THE SUSTAINED DELIVERY OF ANTISENSE OLIGODEOXYNUCLEOTIDES USING BIODEGRADABLE POLYMER MICROSPHERES

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DECEMBER 1999

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SUMMARY

Antisense technology is a novel drug discovery method, which provides an essential tool for directly using gene sequence information to rationally design specific inhibitions of mRNA, to treat a wide range of diseases. The efficacy of naked oligodeoxynucleotides (ODNs) is relatively short lived due to rapid degradation *in vivo*. The entrapment of ODNs within biodegradable sustained-release delivery systems may improve ODN stability and reduce dose required for efficacy.

Biodegradable polymer microspheres were evaluated as delivery devices for ODNs and ribozymes. Poly(lactide-co-glycolide) polymers were used due to their biocompatability and non toxic degradation products. Microspheres were prepared using a double emulsion-deposition method and the formulations characterised. *In vitro* release profiles were characterised by an initial burst effect during the first 48 hours of release followed by a more sustained release. The release profiles were influenced by microsphere size, copolymer molecular weight, copolymer ratio, ODN loading, ODN length, and ODN chemistry.

The serum stability of ODNs was significantly improved when entrapped within polymer microspheres. The cellular association of ODNs entrapped within small spheres (1-2μm) was improved by approximately 20-fold in A431 carcinoma cells compared with free ODNs. Fluorescence microscopy studies showed a more diffuse subcellular distribution when delivered as a microsphere formulation compared with free ODNs, which exhibited the characteristic punctate periplasmic distribution.

For *in vivo* evaluation, polymer microspheres containing fluorescently-labelled ODNs were stereo-taxically administered to the neostriatum of the rat brain. Free ODN resulted in a punctate cellular distribution after 24 hours. In comparison ODN delivered using polymer microspheres were intensely visible in cells 48 hours post administration, and fluorescence appeared to be diffuse covering both cytosolic and nuclear regions. Whole-body autoradiography was also used to evaluate the biodistribution of free tritium labelled ODN and ODN entrapped microspheres, following subcutaneous administration to Balb-C mice. Polymer entrapped ODN gave a similar biodistribution to free ODN. Free ODN was distributed within 24 hours, whereas polymer released ODN was observed still present in organs and at the site of administration seven days post administration.

Keywords: - nucleic acids, controlled release, poly(lactide-co-glycolide), drug delivery, stability.

IN THE NAME OF ALLAH, THE MOST BENEFICIENT, THE MOST MERCIFUL

TO MY FATHER AND MOTHER

FOR ALL YOUR LOVE AND UNDERSTANDING, AND FOR ALL YOUR SUPPORT AND ENCOURAGEMENT THROUGHOUT MY LIFE

ACKNOWLEDGMENTS

The work in this thesis was jointly funded by a case award from the Engineering & Physical Sciences Research Council (EPSRC) and Roche Products Ltd. Consumables were partly funded by Aston university, The Medical Research Council (MRC) and The Cancer Research Campaign (CRC).

I gratefully acknowledge my supervisor at Aston Dr Saghir Akhtar, for his guidance, encouragement and constant support during my studies.

I would also like to thank my external supervisor at Roche Products Ltd Dr. Gordon Beck, for providing the opportunity to carry out the whole body autoradiography studies at Roche Products Ltd, and financial support. Special thanks go to Steve Harris at Roche Products Ltd, for all his technical help and advice.

I am grateful to Dr Wolfgang Sommer, and Kjell Fuxe at the Karolinska Institute, Sweden, for arranging the CNS delivery studies.

I thank Chris Bache for his technical assistance in bringing laboratory equipment back to life.

Special thanks go to all my friends, in particular Pakeeza for her support in the final stages of this thesis, Shaqil and Taeeba, for your patience, and genuine friendship over the last ten years.

Finally and very importantly, special thanks go to all my family, especially my parents, and sister for your continued support throughout my education, and for always being there for me, also my nephews and nieces for making me smile.

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LIST OF ABBREVIATIONS

2'-O-Me	2'-O-methylated
5 Fu	5-fluorouracil
A,G,C,T,U	adenine, guanine, cytidine, thymidine, uridine
AE	adsorptive endocytosis
AIDS	acquired immune deficiency syndrome
ATP	adenosine tri-phosphate
BBB	blood brain barrier
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celcius
cm, mm, μm, nm	centimetre, millimetre, micrometre, nanometre
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
CPG	controlled pore glass
cpm	counts per minute
DABCO	diazobicyclo [2,2,2] octane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSC	differential scanning calorimetry
DTT	dithiothreitol
ECACC	European collection of animal cell cultures
EDTA	ethylenediamine tetra-acetic acid
EGFr	epidermal growth factor receptor

FBS	foetal bovine serum
FCS	foetal calf serum
FDA	Fool and Drug Agency
FITC	flourescein isothiocyanate
FPE	fluid phase endocytosis
g, mg, μg, ng	grams, milligrams, micrograms, nanograms
HIV	human immunodeficiency virus
hpv	human papilloma virus
IC	intra-cereberal
ICV	intra-cerebroventricular
IFN	interferon
i.m.	intra-muscular
i.p.	intra-peritoneal
i.v.	intravenous
KCl	potassium chloride
kDa	kilo Daltons
L,mL,μL	litre, millitre, microlitre
LA	lactic acid
M	molar
M, mM, μM, nM	molar, millimolar, micromolar, nanomolar
mag	objective lens magnification
μCi	micro Curies
mmol,μmol, nmol, pmol	millimoles, micromoles, nanomoles, picomoles
Mg	magnesium
$MgCl_2$	Magnesium chloride
MP	methylphosphonate
mRNA	messenger-ribonucleic acid
Mw	molecular weight
NaCl	sodium chloride
OD	optical density
ODN	oligodeoxynucleotide

o/w	oil-in-water
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PGA	poly(glycolic) acid
PLA	poly(lactic acid)
PLGA	polylactide-co-glycolide
PLLA	poly-L-lactide
PNA	peptide-nucleic acid
PO	phosphodiester
PS	phosphorothioate
PS_2	phosphorodithioate
PVA	poly (vinylalcohol)
®	registered trademark
RES	reticulo-endothelial system
RHS	right hand side
RITC	rhodamine isothiocyanate
RME	receptor mediated endocytosis
RNA	ribonucleic acid
RNase H	ribonuclease H
rpm	revolutions per minute
s.c.	subcutaneous
sd / S.D.	standard deviation
SEM	scanning electron microscope
t 1/2	half life
TBE	tris-borate EDTA buffer
TCA	trichloroacetic acid
TEMED	N,N,N,'N'-trimethylethylenediamine
TETD	Triethylthiuram disulphide
TFO	triplex-forming oligonucleotide
Tg	glass transition temperature
TGA	thermo-gravimetric analysis

Tm	crystalline melting temperature
UV	ultraviolet light
v/v	volume per volume
WBA	whole-body autoradiography
w/o/w	water-in-oil-in-water
w/v	weight per volume
w/w	weight per weight

CHAPTER ONE

INTRODUCTION

1.1 ANTISENSE OLIGODEOXYNUCLEOTIDES

Antisense oligonucleotides (ODN) are short lengths of single stranded deoxyribonucleic acid (DNA) which offer a unique approach to regulating over expressed or undesired genes, and inhibiting viral replication. Antisense ODNs can be designed to complement a region of a particular gene or messenger ribonucleic acid (mRNA), and serve as potential blockers of transcription or translation through sequence specific hybridisation. This hybridisation interferes with the function of the RNA by blocking RNA transport, splicing or translation. In some cases, the RNA-ODN hybrid forms a substrate for RNase H, a cellular enzyme that selectively hydrolyses the RNA portion of the RNA-DNA hybrid and inactivates the RNA, hence interfering with the normal transfer of genetic information in the cell (from DNA to mRNA to protein).

The antisense ODN approach has two advantages over conventional drugs, firstly the mRNA of the disease target gene has a defined sequence, and secondly the antisense ODN interacts with the target gene by Watson-Crick base pairing, providing specificity and affinity. The use of antisense ODNs to regulate specific gene expression allows one to apply the principles determined from the work conducted on one gene target to an indefinite number of genes, thereby providing therapies for many diseases.

Antisense technology has evolved into an intense field of research, and offers considerable promise for novel molecular therapies against various diseases including acquired immune deficiency syndrome (AIDS), cancer and inflammatory disorders. Furthermore, in addition to serving as therapeutic agents, antisense ODNs have been used as experimental reagents in renal and cardiovascular medicine to unravel pathophysiological mechanisms. Viruses are attractive targets for antisense technology

because they harbour genetic information distinct from that of the host cell. Some of the first antisense compounds to enter controlled clinical trials have been developed to inhibit viral pathogens such as human immunodeficiency virus (HIV), cytomegalovirus (CMV) and human papillomavirus (HPV). Several antisense ODNs are undergoing clinical trials evaluation and the first antisense drug has recently been approved for marketing, Vitravene™ (Fomivirsen) is delivered locally to the eye to treat CMV-induced retinitis in AIDS patients. At present other ODN compounds are being screened for anti-cancer, anti-inflammatory and anti-HIV applications (for review see Akhtar and Agrawal, 1997).

The potential of antisense ODNs to inhibit gene expression has been extensively reviewed (Crooke, 1992; Carter and Lemoine, 1993; Stein and Cheng, 1993; Pierga and Magdelenat, 1994; Wagner, 1994; Akhtar 1995; Agrawal, 1996b; Oberbauer, 1997; Wagner, *et al.*, 1997; Bennett, 1998; Juliano *et al.*, 1999). In order to ensure antisense ODNs can selectively inhibit gene expression *in vitro* and *in vivo*, it is important they remain stable at the target site for a sufficient length of time. This requires repeated administration of an antisense compound, given exogenously, at the appropriate intervals to ensure continuous suppression of the target. The use of biodegradable sustained-release delivery systems may obviate the need for repeated administration by facilitating site-specific delivery of the nucleic acid in a controlled manner to the desired site. The entrapment of ODNs within such polymeric matrix systems may also improve ODN stability, reduce the dose required for efficacy and further reduce toxicity or non-specific activities associated with ODNs.

The general structure of an ODN consists of a DNA sugar phosphate backbone as shown in figure 1.1. Unmodified phosphodiester (PO) ODNs are unstable in biological fluids due to cellular and extracellular nucleases (Wickstrom, 1986; Akhtar *et al.*, 1991b), and hence structural modifications have been developed. Chemical modification of each of the PO linkages in an ODN by a variety of methods confers resistance to degradation in a cellular environment (for reviews see Milligan *et al.*, 1993; Wickstrom 1992, 1995; Rojanasakul, 1996). Alternatively, modifications of the ribose moiety can also result in increased resistance to nucleases (see section 1.3).

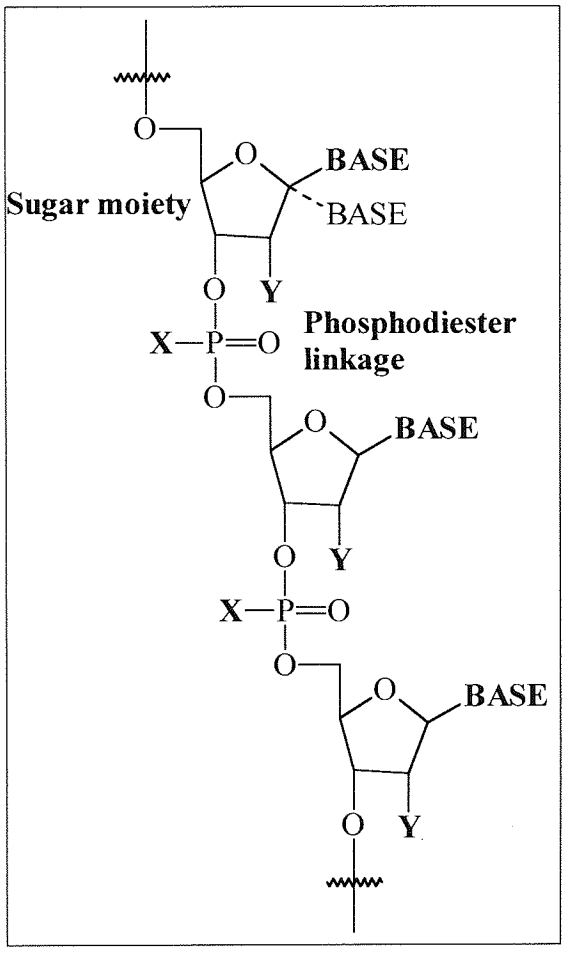


FIGURE 1.1 General Structure of ODNs, with Positions of Structural Modifications.

1.2 MODE OF ACTION OF OLIGONUCLEOTIDES

Antisense ODNs are designed to modulate information transfer from the gene to protein (see figure 1.2) by altering the intermediary metabolism of RNA, and as such, each site along this pathway is a potential site of action for ODNs. Antisense technology aims to inhibit gene expression in a highly sequence-specific and selective manner encompassing three distinct strategies: anti-gene, antisense ODNs and ribozymes(RBZ). The anti-gene strategy relies on short, synthetic ODNs to form triple helical structures with duplex DNA to interfere with gene expression at the level of transcription, whereas antisense ODNs and ribozymes are designed to interfere with gene expression at the level of single stranded RNA (pre-mRNA or mRNA).

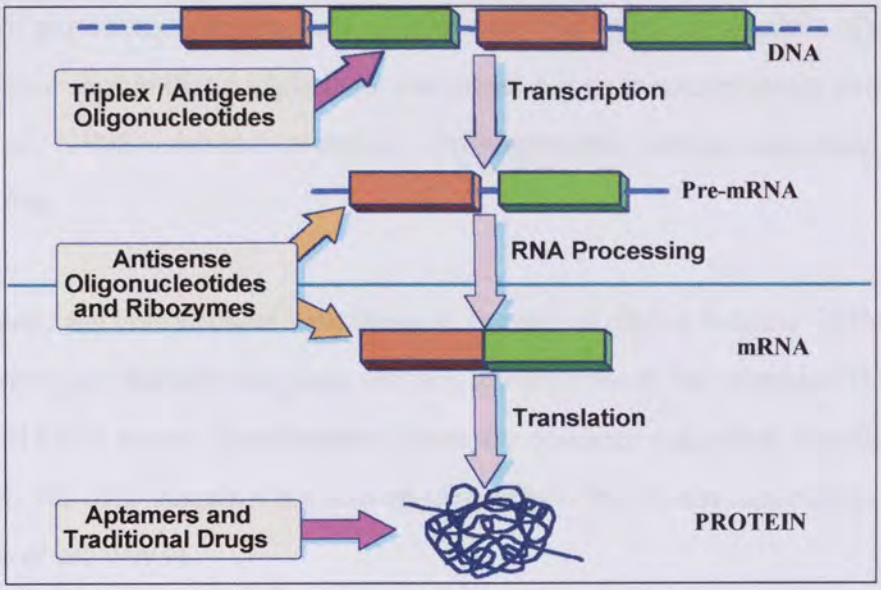


FIGURE. 1.2 Schematic Diagram showing Possible Sites of Action of Oligodeoxynucleotides

1.2.1 The Anti-gene Strategy

Triplex forming ODNs demonstrate sequence-specific recognition of duplex DNA based on the simple principle of Hoogsten pairing rules between a purine strand of the DNA duplex and the ODN bases (Moser and Dervan, 1987). The basic premise of action for triplex DNA is that binding of the ODN to a particular gene should prevent its normal function and expression. The ODN binds to the purine-rich strand of the DNA via Hoogsten hydrogen bonding between the third strand nucleotide and its complement in the purine rich strand. The third strand may contain either polypurines or polypyrimidines. Such hybridisation occurs at the major groove of the duplex where there is sufficient space for the third strand (reviewed by Sun and Helene, 1993).

Triplex holds several advantages over other strategies used to inhibit gene expression. ODNs are capable of targeting the desired gene directly rather than inhibiting at the level of mRNA, i.e. they act at the transcriptional level rather than the translational level (Scanlon *et al.*, 1995). Due to its specificity and DNA-directed mechanism of action, in theory, fewer molecules of ODN are needed compared with the excess antisense or ribozyme molecules needed to bind and cleave at the mRNA level. The stability of DNA

triplexes is of paramount importance to realise their biological application. Cooney *et al.*, (1988) have shown that a triple helix can arrest *c-myc* transcription *in vitro*. Agrawal *et al.*, (1996) inhibited growth of TNF-dependent tumour cells using triplex forming ODNs.

However, there are considerable limitations to the use of triplex forming ODNs since potential targets are limited to regions that are homopurine in one strand of the chromosomal DNA target. Furthermore, there are concerns regarding the affinity of triplex ODNs for their targets since non-specific effects have been reported *in vitro* (Ebbinghaus *et al.*, 1993).

1.2.2 The Antisense Strategy

The inhibition of gene expression exhibited by certain ODNs has been termed an 'antisense' effect because these molecules owe their specificity of action to the fact that they are complementary (antisense) to specific nucleotide sequences within regions of 'target' mRNAs (Akhtar and Juliano, 1991). The targeting of nuclear pre-mRNA or cytoplasmic mRNA with antisense ODNs is referred to as the 'antisense strategy'. Transcription and translation are the two processes that antisense ODNs interfere with to inhibit the synthesis of proteins. The precise mechanism of 'antisense action' remains a debate, although it is known to be dependent upon the type of ODN analogue used (see section 1.3 and figure 1.1). There are several proposed theories concerning the mechanism of action of antisense ODNs. The most popular theory involves either direct binding of the ODN to pre-mRNA to inhibit further RNA processing, or the binding of ODNs to its mRNA target through standard Watson-Crick base-pairing, thereby physically inhibiting the translation by ribosomes (Crooke 1992; Leonetti *et al.*, 1993; Neckers *et al.*, 1992; Agrawal, 1999). Other theories implicate the 5'-capping site attachment to mRNA that inhibits the binding of translational factors (Leonetti *et al.*, 1993)

The binding of the antisense ODNs to the targeted RNA may lead to its degradation via ribonuclease H (RNaseH), a ubiquitous enzyme involved in the degradation of the RNA

strand of an RNA/DNA duplex (Tidd, 1996; Akhtar and Agrawal, 1997). The RNA may be further degraded by cellular exonucleases. The precise recognition elements for RNaseH are not known. However, it has been shown that ODNs with DNA-like properties as short as tetramers can activate RNaseH (Donos-Killer, 1979). Methylphosphonate (MP) ODNs do not activate RNaseH (Kole *et al.*, 1991), nevertheless they are still able to down-regulate protein translation (Wickstrom *et al.*, 1992). Hence physical blocking of mRNA translation at the ribosomes offers a likely explanation for the mechanism by which such ODNs may exert their effects (Akhtar, 1991; Rossi, 1995). In contrast, phosphorothioate (PS) ODNs are excellent substrates (Stein and Cheng, 1993; Mirabelli *et al.*, 1991) for RNaseH.

1.2.3 Ribozymes

Ribozymes (from **ribo**nucleic acid and en**zyme**) are small RNA molecules capable of catalytic activity. Similar to antisense oligonucleotides, ribozymes also rely on complementary base pairing for their action. They can be described as RNA-based antisense oligonucleotides with an incorporated catalytic core (Akhtar, 1998). Ribozymes combine enzymatic processes with the specificity of antisense base pairing (Putnam, 1996), and as such offer two theoretical advantages over antisense oligonucleotides. Ribozymes are capable of cleaving and hence permanently inactivating their target substrate, and they are true catalysts in that they have the ability of multiple turnovers (Curcio *et al.*, 1997).

Many different classes of ribozyme have now been identified. These include Group I and II introns, RNase P, hairpin ribozymes, and hammerhead ribozymes. Of all the various motifs, the hammerhead is the best characterised, possibly due to its small size and simple cleavage mechanism (Scott and Klug, 1996). Occurring in small pathogenic RNAs, the hammerhead motif is a self-cleaving structure that is thought to process multimeric transcripts into monomers during rolling-circle replication of viroid and virusoid genomes (Doudna, 1998).

In the two dimensional representation of the *trans* acting form of the hammerhead (Haseloff and Gerlach, 1988), there are three Watson-Crick helices and a conserved core. In order to facilitate comparison of data from different laboratories, a uniform numbering system for nucleotides was introduced by Hertel *et al.*, (1992), as shown in figure 1.3.

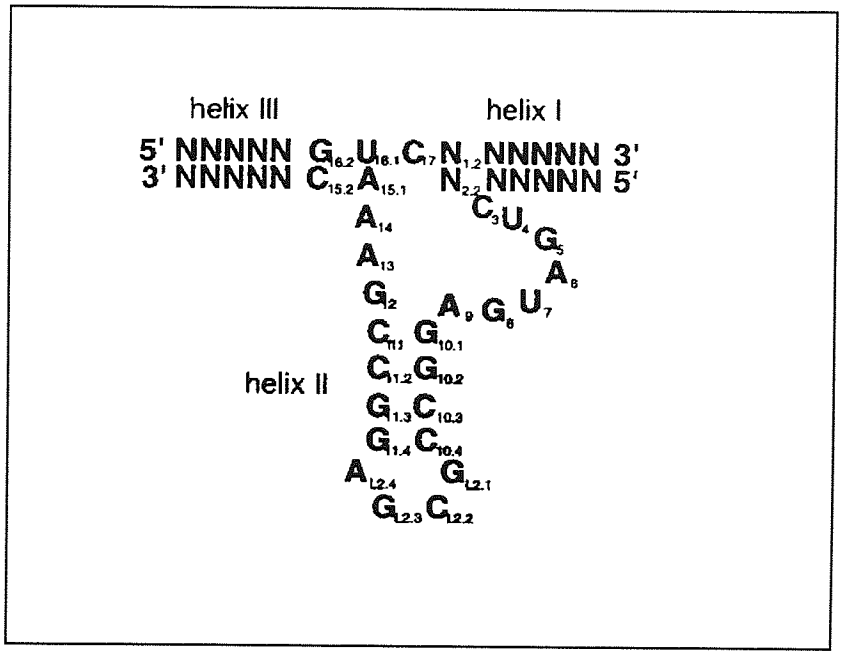


FIGURE 1.3 Standard Numbering System introduced by Hertel *et al.*, (1992) for the Haselhoff and Gerlach (1988) Classic Hammerhead Ribozyme

The 3D structure of the hammerhead ribozyme can be visualised as a wishbone, or Y shape, with stems I and II at the arms and stem III at the base (Sigurdsson and Eckstein, 1995; Scott and Klug, 1996). Nucleotides G5, A6, G8, G12, and A15.1 are deemed conserved nucleotides of the catalytic core and are essential for activity of the hammerhead (Scott and Klug, 1996). Helix II (which comprises a stem and a loop) is the only helix in the hammerhead that is not directly involved in substrate binding (Sigurdsson and Eckstein, 1995), and has thus been targeted in attempts to reduce the size of the hammerhead. Helices I and III flank the catalytic core and they are the substrate-recognition domains. The length and composition of these flanking arms will have an effect on the specificity and turnover of a ribozyme (Hormes *et al.*, 1997).

A major drawback in using RNA is that it is very rapidly degraded in biological fluids. For example, the half-life of an all-RNA hammerhead ribozyme in human serum is less than 0.1 minute (Jarvis *et al.*, 1996). Thus, various chemical modifications are applied in an effort to increase ribozyme stability while retaining catalytic activity. A common approach is to replace RNA in the hybridising arms with DNA (Sawata *et al.*, 1993; Hendry and McCall, 1995). Other chemical modifications include the use of 2'-O-methyl ribonucleotides (Hudson *et al.*, 1999), and the introduction of phosphorothioate DNA bases at the 3'-termini (Fell *et al.*, 1997).

Critical to the success of nucleic acids as pharmaceuticals will be the development of appropriate delivery systems (Hope *et al.*, 1998). There are two principal methods for the delivery of ribozymes into cells, endogenous expression of the ribozyme from an appropriate vector construct, or the exogenous delivery of pre-synthesised ribozymes (reviewed by Juliano and Akhtar, 1992).

Since their discovery ribozymes have come to encompass a wide range of applications, including cancer and viral disease. In search for a novel treatment for glioblastoma multiforme, Ke *et al.*, (1998) constructed two hammerhead ribozymes to target the 5' common region of vascular endothelial growth factor (VEGF) mRNA. Studies of glioblastoma angiogenesis have shown that the expression of VEGF is up regulated in these tumours. Both ribozymes displayed 65-90% efficiency *in vitro*. Expression of tumour necrosis factor α, which is important in certain inflammatory processes, was downregulated with ribozymes (MacKay *et al.*, 1999). With an aim of developing gene therapy for HIV-1 infection, Michienzi *et al.*, (1998) tested the antiviral activity of a ribozyme, which displayed specific and efficient inhibition of viral replication in human T-cell clones. The ribozyme reduced both protein levels and intracellular HIV transcripts.

The success of ribozymes is not confined to the laboratory, and some ribozymes have entered clinical trials. Ribozyme Pharmaceuticals Inc. (RPI), an acknowledged leader in ribozyme therapeutics, currently have some projects underway. Angiozyme™, a ribozyme targeting the VEGF is shortly to enter multi-dose Phase I/II clinical trials.

1.3 STRUCTURAL MODIFICATIONS

The vulnerability of unmodified ODNs to nucleases present in various extracellular fluids and in different intracellular compartments limits their therapeutic potential. Nucleases that degrade DNA or RNA from either the 5' or 3' terminus are known as exonucleases, and those that cleave internally are endonucleases. Numerous nucleases exist and have been shown to degrade ODNs (Wickstrom *et al.*, 1986; Akhtar *et al.*, 1992; Uhlmann *et al.*, 1990; Neckers *et al.*, 1992). Many chemical modifications of the backbone of PO ODNs have been used to circumvent this degradation (Agrawal *et al.*, 1995; Matteucci, 1996), and rapid advances in nucleic acid synthetic chemistry have made it feasible to design ODNs that are highly nuclease-resistant yet retain favourable hybridisation characteristics. Modifications in the base, sugar, and phosphate moieties of ODNs to create ODNs with enhanced affinity, more selectivity for RNA or DNA, enhanced nuclease stability, cellular uptake, and distribution as well as improved pharmacokinetics, have been reported (Agrawal *et al.*, 1995; Cantin *et al.*, 1993).

1.3.1 Modifications to the Phosphodiester Linkage

Phosphorothioates

Phosphorothioates (PS), in which one of the non-bridging oxygens of the internucleotide phosphodiester linkage is replaced by sulphur, have been studied extensively as antisense agents. These analogues have increased resistance to degradation both *in vitro* and *in vivo* (Sands *et al.*, 1994; Iverson *et al.*, 1994; Temsamani *et al.*, 1993), but have decreased affinity for their target nucleic acid. They exhibit both sequence-specific and non-sequence specific activity (Stein *et al.*, 1993), are very stable in serum as well as inside the cell, and are capable of eliciting RNaseH activity. However, they bind in a non-specific manner to both surface and intracellular proteins, resulting in biological effects that are not related to the antisense mechanisms. In general, PS ODNs are synthesised and evaluated as diasteriomeric mixtures, which have potentially different biochemical and biophysical properties from their pure stereoregular counterparts (Tang *et al.*, 1995; Koziokiwickz *et al.*, 1995).

Phosphorodithioates

Substitution of both non-bridging oxygen atoms with sulphur atoms results in the formation of phsphorodithioate (PS_2) ODNs (Gosh *et al.*, 1993). The phosphorodithioate linkage was introduced to eliminate the chiral centre, thereby removing the diastereoisomer problem (Marshall and Caruthers, 1993). PS_2-ODNs are more hydrophobic than PO and PS ODNs, and are more resistant to nuclease degradation. These ODNs are able to form duplexes with unmodified nucleic acids, and are also able to activate RNaseH (Marshall and Caruthers, 1993). PS_2-ODNs have been found to inhibit HIV-1 reverse transcriptase and viral replication (Gosh *et al.*, 1993), although protein binding of these ODNs is reduced compared to PS ODNs. This linkage does not demonstrate any advantage over the aforesaid simple monothioate. Its affinity for the target and ability to activate RNaseH is similar to the diastereoisomeric mixture of monothioates.

Methylphosphonates

Substitution of the non-bridging oxygen of the phosphodiester bond with a methyl group results in the formation of methylphosphonate (MP) ODNs. Unlike PO and PS ODNs, MP ODNs are non-ionic and show poor water solubility, limiting their therapeutic use (Akhtar *et al.*, 1991). Although MPs are stable to nucleases (Agrawal *et al.*, 1979), they are unable to activate the enzyme RNaseH (Neckers *et al.*, 1992) and therefore rely on ribosomal inhibition. MP ODNs have been shown to successfully inhibit gene expression (Maher and Dolnick, 1988). MP ODNs are able to enter cells by passive diffusion (Shoji *et al.*, 1991; Crooke, 1992), do not bind proteins, demonstrate minimal toxicity and posses high stability (Stein *et al.*, 1993). The MP analogue has the advantage of straightforward synthetic methods (Miller, 1991). A deficiency of this linkage is its chiral nature. Different diastereomers have been shown to have significantly different binding affinities (Reynolds *et al.*, 1996).

Phosphoramadites

Substitution of the non-bridging oxygen of the phosphodiester bond with an amine group results in the formation of phosphoramadite ODNs. These ODNs display increased stability (Schultz and Gryaznov 1996) but are unable to activate RNaseH, resulting in reduced antisense activity (Maher and Dolnick, 1988). Such ODNs have

increased lipophilicity and hence enhanced cellular uptake. Preliminary cell culture data suggest that this analogue results in enhanced antisense potency (Gryaznov *et al.*, 1996). Phosphoramidites have been shown to increase antiviral activity compared to PO ODNs (Agrawal *et al.*, 1988). Additionally, this analogue is finding use in the triple helix arena because it greatly enhances third-strand binding to duplex DNA in the parallel pyrimidine-purine-pyrimidine motif (Escude *et al.*, 1996).

Boranephosphite ODNs

Substitution of the non-bridging oxygen of the phosphodiester bond with a BH_3 group results in the formation of boranephosphite ODNs. This analogue is a suprisingly stable, chemically unique isostere of a PO. Zhang and Matteucci (1996), found a 15-mer ODN bearing diastereomerically mixed boranephosphites at all positions, showed poor binding to complementary RNA and DNA. This analogue, despite its elegant simplicity, is not likely to be therapeutically useful.

Chimeric ODNs

Various attempts have been made to combine two modified ODNs in order to generate a 'chimeric' ODN that confers the antisense properties of both molecules. Generally such ODNs have two segments, one that contains an ODN analogue capable of activating RNaseH, and another that does not activate RNaseH. Various studies using chimers of PS/PO ODNs have been conducted to reduce non-specific effects (Stein *et al.*, 1988; Zhao *et al.*, 1993; Ehrlich *et al.*, 1994). These authors found increased stability when in a duplex compared to PS ODNs, however, reduced nuclease stability, and antisense activity was observed. Zhao *et al.*, (1993) found cellular association of the chimeric PO/PS ODN to be lower than the full PS ODN, but higher than the PO ODN counterparts. Chimeric ODNs containing alternating MP and PO internucleoside linkages have increased stability compared to PO ODNs, and also offer the advantage of the activation of RNaseH (Spiller and Tidd, 1992).

1.3.2 Modifications to the Sugar-Phosphate Backbone

Peptide nucleic acids (PNA)

PNAs are nucleic acid analogues in which the sugar phosphate backbone is replaced completely with a chiral polyamide backbone (Nielson *et al.*, 1994). These ODNs hybridise specifically to natural DNA or RNA via base pairing, and are able to bind to a target nucleic acid sequence in either parallel or antiparallel orientations (Hyrup *et al.*, 1994). Stability of PNA/DNA duplexes is significantly greater than DNA/DNA duplexes, and they are stable in biological fluids.

Hairpin and circular ODNs

In order to prevent degradation of ODNs from the 3'-end, two structural modifications have been studied, ODNs containing a 'hairpin loop' at the 3'-end, and 'circular' ODNs. ODNs containing a hairpin loop region at the 3'-end show significantly decreased rates of degradation, and were found to be equally effective antisense agents as their linear counterparts (Khan *et al.*, 1993; Tang *et al.*, 1993). Both PO and PS ODNs containing a hairpin loop region at the 3'-end do not seem to interfere with cellular uptake and affinity for their targets (Tang *et al.*, 1993). Circular ODNs are oligomers in which the 5'-end and the 3'-end have been linked using template-derived enzymatic or non-enzymatic ligation. These circular ODNs have a slower rate of degradation and higher binding efficiency than their linear counterparts (Dolinnaya *et al.*, 1993; Wang *et al.*, 1994; Rubin *et al.*, 1994).

The 2' position of the ribose sugar

The ribose portion of ODNs has been extensively modified, and has been reviewed with a number of modifications showing provocative results (De Measmaeker *et al.*, 1995). The ribose ring has been appended and substituted, conformationally restricted, and even replaced by hexose. The most simple and perhaps most promising substitutions involve modification of the 2' position of the ribose sugar. The 2'-O-methyl group confers stability to single-stranded RNases but PO ODNs are still susceptible to degradation by DNases. Highly electronegative substituents, such as fluorine, virtually lock the ribose ring in the RNA conformation leading to enhanced binding to RNA targets (Kawasaki *et al.*, 1993). The effect of steric bulk at the 2' position has been

investigated with the addition of simple alkyl ethers. The trend seen is that nuclease stability increases and binding affinity decreases with increasing size (Monia *et al.*, 1996). The introduction of the 2'-methoxyethoxy group has resulted in both nuclease stability with POs and excellent binding affinity. This modification resulted in enhanced antitumour activity when incorporated into a *c-raf* kinase ODN (Alleman *et al.*, 1996).

A further modification involves a hexose scaffold. The 1,5-anhydrohexitol PS ODNs hybridise well to RNA (Van Aerschot *et al.*, 1995). This altered sugar residue confers a significantly enhanced affinity to RNA. This modification stabilises a PO linkage towards nucleases. However, this modified hexose is unlikely to improve the cellular permeation of diester ODNs.

The desired properties of an antisense agent cannot be achieved by a single modification. Improvement in one property may often result in a compromise of another. However, the properties of an ODN may be optimised by combining several structural modifications, and as such chimeric ODNs offer many advantages over their single counterparts.

1.4 DESIGN OF OLIGODEOXYNUCLEOTIDES

The success of antisense technology is largely dependent upon the design of the ODNs (Wagner RW, 1994; Stein *et al.*, 1993). The initial step in obtaining antisense-mediated efficacy with ODNs requires the rational design of effective ODNs with sequences complementary to the mRNA of the targeted gene. Once the target mRNA has been decided, the target sequence has to be carefully selected in the nucleotide sequence of the mRNA. This may be achieved by accessible sites for ODN hybridisation on the target mRNA being selected from computer predicted folding of RNA. Antisense ODNs targeting the AUG translation initiation codon have been widely used. Such ODNs are speculated to function through the inhibition of translation, however, the AUG translation initiation codon does not always serve as a good target. Furthermore, poor target selection will also favour the non-specific and non-antisense mediated effects observed with modified-ODN analogues.

Recent studies suggest that simply designing one or two antisense sequences to complementary sites on the target RNA sequence relies on a great deal of luck to predict an accessible site and hence, an efficacious antisense construct (Monia *et al.*, 1996). This is due to the fact that the RNA molecules exhibit a high degree of secondary and tertiary structure, which cannot be readily predicted from currently available computer alogorithms used for assessing RNA folding. In addition to this the presence of RNA-binding proteins inside the cell may also further prevent hybridisation to otherwise single-stranded sites within the target RNA.

Alternative methods for finding the optimal antisense sequence are being investigated. Conventionally, an empirical 'walk-the-gene' strategy has been employed whereby a series of ODNs (up to 50 or more) targeted to multiple sites in the target mRNA are evaluated in cell culture or *in vivo*. Although successful at times, this strategy is extremely costly and time consuming. Recently, two promising, cost efficient and highly elegant strategies based on combinatorial chemistry have been reported:

a) RNaseH mapping of target mRNA with random ODN libraries, and
b) hybridisation of target mRNA to scanning antisense ODN arrays.

The RNaseH mapping technique relies on the ability of the enzyme RNaseH to recognise and selectively cleave the mRNA at sites within the hybridised ODN:mRNA duplex. Sites accessible for hybridisation, as determined by mRNA cleavage fragments produced by the enzyme, are selected by combining the targeted mRNA transcript with a random, or semi-random, library of chemically synthesised ODNs. This strategy has been effectively used to select potent antisense ODNs targeted to the human multiple drug resistance gene (Ho *et al.*, 1996) and to the RNA of hepatitis C virus (Lima *et al.*, 1997). A disadvantage of this strategy is that RNaseH cleavage can occur at many sites within the ODN:mRNA duplex, hence the precise selection of antisense sequence is not possible (Monia *et al.*, 1997). This problem may be overcome by a novel alternative strategy employing 'scanning combinatorial ODN arrays' which has recently been developed (Milner *et al.*, 1997; for review see Southern 1996; Southern *et al.*, 1994).

In this strategy, a series of overlapping ODNs, of defined and variable length, targeting every conceivable site within chosen segments of the target mRNA can be synthesised

by standard phosphoramidite chemistry on a modified polymer membrane or glass surface. Hybridisation of these immobilised antisense ODNs to a radiolabelled target RNA transcript *in vitro* can be used to identify ODNs with precise antisense sequences with accuracy at the level of a single nucleotide. This approach can potentially allow the exhaustive walking of the full-length RNA to identify optimal antisense sequences. However, this can be tedious in practice, and typically only short specified regions of the RNA are normally targeted for combinatorial array design. It is possible to combine RNaseH mapping studies to select an RNA region with good accessibility, with ODN array technology to accurately define target antisense sequences (Milner *et al.*, 1997).

1.5 CELLULAR UPTAKE AND DISTRIBUTION OF ANTISENSE ODNs

Cellular delivery of antisense ODNs is of crucial importance for the successful inhibition of gene expression. In order to exert their pharmacological and biological effects, antisense ODNs must enter cells and interact with pre-mRNA or mRNA in the nucleus and cytoplasm. The delivery process therefore encompasses the targeting of ODNs to cells within specific tissues, their internalisation by these cells and their intracellular trafficking following cell entry.

Exogenously administered ODNs enter cells by a combination of different endocytic mechanisms including fluid phase (pinocytosis) (FPE), adsorptive (AE), and 'binding protein' or 'receptor-mediated' endocytosis (RME) (for reviews see Akhtar and Juliano, 1992; Vlassov *et al.*, 1994a; Rojanasakul, 1996). The precise mechanisms involved are dependent on ODN chemistry, conformation, concentration, and cell type. However, these processes are not efficient enough to deliver antisense ODNs in the quantities required to suppress gene expression (Stein *et al.*, 1994; Akhtar *et al.*, 1995). Thus, the permeability of the cell membrane is a limiting factor in antisense research design.

Antisense ODNs are poorly taken up by cells following direct administration, therefore high concentrations are required for biological activity (Wickstrom *et al.*, 1988; Wu-Pong *et al.*, 1992; Akhtar and Juliano, 1992). The uptake of antisense ODNs is also found to be energy dependant (Loke *et al.*, 1989; Wu-Pong *et al.*, 1992), and the

process is saturable (Beltinger *et al.*, 1995). ODN sequence may also influence cellular uptake, some reports suggest that antisense ODN uptake is largely sequence-independent (Yakobuv *et al.*, 1989; Rojanasakul, 1996). However sequences containing 'G'- tetrads, are found to be cell associated in greater quantities than other ODN sequences due to the formation of distinct secondary structures (Hughes *et al.*, 1994; Agrawal, 1996).

From the numerous studies conducted it is clear that the cellular uptake of antisense ODNs involves an endocytic mechanism (Akhtar and Juliano, 1992; Rojanasakul, 1996). The existence of specific binding proteins for the uptake of antisense ODNs has been reported (Loke *et al.*, 1989; Beltinger, *et al.*, 1995; Gewirtz *et al.*, 1996), although a specific receptor has not been defined. An approximately 80 kDa cell surface protein and a smaller 34kDa protein that function at pH4.5 have been proposed as putative receptor proteins (Loke *et al.*, 1989; Chang *et al.*, 1991). However, other studies suggest that antisense ODNs bind to a range of non-specific, cell surface proteins or lipids (Yakubov *et al.*, 1989; Shoji *et al.*, 1991; Beck *et al.*, 1996).

The precise mechanisms of uptake and distribution of ODNs are poorly understood. Cellular uptake also depends on length, specific sequences, and pendant modifications of ODNs. RME and/or AE are the most common mechanisms of uptake of charged ODNs in most cells (see figure 1.4), although FPE may predominate when these mechanisms become saturated (Beltinger *et al.*, 1995). The uptake of MP ODNs is via a pathway distinct from that of charged ODNs and may involve either passive diffusion or AE (Akhtar, 1991; Neckers *et al.*, 1992).

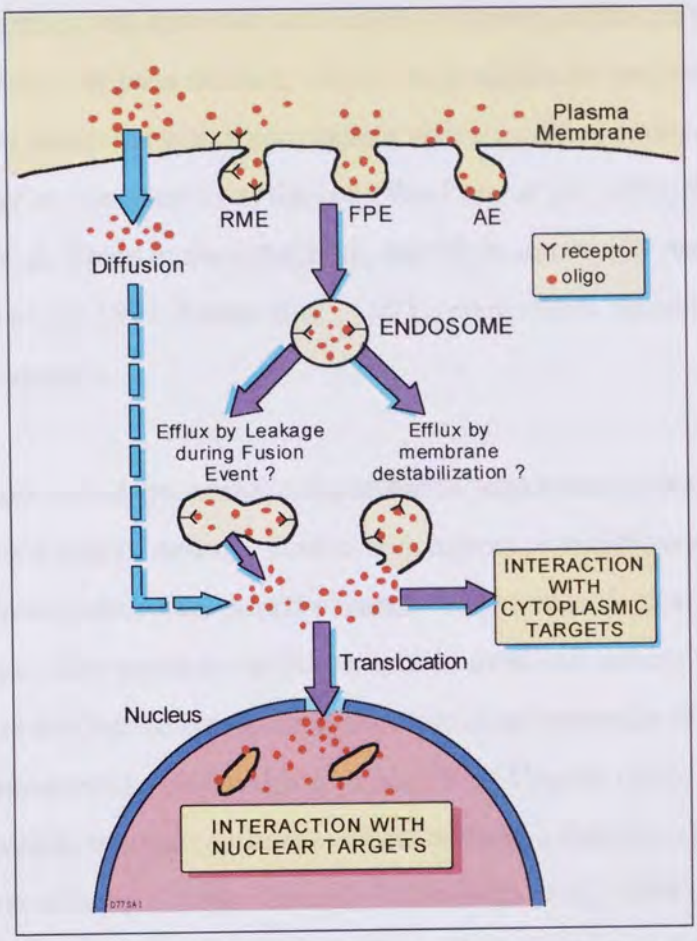


FIGURE 1.4 Schematic Diagram Demonstrating the Cellular Uptake of Oligonucleotides by Endocytosis. Adapted from Akhtar and Juliano (1992). [Receptor mediated endocytosis (RME), adsorptive endocytosis (AE), fluid phase endocytosis (FPE)]

Studies with fluorescently labelled ODNs have indicated that entry by endocytosis invariably leads to endosomal/lysosomal accumulation within the cells. A similar fate is observed for exogenously administered hammerhead ribozymes (Fell *et al.*, 1997), which leads to the characteristic punctate, periplasmic distribution with little or no visible ODN fluorescence in the cytosol, or nucleus. However, some cultured cells, such as keratinocytes, show more avid uptake and generalised sub-cellular distribution (Noonberg *et al.*, 1993; Nestle *et al.*, 1994).

Further evidence for an endocytic mechanism of cellular uptake is provided by ultrastructural studies, which have shown that antisense ODNs become localised within endosomes following cell entry (Shoji *et al.*, 1996; Wagner, 1994; Tonkinson and Stein, 1994; Beltinger *et al.*, 1995). A small proportion of internalised ODNs appear able to

depart endocytic vesicles and enter the nucleus or cytoplasm, although the mechanism for this process has not yet been defined. However, a significant proportion of ODN may be degraded by nucleases which accumulate within acidified endosomes (lysosomes), or may be exported from the cell (Wu-Pong *et al.*, 1992; Stein *et al*, 1993; Hudson *et al.*, 1996a). Once in the cytoplasm, the ODN can rapidly accumulate in the nucleus (Leonettie *et al.*, 1991; Fisher *et al.*, 1993), presumably permitting interactions with nuclear RNA species.

Accumulation in endosomal-lysosomal compartments represents a pharmacologically non-productive site, a sort of detour. Due to this, agents or techniques that promote the release of ODNs from endosomes should enhance the pharmacological effectiveness of antisense molecules. This seems to be the case, as in most cell culture studies, free antisense ODNs are ineffective but become effective in the presence of a facilitator or delivery agent (Bennet *et al.*, 1998; Akhtar *et al.*, 1992; Hughes *et al.*, 1996). In some cases it may be possible to attain antisense effects without a delivery agent by utilising high concentrations of stable ODNs (Gonzalez-Cabrerera *et al.*, 1998). Much effort has been focused on improving the otherwise inefficient and non-targeted delivery of ODNs to living cells. Section 1.6 details some of the major strategies that have been examined for improving ODN delivery.

1.6 OLIGODEOXYNUCLEOTIDE DELIVERY STRATEGIES

The main obstacle to the delivery of antisense agents to cells is their susceptibility to degradation by enzymes in the blood and cells. Therefore, the major goal in developing methods of delivery is to reduce the susceptibility of antisense agents to nucleases while retaining their ability to bind to targeted sites. Modifications have been made to the phosphodiester backbone of ODNs to increase resistance to nuclease degradation as outlined in section 1.3.2. Further strategies to enhance cellular delivery are discussed below.

A major factor influencing the delivery of antisense therapeutic agents is the low permeability of cell membranes to these compounds. Since their sites of activity are in

the cytoplasm or nucleus, penetration of the agent into the cell is essential for the potential of antisense ODNs to be fulfilled. Although antisense sequences are internalised by cells, the extent of uptake may not be adequate for sustained activity *in vivo* (Jaroswezki *et al.*, 1991; Akhtar, 1995). Therefore, it is essential to develop new drug delivery technologies, which will allow controlled and site directed delivery.

An ODN may be delivered by direct insertion into the cell genome or by exogenous administration. A delivery system must protect the ODN from degradation and deliver the ODN to the site of action in therapeutic concentrations. Localised delivery of ODNs may be achieved by the administration of an implant to release the ODN at a pre-determined rate and allow for sustained delivery over an extended time period. Biodegradable systems offer the benefit that on drug exhaustion, these devices degrade to non-toxic products, which are eliminated from the body and therefore do not require any form of removal from the body.

1.6.1 Exogenous Delivery Strategies

1.6.1.1 Increasing Hydrophobicity of ODNs

An option for the exogenous delivery of antisense molecules is their attachment to carrier molecules, which can enhance cellular uptake. The covalent attachment of lipophilic molecules such as cholesterol (Alahari *et al.*, 1996; Kreig *et al.*, 1996), spermine/spermidine-cholesterol (Guy-Caffey *et al.*, 1995), and phospholipids (Shea *et al.*, 1990), can increase cellular uptake and promote activity when attached to antisense ODNs. However, the aforementioned studies conducted using these molecules did not determine whether an endocytic mechanism was involved in the uptake of these conjugates.

Antisense ODNs have also been attached to avidin/biotin complexes via electrostatic attraction to poly-(L-lysine) linkers (Pardridge and Boado, 1990, Boado and Pardridge, 1996). Avidin is thought to enter cells by the adsorptive endocytosis pathway, and

attachment of the avidin/ biotin complex produced a 4-fold increase in cellular uptake *ex vivo* (Pardridge and Boado, 1990). ODNs conjugated with polylysine at either the 5'-or 3'-end are more resistant to serum nucleases and show enhanced antisense activity as compared to unmodified oligomers (Leonetti *et al.*, 1990). Polylysine potentiates the antisense effect by accelerating the uptake of ODNs but it produces toxicity as well.

1.6.1.2 Receptor Mediated Delivery

The conjugation of antisense ODNs to molecules that have a specific receptor has also been examined. This introduces the possibility of receptor mediated delivery which has the potential to increase the cellular uptake of antisense ODNs. Complex formation with receptor ligands or conjugation with receptor antibodies improves cellular association of ODNs, but the cellular fate of ODNs following receptor-mediated targeting probably lies within vesicular compartments. Their escape from these compartments may require active assistance in the form of endosomal disruption agents such as fusogenic peptides, cationic lipids, or molecules which can alter the sub-cellular sorting and trafficking of ODNs (Bongartz *et al.*, 1994; Pichon *et al.*, 1997). However, improved biological activity of ODNs delivered via receptor mediated strategies (Rojanaskul, 1997; Wang *et al.*, 1995) suggests that significant amounts of the nucleic acid can be delivered to the right compartments for efficacy in the absence of endosomal disruption agents.

The transferrin receptor is expressed by a variety of cell types. It is involved in the intracellular transport of iron-bearing transferrin molecules by receptor mediated endocytosis (Friden, 1993). Conjugation of antisense ODNs to transferrin has been shown to improve the cellular uptake of antisense ODNs *ex vivo* (Citro *et al.*, 1992). However, when administered *in vivo*, an antisense ODN / transferrin conjugate would theoretically compete with transferrin for receptor binding, thus compromising uptake. Thus, an alternative approach is to utilise monoclonal antibodies raised against the transferrin receptor antibody, which binds at different sites to the natural ligand (Friden, 1993). Antisense ODNs conjugated to an anti-transferrin receptor monoclonal antibody

achieved a 3-fold increase in cellular uptake *ex vivo*, compared with an unconjugated antisense ODN (Walker *et al.*, 1995).

Targeted delivery of a specific receptor is shown by the targeting of mannose-specific lectins which, mediate the cellular uptake of mannose by macrophage cells (Stahl *et al.*, 1978). The possibility of hijacking this system to deliver antisense ODNs has been investigated (Akhtar *et al.*, 1995b; Liang *et al.*, 1996). The covalent attachment of a mannose conjugate to a radiolabelled antisense ODN during synthesis resulted in a 4-fold increase in cellular uptake by macrophage cells *ex vivo* (Akhtar *et al.*, 1995b), whereas the addition of mannosylated poly-(L-lysine), electrostatically linked to a fluorescent labelled ODN, produced a 17-fold increase in cellular uptake (Liang *et al.*, 1996).

Further examples of receptor targeted delivery include the conjugation of ODNs to epidermal growth factor (EGF) targeting the EGF receptor (Deshpande *et al.*, 1994), galactose (Carmicheal *et al.*, 1995), fucose (Stewart *et al.*, 1996), and folic acid (Ginnobi *et al.*, 1997). The main limitations of all carrier mediated systems are the escape of the conjugate from the endosomes following internalisation by RME, and the release of the carrier molecule from the antisense ODN following delivery.

1.6.1.3 Liposomal Systems

Liposome encapsulation has been the most widely used vehicle for the delivery of antisense ODNs (for reviews see Juliano and Akhtar, 1992; Chonn and Cullis, 1995; Thierry and Tackle, 1995). Liposomes are small spheres of phospholipid bilayers, which can either encapsulate nucleic acids within an aqueous core, or can form lipid nucleic acid complexes due to opposing electrostatic charges. Generally liposomes can be classed as either cationic or non-cationic based upon the type of lipids present in the formulation (Elkins and Rossi, 1995).

Liposomes consist of multiple phospholipid bilayers arranged to create hydrophobic bands alternating with hydrophilic bands (multilamellar), or consist of a single

phospholipid bilayer encasing an aqueous core (unilamellar). Drugs, in this case antisense sequences, can either be partitioned into the hydrophobic layers or be dissolved in the aqueous layers. The drug is released by partitioning back out of the liposome or by disruption of the liposome structure. The composition, type, and size of liposome can be altered to impart different characteristics. A drawback of liposomes is their relatively short circulation time since they are rapidly recognised by the reticulo-endothelial system (RES) and are taken up by endocytosis and deactivated. Advances in liposome technology have created liposomes whose circulation time has been increased by minimising their recognition by the RES (Allen *et al.*, 1989). Adding targeting moieties, such as monoclonal antibodies, to long circulating liposomes can guide the antisense sequence to the desired subset of cells (Leonetti *et al.*, 1990; Zelphati *et al.*, 1994). A liposome-incorporated antisense MP ODN, cytotoxic to CML cells, inhibited the growth of CML cells *in vitro* in a dose dependant manner (Tari *et al.*, 1994).

Although several liposome formulations have been used, the most successful in obtaining biological effects with antisense ODNs in cultured cells is the use of cationic liposomes. Complexation of ODNs with cationic lipids such as Lipofectin® and Lipofectamine® was originally described by Bennett *et al.*, (1992). These lipids spontaneously form complexes with polynulceotides upon mixing in aqueous solution by a condensation reaction (Felgner and Ringold, 1989; Eastman *et al.*, 1997), thus preparation is relatively straightforward.

The mechanism involved in antisense delivery mediated by such cationic lipid complexes is still not fully understood (Hope *et al.*, 1998), although new insights are beginning to emerge (Koltover *et al.*, 1998). The lipid-ODN complex is internalised by endocytosis; thereafter the complex probably induces a 'flip-flop' of anionic phospholipids in the endosome membrane, leading to the neutralisation of the cationic lipid charge, displacement of the bound ODN and release from the endosome (Zelphati *et al.*, 1996). Current observations suggest that the pharmacologically active antisense ODNs are released from the cationic lipid complexes and enter the nucleus as free ODNs prior to the onset of the antisense effect (Marcusson *et al.*, 1998). Modified forms of cationic liposomes are under consideration for *in vivo* delivery of antisense molecules (Gokhale

et al., 1997; Meyer *et al.*, 1998). However, the very large size (Zabner *et al.*, 1995) and high surface charge density of cationic lipid complexes clearly present some major obstacles in terms of systemic use.

The success with commercial cationic lipid formulations is not universal (Bertram *et al.*, 1994) as the effectiveness of cationic lipids is dependant on several variables including cell type, ODN chemistry, and charge ratio, which means that conditions have to be optimised for a given cell nucleic acid system. In addition, cationic lipids are toxic, and generally ineffective in the presence of serum proteins, which consequently has limited their application mainly to cell culture. A serum resistant cationic lipid preparation, termed cytofectin, which is thought to successfully enhance the activity of charged ODNs in the presence of serum has recently been reported (Lewis *et al.*, 1996). However, it is not clear whether this preparation is effective *in vivo*.

Alternatives of the liposomal delivery strategy include the targeting of liposomes to specific cell types by the attachment of antibodies or targeting ligands to the liposomal membrane, rather than attaching these molecules directly to the ODN itself. Antisense ODNs have already been targeted to specific cell types using liposomes bearing monoclonal antibodies (Leonetti *et al.*, 1990).

1.6.1.4 Carrier Peptides

Carrier peptides are able to increase subcellular localisation of ODNs by utilisation of a non-endocytic pathway. ODNs have been successfully delivered to cells bound to polylysine, and conjugated with a signal peptide, which has the potential to interact with lipid bilayers (Dokka *et al.*, 1997). Dokka *et al.*, (1997) reported that the uptake of conjugated ODN showed a dose dependant increase, and rapid accumulation of ODN was seen in the cytoplasm and nucleus. The uptake was unaffected by endocytosis inhibitors, suggesting a non-endocytic mechanism was being utilised.

Morris *et al.*, (1997) enlisted the delivery of ODNs complexed with a chimeric peptide consisting of a hydrophobic fusion domain derived from HIV gp41 and a hydrophilic

nuclear localisation signal derived from SV40 T-antigen. The complex was found to improve stability of the ODN, and efficient delivery to cells was observed. Cellular distribution studies showed the ODN complex to be predominantly in the nucleus, with free ODN present in subcelluar vesicles. Again, a non-endocytic mechanism was proposed.

1.6.1.5 Dendrimer Supermolecular Systems

Dendrimers are repetitively branched polymers. They have proved successful as delivery agents for antisense ODNs (Haensler *et al.*, 1993; Delong *et al.*, 1997; Alahari *et al.*, 1998). One promising aspect of dendrimers is that the delivery complex can be of relatively modest molecular size (Delong *et al.*, 1997). Stable complexes between ODNs and cationic dendrimers form under a variety of conditions e.g. water, PBS, and serum (Delong *et al.*, 1997). These complexes (Mw 5000-10,000) have been shown to enhance ODN uptake and intracellular availability, and also increase delivery to the nucleus (Bielinska *et al.*, 1996).

1.6.1.6 Particulate Systems

The use of microparticles as carrier systems has been extensively reviewed (Arshady 1991; Conti *et al.*, 1992; Couvreur and Puisieux, 1993 and Okada *et al.*, 1995). Microparticles are described as fine spherical particles containing a drug and can be divided into two categories: (1) homogenous or monolithic microspheres in which the drug is dissolved or dispersed throughout the polymer matrix, and (2) reservoir-type microcapsules in which the drug is surrounded by the polymer matrix. Nanoparticles are submicronic polymeric systems containing a drug, which are morphologically equivalent to microspheres but used distinctively to emphasise their small size. The formation of these different sized polymeric systems is dependent on the fabrication process and the polymer used.

Biodegradable polymers have become increasingly important in the development of drug delivery systems. The most widely investigated biodegradable polymers and systems achieving the most success at present are the lactic/glycolic acid polymers (PLGA). The use of biodegradable sustained-release delivery systems may obviate the need for repeated administration by facilitating site-specific delivery in a controlled manner to the desired site in the central nervous system (CNS). The entrapment of ODNs within such polymeric matrix systems may also improve ODN stability, reduce the ODN dose required for efficacy and further reduce toxicity or non-specific activities associated with ODNs. Microspheres offer advantages over other drug delivery devices due to the wide range of possible routes of administration. Microspheres can be administered by injection via the intramuscular (i.m.), subcutaneous (s.c), intravenous (i.v.), or intraperitoneal (i.p) route. Thus far, the targeted routes have been the i.m. and s.c. routes as these have a good potential for sustained release application. Their application in biodegradable delivery devices has been reviewed by Conti *et al.* (1992), and Brannon-Pepas (1995).

