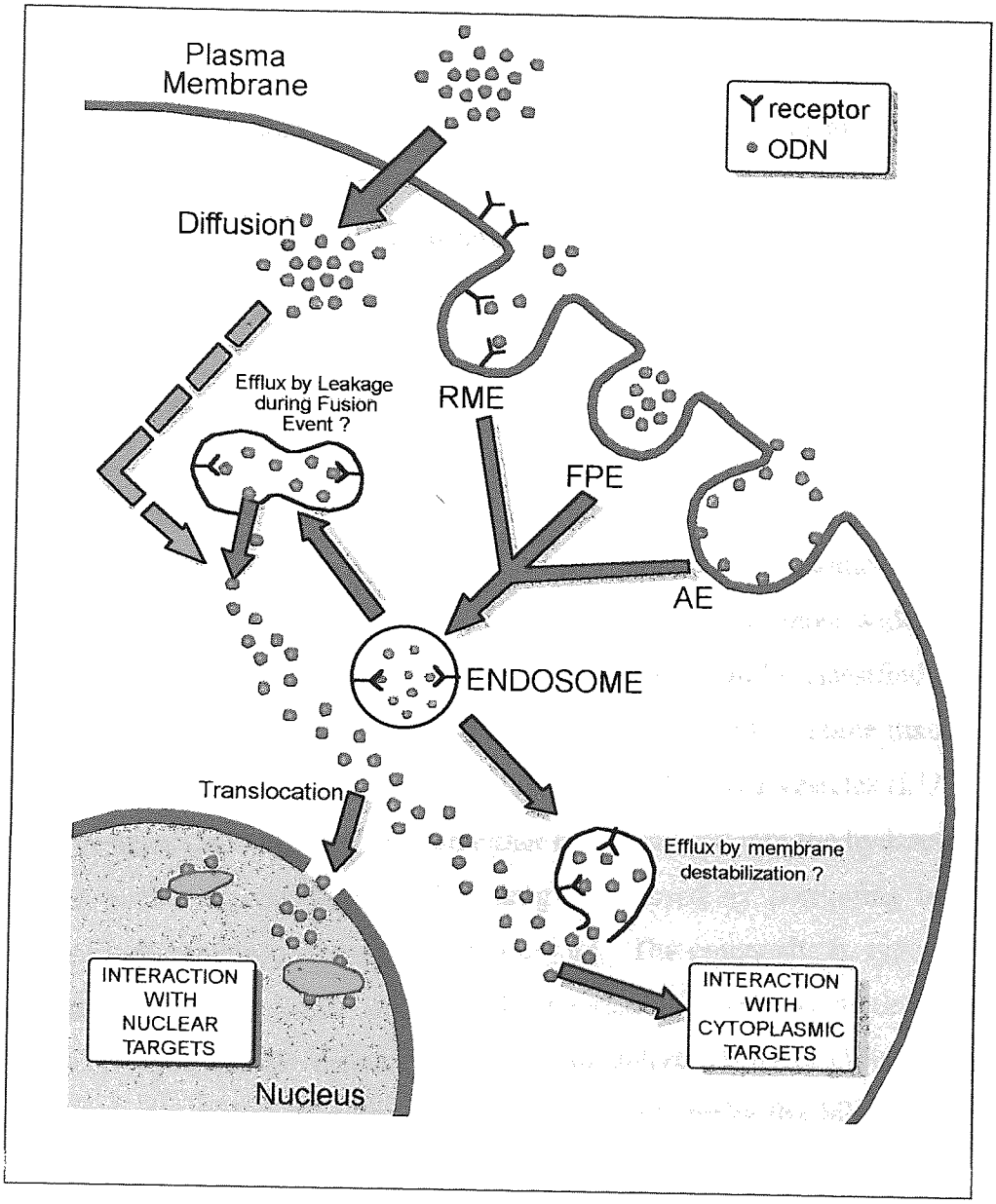


Figure 1.4 A schematic representation of cellular uptake and distribution of ODNs [Receptor-mediated endocytosis (RME), adsorptive endocytosis (AE), fluid-phase endocytosis (FPE)](adapted from Akhtar and Juliano, 1992).

1.4 *IN VITRO* AND *IN VIVO* DELIVERY STRATEGIES

It is clear from the above discussion that one of the main aims for optimal delivery of ODNs is enhanced cellular uptake and improved exit from subcellular compartments. It is therefore no surprise that there are many studies which have focused on improving the efficacy of AODN by utilising different delivery strategies. An optimal delivery system should be able to enhance cellular uptake, improve exit from subcellular compartments and reduce the

susceptibility to nucleases while retaining the ability to bind to targeted sites. Also, the poor pharmacokinetic/pharmacodynamic properties *in vivo* of AODNs (see section 1.5) suggest the need for a delivery system which correctly targets the AODN to the desired site of action and therefore avoid repeated administration for a sustained pharmacological effect, especially for targets with a slow turnover *i.e.* the required time frame needs to be matched to the biological half-life of the target protein and to the desired level of knockdown. Below is a discussion on some of the most popular strategies of AODN delivery.

1.4.1 Liposomes and Lipoplexes

Liposomes are microscopic spheres with an aqueous core surrounded by one or more outer shells consisting of lipids (Chonn *et al.*, 1995). The ability of liposomes to protect nucleic acids and to enhance cellular delivery has led to them being the most widely used delivery system for AODNs in cell-culture experiments. Liposomes can be classified on the basis of size and bilayer number present within a liposome *i.e.* lamellarity. Three main categories of liposomes exist; multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs). AODNs can either be partitioned into the hydrophobic layers or be dissolved in the aqueous layers. The drug is released by portioning back out of the liposome or by disruption of the liposome structure. The composition, type and size of the liposome can be altered to impart different characteristics. The most successful studies have used commercially available cationic liposomes to deliver AODNs (Wielbo *et al.*, 1997b; Chakraborty *et al.*, 1999). The positively charged lipids make the bilayer of the liposome probably increase encapsulation efficiency and enhance uptake due the high affinity for negatively charged cell membranes (Akhtar *et al.*, 1992). The overwhelming conclusion from these studies is that liposomes are able to resolve the problem of extracellular degradation by nucleases and poor permeability that are inherent for AODNs (Zobel *et al.*, 1998; White *et al.*, 1999). The exact mechanism of how these delivery systems function is unknown although it is quiet clear that a significant optimisation in terms of charge ratio between cationic lipid and nucleic acid at a given dose is essential for effective delivery and activity. Cationic lipid complexes (lipoplexes) have also been used to deliver AODNs (Islam *et al.*, 2000). Complexes of ODN and lipid are formed spontaneously upon mixing in aqueous solution by a condensation reaction and therefore preparation of these complexes is relatively easy (Felgner *et al.*, 1989). The effectiveness of lipoplexes is dependent on the type and the nature of the cationic lipid, cell type, ODN chemistry, ODN length and even the method of formation of

complexes. It has been shown that intracellular bioavailabilty and activity of AODNs is improved by utilising cationic lipoplexes (Williams *et al.*, 2000), however cationic lipids are toxic and generally ineffective in the presence of serum proteins (Coulson *et al.*, 1996), which consequently has limited their application mainly to cell culture (Islam *et al.*, 2000).

1.4.1.1 In vivo Delivery

The rapid recognition of liposomes by the reticuloendothelial system (RES) and their consequent uptake by endocytosis leads to a relatively short circulation time *in vivo* (Senior 1987). Long-circulating ODN-liposomes have been observed by modifying liposomes with polyethylene glycol (PEG) to reduce opsonization and phagocytosis by cells of the RES (Klibanov *et al.*, 1990). However, it is possible that local delivery of liposomes and cationic lipoplexes may be more effective. The pharmacokinetics, tissue distribution, stability and cellular uptake of a PS AODN by a human ascites tumour xenograft has been studied in nude mice, in the absence of or complexed with DOTMA. This cationic lipid did not affect ODN uptake or tissue distribution in normal mice. Highest concentrations were observed in the kidney and liver, however, it significantly increased ODN uptake in ascites tumours when delivered intraperitoneally demonstrating its utility for local delivery (Garcia-Chaumont *et al.*, 2000). A recent study attempted to examine the use of cationic lipid-ODN complexes for brain delivery following intracerebroventricular administration. The cationic lipid allowed increased uptake (8-fold) of ODNs in glial cells (Sommer *et al.*, 1998). In respect to liposomal toxicity a risk-benefit analysis will need to be adopted in life threatening conditions where a liposomal delivery system is the only alternative for AODNs.

1.4.2 Receptor-Mediated Delivery

The ability to target AODNs to specific types of cells would reduce adverse effects which may be associated with non-specific drug delivery. Receptor-mediated delivery involves ODNs conjugated to a compound which has a specific cellular receptor *e.g.* transferrin or mannose. This type of delivery system may be particularly appropriate for cells which have an increased requirement for essential nutrients *e.g.* tumour cells.

The transferrin receptor which efficiently transports iron-bearing transferrin molecules into cells by receptor mediated endocytosis represents a potentially useful carrier by which ODNs

may be internalised. This receptor has been reported to efficiently internalise ODNs that have been complexed with its natural ligand transferrin (Citro *et al.*, 1992). This study revealed a dose-dependent inhibitory effect on HL-60 cells by the complexed antisense ODN that was 4-fold more potent than that of the free AODN. When administered *in vivo* however, transferrin-containing conjugates or complexes are likely to be in competition with the uptake of the natural transferrin protein and could potentially compromise normal iron uptake into cells. An alternative approach which has therefore been utilised, is the use monoclonal antibodies to the transferrin receptor. These recognise and bind to regions other than the transferrin binding site of the receptor and thus would not compete for the natural ligand. This principal has been utilised by (Normand-Sdiqui *et al.*, 1998) and it was found that the uptake of AODN conjugated to transferrin into a rat brain endothelial cell line (RBE4) was 2-fold higher than the free ODN and its uptake mechanism was consistent with transferrin receptor-mediated endocytosis.

Mannose is internalised by macrophage cells via mannose specific lectins present on the cell surface (Stahl *et al.*, 1978). Conjugation of ODNs with mannose has therefore been investigated to enhance the delivery in macrophages (Liang *et al.*, 1996). The study utilised a molecular complex consisting of partially substituted mannosylated poly(L-lysine) electrostatically linked to an ODN. The results showed a significant increase in ODN uptake (up to 17-fold) over free ODN-treated controls.

Many tumour cells derived from epithelial tumours over-express the epidermal growth factor receptor (EGFR) which may allow these particular tumour cells to be targeted specifically. It has been shown that a conjugate of EGF covalently linked to poly(L-lysine) which has then been complexed electrostatically to ODN enhanced uptake of ODN, up to 12-fold compared to free ODN (Deshpande *et al.*, 1996).

1.4.2.1 In vivo Delivery

Several studies have looked at using the asialoylglycoprotein receptor (ASGP-R), which is unique to the liver, to target nucleic acids. A study in which a ligand for the ASGP-R was directly linked to an ODN showed more effective delivery (9-fold) to parenchymal liver cells in cell culture compared with free-ODN (Biessen *et al.*, 1999). Furthermore, injection of the ODN and ODN-ligand into male Wistar rats showed that the specific accumulation of ODN in

paranchymal cells was improved almost 60-fold by the ligand. Polyethyleneimine (PEI) conjugated with lactose has been used to attach a chimeric RNA-DNA molecule to ASGP-R. The chimeric ODN was directed to the rat factor IX gene and was designed to introduce a single point-mutation to inactivate factor IX. Injection of the free chimeric molecule into male Sprague-Dawley rats resulted in no detectable mutations but when administered with the PEI-lactose conjugate a dose-related genomic DNA mutation was observed in up to 40% of animals (Biessen *et al.*, 1999).

1.4.3 Carrier-peptide-mediated Delivery

Protein transduction domains are small motifs of approximately 10-16 amino acids in length. They can be used as 'carrier' or 'transport' peptides to promote the delivery of active agents across biological membranes by a receptor- and transporter-independent mechanism. Several of these peptide motifs have now been identified including the Tat protein from the HIV-1 virus, the *Drosophila melanogaster* homeotic transcription factor ANTP and the herpes simplex virus type-1 (HSV-1) VP-22 transcription factor (Schwarze *et al.*, 2000).

There are several examples of these peptides being used for ODN delivery. For example, peptides derived from the Tat and Ant proteins which have been conjugated to a PS-ODN have been used to target the MDR1 gene which encodes p-glycoprotein (Astriab-Fisher *et al.*, 2000). Both peptides were able to efficiently deliver the ODN to cells in culture. The peptide-ODN conjugates inhibited P-glycoprotein expression more effectively in the presence of serum than in the absence of serum. Another group used a chimeric peptide (MPG) consisting of a hydrophobic fusion domain derived from HIV gp41 and a hydrophilic nuclear localization signal derived from the SV40 T-antigen (Morris *et al.*, 1997). They demonstrated that the MPG peptide was able to efficiently bind to both single-stranded and double stranded ODNs. The MPG-ODN complex improved the stability of the ODNs and no significant degradation was observed after 10-hr in medium containing serum. Furthermore, this complex was efficiently delivered with greater than 90% of cells demonstrating uptake of fluorescently labelled MPG-ODN after 1-hr.

1.4.3.1 In vivo Delivery

Although no reports exist on the use of these carrier or transport peptides for ODN or ribozyme delivery *in vivo*, a recent study using a model macromolecular drug, β-galactosidase fused with the membrane-traversing Tat protein resulted in the distribution of β-galactosidase in all tissues, including the brain, following intraperitoneal administration to mice (Schwarze *et al.*, 1999). This study suggested that carrier peptides could be useful for the improved delivery of maromolecules *in vivo* and that their use could also facilitate the delivery of ODNs and ribozymes across the blood-brain barrier. Their efficacy in the presence of serum proteins also suggests a potential for peptide-nucleic acid conjugates for *in vivo* applications.

1.4.4 Transdermal Delivery

Methods for increasing transdermal flux of AODNs have included iontophoresis and electrophoresis (Zewert *et al.*, 1995; Brand *et al.*, 1996). These methods are particularly applicable for charged molecules as they involve an electric driving force. Iontophoresis utilises the application of a low voltage and a constant current to the skin to enable the dermal transfer of charged particles. Iontophoresis has been shown to enhance transdermal flux of intact PS ODN across hairless mouse skin (Brand *et al.*, 1996). Electroporation involves the application of a high voltage pulse for a short time to reversibly permeabilise the lipid bilayer of the dermis. Molecules are able to pass through the cell membrane by pores being opened by electroporation. This method has been shown to increase concentrations of intracellular ODN up to 27-fold in lymphoma U937 cells (Bergan *et al.*, 1993). A study by the same group has shown that AODNs which have been introduced into cells by electroporation led to immediate suppression of targeted *c-myc* protein resulting in rapid cell death of the lymphoma U937, Burkitt's lymphoma ST486, breast carcinoma MCF-7 and Ewing's sarcoma CHP-100 cell lines (Bergan *et al.*, 1996).

1.4.4.1 In vivo Delivery

Rats exposed to 100 μM PS ODN targeting the cytochrome P450 3A2 showed significantly reduced expression of CYP3A2 in the liver following iontophoretic delivery for 3.5-hrs (Brand *et al.*, 2000). The transdermal delivery of ODNs is feasible and offers the flexibility

for either local treatment of skin disease or systemic delivery. The requirement for particularly potent and charged ODNs may limit the overall utility of the transdermal approach, hence neutral oligomers combined with more passive methods which employ penetration enhancers appears to be the most likely direction for transdermal delivery of AODNs.

1.4.5 Polymer Delivery

The use of biodegradable polymeric delivery systems, which provide sustained release of the active compound, could obviate the need for repeated administration by improving both the pharmacokinetics and the pharmacodynamics of ODNs and ribozymes. The entrapment of ODNs within such polymeric matrix systems could improve ODN stability, reduce the ODN required for efficacy, enhance cellular uptake and lower toxicity associated with ODNs (see section 2.1.1 for a more detailed discussion).

1.4.6 Dendrimer Delivery

Dendrimers are new supramolecular delivery systems which have unique features that make them superior to other particulate systems. One advantage is the very defined polymerisation reactions yielding a monodisperse reproducible product. Dendrimers can be synthesised with several functional groups making them one of the more versatile of the particulate systems. Several categories of dendrimers have been synthesised with various functional groups depending on the initial monomer and the building block monomer. Starburst polyamidoamine (PAMAM) dendrimers are by far the most popular types of dendrimers utilised for the delivery of AODNs (Eichman *et al.*, 2000). The cationic surface charge of PAMAM dendrimer provides a suitable surface for the anionic AODNs to bind (see section 3.1.1 for a more detailed discussion).

1.5 *IN VIVO* PHARMACOKINETICS

Pharmacokinetics is an important factor in characterising the relationship between drug administration and pharmacological effect for drug molecules. Pharmacokinetics encompasses the processes involved in achieving and maintaining pharmacological availability at the target site (adsorption, distribution, metabolism and elimination). In

context to AODNs, the factors influencing pharmacokinetics include nuclease degradation, protein binding, plasma clearance, tissue distribution, cellular uptake and subcellular localisation (Figure 1.5).

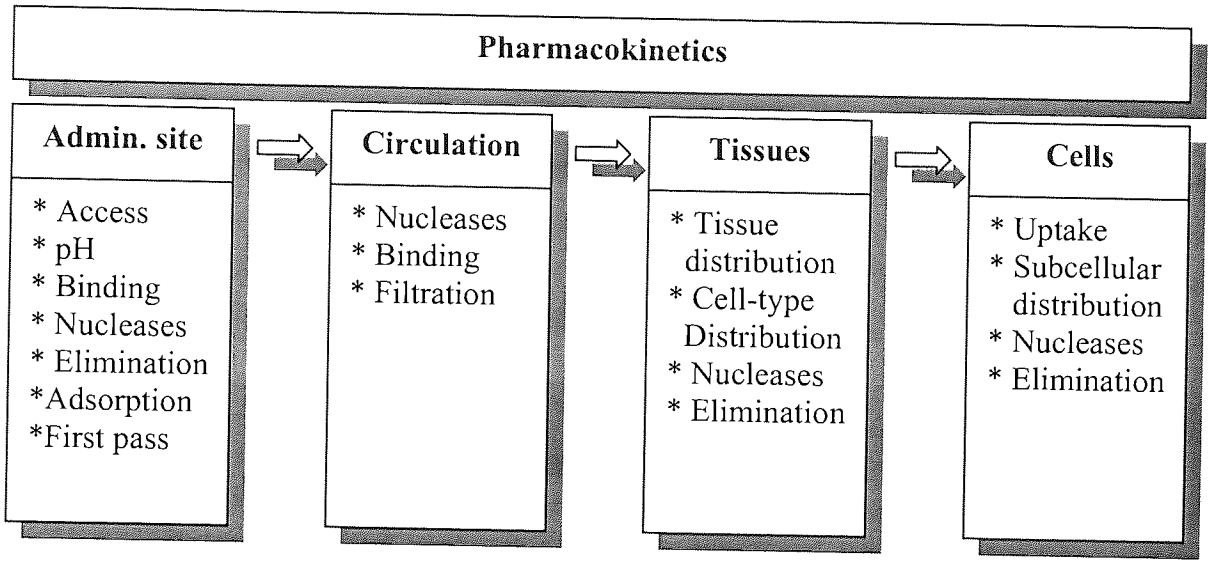


Figure 1.5 Pharmacokinetic barriers to the pharmacological efficacy of ODNs.

Understanding the pharmacokinetics of an ODN provides the basis for its effective use as a therapeutic agent as well as indicating which organs may be sites of toxicity. This section summarizes data on the *in vivo* pharmacokinetics of ODNs introduced by traditional intravenous (i.v.) and intraperitoneal (i.p.) injection as well as studies on delivery of ODNs in animals by various non-invasive delivery systems.

The short degradation half-life (2-5 min) of unmodified PO ODNs following i.v. administration has made the availability of detailed pharmacokinetics of PO ODNs difficult (Akhtar *et al.*, 1997). The increased stability of PS ODNs to nucleases (Sommer *et al.*, 1998) has meant that these ODNs have been studied in detail for their pharmacokinetics (Butler *et al.*, 1997). Following i.v. administration of PS ODNs (20-mer) in mice, rapid elimination from the plasma compartment has been observed (Agrawal *et al.*, 1991; Agrawal *et al.*, 1995). Rats administered with PS ODNs by i.v. have shown biphasic blood kinetics, comprising of a rapid distribution phase followed by a prolonged elimination phase (Nicklin *et al.*, 1999). The same study has shown that PS ODNs were widely distributed in the body and accumulated in most tissues particularly in the liver, kidney, bone marrow and spleen where higher concentrations were observed and retained for an extended period. Similar results have been noted in rats administered i.v. with a 20-mer PS ODN which targets the 3'untranslated region

of human protein kinase C-α (Nicklin *et al.*, 1999). Tissues such as kidney, liver, spleen, pancreas, adrenal gland, salivary gland, mesenteric lymph nodes, duodenum and ileum were able to accumulate the PS ODN suggesting that they bind to tissue matrix and/or that active uptake into cells occurs within these organs, however the principle accumulating organs were the kidney and the liver which concentrated the ODN. Although the ODN was widely distributed it was not observed in the brain and subcutaneous fat cells. A pharmacokinetic profile similar to the one observed with i.v. administration was observed with i.p. administration except that the peak plasma concentration was lower (Agrawal *et al.*, 1991; Agrawal *et al.*, 1995).

The presence of both intact and degraded forms of the PS ODN when extracted from the plasma has been studied in rats (Agrawal *et al.*, 1991; Agrawal *et al.*, 1995). The degradation profile was plasma-, tissue- and time- dependent. Higher molecular weight degradation products of the administered ODN have been shown to be present in the liver and the gastrointestinal tract (Akhtar *et al.*, 1997). Metabolism is complex although it is known that 3'-exonucleases are responsible for the majority of degradation of PS ODNs. However, degradation has also been observed from the 5' –end by PAGE analysis (Temsamani *et al.*, 1997). The major route of elimination of PS ODNs following i.v. or i.p. administration in rats or mice has shown to be in the urine (Agrawal *et al.*, 1991). Recently, a study has been published to develop a whole body physiology-based model of the pharmacokinetics of a PS ODN *in vivo* (Peng *et al.*, 2001). Capillary gel electrophoresis analysis indicated that the predominant species in plasma and all tissues was the intact ODN with some n-1 and n-2 metabolites. The ODN distributed extensively into tissues, but relative affinity varied enormously, being highest for kidney and liver and lowest for muscle and brain.

The pharmacokinetics of a 25-mer PS ODN following i.v. administration in monkeys showed a profile similar to that in mice and rats (Agrawal *et al.*, 1995). Rapid clearance from the plasma was observed with similar tissue distributions. The same PS ODN was then used in humans to analyse the pharmacokinetics following a 2-hr i.v. infusion. Once again the plasma clearance was rapid and the major route of elimination was in the urine (Zhang *et al.*, 1995). Intact PS ODN was detectable in plasma, but not in urine, up to 6-hr after the end of the infusion.

1.5.1 *In vivo* Toxicity Studies

The systemic toxicities of PS ODNs have been studied extensively in rodents, rabbits and monkeys (McIntyre *et al.*, 1993; Galbraith *et al.*, 1994; Henry *et al.*, 1997). It appears that the *in vivo* toxicity of PS ODNs are species-dependent. In rodents the acute toxicity of PS ODNs have been characterised as part of an effort to determine the maximum tolerated doses for *in vivo* genotoxicity assays. A 24-mer PS AODN has been administered i.p. three times weekly for two weeks in CD-1 mice (Sarmiento *et al.*, 1994). Significant toxicity, including mortality of some animals was observed at doses of 100mg kg^{-1} and 150mg kg^{-1}. Acute renal failure was the likely cause of death. Major sites of toxicity were kidneys, liver, spleen and bone marrow. Liver enzyme (aspartate aminotransferase, alanine aminotransferase) levels were also elevated and the major haematological effect was severe thrombocytopaenia.

Studies in monkeys have demonstrated a potential for acute toxicity markedly different than rodents. Bolus i.v. administration of PS ODNs has produced a transient decrease in peripheral total white blood cell and neutrophil counts and a brief increase followed by a prolonged decrease in arterial blood pressure. It is likely that these effects occur due to the activation of the C5 complement. The effects were dose and infusion dependent and were avoided by administering PS ODNs by slow i.v. administration (for review see: Akhtar *et al.*, 1997). Preclinical studies on monkeys have shown that PS ODNs can elicit acute haemodynamic and cardiovascular side-effects once plasma levels exceed a "threshold" concentration (Galbraith *et al.*, 1994; Henry *et al.*, 1997; Levin *et al.*, 1998). These toxicities are thought to be related to the polyanionic nature of the molecules and the binding of these compounds to specific protein factors in the plasma.

1.5.2 Pharmacokinetics of Other ODNs Chemistries

The fate of ODNs with alternating PO and methylposphonate linkages has been investigated (Agrawal *et al.*, 1997). Electrophoretic analysis performed 1-hr postinjection has revealed that the modification afforded a significant protection. The pharmacokinetics of methylphosphonate ODN analogs (a single PO linkage at the 5' end) injected into the vein of a mouse has been investigated (Nicklin *et al.*, 1999). Within a few minutes the compound was distributed among all animal tissues with the lowest level found in the brain. Elimination of the compound from the circulation was rapid with a half-life of 17 mins. Within 2-hr

postinjection, up to 70% of the ODN injected was excreted in the urine. Pharmacokinetic studies have been carried out on mixed-back bone ODNs containing four methylphosphonate linkages at both the 3' and 5' ends of the PS ODN (Zhang *et al.*, 1996). Following i.v. administration this ODN was cleared from the plasma and distributed into tissues rapidly. Analysis of the extracted ODN from plasma and various tissues showed a significant increase in *in vivo* stability of the end-modified mixed-backbones. The major route of elimination of mixed-backbones was in the urine. The relatively long retention times in the RES organs suggested that accumulation of mixed-backbone ODNs and their metabolites upon repeated dosing may account for this toxicity. The tissue disposition of mixed-backbone ODNs is similar following i.p. and s.c. administration, however other modes of administration including delivery formulations facilitating local or site-specific delivery may help to circumvent these problems.

1.6 ANTISENSE THERAPEUTICS IN CLINICAL TRIALS

The two main companies at the forefront of antisense therapeutics are Isis Pharmaceuticals and Hybridon, Inc. The antiviral drug Vitravene™ (fomivirsen) developed by Isis Pharmaceuticals and marketed by CIBAVision has been approved for marketing by the FDA (July, 1998) and has also received approval by European regulatory authorities (August, 1999). Vitravene™ has been licensed for use in the local treatment of CMV retinitis in newly diagnosed or advanced CMV when other therapies are considered unsuitable or have been ineffective e.g. in AIDS patients. Fomivirsen is complementary to the immediate-early transcriptional unit of human CMV mRNA. Isis has five compounds in human clinical trials: ISIS 2302, an inhibitor of ICAM-1, is in Phase II clinical trials for renal transplant rejection and is being explored as an enema formulation for ulcerative colitis (Phase IIa), a topical administration for psoriasis (Phase IIa) and an aerosol administration for asthma, however ISIS 2302 has not demonstrated efficacy in patients with Crohn's disease. ISIS 3521, an antisense inhibitor of protein kinase C-alpha (PKC-a) expression is in Phase II trials as a treatment for cancer (non-small cell lung carcinoma). Due to the success of these trials with ISIS 3521 phase III trials are now in progress. ISIS 5132, targeting *c-raf-1* mRNA, is in Phase II clinical trials as a treatment for cancer. ISIS 2503, a potent selective inhibitor of *H-ras* gene expression has entered Phase II trials as a treatment for cancer.

Most of the clinical trials administered naked ODNs as PS analogues. Since these have several toxicological drawbacks, the use of second generation mixed-backbone analogues has been investigated. Hybridon is in early clinical development with two mixed-backbone oligonucleotide (MBO) antisense drugs, GEM 231 for treatment of solid tumours and GEM 92 for treatment of infection with HIV-1. Both Hybridon drugs are available for licensing to a pharmaceutical development partner.

Ribozyme Pharmaceuticals Inc. (RPI) product pipeline currently includes several products. RPI, in partnership with Chiron Corporation, is developing ANGIOZYME™ a novel inhibitor of angiogenesis that has shown promise in preclinical cancer models. Phase Ia and Ib clinical trials were completed in 1999 and Phase I/II clinical trials have begun. In addition RPI has developed an anti-Hepatitis HEPTAZYME™, which has begun Phase I/II clinical trials. EpiGenesis Pharmaceuticals Inc. in collaboration with Taisho Pharmaceuticals Co. Ltd. have developed EPI-2010 which is a respirable AODN (RASON™) which is in Phase I clinical trials in the U.S. and has just begun trials in the U.K (March 2001). EPI-2010 targets the adenosine A1 receptor which is unique among mediators of asthma in that it is produced in excess in virtually all asthma patients. This one receptor is associated with three aspects of asthma, inflammation, bronchoconstriction and surfactant depletion.

The main barrier to this strategy is now achieving delivery of the nucleic acids in sufficient quantities to the correct sites of action and for the desired level of gene inhibition or functional down-regulation (Akhtar 1998; Juliano *et al.*, 1999). Effective delivery systems may hold the key to overcome these barriers.

1.7 ANTISENSE OLIGONUCLEOTIDES TARGETED TO THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The EGFR is a 170 kDa single-pass transmembrane protein of approximately 1200 amino acids. It is present in most cell types though not haemotopoietic cells. The receptor comprises three major regions: an N-terminus extracellular region, a hydrophobic transmembrane region and a C-terminus intracellular region which contains the tyrosine kinase domain. The extracellular region is a glycosylated portion and is a ligand-binding site for various polypeptide growth factors predominantly epidermal growth factor (EGF) and TGFα. Receptor dimerisation and the activation of the intracellular tyrosine kinase region occurs after the successful binding of the EGF or TGFα to the extracellular ligand-binding region. This process allows the binding of an ATP molecule to the tyrosine kinase domain resulting in receptor autophosphorylation and transphosphorylation of another receptor monomer. The autophosphorylation of the receptor incorporates phosphate into several C-terminus tyrosine residues which then become sites for several *Src* homology 2 (SH2) domain containing signal transducers (i.e. p85, Grb2-Sos, PI(3)K) whose role is to transmit the proliferative and survival signals of the receptor down stream. For example, the newly formed phosphotyrosine residues act as sites for binding Grb2-Sos (a specific SH2 protein), which induces the exchange of GDP for GTP on Ras. The GTP-activated Ras acts as a binding site for Raf, which causes this protein kinase to become localised at the plasma membrane where it triggers the MAP kinase cascade. Once activated the last protein kinase in the cascade (MAPK) translocates to the nucleus where it phosphorylates and activates specific transcription factors such as Elk-1. Elk-1 binds to the promoter regions of a number of genes including *c-fos* and *c-jun*. The products of these genes, fos and jun interact to form a heterodimeric transcription factor called AP-1 that activates genes involved in cell proliferation. Grb2 is not the only protein containing a phosphotyrosine-binding SH2 domain capable of binding to a phosphorylated EGFR. Other SH2 proteins including PI(3)K can also bind to these receptors and become activated as a result of the interaction. This pathway is associated with glucose metabolism and survival signalling. Figure 1.6 shows the basic signal pathway from the EGFR (Raymond *et al.*, 2000). Over-expression of the EGFR has been observed in human breast and glioblastoma tumours (Coulson *et al.*, 1996) and therefore the ability to block the activity of the EGFR would be a logical approach for the treatment of breast and glioblastoma tumours.

Potential new anticancer agents that target the extracellular ligand-binding region of the EGFR include a number of monoclonal antibodies, immunotoxins and ligand-binding cytotoxic agents. Agents that target the intracellular tyrosine kinase region include small molecule tyrosine kinase inhibitors (TKIs), which act by interfering with ATP binding to the receptor and various other compounds that act at substrate-binding regions or downstream components of the signalling pathway (for review see: Ciardiello 2000).

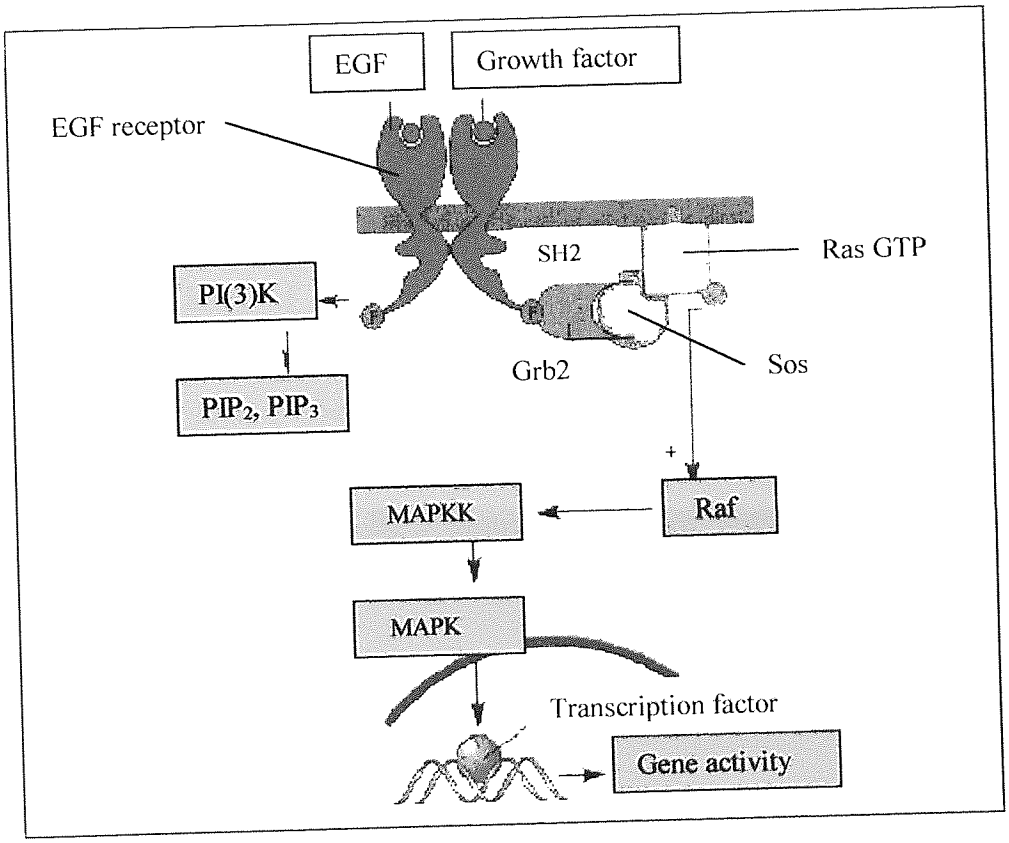


Figure 1.6 The signal pathway from the epidermal growth factor receptor (adapted from Raymond *et al.*, 2000).

AODNs have also been used to target the EGFR, for example, three 20-mer PS ODNs were designed to target different regions of the EGFR mRNA (Witters *et al.*, 1999). These ODNs were added to a human carcinoma cell line (SKOV3) and a human lung carcinoma line (A549) both of which overexpress EGFR. All three AODNs inhibited expression of the EGFR mRNA (range: 22-97% inhibition) compared to control ODNs. A dramatic decrease in endogenous EGFR mRNA and protein levels, reduced proliferation and an induction of apoptosis has been observed with AODNs targeted to the EGFR mRNA of rat C6 glioma cells *in vitro*. As part of the *in vivo* studies survival times were significantly prolonged in two thirds of rats injected with C6 cells followed by the AODN (Pu *et al.*, 2000). Human head

and neck tumours in nude mice have been targeted by EGFR AODNs. Inhibition of tumour growth, suppression of EGFR and an increased rate of apoptosis was observed (He *et al.*, 1998). Altered cell morphology and growth inhibition of a human oral carcinoma KB cell line has been observed with AODNs against the EGFR (Wang *et al.*, 1995). AODN specific for the GRB2 mRNA, a downstream transducer of EGFR has also been utilised in breast cancer cells which has inhibited cellular growth by 76% as compared to the control (Tari *et al.*, 1999).

AODNs complementary to the initiation codon region of the EGFR mRNA have been evaluated on A431 cell line which express amplified levels of EGFR (Coulson *et al.*, 1996). Concentration dependent reduction in proliferation was observed. The effects of EGFR inhibition on A431 cells were not mediated by a true antisense mechanism in that there was no reduction in the level of EGFR mRNA or protein over a 24-hr period, however autophosphorylation of the receptor was significantly reduced by EGFR AODN and not by control ODNs. Other studies show similar results (Rockwell *et al.*, 1997). These studies suggest that AODNs targeted to the EGFR may also have the capacity to inhibit proliferation and alter the morphology of cells by a sequence-selective, but non-antisense mechanism affecting tyrosine kinase activity.

1.8 AIMS

Disease states such as cancer result from gene over-expression and therefore may be affected by nucleic acid drugs such as AODNs which have the potential to down regulate the expression of disease-causing proteins at the level of mRNA. However, problems such as poor cellular penetration and stability have hindered the AODN journey to reach the clinic. To achieve optimal biological effects, AODNs need to be protected from nucleases and enough needs to be delivered at the site of action for extensive time periods. Delivery systems such P(LA-GA) micrsopheres and anionic dendrimers may overcome the limitations of the antisense strategy. The overall aim was therefore to characterise and test each delivery system appropriately.

The aims are outlined below:

- To characterise a P(LA-GA) microsphere delivery system in an attempt to use EGFR AODNs (designed using combinatorial ODN array technology) with a model cytotoxic drug such as 5-FU in an individual formulation of the two agents, as well as co-entrapment of the AODN and 5-FU within a single microsphere formulation.

- To test the optimised formulation in cell culture and determine whether the delivery system has an effect on viable cell number, if so, then is the effect by an antisense mechanism.

- To characterise the cellular uptake properties and stability of a novel anionic dendrimer based on a pentaerythritol structure, covalently linked to EGFR AODNs.

- To test the dendrimer delivery system in cell culture in terms of its ability to cleave the target site, down regulate the target protein and ultimately have an effect on viable cell number.

CHAPTER TWO

FORMULATING AND TESTING SINGLE OR SEPARATE MICROSPHERE DELIVERY SYSTEMS FOR ANTISENSE OLIGONUCLEOTIDES AND 5-FLUOROURACIL

2.1 INTRODUCTION

AODNs need to be delivered to the target site for extended time periods in order to achieve optimal biological effects, especially for targets with a slow turnover (Akhtar *et al.*, 1997a). This can be achieved using biodegradable sustained release polymeric formulations (Chavany *et al.*, 1994; Lewis *et al.*, 1995; Fattal *et al.*, 1998). Of the many options available, the use of biodegradable microspheres of poly (lactide-co-glycolide) [P(LA-GA)] copolymers for the potential delivery of AODNs have been evaluated (Akhtar *et al.*, 1997b; Lewis *et al.*, 1998; Khan *et al.*, 2000). These particulate delivery systems offer several advantages including the potential for localised, site-specific or organ-specific delivery, protection from nucleases that digest ODNs and tailored release profiles that further allow control of the entrapped drug's pharmacokinetics and pharmacodynamic parameters (Lewis *et al.*, 1995; Lewis *et al.*, 1998; Khan *et al.*, 2000).

AODNs have been extensively considered for the down-regulation of oncogenes in cancer therapy and several sequences e.g. those targeting *c-raf* kinase are being tested in ongoing clinical trials (Akhtar *et al.*, 1997a; Akhtar *et al.*, 2000). A more recent approach is the use of AODNs in combination with conventional chemotherapy (Del Bufalo *et al.*, 1996; Tortora *et al.*, 1998; Geiger *et al.*, 1998). For example, a greater inhibition of tumour cell growth was observed *in vitro* and *in vivo* when a *c-myc* AODN was co-administered with cisplatin than with either of the agents alone (Del Bufalo *et al.*, 1996). In other studies, 5-fluorouracil (5-FU), an inhibitor of thymidylate synthase, was shown to be more cytotoxic (by 50-60%) when combined with AODN specific for thymidylate synthase mRNA (Ferguson *et al.*, 1999; Ferguson *et al.*, 2001). An AODN specific for alphafetoprotein mRNA in combination with 5-FU has also shown a significantly enhanced effect on hepatoma cell growth as compared to

either AODN or 5-FU alone (Wang *et al.*, 1999). Thus, therapeutic strategies involving combinations of AODNs with conventional cytotoxics appear promising for potential cancer therapy. Cytotoxic drugs like 5-FU are potent antineoplastic agents but they tend to exhibit side effects in the body (Parker *et al.*, 1990). In the case of 5-FU it is rapidly absorbed through the blood capillaries into systemic circulation (Ardalan *et al.*, 1981). This results in relatively low levels of drug near the site of action with the subsequent loss of efficacy and increased risk of systemic toxicity. By using sustained release formulations of 5-FU the incidence of side-effects may be reduced and therapeutic effects increased (Hagiwara *et al.*, 1996; Brem *et al.*, 1999). Based on the fact that local sustained delivery may enhance therapeutic effects, a pilot study involving eight patients with newly diagnosed glioblastoma, a malignant brain tumour, was conducted in which 5-FU in P(LA-GA) microspheres was delivered locally after surgical resection. Promising results were reported by this study with 2/8 patients achieving disease remission (Menei *et al.*, 1999).

Our laboratory has been examining the use of AODNs for the treatment of cancer by targeting the epidermal growth factor receptor (EGFR), encoded by the *c-erb*B1 proto-oncogene that is commonly over-expressed in glioblastomas and human breast tumours (Coulson *et al.*, 1996; Yarden 2001) (see section 1.7). In an attempt to use EGFR AODNs with conventional cytotoxics, this chapter assesses the possibility of the potential co-delivery of a 21-mer phosphorothioate ODN sequence (complementary to the 5'-coding sequence of *c-erb*B1) and 5-FU, a model cytotoxic drug, using P(LA-GA) microspheres. Individual formulations of the two agents, as well as co-entrapment of the AODN and 5-FU within a single microsphere formulation were characterised and release profiles determined.

2.1.1 Polymer Delivery

The short-lived biological effects of ODNs revealed by *in vivo* and *in vitro* studies prompt the need for repeated administration for sustained efficacy. This may be partly due to the rapid degradation of nucleic acids in the biological environment. Furthermore, the swift redistribution and pharmacokinetic elimination half-life of ODNs [the plasma half-life of 1-hr for phosphorothioate ODNs (Lewis *et al.*, 1998)] is also likely to be a factor. A possible approach to improving both the pharmacokinetics and the pharmacodynamics of ODNs involves the use of sustained-release polymer formulations. Biodegradable polymers afford protection to nucleic acid drugs and depending on the nature of the formulation it is possible

to control the rate of release of the encapsulated drug. These can be fabricated as implantable devices for local delivery or even as parentarally administered systemic formulations. The most widely studied biodegradable polymers for the delivery of nucleic acids are polylactide (PLA), lactic acid-glycolic acid [P(LA-GA)] co-polymers and polyalkylcyanoacrylate (PACA) nanoparticles (Chavany *et al.*, 1992; Lewis *et al.*, 1998; Putney *et al.*, 1999).

2.1.1.1 P(LA-GA) and PLA Polymers

The routine use of lactic acid and glycolic acid co-polymers in resorbable surgical structures (Cutright *et al.*, 1971) and in commercially available sustained-release preparations (Pouton *et al.*, 1996) has demonstrated their biocompatibility and degradation to toxicologically acceptable products. Polylactic acid is an aliphatic polyester. There are three forms of polylactic acid, those deriving from D(-), L(+) and the racemic D,L forms of the parent acid. The L(+) form is metabolised by the body and is more commonly used than the D(-) form. The chirality of the starting monomer leads to the stereoregularity in the polymer resulting in a high degree of crystallinity. Polylactic acid can form a copolymer with glycolic acid, poly (D,L-lactide-co-glycolide) [P(LA-GA)] (Figure 2.1). Polymers based on lactic acid and glycolic acid are thermoplastic crystalline polyesters formed by ring-opening polymerisation catalysed by ZnO of lactide, glycolide or mixtures of these compounds. Low molecular weight (<3000) polymers are produced by direct condensation of lactic acid. Poly L-lactide is highly crystalline (80%) and poly D,L-lactide is amorphous. These are insoluble in water, ethanol and methanol, however they are soluble in organic compounds such as methylene chloride, carbon tetrachloride, chloroform, acetone, dioxane and ethyl acetate.

P(LA-GA) polymers degrade by random bulk hydrolysis of the ester bonds in the polymer chain in an aqueous environment. Degradation produces lactic and glycolic acid monomers and carboxylic acids which along with impurities and humidity catalyse the rate of degradation. The degradation products are finally metabolised to carbon dioxide and water and excreted via the kidneys (Gilding *et al.*, 1979) (Figure 2.2). Degradation of the polymers is biphasic; firstly a decrease in molecular weight of the polymer occurs due to random chain scission which is accompanied by water uptake. The enhanced water uptake increases porosity and soluble monomeric and oligomeric products can be detected. These degradation products leave the bulk polymer through channels resulting in weight loss of the polymer (Hutchinson *et al.*, 1985). The monomers degrade more slowly than the copolymer and the

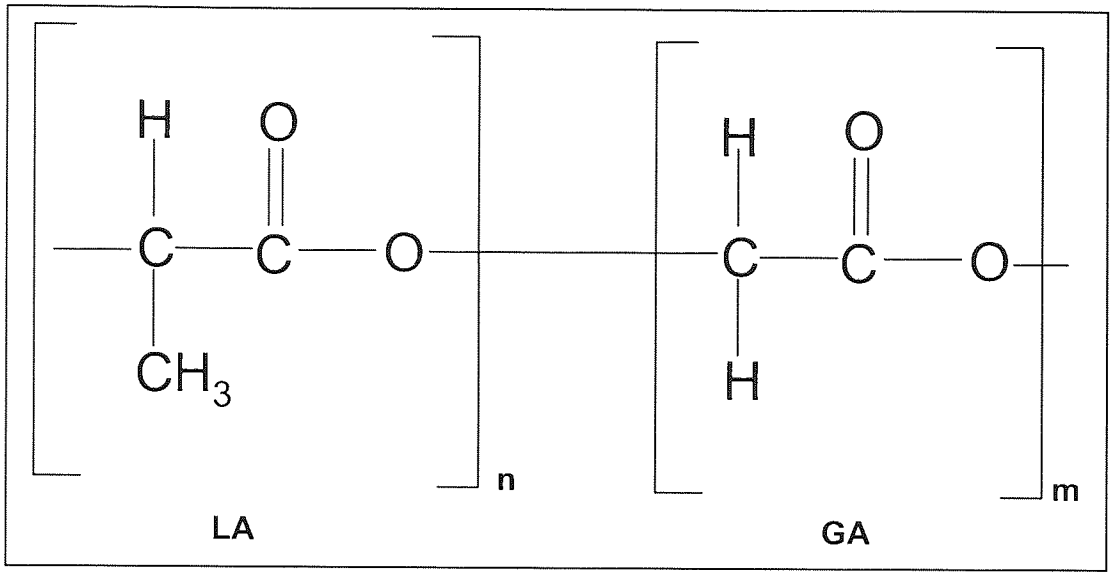


Figure 2.1 The molecular structure of poly (D,L-lactide-co-glycolide). LA = lactic acid, GA = glycolic acid, n = number of lactic acid monomers, m = number of glycolic acid monomers.

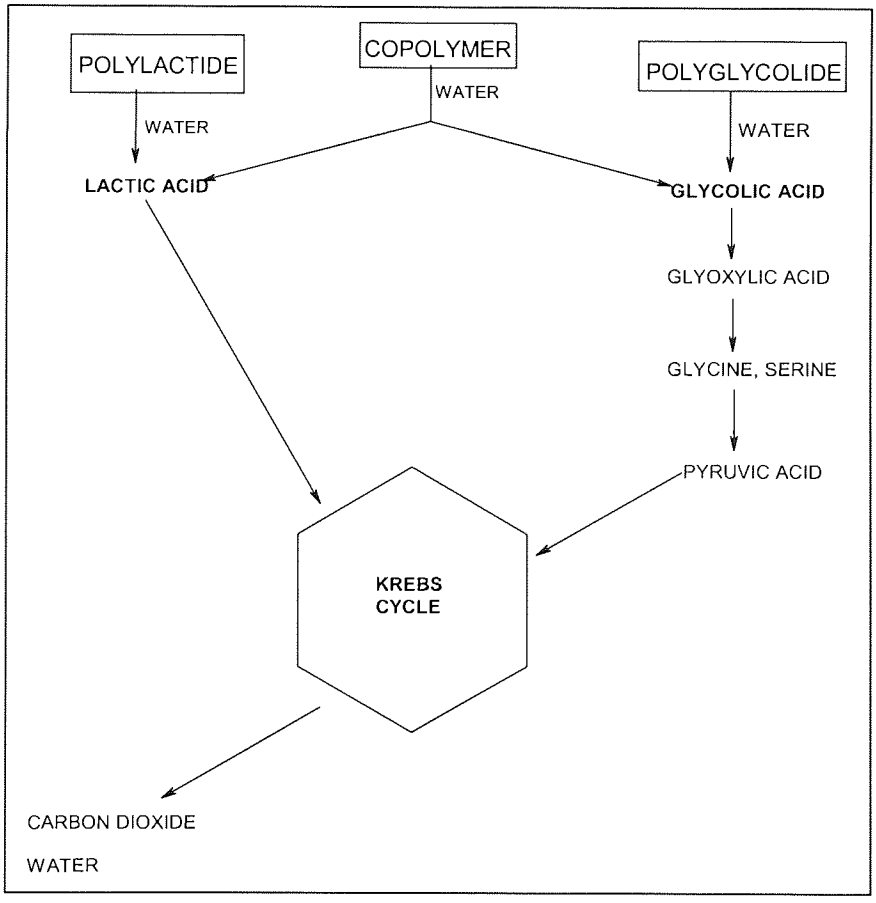


Figure 2.2 Degradation pathway of poly (D,L-lactide-co-glycolide) monomers (adapted from Gilding and Reed 1979).

increased rate of degradation is a result of lower copolymer crystallinity allowing greater absorption of water into the polymer hence faster degradation (Gilding *et al.*, 1979). Increasing the molecular weight of the polymer decreases the rate of degradation (Sanders *et al.*, 1986). Polymer degradation is accelerated in both strongly alkaline and acidic media (Makino *et al.*, 1986). Increasing the ionic strength of the media also increases the rate of degradation (Makino *et al.*, 1986). The presence of tissue lipids can increase the rate of degradation of the polymer by acting as plasticisers (Menei *et al.*, 1999). Adsorption of plasma proteins onto the surface P(LA-GA) microspheres increases the solubility and hence the rate of degradation of the polymer (Makino *et al.*, 1986).

2.1.1.1.1 Tissue Biocompatibilty of Implanted PLA and P(LA-GA) Polymers

Studies to date indicate that PLA and P(LA-GA) microspheres containing bioactive agents are biocompatible and when used in therapeutic applications *in vivo*, do not exhibit untoward reactions either locally or systemically (Anderson *et al.*, 1997). The rate of localized tissue response to PLA or P(LA-GA) microspheres is size-dependent. Microspheres greater than 5 to 10 microns in diameter may not be phagocytosed within macrophages and foreign body giant cells and thus a foreign body response is present at the surfaces of these microspheres. Microspheres smaller than 5 microns may undergo phagocytosis by macrophages, foreign body giant cells and other types of cells. The foreign body reaction with the presence of macrophages and foreign body giant cells is a common tissue response to micropsheres greater than 10 microns in diameter (Anderson *et al.*, 1997). No toxic effects have been observed when P(LA-GA) microspheres were injected directly into the brain (Menei *et al.*, 1993). The absence of systemic side-effects have been observed when P(LA-GA) microspheres loaded with 5-FU were implanted in the wall of the surgical bed after the surgical resection of glioblastoma in 8 people (Menei *et al.*, 1999). The type of administration route is usually associated with tissue responses with different polymers. Schneider observed that small differences in inflammatory reaction occurred relating to molecular weight of the polymer used. The safety of P(LA-GA) polymers in respect to biocompatibility is highlighted from the successful clinical use of Viryl^{Tm} sutures (Schneider 1972).

2.1.1.1.2 P(LA-GA) and PLA Polymers for ODN Delivery

Lewis and coworkers reported on the biological stability, hybridisation potential and *in vitro* release kinetics of PO- and PS-AODNs entrapped within biodegradable PLA film (100 ± 10 μm thickness) matrices (Lewis *et al.*, 1995). Sustained release of ODNs was observed from these devices for >1 month in physiological buffer solutions and was dependent on ODN chemistry (PS-ODNs were released more slowly than PO-ODNs) and on ODN length (a 20-mer was released more slowly than a 7-mer). The polymer-entrapped ODNs were protected from serum nucleases and retained their biological activity for the entire study period. Gel mobility shift analyses and duplex melting-point determinations suggested that the hybridisation capability of antisense ODNs released from the PLA matrices was unaffected by the solvent-casting procedure used for preparing these polymer devices (Lewis *et al.*, 1995).

Studies with P(LA-GA) microsphere devices for entrapping ODNs have shown similar advantages with using biodegradable polymer delivery systems (Akhtar *et al.*, 1997b; Putney *et al.*, 1999). P(LA-GA) microspheres were prepared containing ODNs using a double-emulsion method and the release of the encapsulated ODNs evaluated (Akhtar *et al.*, 1997b). They showed that release-profiles could be controlled and ultimately tailored to specific requirements by altering the size of the microspheres, the amount of ODN loading and the length of the ODN. *In vitro* release-profiles of AODNs from P(LA-GA) matrices have been shown to be triphasic (Lewis *et al.*, 1995): Typically, profiles were characterised by an initial 'burst' effect during the first 48-hr of release (phase 1) followed by a more sustained release (phase 2) with a subsequent additional release (phase 3) resulting from bulk degradation of the microspheres. ODNs have also been encapsulated in pillar shaped P(LA-GA) implants (Yamakawa *et al.*, 1997). The release profile showed sustained release of ODN over 20 days was possible. The release was unaffected by changes in the molecular weight of polymer, however changes in the loading altered the release rate of ODN. A continuous release of intact ODN was observed throughout the 30-day release (Yamakawa *et al.*, 1997).

Polymeric microsphere (or even nanosphere) devices could potentially enhance the delivery of ODNs at the cellular level because of their relatively small size. They could also provide site-specific delivery to a particular tissue or subset of cells and/or provide sustained delivery of the free-ODN into the systemic circulation following implantation (either intramuscularly,

subcutaneously or intraperitoneally). HIV AODNs have been loaded into microspheres for the treatment of cultured murine macrophages to evaluate the potential for delivery at the cellular level (Akhtar and Lewis 1997b). They showed that P(LA-GA) microspheres in the 1-2μm size-range improved the cellular delivery by up to 10-fold compared with free-ODNs. In the same study uptake of ODNs was significantly reduced in the presence of metabolic and phagocytic inhibitors indicating that the microspheres-loaded ODNs were entering cells by an endocytic or phagocytic mechanism. Dose-dependent growth inhibition of smooth muscle cells has been demonstrated with AODN loaded microparticles (Cleek *et al.*, 1997). Release of ODN was characterised by a small initial-burst (20%) followed by controlled release for up to 20 days. It is not clear, as yet, how the polymer-entrapped ODNs are trafficked through the cells and how and when they are released from the delivery system; these issues require further study.

2.1.1.2 Polymer Nanoparticles for ODN Delivery

Biodegradable polyalkylcyanoacrylate (PACA) nanoparticles have also been investigated for the delivery of nucleic acids (Chavany *et al.*, 1992; Chavany *et al.*, 1994; Fattal *et al.*, 1998). PACA nanoparticles are obtained by emulsion polymerisation of various alkylcyanoacrylate monomers in acidic medium. Due to the negative surface charge of the PACA nanoparticles a cationic copolymer or cationic hydrophobic detergent is used to facilitate ODN binding. In contrast to P(LA-GA) microspheres, where ODNs are physically entrapped within the polymer matrix, ODNs are adsorbed onto the charged surface of the PACA nanoparticles. This system has proved to be efficient not only for protecting the ODNs from degradation by exonucleases but also for increasing uptake of ODNs (Chavany *et al.*, 1992; Chavany *et al.*, 1994; Fattal *et al.*, 1998). The uptake of PACA nanoparticles was shown to be temperature dependent suggesting an endocytic/phagocytic process and the nanoparticles accumulated in lysosome/phagosome vesicles (Fattal *et al.*, 1998). AODNs targeted against HA-*ras* adsorbed on to PACA nanoparticles have shown to be effective at 100-fold lower concentration compared to naked AODN (Schwab *et al.*, 1994). However, the toxicity of the hydrophobic cations and the production of formaldehyde on polymer degradation may limit the *in vivo* use of this system. Table 2.1 summarises some of the studies which have been undertaken to deliver ODNs using nanoparticles.

