CNS DELIVERY OF ANTISENSE OLIGODEOXYNUCLEOTIDES USING BIODEGRADABLE MICROSPHERES

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SUMMARY

Antisense oligodeoxynucleotides can selectively inhibit gene expression provided they are delivered to their target site successfully for a sufficient duration. Biodegradable microspheres have previously been developed for the potential systemic delivery of antisense oligodeoxynucleotides and offer an excellent strategy for central administration of antisense oligodeoxynucleotides, providing a sustained-release delivery system. Biodegradable microspheres were formulated to entrap antisense oligodeoxynucleotides for stereotaxic implantation into site-specific regions of the rat brain.

Release profiles of antisense oligodeoxynucleotides from biodegradable microspheres over 56 days that were triphasic were observed with high molecular weight polymers. Antisense oligodeoxynucleotides loaded into microspheres (1-10μm) had a five-fold increase in cellular association with glial and neuronal cells compared to the naked molecule, which was partially due to a greater cellular accumulation as observed by a slower efflux profile.

In vivo distribution studies of antisense oligodeoxynucleotides demonstrated that the use of microspheres provided a sustained-release over more than 2 days compared to 12 hours of the naked molecule. Efficacy of antisense oligodeoxynucleotides was demonstrated during locomotor activity investigations, which significantly reduced cocaine-induced locomotor activity, where no efficacy was demonstrated with microspheres, possibly attributed to antisense loading and measurements being taken during a lag phase of antisense oligodeoxynucleotide release.

Biodegradable microspheres can be delivered site-specifically into the brain and provide sustained-release of antisense oligodeoxynucleotides, offering the potential of *in vivo* efficacy of these reagents in the brain.

Key words microspheres, antisense, oligodeoxynucleotides, poly (D,L-lactide-co-glycolide), D_2 dopamine receptor

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ABBREVIATIONS

AAV	adeno-associated virus
A, C, G, T	adenine, cytosine, guanine, thymidine
ACh	acetylcholine
AIDS	acquired immunodeficiency disease
ANOVA	analysis of variance
AP	anterior/posterior
AS-ODN(s)	antisense oligodeoxynucleotide(s)
AT-1	angiotensin-1 receptor
ATP	adenosine triphosphate
AUG	adenine, uracil, guanine trinucleotide (initiation codon)
BBB	blood-brain barrier
BCNU	1,3-bis(chloro-ethyl)-1-nitrosurea
bins	beam interruptions
c	cortex
^{14}C	^{14}carbon (radionuclide)
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CCK	cholecystokinin
CHO	Chinese hamster ovary
CHOL	cholesterol
CMV	cytomegalovirus
CNS	central nervous system
CPG	controlled-pore glass
cpm	counts per minute
CPu	caudate putamen
CREB	cAMP response element-binding protein

D_1, D_2, D_3, D_4, D_5	dopamine subtypes (1, 2, 3, 4, 5)
DA	dopamine
DAB	diaminobutyric acid
DABCO	diazabicyclo[2.2.2]octane
DAT	dopamine transporter
DCM	dichloromethane
ddH_20	double-distilled water
DDS	drug delivery systems
2-DG	2-deoxyglucose
DIP	N-dodecyl-2-imidazole-propionate
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethoxy sulphoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
DOPE	dioleoylphosphatidylethanolamine
DRG	dorsal root ganglion
DTT	dithiothreitol
DV	dorsal/ventral
EDTA	ethylenediaminetetraacetic acid
ε	extinction coefficient
EGFR	epidermal growth factor receptor
EVAc	poly (ethylene-co-vinyl acetate)
2'-F	2'-fluoro
FCS	foetal calf serum
FDA	Food & Drug Administration
FITC	fluoroisothiocyanate
g	gauge
GA	glycolic acid
^3H	tritium (radionuclide)
HIV	human immunodeficiency virus
HMw	high molecular weight

HPLC	high-performance liquid chromatography
HPV	human papilloma virus
HSV	herpes simplex virus
HVJ	haemagglutinating virus of Japan
ICAM-1	intercellular adhesion molecule-1
i.c.v.	intracerebroventricular
IgG	immunoglobulin G
IHC	immunohistochemistry
i.p.	intraperitoneal
ISH	*in situ* hybridisation
i.v.	intravenous
kDa	kiloDaltons
L	lateral
LA	lactic acid
LH	Lister Hooded
LHRH	leutinising hormone-releasing hormone
LMw	low molecular weight
Log P	partition coefficient
LSC	liquid scintillation counting
LV	lateral ventricles
MFB	median forebrain bundle
MP-ODN(s)	methylphosphonate oligodeoxynucleotide(s)
mRNA	messenger RNA
MTD	maximum tolerated dose
Mw	molecular weight
NA	noradrenaline
NAcc	nucleus accumbens
nAChR(s)	nicotinic acetylcholine receptor(s)
NaN_3	sodium azide
NMDA	*N*-methyl-D-aspartate
OA	oleic acid

OD	optical density
ODN(s)	oligodeoxynucleotide(s)
6-OHDA	6-hydroxydopamine
2'-OMe	2'-O-methyl
^{32}P	^{32}phosphorus (radionuclide)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCPP-SA	poly-[bis(*p*-carboxyphenoxy)propane-sebacic acid (polyanhydrides)
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine
PGA	polyglycolic acid
PHB	polyhydroxybutyrate
PKC	protein kinase C
PKCα	protein kinase C-alpha
PLA	polylactic acid
PLGA	poly (D,L-lactide-co-glycolide)
PLL	poly-L-lysine
PO-ODN(s)	phosphodiester oligodeoxynucleotide(s)
pre-mRNA	pre-messenger RNA
PS-ODN(s)	phosphorothioate oligodeoxynucleotide(s)
PVA	polyvinyl alcohol
RIA	radioimmunoassay
RME	receptor-mediated endocytosis
RNA	ribonucleic acid
RNase H	ribonuclease H
RSV	Rous sarcoma virus
s.d.	standard deviation
SDS	sodium dodecyl sulphate
s.e.m.	standard error of the mean

SEM(s)	scanning-electron micrograph(s)
SN	substantia nigra
SV40-T antigen	Simian virus 40-T antigen
$t_{1/2}$	half-life
TAR	trans-activating response sequence
TBE	tris/boric acid/EDTA
TCA	trichloroacetic acid
TEMED	N,N,N,'N'-Tetramethylethylene diamine
TETD	tetra-ethyliuram disulphide
tk	thymidine kinase
T_m	melting transition
UV	ultra-violet
VTA	ventral tegmental area
v/v	volume/volume
w/o	water-in-oil
w/o/w	water-in-oil-in-water
w/v	weight/volume

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1. ANTISENSE OLIGODEOXYNUCLEOTIDES

Antisense oligodeoxynucleotides (AS-ODNs) may be defined as short lengths of single-stranded deoxyribonucleic acid (DNA) designed to inhibit gene expression. Zamecnik and Stephenson in 1978, proposed the use of synthetic AS-ODNs for therapeutic purposes when they were able to inhibit the growth of the Rous Sarcoma Virus (RSV) in cell culture, by application of a 13-mer ODN that was complementary to the ribonucleic acid (RNA) of RSV.

The potential of AS-ODNs for gene disruption, leading to specific down-regulation of targeted proteins is an attractive strategy for therapy (Nemunaitis *et al.*, 1999; Waters *et al.*, 1999; Stevenson *et al.*, 1999). They also have a great potential as pharmacological tools in assessing the function of genes and their respective proteins (Brussard, 1997; Widnell *et al.*, 1996; Tepper *et al.*, 1997). AS-ODNs provide a means by which the function of a protein can be assessed, providing that there is a biochemical, physiological or behavioural endpoint.

Since their discovery, much research has highlighted numerous problems that may hinder the therapeutic and research potential of AS-ODNs. These problems include lack of stability to nucleases (Akhtar *et al.*, 1992), affinity for their target (Cook, 1992) and permeation into the cytosol and nucleus of cells (Akhtar & Juliano, 1992).

In an effort to overcome these problems, much research has been done on structural modifications (reviewed by Matteucci, 1995) and development of delivery devices (reviewed by Miller & Das, 1998). More recently, research has been done into the development of techniques to investigate optimal targets for antisense activity on the messenger RNA (mRNA) molecule, these include DNA chip technology (Kurian *et al.*, 1999) and ribonuclease H (RNase H) accessibility mapping (Scherr & Rossi, 1998). In order to understand fully the development of structural modifications, delivery devices and determination of optimal target sites, it is important to discuss the modes of action of AS-ODNs and how they interact with their target and cellular permeation of ODNs.

1.1.1. Mechanisms and sites of action

There are several ways to hybridise genes to prevent gene expression, which are outlined below and illustrated in figure 1.1. These mechanisms involve intervention of crucial processes required for protein synthesis, including transcription, post-transcriptional processes and translation and may also involve the disruption and inhibition of critical structures and enzymes.

1.1.1.1. Inhibition of transcription

RNA transcription begins when RNA polymerases randomly collide with DNA at a promoter region, which contains sequence elements that bind RNA polymerase to the first base to be copied. Initiation factors form an active complex with RNA polymerase, allowing for tight binding of this enzyme to the promoter, complete assembly of this complex destabilises the DNA double helix, which unwinds in the promoter region. Once the double helix is unwound, the RNA polymerase is able to move along the DNA template. In this destabilised state, AS-ODNs may have access to the DNA sense strand of the double helix and can hybridise to prevent the progression of transcription and hence the production of pre-messenger RNA (pre-mRNA) (Crooke, 1996a).

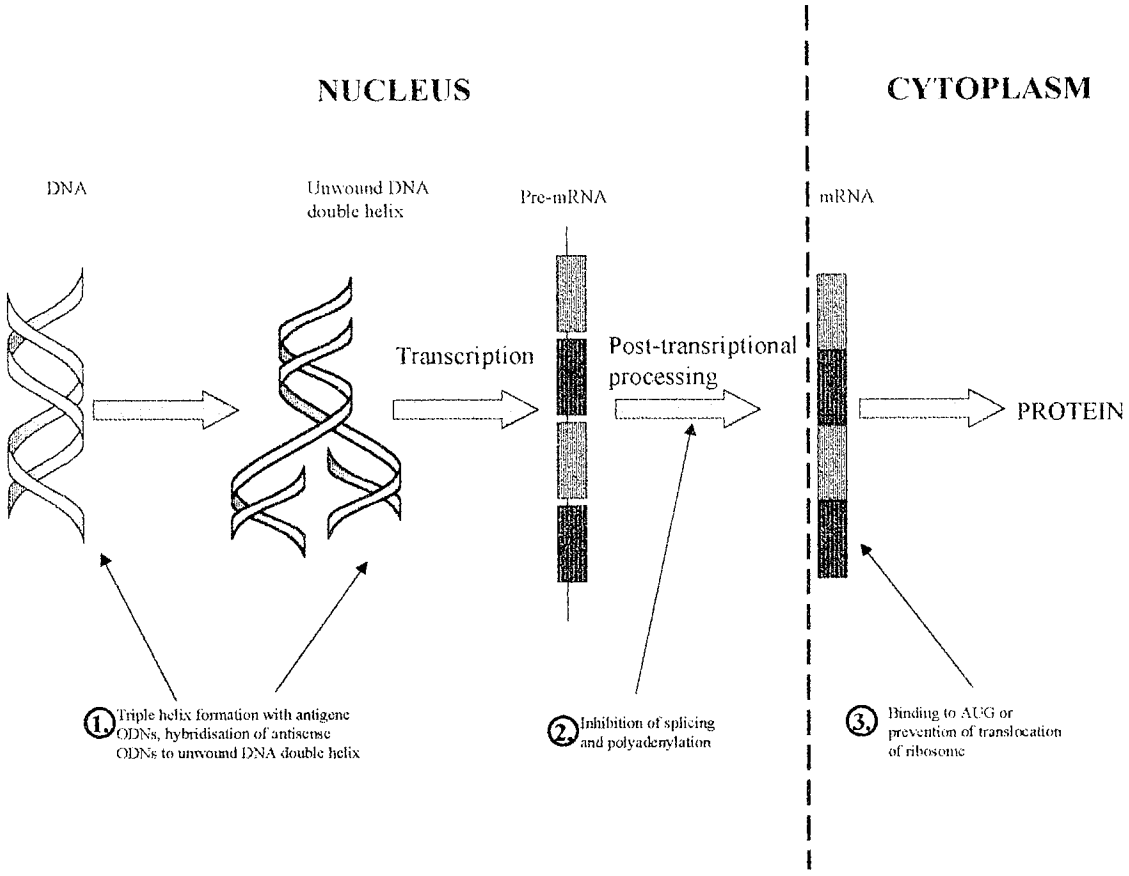


Figure 1.1. A schematic representing the possible sites of interaction of antisense oligodeoxynucleotides. Boxes represent exons on pre-mRNA and mRNA molecules and lines represent introns on pre-mRNA molecule.

ODNs can bind to the DNA double helix *via* Hoogsteen interactions, forming a triple-helix structure where the ODN is referred to as antigene as opposed to antisense (Uhlmann & Peyman, 1990). Binding of single-stranded ODNs to double stranded DNA can sterically hinder the progression of transcription.

1.1.1.2. Inhibition of post-transcriptional processes

Following transcription, there are a number of processes that aid in the progression to translation, termed post-transcriptional processes facilitating in the stabilisation,

nuclear export and maturation of mature mRNA, including such processes as 5'-capping, splicing and 3'- polyadenylation. Intervention of these processes with AS-ODNs may inhibit the production of mature mRNA molecules, thus inhibiting protein synthesis.

Pre-mRNA is stabilised by capping the molecule at the 5'-end. This process also plays a role in binding of the pre-mRNA to the nuclear matrix and transport out of the nucleus. This provides an interesting target for AS-ODNs, due to the unique and characterised structure of the 5'- cap. AS-ODNs that were targeted near to the 5'- cap site of SV40-T antigen mRNA were reported to be sensitive to this ODN (Westerman *et al.*, 1989), but the actual mechanism was not demonstrated. Whereas AS-ODNs that were designed to specifically cleave the 5'- cap structure demonstrated that these 5'- cap targeted AS-ODNs were capable of inhibiting the binding of translation initiation factor eIF-4α in a study by Baker *et al.* (1992).

The excision of introns (non-coding regions) in pre-mRNA molecules is a key step in their metabolism, requiring specific sequences and action of spliceosomes. The hybridisation of AS-ODNs across the splicing junctions of the pre-mRNA can prevent introns from being excised from the exons and thereby blocking the production of mature mRNA. This mechanism has been demonstrated by RNA analyses, where an AS-ODN induced alternative splicing (Dominski & Kole, 1993).

Upstream of the 3'- untranslated region of pre-mRNA polyadenylation takes place. This process acts to stabilise the molecule and may also be involved in the export process out of the nucleus. AS-ODNs complementary to the 3'- terminal region could theoretically inhibit polyadenylation, destabilising the pre-mRNA, although this currently remains a theoretical mechanism, as there is no evidence, to date, for inhibition of polyadenylation.

1.1.1.3. Inhibition of translation

Translation commences when the mature mRNA and a number of initiation factors bind to ribosomes. AS-ODNs may inhibit this process by binding to the site on mRNA where translation begins, the initiation codon (AUG). Alternatively, it may bind further along the mRNA molecule, preventing the necessary translocation of the ribosome along the mRNA. There are some examples of RNA species that have been reported to be inhibited by this mechanism and include human immunodeficiency virus (HIV) (Agrawal *et al.*, 1988), interleukin-1 α (Maier *et al.*, 1990) and *n-myc* (Rosolen *et al.*, 1990).

1.1.1.4. Disruption of RNA structures

RNA adopts numerous three-dimensional structures, most commonly a stem-loop. These structures play a vital role in stability of the RNA and act as recognition motifs for proteins, nucleic acids and ribonucleoproteins for metabolism, and activity of the RNA. Any breakdown in these structures may result in instability and altered metabolism of the RNA. AS-ODNs were designed to a stem-loop structure in RNA of HIV, the TAR (trans-activating response sequence) element. Some of these ODNs successfully bound to TAR, disrupted its structure and inhibited its function of production of a reporter gene (Vickers *et al.*, 1991).

1.1.1.5. Activation of ribonuclease H (RNase H)

Ribonuclease H (RNase H) is a ubiquitous enzyme found in both prokaryotes and eukaryotes. It is an enzyme that breaks down RNA-DNA duplexes by degradation of the RNA strand. It is involved in DNA replication, but may play other roles as it is found in the nucleus and the cytoplasm (Crum *et al.*, 1988). However, its concentration in the nucleus is thought to be greater than in the cytoplasm, and concentrations in the cytoplasm may be due to nuclear leakage.

The precise recognition sites for RNase H are not known, but certain chemistries of ODNs have differing abilities to activate this enzyme. Alterations in the sugar moiety of the ODN can influence activation of RNase H, where sugar modifications resulting in a more RNA-like structure, for example, 2'-fluoro (2'-F) and 2'-O-methyl (2'-OMe), were not a substrate for RNase H (Sproat *et al.*, 1989; Kawasaki *et al.*, 1993). Modifications in the backbone of the ODN also altered the ability to activate this enzyme, where phosphorothioated ODNs (PS-ODNs) are excellent substrates (Mirabelli *et al.*, 1991) and methylphosphonates (MP-ODNs) are not (Maher *et al.*, 1989) (differences between these two modifications are discussed later in section 1.1.3.1.).

RNase H activity has been demonstrated in studies using reverse-ligation polymerase chain reaction (PCR), where cleavage products from *bcr-abl* mRNA in cells treated with phosphorothioate ODNs were identified (Giles *et al.*, 1995).

1.1.1.6. Non-antisense effects

It has become apparent over the last decade that the biological actions of AS-ODNs are complex, and do not always follow the selective mechanisms outlined in figure 1.1. Some of the earlier work on the effects of AS-ODNs in cell culture systems may be flawed, where investigators interpreted inhibition of proteins as being antisense effects, when they were not. A number of biological and biochemical effects by a non-antisense mechanism have been highlighted in studies carried out in recent years (Ellington & Szostak, 1992; Galbraith *et al.*, 1994; Krieg *et al.*, 1995; Benimetskaya *et al.*, 1997).

AS-ODNs that bind receptors, enzymes and other proteins affecting their function are termed aptamers, and occur due to the fact that ODNs can fold into complex three-dimensional structures (Ellington & Szostak, 1992). Aptamers can act in a sequence-selective manner, thus confounding controls, such as sense and scrambled sequences.

ODNs that contain a "G-quartet" are prone to the formation of tetraplexes and higher order structures and to specific interactions with proteins (Burgess *et al.*, 1995; Benimetskaya *et al.*, 1997; Castier *et al.*, 1998). A G-quartet present near the 5'-terminus of a phosphorothioate ODN (PS-ODN) was found to be capable of forming tetraplexes and other higher order structures in a time- and temperature-dependent manner (Benimetskaya *et al.*, 1997). These structures are thought to play an important role *in vivo*, and putative quartet-forming sequences have been identified in telomeric DNA (Sundquist & Klug, 1989), immunoglobulin switch region sequences (Sen & Gilbert, 1988), HIV type I RNA (Sundquist & Heaphy, 1993), and the retinoblastoma gene (Murchie & Lilley, 1992).

There are also sequence-independent aptameric effects, of which phosphorothioate ODNs (PS-ODNs) are known to interact with a wide variety of proteins (Brown *et al.*, 1994; Guvakova *et al.*, 1995). AS-ODNs can also exert non-specific effects on the host immune system; CpG motifs are potent B-cell mitogens (Krieg *et al.*, 1995) and PS-ODNs are known to affect the clotting and complement systems (Galbraith *et al.*, 1994).

1.1.2. Design of antisense oligodeoxynucleotides

There are numerous considerations when designing antisense oligodeoxynucleotides, such as stability, ODN sequence, ODN length and choice of the target site. In order for AS-ODNs to exert their effect *via* an antisense mechanism they need to remain intact and the development of structural modifications (discussed later in section 1.1.3.) and delivery devices (discussed later in section 1.1.5.) have significantly improved the stability of these molecules.

The sequence of the AS-ODN can result in the occurrence of non-antisense effects (described earlier in section 1.1.1.6.), such as the presence of a G-quartet (Burgess *et al.*, 1995; Benimetskaya *et al.*, 1997; Castier *et al.*, 1998) and CpG motifs (Krieg *et al.*, 1995). Modifications to the ODN backbone have also been reported to produce

non-antisense effects, *e.g.* substitution of a sulphur for the oxygen in the phosphodiester linkage, namely phosphorothioate ODNs (PS-ODNs), which are known to interact with proteins, leading to problems with toxicity *in vivo* (Brown *et al.*, 1994; Guvakova *et al.*, 1995).

The length of the ODN should also be considered during the design of antisense reagents, where ODN affinity is increased as the length of the ODN-RNA duplex increases, as determined by melting curves. This has been verified experimentally, where 15- to 25-mers that are typically used in antisense experiments rather than shorter ODNs which may have melting transition (T_m) close to or below 37°C (Cook, 1998). Longer unmodified ODNs may be cleaved in cells and give several smaller sequences that can block the targeted mRNA but also undesired mRNAs (Falker *et al.*, 1994).

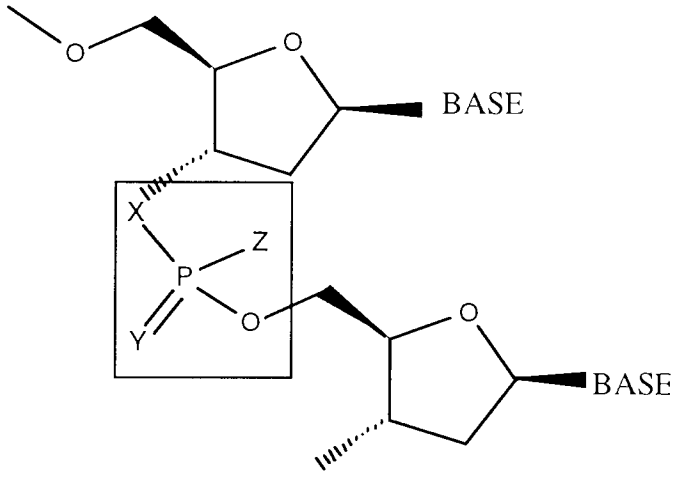
Interactions between the target and AS-ODNs is determined in part by their secondary and tertiary structure, which are difficult to predict (Lima *et al.*, 1992), which has led to the development of techniques such as RNase H accessibility mapping (Scherr & Rossi, 1998) and DNA chip technology (Kurian *et al.*, 1999). RNase H mapping utilises the fact that this enzyme recognises the RNA strand in a RNA/DNA duplex, the combination of the target mRNA with a library of ODNs in the presence of RNase H can elucidate accessible sites by analysis of cleavage products, sequencing of which reveals accessible regions. A DNA chip refers to a regular array of nucleic acid species attached to a flat, solid support, for example glass or silica, where the ordered nature of the nucleic acids allows for parallel analysis of a multitude of hybridisation events (Hacia *et al.*, 1998). The DNA chip technology has been used for selection of effective antisense reagents (Milner *et al.*, 1997). An array of almost 2000 ODNs ranging in length from monomers to 17-mers were fabricated on the surface of a glass plate and incubation with a ^{32}P-radiolabelled β-globin transcript highlighted regions of hybridisation (Milner *et al.*, 1997).

Recently, AS-ODNs designed by the DNA chip technology have demonstrated knockdown of epidermal growth factor receptor (EGFR) in A431 cells in cell culture studies (Petch *et al.*, 1999).

1.1.3. Structural Modifications

ODNs in their natural form have a phosphodiester backbone that renders them susceptible to enzymatic degradation by exonucleases and endonucleases, in a biological milieu. The half-life ($t_{1/2}$) of phosphodiester ODNs (PO-ODNs) in sera and tissue culture media is in the range of minutes (Akhtar *et al.*, 1991a), and in cerebrospinal fluid, is in the range of hours (Szklarczyk & Kaczmarek, 1995). Numerous structural modifications have been designed to overcome these problems in their lack of stability. ODNs were carefully designed so as not to produce such alterations in their structure that may hinder their cellular permeability, affinity for RNA target or ability to activate RNase H. *In vitro* characterisation of these ODNs is achieved by measuring the melting transition (T_m) of the ODN and its RNA target. The T_m is a measure of the temperature at which the bonding between two DNA strands is broken down, thus giving an indication of affinity of lengths of oligodeoxynucleotides for each other. Data representing the cellular permeation of these modified ODNs is not very extensive, where investigations into interactions of modified ODNs in cell culture systems has only been carried out with the more widely used ODNs, such as phosphorothioates, phosphodiesters and methylphosphonates.

Structural modifications have been developed at the phosphodiester linkage (figure 1.2.), ribose moiety or the aromatic bases. This is not an exhaustive list of all modifications that have been synthesised to date, the aim is to highlight the developments made in ODN modification research and their implications in increased stability, recruitment of RNase H and cellular uptake (cellular uptake discussed later in section 1.1.4.).



ANALOGUE	X	Y	Z
Phosphodiester	O	O	O^-
Phosphorothioate	O	O	S^-
Phosphorodithioate	O	S	S^-
Boranephosphate	O	BH_2	O^-
Phosphoramidate	NH	O	O^-
Methylphosphonate	O	O	CH_3

Figure 1.2. The chemical structure of a phosphodiester ODN, tabulating modifications at the phosphodiester linkage. Box highlights the phosphodiester linkage.

1.1.3.1. Phosphodiester linkage modifications

The phosphodiester backbone undergoes rapid degradation by nucleases, the nucleases cleave at the phosphodiester linkage (Halford & Jones, 1968) thus, to overcome the problems with stability, numerous phosphate analogues were designed. These phosphate analogues can be divided into three groups based on their charge anions, cations and neutral analogues (Matteucci, 1995) (figure 1.2.). The anionic analogues bare the closest resemblance to the native phosphodiester linkage.

Phosphorothioate ODNs (PS-ODNs) are anionic phosphate analogues and possess a sulphur atom in place of an oxygen atom on the phosphodiester linkage (see figure 1.2.). They have an increased stability, the ability to recruit RNase H (Mirabelli *et al.*, 1991) and are easily synthesised (Matteucci, 1995). The main disadvantages of PS-ODNs are their lower affinity for RNA compared to PO-ODNs, and that a diastereomeric mixture of 2^n different compounds is obtained, where n is the number of linkages. Non-antisense aptameric effects have been observed with PS-ODNs due to protein binding of this modification (Brown *et al.*, 1994; Guvakova *et al.*, 1995), which has led to toxicity problems *in vivo* (Pezeshki *et al.*, 1996; Schobitz *et al.*, 1997; Ho *et al.*, 1998; Smith *et al.*, 1999). Phosphorodithioates were synthesised to overcome the diastereomer problem, by removal of the chiral centre at the phosphodiester linkage (Marshall & Caruthers, 1993), however the extra sulphur atom does not appear to have any advantage over monothioates in terms of stability, recruitment of RNase H and affinity for its target.

Phosphoramidates possess an amine group in the phosphodiester linkage, and ODNs bearing this modification at all positions are stable to nucleases *in vitro* and have a greatly elevated T_m of interaction with complementary RNA compared with phosphodiesters (Gryaznov *et al.*, 1995).

Despite the anionic linkages increasing stability and the ability to activate RNaseH, cells do not easily take up these analogues. This problem of ineffective cellular permeation led to development of neutral backbones, the most extensively

investigated being methylphosphonates (MP-ODNs). The main aim of this modification was to neutralise the charge on the phosphate leading to a more lipophilic molecule, which could result in membrane permeability by passive diffusion (Matteucci, 1995) (cellular uptake discussed later in section 1.1.4.). Briefly, MP-ODNs appear to be taken up into cells by a different mechanism to PO- and PS-ODNs (Loke *et al.*, 1989), and do not activate RNase H (Maher *et al.*, 1989).

Cationic linkages were developed in order to enhance the affinity of the ODN, by engineering an electrostatic attraction between the ODN and the polyanionic RNA target (Matteucci, 1995), but the issue of permeation has not yet been addressed.

1.1.3.2. Ribose modifications

The ribose portion of ODNs has been extensively modified. The most straightforward and promising substitution involves modification of the 2'- position on the ribose sugar, that is the position that distinguishes between RNA and DNA. Some examples of modifications include 2'-O-methyl (2'-OMe) and 2'-Fluoro (2'-F), where 2'-OMe confers stability to single-stranded RNA nucleases, but is susceptible to degradation by DNA nucleases, unless the phosphodiester is suitably modified (Inoue *et al.*, 1987). Highly electronegative substituents, for example, fluorine in 2'-F result in enhanced binding to RNA targets, by virtually locking the ribose ring in the conformation (Kawasaki *et al.*, 1993), with little protection from nucleases (Matteucci, 1995). Substitutions have also been made at the 1'-, 3'- and 4'- positions, with little success (Matteucci, 1995), offering no more advantages than other substitutions, although a 5'- methyl substituent has shown nuclease stability without compromising affinity (Saha *et al.*, 1995). Another ribose modification is the hexose substitution, which have shown an enhanced affinity to RNA, and stability to nucleases (Augustyns *et al.*, 1993).

1.1.3.3. Heterocyclic analogues

The aromatic heterocyclic bases are the recognition element of the ODN-RNA interaction, numerous modifications to the bases have been made, many of them to the 5'- position of pyrimidines (reviewed by Sanghvi, 1993). Stacking forces or hydrophobic interactions are thought to be the interactions that may be modified to enhance affinity. The propyne series of modifications (pyrimidine analogues) are easily synthesised, enhance stacking interactions, thereby enhancing affinity and have a phosphorothioate backbone (Matteucci, 1995). This series has shown antisense effects in cell culture, but only when delivered to cells *via* microinjection or cationic lipids (Wagner *et al.*, 1993).

1.1.3.4. Ribozymes

This is not a structural modification to the phosphodiester backbone of an oligodeoxynucleotide, but rather a ribonucleotide molecule that has an antisense effect. Ribozymes may be defined as catalytic RNA molecules that have the ability to cleave target RNA substrates in a sequence specific manner, in the absence of proteins or "traditional" enzymes (Hudson *et al.*, 1996a).

1.1.4. Cellular uptake of antisense oligodeoxynucleotides

Despite non-antisense effects occurring *via* protein interactions, and stimulating the host immune system (see section 1.1.1.6.), there are numerous examples of antisense cell culture studies with appropriate controls, where observed effects were due to a true antisense mechanism (Fell *et al.*, 1997; Sogos *et al.*, 1997; Hou *et al.*, 1998). In order for AS-ODNs to exert their effect *via* an antisense mechanism, they must enter cells and interact with pre-mRNA and mRNA in the nucleus and cytoplasm.

1.1.4.1. Mechanisms of cellular uptake

The proposed mechanisms by which AS-ODNs are taken up into cells are illustrated in figure 1.3. Most studies have indicated that ODNs are initially taken up by cells through endocytosis and accumulate in endosomal and lysosomal compartments (reviewed by Akhtar & Juliano, 1992). There are three main types of endocytosis, receptor-mediated, fluid-phase and adsorptive. Receptor-mediated endocytosis occurs when the molecule taken into the cell has bound to a receptor, and the ligand-receptor complex is internalised. Internalisation of a ligand-receptor complex also internalises extracellular fluid, any molecules dissolved in this extracellular fluid are said to be taken up by fluid-phase endocytosis. Adsorptive endocytosis occurs when molecules adsorb onto the membrane surface and are internalised.

The uptake of a fluorescent marker, acridine, at the 5'-end of a PO-ODN into cultured HL60 cells was dependent upon temperature and ODN length, suggesting specificity in the uptake mechanism (Loke *et al.*, 1989). The authors were able to identify a 80kDa receptor-protein by oligo-(dT)-cellulose affinity chromatography. An independent investigation into the cell surface binding and cellular uptake of PO-ODNs, conjugated with a reactive alkylating group at the 5'-end was carried out in a mouse fibroblast cell line, L929 (Yakubov *et al.*, 1989). These cross-linking studies resulted in the labelling of two receptor-proteins of 79kDa and 90kDa. It must be noted that despite these two independent studies being in agreement with each other, the cellular uptake of PO-ODNs was carried out over long periods of time in 10% foetal calf serum, without monitoring stability of the ODNs. PO-ODNs are fairly unstable molecules, being very susceptible to action by nucleases (Akhtar *et al.*, 1991a), thus the uptake may not necessarily be of the intact oligomer, but of shorter degraded fragments. However, later studies have identified membrane proteins that were implicated in ODN uptake, where uptake of ODNs in a human cell line was shown to be dependent on both ODN chain length and ODN sequence (Corrias & Cheng, 1999). Similarly, affinity-labelling studies revealed membrane proteins of 61-63 kDa and 35kDa in size in A431 cells (Laktionov *et al.*, 1999). Both of these

studies demonstrated that the ODNs remained intact throughout the experimental procedure (Corrias & Cheng, 1998; Laktionov *et al.*, 1999).

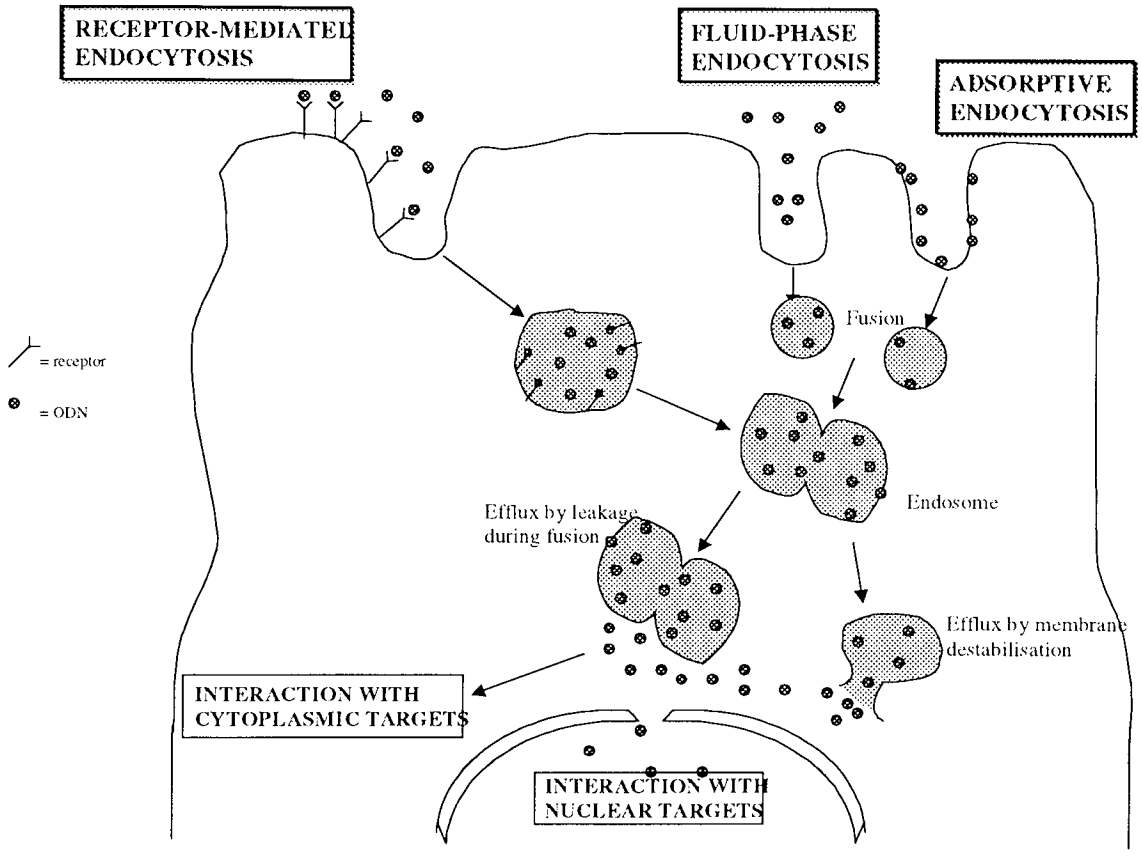


Figure 1.3. Schematic of proposed mechanisms of cellular uptake of AS-ODNs and the intracellular distribution.

The binding of ODN to a receptor at the cellular surface could lead to internalisation of the receptor into the cell *via* clathrin-coated pits, or it could be coupled to cellular signal transducing systems. Ultrastructural studies demonstrated the presence of ODN in clathrin-coated pits and in endosomal and lysosomal compartments (Beltinger *et al.*, 1995). The possible link of receptors to a signal transduction system was investigated in L929 cells, which were previously described to have

receptors for ODNs on their surface. Treatment of cells with ^{32}P-radiolabelled ODN induced an increase in ^{32}P labelling of phosphatidic acid, accompanied by a gradual decrease in diacylglycerol, and protein kinase C activity was found to be lower in membranes isolated from ODN-treated cells compared to control cells (Balakireva *et al.*, 1997).

1.1.4.2. Cellular uptake of different ODN chemistries

Loke *et al.* (1989) investigated uptake of PO-, PS- and MP-ODNs in HL60 cells, where binding of PO-ODN to an 80kDa protein was competed with PS-ODN, but not with MP-ODN. This observation suggested that PO- and PS-ODNs are taken up by similar mechanisms, receptor-mediated endocytosis, requiring the same receptor-protein. MP-ODNs may either be taken up by receptor-mediated endocytosis, but utilising a different receptor-protein, or taken up by an entirely different mechanism.

MP-ODNs are neutral and fairly hydrophobic molecules, so it was initially thought that these entered the cell by simple passive diffusion (Miller *et al.*, 1981). Efflux studies of MP-ODNs across liposomes have shown that they are transported at similar rates to PO-ODNs and PS-ODNs (Akhtar *et al.*, 1991b). A temperature-dependency was observed suggesting an active process may be involved. There was only modest competition with large excess of PO-ODNs, supporting the data reported by Loke *et al.* (1989) that MP-ODNs may be taken up by a different mechanism than PO-ODNs. Examination of subcellular distribution has revealed a punctate distribution of fluorescent-labelled MP-ODN (Shoji *et al.*, 1991), a similar pattern to FITC-dextran, which is known to be taken up by fluid-phase endocytosis (Sandvig *et al.*, 1987). Investigations into the stability of these modified ODNs in a biological milieu have been carried out, where it has been demonstrated that the stability of modified ODNs was greatly improved compared to phosphodiester backbone (Akhtar *et al.*, 1991a; Shoji *et al.*, 1991).

Modifications of ODNs not only enhance stability of the molecules but also offer the potential of altering their cellular uptake, ultimately improving bioavailability. Phosphorothioate modifications enhance the stability of ODNs, but their application *in vivo* has been impaired by non-specific interactions with proteins (Brown *et al.*, 1994; Guvakova *et al.*, 1995), which has caused problems with toxicity.

1.1.4.3. Intracellular trafficking

The endocytic uptake mechanism of ODNs leads to accumulation of these molecules within endosomal or lysosomal compartments (Akhtar & Juliano, 1992), thereafter the efflux of ODNs from these compartments is believed to be through leakage from endosomes during fusion or through endosomal membrane destabilisation. The endosomal and lysosomal compartments are thought of as a pharmacologically non-productive site, ODNs exert no actions in these compartments of the cell and may be degraded by lysosomal enzymes. Attempts have been made to bypass or promote ODN release from these compartments, the use of a number of delivery agents have enhanced the cytoplasmic and nuclear accumulation of AS-ODNs. These delivery agents include cationic lipid complexes, polypeptides, dendrimers, surfactants, liposomes and microspheres, (described later in section 1.1.5.). Once in the cytoplasm ODNs rapidly enter the nucleus and accumulate there (Leonetti *et al.*, 1991; Fisher *et al.*, 1993), permitting these molecules to interact specifically with their pre-mRNA and mRNA targets.

1.1.5. Delivery of antisense oligodeoxynucleotides

As discussed earlier (section 1.1.4.), AS-ODNs cross cell membranes rather poorly (Akhtar & Juliano, 1992), requiring large amounts to allow modest concentrations at the target site and they are also susceptible to action by nucleases (Akhtar *et al.*, 1991a), particularly ODNs with a phosphodiester backbone. Despite the significant improvements reported in the stability of modified ODNs (described earlier in section 1.1.2.), there has been a huge interest in the development of delivery

strategies for AS-ODNs, which have the potential to improve stability, cellular uptake and intracellular trafficking, thus increasing the bioavailability of these molecules.

Issues of stability, cellular uptake, or toxicity are not unique to AS-ODNs many "conventional" drugs also have these problems, which over the past few decades has led to the development of drug delivery systems (DDS). To name a few examples, liposomes, polymeric delivery devices and viral vectors, which have been reviewed by Poznanski and Juliano (1984), Tomlinson (1987) and Juliano (1988). The goal of these systems is to maximise the concentration of active drug, or its duration of action at the target site, and to minimise the concentration of drug, or duration of exposure at other sites where toxic effects may occur.

The development of an AS-ODN drug delivery system would be beneficial for delivery of AS-ODNs into the CNS, as pharmacokinetic studies have revealed that the brain is relatively impervious to systemically administered AS-ODNs (Agrawal *et al.*, 1991).

There have been a number of approaches by researchers into the development of drug delivery systems, including ODN conjugation (Bennett *et al.*, 1992; Akhtar *et al.*, 1995; Walker *et al.*, 1995; Bonora *et al.*, 1997) and carriers (Juliano & Akhtar, 1992; Lewis *et al.*, 1995; Phillips, 1997).

1.1.5.1. ODN conjugates

ODNs can be conjugated to hydrophobic compounds such as cholesterol, alkyl chains, lipids or polycations (Clarenc *et al.*, 1993; Boussif *et al.*, 1995), increasing the hydrophobic nature of ODNs. Conjugation to compounds (ligands) that have a specific cellular receptor *e.g.* transferrin or mannose, manipulating the mechanism of receptor-mediated endocytosis (RME), has also been an area of interest in the development of ODN delivery systems (Wagner *et al.*, 1994; Akhtar *et al.*, 1995).

Conjugation of ODNs to polyethylene glycol (PEG) has the potential to improve the stability and increase the hydrophobicity of these molecules, thus enhancing cellular uptake. Several researchers have synthesised and investigated PEG-ODN conjugates (Rahman *et al.*, 1991; Jäschke *et al.*, 1994; Kawaguchi *et al.*, 1995; Manoharan *et al.*, 1995). Incubation of unconjugated and conjugated ODNs with hydrolytic enzymes revealed that 20-25% of PEG-ODN was intact following 30 minutes incubation, compared to complete degradation of the unconjugated ODN, with little adverse effects on the hybridisation properties (Bonora *et al.*, 1997).

Another major barrier of efficacy of AS-ODNs is the accumulation of ODNs within endosomal and lysosomal compartments. The development of ODNs conjugated with polycations has improved delivery to cytoplasmic and nuclear targets (Bennett *et al.*, 1992; Clarenc *et al.*, 1993). Rhodamine-labelled ODNs were delivered to a primary culture of chicken hypothalamic neurons with polyethylenimine (PEI) and incubated for 2 hours, and fluorescence was observed in predominantly nuclear regions within the neurons (Boussif *et al.*, 1995). However, the authors made no attempt to compare the distribution of PEI-ODNs with the naked ODNs. The use of PEI for transfection of AS-ODNs was also demonstrated in peripheral and central neurons with no disturbance in functionality and morphology of the targeted cells at ODN concentrations at least four-fold lower than naked ODNs (Lambert *et al.*, 1996). The conjugation of poly (L-lysine) (PLL) to ODNs to promote cellular internalisation of AS-ODNs has also been investigated, and a 17-mer-PLL AS-ODN complementary to a HIV-1 splice site inhibited cytopathic effects at much lower concentrations than non-conjugated PO- or MP-ODNs (Stevenson & Iversen, 1989).

