Characterisation of Poly(amidoamine)s and Chitosan as Potential Intracytoplasmic Delivery Systems

by

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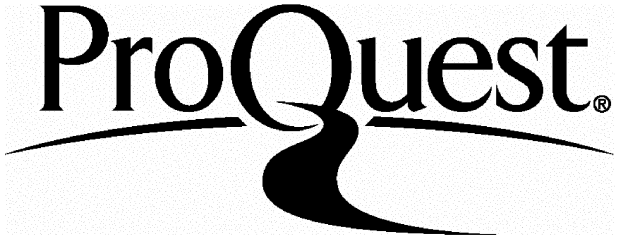

"Why does this magnificent applied science, which saves work and makes life easier, bring us little happiness? The simple answer runs, because we have not yet learned to make sensible use of it."

Albert Einstein (1879-1955)

Address, 1931

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Abbreviations

adapter protein-2	AP-2
adenosine triphosphate	ATP
base pairs	bp
1,4-bis(acryloyl)piperazine	BAP
N, N'-bis(2-hydroxyethyl)ethylenediamine	BHEDA
[2,3-bis(oleoyl)propyl]trimethylammoniumchloride	DOTMA
Boehringer-Mannheim	BM
counts per min	cpm
counts per sec	cps
covalently closed circular	CCC
cystic fibrosis	CF
cystic fibrosis transmembrane conductance regulatory gene	*Cftr*
deoxyribose nucleic acid	DNA
1,2-diacyl-3-trimethylammoniumpropane	DOTAP
diethylaminoethyl	DEAE
3β[*N-(n'-N'*-dimethylaminoethane)-carbamoyl] cholesterol-	DC-Chol
dimethylsulphoxide	DMSO
3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolliumbromide	MTT
dioctadecylamidoglycylspermine	DOGS
dioctadecydimethylammoniumbromide	DODAB
dioleolphosphatidylethanolamine	DOPE
2,3 dioleyloxy-*N*-[sperminecarboxaminoethyl]-*N, N*-dimethyl-1-propanaminium-	
triflurocetate	DOSPA
enzyme linked immunosorbant assay	ELISA
epidermal growth factor	EGF
ethylenediaminetetraacetate	EDTA
enhanced permeability and retention effect	EPR effect
fibroblast growth factor	FGF
foetal calf serum	FCS
galactosamine hydrochloride	GA

gel permeation chromatography	GPC
Gibco Life Technologies	LTI
hexamethyldisilazane	HMDS
N-(2(hydroxypropyl)methacrylamide)	HPMA
interpolyelectrolyte complex	IPEC
intra-muscular	i.m.
intra-venously	i.v.
kilobase pairs	KBp
lactate dehydrogenase	LDH
large unilamella vesicles	LUV
low density lipoprotein	LDL
luteinising hormone releasing hormone	LHRH
melanocyte stimulating hormone	MSH
messenger ribonucleic acid	mRNA
2-methylpiperazine	2-MP
monomethoxypolyethylene glycol	mPEG
multilamella vesicles	MLV
non-ionic surfactant vesicles	NSV
non essential amino acid	NEAA
number average molecular weight	Mn
open circular	OC
phosphate buffered saline	PBS
photon correlation spectroscopy	PCS
poly(amidoamine) dendrimer	PAMAM dendrimer
poly(2-(dimethylamino)ethylmethacrylate)	pDMAEMA
poly(ethyleneglycol) Mw 8 000 Da	PEG 8 000
poly(ethylenimine)	PEI
polymer directed enzyme prodrug therapy	PDEPT
poly(trimethylammonioethylmethacrylatechloride)	pTMAE
poly(vinylalcohol)	PVA

poly(vinylpyrrolidone)	PVP
protein nucleic acid	PNA
Promega	PM
red blood cells	RBC
reticuloendothelial system	RES
R-Gene (Univ. Pittsburgh)	RG
ribose nucleic acid	RNA
scanning electron microscopy	SEM
small unilamella vesicles	SUV
sodium dodecyl sulphate	SDS
standard deviation	S.D.
standard error	S.E.
styrene-co-maleic anhydride-neocarzinostatin	SMANCS
sub-cutaneously	s.c.
N, N, N', N',-tetramethyl-*N, N'*-bis(2-hydroxyethyl)-2,-3-dioleoyloxy-1,4-butanediammoniumiodide	TDA
tissue culture	TC
trans Golgi network	TGN
United Kingdom Co-ordinating Committee on Cancer Research	UKCCCR
un-translated region	UTR
volume for volume	v/v
weight average molecular weight	Mw
weight for volume	w/v
weight for weight	w/w

Abstract

In recent years, gene, antisense and ribozyme therapies have been proposed. These systems all share one common challenge, that of efficient delivery into the cytoplasm of the cell. Immunogenicity is often a significant limiting factor for the viral gene delivery systems used in clinical trials. Synthetic polymers have the advantage of reduced immunogenicity and potentially, they may be tailored, through the application of rational design, to improve cytoplasmic access and modulate cell specific targeting. Consequently, two potential polymeric nucleic acid delivery systems were selected for investigation; highly purified chitosans (three molecular weights) and poly(amidoamine)s. The latter contain amido and amino groups in the polymer chain affording the polymer the ability to change tertiary conformation in response to pH. First polymer biocompatibility was studied by evaluating cytotoxicity *in vitro* and polymer-mediated rat red blood cell (RBC) lysis. Chitosan and poly(amidoamine) polymers were 10-100 fold less toxic than poly(L-lysine) and nearly 1000 times less toxic than poly(ethylenimine). In addition, the poly(amidoamine)s showed the ability to lyse rat RBC in a pH-dependent manner suggesting endosomolytic potential. Chitosan and poly(amidoamine)s formed interpolyelectrolyte complexes with DNA affording protection against DNase II. The body distribution profile of ^{125}I-labelled chitosan and poly(amidoamine), here reported for the first time, showed that certain ^{125}I-labelled poly(amidoamine)s avoid rapid clearance by the liver in the rat and consequently showed time dependent (5h) accumulation in a subcutaneous B16 F10 tumours in the mouse. Preliminary experiments with selected poly(amidoamine)s examined their ability to promote transfection in Hep G2 cells using a pSV-β-galactosidase reporter system. In conclusion, all of the polymers, especially the poly(amidoamine)s warrant further investigation as components of a nucleic acid delivery system.

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Chapter 1

Introduction

Chapter 1 Introduction

There are many potential clinical advantages associated with the treatment of an illness at the molecular level. In recent years, gene, antisense and ribozyme therapies have been proposed (Helene and Toulme, 1990; Sandhu *et al.*, 1997; Thompson *et al.*, 1995). These systems all share the common challenge of effective delivery into the cytoplasm of the cell. Our approach has been to evaluate, systematically, two families of polymeric vehicle, namely chitosan and poly(amidoamine)s as potential vehicles for mediating the intracytoplasmic delivery of nucleic acids *in vivo.*

1.1 The Molecular Basis of Disease

In time, the proportion of diseases that have been elucidated at the molecular level will undoubtedly increase, especially when international initiatives such as the human genome project are considered (Watson, 1990; Bodmer, 1991). Amongst the many initial goals of the human genome project were the generation of three types of genome map (Donis Keller *et al.*, 1987; Billings *et al.*, 1991). The first, a cytogenic map shows the physical locations of specific genes upon their chromosomes. The second is a nucleotide map, indexed through the use of sequence tagged sites (STS) and the third is a genetic linkage map that will allow the location of genes demonstrating Mendelian inheritance (Keith *et al.*, 1987). A map of this resolution (2 centrimorgans) allows the mapping of polygenic diseases and may subsequently be used to refer back to other maps, i.e. the sequence map. It was hoped that using this approach would help elucidate polygenic mechanisms responsible for disease, at a molecular level, as well as providing information about the gene products involved (Trainor, 1990; Green and Waterton, 1991; Watson, 1990; Cantor, 1991).

The pathology of all diseases is thought to be definable at the molecular level, although the exact mechanism(s) responsible for many diseases still remains to be elucidated. During the last decade we have realised that certain diseases can be related to a single gene defect e.g. cystic fibrosis (CF) however, many common diseases, such as cancer and cardiovascular disease are due to

multiple gene (polygenic) defects (Rommens *et al.*, 1989). Currently, where a single gene defect is responsible for a disease, gene therapy may be attempted.

In CF the disease is due to a mutation in the cystic fibrosis transmembrane conductance regulatory (*Cftr*) gene (Koening *et al.*, 1987; Rommens *et al.*, 1989) leading to a dysfunctional chloride ion channel. The CF associated chloride channel is located on the luminal epithelial surface of the lung and, if dysfunctional, causes a build up of mucus. CF patients are then susceptible to bacterially mediated lung disease (reviewed in both Drittanti *et al.*, 1997 and Geddes and Alton, 1998).

In CF patients, gene therapy has been attempted via the delivery of a plasmid encoding the *Cftr* gene to the lung epithelium (Johnson *et al.*, 1992; Rosenfeld *et al.*, 1992). Clinical trials started in December 1992 using both viral, and non-viral (cationic liposome) vectors, (reviewed in Wagner and Gardner, 1997). If we reflect upon the recent progress in the development of gene therapy treatments of CF, it can be seen that there is still much to achieve before we have effective therapy for this lethal illness (Drittanti *et al.*, 1997; Sandhu *et al.*, 1997).

As our understanding of cellular and molecular biology increases so does the opportunity to manipulate cells at the molecular level. Not only can gene therapy be used to introduce a defective gene or modulate transcription, therapeutic strategies such as antisense and ribozymes therapies have also been proposed (Sandhu *et al.*, 1997; Helene and Toulme, 1990; Egholm *et al.*, 1993; Thompson *et al.*, 1995) as well as virally directed antisense (Smith *et. al.*, 1986). Unfortunately, the clinical success of these approaches has also been limited (Felgner and Rhodes, 1991; Ledley, 1994; Crooke, 1995; Douglas and Curiel, 1995; Geddes and Alton, 1998; Sandhu *et al.*, 1997) and highlights the need for a rationally designed delivery system.

1.2 Drug delivery

Drug delivery is a very useful clinical tool and currently accounts for, in the US (1997) a $13 841 million market (Langer, 1998). Drug delivery may be used to maintain continuous levels of drug within a therapeutically desirable range as is seen with systems such as Zoladex®, a controlled release system. Zoladex®, a lactide-co-glycolide matrix in which, a luteinizing hormone releasing hormone (LHRH) analogue is entrapped, is implanted subcutaneous. It is used routinely in the clinic for the treatment of prostate cancer by facilitating the controlled release of the LHRH analogues over a 28 days period (Eichler, 1991). This approach may also decrease the number of doses that are administered and hence increase patient compliance, which has been reported to be responsible for up to 10% (1997) of all hospital admissions in the U.S.A. (Langer, 1998).

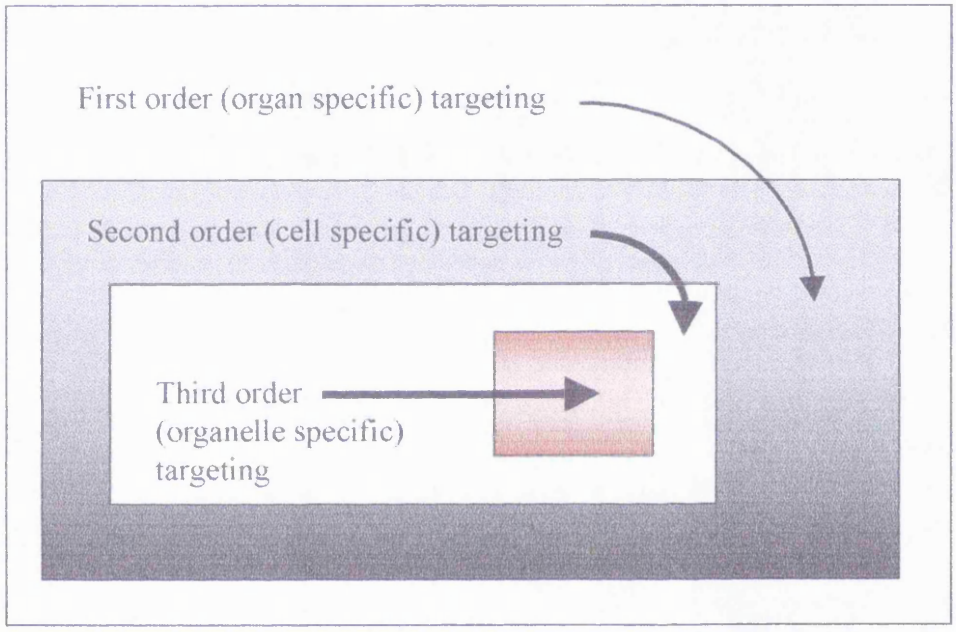
Drug delivery may also reduce cytotoxic side-effects due to the targeting of a drug to a particular cell type, tissue or organ. The idea of drug targeting was first proposed by Ehrlich in 1906 who proposed the concept of the "magic bullet" (reviewed in Basu, 1990; Kataoka, 1997). Designing technologies capable of realising effective, cell-specific targeting has proved very challenging. Herein, the concept of targeting has been divided into active and passive targeting.

Actively targeted drugs normally have a targeting moiety conjugated to the drug or the drug delivery construct, to facilitate an increase in the local concentration of the drug at the designated target site. This approach relies upon the selective localisation of a ligand at a cell specific receptor. Several ligands have been used in this application as described (Table 1.1). It is possible to think of active targeting in relation to first order targeting (organ specific), second order targeting (cell specific) or third order targeting if material can be directed to a specific intracellular compartment within a defined cell type (Figure 1.1). Two fundamental problems have been observed in the instance of active targeting. The proteinacious nature of many targeting moieties such as antibodies often elicits an immune response, which results in the targeting system being degraded prior to target assimilation. The ubiquitous distribution of specific antigens or receptors

Table 1.1 Examples of various targeting moieties

Moiety	Reference
Insulin	Huckett *et al.*, 1990
Transferrin	Zenke *et al.*, 1990; Cotten *et al.*, 1990; Wagner *et al.* 1990; Wagner *et al.*, 1991; Wagner *et al.*, 1992; Wagner *et al.*, 1992[b]
Antibodies	Putman and Kopecek, 1995; Yokoyama and Okano, 1996; Ulbrich *et al.*, 1996; Blakey, 1997
Lectins	Batra *et al.*, 1994; Haltner *et al.*, 1997
Invasins	Paul *et al.*, 1997
Galactose	Duncan *et al.*, 1982; Wu and Wu, 1988; O'Hare *et al.*, 1989
Low density lipoprotein (LDL)	Desmidt and Van Berkel, 1990; Firestone, 1994
Melanocyte stimulating hormone (MSH) Epidermal growth factor (EGF) Fibroblast growth factor (FGF)	Duncan, 1992
Folic acid	Citro *et al.*, 1994

Figure 1.1 First, second and third order targeting
(adapted from Duncan, (1992))



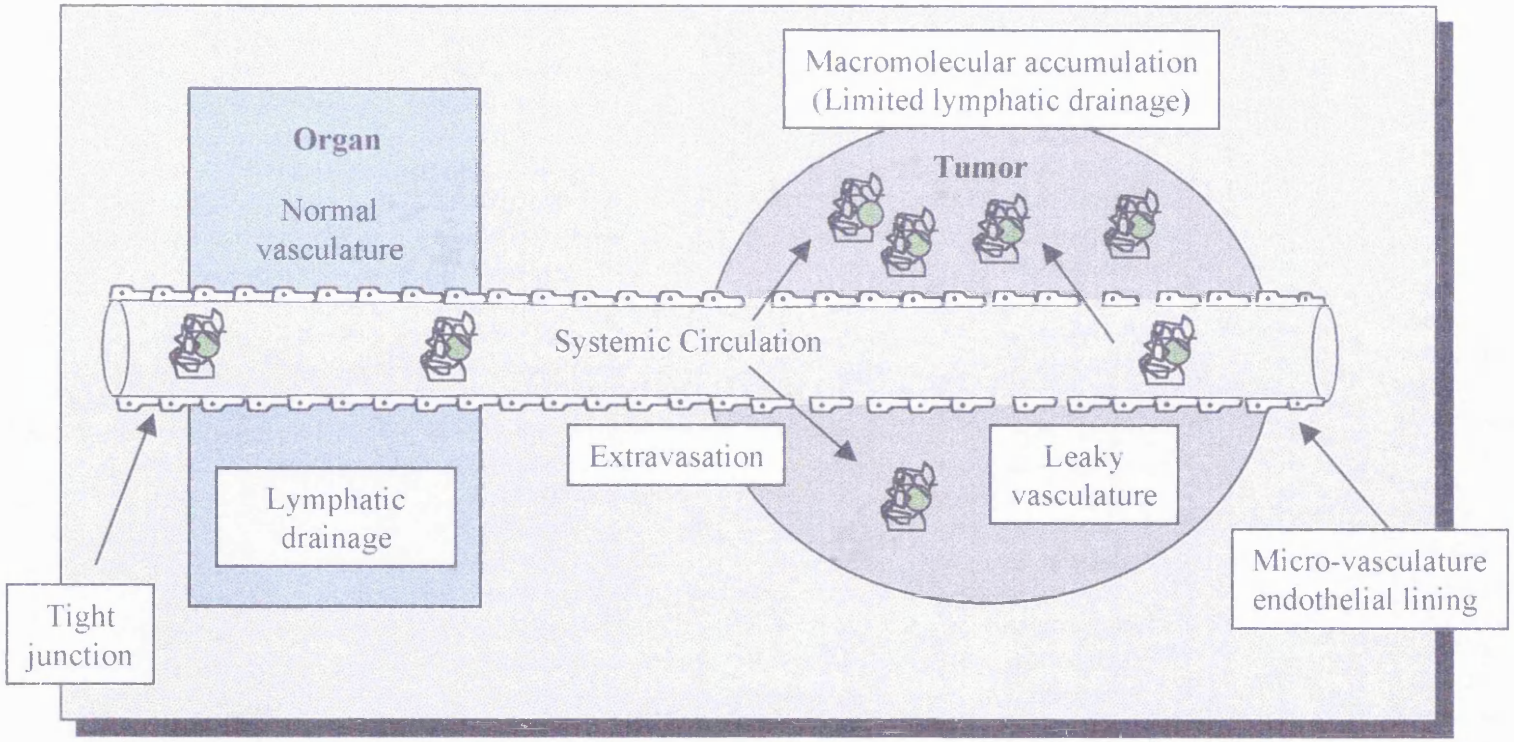
amongst the cellular population has been seen to limit specificity to first order (organ specific) targeting (Yokoyama and Okano, 1996). In the instance of active targeting, organ specificity has been attained using galactose and melanocyte stimulating hormone (MSH) as targeting moieties (reviewed in Duncan, 1992 and Duncan *et al.*, 1996).

Several instances of passive targeting have also been reported. A good example of passive targeting has been seen during the tumour specific accumulation of macromolecular drugs and liposomes via the enhanced permeability and retention (EPR) effect (Duncan, 1992: Maeda, 1994; Yokoyama and Okano, 1996). The EPR effect (Figure 1.2) arises through the angiogenic nature of tumour vasculature, which has a highly permeable endothelial lining. This allows the increased extravasation of circulating macromolecules and particles. As tumours also exhibits limited lymphatic drainage, tumour specific accumulation of macromolecules results and this can lead to a marked targeting effect (reviewed in Duncan, 1992: Maeda, 1994; Maeda and Matsumura, 1986). The entrapment of polystyrene microspheres (15.8μm diameter) in the lung may also provide another example of passive targeting (Illum *et al.*, 1982).

In summary, drug targeting may reduce harmful drug side-effects as well as potentially reducing the amount of drug that is administered. As $136 billion in health costs, 15% of all hospital admissions and 100 000 deaths a year in the US (1997) may be attributable to adverse drug events, the potential for exploiting drug delivery in medicine is evident (reviewed in Langer, 1998).

Historically, several different drug delivery systems have been used in addition to the aforementioned targeting strategies. These technologies have included encapsulating the drug in a liposome, (reviewed in Gregoriadis, 1995), attaching the drug to an antibody, (reviewed in Blakey, 1997) conjugation of the drug to a water-soluble polymeric delivery vehicle (reviewed in Duncan *et al.*, 1996) or the entrapment or encapsulation of the drug in a particle (reviewed in Florence and Salole, 1993; Couvreur *et al.*, 1990). Some examples are given below:

Figure 1.2 Macromolecular accumulation in tumor mass via the enhanced permeability and retention (EPR) effect



Particulate systems

Particulate systems incorporating microcapsules and microspheres can be subdivided according to size. These are the nanoparticles (<1μm), and the microparticles (1-100 μm) reviewed in (Florence and Salole, 1993; Couvreur *et al.*, 1990). These systems are normally designed to swell or degrade following administration so facilitating the controlled release of incorporated substances. This may be in conjunction with a pharmacokinetic modulation as dictated by the particulate size prior to the release of the drug. These systems are not typically designed to facilitate lysosomotrophic drug delivery following intravenous (i.v.) administration and so have not been subject to a full review here in.

Implants

Polymeric implants normally take the form of cross-linked polymeric matrices, which facilitate the controlled release of the drugs they incorporate. This system normally operates through the degradation or swelling of the polymeric matrix after it has been introduced to the patient. This allows the diffusion of the entrapped substance or drug out of the matrix and into the body, either systematically or locally depending upon the site of matrix implantation. The rate of drug release is determined by many factors such as varying the chemical composition of the matrix or altering the degree of matrix cross-linking. (Florence and Salole, 1993). Zoladex® (detailed earlier) is a good example of such a system (Eichler, 1991).

Micelles and liposomes

Probably, the simplest form of lipidic drug delivery system is the micelle. Micelles are formed from hydrophobic/hydrophilic interactions between a strongly amphiphilic molecule and an aqueous environment (Kabanov and Alakhov, 1997; Kabanov and Alakhov, 1994). In relation to vesicular complexity the next, slightly more complex system is the liposome. Liposomes consist of one or more lipid bilayers separated by an aqueous phase. The size, number of

bilayers and chemical composition of the lipid has been used to determine the classification of the liposome. Multilamellar vesicles (MLV- 0.1-10μm), small unilamellar vesicles (SUV- 0.02-0.1μm), large unilamellar vesicles (LUV- 0.1-10μm) and niosomes (NSV- 0.3-0.1μm) have all been reported (reviewed in Florence and Salole, 1993; Lasic and Templeton, 1996; Gregoriadis, 1995). Many advantages associated with using liposomal drug delivery have been reported and these have been detailed (Table 1.2). Liposomes are however, subject to many limitations such as stability in biological fluids, (Lasic and Templeton, 1996) and also clearance by the liver-associated reticuloendothelial system (RES) (Yokoyama and Okano, 1996). One approach that can prevent rapid clearance by the RES has to bind monomethoxypolyethyleneglycol (mPEG) to the liposome surface (Stealth® liposomes) (reviewed in Gregoriadis, 1995). Doxorubicin-containing stealth liposomes are currently in clinical use and are marketed in the USA under the brand name of Doxil™ by Sequus Pharmaceuticals Inc. and in Europe they are called Caelyx® (Gabizon, 1998).

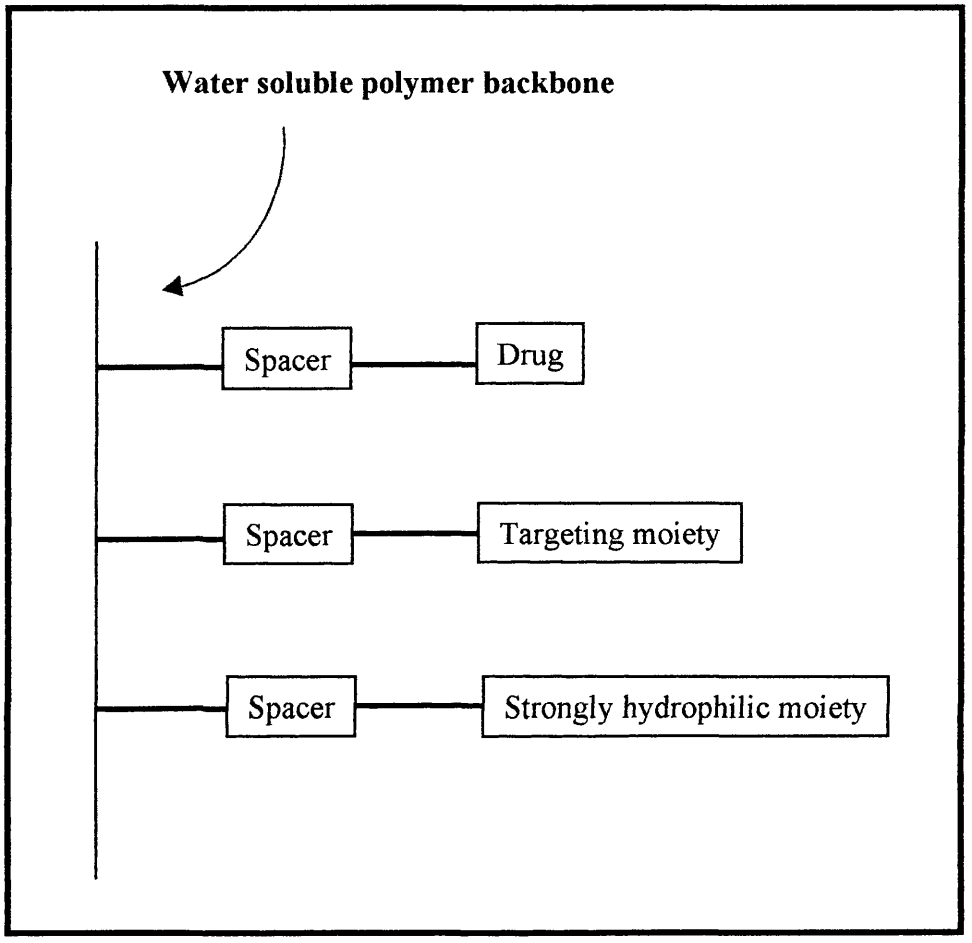
Soluble polymers

The idea of attaching a drug to a polymer was initially proposed to enhance the solubility of a drug in aqueous media (Ringsdorf, 1975) and is shown diagrammatically (Figure 1.3). Soluble polymer-protein conjugates are now in routine clinical use as anticancer treatments. Oncaspar™, an mPEG-L-asparaginase conjugate was approved by the FDA for use in the USA in 1994 for the treatment of patients displaying hypersensitivity to the anti-tumour enzyme L-asparaginase (Muss *et al.*, 1990; Duncan *et al.*, 1996). A conjugate of styrene-co-maleic anhydride and neocarzinostatin (SMANCS) was approved for clinical use in Japan in 1995 for the treatment of primary hepatocellular carcinoma and secondary cancers within the liver. This construct is administered by injection into the hepatic artery and has been reported to undergo tumour specific targeting as a function of the EPR effect (Maeda and Miyamoto, 1994; Maeda, 1994). Several polymer-drug conjugate systems have recently entered clinical trials as potential anticancer treatments such as PK1, an *N*-(2

Table 1.2 Advantages of using a liposomal drug delivery system

Prolonged blood residence time	Lasic and Templeton, 1996; Gabison, 1998
Drug controlled release	Gabizon, 1998
Liposomal targeting	Lasic and Templeton, 1996
Selective local drug release in response to temperature or pH.	Lasic and Templeton, 1996; Zelphati and Szoka, 1996
Liposomal contents protected from enzymatic degradation or humorally mediated attack	Lasic and Templeton, 1996; Zelphati and Szoka, 1996
Intracellular delivery by direct membrane fusion	Zelphati and Szoka, 1996; Lasic and Templeton, 1996; Smith *et al.*, 1997

Figure1.3 The polymer drug conjugate scheme
(modified from (Ringsdorf, 1975)



(hydroxypropyl)methacrylamide) (HPMA) copolymer doxorubicin conjugate (currently in Phase II evaluation (Vasey *et al.*, 1998). PK2, an HPMA copolymer doxorubicin conjugate that also contains a galactose moiety is currently in Phase I evaluation (Pimm *et al.*, 1996; Seymour *et al.*, 1997). HPMA copolymer Taxol® conjugates are currently also in Phase I evaluation (Duncan *et al.*, 1996; Rizzo, 1998). Polymer based systems have also been used to deliver pro-drugs and the appropriate activating agents, a concept termed polymer directed enzyme pro-drug therapy (PDEPT) (Satchi and Duncan, 1998).

1.3 Mechanisms of cellular entry

At the target cell surface, a high molecular weight drug, including polymer therapeutics, proteins and oligonucleotides are unable to diffuse across the cell membrane. Cellular uptake of soluble substances and non-charged particulate matter is, in most instances limited to the endocytic pathway (reviewed by Mellman, 1996). The use of drug delivery systems to target drugs to lysosomes has been termed lysosomotropic drug delivery and has been successfully exploited using constructs such as PK1 (De Duve *et al.*, 1974; Duncan *et al.*, 1996).