2.1.1.3 *In vivo polymer delivery*

Site-specific administration of naked or fluorescently-labelled ODNs and microsphere loaded ODNs in the rat brain has been carried out to examine if P(LA-GA) polymer microspheres could be useful as sustained delivery systems *in vivo* (Khan *et al.*, 2000). Using fluorescently labelled nucleic acids, a single local injection of naked ODNs within an A431 tumour xenograft in nude mice resulted in its rapid elimination from the tumour with only ~20% of the dose remaining after 6-hrs. By contrast, the use of ODN-loaded microspheres maintained ODN delivery for >48-hrs within the tumour. ODNs were stereotaxically administered into the neostriatum of rat brain which further confirmed the sustained-release capability of these devices *in vivo*. It was also found by these authors that ODNs delivered as polymer microspheres in to brain tissue improved the subcellular biodistribution of ODNs that might improve antisense activity. They confirmed that naked PS-ODNs are predominantly taken up by neuronal cells and ultimately localised within vesicular structures as indicated by the punctate distribution of fluorescence in the cells. However, with slowly released PS-ODNs from the P(LA-GA) microsphere formulation, the biodistribution profile was characterised by a more diffuse cytosolic and nuclear fluorescence in neuronal cells. Furthermore, because a 20-fold lower dose of ODNs in microspheres yielded an intensity of signal similar to that generated by free-ODNs, it was suggested that much lower doses of ODNs will be necessary for antisense activity if delivered as biodegradable polymer formulation. A study on the improved efficacy of ODN delivered as polymer formulations has been carried out (Putney *et al.*, 1999). Human melanoma cells were injected in male CD-1 nude mice. The study showed that the subcutaneous delivery of a P(LA-GA) microsphere-encapsulated AODN targeted against the oncogene *c-myc* led to an enhanced suppression of tumour growth when compared with free-ODN administered intravenously. The delivery of ODN subcutaneously in microspheres (6 mg) resulted in ~60% inhibition of tumour growth, whereas the same dose of ODN in solution (administered over 8 days) only achieved 20% inhibition. Western blot analysis also revealed a more prolonged reduction in *c-myc* protein levels (55% after 20 days administration) with microsphere-encapsulated ODN compared with free-ODN administered in solution (no reduction after 20 days). It is clear from the above studies that the use of sustained-release polymers for ODN delivery provide a proven method of improving the pharmacokinetics and pharmacodynamics. This is especially true for long-lived targets that would require repeated administration of ODNs for efficacy *in vivo*. In such cases, careful

Table 2.1 A Summary of Studies Utilising Nanoparticles to Deliver ODNs

Reference	ODN and target gene	Delivery strategy	Cell line	Comments
(Berton *et al.*, 1999)	15-mer PS homopolymer of thymidine	ODNs loaded on to nanoparticles of poly (D,L) lactic acid through the formation of ion pairs using hydrophobic cations (CTAB)	DU145 cells	ODNs loaded nanoparticles did not reside in acidic compartments as compared to naked ODNs. Efflux distribution was biphasic for both ODN loaded nanoparticles along with naked ODNs .
(Fattal *et al.*, 1998)	15-mer oligothymidylate	PACA nanoparticles utilised for ODN adsorption	U937 cells	8-fold increase in cellular uptake for ODNs adsorbed on to nanoparticles compared to naked ODN. ODNs adsorbed on to nanoparticles were protected from nucleases overnight.
(Zobel *et al.*, 1997)	19-mer unmodified PO	ODNs adsorbed on to polyhexylcyanoacrylate nanoparticles (PHCA), which have been stabilised by diethylaminoethyldextran	Vero (African green monkey kidney) cells	ODNs adsorbed nanoparticles were protected against endonucleases, compared to naked ODNs. 20-fold increase in stability was observed with the ODNs adsorbed on to PHCA nanoparticles.
(Nakada *et al.*, 1996)	16-mer oligothymidylate	CTAB used to adsorb ODNs on to polyisobutylcyanoacryrate (PIBCA) nanoparticles	Male OF1 mice	Nanoparticles partially protected ODNs *in vivo* against degradation (in the plasma and in the liver) 5 min after they were specifically delivered to the liver, compared to the total degradation of naked ODN
(Chavany *et al.*, 1994)	16-mer and 15-mer oligothymidylate Vesicular stomatitis virus N protein mRNA initiation codon	CTAB used to adsorb ODNs on to PHCA nanoparticles	U937	Uptake was 8-fold higher compared to naked ODNs, after 24 hour incubation. Also, ODNs were protected from phosphodiesterase for 5- hrs.

Reference	ODN and target gene	Delivery strategy	Cell line	Comments
(Schwab *et al.*, 1994)	Unmodified PO ODN *H-ras* gene	ODNs adsorbed on to PHCA nanoparticles through the formation of ion pairs using CTAB	HB100ras1	Cell proliferation was selectively inhibited by ODNs adsorbed on nanoparticles. The *H-ras* gene was expressed 100-fold lower than compared to free ODN.

selection of the polymer formulation variables such as polymer type, polymer molecular weight and the size and nature of the polymer device may achieve tailored or target-matched sustained delivery.

2.1.2 AODN Selection to the EGFR

Tertiary and secondary structures of the mRNA determine the interaction between the target sequence and the AODN (see section 1.3.1). The AODN complementary to *c-erb*B1 mRNA which encodes for the EGFR was therefore selected using combinatorial ODN array technology (Petch *et al.*, 1999). A series of overlapping ODNs targeting every conceivable site within the chosen segments of the *c-erb*B1 mRNA were synthesised using standard phosphoramadite chemistry on a modified polymer membrane. The radiolabelled *c-erb*B1 mRNA transcript was then introduced to the immobilised ODNs on the polymer membrane which allowed the accessible sites of the mRNA transcript to bind the complementary ODNs. Two AODNs were identified within 100 bases of the start codon which gave good signals when probed with the radiolabelled mRNA. One of these AODN sequences, termed AS1 was used to target the EGFR in further studies using microsphere formulations.

2.1.3 5-Fluorouracil (5-FU)

Since the 1960s 5-FU has been one of the major antimetabolites. It is used for the treatment of a wide variety of carcinomas. 5-FU is a white, odourless, crystalline powder, sparingly soluble in water (1 in 80), very slightly soluble in alcohol (1 in 170) and insoluble in chloroform and ether. It has a pKa of 8.0 (Ardalan *et al.*, 1981). 5-FU interferes with thymidylate synthesis and therefore with synthesis of DNA. It is converted into a 'fraudulent' nucleotide fluorodeoxyuridine monophosphate (FDUMP). This interacts with thymidylate

synthetase and the folate cofactors but cannot be converted into thymidylate because, in FDUMP, fluorine has replaced hydrogen at C5 where methylation would take place and this carbon-fluorine bond is less susceptible to enzymatic cleavage than the carbon-hydrogen bond. The result is inhibition of DNA synthesis (Parker *et al.*, 1990).

5-FU is one of the potent antineoplastic agents available but it tends to exhibit side effects in the body (Parker *et al.*, 1990). Small, sparingly water soluble molecules such as 5-FU are rapidly absorbed through the blood capillaries into the systemic circulation resulting in low levels of drug near the site of action with the subsequent loss of efficacy and systemic toxicity (Ardalan *et al.*, 1981). Incidence of side-effects may be reduced and therapeutic effects increased using sustained release formulations of 5-FU (Hagiwara *et al.*, 1996; Brem *et al.*, 1999). Sustained delivery of 5-FU has been utilised to treat peritoneal carcinomatosis (Hagiwara *et al.*, 1996). Intraperitoneal microspheres distributed higher concentrations of 5-FU to the intraperitoneal tissues for a longer period with lower blood plasma concentrations compared to rats administered with intraperitoneal 5-FU aqueous solution.

2.1.4 AODNs in Combination Therapies

Although studies in a variety of disease models have shown that AODNs are effective *in vivo*, it is likely that they will be more effective if combined with the use of conventional therapies (Citro *et al.*, 1998; Wang *et al.*, 1999; Bilim *et al.*, 2000; Moorehead *et al.*, 2000). The use of AODNs in combination with other cytotoxic agents is among the most recent strategy in AODN based cancer treatment. This approach utilises the specificity of AODNs to target specific genes crucial for tumour cell growth in the presence of effective anticancer agents. Many groups have already shown that an additive or synergistic effect exists between various AODNs in combination with other agents used to treat cancer (for review see: Tortora *et al.*, 1998). In the absence of genes which play a major role in tumour cell growth there seems to be an increase in efficiency of anticancer agents against tumour cells while having few side-effects on normal cells (Mizutani *et al.*, 1994; Blagosklonny *et al.*, 1994). These studies have also confirmed the fact that the additive or the synergistic effects are ODN sequence specific (Del Bufalo *et al.*, 1996; Geiger *et al.*, 1997; Citro *et al.*, 1998). The principal of combination therapy may allow physicians to lower the doses of chemotherapeutic agents currently used to treat malignant cancers and hence reduce organ toxicity or even death associated with

indiscriminate targeting of metabolic pathways of dividing cells. Table 2.2 summarises successful combination strategies.

AODNs have also been used in combination with radiation therapy *in vitro* and *in vivo*. The loss of cellular $p21^{WAF1/CIP1}$ results in increased apoptotic killing by ionising radiation, therefore, *in vitro* treatment of colon cancer cells with a p21 PS AODN (200nM) has been used to inhibit the radiation-induced p21 expression (>95% inhibition) resulting in enhancement of apoptosis (approximately 60% apoptotic cells at 96 hr after 10Gy). AODN in combination with radiation (i.p. ODN for 6 days at 20mg/kg/day and 15Gy) inhibited p21 production and enhanced tumour radiocurability in nude mice compared to scrambled ODNs (Tian *et al.*, 2000). The role of RAD51 protein in cell proliferation and the contribution to radioresistance by repairing DNA damage in malignant gliomas has also prompted research in to combination of AODNs with radiation. Treatment of mouse 203G glioma cells with 100 nM of RAD51 AODN significantly enhanced the radiation-induced cell death compared to scrambled or sense ODNs. When glioma cells were implanted in the cisterna magna the combination of AODNs and irradiation extended the survival time of the glioma bearing mice much longer than could be achieved with radiation alone (Ohnishi *et al.*, 1998).

The possibility of targeting simultaneously two genes involved in chronic myeloid leukemia cell growth, the *bcr-abl* and the transferrin receptor mRNAs has been exploited. The advantage of using a combination of two AODNs is two-fold: firstly, the cells that 'escape' the effect of one AODN may be destroyed by the other. Secondly, simultaneous inhibition of two target genes may potentiate each other causing a synergistic inhibition of cell proliferation. Results have shown that the AODNs were more potent at reducing cell growth when used in combination, showing a synergistic effect i.e. a further inhibition of 20% was achieved in combination as compared to the AODN alone (Vasconcelos *et al.*, 2000).

Combination strategies have not been limited to cancer therapy but have also been applied to HIV (Veal *et al.*, 1998). Results from such studies have suggested a potential role for AODNs in combination with nucleoside analogues for the treatment of HIV infection (see table 2.2 for overview of this study). A plausible hypothesis to explain the chemotherapy-enhancing effect on AODN may be the potentiation of chemotherapy induced apoptosis (Tortora *et al.*, 1998). An enhancement of chemotherapy-induced apoptosis has been described previously for inhibitors of protein Kinase C activity in gastric cancer cells

(Schwartz *et al.*, 1995). This hypothesis of apoptosis induction may therefore explain the synergistic antitumor effects of AODN in combination with standard chemotherapeutic agents *in vivo*.

Loss of chemoresistance when ODNs are combined with chemotherapeutic drugs is an appealing prospect for the future of antisense therapy. This is reflected by promising results in recent literature. For example, AODNs targeted to the c-jun transcription factor which is associated with drug resistance have been shown to reverse cisplatin resistance in human A2780 ovarian cancer cells (Pan *et al.*, 2002). Cisplatin resistance has also been overcome in human bladder cancer model due to the inhibition of the clusterin gene associated with chemoresistance (Miyake *et al.*, 2001). Other studies have shown AODNs to down regulate Bcl-x(L), an anti-apoptotic protein, subsequently enhance the sensitivity of leukemia and pancreatic cancer cells to chemotherapeutic drugs (Xu *et al.*, 2001; Broome *et al.*, 2002).

Table 2.2 Summary of The Recent Work on Combination Therapies

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Citro *et al.*, 1998)	Human melanoma M14	*c-myc* antisense phosphorothioate ODN + cisplatin	15-mer antisense PS ODN (5'-AACGTTGAG GGGCAT-3') complementary to the translation initiation region of *c-myc* mRNA	*In vitro*:- Antiproliferative effects evaluated based on cell number viability as assessed in a coulter counter and by trypan blue dye exclusion respectively. Western Blotting for protein production analysis. Propidium iodide staining and flow cytometry for cell cycle analysis and apoptosis *In vivo*:- CD-1 male nude mice injected in hind leg muscle with cell line.	PS ODN injected iv. alternating doses of 1 and 0.5mg/day for 8 consecutive days. Cisplatin was administered i.p. at 10mg/kg in 3 consecutive daily injections (3.3mg/kg).	Proliferation of melanoma cells were both inhibited by the cisplatin/*c-myc* antisense PS ODN combination to a greater extent than that observed with either agent alone (more than 90% with respect to control cells). Cisplatin produced max. inhibition of 65% and PS ODN produced max. inhibition of 55% compared with controls. Inhibition was most effective when cisplatin was followed by *c-myc* antisense ODN (95% inhibition compared with 75% reduction). Mice treated with combination therapy showed higher inhibition of tumor growth, and increase in life span compared to those treated with either agent alone.
(Del Bufalo *et al.*, 1996)	LoVo Dx from human colon adeno-carcinoma	*c-myb* antisense PS ODN+cisplatin	18-mer antisense PS ODN (5'-GTGCCGGGG TCTTCGGGC-3')	*In vitro*:- antiproliferative effects evaluated by trypan blue exclusion respectively. Western blotting for protein production analysis. *In vivo*:-CD nude mice injec. In nude mice.	PS ODN: - 1mg/mouse day-1 (day 7-day 14) iv. Cisplatin :- Three daily doses 3.3mg/kg day-1 (day 4-6) i.p.	Enhanced inhibition of cell line proliferation *in vitro* and an increase of tumour inhibition *in vivo* with combination compared to two agents administered separately. Combination- 80% inhibition. Cisplatin and ODNs , individually, 50% and 55% inhibition respectively.

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Geiger *et al.*, 1997)	Oestrogen dependent breast cancer MCF-7. Colon cancer HCT-116. Small cell lung carcinoma NCI-H69. Large cell lung carcinoma NCI-H460. Squamous cell carcinoma NCI-H520. Prostate carcinoma PC3.	C-raf Kinase antisense PS ODN+cisplatin or mitomycin C or tamoxifen or adriamycin	20-Mer antisense PS ODN (5'-TCCCGCCTGT GACATGCATT -3')	*In vivo*:- BALB/c nude mice. Tumour fragments (25mg) implanted sc. into left flank of animals. Tumour growth monitored by measuring perpendicular diameters.	PS ODN administered iv. 6mg/kg once daily. Tamoxifen - 20mg/kg p.o. 3 consecutive days weekly. Adriamycin 9mg/kg i.v. once weekly. Cisplatin 11mg/kg i.v. once weekly. Mitomycin-C 3.5mg/kg i.v. once weekly.	Combination with cisplatin or mitomycin-C showed superadditive activities against small lung carcinoma with complete tumour responses (100% reduction). ODN with cisplatin showed super additive anti-tumour effects against human prostate carcinoma with tumour cures and in combination with mitomycin-C, superadditve anti-tumour effects with tumour cures against large cell lung carcinoma.

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Tortora *et al.*, 1997)	LS174T and GEO human colon cancer. MDA MB231 and MDA-MB468 human breast carcinoma. OVCAR-3 human ovarian cancer cells.	Mixed backbone oligonucleotide (MBO) targeting RIα regulatory subunit for protein kinase A type I +palitaxel or ciplatin or doxorubicin or etoposide	18-mer antisense MBO ODN (5'-GCGTGCCTCC TCACTGGC-3') contain phosphoro-thioate and methyl - phosphonate internucleotide linkages	*In vitro*:- cell growth experiments in soft agar 10^4 cells/well evaluated by staining and counting colonies by nitroblue tetrazolium. Flow cytometric analysis of cell cycle and apoptosis by propidium iodide staining. *In vivo*:- BALB/C nude mice injected s.c.with cell suspension.	MBO antisense administered i.p. 10mg/kg (5 days, from day 2 to 6) palitaxel - i.p.20mg/kg (once, on day 1).	Synergistic growth inhibition, which correlated with increased apoptosis, observed with MBO + taxanes, platinium based compounds. e.g. in GEO cells treatment with 1nM paclitaxel which used alone showed 7% growth inhibition caused 60% inhibition in combination with MBO which produced inhibition of 15% on its own. A co-operative effect of MBO and palataxel *in vivo* bearing GEO colon cancer xenografts.
(Mizutani *et al.*, 1994)	Human urinary transitional carcinoma cell line	*c-myc* antisense PS ODN + cisplatin	15-Mer antisense PS ODN (5'-AACGTTGAG GGGCAT-3')	*In vitro*:- cytotoxity determined by a 1 day microculture tetrazolium dye assay (MTT). *c-myc* mRNA was examined by northern blot analysis.	N/A	Treatment with *c-myc* antisense ODN and cisplatin resulted in a synergistic cytotoxic effect on cell line. On combination of ODN which inhibted cell growth by 20% produced inhibition of 60% on combination with cisplatin which inhibits cell growth by 23% on its own. Treatment of cisplatin resistant cell line with *c-myc* ODN and cisplatin reversed the resistance.

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Geiger *et al.*, 1997)	Oestrogen dependent breast cancer MCF-7. Breast carcinoma BT20. Large cell lung carcinoma NCI-H460. Lung carcinoma A549. Small cell lung carcinoma NCI-H69. Prostate carcinoma PC3.	Protein Kinase C-alpha antisense PS ODN + adriamycin or mitomycin-C or cisplatin.	20-Mer antisense PS ODN (5'-GTTCTCGCTG GTGAGTTTCA -3')	*In vivo*:- tumour fragments (25mg) were implanted s.c. into the left flank of BALB/C nude mice. Tumour growth was monitored.	See ref. 3	Combination of ODN with vinblastine cisplatin showed superadditive anti-tumour activities against MCF-7 human breast carcinoma and PC3 prostate carcinomas with complete responses. ODN in combination with adriamycin resulted in superadditive antitumor effects against BT20 human breast carcinoma with complete tumour responses.
(Rao *et al.*, 1997)	K562 human chronic myeloogenous leuemia cell line	*c-myb* PS ODN + 4-hydroxyperoxy cyclophosphamide (4HC)	24-mer antisense PS ODN (5'-TATGCTGTGC CGGGGTCTTC GGGC-3')	*In vitro*:- MTT assay to assess growth inhibition of K562 cells. Reverse transcription polymerase chain reaction in order for gene expression. Flow cytometry analysis of oligonucleotide uptake.	N/A	The combination inhibited the growth of K562 cells *in vitro* when compared to the affects of *c-myb* or 4HC alone. Increase in cellular uptake of *c-myb* oligonucleotide in K562 in the presence of 4HC e.g. growth inhibition of K562 cells by 0.3125µg 4HC and ODN is 50% compared to 25% alone compared with controls.

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Blagosklonny *et al.*, 1994)	U937 and MCF7	BCL-2 and TNF receptor PS ODN + 7-aminoactinomycin D or adriamycin or daunomycin or cycloheximide or mitomycin C, cisplatin	AS TNFR[1]:5'-GCCCATGCCAGACAGCTATGG-3' AS TNFR[2]:5'-GGAGAGGCCCATGCCAGACAG-3'	*In vitro*:- MTT assay to assess growth inhibition. Protection index determined to quantify protective capacity of various ODNs against different drugs	N/A	ODNs protect cells from the cytostatic/cytotoxic action of actinomycin D, adriamycin, daunomycin or quinacrine, but not mitomycin, vincristine, cisplatin, etopside, or cycloheximide. The cytoprotective effect depends on ODN length as well as cytotoxic interaction.
(Wang *et al.*, 1999)	BEL-7404 human hepatoma cell line	Alpha-fetoprotein antisense PS ODN + 5-FU	15-mer antisense PS ODN (5'-ACTTCATGGTTGCTA-3') complementary to the translational initiation region of AFP mRNA	*In vitro* :- AFP gene expression was determined by avidin-biotin-peroxidase complex immunocytochemical method. Cell growth was measured using MTT assay	N/A	The combined treatment with AFP antisense oligomers and 5-fluorouracil showed significantly enhanced hepatoma cell growth inhibition (50%) than either AFP antisense ODN (25%) or 5-FU (20%).

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Ferguson *et al.*, 1999)	Human cervical carcinoma HeLa cell line	Thymidylate synthase (TS) 2'-methoxyethoxylated, PS ODN + 5-FU or Tomudex	20-mer antisense 2'-methoxyethoxylat-ed, PS ODN (5'GCCAGCTGGCAACATCCTTAA-3') complementary to the TS mRNA	*In vitro*:- Antiproliferative effects were based on cell number measurements using a particle counter. Reverse transcriptase-polymerase chain reaction (RT-PCR) was undertaken to measure TS mRNA Cellular content of TS was assayed by the TS binding assay.	N/A	In addition to inhibiting proliferation by up to approximately 40%, ODN enhanced the cytotoxicity of tomudex or 5-FU, added 1 day following transfection, by 50-60%.
(Veal *et al.*, 1998)	MT-4 cells infected with HIV	*gag* antisense PS ODN + 2',3'-dideoxycytidine (ddC) or 3'-azido-2',3'-dideoxythymidine (AZT) or 2',3'-dideoxy-2',3'-didehydrothymidine (d4T)	25-mer antisense PS ODN (5'-CTCTCGCACCCATCTCTCTCCTTCT –3') complementary to the *gag* initiation site of HIV-1	*In vitro* :- Cell viability was measured by MTT assay	N/A	AODN exhibited synergism with ddC, with an approximate 5-fold decrease in ddC IC_{50} value. This interaction was not seen with the mismatch controls. Similar results were seen with the AZT and d4T in combination with the ODN.

Reference	Cell type	Combination utilised	ODN length and type	Method	Dosage details (for *in vivo*)	Comments/Effects
(Moorehead *et al.*, 2000)	Human ovarian carcinoma cell line 2008 and its cisplatin-resistant variant, C13*	*c-fos* antisense PS ODN + cisplatin	18-mer antisense PS ODN complementary to the bases –6 to +14 of *c-fos* mRNA	*In vitro*:- Western blot analysis of *c-fos* protein . Survival assays were carried out using methylene blue and colonies were counted. Cytotoxicity was assessed by DNA fluorochrome assay.	N/A	Combinations of the ODN and cisplatin reduced the amount of cisplatin required to kill 50% of C13* cells by 3-fold.
(Bilim *et al.*, 2000)	T24 cell line from bladder transitional-cell cancer.	*bcl-2* antisense PS ODN + adriamycin	20-mer antisense PS ODN (5'-GTTCTCCCAG CGTGCGCCAT -3' complementary to the AUG translation site of the *bcl-2*	*In vitro*:- Cell viability was measured by MTT assay. Western blot analysis of *bcl-2* protein.	N/A	Combined administration of antisense ODN and adriamycin resulted in increased sensitivity of adriamycin to the T24 cell line, resulting synergistic cytotoxicity.

The demonstration of additive and synergistic antitumor effects of AODN in combination with chemotherapeutic agents warrants the hope that AODNs may increase either the response rate of resistant tumors or the overall effect of chemotherapeutic agents for a variety of different cancers without increasing the toxic side-effects. Various cancers such as prostate cancers, melanomas and non-small cell lung cancers that do not respond effectively to standard chemotherapy may become susceptible when treated with cytotoxins in combination with AODNs.

2.1.5 Specific Aims for Chapter 2

It is widely accepted in the antisense community that poor pharmacokinetic / pharmacodynamic and cellular uptake properties of AODNs can be improved by the use of poly (lacyide-co-glycolide) microsphere delivery systems. Moreover, new strategies to further improve the therapeutic effects of AODNs have been the focus of many antisense studies recently (see section 2.1.4). Amongst these is the use of AODNs in combination with traditional drugs to further enhance therapeutic effects.

In this chapter it was therefore envisaged that the AODN (targeting the epidermal growth factor) in combination with 5-FU (a model cytotoxic drug) encapsulated in poly (lacyide-co-glycolide) microspheres would further improve the therapeutic efficacy of AODNs in a model vulval carcinoma cell line (A431). The aim was therefore to characterise and test the optimised delivery system in cell culture. In terms of characterisation; size, morphology, loading and release profiles would be required for AODN and 5-FU in separate or co-encapsulated microspheres. The optimised delivery system would then be used for testing purposes. In terms of testing; trypan blue dye exclusion assay, MTT assay and Western blotting assays would be undertaken to confirm the enhanced and antisense specific effects.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All chemicals used were of the highest grade available from Sigma Chemical Company (Poole, UK) unless otherwise specified. All reagents were used as received without further purification.

2.2.2 Methods

2.2.2.1 Synthesis of ODNs

Oligodeoxynucleotides were synthesised in phosphorothioate forms on an ABI 392 automated DNA/RNA synthesiser (Applied Biosystems, Warrington, U.K.) using standard phosphoramidite reagents (Cruachem Ltd, Glasgow, U.K.). The sequences used were a 21-mer antisense (5' TTT CTT TTC CTG CAG AGC CCG 3') molecular weight 6293, complementary to *c-erb B1* mRNA and a 21-mer phosphorothioate poly A ODN $[A]_{21}$. This sequence has been shown to be active against the *c-erb B1* mRNA (Petch *et al.*, 1999). The scrambled AODN sequence was (5' CTG ATC CTG CTC TGA TCC TCT 3'). The automated method of solid-phase synthesis has been developed from the technique devised by Merrifield (1963) and has become widely used in the synthesis of oligonucleotides (Brown and Brown 1991). Phosphorothioates were introduced by use of tetraethyl disulphide (TETD) as the sulphurising reagent. The addition of TETD replaced the oxidising step, which would normally be conducted for phosphodiester ODNs. Synthesised PS-ODNs with 5'-trityl groups removed were automatically cleaved from the solid support with 1.5ml of concentrated ammonium hydroxide and incubated at 55°C for 8-hrs to remove the base-protecting groups.

2.2.2.2 Purification of ODNs

After deprotection, the resulting oligodeoxynucleotide was purified through Sephadex G-25 packed (NAP-10) columns (Pharmacia Biotech, St.Albans, U.K.) using gravity separation for maximum product recovery. Briefly, the sephadex column was equilibrated with 15ml of sterile RNase-free water (elution buffer) after which 1ml of the sample was carefully added to the column allowing the sample to enter the gel bed completely. The purified sample was

eluted into a microcentrifuge tube by adding a further 1.5ml of elution buffer to the sephadex column. The purified sample was then dried by vacuum centrifugation using a Savant DNA Speed Vac (Savant,U.K.) and stored at –20°C.

2.2.2.3 Quantification of ODNs

The quantification of oligodeoxynucleotide was determined by UV spectroscopy at 260nm. The purine and pyrimidine bases of DNA strongly absorb light with maxima near 260nm. The following method converts O.D. units into milligrams based on the molecular weight of the sequence and is adapted from that of (Brown and Brown, 1991).

2.2.2.3.1 Estimation of the Molecular Weight (Mw)

Mw = (249 x nA) + (240 x nT) + (265 x nG) + (225 x nC) + (64 x n-1) +2

where:

(i) nA = number of adenine bases in the sequence and n= total number of bases.

(ii) (64 x n-1) accounts for the molecular weight of the phosphate groups

For phosphorothioates, a sulphur (Mw=32) replaces an oxygen (Mw=16) on the phosphodiester side chain. Consequently an adjustment of +16 is made for n-1 bases.

i.e. Mw = (249 x nA) + (240 x nT) + (265 x nG) + (225 x nC) + (**80** x n-1) +2

2.2.2.3.1 Calculation of Micromolar Extinction Coefficient, ε at 264nm

$\varepsilon = \{(8.8 \times nT) + (7.3 \times nC) + (11.7 \times nG) + (15.4 \times nA)\} \times 0.9^*$

* It is necessary to multiply the extinction coefficient of the sum of the individual bases by 0.9 because the base-stacking interactions in the single strand suppress the absorbance of DNA.

2.2.2.3.2 To Convert O.D. Units to Milligrams

$1mg = \varepsilon / (Mw / 1000)$ $O.D._{260}$ units

Therefore 1 $O.D._{260}$ unit = $(Mw / 1000) / \varepsilon$ milligrams

2.2.2.4 3' -End [^{32}P] - Radiolabelling of ODNs

ODNs were radiolabelled with α- [^{32}P][di-deoxy-ATP] (Amersham, Buckinghamshire, U.K.) using a calf thymus terminal transferase kit (Boehringer Mannheim, East Sussex, U.K.) containing 5 × reaction buffer (1M Cocadylate, 125mM Tris-HCl, 1.25mg/ml bovine serum albumin) and 2.5mM cobalt chloride solution.

100pmols of ODN was radiolabelled in a 50µl reaction mixture containing 25 units (1µl) of terminal transferase, 10µl of 5 × reaction buffer, 5µl cobalt chloride and 2µl of α-^{32}P[di-deoxy-ATP]. This reaction was incubated at 37°C for 90 minutes. The reaction mixture was made up to 1ml with ddH_2O, and passed through a NAP^{TM}-10 column (Pharmacia, Buckinghamshire, U.K.) for removal of cocadylate salts. The radiolabelled ODN was collected in 1.5ml of ddH_2O and dried under vacuum in a DNA speed vac. Dried ODN samples were resuspended in 25µl of loading buffer/marker dye (50mg xylene cyanole, 50mg bromophenol blue in 10ml of 10% (v/v) glycerol/1×TBE for PAGE (see section 2.2.2.5.1).

2.2.2.5 Purification of Labelled ODNs by Polyacrylamide Gel Electrophoresis

2.2.2.5.1 Polyacrylamide Gel Electrophoresis (PAGE)

Short-chain nucleic acids such as ODNs can easily be separated according to size difference by polyacrylamide gel electrophoresis. Polyacrylamide gels are formed by the vinyl polymerisation of acrylamide monomers (CH_2=CH-CO-NH_2) into long random chains of polyacrylamide which are cross-linked by the inclusion into the mixture of small amounts of a co-monomer, *N-N*'-methylene-bis-acrylamide, commonly known as "*Bis*". The resulting cross-linked chains form a gel structure, the pore size of which is determined by the concentrations of both acrylamide and *Bis*- acrylamide.

Polyacrylamide gels were prepared as described by (Sambrook, Fritsch, and Maniatis 1989) using Biorad Protean II electrophoresis apparatus (Bio-Rad). Two glass plates (20cm x 20cm) were cleaned with acetone and separated by 1mm spacers were sandwiched together and clamped vertically in the gel stand. The polymerisation reaction was activated by the addition of 0.6ml fresh ammonium persulphate (10%w/v) and 40µl of TEMED to 50ml of a stock gel mix which had previously been prepared and stored at 4°C. The polymerising mix was gently

stirred and carefully poured into the gap between the two plates and a comb inserted. ODNs were separated using 20% polyacrylamide gel solutions.

A minimum period of 30 minutes was allowed for the gel to set, the comb was then removed and the wells washed with sterile 1xTBE. The gel was transferred into a BioRad electrophoresis tank and both upper and lower reservoirs filled with 1xTBE running buffer. Samples were diluted with an equal volume of loading buffer (native: 5% glycerol in 1xTBE) and carefully loaded into the wells using round tipped micropipettes (Costar,U.K.). Marker dyes (0.25% bromophenol blue; 0.25% xylene cyanole in 1xTBE) were used to track the progress of sample migration down the gel. Gels were run at 10-20W for 2-3 hours.

2.2.2.5.2 Autoradiography

Radiolabelled samples, separated by PAGE, were detected by autoradiography. Following electrophoresis the gel was removed from the glass plates, wrapped in a single layer of Saran Wrap and placed in a Hypercassette fitted with an intensifying screen (Amersham Life Sciences, Amersham,U.K.). Under dark room conditions the gel was exposed to a sheet of Kodak HP autoradiograph film for the relevant exposure time (1 minute to 30 minutes). The film was then developed using Kodak photographic reagents and once fixed allowed to dry naturally. The appropriate bands were excised from the gel and the radiolabelled ODN eluted in sterile water and concentrated by drying under vacuum centrifugation.

2.2.2.6 Radiolabelled 5-Fluorouracil

Unlabelled and Tritiated 5-fluorouracil-6-[^{3}H] were obtained from Sigma, Paisley,UK.

2.2.2.7 Liquid Scintillation Counting

Liquid scintillation counting (LSC) was used to quantify the activity of [^{32}P] radiolabelled ODNs or 5-fluorouracil-6-[^{3}H]. Samples were added to 5ml of Optiphase Hisafe III (Pharmacia-Wallace, St Albans, U.K.) and counted for 5 minutes using a Packard 1900TR Scintillation Counter. Adjustments were automatically made for α- [^{32}P][di-deoxy-ATP] radioactive decay during the experimental period by entering the half life and reference date of the radioisotope used. For each experiment counts were compared with background values.

2.2.2.8 Microsphere Preparation

2.2.2.8.1 Polymers

Poly-D,L-co-glycolide 50:50 Mw P(LA-GA) Mw 3,000 (ref RG 502) (Boehringer Lgelheim, Germany) were supplied by Alpha Chemicals (Bracknell, U.K.). Polymers were stored in a desicator at 4°C.

2.2.2.8.2 Preparation of Double Emulsion (w/o/w) Microspheres by the Vortex Method

100µl of internal phase was prepared containing the appropriate amount of 5-FU and/or ODN and 10µl of 4% emulsifying agent polyvinylalcohol (PVA) (87-89% hydrolysed, Mw 13, 000-23, 000 kDa). This internal phase was added to 500mg of P(LA-GA) polymer dissolved in 5ml of dichloromethane (DCM) and vortexed for 5min to form a primary emulsion. The primary emulsion was added to 160ml of external phase (4% PVA, 0.9% NaCl). The emulsion was then stirred at 1000rpm for 3 hours at room temperature using a Heidolph stirrer (Lab Plant, Huddersfield, U.K.). The water in oil in water (w/o/w) emulsion produced was then stirred for a further 3h on a magnetic stirrer plate to allow for complete evaporation of the DCM.

The resulting microspheres were harvested at 4000rpm for 10min (43124-708 rotor,3000g, Mistral 3,000 centrifuge, MSE Leicester LTD), washed three times with distilled water to remove any non-encapsulated ODN and/or 5-FU and surfactant. At each washing stage the supernatants were discarded and the polymer pellet re-suspended in distilled water. The resulting microspheres were re-suspended in 1ml distilled water, frozen at -70°C and freeze-dried for 48h using an Edwards Modulo freeze dryer (BOC Ltd., Sussex, U.K.).

2.2.2.8.3 Preparation of Double Emulsion (w/o/w) Microspheres by the Silverson Method

The double-emulsion method was as described in section 2.2.2.8.2, the volumes were kept the same although a different mixing method was employed. The primary emulsion was mixed at 4000 rpm using a Silverson homogeniser STD2 with a 3/8" mini-micro probe (Silverson Machines, Chesham Bucks, U.K.) for 2 minutes. The resultant emulsion was then further mixed at 6000rpm using a 1" tubular probe (Silverson machines, Chesham, Bucks., U.K.) for 4 minutes. The w/o/w emulsion was stirred on a stirring plate for a minimum of 4 hours to

allow the solvent to evaporate and the spheres were centrifuged and freeze-dried as before (see section 2.2.2.8.2). The resulting spheres were stored in a desicator at room temperature.

2.2.2.9 Characterisation of Microspheres

2.2.2.9.1 Determination of ODN Entrapment Efficiency in Microspheres

ODN entrapment efficiency in microspheres was determined by direct liquid scintillation counting of ODN encapsulated microspheres. 25mg of microspheres were added to 10mL of Optiphase Hi-Safe 3 (Pharmacia-Wallac, St Albans, U.K.) and counted as in section 2.2.2.7. Also, 2µl of free radiolabelled ODN was assessed for counts per minute. All samples were assayed in triplicate and results are the mean of three determinations.

From these results the % encapsulation efficiency was calculated.

$$\text{Encapsulation efficiency} = \frac{\text{Total cpm of microspheres batch}}{\text{Total cpm of radiolabelled ODN}} \times 100$$

2.2.2.9.2 Determination of 5-FU Entrapment Efficiency in Microspheres

5-FU entrapment efficiency in microspheres was determined by dissolving the polymer (25mg of microspheres) in 3 M NaOH followed by liquid scintillation counting in 10mL of Optiphase Hi-Safe 3 (Pharmacia-Wallac, St Albans, U.K.). Also, 2µl of free radiolabelled 5-FU was assessed for counts per minute. All samples were assayed in triplicate and results are the mean of three determinations. The encapsulation efficiency was determined as for ODNs (section 2.2.2.9.1).

2.2.2.9.3 Release Profiles

25mg of the appropriate microspheres were dispersed in 1.5ml of phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl and 10mM phosphate buffer pH 7.4) or DMEM. The microsphere suspensions were shaken in a water bath (Grant OLS 200) at 100 strokes/min at 37°C. At various time points the samples were removed and centrifuged at 13,000rpm for 5min (320g, Sigma 112 centrifuge) to remove any suspended particles. The supernatant was removed and replaced with a further 1.5ml PBS. The amount of ODN and/or 5-FU released from the microspheres was assessed by scintillation counting (see section 2.2.2.7).

2.2.2.9.4 Scanning Electron Microscopy

Samples of microspheres were mounted on carbon adhesive stubs and were gold sputter-coated using an Emscope SC 500 Sputter coater in order to obtain a conducting specimen surface. The surface morphology was viewed using a Cambridge Instruments Steroscan 90 Scanning electron Microscope U.K. connected to a 35 mm camera (Cambridge Instruments U.K.).

2.2.2.9.5 Microsphere Particle-Size Determination

Approximately 10mg of microspheres were resuspended in 10ml of filtered ddH_2O (using a 0.2μM Millipore filter). This was injected into a Malvern Mastersizer E particle-sizer (Malvern Instruments, Malvern, U.K.). the instrument was fitted with a 45mm angle lens and a flow cell and the presentation was for polystyrene in water (2PAD).

2.2.2.10 Gel Electrophoretic Mobility Shift Assay

Samples of the 5'-end labelled ODN (*c-erb B1* AODN and poly A) were mixed with increasing concentrations of 5-FU. Samples were heated to 95°C and cooled slowly to room temperature. An equal volume of gel loading buffer (5% glycerol, 1xTBE and 0.25% bromophenol blue) was added to each of the samples. The samples were then separated on a 20% native polyacrylamide gel. The bands were visualised by autoradiography as before (see section 2.2.2.5.2).

2.2.2.11 Cell Culture

All cell culture procedures were undertaken under aseptic conditions in a Gelaire, biohazard level II laminar flow cabinet from ICN (Thame, Oxfordshire, U.K.).

2.2.2.11.1 Cell Lines

A431 cells were a generous gift from Dr P.L. Nicklin, Ciba-Gigy Pharmaceuticals (Horsham, U.K.). This cell line is derived from a vulval carcinoma (Freshney 1973) and expresses the EGFR at levels 10 to 50-fold higher than seen in other cell lines (Ullrich *et al.*, 1984). U87-MG cell line was purchased from the European Collection of Animal Cell Cultures (ECACC),

(Porton Down, U.K.). These human glioblastoma astrocytoma cells were originally derived from a grade 3 malignant glioma by explant technique (Ponten *et al.*, 1968).

2.2.2.11.2 Culture Media

The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% v/v mycoplasma screened foetal bovine serum (FBS), 1% penicillin/streptomycin and 1mM L-glutamine (all supplied from Gibco, Paisley, U.K.). The same medium, without the addition of the foetal bovine serum, was used in the cell viability studies.

2.2.2.11.3 Stock Cultures

Cells were cultured in 75cm^2 plastic tissue culture vented cap T-flasks, with a non-wetting 0.2μm hydrophobic micro-porous membrane vent (Costar, U.K.), using 25ml of the respective media. The cultures were incubated at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air.

Stock cultures were maintained by changing the media every 48-hrs and passaged when confluent (after approximately 3-4 days depending on cell line). Passaging was carried out using the following procedure:

The medium was removed and the cells washed with 10ml of 0.25% v/v phosphate-buffered saline solution (PBS) (Sigma, Poole, U.K.) in double distilled water. After aspiration of the PBS, 5ml of 1x Trypsin /EDTA solution (0.25% w/v trypsin, 0.2% w/v disodium ethylenediamine tetraacetate in PBS pH 7.2) was added to the monolayers. After one minute, the trypsin /EDTA solution was removed by aspiration and the cells incubated at 37°C for 5 minutes. The flasks were tapped to dislodge the cell monolayer, examined by phase microscopy and re-incubated if necessary until detached from the flask. Fresh medium was added to inactivate the trypsin and cells were pipetted to give a single- cell suspension. For stock cultures the cells were split at a ratio of 1:5 and media was added to a final volume of 25ml. If the cells were to be used in a specific experiment, they were counted by haemocytometry and transferred at the required cell number to the appropriate culture vessel.

2.2.2.11.4 Determination of Cell Number / Viability

The viable cell density of stock cultures was measured by haemocytometry using a trypan blue exclusion test. 100µl of trypan blue (4mg ml^{-1}) was mixed with 400µl of cell suspension (1:1.25 dilution) and incubated at room temperature for 5 minutes. A small amount of the trypan blue cell suspension was then transferred to the counting chamber of a Neubauer haemocytometer (Weber Scientific International Ltd, U.K.). The cells were counted in five large (1mm) squares of the haemocytometer using a light microscope and a mean count per square obtained. Live cells exclude the trypan blue dye and appear with clear cytoplasm, whereas non-viable cells are unable to exclude the stain and appear dark blue. Consequently the number of viable (unstained) cells can be determined. The cell density was calculated using the following equation:

Viable cells per ml = average number of viable cells per square x 10^4 x 1.25 (dilution factor of trypan blue)

% Cell Viability = total viable cells (unstained) / total cells (stained and unstained) x100.

Cells suspensions with a percentage cell viability of < 95% were disregarded.

2.2.2.11.5 Freezing / Thawing of Cell Lines

For long-term storage, frozen stock cultures were prepared in the following manner: Semi-confluent stock cultures were trypsinised as described previously (section 2.2.2.10.3) and neutralised with the addition of 10ml of DMEM medium. The cell suspension was then transferred to a 15ml universal tube (Falcon, U.K.) and centrifuged for 3 minutes at 350 revolutions per minute (Mistral 3000 I centrifuge, Sanyo MSE, Leicester, U.K.). The supernatant was decanted and the cell pellet was re-suspended in 1ml of 'freezing medium' (10% DMSO, 90% heat-inactivated foetal calf serum) and transferred to a 2ml screw capped cryovial (Costar, U.K.). The ampoule was then placed in a –70°C freezer for 4-6 hours before being transferred into a liquid nitrogen (-196°C) cell bank. When required, the cells were recovered by rapid thawing at 37°C and gradual dilution with DMEM medium before seeding in 25cm^2 flasks (Costar, U.K.).

2.2.2.11.6 Cell Viable Studies

A431 cells were seeded in DMEM containing 10% FBS into 24-well plates ($2cm^2$) at 1.25 x 10^4 cells/well or 5.0 x 10^4 cells/well for 72-hrs or 24-hrs treatment times respectively. Following washes with serum free DMEM medium (2 x 0.5ml x 5min), naked AODN/5-FU or microspheres loaded with either AODN/5-FU were added (separately or in combination) in serum free culture medium 24-hrs after seeding. The total volume added per well was 500µl. After incubating over various time periods, cells were washed three times with PBS (2 x 0.5ml x 5min), trypsinised and the number of viable cells counted by trypan blue exclusion assay (section 2.2.2.10.4). The morphology of cells after treatment was also examined by light microscopy and compared to untreated control cells.

2.2.2.11.7 Cell Association Studies

Cells were cultured on plastic 24-well plates (Falcon, U.K.). Confluent stock cultures were trypsinised and counted as described in section 2.2.2.10.4 and the cell density of the stock suspension diluted to 5 x 10^4 cells ml^{-1} with DMEM medium. Each well was seeded with 1ml of the diluted cell suspension to give a final concentration of $5x10^4$ $well^{-1}$. The plates were incubated at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air. After approximately 48-hrs, the cell monolayers had reached 80-90% confluency and were then ready for cell association experiments.

Initially the medium was removed and the monolayer carefully washed twice with PBS (2 x 1ml x 5min) to remove any traces of serum. The washing solution was aspirated and cells treated with 500µl of DMEM containing 2µM AODN and 0.19µM 5-FU with 2pmoles of tritium-labelled 5-FU incubated for 2-hrs. Cellular association of 5-FU was then measured over 4-hrs. Both PBS and serum free medium were equilibrated at 37°C for 1-hr prior to use. The plates were incubated at 37°C, unless otherwise stated, for the duration of the experiment. Once incubated for the desired period of time, the apical media was carefully collected and their radioactive content assessed by liquid scintillation counting. The cells were then washed 3 times (3 x 0.5ml x 5min) with ice cold PBS-azide to inhibit any further cellular metabolism and remove any 5-FU loosely associated with the cell surface. The washings were collected and the radioactive content determined by scintillation counting.

Cell monolayers were solubilised by shaking with 0.5ml of 3% v/v Triton X100 (Aldrich Chemical Company, Gillingham, U.K.) in distilled water for 1-hr at room temperature. The wells were washed twice more (2 x 0.5ml) with Triton X-100 to ensure that all the cells had been harvested and the radioactivity content of the cellular fraction determined by scintillation counting.

For all experiments three extra wells were seeded with cells and the viable cell density was determined by a trypan blue exclusion assay (section 2.2.2.10.4), so that cell association could be normalised to cell number when required.

2.2.2.11.8 MTT Assay

The MTT assay measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide by the mitochondrial dehydrogenase of viable cells. The amount of formazan produced (maximum absorbance at 562nM) is proportional to the number of living cells present in culture (Mossmann 1993). For an MTT assay triplicate wells were seeded with 1.25 $x10^4$ cells/well for 24-hrs after which the cells were treated with AODN and/or 5-FU as before in DMEM (see section 2.2.2.10.4). After a 72-hrs incubation period each well was incubated with 1ml of 5µg/ml MTT for 4-hrs after which the crystals formed were solubilised overnight with 1ml of 10% SDS/0.01M HCl solution. The absorbance of the solution was measured at 562nM.

2.2.2.12 Protein Analysis

2.2.2.12.1 Protein Sample Preparation

Cell samples were prepared under conditions that ensure the denaturing of proteins with minimal aggregation. This is achieved by using the strongly anionic detergent SDS in combination with a reducing agent and heat to dissociate the proteins. The denatured polypeptides bind SDS and become negatively charged enabling separation through gel electrophoresis according to size (Sambrook, Fritsch, and Maniatis 1989).

Cells were trypsinised after washing with PBS (2 x 0.5ml x 5min), (section 2.2.2.10.7) and pelleted by centrifuging at 1000rpm for 5 minutes at 4°C. The cells were re-suspended in 1ml

of PBS, centrifuged at 1000rpm for 5 minutes at 4°C and washed in this manner twice more with PBS. The cells were then lysed by adding 100µl of lysis buffer (0.5M Tris-HCl, pH 6.8; 10% glycerol; 10% SDS; 0.1mM leupeptin and 0.1mM PMSF) for every 1 x 10^6 cells. The cells were vortexed and sonicated on ice until evenly mixed and then centrifuged at 14000rpm for 30 minutes at 4°C. The supernatant was stored at –70°C until required for analysis.

2.2.2.12.2 Protein Determination

Protein content of whole cell lysates was determined by means of a Bio-Rad protein assay. This assay is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs.

Calibration curve was produced using various concentrations of BSA (0µg/ml - 25µg/ml). Whole cell lysate samples of unknown protein concentration were prepared in duplicate by diluting 2µl of the sample in 798µl of distilled water to give a total volume for both standards and samples of 800µl. The assay was initiated by adding 200µl of dye reagent concentrate to each tube of standard and unknown and mixing well before incubating at room temperature for 15 minutes. Absorbance at 595nm was then read using a spectrophotometer (Jenway, U.K.). Calibration curve was constructed by plotting net absorbance vs. the concentration (µg/ml) of the protein standards at 595nm. The calibration curve was then used to determine the amount of protein in the unknown cell lysate samples.

2.2.2.12.3 SDS Polyacrylamide Gel Electrophoresis

All protein electrophoresis was carried out using Bio-Rad Mini-Protean II apparatus (Bio-Rad, California, USA). Two glass plates (7cm x 8cm) were clamped together with spacers (1.5 mm thick) at the sides to form a gel sandwich which was placed in the gel stand and clamped to form a seal at the base of the plates. A 13% polyacrylamide separating gel was prepared [3.2ml 30% acrylamide, 1.9ml resolving buffer (1.5M Tris-HCl, 0.4% SDS pH 8.8), 2.4ml water, 75µl 10% SDS, 7.5µl TEMED, 15µl 10% ammonium persulphate (freshly made)] and poured into the gel sandwich. The gel was overlaid with 1ml of n-butanol and allowed to polymerise for 45 minutes. The n-butanol overlay was poured off and the area

above the separating gel was then dried with filter paper. A 6% stacking gel was prepared [1.25ml 30% acrylamide, 1.56ml stacking buffer (0.5M Tris-HCl, 0.4% SDS pH 6.8), 3.37 ml water, 62.5µl 10% SDS, 6.25µl TEMED, 62.5µl 10% ammonium persulphate (freshly made)] poured on top of the separating gel and a comb inserted between the plates. After the stacking gel had set (after approximately 45 minutes) the comb was removed and the wells rinsed thoroughly with distilled water. The gels were assembled in the electrophoresis tank and both upper and lower reservoirs filled with running buffer (0.3% Tris.HCl; 1.44% glycine; 0.1% SDS).

Protein samples, previously lysed as described in section 2.2.2.11.1, were heated at 90°C for 5 minutes prior to loading. Samples were then loaded into the wells using round ended micropippette tips (Costar, U.K.). In addition to the working samples, a sample of high molecular weight markers of known molecular mass (205 kDa – 45 kDa) (Sigma, Poole, U.K.) was also loaded into one well of each gel. The coloured bands could be visualised on the gel and after transfer of proteins onto nitrocellulose filter, thus allowing comparisons with sample proteins of interest. Gels were run at 100V for about 90 minutes or until the bromophenol blue dye had run off the end of the gel.

2.2.2.12.4 Protein Transfer

Electrophoretic transfer of the proteins on the gel onto the blotting membrane was performed using a Bio-Rad Trans-Blot electrophoretic transfer cell. Following electrophoresis, the stacking gel was carefully removed and the resolving gel was washed twice with distilled water before being equilibrated in transfer buffer (25mM Tris.HCl, 192mM glycine and 20% w/v methanol) for 10 – 15 minutes prior to blotting. In addition, the Hybond-ECL membrane (Amersham, U.K.) and six sheets of filter paper (Whatmann, 3MM) were cut to exactly the same size as the gel and soaked in the transfer buffer for 10-15 minutes. A blotting sandwich was assembled, consisting of three filter papers, nitrocellulose membrane, gel and three further filter papers, and placed in the apparatus of the transfer tank. At each stage of assembly, care was taken to remove any air bubbles by rolling over the surface with a glass pipette. The tank was filled with transfer buffer so that the sandwich was completely submerged and transfer was performed at either 30mV overnight or at 100mV for 1hr 15 minutes. After this the clamps were disassembled and the gels discarded. A ponceau (0.1%

ponceau in 1% acetic acid) stain followed by a destain (1% acetic acid in water) allowed the protein on the nitrocellulose to be visualised to check equal protein loadings.

2.2.2.12.5 Blotting

A commercial ECL immunodetection kit was used to analyse the immunoblots obtained. This is a light-emitting method for detection of a specific antigen, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies (Amersham, Buckinghamshire, U.K.). A primary antibody raised against the protein being studied is added to the membrane followed by a horseradish peroxidase (HRP) conjugated secondary antibody which is targeted to the primary antibody. ECL detection is initiated when detection reagents are added to the membrane and HRP catalyses the oxidation of luminol in the presence of a chemical enhancer (phenol). The light produced by this oxidation (maximum at 428nm) is detected by exposure to blue-light sensitive autoradiography film.

The nitrocellulose obtained from section 2.2.2.11.4 was then blocked with 100mls of 5% milk powder in TBS/Tween (10mM Tris-HCl, 150mM NaCl, 0.2% Tween-20) overnight. The next morning the nitrocellulose was incubated for 3-hrs on a slow spinning wheel at room temperature with 8mls of 1% milk powder in TBS/Tween containing an appropriate dilution of the respective primary antibody in a sealed bag. EGFR antibody (3138) (raised in mouse) (Amersham, Buckinghamshire, U.K.) was used as a 1:1000 dilution i.e. 5µls in 5mls of 1% marvel. After the 3-hrs incubation the nitrocellulose was washed twice in TBS/Tween for 5 minutes each, followed by a wash in 0.5M NaCl for 15 minutes, followed by another wash in TBS/Tween for 10 minutes. The blot was then transferred to 20ml 1% milk powder in TBS/Tween containing 1:2500 dilution of horse peroxidase-conjugated secondary antibody (raised in mouse or rabbit) (Amersham, Buckinghamshire, U.K.), for 1-hr. Washes were repeated as above then sealed in a bag with one end opened.

2.2.2.12.6 ECL

Following the washings the blot was taken into a Dark Room where immunodetection was carried out using ECL reagents (Amersham, U.K.). Firstly, equal volumes of ECL detection solutions 1 and 2 were mixed together (final volume being 0.125 ml / cm^2) and added to the bag containing the blot and incubated for one minute at room temperature without agitation.

The excess reagent was drained out and exposed to a sheet of autoradiography film (Hyperfilm-ECL) for 20 minutes. The film was placed in developer until bands appeared, rinsed in water and then fixed and allowed to dry naturally. Repeat exposures were undertaken to achieve an optimal image. The resulting autoradiographs were then analysed by scanning densitometry as described in section 2.2.2.13.

2.2.2.13 Densitometric Analysis of Autoradiograph Images

Autoradiographs were scanned using a jet scanner (Hewlett Packard ScanJet 5100C) and were saved as TIFF files. The images were then analysed using scanning densitometry. The programme Scion image was used to plot and quantify the relevant image intensities of band patterns shown on the autoradiographs.

2.2.2.14 Statistics

Unless otherwise stated, the determination of significant differences between mean values of data obtained from experimental populations was undertaken by an unpaired students t-test. The Microsoft Excel 5.0a package was used (Fame Software Library, Fame Computers,USA). Data sets were assumed to be significantly different when the two-sided P values were calculated below 0.05. Unpaired t-test assumed that data were randomly sampled, that each value was obtained independently of the others and the populations were scattered according to a Gaussian distribution.

2.3 RESULTS AND DISCUSSION

2.3.1 Formulating Microspheres for AODN Delivery

In an attempt to use P(LA-GA) microspheres for the co-delivery of AODNs and 5-FU for potential combination therapy in cancer, the *in vitro* release profiles of each drug were examined, either individually encapsulated in separate microsphere formulations or from a single formulation co-entrapping both agents. Although P(LA-GA) microsphere formulations allow entrapment of therapeutic levels of drugs (Ciftci *et al.*, 1996; Hagiwara *et al.*, 1996), in this section the aim was to establish proof-of-concept and additionally to minimise costs only very low doses of the two radiolabelled drugs (in the pmolar range; see figure legends for details) were entrapped. However, effective therapeutic doses were also encapsulated in P(LA-GA) microspheres to test the delivery systems on cells (see section 2.3.2.3). As a first step in this evaluation, it was considered necessary to examine the microsphere morphology, size and encapsulation efficiency as well to examine the release profile of each drug individually from separate microsphere formulations. P(LA-GA) microspheres were prepared by the double emulsion (w/o/w) method using the vortex and the silverson protocol (see section 2.2.2.8.2 and 2.2.2.8.3) to compare the formulation characteristics of the two different microsphere sizes produced.

2.3.1.1 Formulating Microspheres using the Vortex Method

2.3.1.1.1 Formulation Characteristics of Separate ODN and 5-FU Loaded Microspheres by the Double Emulsion (w/o/w) Method

The microspheres loaded with each drug exhibited smooth surface morphology (figure 2.3). Mean sizes of 18.4μm and 16.8μm were observed for AODN and 5-FU loaded microspherees when each drug was entrapped in a separate microsphere formulation (figure 2.4) and the mean encapsulation efficiency was 68%±7 and 70%±6 for AODN and 5-FU respectively (table 2.3).

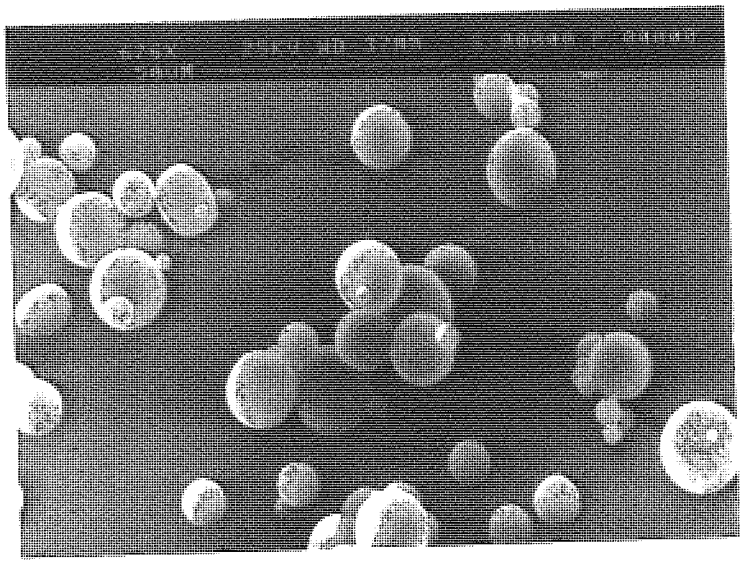
Table 2.3 Formulation Characteristics of Separate ODN and 5-FU Loaded P(LA-GA) Microspheres by the Vortex Method.