Targeting of particulates to tissues is virtually impossible due to the high efficiency of the RES. Site-specific administration of spheres may offer an alternative to uptake of particles by the RES. Davis *et al.*, (1993) have reviewed targeting using microspheres. Following intravenous administration of spheres, particles were taken up to a considerable extent into the bone marrow, endothelial cells, liver, spleen and lymph nodes (Moghimi *et al.*, 1991). Strategies to delay the uptake of spheres by Kupffer cells include the coating of spheres with a hydrophilic polymer, such as poloxamine-908 to prevent opsonization with plasma components, which leads to their rapid uptake by Kupffer cells within the liver (Moghimi *et al.*, 1993). The covalent attachment of spheres to polyethylene glycol was also found to increase blood circulation times (Gref *et al.*, 1994).

The potential of biodegradable microparticles in vaccine delivery has been well documented (Alpar *et al.*, 1994; Johansen *et al.*, 1999). The oral delivery of microspheres has also been investigated for vaccine delivery (Eyles *et al.*, 1997; Norris *et al.*, 1998).

To reiterate, it is clear that repeated administration of ODNs and ribozymes would be required to maintain sustained efficacy against *in-vivo* targets. For example, Yazaki *et al.,* (1996) administered ODNs daily for up to 80 days in their successful inhibition of glioma xenografts in nude mice, implying that although ODNs can gain access to tumours and other tissues *in vivo*, they are eliminated relatively rapidly. Thus a sustained delivery system, whereby controlled and prolonged release of ODNs and ribozymes could be maintained for a period of days to months is indicated.
Various polymeric systems have been investigated for the delivery of antisense ODNs, these delivery systems have been summarised in table 1.1.

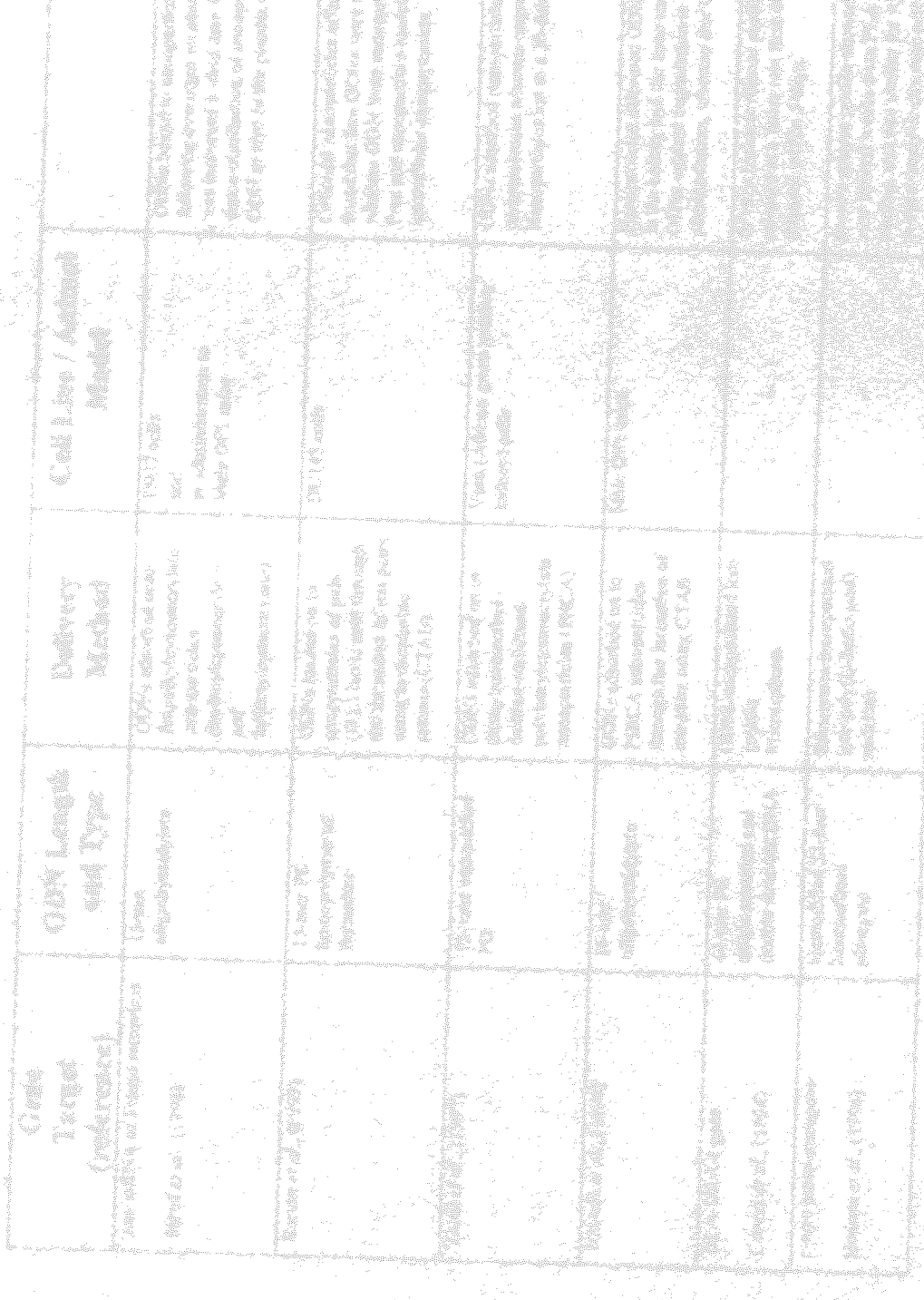


TABLE 1.1 Examples of *In vitro* and *In vivo* Particulate Delivery Systems of Antisense ODNS and Ribozymes

Gene Target (reference)	ODN Length and Type	Delivery Method	Cell Line / Animal Model	Comments (also see text)
Env mRNA of Friend retrovirus Fattal *et al.*, (1998)	15-mer oligothymidylate	ODNs adsorbed onto Polyalkylcyanoacrylate nanoparticles (isobutylcyanocrylate and isohexylcyanocrylate)	U937 cells and iv administration to Male OF1 mice	ODNs bound to nanoparticles were protected from nuclease attack following overnight incubation with phosphodiesterase. Cellular uptake was increased 8 -fold than for free ODN. Pharmacokinetic studies showed tissue distribution of nanoparticles was significantly modified. Stability of ODN *in vivo* in the plasma and liver was also improved for adsorbed ODN.
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	Colloidal nanoparticles affected intracellular trafficking of ODNs. Studies found that free ODNs were resident in an acidic intracellular environment, whereas ODN from nanoparticles did not reside in acidic compartments. Free and nanoparticle-loaded ODNs were effluxed from cells from two intracellular compartments.
Zobel *et al.*, (1997)	19- mer unmodified PO	ODNs adsorbed on to diethylaminoethyl - dextran-stabilised polyhexylcyanoacrylate nanoparticles (PHCA).	Vero (African green monkey kidney) cells	ODNs adsorbed nanoparticles were protected against degradation by endonucleases whereas unprotected ODNs were completely degraded. Nanoparticles led to a 20-fold increase in cellular uptake of FITC-ODNs
Nakada *et al.*, (1996)	16-mer oligothymidylate	ODNs adsorbed on to PIBCA nanoparticles through the formation of ion pairs using CTAB	Male OF1 mice	Nanoparticles delivered ODNs specifically to the liver reducing distribution in the kidney and the bone marrow. Nanoparticles partially protected ODNs against degradation in the plasma and in the liver 5 min after administration, whereas free ODN was totally degraded at the same time.
HLA-DRA 4 gene Cortesi *et al.*, (1994)	44-mer PS single stranded and double stranded DNA	ODNs encapsulated into gelatin Microspheres	–	*In vitro* biphasic release profiles were observed. Single stranded DNA was released at a faster rate than double stranded DNA, with 60% of ODN released within 5 days.
c-myc proto-oncogene Hudson *et al.*, (1996)	Unmodified 32-mer hammerhead ribozyme	Ribozymes incorporated into poly(L-lactic acid) matrices	–	*In vitro* release profile showed entrapped RBZ was released biphasically over several weeks from PLA films. Release was dependent on RBZ loading, and was similar for RNA and DNA ODNs of the same length. The *in-vitro* catalytic cleavage activity of polymer released RBZ was identical to that of free RBZ.

TABLE 1.1 continued

Vesicular stomatitis virus N protein mRNA initiation codon Chavany *et al.*, (1994)	16-mer and 15-mer oligothymidylate	ODNs adsorbed on to PHCA nanoparticles through the formation of ion pairs using CTAB	U937 macrophage like cell line	After 24 hrs incubation, uptake of ODN was 8 times higher when adsorbed to nanoparticles. ODNs were totally protected for 5 hrs when adsorbed, whereas 0.1µg/ml phosphodiesterase degraded free ODN rapidly (half life-2 mins).
H-ras gene Schwab *et al.*, (1994)	Unmodified PO ODN	ODNs adsorbed on to PHCA nanoparticles through the formation of ion pairs using CTAB	HBL100ras1 and subcutaneous administration to nude mice	Nanoparticle-adsorbed ODNs selectively inhibited the proliferation of cells expressing the point-mutated *H-ras* gene at a concentration 100 times lower than free ODN. They also inhibited *H-ras* dependant tumour growth in nude mice.
tat gene in HIV Akhtar *et al.*, (1997)	20-mer PO and PS ODNs	ODNs incorporated into PLGA 50:50 microspheres	RAW 264.7 murine macrophage cells	*In vitro* release profiles were dependent on microsphere size, 1-2µm spheres released 70% of ODN within 4 days compared with 40 days for 10-20µmspheres. Cellular association of ODNs entrapped in small spheres was improved 10-fold in murine macrophages compared with free ODNs.
env mRNA of Friend Retrovirus Aynie *et al.*, (1999)	15-mer PO/poly-L-lysine complex	ODNs encapsulated into alginate nanoparticles	OF1 mice	The alginate system protected the ODN from degradation in bovine serum for 1 hour with 70% of ODN remaining undegraded. Biodistribution was modified following iv administration, compared to free ODN with radioactivity observed in lungs, liver and spleen
c-myc proto-oncogene and *tat*-gene in HIV-RNA Lewis *et al.*, (1998)	20-mer PO and PS ODNs	ODNs incorporated into PLGA co-polymer 50:50 microspheres	–	Triphasic in-vitro release profiles were observed from10-20µm microspheres over a 56 day release period. Release profiles were influenced by microsphere size, molecular weight of polymer, loading of ODN, and pH of release medium. Stability in serum was improved with entrapment of ODN in spheres.
Ayers *et al.*, (1997)	ribozyme	RBZ incorporated into poly acrylic acid polymer gel	Ocular delivery to mice eyes	Results indicated that 3-5% of administered RBZ became localised within the epithelial layers of the cornea. Peak levels were observed in the eye after 30 mins and RBZs were also detected in epithelial cells 3 hours post administration when gel formulation used.
tat gene in HIV-RNA and *c-myc* oncogene exon 2 Lewis *et al* ., (1995)	20-mer PO and PS ODNs 7-mer PS ODN	ODNs incorporated into poly(L-lactic acid) matrices	–	Solvent-cast polymer film matrices (100µm thickness) released entrapped ODN biphasically over a 28 day period, ODN release was dependent upon ODN chemistry, and ODN length. During release, little degradation of polymer film was seen.
Herpes simplex virus Yamakawa *et al.*, (1997)	21-mer PS ODN	ODNs encapsulated into PLGA pillar shaped implants	–	Release profiles showed sustained release of ODN over 20 days was possible. Release was unaffected by changes in Mw of polymer, however changes in loading altered release rate of ODN. Stability in bovine vitreous was assessed.
Rat tenascin mRNA Cleek *et al.*, (1997)	24-mer PS ODN	ODNs encapsulated in to PLGA microparticles	Vascular smooth muscle cells (SMC) from carotid arteries of rats	Release of ODN was characterised by a small initial-burst (20%) followed by controlled release for up to 20 days. SMC proliferation studies exhibited dose-dependent growth inhibition with ODN loaded microparticles.

Initial studies carried out by Chavaney *et al.*, (1992, 1994), utilised polycyanocrylic particles for the delivery of antisense ODNs. Chavaney *et al.*, 1994 adsorbed ODNs onto the surface of polyalkylcyanocrylate nanoparticles by ion pairs between the ODN and hydrophobic cations (tetra phenyl phosphonium salts) that bind to the polymer surface. Chavaney *et al.*, (1994) reported the ODNs were completely protected from degradation by 3' exonucleases for 15 minutes at 37°C. The release of ODN was dependent on the esterase content of the release media (Grangier *et al.*, 1991). Degradation of polymer results in the formation of formaldehyde, which gives rise to toxicity concerns. This, combined with the dose limiting toxicity of the quartenary ammonium salts limits the therapeutic efficacy of this system. Godard *et al.*, (1995) and Fattal *et al.* (1998) also investigated the use of such nanoparticles for the delivery of ODNs. Fattal *et al.*, (1988) reported ODN bound nanoparticles were found to be protected from nuclease attack in cell culture media, and their cellular uptake was increased as a result of the capture of nanoparticles by an endocytic/phagocytic pathway. Also, following *in vivo* administration to mice, stability of ODN in the plasma and in the liver was shown to be improved when ODNs were adsorbed onto the nanoparticles. Zobel *et al.*, (1997), and Zimmer *et al.*, (1999), also reported similar findings using polyalkylcyanocrylate particles. Gelatin microspheres have also been used to encapsulate ODNs (Cortesi *et al.*, 1994). However the release rate is very rapid, with 60% of encapsulated ODN released within 120 hours incubation *in vitro* (Cortesi *et al.*, 1994).

Biodegradable polymer devices, such as poly(L-lactic acid) (PLLA) films have been tested *in vitro* for their ability to provide antisense ODNs with nuclease protection, and permit sustained release of ODNs over periods of several weeks (Lewis *et al.*, 1995). A similar approach has been investigated using polymer film entrapped hammerhead ribozymes *in vitro* (Hudson *et al.*, 1996b). The entrapped ribozyme was released biphasically from the polymer films, followed by a more sustained release over a period of several weeks. Release was dependent on ribozyme loading, but was similar for RNA and DNA ODNs of the same length. Biological stability of the ribozyme was improved from 2 seconds for free ribozyme, to 2 weeks for polymer entrapped ribozyme.

Biodegradable PLGA microspheres have been utilised for the improved delivery of ODNs to macrophage cell lines in culture (Akhtar and Lewis, 1997). The cellular association of ODN entrapped spheres was improved 10-fold in murine macrophages compared with free ODNs. Fluorescence studies of microspheres with macrophages showed a more diffuse subcellular distribution of ODNs compared to free ODNs, which exhibited the characteristic periplasmic distribution. Further studies involving the characterisation of PLGA 50:50 spheres for potential sustained delivery of PO ODNs were undertaken by Lewis *et al.*, (1998).

A further polymer approach has been utilised *in vivo* by Ayers *et al.*, (1996), who formulated chemically stabilised hammerhead ribozymes with poly-(acrylic acid) for ocular delivery in mice. A ribozyme/poly-(acrylic acid) gel was administered to mice eyes, then nuclease susceptibility and autoradiography were used to confirm that ribozymes were internalised within the cornea rather than simply being adsorbed to the surface of the eye. Results indicated that 3 to 5% of the administered ribozyme became localised within the epithelial layers of the cornea following the administration of the gel formulation. In contrast, less than 1% was retained by the eye following administration of the ribozyme in solution. Peak levels were observed in the eye after 30 minutes and ribozyme was detected in the epithelial cells 3 hours post-administration of the gel formulation (Ayers *et al.*, 1996).

Sponge-like alginate nanoparticles were investigated by Aynie *et al.*, (1999) for the delivery of antisense ODNs. *In vitro* characterisation and pharmacokinetics, and tissue distribution of ODN loaded nanoparticles was determined following i.v. administration to mice. ODNs were entrapped due to ionic interactions with alginate particles crosslinked with the aid of poly-L-lysine, resulting in diffusion of ODN or ODN/polylysine complex into the nanoparticle or the alginate gel. This new alginate-based system was found to be able to protect ODN from degradation in bovine serum medium. It also modified biodistribution, as an accumulation of radioactivity was observed in the lungs, liver, and spleen following i.v. administration into mice.

1.6.2 Endogenous delivery

Retroviruses have been used for the endogenous delivery of ODNs. Retroviral vectors possess several properties that are appealing. They can transfect and express therapeutic genes in disease-susceptible cells, have no contaminating helper virus, and integrate into the genome of the cell (Samsulski *et al.*, 1993). The adeno-associated viruses are non-pathogenic, able to transfect diverse cell types, and is an alternative vector for ODN delivery (Samsulski *et al.*, 1993; Cotton *et al.*, 1992). Viral delivery of ODNs is at an early stage of development, and targeting ODN-containing vectors to a particular tissue or cells is difficult.

1.6.3 Physical Methods

The process of microinjection involves the direct insertion of an antisense agent into the cell cytoplasm, therefore bypassing encapsulation into endosomal/lysosomal compartments (Leonetti *et al.*, 1991). Studies conducted by Leonetti *et al.*, (1991), and Fisher *et al.*, (1993) have shown ODN localisation in the nucleus following microinjection into the cytoplasm. The path followed by microinjected ODNs may mimic the events that follow endosomal/lysosomal release into the cytoplasm. The major drawback of this technique is the basis of single cell administration, limiting its potential for therapeutic use.

Iontorophoresis utilises the application of a low voltage and a constant current to the skin, to enable the dermal transfer of charged particles. Various studies of the dermal delivery of ODNs using ionotophoresis have been conducted (Vlassov *et al.*, 1994, Oldenburg *et al.*, 1995, Brand and Iverson *et al.*, 1996, and Brand *et al.*, 1998). Brand *et al.*, (1998) applied the technique of iontorophoresis to deliver ODNs across the dorsal skin of male hairless mice. Significant transfer of ODN across the skin was achieved with intact ODN still present following transfer of ODN (Brand *et al.*, 1998). Electroporation involves the application of a high voltage pulse for a short time to reversibly permeabilise the lipid bilayer of the dermis (Regneir *et al.*, 1999). Electrophoresis is believed to open pores in the cell membrane to allow the transit of

molecules. Bergan *et al.*, (1993) found intracellular concentrations of ODN to increase between 3-fold to 27-fold, following the application of high voltage to cells. Uptake was found to be dependent on cell and ODN type (Bergan *et al.*, 1993). Fraga *et al.*, (1998) reported a 10-fold reduction in the dose of ODNs required to achieve desired effects following utilisation of electroporation to deliver antisense ODNs into *Paramecium tetraurelia*.

1.6.4 Other Methods

Studies using erythrocyte membrane preparations as ODN carrier systems have been reported by Budker *et al.*, (1994), and Vlassov *et al.*, (1994). These erythrocyte 'ghosts' once subject to hypo-osmotic shock, lyse and release free, intact ODN. Cellular uptake of ODNs was found to be enhanced using this method (Vlassov *et al.*, 1994). Cyclodextrins have also been utilised for the delivery of ODNs. These are cyclic structures comprising of glucose polysaccharides with a central cavity region in which molecules may be entrapped. Zhao *et al.*, (1995) entrapped ODNs within such a system, and reported a 2-3-fold increase in uptake of encapsulated PS ODN with no adverse effect on ODN stability. Intracellular association was also shown using fluorescently labelled ODNs.

1.7 *IN VIVO* PHARMACOKINETICS OF ODNs

The pharmacokinetics and tissue disposition profiles of a given ODN are important factors in assessing its therapeutic potential. Most of the *in vivo* observations on the biodistribution of ODNs have dealt with molecules having a PS background, and these can be summarised as follows:

(a) The biphasic plasma half-lives of PS ODNs are in the range of minutes for $t_{1/2}\alpha$ (distributional phase) and many minutes to several hours for $t_{1/2}\beta$ (elimination phase).
(b) The ODNs are widely distributed in the body and are accumulated in most tissues particularly in the liver, but excepting the CNS.
(c) Although PS ODNs are significantly protein bound, particularly at low doses, the primary route of elimination is via the kidneys.
(d) Metablism is complex, but breakdown by 3'-exonucleases is important (Temsamani *et al.*, 1997).
(e) In general, the pharmacokinetic behaviour of PS ODNs in humans is similar to that in lower animals (Agrawal *et al.*, 1997; Glover *et al.*, 1997).

PS ODNs of various sequences have been administered to mice, rats, monkeys and humans, and the pharmacokinetics and metabolism have been studied by numerous authors (Agrawal *et al.*, 1997; Philips *et al.*, 1996; Grindel *et al.*, 1998; Martin *et al.*, 1998; Glover *et al.*, 1997; Nicklin *et al.*, 1998; Agrawal *et al.*, 1998).

In vivo metabolism of PS ODNs has been extensively studied by various techniques. The results show that degradation of PS ODNs is primarily by 3'-exonucleases, although degradation from the 5'-end has also been observed (Temsamani *et al.*, 1997; Cummins *et al.*, 1997). However, the so-called scavenger receptors on living cells and other cell types may play an important role in ODN clearance (Bijsterbosch *et al.*, 1997; Biesson *et al.*, 1998). There has also been considerable interest recently in the enteric uptake and hepatic processing of ODNs (Agrawal *et al.*, 1997; Nolting *et al.*, 1997; Hughes *et al.*, 1995; Graham *et al.*, 1998). In that context, it seems clear that very stable forms of

ODNs have an appreciable permeation rate across the gastro-intestinal epithelium, are cleared or degraded to a modest degree during passage through the portal circulation and liver, and thus these stable compounds have some degree of oral bioavailibility.

Antisense ODNs have short plasma half-lives and are rapidly distributed to various tissues. The bioavailibility of antisense ODNs cannot be accurately determined on the sole basis of plasma pharmacokinetics, and tissue distribution should also be considered. It is to be expected that different types of cells will have different affinities and uptake capacities for various types of ODNs. In addition, the patterns of cellular uptake may also reflect concentration gradients of ODNs between blood and extracellular fluid or between well-perfused and poorly perfused regions in an organ. Studies involving immunofluorescence, immunochemical, and autoradiographic techniques have shown a great variability in ODN uptake into different cell types. (Rifai *et al.*, 1996; Butler *et al.*, 1997).

The *in vivo* toxicity of PS ODNs appears to be species dependent. However, following subchronic i.v. or i.p. administration, dose dependent side effects have been observed with PS ODNs in mice and rats (Sarmiento *et al.*, 1994; Zhang *et al.*, 1995). The relatively long retention times in the RES organs, suggest that accumulation of PS ODNs and their metabolites upon repeated dosing might account for this toxicity. As the tissue disposition of PS ODNs is similar following i.p. and s.c. administration, it is expected that the above toxicity will also be apparent via these routes of administration. Other models of administration, including delivery formulations facilitating local or site-specific delivery, may help to circumvent these problems.

Some of the toxicological observations in mice and rats have been found to be specific to the sequence of PS ODNs. Antisense sequences containing –CG- motifs with appropriate flanking sequences have been shown to be responsible for the induction of interferon (IFN), IFN-mediated natural killer cell activity (Yamomoto *et al.*, 1992; Kuramoto *et al.*, 1992) and for producing immune stimulation (Krieg *et al.*, 1995). PS ODNs exhibit quite different toxicity profiles in monkeys. Bolus i.v. administration of these ODNs in monkeys produced a transient decrease in peripheral total white blood cell and neutrophill counts, and a brief increase followed by a prolonged decrease in

arterial blood pressure. These effects were dose and infusion rate dependent, and were avoided by slow i.v. administration (Galbraith *et al.*, 1994; Cornish *et al.*, 1993). These effects may be PS backbone specific, and by altering the nature of the ODN backbone, these side effects can be minimised (Zhao *et al.*, 1996; Agrawal *et al.*, 1995).

Although favourable pharmacokinetics have been reported in the above mentioned studies, an effective delivery / formulation system may be beneficial to further influence site or target-specific delivery (Akhtar, 1995), and thus the therapeutic index of these molecules.

1.8 OLIGODEOXYNUCLOTIDE CLINICAL TRIALS

Antisense ODNs can selectively inhibit individual gene expression in both cell culture and animal models provided they are designed to target a hybridisation-accessible region on mRNA and gain access to the correct intracellular site(s) of action for an appropriate period of time (Akhtar, 1998; Miller and Das, 1998). Several antisense ODNs are undergoing clinical trials evaluation (see table 1.2), and the first antisense drug has recently been approved for marketing. Vitravene™ (Fomivirsen), which received FDA-approval in July 1998, is an antisense compound delivered locally to the eye to treat cytomegalovirus (CMV)-induced retinitis in AIDS patients. Other ODN compounds are being screened for anti-cancer, anti-inflammatory and anti-HIV applications (for review see Akhtar and Agrawal, 1997).

TABLE 1.2 A Summary of Ongoing Human Clinical Trials with Antisense ODNs

TARGET	DISEASE	STATUS	OWNERSHIP
Vitravene™ Vision	CMV Retinitis (AIDS)	Approved	Isis/CIBA Vision
ISIS 2302 ICAM-1	Crohn's disease Renal transplant Rejection Psoriasis (topical) Ulcerative Colitis	Pivotal Trial Phase II INDCanditate INDCanditate	Isis/Boehringer Ingelheim
ISIS 3521 PKC-alpha	Cancer	Phase II	Novartis
ISIS 5132 *c-raf* kinase	Cancer	Phase II	Novartis
ISIS 2503 *H-ras*	Cancer	Phase II	Isis
GEMO 231	Cancer	Phase II	Hybridon
ISIS 14803 HCV	Hepatitis C	INDCanditate	Isis
TNF-alpha	Inflammation	Preclinical	Isis
VLA-4	Inflammation	Preclinical	Isis

Despite the fact that many of the other ongoing clinical trials with ODNs employ systemic administration (e.g. i.v. infusions), local delivery to sites of disease can offer various advantages. The appropriate choice of an efficient ODN delivery strategy may not improve efficacy, but can help reduce side effects, and ultimately reduce drug costs. Considerable advances in manufacturing ODNs have been made and continued success in this area will undoubtedly make ODN-based therapeutics cost effective. The issue of toxicity has to be balanced with the severity of the condition being treated. Some degree of non-specific toxicity would be tolerable to life threatened AIDS or cancer patients provided that the ODN is efficacious.

1.9 POLYMERS

Over the past two decades, a variety of synthetic polymers have been reported to degrade in mammals. The degradation products of some of these polymers can be eliminated from the body by either metabolism, or kidney filtration. Polymers that undergo degradation in a living environment, through either simple chemical reactions or enzyme catalysed reactions, are designated as biodegradable polymers (Vert *et al.*, 1991). These polymers have become increasingly important in the development of drug delivery systems (Couvreur *et al.*, 1995; Vert *et al.*, 1998). They function either as a matrix to control degradation of the drug, followed by polymer degradation and elimination of degradation products from the body, or they can participate in and control the rate of drug release by polymer hydration and degradation (Dunn *et al.*, 1988). Many polymers have been evaluated for their use as biomedical devices e.g. polylactic acid (PLA), polyhydroxybutyrate, polyanhydrides, polyorthoesters, and poly alkylcayanocrylates. The most widely investigated biodegradable polymers, and indeed the systems achieving the most success at the present time are the lactic/glycolic acid (PLGA) polymers.

1.9.1 Poly(lactic Acid) and Poly(glycolic Acid)

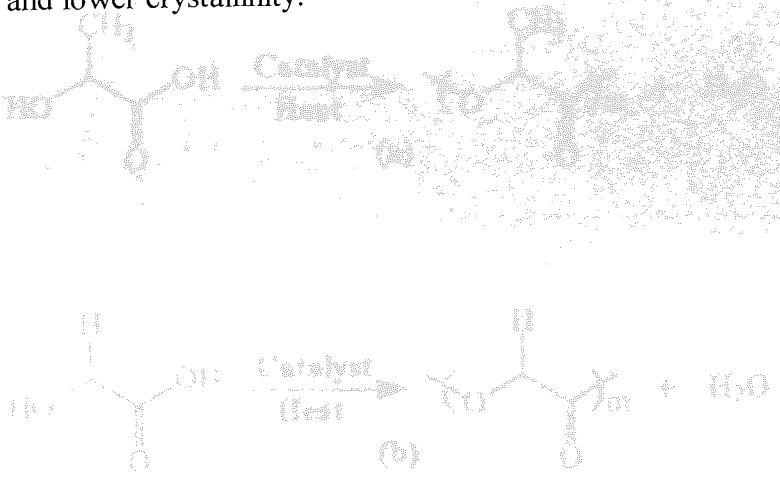
PLGA polymers are homo- and copolymers of lactic acid and glycolic acid. These polymers are generally classified as polyesters, aliphatic polyesters, poly (α-hydroxy acids), or polylactones. PLGA polymers are biodegradable, biocompatible, and bio-absorbable, and in general they do not cause inflammatory reactions in tissues. Their biodegradation products are non-toxic, non-carcinogenic, and non-teratogenic (Jalil and Nixon, 1990). These polymers are easily processed, have good mechanical properties, and are sterilisable. Rates of biodegradation and mechanical properties can be custom-tailored by changing polymer composition (i.e., the copolymerization ratio of lactic acid to glycolic acid) and/or molecular weight (Mw).

In the early 1970s, Ethicon, a Johnson and Johnson company, developed a suture using the copolymer of glycolide and lactide; Vicryl ™(Schneider *et al.*, 1972). The acceptance of PLGA copolymers as sutures made them attractive for a variety of medical and pharmaceutical applications, including wound closure, dental repairs, fracture fixation, ligament reconstruction, and drug delivery (Brekke *et al.*, 1986; King *et al.*, 1985; Vert *et al.*, 1984). PLGA polymer systems have received FDA approval and are in commercial use for the treatment of advanced prostatic cancer and endometriosis. Products include Lupron Depot® (leuprolide acetate), Zoladex®, Zoladex LA® (goserelin acetate), Decapeptyl® (triptorelin), Parlodel LA® (bromocriptine), Suprefact® (buserelin) and Prostap® (leuporelin).

Unlike glycolic acid (GA), which contains only symmetric carbon atoms, lactic acid (LA) has one asymmetric carbon atom and is thus a chiral molecule that exists as two optical isomers, D-lactic acid and L-lactic acid. When two lactic acid molecules combine to form the cyclic dimer lactide, three possible stereoisomers can be formed, D-lactide, which is composed of two D-lactic acid moieties; L-lactide, consisting of two L-lactic acid moieties; and meso-lactide. In addition, an equimolar mixture of D-Lactide and L-lactide forms D,L-lactide (Benicewicz *et al.*, 1991). The lactide isomers give rise to four morphologically distinct polymers: poly (D-lactic acid), and poly(L-lactic acid), which are the two stereoregular polymers; and poly(meso-lactic acid) and poly(D,L-lactic acid),

which are racemic polymers. The polymers derived solely from the optically active D-lactide or from the L-lactide are semi-crystalline, while those derived from the optically inactive meso-lactide or D,L,-lactide are amorphous. Crystallinity is lost in the racemic poly(lactic acid) homopolymers and copolymers of lactic acid and glycolic acid due to the irregularities in the polymer chains. Increased irregularities in polymer chains lead to an increase in the rate of polymer hydration and hydrolysis (Gilding *et al.*, 1979; Reed *et al.*, 1981).

LA is more hydrophobic than GA due to the presence of an extra methyl group. Therefore, LA-rich polymers are more hydrophobic, absorb less water, are more resistant to hydrolytic attack, and degrade more slowly. However, the LA polymers are more soluble in organic solvents than GA polymers because of their greater hydrophobicity and lower crystallinity.



1.9.1.1 Synthesis of PLGA Polymers

Using the property of molecular weight, PLGA polymers can be categorised into low Mw polymers (≅3,000 Da) and high Mw polymers (>10,000 Da). It is relatively easy to synthesise the low molecular weight PLGA polymers. They can be synthesised directly from lactic acid and glycolic acid by condensation polymerisation (Kulkarni *et al.*, 1971; Hutchinson *et al.*, 1985). Figure 1.5 illustrates the chemical reactions for the synthesis of the low Mw PLGA polymers by this method. A high temperature of around 130°-190° C is usually required for the synthesis of such polymers, and a catalyst such as antimony oxide is sometimes used (Avgoustakis *et al.*, 1991).

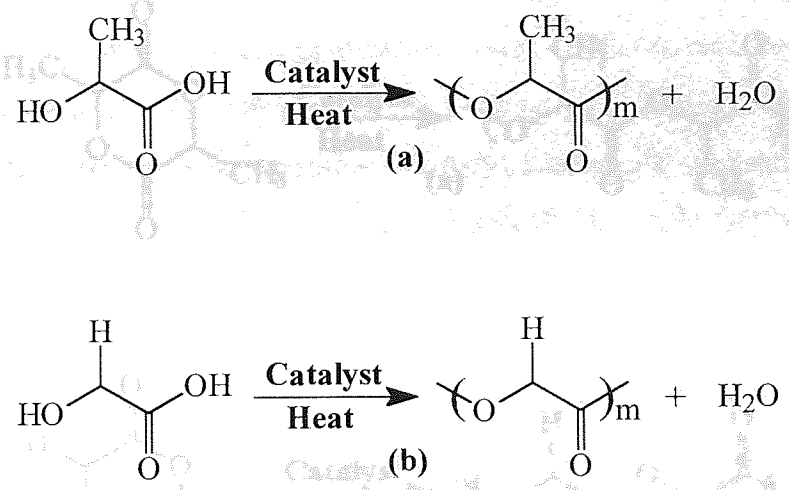


FIGURE 1.5 Formation of PLGA Polymers: (a) from lactic acid; (b), from glycolic acid.

The physicochemical properties of the low Mw polymers limit their biomedical applications, e.g. their mechanical strength is quite low and their degradation is very rapid. These polymers are not suitable for applications in which high mechanical strength is required for the whole duration of usage. They are mainly used in drug delivery systems, where they serve as drug matrices that do not have a high mechanical strength requirement (Sakakura *et al.*, 1992; Wada *et al.*, 1991; Yoshikawa *et al.*, 1988). The preferred method of synthesis for high Mw polymers, is the ring opening

polymerisation of the cyclic diesters (lactides and glycolides) using antimony, lead or tin catalysts (see figure 1.6) (Rak *et al.*, 1985; Marcotte *et al.*, 1989). The copolymer with glycolic acid can involve various molar ratios of monomer components and this controls the degree of crystallinity. PLGA copolymers containing less than 70% of the glycolide are amorphous (Gilding and Reed, 1979). The molecular weight of the polymers can be controlled by the choice of adequate polymerisation methods. The advantages of the ring opening polymerisation method include the fact that there is no water removal or dehydration needed in the polymerisation system. Also, the cyclised monomer(s) and linear forms of the polymer are sufficiently different in physical properties to allow ready purification.

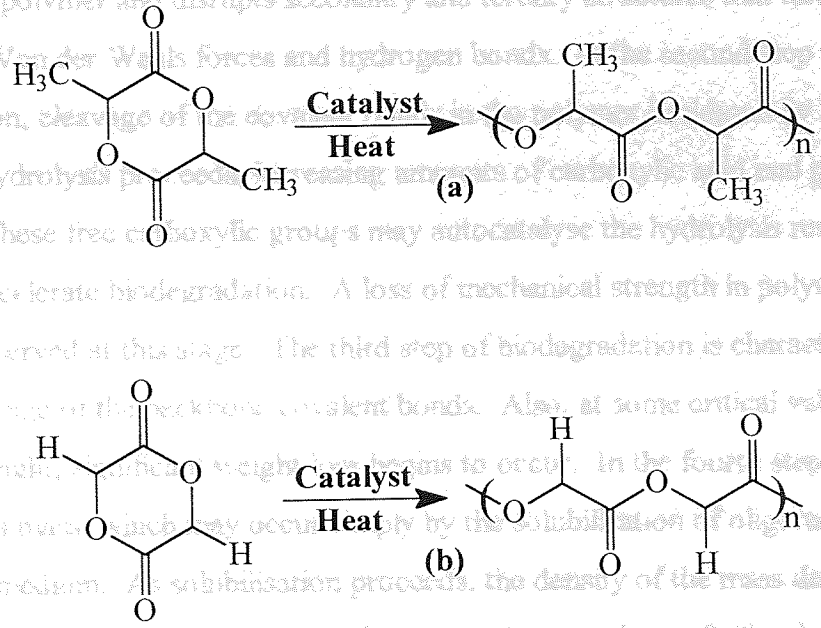


FIGURE 1.6 Formation of PLGA Polymers: (a), from lactide; (b), from glycolide

1.9.1.2 Degradation of Lactic/glycolic acid Polymers

PLGA polymers undergo degradation in an aqueous environment. This hydrodegradation occurs through cleavage of the ester linkages, and occurs both *in vitro* and *in vivo* (Spenlehauer *et al.*, 1989). The kinetics of hydrolysis are strongly influenced by many factors including Mw, pH, temperature, surface structure, crystallinity, and the

presence of impurities or additives. High molecular weight polymers degrade to lower molecular weight polymers, and lower molecular weight polymers degrade with an immediate weight loss. Degradation of the polymer is generally biphasic, initially a decrease in Mw of the polymer occurs due to random chain scission, which is accompanied by water loss. The enhanced water uptake increases porosity and soluble monomeric and oligomeric products can be detected, these degradation products leave the bulk polymer through the various channels and holes resulting in weight loss of polymer (Hutchinson *et al.*, 1985; Vert *et al.*, 1994).

Events responsible for the biodegradation of PLGA polymers have been described in terms of four steps (Kumar *et al.*, 1987). In the first step, a polymer absorbs water and undergoes some swelling (Ginde *et al.*, 1987). The water penetrates into the amorphous region of the polymer and disrupts secondary and tertiary structures that have been stabilised by Van der Waals forces and hydrogen bonds. In the second step of biodegradation, cleavage of the covalent bonds in the polymer backbone by hydrolysis begins. As hydrolysis proceeds, increasing amounts of carboxylic acid end groups are generated. These free carboxylic groups may autocatalyse the hydrolysis reaction, which will accelerate biodegradation. A loss of mechanical strength in polymeric devices is observed at this stage. The third step of biodegradation is characterised by a massive cleavage of the backbone covalent bonds. Also, at some critical value of molecular weight, significant weight loss begins to occur. In the fourth step, the polymer loses mass, which may occur simply by the solubilisation of oligomers into the surrounding medium. As solubilisation proceeds, the density of the mass decreases until the polymer disappears. Any polymeric fragments that remain are further hydrolysed into the free acids.

Degradation produces lactic and glycolic acid monomers, and carboxylic acid. Figure 1.7 depicts a suggested *in vivo* fate of these products (Makino *et al.*, 1985). LA is a natural metabolic product in all higher animals. It enters the tricarboxylic acid cycle, and is metabolised and eliminated from the body as carbon dioxide and water (Wang *et al.*, 1990). GA is a component of several neutral mucopolysaccharides in animal tissues. It can be excreted unchanged by the kidneys or enter the tricarboxylic acid cycle and be eliminated as carbon dioxide and water (Wang *et al.*, 1990).

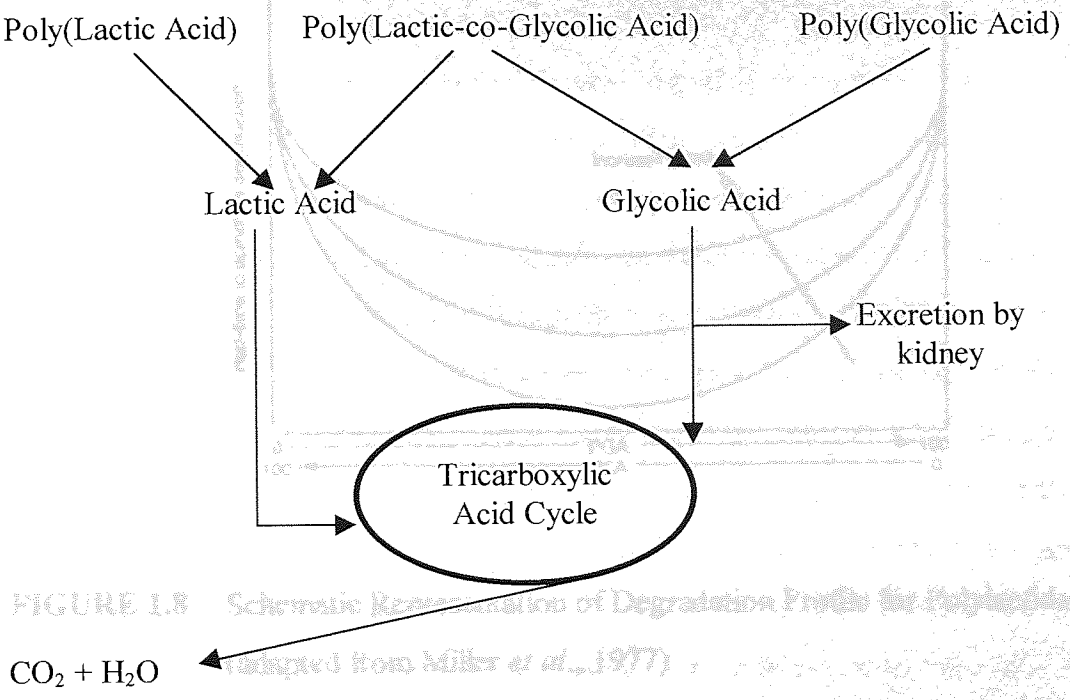


FIGURE 1.7 Metabolism and Excretion of Biodegradation Products of PLGA Polymers *in vivo*.

The rate of hydrolysis of the copolymer increases with increasing glycolide content and reaches a maximum when the lactide glycolide ratio reaches 50:50 (Miller *et al.*, 1977). The reduction in polymer hydrophobicity when the lactide content is reduced from 100% to 80% results in an increased hydrolysis rate (Floy *et al.*, 1993) (figure 1.8). Most hydrophobic and crystalline polymers exhibit a relatively slow degradation rate, related to the low hydration degree in microspheres, related to the water accessibility to the hydrolytically unstable ester linkages in the polymer backbone (Park, 1994).

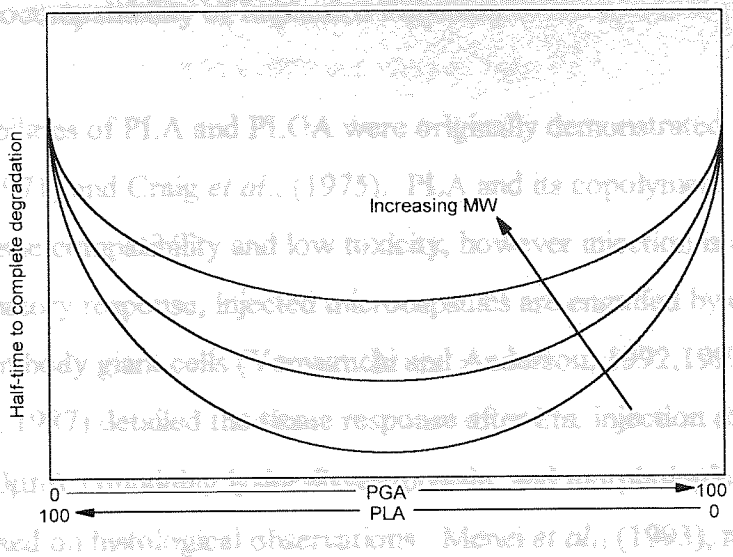


FIGURE 1.8 Schematic Representation of Degradation Profile for Polylactides (adapted from Miller *et al.*, 1977)

The hydrophobic nature of LA causes a steric hindrance to attack by water molecules, and thus the LA moieties are less hydrolytically labile than GA moieties, and therefore degrade faster compared to the former. Biodegradability increases as molecular weight decreases (Sanders *et al.*, 1986; O'Hagan *et al.*, 1994). One reason for this is that the lower molecular weight polymers are more hydrophilic than the corresponding high molecular weight polymers, due to a higher percentage of end-groups. This increasing hydrophilicity permits a more rapid influx of water into the polymer matrix, which speeds up the process of biodegradation. Another contributing factor may be that lower Mw polymers posses more carboxylic end groups that catalyse the biodegradation (Fukuzaki *et al.*, 1991). Polymer degradation is accelerated in both strongly alkaline and acidic media (Makino *et al.*, 1985; Chu, 1981, 1982). The release profile of a drug depends, in part, on the distribution of the drug (Beck *et al.*, 1985). The biphasic degradation process typically results in a triphasic release of entrapped drug from the matrix (Sanders, *et al.*, 1984; Ogawa *et al.*, 1988).

1.9.2 Tissue Biocompatability of Implanted Polymers

The biocompatabilities of PLA and PLGA were originally demonstrated using sutures by Cutrigh *et al.*, (1971) and Craig *et al.*, (1975). PLA and its copolymers have demonstrated tissue compatibility and low toxicity, however injection is accompanied by an acute inflammatory response, injected microcapsules are engulfed by connective tissue and foreign body giant cells (Yamaguchi and Anderson, 1992,1993). Visscher *et al.*, (1985, 1986, 1987) detailed the tissue response after i.m. injection of PLGA or PLA microspheres (30μm), containing lysine-8-vasopressin, and morphologic changes in the microspheres based on histological observations. Menei *et al.*, (1993), reported the absence of toxic effects when PLGA microspheres were injected directly into the brain. Even when traces, as few as 10 parts per million, of polymerisation initiators or solvents such as dichloromethane are present, no significant adverse reactions have been detected after many years of experimentation on animals and humans (Vert *et al.*, 1992). The time course for polymer degradation and the tissue returning to normal is dependent on the polymer selected and the inflammatory response is less for amorphous PDLA. Tissue responses to different polymers may be influenced by the administration route used. Following i.p. injection, only small differences in inflammatory reaction occur related to molecular weight (Van Sliedregt, 1992).

The successful clinical use of Vicryl^{Tm}(PLGA 90:10) and Dexon^{Tm} (PGA homoploymer) sutures has proven that PGA containing polymers can be used safely in soft tissue applications. Severe, late-stage foreign body reactions have been associated with the use of PGA rod implants used for fracture fixation (Botsman *et al.*, 1990; Litsky, 1993). These authors infer that since PLA and PGA are in the same general polyester chemical category, all PLA devices can be expected to elicit the same severe foreign body reactions that are well known for PGA devices. It is important to note that while PLA and PGA are chemically similar, they differ in terms of rate of degradation, which may challenge the body's ability to remove and assimilate the degradation products from the implant site. Also, glycolic acid is metabolised by the cell along a metabolic pathway, which is slightly different from the metabolic pathway for lactic acid (Hollinger and

Battistone, 1986; Botsman, 1991). Thus, it would not be unreasonable to suspect that the body would assimilate PGA in a different manner than PLA.

1.9.3 Sterilisation of Polymers

Sterilisation is an important consideration in the production of drug-loaded microspheres based on the PLGA polymers since these products are administered parenterally. The methods commonly used for producing a sterile pharmaceutical product are gamma irradiation, heat sterilisation, ethylene oxide vapour sterilisation, or aseptic production.

Gamma irradiation is a useful technique for the sterilisation of polymeric implants. However, when this technique is used for sterilisation of drug-loaded microspheres of lactic/glycolic acid polymers, the drugs and/or the polymer matrix may be damaged due to decomposition and cross-linking. Consequently the physical and chemical properties, such as molecular weight and tensile strength, may be changed, and also an alteration in colour of microspheres may be observed (Rozema *et al.*, 1991). Evidence has shown that gamma irradiation can break down polymer chains, decrease molecular weight, lower the mechanical strength, increase the biodegradation rate, and change the drug-release profile (Gilding *et al.*, 1979; Spenleheur *et al.*, 1989).

Heat sterilisation, which consists of exposing objects to a high temperature steam environment for a prolonged period of time, is not appropriate for PLGA polymers as these polymers are heat and moisture sensitive. The heat and moisture can cause chemical and physical changes in the drug loaded polymer products. It has been reported that the molecular weight of the PLGA polymers decreases after steam sterilisation (Rozema *et al.*, 1991).

Ethylene oxide sterilisation is an alternative method if the drug and the polymer are not reactive with the vapour of this agent (Yolles *et al.*, 1976). However, ethylene oxide has been found to soften or plasticize some lactic acid polymers. Residuals of this agent in drug delivery devices can be hazardous to the patient, particularly if the polymer is absorbed with time. It has been shown that ethylene oxide is mutagenic and

carcinogenic (Rozema *et al.*, 1991). Dermatologic and allergic reactions, and haemolysis caused by ethylene oxide have also been encountered (Rozema *et al.*, 1991; Landrigan *et al.*, 1984).

Finally, aseptic production is a safe but more expensive alternative. Since the PLGA polymers are soluble in organic solvents, polymer and drug solutions can be filter sterilised. Therefore the drug delivery system (i.e. microspheres) can be formulated in a clean room environment according to current good manufacturing practice regulations (Lewis *et al.*, 1990).

1.10 AIMS AND OBJECTIVES

The use of biodegradable sustained-release delivery systems may obviate the need for repeated administration, by facilitating site-specific delivery of a nucleic acid in a controlled manner to the desired site. The entrapment of ODNs within such polymeric matrix systems may also improve ODN stability, reduce the ODN dose required for efficacy and further reduce toxicity or non-specific effects associated with ODNs.

The initial aim of this report was to formulate and characterise biodegradable polymeric microspheres for the sustained release of antisense ODNs. Studies were conducted to ensure the maximal loading of antisense ODNs within polymer microspheres was achieved. The influence of polymer properties including polymer Mw, copolymer ratio, sphere size, and ODN loading, chemistry and length on release profiles were also investigated to promote the sustained release of ODNs. A sustained release polymer formulation must protect ODNs from degradation, and maintain a pool of intact biologically active ODN for release from the polymer device. It is critical that polymer-entrapped ODNs are protected from nuclease degradation in biological milieu.

As a consequence of the poor cellular uptake and inappropriate localisation of ODNs, the potential of biodegradable polymer microspheres to enhance the exogenous cellular delivery of antisense ODNs was assessed in A431 cells. Fluorescence microscopy was also utilised to determine the cellular uptake and distribution of FITC-labelled ODNs.

Finally, the *in vivo* distribution of antisense ODNs was also evaluated. Firstly the use of biodegradable polymer microspheres as a site-specific delivery system for targeting ODNs to the neostriatum of the rat brain was assessed. Whereas secondly the *in vivo* biodistribution following subcutaneous administration of free, and encapsulated ^{3}H radiolabeled ODNs as a function of time in mice was analysed, using the technique of whole body autoradiography. The results obtained contribute significantly to our understanding of the movement of ODNs *in vivo*.

CHAPTER TWO

GENERAL METHODS AND MATERIALS

The general methods and materials are described in this chapter. Specific details and variations to the standard procedures are outlined in the relevant chapters.

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.

Cell culture reagents and media were purchased from Life Technologies Inc. (Paisley, UK). Tissue culture flasks, multi-well tissue culture plates, 15mL and 50mL polypropylene tubes were purchased from Beckton Dickinson and Company (Plymouth, UK). Disposable pipettes, microcentrifuge tubes, Finnpippette tubes, Finnpippette tips and 2mL Biofreeze vials were purchased from Starstedt (Leicester, UK)

2.2 GENERAL METHODS

2.2.1 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels were set up as described by Sambrook *et al.*, (1989) and Gait *et al.*, (1991). The gels were run for 2 hours at 10 Watts using a Biorad Protean II electrophoresis system and power source (Biorad, Hemel Hempstead, UK), and were cooled throughout to 10°C using a thermostat controlled water circulator (Sarver Instruments, UK).

1000mL stock solutions of a 20% native polyacrylamide gel mixture solution were prepared [200g acrylamide, 6.6g bis-acrylamide and 200mL 5 x TBE (54g tris base, 27.5g boric acid, 20mL 0.5M EDTA (pH 8) to 1000mL with sterile water)]. The gel mixtures were filtered, de-gassed and stored at 4°C in amber glass bottles. Denaturing gels were prepared by the addition of 8M-urea (480g) to the native gel mixtures.

For each gel a 50mL aliquot of gel mixture was polymerised with 600µL ammonium persulphate (10% w/v) and 40µL N,N,N,N,-tetraethylethylenediamine (TEMED). The polymerising gel mixture was poured between two glass plates (20cm x 20cm and 22cm x 22cm), separated by 1mm spacers (Biorad). A 1mm wide comb with slots of 200mm each was inserted between the plates to make sample wells. The gel was left to set for one hour and pre-run at 10 Watts (Biorad power pack and gel tank) for 30 minutes.

The samples to be run on native gels were added in a loading buffer (50mg xylene cyanole and 50mg bromophenol blue to 10mL with 5% v/v glycerol in 1 x TBE). For denaturing gels, samples were added in a loading buffer (8mL formamide, 100mL 0.5M EDTA, 10mg bromophenol blue made up to 10mL with sterile water). 1 x TBE, diluted from a 5 x stock solution, was used as the running buffer.

2.2.2 Autoradiography

On completion of running the polyacrylamide gel, the small plate was carefully removed, the gel covered with Saran wrap, and placed in a hypercassette (Amersham Life Sciences, Amersham, UK) fitted with an intensifying screen. Under dark room conditions, a Kodak HP autoradiograph film was placed over the gel in order to visualise the radioactive samples, the exposure time varied depending on the activity of the samples. Generally, a five-minute exposure time was adequate for samples labelled with fresh ATP. For longer periods of exposure, the cassette was stored at -70°C. Films were developed using freshly prepared Kodak photographic reagents (Sigma, Poole, UK).

2.2.3 Liquid Scintillation Counting

The specific activities of radiolabelled ODNs were determined by scintillation counting. A known volume of sample was added to 10mL Optiphase Hi-safe 3 scintillation cocktail (Pharmacia Wallac, St Albans, UK) and counted for 5 minutes in a Packard 1900TR Scintillation Counter, using the relevant program for ^{32}P isotope activity. Counts were adjusted for background values and the half-life and reference date was programmed into the counter to account for decay during the experimental period. Specific activities were calculated as cpm / mL. For radiolabelled ODNs, typical specific activities in the region of 200,000 cpm / mL were calculated.

2.3 SYNTHESIS AND PURIFICATION OF OLIGODEOXYNUCLEOTIDES

2.3.1 Preparation of the Automated DNA / RNA Synthesiser

The oligodeoxynucleotides used in this thesis were synthesised on an automated DNA/RNA synthesiser (Model 392, Applied Biosystems (ABI), Warrington, UK). Synthesis reagents, columns, and nucleoside phosphoramidites were supplied by Cruachem Ltd. (Glasgow, UK) unless otherwise stated, and were stored under argon atmosphere at 4°C when not in use.

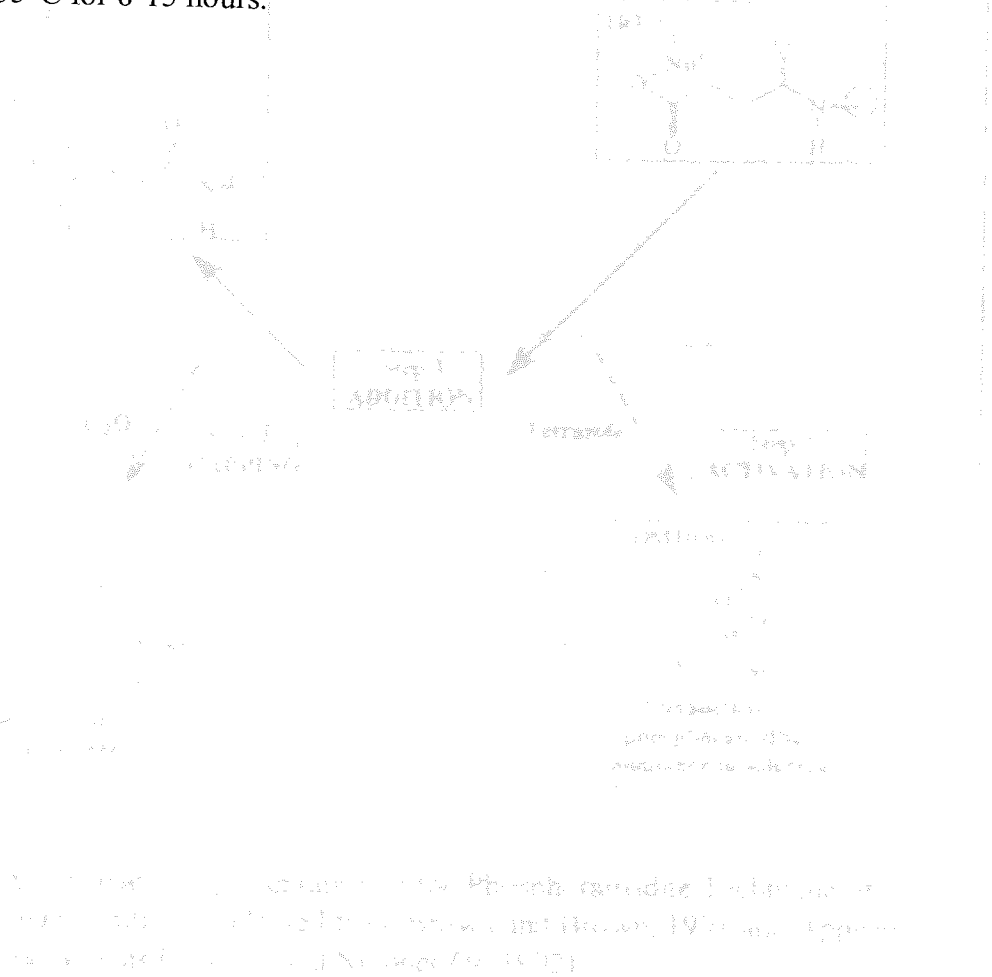
2.3.2 Oligodeoxynucleotide Synthesis

Solid phase phosphoramidite techniques for the automated synthesis of ODNs have been extensively developed (for reviews see Caruthers, 1989; Brown and Brown, 1991). The standard method employed for the synthesis of ODNs involves 2-cyanoethyl chemistry. This method is schematically illustrated in figure 2.1.

The ODNs were synthesised with the 3' end attached to borosilicate CPG support beads, via a succinamide linker. The first step in the synthesis cycle, detritylation, involved removing the dimethyoxyl trityl (DMT) group on the 5' hydroxy group of the support bound nucleotide using trichloroacetic acid (TCA). This resulted in a free 5' hydroxyl, which was able to couple with the next phosphoramadite. The second step, activation, involved the addition of the phosphoramadite nucleoside monomers and tetrazole, a weak acid to the column. The tetrazole protonated the nitrogen of the phosphoramadite making it more susceptible to nucleophilic attack by the 5' hydroxyl of the next nucleoside. This reaction was completed within 30 seconds. The next step, capping, involved the addition of acetic anhydride and 1-methylimidazole to terminate any chains that had not undergone addition. The unreactive chains possessing a 5' OH group were capped by acetylation, hence preventing them playing a part in any further reactions. This procedure minimised the amount of impurities present. The final oxidation step

stabilised the trivalent phosphorous by the formation of the stable pentavalent phosphate triester. Oxidation is achieved with iodine-water-pyridine in basic tetrahydrofuran mixture. At this stage the phosphodiester may be sulphurised to form a phosphorothioate with tetra ethyl thiuram sulphide (TETD).

