BIODEGRADABLE POLYMERS FOR THE SUSTAINED RELEASE OF ANTISENSE NUCLEIC ACIDS

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SUMMARY

Antisense oligodeoxynucleotides can selectively inhibit individual gene expression provided they remain stable at the target site for a sufficient period of time. Thus, the efficacy of antisense oligodeoxynucleotides may be improved by employing a sustained release delivery system which would protect from degradation by nucleases whilst delivering the nucleic acid in a controlled manner to the site of action. Biodegradable polymer films and microspheres were evaluated as delivery devices for the oligodeoxynucleotides and ribozymes. Polymers such as polylactide, polyglycolide, polyhydroxybutyrate and polyhydroxyvalerate were used due to their biocompatability and non toxic degradation products.

Release profiles of antisense nucleic acids from films over 28 days was biphasic, characterised by an initial burst release during the first 48 hours followed by a more sustained release. Release from films of longer antisense nucleic acids was slower compared to shorter nucleic acids. Backbone type also affected release, although to a lesser extent than length. Total release of the nucleic acids is dependent upon polymer degradation, no degradation of the polymer films was evident over the 28 day period, due to the high molecular weight and crystallinity of the polymers required to make solvent cast films. Backbone length and type did not affect release from microspheres, release was generally faster than from films, due to the increased surface area, and low molecular weight polymers which showed signs of degradation over the release period, resulting in a triphasic release profile. An increase in release was observed when sphere size and polymer molecular weight were decreased.

The polymer entrapped phosphodiester oligodeoxynucleotides and ribozymes had enhanced stability compared to free oligodeoxynucleotides and ribozymes when incubated in serum. The released nucleic acids were still capable of hybridising to their target sequence, indicating that the fabrication processes did not adversely effect the properties of the antisense nucleic acids.

Oligodeoxynucleotides loaded in 2μm spheres had a 10 fold increase in macrophage association compared to free oligodeoxynucleotides. Fluorescent microscopy indicates that the polymer entrapped oligodeoxynucleotide is concentrated inside the cell, whereas free oligodeoxynucleotides are concentrated at the cell membrane.

Biodegradable polymers can reduce the limitations of antisense therapy and thus offer a potential therapeutic advantage.

keywords:- oligodeoxynucleotides, film, microsphere, biodegradable, stability.

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LIST OF ABBREVIATIONS

A, C, G, T	adenine, cytosine, guanine, thymidine
Ab	antibody
AIDS	aquired immune deficiency syndrome
ATP	adenosinetriphosphate
bp	base pairs
°C	degrees Celsius
cm,mm,μm,nm	centimetre, millimetre, micrometre, nanometre
CO_2	carbon dioxide
cpm	counts per minute
DABCO	diazobicyclo[2.2.2]octane
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSC	differential scanning calorimetry
DTT	dithiothreitol
EDTA	ethylenediaminetetra acetic acid
FATMLV	freeze and thaw multi lamellar vesicles
FBS	foetal bovine serum
g, mg, μg, ng	gram, milligram, microgram, nanogram
GC	gas chromatography
HIV	Human immunodeficiency virus
HPMC	hydroxypropylmethyl cellulose
IFN-γ	interferon-gamma
im	intramuscular
ip	intraperitoneal
iv	intravenous
L, ml, μl	litre, millilite, microlitre
LPS	lipopolysaccharide
M, mM, μM, nM	molar, millimolar, micromolar, nanomolar
mmol, μmol, nmol, pmol	millimoles, micromoles, nanomoles, picomoles

Mw	weight average molecular weight
MP	methyl phosphonate
mRNA	messenger RNA
MTT	3-[4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide]
N_3 + 2-DG	sodium azide + 2-deoxyglucose
OD_{260}	optical denisity at 260 nm
ODN	oligodeoxynucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PHB	polyhydroxybutyrate
P(HB-HV)	polyhydroxybutyrate/hydroxyvalerate
PHV	polyhydroxyvalerate
PGA	poly (glycolic acid)
PLA	poly (lactic acid)
PLGA	polylactide co-glycolide
PLL	poly(L-lysine)
PO	phosphodiester
PNA	peptide nucleic acid
ppm	parts per million
PS	phosphorothioate
pur	purine (adenine and guanine)
PVA	poly (vinyl alcohol)
PVP	polyvinylpyrrolidone
pyr	pyrimidine (cytosine and thymine)
RES	reticuloendothelial system
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
sc	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	scanning electron micrograph
TBE	tris-borate electrophoresis buffer
TCA	trichloroacetic acid
TEMED	N, N,N',N'-tetramethylethylenediamine
Tm	melting temperature

Tg	glass transition temperature
uv	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER ONE

INTRODUCTION

1.1 ANTISENSE OLIGODEOXYNUCLEOTIDES

Antisense oligodeoxynucleotides (ODNs) are short lengths of single stranded DNA designed to regulate over expressed or undesirable genes and inhibit viral replication. The principle of this method requires an intervention in the normal transfer of genetic information in the cell from DNA to mRNA to protein. Antisense ODNs are designed to be complementary to (sense) pre-mRNA or mRNA molecules of the target gene and interact to form an antiparallel duplex. Once a unique target sequence in the gene or its message of interest is identified, an ODN can be synthesised against the target sequence. Through rational design it is hoped that ODNs will show a dramatic increase in affinity and selectivity for nucleic acid targets compared to traditional drugs.

The potential of antisense ODNs to inhibit gene expression has been extensively reviewed (Zon, 1988; Hélène and Toulmé, 1990; Agrawal and Sarin, 1991; Crooke, 1992; Calabretta *et al.*, 1993; Carter and Lemoine, 1993; Leonetti *et al.*, 1993; Pierga and Magdelenat, 1994). Despite the fact that human antisense clinical trials have already started, questions about the true mechanisms of action and delivery of these antisense nucleic acids still need to be addressed. Studies by Kitajima *et al.*, 1992 ; Higgins *et al.*, 1993; Ropert *et al.*, 1994, have shown that antisense ODNs can selectively inhibit gene expression *in vitro* and *in vivo* provided they remain stable at the target site for a sufficient length of time. This will require repeated administration of an antisense agent given exogenously at appropriate intervals to ensure a continuous suppression of the target.

The general structure of an ODN is shown in Fig.1.1, consisting of the sugar phosphate backbone of a DNA strand. Structural modifications have been developed, due to the nuclease instability (Wickstrom, 1986), which has resulted in a series of ODNs with varying properties. Modifications can be made at the phosphodiester linkage, the base, or the sugar phosphate backbone (section 1.1.3).

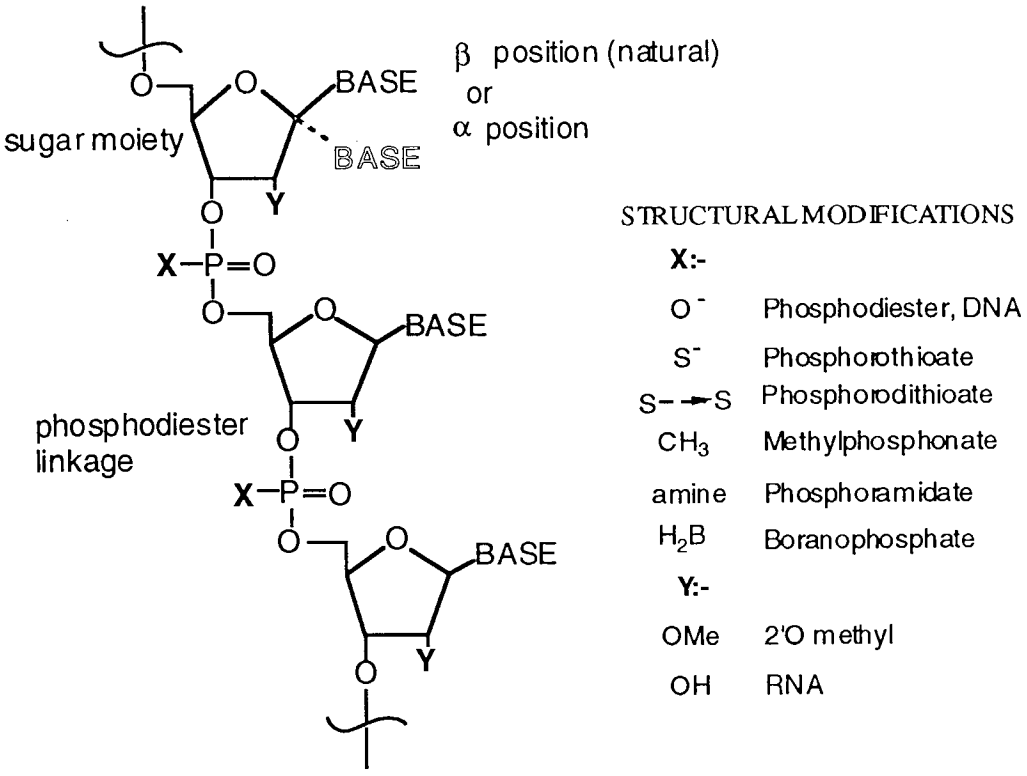


Fig.1.1 General structure of oligodeoxynucleotides, indicating positions of structural modifications.

1.2 INTERACTION OF OLIGODEOXYNUCLEOTIDES WITH NUCLEIC ACID TARGETS AND MODE OF ACTION

Genetic information is encoded by the nucleotide sequence of chromosomal DNA. When a gene is to be expressed, it is transcribed from DNA into the corresponding nucleotide sequence of a mRNA molecule within the nucleus. The processed mRNA enters the cytoplasm and is translated on ribosomes to the amino acid sequence of the protein, the half life of mRNA varies from a few minutes to many hours and is highly regulated. It is the protein which is responsible for producing the biological effects of the gene.

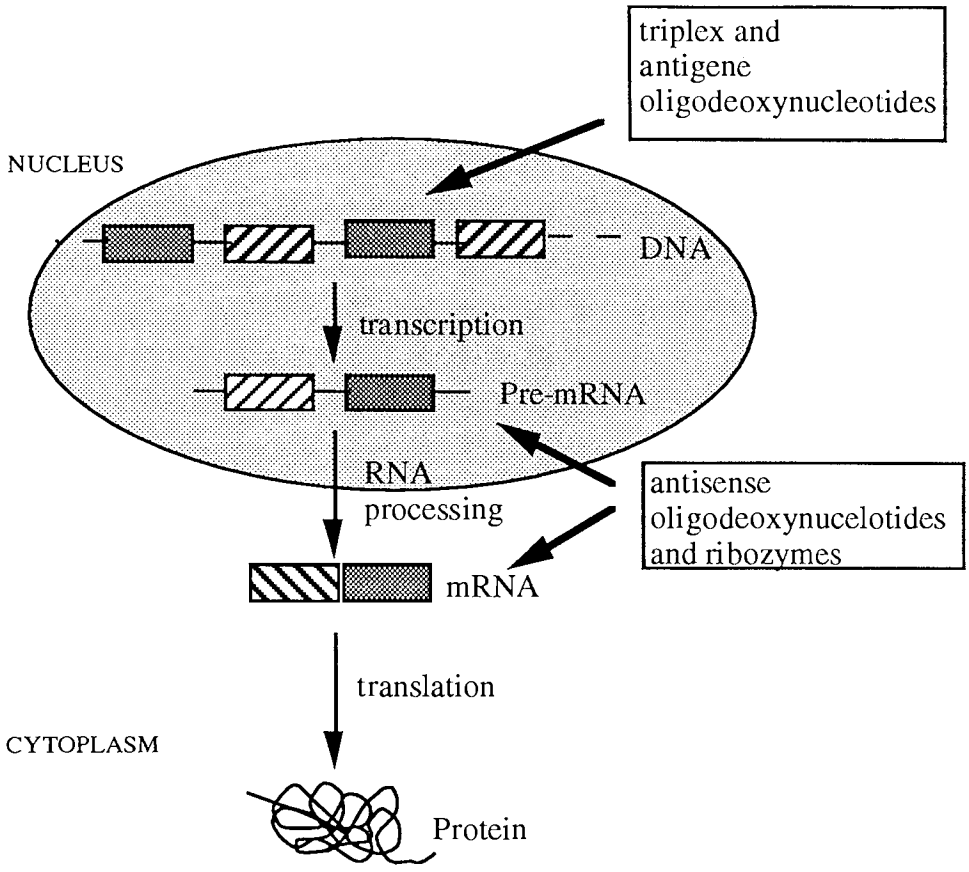


Fig.1.2 Schematic diagram showing possible sites of action of oligodeoxynucleotides.

ODNs are designed to modulate the information transfer from gene to protein (Fig. 1.2), by altering the intermediary metabolism of RNA, each site along this pathway is a potential site of action for ODNs. The ODN can act by antisense or antigene strategies, the antisense effect on single stranded mRNA and the antigene effect on double stranded DNA. The action of ODNs is only transient (Degols *et al.,* 1994) and hence require repeated administration to cause a reduction of the target protein. ODNs can exert their action by several modes of action, structural modifications can also be used to enhance a specific mode of action.

ODNs may bind to DNA and prevent either initiation or elongation of transcription by preventing effective binding of transcription factors. This is done by either seeking transient single stranded regions of DNA, denaturing double stranded DNA or interacting with double stranded DNA to form a triple stranded structure (Moser and Dervan, 1987). The formation of a triplex (reviewed by Sun and Hélène, 1993) occurs when the ODN recognises the hydrogen bonding donor receptor sites on the purine reference strand, lying in the major groove of the DNA double helix. Cooney *et al.*, (1988) have shown that a triple helix can arrest *c-myc*

transcription *in vitro*. Disadvantages of the triple stranded structure is that binding is non specific as runs of homopyrimidines are required, as purine rich ODNs form triple structures most easily. In order to overcome the requirement of purine-pyrimidine runs, ODNs with linkers which stretch over from one strand of the duplex to the other can be used. Relatively long triple strand structures are needed to give sufficient stability to the triplex, addition of intercalators, cross linkers and alkylators can also be used to enhance the stability of the triple helix e.g. psoralen (Takasugi *et al.*, 1991); incorporation of EDTA:Fe to a homopyrimidine ODN resulted in cleavage of the double stranded DNA (Moser and Dervan, 1987). The advantage of the formation of triple stranded structures is that transcription is the first step in the intermediary metabolism of RNA and they may therefore provide substantial leverage for drug therapy.

ODNs can act further along the pathway by binding to pre-mRNA to inhibit further RNA processing. The excision of introns is a key sequence specific step in the mRNA metabolism, binding of ODNs to sequences which need to be spliced, prevents the binding and subsequent action of spliceosomes and therefore cleavage and production of mature mRNA does not occur.

Another mode of action of ODNs is the inhibition of translation: by binding to the translation initiation codon the translational step is prevented. However few studies have shown ODNs to bind to targets for which they were designed. Binding of ODNs near to the 5' cap site of the mRNA is another possible mode of action, as ODNs in this region have been shown to inhibit the binding of proteins required to cap the RNA which cause stability.

Perhaps one of the most important mechanisms of action is the ability of ODNs to activate Ribonuclease H (RNase H), which is an ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. The RNA will become inactivated by rapid and complete degradation regardless of the position of antisense binding (Dash *et al.*, 1987), as this method is catalytic, one antisense molecule can inactivate many target molecules. It has been shown that ODN as short as tetramers, with DNA like properties can activate RNaseH (Donis-Keller, 1979). MP ODNs do not activate RNaseH, however sequences combining MP and PO linkages retain RNaseH activity (Spiller and Tidd, 1992). The induced degradation of mRNA is the objective of catalytic RBZs (see section 1.4).

ODNs may also act as competitive antagonists, by binding to receptors they prevent the natural agonist from binding and therefore affect normal biological responses.

1.3 STRUCTURAL MODIFICATIONS

1.3.1 Modifications to the Phosphodiester Linkage

There are many structural modifications and combinations possible from the original phosphodiester linkage (shown in Fig.1.1). In this section only the most common will be outlined. The modifications are designed to increase nuclease resistance, target binding specificity and cellular uptake.

Phosphodiesters (PO, where X=O), are polar and water soluble. PO ODNs are degraded by endo and exonucleases in tissue culture (Wickstrom, 1986), the dominant nuclease activity in serum is 3' exonuclease. In cells 3' and 5' exonucleases and endonucleases are present, degradation is more rapid in the nucleus compared to the cytoplasm. In serum PO ODNs are rapidly degraded, the rate of degradation depending on the length of the ODN and the serum type. Human, mouse and rat serum have a similar nuclease activity, with rabbit serum having the most activity (Eder *et al.*, 1991). A 14 mer PO in 10 % foetal calf serum (heat inactivated) showed evidence of degradation within 10 minutes.(Akhtar *et al.*, 1991 b). Typically the half life ranges from 15-60 minutes. Poor uptake and rapid degradation will limit bioavailability, and as a result chemical modifications with enhanced stability have been developed.

Phosphorothioates (PS, where X=S), are polar and water soluble. They have increased stability compared to PO in media, cells, serum, most tissues and urine. They are stable to the action of most nucleases (Campbell *et al.*, 1990), with a half life in the range of 7-19 hours in foetal bovine serum and rat cerebrospinal fluid. PS ODNs have decreased affinity for their target sequence compared to PO ODNs (Ghosh *et al.*, 1993 a). Other potential disadvantages of PS modifications include the partial susceptibility to nucleases and the problem of non-specific biological effects (Cazenave *et al.*, 1989). Also, the mononucleotide phosphorothioate degradation product may be incorporated into genomic DNA, as it is known that mononucleotide α thiotriphosphates are substrates for DNA polymerase, and could alter cellular metabolism by influencing the concentration of intracellular mononucleotide pools. Increasing the chain length and concentration of PS ODNs inhibits RNaseH activity (Gao *et al.*, 1991) and may decrease their activity. Combined PO and PS linkages have increased stability compared to PO ODNs and intermediate target affinity between PO and PS ODNs (Ghosh *et al.*, 1993 a)

Methylphosphonates (MP, where X= CH_3) are non ionic and relatively stable to nucleases. They are taken up into cells and are non toxic but have poorer hybridisation properties compared to charged nucleotides (Maher and Dolnick, 1988). Despite poorer hybridisation and inability to activate RNaseH, MP ODNs have still been shown to inhibit gene expression (Maher and Dolnick, 1988). The poor water solubility of MP ODNs may limit their therapeutic usefulness (Akhtar *et al.*, 1991 a). Chimeric ODNs containing alternate MP and PO internucleoside linkages have increased stability compared to PO ODNs and can activate RNaseH (Spiller and Tidd, 1992).

Boron derivatives (where X= H_2B) are modified at the phosphate linkage or at the base. They have good general stability and are nuclease resistant, the P-B bond is more stable to hydrolysis than the P-O bond and they have intermediate electronic properties between MP and PS ODN (Ramsay-Shaw, 1992 personal communication). Boron has no lone pairs and therefore has increased hydrophobicity possibly leading to increased cellular uptake. The properties of these modified ODNs as antisense inhibitors has yet to be explored.

Phosphoramidates (X= amine group), the nonionic phosphate analogue is resistant to nucleolytic attack, however a completely modified analogue, when bound, to its target is not a substrate for RNaseH. Consequently its antisense activity is reduced (Maher and Dolnick, 1991). Phosphoramidate modifications increase lipophilicity and hence cell uptake. The thermal melting temperature (T_m) for a duplex between the partially modified ODN and a complementary unmodified sequence, is decreased by approximately 1.5°C for every phosphate group modification (Dagle *et al.*, 1991). A decrease in T_m results in a decreased hybridisation affinity. Phosphoramidates have increased antiviral activity compared to PO ODNs (Agrawal *et al.*, 1988), possibly as a result of their increased stability.

All mono-substituted analogues at the phosphate linkage, exhibit stereo-isomerism at each P atom. As a result of a chiral centre, an ODN with n singly modified internucleotide linkages has a possibility of 2^n stereoisomers with variable biophysical and biochemical properties. The Rp monophosphorothioate isomer (with the sulphur pointing to the minor groove) is destabilised compared to the Sp isomer (Cohen, 1992). The problems created by isomerism can be avoided by the synthesis of stereo selective isomers, or the use of di-substituted analogues without a chiral centre.

Phosphorodithioates are a further phosphorous modification with enhanced properties (Marshall and Caruthers, 1993). It is achiral, isostructural and isopolar to the natural PO, more hydrophobic compared to PO and PS and resistant to nuclease degradation. It can form stable duplexes with unmodified nucleic acids, however an increased S content results in a reduced T_m, reflecting the ability to hybridise, resulting in lower efficacy and specificity compared to PO and PS (Ghosh *et al.*, 1993 b). Phosphorodithioates also activate RNaseH and inhibit HIV-1 reverse transcriptase and viral replication, however they offer few advantages over PS ODNs (Ghosh *et al.*, 1993 b). They do not appear to bind to cell proteins, but have potential toxicity due to the dithioate inhibition of host cell polymerase, however cell culture studies show little toxicity (Marshall and Caruthers, 1993).

1.3.2 Modifications to the Base

α- ODNs, the α–anomeric form of the nucleoside (shown in Fig.1.1) has been found to be more resistant to both exonuclease and endonucleases compared to the β–anomeric, natural form (Bacon *et al.*, 1988). α–ODNs form parallel rather than anti-parallel duplexes with RNA or DNA strands. These analogues have increased affinity for the target RNA, but do not induce RNase H (Gagnor *et al.*, 1987; Boiziau and Toulmé, 1991).

1.3.3 Modification to the Sugar- Phosphate Backbone

The 2' position of the sugar is also a possible position for modifications to enhance the nuclease stability, the 2'-O-methyl is highly resistant to nucleases in cells and serum. A 2'-O- allyl modification results in an 8 fold increase in stability compared to a 2'-O- methyl and 60-fold increase compared to 2'-deoxynucleotides, (Fisher *et al.*, 1993). Modifications with bulky groups for example nonyl have only a minor negative effect on hybridisation, 2'-fluoro derivatives are nearly as sensitive as unmodified ODNs. Uniformly modified 2'-deoxy -2' fluoro PS ODNs are nuclease resistant with an increased duplex stability (Kawasaki *et al.*, 1993) The 2' hydroxyl in RNA results in the sugar assuming a different conformation than in DNA. RNA-RNA duplexes assume an A-form double helix, whereas DNA-DNA duplexes

assume a B-form double helix. Therefore it is possible to modify ODNs so they bind more tightly to RNA or DNA sequences (Crooke, 1993).

Peptide nucleic acids (PNAs) have the sugar-phosphate backbone replaced with artificial amino acids (Hanvey *et al.*, 1992). The new backbone was designed by computer modeling to be structurally homomorphous to the deoxyribose phosphate backbone. Stability of PNA / DNA duplexes are significantly greater than DNA /DNA duplexes, although PNA_2 / DNA triplexes are the most stable (see section 1.2 for triplex formation). Although there is no data on cellular uptake, PNAs are very stable in biological fluid (review Nielsen *et al.*, 1994).

Circular ODNs are another modification to enhance stability, they are common in nature, the genomes of bacteria and viruses being circular. Circular ODNs are highly resistant to degradation in human serum, by lacking target sites for exonucleases. The circular ODNs bind to single stranded RNA and DNA by pyr*pur*pyr triplex formation, the circular ODN binds the target on 2 sides, making the complex much stronger than Watson-Crick complexes. Circular ODNs display sequence specific selectivities among the highest yet reported for DNA binding molecules. Mismatched complexes are relatively short-lived, while matched complexes have half-lives in the order of years (Prakash and Kool, 1991).

Self-Stabilised ODNs are an alternative modification, which incorporate a hairpin loop structure at the 3' end and have increased nuclease stability, by protecting the 3' end from nuclease attack. The hairpin does not interfere in hybridisation with complementary nucleic acids (Tang *et al.*, 1993).

No single nuclease resistant ODN analogue has fulfilled all the criteria of an ideal antisense agent. Improvement in one property often results in a compromise in another, however the properties of an ODN can be optimised by combining several structural modifications. Chimeric ODNs with terminal MP and internal PO regions have many advantageous over either structure alone (Spiller and Tidd, 1992) as do chimeric PO and PS ODNs (Ghosh *et al.*, 1993 a).

1.4 RIBOZYMES

A further modification, in both structure and mode of action are ribozymes (RBZs), where the DNA backbone is replaced with an RNA backbone. RBZs (Fig.1.3) are catalytic RNA molecules which have the ability to cleave target RNA substrates in a sequence specific manner in the absence of proteins or enzymes and do not require an energy source. For reviews see: Cech and Bass, 1988; Symons, 1992 and 1994. They have enhanced efficacy compared to ODNs (Lange *et al.*, 1994). The hammerhead RBZ, derived from a self-splicing motif originally discovered in plant pathogens, is the most widely studied ribozyme to date for therapeutic applications (for review see: Bratty *et al.*, 1993). The trans acting form based on the design of Haseloff and Gerlach (Haseloff and Gerlach, 1988), consists of two antisense base pairing stems (I and III) combined with a central catalytic core of non-base pairing conserved nucleotides and an internal loop (Stem II) which is responsible for associating with the divalent metal cation (usually Mg^{2+}) required for catalytic cleavage of the substrate (Tuschl and Eckstein, 1993). Trans acting RBZs behave like true enzymes in that they have the ability to cleave substrate RNA sequences without self alteration and can exhibit multiple turnover of substrate RNA molecules.

A standard numbering system has been agreed for the nucleotides within the catalytic core of the hammerhead RBZ (Fig.1.3), (Hertel *et al.*, 1992). Hammerhead RBZs are based around the core sequence of the (+) sTRSV hammerhead, with the other nucleotides determined by the requirements of Watson-Crick base pairing between the RBZ and substrate RNA, enabling sequence specific targeting. As a result RBZs can potentially be targeted against any substrate mRNA provided it contains a specific trio of nucleotides which allow formation of the correct secondary structure to permit cleavage. Rules are not available for reliably predicting all cleavable sites in a given mRNA although the base trios; GUC, CUC, GUA and UUC can generally be cleaved providing incompatible secondary structures are not formed by the bases on either side (Bratty *et al.*, 1993).

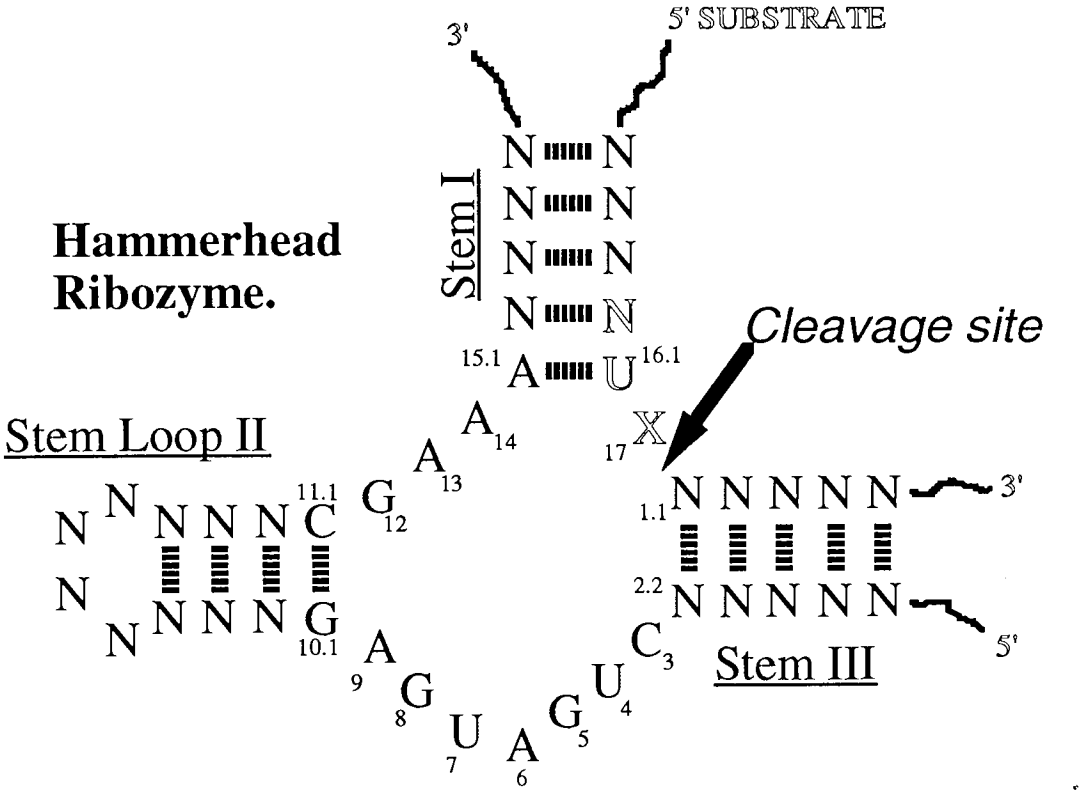


Fig.1.3 General structure of a hammerhead ribozyme and substrate.

Chemical stability is a prime consideration for the exogenous delivery of RBZs. RNA is quickly destroyed by nucleases present in the serum, and cellular uptake of free RNA is poor (Karikó *et al.,* 1994). While a mechanism for cellular uptake of ODNs has been described, nothing has for ribonucleotides (the cellular uptake and distribution is described in section 1.6).

RBZs, like ODNs, can be synthesised by standard solid phase chemistry (Wincott *et al.*, 1995), and chemical modifications can be made at the base, sugar or phosphate backbone to modify stability and cleavage activity. Chemical modifications to RBZs can enhance nuclease stability, but catalytic activity is often compromised, as a result the location of the modification is important. Serious impairment of cleavage activity results when PS linkages were introduced into the hammerhead RBZs at certain sites. (Buzayan *et al.,* 1990; Ruffner & Uhlenbeck, 1990). PS linkages at the 3 and 5' ends protect from degradation as do inverted bases or non bases at the 3' end (Usman, 1995 personal communication). Modifications to the 2' position can enhance nuclease stability, however the T_m of

the RBZ and substrate falls with increasing size of the substituent at the 2' position. 2' fluoro and 2' amino substitutions increase serum nuclease resistance, but decrease their catalytic activity (Pieken *et al.*, 1991). The pyrimidine 2' hydroxyl groups are essential for catalysis and can not be modified (Eckstein, 1992 personal communication). The length of stem II is also important for activity, reduction from four to two base pairs does not alter activity, but a further reduction results in a drastic loss in activity (Tuschl and Eckstein, 1993). Chimeric RBZs with DNA bases in the flanking arms have enhanced nuclease resistance compared to all RNA RBZs (Taylor *et al.*, 1992; Shimayama *et al.*, 1993). These chimeric RBZs also display enhanced cleavage activity (Hendry *et al.*, 1992; Taylor *et al.*, 1992).

Studies have shown that the RBZs needs to be in great excess of the substrate in order to decrease target levels and must remain stable over this period. Unmodified RBZs complexed with lipofectin enhance cellular uptake and increase stability in cell medium (Karikó *et al.*, 1994). Therapeutic applications of RBZs have been reviewed by Rossi *et al.* (1992). Even with improved stability repeated administration remains likely to be necessary for the application of ribozyme therapeutics. Such repeated administration would be clinically undesirable in many cases. Therefore the development of a biodegradable delivery system would be advantageous for the exogenous delivery of both ODNs and RBZs.

1.5 DESIGN OF OLIGODEOXYNUCLEOTIDES

Considerations for the design of antisense molecules for therapeutic application has been reviewed by Hélène (1991) and Magrath (1994). The success of achieving an antisense effect is dependent not only on the ODN stability and the delivery system but also on other factors such as ODN length, sequence and choice of target site.

The sequence length is controlled by the optimum length required to achieve a selective match with the target sequence, bioavailability and production costs. The sequence length governs the specificity of the antisense molecule, decreasing the sequence length results in an increase in the occurrence of complementary sites in the genome. Selectivity for a target increases with increasing sequence length, a single base pair substitution can virtually abolish antisense activity (Maltese *et al.*, 1995). Antiviral activity is both sequence and length dependent (Lisziewicz *et al.*, 1992), decreasing the chain length from a 28mer to 22mer resulted in a 10 fold loss of activity (Kinchington *et al.*, 1992). However the advantages of increased length

are often compromised by a decrease in cellular uptake for longer ODN sequences. The problem of reduced cellular uptake of longer antisense sequences can be overcome by 'tandem targeting' using 2 short sequences with adjacent target sites (Maher and Dolnick, 1988). Antisense molecules are usually in the region of 15-18 nucleotides in length.

The selection of the target site is also important, a 15 mer PS antisense to the loop forming site on the *mdr*-1 mRNA was more effective and specific in modulating multi drug resistance than a 15 mer PS antisense to the 5' end coding region (Thierry *et al.*, 1993). Sequences designed to hybridise to the CAP site (position where ribosomes access RNA) or the AUG site (initiation site), are thought to be most effective. However the secondary structure of the target site determines the accessibility to antisense molecules.

The sequence of the target influences the ODN sequence and certain base combinations are best avoided to reduce the chance of non antisense effects. ODNs containing any 6-base palidromes can induce interferon and natural killer activity. AACGTT are the first six bases of a widely used ODN sequence against *c-myc*, which raises the possibility that some reported findings may be a result of artifacts (Kreig, 1993). Some investigators have reported that PS ODNs containing four consecutive Gs have non-sequence-specific effects (Kreig, 1993). However, murine and human sequences against *Rel* A have 4 and 5 consecutive Gs respectively, however the human sequence or controls had no effect in reducing murine tumour cell growth (M^{c}Intyre *et al.*, 1993), indicating that consecutive Gs alone are not sufficient to reduce tumour cell growth by a non-specific mechanism.

1.6 CELLULAR UPTAKE AND DISTRIBUTION

The antisense strategy relies on the introduction of short sequences of synthetic ODN into cells. Zamecknik and Stephenson showed in 1978 that ODNs do enter cells despite their charged nature, unmodified ODNs as charged molecules do not passively cross the lipid membrane of the cell wall. The cellular uptake and distribution of ODNs has been extensively reviewed by Jaraszewski and Cohen (1991); Budker *et al.* (1992); Juliano and Akhtar (1992); Vlassov *et al.* (1994 a) The potential mechanisms of cellular uptake of ODNs are shown diagramatically in Fig.1.4. The mechanisms of cellular uptake are complex and are not completely understood. The charge and hydrophobicity of the ODN, which are dependent on structural modifications, are proportional to the membrane transport and cellular distribution. Increasing hydrophobicity increases membrane transport and therefore cellular uptake (Temsamani *et al.*, 1994).

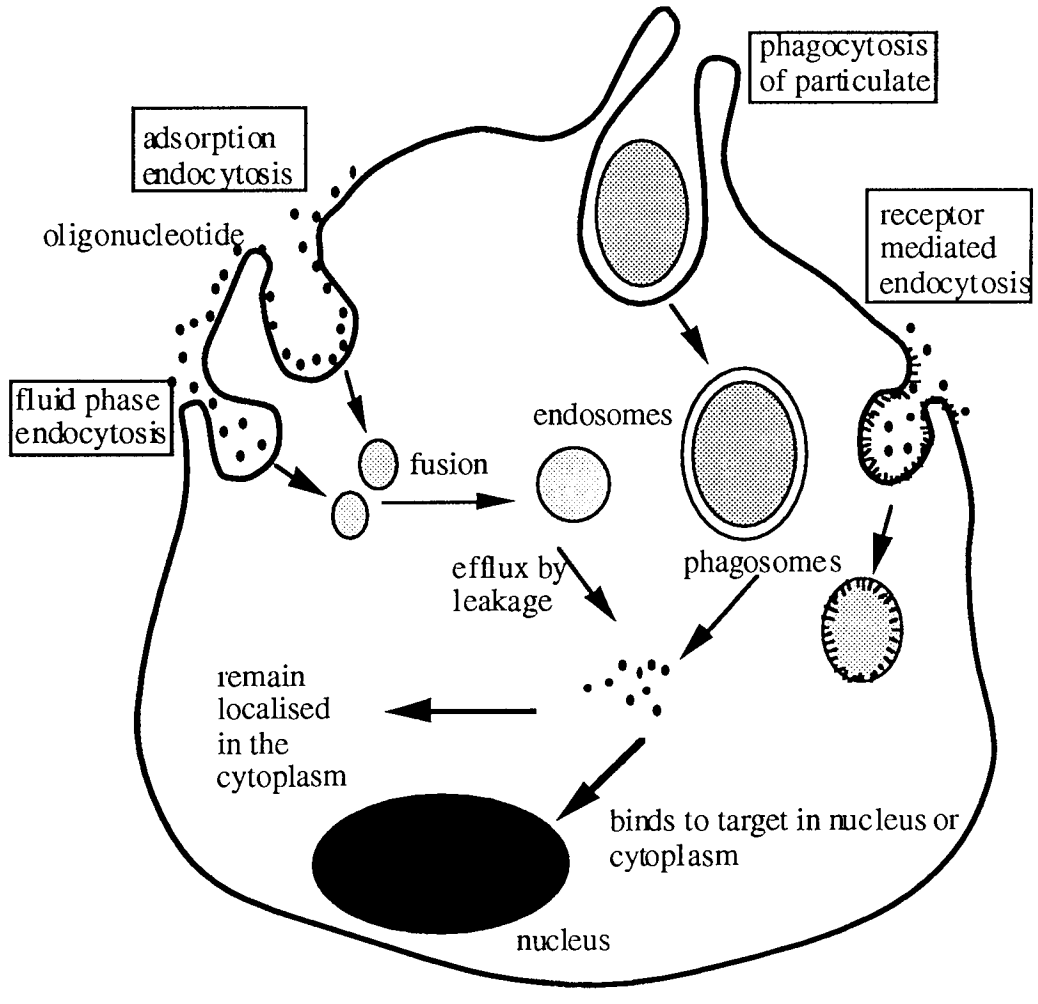


Fig. 1.4 Schematic diagram of cellular uptake and distribution of oligodeoxynucleotides

There are three types of endocytosis shown in Fig.1.4, the first being receptor mediated endocytosis (RME), where a receptor is necessary for the internalisation of ODNs. The second, fluid phase endocytosis (FPE) or pinocytosis, where ODNs may be internalised with the surrounding media. The third method of endocytosis is Adsorptive endocytosis (AE) ODNs adsorbed onto the cell surface are internalised during the normal turnover of the cell membrane. The general efficiency of such uptake is poor.

PO and PS ODNs were thought to enter cells by receptor mediated endocytosis involving a 80-kDa protein (Yakubov *et al.*, 1989), which is saturable process (Loke *et al.*, 1989). However other evidence (Stein *et al.*, 1993 a) suggests that the majority of ODN internalisation is as a result of pinocytosis (fluid phase endocytosis) and is energy dependent (Gao *et al.*, 1992). PS ODNs bind more tightly to the cellular membrane, due to increased affinity for cell surface binding sites (Zhao *et al.*, 1993), although the uptake process occurs more slowly (Loke *et al.*, 1989). MP ODNs are uncharged and lipophilic and do not compete with PO ODNs for uptake, a specific cell surface receptor is not involved (Juliano and Akhtar, 1992) and they are thought to enter cells by either fluid phase endocytosis or adsorptive endocytosis (Shoji *et al.*, 1991). PS ODNs have an increased affinity for the cell membrane ODN binding sites and can completely block PO ODN binding (Zhao *et al.*, 1993). Studies on cellular uptake using flow cytometry (Kreig, 1993 a) have shown, increasing surface binding in the order, MP-PO, PO-, PS-PO, PS- having the highest binding. ODNs bind to the cell membrane to a greater extent at acidic conditions (pH 4.0 - 4.5) compared to the binding at neutral pH. ODN binding at acidic pH may be mediated by a 34kDa protein (Goodarzi *et al.*, 1991). The degree of ODN internalisation is dependent on the cell type (Temsamani *et al.*, 1994; Wu-Pong *et al.*, 1994 a) and is reduced in mature cells. Uptake in keratinocytes does not appear to be dependent on endocytosis (Noonberg *et al.*, 1993). A further study (Kreig *et al.*, 1991) suggests that uptake of ODNs in rapidly growing cells (e.g. malignant cells) is more rapid than in normal cells, the presence of mitogens can also enhance cellular uptake. *In vitro* experiments have found the uptake of a PS 28 mer to be greater in cell monolayers than cells grown in suspension (Gao *et al.*, 1992). Cellular uptake is concentration, temperature and time dependent (Gao *et al.*, 1992; Stein *et al.*, 1993 a ; Temsamani *et al.*, 1994) with shorter ODNs being internalised more readily than longer ODNs (Stein *et al.*, 1988 a; Boiziau and Toulmé, 1991). The uptake process is thought to be more efficient at low concentrations (<1μM), as a larger percentage of the ODN is adsorbed onto the cell surface and internalised by a more efficient adsorptive

endocytosis process (Yakubov *et al.*, 1989). Once transported across the cell membrane, all the ODNs are thought to be enclosed in endosomes which are membrane vesicles. ODNs are released from these endosomal compartments as biological effects do occur, efflux from these acidic endosomes is probably by transient membrane destabilisation, simple leakage during endosome fusion or by diffusion through the membrane, although the exact mechanism is unknown. Free cytoplasmic ODNs rapidly diffuse to the nucleus, PO and PS ODNs colocalise with concentrated regions of small nuclear proteins while MP ODNs colocalise with concentrated regions of genomic DNA (Chin *et al.*, 1990; Leonetti *et al.*, 1991). Endocytosis may have the disadvantage of long-term isolation of ODNs in endosomes, reducing their intracellular availability and stability (Cohen, 1992). The degraded metabolites are exocytosed from the cell (Fisher *et al.*, 1993).

Despite the relatively low *in vitro* cellular uptake of these molecules, sufficient cellular uptake must occur, as *in vivo* studies show that antisense ODNs do exert a pharmacological effect (see section 1.8). Despite these proposed mechanisms of cellular uptake, *in vivo* DNA molecules can enter cells by completely unknown mechanisms (Zhu *et al.*, 1993).

The access of antisense nucleic acids to their target cells at adequate concentrations is a prerequisite for their successful use as therapeutic agents, Therefore new delivery devices which can improve the uptake of nucleic acids into cells will represent a major step forward in their clinical application.

1.7 OLIGODEOXYNUCLEOTIDE DELIVERY STRATEGIES

There are two main approaches to the delivery of antisense molecules to cells, the first is intracellular expression of an antisense gene, where a DNA sequence is inserted into the cell genome, from which the antisense molecule is permanently transcribed. The second approach is exogenous delivery, where the antisense molecule is treated as a drug for administration. It is this second delivery approach which is the main subject of interest in this thesis. Strategies for the delivery of ODNs are equally relevant for the delivery of RBZs. New approaches for the targeting of ODNs have been reviewed by Kabanov and Alakhov (1994).

The potential of ODNs will remain unfulfilled without the parallel development of new drug delivery technologies which allow controlled and site directed delivery. The large molecular weights, relative instability in biological milieu, rapid elimination and charged nature of many nucleic acid derivatives pose a major challenge to the delivery of nucleic acids to their sites of action. Exogenous delivery systems which can protect nucleic acids from digestion and simultaneously provide sustained delivery over extended time periods may be useful for the biopharmaceutical application of nucleic acids. Localised delivery by use of implants counteracts the 'loss of drug' to non target areas of the body, the biodegradable nature of the device eliminates the need to remove the delivery device, although problems may arise if cessation of treatment is necessary. Further structural modifications have been made to ODNs to enhance transport into cells. Without specific delivery strategies adequate intracellular concentrations may only be reached after administration of potentially toxic levels of ODNs.

In an attempt to characterise the different strategies for ODN delivery, there is some overlap as some of the methods are not mutually exclusive.

1.7.1 Increasing Hydrophobicity of the Oligodeoxynucleotide

Attachment of hydrophobic compounds such as cholesterol (Boutorine *et al.*, 1992), alkyl chains (Kabanov *et al.*, 1990), lipids (Shea *et al.*, 1990) or polycations (Leonetti *et al.*, 1990) to ODNs increases the hydrophobic nature of the ODNs. Increased adsorption to the cell membrane has been shown to increase cellular uptake (Temsamani *et al.*, 1994). The attachment is usually by means of

biodegradable linkers, containing disulphide or ester bonds, which enables the ODN to be released from the conjugate.

Conjugation of ODNs with a 5' cholesterol moiety (chol-ODN), which binds to low density lipoprotein has substantially increased cellular uptake, locating predominately in the nucleus (Kreig, 1993 b). However the addition of cholesterol will dramatically reduce the T_m of the molecule and so alter its hybridisation ability. The addition of porphyrin (an organic compound consisting of 4 pyrrole rings linked by CH bridges with a heavy metal in the centre) to the terminal phosphate of a 17 mer caused a 6 fold increase in uptake in T24 cells, while a 30-100 fold increase in cell binding was achieved by the addition of cholesterol to the ODN (Boutorine *et al.,* 1992).

Polycation (polylysine L-lysine, PLL) conjugation with ODNs has resulted in a higher resistance to exonucleases and increased ODN penetration into cells (Verspieren *et al.*, 1987; Leonetti *et al.*, 1990; Degols *et al.,* 1994). Some cell lines do not take up PLL conjugates for unknown reasons, cytotoxicity can limit the usefulness of PLL. Conjugates are often administered with heparin (a sulphated polyanion, with antiviral properties against HIV-1), heparin interferes with early steps of HIV multiplication cycle, preventing virus adsorption onto the cell membrane, and therefore decreasing the initial virus load. Secondly it decreases PLL toxicity and also modifies the release of conjugated material without altering sequence specificity (Degols *et al.,* 1994). This delivery modification is therefore especially useful for the delivery of ODNs used against HIV. PLL itself does not enable cell targeting and conjugates with antibodies or transferrin have been used. Conjugation with asialoglycoprotein-polylysine carriers enhances cellular delivery and protects from degradation (Chiou *et al.*, 1994). The presence of polycations condenses DNA, which facilitates cellular delivery.

Lipofectin, a cationic lipid mixture can significantly increase the uptake and activity of phosphorothioate ODNs in several cell lines and also alters the intracellular distribution of these nucleotides. Cellular delivery of PO ODNs increased 25 fold when administered with a cationic lipid (DOTAP), and protects the ODN from degradation in human serum (Capciccioli *et al.*, 1993).

1.7.2 Receptor Mediated Delivery

Another option for the enhanced cellular delivery of ODNs is conjugation with a compound which has a specific cellular receptor e.g. transferrin and mannose. Receptor-specific macromolecular carriers are good candidates to target ODNs to selected cells and increase their intracellular delivery. Site specific delivery decreases adverse effects associated with with non specific drug delivery. The rational of this approach has been well established, however there have been few sucessful *in vivo* studies

Transferrin receptors are abundantly expressed on the surface of many cell lines and therefore provide a simple mechanism to deliver ODNs to cells. The transferrin receptor efficiently transports iron bearing transferrin molecules into cells by RME. An ODN/transferrin-polylysine complex showed greater biological effect than unmodified ODNs, or polylysine ODNs (Citro *et al.*, 1992). The presence of polycations condenses the DNA structure, facilitating endocytosis, enabling RME as a result of the transferrin group (Wagner *et al.*, 1991). Walker *et al.* (1995), report improved cellular delivery of ODNs using a transferrin receptor antibody ODN conjugate, the conjugate being internalised 3 fold greater than unmodified ODNs. However the abundance of transferrin receptors in the body prevents cell specific targeting of the ODNs. The ideal situation would be to take advantage of receptors unique to the target site, thereby concentrating the distribution of the ODN to a specific site in the body.

Mannose specific lectins on the surface of macrophages mediate the cell uptake of mannose and fucose terminated glycoproteins (Stahl *et al.*, 1978). As a result conjugation of ODNs with mannose has been investigated as a method to improve delivery to macrophages. An ODN conjugated with streptavadin substituted with mannose residues increased the intracellular delivery of the ODN 20 fold compared to the non mannose conjugated ODN (Bonfils *et al.*, 1992 a). The protein incorporates 12 mannose residues and is attached to the ODN via a biodegradable biotin linker. The mannose conjugates are associated with endosomes (Bonfils *et al.*, 1992 b). The synthesis of a monomannoside phosphoramidite linked to the 5' end of an ODN sequence, has shown a 2 to 4 fold increase in uptake compared to unconjugated ODNs (Akhtar *et al.*, 1995). Increasing the number of mannose residues should enable an increased cellular delivery, however it is important that the ODN can be released from the conjugate in order to exert its biological effect.

1.7.3 Membrane Carriers

An alternative approach to cellular delivery of ODNs is the use of carriers. The carriers can deliver ODNs to cells irrespective of the ODNs natural ability to interact with the cell membrane and enter cells. Another main advantage for the use of carriers for ODN delivery, is that the central core of the carrier is protected from the action of enzymes and therefore offers stability to the entrapped ODN. Such membrane carriers include viral envelopes, liposomes and microspheres. The main disadvantage of membrane carriers is that they are naturally targeted to the reticuloendothelium sysyem organs, locating mainly in the liver and spleen.

1.7.3.1 Viral Envelopes

Another method to enhance cellular delivery is by conjugation of an ODN with a fusiogenic peptide by means of a disulfide bond, enabling a 5-10 fold improvement in potency (Bongartz *et al.,* 1994). The peptide changes conformation in acidic pH and destabilises the endosomal membranes, resulting in increased cytoplasmic delivery. The peptide used was an influenza hemaglutinin envelope protein, as enveloped viruses have efficient transfer of their genome from endosomes into cytoplasm of the host cell.

1.7.3.2 Liposomes

To date liposome encapsulation is the most popular vehicle for delivering DNA and RNA to target cells (review Juliano and Akhtar, 1992; Clarenc *et al.*, 1993). Liposomes are microscopically small spheres of phospholipid bilayers which can encapsulate nucleic acids within the aqueous centre or form lipid nucleic acid complexes which protect DNA and RNA from nuclease attack. Traditional liposome preparation methods can not be used with ODNs as sonication of the lipid/ODN suspension results in degradation of the ODN (Byrn *et al.*, 1991). An unmodified 15mer encapsulted in liposomes was stable for up to 7 days when incubated in 10% serum containing media compared to free ODN which rapidly degraded in 30 minutes (Thierry and Dritschilo, 1993). Liposomes are classified into subtypes, depending on morphology (e.g. MLV, FATMLV, SLV) and lipid used (cationic or nonionic, cationic lipids do not encapsulate nucleic acids but form complexes with them, based on the negative charged phosphate groups of the nucleic acid and the positively charged lipid). Cationic liposomes improve ODN stability and

intracellular delivery (Lappalainen *et al.*, 1994 b), fusion with the cell membrane and the ODN is released into the cytoplasm. Unfortunately at high concentrations (40μM) cationic lipid are toxic. The enhanced uptake depends on the cell type used, a 4 fold increase was seen in SKVLB cells and an 18 fold increase in MOLT-3 cells compared to free ODN (Thierry and Dritschilo, 1993). Wang *et al.*, (1995) report that the use of folate-PEG-liposomes increase cellular uptake nine fold compared to non targeted liposomes and a sixteen fold increase compared to an unencapsulated 15 mer (PS or PO).

Encapsulation efficiency in neutral uncharged liposomes is generally low, with MP having the greatest entrappment efficiency (25-30%) in FATMLV (Akhtar *et al.*, 1991 a) as a result of their increased hydrophobicity compared to PO and PS ODNs. Release of PS from neutral FATMLV is relatively slow with 50% released over a 6-9 day period (Akhtar and Juliano, 1992). pH sensitive liposomes promote efflux from the endosomes into the cytoplasm, as protonation causes destabilisation of the membrane.

Immunoliposomes have been reviewed by Mori and Huang, (1995), incorporation of monoclonal antibodies coupled to the vesicles enables targeting to a specific cell population, reducing the need to chemically modify the ODN. The antibody (Ab) conjugate with cationic liposomes form a ternary electrostatic complex, addition of liposomes enhances the transfection efficiency of Ab-PLL/DNA by 10-20 fold in mouse lung endothelial cells in culture (Trubetskoy *et al.*, 1992).

1.7.3.3 Microparticles

The use of nano and microparticles as delivery devices has been extensively reviewed by Watts *et al.*(1990), Arshady (1991) and Couvreur and Puisieux (1993). Microparticles are ideally in the range of 1-125μm and include microcapsules and microspheres. Microcapsules are vesicular systems, where the drug is confined to a central cavity surrounded by a polymeric membrane. Microspheres can be defined as microporous matrix systems, where the drug is distributed uniformly within the polymer matrix. Nanoparticles are submicronic polymeric systems. The formation of these different sized polymeric systems is dependent on the fabrication process. They can be fabricated from various materials, e.g. polyesters, albumin, gelatin, cellulose.

Microsphere delivery systems have the ability to enhance the pharmacokinetic profile of nucleic acids and in some cases enhance transport across biological barriers. The nature of the device can also offer stability against degradation to the entrapped drugs (Cohen *et al.*, 1991). Particulates of biodegradable polymers provide a diffusion barrier with a predictable release pattern, allowing the polymer to be adsorbed harmlessly after drug release. Microspheres have an advantage over other delivery devices, due to their wide range of possible routes of administration, they are easily administered by a single injection, whereas other controlled release delivery forms larger in size require surgical incision. However their potential for erratic release and dose dumping must also be considered. Their applications in biodegradable delivery devices has been reviewed by Conti *et al.*, (1992); Brannon-Peppas (1995). Intrathecal and intraperitoneal administration of bupivacaine loaded PLA and PLGA microspheres has illustrated controlled drug release (Le Corre *et al.*, 1994).

The efficiency of the mononulcear phagocyte system is so great that, that targeting of particulates to tissues is virtually impossible. After intravenous administration of ^{14}C PLA nanospheres, the majority of the radioactivity is found in the liver, spleen, bone marrow, lymph nodes and lungs, where a high density of macrophages are present (Bazile *et al.*, 1992). Coating of microspheres with poloxamine (propylene oxide/ethylene oxide) decreases the localisation in the liver and extends the circulation time, resulting in increased uptake of the spheres in the bone marroe and the spleen. The thickness of the hydrophilic coating is inportant in avoiding uptake by the liver (Illum *et al.*, 1987; Davies *et al.*, 1993). Gref *et al.* (1994) report that attaching PEG to PLGA in a one step procedure increases the circulation time. Coating the microspheres with albumin (a hydrophilic protein) decreases their capture by macrophages, as the surface becomes less hydrophobic (Tabata *et al.*, 1989), however the effect is difficult to interpret as the spheres have already been stabilised by emulsifiers used in their manufacture. Le Ray *et al.* (1994) report that ^{14}C PLGA nanospheres with a high residual PVA surfactant concentration (50%) have a high uptake by RES organs. Intestinal uptake of nanospheres is poor after oral administration, however uptake can be increased by co-administration with concentrated milk (Le Ray *et al.*, 1994).

Polymeric carriers have been reported for the delivery of ODNs (Chavany *et al.*, 1992), the polymeric nature of these nanospheres makes them more stable than liposomes in biological fluids. The ODN is adsorbed onto the surface of preformed polycyanoacrylic nanoparticles by ion pairs between the ODN and hydrophobic cations (e.g. tetraphenylphosphonium salts, TPP) that bind to the polymer surface.

Increasing the ODN chain length increases the adsorption efficiency, although presumably this can be saturated. The ODN is resistant to degradation by 3' exonucleases for 15 minutes at 37°C. The toxicity of the quarteneray ammonium salts limits the dose of spheres which can be given. The release of the surface bound ODN is dependent on the degradation of the polyalkylcyanoacrylate polymer, which in turn is dependent on the esterase content of the release media (Grangier *et al.*, 1991). However the formation of formaldehyde on degradation of the polymer creates toxicity concerns (Çiçek *et al.*, 1994), this combined with the dose limiting toxicity of the quartenary ammonium ions limits the therapeutic utility of this delivery system. Gelatin microspheres (Cortesi *et al.*, 1994) have also been used to encapsulate ODN, but the release rate of the ODN from the spheres is very rapid, with 60% released within 120 hours incubation *in vitro*. Both Gelatin and polyalkylcyanoacrylate are enzymatically degradable, therefore degradation will vary at different sites *in vivo* due to varying enzyme levels. Therefore there is a need to formulate a device for the delivery of ODNs which is non toxic, can protect the ODNs from degradation and can provide sustained release.

1.8 *IN VIVO* PHARMACOKINETICS

ODNs are relatively non toxic, they do not generally exhibit toxicity at doses which are effective for the down regulation of gene expression.

Pharmacokinetic studies to date (Agrawal *et al.,* 1992; Goodchild *et al.,* 1991; Iverson, 1991; Cossum *et al.,* 1993; Yakubov *et al.,* 1993;) have concentrated on PS and PO and MP ODNs, different behavior can be expected from heavily modified ODNs. Preliminary toxicological studies have shown that a dose of 100 mg kg^{-1} body weight of PS for 14 days is non toxic in mice (Agrawal *et. al.,* 1992). Concentrations up to 100μM of ISIS 1082 (a PS 21 mer) had no effect on cell viability (Crooke *et al.,* 1992). Cellular uptake and interactions with cell surface and blood proteins affect their biodistribution. PS ODNs have a biphasic plasma elimination with an initial half life of 15 to 25 minutes, which represents distribution out of the plasma compartment, a second half life of 20-40 hours represents elimination from the body. Repeated daily injections of a 27 mer PS ODN reaches a steady state concentration in 6-9 days (Iverson, 1991).

After intravenous administration PO rapidly distribute to most organs in mice, high concentrations are seen in the liver, spleen, kidneys and muscle, with the brain being the least accessible site for distribution. Approximately 50% of the dose (partially degraded) is excreted by the kidneys in 24 hours. The ODNs reach the bloodstream 30 minutes after intraperitoneal injection and have a similar distribution to intravenous administration, distribution is considerably slower after subcutaneous administration. Intranasal and oral administration provide 5-20% delivery efficiency compared to intraperitoneal, with vaginal and rectal delivery 5-10 times less efficient than the oral route, topical delivery was the least efficient. Topical delivery may have a use in the treatment of topical disease states e.g. Herpes, psoriasis and melanomas, delivery can be improved by the use of penetration enhancers and iontophoresis. Transport is probably by transcytosis through the mucosa cells (Vlassov *et al.,* 1993), an increase in ODN length decreases the rate of penetration (Nolen *et al.,* 1994), with MP ODNs having the highest rate of percutaneous penetration. Degradation of the ODN was greatest following intraperitoneal administration, due to the increased activity of macrophages in the peritoneum and nucleases present, nucleases in the intestines also degrade ODN before they reach the blood stream.

Association of ODN with lipoproteins *in vivo* prolongs plasma half-life and modifies tissue distribution (de Smidt *et al.,* 1991), plasma half life of cholesterol linked ODNs is increased compared to control PO ODNs due interaction with plasma lipoproteins. Delivery of ODNs with cationic lipid (DOTMA, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride) does not appear to alter the tissue distribution in normal mice (Saijo *et al.,* 1994).

After intraventricular administration ODNs were resistant to degradation in the CNS and were cleared by bulk flow (Whitesell *et al.,* 1993). Continuous infusion of a PS ODN at 1.5nmol/hour over a 7 day period resulted in no obvious neurological or systemic toxicity.

Following initial pharmacokinetic studies, in vivo antisense experiments were performed. Table 1.1 gives some examples of successful in vivo antisense therapy. Caution must be applied when interpreting the results from *in vitro* and *in vivo* use of antisense ODNs (Stein and Kreig, 1994), due to the lack of controls to eliminate non specific effects of PS ODNs, the polyanionic nature of ODNs and the presence of G quartets. Studies suggest that PS ODN have non specific effects (Cazenave *et al.,* 1989) Three antisense ODNs against *rev*, *gag* and *pol* mRNA failed to suppress HIV-1 replication in long term culture, however they were effective in acute infection probably as a result of non antisense effects preventing HIV entry into the cells (Kinchington *et al.,* 1992). Purity of the ODNs is also significant as the presence of trityl ammonium ions (due to the removal of the trityl group during synthesis) can also cause toxicity. Another non specific effect following the rapid infusion of PS 20-33mer is transient decrease in white blood cell and neutrophil counts, haemoconcentration and a brief increase followed by a prolonged decrease in arterial blood pressure. These non specific effects were reported by Galbraith *et al.*, (1994) after GEM 91 (a 25mer PS ODN complementary o the *gag* site of HIV) infusion in monkeys, and are possibly mediated by activation of C5 complement, the adverse effects can be avoided by using a slow infusion. The time of ODN treatment is also important for analysis of these results as often the initiation of ODN treatment is prior to or coincides with the injection of tumour cells, which is not a situation likely to occur in a clinical environment ! All the above factors need to be considered when assessing the true degree of an antisense effect.