Drug Loaded	Mean Particle Size (μm)	% Mean Encap. Efficiency (n=3±SD)	Loading (pmoles/mg)
AODN	18.4	68±7	40
5-FU	16.8	70±6	30

2.3.1.1.2 In-vitro Release Studies of AODN and 5-FU from Separate Microsphere Formulations

The release profiles of AODN and 5-FU from separate P(LA-GA) microsphere formulations are shown in figure 2.5. Release appears biphasic for both drugs which is characterised by an initial 'burst effect' (phase 1) followed by sustained release (phase 2). Phase 1 is usually attributed to the drug being present at or near the surface of the microspheres whereas the slower second phase of release represents the movement of drug which is entrapped deeper in the polymer matrix. Similar release profiles have been reported for AODNs previously (Lewis *et al.*, 1998). The comparative release profiles for the two agents from separate formulations suggest that the burst effect is similar for both drugs e.g. after the first hour time point, 10% of the 5-FU entrapped was released compared to 7% for the 21-mer anti-EGFR AODN (table 2.5). These data suggest that despite the major differences in molecular weight between 5-FU (130) and the AODN (6300) entrapment and distribution of drugs within the microsphere matrices appear similar. However, consistent with molecular weight differences the subsequent release profile highlights the more rapid (~2-fold) release of 5-FU compared to AODN. For example, the release rate between days 2 and 7 for 5-FU (3.2% day $^{-1}$) was greater than for AODN (1.6% day $^{-1}$) reflecting the easier movement of the lower molecular weight of 5-FU molecule through the polymer matrix as compared to the larger AODN (table 2.5). After 7 days about 35% of the entrapped 5-FU and 18% of the AODN was released but the release rates tend to tail off in the subsequent "slower" phases for both agents. Indeed, after 35 days about 80% of the entrapped 5-FU was released compared to only about 40% for the AODN. This showed that it takes approximately a further 28 days to double the average cumulative % release of each agent. This slowing of the release rate was observed with both agents and probably represents the difficulty of progressively fewer drug particles having progressively less access to matrix pore networks that allow efflux from the microsphere.

A



B

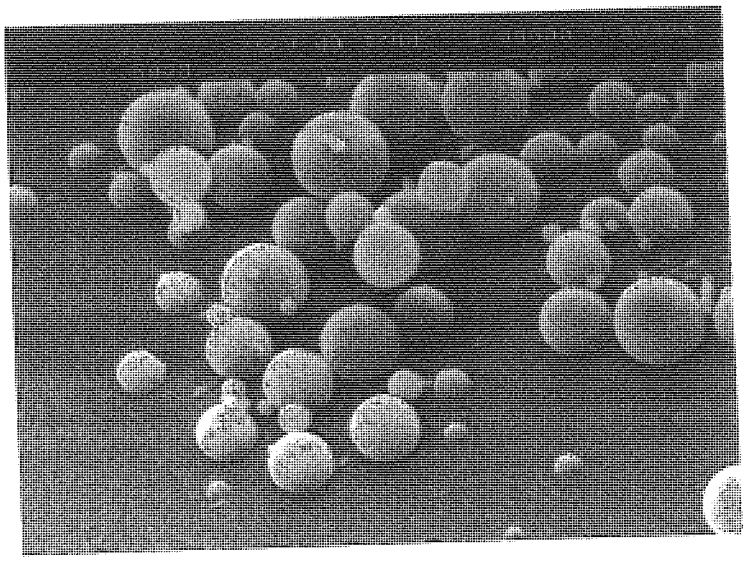
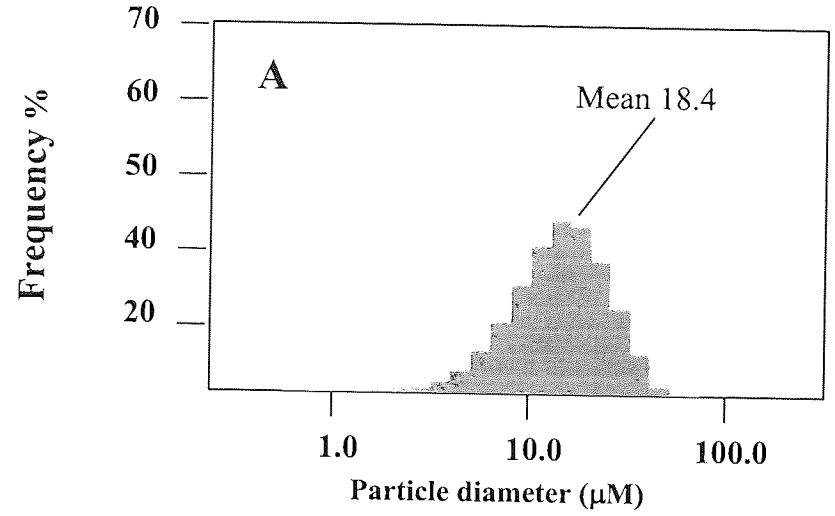


Figure 2.3 Scanning electron micrograph showing the morphology of (A) 5-FU loaded and (B) AODN P(LA-GA) copolymer microspheres prepared by the vortex, double-emulsion method (see methods section).



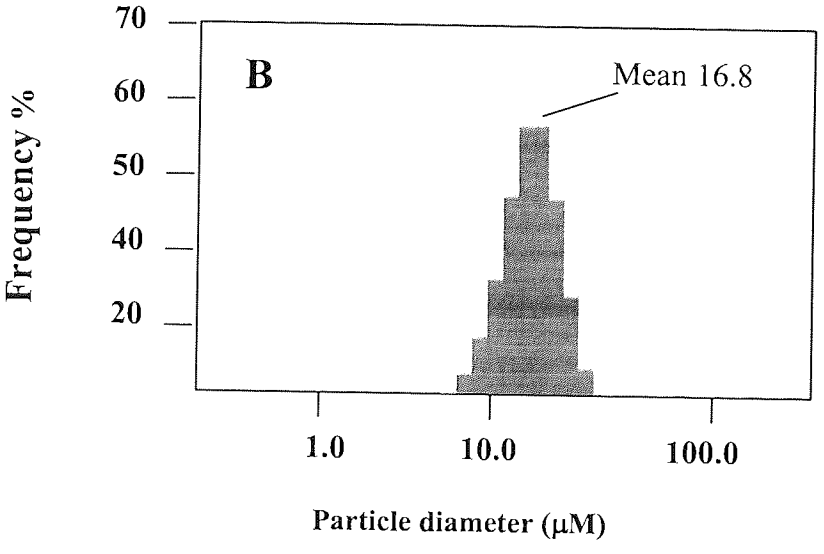


Figure 2.4 Particle-size distribution of (A) AODN and (B) 5-FU loaded microspheres prepared by the vortex, double-emulsion method. The % frequency versus particle diameter (log scale) distribution profile is given.

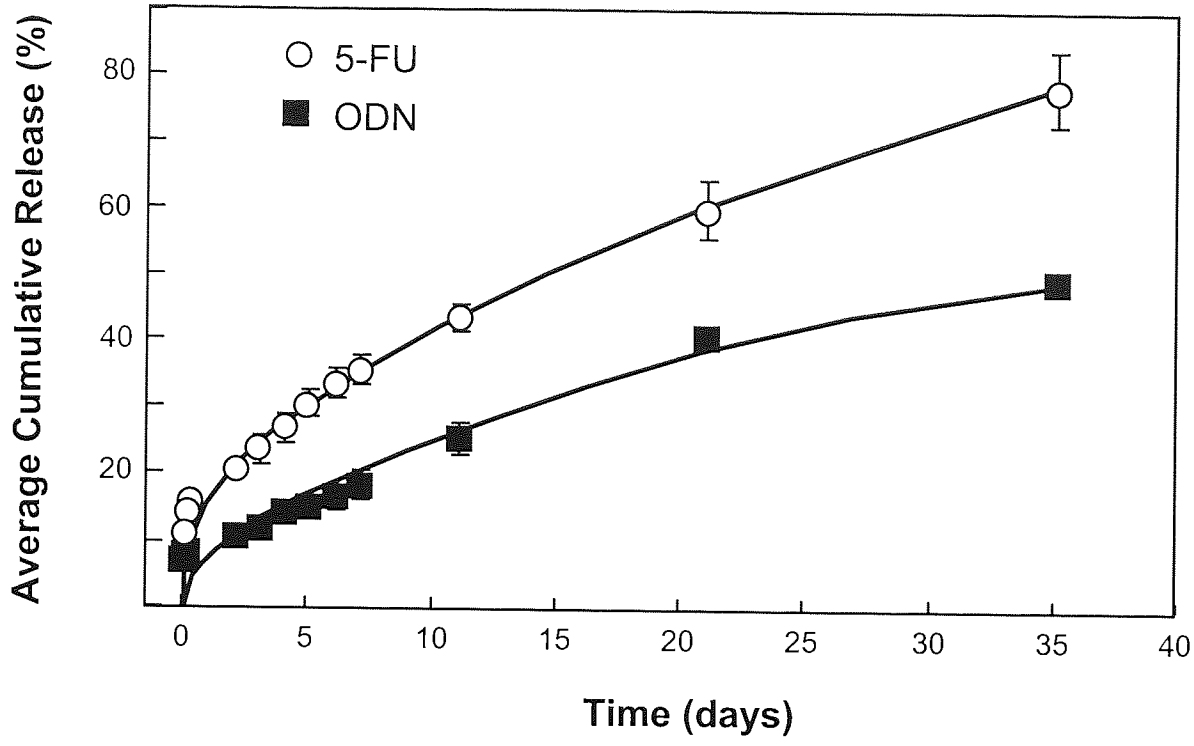


Figure 2.5 *In vitro* release profile of AODN (Loading ~ 40pmoles/mg polymer) and 5-FU (Loading ~30pmoles/mg polymer) from individual microsphere formulations (18.4μm and 16.8μm sizes for AODN and 5-FU respectively). Data points represent percentage average cumulative release. Data represents the mean n=4±SD.

2.3.1.1.3 In vitro Release Studies and Formulation Characteristics of AODN and 5-FU Loaded in a Single Microsphere Formulation by the Double-Emulsion (w/o/w) Method

Similar microsphere sizes were produced with co-encapsulation formulations as compared to separate formulations (17.8μm and 15.6μm for AODN and 5-FU respectively) suggesting that microsphere characteristics (at the doses of drug used) were not adversely affected when both drugs were co-entrapped in a single formulation compared to having them in separate formulations. This was further supported by the fact that entrapment efficiencies of the two agents were similar (~70%) in co-entrapped and individual microsphere formulations (table 2.4)

Table 2.4 Formulation Characteristics of ODN and 5-FU Loaded in a Single P(LA-GA) Microsphere Formulation by the Vortex Method.

Drug Loaded	Encapsulation Efficiency (%)	Loading (pmoles/mg)
AODN	69±6	42
5-FU	74±5	33

Figure 2.6 shows the release profiles of both AODNs and 5-FU from a single microsphere formulation. Indeed for comparative purposes, the drug loadings and microsphere particle size used in the co-entrapped single formulation (figure 2.6) were selected to be similar to those used in the separate formulations given in figure 2.5.

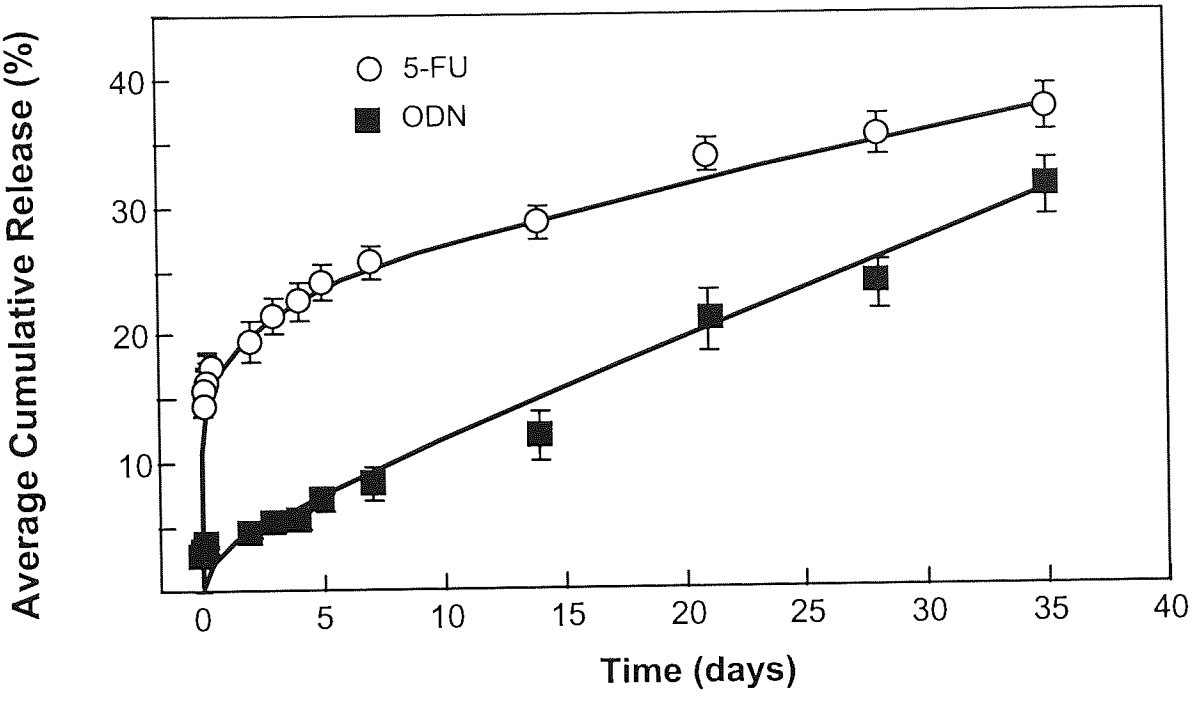


Figure 2.6 *In vitro* release profiles of 5-FU and AODNs from a microsphere formulation containing both agents. Mean particle diameter and drug loadings were similar for both agents to those used in Figure 2.5; (AODNs : 42pmoles/mg polymer and 5-FU : 33pmoles/mg polymer). Data points represent percentage average cumulative release. Data represents the mean n=4±SD.

The release profiles for the co-entrapped formulation appear to have different shapes than those observed for the separate formulations (compare figure 2.5 and 2.6). Firstly, the burst effect for AODNs from the co-entrapped microspheres was reduced (~ 3-fold) from about 7% to 2.5% whereas for 5-FU the burst effect increased slightly (~1.5-fold) from 10% to 15%. In addition the amount, and thus the overall average rate of drug released over 35 days for both agents was reduced. Only about 35% of the entrapped 5-FU was released after 35 days compared to about 80% for the separate formulation; a change of over 55%. Similarly only about 30% of the AODN was released from the co-entrapped formulation compared to over 40% from the separate formulation; a change of about 30% from the separate formulation. These data suggest that efflux of 5-FU was affected to a greater extent when co-entrapped

with ODN than was the case for the 21-mer AODN. It is possible that these markedly reduced release rates for both agents arise due to differences in drug distribution within the co-entrapped versus separate microsphere formulations. To some extent this is suggested by the observed differences in the burst effects of the two drugs in each of the formulations. Alternatively (and/or additionally), these differences, especially noting the marked change in the 5-FU release profile, may be due to an interaction with the AODN. It is possible that since 5-FU is a thymine analogue, it may hydrogen bond with purine residues (*e.g.* adenine) within the 21-mer anti-EGFR ODN. A potential hydrogen-bonding interaction is depicted in figure 2.7. To investigate this hypothesis further electrophoretic mobility shift assays (EMSA) were performed.

Table 2.5 A summary of Percent average Cumulative Release and Release Rates of Drugs from Separate and Co-entrapped Microsphere Formulations (n=4±S.D.).

Microsphere Formulation	**Drug**	**% Average Cumulative Release**			**Release Rate from day 2 to day 7 expressed as % release/day**
		1 h (burst)	**7 days**	**35 days**	
Separate	ODN	7 ± 0.4	18 ± 2.3	41 ± 1.7	1.6± 0.2
	5-FU	10 ± 0.8	36 ± 2.3	77 ± 4.3	3.2± 0.3
Co-entrapped	ODN	2.5 ± 0.1	8 ± 1.4	30 ± 2.4	0.8± 0.1
	5-FU	15 ± 1.1	26 ± 1.3	34 ± 1.3	1.2± 0.2

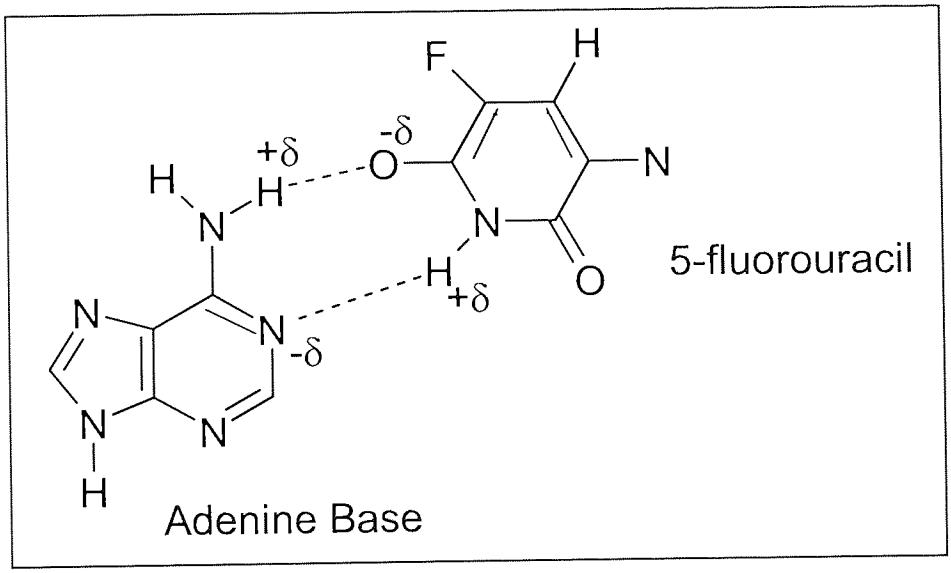


Figure 2.7 Putative hydrogen bonding interaction between 5-FU and the adenine residues in the AODN.

2.3.1.1.4 Gel Mobility Shift Assay for Detecting an Interaction

Previous studies have used electrophoretic mobility shift assay (EMSA) to detect single nucleotide changes in ODN sequence as well as modest conformation changes in ODNs of the same length as such differences resulted in altered migration of the ODN in polyacrylamide gels (Coulson *et al.*, 1996). Thus, it was postulated that if enough of the 5-FU molecules were hydrogen bonding to the AODN then one should be able to detect a change in the migration of the AODN. Figure 2.8 shows the 5'-end [^{32}P]-radiolabelled 21-mer anti-EGFR ODN incubated with increasing concentrations of unlabelled 5-FU and the samples run on a non-denaturing (native) polyacrylamide gel (see section 2.2.2.10). Lane 4 shows a modest, but reproducible, retardation in the migration of the radiolabelled ODN upon incubation with highest 5-FU concentration used in the study, when compared to the control ODN in Lane 1. This modest effect is to be expected as there are only two adenine residues in the 21-mer anti-EGFR ODN (see section 2.2.2.1) to which the 5-FU can potentially hydrogen bond. So the predicted complex would only have slightly higher Mw (increase of only 260 daltons corresponding to 2 molecules of 5-FU) compared to the free AODN.

As a further test of the hypothesis a 21-mer poly (A) ODN was prepared to see if this resulted in a more pronounced gel shift upon incubation with 5-FU. Figure 2.8 shows a more noticeable gel shift when poly (A) ODN is incubated with increasing concentrations of 5-FU. For example compare Lane 4, where the poly (A) ODN is incubated with 1.6 pmoles of 5-FU, with the migration of the control ODN shown both in Lanes 1 and 5. Taken together these results suggest that 5-FU can hydrogen bond with adenine residues in ODNs and that this drug-drug interaction may contribute to the slower release profiles of the two agents from the co-entrapped P(LA-GA) microsphere formulation.

2.3.1.1.5 In vitro Release Profiles of AODNs and 5-FU from Different Mixtures of Single Formulations

To evaluate the merits of utilising a single P(LA-GA) microsphere containing both agents over separate microsphere formulations of each agent, *in vitro* release profiles of AODNs and 5-FU from different mixtures of single formulations was carried out. Different microsphere masses containing either 5-FU (at a loading of 0.5pmoles/mg polymer) or ODN (at a loading of 1.0pmoles/mg) were mixed in varying ratios and their release profiles investigated (figure 2.9). Since the drug loadings used in this study were lower than used in figure 2.5, the overall

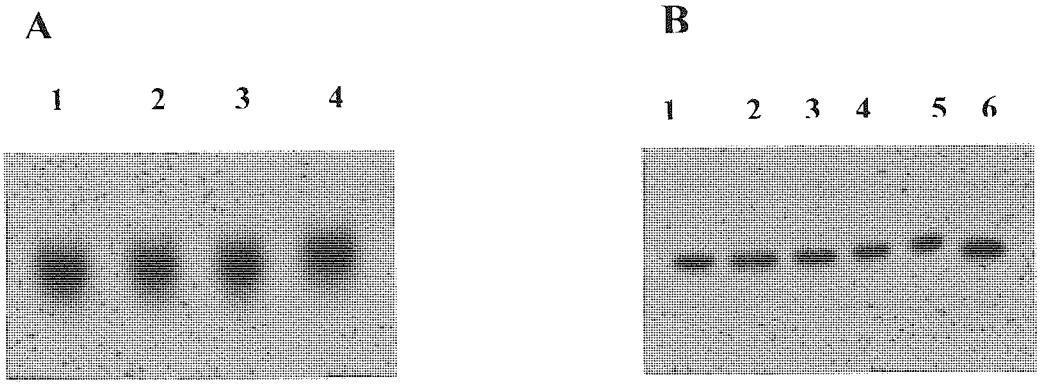


Figure 2.8 Electrophoretic mobility shift assays for detecting a potential interaction between 5-FU and 5-end [γ-^{32}P] labelled AODN or Poly (A) ODN. **Panel A**: Mobility shift of trace amounts of radiolabelled 21-mer anti-EGFR AODN when combined with increasing concentrations of 5-FU (see methods). Lane 1= control (AODN alone); Lanes 2-4 represent incubation of AODN with increasing amounts of unlabelled 5-FU at 0.4, 0.8 and 1.6 pmoles respectively. **Panel B**: Mobility shift of trace amounts of radiolabelled 21-mer Poly (A) (1.3 pmoles) when combined with increasing concentrations of 5-FU. Lanes 1 and 6 represent migration of the control Poly (A) ODN whereas Lanes 2-5 represent incubation of AODN with increasing amounts of unlabelled 5-FU at 0.4, 0.8 and 1.6 pmoles respectively and lane 6 representing the same concentration as lane 4 to confirm gel shift is not due to running of the gel (control).

amount of drug released was also lower for both agents. This is consistent with our previous reports where drug release from polymer matrices was proportional to amount of ODN entrapped (Lewis *et al.*, 1995; Khan *et al.*, 2000). The purpose of this study, however, was to show that by changing the mass ratio of microspheres containing each drug at any fixed loading, one could easily modify the release rate of AODNs or 5-FU. Using 30mg of each microsphere formulation as a standard, figure 2.9A shows that AODN release was faster than 5-FU. This is in contrast to that seen in figures 2.5 and 2.6 but is clearly due to the two-fold higher AODN loading in the microspheres relative to 5-FU. Thus, by using an equivalent mass ratio of microspheres containing twice the drug loading for AODN compared to 5-FU, one can essentially reverse the influence of molecular weight on release rates. Considering the wide difference in molecular weight of AODN (~6300 Da) and 5-FU (~130 Da), it further suggests that drug loading may be a greater influence than molar mass of drugs on release rates from P(LA-GA) microsphere formulations. Figure 2.9B shows that on increasing the mass of microspheres containing AODNs from 30 to 70mg and fixing the 5-FU microsphere mass at 30mg a proportional increase in the amount of ODN released at a given time point

was observed. For example, at day 21 average cumulative release of AODN increased from 19 pmoles to 45 pmoles, an approximate 2-fold increase. Conversely, increasing the mass of microsphere containing 5-FU to 70mg and keeping AODN microsphere mass fixed at 30mg in the release experiment yielded an increase in the amount of 5-FU released at day 21 from 11 to 20 pmoles, an approximate 2-fold increase (Figure 2.9C). Furthermore, this particular mass ratio of the two formulations as presented produced a final formulation in which the release profiles of 5-FU and AODN were similar. Thus, if a matched (similar) release profile of the two agents was required for a particular *in vivo* condition this mass combination of the separate microsphere formulations would be appropriate. These data suggest that drug release can be tailored by simply altering drug loading and mass ratio of the separate microsphere formulations of the two (or more) drugs. Of course, the exact release profiles necessary *in vivo* will be dependent on many factors including the nature and condition of the disease state, the biological half-life of the drugs being delivered and the half-life of the antisense-targeted gene-product. Such variables need consideration in the selection of the 'correct' formulation mix. Taking these results into consideration it was therefore decided that separate microsphere formulations containing each drug would be made for subsequent formulations as well as testing these delivery systems on cells.

2.3.1.2 Formulating Microspheres using the Silverson Method

2.3.1.2.1 Formulation Characteristics of ODN and 5-FU Loaded Microspheres by the Double Emulsion (w/o/w) Method

The microspheres loaded with each drug exhibited smooth surface morphology (figure 2.10). Mean sizes were 4.63μm and 3.93μm for microspheres loaded with AODN and 5-FU respectively, when each drug was entrapped in a separate microsphere formulation (figure 2.11) and the encapsulation efficiency was 53% and 40% for AODN and 5-FU respectively (table 2.6). The smaller microsphere sizes produced were a result of higher stirring rates in the primary and secondary emulsion. A more constant speed of the silverson method (see section 2.2.2.8.3) produced a narrower size distribution compared to the vortex method (see figure 2.11). The smaller encapsulation efficiencies observed with smaller microspheres were consistent with previous studies (Akhtar *et al.*, 1997; Lewis *et al.*, 1998).

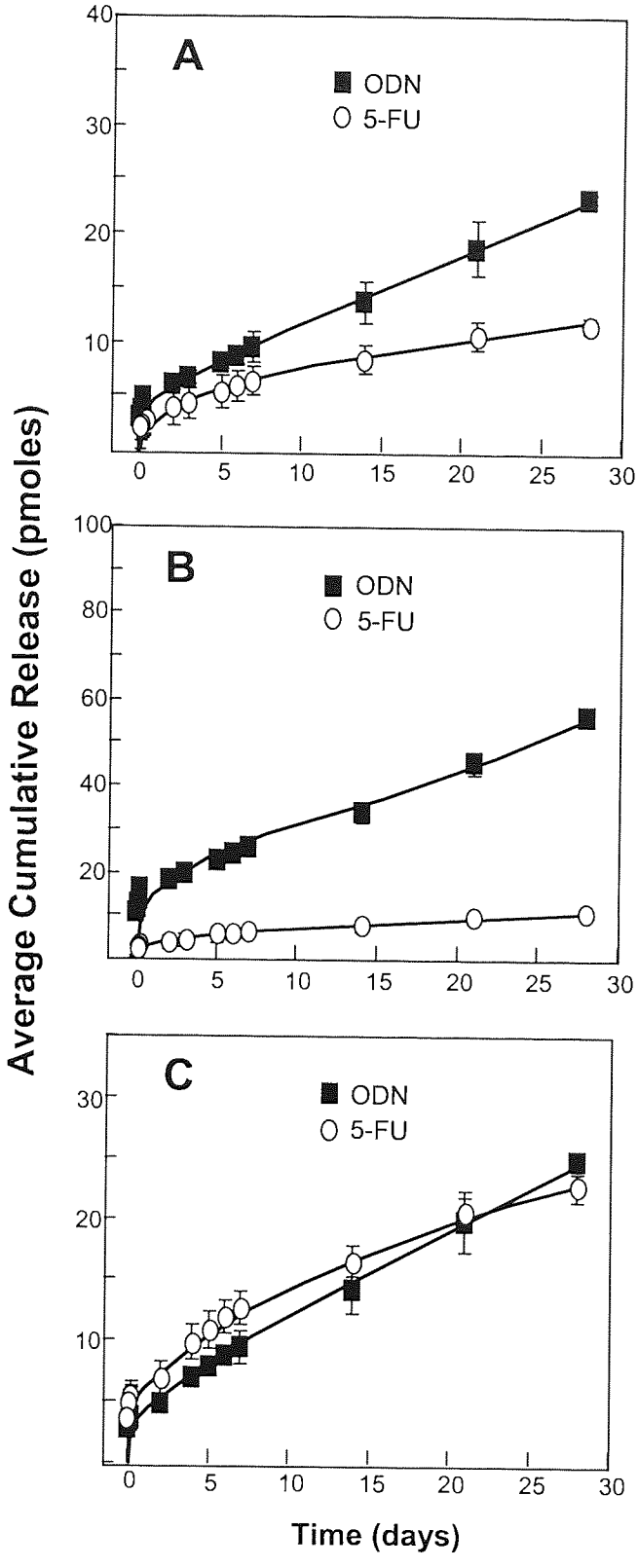
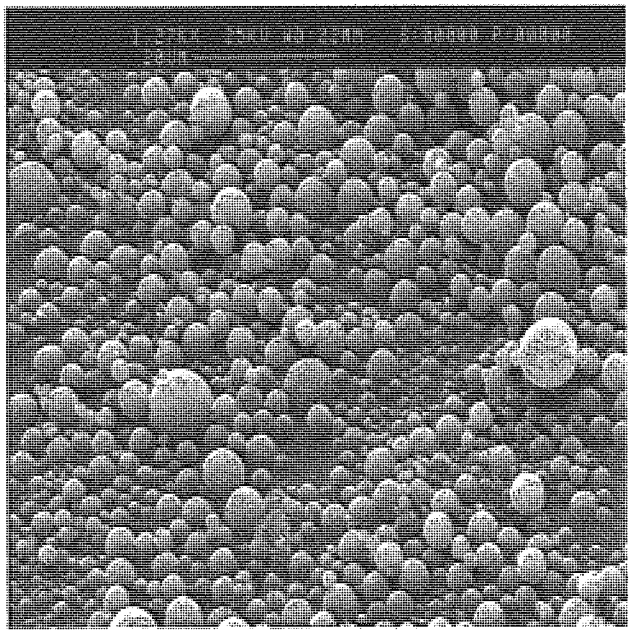


Figure 2.9 Release profiles on co-delivery of separate AODN and 5-FU microsphere formulations. Release profiles of radiolabelled AODN and 5-FU (loadings were similar to figure 2.5 and 2.6) were assessed upon combining different mass ratios of the separate microsphere formulations of each drug. Panel A: 30mg of each formulation was mixed in the *in vitro* release experiments (see Methods); Panel B: 70mg of AODN microspheres combined with 30mg of 5-FU formulation; and Panel C: 30mg of AODN microspheres combined with 70mg of 5-FU. Data represents the mean n=4±SD

A



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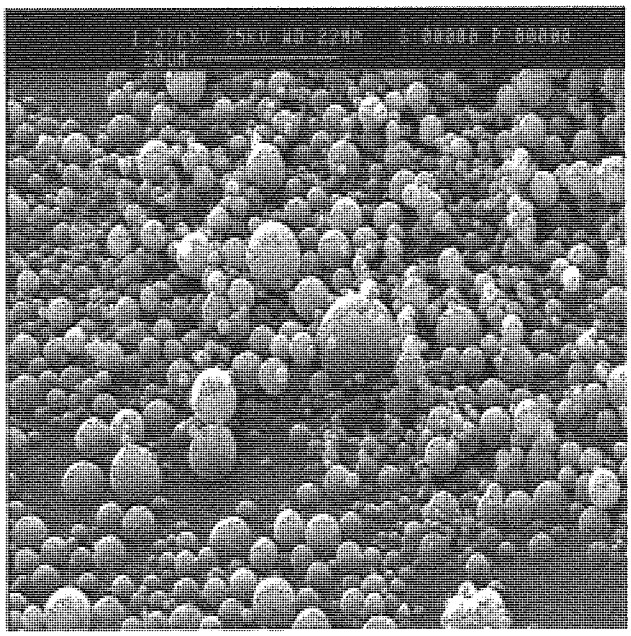
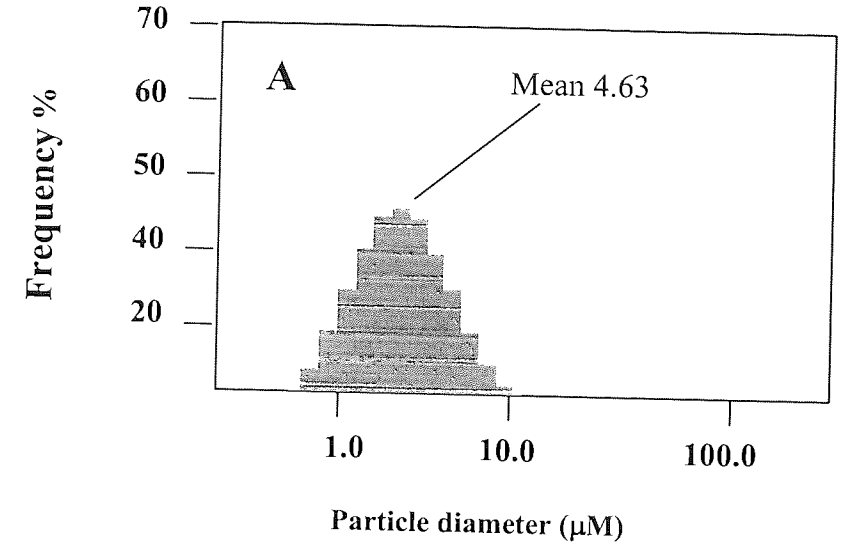


Figure 2.10 Scanning electron micrograph showing the morphology of (A) 5-FU loaded and (B) P(LA-GA) copolymer microspheres prepared by the Silverson, double emulsion method (see section 2.2.2.8.3).



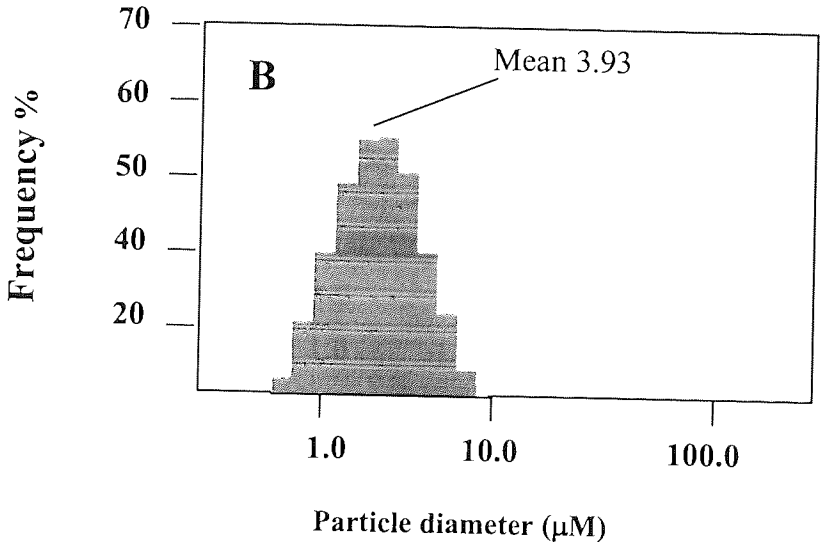


Figure 2.11 Particle-size distribution of (A) AODN and (B) 5-FU loaded microspheres prepared by the Silverson, double emulsion method. The % frequency versus particle diameter (log scale) distribution profile is given.

Table 2.6 Formulation Characteristics of Separate ODN and 5-FU Loaded P(LA-GA) Microspheres by the Silverson, Double Emulsion Method.

Drug Loaded	Mean Particle Size (μM)	% Mean Encapsulation Efficiency (n=3±SD)	Loading (pmoles/mg)
AODN	4.63	53±3	24
5-FU	3.93	40±5	18

2.3.1.2.2 In vitro Release Studies of AODN and 5-FU from Microsphere Formulations

The release profiles of AODN and 5-FU from separate P(LA-GA) microsphere formulations by the Silverson method (see section 2.2.2.8.3) are illustrated in figure 2.12. Similar release patterns were observed to the vortex method (see section 2.2.2.8.2). However, the release of AODNs and 5-FU from microspheres made by the Silverson method (see section 2.2.2.8.3) was faster compared to the burst from the microspheres produced by the vortex method. Increased surface area to volume ratio of the small spheres formulated by the silverson method compared to the large microspheres made by the vortex method could be the reason for the difference in release rates.

The Silverson, double-emulsion method (see section 2.2.2.8.3) produced small microspheres which were able to release the drugs over 28 days. The small size of the microspheres (1-8μm) would enable them to enter cells more efficiently as compared to larger microspheres (10-20μm) (Akhtar *et al.*, 1997; Khan *et al.*, 2000). The Silverson double emulsion method (see section 2.2.2.8.3) was therefore chosen for cell culture efficacy studies.

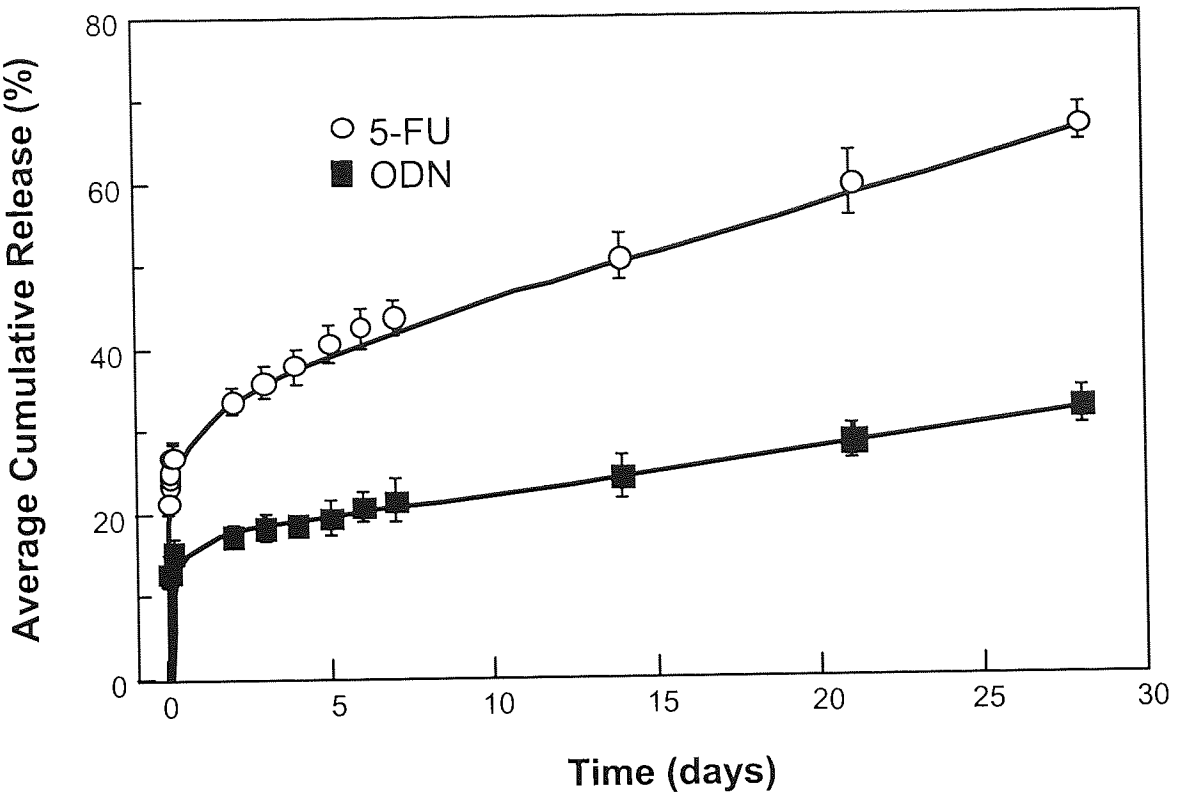


Figure 2.12 *In vitro* release profile of AODN (Loading = 24pmoles/mg) and 5-FU (Loading = 18pmoles/mg) from individual microsphere formulations prepared by the silverson, double emulsion method. Small spheres (4.63μm and 3.93μm for AODN and 5-FU loaded microspheres respectively). Data points represent average

2.3.2 Testing AODN and 5-FU Microsphere Delivery System in A431 Vulval Carcinoma Cell Line

In vitro cell culture studies were undertaken to evaluate the efficacy of an AODN (targeting *c-erb*B1) and 5-FU microsphere delivery system in an A431 vulval carcinoma cell line. The A431 cell line was derived from a human epidermoid carcinoma of the vulva and is known to over-express an unusually large number of EGF receptors (EGFR) (1-3 $\times$ 10^6 per cell) (Ullrich *et al.*, 1982). It was concluded from section 2.3.1.2 that separate microsphere formulations made by the Silverson method (1-8μm in diameter) (see section 2.2.2.8.3), containing each drug would be used for the efficacy studies. Viable cell number and EGFR expression studies using trypan blue dye exclusion, MTT assay and Western blotting assays were used. Initially, efficacy studies were evaluated using naked AODN and 5-FU and then subsequently encapsulated in microsphere delivery systems. As a first step in this evaluation, it was considered necessary to characterise A431 cell growth.

2.3.2.1 Characterisation of A431 cell growth

A431 cell growth was characterised to assess optimal growth conditions for efficacy studies in serum-free medium. Cells were seeded onto 24-well plates at a density of 1.25×10^4 cells/ml (time 0) and 5×10^4 cells/ml (time 0) in serum-free medium. Cells were then incubated at 37°C for the time periods indicated at which point viable cell number was determined by trypan blue dye exclusion assay. The growth curves obtained are shown in figure 2.13. Cells entered a lag period of at least 12-hrs, followed by a period of exponential growth. These results indicated that efficacy studies conducted over 24-hrs as well as 72-hrs could be performed in serum-free medium.

2.3.2.2 Effect of Naked AODN and/or 5-FU on viable cell number and EGFR expression of A431 Cells

Down-regulation of a target protein such as the EGFR by AODNs can result in lower viable cell numbers by inhibition of proliferation or by an increase in apoptosis (Witters *et al.*, 1999). However, small amounts of protein down-regulation may not be sufficient to reduce

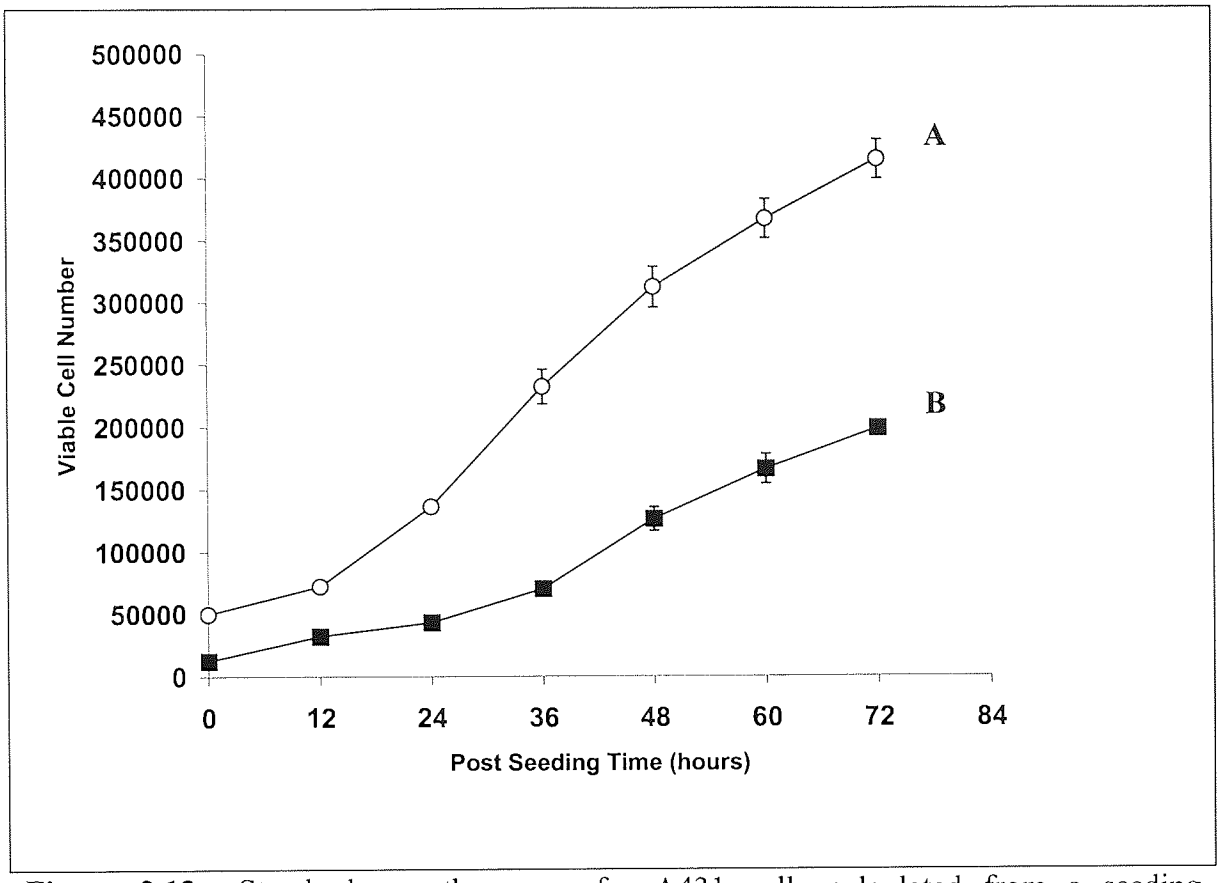


Figure 2.13 Standard growth curves for A431 cells calculated from a seeding concentration of (A) 5×10^4 cells/ml/well and (B) 1.25×10^4 cells/ml/well at 37°C (n=3±SD).

viable cell number. Also, genetic disruption of the target may induce cellular changes that compensate for the loss of function, therefore, combining AODN with 5-FU may overcome this potential problem. The effect of naked AODN and 5-FU separately on viable cell number was first evaluated followed by the effect of these naked drugs in combination on A431 cells.

The efficacy of the naked drugs in reducing viable cell number was assessed by using the trypan blue dye exclusion assay (see section 2.2.2.11.4). 24-hr and a 72-hr time points were used to determine viable cell number. The half-life of EGFR protein is reported to be approximately 7 days (King *et al.*, 1980). Thus, the reason for using the longer time point (72-hrs) was due to the rationale that reduction in the target protein levels and the subsequent change in the viable cell number may require a longer incubation time with the drugs.

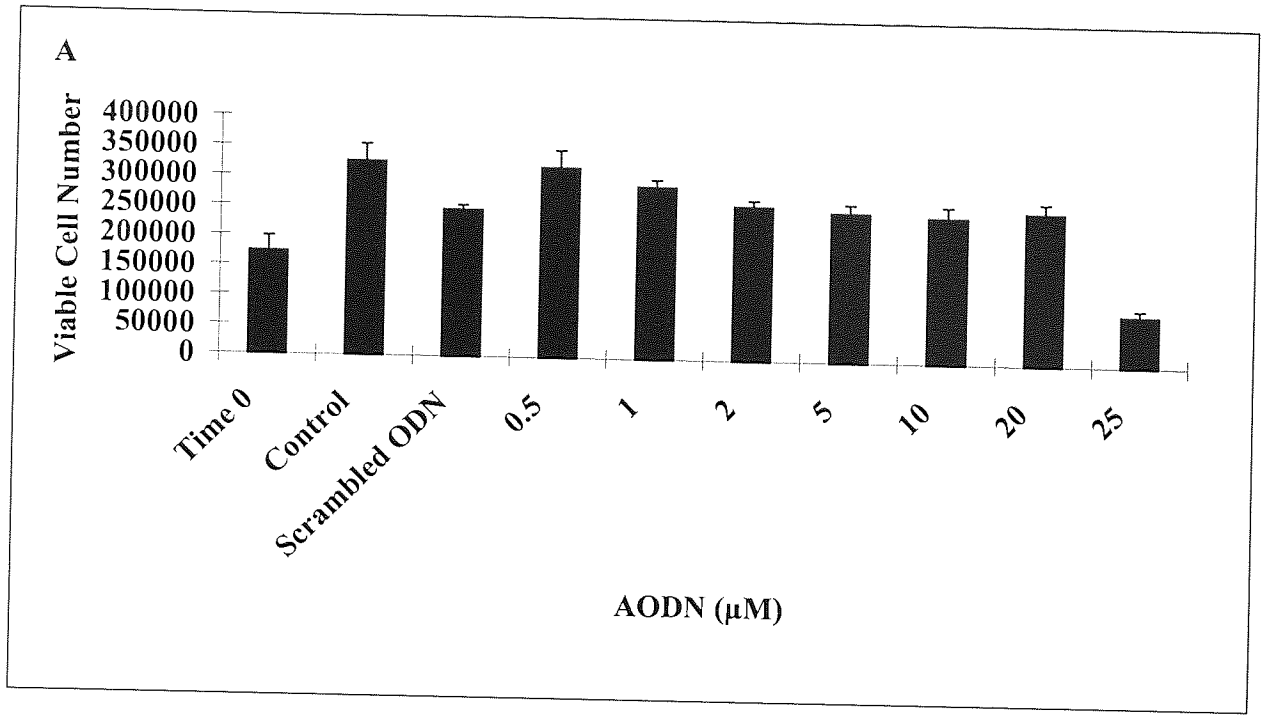
2.3.2.2.1 Effect of Naked AODN over 24-hrs or 72-hrs

The AODN, AS1, has been shown to reduce the growth of A431 cells when lipofectin was used as a delivery agent (Petch *et al.*, 1999). In that study, the AODN was used in the μM range, therefore it was decided that similar concentrations would be used. A431 cells were treated with AODN for a period of 24-hrs and 72-hrs at 37°C in serum-free medium (see section 2.2.2.11.6; figure 2.14).

Although no reduction in viable cell number was observed at either time point for 0.5μM AODN, *i.e.* there was no significant difference in viable cell number with 0.5μM AODN as compared to the untreated control ($P>0.05$). Statistical analysis (ANOVA; Dunnett's test) showed a significant difference ($P<0.05$) in viable cell number following 1μM AODN and higher concentration treatments (e.g. approx. 20% average reduction in cell number for 5μM AODN) when compared to the untreated control. A similar effect was seen with the scrambled ODN (approx. 20% average reduction in cell number) and therefore a non-antisense effect may be responsible at the higher concentrations, however, one cannot rule out an antisense effect due to the possibility of both antisense and non-antisense effects occurring simultaneously. The effect of AODN on EGFR expression was therefore evaluated to assess this possibility (section 2.3.2.2.4). At the higher AODN concentration (25μM) cytotoxiciy was observed, possibly due to the phosphorothioate nature of the AODN (Crooke 1991) and was consistent with other studies undertaken (Petch et al., 1999).

2.3.2.2.2 Effect of Naked 5-FU Over 24-hrs or 72-hrs

The concentrations of 5-FU used in this study were obtained from Ciftci *et al.*, 1996. A431 cells were treated with 5-FU for a period of 24-hrs and 72-hrs at 37°C in serum-free medium (see section 2.2.2.11.6). Incubation of 5-FU with A431 cells over 24-hrs produced a dose-dependent effect on viable cell number (figure 2.15 A), with an IC_{50} value at 0.19μM. However, after 72-hrs only a small number of viable cells remained due to the long exposure time of the 5-FU to cells (figure 2.15 B). In order to assess the efficacy of AODNs in combination with 5-FU, a dose response effect would be required for individual drug treatments and therefore a 72-hr treatment period would be unsuitable for subsequent combination studies with naked drugs on A431 cells.



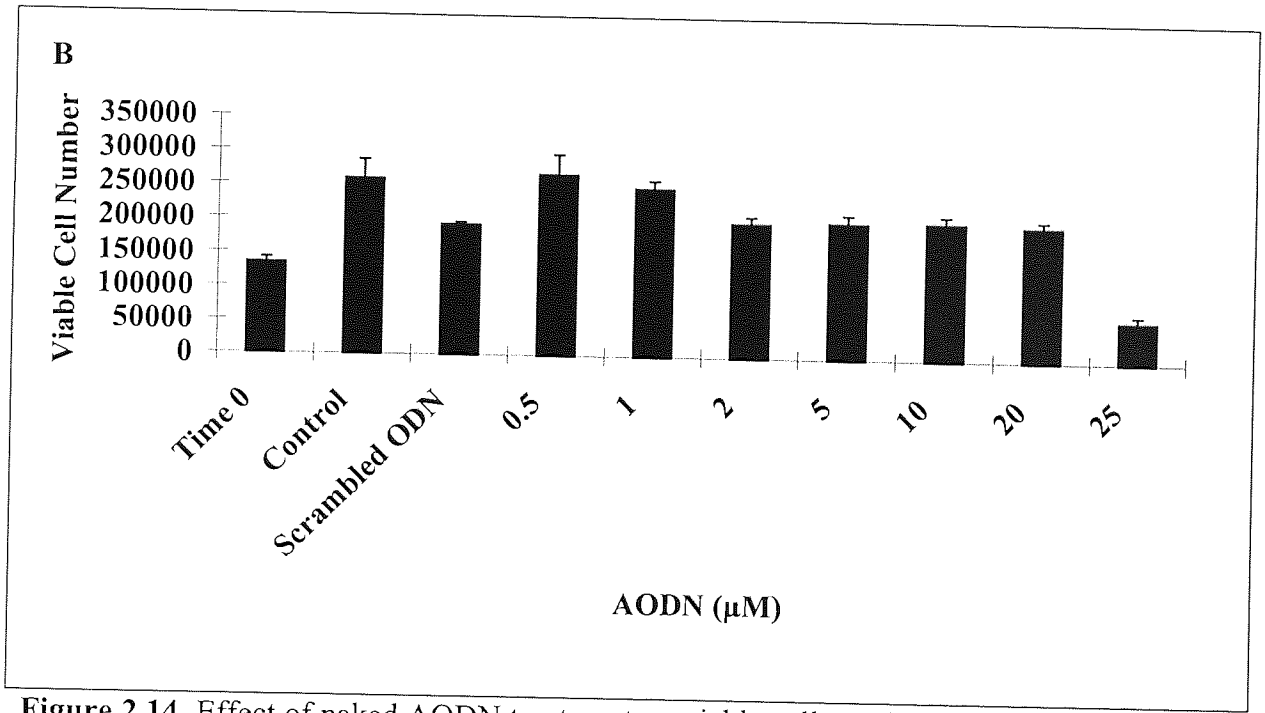


Figure 2.14 Effect of naked AODN treatment on viable cell number of A431 cells over (A) 24-hrs and (B) 72-hrs in serum free medium. Time 0= cell number at treatment time; Control= no treatment; scrambled ODN=2μM. Data represents the mean n=3±SD.