The conjugation of ODNs to ligands may serve to enhance cellular uptake of these molecules by manipulating the process of receptor-mediated endocytosis (RME) where ODNs are internalised into the cell following the ligand binding to its receptor at the cell surface. ODNs conjugated to transferrin were used to enhance their cellular uptake, and these conjugates led to efficient internalisation of the ODNs compared to their unconjugated counterparts (Wagner *et al.*, 1994). A major

disadvantage of this delivery strategy was the possible competition that the transferrin/ODN conjugates may have with the natural ligand in the *in vivo* situation, thus compromising normal iron uptake into cells. An alternative strategy was designed utilising monoclonal antibodies specific for the transferrin receptor, with an antibody recognition site away from the transferrin-binding site (Walker *et al.*, 1995). This transferrin receptor antibody-ODN conjugate was applied to a glioma cell line, U87-MG and an endothelial cell line, ECV304, where a three-fold increase in cellular association, compared to naked ODN was observed. Transferrin antibody-ODN conjugates may also be used for enhancing delivery across the blood-brain barrier (BBB), which is relatively impervious to ODNs without a delivery device (Agrawal *et al.*, 1991). The development of ODN-ligand/antibody conjugates directed against a receptor that is unique to the cellular target would be a more specific delivery system, as transferrin receptors are found on numerous cell surfaces in the body. Indeed, improved *in vitro* and *in vivo* delivery was observed with a 20-mer ODN conjugated to a ligand for the parenchymal cell-specific asialoglycoprotein receptor, which resulted in a sixty-fold increase in ODN accumulation into parenchymal liver cells (Biessen *et al.*, 1999). However, it must be noted that the pharmacokinetic investigations by Agrawal *et al.* (1991) and Crooke *et al.* (1996b) revealed that unconjugated ODNs are naturally targeted to organs of the reticuloendothelial system, which includes the liver (Agrawal *et al.*, 1991; Crooke, 1996b). Thus the sixty-fold increase in ODN-asialoglycoprotein ligand accumulation observed may be partially due to a natural accumulation of ODNs in the liver.

Cellular uptake of mannose- and fucose- terminated glycoproteins in macrophages are mediated by specific lectins on the cellular surface (Stahl *et al.*, 1978). This observation has raised interest in the conjugation of ODNs to mannose residues to improve ODN delivery to macrophages. A monomannoside phosphoroamidite linked to the 5'-end of an ODN sequence, showed a two- to four-fold increase in macrophage uptake compared to unconjugated ODN (Akhtar *et al.*, 1995). The uptake of a biotinylated AS-ODN was enhanced in bovine brain capillaries incubated

with avidin and the basis for this uptake was thought to be the cationic nature of the avidin protein, due to competitive inhibition by the polycationic proteins, protamine, streptavidin and α-methylmannoside (Pardridge & Boado, 1991).

Cellular uptake of ODNs has been improved by development of these ODN conjugates, however there still may be an accumulation of ODNs into endosomal and lysosomal compartments within the cytosol of the cell. In this case, these conjugates could be used in combination with compounds that promote release of ODNs from endosomal compartments. Certain adjuvants have significantly enhanced the effect of AS-ODNs, where PS-ODNs targeted to chloramphenicol acetyltransferase (CAT) were delivered to a Chinese hamster ovary (CHO) cell line expressing CAT, in combination with a number of adjuvants (Hughes *et al.*, 1996).

Cationic lipids are amphiphilic molecules that enable the delivery of ODNs across the cell membrane by the formation of ion pairs between the negatively charged phosphate groups in the ODN backbone and the polar head groups of the membrane bilayer. The use of these reagents for the delivery of nucleic acids was first described by Felgner *et al.* (1987) during the development of transfection procedures. There are now commercially available transfection reagents including Lipofectin® and Lipofectamine™ and some researchers have utilised these reagents to enhance delivery of ODNs in cell culture systems (Bennett *et al.*, 1992; Fell *et al.*, 1997). The mechanisms of these reagents are not fully understood, although new insights are emerging (Koltover *et al.*, 1998). In terms of their use systemically, there are some major obstacles due their large size and a high surface charge density.

1.1.5.2. Carriers

Liposomes, viral and plasmid vectors and microparticles have been investigated as potential delivery systems for ODNs. The use of these carriers has proved to be an excellent strategy for cellular delivery (Juliano & Akhtar, 1992; Lewis *et al.*, 1995; Phillips, 1997), causing a significant increase in cellular uptake of ODNs. They also

serve to protect the ODN against the action of nucleases that may be present in cell culture and *in vivo* systems. The particulate nature of these carriers creates a disadvantage in that they are naturally targeted to the reticuloendothelial system, located mainly in the liver and spleen.

1.1.5.2.1. *Liposomes*

Liposomes are composed of a phospholipid bilayer membrane surrounding an internal aqueous compartment. Highly polar, water-soluble drugs may be entrapped in the aqueous compartment, whereas lipophilic molecules can become part of the lipid bilayer (Juliano & Akhtar, 1992).

The efficiencies of ODN incorporation into liposomes is dependent upon the fabrication process used and the type of modified ODN incorporated (Juliano & Akhtar, 1992). Incorporation of methylphosphonates was always higher compared to PO- and PS-ODNs because of their tendency to bind to lipids (Juliano & Akhtar, 1992). Liposomes have become widely used for the delivery of ODNs and enhanced cellular delivery has been observed (Abe *et al.*, 1998; Alahari *et al.*, 1998). The development of pH-sensitive fusogenic liposomes was developed to overcome accumulation of ODNs in endosomal compartments of the cell. Investigations into the use of pH-sensitive liposomes composed of dioleoylphosphatidylethanolamine (DOPE)/oleic acid (OA)/cholesterol (CHOL) demonstrated their potential as carriers of ODNs (DeOliveira *et al.*, 1998). Upon entry into the cell the amphiphile maintains its structure at pH 7, but as they move into endosomes where the pH drops, the amphiphile becomes protonated and fuses with the endosomal membrane releasing the ODN into the cytoplasm. Despite improved cellular delivery, the anionic nature of these liposomes resulted in poor ODN encapsulation.

1.1.5.2.2. *Viral vectors*

Viruses must efficiently transfect their DNA or RNA into their host cells so that they can replicate further and the inherent ability of viruses to achieve this has been manipulated in the development of gene delivery strategies. A number of viral vectors have been constructed in the development of gene delivery strategies, such as herpes simplex virus (HSV), adenoviruses, retroviruses and adeno-associated viruses (AAV). Improved efficacy of AS-ODNs was observed in spontaneously hypertensive rats following a direct injection of recombinant adeno-associated virus containing AS-ODN directed to the angiotensin-1 (AT-1) receptor (Phillips, 1997). The combination of viral vectors with liposomes also has great potential for cellular delivery of ODNs, where FITC-ODNs delivered with a haemagglutinating virus of Japan (HVJ)-liposome vector showed a strong persistent fluorescent signal for 2 weeks in comparison to 3 days with naked ODN (Yamada *et al.*, 1996). However the use of adeno- and retroviral vectors can pose problems with stimulation of the hosts immune system, which led to the development of adeno-associated viral vectors due to their non-pathogenic nature.

1.1.5.2.3. *Plasmid vectors*

Plasmid vectors can be engineered to incorporate a DNA sequence encoding the target antisense RNA, replication of the plasmid produces multiple copies of the antisense RNA gene, which is then transcribed into the antisense RNA molecule. These vectors can provide a means of long-term delivery of antisense molecules due to the replication of the plasmids producing multiple copies of the target antisense molecule.

The use of plasmid vectors for the long-term delivery of antisense RNA has been carried out with a degree of success (Weiss *et al.*, 1997a; Davidkova *et al.*, 1998.) A long-term cataleptic response was observed up to 1 month following treatment with

the D_2 antisense vector compared to a cataleptic response lasting 2 days following a single haloperidol injection (Davidkova *et al.*, 1998).

1.1.5.2.4. *Microparticles*

Microparticles are polymeric delivery systems including microcapsules and microspheres. Microcapsules are polymeric membranes, where the drug is enclosed in the central core, whereas microspheres are microporous matrix systems where the drug is dispersed uniformly throughout the polymer matrix, sub-micronic polymeric systems are defined as nanoparticles. These delivery devices entrap a drug within the core or matrix of the microparticle and can provide sustained-release of the entrapped drug depending on the microparticle characteristics.

Both natural and synthetic polymers have been used for the fabrication of microparticles, including gelatin, polylactides, polyanhydrides, albumin, copolymers and cellulose. Alterations in the fabrication process of microparticles can tailor the profile of drug release from this delivery system, resulting in sustained-release over altering lengths of time and they can also serve to protect the entrapped drug from enzymatic degradation.

The use of biodegradable polymers provides a means of sustained-release where breakdown of the particulate system occurs naturally, producing non-toxic products that are excreted *via* normal physiological processes.

The use of microparticles for delivery systems of AS-ODNs is discussed in detail in section 1.2.

1.1.6. *In vivo* pharmacokinetics

Prior to the application of AS-ODNs in therapy, it is imperative to evaluate the distribution of these molecules in the whole animal, monitoring the *in vivo*

pharmacokinetics in the fate of ODNs, stability in different tissues and plasma and elimination of ODNs from the animal. The chemistry of modified ODNs, route of administration and the use of delivery systems can all affect the bioavailability of ODNs, so must be considered in each instance. Detailed pharmacokinetic data are not available for PO-ODNs due to their half-life ($t_{1/2}$) in plasma following intravenous (i.v.) injection ranging from two to five minutes (Agrawal *et al.*, 1995), whereas PS-ODNs are more resistant to degradation by nucleases (Akhtar *et al.*, 1991a) and their pharmacokinetics have been studied quite extensively.

Generally, PS-ODNs are distributed to all tissues, except the brain, with the greatest accumulation observed in organs of the reticuloendothelial system, including the liver, kidneys, spleen and bone marrow (Agrawal *et al.*, 1991; Cossum *et al.*, 1993). The plasma half-lives of PS-ODNs range from minutes to hours depending on dose and species (Cossum *et al.*, 1993; Zhang *et al.*, 1995). The plasma levels of PS-ODNs were dependent upon the route of administration where higher plasma concentrations were found after intravenous (i.v.) administration than after intraperitoneal (i.p.) administration (Agrawal *et al.*, 1991). PS-ODNs have been shown to bind to BSA *in vitro* more avidly than their oxygen congeners (Crooke *et al.*, 1996b), it may be that the elevated plasma levels of PS-ODNs following i.v. injection is due to PS-ODNs binding to serum proteins. The binding of phosphorothioated ODNs to serum proteins may hinder their therapeutic potential, as these analogues remain in the plasma for longer periods of time, so despite PS-ODNs being more stable than PO-ODNs, degradation can still occur due to longer exposure times to nucleases.

The major route of elimination of PS-ODNs is *via* the urine (Agrawal *et al.*, 1991; Zhang *et al.*, 1995) with approximately 30% of PS-ODN excreted 24 hours post-injection and an additional 10% excreted between 24-48 hours post-injection, independent of route of administration (Agrawal *et al.*, 1991). Most degradation was observed in the liver and kidneys, with 30-40% observed at 24 hours and up to 50% at 48 hours. Chain extension of ODNs was observed in the liver and kidney and high

molecular weight bands were observed in the small and large intestines, which were thought to be due to interaction of PS-ODNs with large macromolecules (Agrawal *et al.*, 1991). However, problems of stability may be overcome by route of administration, where ODNs remained intact following intranasal, intravaginal and intrarectal injection, whereas 50% of ODNs were degraded following i.p. injection (Vlassov *et al.*, 1995).

The use of drug-delivery systems can further enhance the bioavailability of ODNs and may serve to protect ODNs from degradation (Lewis *et al.*, 1995), produce a depot effect providing localised administration and reduce binding to plasma proteins. The use of biodegradable microspheres for delivery of an AS-ODN targeted to human papilloma virus (HPV) resulted in significant levels of ODN at the site of injection 7 days after administration, whereas ODN administered as the naked molecule was eliminated within 24 hours (Khan *et al.*, 1999).

1.1.7. Application of antisense oligodeoxynucleotides

Advances in molecular cloning and the near completion of the human genome project have created an abundance of knowledge of specific genes and their products. Such advances have enabled a new target for disease and also investigative tools for research into the function of specific proteins when no standard pharmacological agents were available. The concurrent advances in antisense research have enabled the application of AS-ODNs in therapy and as investigative tools in research and development.

1.1.7.1. Research tools

The knowledge of different molecular forms of receptor subtypes or peptides of a same family derived from cloning studies allows for the use of AS-ODNs in functional studies of these molecularly distinct proteins. This is an advantage over conventional pharmacological agents due to their lack of selectivity.

Antisense technology is already making contributions to the understanding of gene and protein function, with alterations in behaviour, biochemistry and physiology implicating their function. For many neurotransmitter receptors, there are multiple subtypes encoded by a number of related genes, such as nicotinic receptors (Bean *et al.*, 1995), and $GABA_A$ receptors (Rabow *et al.*, 1995) of which specific pharmacological agents are not available. AS-ODNs have been successfully used for reducing the expression of specific receptor subtypes, α1A antisense knockdown confirmed observations that this subtype of neuronal calcium channels was a component of a P-type as well as Q-type channels (Gillard *et al.*, 1997). A significant knockdown of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) provided evidence supporting a role for CREB in autoregulation of the cAMP pathway in the CNS, as well as in mediating some of the effects of morphine on this pathway in the nucleus accumbens (Widnell *et al.*, 1996). It was also determined by antisense knockdown that nigrostriatal neurons express both D_2 and D_3 autoreceptors at their somatodendritic and axon terminal regions (Tepper *et al.*, 1997).

1.1.7.2. Clinical trials

AS-ODNs are currently undergoing clinical investigation and there are several trials taking place at various phases. These AS-ODNs are targeted to such disease as cancer, viral infections and inflammatory diseases.

Three independent phase I clinical trials are currently taking place for treatment of non-Hodgkin's lymphoma with AS-ODNs targeted against protein kinase C-alpha (PKC-α), BCL-2, and *c-raf-1* (Nemunaitis *et al.*, 1999; Waters *et al.*, 1999; Stevenson *et al.*, 1999). Phase I clinical trials involve the determination of a dosing regimen including the maximum tolerated dose (MTD), toxicological profile, pharmacokinetic and pharmacodynamic data. A dosing regimen ranging from 0.15-6.0mg/kg/day of AS-ODN against PKC-α were administered by intravenous infusion three times a week over a three week period to a total of 36 patients with advanced

cancer (Nemunaitis *et al.*, 1999). Despite mild clinical toxicity being observed in these patients including mild nausea, vomiting, fever, chills and fatigue, this toxicity was not dose limiting with observed clinical activity. A short elimination half-life was observed ranging from 18-92 minutes, suggesting that prolonged administration may be necessary for further clinical development, which could be provided by the use of a drug-delivery system such as microspheres. Similar observations were made in a phase I clinical study of an AS-ODN target against the *c-raf-1* oncogene for treatment of non-Hodgkin's lymphoma, with mild clinical toxicity, suppression of the target gene and the dose well tolerated up to 6mg/kg (Stevenson *et al.*, 1999). Another phase I clinical trial for treatment of HIV-positive patients targets the *gag* site of the HIV gene, where a 25-mer PS-ODN, Trecovirsen (GEM 91), was well tolerated following single i.v. doses up to 2.5mg/kg (Sereni *et al.*, 1999).

An antisense compound (ISIS 2302) that inhibits the expression of intercellular adhesion molecule (ICAM-1) is now in phase II trials for renal transplant rejection, psoriasis (topical) and ulcerative colitis (enema). However, a pivotal study into the use of ISIS 2302 for Crohn's Disease has recently shown disappointing results where efficacy was not demonstrated. Phase II trials of ISIS 3521 that is antisense to protein kinase C-alpha (PKC-α) has shown encouraging results with a range of solid tumours including non-small cell lung carcinoma, ovarian cancer and non-Hodgkin's lymphoma. Phase III trials for the use of ISIS 3521 in the treatment of non-small cell lung carcinoma are to begin this year.

The world's first antisense drug was approved for marketing in August 1998 and since then is being sold in the U.S.A., Brazil and Europe. Vitravene™ (fomiversin) is a phosphorothioate for treatment of cytomegalovirus (CMV)-retinitis in AIDS patients. In comparison with all of the currently available treatments of CMV-retinitis, Vitravene™ provides the best patient compliance, as administration involves a local intravitreal injection as opposed to surgical implant or i.v. infusion.

1.2. BIODEGRADABLE POLYMERS

Biodegradable polymers are natural or synthetic compounds, which degrade either enzymatically or non-enzymatically into biocompatible or non-toxic products, which are metabolised or excreted *via* normal physiological pathways (Jalil & Nixon, 1990). They have been studied extensively over the past few decades, for their potential as drug delivery systems (Brem, 1990; Langer, 1991; Couvreur *et al.*, 1997; Hanes *et al.*, 1997). Degradation of polymeric drug-delivery systems (DDS) provide a controlled-release system that may be tailored to suit the requirements of the entrapped drug by altering polymer and drug variables during formulation. Many polymers have been investigated for their use as drug delivery systems, for example polylactic acid (PLA) (Lewis *et al.*, 1995), polyanhydrides (Brem *et al.*, 1991) and polyalkylcyanoacrylates (Chavany *et al.*, 1992). Polyesters of lactic and glycolic acids are the most extensively investigated synthetic polymers due to their use for over 20 years as resorbable sutures. Copolymers of these polyesters are discussed here as their Food & Drug Administration (FDA) approval and commercialisation makes them desirable as a novel delivery device for AS-ODNs. Their wide acceptance, long history of biocompatibility and degradation to toxicologically accepted products gives such polymers an excellent profile for antisense delivery. Current therapies using this drug delivery system include depot formulations of leutinising hormone-releasing hormone (LHRH) analogues for treatment of prostate cancer and endometriosis, for example, Prostap SR®, De-Capeptyl SR®, Lupron Depot® and Zoladex® which are all entrapped within poly (D,L-lactide-co-glycolide) (PLGA) microspheres.

Research into the use of biodegradable polymers for sustained drug delivery has been ongoing since the mid-1970s when it the potential of these polymers for providing sustained-release drug delivery formulations was first realised (Langer & Folkman, 1976). However, the use of these devices as a sustained-release mechanism for AS-ODNs is not extensively described (Cortesi *et al.*, 1994; Lewis *et al.*, 1995; Cleek *et al.*, 1997; Yamakawa *et al.*, 1997). Advantages for the use of microparticulate

systems as a delivery device for ODNs include increased stability, localised delivery of the ODN, controlled release, reduced number of administrations and reduced concentration of ODN employed.

1.2.1. Poly (D,L-lactide-co-glycolide) (PLGA)

Polylactides, polyglycolides and poly (lactide-co-glycolide) are polyesters of lactic and glycolic acids. Poly (D,L-lactide-co-glycolide) is a copolymer composed of lactic acid (LA) and glycolic acid (GA) units (figure 1.4.), synthesised by ring-opening polymerisation of D,L-lactide and glycolide using zinc catalysts of lactic acid and glycolic acids for high molecular weight (HMw) copolymers. However, low molecular weight (LMw) (Mw $\leq$ 3000) copolymers are synthesised by direct condensation of lactic and glycolic acid (Gilding & Reed, 1979).

The chemical configuration of the polymer alters the ability to crystallise, leading to either a more crystalline or amorphous polymer (Kissel *et al.*, 1991). As the copolymer approaches 100% of polylactic acid (PLA) or polyglycolic acid (PGA), the degradation rate decreases with the copolymer composition of 50:50 degrading the most rapidly, as it is less likely to contain crystalline blocks of either of the monomers. More crystalline polymers degrade slowly, as the crystalline regions are less accessible to water. The molecular weight of the copolymers also affects the degree of crystallinity and thus the degradation of the copolymer.

1.2.1.1. Erosion of poly (D,L-lactide-co-glycolide)

The erosion of PLGA biodegradable polymers begins with degradation, in a process of random chain scission, followed by the release of water-soluble degradation products, such as oligomers and monomers. The loss of water-soluble products leads to a mass loss of the polymer matrix which is characteristic of erosion.

The degradation of PLGA biodegradable polymers is a chemical process because the functional groups that constitutes these polymers hydrolyse in an aqueous environment. Hydrolysis starts with the intrusion of water into the polymer bulk, for PLGA polymers the hydrolysis is slow, affecting the complete cross-section of the polymer matrix, named polymer bulk erosion (Langer & Peppas, 1983). The rate of hydrolysis of PLGA polymers is affected by monomer structure, polymer molecular weight and copolymer ratio (Wang *et al.*, 1990). Copolymers with a higher GA content have a greater rate of hydrolysis than copolymers containing a higher LA content. This is due to the structure of these monomers, the methyl group on lactic acid makes the monomers more hydrophobic than glycolic acid (figure 1.4.) causing steric hindrance to attack by water molecules. Molecular weight decreases before any significant mass loss of the polymer, suggesting that hydrolysis occurs to a high degree before soluble oligomers and monomers are produced (Wang *et al.*, 1990). These monomeric components are ultimately excreted in humans, *via* the Krebs' cycle, a normal biochemical pathway of the cell (figure 1.5.).

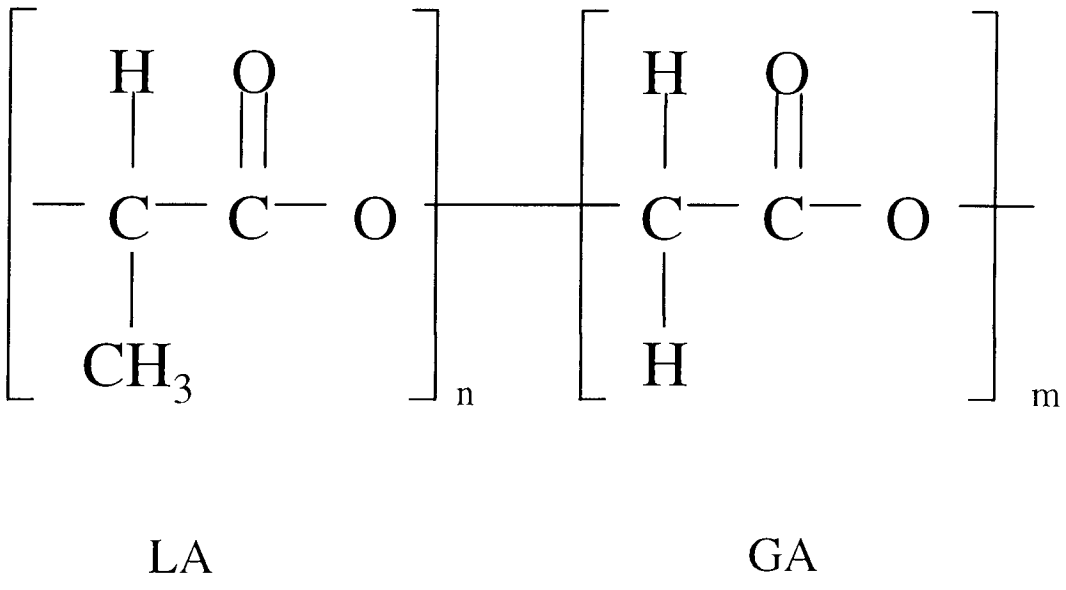


Figure 1.4. The composition of poly (D,L-lactide-co-glycolide). LA = lactic acid, GA = glycolic acid, n = number of lactic acid monomers, m = number of glycolic acid monomers.

1.2.2. Formulation of PLGA microspheres

The formulation of biodegradable microspheres can be carried out by a number of different methods including solvent extraction and evaporation, coacervation phase separation and spray coating and drying by a melting method. There are two types of solvent evaporation, the single-emulsion and double-emulsion solvent evaporation.

1.2.2.1. Single-emulsion solvent evaporation

This involves the mixing of a polymer/drug solution into an immiscible phase containing an emulsifier to form droplets. In this technique, the drug is dissolved in an organic solvent, usually dichloromethane or chloroform, containing the dissolved polymer. This solution is emulsified into an aqueous phase, creating an oil-in-water (o/w) emulsion.

The solvent is allowed to evaporate by stirring, applying heat or a vacuum. As the solvent evaporates, a suspension of hardened drug containing microspheres is formed. This suspension becomes more viscous as the solvent evaporates, and under these conditions, the droplets have a tendency to coalesce, the addition of a stabilising agent, for example, polyvinyl alcohol (PVA), gelatin or methylcellulose, provides a protective layer surrounding the droplets, reducing coalescence (Arshady, 1991).

1.2.2.2. Double-emulsion solvent evaporation

The double-emulsion solvent evaporation technique is of particular interest for drugs that are sensitive to organic solvents and have a high aqueous solubility. This is a water-in-oil-in-water (w/o/w) emulsion method. The main difference between the o/w and w/o/w is that the drug is dissolved in water in the w/o/w, not solvent. The aqueous drug solution is mixed with an organic solution of polymer dissolved in

solvent. The resultant w/o emulsion is further emulsified into an external aqueous solution of PVA.

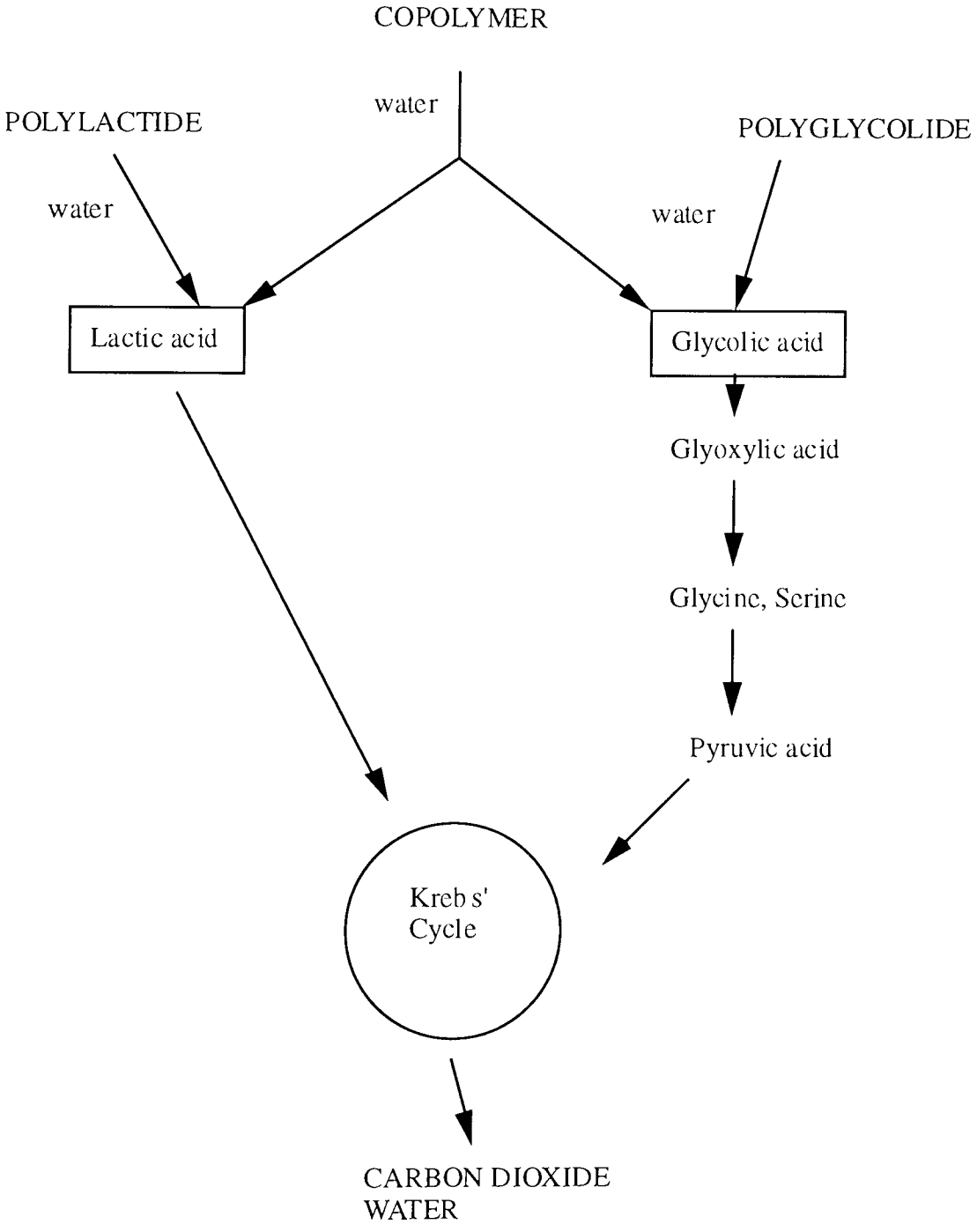


Figure 1.5. The excretion pathway of PLGA monomers *via* the Krebs' cycle following polymer bulk erosion.

1.2.3. Release of oligodeoxynucleotides from microspheres

The release of ODNs from microspheres has been shown to be a triphasic or biphasic process depending upon the composition and molecular weight of the polymer used in the formulation (Lewis *et al.*, 1995). ODNs that are at or near the surface of microspheres diffuses into the surrounding media upon initial contact with an aqueous environment, termed the "burst effect". The second phase of release, termed the lag period, involves the hydrolysis to lower molecular weight polymers, which are still water-insoluble, thus there is no weight loss during this phase. The use of low molecular weight polymers may reduce the duration of this phase or it may overlap with the third phase of release, where there is a steady release of ODNs. The tertiary phase of release occurs with a significant weight loss, as the lower molecular weight polymers are further hydrolysed into water-soluble monomers.

Microspheres as a delivery system for ODNs may overcome many hurdles encountered by researchers into AS-ODNs. The microspheres act as a protective barrier of the ODN against nuclease action, thus enhancing the stability of the molecule (Lewis *et al.*, 1995), (see also sections 3.3.1. and 4.3.1.3.) whilst providing localised delivery of the ODN to the target site. Due to their hydrophobic nature, cells take up microspheres more efficiently than ODN alone, resulting in increased cellular uptake of ODN *via* microspheres (Chavany *et al.*, 1994; Akhtar & Lewis, 1997). The development of a microsphere formulation could provide prolonged administration without the need for repeated administration, therefore increasing patient compliance. In terms of investigating the function of specific proteins, microspheres would be advantageous for the investigation into proteins of long half-lives ($t_{1/2}$). The use of polymeric microspheres for delivery of AS-ODNs into the CNS could help overcome the problem of blood-brain barrier (BBB) penetration (Agrawal *et al.*, 1991). However, the development of microspheres for delivery of AS-ODNs is in its infancy (Cortesi *et al.*, 1994; Lewis *et al.*, 1995; Cleek *et al.*, 1997; Yamakawa *et al.*, 1997; Khan *et al.*, 1999; Smith *et al.*, 1999) and there is a need for extensive investigations into the formulation of these delivery systems, their

interactions with particular cell types, pharmacokinetic evaluation and efficacy of ODN-loaded microspheres.

1.3. DELIVERY OF AS-ODNs INTO THE CNS

AS-ODNs have been used in the CNS for a number of years for the investigation into the function of specific subtypes or neurotransmitter receptors (reviewed by Neumann, 1997; Nicot & Pfaff, 1997; Weiss *et al.*, 1997b). The use of polymers for delivery of certain neuroactive agents, which are otherwise impervious to the brain, has been employed over recent years with a huge degree of success (Brem *et al.*, 1991; Menai *et al.*, 1997), which has led to FDA approval of the first polymeric delivery device for treatment of brain tumours, Gliadel® wafers. However, the use of polymers for delivery of AS-ODNs into the CNS has only recently been reported (Smith *et al.*, 1999), where *in vivo* sustained-release was observed over a period exceeding 2 days compared to 12 hours of the naked molecule. The current data available for the use of polymeric microspheres for delivery of AS-ODNs *in vivo* is limited, with this technology remaining predominantly at the formulation stage (Cortesi *et al.*, 1994; Lewis *et al.*, 1995; Cleek *et al.*, 1997; Yamakawa *et al.*, 1997). There has been relatively little research to date into the interaction of these systems with cell culture systems (Akhtar & Lewis, 1997) and for peripheral *in vivo* delivery (Khan *et al.*, 1999).

1.3.1. Polymeric drug delivery devices for CNS delivery

Poly (ethylene-co-vinyl acetate) (EVAc) was the first biocompatible polymer to provide successful controlled release of high molecular weight substances (Langer & Folkman, 1976). However, despite EVAc having the advantageous property of biocompatibility, there was one major property lacking, biodegradability, thus necessitating surgical removal of the polymeric implants. A search for synthetic biodegradable polymers began in the 1970s, where synthetic polymers were favoured because the cost, availability and processing conditions which can be efficiently

controlled. The first application of synthetic polymeric devices in the brain was aimed at improving the labelling of perivascular meningeal projections from cat trigeminal ganglia (Mayberg *et al.*, 1981). Since this study, other research groups have demonstrated the use of polymeric devices in the brain (table 1.1. for examples of these studies) for delivery of dopamine, noradrenaline (McRae & Dahlstrom, 1994), bethanechol (Howard *et al.*, 1989) and 1,3-bis(chloro-ethyl)-1-nitrosurea (BCNU) (Brem *et al.*, 1991; Grossman *et al.*, 1992; Tamargo *et al.*, 1993).

Polymeric delivery systems have been investigated within the CNS for their potential therapeutic use for Parkinson's disease, Alzheimer's disease and brain tumours. Models used for *in vivo* evaluation of efficacy of these treatments include lesioning of nigrostriatal pathway leading to striatal denervation, lesioning of fimbria-fornix leading to hippocampal denervation and implantation of a gliosarcoma cell line for Parkinson's disease, Alzheimer's disease and malignant brain tumours respectively.

The main neurpatholgical pathway of Parkinson's disease is the nigrostriatal dopamine pathway (McRae *et al.*, 1994). The animal model for Parkinson's disease involves a unilateral lesion using 6-hydroxydopamine (6-OHDA) in the ascending median forebrain bundle (MFB) of monoamine neurons, causing up-regulation of the postsynaptic dopamine receptors, which after dopamine agonist administration, is functionally manifested as contralateral rotational behaviour (Ungerstedt, 1971a). Degeneration of cortical cholinergic processes has been implicated as an important feature of Alzheimer's disease (Collerton, 1986). According to the "Cholinergic hypothesis", it is inadequate cortical levels of acetylcholine (ACh) that produce deficits in memory and learning. An animal model for Alzheimer's disease involves lesions in the fimbria-fornix, which causes cholinergic denervation of hippocampus, with marked impairment of spatial memory (Howard, *et al.*, 1989). The implantation of 9L gliosarcoma cell line provides an animal model for malignant brain tumours (Tamargo *et al.*, 1993).

1.3.1.1. Biocompatibility of polymer delivery systems in the brain

As much research into the central use of polymeric delivery devices has been carried out using polyanhydrides which are poly-[bis(*p*-carboxyphenoxy)propane-sebacic acid (PCPP-SA) and poly (D,L-lactide-co-glycolide) (PLGA). There have been a number of studies carried out into the biocompatibility of these polymers in the brain (Tamargo *et al.*, 1989; Menai *et al.*, 1993; Kou *et al.*, 1997).

In vitro analysis of PLGA 50:50 (Mw 4.5 kDa) rods in PBS, simulated CSF and goat CSF revealed that the rods were completely eroded over a period of 28 days as determined by HPLC analysis of lactic acid and glycolic acid units (Kou *et al.*, 1997). Following implantation of these polymer rods into rats' brains, the polymer rods and breakdown products were well tolerated by the brain tissue, with a brain tissue response similar to that following mechanical trauma and foreign body exposure, which was more pronounced after 14 days and largely subsided by 28 days. The brain tissue response observed in the study by Kou *et al.* (1997) was similar to that observed by Menai *et al.* (1993) following implantation of PLGA microspheres. The similarity between these two studies suggests that the formulation of microspheres does not affect the biocompatibility of these PLGA delivery devices (Menai *et al.*, 1993; Kou *et al.*, 1997). A comparison between PCPP-SA and standard neurosurgical implants was carried out in the brains of Sprague Dawley rats, where no behavioural changes or neurological deficits indicative of systemic or localised toxicity was observed (Tamargo *et al.*, 1989). A localised inflammatory response was observed, which resided as the polymer degraded over 5 weeks. These biocompatibility studies suggested that these polymers would be suitable for use as drug delivery devices, indeed PCPP-SA is currently available as a DDS for delivery of BCNU in the treatment of brain tumours, Gliadel® wafers, approved in 1996.

1.3.2. Alternative drug delivery systems for CNS delivery of AS-ODNs

The development of drug delivery systems (DDS) for AS-ODNs has been the focus of a number of researchers over the past decade (Bennett *et al.*, 1992; Akhtar *et al.*, 1995; Lewis *et al.*, 1995; Walker *et al.*, 1995; Bonora *et al.*, 1997; Phillips & Gyurko, 1997) (discussed earlier in section 1.1.5.). Many of these approaches have involved ODN conjugation and the development of certain carrier systems including liposomes, viral vectors and microparticles. The delivery of AS-ODNs into the CNS requires further evaluation due to the blood-brain barrier. Certain drug delivery systems can be administered systemically, such as hydrophobic ODN conjugates (Walker *et al.*, 1995), whereas direct implantation into the brain is required for other systems, such as microparticles.

Current approaches for the delivery of AS-ODNs into the CNS includes osmotic mini-pumps (Nissbrandt *et al.*, 1995; Ekman *et al.*, 1998: Tremblay *et al.*, 1998), repeated injections *via* cannulae (Karle & Nielsen, 1995; Zhou *et al.*, 1996; Rajakumar *et al.*, 1997) and genetically engineered cells. The frequently used cannulae and osmotic mini-pumps require cumbersome and time-consuming surgery and are not the most suitable for clinical applications, in terms of patient compliance. Implantation of large implants may be inconvenient, while implantation of tissue poses a number of ethical questions.

Polymer	Entrapped drug	Animal model	Mode & site of administration	Effect of drug	Ref.
PLGA 75:25	pBC 264 (a cholecystokinin (CCK)-B-selective CCK peptidomimetic agonist)	Male Wistar rats (200-220g). Open-field test	1µl of microsphere suspension (1mg or 4mg) stereotaxically injected into the nucleus accumbens	1 hour post-injection showed a decrease in spontaneous alternation behaviour.	Blanco-Príeto *et al.*, 1996
PLGA 50:50	Dopamine (DA) Noradrenaline (NA)	Male Sprague Dawley rats (200-250g). Unilaterally lesioned using 6-OHDA. Contralateral rotational behaviour	3µl microsphere suspension stereotaxically injected into the denervated striatum	29% decrease in rotations from pre-microsphere baseline within 4 weeks.	McRae & Dahlstrom, 1994
PCPP-SA 50:50	Bethanechol (a cholinomimetic agent)	Male Sprague Dawley rats (250-300g). Fimbria-fornix lesion. Radial maze test	Cylindrical implants stereotaxically implanted into the denervated hippocampus	Significant improvement of maze performance ($p < 0.005$). Beneficial effects persisted > 40 days.	Howard *et al.*, 1989
PCPP-SA 20:80	BCNU (a chemotherapeutic agent for treatment of brain tumours	Male Fischer 344 rats (~250g). Implanted with 9L gliosarcoma cell line.	Polymeric discs administered 4 days post-implantation of 9L cells	17% of group implanted with BCNU/PCPP-SA showed no viable tumours at the end of the experiment.	Tamargo *et al.*, 1993
PCPP-SA 20:80	BCNU	Human 21 patients with recurrent malignant glioma	BCNU-loaded wafers. 3 groups were studied with increasing concentration of BCNU (1.93%, 3.85% and 6.35%).	86% of patients lived more than 1 year from the initial diagnosis. 8% lived more than 1year after implantation.	Brem *et al.*, 1991
PCPP-SA 20:80	BCNU	New Zealand white rabbits Autoradiographic studies on coronal sections	BCNU-loaded discs injected stereotaxically.	50% BCNU 3 days post-implantation, 15% BCNU 7 days post-implantation <10% BCNU 14-21 days post-implantation.	Grossman *et al.*, 1992

Table 1.1. *In vivo* studies of the use of biodegradable polymeric drug delivery devices in the CNS. BCNU = 1,3-bis(chloro-ethyl)-1-nitrosurea, CCK = cholecystokinin, DA = dopamine, NA = noradrenaline, 6-OHDA = 6-hydroxydopamine, PCPP-SA = poly-[bis(*p*-carboxyphenoxy)propane-sebacic acid, PLGA = poly (D,L-lactide-co-glycolide).

1.4. DOPAMINE

The *in vivo* investigations in this current study were targeted to a receptor subtype of the dopaminergic system, the D_2 dopamine receptor (D_2 receptor, see section 1.4.2. for further discussion). In order to discuss this molecular target, it is important to outline the role of dopamine within the CNS, location of dopaminergic systems, nomenclature of the dopamine receptor subtypes and localisation of receptor mRNA.

1.4.1. Central dopaminergic systems

Until the mid-1950s, dopamine was always considered to be an intermediate in the biosynthesis of the catecholamines, noradrenaline and adrenaline, however the discovery of significant tissue levels of dopamine led scientists to believe that dopamine was a neurotransmitter in its own right (Ungerstedt, 1971b).

Dopaminergic pathways in the brain have been mapped by behavioural and histological studies (Lindvall, 1979; Cooper *et al.*, 1991) and these pathways have been defined in terms of their length, short, medium and long pathways. The major pathways of interest in this thesis are the longest dopaminergic pathways, the nigrostriatal and mesolimbic pathways. The nigrostriatal pathway accounts for approximately 75% of dopamine in the brain. The cell bodies originate in the substantia nigra where the fibres project through the medial forebrain bundle (MFB) with the axons terminating in the corpus striatum. The mesolimbic pathway originates mainly in the ventral tegmental area (VTA) with the fibres projecting through the medial forebrain bundle (MFB) terminating in parts of the limbic system, especially the nucleus accumbens (NAcc) (figure 1.6.).

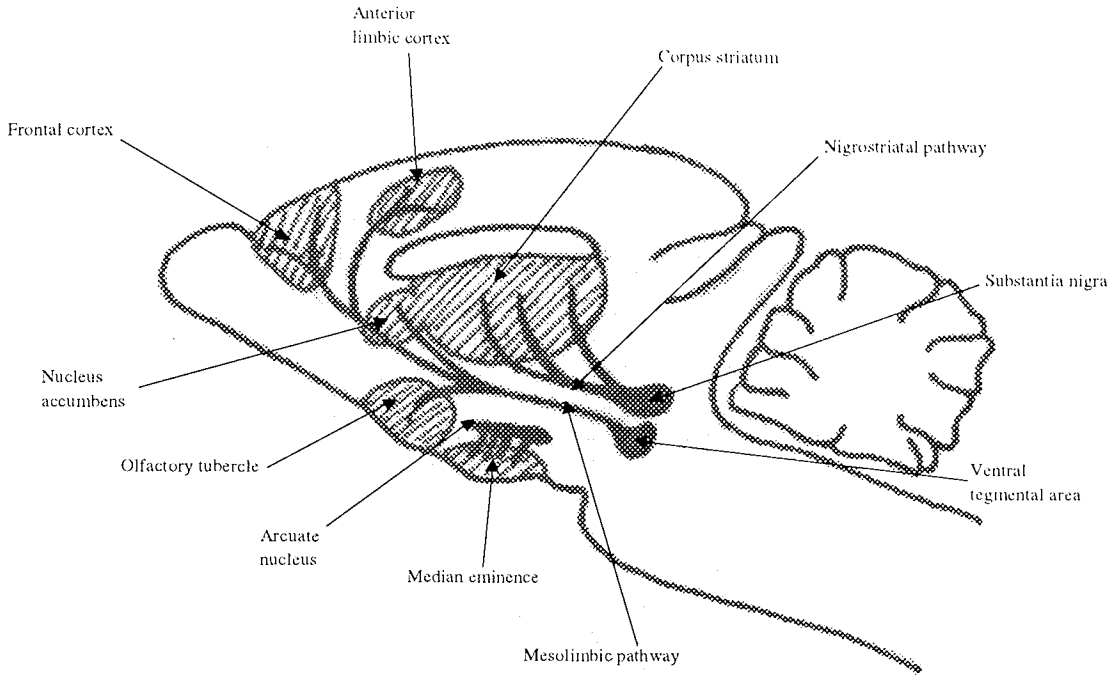


Figure 1.6. Dopaminergic pathways in the rat brain, redrawn from Rang, Dale & Ritter (1995). Cell bodies and fibre tracts are shown in bold type, and striped regions show the location of dopaminergic terminals.