TARGET GENE	ANIMAL MODEL	ODN TYPE AND LENGTH	DELIVERY METHOD	COMMENTS
N-*myc* Whitesell *et al.*, (1991)	neuroeoithe-lioma S.C. xenografts grown in athymic mice	PO 15mer	S.C. osmotic pump	50% reduction in tumour mass after 14 days
p53 Bayever *et al.*, 1992	Rhesus monkey	PS 20mer	50-150mg/kg	remission from acute myeloid leukaemia
C-*myb* Ratajczak *et al.* (1992)	K562 human leukemia cells in mice	PS 24mer	infusion pump 100μg/day for 7 or 14 days treatment commenced 4 weeks after cell transplantation	Antisense treated animals survived 3.5 times longer than control animals
c-*myc* Wickstrom *et al.*, (1992)	Eμ c-myc transgenic mice	MP 15mer	300nmoles as single I.V. injection	significant reduction in c-myc protein levels in lymphocytes of antisense treated mice
NK-κB Higgins *et al.*, (1993)	K-BALB (fibrosarcoma) and B-16 (melanoma) murine tumour cells in nude mice	PS 24mer	2.8mg over 14 days by osmotic pump and 1.4mg S.C. twice weekly for 2 weeks	tumour regression in 1-2 weeks
neuropeptide Y Y_1 receptor Wahlestedt *et al.*, (1993)	rats	PO 18mer	50-100μg twice daily for 2-3 days	reduced receptors
c-Ha-Ras Gray *et al.*, (1993)	NIH-3T3 cells transformed by the activated c-Ha-ras oncogene from T24 human bladder cancer cells	PO 15mer	50μM added to cells for 3 days prior to injection of cells into mice	pretreatment reduced ras p21 cellular levels by 90% for up to 14 days

Table 1.1 Examples of successful *in vivo* antisense therapy.

The use of repeated administration, infusion pumps and the need for slow infusion rates further confirms the need for the development of a sustained delivery device for ODNs.

1.9 OLIGODEOXYNUCLEOTIDE CLINICAL TRIALS

Clinical trials with ODNs have already begun and are showing promising results, despite the problems of stability and cellular delivery already discussed.
ISIS-2302 an antisense ODN against mRNA of ICAM-1 is currently in Phase I trials to asses its potential for the treatment of inflammatory diseases. In phase II trials is a 20 mer PS (ISIS-2105) complementary to the mRNA sequence for the translational initiation codon of the E2 protein, which regulates viral transcription. 1mg /lesion has been administered intralesionally to genital warts caused by human papilloma virus type 2 (HPV-2). Peak plasma concentrations were achieved within 1 hour and levels remained above the minimum inhibitory concentration for 72 hours. As a result a twice weekly topical application is being developed. ISIS-2922 complementary to the immediate-early transcriptional unit of human Cytomegalo virus (CMV) mRNA, is currently in Phase III clinical trials for the treatment of cytomegalovirus retinitis. Immediate and delayed treatment are being compared, as well as combined therapy with ganciclovir (Henahan, 1995).
A PS antisense *c-myb* has been used in chronic myelogenous leukaemia with encouraging results when used as a bone marrow purging agent. A 20 mer PS complementary to p53 mRNA (a tumour supressor gene) is in Phase I trials in patients with relapsed acute mylelogenous leukaemia, 17 patients received a 10 day continuous IV infusion of ODN at 0.05-0.25 mg/kg/hour, bone marrow samples were collected one day before treatment began and after 7 days of therapy. Increasing dose lead to greater changes in cellularity, with no toxic effects observed (Short, 1995).

Gem-91 (Hybridon Inc.) a PS 25mer ODN complementary to the AUG site of the *gag* region of the HIV-1 genome is the first antisense therapeutic agent for AIDS. It is in clinical trials in both France and the United States, following successful *in vitro* inhibition of HIV-1 replication in macrophages (Agrawal, 1995 personal communication).

1.10 POLYMERS

Biodegradable polymers have been studied with great interest over the last twenty years for use as carriers in drug delivery and have been extensively reviewed by Langer and Peppas, 1981; Holland *et al.*, 1986; Duncan, 1992; Floy *et al.*, 1993; Heller, 1993; Hayashi, 1994. Polymers can be used to optimise the rate of drug release and provide constant plasma concentrations, as well as being used to target a drug to a specific site of action. Nucleic acids are biologically labile and therefore must be effectively protected at the depot site if the active drug is to be continually released. Polymers employed as controlled release delivery devices should be biodegradable, non toxic and give predictable release kinetics. Following drug release, the empty device remains at the site of administration where it is completely degraded to inert, biocompatible degradation products. The desired properties of the polymer can be obtained by altering the molecular weight, copolymer ratio, crystallinity or the addition of excipients e.g. plasticisers.

Many polymers have been evaluated for their use as biomedical devices (e.g. polylactic acid, polyhydroxybutyrate, polyanhydrides, polyorthoesters, poly alkyl cyanoacrylates). However only polylactic acid and polyhydroxybutyrate with their copolymers will be discussed in this section.

1.10.1 Poly(lactic Acid) and Poly(glycolic Acid)

The long history of use of poly lactic acid and its copolymer with glycolic acid has demonstrated their biocompatability and degradation to toxicologically acceptable products. The polymer is commercially available in a variety of copolymer ratios and molecular weight. There is great interest for their use in the development of controlled release polypeptide (Sanders *et al.*, 1984) and vaccine (O'Hagan *et al.*, 1994 a) preparations. The polymer system has FDA approval and is in commercial use in several products. The first controlled-release peptide microcapsule formulation to become available on the market was Decapeptyl (triptorelin, Ipsen-Beafour) used in the treatment of testosterone dependent prostate cancer, using a 54,000 molecular weight PLGA at a 50:50 LA:GA ratio. Other microsphere preparations using similar polymer matrices include Lupron Depot - (leuprorelin, Takeda-Abbott) PLGA ratio 75:25; Somatuline (lanreotide, Ipsen-Beafour);

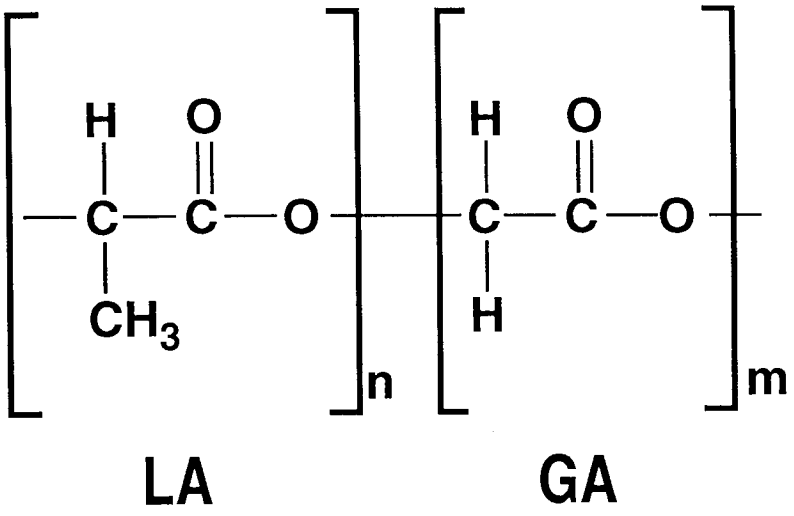


Fig.1.5 Structure of polylactide co-glycolide, PLGA.
(LA = lactic acid, GA = glycolic acid)

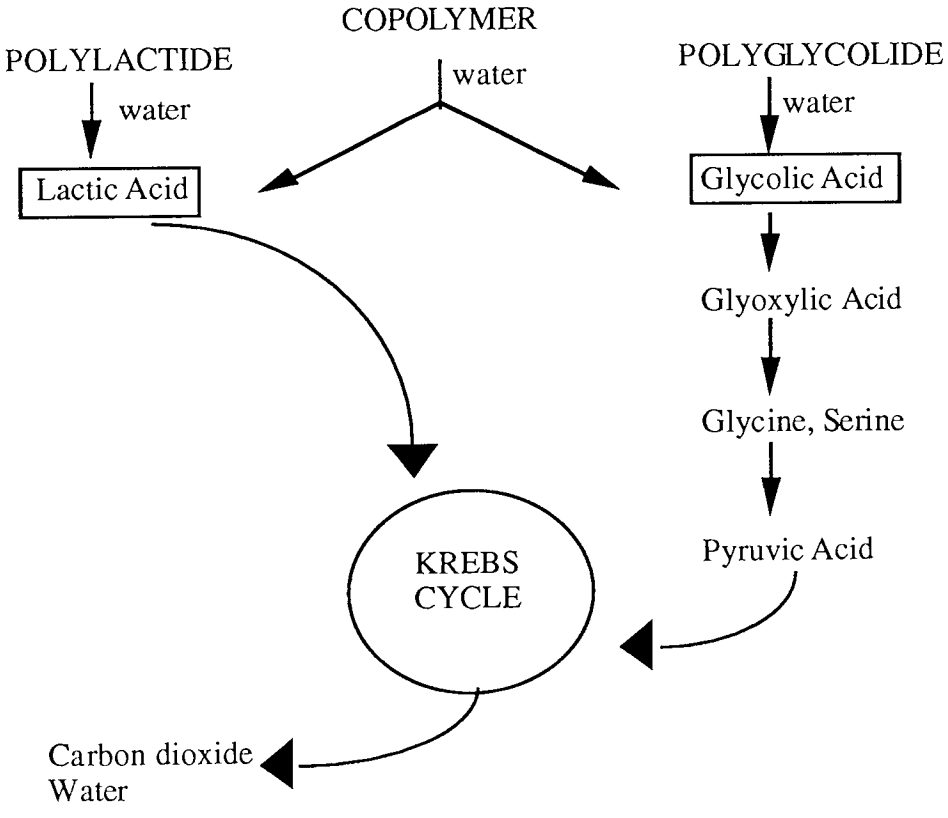


Fig.1.6 Degradation of lactic and glycolic acid in living systems. Adapted from Literature Catalogue BI 26, Alpha Chemicals.

Parlodel LA and Parlodel LAR 50:50 (bromocriptine, Sandoz). Non microsphere PLGA preparations include Zoladex (goserelin, Zeneca) a cylindrical polymer depot releasing over a 1 or 3 month period and Suprefact 75:25 (buserilin, Hoescht) which releases over a 3 month period.

Polylactic acid (PLA) is an aliphatic polyester, due to the presence of a chiral center there are three forms, those deriving from D(-), L(+) and the racemic D,L forms of the parent acid, the L(+) form is metabolised by the body and is more commonly used than the D(-) form, The chirality of the starting monomer leads to stereoregularity in the polymer, resulting in a high degree of crystallinity. PLA can form a copolymer with glycolic acid, PLGA (Fig.1.5). Polymers based on lactic and glycolic acid are thermoplastic crystalline polyesters formed by ring opening polymerisation catalysed by ZnO, of lactide, glycolide or mixtures of these compounds. Low molecular weight (< 3000) polymers are produced by direct condensation of lactic acid. Poly L-lactide is highly crystalline (80%) and poly D,L-lactide is amorphous. They are insoluble in water, ethanol and methanol and soluble in organic solvents such as methylene chloride, carbon tetrachloride, chloroform, acetone , dioxane and ethyl acetate. It is a non toxic biodegradable polymer, the degradation cycle is shown in fig.1.6.

The kinetics of hydrolysis are strongly influenced by molecular weight, presence of residual monomers, pH, temperature, particle size and distribution, surface structure, crystallinity and presence of impurities or additives. PLGA polymers degrade by random bulk hydrolysis of the ester bonds in the polymer chain in an aqueous environment. Degradation produces lactic and glycolic acid monomers and carboxylic acids which along with impurities and humidity catalyse the rate of degradation. The degradation products are finally metabolised to carbon dioxide and water and excreted via the kidneys. Degradation of the polymers is biphasic; firstly a decrease in molecular weight of the polymer occurs due to random chain scission, which is accompanied by water uptake. The enhanced water uptake increases porosity and soluble monomeric and oligomeric products can be detected, these degradation products leave the bulk polymer through channels and holes resulting in weight loss of the polymer (Hutchinson *et al.*, 1985). Degradation times are listed in Table 1.2. The monomers degrade more slowly than the copolymer, the increased rate of degradation is as a result of lower copolymer crystallinity, allowing greater absorption of water into the polymer and hence faster degradation (Gilding and Reed, 1979; Holland *et al.*, 1986). The rate of hydrolysis of the copolymer increases with increasing glycolide content and reaches a maximum

POLYMER	TIME FOR COMPLETE HYDROLYSIS *in vitro* pH 9
poly L-lactide molecular weight > 20 000	months
polyglycolide molecular weight > 20 000	months
poly DL-lactide molecular weight > 20 000	weeks
copolymers	depends monomer ratio
poly L-lactide molecular weight > 2 000	few weeks
polyglycolide molecular weight > 2 000	few days

Table 1.2 Comparison of degradation of different resorbable polyesters. Adapted from Literature catalogue No BI 26, Alpha Chemicals.

when the lactide glycolide ratio reaches 50:50 (Miller *et al.*, 1977). Glycolic acid is formed at an initially higher rate than lactic acid, the methyl group on lactic acid makes it more hydrophobic compared to glycolic acid (Fig.1.5) and causes steric hindrance to attack by water molecules, thereby explaining the increased rate of degradation of copolymers. With increasing degradation time the release of lactic acid predominates (Wang *et al.*, 1990). Increasing the molecular weight of the polymer decreases the rate of degradation (Sanders *et al.*, 1986). The PLGA 50:50 copolymer microspheres degrades more rapidly than the 75:25 copolymer (O'Hagan *et al.*, 1994). A PLGA copolymer implant varying in PLA composition from 50 to 100% would have an *in vivo* half life in the range of 1week to 6months (Miller *et al.*, 1977). Initially degradation is faster at the polymer surface (Vert *et al*, 1994), after the first day further hydration of the microsphere occurs and degradation is fastest at the centre. Intermediate degradation products have a small diffusion coefficient and can be removed from the surface, but not the central core, as a result the central region is more acidic compared to the outside, as ions in the media can decrease the relative acidity at the surface. The acidity of the matrix core catalyses the polymer degradation. Once the central degradation products are small enough, they diffuse out creating a hollow shell, as degradation at the central region occurs more quickly than the outer surface can erode. As degradation occurs both internally and externally, this is an important problem in the delivery of nucleic

acids, as interaction with the biological milieu causes degradation. Polymer degradation is accelerated in both strongly alkaline and acidic media (Makino *et al.*, 1985, Chu, 1981, 1982). Increasing the ionic strength of the media also increases the rate of degradation (Makino *et al.*, 1986 b). The presence of tissue lipids can also increase the rate of degradation of the polymer by acting as plasticisers (Menei *et al.*, 1994 a). Adsorption of plasma proteins onto the surface of PLA microspheres increases the solubility and hence the rate of degradation of the polymer (Makino *et al.*, 1987).

The biphasic degradation process typically results in a triphasic release of entrapped compounds from the matrix (Sanders *et al.*, 1984; Ogawa *et al.*, 1988). Similar degradation and release occurs *in vivo* and *in vitro*, with *in vitro* degradation being slightly faster (Pitt *et al.*, 1981; Sanders *et al.*, 1986; Fournier *et al.*, 1991), penetration of the delivery system by macrophages may alter the biodegradation. Formation of a collagenous capsule around the implant will reduce the rate of release *in vivo*. The presence of enzymes does not effect the *in vivo* degradation of the polymer device (Pitt *et al.*, 1981), but enzymes are known to have a role in the degradation of primary hydrolysis products (Salthouse *et al.*, 1976). *In vitro* experiments to investigate the role of enzymes on polymer degradation have shown that carboxylic esterases (Makino *et al.*, 1985) accelerate molecular weight loss, enzymatic degradation is predominately at the surface in amorphous domains (Reeve *et al.*, 1994).

In vivo degradation studies show that implanted injection molded rods initially transparent with a homogenous surface, become friable and broke into whitish brown fragments with a porous surface. Mechanical stability decreased by half in 3 weeks *in vivo* (Pistner *et al.*, 1994 a & b). The rate of degradation is independent of PLGA microsphere size in the size 30-130μm (Visscher *et al.*, 1988), however Mehta *et al.* (1994) report that increasing the particle size from 100-200μm decreased the onset of mass loss. PLGA 75:25 microspheres of molecular weight 14,000 had disappeared from a subcutaneous injection site 28 days post administration (Ogawa *et al.*, 1989).

1.10.2 Poly (β- Hydroxybutyrate), (PHB) and copolymer with poly(hydroxyvalertae), P(HB-HV)

The uses of PHB for biodegradable delivery devices have been reviewed by Müller and Seebach, (1993); Koosha *et al.*, (1989); Akhtar and Pouton, (1989). PHB, is a naturally occurring, optically active crystalline aliphatic polyester, produced from various strains of micro organisms e.g. *Alcaligenes eutrophus* which is a gram negative rod. It serves as a carbon and energy source and is found to be abundant in nature. Biosynthesis of PHB in batch-fed cultures of methylobacterium yield 70-80% of dry cell weight. Increasing the amount of methanol in the culture increases the amount of PHB produced, but this results in a wide range of molecular weights produced. Research is being carried out by plant scientists to engineer a variety of oil seed rape that can produce PHB and so simplify the isolation procedure. The main advantage of PHB and its copolymers is that production from a living organism results in high purity, in contrast to synthetic polymers e.g. polylactide co-glycolide polymers which contain inorganic catalyst residues even after extensive purification. High purity is essential when aiming to achieve a non toxic product.

PHB is a highly crystalline thermoplastic with a melting point range of 160-175°C. The copolymer with hydroxyvalerate (Fig.1.6) is also microcrystalline with a slightly lower melting point. Density of wide angle X-ray scattering measurements have shown that the copolymer is less crystalline than the homopolymer. The copolymer is tougher, less brittle and more pliable than the homopolymer, with a typical crystallinity of 80%, a glass transition temperature of 0°C and a low density of 1.2252g / cm^3 (due to the presence of internal voiding, Owen, 1992). It can be precipitated from solution to give thin lamellar crystals. The high degree of crystallinity of PHB homopolymer is a disadvantage which limits fabrication. Modification of the crystallinity can be achieved by incorporating up to 50 mol % β - hydroxyvalerate (fig.1.7).

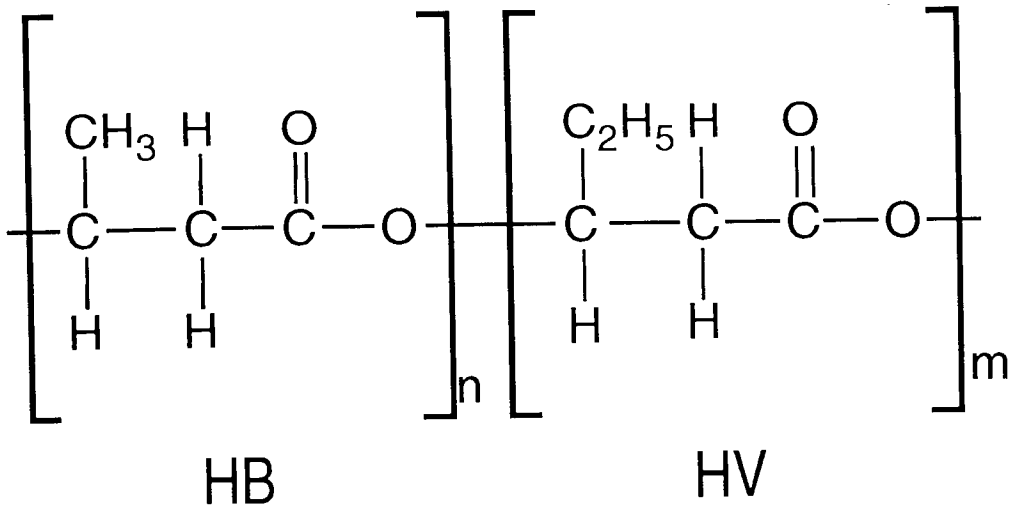


Fig.1.7 Structure of poly(hydroxybutyrate co-valerate), P(HB-HV)

In vitro degradation of P(HB-HV) polymers is by a surface erosion mechanism, producing the monomeric acid 3-hydroxybutyric acid which is a normal constituent of blood. Molecular weight analysis of the degraded polymer samples confirms the theory of surface erosion, the molecular weight of PHB remained unchanged, with no mass loss during a 6 month in vitro hydrolisis study (Gilding, 1981). PHB is sensitive to microbial degradation but biodegradation is slow under physiological conditions of 37°C and pH 7.4 and has a suggested half life of 152 weeks, the water uptake of these polymers is too low to be measured precisely. The degradation of the copolymer is slightly more rapid than the homopolymer, although this relationship is not linear. Holland *et al.* (1987) studied the rate of degradation and found it to be dependent on copolymer composition, molecular weight, pH and temperature of media and presence of plasma. Maximum degradation was found to occur by increasing pH from 2.3 to 10.6 and temperature from 37 to 70°C. Increasing the number of hydroxyvalerate units results in a faster rate of degradation although this is not linear. Lower molecular weight polymers degrade faster than higher molecular weights and amorphous regions degrade faster than crystalline regions, *in vivo* PHB exists in an amorphous state (de Koning, 1992 a). As a result PHB has along retention time in the body. *In vivo* degradation is faster than *in vitro* due to the presence of enzymes. The rate of degradation of the polymer can be increased by the addition of excipients. Incorporation of bases increases the rate of degradation, which is dependent on the basicity and degree of loading. The rate of hydrolysis depends on the water solubility and the polymer:water partition of the incorporated base (Yoshioka *et al.*, 1991).

Polysaccharides e.g. amylose, dextran and sodium alginate increase the rate of conventional degradation by increasing the internal porosity due to polysaccharide erosion from the matrix, resulting in the eventual collapse of the matrix (Yasin *et al.*, 1989,1990). Blending with a plasticiser e.g. ethylene vinyl acetate (EVA) decreases the crystallinity of P(HB-HV) (Gassner and Owen, 1992), however EVA is nonerodible matrix. Blending with polycaprolactone (PCL) has the advantage that it is biodegradable (Yasin and Tighe, 1992).

Degradation by surface erosion enables predictable drug release kinetics, as there is little change in molecular weight with mass loss. The release rate of an incorporated drug in a polymer which undergoes surface erosion is proportional to the surface area of the delivery device. Surface eroding polymers protect the remaining incorporated drug from degradation prior to release as the polymer does not take up water.

1.10.3 Tissue Biocompatability of Implanted Polymers

Tissue biocompatability is an essential prerequisite for polymers for sustained release implants. Poly α- hydroxy acids generally have good biocompatability, even when trace amounts of ethylene oxide or solvents are present. No significant adverse reaction has been detected after 12 years of experimentation with resorbable polymers in animals and humans.

Polylactic acid 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, any inflammatory response subsides 70 days post injection (Visscher *et al.*, 1988; Yamaguguchi and Anderson, 1993). Menei *et al.*, (1993) reported the absence of toxic effects when PLGA microspheres were injected directly in to the brain. Subcutaneously implanted discs and cylinders of PLA, PHB and P(HB-HV) are well tolerated, with no acute inflammation, abscess formation or tissue necrosis observed in adjacent tissue. Mononuclear macrophages, proliferating fibroblasts and mature vascularised fibrous capsules were a typical response (Gogolewski *et al.*, 1993 a). After a 4 week period the D-unit polymer chain of PLA caused less inflammation compared to the L-unit polymer chain. Increasing the content of HV in

PHB/HV increased the inflammatory response (Gogolewski *et al.*, 1993 a). Over a period of 1-3 months slightly more tissue reaction was seen with PHB and PHB/HV implants compared to PLA, although the tissue response 6 months post implantation was similar. Implantation of PHB into bone tissue for up to 12 months showed no evidence of a chronic inflammatory response.

After intraperitoneal injection of poly L-lactide particulates there is an increase in numbers of neutrophilic granulocytes present up to 48 hours post injection, which decrease with time. The particles aggregate and intermingle with the inflammatory cells, the degree of the inflammatory response is proportional to the size and form of the particles but not the molecular weight (Visscher *et al.*, 1988; van Sliedregt *et al.*, 1992). The shape of the implanted polymer is also important, with triangular implants giving the highest enzyme activity, followed by pentagonal and circular smooth implants gave the least tissue response (Matlaga *et al.*, 1976). Epithelial cells grown on PLA films showed normal morphology, although confluency and differentiation over a 14 day period showed differences compared to control cultures (van Sliedregt *et al.*, 1993). The presence of lactic acid monomers (0.5%) reduced the metabolic activity of human mammary fibroblasts (reduced cell proliferation and less densely packed) (van Sliedregt *et al.*, 1994). The *in vivo* effects of monomer release from the implanted device is influenced by the draining capacity of the surrounding tissues.

Long term implantation into rodents highlights the problem of foreign body tumorigenesis (Oppenheimer effect) which is independent of the chemical nature of the implant (Pistner *et al.*, 1994). Therefore it is important to employ polymers with relatively short *in vivo* half-lives for drug delivery applications.

1.10.4 Sterilisation of Polymers for Biomedical Use

PLA can be easily sterilised for parenteral use, however the use β and γ rays results in some polymer degradation. The use of γ rays can result in simultaneous chain scission and cross linking of the polymer chains (Horácek *et al.*, 1993). Scission of the polymer chains are more pronounced in the amorphous region compared to the crystalline regions, aromatic regions have a greater ability to absorb irradiation energy compared to aliphatic regions (Chu *et al.*, 1992). A normal dose of γ rays (25KGY) can cause a total loss of strength during a 10 day period after implantation (Gilding and Reed, 1979). The degradative effect of the γ rays shortens the lag

phase in the release profiles (Sanders *et al.*, 1984), the degradative effect continues on storage of the sterilised polymer (Spenlehauer *et al.*, 1989).Tsai *et al.* (1986) report that sterilisation of PLA with ^{60}Co γ-rays (100 GY) did not affect microcapsule structure, release rate or drug stability.

Ethylene oxide sterilisation causes no adverse effects to the polymer used at 12% ethylene oxide at 54°C and 18lbs in^2 for 105 minutes. Evacuation at 60°C and 0.2bar for 5 hours reduces the ethylene oxide content of the polymer.

Wu *et al.* (1994) report the use of UV sterilisation for polyanhydride films (one hour exposuire on each side), however UV light could possibly cause cross linking of the entrapped ODNs strands (Takasugi *et al.*, 1991), making sterilisation by this method a non viable option.

1.11 THE MACROPHAGE

The macrophage is a complex cell with varied roles (extensively reviewed by Tabata and Ikada, 1990 a ; Auger and Ross, 1992). It is the major differentiated cell in the mononuclear phagocyte system, they are widely distributed in the body (lymphoid organs, liver spleen, bone marrow, lungs, gastrointestinal tract, CNS and skin) and have a wide range of physiological and pathological functions. Macrophages originate in the bone marrow as monoblasts, develop into monocytes and differentiate into macrophages in tissues. They are unable to renter the circulation and remain in the tissue for several months, before being destroyed in the lymph nodes. The presence of phagocytosable particles stimulates the production of macrophages .

Macrophages are generally large 25-50μm in diameter and irregularly shaped with an eccentric nucleus of variable shape. Actin microfilaments immediately beneath the cell membrane are responsible for the ruffled cell surface, locomotion, as well as influencing endocytotic events. Macrophages engulf foreign material by moving to surround the particulate, this process may take only a few minutes. Lysosomal enzymes involved in the degradation of phagocytosed material are synthesised in the endoplasmic reticulum and are packaged by the golgi apparatus into primary lysosomes. Membrane bound lysosmes fuse with phagosomes to form secondary lysosmes which contain the ingested material. If the particle is too large for one cell to ingest, several cells surround the particle to form a capsule.

Activated macrophages have enhanced cellular metabolism, mobility, lysosomal enzyme activity, phagocytic activity, cytocidal capacity , and increased size. Interferon gamma promotes responsive macrophages to primed macrophages, addition of lipopolysaccharide enables primed macrophages to reach an activated state.

1.12 AIMS AND OBJECTIVES

A delivery device for ODNs and RBZs is essential for the successful therapeutic use of these nucleic acids, due to their short biological half-life requiring repeated administration and poor stability of unmodified sequences. A polymeric device which is non toxic, protects the entrapped nucleic acids from degradation and releases the entrapped drug at a predetermined rate over an extended time period would be ideal.

Therefore the aim of the project was firstly, to design and characterise biodegradable polymeric carriers which are suitable as sustained release dosage forms for ODNs and RBZs. Secondly, to ensure that the dosage form is capable of protecting the nucleic acid from degradation in the biological milieu prior to release, whilst the fabrication procedure must not adversely effect the nucleic acids ability to hybridise to its target sequence. The final aim was to assess the potential for enhanced cellular delivery of ODNs by use of microspheres.

CHAPTER TWO

MATERIALS AND METHODS

The general materials and methods are detailed in this chapter, any variations to the standard procedures outlined here are detailed in the relevant chapters.

2.1 MATERIALS

Double distilled de-ionised water was used throughout and sterilised by autoclaving at 15 pounds per square inch above atmospheric pressure at 120°C for 20 minutes. All glass and plasticware not purchased sterile was autoclaved prior to the handling of DNA and RNA. For the handling of RNA all glassware was soaked in 3% hydrogen peroxide for 2 hours before rinsing in RNase free water (0.1% diethyl pyrocarbon in distilled water and autoclaved).The plasticware was also rinsed with RNase free water prior to autoclaving.

All reagents were cell culture grade or the highest purity available from Sigma Chemical Company (Poole, UK) unless otherwise specified. Cell culture media and reagents were purchased from Gibco (Paisley, UK), tissue culture flasks, multiwell tissue culture plates, 15ml and 50ml polypropylene Falcon tubes were purchased from Falcon Labware (Beckton Dickinson and Co., Plymouth, UK). Disposable pipettes, microcentrifuge tubes, finpipette tips and 2ml Biofreeze vials were purchased from Starstedt (Leicester, UK).

2.2 GENERAL METHODS

2.2.1 Polyacrylamide Gel Electrophoresis

The polyacrylamide gels were set up and run as described in Sambrook *et al.* (1989), using a Biorad gel electrophoresis system (Biorad, Hemel Hempstead, UK).

A 1000ml stock solution of native 20% polyacrylamide gel mixture was prepared (200g acrylamide, 6.6g bis-acrylamide and 200ml TBE x5). The gel mixture was filtered and degassed and stored at 4°C, protected from the light. To make a denaturing gel 7M urea (420g) was added to the above gel mixture, RBZs were run on a denaturing polyacrylamide gel containing 8M urea. A 50ml aliquot of the stock gel mixture was polymerised with 0.6ml 10% ammonium persulphate (freshly prepared) and 40µm TEMED (N,N, N',N'-tetramethylethlenediamine) for each gel. The polymerising gel mixture was poured between two glass plates (20 x 20cm and 22 x 20cm) with 1mm spacers (Biorad). A 15 well comb was inserted at the top of the plates to form the sample wells, the gel was allowed to set for one hour at room temperature. The samples to be run on the gel were added in a loading buffer (50mg xylene cyanole, 50mg bromophenol blue to 10ml with TBE in glycerol). To denature the samples for a denaturing gel, 4.2g of urea was added to the loading buffer, omitting the glycerol. Tris borate buffer was used as a running buffer and was diluted from a 5x stock solution (54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA (pH8) /1000ml). The gel was run for approximately 2 hours at 300 volts using a Biorad power pack, and cooled with tap water.

2.2.2 Autoradiography

After running a polyacrylamide gel with radioactive samples, the small plate was removed and the gel covered with a single layer of cling film. The gel was exposed to Kodak film in order to visualise the radioactive samples in a Hypercassette (Amersham) fitted with an intensifying screen. The time of exposure varied from 5 minutes for fresh ^{32}P to 48 hours depending on the activity of the samples,

exposure for longer than 5 minutes was at -70°C to prevent migration of the samples. The film was developed and fixed using Kodak reagents (Sigma).

2.2.3 Scintillation Counting

The specific activity of the radiolabelled ODNs was determined by scintillation counting. A known volume (5µl) of sample was added to 10 ml Optiphase Hi-Safe 3 (Pharmacia-Wallac) and counted in a Packard 1900TR scintillation counter using appropriate [^{35}S] and [^{32}P] activity programmes for 5 minutes, against a background. The half life and reference date of the radionucleotides were used to account for decay during the experimental period. Both [^{35}S] and [^{32}P] are β emitters having half lives of 89 and 14.2 days respectively. Specific activities of ODN solutions were calculated as cpm/µl, specific activities were in the region of 200,000 cpm/µl. A volume of 50µl was used in all polymer loading, with unlabelled ODN added to adjust the total concentration.

2.3 OLIGODEOXYNUCLEOTIDE SYNTHESIS AND PURIFICATION

2.3.1 Oligodeoxynucleotide Synthesis

The various methods of ODN synthesis have been reviewed by Caruthers, (1989). The ODNs for use in this thesis were synthesised on an automated DNA/RNA synthesiser (Model 392, Applied Biosystems (ABI), Warrington, UK) using standard cyanoethyl chemistry (reagents from Cruachem, Glasgow, UK). Only PO and PS ODNs were used for experimentation in this thesis, due to their simplified synthesis compared to other modifications discussed in section 1.3.

The ODNs were synthesised with the 3' end attached to a solid silica CPG bead support via a succinamide linker. The synthesis cycle in schematic form is shown in Fig. 2.1. The first step was detritylation, the dimethoxyltrityl (DMT) group on the 5' hydroxyl group of the support bound nucleotide was removed with trichloroacetic acid (TCA), leaving a reactive 5' hydroxyl, which was coupled to the next phosphoramidite. Quantification of the released trityl cation gave an indication of the step-wise coupling efficiency and overall yield (see 2.3.2). The second step, activation, was achieved by the simultaneous addition of the phosphoramidite nucleoside monomer and tetrazole (a weak acid) to the column. The tetrazole protonated the nitrogen of the phosphoramidite, making it susceptible to nucleophilic attack by the 5' hydroxyl of the next nucleoside. The addition of the phosphoramidite was completed in 30 seconds. Further additions in each cycle were prevented by the DMT group at the 5' end of the last nucleoside. In the fourth step, capping, addition of acetic anhydride and 1-methylimididazole terminates any chains that have not undergone addition, thereby minimising the length of impurities (i.e. failed sequences) present, as they were unable to undergo further addition steps. Step five, oxidation, where the unstable trivalent phosphorous was stabilized by oxidation with iodine-water-pyridine in basic tetrahydrofuran mixture to form the stable pentavalent phosphate triester. Alternatively the triester was sulphurised to form a phosphorothioate with tetraethylthiuram sulphide. The complete structure is shown in Fig.1.1.

The synthesis cycle was repeated until the ODN reached the required length. The ODN was cleaved from the column by treatment with concentrated ammonium hydroxide (d=0.88) and transferred to a collecting vial. The cyanoethyl phosphorous protecting groups were removed in the ammonium hydroxide when heated to 55°C for 8-15 hours.

All reagents were kept under argon on the synthesiser and once diluted had a shelf life of 14-28 days.

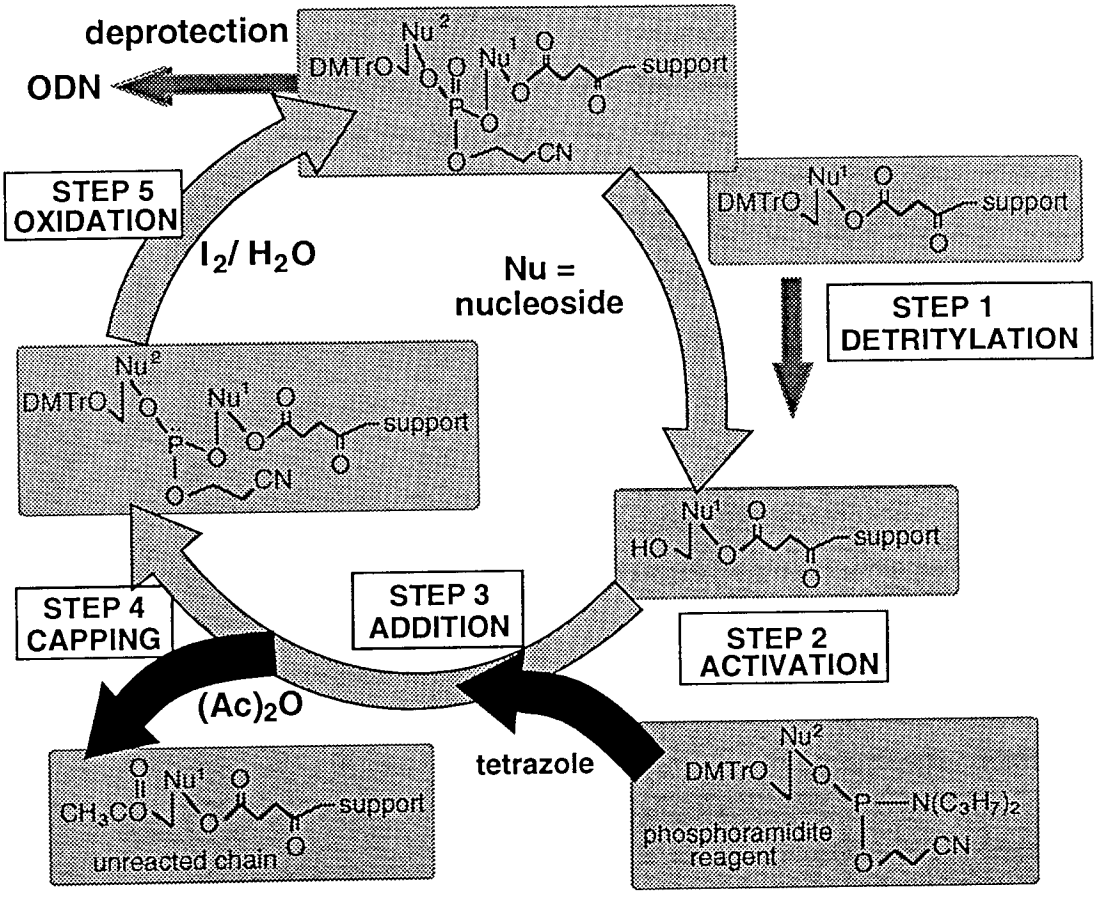


Fig. 2.1 General scheme of oligodeoxynucleotide synthesis (reproduced from ABI user manual)

2.3.2 Coupling Efficiency

Quantification of the trityl cation released after detritylation was used to determine the coupling efficiency (ABI user manual). The trityl fraction was diluted to 25ml

with 0.1M p-toluenesulfonic acid in acetonitrile and the absorbance measured at 498nm (Phillips UV/Vis spectrophotometer PU 8730).

overall yield = OD_{498} last or lowest trityl / OD_{498} second or highest trityl

stepwise yield = overall yield $^{1/couplings}$

NB. The number of couplings is 1- the number of bases in the sequence.

The absorbance of the first trityl was ignored as this represents the first base, which was already attached to the column. The stepwise yield was typically 98% and the overall yield for a PO 20mer ODN was typically 70%.

2.3.3 Oligodeoxynucleotide Purification

After deprotection, the resulting ODN, in concentrated ammonium hydroxide, was purified using a NAP 10 column (Pharmacia Biotech) containing sephadex G25. The column was washed with 15ml sterile distilled water, before the addition of the ODN in a 1ml volume. A further 1.5ml of sterile water was added following the addition of the ODN solution to the column and this fraction (containing the ODN) was collected in a microcentrifuge tube. The eluted ODN in sterile water was dried under vacuum (DNA Speed Vac, Savant, UK) and stored at -20°C. The purification procedure removes any salts and short failed sequences (less than 10mer) present. The purity of the ODNs was not quantified.

2.3.4 Quantification of the Oligodeoxynucleotide

One synthesis of a 20mer yields approximately 1mg of ODN before purification. The amount of ODN was determined by UV spectroscopy at 260nm. One optical density unit of single stranded ODN is equivalent to approximately 33µg (Sambrook *et al.*, 1989). A more accurate method (Brown and Brown, 1991) converts OD units into µg based on the molecular weight of the sequence, this method does not allow for the differences in molecular weight between PO and PS ODNs.

A. Molecular Weight

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

where:-

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

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

B. Calculation of Micromolar Extinction Coefficient, ε at 260nm

$\varepsilon = \{(8.8 \times nT) + (7.3 \times nC) + (11.7 \times nG) + (15.4 \times nA)\} \times 0.9\ \mu mol^{-1}$

It is necessary to multiply by 0.9 to account for the suppression of absorbance of the DNA due to base stacking interactions in the single strand.

C. To Convert OD_{260} into milligrammes

$1mg\ \varepsilon / (\text{mol wt} / 1000) = x\ OD_{260}$

$\therefore\ 1\ OD_{260} = 1 / x = y\ \mu g$

Appendix 3 lists all the sequences and OD conversions used throughout this thesis.

2.4 OLIGODEOXYNUCLEOTIDE LABELLING

2.4.1 5' End [^{32}P] Labelling

The ODNs were labelled at the 5' end with [^{32}P]-γ-ATP (Amersham, UK) with a specific activity > 185 TBq/mmol at the reference date, using bacteriophage T4 polynucleotide kinase (Sambrook *et al.*, 1989) 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 30 minutes. Approximately 1nmole of ODN was labelled in a 20µl reaction mixture of 20 units T4 Kinase (Gibco), 4µl reaction buffer and 5µl [^{32}P]-γ-ATP.

2.4.2 3' End [^{35}S] Labelling

ODNs were also labelled at the 3' end with [^{35}S] dATPαS (Amersham, UK) with a specific activity > 37 TBq/mmol at the reference date, using a terminal transferase kit (Boehringer Inglheim, Germany) at 37°C for 60 minutes (Sambrook *et al.*, 1989). The nature of labelling results in the addition of an extra base at the 3' end of the sequence. Approximately 1nmole of ODN was labelled in a 50µl reaction mixture of 1µl terminal transferase, 5µl Cobalt chloride, 10µl reaction buffer and 3µl [^{35}S] dATPαS.

2.4.3 5' End Fluroscein Labelling

A fluorescent label was added to the 5' end of the ODN during the automated synthesis cycle. Fluoroscein phosphoramidite (Applied Biosystems) was diluted in acetonitrile (according to manufacturers instructions) and added to a spare port on the synthesiser. Coupling times of the standard cycle were doubled to ensure complete addition. The labelled ODN was deprotected at 55°C for 4 hours, dried and stored in a microcentrifuge tube at -20°C protected from the light, to prevent degradation of the label.

2.5 PURIFICATION OF LABELLED OLIGODEOXYNUCLEOTIDES

2.5.1 Polyacrylamide Gel Electrophoresis

The radiolabelled ODNs were purified by polyacrylamide gel electrophoresis, an equal volume of 10% glycerol solution was added and the samples run on a 20% native polyacrylamide gel. A denaturing gel was not used due to contamination of the labelled ODN with urea which was difficult to separate. Loading markers; bromophenol blue and xylene cyanole were used in the glycerol solution as marker dyes in one lane only, as the dye runs at the same level as a 20mer ODN and so would contaminate the labelled ODN with dye. The position on the gel of the labelled full length ODN was visualised by autoradiography as in section 2.2.2. The appropriate bands were cut from the gel and the radiolabelled ODN was eluted in water and concentrated by drying under vacuum. The concentration of the radioactive ODN solution was determined as described in section 2.3.4.

2.5.2 Column Purification

As an alternative approach, the radiolabelled ODN was separated from the unreacted label and short failed sequences by purification through NENSORB-20 columns (NEN-DUPONT). The column was rinsed with 100% methanol, followed by 2ml of Reagent A (10ml of 0.1M Tris HCl and 1mM EDTA pH7.7 with 14µl of triethylamine). The solutions were pushed through the column using a 10ml syringe. Reagent A (300µl) was added to the labelling mixture before addition to the column with a pipette. The column was then washed with 3ml of reagent A followed by 3ml sterile water. The ODN was eluted from the column in 300µl of 20% ethanol. Care was taken to prevent the column from drying out, the maximum load for the column was 20µg. The purity of the ODN was then confirmed by gel electrophoresis and autoradiography.

2.6 RIBOZYME SYNTHESIS AND LABELING

The hammerhead ribozyme (RBZ) sequences used in this thesis were a gift from Cruachem (Glasgow, UK).

2.6.1 Choice Of Target mRNA Site

This site for the 32 *myc* RBZ designed against the human *c-myc* oncogene, exon 2 (Mw 10400), was chosen due to its proximity to the AUG codon and the 5'-end of the gene, the presence of a GUC base trio and the absence of four adjacent guanosine residues within either the RBZ or corresponding substrate sequences. The presence of 'G'-quartets have the potential to cause non-specific effects, highlighted by studies using antisense ODNs (Yaswen *et al.*, 1992).

2.6.2 Ribozyme Synthesis

A 32mer unmodified RNA hammerhead RBZ 5' GGU GUC UGA UGA GGC CGU UAG GCC GAA ACC GC (Fig.2.2) and a 27mer region of the target mRNA substrate molecule 5' UCC UAC GUU GCG GUC ACA CCC UUC CUC were synthesised in order to assess the *in vitro* stability and catalytic activity of the polymer released RBZ. The sequences were synthesised using 1.0μM scale Fpmp chemistry on an automated DNA/RNA synthesiser (Cruachem, Model PS250) using standard RNA phosphoramidite reagents (Cruachem Ltd). The RNA was removed from the CPG support manually by treating the contents of the column with 1ml ammonium hydroxide (d=0.88) at 55 °C for 12 hours. The synthesised RNA bearing the 2'-O-Fpmp and 5'-O-DMTr protecting groups, was purified by reverse phase HPLC. RNA was deprotected with 0.01M HCl (pH2) at 30°C for 38 hours and neutralised with dilute ammonium hydroxide. The deprotected RNA sequences were then desalted by precipitation from n-butanol at -70°C and evaporated to dryness in ethanol under vacuum (Cruachem Technical Bulletins Nos. 37 & 42).

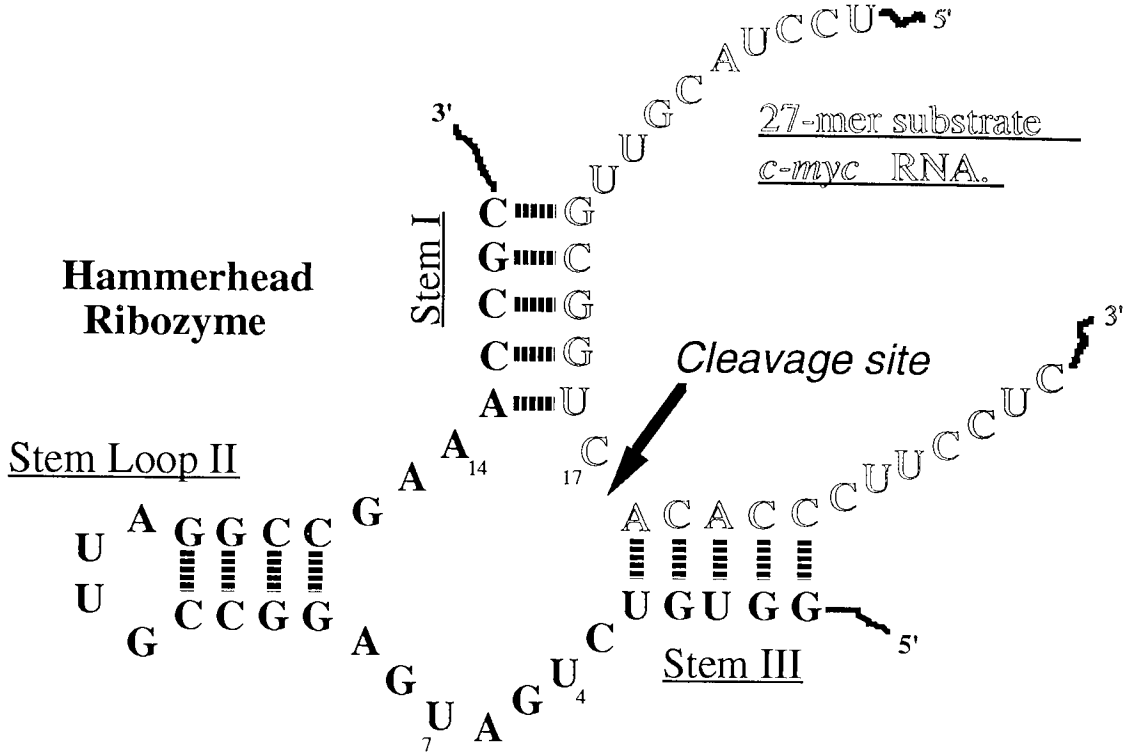


Fig. 2.2 Structure of hammerhead ribozyme and substrate

2.6.3 Ribozyme Radiolabelling

The deprotected RBZ and substrate were labelled at the 5'-end with [^{32}P]-γ-ATP (Amersham, UK), as for DNA ODNs (section 2.4.1). The radiolabelled RNA was purified using 20% native polyacrylamide gels (section 2.2.1) and eluted from the gel as in section 2.5.1, using RNase free water. The RNA was concentrated under vacuum and the concentration of the RBZ and substrate solutions were determined as in section 2.3.4 by UV absorption at 260nm.

2.7 POLYMERIC DEVICES

2.7.1 Polymers

Poly L-lactic acid and copolymers (Boehringer Inglheim, Germany) were distributed by Alpha Chemicals (Bracknell, UK). The polymers were stored in a dessicator at 4°C.

Poly L-lactic acid (PLA) Mw 690,OOO (ref L 214) was received as almost white granules, almost odourless, Poly D,L-lactide co-glycolide 50:50 P(LA:GA) Mw. 30,000 (ref RG 503) a white lumpy powder, almost odourless and Mw 3,000 (ref RG 502) a white/brownish powder almost odourless. The certificate of analysis states:-monomers < 0.5%, acetone < 0.1%, heavy metals < 10ppm, tin < 37 ppm and water < 0.4%.

Polyhydroxybutyrate (PHB, BIOPOL, ref S100P) Mw 540,000 and polyhydroxybutyrate co-valerate (PHB/12-HV) Mw 680,000 (ref D600P) were purchased as almost white powders from Zeneca (UK). The polymers were stored in a cool dry place.

2.7.2 Preparation of Solvent Cast Films

Solvent cast films were made from PHB, PHB/HV and PLA. High molecular weight crystalline polymers were needed to form solvent cast films. The polymers were dissolved in an excess of chloroform, refluxing for several hours was required to dissolve the PHB and PHB/HV, any of the PHB which had not dissolved was removed by filtering. The polymer solvent solutions were concentrated using a rotary evaporator to produce solutions of 10% w/w for PHB and PHB/HV and 2% w/w for PLA. Approximately 50µl of labelled ODN or RBZ with a specific activity in the region of 1 million cpm was mixed with unlabelled ODN or RBZ to give the required concentration. The ODN or RBZ to be incorporated in the film was added to the polymer (approx 250mg) in 60µl of aqueous solution and mixed well. The polymer/ODN mixtures were cast on degreased plates, the chloroform was allowed

to evaporate slowly overnight in a covered chamber. The films were dried at 40°C for 48 hours and stored in a dessicator before use.

2.7.2 Preparation of Microspheres

The methods used to produce ODN loaded microspheres are detailed in section 4.2.

2.8 CHARACTERISATION OF POLYMER DEVICES

2.8.1 Microsphere Batch Yield Determination

The freeze dried microsphere batches were weighed in order to asses the yield of each sphere preparation.

2.8.2 Extraction of Oligodeoxynucleotide from the Polymer and Determination of Loading

The ODN was extracted from the polymer by dissolving the delivery device (film or microsphere) in 2ml chloroform, vortexing with 2ml sterile water and centrifuging. The aqueous layer containing the ODN was removed and concentrated under vacuum. The extraction procedure was repeated three times. The ODN concentration was assessed by scintillation counting. The RBZ was extracted from the polymer by the same method but using sterile RNase free water.

2.8.3 Release Experiments from Solvent Cast Films

ODN and RBZ loaded polymer films, approximately 1cm^2 and weighing 10mg were placed in 1.5ml of media (in a 2ml capped vial). The release media used were either serum, phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl and 10mM phosphate buffer pH 7.4) or citrate phosphate buffer (CPB) (21mM citric acid and 58mM sodium phosphate pH 5.5). The vials were shaken on a simple to and fro shaker at 400 rpm at 37°C. Release of radiolabelled ODN and ribozyme from the films was monitored at intervals over a period of 28 days, daily over the first 7 days then every 2nd or 3rd day for the following 21 days. At each time interval 1.5ml of the release media was removed for scintillation counting as described in section 2.2.3. An equivalent volume of fresh release media was replaced at each sampling time. A ^{35}S label was used for the long term release studies due to the short half life of ^{32}P. The scintillation counter allowed for the decay of the radioisotope from the reference date, to enable comparison of the released labeled ODN over the release period studied.

2.8.4 Release Experiments from Microspheres

Release of ODNs from microspheres was determined in a similar way to the films. Approximately 25 mg of micropsheres were suspended in 1.5ml of release media. The release media used were either serum, DMEM, DMEM 10 % serum, phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl and 10mM phosphate buffer pH 7.4), citrate phosphate buffer (CPB, 21mM citric acid and 58mM sodium phosphate pH 5.5) or pH 10 buffer (27.5mM sodium carbonate and 22.5mM sodium bicarbonate). The vials were shaken on a simple to and fro shaker at 400 rpm at 37°C. Release of radiolabelled ODN was monitored at intervals over a period of 28 days, daily over the first 7 days then every 2nd or 3rd day for the following 21 days. At each time interval the release media was removed and centrifuged for 5 minutes at 13,000 rpm to remove any suspended microspheres. The supernatant was removed for scintillation counting as in section 2.2.3, and the sphere pellet was resuspended in 1.5ml of fresh release media and replaced into the release vial.

2.8.5 Scanning Electron Microscopy

The surface morphology of polymer films and microspheres were examined using a Cambridge Instruments Stereoscan 90 Scanning Electron Microscope connected to a 35mm camera. The samples were thoroughly dried and embedded in carbon adhesive on aluminium stubs and gold coated in an Emscope SC 500 sputter coater, in order to obtain a conducting specimen surface.

2.8.6 Differential Scanning Calorimetry

Differential scanning calorimetry was undertaken with a Perkin-Elmer DSC-4 instrument using the Thermal Analysis Data Station (TADS) for data collection, handling and presentation. Samples for thermal analysis were accurately weighed (1-4 mg) into an aluminium pan and covered with an aluminium lid which was then crimped into position. The pan was placed in the DSC oven together with a blank, prepared in exactly the same way but without the sample. The sample and blank were continuously purged with nitrogen gas at a flow-rate of 25 cm^3 min^{-1} (1.4 kg cm^{-2}) and the thermograms were recorded over a temperature range of 40-290°C with a programmed heating rate of 10°C min^{-1}. Temperature calibration was made with an indium standard (onset temperature 156.6°C) and temperatures are quoted as those of transition onset (T_{onset}) or of peak maximum (T_{max}).

2.8.7 Microsphere Particle Size Determination

Approximately 10mg of spheres were added to 10ml of 0.2µm filtered water and injected into a Malvern Mastersizer E particle sizer (Malvern Instruments, Malvern, UK). The average size of the spheres and sphere range were plotted using a software package (Malvern).

2.8.8 Microsphere Surface Charge Determination

The zeta potential of the microspheres was determined using a Zetamaster (Malvern) and PCS windows data handling software package. Approximately 10mg of spheres were added to 2 ml 10mM KCl, and injected into the instrument, three values were calculated for each run.

2.8.9 Solvent Retention in Polymeric Devices

Freshly prepared polymer films and microspheres dried in the usual way, were analysed for solvent content by gas chromatography (GC). Analysis was done using a PYE Unicam series 304 Chromatograph and a 0.8% THEED on CARBOPACK C 80/100 column 1m in length, under the following experimental conditions:- injection temperature = 70°C, column temperature = 60°C, detector temperature = 120°C, detector sensitivity = $8x10^2$, hydrogen flow = 1.0 kg/cm^2, air flow = 0.5kg/cm^2 and nitrogen flow = 0.5kg/cm^2

A 20mg piece of polymer film was dissolved in 2ml of dichloromethane and the chloroform content was determined by a chloroform in dichloromethane calibration graph. A sample volume of 1µl was injected onto the column in each case, producing smooth symmetrical curves. The method was repeated for the microspheres, dissolving 20 mg in 2 ml of chloroform and assaying for dichloromethane content.

Boiling point of dichloromethane = 41°C
Boiling point of chloroform = 61°C

2.8.10 Polyvinyl Alcohol (PVA) Assay

The amount of PVA remaining in the microspheres was quantified using a modified method described by Alléman *et al.*, (1993), based on the complex formed between iodine and PVA in the presence of boric acid (Finley, 1961).

Approximately 40mg of spheres were accurately weighed and dissolved in 5ml of chloroform. The sphere solution was sonicated in a water bath for 10 minutes, filtered on a cellulose filter (1.2μm pore size, Millipore) and washed with a further 10ml of chloroform before drying. The filter was boiled in 20ml distilled water to dissolve the PVA. A 1ml aliquot of the PVA solution was added to 3ml boric acid (4%) and 0.6ml iodine solution (1.27% iodine and 2.5% potassium iodide). The volume was adjusted to 10ml with distilled water.

The absorbance of the solutions was measured at 690nm (Phillips UV/Vis spectrophotometer PU 8730). The percentage of associated PVA remaining with the microspheres was calculated from a calibration curve.

2.9 ACTIVITY OF NUCLEIC ACIDS RELEASED FROM POLYMER DEVICES

2.9.1 Stability of Polymer Entrapped Oligodeoxynucleotide and Ribozyme

A 5' end [^{32}P] labelled PO 20 mer ODN or a 5' end [^{32}P] labelled 32 mer RBZ were loaded into polymer films or microspheres and incubated in different release media as detailed in section 2.8.3 and 2.8.4. The entrapped nucleic acid was extracted from the polymer film after various incubation times over a 28 day period, as described in section 2.8.2. The stability of the polymer extracted ODN and RBZ sample was assessed by running the samples on a 20% denaturing polyacrylamide gel as described in section 2.2.1 and visualised by autoradiography (section 2.2.2). The relative intensity of the 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.

2.9.2 Oligodeoxynucleotide Gel Mobility Shift Assay

In order to determine if the ODN released from the polymer film was still capable of hybridising to its complementary "sense" sequence, a gel shift assay was performed (Sambrook *et al.*, 1989). The 5' end [^{32}P]-γ-ATP labelled PO 20 *tat* was extracted from the PLA film as in section 2.8.2 and added to an excess (x3) of the complementary sense ODN in 100mM NaCl, 10mM sodium dihydrogen phosphate and 0.1mM EDTA at pH7.0. The solution was heated to 90°C to destroy any secondary structure and then slowly cooled to room temperature overnight to allow the duplex formation. An equal volume of 5% glycerol (in 1x TBE) was added to the sample and run on a 20% native polyacrylamide gel at 4°C (2.2.1) and visualised by autoradiography (2.2.2).

2.9.3 Duplex Melting Point (T_m) Determinations

In order to confirm that the polymer entrapped ODN is still capable of hybridising to its target sequence, T_m determinations were performed on a duplex obtained with 20 *tat* PO ODN extracted from polymer films and microspheres and a complementary 20mer sense strand. Antisense and sense ODNs were mixed in a 1:1 ratio in a buffer containing 1 M NaCl and 0.01M Tris HCl pH 7.0 to give an approximate concentration of 1 OD_{260} (Sambrook *et al.*, 1989; Hoke *et al.*, 1991) The samples were pre-melted at 90°C to destroy any secondary structure, and allowed to cool to room temperature overnight. The resulting duplex was heated from 0 to 90°C at a rate of 0.5°C/min and cooled 6 times (with a total of 180 data points per run). The absorbance at 260nm was recorded, using a Cary IE UV-Visible Spectrophotometer connected to a Star LC 24-300 colour printer. The data was processed using a Cary Thermal Easy software package. The midpoint on a sigmoidal plot of absorbance against temperature corresponds to the T_m of the ODN (i.e. the temperature at which 50% of the oligonucleotide is still in a duplex) The T_m was calculated using three different methods derivative, average and hyperchromic.

2.9.4 Biological Activity of Released Ribozyme

The RBZ was extracted from the polymer film as described in section 2.8.2. A 50μM stock solution of released RBZ was prepared in 50mM Tris (pH7.5), 10mM $MgCl_2$ which had previously been heated to 90°C. A 2μM stock solution of the all RNA substrate was prepared in 50mM Tris (pH7.5), 10mM $MgCl_2$ which had previously been heated to 90°C. This yielded a 25:1 ratio of ribozyme to substrate in the final reaction mixture. The cleavage reaction was initiated by the addition of an equal volume of RBZ stock solution to the substrate stock solution, both previously incubated at 37°C for 15 minutes. The reaction was stopped after the required time interval by the addition of an equal volume of 8M urea and storage at -20°C. The RBZ, substrate and any cleavage product(s) were separated by 20% polyacrylamide (8M urea) gel electrophoresis as in section 2.2.1 and visulised by autoradiography (section 2.2.2). The relative intensity of bands on the autoradiographs was 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 CULTURE TECHNIQUES

2.10.1 Cell Lines

Murine macrophages, Raw 264.7 were purchased from the European collection of animal cell culture catalogue (ECACC). They were established from the ascites of a tumour induced in a male mouse by the intraperitoneal injection of Abelson leukaemia virus (A-MuLV).