The synthesis cycle was repeated until the required length of ODN was reached. On completion of synthesis, the solid support material bearing the synthesised ODN was removed from the column. The ODNs were cleaved from the solid support material and the base protecting groups removed by treatment with concentrated ammonium hydroxide, at 55°C for 8-15 hours.



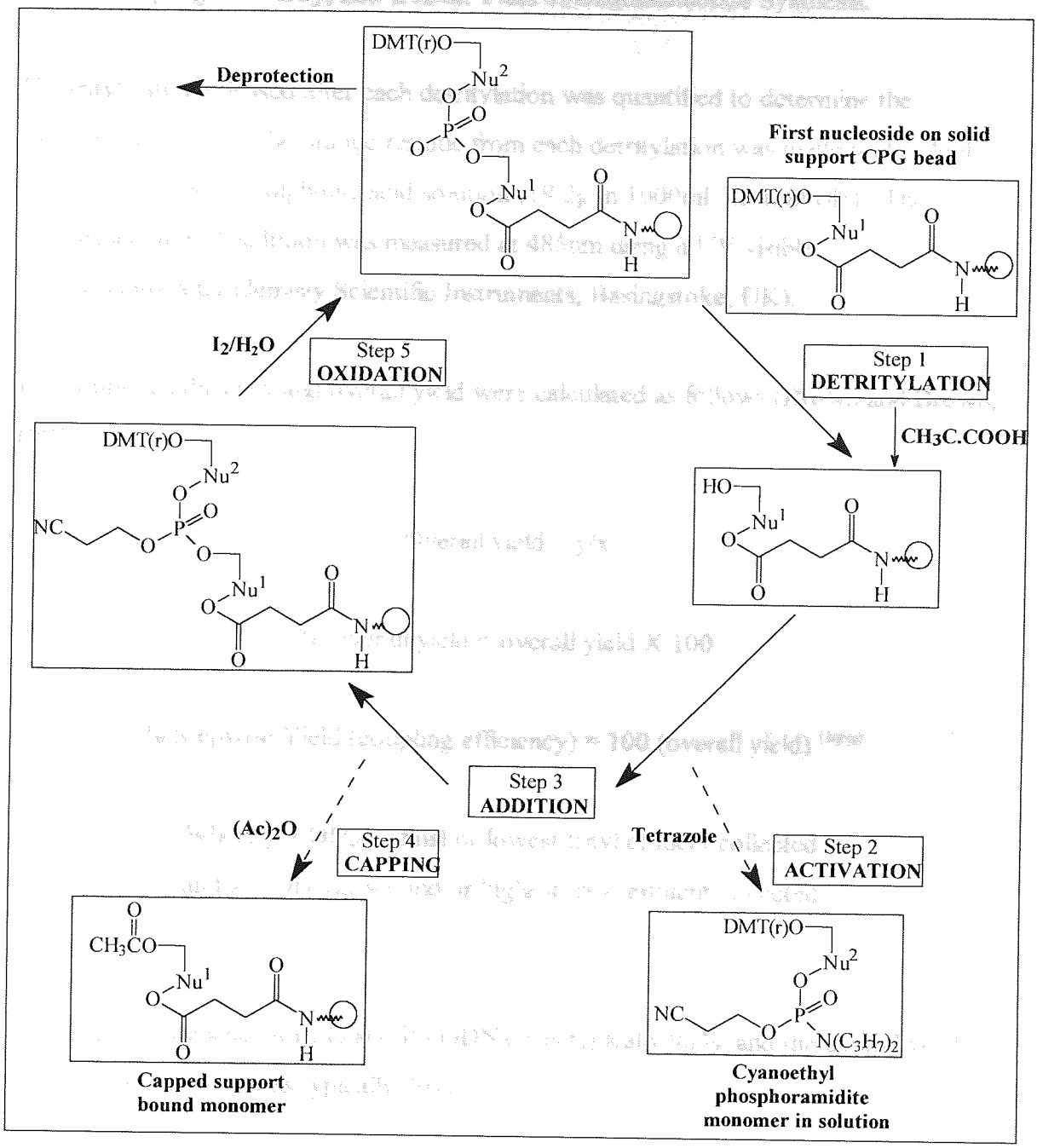


FIGURE 2.1 A Schematic Representation of the Phosphoramadite Technique of ODN Synthesis (adapted from Brown and Brown, 1991 and Applied Biosystems User Manual Number 69. 1992)

2.3.3 Coupling Efficiency, and Overall Yield of Oligonucleotide Synthesis.

The trityl cation released after each detritylation was quantified to determine the coupling efficiency. The orange residue from each detritylation was made up to 25mL with 0.1M p-toluene sulphonic acid solution (19.2g in 1000mL acetonitrile). The absorbance of each solution was measured at 485nm using a UV visible spectrophotometer (Jenway Scientific Instruments, Basingstoke, UK).

The coupling efficiency and overall yield were calculated as follows (Brown and Brown, 1991):

$$\text{Overall yield} = y/x$$

$$\% \text{ Overall yield} = \text{overall yield} \times 100$$

$$\% \text{ Stepwise Yield (coupling efficiency)} = 100\ (\text{overall yield})^{(1/y-x)}$$

Where $y = OD_{495nm}$ final or lowest trityl effluent collected

and $x = OD_{495nm}$ second or highest trityl effluent collected

The coupling efficiency for PO and PS ODNs was typically 98%, and the overall yield for a 20mer PO ODN was typically 70%.

2.3.4 Oligodeoxynucleotide Purification

Following deprotection, ODNs were purified using DNA grade Sephadex G-25 Columns (Nap-10 columns, Pharmacia Biotech, St Albans, UK). The column was washed with 3 x 5mL sterile water, prior to the addition of the ODN solution in a 1mL volume. A further 1.5mL of sterile water was added to the column. The latter fraction containing the purified ODN was collected in a microcentrifuge tube. The eluted ODN

samples were dried by vacuum centrifugation using a Savant DNA Speed Vac (Savant, Basingstoke, UK) and stored at -70°C. This purification procedure removed salts and other impurities such as failed sequences less than 10 bases in length. ODNs were further purified by PAGE (see section 2.5.1).

2.3.5 Quantification of Oligodeoxynucleotides

The amount of ODN was quantified by UV spectroscopy at 260nm (Jenway Scientific Instruments, U.K). The method employed was adapted from Brown and Brown (1991) using OD_{260nm} measurements.

2.3.5.1 Estimation of the Molecular Weight of ODNs (Brown and Brown, 1991):

Mol wt = {(251 x nA) + (245 x nT) + (267 x nG) +(230 x nC) +(61 x n-1) + (54 x n) + (17 x n-1) + 2}

Where; (1) nA the number of adenine bases in the ODN sequence, etc.

(2) n is the total number of bases

(3) (61 x n-1) accounts for the molecular weight of the phosphate groups (this adjustment is (78 x n-1) for PS ODNs)

(4) (54 x n) accounts for the hydration of 3 water molecules per base.

(5) (17 x n-1) accounts for ammonium cations associated with the phosphate groups.

2.3.5.2 Estimation of the Molecular Extinction Coefficient (ε) (Brown and Brown, 1991):

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

Conversion of OD_{260nm} units into milligrams (Brown and Brown, 1991):

$$1mg \approx [(\varepsilon) / (Mol.wt/1000)]\ OD_{260nm} \text{ units}$$

$$\therefore\ 1\ OD_{260nm} \text{ unit} \approx (Mol.wt/1000) / \varepsilon \text{ milligrams}$$

2.4 LABELLING OF OLIGODEOXYNUCLEOTIDES

2.4.1 5'-End [^{32}P] -Radiolabelling

ODNs were labelled at the 5'-end with (^{32}P)-γ-dATP (Supplied by Amersham Life Sciences, Bucks, UK, DuPont NEN and ICN Biomedicals, Hampshire, UK), with a specific activity greater than 185 TBq / mmol at the reference date. The method employed is described by Sambrook *et al.,* (1989), using bacteriophage T4 polynucleotide kinase (Life Technologies and Bioline, Paisley, UK) in 5x reaction buffer (100mM Tris pH 7.5, 20mM $MgCl_2$, 10mM DTT, 0.2mM spermidine and 0.2mM EDTA) at 37°C for 45 minutes.

Approximately 100 picomoles of ODN were labelled in a 20μL reaction mixture of 20 units T4 kinase (Gibco, Paisley, UK), 4μL reaction buffer and 2μL ^{32}P-γ-ATP, to 20μL with double distilled water. Radiolabelled ODNs were purified by PAGE (see section 2.5.1).

2.4.2 3'-End [^{35}S]- Radiolabelling

ODNs were also labelled at the 3'-end with [^{35}S] dATPαS (Amersham Life Sciences, Bucks, UK), with a specific activity greater than 37 TBq / mmol at the reference date. The reaction involving the addition of a dideoxynucleotide at the 3' end was carried out according to the manufacturer's protocol, using calf thymus terminal deoxynucleotidyl transferase (Boehringer Manheim, Germany). Approximately 1 nanomole of ODN was labelled in a 50mL reaction mixture containing 10mL of the supplied reaction buffer (1M potassium cacodylate, 125mM Tris-HCl, 1.25mg/mL BSA, 25mM cobalt chloride), 5mL cobalt chloride (100mM), 3 mL of [^{35}S] dATPαS, and 2mL terminal deoxynucleotidyl transferase. The reaction mixture was incubated at 37°C for 60 minutes, then purified by filtering the mixture through a Sephadex G-25 column (Nap-10 columns, Pharmacia Biotech, St. Albans, UK) as described in section 2.3.4. The eluted fraction was dried by vacuum centrifugation using a Savant DNA Speed Vac (Savant, Basingstoke, UK). This stage was included to ensure removal of potassium cacodylate and any un-incorporated [^{35}S] dATPαS, both of which distort ODN migration by PAGE. Dried ODNs were reconstituted in 40μL glycerol (50% containing 0.1% bromophenol blue and 0.1% xylene blue) and separated using native 20% PAGE (see section 2.5.1).

2.4.3 Internal Oligodeoxynucleotide Radiolabelling

Internal ODN radiolabelling involved a 2 stage process. The ODN to be radiolabelled was synthesised in two halves, (termed primer 1 and primer 2, from the 5'- to the 3'-end). For a 20mer ODN sequence, the ODN was synthesised as two 10mers, and a complementary template sequence was synthesised (see section 2.3.2). Using a protocol developed by Dr.N.Normund (personal communication, Aston University), 10 picomoles of primer 1 which had been previously 5'-end labelled and purified (see sections 2.4.1 and 2.5) was mixed with 10 picomoles of primer 2, and 100 picomoles of the template ODN in 1mL ligation buffer (660mM Tris-HCl pH7.6, 50mm Magnesium Chloride, and 10mm DTT) (Life Technologies and Bioline UK), 0.5mL ATP solution

(10mM ATP in 50mM Tris-HCl pH 7.5) (Life Technologies and Bioline, Paisley, UK) and 5mL Sodium Chloride solution (0.8M). The resulting mixture was heated at 95°C for 5 minutes, and then cooled to room temperature prior to incubation at 4°C for 3 hours. DNA Ligase enzyme (Life Technologies and Bioline, Paisley, UK) was subsequently added to make a final volume of 10mL, with a final 3 hour incubation at room temperature. Internally radiolabelled ODNs were purified by PAGE (see section 2.5.1).

2.4.4 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.

2.5 PURIFICATION OF LABELLED OLIGODEOXYNUCLEOTIDES

2.5.1 Polyacrylamide Gel Electrophoresis

The radiolabelled ODNs were purified by PAGE as described in section 2.2.1. Radiolabelled ODNs were mixed with an equal volume of non-denaturing loading buffer (10mg xylene cyanole, 10mg bromophenol blue, 10% v/v glycerol in 10mL TBE). The samples were run on a 20% native polyacrylamide gel. The position of the purified radiolabelled ODN within the gel was visualised by autoradiography as described in section 2.2.2. The appropriate bands were excised from the gels, and the radiolabelled

ODN were eluted in sterile water, and concentrated by drying under vacuum centrifugation.

2.5.2 Column Purification

As an alternative to PAGE, radiolabelled ODNs were also purified using Nensorb-20® chromatographic cartridge columns (Dupont NEN Research products, Boston, USA) Johnson *et al.*, (1990). This method separates salts and short failed sequences of less than 10 nucleotide bases in length. ODNs of greater than 10 nucleotide bases were eluted from the column using short chain alcohols, which successfully removed the bound ODNs from the column. Initially, the columns were rinsed with 2mL of methanol, followed by the addition of 5mL of 'Reagent A buffer' (0.1M Tris HCl, 1mM EDTA pH 7.4, triethylamine 14mL/10mL). The solutions were pushed through the column in a drop-wise manner using a 20mL syringe. Reagent A (300µL) was added to the labelling sample before drop-wise addition to the column resin bed, and then pushed into the resin using the syringe. The column was further washed with 10mL Reagent A buffer, followed by 5mL sterile water. The column bound ODNs were eluted with 15% methanol, and dried by vacuum centrifugation. The capacity of each cartridge was 20µg of nucleic acid and proteins.

2.6 RIBOZYME SYNTHESIS AND LABELLING

The RBZ sequence used in this thesis was a 36-mer chimeric RBZ containing U4/U7 amino groups in a mostly 2'-O-methyl-modified hammerhead RBZ sequence targeting the 3'-untranslated region (3'UTR) of the human EGFR mRNA. The specific chemical modifications applied to the RBZ consisted of a nuclease-stable, catalytically active hammerhead motif containing 5 ribose residues, 2-amino (2'NH2) modifications at position U4 and U7 with all remaining residues being 2'-O-methyl nucleotides except for a 3'-terminal inverted thymidine residue. The RBZ was synthesised on an automated ABI 394/DNA/RNA synthesizer using phosphoramadite chemistry according to the method of Wincott *et al.*, (1995).

2.6.1 Ribozyme Radiolabelling

For internal labelling RBZs were synthesised in two halves with the junction 5' to the GAAA sequence in loop II. The 3'-half RBZ portion was 5'-end labelled using T4 polynucleotide kinase and [^{32}P] ATP as in section 2.4.1, and then ligated to the 5'-half RBZ portion using T4 RNA ligase (Pharmacia Biotech, Sweden) in 50mM Hepes, pH 8.3, 3mM dithiothreitol, 5mg/mL BSA, and 10mM $MgCl_2$ at 37°C for 2 hours. Following radiolabelling, RBZs were purified by native 20% PAGE (see section 2.2.1). Excised bands were eluted in 0.1% diethyl pyrocarbonate-treated water, desalted on Nap10 columns (Pharmacia Biotech, St Albans, UK), and lyophilized (Savant DNA Speed Vac).

2.7 MICROSPHERE PREPARATION

2.7.1 Polymers

Poly-D,L-lactide-co-glycolide 50:50 PLGA Mw 3,000 (ref RG 502) and Mw 40,000 (ref RG 503) (Boehringer Ingelheim, Germany) were supplied by Alpha Chemicals (Bracknell, U.K.). The polymers were stored in a dessicator at 4°C.

2.7.2 Preparation of Double Emulsion (w/o/w) Microspheres (10-20μm)

The primary emulsion was prepared using an aqueous solution of ODN. A known quantity of radiolabelled ODN (30 picomoles) in water was mixed with unlabelled ODN to give the desired concentration. A 10μL aliquot of 0.4% w/v emulsifying agent polyvinylalcohol (PVA) (87 -89% hydrolysed, Mw 13,000-23,000 kDa) was added to give a final volume of 100uL. The ODN solution was added to 500mg of polymer dissolved in 5mL of dichloromethane, and vortexed for 5 minutes. The resultant emulsion was then emulsified into 80mL of aqueous external phase (saline 0.9% w/v, and PVA 4% w/v), for 3 hours at 1000rpm at room temperature using Heidolph stirrers (Lab-Plant, Huddersfield, UK). The w/o/w emulsion was stirred on a stirring plate, for a minimum of 4 hours to allow for evaporation of solvent. The resulting microspheres were collected by centrifugation at 4,000rpm for 10 minutes (43124-708 rotar, 3000g, Mistral 3,000 centrifuge, MSE Leicester Ltd), and washed three times in sterile distilled water to remove any non-capsulated ODN and emulsifying agent. 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 overnight (Edwards / Modulyo, BOC Ltd, Sussex, U.K.), and stored in a dessicator at room temperature.

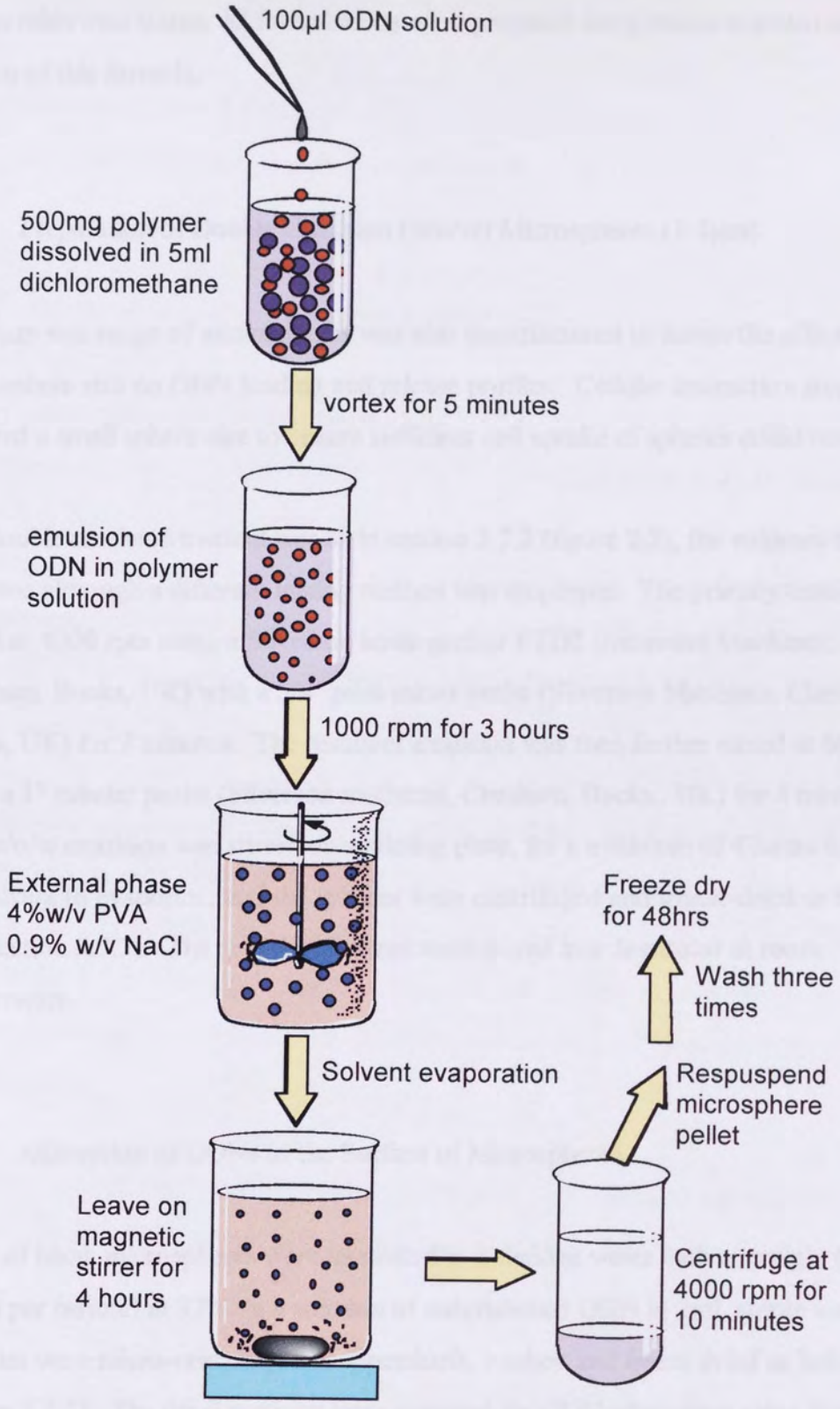


FIGURE 2.2 Schematic Diagram of Double Emulsion Microsphere Preparation

Unless otherwise stated, all formulations were prepared using this or a scaled down version of this formula.

2.7.3 Preparation of Double Emulsion (w/o/w) Microspheres (1-5μm)

A 1-5μm size range of microspheres was also manufactured to assess the effects of microsphere size on ODN loading and release profiles. Cellular interaction studies also required a small sphere size to ensure sufficient cell uptake of spheres could occur.

The double emulsion method was as in section 2.7.2 (figure 2.2), 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 (Silverson Machines, Chesham, Bucks, UK) with a 3/8" mini-micro probe (Silverson Machines, Chesham Bucks, UK) for 2 minutes. The resultant emulsion was then further mixed at 6000rpm using a 1" tubular probe (Silverson machines, Chesham, Bucks., UK) 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 2.7.3). The resulting spheres were stored in a dessicator at room temperature.

2.7.4 Adsorption of ODNs to the Surface of Microspheres

50mg of blank microspheres were incubated in a shaking water bath overnight (100 cycles per minute) at 37°C in a solution of radiolabelled ODN in 2mL sterile water. The particles were micro-centrifuged in eppendorfs, washed and freeze dried as before (see section 2.7.3). The dried particles were assessed for ODN adsorption using liquid scintillation counting as in section 2.2.3.

2.8 CHARACTERISATION OF MICROSPHERES

The yield was calculated from the ratio of the weight of freeze-dried microspheres obtained to the total amount of polymer used in the preparation.

2.8.1 Determination of Oligodeoxynucleotide Loading in Microspheres

Two methods were used to determine ODN loading. The first method involved extraction of ODN from microspheres. The ODN was extracted from the polymer by dissolving 30mg of microspheres in 2mL chloroform, vortexing with 2mL sterile water and then centrifuging. The aqueous layer containing the ODN was removed and concentrated under vacuum centrifugation (Savant UK). The extraction procedure was repeated three times. The concentration of ODN was determined by liquid scintillation counting (see section 2.2.3).

The second method to determine ODN loading in microspheres involved direct liquid scintillation counting of ODN encapsulated microspheres. 30mg of microspheres were added to 10mL of Optiphase Hi-Safe 3 (Pharmacia-Wallac, St Albans, UK) and counted as in section 2.2.3. 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 % w/v ODN encapsulated per dry weight of microspheres could be determined and the encapsulation efficiency calculated.

$$\text{Encapsulation efficiency} = \frac{\text{Actual ODN loading \% w/w}}{\text{Theoretical ODN loading \% w/w}} \text{x } 100$$

2.8.2 *In Vitro* Release Studies from Microspheres

The release of ODNs from microspheres was performed by incubation of 30mg of spheres in 1.5mL release media in glass sample tubes. The release media used was phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCL and 10mM phosphate buffer pH 7.4). The tubes were incubated at 37°C in a shaking water bath. Release of radiolabelled ODN was monitored at intervals over a 28-day period. Samples were taken at hourly intervals on the first day, then daily for the first 7 days, then every 2nd or 3rd day for the following 21 days. At each sampling time, the release media was removed and centrifuged at 13,000rpm for 5 minutes to remove any suspended microspheres. The supernatant was removed for liquid scintillation counting as in section 2.2.3. A 1.5mL aliquot of fresh release media was added to the microsphere pellet, which was re-suspended and replaced into the release vial.

2.8.3 Scanning Electron Microscopy

The surface morphology of the microspheres was examined using scanning electron microscopy. Samples were prepared by mounting on aluminium stubs using carbon tape, and coated with gold for 1.5 minutes under an argon atmosphere (Emscope SC500). Under magnification using a Cambridge Instruments Stereoscan 90B, the surface of the particles was studied. Photomicrographs were taken using a Nikon 35mm camera.

2.8.4 Microsphere Particle Size Determination

10mg of microspheres were redispersed in double distilled, 0.2μm filtered water, and sized by laser diffractometry using a Malvern Mastersizer E particle sizer (Malvern Instruments, Malvern, UK). The instrument was fitted with a 45mm angle lens and a flow cell. The average size of spheres and size range was plotted using a software package (Malvern).

2.8.5 Differential Scanning Calorimetry (DSC)

Thermal characteristics of polymer/microspheres were performed using a Perkin-Elmer DSC-4 system for differential scanning calorimetry, using the Thermal Analysis Data Station (TADS) for data collection. Temperature calibration of the instrument was carried out using an Indium standard. Samples (4-8mg) were sealed into aluminium pans and DSC measurements were run from 25°C to 400°C against an empty pan. All tests were run under nitrogen at a flow-rate of 25cm^3 min^{-1}, heating rate of 10°C min^{-1}, and cooling rate of 320°C min^{-1}. Samples were generally heated over a temperature range of 40°C to 250°C. For quench cooled samples, samples were heated to 200°C, then rapidly cooled to -40°C and reheated from -40°C to 250°C. Temperatures were quoted as those of onset (T_{onset}) or of peak maximum (T_{max}).

2.8.6 Microsphere Surface Charge Determination

The relationship between particle surface charge and phagocytosis is complicated but it is generally recognised that uncharged microspheres display reduced uptake by the RES (Tabata & Ikada, 1990). The surface charge carried by microspheres can be determined by zeta potential measurements, which can be taken as an indirect measure of the surface charge (Muller *et al.*, 1986).

Zeta potential measurements were analysed by laser Doppler anemometry using a Zetamaster (Malvern Zetamaster, Malvern instruments Ltd., Malvern, UK) provided with a photon correlation spectrometer. Approximately 10mg of microspheres were dispersed in 2mL 10mM KCl, and injected into the instrument. Five separate values were collected for each run.

2.8.7 Determination of Residual PVA Content in Microspheres

Residual PVA in microspheres was determined using a modified method derived by Alleman *et al.*, (1993). This method was based on the stable coloured complex formed between iodine and PVA in solution in the presence of boric acid (Finley, 1961).

Approximately 40mg of microspheres were weighed and dissolved in 5mL of chloroform (HPLC grade). The sphere solution was sonicated for 10 minutes and filtered through a cellulose filter (3.0 mm pore size, Whatman), washed with a further 10mL of chloroform and dried. In order to dissolve the PVA on the filter, the filter was boiled in 20mL distilled water. A 1mL aliquot of the PVA solution was added to 3mL boric acid (4% w/v) and 0.6mL iodine solution (1.27% w /v iodine, and 2.5% w/v potassium iodide). The volume was adjusted to 10mL with double-distilled water.

The absorbance of the solutions was measured at 690nm (Philips UV/Vis spectrometer PU 8730). The percentage of PVA remaining was calculated from a calibration curve.

2.9 STABILITY OF POLYMER ENTRAPPED OLIGODEOXYNUCLEOTIDES

Radiolabelled ODNs were encapsulated into microspheres as in section 2.7.2. The microspheres were incubated in release media as detailed in section 2.8.2. The entrapped ODN was extracted from the microspheres after various incubation times over a 28 day period, as described in section 2.8.1. The stability of the extracted ODN was assessed by running the samples on a 20% denaturing polyacrylamide gel as described in section 2.2.1, and visualised by autoradiography as in section 2.2.2. The relevant intensity of single intact bands on the autoradiographs were estimated by scanning laser densitometry using a scanner (Apple Macintosh) and NIH image 1.54, a programme for densitometric analysis of 1-D gels package.

2.10 CELL LINES AND CULTURE TECHNIQUES

2.10.1 A431 Cell Line

A431 epithelial cells are derived from a vulval carcinoma (Freshney, 1973). These were purchased from the European collection of animal cell culture catalogue (ECACC). A431 cells express the epidermal growth factor receptor at levels 10 to 50 fold higher than is seen in other cell lines (Uhlrich *et al.*, 1984), and also express the human transferrin receptor (Gheradi, 1996). The cells were maintained at 37°C, in a 5% CO_2 atmosphere, in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % v/v mycoplasma-screened FCS, 1% v/v penicillin / streptomycin and 2mM L- glutamine (All supplied by Life Technologies Inc, Paisley, UK). Cells were maintained in 75 cm^2 flasks (Falcon, UK) containing 30mL maintenance media. Cells were passaged every 2 days by diluting with fresh media.

The number of cells to be seeded in multiwell cell culture plates was determined using a Nebhauer heamacytometer (as described in section 2.10.3), and diluting to the required concentration. For cell association studies, A431 cells were seeded at 1 x 10^5 cells per

well in 24 well plates (Costar, Cambridge, USA) unless otherwise stated, resulting in 70% confluent cell cultures after 24 hours.

2.10.2 Long Term Storage of Cells

All cell lines were prepared for long time storage by trypsinising a semi-confluent 75cm^2 flask with 2mL trypsin (2% v/v) in PBS / EDTA (0.2% w/v EDTA in PBS, pH 7.2) and neutralising with 10mL DMEM. The cells were pelletted by centrifugation at 1,000 rpm for 3 minutes (Mistral 3000 1 centrifuge, Sanyo MSE, Leicester, UK). The supernatant media was removed, and the cell pellet was re-suspended in 1mL DMEM containing 90% v/v FCS and 10% DMSO in a 2mL screw capped cryo-vial (Costar, Cambridge, USA). The cells were frozen slowly at -70°C before transfer to a liquid nitrogen storage vessel at -196°C. The cells were recovered by rapid thawing at 37°C and gradual dilution with media. The cells were pelletted and resuspended in media before seeding in 25cm^2 flasks.

2.10.3 Determination of Cell Number

The cell density was determined using the counting chamber of a Neubauer haemocytometer (Weber Scientific International Ltd, UK). Cells were removed from culture flasks with 2mL trypsin (2% v/v) in PBS / EDTA (0.2% w/v EDTA in PBS, pH 7.2) and diluted to 10mL with PBS. The cells were immediately pelletted by centrifugation at 1,000rpm for 3 minutes (Mistral 1 centrifuge). The supernatant was removed and the cells were re-suspended in 10mL of media. The counting chamber of the haemacytometer was filled with a small aliquot of cell suspension. The haemocytometer was placed under the light microscope and the cell number was determined by obtaining the mean count per large square on the haemocytometer. The mean count value indicated the number of cells x 10^4 present in the sample per mL.

2.10.4 Determination of Viable Cell Number by Trypan Blue Exclusion Assay

Cell viability was measured using a trypan blue exclusion assay. To perform this assay 100μL of trypan blue (4mg/mL) was mixed with 400μL of re-suspended, trypsinised cells and counted as described in section 2.10.3. Live cells exclude trypan blue, whereas non-viable cells are stained by trypan blue, hence the two can be distinguished when viewed under the microscope.

The mean total amount count of viable cells per square and the total mean cell count [viable + non-viable (blue)] can be used to calculate the number of viable cells per mL and the percentage of viable cells:

Viable cells per mL = mean viable count per square x 10^4 x 1.25 (dilution factor)
% Viable cells = mean viable cell count / total cell count x 100

2.11 CELL ASSOCIATION STUDIES

2.11.1 Cell Association of Free Oligodeoxynucleotides

Cells were seeded at the required concentration in 24 well plates as described in section 2.10.3. Radiolabelled ODNs were diluted to the required concentration in serum free media, unless otherwise stated. Following incubation, cells were washed with 1mL sterile PBS (37°C) per well. The PBS was removed, and ODN added in serum free culture media. Cells were incubated at 37°C for the required length of time, unless otherwise stated. Following incubation, the apical serum-free media was removed from the cells and collected. The cells were further washed with ice cold PBS / azide (0.05%w/v sodium azide in PBS) and washings collected. Cell monolayers were solubilised by the addition of 1mL triton-X 100 (1% in distilled water) for 2 hours at 37°C. The cell suspension was collected, and the wells further washed twice with 1mL triton-X100 to remove any remaining cells. The cell suspension and triton-X100 washes were pooled together.

The quantity of radiolabelled ODNs in each of the following fractions, apical media, PBS washes and cell suspensions were assessed by liquid scintillation counting using a Packard 1900TR Scintillation Counter. Samples were collected in scintillation vials, and 10mL of Optiphase Hi-safe 3 (Pharmacia-Wallac, St Albans, UK) added. Samples were counted for 5 minutes using the relevant ^{32}P isotope activity program. The half life and reference date of ^{32}P radionucleotide were used to account for decay during the experimental period. Counts per minute were recorded.

2.11.2 Cell Association of Microspheres

The method used was the same as given above. Microspheres were suspended in serum free culture media, and added to the cell monolayers at required concentrations in a total volume of 0.5mL. Following required incubation periods, apical media was removed, and cell monolayers washed with PBS /azide to ensure all non-associated spheres were

removed from the cell surface. Cell monolayers were solubilised as outlined previously using triton-X100 (1% in distilled water). Liquid scintillation counting was used to quantify radiolabelled ODNs present in each fraction.

2.11.3 Efflux of Oligodeoxynucleotides from cells

The efflux of radiolabelled ODNs from cells was assessed. Routine cell associations were performed as in section 2.11.1. Following removal of the apical supernatant media and PBS /azide washes, serum free media (warmed at 37°C) was added to the cell layer, and incubated at 37°C for a further 30 minutes. Following incubation the apical media was removed and placed into a separate scintillation vial, along with the PBS / azide washes. This procedure was repeated at defined time intervals after the initial incubation period. Finally, the cells were solubilised as in section 2.11.1 and placed in separate vials. The quantity of radiolabelled ODN in each efflux sample was assessed by liquid scintillation counting, as in section 2.2.3.

2.11.4 Efflux of ODNs / ODN loaded Microspheres from cells

The method used was the same as given above. Cell association of cells with microspheres was performed as in section 2.11.2. Following removal of the apical supernatant media and PBS /azide washes, serum free media (warmed at 37°C) was added to the cell layer, and incubated at 37°C for a further 30 minutes. Following incubation the apical media was removed and placed into a separate scintillation vial, along with the PBS / azide washes. This procedure was repeated at defined time intervals after the initial incubation period. Finally the cells were solubilised as in section 2.11.1 and placed in separate vials. The quantity of radiolabelled ODN in each efflux sample was assessed by liquid scintillation counting, as in section 2.2.3.

2.11.5 Assay to Determine The Effect of PBS / Azide washes

A cell association study was conducted as described in section 2.11.1 to assess the number of PBS / azide washes necessary to ensure all loosely bound ODN was removed from A431 cell surfaces. Following incubation of radiolabelled ODN, the apical media was removed and several PBS / azide (0.05 % w/v sodium azide in sterile PBS) washes were performed. Each wash was collected in separate scintillation vials. The quantity of radiolabelled ODN in each wash sample was detected separately in order to estimate the number of washes necessary to remove all loosely bound ODN.

2.11.6 Cell Association of Radiolabelled Mannitol

Mannitol is known to enter cells by fluid phase endocytosis (Luby-Phelps, 1989) and hence it is a well established marker for this process. Cell association studies were performed as in section 2.11.1, however ODNs were replaced with D-[^{14}C] Mannitol (Amersham Life Sciences, Amersham, Bucks, UK). The quantity of D- [^{14}C] Mannitol in each fraction (apical media, PBS / azide washes and cell suspension) collected was assessed by liquid scintillation counting, using an appropriate program for the detection of ^{14}Carbon as in section 2.2.3.

2.11.7 The Effect of Temperature on Cell Association of Oligodeoxynucleotides

The effect of temperature on cell association of ODNs was assessed at 37°C and 4°C. Standard cell association studies were performed as in section 2.11.1 at 37°C. At 4°C, following the growth period, the cells in 24 well plates were incubated in serum free media in the refrigerator for 15 minutes. The media was removed and radiolabelled ODN in serum free media (4°C) was added to each sample well and incubated for the required time at 4°C. Following this, the standard cell association protocol (section 2.11.1) was performed.

2.11.8 The Effect of Metabolic Inhibitors on Cell Association of Oligodeoxynucleotides

To assess the effect of metabolic inhibitors on the cell association of ODNs, cells were pre-treated with sodium azide and 2'-deoxy-glucose, which reduce cellular ATP production by greater than 60%, as described by Wu-Pong *et al.*, (1992). Sodium azide is an inhibitor of the enzyme cytochrome oxidase, whereas 2'-deoxy glucose is a glycolytic inhibitor.

Cells were seeded in 24-well plates, and following initial washing with sterile PBS, cells were pre-treated for 30 minutes at 37°C with serum free media containing the metabolic inhibitors (10mM sodium azide and 50mM 2'-deoxy-glucose). The media was removed and radiolabelled ODN in serum free media containing the inhibitors was added to the wells, and cell associations were conducted as described in section 2.11.1.

2.11.9 The Effect of Competitors on the Cell Association of Oligodeoxynucleotides

Self-competition studies were performed to assess the uptake of a radiolabelled ODN in the presence of an excess of unlabelled ODN of the same type. In competition studies an excess of PS ODN / blank PLGA microspheres was also used to compete with radiolabelled ODNs for cellular association. Experiments were performed as described in section 2.11.1. However, following initial washing of the cells with sterile PBS, cells were pre-treated for 15 minutes with media containing the unlabelled, competing ODN at 37°C. The media was removed, and radiolabelled ODN in serum free media containing an excess of unlabelled competitor was added to the cells. Cells were incubated at 37°C and studies completed as described in section 2.11.1.

2.12 CELLULAR LOCALISATION OF FLUORESCENT LABELLED ODNS AND DEXTRANS

2.12.1 Preparation of Fluorescent Labelled Nucleic Acids and Dextrans

Rhodamine B isothiocyanate-dextran (0.002-0.01 moles RITC per mole of glucose, approximately Mw 10,000) was obtained from Sigma and purified through DNA Grade Sephadex G-25 Columns (Nap-10 columns, Pharmacia Biotech, St Albans, UK) as described in section 2.3.4 to remove any free rhodamine present. Fluorescent labelled dextrans are well characterised markers of fluid phase endocytosis and are known to reside in endosomal / lysosomal vesicles following cell entry (Berlin and Oliver, 1980). 5'-FITC labelled nucleic acids were synthesised and purified as described in section 2.3. Free FITC (mixed isomers, HPLC purified) were obtained from Sigma.

2.12.2 Cell Association of Fluorescent Labelled Probes

A431 cells were seeded onto 8 well plastic chamber slides (Nunc-Gibco, Paisley, UK) and incubated at 37°C for 24 hours. A431 cells were seeded at a density of 3 x 10^4 cells per well. Following incubation, cells were washed with sterile PBS (37°C).

5'-FITC labelled ODNs were diluted to a concentration of 5μM in serum free culture media. The media was added to the cells and incubated for the required time period. For control studies, cells were also incubated with free 5μM free FITC, as for the ODN samples, at the same time periods. After incubation, cells were washed six times with sterile PBS to remove any non-cell associated fluorophores. The cells were fixed using 2% glutaraldehyde (freshly prepared in PBS) for 30 minutes in ice. The fixative was removed and the plastic chamber gasket was separated from the slide. Slides were mounted in glycerol (50%) in PBS containing 1% v/v DABCO an anti-fading agent (Johnson *et al.*, 1982) and a cover slip was added.

2.12.3 Fluorescent Microscopy

Cell associated fluorescence was visualised using a Jenamed fluorescence microscope (Jena Instrumernts, Oberkochen, Germany) and a high-pressure mercury HBO-50 light source (C-Z Scientific, Basingstoke, UK). According to the UV emission spectra of fluorescein and rhodamine (Lansing-Taylor and Salmon 1989), a 510nm wavelength blocking filter was used for the detection of fluorescein and FITC labelled probes, as such a filter would totally prevent any cross-over excitation from rhodamine. For rhodamine dextran detection a 590nm wavelength narrow band blocking filter was used (Holz, 1982). Cells were photographed using an Olympus camera with a Jenamed adapter

2.13 STATISTICS

Unpaired students t-tests, 95% confidence intervals and two-sided P values were calculated using the Instat 2 Statistical Software package (Graph pad Software, San Diego, USA). Tests were performed on cell association data from different experimental populations to determine statistically significant differences between mean values obtained in experiments.

Low P values indicated that experimental populations were unlikely to be sampled from populations with equal mean values. Data sets were assumed to be significantly different when P values below 0.05 were calculated. Unpaired t-tests assumed that data were randomly sampled, that each value was obtained independent of the others, that the populations were scattered according to a Gaussian distribution and that the standard deviations (SD) of the two populations were not significantly different.

CHAPTER THREE

ENCAPSULATION AND RELEASE OF OLIGODEOXYNUCLEOTIDES FROM POLYMER MICROSPHERES

3.1 INTRODUCTION

Biodegradable polymers may be defined as synthetic or natural polymers, which are degraded *in vivo*, either enzymatically or non-enzymatically, to produce biocompatible or non-toxic by-products. These can be further metabolised or excreted via normal physiological pathways. PGA is the simplest linear, aliphatic polyester. Its most characteristic property is its high crystallinity which gives rise to a high melting point and low solubility in organic solvents. PLA is more hydrophobic than PGA, due to the presence of an extra methyl group. These polymers and their copolymers have been proven to be safe for use in humans, and are known to be biodegradable (Cutrigh *et al.*, 1971; Anderson and Shive, 1997). They are currently used and are being further developed for many medical applications, such as drug carriers in controlled drug release systems (Okada and Toguchi, 1995; Couvreur *et al.*, 1995). These polymers are known to degrade by simple hydrolysis of the ester backbone in aqueous environments such as body fluids. Furthermore, the degradation products are ultimately metabolised to carbon dioxide and water or are excreted via the kidneys.

Microspheres are microporous spherical matrix systems where the drug is uniformly dispersed within the polymer. The use of microspheres as carrier systems has been reviewed by Conti *et al.*, (1992), Okada *et al.*, (1995), Crofts and Park, 1998). Microspheres have the advantage of a wide range of potential delivery routes depending on their desired application, and have been fabricated using various methods, including solvent evaporation, solvent extraction, interfacial deposition, and spray drying (Pavanetto *et al.*, 1992). The most widely used method for microencapsulation into PLGA polymers is w/o/w solvent evaporation (Uchida *et al.*,

1996; O'Donnell and M^{c}Ginity, 1997). The w/o/w technique is very popular for preparing microspheres of water-soluble peptides, proteins and other macromolecules; for example, leutinizing hormone derivatives, serum albumin, and tetanus or diphtheria toxoids have been successfully encapsulated using PLA or PLGA polymers (Bodmer *et al.,* 1992; Hanes *et al.*, 1997; O'Hagan, 1998; Johansen *et al.*, 1999). Poorly water-soluble drugs are generally encapsulated using a single o/w emulsion. A typical w/o/w production procedure can be summarised as follows; polymers are dissolved in an organic solvent such as methylene chloride and emulsified into an aqueous drug solution to form a w/o emulsion. This primary emulsion is then re-emulsified into aqueous solution containing an emulsifier, usually polyvinyl alcohol, to produce a multiple w/o/w dispersion. Subsequent evaporation of the organic solvent leads to hardened microspheres that can be filtered and dried. The common process parameters that have to be considered when developing such a procedure are the selection of solvents, the rate of solvent removal, polymer type and concentration, ratio of solvent to aqueous drug solution, emulsifier type, emulsion stability, and mixing conditions of a w/o/w dispersion. The effects of these process parameters on the characteristics of microspheres have been well presented in several review articles (Jalil and Nixon 1990; Watts *et al.,* 1990; Arshady 1991; Nihant *et al.,* 1994).

PLGA is known to undergo homogenous bulk erosion, hence the release of encapsulated drugs from the polymer matrix is often multiphasic. Release occurs due to a combination of several factors including diffusion from the surface, diffusion through pores in the polymer matrix, and polymer erosion or degradation (Anderson and Shive, 1997). The rate of drug release from the polymer microsphere matrix is influenced by the type and composition of polymer used, and also by drug loading (Oh *et al.*, 1999; Urata *et al.*, 1999). Events responsible for the biodegradation of PLGA polymers have been defined as consisting of four steps (see figure 3.1). In the first step, the polymer absorbs water and undergoes a little swelling. The water penetrates into the amorphous region of the polymer and disrupts secondary and tertiary structures that have been stabilised by Van der Waals forces and hydrogen bonds. The initial phase of drug diffusion from the polymer surface can result in a significant burst release due to surface bound or loosely associated drug. The second step of biodegradation is due to the cleavage of the covalent bonds in the polymer backbone as hydrolysis begins. At this stage drug release enters a second, slower phase. The third

step is characterised by the massive cleavage of the backbone covalent bonds. An auto-accelerated, random chain cleavage process occurs throughout the bulk of the polymer matrix, and the polymer is degraded to a lower molecular weight. As the molecular weight decreases, the polymer matrix becomes porous thus facilitating drug release. In the fourth step, the polymer loses mass by the solubilization of oligomers into the surrounding medium. As solubilization proceeds, the density of the mass decreases until the polymer disappears.

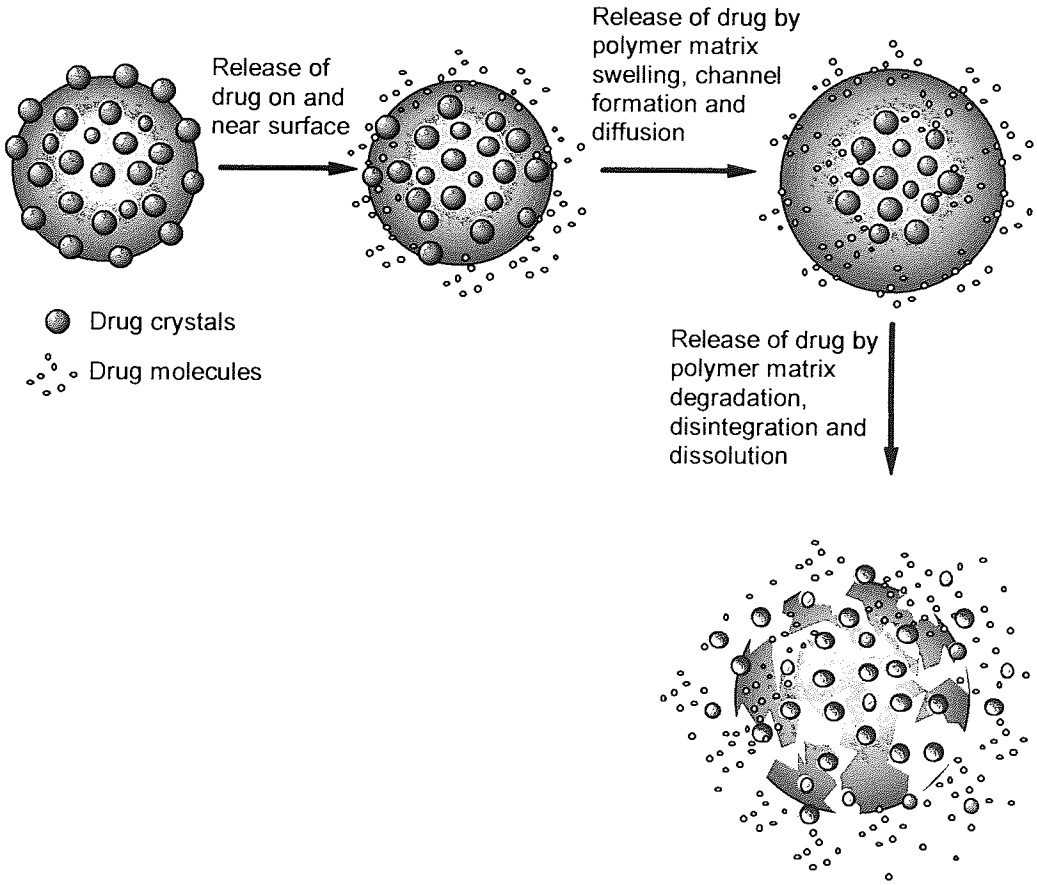


FIGURE 3.1 Schematic Representation of Drug Release from Biodegradable Microspheres

The aim of the work outlined in this chapter was to entrap ODNs into polymer microspheres using a double emulsion w/o/w technique. Microsphere properties were characterised and major consideration was given to ensure maximum loading of ODN per mg of polymer. To increase loading of ODNs the possibility of saturating the aqueous phase in the w/o/w emulsion with salt was also investigated. The influence of

polymer properties including polymer Mw, copolymer ratio, sphere size, and release conditions on *in vitro* release profiles were investigated.

PLGA 50:50 of Mw 3,000 or 40,000 were chosen as polymers for the formulations. These copolymers have short half-lives allowing all the entrapped ODN to be released. The amorphous nature of these polymers results in better sustained release of ODN compared to crystalline polymers, and also more efficient loadings (Cohn *et al.*, 1987).

3.2 PREPARATION OF OLIGODEOXYNUCLEOTIDE LOADED MICROSPHERES

3.2.1 Microsphere Formulation and Loading

3.2.1.1 Double Emulsion Microspheres 10-20μm size range

The double emulsion process is a popular method for entrapping water-soluble drugs into polymer microspheres (O'Donnell and M^{c}Ginity, 1997). Microspheres were prepared as described in section 2.7.2 adapted from Lewis *et al.*, (1996). The microsphere preparations were characterised to assess batch to batch reproducibility, sphere size, surface charge, solvent retention, and ODN loading. For the 10-20μm microsphere size range, this method appeared to produce reproducible smooth, spherical microspheres. Following preparation of microspheres, four washes were adequate to ensure that all loosely associated ODN was removed from the particles. As can be seen from figure 3.2, minimal radioactivity was detected in the fourth wash, hence it may be assumed that all remaining ODN was encapsulated within the microspheres, reducing the probability of a high burst effect.

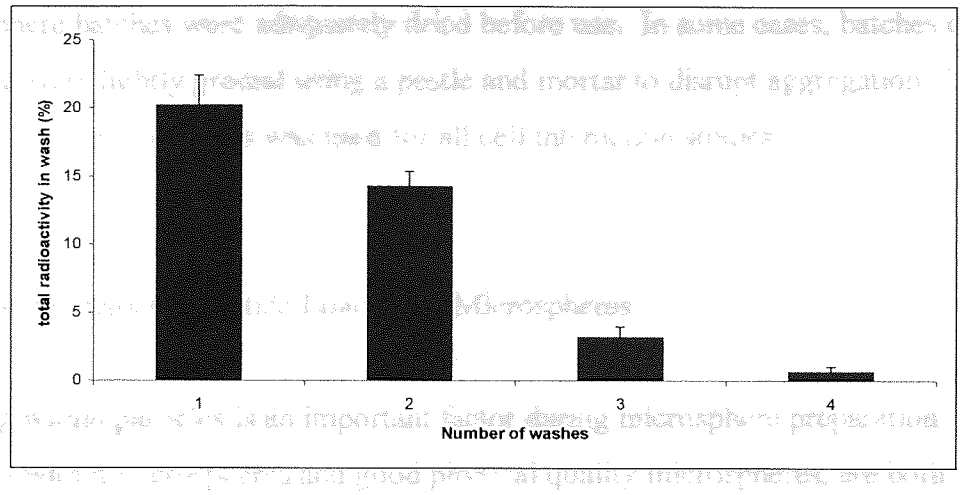


FIGURE 3.2 Number of washes required for the removal of free radiolabelled ODN from the surface of microspheres following fabrication. PLGA Mw 3,000 10-20μm microspheres. (n=4±S.D.)

Particle size is controlled by the speed of mixing (O'Donnell and McGinity, 1997), hence this was maintained at 1000rpm for this method. This method resulted in high ODN loadings with maximal encapsulation efficiency of over 70% being incorporated into the particles.

Other process parameters were investigated to ensure optimal conditions were achieved. Reduction of the internal organic phase led to a decrease in encapsulation efficiency from over 60% to 42% for PLGA particles with a theoretical load of 0.3%w/w. An increase in the internal aqueous phase volume resulted in microspheres of poor morphology with evident pores and cracks.

3.2.1.2 Double Emulsion Microspheres 1-5μm size range

Smaller size microspheres were prepared as described in section 2.7.3. A uniform particle size range was seen, due to the efficient mixing technique employed using the Silverson Homogeniser. A constant mixing speed was achieved, hence giving rise to a narrow size range. Aggregation of spheres was present because the small particle size results in an increase in inter-particle static charge, creating a high degree of attraction.

However, aggregation was minimised using an anti-stat gun, and also ensuring microsphere batches were adequately dried before use. In some cases, batches once prepared were lightly ground using a pestle and mortar to disrupt aggregation. This size range of microspheres was used for all cell interaction studies.

3.2.2 Oligodeoxynucleotide Loading in Microspheres

Loading within particles is an important factor during microsphere preparation. High loadings within microspheres and good physical quality microspheres, are both important properties for successful delivery systems. With increasing primary emulsion viscosity encapsulation efficiency was increased. The combination of vigorous stirring and the mechanical strength from the high viscosity prevents migration of the inner water phase to the external phase (Bodmeier and McGinity, 1988; Ogawa *et al.,* 1988). With the w/o/w technique, the majority of drug loss is seen into the external aqueous phase during preparation, however the organic barrier provided by the organic solvent minimises this (O'Donnell and M^{c}Ginity, 1997).

Saturation of the external phase was undertaken to limit the solubility and entry of water-soluble ODNs into the aqueous external phase. Previous studies showed little effect on ODN loading by increasing sodium chloride (NaCl) concentration from 0.9 to 6.6%w/v (Lewis, 1996). Further attempts to increase NaCl concentration was investigated to saturate the external phase, however above 10%w/v of salt concentration, the NaCl was seen to rapidly precipitate out of solution on cooling. Therefore a nominal concentration of 0.9%w/v NaCl was maintained in the formulation.

The physico-chemical properties of the polymer systems and ODNs used are summarised in table 3.1. It can been seen that PS-modified ODNs could be entrapped with relatively high encapsulation efficiencies (up to about 70%) for large microspheres, but less efficiently (~30%) in small microspheres, which was consistent with earlier findings for unmodified PO ODNs (Lewis *et al.,* 1998). In each case, the production yields were greater than 80%. Table 3.1 shows that changing polymer Mw had no significant difference in loading. In theory, an increase in polymer Mw

increases the viscosity of the polymer dissolved in the organic phase, which can result in an increased droplet size, and hence result in an increased amount of ODN entrapped.

Table 3.1 shows the effect of two different loadings, highlighting the differences between a low loading of 3.34nmoles/mg in comparison to higher loading of 6.63 nmoles/mg. A decrease in encapsulation efficiency was observed for the high loading of 43.1%, due to the increased diffusion of the ODN into the external aqueous phase. The release profiles obtained for these particles (see section 3.3.1) give an indication to the distribution of ODN within the polymer.

TABLE 3.1. Physico-chemical Properties of Antisense (*hpv* target) ODN-Loaded PLGA 50:50 Copolymer Microsphere Delivery Systems

Mw (Da)	PARTICLE SIZE RANGE	NOMINAL LOADING[1]	ENCAPSULATION EFFECIENCY[2]	ACTUAL LOADING	
3,000	10-20μm	0.53 nmoles/mg	62.5%	0.33 nmoles/mg	2.14μg/mg
40,000	10-20μm	1.23 nmoles/mg	66.2%	0.82 nmoles/mg	5.32μg/mg
40,000	10-20μm	15.4 nmoles/mg	43.1%	6.63 nmoles/mg	43.04μg/mg
40,000	10-20μm	4.86 nmoles/mg	68.7%	3.34 nmoles/mg	21.7μg/mg
40,000	10-20μm	1.23 nmoles/mg	66.2%	0.82 nmoles/mg	5.32μg/mg
40,000	1-5μm	2.89 nmoles/mg	27.5%	0.79 nmoles/mg	5.13μg/mg

[1]The amount of ODN per mass of polymer added to the formulation in the primary emulsion phase

[2]The ratio (expressed as a percentage) of the actual amount of ODN incorporated into microspheres to the nominal ODN loading

3.2.2.1 Determination of Range of Loading

Microspheres were prepared using a double emulsion system as described in section 2.7.2. The effect of ODN loading was determined by increasing the concentration of [^{32}P]-labelled 20-mer PS ODN added to the polymer solution in a 100μL volume. ODN was added in the range from 2μg/mg to 200 μg/mg of PLGA Mw 3,000 polymer.

TABLE 3.2 The Effect of ODN Concentration (2-200μg/mg polymer) on Loading in PLGA (50:50) Mw 3,000 Microspheres size 10-20μm. (n=4±S.D.)

Nominal Loading μg/mg	Encapsulation Efficiency (%)	Actual Loading (μg/mg)
2	72.4 ±1.2	1.45
4	73.9 ±2.5	2.96
8	68.6 ± 2.4	5.49
20	63.3 ± 3.2	12.66
40	60.5 ± 1.8	24.2
80	52.4 ± 2.2	41.92
200	23.4 ± 3.6	46.8

Over the concentration range 2μg/mg-200μg/mg polymer studied, actual loading of ODN in the polymer spheres was increased. Up to a concentration of 40μg/mg the percentage loading was still over 60%, further increases in loading were seen with 80μg/mg and 200μg/ mg although encapsulation efficiency was decreased. It is clear that PLGA microspheres are capable of entrapping high amounts of ODN, necessary for achieving sustained delivery over periods of time. Increasing ODN concentrations increases loading but this is limited by saturation of the primary aqueous emulsion phase. With high concentrations of ODN, the viscosity of primary emulsion was increased due to the presence of ODN, resulting in an inadequate mixing and coating of

ODN with emulsifying agent. Higher concentrations of ODN were attempted but microspheres formed were of poor quality, with minimal loading. Further formulation development is necessary if further increases in loadings are required, and this is an opportunity for further work to be conducted.

3.2.2.2 Effect of ODN Chemistry and Length on Loading

The effect of ODN chemistries on loading was determined using equivalent concentrations of unmodified DNA, 2'-O-methyl-modified RNA, and PS ODNs. Microspheres were prepared using a double emulsion system as described in section 2.7.2.

Table 3.3 summarises the physico-chemical properties of the polymer systems and ODNs used. A 36-mer PS ODN was compared to an identical sequence 2'-O -methyl-modified ribozyme, and a 20-mer PS ODN was compared to the same sequence PO ODN. In terms of chemistry, no significant effect on percentage loading of ODN in the polymer microspheres was observed. Varying the ODN length from a 7-mer to a 32-mer, also had no apparent effect on percentage loading, with both longer and shorter sequences being efficiently entrapped within the polymer matrix.