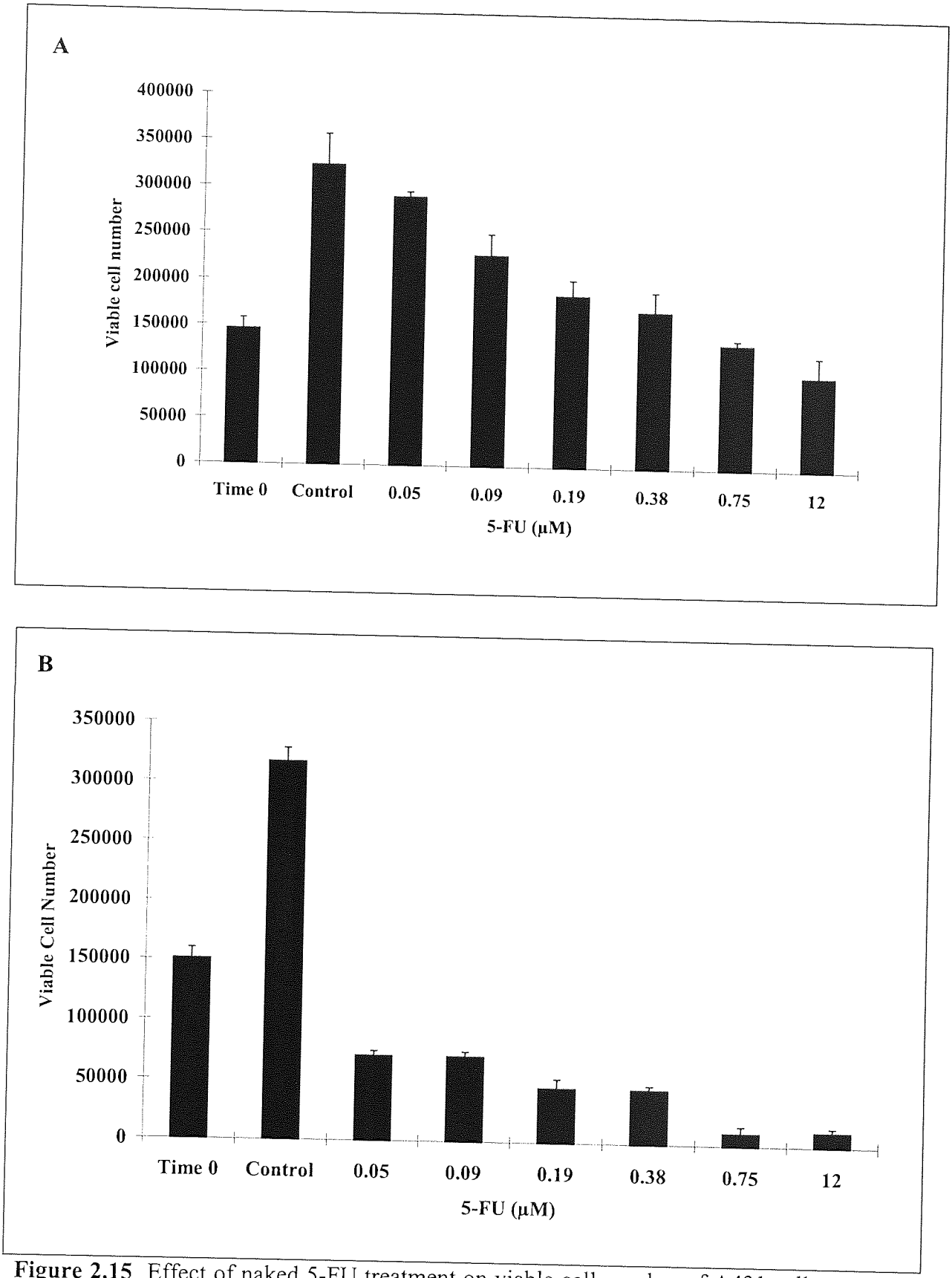


Figure 2.15 Effect of naked 5-FU treatment on viable cell number of A431 cells over (A) 24-hrs and (B) 72-hrs in serum-free medium. Time 0=cell number at treatment time; control=no treatment. Data represents the mean n=3±SD.

2.3.2.2.3 Effect of Naked AODN in Combination with 5-FU Over 24-hrs or 72-hrs

Various concentrations of AODNs discussed in section 2.3.2.2.1 were combined with the 5-FU at an IC_{50} value obtained from section 2.3.2.2.2 (0.19μM). The combination treatments were incubated with A431 cells at 37°C for 24-hrs. The study was not conducted over 72-hrs because of the inability of the 5-FU to produce a dose response effect as well as the small numbers of viable cells remaining after treatment, which could produce inaccurate results for subsequent experiments (see section 2.3.2.2.2). The results are shown in figure 2.16.

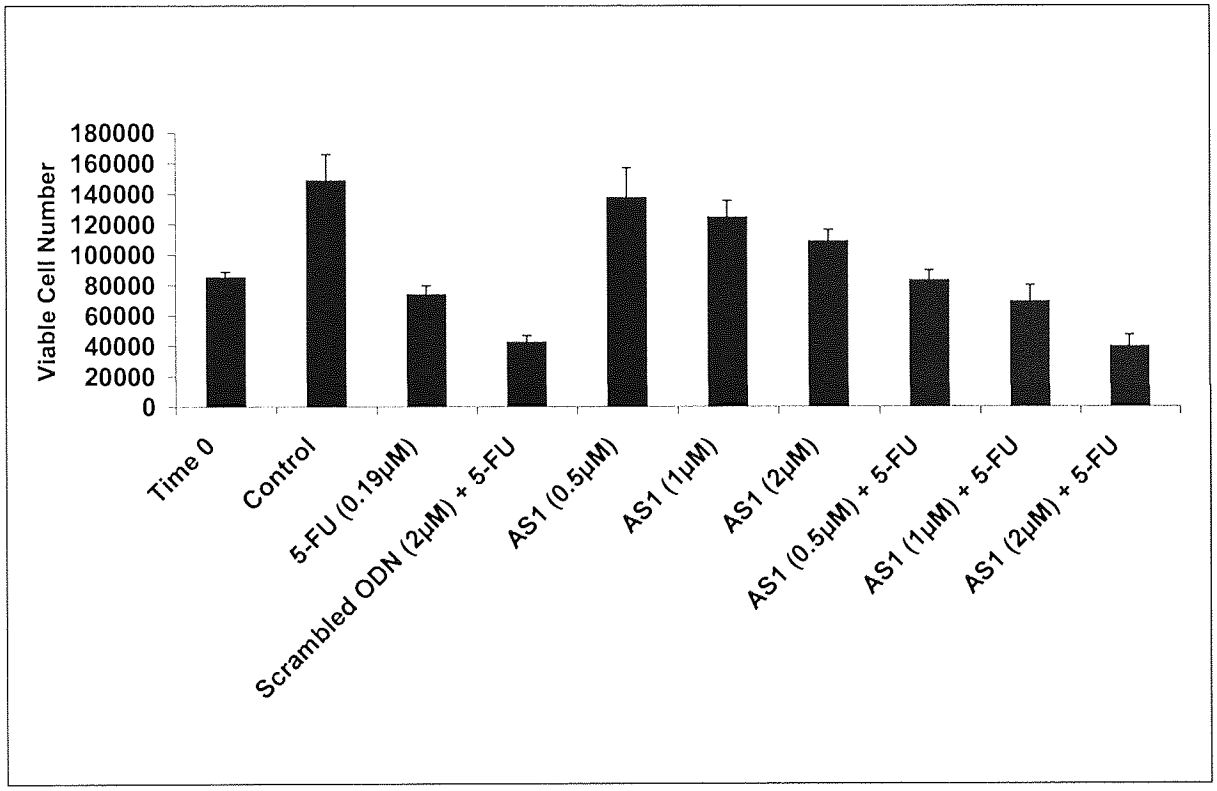


Figure 2.16 Effect of naked IC_{50} 5-FU (0.19μM) in combination of various concentrations of AODN treatment on viable cell number of A431 cells over 24-hrs in serum-free medium. Time 0= cell number at treatment time; control=no treatment. Data represents the mean n=3±SD.

There was no change in viable cell number when the cells were treated with 0.5μM AODN as compared to the control (P>0.05). This confirmed the results obtained in section 2.3.2.2.1. However, 1μM AODN, which caused an average reduction of 16% ± 6 in A431 viable cell number, when combined with 0.19μM 5-FU (IC_{50}) resulted in an average reduction of 53% ± 7. Greater effects were seen with higher AODN concentrations e.g. 2μM AODN caused an

average 27% ± 5 reduction of A431 cells, but when combined with 0.19μM 5-FU (IC_{50}) an average 73% ± 5 reduction was observed. Table 2.7 summarises the data obtained.

Table 2.7 The Effect of AODN in Combination with IC_{50} 5-FU on the Average % Reduction in Cell number of Control by Trypan blue dye exclusion Assay.

Treatment of A431 cells (μM)		**Average reduction in % cell number of control**	**Statistical Analysis (Dunnett's Test, compared to Control)**
AODN	**5-FU**		
0	0.19	50 ± 4	$p<0.05$
0.5	0	7 ± 8	$p>0.05$
1	0	16 ± 6	$p<0.05$
2	0	27 ± 5	$p<0.05$
0.5	0.19	44 ± 4	$p<0.05$
1	0.19	53 ± 7	$p<0.05$
2	0.19	73 ± 5	$p<0.05$

Similar additive effects were seen with scrambled ODN (2μM) in combination with 5-FU (0.19μM), suggesting that these additive effects may be brought about by a non-antisense cytotoxic effect of the ODN. To confirm the results obtained by the trypan blue dye exclusion assay, an MTT colorimetric assay was undertaken (see section 2.2.2.11.8).

For MTT assay, cells were treated with the drugs in the same way as for the trypan blue dye exclusion assay (see section 2.2.2.11.6). To determine the validity of the MTT assay, absorbance was measured over 5-days for A431 cells. Cells were seeded as before (see section 2.2.2.11.3) and at each time point triplicate wells were treated with MTT and the absorbance measured. The calibration graph (figure 2.18) demonstrated a linear relationship between cell number and absorbance at 562nm, which indicated that this assay was valid to use. Figure 2.17 showed that the MTT assay gave similar results for the growth of A431 cells as would be observed using the trypan blue dye exclusion assay.

The results obtained from the MTT assay were similar to those from the trypan blue dye exclusion assay (figure 2.19). Similar effects were observed when higher concentrations were combined with 5-FU (IC_{50}) as seen with the trypan blue dye exclusion assay. Table 2.8 summarises the data obtained by the MTT assay.

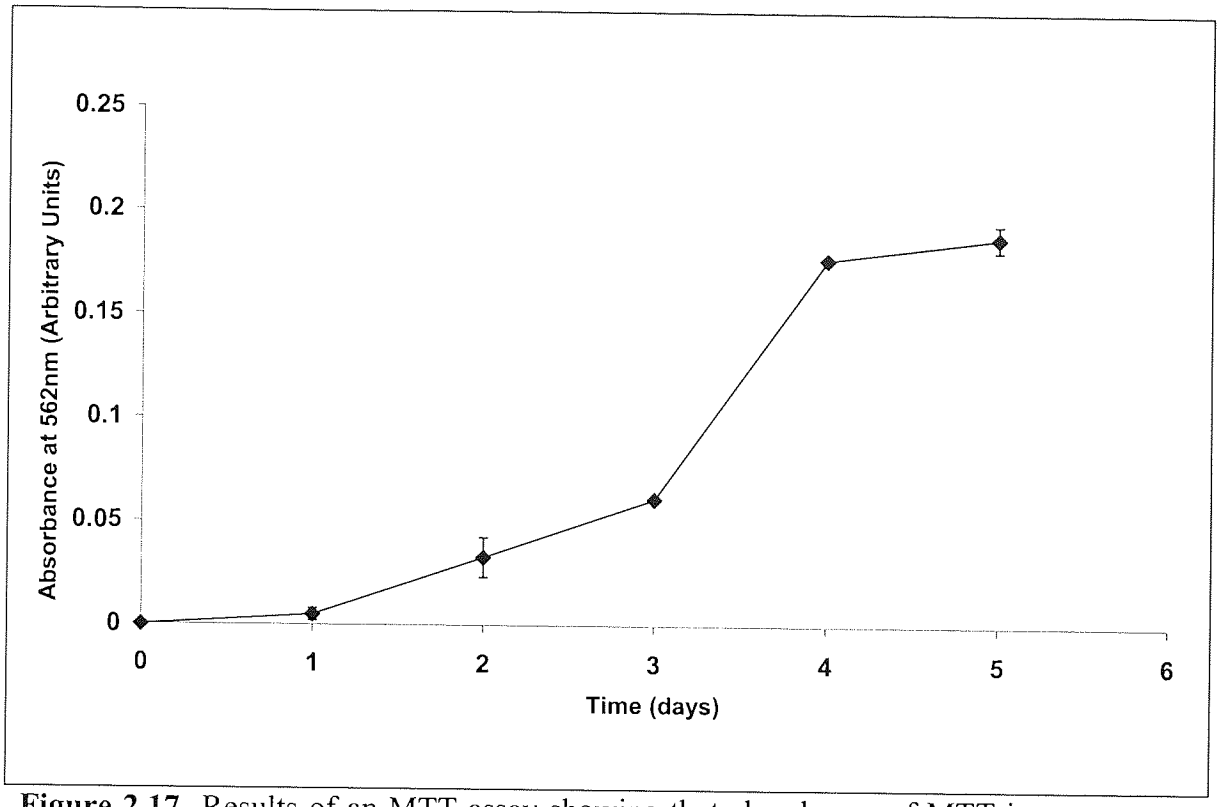


Figure 2.17 Results of an MTT assay showing that absorbance of MTT increases over time indicating that there is increasing cell number. Data represents the mean n=3±SD.

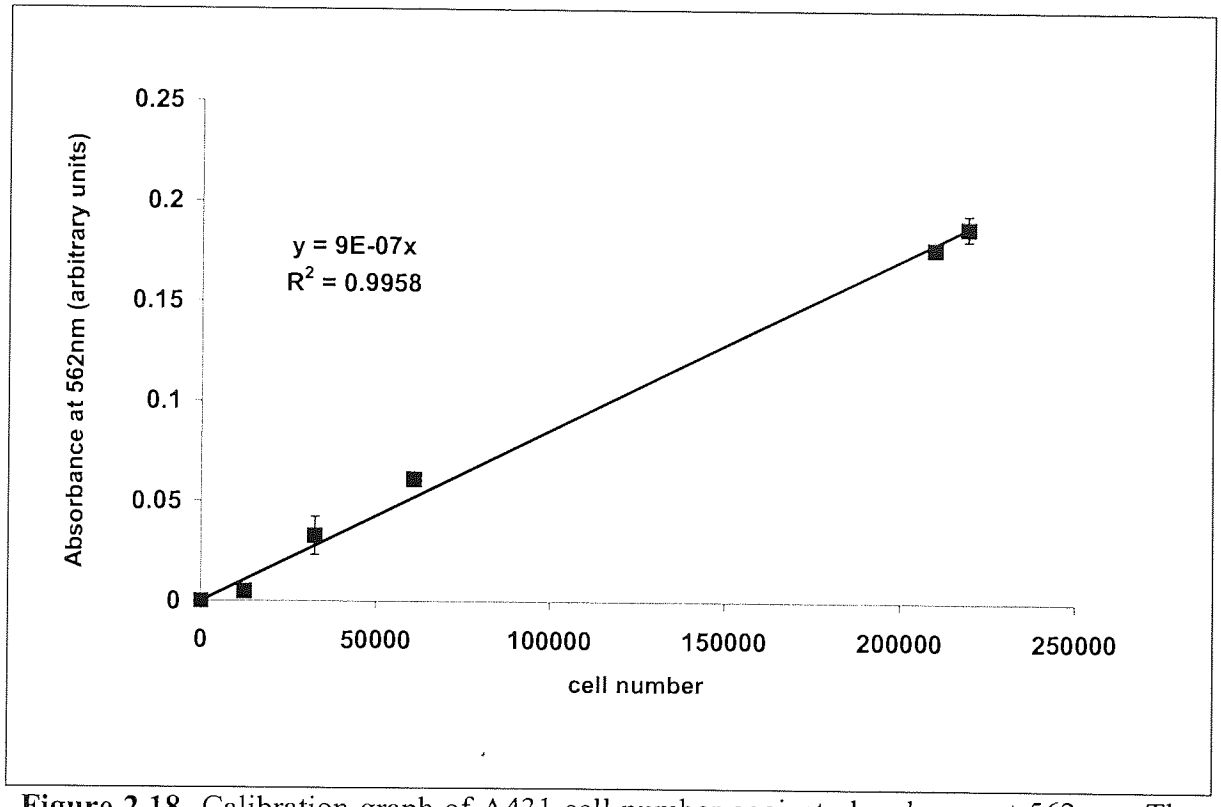


Figure 2.18 Calibration graph of A431 cell number against absorbance at 562nm. The cells were incubated for 24-hrs at 37°C. Data represents the mean n=3±SD.

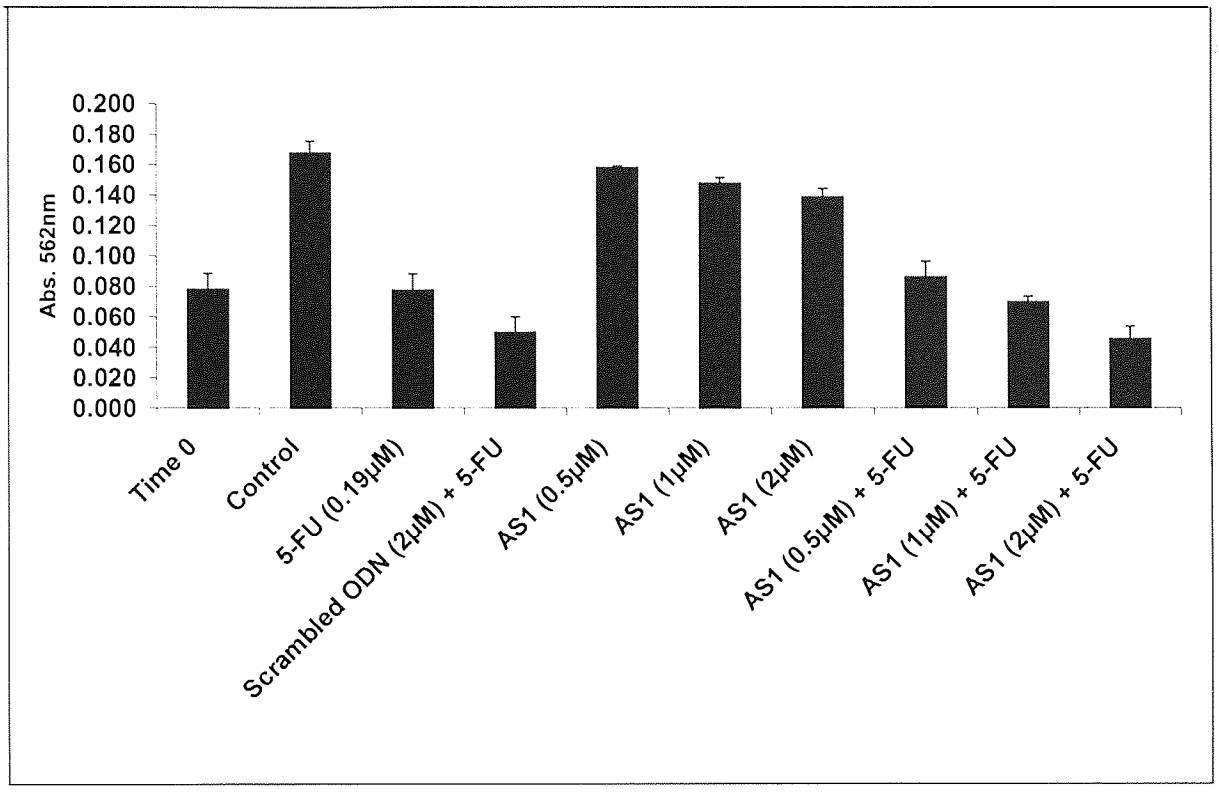


Figure 2.19 Effect of naked IC_{50} 5-FU (0.19μM) in combination with various concentrations of AODN treatment on viable cell number of A431 cells over 24-hrs in serum free medium as shown by the MTT assay. Time 0= cell number at treatment time; control=no treatment. Data represents the mean n=3±SD.

Table 2.8 The Effect of AODN in Combination with IC_{50} 5-FU on the Average % Reduction in Cell number of Control MTT assay.

Treatment of A431 cells (μM)		Average reduction in % absorbance of control	Statistical Analysis (Dunnett's Test, compared to Control)
AODN	5-FU		
0	0.19	54 ± 6	$p<0.05$
0.5	0	6 ± 0.6	$p>0.05$
1	0	12 ± 1	$p<0.05$
2	0	17 ± 3	$p<0.05$
0.5	0.19	49 ± 6	$p<0.05$
1	0.19	58 ± 7	$p<0.05$
2	0.19	73 ± 5	$p<0.05$

It was clear from the above data that combining AODN with 5-FU had an additive effect on the percentage reduction in A431 cell number as compared to either drug on its own. To assess if these results were due to an antisense effect EGFR protein expression was performed by western blotting, in A431 treated cells.

2.3.2.2.4 The Effect of EGFR Expression in A431 Cells Treated with Naked AODN and/or 5-FU

Assessment of EGFR protein expression was undertaken to further investigate the effects of combining AODN with 5-FU on A431 cells. The cells were treated with the relevant drugs as before (see section 2.2.2.11.7) and a Western blot was conducted as described in the methods section 2.2.2.12. The greatest percentage reduction in cell number was achieved with 2μM AS1 combined with 0.19μM 5-FU and therefore this combination was used for the western blot. From the Western blot no significant decrease in EGFR protein expression was evident with the relevant treatments (figure 2.20).

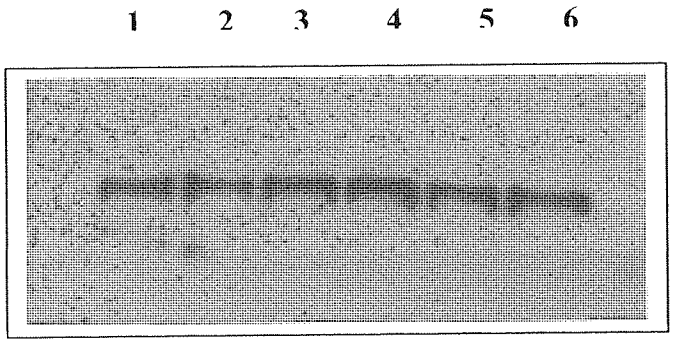


Figure 2.20 Effect of ODN and 5-FU treatments over 24-hrs on EGFR expression in A431 cells. Lane 1= control (no treatment), lane 2= scrambled ODN (2μM), lane 3= AODN (2μM), lane 4= 5-FU (0.19μM), lane 5= scrambled ODN (2μM) + 5-FU (0.19μM), lane 6= AODN (2μM) + 5-FU (0.19μM). Images depict representative blots from duplicate experiments.

The trypan blue dye exclusion assay suggested that combining naked AODN with 5-FU had a greater effect on reducing the percentage viable cell number of A431 cells as compared to either drug on its own. However, this effect is unlikely to be an antisense mechanism because no differences in EGFR expression was observed by Western blotting as compared to the controls.

Studies have shown that AODN uptake is enhanced in the presence of cytotoxic drugs which may alter the lipid or protein components of the membrane and consequently lead to increased membrane fluidity and permeability (Skorski *et al.*, 1997; Rao *et al.*, 1997). Such changes are primarily likely to affect AODN uptake by fluid-phase endocytosis. It has also been

demonstrated that an increase in cisplatin accumulation within urinary bladder tumour cells was induced following treatment of the cells with *c-myc* AODN (Mizutani *et al.*, 1994). It may be likely therefore that the uptake of 5-FU is influenced by the AODN, which could explain the results obtained. Cell association of 5-FU in the presence of the AODN was undertaken to confirm this hypothesis.

2.3.2.2.5 Cellular Association of 5-FU on A431 Cells Treated with AODN

A431 cells were seeded in 24-well plates as before (see section 2.2.2.11.7) and were treated as in section 2.2.11.7. Cellular association of 5-FU was measured over 4-hrs in serum-free media and the results are shown in figure 2.21. AODN had no significant effect on the cellular association of 5-FU in A431 cells over 4-hrs ($P>0.05$). This result suggested that the AODN does not modulate the cellular association or the uptake of 5-FU in A431 cells and it is unlikely therefore that the AODN is altering membrane permeability to allow increased amounts of 5-FU to enter the cytosol. It can be argued that cellular association of the 5-FU in the presence of AODN did not fully mimic the efficacy studies due to the shorter incubation time period. However, this was not possible due to the concentrations used of each drug, which reduced viable cell number (for efficacy studies) and consequently would invalidate the results obtained if cellular association studies were to be carried out at 24-hrs and 72-hrs.

Overall, combination of AODN and 5-FU produced an additive effect on cell number as compared to either AODN or 5-FU alone. The results indicated that there was no increased uptake of 5-FU when used in combination with the AODN. The Western blot analysis indicated no EGFR down-regulation. This, in addition to the fact that scrambled ODNs also had a significant effect when combined with 5-FU, suggested that a non-antisense cytotoxic effect was occurring. It is likely that the phosphorothioate ODN was binding non-specifically to cellular proteins resulting in some degree of cytotoxicity which on its own was not enough to have an effect on cell number. However, the addition of 5-FU caused further cytotoxicity which ultimately resulted in reduction of cell number. Non-specific binding of phosphorothioate ODNs to cellular proteins is likely to become more pronounced with increasing amounts of ODN concentrations resulting in further cell number reduction in combination with 5-FU. It is a well documented fact that ODNs, especially phosphorothioates interact with a wide variety of proteins in a sequence-independent manner (Gao *et al.*, 1993; Stein *et al.*, 1993; Weidner *et al.*, 1995; Veal *et al.*, 1998; Juliano *et al.*,

1999). Phosphorothioate ODNs have been shown to interact with nuclear and cytoplasmic proteins in a non-antisense manner (Gao *et al.*, 1993; Weidner *et al.*, 1995). Similar effects have been noticed when an AODN has been combined with a nucleoside analogue reverse transcriptase inhibitor in HIV-1 infected cells (Veal *et al.*, 1998). In this study, the mismatch controls interacted synergistically with a nucleoside analog reverse transcriptase inhibitor to reduce viable cell number, suggesting a non-antisense effect. Such AODN interactions with cellular receptors, enzymes and other proteins can cause changes in cell metabolism and ultimately can lead to antiproliferation, apoptosis and cellular toxicity (Burgess *et al.*, 1995; White *et al.*, 1996; Castier *et al.*, 1998; Juliano *et al.*, 1999). Results from the control experiments and the phosphorothioate nature of the AODN therefore strongly indicate that a similar phenomenon is occurring in this case i.e. the cytotoxic effects of the AODN and 5-FU in combination allow the cells to reach a certain threshold level before a reduction in viable cell number is noticed.

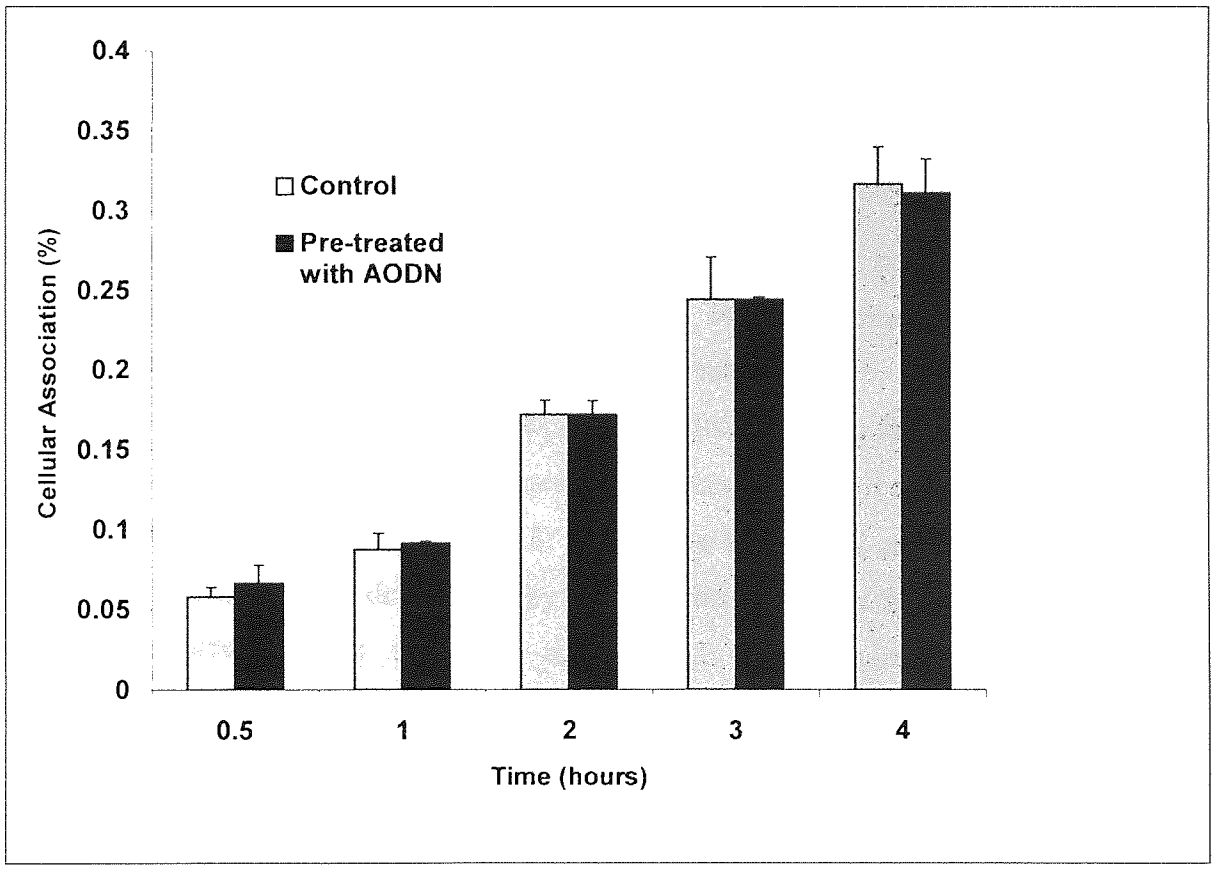


Figure 2.21 Cellular association of 0.19μM 5-FU over 4-hrs in A431 cells pre-treated with 2μM AODN. Data represents the mean n=3±SD.

2.3.2.3 Effect of AODN and/or 5-FU in P(LA-GA) Microsphere Delivery System on Viable Cell Number and EGFR expression of A431 Cells

Studies undertaken with naked AODN and/or 5-FU in section 2.3.2.2 were repeated using a P(LA-GA) microsphere delivery system. The aim for carrying out such a study was to assess if a delivery system is able to improve the efficacy of AODN and/or 5-FU compared to naked drugs. Properties of P(LA-GA) microspheres should have the potential to reduce toxicity, enhance cellular association as well as to provide sustained delivery to ultimately improve efficacy (see section 2.1.1). As a first step in this evaluation, it was considered necessary to examine the toxicity effects of unloaded P(LA-GA) microspheres in A431 cell line.

2.3.2.3.1 Toxicity Effects of Unloaded P(LA-GA) Microspheres

Although P(LA-GA) have been widely used for delivering many drugs without any toxicity effects, it was still considered important to determine their effects in A431 cell line due to the possibility of toxicity caused by the excipients in the microsphere formulation such as polyvinylalcohol and dichloromethane residues which could invalidate the results.

Trypan blue dye exclusion assay was used to measure viable cell number of A431 cells to assess microsphere toxicity (see section 2.2.2.11.4). Increasing mass (5mg to 200mg) of unloaded microspheres, made by the Silverson double-emulsion method (see section 2.2.2.8.3), were incubated with A431 cells in serum-free medium for 24-hrs and 72-hrs at 37°C. After incubation for the relevant times the medium was removed and the cells washed with PBS as described in section 2.2.2.11.6. Cells were trypsinised and the total viable cells remaining were immediately determined by trypan blue dye exclusion assay. Control cell samples were incubated for the same time periods in the same conditions without microspheres.

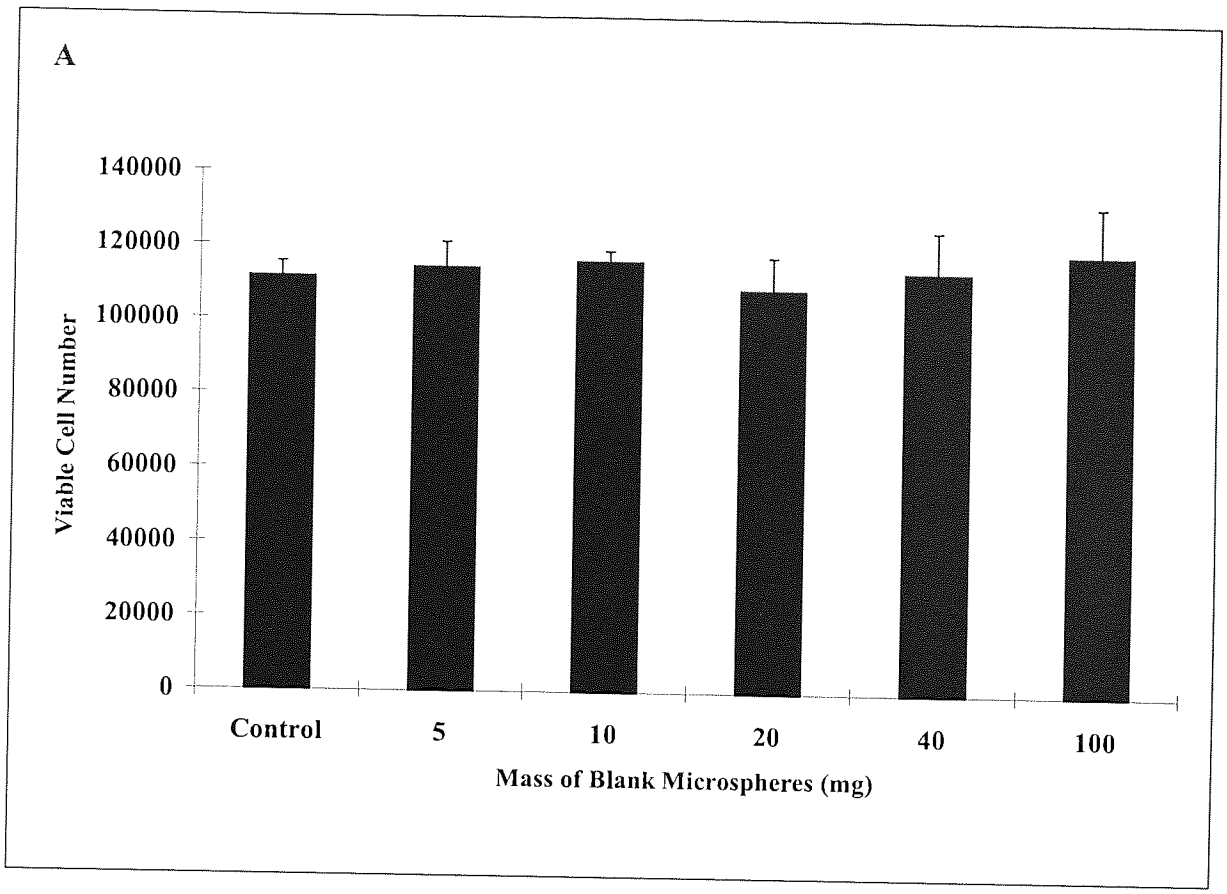
Figure 2.22 shows the effect of various amounts (mg) of unloaded P(LA-GA) microspheres on A431 viable cell number after 24-hrs and 72-hrs at 37°C compared to the control population. There was no significant change in the viable cell number after 24-hrs ($P>0.05$). However, after 72-hrs the viable cell number was partially reduced when incubated with 100mg of microsphere mass compared to the control samples ($P<0.05$). The reduction in

viable cell number is possibly due to the large concentration of microspheres taking up space within the cell culture wells and subsequently preventing further cellular growth. It has been shown that polymer monomers are able to reduce cellular confluency in a similar manner (van Sliedregt *et al.*, 1994), however it is more likely that the micospheres are preventing confluency rather than the monomers, because it may not possible for the polymer to degrade and produce monomers within the incubation time in this particular case. Alternatively, the large microsphere mass may prevent nutrients (from the serum-free medium) from being taken up by the cells which could also affect viable cell number. All efficacy studies carried out were done with much smaller microsphere masses, therefore the experiment was used to design appropriate contact conditions.

2.3.2.3.2 Effect of Encapsulated AODN in a P(LA-GA) Microsphere Delivery System on A431 Cells

2.3.2.3.2.1 Cellular Association of AODN in P(LA-GA) Microspheres Compared with Naked AODN

A431 cellular association of AODN encapsulated in microspheres was compared with naked AODN. Cellular association was investigated at a 24-hr and 72-hr incubation period (see section 2.2.2.11.7) in serum-free medium and the results are shown in figure 2.23. After 24-hrs, the average cellular association of naked AODN was 3% as compared to 14% with AODN encapsulated in microspheres. Surprisingly, after a 72-hr incubation period similar cellular association was observed to 24-hrs. One would expect greater uptake with larger incubation time periods. However, it is possible that the AODN or the microspheres were binding to specific components on the cell membrane, resulting in a saturation point being achieved at or before the 24-hr time point. This was consistent with other studies which have shown cellular association profiles of ribozymes and microspheres to plateau after a few hours (Fell *et al.*, 1997; Khan 1999).



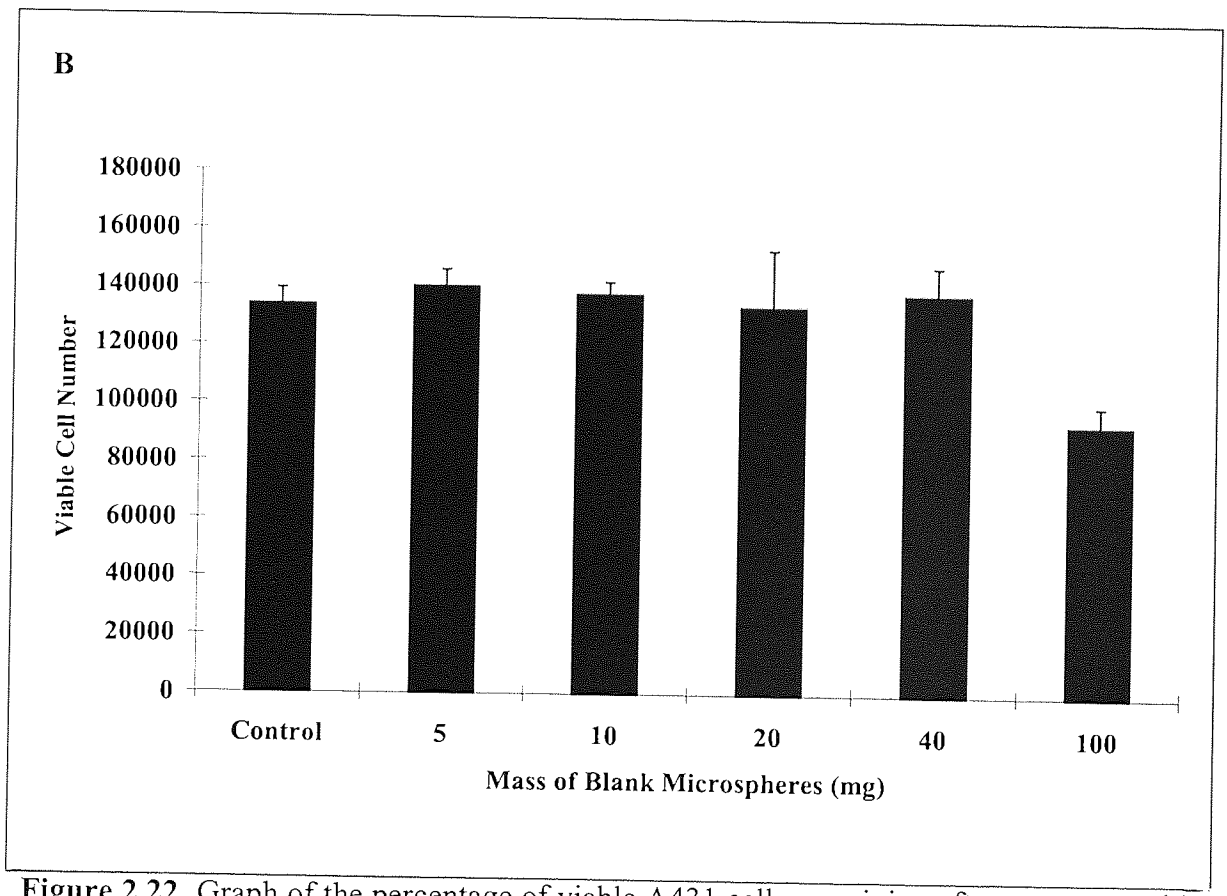


Figure 2.22 Graph of the percentage of viable A431 cells remaining after treatment with various amounts of P(LA-GA) microspheres size 3.3μm in 300μl DMEM, for (A) 24-hrs (B) 72-hrs compared with control (n=3±SD).

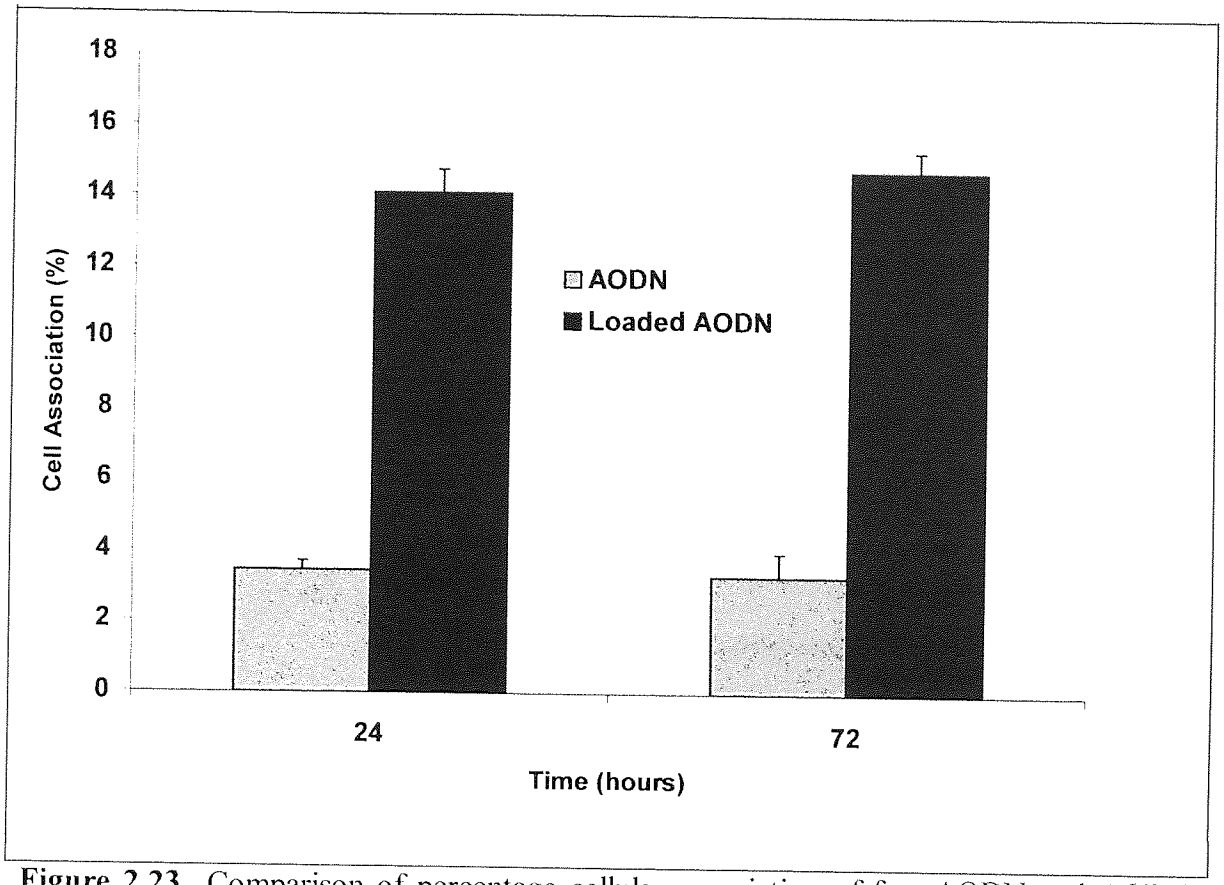
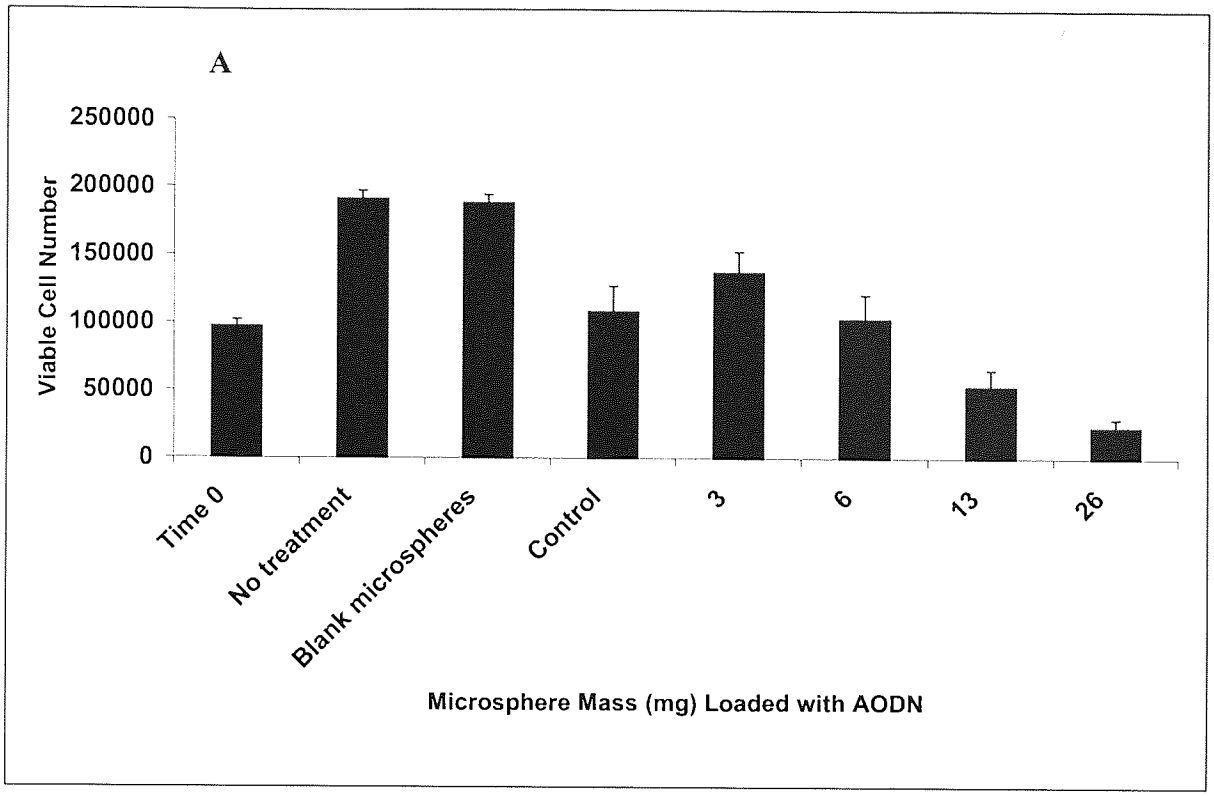


Figure 2.23 Comparison of percentage cellular association of free AODN and AODN encapsulated in P(LA-GA) microspheres at a 24-hr and 72-hr incubation period at 37°C. The free AODN was added in equal amounts (1.5pmoles) to the entrapped AODN. Data represents the mean n=3±SD.

2.3.2.3.2.2 Effect of AODN Encapsulated in P(LA-GA) Microspheres over 24-hrs or 72-hrs

Various microsphere masses loaded with AODN (prepared by the Silverson method; section 2.2.2.8.3) were used to treat A431 cells until a dose-response was achieved. 150nmoles of AODN was added to the formulation and the microspheres were made as before (see section 2.2.2.8.3). Increasing microsphere masses containing AODN (3-26mg) were incubated with A431 cells at 37°C for 24-hrs and 72-hrs. Cells were treated with microspheres containing AODN in the same way as for naked AODN (see section 2.2.2.11.6). However, with the microsphere treatments, after the relevant incubation times, the cell monolayer was carefully washed four times with PBS-azide to make sure all the microspheres had come off the cells. Trypan blue dye exclusion was carried out as before (see section 2.2.2.11.4) to measure viable cell number. The results are shown in figure 2.24.



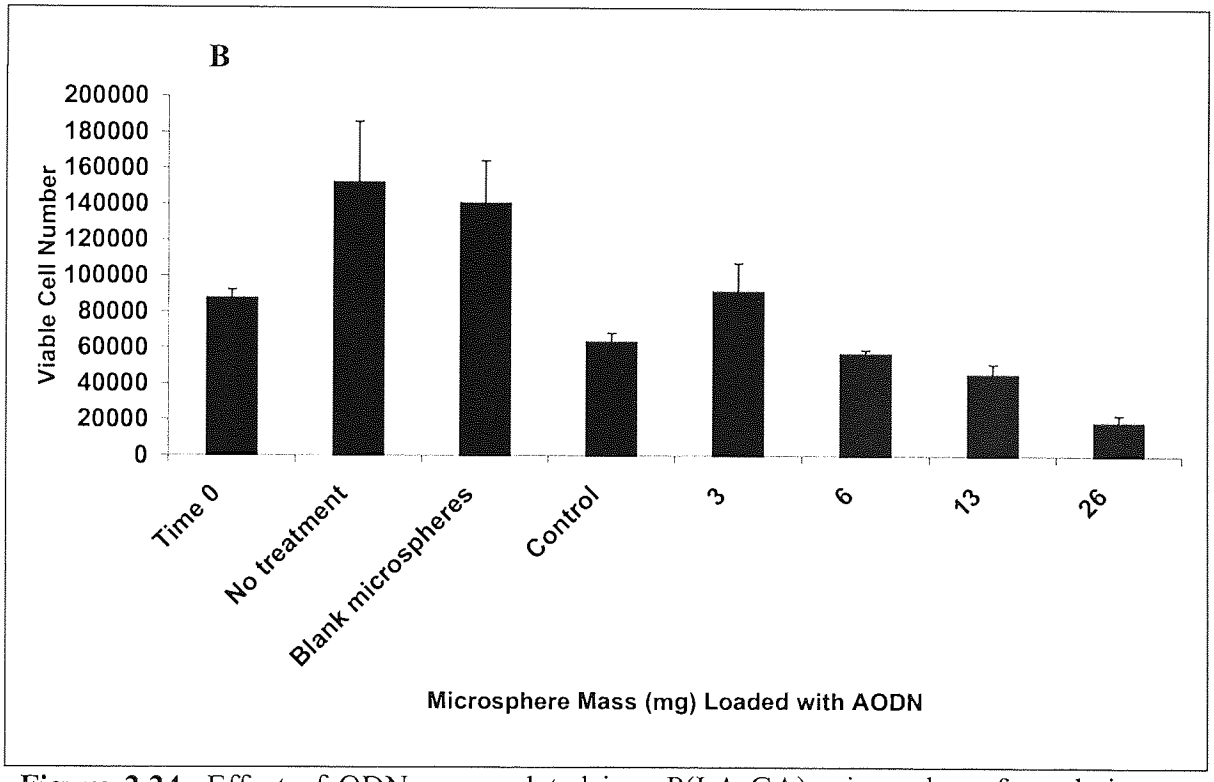


Figure 2.24 Effect of ODN encapsulated in a P(LA-GA) microsphere formulation on viable cell number of A431 cells over (A) 24-hrs and (B) 72-hrs in 300µl DMEM. Time 0=cell number at treatment time; blank microspheres=26mg; control=6mg microsphere mass loaded with scrambled ODN at same conc. as AODN. Data represents the mean n=3±SD.

The amount of AODN being released from each microsphere mass was determined before the results could be compared and evaluated. Table 2.9 shows the formulation characteristics of microspheres loaded with AODN which produced a dose-response effect. The release of AODN from the microspheres was also assessed in serum free medium to determine the amount of AODN released on A431 cells (figure 2.25).

Table 2.9 Formulation Characteristics of ODN Loaded P(LA-GA) Microspheres for a Dose Response Effect on A431 Cells.

Drug	% Average Cumulative Release (hours)			% yield	% Encapsulation efficiency
	0 (burst)	24	72		
AODN	22±2	26±2	30±2	60	60±7

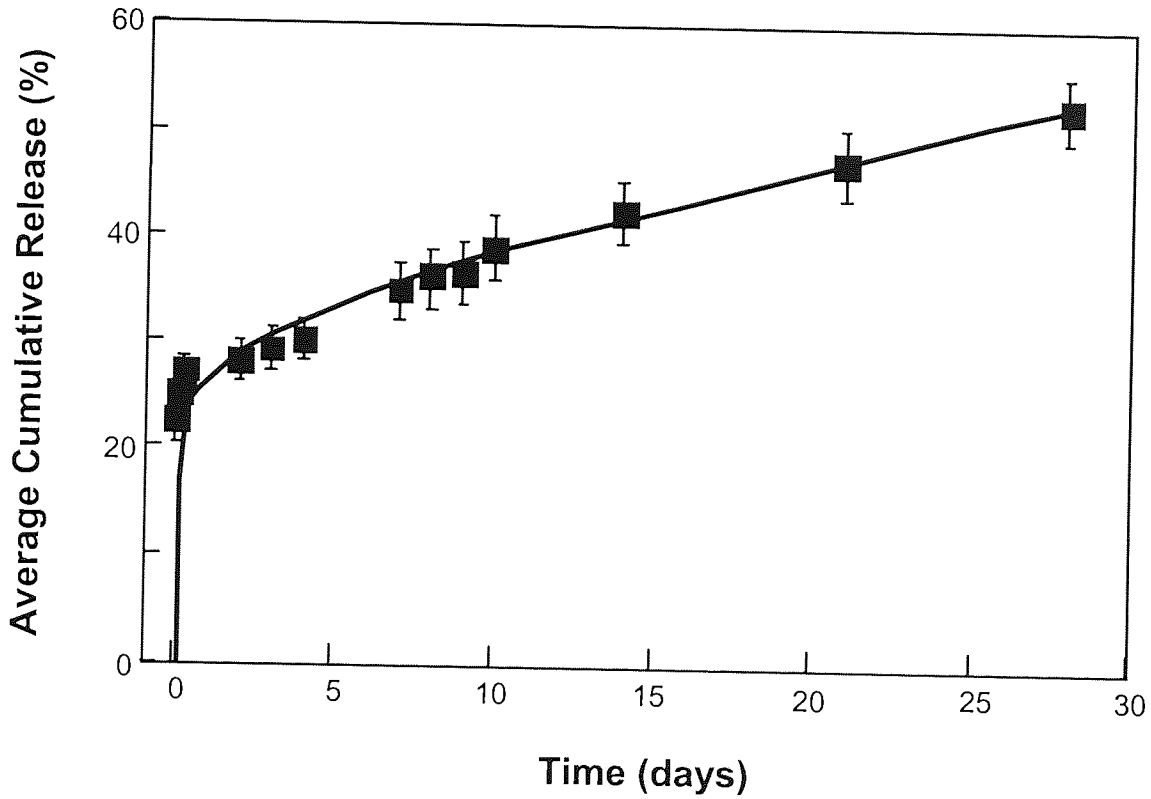


Figure 2.25 *In vitro* release profile of AODN from a P(LA-GA) microsphere formulations prepared for the dose response effect on A431 cells. Data represents the mean n=3±SD.

The amount of AODN encapsulated within a microsphere formulation was calculated from the percentage yield and encapsulation efficiency. 150mg of microsphere sample was obtained from a 60% yield and therefore the mass of microspheres obtained had a total of

90nmoles of AODN from an encapsulation efficiency of 60%. It was deduced from the release data that approximately 26% and 30% of AODN was released from microspheres at 24-hrs and 72-hrs respectively. From these findings, the amounts of AODN released from each mass at the relevant time points was determined (table 2.10).

Table 2.10 The Amount of AODN Released from P(LA-GA) Microspheres Masses at the Relevant Incubation Time Points on A431 Cells to Produce a Dose Response Effect.

	AODN Release (nmoles)	
Mass of Microsphere (mg) encapsulating AODN (nmoles)	**24-hrs**	**72-hrs**
3 (1.8)	0.47	0.54
6 (3.6)	0.94	1.08
13 (7.8)	2.01	2.34
26 (15.6)	4.00	4.68

Percentage viable cell number of A431 cells was determined at 24-hr and 72-hr incubation times from figure 2.24. The values obtained are shown in table 2.11.

Table 2.11 The Effect of AODN Encapsulated in a P(LA-GA) Microsphere Formulation on the Average % Reduction in Viable Cell Number of Control by Trypan blue dye exclusion Assay.

Microsphere Mass (mg)	**Average Reduction in % Cell Number of Control**		**(Dunnett's Test, compared to Blank Microspheres)**
	24-hrs	**72-hrs**	
3	17±9	40±10	$p<0.05$
6	38±10	62±1	$p<0.05$
13	68±7	70±4	$p<0.05$
26	86±4	88±3	$p<0.05$

A dose-dependent effect was produced with increasing microsphere masses encapsulated with AODN, for 24-hrs and 72-hrs in A431 cells. Microsphere mass of 3mg produced a 17%±9 reduction in A431 cell number at 24-hr incubation time as compared to blank microsphere treatment ($P<0.05$). For a 72-hr incubation time, the difference in average reduction in percentage cell number was greater, compared to 24-hrs (40%±10) and was also significantly different as compared to the blank microsphere treatment ($P<0.05$). For 6mg microsphere mass the average reduction in percentage cell number was 38%±10 and 62%±1 for a 24-hr

and a 72-hr incubation time respectively. Both differences were significant compared to the blank microsphere treatments ($P<0.05$). The difference in viable cell number between 24-hrs and 72-hrs was also significant for 3mg and 6mg microsphere masses encapsulating AODN ($P<0.05$). For larger microsphere masses (13mg and 26mg) the differences in average reduction in percentage cell number between 24-hrs and 72-hrs was insignificant ($P>0.05$). However, significant reduction in percentage cell number was observed with each corresponding blank microsphere treatment ($P<0.05$). For a 26mg microsphere mass the average reduction in percentage cell number was 86%±4 and 88%±3 for a 24-hr and a 72-hr incubation time respectively. This overall data correlated well with the release of AODN from the different masses of microspheres at the 24-hr and 72-hr time points (table 2.11). The insignificant difference in percentage viable cell numbers between 24-hr and 72-hr time periods for larger microsphere masses (13mg and 26mg) may be attributed to extensive cytotoxicity due to the large amounts of AODN being released during the burst phase.