1.4.2. Dopamine receptors

Biochemical and pharmacological data have classified dopamine receptors into two main groups. D_1-type receptors that stimulate the formation of intracellular cyclic adenosine monophosphate (cAMP), while D_2-type receptors inhibit the formation of intracellular cAMP (Kebabian & Calne, 1979; Swennan & Denef, 1982). Dopamine antagonists have been used in receptor binding studies, advancing knowledge into function of receptors and interaction of drugs with receptors, also leading to the development of effective therapies (Kopp *et al.*, 1992). However, this classic pharmacological approach does have its disadvantages, in that drug design can be tedious and time-consuming, and prolonged use of dopamine antagonists causes up-regulation of dopamine receptors. This has led to tolerance of drugs and serious

side-effects (Angulo *et al.*, 1991; Rogue *et al.*, 1991; Kopp *et al.*, 1992). These changes in receptor function have been shown to be due to alteration in the expression of mRNAs encoding the receptors. Thus, the use of AS-ODNs targeted to the specific mRNA may obviate the problem of receptor up-regulation.

1.4.2.1. Localisation of dopamine receptor subtype mRNA

Molecular biological techniques have advanced the field of dopamine receptors, leading to discovery of many new subtypes of receptors. Classic pharmacological data indicated two main types of dopamine receptors, D_1-type and D_2-type receptor subtypes (Kebabian & Calne, 1979). Analysis of cloned sequences has revealed the D_1-type receptors include both D_1 and D_5 subtypes, whereas the D_2-type receptors include D_2, D_3 and D_4 subtypes (Civelli *et al.*, 1991; Mack *et al.*, 1991; Gingrich & Caron, 1993).

These different subtypes of dopamine receptors are distributed differentially throughout the brain. D_1 mRNA is localised in the caudate putamen (CPu), nucleus accumbens (NAcc) and olfactory tubercle (Weiner *et al.*, 1991). High concentrations of D_2 dopamine receptor mRNA are also found in these regions, particularly in the dorsolateral and ventrolateral portions of the caudate putamen. Lower concentrations of D_2 mRNA are located in the globus pallidus, substantia nigra (SN) and ventral tegmental area (VTA). As cell bodies in the ventral tegmental area and substantia nigra innervate the caudate putamen, nucleus accumbens and prefrontal cortex (Lindvall & Björklund, 1974; Fallon & Moore, 1978), this suggests both a presynaptic and postsynaptic role for the D_2 transcript. D_3 mRNA has a more differential localisation than the D_1 and D_2 mRNA, low levels are found in the caudate putamen, with high expression in the olfactory tubercle, nucleus accumbens and islands of Calleja. D_3 receptors are associated with cognitive, emotional and endocrine functions, due to a major distribution in the limbic system, in areas such as hippocampus, septum and mammillary nuclei of the hypothalamus (Sokoloff *et al.*, 1990; Bouthenet *et al.*, 1991; Landwehrmeyer *et al.*, 1993). The mesocorticolimbic

system (frontal cortex, midbrain, hypothalamus and medulla) has a high expression of D_4 dopamine receptors (Van Tol *et al.*, 1991). D_5 receptor mRNA shows relatively high expression in the hippocampus, hypothalamus and mammilary and pretectal nuclei, with low expression in the striatum and frontal cortex (Sunahara *et al.*, 1991).

Classic pharmacological approaches have advanced our knowledge of dopamine receptors and will continue to do so, however in the absence of subtype-specific pharmacological agents, molecular biological approaches have the potential of specifically genetically manipulating the activity of receptor-proteins. These genetic manipulations can aid research into the function of specific dopamine receptor subtypes due to their specificity.

1.4.3. Antisense oligodeoxynucleotides as a research tool of dopamine receptors

The implication of the D_2 receptor in the pathophysiology of Parkinson's disease (Burns *et al.*, 1983), schizophrenia (Carlsson, 1988) and tardive dyskinesia (Waddington *et al.*, 1983) has made research into this particular receptor of clinical importance. The molecular basis of these neurological and psychiatric disorders is not entirely understood, although changes in the function of dopaminergic receptors has been implicated in their aetiology, as well as in their treatment.

The use of molecular strategies such as cloning techniques and *in situ* hybridisation (ISH) has resulted in a more detailed classification of dopamine receptors, highlighting subtypes of these receptors (section 1.4.2.). AS-ODNs have become a useful tool in neuropharmacological research over recent years, where specific down-regulation of receptor subtypes has outlined details in neurochemical pathways that could not have been defined by pharmacological agents due to their lack of specificity for certain receptor subtypes.

1.4.3.1. *In vivo* efficacy of AS-ODNs targeted to dopaminergic proteins

In vivo investigations into the efficacy of antisense reagents targeted to the D_2 dopamine receptor assess levels of proteins (Nissbrandt *et al.*, 1995) and mRNA (Hadjiconstantinou *et al.*, 1996) and also utilise certain behavioural pharmacological models (Weiss *et al.*, 1993; Zhang & Creese, 1993). Contralateral rotational behaviour (Weiss *et al.*, 1993), stereotypy and grooming behaviours (Zhang *et al.*, 1994; Weiss *et al.*, 1997b), induced locomotor activity (Zhang & Creese, 1993), induced decreased body temperature (Weiss *et al.*, 1997b), catalepsy (Zhang & Creese, 1993; Weiss *et al.*, 1997b) are examples of behavioural studies that may be carried out to monitor function of dopamine receptors. Antisense studies carried out *in vivo* into dopaminergic pathways are summarised in table 1.2.

A 20-mer phosphorothioate AS-ODN bridging the initiation codon of D_2 mRNA was administered intracerebroventricularly (i.c.v.) by repeated injection every 12 hours for 3 injections to mice with unilateral 6-hydroxydopamine (6-OHDA) lesions in the corpus striatum (Weiss *et al.*, 1993). AS-ODNs directed towards D_2 dopamine mRNA showed a significant reduction in contralateral rotation upon challenge with the D_2 agonist (quinpirole) with no reduction in contralateral rotational behaviour upon challenge with the D_1 agonist (SKF38393) (Weiss *et al.*, 1993). There was no significant reduction in contralateral rotational behaviour following injection of vehicle or random ODN.

Stereotypy and grooming behaviours are mediated by D_2 and D_1 dopamine receptors respectively, in normal animals (Weiss *et al.*, 1997b). Weiss and colleagues (1997b) observed that treatment with D_2 antisense inhibited the stereotyped behaviour induced by quinpirole, but failed to block SKF38393-induced grooming behaviour. Conversely, treatment with D_1 antisense inhibited the grooming behaviour induced by SKF38393, failing to block quinpirole-induced stereotypy. Similar observations were made by Zhang *et al.* (1994), both groups failing to alter stereotypy or grooming behaviours with random sequence controls. These data indicated a

selective action of both D_1 and D_2 dopamine receptors, providing an ideal tool to investigate the functions of other subtypes of dopamine receptors in the central nervous system.

Zhang and Creese (1993) demonstrated *in vivo* efficacy with ODNs that were antisense to the D_2 dopamine receptor, investigating their effect on locomotor activity, grooming behaviour and induction of catalepsy. Levels of receptor-protein were monitored in autoradiographic ligand binding assays using [^{3}H]-spiperone or [^{3}H]-SCH23390. Phosphorothioated AS-ODNs were continuously delivered *via* subcutaneously implanted osmotic minipumps, delivering at a rate of 1µl/hour over 3 days into the lateral ventricles. Treatment with D_2 antisense inhibited quinpirole-induced locomotor activity, without altering SKF38393-induced grooming behaviour, and also gradually induced a strong cataleptic response during the 3 days of antisense treatment, a reduction in spontaneous locomotor activity was also observed. Silvia and colleagues (1997) used a 17-mer phosphorothioated AS-ODN, targeted to the translated region of the dopamine transporter (DAT) to investigate their effect on contralateral rotational behaviour in rats. Psychostimulants showed differential effects on contralateral rotational behaviour in antisense-treated animals. The antisense-treated animals consistently rotated contralaterally in response to amphetamine (2mg/kg), but not to cocaine (10mg/kg) or nomifensine (10mg/kg). This indicated a different mode of action for amphetamine and cocaine, supporting the view that amphetamine acts as dopamine releaser, whereas cocaine acts by blocking dopamine transport.

The use of AS-ODNs for the investigation of dopamine receptors in the brain has been quite extensively investigated (Weiss *et al.*, 1993; Zhang & Creese, 1993; Silvia *et al.*, 1994; Zhang *et al.*, 1994; Nissbrandt *et al.*, 1995; Hadjiconstantinou *et al.*, 1996; Rajakumar *et al.*, 1997). Studies using AS-ODNs targeted to dopamine receptors use well-characterised behavioural pharmacological experiments such as induced-locomotor activity, catalepsy and induced-stereotypy and grooming. Thus, the combined knowledge of the use of AS-ODNs targeted to dopamine receptors and

the behavioural pharmacological experiments used to determine efficacy of these AS-ODNs makes the target of dopamine receptors ideal, in terms of the proof of concept of the microsphere delivery system.

Target	Species	Sequence	Behaviour	Comments	Ref.
D_2 dopamine receptor mRNA	Mouse	PS 20-mer 5'-gTg gAT CCA TTg ggg CAg Tg- (-10 to +10)	Induced contralateral rotational behaviour.	i.c.v. repeated injection 3 times at 12hr intervals. $IC_{50} \cong$ 0.5nmol /injection. Selective blockade of D_2 receptors.	Weiss *et al.*, 1993.
D_2 dopamine receptor mRNA	Male Swiss-Webster mice	PS 20-mer 5'-gTg gAT CCA TTg ggg CAg Tg-3' (bridging initiation codon -10 to +10).	Induced contralateral rotational behaviour.	i.c.v. injection into unilaterally lesioned mice (corpus striatum). Repeated injections 3 times at 12hr intervals.	Zhou *et al.*, 1994.
D_2 dopamine receptor mRNA	Male Sprague Dawley rats	PS 19-mer 5'-Agg ACA ggT TCA gTg GaT C- (codons 2-8 of rat mRNA).	Induced locomotor activity. Induced grooming behaviour. Catalepsy.	i.c.v. Alzet osmotic minipumps over 3 days (1µl/hour). Significant catalepsy ($P<0.001$). Significant reduction in locomotor activity ($P<0.01$).	Zhang & Creese, 1993.
D_2 dopamine receptor mRNA	Male Sprague Dawley rats	PS 17-mer 5'-AgA TTC AgT ggA TCC AT-3' (complementary to first 17 nucleotides).	Induced motor behaviour	Substantia nigra Repeated injection every 12 hrs for 5 doses. Significant increase in rotation, contralaterally to treated side.	Silvia *et al.*, 1994.
D_1 dopamine receptor mRNA	Male Swiss Webster mice	PS 20-mer 5'-gTT AgC CAT CTT CCA gA-3' (bridging initiation codon -8 to +12).	Induced grooming behaviour	i.c.v. repeated injection every 12hrs. For 3 injections. Dose-related decrease in quinpirole induced grooming behaviour; $IC_{50} \cong$ 6nmol/ injection ($P<0.001$)	Zhang *et al.*, 1994.
Dopamine transporter	Male Sprague Dawley rats	PS 18-mer 5'-AgA TTC AgT ggA TCC AT-3' (translated region of DAT)	Induced contralateral rotational behaviour.	Reticular substantia nigra. Repeated injection 5 times at 12hr intervals. Significant ($P<0.01$) rotation at 500pmol/day	Silvia *et al.*, 1997.

Table 1.2. Summary of *in vivo* investigation into the effects of antisense oligodeoxynucleotides on dopaminergic systems. IC_{50} = inhibitory concentration (50%), i.c.v. = intracerebroventricular, PO = phosphodiester, PS = phosphorothioate

1.1.5. AIMS OF THESIS

Advances in molecular cloning techniques have revealed molecular structures of new subtypes of neurotransmitter receptors, where their biological and pharmacological properties remain relatively unknown. The development of the antisense strategy provided a means of investigating these new receptor subtypes, when pharmacological agents were not available. AS-ODNs have already proved their potential as therapeutic agents with the first antisense therapy released onto the market in 1998, Vitravene™ for the treatment of CMV-retinitis in AIDS patients. The use of AS-ODNs within the CNS requires chronic treatment to allow for an adequate reduction in gene expression (Wahlestedt, 1994) also bypassing the blood-brain barrier and increased stability are further requirements for their central administration. The development of drug-delivery systems such as biodegradable microspheres can fulfil all of the above criteria for increased CNS bioavailability, providing sustained-release of ODNs following implantation into site-specific regions of the brain.

The aims of this thesis are outlined below:

1. To develop a formulation of biodegradable microspheres entrapping AS-ODNs for application within the CNS. Initial investigations involving the effect of altering formulation parameters such as polymer molecular weight, particle-size and ODN sequence on the *in vitro* release of ODNs into PBS are to be carried out.

2. To test the developed microsphere formulation in cell-culture systems, determining whether the microspheres enhance the cellular association of AS-ODNs in glial and neuronal cells. Investigations into the possible mechanisms that may be involved with the association of naked and entrapped ODNs will also be addressed.

3. To assess the distribution and efficacy of AS-ODNs following stereotaxic implantation into site-specific regions of the rat brain. Distribution studies of naked and entrapped ODNs into the caudate putamen and lateral ventricles will address the question of whether microspheres have the potential of providing a sustained-release of ODNs over a defined length of time. Efficacy studies will address the question of whether microspheres can provide sustained-release of AS-ODNs in a sufficient dose to demonstrate an antisense effect.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

All general methods used throughout this thesis are outlined in this chapter, any methods specific to a particular chapter will be described therein.

2.1. OLIGODEOXYNUCLEOTIDE PREPARATION

All oligodeoxynucleotides (ODNs) were synthesised on an automated DNA/RNA synthesiser Model 392 (Applied Biosystems, Cheshire, U.K.), using standard phosphoroamidite chemistry (0.2μM scale). All reagents were purchased from Cruachem (Scotland, U.K.). All sequences used throughout this thesis are listed in appendix one.

2.1.1. Chemistry of oligodeoxynucleotide synthesis

The synthesis of single-stranded ODNs involves a series of chemical reactions, outlined in figure 2.1.

The synthesis cycle for phosphodiester ODNs is composed of five steps, detritylation, activation, addition, capping and oxidation. The synthesis cycle was repeated until the ODN reached the desired length. Cleavage of the ODN from the controlled-pore glass (CPG) column was achieved by a 1 hour treatment with concentrated ammonium hydroxide, and transferred to a collecting vial. This

treatment also removed the cyanoethyl phosphate protecting groups. The protecting groups on the exocyclic amines of the bases adenine (A), guanine (G) and cytosine (C) were removed by treatment in the ammonium hydroxide solution at 55°C for 8-15 hours. The ammonium hydroxide was removed under vacuum (DNA Speed Vac, Savant, U.K.).

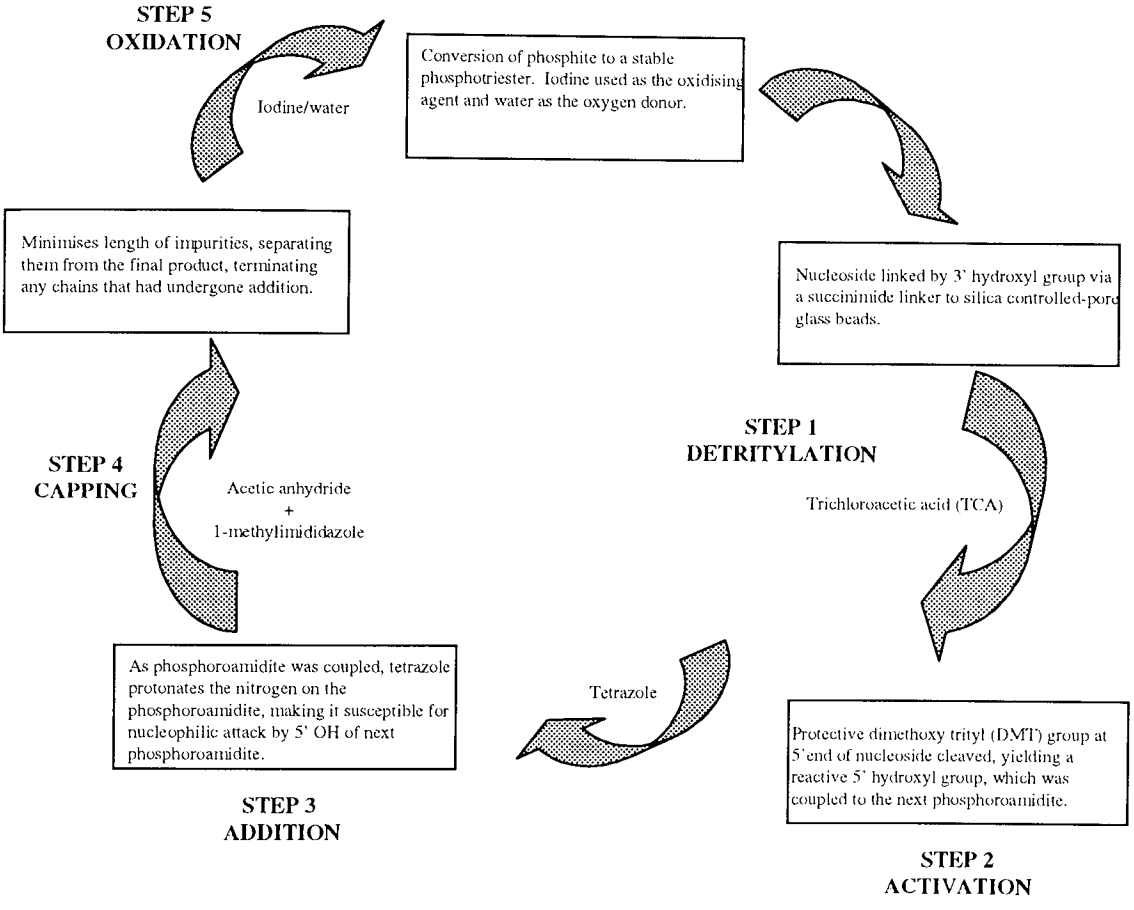


Figure 2.1. Schematic of phosphodiester oligodeoxynucleotide synthesis. DMT = dimethoxy trityl, TCA = trichloroacetic acid.

2.1.2. Phosphorothioate synthesis

For synthesis of phosphorothioates, the oxidation step illustrated in figure 2.1. was replaced with a sulphurisation step. A sulphurising reagent, tetra-ethyliuram

disulphide (TETD) in acetonitrile, converts the phosphite group into a phosphorothioate triester at room temperature.

2.1.3. Fluoresceinated ODN synthesis

A fluorescent label was added to the 5'-end of the ODN during automated synthesis. A fluoroisothiocyanate (FITC)-phosphoroamidite (Cruachem, Scotland, U.K.) was diluted in anhydrous acteonitrile (DNA grade, Applied Biosystems, Warrington, U.K.) according to the manufacturers instructions, and inserted into a spare port on the DNA/RNA synthesiser. The labelled ODN was deprotected at 55°C for 8-15 hours and dried under vacuum in a DNA speed vac (Model DNA 110, Savant, Leicestershire, U.K.). The lyophilised ODN was stored at -20°C protected from the light, to prevent quenching of the fluorescent label.

2.1.4. Quantification of ODNs

The concentration of ODNs was determined by ultra-violet (UV) spectroscopy at a wavelength of 260nm. A method devised by Brown and Brown (1991) converts optical density (OD) units into μg, based on the molecular weight (Mw) of the sequence.

2.1.4.1. Determination of molecular weight

$$Mw = (249 \times nA) + (240 \times nT) + (265 \times nG) + (225 \times nC) + (64 \times n\text{-}1) + 2$$

Where;

nA, T, G, C = number of adenine, thymidine, cytosine, guanine bases respectively

n = total number of bases

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

Note: For phosphorothioates, 16 was added to the molecular weight of the phosphate group, (i.e. 80 x n-1).

2.1.4.2. Determination of extinction coefficient (ε)

Extinction coefficient (ε) = (8.8 x nT) + (7.3 x nC) + (11.7 x nG) + (15.4 x nA) x 0.9

The value of 0.9 accounts for suppression of absorbence of the DNA due to base stacking interactions in the single strand.

2.1.4.3. Conversion of OD_{260} into milligrams

1 mg ε / (Mw/1000) = x OD_{260}

1 OD_{260} unit = 1 / x = y μg

2.1.5. Storage of the ODNs

The dried pellet of synthesised ODNs was resuspended in 200μl of sterile double-distilled water (ddH_2O). This stock solution of ODN was divided into 30μl aliquots in microcentrifuge tubes and dried under vacuum in a DNA speed vac (Model 110, Savant, Leicestershire, U.K.) which were stored at -70°C.

2.2. OLIGODEOXYNUCLEOTIDE RADIOLABELLING

ODNs were radiolabelled at either the 5'- or 3'-end of the molecule using a ^{32}P[ATP] radiolabel. Following radiolabelling, ODNs were purified by polyacrylamide gel electrophoresis (PAGE), as described in the next section, 2.3.1.

2.2.1. 5'-end radiolabelling

The ODNs were radiolabelled with γ-^{32}P[deoxy-ATP] (ICN, Oxfordshire, U.K.) using bacteriophage T4 polynucleotide kinase in 5 x reaction buffer (100mM Tris pH 7.5, 20mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.2mM spermidine and 0.2mM EDTA) (all Bioline Ltd., London, U.K.). The bacteriophage T4 polynucleotide

kinase catalyses the transfer of a phosphate from the γ-^{32}P[deoxy-ATP] to the 5'-end of the ODN, which has had its terminal dimethoxy trityl (DMT) group removed during automated synthesis (section 2.1.).

100pmols of ODN was radiolabelled in a 20µl reaction mixture of 20 units (1µl) of bacteriophage T4 polynucleotide kinase, 2µl of reaction buffer and 2µl γ-^{32}P[deoxy-ATP]. This reaction mixture was incubated at 37°C for 1 hour. An equal volume of loading buffer/marker dye (50mg xylene cyanole, 50mg bromophenol blue in 10ml of 10% (v/v) glycerol/1xTBE) was added to each radiolabelled sample for PAGE (section 2.3.1.).

2.2.2. 3'-end radiolabelling

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

100pmols of ODN was radiolabelled in a 50µl reaction mixture containing 25 units (1µl) of terminal transferase, 10µl of 5 x reaction buffer, 5µl cobalt chloride and 2µl of α-^{32}P[di-deoxy-ATP]. This reaction mixture was incubated at 37°C for 90 minutes. The reaction mixture was made up to 1ml with ddH_2O, and passed through a NAP™-10 column (Pharmacia, Buckinghamshire, U.K.) for removal of cocadylate salts. The radiolabelled ODN was collected in 1.5ml of ddH_2O and dried under vacuum in a DNA speed vac (Model 110, Savant, Leicestershire, U.K.). Dried ODN samples were resuspended in 25µl of loading buffer/marker dye (50mg xylene cyanole, 50mg bromophenol blue in 10ml of 10% (v/v) glycerol/1xTBE) for PAGE (section 2.3.1.).

2.3. PURIFICATION OF RADIOLABELLED ODNs

2.3.1. Polyacrylamide gel electrophoresis (PAGE)

There are two types of PAGE, non-denaturing (native) or denaturing (7M urea). Native PAGE was used for separation of radiolabelled ODNs from free label and bi- and mono-phosphates (Sambrook *et al.*, 1989). Denaturing PAGE was used for stability experiments (section 3.2.3. and 4.2.2.). Stock solutions of native and urea gel mixtures were prepared as described in appendix two.

For purification of radiolabelled ODNs, a 50ml aliquot of stock 20% (v/v) polyacrylamide native gel mixture (appendix two) was polymerised with 0.6ml of freshly prepared 10% (w/v) ammonium persulphate (Sigma, Dorset, U.K.) and 40µl of N, N, N, 'N'-tetramethyehtlene (TEMED) (Sigma, Dorset, U.K.). For stability experiments, a 50ml aliquot of stock 20% (v/v) polyacrylamide urea gel mixture was used. The polymerising gel mixture was poured between two clean glass plates (20 x 20 cm and 22 x 20 cm) (The Gel Company, Cheshire, U.K.) with 1 mm spacers (Bio-Rad, Hertfordshire, U.K.). A 15-well comb was inserted at the top of the plates to form the sample wells. The gel was allowed to set for 30 minutes at room temperature.

The gel was placed in a Bio-Rad electrophoresis tank, cooled with tap water, containing 1 x TBE, diluted from a stock solution of 10 x TBE (appendix two). Residual salts in the gel following polymerisation were removed by pre-running for 30 minutes at 300V, using a Bio-Rad power pack. Samples were added to an equal volume of loading buffer/marker dye (50mg xylene cyanole, 50mg bromophenol blue in 10ml of 5% (v/v) glycerol/1xTBE) and were loaded onto the gel in a volume of 20-50µl and underwent electrophoresis for 3 hours at 300V. Samples for denaturing gels were suspended in an equal volume of marker dye containing 4.2g urea, without glycerol.

2.3.2. Autoradiography

Following PAGE (section 2.3.1.), autoradiography was used for visualisation of radiolabelled bands. After electrophoresis, one of the glass plates was removed and the gel was covered in Saranwrap. The gel was exposed to Hyperfilm (Genetic Research Instrumentation Ltd.) in an autoradiography cassette (Amersham, Buckinghamshire, U.K.). Exposure times varied depending on activity of the samples, freshly radiolabelled samples were exposed for 30-60 seconds, whereas stability samples with a lower activity were exposed for several days. Exposure times exceeding 5 minutes were incubated at -70°C, to prevent diffusion of ODNs through the gel. Hyperfilm was developed and fixed using GBX Developer and GBX Fixer solutions as recommended by the manufacturer (Sigma, Dorset, U.K.).

2.3.3. Gel extraction from ODNs

Bands on the Hyperfilm corresponding to radiolabelled ODNs were aligned on the gel and excised. ODN was extracted from the gel fragments by a "Crush-and-Soak" method (Sambrook *et al.*, 1989). Excised bands were suspended in 1.5ml of ddH_2O and shaken overnight at room temperature. 1.5ml of ddH_2O containing the radiolabelled ODN, was transferred to a microcentrifuge tube, and dried under vacuum in a DNA speed vac (Model 110, Savant, Leicestershire, U.K.). A further 1.5ml of ddH_2O was added to the gel fragments and shaken for 2-4 hours, ddH_2O containing the radiolabelled ODN, was transferred to a microcentrifuge tube and dried under vacuum, this was repeated twice, and all radiolabelled ODN was pooled into 1 microcentrifuge tube. Radiolabelled ODNs were passed through a NAP™-10 column (Pharmacia, Buckinghamshire, U.K.) to remove minuscule amounts of gel present in the radiolabelled sample, then dried under vacuum in a DNA speed vac (Model 110, Savant, Leicestershire, U.K.). The radiolabelled ODNs were stored in a lead pot at -20°C.

2.4. LIQUID SCINTILLATION COUNTING (LSC)

The specific activity of radiolabelled ODN was determined by liquid scintillation counting (LSC.). A known volume or weight of sample was added to 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Fischer, Leicestershire, U.K.) and counted in a Packard 1900TR scintillation counter using ^{32}P and ^{14}C activity programmes for 5 minutes, against background (5ml of scintillation cocktail only). The half-life and reference dates of the radionuclides were used to account for decay during the experimental period. ^{32}P is a β emitter with a half-life of 14.2 days.

2.5. MICROSPHERE PREPARATION

2.5.1. Preparation of double-emulsion (w/o/w) microspheres by Silverson method

Table 2.1. summarises the batches of microspheres (prepared by this particular method) used throughout this thesis describing the ODNs loaded into each batch of microspheres for each particular study and the type of label used.

An aqueous solution containing the ODN (2μg-100μg) and an emulsifying agent of 0.4% (w/v) polyvinyl alcohol (PVA), Mw 13-23000, 88% hydrolysed (Sigma, Dorset, U.K.) was mixed with an organic solution containing the polymer, to prepare the primary emulsion. The organic solution consisted of 500mg of PLGA 50:50 (Mw 45 kDa) dissolved in 5ml of dichloromethane (DCM) (HPLC grade). The aqueous ODN solution was mixed with the organic polymer solution at 4000rpm for 2 minutes using a Silverson homogeniser STD2 (Silverson Machines, Buckinghamshire, U.K.) with a 3/8" mini-micro probe (Silverson Machines, Buckinghamshire, U.K.). The resultant water-in-oil (w/o) emulsion was then emulsified into 160ml of an aqueous 4% (w/v) solution of PVA at 6000rpm for 6 minutes, using a 1" tubular probe (Silverson Machines, Buckinghamshire, U.K.).

The w/o/w emulsion was stirred on a magnetic stirring plate overnight, to allow for the solvent to evaporate. The particles were collected by centrifugation at 2700g for 10 minutes (Model LC1, Sarstedt, Leicestershire, U.K.) and washed twice in ddH_2O. The microspheres were freeze-dried for 48 hours using an Edwards Modulo freeze-drier (Boc Ltd., Sussex, U.K.) and stored in a desiccator at room temperature.

Investigation	ODN(s)	Label
In vitro release (section 3.2.3.)	Poly (A) 15-mer Poly (A), (C), (G), (T) 10-mers α1A antisense (18-mer)	5'-end γ-^{32}P radiolabel (section 2.2.1.)
Cellular association, efflux and stability (sections 4.2.2., 4.2.3. and 4.2.4.)	Poly (A) 15-mer	3'-end α-^{32}P radiolabel (section 2.2.2) 5'-end fluorescein label (section 2.1.3.)
In vivo distribution (section 5.2.4.)	Poly (A) 15-mer	5'-end fluorescein label (section 2.1.3.)
In vivo efficacy (section 5.2.5.)	D_2 dopamine antisense 19-mer D_2 dopamine random 19-mer	No label

Table 2.1. Microsphere batches used throughout investigations. All ODNs are phosphorothioates.

2.5.1.1. Determination of entrapment efficiency

The percentage entrapment efficiency was expressed as:

$$\frac{\text{Total cpm of microsphere batch}}{\text{Total cpm of radiolabelled ODN}} \times 100$$

2µl of radiolabelled ODN and 30mg of microspheres were each added to 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Fischer, Leicestershire, U.K.). The radioactivity of each sample was determined by LSC (section 2.4.).

2.6. SCANNING-ELECTRON MICROSCOPY

The surface morphology of the particles was examined using a scanning-electron microscope. The dry microsphere samples were mounted on carbon discs on aluminium stubs and coated with gold under an argon atmosphere (Emscope Sputter Coater SC500). The gold coating provided a conducting surface for the electrons. The surface morphology was examined under magnification using a Cambridge Instruments Stereoscan 90 scanning-electron microscope. Electronmicrographs were taken using a PIXIE Image Processing and Archiving software package for Windows® 95.

2.7. PARTICLE-SIZE DETERMINATION

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

2.8. CELL CULTURE

2.8.1. Materials

Cell culture media and supplement reagents were purchased from Gibco (Paisley, Scotland, U.K.) unless otherwise stated. Media was stored at 4°C and supplement reagents (foetal calf serum (FCS), L-glutamine and penicillin/streptomycin) were stored in aliquots at -20°C. Tissue culture flasks ($25cm^3$ and $75cm^3$), multiwell

plates, 15ml and 50ml polypropylene tubes and disposable pipettes were purchased from Corning Costar (Buckinghamshire, U.K.). Microcentrifuge tubes, finipipette tips, scintillation vials and 2ml cryovials were purchased from Sarstedt (Leicestershire, U.K.).

2.8.2. Cell lines

Two cell lines were used throughout the cellular association studies, a glial and neuronal cell line both originating from the rat. The glial cell line was a gift from Knoll Pharmaceuticals, Nottinghamshire, U.K., called C6 glioma. The neuronal cell line was a gift from Eli Lilly & Co., Surrey, U.K., called GT_1 neuronal. Both cell lines are adherent and grow as monolayers.

2.8.3. Culture media

The maintenance media used for C6 rat glioma cells was Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 1% (w/v) L-glutamine and 1% (w/v) penicillin/streptomycin. The same media was used for cellular association and stability studies either with or without serum.

The maintenance media used for GT_1 rat neuronal cells were 1:1 DMEM/F-12. DMEM (high glucose, without sodium pyruvate, without L-glutamine) and F-12 HAMS nutrient medium (both purchased from Sigma, Dorset, U.K.) supplemented with 10% (v/v) FCS, 1% (w/v) L-glutamine and 1% (w/v) penicillin/streptomycin. The same media was used with or without serum for cellular association and stability studies.

2.8.4. Maintenance of cell lines

Both cell lines were cultured in $75cm^3$ tissue culture flasks in 20ml of media in a humidified incubator at 37°C with 5% (v/v) CO_2. When cells reached confluence

they were passaged. To passage, cells were washed with pre-warmed PBS, and trypsinised with 2.5ml of 1% (v/v) trypsin (Gibco, Paisley, Scotland, U.K.) in PBS/EDTA and incubated for 5 minutes at 37°C. Trypsinised cells were made up to 10ml with media and centrifuged (Mistral 3000I, MSE, Leicestershire, U.K.) at 210g for 5 minutes. Supernatant was removed and the cell pellet was resuspended in 10ml of media, C6 glioma cells were diluted at 1 in 20 and GT_1 neuronal cells were diluted at 1 in 10.

2.8.5. Counting and seeding cells

For cellular association, viability and stability studies, cells were seeded into 24 well plates and for fluorescent microscopy studies, cells were seeded into 8 well Lab-Tek® Chamber slides™ (NUNC, Gibco, Paisley, Scotland, U.K.) at appropriate densities. A haemocytometer (enhanced Neubauer) (Weber Scientific International, Middlesex, U.K.) was used to determine cell number. Cells were seeded at 5 x 10^4/ml (1ml/well) and 3 x 10^4/ml (300µl/well) for 24-well plates and chamber slides respectively. Plates and chamber slides were incubated overnight (18 hours) at 37°C in a humidified incubator with 5% (v/v) CO_2. Prior to any experiments plates were washed in pre-warmed PBS, to remove any residual serum in the wells.

2.8.6. Long-term storage

Cells were stored long-term in liquid nitrogen. A confluent 75cm^3 flask of cells was trypsinised, and the cells were pelleted by centrifugation (Mistral 3000I, MSE, Leicestershire, U.K.) at 210g for 5 minutes. The pellet was resuspended in 2ml of freezing media (complete FCS containing 10% (v/v) dimethlyl sulphoxide (DMSO) (Sigma, Dorset, U.K.)) and dispensed into 2 cryovials, 1ml per vial. The cryovials were wrapped in tissue and stored in a polystyrene box at -70°C for 24 hours. The cryovials were transferred to liquid nitrogen. The cells were recovered by rapid thawing at 37°C and gradual dilution with media supplemented with 20% (v/v) FCS and seeded into a 25cm^3 flask in a final volume of 10ml.

CHAPTER THREE

IN VITRO PREPARATION AND CHARACTERISATION OF OLIGODEOXYNUCLEOTIDE-LOADED MICROSPHERES

3.1. INTRODUCTION

A current problem concerning the use of ODNs is that they are quickly degraded in plasma (Akhtar *et al.*, 1991a; Akhtar *et al.*, 1992; Hudson *et al.*, 1996b) and peripheral tissues (Agrawal *et al.*, 1991), in areas where endogenous nuclease activity is very high. Although chemical modifications of ODNs have improved the stability, degradation products were still observed following a 60 minute incubation in cytoplasmic and nuclear extract, human and calf serum, for both PS- (14-mer) and MP-ODNs (14-mer) (Akhtar *et al.*, 1991a). PS-ODNs have been the most widely used chemistry, because of their superior stability compared to PO-ODNs and their ability to recruit RNase H (Mirabelli *et al.*, 1991). The stability of PS-ODNs in nervous tissue is greater than in peripheral tissue, and a 15-mer (Szklarczyk & Kaczmarek, 1995) and 20-mer (Zhang *et al.*, 1996) were stable in the brain for up to 24 hours. Although the stability of ODNs in nervous tissue is increased, there is still the requirement for further improvement of their stability. A possible method of enhancing their stability is entrapment within polymeric delivery devices as PO-ODNs entrapped within PLA film matrices were shown to be fully intact after 28 days incubation in foetal calf serum (Lewis *et al.*, 1995).

The use of biodegradable microspheres have the potential to enhance the stability, whilst providing sustained-release of intact ODNs thus potentially improving

efficacy with target proteins of longer half-lives. A single bolus dose of microspheres could provide long-term efficacy, where current modes of long-term delivery of AS-ODNs, such as repeated injections (Weiss *et al.*, 1993; Rajakumar *et al.*, 1997) and osmotic mini-pumps, (Zhang & Creese, 1993; Ekman *et al.*, 1998) are time-consuming and impractical.

Poly (lactide-co-glycolide) (PLGA) is a well characterised polymer and its biocompatibility and biodegradability as microspheres has been extensively investigated, both peripherally and centrally (reviewed by Kou *et al.*, 1997), making this polymer ideal for incorporation of novel ODNs. Research carried out to date on the encapsulation of ODNs into PLGA delivery systems (Lewis *et al.*, 1998; Cleek *et al.*, 1997; Yamakawa *et al.*, 1997) is limited, but promising (outlined in table 3.1.).

Mw & composition	Characteristics	ODN	Comments	Ref.
Mw = 3kDa 50:50 ratio	10-20μm microspheres	PO-ODN 20-mer targeted to the *tat* gene of HIV	A triphasic release profile was observed with a significant improvement in serum stability, compared to naked ODN.	Lewis *et al.*, 1998
Mw = 45kDa 50:50 ratio	Microspheres sieved below 100μm	PS-ODN 24-mer targeted against rat tenascin mRNA	A biphasic profile was observed and smooth muscle cell proliferation was inhibited in a dose-dependent manner	Cleek *et al.*, 1997
A number of different Mw PLGA were used including 5kDa, 10kDa, 15kDa and 20kDa 75:25 ratio	Pillar shaped implants with a diameter of 0.8mm	PS-ODN 21-mer targeted to the initiation codon on Herpes simplex virus mRNA	Both biphasic and triphasic release profiles were observed from these implants into buffer and bovine vitreous fluid, dependent upon molecular weight of the implant.	Yamakawa *et al.*, 1997

Table 3.1. Investigations into the use of PLGA delivery devices for delivery of AS-ODNs. PLGA = poly (lactide-co-glycolide), PO-ODN = phosphodiester oligodeoxynucleotide, PS-ODN = phosphorothioate oligodeoxynucleotide

The investigations into the formulation of PLGA devices for delivery of AS-ODNs remain predominantly at the formulation stage with relatively little investigation into their *in vivo* systemic application (Khan *et al.*, 1999). The application of PLGA devices for the CNS delivery of AS-ODNs had not yet been investigated, although the success of these devices for delivery of proteins and peptides has been reported extensively (McRae & Dahlstrom, 1994; Menai *et al.*, 1994; Blanco-Príeto *et al.*, 1996).

When developing a microsphere formulation for entrapment of ODNs, one must consider the intended application, the duration of release and the type of polymer used (biocompatibility and biodegradation). The intended application in the current investigations is for stereotaxic implantation into the brain, thus particle-size is an important issue as smaller particles (sub-micronic) have been shown to move away from the site of injection (Menai *et al.*, 1994). The target protein in these investigations is the D_2 dopamine receptor, which has demonstrated 50% down-regulation of protein over a period of 5 days in earlier antisense experiments (Zhang & Creese, 1993), thus a release profile over at least one month was investigated. PLGA has demonstrated biocompatibility in brain tissue (Menai *et al.*, 1993) and has been used in controlled release formulations in such pharmaceuticals as Prostap SR®, De-Capeptyl SR®, Lupron Depot® and Zoladex®. Also, PLGA has been the most investigated polymer in terms of entrapment of ODNs (see table 3.1.).

The ODN release profile can be manipulated by altering formulation parameters such as polymer molecular weight, particle-size and ODN characteristics (Arshady, 1991). These parameters can affect the release profile by altering polymer degradation rates, particle surface interactions with the release media and interactions between the ODNs and polymer respectively (Lewis *et al.*, 1998).

There are a numerous techniques for the formulation of microspheres, including coacervation phase separation, solvent evaporation and extraction, spray coating and drying (Pavenetto *et al.*, 1992). The selection of a particular method is dependent on

the nature of the polymer, the type of drug and the intended use. The double-emulsion solvent evaporation method is the method of choice for this project, as ODNs are water-soluble and sensitive to organic solvents. The polymer is dissolved into a volatile organic solvent (*e.g.* dichloromethane) and an aqueous solution of the drug is mixed with the organic phase to form a primary emulsion. The resultant water-in-oil (w/o) emulsion is further homogenised into an external aqueous phase containing PVA to form a water-in-oil-in-water (w/o/w) emulsion. PVA coats the surface of the hardening microspheres to prevent coagulation of droplets, which may occur during the early phases of solvent removal, when there is an increase in viscosity in the double-emulsion (Arshady, 1991).

In this study, the aim was to produce an optimal formulation of PLGA 50:50 biodegradable microspheres for delivery of AS-ODNs into the CNS. Parameters including polymer molecular weight, particle-size and ODN sequence were investigated to tailor the release profile. These parameters were investigated to provide a release profile over at least one month, with a formulation of particles in the micrometer range for facile stereotaxic implantation into discrete brain regions.

3.2. MATERIALS AND METHODS

All microsphere batches used throughout this chapter were prepared by a double-emulsion solvent evaporation technique using a Silverson homogeniser (section 2.5.1.), unless otherwise stated. Two different molecular weights of the co-polymer, PLGA 50:50 were used, low molecular weight (LMw) 3kDa and high molecular weight (HMw) 45kDa. All microsphere batches were loaded with a 5'-end γ-^{32}P[ATP] radiolabelled poly adenine (A) 15-mer PS-ODN, unless otherwise stated. Microspheres prepared for particle-size determination and scanning-electron microscopy were loaded with an unlabelled poly (A) 15-mer PS-ODN, and are described as "cold" batches of microspheres.

3.2.1. Preparation of double-emulsion (w/o/w) microspheres by the vortex method

An aqueous solution containing PS-ODN (25µg) and an emulsifying agent, 0.4% (w/v) polyvinyl alcohol (PVA) (Mw 13-23000, 88% hydrolysed, Sigma, Dorset, U.K.) was mixed with 5ml of dichloromethane (DCM) (HPLC grade) containing 500mg of LMw (3kDa) PLGA 50:50. The two phases were vortexed (at maximum speed) for 5 minutes to form a primary emulsion, which was then added to 160ml of an aqueous 4% (w/v) solution of PVA and with a Heidolph homogeniser (1000 rpm) for 3 hours at room temperature to form a (w/o/w) double-emulsion.