These results showed that neither ODN chemistry nor length affect the entrapment efficiency of the preparation procedure with the variables studied. The distribution of the ODN within the polymer matrix was not evaluated, but the release profiles (see section 3.3.1) may give a greater insight into the distribution of ODN within the polymer. Although no effect on encapsulation efficiency was observed, there may be differences in the drug distribution within the microsphere.

TABLE 3.3. Physico-chemical Properties of ODN and RBZ-Loaded PLGA (Inherent Viscosity 0.2) Copolymer Microsphere Delivery Systems (Particle Size 10-20μm)

CO-POLYMER RATIO	ODN TARGET[1]	CHEMISTRY	ANTISENSE SEQUENCE	NOMINAL LOADING[2] (mg^{-1})	ENCAPSULATION EFFECIENCY	ACTUAL LOADING (mg^{-1})
50:50	–	36-mer PS	5' TGT GTG TCT GAT GAG GCC GAA AGG CCG AAA CTG AAC3'	3μg	68.5%	2.06μg
50:50	EGFR mRNA	36-mer RBZ	5'UGU GUG UCU GAU GAG GCC GAA AGG CCG AAA CUG AAC3'	3μg	63.24%	1.89μg
75:25	*hpv*	20-mer PS	5'GTA CCT GAA TCG TCC GCC AT3'	1μg	58.89%	589ng
85:15	*hpv*	20-mer PS	5'GTA CCT GAA TCG TCC GCC AT3'	1μg	64.44%	644ng
90:10	*hpv*	20-mer PS	5'GTA CCT GAA TCG TCC GCC AT3'	1μg	59.16%	592ng
50:50	*hpv*	20-mer PS	5'GTA CCT GAA TCG TCC GCC AT3'	1μg	68.5%	0.69μg
50:50	*hpv*	20-mer PO	5'GTA CCT GAA TCG TCC GCC AT3'	1μg	73.26%	0.74μg
50:50	*c-myc*	7-mer PS	5'GGG GCA T3'	10ng	60.44%	6.04ng
50:50	*c-myc*	15-mer PS	5'GGT GTC TGA TGA GGC 3'	10ng	63.77%	6.38ng
50:50	*c-myc*	32-mer PS	5'GGT GTC TGA TGA GGC CGT TAG GCC GAA ACC CGC3'	10ng	66.43%	6.64ng

[1]Human Papiloma virus, *c myc*, Human epidermal growth factor receptor mRNA

[2]The amount of ODN per mass of polymer added to the formulation in the primary emulsion phase

[3]The ratio (expressed as a percentage) of the actual amount of ODN incorporated into microspheres to the nominal ODN

3.2.2.3 Effect of Copolymer Ratio on Loading

LA is more hydrophobic than GA due to the presence of an extra methyl group. Therefore lactic acid rich polymers are more hydrophobic, absorb less water, are more resistant to hydrolytic attack, and degrade more slowly. In addition, the LA polymers are more soluble in organic solvents than glycolic acid polymers because of their greater hydrophobicity and lower crystallinity. For the copolymer ratios studied, encapsulation efficiencies ranged from 58.89% to 64.44% (see table 3.3) i.e. no significant differences were observed. Again saturation of loading was not investigated, but the release profiles obtained (section 3.3.1) gives an indication of difference in release from the polymer matrix.

3.3 *IN-VITRO* RELEASE STUDIES

3.3.1 Release of Oligodeoxynucleotides from Double Emulsion Microspheres

The microspheres were made by a double emulsion system as described in section 2.7.2. In order to assess whether ODNs entrapped within microspheres by the double-emulsion method were capable of sustained release from these polymer devices, *in vitro* release profiles were performed in physiological buffer, at 37°C (see section 2.8.2).

The double emulsion method for preparing particles resulted in microspheres with smooth morphology in the size range of 10-20μm. To study inter-batch variation, several batches were made. All batches gave similar encapsulation efficiencies and loadings, resulting in similar release profiles produced from each batch (illustrated in figure.3.3).

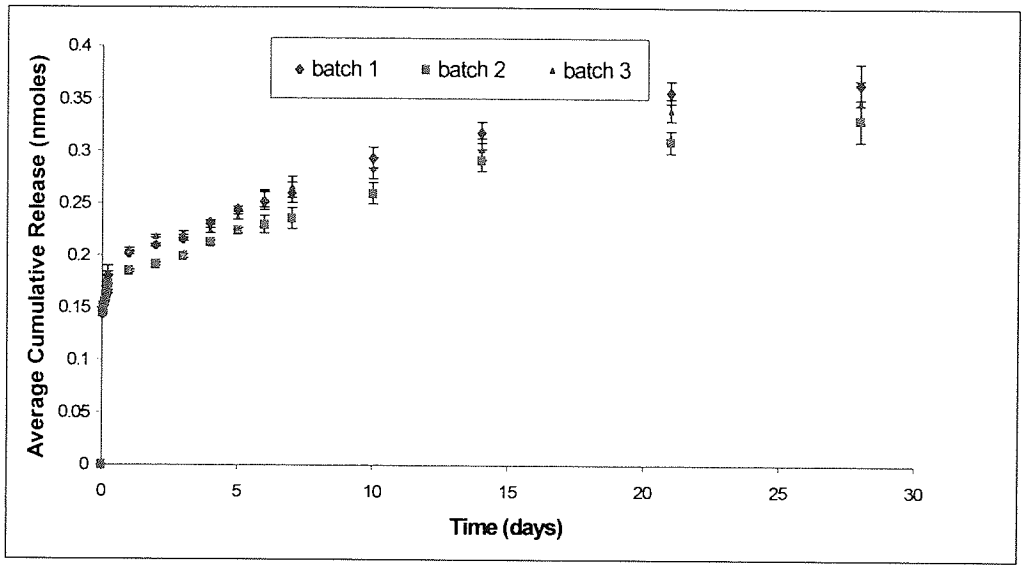


FIGURE 3.3 The effect of batch to batch variation of 20-mer phosphorothioate-modified ODN on average cumulative release (nmoles) from 10-20μm sized PLGA (Mw 3,000) microspheres in PBS, pH 7.4 at 37°C over a 28 day release period. Batch 1, 2 and 3 are identical, independent batches. Loading = 0.47nmoles. (n=4±S.D.)

The following graphs highlight the typical *in vitro* release profiles of phosphorothioate-modified ODNs as a function of drug loading (figure 3.4), particle size (figure 3.5) and polymer molecular weight (figure 3.6).

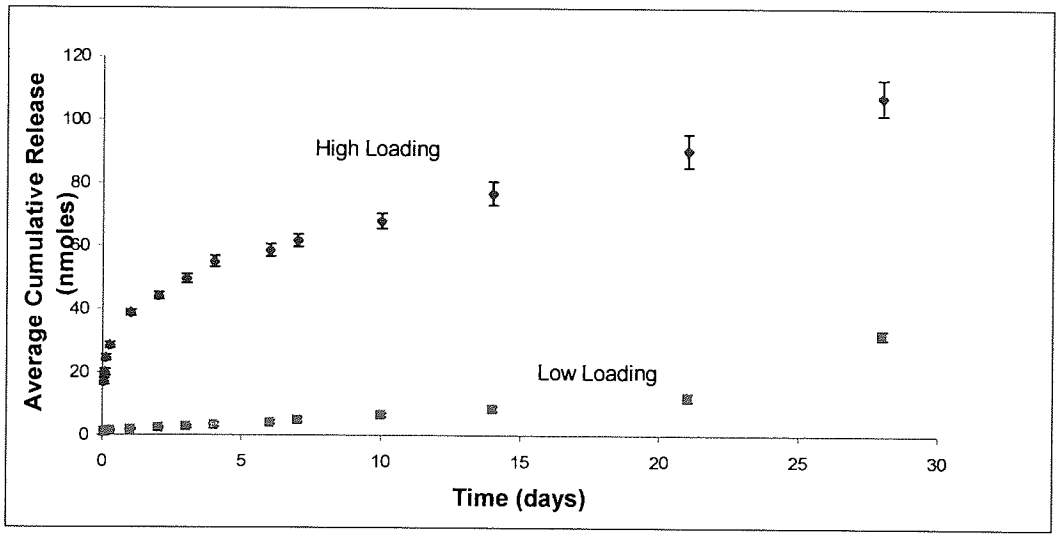


FIGURE 3.4 The effect of loading of a 20-mer phosphorothioate-modified ODN on average cumulative release (nmoles) from 10-20μm sized PLGA (Mw 3,000) microspheres in PBS, pH 7.4 at 37°C over a 28 day release period. High loading = 6.63nmoles/mg of polymer, Low loading =3.34 nmoles/mg of polymer. (n=4±S.D.)

Release from polymer microspheres encapsulated with relatively high concentrations of ODN loading (6.6nmoles/mg), occurred more rapidly than from polymer microspheres entrapping a lower concentration (3.3nmoles/mg). The release profiles obtained for the two loadings (figure 3.3) also exhibited a different shape indicative of the different release mechanisms involved. The microspheres with high ODN loading exhibited an essentially biphasic release profile, where the initial rapid release of about 10% of the dose (the so-called 'burst effect') is followed by a much slower period of "sustained release" from the polymer over a 28 day period. The burst effect (or Phase I) is thought to reflect release of ODN present at, or entrapped close to the surface of the particles. The slower, Phase II of release reflects diffusion of ODN through pores or aqueous channel networks formed within the polymer matrix upon hydration. In the case of the low ODN loaded microparticles the burst effect was not very significant but a third, and relatively more rapid phase of release (Phase III) is evident at the terminal stages of this release profile. This third phase probably represents the onset of bulk degradation of the polymer microspheres liberating more of the entrapped ODN after about 21 days. This result is similar to that reported previously for the release of many different drugs including proteins and anti-cancer agents from these and related biodegradable polymers (Crotts and Park, 1998; Okada and Toguchi, 1995; Ogawa *et al.,* 1988a,b).

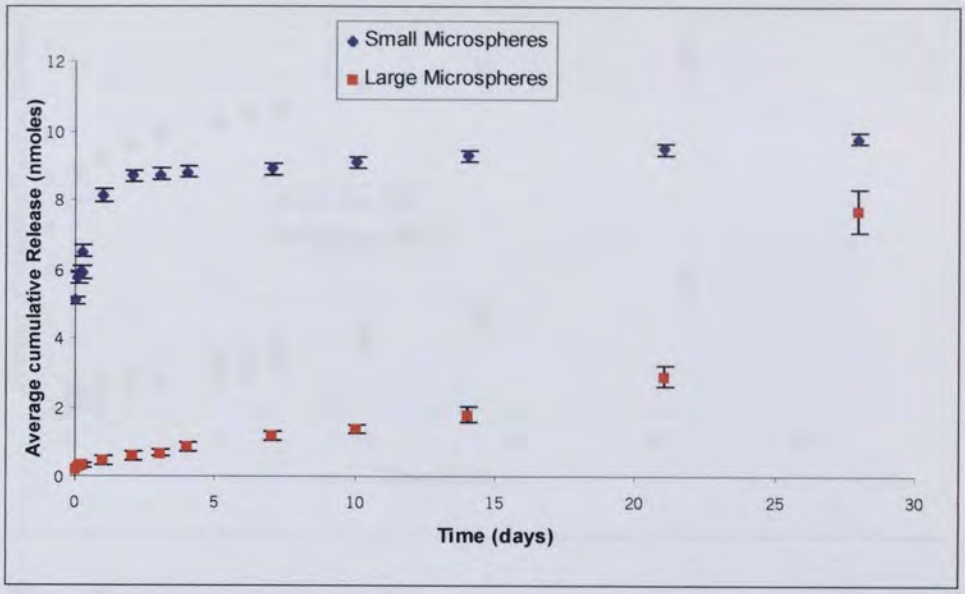


FIGURE 3.5 Comparative release of ODN from small (1-5μm) and large (10-20μm) PLGA (Mw 3,000) microspheres in PBS pH 7.4, at 37°C is shown over a 28 day release period. Small PLGA microspheres (1-5μm) and large microspheres (10-20μm) were loaded with 0.79nmoles and 0.82nmoles of ODN per mg of polymer respectively.

The rate and amount of ODN release was also faster for small (1-5μm) microspheres compared to the large (10-20μm) microspheres when entrapped with a similar low ODN loading, although much of this could be attributed to the significantly greater burst effect with smaller particles (Figure 3.5). This phenomenon has previously been attributed to the greater surface area to volume ratio of the smaller microspheres, which results in a greater contact with the release medium (Lewis *et al.*, 1998). *In vitro* release of ODN from low molecular weight (Mw ~3,000) polymer also exhibited a greater burst effect (approx. 10-15-fold) compared to ODNs entrapped within a higher molecular weight (Mw ~40,000) polymer microsphere. The reason for this is not entirely clear, but may reflect differences in hydrophobicity and degree of crystallinity within these polymer molecular weights having an influence on ODN entrapment and release (Okada and Toguchi, 1995; Pouton and Akhtar, 1996).

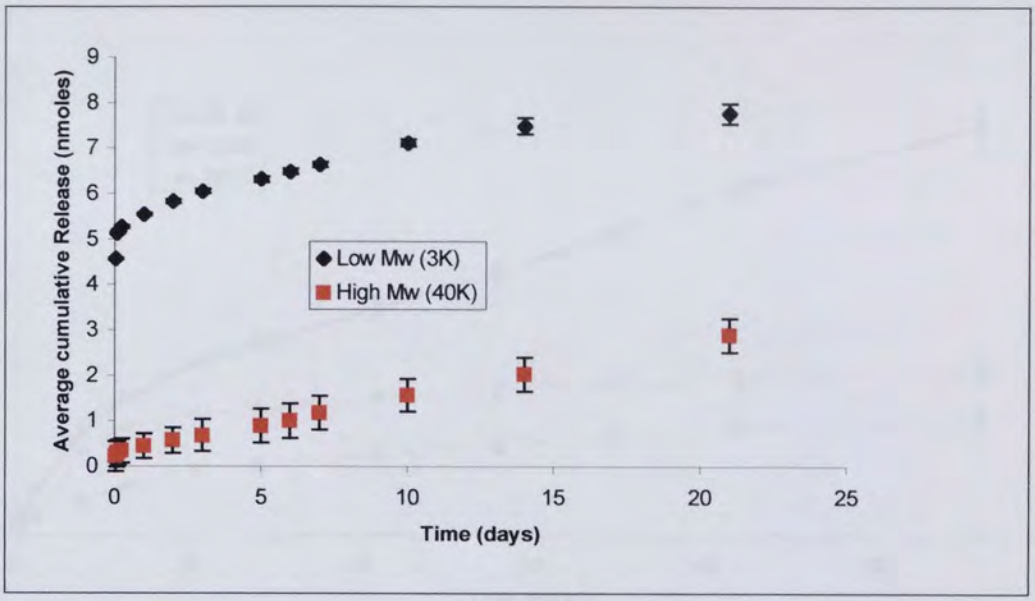


FIGURE 3.6 The effect of polymer Mw. Comparative release profiles of ODN from low weight average molecular weight (Mw 3,000) and high Mw (40,000) microspheres with equivalent ODN loading of 0.33 nmoles/mg were determined in PBS pH 7.4, at 37°C over a 21 day release period. Microsphere size 10-20μm. (n=4±S.D.)

Polymer properties play a crucial role in the release of encapsulated drug from microspheres. Polymer Mw and polymer ratios are important in controlling the rate of release of entrapped drug (Cohen *et al.,* 1991; Park, 1994). Combinations of polymer characteristics are utilised to tailor the release of drugs to the required use. Copolymer ratio plays an important role in controlling degradation of polymer. Slow degradation of polymer can ensure a slow rate of release of entrapped drug is seen. The hydrophobic nature of lactic acid, rendering the polymer more resistant to hydrolytic attack, and also the crystalline nature of these polymers result in a slower degradation profile of the polymer. Therefore microspheres produced from copolymer ratios with high lactic acid composition are seen to release entrapped drugs at a much slower rate (see figure 3.7).

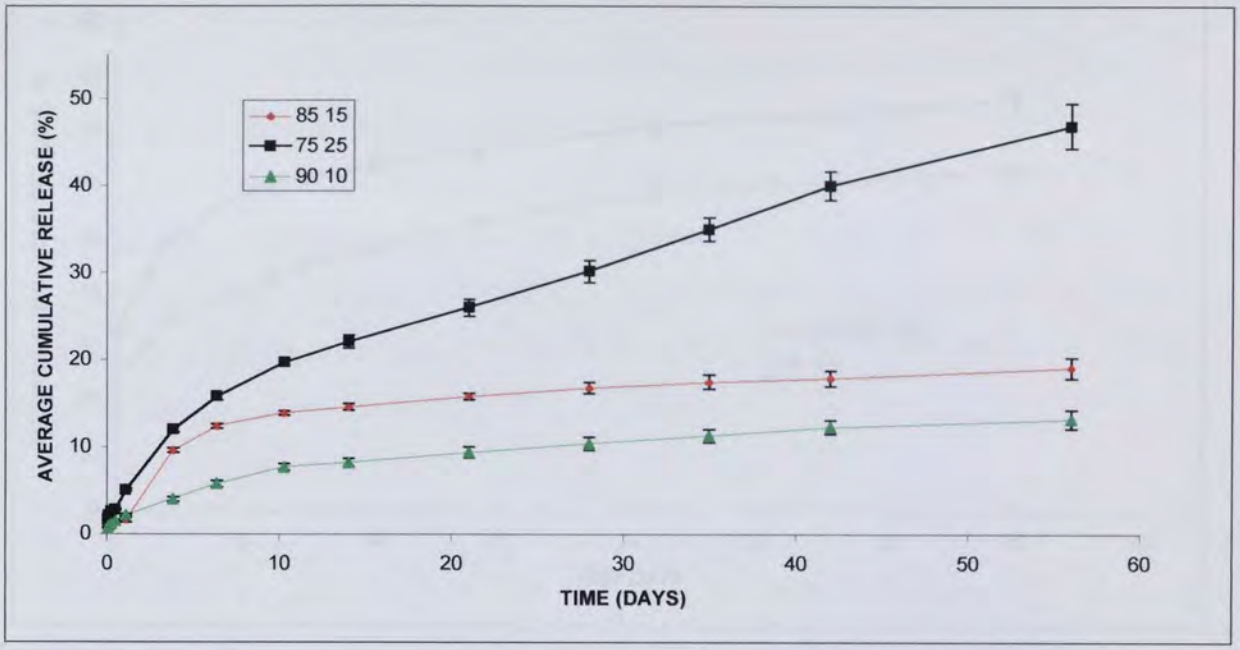


FIGURE 3.7 Comparative release profiles of ODN from microspheres prepared from different PLGA copolymer ratios (75:25), (85:15), (90:10) of lactic acid: glycolic acid respectively, with equivalent ODN loading of ~0.33nmoles/mg determined in PBS pH 7.4, at 37°C over a 56 day release period. Microsphere size 10-20μm. (n=4±S.D.)

Figure 3.7 clearly demonstrates that with an increase in PLA ratio, a significant reduction in release rate of entrapped ODN is seen. Of the three copolymer ratios used to fabricate microspheres, the 75:25 ratio depicts the most rapid rate of ODN release. All three polymer preparations produced reduced burst effects, in comparison to 50:50 ratio profiles. However, the 75:25 ratio profile demonstrates a triphasic release profile with a secondary stage of slower sustained release, due to diffusion of ODN through pores in the polymer matrix. A further increase in release rate is seen after 35 days corresponding to the increased rate of degradation of the polymer. Similar findings were reported by Yamakawa *et al.*, (1997) who investigated the release of PS ODNs from PLGA implants.

ODN characteristics were also investigated to determine any differences on release properties from PLGA microspheres. Figures 3.8 and 3.9 demonstrate the effect of ODN chemistry on release. Figure 3.8 compares the release of a 36-mer hammerhead RBZ, to a 36-mer ODN of the same sequence. Both profiles show similar biphasic profiles, with an initial burst release followed by a second phase of slower release.

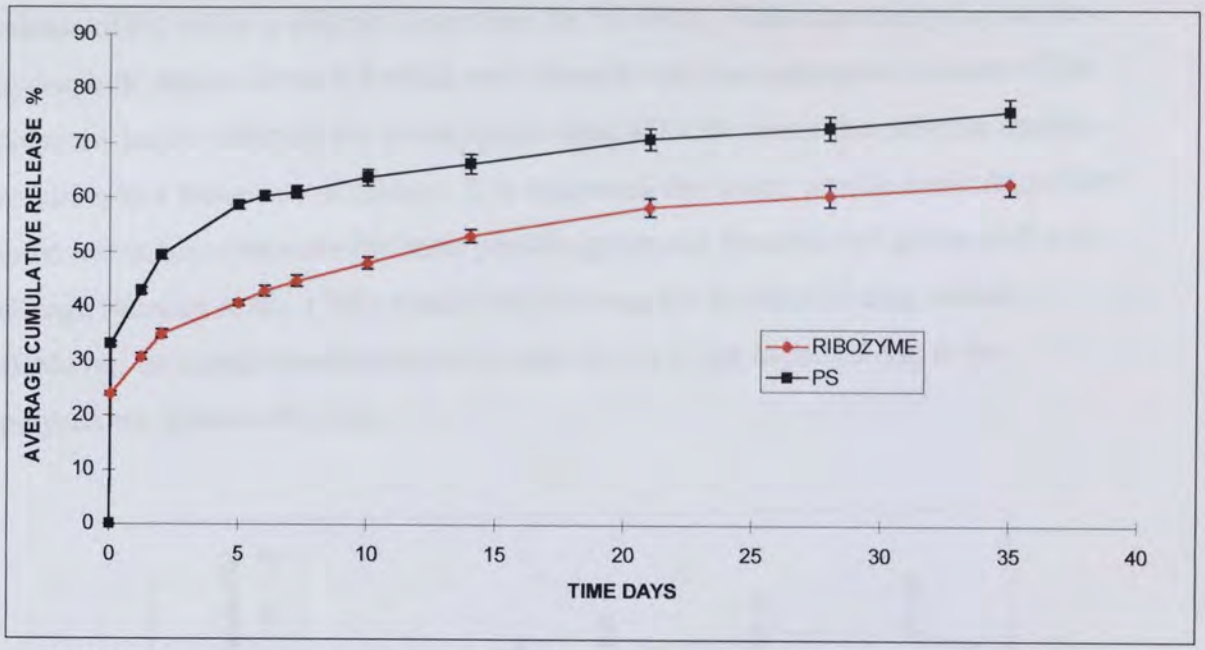


FIGURE 3.8 Comparative release profiles of a 36-mer PS ODN and a 36-mer hammerhead RBZ from microspheres prepared from PLGA Mw 3,000 with equivalent ODN and ribozyme loading of ~1.98μg /mg in PBS pH 7.4, at 37°C over a 35 day release period. Microsphere size 10-20μm. (n=4±S.D.)

As evident from figure 3.8, release profiles for the RBZ and ODN are similar. However, the RBZ is released at a slower rate than the ODN, with a 10% reduction in burst effect observed. This may be attributed to the difference in molecular weights of ribozyme (12211) and ODN (10167). The higher molecular weight of the RBZ results in the RBZ having a slower rate of diffusion through the polymer matrix, hence giving rise to a slower rate of release. The difference in burst effect is generally attributed to the presence of poorly encapsulated drug molecules located at the surface (or near the surface) of the particles. The desorption of such molecules may be the cause of high burst effects, as adsorption may result from the fraction of ODN that leaks from the inner aqueous phase towards the external aqueous phase during the final emulsion process, and may be readsorbed on the dichlorometane/water interface due to high tensio-active properties. In microspheres stabilised with PVA, leakage from the internal to the external phase of the w/o/w emulsion is limited, as the ODN content present in the external aqueous phase is not able to anchor at the dichloromethane / water interface and displace the PVA macromolecules during the emulsion process. Figure 3.9 compares the release of a 20-mer PO and a 20-mer PS ODN, both of the same sequence. Both ODNs appear to be released at very similar rates, albeit the

release of PO ODN is slightly faster than the PS ODN. This is probably due to the hydrophilic nature of the PO which may interact with the hydrophobic nature of the polymer, hence affecting the actual distribution of ODN within the polymer matrix, resulting in a faster rate of release. It is suggested that many peptide basic drugs have ionic interactions between the basic peptide group and the carbonyl group of PLGA (Kragh Hanssen *et al.*, 1990), which may prolong the duration of drug release. However, an interaction between ODN and PLGA is not expected due to the polyanionic feature of ODNs.

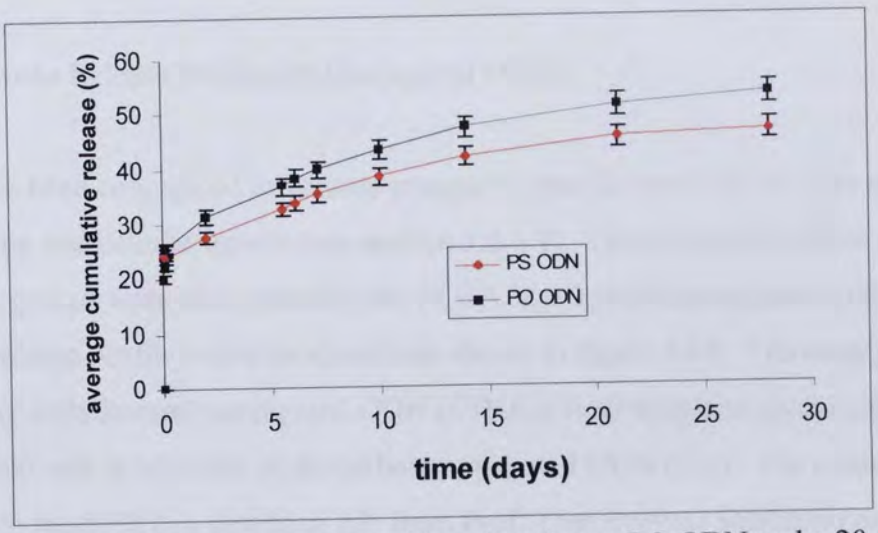


FIGURE 3.9 Comparative release profiles of a 20-mer PO ODN and a 20-mer PS ODN from microspheres prepared from PLGA (Mw 3,000) with equivalent ODN loading of ~0.7μg /mg in PBS pH 7.4, at 37°C over a 35 day release period. Microsphere size 10-20μm. (n=4±S.D.)

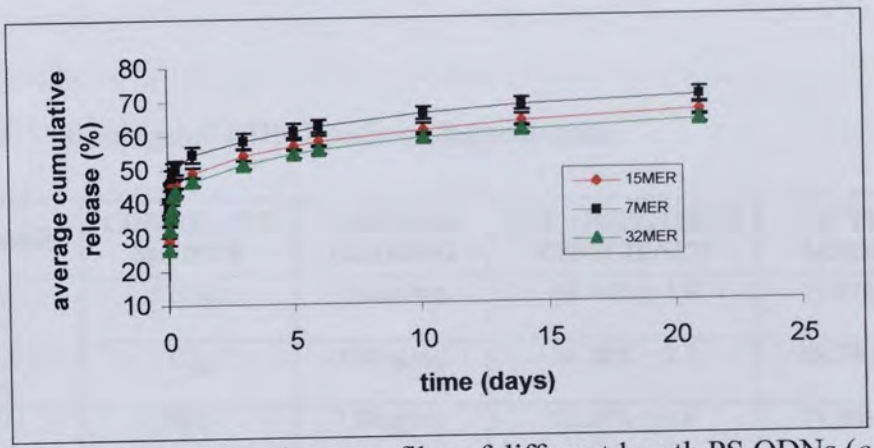


FIGURE 3.10 Comparative release profiles of different length PS ODNs (*c-myc*). A 7-mer, 15-mer, and 32-mer were encapsulated in PLGA (Mw 3,000) microspheres with equivalent ODN loadings of ~6 ng /mg in PBS pH 7.4, at 37°C over a 21 day release period. Microsphere size 10-20μm. (n=4±S.D.)

Figure 3.10 evaluates the effect of ODN length on the release pattern of ODNs from PLGA microspheres. It can be seen that the 7-mer is released most rapidly, followed by the 15-mer and then the 32-mer. The actual differences in the release rates are not proportional to ODN length, as even the release profiles for the 7-mer and 32-mer are still similar. The apparent differences in release may be due to the differences in Mw of ODNs: 7-mer (1895) 15-mer (4654) and 32-mer (10444), resulting in the lower Mw ODNs diffusing through the polymer matrix faster than higher Mw ODNs. Differences may also be due to the actual distribution of ODN within the polymer matrix.

3.3.2 *In vitro* Release Profiles of Conjugated ODNs

ODNs have been conjugated to various groups by specific methods in order to enhance their binding and cellular uptake (see section 1.6.1.1). ODNs linked to three different lipophillic groups were encapsulated into PLGA Mw 3,000 microspheres (table 3.4), and their release profiles were produced are shown in figure 3.11. The conjugated ODNs were a cholesterol conjugated ODN (CHOL), hexa-ethylene glycol conjugated ODN (HEG) and an aliphatic hydrocarbon conjugated ODN (C_{16}). The conjugated ODNs were received as a generous gift from Prof. Tom Brown (Southampton University). The synthesis, purification and identification of the CHOL, C_{16}, and HEG 28-mer *rev* ODN conjugates is described by MacKellar *et al.,* (1992). The conjugated ODNs were 3'-end labelled with ^{32}P-ATP (See Section 2.4.2).

TABLE 3.4 Conjugated ODN Loading Characteristics

POLYMER	CONJUGATE MOIETY	NOMINAL LOADING	ENCAPSULATION EFFECIENCY	ACTUAL LOADING
PLGA 50:50	CHOL	150ng/mg	46.54% ± 1.8	69.81ng/mg
PLGA 50:50	C_{16}	150ng/mg	56.26% ± 2.3	84.39ng/mg
PLGA 50:50	HEG	150ng/mg	52.24% ± 2.6	78.36ng/mg

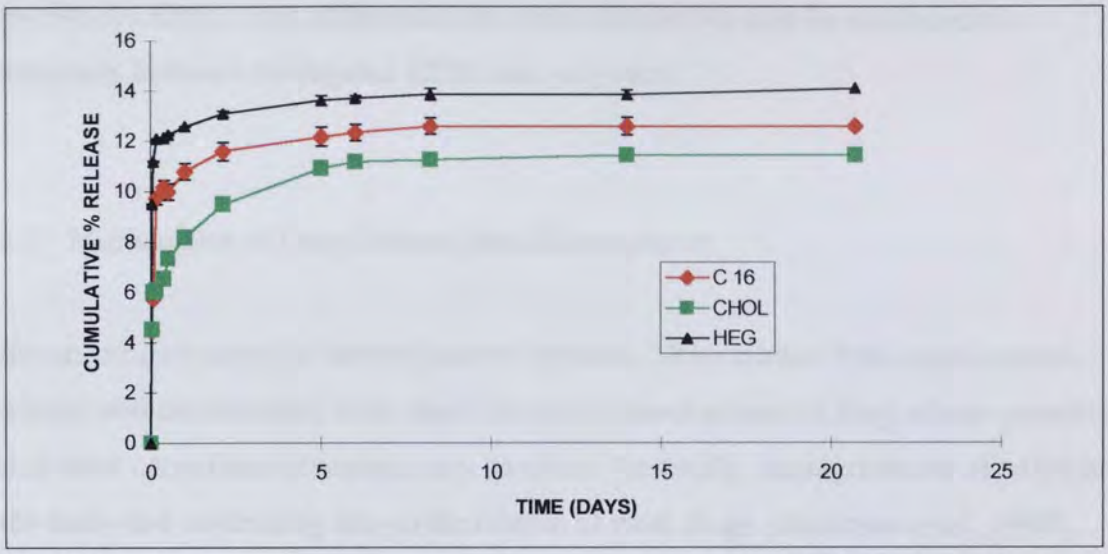


FIGURE 3.11 The effect of conjugated 28-mer *s-rev* ODNs (CHOL, C16, HEG) on release from PLGA microspheres (Mw 3,000) with equivalent ODN loadings in PBS pH 7.4 at 37°C. Microsphere size 10-20μm (n=4±S.D.)

Conjugation of lipophillic moieties to ODNs is performed to enhance cellular uptake across the plasma membrane (Beck *et al.*, 1996). The conjugate moieties, CHOL, C_{16}, and HEG, decrease the overall negative charge of the ODN making the molecule more hydrophobic (Dagle *et al.*, 1991). Cholesterol has been shown to bind to low -density-lipoproteins (LDLs) in plasma. Therefore, ODNs conjugated to cholesterol can potentially bind LDL receptors and be internalised by RME (Vlassov *et al.*, 1994). Conjugation of ethylene-glycol units to ODNs also increases lipophilicity and exonuclease stability (Jaschke *et al.*, 1994). Increased ODN lipophilicity is also achieved by conjugation to aliphatic hydrocarbon chains (Mackellar *et al.*, 1992; Temsamani *et al.*, 1994).

Figure 3.11 compares the release profiles of the three, above mentioned conjugated ODNs. Similar biphasic release profiles were displayed by each ODN conjugate. However, it was evident that over the time frame examined, not all the encapsulated conjugated ODNs had been released from the microspheres. In comparison to unconjugated ODN release characteristics, a markedly reduced burst effect was seen for all the conjugated ODNs. The differences in release rate of each conjugated ODN may be attributed to differences in Mw of conjugated ODN. HEG (Mw 9171) ODNs was released from PLGA spheres at a faster rate than C_{16} (Mw 9195), and CHOL

(Mw 9431) ODNs. Also, differences in release properties may be attributed to interactions between conjugated ODNs and polymers.

3.3.3 Mechanisms of Drug Release from Microspheres

Microspheres are complex heterogeneous systems. Drug release from microspheres has been well documented, with many theoretical mechanisms of drug release possible, and in most cases these processes may co-exist. Generally, biodegradation of polymer is the main rate controlling step in the release of most drugs (Anderson *et al.*, 1997), but other factors also may affect the rate of degradation, e.g. the thickness of polymer microsphere walls may decrease the rate of degradation (Cowsar *et al.*, 1985). Diffusion plays a key role in release of drug at various stages. As the polymer erodes, the diffusion pathlength of the matrix increases, resulting in diffusion of drug through the pores created. Therefore the initial inner structure of all microspheres is important in controlling the release process. The shear forces used in formation of the primary emulsion process also are important in controlling microsphere properties. High shear forces result in the production of microspheres with reduced porosity, resulting in a matrix like structure with an even distribution of drug throughout the microsphere.

The aqueous channels formed during the release of entrapped drug are the main points of release of drug from polymer matrix to the surrounding medium. These channels or passages are initially formed due to the presence of drug either at, or near to the surface of the microsphere. Drug release occurs due to a dissolution/diffusion process, which is dependent on drug loading and drug/microsphere morphology. As the polymer degrades, micropores appear within the polymer resulting in increased water uptake degradation properties of the polymer.

Drug loading also determines the release rate from microspheres. At low loadings, drug molecules exist as solid crystals embedded within the polymer matrix, and due to the low concentration of drug molecules not in contact with each other. Therefore drug molecules must diffuse through the matrix before they can be released, hence offering slow release. With an increase in loading, drug molecules form a network between the matrix, and act as a drug reservoir resulting in a faster release rate, hence the nature of a

drug e.g. hydrophobicity entrapped within a polymer matrix, can affect degradation (Vert *et al.,* 1991).

For PLGA, the Mw and co-polymer ratio of lactide/glycolide, has great influence on the degradation rate of the polymer (Anderson and Shive, 1997; Crofts and Park, 1998). The release rate may also be increased by the presence of a more basic drug in the matrix because the hydrolytic rate of PLA increases in alkaline pH. The rate of PLGA degradation increases with a decrease in pH of the receiving medium (Heya *et al.,* 1994).

A number of *in vitro* release profiles for different PLGA polymers containing ODNs were fitted to the main models which have been proposed to describe drug release kinetics from microspheres and other polymer matrices (Malmataris and Avgerinos, 1990).

Zero order	$100\text{-}M = k_0 t$
First order	$\ln (100 - M) = k_1 t$
Cube root	$\sqrt[3]{100} - \sqrt[3]{M} = k_2 t$
Square root	$100 - M = k_3 \sqrt{t}$

Where M = the percentage of unreleased drug; k = rate constant for the corresponding best fit line and t= time in hours.

The values of the release rate constants and the corresponding correlation coefficients (r^2) are shown in table 3.5.

TABLE 3.5 Correlation Coefficients for Fit of Dissolution Results for Various PLGA 50:50 Microsphere Systems Loaded with ODN.

Polymer Mw	Particle Size Range (μm)	ODN	Load μg/mg	Zero Order r^2	First Order r^2	Cube root r^2	Square root r^2
3kDa	10-20	PS 20-mer	1.6	0.937	0.936	0.917	0.967
40kDa	10-20	PS 20-mer	1.8	0.939	0.936	0.929	0.975
3kDa	10-20	PS 20-mer	2.2	0.941	0.985	0.973	0.988
3kDa	1-5	PS 20-mer	1.9	0.934	0.914	0.965	0.977
3kDa	10-20	PS 20-mer	1.7	0.999	0.998	0.998	0.959
3kDa	10-20	PS 20-mer	9.2	0.966	0.983	0.985	0.996
3kDa	10-20	PO 20-mer	2.6	0.921	0.939	0.979	0.967
3kDa	10-20	PS 20-mer	2.7	0.976	0.912	0.942	0.997
3kDa	10-20	RBZ	2.5	0.956	0.976	0.912	0.983

The release profiles from microspheres with different characteristics can be best described using different kinetic models (Narasimhan and Langer 1997). The initial phase of drug release is due to loss of superficial drug from the particle surface, and so it was the second phase of release, after this burst effect, that was examined in further detail. Cube root release kinetics are characteristic of the dissolution of a spherical particle, with geometric disappearance of the delivery system occurring. The cube root release kinetics are characteristic of the release from a matrix system. The square root time plots generally showed higher r^2 values than the first order plots. This indicates that release of ODN from the microspheres is mainly diffusion controlled with the drug leaving the matrix through pores and channels (Higuchi, 1963). Drug release is

described as a three stage process; initial diffusion from superficial regions, followed by a slower release due to polymer hydrolysis, and finally release resulting from polymer erosion (Sanders *et al.*, 1984). This theory may apply to ODN release during polymer hydrolysis with diffusion through the pores formed during polymer hydrolysis. A third phase of release due to erosion of polymer is not always exhibited. Therefore the cumulative release from PLGA particles shows best correlation with respect to the square root of time data during the diffusional phase. The release of drugs from matrix systems is affected by the degree of porosity of spheres, and degradation of polymer. Particles with increased porosity have an increased release rate (Sato *et al.*, 1988).

3.4 ADSORPTION OF ODNs TO THE SURFACE OF PLGA MICROSPHERES

As an alternative to encapsulation of ODNs, and to determine what role adsorbed ODN may have on the initial burst effect, adsorption of ODN to the surface of microspheres was investigated *in vitro*. ODNs were adsorbed onto blank microspheres (see section 2.7.4), to assess the quantity of ODNs adsorbed during the encapsulation procedure, hence being responsible for the initial burst effect. Initially 50mg of blank PLGA microspheres were shaken in solutions of ODN over a range of time frames (see figure 3.12). A steady increase of ODN adsorption was seen with increasing time, and maximum adsorption was seen after 24 hours. Therefore all adsorption experiments herewith were subject to 24 hours shaking time. Figure 3.12 indicates the relative amount of ODN removed, following the 24 hour adsorption process. It can be clearly seen that greater than 60% of initial ODN concentration was removed from the surface of the microspheres after the first wash. Trace amounts of ODN were removed with subsequent washing.

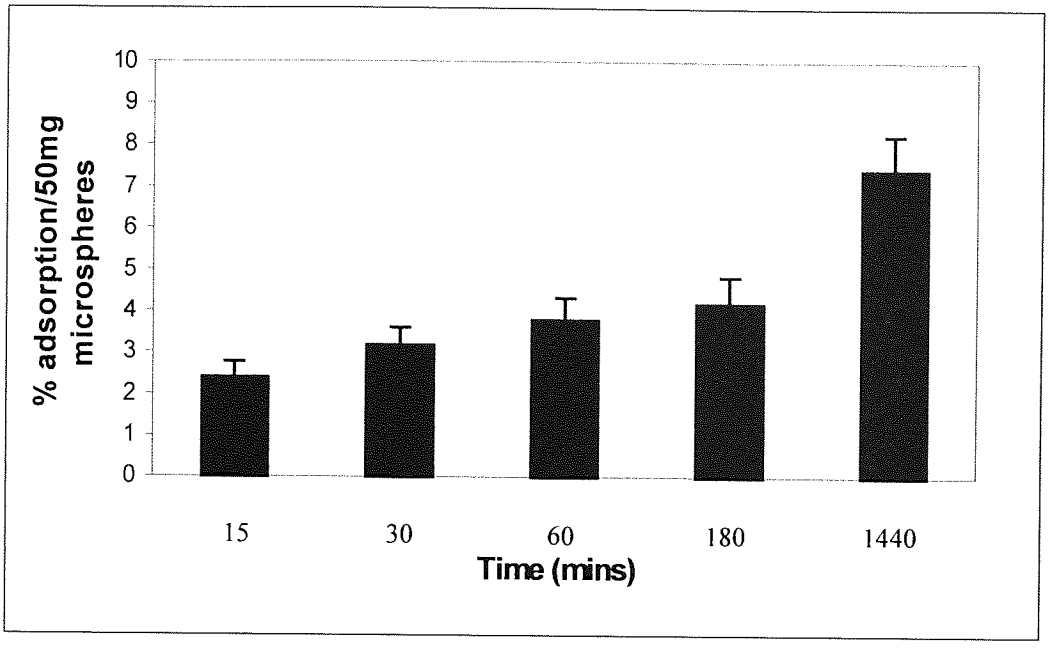


FIGURE 3.12 The effect of increasing shaking time on adsorption of PS ODN onto blank PLGA (Mw 3,000) microspheres over a time period up to 24 hours in sterile double distilled water. Microsphere size =10-20μm. (n=4±S.D.)

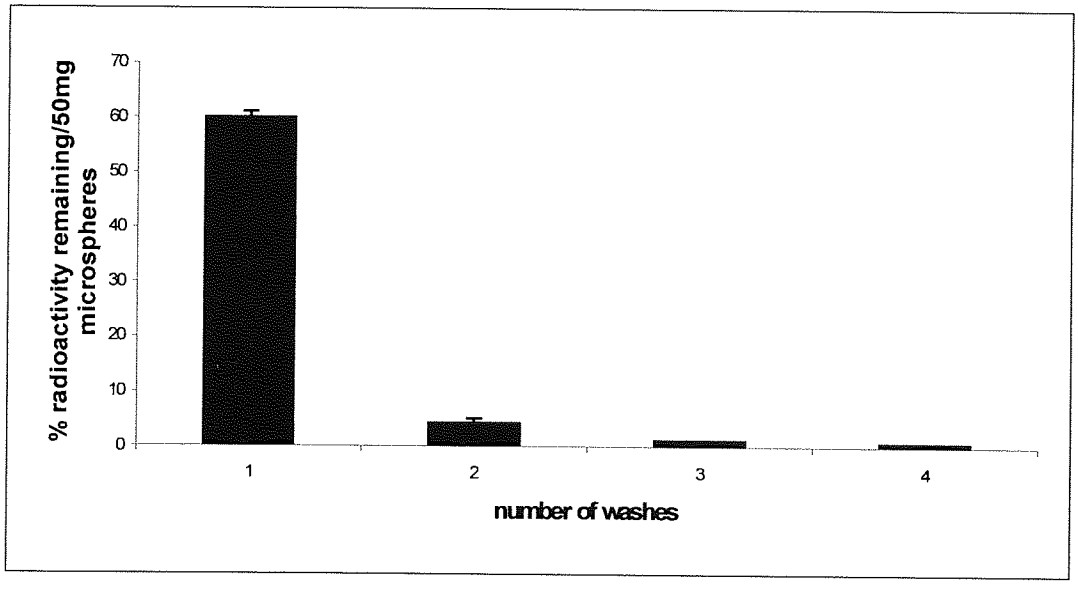


FIGURE. 3.13 The % radioactivity remaining in each wash cycle following adsorption of PS ODN onto blank PLGA (Mw 3,000) microspheres following a 24 hour shaking period. (n=4±S.D.)

Further attempts were made to increase ODN adsorption. Figures 3.14 and 3.15 demonstrate the effect of increasing ODN concentration in the media on adsorption.

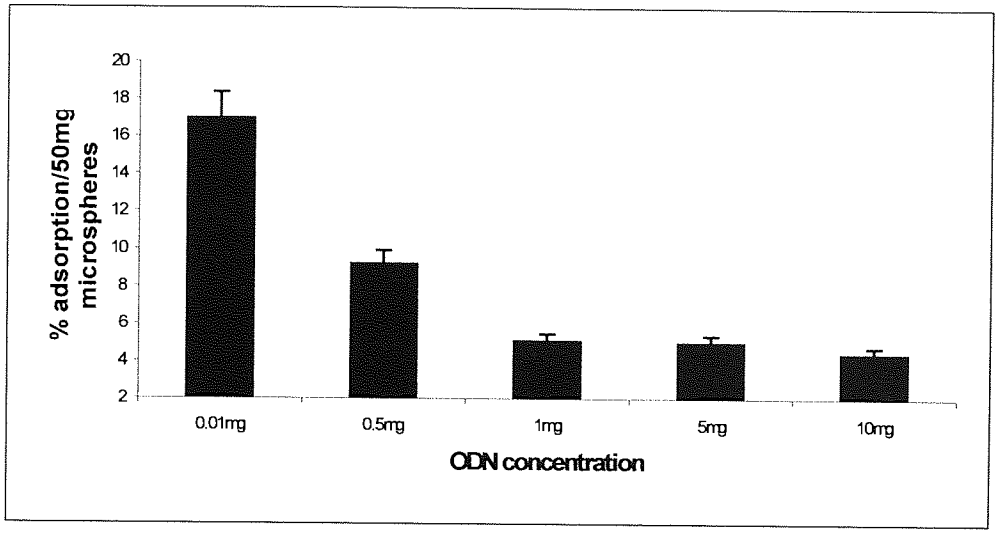


FIGURE 3.14 Effect of increasing ODN concentration on adsorption of PS ODN onto blank PLGA (Mw 3,000) microspheres following 24 hour shaking period. Microsphere size = 10-20μm. (n=4+S.D.)

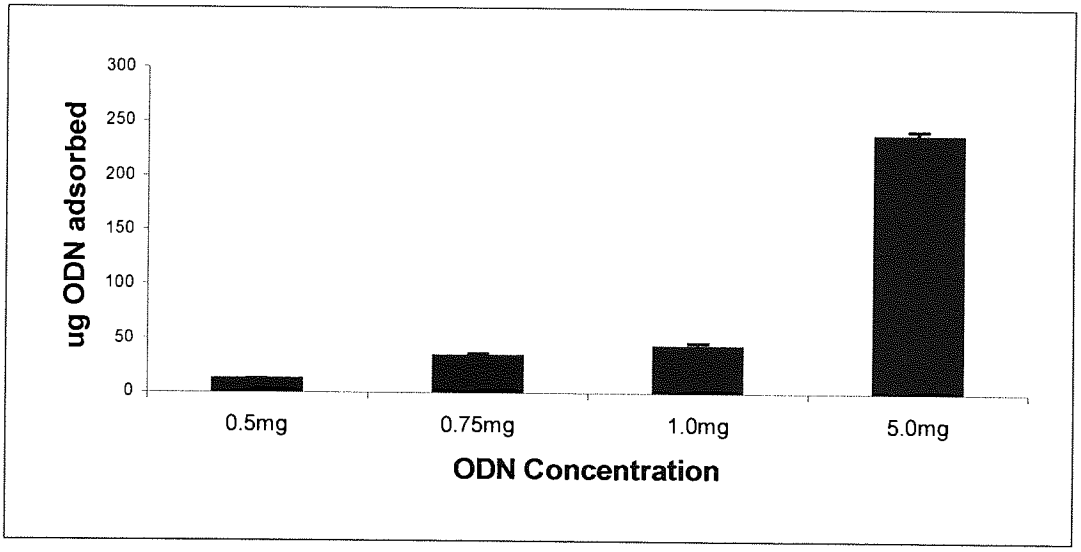


FIGURE 3.15 Determination of actual concentration PS ODN adsorbed onto blank PLGA (Mw 3,000) microspheres, following adsorption after 24 hours in increasing PS ODN concentration. Microsphere size = 10-20μm. (n=4+S.D.)

Figure 3.15 illustrates that with increasing initial ODN concentration in the shaking media, an increase in adsorbed ODN was seen. With an increase in ODN concentration from 0.5mg to 1.0mg, a linear increase in adsorption was achieved from approximately

20μg to 40μg ODN being adsorbed. However, with the 5mg concentration a greater extent of adsorption was seen, although similar adsorption was achieved for both 5mg and 10mg ODN concentrations. An increase in adsorption levels can be achieved, using this technique, although significantly high initial quantities of ODN are required, resulting in relatively low ODN loadings. In comparison to ODN encapsulation, adsorption of ODNs appeared to present an insufficient, costly, and impractical procedure for ODN entrapment into spheres.

Polymer characteristics were also investigated, to determine whether polymer properties influence the degree of adsorption. Batches of PLGA microspheres of Mw 3,000 and 40,000, and PLA of Mw 50,000 were produced as in section 2.7.2, and adsorption of ODN to spheres was assessed as described in section 2.7.4 (see figure 3.16).

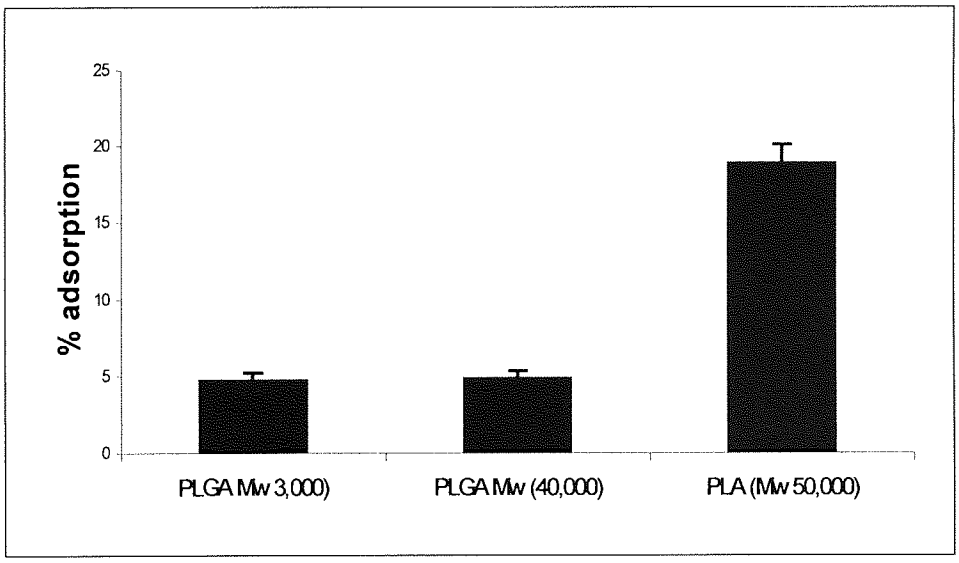


FIGURE 3.16 Effect of polymer and polymer Mw, on % adsorption of PS ODN following 24 hours shaking period in sterile water. Microsphere size 10-20μm. (n=4±S.D.)

For Mws of PLGA microspheres of 3,000 and 40,000, the degree of adsorption was similar, however for PLA microspheres, significantly higher adsorption was seen than with PLGA microspheres. This may be attributed to the increased hydrophilic nature of the PLGA polymer, and the hydrophobic nature of the PLA polymer, resulting in interactions occurring between ODN and polymer. The effect of solution properties on

the adsorption of peptides to PLGA microspheres has been examined by Tsai *et al.*, (1996), the dependency on solvent ionic strength and polarity suggest that hydrophobic interactions play an important role in the adsorption process. The effect of adsorption media solution was also studied. The aqueous vehicle was compared to solutions of the divalent cations magnesium (Mg) and calcium (Ca), to investigate whether the positive nature of the cationic solutions could increase the amount of adsorption of ODN. Figure 3.17 shows that no significant difference in adsorption of ODN onto PLGA microspheres was seen with water and 1M Mg solution, although the degree of adsorption was increased three-fold in 1M Ca solution. In a combination of 1M Ca and 1M Mg solutions, adsorption was greater than seen in 1M Mg solution alone, but less than for the 1M Ca solution.

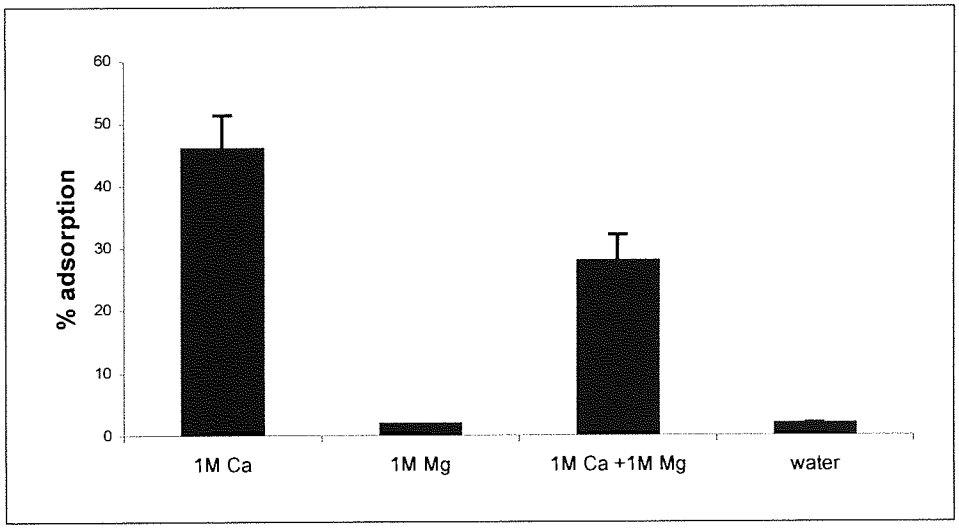


FIGURE 3.17 Determination of % adsorption of PS ODN in different adsorption solutions, on blank PLGA Mw 3,000 microspheres. Size =10-20μm. (n=4±S.D.)

Figure 3.18 demonstrates the effect of increasing Ca molarity in the shaking media, to determine any differences in ODN adsorption. Concentrations between 1M and 4M were employed. Similar adsorption levels of ODNs onto PLGA microspheres was obtained for each sample, indicating media concentrations within the range studied had negligible influence on loading, which may be due to Ca being present in excess concentration over ODN.

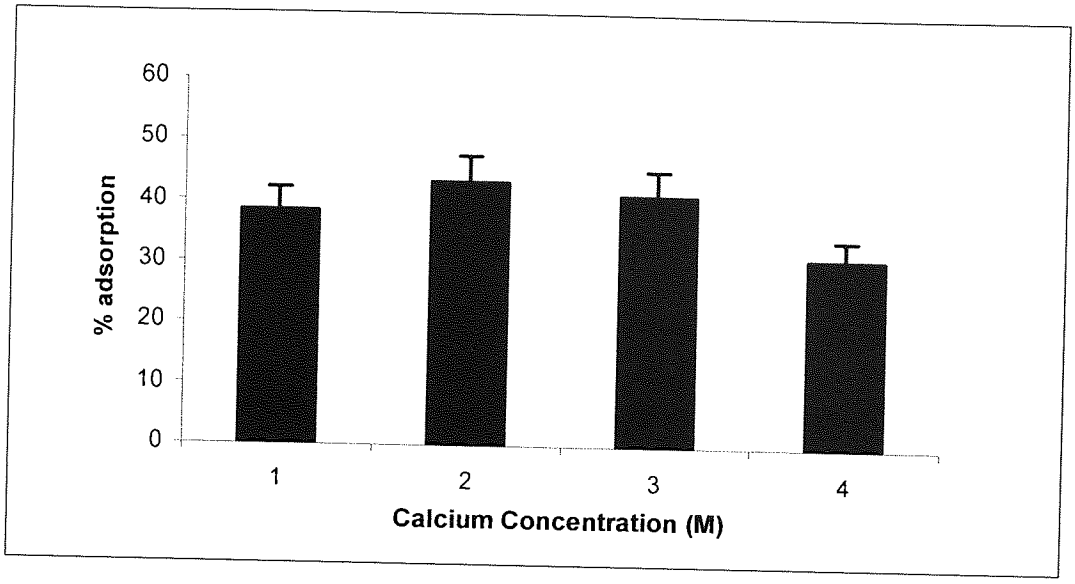


FIGURE 3.18 Effect of increasing Ca molarity of adsorption solution on % PS ODN adsorbed onto blank PLGA Mw 3,000 microspheres. Size =10-20μm (n=4+S.D.)

Release profiles produced by adsorbed ODNs were generally erratic, giving a rapid rate of release. An excessively high burst effect was seen, with the remaining ODN released rapidly, with complete ODN released within two days (see figure 3.19). Therefore the adsorption of ODN onto blank microspheres appeared to be not very useful for sustained delivery formulation studies. The high burst effect may be attributed to the poor binding of ODN to the surface of the spheres. Solvent extraction methods are known to form more porous spheres, providing a larger surface area for adsorption (Sato *et al.*, 1988), however this fabrication method was not attempted in this study.