2.10.2 Culture Media

The maintenance media used was Dulbecco's modified Eagle's media (DMEM) supplemented with 10% v/v foetal bovine serum (FBS) (mycoplasma screened) 1% L-glutamine and 1% v/v penicillin and streptomycin (Gibco). FBS (Gibco 011-06290H), L-glutamine (Gibco 041-01965M) and penicillin/streptomycin (Gibco

043-05140D) were stored at -20°C and thawed at 37°C immediately prior to use, the DMEM was stored at 4°C. The same media with or without serum was used in the stability and uptake studies.

2.10.3 Cell Culture

Cells were grown in 75cm^2 flasks with 25ml of media in a humidified (95%) incubator at 37°C with 5% carbon dioxide in air. The cells were maintained by changing the media every 48 hours and passaged by diluting 1 to 5, at weekly intervals. To passage, cells were washed with PBS, and trypsinised with 2% trypsin in PBS/EDTA. Fresh media was added and the cells seeded into wells or flasks at the appropriate density.

The number of cells seeded in multiwell plates was determined by counting the cells using a haemocytometer and diluting to the appropriate number before plating. Cells were plated at 5 x10^5 well^{-1} in a 24 well plate for uptake studies (unless otherwise stated). A fifth of the number were plated in 96 well plates for assays.

2.10.4 Long Term Storage

Cells were prepared for long term storage by trypsinising a semi confluent 75cm^2 flask, the cells were pelleted by centrifuging at 1,000 rpm for 2 minutes (43124-708 rotor, 150g, Mistral 3000 I centrifuge, MSE, Leicester, UK). The pellet was resuspended in 1ml DMEM containing 10% FCS and 10% DMSO in a 2ml Biofreeze tub and frozen slowly at -70°C before transferring to a liquid nitrogen store. The cells were recovered by rapid thawing at 37°C and gradual dilution with media, the cells were pelleted and resuspended in media before seeding in 25 cm^2 flasks.

2.11 CELLULAR ASSOCIATION

2.11.1 Cellular Association of Free Oligodeoxynucleotides

A 5' end labelled [^{32}P] hairpin ODN (Khan and Coulson, 1993) was diluted to the appropriate concentration with the corresponding cold ODN before adding to the cells. Cells were plated at 5 x10^5 well^{-1} in a 24 well plate, and the ODN was added approximately 12 hours later, uptake was monitored at intervals over a 24 hour period. The media was removed from the plate and placed in a scintillation vial. The cells were washed five times with PBS/sodium azide (0.05%) to remove any ODN loosely associated with the cell surface and inhibit any further cellular metabolism. PBS washings were collected in a separate scintillation vial. The cell monolayer was removed by the addition of 3% Triton X-100 at 37°C, this was repeated twice to ensure that all the cells had been removed from the well. Counts in the cellular fraction represent ODN that was associated with the cell, either at the surface or internalised. The [^{32}P] associated with the media, washes or cellular fractions was determined by scintillation counting as in section 2.2.3.

2.11.2 Cellular Uptake of 2μm Microspheres

The method was repeated as above, the microspheres were added to the cells at various concentrations, by dispersing in media, a total volume of 0.5ml was added to each well. The 5 washes with PBS/sodium azide ensured that all non associated spheres were removed from the cell surface. The cells and spheres were removed from the plate for scintillation counting with 3% Triton X-100 as before. Efflux from the cells was determined by incubating the cells with fresh media for a further 24 or 48 hours after washing the cells to remove the non associated spheres.

2.11.3 Determination of Cellular Association, Uptake and Release of Oligodeoxynucleotides from the Sphere within the Cell.

The experiment was repeated as before, however after the washes, the cells were removed from the plate with 2% trypsin and placed in a 1.5ml microcentrifuge tube, the cells and spheres were then centrifuged at 13,000 rpm for 3 minutes, counts in the supernatant represented any non-entrapped ODN associated with the cell surface. The cells were lysed by resuspending in 500µl distilled water for 5 minutes, the tubes were then vortexed to aid lysis, before centrifugation to remove the membrane fraction and the spheres, this process was repeated twice. The concentration of ODN associated at the cell surface (in the trypsin fraction), present in the cytoplasm (in the water fraction after lysis) or entrapped in spheres (in the pellet with the cell membrane after centrifugation) was assessed and thereby the exact uptake, release and association with the cell surface calculated. Efflux from the cells was determined by incubating the cells with fresh media for a further 24 or 48 hours after washing the cells to remove the non associated spheres.

2.11.4 Localisation of Oligodeoxynucleotide inside the Cell

A 5' fluorescent labelled 15 mer PS ODN was entrapped in the polymer spheres at a concentration of 0.2µg/mg polymer. Raw 264.7 cells were plated at a concentration of 1.5 $x10^5$ onto plastic chamber slides, 8 wells per slide (Nunc, Gibco) and incubated overnight. A sphere suspension (300µl) of 5mg polymer/ml, in DMEM serum free media was added to wells in duplicate, the equivalent free fluorescent ODN was added to the control wells. The chamber slide was incubated for a further 24 hours. The wells were washed five times with PBS to remove the media and excess spheres, and fixed in 1% paraformaldehyde (in PBS) for 1 hour at 4°C. The wells were washed with PBS, before removing the sides from the wells. The slide was mounted in PBS/glycerol mixture (1:1) containing 1% diazabicyclo[2.2.2] octane (DABCO) as an antifading agent (Johnson *et al.* 1982), and covered with a coverslip. Fluorescence in the cells was visualised using a fluorescence microscope (Jenamed) and a 510nm filter. The cells were also counterstained with propidium iodide. The cell nucleus stained with propidium iodide was used to aid in the determination of the cell localisation of encapsulated and free ODNs. The cells were grown in chamber slides and the spheres added, incubated and washed as above. The cells were fixed in 200µl acid alcohol (99 parts 70 % ethanol and 1 part 1M HCl) for 2 minutes, then washed with PBS four times to remove the acid, before

addition of 200µl of propidium iodide (100µg/ml in PBS). The cells were visualised using a fluorescence microscope (Jenamed) and a filter at 570nm.

2.11.5 Effect of Microspheres on Cell Number

A crystal violet staining assay was used to determine the number of cells present on a 96 well plate colorimetrically. Cells were plated in the range 10^3 to 10^5 cells per well (n=6) in order to produce a standard curve. After seeding in 200µl the plate was incubated in a humidified incubator at 37°C and 5% CO_2 for 4 hours, to allow the cells to attach to the plate but not divide. The wells were washed carefully with PBS containing 3% sucrose at 37°C, by removing 150µl of media and replacing with 50 µl of wash solution, five times in total. The cells were then fixed in 2% paraformaldehyde by addition of 50µl of fix solution (4% paraformaldehyde in PBS). After 30 minutes the wells were emptied and 4% paraformaldehyde was added to fix the cells for one hour, the wells were emptied and washed three times with 200µl of PBS. After blotting the wells dry 50µl of 0.1% crystal violet in PBS was added. The plate was shaken gently on a mechanical shaker to stain the cells for 30 minutes, the stain was removed by washing 6 times by total immersion in water. The plate was blotted dry and the stain solubilised in 50µl of 1% SDS. The absorbance was measured at 550nm against a reference of 690nm on an anthos plate reader 2001 (lab tec instruments) connected to an Epson LX-850 printer.

The effect of spheres on the cell number of RAW 264.7 macrophages was determined by the addition of 200µl of unloaded (blank) sphere suspension (5mg polymer /ml in DMEM containing serum) to each well of a 96 well plate, and incubated for a further 24 hours. The absorbance at 540nm for was converted into cell number using the standard curve.

2.11.6 MTT Assay

This colorimetric assay relies on the metabolism of 3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) in the mitochondria of living cells to a formazan dye which has a maximum absorbance at 540nm (Mosman, 1983; Denizot and Lang, 1986). The cells were plated in 96 well plates, and incubated overnight. A 200µl volume of sphere suspension (5mg/ml in DMEM containing 10% FBS) was added after washing the wells with PBS, fresh media was added to the control

wells. The plates were incubated for a further 24 hours. The wells were washed with PBS five times to insure all the free spheres had been removed and 100µl of fresh media was added to each well. A 20µl volume of 5mg/ml MTT solution was added per well and the cells were incubated for a further 4 hours. The plate was then centrifuged for 10 minutes at 1,000rpm (244 rotor, 150g IEC Centra-3c, Runcorn, UK). The media was aspirated from the wells and the dye solubilised by addition of 100µl DMSO per well and shaken on a mechanical shaker for 10 minutes. The absorbance was measured at 550nm against a reference of 690nm on an anthos plate reader 2001 (lab tec instruments) connected to an Epson LX-850 printer.

2.12 STATISTICS

An independent t-test was performed on the release profiles of ODNs released from polymer films to assess the significance of any differences seen in the release profiles. The slope of the linear portion (approximately from day 5 to day 20) of the release profiles was calculated for each release profile (n=4). The slopes were calculated using a linear regression programme written by W.J. Irwin and the t-tests were calculated using a StatWorks programme.

CHAPTER THREE

RELEASE OF OLIGODEOXYNUCLEOTIDES AND RIBOZYMES FROM SOLVENT CAST FILMS

3.1 INTRODUCTION

The poor biological stability of ODNs (Wickstrom, 1986) coupled with their rapid *in vivo* elimination kinetics (Agrawal *et al.*, 1992), has required the repeated administration of antisense nucleic acids for a sustained pharmacological effect especially for targets with a slow turnover (see section 1.8 for examples). For example, tumour regression in mice was observed after 1 to 2 weeks, following repeated subcutaneous injection of a PS ODN (Higgins *et al.*, 1993). In the case of peripherin, a neurone-specific intermediate filament protein, which has a slow turnover (half-life of about 7 days), repeated administration of antisense ODNs for up to 40 days was required to observe a reduction in protein levels of 90% in cultured PC12 phaeochromocytoma cells (Troy *et al.*, 1992). To reduce the need for repeated administration, a sustained delivery device would be advantageous. In recent *in vivo* studies, mini-osmotic pumps have been employed for the sustained delivery of ODNs (e.g. Whitesell *et al.*, 1991; Kitajima *et al.*, 1992; Ratajczak *et al.*, 1992; Higgins *et al.*, 1993). Although such infusion devices eliminate the problems caused by repeated administration, they suffer the disadvantage that the drug-depleted device has to be surgically removed. Thus, biodegradable delivery systems which can protect ODNs from degradation in the biological milieu and simultaneously, provide sustained delivery of the nucleic acid, thus avoiding the need for repeated parenteral administration, will be useful from a therapeutic viewpoint.

Biodegradable implantable polymers have been used for controlled drug delivery, Zoladex (goserelin) in PLGA (see section 1.10 for further examples), is in clinical use, with carmustine in polyanhydride wafers Brem, (1991) in clinical trials. Implantable

biodegradable polymer devices probably have optimal therapeutic potential to enhance substance delivery, if they are implanted directly at, or close to an accessible target site, enabling delivery in a sustained release manner close to the tumour site. Implantation close to the site of action solves some of the problems associated with targeting a drug to a specific body site by systemic administration. The polymeric delivery devices produce significantly lower blood levels and therefore result in fewer side effects as a result of localised delivery. The slow release mechanism not only reduces the need for repeated administration but also offers protection to the entrapped drug prior to release.

Fournier *et al.* (1991) used biodegradable cylindrical implants of PLGA loaded with vinorelbine for the local treatment of solid tumours. The *in vivo* release was found to be slower compared to *in vitro*, this may be due to the formation of a collagenous capsule around the implant *in vivo*. A dose dependent antitumour affect was seen in mice in a solid P388 leukemia model, when the implants were administrated into or in contact with the tumour site. Implants were more effective than intravenous administered vinorelbine, the treatment was successful in 8 out of 9 patients with head and neck cancer.

The treatment of brain tumours is a potential use of ODNs, however the blood brain barrier limits the usefulness of many drugs to the central nervous system and pharmacokinetic studies with ODNs (Agrawal *et al.*, 1992) showed only low concentrations reaching the brain following intravenous administration. After intraventricular administration PS ODNs are cleared by bulk flow. Continuous infusion at 1.5nmol/hr by mini-osmotic pump can maintain micromolar levels of intact PS ODN for at least a week without obvious neurological or systemic toxicity (Whitesell *et al.*, 1993). Therefore polymeric devices could be used to deliver higher concentrations of drug to the brain than can be achieved by systemic delivery . Implantation after surgery to remove the initial tumour mass, would prevent the need for further surgery and would avoid the need for continuos infusions to maintain the ODN concentration in the brain. Tumour reoccurrence would hopefully be prevented by the sustained release of the relevant antitumour agent. Carmustine when impregnated in polymers appears was more effective in treating brain tumours compared to standard methods of delivery (Brem, 1990). Copolymers of poly lactide co-glycolide (PLGA) are biocompatible with brain tissue (Menei *et al.*, 1993) and would be an ideal starting point for the development of a sustained release delivery device, with the potential for implantation into brain tissue. Macroscopic implants require open surgery for implantation, however

the development of micro and nanospheres enables them to be stereotactically implanted into the brain due to their size, causing no damage to the surrounding tissue (Menei *et al.*, 1994 b).

The potential of PLA solvent cast films was evaluated as sustained release matrices for ODNs and RBZs. Films were chosen as the first preparation due to their relative ease of fabrication.

3.2 RELEASE OF OLIGODEOXYNUCLEOTIDES FROM SOLVENT CAST FILMS

3.2.1 Effect of Oligodeoxynucleotide Loading, Length and Backbone type on the Release Profiles

The PLA films were prepared as described in section 2.7.2. The nature of the solvent casting procedure enabled all the ODN added to be entrapped/associated with the PLA matrix. Production yields were approximately 90% with some polymer loss to the mixing vesicle during the casting of the polymer solution.

The resulting films were smooth, flexible, transparent and approximately 100μm in thickness. The film was cut in to $1cm^2$ pieces with an approximate mass of 10mg for further experiments.

Figure 3.1. shows the influence of drug loading on release of PO 7mer ODN from PLA thin-film matrices in PBS at pH 7.4 and 37°C. The higher the initial loading of the ODN in PLA matrices, the greater the amount of the ODN released over the 28 day study period. For any given loading, the ODN release appears to be biphasic which is characterised by an initial rapid phase of release ('burst effect') of approximately 10% followed by a second phase of slower release. These biphasic release profiles obtained with antisense ODNs were similar to those observed for macromolecular protein drugs and some low molecular weight species from related polymer devices (Heller, 1993). In all instances, the burst effect is largely attributed to release of drug present at and/ or entrapped close to the surface of the polymer device, whereas the second phase of

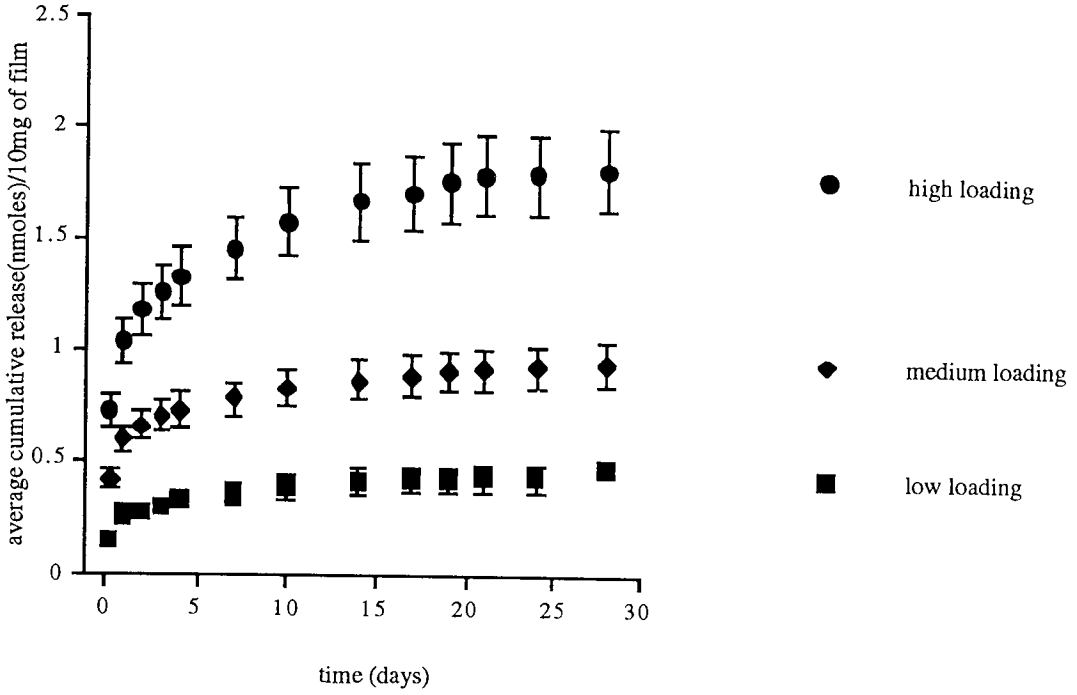


Fig.3.1. The influence of the initial ODN loading on the release of PO 7 mer poly A ODN from PLA films in PBS at 37°C. Loading of the ODN per 10mg film of PLA = low = 0.7 nmoles, medium = 1.4 nmoles, high = 2.8 nmoles. n = 4 ± S.D.

slower release probably represents efflux of ODN entrapped further inside the polymer matrix. The magnitude of the burst effect increased with increased loading which suggested that as the ODN loading is increased, progressively greater amounts of the ODN is associated with and/ or entrapped close to the surface of the polymer matrices. Statistical analysis of the results was determined as in section 2.12. An independent t-test using the slopes of the release profiles, indicated that the rates of release from the films of different loading were significantly different at the 95% confidence interval. Not all the entrapped ODN is released from the polymer during the 28 day period of the study, as the remaining ODN is probably trapped in the polymer core and has further to diffuse before release can occur. The rate of release could be increased in the secondary slower phase of release by the use of ultrasound (Kost *et al.*, 1989, Supersaxo *et al.*, 1993). Ultrasound enhances the release of entrapped substances by causing reversible polymer degradation. Incorporation of plasticisers, ethyl vinyl acetate (EVA) or glycerol can also be employed to enhance the release rate of entrapped drugs by accelerating the breakdown of the polymer. Blending with a EVA has been shown to

decrease the crystallinity of the polymer (Gassner and Owen, 1992), however the incorporation of a non erodible substance is a disadvantage in a degradable system. Incorporation of glycerol in a silicone matrix has been shown to enhance the release of entrapped small molecules (Hsieh *et al.*, 1985) It is thought that the dispersed glycerol helps absorb water, therefore aiding drug release. The release rate of ODNs from PLA films could also be increased by the incorporation of a lower molecular weight polymer (von Recum *et al.*, 1995). Copolymerisation with a water soluble moiety such as poly ethylene glycol (PEG), will enhance release of the entrapped substance (Hu and Liu, 1994). The addition of PEG decreases the mechanical strength polymer and hydrophobicity of the polymer.

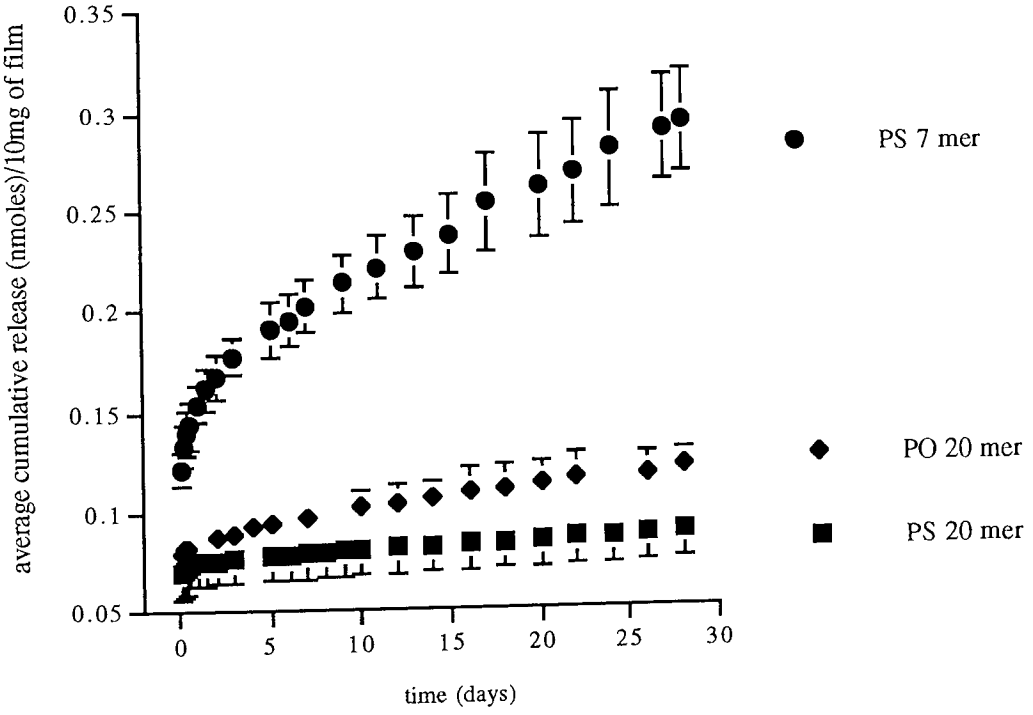


Fig.3.2 The influence of ODN chemistry and sequence length on release from PLA films in PBS at 37°C. Loading of the ODNs per 10mg PLA film = 0.5nmoles. n = 4± S.D.

ODN chemistry and sequence length do affect the *in vitro* release profiles of antisense ODNs from PLA matrices, as shown in Fig 3.2. The rates of release in each profile are significantly different at the 95% confidence interval using an independent t-test. The

extent of the burst effect and the rate of release in the subsequent "slow" phase was dependent on both ODN length and backbone chemistry of the antisense nucleic acid. Two ODNs of equal length (20mer) but of different backbone types, either phosphodiester or phosphorothioate, similarly exhibited biphasic release profiles.

Release of the ODN from PLA films continues steadily with time as shown in Fig. 3.3, the ODN loaded films therefore have the potential for long term treatment with prolonged sustained release of ODNs. However even after 100 days incubation in PBS at only 50% of the entrapped ODN was released.

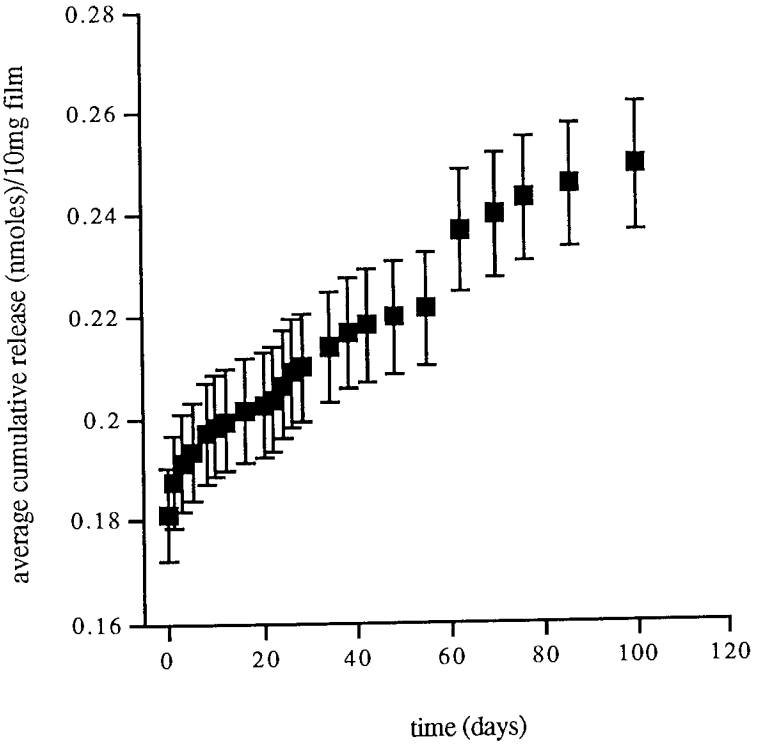


Fig.3.3 Long term release profile of PO 20 *tat* from PLA films in PBS at 37°C. Loading of ODN per 10mg PLA film = 0.5nmoles. n=4 ±S.D.

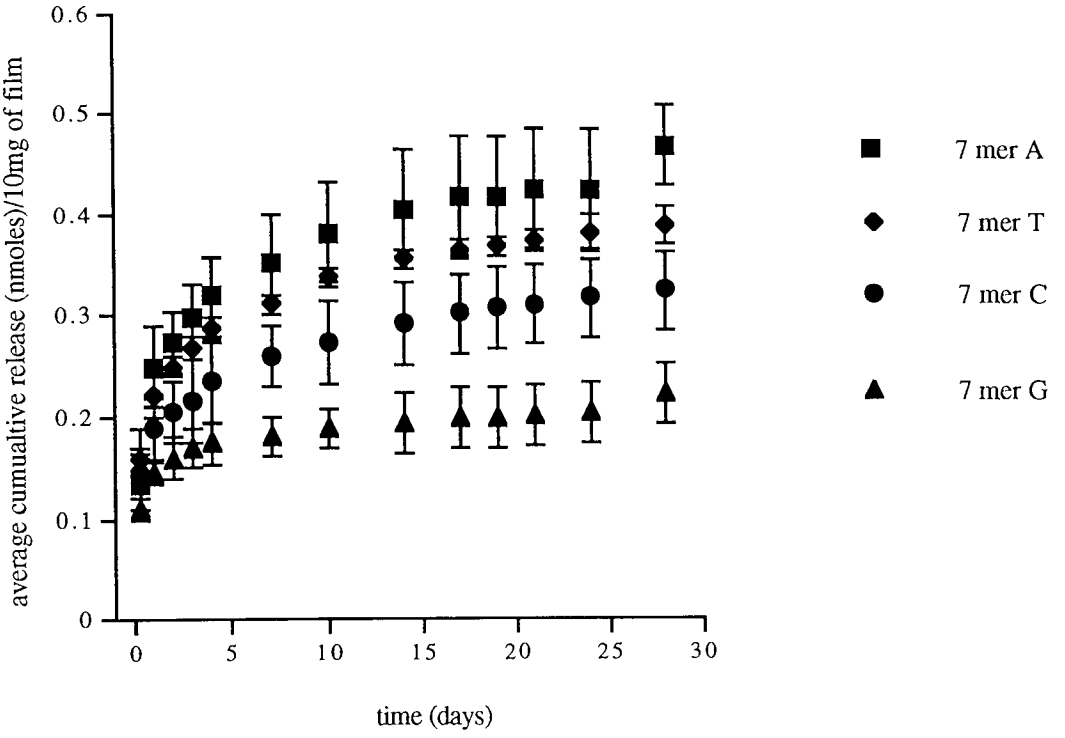


Fig.3.4 Effect of sequence on release of a 7mer PO ODN from PLA films in PBS pH 7.4 at 37°C. Loading of 7mer ODNs per 10mg PLA film = 0.7nmoles, n=4 ±S.D.

Figure 3.4 indicates that the initial burst release of homo PO ODN sequences (A,G,C,T) are almost identical, with the ODN sequence having little effect, as the burst release is represented by ODN present at the polymer film surface. The release profiles of the different sequences are also similar which would be expected from the results in Fig.3.2. However differences (albeit slight) are seen during the second period of slower release, with the release rate increasing in the order G<C<T<A. Statistical analysis by means of an independent t-test on the slopes of each of the release profiles shows there is a significant difference in the release of a 7mer homo G ODN compared to the release of 7mer homo A, C or T ODNs at the 99% confidence interval. The PO homo G ODN is released more slowly than the other sequences, possibly as a result of its increased hydrophobicity compared to the other bases, or tertiary structure.

Hughes *et al.*, (1994) report that cellular uptake of a 10mer PS homo G ODN is greater than other PS homo ODNs. This is probably due to a greater initial association with the plasma membrane, homo-G ODNs show the greatest binding to liposome membranes. Peyman *et al.*, (1995) reported similar findings, with the uptake of a 15mer PS homo sequences being in the order of G>T>A>C. Care must be taken in the analysis of these

results due to the known tertiary structures as identical bases can associate in a great variety of arrangements even though these might be less thermodynamically stable than complementary base pairing. The use of a 7mer sequence with no complex tertiary structure due to lack of sequence length confirms that the differences seen must be as a result of differences in hydrophillicity and not as a result of complex tertiary structures which may enhance cellular uptake or hinder release from a polymer film.

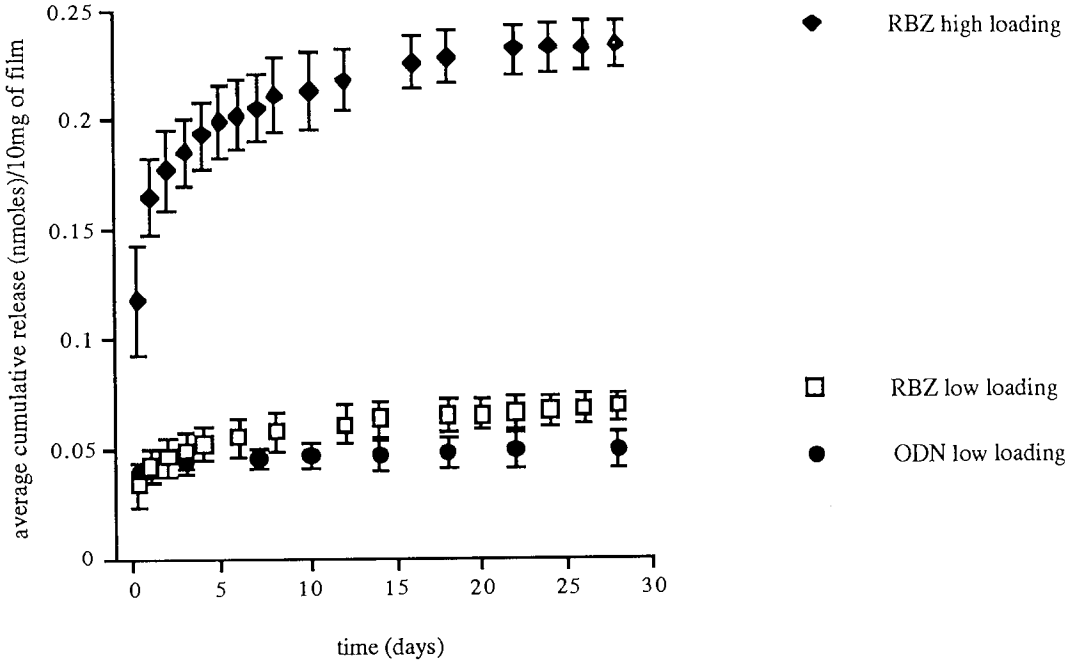


Fig. 3.5 Effect of a 32mer RBZ loading on release from PLA films and comparison with a 32mer ODN release in PBS at 37°C. Loading of ODN/RBZ per 10mg PLA film:- High = 0.67nmoles, Low = 0.24nmoles (RBZ and ODN). n=4 ±S.D.

The release of an unmodified 32mer hammerhead ribozyme (RBZ) from PLA films appears to be similar to that of the release of an unmodified 32mer ODNs from PLA films (Fig.3.5), again characterised by an initial burst release followed by a second phase of slower release. Increasing the initial loading results in an increased rate of RBZ released from the polymer films in PBS at 37°C , showing a similar trend to ODN loading as seen in Fig.3.1. By comparing Fig.3.1 and 3.5 the release of a high loading 20mer appears to plateau after 20 days incubation in PBS at 37°C compared to a plateau around 14 days for the RBZ, the increased molecular weight of the 32mer RBZ (10400) compared to the 20mer ODN (6090) results in a slower diffusion through the polymer matrix and hence a slower rate of release. This same effect is demonstrated in

Fig.3.2, where the shorter 7mer ODN is released from the PLA film more rapidly than a 20mer ODN. The release of the RBZ from the PLA film appears to be slightly greater than that of a 32mer ODN (fig 3.5), this may be as a result of the RBZ being less stable compared to the ODN and undergoing greater degradation in the polymer film and hence the presence of smaller degradation products appearing to slightly enhance the release rate. The stability of ODNs and RBZs entrapped in polymer films is discussed in greater detail in Chapter Six.

3.2.2 Effect of Release Media on Release of Oligodeoxynucleotides from Polymer films

To further examine the role of pH and type of release medium used on the rate release of ODNs from PLA matrices, we compared release at pH 5.5 (citrate-phosphate buffer, CPB), pH 7.4 (phosphate-buffered saline, PBS), and in foetal calf serum. The degradation of PLA has been reported to be faster at a higher pH (Menei *et al.*, 1993), or a lower pH (Chu, 1982; Heya *et al.*, 1991).

The pH of the media did not result in any marked differences in the release profiles of a 32mer HH RBZ from PLA matrices when incubated in buffer at pH 5.5 or pH 7.4 (phosphate-buffered saline, PBS) for 28 days (fig.3.6). The lack of effect may have been as a result of the higher molecular weight of polymer employed for film fabrication compared to the studies by Chu, (1982) and Heya *et al.* (1994). The presence of serum (fig.3.7) did not alter the release of a PO 20 tat ODN from PLA films over the 28 day period studied.

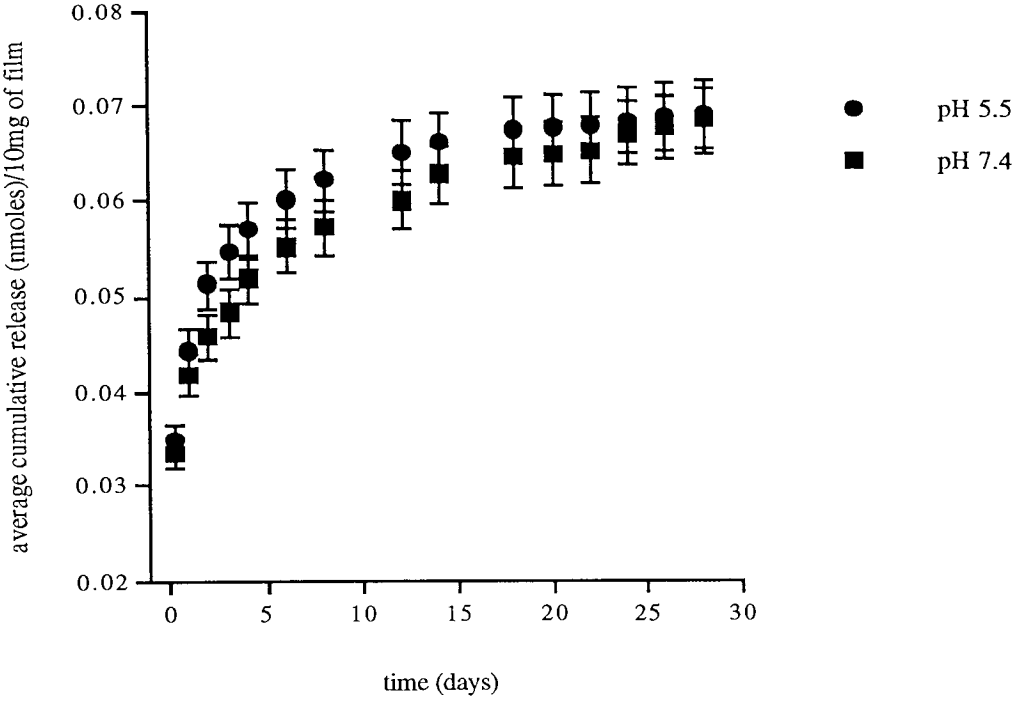


Fig. 3.6 Effect of media pH on the release of a 32mer RBZ from PLA films at 37°C. Loading = 0.24nmoles/10mg film, n=4 ±S.D.

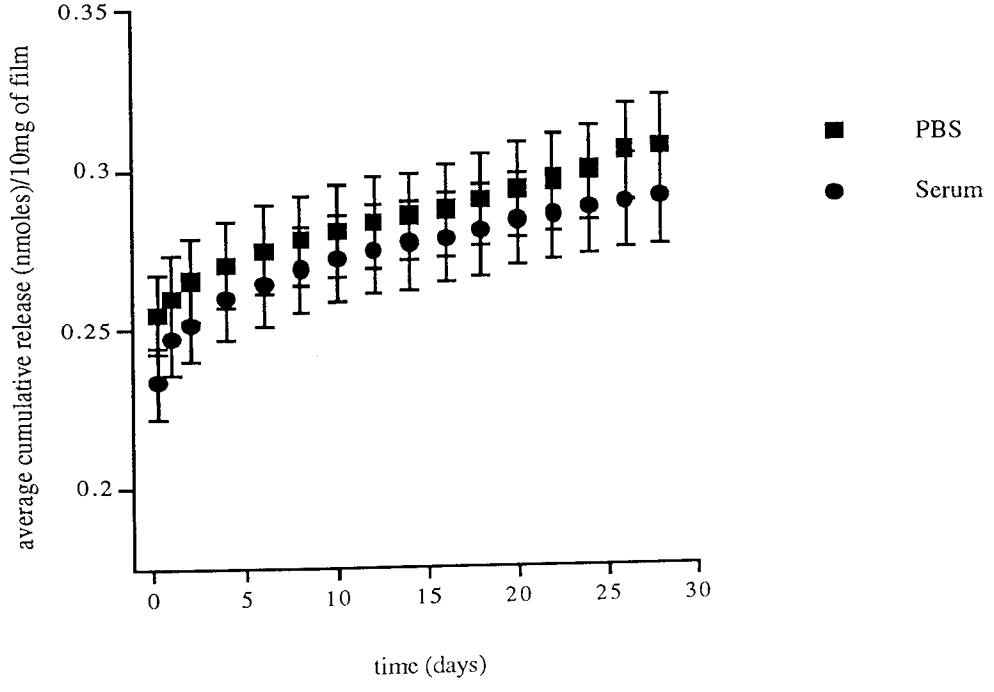


Fig.3.8 Effect of serum on the release of a PO 20mer *tat* ODN from PLA films at 37°C. Loading = 0.65nmoles/10mg film, n=4 ±S.D.

3.2.3 Effect of Polymer on Release of Oligodeoxynucleotide from Solvent Cast Films

PLA, PHB and P(HB-HV) (12mol% HV) polymers were compared to see if polymer type had any effect on the release of ODNs from thin solvent cast films in PBS at 37°C. The polymer films were prepared as in section 2.7.1. and the release experiments are detailed in section 2.8.4.

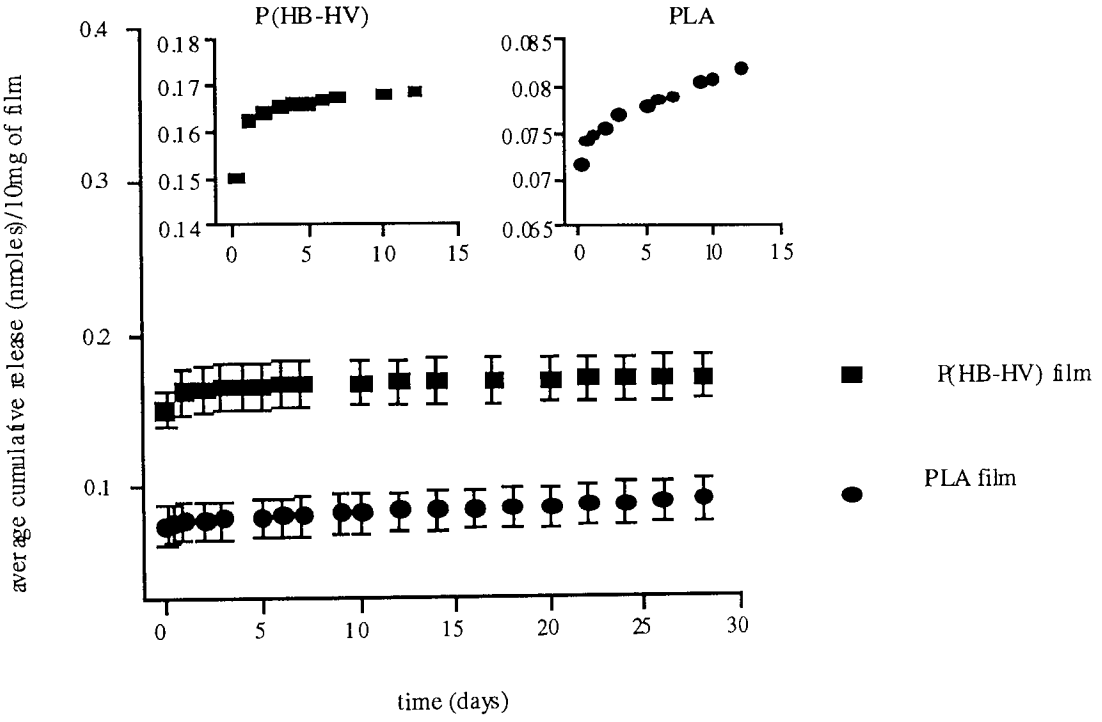


Fig.3.8 Comparison of release of PO 20 *tat* ODN from P(HB-HV) and PLA solvent cast films in PBS at 37°C. Loading of ODN per 10mg PLA film = 0.5nmoles. n=4 ±S.D.

The release of a PO 20 *tat* ODN from both PLA and P(HB-HV) films is biphasic as shown in Fig 3.8, however the burst release is greater from PHB:HV films (35%) compared to the release from a PLA films (20%). This suggests that the ODN is not as well incorporated in to the P(HB-HV) matrix compared to the PLA matrix, resulting in the higher burst release. However the rate of release of the ODN following the initial burst release is not significantly different at the 95% confidence interval as determined by an independent t-test. However on examination of the release profiles in the early stages, the inset to Fig.3.8 shows that the secondary phase of release appears to plateau

earlier in the P(HB-HV) films at approximately 10 days compared to release from the PLA which has not reached a plateau by day 15. A greater rate of release of ODNs from PLA films compared to P(HB-HV) films may be expected, possibly due to the absorption of water in the PLA matrix aiding the diffusion of the ODN through the polymer. In comparison the P(HB-HV) polymer is expected to take up little water during the short period of incubation and hence there may be reduced diffusion of the ODN through the polymer.

Fig.3.9 shows that the release of a PO 20 *tat* ODN is slightly faster from the copolymer compared to the PHB homopolymer, although both polymers have similar degrees of crystallinity, the copolymer crystallites do not appear to entrap the ODN as well as the homopolymer (Akhtar *et al.*, 1992 c). However the release rates are not significantly different at the 95% confidence interval as determined by an independent t-test.

Glycerol was added to the polymer before addition of the ODN and film casting at a loading of 5 and 10% w/w of dry polymer mass, with the aim of increasing the rate of release from the P(HB-HV) polymer matrices. The results in Fig.3.10 show that increasing the amount of glycerol increases the burst release of the ODN, although it does not affect the rate of release during the slower secondary phase of release. The glycerol appeared to concentrate at the polymer surface making the film sticky to the touch. Blending of the polymer with polysaccharides could be used to accelerate the development of porosity and enhances the degradation process (Holland *et al.*, 1990)

The slow rate of release of the ODNs from the PHB and P(HB-HV) polymer film matrices may preclude their use as sustained delivery systems for ODNs without further modifications to enhance the rate of release. However due to the very slow *in vivo* degradation of these polymers, the films may have a role where very long term release is required.

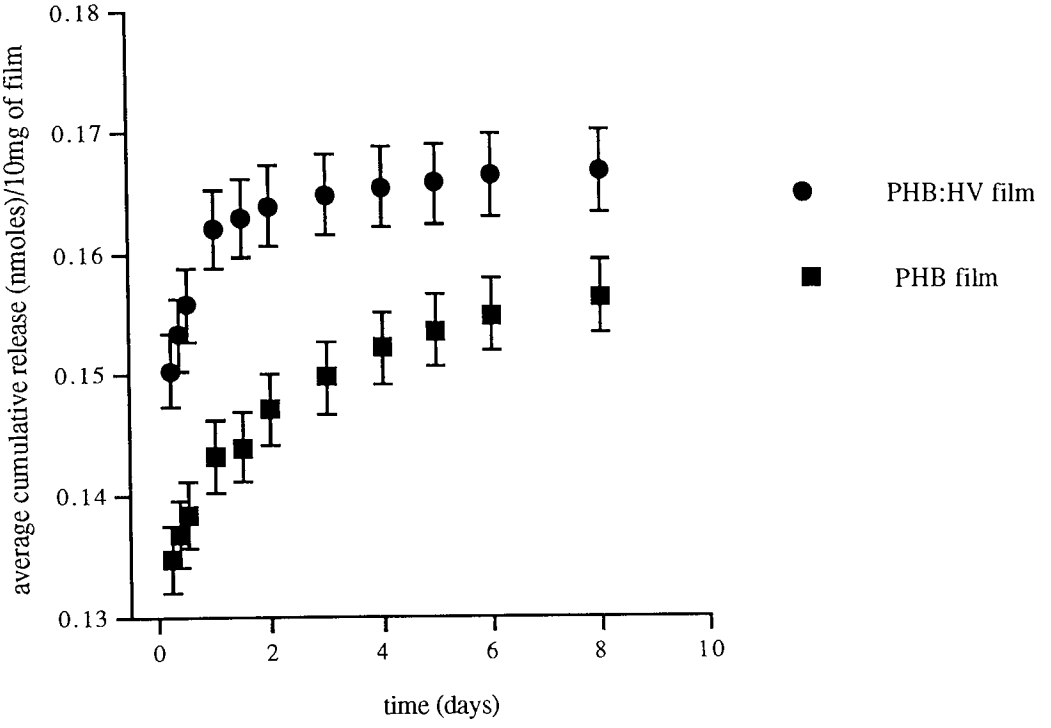


Fig.3.9 Comparison of release of PO 20 *tat* ODN from PHB and PHB:HV solvent cast films in PBS at 37°C. Loading of the ODN per 10mg PLA film = 0.5nmoles. n=4 ± S.D.

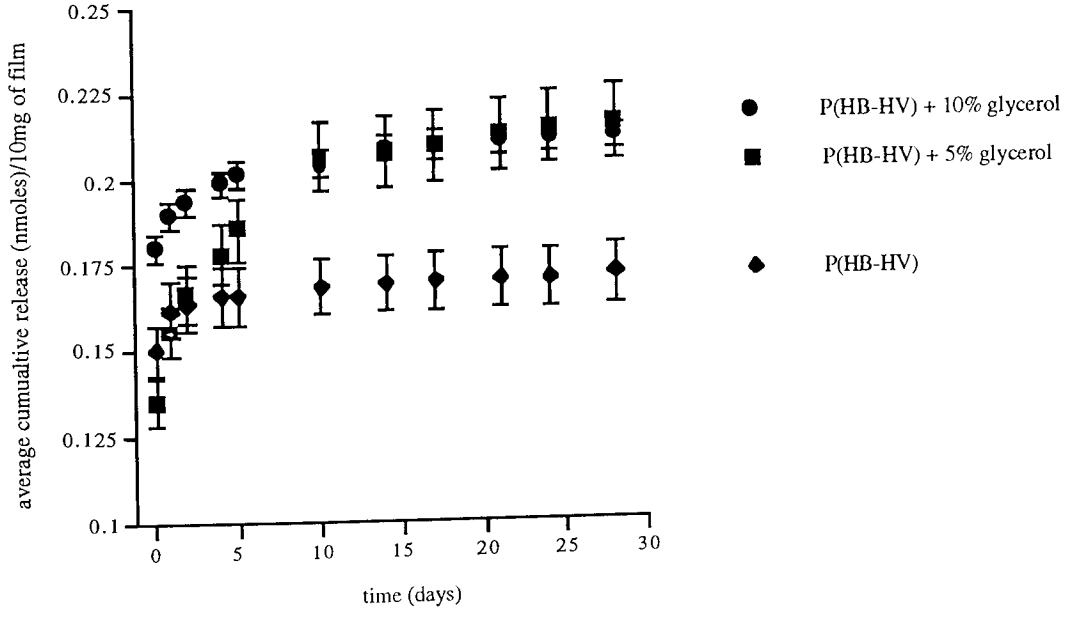


Fig.3.10 Effect of incorporation of glycerol in the polymer matrix on release of PO 20mer ODN from polymer films in PBS at 37°C. Loading of ODN per 10mg PLA film = 0.5nmoles. n=4 ± S.D.

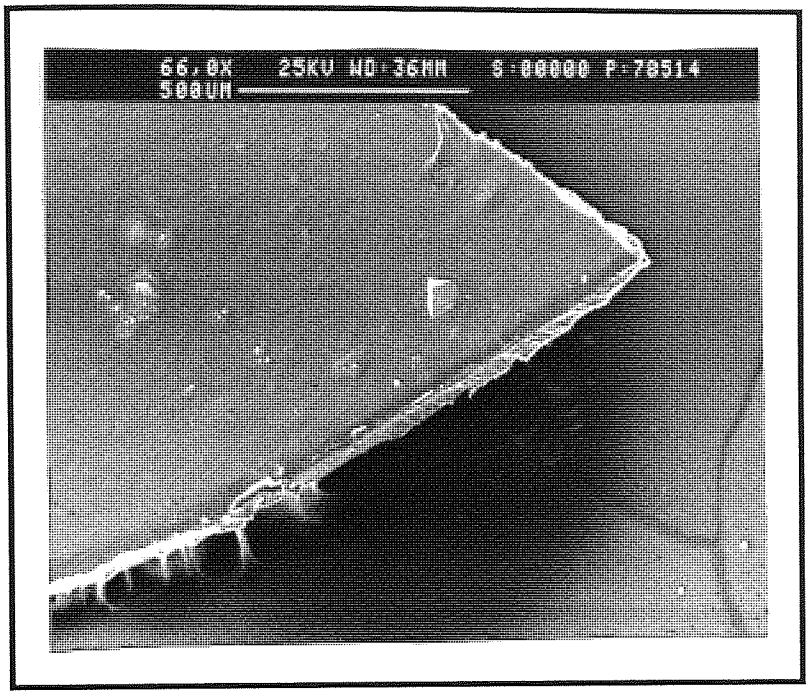
3.3 POLYMER DEGRADATION

Degradation of the polymer films was assessed by differential scanning calorimetry (DSC) and by scanning electron microscopy (SEM) as described in section 2.8. The effect of any degradation of the polymer films could then be correlated with the release of ODNs in section 3.2.

Polymer Sample	Description	T_m onset (°C)	T_m peak (°C)	Enthalpy of fusion (J/g)
PLA	white crystalline solid granules, almost odourless	177.5 ±1.2	187.3 ±1.8	46.7 ±6.5
PLA film	flexible, smooth and transparent. Occasionally air bubbles are trapped in the film during incorporation of the ODN due to the viscous nature of the polymer solution	170.8 ±0.6	177.5 ±0.5	20.5 ±1.59
PLA film +5% glycerol	opaque film	163.7	168.9 ±0.8	4.45 ±0.9
PLA film PBS 28 days	intact, no visible change	171.6 ±0.7	176.4 ±0.3	12.8 ±0.3
PLA film CPB 28 days	intact, no visible change	171.4 ±1.2	175.9 ±0.6	13.6 ±0.9
PLA film serum 28 days	intact, no visible change	171.8 ±0.5	177.5 ±0.3	20.7 ±5.3
PLA film PBS 98 days	intact, no visible change	172.1 ±0.5	172.2 ±0.4	32.15 ±9.0
PLA serum 98 days	film has disintegrated into small brittle fragments	sample size too small for DSC analysis		

Table 3.1. Polymer sample characteristics and Differential Scanning calorimetry (DSC) data, n=4 ±S.D.

(A)



(B)

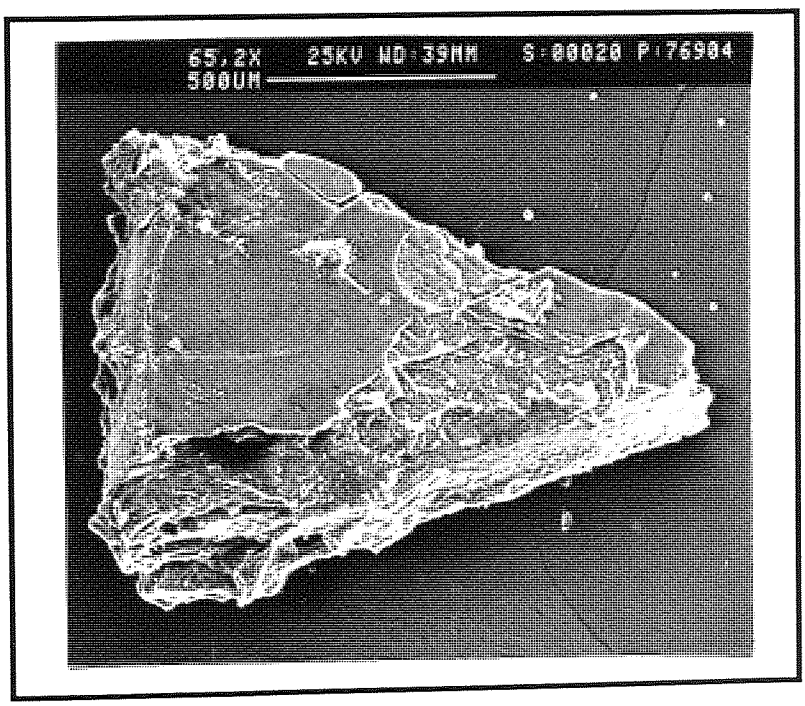


Fig.3.11 Scanning Electron Micrographs of PLA films (A) before, (B) after 98 days incubation in serum at 37°C.

No degradation of the films was observed by SEM after 28 days in serum or phosphate buffered saline (Figure 3.12). After 28 days the films remained intact, flexible and opaque. Data from DSC profiles (Table 3.1) did not show any change in the film crystallinity or melting point compared to films which have not been incubated in PBS, CPB or serum (at pH 7.4 and 37°C).The lack of any evidence of degradation in these different release media explains why no difference in the release profiles was evident between the 3 conditions.

These results indicate that there was no enzymatic degradation of the polymer in serum during the 28 day release period which may have caused an enhanced release of the ODN. However, morphological evidence from scanning electron micrographs suggested that serum enzymes were capable of degrading PLA films incubated in serum for 98 days (See Figure 3.12). At this stage, the $1cm^2$ film had broken into many small fragments which were opaque and brittle. Scanning electron micrographs showed evidence of film fracture and mass loss of the polymer. Polymer degradation may therefore effect ODN release from the polymer films over extended release periods.

3.4 KINETICS OF OLIGODEOXYNUCLEOTIDE RELEASE FROM POLYMER FILMS

The nature of the interactions of the ODNs with the polymer matrices, and the factors determining their release were unknown, as the results in section 3.3. indicate that polymer degradation does not appear to influence release of ODNs from the polymer films. To further investigate the mechanism of ODN release from PLA matrices the release data from PLA films were fitted to mathematical models.

The ODN concentration within the polymer films for these experiments is very low (2nmoles per film of mass 10mg). At such concentrations, it is probable that the ODN may be regarded as being distributed intimately throughout the polymer matrix and the device may behave as a solution system subsequent to the initial burst phase. To test this hypothesis, release data were fitted to the non-linear model describing the diffusional release of a dissolved drug from a monolithic polymer slab (Baker, 1987), suitably modified for a burst effect as indicated in Equation 1 (Fig. 3.13). Curve fitting was undertaken using iterative non-linear least squares regression employing the

BASIC program NONREG (Irwin, 1990) with the subroutine listed in Table 3.2 and the parameter variation variable set to 10^{-5} (line 1140). In general, the initial estimates of the parameters (D and M_b, with a fixed value of h = 0.01 cm) rapidly converged to provide an excellent fit to the model and gave the estimates which are recorded in Table 3.2. Calculations were repeated to further allow the estimation of the film thickness as a third parameter; these gave essentially the same values with the estimate for h always being close to the expected 100 μm (approx thickness of the films produced), confirming the validity of the analysis and the measurement of the film thickness. Equation 1 is continuous over the full release profile, but it may be simplified by the use of two approximations; an early-time approximation when less than 60% of drug has been released and a late-time approximation when over 40% of release has occurred. During the present experiments, release over the 4 week period studied was usually <60% . Thus, the approximate model shown in Equation 2, should also correspond to the release profile - especially as the initial release of ODN does not represent diffusional loss and, hence, further limits the fraction released by diffusional processes. Release data were similarly fitted to this model using NONREG or FigP (Biosoft Ltd.) with good agreement with the earlier parameter estimates.

EQUATIONS

$$M_t = (M_o - M_b).\left(1 - \sum_{n=0}^{\infty} \frac{8.\exp\left[\frac{-D.(2n+1)^2.\pi^2.t}{h^2}\right]}{(2n+1)^2.\pi^2}\right) + M_b \qquad \text{[Eq.1]}$$

$$M_t = A.\sqrt{D.t.C_m.\left(\frac{4.(M_o - M_b)}{A.h} - C_m\right)} + M_b \qquad \text{[Eq.2]}$$

$$M_t = 4.(M_o - M_b).\sqrt{\frac{D.t}{\pi.h^2}} + M_b \qquad \text{[Eq.3]}$$

Fig.3.12 Equations for calculations of kinetics of ODN release from the PLA matrix where:-

M_t = mass of drug released at time t
M_o = the total mass of drug in the device
M_b = the mass of drug released in the initial burst
D = the diffusion coefficient of the drug in the device
h = the thickness of the device
n = an integer which increases from 0 to infinity but requires progressively fewer terms as t increases
Cm = solubility of drug in the matrix

ODN	Medium [pH]	Amount ODN (nmol film^{-1})	D ($cm^2 d^{-1}$) x10^9 [s.e.]	M_b (%) [s.e., n]	Release rate (k) (nmol d^{-1}) x10^{-3} [r^2, n]
PO 20 *tat*	PBS [7.4]	0.68	11.39 [0.736]	36.6 [0.17,23]	1.425 [0.990, 16]
PO 20 *tat*	FCS [7.4]	0.68	11.85 [0.876]	34.6 [0.22,16]	1.09 [0.993, 10]
PS 20 *tat*	PBS [7.4]	0.58	0.794 [0.043]	12.2 [0.05,23]	0.448 [0.994, 16]
PS 7 *c-myc*	PBS [7.4]	0.55	110.8 [2.52]	21.6 [0.26,21]	4.56 [0.996, 13]

Table 3.2 Parameters for the release of ODN from PLA film matrices.
[D, apparent diffusion coefficient calculated from Eq. 1; M_b, percentage of release in burst phase of release; release rate is the slope of the later, linear portion of the release profile; PBS, phosphate-buffered saline, FCS, foetal calf serum; CPB, citrate-phosphate buffer; s.e., standard error of estimate; r^2, fraction of variance explained by linear regression; n, number of data points in analysis].

```
890 PI=3.141593 : AM0=100 : H=100: 'M0 as %; h in µm to avoid overflow errors
900 AD=P(1) : AMB=P(2) : 'H=P(3) : 'Use if estimation of h is required
910 'Expressions here
920 ON J GOTO 930 , 950 , 970 , 1000 , 1020 : 'Select equation
930 SUM1=0 : 'Summation variable
934 AN=-1 : 'Counter for n
935 AN=AN+1 : SUM2=SUM1 : 'New value of n; current sum
936 AN1=(2*AN+1)^2
937 ANUM=8*EXP(-AD*AN1*PI*PI*T/H/H) :' and replace H by P(3) here
938 DENOM=AN1*PI*PI : SUM1=SUM1+ANUM/DENOM
939 IF ABS(SUM1-SUM2)/SUM1>1E-08 THEN 935 ELSE 940
940 C=(AM0-AMB)*(1-SUM1) + AMB : 'Estimated amount released
945 RETURN
```

Table 3.3 BASIC subroutine for the non-linear estimation of release parameters D (P(1)) and M_b (P(2)) using program NONREG.

In view of the fact that this simpler $t^{1/2}$ model also fits the concentration-time data, a further release mechanism is possible; that from a polymer matrix in which the drug is suspended. The model for the appearance of drug in solution from a monolithic dispersion is shown in Equation 3, again modified for a burst effect. Attempts to fit the release profile to this model were less successful for, although the calculated line fitted the experimental points reasonably well and close estimates of the extent of the burst effect (M_b) were obtained, values for D and C_m (the solubility of drug in the matrix) were not statistically significant. This may be due to our failure to extract the parameters adequately but perhaps may also support the conjecture that release from a polymeric solution (Eqs. 1 and 2) is an appropriate model for ODN release from the PLA films used in this work. Over the time period studied, the slow rates of release allow the terminal portions of the plot to approximate to a linear release of ODN with little error. To provide a further comparison of the release kinetics, these values are also recorded in Table 3.2.