The w/o/w emulsion was stirred on a magnetic stirring plate for 4 hours at room temperature, allowing for evaporation of the DCM through punctured Parafilm™. The particles were collected by centrifugation at 2700g for 10 minutes (Model LC1, Sarstedt, Leicestershire, U.K.) and washed twice in double-distilled water (ddH_2O). The microspheres were freeze-dried for 48 hours (Edwards Modylo freeze-drier, Boc Ltd., Sussex, U.K.) and stored in a dessicator at room temperature.

3.2.2. Particle-size and morphology

Particle-size was determined by laser diffractometry as described in section 2.7. The morphology of each batch of microspheres was observed by scanning-electron microscopy, see section 2.6.

3.2.3. Stability of ODN in microspheres

30mg of microspheres loaded with 5'-end γ-^{32}P[ATP] poly (A) 15-mer ODN were suspended in 1.5ml of phosphate-buffered saline (PBS) in glass sample tubes (Fischer, Leicestershire, U.K.). These were incubated in a to-and-fro shaking water bath at 37°C. At time-points (0, 1, 3, 7, 14, 28, and 56 days. 1 vial per time-point), 1.5ml of microsphere suspension was transferred to a microcentrifuge tube and

centrifuged at 10500g for 5 minutes (MSE, Leicestershire, U.K.). To extract the ODN entrapped within the microspheres, the supernatant was discarded and the pellet was resuspended in 0.75ml DCM and 0.75ml ddH_2O. The DCM was used for solubilisation of the polymer, and the ddH_2O for dissolving the ODN. The DCM/H_2O mixture was vortexed, then centrifuged at 10500g for 5 minutes (MSE, Leicestershire, U.K.). The water fraction was collected, and a further 0.75ml of ddH_2O was added to the solvent fraction. The DCM/H_2O mixture was vortexed and centrifuged as before, this was repeated four times with the water fraction collected each time. All water fractions were pooled and dried under vacuum in a DNA Speed vac (Model DNA 110, Savant, Leicestershire, U.K.). All samples of ODN extracted from microspheres at time points were stored in gel loading buffer containing urea and marker dyes (4.2g urea, 50mg xylene cyanole, 50mg bromophenol blue 10ml of ddH_2O) and stored at -20°C. The stability of these samples was determined by means of polyacrylamide gel electrophoresis using a 20% denaturing gel containing 7M urea (section 2.3.1.).

3.2.4. *In vitro* release studies

30mg of microspheres loaded with 5'-end γ-^{32}P[ATP] radiolabelled ODN were suspended in 1.5ml of PBS in glass sample tubes (Fischer, Leicestershire, U.K.). These were incubated at 37□C in a to-and-fro shaking water bath. Samples were taken at time points, every hour for the first 8 hours on day 1, then daily up to the end of the first week, and weekly thereafter. To sample, 1.5ml of suspended microspheres was transferred to a microcentrifuge tube and centrifuged at 10500g for 5 minutes in a microcentrifuge (MSE, Leicestershire, U.K.). The whole supernatant was added to 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Fischer, Leicestershire, U.K.) for analysis by liquid scintillation counting (LSC) (section 2.4.). The microsphere pellet was resuspended in 1.5ml of fresh PBS and returned to the sample tube.

All release studies were performed in quadruplicate for each batch and the results were expressed as the mean cumulative release (nmoles/30mg microspheres) ± s.d.

3.2.5. Polymer degradation studies

30mg of cold microspheres loaded with poly (A) 15-mer ODN were suspended in 1.5ml of PBS in glass sample tubes (Fischer, Leicestershire, U.K.). These were incubated in a to-and-fro shaking water bath at 37°C. At time points (7, 14, 28 days), 1.5ml of microsphere suspension was transferred to a microcentrifuge tube and centrifuged at 10500g for 5 minutes (MSE, Leicestershire, U.K.). The supernatant was discarded and the pellet was dried for 24 hours on a freeze-drier (Edwards Modylo freeze-drier, Boc Ltd., Sussex, U.K.). All samples were observed for any morphological changes by scanning-electron microscopy (section 2.6.).

3.2.6. Octanol/water partition coefficients of ODNs

5'-end γ-^{32}P[ATP] radiolabelled poly adenine (A), cytosine (C), guanine (G) or thymidine (T) 10-mer ODNs measuring 100000 cpm by LSC (section 2.4.) were each suspended in 0.5ml of ddH_2O (aqueous phase) and 0.5ml of octanol (Sigma, Dorset, U.K.) (organic phase). The mixture was shaken vigorously for 3 hours at room temperature, and centrifuged at 10500g for 10 minutes (MSE, Leicestershire, U.K.) to separate the two phases. 300µl from each phase was transferred to a separate scintillation vial (Sarstedt, Leicestershire, U.K.) and added to 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Fischer, Leicestershire, U.K.). Radioactivity in each phase for each ODN was determined by LSC (section 2.4.).

The partition coefficient (log P) was expressed as the log of the amount of ODN present in the organic phase compared to the amount present in the aqueous phase.

$$\log P = \log \frac{[ODN]_{octanol}}{[ODN]_{ddH2O}}$$

Log P was expressed as a mean of four replicates (± s.d.).

3.3. RESULTS AND DISCUSSION

3.3.1. Effect of polymer molecular weight

Differences in polymer molecular weight can alter the viscosity of the organic phase during the formulation of microspheres. Alterations in particle-size, partitioning into polymer or drug phase during fabrication and the release profile can all be affected by such changes in viscosity. Molecular weight of the polymer affects its degradation, where high molecular weight polymers degrade slower than low molecular weight polymers. Such alterations in the rate of degradation of different molecular weight polymers can in turn affect the release profile of the ODNs.

The particle-size, morphology and release profiles of two different molecular weight PLGA 50:50 microsphere preparations, 3kDa and 45kDa were investigated. The characteristics of these two preparations of microspheres are summarised in table 3.2.

Polymer type	ODN sequence	Nominal load (pmols/mg)	Actual load (pmols/mg)	Entrapment efficiency (%)	Particle-size distribution (μm)
LMw (3kDa) PLGA 50:50	Poly (A) PS 15-mer	10.30	5.05 ± 0.28	48.98 ± 2.66	2-22
HMw (45kDa) PLGA 50:50	Poly (A) PS 15-mer	10.30	5.28 ± 0.21	51.25 ± 2.02	2-28

Table 3.2. Summary of microsphere characteristics for low and high Mw PLGA 50:50 microspheres.

The particle-size distribution increased slightly with an increase in polymer molecular weight (figure 3.1.). An increase in polymer molecular weight increases the viscosity in the organic phase that may result in an increase in droplet size and thus a larger particle diameter. Both molecular weights showed a narrow size distribution, between 2 and 28μm, and produced particles of a similar morphology, spherical with a smooth surface (figure 3.2.).

Both of these preparations were prepared by a double-emulsion technique using a Silverson homogeniser (section 2.5.1.). This technique produces spheres with a narrow particle-size distribution, in comparison to the vortex method using a Heidolph homogeniser (section 3.2.1.), that is more commonly used for the preparation of larger particles. The effect of particle-size is discussed later in section 3.3.2.

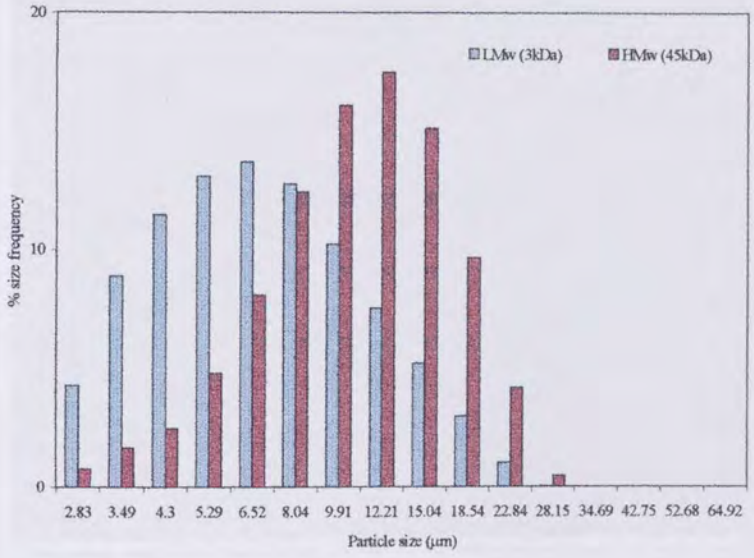
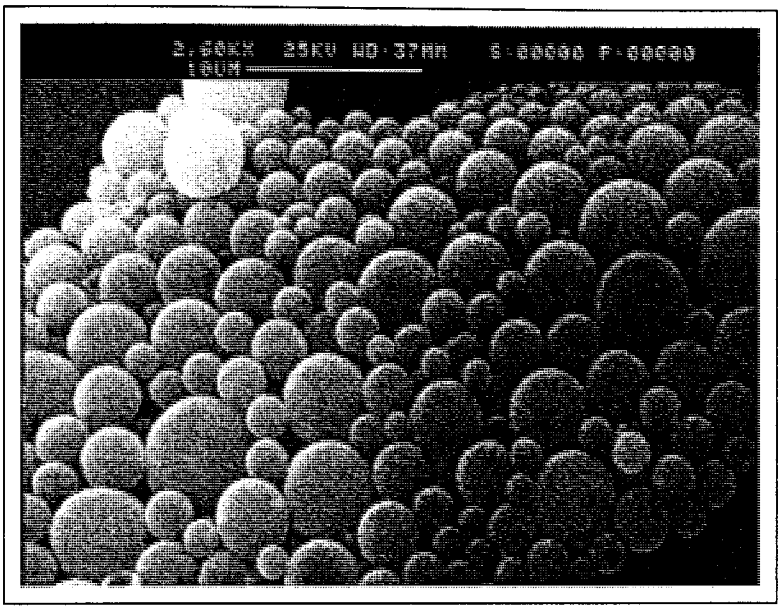


Figure 3.1. Particle-size distribution of LMw (3kDa) and HMw (45kDa) PLGA 50:50 microspheres loaded with PS-ODN poly (A) 15-mer, as measured by laser diffractometry on a Malvern Mastersizer (section 2.7.).

A.



B.

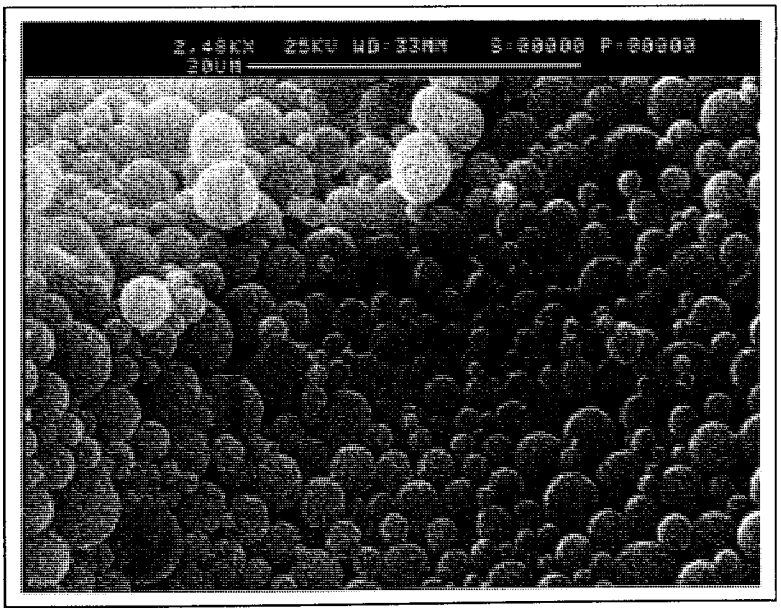


Figure 3.2. Scanning electron micrographs of LMw (3kDa) (A) and HMw (45kDa) (B) PLGA 50:50 microspheres loaded with PS-ODN poly (A) 15-mer, as determined by scanning electron microscopy (section 2.6.).

In order to determine that the release studies were measuring the release of intact ODN and not shorter degraded fragments, the stability of PS-ODNs entrapped within microspheres was investigated. PS-ODNs extracted from HMw PLGA 50:50 microspheres (section 3.2.3.) were stable in PBS over a period of 56 days, as determined by urea PAGE (section 2.3.1.) (figure 3.3), that is, throughout the duration of the *in vitro* release experiments. The stability of ODNs in foetal calf serum was not investigated in these studies, as this has been previously reported for polymeric matrices showing that 5' end γ-^{32}P-radiolabelled 20-mer *tat* PO-ODN are stable in PLA polymer films for up to 28 days incubation (Lewis *et al.*, 1995). A further investigation into the stability of ODNs in cell culture systems is addressed later in section 4.3.1.3.

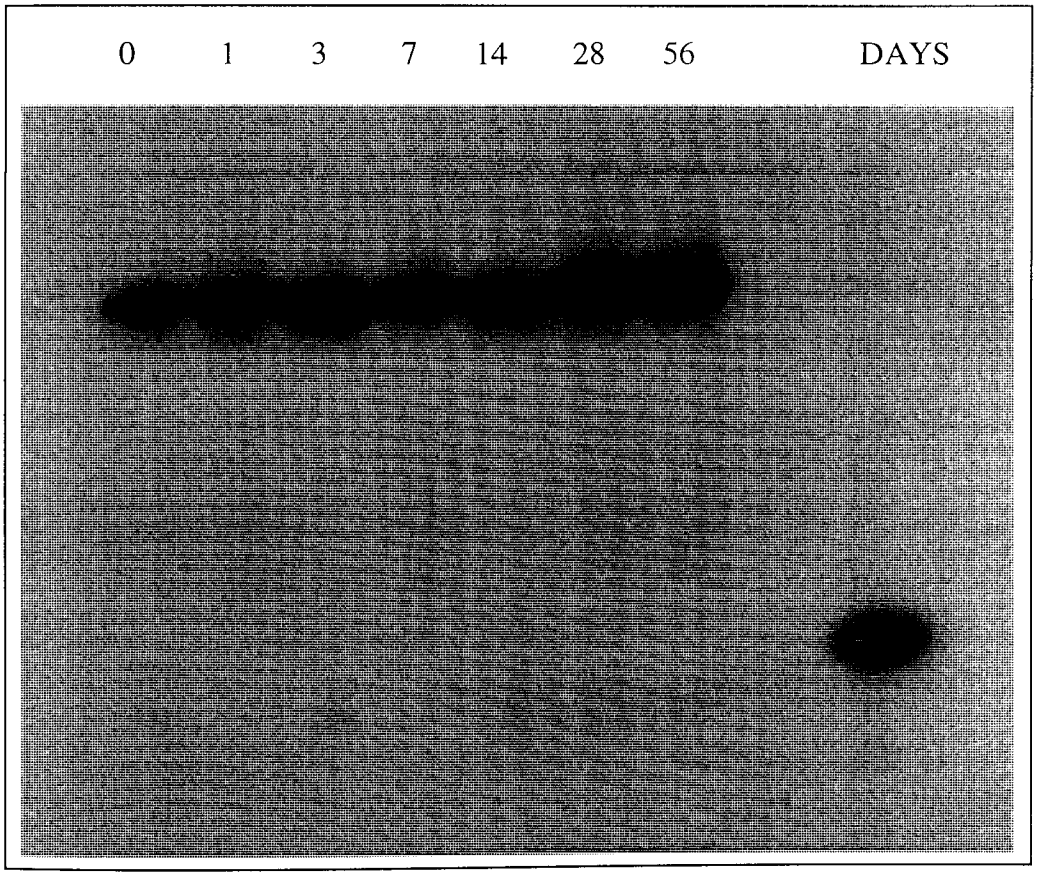


Figure 3.3. Stability of 5' end γ-^{32}P-radiolabelled PS-ODN poly (A) 15-mer extracted from HMw (45kDa) PLGA 50:50 microspheres incubated in PBS at 37°C over 56 days. Zero time represents minimal exposure to degradation condition before sample removal and Ω represents free label.

The actual load of ODN incorporated into low and high molecular weight microspheres were similar, 5.05 ± 0.28 and 5.28 ± 0.21 pmols/mg of polymer respectively (table 3.2.). During these preliminary investigations, low loading of ODNs were used due to financial limitations within the project.

The release profiles are illustrated in figure 3.4.A, with linear regression analysis detailed in figure 3.4.B. LMw microspheres showed a biphasic release profile, with approximately 57% of ODN released within the first 24 hours, and a secondary phase of steady sustained-release. The release profile of HMw microspheres was triphasic, with a lower burst effect than the LMw microspheres, with approximately 9.5% of ODN released within the first 24 hours. There was a steady sustained secondary phase of release over 28 days, then the rate of release increased after 28 days as it entered a tertiary phase of release. This was highlighted in the differences in the slopes of the lines for HMw microspheres, 0.0002 and 0.0011 for 10-28 and 28-56 days respectively (figure 3.4.B.).

In a study where the release of ODNs from three different molecular weight PLGA 75:25 implants was monitored, a biphasic release profile was observed for the lowest molecular weight 10kDa PLGA, whereas a triphasic profile was observed with the 15kDa and 20kDa PLGA implants (Yamakawa *et al.*, 1997). A burst effect was observed for the 10kDa PLGA implants, whereas no burst effect was observed for 15kDa and 20kDa PLGA implants, only a lag period of 7-12 days.

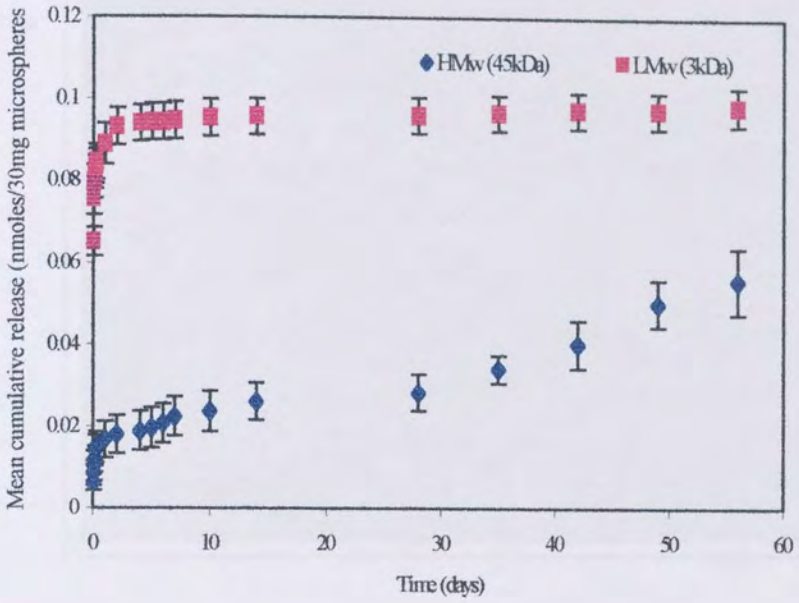
In these investigations, the initial burst effect may be attributed to loosely bound ODN at the surface of the microspheres and diffusion of ODN from within the microspheres. The increased burst effect observed with LMw microspheres compared to HMw microspheres may be due to more ODN on the surface of the LMw microspheres and less entrapped within the microsphere matrix. Differences in viscosity between the LMw and HMw PLGA may alter the partitioning of ODN into the polymer or drug phase during fabrication, resulting in differences in the amount of ODN at the microsphere surface or within the matrix. HMw polymers are more

viscous than LMw polymers, thus resulting in less diffusion of ODN from the HMw microspheres compared to LMw microspheres. Similar observations were made by Yamakawa *et al.* (1997), where the burst effect decreased with increasing molecular weight.

The secondary phase of release occurred at a slower rate than the initial burst effect and the tertiary phase of release. During this phase, hydrolysis may have been occurring at the surface, with simultaneous diffusion of water into the polymer bulk (Göpferich, 1997). Bulk erosion occurs when hydrolysis of the polymer is slower than diffusion of water into the polymer bulk, thus the complete cross-section of the polymer matrix is affected by erosion (Langer & Peppas, 1983). Polymer bulk erosion proceeds with an initial decrease in molecular weight prior to any significant mass loss of polymer suggesting that hydrolysis proceeds to a high degree before soluble oligomers and monomers are produced (Wang *et al.*, 1990). This finding correlates with what was observed during this release study, the secondary phase may be accounted for by hydrolysis, without any mass loss, resulting in no or little formation of pores on the surface of the microspheres. At the point where there is significant mass loss, pore formation at the surface may have resulted in channelling of ODNs out of the microspheres leading into the tertiary phase of release.

The tertiary phase of release observed with the HMw microspheres begins from around day 28 (figure 3.4.A. and B). The morphology of the microspheres was examined during the *in vitro* release investigations, to compare the surface characteristics of the microspheres with the phases of release of ODN. The microspheres were incubated in PBS over time (section 3.2.5.) and examined by scanning-electron microscopy (section 2.6.). Following 28 days incubation in PBS, pitting was observed on the surface (figure 3.5.) and there also appeared to be a degree of swelling of the microspheres, which may have occurred due to diffusion of water into the microsphere matrix. There were no differences in morphology between 7 and 14 days, thus SEMs are shown for 7 and 28 days (figure 3.5.).

A.

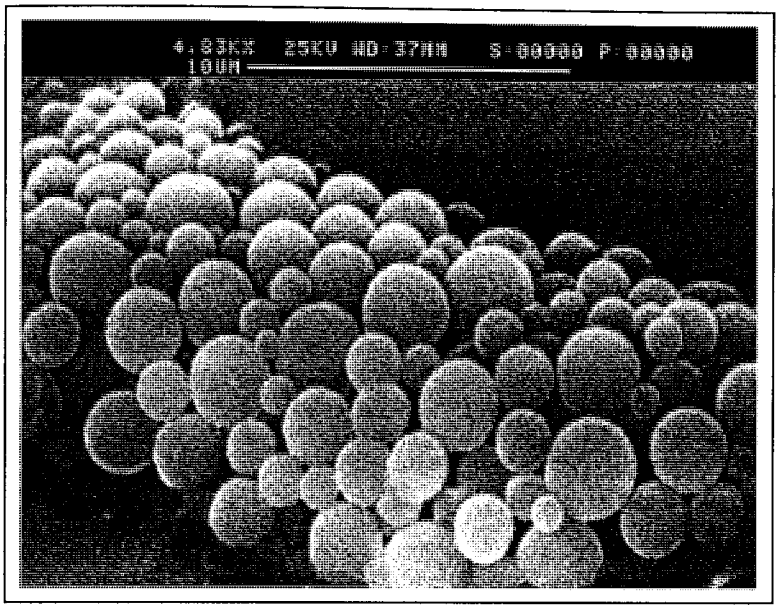


B.

Phase of release profile	Microsphere preparation	Slope of line	R^2
Burst (0-8hrs)	LMw (3kDa)	0.0838	0.8231
	HMw (45kDa)	0.0416	0.9874
1-7 days	LMw (3kDa)	0.0080	0.6752
	HMw (45kDa)	0.0080	0.9530
10-28 days	LMw (3kDa)	0.0001	0.9268
	HMw (45kDa)	0.0002	0.8881
28-56 days	LMw (3kDa)	0.0001	0.8825
	HMw (45kDa)	0.0011	0.9872

Figure 3.4. Effect of polymer molecular weight on the mean cumulative release of PS-ODN poly (A) 15-mer from PLGA 50:50 microspheres in PBS pH 7.4 at 37°C (A) and linear regression analysis of phases of release profile (B). n = 4 ± s.d. Loading of ODN per 30mg of LMw (3kDa) and HMw (45kDa) microspheres respectively are 0.150 nmols and 0.157 nmols.

A.



B.

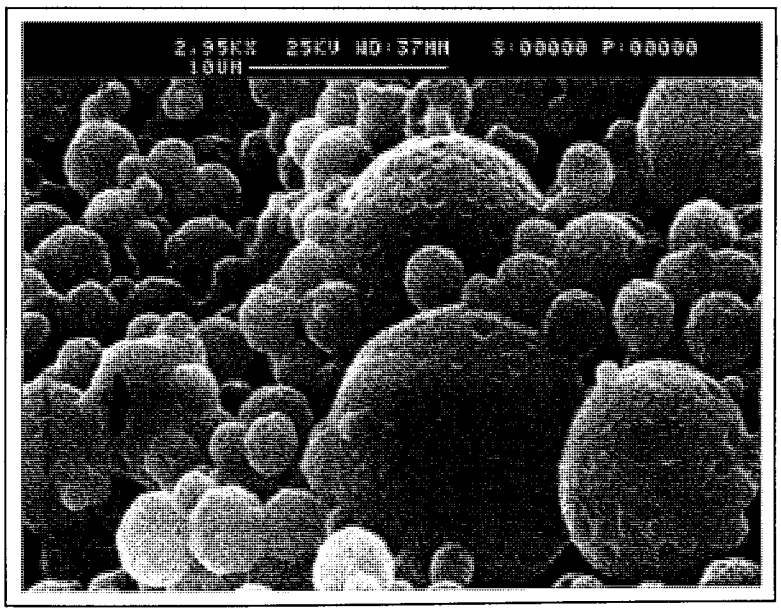


Figure 3.5. Scanning electron micrographs of HMw (45kDa) PLGA 50:50 microspheres after incubation in PBS at 37°C for 7 days (A) and 28 days (B), as determined by scanning electron microscopy (section 2.6.).

The biphasic profile of the LMw microspheres may have been a result of high concentrations of ODNs at the surface, and less within the microsphere matrix, which correlates with the huge burst release of approximately 57%. A reduced amount of ODN entrapped within the microspheres may have resulted in no tertiary phase being observed, as a majority of the ODN is at, or just below the surface of the microspheres. Lower molecular weight copolymers degrade faster than higher molecular weight copolymers because they are less crystalline, with crystalline regions being less accessible to water (Wang *et al.*, 1990). The faster rate of degradation of LMw PLGA would result in a quicker reduction in the loss of polymer mass, and pores at the surface of the microspheres may have formed relatively quickly so that ODN release coincides with the end of the initial burst effect.

3.3.2. Effect of particle-size

The double-emulsion Silverson method (section 2.5.1.) produced microspheres with a narrow particle-size distribution, between 2-8μm, whereas microspheres prepared by the vortex method (section 3.2.1.) produced particles of a much wider size distribution (figure 3.6.), with some particles up to 65μm in diameter. The Silverson method of preparing microspheres provided homogenous mixing of the primary emulsion, compared with manual vortexing. Increasing the stirring rate in the primary emulsion decreases the particle-size and the faster the stirring rate, the narrower the particle-size distribution (Lee *et al.*, 1999). For investigation into the release of ODN from particles of different size, the microspheres prepared by the vortex method were sieved to a defined size (between two sieves 38μm and 90μm), whereas, sieving of the Silverson-prepared particles was not required (2-8μm). The particles produced by both methods showed similar morphology, spherical with a smooth surface (figure 3.7.).

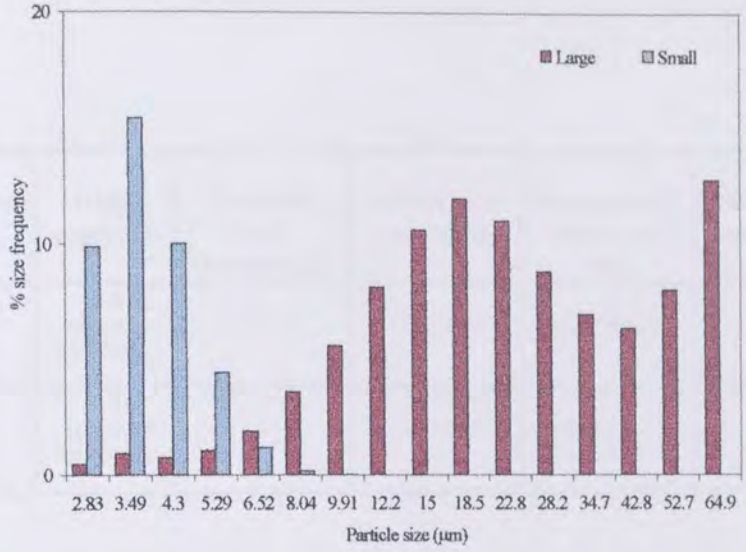


Figure 3.6. Particle-size distribution of large (prepared as in section 3.2.1.) and small (prepared as in section 2.5.1.) LMw (3kDa) PLGA 50:50 microspheres, as measured by laser diffractometry on a Malvern Mastersizer (section 2.7.).

Similar loadings were recorded for both small (2.08 ± 0.23 pmols/mg of polymer) and large (2.43 ± 0.06 pmols/mg of polymer) particles prepared by the Silverson and vortex method respectively (table 3.3.). The release of these particles into PBS was monitored over 14 days (figure 3.8.A. and B). Both small and large particles showed a biphasic release profile over 14 days, with an initial burst effect occurring within the first 24 hours, then followed by sustained-release of the ODN. A greater burst release of 62.3% in the first hour was observed for the smaller particles than the large particles, which released 6% within the first hour. The greater burst release for the small particles may be attributed to the increased surface area, which results in a higher concentration of ODN at the surface. In an investigation of methotrexate-release from gelatin microspheres, particle-size was shown to affect the rate of drug release, where increasing the particle-size decreased the rate of drug release (Narayani & Panduranga Rao, 1994). The smaller the particle-size, the higher the dissolution rate of the entrapped drug, due to the larger surface area of the smaller particles, hence release rate increases with a decrease in particle-size.

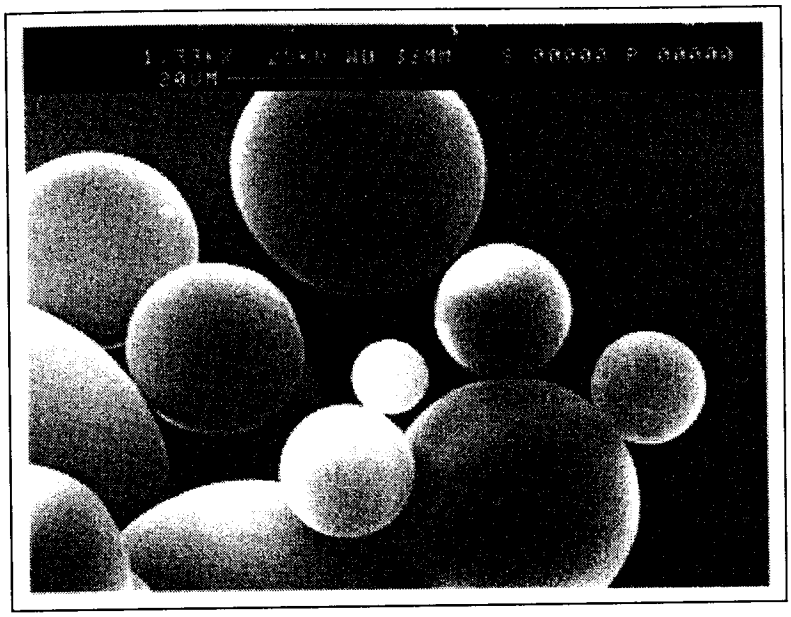
Polymer type	ODN sequence	Nominal load (pmols/mg)	Actual load (pmols/mg)	Entrapment efficiency (%)	Particle-size distribution (μm)
LMw (3kDa) PLGA 50:50	α1A antisense PS 18-mer	3.47	2.08 ± 0.23	59.94 ± 6.52	2-8μm
LMw (3kDa) PLGA 50:50	α1A antisense PS 18-mer	3.47	2.43 ± 0.06	70.03 ± 1.87	2-65μm

Table 3.3. Summary of microsphere characteristics for small and large LMw PLGA 50:50 microspheres.

Particle-size distribution is a vital factor in the characterisation of microspheres, as the surface area of particles can determine the rate and duration of drug release. A smaller particle-size leads to a greater surface area/volume ratio resulting in a faster release, whereas a larger particle-size reduces the surface area/volume ratio resulting in a slower release. A controlled drug delivery system could be tailored by appropriate mixing of microspheres of different sizes to suit the requirements of their application. It has been demonstrated that the mixing of small and large gelatin microspheres produced release profiles intermediate between small and large microspheres alone (Narayani & Panduranga Rao, 1996).

The intended application of the microspheres is an important consideration, as well as the duration of the release profile. The stereotaxic injection of microspheres into discrete tissue regions in the rodent brain may be affected by particle-size, where nanospheres have been used for retrograde labelling of neurons (Katz *et al.*, 1984). Thus, in terms of drug release into discrete tissue regions, microspheres may be more suitable than nanospheres producing a more localised distribution of drug, where nanospheres may be taken up by neuronal fibres and transported away from the site of injection (Menai *et al.*, 1994).

A.



B.

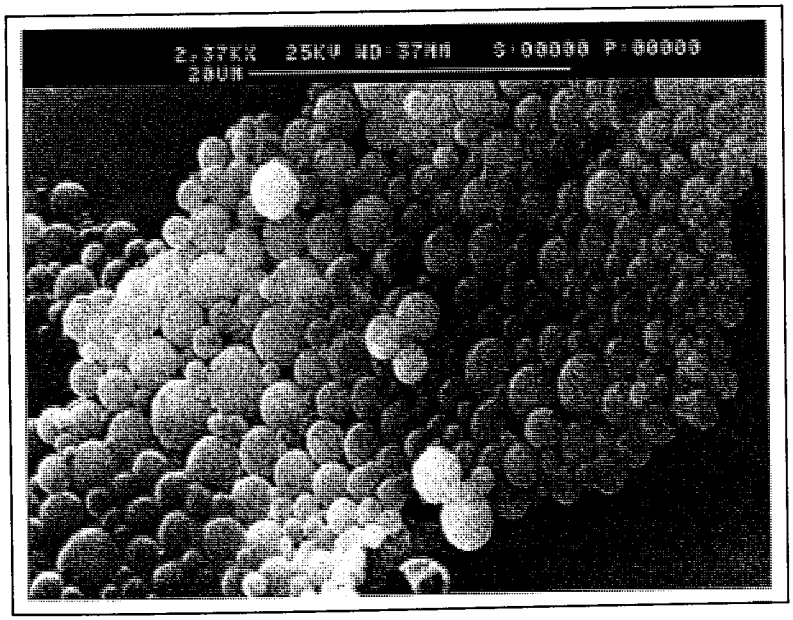
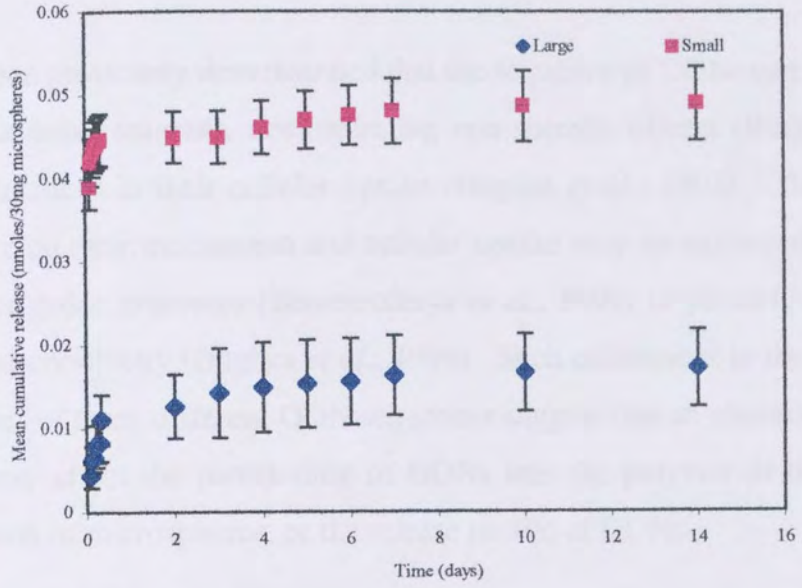


Figure 3.7. Scanning electron micrographs of large (A) and small (B) LMw PLGA 50:50 microspheres loaded with PS-ODN 18-mer, as determined by scanning electron microscopy (section 2.6.).

A.



B.

Phase of release profile	Microsphere preparation	Slope of line	R^2
Burst (0-8hrs)	38-90μm	0.0224	0.9136
	1-10μm	0.0199	0.7847
1-14 days	38-90μm	0.0007	0.9059
	1-10μm	0.0008	0.9707

Figure 3.8. Effect of particle-size on the mean cumulative release of PS-ODN from LMw (3kDa) PLGA 50:50 microspheres in PBS pH 7.4 at 37°C (A). Linear regression analysis of phases of release profile (B). n = 4 ± s.d. Loading of ODN per 30mg of small (1-10μm) and large (38-90μm) microspheres respectively are 0.0624 nmols and 0.0728 nmols.

3.3.3. Effect of ODN sequence

It has been previously demonstrated that the sequence of ODNs can affect efficacy of these antisense reagents, demonstrating non-specific effects (Burgess *et al.*, 1995) and differences in their cellular uptake (Hughes *et al.*, 1994). The effect of ODN sequence on their mechanism and cellular uptake may be attributed to the formation of higher order structures (Benimetskaya *et al.*, 1997) or possibly by differences in their hydrophobicity (Hughes *et al.*, 1996). Such differences in the physicochemical properties of these different ODN sequences suggest that an alteration in sequence of ODN may affect the partitioning of ODNs into the polymer or drug phase during fabrication of microspheres, or the release profile of ODNs.

Microspheres (HMw PLGA 50:50) were prepared loaded with homopolymer 10-mer PS-ODNs, poly (A), poly (C), poly (G) and poly (T). All batches were of similar size distribution (table 3.4.) and showed similar morphology, spherical with a smooth surface. Similar loadings were determined for all four batches of microspheres (table 3.4.).

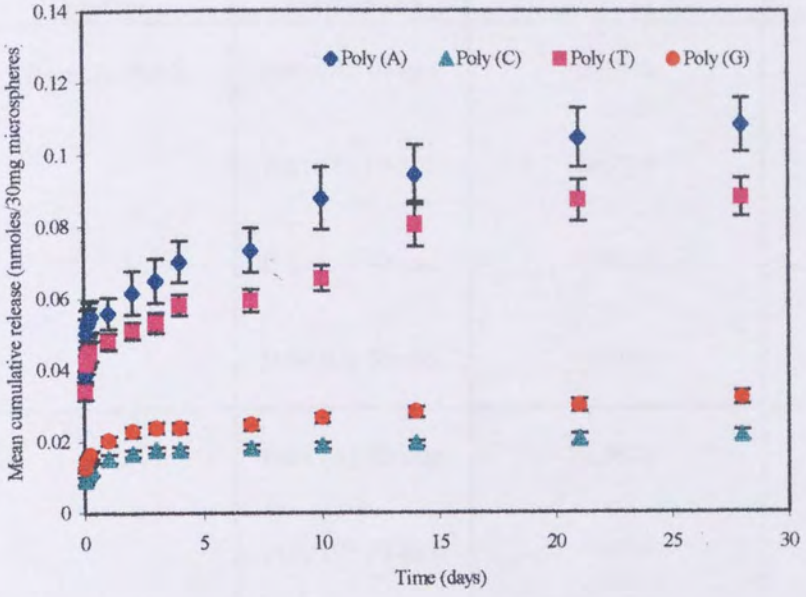
Polymer type	ODN sequence	Nominal load (pmols/mg)	Actual load (pmols/mg)	Entrapment efficiency (%)	Particle-size distribution (μm)
HMw (45kDa) PLGA 50:50	Poly (A) PS 10-mer	15.57	3.22 ± 0.13	20.66 ± 0.81	2-10
HMw (45kDa) PLGA 50:50	Poly (C) PS 10-mer	16.82	2.45 ± 0.63	14.57 ± 3.75	1-10
HMw (45kDa) PLGA 50:50	Poly (G) PS 10-mer	14.83	3.59 ± 1.23	24.23 ± 8.29	2-10
HMw (45kDa) PLGA 50:50	Poly (T) PS 10-mer	16.02	2.93 ± 0.59	18.29 ± 3.71	2-7

Table 3.4. Summary of microsphere characteristics for HMw PLGA 50:50 microspheres loaded with four homopolymer 10-mer PS-ODNs

The release of ODN from the microspheres was monitored over 28 days (figure 3.9.A.). Similar triphasic release profiles were observed for poly (A) and poly (T), and biphasic profiles for poly (C) and poly (G). Similar slopes of burst release were observed for all four samples of microspheres (table 3.5.). Poly (A), poly (T), poly (C) and poly (G) had slopes of 0.0264, 0.0127, 0.0128 and 0.0167 and correlation coefficients of 0.9383, 0.9848, 0.8488 and 0.8778 respectively. A greater amount of ODN was released from microspheres loaded with poly (A) and (T) during the burst phase of release, than poly (C) and (G) microspheres. Poly (A) and (T) were released at a greater rate than poly (C) and (G) during the secondary phase of release, with slopes of 0.0029, 0.0023, 0.0003 and 0.0005 respectively (table 3.5.). A tertiary phase of release was apparent for poly (A) and poly (T) (slopes of 0.001 and 0.006), whereas there were no apparent differences in slopes for poly (C) and poly (G) between 1-14 days (0.0003 and 0.0005) and 14-28 days (0.0002 and 0.0003), suggesting a biphasic release profile.

The amount of homopolymer 10-mer ODNs released during the initial burst effect and throughout the whole release study occurred in the following order A>T>G>C (figure 3.9.A). The release of homopolymer 7-mer PO-ODNs was investigated in polylactic acid (PLA) films, where ODN was released over 28 days in the order A>T>C>G (Lewis, 1996), similar to the observations made during these investigations. During the studies by Lewis (1996), it was suggested that the observed differences in release of homopolymer ODNs might be attributed to hydrophobicity or the development of tertiary structures, although this was never investigated. Thus, the hydrophobic nature of the PS-ODNs was investigated in these studies, in an attempt to elucidate whether the hydrophobic properties of the ODNs can affect the release profile.

A.



B.

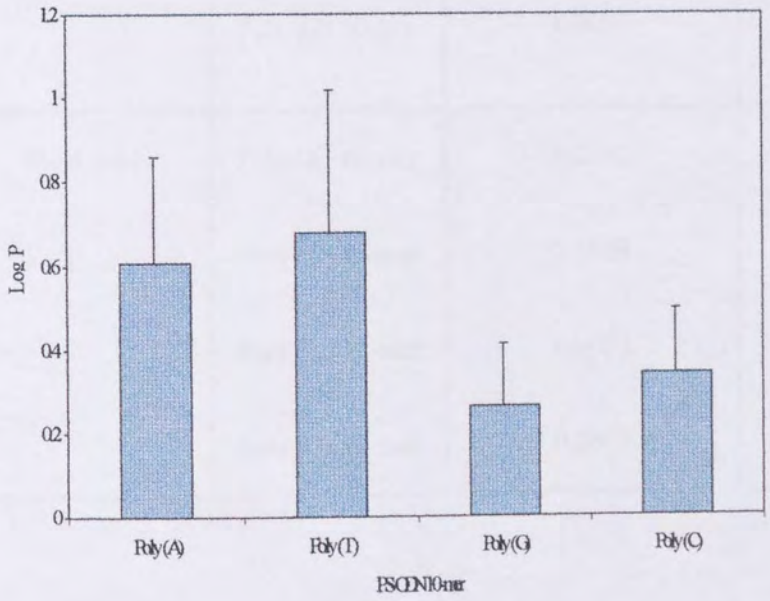


Figure 3.9. Effect of ODN sequence on the mean cumulative release of homopolymer 10mer PS-ODNs from HMw (45kD) PLGA 50:50 microspheres (1-10μm) in PBS pH 7.4 at 37°C (A). Log P values of homopolymer 10mer PS-ODNs (B). n = 4 ± S.D. Loading of ODN per 30mg of poly A, poly T, poly C and poly G microspheres respectively are 0.0966, 0.08776, 0.07335 and 0.1079 nmols.