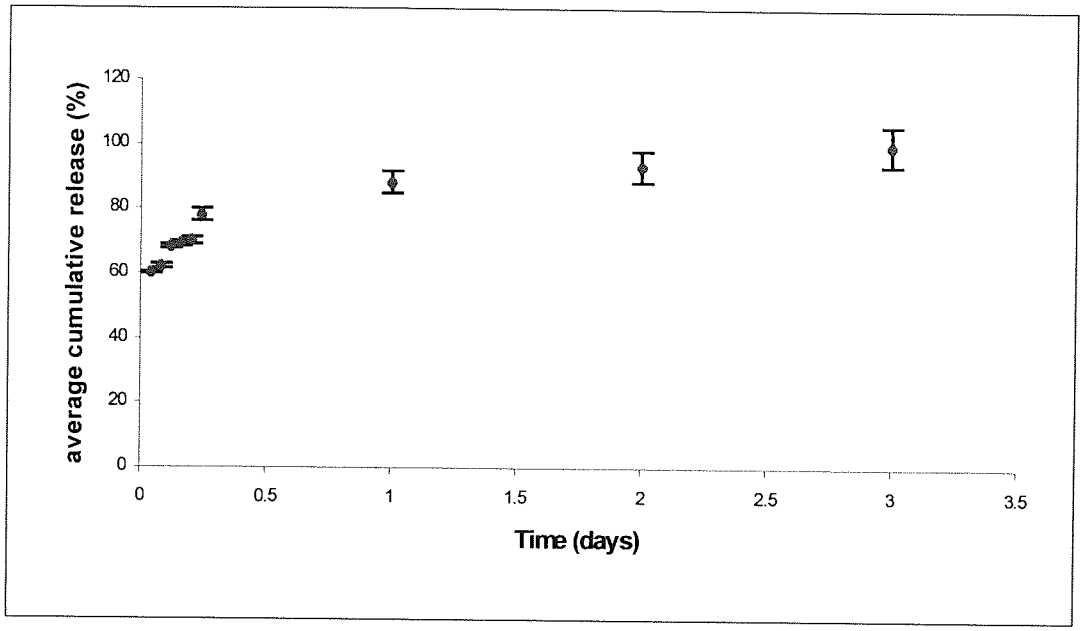


FIGURE 3.19 Release profile for PS ODN desorption from the surface of PLGA Mw 3,000 microspheres, following adsorption for 24 hours in PBS pH 7.4, at 37°C. (n=4±S.D.)

3.5 ENCAPSULATION OF BOVINE SERUM ALBUMIN (BSA) INTO PLGA MICROSPHERES

BSA has been used as a model protein for many studies. It has been well characterised (Peters, 1975), having a molecular weight of 66kDa and consisting of 581 amino acid residues. Albumin is a model antigen, producing good titres and its stability, flexibility and charge have been extensively examined (Lemoine *et al.*, 1998; Hanes *et al.*, 1997). BSA has been widely studied as a model antigen in order to study microsphere formation using various techniques and polymers (Boury *et al.*, 1997). PLA and PLGA have been predominantly used for the preparation of microspheres by solvent evaporation techniques. This section compares the effects of BSA co-encapsulation with ODNs.

3.5.1 Bicinchoninic Acid (BCA) Protein Assay

For accurate determinations of low protein content, a BCA assay procedure was followed (Smith *et al.*, 1985). Proteins react with alkaline copper (II) to produce copper (I). Two molecules of the BCA reagent react with Cu^{1+} to form a copper/peptide chelate.

The product of the reaction is water-soluble and has an intense purple colour. 500mL of Reagent A was prepared, the solution being stable for up to 6 months at room temperature (table 3.6).

TABLE 3.6 Formula for BCA Reagent A

INGREDIENT	QUANTITY
BCA disodium salt	5.00g
Na_2CO_3.H2O	10.00g
Na_2 tartrate	0.80g
NaOH	2.00g
$NaHCO_3$	4.75g
double-distilled water	to 500mL

The pH of the solution was adjusted to 11.25 with stepwise addition of 50% w/v NaOH or $NaHCO_3$ for maximum rate of colour development. Reagent B consists of 4% CuSO4.5H2O. These reagents are stable for up to six months at room temperature. To prepare the working reagent, 50 parts of Reagent A are added to 1 part of Reagent B. This solution is initially apple green but develops a purple colour on standing. 200µL of the working reagent was added to 10µL of the protein sample on a 96 well plate. The solutions were mixed for 1 minute, and incubated at 60°C for one hour. The sensitivity of the assay was heightened by extending the incubation period from the recommended 30 minutes to one hour (Smith *et al.*, 1985).

A series of protein standards were prepared in the same solution as the sample for each assay run. The concentrations of protein used were in the range 10-200μg/mL. After cooling to room temperature, the absorbances were read using an Anthos 2000 Plate reader (Anthos Labtec Instruments, Austria) at 570nm. Blanks were run under the same conditions and these values were subtracted from the absorbance of the standard or the unknown. Calibration curves were constructed by plotting net absorbance at 570nm versus protein concentration, and used to determine the concentrations of the unknowns.

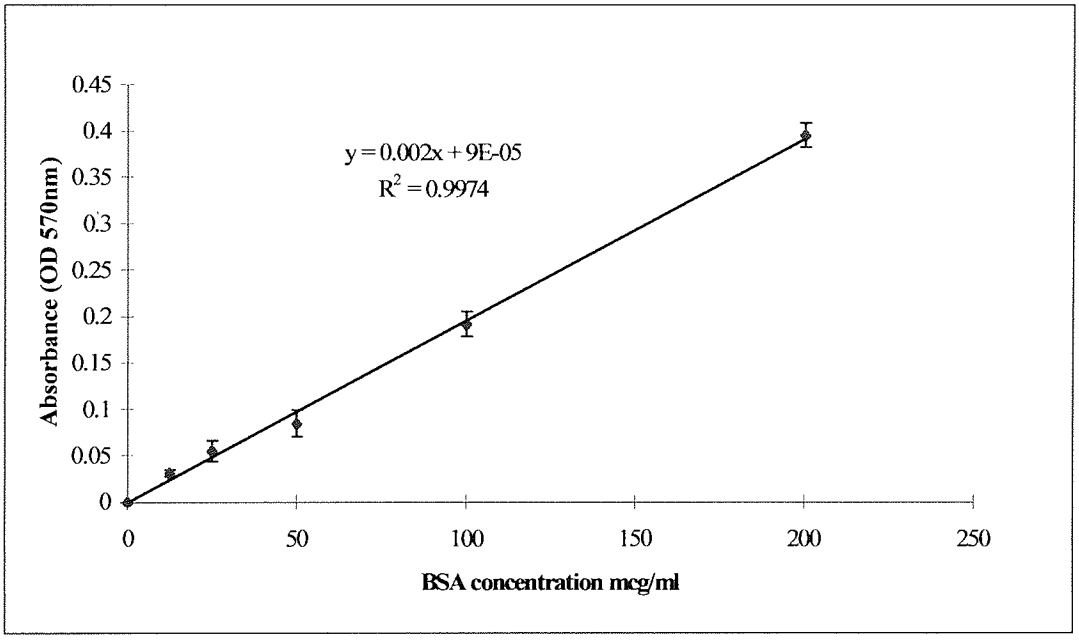


FIGURE 3.20 Calibration graph of BSA concentration μg/mL against absorbance at 570nm, for the determination of BSA content in microsphere preparations

3.5.2 The Effect of BSA on Loading

TABLE 3.7 The Effects of BSA and 20-mer PS ODN Load on PLGA Encapsulation Efficiency (n=4±S.D.).

Theoretical Load BSA (% w/w)	Theoretical Load ODN (% w/w)	Yield Efficiency (%)	Encapsulation efficiency (%)	Actual Load BSA (% w/w)	Actual Load ODN (% w/w)
0.2	2	83.3±2.4	16.1±2.2	0.03	0.32
0.4	2	74.7±1.7	13.7±1.8	0.05	0.27
2	2	76.6±1.5	12.1±1.6	0.24	0.24
4	2	82.6±1.9	11.24±1.8	0.45	0.22
10	2	81.3±3.2	23.31±2.6	1.87	0.46
20	2	77.4±2.8	4.62±2.8	0.92	0.09
40	2	-	-	-	-
0	2	84.2±1.4	62.2±1.6	0	1.24
10	0	76.4±1.8	62.4±2.3	6.24	0

Table 3.7 summarises the effects of combined encapsulation of a 20-mer PS ODN and increasing percentages of BSA. Yields in the range 70-80% depending on theoretical load were obtained. The most efficient loading is seen with 10% w/v theoretical load BSA, and 2%w/w PS ODN. However, the co-encapsulation efficiencies were much lower than for encapsulation of sole ODN, where the maximum efficiency of 62.2% was seen. Microsphere formation was not possible for particles with a theoretical load of 40% w/v BSA, and even at 20 % w/v poor microspheres were formed, with an encapsulation of only 4.62 %. High percentages of protein relative to polymer cause formation of collapsed and pitted microspheres (Jeffrey *et al.*, 1993). This may be due to insufficient polymer being present to coat the protein, and leakage of BSA during the formation of the final w/o/w emulsion as seen by Boury *et al.*, (1997). High ratios of proteins are also known to disturb the stability of the w/o/w emulsion, resulting in poor microsphere formation (Heya *et al.*, 1991a). The encapsulation efficiency may also fall as drug level is increased due to increased diffusion of the drug into the external

aqueous phase. Generally, by increasing the concentration of BSA in the aqueous phase of the primary emulsion, one markedly increases the BSA content while only a slight increase in the encapsulation efficiency is noticed.

ODNs are known to readily bind to BSA (Geselowitz and Neckers, 1995), hence we may assume binding of these molecules in the primary emulsion phase may give rise to a larger complex, with increased Mw. Thereby the nature of the ODN/BSA complex may be the reason of the low encapsulation efficiencies. Distribution within the polymer matrix may also be limited, due to size and configuration of complexes. As the Mw of the drug increases, the diffusion coefficient decreases and the polymer is unlikely to permit partition-dependent diffusion through the polymer. The release profile obtained for the co-encapsulated BSA/ODN is shown in figure 3.21, which clearly demonstrates that BSA/ODN is released at a slower rate in comparison to the sole ODN encapsulated microspheres. Although the general profile is similar, a much lower burst effect is visible, followed by a secondary slower phase of release where molecules diffuse through the polymer matrix as the polymer erodes.

The release of peptides and proteins from biodegradable polymers has been extensively studied (Alonso *et al.*, 1993; Hora *et al.*, 1991). The release being affected by pore diffusion and bioerosion (Bodmer *et al.*, 1992). Cohen *et al.*, (1991) suggest low Mw polymers are more hydrophilic, and thus the relative solubility of the BSA in PLGA is enhanced, leading to stable protein contents. Ionic interactions between the BSA (positively charged amine groups) and the carboxylic polymers end groups (negatively charged) are possible. In addition, the hydrophillicity of the RG502 polymer probably enhances the penetration of the BSA in the PLGA monolayer at the dichloromethane / water interface in the primary emulsion.

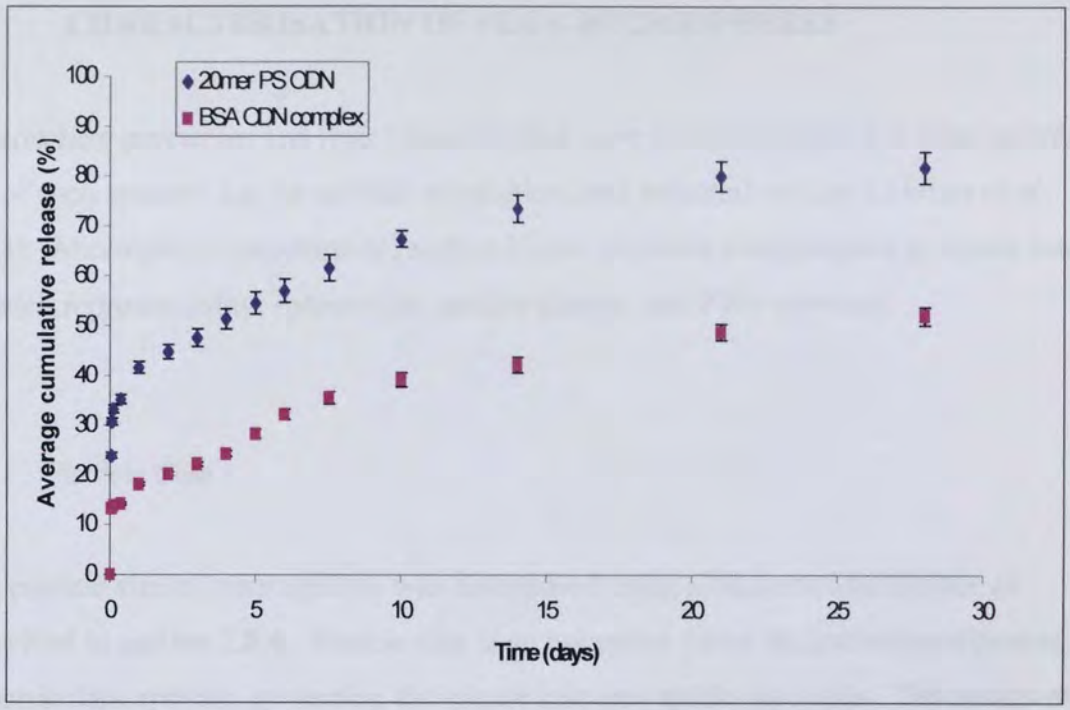


FIGURE 3.21 Comparative release profiles of a PS ODN and co-encapsulated PS ODN and BSA complex from PLGA (Mw 3,000) microspheres in PBS pH 7.4, at 37°C is shown over a 28 day release period. Microsphere size = 10-20μm, ODN loading = 0.47nmoles of ODN per mg of polymer.

The important decrease of the burst effect, as discussed before may be related to the increase in density of the terminal carboxylic acid end groups in the Resomer RG 502, and the physical interaction resulting from interpenetration of BSA and PLGA at the core of the microsphere. The release of BSA by the classical model of diffusion through water-filled networks of pores and channels promoted by degradation of the polymer (Spenleheur *et al.*, 1989) is probably coupled with other processes. During the degradation process, the appearance of ionised carboxylic acid groups is enhanced with the low Mw polymer. The interactions of these charged functional groups with the positively charged groups of the protein could lead to the retention of BSA in the polymer matrix, and consequently may explain the slower release of the protein. This, in conjunction with the binding to ODN, may explain the slower release rate.

3.6 CHARACTERISATION OF PLGA MICROSPHERES

Microsphere properties and their characteristics have a major influence for the potential use of such systems e.g. on cellular association, and potential toxicity (Alonso *et al.*, 1993). Microsphere preparations produced were therefore characterised to assess batch to batch reproducibility, sphere size, surface charge, and PVA retention.

3.6.1 Particle Size

The particle size of microspheres was determined using a Malvern Mastersizer as described in section 2.8.4. Particle size is an important factor in predicting behaviour of particulate systems, governing the release rate and uptake into cells. The results of the particle size distributions for the different methods of production are displayed in figures 3.22a and 3.22b. The sphere size is quoted in μm, with the mean value highlighted.

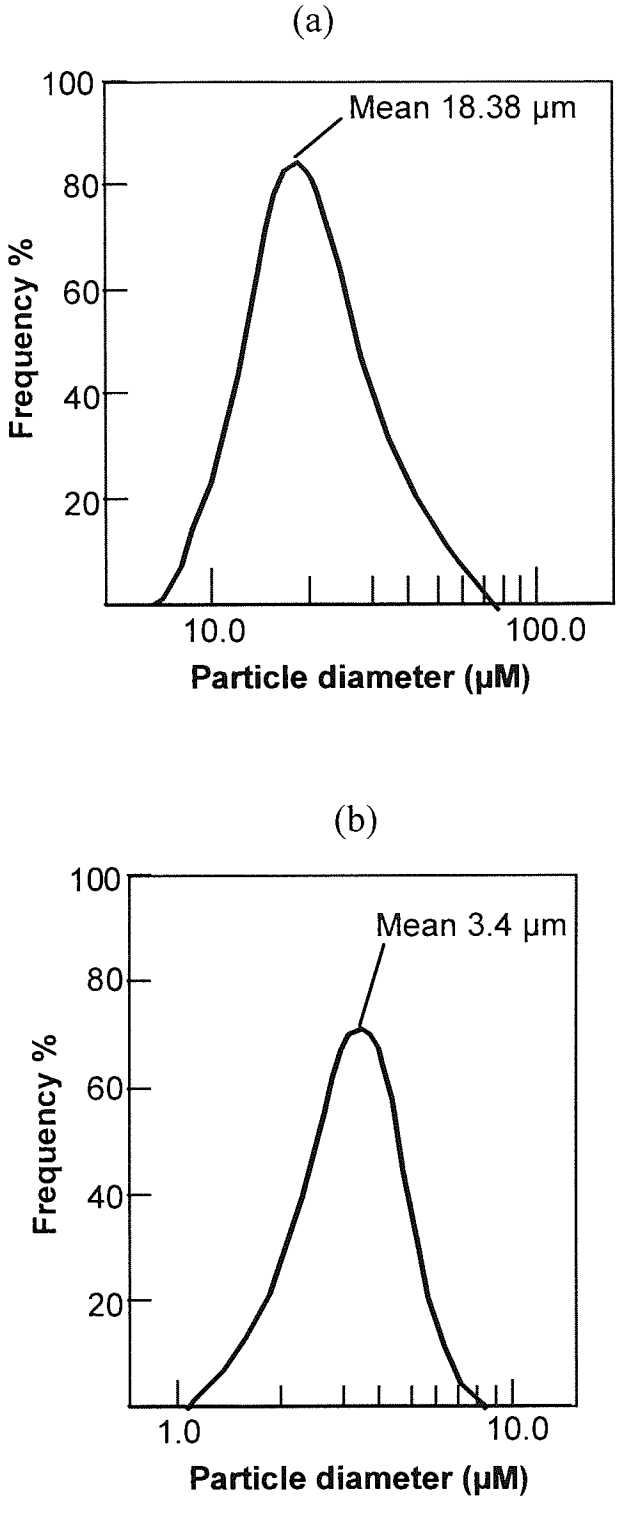


FIGURE 3.22 Particle size distribution profiles of PLGA microspheres formed using a double emulsion method for large microspheres **(a)**, and small microspheres **(b)**. The % frequency versus particle diameter (log scale) is given.

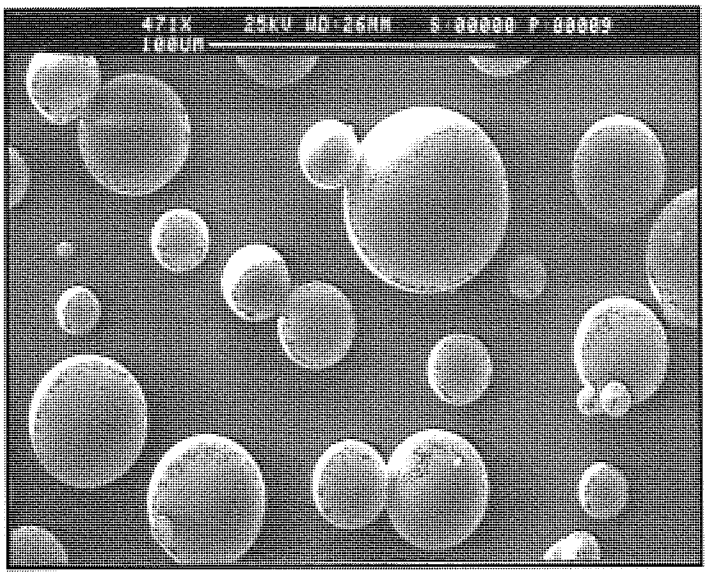


FIGURE 3.23 Scanning electron micrograph of PLGA microspheres formed by a double emulsion method. (Microsphere size =10-20μm.)

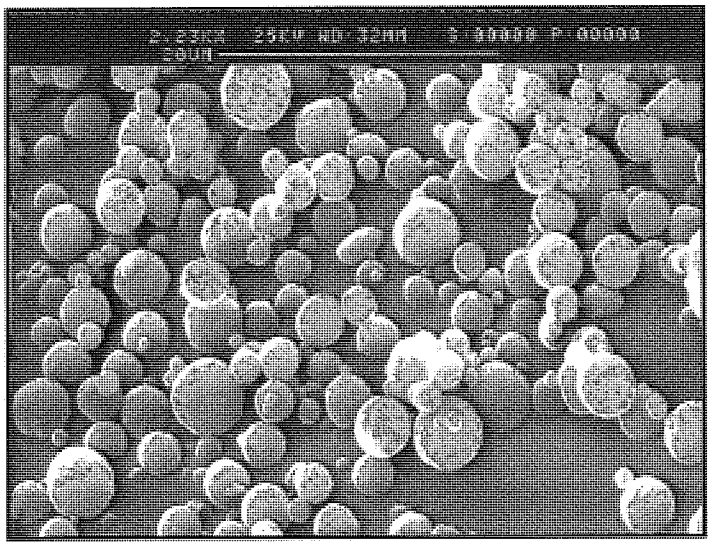


FIGURE 3.24 Scanning electron micrograph of PLGA microspheres formed by a double emulsion method. (Microsphere size =1-5μm)

3.6.2 Surface Charge Determination

The fate of particulate systems in the body depends on various factors including particle size, hydrophobicity and charge. It has been demonstrated that phagocytosis occurs more readily with increasingly negative zeta potentials of the microsphere surface (Tabata and Ikada, 1989). The zeta potential of microspheres is affected by the pH and ionic strength of the medium (Makino *et al.*, 1986). The zeta potentials of various microsphere suspensions were determined, and these are summarised in table 3.8.

TABLE 3.8 Summary of the Effect of Particle Size, Polymer Mw, and ODN Loading on the Zeta Potential of Microspheres. (n=4±S.D.)

Formulation	**Zeta Potential (mV)**
Latex 0.2μm	-45.2±1.2
Latex 1.0μm	-49.5±0.8
Latex (carboxylated) 1.0 μm	-42.7±1.4
PLGA (Mw 3,000) 1-5μm	-2.6±0.4
PLGA (Mw 40,000) 1-5μm	-3.2±0.6
PLGA (MW 3,000) 10-20μm	-3.8±0.7
PLGA (Mw 40,000) 10-20μm	-3.4±0.4
PLGA (Mw 3,000) 1-5μm 0.1%w/w ODN	-2.8±0.3
PLGA (Mw 3,000) 1-5μm 1%w/w ODN	-3.3±0.2
PLGA (Mw 3,000) 10-20μm 0.1%w/w ODN	-3.8±0.9
PLGA (Mw 3,000) 10-20μm 1%w/w ODN	-3.6±0.7
PLGA (Mw 40,000) 1-5μm 0.1%w/w ODN	-4.1±0.5
PLGA (Mw 40,000) 1-5μm 1%w/w ODN	-4.4±0.3
PLGA (Mw 40,000) 10-20μm 0.1%w/w ODN	-3.7±0.5
PLGA (Mw 40,000) 10-20μm 1%w/w ODN	-3.9±0.2

The zeta potentials for spheres of different polymer Mw, ODN loading, and size were determined. Any differences in surface charge may give an indication to how each formulation may be affected by the cellular uptake. Table 3.8 lists the zeta potentials of microspheres examined. Generally, the biodegradable microspheres do not show any consistent trends in zeta potentials, and all appear less negatively charged than the polystyrene standards. Sphere size (large 10-20μm and small 1-5μm), and polymer Mw appear to have minimal effect on the surface charge. Spheres containing encapsulated ODN are not significantly more charged than empty particles. With an increase in loading, a minimal difference in surface charge is seen. As ODNs are negatively charged, a difference in surface charge is possible if ODNs are concentrated at the surface of spheres. The process of microsphere formation itself can reduce the zeta potential of the polymer, due to adsorption of residual surfactant, as residual PVA may reduce the negative charge of the microsphere surface (Yamaouka *et al.*, 1993).

3.6.3 Differential Scanning Calorimetry (DSC)

Thermal analysis is a widely used instrumental technique in pharmaceutical formulation. Two most commonly used techniques are thermogravimetric analysis (TGA) and differential scanning calorimetry. A DSC thermal profile provides information on the Tm of a formulation, glass transition temperatures (Tg) (and associated energy changes), purity, compatibility, decomposition kinetics, polymorphic transitions of a sample, *etc.* DSC may provide an insight into the distribution of drug within the polymer matrix, and the effects of drug loading and other manufacturing parameters on the properties of the polymers employed to form microparticles.

PLGA 50:50 polymers are amorphous, exhibiting only Tg temperatures, (see figure 3.25 for thermal profile). The Tg generally decreases with increasing glycolide content (Asano *et al.*, 1989). The poly(L)lactide polymer is completely crystalline, and only exhbits a melt endotherm (Tm). However, on formulation to microspheres the polymer becomes semi-crystalline with both amorphous and crystalline regions (figure 3.27), hence altering thermal properties. The process of microsphere formation may alter the

organisation of polymer chains, therefore leading to alterations in Tg and polymer crystallinity.

The presence of any residual solvent in the microspheres can also be assessed by DSC with the presence of a characteristic endotherm at around 60-80°C. The absence of such an endotherm indicates that the drying process was successful in removal of solvent.

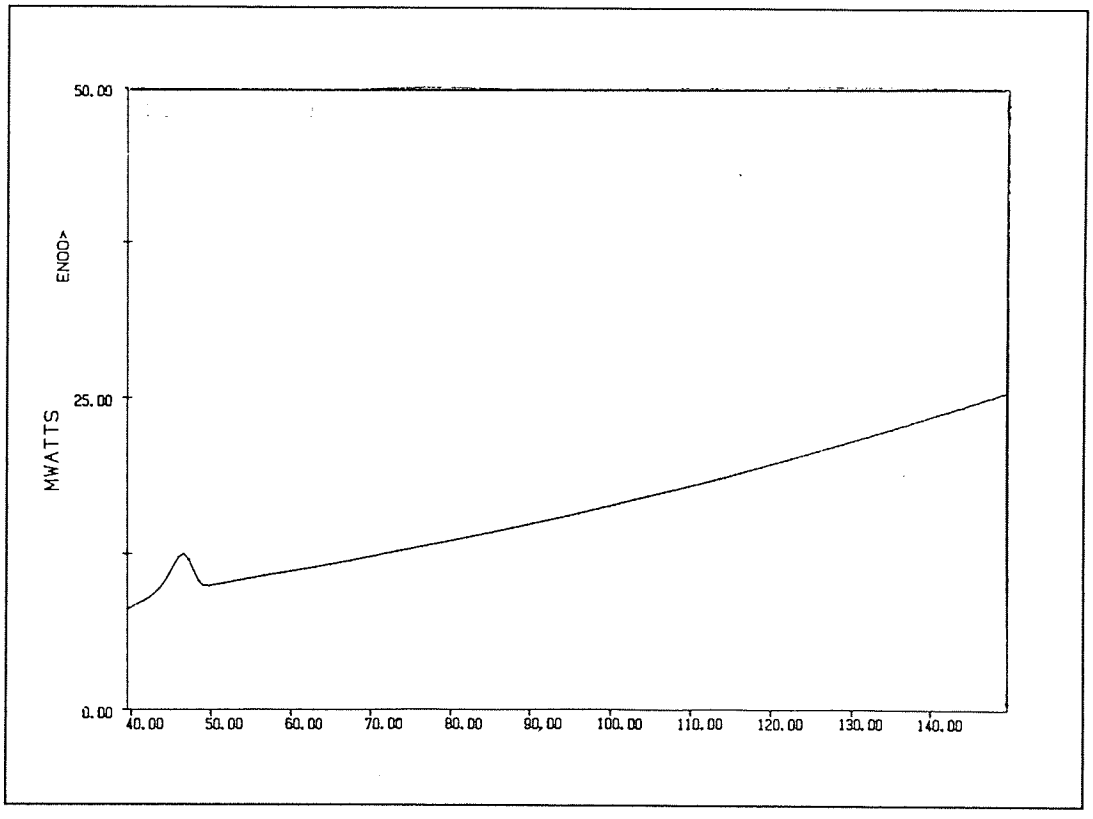


FIGURE 3.25 Thermal Profile for PLGA Polymer (Mw 3,000)

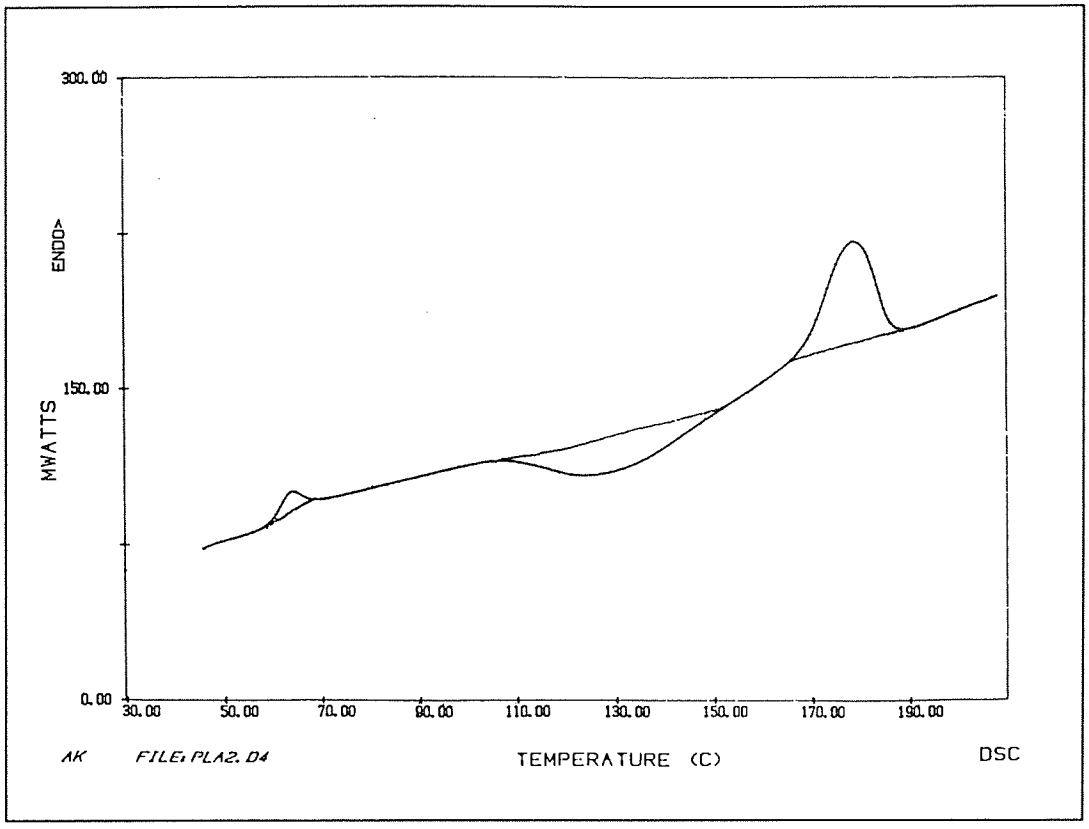


FIGURE 3.26 Thermal Profile for PLLA Polymer (Mw 2,000)

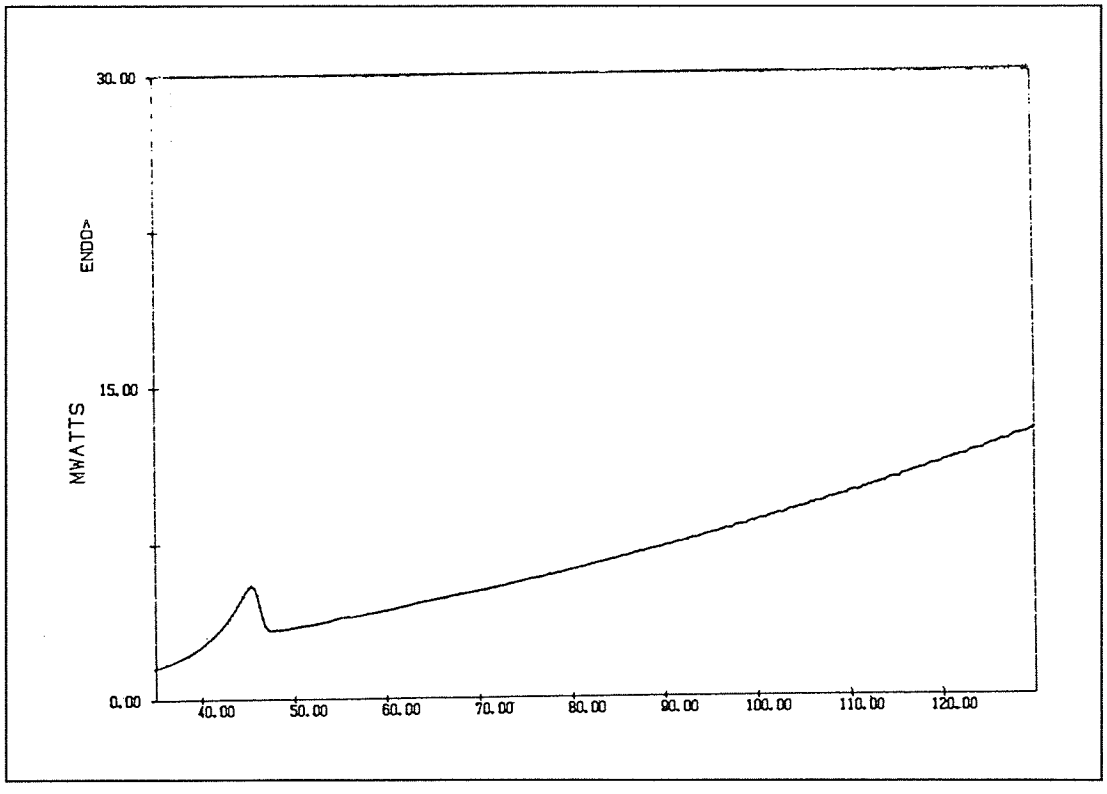


FIGURE 3.27 Thermal Profile for Empty PLGA (50:50) microspheres

The effect of encapsulation of ODNs on the glass transition temperature is shown in table 3.9. The process of microsphere formation results in a reduction in the Tg, the effect of encapsulation results in further reduction of the Tg, and the Tg also falls as the loading is increased. Drugs, which have no affinity for the polymer, have no effect on the Tg of the polymer. However, drugs that interact with the polymer to form a true or partial solution, will plasticise the polymer and reduce the Tg.

TABLE 3.9 Glass Transition Temperatures for PLGA Microspheres (n=4±S.D.)

Description	T_g (°C)
PLGA polymer	48.86±0.02
PLGA empty microspheres	46.38±0.02
PLGA loaded microspheres (0.1%w/w)	45.37±0.01
PLGA loaded microspheres (0.6%w/w)	44.56±0.03

DSC was also employed to investigate the thermal properties of ODNs. A 20-mer PS ODN was sealed in a DSC pan and heated. The sample was heated to 600°C (figure 3.28). The thermal profile produced displayed little thermal characteristics within this range.

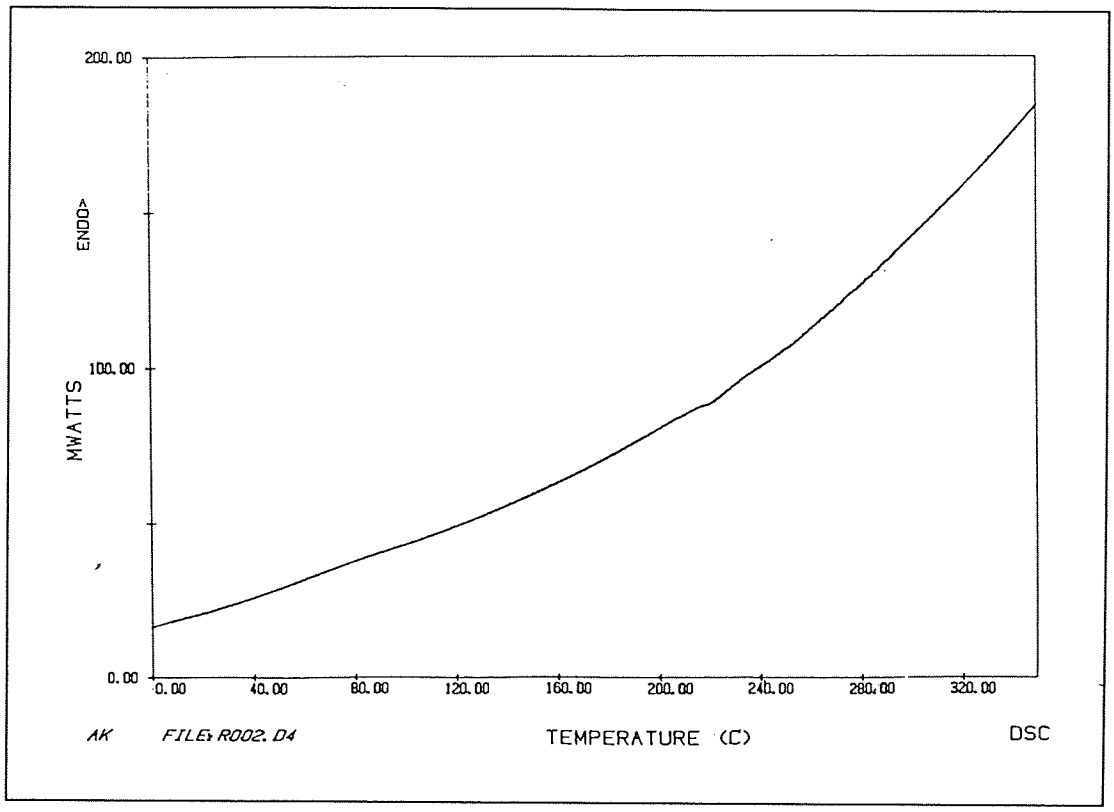


FIGURE 3.28 Thermal Profile for 20-mer PS ODN

TGA is the standard technique recommended by the United States Food and Drug Administration (FDA) to test for moisture levels in dried formulations, and can hence also be used for determining the moisture content of polymers/microspheres. A high moisture content indicates poor storage conditions, or a poor manufacturing process with inefficient drying that may lead to degradation of polymer/microspheres. Table 3.10 shows that the moisture content for loaded and unloaded microspheres is below 1%. Thus, it can be inferred that the manufacturing process was adequate with efficient drying, and that the microspheres were stored correctly.

TABLE 3.10 Percentage Moisture Content of PLGA Mw 3,000 microspheres (n=4±S.D.)

PREPARATION	MOISTURE CONTENT (%)
PLGA EMPTY MICROSPHERES	0.72±0.08
LOADED MICROSPHERES (0.1 %w/w)	0.86±0.06
LOADED MICROSPHERES (0.5 %w/w)	0.95±0.09
PLGA MICROSPHERES AFTER 30 DAYS STORAGE	0.92±0.05

3.6.4 Determination of Residual PVA in Microspheres

Microsphere preparation methods generally involve the emulsification of aqueous and organic phases, followed by removal of the solvent. Quantification of residual surfactants is essential as PVA, though generally considered safe and non-toxic, may influence the final sphere characteristics. The presence of PVA on the sphere surface reduces the particle surface hydrophobicity, which can result in a reduced cellular uptake by macrophages (Muller and Wallis, 1993). Also, long contact times with PVA can result in incorporation of PVA into the polymer matrix, increasing the burst effect (Kwong *et al.*, 1986). The assay method for determining residual PVA in microspheres is described in section 2.8.7. The first step in this assay was the construction of a calibration curve as illustrated in figure 3.29.

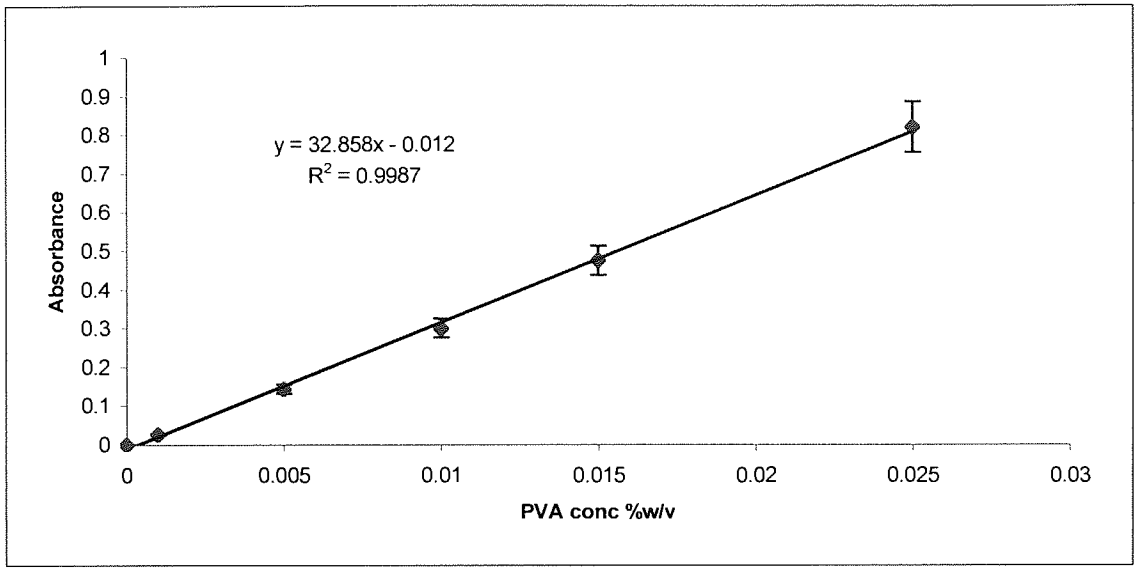


FIGURE 3.29 Calibration graph of PVA concentration (%w/v against absorbance at 690nm, for the determination of residual PVA in microsphere preparations (n=6±S.D.)

TABLE 3.11 The Effect of Polymer Properties on Residual PVA Content in PLGA Microspheres

Polymer Mw PLGA	Microsphere size (μm)	Residual PVA (%w/w)
3,000	1-5	0.145±0.02
3,000	10-20	0.189±0.03
3,000	10-20 unwashed	1.682±0.03
40,000	1-5	0.154±0.06
40,000	10-20	0.188±0.04
40,000	10-20 unwashed	1.724±0.04

The results of the PVA assay are listed in table 3.11, the microsphere size or polymer molecular weight did not appear to affect the amount of PVA retained. For microspheres that were dried without washing, the value for residual PVA was 1.68 % and 1.72 %w/v for Mws 3,000 and 40,000. These high values of residual emphasise the importance of a standardised washing procedure to ensure removal of all surface associated PVA.

3.7 STABILITY OF OLIGODEOXYNUCLEOTIDES IN POLYMER MICROSPHERES

Naked ODNs, even those containing phosphorothioate linkages, can be degraded within a few hours by serum nucleases (Akhtar *et al.*, 1991; Sands *et al.*, 1995). Therefore a sustained release polymer formulation must protect ODNs from degradation and maintain a pool of intact, biologically active ODN for release from the polymer device. In order to assess the stability of naked ODN and ODN encapsulated within microspheres, in serum and release media, autoradiography was used as described in section 2.2.2.

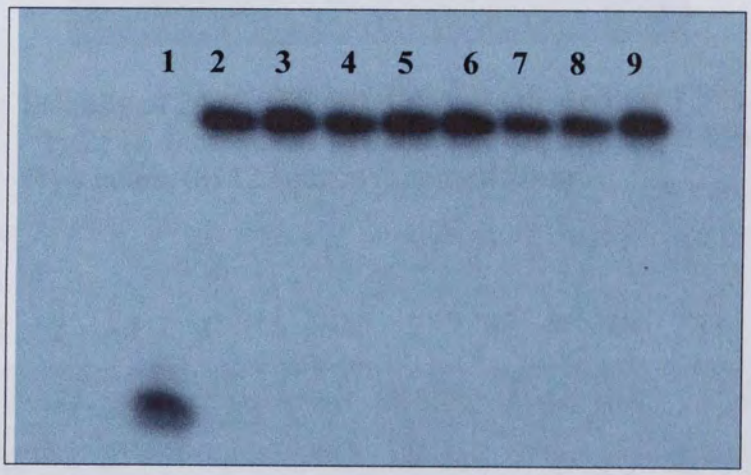


FIGURE 3.30 Stability of 20-mer PS ODN exposed to PBS at 37°C. (1) control free ^{32}P (2) 15 minutes, (3) 30 minutes, (4) 1 hour, (5) 2 hours, (6) 4 hours, (7) 8 hours, (8) 12 hours, (9) control 20-mer.

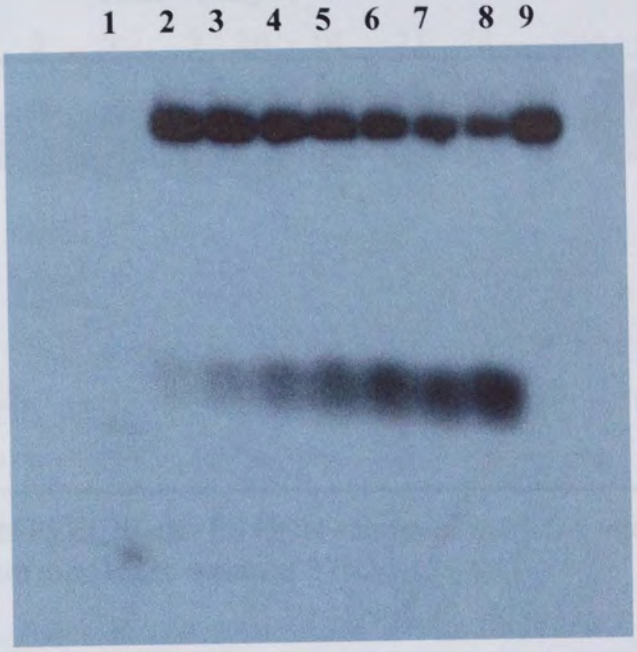


FIGURE 3.31 Stability of 20-mer PS ODN exposed to serum at 37°C. (1) control free ^{32}P (2) 15 minutes, (3) 30 minutes, (4) 1 hour, (5) 2 hours, (6) 4 hours, (7) 8 hours, (8) 12 hours, (9) control 20-mer.

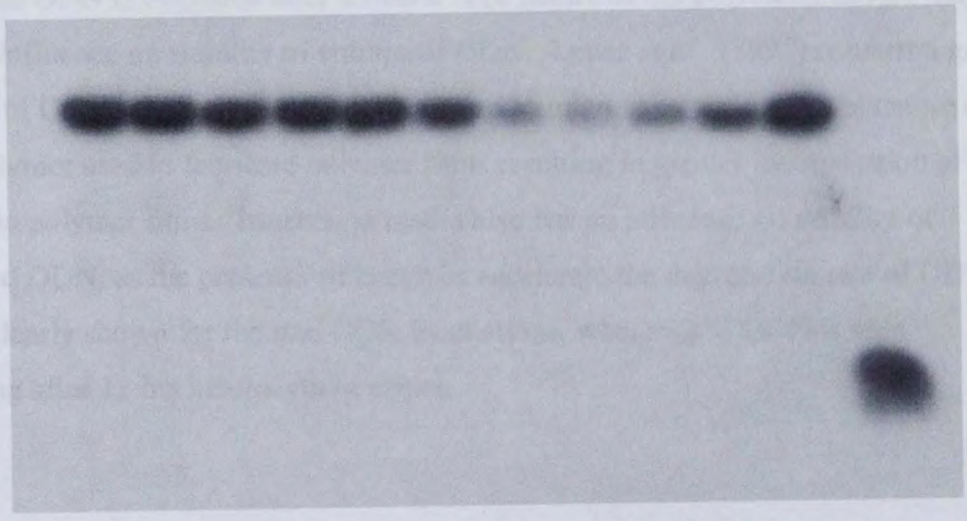


FIGURE 3.32 Stability of 20-mer PS ODN entrapped in PLGA microspheres 10-20μM exposed to PBS at 37°C. (1) 1 day, (2) 2 days, (3) 3 days, (4) 4 days, (5) 5 days, (6) 7 days, (7) 10 days, (8) 14 days, (9) 21 days, (10) 28 days, (11) control 20-mer, (12) control free ^{32}P.

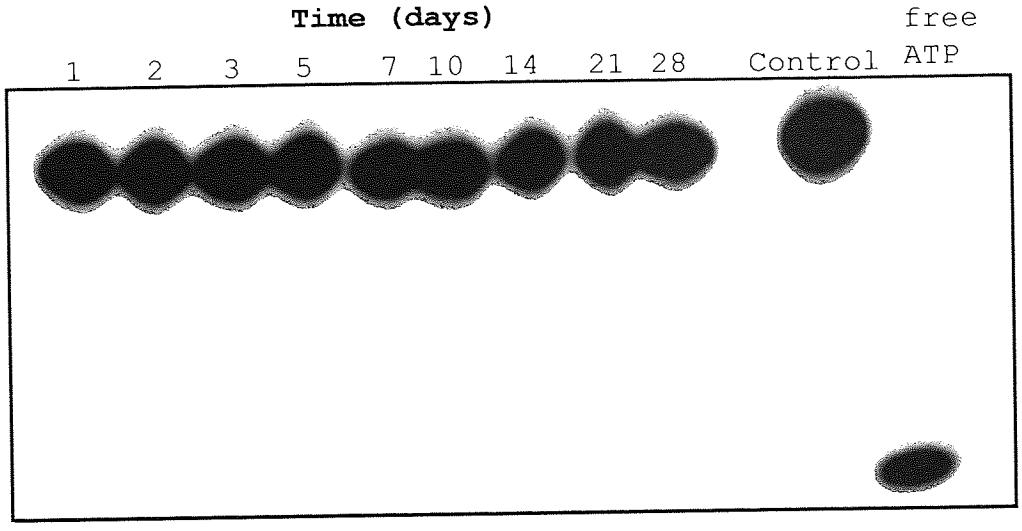


FIGURE 3.33 Stability of 20-mer PS ODN entrapped in PLGA microspheres 10-20μm exposed to serum at 37°C.

Figure 3.33 shows that undegraded, full-length PS modified ODN can be recovered from polymer microspheres after at least 28 days incubation in serum, suggesting that polymer-entrapped ODNs are protected from nuclease degradation in biological milieu. The free ODN is seen to be stable in PBS over 12 hours, however on exposure to serum, the ODN is degraded after 2 hours. The nature of the polymeric device may have an influence on stability of entrapped ODN. Lewis *et al.*, (1995) conferred greater stability of ODN with polymer films compared to microspheres, due to the nature of the homopolymer used to fabricate polymer films resulting in greater incorporation of ODN into polymer films. Incubation media also has an influence on stability of entrapped ODN, as the presence of enzymes accelerate the degradation rate of ODNs. This is clearly shown for the free ODN incubations, where 22 % ODN is seen remaining after 12 hrs incubation in serum.

3.8 POLYMER DEGRADATION STUDIES

Microspheres formed were generally smooth and spherical with an overall intact surface. As PLGA microspheres are degraded, initially the particle size increases due to the uptake of water and pores are visible on the now uneven surface. The low Mw 3,000 microspheres are seen to degrade more rapidly. Figure 3.34 shows that at day 7 the microspheres appear porous, and polymer debris is present. After 14 days, the extent of degradation is greater, with the spherical structure of the microspheres clearly broken down. After 56 days, the low Mw sphere structure has totally collapsed. For the higher Mw 40,000 microspheres, the pattern of results was similar with the appearance of pores, however after 35 days, although the surfaces of the spheres appeared uneven, the microsphere structure still remained intact in comparison to the totally collapsed low Mw microspheres.

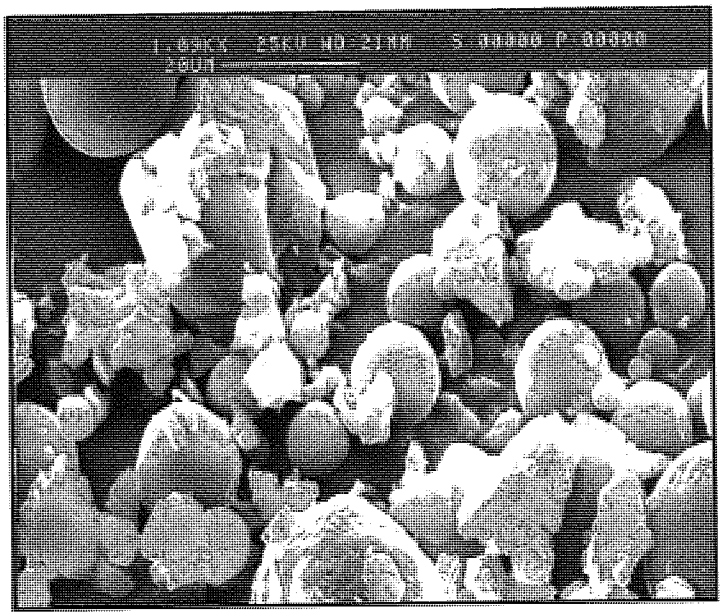


FIGURE 3.34 Scanning Electron Micrograph of large (10-20μm) PLGA Mw 3,000 after exposure to PBS pH 7.4 release medium at 37°C for 7 days

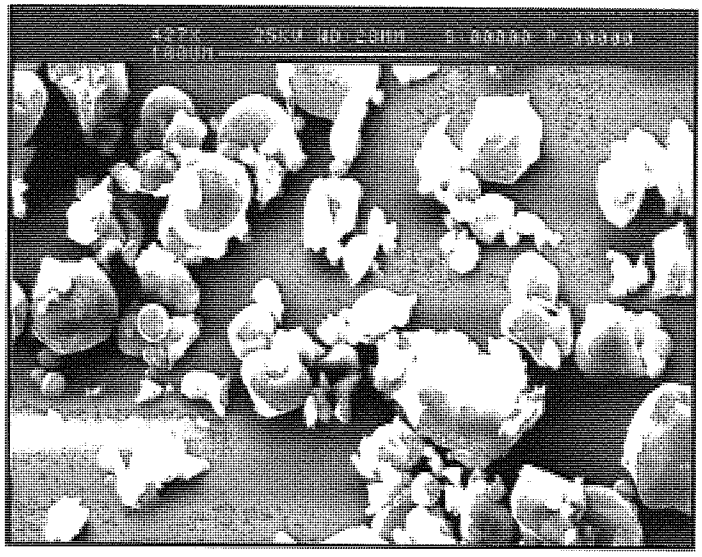


FIGURE 3.35 Scanning Electron Micrograph of large (10-20μm) PLGA Mw 3,000 after exposure to PBS pH 7.4 release medium at 37°C for 14 days

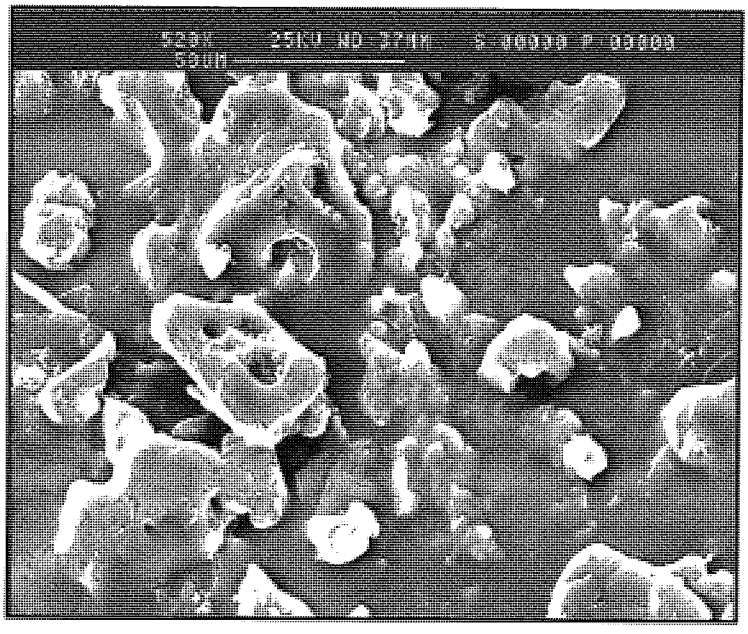


FIGURE 3.36 Scanning Electron micrograph of large (10-20μm) PLGA Mw 3,000 after exposure to PBS pH 7.4 release medium at 37°C for 56 days

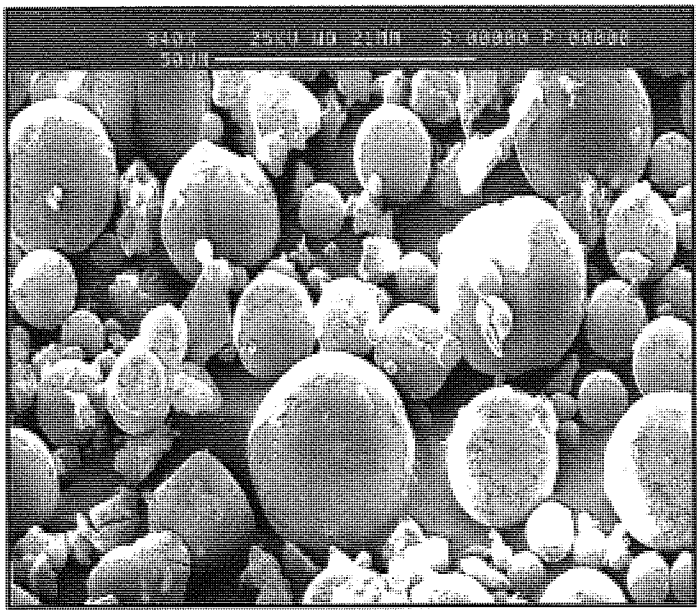


FIGURE 3.37 Scanning Electron Micrograph of large (10-20μm) PLGA Mw 40,000 after exposure to PBS pH 7.4 release medium at 37°C for 7 days

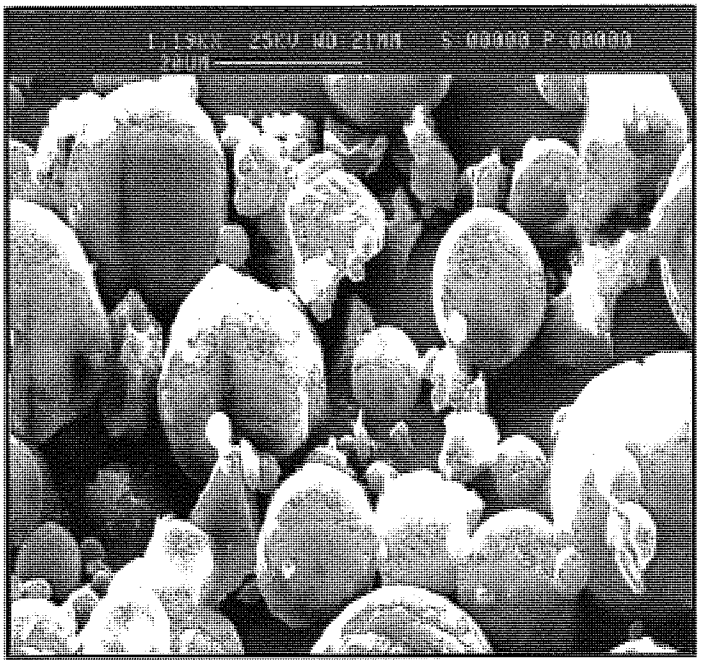


FIGURE 3.38 Scanning Electron Micrograph of large (10-20μm) PLGA Mw 40,000 after exposure to PBS pH 7.4 release medium at 37°C for 14 days

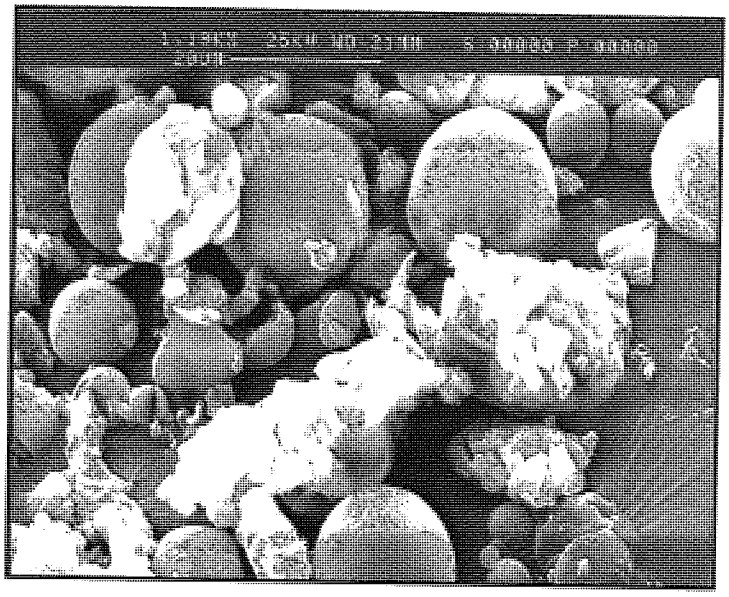


FIGURE 3.39 Scanning Electron Micrograph of large (10-20μm) PLGA Mw 40,000 after exposure to PBS pH 7.4 release medium at 37°C for 35 days.