However, the rate of release of the PO 20mer ODN ($k= 1.425 \times 10^{-3}$ nmol d^{-1}) from the polymer matrix was faster compared to the PS 20mer ODN ($k=4.48 \times 10^{-4}$ nmol d^{-1}; See Table 3.1 for summary of kinetics data). Although both ODNs are polyanionic, the fact that phosphorothioates have been shown to exhibit higher octanol: water partition coefficients and greater lipid binding (Akhtar *et al.*, 1991a) suggests that they are more lipophilic compared to phosphodiesters and this may provide an explanation for their slower efflux from the hydrophobic polymer matrices. The shorter PS 7mer ODN was released more rapidly ($k = 4.56 \times 10^{-3}$ nmol d^{-1}) compared to the longer PS 20mer ODN ($k = 4.48 \times 10^{-4}$ nmol d^{-1}) suggesting that longer ODNs interact more with the hydrophobic polymer matrices thereby retarding their efflux.

The release profiles of a PO 20mer *tat* ODN from PLA films (fig.3.7) were similar in phosphate buffered saline or foetal calf serum (both at pH 7.4; kinetic data given in Table 3.2) suggesting that release did not vary with a change in release medium. There was good agreement in the values obtained for the apparent diffusion coefficient, burst effects and release rates for ODN released in PBS or foetal calf serum as shown in Table 3.2.

3.5 SOLVENT RETENTION IN SOLVENT CAST FILMS

Chloroform is hepatotoxic and nephrotoxic, decreases respiration, produces hypotension and decreased cardiac output (Martindale, 29). Poisoning leads to respiratory depression and cardiac arrest. Liquid chloroform is irritant to mucous membranes. In the UK medicinal products are limited to not more than 0.5% w/w, exceptions are for external or anaesthetic preparations. However in the USA the FDA have banned the use of chloroform in medicines and cosmetics because of reported carcinogenicity in animals (Martindale, 29).

As a result of the known toxicity of chloroform and maximum levels allowed in a preparation, it was very important to characterise the levels of chloroform in the PLA matrices. Solvent retention was determined by gas chromatography as described in section 2.8.6. and found to be 2.5%w/w, a graphical result as comparison with solvent retention in micropsheres is shown in fig.4.7.

The levels of chloroform retained in the films exceeds the recommended maximum levels of chloroform and steps will be needed to reduce these levels if ODN and RBZ loaded films are going to have a clinical use. In order to further reduce the levels of solvent retention the films could be dried for a longer period and at a higher temperature before storage in a dessicator. Preparation of thinner films (less than 100μm in thickness may enable more efficient removal of the residual chloroform. In view of the fact that the FDA have banned the use of chloroform in medicinal products, the use of dichloromethane as a solvent, which has a lower boiling point compared to chloroform and hence easier removal of the solvent may be more suitable. A melt processing method may also be employed which avoids the problems associated with the use of solvents.

3.6 CONCLUDING REMARKS

The results in this chapter demonstrate that ODNs can be entrapped into and released from biodegradable polymer films. The *in vitro* release of antisense ODN from PLA matrices was dependent on the ODN chemistry, sequence length and type as well as the loading of the ODN in the PLA films. The release of and all RNA hammerhead RBZ was similar to that of an identical DNA sequence. Although the actual amount of ODN entrapped per film in this study was relatively low, the required dose for any given application may be achieved by either a) incorporating a higher loading, b) increasing the dimensions of the matrix or 3) by using multiples of the smaller film matrices. It should be noted that with the very potent anti-HIV phosphorothioate ODNs, only a low maintenance dose (of 10% of bolus) is usually required to maintain the antiviral effect (Lisiewicz *et al.*, 1993). As such, even the low doses that are delivered by films produced in this study may be potentially useful in instances where very low maintenance doses are sufficient for a sustained pharmacological effect *in vivo* .

The *in vitro* release profiles in this chapter can only be used as a guide for the expected in *vivo* release. A study by Higgins *et al.* (1993) delivered 2.8mg of a 24mer PS ODN by means of an infusion pump over a 14 day period, a rough approximation to provide an equivalent dose by the use of biodegradable polymer films would require the implantation 20 pieces of 10mg PLA film (200mg) loaded with 5nmoles ODN/10mg of film. The mass of the polymer to be implanted could be further reduced if the ODN loading was further increased, or all the ODN could be released over the required period, by enhancing the polymer degradation. Therefore the use of polymer films have a realistic potential for the delivery of ODNs.

The effect of the fabrication procedure on the stability of the entrapped nucleic acid on incubation in serum and the affinity for the target sequence are discussed in Chapter Six. The fabrication and release of ODNs from polymer microspheres are discussed in Chapters Four and Five.

CHAPTER FOUR

OLIGODEOXYNUCLEOTIDE-LOADED MICROSPHERE PREPARATION AND CHARACTERISATION

4.1 INTRODUCTION

Microspheres can be defined as microporous spherical matrix systems where the drug is uniformly dispersed throughout the polymer. They have the advantage of a wide number of delivery routes depending on their desired application.

Microspheres can be prepared by several different methods, emulsion solvent evaporation, emulsion solvent extraction, interfacial deposition, spray drying and a melting method. Solvent evaporation is the most common where the polymer is dissolved in a volatile organic solvent (e.g. dichloromethane). The drug to be incorporated is either dissolved or suspended in the polymer solution and the resulting mixture is emulsified in an aqueous phase containing an emulsifier. Evaporation of the solvent occurs during stirring, forming solid drug-loaded microparticles, either at atmospheric pressure, or at reduced pressure and at various temperatures. The resultant particle size depends on the stirring rate (Jefferey *et al.*, 1991) stability of the emulsion and molecular weight (Mw) of the polymer . Crystalline polymers give a decreased loading as on crystallisation the internal aqueous droplets are rejected from the polymer matrix. High molecular weight polymers form porous particles due to the coarseness and instability of the primary emulsion due to increased viscosity. Therefore to optimise loading and sphere properties, crystalline high molecular weight polymers are best avoided (Schugens *et al.*, 1994). Problems associated with microsphere preparation include particle aggregation and the loss of drug to the aqueous phase and the solvent must not be miscible in water (e.g. DMSO and acetone) as the rapid solvent exchange results in the formation of irregular particles. The standard oil in water (O/W) emulsion is the most suitable method for drugs which are insoluble in the aqueous medium. Loss of hydrophilic drugs can be minimised by, saturating the aqueous phase with the drug (Jalil and Nixon, 1989), inverting the emulsification phase O/W to W/O (Jalil and Nixon, 1989), production of a double emulsion e.g. W/O/W (Ogawa *et al.*, 1988)

or multiple emulsion W/O/W/O (Iwata and M^{c}Ginity, 1993). A high loading of a hydrophilic drug can only be achieved with a stable primary emulsion (Nihant *et al.*, 1994).

The aim of this chapter was to entrap ODNs into polymer microspheres as efficiently as possible, to enable a high loading of the ODN per mg of polymer and minimise ODN loss during the preparation. A single W/O emulsion was not tried due to the expected poor entrappment of water soluble drugs. Saturation of the aqueous phase with ODN was too expensive to try. The other two approaches to maximise the loading of ODNs into polymer matrices were tried; O/W emulsion and a W/O/W emulsion.

PLGA 50:50 of molecular weight 3,000 and 30,000 were chosen as polymers for the formulation of ODN-loaded micropsheres. These copolymers have comparatively short biological half-lives in the range of a few weeks to months (see section 1.10.1), thus enabling all the entrapped ODN to be released. Their amorphous nature enables better sustained release compared to crystalline polymer microspheres and the low molecular weight used are thought to result in more efficient loading (Schugens *et al.*, 1994).

Characterisation of the release profiles and solvent retention of the resulting ODN loaded microspheres was important to confirm the success of the various formulations.

4.2 PREPARATION OF OLIGODEOXYNUCLEOTIDE LOADED MICROSPHERES

4.2.1 Single Emulsion Micropsheres

The first method was based on a non aqueous external phase system, adapted from Jalil and Nixon (1989) to produce single emulsion micropsheres.

A 50µl aliquot of radiolabelled ODN in water was mixed with unlabelled ODN to give a final volume of 100µl of known ODN concentration (approximately 25nmoles of a 20mer ODN). The procedure for radiolabelling of ODNs is outlined in section 2.4. The ODN solution (100µl) was added to 500mg of polymer

dissolved in 5ml of acetonitrile and vortexed with 50ml light liquid paraffin containing 2% span 40 as the continuos phase, for 5 minutes and then stirred at 1,000rpm (the revolutions were checked by a hand held digital tachometer) at room temperature using Heidolph stirrers for 3 hours to allow evaporation of the solvent and sphere formation. The microspheres were collected by vacuum filtration and washed four times with 150ml of petroleum ether to remove traces of paraffin and stored under vacuum.

Despite washing the spheres thoroughly in ether, they remained slightly greasy and were difficult to resuspend in PBS.

4.2.2 Double Emulsion Microspheres

The use of a double emulsion procedure is a popular method for encapsulating water soluble drugs into polymer microspheres. The method described was adapted from Splenlehauer *et al.*, 1989. A small volume of aqueous internal phase was used to minimise the resulting particle size (Jefferey *et al.*, 1993).

4.2.2.1 - A 100µl aliquot of aqueous ODN solution (prepared as in section 4.2.1) was added to 500mg of polymer dissolved in 5ml of dichloromethane and vortexed for 5 minutes. This primary emulsion was added to 80ml of aqueous external phase at 4°C (saline 0.9%w/v, methylcellulose 0.05%w/v and PVA 4%w/v [87-89% hydrolyzed, Molecular weight 13,000-23,000]) and stirred at 1,000 rpm at room temperature using Heidolph stirrers for 3 hours to allow evaporation of the dichloromethane. The resulting spheres were centrifuged at 4,000 rpm for 10 minutes (43124-708 rotor, 3,000g, Mistral 3,000 I centrifuge, MSE Leicester Ltd), and washed three times with distilled water to remove any emulsifier and non encapsulated ODN, the supernatants were discarded and the polymer pellet was resuspended in distilled water at each washing step. The microsphere pellet was frozen at -20°C and freeze dried overnight (Edwards/Modulyo, BOC Ltd, Sussex, UK).

The above basic method appeared promising in preliminary experiments, however various parameters were changed to see if further optimisation of ODN loading could be achieved. An emulsifier was added to the primary emulsion (PVA 4%) as

Nihant *et al.*, (1994) report that a high loading of a hydrophilic drug requires a stable primary emulsion. The concentration of NaCl in the external aqueous phase was increased, with the expectation that saturation of the external phase would increase the percentage of ODN loading in the polymer microspheres.

Increasing the salt concentration from 0.9 to 6.6% w/v in the external phase had little effect on PO ODN 20 *tat* loading in the polymer spheres (Fig. 4.1.). The salt concentration was not further increased to obtain a saturated external phase, as the presence of increased amounts of NaCl caused the stirring blades to rust, producing an inelegant product.

ODN loading was increased to approximately 65% (Fig.4.1) when 4% PVA was added to the primary emulsion, therefore increasing the stability of the primary emulsion and enabling an increased loading efficiency (Schugens *et al.*, 1994). A further increase in PVA in the primary emulsion did not further enhance the ODN loading in the polymer spheres.

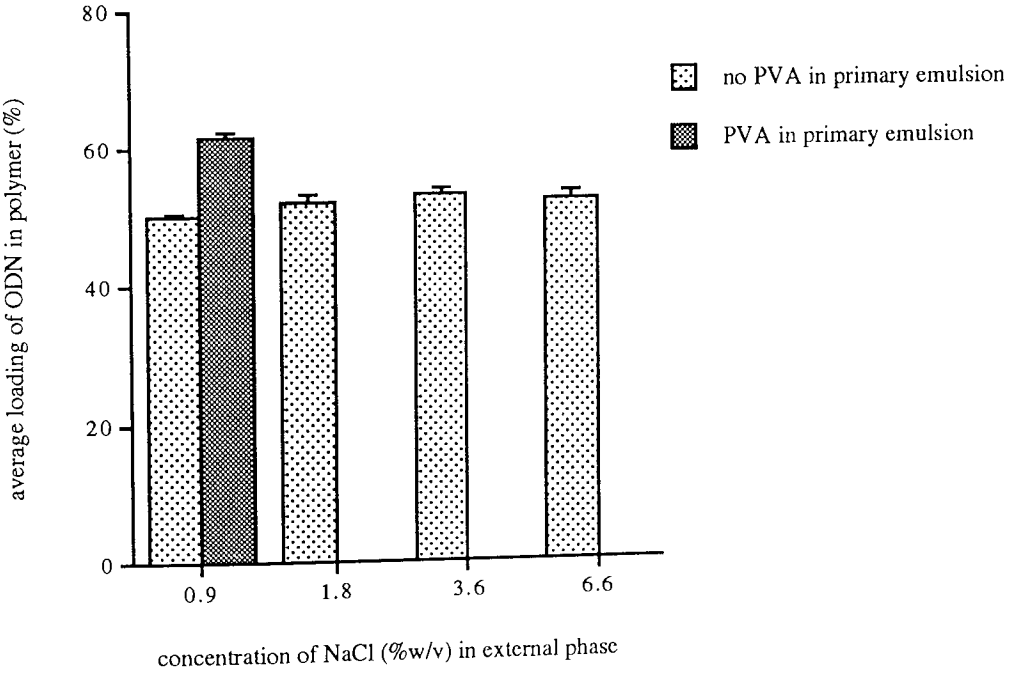


Fig.4.1 Effect of increasing concentration of NaCl on PO 20mer ODN loading in PLGA microspheres (Mw 3,000). The effect of addition of PVA to the primary emulsion is shown with a NaCl concentration of 0.9 %w/v. n = 4 ± S.D.

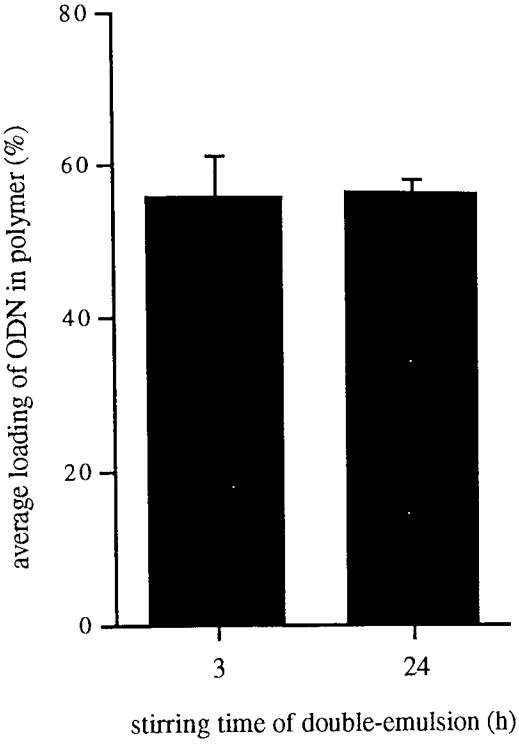


Fig.4.2 Effect of stirring time on loading of PO 20mer ODN into PLGA microspheres (Mw 30,000). n=3 ± S.D.

Stirring times between 3 and 24 hours did not effect the percentage loading as shown in fig.4.2. However short stirring times were chosen to reduce the contact time of the ODN with an aqueous medium to minimise the possibility of degradation or release of the ODN from the polymer surface.

A revised double emulsion method (described in section 4.2.2.4. and diagramatically in Fig.4.3) was used for all further microsphere batches.

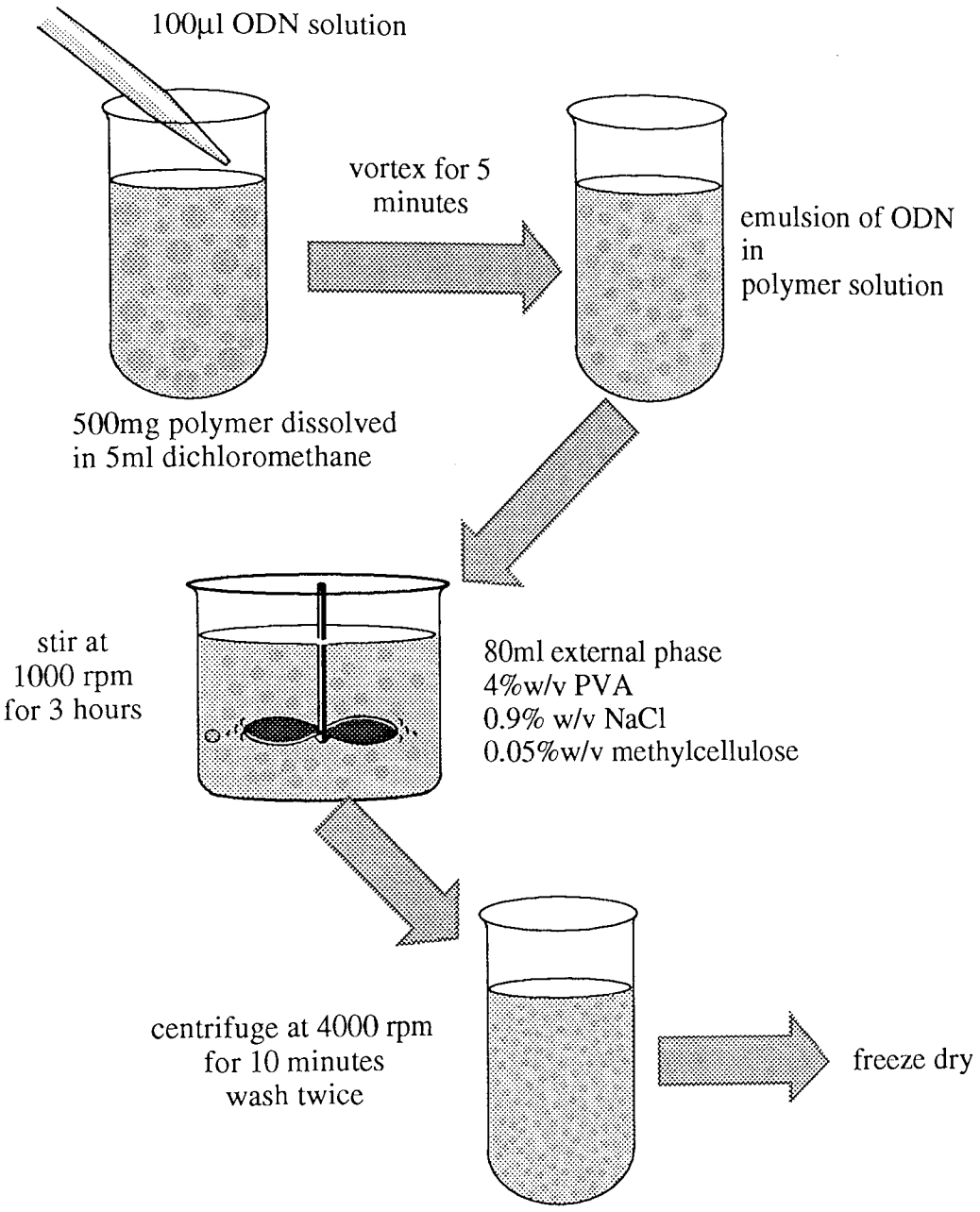


Fig 4.3 Schematic diagram of double-emulsion microsphere preparation

4.2.2.2 - An aliquot (100µl) of aqueous ODN solution was prepared as in section 4.2.1 (with the addition of 0.4% w/v PVA), was added to 500mg of polymer dissolved in 5ml of dichloromethane and vortexed for 5 minutes. This primary emulsion was added to 80ml of aqueous external phase at 4°C (saline 0.9%w/v, methylcellulose 0.05%w/v and PVA 4%w/v) and stirred at 1,000 rpm at room temperature using Heidolph stirrers for 3 hours to allow evaporation of the dichloromethane. The resulting spheres were centrifuged at 4,000rpm for 10 minutes using a Starstedt LCI centrifuge, and washed three times with distilled

water to remove any emulsifier and non loaded ODN. The pelleted spheres were washed and freeze dried as before.

An attempt was made to produce spheres of a smaller size, in order to characterise the effect of loading and release of ODNs from different sized spheres. Both increased solvent volume and stirring rate are known to decrease the final sphere size (Sansdrap and Moës, 1993) and these methods were attempted to produce smaller 1μm particles.

4.2.2.3 - The volume of dichloromethane used in section 4.2.2.2 was increased to 10ml and the volume of the aqueous external phase was increased to 160ml. The rest of the preparation procedure remained unchanged. The spheres were centrifuged for 20 minutes at 4,000rpm (to ensure capture of the smaller spheres), and were washed and freeze dried as before.

4.2.2.4 - The volumes were kept the same as in section 4.2.2.2., but a different mixing method was employed. The primary emulsion was mixed using a Silverson Homogeniser STD 2 (Silverson Machines, Chesham, UK) at high speed (16,000rpm) for 2 minutes, with a 3/8" mini micro probe. The double emulsion was mixed for 5 minutes using a 1" tubular probe (at 16,000rpm), prior to stirring at 1000rpm for 3 hours to allow evaporation of the dichloromethane (as in section 4.2.2.2). The spheres were centrifuged and dried as before.

The resulting dry spheres were ground in a pestle and mortar to a fine powder and stored in a dessicator at 4°C. Any static charge on the surface of the microspheres was reduced using a hand held anti-stat gun (zero-stat). All the microsphere preparations were stored at 4°C in a dessicator to prevent degradation of the polymer. Storage at elevated temperatures and humidity can alter the drug release characteristics (Aso *et al.*, 1993).

4.3 CHARACTERISATION OF THE MICROSPHERES

The microsphere preparations were characterised to assess batch to batch reproducibility. Sphere size, surface charge, solvent retention and ODN loading were determined as these characteristics may greatly influence cellular association, ODN release, as well as potential toxicity of the particulate preparation (Tabata and Ikada, 1990 a; Alonso *et al.*, 1993)

4.3.1 Scanning Electron Microscopy

The spheres samples were visualised by scanning electron microscopy as described in section 2.8.1. Microscopic evaluation of the sphere morphology would enable differences in the preparative methods to be highlighted.

The SEM photograph in Fig.4.4.A shows the spheres produced in section 4.2.2.2 from a double emulsion method, the spheres produced are homogenous with smooth non porous surfaces with a good yield of spherical particles in a narrow size range. Little to no polymer debri is present in the freshly made batches, indicating that all the polymer has been incorporated into the spheres. Spheres produced from PLGA 50:50 molecular weight 30,000 and 3,000 had an identical morphology as seen by SEM. Any dents or pitting at the sphere surface are a probable consequence of collision between particles during the mixing process, before the spheres have fully hardened. Method 4.2.2.4 produced similarly homogenous spheres (Fig.4.4.B) with smooth non porous surfaces but reduced in size compared to those in Fig.4.4.A. Aggregation of the spheres may be due to residual solvent present at the sphere surface making them tacky.

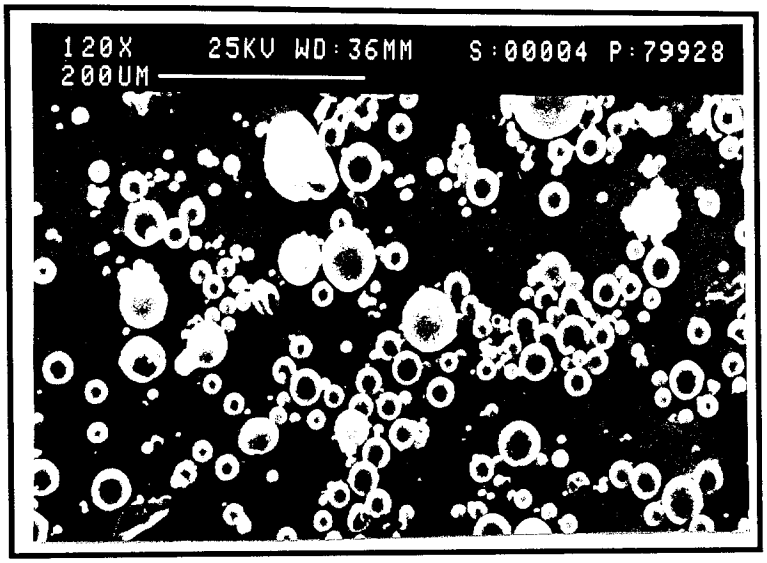


Fig 4.4.A Double emulsion spheres PLGA Mw 3,000, made as in section 4.2.2.2.

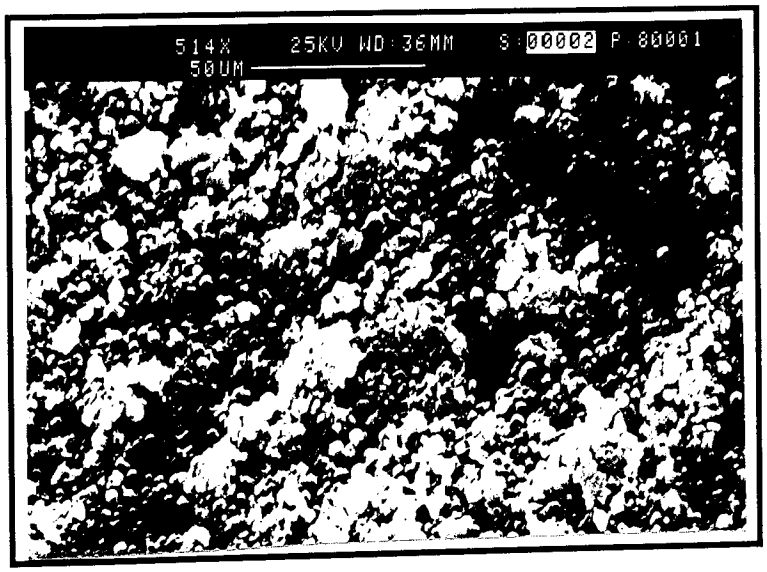


Fig. 4.4.B Double emulsion spheres PLGA Mw 3,000, made as in section 4.2.2.4.

4.3.2 Particle Size

The particle size of the spheres produced was determined by using a Malvern Mastersizer as described in section 2.8.2. Particle size analysis is important in the prediction of release profiles of the entrapped drug, with a greater release rate expected from smaller spheres (Bhardwaj *et al.*, 1995). Cellular uptake of micropsheres is also dependent on microsphere size (Tabata and Ikada, 1988 a).

The results of the particle size distribution studies for the different sphere preparative methods are detailed in fig.4.5 A-D. The sphere size is quoted in diameter (μm), with the most common value (modal) being quoted in the discussion.

The polymer molecular weight did not affect the resulting size range, with both PLGA molecular weight 3,000 (fig.4.5.A) and 30,000 (fig.4.5.B) giving modal values for the sphere diameter of 15.04μm. These spheres made as in section 4.2.2.2. are referred to as in the size range of 10-20μm for further experiments. Increasing the solvent volume (fig. 4.5.C) did reduce the particle size with a modal value of 12.21μm, but produced an increase in the microsphere size distribution. Increasing the stirring rate of the primary emulsion (fig.4.5.D) produced smaller spheres with a modal value for sphere diameter being 1.51μm with a narrower size distribution. These spheres made as in section 4.2.2.4 are referred to as in the size range of 1-2μm in all further experiments. A method which produces a narrow size distribution is the ideal procedure for the characterisation of ODN loading and release, however a combination of sizes may be employed to tailor the release profiles to the levels required. As a result methods 4.2.2.2 and 4.2.2.4 were employed to produce spheres in the range of 10-20μm and 1-2 μm respectively.

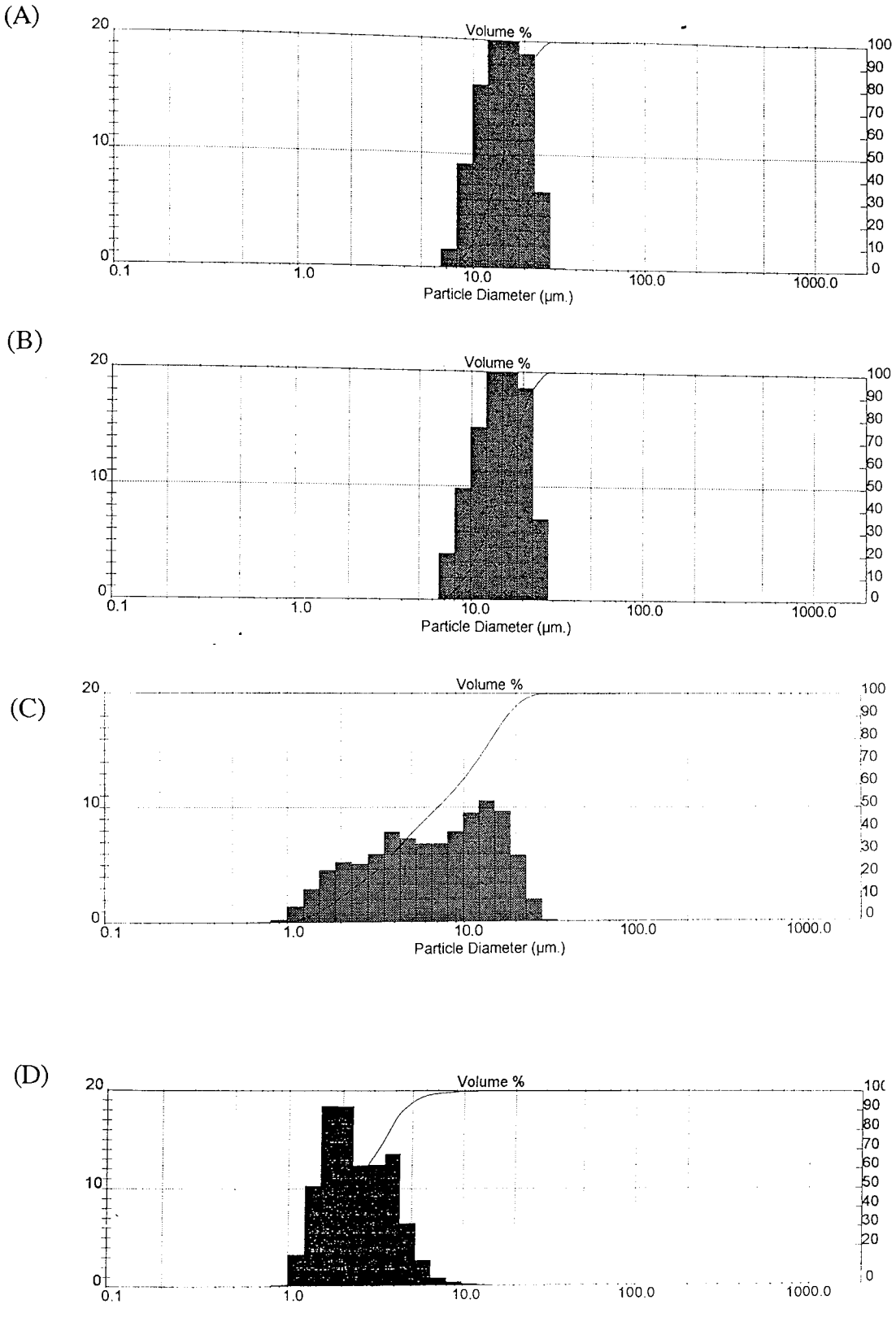


Fig. 4.5 Particle size distribution determined by a Malvern Mastersizer for PLGA double emulsion spheres (A) Mw 3,000 (4.2.2.2.) (B) Mw 30,000 (4.2.2.2.), (C) Mw 3,000 intermediate (4.2.2.3) (D) Mw 3,000 small (4.2.2.4.)

4.3.3 Zeta Potential

The zeta potential of the spheres can be used to give an indication of the surface charge on the spheres. Surface charge can be used to predict the capture of the spheres by the reticular endothelial system (RES), increasing the degree of surface charge (either positively or negatively) results in an enhanced cell uptake (Tabata and Ikada, 1988 a). It is thought that the negative charge of the macrophage membrane and the presence of divalent cations (Mg^{2+} and Ca^{2+}) in the culture media are closely related to the dependence of phagocytosis on the zeta potential of the microspheres.

The zeta potential for spheres of different polymer molecular weight, ODN loading and size was assessed to determine if these differences in formulation would effect cellular uptake by RES cells. If the loaded ODN is concentrated at the surface of the spheres, this may alter the sphere surface charge, and so increasing the sphere loading may alter the surface properties of the spheres. The zeta potential of the sphere preparations was determined as in section 2.8.3.

Table 4.1. lists the zeta potentials of micropsheres of different sizes, polymer molecular weight and ODN loading produced by methods 4.2.2.2. and 4.2.2.4. Sphere size (large 10-20μm and small 1-2μm), polymer molecular weight (PLGA 3,000 and 30,000) and ODN loading (1-8μg/mg polymer) did not appear to have a significant effect on the surface charge of the spheres. Reference values of zeta potentials of spheres 1.5μm in diameter (Tabata and Ikada, 1988 a) are listed in table 4.3. for comparison.

Therefore it can be concluded that the sphere size (1-2μm and 10-20μm), polymer molecular weight (3,000 and 30,000) and ODN loading (1-8μg/mg polymer) did not affect the sphere surface charge, and therefore differences in surface charge would not be responsible for any differences in cellular uptake of the different types of microsphere produced.

Sample	Zeta potential (mV)
large (Mw 30,000)	1.8 ± 0.2
small (Mw 3,000)	2.3 ± 0.4
small (Mw 30,000)	1.8 ± 0.1
large (Mw 3,000) 1μg ODN/mg polymer	1.6 ± 0.1
large (Mw 3,000) 2μg ODN/mg polymer	2.8 ± 0.5
large (Mw 3,000) 8μg ODN/mg polymer	2.7 ± 1.2

Table 4.1 Summary of the effect of particle size, polymer Mw, and ODN loading on the zeta potential of the microspheres. n = 3 ± standard deviation

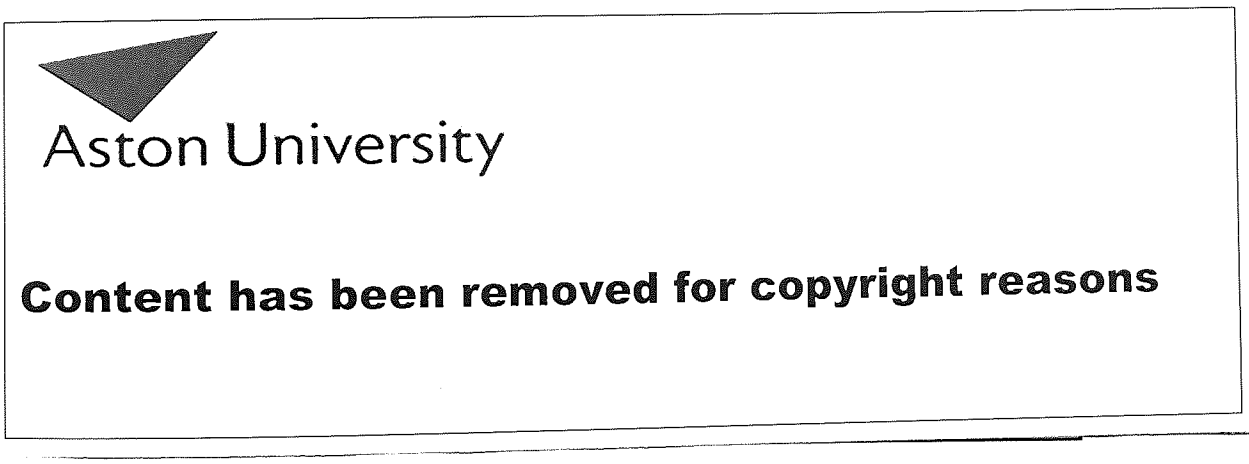


Table 4.2 Zeta potential references values for 1.5μm cellulose and surface modified spheres (Tabata and Ikada, 1988).

4.3.4. Solvent Retention

Chlorinated solvents and surfactants are an important part of microsphere preparation which rely on the emulsification of aqueous and organic phases followed by solvent removal. Quantification of these contaminants is important, in order to identify the toxicity of the preparations. Dichloromethane is toxic, it is partially metabolized to carbon dioxide and carbon monoxide, increasing the blood concentration of carboxyheamoglobin. Small amounts of unchanged dichloromethane are exhaled and excreted in the urine (Martindale, Edition 29). Solvent retention was determined by gas chromatography, outlined as in section 2.8.9.

Solvent	Retention Time (min) (major peak)
dichloromethane	10
chloroform	30

Table 4.3 GC retention times of chloroform and dichloromethane. Experimental conditions:- injection temperature = 70°C, column temperature = 60°C, detector temperature = 120°C, detector sensitivity = 8x 10^2, hydrogen flow = 1.0 kg/cm^2, air flow = 0.5kg/cm^2 and nitrogen flow = 0.5 kg/cm^2.

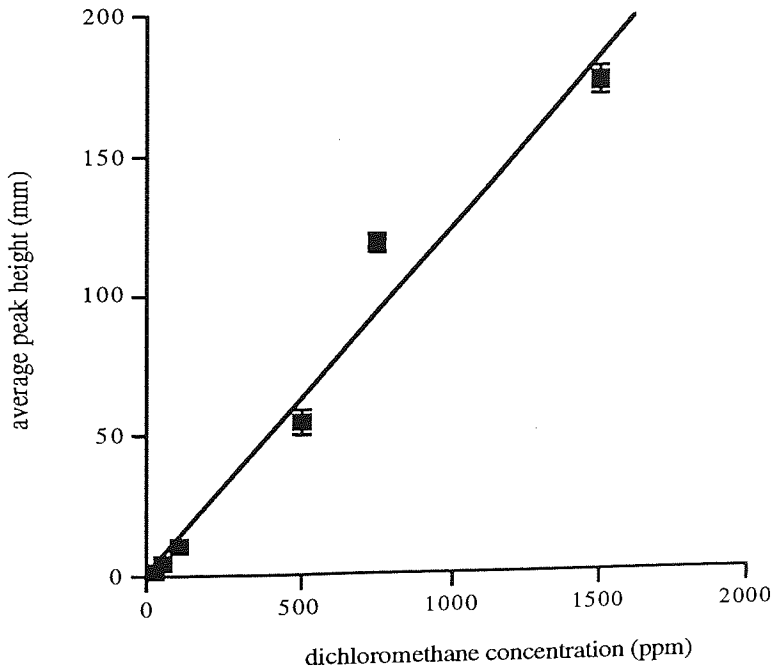


Fig.4.6 Calibration graph of peak height against dichloromethane concentration (parts/1000). n= 3 ± S.D. Equation of the line y = 0.123x + 1.382 Correlation coefficient r = 0.984.

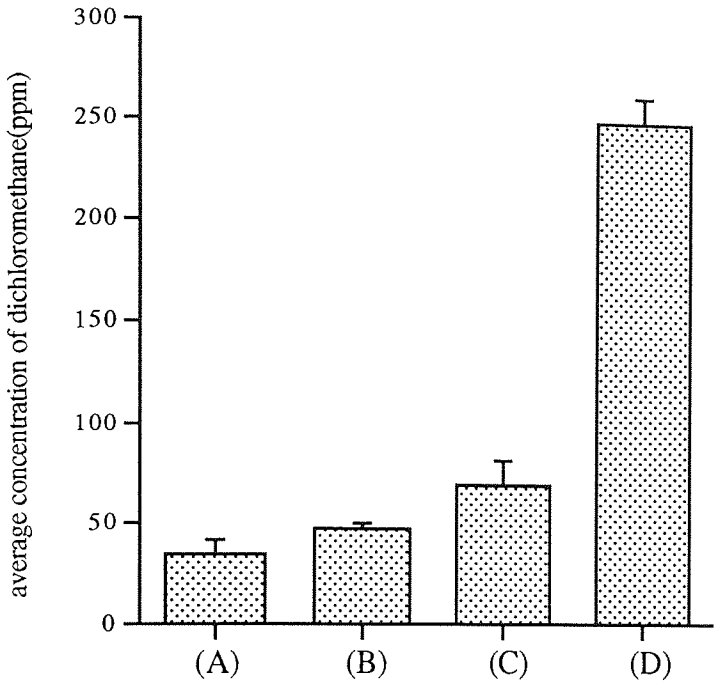


Fig.4.7 Dichloromethane retention in PLGA microspheres (ppm)
(A) = 1-2μm (3,000), (B) = 10-20μm (Mw 3,000), (C) = 10-20μm (Mw 30,000), (D) = PLA film (Mw 690,000). n = 3 ± S.D.

The calibration graph of dichloromethane concentration in Fig.4.6 was linear over the range of 0 to 2000ppm and can therefore be used to calculate the dichloromethane retention in the spheres. The solvent retention in the spheres ranged from 35ppm for the small spheres increasing to 70ppm for the larger spheres. An increase in polymer molecular weight and sphere size results in an increased solvent retention as shown in Fig.4.7. The solvent retention was similar in freshly prepared spheres and spheres which had been stored in a desssicator for various time periods. The greatest solvent retention is seen for the PLA films (shown as a comparison with chloroform as the solvent) with approximately 5 times greater concentration of solvent compared to the small microspheres. The increased solvent retention is possibly due to the reduced surface area per mass of the polymer film compared to the microspheres. The solvent retention results obtained from the double-emulsion preparations described in this chapter are similar to Lupron Depot (19μm sphere preparation) with a dichloromethane retention of 27ppm. Solvent retention increased for larger spheres e.g. Decapeptyl depot (50μm sphere preparation) had a concentration of 480ppm (Kamei *et al.*, 1995). Martindale lists

the recommended exposure limits of dichloromethane as being 100ppm (long term) and 250ppm (short term) in the UK and in the USA levels of 500ppm are permissible and 75ppm are recommended. Therefore from this information the levels of solvent retention are within acceptable levels, however complete removal of the solvent would be advantageous to avoid the problems if long-term treatment with these polymeric devices was necessary.

4.3.5 Residual PVA Content of Spheres

Polyvinyl alcohol is generally considered safe and non-toxic (Martindale, 29) and is used in ophthalmic preparations. However any contamination of the final product with PVA will influence the final sphere surface characteristics. The presence of surfactants on the sphere surface has the potential to make them more hydrophilic, which can result in reduced cellular uptake of the spheres by macrophages (Müller and Wallis 1993). The assay method (Allémann *et al.*, 1993) used is detailed in section 2.8.10 and is based on the colour complex formed between PVA and iodine in the presence of boric acid.

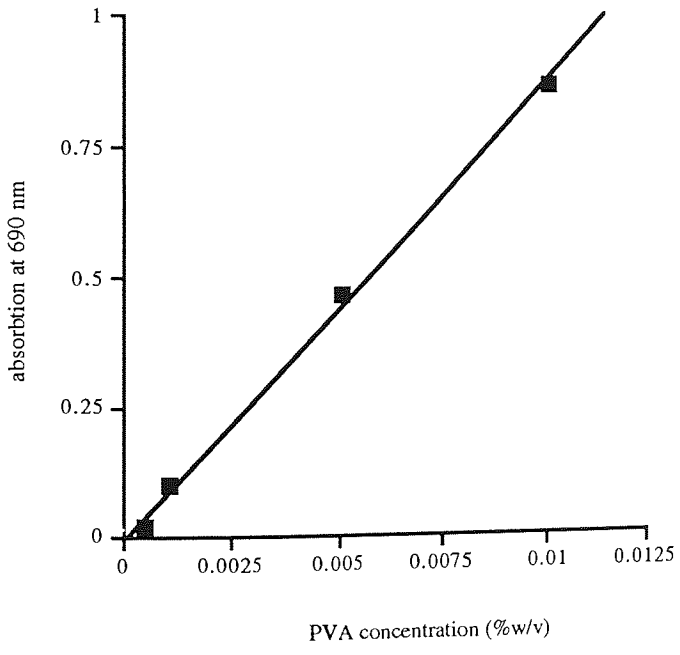


Fig.4.8 Calibration graph of PVA concentration (%w/v) against absorbance @ 690nm of the PVA/iodine complex. Equation of the line y = 88.286x - 0.003 correlation coefficient r = 0.998.

The calibration graph (fig.4.8) of PVA concentration against absorbance at 690nm is linear over the range of 0.001 to 0.01%w/v PVA, enabling the residual PVA concentration to be determined. The results of the PVA assay are listed in Table 4.5, with only traces of PVA remaining. The microsphere size or polymer molecular weight did not appear to effect the amount of PVA retained at approximately 0.16%w/w. Microspheres washed only once have approximately double the amount of residual PVA, this emphasises the importance of a standardised washing procedure to remove all the surface associated PVA.

Polymer Mw PLGA	Microsphere size (μm)	Residual PVA (%w/w)
3,000	1-2	0.158±0.0025
3,000	10-20	0.167±0.0052
3,000	1-2 (washed once)	0.362±0.0034
30,000	1-2	0.162±0.0044
30,000	10-20	0.167±0.0043
30,000	10-20 (washed once)	0.242±0.0051

Table 4.4 The effect of polymer Mw, microsphere size and washing on content of residual PVA (%w/w of microsphere) in PLGA micropsheres. n = 3 ± S.D.

4.4 OLIGODEOXYNUCLEOTIDE LOADING IN MICROSPHERES

The production yields and ODN loading are also an important aspect of microsphere production due to the expense of the ODNs. The results of each preparative method are summarized in Table 4.5.

	Yield (%)	Loading (%)	Size range (μm)
Method 4.2.2.(A)	95.1 ± 2	51.75 ± 2.2	10-20
Method 4.2.2.(B)	94.4 ± 1.9	66.8 ± 3.2	10-20
Method 4.2.2.(C)	42.2 ± 4.2	12.3 ± 1.4	1-10
Method 4.2.2.(D)	50.3 ± 2.8	26.9 ± 4.3	1-2

Table 4.5. Summary table of the effect of preparation method on microsphere production yields, loading (±S.D.) and size range (mean diameter). Polymer Mw did not effect the yield, loading or size of the microspheres.

The greatest yields and ODN entrapment were produced by the double emulsion method of 4.2.2.2. Decreasing the sphere size caused a reduction in both the batch yield and ODN loading. As a result of the loading and production yield study, the majority of the experiments were based on the double emulsion method of 4.2.2.2. No attempt was made to further enhance the loading in the smaller spheres (4.2.2.4), this is an opportunity for further work.

4.4.1. Determination of Range of Loading

The ODN loading in the polymeric microspheres was determined as in section 2.7.3. Microspheres were made by a double-emulsion method as described in 4.2.2.2, with increasing concentration of PO ODN ([^{35}S] 20 *tat*) added to the polymer solution in a 100μl volume from 0.085μg to 8μg/mg of PLGA (molecular weight 3,000) polymer. Financial constraints determined the maximum concentration for the loading experiments.

PO 3' [^{35}S] labelled 32, 15 and 7 mer *c-myc* ODNs were loaded into PLGA (molecular weight 30,000) microspheres as in 4.2.2.2 to determine the effect of sequence length on loading.

Over the concentration range 0.085 - 8μg/mg polymer, loading of the ODN in the polymer spheres had not been saturated (fig.4.10), with the percentage loading being approximately 65%. These results indicate that the polymer microspheres are

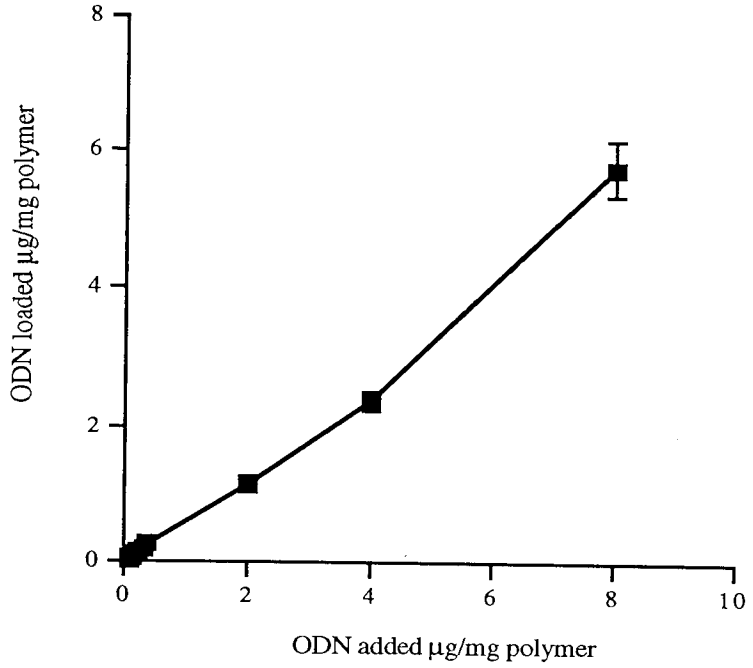


Fig 4.9 The effect of ODN concentration (0.8-8µg/mg polymer) on loading in PLGA microspheres (Mw 3,000). n = 3 ± S.D.

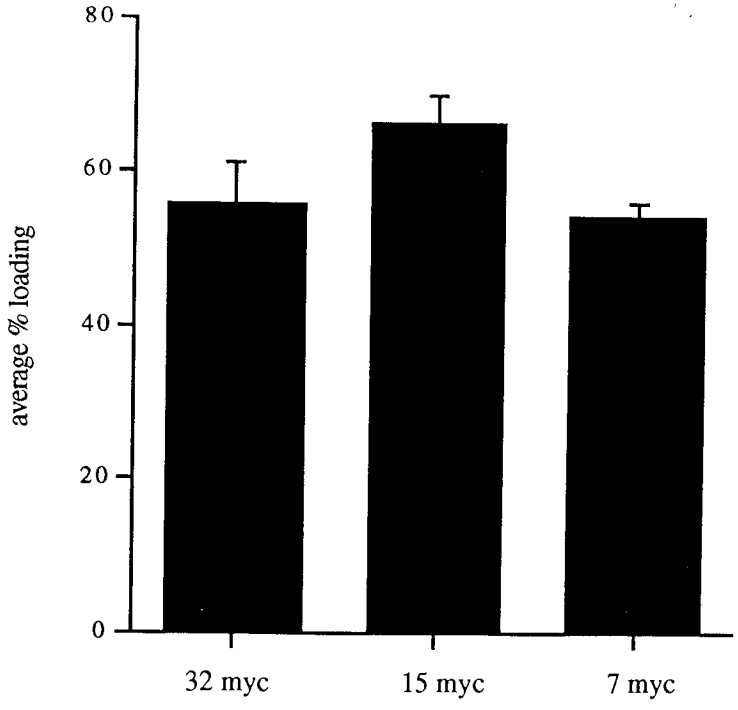


Fig.4.10 Effect of ODN length (7-32 mer) on loading in polymer micropsheres. ODN was added at 100µg/250mg polymer. P(LA:GA) microspheres 10-20µm, Mw 30,000. n = 4 ± S.D.

capable of entrapping high amounts of ODN necessary for sustained delivery over a period of time.

ODN length from 7 to 32 mer, with a corresponding molecular weight of 1895 to 10400, does not effect the percentage loading of the ODN in the polymer microspheres (Fig.4.11). Longer ODN sequences appeared to be equally as efficiently entrapped in the polymer matrix compared to shorter ODNs.

The results suggest that the amount and length of the ODN does not effect the entrapment efficiency of the preparation procedure over the ranges studied, although loading under these conditions has not been saturated. The distribution of the ODNs within the polymer matrix has not been determined, however the release profiles in Chapter Five may give an indication as to the ODN distribution within the polymer.

4.5 CONCLUDING REMARKS

In this chapter formulation methods have been characterised in terms of sphere size, solvent retention and ODN loading. It is hoped that this information can be used to aid predication and understanding of release profiles and cellular uptake of ODN loaded microspheres.

The single emulsion procedure resulted in a poor loading and gave a greasy unacceptable sphere preparation. ODN loaded microspheres of size 10-20μm have been successfully made from a double emulsion procedure from PLGA of molecular weight 3,000 and 30,000. It can also be concluded that increasing the stirring rate as compared to increasing the solvent volume was more successful in producing smaller spheres in a narrow size range. Production yields of approximately 95% were achieved for the larger spheres with an approximate loading of 65%, this relatively high loading and yield is important in the formulation of a delivery device for an expensive product such as antisense ODNs and RBZs. Unfortunately the loading and yields were not as great with the smaller spheres. Loading in the smaller spheres will have to be optimised to enhance their therapeutic potential, which was not investigated as part of this thesis.

Dichloromethane and PVA retention values have not been widely reported in the literature pertaining to similar spheres, however quantification of their presence is an important factor consideration if the spheres are to be used parenterally without

adverse effects. The retention of both dichloromethane and PVA in the polymeric spheres produced in this section was minimal, although further attempts may be necessary to eliminate their retention completely.

The capacity for entrapment of ODNs into the microspheres appears to be high, with the loading not being saturated upto 8μg/mg polymer. The ODN sequence and length did not appear to effect the loading efficiency over a range of 7 to 32mer, it is therefore expected that the 32mer RBZ will have a similar loading efficiency as the 32mer ODN.

Therefore polymeric microspheres have the potential to entrap nucleic acids for a therapeutic application. Their potential for sustained release delivery devices is discussed in Chapter Five.

CHAPTER FIVE

RELEASE OF OLIGODEOXYNUCLEOTIDES FROM POLYMER MICROSPHERES

5.1 INTRODUCTION

Release of the matrix entrapped drug may be as a result of several factors; diffusion from the surface or through pores in the polymer matrix; polymer erosion or degradation; or pulsed release initiated by a magnetic or sonic field. Drug loading and type of polymeric device also have a role in effecting drug release from polymer matrixes. The total release profile may be due to a combination of these factors.

PLGA is known to undergo homogenous bulk erosion (described in section 2.10), and as a result the release of entrapped drugs from the polymeric devices is often multiphasic. Due to the relatively short half-lives of PLGA polymers, their degradation influencing the release of entrapped substances, unlike the release of ODNs and RBZs from high molecular weight solvent cast PLA films described in Chapter Three, where there was no evidence of polymer degradation over a 28 day period. The degradation process and typical release profiles are described in section 1.10. Drug release from the PLGA polymer matrix has a triphasic profile (Sanders *et al.*, 1986; Wang *et al*, 1990), which is typical of bulk eroding controlled release systems. It is characterised by a secondary phase of lower release preceded and followed by phases of higher release. Duration of the secondary phase is proportional to the molecular weight of the copolymer. The three phases of release can be described in relation to the matrix erosion, firstly diffusion of drug from the polymer surface can create a significant burst release due to either surface bound or loosely associated drug. The molecular weight of PLGA 50:50 decreases quickly during the first three days of hydrolysis, creating a porous structure throughout the polymer matrix facilitating drug release. Thus any cracks, pores and malformation of spheres during production will lead to increased rates of drug release. At this stage the molecular weight has not reduced sufficiently and the degradation products were not small enough to be soluble, this is responsible for the second slower phase

of release. The final stage of high release is due to a complex diffusional mechanism from an increasingly permeable matrix and eventual matrix collapse.

It has been widely reported that the polymer composition and fabrication technique closely relate to the drug release (Pavenetto *et al.*, 1993; Sah and Chien, 1993; Sansdrap and Moës, 1993). Therefore the release profiles of ODNs from PLGA microspheres from the different preparative methods were determined as well as the effect of ODN length, loading, sphere size and the release conditions. Therefore it is important to characterise the release profiles for each individual fabrication system.

5.2. RELEASE OF OLIGODEOXYNUCLEOTIDES FROM SINGLE EMULSION MICROSPHERES

The microspheres were made by a single W/O emulsion as described in section 4.2.2.1 and the release experiments were carried out as detailed in section 2.8.4.

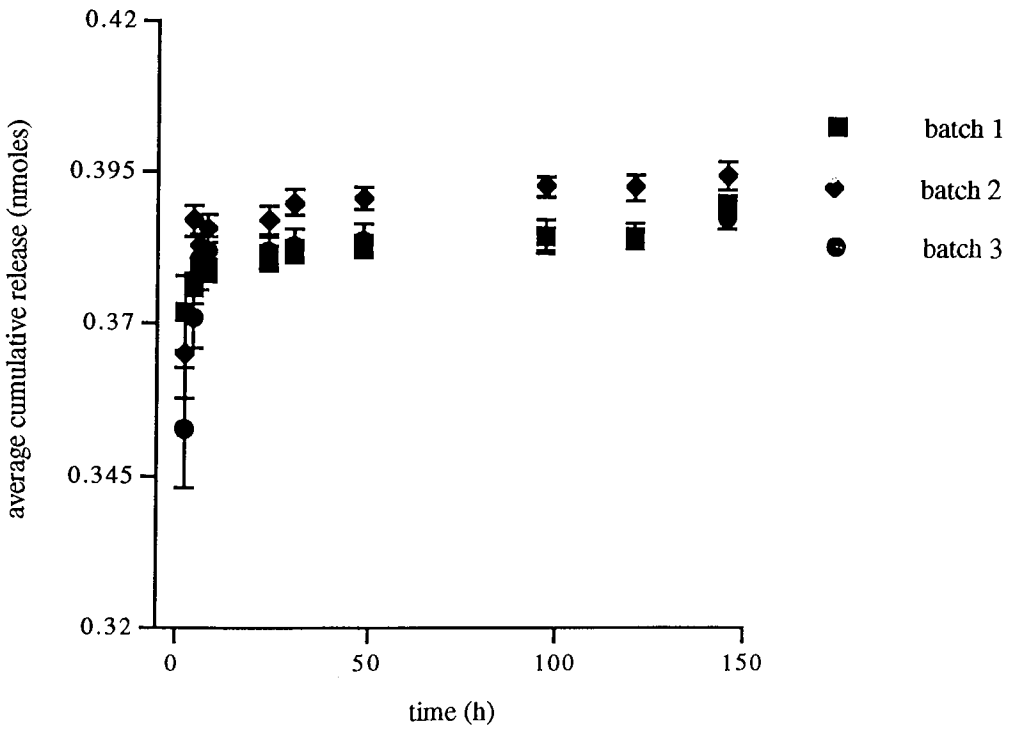


Fig.5.1 Average cumulative release (nmoles) of PO ODN 20 *tat* from PLGA (Mw 3,000) microspheres (25mg) in PBS, pH7.4 at 37°C over a 144 hour release period. Sphere size 10-20μm made from a single emulsion 4.2.2.1. Batch 1, 2 and 3 are identical independent batches. Loading = 0.40 nmoles, n = 4 ± S.D.

The release profile shows that the majority of the ODN was released during the first 24 hours, with a burst release in excess of 90% of the polymer associated 20mer ODN. This release profile indicates that the majority of the ODN is associated with the surface of the spheres, and not encapsulated within the polymer matrix. The spheres did not resuspend easily in PBS, due to their sticky surfaces as a result of the incomplete removal of the oil external phase with hexane or ether washes.

The high burst release, was undesirable for a sustained release product for nucleic acid delivery and other fabrication procedures need to be investigated. The relatively poor ODN entrapment efficiency (30%), the slightly sticky nature of the spheres and the fact that they did not resuspend easily in PBS confirmed the unsatisfactory nature of the preparation. As a result of these findings an aqueous double emulsion method was tried (as described in section 4.2.2).

5.3. RELEASE OF OLIGODEOXYNUCLEOTIDES FROM DOUBLE EMULSION MICROSPHERES

The double emulsion microspheres were made as in section 4.2.2.2 in an attempt to produce free flowing microsphere preparations with enhanced efficiency of ODN loading with more reproducible and sustained release profiles compared to those of the single emulsion microspheres. The release experiments were carried out as in section 2.8.4.

The preparation procedure outlined in section 4.2.2.2. for large 10-20μm PLGA microspheres gives reproducible ODN loading (see section 4.4) resulting in similar release profiles for each microsphere batch, as shown in Fig.5.2. The washing procedure in the sphere preparation aims to remove all the non entrapped ODN associated with the sphere surface and thereby reduce inconsistency in the burst release for batches of similar ODN loading.