Endocytosis has been subdivided into two categories, depending upon the nature of the substance being ingested, pinocytosis and phagocytosis. Phagocytosis or 'cell eating' has been reported to be an actin-dependent process used to internalise particulate matter (>0.5μm in diameter). Phagocytosis has been associated with cells of the RES e.g. macrophages. This limits the usefulness of phagocytosis as a drug delivery route, as non-phagocytic cells may not be able to ingest large particles. A mechanism of particulate forced entry has also been proposed that may be implicated in cytotoxic cell death. In this study we have been trying to design delivery systems that can exploit pinocytic uptake (reviewed in Duncan, 1992; Mellman, 1996).

Pinocytosis or 'cell drinking' involves the internalisation of soluble material as well as particles of less than 0.2 μm in diameter. Pinocytosis has been

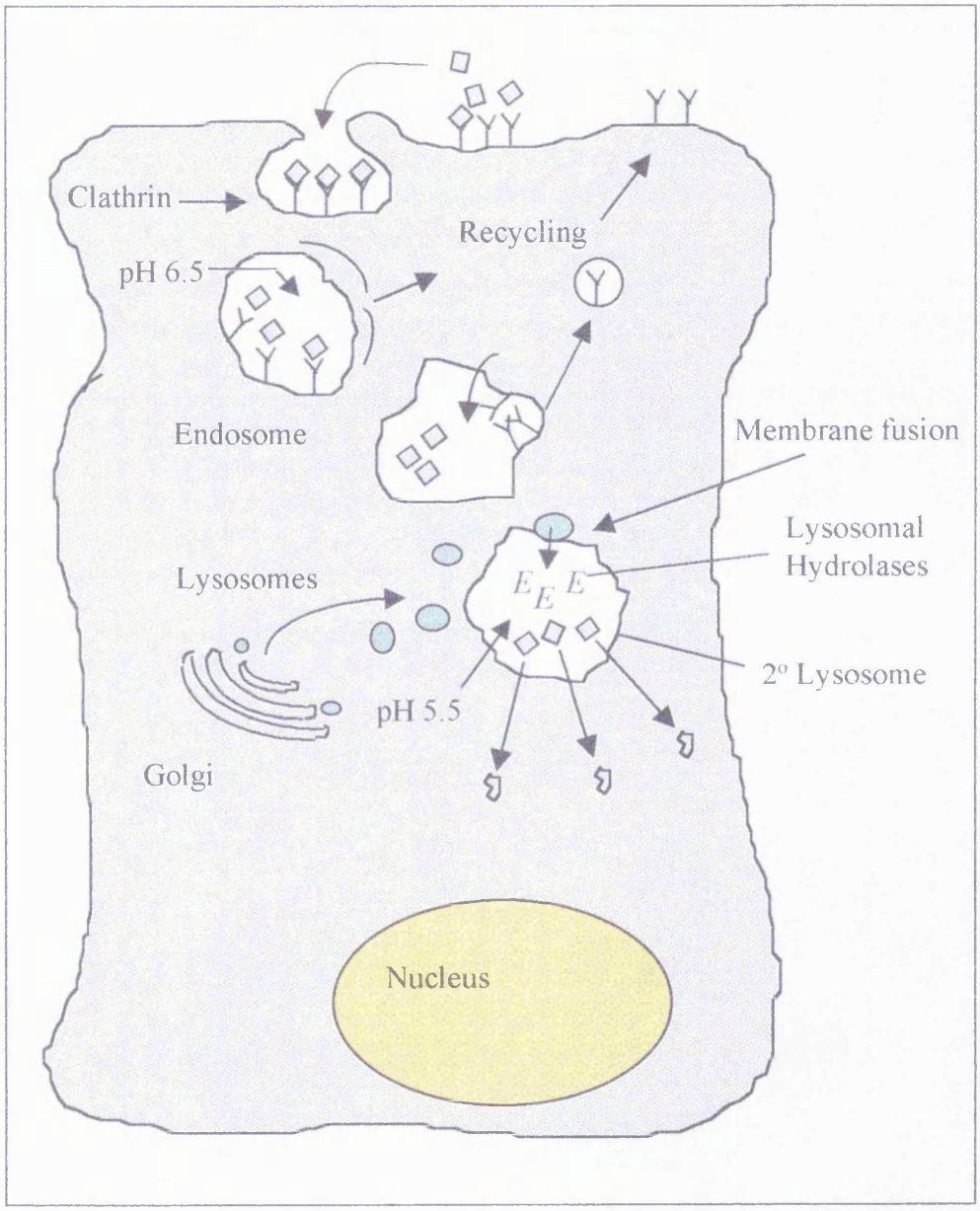
reported in most mammalian cell types and has been subdivided into three categories: fluid-phase pinocytosis, adsorptive pinocytosis and receptor-mediated pinocytosis (Duncan, 1992; Mellman, 1996). Although terminology and specific mechanisms are still the subject of some debate the general principles of these processes are widely agreed. For the purposes of this thesis the following classification scheme has been adopted though this does not detract from the validity of concepts such as macropinocytosis and caveolae-mediated transcytosis (reviewed in Mellman, 1996).

Fluid-phase pinocytosis is mediated through a process of membrane invagination and the concomitant capture of extracellular fluids. Neutral macromolecules that do not display inherent membrane binding, such as poly(vinylpyrrolidone) (PVP), dextran and HPMA are internalised by this route (Pratten *et al.*, 1978; Duncan *et al.*, 1979; Duncan *et al.*, 1981; Duncan *et al.*, 1981[b]; McCormick *et al.*, 1986). Following internalisation the vesicle undergoes a series of intracellular fusion events, traversing the endosomal compartment and transferring to the secondary lysosome. This method of uptake has been reported to be inefficient, as the rate of substance uptake is dependent upon its extracellular concentration.

Non-specific, adsorptive pinocytosis is mediated via the binding of a molecule to the cell surface by ionic interaction between the molecule and the plasma membrane or through hydrophobic/hydrophilic interactions between non-polar moieties and the lipid bilayer. This interaction has been used to enhance the cellular uptake of antisense oligonucleotide analogues via the conjugation of cholesterol to the oligonucleotide (Stein *et al.*, 1991). Following the binding of molecules, events such as local membrane depolarisation induce membrane invagination, budding and vacuole formation (reviewed in Mellman, 1996).

Receptor-mediated pinocytosis (Figure 1.4) occurs in a specialised region of the mammalian plasma membrane that contains a high concentration of receptors called a coated pit. The proteinacious coating on the cytoplasmic face of the pit consists mainly of clathrin, though other proteins such as the adaptin,

Figure 1.4 Receptor-mediated pinocytosis



adapter protein-2 (AP-2) and dynamin are also present. Following receptor binding the area defined as a coated pit undergoes invagination mediated by hexamer and pentamer triskelions formed from a clathrin/AP-2 complex. Vesicle budding is then mediated by dynamin and the protinatious vesicle coating is shed. Following this the pH of the early endosome acidifies under the auspices of an ATP driven proton pump present in the endosomal membrane (Al-Awqati, 1986; Mellman *et al.*, 1986; Forgac, 1992).

Vesicle trafficking has been extensively studied and is regulated by many proteins bound directly and indirectly to the cytoplasmic surface of the vesicle and the cytoskeleton. These proteins include anexins, (reviewed in Moss, 1997) AP-2 (Mellman, 1996) and COP I and II proteins (Robinson, 1997). The exact molecular mechanisms responsible for the intracellular trafficking of vesicles has been reviewed extensively (Mellman *et al.*, 1993; Nuoffer and Balch, 1994; Mellman, 1996; Mellman, 1996[b]). After internalisation, the drop in endosomal pH can affect a disassociation between receptor/ligand complexes inside the endosome. This allows receptor and membrane recycling between the primary endosome and the plasma membrane via the formation of recycling vesicles (Figure 1.4). Following further intracytoplasmic translocation, the fusion of primary lysosomes, derived from the trans Golgi network (TGN), with the late endosome has been documented (Moss, 1997; Mellman, 1996). The resulting vesicle has been termed the secondary lysosome. The products of lysosomally mediated catalysis cross the lysosomal membrane and enters the cytosol by passive diffusion or via interaction with membrane bound porters (Bird and Lloyd, 1991).

There are many limiting factors associated with the cytosolic delivery of substances via a pinocytic pathway. The first of these are the battery of lysosomal enzymes (Table 1.3) that may degrade therapeutic substances in the secondary lysosome prior to a therapeutic effect being seen. A physical barrier, which takes the form of the vesicular limiting membrane has also been reported to limit cytosolic access (Bird and Lloyd., 1991; Klemm *et al.*, 1998). This membrane

Table1.3. Lysosomal enzymes (from Barret and Heath, 1977)

Oxidoreductases	Enzymes acting on bonds involving phosphorus	Endoglycosidases and related enzymes	Exoglycosidases and related enzymes I
$NADPH_2$ oxidase	Phosphatidate phosphatase	Hyaluronate endoglucosaminidase	Neuraminidase
Peroxidases	Phosphoprotein phosphatase	Heparin endoglucuronidase	α-Glucosidase
Carboxylic esterases	Deoxyribo-nuclease II	Glycopeptide endoglucosaminidase	β-Glucosidase
Arylesterases	Sphingomyelin phospho-diesterase	Heparin sulphate endoglycosidase	α-Galactosidase
Triacylglycerol lipase	Phospho diesterase II	Aspartylglucosyl-aminase	β-Galactosidase
Phospholipase A_2	Ribonuclease II	Lysozyme	α-Mannosidase
Phospholipase A_1	Acyl di(glycerophosphoryl) glycerol phosphodiesterase		β-Mannosidase
Cholesterol esterase	Nucleoside triphosphatase		
Acylsphingosine deacylase	Acid phosphatase		

Exopeptidases	Endopeptidases	Enzymes releasing inorganic sulphate	Exoglycosidases and related enzymes II
Lysosomal aminopeptidase	Cathepsin D, E, G, B, H and L	Sulphatase A	α -N-acetyl-glucosaminidase
Lysosomal carboxy-peptidase A	γ-Glutamyl hydrolase	Sulphatase B	β- N-acetyl-glucosaminidase
Tyrosine acid carboxypeptidase	Acrosin	Chondratin-6-sulphatase	α-N-acetyl-Galactosaminidase
Lysosomal carboxy-peptidase B	Lysosomal elastase	Iduronosulphatase	β-Glucuronidase
Lysosomal carboxy-peptidase C		Adenylylsulphatase	α-L-Fucosidase
Lysosomal dipeptidase		Phosphoadenylyl-sulphatase	α-L-Iduronidase
Dipeptidylpeptidase I		Heparin Sulphamatase	$NAD(P)^+$ nucleosidase
Dipeptidylpeptidase II			

may pose a physical constraint limiting the passage of macromolecules from the secondary lysosome into the cytosol.

1.4 Therapeutic strategies: antisense, antigene and ribozyme strategies

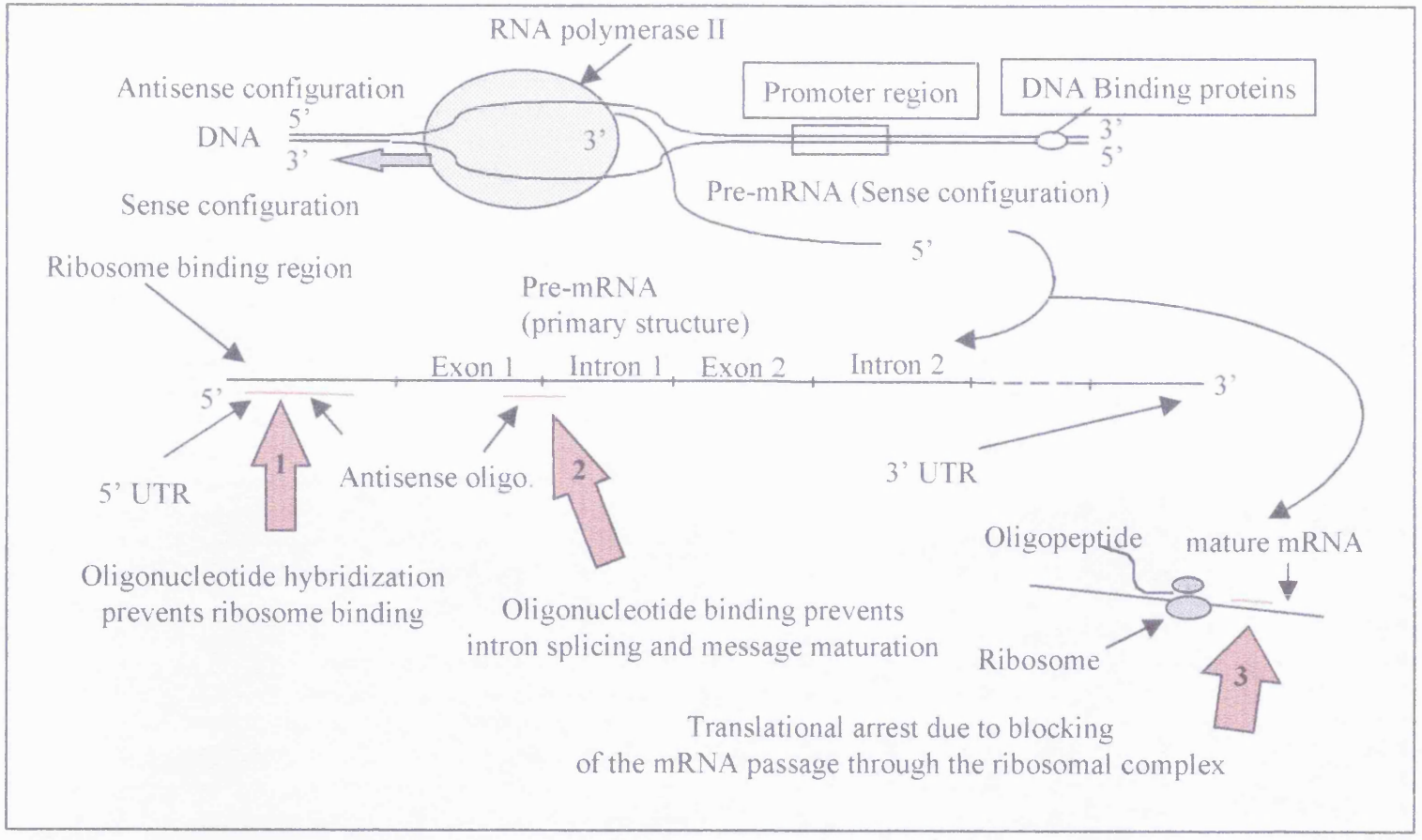
Over recent years many new therapeutic strategies have peen proposed that modulate gene expression and these will be detailed in the following sections. The down regulation of an over-expressing or mutated gene has been reported to be one of the simplest methods of modulating cellular regulation. This strategy requires the intracytoplasmic delivery of a molecule that can selectively bind to either DNA or RNA in a sequence specific manner causing transcriptional or translational arrest. The most well documented instances of this phenomenon have been termed ribozyme, antisense and antigene technologies (reviewed in Helene and Toulme, 1990).

Antisense

Antisense has been used to down regulate a specific gene product through the formation of a messenger ribonucleic acid (mRNA):antisense oligonucleotide analogue hybrid utilising either Watson-Crick base pairing or Watson-Crick-Hoogsteen/reverse Hoogsteen bonding (Helene and Toulme, 1990). The formation of the nucleic acid-hybrid may produce translational arrest at one of several levels following transcription (Figure 1.5).

To augment antisense-gene down regulation, one strategy has been to design an oligonucleotide that can hybridise over the pre-mRNA intron/exon junction. This interaction should prevent the removal of introns and the subsequent maturing of the RNA prior to translation, thus rendering the pre-mRNA transcript nonsensical. Also, it is probable that the formation of a mRNA:oligonucleotide hybrid may inhibit the passage of the transcript through the ribosome complex during translation. Many other target sites within the mature mRNA have also been proposed ranging from the inhibition of mRNA binding to the ribosome, via the inhibition of binding and initiation factors, to the inhibition of mRNA translocation following intron splicing and hence

Figure 1.5 Possible sites of oligonucleotide binding during antisense mediated modulation of gene expression

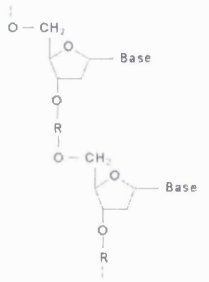
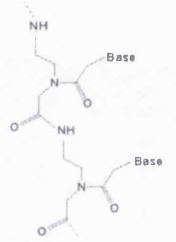


translational arrest. Following the hybridisation of the antisense oligonucleotide to the mRNA, the mRNA portion of the mRNA:oligonucleotide complex is degraded by ribonuclease H freeing the oligonucleotide to bind to further transcription copies and so perpetuating further translational arrest (reviewed in Helene and Toulme, 1990).

Although antisense has been seen to work both *in vitro* and to a limited degree *in vivo,* (Miribelli *et al.*, 1991; Woolf *et al.*, 1990; Webb *et al.*, 1997; Webb *et al.*, 1997[b]), many fundamental problems associated with this phenomenon have been reported. These include oligonucleotide instability in biological fluids (Hudson *et al.*, 1996; Crook, 1993), oligonucleotide pharmacokinetics and oligonucleotide bioavailability (Crook, 1995; Wagner, 1995; Matteucci and Wagner, 1996). Further, it has been suggested that the secondary structure associated with the mRNA transcript may inhibit oligonucleotide mRNA binding (Milner *et al.*, 1997). In spite of these difficulties antisense therapy has still passed into clinical trial (Webb *et al.*, 1997; Webb *et al.*, 1997[b]) for the treatment of leukaemia and cytomegalovirus mediated retinitis (Cohen, 1995).

Oligonucleotide stability can be enhanced through the use of oligonucleotide analogues incorporating substitutions of a non-bridging oxygen atom on the phosphodiester bond (Zon and Geiser, 1991; Akhtar and Juliano, 1991). The more common substitutions are shown (Table 1.4) though more esoteric substitutions have also been reported (Zhang *et al.*, 1997). Antisense oligonucleotide modification has been developed to an extreme in the example of protein nucleic acid, (PNA) where the entire sugar phosphate backbone has been substituted by a synthetic nitrogenous macromolecular chain, demonstrating similar steric properties to the DNA sugar phosphate backbone (Nielsen *et al.*, 1991; Egholm *et al.*, 1993; Lowe and Vilaivan, 1997; Lowe *et al.*, 1996). Most of these substitutions have been reported to increase oligonucleotide half-life in biological fluid, however the pharmacokinetic problems associated with antisense remain to be resolved.

Table 1.4 Antisense oligonucleotide analogues: structure and nuclease resistance

Molecule	R Group	Nuclease Resistant	Parental Structure
Phosphodiester	O=P-O^-	No	
Phosphorothioate	O=P-S^-	Yes	
Methylphosphonate	O=P-CH_3	Yes	
Phosphorodithioate	S=P-S^-	Yes	
PNA	N/a	Yes	

Antigene

Antigene strategies operate via the hybridisation of a macromolecule to the DNA duplex in a sequence specific manner blocking either the binding of transcriptional initiation factors, or the opening of the DNA duplex by RNA polymerase II. Several methods of achieving gene-specific down regulation via macromolecular hybridisation have been reported though the most widely exploited form is that of the Hoogsteen/reverse Hoogsteen triplex (Helene, 1990 and reviewed in Helen and Toulome, 1990). Alternatively, synthetic molecules have been developed that bind to the minor groove of the DNA duplex and exert an antigene effect in a manner similar to that of the Hoogsteen triplex (Trauger *et al.*, 1996; Trauger *et al.*, 1996[b]; Pilch *et al.*, 1996).

Ribozymes

RNA structures that catalyse the cleavage of many forms of RNA, (i.e. specific introns and tRNA precursors) have been termed "ribozymes" (reviewed in Helene and Toulme, 1990; Thompson *et al.*, 1995). Artificial ribozymes have been synthesised that cleave mRNA in a sequence specific manner, so inducing translational arrest (Symons, 1992; Ayers *et al.*, 1996). As RNA has been documented to be considerably more susceptible to nuclease degradation than DNA, (Maniatis *et al.*, 1986) two separate strategies have been adopted to facilitate the intracellular delivery of ribozymal RNA.

The first has been to encode the ribozyme' RNA in the form of a gene located in a plasmid. Once the plasmid, containing a gene encoding the ribozyme is delivered, it may be expressed allowing the ribozyme transcripts to facilitate the transitional arrest of the target gene with in the cell. An alternative approach to ribozyme delivery has been attempted through the chemical modification of the ribozymal RNA. This has been seen to increase ribozyme stability in serum, but limit ribozyme cellular entry (Marschall *et al.*, 1994).

Many forms of ribozyme have been synthesised, (reviewed in Symons, 1992) such as the hairpin, double hammerhead and the hammerhead ribozyme. The latter is the smallest and also the most widely utilised (Thompson *et al.*, 1995). Although this system has been seen to be more biologically active than

conventional antisense (Helene and Toulme, 1990), it has been subject to all of the limitations associated with gene and antisense delivery *in vivo*.

1.5 Gene therapy and techniques used to facilitate gene delivery

Historically, the term gene therapy has referred to the replacement of a missing or dysfunctional gene, so restoring normal cellular function. The treatment of cystic fibrosis (Section 1.1) has provided a good example of gene therapy, however many limiting factors have been reported (Smith *et al.*, 1997; Zabner, 1997; Zelphati and Szoka, 1996; Lasic and Templeton, 1996; Sandhu *et al.*, 1997). Table 1.5 summarises the criteria that are thought most likely to influence the successful delivery of a gene *in vivo*. Genes may also be used to mediate vaccination. Genetic vaccines have been administered in the form of a plasmid encoding a gene that will express specific antigenic proteins. These proteins then raise an immunogenic response so mediating vaccination (Wahren, 1996; Thomas *et al.*, 1997). This form of vaccination is subject to many of the limitations associated with gene delivery (Wahren, 1996). The various methods have been used *in vitro* and *in vivo* to introduce genes to cells and some of the more common ones are listed below.

Electroporation

The cell membrane can be viewed as a thin, insulating film surrounding the cytoplasm and when cells are placed in an electric field (low intensity, direct current) in an aqueous environment, the cytoplasm is seen to polarise in phase with the electrodes. When a slightly higher current is applied, the formation of hydrophilic membrane pores has been reported (Deuticke and Schwister, 1989). This leads to a dramatic increase in membrane conductance and non-specific membrane permeability (Tsong, 1992). This event is reported to allow the entry of DNA into the cytoplasm as DNA is also being forced to move towards the cathode in a manner consistent with electrophoretic migration (Tsong, 1992). Once the current is switched off, the pore size is seen to dramatically decrease. It has been noted that membrane damage is evident for minutes to hours following

Table 1.5 Criteria essential for the successful delivery of genes *in vivo* using a polymeric vector

Criteria	Likely result of criteria not being met	References
Vector biocompatibility	Toxicity	Ringor and Van Holder, 1986; Rihova, 1996; Sgouras, 1990
Plasmid nucleases stability	Gene degraded	Barret and Heath, 1977; Hudson *et al.*, 1996; Tuduri and Englebienne, 1988 Chiou *et al.*, 1994, Sgouras, 1990; Bielinska *et al.*, 1997
Plasmid/vector system pharmacokinetic profile	Gene will not reach target	Abdallah *et al.*, 1996; Wolfert and Seymour, 1996; Seymour *et al.*, 1998
Plasmid/vector internalization	Gene will not reach target	Wu and Wu, 1988; Wagner *et al.*, 1991
Plasmid cytosolic access following internalization	Gene will not reach target	Boussif *et al.*, 1995; Smith *et al.*, 1997; Kichler *et al.*, 1997
Plasmid/vector disassociation following endosomal exit	Gene expression not seen	Zelphati and Szoka, 1996; Smith *et al.*, 1997
Plasmid nuclear localization	Plasmid will not come into contact with RNA polymerase II	Langford *et al.*, 1986; Smith *et al.*, 1997
Expression of gene at an appropriate level	Cellular dysfunction	Sandhu *et al.*, 1997
In expensive, reproducible well characterised, large scale production	Expense may prohibit routine use in the clinic	Duncan, 1992

electroporation depending upon cell type and the strength of the electric treatment (Neumann, 1989; Deuticke and Schwister, 1989: Tsong, 1992; Tsong, 1989).

Particle bombardment

Gene transfer by particle bombardment was originally developed for the transfection of plants (Christou *et al.*, 1990) though it has also been used to transfect mammalian cells (Fitzpatrick-McElligott, 1992; Yang, 1992). This technique uses sterile, ballistic, microscopic particles coated with the gene of interest which are fired at the target cells. The gene-coated particles are reported to penetrate the plasma membrane of cells organised as a tissue, organ or monolayer thus delivering the gene to the cytosol (Yang, 1992). The motive force used to accelerate the particles may be produced by various means. The most common methods include high voltage electrical discharge, 15-22KV for the Accel® "Gene Gun" (Christou *et al.*, 1990) and by helium pressure in the case of Biolistics® (Fitzpatrick-McElligott, 1992). Both electroporation and particle bombardment have been used *ex vivo* following the surgical removal of tissue, which is subject to transfection prior to re-implantation (Titomirov *et al.*, 1991).

Microinjection

Transfection by microinjection is facilitated by the direct injection of exogenous DNA into the nucleus of the target cell (Capecci, 1980; Sih *et al.*, 1994). This form of transfection is very labour intensive and requires a high degree of user skill (Maniatis *et al.*, 1986). The application of this technique to the *in vivo* milieu has been reported to be minimal as the density of cells that would require injection is simply too large.

Calcium phosphate

The use of calcium phosphate to co-precipitate DNA into discreet flocculent particles was one of the first systems used to transfect cells (Graham and Van der Eb, 1973). This system is subject to many limitations, which can be detailed as follows;

1) Calcium phosphate-mediated transfection is cytotoxic after long periods of exposure (Muller *et al.*, 1990).
2) This method is unable to transfect particular cell types such as cells in suspension, (Pahl *et al.*, 1991) or differentiated cells (Muller *et al.*, 1990).
3) Transfection efficiency associated with this technique has been reported to be low, in the range of 1 in 10^{3-5} (Graham and Van der Eb, 1973).

In-spite of the aforementioned limitations, calcium phosphate co-precipitation has been used to increase understanding of the cellular mechanisms responsible for transfection. These studies have provided a platform on which improved techniques could be developed. The variables that were found to influence the efficiency of calcium phosphate co-precipitation can be summarised as follows;

1) Preparation and composition of co-precipitate (Graham and Bacchetti, 1983).
2) Pre-transfection cell density (Graham and Bacchetti, 1983).
3) Duration of cellular exposure to precipitate (Rippe *et al.*, 1990).
4) Specific biology of the cell type (Burke *et al.*, 1984; Basolo *et al.*, 1990).

In addition, some mammalian cells demonstrate cell-specific, non-heritable competence with regard to their ability to receive exogenous DNA (Wigler *et al.*, 1979).

The mechanisms of calcium phosphate-mediated transfection can be described as follows. The co-precipitate is first internalised by endocytosis. (Orrantia and Chang, 1990). There has been much discussion as to the mechanism of co-precipitate export from the endosome or secondary lysosome into the cytosol and the mechanism(s) of subsequent DNA transport to the nucleus. It has been proposed that the co-precipitates have the ability to disrupt the endosome or secondary lysosome membrane (Loyter *et al.*, 1982). Subsequent nuclear localisation of the DNA by diffusion has also been postulated (Zabner, 1997). Alternatively a vesicular-based nuclear transport mechanism may exist (Orrantia and Chang, 1990). Following nuclear translocation, the DNA, in most instances,

dose not integrate with the host genomic chromosomal DNA (Sinclair *et al.*, 1983; Orrantia and Chang 1990) and forms mini-chromosomes in the nucleus (Reeves *et al.*, 1985). In a few instances, the stable integration of transfected genes into the chromosomal DNA has been reported (Wigler *et al.*, 1979). Why this event should happen in some instances and not others remains unclear, as are the mechanisms by which this process takes place. The routine application of calcium phosphate-mediated transfection to the clinic is not likely, as the transfection efficiency of this technique is too low (Chang, 1994).

Viral vectors

The specific mechanisms of virus-mediated infection and transfection have been well documented (reviewed in Dimmock and Primrose, 1996; Douglas and Curiel, 1995; Davis, 1997; During, 1997). Viruses would appear to be the perfect vector for gene delivery; however, they have a number of limitations. Viral vectors can be cytotoxic and immunogenic, thus limiting their efficacy and the number of times they can be administered. The prospect of viral wild-type reversion could also be dangerous (Douglas and Curiel, 1995) and in addition, the point of gene insertion mediated by particular viral vectors could cause malignancies (Laic and Templeton, 1996). Although successful virus-mediated transfection has been reported *in vitro*, *in vivo* and also in the clinic, (Wagner and Gardner, 1997) the aforementioned problems still present a significant barrier to optimal system performance (Lasic and Templeton, 1996). In spite of this, virus-mediated gene delivery is still the most commonly used delivery system in the clinic to date (Felgner, 1990; EWGT News letter No. 5 (1996); SCRIP, 1997; Sandhu *et al.*, 1997).