In comparison to the naked AODN, a greater effect on viable cell number reduction was achieved with encapsulated AODN. It is therefore likely that the microsphere delivery system was enhancing the effect of AODNs. However, it seems that the effect is not due to an antisense mechanism because a similar effect on viable cell number was observed by scrambled AODN encapsulated in P(LA-GA) microspheres. This needs to be confirmed by Western blotting to analyse protein expression. It is worth noting that when utilising the microsphere delivery system, 1.08nmoles of AODN reduced viable cell number by 55% with 6mg microsphere mass, compared to 15% with naked 6nmoles of AODN over 72-hrs. This indicated that the delivery system allowed a much smaller amount of AODN to have a greater effect on viable cell number of A431 cells, compared to naked AODN and thus confirms the ability of microspheres to reduce ODN dose required for efficacy (Khan 1999). This may be attributed to the fact that the microspheres, which have not entered the cells allow the AODNs (which have been released during the burst and sustained phase) to be released in close proximity to the cell membrane as compared to the naked AODNs and therefore enhance the effectiveness of the encapsulated AODNs on viable cell number.

2.3.2.3.3 Effect of Encapsulated 5-FU in a P(LA-GA) Microsphere Delivery System on A431 Cells

2.3.2.3.3.1 Cellular Association of 5-FU in P(LA-GA) Microspheres Compared with Naked 5-FU

A431 cellular association of 5-FU encapsulated in microspheres was compared with naked 5-FU. Cellular association was investigated at a 24-hrs and 72-hrs incubation period (see section 2.2.2.11.7) in serum free medium. The results are shown in figure 2.26.

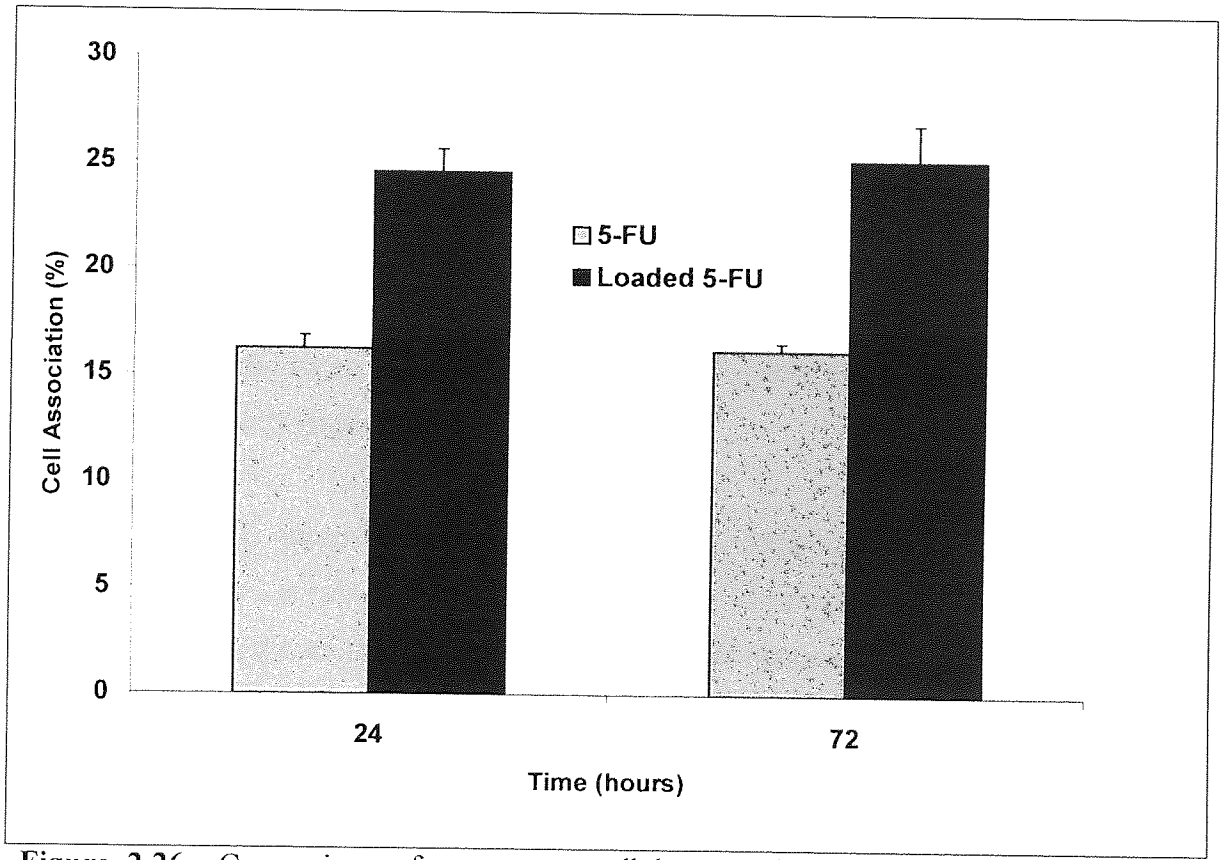


Figure 2.26 Comparison of percentage cellular association of free 5-FU and 5-FU encapsulated in P(LA-GA) microspheres at a 24-hr and 72-hr incubation period at 37°C. The free 5-FU was added in equal amounts to the entrapped 5-FU. Data represents the mean n=3+SD.

After 24-hrs the average cellular association of naked 5-FU was 16% as compared to 24% with 5-FU encapsulated in microspheres. For 72-hrs similar cellular association results were obtained to AODNs i.e. there was no significant change in cellular association after 24-hrs ($P>0.05$). The lack of difference in cellular association between 24-hrs and 72-hrs is likely to do with maximum saturation of naked or encapsulated drug with A431 cells before 24-hrs and

therefore incubation after this time point may not have any effect on cellular uptake (see section 2.3.2.3.2).

2.3.2.3.3.2 Effect of 5-FU Encapsulated in P(LA-GA) Microspheres Over 24-hrs or 72-hrs

Various microsphere masses loaded with 5-FU (prepared by the Silverson method; section 2.2.2.8.3) were used to treat A431 cells until a dose-response was achieved. 11.5μmoles of 5-FU was added to the formulation and the microspheres were made as before (see section 2.2.2.8.3). Increasing microsphere masses containing AODN (2.5-23mg) were incubated with A431 cells at 37°C for 24-hrs and 72-hrs. Cells were treated with microspheres containing 5-FU and the viable cell number was measured in the same way as for encapsulated AODN. The results are shown in figure 2.27.

The amount 5-FU being released from each microsphere mass was determined before the results could be compared and evaluated. Table 2.12 shows the formulation characteristics of microspheres loaded with 5-FU which produced a dose response effect. The release of 5-FU from microspheres was also assessed in serum-free medium to determine the amount of 5-FU released on A431 cells (figure 2.28).

The amount of 5-FU encapsulated within a microsphere formulation was calculated from the percentage yield and encapsulation efficiency. 150mg of microsphere sample was obtained from a 62% yield and therefore the mass of microspheres obtained had a total of 6.9μmoles of 5-FU from an encapsulation efficiency of 60%. It was deduced from the release data that approximately 38% and 43% of 5-FU was released from microspheres at 24-hrs and 72-hrs respectively. From these findings the amounts of 5-FU released from each mass at the relevant time points was determined (table 2.13). Percentage reduction in viable A431 cell number was determined at 24-hr and 72-hr incubation times from figure 2.27. The values obtained are shown in table 2.14.

A dose-dependent effect was produced with increasing microsphere masses encapsulated with 5-FU, for 24-hrs and 72-hrs in A431 cells. Microsphere mass of 2.5mg produced a 15%±5 reduction in A431 cell number at 24-hr incubation time as compared to the control. For a 72-hr incubation time the difference in average reduction in percentage cell number was greater

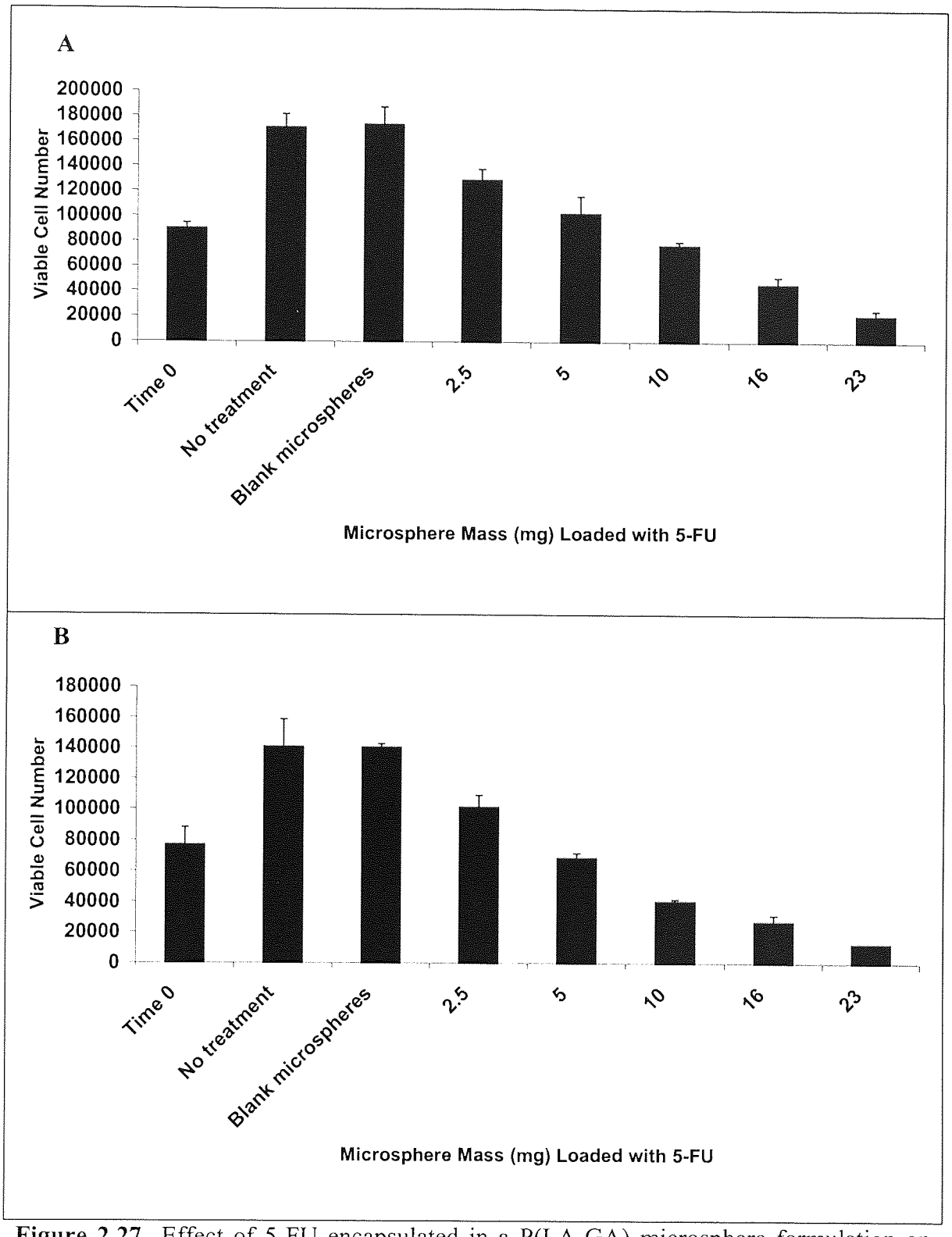


Figure 2.27 Effect of 5-FU encapsulated in a P(LA-GA) microsphere formulation on viable cell number of A431 cells over (A) 24-hrs and (B) 72-hrs in 300µl DMEM. Time 0=cell number at treatment time; blank microspheres=23mg. Data represents the mean n=3±SD.

Table 2.12 Formulation Characteristics of 5-FU Loaded P(LA-GA) Microspheres for a Dose Response Effect on A431 Cells.

Drug	% Average Cumulative Release (hours)			% yield	% Encapsulation efficiency
	0 (burst)	24	72		
5-FU	33±2	38±2	43±2	62	60±5

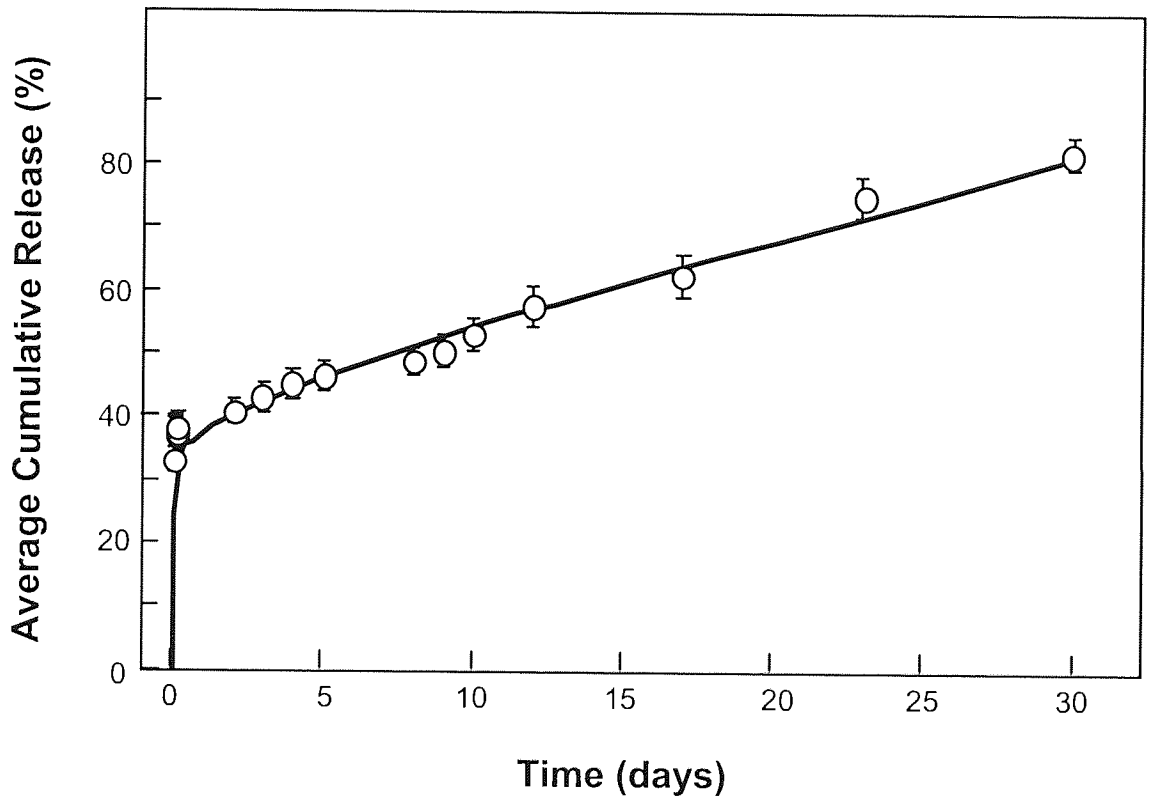


Figure 2.28 *In vitro* release profile of 5-FU from a P(LA-GA) microsphere formulations prepared for the dose response effect on A431 cells. Data represents the mean n=3±SD.

for 2.5mg of microsphere mass as compared to 24-hrs (28%±2) and which was significantly different as compared to the control ($P<0.05$). For 5mg microsphere mass the average reduction in percentage cell number was 33%±9 and 51%±10 for 24-hr and 72-hr incubation times respectively. Both differences were significant compared to the controls ($P<0.05$). Unlike AODN encapsulated microspheres, the percentage viable cell number between 24-hrs and 72-hrs remained significant even for larger microsphere masses ($P<0.05$). For 23mg microsphere masses the average reduction in percentage cell number was 86%±4 and 91%±3 for 24-hr and 72-hr incubation times respectively. Overall, data correlated well with the

release of 5-FU from the different masses of microspheres at the 24-hr and 72-hr time points (table 2.14).

Table 2.13 The Amount of 5-FU Released from P(LA-GA) Microspheres Masses at the Relevant Incubation Time points on A431 Cells to Produce a Dose Response Effect.

	5-FU Release (µmoles)	
Mass of Microsphere (mg) encapsulating 5-FU (µmoles)	**24 hrs**	**72hrs**
2.5 (0.015)	0.04	0.05
5 (0.23)	0.08	0.10
10 (0.46)	0.17	0.20
16 (0.74)	0.27	0.31
23 (1.06)	0.40	0.45

Table 2.14 The Effect of 5-FU Encapsulated in a P(LA-GA) Microsphere Formulation on the Average % Reduction in Cell Number of Control by Trypan blue dye exclusion Assay.

Microsphere Mass (mg)	**Average Reduction in % Cell Number of Control**		**(Dunnett's Test, compared to Blank Microspheres)**
	24 hrs	**72 hrs**	
2.5	15±5	28±9	$p<0.05$
5	33±9	51±10	$p<0.05$
10	49±2	71±7	$p<0.05$
16	70±4	81±4	$p<0.05$
23	86±3	91±3	p<o.05

The encapsulated 5-FU produced a greater reduction in viable cell number for a 24-hr and a 72-hr treatment period as compared to the naked 5-FU, probably due to the release of the 5-FU in close proximity to the cell monolayer during the burst and sustained release phase. The microsphere delivery system allowed a dose response effect to be achieved even after 72-hrs, contrary to the naked 5-FU which resulted in only a small number of viable cells remaining after treatment. These results suggested that 5-FU cytotoxicity could be controlled over 72-hrs using the microsphere delivery system (dose-response effect achieved) and therefore combination experiments could also be undertaken over extended time periods, unlike the naked drugs which could only be carried out over 24-hrs for the reasons discussed in section 2.3.2.2.3. This result also has important implications in the clinic where the ability to control

cytotoxicity at the target site can reduce side-effects, which is especially important for drugs such as 5-FU that cause a great deal of systemic toxicity (Ardalan *et al.*, 1996).

2.3.2.3.4 Effect of AODN in Combination with 5-FU in Separate P(LA-GA) Microsphere Delivery Systems over 72-hrs

Various microsphere masses loaded with AODNs, were combined with a 5mg microsphere mass loaded with 5-FU (a concentration which inhibited approximately 50% cell growth after a 72-hr incubation period, table 2.14). The relevant mixtures of microsphere masses containing each drug were incubated for 72-hrs with A431 cells at 37°C. The study was conducted for a 72-hr time period because previous results indicated that this time-point produced the most significant effects on viable cell number (table 2.14). The results are shown in figure 2.29.

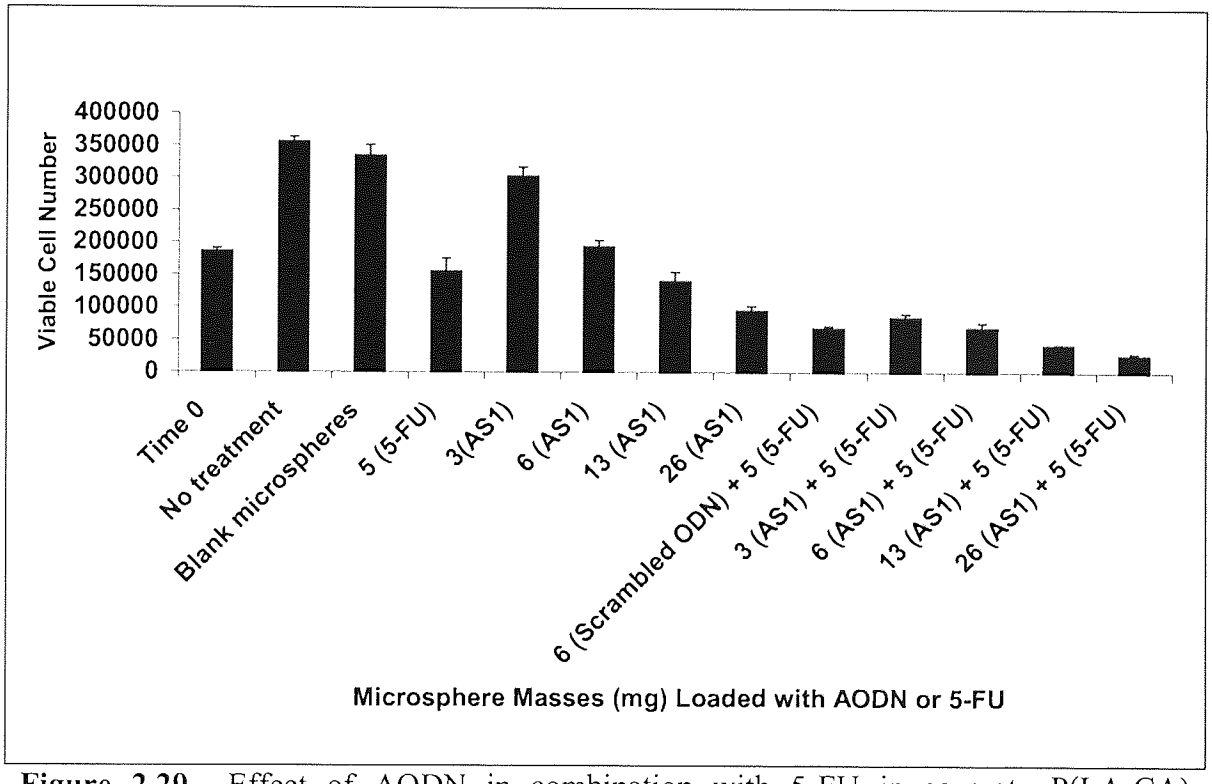


Figure 2.29 Effect of AODN in combination with 5-FU in separate P(LA-GA) microsphere delivery systems on viable cell number of A431 cells over 72-hrs in 300µl DMEM. Time 0=cell number at treatment time; blank microspheres=31mg. Data represents the mean n=3±SD.

Combining separate microsphere formulations loaded with either AODN or 5-FU resulted in an additive response in reducing viable cell number as compared to either encapsulated drug alone. 5-FU encapsulated in 5mg of microspheres resulted in a 56%±6 reduction in cell

number as compared to the controls. AODN encapsulated in 3mg of microsphere mass resulted in a 14%±4 reduction in cell number as compared to the controls. However, a 75%±1 reduction in cell number was observed when these microsphere masses containing each drug were combined. Additive effects were also observed with larger microsphere masses containing AODN. For example, AODN encapsulated in 6mg of microsphere mass resulted in a 45%±3 reduction in cell number. However, an 80%±2 reduction in cell number was observed when the AODN encapsulated microsphere mass (6mg) was combined with 5mg of microsphere mass containing 5-FU. Table 2.15 summarises the data obtained.

Table 2.15 The Effect of AODN in Combination with 5-FU in Separate P(LA-GA) Microsphere Delivery Systems on the Average % Reduction in Cell number of Control by Trypan blue dye exclusion Assay.

Separate microsphere masses (mg) encapsulated with each drug		**Average reduction in % cell number of control when microsphere masses combined**	**(Dunnett's Test, compared to Blank Microspheres)**
AODN	**5-FU**		
0	5	56±6	p<0.05
3	0	14±4	p<0.05
6	0	45±3	p<0.05
13	0	60±4	p<0.05
26	0	72±2	p<0.05
3	5	75±1	p<0.05
6	5	80±2	p<0.05
13	5	87±0.2	p<0.05
26	5	92±1	p<0.05

Similar effects were seen with 6mg and 5mg of microsphere masses encapsulated with scrambled AODN and 5-FU respectively (figure 2.29), suggesting that the additive effect is brought about by a non-antisense cytotoxic effect. The percentage reduction in viable cell numbers observed with AODN and 5-FU combined in separate microsphere formulations on A431 cells was greater than combining naked AODNs and 5-FU after 72-hrs. This indicated that combining AODN and 5-FU in separate microsphere systems enhanced the cytotoxic effects of the drugs as compared to naked drug combinations (discussed in section 2.3.2.3.5).

It is clear from the above data that combining encapsulated AODN with 5-FU in separate microsphere masses have an additive effect on the percentage reduction in A431 cell number

as compared to either encapsulated drug on its own. To assess if these results were due to an antisense effect, protein EGFR expression of A431 treated cells was required for analysis.

2.3.2.3.5 The Effect on EGFR Expression in A431 Cells Treated with AODN in Combination with 5-FU in Separate P(LA-GA) Microsphere Delivery Systems over 72-hrs

Assessment of EGFR protein expression was undertaken to further investigate the effects of combining separate microsphere formulations encapsulating either AODN and/or 5-FU on A431 cells. The cells were treated with the relevant microsphere masses loaded with each drug as before (see section 2.2.2.11.3) and a Western blot was conducted as described in the methods section 2.2.2.12. AODN and 5-FU encapsulated in 6mg and 5mg microsphere masses respectively were used on A431 cells. Scrambled AODN with the same loading as AODN was also encapsulated in 6mg of microsphere mass and was used as a control. From the western blot no significant decrease in EGFR protein expression was evident with the relevant treatments (figure 2.30).

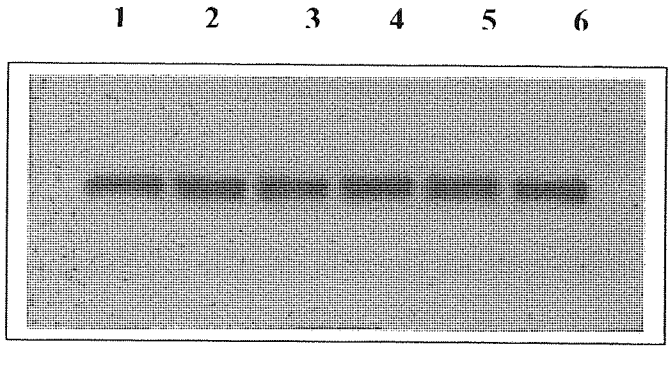


Figure 2.30 Effect of EGFR expression in A431 cells treated with AODN in combination with 5-FU in separate P(LA-GA) microsphere delivery systems over 72-hrs. Lane 1= control (no treatment), lane 2= 6mg microspheres (scrambled AODN), lane 3= 6mg microspheres (AODN), lane 4= 5mg microspheres (5-FU), lane 5= 6mg microspheres (scrambled AODN)+ 5mg microspheres (5-FU), lane 6= 6mg microspheres (AODN)+ 5mg microspheres (5-FU). Images depict representative blots from duplicate experiments.

The trypan blue dye exclusion assay suggested that combining AODN with 5-FU in separate microsphere formulations had a greater effect on viable cell number in A431 cells as compared to either encapsulated drug on its own. However, the effect was unlikely to be an antisense mechanism because no differences in EGFR expression was observed by Western

blotting. To further confirm that the dose response effect obtained with AODN encapsulated in P(LA-GA) microspheres was not due to an antisense effect, a Western blot analysis was undertaken to assess EGFR expression in A431 cells with a range of microsphere masses encapsulated with AODN (figure 2.31). Even at higher microsphere masses no differences in EGFR expression was observed by western blotting.

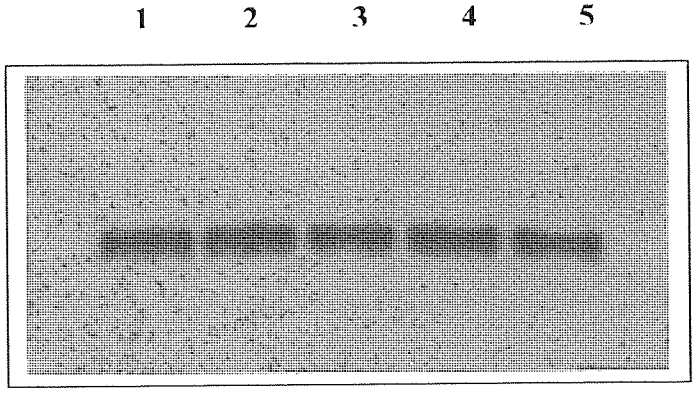


Figure 2.31 Effect of EGFR expression in A431 cells treated with increasing microsphere masses encapsulating AODN over 72-hrs. Lane 1= control (no treatment), lane 2= 2.5mg microspheres, lane 3= 5mg microspheres, lane 4= 10mg microspheres, lane 5= 23mg microspheres. Images depict representative blots from duplicate experiments.

Overall, there was no change in the expression of EGFR in A431 cells with a variety of microsphere masses encapsulating AODN. The successful delivery of AODNs to the target site by microspheres is likely to be dependent upon the release of intact AODNs into the cytosol from the microspheres, which are taken up by an endocytic pathway and are present within endosomal vesicles (Chavany *et al.*, 1994; Lewis *et al.*, 1995; Akhtar *et al.*, 1997; Hughes *et al.*, 2001). It is possible therefore that a 72-hr incubation period of microspheres with cells was not sufficient for enough AODN molecules to be released into the cytosol or the nucleus to have an effect. This is contrary to delivery systems such as lipids where the majority of the AODN is released in to the cytosol from the endosomal vesicles, rather than the release of small amounts of drug over a longer time period as in microsphere delivery systems (Guise *et al.*, 1987; Lappalainen *et al.*, 1996; Wielbo *et al.*, 1997). Incubation of microspheres with cells for longer time periods would result in over-confluency of cells and subsequently cell death, ultimately leading to invalidation of results. It is not surprising therefore that the majority of successful studies to date on the efficacy of AODN microsphere delivery systems are restricted to *in vivo* (Putney *et al.*, 1999; Khan *et al.*, 2000). Animal

studies allow microspheres to fulfil their requirements as delivery systems such as improving pharmacokinetic, sustained release and stability properties of AODNs, with the subsequent ability to show their full potential (Khan *et al.*, 2000).

These results suggested that the growth inhibition of A431 cells seen by the trypan blue dye exclusion assay was due to a non-antisense cytotoxic effect (see section 2.3.2.2.3). The results also indicated that utilising the microsphere delivery system enhanced the effectiveness of the mechanism involved for the reduction of A431 viable cell number. It is likely that the effects and mechanisms of action seen by encapsulated AODN and 5-FU are similar to that of naked drugs (see section 2.3.2.2.3). However, the greater effects of encapsulated AODN and 5-FU is likely to do with the greater number of AODN and 5-FU molecules being released in close proximity to the surface of the cell monolayer (micospheres binding to cell membrane), during the burst phase as well as the sustained phase (for those microspheres which have not entered the cells) as compared to naked drugs.

2.4 CONCLUDING REMARKS

The use of P(LA-GA) microspheres for the delivery of AODNs and 5-FU have been evaluated in this chapter. Microsphere formulations loaded with each drug individually or together were characterised in terms of microsphere morphology, size and encapsulation efficiency as well release profile. Microspheres were prepared using the silverson and the vortex w/o/w double emulsion methods. The resultant optimised formulation was then tested on A431 vulval carcinoma cell line.

Using a double-emulsion method for preparing the P(LA-GA) microspheres, successful entrapment and sustained release over 28 days was observed in both types of formulation (separate or co-entrapped microspheres). Release of AODN and 5-FU from all formulations appeared to be biphasic. However, the release rates of the two agents were slower when co-entrapped as a single microsphere formulation compared to those obtained with the separate formulations. Electrophoretic mobility shift assays (EMSA) suggested that this might be, in part, due to an interaction of 5-FU with the ODN or alternatively the difference in drug distribution within the microsphere matrix. Further, the data suggested that by mixing individual formulations of 5-FU and ODNs at different mass ratios allowed greater flexibility in achieving the desired release profile as well as avoiding potential drug-drug interactions. The Silverson method produced smaller microspheres as compared to the vortex method.

Thus, co-administration of Silverson-prepared individual P(LA-GA) microsphere formulations of AODNs and 5-FU, at appropriate mass ratios were utilised for efficacy studies.

The trypan blue dye exclusion assay demonstrated a reduction in viable cell numbers of A431 cells treated with naked AODN and 5-FU in combination. However, Western blots showed no reduction in EGFR expression. Control experiments (scrambled AODN) suggested a likely non-antisense cytotoxic effect. Trypan blue dye exclusion data also indicated that a more controlled reduction in viable cell number (dose-response effect) could be achieved over 72-hrs with the microsphere delivery system as compared to naked 5-FU. The reduction of A431 viable cell number was more pronounced for AODN and 5-FU loaded in microsphere delivery systems as compared to naked drugs, but western blots showed no EGFR down-regulation. The enhanced non-antisense cytotoxic effects of the AODN, encapsulated in the microsphere delivery system, was likely to do with the greater number of AODN and 5-FU molecules released in close proximity to the surface of the cells, due to electrostatic binding between the cationic microspheres and the anionic glycoprotein on the cell surface membranes, during the burst phase as well as the sustained phase. The study also highlighted the limitation of using the microsphere delivery system in cell culture where the time available for incubation restricts the full potential of the delivery system in terms of enhancing the pharmacokinetic/pharmacodynamic properties of AODNs.

CHAPTER THREE

CELLULAR UPTAKE PROPERTIES AND EFFICACY OF AN ANIONIC DENDRIMER DELIVERY SYSTEM FOR ANTISENSE OLIGONUCLEOTIDES

3.1 INTRODUCTION

Dendrimers, which are highly branched, three- dimensional, macromolecular structures, may be good candidates for the increased uptake and stability of AODNs, because their defined polymerisation reactions yield a monodispersed globular shape with a large number of controllable 'peripheral' functional groups which are characteristics of an attractive delivery system. The type of dendrimer synthesised is strongly dependent upon the monomer, building block monomer and the functional groups. Starburst polyamidoamine (PAMAM) dendrimers are currently the most popular types of dendrimers utilised for the delivery of AODNs (for reviews see: Eichman *et al.*, 2000; Liu *et al.*, 1999). The cationic surface charge of PAMAM dendrimer provides a suitable surface for the anionic AODNs to bind. PAMAM dendrimers have mediated high-efficiency transfection of DNA in to a variety of cultured mammalian cells (Haensler *et al.*, 1993; Bielinska *et al.*, 1996). Pharmacological effectiveness of anti-*MDR1* AODNs has been enhanced by utilising PAMAM dendrimers as carrier systems (Alahari *et al.*, 1998). Metabolic degradation of the AODNs has also shown to be reduced by 75% compared to controls when complexed with dendrimers in serum and in lysosomes (Poxon *et al.*, 1996). However, a major drawback of these cationic dendrimers is their haemolytic and cytotoxic effects on cells which are dependent upon the molecular weight and the number of surface groups (Malik *et al.*, 2000). Anionic dendrimers on the other hand, have shown no cytotoxic effect on cells over a broad concentration range (Malik *et al.*, 2000) but their use has been limited because cell charge may preclude cell surface binding. This is contrary to the results of a study conducted in which a PAMAM dendrimer was modified to provide a net negative charge, which resulted in greater cellular uptake as compared to a cationic PAMAM dendrimer (Wiwattanapatapee *et al.*, 2000). Enhanced

efficacy has also been demonstrated by AODNs encapsulated in anionic liposomes as compared to cationic liposomes (Lakkaraju *et al.*, 2001).

It is envisaged that the ability to covalently link AODNs to dendrimers would allow tighter control over the release of AODNs once inside the cells i.e. modifying the covalent link would enable AODNs to be released within the cell at the desired site. Although drugs such as 5-FU have been covalently linked to dendrimers (Zhuo *et al.*, 1999), there are no reports of AODN attached to dendrimers in a similar manner. This principal may open new avenues to further improve the efficacy of AODNs e.g. covalently linking other drugs in conjunction with AODNs on the same dendrimer molecule to control the release of each drug at the desired site within the cell by modified covalent links. Indeed, it may not even be necessary for covalently linked AODN to detach from the dendrimer to have an effect at the target site. In this chapter we examine intracellular stability, mechanism of uptake and efficacy of an anionic dendrimer based on a pentaerythritol structure (Shchepinov *et al.*, 1997), covalently linked to AODNs, complementary to *c-erb*B1 mRNA which encodes for the EGFR (see section 1.7).

3.1.1 Synthetic Perspectives

Control over the physical properties of dendrimers is an important characteristic, with the manufacturer having full control over almost every aspect of the dendritic design (for reviews see: Moorefield *et al.*, 1994; Liu *et al.*, 1999; Eichman *et al.*, 2000). The concept of directionality is an important factor in the dendrimer structure (Figure 3.1). The direction in which the polymer will grow is dependent upon the number and position of reactive groups (which attach to the initial core or monomer). For example, a monomer with one reaction site will grow in one direction, whereas a monomer with three attachment sites will grow in three directions. Directionality is also dependent upon the building block monomers. A layer of monomer building blocks are linked to the initial monomer (core) via the reactive sites. The number of building blocks that can be added is dependent upon the number of available reactive sites on the particular core. An important consideration is the nature of the branching centre e.g. a pyramidal branching centre (e.g. NH_3) will direct polymer growth differently than a planer tri-directional branching centre (e.g. 1,3,5-trisubstituted benzene) (figure 3.1 and 3.2).

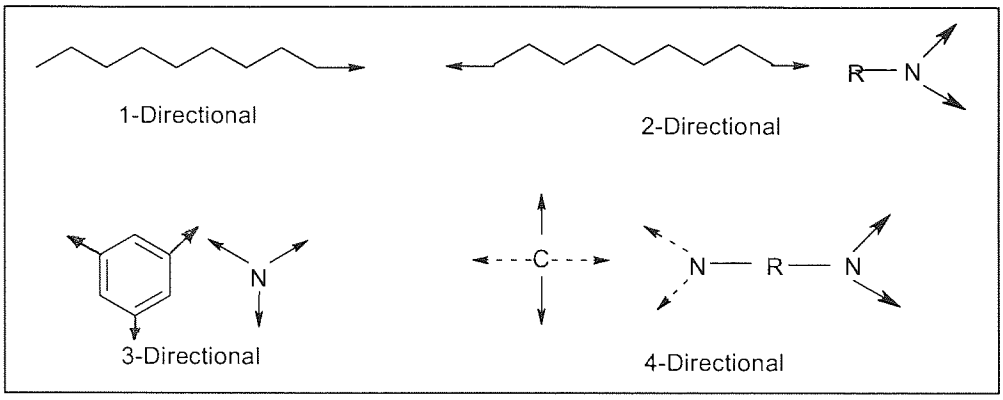


Figure 3.1 Monomers illustrating the concept of directionality associated with dendrimer growth (adapted from Moorefield and Newkome, 1994).

The number of surface groups, the shape, porosity, density, hydrophobic/hydrophilic nature and stability is dependent upon the choice of the initial core and the building block (Moorefield *et al.*, 1994). It is not surprising, therefore, that the overall shape of the end dendritic polymer is dicatated by the choice of core and building block.

3.1.2 Polyamidoamine Dendrimers

To date, most studies regarding dendrimer delivery of ODNs have been performed using the polyamidoamine (PAMAM) starburst dendrimers (Haensler *et al.*, 1993; Bielinska *et al.*, 1996; DeLong *et al.*, 1997). PAMAM starburst dendrimers possess a hydrocarbon core, charged surface amino groups and have a well-controlled chemistry. The cationic surface groups facilitate the binding of anionic ODNs by electrostatic interactions (figure 3.3). The growth of dendrimer emanates from an initiator core molecule such as ammonia or ethylenediamine (EDA). The overall molecular and surface charge density depends upon the initiator core molecule. A series of stepwise polymerisation reactions allow the polymer to grow outwards to form layers (i.e. generations) from the initiator core and ultimately to form a tree-like structure. (Bielinska *et al.*, 1996). Dendrimer synthesis requires an iterative multistep reaction sequence that involves a Michael addition of methyl acrylate followed by amidation with ammonia. Each iteration leads to a new generation that is identified by successive integers (e.g. generation 0, generation 1, generation 2). This gives a high density of primary amino groups located on the external surface of the dendrimer that are positively charged at the physiological pH (Figure 3.4).

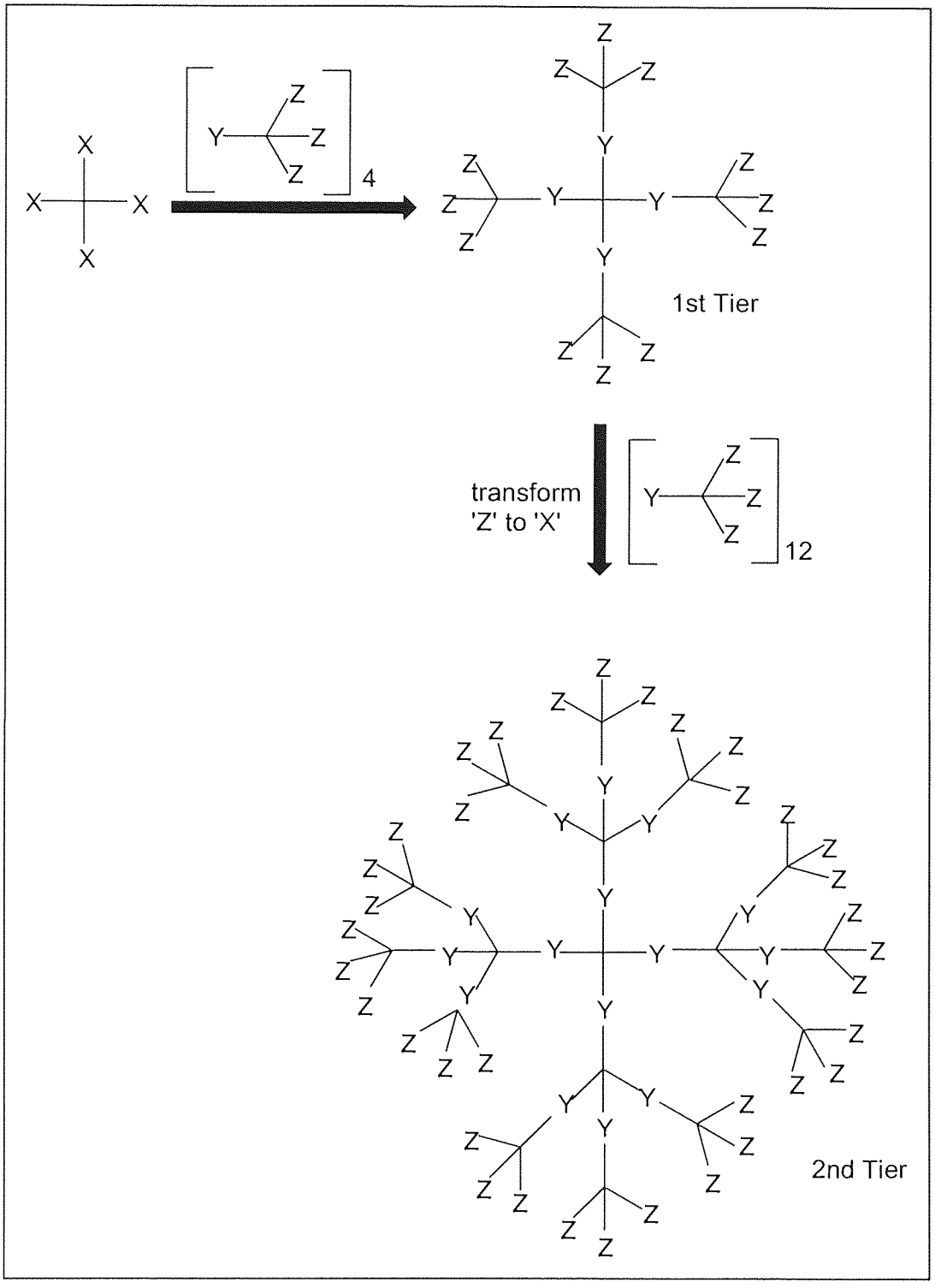


Figure 3.2 An illustration of divergent iterative strategy used for dendrimer preparation (adapted from Moorefield and Newkome, 1994).

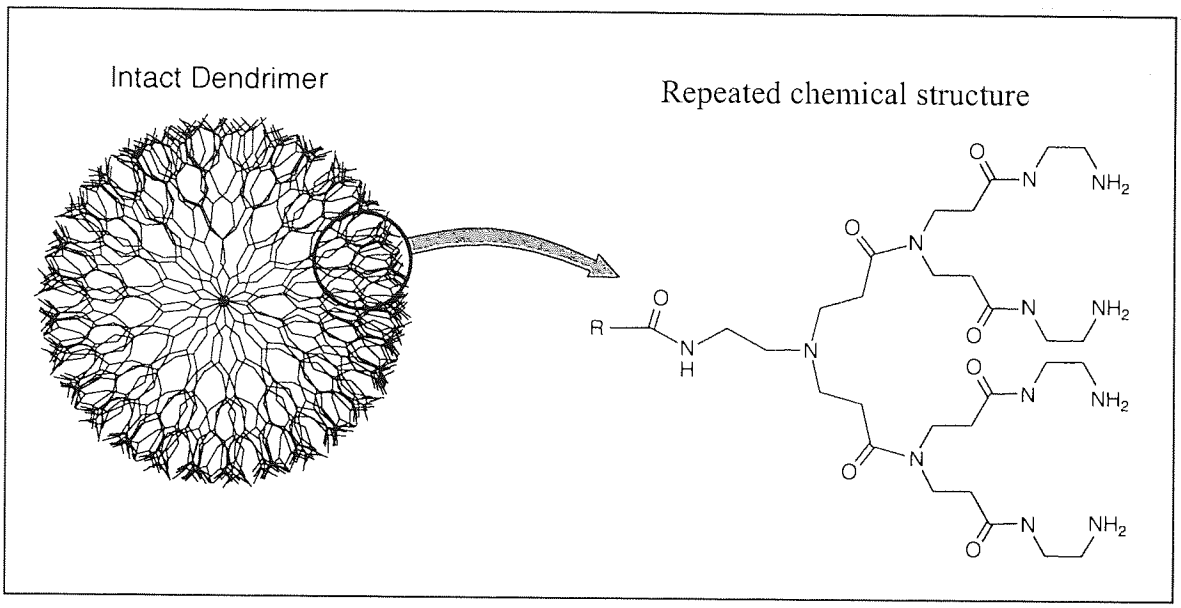


Figure 3.3 Representation of the globular structure of the PAMAM dendrimer. Enlargement shows the regular chemical structure and the terminal amino groups that facilitate nucleic acid binding (Hughes *et al.*, 2001).

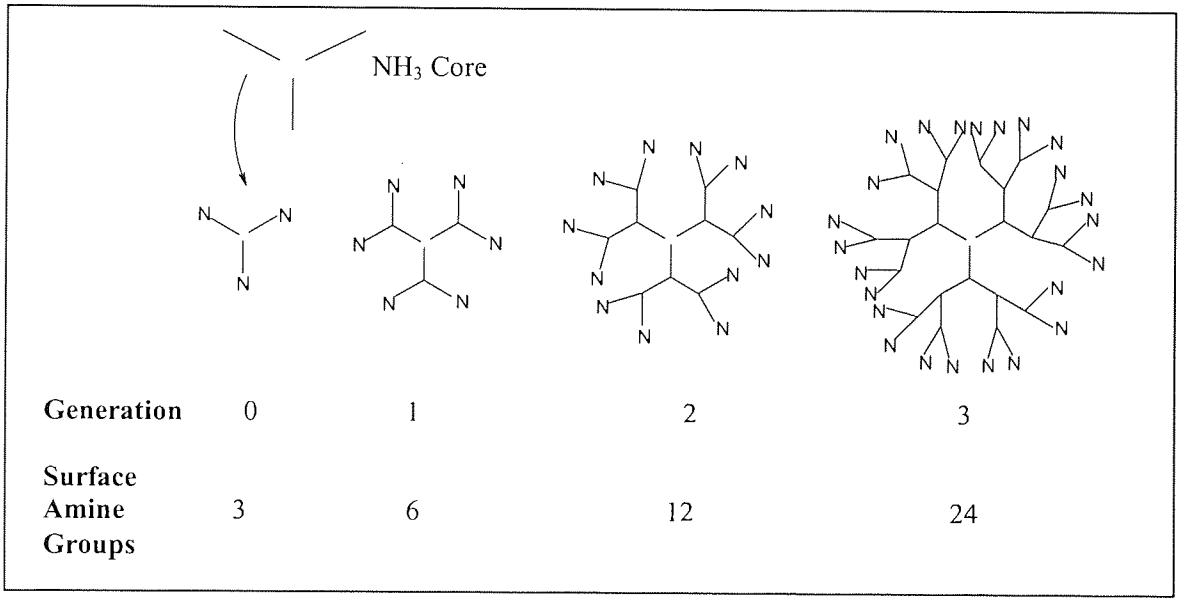


Figure 3.4 Schematic presentation of PAMAM dendrimer structure and growth (adapted from Bielinska *et al.*, 1996).

3.1.2.1 PAMAM Dendrimer Characterisation

Dendrimers range from 10 Å to 130 Å in diameter for generation 0 (G0) through to generation 10 (G10) and therefore are classified as nanoscale molecules. General nomenclature for PAMAM dendrimers with an initiator core e.g. ethylenediamine (EDA) is Gx EDA, where x is the particular generation and $GxNH_3$ for ammonia core molecules. The molecular weight is

increased exponentially with each new layer synthesised, the number of primary amine surface groups are doubled and the diameter is increased by approximately 10 Å. Polymer shapes may be affected from lower generation to higher generation dendrimers i.e. planer polymer shapes are formed for G0 to G4 dendrimers and spherical shapes are seen for G5-G10 dendrimers (for review see: Baker *et al.*, 1994).

3.1.2.2 PAMAM Dendrimer Delivery of DNA

Stable complexes between ODNs and cationic dendrimers form in a variety of solutions, such as water, phosphate-buffered saline and serum (DeLong *et al.*, 1997). PAMAM dendrimers form complexes with DNA through electrostatic interactions between negatively charged phosphate groups of the nucleic acid and the protonated (positively charged) primary amino groups on the dendrimer surface. Charge neutralisation of both components and alterations of the net charge of the complex lead to changes in its physiochemical and biologically relevant properties (Tang *et al.*, 1997; Bielinska, 1999). The formation of high molecular weight and high-density complexes depends strongly on the DNA concentration. The precipitate formation increases as the DNA concentration rises from 10 ng ml^{-1} to 1 mg ml^{-1}. Insoluble, high density aggregates are not formed with DNA concentrations of less than 10 ng ml^{-1}, complexed to G5, G7, or G9 generations for both NH_3 and EDA cores. Increasing the dendrimer-DNA charge ratio facilitates complex formation. Studies suggest that greater than 90% of transfection is dependent upon soluble and low-density sub-populations of complexes and therefore progressive increases in the dendrimer-DNA charge ratio would allow soluble and low-density complexes to be formed leading to enhanced transfection (Wolfert, 1996). Complexes formed at higher DNA concentrations usually result in a non-uniform distribution of larger, higher-density aggregates and precipitates.

PAMAM dendrimer complexes (5000-10000 Mw) have shown enhanced ODN uptake and intracellular availability with increased delivery to the nucleus (Bielinska *et al.*, 1996). PAMAM dendrimers mediate the high efficiency transfection of several cultured mammalian cell lines such as the CV-1 (monkey fibroblast), HeLa (human carcinoma) and HepG2 (human hepatoma) (Haensler *et al.*,1993). Dendrimer-ODN complexes cause a 75% reduction in metabolic degradation of phosphodiester ODNs in serum and also afford protection from the lysosome (Poxon *et al.*, 1996). A study which examined the delivery of ODNs with PAMAM dendrimers found that the dendrimer remains complexed with the ODN

as it enters the cell nucleus (Yoo *et al.*, 2000). It was also reported that conjugation of the dendrimer to a small fluorescent dye enhanced its ability to deliver ODNs and increased its effectiveness in serum. This offers the potential for tailoring the dendrimer surface structure to achieve the desired uptake properties. Table 3.1 gives some examples of successful cellular uptake experiments. It is clear from table 3.1 that PAMAM dendrimers have significant potential for use as drug delivery agents, however, the biocompatibility and toxicity of the dendrimers must be fully understood before proceeding with more relevant third-generation designs.

Table 3.1 A Summary of Studies Utilising Dendrimers to Successfully Deliver AODNs.

Reference	Dendrimer description	Cell line	Genteic material complexed to the dendrimer	Comments
(Haensler *et al.*,1993)	Ammonia initiator core (generation 5 and 6)	CV-1 (monkey fibroblasts), HeLa (human carcinoma) and HepG2 (human hepatoma)	Plasmids encoding firefly luciferase and β-galactosidase	Dendrimers mediate high efficiency transfection of a variety of cultured mammalian cells. Maximum transfection efficiency was noted with generation 5 dendrimers (54 A). There was a 2-fold decrease in expression of luciferase in 10% serum.
(Bielinska *et al.*, 1996)	Ammonia initiator core (generation 6, 7 and 10)	D5 mouse melanoma and Rat2 embryonal fibroblasts	Luciferase reporter plasmid and antisense luciferase mRNA expressing plasmids	The specific reporter luciferase gene expression was inhibited by 30 to 60%. Generation 6 provided greatest luciferase inhibition.
(Poxon *et al.*, 1996)	Ethylenediamine core (generation 2, 4 and 5)	Chinese hamster ovary	AODN against the initiation codon of firefly luciferase	15-fold uptake of antisense oligonucleotides
(DeLong *et al.*, 1997)	Ethylenediamine core (generation 3)	Human astrocytoma (U251)	AODN	50-fold enhancement in cell uptake of ODN. An enhanced cytosolic and nuclear availability was observed.
(Alahari *et al.*, 1998)	Ethylenediamine core (generation 7)	NIH 3T3	AODN targeted against the MDR1 gene	8-fold increase in oligonucleotide uptake. Nuclear availability was observed.

Reference	Dendrimer description	Cell line	Genteic material complexed to the dendrimer	Comments
(Qin *et al.*, 1998)	Ethylenediamine core (generation 5)	Murine cardiac grafts	Plasmid encoding viral interleukin-10	Dendrimer induced a >1000-fold increase in gene transfer efficiency in myocytes and graft infiltrating cells, compared with naked plasmid. Plasmid encoding viral interleukin-10 complexed to dendrimer led to increased graft survival days.
(Yoo *et al.*, 1999)	Ethylenediamine core (generation 4, 5 and 7)	HeLa cells	AODN directed against the beta-globulin 705 splice site to correct splicing and allow luciferase expression	Most promising results were observed with generation 5 and 7. Moderate activity of antisense oligonucleotide activity remained in 10% serum and some activity even remained in the presence of 70% serum.
(Yoo *et al.*, 2000)	Ethylenediamine core (generation 5) conjugated with fluorescent dye Oregon green 488 (hydrophobic small molecule	HeLa cells	A~ODN directed against the beta-globulin 705 splice site to correct splicing and allow luciferase expression	The dendrimer-oligonucleotide complex remained associated during the process of uptake into vesicular compartments and eventual entry into the nucleus. The Oregon green 488-conjugated dendrimer was a much better delivery agent.

3.1.2.2.1 PAMAM Dendrimer Delivery of DNA In vivo

Studies that allow us to observe the pharmacokinetic and biocompatibility profiles of dedrimers are important if they are to be utilised for *in vivo* delivery systems. A few reports concerning the biocompatibility of dendrimers have been published. The cytotoxicity of PAMAM dendrimers have shown to be concentration- and generation-dependent (Roberts *et al.*, 1999). In this study, three dendrimer generations were evaluated for their toxicity effects i.e. generations 3, 5 and 7 in male Swiss-Webster mice. PAMAM dendrimers did not show any significant toxicity or evidence of any immune response when the polymers were administered alone or in combination with an adjuvant. However, in a recent study (Malik *et al.*, 2000) toxicity in rats was observed at concentrations of PAMAM dendrimers greater than 1.0 mg ml^{-1}. Several parameters such as the dendrimer generation and concentration were

important for the determination of hemolytic and cytotoxic effects. The same study also evaluated anionic PAMAM dendrimers for their toxicity properties. These dendrimers showed less toxic effects as compared to cationic PAMAM dendrimers (Malik *et al.*, 2000).

In vivo studies are presently being conducted to deliver dendrimer-DNA complexes intravenously in mice. The greatest quantity of gene expression in Balb/c mice has been obtained in the bronchial and alveolar regions of the lung . It is also possible that *in vivo* targeted gene delivery using antibody conjugates may also be eventually achieved. The reproducible and defined small size of dendrimers offers a significant advantage over other particulate systems. However, preliminary toxicity data *in vivo* suggests that extensive testing is necessary to establish biocompatibility and to fully evaluate the potential use of dendrimers for delivering antisense molecules (Roberts *et al.*, 1999; Malik *et al.*, 2000).

3.1.3 Anionic Dendrimer Based on a Pentaerythritol Structure

In this chapter we examine intracellular stability, mechanism of uptake and efficacy of a novel anionic dendrimer, based on a pentaerythritol structure (Shchepinov *et al.*, 1997; Shchepinov *et al.*, 1999), covalently linked to AODNs (the molecular weight of the complex was ~59 kDa), complementary to *c-erb*B1 mRNA which encodes for the EGFR. The dendrimer-AODN was a generous gift from Dr. M.S. Shchepinov (see section 3.2.2.1).

3.2 MATERIALS AND METHODS

3.2.1 Materials

All chemicals used were of the highest grade available from Sigma Chemical Company (poole, UK) unless otherwise specified. All reagents were used as received without further purification.