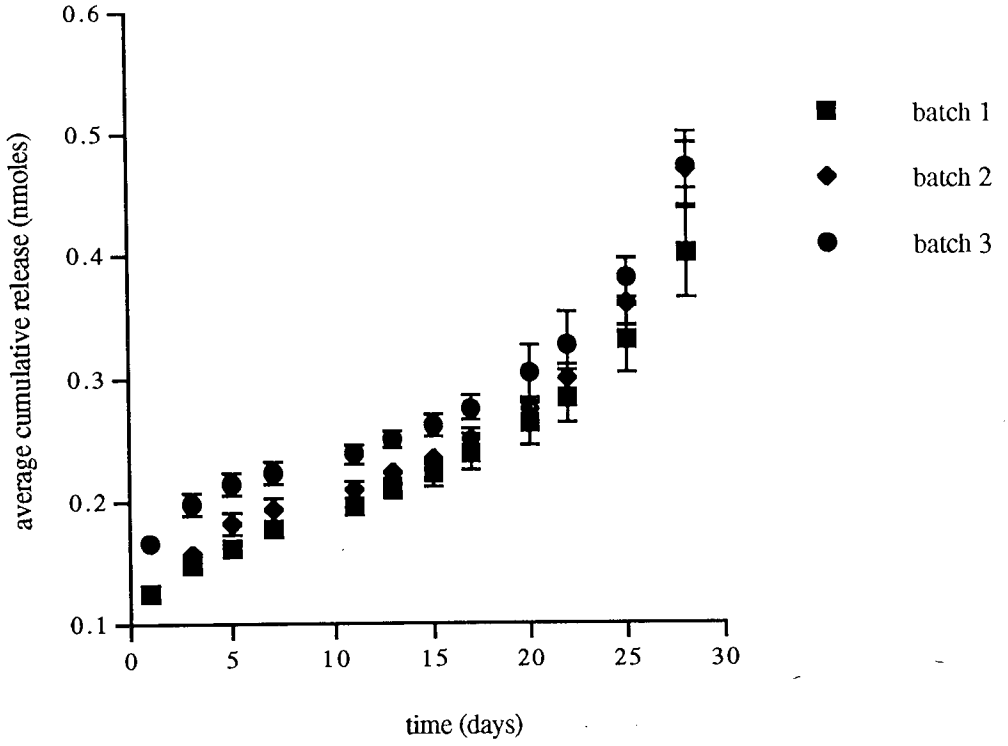


Fig.5.2 Average cumulative release (nmoles) of PO ODN 20 *tat* PLGA (Mw 3,000) microspheres (25mg) in PBS pH 7.4 at 37°C over a 28 day release period. Sphere size 10-20μm, made from a double emulsion (4.2.2.2) Batch 1, 2 and 3 were identical independent batches. Loading = 0.74 nmoles . n = 4 ± S.D.

Increasing the loading of PO ODN 20 *tat* from 50ng/mg polymer to 300ng/mg of polymer in Fig 5.3 results in an increase in the percentage burst release, from approximately 10% to 20% respectively. The increased loading also results in an increased rate of ODN release with time. These findings agree with those of Tsai *et al.*, (1986); Alonso *et al.*, (1993), who report that the loading of water soluble drugs strongly affects the initial burst release and the rate of drug release. The initial burst release corresponds to the release of ODN present at the sphere surface, either entrapped or loosely associated.

Increasing the molecular weight of the polymer in the delivery device reduces the rate of release of the entrapped drug (Kamei *et al.*, 1995; Alonso *et al.*, 1993) as demonstrated in Fig.5.4. The release of PO ODN 20 *tat* 140ng/mg was slightly slower from PLGA matrix of molecular weight 30,000 compared to that of molecular weight 3,000 (Fig.5.4). The difference in the release profiles is only slight, with the greatest effect seen after 25 days incubation in PBS at 37°C, when sphere degradation had occurred. The rate of degradation of PLGA increases with a decrease in the molecular weight, as a result of faster mass loss, and hence faster release (Ogawa *et al.* 1988) where decreasing the polymer molecular weight resulted

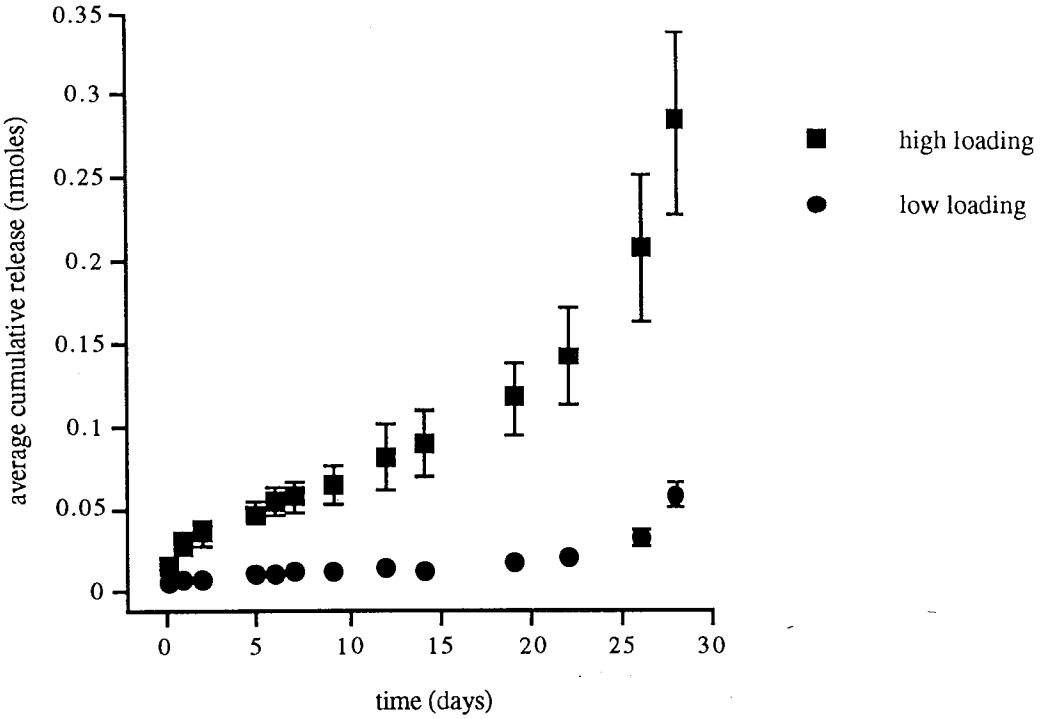


Fig 5.3 Effect of ODN loading on average cumulative release of 20 D *tat* (nmoles) from PLGA (Mw 3,000)microspheres (25mg) in PBS pH 7.4 at 37°C over a 28 day release period. Sphere size 10-20μm, made from a double emulsion (4.2.2.2). High loading= 1.23 nmoles, low loading= 0.20 nmoles. n=4±S.D.

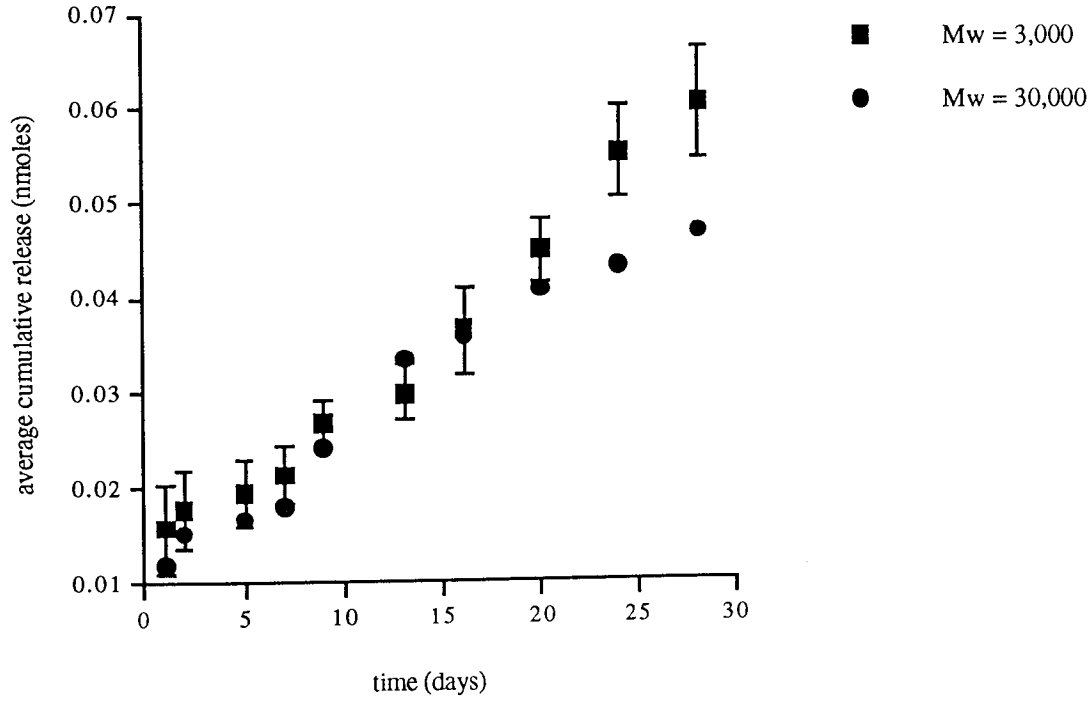


Fig.5.4 Effect of polymer molecular weight on the average cumulative release of PO ODN 20 *tat* from PLGA microspheres (25mg) in PBS pH 7.4 at 37°C over a 28 day release period. Mw 3,000 and 30,000, size 10-20μm, made from a double emulsion (4.2.2.2). Loading = 0.57 nmoles. n = 4 ± S.D.

in an increased rate of release. Increasing the polymer molecular weight can be used to decrease the degree of burst release (Cohen *et al.*, 1991).

Although it has been widely reported that polymer ratio and molecular weight are important in controlling the rate of release of the entrapped drug, these differences may not be significant in effecting the pharmacological effect. For example, two microsphere preparations containing LHRH analogues have similar pharmacological effects despite differences in polymer molecular weight, ratio and sphere size. Lupron incorporates 20μm spheres of PLGA 70:30 and molecular weight 14,000, compared to Decapeptyl which utilises 50μm spheres of PLGA 50:50 and molecular weight 54,000 (Kamei *et al.*, 1995).

ODN length does not have a great effect on the release pattern of ODNs from PLGA microspheres, although Fig.5.5 does shows the longer 32mer ODN of molecular weight 10400 is released marginally faster than a 7mer ODN of molecular weight 1895. The release rate does not appear to be proportional to the ODN length and corresponding molecular weight. The greater release of the 32mer may be as a result of less efficient entrapment in the polymer matrix compared to shorter ODNs.

Fig.5.5 clearly demonstrates the expected triphasic release profile of ODNs from PLGA matrices agreeing with the findings of Wang *et al.*, (1990).The ODN released during the secondary stage of slower more sustained release probably diffuses through pores in the polymer matrix, as a result of polymer hydration before being released. The release rates increase after 25 days incubation in PBS at pH 7.4 and 37°C, corresponding to the increased rate of degradation of the polymer. These results agree with Kamei *et al.*, (1995) who report that the release of LHRH analogues increases 2 weeks after administration as a result of polymer

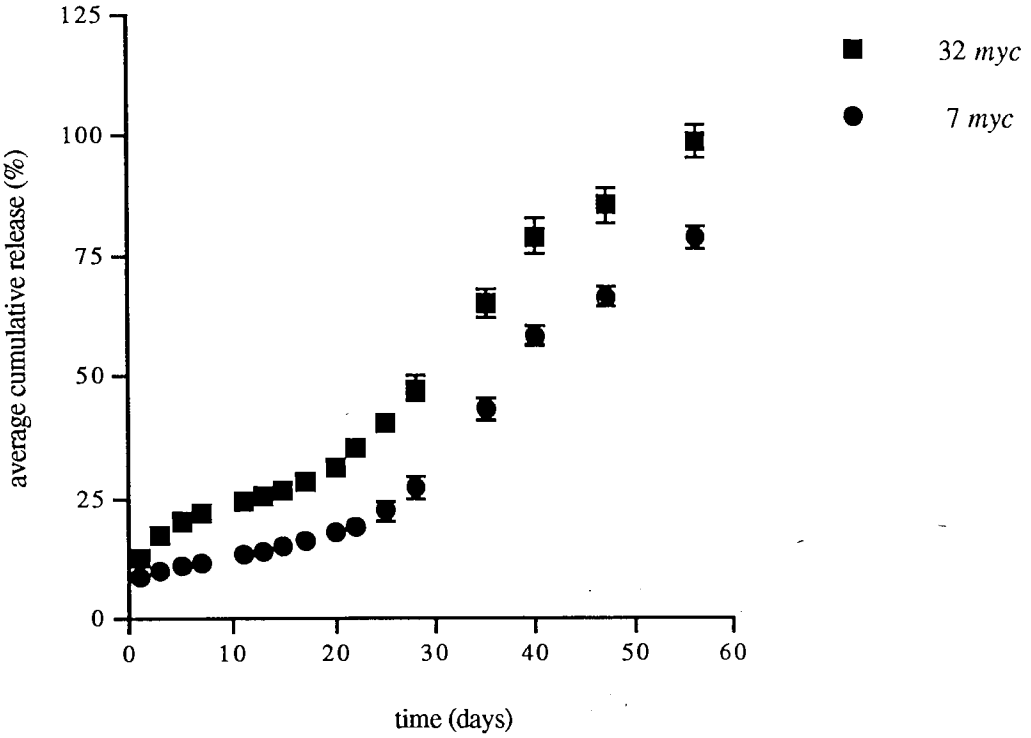


Fig.5.5 Average cumulative release of PO ODN 32 and 7*myc* (%) from (25mg) microspheres in PBS pH 7.4 at 37°C over a 56 day release period. PLGA 50:50, Mw 3,000, size 10-20μm, made from a double emulsion (4.2.2.2). Loading = 110 ng/mg polymer. n = 4 ± S.D.

degradation. Total ODN release coincides with the complete collapse of the polymer matrix at around 56 days incubation in PBS at 37°C.

Scanning electron micrographs (fig.5.6) confirm that degradation of the spheres has occurred. The spheres produced in section 4.2.2.2. produced solid and not hollow spheres as is demonstrated by the SEM in Fig.5.6 A. The broken sphere indicates a porous degraded central core, the amount of polymer debri present in the SEM confirms the degradation to be by bulk erosion, with the spherical solid structure beginning to collapse at around 28 days incubation in PBS at 37°C. The scanning electron micrographs in Fig.5.6 A-D clearly indicate that degradation of the PLGA spheres of both sizes and Mw have degraded in PBS at 37°C after 28 days, this coincides with the increased rate of release seen in the release profiles which occurs at about 25 days (see Fig.5.5). A greater degree of degradation is seen in the 1-2μm spheres of molecular weight 3,000 as compared to the larger 10-20μm spheres of molecular weight 30,000, as expected due to the reduced molecular weight and

particle size, with an increased surface area. The small 1-2μm spheres have aggregated following incubation in PBS to form larger polymeric clumps (fig.5.6 B) loosing the definition of their structure, however some individual spheres are still evident.

The microsphere structure has completely collapsed after 56 days in PBS at 37°C as shown by the SEM in Fig.5.6 C and D, this coincides with the period of complete release of the ODN from the polymer. Less polymer mass is evident in Fig.5.6 C compared to that in 5.6 D, again explained by the fact that the smaller spheres have a greater surface area and hence a faster rate of degradation. After 56 days incubation in PBS at 37°C the remaining polymer (as mass loss had occurred, but was not quantified), once washed and freeze dried was no longer a free flowing powder but remained sticky and difficult to handle, therefore limiting any further analysis at this stage.

Fig. 5.6. A SEM of 10-20μm PLGA 50:50 Mw 30,000 after incubation in PBS for 28 days at 37°C. (Control Fig. 4.4.A)

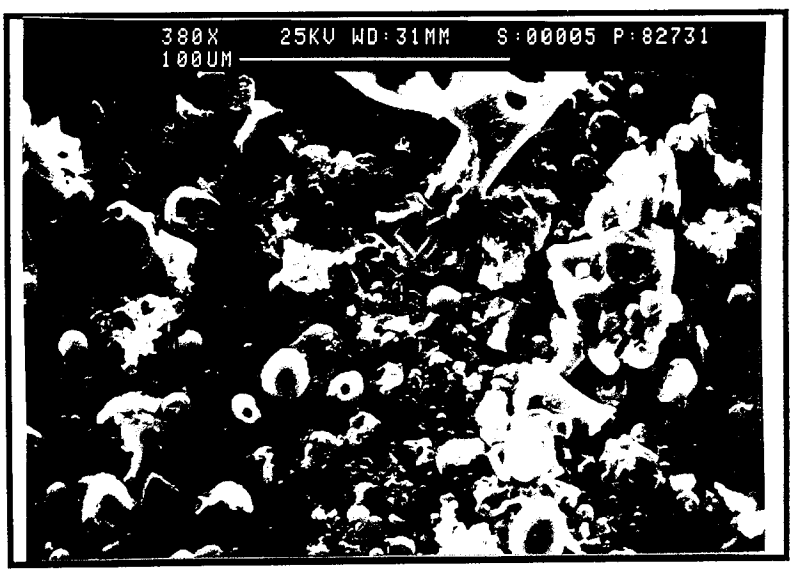


Fig.5.6.B SEM of 1-2μm PLGA 50:50 Mw 3,000 after incubation in PBS for 28 days at 37°C. (Control Fig. 4.4.B)

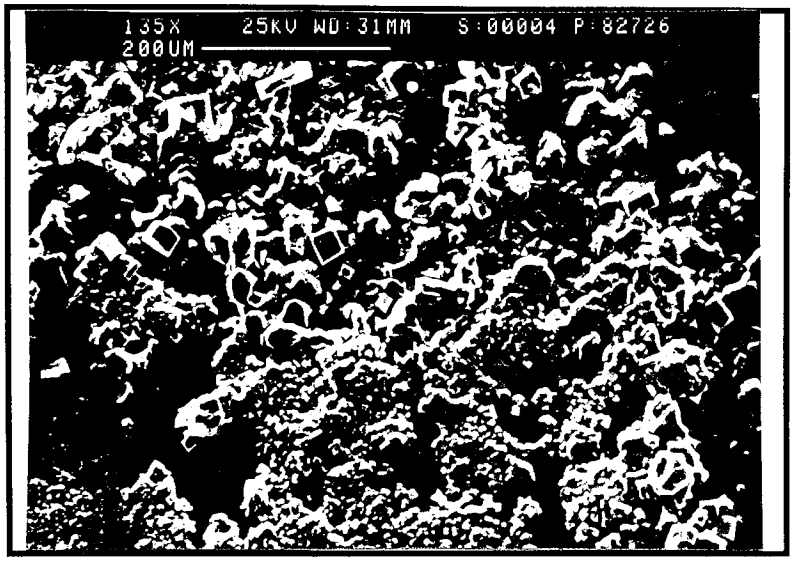


Fig. 5.6.C SEM of 10-20µm PLGA 50:50 Mw 30,000 after incubation in PBS for 56 days at 37°C.

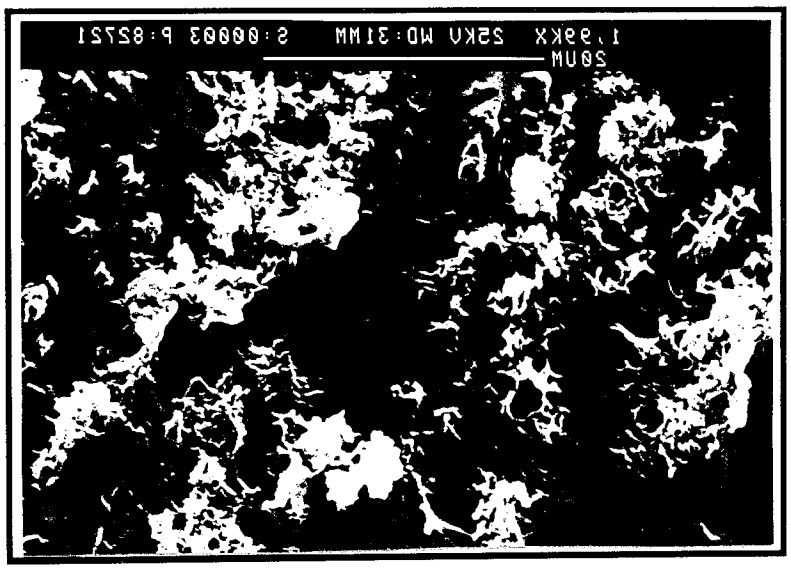


Fig. 5.6.D SEM of 1-2µm PLGA 50:50 Mw 3,000 after incubation in PBS for 56 days at 37°C.

Table 5.1 lists a range of ODN lengths and their resultant burst release and final release, showing no correlation to the length of the entrapped ODN. These results differ from release of ODNs from polymer films (Fig.3.2) where shorter ODNs were released from the polymer matrix at a faster rate compared to longer ODNs. These results could possibly be explained by degradation and water uptake in low molecular weight PLGA microspheres having a greater effect on release than diffusion of the ODN through the surface layers of the polymer matrix. In contrast there is little degradation and water uptake in the crystalline PLA films and release is largely dependent on the diffusion of the ODN from the surface layers of the film. Cortesi *et al.* (1994) report that the release of a 144mer double stranded DNA from gelatin microspheres is slower compared to that of a 44mer ODN. The ODN release was biphasic, with 60% of the entrapped 44 mer ODN released after 120 minutes, followed by a plateau in release. In comparison the use of PLGA as a microsphere matrix has the advantage of a more sustained release profile.

Figure 5.7 indicates that the sphere size has a dominant effect on the release profile of ODNs. The release of ODNs from small spheres (1-2μm) (section 4.2.2.4) is significantly faster compared to release from the larger spheres (10-20μm) (section 4.2.2.2). The dramatic difference in release profiles is as a result of the increased surface area to volume ratio of the small spheres compared to the larger spheres, giving a larger contact with the release media The larger surface area results in a greater concentration of ODN on or near the surface causing an increased burst release. Similar results have been reported that a reduction in the sphere size results in an increased release (Bhardwaj *et al.*, 1995; Narayani and Rao, 1994; Alonso *et al.*, 1993). However complete degradation of the polymer is independent of sphere size (Visscher *et al.*, 1988) and after 56 days both sphere sizes have released 100% of the entrapped ODN, corresponding to collapse of the polymer matrix (fig.5.6)

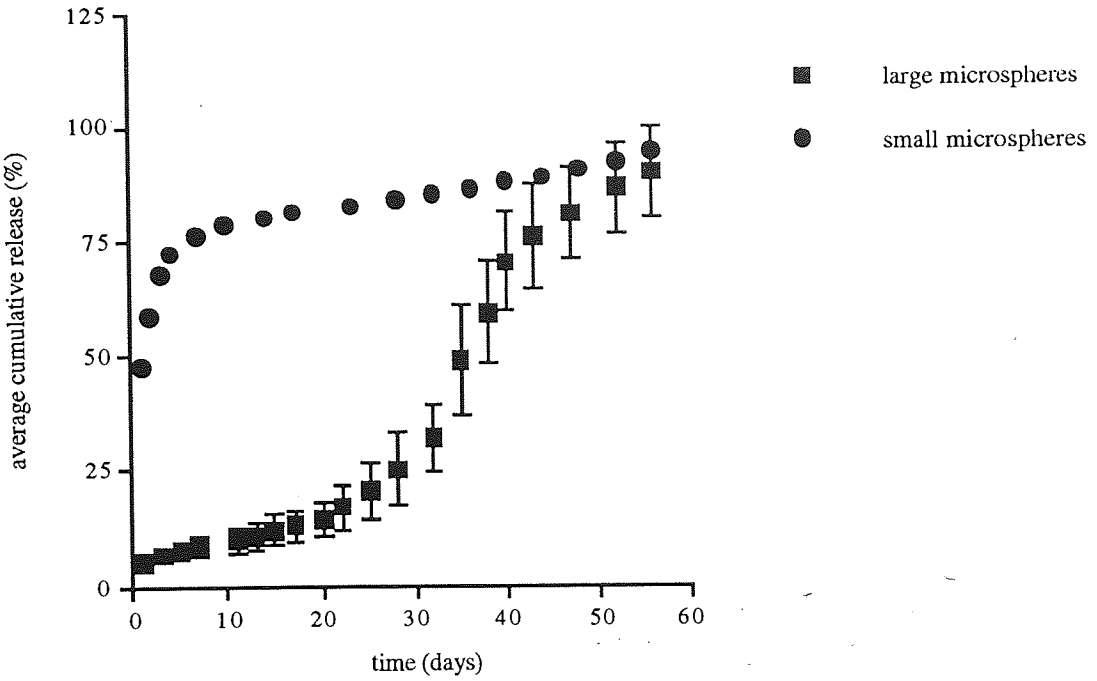


Fig.5.7 Effect of sphere size on the average cumulative release(%) of PO ODN 20 *tat* from PLGA (Mw 3,000) microspheres (25mg) in PBS pH 7.4 at 37°C over a 56 day release period. Small spheres = 1-2μm and large spheres = 10-20μm. Loading = 0.20 nmoles, n = 4 ± S.D.

The large burst release of ODNs from small microspheres may preclude their use in sustained delivery, despite the large initial release the remaining release profile is more consistent than the release profile from the larger spheres. To reduce the initial burst release the microspheres could be coated to provide a barrier for the surface bound ODN to diffuse through. Alternatively double walled microspheres could be prepared following the same principle (Pekarek *et al.*, 1994). Increasing the amount of low molecular weight polymer in a polymer mix will increase the rate of initial burst release (Le Corre *et al.*, 1994), therefore conversely increasing the proportion of higher molecular weight polymer should decrease the initial burst release.

Length	Loading (ng/mg)	Burst (%)	Burst (ng/ 25mg)	Release 28 days (%)	Release 28 days (ng/25mg)
20 D *tat* polymer Molecular weight =3,000	80	7 ± 1.14	5.6	24.8± 1.9	19.8
20 D *tat* polymer Molecular weight = 30,000	80	2.1 ± 0.39	1.68	11.3 ± 3.5	9.1
7 *myc* polymer Molecular weight = 30,000	20	10.5 ± 0.18	0.21	28.2 ± 1.9	5.6
7 *myc* polymer Molecular weight = 30,000	110	11.5 ± 0.34	12.65	32.9 ± 1.4	36.2
15 *myc* polymer Molecular weight = 30,000	110	6.0 ± 0.4	6.6	31.4 ± 3.7	34.54
32 *myc* polymer Molecular weight = 30,000	110	7.36 ± 0.11	8.1	38.3 ± 3.4	42.13

Table 5.1 Summary of the effect of polymer molecular weight and ODN length on release from microspheres 10-20μm. n = 4 ± S.D.

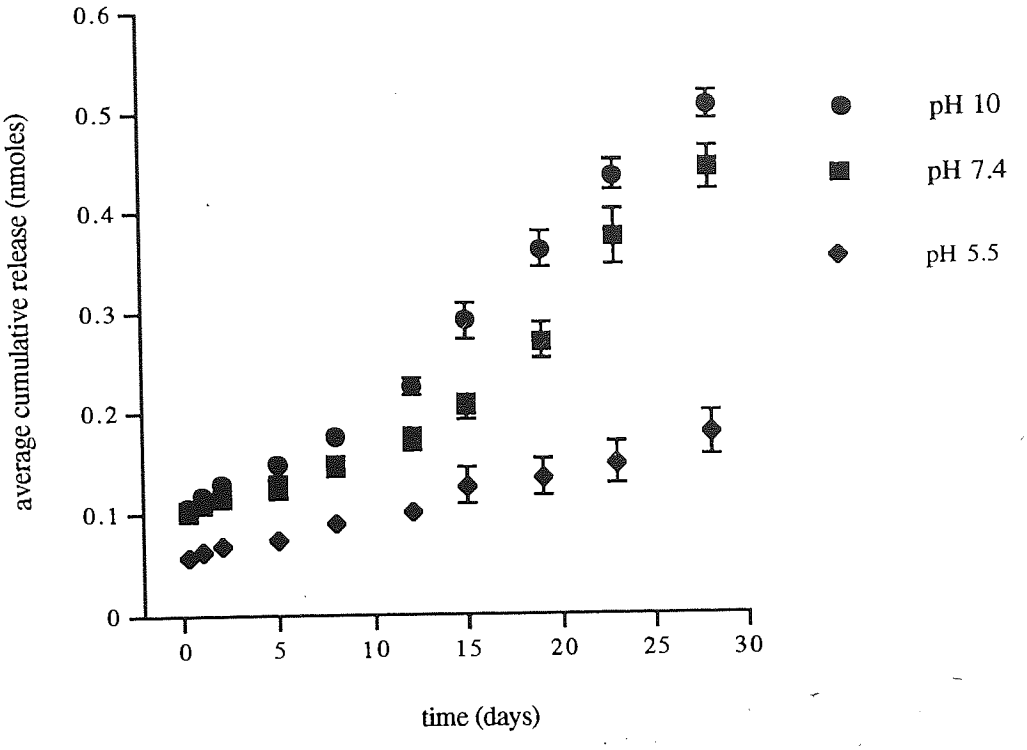


Fig.5.8 Effect of pH of release media on the average cumulative release of PO ODN 20 *tat* (%) from microspheres (25mg) at 37°C over a 28 day release period. PLGA 50:50, Molecular weight 3,000, size 10-20μm, made from a double emulsion (4.2.2.2). Loading = 1.10 nmoles. n = 4 ± S.D.

The release profile from the larger spheres are not necessarily ideal, due to the secondary stage of very slow release and the collapse of the polymer matrix causing a secondary burst release which is of greater magnitude than the initial release. The rate of drug release from the polymer matrix could be further increased by the use of ultrasound (Kost *et al.*, 1989; Supersaxo *et al.*, 1993). For specific applications careful combination of sphere size and polymer Molecular weight could be used to tailor ODN release to fit zero order kinetics.

Release of ODNs from PLGA microspheres increases with increasing pH of the release media (Fig.5.7). Degradation of polymer alone is not sufficient to explain these differences in release, degradation of PLGA has been reported to be enhanced at both pH 10 and pH 5 compared to pH 7 (Chu, 1981 and 1982). Therefore the effect of pH on the entrapped ODNs appears to be having a greater effect on the ODN release from the polymer than can be explained by polymer degradation alone. The actual influence of pH on ODN release from polymer matrices is unknown, however a report by Jaishree and Wang (1993) report that ODNs undergo conformational changes over a pH range from 3-7, which could possibly explain the differences in release.

These results could imply that the release of ODN from PLGA microspheres implanted directly into a tumour would be released more slowly due to the acidic nature of the environment, compared to microspheres implanted close to the tumour requiring the ODN to diffuse out of the polymer matrix and into the tumour mass.

5.4. CONCLUDING REMARKS

The results in this section indicate that ODNs are released from PLGA microspheres in a triphasic manner. Increasing the loading of the ODN in the polymer microsphere increases the burst and rate of release of the ODN as does decreasing the microsphere size. Total release of the entrapped ODN coincides with complete degradation of the polymer matrix. A difference in the release from PLGA microspheres of molecular weight 3,000 and 30,000 was only evident after approximately 20 days incubation as a result of the lower molecular weight polymer degrading faster than the higher Molecular weight polymer and therefore resulting in a greater rate of ODN release. Release of the ODN from the polymer was affected by pH of the media. Although release experiments with ribozymes were not studied, there are no reasons to suggest that the release of a stable 32 mer RNA would be any different from the equivalent sized DNA.

The *in vitro* release profiles presented in this chapter can only act as a guide to the expected release *in vivo*. Encapsulation of the injected polymer *in vivo* can reduce the release rate, as will varying mechanical movement depending on the site of injection and volume of fluid for release. Clearly microsphere size, ODN loading and localised pH will have an effect on the *in vivo* release profiles. It would be expected that the more hydrophobic modifications of ODNs (e.g. MP ODNs and Chol-ODNs) would be released more slowly from the polymer matrix compared to the hydrophilic PO ODNs, due to a greater interaction of the phosphate backbone with the hydrophobic polymer. Incorporation of fatty acid esters as additives into the polymer microspheres can be used to increase the release rates of hydrophobic drugs (Juni *et al*, 1985). Adjusting the kind of fatty acid and use of different alkyl chain lengths enables the release rates to be adjusted. The formulation of ODN-loaded micropsheres appear promising for ODN delivery. Their use *in vivo* is discussed in Appendix One.

CHAPTER SIX

STABILITY OF NUCLEIC ACIDS IN POLYMER DELIVERY DEVICES

6.1 INTRODUCTION

In order to asses that the film and microsphere polymer devices made in Chapters Three and Four are suitable delivery systems for nucleic acids, it is imperative to demonstrate that the released nucleic acid is stable and capable of hybridising to its target sequence. Stability of the ODN is important as it is proportional to the antisense effects (Hoke *et al.*, 1991; Ropert *et al.*, 1994). Hybridisation of the nucleic acids to the target sequence is a requirement for the desired mechanism of action. Any formulation process which adversely affects either nucleic acids stability or hybridisation would render the device useless for the delivery of nucleic acid therapeutics.

In all of the following experiments unmodified ODNs and RBZs were used as they have little intrinsic stability. Any chemical modification would mask the stabilising effect of the polymer.

6.2 STABILITY OF NUCLEIC ACIDS IN POLYMER FILMS AND MICROSPHERES

The stability of the ODN and RBZs loaded into polymer films and microspheres, incubated in serum and release media, was assessed by autoradiography as described in section 2.9.1.

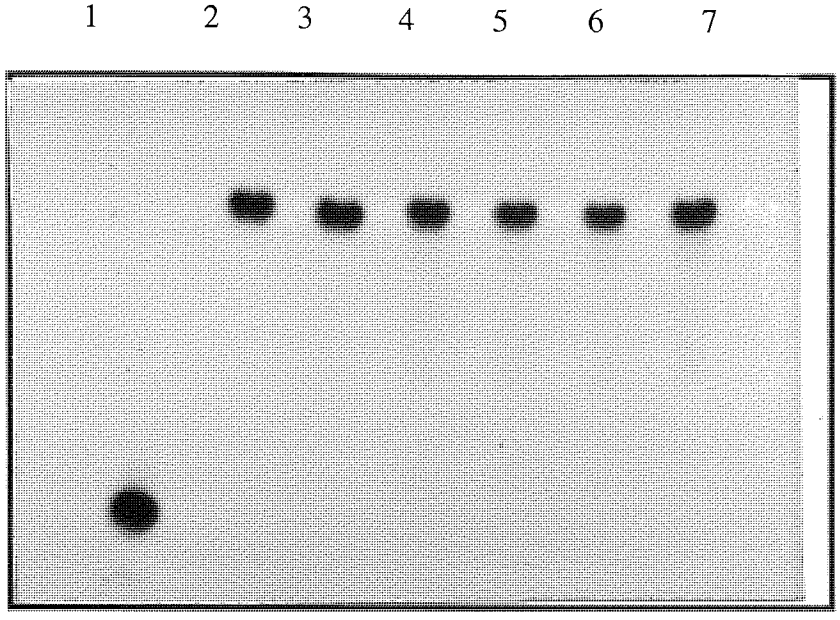


Fig.6.1 Stability of 20mer ODN-loaded in P(HB-HV) film (Mw 680,000) exposed to serum at 37°C over a 28 day period. (1) control free ^{32}P, (2) control 20mer, (3) day 0, (4) day 7, (5) day 14, (6) day 21, (7) day 28.

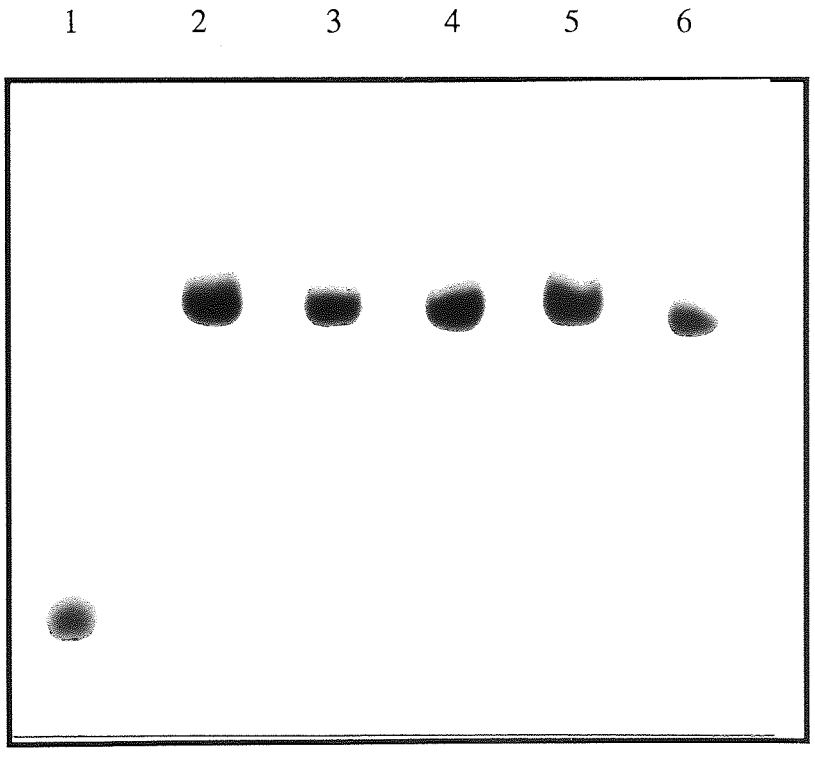


Fig.6.2 Stability of 20mer ODN-loaded in PLA film (Mw 690,000) exposed to serum at 37°C over a 28 day period. (1) control free ^{32}P, (2) control 20mer, (3) day 7, (4) day 14, (5) day 21, (6) day 28.

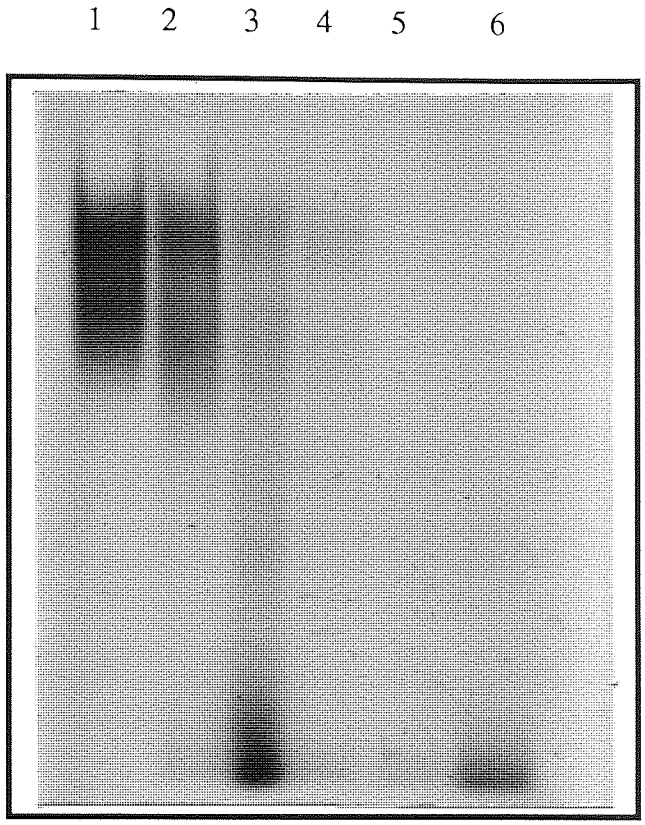


Fig 6.3 Stability of 32mer HH RBZ-loaded in PLA film (Mw 690,000) exposed to serum at 37°C over a 28 day period. (1) control 32mer RBZ, (2)day 0, (3) day 7, (4) day 14, (5) day 21, (6) day 28.

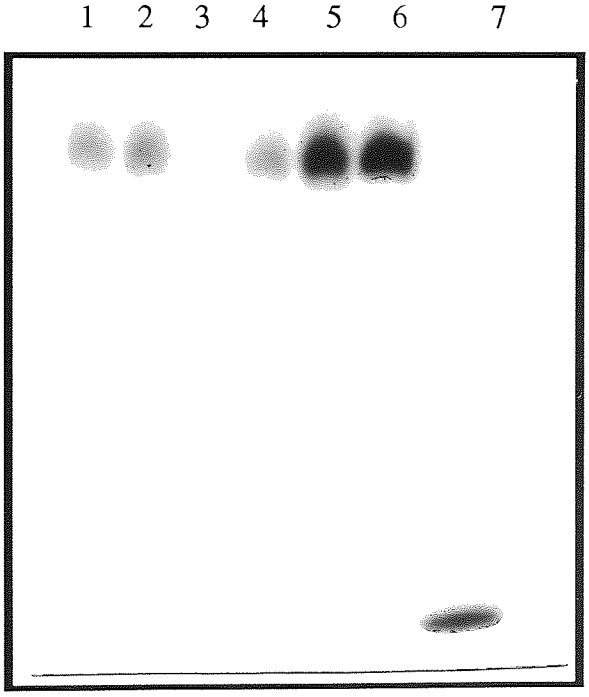


Fig.6.4 Stability of 20mer ODN entrapped in PLGA microspheres 10-20μm (Mw 30,000) exposed to DMEM (10% serum) at 37°C over a 28 day period. (1) day 21, (2) day 14, (3) day 7, (4) day 1, (5) day 0, (6) control 20 mer, (7) control free ^{32}P.

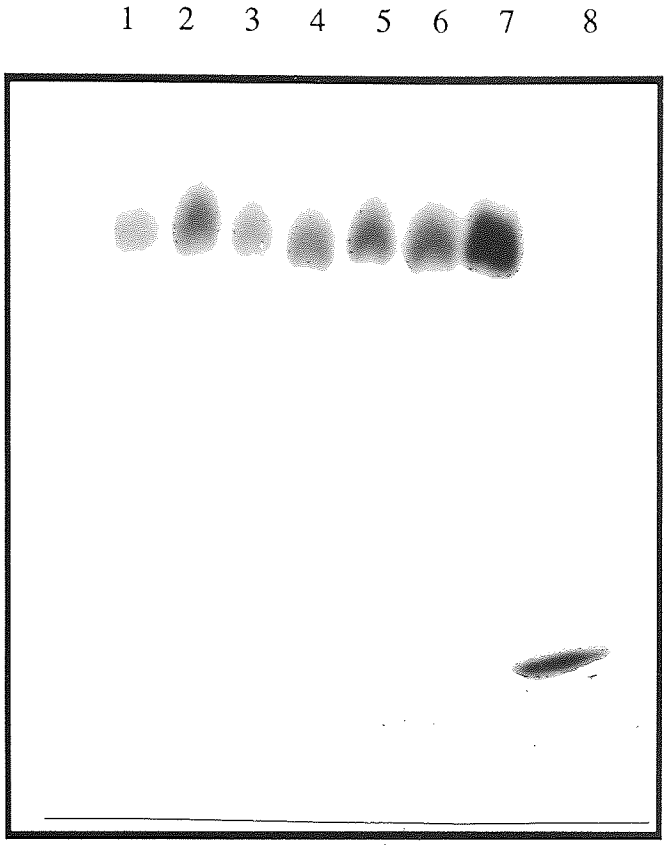
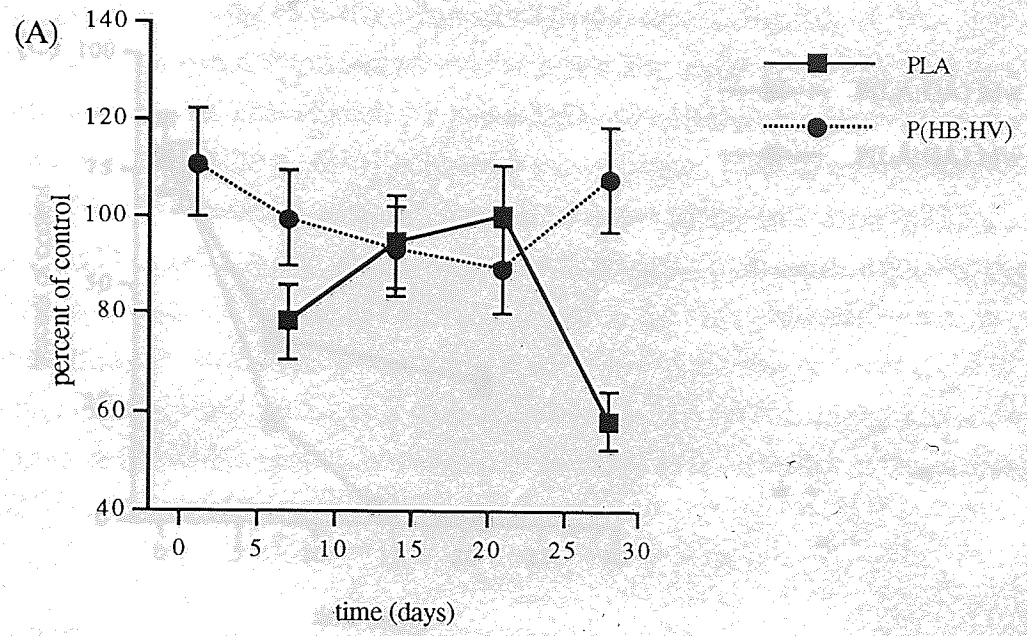
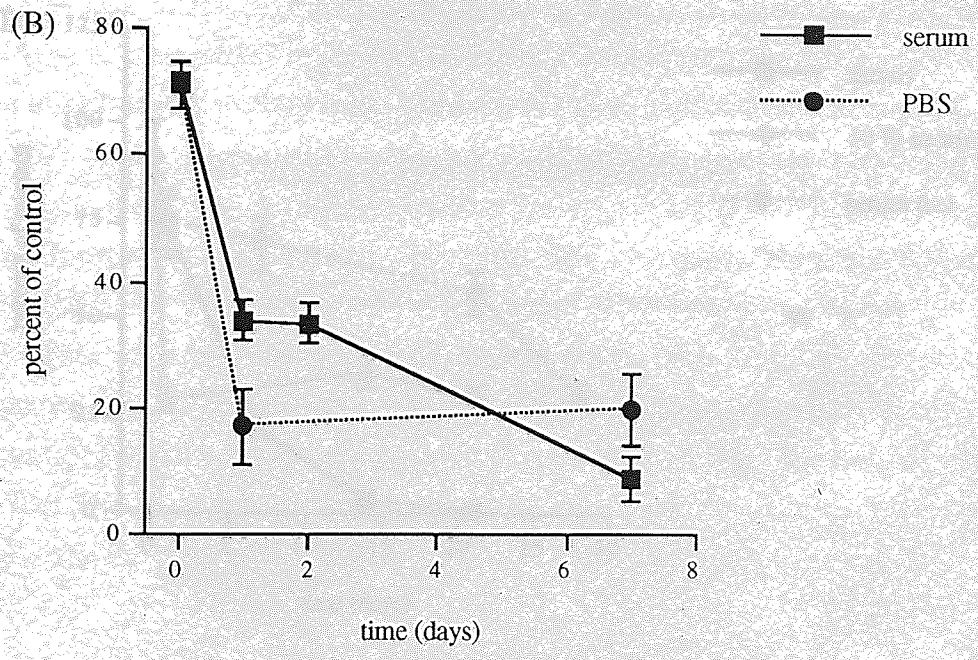


Fig.6.5 Stability of 20mer ODN-loaded in PLGA microspheres 10-20μm (Mw 30,000) exposed to DMEM (serum free) at 37°C over a 28 day period. (1) day 28, (2) day 21, (3) day 14, (4) day 7, (5) day 1, (6) day 0, (7) control 20mer, (8) control free ^{32}P.

The results shown in the stability autoradiographs of Figures 6.1 to 6.5 are summarised graphically in Figure 6.6. In the experiments all the nucleic acids were end labelled, as a result, a decrease in the intensity of the band may denote dephosphorylation and not necessarily 5' end degradation. The degree of degradation could be fully determined by the use of an internally labelled ODN or RBZ sequence, the degradation products would appear as bands with intermediate mobility on the autoradiographs, between the full length sequence and the free label control.

Entrapment of nucleic acids within the polymer devices, when incubated in serum or cell culture media have enhanced stability compared to free nucleic acids which degrade almost immediately (Wickstrom *et al.*, 1986).


(A)
140
120
100
80
60
40
percent of control
0
5
10
15
20
25
30
time (days)
PLA
P(HB:HV)


(B)
80
60
40
20
0
percent of control
0
2
4
6
8
time (days)
serum
PBS

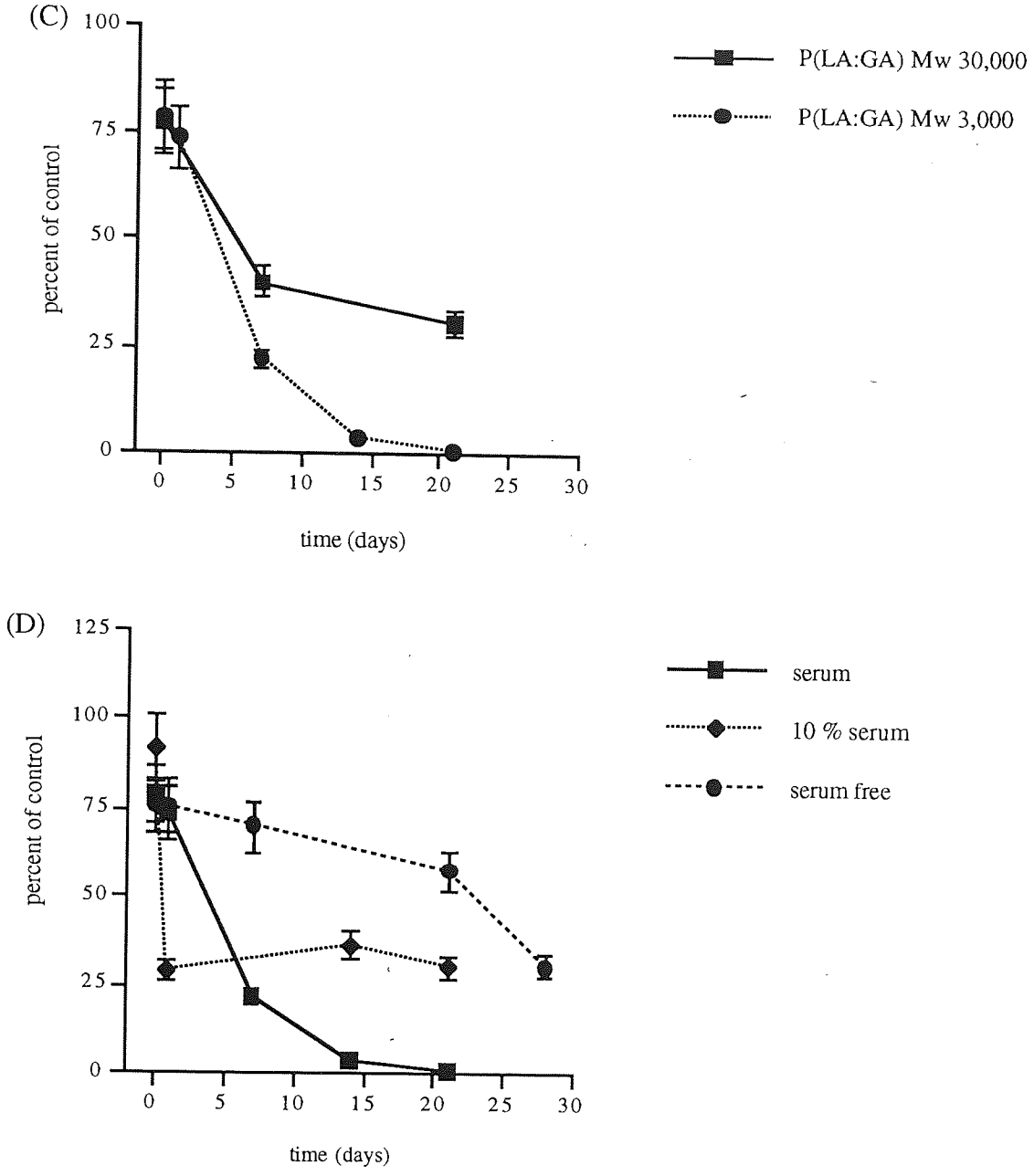


Fig.6.6 Stability of polymer entrapped ODNs and RBZs determined from densitometry of autoradiographs. (A): PO 20 mer ODN in PLA and P(HB-HV) films incubated in serum at 37 °C. (B) 32 mer RBZ in PLA films incubated in PBS and serum at 37°C (C): Effect of Mw of PLGA microspheres incubated in serum at 37°C. (D): Effect of serum on the stability in PLGA microspheres Mw 3,000 at 37 °C. Error bars represent 10% of band intensity.

Intact full length PO 20 *tat* ODN is present in P(HB-HV) and PLGA films after incubation in FBS at 37°C for 28 days. The P(HB-HV) films appear to confer greater stability to the entrapped polymer ODN, with approximately all the entrapped ODN being stable after 28 days incubation compared to only 60% remaining intact in the PLA films compared to the control (Fig 6.6.A). The difference in polymer degradation could be a possible explanation for this, water uptake from the incubation media is low in P(HB-HV) films and degradation is by surface erosion (Gilding, 1981), as a result the entrapped ODN is not exposed to the media and is therefore protected from degradation by the biological milieu. However PLA is known to absorb water and degrade by bulk erosion (Miller *et al.*, 1977), as a result the entrapped ODN is exposed to the biological milieu and so degradation can occur. The greatest difference between the two polymer films of comparative size and thickness is seen at 28 days, which confirms hydration of the polymer as a possible explanation for the differences in the stability of the entrapped ODN.

The RBZs entrapped in polymer films do not appear to be as stable compared to the all DNA ODNs. The increased susceptibility of RNA degradation compared to DNA, or that the longer sequence is not as well entrapped in the polymer matrix may offer possible explanations. The most probable explanation is degradation of the RBZ during the extraction process from the polymer film prior to running on a gel, as the stability of the RBZ appears to be very similar in both PBS and serum (Fig 6.6.B), as at time zero only 70% of the RBZ was intact. There is still some intact RBZ after 14 days incubation in serum at 37°C, however complete degradation occurred after longer incubation periods (Fig. 6.3). This demonstrates enhanced stability compared to free RBZ which is known to degrade virtually instantaneously when exposed to serum (Karikó *et al.*, 1994). The stability during the first 14 days of incubation coincides with the period of maximum release of the RBZ from the PLA film (Fig.3.5).

The nature of the polymeric device also appears to influence the stability of the entrapped ODN, with polymeric films conferring greater stability to the ODN, compared to micropsheres, with less than 50% of the full length ODN remaining after 7 days in PLGA 50:50 microspheres compared to approximately 80% remaining in PLA films. Several factors could be responsible for this difference in stability, the lower molecular weight and copolymer used to produce the microspheres result in greater degradation (Bodmeier *et al.*, 1987) compared to the

higher molecular weight homopolymer used in the films. This is further confirmed by scanning electron micrographs in Chapter Five showing evidence of degradation in PLGA micropsheres at 28 days, whereas no degradation was evident at the same time period for PLA films. The increased surface area of the microspheres compared to the films will also affect the degradation rate due to a greater contact with the biological milieu and hence a greater possibility for degradation. Complete incorporation of ODNs in microspheres may not be as great as for the polymer films, resulting in a higher concentration of ODN at the sphere surface and therefore in contact with the media.

Polymer molecular weight also has an influence on the stability of the entrapped ODN as seen by a direct comparison of device type and size (Fig.6.7.C), less than 50% full length ODN is present in PLGA 30,000 microspheres compared to less than 25% in PLGA 3,000 microspheres after incubation in serum for 7 days at 37°C. Stability studies by Chavany *et al.* (1992 and 1994) are only over a very short period (hours), so do not allow for comparison.

The incubation media also influences the stability of the entrapped ODN, with the entrapped ODN being least stable in serum compared to serum free cell culture media (Fig.6.4, 6.5 and 6.6.D), as expected the enzymes in the incubation media effect the rate of degradation of the ODNs.

MALDI-TOF (matrix assisted laser desorption ionisation time of flight) mass spectrometry (Pieles *et al.,* 1993) could be used as an alternative method to autoradiography for assessing the integrity of the ODN and RBZ. It offers a more quantitative and qualitative method compared to the visual analysis of autoradiography. Capillary electrophoresis would also enable the amount of full length product to be determined.

6.3 HYBRIDIZATION TO TARGET SEQUENCE - OLIGODEOXYNUCLEOTIDE GEL MOBILITY SHIFT ASSAY

Stability of the entrapped nucleic acid is not the only consideration in the formulation of a polymeric delivery device, as the released nucleic acid must be capable of hybridising to its target sequence in order to exert its antisense effects. Hybridisation to the target complementary sequence can be assessed by a gel mobility shift assay or by determining the melting temperature (T_m) of the duplex.

Hybridisation of a PO 20 *tat* ODN released from the polymer film to its PO 20 mer complementary "sense" sequence was determined by a gel shift assay, detailed in section 2.9.2.

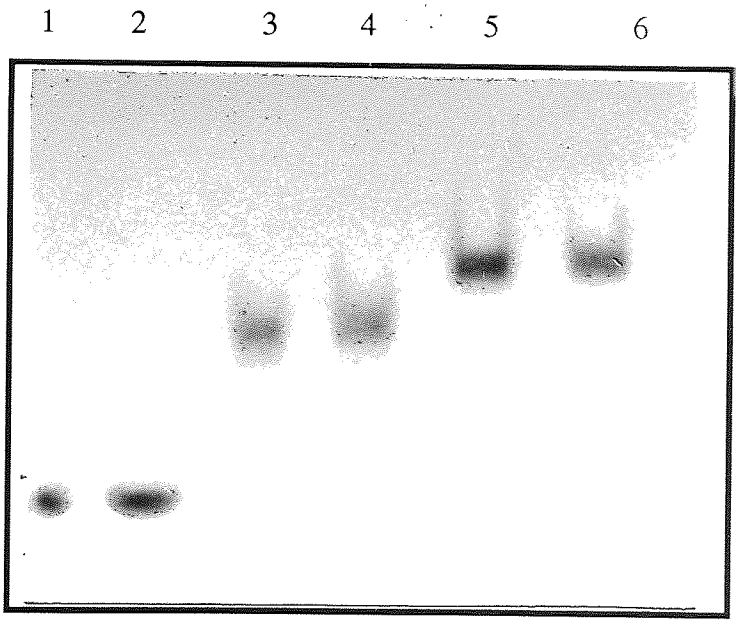


Fig. 6.7. Autoradiograph of gel mobility assay (1) free ^{32}P, (2) free ^{32}P + sense strand, (3) PO 20 *tat* ODN(control), (4) PO 20 *tat* ODN (extracted from PLA film), (5)PO 20 *tat* ODN (control)+ sense strand, (6) PO 20*tat* ODN (extracted from PLA film) + sense strand.

The principle of a gel shift assay is to determine if the antisense and sense sequences can hybridise under the appropriate conditions to form a duplex. The resulting duplex will have a doubled molecular weight as compared to the single antisense or sense sequences and therefore would be expected to have less mobility

on a native polyacrylamide gel compared to the single stranded sequences. The released PO 20 *tat* ODN was still capable of hybridising to its complementary 20 mer sense sequence *in vitro* (fig 6.7), as seen by a retardation in the migration of the radiolabelled duplex band (lane 6) as compared to the migration of a single strand (lane 4, non-duplex). The similarity of lanes 5 and 6 indicates that the fabrication procedure does not alter the hybridisation of the ODN to the sense strand compared to an ODN which has not been entrapped in the polymer film. A negative control of addition of free label to the sense strand (lane 2) did not lead to a band shift when compared to the migration of free label alone, as no hybridisation occurred.

These results demonstrate that the PO 20 *tat* ODN released from the PLA film is still capable of hybridising to its target sequence to form a duplex.

6.4 DUPLEX MELTING TEMPERATURES (T_m)

Although the gel mobility shift assay suggests that the ODN released from the polymer films is capable of hybridising to its complementary sequence, the affinity of the binding may have been impaired by the solvent casting fabrication procedure, resulting in partial hybridisation which may be sufficient to result in a band shift in a gel shift assay as in section 6.3. The affinity of hybridisation can be assessed by thermal melting data.

The affinity of ODNs to their receptor sequences results from hybridisation interactions, the two major contributions to the free energy of binding are hydrogen bonding (Watson-Crick base pairing) and base stacking in the double helix. Affinity is affected by ionic strength and results from hydrogen bonding between complementary base pairs and increases with increasing length of the ODN receptor complex. The thermal melting temperature (T_m) is defined as the temperature where 50% of the ODN is present as a duplex and 50% is a single strand. In order to further clarify if the fabrication procedure affects the hybridisation, the T_m were compared for a PO 20 *tat* ODN with its complementary strand before and after solvent casting, therefore by use of this technique, the effect of fabrication into a polymer device can be determined. The method for the T_m determination is detailed

in section 2.9.3. The experimentally determined T_m results were compared with theoretical values to confirm that the values were in the expected range.

6.4.1 Theoretical Calculation of T_m

The T_m value was theoretically calculated by 2 methods to confirm the accuracy of the results from the experimental procedure.

PO 20 *tat* antisense 3' ACA CCC AAT TCT GAA AAT GG
PO 20 *tat* sense 5' TGT GGG TTA AGA CTT TTA CC

Method 1 - $T_m = 2°C (A + T) + 4°C (G + C)$
$T_m = 56°C$

where A,G,C,T are the number of base residues in the hybrid, however this method overestimates the T_m of hybrids with oligonucleotides longer than 18 nucleotides (Itakura *et al.*, 1984).

Method 2 - $T_m = 81.5 + 16.6(\log_{10} [Na^+]) + 0.41$ (fraction G+C)- (600/N)
$T_m = 54.22°C$

where N = chain length and Na^+ concentration is 1 M or less, this method works for chain lengths between 14-70 nucleotides (Bolton and M^{c}Carthy, 1962).