Synthetic non-viral vectors

As viral vectors do have certain limitations as outlined above, the synthetic viral mimetic is being investigated in an attempt to circumvent these problems. Two main approaches have been used, utilising either polymer or lipidic systems.

Lipidic vectors

Two categories of lipidic DNA delivery systems have been reported. The first uses cationic lipids to condense, and coat, the plasmid and the second uses cationic liposomes to entrap and condense plasmid DNA.

Considerable research effort has been directed towards the development of virally mimetic liposomes within the context of gene delivery (Lasic and Templeton, 1996; Zelphati and Szoka, 1996; Zabner, 1997). Lipidic systems have been used that can protect nucleic acids from enzymatic degradation (Morrison-Perrie and Gregoriadis, 1997) and may promote membrane fusion, either at the plasma membrane or within the endosome or lysosome (Zelphati and Szoka, 1996). Although there are many advantages associated with liposomal delivery systems, they do have disadvantages. The mathematics governing the shape of spherical liposomes has been reported to render the liposome unstable (reviewed in Lasic and Templeton, 1996). This can be a major problem in the presence of serum proteins, which can cause the liposomal components to disassociate (Lasic and Templeton, 1996; Zelphati and Szoka, 1996). Problems have also been seen with liposomal pharmacokinetics as after i.v. administration they are often subject to RES capture in the liver, limiting the opportunity to direct to other cell types.

An alternative form of lipid-mediated DNA delivery utilises combinations of cationic lipids such as dioctadecylamidoglycylspermine (DOGS) or [2,3-*bis*(propyl]trimethylammoniumchloride (DOTMA) to condense plasmid DNA (Lasic and Templeton, 1996; Zabner, 1997). Although cationic lipids have very high transfection efficiency, they are particularly haemolytic and are therefore unsuitable for systemic administration. They have however found a wide application *in vitro* as research tools and have been used *in vivo* (Li and Huang, 1997). The commercially available lipidic preparations are listed (Table 1.6, adapted from (Lasic and Templeton, 1996)).

Table 1.6 Commercially available lipidic systems used for transfection. adapted from Lasic and Templeton, (1996)

Name	Composition	Conc. Mg/ml	Conc. mM	Producer	Mw	+cholesterol/mol
Lipofectin	DOTMA:DOPE (1:1)	1	1.45	LTI	687	0.53
Lipofecamine	DOSPA:DOPE (3:1)	2	2.04	LTI	977	3.36
Lipofectace	DODAB:DOPE (1:2.5)	1	1.41	LTI	708	0.32
DOTAP	DOTAP	1	1.36	BM	732	1.0
Transfectam	DOGS	1	1.11	PM	902	4
TFX-50	TDA:DOPE (1:1)	-	2.1	PM	891	1
DC-Chol	DC-Chol:DOPE (3:2)	-	2	RG	606	0.62

LTI = Gibco Life Technologies
BM = Boehringer-Mannheim
PM = Promega
RG = R-Gene (Univ. Pittsburgh)

Polymeric vectors

Cationic polymers have been used as nucleic delivery vehicles both *in vitro* and *in vivo* (Wu and Wu, 1998; Kabanov *et al.*, 1993; Kabanov and Kabanov, 1995). These systems utilise the phenomenon of co-operative polyion binding to form a condensed, complex consisting of the cationic polymer and the anionic DNA (Shapiro *et al.*, 1969).

The tertiary structure of double stranded α helical DNA is governed by many forces, such as the repulsion between negative charges associated with the non-bridging oxygen on the phosphate groups, inter- and intra-molecular hydrogen bonding and hydrophobic/hydrophilic interactions associated with the purine and pyrimidine bases. The result, at physiological pH and salt concentration is a rigid structure, forming a 'worm like' random coil (reviewed in Manning, 1978; Lasic *et al.*, 1998). This structure is not seen *in vivo* as DNA normally exists in a condensed form, the DNA being wrapped around positively charged histones to form chromatin which, in turn form chromosomes (Bohinski, 1987).

The phenomenon of DNA condensation by histones is, in part, a function of charge. This is due to interactions between the positively charged arginine and lysine residues found in the histone primary structure and the negatively charged phosphate groups of the DNA backbone. Once the electrostatic repulsion between the non-bridging oxygen has been neutralised, a much less rigid DNA tertiary structure is evident. DNA, in the condensed form is more enzymatically stable than in the non-condensed form (Mumper *et al.*, 1998; MacLaughlin *et al.*, 1998). Consequently, it is hoped that the phenomena of complex formation, DNA condensation and stabilisation can be utilised in the field of drug delivery. This phenomenon may help facilitate the transit of DNA through different body compartments which may contain a high concentration of nucleases (Bielinska *et al.*, 1997; Smith *et al.*, 1997).

If histones are exchanged for an alternative polybase such as poly(L-lysine), DNA condensation is again observed (Chapter 4). Many factors have been

reported to influence the physical properties of the complex, (Kabanov and Kabanov, 1995; Shapiro *et al.*, 1969) and it is advantageous to have a screening process that may indicate the optimal complex formation rather than use a pharmacological or therapeutic end point (Kabanov *et al.*, 1993: Kabanov and Kabanov, 1995).

Several methods of assessing DNA condensation and complex formation are available. These include monitoring the degree of ethidiumbromide exclusion, (Kortenkamp *et al.*, 1992; Wolfert and Seymour, 1996) monitoring the susceptibility of DNA to enzymatic degradation (Chiou *et al.*, 1994; Bielinska *et al.*, 1997), and examining changes in DNA electrophoretic mobility as a function of complex formation (Wolfert *et al.*, 1996; Tang *et al.*, 1996). *In vitro* screens for transfection potential are also available using marker genes such as *Escherichia coli* β-galactosidase or fire fly Luciferase (Mumper *et al.*, 1998; Zanta *et al.*, 1997). When comparing transfection efficiencies it is important to remember that no "gold standard" exists, however this problem is discussed in Chapter 7.

Unfortunately, there are also disadvantages associated with polycationic systems. These include vector toxicity and rapid liver clearance following systemic administration (Pimm *et al.*, 1995; Seymour *et al.*, 1998). Polycation cytotoxicity has been observed in a molecular weight- and concentration-dependent manner (Wolfert and Seymour, 1996; Sgouras, 1990). This has been reported to be a function of polymer charge, molecular weight and counter ion (Carreno Gomez and Duncan, 1997; Sgouras, 1990). The specific mechanisms of toxicity are well documented and are as varied as polymer-mediated ion channel dysfunction, at very low concentrations of polybase (Rink *et al.*, 1994) to polymer-mediated membrane perturbation (Kachalsky *et al.*, 1959). In the instance of poly(L-lysine), membrane damage has been reported to be due to a polymer coil–α helix transition following membrane association leading to membrane rupture as a result of sheer stress (Hartmann and Galla, 1978).

In view of the aforementioned disadvantages, synthetic polymeric vectors have many potential advantages over viral and lipidic vectors. It is possible to

reduce the toxicity of the cationic vector by altering the chemical backbone of the polymer (Heller *et al.*, 1996; Ferruti *et al.*, 1997). It is also possible to manipulate the way in which the polymer interacts with the DNA by altering the chemical composition of the polymer, possibly allowing a modulation of the physical properties (Wolfert *et al.*, 1996) and possibly the pharmacokinetics of the complex. The inclusion of a targeting moiety (Wu and Wu, 1998) may also enhance cellular uptake *in vitro* (Zanta *et al.*, 1997; Wagner *et al.*, 1990; Wagner *et al.*, 1991). As polymeric vectors are synthetic, the problems associated with immunogenicity or wild-type reversion may also be circumvented. Table 1.7 has detailed the polymers most commonly used to facilitate the intracellular delivery of DNA. Fusogenic molecules have also been included in polymeric delivery systems. These molecules are thought to facilitate the endosomal release of the DNA into the cytosol and some of the more common ones are listed (Table 1.8) (Kichler *et al.*, 1997).

One possible way of circumventing many of the difficulties associated with polymer-mediated gene delivery is to abandon the idea of forming an ionic complex using polycations and attempt to utilise hydrogen bonding as the force mediating complex formation. Non-condensing complexes formed between PVP or PVA and DNA have been reported (Mumper *et al.*, 1998). As neutral polymers are less toxic and membrane lytic than polycations they may make better candidates as delivery vectors. Non-ionic, non-condensing complexes have been used to protect DNA from enzymatic degradation and facilitate transfection following intra-muscular (i.m.) injection. This route of administration achieves only local transfection, which has also been shown using naked plasmid DNA (Dowty and Wolf, 1994). This system (Mumper *et al.*, 1998) is, as it exists at this time, not suitable for systemic administration.

1.6 Chitosan and poly(amidoamine)s

Chitin or poly-β-(-1-4-*N*-acetyl-D-glucosamine) is a biologically abundant naturally occurring polysaccharide, which has been reported to be hydrolysed *in*

Table 1.7 Polymers used for transfection

Polymers	Comments	References
Polycations		
Histones	Immunogenic, not very efficient	Wagner *et al.*, 1991
DEAE Dextran	Toxic Not very efficient	McCutchan and Pagano, 1968
Poly(L-lysine)	Toxic	Wagner *et al.*, 1990; Wu and Wu, 1988; Wolfert and Seymour, 1996
Poly(D-lysine)	Toxic	Wagner *et al.*, 1991
Poly(ethyleneimine)	Toxic	Boussif *et al.*, 1995
PAMAM dendrimers	Toxic	Tang *et al.*, 1996
pDMAEMA	Toxic	Van de Wetering *et al.*, 1997
Polybrene	Not very efficient	Wagner *et al.*, 1990
Protamine	Not very efficient	Wagner *et al.*, 1990
HPMA-pTMAE	Reduced toxicity	Wolfert *et al.*, 1996
PEG-poly(L-lysine)	Reduced toxicity	Wolfert *et al.*, 1996
Poly(L-lysine) - grafted Dextran	Reduced toxicity	Maruyama *et al.*, 1996
Terplex Systems	(LDL/poly(L-lysine)	Kim *et al.*, 1997
Neutral polymers		
PVA and PVP	Hydrogen bonding rather than ionic interactions	Mumper *et al.*, 1998

Table 1.8 Membrane disruptive agents

Substance	Reference
Melittin	Perez-Paya *et al.*, 1997; Kitchler *et al.*, 1997
Immunotoxins i.e. ricin	Oeltmann and Heath, 1979
Bacterial toxins i.e. diptheria toxin	Pappenheimer and Gill, 1973
Viral capsid proteins i.e adenovirus	Wagner *et al.*, 1992[b] Kitchler *et al.*, 1997
Synthetic peptides (i.e. GALA)	Nicol *et al.*, 1996 Kitchler *et al.*, 1997 Thomas *et al.*, 1995 Fattal *et al.*, 1994
Poly(ethylenimine)	Boussif *et al.*, 1995

vivo by lysozyme (Muzzarelli *et al.*, 1988). Chitosan is produced by the deacetylation of chitin and has been seen to be composed of the monomer units *N*-acetyl glucosamine and glucosamine, (Aspden *et al.* 1996) (Figure 1.6).

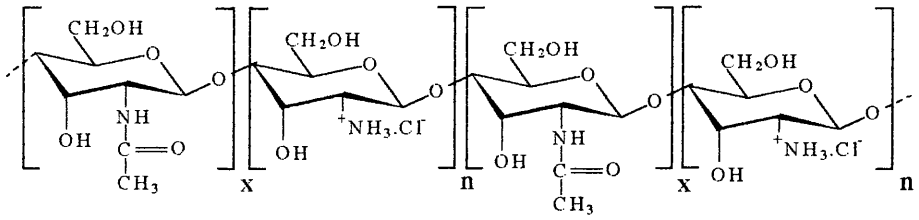
Although chitosan is polycationic, it has been widely reported to be biocompatible within the context of oral delivery and has been approved as a pharmaceutical excipient in Japan. It has also been reported to be suitable as a wound dressing and also as a part of hair and skin care products (Bersch *et al.*, 1995; Hirano *et al.*, 1988; Muzzarelli *et al.*, 1988). In the instance of systemic administration very highly deacetylated, high molecular weight products have been reported to be toxic in a concentration, charge, counter-ion and molecular weight dependent manner (Carreno-Gomez and Duncan, 1997). If the molecular weight and degree of deacetylation is reduced then the cytotoxicity of the molecules is also decreased (Ferruti *et al.*, 1997; Heller *et al.*, 1996). In view of this, the low molecular weight, highly purified chitosan molecular weight fractions, demonstrating a low degree of deacetylation were prepared and characterised by TRANSGENE SA for use herein and are described in Table 1.9.

Poly(amidoamine)s

Historically, poly(amidoamine)s have been used as heparin-complexing agents during hemodialysis as poly(amidoamine)s have been used in de-heparinising filters (Ferruti *et al.*, 1985; Ferruti, 1996). More recently the possibility of using a biocompatible poly(amidoamine) as a drug delivery vehicle has been proposed (Duncan *et al.*, 1994; Sgouras, 1990).

Poly(amidoamine)s are synthetic, water soluble, non-branching, biodegradable polymers having defining amido and amino groups arranged regularly along a macromolecular chain (Duncan *et al.*, 1994; Ranucci *et al.*, 1991). This attribute can mediate a change in the tertiary conformation of the polymer in a pH-dependent manner and has been shown by viscometry. The mechanisms responsible for the change in poly(amidoamine) tertiary conformation in response to a change in pH are as follows:

Figure 1.6 Structure of chitosan



N.B. Ratio of n:x determines the degree of deacetylation

Table 1.9 Physical properties of chitosans N1-3

Code Number	Substance.	Degree of deacylation	Mw (Da)
N1	Chitosan	65.36 %	< 5 000
N2	Chitosan	55.25 %	5 - 10 000
N3	Chitosan	55.25 %	>10 000

1) The poly(amidoamine) polymer chain in aqueous neutral solution exists as a random coil (Ferruti *et al.*, 1985). Following the first protonation, strong hydrogen bonding between charged amonium ions and carbonyl groups belonging to the same monomeric unit has been reported to limit conformational freedom of the polymer coil.

2) Then a second dramatic increase in viscosity is observed after the second protonation when positively charged amonium ions repel each other increasing the hydrodynamic volume of the polymer coil (Ferruti *et al.*, 1985).

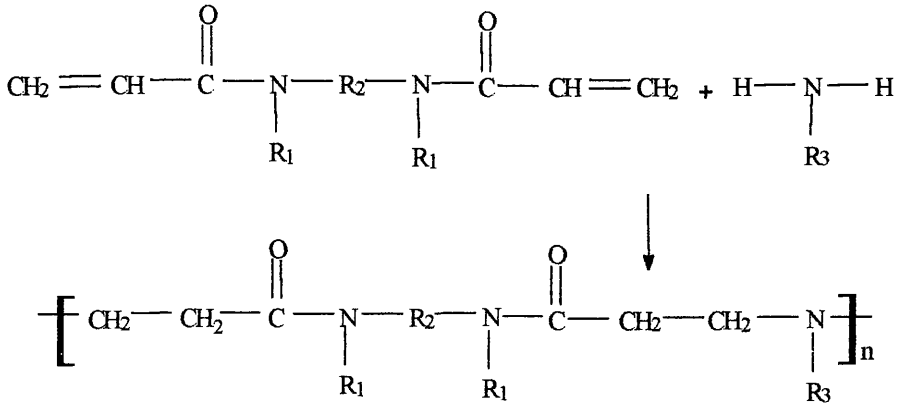
This attribute may provide an ideal opportunity to design polymer conjugates which can be triggered to undergo a pH-dependent conformational change within the endosomal and lysosomal compartments of the cell (pH 5.5-6.5) (Richardson *et al.*, 1998; Duncan *et al.*, 1994; Ranucci *et al.*, 1991). This may in turn provide the opportunity to hide or protect molecules from enzymatic or immunogenically mediated attack as previously described in Duncan *et al.*, (1994).

Poly(amidoamine)s are formed through the polyaddition of primary monoamines or bis-secondary amines to bis-acrylamides as shown (Figure 1.7). The anhydrous poly(amidoamine)s salt are reported to be stable in air and the presence of hydrolytically labile bonds in the main chain may enable the renal excretion of high molecular weight poly(amidoamine)s. The structures of the poly(amidoamine)s investigated here in are shown (Figure 1.8 and 1.9) along with there specific physical properties (Tables 1.10 and 1.11). The pKa's associated with these molecules have been documented (Ferruti, 1996; Ferruti *et al.*, 1998) ISA 1, 4 and 9 are more cationic at neutral pH than ISA 22 and 23. This is a consequence of the spatial arrangement and acyclic nature of some of the secondary amines in the ISA 1, 4 and 9 backbone relative to ISA 22 and 23.

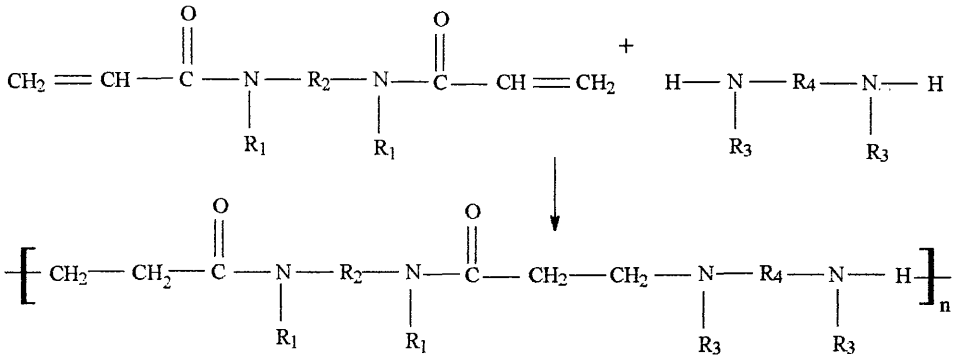
1.7 Aims of this thesis

The experimental aims of this thesis may be summarised as follows. The first objective was to evaluate the biocompatibility of both the poly(amidoamine)s

Figure 1.7 Poly(amidoamine) synthesis by polyaddition



A) Polyaddition of monoamines to bis-acrylamides



B) Polyaddition of bis-secondary amines to bis-acrylamides

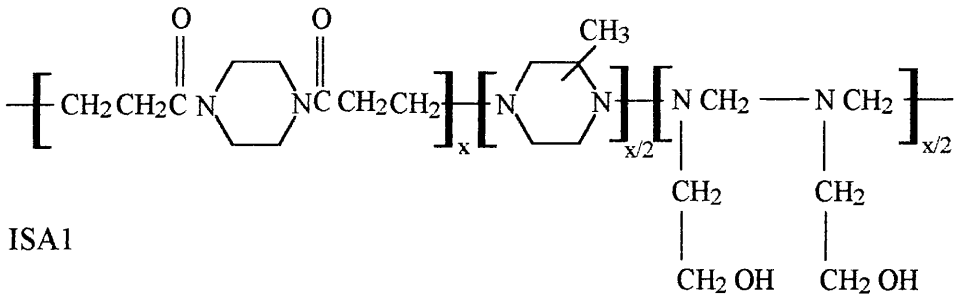
Table 1.10 Physical properties of ISA1, 4 and 9

Polymer	Mw (Da)	Mn (Da)	Monomers
ISA 1	8700	5000	(BAP) (1 Mol) (2-MP) (0.5 Mol) (BHEDA) (0.5 Mol)
ISA 4	8000	4700	(BAP) (1 Mol) (2-MP) (0.47 Mol) (BHEDA) (0.5 Mol) (Tyramine) (0.03 Mol)
ISA 9	6300	3900	(BAP) (1 Mol) (2-MP) (0.5 Mol) (GA) (0.075 Mol) (Tyramine) (0.03 Mol) (BHEDA) (0.5 Mol)

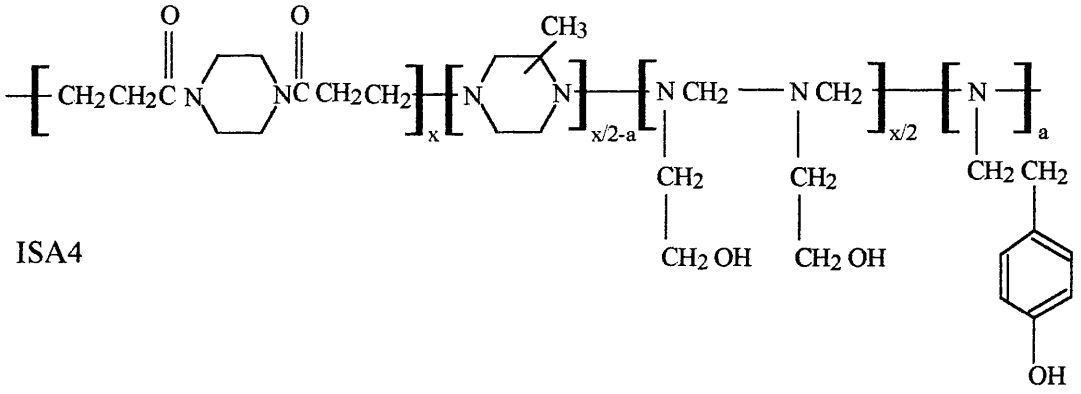
Mw (weight average molecular weight) and Mn (Number average molecular weight) were determined by gel permeation chromatography (GPC)

1,4-bis(acryloyl)piperazine (BAP)
2-methylpiperazine (2-MP)
N,N'-bis(2-hydroxyethyl)ethylenediamine (BHEDA)
galactosamine hydrochloride (GA)

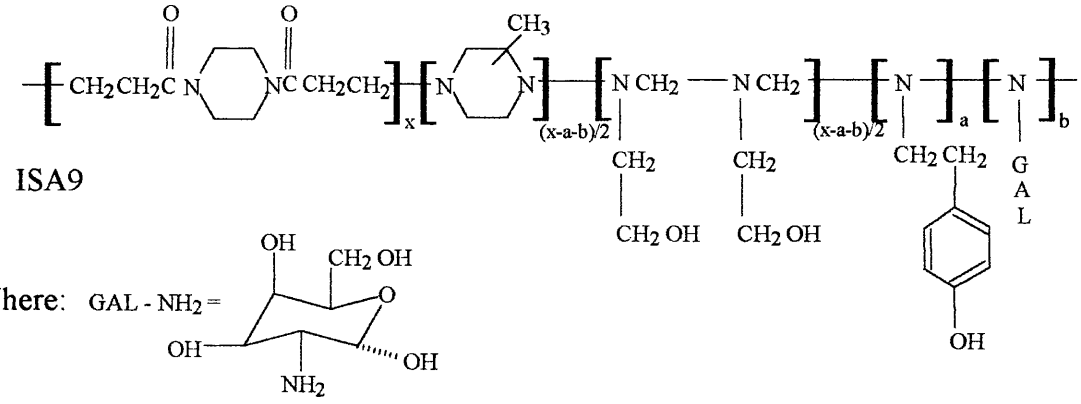
Figure 1.8 Structures of poly(amidoamine)s ISA1, 4 and 9



ISA1



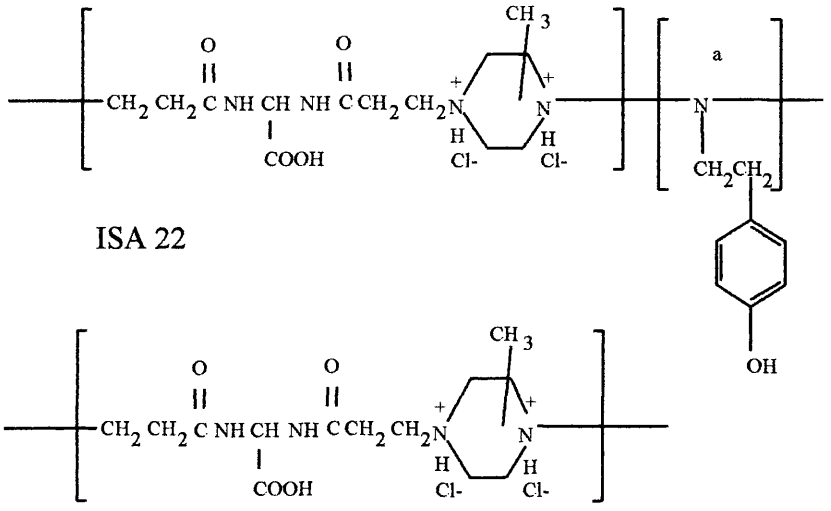
ISA4



ISA9

Where: GAL - NH_2 =

Figure 1. 9 Structure of poly(amidoamine)s ISA 22 and 23



ISA 22

ISA 23

Table 1.11 Characteristics of poly(amidoamine)s ISA22 and 23

Name	Mn (Da)	Monomers
ISA 22	8 500	2,2 bis(acrylamide)acetic acid morpholine Tyramine (2-3 Mol%)
ISA 23	10 500	2,2 bis(acrylamide)acetic acid morpholine

Mn (Number average molecular weight) calculated by GPC

and also the chitosan molecules *in vitro* (Chapter 3). This is the most vital part of the evaluation of any potential therapeutic carrier, as it may define the subsequent application of the material. Following this the membrane lytic properties of the poly(amidoamine)s were evaluated at pH 7.4 6.5 and 5.5 indicative of the systemic circulation, primary endosome and the secondary lysosomes respectively. This study was undertaken to evaluate the possibility of polymer mediated pH-dependant membrane lysis so affecting cytosolic access via endosomolytic delivery (Chapter 3). For this possibility to be realised a polymer candidate would have to show little membrane lytic activity at neutral pH and become very lytic at pH 6.5 following internalisation.

After the evaluation of polymer biocompatibility, it was considered important to determine whether the polymers could mediate the formation of a polymer:DNA complex capable of protecting the DNA portion from nuclease degradation (Chapter 4). This may allow the delivery of a functional portion of DNA to the target cell type via the systemic circulation. Further to this the nuclear delivery of the gene may be possible via a lysosomotropic pathway.

If a drug delivery system is to be directed in a tissue-tropic manner, it must display an appropriate body distribution, as rapid clearance into liver or lung often restricts the ability to target to other tissues. In view of this, the inherent body distribution of ^{125}I-labelled chitosans N1-3 (Chapter 5) and ^{125}I-labelled poly(amidoamine)s was determined in the rat (Chapter 6). In the case of the poly(amidoamine)s, experiments were performed using both the ^{125}I-labelled poly(amidoamine) and also their DNA complexes. As a poly(amidoamine) (^{125}I-labelled ISA 22) was identified which did not immediately localise in the liver, the tumour accumulation of ^{125}I-labelled ISA 22, was also examined in a mouse model (Chapter 6). The tumour accumulation experiments were performed using either native ^{125}I-labelled ISA 22 or ^{125}I-labelled ISA 22 administered as a DNA containing complex.

Finally, as poly(amidoamine) were identified that met some of the fundamental criteria needed for a non-viral vector, it was considered important to obtain preliminary data on their transfection efficiency using a standard *in vitro* assay. Briefly, this assay monitored the transfection of Hep G2 with a marker gene (β-galactosidase). Cytotoxicity in response to transfection was also evaluated by trypan blue exclusion (Chapter 7).

Chapter 2

Materials and Methods

2.1 Materials

Equipment

The UV transilluminator was supplied by Scientific Laboratory Supplies, (Nottingham, UK) and Sigma, (Dorset, UK) supplied the Polaroid Gel Documenting System and the benzoylated dialysis tubing. The Rio-Gamma 1274 counter was supplied by LKB-Wallack (London, UK) and the orbital shaker by Fischer (Leicestershire, UK). Amersham Pharmacia Biotech, (Hartfordshire, UK) supplied the PD10 columns and the paper electrophoresis was performed in a Shandon paper electrophoresis tank supplied by Scientific Laboratory Supplies (Nottingham, UK). The microtitre plate reader was a Titerteck Multiskan plus, supplied by EFLAB (Finland). L.I.P services and equipment Ltd., (West Yorkshire, UK) supplied the heparin/lithium blood tubes. The gold deposition for the scanning electron microscopy was performed using a K550 supplied by EMETECH Ltd. (Kent, UK). The scanning electron microscopy was performed using a XL 20 series scanning electron microscope supplied by PEO (Eindhoven, The Netherlands). All general reagents were from BDH (Ontario, Canada) or Sigma (Dorset, UK) and were of analytic reagent grade.

Organisms and cells

Gibco BRL (Paisley, UK) supplied competent *Escherishia coli* DH5α cells and Bantin and Kingman Ltd., (Hull, UK) supplied the C57 black, male, mice and the male Wistar rats. The B16 F10 cells were kindly donated by Prof. I. Hart (St. Thomas's Hospital London, UK) and the L132, Hep G2, CCRF-CEM and Mewo cells were from ECCAC (Wiltshire, UK). The following tissue culture grade items were supplied by Sigma (Dorset, UK): DMSO, MTT, Trypan blue and optical grade DMSO. Gibco BRL, (Paisley, UK) supplied all foetal calf serum, RPMI 1640, MEM, E199 and the non-essential amino acids (NEAA).