3.2.2 Methods

3.2.2.1 Dendrimer Synthesis

Synthesis, characterisation and quantification of the dendrimer delivery system was undertaken by Shchepinov *et al.*, 1997. The dendrimer was a generous gift from Dr. M.S. Shchepinov, Department of Biochemistry at the University of Oxford. A Dendrimer-AODN complex containing nine ODN molecules of the same sequence (5' TTT CTT TTC CTG CAG AGC CCG 3') was used for the study (figure 3.5). The AODNs were linked to the dendrimer by normal phosphoramidite chemistry and were labelled with [γ-^{32}P]ATP at the 5' end. The labelling of Dendrimer-AODN with [γ-^{32}P]ATP was performed in our own laboratory at Aston University (see section 3.2.2.2).

3.2.2.2 5' –End [^{32}P] -Radiolabelling of ODNs Attached to the Dendrimer

The dendrimer-linked AODNs were labelled at the 5'-end with [γ-^{32}P]ATP (Amersham, UK) in a 20µl reaction containing 4µl 5x reaction buffer (100mM Tris pH 7.5, 20mM $MgCl_2$, 10mM DTT, 0.2mM spermidine and 0.2mM EDTA), 5µl of [γ-^{32}P]ATP (4500Ci /mmol) (Amersham, UK), 20 units of T4 polynucleotide kinase (GibcoBRL, Paisley, Scotland), 5µl of the relevant dendrimer-AODN and made to 20µl with double distilled water. The reaction was incubated at 37°C for 45min. The products were separated on 6% native polyacrylamide gels for 3h at 10 watts. The products were visualised by autoradiography and the appropriate region of the gel excised and purified as described previously (see section 2.2.2.5).

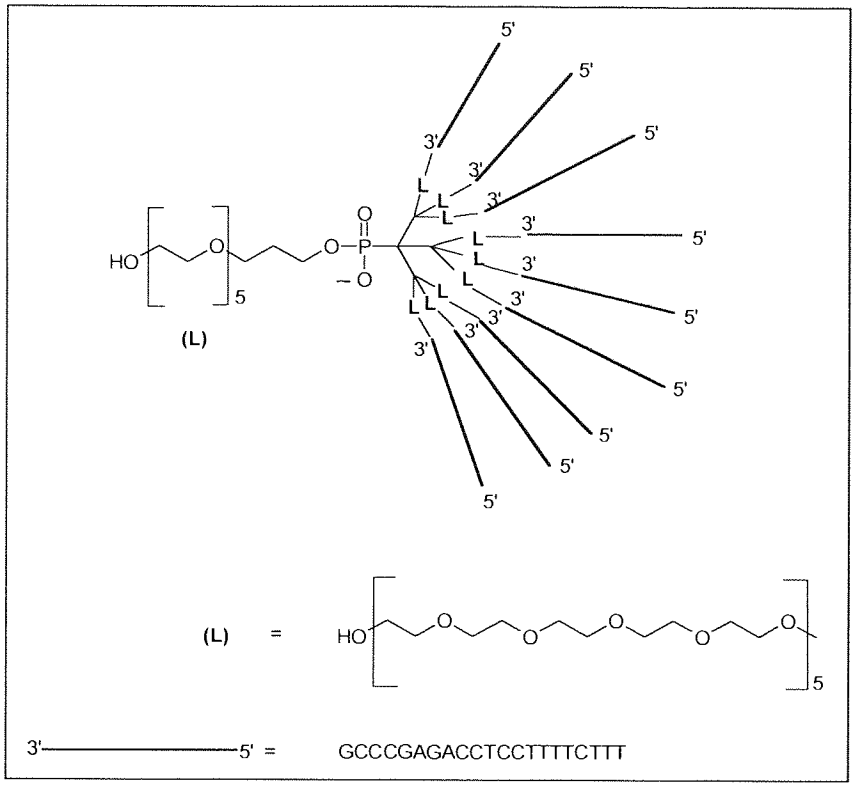


Figure 3.5 Anionic dendrimer based on a pentaerythritol structure, covalently linked to AODNs by phosphoramidite chemistry (adapted from Shchepinov *et al.*, 1997).

3.2.2.3 5'- End Fluorescein Labelling

A fluorescent label was attached to the 5'-end of ODNs during automated synthesis. Fluorescein cyanoethyl phosphoramidite (Cruachem) was reconstituted in DNA grade acetonitrile to a concentration of 0.1M and added to a spare position on the DNA/RNA synthesiser. The custom synthesis cycle for use with ribonucleoside phosphoramidites was used due to the longer coupling times allowed with this cycle, to ensure complete addition. 5'-FITC labelled ODNs were deprotected as previously described for synthesised ODNs (see section 2.2.2.1).

A fluorescent label attached to the 5'end of ODNs linked to the dendrimer was synthesised, characterised and quantified by Dr. Shchepinov at Oxford University.

3.2.2.4 Stability Studies

3.2.2.4.1 Stability in DMEM (Serum Free)

Radiolabelled AODNs / Dendrimer-AODNs were incubated in 300µl of DMEM (serum-free) at 37°C to give a final concentration of 100nM. Samples (10µl aliquots) were removed at timed intervals, mixed with a loading buffer containing 9:1 v/v formamide:1 x TBE, 0.25% xylene cyanol, 0.25% bromophenol blue and frozen at –20°C prior to gel loading. Degradation profiles were analysed on a denaturing gel electrophoresis prepared by the addition of 8M-urea (480g) to the native gel mixtures (20% and 6% polyacrylamide for AODN and dendrimer-AODN respectively). Native gel preparation and autoradiography of wet gels was carried out as before (see section 2.2.2.5.2).

3.2.2.4.2 Stability in Foetal Bovine Serum (FBS)

Radiolabelled AODNs / Dendrimer-AODNs were incubated in 300µl of 10% FBS in DMEM at 37°C to give a final concentration of 100nM. Samples (10µl aliquots) were removed at timed intervals, mixed with a loading buffer containing 9:1 v/v formamide 1 x TBE, 0.25% xylene cyanol, 0.25% bromophenol blue and frozen at –20°C prior to gel loading. Samples were treated with 5µl proteinase K (0.1mg) and heated at 60°C before loading the sample on to the gel. Degradation profiles were analysed by denaturing gel electrophoresis as described in section 2.2.2.5.1.

3.2.2.4.3 Stability in U87-MG/A431 Cell Supernatants

U87-MG and A431 cells (see section 2.2.2.11.1) were seeded onto 24-well plates at a density of $5x10^4$ cells/well as previously described in section 2.2.2.11.7 and used approximately 48-hrs post seeding. Radiolabelled AODNs/Dendrimer-AODNs were added to 300µl of serum-free DMEM medium to give a final concentration of 100nM and incubated at 37°C. 10µl aliquots of the apical solution were collected at variable time points over a period of 4-hrs, mixed with an equal volume of formamide loading buffer (9:1 v/v formamide: 1 x TBE). Samples were loaded and bands were detected by autoradiography of wet gels as described in section 2.2.2.5.1.

3.2.2.5 Cell Association Studies

Cell culture conditions and the experimental procedure undertaken for all cell association studies are detailed in sections 2.2.2.11.

3.2.2.5.1 Assay to Determine the Number of PBS-Azide Washes

Cells were seeded onto 24-well plates at a density of $5x10^4$ cells/well and used approximately 48-hr post-seeding. Cells were incubated for 3-hrs at 37°C with either [^{32}P]-labelled 0.1nM AODN or 1.3nM dendrimer-AODN in 300µl of DMEM. Following incubation, cells were washed with ice-cold PBS-azide (0.05% w/v sodium azide in sterile PBS) (0.5ml x 5min) an increasing number of times i.e. from 1 wash to 7 washes, The radioactivity associated with the apical samples, respective wash samples and cell fractions from each assay were counted by the scintillation counter (section 2.2.2.7).

3.2.2.5.2 Cell Association of Radiolabelled Mannitol

To assess the cell association of the fluid phase D-[1-^{14}C] Mannitol marker, cell association studies were performed as described in section 3.2.2.5.3 except radio-labelled AODN or dendrimer-AODN was replaced with D-[1-^{14}C] Mannitol (Amersham Life sciences, Amersham, U.K.). Quantities of radiolabelled mannitol in each of the three fractions collected were assessed by scintillation counting. In this case, however, fractions were added to 10ml of Optiphase Hi-safe 3 (Pharmacia-Wallace, St.Albans, U.K.) and counted for 10 minutes using an appropriate programme for the detection of ^{14}Carbon.

3.2.2.5.3 The Effect of Time on Cellular Association

The cells were seeded at a density of $5x10^4$ cells/well in DMEM containing 10% FBS onto 24-well plates as previously described and used approximately 48-hr post-seeding (see section 2.2.2.11). U87-MG/A431 monolayers were incubated with either [^{32}P]-labelled 0.1nM AODN or 1.3nM dendrimer-AODN in 300µl of DMEM at 37°C for varying time periods (30-180 minutes) and cellular association studies were conducted as described in section 2.2.2.11.7.

3.2.2.5.4 The Effect of Temperature on Cellular Association

For low temperature experiments (at 4°C), the following modifications were adopted. After the required growth period (48-hr post seeding) cells were washed twice with ice-cold sterile PBS (2 x 0.5 x 5min at 4°C). The washing solution was aspirated and replaced with 300µl of serum-free DMEM medium containing the radiolabelled 0.1nM AODN or 1.3nM dendrimer-AODN. The serum free medium containing the 0.1nM AODN or 1.3nM dendrimer-AODN had been equilibrated at 4°C for 15 minutes prior to addition to the cells. Cells were then incubated at 4°C for the required time and cell association studies were undertaken as before (see section 2.2.2.11.7).

3.2.2.5.5 Efflux of Intracellular AODN and Dendrimer-AODN

The cells were seeded at a density of $5x10^4$ cells/well onto 24-well plates as previously described and used 48-hrs post-seeding (see section 2.2.2.11.4). For efflux studies, cells were incubated for 3 hours at 37°C with 0.1nM AODN or 1.3nM dendrimer-AODN in serum-free medium. After incubation, the apical medium was removed, the cells washed with PBS (3 x 0.5ml) and 0.5ml of fresh serum-free medium (without the addition of AODN or dendrimer-AODN) was added. The efflux of internalised dendrimer-AODN was monitored at fixed intervals over 4-hrs. At each timed interval 0.5ml of serum-free medium was removed and equivalent of fresh serum-free medium was added. The radioactivity pertaining to all fractions collected was determined by scintillation counting as described in section 2.2.2.7.

3.2.2.5.6 The Effect of Metabolic Inhibitors on Cellular Association

The cells were seeded at a density of $5x10^4$ cells/well onto 24-well plates as previously described and used approximately 48-hrs post-seeding (see section 2.2.2.11.4). U87-MG/A431 monolayers were pre-incubated with 10mM sodium azide/20mM 2-deoxyglucose (Sigma, Poole, U.K.) for 60 minutes at 37°C in serum-free medium. After pre-treatment, the cells were incubated with radiolabelled 0.1nM AODN or 1.3nM dendrimer-AODN for a further 90 minutes in the continuing presence of the inhibitors. Cell association studies were undertaken as before (see section 2.2.2.11.7).

3.2.2.5.7 The Effect of Post-uptake Trypsin Washing

U87-MG/A431 cells at a density of $5x10^4$ cells/well were seeded onto 24-well plates as previously described and used 48-hr post-seeding (see section 2.2.2.11.4). After the required growth period, cell monolayers were incubated with radiolabelled 1.3nM dendrimer-AODN for a period of 180 minutes. To remove surface-bound dendrimer-AODN, after washing with ice-cold PBS-azide, cells were treated with 200µl of trypsin-EDTA until all cells came off the wells. PBS (1ml) was then added, and the cells were recovered by centrifugation at 1000rpm for 5 minutes. The supernatant was collected, and cells were then resuspended in a further 1ml of PBS. The cell-associated radioactivity pertaining to both fractions (washing solution and cell) was determined by LSC.

3.2.2.5.8 The Effect of Competitors on Cellular Association

Competition studies were performed as described in section 2.2.2.11.7 with the following additions. Following initial washings with PBS cells were pre-incubated with potential competitors for a period of 15 minutes at 37°C. Cells were incubated at 37°C with radiolabelled 1.3nM dendrimer-AODN for 90 minutes (washings were undertaken with PBS before the addition of competitors). Salmon testes DNA (molecular biology grade), the nucleotide monomer dATP (molecular biology grade), dextran sulphate and heparin (sodium salt, Grade I-A, cell culture tested) were all purchased from Sigma (Poole, U.K.). The phosphorothioate ODN was synthesised as described in section 2.2.2.1.

3.2.2.5.9 The Effect of Cell Line on Cellular Association

Cellular association was investigated in a range of cell lines and the procedure described in section 2.2.2.10.7 followed. U87-MG and A431 cells were seeded at a density of $5x10^4$ cells/well on 24-well plates, 48-hrs prior to the experiment in order to achieve 70-90% confluency. C6 glial and SY5Y cells were seeded at a density of $1x10^5$ cells/well to achieve 70-90% confluency.

3.2.2.5.10 Subcellular Distribution of Fluorescently Labelled AODN and dendrimer-AODN

For experiments concerning the subcellular distribution of AODN and dendrimer-AODN, cells were seeded at a density of $2x10^4$ cells per well onto plastic chamber slides (Nunc, Gibco, U.K.) and incubated at 37°C in DMEM medium containing 10% FBS for 24-hrs. Following the required growth period cells were washed carefully with serum-free medium at 37°C (2 x 100µl per well).

The FITC-labelled AODN or dendrimer-AODN was diluted to the required concentration (10µM) in serum-free DMEM and equilibrated to 37°C prior to the experiment. The AODN or dendrimer-AODN containing medium was added to the wells in a total volume of 300µl per well and the cells incubated at 37°C for the required time period. Following incubation cells were carefully washed six times with serum-free medium (6 x 100µl x 1min) to remove all traces of non-associated fluorophores. Cells were then fixed with 2% v/v paraformaldehyde in PBS (1 x 100µl per well) for 30 minutes at room temperature. The fixative was then removed, the cells washed twice with PBS (2 x 100µl per well) and the plastic chamber gasket separated from the slide. Cells were mounted in a drop (10µl) of Vector Shield®, an anti-fading agent which enhances fluorescein detection, and a cover slip added.

Cells were then observed under an inverted Jenamed fluorescence microscope (Jena Instruments, Oberkochen, Germany). A 510nm wavelength blocking filter was used for detection of fluorescein as supplied by the microscope manufacturers. Cells were photographed using an Olympus camera with Jenamed adapter and Kodak colour film (iso 200).

3.2.2.6 Cell Viability Studies

A431 cells were seeded in DMEM medium containing 10% FBS into 24-well plates ($2cm^2$) at $1.25x10^4$ cells/well and $5x10^4$ cells/well for a 72-hr and 24-hr treatment times respectively. The cells were treated with various concentrations of AODN or dendrimer-AODN in serum free DMEM, 24-hrs after seeding. After the relevant time periods the cells were trypsinised and the number of viable cells counted by trypan blue exclusion assay as before (see section

2.2.2.11.4). The morphology of cells after treatment was also examined by light microscopy and compared to untreated control cells.

3.2.2.7 RNase H Assay

The *in vitro* transcription was performed using the MAXIscript™ *in vitro* transcription kit (Ambion, Austin Texas) as per the manufacturers recommended conditions. Briefly, 1μg of template DNA was mixed with 5μM CTP, GTP and UTP; 3.125μM 800 Ci/mmol α^{32}P-[ATP] and 1xTranscription buffer in a total volume of 19μl. Following addition of 1μl RNA polymerase (15U/μl) the reaction was allowed to proceed at 37°C for 1-hr. After 1-hr, 1μl of RNase-free DNase was added and the reaction incubated for a further 15 min. The RNA products were then recovered by NH_4OAc/Ethanol precipitation.

The reaction consisted of the RNA transcript 1nM, reaction buffer (100mM Tris pH7.4, 500mM KCl and 50mM $MgCl_2$), 10mM DTT and the AODN and dendrimer-AODN at 1μM in a total volume of 9μl. The reaction was pre-incubated at 37°C for 15min prior to addition of 1μl Ribonuclease H (2U/μl) (Gibco BRL Paisley, Scotland). The reaction was then allowed to proceed at 37°C for 1-hr. The reaction was stopped by the addition of 10μl of gel loading buffer (80% formamide v/v, 1xTBE and 0.25% w/v bromophenol blue. The products were then separated by 6% denaturating PAGE at a constant 25W for 2½-hr. The separated products were visualised by autoradiography (see section 2.2.2.5).

In addition to the working samples, a sample of RNA Century™ marker of known base lengths (1000b to 100b) was also loaded into one well of each gel. The RNA markers were labelled using the RNA Century™ labelling system kit (Ambion, Austin Texas) as per the manufacturers recommended conditions.

3.2.2.8 Protein Analysis

Western blotting was undertaken for protein analysis of A431 cells treated with various concentrations of AODN or dendrimer-AODN. The procedure is described in section 2.2.2.12.

3.2.2.8.1 β -actin controls

The nitrocellulose blot was stripped after the EGFR analysis was undertaken so that β-actin blotting could be performed. The blot was washed three times with TBS-tween and then incubated in 20mls of stripping buffer (6.25ml of 0.5M tris pH 6.7, 10ml of 10% SDS and 0.36ml of mercatoethanol to a final volume of 50ml with distilled water) at 55°C for 30 minutes in a shaking oven. The blot was washed again three times with TBS-tween and then blocked as normal. The blots were treated with primary β -actin antibody (anti-rabbit) in a 1:100 dilution. The blot was then treated with 1:2500 dilution of horse peroxidase-conjugated secondary antibody (anti-mouse), for 1-hr as before (section 2.2.2.12.5).

3.3 RESULTS AND DISCUSSION

3.3.1 Stability of AODNs Attached to the Dendrimer

An important role of a delivery system is its ability to protect AODNs from degradation and hence maintain AODN efficacy. Degradation of naked phosphorothioate AODN within a few hours by serum nucleases reflects the importance of a delivery system to protect AODNs (Sands *et al.*, 1994; Hudson *et al.*, 1996). The stability of naked AODN and AODNs attached to the dendrimer was assessed by incubating them (see section 2.2.2.5.2) in a variety of biological milieu and visualised by autoradiography. Naked AODN was radiolabelled at the 3'-end of the molecule by addition of a α-^{32}P [di-deoxy-ATP] catalysed by terminal deoxynucleotidyl transferase (see methods section 2.2.2.4). The attachment of ATP to the 3'-position on the ribose sugar may prevent 3'- exonuclease enzymes to cleave the first site at the 3'-end of the AODN and therefore enhance stability (Stein *et al.*, 1988). The AODN attached to the dendrimer was labelled at the 5'-end with γ-^{32}P-labelled ATP (see section 3.2.2.2). Labelling at the 3'-end was not possible because the 3'-end of the AODN was attached to the dendrimer. Autoradiography would detect labelled fragments of different lengths or free ^{32}P-ATP due to ODN fragments as a result of degradation. The stability experiments would also indicate the time point when either naked AODN or AODN attached to the dendrimer would degrade and therefore allow a time frame within which cellular associations studies could be conducted.

3.3.1.1 Stability of AODN and AODN Attached to Dendrimer in Dulbecco's Modified Eagle's Medium (Serum Free)

A comparative stability study of AODN and AODN attached to dendrimer was conducted in Dulbecco's modified Eagle's medium (DMEM) supplemented by 1% penicillin/streptomycin and 1mM L-glutamine (see section 3.2.2.4.1). The results obtained were compared to incubation in DMEM containing foetal bovine serum. Naked AODN and AODN attached to the dendrimer were both stable throughout the 24-hr incubation period in DMEM (serum-free) (figure 3.6 and 3.7).

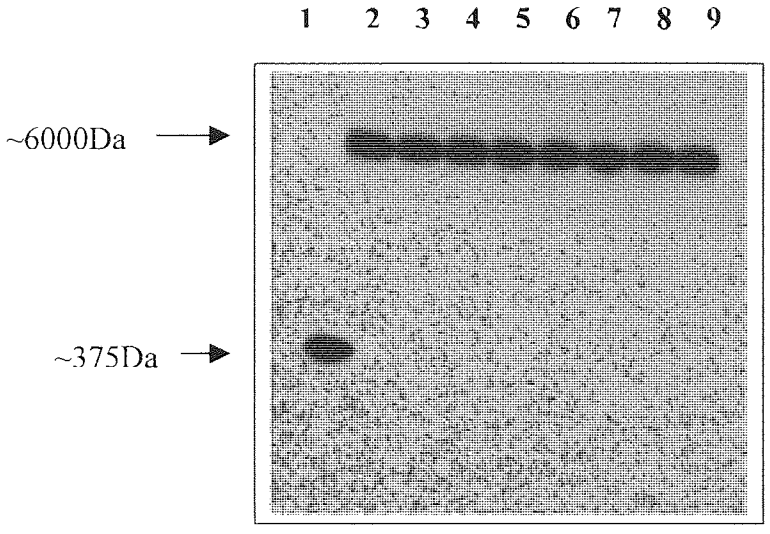


Figure 3.6 Stability of naked AODN exposed to DMEM (serum free) at 37°C. (1) control free α- [^{32}P][di-deoxy-ATP], (2) 0 hour, (3) 1 hour, (4) 2 hours, (5) 3 hours, (6) 4 hours, (7) 5 hours, (8) 6 hours, (9) 24 hours.

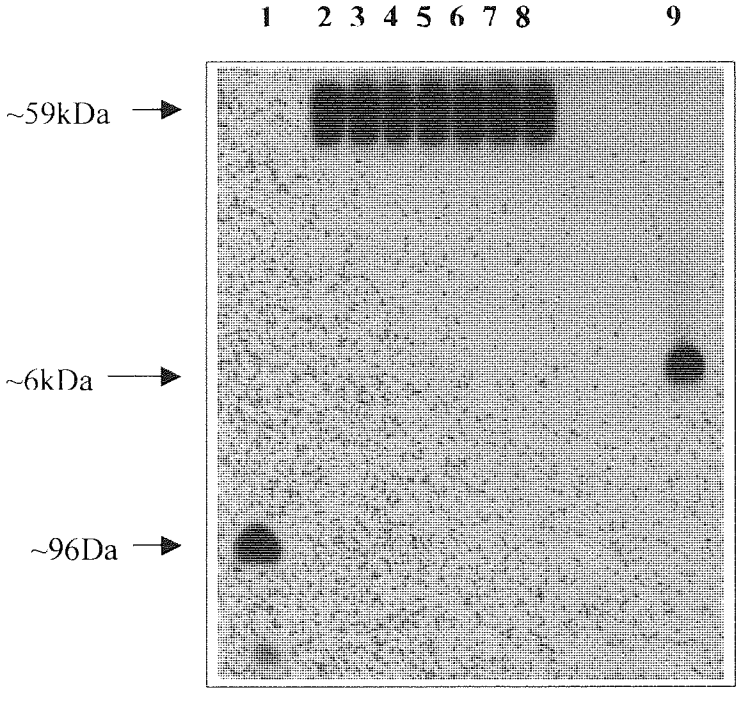


Figure 3.7 Stability of AODN attached to dendrimer exposed to DMEM (serum free) at 37°C. (1) control free [γ-^{32}P]ATP, (2) 0 hour, (3) 1 hour, (4) 2 hours, (5) 4 hours, (6) 5 hours, (7) 6 hours, (8) 24 hours, (9) control AODN.

3.3.1.2 Stability of AODN and AODN Attached to Dendrimer in Foetal Bovine Serum (FBS) Media

A comparative stability study of AODN and AODN attached to dendrimer was conducted in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% v/v foetal bovine serum, 1% penicillin/streptomycin and 1mM L-glutamine (see section 3.2.2.4.2). Serum displays substantial nucleases activity and serum stability is frequently used in the antisense field as a general indication of the extracellular stability *in vivo* (Crooke 1992). Depending on the species, the activity of nucleases in sera vary, however, foetal bovine has been reported to be more active than other species such as mouse or human (Crooke 1992).

Naked AODN exposed to 10% v/v foetal bovine serum in DMEM started to show degradation products at 2-hr time point (figure 3.8). However, at the 2-hr time point 92% of the AODN was still intact compared to the control. The half-life of the naked AODN was approximately 4-hrs and at the 24-hr time point approximately 11% of the intact AODN remained compared to the control (table 3.2). In comparison to the naked AODN, the AODN attached to the dendrimer showed no degradation products throughout the 24-hr incubation period in 10% v/v foetal bovine serum in DMEM (figure 3.9). The results suggested that the dendrimer was protecting the AODN from nucleases in the serum. These result were consistent with other studies involving PAMAM dendrimers (Poxon *et al.*, 1996; DeLong *et al.*, 1999). It is possible that the 3'-end of the AODN attached to the dendrimer is protected from exonucleases which are likely to cleave the 3'-end and which are also more predominant in the serum media and cellular secretions compared to 5'-end nucleases (Dagle *et al.*, 1991; Peyman *et al.*, 1997). Previous studies have shown that protecting the 3'-end of the ODN prevents degradation and is also more effective than 5'-end protection for nuclease stability in foetal calf serum and in human blood serum (Boado *et al.*, 1999; Maier *et al.*, 1995). However, structural hindrance may also be an explanation for the enhanced stability of AODN attached to the dendrimer as compared to the naked AODN.

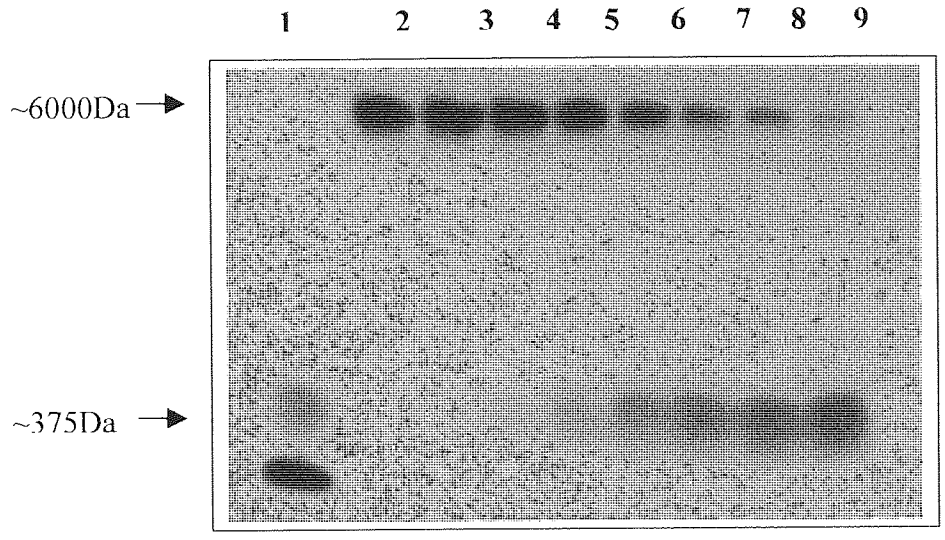


Figure 3.8 Stability of naked AODN exposed to DMEM supplemented by 10% v/v foetal bovine serum at 37°C. (1) control free α- [^{32}P][di-deoxy-ATP], (2) 0 hour, (3) 1 hour, (4) 2 hours, (5) 3 hours, (6) 4 hours, (7) 5hours, (8) 6 hours, (9) 24 hours.

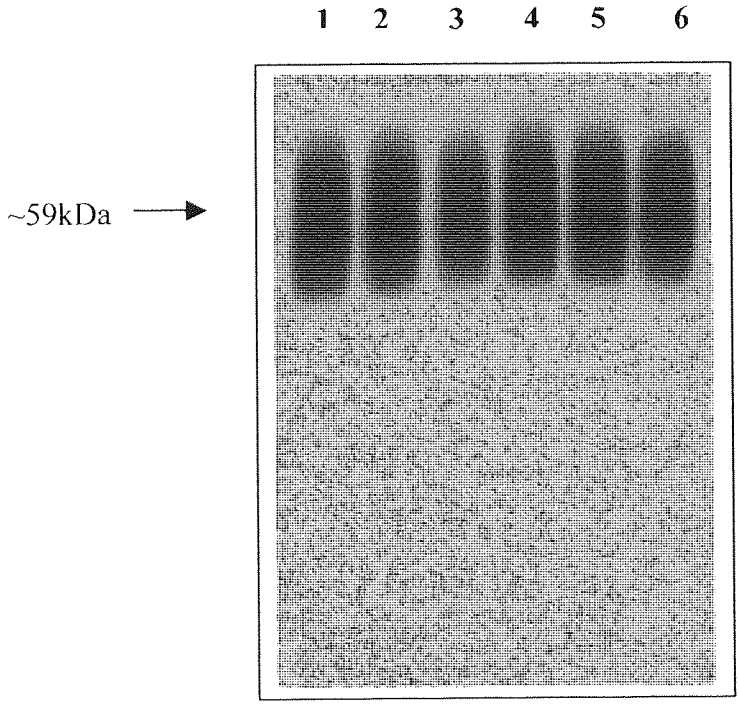


Figure 3.9 Stability of AODN attached to dendrimer exposed to DMEM supplemented by 10% v/v foetal bovine serum at 37°C. (1) 0 hour, (2) 1 hour, (3) 2 hours, (4) 4 hours, (5) 6 hours, (6) 24 hours.

3.3.1.3 Stability of AODN and AODN Attached to Dendrimer in U87-MG and A431 Cells Supernatants

Stability was undertaken in U87-MG and A431 cell supernatants (see section 2.2.2.11.1) in DMEM (serum-free) to make sure that the results obtained from the cellular association studies represent the uptake of intact AODN or AODN attached to the dendrimer and not that of the degraded ODNs or free [^{32}P] label (see section 3.2.2.4.3). This would allow a time frame within which cellular associations studies could be conducted.

Naked AODN exposed to U87-MG cells in DMEM (serum-free) started to show degradation products at the 3-hr time point (see figure 3.10). At the 3-hr time point approximately 69% of the AODN was still intact compared to the control. The half-life of the naked AODN was approximately 24-hrs compared to the control (table 3.2). In comparison to the naked AODN, the AODN attached to the dendrimer showed no degradation products throughout the 24-hr or 48-hr incubation period, when exposed to U87-MG cells in DMEM (serum-free) (figure 3.11). However, naked AODN exposed to A431 cells in DMEM (serum-free) was less stable than exposed to U87-MG cells in the same conditions (figure 3.12). Naked AODN exposed to A431 cells started to show degradation products at the 2-hr time point. At this point approximately 70% of the AODN was still intact compared to the control. The half-life of the naked AODN was approximately 6-hrs compared to the control (table 3.2). The difference in degradation profiles of AODN exposed to U87-MG and A431 cells is possibly due to the amounts of nucleases secreted by each cell line. Although a confluent monolayer was used for each cell line these results cannot be conclusive due to differences in cell number. The AODN attached to the dendrimer showed no degradation products throughout the 24-hr incubation period exposed to A431 cells in DMEM (serum-free) (figure 3.13), in comparison to the naked AODN. The results suggested that the dendrimer was protecting the AODN from nucleases released by each cell line in DMEM (serum-free) for reasons explained above.

It was decided from these stability studies that cellular association studies would be conducted over 3-hrs in DMEM (serum-free) for naked AODNs in U87-MG and A431 cells. Although dendrimer-AODN was stable up to 24-hrs the cellular association studies were also conducted up to 3-hrs so that the results could be compared easily. Also, it is likely that the majority of cellular association will occur in the first 3-hrs due to cellular surface saturation (Fell *et al.*, 1997).

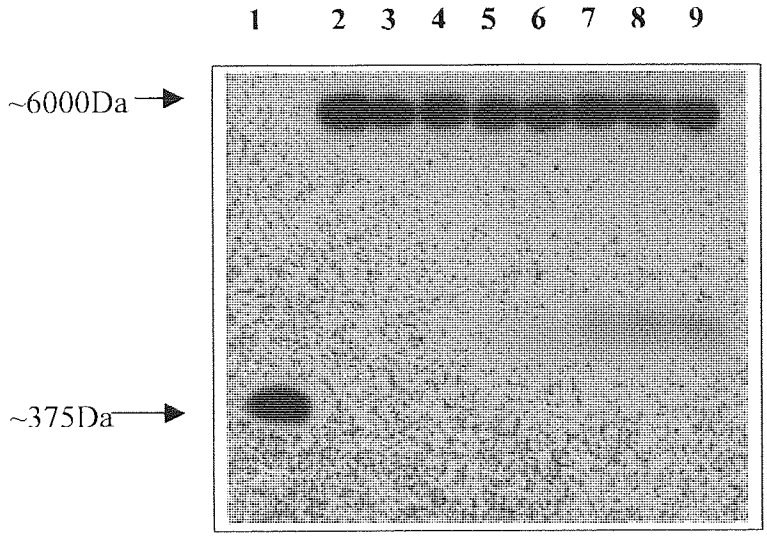


Figure 3.10 Stability of naked AODN exposed to U87-MG cells in DMEM (serum free) at 37°C. (1) control free α-[^{32}P][di-deoxy-ATP], (2) 0 hour, (3) 1 hour, (4) 2 hours, (5) 3 hours, (6) 4 hours, (7) 5hours, (8) 6 hours, (9) 24 hours.

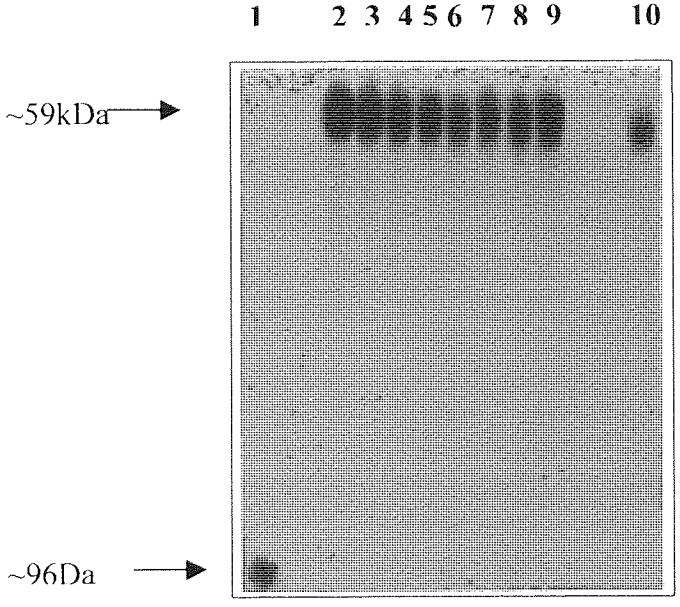


Figure 3.11 Stability of AODN attached to dendrimer exposed to U87-MG cells in DMEM (serum free) at 37°C. (1) control free [γ-^{32}P]ATP, (2) 0 hour, (3) 1 hour, (4) 2 hours, (5) 3 hours, (6) 4 hours, (7) 5 hours, (8) 6 hours, (9) 24 hours, (10) 48 hours.

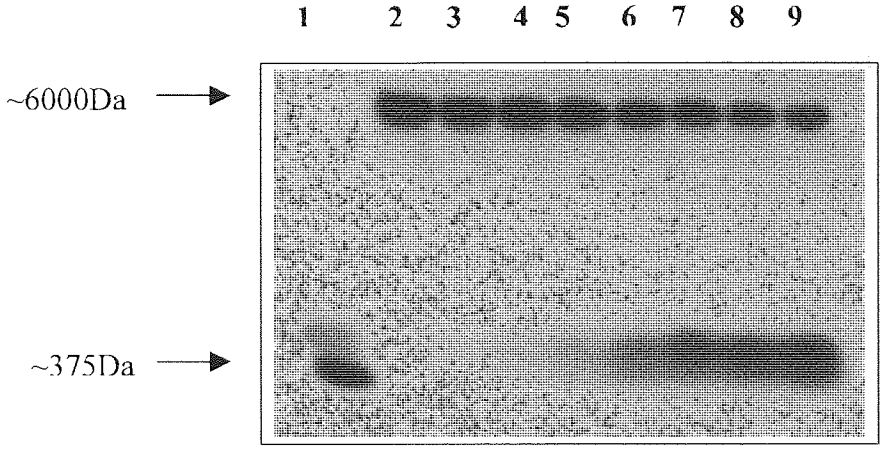


Figure 3.12 Stability of naked AODN exposed to A431 cells in DMEM (serum free) at 37°C. (1) control free α-[^{32}P][di-deoxy-ATP], (2) 0 hour, (3) 1 hour, (4) 2 hours, (5) 3 hours, (6) 4 hours, (7) 5hours, (8) 6 hours, (9) 24 hours.

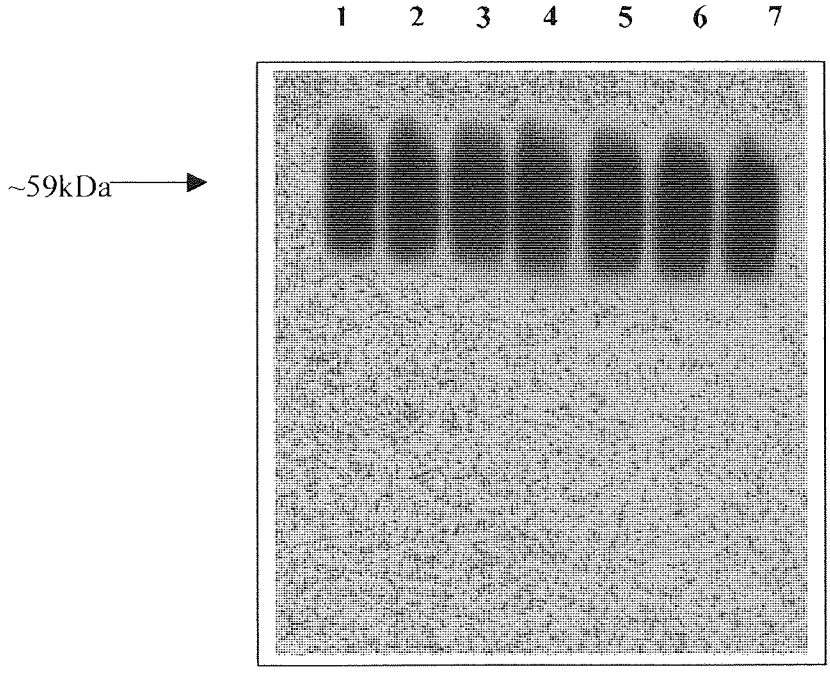


Figure 3.13 Stability of AODN attached to dendrimer exposed to A431cells in DMEM (serum free) at 37°C. (1) 0 hour, (2) 1 hour, (3) 2 hours, (4) 4 hours, (5) 5 hours, (6) 6 hours, (7) 24 hours.

Table 3.2 Degradation Profiles of Naked AODN in different Biological Milieu.

Time (hours)	Intact AODN (% of control)		
	10% FBS in DMEM	**Exposed to U87-MG in DMEM (serum free)**	**Exposed to A431 in DMEM (serum free)**
0	100	100	100
1	95	80	80
2	92	71	70
3	73	69	64
4	53	66	62
5	40	62	57
6	27	60	52
24	11	58	42

3.3.2 Cell Association Studies of Dendrimer-AODN

Cellular mechanisms for the uptake of AODN using PAMAM dendrimers have been investigated (Bielinska *et al.*, 1996). These studies suggest that active transport is likely to be involved in the uptake of PAMAM dendrimer-AODN complexes. Anionic glycoproteins and phospholipids that reside on the cell surface membrane have also shown to be important for the cationic dendrimer-AODN complex to bind by electrostatic interactions and subsequently enter the cell (Tang *et al.*, 1997). Due to the unique nature of the anionic dendrimer complex it was therefore important to study the extent and mechanism by which they enter cells. Such information will be vital to modify the existing delivery system to further enhance the uptake and efficacy of AODNs. To address this issue we examined the cell surface interaction and uptake of the dendrimer-AODN. The cell association/uptake of the dendrimer-AODN was examined using the human glioma cell line U87-MG as a model. These cells are derived from a grade III glioblastoma and have many distinguishing phenotypic features of malignant gliomas (Ponten *et al.*, 1968). The A431 cell line was also used as a model, which was derived from a human epidermoid carcinoma of the vulva (see section 2.2.2.11.1).

3.3.2.1 Cell Association Studies

3.3.2.1.1 Optimisation of Cell Association Study Protocol

U87-MG cell growth was characterised to assess optimal growth conditions for cellular association and efficacy studies to be carried out in serum free medium. Cells were seeded onto 24-well plates at a density of 1.25×10^4 cells/ml/well (time 0) and 5×10^4 cells/ml/well (time 0). Cells were then incubated at 37°C for the time periods indicated, at which point viable cell number was determined by trypan blue dye exclusion assay. The growth curves obtained are shown in figure 3.14. Cells entered exponential growth for at least 60-hrs. These results indicated that efficacy studies conducted over 24-hrs as well as 72-hrs could be performed in serum-free medium. For cellular association studies, the cells were seeded at 5×10^4 cells/ml/well for 48-hrs before treatments and the data obtained was normalised to 1×10^5 cells/ml/well. A431 cell growth in section 2.3.2 was also characterised to assess optimal growth conditions for cellular association and efficacy studies in serum free DMEM.

Similar results to the U87-MG cell line allowed A431 cells to be seeded at the same concentrations.

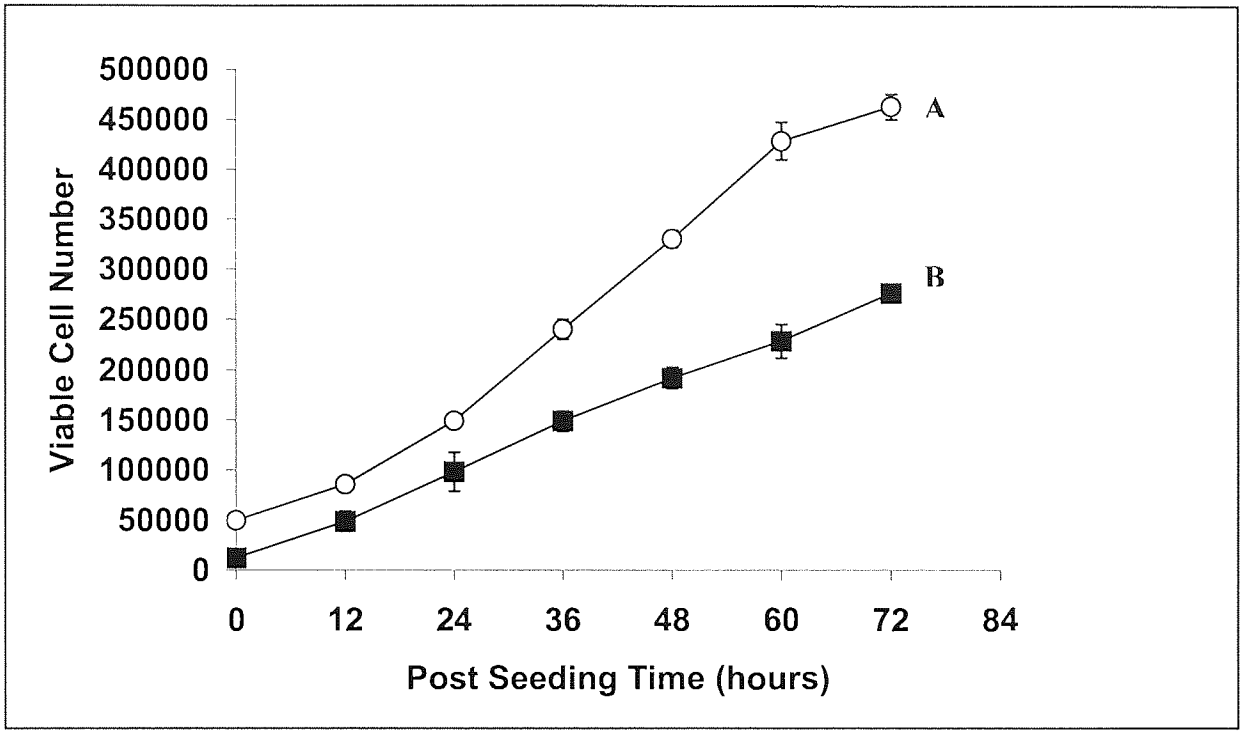


Figure 3.14 Standard growth curves for U87-MG cells calculated from a seeding concentration of (A) 5×10^4 cells/ml/well and (B) 1.25×10^4 cells/ml/well at 37°C (n=3±SD).

To optimise the number of PBS-azide washings required to remove unbound and loosely bound AODN or dendrimer-AODN a post-incubation assay was undertaken in U87-MG and A431 cell lines (see section 3.2.2.5.1). Figure 3.15 A and 3.15 B shows the percentage AODN or dendrimer-AODN removed from the U87-MG and A431 cell surfaces at each wash respectively. The results showed that approximately 95% and 98% of loosely bound or non-cell associated AODN was removed after 3 × 1 ml washes on U87-MG and A431 cells respectively. For dendrimer-AODN, 85% and 90% of loosely or non-cell associated AODN was removed after 5 × 1 ml washes on U87-MG and A431 cells respectively. Although higher washes further removed AODN and dendrimer-AODN, the cell layers were also being removed (visualised using an inverted microscope), which can potentially invalidate the data.

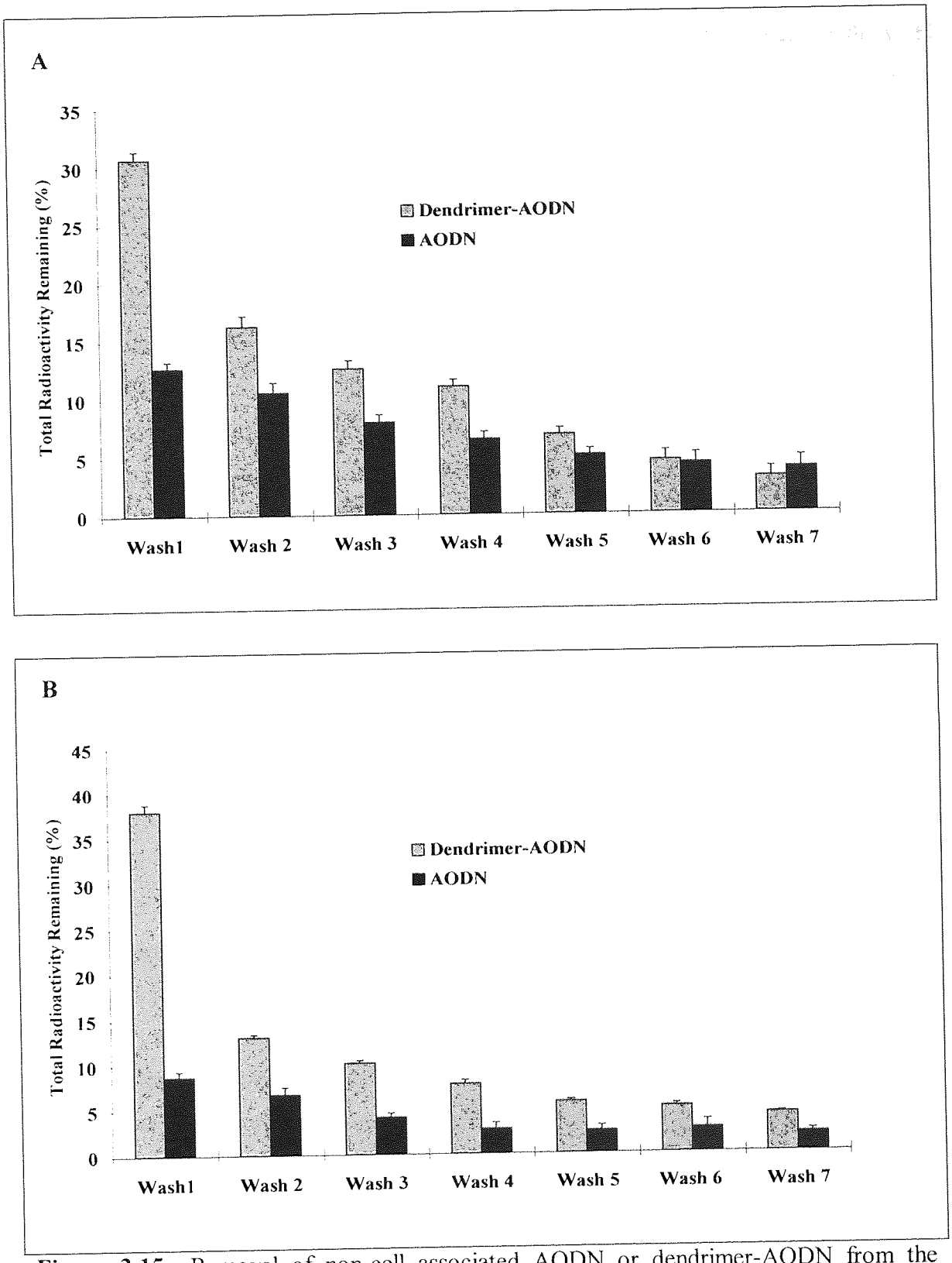


Figure 3.15 Removal of non-cell associated AODN or dendrimer-AODN from the surface of (A) U87-MG (B) A431 cells by consecutive PBS-azide washes after 24 hours (n=3±SD).

Before proceeding with the cellular association studies, it was important to assess the viable cell number of U87-MG and A431 cells when treated with various concentrations of AODN and dendrimer-AODN, which would ultimately be used for cellular association studies and also would give an indication of cytotoxicity. The importance of carrying out this study was highlighted by the fact that cellular uptake becomes more pronounced in dead, non-viable cells as the membranes lose their selective properties (Freshney 1973). Increasing concentrations of either AODN (0.5nM-200nM) or dendrimer-AODN (0.5nM-400nM) were incubated with U87-MG or A431 cells which were seeded at 5×10^4 cells/ml/well, 24-hr prior to treatments in serum free DMEM. After an incubation period of 4-hrs cells were trypsined and viable cells were counted using trypan blue dye exclusion assay (see section 2.2.2.11.4). The results are shown in figure 3.16 and 3.17. The results showed no significant differences in viable cell number with AODN or dendrimer-AODN concentrations as compared to the control (no treatment) with U87-MG or A431 cells ($P>0.05$). This indicated that AODN and dendrimer-AODN were non-toxic to U87-MG and A431 cells under the conditions conducted.

3.3.2.1.2 Temperature and Time Dependence of Cellular Association

The cell association of dendrimer-AODN and AODN was determined at selected time intervals over 180 minutes for U87-MG or A431 cells. Efflux was also measured for U87-MG cells. As figure 3.18 A demonstrates, at 37°C, U87-MG cell association of dendrimer-AODN was approximately 2-fold higher than AODN over a 180 minute incubation time. For the A431 cell line, cell association of dendrimer-AODN was also approximately 2-fold higher than AODN over a 90 minute incubation time (figure 3.18 B). However, at 120 minute and 180 minute time points, cellular association was approximately 3.5-fold higher for dendrimer-AODN as compared to the naked AODN for the A431 cell line. Similar cellular association-time profiles were observed between U87-MG and A431 cell lines for dendrimer-AODN and AODN i.e. for both cell lines the first 2-hrs showed rapid cellular association, after which time the rate of cellular association began to level off. Statistical analysis showed insignificant differences between 120 and 180 minutes ($P>0.05$) for both cell lines. These profiles were consistent with other studies where cellular association had shown to plateau after a few hours (Yakubov *et al.*, 1989; Fell *et al.*, 1997; Khan 1999). Such a pattern was indicative of the fact that after 2-hrs of incubation the dendrimer-AODN was in equilibrium

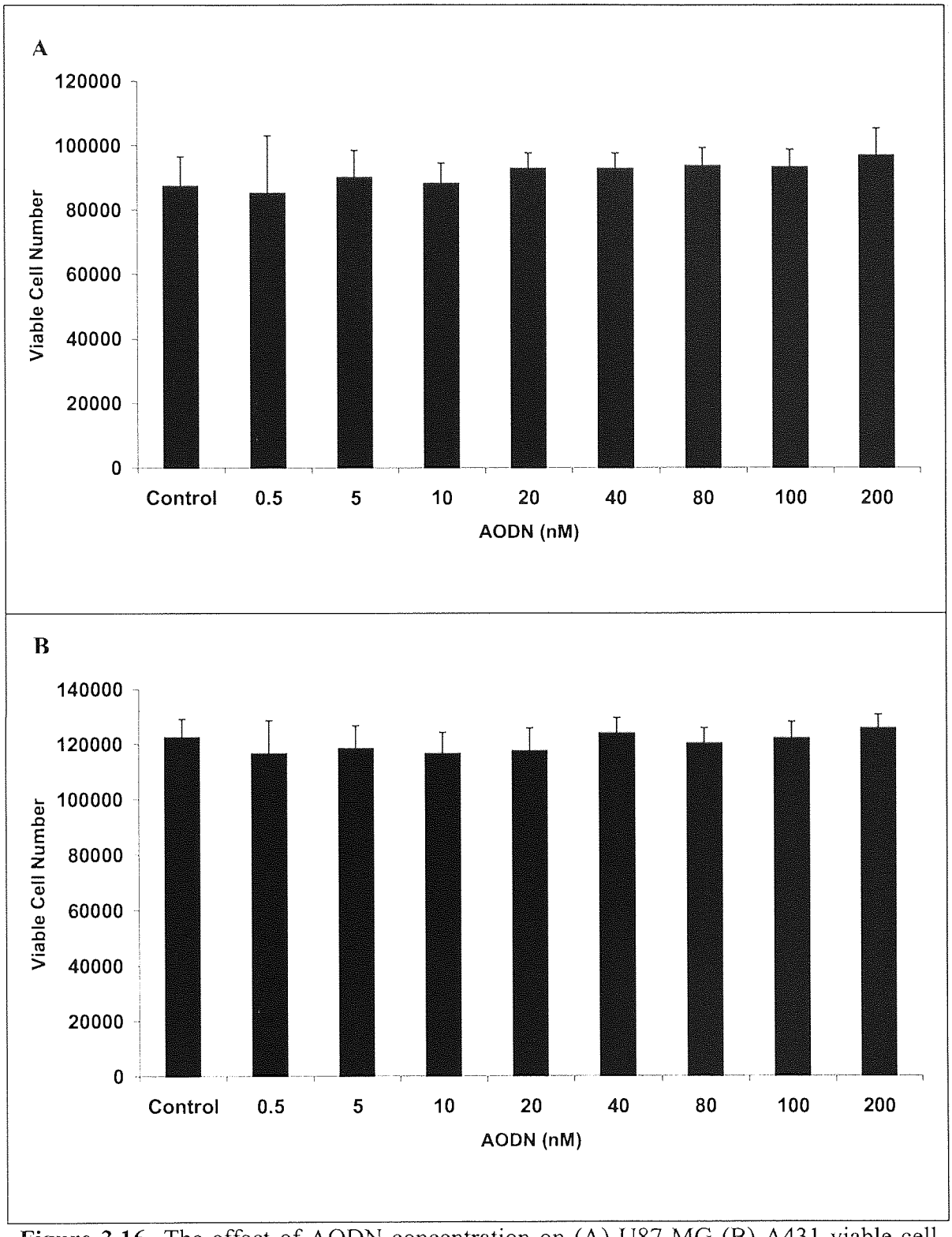


Figure 3.16 The effect of AODN concentration on (A) U87-MG (B) A431 viable cell numbers after four hour incubation at 37°C (n=3±SD).