6.4.2 Experimental determination of T_m values

The experimental procedure used to determine duplex melting temperatures focuses on the change in UV absorbance at 260nm with an increase in temperature, to produce UV melting curves. At low temperatures in solution, 2 complementary single stranded oligomer sequences will associate to form a based paired and base stacked duplex structure (Fig. 6.8). As the temperature is increased the thermal energy disrupts the helix stabilising forces and the duplex structure melts. Unstacked base pairs in a single stranded state have a higher absorbance at 260nm compared to the stacked duplex, this increase in absorbance is a hyperchromic shift with the T_m at the point of greatest change (fig 6.9).

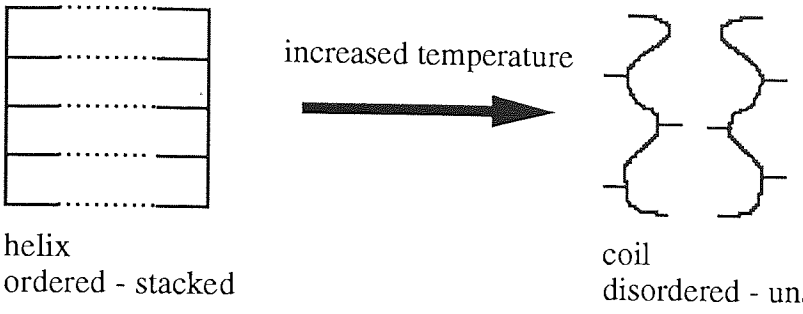


Fig. 6.8 Schematic diagram showing the effect of heat on a DNA helix.

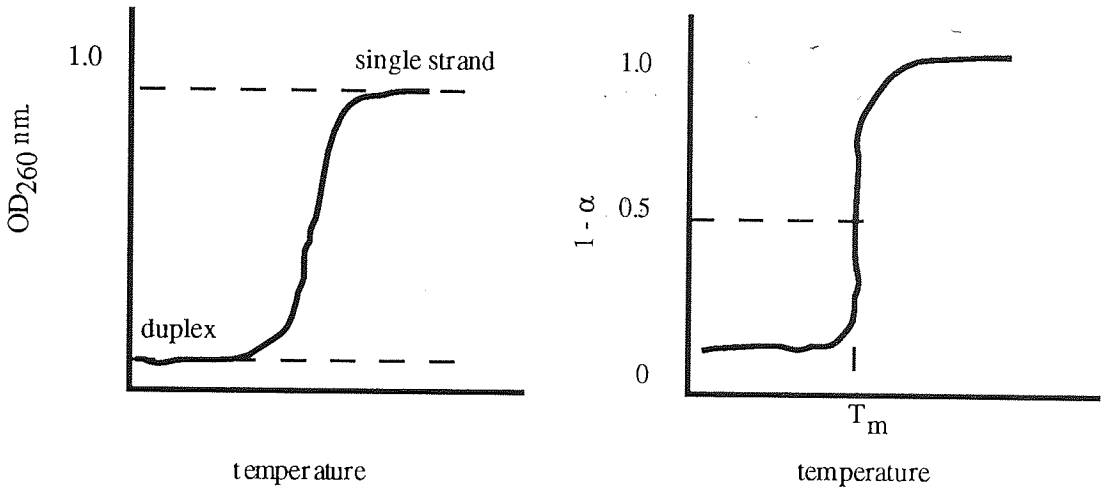


Fig.6.9 Schematic diagram of UV melting curves adapted from Breslauer, (1986).

The experimental UV temperature profile of the ODN duplex in Figure 6.10, shows a smooth absorbance profile with a maximum absorbance at 260nm consistent with that expected of a uncontaminated ODN sample. The absorbance of the duplex increases with increased temperature, with the T_m value falling between the absorbance profiles of greatest change. The thermal melting curve (Fig.6.11) shows non-cooperative binding, the transition from duplex to single strand proceeds with a significant population of intermediates, as a result the 1-α plot (Fig.6.12) is broader than that of cooperative binding, where the transition proceeds in a 2 step manner. Broadening of the plot leads to a reduction in the calculated enthalpy transition. The T_m results were calculated via the software package by 3 methods, average, derivative and hyperchromicity methods. The values from the average and hyperchromicity methods were similar, the results are not the same for all three

methods as non-cooperative binding does not perfectly match the equations for the calculations.

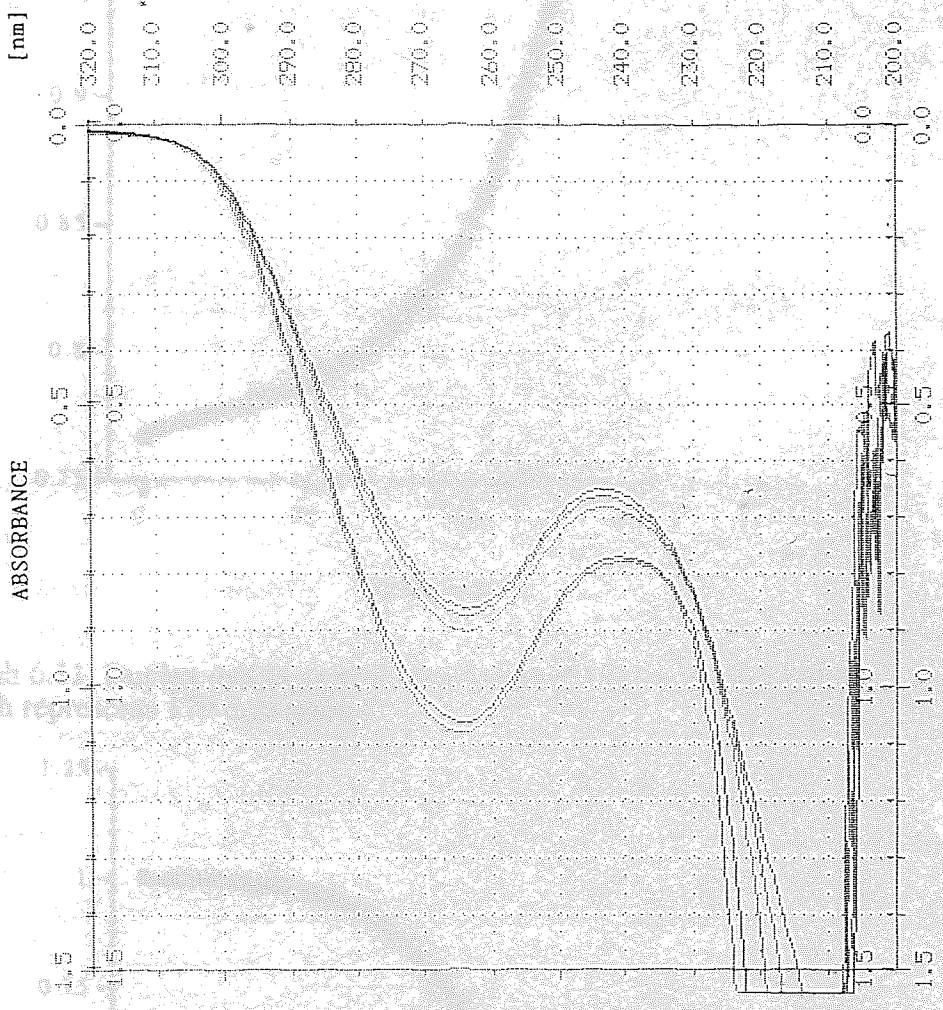
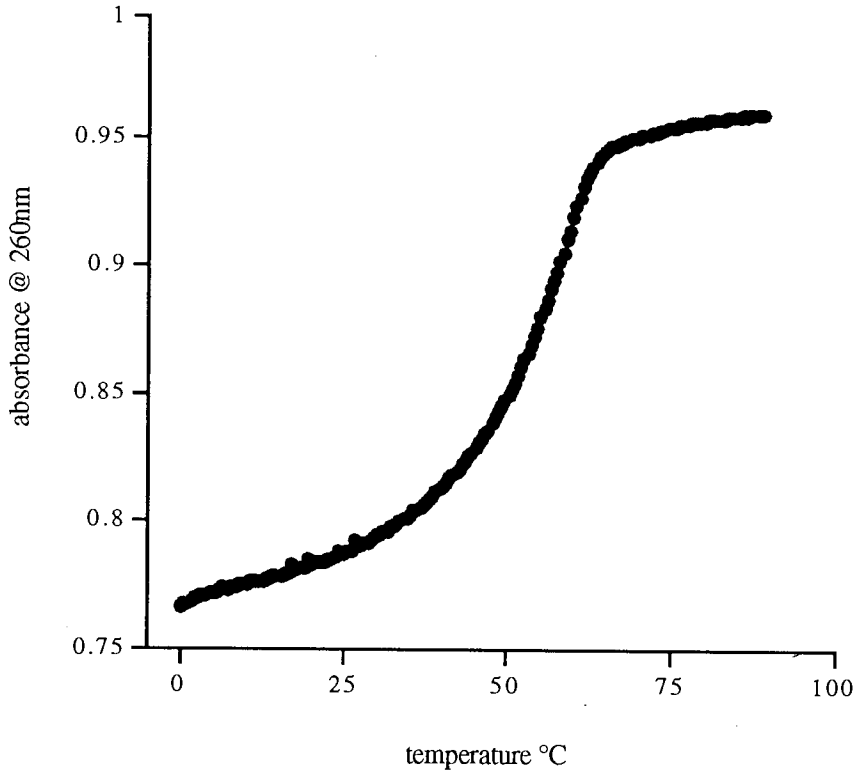
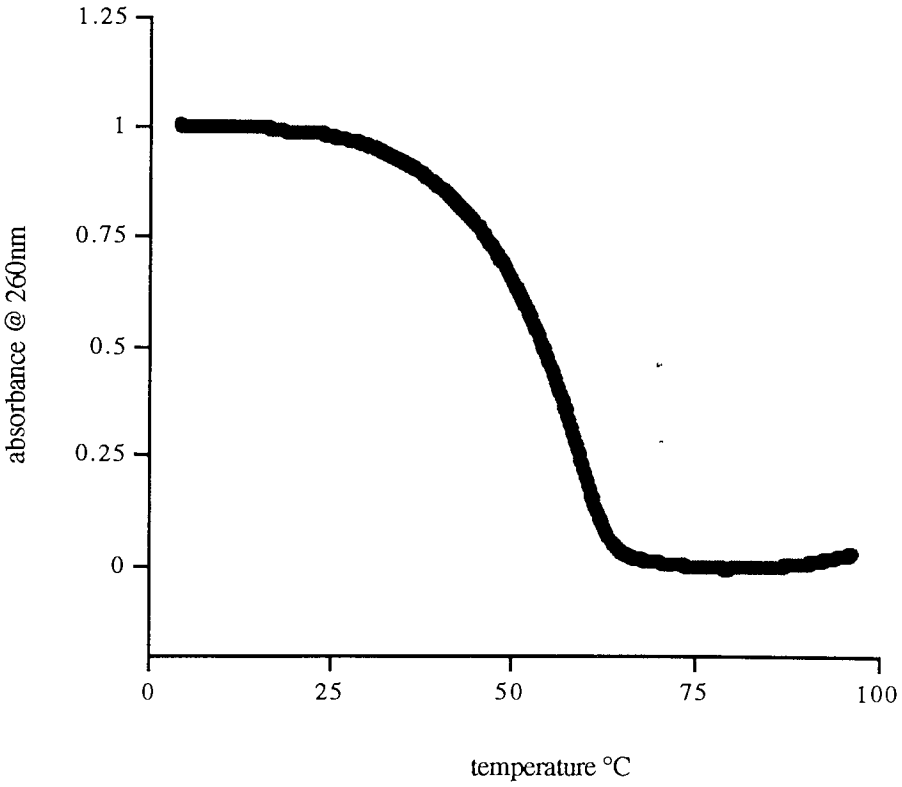


Fig.6.10 UV absorbance trace of ODN duplex showing increased absorbance with an increase in temperature. The bottom curve is at 0°C, with increasing temperature 25,50,75,100°C for the top curve.



Graph 6.11 Duplex melting curve absorbance at 260nm against temperature, the graph represents 179 data points.



Graph 6.12 A 1-α plot, the graph represents 922 data points.

SAMPLE	HYPERCHROMICITY METHOD T_m
CONTROL	54.88 (0.29)
FILMS	54.85 (0.18)
SPHERES	54.61 (0.57)

Table 6.1 Summary of the T_m results calculated from the hyperchromicity method for PO 20 *tat* ODN control and extracted from PLA films and PLGA 50:50 spheres (Mw 3,000) ±S.D.

The results in table 6.1 show that the T_m values for control ODN and ODN extracted from polymer films and microspheres are 54.88, 54.85 and 54.61°C respectively, and are consistent with the theoretically calculated values, indicating that the fabrication procedure has not adversely effected the affinity of the ODN for its target sequence. The T_m values obtained theoretically and experimentally are not absolute values for the affinity of the ODN for its target sequence. *In vivo* values may be reduced due to the secondary structure of the complementary sequence (Freier *et al.*, 1992) as the target site is not of an equivalent length but part of a larger structure.

6.5 BIOLOGICAL ACTIVITY OF RELEASED RIBOZYME

The HH RBZ released from the polymer film is required to retain its activity and be capable of hybridising to its complementary substrate sequence, causing cleavage. Determination of the activity of the 32mer RBZ released from PLA films was assessed as in section 2.9.4.

Figures 6.13 and 6.14 indicate that the 32mer HH RBZ released from the PLA film was still capable of hybridising to its corresponding 27 mer substrate *in vitro* and causing cleavage. There is an increasing concentration of the radiolabelled 15mer cleavage product with increased reaction time, with a proportional decrease in the substrate concentration. The catalytic activity of the polymer released RBZ appears to be unaffected by the entrapment process as the cleavage ability of the polymer released RBZ is similar to that of unentrapped RBZ, with 50% of the substrate molecule being cleaved within approximately 6 hours *in vitro* in both cases (Table 6.2).

	Substrate $t_{1/2}$ (hours)
Free RBZ	6
Polymer entrapped RBZ	6

Table 6.2 Summary of substrate $t_{1/2}$ comparing the activity of the RBZ with polymer entrapped RBZ.

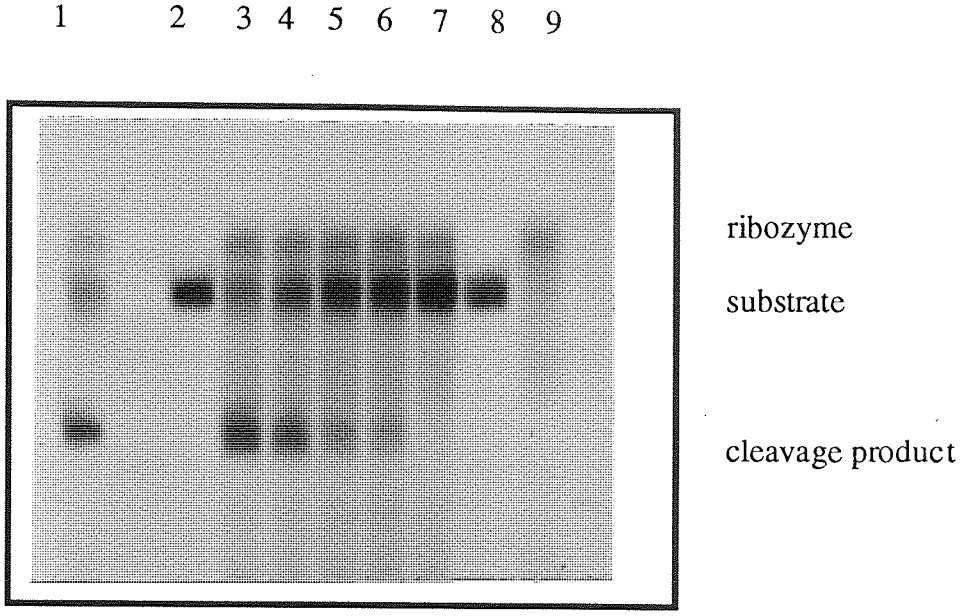


Fig. 6.13 Activity of released HH RBZ from PLA films showing cleavage of substrate. Lanes show increasing incubation time with substrate at 37°C. Lane (1) positive control of substrate and ribozyme, (2) substrate 18hrs, (3) reaction 18hrs, (4) reaction 8hrs, (5) reaction 4hrs, (6) reaction 2hrs, (7) reaction 0 hrs, (8) substrate control, (9) ribozyme control. Lanes 3-9 at 37 °C. [Mg^{2+}] = 10mM, for details of reaction see 2.9.4.

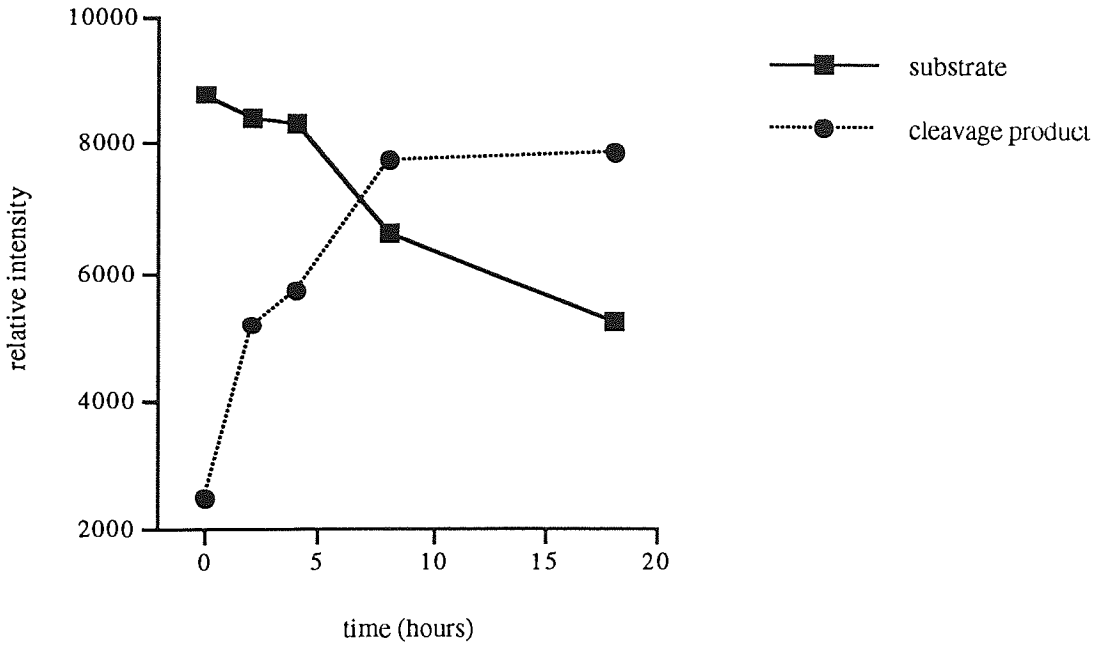


Fig.6.14 Cleavage of 27mer RNA substrate and formation of 15mer cleavage product by 32mer HH RBZ released from the PLA film, as determined by densitometry of the autoradiograph in Fig.6.13.

6.5 CONCLUDING REMARKS

The results in this chapter have shown that the use of biodegradable polymers has enhanced the stability half-life of ODNs and RBZs when incubated in serum and cell culture media compared to free nucleic acids. A combined approach using chemically modified ODNs and entrapment within a polymer matrix offers the optimum stability of the ODN or RBZ prior to its release at the target site.

The larger surface area of microspheres as compared to films, possible high concentration of ODN at the surface along with degradation of the spheres from the central core (Vert, 1994) limits the time period during which the entrapped ODN will remain stable, therefore microspheres are perhaps not as advantageous as films for the long term controlled release of nucleic acids to their target site. However the combination of chemically modified ODNs entrapped within polymer micropsheres will convey stability over a longer period.

Possibly more important than the stability of the entrapped ODN is its ability to retain activity by hybridising to its complementary target sequence as in the gel mobility assay and in the case of the RBZ result in substrate cleavage. The T_m values for ODNs released from films and spheres indicate that the fabrication process does not adversely effect the ODN affinity for its complementary sequence. This is an important result as the use of chemical modifications to the PO backbone to enhance stability have been shown to reduce their affinity for the target sequence (Dagle *et al.*, 1991; Ghosh *et al.*, 1993).

Therefore polymer matrices offer a true potential for the delivery of nucleic acids for therapeutic applications.

CHAPTER SEVEN

ASSOCIATION OF MICROSPHERES INTO CELLS

7.1 INTRODUCTION

Microspheres are known to accumulate in phagocytic cells, these mechanism can be exploited to enhance the delivery of ODNs to certain cell types.

Encapsulation of a drug in microspheres has been shown to increase the delivery and activity of the drug to macrophages (Tabata and Ikada, 1987). Macrophages can phagocytose microspheres in the size range of 1 to 20μm (Yamaguchi and Anderson, 1993), however the degree of phagocytosis of particulates is dependent on size, surface charge and hydrophobicity. Studies investigating the cellular delivery of various types of microspheres have demonstrated that the optimum size for phagocytosis of microspheres by macrophages is 1-2μm (Tabata *et al.*, 1988 a; Kimura *et al.*, 1994). Aggregation of particles may be the reason for a lower association of smaller spheres, as well as the possibility of a minimum threshold particle size for recognition by the macrophages. Particle charge and hydrophillicity are also important factors effecting phagocytosis. Charged hydrophobic particles (anionic or cationic) are taken up to a greater degree than non charged hydrophilic particles (Tabata and Ikada, 1989). Kimura *et al.* (1994), report that retinal pigment endothelial cells can phagocytose 1-2 microspheres / cell over a 12 hour period, phagocytosis of particulate material increases with time regardless of the surface properties. Peritoneal macrophages are capable of degrading PLA intracellulary (Tabata *et al.*, 1988 b), degradation occurs at a faster rate due to localisation in acidic and enzyme rich lysomal compartments after phagocytosis, their large surface area and low molecular weight. PLGA 50:50 spheres of Molecular weight 5,000 remained in the cells for at least 14 days, however, the intracellular fate was not identified (Kimura *et al.*, 1994). However PLGA 50:50 spheres of Molecular weight 3,000 had completely degraded after 7 days, indicating that the rate of degradation can be controlled by the polymer Molecular weight (Tabata and Ikada, 1988 b). Delivery of ODNs using polycyanoacrylate nanoparticles has already been reported (Chaveny *et al.*, 1992 and 1994) in a system where ODNs are adsorbed

onto the nanoparticle surface by hydrophobic quartenary ammonium salts. These authors reported enhanced delivery of ODNs using nanoparticles compared to free ODN. However the amount of ODN that can be delivered using this type of nanosphere preparation is limited due to the toxicity of the hydrophobic quartenary ammonium salts.

The macrophage is also of particular interest for the delivery of ODNs as they are involved in the development and latency period of acquired immunodeficiency syndrome (AIDS) (Roy and Wainberg, 1988; Meltzer *et al.*, 1990) the clinical latency in asymptomatic HIV infected patients may be as a result of the sequestration of the virus by macrophages. AZT and ddC loaded albumin and polyhexylcyanoacrylate micropsheres have been shown to reduce HIV infection in macrophages (Bender *et al.*, 1994).

Therefore the aim of this section was to asses the association of free ODN and ODN-loaded polymer microspheres in cultured cells as a method of improving delivery of ODNs to target cells. The main cell line studied was a murine macrophage, Raw 264.7 due to macrophage ability to phagocytose particulate matter. The macrophage is the primary cell injected material will encounter, as a result of its role in the elimination of invading foreign materials (see section 1.5).

The details of the experimental procedure for cell association studies are outlined in section 2.9, a schematic diagram of the method is shown in Fig.7.1. Due to the known instability of PO ODNs in cell culture media (Akhtar *et al.*, 1991 b), a 5'end[^{32}P] modified PO hairpin ODN (3' CG AAA GCG TAC GGG GAG TTG 5'), complementary to the AUG initiation codon of human *c-myc* was used. The formation of a 3' stabilising hairpin loop is known to covey stability in cell culture media over a 24 hour period (Khan & Coulson, 1993). In each case approximately 0.2nmoles of ODN was added to each well, either free or loaded into microspheres. The microsphere size and surface charge was analysed in Chapter Four, to ensure that the addition of ODN to the micropsheres did not alter the size and surface charge and hence alter cellular association. The results in Chapter Four indicate that ODN loading does not alter the microsphere size or surface charge.

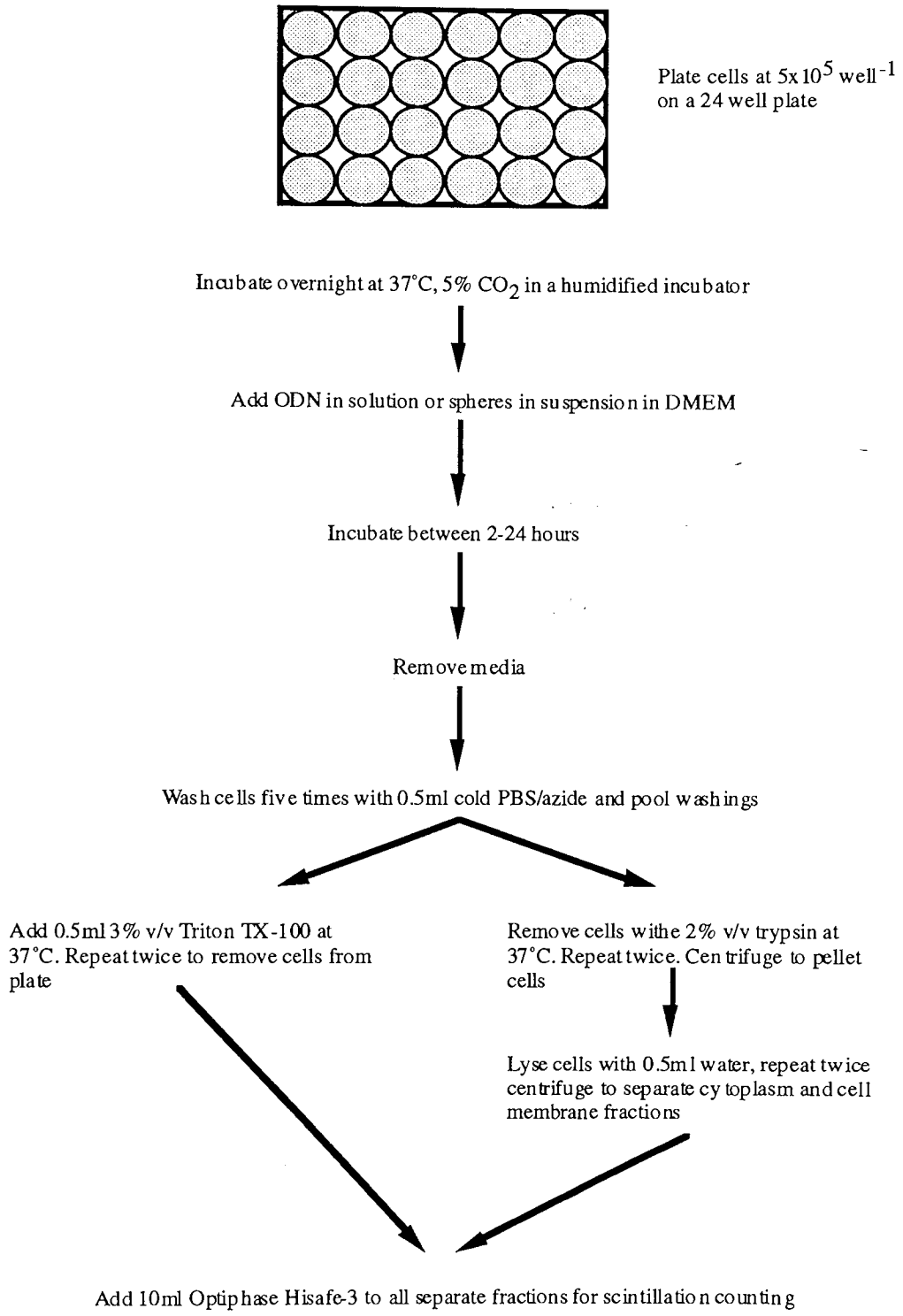


Fig.7.1 Schematic diagram of the experimental procedure for assessing cellular association.

7.2 TOXICITY OF PLGA MICROSPHERES ON MURINE MACROPHAGES

Polylactide and its copolymer with polyglycolide have widely been reported as being non toxic (Vissher *et al.*, 1988; Yamaguchi and Anderson, 1993, section 1.10.3). However it is important to assess the effects of the spheres on the cells due to the presence of residual solvents and emulsifiers used during preparation which may be toxic and have an adverse effect of the cells.

To assess the cellular toxicity of 2μm spheres of PLGA, unloaded 1 to 2μm spheres were made by a double emulsion method as in section 4.2.2.4, and added to murine macrophages (RAW 264.7) in 96 or 24 well plates, in order to determine their effect on cell number and cell metabolism. The microspheres were not sterilised prior to the cell association studies, although they were added to antibiotic containing media detailed in section 2.10. A crystal violet staining assay was used to determine the number of cells present on a 96 well plate colorimetrically detailed in section 2.11.6. In each case the cells were incubated in DMEM containing 10% FBS. The effect of the spheres on the cell number during a 24 hour association experiment was determined by removing the cells from a 24 well plate with 1ml cell dissociation media and counting the cells using a haemocytometer.

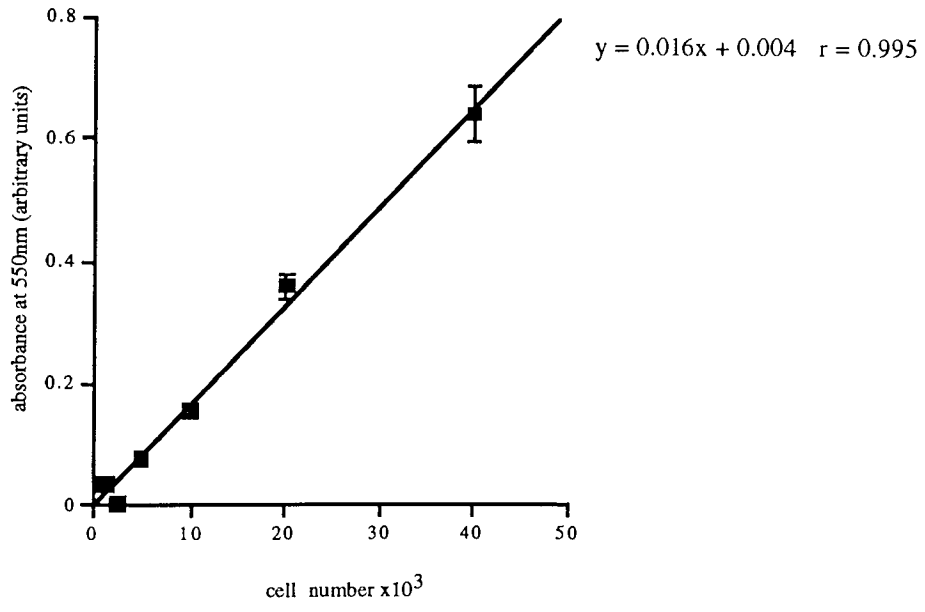


Fig.7.2 Calibration graph of RAW 264.7 cell number against absorbance at 550nm. The cells were incubated for 24 hours at 37°C. n= 4 ± S.D.

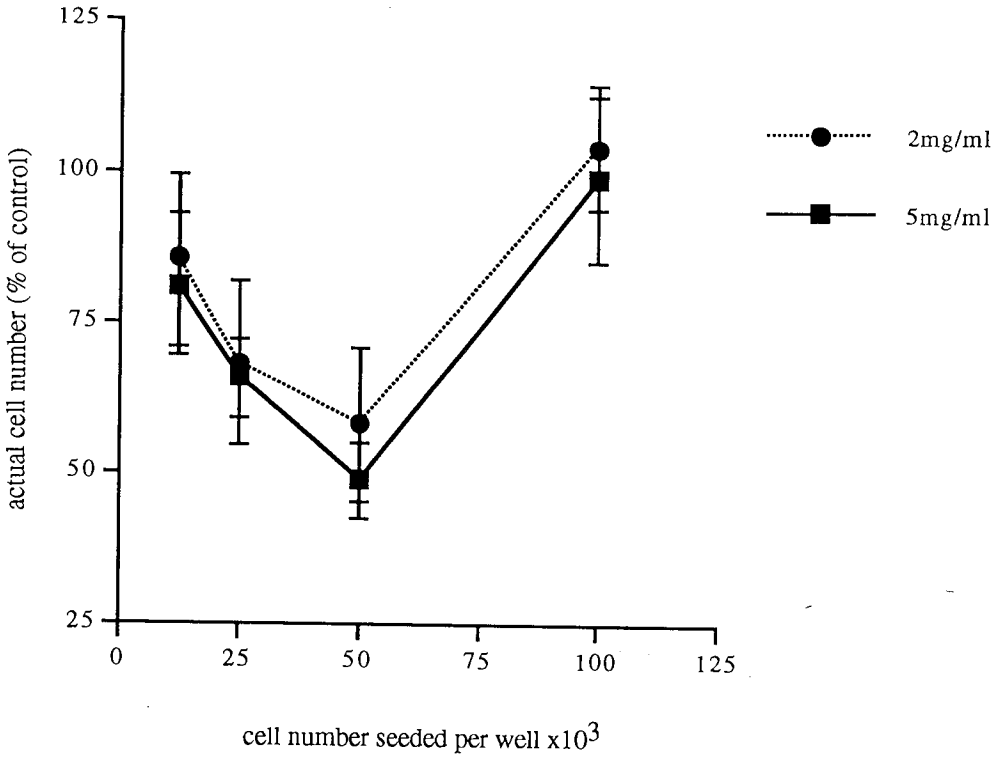


Fig.7.3 The effect of sphere concentration on cell number, in RAW 264.7 cells after 24 hours incubation at 37°C. Spheres are blank 2μm PLGA of Mw 3,000. Results are calculated from the difference in absorbance readings from the crystal violet assay between the control of cells alone and cells with spheres. n = 4 ± S.D.

The presence of blank spheres did not effect the absorbance at 540nm, this was confirmed by the addition of blank spheres before measuring the absorbance of control cells. The calibration graph (Fig.7.2) demonstrates a linear relationship between cell number and absorbance at 550nm. The results in graph (Fig.7.3) show that the presence of spheres have an effect on the cell numbers of murine macrophages, although there is little difference in the effect between the concentration of the spheres added to the cells. The greatest reduction in cell number is seen when the cells are sub confluent, the presence of the spheres possibly prevented the cells growing further due to the shortage of space inside the well. The presence of polymer monomers can reduce cell confluency (van Sliedregt *et al.*, 1994), however in this experiment the reduced cell number is possibly due to reduced area in the well, due to the presence of spheres, as it is unlikely that the polymer spheres would have degraded sufficiently to produce monomers over this short time period. The spheres have least effect at the extreme cell numbers, possibly due to increased space in the well at low cell numbers, and the cells already being confluent at the higher cell number before the addition of the spheres. Fig. 7.4 indicates that the presence of spheres does not reduce the macrophage cell

numbers significantly in a 24 well plate. All the cellular association experiments were done in 24 well plates, it was therefore assumed that the presence of the spheres was not adversely effecting the cell numbers during the course of the cell association experiments.

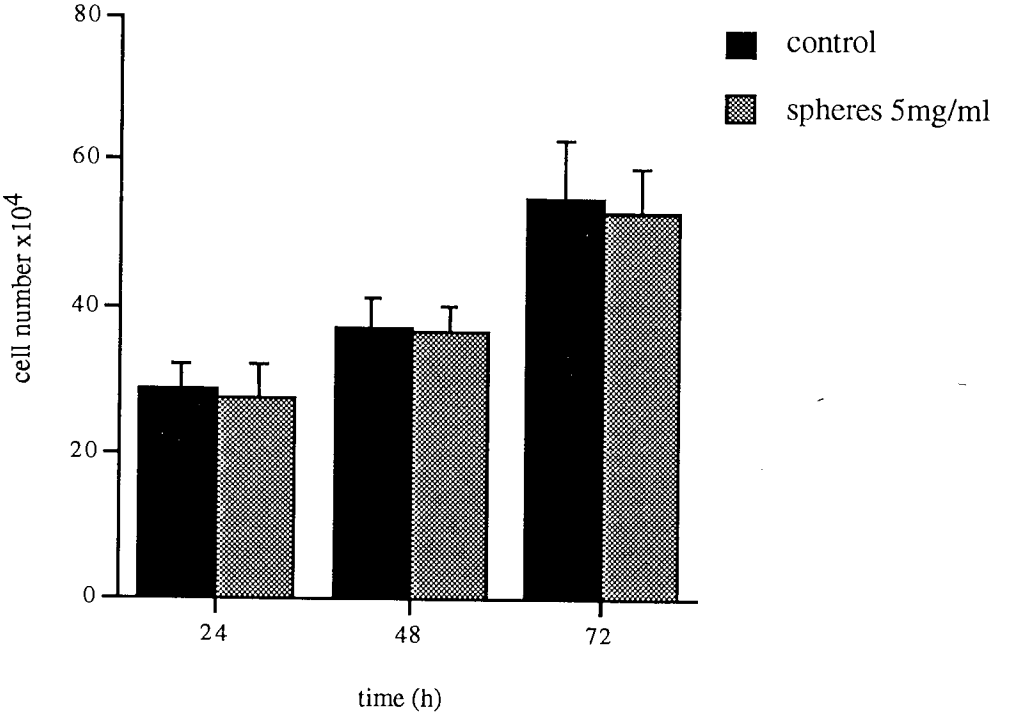


Fig.7.4 Effect of unloaded PLGA 2μm spheres (Mw 3,000) on cell number, in RAW 264.7 cells after 24, 48 and 72 hours incubation at 37°C. Results are calculated from direct counting of the cells in a 24 well plate. n = 4 ± S.D.

The effect of PLGA microspheres on cell metabolism was determined by a MTT assay, described in section 2.11.7.

The presence and association of spheres with murine macrophages did not adversely effect the metabolism of the cells, in this assay (fig.7.5). From this data it can be concluded that the presence of spheres does not adversely effect the cell number or metabolism of the macrophages under the conditions that were used for the cell association experiments. Lappalainen *et al.*, (1994) report that microscopy is a better tool to determine cellular damage compared to the MTT assay. The MTT assay measures mitochondrial dehydrogenase of viable cells, as mitochondria are known to be the last organelles to survive cell damage after injury, this does not mean that no damage has been caused to the cells.
However from the results of cell number experiments, the MTT assay and microscopical observations, no adverse effects are seen on the cells by the addition

of PLGA microspheres over the 24 hour period studied for cell association experiments.

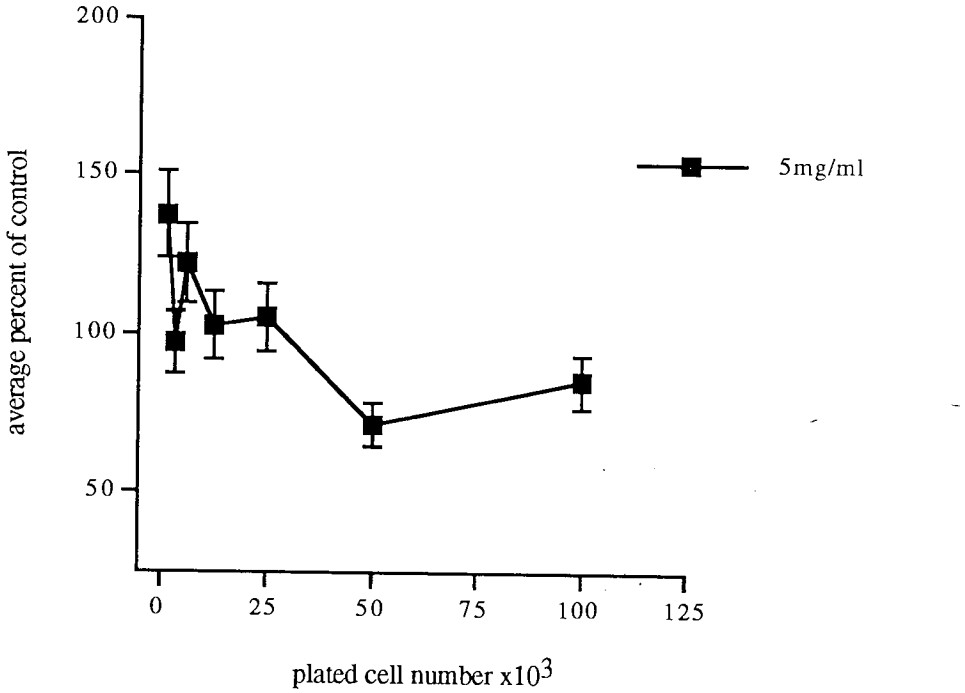


Fig.7.5 Effect of spheres on MTT assay, in RAW 264.7 cells after 24 hours incubation at 37°C. Spheres are blank 2μm PLGA Mw 3,000. n= 4 ± S.D.

7.3 OPTIMISATION OF CELL ASSOCIATION STUDIES

7.3.1 The Influence of Cell Washing on Cell Associated Radiolabel after Incubation with Oligodeoxynucleotides and Spheres

In order to standardise the cell association method and enable direct comparisons between experiments, it was important to asses the number of washes necessary to remove loosely associated ODNs and spheres from the cell surface.
The total radioactivity of free ODN was approximately 1 million cpm and for the spheres at 5mg/ml 500,000cpm. This level of radioactivity was kept constant throughout all the association experiments.

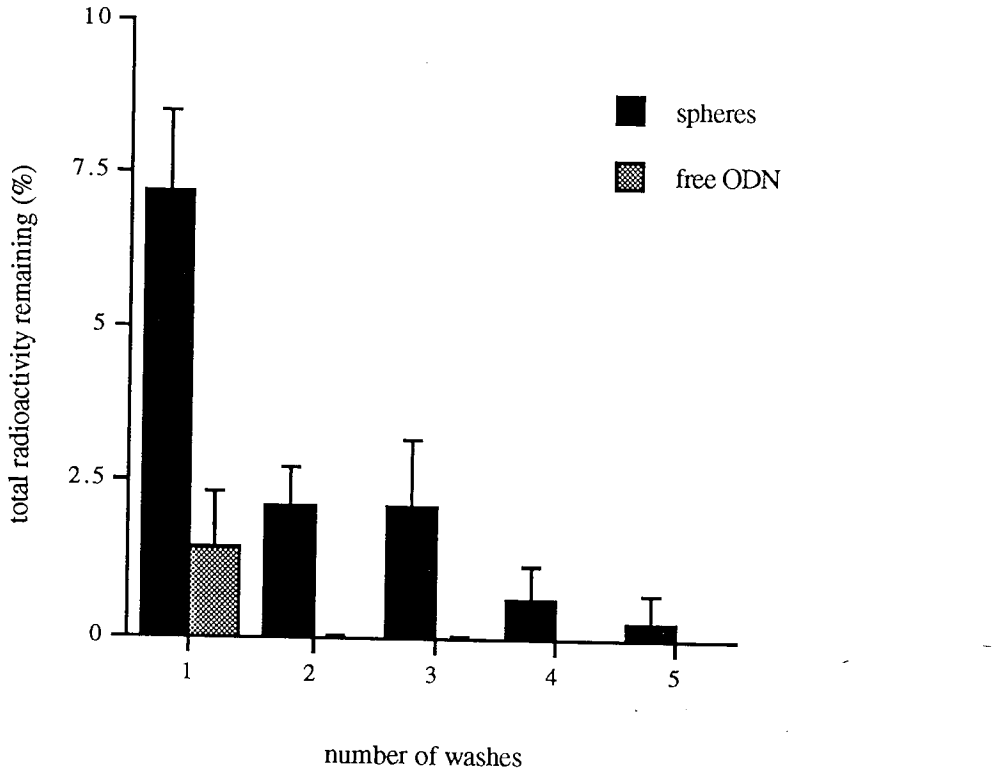


Fig.7.6 Removal of free radiolabel from the surface of RAW 264.7 cells after treatment with free ODN or ODN loaded microspheres. PLGA 2μm Spheres Mw 3,000. The cells were plated at 5 x 10^5 $well^{-1}$. n = 4 ± S.D.

Approximately 0.3% of the total radioactivity added remained after 5 successive cell washings with 0.5ml PBS/azide following treatment with ODN loaded microspheres (Fig.7.6). After 5 washes all the non associated spheres appeared to have been removed the from the wells. All loosely associated radioactivity from free ODNs was removed in only two washes.

As a result of these findings a five wash step protocol was used in all the association experiments, to ensure that loose spheres were being removed from the cell surface and thus preventing artificially high association results.

7.3.2 The Influence of Sphere Dose on Cellular Association

In order to investigate the amount of spheres which can be internalised by RAW 264.7 macrophages, a wide range of polymer sphere concentrations were added from 1 to 20 mg/ml. A volume of 0.5ml of each sphere suspension was added to the cells (5x10^5 per well) and incubated with the cells for 24 hours at 37°C.

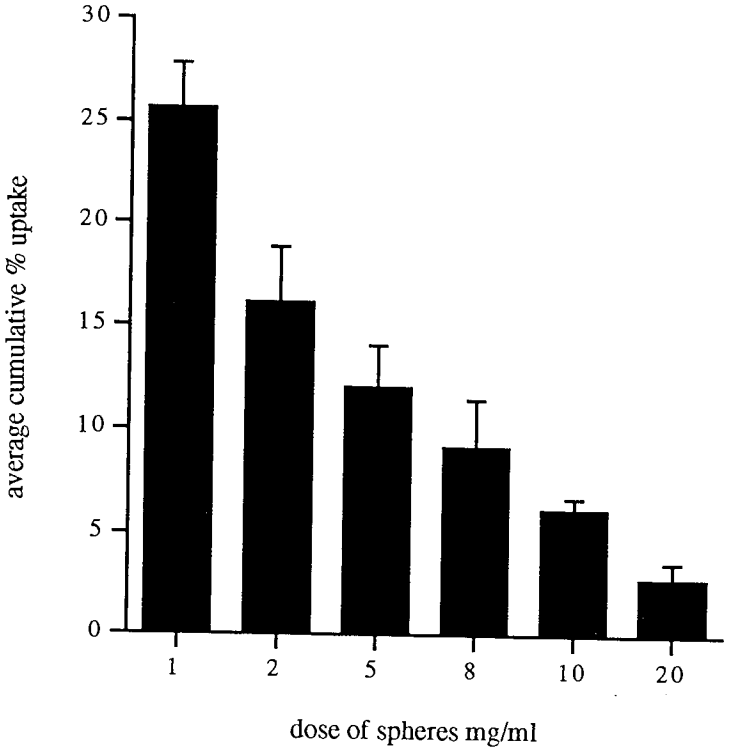


Fig.7.7 The effect of the polymer dose on the % association of 2μm PLGA 50:50 spheres (Mw 3,000) in RAW 264.7 cells plated at $5x10^5$ cells $well^{-1}$. Association was determined in serum containing media at 37°C after 24 hours. n = 4 ± S.D.

Increasing the sphere concentration did not proportionally increase the cellular association (Fig.7.7), indicating that the association process may be saturable. This result agrees with the findings of Chaveny *et al.*, (1994) who reported that doubling the dose of spheres added to the cells did not result in doubled cellular association. The results indicate that there is a maximum polymer load that can associate with the macrophages. In this experiment $5x10^5$ cells internalised approximately 250μg of polymer spheres. In order to internalise the greatest possible amount of ODN it would be advantageous to have the highest ODN : polymer ratio as possible.

In all subsequent experiments, spheres were added at a concentration of 5mg/ml of media to enable a compromise of maximum percentage association with high specific activity of the spheres.

7.3.3 The Effect of Cell Number on Microsphere Association

A range of cell numbers were plated in order to determine the optimum cell number for maximum cell association of microspheres. Cell numbers from 1 to 120 x10^4 cells were plated in a 24 well plate, and a volume of 0.5ml of a 5mg/ml sphere suspension was added. The spheres were incubated with the cells for 24 hours at 37°C.

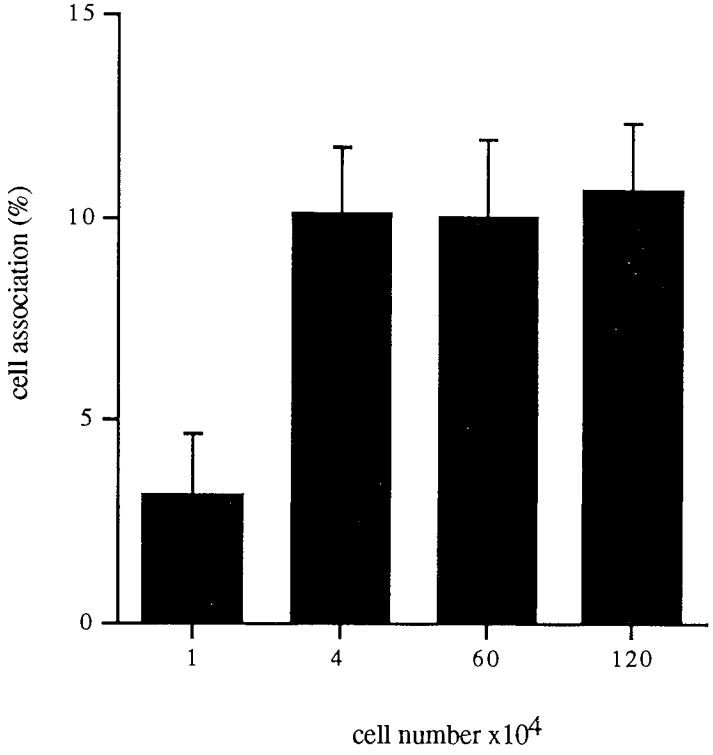


Fig.7.8 The effect of RAW 264.7 macrophage cell number on the percentage association of 2µm PLGA 50:50 spheres (Mw 3,000). Association was determined in serum containing media at 37°C after 24 hours. n = 4 ± S.D.

The results do not indicate a significant difference in sphere association over the range 4 to 120 x10^4 cells per well, indicating that not all cells are internalising the spheres. Hughes *et al.*, (1995) report that the association of free radiolabelled ODNs in cultured murine spleen and lymph node cells was heterogeneous, some cells having high levels while others having no detectable ODN, suggesting that the cells are not equally phagocytic. A second possible reason for similar association of spheres over the cell range of 4 to 120 x10^4 cells per well could be that the spheres appeared to settle and concentrate in the central regions of the well during

incubation, only cells in direct contact with the spheres are capable of being phagocytosed by the macrophages.

In all subsequent experiments, cells were plated at $5x10^5$ cells $well^{-1}$, at approximately 80% confluency at the time of sphere addition.

7.4 INHIBITORS OF MICROSPHERE ASSOCIATION

7.4.1 TEMPERATURE

Internalisation of spheres at a dose of 0.5ml of 5mg/ml was assessed at 37°C and 4°C over 24 hours, to determine if the spheres were actually being internalised or just adsorbed to the cell surface. Phagocytosis of spheres by macrophages is known to be an active process (Auger and Ross, 1992) and is greatly suppressed at low temperatures (Silverstein *et al.*, 1977).

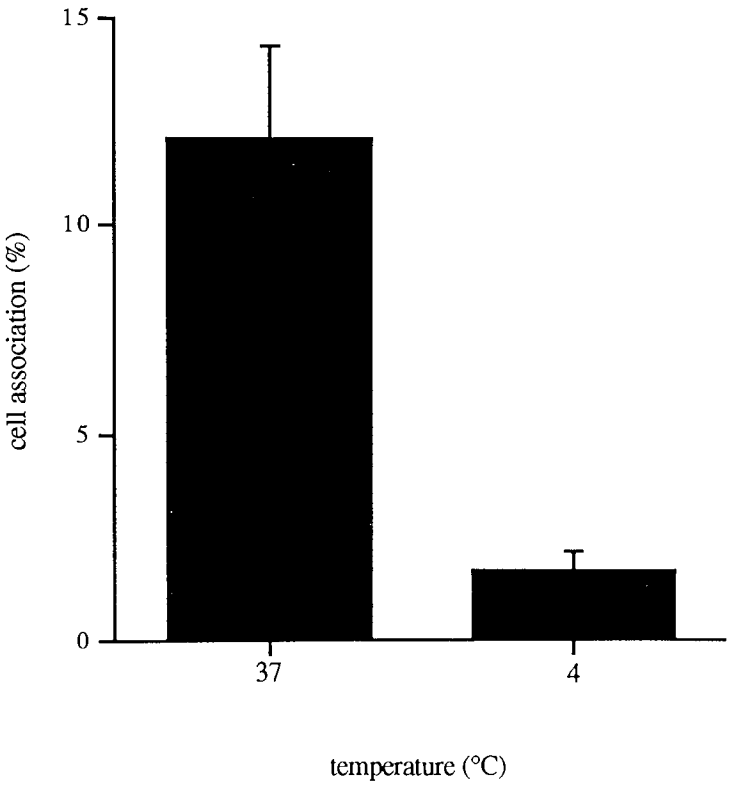


Fig.7.9 The effect of temperature 37 and 4°C on the association of 2µm PLGA 50:50 spheres (Mw 3,000) in RAW 264.7 cells at $5x10^5$ cells $well^{-1}$. n = 4 ± S.D.

The cellular association of the 2μm spheres was analysed at 4°C and 37°C (Fig. 7.9). The results showed that the association of the ODN was reduced at 4°C compared to 37°C, these results agree with those of Tabata and Ikada (1988 a), indicating that the internalisation of the spheres by the cells is temperature dependent and probably occurs by an active mechanism such as an endocytic/phagocytic process.

The magnitude of association at 4°C (approx 2%), probably represents cell associated spheres, or released ODN from spheres which were tightly associated with the cell surface, and could not be removed during the washing process. These results also suggest that the cells remain viable during the 24 hour incubation with the spheres, as association in dead cells is not an active process and would not be expected to differ between 4°C and 37°C, this further confirms the results found in the MTT and crystal violet assays.

7.4.2 CHEMICAL INHIBITION

Metabolic inhibitors were added to the cells to gain further evidence that the spheres were being internalised by an active mechanism and not simply adsorbed on to the cell surface. The inhibitors were added to the media at a concentration of (1) colcemid 50μg/ml, (mitotic inhibitor) it is a cholchicine derivative with a faster rate of action (Ray *et al.*, 1984) and (2) 10mM sodium azide and 50mM 2-deoxyglucose (metabolic inhibitor) (Silverstein *et al.*, 1977). The cells were pretreated for 3 hours before addition of 0.5ml of 5mg/ml sphere suspension in media containing the inhibitors and incubated at 37°C for 24 hours.

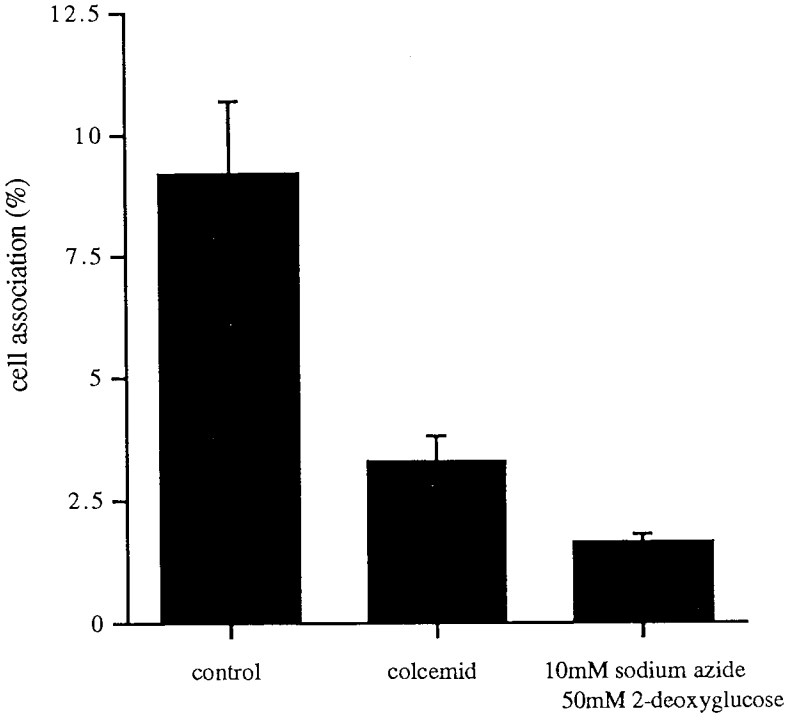


Fig.7.10 The effect of phagocytosis inhibitors on association of PLGA 50:50 spheres, (Mw 3,000) in RAW 264.7 macrophages after 24 hours at 37°C. Concentration of colcemid = 50μg/ml, 10mM sodium azide and 50mM 2-deoxyglucose in serum containing media. The cells were incubated for 3 hours in media with inhibitor before addition of spheres in inhibitor containing media. n = 4 ± S.D.

The results in Fig.7.10 indicate that the addition of metabolic inhibitors dramatically reduces the percentage association of the spheres. Sodium azide and 2-deoxy-glucose resulted in the greatest degree of inhibition compared to colcemid. These results provide supporting evidence to suggest that the spheres are being internalised by an active process and are not just being adsorbed onto the cell surface.

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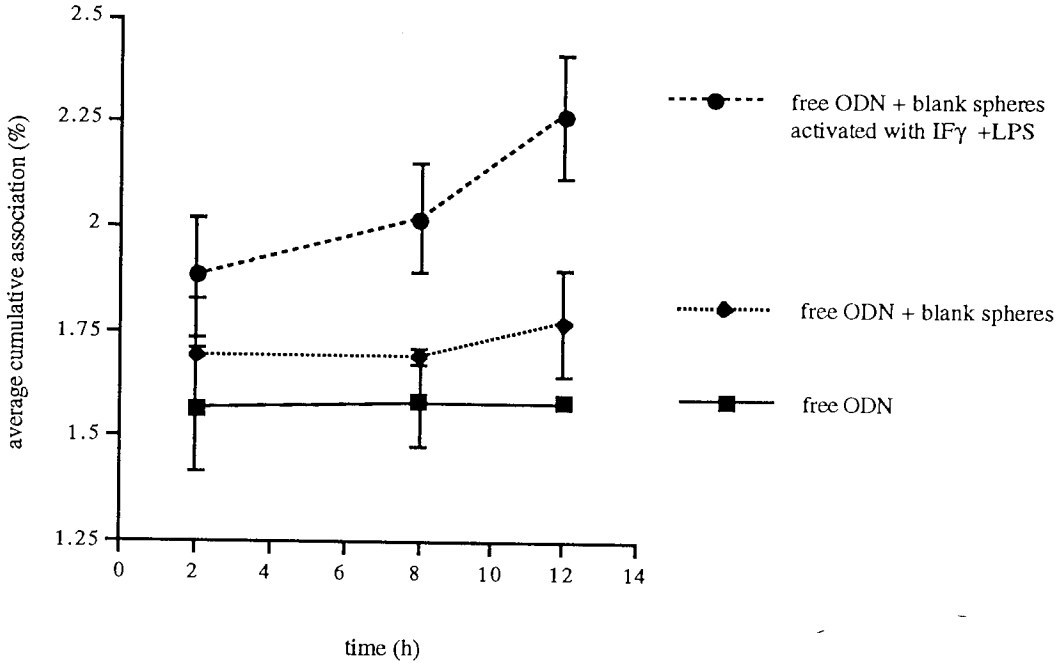

Fig.7.12 The effect of 2μm PLGA 50:50 blank spheres (Mw 3,000) and macrophage activation on the cellular association of unencapsulted ODN in Raw 264.7 macrophages at 37°C. A volume of 0.5ml of 5mg polymer /ml was added with or without Ifγ and LPS, in serum free media with free stabilised ODN. n = 4 ± S.D.

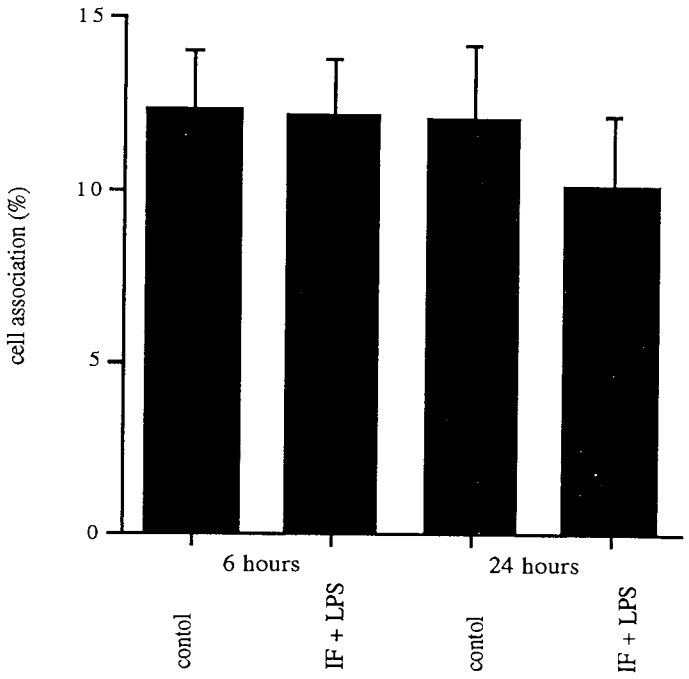

Fig.7.13 The effect of addition of Ifγ and LPS on macrophage association of microspheres after 6 and 24 hours. n = 4 ± S.D.