Specialised reagents

The following reagents and enzymes were purchased as molecular biology grade products from Sigma (Dorset, UK): glucose, isopropanol, tris-base, tris-Cl,

chloroform, lithium chloride, EDTA (di-sodium salt), glycerol, agarose, sodium hydroxide, SDS, sodium chloride, potassium acetate, ethidiumbromide, ammonium acetate, phenol: chloroform: isoamyl alcohol (25:24:1), RNase A and lysozyme. Restriction buffer C, *Bam* H1 and *Vsp* I were supplied by Promega (Southampton, UK) as were the β-galactosidase assay kit and the plasmid pSV-β-galactosidase. The LipofectACE and λ *Hind* III DNA were from Gibco BRL (Paisley, UK) and the calf thymus DNA from Sigma, (Dorset, UK). Medical grade oxygen, nitrogen and carbon dioxide (All 95% (v/v)) and the liquid nitrogen were supplied by BOC (Surrey, UK). The Isoflurane was supplied by Abbott Labs (Kent, UK) and Amersham Pharmacia Biotech (Hartfordshire, UK) supplied all of the radioisotopes.

Polymers

The following polymers were all from Sigma (Dorset, UK): PEG Mw 8 000 Da, Dextran Mw 72 000 Da, poly(L-lysine) Mw 56 500 Da, poly(L-lysine) Mw 47 700 Da and poly(L-lysine) Mw 260 000 Da. The HPMA copolymer tyrosinamide was a kind gift from Dr K. Ulbrich, (Prague, Czech Republic). Chitosans N1-3 were supplied by TRANSGENE SA (Strasbourg, France) and all the poly(amidoamine)s were supplied by Prof. P. Ferruti, (Milan, Italy).

2.2 Methods

2.2.1 Cell culture and viability techniques

Cells were maintained in an atmosphere of 5%(v/v) CO_2 at 37°C in a humidified CO_2 culture incubator and these conditions were defined as standard. All manipulations were carried out aseptically in a Class II tissue culture hood. All materials added to cell cultures were sterile, osmotically balanced and heated to 37°C. The cells were maintained in 75cm^2 tissue culture treated, cantered neck flasks with vented (0.2μm) tops and were passaged once every seven days. This maintained the cells in the exponential phase of growth.

For cells grown as a monolayer, the media was removed by aspiration and the cells washed twice with phosphate buffered saline (PBS) (10ml). Cells were

removed from the flask by the addition of 1x trypsin, ethylenediaminetetraacetate (EDTA) (1ml). After incubation (approximately 3-5min) and gentle agitation, the monolayer was examined microscopically (x100) using an inverted microscope. Once the majority of cells were seen in suspension, media (9ml) containing 10% foetal calf serum (FCS), was added to dilute the trypsin (Table 2.1 lists cell culture conditions). The culture was then disaggregated by gently passing the suspension through a sterile 21-gauge needle attached to a sterile 20ml syringe. This was done three or four times prior to microscopic examination. Care was taken to avoid the over exposure of cells to shear stress. Aliquots of the suspension were used to seed further flasks at a density dictated by the generation time and hence split ratio of the cell line and residual cells were autoclaved. Suspension cultures were split by removing and discarding a proportion of the culture by aspiration. The media that was removed was then replaced with fresh media. The proportion of the cultures that was discarded was dictated by the split ratio of the cell line (Table 2.1).

Cell bank

A stock of cells was stored frozen at –80°C or in liquid nitrogen vapor. First $5x10^6$cells/ml were prepared in 90%(v/v) FCS and 10%(v/v) dimethylsulphoxide (DMSO), sterilised by filtration (0.2 μm sterile filter). The cells were then disaggregated as detailed previously. A volume of 1-1.6ml of suspension was then transferred into a sterile cryogenic vial and this was placed in an insulating polystyrene box and placed at -80°C for 18h insuring a uniform drop in temperature (~1°C/min). Following this the vials were either placed in liquid nitrogen vapour (-192°C) or left at -80°C.

To recover cryogenically preserved cells the vials, left with the lid one-quarter turn open, were placed at 37°C inside a 30ml sterile universal bottle. This was left until the preparation was visually seen to thaw (5min). Following this the suspension was subject to centrifugation at 1 500xg for 10min at 22°C. The supernatant was then removed and the cells re-suspended in the appropriate cell culture media (10ml) (Table 2) and used to seed a 75cm² flask.

Table 2.1 Cell lines and culture conditions

Cell line	Name	Adherent	Split Ratio	Supplier	Culture media (All from Gibco)
B16 F10	Murine Malignant Melanoma	Yes	1:20	Prof. I. Hart, St Thomas's Hospital London UK.	10% (v/v) FCS, RPMI 1640 25mM HEPES 5mM L-glutamine
Mewo	Human Malignant Melanoma	Yes	1:7	ECCAC	E-MEM, 10% (v/v) FCS 5mM L-glutamine
Hep G2	Human Hepatocellular Carcinoma	Yes	1:5	ECACC	1 % (v/v) NEAA 25mM HEPES, 10% (v/v) FCS E-MEM, 5mM L-glutamine 400µl 1N NaOH / 100ml media
CCRF-CEM	Human Lymphoblastic leukaemia	No	1:10	ECCAC	RPMI 1640 10% (v/v) FCS 5mM L-glutamine
L132	Human Embryonic Lung	Yes	1:10	ECCAC	E199, 5mM L-glutamine 5% FCS (v/v)

Evaluation of cell viability by trypan blue exclusion

The trypan blue, supplied as a sterile 4%(w/v) solution in PBS, was first diluted 1:1 with PBS to a concentration of 2%(w/v). One volume of 2%(w/v) trypan blue solution (in PBS) was added to a 20µl aliquot of the cell suspension. The average number of blue (dead) and clear (viable) cells was estimated using an improved Neubauer haemocytometer. The number of cells in a known volume (0.1 x 0.1 x 0.1mm) was then recorded. The percentage of viable cells was then calculated as well as the total number of viable cells/ml (Cells/ml = No. Cells in 0.1 x 0.1 x 0.1mm volume x dilution factor (from stock) $x10^4$). Care was taken not to overload the haemocytometer and the mean of five samples was used when estimating cell number.

MTT assay to assess cell viability

Adherent cells were seeded into separate, sterile, flat-bottomed 96 well, tissue culture treated plates at a density of $5x10^4$ cells/well (B16 F10 cells at $1x10^4$ cells/well). The culture was then left to incubate for 24h in standard conditions. Prior to the end of the 24h incubation period, the polymers to be assayed were dissolved in fresh culture medium as described in Chapter 3. This was used to replace the existing media covering the cells after the designated incubation period and concentrations between 0-5mg/ml were used. The cultures were then incubated using standard conditions for 67h. During this time, 250mg of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazollium bromide (MTT) was dissolved in 50ml of PBS and filter sterilised. After the 67h incubation, 20µl of MTT stock was added to each well giving a final concentration of 833µg/ml of MTT in the media. The experiment was then left to incubate for a further 5h again using standard conditions. After a further incubation period of 5h the culture media was removed and 100µl of optical grade DMSO was added to each well. The cultures were left for 1h in DMSO and the plates were read at 550nm using a microtitre plate reader. The results were expressed as viability (%) (± standard deviation (S.D.)) against polymer concentration.

Cultures in suspension were incubated for 24h after seeding at the same density as adherent cultures. Prior to the addition of the polymer solution, the cells were centrifuged at 1 000xg for 10min at room temperature and the supernatant removed. The pellet of cells was then re-suspended in media containing the appropriate concentration of polymer and replaced in the incubator for 67h prior to the addition of MTT as before.

Following the 72h incubation the plate was again subject to centrifugation as before and the supernatant removed. The cell pellet was then re-suspended in optical grade DMSO and treated as a monolayer culture. The results were expressed as before.

2.2.2 Red Blood Cell (RBC) lysis assay

Fresh blood was obtained from Male Wistar rats (~250g body weight) through cardiac puncture following CO_2 asphyxiation and was collected in heparin/lithium blood tube. Erythrocytes were isolated by centrifugation at 1 000xg for 10min at 4°C and the supernatant discarded along with the very top of the pellet i.e. the top 3-5mm (Sgouras, 1990). Next, a fresh disposable centrifuge tube was weighed and the weight recorded. Following this the RBC were re-suspended in PBS which had been pre-chilled to 4°C, transferred to the pre-weighed centrifuge tube and re-pelleted by centrifugation. The supernatant was again discarded and the washing process repeated. Following the final centrifugation step the supernatant was again removed. As the weight of the pellet was known, it was possible to re-suspend the cells in a volume of PBS appropriate to a 2%(w/v) RBC suspension.

Following this a 100µl volume of a x2 concentration of polymer solution was added to one volume of the 2%(w/v) RBC suspension. This was performed in 96 well ELISA plates. Following this the preparation was incubated for the desired time period at 37°C in a humidified environment. Using PBS instead of polymer solution generated the negative control. A 1%(v/v) solution of triton-X-100 was used to solublise the RBC membranes and release 100% of the haemoglobin present (Duncan *et al.*, 1994).

After the incubation period, the degree of haemoglobin release was assessed by subjecting the ELISA plates to centrifugation at 1 500xg for 15min at room temperature. Next the supernatant was removed and placed into a clean ELISA plate. The absorbency of the supernatant was then measured at 550nm to assess the degree of haemoglobin release relative to the triton-X-100 control. The results were expressed as haemoglobin released (%±S.D.) relative to polymer concentration.

2.2.3 Scanning electron microscopy of cells in culture and RBCs

Cells were grown on sterile glass coverslips in 6 well sterile, cell culture coated plates and were exposed to the polymers at various concentrations over the specified time periods. The cells were then examined using scanning electron microscopy (SEM) after a preparatory step as follows: Cells incubated with varying concentrations of polymer were washed in PBS and incubated overnight in 0.25%(w/w) electron microscopy grade glutaraldehyde diluted in PBS. After this the cells were subject to a further incubation in 1%(w/v) osmium tetroxide also diluted in PBS for 1h at room temperature. Following the removal of the osmium tetroxide, the cells were incubated in 50%(v/v) ethanol made up in sterile distilled water for 30min. This step was then repeated with 60%(v/v) ethanol, 70%(v/v) ethanol, 80%(v/v) ethanol, 90%(v/v) ethanol, absolute ethanol, acetone and hexamethyl disilazane (HMDS). The HMDS was allowed to evaporate and the samples were mounted upon SEM platform using carbon cement and coated in gold. This was performed using an EMETECK E550 set at 50μA for 5min. The samples were examined using SEM.

RBCs were incubated with various concentrations of polymer (0-5mg/ml) as previously described. The preparations were then transferred into microfuge tubes and subject to centrifugation (1200xg for 1min at room temperature) and the supernatant discarded. A 0.25%(v/v) solution of glutaraldehyde diluted in PBS was then added to the pellet, which was re-suspended by gentle aspiration. The preparation was then left at room temperature for 18h. The cells were then subject

to centrifugation as before and the supernatant removed. After this the cells were incubated in 1%(w/v) osmium tetroxide also diluted in PBS. The cells were then subject to gentle aspiration and left for 1h at room temperature. The cells were then dehydrated as before with the omission of the acetone step, coated in gold and examined as before (Audrey-Glavert, 1975).

2.2.4 Gel retardation assay to measure DNA:polymer interpolyelectrolyte complex formation

Except where otherwise stated, all polymers were dissolved in 0.9%(w/v) sterile sodium chloride at room temperature and left for 5min to dissolve. The λ *Hind* III DNA was also diluted in 0.9%(w/v) sterile saline to a concentration of 0.1mg/ml and was stored at –20°C. The polymer samples were prepared on the day of the experiment. In each instance, where charge or weight ratios are quoted, the proportion of DNA is quoted first in each instance. The formation of interpolyelectrolyte complexes (IPEC) was performed at room temperature and the complexes were allowed to form for 30min prior to evaluation. In the case of chitosan, the complexes were formed on the basis of charge to charge ratio. Poly(amidoamine):DNA complexes were prepared in exactly the same manner as the chitosan:DNA complexes with the exemption of the charge ratio. Poly(amidoamine):DNA complexes were formed on the basis of weight. Poly(L-lysine) complexes were formed on a charge to charge basis. Dextran complexes were evaluated on a weight to weight basis.

Electrophoresis was performed using a 0.5%(w/v) agarose gel containing 0.25μg/ml ethidiumbromide. The agarose gel was made in 1xTAE buffer (50xTAE: 242g Tris base, 57.1ml glacial acetic acid, 100ml EDTA pH 8, adjust to pH 7.2 and bring final volume to 1000ml with distilled water, autoclave) and the samples were subject to electrophoretic separations at 100V for 60min. They were then visualized using a UV transilluminator and photographed using a Polaroid gel documenting system. A λ *Hind* III digest (1μg/well) was used as a marker. Reference polymers poly(L-lysine) (Mw 260 000Da, 47 700Da and 3 900Da) and dextran (Mw 72 000Da) were also used. In each instance 1μg DNA

equivalent/well was loaded into the gel. Loading was facilitated using a gel loading buffer (6x gel loading buffer, 10mM Tris-HCl, pH 7.5, 50mM EDTA, 10% ficol 400, 0.25%(w/v) bromophenol blue and 0.25%(w/v) xylene cyanol).

2.2.5 Evaluation of DNA nuclease stabilisation by complex formation

Calf thymus DNA was dissolved at a concentration of 1mg/ml in 0.2N sodium acetate buffer pH 5.5 supplemented with 0.2N potassium chloride and left for 24h at 4°C. The following day 1ml of the DNA solution was diluted to 100μg/ml also in sodium 0.2N sodium acetate buffer pH 5.5 supplemented with 0.2N potassium chloride and heated to 37°C. To this, 300U/ml of DNase II (EC 3.1.4.6 isolated from porcine spleen) was added. The incubation mixture was then re-placed at 37°C. Immediately, i.e. at time 0, 2x500μl aliquots were removed which served as blanks. To each of these, 500μl of 4°C 10%(v/v) perchloric acid was added and the samples incubated for 30min on ice prior to centrifugation (1 200xg for 20min at room temperature). The supernatant was removed and its absorbency (260nm) recorded, denoting the quantity of degraded DNA in the sample. Samples were taken at 1, 5, 15, 30, 45 and 60min from the beginning of the incubation period, processed as before and the proportion of degraded DNA measured spectrophotometrically (Barret and Heath, 1977). Following this the experiments was repeated with the addition of the polymer. In this instance polymer:DNA complexes were allowed to form as previously detailed prior to the addition of the DNase II. Constant reaction volumes and proportions were maintained between all experiments.

Results were expressed as DNase II inhibition (%) relative to a control containing no polymer. This was done by expressing the area under the curve (calculated by trapezoid approximation and extrapolation to zero) of the polymer containing experiment as a percentage of the area under the curve of a control and subtracting the result from 100%.

2.2.6 Radioiodination of polymers

The chloramine T method

HPMA copolymer tyrosinamide, ISA 4, 9 or 22 were individually dissolved (10mg/ml) in 0.1M phosphate buffer pH 7.4 (0.5 N Na_2HPO_4 and 0.5 N NaH_2PO_4) (0.5ml) and placed in separate glass vessels. Sodium [^{125}I]iodide (500μCi) (5μl) was added to each preparation and allowed to stand for 2min to equilibrate. The viability of the chloramine T solution (2mg/ml in phosphate buffer pH 7.4) was checked by placing a drop on a crystal of potassium iodide and watching for a brown colour change. If this was observed then chloramine T solution (75μl in each) was added to the reaction and left for 15min under gentle stirring conditions. Then sodium metabisulphate (500μl of a 2mg/ml solution in phosphate buffer pH 7.4) and a crystal of potassium iodide was added and the reaction mixture left for a further 2min. An aliquot (5μl) of the reaction mixture was then removed for the subsequent determination of labelling efficiency of the preparation. The reaction mixture was placed in benzoylated dialysis tubing and dialysed against 1%(w/v) sodium chloride until no radioactivity (< 20cps) was found in the dialisate. The purity and labelling efficiency of the final preparation were then determined by paper electrophoresis. The specific activity (μCi/mg) of the preparation was then calculated.

The Bolton and Hunter reagent method

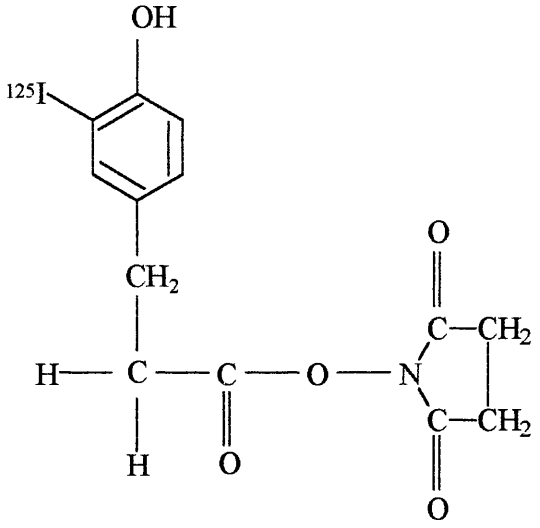
First, Bolton and Hunter reagent (500μCi) was dispensed into a glass vial and this process repeated for each polymer preparation to be labelled. Following this the solvent (dry benzene/2%(v/v) dimethylformamide) was removed from the Bolton and Hunter reagent in a fume hood using a stream of nitrogen. Next, 5mg of the chitosans N1-3 were dissolved separately in three 500μl aliquots of 0.1M borate buffer pH 6.5 (3.094g boric acid, 3.728g potassium chloride and made up to 1000ml with distilled water (pH correction with sodium hydroxide)). Following this 97.5μl of trimethylamine was added to each preparation. The polymer preparations were then added to the dry Bolton and Hunter reagent and allowed to stir on ice for 15min. The preparation was then saturated with potassium iodide

and 5µl of the reaction mixture removed for the determination of labelling efficiency. The labelled polymer was then placed in dialysis tubing as previously described and dialysed against 1%(v/v) hydrochloric acid until no radioactivity was found in the dialysate. The labelling efficiency and purity of the ^{125}I-labelled polymers was then calculated using paper electrophoresis and the specific activity of each polymer determined (µCi/mg). The polymers were then bottled and stored at 4°C. The structure of Bolton and Hunter reagent and the predicted structure of the ^{125}I-labelled chitosan are shown in Figure 2.1 and 2.2 respectively.

Characterisation of radioiodinated polymers by paper electrophoresis

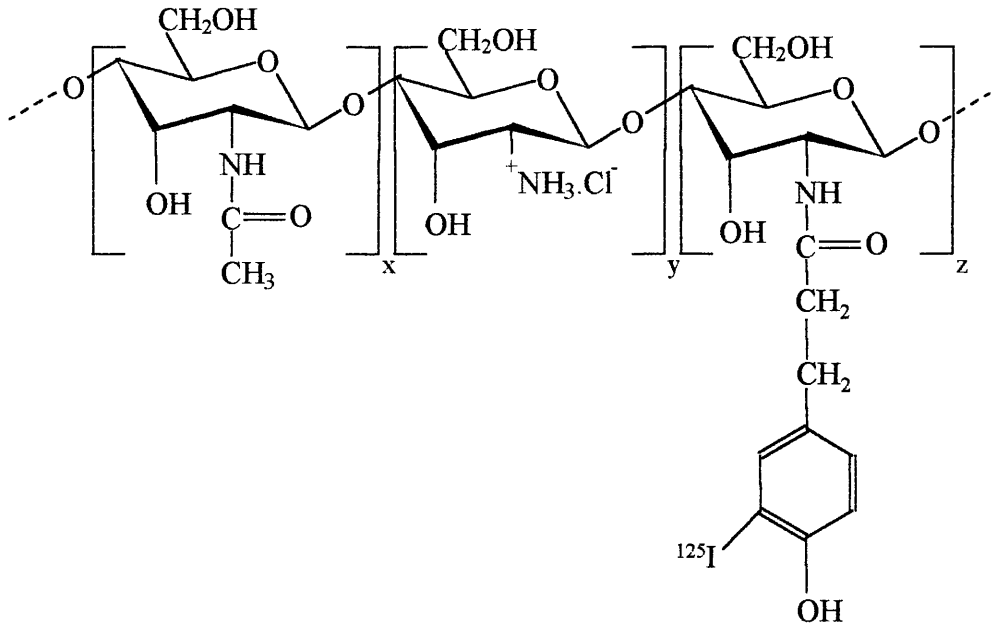
First, a 5x30cm strip of chromatography paper was cut to size and the central portion divided into 5mm strips by pencil lines (40 strips). The fifth strip was marked as the point for sample application and denoted the origin. Barbitone buffer (50mM sodium barbital and 10mM barbital) was then used to soak the chromatography paper, which was then blotted dry. The same buffer was then placed in the paper electrophoresis tank and the paper strips put in place across the supporting bars. The sample application point was placed nearest the anode. As a reference control, either very dilute free sodium [^{125}I]iodide (4µl) (Figure A1.2) or Bolton and Hunter reagent left in aqueous solution for 24h+ (Figure A1.3) was loaded onto the origin of the first strip. Similarly, for each polymer (crude reaction mixture and the purified preparation) 4µl was loaded onto the origin of individual paper strips. The tank was then connected to a power supply and the samples were run at 400V for 30min. The chromatography paper strips were removed, the marked 5mm strips cut out and placed into counting tubes with 1ml of water. These samples were then assessed for radioactivity using a gamma counter. The results were plotted as counts per minute (cpm) against distance migrated and the amount of [^{125}I]iodide present as free and bound radioactivity calculated in each instance. The area under the curve for each peak was calculated using trapezoid approximation and extrapolation to zero. Specific activities were calculated for each polymer preparation and expressed as µCi/mg assuming a 100% recovery from dialysis.

Figure 2.1 Structure of Bolton and Hunter reagent



N-succinimidyl 3-(hydroxy 5-[^{125}I] iodophenyl) propionate

Figure 2.2 Proposed structure of the radioiodinated chitosan



Evaluation of the stability of ^{125}I-labelled polymers by GPC (Gel permeation chromatography)

The stability of the ^{125}I-labelled poly(amidoamine)s ISA 4, 9 and 22 was examined by first incubating the ^{125}I-labelled polymers in MEM containing 10%(v/v) FCS at 37°C. At the appropriate time points, aliquots (1ml) were taken and applied to a PD10 column equilibrated in distilled water. Next, 40x0.5ml fractions were eluted from the column and collected in individual counting tubes containing 0.5ml of distilled water. The amount of radioactivity present in each fraction was then assayed for as before. The radioactivity of each fraction (cpm) was then plotted against retention volume (ml). Stability was further examined by freezing (-20°C) a sample of ^{125}I-labelled ISA 4, 9 or 22 in 1%(v/v) sodium chloride for 30 days, thawing the sample and analysing it, using PD10 GPC as before.

2.2.7 Body distribution of ^{125}I-labelled polymers

For chitosans N1-3, $5x10^6$ cpm of polymer was diluted into 1.5ml of 0.9%(w/v) sterile sodium chloride. The pH was then neutralised with saturated sodium hydroxide and the final volume made up to 2ml with 0.9(w/v) sterile sodium chloride. Of this sample, 200µl were withdrawn using a 1ml syringe and 12 gauge needle and counted as a measure of injected dose. Next 200µl ($5x10^5$ cpm) of each polymer was injected i.v. into the caudal tail vein of individual rats (3 rats per time point per polymer), anaesthetised using a mixture of 4%(v/v) oxygen and 2%(v/v) isoflurane. The tail of each rat was then checked for localised radioactivity and the rat placed in metabolic cage for 5, 60 or 300min. After the specified time, each rat was weighed and killed by CO_2 asphyxiation. The major organs (liver, lungs, heart, kidneys, spleen, thyroid, urine and blood) were then removed and dissolved in 5 or 10ml of 10N sodium hydroxide with 0.3%(v/v) Triton-X-100. The samples were then left to digest for 5 days or until the organs had dissolved. Triplicate samples (1ml) from each organ were then assayed for radioactivity. The results obtained were expressed as the quantity of [^{125}I]iodide

recovered (%)/organ and the blood volume of the rat was calculated assuming that there is 7.2ml of blood/100g body weight of rat (Duncan *et al.*, 1982).

2.2.8 Preparation of pSV-β-galactosidase

Transformation of E.coli DH5α cells with pSV-β-galactosidase

First the plasmid, (10ng) was diluted into 10μl of sterile TE pH 8 (10mM Tris pH 8, 1mM EDTA) and placed in a sterile 15ml round bottomed centrifuge tube cooled to 4°C. Then 200μl of rapidly thawed (heat pulsed) competent *Escherishia coli* DH5α cells were added and this preparation placed on ice for 30min. The cells were then incubated at 37°C for 5min prior to being placed on ice for a further 1min. After this procedure sterile LB broth (1ml) pre-heated to 37°C was added to the preparation, which was then left to incubate at 37°C for 1h. A control was also prepared as above, substituting the plasmid for TE buffer pH 8. After the final incubation period, the transformed preparation and the control were used to inoculate separate agar plates containing ampicillin (50μg/ml). These were then left at 37°C for 18h. A streak of colonies from the plate containing the transformed cultures were then stored in 70 %(v/v) sterile glycerol and 30%(v/v) LB broth and frozen (-70°C). The agar stock plates were then sealed and placed at 4°C.

Growth of transformed E.coli

A sterile aliquot of LB media (10ml) containing ampicillin (50μg/ml) was prepared and was used as a starting culture. Next LB media (1l) was prepared for the final plasmid culture and the starting culture (10ml) was inoculated by aseptically transferring 50μl of colonies from the previously prepared agar plate. This was left to incubate at 37°C until the optical density (600nm) reaches approximately 0.6 (6-8h at 37°C). The starting culture was then aseptically transferred into a baffled flasks (2l) containing the previously prepared, sterile LB broth (1l). This was incubated overnight in an orbital shaker set at 180rpm and 37°C.

Isolation of plasmid DNA from E.coli cultures

The cells were first pelleted by centrifugation at 4°C for 30min at 3 000xg. The cell pellets were re-suspended in 1xSTE buffer (1l) (0.1mM sodium chloride, 10mM Tris pH 8 and 1mM EDTA pH 8) (Maniatis *et al.*, 1986) and re-pelleted through centrifugation as before. Each pellet was then suspended (final volume of 10ml) in a solution of 50mM glucose, 25mM Tris pH 8 and 10mM EDTA pH 8. To this suspension 2ml of a lysozyme solution (25mg/ml made in 10mM Tris pH 8) was added followed by 20ml of freshly prepared 0.2N sodium hydroxide, 1%(w/v) sodium dodecyl sulphate (SDS) to produce a lysate. The lysate was then gently mixed and left to incubate at room temperature for 10min. Next 15ml of an ice cold preparation of solution A (60ml 5M potassium acetate, 11.5ml glacial acetic acid, and 28.5ml de-ionised double distilled water) was added and the preparation mixed and placed on ice for 10min. Following this the lysed bacterial preparation was centrifuged at 20 000xg at 4°C for 15min. The supernatant was then decanted, filtered through sterile cheesecloth, subject to precipitation with isopropanol (0.6 vol.) and left for 10min at room temperature.

The nucleic acids were then pelleted by centrifugation at 15 000xg for 30min at room temperature. The supernatant was disposed of and the pellet dissolved in TE pH 8 (3ml) after the remainder of the isopropanol had been removed.

The high molecular weight DNA was then precipitated by adding 1x volume of ice cold 5M lithium chloride followed by centrifugation at 15 000xg for 30min at 4°C. The supernatant was decanted into a fresh sterile tube, subject to precipitation with isopropanol and centrifuged as before. Following centrifugation the supernatant was removed, disposed of and the resulting pellet re-suspended in TE pH 8 (500µl) containing 20µg/ml RNase A. The sample was then transferred into a sterile 1.5ml Eppendorf tube and left at room temperature for a further 30min. Following this 1.6M sodium chloride containing 13%(w/v) PEG 8000 (500µl) was added and the plasmid DNA harvested by centrifugation at 12 000xg for 5min at 4°C. The supernatant was removed and disposed of and the pellet dissolved in TE pH 8 (400µl). The solution was then subject to phenol

extraction. This was carried out by first extracting with an equal volume of equilibrated phenol: chloroform: isoamyl alcohol (25:24:1) and finally with an equal volume of chloroform. The next step was to transfer the aqueous phase into a fresh sterile Eppendorf and ethanol precipitate. This was performed by adding a 0.1x volume of 10M ammonium acetate with 2.5x volume of absolute ethanol and precipitating for 1h at -20°C. The precipitate was then collected by centrifugation at 1 200xg for 15min at 4°C. Finally the pellet was re-suspended in TE pH 8 (500µl) and the concentration of DNA assayed by measuring the optical density (260nm) of the sample. The sample was then further diluted (1mg/ml) through the addition of TE pH 8.