CONCLUDING REMARKS

In this chapter microsphere formulation studies have been characterised. The solvent evaporation technique employed was suitable for producing microspheres in the range of both 10-20μm, and 1-5μm with controllable ODN loading and yields. PLGA Mws of 3,000 and 40,000 both produced successful batches of microspheres. For the larger spheres production yields of up to 90% were achieved, with loadings of up to 60%. However, for the smaller spheres loadings of up to 30% were achieved, further studies are necessary which includes optimisation of loadings for these spheres to enhance their therapeutic potential. Analysis of the physico-chemical characteristics of the microspheres produced allows detection of differences between different formulations in terms of crystallinity, hydrophobicity, surface charge residual solvents and surfactant levels. The retention of residual and PVA in the microspheres was also minimal.

The entrapment of ODNs into the large microspheres appeared to be high with loadings up to 46.8μg/mg polymer achieved. ODN length, sequence, and chemistry were also studied and found to have no significant effect on loading efficiency. The effects of various parameters on release profiles were also studied. Increasing the loading of ODNs in the polymer microspheres increased the burst and the rate of release of the ODN. This was also seen with decreasing particle size of spheres. A difference in the release from PLGA microspheres of Mw 3,000 and 40,000 was evident, due to faster degradation of the low Mw polymer resulting in a greater rate of ODN release. Release of ODNs of different chemistries was compared, however little difference in release rates was seen. With ODN length, the smaller 7-mer was released faster than longer ODNs.

Adsorption of ODNs onto blank PLGA microspheres was also assessed to determine any effects on the initial burst effect, and also as an alternative to encapsulation within microspheres. Minimal quantities of adsorbed ODN were achieved, with the majority of ODN released within two days following incubation. The stability of ODNs in PLGA microspheres was also confirmed. The biodegradable polymers enhanced the stability half life of ODNs when incubated in serum and cell culture media compared to free nucleic acids.

CHAPTER FOUR

CELLULAR INTERACTIONS OF PLGA MICROSPHERES

4.1 INTRODUCTION

In order for antisense ODNs to fulfil their potential in the treatment of diseases caused by undesirable gene expression, it is essential to deliver them to their intracellular target sites (mRNAs) within the cell nucleus and /or cytoplasm. Problems associated with delivery involve poor biological stability of antisense ODNs. Strategies to improve delivery include the use of chemically modified ODNs with enhanced stability, which have been discussed in chapter one (see section 1.3). Delivery and stability can also be enhanced by the use of delivery systems e.g. microspheres which protect the entrapped ODN from the extracellular environment. It is important to understand the behaviour and intracellular fate of such delivery systems.

The mechanisms of cellular uptake of antisense ODNs have been thoroughly investigated, and a number of mechanisms have been proposed (for reviews see Akhtar and Juliano, 1992; Rojanasakul, 1996). Cellular uptake of antisense ODNs is an energy dependant process, which is inhibited at low temperatures and by metabolic inhibitors (Wu-Pong *et al.*, 1992). Certain studies have reported that PO ODNs bind to a specific surface protein and enter cells by RME (Loke *et al.*, 1989). However, others have reported concentration dependant binding to a wide range of cell surface proteins or lipids, a non-specific process thought to involve ionic interactions between the anionic ODNs and surface proteins (Yakubov *et al.*, 1989; Shoji *et al.*, 1991; Beck *et al.*, 1996). These studies indicate a non-specific uptake mechanism of AE at low ODN concentrations, with FPE occurring at higher ODN concentrations when the AE mechanism becomes saturated.

As a consequence of the poor cellular uptake and inappropriate localisation of ODNs, it is necessary for either high concentrations of ODNs to be administered to cells, or for

alternative strategies, which can enhance delivery, to be used. For localised administration, repeated administration of high concentrations is not a viable prospect, therefore strategies to enhance cellular delivery are required.

In this chapter, cellular delivery of antisense ODNs encapsulated in biodegradable PLGA microspheres has been investigated. The encapsulation of a drug into microspheres has been shown to increase the delivery and activity of the drug to macrophages (Tabata and Ikada, 1987). Microspheres are known to be taken up by the reticulo-endothelial system and accumulate in phagocytic cells. The extent to which particles are taken up depends on various factors including their size, surface charge and hydrophobicity. Macrophages can phagocytose microspheres in the size range of 1 to 20μm (Yamaguchi and Anderson, 1993). The optimum size for phagocytosis has been reported by Kimura *et al.,* (1994) as being 1 to 2μm. Charged hydrophobic particles (anionic and cationic) are taken up to a greater degree than non-charged hydrophilic particles (Tabata and Ikada, 1989). Kimura *et al.*, (1994) investigated the cellular association of 1-2μm microspheres with retinal pigment endothelial cells, and found phagocytosis of microspheres to occur over a 12 hour period.

Akhtar and Lewis, (1997) evaluated the use of PLGA microspheres for the improved delivery of anti-HIV ODNs to macrophage cells. The cellular association of ODNs entrapped within 1-2 μm microspheres was improved 10-fold in murine macrophages compared with free ODNs. Uptake was also enhanced when macrophages were activated with interferon-γ and lipopolysaccharide. Intracellular trafficking studies revealed a punctate distribution of fluorescently labelled ODN encapsulated microspheres indicative of an endocytic mechanism (Akhtar and Lewis, 1997).

The aim of this section of study was to assess the association of free ODN and ODN encapsulated in polymer microspheres, in cultured cells as a method of improving delivery of ODNs to target cells. The main cell line studied was A431 cells.

4.2 MATERIALS AND METHODS

General materials and methods are outlined in Chapter Two. ODN synthesis, purification, labelling, microsphere preparation, and cell culture techniques are all described in detail in Chapter Two; alterations to the methods are outlined in the sections below.

In each experiment, free ODN or microsphere-encapsulated ODN was added to each well. A schematic diagram of the method is shown in figure 4.1. Microsphere characteristics in terms of size, surface charge have been assessed in Chapter Three.

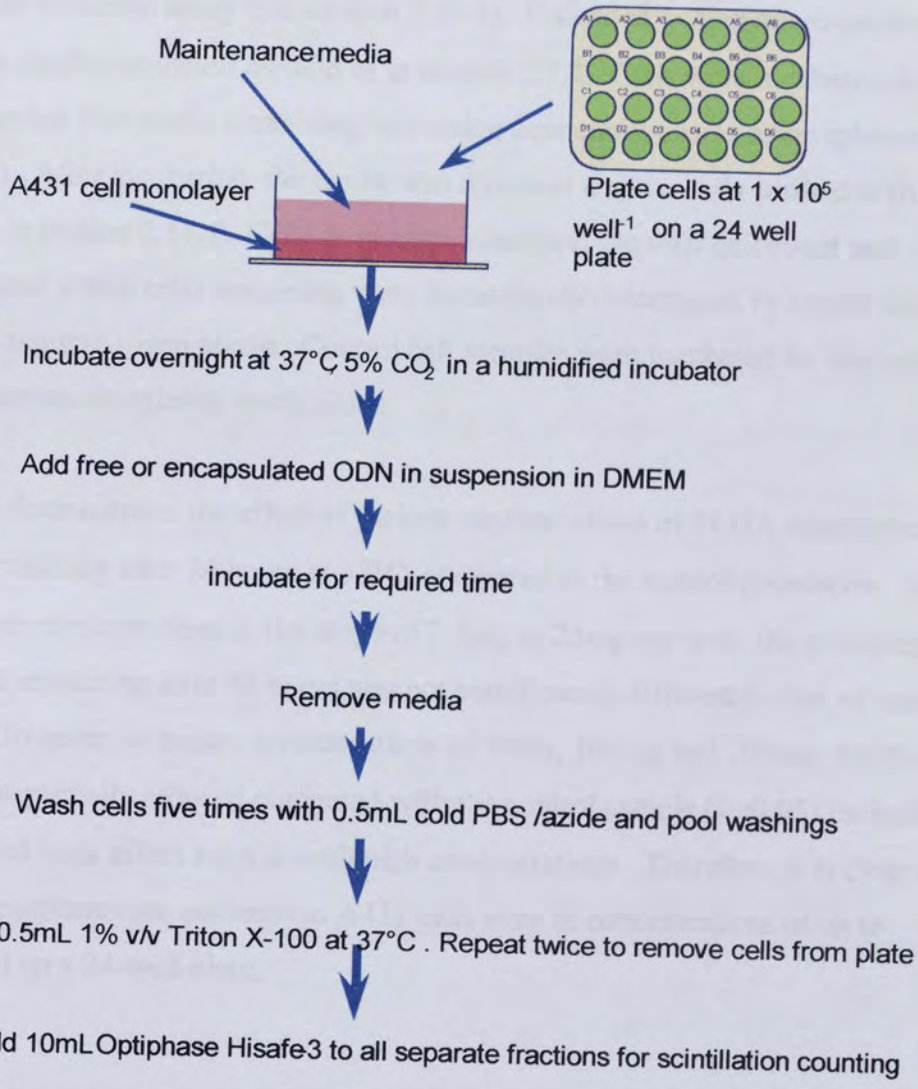


FIGURE 4.1 Schematic Diagram of the Experimental Procedure for Assessing Cellular Association

4.3 RESULTS

4.3.1 Toxicity of PLGA Microspheres on A431 Cells and Characterisation of Cells

PLA and its copolymers have been widely used in the delivery of many drugs. Although their non-toxicity has been reported, it is essential to determine their effects on cells.

To assess toxicity of microspheres, cell viability of A431 cells was measured using the trypan blue exclusion assay (see section 2.10.4). Unloaded 1-2μm microspheres were made by a double emulsion method as in section 2.7.3. Cells were incubated for twelve hours in serum free media containing increasing concentrations of microspheres (2.5mg to 200mg). After incubation, the media was removed and the cells washed with PBS as described in section 2.11.1. Cells were trypsinised and the total cell count and percentage of viable cells remaining were immediately determined by trypan blue exclusion assay as given above. Control cell samples were incubated for the same time period in serum-containing media alone.

Figure 4.2 demonstrates the effect of various concentrations of PLGA microspheres on A431 cell viability after 24 hours at 37°C, compared to the control population. At microsphere concentrations in the range of 2.5mg to 25mg per well, the percentage of viable cells remaining after 24 hours was not significantly different to that of control samples. However, at higher concentrations of 50mg, 100mg and 200mg, viable cell number was partially reduced compared with the control sample ($P<0.05$) indicating only a partial toxic effect even at such high concentrations. Therefore, it is clear that PLGA microspheres are not toxic to A431 cells even at concentrations of up to 100mg/well on a 24-well plate.

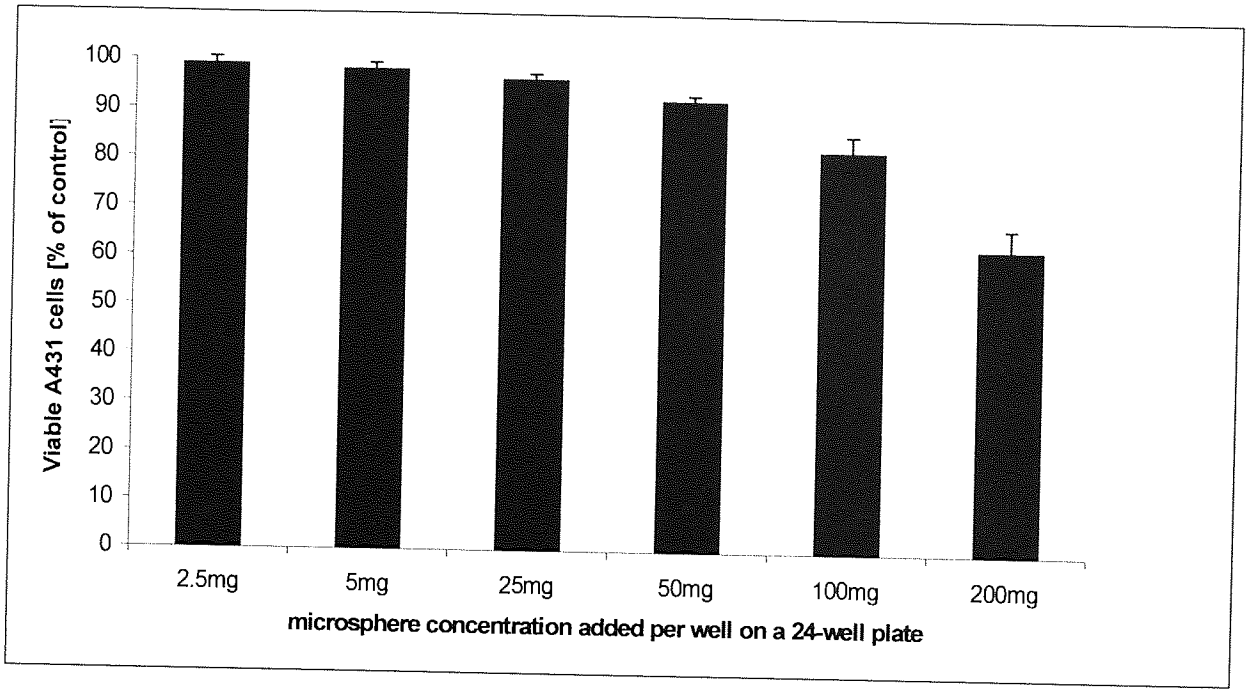


FIGURE 4.2 Graph of the Percentage of Viable A431 cells Remaining After Treatment with Various Concentrations of PLGA Mw 3,000 Microspheres size 1-2μm, for 24 hours at 37°C Compared with Control. (n=6 ±SD)

The total viable cell count of cells incubated in serum free media was not significantly different to the control sample group grown in serum containing media over the same time frame. Also, the cell viability for samples exposed for a four hour time period to PLGA microspheres as above, showed minimal toxicity after the 4 hour exposure time.

With reference to the growth curve obtained for A431 cells (see figure 4.3), the doubling time was in excess of 12 hours. Therefore a cytotoxic, rather than anti-proliferative effect would appear to offer the most likely explanation of the reduction in cell number following exposure to high microsphere concentrations. The aim of this study was to establish the maximum, non-toxic concentration of microspheres that could be administered during cell association studies, and also to gain an insight into any *in vivo* toxicity, which may occur.

To characterise the A431 cell line, a growth curve was established in serum containing media. In order to calculate the growth curve, cells were seeded as described in section 2.10.1 for various time periods at 37°C. Cells were trypsinised and viable cell counts were calculated by trypan blue exclusion assay as described in section 2.10.4.

The growth curve (figure 4.3) indicates that following seeding, A431 cells enter a lag period of at least 12 hours, followed by a period of exponential growth. Cell number increased by a mean value of 52% after 24 hours. The cell doubling time during the exponential growth phase, estimated using the method of Grittiths (1992), was approximately 34 hours, a doubling time typical of this cell line (Gheradi, 1996).

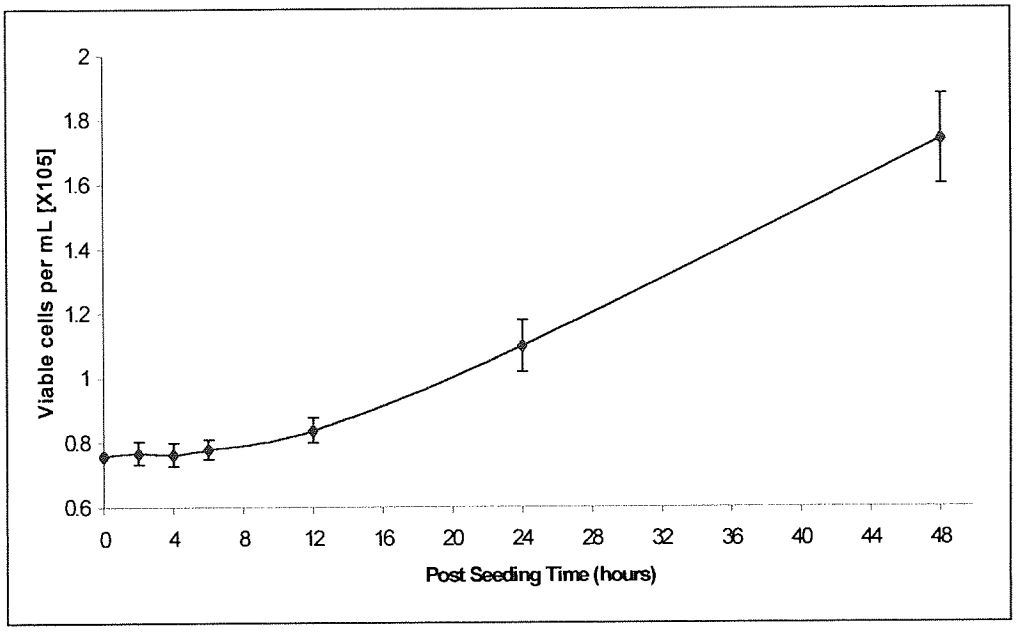


FIGURE 4.3 Standard Growth Curve for A431 Cells Calculated from a Seeding Concentration of 7.5 x 10^4 Cells / mL at 37°C. (n=6±SD)

4.3.2 Optimisation of Cell Association Studies

4.3.2.1 The Influence of Cell Washing on Cell Association after Incubation with ODNs and PLGA Spheres

In order to establish the number of PBS / azide washes required to remove loosely bound or non-cell associated ^{32}P labelled ODNs from the cell surface, assays were performed as detailed in section 2.11.5. The percentage of non-cell associated ODN and microspheres, which were removed by each of five PBS / azide washes was calculated and the results are shown in figure 4.4.

The results indicate that over 99% of non-cell associated radioactivity i.e. that which can be removed from cells by apical washing, was removed by 4 x 1mL washes for free ODN; whereas for ODN loaded microspheres, five washes were sufficient for removal of loosely associated radioactivity.

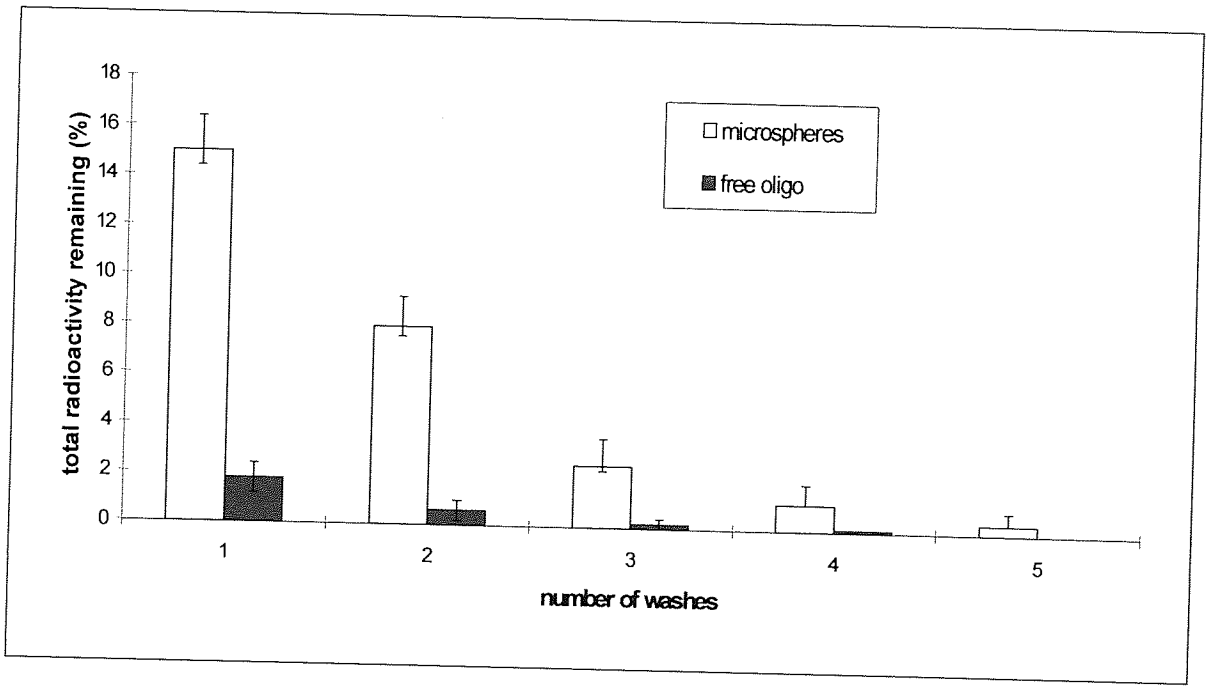


FIGURE 4.4 Removal of Non-Cell Associated ODN or ODN Loaded Microspheres from the Surface of A431 Cells by Consecutive PBS / Azide washes PLGA 2μm Spheres Mw 3,000 after 24 hours. (n =4± SD)

As a result of these findings, in subsequent cell association studies a five step washing protocol was used to remove non-bound or loosely cell associated radioactive material. Any remaining radioactivity would be assumed to be cell associated.

4.3.2.2 The Effect of Cell Number on Microsphere Association

A range of cell concentrations were plated out to determine the optimum cell number for maximum cell association of microspheres. Cell numbers in the range from 1 x 10^4 to 1 x 10^6 were plated out in a 24 well plate, and a volume of 0.5mL of a 5mg/mL sphere suspension added. The spheres were incubated with the cells for 24 hours at 37°C.

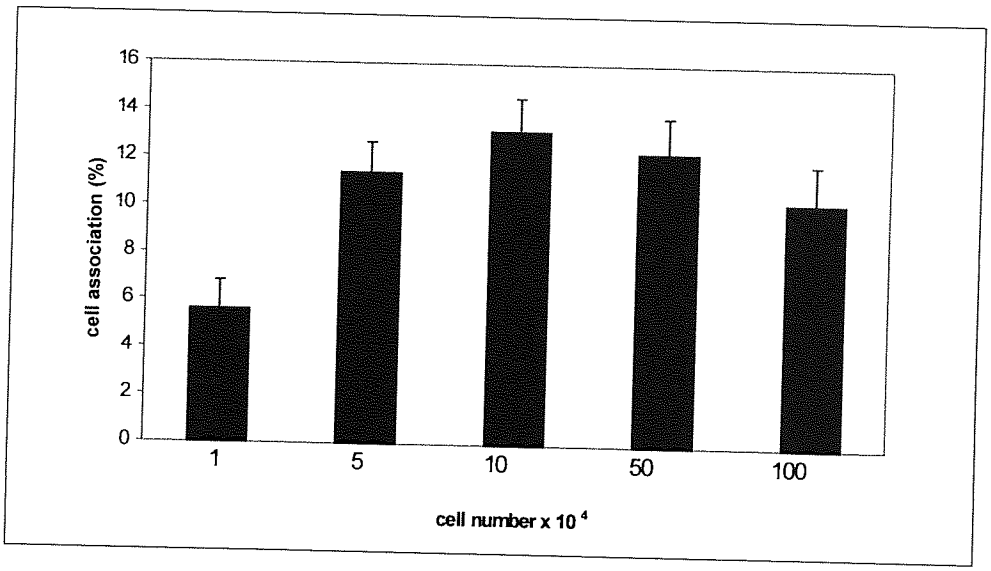


FIGURE 4.5 The Effect of A431 Cell Number on the Percentage Cellular Association of 2μm PLGA Mw 3,000 spheres. Association was Determined in Serum Containing Media at 37°C after 24 hours. (n=4±SD)

The results do not show a significant difference in sphere association from 5 x 10^4 to 1 x 10^6 cells per well (see figure 4.5), however optimum cell association is seen at the cell seeding of 1 x 10^5. Differences in cell associations may be due to not all cells internalising spheres or the spheres being unevenly distributed over the wells. In all further cell association experiments, cells were plated at 1 x 10^5 cells per well.

4.3.2.3 Influence of Sphere Dose on Cellular Association

To determine the amount of polymer spheres, which can be internalised by A431 cells, a range of polymer sphere concentrations were investigated. Sphere suspensions of concentrations from 1 to 20mg/mL, in a volume of 0.5mL, were added to each well of a 24 well plate (1 x 10^5 cells/well). The suspensions were incubated with the cells for 24 hours at 37°C.

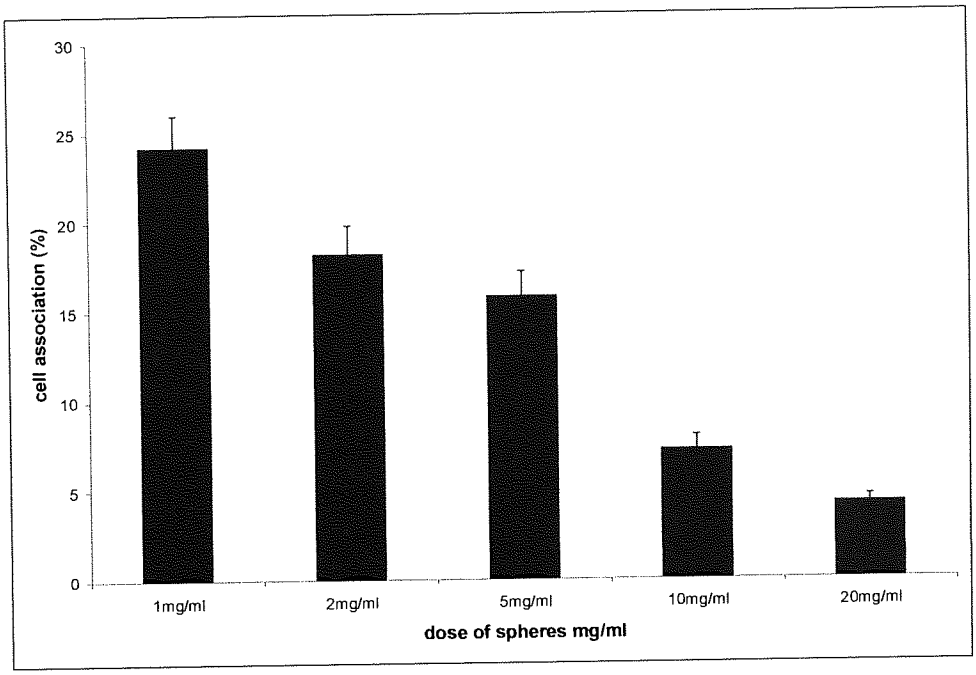


FIGURE 4.6 The Effect of the Polymer Dose on Percentage Cell Association of 2μm PLGA Mw 3,000 spheres in A431 cells. Association was determined in Serum Containing Media at 37°C for 24 hours. (n=4±SD)

With increase in sphere concentration, a proportional increase in percentage cellular association was not seen (see figure 4.6). Thereby it can be assumed that the association process may be saturable. Chavaney *et al.*, (1994) reported that doubling the dose of spheres did not result in doubled cellular association. The results show that a maximum polymer association is possible for A431 cells. At a 5mg /mL dose of spheres, approximately 375μg of polymer spheres were internalised.

In all subsequent experiments spheres were added at a concentration of 5mg/mL to ensure maximum association was possible.

4.3.3 Time Profile of Cell Association of Polymer Microspheres Compared with a Phosphorothioate ODN and Markers for Fluid Phase Endocytosis

To investigate the cellular association of PLGA microspheres over a 24 hour period in A431 cells, the protocol described in section 2.11.2 was used. A time profile of the cell association of small (1-2μm) and large (10-20μm) microspheres was obtained, and compared to that of D-[^{14}C] mannitol which enters cells exclusively by FPE and can therefore be used as a marker for this process (Levis *et al.*, 1995). Cellular association of microspheres was also compared with PS ODNs, which are generally considered to enter cells by a process of AE and / or RME. Cell association of a 20-mer internally radiolabelled PS ODN as described in section 2.11.1 was tested in A431 cells.

The results of these studies are illustrated in figure 4.7. Association of free ODN and ODN-loaded microspheres increased with time over the 24-hour period of the study. After 24 hours, approximately 17% of small microspheres were internalised, whereas approximately only 4% of the larger spheres were internalised over the same time period, indicating that size is an important factor for optimum cellular association. Tabata *et al.*, (1988a) reported an optimum size of 1-2 μm for particulate internalisation by macrophages. Cell association of the free radiolabelled PS ODN was significantly lower than for particulate association, a maximum cell association of approximately 1.5 % was seen after the 24 hours. Hence all cell association studies using microspheres were incubated for 24 hours and ODNs were incubated for 4 hours to ensure maximal association.

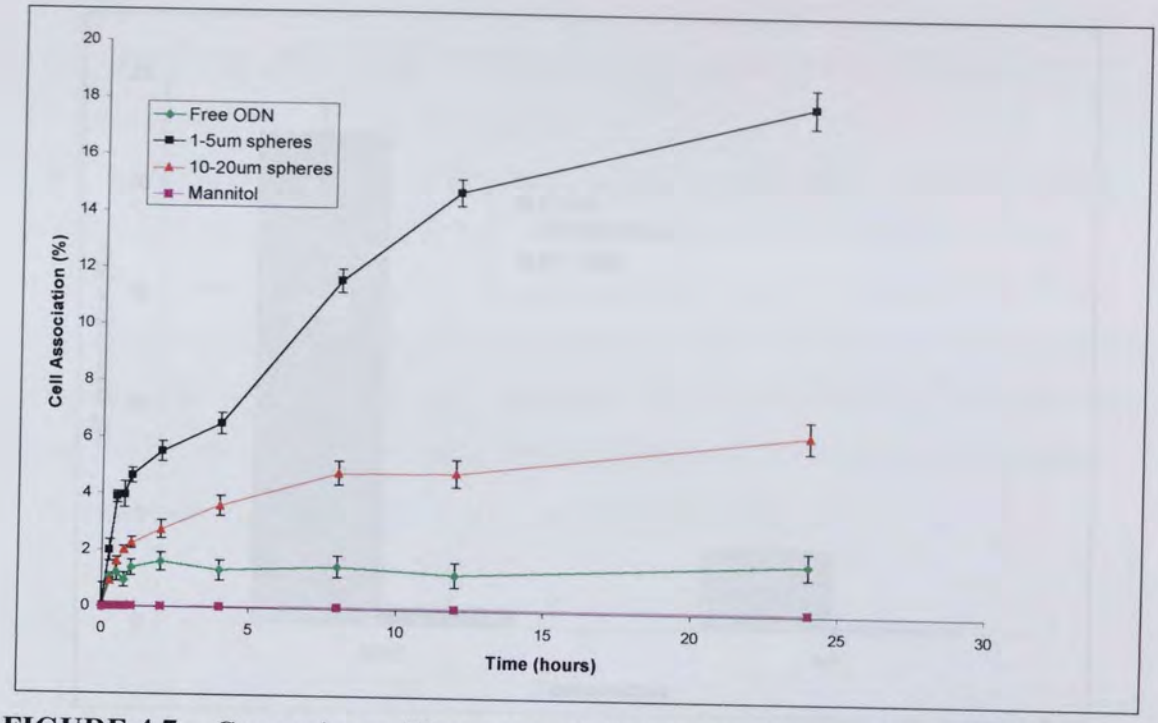


FIGURE 4.7 Comparison of Percentage Cellular Association of Free PS ODN and ODN-loaded PLGA Mw 3,000 microspheres size range (1-2μm and 10-20μm) in A431 cells at 37°C. The free ODN was added in equal concentration to the entrapped ODN. (n=4± SD)

4.3.4 Inhibitors of Microsphere Association

4.3.4.1 The Effects of Temperature on Cell Association in A431 cells

To assess the effect of temperature on the cellular association of radiolabelled ODNs and ODN-loaded microspheres, cell association was tested in A431 cells as described in sections 2.11.7. Spheres were added in a volume of 0.5mL at a dose of 5mg/mL at 37°C, and 4°C. The results are shown in figure 4.8. It is clear that at 4°C, cell association of both ODN and PLGA microspheres was markedly reduced ($P<0.006$) compared to 37°C. These results are similar to those reported for ODNs by Wu-Pong *et al.*, (1992) and Shoji *et al.*, (1996). Akhtar and Lewis, (1997) reported similar cell association reduction at similar temperatures for PLGA microspheres in a murine macrophage cell line.

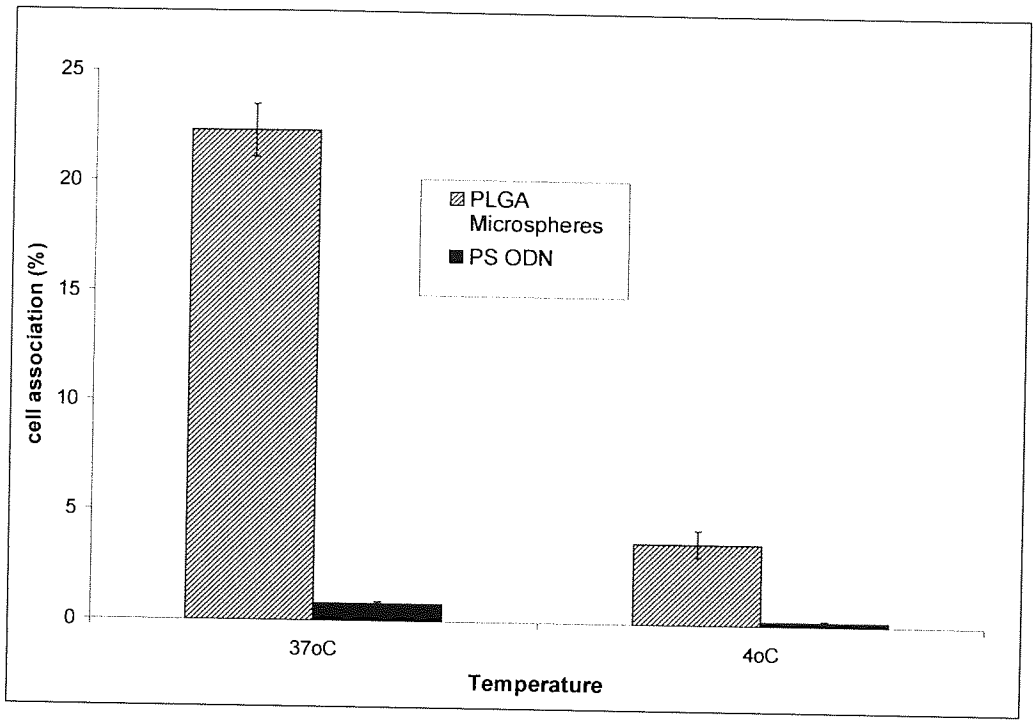


FIGURE 4.8 The Effects of Temperature 37°C and 4°C on the Cell Association of 20mer PS ODN and 2μm PLGA Mw 3,000 microspheres in A431 cells. (n=4±SD)

The reduced association for both ODN and microspheres at 4°C indicates that the internalisation of microspheres is temperature dependent, and probably occurs by an active mechanism requiring cellular energy such as an endocytic / phagocytic process.

4.3.4.2 The Effects of Metabolic Inhibition on Cell Association in A431 cells

To assess the effect of metabolic inhibitors on the cellular association of radiolabelled ODNs and ODN-loaded microspheres, cell association was tested in A431 cells as described in section 2.11.8. Metabolic inhibitors were assessed to further demonstrate that microspheres were being internalised by an active mechanism and not by simple adsorption. The inhibitors used were 10mM sodium azide and 50mM 2-deoxyglucose. The cells were pre-treated for 4 hours before addition of spheres at a dose of 5mg/mL in a 0.5mL volume at 37°C. The results are illustrated in figure 4.9.

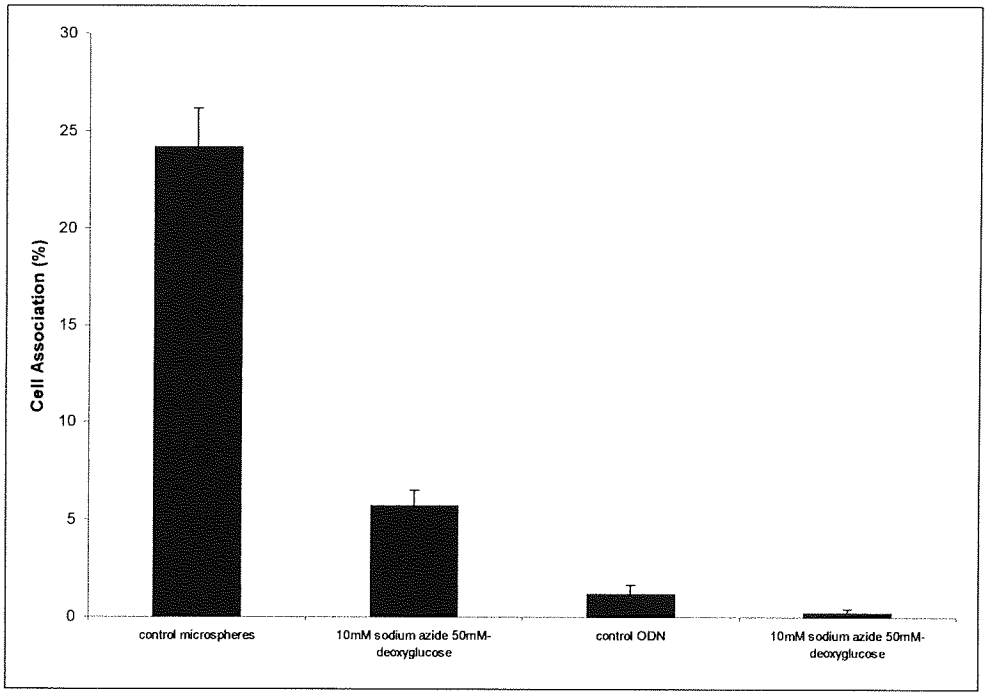


FIGURE 4.9 Graph Showing the Effect of Metabolic Inhibitors on the Percentage Cell Association of PLGA Mw 3,000 spheres, and 20mer PS ODN in A431 Cells as a Function of Time, Compared with Control. (n=6 ±SD)

Fig 4.9 shows that metabolic inhibition reduced the extent of ODN and microsphere cell association compared with the control. Metabolic inhibition reduced cell association by approximately 70% for both ODN and spheres ($P<0.006$). Similar effects of metabolic inhibitors on cell association of antisense ODNs in other cell lines has been reported by Wu Pong *et al.*, (1992) and Beck *et al.*, (1996). Akhtar *et al.*, (1997), have previously reported similar reduction for PLGA spheres in a murine macrophage cell line.

The reduction in cell association further indicates the requirements for cellular energy, which may arise from the demands of an active process. This provides further evidence that spheres and ODN are being internalised by an active process such as RME. The reason for incomplete inhibition of cell association may be incomplete ATP depletion by the sodium azide/2-deoxyglucose combination, or energy independent surface binding of ODN and microspheres.

4.3.5 The Effect of ODN Competition on Cell Association of PLGA Microspheres

To assess the effect of competition of microspheres with ODNs, an increasing excess of unlabelled ODN was added both prior to, and during cell association studies. The results shown in figure 4.10 were obtained after 24 hours incubation with 0.5mL of a 5mg/mL sphere suspension at 37°C. The control represents cell association of 0.5mL of 5mg/mL sphere suspension.

The addition of a 1μM concentration of unlabelled ODN demonstrated no significant difference in cell association of spheres after 24 hours. However, with increasing concentration of competing excess unlabelled ODN, a reduction in cell association of spheres was seen ($P<0.006$). With an excess concentration of 100mM ODN, cell association was reduced by 10%, as compared to the control after 24 hours. This reduction indicated that to a certain degree the PLGA microspheres were competing with the excess unlabelled ODN for cell association. It is most likely that the competition is on the surface of the cell, possibly with both microspheres and ODN competing for binding sites on the cell surface.

PS ODNs are generally considered to enter cells by either AE or RME, therefore the spheres may be competing for cellular uptake via one of these mechanisms. Saturable and self competitive cell association for PS ODNs has been demonstrated by a number of studies, and is due to a specific cell surface binding mechanism which may be possibly mediated by surface proteins or lipids (Zhao *et al.*, 1993; Beltinger *et al.*, 1995; Shoji *et al.*, 1996).

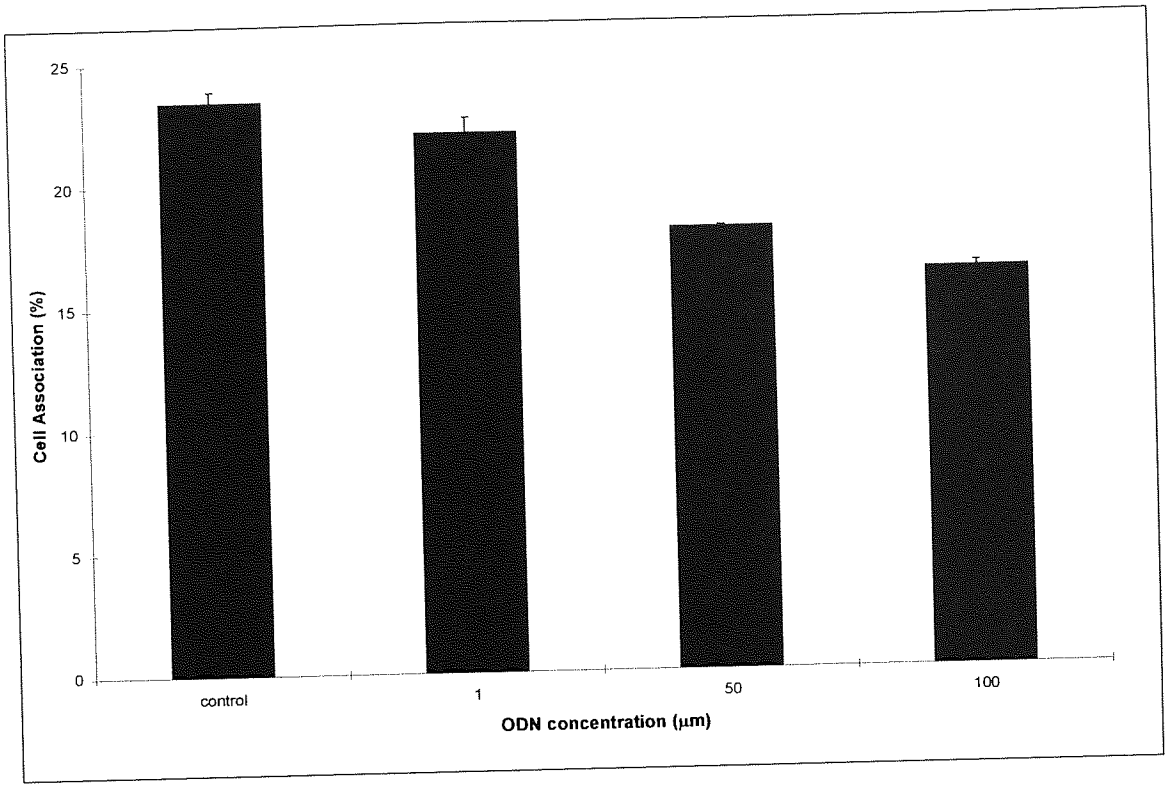


FIGURE 4.10 Graph Showing Percentage Cell Association of PLGA Mw 3,000 Microspheres (Size 1-2µm) with A431 Cells in the Presence of Excess Concentrations of Oligonucleotides. (n=4±SD)

4.3.6 The Effect of PLGA Mw 3,000 Microspheres Self Competition on Cellular Association of PLGA Microspheres

Further attempts were made to demonstrate the effect of 'self'-competition of spheres on cell association. Experiments were performed as described in section 2.11.4; excess concentrations of unloaded PLGA microspheres were added both prior to and during cell association studies at 37°C. The results are demonstrated in figure 4.11, which were obtained after 12 hours incubation with 0.5mL of 5mg/mL sphere suspension.

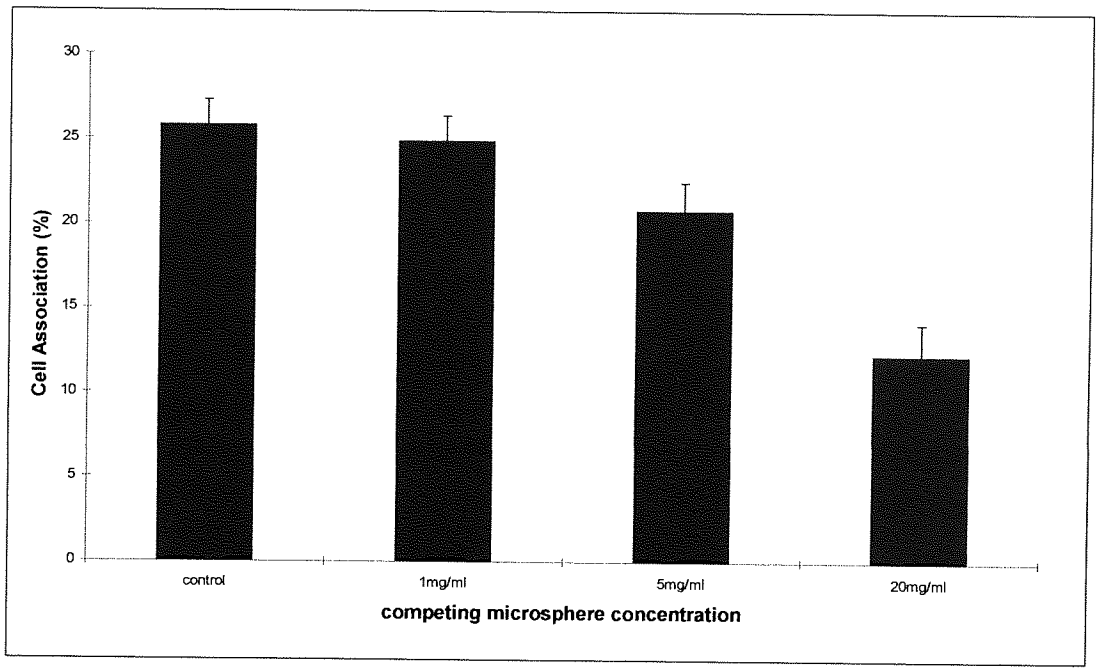


FIGURE 4.11 Graph Showing Percentage Cell Association of PLGA Mw 3,000 Microspheres (Size 1-2μm) with A431 Cells in the presence of Excess Concentrations of PLGA Mw 3,000 Microspheres. (n=4±SD)

The addition of excess microspheres showed minimal difference in cell association for competition with 1mg/mL concentration of microspheres. However, approximately 2% reduction in association was seen for the 5mg/mL concentration, and an approximately 12 % reduction resulted from the 20mg/mL concentration. This indicated that the radiolabelled, ODN loaded microspheres were competing with the excess spheres, although a significant reduction ($P<0.006$) in association is only seen after addition of a 20mg/mL concentration of spheres. Thereby we may assume the spheres are also competing for the same uptake mechanism, and competition appears to be

concentration dependent. A similar reduction in association is seen for excess ODN competition as in section 4.3.5.

4.3.7 The Effect of 'Competitor' Chain Length on Cell Association of PLGA Microspheres

Further investigations were conducted to determine whether ODN chain length had any effects on sphere cell association. Competition studies were performed as previous, using 20mM of salmon testes DNA and 20mM dATP, as competing agents. The salmon testes DNA (587-831 base pairs, Sigma D-9156) was used to demonstrate the effect of competition with a long chain DNA sequence. The dATP was used to demonstrate the effect of competition with a single base monomer.

Cell association was determined after a 24-hour incubation with a 0.5mL dose of 5mg/mL sphere suspension both with and without excess competing agent at 37°C. The results are displayed in Figure 4.12.

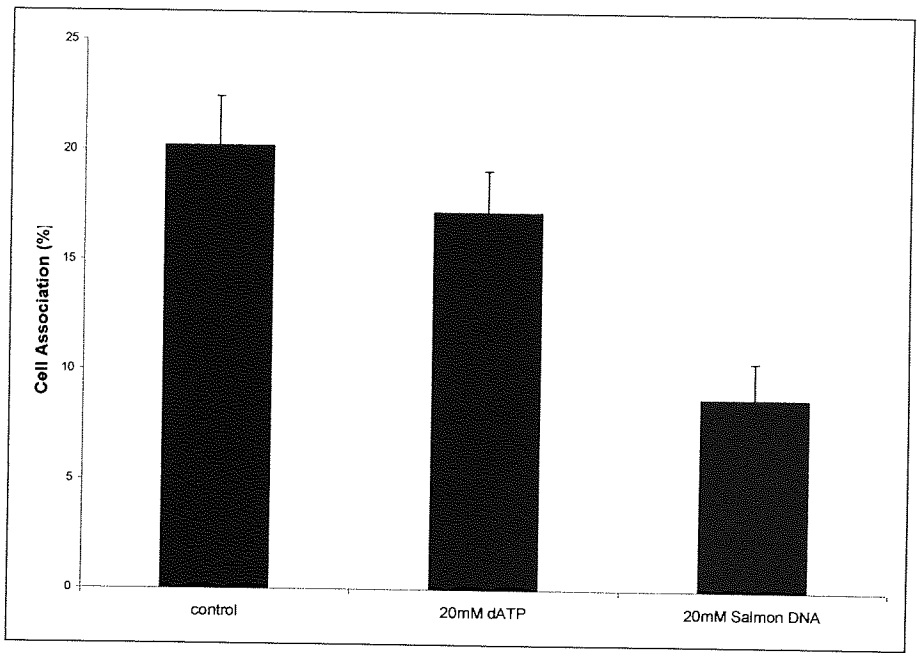


FIGURE 4.12 Graph Showing Percentage Cell Association of PLGA Microspheres with A431 Cells in the Presence of Excess Concentrations of Salmon Testes DNA and dATP after 12 hours. (n=4±SD)

Cell association of spheres was significantly reduced ($P<0.006$) in the presence of excess salmon testes DNA, however, no significant difference in cell association was seen in the presence of dATP. This indicates competition between the spheres and nucleic acids for cellular uptake and / or binding. Clearly, competitive inhibition by salmon testes DNA was significantly greater than both that produced by a 20mer PS ODN, and 20mM dATP. These results indicate that nucleotide chain length may increase inhibition of sphere cell association.

The inhibitory effects of salmon testes DNA and dATP on cell association has been shown with PO ODNs by Wu-Pong *et al.*, (1996). An increase in ODN length was shown to increase cellular association. This may be as a result of an increase in the anionic character of the ODN allowing greater ionic interaction with cell surface proteins (Beck *et al.*, 1996).

4.3.8 The Effect of High Molecular Weight Polyanions on Cellular Association of PLGA Mw 3,000 Microspheres

To investigate the competitive effect of non-nucleic acid polyanions on cellular association of spheres, studies were carried out as described in section 2.11.9. Heparin is a polyanionic compound that can potentially compete with ODNs for cellular association, if the process was mediated by ionic interactions with cell surface structures such as proteins.

The results are shown in figure 4.13, and represent cell association of 0.5mL of 5mg/mL spheres in the presence of a competing polyanion. The control sample represents cell association of 0.5mL of 5mg/mL spheres in the absence of any competing polyanion. Cell association of spheres was significantly reduced ($P<0.006$) in the presence of heparin.

Heparin has been previously reported to competitively inhibit cellular association of antisense ODNs *in vitro* (Gewirtz *et al.*, 1996).

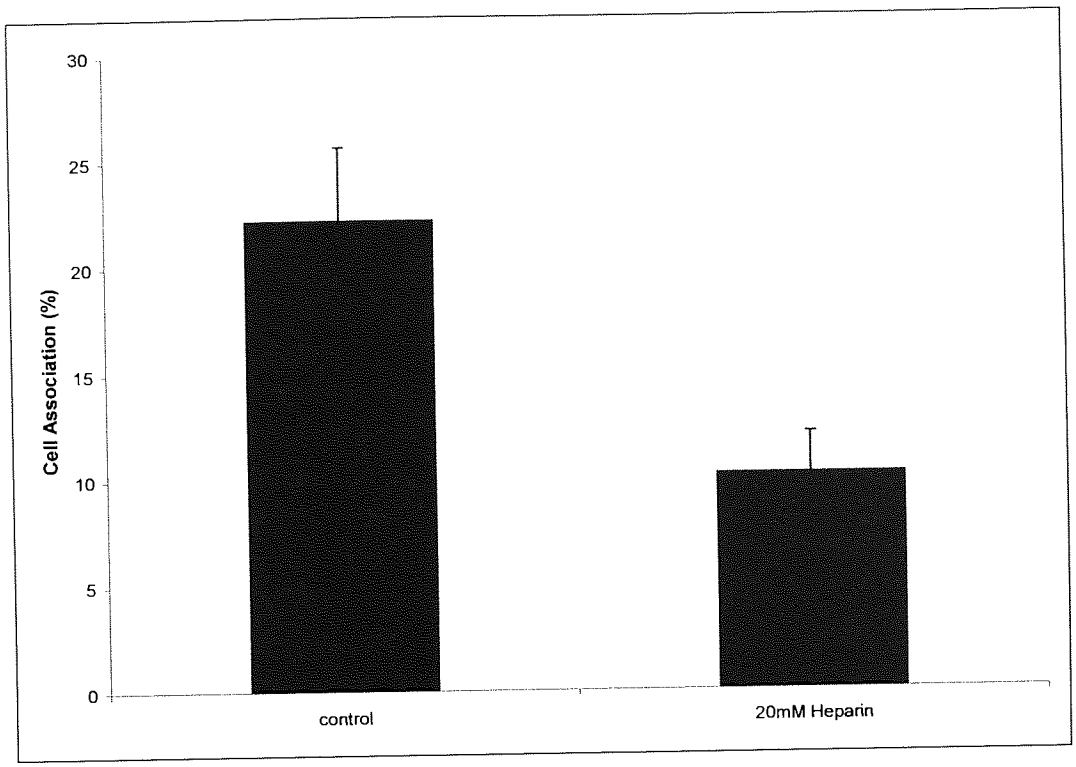


FIGURE 4.13 Graph Showing Percentage Association of PLGA Mw 3,000 Micropheres with A431 Cells in the Presence of Excess Concentrations of Heparin after 24 hours Incubation at 37°C. (n=4±S.D)

4.3.9 Comparison of PLGA Microsphere Association in A431 cells with Other Cell Lines

In order to compare the level of cell association of PLGA microspheres in A431 cells to other cell lines, cell association studies were performed using the method outlined in section 2.11.2, using A431 (vulval) epithelial cells, U87-MG (glial) cells, C6 (glial) cells and Caco-2 (colonic) cells.

The results of these studies, which are normalised to cell number, are displayed in figure 4.14. The greatest cell association for microspheres was seen for U87-MG cells. Generally a lower degree of association was seen with C6 and Caco-2 cells, with C6 cells showing the least association. However, no significant difference was seen between the cell lines for cell association.

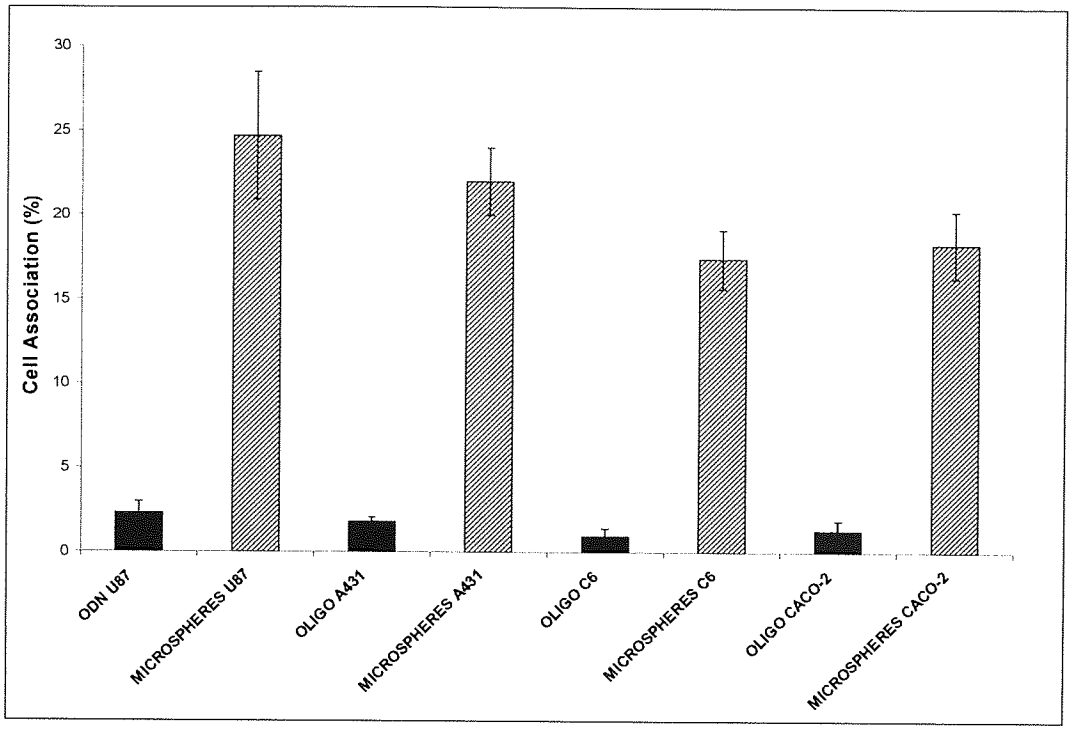


FIGURE 4.14 A Comparison of the Cellular Association of PLGA Mw 3,000 Microspheres and PS ODNs in Various Cell Lines. (n=4±S D)

It is important to realise the difficulties in comparing results from different cell lines, although general trends may be obtained. Variation in cellular association characteristics between each cell line is expected as each cell line varies in shape, size, and morphology. The uptake properties of PO and PS ODNs are also known to differ using different cell lines *in vitro* (Akhtar and Rossi, 1996).