1.5% to 1.75% in the presence of blank spheres to 2.25% with the addition of IFγ and LPS with blank spheres after 12 hours. The increased association could be explained by the presence of spheres stimulating phagocytosis and the ODN being co-internalised with the spheres. IFγ and LPS stimulate the phagocytotic mechanism of the spheres (Auger and Ross, 1992) and as a result an increased amount of free ODN was internalised with the spheres. The addition of Ifγ and LPS to ODN encapsulated microspheres (Fig.7.13) did not appear to further enhance the association of the spheres.

7.6 RELEASE OF OLIGONUCLEOTIDE FROM SPHERES AFTER PHAGOCYTOSIS BY MURINE MACROPHAGES

After internalisation the particles accumulate in phagosomes, it is important to determine how much of the ODN loaded into the spheres is free inside the cell after internalisation, and how much is released with time. Only free ODN is capable of escaping from the phagosome and hybridising to its target sequence inside the cell.

The experimental procedure is detailed in section 2.11.3, a modification of the association method was used in order to distinguish between the amount of ODN loosely associated with the cell, internalised in spheres or free internalised ODN.

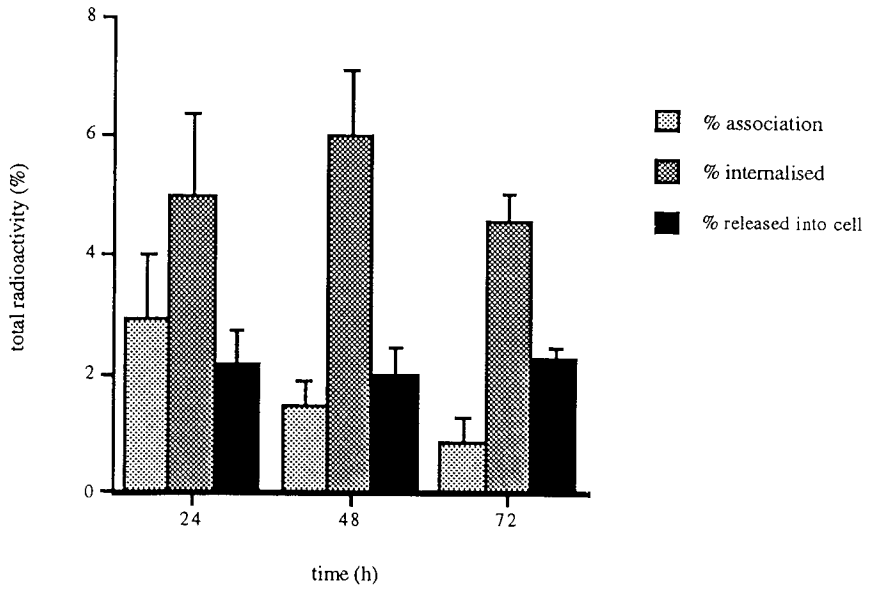

Fig.7.14 Determination of the percentage of radioactivity associated with the cell surface, internalised and released. 2μm spheres PLGA, (Mw 3,000) and RAW 264.7 cells at $5x10^5$ cells $well^{-1}$. n = 6 ± S.D.

The percentage of free ODN inside the cell does not appear to increase with time. Cells can release internalised ODNs, and efflux occurs at a similar rate to the binding process (Yakubov *et al.*, 1989). If release of ODNs from microspheres does occur inside the cell, this could be explained by the possibility that release of ODN from the sphere is proportional to efflux of ODN out of the cell. Hence the concentration of ODN in the cell remains constant over the 72 hour studied. Cellular association decreased with time, possibly due to either further internalisation of the spheres or removal of associated spheres by the addition of fresh media after 24 and 48 hours. Changes in the percentage of ODN internalised increases at 48 hours compared to 24 hours, as a possible result of internalisation of the spheres associated with the sphere surface. The decreased value at 72 hours could be explained as a result of efflux of released ODN out of the cell (Temsamani *et al.*, 1994), or removal of degraded ODN from within the cell.

The cellular association and internalisation of ODN-loaded microspheres spheres is not clearly defined, as the resulting association of the ODN with the cell is a combination of (1) internalisation of the microspheres, (2) internalisation of ODN released from microspheres in the media, (3) efflux of either free ODN or (4) ODN released from the microsphere inside the cell. This concept as a result of fig.7.14 is explained diagramatically in fig.7.15.

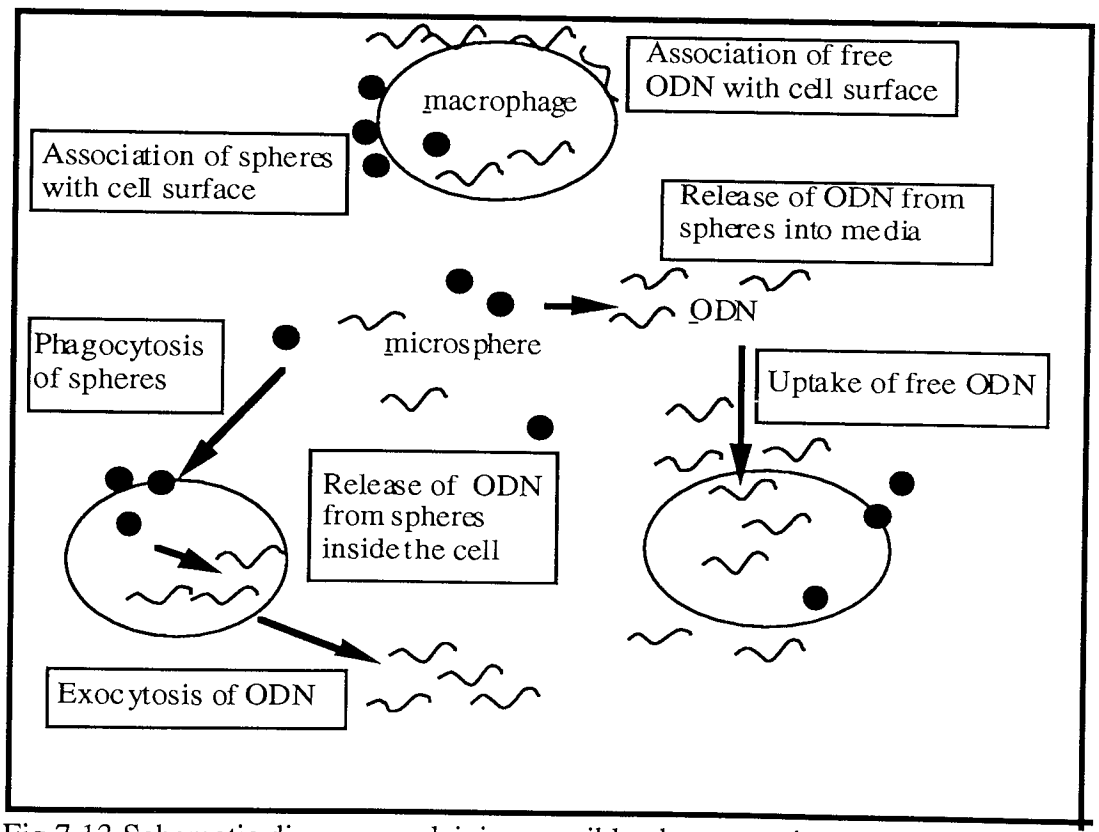

Fig.7.13 Schematic diagram explaining possible phagocytosis of microspheres by murine macrophages and release of entrapped oligodeoxynucleotide.

7.7 LOCALISATION OF OLIGONUCLEOTIDE IN CELL

A fluorescently labelled PS 15mer ODN was used to visually determine the localisation of free ODN and ODN-loaded polymer microspheres in murine macrophages as in section 2.11.4. To further determine the cell localisation of encapsulated and free ODN, the cell nucleus was stained with propidium iodide.

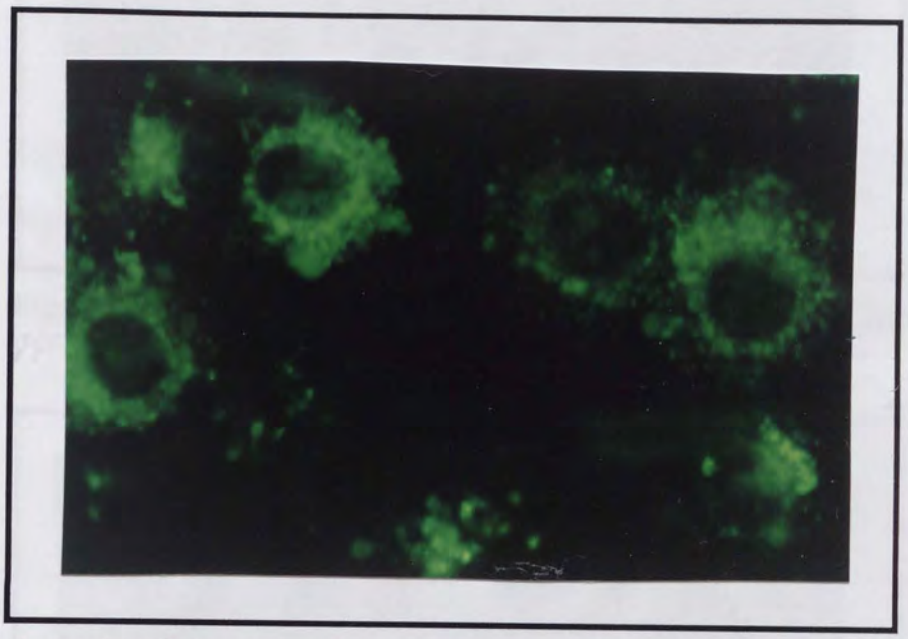

Fig.7.16 Cellular association of free fluorescent 15 PS ODN with RAW 264.5 macrophages, magnification X100

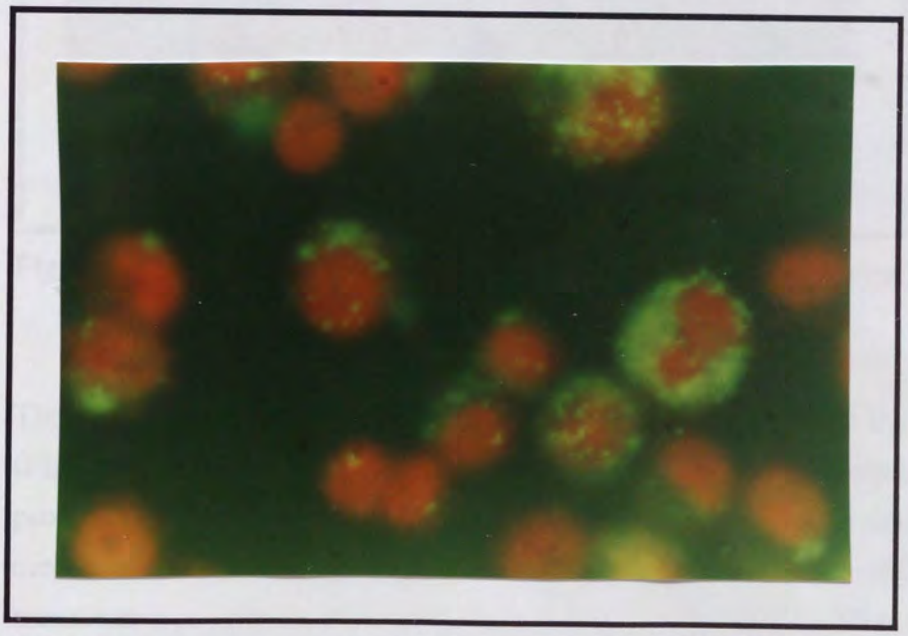

Fig 7.17. Cellular association of fluorescent 15 PS ODN in RAW 264.7 macrophages, stained with propidium iodide, 510nm filter. Magnification X100.

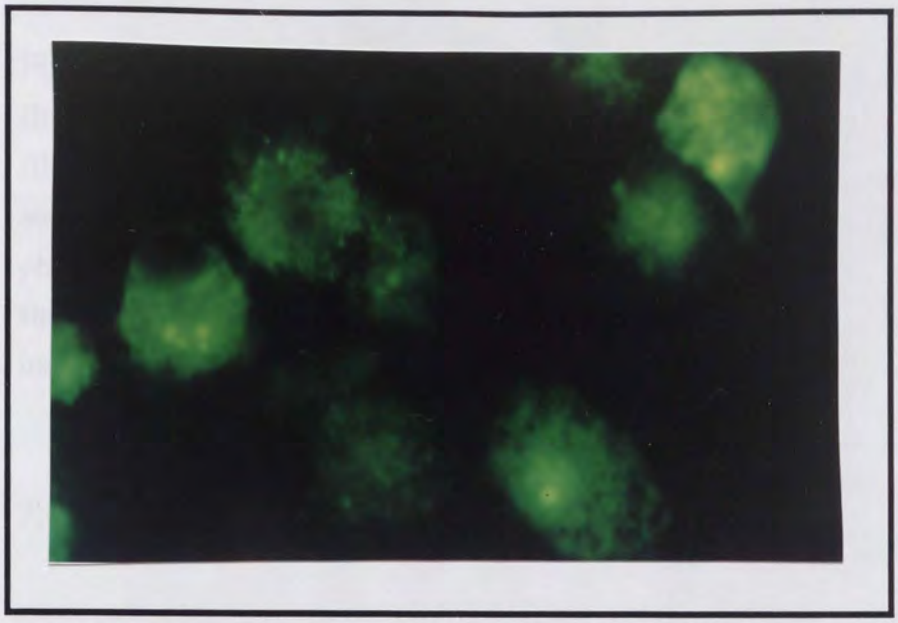

Fig.7.18 Cellular association of fluorescent 15 PS ODN-loaded 2µm PLGA microspheres in RAW 264.7 macrophages. Magnification X100.

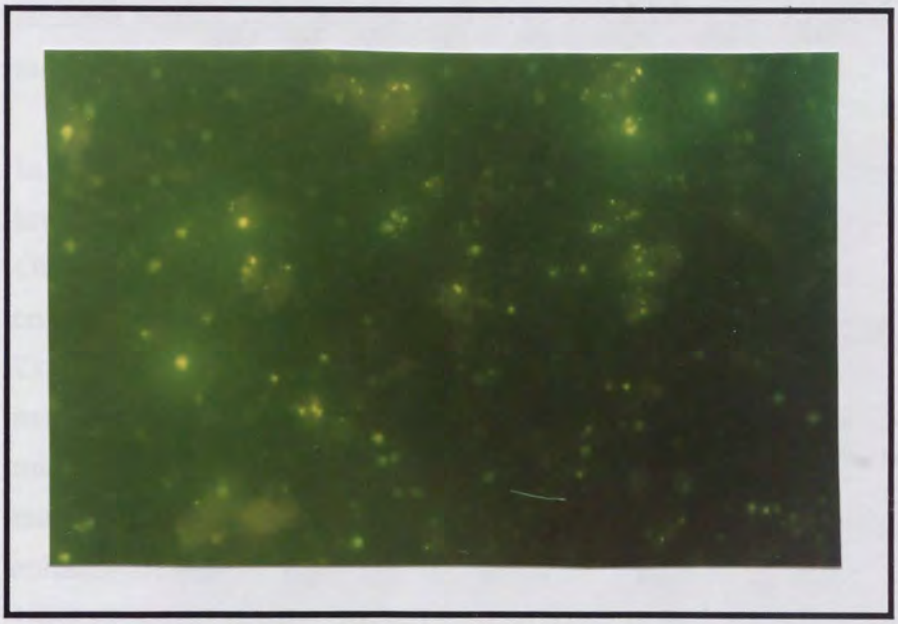

Fig 7.19 Fluorescence of PLGA 2µm microspheres. Magnification X 50

The free PS 15 mer fluorescently labelled ODN is concentrated at the cell membrane (Fig.7.16), with no fluorescence evident within the cell itself. Staining with propidium iodide (Fig.7.17) confirms that the ODN is associated with the cell membrane and not the nucleus. PS ODNs are known to associate with cell membranes prior to their internalisation (Zhao *et al.*, 1993). Cells exposed to spheres (fig.7.18), show a greater diffused fluorescence across the cell compared to the control (fig.7.16), where the fluorescence appears to be concentrated at the cell membrane.

Fig.7.19 shows that the microspheres produced in section 4.2.2.4 with fluorescently labeled ODN were fluorescent when visualised under a fluorescent filter, indicating that some of the entrapped ODN is located near or on the sphere surface. Any fluorescence seen in macrophages exposed to spheres, represent phagocytosed or associated microspheres, however as the spheres were naturally fluorescent this does not indicate that all the fluorescence is due to ODN released inside the cells from the spheres following internalisation.

7.8 CONCLUDING REMARKS

In all the studies described in this chapter it was assumed that the label is predominately associated with the ODN at all time points (verified by stability studies Chapter Six). The presence of the spheres does not adversely effect the cell number or the cell metabolism.

In this chapter it has been shown that 2μm spheres loaded with ODN are internalised by murine macrophages by phagocytosis, and some of the entrapped ODN is released inside the cell. It has also been shown that the use of spheres can enhance the delivery of ODNs to macrophage cells compared to free ODN alone. Coating microspheres with gelatin (Tabata *et al.*, 1989) has been shown to enhance macrophage association of microspheres, as a result ODN loaded gelatin microspheres (Cortesi *et al.*, 1994) may have potentially greater association in macrophage cells. Surface modification of the microspheres could be used to further enhance the cellular association of the ODN-loaded microspheres (Tabata *et al.*, 1988 a).

Spheres less than 10μm in size can be adsorbed via the peyer's patches following oral administration (Eldridge *et al.*, 1990) and therefore micropsheres can have a potential use in the delivery ODNs to specific areas in the gut.

Therefore entrapment of ODNs in microspheres offers the potential for enhancing cellular delivery of ODNs and therefore efficacy.

GENERAL DISCUSSION

The *in vivo* therapeutic applications of ODNs and RBZs are severely limited by their poor biological stability (Wickstrom, 1986), short half life in the circulation (Agrawal *et al.*, 1992) and limited cellular uptake (Stein *et al.*, 1988). Studies have shown (Troy *et al.*, 1992; Higgins *et al.*, 1993) that ODNs need to be incubated at the target site for extended time periods inorder to achieve a biological effect, especially for targets with a turnover. Chemical modifications to enhance stability and *in vivo* half lives, often lead to a reduction in antisense activity or toxicity (Maher and Dolnick, 1991; Dagle *et al.*, 1991).Therefore a sustained release delivery system which protects the nucleic caid from degradation enabling minimum chemical modification, with the potential to enhance cellular delivery and uptake to certain cell lines would be advantageous. A further need for a sustained delivery device was highlighted in a study by Galbraith *et al.*(1994), where a rapid IV infusion resulted in unwanted side effects.

Biodegradable polymers have a potential role in the delivery of antisense nucleic acids *in vivo* as they have the ability to remove some of the delivery problems associated with antisense therapy. Injection of a polymeric device close to a tumour site would enable localised sustained delivery (Brem, 1990; Fournier *et al.*, 1991), alternatively IV delivery of polymer microspheres would enable passive targeting to the liver and other reticuloendothelial organs (Illum *et al.*, 1987; Davies *et al.*, 1993).

The aim of the research in this thesis was to make and optimise biodegradable polymer delivery devices using various polymers, PLA, PLGA, PHB and P(HB-HV). Antisense ODNs and RBZs were entrapped in polymer films and microspheres, entrapment efficiency, subsequent release and stability of the nucleic acids were assessed.

The only published study to date which incorporates the use of a polymer for ODN delivery, are polycyanoacrylate nanospheres described by Chavany *et al.* (1992 and 1994). However the loading of the ODN on the spheres and the subsequent dose to be delivered are limited by the toxicity of the ion pairs used to adsorb the ODNs onto the surface of the polymer. Therefore a double emulsion method to entrap the ODN within a PLGA matrix was investigated as part of this thesis, with the

proposed advantage of reduced potential toxicity due to the absence of ion pairs and the use of a biodegradable polymer with an established safety profile. The double emulsion method adapted from Spenlehauer *et al.* (1989) enabled a high ODN entrappment within the polymer. Loading was not saturated over a range of 0.08 to 8μg of added ODN, with an entrappment efficiency of over 60%, independent of ODN sequence or molecular weight in spheres of 10 to 20μm in diameter. Smaller spheres of size 1 to 2μm resulted in a reduced efficiency of loading of approximately 30%. The release of ODNs from PLGA microspheres was typical of release of a hydrophilic drug from a microsphere preparation. The release profile was triphasic, with an initial burst release, a secondary stage of slower sustained release followed by an increased rate of release corresponding to the collapse of the polymer matrix. An increased loading resulted in an increased burst and subsequent rate of release (Tsai *et al.*, 1996; Okada *et al.*, 1994), a reduction in polymer molecular weight caused a slight decrease in burst and release rate (Cohen *et al.*, 1991). The most dramatic effect was seen with reduced microsphere size giving a greatly enhanced burst release, as a result of the increased surface area (Alonso *et al.*, 1993). Release of ODNs from PLGA microspheres could be tailored to meet specific requirements by incorporating a combination of different microsphere sizes, polymer molecular weight and ODN entrappment. The release from the polymer microspheres could be further controlled by the formation of double walled microspheres (Pekarek *et al.*, 1994). Chavany *et al.* (1992 and 1994) did not report on the release of the ODNs from the nanospheres. Entrapment of ODNs in gelatin microspheres has been reported by Cortesi *et al.* (1994), however release of the ODN from the gelatin microspheres was rapid, providing little sustained release.

Polymeric spheres have the potential to enhance cellular delivery of nucleic acids compared to free nucleic acids in certain cell types. Chavany *et al.* (1994) report that the use of nanospheres increased the delivery of ODNs to macrophage like cell lines compared to free ODNs. Kimura *et al.* (1994) report that the optimum sphere size for macrophage phagocytosis is 1-2μm, as a result the macrophage uptake of ODN loaded 1-2μm spheres was investigated. The sphere suspension in the culture media associated with the cells over a 24 hour incubation period. The cells appeared to have a maximum load for polymer uptake, $5x10^5$ cells taking up approx 250μg of polymer independent of the amount of polymer added. A dose of 2.5 mg polymer (5mg/ml) resulted in approximately 10% cell association compared to only 1% of the equivalent free ODN over 24 hours at 37°C. Uptake of the spheres at 4°C was only 2% and phagocytosis inhibitors (10mM sodium azide and 50mM 2-deoxyglucose) inhibited cellular association by 75%. These results indicate that the

microspheres were being internalised and not simply absorbed to the cell surface. The polymer molecular weight did not effect the association of the spheres with the cells. Fluorescent microscope pictures show that the PS ODNs entrapped in small microspheres were concentrated inside the cell, compared to the unencapsulted PS ODNs which are concentrated at the cell membrane. Although the spheres have entered the cell the ODN must first be released from the microsphere and reach its target site before it can exert its action. Only 3% of the total ODN associated with the cell in the microspheres is present in an unencapsulted form inside the cell after 24 hours incubation, although this figure is low, it is still greater than the internalisation of unencapsulated ODNs. The main disadvantage of this type of delivery system is that the ODN associated with the polymer may become entrapped within endosomes and be unable to reach its appropriate target site.

The solvent cast films produced from PLA, PHB and P(HB-HV)were approximately 100μm in thickness, release from the films was biphasic, characterised by an initial burst release corresponding to the ODN concentrated at the film surface. The secondary phase of release was sustained over a longer period. An increase in the initial loading of ODN in the polymer film resulted in an increased burst release and the subsequent rate of release. The total release of the ODN will be dependent on the degradation of the polymer films. No degradation was evident over the 28 days of the study, when incubated in PBS, foetal calf serum and citrate phosphate buffer (as determined by electron microscopy and DSC), this is probably due to the high molecular weight and crystallinity of the polymers needed to form films by a solvent casting method. Release in the secondary phase could be enhanced by the use of ultrasound (Kost *et al.*, 1989; Supersaxo *et al.*, 1993) or by blending with polysaccharides (Holland *et al.*, 1990) to accelerate the rate of polymer degradation. During the second period of release, the rate of release of shorter lower molecular weight ODNs (e.g. 7 *myc*, molecular weight 1895) was faster than ODNs with a higher molecular weight (e.g. 32 *myc*, molecular weight 10400). The shorter sequences can diffuse through the polymer matrix more easily compared to the longer sequences which are entraped to a greater extent within the polymer matrix. Increasing the sequence length resulted in an early plateau in the release profile. The backbone type also effected the release profile, although to a lesser extent than backbone length. PS ODNs were released more slowly compared to an equivalent PO ODN, possibly due to the increased lipophilicity of the PS ODN having a greater affinity for the hydrophobic polymer matrix. The ODN sequence also effects the release of ODNs from PLA films, the release of a 7mer homo G sequence was released more slowly than equivalent homo

A, C or T sequences. The greater hydrophobicity of the G bases (Peyman *et al.*, 1995) appear to have an increased affinity for the polymer matrix, which may serve as a possible explanation for the differences in release. The burst release of a PO 20mer ODN from PHB and P(HB-HV) films was greater than from PLA films, possibly indicating that the ODN entrapment is not as efficient in PHB polymer matrices compared to PLA. The subsequent rate of release of the ODN from PHB and P(HB-HV) films was slower compared to the release from PLA films, the reduced water uptake by the PHB polymers compared to the PLA polymer may reduce the ability of the ODN to diffuse through the polymer matrix. The release media either PBS, serum or citrate phosphate buffer did not effect the release profiles.

The release of a 32 mer all RNA hammerhead RBZ from PLA films was also biphasic, again the initial loading in the polymer film effected the release profile. The release profile of the RBZ was similar to that of an equivalent DNA sequence, indicating that the differences between RNA and DNA were not sufficient to alter the release from PLA polymer matrices. As a result, it can be concluded that ODN chemistry, sequence and length must be taken into account when predicting the release of ODNs from polymeric films.

The stability of the polymer entrapped nucleic acid is a prerequisite for the success of polymer delivery systems for antisense nucleic acids. The polymer entrapped unmodified 20mer ODN was used as a model ODN due to the lack of inherent stability, had enhanced stability compared to non polymer entrapped ODNs. The polymer (P(HB-HV) and PLA) film entrapped PO 20mer *tat* ODN remained stable, with the full length ODN present after 28 days incubation in foetal calf serum. The RBZ had increased stability when entrapped in PLA films and incubated in serum compared to the free RBZ which degrades rapidly within minutes. However there was no detectable intact RBZ present in the polymer film after 14 days, suggesting that the stability of the RNA is not as great as DNA in the polymer films. ODN stability in microspheres 10-20μm in size was not as great as in the high molecular weight polymer films with degradation products evident after 14 days incubation in serum containing media. The reduced stability of the ODNs in the microspheres is most probably as a result of degradation of the lower molecular weight polymer and the larger surface area compared to the films, giving rise to greater exposure to degradation by the incubation media. The stability of the microsphere loaded ODN could be enhanced by the use of a chemical modifications if longer incubation periods were necessary.

One of the main advantages of the use of biodegradable polymer delivery devices is the stability offered by entrapment within a polymer matrix, reduces the need for drastic chemical modifications to the nucleic acids, which often lead to reduced antisense effects. It is known that some chemical modifications reduce the affinity of the ODN to its target sequence (Marshall and Caruthers, 1993). Therefore it is important to confirm that the formulation process of films and micropsheres does not adversely effect the action of the released nucleic acids. The released PO ODN from either polymer films or microspheres was still capable of hybridising to its target sequence as determined by a gel shift mobility assay and T_m measurements. The RBZ released from the polymer film was also still capable of hybridising to and cleaving its target sequence. Therefore the affinity of the ODN and RBZ for the target sequences was not affected by the fabrication procedure.

In order to maximize the delivery of ODNs by polymer matrices, a high loading of ODN / polymer would be beneficial. Microspheres were loaded with a PS 24 mer ODN against the p65 subunit of NF-kB 5µg/mg of polymer for *in vivo* studies (appendix 1), with the aim of reducing tumour size. Unfortunately the ODN loaded microspheres had no effect on the tumour size. However, the lack of an antisense effect may be as a result of errors in the experimental protocol.

Entrapment of ODN conjugates within poymer devices may provide the ultimate delivery system, with the polymer device providing sustained delivery and protecting the entrapped nucleic acid from degradation. Following the release the conjugated targeting moiety (for example antibodies, transferrin, mannose), would enbale internalisation in specific cells. Capping of the ODN at the exposed unconjugated end (3') could be used to enhance stability once the conjugate is released from the polymeric device. Specific targeting of micropsheres could be an alternative approach, coating of the spheres with a hydrophilic polymer (poloxamine) could be used to extend circulation times, while a targeting moiety attched to the sphere surface directs the polymeric device to a specific cell population. However this approach does not overcome the problems associated with subsequent release of the microsphere loaded nucleic acid from endosomal compartments once inside the cell.

In conclusion ODNs and RBZs were successfully incorporated into polymer films and microspheres and were released in a sustained manner over the 28 day period studied. The polymer device protected the entrapped nucleic acids prior to release

without affecting their ability to hybridise to their target sequence. Entrappment and release is dependent on both polymer and nucleic acid properties, these factors will need to be considered in predicting release from biodegradable polymer delivery devices. Therefore biodegradable polymers have the potential to play a role in the successful therapeutic application of antisense ODNs and RBZs.

SUGGESTIONS FOR FURTHER WORK

Although the results in this thesis appear promising, further research is necessary to optimise polymer film and micropsheres for use as ODN and RBZ delivery devices.

Optimisation of a higher loading in polymer microspheres of different sizes would be advantageous, as the initial *in vivo* studies highlighted the problems of having a high polymer mass for the delivery of the required dose of ODN. Formulation of microspheres with a high nucleic acid entrapment and a reduced burst release would also be a major advantage.

It would be interesting to investigate the degree of cellular association and internalisation of the microspheres in other cell types, as cellular association of ODNs is known to vary between cell types. Evidence of an *in vitro* antisense effect following release from a polymeric device would be needed to confirm the non destructive nature of these delivery systems on the entrapped nucleic acids.

Further experiments assessing the *in vivo* release and distribution of the ODN and the microspheres following administration at various injection sites would be necessary before repeating the experiments with sterilised spheres in close proximity to the growing tumour.

REFERENCES

Agrawal, S. and Sarin, P. S. (1991) Antisense oligonucleotides: gene regulation and chemotherapy of AIDS. Advanced Drug Delivery Reviews 6, 251-270.

Agrawal, S., Temsamani, J., and Tang, J.Y. (1992) Pharmacokinetics, biodistribution and stability of oligodeoxynucleotides phosphorothioates in mice. Proceedings of the National Academy of Scieces USA, 88, 7595-7599.

Akhtar, S. and Pouton, C. (1989) Polyhydroxybutyrate: Potential in drug delivery of macromolecular peptides. Drug News and Perspectives, 2, 89-93.

Akhtar, S., Basu. S., Wickstrom, E. and Juliano, R. L. (1991a) Interactions of antisense DNA oligonucleotide anologs with phospholipid membranes (liposomes). Nucleic Acids Research 19, 5551-5559.

Akhtar, S., Kole, R., and Juliano, R.L. (1991b) Stability of antisense DNA oligodeoxynucleotide analogs in cellular extracts and sera. Life Sciences, 49, 1793-1801.

Akhtar, S., Pouton, C. and Notariannni, L. J. (1991c) The influence of crystalline morphology and copolymer composition on drug release from solvent cast and melt processed P(HB-HV) copolymer matrixes. Journal of Controlled Release, 17, 225-234.

Akhtar, S. and Juliano, R.L. (1992 a) Liposome delivery of antisense oligonucleotides: adsorption and efflux characteristics of phosphorothioate oligodeoxynucleotides. Journal of Controlled Release 22,47-56.

Akhtar, S. and Juliano, R. L. (1992) Cellular uptake and intracellular fate of antisense oligonucleotides. Trends in Cell Biology, 2, 139-144.

Akhtar, S., Pouton, C. and Notariannni, L. J. (1992) Crystallization behaviour and drug release from polyhydroxyalkanoates. Polymer, 33, 117-126.

Akhtar, S., Routledge, A., Patel, R. and Gardiner, J.M. (1995) Synthesis of mono- and dimannoside phosphoramidite derivatives for solid-phase conjugation to oligonucleotides. Tetrahedron Letters, 36, 7333-7336.

Alléman, E., Doelker, E. and Gurny, R. (1993) Drug loaded poly(lactic acid) nanoparticles produced by a reversible salting-out process: purification of an injectable dosage form. European Journal of Pharmaceutics and Biopharmaceutics, 39, 13-18.

Alonso, M.J., Cohen, S., Park. T.G., Gupta, R.K., Siber, G.R. and Langer, R. (1993) Determinants of release rate of tetanus vaccine from polyester microspheres. Pharmaceutical Research, 10, 945-953.

Arshady, R. (1991) Preparation of biodegradable microspheres and microcapsules: 2. Polylactides and related polyesters. Journal of Controlled Release, 17, 1-22.

Aso, Y., Yoshioka, S. and Terao, T. (1993) Effects of storage on the physicochemical properties and release characteristics of progesterone-loaded poly (l-lactide) microspheres. International Journal of Pharmaceutics, 93,153-159.

Aso, Y., Yoshioka, S., Li Wan Po, A. and Terao, T. (1994) Effect of temperature on poly(D,L-lactide) microspheres. Journal of Controlled Release, 31, 33-39.

Auger, M.J. and Ross, J.A. (1992) The Biology of the macrophage. In: The Macrophage, The natural immune system. Lewis, C.E. and O'D McGee, J. (Eds) IRL Press at Oxford University Press.

Baker, R.W. (1987) Controlled release of bioactive agents, 50-75. John Wiley and Sons, New York. (Equations 3.14, 3.16, 3.35).

Bacon, T.A., Morvan, F., Rayner, B., Imbach, J.L. and Wickstrom, E. (1988) α-Oligonucleotide stability in serum, subcelluler extracts and culture media. Journal of Biochemical and Biophysical Methods 16, 311-318.

Bayever, E., Iverson, P., Smith, L., Spinolo, J. and Zon, G. (1992) Systemic Human Antisense Therapy Begins. Antisense Research and Development, 2, 109-110.

Bazile, D.V., Ropert, C., Huve, P., Verrecchi, T., Marlard, M., Frydman, A., Veillard, M. and Spenlehauer, G. (1992) Body distribution of fully biodegradable (^{14}C)-poly(lactic acid) nanoparticles coated with albumin after parental administration to rats. Biomaterials, 13, 1093-1102.

Bender, A., Schafer, V., Steffan, A.M., Royer, C., Kreuter, J., Rubsamen-Waigman, H., von Briesen, H. (1994) Inhibition of HIV *in vitro* by antiviral drug-targeting using nanoparticles. Research in Virology, 145, 215-220.

Bennet, C.F., Chiang, M-Y., Chan, H., Shoemacer, J.E.E. and Mirabelli, C.K. (1992) Molecular Pharmacology, 41-1023-1033.

Bhardwaj, S.B., Shukla, A.J. and Collins, C.C. (1995) Effect of varying drug loading on particle size distribution and drug release kinetics of verapamil hydrochloride microspheres prepared with cellulose esters. Journal of Microencapsulation, 12, 71-81.

Bodmeier, R., Oh, K.H. and Chen, H. (1987) The effect of the addition of low molecular weight poly(DL-lactide) on drug release from biodegradable poly (DL-lactide) drug delivery systems. International Journal of Pharmaceutics, 51, 1-8.

Bodmeier and McGinity (1988) Solvent selection in the preparation of poly(DL-lactide) microspheres prepared by the solvent evaporation method. International Journal of Pharmaceutics, 43, 179-186.

Boiziau, C. and Toulmé, J.J. (1991) Modified oligonucleotides in rabbit reticulocytes: uptake, stability and antisense properties. Biochimie, 73, 1403-1408.

Bolton, E.T. and M^{c}Carthy, B.J. (1962) A general method for the isolation of RNA complementary to DNA. Proceedings of the National Academy of Sciences USA, 48, 1390-1397.

Bonfils, E., Mendes, C., Roche, A.C., Monsigny, M. and Midoux, P. (1992 a) Uptake by macrophages of a biotinylated ODN-α-deoxythymidylate by using mannosylated steptavidin. Bioconjugate Chemistry, 3, 277-284.

Bonfils, E., Depierreux, C., Midoux, P., Thuong, N.T., Monsigny, M. and Roche, A.C. (1992 b) Drug targeting: synthesis and endocytosis of

oligonucleotide-neoglycoprotein conjugates. Nucleic Acids Research, 20, 4621-4629.

Bongartz, J., Aubertin, A., Milhaud, P.G., and Lebleu, B. (1994) Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide. Nucleic Acids Research, 22, 4681-4688.

Boutorine, A.S., Boiziau, C., Le Doan, T., Toulme, J.J . and Hélène, C. (1992) Effect of the terminal phosphate derivitization of β and α -oligonucleotides on their antisense activity in protein biosynthesis, stability and uptake by eukaryotic cells. Biochemie, 74, 485-489.

Brannon-Peppas, L. (1995) Recent advances on the use of biodegradable micro-particles and nanoparticles in controlled drug delivery. International Journal of Pharmaceutics, 116, 1-9.

Bratty, J., Chartrand, P., Ferbeyre, G., and Cedergren, R. (1993) The hammerhead RNA domain, a model ribozyme (review). Biochimica et Biophysica Acta, 1216, 345-359.

Brem, H. (1990) Polymers to treat brain tumours. Biomaterials, 11, 699-701

Brem, H., Mahaley, M.S., Vick, N.A., Black, K.L., Schold, C., Burger, P.C., Friedman, A., Ciric, I.S., Eller, T.W., Cozzens, J.W. and Kenealy, J.N. (1991) Intersititial chemotherapy with drug polymer implants for the treatment of recurrent gliomas. Journal of Neurosurgery, 74, 441-446.

Breslauer, K.J. (1986) Methods for obtaining thermodynamic data on oligonucleotide transitions. In: Thermodynamic data for biochemistry and biotechnology. (Hinz, H. Ed.) Springer-Verlag, Berlin, Heidelberg, New York and Tokyo.

Brown, T. and Brown, D.J.S. (1991) Modern machine-aided methods of synthesis. In: Oligonucleotides and Analogues. A Practical Approach, Eckstein, F (Ed) IRL Press, Oxford University Press.

Budker, V.G., Knorre, D.G. and Vlassov, V.V. (1992) Cell membranes as barriers for antisense constructions. Antisense Research and Development, 2, 177-184.

Buzayan, J.M., van Tol, H., Feldstein, A. and Brueing, G. (1990) Identification of a non-junction phosphodiester that influences an autocatalytic processing reaction of RNA. Nucleic Acids Research, 18, 4447-4451.

Byrn, S.R, Carlson, D.V., Chen, J.K., Cushman, M.S., Goldman, M.E., Ma, W.P., Pidgeon, C.L., Ray, K.A., Stowell, J.G. and Weith, H.L. (1991) Drug-oligonucleotide conjugates. Advanced Drug Delivery Reviews, 6, 287-308.

Calabretta, B., Skorski, T., Szczylik, C . and Zon, G. (1993) Prospects for gene-directed threapy with antisense oligodeoxynucleotides. Cancer Treatment Reviews, 19, 169-179.

Campbell, J.M., Bacon, T.A. and Wickstrom, E. (1990) Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera, and cerebrospinal fluid. Journal of Biochemical and Biophysical Methods, 20, 259-267.

Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T. and Quattrone, A. (1993) Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. Biochemical and Biophysical Research Communications, 197, 818-825.

Carter, G. and Lemoine, N.R. (1993) Antisense technology for cancer therapy: does it make sense ? (review) British Journal of Cancer 67, 869-876.

Caruthers, M.H. (1989) Synthesis of oligonucleotides and oligonucleotide analogues. In: Oligodeoxynucleotides - Antisense Inhibitors of Gene Expression. Topics in Molecular and Structural Biology 12. Cohen, J.S. (Ed). The Macmillan Press Ltd., London.

Cazenave, C., Stein, C.A., Loreau, N., Thuong, N.T., Neckers, L.M., Subasinghe, C., Hélène, C., Cohen, J.S. and Toulme, J. (1989) Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides. Nucleic Acids Research, 4255-4273.

Cech, T.R. and Bass, B.L. (1986) Biological catalysis by RNA. Annual Review of Biochemistry 55, 599-629.

Cech, T.R. and Uhlenbeck, O.C. (1994) Hammerhead nailed down. Nature, 372, 39-40.

Chavany, C., Le Doan,T., Couvreur, P., Puisieux, F. and Hélène,C. (1992) Polyalkylcyanoacrylate nanoparticles as polymeric particles for antisense oligonucleotides. Pharmaceutical Research 9, 441-449.

Chavany, C., Saison-Behmoaras, T., Le Doan, T., Puisieux, F., Couvreur, P., and Hélène, C. (1994) Adsorption of oligonucleotides onto polyisohexylcyanoacrylate nanoparticles protects them against nucleases and increases their cellular uptake. Pharmaceutical Research, 11, 1370-1378.

Chen, Y. and Willmott, N. (1987) Comparaison of albumin and casein microspheres as carriers for doxrubicin. Journal of Pharmacy and Pharmacology, 39, 978-985.

Chin, D.J., Green, G.A., Zon, G., Szoka, F.C., Straubinger, R.M. (1990) Rapid nuclear accumulation of injected oligonucleotides. The New Biologist, 2, 1091-1100.

Chiou, H.C., Tangco, M.V., Levine, S.M., Robertson, G., Kormis, K., Wu, C.H. and Wu, G.Y. (1994) Enhanced resistance to nuclease degradation of nucleic acids complexed to asialoglycoprotein-polylysine carriers. Nucleic Acids Research, 22, 5439-5446.

Chu, C.C. (1981) The in-vitro degradation of poly(glycolic acid) sutures-effect of pH. Journal of Biomedical Materials Research, 15, 795-804.

Chu, C.C. (1982) The effect of pH on the in vitro degradation of poly(glycolide lactide) copolymer absorbable sutures. Journal of Biomedical Materials Research, 16, 117-124.

Çiçek, H., Tuncel, A., Tuncel, M. and Piskin, E. (1994) Degradation and drug release characteristics of monosize polyethylcyanoacrylate microspheres. Journal of Biomaterials Science Polymer Edition, 6, 845-856.

Citro, G., Perrotti, D., Cucco, C., D'Agnano, I., Sacchi, A., Zupi, G. and Calabretta, B. (1992) Inhibition of leukemia cell proliferation by receptor-mediated uptake of c-myb antisense oligodeoxynucleotides. Proceedings of the National Academy of Sciences, USA, 89, 7031-7035.

Clarenc, J.P., Degols, G., Leonetti, J.P., Milhaud, P. and Lebleu, B. (1993) Delivery of antisense oligonucleotides by poly(L-lysine) conjugation and liposome encapsulation. Anti-Cancer Drug Design 8, 81-94.

Cohen, I., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R. (1991) Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharmaceutaical Research, 8, 713-720.

Cohen, J.S. (1992) Selective anti-gene therapy for cancer: principles and prospects. Tohoku Journal of Experimental Medicine 168, 351-359.

Conti, B., Genta, I., Modena, T., Pavanetto, F. (1995) Investigation on process parameters involved in polylactide-co-glycolide microspheres preparation. Drug Development and Industrial Pharmacy, 21, 615-622.

Conti, B., Pavanetto, F. and Genta, I. (1992) Use of polylactic/ acid for the preparation of microparticulate drug delivery systems (review). Journal of Microencapsulation, 9, 153-166.

Cooney, M., Gzernuszewicz, G., Postel, E.H., Flint, S.J. and Hogan, M.E. (1988) Site-specific oligonucleotide binding represses transcription of the human *c-myc* gene in vitro. Science, 241, 456-459.

Cortesi, R., Esposito, E., Menegatti, E., Gambari, R. and Nastruzzi, C. (1994) Gelatin microspheres as a new approach for the controlled delivery of synthetic oligonucleotides and PCR-generated DNA fragments. International Journal of Pharmaceutics. 105, 181-186.

Cossum, P.A., Sasmor, H., Dellinger, D., Truong, L., Cummins, L., Owens, S.R., Markham, P.M., Shea, J.P. and Crooke, S. (1993) Dispostion of the ^{14}C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats. The Journal of Pharmacology and Experimental Therapeutics, 267, 1181-1190.

Couvreur, P. and Puisiex, F. (1993) Nano and microparticles for the delivery of polypeptides and proteins. Advanced Drug Delivery Reviews. 10, 141-162.

Crooke, R.M. (1991) *In vitro* toxicology and pharmacokinetics of antisense oligonucleotides. Anticancer Drug Design. 6, 609-646.

Crooke, R.M., Hoke, G.D. and Shoemaker, J.O.E. (1992) *In vitro* toxicological evaluation of ISIS 1082, a phosphorothioate oligonucleotide inhibitor of herpes simplex virus. Antimicrobial Agents and Chemotherapy, March, 527-532.

Crooke, S.T. (1992) Therapeutic applications of oligonucleotides. Annual Reviews in Pharmacology and Toxicology, 32, 329-376.

Crooke, S.T. (1993) Progress towards oligonucleotide therapeutics: pharmacodynamic properties. Faseb Journal 7, 533-539.

Dagle, J.M., Andracki, M.E., DeVine, R.J. and Walder, J.A. (1991) Physical properties of oligonucleotides containing phosphoramidate-modified internucleoside linkages. Nucleic Acids Research, 19, 1805-1810.

Dash, P., Lotan, I., Knapp, M., Kandel., E.R. and Goelet, P. (1987) Selective elimination of mRNA *in vivo*: Complementary oligodeoxynucleotides promote RNA degradation by an RNase H-like-activity. Proceedings of the National Academy of Sciences USA, 84, 7896-7900.

Davis, S.S., Illum, L., Moghimi, S.M., Davies. M.C., Porter, C.J.H., Muir. I.S., Brindley, A., Christy, N.M., Norman, M.E.,Williams, P. and Dunn S.E. (1993) Microspheres for targeting drugs to specific body sites. Journal of Controlled Release. 24, 157-163.

Degols, G., Leonetti, J. and Lebleu, B. (1992) Sequence-specific activity of antisense oligonucleotides conjugated to poly(L-Lysine) carriers. Annals of the New York Academy of Sciences 660, 331-333.

Degols, G., Devaux, C. and Lebleu, B. (1994) Oligonucleotide-poly(L-lysine)-heparin complexes: Potent sequence-specific inhibitors of HIV-1 infection. Bioconjugate Chemistry, 5, 8-13.

Denizot, F. And Lang, R. (1986) Rapid colourimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods, 89, 271-277.

Dervan, P. (1989) Oligonucleotide recognition of double-helical DNA by triple-helix formation. In: Oligodeoxynucleotides - Antisense Inhibitors of Gene Expression. Topics in Molecular and Structural Biology 12. Cohen, J.S. (Ed). The Macmillan Press Ltd., London.

Donis-Keller, H. (1979) Site specific enzymatic cleavage of RNA. Nucleic Acids Research, 7, 179-192.

Duncan, R. (1992) Drug-polymer conjugates: potential for chemotherapy. Anti-Cancer Drugs, 3, 175-210.

Eder, P.S., DeVine, R.J., Dagle, J.M. and Walder, J.A. (1991) Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3' exonuclease in plasma. Antisense Research and Development, 1, 141-151.

Eldridge, J.H., Hammond, C.J., Meulbroek, J.A., Staas, J.K., Gilley, R.M. and Tice, T.R. (1990) Controlled vaccine release in the gut-associated lymphoid tissues I. Orally administered biodegradable microspheres target the peyer's patches. Journal of Controlled Release, 11, 205-214.

Finley, J.H. (1961) Spectrophometric determinations of polyvinyl alcohol in paper coatings. Analytical Chemistry, 33, 1925-1927.

Fisher, T.L., Terhorst, T., Cao, X. and Wagner, R.W. (1993) Intracellular disposition and metabolism of flourescently labeled unmodified oligonucleotides microinjected into mamalian cells. Nucleic Acids Research, 21, 3857-3865.

Floy, B.J., Visor, G.C. and Sanders, L. (1993) Design of biodegradable polymer systems for controlled release of bioactive agents. Polymeric Delivery Systems, 10, 154-167.

Fournier, C., Hecquet, B., Bouffard, P., Vert, M., Caty, A., Vilian, M-O., Vanseymortier, L., Merle, S., Krikorian, A., Lefebvre, J., Delobelle, A. and Adenis, L. (1991) Experimental studies and preliminary clinical trial of vinorelbine-loaded polymeric bioresorbable implants for the local treatment of solid tumours. Cancer Research, 51, 5384 - 5391.

Freier, S.M., Lima, W.F., Sanghvi, Y.S., Vickers, T., Zounes, M., Cook, P.D. and Ecker, D.J. (1992) Thermodynamics of antisense oligonucleotide hybridisation. Gene Regulation: Biology of antisense RNA and DNA. Erickson, R.P. and Izant, J.G. (eds.) Raven Press Ltd., New York.

Gagnor, C., Bertrand, J., Thenet, S., Lemaître, M., Morvan, F., Rayner, B., Malvy, C., Lebleu, B., Imbach, J. and Paoletti, C. (1987) α-DNA VI: comparative study of α- and β- anomeric oligodeoxyribonucleotides in hybridization to mRNA and in cell free translation inhibition. Nucleic Acids Research, 15, 10419-10436.

Galbraith, W.M., Hobson, W.C., Giclas, P.C., Schechter, P.J. and Agrawal, S. (1994) Complement activation and hemodynamic changes following intravenous administration of phoshorothioate oligonucleotides in the monkey, Antisense Research and development, 4, 201-206.

Gao, W., Han, F., Storm, C., Egan, W. and Cheng, Y. (1991) Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNaseH implications for antisense technology. Molecular Pharmacology, 41, 223-229.

Gao, W., Storm, C., Egan, W. and Cheng, Y. (1992) Cellular pharmacology of phosphorothiate homooligodeoxynucleotides in human cells. Molecular Pharmacology 43, 45-50.

Gassner, F. and Owens, A.J. (1992) On the physical properties of Biopol/ethylene-vinyl acetate blends. Polymer, 33, 2508-2512.

Ghosh, M.K., Ghosh, K., Dahl, O. and Cohen, J.S. (1993 a) Phosphorothioate-phosphodiester oligonucleotide co-polymers: assessment for antisense application. Anti-Cancer Drug Design, 8, 15-32.

Ghosh, M.K., Ghosh, K., Dahl, O. and Cohen, J.S. (1993 b) Evaluation of some properties of phosphorodithioate oligodeoxyribonucleotide for antisense application. Nucleic Acids Research, 21, 5761-5766.

Gilding, D.K. and Reed, A.M. (1979) Biodegradable polymers for use in surgery - polyglycolic / poly(lactic acid) homo and copolymers. Polymer, 20, 1459-1464.

Gilding, D.K. (1981) Biodegradable polymers. Biocompat. Clin. Implan. Mat., 2, 209-232.

Gogolewski, S., Jovanovic, M., Perren, S.M., Dillon, J.G. and Hughes M.K. (1993) Tissue response and in vivo degradation of selected polyhydroxyacids: Polyactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/VA). Journal of Biomedical Materials Research, 27, 1135-1148.

Gogolewski, S., Jovanovic, M., Perren, S.M., Dillon, J.G. and Hughes, M.K. (1993) The effect of melt processing on the degradation of selected polyhydroxyacids: polylactides, polyhydroxybutyrate and polyhydroxybutyrate-co-valerates. Polymer Degradation and Stability, 40, 313-322.

Goodarzi, G., Watabe, M. and Watabe, K. (1991) Binding of oligonucleotides to cell membranes at acidic pH. Biochemical and Biophysical Research Communications, 181, 1343-1351.

Goodchild, J., Kim, B., Zamecnik, P.C. (1991) The clearance and degradation of oligodeoxynucleotides following intravenous injection into rabbits. Antisense Research and Development, 1, 153.

Grangier, J.L., Puygrenier, M., Gauthier, J.C. and Couvreur, P. (1991) Nanoparticles as carriers for growth hormone releasing factors (GRF). Journal of Controlled Release, 15, 3-13.

Gray, G., Hernandez, O.M., Hebel, D., Root, M., Pow-Sang, J.M. and Wickstrom, E. (1993) Antisense DNA inhibition of tumour growth induced by c-Ha-*ras* oncogene in nude mice. Cancer Research, 53, 577-580.

Gref, R., Minamitake, I., Peracchia, M.T., Trubetskoy. V., Torchilin, V. and Langer, R. (1994) Biodegradable long-circulating polymeric nanospheres. Science 263, 1600-1603.

Hanvey, J.C., Peffer, N.J., Bisi, J.E., Thompson, S.A., Cadilla, R., Josey, J.A., Ricca, D.J., Hassman, C.F., Bonham, M.A., Au, K.G., Carter, S.G., Bruckenstein, D.A., Boyd, A. L., Noble, S.A. and Babiss, L.E. (1992) Antisense and antigene properties of peptide nucleic acids. Science, 258, 1481-1485.

Haseloff, J. and Gerlach, W.L. (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature, 334, 585-591.

Hayashi, T. (1994) Biodegradable Polymers for Biomedical Uses. Progress in Polymer Science, 19, 663-702.

Hélène, C. (1991) Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides, 27, 1466-1471.

Hélène, C. and Toulme, J-J. (1990) Specific regulation of gene expression by antisense, sense and antigene nucleic acids (Review). Biochimica et Biophysica Acta, 1049, 99-125.

Heller, J. (1993) Polymers for the controlled parenteral delivery of peptides and proteins. Advanced Drug Delivery Reviews, 10, 163-204.

Henahan, S. (1995) Oligonucleotides a sensible approach to gene therapy. Inpharma 8 April 11-12.

Hendry, P., M^{c}Call, M.J., Santiago, S. and Jennings, P.A. (1992) A ribozyme with DNA in the hybridising arms displays enhanced cleavage ability. Nucleic Acids Research, 20, 5737-5741.

Hertel, K.J., Pardi, A., Uhlenbeck, O.C., Koizumi. M., Ohtsuka, E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W.L., Hodgson, R. And Symons, R.H. (1992) Numbering system for the hammerhead. Nucleic Acids Research, 20, 3252.

Heya, T., Okada, H., Ogawa, Y. and Toguchi, H. (1994) *In vitro* and *in vivo* evaluation of thyrotrophin releasing hormone release from copoly(D,L lactic/glycolic acid) microspheres. Journal of Pharmaceutical Sciences, 83, 636-640.

Higgins, K.A., Perez, J.R., Coleman, T.A., Dorshkind, K., M^{c}Comas, W.A., Sarmiento, U.M., Rosen , G.A. and Narayanan, R. (1993) Antisense inhibition

of the p65 subunit of NF-kB blocks tumorigenicity and causes tumor regression. Proceedings of the National Academy of Sciences USA, 90, 9901-9905.

Hoke, G.D., Draper, K., Freier, S.M. Gonzalez, C., Driver, V.B., Zounes, M.C. and Ecker, D.J. (1991) Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridisation and antiviral efficacy versus herpes simplex virus infection. Nucleic Acids Research. 19, 5743-5748.

Holland, S.J. and Tighe, B.J. (1986) Polymers for biodegradable medical devices. 1. The potential of polyesters as controlled macromolecularl release systems. Journal of Controlled Release, 4, 155-180.

Holland, S.J., Jolly, A.M., Yasin, M. and Tighe, B.J. (1987) Polymers for biodegradable medical devices. II. Hydroxybutyrate-hydroxyvalerate copolymers: hydrolytic degradation studies. Biomaterials, 8, 2890-295.

Holland, S.J., Yasin, M. and Tighe, B.J. (1990) Polymers for biodegradable medical devices. VII. Hydroxybutyrate-hydroxyvalerate copolymers: degradation of copolymers and their blends with polysaccharides under in vitro physiological conditions. Biomaterials, 11, 206-215.

Horácek, I. and Kudlácek, L. (1993) Influence of Molecular Weight on the Resistance of Polylactide Fibers by Radiation Sterilization. Journal of Applied Polymer Science, 5O, 1-5.

Hseih, D.S.T., Mann, K. And Chiang, C.C. (1985) Enhanced release of drugs from silicone elastomers. I. Release kinetics of pineal and steroidal hormones. Drug Development in Industrial Phramcy, 11, 1391-1410.

Hu, D.S-G. and Liu, H-J (1994) Structural analysis and degradation behavior in polyethylene glycol/poly(L-Lactide) copoloymers. Journal of Applied Science, 51, 473-482.

Hughes, J.A., Avrutskaya, A.V. and Juliano, R.L. (1994) Influence of base composition on membrane binding and cellular uptake of a 10 mer phosphorothioate oligonucleotides in chinese hamster ovary (CH^RC5) cells. Antisense Research and Development, 4, 211-215.

Hughes, J.A., Avroutskaya, A., Sasmor, H. M., Guinosso, J., Cook, P.D. and Juliano, R.L. (1995) Oligonucleotide transport across membranes and into cells: Effects of chemical modifications. In: Delivery Strategies for Antisense Oligonucleotide Therapeutics (Akhtar, S. Ed.) CRC Press, Boca Raton, Florida, USA.

Hutchinson, F.G. and Furr, B.J.A. (1985) Biodegradable polymers for the sustained release of peptides. Biochemical Society Transactions, 13, 520-523.

Illum, L., Davis, S.S., Muller, R.H., Mak, E. and West, P. (1987) The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilized with a blockcopolymer - Poloxamine 908. Life Sciences, 40, 367-374.

Irwin, W.J. (1990) Kinetics of Drug Decomposition: BASIC Computer Solutions, Elseveir, Amsterdam, 82-84 and 175-181.

Itakura, K., Rossi, J.J. and Wallace R.B. (1984) Synthesis and use of synthetic oligonucleotides. Annual Review of Biochemistry 53, 323-356.

Iversen, P. (1991) *In vivo* studies with phosphorothioate oligonucleotides: pharmacokinetics prologue. Anti-Cancer Drug Design, 6, 531-538.

Iwata, M. and McGinty, J. W. (1993) Dissolution, stability and morphological properties of conventional and multiphase poly (DL-lactic-co-glycolic acid) microspheres containing water-soluble compounds. Pharmaceutical Research, 10, 1219-1227.

Jaishree, T.N. and Wang, A. H-J. (1993) NMR studies of pH-dependent conformational polymorphism of alterating (C-T)n sequences. Nucleic Acids Research, 21, 3839-3844.

Jalil, R. and Nixon, J.R. (1989) Microencapsulation using poly (L-lactic acid) I: Microcapsule properties affected by the preparative techniques. Journal of Microencapsulation, 6, 473-484.

Jaroszewski, J.W. and Cohen, J.S. (1991) Cellular uptake of antisense oligonucleotides. Advanced Drug Delivery Reviews, 6, 235-250.

Jefferey, H., Davis, S.S. and O'Hagen, D. (1991) The preparation and characterisation of poly(lactide-co-glycolide) microparticles. I. Oil-in-water emulsion solvent evaporation. International Journal of Pharmaceutics, 77, 169-175.

Jefferey, H., Davis, S.S. and O'Hagen, D. (1993) The preparation and characterisation of poly(lactide-co-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil)-in water emulsion solvent evaporation technique. Pharmaceutical Research. 10, 362-368.

Jefferies, W.A., Brandon, M.R., Hunt, S.V., Williams, A.F., Gater, K.C. and Mason, D.Y. (1984) Transferrin receptors on endothelium of brain cappilaries. Nature, 321, 162-183.

Johnson, G.D., Davidson, R.S., McNamee, K.C., Russell, G., Goodwin, D. and Holborow, E.J. (1982) Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. Journal of Immunology Methods 55, 231-242.

Juliano, R.L. and Akhtar, S.(1992) Liposomes as a drug delivery system for antisense oligonucleotides. Antisense Research and Development 2, 165-176.

Juni, K., Ogata, J., Matsui, N., Kubota, M. and Nakano, M. (1985) Modification of the release rate of aclarubicin from polylactic acid microspheres by using additives. Chemical and Pharmaceutical Bulletin, 33, 4. 1734-1738.

Kabanov, A.V., Vinogradov, S.V., Ovcharenko, A.V., Krivonos, A.V., Melik-Nubarov, N.S., Kiselev. V.I. and Severin, E.S. (1990) A new class of antivirals: antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus production and synthesis of virus-specific protiens in MDCK cells. FEBS Letters, 259, 327-330.

Kabanov, A. and Alakhov, V.Y. (1994) New approaches to targeting bioactive compounds. Journal of Controlled Release, 28, 15-35.