The plasmid was then transferred into 50µl aliquots contained in sterile Eppendorf tubes and stored at –20°C. Next, 1µg of recovered plasmid was subject to electrophoresis on a 0.7%(w/v) agarose gel. Pre-prepared plasmid (1µg) and λ *Hind* III digest (1µg) were used as molecular weight markers. Following this restricting analysis was used as a measure of plasmid purity.

Characterisation of recovered plasmid

Restriction analysis of the plasmid was undertaken as follows: Plasmid (10µg) was placed in a sterile Eppendorf tube and to this 5µl of restriction buffer C (10mM Tris, 10mM magnesium chloride, 50mM sodium chloride, 1mM dithiothreitol) was added as well as 10U of *Bam* H1. The final volume was then made up 50µl with sterile de-ionised water and incubated at 37°C for 1h. Next, the digest was removed and placed at 65°C for 15min prior to being placed on ice. From this digest 25µl (5µg) was removed, and to this, 5 units of *Vsp* I was added. The digest was again placed at 37°C for 1h. The second digest was then heated to 65°C for 15min and stored at 4°C. The result of the first digest and the result of the second digest were then subject to electrophoretic separation in a 0.8%(w/v) agarose gel (1xTAE) containing 0.25µg/ml ethidiumbromide. Plasmid DNA (1µg) and λ *Hind* III digest (1µg) were used as molecular weight markers. The gel was then photographed using a gel documenting system. If the plasmid was seen to digest successfully in both instances it was deemed pure, if not, the phenol

extraction and ethanol precipitation steps detailed in the previous section were repeated.

2.2.9 *In vitro* transfection experiments

Transfection in vitro

Hep G2 cells were grown in 75mm^2 tissue culture flasks as previously detailed. Once ~70% confluence was attained, the cells were passaged and 1x 10^6 cells/well were used to seed 6 well tissue culture treated plates. After a further 24h incubation the transfection reagents were added to the cultures established in the 6 well plates. The complexes were formed at 1:10 vector weight excess under sterile conditions 30min prior to their addition to the culture media. The following samples were evaluated:

1. Sample 1 Plasmid (10ng) only
2. Sample 2 Polymer (100ng) only (Either ISA 23, ISA 22 and LipofectACE)
3. Sample 3, Plasmid (10ng)/polymer (100ng) complex
4. Sample 4 control (PBS)

The efficiency of the transfection was assayed 48h after the addition of the plasmid, the polymer or the plasmid/polymer complex. After this incubation time a commercially available kit (Promega E2000) was used to assay for increases in β-galactosidase activity. The results were expressed as units of β-galactosidase activity/cell.

Evaluation of transfection-associated cytotoxicity

As transfection-associated cytotoxicity is likely to involve membrane destabilisation, trypan blue exclusion as opposed to MTT was used to assess transfection mediated-toxicity. First cell cultures identical to those described in the previous section were established. The vector, plasmid, plasmid:polymer complex or controls were prepared as previously described. Following the incubation of the plasmid, polymer, plasmid/polymer complex or control with the culture for 48h, instead of assaying for β-galactosidase activity, cell viability

using trypan blue exclusion was performed. Results were expressed as viability (%± S.D.) relative to cells with out added polymer or plasmid.

Chapter 3

Biocompatibility of Chitosan and Poly(amidoamine)s

3.1 Introduction

Many different types of polymer, either as solid appliances or as water-soluble systems, have found application as biomaterials. Materials as diverse as poly(methyl-methacrylate) intra-ocular lenses and HPMA copolymer drug conjugates have been described (Rihova, 1996; Ringoir and Van Holder, 1986; Vasey *et al.*, 1998). Because of the varied nature of both the biomaterials and their application, care must be taken when defining biocompatibility. Williams, (1986) defined biocompatibility as, "*The ability of a material to perform, with an appropriate host response in a specific application.*" From this definition, it is important to understand that any assay used for screening the biocompatibility profile of a substance must be specific to an intended application. Fortunately, many studies have been undertaken that describe the biocompatibility of soluble polymers and many of the important *in vitro* reference systems have already been established (Sgouras and Duncan, 1990; Sgouras, 1990; Carreno Gomez and Duncan, 1997).

In this study, it was considered important to undertake a preliminary investigation of the likely biocompatibility profile of the poly(amidoamine)s ISA 1, 4, 9, 22 and 23 and the chitosan polymers N1, 2 and 3. The *in vitro* cytotoxicity and haematoxicity assays used herein examine whether these polymers are likely to be tolerated once administered systemically *in vivo*. Obviously, such simple tests can not be extrapolated to the likely *in vivo* toxicological profile of the polymer, but instead help to asses whether further evaluation of the polymer, as a systemically administered potentially drug carrier is warranted (Sgouras, 1990). In order to standardise the *in vitro* biocompatibility screens, the concept of reference controls also needs to be addressed. These reference polymers have been well characterised *in vitro* and in some case *in vivo* and provide a benchmark against which any observed polymer toxicity may be compared. (Duncan *et al.*, 1996; Sgouras, 1990). This library of polymers includes poly(L-lysine), HPMA, dextran, alginates, poly(ethylenimine), chitosan and poly(amidoamine) (PAMAM) dendrimers (Sgouras,1990; Duncan *et al.*, 1996; Malik *et al.*, 1997).

Based on the earlier work of Sgouras, (1990), the following assays were selected to assess the likely biocompatibility profiles of poly(amidoamine)s ISA 1, 4, 9, 22 and 23 and the chitosans N1, 2 and 3 *in vitro*. Evaluation of cytotoxicity using the MTT assay and through observing cell morphology using SEM

1) Evaluation of polymer RBC interactions using the RBC lysis assay
2) Evaluation of poly(amidoamine) RBC interactions in response to pH using the RBC lysis assay and observation of RBC morphology using SEM

Throughout poly(L-lysine) Mw 56 500 Da and dextran Mw 72 000 Da were used as reference polymers for comparison.

There are various methods for assessing cytotoxicity and each reveals specific information about the molecular or cellular mechanisms responsible for cell damage. For example, the comet assay may establish the integrity of gnomic DNA (Olive *et al.*, 1997). Trypan blue exclusion or assays for lactate dehydrogenase (LDH) release evaluate cell membrane integrity (Rihova, 1996; Sgouras, 1990).

The MTT assay has been used herein as it has been shown to be comparable with the other standard assays for cytotoxicity and cell viability such as [^{3}H]leucine or [^{3}H]thymidine incorporation (Mossmann, 1983; Twentyman and Luscombe, 1987; Sgouras and Duncan, 1990; Carreno Gomez and Duncan, 1997). MTT has been postulated to measure the activity of specific enzymes in the mitochondrial succinoxidase system (Mossmann, 1983; Slater *et al.*, 1963), although other mechanisms have also been implicated (Altman, 1972; Dhanjal and Fry, 1997). During the MTT assay a water-soluble (yellow) tetrazolium dye is metabolised to a non-soluble (blue) formazan salt which may later be dissolved in optical grade DMSO. As formazan salt production is directly proportional to viable cell density, viable cell number can be assessed by spectrophotometric quantitation of the formazan product at OD_{550}. It has however also been noted that the rate of formazan salt production is specific to each individual cell line and the

specific culture conditions used (Sgouras, 1990). Consequently, the results obtained (OD_{550}) are relative to a control as opposed to absolute values. Other independent studies have also found the MTT assay to be suitable for assaying synthetic molecules for possible cytotoxicity (Olivier *et al.*, 1995), in a reproducible manner (Sieuwerts *et al.*, 1995).

The first tissue type that the polymer is going to interact with following systemic administration is the blood and consequently polymer-RBC interactions were examined. This is only a preliminary investigation related to the likely haematocompatibility profile of the polymers as many extremely important issues, such as complement interaction, protein binding and immunogenisity that would contribute to polymer toxicity *in vivo* remain to be addressed. As with the cytotoxicity experiments, an insight into the likely effects of the i.v. administration of the polymer may be gained (Sgouras, 1990; Duncan *et al.*, 1991; Duncan *et al.*, 1994). SEM was also used to monitor any charge in RBC morphology.

As described in Chapter 1, poly(amidoamine)s undergo a pH-dependent conformational change in response to a drop in the pH of their environment (Duncan *et al.*, 1994). This is a direct result of the protonation of amino and amido groups in the main chain of the polymer and the subsequent electrostatic repulsion between positively charged ions (Ferruti *et al.*, 1985). In view of this, it was considered interesting to determine the effect of pH on the ability of poly(amidoamine)s to lyse RBC membranes. These experiments provided a model for the study of pH-dependent membrane rupture, such as may be expected as a consequence of polymer internalisation by endocytosis.

3.2 Methods

Please also refer to appropriate section in Chapter 2, Dextran (Mw 72 000 Da) and poly(L-lysine) (Mw 56 500 Da) were used as negative and positive reference controls respectively in all biocompatibility experiments. Prior to use all

polymer solutions were filter sterilised through 0.2μm filters that had been first saturated with the appropriate polymer (Sgouras and Duncan, 1990).

Cytotoxicity

Media pH was initially checked by looking for a colour change in the phenol red indicator present in all the tissue culture media used. If a colour change was observed after the polymers were dissolved at a concentration of 5mg/ml a polymer concentration/media pH relationship was established. This was done by monitoring the pH of the media in response to a dilution of the polymer concentration. Fresh polymer solutions were made (daily) before each experiment was performed. Standard cell culture protocols were followed to establish the cytotoxic profile of poly(amidoamine)s ISA1, 4, 9, 22, and 23 and chitosans N1, 2 and 3 as described in Chapter 2. SEM was also used to substantiate the results generated using this experimental model. IC_{50} values were established by finding the mean(±S.D.) of 3 sets of 6 replicate using linear regression analysis. Statistical comparisons were made using a paired Student's t-test incorporated into Prism v2.01 by GraphPad software.

RBC lysis experiments

The poly(amidoamine)s, incubation times of 1h and 24h were used over a concentration range of 0-5mg/ml. The chitosans were assayed over 1 and 5h time periods using a concentration range of 0-5mg/ml. SEM was also used to examine the chitosan–RBC interactions.

RBC lysis in response to pH

Poly(amidoamine)-RBC interactions were further examined by studying the effect of both time (1h, or 24h) and pH (5.5, 6.5 or 7.4) on haemoglobin release using the RBC-lysis assay. All experiments used a polymer concentration of 1mg/ml. Polymer solutions and RBC suspensions were generated using PBS with the pH adjusted to 5.5, 6.5 and 7.4 using hydrochloric acid (1N). With the exception of the aforementioned detail, the experiments were performed as

previously detailed. Moreover, SEM was also used to examine changes in cell morphology induced by pH and/or increasing incubation time.

3.2 Results

Poly(amidoamine) cytotoxicity was examined using B16 F10, Mewo and Hep G2 cells after a 72h incubation. It was seen (Figure 3.1) that ISA 1, 4 and 9 showed concentration-dependent cytotoxicity when incubated with B16 F10 cells. This trend was repeated (Figure 3.2 and 3.3) in the case of Mewo and Hep G2 cells respectively. Examination of the cytotoxicity profile of the poly(amidoamine)s ISA 22 and 23 against B16 F10 cells after a 72h incubation showed that ISA 22 and 23 are apparently less toxic than ISA 1, 4 and 9 (Figure 3.4). This is reflected in the IC_{50} values (Table 3.1). The IC_{50} values obtained for ISA 1, 4 and 9 against B16 F10 were 3.05±0.70, 3.45±1.18 and 4.58±0.67 mg/ml respectively. This result is nearly 100 times greater than that of poly(L-lysine) (0.05±0.01mg/ml). Table 3.1 shows that a similar trend is observed when the IC_{50} values for all of the poly(amidoamine)s tested are compared with poly(L-lysine) in all of the cell lines used. No significant difference was observed between the IC_{50} values obtained using B16 F10 and Mewo cell lines to assess poly(amidoamine) cytotoxicity. These experiments were compared using a paired Students t-test with a confidence limit set at 95% (P=0.0558).

Figures 3.5 and 3.6 show the effect of ISA 22 and 23 on Mewo and Hep G2 cells respectively. When SEM was used to examine the morphology of the Mewo cells after exposure (72h) to ISA 22, little change in morphology was evident compared to the control cells.

The RBC lysis profile of ISA 1, 4 and 9 after 1h exposure to rat RBCs is shown (Figure 3.8). In this instance both ISA 1 and 4 show more lysis than poly(L-lysine) at higher concentrations (above 3mg/ml). Figure 3.9 shows the RBC lysis profile of ISA 1, 4 and 9 following 24h exposure to the RBCs. All of the polymers tested are much more lytic than poly(L-lysine) at all concentrations.

Figure 3.1 Effect of ISA 1,4 and 9 upon the viability of B16 F10 cells following an incubation time of 72h using the MTT assay (Mean (± S.D.) of 3 experiments (n=6)).

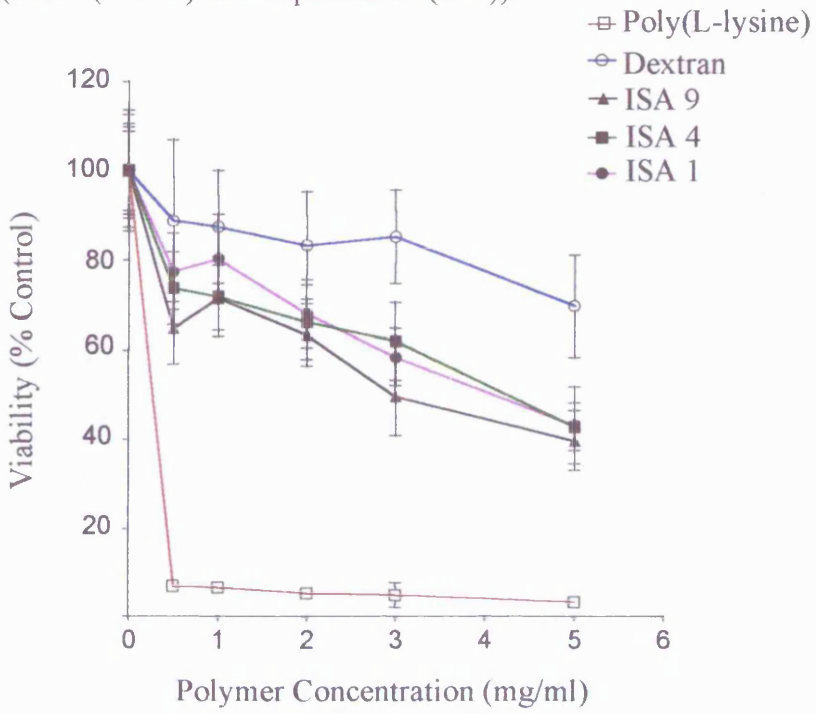


Figure 3.2 Effect of ISA 1,4 and 9 upon the viability of Mewo cells following an incubation time of 72h using the MTT assay (Mean (± S.D.) of 3 experiments (n=6)).

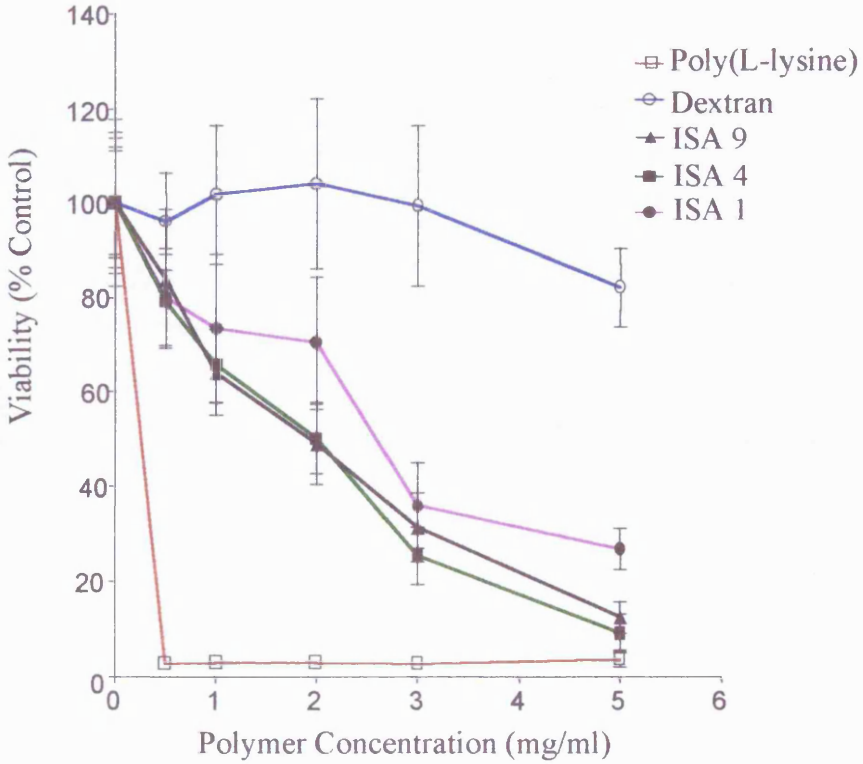


Figure 3.3 Effect of ISA 1,4 and 9 upon the viability of Hep G_2 cells following an incubation time of 72h using the MTT assay (Mean (± S.D.) n=4)

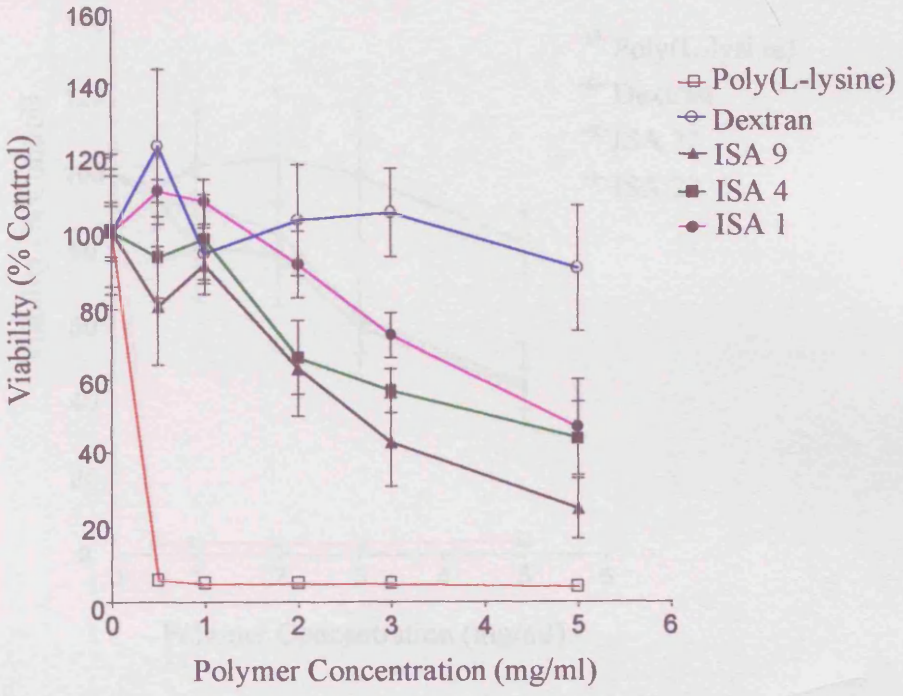


Figure 3.4 Effect of ISA 22 and 23 upon the viability of B16 F10 cells following an incubation time of 72h using the MTT assay (Mean (± S.D.) of 3 experiments (n=6)).

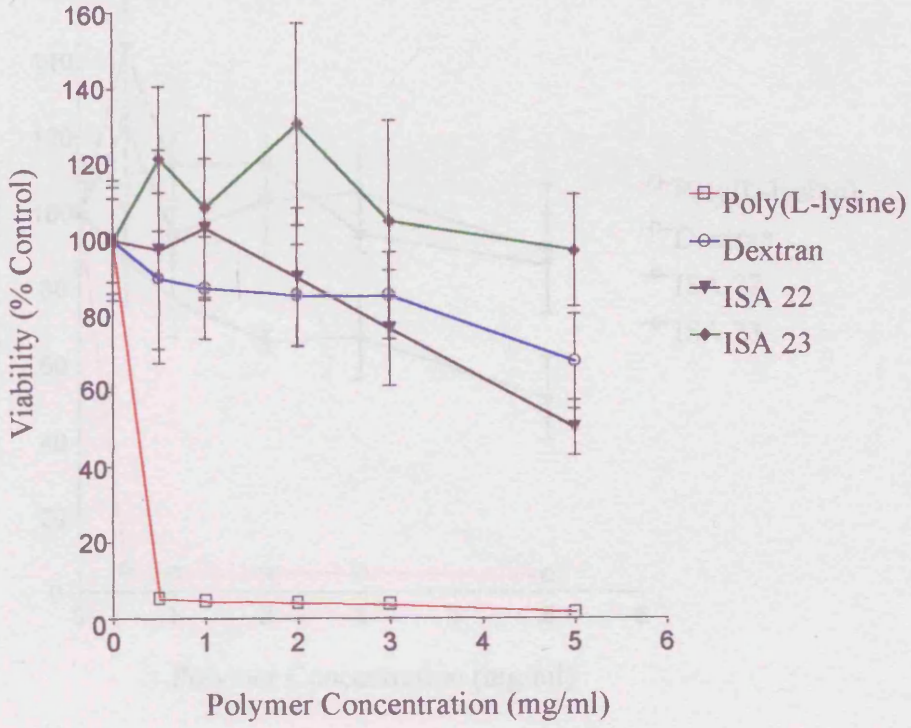


Figure 3.5 Effect of ISA 22 and 23 upon the viability of Mewo cells following an incubation time of 72h using the MTT assay (Mean (± S.D.) of 3 experiments (n=6)).

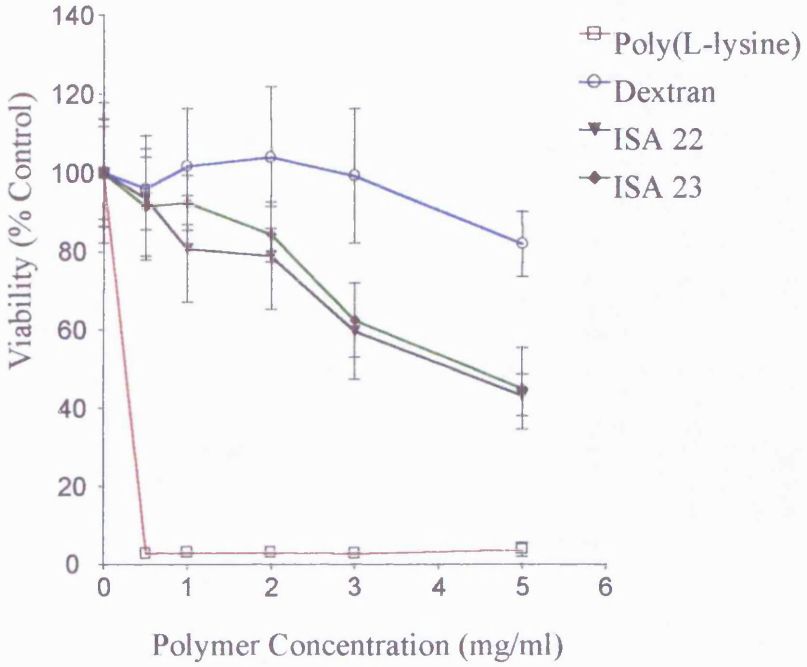


Figure 3.6 Effect of ISA 22 and 23 upon the viability of Hep G_2 cells following an incubation time of 72h using the MTT assay (Mean ± S.D. n=4)

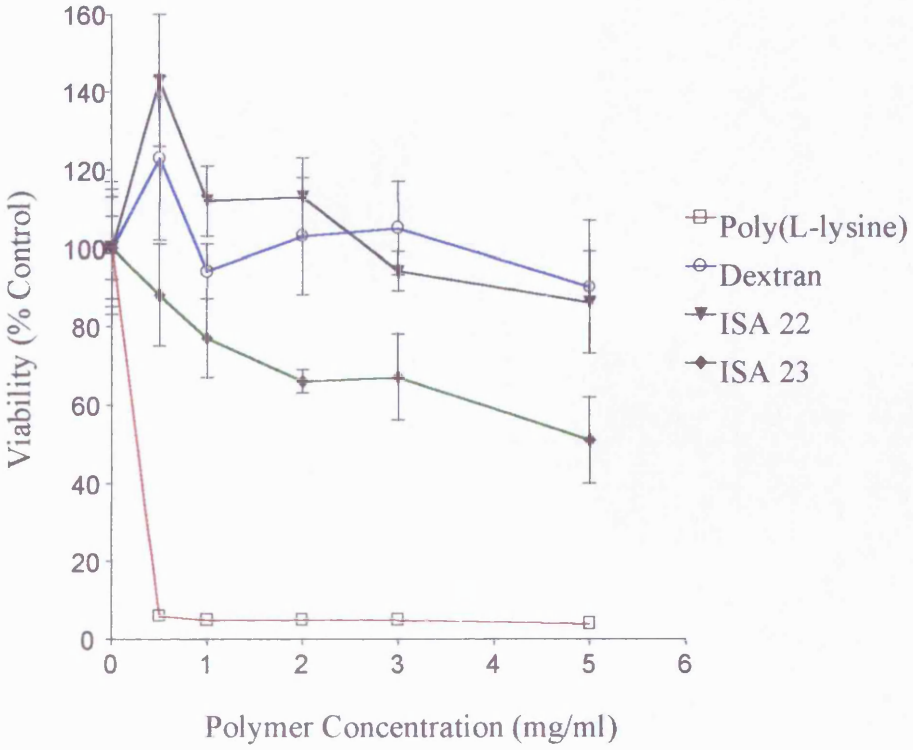
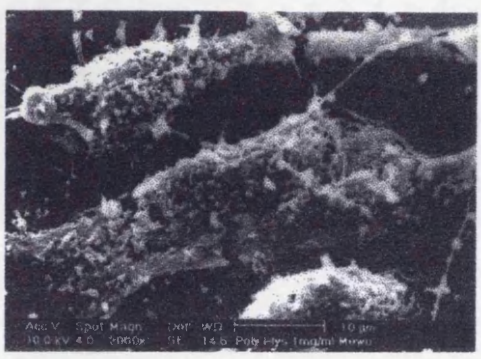


Table 3.1 The cytotoxicity of poly(amidoamine)s and chitosans against all cell lines

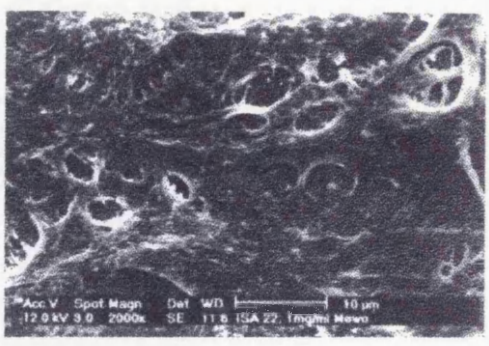
Polymer	Molecular Weight (Da)	Cytotoxicity† Cell line B16 F10 (N=18)	Mewo (n=18)	Hep G2 (n=4)	CCRF-CEM (n=18)	L132 (n=18)
ISA 1	5 000 (Mn)	3.05 ± 0.70	2.24 ± 0.36	2.80	Not tested	Not tested
ISA 4	4 700 (Mn)	3.45 ± 1.18	1.89 ± 0.51	4.60	Not tested	Not tested
ISA 9	3 900 (Mn)	4.58 ± 0.67	2.86 ± 0.11	>5.00	Not tested	Not tested
ISA 22	8 500 (Mn)	4.00 ± 1.41	4.63 ± 0.53	>5.00	Not tested	Not tested
ISA 23	10 500 (Mn)	>5.00	4.23 ± 1.10	>5.00	Not tested	Not tested
N1	<5000 (Mw)	Not tested	Not tested	Not tested	>1.00	>1.00
N2	5-10 000 (Mw)	Not tested	Not tested	Not tested	>1.00	>1.00
N3	>10 000 (Mw)	Not tested	Not tested	Not tested	>1.00	>1.00
Poly(L-lysine)	56 500 (Mw)	0.05 ± 0.01	0.01 ± <0.01	0.05 ± 0.01	0.012 ± 0.01	0.012 ± 0.007
Dextran	70 000 (Mw)	>5.00	>5.00	>5.00	>1.00	>1.00

† Cytotoxicity is expressed as IC_{50} (mg/ml) ±S.D. Mean of 3 experiments (n=6) unless otherwise indicated
Mn = number average molecular weight
Mw = weight average molecular weight

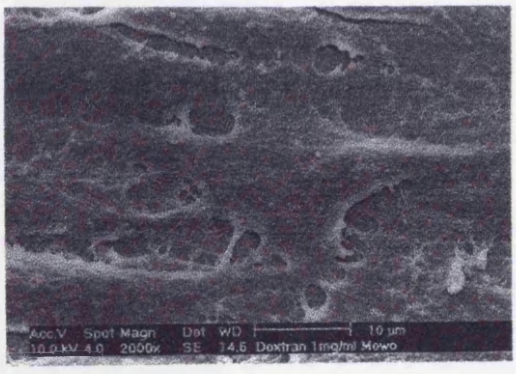
Figure 3.7 Scanning electron micrographs showing typical Mewo cell morphology after exposure to specific polymers for 72h at a concentration of 1mg/ml



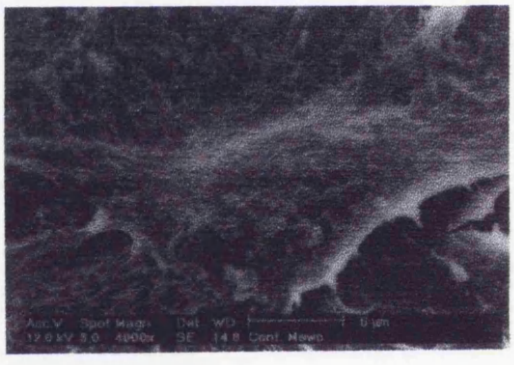
Panel (a) Mewo cells after exposure to poly(L-lysine) at 1mg/ml for 72h (x 2 000)



Panel (b) Mewo cells after exposure to ISA 22 at 1mg/ml for 72h (x 2 000)



Panel (c) Mewo cells after exposure to Dextran at 1mg/ml for 72h (x 2 000)



Panel (d) Mewo cells after 72h culture (x 4 000)

Figure 3.8 Effect of Poly(amidoamine)s ISA1,4 and 9 upon rat RBCs following 1h incubation (Mean (± S.D.) of 3 experiments (n=6)).