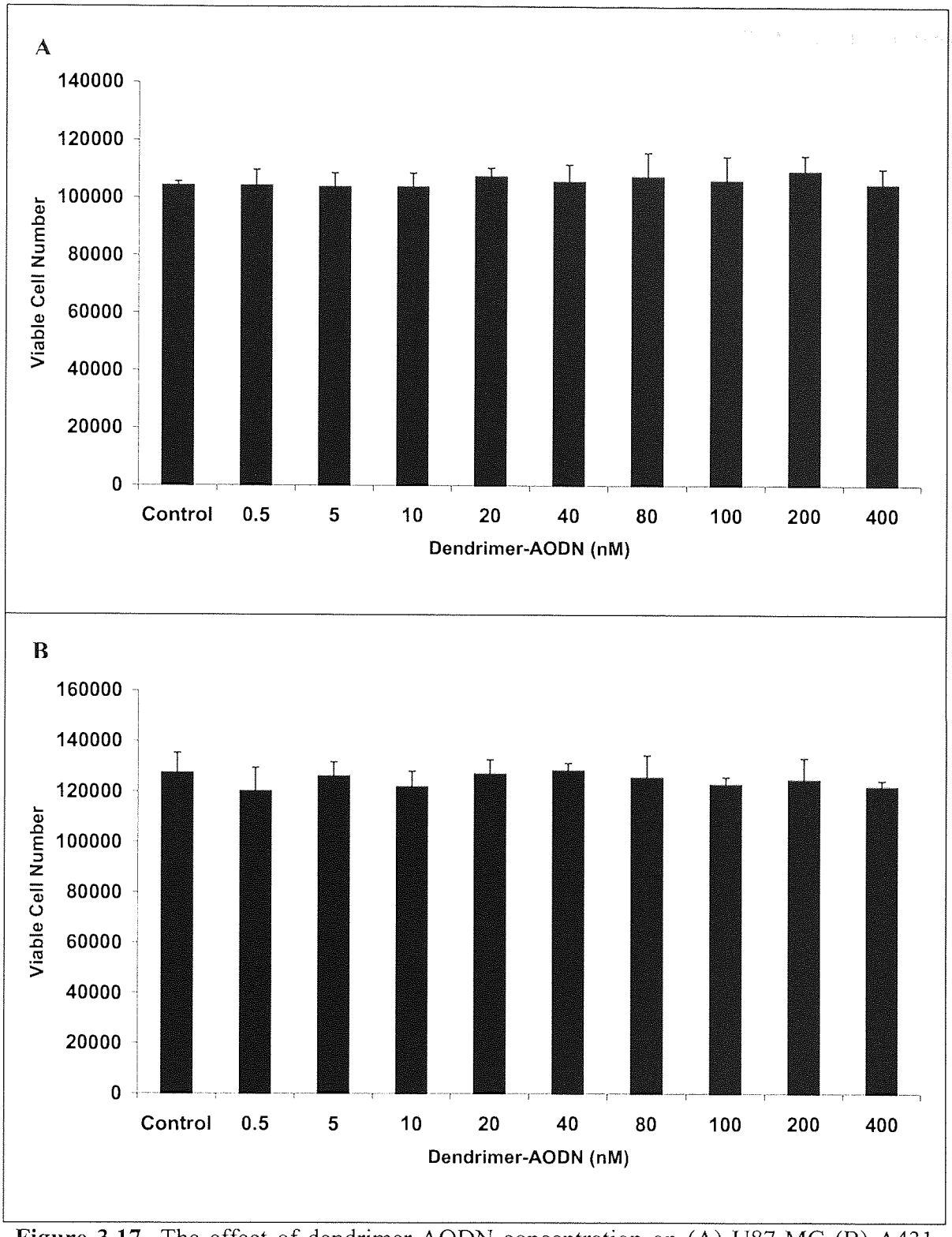


Figure 3.17 The effect of dendrimer-AODN concentration on (A) U87-MG (B) A431 viable cell numbers after four hour incubation at 37°C (n=3±SD).

with dendrimer-AODN exocytosis (Beck *et al.*, 1996; Fell *et al.*, 1997). PAMAM dendrimers have also shown to enhance cellular association of DNA by 6-fold, however concentrations to achieve this resulted in cellular toxicity due to the cationic nature of the complexes (Tomlinson *et al.*, 1996; Malik *et al.*, 2000). In an attempt to determine the extent of fluid-phase endocytosis (or pinocytosis) in U87-MG and A431 cells, the uptake of [^{14}C]-labelled mannitol, a classic non-metabolisable marker for fluid-phase pinocytosis (Chonn *et al.*, 1995) was measured over a 3-hr period at 37°C. Compared with the uptake of radiolabelled dendrimer-AODN the basal rate of pinocytosis in these cells remained extremely low (0.09%) throughout this period for both U87-MG and A431 cells and was unlikely, therefore, to account for a significant fraction of dendrimer-AODN uptake at the concentration used in this cell line. In contrast, however, cellular association at 4°C of dendrimer-AODN and AODN was reduced compared with 37°C and remained at relatively constant low levels for U87-MG cells (0.9% and 0.5% respectively) after a 1.5-hr incubation period (figure 3.19 A). Similar results were obtained with the A431 cells i.e. at 37°C cellular association was 3% as compared to 0.1% when incubated at 4°C for 2-hrs (figure 3.19 B). The difference in percentage cellular association between the two temperatures was greater in A431 cells as compared to U87-MG cells. This may reflect variation in types of cell surface proteins which differ in their sensitivity to temperature. Previous studies have shown binding processes in cells involved both temperature-sensitive and insensitive component (Akhtar *et al.*, 1996). These results demonstrated that dendrimer-AODN uptake was dependent upon temperature in both U87-MG and A431 cells. The temperature dependent cellular association studies suggested that endocytic/phagocytic mechanisms were taking place, which require cellular energy. This result was consistent with other studies which involved microsphere delivery systems (Khan 1999 ; Smith 2000).

Cell association in U87-MG cells of both dendrimer-AODN and AODN were also found to be dynamic, representing both uptake and efflux processes. As shown in figure 3.20, efflux of both the dendrimer-AODN and AODN was rapid in the first 60 minutes, when approximately 27% of the internalised dendrimer-AODN was released, compared to the release of 34% of AODN. Slower efflux over 3-hrs then followed, during which a further 48% and 54% of dendrimer-AODN and AODN was released respectively. After 4-hrs, 44% of the dendrimer-AODN and 40% of the AODN remained sequestered within the cells.

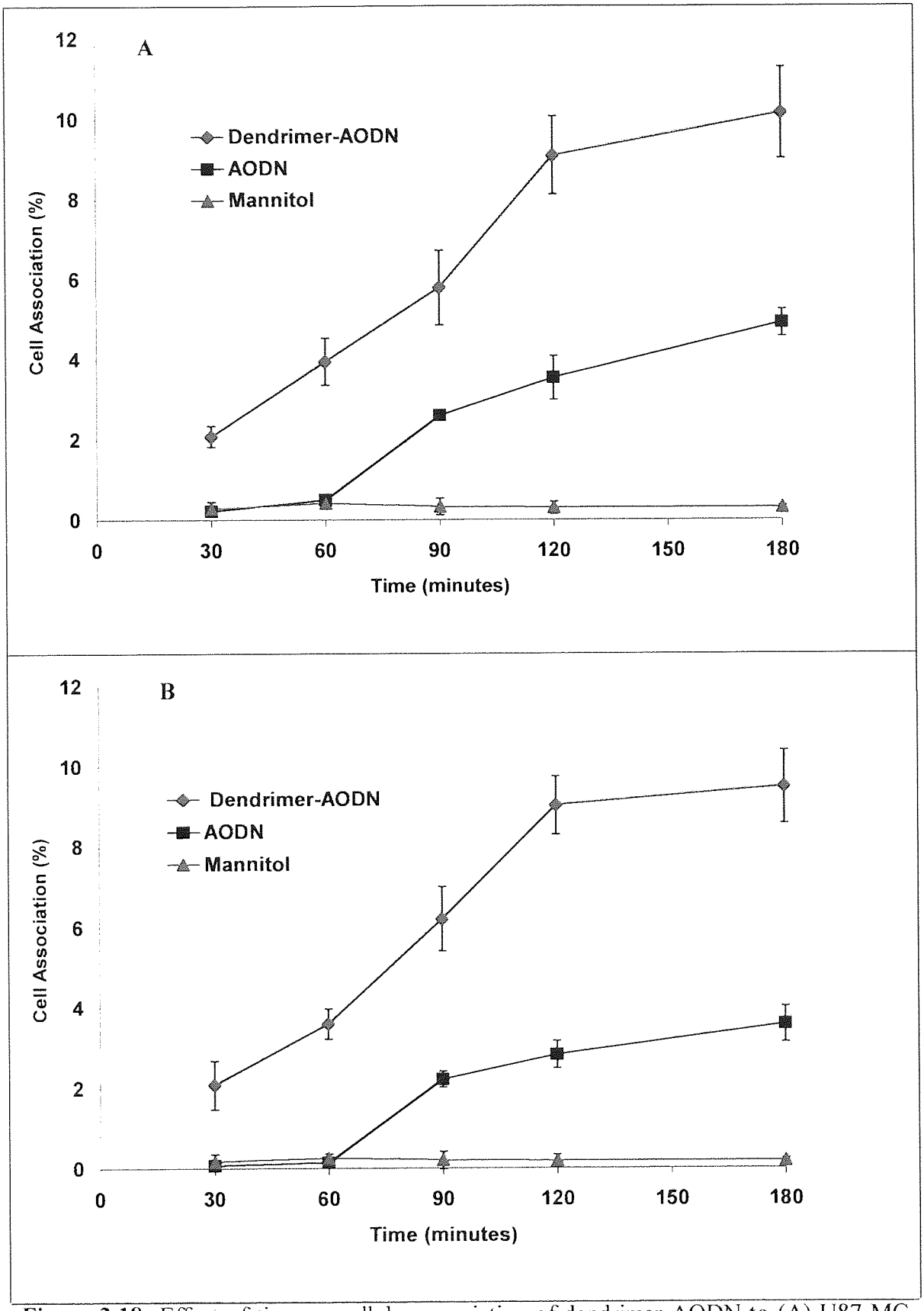


Figure 3.18 Effect of time on cellular association of dendrimer-AODN to (A) U87-MG (B) A431 cells. Cell monolayers were incubated with [^{32}P]-labelled dendrimer-AODN, AODN and [^{14}C]-labelled mannitol in serum free media DMEM media at 37°C (n=3±SD).

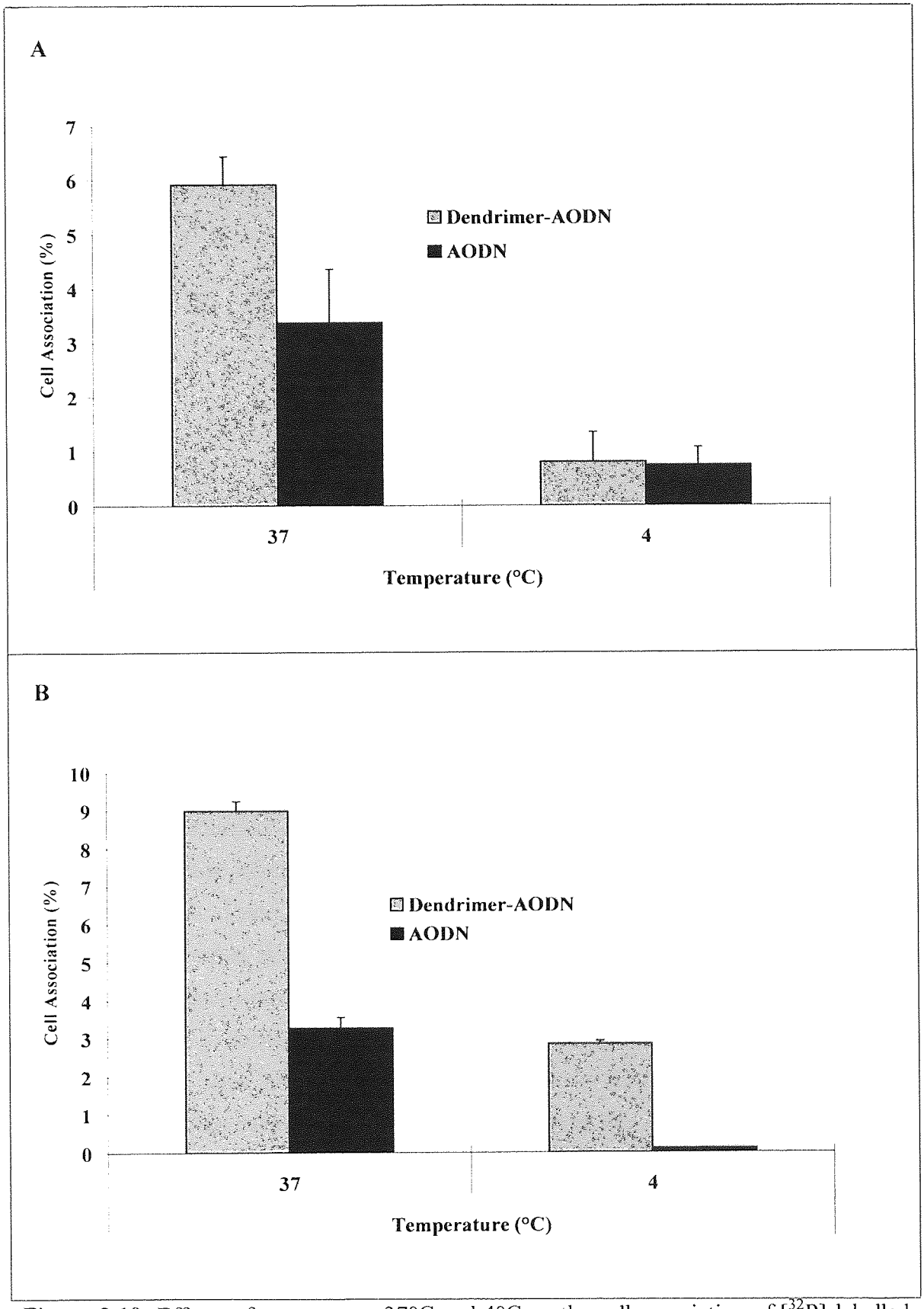


Figure 3.19 Effects of temperature 37°C and 4°C on the cell association of [^{32}P]-labelled dendrimer-AODN and naked [^{32}P]-labelled AODN in (A) U87-MG (B) A431 cells (n=3±SD).

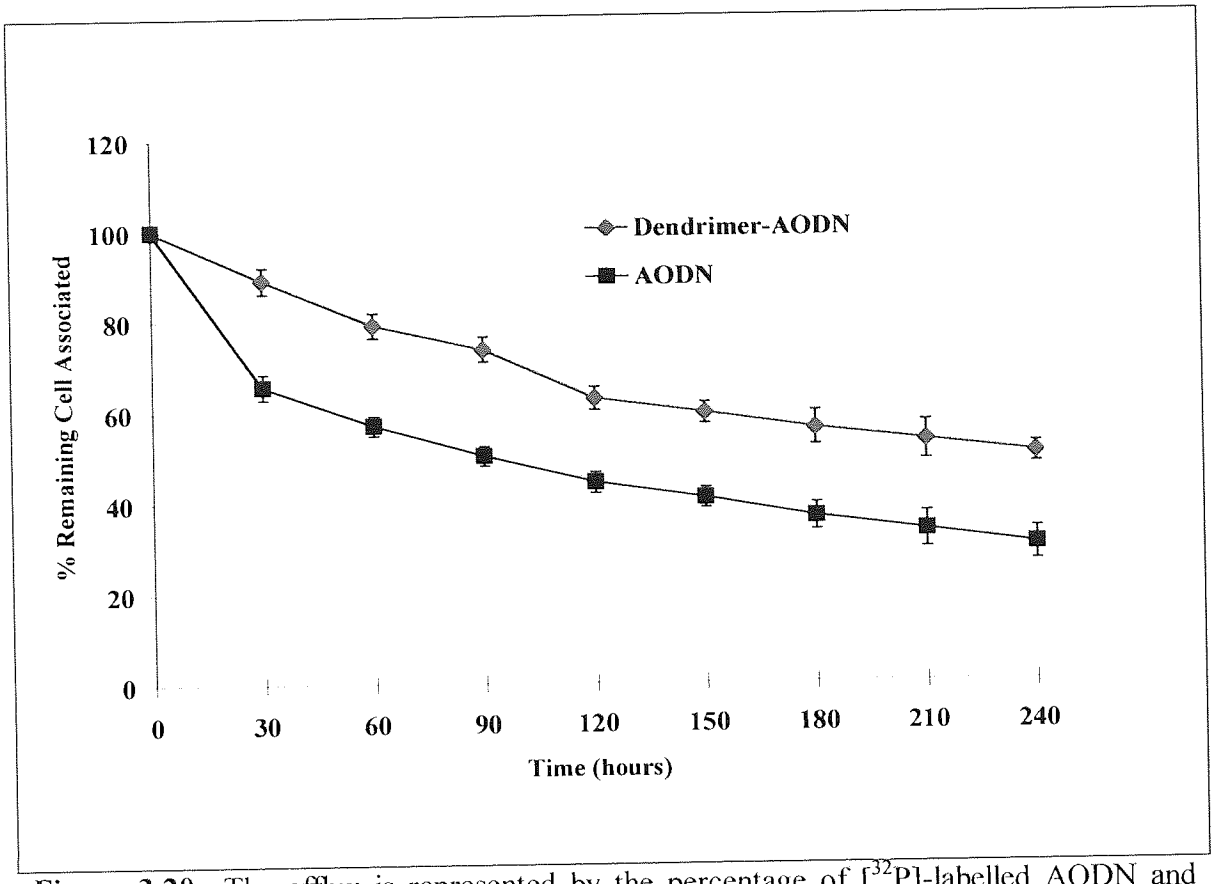


Figure 3.20 The efflux is represented by the percentage of [^{32}P]-labelled AODN and dendrimer-AODN remaining in U87-MG cells (n=3±SD).

Similar results have been observed in other studies for AODNs (Gao *et al.*, 1993; Tonkinson *et al.*, 1994; Temsamani *et al.*, 1994) and it has been suggested that this biphasic pattern of efflux is representative of exocytosis from two intracellular compartments. Thus the initial fast efflux of both dendrimer-AODN and AODN may represent release from shallow compartments situated at or near the surface of the cell (e.g. strongly bound to the cell surface or within primary endosomal vesicles), whereas the slower efflux from dendrimer-AODN or AODN sequestered in deeper compartments (e.g. late endosomes or lysosomes or from other, as yet unidentified sites within the cell). Such patterns are thought to be indicative of intracellular trafficking subsequent to endocytosis (Stein *et al.*, 1993). The rate of efflux of the dendrimer-AODN was greater than AODN over 240-hrs. This suggested that dendrimer-AODN may have either been sequestered within deeper endosomal vesicles or bound more strongly to cell surface as compared to the AODN.

3.3.2.1.3 Energy Dependence of Cell Association

The temperature-dependence of dendrimer-AODN uptake in U87-MG and A431 cells suggested that an active process was involved and largely discounted the possibility that dendrimer-AODN was taken up by passive diffusion. This assertion was further supported by energy depletion experiments where U87-MG and A431 cells were preincubated with 10mM NaN_3 (a cytochrome oxidase inhibitor) and 20mM 2-deoxyglucose (a glycolytic inhibitor) for 1-hr before the addition of the AODN or dendrimer-AODN. U87-MG cell association of the dendrimer-AODN was reduced significantly by 67% compared to 90% inhibition with AODN alone in the presence of these inhibitors ($P<0.05$) (figure 3.21 A). A431 cell association of dendrimer-AODN and AODN was also reduced significantly by 67% as compared to the control ($P<0.05$) (figure 3.21 B). This clearly indicated that an energy-dependent process was involved in dendrimer-AODN uptake. Similar effects have been noted for phosphodiester ODNs and phosphorothioate ODNs by other workers (Shoji *et al.*, 1996).

The temperature and energy-dependence of cellular association in U87-MG and A431 cells suggested that endocytosis may account for the uptake of dendrimer-AODN, as is presumed for AODNs (Akhtar *et al.*, 1992). PAMAM dendrimers also appear to be internalised through energy-dependent endocytosis, because pre-incubation of the rat embryonal fibroblast cell line (Rat2) with metabolic inhibitors reduced cellular association (Kukowska-Latallo *et al.*, 1996).

3.3.2.1.4 Inhibition of Uptake by Competitors

Dendrimer-AODN uptake was found to be inhibited by competition with unlabelled dendrimer-AODN in a dose-dependent manner, with a 250-fold excess of the competitor significantly reducing cellular uptake by 88% after 1.5-hrs of incubation in the U87-MG cell line ($P<0.05$) (figure 3.22). The self-competing nature of dendrimer-AODN uptake is indicative of a specific cell surface binding mechanism possibly mediated by binding proteins. Thus, to assess the extent of protein-bound component of dendrimer-AODN association, trypsin was used to strip off surface-bound dendrimer-AODN by digesting cell surface protein-binding sites of the U87-MG cell line. Trypsinization, following incubation of the dendrimer-AODN for 3-hrs at 37°C, removed approximately 83% of cell-associated radioactivity (figure 3.23), showing that a large proportion of binding occurred via cell

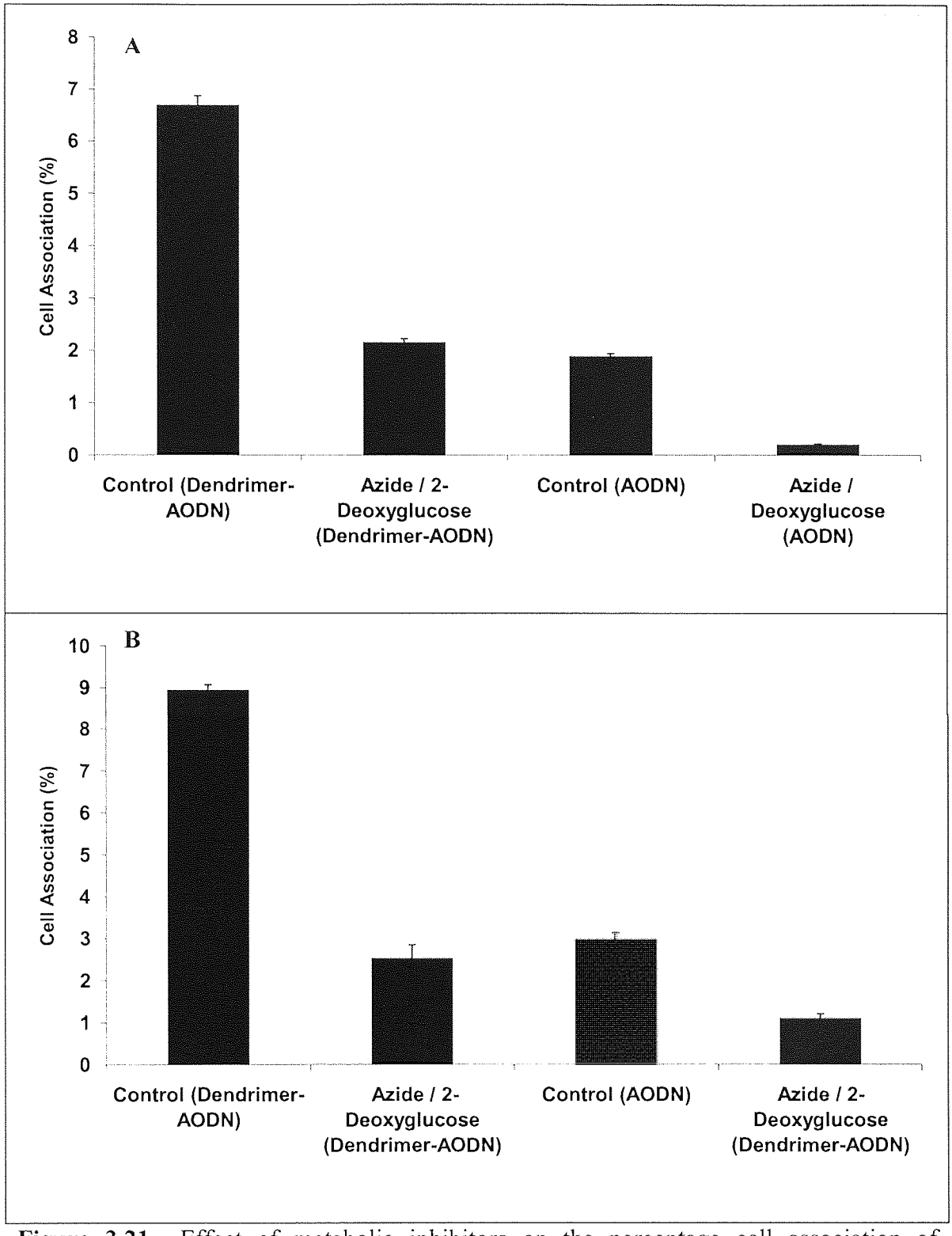


Figure 3.21 Effect of metabolic inhibitors on the percentage cell association of dendrimer-AODN to (A) U87-MG (B) A431 cells at 37°C in serum free DMEM medium for 1.5 hours (n=3±SD).

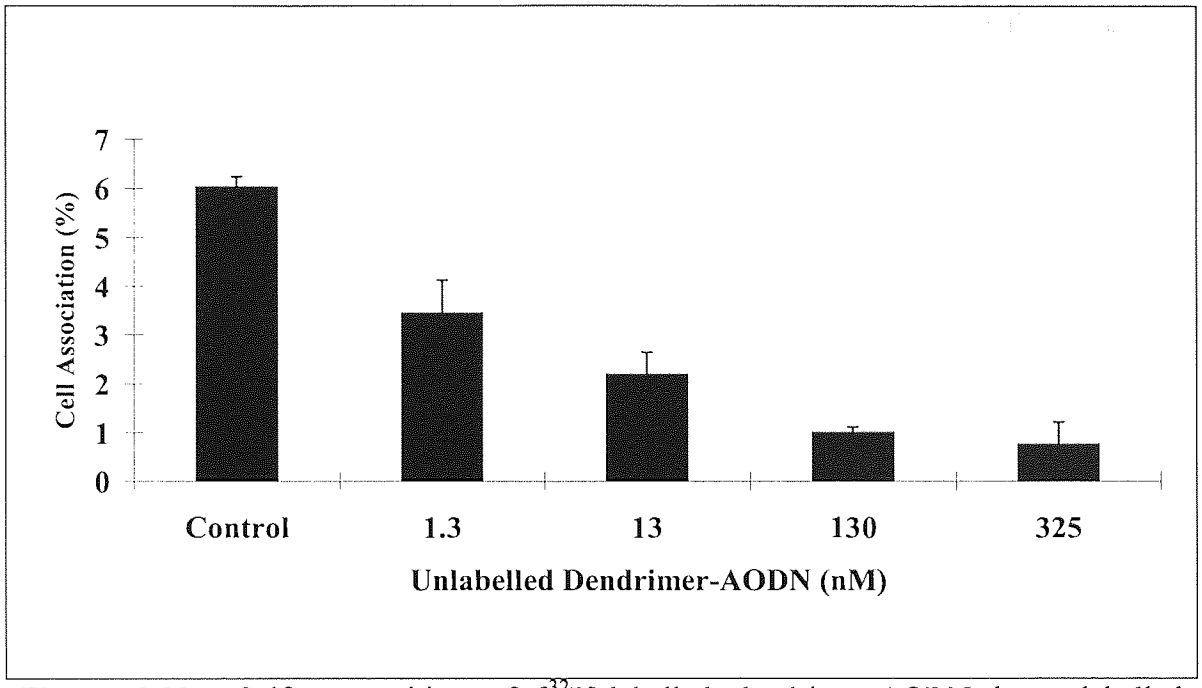


Figure 3.22 Self competition of [^{32}P]-labelled dendrimer-AODN by unlabelled dendrimer-AODNat 37°C in serum-free medium for 1.5 hours along with varying concentrations of unlabelled dendrimer-AODN in the U87-MG cell line (n=3±SD).

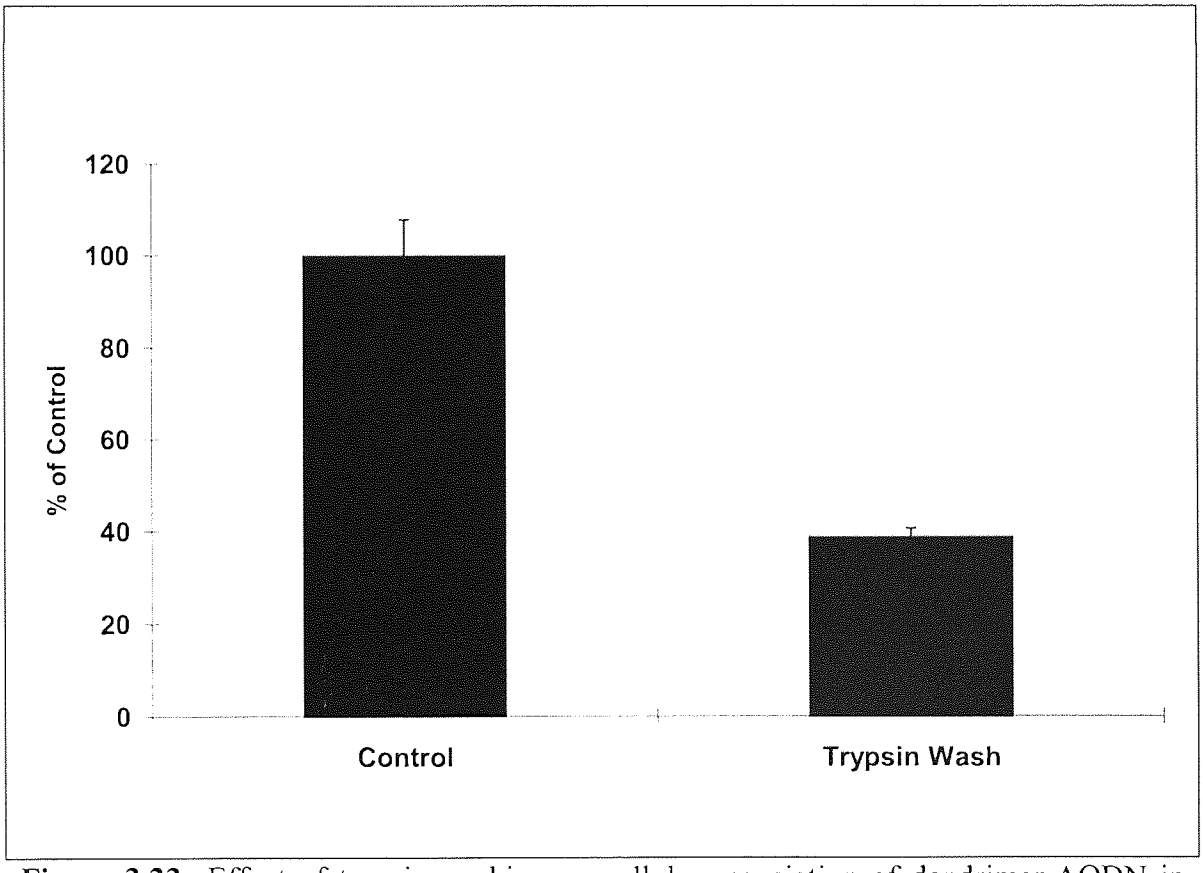


Figure 3.23 Effect of trypsin-washing on cellular association of dendrimer-AODN in U87-MG cells; control : total associated dendrimer before addition of trypsin (n=3±SD).

surface proteins. The remaining components may have become internalised or could represent tight binding to either non-trypsinsensitive (glyco) proteins or even cell surface lipids (Beck *et al.*, 1996).

To further characterize the specificity of dendrimer-AODN in U87-MG cell association, the effect of nucleic acid and anionic competitors was examined. Potential competitors, including unlabelled ODNs, salmon sperm DNA, ATP, and polyanions, such as dextran sulphate were preincubated with cells for 15 minutes at 37°C. After pre-treatment and washing (see section 3.2.2.5.8), [^{32}P]-labelled dendrimer-AODN was added to the cells and incubated for 60 minutes. Figures 3.24 A and 3.24 B show that dendrimer-AODN uptake also involved a non-specific component, as other nucleic (salmon sperm DNA, PS-ODNs) and non-nucleic acid molecules (dextran sulphate, heparin) decreased dendrimer-AODN uptake by up to 77%. As PO-ODN and PS-ODN are generally considered to enter cultured cells via receptor mediated, adsorptive (binding protein-mediated) or fluid phase endocytosis (Akhtar *et al.*, 1992; Hughes *et al.*, 2001), the fact that uptake of dendrimer-AODN was subject to competition with PS-ODN indicated that there could be an overlap in their mechanisms of entry and that dendrimer-AODNs may compete for cell uptake via one or more of these processes. The non-specific competition by unrelated polyanions has also been demonstrated for AODNs and highlights the importance of ionic interaction with cell surface structures in the cellular uptake of these entities. King *et al.* reported a 30-kDa DNA binding protein that could be inhibited by heparin and Loke *et al.* described an 80-kDa surface protein to which binding could be inhibited by single nucleotides as well as tRNA and plasmid DNA. More recently, a 46-kDa binding protein has been identified in several cell lines that can be competed with a variety of polyanions, including ssDNA, ds DNA, RNA, and heparin (Akhtar *et al.*, 1996; Hawley *et al.*, 1996).

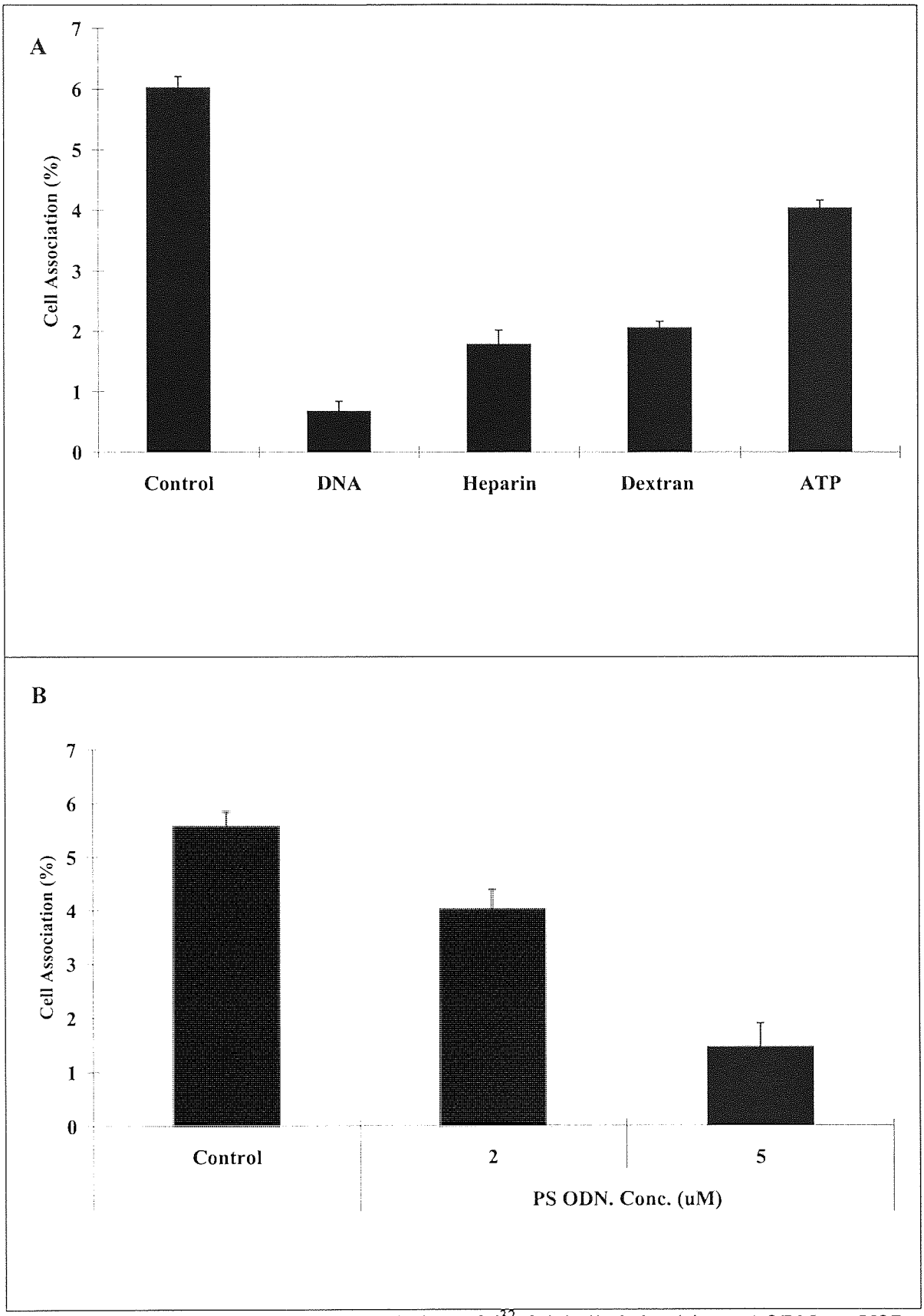


Figure 3.24 Effect of cellular association of [^{32}P]-labelled dendrimer-AODN on U87-MG cells after treatment with (A) with potential competitors (B) PS AODN at 37°C in serum free DMEM medium. (n=3±SD).

3.3.2.1.5 Subcellular Distribution of Dendrimer-AODN

To study the uptake and intracellular distribution of dendrimer-AODNs, U87-MG or A431 cells were incubated with FITC-labelled dendrimer-AODN (dendrimer-AODN-FITC), the FITC linked to the 5'-end of AODN and visualised using an inverted fluorescence microscope (see section 3.2.2.5.10). The majority of the U87-MG cells (approximately 90%) produced extensive, diffused fluorescence within the cytosol and the nucleus (figure 3.27 A.). However, some cells showed more intense fluorescence within the nucleus as compared to the cytosol (figure 3.27 B). The distribution may be due to the dendrimer-AODN being entrapped in larger vesicles within the cytosol. Alternatively, the distribution may be due to the dendrimer-AODN and/or the cleaved AODN being released into the cytosol and the nucleus from the endosomes. The distribution of dendrimer-AODN-FITC was compared with that of FITC labelled AODN at the 5'-end (figure 3.26). A punctate and a less intense fluorescence distribution was evident with naked AODNs, indicative of entry through endocytosis and subsequent compartmentalisation within vesicles, however they do not provide sufficient information to the exact uptake mechanism i.e. fluid phase endocytosis, adsorptive endocytosis or receptor mediated endocytosis (Shoji *et al.*, 1991). A431 subcellular distribution studies showed similar distributions as U87-MG cells, however a greater percentage of A431 cells (approximately 50%) showed more intense fluorescence within the nucleus as compared to the cytosol (figure 3.28 A). The other approximately 50% of the cells produced extensive, diffused fluorescence within the cytosol with a small proportion in the nucleus (figure 3.28 B). The higher proportion of A431 cells containing large amounts of fluorescence in the nucleus as compared to U87-MG cells suggested that the dendrimer-AODN or the AODN cleaved from the dendrimer in the A431 cells was able to move more readily from the endosomes within the cytosol and into the nucleus as compared to U87-MG cells. The difference in distribution between the two cell lines is likely to do with the variability of transport proteins and other trafficking mechanisms (Akhtar *et al.*, 1992). The distribution of dendrimer-AODN-FITC was compared with that of FITC labelled AODN at the 5'– end in A431 cells (Figure 3.26). A similar distribution was observed with A431 cells as compared to U87-MG cells i.e. a punctate and a less intense fluorescence distribution was evident with naked AODNs. The accumulation of the AODN in the nucleus and the cytosol is important for antisense activity and therefore these distribution results provide some optimism for efficacy studies (Akhtar *et al.*, 1992). Similar distribution studies have been

observed with AODNs complexed to PAMAM dendrimers (DeLong *et al.*, 1997; Alahari *et al.*, 1998; Yoo *et al.*, 2000).

3.3.2.2 Cell Type-Specific Uptake

AODN uptake has been reported to be cell type dependent (Noonberg *et al.*, 1993; Nakai *et al.*, 1996; Akhtar *et al.*, 1996; Hawley *et al.*, 1996) which may reflect differences in, among others, the relative expression levels of the putative binding proteins. In an attempt to compare the magnitude of dendrimer-AODN association observed in U87-MG cells with other cell lines, we also examined dendrimer-AODN association with A431 (vulval epithelial), C6 glial (rat glial) and SY5Y (human neuroblastoma) cells. When normalised to cell number, cell association of dendrimer-AODN was also found to be cell type dependent (figure 3.29) with at least 1.3-fold increase in cellular association of dendrimer-AODN between those showing the greatest association (A431) and at least 6-fold increase between cells showing the least uptake (SY5Y). Similar trends were observed with AODNs. Although these results are useful in demonstrating that like AODN, cell association of dendrimer-AODN is cell type specific, the limitations of this type of comparison are acknowledged. Various factors, such as differences in cell size, gross morphology, and varying stages in cell cycle, could influence cell binding. Thus, quantitatively accurate comparisons between different cell types are difficult to obtain.

Overall, it was evident from these results that the cellular uptake properties of this anionic dendrimer delivery system in U87-MG and A431 cells showed many similarities to those observed by the uptake of naked AODNs and ribozymes (Fell *et al.*, 1997). However, cellular uptake and distribution studies suggested that the uptake of dendrimer-AODN was 2-fold and 3.5-fold greater than naked AODNs in U87-MG and A431 cells respectively, at 180 minute incubation time. Although uptake mechanisms are likely to be similar, it seems that once inside the cell, the dendrimer was able to destabilise the endosome and carry or release AODNs into the cytosol and the nucleus. The mechanism of how PAMAM dendrimers enter cell nuclei is as yet unexplained but may involve the interaction and coating of the complex with intracellular lipids during trafficking, subsequently allowing the dendrimer to fuse with the nuclear envelope and release the complex in to the nucleus (Godbey *et al.*, 1999a). A similar mechanism may account for the anionic dendrimer.

Proposed mechanisms of dendrimer-mediated cell entry for PAMAM dendrimers involves the net cationic surface charge of the dendrimer-AODN to interact with the anionic glycoproteins and phospholipids that reside on the cell membrane surface which leads to the subsequent movement of dendrimer-AODN complex into the cytosol by endocytosis (for review see: Eichman *et al.*, 2000). Similarly to the proposed theory for the uptake of cationic dendrimers, the mechanism by which anionic dendrimers bind to cell surface membrane may involve the anionic nature of the complex to interact with cationic charged surface membrane components. An overlap of specific and non-specific protein binding is likely to be occurring on the cell surface before internalisation into the cytosol occurs. The results obtained have shown that the predominant mechanism of uptake of the dendrimer-AODN is likely due to adsorptive and/or receptor mediated endocytisis, through a variety of cell surface proteins and possibly other membrane components. Once inside the cell the AODN may exit from the endosome into the cytosol with or without the dendrimer. Studies have shown that dendrimer-DNA complexes have shown to be localised within the cytosol and the nucleus where its pharmacological effects are manifested (Godbey *et al.*, 1999a; Godbey *et al.*, 1999b; Yoo *et al.*, 2000).

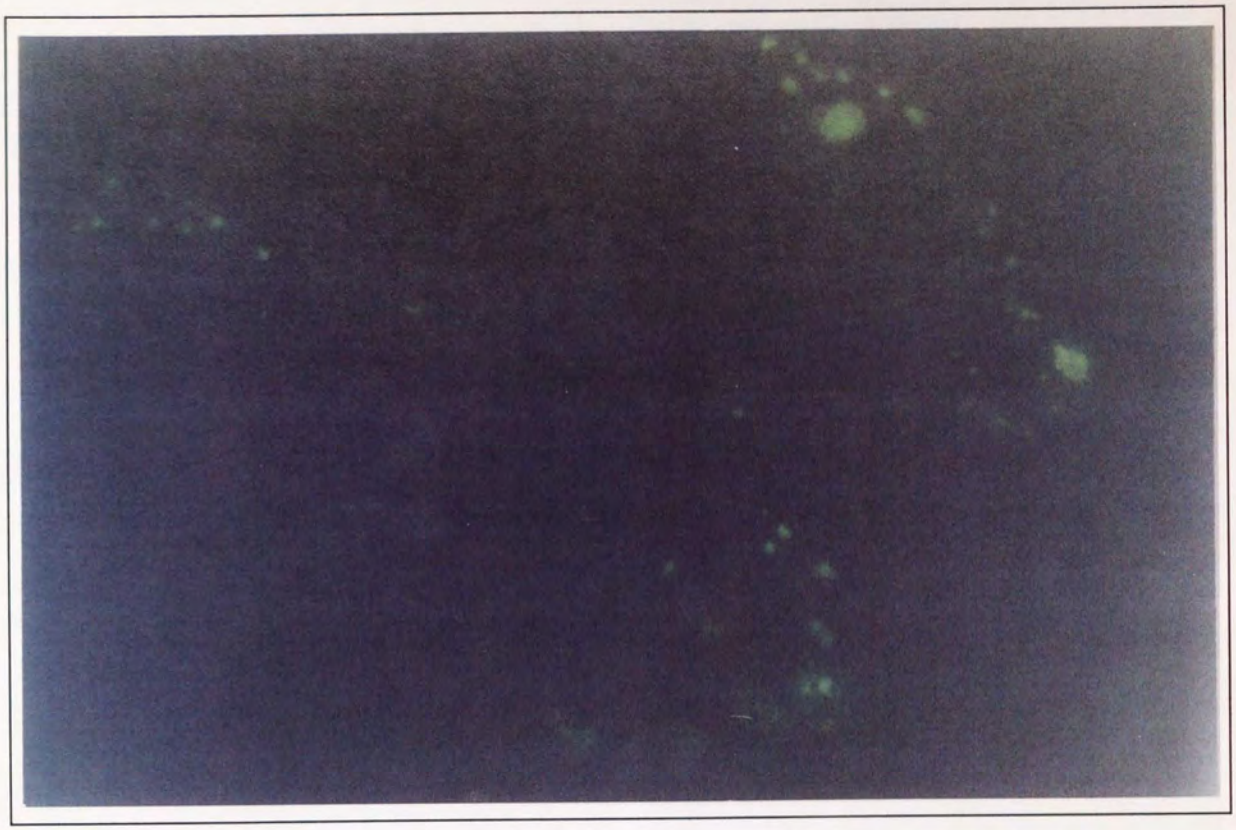


Figure 3.25 Fluorescence detection of AODN-FITC associated with U87-MG cells (magnification × 100).

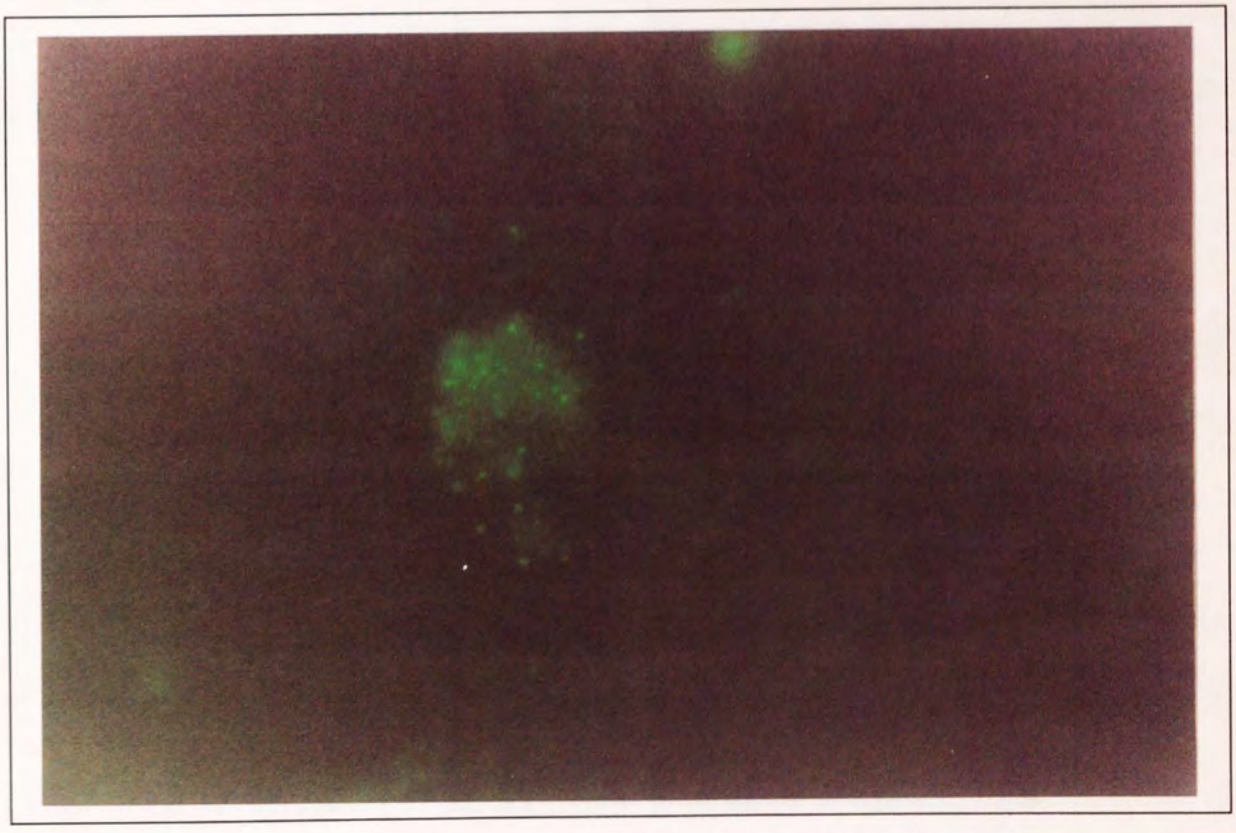
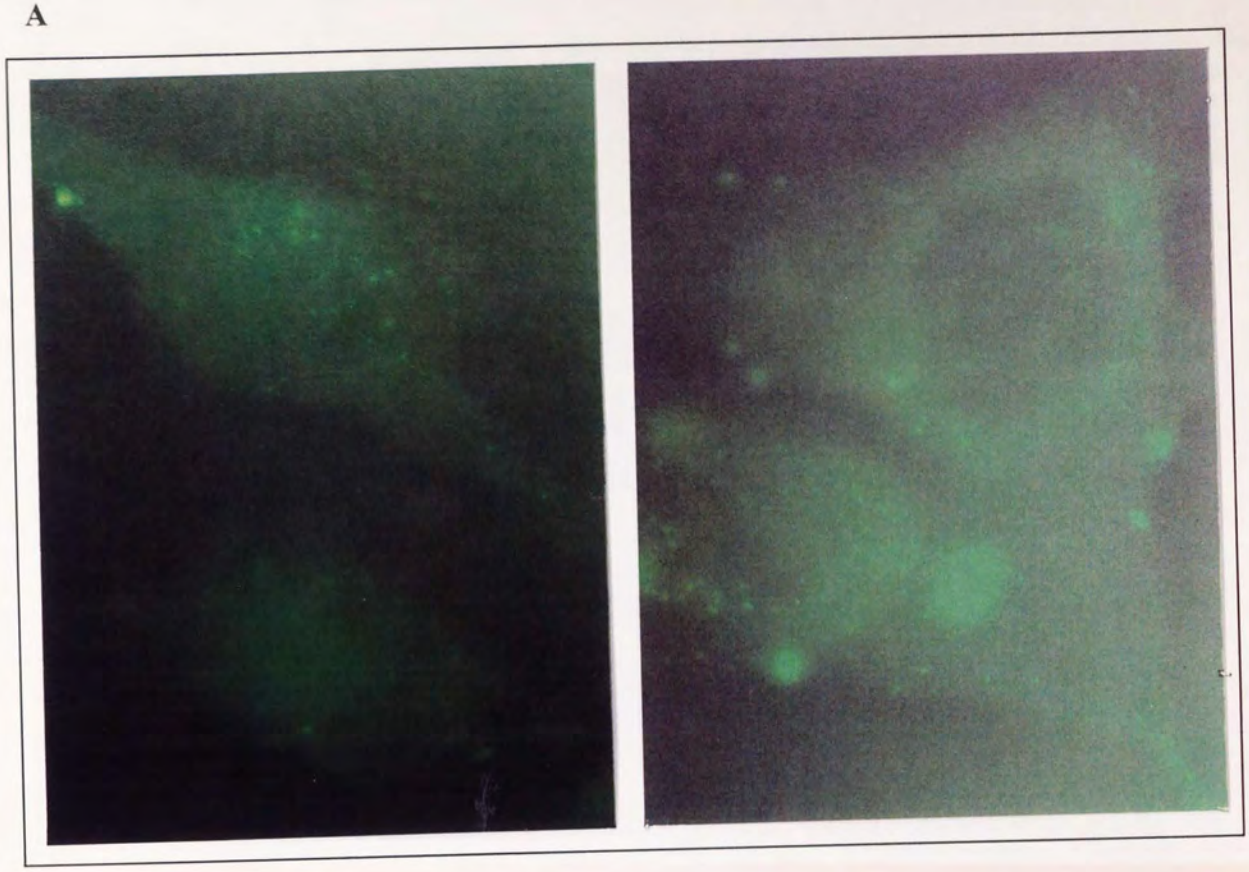


Figure 3.26 Fluorescence detection of AODN-FITC associated with A431 cells. (magnification × 100).

A



B

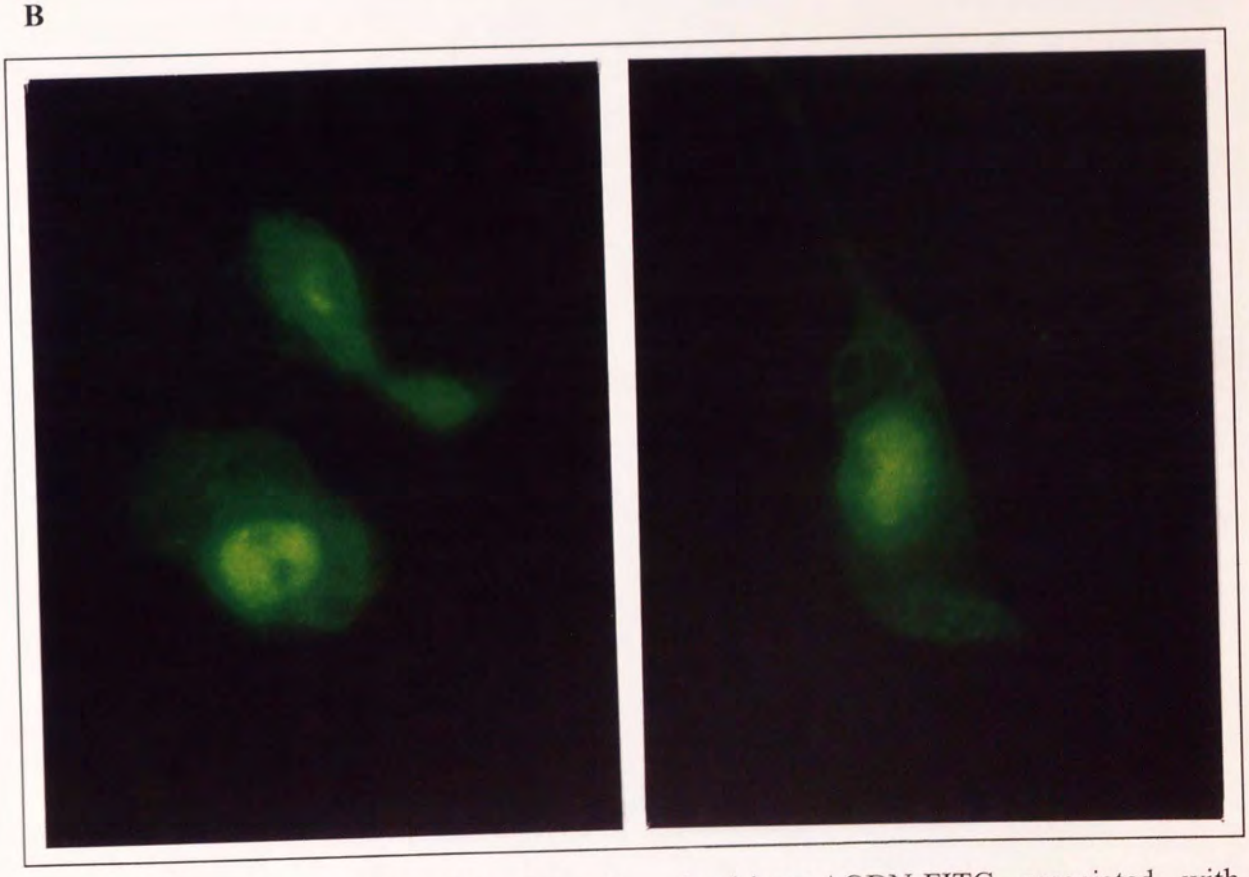
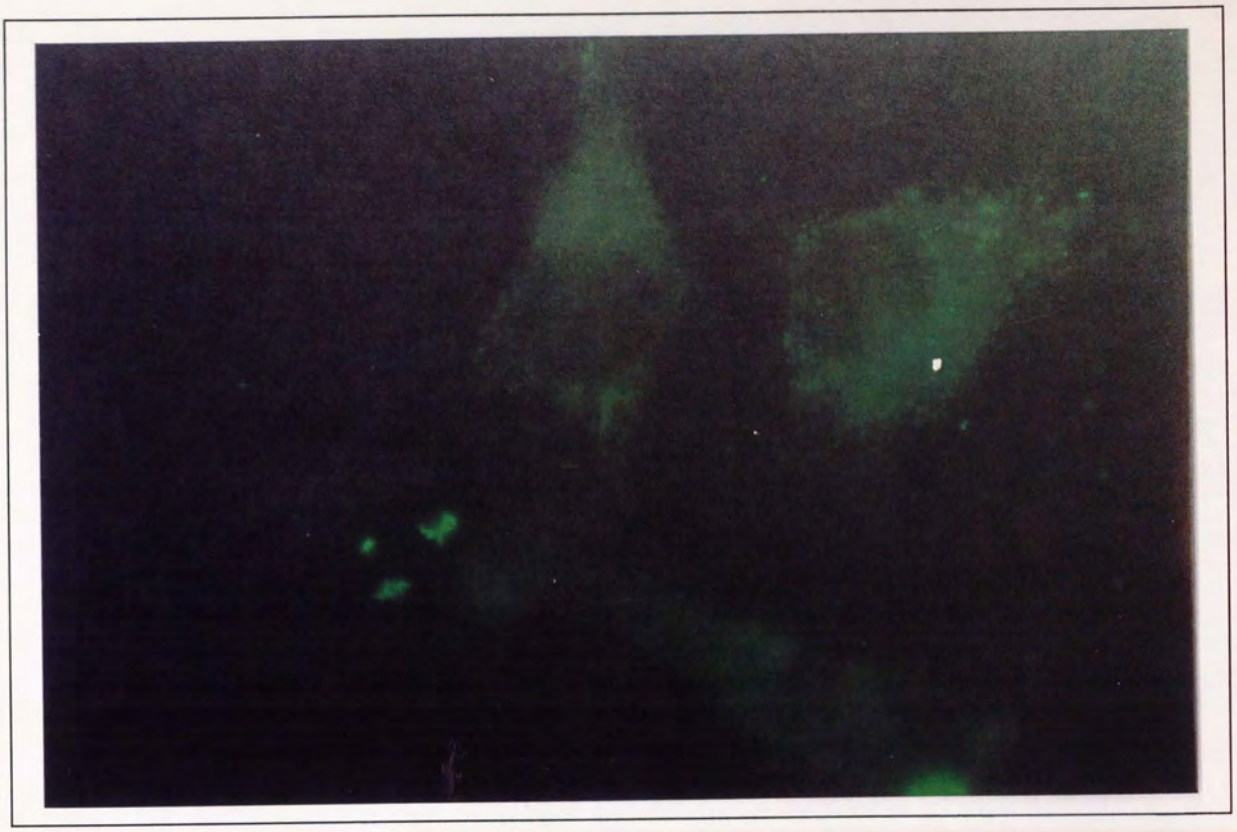


Figure 3.27 Fluorescence detection of dendrimer-AODN-FITC associated with approximately (A) 90% and (B) 10% of U87-MG cells. (magnification × 100).