Kamei, K., Linuma, Y., Yuasa, Y., Saikawa, A., Igari, Y. and Ogawa, Y. (1995) LHRH agonist depot formulations. Comparative studies of Decapeptyl with Enantone. European Hospital Pharmacist, 1, 113-120.

Karikó, K., Megyeri, K., Xiao, Q., Barbathan, E.S. (1994) Lipofectin-aided cell delivery of ribozyme targeted to human urokinase receptor mRNA. FEBS Letters 352, 41-44.

Kawasaki, A.M., Casper, M.D., Freier, S M., Lesnik, E.A., Zounes, M.C., Cummins, L.L., Gonzalez, C. and Cook, P.D. (1993) Uniformly modified 2'-Deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specifity for RNA targets. Journal of Medicinal Chemistry, 36, 831-841.

Khan, I.M. and Coulson, J.M. (1993) A novel method to stabilise antisense oligonucleotides against exonuclease degradation. Nucleic Acids Research, 21, 2957-2958.

Kimura, H., Ogura, Y., Moritera T., Honda, Y., Tabata Y. and Ikada, Y. (1994) *In vitro* phagocytosis of polylactide microspheres by retinal pigment epithelial cells and intracellular drug release. Current Eye Research, 353-360.

Kinchington, D. Galpin, S, Jaroszewski, J.W., Ghosh,K., Subasinghe, C. and Cohen,S. (1992) A comparison of gag, pol, and rev antisense oligodeoxynucleotides as inhibitors of HIV-1. Antiviral Research, 17, 53-62.

Kissel, T., Brich, Z., Bantle, S., Lancranjan, I., Nimmerfall, F. and Vit, P. (1991) Parenteral depot-systems on the basis of biodegradable polyesters. Journal of Controlled Release, 16, 27-42.

Kitajima, I., Shinohara, T., Bilakovics, J., Brown. D.A., Xu, X., Nerenberg, M. (1992) Abalation of transplanted HTLV-1 Tax-transformed tumors in mice by antisense inhibition of Nf-κB. Science, 258, 1792-1795.

de Koning, G.J.M. and Lemstra, P.J. (1992) The amorphous state of bacterial poly((R)-3-hydroxyalkonate) *in vivo*. Polymer 33, 3292-3294.

Koosha, F, Muller, R.H. and Davis, S.S. (1989) Polyhydroxybutyrate as a Drug Carrier. Critical Reviews in Therapeutic Drug Carrier Systems.6, 117- 130.

Kost, J., Leong, K. and Langer, R. (1989) Ultrasound-enhanced polymer degradation and release of incorporated substances. Proceedings of the National Academy of Sciences USA., 86, 7663-7666.

Kreig, A.M. (1993) Uptake and efficacy of phosphodiester and modified oligonucleotides in promary cell cultures. Clinical Chemistry 39, 710-712.

Kreig, A.M., Tonkinson, J., Matson, S., Zhao, Q., Saxon, M., Zhang, L., Bhanja, U., Yakubov, L. and Stein C.A. (1993) Modification of antisense phosphodiester oligodeoxynucleotides by a 5' cholesterol moiety increases cellular association and improves efficacy. Proceedings of the National Academy of Sciences, 90, 1048-1052.

Lalla. J.K. and Sapna, K. (1993) Biodegradable microspheres of poly(DL-lactic acid) containing piroxicam as a model drug for controlled release via the parenteral route. Journal of Microencapsulation, 10, 4, 449-460.

Lange, W., Daskalakis, M., Finke, J. and Dölken, G. (1994) Comparison of different ribozymes for efficient and specific cleavage of BCR/ABL related mRNAs. FEBS Letters, 338, 175-178.

Langer, R.S. and Peppas, N.A. (1981) Present and future applications of biomaterials in controlled drug delivery systems. Biomaterials, 2, 201-214.

Lappalainen, K., Urtti, A., Soderling, E., Jaaskelainen, I., Syrjanen K. and Syrjanen, S. (1994) Cationic liposomes improve stability and intracellular delivery of antisense oligonucleotides into CaSki cells. Biochimica et Biophysica Acta, 1196, 201-208.

Le Corre, P., Le Guevello, P., Gajan, V., Chavanne, F. and Le Verge, R. (1994) Preparation and characterisation of bupivacaine-loaded polylactide and polylactide-co-glycolide microspheres. International Journal of Pharmaceutics, 107, 41-49.

Leonetti, J.P., Degols, G. and Lebleu, B. (1990) Biological activity of oligonucleotide-poly(L-lysine) conjugate : Mechanism of cell uptake. Bioconjugate Chemistry, 1, 149-153.

Leonetti, J.P., Mechti, N., Degols, G., Gagnor, C. and Lebleu, B. (1991) Intracellular distribution of microinjected antisense oligonucleotides. Proceedings of the National Academy of Sciences USA, 88, 2702-2706.

Leonetti, J.P., Degols, G., Clarenc, J.P., Mechti, N. and Lebleu, B. (1993) Cell delivery and mechanisms of action of antisense oligonucleotides. Progress in Nucleic Acid Research and Molecular Biology, 44, 143-165.

Le Ray, A.M., Vert, M., Gautier, J.C., Benoit, J.P. (1994) Fate of [^{14}C]poly(DL-lactide-co-glycolide) nanoparticles after intravenous and oral administration to mice. International Journal of Pharmaceutics, 106, 201-211.

Lisiewicz, J., Sun, D., Klotman, M., Agrawal, S., Zamecnik, P. and Gallo, R. (1992) Specific inhibition of humman immunodefieciency virus type 1 replication by antisense oligonucleotides: An in vitro model of treatment. Proceedings of the National Academy of Sciences USA, 89, 11209-11213.

Lisiewicz, J., Sun, D., Metelev, V., Zamecnik, P., Gallo, R. and Agrawal, S. (1993) Long-term treatment of human immunodefiency virus-infected cells with antisense oligonucleotide phosphorothioates. Proceedings of the National Academy of Sciences USA, 90, 3860-3864.

Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. and Neckers, L.M. (1989) Characterisation of oligonucleotide transport into living cells. Proceedings of the National Academy of Sciences USA, 86, 3474-3478.

Magrath, I.T. (1994) Prospects for the therapeutic use of antisense oligonucleotides in malignant lymphomas. Annals of Oncology 5 (Suppl.1.): S67-S70.

Maher, L.J. and Dolnick, B.J. (1988) Comparative hybrid arrest by tandem antisense oligodeoxyribonucleotides or oligodeoxyribonucleoside methylphosphonates in a cell-free system. Nucleic Acids Research, 16, 3341-3358.

Maher, L.J., Wold, B. and Dervan, P.B. (1989) Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation. Science, 245, 725-729.

Makino, K., Arakawa, M. and Kondo, T. (1985) Preparation and the *in vitro* degradation properties of polylactide microcapsules. Chemical and Pharmaceutical Bulletin, 33, 1195-1201.

Makino, K., Ohshima, H. and Kondo, T. (1986) Transfer of protons from bulk solution to the surface of poly(L-lactide) microcapsules. Journal of Microencapsulation, 3, 195-202.

Makino, K., Ohshima, H. and Kondo, T. (1986) Mechanism of hydrolytic degradation of poly(L- lactide) microcapsules: effects of pH, ionic strength and buffer concentration. Journal of Microencapsulation, 3, 203-212.

Makino, K., Ohshima, H. and Kondo, T. (1987) Effects of plasma proteins on degradation properties of poly(L-lactide) microcapsules. Pharmaceutical Research, 4, 62-65.

Maltese, J-Y., Sharma, H.W., Vassilev, L. and Narayanan, R. (1995) Sequence context of antisense RelA/NF-κB phosphorothioates determines specificity. Nucleic Acids Research 23, 1146-1151.

Marshall, W.S. and Caruthers, M.C. (1993) Phosphorodithioate DNA as a potentail therapeutic drug. Science 259, 1564-1570.

Martindale, The Extra Pharmacopoeia, 29th Edition. The Pharmaceutical Press, London, 1989.

Matlaga, B.F., Yasenchak, L.P. and Salthouse, T.N. (1976) Tissue response to implanted polymers: The significance of sample shape. Journal of Biomedical Materials Research, 10, 391-397.

M^{c}Intyre, K.W., Lombard-Gillooly, K., Perez, J.R., Kunsch, C., Sarmiento, U.M., Larigan, J.D., Landreth, K.T. and Narayanan, R. (1993) A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-κB p65 causes sequence-specific immune stimulation. Antisense Research and Development, 3, 309-322.

Mehta, R.C., Jeyanthi, R., Calis, S., Thanoo, B.C., Burton, K.W., De Luca , P.P. (1994) Biodegradable microspheres as depot system for parenteral delivery of peptide drugs. Journal of Controlled Release, 29, 375-384.

Meltzer, M.S., Skillman, D.R., Hoover, D.L., Hanson, B.D., Turpin, J.A., Kalter, C. and Gendleman, H.E. (1990) HIV and the immune system. Immunology Today, 11, 217-223.

Menei, P., Daniel, V., Montero-Menei, C., Brouillard, M., Pouplard-Barthelaix, A. and Benoit, J.P. (1993) Biodegradation and brain tissue reaction to poly(D,L-lactide-co-glycolide) microspheres. Biomaterials, 14, 470-478.

Menei, P., Croue, A., Daniel, V., Pouplard-Bartgekaux, A., and Benoit, J.P. (1994 a) Fate and biocompatibility of three types of microspheres implanted into the brain. Journal of Biomedical Materials Research, 28, 1079-1085.

Menei, P., Benoit, J-P., Boisdron-Celle, M., Fournier, D., Mercier, P. and Guy, G. (1994 b) Drug targeting into the central nervous system by stereotactic implantaion of biodegrable microspheres. Neurosurgery, 34, 1058-1064.

Miller, R.D., Brady, J. M. And Cutright, D.E. (1977) Degradation rates of oral resorbable implants (polylactates and polyglycolates): rate modification with changes in PLA/PGA copolymer ratios. Journal of Biomedical Materials research, 11, 711-719.

Mori A., and Huang L. (1995) Targeted Immunoliposomes. Potential Role in the Delivery of Cytotoxic Drugs. Clinical Immunotherapeutics, 3, 227-240.

Moser, H.E. and Dervan, P.B. (1987) Sequence-specific cleavage of double helical DNA by triple helix formation. Science, 238, 645-650.

Mosmann, T. (1983) Rapid colorimetric assay foe cellular growth and survival: Application to proliferation and cytotoxicty assays. Journal of Immunological Methods, 65, 55-63.

Müller, H-M. and Seebach, D. (1993) Poly(hydroxyalkanoates): A fifth class of physiologically important organic biopolymers. Angewandte Chemie: International Edition, 32, 477-502.

Müller, R.H. and Wallis, K.H. (1993) Surface modification of i.v. injectable biodegradable nanoparticles with poloxamer polymers and poloxamine 908. International Journal of Pharmaceutics, 89, 25-31.

Narayani, R. and Rao, K.P. (1994) Controlled release of anticancer drug methotrexate from biodegradable gelatin microspheres. Journal of Microencapsulation, 11, 69-77.

Neckers, L.M., Geselowitz, D.A., Chaveny, C. (1995) Pharmacokinetics and delivery of oligonucleotides to the brain. In: Delivery Strategies for Antisense Oligonucleotide Therapeutics (Akhtar, S. Ed.) CRC Press, Boca Raton, Florida, USA.

Nihant, N., Stassen, S., Grandfils, C., Jérôme, R. and Teyssié, P. (1993) Microencapsulation by coacervation of poly(lactide-co-glycolide)-II: Encapsulation of a dispersed aqueous phase. Polymer International. 32, 171-176.

Nihant, N., Schugens , C., Grandfils, C., Jérôme, R. and Teyssié, P. (1994) Polylactide microparticles prepared by double emulsion/evaporation technique. I. effect of primary emulsion stability. Pharmaceutical Research, 11, 1479-1484.

Nihant, N., Stassen, S., Grandfils, C., Jérôme, R. and Teyssié, P. (1994) Microencapsulation by coacervation of poly(lactide-coglycolide) III. Characterisation of the final microspheres. Polymer International, 34, 289-299.

Nielsen, P.E., Egholm, M. and Buchardt, O. (1994) Peptide Nucleic Acid (PNA). A DNA mimic with a peptide backbone. Bioconjugate Chemistry, 5, 3-7.

Nolan, H.W., Catz, P. and Friend, D.R. (1994) Percutaneous penetration of methyl phosphonate antisense oligonucleotides. International Journal of Pharmaceutics, 107, 169-177.

Noonberg, S.B., Garovoy, M.R. and Hunt, C.A. (1993) Characteristics of oligonucleotide uptake in human ketainocyte cultures. The Journal of Investigative Dermatology, 101, 727-731.

Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T. and Shimamoto, T. (1988a) A new technique to efficiently entrap leuprolide acetate into micropsheres of copoly(lactic/glycolic acid). Chemisrty and Pharmacy Bulletin, 36, 1095-1103.

Ogawa, Y., Okada, H., Yamamoto, M. and Shimamoto, T. (1988b). *In vivo* release profile of leuprolide acetate from microcapsules prepared with polylactic acids or copoly(lactic/glycolic) acids and *in vivo* degradation of these polymers. Chemistry and Pharmaceutical Bulletin, 36, 2576-2581.

O'Hagan ,D.T., Jeffery, H. and Davis, S.S. (1994a) The preparation and characterization of poly(lactide-co-glycolide) microparticles: III. Microparticle/polymer degradation rates and the *in vitro* release of a model protein. International Journal of Pharmaceutics, 103, 37-45.

O'Hagan, D.T., Rahman, D., Jeffery, D., Sharif, S. and Challacombe, S.J. (1994b) Controlled release microparticles for oral immunization. International Journal of Pharmaceutics 108, 133-139.

Okada, H., Doken, Y., Ogawa, Y. and Toguchi, H. (1994) Preparation of a three-month depot injectable microspheres of Leuproelin acetate using biodegrabale polymers. Pharmaceutical Research, 11, 1143-1147.

Oppenheimer, B.S., Oppenheimer, E.T., Danishefsky, I., Stout, A.P. and Eirich, F.R. (1955) Further studies of polymers as carcinogenic agents in animals. Cancer Research, 15, 333- 354.

Owen, A.J. and Heinzel, J. (1992) Crystallisation and melting behaviour of PHB and PHB/HV copolymer. Polymer 33, 1563-1567.

Pardridge, W.M. (1992) Recent Developments in Peptide Drug Delivery to the Brain. Pharmacology & Toxicology, 71, 3-10.

Pavanetto, F., Genta, I., Giunchedi, P. and Conti,B. (1993) Evaluation of spray drying as a method for polylactide and polylactide co-glycolide microsphere preparation. Journal of Microencapsulation, 10, 487-497.

Pekarek, K.J., Jacob, J.S. and Mathiowitz, E. (1994) Double-walled polymer microspheres for controlled drug release. Nature, 367, 258-26.

Peyman, A., Ryte, A., Helsberg, M., Kretzschmar, G., Mag, M. and Uhlmann, E. (1995) Enhanced cellular uptake of G-rich oligonucleotides. Nucleosides and Nucleotides 14, 1077-1081.

Pieken, W.A., Olsen, D.B., Benseker, F., Aurup, H. and Eckstein, F. (1991) Kinetic Characterization of Ribonuclease-Resistant 2'-Modified Hammerhead Ribozymes. Science, 253, 314-317.

Pieles, U., Zurcher, W., Schlar, M. and Moser, H. (1993) Matrix-assisted laser desorption ionization time-of-flight mass specrometry: a powerful tool for the mass and sequence analysis of natural and modified oligonucleotides. Nucleic Acids Research, 21, 3191-3196.

Pierga, J.Y. and Magdelenat, H. (1994) Applications of antisense oligonucleotides in oncology (review). Cellular and Molecular Biology, 40, 237-261.

Pistner, H., Gutwald, R., Ordung, R. and Reuther, J. (1993) Poly(L-lactide): a long-term degradation study *in vivo*. Part I. logical results. Biomaterials, 14, 671-677.

Pistner, H., Stallforth, H., Gutwald, R., Muhling, J., Reuther, J. and Michel C. (1993) Poly(L-lactide): a long-term degradation study *in vivo*. Part II: physico-mechanical behaviour of implants. Biomaterials, 15, 439-450.

Pistner, H., Bendix, D.R., Muhling, J. and Reuther, J.R. (1994) Poly(L-lactide): a long-term degradation study *in vivo*. Part III. Analytical characterisation. Biomaterials 14, 291-299.

Pitt, C.G., Gratzl, M.M., Kimmel, G.L., Surles, J. and Schindler, A. (1981) Aliphatic polyesters II. The degradation of poly(D,L-lactide), poly (ε-caprolactone) and their copolymers *in vivo*. Biomaterials, 2, 215-220.

Prakash, G. and Kool, T. (1991) Molecular recognition by circular oligonucleotides. Strong binding of single-stranded DNA and RNA. Journal of the Chemical Society, Chemistry Communication, 1161-1163.

Ratajczak, M.Z., Kant, J.A., Luger, S.M., Hijiya, N., Zhang, J., Zon, G. and Gewirtz, A.M. (1992) *In vivo* treatment of human leukaemia in a scid mouse model with *c-myb* antisense oligodeoxynucleotides. Proceedings of the National Academy of Sciences USA, 89, 11823-11827.

Ray, K., Bhattacharyya, B. and Biswas, B.B. (1984) Anion-induced increases in the affinity of colcemid binding to tubulin. European Journal of Biochemistry, 142, 577-581.

Reeve, M.S., M°Carthy, S.P., Downey, M.J. and Gross, R.A. (1994) Polylactide stereochemistry: Effect of enzymatic degradabilty. Macromolecules 27, 825-831.

Richardson, M.J. (1989) Thermal Analysis in 'Comprehensive Polymer Science' Vol 1.Allen. G. and Bevington J.C., Booth. C. and Price. C. (Eds.) Pergamon Press, 867-899.

Ropert, C., Okruszek, P., Couvreur, P. and Malvy, C. (1994) Retroviral inhibition by antisense oligonucleotides determined by intracellular stability. Antisense Research and Development, 4, 207-210.

Rossi, J.J., Elkins. D., Zaia, J.A. and Sullivan, S. (1992) Ribozymes as anti-HIV-1 therapeutic agents: principles, applications and problems. AIDS Research and Human Retroviruses, 8, 183-189.

Roy, S. and Wainberg, M.A. (1988) Role of the mononuclear phagocyte system in the development of aquired immunodeficiency syndrome (AIDS). Journal of Leukocyte Biology, 43, 91-97.

Ruffner, D. and Uhlenbeck, O. (1990) Thiophosphate interference experiments locate phosphates important for the hammerhead RNA self-cleavage. Nucleic Acids Research, 18, 6025-6029.

Sah, H. and Chien, Y.W. (1993) Evaluation of microreservoir-type biodegradable microcapsule for controlled release of proteins. Drug Development and Industrial Pharmacy, 19, 1243-1263.

Saijo, Y., Perlaky, L., Wang, H. and Busch, H. (1994) Pharmacokinetics, tissue distribution and stability of antisense oligodeoxynucleotide phosphorothioate ISIS 3466 in mice. Oncology Research, 6, 243-249.

Salthouse, T.N. (1976) Cellular enzyme activity at the polymer-tissue interface. Journal of Biomedical Materials Research, 10, 197-229.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (second edition). Cold Spring Harbour Laboratory Press.

Sanders, L.M., Kent, J.S., M°Rae, G.I., Vickery, B.H., Tice, T.R. and Lewis, D.H. (1984) Controlled release of a leteinizing hormone-releaseing hormone

analogue from poly(D,L-lactide-co-glycolide) microspheres. Journal of Pharmaceutical Sciences, 73, 1294-1297.

Sanders, L.M., Kell, B.A., M^{c}Rae, G.I. and Whitehead, G.W. (1986) Prolonged-controlled release of Nafarelin, a luitenizing hormone-releaseing hormone analogue, from biodegradable polymeric implants: Influence of composition and molecular weight of polymer. Journal of Pharmaceutical Sciences, 75, 356-360.

Sansdrap, P. and Moës, A. J. (1993) Influence of manufacturing parameters on the size of characteristics and the release profiles of nifedipine from poly(D,L-lactide - co-glycolide) microspheres. International Journal of Pharmaceutics, 98, 157 - 164.

Sarver, N., Cantin, E.M., Chang, P.S., Zaia, J.A., Ladne, P.A., Stephens, D.A. and Rossi, J.J. (1990) Ribozymes as Potential Anti-HIV-1 Therapeutic Agents. Science, 247, 1222-1225.

Schugens, C., Laruelle, N., Nihant, N., Grandfils, C.H., Jerome, R. and Teyssie P.H. (1994) Effect of the emulsion stability on the morphology and porosity of semicrystalline poly l-lactide microparticles prepared by w/o/w double emulsion-evaporation. Journal of Controlled Release, 32, 161-176.

Shea, R.G., Marsters, J.C. and Bischofberger, N. (1990) Synthesis, hybridisation properties and antiviral activity of lipid-oligodeoxynucleotide conjugates. Nucleic Acids research, 18, 3777-3783.

Shimayama, T., Nishikawa, F., Nishikawa S. And Taira, K. (1993) Nuclease-resistant chimeric ribozymes containing deoxribonucleotides and phosphorothioate linkages. Nucleic Acids Research, 21, 2605-2611.

Shoji, S., Akhtar, S., Periasamy, A., Herman, B. and Juliano, R.L. (1991) Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. Nucleic Acids Research, 19, 5543-5550.

Short, R. (1995) Antisense oligonucleotides a novel approach to blasting leukaemia. Inpharma 1 April 9-10.

Silverstein, S.C., Steinman, R.M. and Cohn, Z.A. (1977) Endocytosis. Annual Review of Biochemistry, 46, 669-722.

de Smidt, P.C., Le Doan, T., de Falco, S. and van Berkel, T.J.C. (1991) Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. Nucleic Acids Research, 19, 4695-4700.

Spenlehaur, G., Vert, M., Benoit, J.P. and Boddart, A. (1989) In vitro and in vivo degradation of poly(D,L lactide/glycolde) type microspheres made by solvent evaporation method. Biomaterials 10, 557-563.

Spiller, D.G. & Tidd, D.M. (1992) The uptake kinetics of chimeric oligodeoxynucleotide analogues in human leukaemia MOLT-4 cells. Anti-Cancer Drug Design, 7, 115-129.

Stahl, P.D., Rodman, J.S., Miller, M.J. and Schlesinger, P.H. (1978) Evidence for receptor-mediated binding of glycoproteins, glycoconjugates and lysosmal glycosidases by alveolar macrophages. Proceedings of the National Academy of Sciences USA, 75, 1399-1412

Stein, C. A. , Mori, K., Loke, S.L., Subasinghe, C., Shinozuka, K., Cohen, J.S. and Neckers, L.M. (1988) Phosphorothioate and normal oligodeoxyribonucleotides with 5'-linked acridine: characterization and preliminary kinetics of cellular uptake. Gene, 72, 333-341.

Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen,J. (1988) Physiochemical properties of phosphorothioate oligodeoxynucleotides. Nucleic Acids Research, 16, 3209-3221.

Stein, C.A., Tonkinson, J. L., Zhang, L-M., Yakubov, L., Gervasoni, J., Taub, R. and Rotenberg, S.A. (1993) Dynamics of the internalization of phosphodiester oligonucleotides in HL60 cells. Biochemistry, 32, 4855-4861.

Stein, C.A. and Krieg, A.M. (1994) Problems in the interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides. Antisense Research and Development, 4, 67-69.

Sun, J. S. and Hélène, C. (1993) Oligonucleotide-directed triple helix formation Current Opinion of in Structural Biology, 3, 345-356.

Supersaxo, A., Kou, J. H., Teitelbaum, P. and Maskiewicz. (1993) Preformed porous microspheres for controlled and pulsed release of macromolecules. Journal of Controlled Release, 23, 157-164.

Symons, R.H. (1992) Small catalytic RNAs. Annual Review of Biochemistry, 61, 641-671.

Symons, R.H. (1994) Ribozymes. Current Opinion in Structural Biology, 4, 322-330.

Tabata, Y. and Ikada, Y. (1987) Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. Journal of Pharmacy and Pharmacology, 39, 698-704.

Tabata, Y. and Ikada, Y. (1987) Activation of macrophage in vitro to acquire antitumor activity by a muramyl dipeptide encapsulated in microspheres composed of lactide copolymer. Journal of Controlled Release, 6, 189-204.

Tabata, Y. and Ikada, Y. (1988 a) Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophages. Biomaterials, 9, 356-361.

Tabata, Y. and Ikada, Y. (1988 b) Macrophage phagocytosis of biodegradable microspheres composed of L-lactic acid / glycolic acid homo- and copolymers. Journal of Biomedical Materials Research, 22, 837-858.

Tabata, Y. and Ikada, Y. (1988) Potentiation of antitumor activity of macrophages by recombinant interferon alpha A/D contained in gelatin microspheres. Japanese Journal of Cancer Research, 79, 636-646.

Tabata, Y. and Ikada, Y. (1989) Effect of surface wettability of microspheres on phagocytosis. Journal of Colloid and Interface Science, 127, 132-140.

Tabata, Y. and Ikada, Y. (1990) Phagocytosis of polymer microspheres by macrophages. In: Advances in Polymer Science, 107-141.

Tabata, Y. and Ikada, Y. (1993) Targeting of muramyl dipeptide to macrophages by gelatin conjugation to enhance their in vivo antitumor activity. Journal of Controlled Release, 27, 79-88.

Tabata, Y., Takebayashi, Y., Ueda, Y. and Ikada, Y. (1993) A formulation method using D,L- lactic acid oligomer for protein release with reduced initial burst. Journal of Controlled Release, 23, 55 - 63.

Takasugi, M., Guendouz, A., Chassignol, M. (1991) Sequenec-specific photo induced cross-linking of the 2 strands of double-helical DNA by a psoralen covalently linked to a triple helix-forming oligonucleotide. Proceedings of the National Academy of Sciences USA, 88, 5602-5606.

Taylor, N.R., Kaplan, B.E., Swiderski, P., Li, H. and Rossi, J.J. (1992) Chimeric DNA-RNA hammerhead ribozymes have enhanced in vitro catalytic efficiency and increased stability in vivo. Nucleic Acids Research, 20, 4559-4565.

Tamada, J.A. and Langer, R. (1993) Erosion Kinetics of hydrolytically degradable polymers. Proceedings of the National Academy of Sciences USA. 90, 552-556.

Tang, J., Temsamani, J., and Agrawal, S. (1993) Self-stabilised antisense oligodeoxynucleotide phosphorothioates: properties and anti-HIV activity. Nucleic Acids Research, 21, 2729-2735.

Temsamani, J., Kubert, M., Tang, J., Padmapriya, A. and Agrawal. S. (1994) Cellular uptake of oligodeoxynucleotide phosphorothioates and their analogues. Antisense Research and Development, 4, 35-42.

Thierry, A.R., Rahman, A. and Dritschilo, A. (1993) Overcoming multidrug resistance in human tumour cells using free and liposomally nencapsulated antisense oligodeoxynucleotides. Biochemical and Biophysical Research Communications, 190, 952-960.

Troy, C. M., Greene, L. A. and Shelanski, M. L. (1992) Neurite outgrowth in peripherin-depleted PC12 cells. The Journal of Cell Biology 117, 1085-1092.

Trubetskoy, V.S., Torchilin, V.P., Kennel, S. and Huang, L. (1992) Cationic liposomes enhance targeted delivery and expression of exogenous DNA mediated by N-terminal modified poly(L-lysine)-antibody conjugate in mouse lung endothelial cells. Biochimica et Biophysica Acta, 1131, 311-313.

Tsai, D.C., Howard, S.A., Hogan, T.F., Malanga. C.J., Kandzari. S.J. and Ma, J.K.H. (1986) Preparation of in vitro evaluation of polylactic acid-mitomycin C microcapsules. Journal of Microencapsulation, 3, 181-193.

Tuschl, T. and Eckstein, F. (1993) Hammerhead ribozymes: Importance of stem-loop II for activity. Proceedings of the National Academy of Sciences USA, 90, 6991-6994.

Uhlmann, E. and Peyman, A. (1990)Antisense Oligonucleotides: A new therapeutic principle. Chemical Reviews, 90, 543-584.

van Sliedregt, A., de Groot, K. and van Blitterswijk, C.A. (1993) *In vitro* biocompatability testing of polylactides. Part II Morphological aspects of different cell types. Journal of Materials in Medicine, 4, 213-218.

van Sliedregt, A., Knook, M., Hesseling, S.C., Koerten, H.K., de Groot, K. and van Blitterswijk, C.A. (1992) Cellular reaction on the intraperitoneal injection of four types of polylactide particulates. Biomaterials, 13, 819-824.

van Sliedregt, A., van Loon, J.A., van der Brink, J., de Groot, K. and van Blitterswijk, C.A. (1994) Evaluation of polylactide monomers in an *in vitro* biocompatability assay. Biomaterials, 15, 251-256.

Verspieren, P., Cornelissen, A.W.C.A., Thuong, N.T., Hélène,C . and Toulme, J.J. (1987) An acridine-linked oligodeoxynucleotide targeted to the common 5' end of trypanosome m RNAs kills cultured parasites. Gene 61, 307-315.

Vert, M., Mauduit, J and Li, S. (1994) Biodegradation of PLA/GA polymers: increasing complexity. Biomaterials, 15, 1209-1213.

Visscher, G.E., Pearson, J.E., Fong, J.W., Argentieri, Robinson, R.L. and Maulding. H.V. (1988) Effect of particle size on the in vitro and in vivo degradation rates of poly(D,L-lactide-co-glycolide) microcapsules. Journal of Biomedical Materials Research, 22, 733-746.

Vlassov, V.V., Karamyshev, V.N. and Yakubov, L.A. (1993) Penetration of oligonucleotides into mouse organism through mucosa and skin. FEBS, 327, 271-274.

Vlassov, V.V., Balakireva, L.A. and Yakubov, L.A. (1994 a) Transport of oligonucleotides across natural and model membranes. Biochimica et Biophysisca Acta, 1197, 95-108.

Vlassov, V.V., Nechaeva, M.V., Karamyshev, V.N. and Yakubov, L.A. (1994-b) Iontophoretic delivery of oligonucleotide derivatives into mouse tumour. Antisense Research and Development, 4, 291-293.

Von Recum, H.A., Cleek, R.L., Eskin, S.G. and Mikos, A.G. (1995) Degradation of polydispersed poly(L-lactic acid) to modulate lactic acid release. Biomaterials, 16, 441-447.

Wada, R., Hyon, S., Nakamura, T, and Ikada, Y. (1991) *In Vitro* Evaluation of sustained drug release from biodegradable elastomers. Pharmaceutical Research 8, 1292-1296.

Wagner, E., Cotten, M., Foisner, R. and Birnstiel, M.L. (1991) Transferrin-polycation -DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. Proceedings of the National Academy of Sciences, USA, 88, 4255-4259.

Wagner. R.W. (1994) Gene inhibition using antisense oligodeoxynucleotides. Nature, 372, 333-335.

Wahlestedt, C., Pich, E.M., Koob, G.F., Yee, F. and Heilig, M. (1993) Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeocynucleotides. Science, 259, 528-531.

Walder, R.Y. and Walder. J.A. (1988) Role of RNase H in hybrid arrested translation by antisense oligonucleotides. Proceedings of the National Academy of Sciences USA, 85, 5011-5018.

Walker, I., Irwin., W.J. and Akhtar, S. (1995) Improved cellular delivery of antisense oligonucleotides using transferrin receptor antibody-oligonucleotide conjugates. Pharmaceutical Research, 12, 1548-1553.

Wang, H.T., Palmer, H., Linhardt, R.J., Flanagan, D.R. and Schmitt, E. (1990) Degradation of poly(ester) microspheres. Biomaterials, 11, 679-685.

Wang, S., Lee, R.J., Cauchon, G., Gorenstein, D.G. and Low, P.S. (1995) Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. Proceedings of the National Academy of Sciences USA, 92, 3318-3322.

Watrous-Peltier, N., Uhl, J., Steele, V., Brophy, L. and Merisko-Liversidge, E. (1992) Direct suppression of phagocytosis by amphipathic polymeric surfactants. Pharmaceutical Research, 9, 1177-1183.

Watts, P.J., Davies, M.C. and Melia, C.D. (1990) Microencapsulation using emulsification/solvent evaporation: An overview of techniques and applications. Critical Reviews in Therapeutic Drug Carrier Systems, 7, 235-259.

Whitesell, L., Rosolen, A. and Neckers, L. (1991) *In vivo* modulation of N-myc expression by continuous perfusion with an antisense oligonucleotide. Antisense Research and Development, 1, 343-350.

Whitesell, L., Geselowitz,D., Chaveny,C., Fahmy,B., Walbridge,S., Alger,J.R. and Neckers.L.M. (1993) Stability, clearance and disposition of intraventricularly administered oligodeoxyribonucleotides: Implications for theraputic apllication within the central nervous system. Proceedings of the National Academy of Sciences, 90, 4665-4669.

Wickstrom, E. (1986) Oligodeoxynucleotide stability in subcellular extracts and culture media. Journal of Biochemical and Biophysical Methods, 13, 97-102.

Wincott, F., DiRenzo, A., Shaffer, C., Grimm, S., Tracz, D., Workman, C., Sweedler, D., Gonzalez, C., Scaringe, S. and Usman, N. (1995) Synthesis, deprotection, analysis and purification of RNA and ribozymes. Nucleic Acids Research, 23, 2677-2684.

Wu, M.P., Tamada, J. A., Brem, H. and Langer, R. (1994) *In vivo* versus *in vitro* degradtion of controlled release polymers for intracranial surgical therapy. Journal of Biomedical Materials Research, 28, 387-395.

Wu-Pong, S., Weiss, T. L. and Hunt, C. A. (1992) Antisense *c-myc* oligoribonucleocleotide cellular uptake. Pharmaceutical Research, 9, 1010-1017.

Wu-Pong, S., Weiss, T. L. and Hunt, C. A. (1994) Antisense c-myc oligonucleocleotide cellular uptake and activity. Antisense Research and Development, 4, 155-163.

Wu-Pong, S., Weiss, T.L., Hunt, C.A. (1994) Calcium Dependent Cellular uptake of a *c-myc* antisense oligonucleotide. Cellular and Molecular Biology, 40, 843-850.

Yakubov, L.A., Deeva, E. A., Zarytova, V. F., Ivanova, E. M., Ryte, A.S., Yurchenko, L. V., Vlassov, V. V. (1989) Mechanism of oligonucleotide uptake by cells: Involvement of specific receptors? Proceedings of the. National Academy of Sciences USA. 86, 6454-6558.

Yamaguchi, K. and Anderson, J.M. (1993) *In vivo* biocompatability studies of medisorb 65/35 D,L-lactide/glycolide copolymer microspheres. Journal of Controlled Release, 24, 81-93.

Yasin, M., Holland, S.J., Jolly, A.M. and Tighe, B.J. (1989) VI. Hydroxybutyrate - hydroxyvalerate copolymers: accelerated degradation of blends with polysaccharides. Biomaerials, 10, 400 - 412.

Yasin, M., Holland, S.J., Jolly, A.M. and Tighe, B.J. (1990) V. Hydroxybutyrate - hudroxyvalerate copolymers: effects of polymer processing on hydrolytic degradation. Biomaterials, 11, 451 - 454.

Yasin, M. and Tighe, B.J. (1992) Polymers for biodegradable medical devices. VIII. Hydroxybutyrate-hydroxyvalerate copolymers: physical and degradative properties of blends with polycaprolactone. Biomaterials, 13, 9-16.

Yasin, M. and Tighe, B.J. (1993) Strategies for the design of biodegradable polymer systems: Manipulation of polyhydroxybutyrate-based materials. Plastics, Rubber and Composites Processing and Applications. 19, 15-27.

Yoshioka, S., Kishida, A., Izumikawa, S., Aso,Y. and Takeda, Y. (1991) Base-induced polymer hydrolysis in poly(β-hydroxybutyrate / β- hydroxyvalerate) matrices. Journal of Controlled Release, 16, 341-348.

Zamecknik, P.C. and Stephenson, M.L. (1978) Inhibition of rous sarcoma virus replication and cell transformation by a specific oligodeoxyribonucleotide. Proceedings of the National Academy of Sciences, USA, 75, 280-284.

Zhao, Q., Matson, S., Herrera, C., Fisher, E., Yu, H. and Kreig, A.M. (1993). Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate and mixed phosphorothioate and methylphosphonate oligonucleotides. Antisense Research and Development, 3, 53-66.

Zhu, N., et al., (1993) Systemic gene expression after intravenous DNA delivery into adult mice. Science, 261, 204-211.

Zon, G. (1988) Oligonucleotide analogues as potential chemotherapeutic agents. Pharmaceutical Research, 5, 539-549.

APPENDIX ONE

ANTI-TUMOUR EFFICACY IN VIVO USING OLIGODEOXYNUCLEOTIDE-LOADED MICROSPHERES

A1.1 INTRODUCTION

The work for this chapter was done in collaboration with Hoffmann-La Roche Inc., Nutley, New Jersey, USA, with special thanks to Dr Ramaswamy Narayanan for providing the ODNs for loading into micropsheres after the initial studies and organising the animal studies.

NF-κB is a transcription factor composed of two subunits p50 (NF-κB1) and p65 (RelA), which has a role in mediating cell adhesion and neoplastic cell growth. NF-κB binding sites are present on various adhesion molecules, cytokines and growth factor receptors. The inhibition of p65 inhibits tumour cells *in vitro* and growth of tumour cells both *in vitro* and *in vivo* (Higgins *et al.* 1993).

Previous *in-vivo* experiments at Roche dosed mice with either a subcutaneous infusion pump releasing 2.8 mg of ODN (PS 24mer RelA) over a 2 week period, or a 1.4mg ODN dose administered subcutaneously twice weekly for 2 weeks. A significant reduction in tumor mass was observed in 1-2 weeks compared to control animals, which did not receive ODN treatment. Pretreatment with the ODNs before injection of the tumour cells K-BALB (fibrosarcoma) or B-16 (melanoma) did not enhance the degree of tumour regression observed.

In theory the use of a biodegradable delivery device, to provide sustained release of ODNs over a two week period would have the advantage over an infusion pump as the drug depleted polymer erodes and does not need to be surgically removed unlike the infusion pump.

A1.2 AIM OF STUDY

As a result of the successful *in vivo* experiments with ODNs (scetion 1.8), the aim of this section was to formulate ODNs into PLGA microspheres and develop an administration protocol. The use of ODN-loaded microspheres as a delivery device could then be assessed, and the degree of tumour regression compared with either an infusion pump or ODN alone.

A1.3 FORMULATION OF MICROSPHERES FOR *IN VIVO* STUDIES

The murine PS 24 mer antisense and sense ODN sequences targeted to the p65 subunit of NF-κB (Rel A) are listed in appendix 2. The antisense and sense ODNs were kindly provided Hoffmann La Roche in sterile PBS (calcium and magnesium free) at a concentration of 14mg/ml.

The microspheres were initially made as in section 4.2.2.2, adding 2 μg of ODN to 500mg of polymer (PLGA 50:50 molecular weight 3,000) to produce spheres of approximately 10 to 20 μm in size. This size was chosen to enable a depot release preparation and limit internalisation by peritoneal macrophages. Certain changes to the procedure had to be made in order to produce sufficient amounts of ODN loaded micropsheres, in a formulation which could be injected easily through a 21G needle. Although initial batches of spheres would pass through a 21G needle when resuspended in PBS, sedimentation and aggregation of the spheres in the syringe barrel prior to injection caused the syringe barrel and needle to become blocked. The major limitations to the administration of the spheres were, the large mass of spheres to give 1.4mg ODN and the small maximum volume which could be injected into a mouse without adverse effects. Therefore, it was important to achieve the highest possible ODN loading in the polymer microspheres to reduce the polymer mass to be injected.

The loading experiments in section 4.4 indicate that the loading of ODN in the polymer microspheres is linear over the range 0.8-8μg/mg of polymer, the amount of ODN added per batch was not increased above this range. The total loading of PS 24 mer ODN was 5μg /mg polymer microspheres. Increasing the loading of the

ODN in the polymer spheres did not effect the sphere size or surface charge (see section 4.3.5).

The main formulation problems were not associated with increasing the ODN loading but were found in the process of injecting the spheres into the mice. Although in practice the spheres would suspend in PBS and pass through a 21G needle immediately after drawing into a syringe barrel. However in the practicalities of the animal experiments immediate injection into the mice was not possible and the spheres sedimented in the barrel, before they could be injected into the mice. The rapid sedimentation was probably as a result of the high polymer mass per injection volume (500mg/ml). As a result, a different injection vehicle was required which would enable the spheres to remain in suspension in the syringe barrel during the period prior to injection. Some of the problems encountered and the solutions are listed in Table A1.1

PROBLEMS	SOLUTIONS
The freeze dried pellet of spheres required grinding to produce a free flowing powder. Grinding induced a greater charge on the spheres.	The spheres were freeze dried as a suspension, to provide a free flowing powder without grinding.
Static charge associated with the spheres.	Charge reduced by a zero-stat gun.
Presence of aggregates blocks the syringe and the needle.	The spheres were sieved through a 125μm sieve to remove any large aggregates.
Sedimentation of the spheres in the syringe barrel due to their large relative size and number, blocking the syringe barrel and the needle.	Addition of suspending agents/surfactants to keep the spheres suspended in the syringe barrel.
The polymer mass is too large to inject into mice.	Increase the loading of ODN per mg of polymer microspheres.

Table A1.1 Problems and solutions of microsphere manufacture for animal experiments

A suspension of the microspheres in an appropriate vehicle was required for the administration of the ODN loaded spheres. The suspension must ideally remain sufficiently homogenous for at least the time period it takes to fill the syringe barrel and inject into the mouse, with any sediment being easily resuspendable. Suspensions can be of two types, flocculated or deflocculated. A flocculated suspension sediments rapidly, however the large sediment volume is easily resuspended on gentle agitation. A deflocculated suspension sediments very slowly, however the sediment forms a hard cake and is difficult to resuspend. A deflocculated system with a sufficiently high viscosity to prevent sedimentation would be an ideal situation. Sedimentation of the particles depends on the particle size and the viscosity of the medium. In this instance the particle size could not be reduced so the suspension was formed by modifying the viscosity of the medium and the wetting agents employed. Many of the delivery formulations mentioned in the literature require the administration of low concentrations of spheres in a relatively high volume. For example Lalla and Sapna (1993) report the delivery of a 100mg of microspheres in 2ml of normal saline 0.1% methylcellulose as a suspending agent and 1% tween-20 as a wetting agent.

Surfactants, hydrophilic colloids and solvents were used to determine the ideal vehicle for administration of the spheres.

Each vehicle was assessed by successful suspension formation of 100mg polymer spheres in 200µl of vehicle, drawing into the syringe barrel and injection through a 21G needle either immediately or after standing for 5 minutes.

Experiments were performed on a small scale in order to test the suitability of the surfactants in the vehicle. In each case 200µl of vehicle was added to 100mg of polymer spheres. All suspending agents were made up in PBS. The results are shown in Table A1.2.

FORMULATION VEHICLE	EASE OF INJECTION	
	immediate injection	injection after 5mins
PBS	√	X
0.5 -5% PVP	√	X
0.1-0.5 % HPMC	X	X
0.5-2% Tween 80	√	X
O.5-5% Sodium pyrophosphate	√	X
PEG 400	very sticky	X
50% PEG 400	√	X
1% PVP + 50 % PEG	√	√
2.5% PVP + 50% PEG	√	√
50% PEG + 2% Tween	√	√

Table A1.2 Choice of administration vehicle for polymer microspheres. √ denotes that the spheres were easily injected through a 21G needle, X denotes that the spheres could not easily be injected through a 21G needle.

A sphere suspension in phosphate buffered saline (PBS) was easily injected through a 21G needle immediately after drawing into the syringe barrel, however on standing, the sphere sediment in the syringe barrel prevented injection of the spheres via the needle. A similar result was seen when the suspension included 0.5 to 5% polyvinyl pyrolidine (PVP), 0.5 to 2% Tween-20, 0.5 to 5% sodium pyrophosphate. The suspension formed with 0.1 to 0.5% hydroxypropylmethyl cellulose (HPMC) could not be injected through the 21G needle as the suspension formed a solid 'paste' in the syringe barrel thus preventing release. An injection vehicle of 100% polyethylene glycol 400 (PEG) was too sticky and viscous for easy handling and injection. Dilution to 50% with PBS didn't appear to solve the problem on injection after standing for 5 minutes, however incorporation of PVP or 2% tween-20 produced a suspension which remained stable for the 5 minutes necessary between drawing into the syringe and injecting into the mice. This suspension had a very large sedimentation volume and was easily resuspended.

The rate of sedimentation of the successful flocculated suspensions were assessed by measuring the depth of clear supernatant in a 1ml pipette over time, of a suspension of 50mg polymer spheres in 500µl of vehicle. The results comparing PBS with 50% PEG and 2.5% PVP are shown in fig A1.1. The results show that the rate sedimentation of the spheres is clearly reduced in the PEG/PVP vehicle compared to PBS alone. Only the 50% PEG and 2.5% PVP is shown graphically, but the results for 1% PVP or 2% Tween were similar.

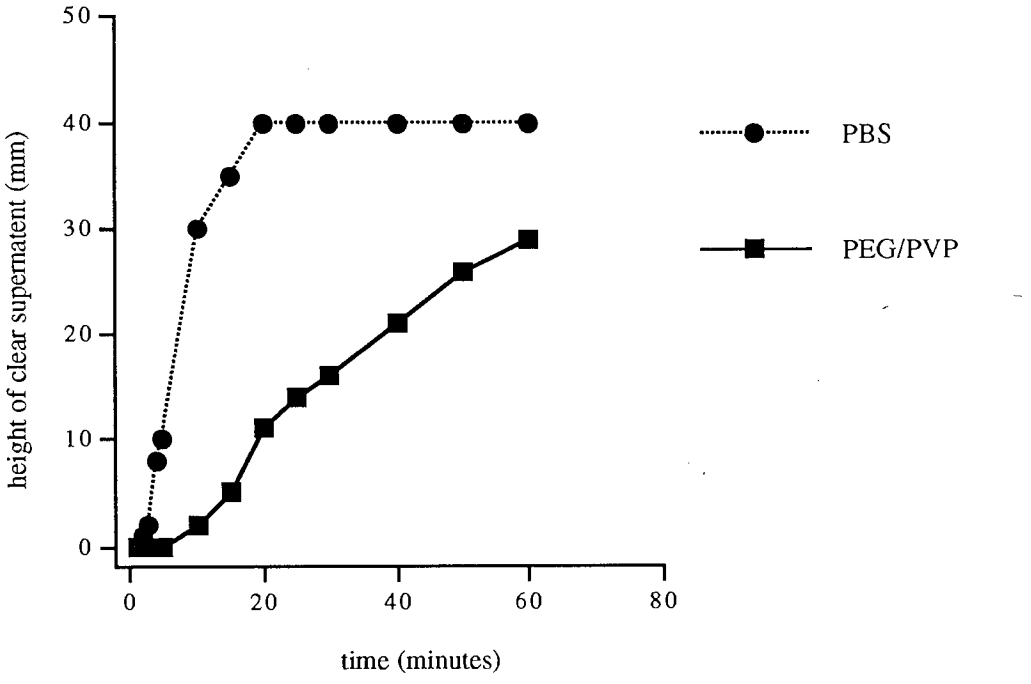

Fig.A.1. Sedimentation of PLGA (Mw 3,000, 10-20µm) microspheres (50mg) in delivery vehicle (500µl) at room temperature, shown as the formation of a clear supernatant over time.

A1.4 SCALE UP PRODUCTION

Microsphere batches were made which contained either antisense or sense ODNs, control spheres were also produced which were unloaded. The PS 24mer ODN (4mg) in PBS was added to 5ml polymer (500mg) dichloromethane solution and vortexed to produce the primary emulsion as detailed in section 4.2.2.2. However, two batches of the primary emulsion were added to 160ml of external phase and stirred at 1,000 rpm for 4 hours to allow evaporation of the solvent. The spheres were made simultaneously on 4 identical stirrers. The method is shown diagramatically in fig.4.1. The spheres were washed three times with sterile distilled water and freeze dried in a suspension to create a free flowing powder. The resulting spheres were put through a 125μm sieve to remove any aggregates. The scaled up production procedure did not affect the sphere size, surface charge or the batch yields. The spheres were packed in a universal tube with a dessicator and sent to Roche for the animal experiments.

Antisense and sense loaded microspheres were made, as well as blank microsphere sphere batches as a control.

A1.5 PROTOCOL FOR THE ADMINSTRATION OF OLIGODEOXYNUCLEOTIDE-LOADED MICROSPHERES

1. The microspheres (500mg) either blank, loaded with antisense or sense ODN were weighed into a 2ml vial and 1ml of the vehicle was added and vortexed to form a smooth suspension.

2. The sphere suspension was drawn into a 2ml syringe, any air bubbles were removed before attaching a 21 G needle. The syringe barrel was rotated as much as possible to prevent any sedimentation prior to injecting 500μl of the suspension intraperitoneally. In each experimental group there were 5 nude mice.

3. The spheres were injected subcutaneously 7 days before injection of the K-BALB tumour cells (fibrosarcoma) due to the expected lag phase of *in-vivo* release. A further dose of spheres was administered 7 days later.

4. The tumour size was monitored 2 weeks after the injection of the tumour cells.

A1.6 *IN VIVO* RESULTS

Injection of the 500µl of sphere suspension, or vehicle alone into the mouse peritoneum resulted in acute peritonitis, with 2 animals in each group dying overnight and the remainder dying within 48-72 hours post injection. Death was assumed to be as a result of the large volume of injection. As a result a reduced volume of 250µl was injected in 2 animals, no peritonitis occurred and the experiments were then continued with a reduced injection volume.

All the mice were alive and well on day 21, i.e. 14 days after the injection of the tumour cells, with no peritonitis or toxicity observed. Unfortunately the ODN loaded microspheres delivered in this way did not have a positive effect on reducing the size of the developing tumour as shown graphically in fig A1.2. Unfortunately no statistical data was available from Roche.

The acute peritonitis which occurred following the injection of a 500µl dose of microsphere suspension meant that the dose given to the animals was reduced by a half, and this reduction was not compensated by additional injections when the protocol was changed. The reduced dose of ODN given in the PLGA microspheres as compared to the infusion pump or the subcutaneous injection of free ODN could be the major contributing factor which caused the polymeric delivery device containing the antisense ODN to have no effect on the tumour size.

Increasing the loading of the ODN results in an increased burst effect as demonstrated in the release experiments in section 5.3, as a result of an increased concentration of ODN present at the microsphere surface and not an even incorporation into the polymer matrix. It is possible that the loading could be increased to such an extent, that the majority of the dose is released during the initial burst release as the majority of the ODN is present at the sphere surface. This appears to be the explanation for the results in fig A1.3, where the burst release is very high (90%). The *in vivo* release from PLGA microspheres is expected to be similar to *in vitro* release (Pitt *et al.*, 1991). However the *in vitro* release profiles

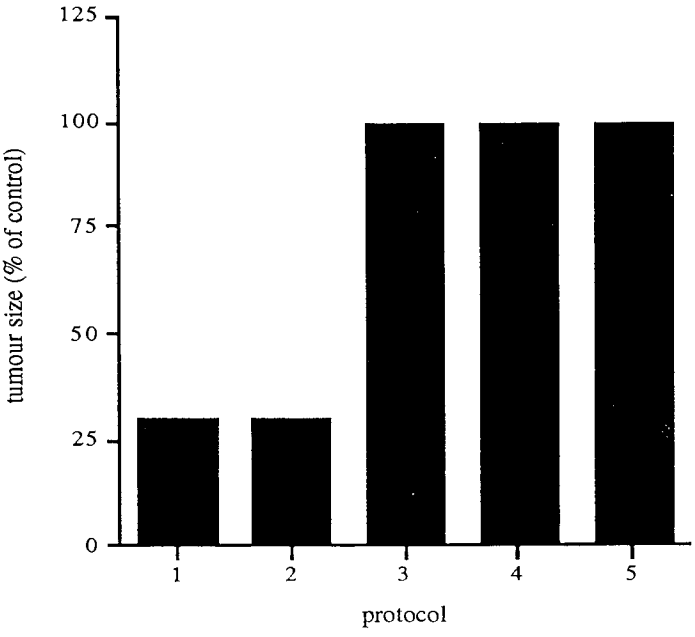

Fig.A1.2. Tumour size as a result of *in vivo* experiments, shown as percentage of the control, where tumour cells alone were injected. Protocol 1: 1.4mg PS ODN injected subcutaneously twice weekly for 2 weeks, Protocol 2: 2.8mg PS ODN released from a subcutaneous implant over a 2 weeks period, Protocol 3: 125mg antisense ODN loaded microspheres injected on day 1 and day 7, Protocol 4: 125mg sense ODN loaded microspheres injected on day 1 and day 7, Protocol 5: 125mg control (blank) microspheres injected on day 1 and day 7.

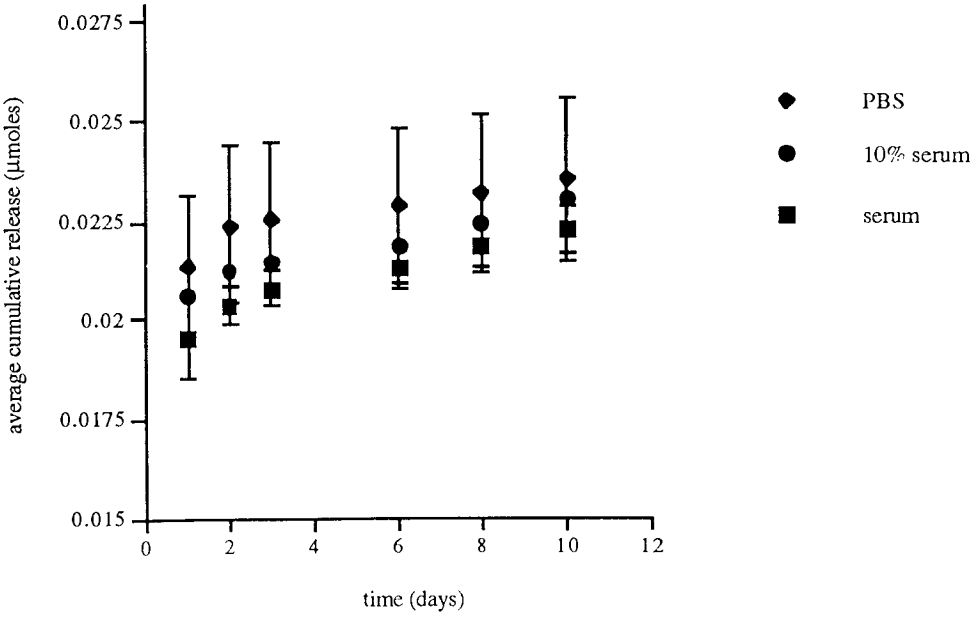

Fig.A1.3. Effect of serum on the average cumulative release of PS 24mer ODN (µmoles) from microspheres (25mg) at pH 7.4 and 37°C over a 10 day release period. PLGA 50:50, Mw 3,000, size 10-20µm, made from a double emulsion (4.2.2.2).Loading = 8µg/mg of polymer. n = 4 ± S.D.

can only serve as a guide to the release *in vivo*, due to different fluid volumes as well as different degrees of movement or agitation at various injection sites compared to the *in vitro* set up. If the high burst release of the ODN from the polymer was a contributing factor in the failure of the polymeric delivery device to reduce tumour growth, the spheres could be coated to reduce the initial burst release (Pekarek *et al.*, 1994).

The fact that the spheres were not injected as close to the tumour site as the free ODN or the subcutaneous infusion pump, could be a contributing factor as to why no tumour regression was observed. In future experiments it may be better to sterilise the microspheres by γ-irradiation or ethylene oxide and then inject the spheres in an identical location as the infusion pump.

A1.7 CONCLUDING REMARKS

Microspheres were successfully prepared with sufficient ODN/mg of polymer to give an equivalent ODN dose compared to the subcutaneous infusion pump in a relative low polymer mass. A suspension was successfully formulated which enabled delivery of the spheres through a 21G needle without sedimentation in the syringe barrel. Despite the initial acute peritonitis with a 500µl dose, a reduced dose of 250µl had no adverse effects.

The *in vivo* studies detailed in this chapter were performed towards the latter stages of my research which unfortunately did not leave any time to modify the procedures and repeat the experiments. Assessment of the *in vivo* release of the ODNs from the microsphere and the *in vivo* distribution following administration at various injection sites would be useful information before repeating the experiments to investigate the role of antisense ODN and polymeric delivery devices on tumour size regression.

This study has highlighted some of the problems associated with a multi centre experiment. Although the *in vivo* results were disappointingly negative, many important factors have been raised during this initial study, which will hopefully aid subsequent *in vivo* experiments using ODN or RBZ loaded biodegradable polymer devices.

APPENDIX TWO

NUCLEIC ACID SEQUENCES, MOLECULAR WEIGHT AND EXTINCTION COEFFICIENTS

Molecular weights and extinsion coefficients have been calculated according to the method detailed in section 2.3.4. The differences in the Mw between PO and PS have been ignored for these calculations. The Mw of the RNA RBZs was calculated as for the DNA equivalent. All the sequences are antisense unless otherwise stated.

1. 20 mer *tat*

3' ACA CCC AAT TCT GAA AAT GG 5'
Mw = 6090 $\varepsilon = 179.1 cm^2 \mu mol^{-1}$ 1 OD_{260} = 29.4μg

2. 20 mer *tat* sense

3' CCA TTT TCA GAA TTG GGT TG 5'
Mw = 6134 $\varepsilon = 196.8\ cm^2\ \mu mol^{-1}$ 1 OD_{260} = 32.08μg

3. 32 mer *c-myc*

3' CGC CAA AGC CGG ATT GCC GGA GTA GTC TGT GG 5'
Mw = 10444 $\varepsilon = 310.2\ cm^2\ \mu mol^{-1}$ 1 OD_{260} = 29.7μg

4. 32 mer *c-myc* sense

3' CCA CAG ACT ACT CCG GCA ATC CGG CTT TGG CG 5'
Mw = 9900 $\varepsilon = 309.6\ cm^2\ \mu mol^{-1}$ 1 OD_{260} = 31.27μg

5. 15 mer *c-myc*

3' TAC GGG GAG TTG CAA 5'
Mw = 4654 $\varepsilon = 155.9\ cm^2\ \mu mol^{-1}$ $1\ OD_{260} = 33.5\mu g$

6. 7 mer *c-myc*

3' TAC GGG G 5'
Mw = 1895 $\varepsilon = 48.5\ cm^2\ \mu mol^{-1}$ $1\ OD_{260} = 25.6\mu g$

7. 7 mer poly A

3' AAA AAA A 5'
Mw = 2129 $\varepsilon = 46.71\ cm^2\ \mu mol^{-1}$ $1\ OD_{260} = 21.94\mu g$

8. 7 mer poly C

3' CCC CCC C 5'
Mw = 1961 $\varepsilon = 83.5\ cm^2\ \mu mol^{-1}$ $1\ OD_{260} = 42.6\mu g$

9. 7 mer poly G

3' GGG GGG G 5'
Mw = 2241 $\varepsilon = 68.1\ cm^2\ \mu mol^{-1}$ $1\ OD_{260} = 30.4\mu g$

10. 7 mer poly T

3' TTT TTT T 5'
Mw = 2066 $\varepsilon = 76.9\ cm^2\ \mu mol^{-1}$ $1\ OD_{260} = 37.2\mu g$

11. 24 mer Rel A

3' TGG TAC CTG CTA GAC AAA GGG GAG 5'
Mw = 7462 $\varepsilon = 222.4\ cm^2\ \mu mol^{-1}$ 1 OD_{260} = 29.8 μg

12. 24 mer Rel A sense

3' CTC CCC TTT GTC TAG CAG GTA CCA 5'
Mw = 7265 $\varepsilon = 214.8\ cm^2\ \mu mol^{-1}$ 1OD_{260} = 29.6μg

13. 32 mer *c-myc* RNA RBZ

3' CUC CAA AGC CGG AUU GCC GGA GUA GUC UGU GG 5'
Mw = 10444 $\varepsilon = 310.2\ cm^2\ \mu mol^{-1}$ 1 OD_{260} = 29.7μg

14. 27 mer *c-myc* RNA substrate

3' CUC CUU CCC ACA CUG GCG UUG CAU CCU 5'
Mw = 5843 $\varepsilon = 164.2\ cm^2\ \mu mol^{-1}$ 1 OD_{260} = 28.1μg