4.3.10 Efflux of ODNs from A431 Cells

Following cellular association of antisense ODNs, it has been demonstrated that some of the ODNs having been internalised within cells are later exported to the extracellular environment (Wu Pong *et al.*, 1992; Stein *et al.*, 1993; Agrawal and Akhtar, 1995). The efflux of PS ODNs, microspheres containing ODNs, and D[1-^{14}C] mannitol was conducted as described in section 2.11.3.

A significant proportion of internalised ODN were exported from A431 cells in a biphasic manner following steady state accumulation (see figure 4.15). Approximately 30% of the internalised ODN was rapidly effluxed. This phase is known as the 'shallow compartment'. The remaining ODN, approximately 70%, was effluxed slower from the second phase known as the deeper 'B' compartment.

With regard to the PLGA microspheres, it can be seen that the efflux of microspheres / ODN also followed a similar biphasic trend. Approximately 35% being effluxed in the initial rapid phase, and the remaining 65% release over the deep second phase. The rate of efflux of microspheres appears to be faster than the free ODN. This may be due to the free ODN being internalised to deeper compartments in the cell over the same time period, whereas the spheres are not as internally associated, hence are effluxed at a faster rate.

Figure 4.15 also displays the cellular efflux of D- [^{14}C] mannitol. It must be noted that the overall rate of loss of mannitol from A431 cells is only representative, as the initial concentration of mannitol was added at a much lower concentration than ODN. However, this study does provide information regarding the rates of cellular efflux, to enable us to describe exocytosis from particular compartments.

Fluid phase markers e.g. mannitol, are known to vary in residence times in both compartments according to the load time used (Besterman *et al.*, 1981). Mannitol appeared to be distributed evenly between both the 'shallow' and 'deep' compartments following a load time of 4 hours. The amount of mannitol in the 'shallow' compartment after 4 hours appeared to be higher than that for both PS ODNs and ODN loaded spheres.

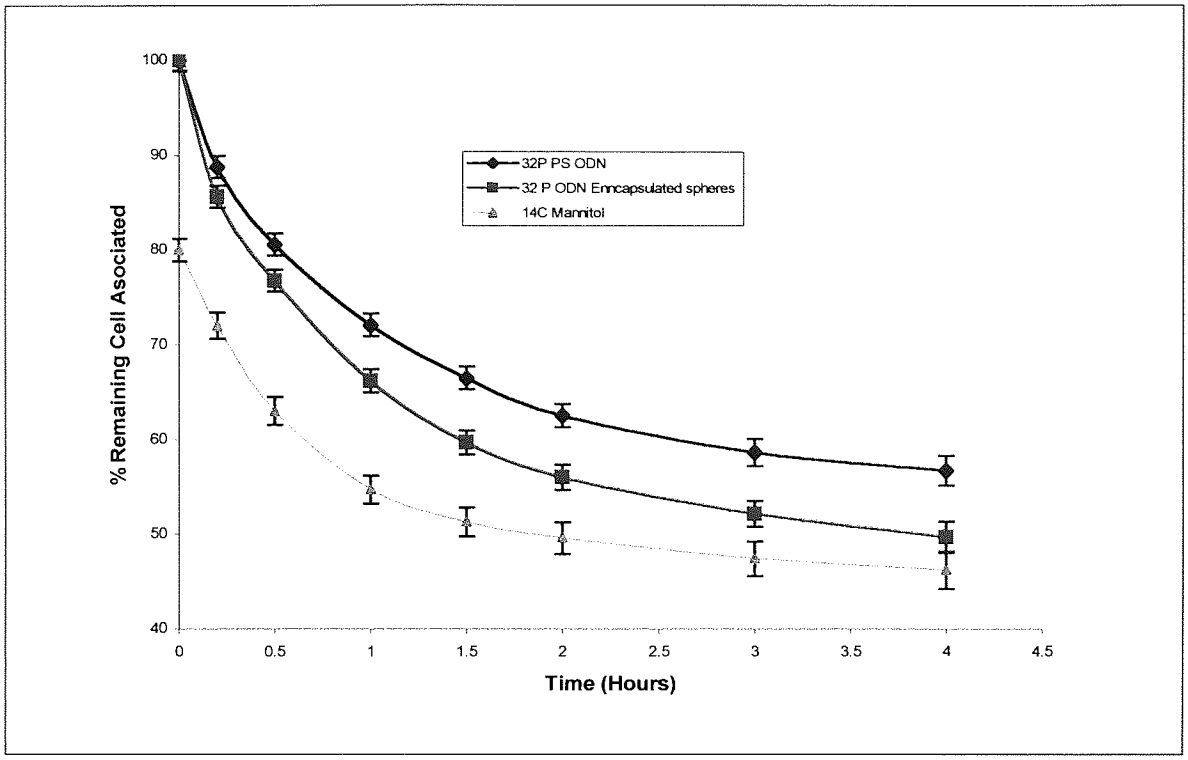


FIGURE 4.15 Graph Demonstrating the Rate of Loss of Radiolabelled Compounds from A431 Cells as Function of Time

4.3.11 Fluorescent Localisation Studies

A431 cells grown on chamber slides, as described in section 2.12.2, were incubated for 4 hours in serum free media and viewed under the fluorescent microscope. No auto-fluorescence was observed which indicated that fluoresence detected in subsequent studies would be due to the presence of exogenously delivered flurophores. Furthermore, phase contrast microscopy of untreated cells indicated that the appearance and morphology of these cells was similar to that of cells which were incubated with either 5μM FITC-labelled PS ODNs, FITC-labelled PS ODNs-encapsulated into PLGA spheres, RITC-dextran or free fluorescein.

Viable cell counts indicated that treatment with 5μM FITC ODN, and free fluorescein had no effect on viable cell numbers when compared with control samples of cells which were not exposed to fluorescent compounds.

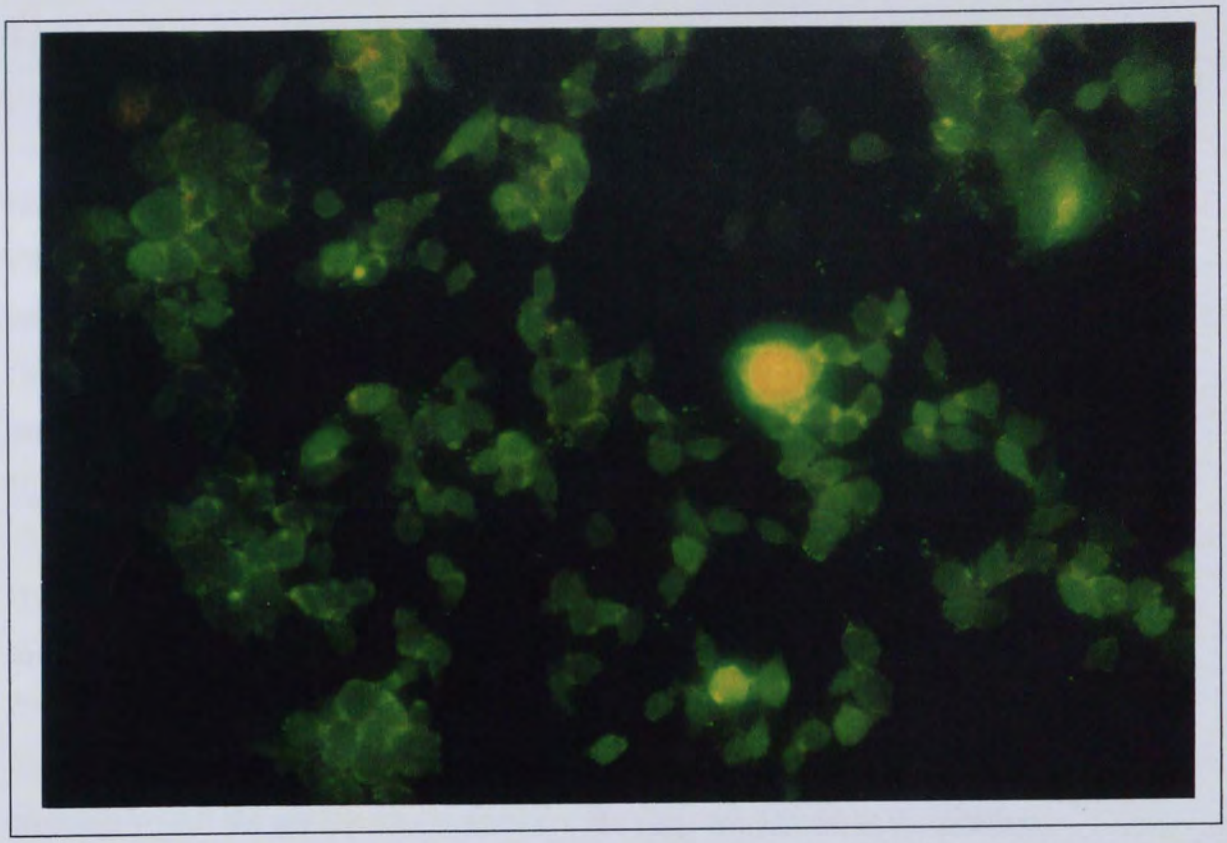


FIGURE 4.16 Fluorescence Detection of 'Free' FITC Label Associated with A431 Cells. (magnification x 10)

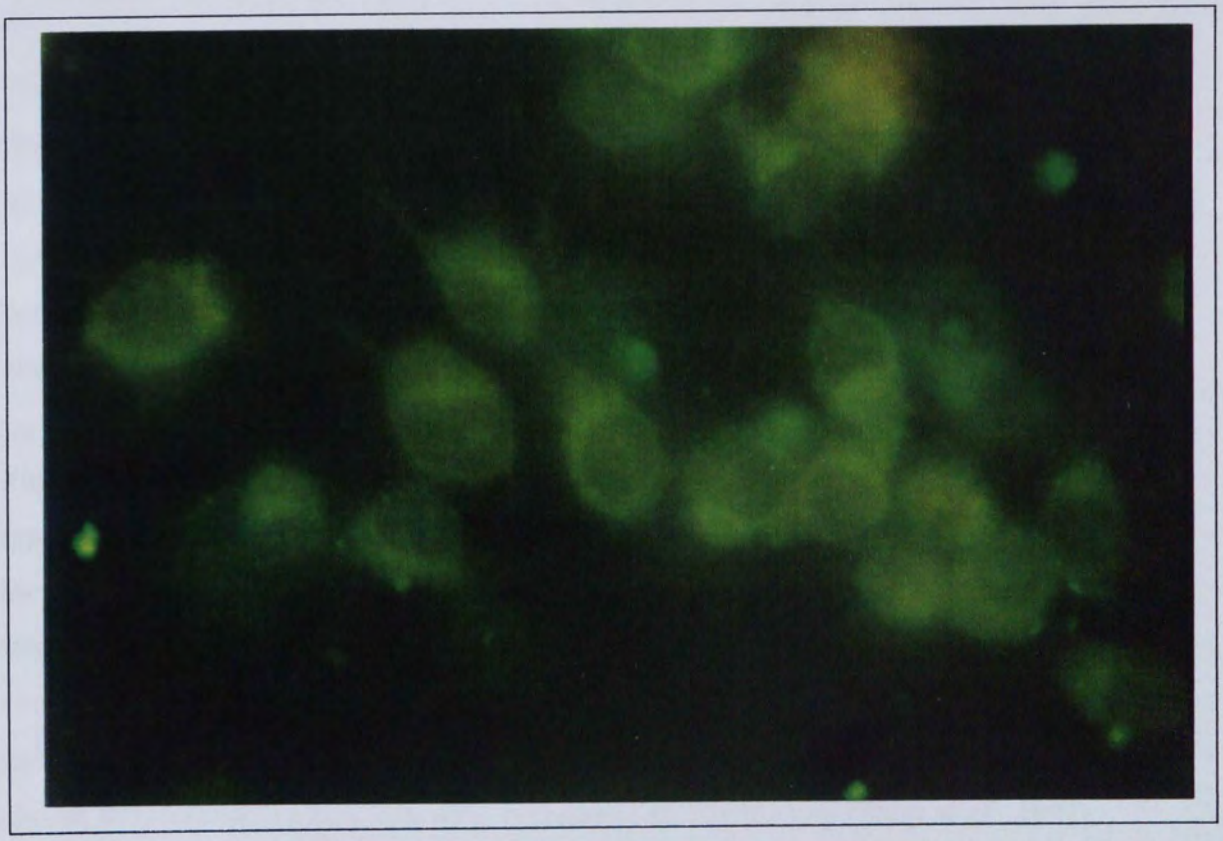


FIGURE 4.17 Fluorescence Detection of FITC-ODN Associated with A431 Cells. (magnification x 50)

4.3.11.1 Cellular Uptake and Distribution of FITC-Labelled ODNs Compared with Free FITC.

Incubation with 'free ' FITC label produced an intense fluorescence within A431 cells. Fluorescence was widely distributed throughout the cells with a considerable degree of intra-nuclear localisation apparent (see figure 4.16). This widespread pattern of fluorescence was largely expected, as such molecules have been shown to diffuse freely across biological membranes, and thus, penetrate diverse regions of living cells (Lansing-Taylor and Salmon, 1989).

By contrast, the cellular distribution of the FITC-labelled ODN was distinctly different to that of the free fluorescent labels (see figure 4.17). The intensity of cell associated fluorescence appeared to be considerably lower than that observed with the free fluorescent label. The majority of fluorescence was localised at the periphery of cells, probably within the cytoplasm. In addition, the punctate pattern of distribution observed would be consistent with a predominantly endosomal localisation of the FITC-ODN.

The clear differences in cellular localisation patterns of the FITC-ODN and free fluorophores indicated that the FITC label remained attached to the ODN in the intracellular environment for at least 4 hours and could therefore, be used as a specific label for fluorescence localisation studies. Similar punctate distribution patterns have been observed for the subcellular distribution of fluorescent labelled ODNs (Tonkinson and Stein, 1994; Tarrason *et al.*, 1995; Shoji *et al.*, 1991 & 1996).

Fluorescently labelled dextrans are known to reside in endocytic vesicles of various types following cell entry (Berlin and Oliver, 1980). In this study, A431 cells were incubated with rhodamine-labelled dextrans as a control. When the subcellular localisation of rhodamine-dextran was compared with that of FITC-labelled PS ODN, similar distribution patterns were observed (see figures 4.17 and figure 4.18). This indicated that the majority of FITC-PS ODNs and RITC-dextran were present within similar intracellular regions following cell entry / and or binding (Shoji *et al.*, 1991).

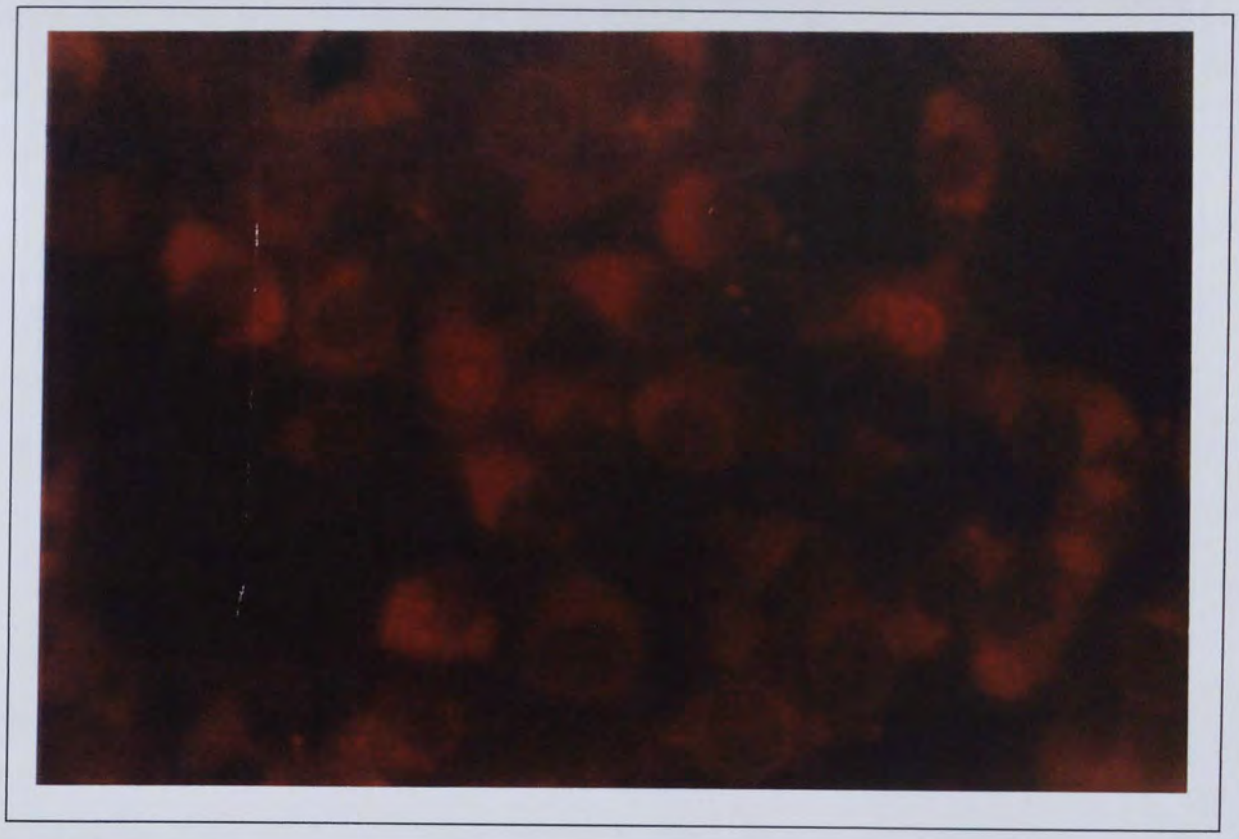


FIGURE 4.18 Fluorescence Detection of RITC-dextran in A431 Cells after 4 hours Incubation at 37°C. (magnification x 50)

The punctate pattern of subcellular distribution, combined with the considerable degree of colocalisation with an endosomal marker (RITC-dextran), suggested that ODNs were mainly sequestered into endosomal vesicles following cell entry. However, these observations alone, provided little information regarding the exact nature of the cellular uptake mechanism, other than it appeared to be mediated by an endocytic process. Molecules entering cells by either FPE, AE or RME would become localised within some type of endosomal vesicle following cell entry. Hence similar patterns of subcellular localisation would be expected if any of these mechanisms were involved in the cellular uptake of ODNs.

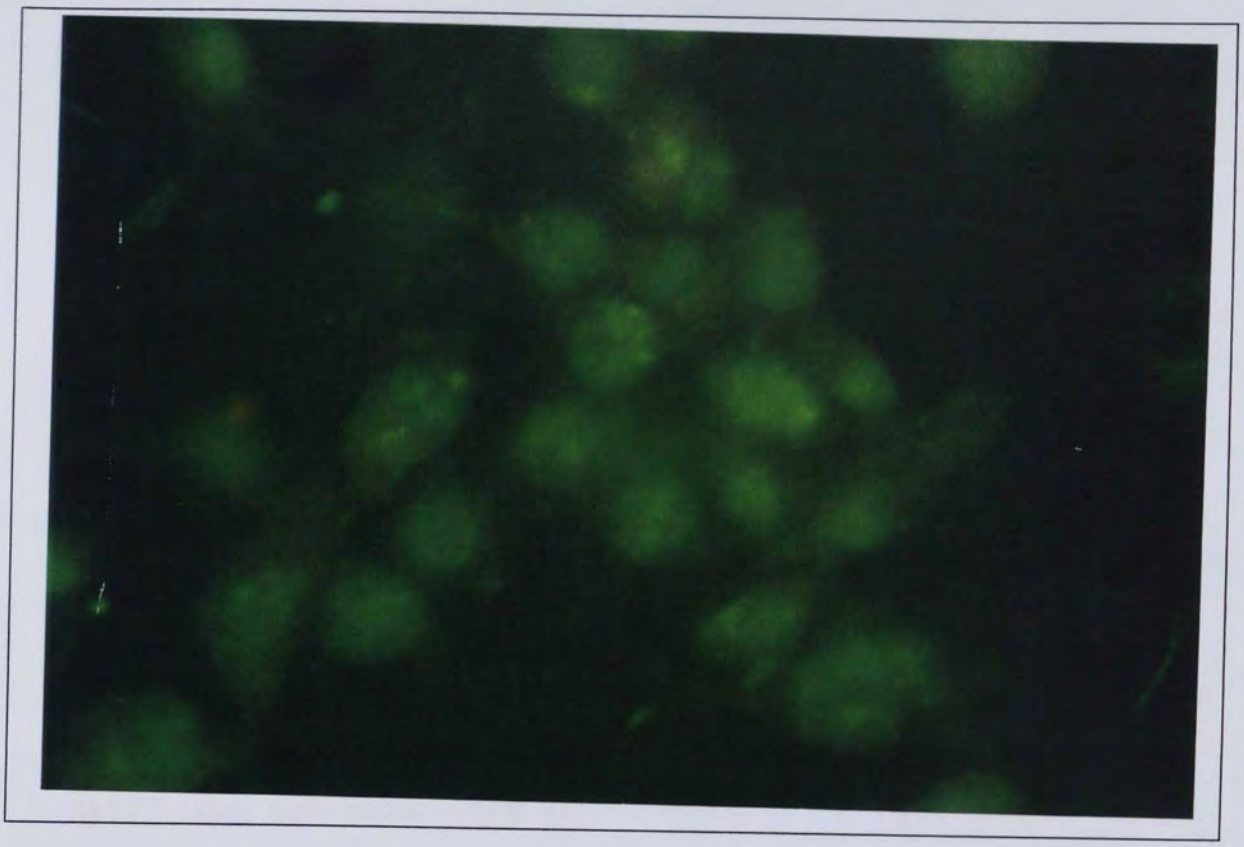


FIGURE 4.19 Fluorescence Detection of FITC-PS ODN encapsulated 2μm PLGA Microspheres in A431 Cells. (magnification x 50)

Incubation of A431 cells with FITC-PS encapsulated microspheres, produced widespread fluorescence, within the cytosol and also the nucleus (see figure 4.19). However, it is not yet clear whether this diffuse fluorescence simply arises as a consequence of the fluorescent microspheres becoming entrapped within larger vesicles (e.g. phagosomes), or whether more of the ODN had become released into the cytosol. The proposed greater release from phagosomes or phagolysosomal compartments may result from the improved stability of ODNs within the microspheres (Lewis *et al.*, 1995) compared with free ODNs which are thought to degrade rapidly in lysosomal enzyme containing compartments (Hudson *et al.*, 1996a).

4.4 CONCLUDING REMARKS

The cellular association of PLGA microspheres containing entrapped ODNs has been studied in this chapter. The ODNs used were either internally radiolabelled, or 3' labelled to reduce experimental inaccuracies, due to the fact that 5'-end labels are prone to be removed by phosphatase enzymes.

Free ODN added to cells resulted in inefficient cell association, with approximately 1% of the added quantity becoming cell associated after 12 hours. The presence of spheres on cells did not appear to affect the cell number or cell morphology, however it was shown that the use of spheres enhanced the delivery of ODNs to A431 cells compared to free ODN alone, with approximately 20% cell association achieved. The level of cell association was significantly higher than that of mannitol, a fluid phase endocytosis marker, thereby indicating that neither ODN nor spheres were likely to enter the cells via FPE alone. Cellular association was also reduced at low temperature and in the presence of metabolic inhibitors, indicating passive diffusion was not the sole reason for cell entry.

Cellular association of spheres appeared to be concentration dependent, and self-competitive association was also demonstrated. These characteristics indicate the presence of a specific cell surface binding mechanism, such as RME or AE. Competition studies with PS ODNs and spheres indicated a similar mechanism of cell association between ODNs, as these appeared to compete with spheres for cellular association to a certain extent. However, polyanions such as heparin also competed with spheres for cellular association with A431 cells. Thus the presence of a specific receptor for sphere uptake is unlikely.

Generally, these findings suggest that a process of RME and / or AE would most likely be the predominant mechanism by which PLGA spheres enter cells. The resulting association of ODN with the cell may be a combination of several mechanisms such as the internalisation of spheres, the internalisation of ODN released from spheres in media, the efflux of free ODN or the release of ODN from the microspheres inside the cell. Cellular association of spheres was cell-line specific, therefore, difficulties may

arise in drawing direct comparisons between the results obtained for A431 cells and those obtained for other cell lines.

Fluorescence microscopy studies showed that a more diffuse subcellular distribution of ODNs was observed when delivered as a microsphere formulation compared with free ODNs, which exhibited the characteristic punctate periplasmic distribution.

The coating of microspheres can offer the potential for greater association of spheres. Cortesi *et al.*, (1994) investigated the coating of spheres with gelatin, which resulted in greater association with macrophage cells. Also, surface modification of the spheres may enhance the cellular association of ODN loaded spheres (Tabata and Ikada 1988).

Entrapment of ODNs within microspheres offers the potential for enhancing cellular delivery of ODNs, and hence their efficacy.

CHAPTER FIVE

IN VIVO SUSTAINED DELIVERY OF ANTISENSE OLIGODEOXYNUCLEOTIDES

5.1 INTRODUCTION

In recent years, antisense ODNs have been increasingly used in the CNS as biological tools for understanding gene expression in relation to downstream behavioural consequences, as rapid screens in drug-target validation, and as potential therapeutic agents (for reviews see Chiasson *et al*, 1996; Wahlestedt, 1994). However, since ODNs can not normally traverse the BBB (Agrawal *et al.*, 1992; Akhtar and Agrawal, 1997), these studies have necessitated the use of localised intracerebral or intracerebroventricular (ICV) injections. Although local administration has been reported to result in a sufficient ODN uptake into neuronal and glial cells for pharmacological activity *in vivo* (Sommer *et al.*, 1996; Wan *et al.*, 1998; Chiasson *et al.*, 1998; Peris *et al.*, 1998; Zhu and Ho, 1998), the effect is often short-lived due to the relatively rapid degradation and elimination of ODNs (Chiasson *et al.*, 1996; Grzanna *et al.*, 1998). The delivery of ODNs to the brain is further complicated by the fact that repeated injections to this sensitive organ, especially of naked PS ODNs, can result in toxicity and tissue damage ranging from gliosis to large lesions (Chiasson *et al.*, 1994, 1996; Ho *et al.*, 1998). In order to avoid the problems associated with this method of delivery, biodegradable systems are currently under investigation. These systems are advantageous in that they obviate the need for repeated administration by facilitating site-specific delivery of the nucleic acid in a controlled manner to the desired site in the CNS. The entrapment of ODNs within such polymeric matrix systems also improve ODN stability, reduce the ODN dose required for efficacy and further reduce toxicity or non-specific activities associated with ODNs.

Biodegradable PLGA polymers are thermoplastic polyesters, which have long been used in resorbable surgical sutures (Cutrigh *et al.*, 1971), and in commercially available

sustained release preparations such as Zoladex (Zeneca Pharmaceuticals, UK) (Crotts and Park, 1998; Pouton and Akhtar, 1996). Furthermore, they are reported to be biodegradable and biocompatible in the CNS (Menei *et al.*, 1993), and thus appear as attractive candidates for drug delivery to the brain. No reports exist on the use of biodegradable PLGA copolymers for delivering ODNs to the CNS *in vivo*, hence in the first section of this chapter, the use of implantable biodegradable PLGA microspheres as sustained-release delivery systems for the site-specific administration of antisense ODNs to the neostriatum of the rat brain was evaluated. *In vitro* characterisation of these systems suggests that ODNs can be adequately entrapped to provide sustained delivery in a manner that is dependent on microsphere size, drug loading and polymer molecular weight (see Chapter 3).

The second section of this chapter investigates body distribution following s.c. administration of antisense ODNs using the technique of whole body autoradiography (WBA). WBA is a technique for studying the tissue distribution of radiolabelled molecules in the whole animal. The results obtained contribute significantly to our understanding of the movement of substances and their metabolites *in vivo*. Moreover, the sites of accumulation of radioactivity may be related to the metabolic fate of a compound, the physiological response, pharmacological parameters, and therapeutic and toxic effects.

The original technique of WBA was later modified by Martin *et al.*, (1962). The development of alternative methods for preparing autoradiographs has been accompanied by an increasing awareness of the potential of the technique as an investigative tool for a variety of purposes. Whole body autoradiographs can provide a picture of the distribution of isotopically labelled compounds *in vivo* extending from the principal organs down, even to the cellular level in some cases. The picture is an essentially static one, providing information on the distribution at a precise moment after the isotope is administered. However, a series of distribution patterns obtained at various times after administration can be interpreted to provide a dynamic view of the movement of the labelled molecules. Visual scanning of autoradiographs obtained at different times after administration can provide useful preliminary information on absorption, general distribution and routes of excretion. In some instances the method reveals deposition in tissues which would remain undetected by other methods, and on

those frequent occasions when quantitative analysis of excreta shows incomplete recovery of radioactivity, the method will usually reveal the site of the 'lost' radioactivity remaining in the animal.

WBA, whilst providing extremely valuable information, cannot and should not be regarded as an end in itself. Quite clearly, for a complete understanding of the movement of molecules *in vivo*, the results of many other techniques, such as biochemical, physiological, and pharmacological are required. However, these can be more clearly understood when viewed together with whole body distribution patterns.

5.2 METHODS AND MATERIALS

5.2.1 CNS Delivery Studies

An antisense ODN complementary to the *c-fos* proto-oncogene (5'-GAA CAT CAT GGT CGT-3') with a 3'-C6 amino-linker was synthesised as a PS, on an Applied Biosystems model 380B synthesiser by BIOTEZ (Berlin, Germany). After coupling to the fluorescein isothiocyanate (FITC), the ODN was purified by HPLC. The deprotected ODNs were purified using a NAP 10 column (Pharmacia Biotech, St Albans, UK) containing Sephadex G25. The ODNs were eluted in sterile water, dried under vacuum (DNA Speed Vac, Savant, UK), and then stored at -20°C until required. The concentration of ODNs was assessed by optical density measurements at 260nm. Microspheres were prepared as described in section 2.7.

5.2.1.1 Animals and Treatment

Male Sprague Dawley rats (ALAB, Stockholm, Sweden) weighing 200-230 g were maintained under a standard light/dark cycle, and allowed free access to food and water. For surgery, rats were anaesthetised with halothane (1.5% in an airflow of 1.5 L/min) and placed in a Kopf stereotaxic frame. Injections (2μL) were made either via a 32 gauge syringe for ODNs (12nmole), or via a 26 gauge syringe for microspheres

(approximately 250μg, loaded with ODNs at 0.28 nmole/mg); in the neostriatum at the co-ordinates: Bregma A +0.5, L 3.1, V -5.0 according to the atlas of Paxinos and Watson (1986).

5.2.1.2 Histological evaluation

Two days after injection, the rats were terminally anaesthetised with pentobarbital (100mg/kg) and perfused through the left cardiac ventricle with 200mL of 0.9% saline, followed by 300mL of a fixative containing 4% paraformaldehyde and 0.25 % v/v glutaraldehyde in 0.1M phosphate buffered saline. The addition of glutaraldehyde to the paraformaldehyde fixative provides a stable fixation to the ODNs (Sommer *et al.*, 1996, 1998). The brains were kept in the fixative for two hours and then transferred to 10% phosphate buffered sucrose for 1-2 days, after which they were frozen. Coronal sections (14 μm thick) through the striatum were cut on a freezing microtome and evaluated with an epifluorescence microscope.

5.2.1.3 Dual-labelling immunohistochemistry

Sections were incubated overnight in (i) rabbit antiserum to glial fibrillary acidic protein GFAP (1: 500, DAKO), or (ii) the mouse monoclonal antibody A60 recognising neuron specific nuclear protein NeuN (1: 50) (Mullen *et al.*, 1989; Sommer *et al.*, 1996). All steps of the indirect immunofluorescence (rhodamine conjugated anti-mouse or anti-rabbit antibody, 1: 40, Amersham) were carried out in phosphate buffered saline containing 0.25 % Triton at 4 °C. The sections were coverslipped with a mixture of glycerol and buffer containing 0.1% para-phenylenediamine. Examination of the fluorescence labelled ODN and the immunofluorescence of the cellular markers was made in an epifluorescence microscope (Nikon) equipped with filter holders that allowed switching between 365 and 550-580 nm illumination.

5.2.2 WBA STUDIES

5.2.2.1 Methods

The PS-modified, 20-mer antisense ODN sequence -5' GTA CCT GAA TCG TCC GCC AT 3'- targeting the *Hpv* was used for the peripheral studies. The antisense molecules were obtained from Sigma and were internally ^{3}H labelled by tritium exchange as described by Graham *et al.*, (1993). ^{3}H 5-Flurouracil (5FU) was obtained from Amersham Life Sciences (UK) with a specific activity of 1.54mCi. ODN entrapped microspheres were prepared as described in section 2.7.

Balb C mice of a target weight 15-30g were obtained from Charles River, Margate, Kent. Four groups of three females were used for the study, and were assigned individual code numbers identified by temporary experimental tail markings. The mice were housed on paper pellets in labelled plastic and wire cages. Tap water and rodent diet were available at all times.

Dose formulation:
Group 1 *hpv* ODN solution
Group 2 *hpv* ODN encapsulated in PLGA spheres
Group 3 5-FU solution

Each formulation was administered in sterile isotonic saline by a single subcutaneous dose. Animal body weights were recorded before drug administration on the day of dosing.

5.2.2.2 Sample Collection

One animal from each group was killed in a rising concentration of carbon dioxide at the following times post dose times: 1 hour, 24 hours and 7 days. Immediately following confirmation of death, each carcass was plunged into a freezing mixture of hexane and cardice (-70°C).

WBAs were performed according to the methods described by Ullberg, (1977) and Curtis *et al.*, (1981). Once frozen, each mouse was placed on its RHS in a metal frame. Chilled 2%w/w carboxymethylcellulose (CMC) was poured over the carcass, and the whole frame was submerged into the freezing cardice/hexane mixture. Once solid, the metal frame was removed, and attatched to the microtome stage, and the mouse was ready for sectioning.

The frozen carcass was allowed to equilibrate to the temperature of the Leica 1210 Macrocut (-20°C). Initial sections of 90μm were taken to trim the CMC block until a plane was reached containing the organs of interest. Sagittal sections of 20μm each were taken at different levels through each animal using the sledge microtome. Sections were cut onto adhesive tape (3M 800), and left to freeze dry in the cryostat at -20°C for 48hours. Section tapes were placed into a Molecular Dynamics cassette, and covered by a Kodak phosphor screen. After exposure time, the screen was placed into the Molecular Dynamics (STORM 860) PhosphorImager system and scanned using the software package. Alternatively, sections were also analysed using a Digital X-Ray Imaging System (Biospace Measures). Samples of tissue (liver, kidney and site of injection) were punched out of the carcass following sectioning, and stored at -80°C for microautoradiography analysis.

5.2.2.3 Microautoradiography

Tissue samples were excised from the remainder of the carcass following sectioning. The blocks of tissue were the transferred to buffered formol saline (BFS) and left to fix for 48 hours. Following fixation, the samples were transferred to an automatic tissue processor (Shandon Hypercentre 2) and processed through to wax on an overnight cycle. The samples were mounted into wax blocks and floated out onto glass microscope slides following sectioning at 4μM using a Shandon AS325 retracting microtome. The slides were dried overnight and prepared for autoradiography by dewaxing in 2 changes of fresh xylene (20 minutes per change). Under safelight conditions (Ilford 902 filter), Amersham EM-1 nuclear emulsion was melted in a water bath set at 45°C and transferred to an Amersham dipping vessel. The sections were dipped in the emulsion for 5 seconds, slowly removed, and held vertical on a tissue for a further 5 seconds. Excess emulsion on the back of the slide was removed using a tissue and the slide transferred to a cold plate set at 10°C to allow the emulsion to set.

The samples were removed from the cold plate, dried at ambient temperature, transferred to sealed, lightproof boxes containing silica gel and exposed for up to 8 weeks at 4°C. The latent images formed during exposure were converted to silver grains by photographic development. The emulsion coated slides were placed in Ilford phenisol developer diluted 1 in 5 with ultrapure water (milli-Q) for 5 minutes, transferred to 1% glacial acetic acid for 1 min and fixed in 30% sodium thiosulphate for 10 minutes. Following a 20 minutes wash in running water the samples were stained in Gills haematoxylin for 3 minutes, blued in running water and transferred to1% aqueous Eosin (both Raymond A Lamb) for 10 seconds. The tissue sections were then dehydrated in absolute alcohol and transferred to xylene. The slides were then mounted and coverslipped using Pertex mounting media (Cellpack).

5.3 RESULTS

5.3.1 *In vivo* CNS Distribution Studies of ODNs Released from Biodegradable Polymer Microspheres

In this study, the distribution of naked fluorescently (FITC)-labelled ODN that had been directly injected into the neostriatum of the rat brain, was compared to the identical ODN delivered to the same site when encapsulated in PLGA microspheres. FITC-labelled ODNs were entrapped in PLGA microspheres (10-20μm), and were stereo-taxically implanted into the neostriatum of rats as a saline solution using a 26-gauge syringe needle. The distribution after two days release of the FITC-labelled ODN from polymer microspheres in the neostriatum is shown in figure 5.1.

The polymer microspheres appeared to remain at the site of injection, visible as an intense fluorescent signal at the tip of the needle tract (see figure 5.1), albeit, fluorescently-labelled cells could be observed as far as 1 mm from the site of injection of the microspheres, indicative of ODN release and migration within a localised region of the CNS. In one animal, the signal was distributed throughout the entire neostriatum after 48 hours. A close examination of the fluorescent cells within the boxed region of the neostriatum (figure 5.1a) is shown at higher magnification in figure 5.1b. Not all cells were able to accumulate ODN as evidenced by a heterogeneous distribution of the fluorescent stain in this section (figure 5.1b).

The microsphere delivered ODNs appeared to be taken up mostly by cells with a neuronal morphology, and the identity of fluorescently labelled cells was confirmed by staining with a neuronal cell-specific marker. Double label immunohistochemistry revealed the nature of these cells as NeuN immunopositive neurons (figure 5.1c). Over the 48 hour time course of this study, significant double labelling for GFAP was not found, thus indicating that the astroglia were not, or were only weakly involved in the uptake of the ODNs.

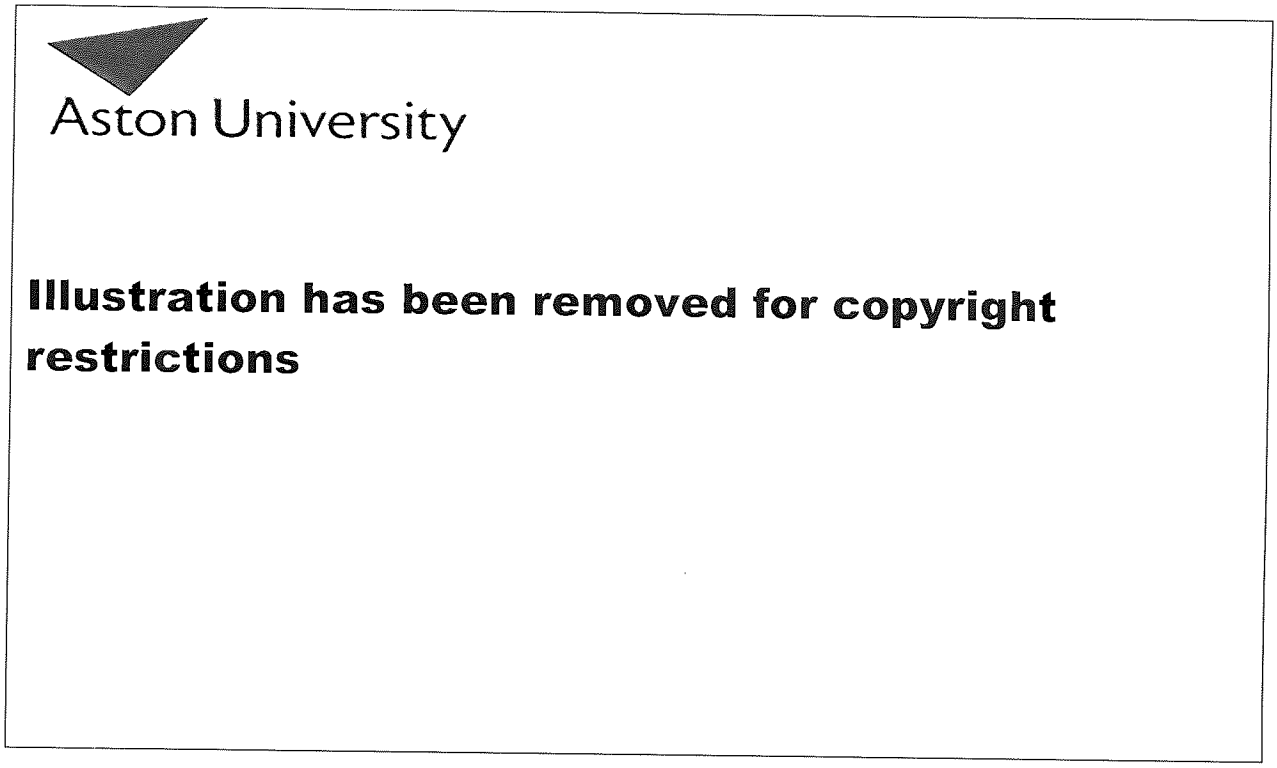


FIGURE 5.1 Photomicrographs of FITC 3'-conjugated c-fos phosphorothioate-modified antisense ODN released from 10-20μm size PLGA (Mw 3,000) microspheres injected into the neostriatum over a period of 2 days (**a**). At higher magnification the uptake of the FITC labelled ODN is shown with a diffuse staining of neuronal cell bodies and nuclei (**b**). In the same sections the neurons were identified by staining with the mouse monoclonal antibody A-60 followed by detection with a rhodamine conjugated anti-mouse antibody (**c**). The injection was placed at the co-ordinates: Bregma A +0.5, L 3.1, V -5 according to the atlas of Paxinos and Watson, (1986). [Magnification x 40 (a), x 200 (b, c)}

The sub-cellular biodistribution of naked ODNs and ODNs entrapped within microspheres that had been administered to the same site in the neostriatum were next compared.

Directly injected, FITC labelled, PS naked ODNs were distributed throughout most of the dorsal part of the neostriatum within 20 minutes (data not shown), however, 24 hours after the intrastriatal injection, the FITC labelled ODNs appeared to exhibit a punctate distribution within the cytosol (figure 5.2a), indicative of vesicular localisation. Only weak fluorescence, associated with very little or no naked ODN, was visible 48 hours post-administration (data not shown). In contrast, the same ODN (in sequence and chemistry) when administered as a polymer microsphere formulation, was still visible with a strong fluorescent intensity after the 48 hour study period. FITC-labelled ODNs that had been slowly released from the microspheres for about two days appeared to produce a generalised, uniform distribution of fluorescence that was visible within the cytosol and nuclei of these neuronal cells (Figure 5b).

It is seen that despite the large differences in the total amount of FITC labelled ODNs administered to the animals (about 20-fold lower within microspheres), the intensity of the fluorescent staining appeared to be similar for polymer delivered ODNs after 48 hours as compared to free ODN at 24 hours (figures 5.2a and 5.2b).

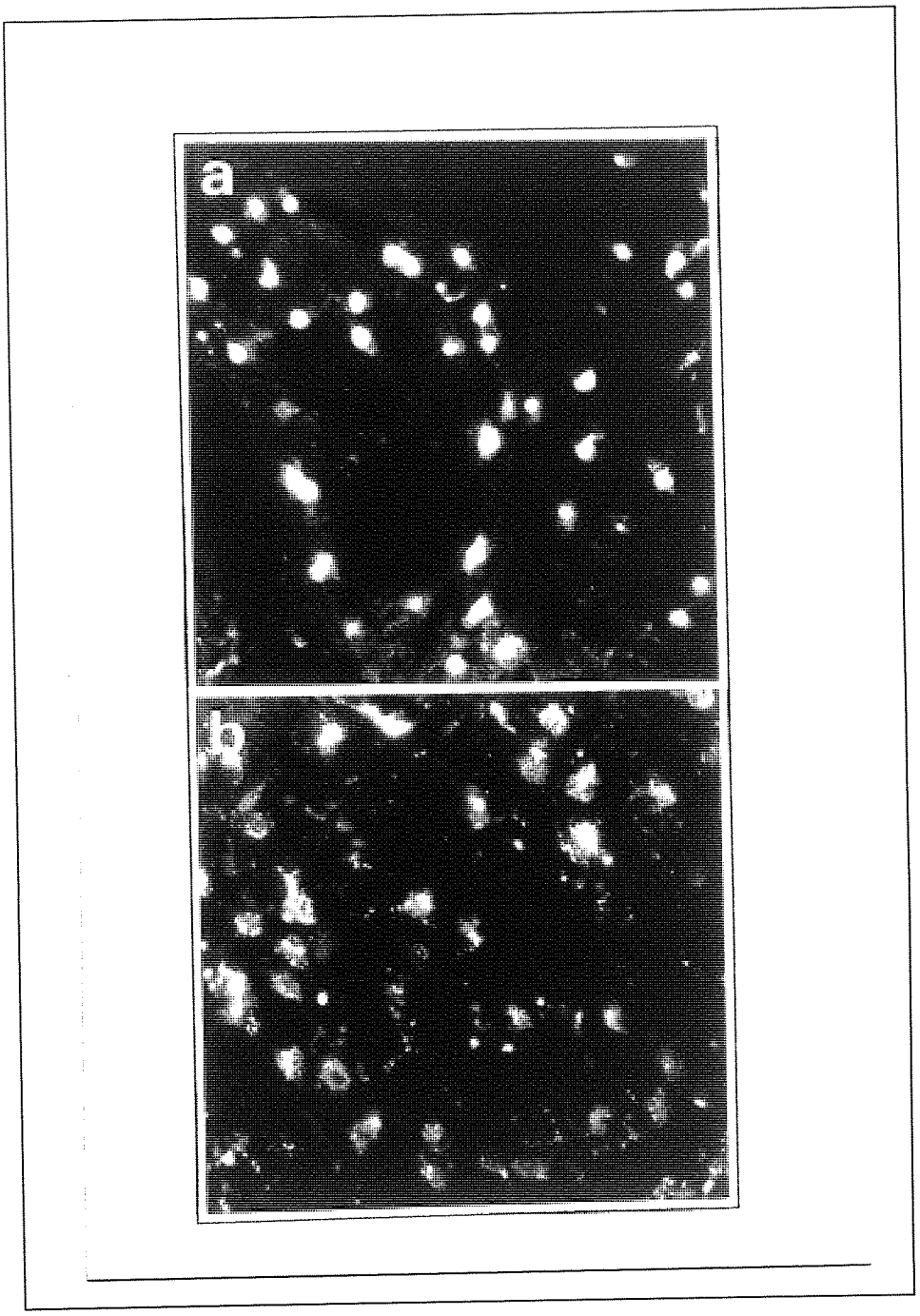


FIGURE 5.2 Photomicrographs of FITC 3'-conjugated *c-fos* antisense phosphorothioate oligonucleotide labelled neurons (**a**) 2 days after the release from 250μg microspheres loaded with ca. 0.07nmol ODN or (**b**) 1 day after the injection of 1nmol of the same ODN dissolved in saline. Note the differences in the intracellular appearance of the staining and the nearly equal strength in signal intensity. (Magnification x 400)

Direct, single-dose administration (i.c.v. or intracerebral injection) of antisense ODNs appears to be effective for some CNS applications targeting short-lived proteins, such as the immediate-early gene family (Sommer *et al.*, 1993; Chiasson *et al.*, 1994; Grzanna *et al.*, 1998), but repeated administration is usually required for long-lived proteins and/or for mRNA targets exhibiting a slow-turnover. For example, the reported *in vivo* half-lives of the 7-transmembrane family of receptors such as dopamine D1 and serotonin 1A can be as high as 5-8 days (reviewed in Ho *et al.*, 1998). Thus, in order to achieve a significant level of antisense activity (>50%) against these targets, delivery of ODNs needs to be maintained for at least 1-2 half-lives (> 10-16 days). To overcome the problems of repeated administration over such lengths of time, and the resultant toxicity previously observed with repeat-dosing of PS ODNs (Chiasson *et al.*, 1996; Ho *et al.*, 1998), the proposed use of biodegradable polymer formulations such as PLGA microspheres may offer significant advantages. Previous studies (Chapter 3) have shown that homo- and copolymers of LA and GA may be suitable for the sustained delivery of unmodified antisense ODNs and ribozymes, as also reported by other authors (Lewis *et al.*, 1995; Hudson *et al.*, 1996; Akhtar and Lewis, 1997; Lewis *et al*,1998; Cleek *et al*, 1997; Yamakawa *et al*, 1997).

5.3.2 WBA Studies

For this study, the details of the formulations used and the protocol of the study are summarised in table 5.1. Having achieved sustained delivery to the brain, the focus of this study was to investigate sustained release of ODNs from PLGA spheres following subcutaneous administration.

TABLE 5.1 Table Summarising *in vivo* Parameters Required Following s.c. Administration of PS ODN, PS ODN Encapsulated in PLGA Spheres and free 5-FU for WBA study.

SAMPLE	ACTIVITY OF SAMPLE	VEHICLE	ACTIVITY ADMINISTERED	ACTUAL DOSE
^{3}H PS ODN	0.08μCi/μg ODN	SALINE	96.58 μCi / mL	11mg/kg
^{3}H ODN LOADED PLGA SPHERES	0.35μCi/mg spheres	SALINE	46.68 μCi /mL	5.32mg/kg
^{3}H 5FU	0.5μCi/μL	SALINE	176.5μCi /mL	11mg /kg

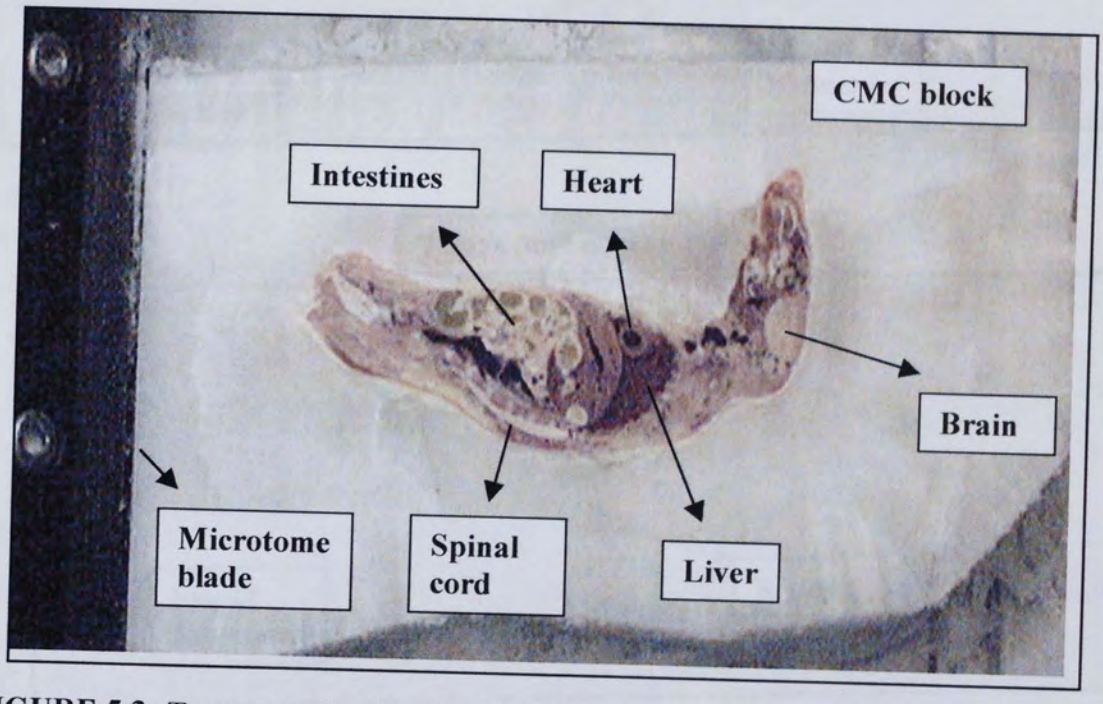


FIGURE 5.3 Transverse Section Showing Mouse Embedded in CMC Block

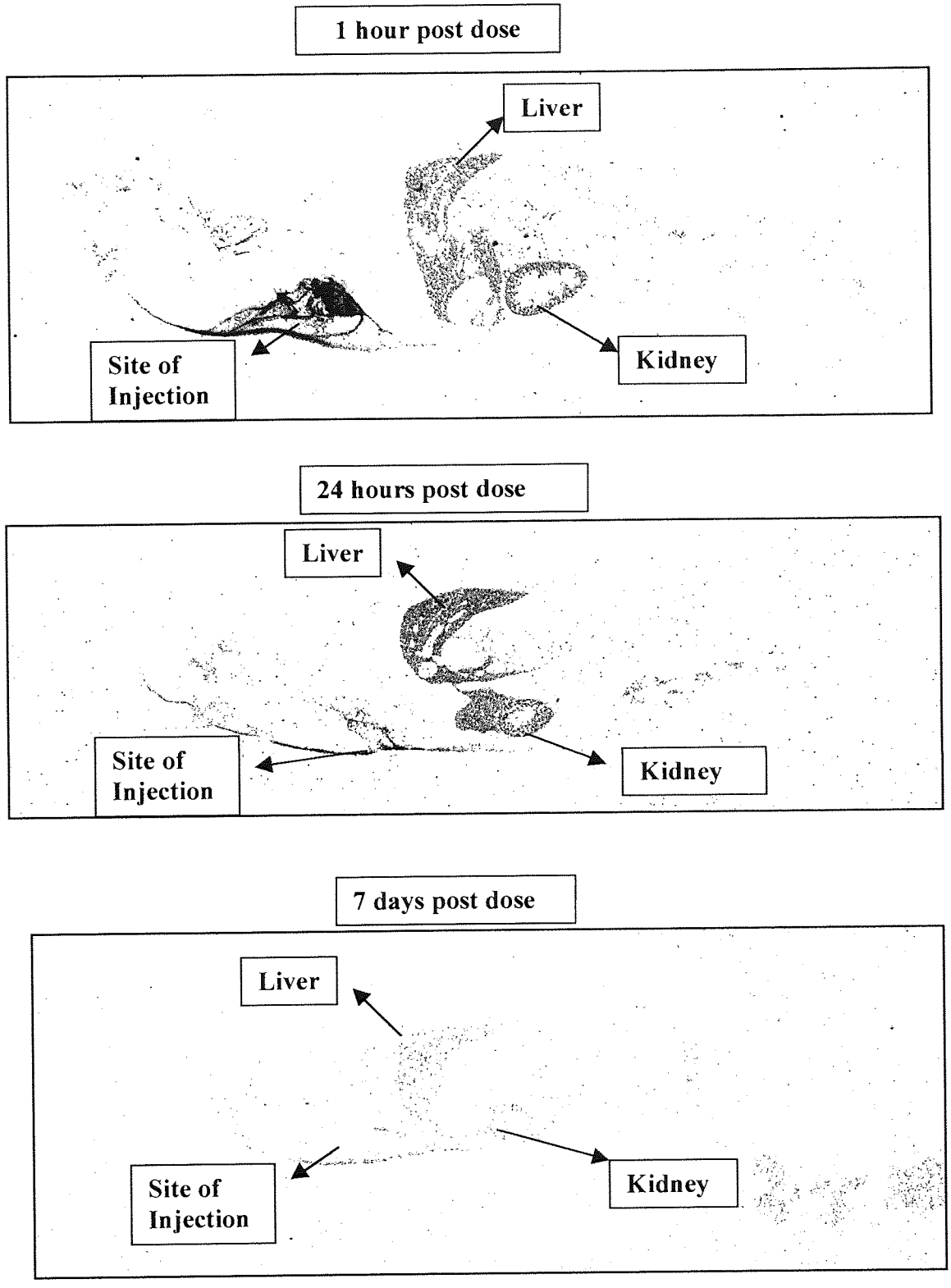


FIGURE 5.4 Comparison of Autoradiographic Biodistribution Images following s.c. Administration of Free ODN in Mice using the Molecular Dynamics PhosphorImager (dose of free ODN=11mg/kg)

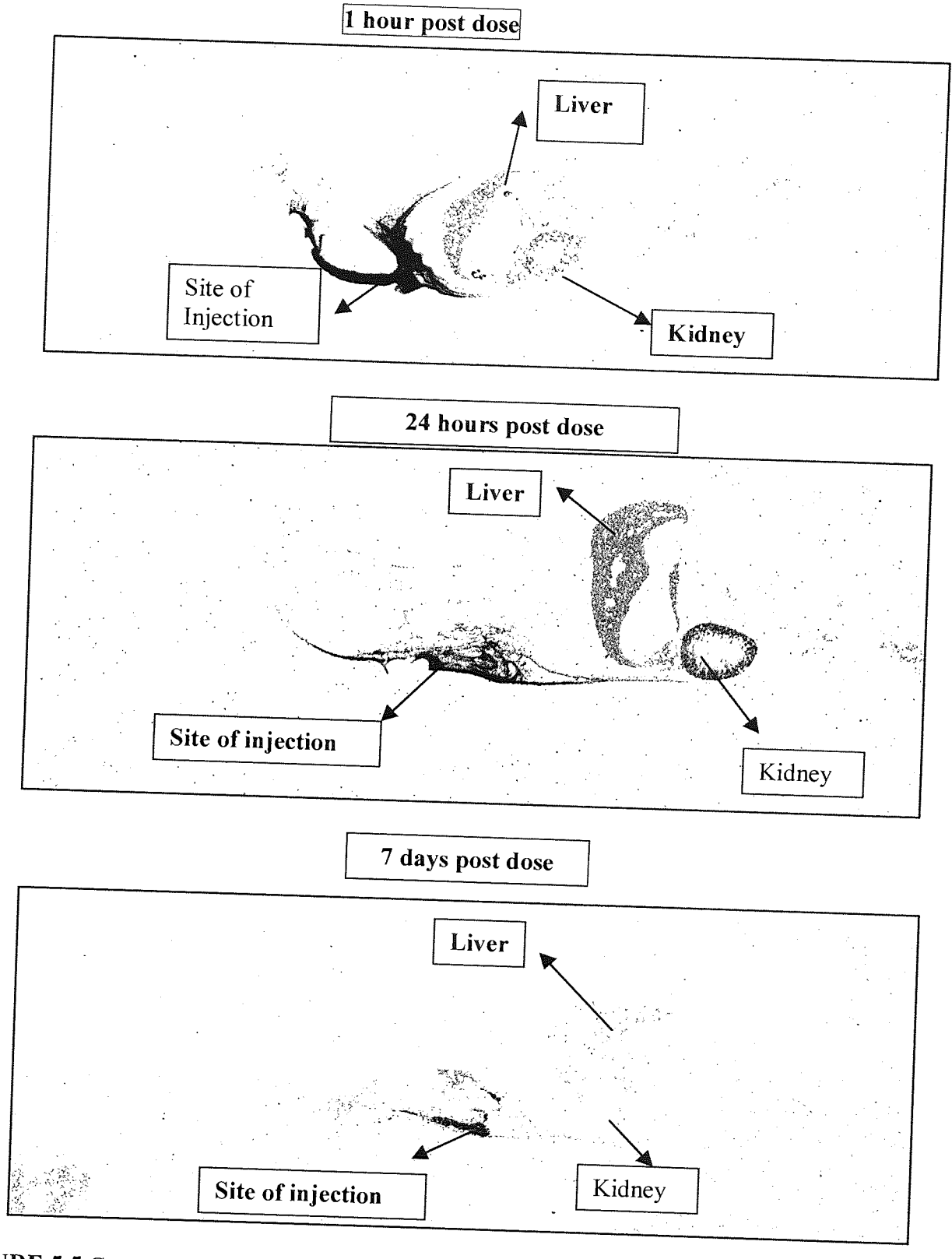


FIGURE 5.5 Comparison of Autoradiographic Biodistribution Images following s.c. Administration of ODN Encapsulated PLGA Spheres in Mice using the Molecular Dynamics PhosphorImager (Dose of ODN =5.32mg/kg)