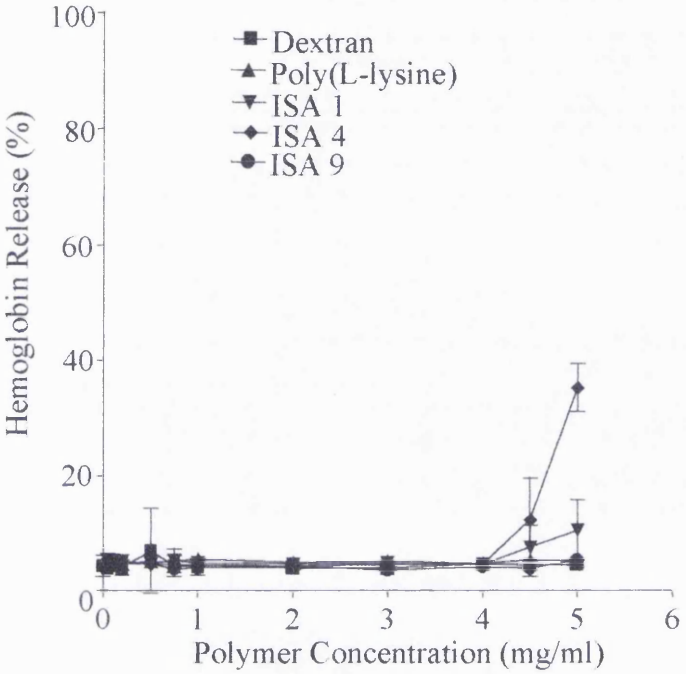
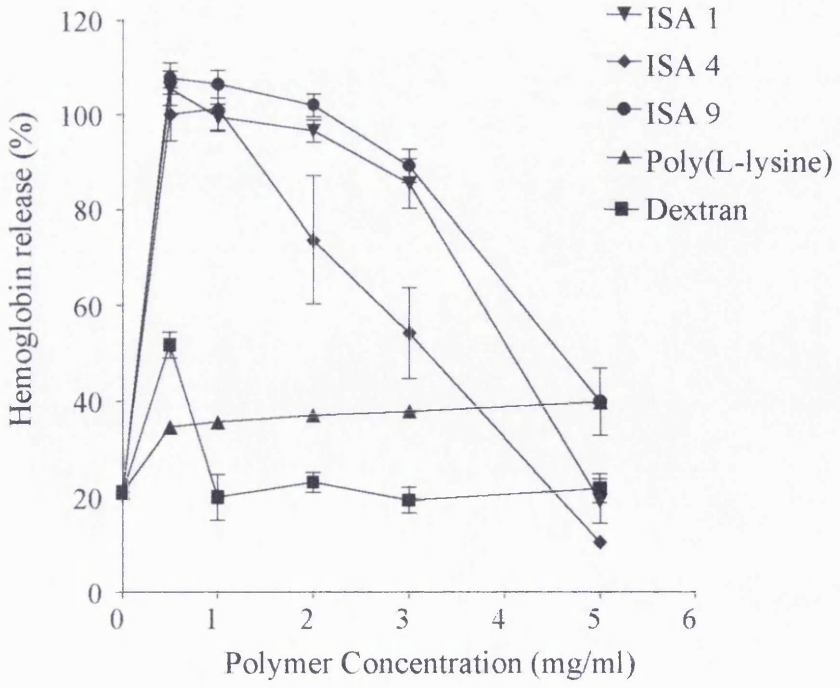


Figure 3.9 Effect of Poly(amidoamine)s ISA1,4 and 9 upon rat RBCs following 24h incubation (Mean (± S.D.) n=4)



The precipitation of haemoglobin by the poly(amidoamine)s at concentrations over 1mg/ml is evidant. ISA 22 shows a much less lytic profile than ISA 1, 4 and 9 after 1h (Figure 3.10) and after 24h (Figure 3.11) at concentrations below 3mg/ml. ISA 23 however shows a much more lytic profile than both ISA 22 and poly(L-lysine).

Poly(amidoamine) membrane disruption in response to pH was investigated. Figure 3.12 shows the lytic profile of ISA 1, 4 and 9 at pH 7.4, 6.5 and 5.5 following a 1h incubation. RBC membrane integrity in the presence of PBS buffer and Dextran controls is indicative of membrane stability at pH values as low as 5.5. Figure 3.13 shows the same experiment with a 24h incubation time and it was seen that ISA 1, 4 and 9 demonstrated time- and pH-dependent membrane lytic activity. Figure 3.14 and 3.15 show the pH-responsive, lytic profile of ISA 22 and 23 over a time period of 1h and 24h respectively. ISA 22 and 23 showed an increased lytic profile of at pH 5.5 even after 1h incubation. SEM was also used to observe changes in RBC morphology in response to poly(amidoamine) ISA 23 (1mg/ml)) over the above pH ranges and incubation times (Figure 3.16). The observation that RBC morphology has been completely destroyed by ISA 23 at pH 5.5 after 24h and not at pH 7.4 is consistent with the haemolysis study.

Prior to the evaluation of the cytotoxicity of the chitosans N1-3, the relationship between media pH and chitosan concentration was established. A change in the pH of the media is evident at polymer concentrations above 1mg/ml (Figures 3.17 and 3.18). Figure 3.19 shows the relationship between chitosans N1, 2 and 3 concentration and L132 cell viability. Within the concentration range used, no IC_{50} value could be determined (Table 3.1) though some evidence for cytotoxicity at concentrations above 1mg/ml was observed (Figure 3.20).

The RBC lysis profiles for the chitosan molecular weight fractions are shown for 1h and 5h incubation periods (Figure 3.21 and 3.22 respectively). Of note is the lack of lysis caused by the chitosan molecular weight fractions relative to the poly(L-lysine) control after a 5h incubation. Poly(L-lysine) can cause the

Figure 3.10 Effect of Poly(amidoamine)s ISA22 an 23 upon rat RBCs following 1h incubation (Mean (± S.D.) of 3 experiments (n=6)).

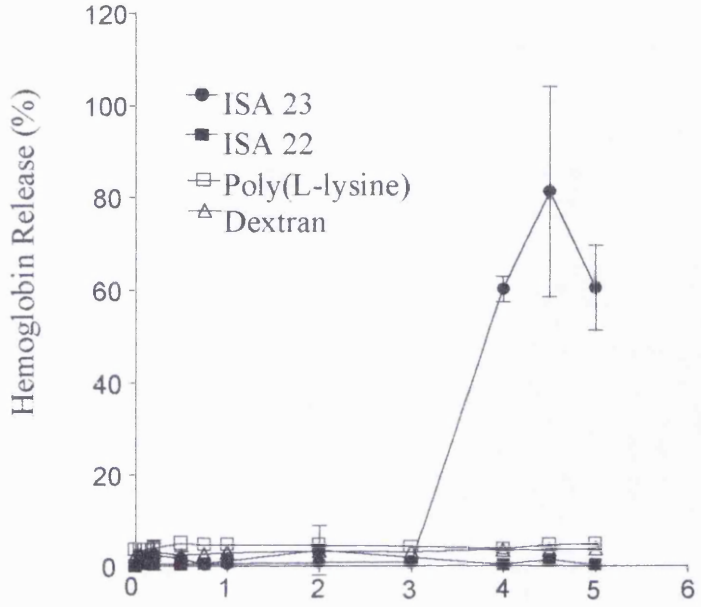


Figure 3.11 Effect of Poly(amidoamine)s ISA22 an 23 upon rat RBCs following 24h incubation (Mean (± S.D.) of 3 experiments (n=6)).

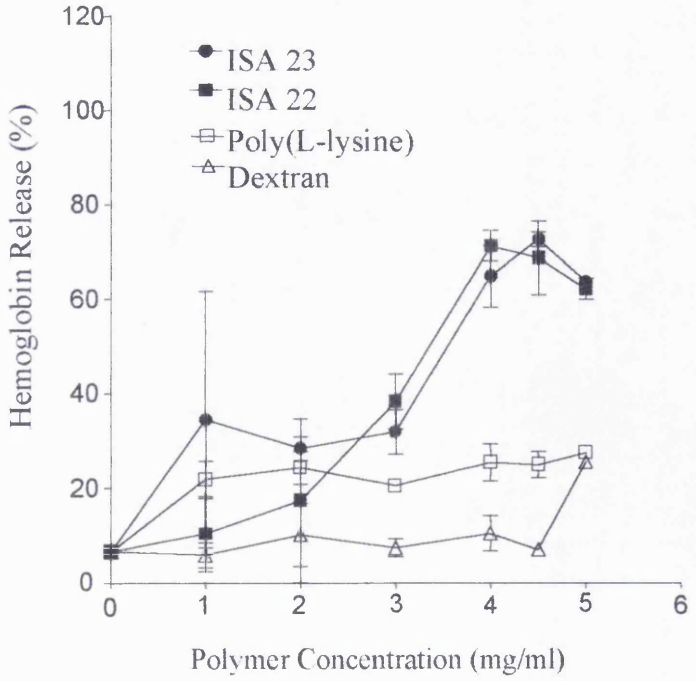


Figure 3.12 Effect of pH on poly(amidoamine) ISA 1, 4 and 9 (1mg/ml) rat RBC interaction at a after 1h (Mean (± S.D.) of 2 experiments (n=6)).

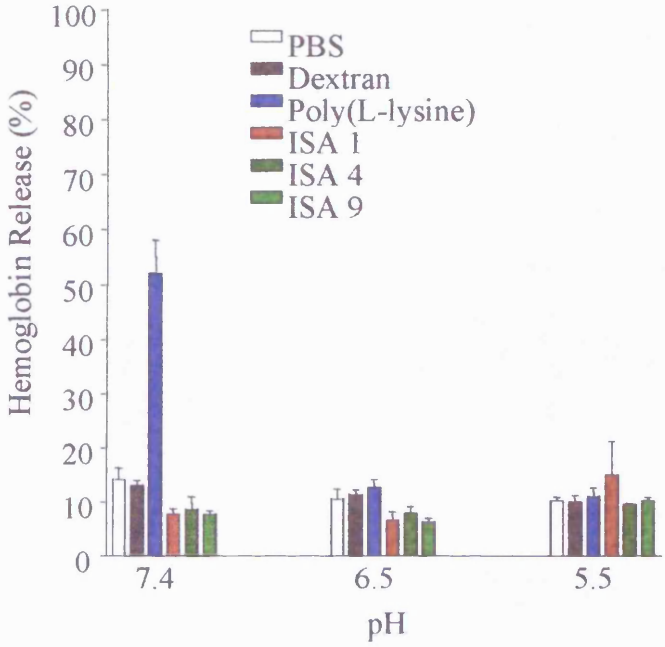


Figure 3.13 Effect of pH on poly(amidoamine) ISA 1, 4 and 9 (1mg/ml) rat RBC interaction at a after 24h (Mean (± S.D.) of 2 experiments (n=6)).

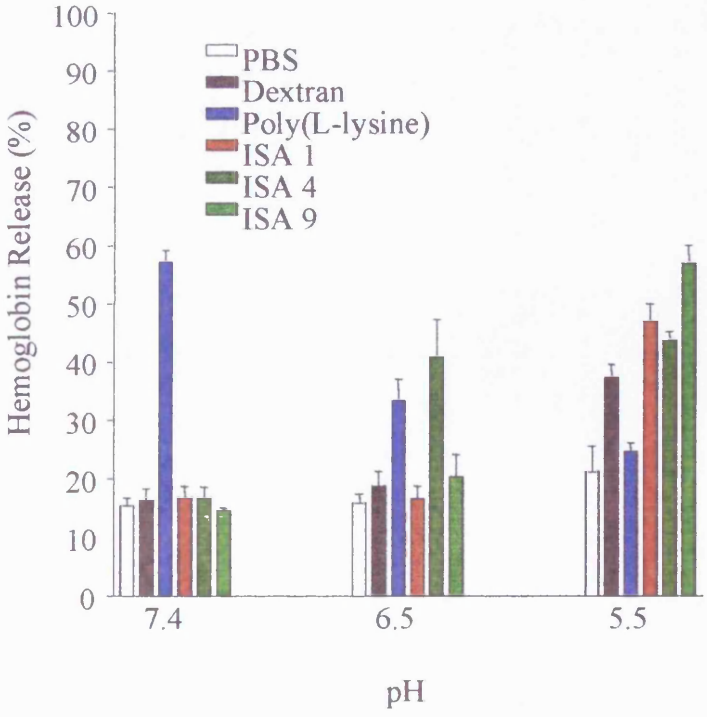


Figure 3.14 Effect of pH on poly(amidoamine) ISA22 and 23 (1mg/ml) rat RBC interaction at a after 1h (Mean (± S.D.) of 2 experiments (n=6)).

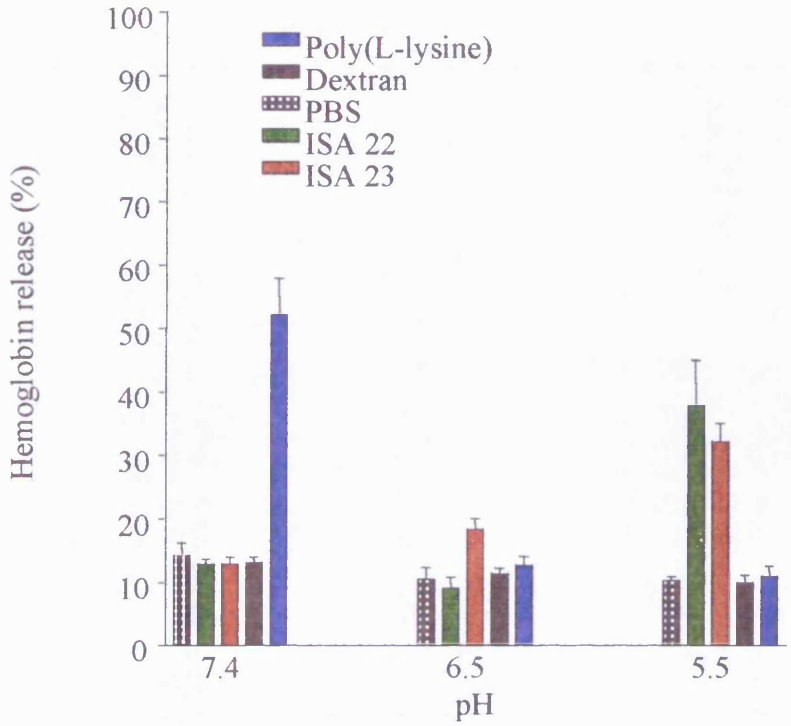


Figure 3.15 Effect of pH on poly(amidoamine) ISA22 and 23 (1mg/ml) rat RBC interaction at a after 24h (Mean (± S.D.) of 2 experiments (n=6)).

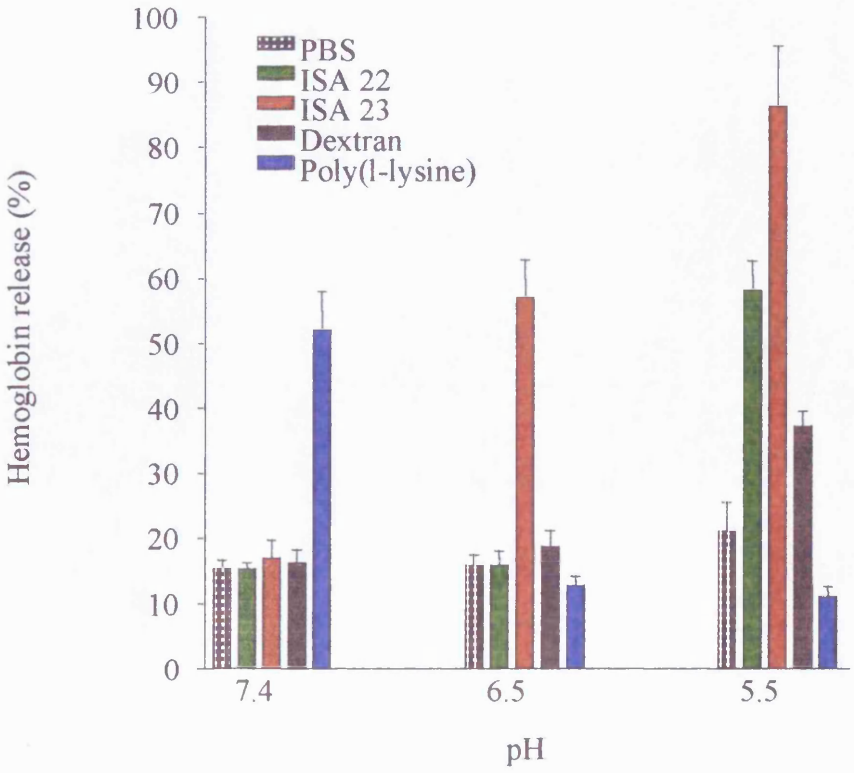
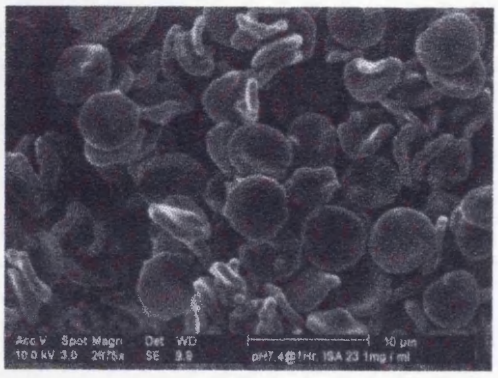
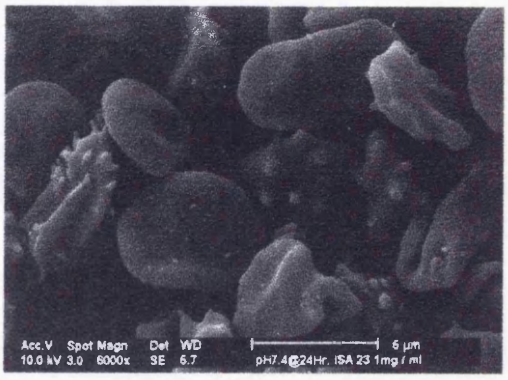


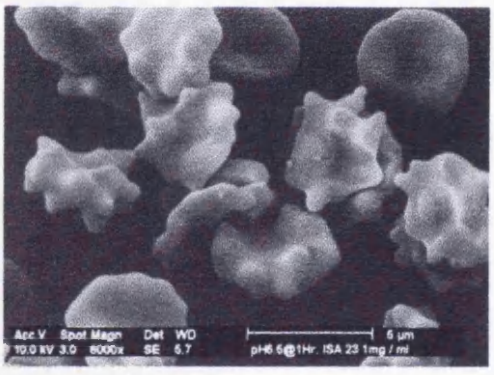
Figure 3.16 Typical RBC morphology in response to exposure to 1mg/ml ISA 23 for 1 and 24h at pH 7.4 and 5.5 by SEM



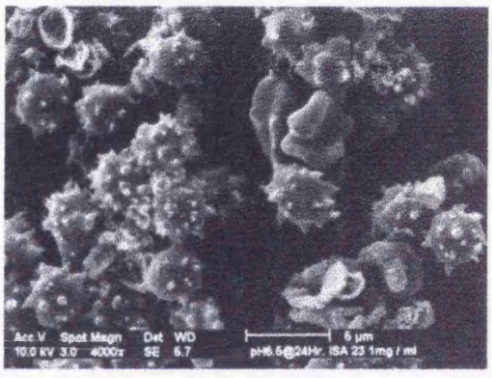
Panel (a) Rat RBCs at pH 7.4 after 1h exposure to ISA 23 at 1mg/ml (x 2 875)



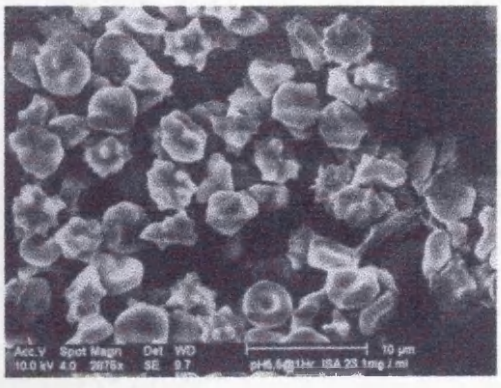
Panel (b) Rat RBCs at pH 7.4 after 24h exposure to ISA 23 at 1mg/ml (x 6 000)



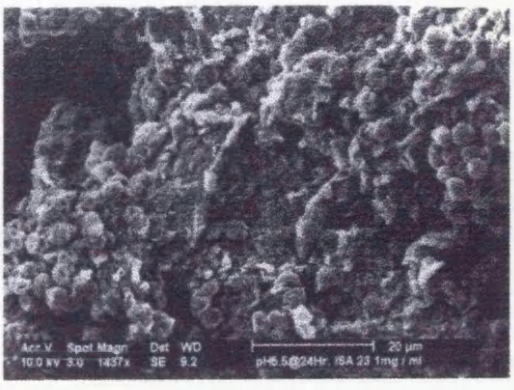
Panel (c) Rat RBCs at pH 6.5 after 1h exposure to ISA 23 at 1mg/ml (x 6 000)



Panel (d) Rat RBCs at pH 6.5 after 24h exposure to ISA 23 at 1mg/ml (x 4 000)



Panel (e) Rat RBCs at pH 5.5 after 1h Exposure to ISA 23 at 1mg/ml (x 2 876)



Panel (f) Rat RBCs at pH5.5 after 24h Exposure to ISA 23 at 1mg/ml (x 1 437)

Figure 3.17 Effect of chitosan N1-3 concentration on media (RPMI 1640) pH

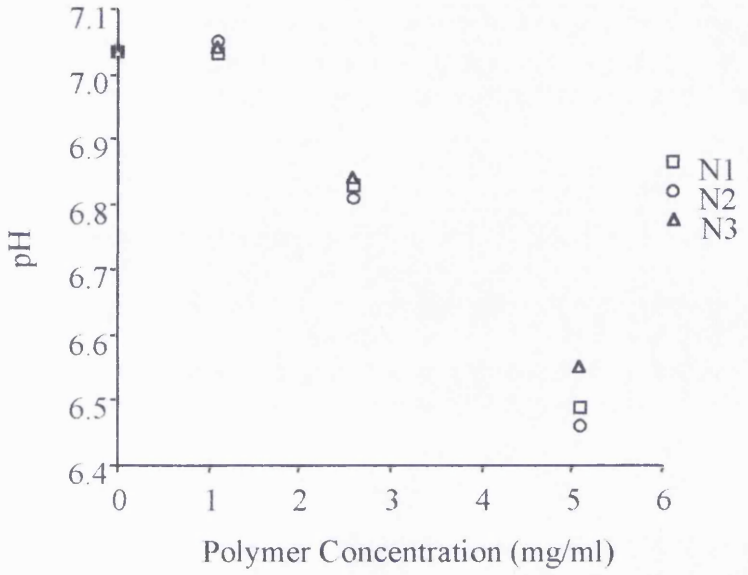


Figure 3.18 Effect of chitosan N1-3 concentration on media (E199) pH

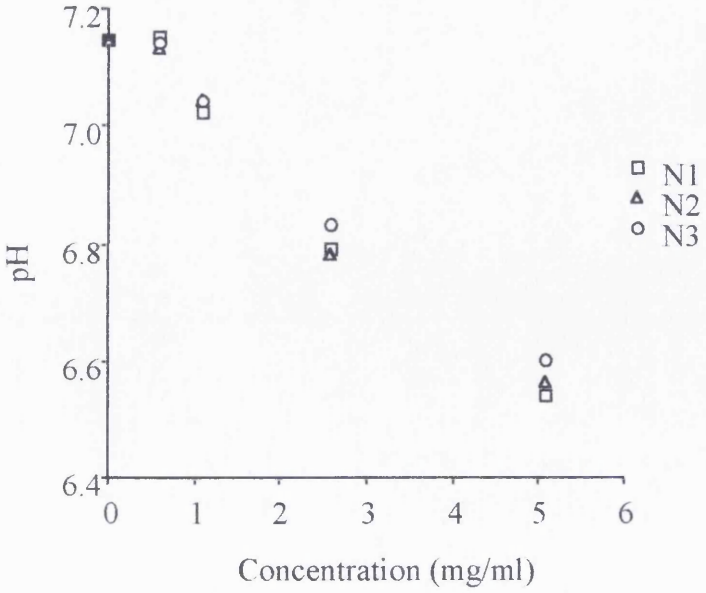


Figure 3.19 Effect of chitosans on the viability of L132 cells using the MTT assay (Mean ± S.D. of 3 experiments (n=6)).

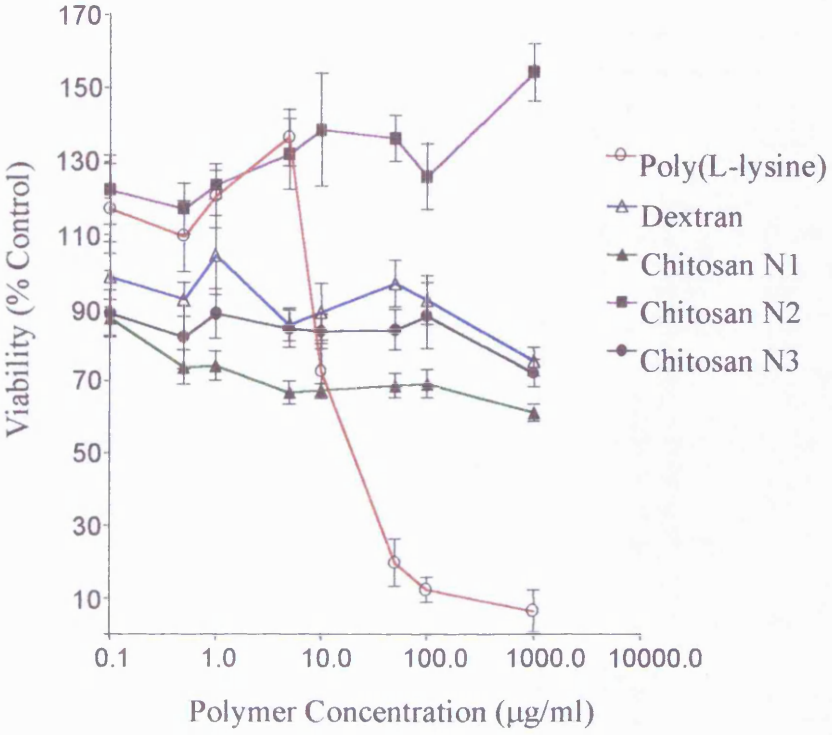


Figure 3.20 Effect of chitosans on the viability of CCRF-CEM cells using the MTT assay (Mean ± S.D. of 3 experiments (n=6)).