Phase of release profile	Microsphere preparation	Slope of line	R^2
Burst (0-8hrs)	Poly (A) 10-mer	0.0264	0.9383
	Poly (T) 10-mer	0.0127	0.9848
	Poly (C) 10-mer	0.0128	0.8488
	Poly (G) 10-mer	0.0167	0.8778
1-14 days	Poly (A) 10-mer	0.0029	0.9690
	Poly (T) 10-mer	0.0023	0.9539
	Poly (C) 10-mer	0.0003	0.8223
	Poly (G) 10-mer	0.0005	0.9076
10-28 days	Poly (A) 10-mer	0.0010	0.9268
	Poly (T) 10-mer	0.0060	0.8362
	Poly (C) 10-mer	0.0002	0.9949
	Poly (G) 10-mer	0.0003	0.9966

Table 3.5. Table of linear regression analysis of phases of ODN sequence release profile (see figure 3.9.).

The hydrophobic nature of the ODN homopolymers was investigated by measuring differences in their partition coefficients (Log P), that is the ability of the ODN to partition between an organic phase (octanol) and an aqueous phase (water) (section 3.2.6.). The higher the Log P value the higher the concentration of ODN partitioning into the organic octanol phase, indicating an increased hydrophobicity. Figure 3.9.B. illustrates the differences in partition coefficients (Log P) between the four homopolymer 10-mer ODNs. These data indicate that poly (C) and poly (G) are less hydrophobic than poly (A) and poly (T), with Log P values of 0.34, 0.26, 0.61 and 0.68 respectively. The rank order of hydrophobicity is T>A>C>G (albeit the differences between (A) and (T) and (C) and (G) are minimal). The differences in hydrophobicity between poly (C) and (G) and poly (A) and (T) may have affected the release profile due to alterations in partitioning into the organic or aqueous phase occurring during the fabrication of the microspheres. More hydrophobic molecules would partition into the organic (polymer) phase than the aqueous phase, leading to more surface ODN and leakage manifested as an increased burst effect (figure 3.9.A) as was observed with poly (A) and (T). Less hydrophobic molecules would partition more into the aqueous phase of the emulsion leading to more ODN entrapped with a lower burst effect (figure 3.9.A) as was observed for poly (C) and (G).

The presence of tertiary structures was not investigated in these studies but cellular studies have revealed that there were differences in the uptake of homopolymer PS-ODNs, where homopolymer 10-mers were taken up in the rank order G>T>C>A in Chinese hamster ovary cells (Hughes *et al.*, 1994). Similar investigations carried out by Peyman *et al.* (1995) revealed that cellular uptake of homopolymer 16-mers was in the order of G>T>A>C in Vero cells. In both of these studies it was believed that the propensity for poly (G) 10- and 16-mers to be preferentially taken up by cells was attributed to higher structures of poly (G) ODNs (Hughes *et al.*, 1994; Peyman *et al.*, 1995). Indeed, the presence of a G-quartet near the 5'- terminus of a PS-ODN promoted formation of tetraplexes and other higher order structures in a time and temperature-dependent manner with tetraplexes forming preferably at lower temperatures (Benimetskaya *et al.*, 1997) (discussed further in section 1.1.1.6.). The

current lack of research into the effect of ODN sequence on release from biodegradable microspheres, requires that comparisons to be made are with, the effect of ODN sequence in cellular studies, however the variables between these two modes of investigation limit the validity of these comparisons. Cellular uptake studies may involve the interaction of certain proteins (Fennewald & Rando, 1995; Bates *et al.*, 1999) also ODNs are less stable in biological fluids than in water or PBS, due to the presence of nucleases (Akhtar *et al.*, 1992). Differences in the higher order structures of these homopolymer sequences may result in alterations in entrapment of the ODNs within the microspheres, with differences in concentrations of ODNs at the surface or within the microsphere matrix.

3.4. CONCLUSIONS

The development of a microsphere formulation for the delivery of AS-ODNs can be tailored by altering such formulation parameters as polymer molecular weight, particle-size and sequence of ODN. Such changes in the formulation of microspheres can affect the particle-size and partitioning into polymer or drug phase, which in turn can affect the release profile of ODNs.

High molecular weight microspheres released entrapped ODN over a longer period of time, with a triphasic profile, than low molecular weight microspheres, with a biphasic profile. For LMw microspheres most of the ODN was released within the first 24 hours, whereas HMw microspheres released the ODNs over a period of 56 days, with a steady sustained-release between 1-14 days and an increase in the rate of release from 28 days onwards (figure 3.4.). The fact that the LMw microspheres released most of the ODN during the burst effect (within 24 hours), and then showed a lag phase of release resulted in further investigations (cell culture and *in vivo*) being carried out with the HMw microsphere formulation.

The release of ODNs from small and large microspheres demonstrated that the particle-size of microspheres could alter the release profile, with larger microspheres,

decreasing the dissolution rate (figure 3.8.). However, the intended application of the microsphere formulation is also an important consideration. The intended application of this formulation is for stereotaxic implantation into discrete tissue in the brain, where it has been suggested that microparticles would be more suited to drug delivery than nanoparticles, as nanoparticles can be transported away from the site of injection (Menai *et al.*, 1994).

The sequence of the ODNs had an effect on the release from HMw PLGA 50:50 microspheres, which may have been attributed to differences in hydrophobicity of the different homopolymers (figure 3.9.A. and B.). The differences in hydrophobicity may have resulted in the different sequences partitioning differentially into the organic (polymer) phase during fabrication of the microspheres. The formation of higher structures in G-quartet containing ODNs may have affected entrapment of the ODNs within the polymeric microspheres altering the release profile, however the tertiary structures of ODNs was not investigated and requires further investigation to support this hypothesis.

A formulation using HMw PLGA 50:50 prepared by the Silverson method (section 2.5.) was chosen for further cell culture and *in vivo* investigations for its long duration of release and small particle-size. For cell culture (chapter four) and *in vivo* distribution and efficacy studies (chapter five), the microspheres were loaded with a poly (A) 15-mer PS-ODN, so any differences between these studies could not be attributed to differences in ODN sequence.

CHAPTER FOUR

CELLULAR ASSOCIATION OF BIODEGRADABLE MICROSPHERES WITH GLIAL AND NEURONAL CELLS

4.1. INTRODUCTION

The reported problems of ODNs with stability (Akhtar *et al.*, 1992), cellular uptake and intracellular trafficking (reviewed by Akhtar & Juliano, 1992) has fuelled interest into the development of delivery strategies that have the potential to overcome these challenges. Such delivery strategies include polycations, cationic lipids, liposomes and biodegradable microparticles (reviewed by Miller & Das, 1998), expression vectors (reviewed by Weiss *et al.*, 1999) and viral vectors. The use of biodegradable microspheres is an attractive strategy. They have been reported to enhance the stability of ODNs (Lewis *et al.*, 1995), whilst improving cellular uptake (Akhtar & Lewis, 1997) and providing sustained-release over periods of time defined by different formulation parameters of the microspheres. The delivery of an intact ODN to its target at efficacious concentrations over a sustained period of time would significantly improve the pharmacological potential of these molecules. In terms of CNS delivery, PLGA biodegradable microspheres offers the advantage of biocompatibility with brain tissue (Menai *et al.*, 1993), and degradation of the polymer into non-toxic monomers that are excreted *via* normal physiological pathways.

The use of biodegradable microspheres for cellular delivery of ODNs has not been extensively investigated. Adsorption of ODNs onto polyalkylcyanoacrylate

nanoparticles in the presence of an ion-pair former, such as quaternary ammonium salts, protected the ODNs from 3'- exonuclease degradation (Chavany *et al.*, 1992). The cellular uptake of ODNs was increased as a result of capture of these nanoparticles by an endocytic/phagocytic pathway (Chavany *et al.*, 1994). However, the use of polyalkylcyanoacrylate has been criticised because of its cytotoxicity and the general toxicity of quaternary ammonium salts. Despite the potential of polyalkylcyanoacrylate particles as convenient carriers for the protection and delivery of ODNs to cells, their cytotoxic nature renders them a less desirable delivery system for cellular delivery of ODNs. The cellular association of ODNs entrapped within PLGA nanoparticles was improved ten-fold in murine macrophages compared to the naked molecule (Akhtar & Lewis, 1997), and the use of PLGA poses no problems with toxicity as they have been used in FDA approved biomaterials for years. More recently the incorporation of an 18-mer directed to the rat serotonin transporter into PLGA 85:15 nanoparticles resulted in significant uptake of ODN into rat basophilic leukaemia cells with observed protection from nuclease action (Das *et al.*, 1998).

The use of ODNs within the CNS has highlighted a number of problems, which can impair their use as research tools and in therapy (reviewed by Neumann, 1997; Nicot & Pfaff, 1997; Robinson *et al.*, 1997), discussed later in section 5.1. and 5.4. Problems of toxicity arise due to the therapeutic index being extremely narrow, where high concentrations of ODN are required for efficacy, but these high concentrations of ODN can cause toxicological problems. Currently, continuous levels of ODN may be administered into the brain by osmotic minipumps (Tremblay *et al.*, 1998; Zhang & Creese, 1993) or the use of repeated injections (Zhou *et al.*, 1996; Karle & Nielsen, 1995). Biodegradable microspheres provide a means of continuous release of ODN at the target site with a single dose. The interaction of ODNs when delivered by biodegradable microspheres, with neuronal and glial cells has not been extensively investigated, with most research carried out in glial cells (Beck *et al.*, 1996; Fell *et al.*, 1997). The mechanism of the delivery of ODNs via

particulate systems is not well understood, although it is believed to occur *via* endocytosis (Zobel *et al.*, 1997).

The development of a microsphere formulation can be tailored to suit the requirements of the intended application as evidenced in chapter three, however, further characterisation is required prior to its use *in vivo* for ethical reasons. The aims of this chapter were to investigate any differences in cellular association and uptake of ODNs delivered as the naked molecule or entrapped within microspheres, investigating their association in both glial and neuronal cells. The mechanisms of cellular uptake of ODN-loaded microspheres were also investigated and in addition the effects of inhibitors, competitors and temperature were examined.

4.2. MATERIALS AND METHODS

A poly (A) 15-mer phosphorothioate ODN was used throughout this chapter, radiolabelled at the 3'-end of the molecule (as described in section 2.2.2.), either as the naked molecule or entrapped within HMw (45kDa) PLGA 50:50 microspheres (prepared as in section 2.5.1.). Non-radiolabelled reagents are referred to as 'cold'.

Two different cell lines were used throughout these investigations, a rat glial cell line (C6 glioma) and a rat neuronal cell line (GT_1 neuronal) (see section 2.8.2.).

4.2.1. Cell viability assay

The viability of cells was determined by a trypan blue exclusion assay. Cells were seeded in 24-well plates in quadruplicate at 5 x 10^4/ml (1ml/well) (section 2.8.5.), and incubated overnight at 37°C in a humidified incubator with 5% (v/v) CO_2. Cells were incubated with unloaded microspheres at varying doses (5, 10, 50mg/ml) in serum-free media for 24 hours at 37°C. Microspheres were removed and wells were washed three times with pre-warmed PBS. 200µl of trypsin (1mg/ml) (Gibco, Paisley, Scotland, U.K.) was added to each well and incubated for 5 minutes at 37°C.

An equal volume of trypan blue (Gibco, Paisley, Scotland, U.K.) was added to each well and cells were counted using a haemocytometer (enhanced Neubauer) (Weber Scientific International, Middlesex, U.K.). The % viability was determined by the following calculation.

$$\% \text{ Viability} = \frac{\text{No. of viable cells}}{\text{No. of total cells}} \times 100$$

4.2.2. Stability of ODNs in culture

A 3'-end α-^{32}P radiolabelled ODN (section 2.2.2.) was diluted to 100 000 cpm per ml in serum-free media, and ODN-loaded microspheres were made up to 5mg/ml in serum-free media.

Cells were seeded into 24-well plates at 5 x 10^4/ml (1ml/well) (section 2.8.5.) and incubated overnight at 37°C in a humidified incubator with 5% (v/v) CO_2. The following day, 0.5ml of naked ODN and ODN-loaded microspheres were added to wells in triplicate. At time points (0, 1, 2, 4, 6, 8, 12, 24 hours), a 10µl aliquot of media was removed from the well and transferred to a microcentrifuge tube, 10µl of urea marker dye (4.2g urea, 50mg xylene cyanole, 50mg bromophenol blue in 10ml of ddH_2O) was added to each sample and stored at -20°C. Once all samples were collected, their stability was determined by denaturing PAGE (section 2.3.1.).

4.2.3. Cellular association experiments

A 3'-end α-^{32}P radiolabelled ODN (section 2.2.2.) (within 1 week of radiolabelling procedure) was diluted to the appropriate concentration (corresponding to the total concentration of ODN in 5mg/ml of microspheres) with cold ODN in serum-free media. ODN-loaded microspheres (section 2.5.1.) were made up to a concentration of 5mg/ml in serum-free media.

Cells (C6 glial and GT_1 neuronal) were seeded into 24 well plates at 5 x 10^4/ml (1ml/well) (section 2.8.5.) and incubated overnight (18 hours) at 37°C in a humidified incubator with 5% (v/v) CO_2. The following day, wells were washed in 0.5ml of pre-warmed PBS and incubated with ODN and microspheres in serum-free media. Cellular association was monitored at time points over a 24 hour period, unless otherwise stated, the time points being, 0.5, 1, 2, 4, 8, 12 and 24 hours. To sample, the media was transferred to a scintillation vial and cells were washed three times with 0.5ml of PBS to remove any ODN loosely associated with the cell surface. The PBS washings were pooled along with the media in a scintillation vial. The cell monolayer was removed by addition of 1% (v/v) Triton X-100 (Sigma, Dorset, U.K.) for 5 minutes at room temperature. The wells were washed three times with 1% (v/v) Triton X-100 to ensure complete removal of cells from the wells, and the cell fraction was transferred to a scintillation vial. 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Pharmacia, Leicestershire, U.K.) was added to all samples and radioactivity was determined by LSC (section 2.4.).

There were six replicates for each variable and the amount of ODN associated with the cells was expressed as the mean % cellular association/10^5 cells ± s.d.

For studies investigating the effect of temperature, metabolic inhibitors, self-competitors (section 4.2.5.) and for efflux experiments (section 4.2.4.), cells were incubated with naked ODN and ODN-loaded microspheres for 2 hours, prior to sampling.

4.2.4. Efflux studies

Naked ODN and ODN-loaded microspheres were incubated with neuronal (GT_1) cells (as described in section 4.2.3.) for 2 hours at 37°C in a humidified incubator with 5% (v/v) CO_2. After incubation, the apical medium was transferred to a scintillation vial along with three PBS washes. The wells were incubated with 0.5ml of fresh serum-free media. Following 30 minutes incubation the apical medium was

transferred to a scintillation vial and wells were replaced with fresh media until the next time point. This was repeated at defined time points (60, 120, 240, 360 minutes), with each apical solution of medium being transferred to a separate scintillation vial per time point. 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Pharmacia, Leicestershire, U.K.) was added to all samples and radioactivity was determined for each sample by LSC (section 2.4.). There were six replicates for both naked ODN and ODN-loaded microspheres, and the amount of ODN exocytosed from the cell was expressed as efflux (mean % ODN remaining) ± s.d.

4.2.5. Competition and inhibition studies

Investigations into the possible mechanisms involved in cellular association of ODN and ODN-loaded microspheres were carried out by monitoring cellular association at two different temperatures, 4°C and 37°C and following pre-incubation with the metabolic inhibitors sodium azide (NaN_3) + 2-deoxyglucose (2-DG) and competitors adenosine triphosphate (ATP), heparin, PO-ODN, PS-ODN and cold microspheres.

GT_1 neuronal cells were seeded into 24 well plates at 5 x 10^4/ml (1ml/well) (as in section 2.8.5.) and incubated overnight (18 hours) at 37°C in a humidified incubator with 5% (v/v) CO_2. The following day, plates were incubated with naked ODN or ODN-loaded microspheres (as described in section 4.2.3.) and incubated for 2 hours at either 4°C or 37°C. Plates were incubated with NaN_3 (10mM) (Sigma, Dorset, U.K.) + 2-DG (20mM) (Sigma, Dorset, U.K.) for 15 minutes at 37°C in a humidified incubator with 5% (v/v) CO_2. Following pre-treatment with metabolic inhibitors, the plates were incubated with naked ODN and ODN-loaded microspheres for 2 hours at 37°C in a humidified incubator with 5% (v/v) CO_2. Competition studies required a 15 minute pre-treatment of ATP (5μM) (Sigma, Dorset, U.K.), heparin 10μM) (Sigma, Dorset, U.K.), PO-ODN (5μM), PS-ODN (5μM) (prepared as in section 2.1.) and cold microspheres (10mg/ml) (prepared as in section 2.5.1.), prior to a 2 hour incubation with naked ODN and ODN-loaded microspheres.

There were six replicates for each inhibitor and competitor and different temperature, results are expressed as the mean percent of control ± s.d. Significant differences from untreated cells was determined using a paired students *t*-test.

4.2.6. Determination of sites of cellular interaction

Naked ODN and ODN-loaded microspheres were incubated with GT_1 neuronal cells at 5 x 10^4/ml (1ml/well) (as described in section 4.2.3.) for 2 hours at 37°C in a humidified atmosphere with 5% (v/v) CO_2. Following incubation, the wells were washed three times with 0.5ml of pre-warmed PBS, the cells were removed from the wells by incubating with 200µl of trypsin (1mg/ml) (instead of 1% (v/v) Triton X-100) for 5 minutes at 37°C and placed in a 1.5ml microcentrifuge tube. Cells were centrifuged for 3 minutes at 17900 g, and the supernatant was added to a scintillation vial, this crude fraction contained ODN that was protein-bound (fraction 1), thus washed off during trypsinisation. The pellet was re-suspended in 0.5ml of ddH_2O and vortexed for 5 minutes to aid lysis, then centrifuged for 3 minutes at 17900 g, this step was repeated again, and both supernatants were retained and added to a scintillation vial. This was termed fraction 2 crudely containing ODN from the cytoplasm of the cell that was released during lysis of the cell fraction. The pellet was added to a scintillation vial, which crudely corresponds to the cell membrane fraction (fraction 3). 5ml of scintillation cocktail (Optiphase Hi-Safe 3, Wallac, Fischer, Leicestershire, UK) was added to all samples and radioactivity was determined by LSC (section 2.4.). There were six replicates for both ODN and microspheres and results were expressed as the % fraction of total cellular associated ODN within the protein-bound, cytoplasmic and cell membrane fractions ± s.d.

4.2.7. Fluorescence microscopy

Cells were seeded into 8 well Lab-Tek® Chamber slides™ (NUNC, Gibco, Paisley, Scotland, U.K.) at 3 x 10^4 cells/ml, 300µl of cell suspension per well and incubated overnight at 37°C in a humidified atmosphere with 5% (v/v) CO_2 (section 2.8.5.).

The wells were carefully washed with pre-warmed serum-free media and incubated with naked FITC-ODN (1μM) and FITC-ODN-loaded microspheres (5mg/ml) for 24 hours at 37°C (wrapped in aluminium foil to avoid quenching of fluorescence) in a humidified atmosphere with 5% (v/v) CO_2. Following incubation, the wells were carefully washed twice in pre-warmed serum-free media. The cells were fixed in 200μl of 1% (v/v) paraformaldehyde/PBS for 1 hour at 4°C. The walls of the chamber slides were removed according to the manufacturer instructions. The slides were mounted in 1:1 PBS/glycerol containing an anti-fading agent diazabicyclo[2.2.2]octane (DABCO) (Sigma, Dorset, U.K.) at a concentration of 25mg/ml and coverslipped. Localisation of ODN in the cells was visualised using a UV microscope (Zeiss, West Germany) and a 510nm filter. The cells were photographed using a camera (Nikon).

4.3. RESULTS AND DISCUSSION

4.3.1. Optimisation of cell lines for cellular association studies

Prior to any investigations carried out on the two rat cell lines, glial cells (C6 glioma) and neuronal cells (GT_1), optimisation studies were performed. These optimisation studies included viability of cells following incubation with microspheres, the number of washes required for removal of radioactivity and stability of both naked and entrapped ODNs following incubation with both cell lines.

4.3.1.1. Viability of C6 and GT_1 cells with microspheres

The biocompatibility of PLGA is well established and has been used extensively in biomaterial applications and drug-delivery systems (Wang *et al.*, 1996; Pettit *et al.*, 1997). For the cellular association studies, the viability of each cell line was investigated for possible toxicity that may be caused by excipients in the formulation of the microspheres, such as DCM and PVA. Cell viability was determined for both glial and neuronal cells (section 4.2.1.) following incubation with unloaded HMw

(45kDa) PLGA 50:50 microspheres of varying concentrations (5, 10 and 50mg/ml). Cells were viable when incubated with high concentrations of microspheres (figure 4.1.). Incubation with 5mg/ml of microspheres showed greater than 90% viability in both cell lines, this was the concentration of microspheres used throughout the cellular association experiments.

4.3.1.2. Determination of number of washes for removal of radioactivity

For the cellular association experiments the number of PBS washes were determined, to ensure removal of radioactivity (figure 4.2.). In order to determine that the monolayers were not disrupted during the washing procedure, they were observed by light microscopy in between each washing step. Monolayers remained undisrupted for up to three washes, but after three washes monolayers became slightly disrupted. After three PBS washes there was almost complete removal of radioactivity following incubation with the naked ODN, with residual amounts of radioactivity following incubation with ODN-loaded microspheres. For all cellular association experiments, three PBS washes were used as any further washing steps caused disruption of the monolayers.

4.3.1.3. Stability of ODNs with C6 and GT_1 cells

The stability of ODNs has greatly improved over recent years with modifications in their chemistry (reviewed by Matteucci, 1995). PS-ODNs are more widely used because of their enhanced stability compared to PO-ODNs, and also their ability to recruit RNase H (ODN chemistries discussed earlier in section 1.1.3.). However, the stability of PS-ODNs in different media still requires improvement, where PS-ODNs are stable in S100 cytoplasmic extract for 60 minutes, but degradation begins within a 60 minute incubation with nuclear extract and normal human serum (Akhtar *et al.*, 1992). The use of 3'-end α-^{32}P-radiolabelled ODNs for cellular association experiments was preferable, as 5'-end γ-^{32}P-radiolabelled ODNs are known to rapidly undergo cleavage by phosphodiesterases (Wu-Pong *et al*, 1994).

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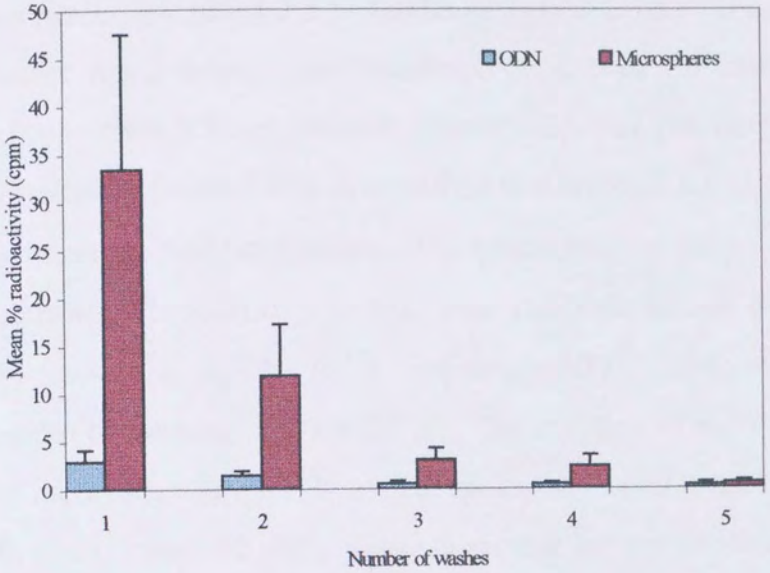

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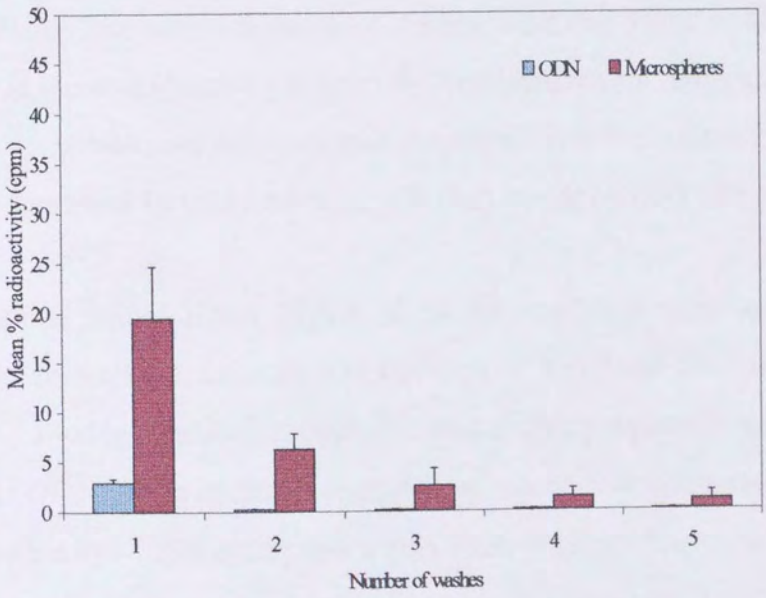

Figure 4.2. Number of PBS washes required for removal of radioactivity from the surface of C6 (glial) (A) and GT_1 (neuronal) (B) cells after treatment with naked ODN or ODN-loaded microspheres. Cells seeded at 5 x 10^4/well. n = 6 ± s.d.

Naked and entrapped ODNs were incubated with C6 glioma and GT_1 neuronal cells in serum-free media (section 4.2.3.). ODNs delivered as the naked molecule were only fully intact for 2 hours when incubated with both C6 and GT_1 cells, and degradation began from 2 hours onwards (figure 4.3.), data not shown for GT_1 cells. Only one degradation product was observed in this study (λ), and this product was slightly larger than the free label control (Ω), which may be due to the nature of the 3'-end radiolabelling procedure. ODNs were radiolabelled at the 3'-end of the molecule by addition of a α-^{32}P [di-deoxy-ATP] catalysed by terminal deoxynucleotidyl transferase (section 2.2.2.). The addition of α-^{32}P [di-deoxy-ATP] may alter the recognition site for 3'- exonucleases, as the ATP has hydrogen at both the 2'- and 3'- positions on the ribose sugar to prevent further additions of the adenine base during the radiolabelling procedure. This alteration in structure of the radiolabelled ODN at the 3'-end of the molecule may render this portion of the molecule 'unrecognisable' to the 3'- exonuclease enzymes, thus leading to cleavage at the next available site which is one base further from the 3'-end of the molecule. 3'-exonuclease is the major activity present in foetal calf serum, consequently any ODN containing a 3'- substituent may enhance the stability, it was reported that 3'-labelled ODNs were degraded 10 times more slowly than normal ODNs (Stein *et al.*, 1988).

ODNs entrapped within HMw PLGA 50:50 microspheres were fully intact for 24 hours, that is, throughout the complete duration of a cellular association experiment (figure 4.4.). Biodegradable microspheres as a delivery system for ODNs can serve to protect the ODNs from nuclease degradation, where it was reported that a 5'-end γ-^{32}P-radiolabelled PO-ODN entrapped within PLA polymer films was stable for up to 28 days following incubation in foetal calf serum (Lewis *et al.*, 1995).

Due to the lack of integrity of naked ODNs from 2 hours onwards, any investigative experiments into the mechanisms of cellular association were incubated with naked and entrapped ODNs for 2 hours.

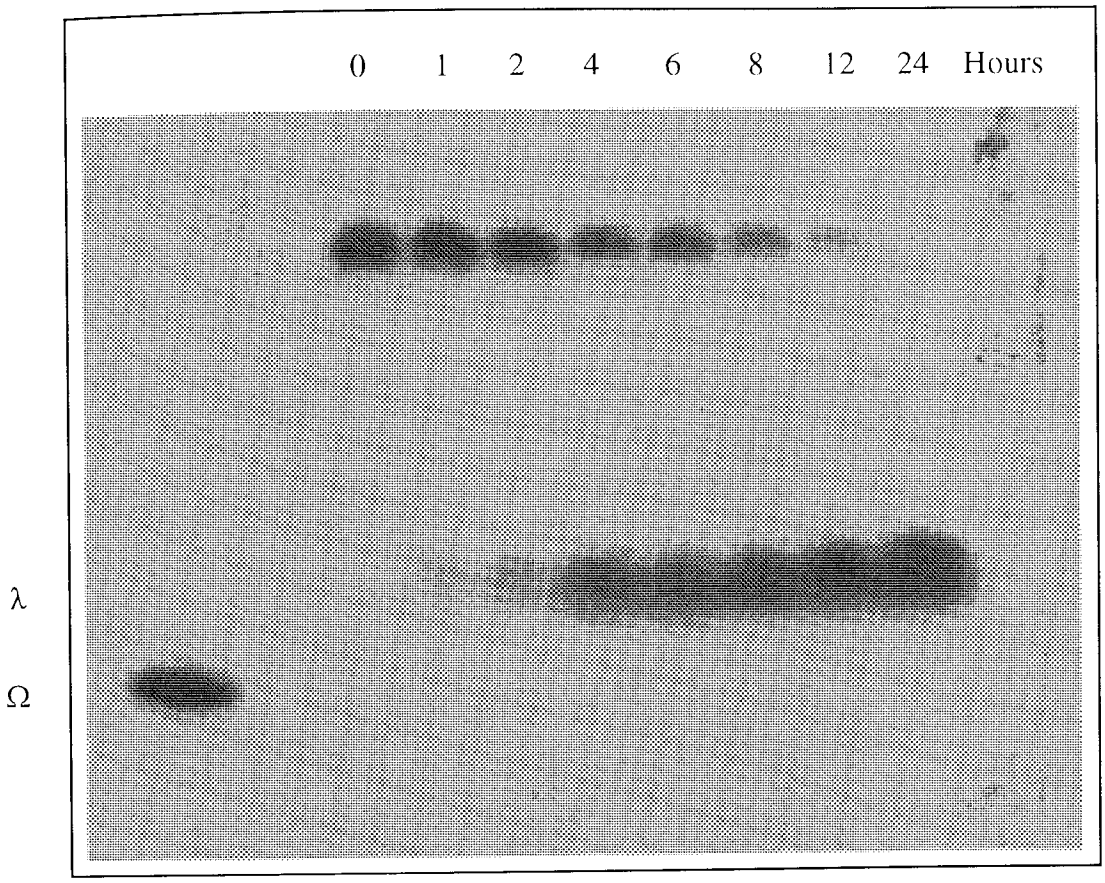

Figure 4.3. Stability of naked 3'-end α-^{32}P-radiolabelled PS-ODN poly (A) 15-mer incubated with C6 rat glioma apical solutions in serum-free media for 24 hours analysed by 20% denaturing PAGE. Zero time represents minimal exposure to degradation condition before sample removal, λ represents the degradation product and Ω represents the free label control.

4.3.2. Cellular association of naked and entrapped ODNs

The cellular association of both naked and entrapped ODNs with glial (C6) and neuronal (GT_1) cells was investigated by monitoring the association of radiolabelled ODNs that had either been delivered as the naked molecule or in a microsphere delivery system. Controls used in these investigations were free radiolabel to demonstrate that in the event of any cleavage of the radiolabel, the association of radioactivity with the cells was the radiolabelled ODN and not the radiolabel alone, and a fluid-phase marker, ^{14}C-mannitol.

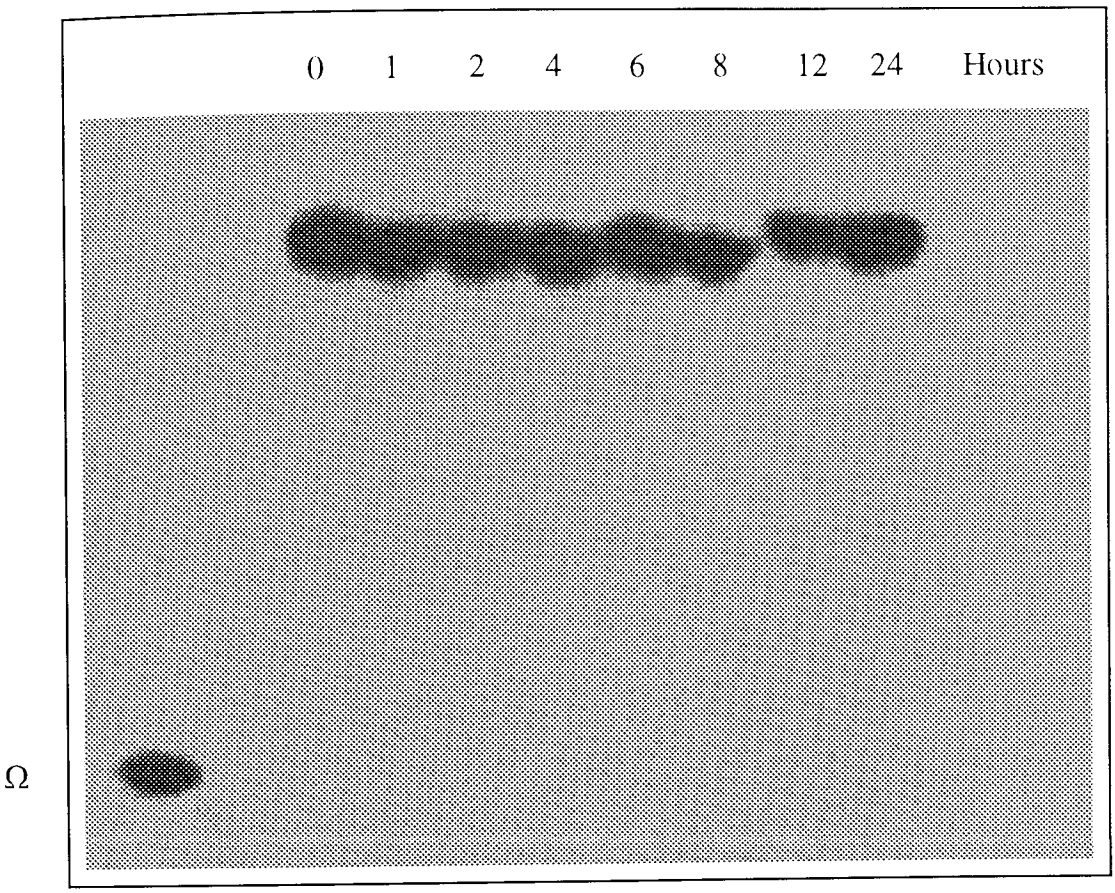

Figure 4.4. Stability of 3'-end α-^{32}P-radiolabelled PS-ODN poly (A) 15-mer entrapped within HMw PLGA 50:50 microspheres incubated with C6 rat glioma apical solutions in serum-free media for 24 hours analysed by 20% denaturing PAGE. Zero time represents minimal exposure to degradation condition before sample removal, Ω represents the free label control.

The amount of naked ODN associated with GT_1 neuronal cells was slightly greater than with C6 glioma cells (figure 4.5.), where over a period of 24 hours approximately 0.7% and 0.4% of ODN was associated with GT_1 neuronal and C6 glioma cells respectively. There is very little evidence available investigating differences between neuronal and glial cells in cell culture studies, although other cell types have been compared (discussed later) (Walker *et al.*, 1995; Beck *et al.*, 1996; Hawley & Gibson, 1996). It is worth noting that there was differential association of ODNs with neuronal and glial cells observed during *in vivo* studies (Sommer *et al.*, 1993; Zhang *et al.*, 1996), although these observations can not be directly compared with the observations made during these current investigations. A greater degree of ODN uptake was observed in neuronal cells than glial cells in an *in*

vivo study (Sommer *et al.*, 1993). Highest amounts of ODN were found in dopamine and cyclic AMP-regulated, phosphoprotein-positive nerve cell bodies and some dendrites, but not axon terminals, and a much lesser degree of uptake was observed in glial cells. Another *in vivo* study investigating the distribution of FITC-ODN following intracerebroventricular (i.c.v.) injection into mouse brain showed differential labelling of different cell types, further supporting that there may be a selective uptake of ODNs in certain cell types (Zhang *et al.*, 1996).

The cellular binding and uptake of PS-ODNs has been shown to be cell type-dependent (Beck *et al.*, 1996), association of ODNs with epithelial cell lines from the breast, liver, amnion, bladder and vulva were investigated, with greatest association observed for cells derived from the liver and amnion. Differences between endothelial and glioma cells were also seen where there was a greater amount of cellular association with the latter (Walker *et al.*, 1995). Variations in the cellular association may be explained by differences in expression levels of ODN binding proteins, such as those described by Yakubov *et al.* (1989) and Loke *et al.* (1989) (discussed earlier in section 1.1.3.). Another important consideration when comparing association of ODNs in different cell lines, especially between independent studies, is the cell cycle. If the cell lines being investigated are at different stages in the cell cycle, proteins may be at different stages of their turnover, which could affect the amount of ODN binding and internalisation into the cell. It was observed that rapid uptake of ODNs into mouse fibroblasts, with no detectable membrane binding was most active in the G_1/S phase of the cell cycle (Zamecnik *et al.*, 1994).

Earlier studies of ODN cellular uptake have shown a biphasic pattern of ODN interaction (Beck *et al.*, 1996; Hawley & Gibson, 1996). In Caco-2 cells a rapid initial binding phase complete within 15 minutes was followed by a slower phase (Beck *et al.*, 1996). This was consistent with the data from a parallel study using a pre-monocytic cell line, THP-1 (Hawley & Gibson, 1996). No distinct phases of cellular association were observed in this instance (see inset in figure 4.5.), however, the earliest time point recorded was at 30 minutes and the studies by Beck *et al.*

(1996) and Hawley and Gibson (1996) report this initial rapid binding phase occurring within 15 minutes. The cellular association in this study was more consistent with the slower secondary phase observed in the previous studies by Beck *et al.* (1996) and Hawley and Gibson (1996). The data here was more consistent with that reported by Li *et al.* (1997), where the uptake of FITC-ODN in bovine adrenal cortex cells was time dependent, with intracellular accumulation between 4 and 24 hours, then a slower increase with maximal uptake observed at 72 hours.

In the current study, there was an approximate five-fold increase in the association of ODN with neuronal and glial cells when delivered by biodegradable microspheres (figure 4.5.). Over a period of 24 hours approximately 15% and 8% of ODN was associated with GT_1 neuronal and C6 glioma cells respectively (figure 4.5.). In these experiments the amount of ODN associated with the cells was determined by measuring the amount of radioactivity associated with the cell fraction, thus it can be assumed that the radioactivity associated with the cell fraction could correspond to radiolabelled ODN-loaded microspheres or released radiolabelled ODN. A slightly greater degree of cellular association of naked ODN was observed with GT_1 cells than C6 cells (inset in figure 4.5.), thus a greater degree of association of released ODN may be expected with GT_1 neuronal cells compared to C6 glioma cells. For both cell types the increased association of ODN was five-fold, so the microspheres enhanced the delivery of ODN, but to the same degree in both cell lines, suggesting that the association of microspheres is not cell type dependent. The increased cellular association of ODN when entrapped within microspheres may be due to a greater initial binding of microspheres to cells or to differences in efflux between naked and entrapped ODN.

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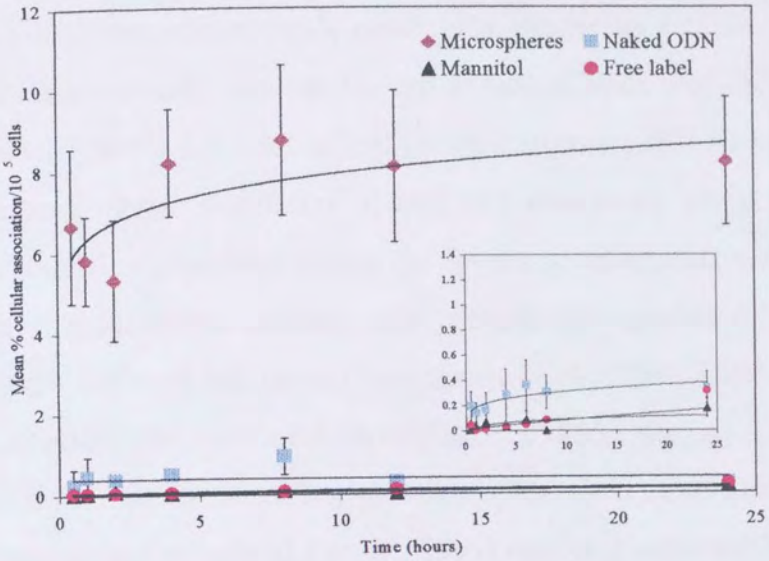

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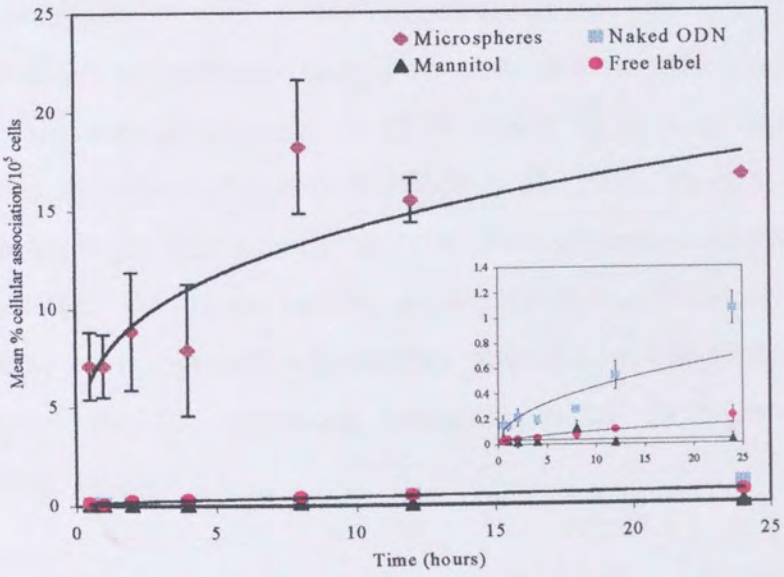


Figure 4.5. Cellular association of naked and entrapped ODNs over time with C6 (glial) (A) and GT_1 (neuronal) (B) cells. Incubated with poly (A) 15-mer-loaded microspheres (5mg/ml), naked poly (A) 15-mer PS-ODN (50pmol/well), ^{14}C-mannitol (50pmol/well) and α–^{32}P[ATP] (10000cpm/well). Inset is a close-up of ODN, mannitol and free radiolabel. Results expressed as the mean % cellular association/10^5 cells ± s.d. (n = 6).