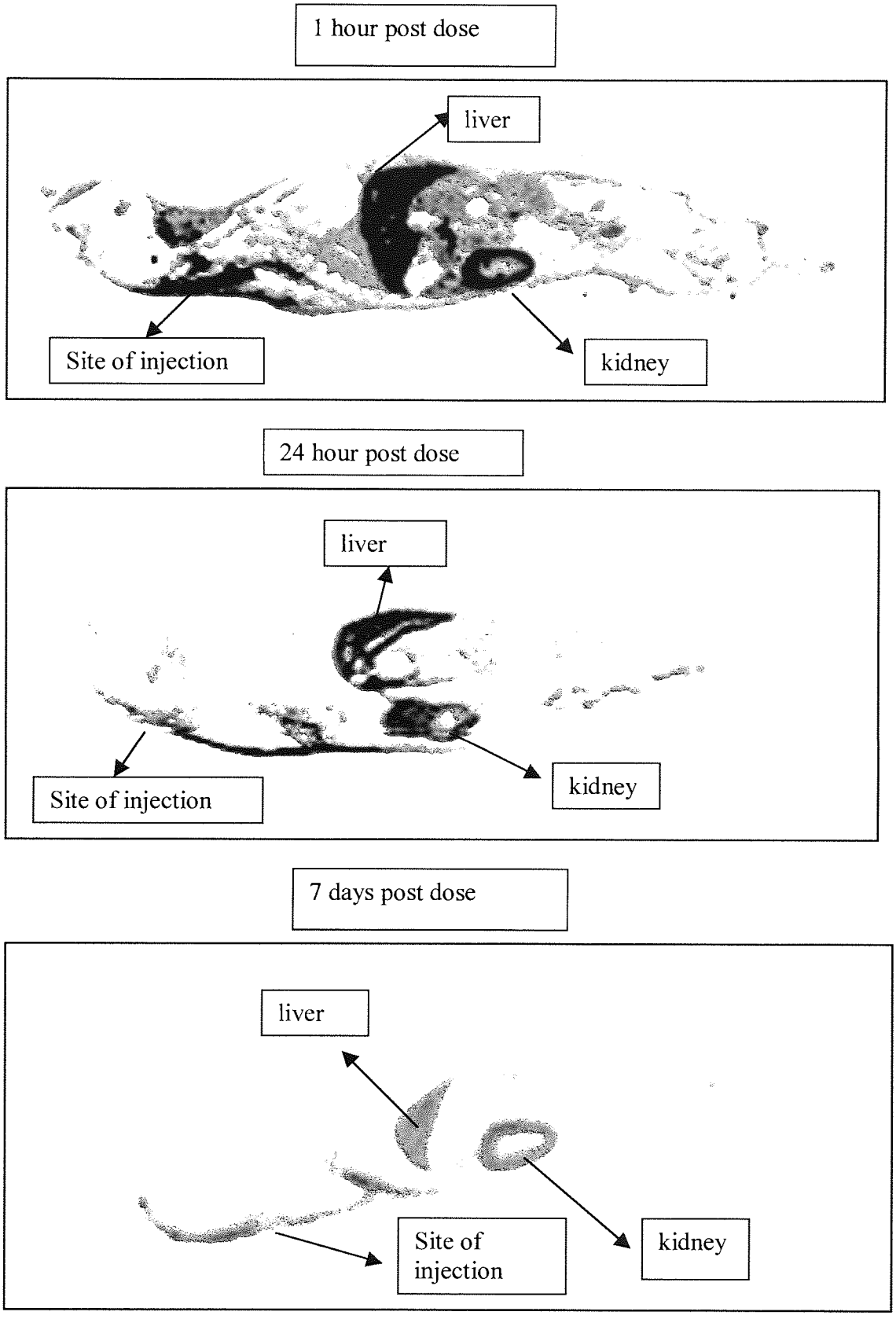


FIGURE 5.6 Comparison of Autoradiographic Biodistribution Images Following s.c. Administration of Free ODN in Mice using the Biospace Measures Digital Imager (Dose of free ODN=11mg/kg)

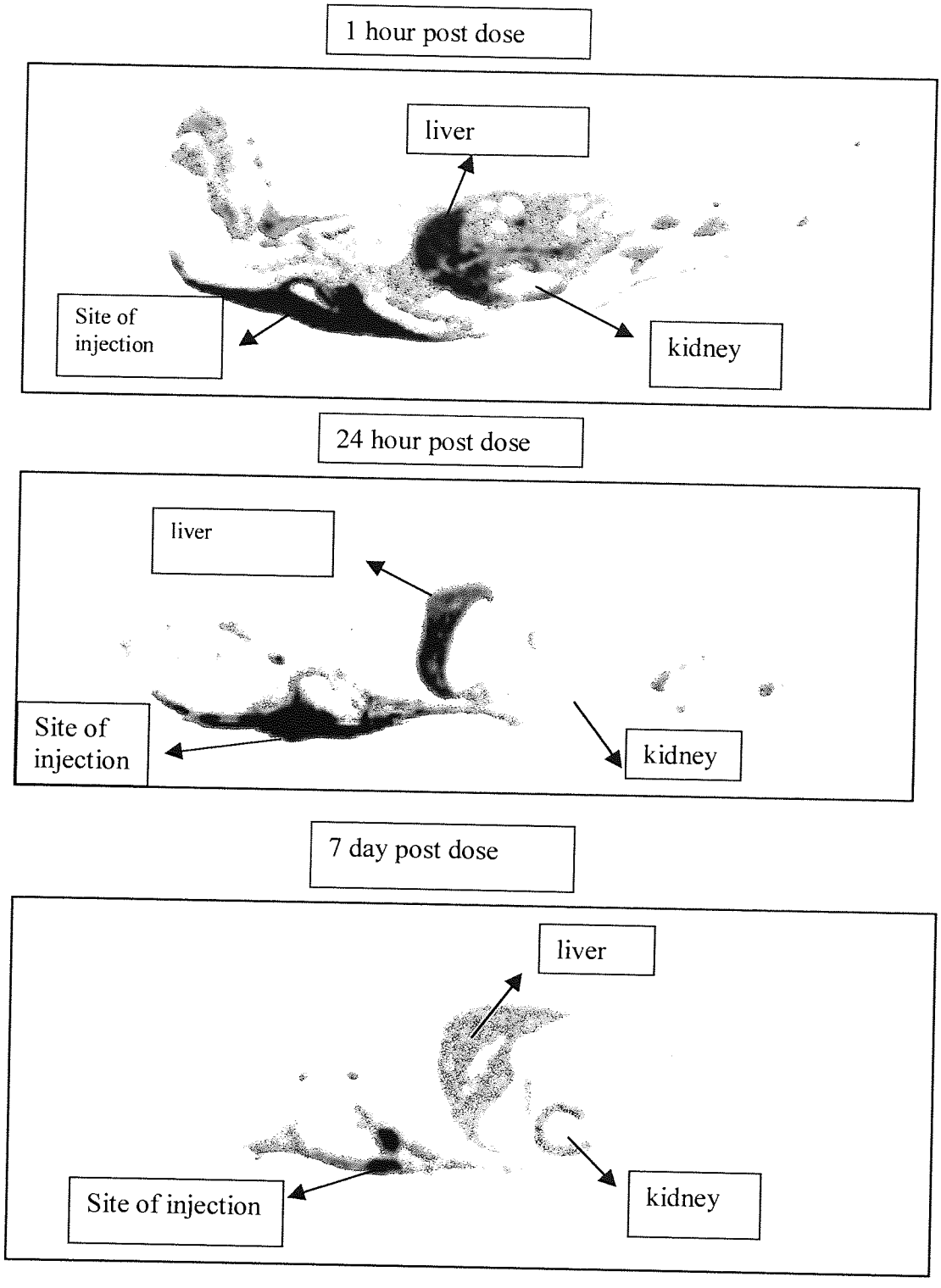


FIGURE 5.7 Comparison of Autoradiographic Biodistribution Images Following s.c. Administration of ODN Encapsulated PLGA Spheres in Mice using the Biospace Measure Digital Imager (Dose of ODN=5.32mg/kg)

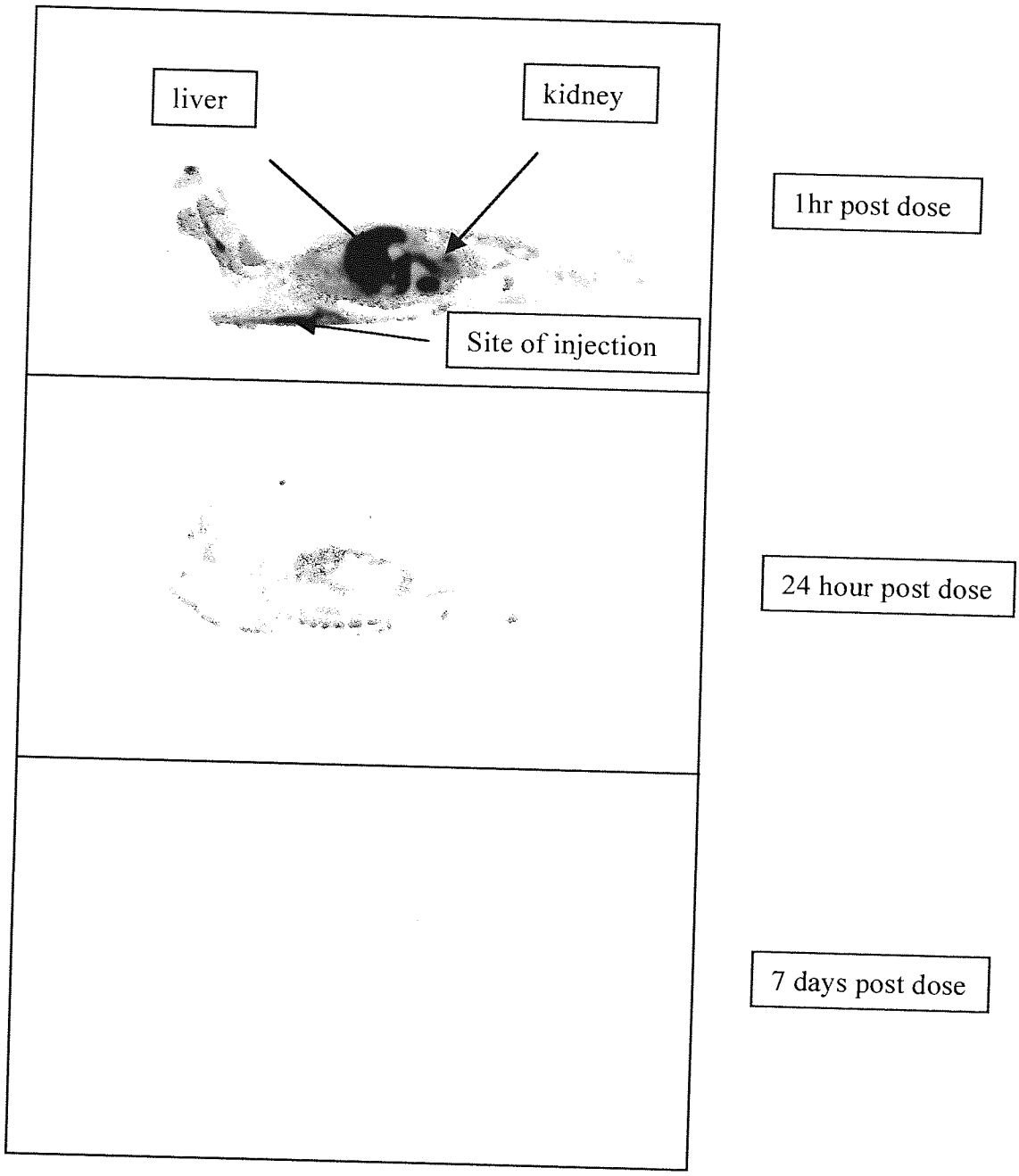


FIGURE 5.8 Comparison of Autoradiographic Biodistribution Images following s.c. Administration of 5-FU in Mice using the Biospace Measure Digital Imager (dose of free 5-FU=11mg/kg)

Autoradiographic images produced by both systems as shown in figures 5.4 to 5.7 show comparative results. The image quality was significantly greater with the Biospace Measures System, with images of high precision produced after 24 hours, whereas with the Molecular Dynamics Phosphor Imager, a twelve week exposure period was necessary. These findings were similar to the findings of Kalifa *et al.*, (1998).

Figures 5.4 and 5.6 showed that administration of free ODN resulted in a uniform distribution to several organs within an hour, with high levels of radioactivity present in both the kidney and liver. A similar distribution was observed after 24 hours, with a lower level of radioactivity present at the site of administration. After 7 days, minimal radioactivity was seen remaining within the body, following a single dose of ^{3}H ODN.

Figures 5.5 and 5.7 showed administration of ODN encapsulated microspheres after one hour resulted in a high level of radioactivity at the site of administration, with a lower level distributed within the body. Similar distribution was observed after 24 hours, with significant levels of activity remaining at the site of administration, which was present even after 7 days indicating that the biodegradable microspheres were still releasing the encapsulated ODN in a sustained manner. The free 5-FU, a low molecular weight compound with a well-characterised biodistribution pattern, was seen to be distributed and eliminated within the first 24 hours as shown in figure 5.8.

Nicklin *et al.*, (1998) investigated the *in vivo* distribution of a 20-mer PS ODN following a single i.v. dose (3.6mg/kg) using WBA. Comparable results were obtained for PS ODNs, with low levels of radioactivity still remaining after 10 days. Clearance of a ^{35}S labelled, 25-mer PS ODN appeared to occur more slowly, particularly from the kidney. Similar findings were seen with whole body tissue associated radioactivity being much reduced after 7 days, with low levels present in the kidney cortex and only traces in the liver.

Gupta *et al.*, (1996) reported similar distribution results for PLGA spheres. This group investigated the *in vivo* distribution of radioactivity in mice following subcutaneous administration of PLGA 50:50 microspheres containing ^{14}C labelled tetanus toxoid. The authors found that the majority of the soluble tetanus toxoid administered disappeared from the site of injection within one hour of injection. In contrast,

approximately 20% of radioactivity persisted at the injection site for up to four weeks for mice injected with microspheres encapsulating tetanus toxoid. Significant radioactivity levels were found for spheres in the kidney, liver, and draining lymph nodes. A similar study using WBA was carried out by Landry *et al.*, (1998), who investigated the peroral administration of ^{14}C PLA nanoparticles coated with human serum albumin to guinea pigs. Although a different administration route was employed, a similar WBA procedure was used. The authors reported that 24 hours post administration, the major part of radioactivity was located in the gastro-intestinal tract.

5.3.2.1 Tissue Distribution OF ^{3}H ODN

The body distribution of the two preparations, as visualised by WBA, has been detailed in section 5.3.2. In order to determine a more accurate representation of radioactivity in various organs, the quantification of radioactivity in the liver, kidney, spleen, and site of injection was carried out using microsoft excel. Unknown values were back calculated from a standard curve fitted to an unweighted linear regression. The standard curve was produced using Amersham tritium microscales (RPA 510 Batch 13) and standard concentrations were corrected for isotopic decay using the standard half life tables provided.

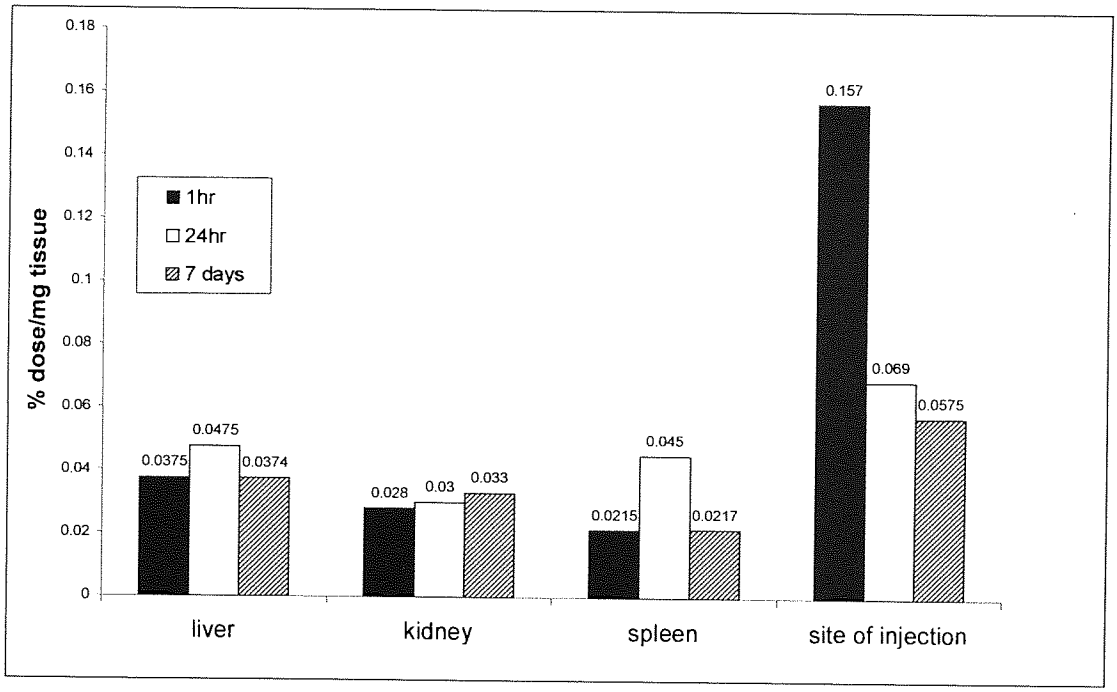


FIGURE 5.9 Tissue Distribution of ^{3}H PS ODN following s.c. Administration to Mice (dose of free ODN = 11mg/kg)

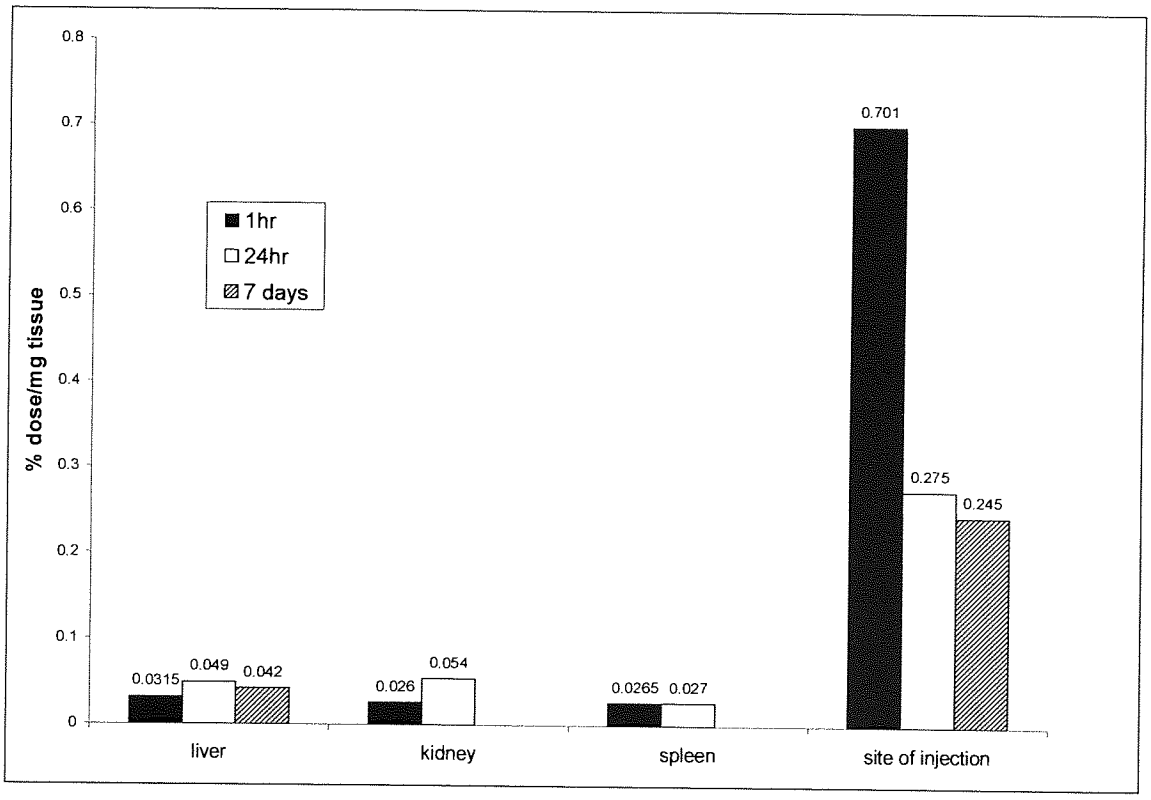


FIGURE 5.10 Tissue Distribution of ^{3}H PS ODN loaded PLGA Microspheres following s.c. Administration to Mice (dose of microspheres=10g/kg = 5mg/kg ODN)

The 20-mer ^{3}H PS was widely distributed to various tissues. Figure 5.9 shows the % dose/mg of tissue associated with each organ following administration of free ODN, 1 hour, 24 hours, and 7 days post administration; whereas figure 5.10 displays the % dose/mg tissue following administration of ODN encapsulated microspheres. The organs in which the highest levels of ODN were detected were the liver, kidney, and spleen. It is evident, from both figures 5.9 and 5.10, that similar tissue distributions are seen in these organs for both free ODN and ODN released from microspheres. These tissues are able to accumulate PS ODNs, suggesting that they bind to tissue matrix and/or that active uptake into cells occurs within these organs. The extent of uptake into these organs can be visually observed with reference to WBA images produced (see figures 5.4-5.7). The highest concentration was achieved in the liver for both free ODN and spheres, although the liver does account for the greatest proportion of the dose partly by virtue of its greater tissue mass.

In this study, the comparison of free ODN and the effect of encapsulation into PLGA microspheres following s.c. administration was the key concern, with sustained release of ODN from PLGA spheres being examined. From figure 5.9 it can be seen that the concentration of free ODN present at the site of administration 7 days post administration is 3-fold less i.e. much reduced from the concentration of free ODN following initial administration. The rapid body distribution of free ODN following administration can also be depicted by WBA images. However, on administration of sphere encapsulated ODN, concentration of ODN at the site of administration was sufficiently higher than for free ODN (figure 5.10). One hour post administration, 0.701% dose/mg of tissue was present at the site of injection for PLGA microspheres, compared to 0.157% dose/mg of tissue of free ODN. Seven days after administration of free ODN, trace levels of 0.0575% dose/mg were detected, however for ODN loaded microspheres, after 7 days, 0.275 % dose/mg was still present. It is clear that the spheres are still present at the site of injection seven days post administration, thus it can be concluded that they are capable of releasing ODN in a sustained manner. The levels of radioactivity quantified in the liver, kidney, and spleen, following each time-point are considerably lower than for that at the site of injection, hence it may be assumed that the spheres were still present at the site of administration as a depot system, from which sustained release of ODN over the time frame was possible.

Comparison of these organ observations with those for other PS ODNs is complicated by numerous experimental variables such as ODN length and sequence, radiolabel type, detection methods, dose and animal species. Nevertheless, similar results have been displayed by Agrawal *et al.*, (1991); Goddarzi *et al.*, (1992); Sands *et al.*, (1994); Zhang *et al.*, (1995 a); Rifai *et al.*, (1996) and Philips *et al.*, (1997).

The blood kinetics and tissue distribution of PS ODNs are dose dependant (Philips *et al.*, 1996; Rifai *et al.*, 1996). The PS backbone is the dominant characteristic governing the pharmacokinetic handling of PS ODNs. However, tissue distribution is also affected by their base sequence. It is difficult to appreciate this sequence dependence since it is masked by other experimental variables such as the detection method, dose and animal species. Metabolic chain shortening of PS ODNs *in vivo* has been widely observed (Agrawal *et al.*, 1991, 1995a; Zhang *et al.*, 1995a; Crooke *et al.*, 1996) and attributed to 3'-exonuclease-mediated cleavage through indirect experimental evidence. Temsamani *et al.*, (1993) showed the 3' capping, but not the 5' capping, of a PS ODN increased its *in vivo* stability compared to the uncapped compound.

Many studies have been conducted investigating the tissue distribution pattern of PS ODNs. Many different routes of administration of ODNs have been analysed, and different *in vivo* models have also been used (Akhtar and Agrawal 1997; Nickin *et al.*, 1988). Agrawal *et al.*, (1995) investigated the pharmacokinetics of PS ODNs and MP ODNs following i.v. administration into mice. The authors found that approximately 70% radioactivity was excreted in the urine, between 60 and 120 minutes after administration of the MP ODN. Peak concentrations of radioactivity were observed in most tissues five minutes after administration of the ODN. The highest concentrations were found in renal tissue, whereas lower concentrations were detected in the lungs, liver, spleen, muscle and brain. Temsamani *et al.*, (1997) evaluated the tissue distribution following i.v. administration a 20-mer PS ODN. Following administration, the ODN was taken up by most tissues, in particular the liver and kidneys. The PS ODN concentration increased in these two organs for up to 48 hours (the end-point of the study). Gel electrophoresis of the 20-mer PS ODN recovered from the tissues displayed a tissue specific degradation profile. This PS ODN was found to be very stable in most tissues except the kidneys and liver. Temsamani *et al.*, (1997) showed the degradation rate in these tissues was time dependent, and 50% of the PS ODN was

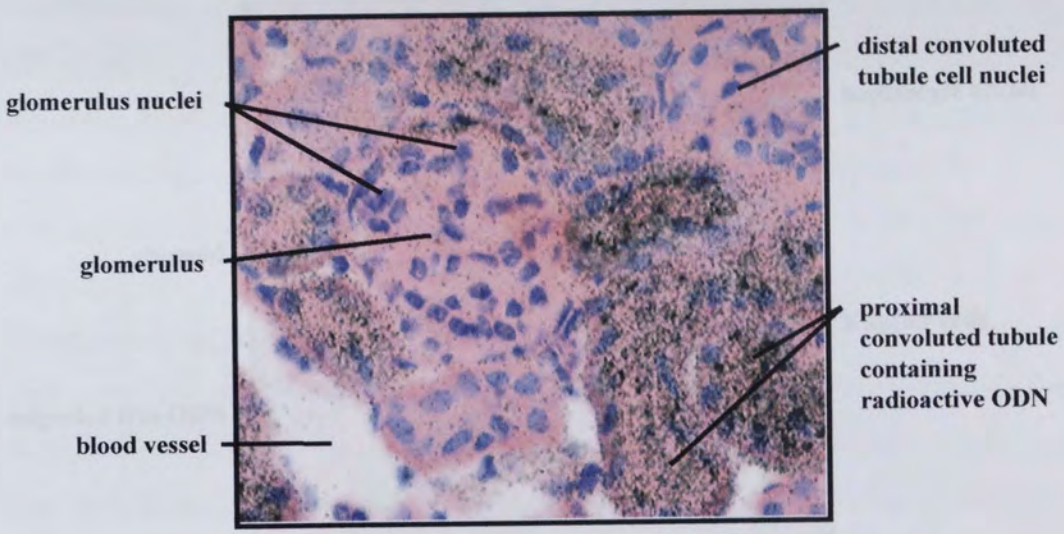
found to be degraded in 48 hours. About 30% of the administered dose was excreted in urine in the first 24 hours after administration, and most of the excreted PS ODN was found to be degraded.

Agrawal *et al.*, (1997) examined the distribution and metabolism of a 25-mer PS ODN following i.v. and s.c. administration into Sprague Dawley rats. Tissue distribution of PS ODN in rats was similar to that reported in the mice study above. The liver and kidneys were the organs with the highest uptake of ODN. After s.c. administration, tissue distribution of the PS ODN was very similar to that obtained after intravenous administration. Goddarzi *et al.*, (1991) tested the stability, organ distribution, and toxicity of a 20-mer PS in mice by i.v., i.p., and s.c. administration of ODN. The liver retained the maximum amount of ODN after i.v. administration, closely followed by the kidneys. During i.p. and s.c. injection, both the liver and kidneys retained almost equal amounts, both slightly greater than that observed after i.v. administration.

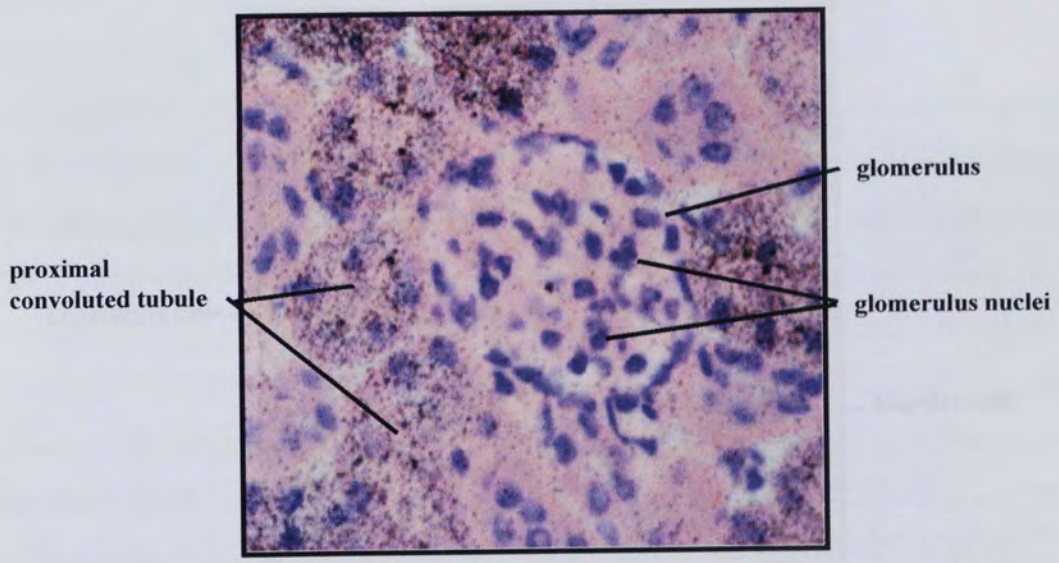
Bolus i.v. administration of antisense ODNs for the treatment of systemic conditions is the most straightforward delivery mode from a pharmacokinetic perspective. Unfortunately, PS ODNs cannot be administered this way, since clinically relevant doses would result in peak plasma levels exceeding the putative threshold concentration for acute side effects. Slow i.v. infusion with PS ODNs results in much lower, but sustained peak levels and potentially circumvents these effects. However, it incurs additional inconvenience to patients and health-care professionals. Subcutaneous administration of PS ODNs (Agrawal *et al.*, 1995a; Raynaud *et al.*, 1997; Philips *et al.*, 1997) is an alternative approach to reducing the rate of dose input. Nicklin *et al.*, (1998) reported that when compared to the i.v. route, the s.c. dose route had peak blood concentrations which were reduced five-fold, and the tissue distribution pattern was identical. In addition, bioavailablility was increased with higher doses. The systemic appearance of metabolites was only marginally increased following s.c. administration (Philips *et al.*, 1997). The s.c. route may therefore offer a viable alternative to i.v. infusion, and its clinical application warrants further consideration.

5.3.2.2 Cellular Uptake *in vivo*

Cellular uptake of ^{3}H ODN *in vivo* was provided by light microscopy autoradiography of tissues as described in section 5.2.2.3. Figure 5.11a displays cellular localisation of free ODN in the kidney after 24 hours, and fig 5.11b displays the distribution of encapsulated ODN. High levels of ODN were present in the cells of the proximal convoluted tubules, but levels were minimal in the distal tubules and glomeruli. For ODN loaded spheres, a similar distribution was seen compared to free ODN, although the ODN concentration was much lower than that exhibited by free ODN. The findings for free ODN were similar to those of other authors: Nicklin *et al.*, (1998), Rifai *et al.*, (1996), Oberbrauer *et al.*, (1996) and Carome *et al.*, (1997). Nicklin *et al.*, (1998) stated high concentrations of ODN radioactivity were found in the Bowmans capsule two minutes after i.v. administration. However, minimal amounts were found in the glomeruli at 360 minutes, which provides direct evidence for renal filtration of PS ODNs. Sands *et al.*, (1996) reported that after ^{3}H labelled PS ODNs were infused into mice, radioactivity was primarily located in the renal cortex on autoradiography of whole kidney sections. Oberbauer *et al.*, (1996) who performed similar studies in rats, showed that the proximal tubule was the primary site for uptake of radiolabelled PS ODNs. Furthermore, PAGE analysis of renal extracts obtained up to seven days after infusion of radiolabelled PS ODN into these rats revealed full-size intact ODNs. In contrast, intact full size ODN was not found in the urine at any time point.



(a)



(b)

FIGURE 5.11 Uptake and Distribution Pattern of **(a)** [^{3}H] ODN, **(b)** ODN encapsulated microspheres in kidney tissue (24 hours post dose, magnification x100 oil emulsion)

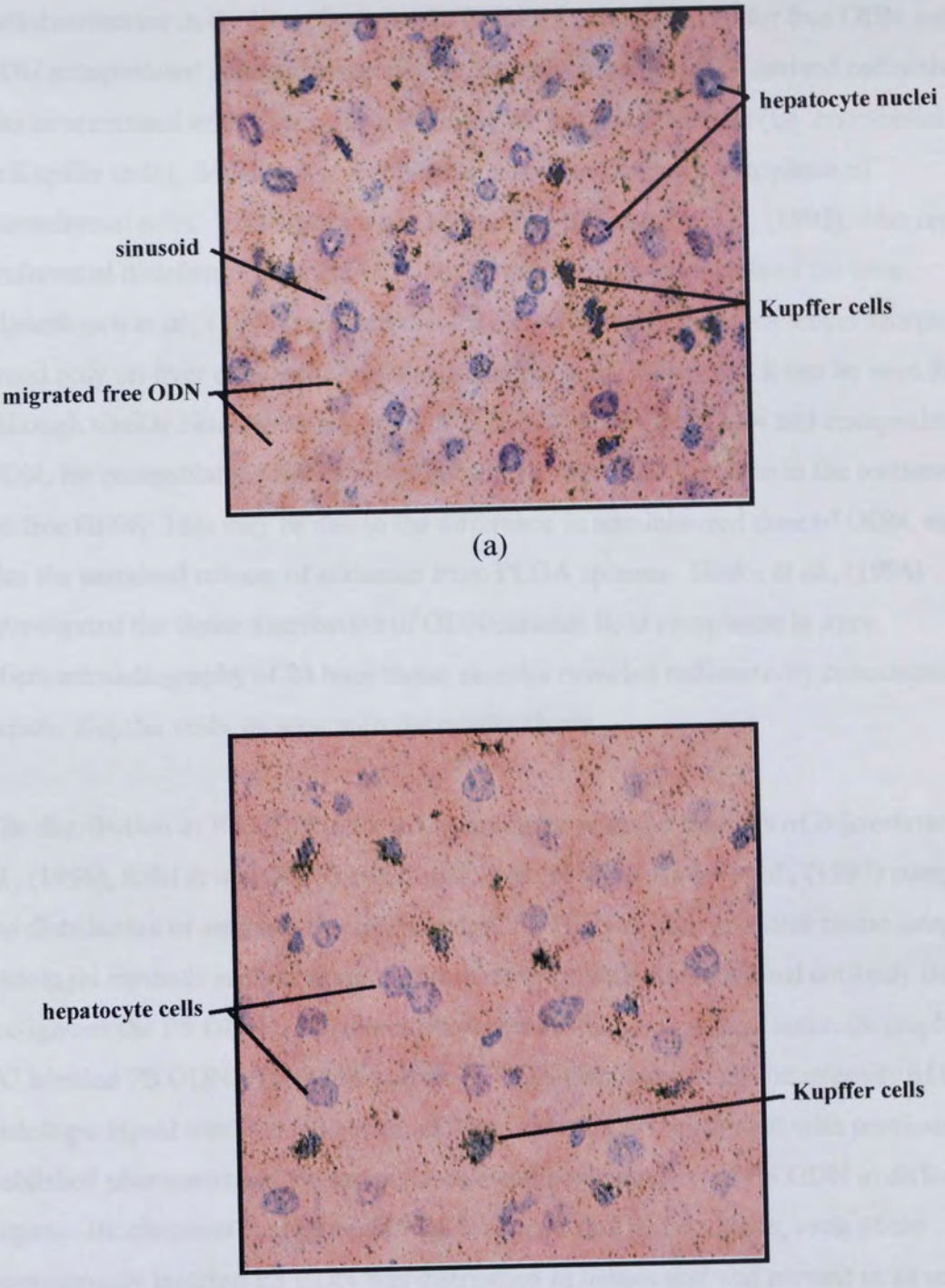


FIGURE 5.12 Uptake and Distribution Pattern of **(a)** [^{3}H] ODN, **(b)** ODN encapsulated microspheres in liver tissue (24 hours post dose, magnification x 100 oil emulsion)

Cell distribution in the liver, is shown in figures 5.12a and 5.12b for free ODN and ODN encapsulated spheres, respectively. In both cases, ^{3}H ODN derived radiolabel was concentrated within specific cells lining the hepatic sinusoids (i.e. endothelial cells or Kupffer cells). Much lower levels were associated with the cytoplasm of parenchymal cells. This agrees with the findings of Inagaki *et al.*, (1992), who reported preferential distribution of a PS ODN to the non-parenchymal cells of the liver. Bijsterbosch *et al.*, (1996) reported that PS ODNs bind to type 1 scavenger receptors found only on liver endothelial cells and Kupffer cells. However, it can be seen that although similar biodistribution of ODN is seen for both free ODN and encapsulated ODN, for encapsulated ODN a lower concentration of ODN is seen in the sections than for free ODN. This may be due to the difference in administered dose of ODN, and also the sustained release of antisense from PLGA spheres. Osaka *et al.*, (1996) investigated the tissue distribution of ODN/cationic lipid complexes in mice. Microautoradiography of 24 hour tissue samples revealed radioactivity concentrated in hepatic Kupffer cells, as seen with the results above.

The distribution of PS ODN in these tissues agree with the findings of Bijsterbosch *et al.*, (1996), Rifai *et al.*, (1996) and Butler *et al.*, (1997). Butler *et al.*, (1997) compared the distribution of an intravenously injected, PS ODN *in vivo* in rodent tissue using histologic methods such as immunohistochemistry with a monoclonal antibody that recognised the PS ODN used, direct fluorescence microscopy, and autoradiography of ^{14}C labelled PS ODN. The same pattern of ODN distribution and the intensity of the histologic signal was observed with all three methods, which agreed with previously published pharmacokinetic data on the relevant concentration of PS ODN in different organs. Biochemical studies performed indicated that by two hours, most of the intravenously injected PS ODN was distributed in tissues and was present in its original form. Although degradation occurs within the tissues, more than 50% of the drug was intact at 24 hours in most organs.

Schwab *et al.*, (1994) investigated the inhibition of mutated *H-ras*-mediated cell proliferation and tumorigenicity in nude mice, following s.c. administration of antisense ODNs adsorbed onto polyalkylacyanocrylate nanoparticles. The authors found that the nanoparticle adsorbed ODNs selectively inhibited the proliferation of

cells at a concentration 100 times lower than that of free ODNs and also markedly inhibited tumour growth after s.c. injection.

Sato *et al.*, (1996) investigated targeted delivery to the lungs in mice of taxol-loaded PLGA microspheres containing isopropyl myristate. PLGA (Mw 10,000), in a 75:25 copolymer ratio was used for fabrication of spheres. The sphere suspension was administered by a single i.v. bolus injection through the jugular vein. The authors observed sustained release of taxol in the lungs compared to other tissues and plasma. High levels in the lungs were observed for three weeks following administration of spheres, whereas free taxol solution disappeared from plasma within 4-6 hours, with extensive tissue distribution.

Eyles *et al.*, (1997) studied the oral delivery and fate of PLA microsphere encapsulated Interferon in rats. The effects of encapsulated and unencapsulated interferon were determined. The authors concluded that microencapsulation markedly affected the oral uptake, and possibly post-absorption pharmacokinetic parameters.

Stjarnkvist, (1993) investigated the effect of microparticle-bound primaquine in mice. In this study, primaquine was coupled to polyacryl starch microparticles via a biodegradable tetrapeptide spacer arm. The microparticle bound primaquine was found to be in excess of 100 times more efficient than free drug in reducing parasite load. This was probably due to the targeting of the primaquine-loaded microparticles mainly to the liver in the infected animals.

Yang *et al.*, (1997) evaluated the intratumoral injection of PLA cisplatin microspheres in rats with breast tumours using ^{131}I Iodomisonidazole. This study found a decrease in tumour size after therapy with both free cisplatin and spheres. The advantage of significantly less renal toxicity following treatment with spheres was apparent.

5.4 CONCLUDING REMARKS

Various studies on the pharmacokinetics of ODNs have confirmed that these molecules are unable to traverse the BBB (for review see Agrawal and Akhtar, 1997). Although some investigators have attempted to improve the systemic delivery of ODNs across the BBB e.g. by transferrin receptor-mediated endocytosis (Walker *et al.*, 1995; Boado, 1995; Partridge *et al.*, 1995; Normand-Sidiqui and Akhtar, 1998), these strategies have largely been unsuccessful in whole animal studies. Therefore, local delivery strategies that are able to bypass this formidable barrier and also deliver ODNs in a controlled manner to a specific-region of the brain are required in order to facilitate a more widespread use of ODNs in the CNS.

Antisense ODNs are increasingly being used in the CNS as biological tools, drug-target validation agents and potential therapeutic agents. Although the local delivery of naked ODNs to the brain can result in the desired biological effects, the duration of efficacy is relatively short lived due to the combined effects of rapid ODN degradation and elimination half-lives *in vivo*. In this study, the use of biodegradable polymer microspheres as a site-specific delivery system for targeting ODNs to the neostriatum of the rat brain was investigated. For *in vivo* evaluation, PLGA microspheres containing fluorescently-labelled ODNs were stereo-taxically administered to the neostriatum of the rat brain and the biodistribution of ODNs was monitored after 48 hours. Administration of free, fluorescently-labelled ODNs to the neostriatum resulted in a punctate cellular distribution of ODNs after 24 hours, with little or no ODN remaining in the neostriatum after 48 hours. In comparison, fluorescently-labelled ODNs delivered using polymer microspheres, were intensely visible in cells 48 hours post-administration and the fluorescence appeared to be diffuse covering both cytosolic and nuclear regions. Dual-label immunohistochemical analyses suggested that ODNs were distributed mainly to neuronal cells. These data indicate that site-specific administration of ODNs using biodegradable polymer microspheres provide not only sustained delivery of nucleic acids, but can also improve the cellular distribution of ODNs to brain cells. Sustained or controlled-release biodegradable polymer formulations, therefore, represent an attractive strategy for improved local delivery of ODNs to the CNS.

WBA offers a sensitive, accurate and reproducible method for the quantitative measurement of the tissue distribution of radiolabelled compounds (Wilson *et al.*, 1995; Shamada *et al.*, 1995; Mumtaz *et al.*, 1997; Zane *et al.*, 1997; Labarre *et al.*, 1998). Such methodology evaluates biodistribution, provides pharmacokinetic data for predicting the potential tissue deposition of an absorbed dose of radioactivity, thus allowing visual and quantitative evaluation of radioactivity in small anatomical structures that otherwise could not be detected by conventional tissue combustion technology. This novel method of WBA, should prove valuable information for pharmacodynamic studies, and afford a predictive tool for nuclear medicine by assessing specific targeting of selected tissues.

Following s.c. implantation of PLGA microspheres containing radiolabelled ODNs in Balb-C mice, and analysis using WBA, polymer encapsulated ODNs gave a similar biodistribution to free ODN. However, the majority of free ODN was distributed within 24 hours, whereas polymer released ODN was observed still present in organs and at the site of administration to a greater extent even after seven days. Microautoradiography studies of the liver and kidneys showed similar biodistribution of antisense compounds with the majority of radioactivity concentrated in the Kupffer cells of the liver and the proximal convoluted tubules of the kidney.

The pharmacokinetics of PS ODNs are well characterised. PS ODNs are primarily metabolised by 3'-exonucleases, however 5'-exonuclease-mediated degradation also occurs. It appears that the modulation of pharmacokinetc parameters by alternative delivery routes may offer attractive opportunities for their use in the clinical setting. The stability of antisense ODNs is a key factor in all *in vivo* studies. Due to a limitation of facilities and time available, the stability of ODNs in the excised tissues from mice was not conducted in the above WBA studies. However, the *in vitro* stability of the same PS ODN microspheres following incubation in serum for 28 days was conducted (see section 3.7). PLGA microspheres provided stability of the entrapped ODN, with intact ODN still present after a 28 day exposure period.

The data obtained collectively suggests that biodegradable, sustained release, PLGA microsphere delivery systems offer a method of reducing the dose of ODN required whilst improving cellular biodistribution and pharmacodynamics. Furthermore, by

tailoring the variables of polymer molecular weight, ODN loading and microsphere size, sustained release profiles can potentially be matched to the half-life of a given target. Therefore, PLGA copolymer delivery systems are worthy of further investigation as a safe method of providing site-specific and sustained antisense efficacy.

DISCUSSION

Antisense ODNs can selectively inhibit individual gene expression in both cell culture and animal models, provided they are designed to target a hybridisation-accessible region on mRNA and gain access to the correct intracellular site(s) of action for an appropriate period of time (Akhtar, 1998; Miller and Das, 1998). The use of biodegradable sustained-release delivery systems may obviate the need for repeated administration by facilitating site-specific delivery of nucleic acids in a controlled manner to the desired site. The entrapment of ODNs within polymeric matrix systems may also improve ODN stability, reduce the ODN dose required for efficacy and further reduce toxicity or non-specific activities associated with ODNs.

The overall aim of the research conducted in this thesis was to optimise, characterise and investigate biodegradable PLGA microspheres as potential drug delivery devices for the sustained delivery of antisense ODNs and RBZs. The double emulsion method was deemed suitable for the efficient entrapment of ODNs within defined spherical microspheres, of a reproducible smooth morphology in the diametrical size ranges of 1-5 and 10-20μm. Lewis *et al.*, (1995, 1998) had shown that the fabrication procedures typically used in making PLGA sustained release devices did not adversely affect the biological properties of antisense nucleic acids. Nucleic acids that were entrapped and subsequently released from polymer matrices retained their ability to hybridise, as evidenced by duplex melting temperature determinations (Lewis *et al.*, 1995; 1998) and in the case of a hammerhead ribozyme, by its ability to cleave a RNA substrate as effectively as the control (Hudson *et al.*, 1996). This suggested that unlike protein and peptide-based drugs where solvent-induced structural changes have been observed to render them biologically inactive (for reviews see Crotts and Park, 1998; Okada and Toguchi, 1995), the conformation of ODNs and ribozymes and hence their biochemical activity, had not been altered by the use of organic solvents in the double emulsion methodology of microsphere fabrication.

In vitro studies with PLGA microspheres presented in this study indicated that PS ODNs could be released in a sustained manner and this was controlled by the extent of

ODN loading, microsphere size and polymer molecular weight. The mechanism of release appeared to be a combination of diffusion, matrix release, and in some cases polymer degradation which is consistent with results obtained for unmodified PO ODNs (Lewis *et al.*, 1998). These data suggest that by careful selection of these parameters, and others such as ODN length and chemistry, copolymer composition, and polymer type, it may be possible to achieve the desired target-matched release profile necessary for activity against targets with any given half-life.

The potential for long term delivery was further supported by the enhanced nuclease stability of PS ODNs entrapped within PLGA microspheres. Polymer-entrapped ODNs remained resistant to serum nucleases for at least 28 day, this suggested that biodegradable sustained systems designed to provide ODN release for up to one month would be releasing intact, full-length PS ODNs over the entire duration of the device.

Polymeric spheres have the potential to enhance cellular delivery of antisense ODNs. In this report, the cellular associations of free ODNs and ODN encapsulated microspheres were studied in cell culture models. The A431 epithelial cell line was chosen for mechanistic studies. The sphere suspension in cell culture media was allowed to associate with the cells over a 12-hour incubation period. The cells appeared to have a maximum load for polymer uptake, $1x10^5$ cells taking up approximately 375 μg of polymer spheres independent of the amount of spheres added. Cellular association was highly efficient, a dose of 2.5mg polymer (5mg/ml) resulted in approximately 20% cell association compared to only 1-2% of the equivalent free ODN over 12 hours at 37°C.

At reduced temperature (4°C) and in the presence of metabolic inhibitors, cellular association was significantly reduced for both free ODN and microspheres. These results suggested that the spheres were being internalised, with the involvement of endocytosis, and not simply being adsorbed to the cell surface or passive diffusion occurring alone. Cellular entry via fluid phase endocytosis may also be unlikely, as levels of cell associated spheres and free ODN were considerably higher than those of mannitol, which is a marker of FPE (Luby-Phelps, 1989). Similar findings have been reported by Lewis *et al.*, 1998. Cellular association of spheres was demonstrated to be

concentration dependent and saturable. These characteristics have also been described for ODNs (Loke *et al*, 1989; Hawley and Gibson, 1996).

Competition studies between PS ODNs and spheres were also conducted, and spheres appeared to compete with ODNs for association in A431 cells. This suggests an overlap in their mechanism(s) of cell entry. However, polyanions such as dextran sulphate and heparin were also shown to compete with ODNs for cell association. As these polyanions and polymer microspheres are chemically unrelated to ODNs, this casts doubt upon the existence of a specific protein receptor being responsible for the cellular uptake of ODNs. Alternatively if specific binding protein(s) do exist, they exhibit low levels of affinity for their ODN ligands.

The overall trends in cellular association in a variety of unrelated cell lines confirmed that the levels of cell association were cell-type dependent (when normalised to cell number). A variety of factors including cell size, morphology, and availability of binding sites may have influenced levels of cell association. Similar cell-type dependency for the association of ODNs has been reported by various authors (Loke *et al.*, 1989; Beck *et al.*, 1996; Akhtar and Rossi, 1996). This highlights the fact that it is difficult to draw quantitatively accurate comparisons between cell association studies performed in different cell-types.

Cellular efflux studies, in A431 cells, confirmed that cell association of both ODNs and ODN encapsulated spheres was dynamic, and represented both the uptake and efflux processes. The establishment of an equilibrium between cellular uptake and export offered a likely explanation for the 'plateau effect' which was observed after 2-3 hours in the initial cell association studies. Efflux appeared to be biphasic, with approximately 50% of cell associated spheres being exported from cells within 4 hours. Similar results for ODNs have been observed by other investigators (Stein *et al.*, 1993; Tonkinson and Stein, 1994).

Since the ultimate goal of the research group is to apply the use of antisense ODNs and ribozymes to the treatment of malignant diseases, the *in vivo* distribution of ODN encapsulated spheres was assessed following localised delivery to the brain. In recent years, antisense ODNs have been increasingly used in the CNS, as biological tools for

understanding gene expression in relation to downstream behavioural consequences, as rapid screens in drug target validation and as potential therapeutic agents (for reviews see Chiasson *et al*, 1996; Wahlestedt, 1994). However, since ODNs can not normally traverse the BBB (Agrawal *et al.*, 1992; Akhtar and Agrawal, 1997), these studies have necessitated the use of localised i.c. or i.c.v. injections. The use of implantable biodegradable PLGA microspheres as sustained-release delivery systems for the site-specific administration of antisense ODNs to the neostriatum of the rat brain were evaluated.

The time course of intracellular distribution following intrastriatal injection of naked FITC-labelled ODNs has been studied (Sommer *et al*, 1998), in which the stability of PS ODNs over the time-frames used in this study were confirmed (see also Ho *et al*, 1998). The studies also found a major switch in the pattern of intracellular fluorescence, from a diffuse cytoplasmic and nuclear staining within thirty minutes post-injection to a punctate cytoplasmic staining that appeared gradually at later time points (6, 24 and 48 hours) which by electron microscopic analysis was shown to be due to the presence of the ODNs in intracellular vesicles (Sommer *et al.*, 1998). In the present study, we confirmed that naked PS ODNs are predominantly taken up by neuronal cells, and ultimately localise within vesicular structures as indicated by the punctate distribution of fluorescence in these cells. However, in the case of the slowly released PS ODNs from the PLGA microsphere formulation, the pharmacodynamics and pharmacokinetic profile of ODNs appeared to have been favourably modified. This resulted in an improved ODN biodistribution in these cells following sustained release and was characterised by the more diffuse cytosolic and nuclear fluorescence in neuronal cells. Although the mechanism of uptake and intracellular trafficking was not addressed in this study, the current hypothesis is that uptake of ODNs released from microspheres still occurs via endocytosis but the continuous release and exposure of ODNs to neuronal cells allows for a greater release from vesicular compartments culminating in a greater subsequent accumulation within the cytosol and nucleus of these cells. This altered intracellular distribution following sustained polymeric delivery of ODNs, as opposed to that observed with a single bolus administration, may result in an increased and prolonged antisense activity.

It is important to note that although polymeric delivery although improved the biodistribution in neuronal cells, it did not alter the cellular tropism of ODN uptake during the study period. It is not entirely clear why neuronal cells have a greater affinity to take up ODNs compared to other cell populations, such as the astrocytes and astroglia, but similar findings have been reported previously (Sommer *et al.*, 1993; Sommer, 1998, Zhang *et al.*, 1996; Szklarczuk and Kaczmarek, 1997). It may reflect differences in cell surface binding proteins known to facilitate ODN uptake, or simply the rate of endocytosis or vesicular transport and/or intracellular trafficking. However, it may be possible to alter cell tropism by entrapping ODN conjugates that contain a cell-specific targeting moiety (Juliano *et al.*, 1999).

It is also notable, that the strength of the fluorescent signal in cells seems to be almost identical despite the nearly 20-fold lower dose of ODN injected into the animals as polymer microspheres compared to the naked ODN. This finding strengthens earlier observations that after bolus injections most of the ODNs seem to disappear from the injected region, probably reflecting a rapid clearance (Sommer *et al.*, 1998; Szklarczuk and Kaczmarek, 1997; Grzanna *et al.*, 1998). It also suggests that much lower doses of ODNs will be required for activity if delivered as a biodegradable polymer formulation.

WBA is a technique for studying the tissue distribution of radiolabelled molecules in the whole animal. The results obtained contribute significantly to the understanding of the movement of substances and their metabolites *in vivo*. Moreover, the sites of accumulation of radioactivity may be related to the metabolic fate of a compound. WB autoradiographs can provide a picture of the distribution of isotopically labelled compounds *in vivo,* extending from the principal organs down, to even the cellular level in some cases.

In this report, two different methods were used for the analysis of whole body images. A PhosphorImager was compared to a Biospace Digital imaging system. Images produced by the Biospace system were of greater precision, with images produced after a 24 hour exposure period. Similar images were produced on exposure to the Phosphor imaging system, however a 12 week exposure period was necessary for tritium analysis

Following s.c. implantation of PLGA microspheres containing radiolabelled ODNs in Balb-C mice, and analysis using WBA, polymer encapsulated ODN gave a similar biodistribution to free ODN. However, the majority of free ODN was distributed within 24 hours, whereas polymer released ODN was observed to be present in organs and at the site of administration to a greater extent after seven days. In comparison, 5-FU, a low molecular weight compound was cleared within 24 hours post administration. Quantification of radioactivity present in the liver, kidney, spleen and the site of injection were also conducted. High levels were found in the liver and kidney at each time point. The sustained release capacity of encapsulated ODN was the main focus of this study. From quantification analysis, it was evident that the concentration of radioactivity at the site of administration was significantly higher for encapsulated spheres than free ODN. Seven days post administration, spheres were still resident at the site of injection at a higher concentration, compared to organ levels of radioactivity. Hence, it may be assumed that spheres are still present at the site of administration and thus offer the potential for sustained release of ODN. WBA whilst providing extremely valuable information, cannot and should not be regarded at an end in itself. Quite clearly, for a complete understanding of the movement of molecules *in vivo*, the results of many other techniques, for example biochemical, physiological and pharmacological, are required. However these can be more clearly understood when viewed together with WB distribution patterns.

Microautoradiography studies of the liver and kidney showed similar biodistribution of antisense compounds, with the majority of radioactivity concentrated in the Kupffer cells of the liver and the proximal convoluted tubules of the kidney.

In conclusion, these studies suggest that biodegradable, sustained release PLGA microsphere delivery systems offer a method of reducing the dose of ODNs and RBZs required, whilst improving cellular biodistribution and pharmacodynamics. Furthermore, by tailoring the variables of polymer molecular weight, ODN loading and microsphere size, sustained release profiles can potentially be matched to the half-life of a given target. Therefore, PLGA copolymer delivery systems are worthy of investigation further as a safe method of providing site-specific and sustained antisense efficacy.

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