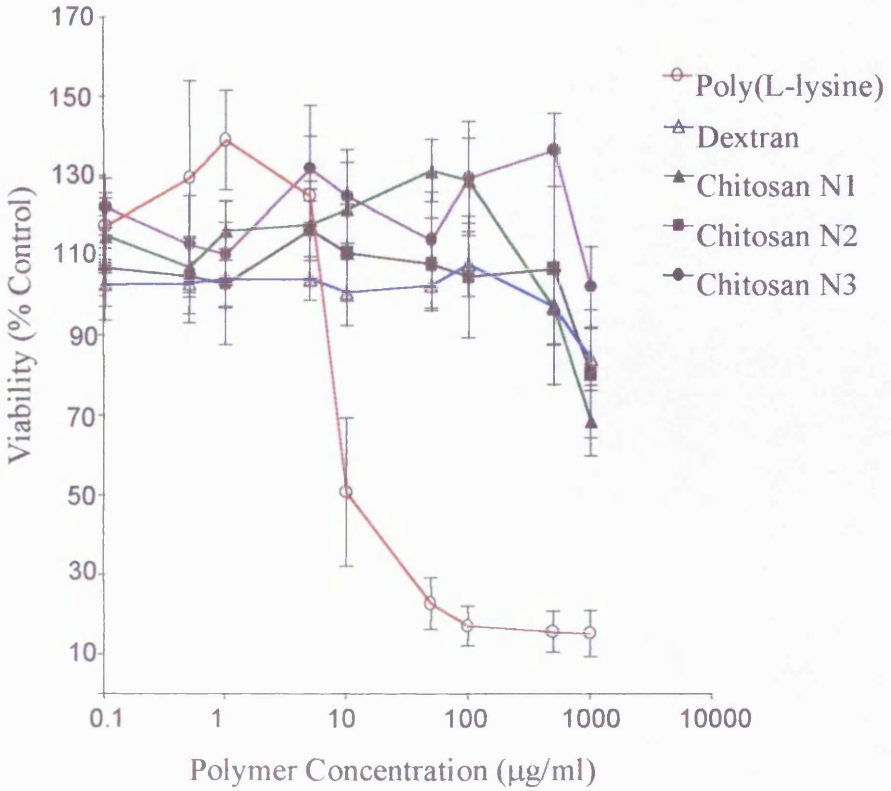


Figure 3.21 Effect of chitosans upon rat RBCs following 1h incubation (Mean ± S.D. of 3 experiments (n=6)).

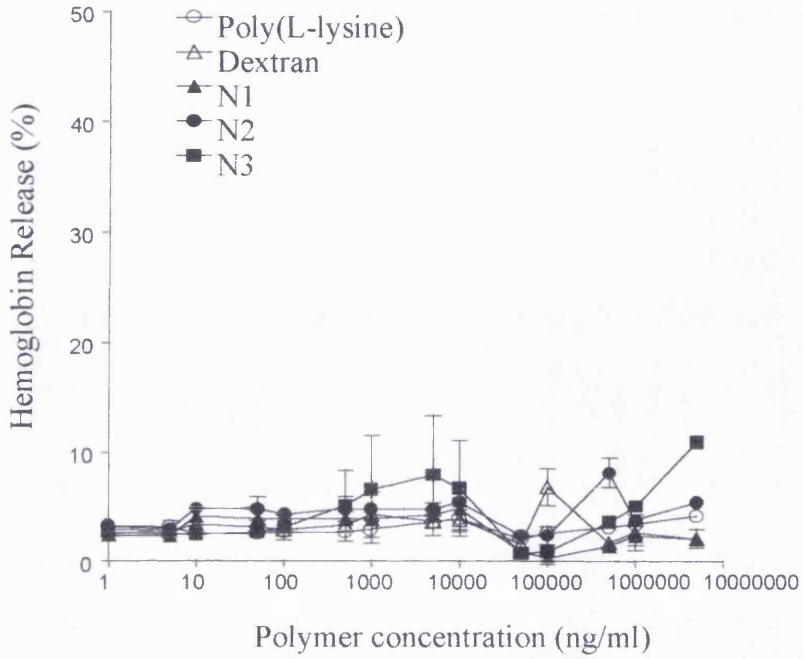
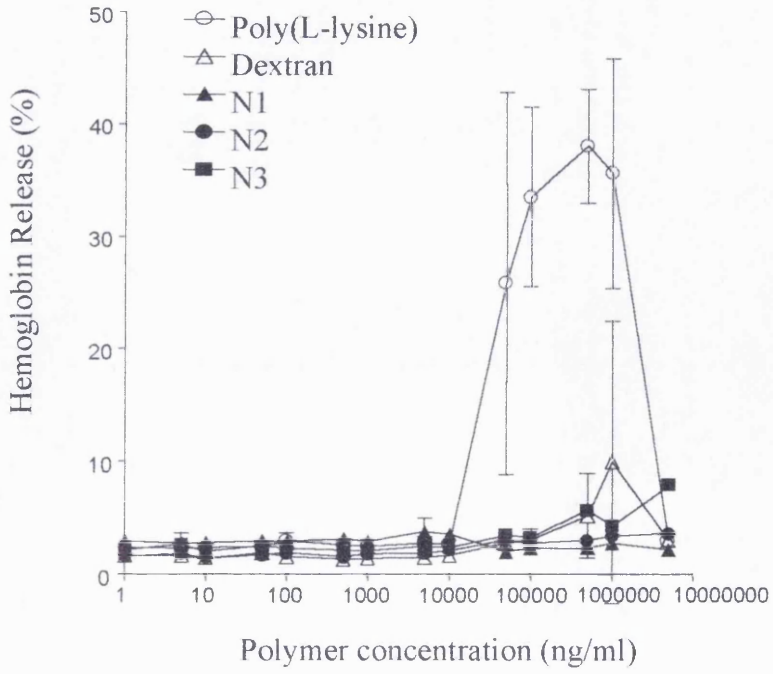


Figure 3.22 Effect of chitosans upon rat RBCs following 5h incubation (Mean ± S.D. of 3 experiments (n=6)).



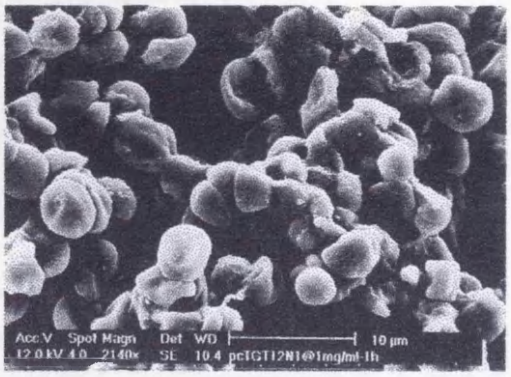
haemoglobin released to precipitate following an incubation time of 5h at concentrations above 1mg/ml. The investigation into chitosan – RBC interactions was furthered by, a morphological study using SEM (Figures 3.23 and 3.24). No significant changes in RBC morphology were evident other than a little crenation and aggregation. The reference controls are shown (Figure 3.24) and poly(L-lysine) mediated membrane fusion is evident after 1h (Panel 3.24 (a)) and 5h (Panel (b)).

3.4 Discussion

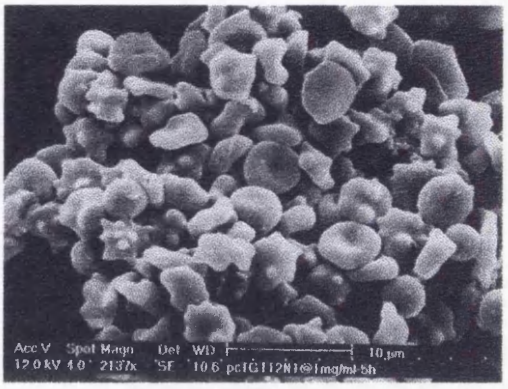
IC_{50} values can only be estimated for the poly(L-lysine) samples during the evaluation of the poly(amidoamine)s and the results obtained are comparable with the literature (Sgouras, 1990), consequentially, its usefulness as a control is not compromised. When CCRF-CEM cells are used to evaluate the IC_{50} of poly(L-lysine), (Mw 57 000 Da) Sgouras, (1990) reported a value of 23.90μg/ml. The IC_{50} value obtained herein using the same experimental conditions gave a value of 12.00±80.00μg/ml. If the experiment is repeated using Hep G2 cells, Sgouras (1990) reports an IC_{50} value of 59.90μg/ml and the value obtained herein is 50.00±10.00μg/ml.

In general the human melanoma cell line seemed to be more sensitive to the effects of poly(amidoamine)s than the mouse melanoma B16 F10 cells, though, the apparent differences were not statistically significant. When the mechanisms of polycation mediated membrane rupture documented in Nevo *et al.*, (1955), Kachalsky *et al.*, (1959) and Hartmann and Galla, (1978) are considered (Chapter 1), this result is consistent with the literature (Sgouras, 1990). The IC_{50} values for poly(L-lysine) are approximately 100 times less than those of ISA 22 and ISA 23. Poly(ethylenimine) (Mw 70 000) has been shown to have an IC_{50} value of 6.5μg/ml when incubated with Hep G2 cells (Sgouras, 1990) and it is nearly 1000 times more toxic than the poly(amidoamine)s assayed against Hep G2 cells herein. When PAMAM dendrimers are compared with the linear poly(amidoamine)s evaluated herein, generation 3 and 4 dendrimers have been reported to be cytotoxic at concentrations above 100μg/ml against B16 F10

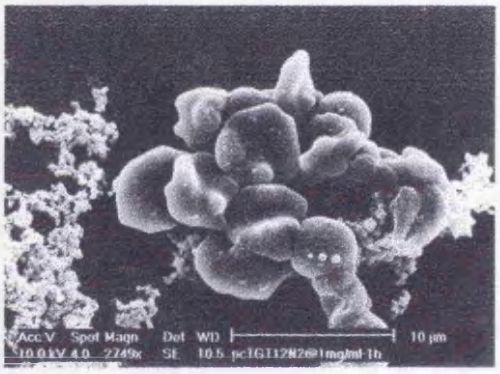
Figure 3.23 Scanning electron micrographs sowing typical RBC morphology following either 1 or 5h exposure to chitosans at a concentration of 1mg/ml



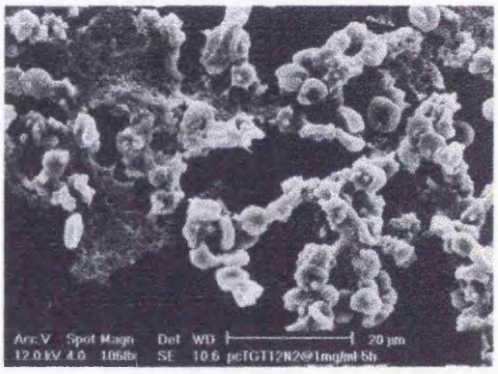
Panel (a) RBCs following 1h exposure to N1 at 1mg/ml (x2 140)



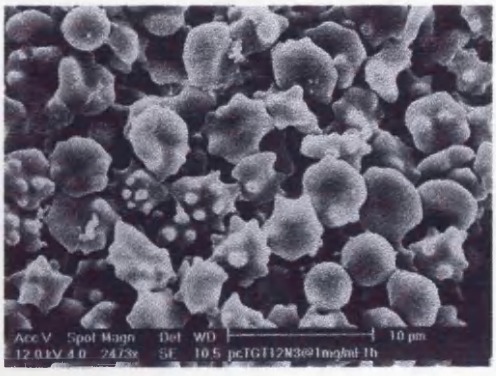
Panel(b) RBCs following 5h exposure to N1 at 1mg/ml (x2 137)



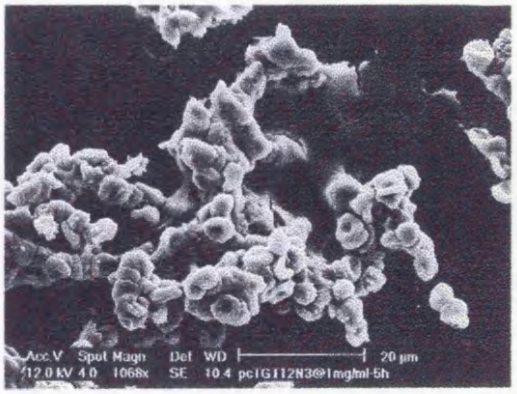
Panel (c) RBCs following 1h exposure to N2 at 1mg/ml (x2 749)



Panel (d) RBCs following 5h exposure to N2 at 1mg/ml (x1 068)

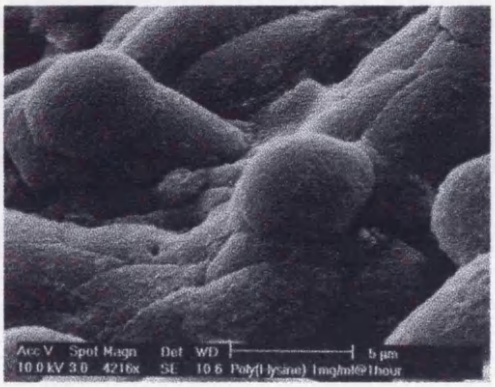


Panel (e) RBCs following 1h exposure to N3 at 1mg/ml (x2 473)

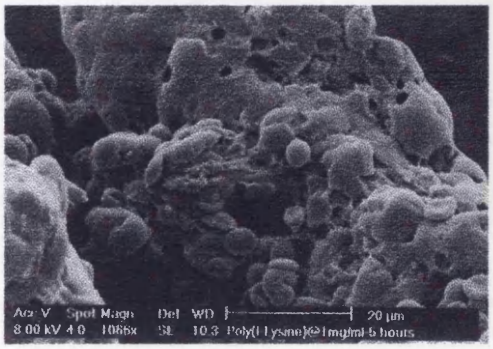


Panel (f) RBCs following 5h exposure to N3 at 1mg/ml (x1 068)

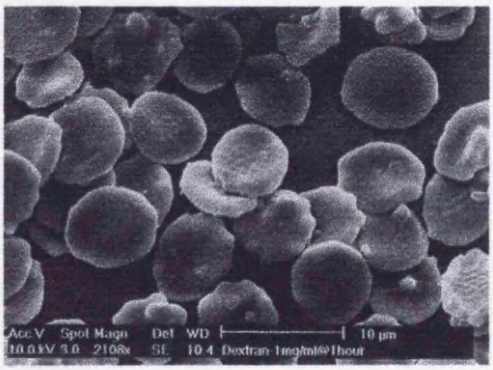
Figure 3.24 Scanning electron micrographs showing typical control RBC morphology



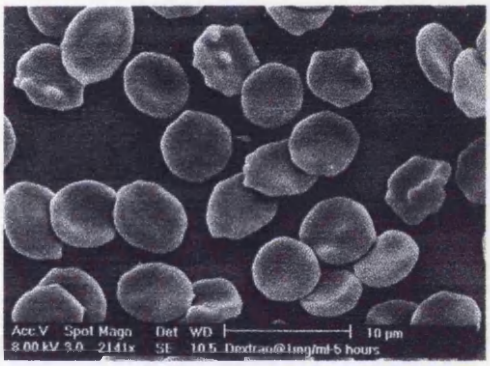
Panel (a) RBCs following 1h exposure to poly(L-lysine) at 1mg/ml (x4 216)



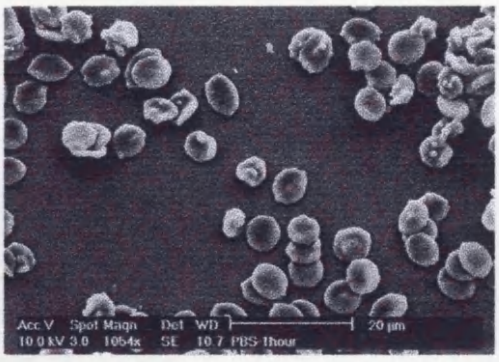
Panel (b) RBCs following 5h exposure to poly(L-lysine) at 1mg/ml (x1 066)



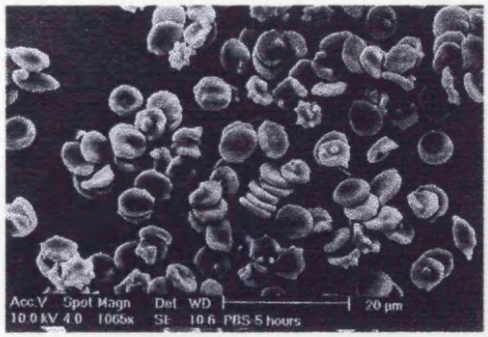
Panel (c) RBCs following 1h exposure to dextran at 1mg/ml (x2 108)



Panel (d) RBCs following 5h exposure to dextran at 1mg/ml (x2 141)



Panel (e) RBCs following 1h exposure to PBS pH 7.4 (x1 054)



Panel (f) RBCs following 5h exposure to PBS pH 7.4 (x1 065)

cells (Malik *et al.*, 1997). Other families of poly(amidoamine)s such as BPI (Mn 6 200) have been found to be quite cytotoxic (IC_{50} of 50μg/ml using CCRF-CEM cells) at relatively low concentrations, however poly(amidoamine)s such as BPP (Mw 6 300) have been shown to have IC_{50} values in excess of 500μg/ml when tested on both CCRF-CEM cells and Hep G2 cells (Sgouras, 1990). This is encouraging as it has shown that it is possible to alter the degree of cytotoxicity associated with poly(amidoamine)s by altering the structure and relative charge of the poly(amidoamine) backbone.

Poly(amidoamine) ISA 1, 4 and 9 red blood cell interactions at pH 7.4 were concentration- and time-dependant. Figure 3.8 and 3.9 show a high degree of haemoglobin release relative to poly(L-lysine). This result is likely to indicate that these polymers may not be suitable for systemic administration at high doses. ISA 22 and 23 were less lytic than ISA 1, 4 and 9 at concentrations below 3mg/ml after 24h incubation (Figure 3.10 and 3.11). This is likely to be due to the differences in the structure of the polymer backbone and the less cationic nature of ISA 22 and 23 relative to ISA 1, 4 and 9 as described in Chapter 1.

At concentrations of 1mg/ml all of the poly(amidoamine)s tested demonstrated pH-responsive haemoglobin release. This is best exemplified with ISA 22 and 23, which caused approximately 65% and 90% haemoglobin release, respectively after 24h incubation at pH 5.5 (Figure 3.15). As this response is not seen with cationic polymers such as poly(L-lysine), it may be attributable to the protonation of the poly(amidoamine) backbone. This observation was supported morphalogically with SEM (Figure 3.16). The difference in morphology between the rat RBCs that have been exposed to ISA 23 at 1mg/ml for 24h at pH 7.4 (Figure 3.16 panel (b)) and those at pH 5.5. (panel (c)) is apparent. Panel (c) shows a total loss of RBC morphology as well as a high degree of membrane fusion mediated by ISA 23 in response to a drop in pH (5.5) after 24h incubation. This is not evident in panel (b) (pH 7.4, ISA 23 1mg/ml, 24h incubation) where only a small degree of crenation is observed.

Chitosan has been widely reported to be a biocompatible substance though again the intended application of the material needs to be carefully considered

(Hirano *et al.*, 1988; Aspden *et al.*, 1996; Lee *et al.*, 1995; Heller *et al.*, 1996). Although chitosan is an approved pharmaceutical excipient in Japan, this does not necessarily mean that it will not demonstrate toxicity if administered i.v. (Bodmer *et al.*, 1989; Carreno Gomez and Duncan, 1997). Although specific chitosan molecules have been reported to be cytotoxic in a concentration-, molecular weight-, charge- and counter-ion- dependent manner (Carreno Gomez and Duncan, 1997), the molecular weight fractions examined here in did not demonstrate any significant toxicity within the experimental parameters examined (Figure 3.19, 3.20 and Table 3.1). This may be because of the molecules reduced degree of deacetylation and molecular weight in comparison to the experimental molecules previously examined (Carreno Gomez and Duncan, 1997) which in the instance of CL210 had a 100% degree of deacetylation and a molecular weight of approximately 100 000 Da. It is also of note that only one polymer, of the five compounds tested in Carreno Gomez and Duncan, (1997) demonstrated any cytotoxicity below 1mg/ml (Cl 210). Figure 3.17 and 3.18 show that there is a pronounced alteration in media pH at chitosan concentrations above 1mg/ml. This could possible be responsible for the drop in cellular viability seen (Figure 3.20) at high chitosan concentrations both here and in the study of Carreno Gomez and Duncan, (1997).

All the chitosan preparations showed very little haemolysis at concentrations up to 5mg/ml at all of the time points tested (Figures 3.21 and 3.22). These findings are in agreement with previous experiments (Heller *et al.*, 1996) and these molecules (N1-3) demonstrate a better biocompatibility profile than chitosan Cl 210 manufactured by Pronova. This is because chitosans N1-3 show a reduction in molecular weight and degree of deacetylation. Both these polymer attributes have been reported to be responsible for the red blood cell lysis and cytotoxicity. The RBC lytic profile of the chitosans N1-3 examined herein were assayed at time points 1h and 5h as opposed to 1h and 24h as reported by (Carreno Gomez and Duncan, 1997). This is because of the likely plasma residence time of these molecules as detailed in Chapter 5.

Figure 3.23 and 3.24 serve to reaffirm the observations made by the spectrophotometric RBC lysis model. This study also served to examine whether the low degree of haemoglobin release was due to haemoglobin co-precipitation though this phenomenon should not have gone undetected within the concentration range examined. A small degree of amorphous matter is observed (Figure 3.23 panel (c) and (d)). This is also consistent with Carreno Gomez and Duncan, (1997), and is thought to be glutaraldehyde cross-linked chitosan, precipitated during the sample preparation. Of note is the increased sensitivity of the morphological examination with reference to the haemoglobin release assay. RBC morphology is seen to alter before haemoglobin is released in any large quantity. This is consistent with the stated methods of polycation mediated membrane rupture as described previously.

3.5 Conclusions

All the poly(amidoamine)s and chitosans N1-3 examined were not toxic relative to poly(L-lysine) Mw 56 500 Da. The poly(amidoamine)s ISA 1, 4 and 9 are possibly too membrane lytic for systemic administration at high doses, though they do provide a reference point for further experimental work. ISA 22 and 23 showed very little membrane activity at pH 7.4 at concentrations below 3mg/ml, however some RBC lysis was evident above this concentration. ISA 22 and 23 and chitosans N1-3 were not seen to be as lytic as ISA 1, 4 and 9 at concentrations below 3mg/ml. The chitosans N1-3 demonstrated little haemolysis and this warranted further investigation into their potential as systemically administered delivery vehicles.

If a poly(amidoamine) can be designed whose structure is tailored to effect membrane rupture in response to the change in pH during transport from the extracellular space (pH 7.4) to the endosome (pH 6.5), it may be possible to design an endosomolytic polymer that can facilitate cytosolic access via the rupture of the endosomal membrane. Such a polymer would be non-membrane lytic in the systemic circulation and may bypass the secondary lysosome. It is important the enzymes contained in the secondary lysosome are not released into

the cytosol, as many catabolic lysosomal enzymes such as cathepsin B are still active at pH 7.4 (Barret and Heath, 1977) and could be damaging to the cell. If the secondary lysosome could be bypassed and cytosolic access for the therapeutic achieved, there may be further ramifications with regard to the half-life and bioavailability of biological therapeutics.

In view of the acceptable biocompatibility of the poly(amidoamine)s and chitosans observed here, the next step was to investigate their capacity to form an intermolecular complex with DNA. This may serve to protect the DNA from enzymatic degradation (Chapter 4).

Chapter 4

Chitosan and Poly(amidoamine):DNA Interactions

4.1 Introduction

The interaction between DNA and a polybase such as poly(L-lysine) is initially due to the co-operative binding of polyions to form what has been termed an interpolyelectrolyte complex (IPEC) (Kabanov and Alakhov, 1994) or polyplex (Felgner *et al.*, 1997; Midoux *et al.*, 1998). It is of note that hydrophobic and hydrophilic interactions as well as hydrogen bonding may contribute to IPEC formation (Kabanov and Alakhov, 1994) as well as solvation effects as reviewed by Tang and Szoka, (1998). In the form of an IPEC, DNA may or may not exists in a condensed form depending upon the ratio of polycation to DNA. DNA condensation may be defined as the transition of DNA tertiary structure from a random coil to a globular form and has been reviewed in Lasic *et al.*, (1998). Protective interactive non-condensing (PINC) complexes formed from DNA and PVA or PVP have also been reported (Mumper *et al.*, 1998).

The physical and biological properties of the IPEC are dictated by the structure of the polymer and the amount of polymer used relative to the amount of DNA (Shapiro *et al.*, 1969; Kabanov and Alakhov, 1994). Previous studies have reported that salt concentration and pH are also important factors when considering the formation of an IPEC particle (Shapiro *et al.*, 1969). Many of the physical characteristics of IPECs have been related to IPEC transfection efficiencies (Chapter 1) and the intracellular events resulting in transfection (Kabanov and Alakhov 1994; Kabanov and Kabanov, 1995; Smith *et al.*, 1997).

Historically there has been much interest in polycation:DNA interaction. Poly(L-lysine) was used to model DNA:protein interactions (Haynes *et al.*, 1970) and DEAE dextran was one of the first transfection vectors to be used *in vitro* (McCutchan and Pagano, 1968). As has been discussed (Chapters 1 and 3) polycationic molecules such as poly(L-lysine) are toxic at relatively low concentrations (<50μg/ml), however chemical modification and a reduction in the density of the cationic charges along the polymer backbone may reduce the toxicity of the molecule (Ferruti *et al.*, 1997; Heller *et al.*, 1996). The poly(L-lysine):DNA transfection system has been adapted and extensively researched with a view to analysing some of the cellular mechanisms responsible for, and the

optimisation of transfection. Some of the more significant studies have examined the mechanisms of cellular entry using receptor specific ligands, (Wu and Wu, 1998; Cotton *et al.*, 1990; Wagner *et al.*, 1991; Wagner *et al.*, 1990; Zanta *et al.*, 1997), cytosolic access (Wagner *et al.*, 1992; Wagner *et al.*, 1992[b]; Kichler *et al.*, 1997), as well as a variation in the Mw, size and chemical nature of the polycation used (Wagner *et al.*, 1991; Wolfert and Seymour, 1996; Wolfert *et al.*, 1996; Boussif *et al.*, 1995) reviewed in (Kabanov and Kabanov, 1995; Smith *et al.*1997).

It is hoped that the transfection of cells can be emulated using IPECs formed using biocompatible polymers however, before transfection can occur, the DNA must be protected from nucleases. Here, the ability of poly(amidoamine)s ISA 23, chitosans N1-3, poly(L-lysine) (Mw 260 000 Da, 47 700 Da and 3 900 Da) and dextran (Mw 72 000) to stabilise DNA with regard to nuclease (DNase II) degradation has been investigated. Prior to this, complex formation was examined using gel retardation.

The electrophoretic migration of DNA within an agarose gel is due to the negative charges of the DNA molecule at pH 7.2 (pH of TAE electrophoresis buffer) being drawn towards the cathode in a DC electric field. Factors that influence the electrophoretic separation of DNA molecules are the molecular weight of the DNA, its tertiary conformation and the matrix pore size. Different molecular weights of DNA are separated due to varying resistance to passage through a polysaccharide matrix (Maniatis *et al.*, 1986).

Gel retardation in this instance examines both the apparent change in molecular weight of the DNA molecule, (as a contiguous fragment may be formed (Hud *et al.*, 1995)) as well as the neutralisation of the net charge of the DNA molecule as a function of IPEC formation (Dash *et al.*, 1997; Wolfert *et al.*, 1996; Citro *et al.*, 1994). The change in DNA tertiary structure as a function of condensation will also influence the electrophoretic migratory pattern of the complex (Maniatis *et al.*, 1986).

The condensation of DNA has also been reported to exclude intercalating dyes such as ethidiumbromide (Chapter 1) as a result of the loss of rigidity of the

DNA duplex. A consequence of this is that if condensed DNA is subject to electrophoresis, detection with ethidiumbromide may result in no DNA being evident. It is important to emphasise that this assay is only semi-quantitative, though does give an indication of the degree of IPEC formation. DNA gel retardation may be defined as an alteration in either the detection or the mobility of the DNA as a result of polymer:DNA interactions following agarose gel electrophoresis. Varying degrees of retardation has been reported. In the most extreme incidences no DNA is detected either in the gel or the gel loading well. The extent of the retardation may be seen to diminish to a slight distortion in the migration patens as defined by a shift in the apparent molecular weight of the DNA (Dash *et al.*, 1997; Wolfert *et al.*, 1996; Citro *et al.*, 1994).

IPEC formation may result in the inhibition of nucleases (Kabanov and Alakhov, 1994; Kabanov and Kabanov, 1995). DNase II (EC 3.1.4.6) is a lysosomal enzyme and has been reported to be widely distributed amongst many species, from the mammals to the insects. Human DNase II (Mw 30 000 Da) has been reported to be an endonuclease with no exonuclease activity and the molecule is a dimeric glycoprotein composed of two identical subunits. DNase II has been seen to have one of two mechanisms of cutting DNA. DNase II isolated from the human gastric mucosa has been reported to cut using a rapid, sequential, double stranded mechanism whereas the uterine and pancreatic enzymes cut only one strand of the duplex. Both forms of the enzyme leave 3' phosphate end groups. The pH optimum is reported to be in the range of pH 3.8 to 5.5 and the process of cleavage is Mg^{2+} independent (reviewed in Barret and Heath, 1977). For these experiments porcine spleen DNase II was used indicating that a double stranded cut may be expected if no DNA protection is afforded (Barret and Heath, 1977; Kabanov and Kabanov, 1995; Mumper *et al.*, 1998).

In this study, the interaction of λ *Hind* III and calf thymus DNA with poly(amidoamine)s ISA 1, 4, 9, 22 and 23 as well as chitosans N1-3 were examined. These experiments were conducted with a view to investigating

whether any interactions between the polymers and the λ *Hind* III DNA were evident (as indicated by gel retardation) and whether these interactions could prevent the degradation of calf thymus DNA from DNase II.