The efflux of entrapped ODN from GT_1 cells was slower than efflux of naked ODN (figure 4.6.), a slower efflux would result in a greater accumulation of ODN in the cells, which may partially account for the enhanced uptake of ODN observed with microspheres (figure 4.5.). An efflux process suggests that there is a continuous dynamic balance between cellular uptake and exocytosis of ODNs, and altering ratios of influx/efflux processes affects the degree of cellular accumulation of ODNs. Altering rates of the efflux process may account for reported differences in ODN uptake amongst different cell types (Temsamani *et al.*, 1994; Zhao *et al.*, 1994; Li *et al.*, 1997) affecting the ratio of influx/efflux. Cellular studies have demonstrated differences in the rate of the efflux process, where the rate of efflux of ODNs was slower in adrenal cortex cells (Li *et al.*, 1997) than that reported in marrow B cells (Zhao *et al.*, 1994) and human 293 cells (Temsamani *et al.*, 1994). The accumulation of ODNs in the investigations with marrow B cells and human 293 cells (Temsamani *et al.*, 1994; Zhao *et al.*, 1994) peaked earlier than the reported ODN accumulation in adrenal cortex cells (Li *et al.*, 1997). However, it must be noted that direct comparisons cannot be made due to differences in cell type and species. Although differences in ODN uptake have been reported by authors investigating a number of cell lines (Beck *et al.*, 1996; Hawley & Gibson, 1996), where altering influx/efflux ratios may contribute towards these observed differences in ODN uptake. During the current investigations, the ODNs entrapped within the microspheres have a greater influx/efflux ratio than naked ODN (figure 4.6.) that may account for the increased accumulation of ODN when delivered by microspheres (figure 4.5.).

The pattern of efflux observed here had a biphasic pattern for both naked and entrapped ODN (figure 4.6.), although the initial phase for naked ODN was greater than for entrapped ODN. It has been suggested that the biphasic pattern of efflux is representative of exocytosis from two intracellular compartments (Tonkinson & Stein, 1994). The initial rapid efflux of ODN may represent release of ODN from shallow compartments situated at or near the surface of the cell (*e.g.* strongly bound to the cell surface or within primary endosomal vesicles). Whereas the slower efflux

represents ODN sequestered in deeper compartments within the cell (e.g. late endosomes or lysosomes). In this instance, a biphasic pattern of efflux was observed for both naked and entrapped ODN (figure 4.6.), but at a slower rate for entrapped ODN compared to naked ODN. The differences in rate of efflux may be attributed to a greater amount of entrapped ODN residing within deeper compartments compared to naked ODN.

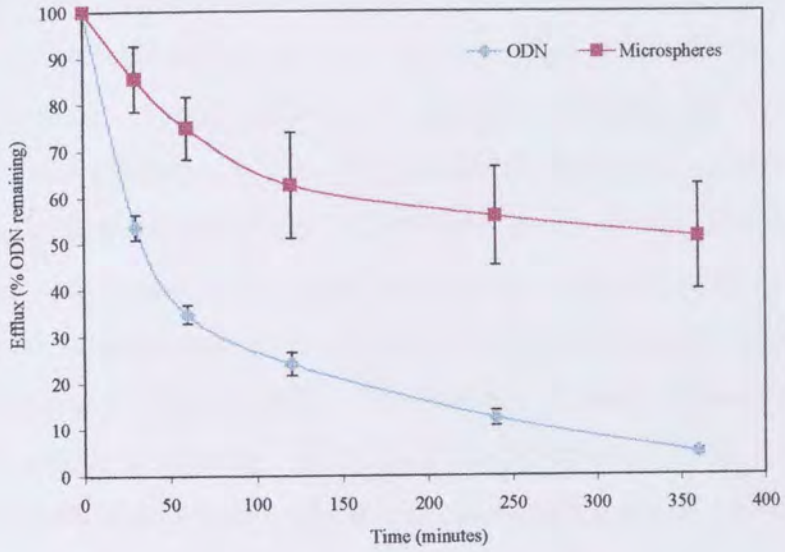


Figure 4.6. Efflux of naked and entrapped poly (A) 15-mer PS-ODN. α-^{32}P[ATP] poly (A) 15-mer was incubated with GT_1 cells; naked ODN (50pmol/well) and microspheres (5mg/ml) for 2 hours at 37°C. The amount of ODN exocytosed from the cell was expressed as efflux (mean % ODN remaining) ± s.d. (n = 6).

The hydrophobic nature of the microspheres may facilitate their interaction with cell membranes, further enhancing the association with cells. The increased association may be due to a combination of an increased influx of ODN due to a greater interaction of microspheres at the cellular surface (figure 4.5.) and also decreased efflux of ODN (figure 4.6.) caused by association of microspheres with intracellular membranes.

Throughout the cellular association experiments the monolayers were examined by light microscopy, for observation of any morphological changes that may have occurred. Towards the end of the cellular association of ODNs and microspheres with GT_1 neuronal cells (up to 24 hours), a morphological change was observed where the cells looked more 'neuronal-like', no morphological change was observed for C6 glioma cells. This alteration in morphology may have been due to a change in the media, where monolayers were seeded in 10% (v/v) foetal calf serum-containing media, whereas they were incubated with the ODNs and microspheres in serum-free media. Serum starvation has been a widely used method for inducing cell differentiation in cell culture systems, for example inducing the transition from a blast cell to a mature neuron in NG-108 neuroblastoma cells (Seidman *et al.*, 1996) and stimulating differentiation in GT_1 cells (Mores *et al.*, 1996). The binding of a 3'-phosphorothioate end-capped PO-ODN in a human large cell lung carcinoma line, COR-23, was investigated in cells grown in both serum-containing (10%) and serum-free media (Hawley & Gibson, 1996). The absence of serum during growth led to a considerable increase in binding. Geselowitz and Neckers (1995) blocked cellular association of ODN with albumin and it was concluded that this was the membrane-associated surface protein binding the ODN. Serum-free media was used in these investigations because PS-ODNs were reported to be unstable in the presence of foetal calf serum (Akhtar *et al.*, 1992). However the morphological change observed with GT_1 neuronal cells but not C6 glioma cells, caused direct comparisons between these two cell lines to become more complex.

4.3.3. Mechanisms of cellular association

Investigations into the possible mechanisms of cellular association of ODNs when delivered entrapped within microspheres or as the naked molecule were carried out in GT_1 neuronal cells. Time constraints limited these investigations to be carried out in one cell line. Further investigations comparing both glial and neuronal cell lines may have aided in the elucidation of possible mechanisms attributing to the slight differences in their ODN cellular association (figure 4.5.).

The cellular uptake mechanisms of three analogues (PO-, PS- and MP-) of ODNs targeted to HSV were investigated in African green monkey kidney cells. Temperature dependence was observed with all three analogues, although there was a degree of uptake with PS-ODNs at 4°C, which reflects association with cell-bound substances (Shoji *et al.*, 1996). PS-ODNs were used in the current study, and it has been suggested that the major interactions during the initial phase of cell association of PS-ODNs are non-energy requiring mechanisms (Beck *et al.*, 1996). Cellular association of PS-ODNs (but not PO-ODNs) with Caco-2 monolayers was temperature independent following 15 minutes incubation at varying temperatures. In a study comparing incubation of 3'-phosphorothioate end-capped PO-ODN with T15 cells at 4°C and 37°C over time, the initial ODN binding was shown to be rapid at both temperatures, suggesting a non-energy requiring process (Hawley & Gibson, 1996). However, at later time points increased cellular association was observed only at the higher temperature, suggesting an active uptake mechanism, in agreement with our findings (figure 4.8.A).

Pre-incubation of GT_1 cells with sodium azide/2-deoxyglucose (section 4.2.5.) significantly reduced cellular association of ODN delivered as both the naked molecule and entrapped within microspheres (figure 4.8.B), suggesting that an active process is involved in ODN cellular association. A comparison with a similar study carried out with Caco-2 cells demonstrated no inhibition of cellular association (Beck *et al.*, 1996). However, the investigations by Beck *et al.* (1996) were carried out following a 15 minute incubation with PS-ODN, as opposed to a 2 hour incubation in the current study. The dependence of temperature at later time points for PS-ODNs suggests that an active process comes into play later, following a rapid non-active uptake of PS-ODN, as demonstrated by Shoji *et al.* (1996). This is supported by our finding that sodium azide/2-deoxyglucose can inhibit association of PS-ODN and PS-ODN-loaded microspheres over 2 hours.

The use of PS-ODNs has demonstrated that there is a non-specific component in their cellular association (Beck *et al.*, 1996; Shoji *et al.*, 1996), which is believed to

be mediated through proteins (Guvakova *et al.*, 1995). In an effort to determine the sites of ODN interaction within the cell, experiments were carried out to separate the protein-bound fraction from the cytoplasmic and cell membrane fraction (section 4.2.6.). Incubation of GT_1 cells with naked PS-ODN was predominantly protein-bound, where 75.91% ± 6.58% of naked PS-ODN was present in the trypsinised fraction (figure 4.7.). However, incubation of PS-ODN-loaded microspheres with GT_1 cells showed a different distribution of ODN in the protein-bound, cytoplasmic and cell membrane fraction (figure 4.7.). 20.20% ± 10.04% of the ODN from microspheres was in the protein-bound fraction, and 43.24% ± 22.65% and 36.56% ± 21.98% were in the cytoplasmic and cell membrane fraction respectively. The different proportions of entrapped ODN in the protein-bound, cytoplasmic and cell membrane fractions compared with naked ODN suggests that the microspheres are associating with the cells by a different mechanism to the naked ODN.

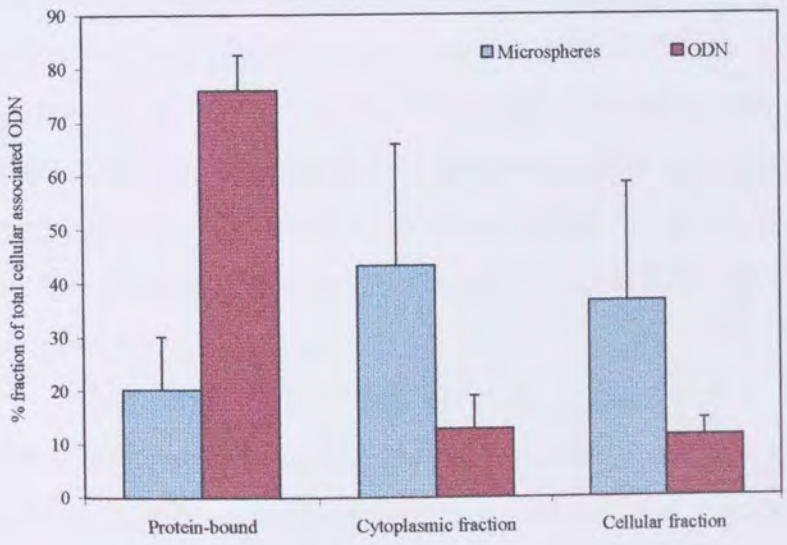


Figure 4.7. Determination of sites of cellular interaction of naked ODN (50pmol/well) and ODN entrapped in microspheres (5mg/ml) following a 2 hour incubation with GT_1 neuronal cells at 5 x 10^4 cells/ well. Results are expressed as % fraction of total cellular associated ODN ± s.d. (n = 6).

Competition studies can aid in the determination of the nature of the cellular association, self-competition suggests specificity in the cellular association, competition with a polyanion such as heparin suggests an ionic component and competition with ATP suggests the involvement of an active mechanism. Both PO- and PS-ODNs inhibited the cellular uptake of a radiolabelled PS-ODN (naked molecule) (figure 4.9.A) suggesting an overlap in the uptake mechanisms for PO- and PS-ODNs. However, competition studies carried out with PS-ODN-loaded microspheres demonstrated that PS-ODN delivered to cells entrapped within microspheres were inhibited in the presence of 'cold' PS-ODN but not 'cold' PO-ODN (figure 4.9.B). Inhibition of cellular association of PS-ODN-loaded microspheres was also inhibited by 'cold' microspheres to a similar degree as 'cold' PS-ODN. The differences in inhibition observed between naked PS-ODN and PS-ODN-loaded microspheres suggests that they are being taken up into cells by different mechanisms, inhibition with heparin, as observed with both naked PS-ODN and PS-ODN-loaded microspheres suggests that there is a non-specific component in their uptake mechanism. Heparin is a polyanion, and inhibition with this molecule suggests that there is a degree of ionic interaction involved in the uptake of ODN when delivered as the naked molecule and entrapped within microspheres. Inhibition with ATP suggests that the uptake mechanism is part of an active process, as was suggested by the temperature dependence and inhibition by metabolic inhibitors (figure 4.8.A and B).

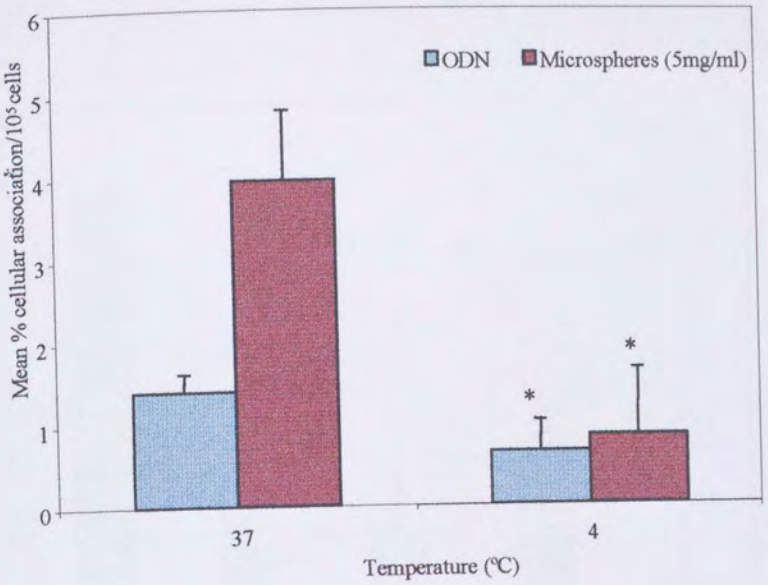
When ODN entrapped within microspheres is incubated with cells, some ODN could be taken up as the naked molecule once it has been released from the microspheres, or ODN-loaded microspheres may be internalised. When ODN-loaded microspheres enter an aqueous environment a burst effect occurs (as described earlier in chapter 3, see figure 3.4.), thus releasing naked ODN into the cell culture system. It is likely that there are both mechanisms occurring following incubation with cells, that is association of ODN-loaded microspheres and of naked ODN. The fact that PO-ODN causes no significant inhibition of cellular association of PS-ODN-loaded microspheres (figure 4.9.B), but does for naked PS-ODN suggests that cellular

uptake of PS-ODNs when delivered by microspheres is occurring mainly by uptake of microspheres containing ODN, as opposed to uptake of released naked ODN.

The distribution of FITC-ODN delivered to C6 glioma cells over 24 hours as a naked or entrapped molecule was determined by fluorescence microscopy (section 4.2.7.). Punctate distribution was observed in cells incubated with naked ODN, with no observed nuclear distribution (figure 4.10.). This punctate distribution is indicative of vesicular localisation following endocytic entry into these cells, and is similar to that observed for fluid-phase markers such as fluorescently-labelled dextrans (Shoji *et al.*, 1991) and to many other types of ODN chemistries (Beltinger *et al.*, 1995; Iversen *et al.*, 1992; Spiller & Tidd, 1992). The uptake of ^{35}S-radiolabelled PS-ODN occurred *via* receptor-mediated endocytosis at concentrations of <1μM, whereas fluid-phase endocytosis prevailed at higher concentrations (Beltinger *et al.*, 1995), suggesting that the type of endocytic mechanism is dependent upon concentration. In these experiments a concentration of 1μM of FITC-ODN was used, the use of a fluorescent-labelled fluid-phase marker such as dextran, could have helped define the particular type of endocytosis occurring in this instance, however time constraints limited the reagents investigated in these experiments.

The fluorescent distribution of entrapped ODNs was heterogeneous across the field of view, where some cells possessed the distinct punctate distribution similar to that observed following incubation with naked ODNs. However, some of the cells (figure 4.11.) showed a much more diffuse distribution of fluorescence, but the ODNs still appeared to be unable to access the nuclear compartment. The heterogeneous fluorescent distribution that was observed following incubation with microspheres (figure 4.11.) demonstrates the complexity of the mechanisms that may be involved with uptake of ODNs delivered by polymeric microspheres. Over the 24 hour incubation with cells there may be a proportion of ODN molecules that are not associated with the microspheres (*i.e.* they are released as naked molecules), some may be loosely associated at the surface of the microspheres, and some may be entrapped within the microsphere matrix.

A.



B.

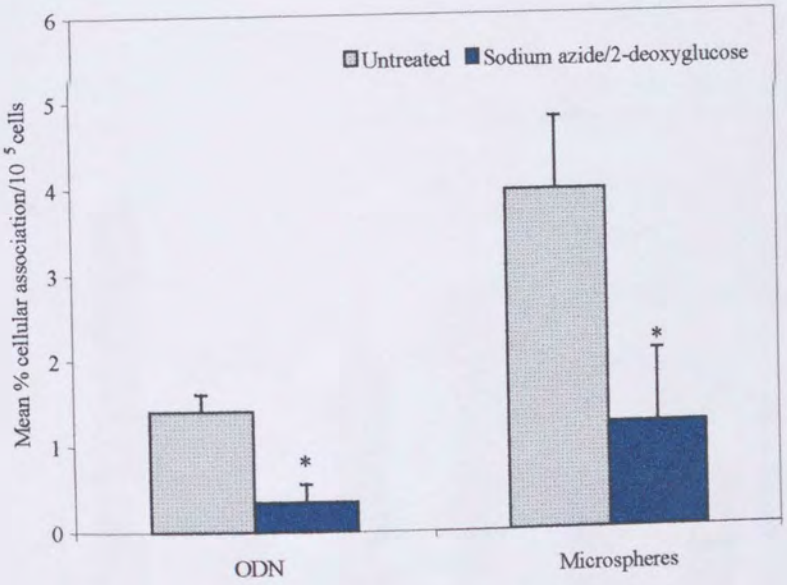
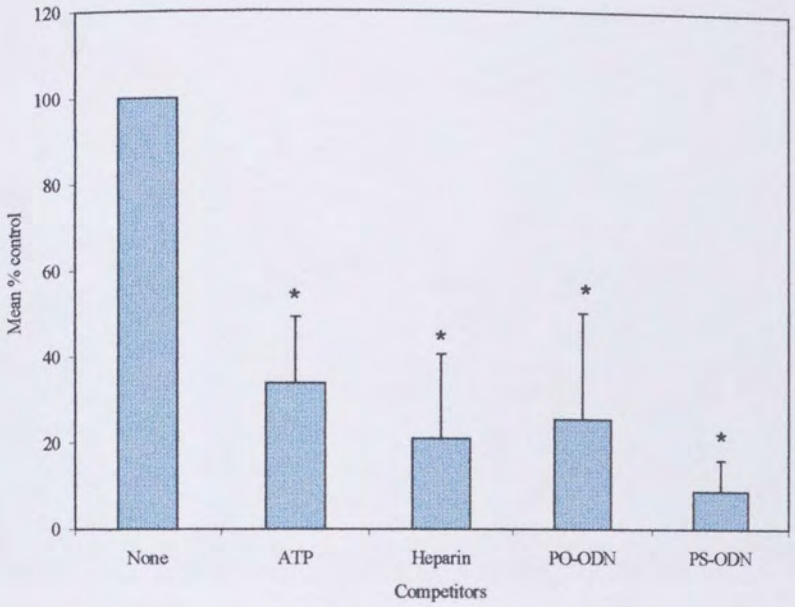


Figure 4.8. Effect of temperature, 4°C and 37°C (A) and metabolic inhibitors, NaN_3 (10mM) + 2-DG (20mM) 15 minute pre-treatment (B) on cellular association with GT_1 cells. Radiolabelled poly (A) 15-mer PS-ODN (50pmol/well) and radiolabelled ODN-loaded microspheres (5mg/ml) incubated for 2 hours. n = 6 ± s.d. where * denotes a significant reduction ($p \leq 0.05$) from cells incubated at 37°C for (A) and untreated cells for (B).

A.



B.

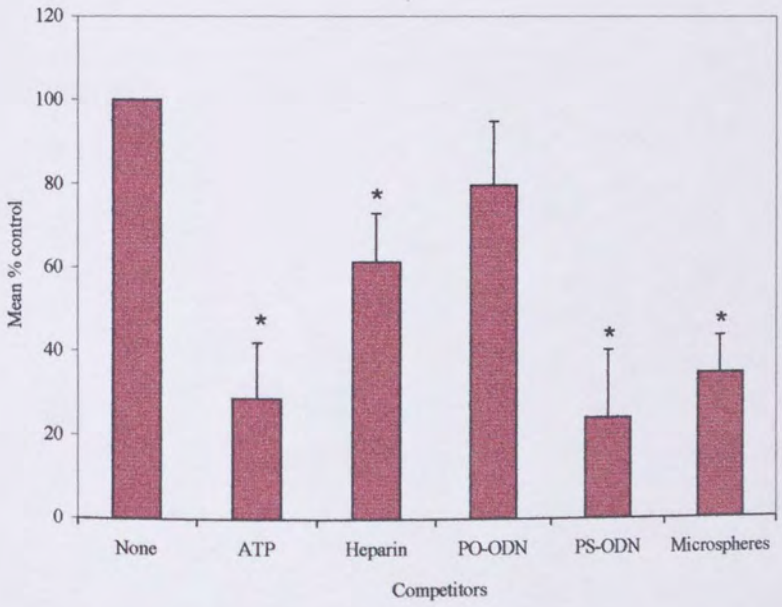
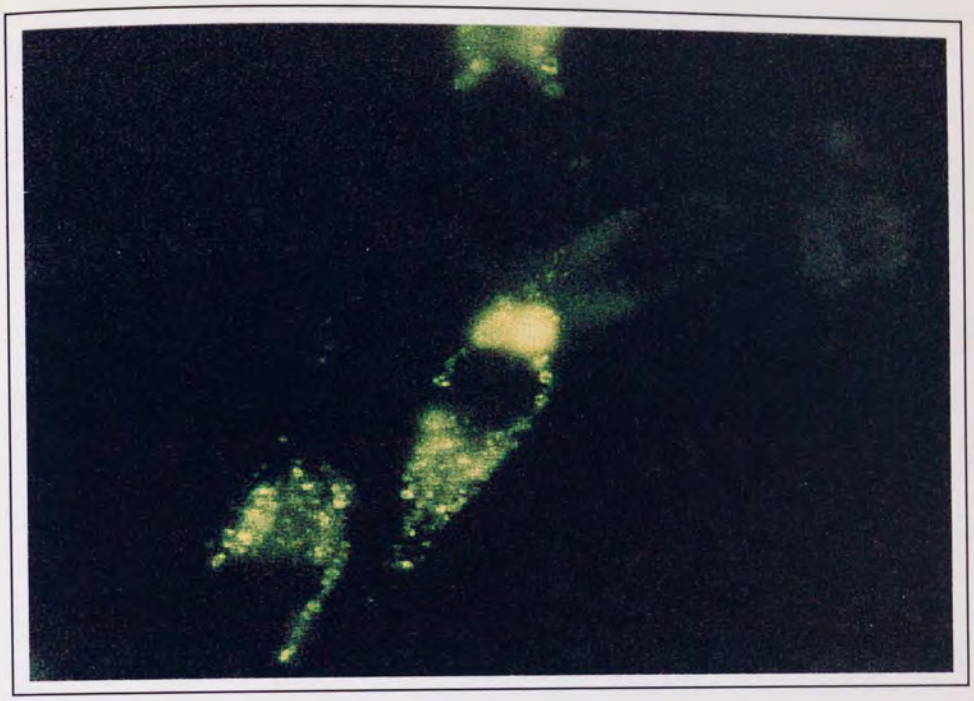


Figure 4.9. Effect of competitors and self-competitors on GT_1 cellular association with naked ODN (A) and ODN-loaded microspheres (B). Cells were pre-incubated with potential competitors for 15 minutes, then incubated with radiolabelled naked ODN (50pmol/well) or radiolabelled ODN-loaded microspheres (5mg/ml). $n = 4 \pm$ s.d. where * denotes a significant reduction ($p \leq 0.05$) from the control.

A.



B.

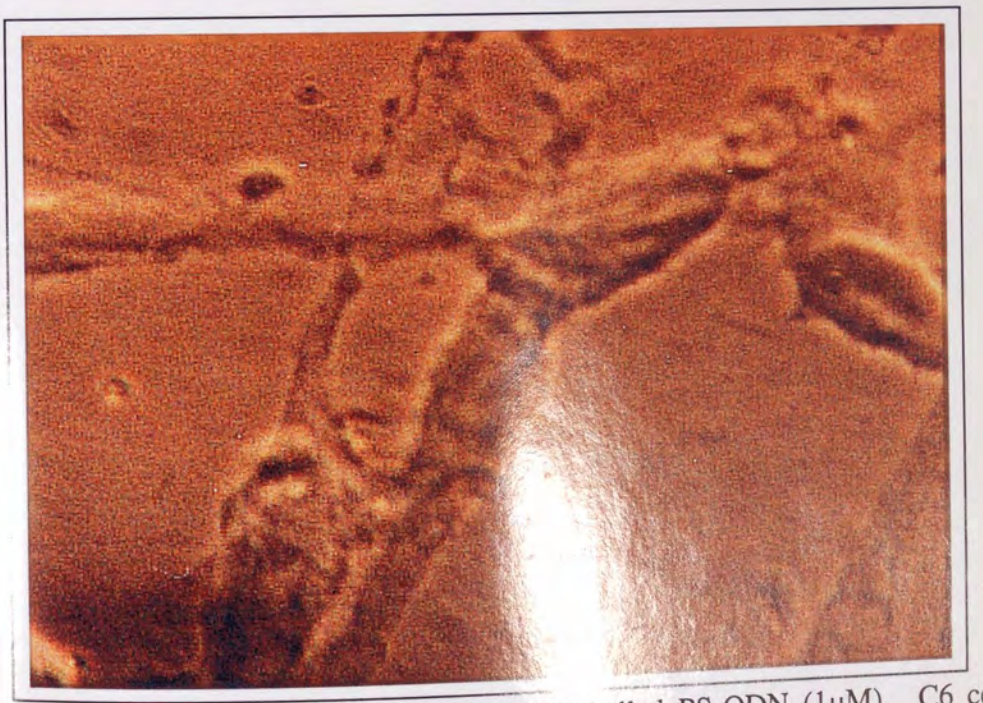
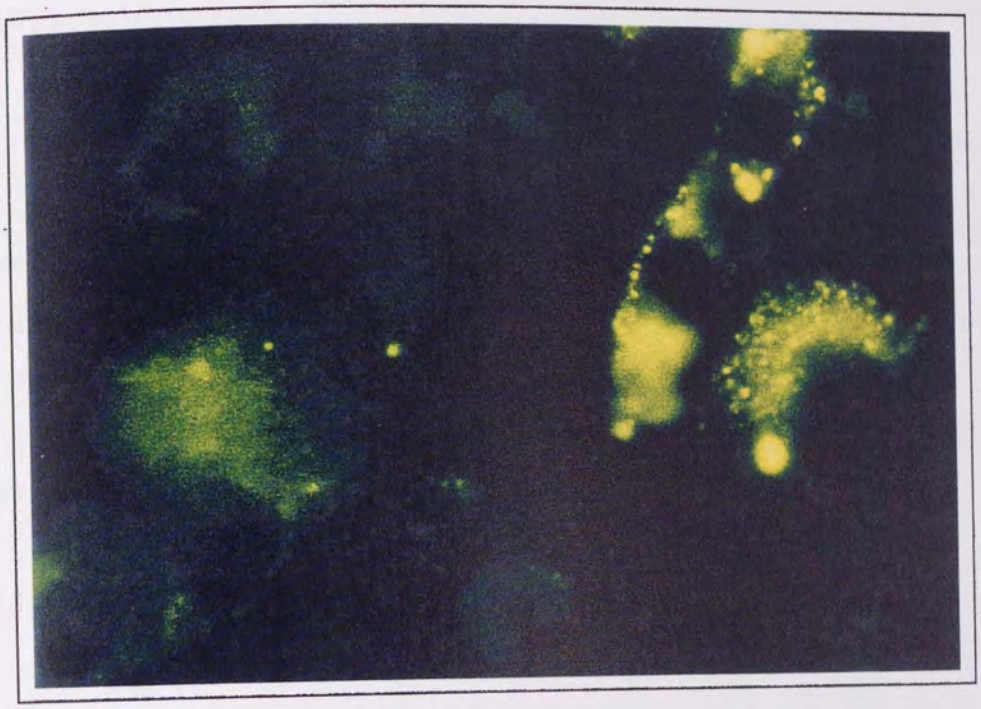


Figure 4.10. Subcellular distribution of FITC-labelled PS-ODN (1μM). C6 cells were incubated for 24 hours at 37°C, fluorescent image at x 200 magnification (A) and phase contrast image at x 200 magnification (B).

A.



B.

Figure 4.11. Subcellular distribution of FITC-labelled PS-ODN entrapped within HMw PLGA 50:50 microspheres (5mg/ml). C6 cells were incubated for 24 hours at 37°C, fluorescent image at x 200 magnification (A) and phase contrast image at x 200 magnification (B).

4.4. CONCLUSIONS

The formulation of biodegradable microspheres for delivery of AS-ODNs was described and characterised in chapter three. The formulation developed was intended for application within the CNS by direct injection into site-specific regions of the brain. Prior to the *in vivo* use of this microsphere formulation, cell culture experiments were carried out in an attempt to determine any differences in cellular association between ODNs delivered as either the naked molecule or within microspheres i.e. do the microspheres facilitate the cellular delivery of AS-ODNs?

The optimisation of the cellular association experiments revealed that the entrapment of PS-ODNs within polymeric microspheres protected them from degradation when incubated with both C6 and GT_1 cells. Degradation of the naked molecule began within 2 hours of incubation with these cell lines (figure 4.3.), whereas the entrapped ODN remained stable over a period of 24 hours (figure 4.4.), throughout the duration of the experiment. The integrity of entrapped ODN throughout the duration of the experiment ensured that any measurements made with cellular association of ODN-loaded microspheres were of intact ODN and not degraded ODN fragments. Any investigations carried out into the mechanisms of naked and entrapped ODN cellular association were carried out over a 2 hour incubation period due to the lack of integrity of naked ODN after 2 hours (figure 4.3.). No further stability data was determined during these investigations, however these preliminary investigations demonstrated the potential of microspheres within a biological environment to protect ODNs from enzymatic degradation.

Investigations into the cellular association of ODNs delivered to glial and neuronal cells as either the naked molecule or entrapped within microspheres demonstrated that microspheres enhanced the delivery of ODNs five-fold (figure 4.5.). These findings highlighted the potential of biodegradable microspheres as a delivery system for AS-ODNs to CNS targets. A general observation during these investigations was that there was a slight difference in the degree of cellular association of naked ODN

between glial and neuronal cells, with a greater cellular association found with the latter. This finding was in agreement with previous cellular association studies, where the cellular association of ODNs has been shown to be cell type dependent (Beck *et al.*, 1996; Hawley & Gibson, 1996). The preferential association of ODN with particular cell types may pose problems when applying ODNs to a particular target cell type that has demonstrated poor uptake of ODNs. However, microspheres have the potential of being formulated with derivatives on their surface for targeting specific cell types, for example 5-FU loaded chitosan-gel nanospheres coated with polysaccharides caused cell specific cytotoxicity (Ohya *et al.*, 1994). The formulation of derivatives on the surface of microspheres may be applied here to target predominantly glial or neuronal cells, thus further enhancing the uptake of ODNs.

The limited data available describing the cellular association of AS-ODN-loaded microspheres (Akhtar & Lewis, 1997; Cleek *et al.*, 1997) warranted the need for investigation into the possible mechanisms that may be involved in their cellular association. The efflux of entrapped ODN had a slower profile than naked ODN, and it has been suggested that the greater the influx/efflux ratio the greater the accumulation of ODN (figure 4.6.). Thus, the slower efflux of entrapped ODNs may have contributed towards the greater degree of association observed with entrapped ODN compared to naked ODNs. The greater influx/efflux ratio for entrapped ODNs would ultimately lead to a greater cellular accumulation of ODNs, increasing bioavailability, thus increasing their pharmacological potential.

The cellular association of PS-ODNs was shown to be dependent upon temperature and inhibited by metabolic inhibitors for both naked and entrapped ODNs, indicative of an active process (figure 4.8.A and B). The determination of sites within the cell where naked and entrapped ODNs may be associated revealed a differential localisation of naked and entrapped ODNs (figure 4.7). Naked ODNs were predominantly associated with the protein-bound fraction, whereas entrapped ODNs demonstrated no preference for a particular fraction and were found in the protein-

bound, cytoplasmic and cell membrane fraction (figure 4.7.). Overall, there was a greater accumulation of entrapped ODNs within intracellular compartments compared to naked ODNs, further increasing bioavailability for interaction with their intracellular targets. Differential patterns of fluorescent distribution were observed between naked and entrapped ODNs, where a punctate distribution indicative of an endocytic mechanism was observed for naked ODNs (figure 4.10.). There was a heterogeneous pattern of distribution observed for entrapped ODNs (figure 4.11.), being both punctate and diffuse. This suggested that there may be more than one mechanism involved in the uptake of ODNs, when delivered by polymeric microspheres. The involvement of more than one type of uptake mechanism for ODNs delivered by microspheres was not surprising, as there would be a combination of released ODN, ODN loosely associated at the microsphere surface and ODN entrapped within the microsphere matrix. This may result in uptake of naked ODN and possible internalisation of microspheres.

The enhanced cellular association (five-fold) of ODNs when delivered within biodegradable microspheres offers an excellent delivery system for CNS delivery of AS-ODNs. These data suggest that this delivery system may aid in sustained delivery of AS-ODNs within the brain, allowing for adequate reduction in gene expression (Wahlestedt *et al.*, 1993), enhance stability and facilitate in delivery to intracellular targets.

CHAPTER FIVE

IN VIVO DISTRIBUTION AND EFFICACY OF NAKED ODN AND ODN-LOADED MICROSPHERES

5.1. INTRODUCTION

5.1.1. Delivery of AS-ODNs into the CNS

The use of AS-ODNs within the CNS often requires chronic treatment to allow for adequate reduction in gene expression (Wahlestedt, 1994). Multiple injections of AS-ODNs (Karle & Nielsen, 1995; Rajakumar *et al.*, 1997) or osmotic mini-pumps (Nissbrandt *et al.*, 1995; Tremblay *et al.*, 1998) are currently routinely employed to provide chronic treatment. However, the use of minipumps requires that the tip of the infusion cannula is located directly within the targeted brain region over the duration of the experiment (3-7 days), which can cause infiltration of astrocytes/macrophages manifested as a local tissue irritation (Neumann, 1997). Repeated administration of AS-ODNs is time-consuming and fairly impractical. Despite these disadvantages of osmotic mini-pumps and repeated injections for AS-ODN CNS delivery, such approaches have been used successfully, demonstrating efficacy of antisense reagents (Weiss *et al.*, 1993; Rajakumar *et al.*, 1997 for multiple injections and Zhang & Creese, 1993; Ekman *et al.*, 1998 for osmotic mini-pumps). If the use of AS-ODNs within the CNS is to become a reality for therapeutic purposes, there is a need to improve the CNS delivery of AS-ODNs, one possible delivery system being the use of polymeric drug-delivery devices.

Polymeric drug-delivery devices have been used within the CNS with a degree of success for delivery of otherwise impervious neuroactive agents (see table 1.1.). Indeed, the first polymeric delivery of a chemotherapeutic agent (BCNU) for brain tumours, Gliadel® wafers, was released onto the therapeutic market in 1996. A number of researchers have been investigating the potential of polymeric microspheres as a delivery system for AS-ODNs (Cortesi *et al.*, 1994; Akhtar & Lewis, 1997; Cleek *et al.*, 1997; Yamakawa *et al.*, 1997). However, their application within the CNS has not yet been investigated.

The use of polymeric delivery systems not only provides a means of chronic treatment with sustained-release, but they have the potential of enhancing stability of AS-ODNs whilst also overcoming the problem of blood-brain barrier penetration. Pharmacokinetic studies have demonstrated that the brain is relatively impervious to AS-ODNs (Agrawal *et al.*, 1991; Crooke *et al.*, 1996b) and implantation of polymeric delivery devices into the brain overcomes this barrier. Despite there being evidence that PS-ODNs are more stable when administered centrally than systemically, degradation products have been observed as early as 6 hours (Liu *et al.*, 1994). Enhanced stability following central administration is believed to be because cerebrospinal fluid and brain tissue contain low levels of nucleases (Brysch & Schlingensiepen, 1994) compared to plasma and peripheral tissues, where endogenous nuclease activity is very high (Akhtar *et al.*, 1992). The use of biodegradable microspheres *in vitro* have demonstrated enhanced stability of PS-ODNs (see section 3.3.1. and 4.3.1.4.) and it was reported that PO-ODNs entrapped within PLA films were stable for up to 28 days in foetal calf serum (Lewis *et al.*, 1995). The use of PS-ODNs in the CNS entrapped within biodegradable microspheres offers the potential of greatly improving their stability within the CNS when targeting proteins of long half-lives.

Another advantage of the use of microsphere formulations is demonstrated in earlier studies on the targeting of drugs into the CNS using polymeric devices. These devices were macroscopic requiring open surgery for implantation (Becker *et al.*,

1990; Reinhard *et al.*, 1991). Microsphere formulations can be easily implanted by stereotaxy into deep regions of the brain (Benoit *et al.*, 1994; McRae & Dahlstrom, 1994; Blanco-Príeto *et al.*, 1996), avoiding the inconvenience of insertion of large implants by open surgery and can be repeated if necessary. The biocompatibility of polymers in the brain, particularly poly (lactide-co-glycolide) (PLGA) has already been demonstrated with both PLGA polymer (Kou *et al.*, 1997) and PLGA microspheres (Menai *et al.*, 1993).

5.1.2. Behavioural tests for efficacy of D_2 dopamine receptor antisense reagents

The molecular target in the current investigations is the D_2 dopamine receptor (for background research on dopamine, see section 1.4.). The cloning of the D_2 dopamine receptor (Bunzow *et al.*, 1988) has enabled research into the distribution of D_2 mRNA throughout the brain (Bunzow *et al.*, 1988; Meador-Woodruff *et al.*, 1989; Mengod *et al.*, 1989). More recently, research has been carried out into the use of AS-ODNs for assessing the function of dopaminergic receptor subtypes, including the D_2 dopamine receptor (Weiss *et al.*, 1993; Zhang & Creese, 1993; Zhou *et al.*, 1994; Silvia *et al.*, 1997) (summarised in table 1.2.). These groups have used a number of animal models for assessing the function of dopamine receptors using AS-ODNs, such as locomotor activity (Zhang & Creese, 1993; Silvia *et al.*, 1994), quinpirole-induced-stereotypy (Davidkova *et al.*, 1998), induced-grooming (Zhang & Creese, 1993; Zhang *et al.*, 1994) and induction of catalepsy (Zhang & Creese; 1993; Davidkova *et al.*, 1998).

5.1.3. Specificity of antisense reagents

When using AS-ODNs centrally or systemically it is important to use the appropriate controls in order to determine sequence specificity of the antisense reagent. Such controls include sense ODN, which is identical to the target sequence, scrambled ODN, which has the same base composition as the antisense but in a scrambled/random order or mismatch ODN, which is similar to the antisense but

contains 3-5 mismatches while maintaining the same composition. It is sometimes recommended to use vehicle controls since some ODNs can exert non-specific effects, in particular modified ODNs such as PS-ODNs at high concentrations or following repeated or continuous administration (Brown *et al.*, 1994; Guvakova *et al.*, 1995). ODNs can exert their effects *via* non-antisense mechanisms, including aptameric action (Ellington & Szostak, 1992) and the formation of G-quartet higher structures (Burgess *et al.*, 1995). Non-specific effects have also been observed on the hosts defence system where CpG motifs are potent B-cell mitogens (Krieg *et al.*, 1995) and PS-ODNs are known to affect the clotting and complement systems (Galbraith *et al.*, 1994) (see section 1.1.1.6.).

It is important during the design of these antisense experiments to assess the specificity of any observed antisense effects by either assessing the levels of targeted protein or the levels of mRNA. Both of these parameters can aid in the elucidation of possible mechanisms that may have been involved in the efficacy of the antisense reagents tested. Discrepancies between protein levels and mRNA levels have been observed on a number of occasions, for example PO-ODN antisense to *N*-methyl-D-aspartate (NMDA) receptor, reduced receptor-protein binding in the cerebral cortex but not the levels of mRNA following i.c.v. administration (Wahlestedt *et al.*, 1993), whereas PO-ODN targeted to the D_2 dopamine receptor reduced both protein and mRNA levels (Zhou *et al.*, 1994) and an increase in mRNA levels of V1 vasopressin receptor, but a reduction in protein levels was observed following infusion of end-capped PS-ODNs into the septum (Landgraf *et al.*, 1995).

5.1.4. Aims of chapter

There were two main aims of this chapter, as outlined below:

1. To investigate the distribution of AS-ODNs in the brain following an injection into the caudate putamen (CPu) and lateral ventricles (LV) delivered as the naked molecule or entrapped within PLGA 50:50 biodegradable

microspheres. During these distribution studies the animals were observed for any signs of behavioural toxicity. The distribution of fluoresceinated ODN reagents was monitored by fluorescence microscopy.

2. To determine the efficacy of the antisense reagents targeted to the D_2 dopamine receptor. Are the antisense reagents efficacious when administered by repeated injection of the naked molecule and does entrapment of the antisense reagents in biodegradable microspheres provide efficacy? During antisense treatment, animals were observed for behavioural toxicity. Efficacy was monitored following antisense treatment by investigating spontaneous and cocaine-induced locomotor activity. Specificity of antisense reagents was tested by the use of appropriate controls and assessing levels of D_2 dopamine receptor-protein by immunohistochemistry.

5.2. MATERIALS AND METHODS

5.2.1. Subjects and housing conditions

All subjects were male Lister Hooded (LH) rats (Harlan, U.K.), weighing between 250-350g and following their arrival, were housed 5 rats per cage and were maintained on a 12 hour light/dark cycle (with lights on between 07:00 and 19:00 hours GMT). Food and water were available *ad libitum.*

5.2.2. Surgical implantation of guide cannulae

Two separate sets of studies were carried out, ODN distribution and antisense efficacy studies. Rats for distribution studies were injected unilaterally into the caudate putamen (CPu) (+0.7mm anterior/posterior (AP), 3mm lateral (L), 3.5mm dorsal/ventral (DV)) or the lateral ventricles (LV) (-0.8mm AP, 1.5mm L, 3.3mm DV) (Paxinos & Watson, 1997). Rats used in antisense efficacy studies were injected bilaterally into either the caudate putamen or lateral ventricles. Bilateral

guide cannulae were held 3mm apart for lateral ventricle implantation or 6mm apart for caudate putamen implantation in a steel clamp. Guide cannulae used for implantation were manufactured 'in house' from 23gauge (23g) syringe needles (manufacture of guide cannulae is illustrated in appendix three).