A



B

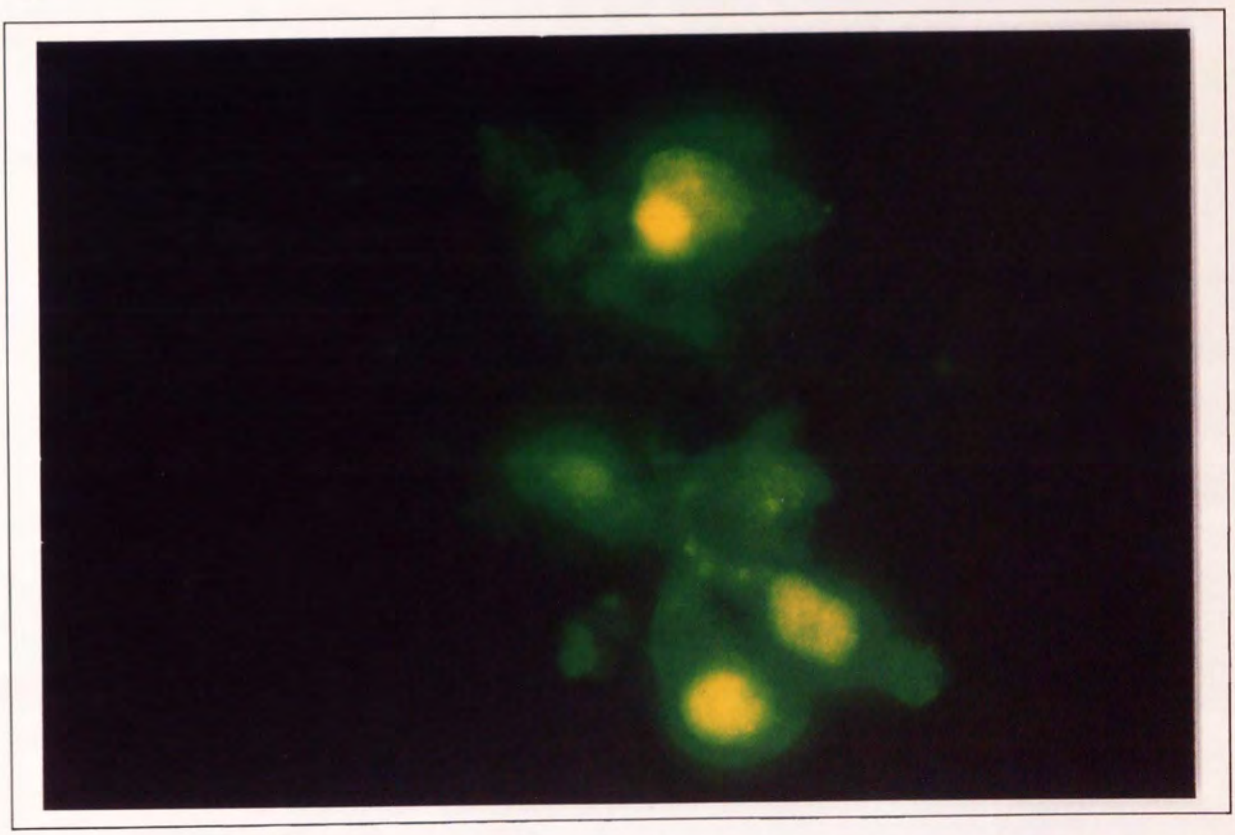


Figure 3.28 Fluorescence detection of dendrimer-AODN-FITC associated with approximately (A) 50% and (B) 50% of A431 cells. (magnification × 100).

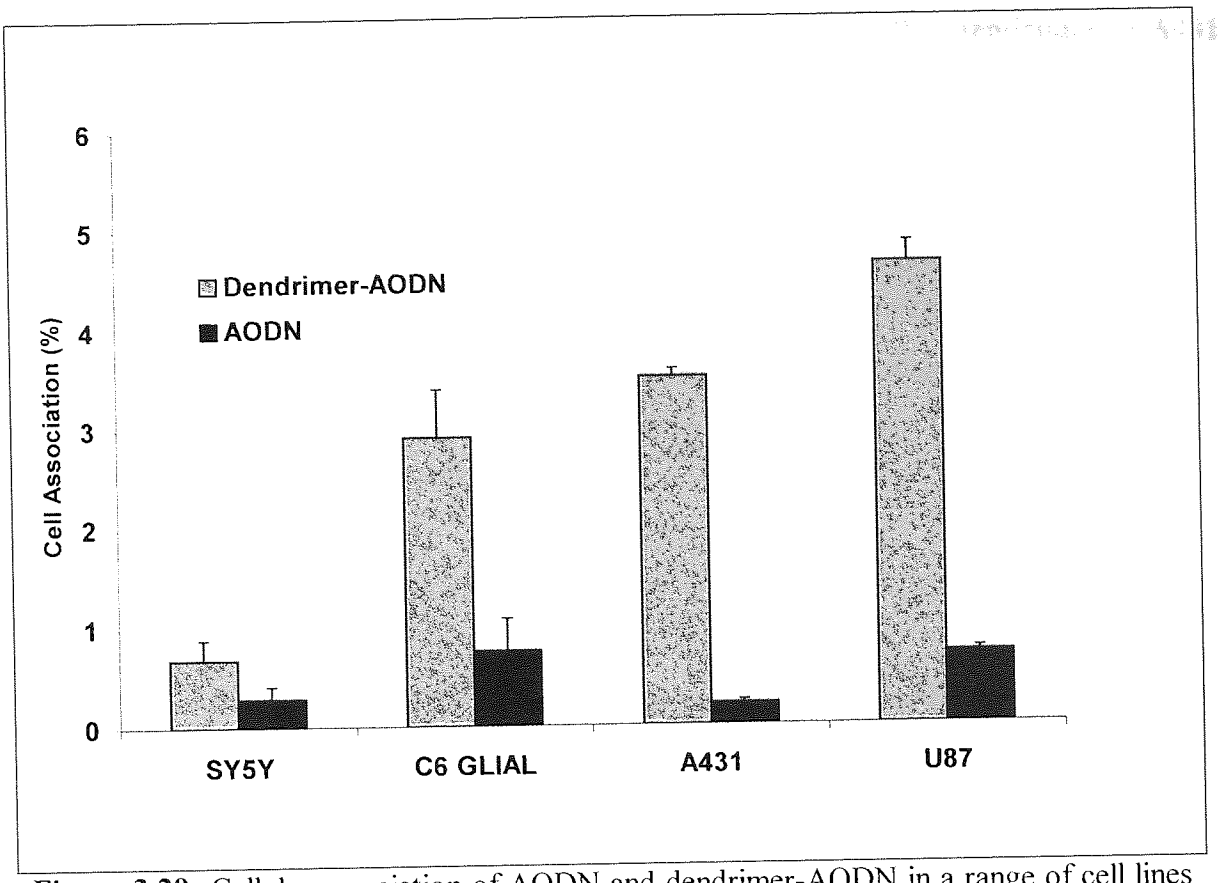


Figure 3.29 Cellular association of AODN and dendrimer-AODN in a range of cell lines in serum-free DMEM medium for 60 minutes at 37°C (n=3±SD).

3.3.3 *In vitro* and Biological Efficacy of AODN Attached to the Dendrimer in A431 Cells

In vitro and biological efficacy studies have been carried out in this section for assessing the ability of the AODN attached to the dendrimer to cleave the *c-erb*B1 mRNA and ultimately to down-regulate EGFR expression with the subsequent reduction in viable cell number of A431 cells. The A431 cell line was chosen for its ability to express high levels of EGFR as compared to U87-MG cells (Hoi Sang *et al.*, 1995a; Hoi Sang *et al.*, 1995b) and thus make EGFR expression easier to visualise.

3.3.3.1 RNase H Assay to Assess In vitro Activity of Dendrimer-AODN

The ability of the AODN attached to the dendrimer to cleave the *c-erb*B1 mRNA was initially assessed *in vitro*. The importance of this study was highlighted by the possibility of steric hindrance caused by the dendrimer-AODN, which could affect the AODNs ability to bind with the target or the capability of RNase H to efficiently cleave the duplex. Thus, RNase H assay was undertaken to assess this possibility. The *c-erb*B1 transcript labelled with α-^{32}P-ATP was incubated with the dendrimer-AODN in the presence of RNase H at 37°C for 1-hr. The samples were resolved on a 6% denaturing gel to analyse cleavage (see section 3.2.2.7). The results are shown in figure 3.30.

Cleavage products were observed at ~480b implying that the AODN attached to the dendrimer in the presence of RNase H was able to cleave the *c-erb*B1 transcript. No cleavage products were observed with the AODN attached to the dendrimer without RNase H. These results suggested that the AODN was able to bind and cleave the target site while attached to the dendrimer. It is therefore improbable that the dendrimer will prevent the AODN from binding to the mRNA and rendering it inactive due to structural hindrance. This is contrary to a study involving PAMAM dendrimers complexed to DNA which appeared to be less efficient at mediating transfection due steric hindrance at the level of transcription (Kukowska-Latallo *et al.*, 1996). However, it is important to note that the cleavage was undertaken in an environment where no metabolic activities and nuclear proteins existed which could bind to charged species and consequently inactivate the complex or even cause non-sequence-specific biological effects (Weidner *et al.*, 1995; Brukner *et al.*, 2000).

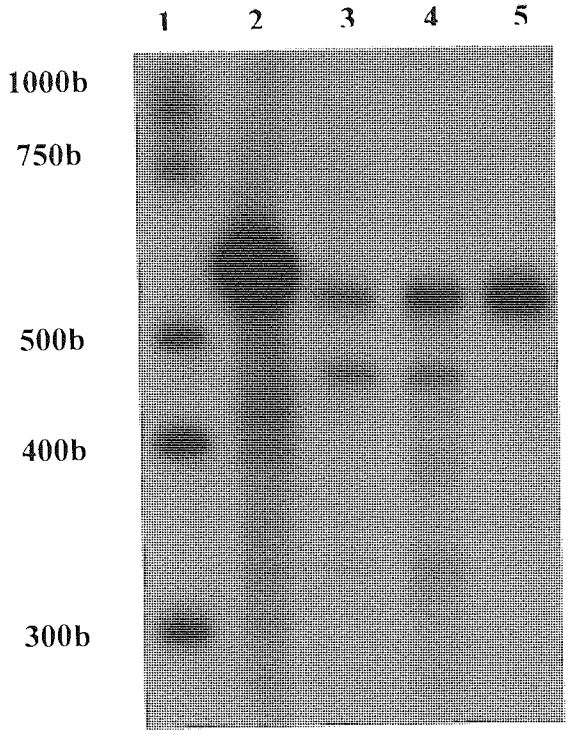


Figure 3.30 *In vitro* activity of dendrimer-AODN incubated with α-^{32}P-ATP labelled *c-erb*B1 in the presence of RNase H at 37°C. (1) RNA markers, (2) *c-erb*B1 transcript (1μM), (3) AODN (1μM) + *c-erb*B1 transcript (1μM), (4) dendrimer-AODN (1μM) + *c-erb*B1 transcript (1μM), (5) dendrimer-AODN (1μM) + *c-erb*B1 (1μM) without RNase H.

3.3.3.2 Effect of dendrimer-AODN on Viable Cell Number and EGFR Expression of A431 Cells

The ability of the dendrimer-AODN to successfully cleave the target site initiated cell culture work to assess the ability of dendrimer-AODN in down-regulating EGFR expression and the possibility of affecting viable cell number of A431 cells.

The efficacy of the dendrimer-AODN in affecting A431 viable cell number was assessed by using trypan blue dye exclusion assay (see section 2.2.2.11.4). A 72-hr time point was used to assess viable cell numbers. Cells were seeded at 1.25×10^4 cells/well and after 24-hrs treated with varying concentrations of dendrimer-AODN for 72-hrs. The results are shown in figure 3.31.

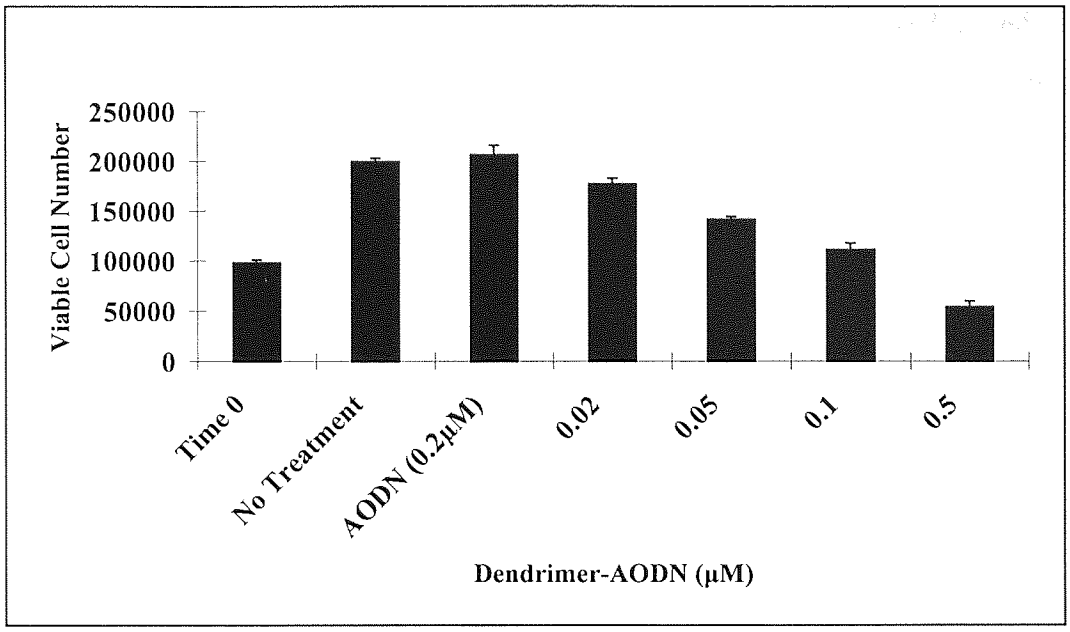


Figure 3.31 Effect of dendrimer-AODN treatment on viable cell number of A431 cells over 72-hrs in serum free medium. Time 0= cell number at treatment time. Data represents the mean n=3±SD.

Incubation of dendrimer-AODN with A431 cells over 72-hrs produced a dose-dependent effect. The percentage growth inhibition with each dendrimer-AODN concentration was evaluated in table 3.3.

Table 3.3 The Effect of Dendrimer-AODN on the Average % Reduction in Cell Number of Control by Trypan blue dye exclusion Assay.

A431 cells treated with dendrimer-AODN (μM)	Average reduction in % cell number of control
0.02	7 ± 2.5
0.05	26 ± 1.4
0.1	41 ± 3
0.5	71 ± 2.7

The average reduction of A431 cell number was 7% ± 2.5 when treated with 0.02μM of dendrimer-AODN as compared to untreated cells. At a concentration of 0.5μM of dendrimer-AODN the average reduction in percentage cell number was 71% ± 2.7 as compared to the untreated cells. It is clear from the above data that the AODN-dendrimer was having an effect on viable cell number in comparison to naked AODN and untreated cells. However, to assess if these results were due to an antisense effect EGFR protein expression was analysed in A431 treated cells. The cells were treated with the concentrations obtained from the trypan blue dye exclusion data, and a Western blot was conducted as described in the methods

section 2.2.2.12. The results are shown in figure 3.32. Naked AODN showed no reduction in EGFR expression which was expected from cellular association, subcellular distribution and trypan blue dye exclusion studies. However, a dose-dependent decrease in EGFR protein expression was observed when A431 cells were treated with dendrimer-AODN over 72-hrs in DMEM (serum free) as compared to untreated cells. A reduction of 20%, 28% and 45% in EGFR expression was observed with 0.05μM, 0.1μM and 0.5μM dendrimer-AODN treatments respectively, as compared to untreated cells. The effects of dendrimer-AODN on EGFR protein expression levels correlated with the reduction in the A431 viable cell number. These results suggested that the dendrimer successfully delivered the AODN to the target site where it was able to bind with the *c-erb*B1 mRNA and consequently down-regulate EGFR protein expression (see section 1.7). The ultimate result would be a decrease in viable cell number as compared to the untreated cells. Indeed, an antisense RNA has shown to reduce expression of the EGFR and the malignant behaviour of a human carcinoma cell line (Moroni *et al.*, 1992). These results illustrate the potential efficacy of antisense mechanisms when the antisense molecule reaches the target mRNA. Scrambled AODNs attached to the dendrimer is required as a control to confirm the antisense effect, however it was not possible to obtain these due to unforeseen circumstances. To further confirm an antisense effect the level of EGFR mRNA expression needs to be investigated by Northern blotting because the transcript may be depleted or cleaved if an RNase H-mediated mechanism was operating (Pu *et al.*, 2000). Unfortunately, time restraints prohibited further research on this matter.

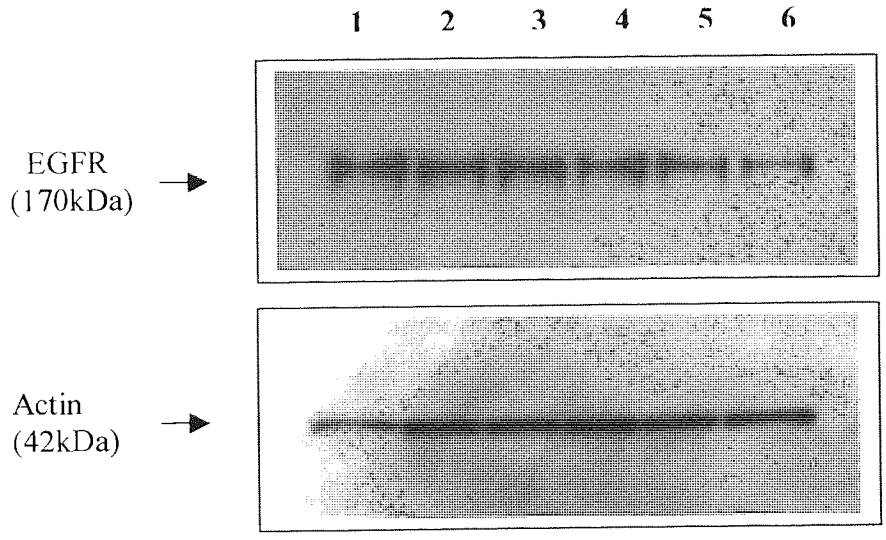


Figure 3.32 Effect of dendrimer-AODN on EGFR expression in A431 cells. Lane 1= no treatment, lane 2= 0.2 μM AODN, lane 3= 0.02μM dendrimer-AODN, lane 4= 0.05μM dendrimer-AODN, lane 5= 0.1μM dendrimer-AODN, lane 6= 0.5μM dendrimer-AODN. Images depict representative blots from duplicate experiments.

3.3.4 Cell Association Studies of Dendrimer-AODN in DMEM Containing Foetal Bovine Serum

The experiments so far have been undertaken in DMEM (serum-free) conditions to determine if the anionic dendrimer was capable of delivering AODNs into cells and down-regulating the target site. However, to determine if this delivery system could be effective *in vivo* where proteins and nucleases are present, cellular association experiments were undertaken in the presence of DMEM containing foetal bovine serum. U87-MG cells were used as a model cell line.

3.3.4.1 Temperature and Time-Dependence of Cellular Association in Foetal Bovine Serum in DMEM

Cellular association of dendrimer-AODN in U87-MG cells in 10% foetal bovine serum DMEM was carried out (see section 3.3.2.2). The cell association of dendrimer-AODN was determined at selected time intervals over 180 minutes at 37°C and 4°C. As figure 3.33 demonstrates, at 37°C and 4°C cell association of dendrimer-AODN increased constantly to approximately 1% and 0.7% respectively, after which the rate of uptake began to level out. The cellular association of the dendrimer-AODN was insignificantly different between 37°C and 4°C ($P>0.05$), thereby demonstrating that the two uptake profiles were similar and that only a minor difference in uptake process is observed at 4°C. In an attempt to determine the extent of fluid-phase endocytosis (or pinocytosis) in U87-MG cells, the uptake of [^{14}C]-labelled mannitol, a classic nonmetabolizable marker for fluid phase pinocytosis (Chonn *et al.*, 1995) was measured over a 3-hr period at 37°C. Compared with the uptake of radiolabelled dendrimer-AODN at 37°C the basal rate of pinocytosis in these cells remained extremely low (0.1-0.4%) throughout this period and is unlikely to account for a significant fraction of dendrimer-AODN uptake at the concentration used in this cell line. The rate of association was constant over the duration of the experiment. However, it is clear from the data that cellular association of dendrimer-AODN was considerably reduced in the presence of 10% serum as compared to serum free medium (see section 3.3.2.2.2). The effect of serum on cellular association was confirmed by the reduction in cellular association with increasing serum concentrations in DMEM at 37°C for 1.5-hrs incubation period. The results are shown in figure 3.34.

Increasing serum concentration in DMEM (serum-free) resulted in the gradual reduction in cellular association as compared to the control (no serum). Table 3.4 shows percentage reduction in cellular association of control. 2% serum in DMEM inhibited cellular association by 34% ± 0.7 and increasing serum concentration to 5% and 10% resulted in cellular inhibition of 62% ± 0.5 and 82% ± 0.1 respectively.

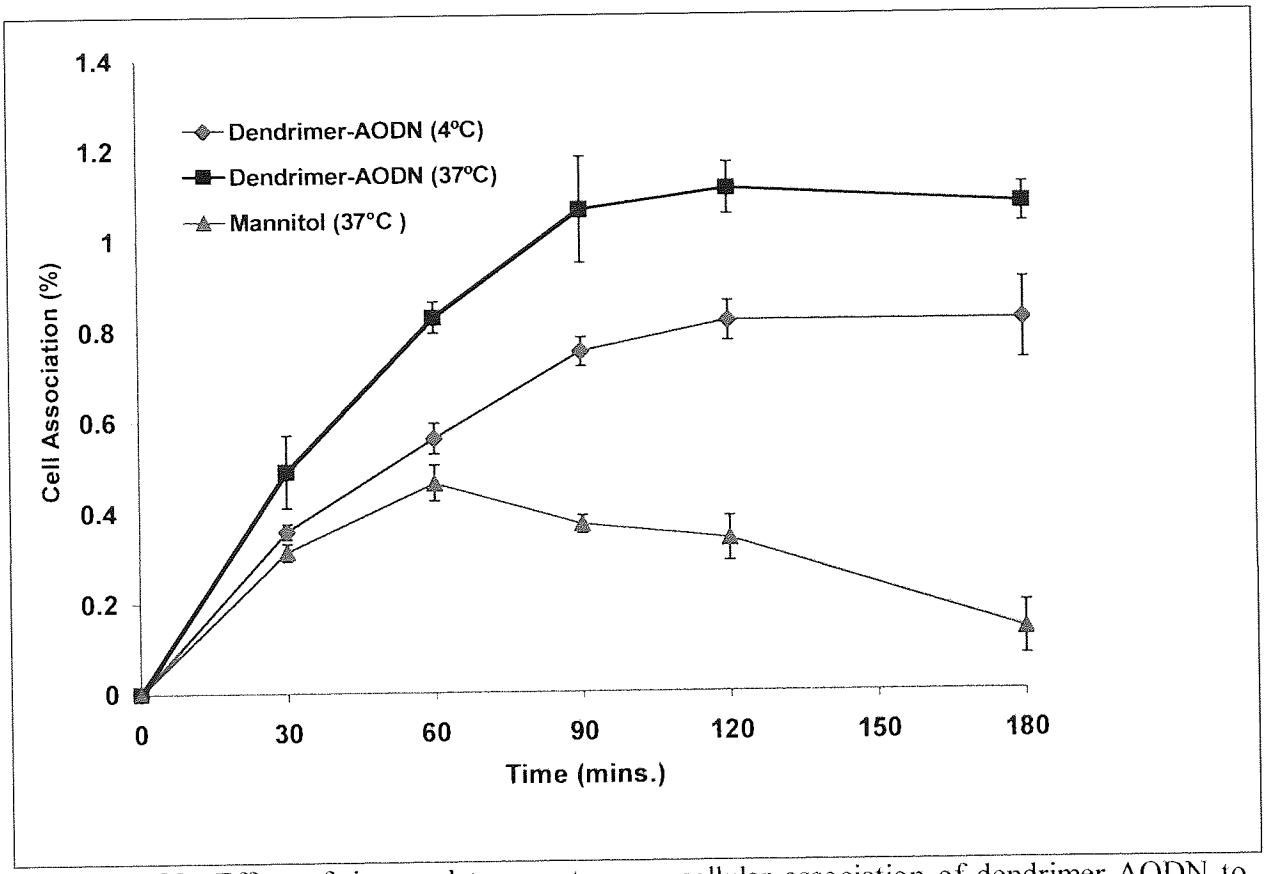


Figure 3.33 Effect of time and temperature on cellular association of dendrimer-AODN to U87-MG cells. Cell monolayers were incubated with [^{32}P]-labelled dendrimer-AODN and [^{14}C]-labelled mannitol in DMEM containing 10% foetal bovine serum at 37°C and 4°C (n=3±SD).

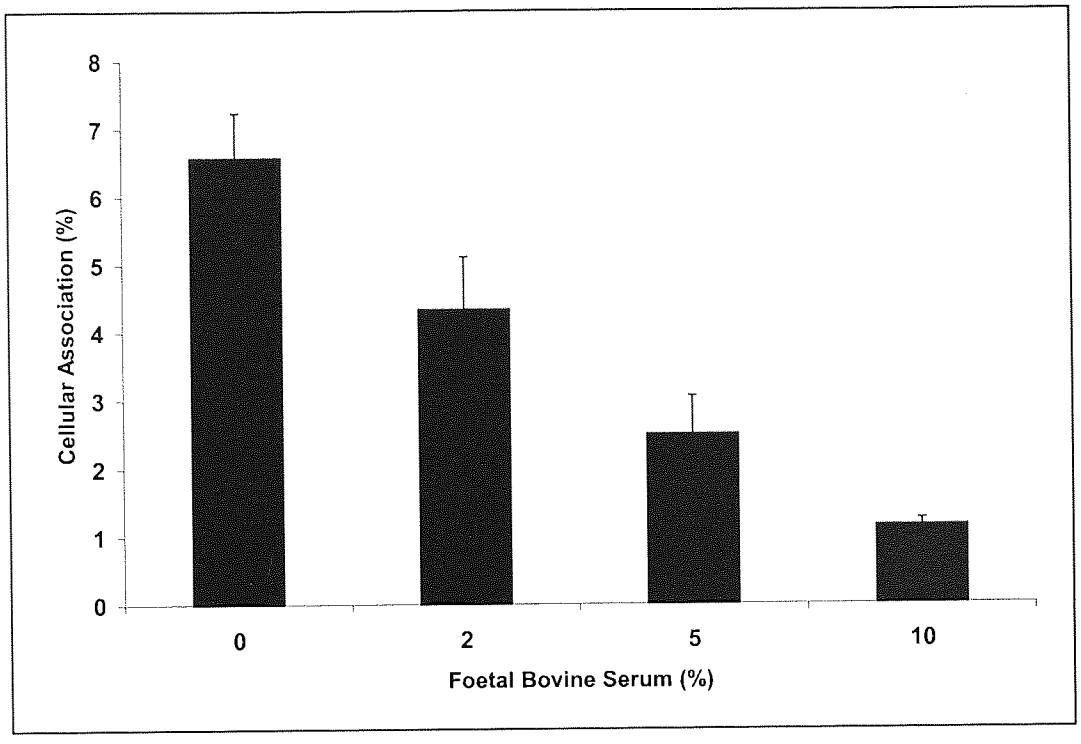


Figure 3.34 The effect of dendrimer-AODN on U87-MG cellular association with increasing concentrations of serum in DMEM at 37°C (n=3±SD).

Table 3.4 The effect of dendrimer-AODN on the percentage reduction of A431 cellular association with increasing concentrations of serum in DMEM at 37°C.

Serum concentration in DMEM (%)	Reduction in cellular association of control (%)
2	34% ± 0.7
5	62% ± 0.5
10	82% ± 0.1

These results suggested that serum inhibited cellular association of dendrimer-AODN. It is likely that proteins present in the serum were binding to the anionic dendrimer and thus prevented cellular association and subsequent uptake. It was not surprising therefore that proteinase K was required to digest the proteins in the DMEM containing 10% foetal bovine serum before the dendrimer-AODN was successfully able to run down the polyacrylamide gel, whilst carrying out the experiments for stability studies (see section 3.2.2.4.2). In the absence of proteinase K it is likely that the large molecular weight of the dendrimer-AODN/protein complex prevented the migration of the complex down the polyacrylamide gel. The cellular association results were consistent with other studies which have shown that most of the delivery systems do not function well in the presence of serum (Juliano *et al.*, 1999; Astriab-Fisher *et al.*, 2000). Similar results have been observed with PAMAM

dendrimers complexed to AODNs where serum significantly reduced the efficacy of AODNs (Yoo *et al.*, 1999). Also, in the same study lipofectamine, a cationic lipid cytofectin of AODNs displayed no activity at all. Subcellular distribution studies were undertaken in different serum concentrations to analyse differences in distribution in DMEM containing serum as compared to serum free DMEM.

3.3.4.2 Subcellular Distribution of Dendrimer-AODN in DMEM containing Serum

To study the uptake and intracellular distribution of dendrimer-AODNs in the presence of different serum concentrations cells were incubated with FITC-labelled dendrimer-AODN (dendrimer-AODN-FITC) with the FITC linked to the 5'-end of the AODN and visualised using an inverted fluorescence microscope (see methods section 3.2.2.5.10). The results are shown in figure 3.35. The distribution studies showed reduced dendrimer-AODN-FITC associated with U87-MG cells with increasing serum concentrations. With 2% serum in DMEM the majority of the dendrimer-AODN-FITC was associated on the cell membrane with small amounts within the cytosol (figure 3.35 A). Increasing serum concentrations reduced the dendrimer-AODN-FITC associated with the cell membrane i.e. for 20% serum in DMEM, no fluorescence was seen within the cytosol, however, small amounts of fluorescence was associated with the cell membrane (figure 3.35 D). Globules of fluorescence were noticed around the cells as well as on the surface of the cell membranes. It is likely that the observed globules are products of dendrimer-AODN/serum protein complexes. This can have two possible consequences; first, neutralisation of the anionic charge of the dendrimer which may prevent the complex from binding to the cell surface and consequently entering the cell and second, an increase in the size of the complex, resulting in the dendrimer complex being too large for it to be taken up by the cell (Zimmer 1999). A combination of both of these processes taking place in the presence of serum may also be a possibility. The binding of the serum proteins to the dendrimer-AODN can be confirmed by a gel mobility shift assay in which the dendrimer-AODN would be incubated with the relevant serum concentrations and then resolved on a non-denaturing (native) polyacrylamide gel (see section 2.3.1.1.4). Binding would show a change in migration as compared to controls. Due to time constraints it was not possible to carry this out. However, whilst conducting stability experiments of the dendrimer-AODN in DMEM containing serum, retardation in the migration of the dendrimer-AODN was noticed on polyacrylamide gels (discussed in section 3.3.4.1).

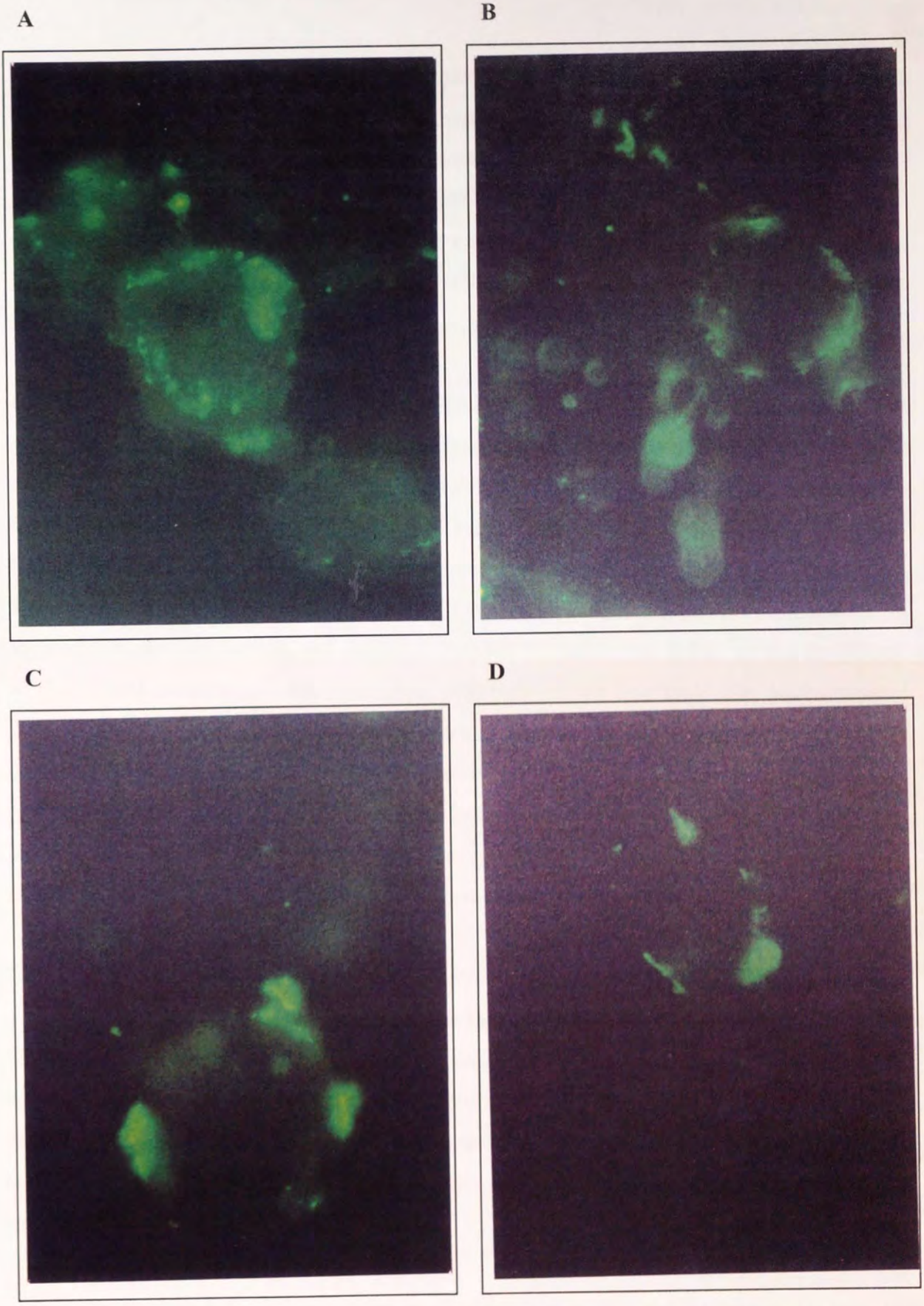


Figure 3.35 Fluorescence detection of dendrimer-AODN-FITC associated U87-MG cells. (magnification × 100) in (A) 2% serum in DMEM, (B) 5% serum in DMEM, (C) 10% serum in DMEM, (D) 20% serum in DMEM.

3.4 CONCLUDING REMARKS

Little is known about the mechanisms of dendrimer-mediated cellular uptake of AODNs. In this chapter we have characterised the cell association/uptake properties of a novel anionic dendrimer based on a pentaerythritol structure, covalently linked to AODNs in U87-MG glioma and A431 vulval carcinoma cells in DMEM (serum-free). RNase H assay, trypan blue dye exclusion assay and western blotting was undertaken to assess the efficacy of the delivery system. Metabolic stability of dendrimer-AODN was also assessed in a variety of biological medium.

At the 180 minute incubation time point cell association of the dendrimer-AODN was approximately 2-fold and 3.5-fold higher as compared to the naked AODN for U87-MG and A431 cells respectively. The cellular association of the 5' labelled AODN linked to the dendrimer in U87-MG glioma cells and A431 cells was temperature and energy dependent and involved an active process. The active process could be competed with cold dendrimer-AODN of the same chemistry, antisense PS-ODNs, and a variety of other polyanions (salmon sperm DNA, dextran sulfate, and heparin) in U87-MG cells. The ability of the dendrimer-AODN to self compete and also to compete with both nucleic acid molecules (salmon sperm DNA, PS-ODNs) and non-nucleic acid molecules (dextran sulphate, heparin) suggested an overlap of specific and non-specific cellular association. The results indicated that the predominant mechanism of uptake was by adsorptive and/or receptor mediated endocytosis through a variety of cell surface membrane components. Stability studies showed that AODNs were more stable when linked to the dendrimer as compared to the naked AODN.

DMEM containing 10% Foetal calf serum inhibited cellular association of dendrimer-AODN. The inhibition was proportional to the percentage of foetal bovine serum in DMEM. The binding of proteins within the foetal bovine serum to the dendrimer-AODN is likely to be inhibiting cellular uptake. Subcellular distribution studies showed globules of fluorescence forming in the presence of DMEM containing serum, which may be the result of proteins in the serum binding to the dendrimer-AODN by electrostatic forces and hence preventing cellular uptake of the dendrimer-AODN.

The ability of the AODN attached to the dendrimer to cleave the *c-erb*B1 mRNA was initially assessed *in vitro*. Dendrimer-AODN in the presence of RNase H was able to cleave the *c-*

*erb*B1 transcript. This result suggested that the AODN was able to bind and cleave the target site while attached to the dendrimer. Trypan blue dye exclusion assay suggested a reduction in viable cell number. Western blots showed EGFR down regulation with dendrimer-AODN, suggesting the dendrimer-AODN may be specifically inhibiting EGFR expression and consequently having an effect on viable cell number.

DISCUSSION

Human glioblastomas and breast tumours have been shown to over-express the *c-erb*B1 proto-oncogene which encodes for the epidermal growth factor receptor (EGFR). The ability to block the *c-erb*B1 proto-oncogene and subsequently down-regulate EGFR expression would therefore be a logical approach to treat such cancers. Synthetic, short, single-stranded DNA called antisense oligonucleotides (AODNs) that are complementary to the 5'-coding sequence of *c-erb*B1 may confirm this approach of rational drug design and ultimately be used as therapeutic agents. Poor pharmacokinetic/pharmacodymanic and cellular uptake properties are amongst the main problems for AODNs, which need to be overcome if they are to become successful therapeutic agents. For AODN molecules to specifically down-regulate the target protein they need to be delivered at the target site, be protected from digestive enzymes and be able to enter the cells readily. To further enhance the therapeutic efficacy of AODNs other strategies such as combination therapies with cytotoxic drugs may need to be employed. To address these issues, the use of combination therapy of AODNs (complementary to the 5'-coding sequence of *c-erb*B1) and cytotoxic agents such as 5-fluorouracil (5-FU) in poly (lactide-co-glycolide) P(LA-GA) polymeric formulations as well as a novel anionic dendrimer delivery system have been evaluated in this thesis. It seemed reasonable to focus on each potential delivery system i.e. dendrimer and polymer in two separate chapters which in turn would comprise of characterisation and efficacy.

In an attempt to use EGFR AODNs with conventional cytotoxics, the first chapter assessed the possibility of the potential co-delivery of a 21-mer phosphorothioate ODN sequence and 5-FU, a model cytotoxic drug, using P(LA-GA) microspheres. Individual formulations of the two agents, as well as co-entrapment of the AODN and 5-FU within a single microsphere formulation were characterised and release profiles determined. Sustained release over 35 days was observed in all types of formulation as well as the loaded microspheres exhibiting smooth surface morphology. These results were consistent with other studies (Lewis *et al.*, 1998; Khan *et al.*, 2000). However, the release profiles for the co-entrapped formulations appeared to have different shapes than those observed for the separate formulations. Firstly the burst effect for AODNs from the co-entrapped microspheres was reduced (~ 3-fold) from about 7% to 2.5% whereas for 5-FU the burst effect increased slightly (~1.5-fold) from 10%

to 15%. In addition the amount, and thus the overall average rate of drug released over 35 days for both agents was reduced. It is possible that these markedly reduced release rates for both agents arise due to differences in drug distribution within the co-entrapped versus separate microsphere formulations. To some extent this is suggested by the observed differences in the burst effects of the two drugs in each of the formulations. Alternatively (and/or additionally), these differences, especially noting the marked change in the 5-FU release profile, may be due to an interaction with the AODN. A gel mobility shift assay suggested that 5-FU can bind to adenine residues in ODNs and that this drug-drug interaction may contribute to the slower release profiles of the two agents from the co-entrapped P(LA-GA) microsphere formulation. To evaluate the merits of utilising a single P(LA-GA) microsphere containing both agents over separate microsphere formulations of each agent, *in vitro* release profiles of AODNs and 5-FU from different mixtures of single formulations was carried out. For example, increasing the mass of microsphere containing 5-FU to 70mg and keeping AODN microsphere mass fixed at 30mg in the release experiment yielded an increase in the amount of 5-FU released at day 21 from 11 to 20 pmoles, an approximate 2-fold increase. Similar results can be obtained for AODN by keeping the 5-FU mass fixed and increasing the mass of microsphere containing AODN. These data suggested that drug release could be tailored by simply altering drug loading and mass ratio of the separate microsphere formulations of the two (or more) drugs. Combination studies have shown phosphorothioate AODNs to interact with cytotoxic drugs and subsequently reduce their efficacy (Stull *et al.*, 1993; Blagosklonny *et al.*, 1994). Thus, the co-entrapped single formulation was deemed unsuitable for the co-delivery of these two anti-cancer agents. Separate microsphere formulations of the two drugs were considered superior as they avoided potential drug-drug interactions (due to the physical separation of the two drugs) and, by simply mixing different mass ratios, provided a greater flexibility in achieving the required release kinetics. Taking these results into consideration it was therefore decided that separate microsphere formulations containing each drug would be made for subsequent formulations as well as testing these delivery systems on cells. To test the delivery system microspheres were prepared by the Silverson double-emulsion method. This method produced smaller microspheres (1-8μM) which would enable them to enter cells more efficiently as compared to larger microspheres (10-20μM) (Akhtar *et al.*, 1997; Khan *et al.*, 2000).

In vitro cell culture studies were undertaken to evaluate the efficacy of an AODN (targeting *c-erb*B1) and 5-FU microsphere delivery system in an A431 vulval carcinoma cell line. The

delivery system may improve the efficacy of AODN and/or 5-FU compared to naked drugs due to increased cellular uptake and sustained release of the drugs. This hypothesis is reflective of a recent study which demonstrated enhanced cellular uptake of AODNs/liposome complex with the subsequent improved efficacy in combination with idarubicin in K-562 cell line (Vellon *et al.*, 2002). Initially, efficacy studies were evaluated using naked AODN and 5-FU and then subsequently encapsulated in microsphere delivery systems. Viable cell number and EGFR expression studies were carried out using trypan blue dye exclusion, MTT assay and western blotting assays. Various concentrations of AODNs were combined with the 5-FU at an IC_{50} value (0.19μM). The combination treatments were incubated with A431 cells at 37°C for 24-hrs. 1μM of AODN which caused an average reduction of 16% ± 6 in A431 viable cell number, however when combined with 0.19μM 5-FU (IC_{50}) an average reduction of 53% ± 7 was noticed. Additive effects were also seen with higher AODN concentrations e.g. 2μM of AODN caused an average 27% ± 5 reduction of A431 cells, but when combined with 0.19μM 5-FU (IC_{50}) an average 73% ± 5 reduction was observed. Similar additive effects were seen with scrambled AODN (2μM) in combination with 5-FU (0.19μM), suggesting that the effect was brought about by a non-antisense cytotoxic effect of the ODN. From the Western blot no significant decrease in EGFR protein expression was evident with the relevant treatments. This result confirmed that the effect was unlikely to be an antisense mechanism because no differences in EGFR expression was observed by Western blotting as compared to the controls. Overall, these results indicated that a non-antisense cytotoxic effect was likely to be occurring. It is probable that the phosphorothioate ODN was binding non-specifically to cellular proteins resulting in some degree of cytotoxicity which on its own was not enough to have an effect on cell number. However, the addition of 5-FU caused further cytotoxicity which ultimately resulted in reduction of cell number. It is a well documented fact that ODNs, especially phosphorothioates interact with a wide variety of proteins in a sequence-independent manner (Gao *et al.*, 1993; Stein *et al.*, 1993; Weidner *et al.*, 1995; Veal *et al.*, 1998; Juliano *et al.*, 1999). Studies undertaken with naked AODN and/or 5-FU were repeated using a P(LA-GA) microsphere delivery system. Various microsphere masses encapsulated with AODNs were combined with a 5mg microsphere mass encapsulated with 5-FU (a concentration which reduced 50% of viable cell number after a 72-hr incubation period). Combining separate microsphere formulations loaded with either AODN or 5-FU resulted in an additive response as compared to either encapsulated drug alone. For example, AODN encapsulated in 6mg of microsphere mass

resulted in a 60%±4 reduction in cell number. However, an 87%±0.2 reduction in cell number was observed when this AODN encapsulated microsphere mass was combined with 5mg of microsphere mass containing 5-FU. Similar additive effects were seen with 6mg and 5mg of microsphere masses encapsulated with scrambled AODN and 5-FU respectively, suggesting that the additive effect was brought about by a non-antisense cytotoxic effect of the ODN. This was confirmed by western blot which showed no significant decrease in EGFR protein expression with relevant microsphere combination treatments. The successful delivery of AODNs to the target site by microspheres is likely to be dependent upon the release of intact AODNs into the cytosol from the microspheres which are taken up by an endocytic pathway and are present within endosomal vesicles (Chavany *et al.*, 1994; Lewis *et al.*, 1995; Akhtar *et al.*, 1997; Hughes *et al.*, 2001). It is possible therefore that a 72-hr incubation period of microspheres with cells was not sufficient for enough AODN molecules to be released into the cytosol or the nucleus to have an effect. It is likely that the effects and mechanisms of action seen by encapsulated AODN and 5-FU are similar to that of naked drugs i.e. non-antisense cytotoxic effect. However, the greater effects of encapsulated AODN and 5-FU is likely to do with the greater number of AODN and 5-FU molecules being released in close proximity to the surface of the cell monolayer (micospheres binding to cell membrane), during the burst phase as well as the sustained phase (for those microspheres which had not entered the cells) as compared the naked drugs. This study highlights the problems associated with AODNs binding non-specifically to intracellular proteins, subsequently leading to toxicity and also the importance of controls to confirm sequence-specific antisense effects. The study also highlighted the limitation of using the microsphere delivery system in cell culture where the time available for incubation restricts the full potential of the delivery system in terms of enhancing the pharmacokinetic/pharmacodynamic properties of AODNs. For example, incubation of microspheres with cells for longer time periods would result in over-confluency of cells and subsequently cell death, ultimately leading to invalidation of results. It is not surprising therefore that the majority of successful studies to date on the efficacy of AODN microsphere delivery systems are restricted to *in vivo* (Putney *et al.*, 1999; Khan *et al.*, 2000). Animal studies allow microspheres to fulfil their requirements as delivery systems such as improving pharmacokinetic, sustained release and stability properties of AODNs, with the subsequent ability to show their full potential.

The mechanism of uptake, stability and efficacy of a novel anionic dendrimer, based on a pentaerythritol structure (Shchepinov *et al.*, 1997; Shchepinov *et al.*, 1999), covalently linked to AODNs (complementary to *c-erb*B1 mRNA which encodes for the EGFR) was examined in chapter 3. In comparison to the naked AODN, the AODN attached to the dendrimer showed no degradation products throughout the 24-hr incubation period in 10% v/v foetal bovine serum in DMEM. The results suggested that the dendrimer was protecting the AODN from nucleases in the serum. Similar results were seen when exposed to A431 cells in DMEM (serum free). This study highlighted the ability of the dendrimer to protect AODNs from digestive enzymes. A combination of structural hindrance and the protection of the 3'-end attached to the dendrimer (which is more prone to exonucleases) was the likely explanation for the enhanced stability.

Due to the unique nature of this dendrimer delivery system it was important to evaluate its cellular association properties before efficacy studies could be carried out. At 120 minute and 180 minute time points, cellular association of the dendrimer-AODN was approximately 3.5-fold higher than naked AODN for A431 cells. Cellular association at 4°C of dendrimer-AODN and AODN was reduced compared with 37°C and remained at relatively constant low levels for U87-MG cells (0.9% and 0.5% respectively) after a 1.5-hr incubation period. Similar results were obtained with the A431 cells. Cellular association studies indicated that endocytic/phagocytic mechanisms were taking place, which require cellular energy. This result was consistent with other studies which involved microsphere delivery systems (Khan 1999; Smith 2000). These conclusions were further confirmed by energy depletion experiments which showed a significant reduction in cellular association of dendrimer-AODN and AODN in the presence of metabolic inhibitors. Dendrimer-AODN uptake in U87-MG cells was found to be inhibited by competition with unlabelled dendrimer-AODN as well as other molecules which were chemically unrelated to the dendrimer-AODN such as salmon sperm DNA, ATP, and polyanions, such as dextran sulphate. These results suggested that there could be an overlap in specific and non-specific protein binding before the dendrimer-AODN enters the cell by endocytosis. Additionally, cellular association of dendrimer-AODN was competed with unlabelled phosphorothioate AODN which indicated an overlap of adsorptive (binding protein-mediated), receptor mediated or fluid phase endocytosis due to the fact that phosphorothioate AODNs are considered to enter cells by these mechanisms (Akhtar *et al.*, 1992). However, fluid-phase endocytosis was unlikely to account for a

significant fraction of dendrimer-AODN uptake since the cellular association of the fluid phase marker, mannitol, was significantly lower than cellular association of the dendrimer-AODN. The extent of protein binding was assessed by trypsin washing experiments which showed that approximately 83% of the dendrimer-AODN was bound to the cell via surface proteins. Differences in subcellular distribution studies were observed between fluorescently labelled dendrimer-AODN and naked AODN. Dendrimer-AODN produced a more diffused distribution throughout the cytosol and nucleus with some cells showing strong fluorescence within the nucleus as compared to the less intense and punctate distribution within the cytosol for naked AODN. These results suggested that the dendrimer-AODN was being entrapped in larger vesicles within the cytosol. Alternatively, the distribution may be due to the dendrimer-AODN and/or the cleaved AODN being released into the cytosol and the nucleus. Proposed mechanisms of dendrimer-mediated cell entry for PAMAM dendrimers involves the net cationic surface charge of the dendrimer-AODN to interact with the anionic glycoproteins and phospholipids that reside on the cell membrane surface which leads to the subsequent movement of dendrimer-AODN complex into the cytosol by endocytosis (for review see: Eichman *et al.*, 2000). Similarly to the proposed theory for the uptake of cationic dendrimers, the mechanism by which anionic dendrimers bind to cell surface membrane may involve the anionic nature of the complex to interact with cationic charged surface membrane components. Overall, these results showed that dendrimer-AODN was likely to enter cells by binding specifically or non-specifically to cell surface proteins and consequently enter cells by a combination of adsorptive (binding protein-mediated) and receptor mediated endocytosis.

In vitro and biological efficacy studies were carried out for assessing the ability of AODN attached to the dendrimer to cleave the *c-erb*B1 mRNA and ultimately to down-regulate EGFR expression with the subsequent reduction in viable cell number of A431 cells. *In vitro* RNase H assay highlighted the fact that the AODN was able to bind and cleave the target site while attached to the dendrimer. It was therefore improbable that the dendrimer would prevent the AODN from binding to the mRNA and rendering it inactive due to steric hindrance. This was contrary to a study involving PAMAM dendrimers complexed to DNA which appeared to be less efficient at mediating transfection due steric hindrance at the level of transcription (Kukowska-Latallo *et al.*, 1996). However, it must be appreciated that cleavage was undertaken in an environment where no metabolic activities and nuclear proteins are present which may bind to the dendrimer complex and render it inactive. The

effect of dendrimer-AODN on viable cell number and EGFR expression was assessed in cell culture. Increasing the concentration of the dendrimer-AODN produced a dose-response effect on A431 viable cell number. For example, the average reduction of A431 cell number was 7%±2.5 when treated with 0.02μM of the dendrimer-AODN as compared to the untreated cells. At a concentration of 0.5μM of the dendrimer-AODN the average reduction in percentage cell number was reduced to 71%±2.7 as compared to the untreated cells. EGFR expression was also down regulated with similar concentration in a dose-dependent manner. Overall, these results showed that the dendrimer may be able to deliver the AODN at the target site where the AODN was likely to down-regulate EGFR expression via an antisense mechanism and ultimately have an effect on A431 viable cell number. However, these results need to be confirmed by controls such as scrambled AODN attached to the dendrimer and also the dendrimer without any AODNs attached. Due to problems in obtaining these controls it was not possible to carry this out.

To determine if the dendrimer-AODN was able to retain its activity in serum and subsequently be effective in *in vivo,* cellular association studies were undertaken in DMEM containing foetal bovine serum. U87-MG cells were used as a model cell line. These studies showed that cellular association was reduced with increasing serum concentrations. These results were consistent with other studies which have shown that most carrier or delivery systems do not function well in the presence of serum (Juliano *et al.*, 1999; Astriab-Fisher *et al.*, 2000). Subcellular distribution studies of fluorescently labelled dendrimer-AODN showed reduced dendrimer-AODN-FITC associated with U87-MG cells with increasing serum concentrations. Globules of fluorescence were noticed around the cells as well as on the surface of the cell membranes. It may be that the anionic charge of the dendrimer complex allows it to bind to proteins in serum by electrostatic forces. This could have two possible consequences; first, neutralistaion of the anionic charge of the dendrimer which may prevent the complex from binding to the cell surface and consequently entering the cell and second, an increase in the size of the complex, resulting in the dendrimer complex being too large for it to be taken up by the cells (Zimmer 1999). Overall, these results suggested that dendrimer-AODN in serum medium was unable to enter the cells and may not be appropriate in an *in vivo* scenario. However, this does not discount the possibility of using the anionic dendrimer as a tool for functional genomics and target validation purposes. Although the efficient delivery of AODNs into cell *in vitro* is generally straightforward using cationic lipid

transfection approaches, some cell types can be problematic and might require alternative delivery approaches. For example, dendrimers have shown to be better suited for the delivery of AODNs into certain cell types (Koller *et al.*, 2000).

It is becoming apparent that for AODNs to be successful therapeutic agents they will ultimately need to be administered in combination with existing drug therapies. This is reflected by the large number of publications in recent years which have shown very encouraging results for combination therapies in comparison to AODNs alone (see section 2.1.4). Additionally, there is no doubt that antisense technology has reserved its place as the tool for target validation and gene function determination in the post-genomic era (for reviews see: Taylor *et al.*, 1999; Koller *et al.*, 2000; Dean 2001).

SUGGESTIONS FOR FURTHER WORK

It would be interesting to screen a number of cytotoxic agents in combination with modified back-bone AODNs, with or without a delivery system, on a variety of cell models. Studies have shown varied effects depending on drug combinations and cell lines used (Benner *et al.*, 1997). AODNs combined with more than one drug would also be an appealing study to carry out (Tortora *et al.*, 2001). Modification of AODN back-bone is also an important factor which needs to be considered to reduce the binding of AODN with intracellular protein and consequently leading to non-antisense cytotoxic effects (Smetsers *et al.*, 1997). It is envisaged that the microsphere delivery system would show its full potential in *in vivo* (see discussion) and therefore essential that the microsphere studies are conducted in animals.

Although promising results have been obtained with the dendrimer delivery system it needs to be appreciated that this was a novel delivery strategy and therefore further characterisation studies need to be carried out e.g. size, stability in variable conditions and stoichiometry. As regards to the mechanistic studies, more experiments will need to be undertaken to characterise specific receptor proteins (molecular weights) involved for cellular association and uptake. This could be carried out using techniques such as South-Western blotting. Efficacy studies need to be confirmed by the addition of rigorous controls e.g. dendrimers linked to scrambled AODNs. Also, antisense effects need to be confirmed by analysing RNA levels by Northern blotting. It is recommended that mechanistic cellular association and activity studies are undertaken on a variety of cell models. Also, it would be interesting to evaluate delivery and activity of cleavable ODN linkage to the dendrimer (e.g. disulphide bonds).

Once the delivery systems are fully characterised and tested *in vitro*, particularly for the dendrimers, then *in vivo* studies could be carried out in terms of toxicity, distribution, and efficacy.

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APPENDIX I

Publications

- **Hussain,M.**, Beale,G., Hughes,M. and Akhtar,S. (2002) Co-delivery of an antisense oligonucleotide and 5-fluorouracil using sustained release poly(lactide-co-glycolide) microsphere formulations for potential combination therapy in cancer. *International Journal of Pharmaceutics.* **234**, 129-138.

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