Male Lister Hooded rats (250-350g) were anaesthetised by an intraperitoneal (i.p.) injection of pentobarbitone (Sagatal™, Vet Drug, Gwent, U.K.) at 90mg/kg. Some rats were also given 3mg/kg of atropine methyl nitrate (Sigma, Dorset, U.K.) to curb bronchial secretions. Completely anaesthetised rats were placed into a Kopf stereotaxic frame (David Kopf instruments, Harvard apparatus Ltd., Kent, U.K.), the incisor bar set at -3 to -3.5mm, and the ear bars equidistant, ensuring the flat skull position (Paxinos & Watson, 1997). A midline incision was made down the head and the skin was held back with four vascular clamps (Interfocus Ltd., Suffolk, U.K.). The periosteum was removed with a chisel, to expose the skull highlighting bregma. A 1% (w/v) solution of Virkon (Vet Drug, Gwent, U.K.) was used to wipe and sterilise the skull and surrounding tissue. Baseline co-ordinates were taken using the guide cannula, with bregma as the reference point and co-ordinates for each site of injection were determined from bregma (positioning of bregma illustrated in figure 5.1.). The correct positions were marked on the skull and small burr holes were made in the skull using a hand held dentist drill model 850 (Dremel, Racine, WI, U.S.A.) and 4 further burr holes were drilled for screws. Guide cannulae (23g, 10mm in length) were implanted into the brain through the burr holes and held in place by dental cement (ESA Analytical Ltd., Cambridgeshire, U.K.) and four 10BA 3/32 Cheesehead stainless steel screws (Kemps Stainless Fasteners Ltd., Surrey, U.K.). Cement was allowed to dry and the incision was sutured with 4/0 Merslik black silk braided non-absorbable suture (Ethicon Ltd., Edinburgh, U.K.) with two stitches either side of the guide cannula. Anaesthetised animals were placed in a thermacage (Datesand Ltd., Cheshire, U.K.) until awaking from anaesthesia. Rats were returned to individually housed cages and allowed at least 7 days to recover with food and water *ad libitum.*

5.2.3. Direct injection procedure

Injection cannulae were manufactured 'in house' from 23g and 31g metal tubing (Coopers Needleworks, Birmingham, U.K.). Injection cannulae for CPu injections were made to protrude 2mm below the tip of the guide cannula, and for LV injections to protrude 1mm below the tip of the guide cannula.

The injection cannula was attached to fine bore polythene tubing (0.4mm inner diameter, 0.8mm outer diameter) (Fischer, Leicestershire, U.K.) which was connected to one or two 10µl Hamilton microinjection syringes for unilateral and bilateral injections respectively (Fischer Instruments, Sussex, U.K.). These were mounted on a syringe pump (Instech, model 2000, Pre-Search, Hertfordshire, U.K.). The polythene tubing was cut to a length such that the rat was able to move freely, whilst being lightly restrained.

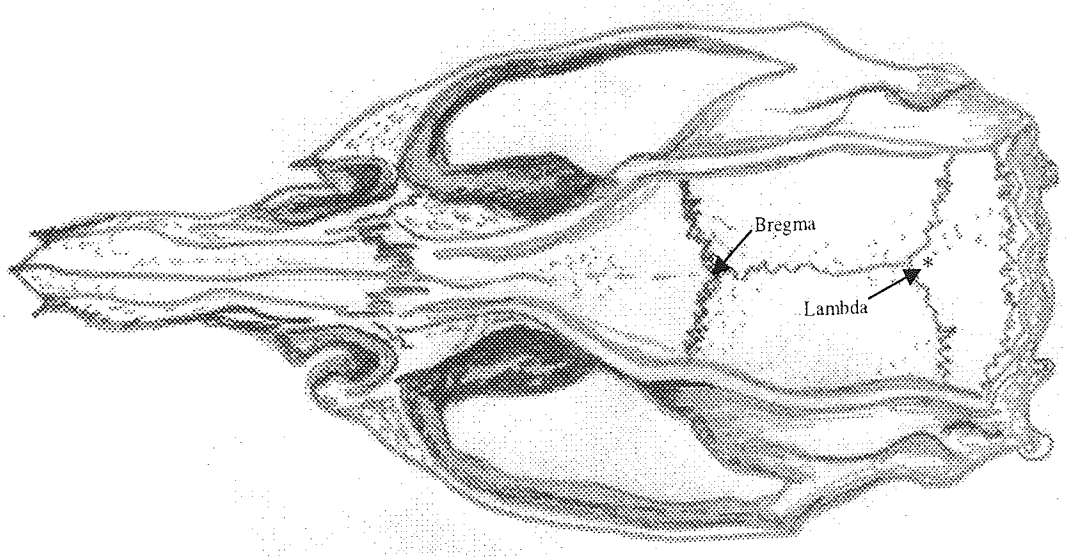


Figure 5.1. Dorsal view illustration of the rat brain with relation to bregma and lambda. Redrawn and modified by J.Heymans from diagrams in Paxinos & Watson (1997).

All reagents injected directly into the brain were made up in double-distilled water (ddH_2O) (filtered using a 0.2μm millipore filter) and were administered at a rate of 1μl/minute, unless otherwise stated. Injections into the caudate putamen were administered as a 1μl bolus injection. Injections into the lateral ventricles were administered as a 5μl bolus (rate of 2.5μl/minute) with a holding time of 2-3 minutes for distribution studies and 1μl per side (1μl/minute) for efficacy studies. Haloperidol was initially dissolved in a few drops of 10% (v/v) acetic acid, and ddH_2O (filtered using a 0.2μm millipore filter) was added. The pH was increased to approximately 4.0 with 1M NaOH, this was then made up to a final concentration of 20mg/ml for direct injection into the brain of 20μg/μl (a 1μl bolus).

5.2.4. Distribution studies

Fifty-four male LH rats (350-450g) with previously implanted unilateral guide cannula (section 5.2.2.) were injected with either naked FITC-PS-poly (A) 15-mer or FITC-PS-poly (A) 15-mer-loaded microspheres. The distribution of fluorescence over time was observed by fluorescence microscopy, for comparison of distribution between naked and entrapped ODNs.

5.2.4.1. Injection of fluorescent reagents

All FITC-PS-ODN reagents were prepared on an automated DNA synthesiser (section 2.1.3.) and FITC-PS-ODNs were loaded into HMw (45kDa) PLGA 50:50 microspheres (section 2.5.1.). For direct injection of all FITC-PS-ODN reagents (naked molecule or entrapped within microspheres), animals were observed throughout the duration of the experimental period. Animals were sacrificed at 2, 8, 12, 24, 36 and 48 hours and 5 days for CPu animals. For rats with cannula implanted into the LV, distribution was monitored at 2, 8 and 24 hours.

The dose of naked FITC-PS-ODN was 0.7nmol/μl (1μl bolus) and 0.5mg/μl (2μl bolus, approximately 0.7nmol final dose of FITC-PS-ODN) for FITC-PS-ODN-

loaded microspheres. The total loading of ODN in the microsphere bolus was of similar dose to the final bolus dose of naked ODN. The loading of fluoresceinated reagents into microspheres was determined by production of batches with radiolabelled ODN and determining entrapment by measuring the amount of radioactivity in the microsphere batches by liquid scintillation counting (section 2.4.). The loading of fluoresceinated ODN into microspheres was estimated from loading of radiolabelled batches of microspheres.

5.2.4.2. Cryostat sectioning

Freshly removed brains were cut into blocks using a rodent brain matrix for coronal sections in rats 200-400g (ASI instruments, MI, U.S.A.). For CPu distribution studies, 8mm blocks were cut around the site of cannula implantation and for LV distribution studies, 18mm blocks were cut around the site of cannula implantation. Brain blocks were snap frozen in pre-cooled *iso*-pentane and were mounted on a chuck using OCT mountant (Sakura Tissue-Tek, Raymond A Lamb Ltd., East Sussex, U.K.). Coronal 10μm sections were prepared on a cryostat (Bright Instruments U.K. Ltd., Cambridgeshire, U.K.), and mounted onto pre-coated Snowcoat Xtra™ microscope slides (Surgipath, IL, U.S.A.).

5.2.4.3. Fluorescence microscopy

To establish the distribution of FITC-PS-ODNs in the rat brain following direct administration, cryostat sections were examined by fluorescence microscopy on a UV microscope (Leica DM IRB U.K. Ltd., Buckinghamshire, U.K.). Images were taken using the Optimas® 6 program for Windows® 95.

5.2.5. Efficacy studies

Forty male LH rats (350-450g) with previously implanted bilateral guide cannulae (see section 5.2.2.) were assessed for spontaneous and cocaine-induced locomotor

activity following direct injection of either AS-ODNs, random control ODNs (appendix one for sequences), vehicle, AS-ODN-loaded microspheres and haloperidol into either the CPu or LV to assess efficacy of these reagents. Antisense and random control ODN sequences were identical to those reported in an investigation by Zhang and Creese (1993) where efficacy of these reagents was observed in behavioural studies of locomotor activity, grooming behaviour and induction of catalepsy. The antisense and random control reagents were fully phosphorothioated molecules, with the antisense recognising codons 2-8 on rat mRNA, bridging the initiation codon.

5.2.5.1. Direct injection of antisense reagents

Antisense and random PS-ODN 19-mers (for sequences, see appendix one) were injected bilaterally into the CPu or LV (as described in section 5.2.3.) at a dose of 20μg/μl twice daily (every 12 hours) for a total of 9 doses over a period of 5 days. Vehicle (ddH_2O, filtered using a 0.2μm millipore filter, 1μl/side) was injected either as a single dose or as repeated dose, twice daily (every 12 hours) for a total of 9 doses over a period of 5 days, and microspheres were administered as a single dose (0.5mg/μl; 2μl/side). Following each injection, the animals (n = 4) were returned to individually housed cages, and were monitored throughout the dosing regimen.

5.2.5.2. Apparatus

Experiments were carried out in 8 black circular Perspex boxes (manufactured 'in house' at Eli Lilly & Co., Erl Wood Manor R&D site, Surrey, U.K.). Each box had a black circular Perspex outer chamber (diameter 420mm, height 300mm) and an inner clear Perspex chamber (200mm diameter) containing a central light source. The central light source consisted of 3 evenly arranged light beams that crossed the runway between the inner and outer chamber. The animal's behaviour was relayed to a computer in a separate room to the activity boxes and data was recorded using a computer program (Paul Fray Ltd, Cambridge, U.K.), measuring total beam

interruptions every 10 minutes (bins/10 minute). The experiments were completed in a dark and quiet room with little disturbance from the experimenter.

5.2.5.3. Procedure

5-6 hours after the administration of the last dose of antisense reagents, including controls and vehicle (dose 8, day 5) (see section 5.2.5.1.), animals were habituated in the locomotor activity boxes for a total of four 10 minute habituation periods. After 3 habituation periods (30 minutes), the rats were administered with cocaine (Sigma, Dorset, U.K.) intraperitoneal (i.p.) (40mg/kg) and locomotion was recorded every 10 minutes for a period of 90 minutes.

For experiments with a direct injection of haloperidol (20µg/µl), rats were injected directly into the CPu (as described in 5.2.3.) and immediately after were introduced to the locomotor activity boxes for habituation, then administered with cocaine (40mg/kg i.p.) (Sigma, Dorset, U.K.) at 30 minutes.

5.2.5.4. Statistical analysis

The locomotor counts were analysed using a SAS Proc. GLM (SAS, 1988) program for Windows® 95. Comparisons were made between groups using a one-way analysis of variance (ANOVA) for independent groups, followed by a *post-hoc* least square means multiple comparison test.

5.2.6. Immunohistochemistry (IHC)

Following analysis of locomotor activity, all rats were killed using Schedule 1 procedures (Codes of Practice for Animal's (Scientific Procedures) Act 1986) with exposure to CO_2 gas. The brains were removed and immersed in 10% (v/v) formalin for at least 24 hours prior to mounting in paraffin blocks. Coronal 5µm sections

were cut around the site of injection and mounted onto Snowcoat Xtra™ (Surgipath, IL, U.S.A.) slides for immunohistochemical (IHC) analysis.

IHC analysis was carried out in order to assess any differences in levels of D_2 dopamine receptor-protein at the site of injection following treatment with antisense, random control, vehicle and antisense-loaded microspheres. All antibody reagents were part of a kit (Vectrastain Elite ABC, Vectra laboratories, Peterborough, U.K.), with exception to the primary antibody (Chemicon, Essex, U.K.). Each washing step with PBS or ddH_2O, or dehydration/rehydration step with xylene or IMS was for 5 minutes.

Coronal 5μm sections mounted on Snowcoat Xtra™ microscope slides (Surgipath, IL, U.S.A.) were dewaxed and rehydrated in a Coplin staining jar (Fischer Scientific equipment, Manchester, U.K.) in 2 changes of xylene (Fischer Scientific U.K. Ltd., Manchester, U.K.), followed by 2 changes of IMS (Fischer Scientific U.K. Ltd., Manchester, U.K.) and one 5 minute wash in ddH_2O. Endogenous peroxidase activity was quenched by incubation of the slides in 0.3% (v/v) H_2O_2 in ddH_2O for 5 minutes, followed by a 5 minute wash in ddH_2O. The ddH_2O was poured off the slides and the slides were incubated in 2 changes of PBS.

The slides were immersed in pre-heated (90°C) target retrieval solution (DAKO Ltd., Cambridgeshire, U.K.) and incubated for 20 minutes in a water bath, after which they were removed, allowed to cool for 20 minutes and the retrieval solution was poured off and slides were washed in 3 changes of PBS. The slides were transferred to an incubation tray, and a ring drawn carefully around each section using a DAKO pen (DAKO Ltd., Cambridgeshire, U.K.) to maintain position of all solutions. 300μl of blocking solution (5% (v/v) goat serum in PBS) was added to each slide and incubated for 30 minutes. The blocking solution was poured off and 300μl of the primary antibody (rabbit anti-human polyclonal recognising the D_2 dopamine receptor (Chemicon, Essex, U.K.) or rabbit IgG (Southern Biotech, Birmingham, AL)) at a concentration of 2.5μg/ml was incubated with the slides overnight at 4°C.

The slides were washed in 3 changes of PBS, then the secondary antibody (biotinylated goat anti-rabbit polyclonal antibody) was added to each slide at a dilution of 1/200 and incubated for 45 minutes at room temperature. The slides were washed in 3 changes of PBS, and then the slides were incubated with the enzyme-conjugate (made 30 minutes prior to incubation), 300µl per slide for 30 minutes at room temperature. The slides were washed in 3 changes of PBS, then washed once for 5 minutes in ddH_2O. The chromagen solution, $DAB/H_2O_2/Ni$ was added to each slide in turn and the colour was allowed to develop for 8 minutes, or until the positive control had developed sufficient colour. The $DAB/H_2O_2/Ni$ was washed off and the slides were immersed in ddH_2O, the water was poured off and slides were washed in clean ddH_2O for 5 minutes. The slides were dehydrated in 2 changes of IMS, followed by 2 changes of xylene and mounted using DPX.

Localisation of D_2 dopamine receptors was observed by light microscopy and images were taken using an Optimas® 5 program for Windows® 95.

5.3. RESULTS AND DISCUSSION

5.3.1. Distribution studies

All injections of fluoresceinated naked ODN and ODN-loaded microspheres into the CPu and LV were administered *via* 31g injection cannula, as described earlier in section 5.2.3. Some practical problems were noted during the injection procedure (see section 5.3.1.1.) and animal behaviours were observed throughout the duration of the experiment (see section 5.3.1.2.), at the end of each time-point fluorescent distribution was then monitored by fluorescence microscopy (see section 5.3.1.3.).

5.3.1.1. Injection procedure of naked ODN and ODN-loaded microspheres

Rats were injected with 0.7nmol/µl of FITC-poly (A) 15-mer and 0.5mg/µl of poly (A) 15-mer-loaded microspheres. A concentration of 0.7nmol of FITC-poly (A) 15-

mer was chosen as preliminary pilot investigations revealed that concentrations below this showed a poor fluorescent signal. Concentrations above 0.7nmol/μl would not have been a directly comparable dose with that loaded into the microspheres, as no greater than 0.5mg/μl of microspheres could have been administered by direct injection into the brain.

It must be noted that there were some practical problems with the injection of microspheres at higher concentrations, as it was a suspension being administered in water, the injection was not homogenous and occasionally caused blocking of the 31g injection cannula. At this point in the investigations, the numbers of operated animals were limiting, and one of the main aims was to compare distribution of naked FITC-ODNs and FITC-ODN-loaded microspheres. Thus, a lower polymer load of 0.5mg/μl was chosen, despite there being a weaker fluorescent signal leading to observations being made at a higher magnification. Observations at higher magnifications made it more difficult to determine overall distribution of the ODNs throughout the tissue, however when observing different 10μm sections, there was generally little fluorescence observed at distances greater than approximately 1mm from the site of injection.

During the injection procedure, there was a defined holding time (section 5.2.3.), 1 minute for a 1μl bolus and 2-3 minutes for a 5μl bolus. The purpose of the holding time was to allow the bolus dose to diffuse away from the tip of the injection cannula. Ideally, longer holding times would ensure that there had been sufficient diffusion of the bolus dose away from the tip of the injection cannula, however the direct injection procedure was carried out on awake, freely moving rats, making longer holding times very difficult. Insufficient diffusion of the bolus from the site of injection led to a great amount of fluorescent ODN present in the cannula tract (figure 5.2.). The fluorescence in the cannula tract was probably due to residual amounts of fluorescence present at the tip of the injection cannula, which resulted in backflow as the injection cannula was pulled out of the guide cannula. However, fluorescent distribution of ODNs was observed at higher magnifications.

5.3.1.2. Behavioural observations

All rats that were injected with both naked FITC-poly (A) 15-mer and FITC-poly (A) 15-mer-loaded microspheres into both the CPu and LV were observed for any behavioural abnormalities throughout the duration of the distribution experiments, for any signs of behavioural toxicity. Behavioural manifestations of toxicity include decreased alertness, passivity, impaired grooming, restlessness, piloerection, lachrymation and irritability. Neurological deficits may also be seen due to toxicity, manifested by focal motor changes or gait disturbances (Crossland, 1980).

No toxicity was observed following injection of naked FITC-poly (A) 15-mer or FITC-poly (A) 15-mer-loaded microspheres into discrete tissue *i.e.* caudate putamen throughout the duration of the experiment (up to 5 days). However, when naked FITC-poly (A) 15-mer was injected into the lateral ventricles, severe toxicity (decreased alertness, impaired grooming, piloerection and irritability) was observed, with some animals not surviving the duration of the experiment (24 hours).

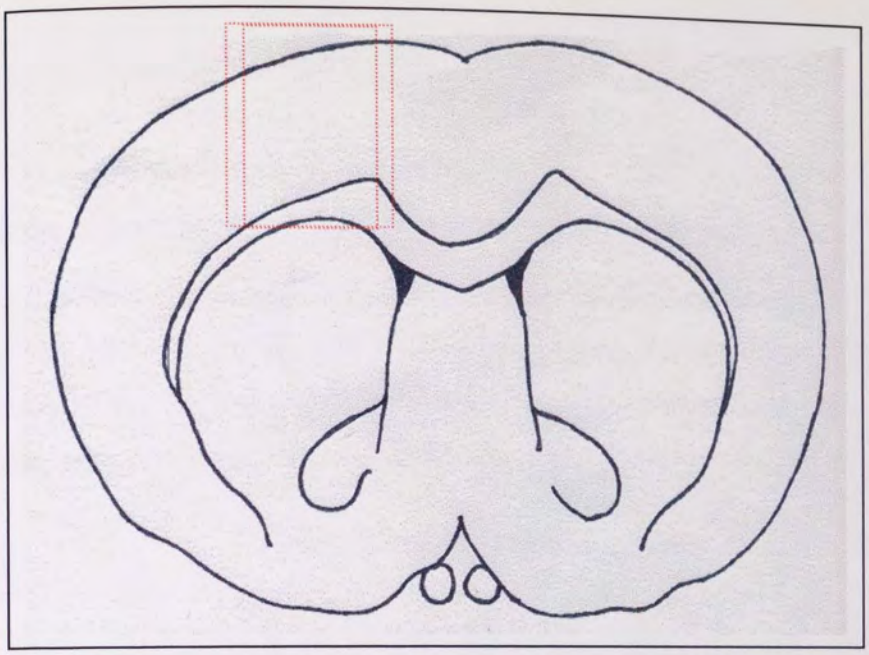
Toxicity of PS-ODNs has been observed within the CNS during other investigations, manifested as weight loss (Ho *et al.*, 1998) (see also figure 5.5.) and other behavioural abnormalities (Pezeshki *et al.*, 1996; Schobitz *et al.*, 1997). A fourteen-fold higher dose of a fully phosphorothioated ODN administered intravenous (i.v.) as opposed to intracerebroventricular (i.c.v.), did not produce any symptoms of toxicity or weight loss in rats (Ho *et al.*, 1998). The observed lack of systemic toxicity of PS-ODNs combined with the reported inability of ODNs to penetrate the blood-brain barrier (Agrawal *et al.*, 1991; Crooke *et al.*, 1996b) suggests that toxicity observed following i.c.v. injection of PS-ODNs may be centrally mediated. The non-specific binding of phosphorothioates to proteins may be the underlying cause of toxicity (Brown *et al.*, 1994; Guvakova *et al.*, 1995). Cell culture studies have indicated that binding can occur with proteins that may be involved in replicational, transcriptional and translational processes (Shoeman *et al.*, 1997).

Most of the reported toxicity of ODNs following central administration has occurred with those ODNs administered intracerebroventricularly (Pezeshki *et al.*, 1996; Schobitz *et al.*, 1997; Ho *et al.*, 1998) and not following a site-specific injection in a discrete brain region. It was reported that after acute bolus administration, ODNs flowed readily through the ventricular system demonstrated by the rapid appearance of ODN in CSF sampled from the extracranial cisterna magna (Whitesell *et al.*, 1993). As the ODN passes through the ventricular system, there may be leakage into surrounding tissues and as toxicity is thought to arise through non-specific interactions of PS-ODNs with proteins (Brown *et al.*, 1994; Guvakova *et al.*, 1995), an i.c.v. injection provides a greater access of ODNs with proteins compared to a site-specific ODN injection.

Entrapping the FITC-poly (A) 15-mer into biodegradable microspheres appeared to mask the toxicity that was observed in the lateral ventricles. The apparent masking of toxicity by the microspheres may be attributed to only part of the final dose of ODN released into the surrounding tissue during the 'burst' effect (for release profile, see figure 3.4.). It may be that the bolus dose of naked ODN is within a dose range that causes toxicity, whereas ODN loaded into microspheres is released over time, resulting in a lower bolus dose at the time of injection.

Any further distribution studies were not carried out in the lateral ventricles due to the observed behavioural toxicity up to 24 hours following injection. There is further discussion of behavioural toxicity in section 5.3.2.1.

A.



B.

Figure 5.2. Coronal schematic at +0.7mm bregma at site of cannula implantation, redrawn from Paxinos & Watson, 1997 (A) and fluorescent image of guide cannula tract (B) at +0.7mm bregma following a unilateral injection of naked FITC-PS-ODN into the caudate putamen (+0.7mm AP, 3mm L, 3.5mm DV bregma, Paxinos & Watson, 1997). x 25 magnification.

5.3.1.3. Distribution of PS-ODNs

These investigations were originally aimed at comparing the distribution of ODNs, when delivered as the naked molecule or entrapped within microspheres, within the caudate putamen, determining whether the microsphere delivery system had the potential of providing long-term efficacy of ODNs. Later investigations into the distribution of ODNs following an intracerebroventricular injection (i.c.v.) (i.e. into the lateral ventricles) were aborted due to the observed behavioural toxicity up to 24 hours (see section 5.3.1.2.).

In summary, preliminary observations of coronal sections at earlier time points of i.c.v. injection (2 and 8 hours) showed a general diffuse fluorescent distribution (for naked ODN) in regions throughout the brain which lay adjacent to the ventricular system, whereas microsphere injections remained more localised to the site of injection. Injections of naked ODNs at 24 hours showed extensive tissue damage (particularly at -3.8mm bregma) in the coronal sections, whereas with microsphere i.c.v. injections at this time point, the ODNs were distributed throughout the brain. The extensive tissue damage at -3.8mm bregma may have been because this particular area of the brain has a large part of the ventricular system passing through it, thus large amounts of ODNs could have leaked into surrounding tissue causing this extensive tissue damage.

Injections of naked ODNs into the caudate putamen showed a diffuse pattern of distribution within the caudate putamen at the earlier time points (2 hours). This diffuse pattern became more punctate between 8 and 12 hours, which may be indicative of cellular uptake, with fluorescence disappearing at 24 hours (figure 5.3. for distribution of naked ODN) (see figure 4.10. and 4.11. for punctate cellular distribution).

Intracerebroventricular investigations into the CNS of rodents have revealed similar patterns of distribution which were diffuse and predominantly interstitial at earlier time points, which then became more punctuated at later time points, indicative of

cellular uptake (Zhang *et al.*, 1996; Broaddus *et al.*, 1998). However, these reported studies described the distribution of ODNs following intracerebroventricular injections, not intrastriatal, as in these current investigations. The distribution of fluoresceinated PS-ODNs injected into the rat striatum and lateral ventricles showed that a vast majority of fluorescent signal was confined to interstitial spaces at 1, 6 and 12 hours (Grzanna *et al.*, 1998). Neuronal nuclei displayed a faint labelling along the outer portion of the nucleus at 1 and 6 hours and fluorescence was observed in large cytoplasmic granules at 16 hours. Efficacy studies with antisense targeting *c-fos* showed suppression of *c-fos* at 3-12 hours but not 16-24 hours. The combined analysis of efficacy experiments and distribution studies revealed that sequestration of AS-ODNs into large cytoplasmic granules coincided with their loss of biological activity (Grzanna *et al.*, 1998).

All of these distribution experiments, including those described here, into either the striatum or lateral ventricles assumed cellular uptake due to the presence of fluorescent punctate structures. Further analysis into the specific cell types needs to be carried out in order to determine the exact cellular interaction of ODNs following direct injection into the brain.

Injections of ODN-loaded microspheres showed a localised distribution at the site of the injection, with fluorescence observed at or near the cannula tract, whereas distribution of naked ODN diffused further away from the site of injection. There was an obvious *in vivo* release profile observed for ODN-loaded microspheres. The greatest amount of fluorescence was observed at 24 hours and fluorescence still remained up to 48 hours (figure 5.4. for distribution of ODN-loaded microspheres). There was no fluorescence observed at 5 days following injection of ODN-loaded microspheres (data not shown) and as no time points were taken between 2 and 5 days, it was not clear at what time point the fluorescence disappeared.

The lack of fluorescence at 5 days may be due to low entrapment of ODN into the microspheres or it could be that the release of ODN from the microspheres is in a

steady phase of release (see figure 3.4.A). Earlier *in vitro* release studies of poly (A) 15-mer revealed that this ODN was released over a period of two months into PBS with at least three distinct phases of release (see section 3.3.1. for release profile). The initial burst effect occurred within the first 24 hours, then there was a slower phase of release up to approximately 28 days, then the final phase of release up to two months. The third phase of release appeared to be occurring at a faster rate than the second phase, as determined by linear regression analysis (figure 3.4.B). At 5 days *in vitro*, the ODN was in a steady phase of release, that is, that there was very little release of ODN during this period of the release profile. It may be that the release profile needs to be tailored to show a release profile that adheres more to zero order kinetics, that is, at a gradual increase of ODN released over time. A release profile that has less defined phases of release may provide a more steady release of ODN in an *in vivo* environment. Comparisons of the *in vivo* and *in vitro* release profiles can not provide definitive conclusions due to the different nature of these two environments. However, the *in vitro* release profiles of this formulation can help provide a possible explanation as to what may be occurring in the *in vivo* situation.

There have been no investigations into the *in vivo* CNS delivery of AS-ODNs using biodegradable microspheres, and the data reported here is promising, where fluorescent ODNs delivered by microspheres remained at the site of injection up to 2 days, compared to 12 hours for naked ODNs. Further investigations into the formulation of the microspheres may provide greater sustained-release *in vivo*, tailoring the release profile and optimising the entrapment efficiency of ODN into the microspheres. The release profile can be tailored by altering such parameters as the polymer molecular weight, particle-size and ODN sequence (see chapter three). Other parameters that may affect the release profile include copolymer composition, ODN loading and surfactants used during formulation.

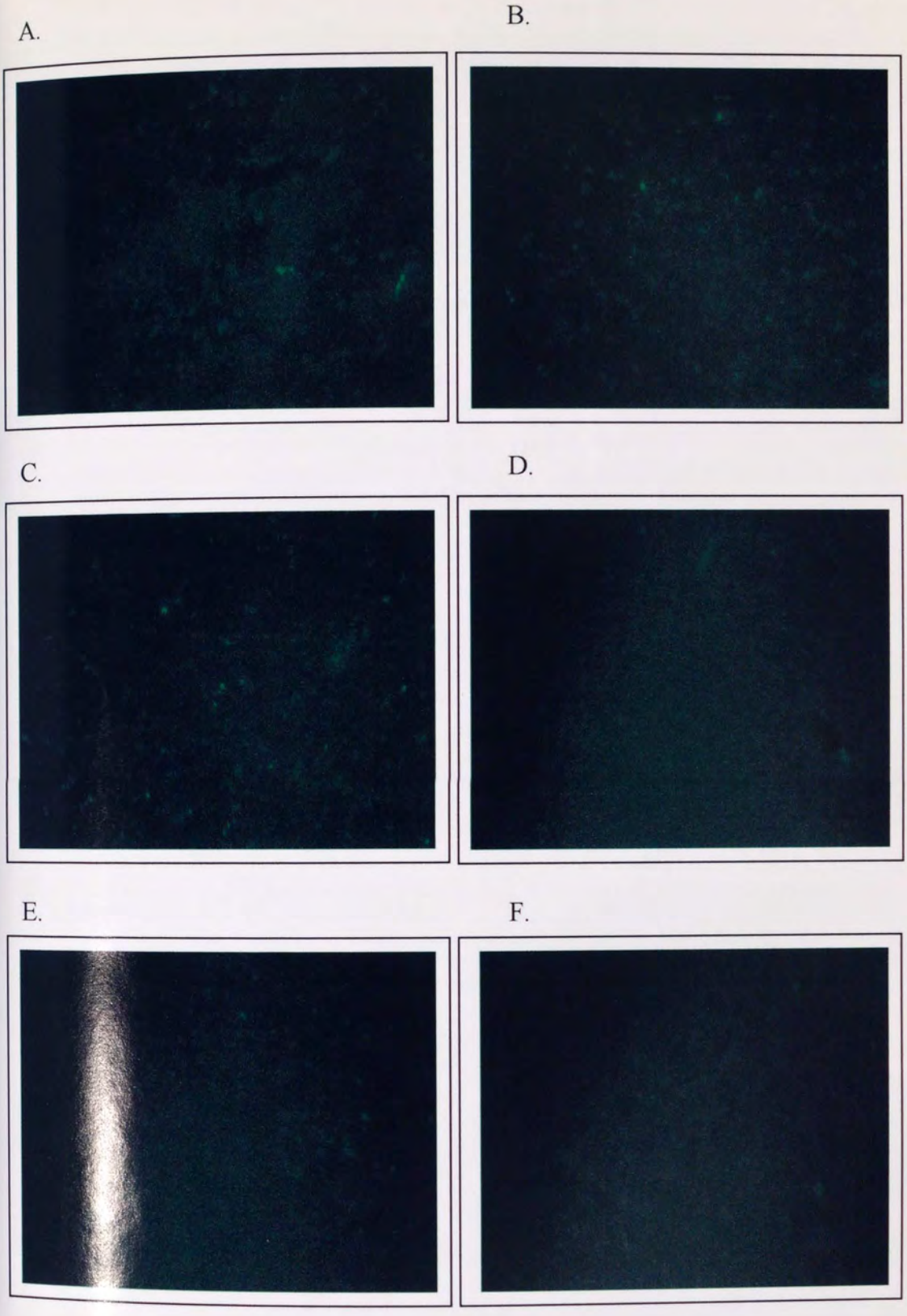


Figure 5.3. Distribution of naked FITC-poly (A) 15-mer delivered to the caudate putamen as naked molecule (0.7nmol/μl) at 2 hours (A), 8 hours (B), 12 hours (C), 24 hours (D), 36 hours (E) and 48 hours (F).

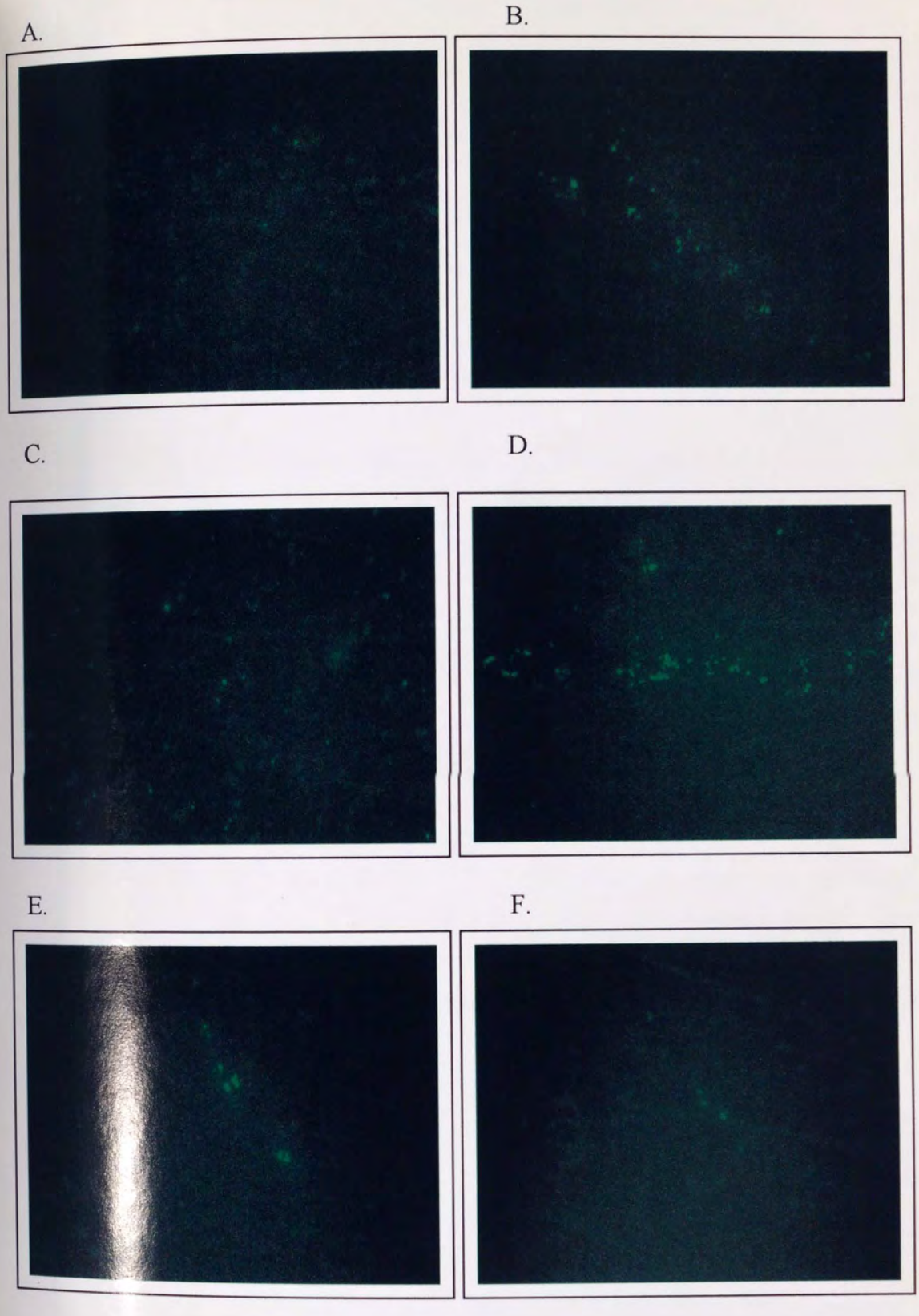


Figure 5.4. Distribution of FITC- poly (A) 15-mer-loaded microspheres (0.5µg/µl) delivered to the caudate putamen at 2 hours (A), 8 hours (B), 12 hours (C), 24 hours (D), 36 hours (E) and 48 hours (F).

5.3.2. Efficacy Studies

5.3.2.1. Behavioural observations

All rats administered with antisense, random control or vehicle were dosed every 12 hours over a period of 5 days for 9 doses (section 5.2.5.1.) and following each injection were returned to their singly housed cages. The rationale for this dosing regimen was further supported by the observations made during the distribution studies (section 5.3.1.), where the fluorescence disappeared after 12 hours following injection of naked ODN into the CPu (figure 5.3.). All rats were observed intermittently throughout the experimental period and were also weighed before each dose was administered.

A significant weight loss was observed in animals that received a repeated dose of both antisense and random control ODNs into the caudate putamen when compared to vehicle (figure 5.5.A), in agreement with a dose-dependent weight loss observed in rats, following i.c.v. administration of PS-ODNs (Ho *et al.*, 1998).

Throughout the experimental period, the antisense and random control animals showed signs of hypomotility and had abnormal gait. Hypomotility may occur when there is blockade of D_2 dopamine receptors, as in a cataleptic state, however muscular rigidity is also manifested in a cataleptic state, which was not observed in these experiments (Hoffman & Donovan, 1995; Lorenc-Koci *et al.*, 1996). Gait disturbances, which was the other behavioural symptom of these animals, can occur when there are neurological deficits during behavioural toxicity (Crossland, 1980). Upon initial analysis of behaviours of these animals, they show some symptoms of catalepsy and some symptoms of behavioural toxicity, any efficacious actions of antisense treatment can only be determined by further analysis into their pharmacology (section 5.3.2.2.) and distribution of D_2 dopamine receptors following antisense treatment (section 5.3.2.3.). It is important to note that animals were not allowed to undergo a procedure to assess catalepsy due to limitations in the project

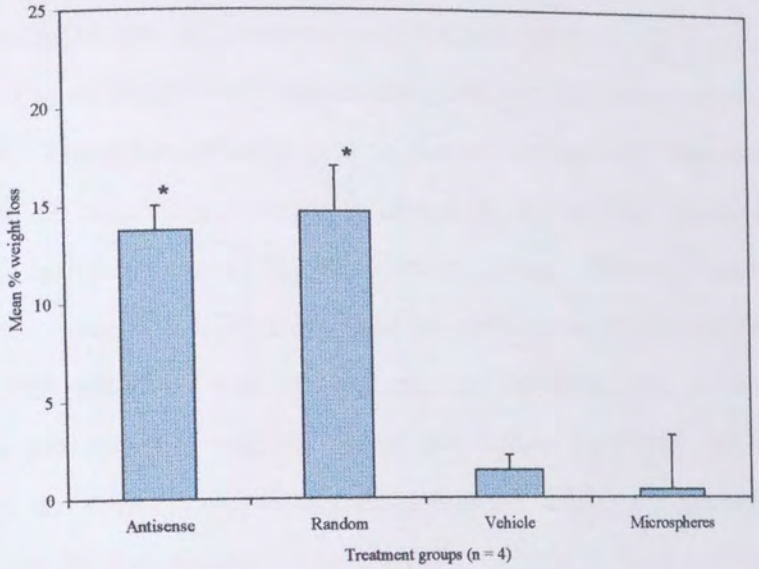
licence, only allowing one procedure per animal, which in this case was locomotor activity (section 5.3.2.2.).

During the direct injection procedure of antisense and random control reagents into the caudate putamen, the rats were very docile, however as soon as these animals were restrained for i.p. injections during cocaine administration in the locomotor activity study, they became extremely irritable, making dosing very difficult. The combination of behaviours of both passivity and irritability are further symptoms of behavioural toxicity.

The behaviours of these two groups of animals (antisense and random control in the CPu) suggests that a knockdown in D_2 dopamine receptors could be occurring because of the reduced motor activity observed, however a true cataleptic state was not observed, where there was no observation of muscular rigidity. Also, similar behaviours were observed for both antisense and random control groups, which suggests non-specificity (see section 5.3.2.3. for further discussion). There is a fine line between dopamine-mediated or toxicity-mediated behaviours, where decreased alertness and passivity that occurs during behavioural toxicity could be masked by hypomotility that occurs during down-regulation of D_2 dopamine receptors, and vice versa. The weight loss observed could also be due to hypomotility, as the animal moves slower and it becomes an effort to eat food and drink water, but again this hypomotility could be due to behavioural toxicity or due to a knockdown of D_2 receptors. This highlights further, the need for locomotor activity studies and analysis of protein levels following antisense treatment.

There were no changes in animal behaviour during dosing, for vehicle and microsphere-treated groups into the caudate putamen or the lateral ventricles.

A.



B.

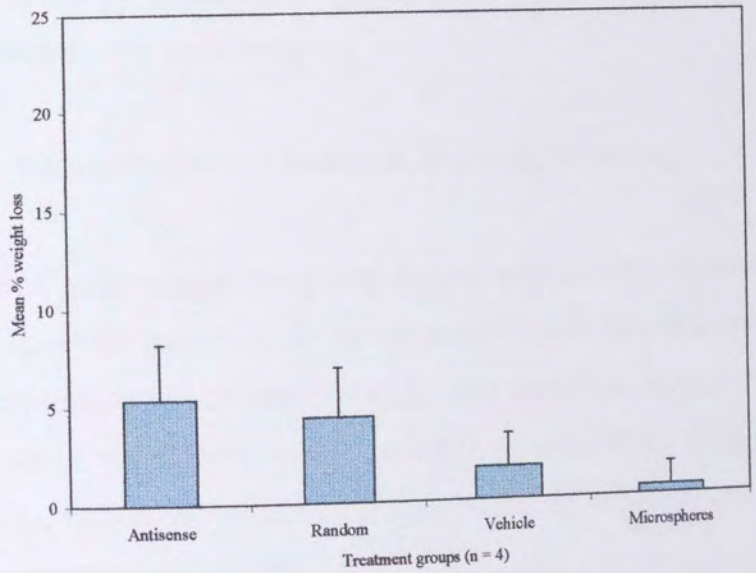


Figure 5.5. Determination of weight loss in rats that had received a repeated dose of antisense (20μg/μl), random control (20μg/μl), vehicle (1μl bolus) and single dose of microspheres (0.5mg/μl, 2μl bolus) in the caudate putamen (A) or lateral ventricles (B). Results are the mean % weight loss (± s.d.) in animals between the beginning (before first dose) and end (after last dose) of the experiment. n = 4 and * denotes a significant difference ($p \leq 0.05$) from vehicle-treated animals.

Repeated administration of antisense and random control ODNs into the lateral ventricles showed no significant differences in weight loss when compared to vehicle (figure 5.5.B). These two treated groups showed no signs of hypomotility and no obvious signs of behavioural toxicity, although during i.p. dosing they showed increased irritability compared to the vehicle group. These observations of no weight loss, no signs of hypomotility and no obvious signs of behavioural toxicity suggests that the antisense and random control treatment had no effect on these animals when administered into the lateral ventricles. However, this assumption is based only on the behavioural observations and the effect of antisense and random control into the lateral ventricles is clarified further in later sections, following investigations into cocaine-induced locomotor activity (section 5.3.2.2.) and D_2 dopamine receptor-protein levels (section 5.3.2.3.). However, it is possible that there may be an element of behavioural toxicity observed due to the irritability of the animals observed during i.p. dosing.

5.3.2.2. Effect of antisense treatment on locomotor activity

Cocaine-induced locomotor activity experiments were carried out in order to define whether the observed reduction in motor function was due to a reduction in D_2 dopamine receptors or behavioural toxicity. Any reduction in concentration of D_2 receptors would be highlighted in such a study, whereas if the reduction in motor function was due to behavioural toxicity, cocaine should be able to induce locomotor activity in antisense-treated groups.

Initially, animals were administered with haloperidol (20μg) by direct injection into the caudate putamen. Haloperidol is a D_2 antagonist and upon administration caused a significant reduction in spontaneous motor activity. Subsequent administration of cocaine caused no increase in locomotor activity, which was due to the antagonism by haloperidol of the D_2 dopamine receptor (figure 5.6.). Treatment with haloperidol demonstrates the effects of a D_2 antagonist on cocaine-induced locomotor activity, and is similar to the profile one would expect if D_2 receptor number were reduced.

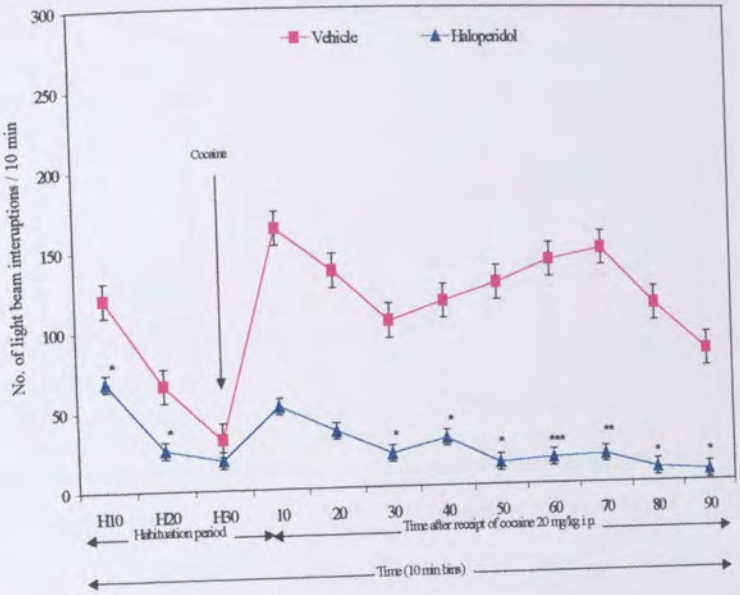
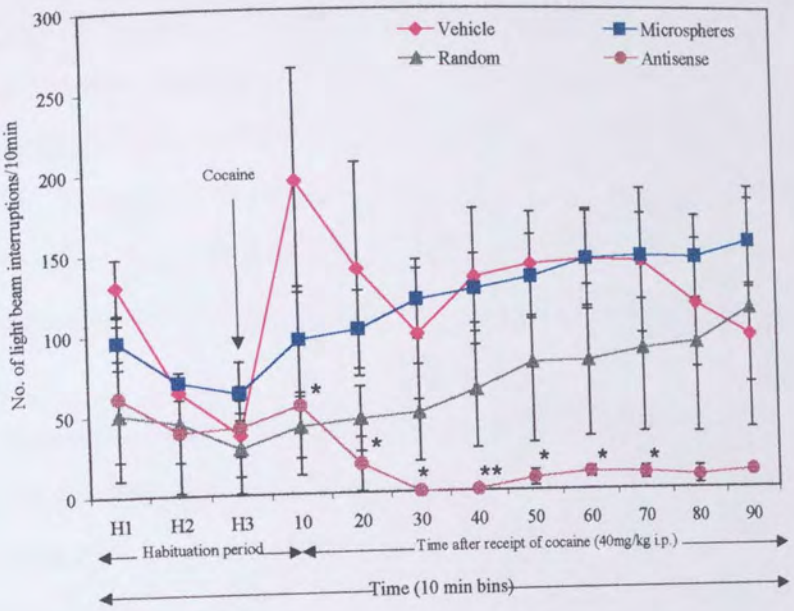


Figure 5.6. Effect of a single injection into the caudate putamen of haloperidol (20µg, 1µl bolus) on cocaine (40mg/kg i.p.)-induced locomotor activity in male Lister Hooded rats. Haloperidol injection administered immediately before habituation to activity cages. Results are the mean number of light beam interruptions/10 minutes (bins/10 min) ± s.e.m. (n = 4) where * ≤ 0.05, ** ≤ 0.01 and ***≤ 0.001.

There was a significant reduction in cocaine-induced locomotor activity following repeated administration of antisense into the caudate putamen, whereas there was no significant reduction in locomotor activity observed following repeated administration of random control ODN or single administration of antisense ODN-loaded microspheres (figure 5.7.A). Although it must be noted that the random control ODN-treated animals were consistently lower than vehicle, albeit not significant, which may be attributed to the small number of animals in each group (n = 4). The reduction in cocaine-induced locomotor activity following antisense treatment suggests that there may be a down-regulation of D_2 dopamine receptors.

A.



B.

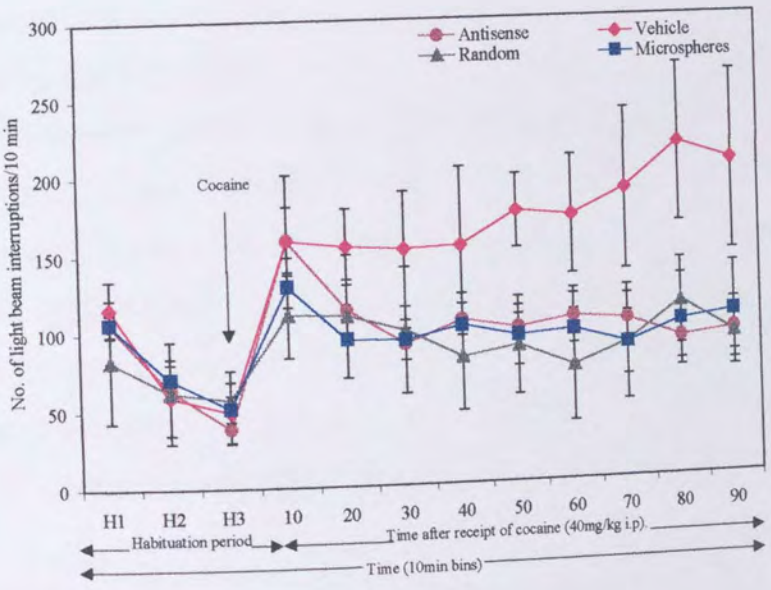


Figure 5.7. Effect of repeated administration of D_2 antisense (20μg/μl), random control (20μg/μl), vehicle (1μl bolus) and single administration of D_2 antisense-loaded microspheres into the caudate putamen (A) and lateral ventricles (B) on cocaine (40mg/kg i.p.)-induced locomotor activity in male Lister Hooded rats. Animals habituated 4-6 hours following last dose. Results are the mean number of light beam interruptions (bins) ± s.e.m. (n = 4) where * ≤ 0.05, ** ≤ 0.01 and ***≤ 0.001.

Cocaine is a dopamine reuptake inhibitor, thus it increases the levels of dopamine within the synaptic cleft, which in turn leads to an increase in locomotor activity due to the increased dopamine binding to the D_2 receptors on the post-synaptic membrane. If there were a reduction in the number of receptors at the post-synaptic membrane then this would result in a reduction in the stimulation of cocaine, as the increased concentration of dopamine has fewer receptor binding sites.

Similar observations to those seen in the current experiments were made by Zhang and Creese (1993) where the i.c.v. administration of PS-ODNs showed a significant reduction in quinpirole-induced locomotor activity and an increase in catalepsy. However, a significant increase in locomotor activity was observed in rats following direct injections of PS-ODNs into the substantia nigra (Silvia *et al.*, 1994). The site of administration of ODNs, the substantia nigra, may explain the conflicting observations made by Silvia *et al.* (1994) and Zhang and Creese (1993). The D_2 dopamine receptors found on dopaminergic neurons of the substantia nigra have been reported to play a regulatory role in dopamine release, tyrosine hydroxylase activity and neuronal firing rate, and are termed autoreceptors. Site-specific down-regulation of these autoreceptors would inhibit their regulatory role, causing a reduction in the inhibition of dopamine release. This would result in an increase in dopamine levels in this particular area of the brain, resulting in an increase in locomotor activity in antisense-treated animals when compared to vehicle.

The reduction in locomotor activity observed in these experiments (figure 5.7.A) may be due to knock down of post-synaptic receptor mRNA, which correlates with the anatomy of the nigrostriatal pathway (see figure 1.6.). The substantia nigra contains the dopaminergic cell bodies that release dopamine to innervate the striatum, including the caudate putamen.

There was no significant differences in cocaine-induced locomotor activity recorded between random control ODN and vehicle, although random control-treated animals did show a consistently lower cocaine-induced locomotor activity compared to

vehicle. The lack of a significant difference in cocaine-induced locomotor activity between these two groups suggests that there may be a degree of selectivity of the antisense ODN. The selectivity of the antisense ODN compared to random control ODN was investigated further by assessing the D_2 dopamine receptor-protein levels by immunohistochemical analysis (see section 5.2.3.3.).

The locomotor activity of animals treated with antisense, random control and antisense-loaded microspheres in the lateral ventricles showed no significant differences compared to vehicle (figure 5.7.B). Although it is worth noting that all treated groups were slightly lower than the vehicle-treated group, albeit not significantly, which again could be attributed to the low number of animals in each group. For these preliminary investigations into the lateral ventricles, the same dose was used for both the site-specific (intrastriatal) injections into the caudate putamen and i.c.v. injections into the lateral ventricles. The reported rapid clearance of ODNs following administration into the lateral ventricles (Whitesell *et al.*, 1993) suggests that these observed differences between intrastriatal and i.c.v. injections may be attributed to site of ODN administration. A higher dose of ODNs (following i.c.v. injection) may be required to provide an efficacious dose due to the reported rapid clearance form the ventricular system. Whereas, injections of ODNs into site-specific regions of the brain, where there is a reported abundance of the target mRNA may provide greater efficacy.

The lack of efficacy of antisense-loaded microspheres conformed to the observations made during the ODN distribution studies (section 5.3.2.1.), where ODNs were present in the tissue up to 2 days, but not 5 days. There could be a number of factors that may have caused this lack of efficacy, some of which were discussed earlier (section 5.3.1.3.). There was only one dosing regimen (5 days) that was tested in these preliminary efficacy investigations. It has been reported that following antisense treatment there is restoration to normal behaviours (Weiss *et al.*, 1993; Zhou *et al.*, 1994; Zhou *et al.*, 1996) within 2-3 days after cessation of antisense treatment. Microspheres were given as a single bolus dose due to their sustained-

release profile. If there was a sufficient dose at the injection site at the beginning of the dosing regimen (i.e. occurring during the burst phase of release), the duration of 5 days may have been a sufficient duration for restoration to normal protein levels.

Further studies could be carried out investigating the effect of naked antisense and antisense-loaded microspheres on locomotor activity over different time periods of treatment, for example 2, 3 and 4 days. Experiments could also be carried out to determine the time taken for restoration to normal behaviours. Also, further experiments may include testing different formulations of microspheres with different ODN loadings.

5.3.2.3. D_2 dopamine receptor-protein levels following antisense treatment

The levels of D_2 dopamine receptor-protein were determined by immunohistochemistry (IHC) using a polyclonal antibody specific for the D_2 dopamine receptor (section 5.2.6.1.). Staining with IgG antibody during IHC studies was carried out to determine any non-specific binding sites on the IgG molecule itself, which may interact with the coronal tissue sections (i.e. IgG is the negative control). There was residual background staining with IgG (figure 5.8.), therefore any staining with the anti-D_2 dopamine receptor antibody that showed a greater intensity of staining than that observed with the control IgG may be attributed to a specific staining of the D_2 dopamine receptor.

A.

B.

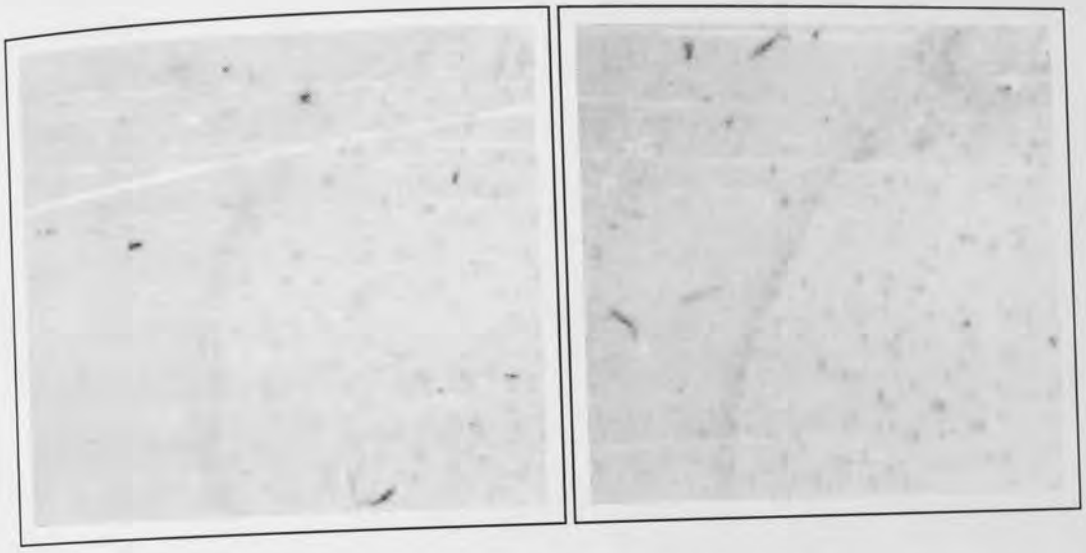


Figure 5.8. IgG (2.5μg/ml) controls for IHC analysis of D_2 dopamine receptor distribution in the caudate putamen (A) and lateral ventricles (B). (A) is the control for figure 5.9. and (B) is the control for figure 5.10. Staining carried out in 5μm coronal sections of brains treated with vehicle.

There was a qualitative reduction in the level of D_2 dopamine receptor in coronal sections of antisense-treated animals into the caudate putamen (figure 5.9.A), however a reduction in protein levels was also observed in the random control-treated group, although to a lesser degree (figure 5.9.D). The staining of the vehicle-treated animals demonstrated a distinct definition between the cortex and caudate putamen (figure 5.9.A). The definition between these two brain regions of the coronal sections was observed, to a lesser degree, in the random control-treated group (figure 5.9.D) and the microsphere-treated group (figure 5.9.C), but was lost entirely with the antisense-treated group (figure 5.9.B). The lack of reduction of protein levels in the microsphere-treated group was in keeping with the lack of effect observed during the efficacy studies, as there was no alteration in the behaviour and cocaine-induced locomotor activity.

A reduction in D_2 dopamine receptor protein levels following antisense treatment was observed by several researchers (Weiss *et al.*, 1993; Zhang & Creese, 1993; Zhou *et al.*, 1994), although to varying degrees. This was probably due to the

differences in these studies, where there was a discontinuous antisense administration in mice (Weiss *et al.*, 1993; Zhou *et al.*, 1994) and a continuous antisense administration in rats (Zhang & Creese, 1993).

The reduced protein levels of the random control-treated group may have occurred due to non-specific interaction of PS-ODNs with proteins that may be involved in replicational, transcriptional and translational processes, as was observed in cell culture studies (Shoeman *et al.*, 1997). Any non-specific interaction with such proteins could lead to a reduction in protein synthesis, hence the observed reduction in protein levels in the random control-treated group. The possible non-specific reduction in protein synthesis may also explain why there was a consistently lower cocaine-induced locomotor activity, albeit not statistically significant (figure 5.7.A).

Immunohistochemical analysis of coronal sections of all treated groups injected into the lateral ventricles demonstrated no distinctive reduction in D_2 dopamine receptor levels (figure 5.10.A-D). However, there did appear to be a slight reduction in the levels of D_2 dopamine receptor-protein in the antisense-treated group, which correlates with the slightly lower cocaine-induced locomotor activity observed during the locomotor experiments (figure 5.7.B).

Specificity of these ODNs could have been analysed further by assessing the levels of other dopamine receptor subtypes (such as D_1, D_3 etc.) following D_2 antisense treatment, but were not carried out due to time constraints.

The advantage of using protein levels as a marker and not mRNA levels is that the mRNA levels rely on antisense working *via* hydrolysis of mRNA by recruitment of RNase H causing down-regulation of mRNA, which is not always the case. Both specific and non-specific antisense mechanisms are described in section 1.1.1. Time constraints in this project resulted in the measurement of protein levels only, measurement of the mRNA levels as well as the protein levels could aid in the elucidation of what particular mechanisms may be involved in the antisense action.

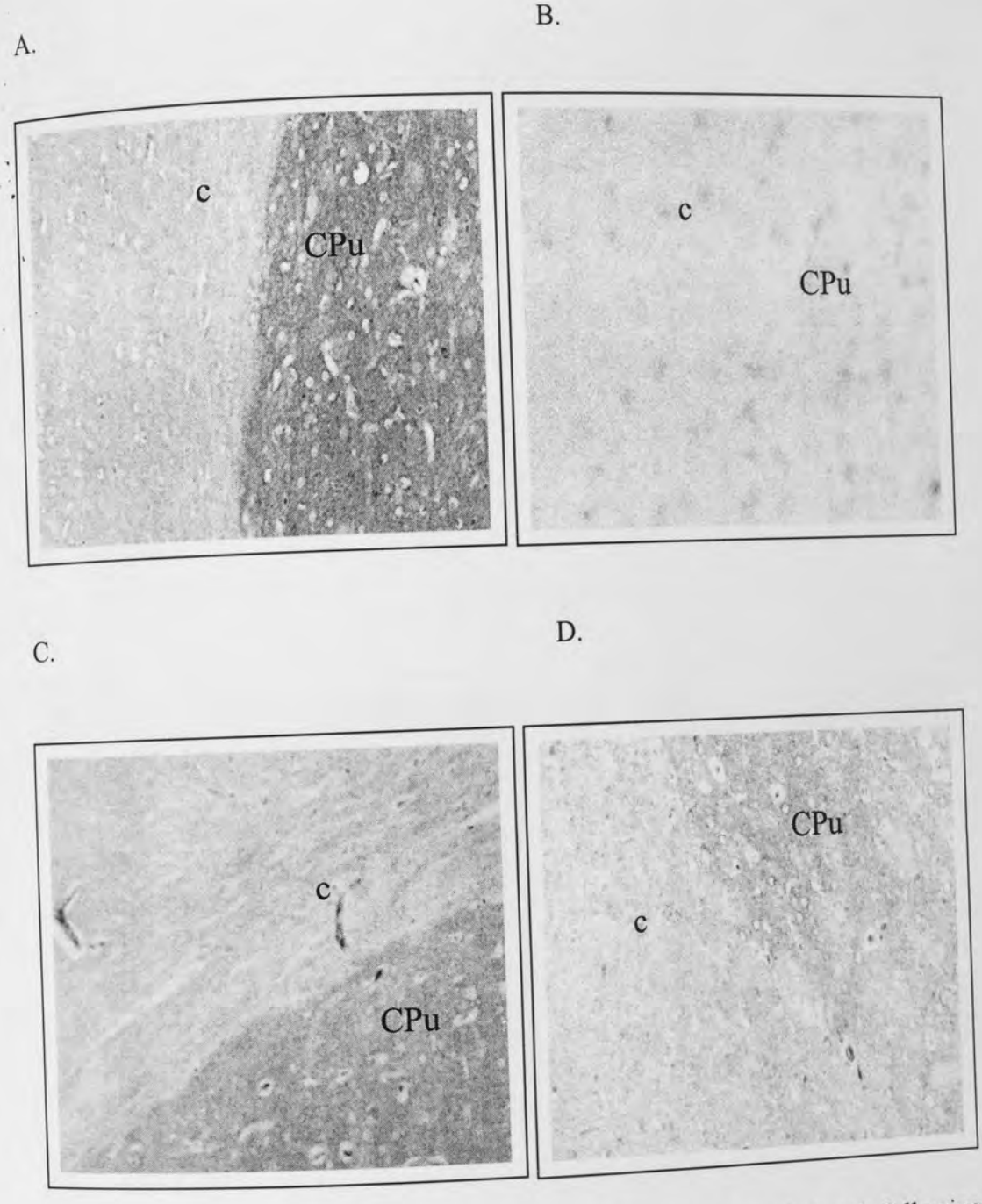


Figure 5.9. Effect of D_2 AS-ODN on D_2 dopamine receptor protein levels following intrastriatal (into CPu) bilateral injection, as determined by IHC analysis. Rats with bilateral guide cannulae received intrastriatal injections of vehicle (ddH2O (filtered using a 0.2μm millipore filter) 1μl/side) (A), D_2 antisense PS-ODN (20μg/μl) (B), D_2 antisense-loaded microspheres (0.5mg/μl, 2μl bolus) (C) and random control PS-ODN (20μg/μl) (D). All doses administered twice daily for 9 injections (5 days), except for microspheres administered as a single bolus. Brains removed immediately after locomotor activity analysis (approximately 6 hours after the last injection) and 5μm coronal sections were prepared at the level of the caudate putamen for IHC using a rabbit polyclonal D_2 antibody (2.5μg/ml). n = 4. CPu = caudate putamen, c = cortex.

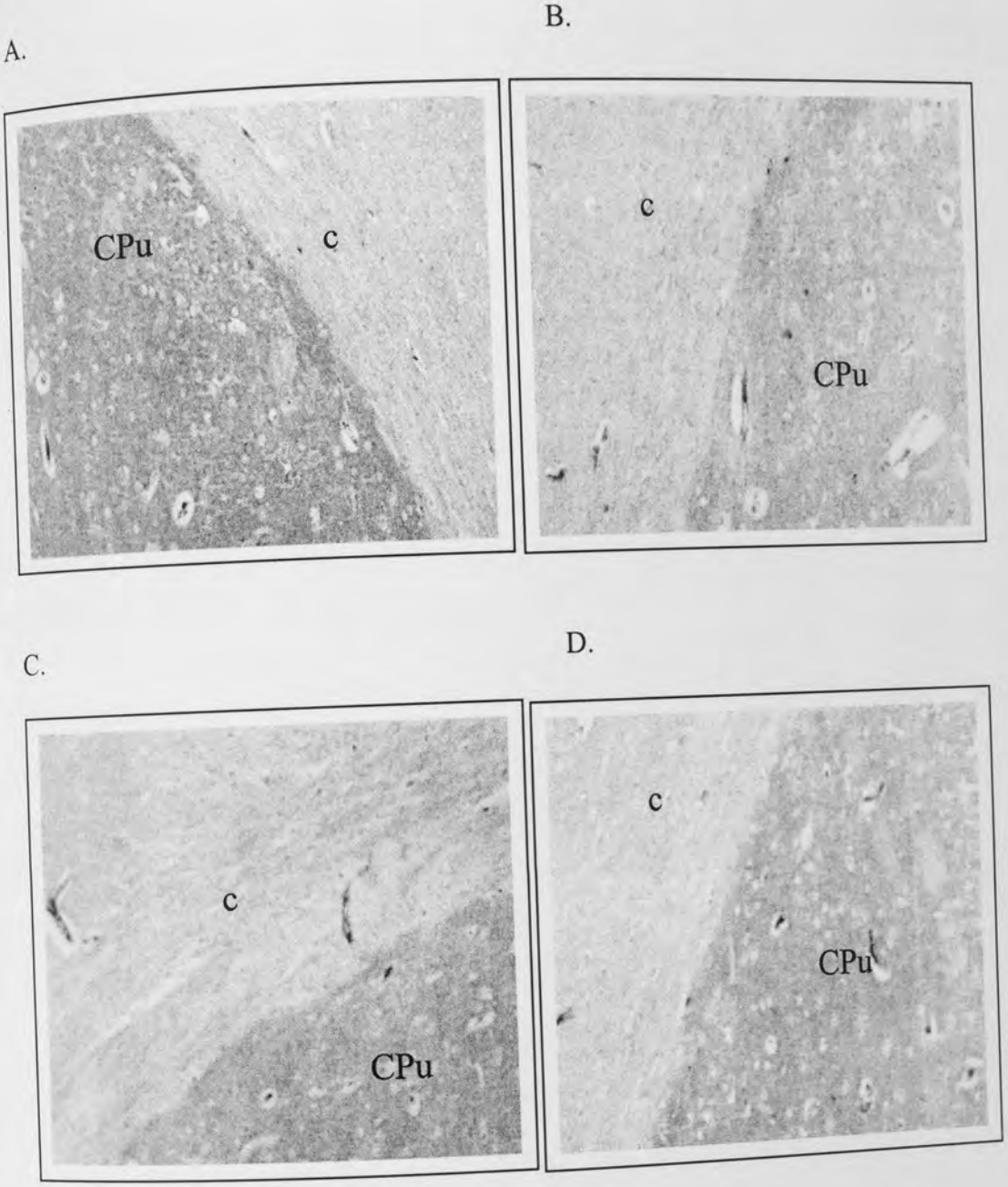


Figure 5.10. Effect of D_2 AS-ODN on D_2 dopamine receptor protein levels following intracerebroventricular (into LV) bilateral injection, as determined by IHC analysis. Rats with bilateral guide cannulae received intracerebroventricular (i.c.v.) injections of vehicle (ddH_20 (filtered using a 0.2μm millipore filter) 1μl/side) (A), D_2 antisense PS-ODN (20μg/μl) (B), D_2 antisense-loaded microspheres (0.5mg/μl, 2μl bolus) (C) and random control PS-ODN (20μg/μl) (D). All doses administered twice daily for 9 injections (5 days), except for microspheres administered as a single bolus. Brains removed immediately after locomotor activity analysis (approximately 6 hours after the last injection) and 5μm coronal sections were prepared at the level of the lateral ventricles for IHC using a rabbit polyclonal D_2 antibody (2.5μg/ml). n = 4. CPu = caudate putamen, c = cortex.

5.4. CONCLUSIONS

It was demonstrated in cell culture studies (chapter four) that the microsphere formulation developed in chapter three showed a five-fold increase in the accumulation of ODNs within glial and neuronal cells when compared to the naked ODN molecule. These findings highlighted the potential of this microsphere formulation for the delivery of AS-ODNs into site-specific regions of the rat brain. The distribution of fluorescent ODNs was investigated as well as the efficacy of AS-ODNs targeted to the D_2 dopamine receptor delivered as the naked molecule or entrapped within biodegradable PLGA microspheres.

The intracerebroventricular injection of naked FITC-PS-ODNs demonstrated a general behavioural toxicity with behaviours such as decreased alertness, impaired grooming, piloerection and irritability up to 24 hours, with some animals not surviving the duration of the experiment. This observed toxicity was in agreement with several other researchers (Pezeshki *et al.*, 1996; Schobitz *et al.*, 1997; Ho *et al.*, 1998). Comparisons of data of experiments with PS-ODNs administered both systemically and centrally (Agrawal *et al.*, 1991; Crooke *et al.*, 1996b; Ho *et al.*, 1998) suggest that the toxicity observed following i.c.v. injection may be centrally mediated, possibly by non-specific interactions with proteins (Brown *et al.*, 1994; Guvakova *et al.*, 1995).

The most striking observation during the current studies, following i.c.v. injections of PS-ODNs was that the entrapment of these ODNs into biodegradable microspheres appeared to mask toxicity with animals showing no signs of abnormal behaviours and surviving the duration of the experiment. The masking of the toxicity when entrapped within microspheres may have been due to the sustained-release nature of the microspheres, providing a sustained concentration of ODN at the site of injection, as opposed to a higher bolus dose of naked ODN. This masking of toxicity demonstrates the potential of this delivery system, where an ODN dose

that would otherwise be considered as toxic (as a single bolus), could be loaded into microspheres to be slowly delivered over time.

The distribution of PS-ODNs following injection into the caudate putamen showed that naked ODNs were only present in the tissue for 12 hours (figure 5.3.). However, sustained-release of the fluorescent ODNs was observed up to 48 hours (at least) when delivered into the caudate putamen by biodegradable microspheres (figure 5.4.). These observations demonstrated the potential of microspheres for long-term delivery of AS-ODNs into the brain.

Efficacy of AS-ODNs targeted to the D_2 dopamine receptor administered by repeated injection into the caudate putamen was demonstrated. There was a significant reduction in cocaine-induced locomotor activity in antisense-treated animals and a slight reduction (albeit not statistically significant) in random control ODN-treated animals (figure 5.7.A) with a concomitant reduction in D_2 dopamine receptor-protein levels (figure 5.9.B). Unfortunately, no efficacy of ODN was observed in microsphere-treated animals, which may be attributed to loading of ODN in the microspheres or the duration of efficacy experiments. Further studies using microspheres of different ODN loadings and alterations in the duration of the efficacy experiments need to be carried out.

The locomotor activity and immunohistochemical studies suggested that the naked antisense reagents are efficacious, however a reduction in protein levels observed with the random control sequence (figure 5.8.) suggested that there might be a non-specific component in their mechanism. This non-specific mechanism may have occurred through interactions with proteins, which is not uncommon for PS-ODNs, which can also lead to behavioural toxicity. The random control sequence possessed a G-quartet (see appendix one for sequence), and previous studies have demonstrated that such structures are prone to the formation of tetraplexes and specific interactions with proteins (Benimetskaya *et al.*, 1997). The passivity of these animals during direct injection and irritability during i.p. dosing indicates that there may have been

an element of behavioural toxicity during PS-ODN administration of both antisense and random control sequences. Further studies may be carried out that incorporate the use of chimeric PS-ODNs, which have shown marked reductions in behavioural toxicity (Ho *et al.*, 1998). Also, improvements in the formulation of antisense-loaded biodegradable microspheres may lead to a greater efficacy and reduced behavioural toxicity due to the sustained-release of ODNs.

The masking of behavioural toxicity and sustained-release of ODNs over a period of at least 2 days highlighted the potential of biodegradable microspheres for CNS delivery of AS-ODNs. Further development into the formulation of the microspheres with improved ODN loading and a release profile tailored nearer to zero order kinetics may prolong the duration of ODN release following stereotaxic implantation of ODN-loaded microspheres. The efficacy of naked AS-ODNs observed during locomotor activity experiments suggest that AS-ODNs targeted to the D_2 dopamine receptor may be a good behavioural model as a baseline for comparison with antisense-loaded microsphere formulations.

CHAPTER SIX

GENERAL DISCUSSION

6.1. DISCUSSION

Since the discovery of AS-ODNs in 1978 (Zamecnik & Stephenson, 1978), it took two decades for their therapeutic potential to become a reality, with the first antisense therapy released onto the market in 1998, Vitravene™. The major area of antisense research over the past two decades has focused on cancer and viral infections (Agrawal *et al.*, 1988; Rosolen *et al.*, 1990), although recently there has been developments into the use of AS-ODNs as an experimental tool in neuroscience (Chiasson *et al.*, 1992; Zhang & Creese, 1993). Improvements in molecular cloning techniques has led to further classification of receptor classes with the elucidation of more subtypes of receptors, making it increasingly more difficult to find truly selective agonists/antagonists. Antisense techniques as a tool in neuroscience have the potential of selective pharmacological definition of subtypes of receptors of the same family, where sequence homology may be high. Such selective intervention of receptor subtypes has contributed to the elucidation of the function of specific subtypes of a certain receptor class. For example, in defining specific functions of the D_1, D_2, D_3, D_4 and D_5 dopamine receptors (Zhang & Creese, 1993; Zhang *et al.*, 1994; Nissbrandt *et al.*, 1995; Apostolakis *et al.*, 1996).

The current data on the application of AS-ODNs within the CNS limits their use to that of a research tool and not therapy due to problems of antisense specificity,

modes of administration and toxicity (reviewed by Neumann, 1997 and Robinson *et al.*, 1997). There is a need to improve the delivery and efficacy of antisense reagents in the brain and current delivery techniques available for systemic application of AS-ODNs may not be entirely transferable to a central application. Indeed, the current antisense treatment for CMV-retinitis in AIDS patients, Vitravene™, is administered by a local intravitreal injection once a month, such treatment regimens for a neurological disorder are impractical. It would not be feasible for current routine modes of central AS-ODN administration, repeated injections and osmotic mini-pumps, to move into the clinic. Some researchers have addressed this issue of CNS delivery with the development of RNA vectors (Weiss *et al.*, 1997a) and water-absorbent polymers (Bannai *et al.*, 1998) for long-term delivery of AS-ODNs into the brain. The application of antisense-loaded biodegradable microspheres in the brain has not been investigated. These delivery systems have been investigated in *in vitro* models (Lewis *et al.*, 1995; Cleek *et al.*, 1997; Yamakawa *et al.*, 1997) and systemically *in vivo* (Khan *et al.*, 1999). In the current study the use of biodegradable microspheres was investigated for CNS delivery of AS-ODNs.

There has been extensive research into the use of polymeric delivery devices for the administration of numerous neuroactive agents into the brain, for example neurotransmitters (McRae & Dahlstrom, 1994) and chemotherapeutic agents (Brem *et al.*, 1991). Significant improvements in their efficacy, as compared to the naked molecule (see table 1.1.), were observed. This research led to FDA approval of the first polymeric delivery device for the treatment of brain tumours, Gliadel® wafers. Gliadel® wafers are administered during the operative procedure of resection of brain tumours and are placed in the wall of resection to provide a sustained local administration of the chemotherapeutic agent, BCNU, so reducing systemic toxicity. The property of sustained and local CNS administration of polymeric delivery devices makes them an attractive delivery system for AS-ODNs into the brain. Biodegradable microspheres as a delivery system provide the advantage of their microscopic size enabling facile stereotaxic implantation into deep regions of the brain (Benoit *et al.*, 1994; McRae & Dahlstrom, 1994; Blanco-Príeto *et al.*, 1996).

PLGA has been extensively used in biomaterials and is in use for current controlled release formulations and biocompatibility studies have revealed that PLGA used within the brain caused no adverse reactions, other than a local inflammatory response (Kou *et al.*, 1997; Menai *et al.*, 1993).

The formulation of antisense-loaded biodegradable microspheres has proved to be the most crucial component in the development of these devices for CNS delivery. There are a number of parameters during the formulation that can alter the release profile, such as the polymer molecular weight, ODN sequence and particle-size (investigated in chapter three). Studies detailed in chapter three showed that differences in the polymer molecular weight determined whether the release profile was biphasic or triphasic, as observed with low (3kDa) and high (45kDa) molecular weight PLGA respectively (figure 3.4.). During the *in vivo* distribution studies, the fluorescent signal of ODNs in coronal sections was present at 48 hours post-implant (figure 5.4.). The *in vitro* release profile of these HMw microspheres being in an almost lag phase at this time (figure 3.4.A), may have been a contributory factor in this pattern of distribution. However, low entrapment efficiencies of ODN into the microspheres may have also contributed to the shorter duration of *in vivo* release, where low loadings of ODN could result in a sub-efficacious dose. Improved entrapment efficiencies would lead to a higher loading, which in turn may result in a higher final concentration of ODN at the site of injection.

The particle-size affected the *in vitro* release of ODNs with a greater release observed in small particles than large particles (figure 3.8.). A cocktail of different size particles could tailor the release profile to be intermediate between the two different sizes, as has been done previously with a degree of success (Narayani & Panduranga Rao, 1996). Also, during formulation procedures it is important to consider the sequence of the ODN, where different release profiles were observed with poly (A), (T), (G) and (C) 10-mers (figure 3.9.A) which could be attributed to hydrophobicity (figure 3.9.B) or to the formation of higher order structures (Benimetskaya *et al.*, 1997).

The cellular investigations, described in chapter four, highlighted the potential of the microsphere formulation chosen for these studies, HMw (45kDa) PLGA microspheres (1-10μm), where a five-fold increase in cellular association was observed with both glial and neuronal cells compared to the naked molecule (figure 4.5.). Further studies revealed that the increased association observed with microspheres might be due to a slower efflux of entrapped ODN from cells (figure 4.6.), and a greater proportion of entrapped ODN associated within the cytoplasmic and cell membrane fraction (figure 4.7.) resulting in a greater accumulation of ODN.

During the *in vivo* investigations, described in chapter five, a degree of behavioural toxicity was observed with naked ODN in the distribution studies following injection into the lateral ventricles and in the efficacy experiments with both antisense and random control injected into the caudate putamen. Such toxicity has been described previously with the use of PS-ODNs (Pezeshki *et al.*, 1996; Schobitz *et al.*, 1997; Ho *et al.*, 1998). Development of chimeric ODNs containing both phosphodiester and phosphorothioate linkages showed an elimination in behavioural toxicity, demonstrating that these phosphorothioate linkages may be involved in the observed toxicity (Ho *et al.*, 1998). The most striking observation throughout the *in vivo* investigations was that incorporating PS-ODNs into biodegradable microspheres masked any behavioural toxicity. This was an encouraging finding in terms of the application of microspheres for the delivery of PS-ODNs into the CNS.

The efficacy of the AS-ODN targeted to the D_2 dopamine receptor in the locomotor activity studies (figure 5.7.A) was an excellent preliminary finding during these investigations, demonstrating that these experiments may be a good model to test the efficacy of microspheres for the delivery of antisense reagents. However, a reduction in protein levels (though not as marked as observed with antisense) was also observed with the random control sequence. This was believed to occur through non-specific protein interactions with crucial proteins involved in transcriptional or transitional processes, as has been observed in previous cell culture studies (Shoeman *et al.*, 1997). An additional point to be aware of, is the presence of a G-

quartet in the sequence of the random control ODN (see appendix one). Such sequences have demonstrated non-specific interactions (Benimetskaya *et al.*, 1997; Saijo *et al.*, 1997) which are believed to be mediated by proteins (Bates *et al.*, 1999).

Such non-specific observations of behavioural toxicity and non-specific reduction in protein levels during the locomotor activity experiments and the IHC analysis clarify the need to optimise this model for investigations into the efficacy of antisense-loaded microspheres. The results derived from treatment with naked antisense are considered to be a baseline, from which to compare the efficacy of the antisense-loaded microsphere treated group of animals. Such optimisation of this model may include the utilisation of ODNs of different chemistries possibly by reduction in the number of phosphorothioate linkage. The selection of accessible sites on the mRNA target, using such techniques as RNase H accessibility mapping and DNA chip technology, determining a more efficacious antisense sequence may also optimise this model.

6.2. SUGGESTIONS FOR FURTHER WORK

Further development into the formulation of antisense-loaded microspheres with the aim of improving entrapment efficiency and tailoring the release profile to zero-order kinetics needs to be carried out in an attempt to produce an efficacious formulation. A number of different batches of microspheres of different ODN loadings and release profiles are recommended to be tested in *in vivo* efficacy investigations.

Improvement in the efficacy of the naked antisense molecule could in turn improve efficacy of microspheres as a delivery system. Further work into the design of the antisense molecule itself using such methodology as RNase H accessibility mapping (Scherr & Rossi, 1998) and the DNA chip technology (Kurian *et al.*, 1999) could be investigated to determine the most accessible sites on the target mRNA molecule.

6.3. FINAL CONCLUSIONS

The formulation developed for this thesis demonstrated a sustained-release profile during *in vitro* release studies into PBS over a period of two months, which provided a five-fold increase in cellular association with both glial and neuronal cells. The enhanced stability observed during the *in vitro* investigations highlighted the potential of this formulation, along with the sustained-release profile and enhanced cellular uptake, to increase the bioavailability of ODNs when delivered into the brain.

During the *in vivo* investigations a sustained-release of ODN was observed over a period of 2 days (compared to 12 hours for the naked ODN) when delivered using this microsphere formulation. The microsphere formulation also masked behavioural toxicity that was observed with naked ODNs during distribution studies into the lateral ventricles, demonstrating a further advantage of this delivery system. Significant efficacy of antisense reagents targeted to the D_2 dopamine receptor demonstrated by cocaine-induced locomotor activity and ISH studies, highlighted the potential of this target as a suitable behavioural model to provide a baseline for which to compare future antisense-loaded microsphere formulations.

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APPENDICES

A1. Appendix one: Oligodeoxynucleotide sequences

1. Poly (A) 15-mer

5'- AAA AAA AAA AAA -3'

Mw = 4857

2. Poly (A) 10-mer

5'- AAA AAA AAA A -3'

Mw = 3212

3. Poly (C) 10-mer

5'- CCC CCC CCC C -3'

Mw = 2972

4. Poly (G) 10-mer

5'- ggg ggg ggg g- 3'

Mw = 3372

5. Poly (T) 10-mer

5'- TTT TTT TTT T -3'

Mw = 3122

6. Antisense sequence (18-mer) targeted to α1A subunit of the voltage-dependent calcium channel

5'- gTC TCC AAA gCg ggC CAT -3'

Mw = 5753

7. Antisense sequence (19-mer) targeted to the D_2 dopamine receptor (Zhang & Creese, 1993)

5'- Agg ACA ggT TCA gTg gAT C -3'

Mw = 6177

8. Random sequence (19-mer) for D_2 dopamine receptor (Zhang & Creese, 1993)

5'- AgA ACg gCA CTT ATg ggg T -3'

Mw = 6177

A2. Appendix two: Experimental Buffers

1. Stock solution 20% native polyacrylamide gel mixture (500ml)

330ml polyacrylamide gel mix (Severn Biotech Ltd., Worcestershire, U.K.)
50ml 10 x TBE (for preparation see below A2.2)
120ml ddH_2O
For a denaturing gel mixture, 7M (420g) of urea was added to the above gel mixture.
Stock solutions were stored at 4°C, protected from light.

2. 10 x Tris/Boric acid/EDTA (TBE) buffer (1000ml)

This buffer was used as a running buffer (diluted to 1 x TBE) during polyacrylamide gel electrophoresis and as a diluent when preparing gels for PAGE.

108g Tris base (Bio-Rad, Hertfordshire, U.K.)
55g Boric acid (ICN, Oxfordshire, U.K.)
50ml 0.2M EDTA pH 8.0 (Sigma, Dorset, U.K.)

A3. Appendix three: Manufacture of guide cannulae

Guide cannulae were manufactured from 23-gauge (blue) syringe needles. The dimensions and method of manufacture are illustrated in figure A3.1. Initially, the measurements were made 6mm for the outer case and 4mm for the needle. Syringe needles were cut using a saw and edges were smoothed.

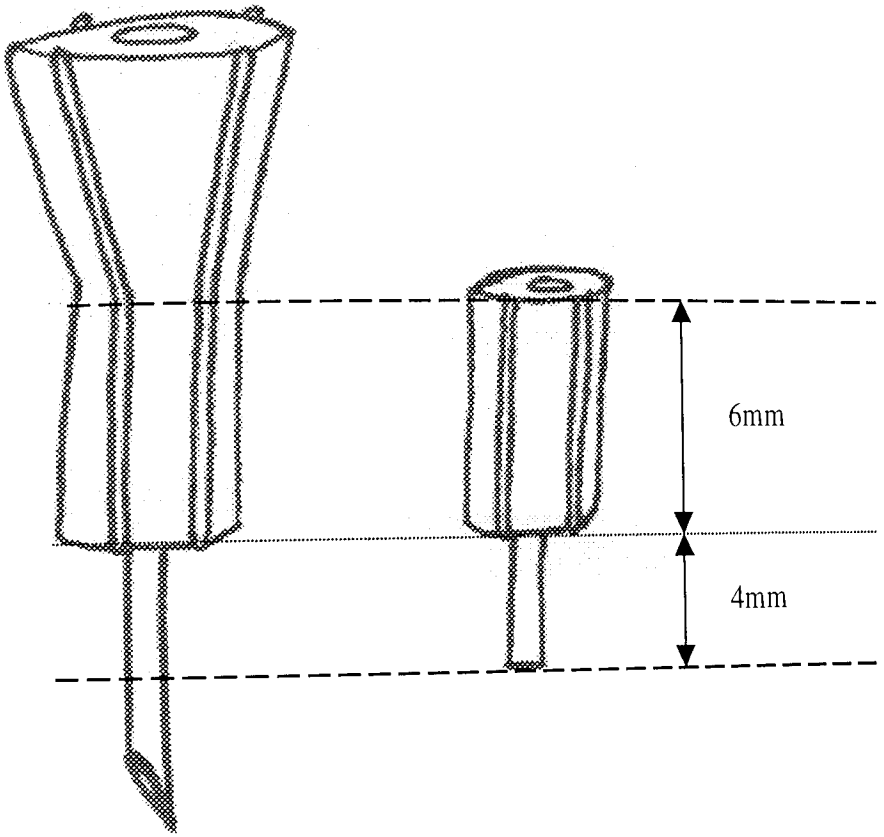

Figure A3.1. Manufacture of 23-gauge guide cannula (not drawn to scale).