4.2 Methods

Chitosan N1-3 and poly(L-lysine); Mw 260 000 Da, 47 700 Da and 3 900 Da were used to form IPECS with λ *Hind* III DNA using charge:charge ratios whereas dextran and the poly(amidoamine)s were mixed with DNA on a weight to weight basis. The quantities of each substance required to form IPECs were calculated, assuming that all polybasic molecules were fully protonated at neutral pH (Table 4.1);

Poly(L-lysine)

As poly(L-lysine) is a homopolymer with the monomer having a Mw of 129 Da, 129g of monomer may be seen to contain 6.023×10^{23} positive charges. Thus $6.023 \times 10^{23} / 129\,000 = 4.67 \times 10^{18}$ positive charges/mg.

Calf thymus and λ Hind III DNA

As the mean molecular weight of the DNA monomer may be calculated, (322Da) (Maniatis *et al.*, 1986) DNA may be seen to contain 1.87×10^{20} negative charges/mg.

Chitosan

Fully deacetylated chitosan hydrochloride contains 3.01×10^{18} ($^{+}NH_3.Cl^{-}$) groups per mg of solid. Once the degree of polymer deacetylation is taken into consideration the following may be seen.

N1 $3.0115 \times 10^{18} / 65.36\,\% = 1.968 \times 10^{18}$ positive charges/mg

N2 and 3 $3.0115 \times 10^{18} / 55.25\,\% = 1.664 \times 10^{18}$ positive charges/mg

Gel retardation assays were performed as described in Chapter 2. The DNase II protection assays were performed as described (Chapter 2) with the

Table 4.1 Quantity (μg) of polymer used in a 1:1 charge ratio (add to 1μg DNA)

Polymer	(Quantity μg)
N1	9.5
N2 and 3	11.3
Poly(L-lysine)	0.4
ISA 1, 4 and 9	1.0
ISA 22 and 23	1.0
Dextran	1.0

following additions. The calf thymus DNA was mixed with chitosan also dissolved in sodium acetate buffer (0.2N) pH 5.5 containing potassium chloride (0.2N) to give the following charge ratios; 0, 1:1, 1:0.1 and 1:0.05. This was done prior to the addition of the DNase II enzyme. The results obtained were expressed as DNase II inhibition (%) relative to a control containing no polymer and normalized to the point of maximum DNA degradation against time (min) in triplicate.

ISA 23 was dissolved as above and IPECs were formed on a weight to weight basis at a ratio of 1:100 (DNA:polymer). The experiments were again performed in triplicate and the results were expressed as above. Poly(L-lysine) and dextran were also used as reference controls. The following samples were tested in triplicate and the results expressed as above;

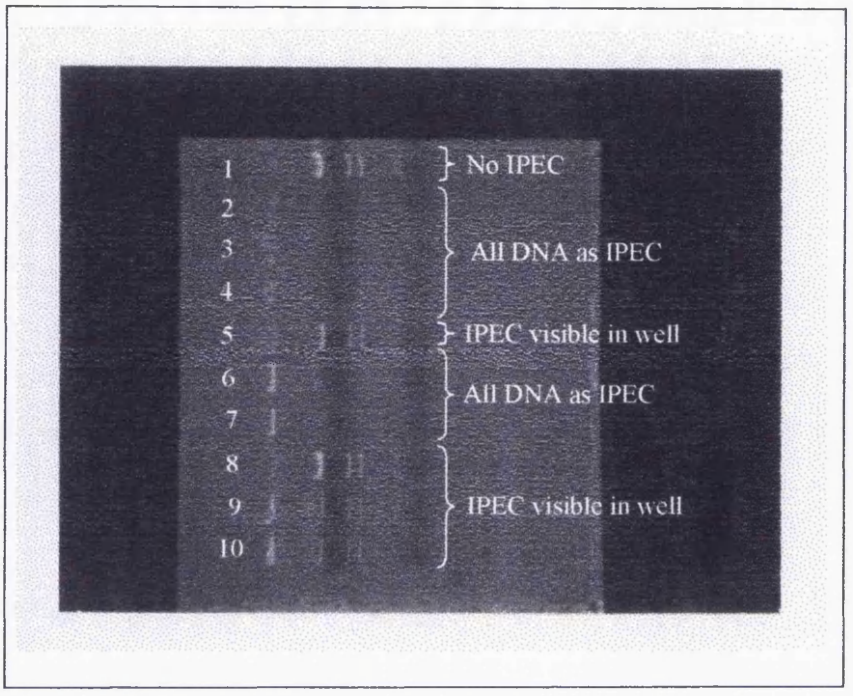
Poly(L-lysine) Mw 260 000 Da	1:10, 1:1, 1:0.01 and 1:0.05 (charge ratios)
Poly(L-lysine) Mw 4 700 Da	1:10, 1:1, 1:0.01 and 1:0.05 (charge ratios)
Dextran	1:1000, 1:100 and 1:10 (weight ratios)

4.3 Results

The ability of chitosans N1-3 to retard the electrophoretic mobility of λ *Hind* III DNA is shown (Figure 4.1) and at a charge ratio of 1:1 DNA is visible only in the well of the gel in all instances. This is indicative of a high degree of association between the polymer and the DNA. When the quantity of polymer is reduced relative to the amount of DNA used, retardation is still evident apparently in a molecular weight-dependent manner.

DNA mixed with the poly(amidoamine)s ISA 1, 4 and 9 (Figure 4.2) displayed a similar pattern of retardation as the DNA mixed with the chitosan molecules (Figure 4.1). In contrast, the poly(amidoamine)s ISA 22 and 23 (Figure 4.3) showed a much lower ability to retard DNA, though at higher polymer concentrations retardation was still evident (Figure 4.3, lanes 2 and 3). Figure 4.4 shows the degree of retardation mediated by mixing λ *Hind* III DNA with the

Figure 4.1 Gel retardation by chitosans N1,2 and 3



1) λ *Hind* III DNA control (1μg DNA)

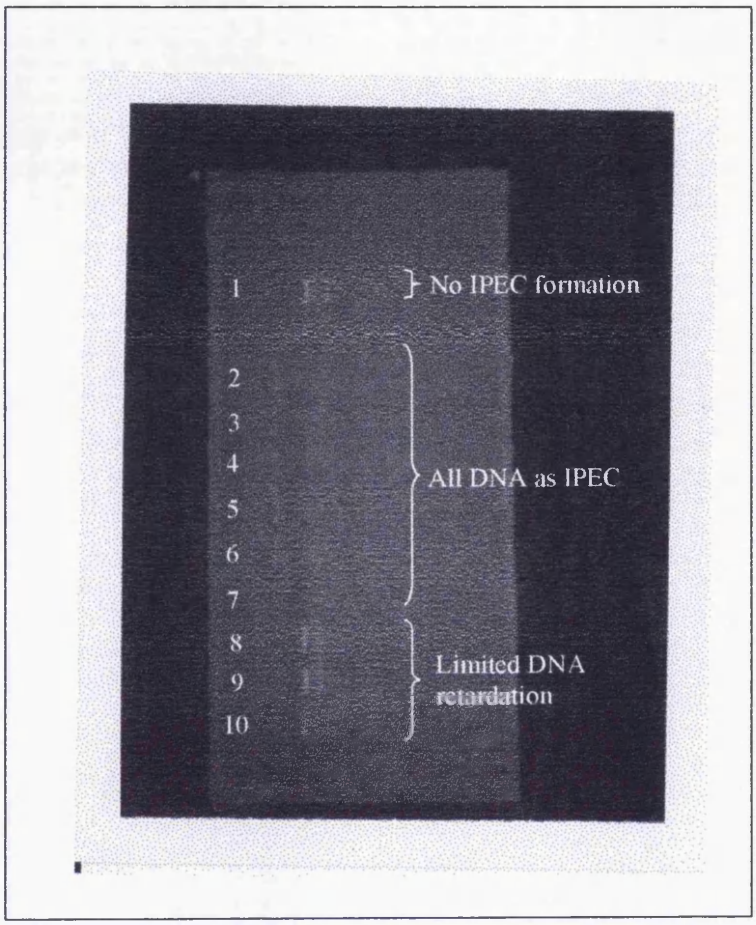
2) 1:1 complex (N1)
3) 1:1 complex (N2)
4) 1:1 complex (N3)

5) 1:0.1 complex (N1)
6) 1:0.1 complex (N2)
7) 1:0.1 complex (N3)

8) 1:0.05 complex (N1)
9) 1:0.05 complex (N2)
10) 1:0.05 complex (N3)

Ratios refer to the quantity of DNA:polymer
1μg of DNA used in each instance
All ratios refer to charge:charge interactions

Figure 4.2 Gel retardation by poly(amidoamine)s ISA 1, 4 and 9



1) λ *Hind* III DNA control (1μg DNA)

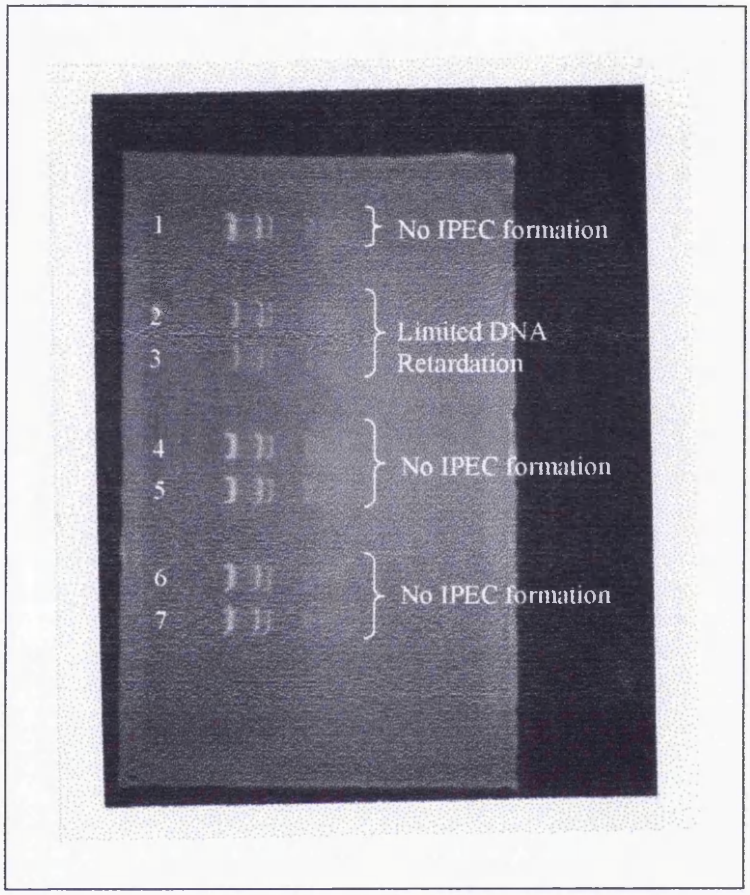
2) 1:10 complex (ISA 1)
3) 1:10 complex (ISA 4)
4) 1:10 complex (ISA 9)

5) 1:1 complex (ISA 1)
6) 1:1 complex (ISA 4)
7) 1:1 complex (ISA 9)

8) 1:0.1 complex (ISA 1)
9) 1:0.1 complex (ISA 4)
10) 1:0.1 complex (ISA 9)

Ratios refer to the quantity of DNA:polymer 1μg of DNA used in each instance. All ratios refer to weight:weight interactions

Figure 4.3 Gel retardation by poly(amidoamine)s ISA22 and 23



1) λ *Hind* III DNA control (1μg DNA)

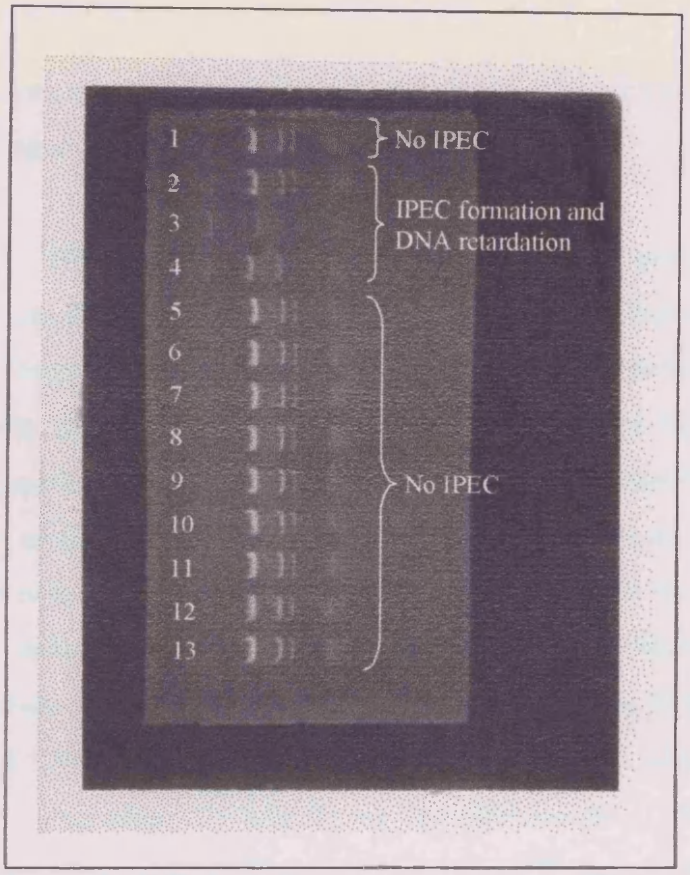
2) 1:100 complex (ISA23)
3) 1:100 complex (ISA22)

4) 1:10 complex (ISA23)
5) 1:10 complex (ISA22)

6) 1:1 complex (ISA23)
7) 1:1 complex (ISA22)

Ratios refer to the quantity of DNA:polymer 1μg of DNA used in each instance. All ratios refer to weight:weight interactions

Figure 4. 4 Gel retardation by poly(L-lysine) (complex by charge) and dextran (complex by weight)



1) λ *Hind* III DNA control (1μg DNA)

2) 1:1 complex (poly(L-lysine) Mw 260 000 Da)
3) 1:1 complex (poly(L-lysine) Mw 47 700 Da)
4) 1:1 complex (poly(L-lysine) Mw 3 900 Da)

6) 1:0.1 complex (poly(L-lysine) 260 000 Da)
7) 1:0.1 complex (poly(L-lysine) 47 700 Da)
8) 1:0.1 complex (poly(L-lysine) 3 900 Da)

10) 1:0.05 complex (poly(L-lysine) 260 000 Da)
11) 1:0.05 complex (poly(L-lysine) 47 700 Da)
12) 1:0.05 complex (poly(L-lysine) 3 900 Da)

Ratios refer to the quantity of polymer:DNA:. 1μg of DNA was used in each instance. All ratios refer to charge:charge interactions

5) 1:1 Dextran complex
9) 1:0.1 Dextran complex
13) 1:0.05 Dextran complex

Ratios refer to the quantity of DNA: polymer. 1μg of DNA was used in each instance. All ratios refer to weight:weight interactions

reference polymers, poly(L-lysine) and dextran at specified charge ratios. No retardation was evident when dextran was used. Poly(L-lysine)-mediated retardation was observed in a poly(L-lysine) concentration- and molecular weight-dependent manner.

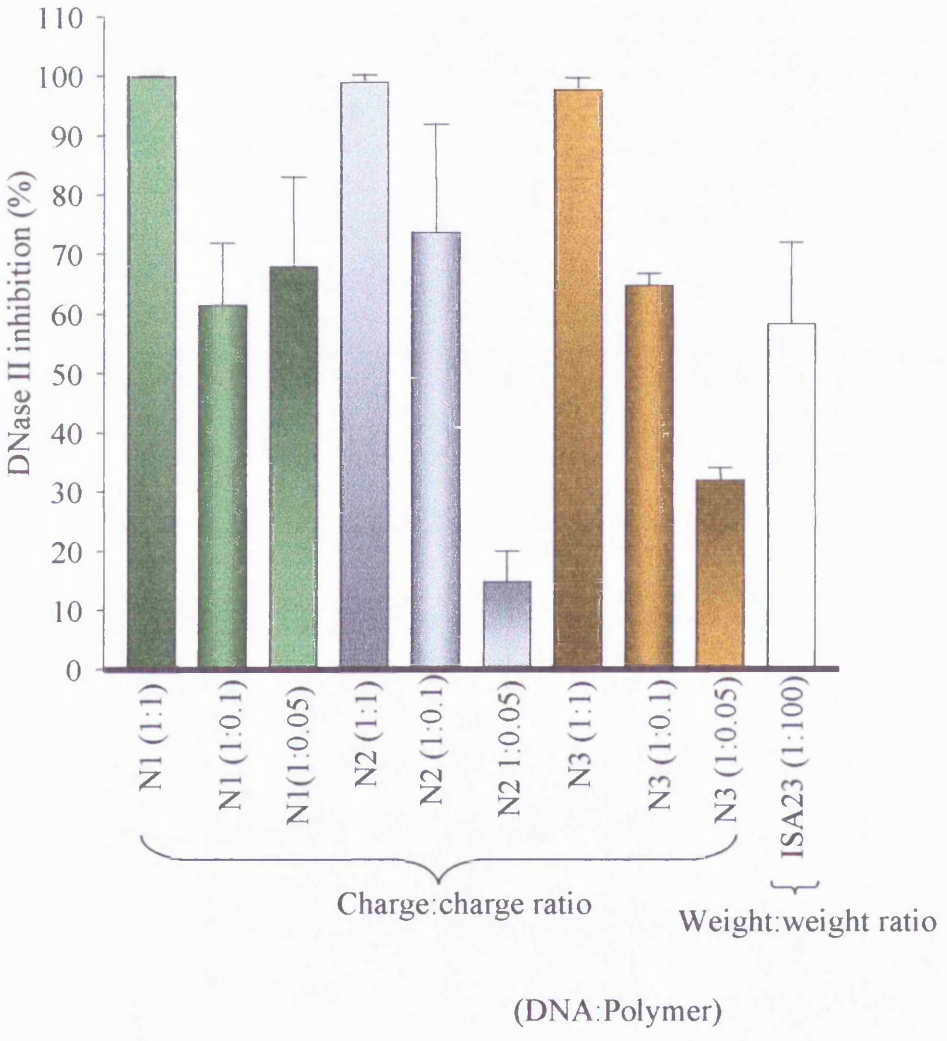
DNase II was shown to rapidly degraded calf thymus DNA (Figure 4.5 and 4.6) and all of the polymers tested (chitosan, poly(L-lysine), poly(amidoamine)s and dextran) demonstrated the ability to inhibit the degradation of DNA by DNase II. The degree of DNase II protection was effected by the chemistry of the polymer backbone and the DNA:polymer ratio (Figure 4.5). Where a 1:1 charge ratio of the chitosans was employed >95% inhibition of DNase II was seen in each instance. When the physical quantity of chitosans N2 and N3 in the reaction was reduced, so was the degree of DNase II inhibition though this was not observes with N1. When ISA 23 was mixed with the DNA at a weight ratio of 1:100 (DNA:polymer) >60% DNase II inhibition was evident (Figure 4.5).

The effect of adding defined quantities of poly(L-lysine) or dextran to calf thymus DNA prior to performing the DNase II assay is shown in Figure 4.6. At charge ratios of 1:1 and above ~75% inhibition of DNase II is evident for both poly(L-lysine) molecular weights. At lower concentrations of poly(L-lysine) and dextran a synergy between the polymer and DNase II is observed.

4.3 Discussion

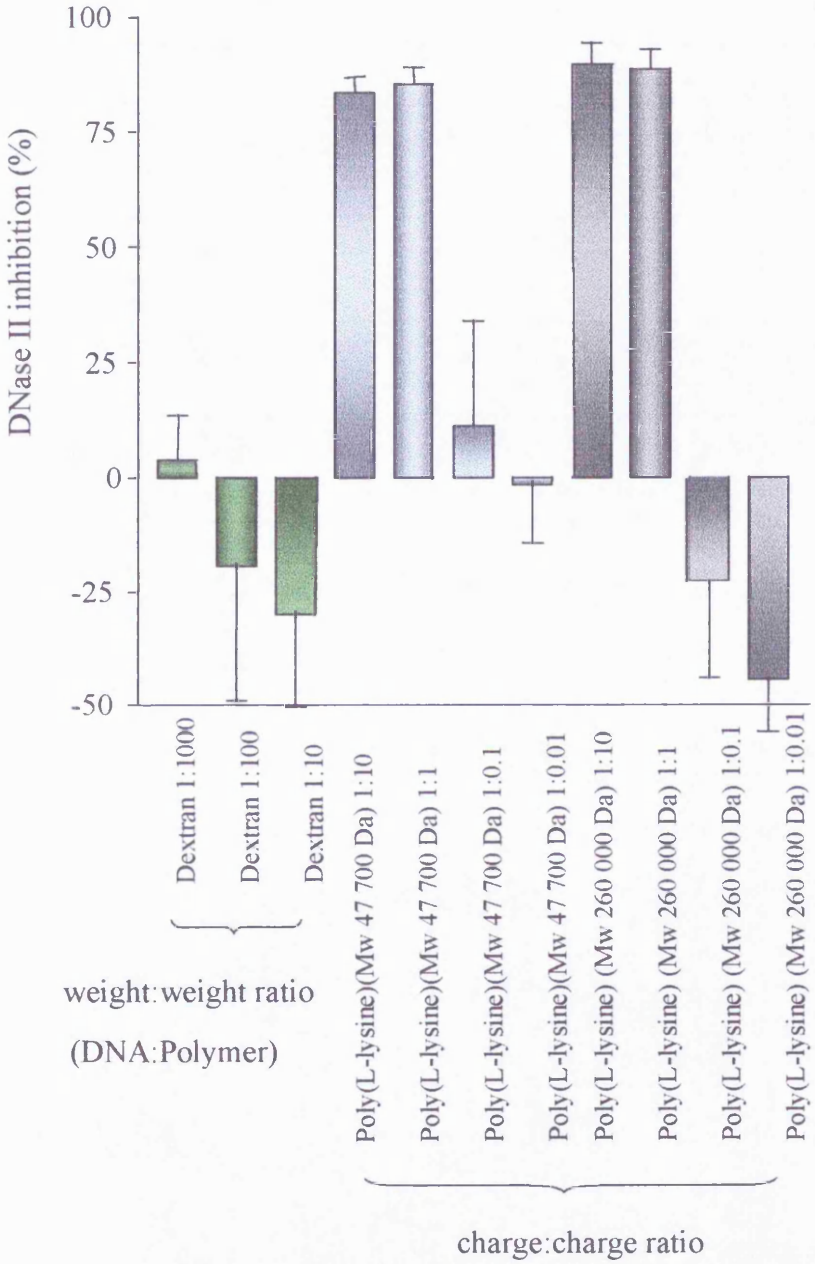
When compared on charge ratio basis, the chitosans were found to be more efficient at forming IPECs than the poly(L-lysine) molecules (Figures 4.1 and 4.4). When the quantity (weight) of polymer used to form an IPEC, relative to the amount (weight) of DNA is considered (Table 4.1) the comparison of polymers using charge ratios may be misleading. Comparison on a weight basis shows that chitosans N1-3 have an efficiency similar to poly(L-lysine) with regard to the electorphoretic retardation of DNA. This is surprising as the distribution of charged groups along the macromolecular backbone of the chitosan molecules is much less dense than poly(L-lysine) (Table 4.1). As the chitosan molecules have a

Figure 4.5 Inhibition of DNase II mediated DNA degradation by the chitosans N1-3 and ISA 23 (Mean ± S.E. n=3)



Results were expressed as DNase II inhibition (%) relative to a control containing no polymer. This was done by expressing the area under the curve (calculated by trapezoid approximation and extrapolation to zero) of the polymer containing experiment as a percentage of the area under the curve of a control and subtracting the result from 100%

Figure 4.6 Inhibition of DNase II mediated DNA degradation by poly(L-lysine) (Mw 260 000 Da and 47 700 Da) and dextran (Mw 72 000Da)(Mean ± S.E. n=3)



Results were expressed as DNase II inhibition (%) relative to a control containing no polymer. This was done by expressing the area under the curve (calculated by trapezoid approximation and extrapolation to zero) of the polymer containing experiment as a percentage of the area under the curve of a control and subtracting the result from 100%

polysaccharide backbone (Figure 1.7) as opposed to a peptide backbone, the chitosan molecules have a much higher capacity for hydrogen bonding relative to poly(L-lysine) and this may explain the high degree of chitosan mediated DNA gel retardation.

When compared on a charge to charge basis (Figure 4.1 and 4.4) the low molecular weight chitosan N1 was not as efficient at retarding DNA as the higher molecular weight chitosans (N2 and N3) (Figure 4.1). Chitosan N1 has a higher degree of deacetylation than chitosan N2 and N3 (Table 1.9) and consequently less chitosan (weight) was used during the preparation of an IPECs at identical charge ratios relative to N2 and N3 (Table 4.1).

ISA 1, 4 and 9 form IPECs much more efficiently than ISA 22 and 23 (Figures 4.2 and 4.3). This is because of the cationic nature of ISA 1, 4 and 9 at pH 7.4 relative to that of ISA 22 and 23, (Chapter 1). It is however of note that both ISA 22 and ISA 23 do retard the electrophoretic migration of DNA at very high concentrations of polymer relative to the quantity of DNA used, as is evident (Figure 4.3).

When the physical quantities (weight) of all of the polymers examined here-in (poly(amidoamine)s, chitosans N1, N2 and N3, poly(L-lysine) and dextran) required to retard DNA is considered and compared, the polymers may be ranked thus: poly(L-lysine) (Mw 47.7 K Da) > ISA1, 4 and 9 > Chitosan N1, N2 and N3) > ISA 22 and 23 > Dextran. When this comparison is made from the perspective of charge the following rank retardation efficacy was seen. The most efficient polymer was N2 and N3 > N1> poly(L-lysine). Comparisons of poly(amidoamine)s with chitosan are difficult because of the very different chemical structures and physical properties of these molecules.

The formation of DNA toroidal condensates by poly(L-lysine) is well documented (Hud *et al.*, 1995; Shapiro *et al.*, 1969; Manning, 1978) and these structures may also be viewed using electron microscopy (Wagner *et al.*, 1991;

Wolfert *et al.*, 1996) and have been termed "doughnuts" (Manning, 1980). These reports document a change in the tertiary structure of DNA indicative of condensation. It is this change in DNA structure that may, in part, account for the inability of nucleases such as DNase II to reach or recognise the DNA as a substrate for catabolism. Kabanov and Alakhov, (1994) reported that nucleases would only degrade DNA at sites where no polybase is present. This is supported experimentally here as the proportion of polymer- associated with the IPEC is reduced, more nuclease degradation occurs (Figures 4.5 and 6).

Limited ionic association may drive the formation of IPECs using ISA 23 and it is this association that is thought to be responsible for the inhibition of DNase II (Figure 4.5) and also the gel retardation (Figure 4.3). The inhibition of DNase II activity exerted by dextran (Figure 4.6) may be due to polymer mediated DNA phase separation and subsequent DNA condensation (reviewed in Lasic *et al.*, 1998).

4.5 Conclusions

Here it has been shown that the chitosans N1-3 can retard DNA with an efficiency similar to that of poly(L-lysine) when compared by weight basis, whilst demonstrating a biocompatibility profile suited to systemic administration (Chapter 3).

The most efficient DNA retarding polymer when compared by weight as opposed to charge (Table 4.1) was poly(L-lysine). All the chitosans N1-3, ISA 1, 4, 9, 22 and 23 all mediated varying degrees of gel retardation, the efficiency of which could be compared on the basis of polymer weight as opposed to charge. Dextran did not produce any DNA gel retardation even at 1:1000 polymer excess by weight. All of the polymers examined herein can, under specific conditions protect DNA from enzymatic degradation by DNase II.

The usefulness of polymer:DNA complexes *in vivo* is very much dependent on their biodistribution after i.v. administration. Consequently, the next

step in the systematic evaluating the potential of both poly(amidoamine)s and chitosan polymers as potential nucleic acid delivery vehicles was to examine the body distribution of ^{125}I-labelled chitosans N1-3 (Chapter 5) and ^{125}I-labelled ISA 4, 9 and 22 (Chapter 6).

Chapter 5

Body Distribution of ^{125}I-Labelled Chitosan

5.1 Introduction

As discussed in Chapter 1, the attachment of a therapeutic agent to a polymeric vector may modulate the pharmacokinetics of the agent in a therapeutically advantageous manner (Duncan, 1992). This is particularly relevant from the perspective of nucleic acid delivery, as an increase in the local concentration of nucleic acids or IPEC may lead to an increase in local transfection efficiency. If a beneficial change in pharmacokinetics is to be achieved it is vitally important that the vector itself displays an appropriate body distribution. In view of this, it was considered important to establish the body distribution of ^{125}I-labelled chitosans N1-3.

Although chitosan has been proposed as a component of many drug delivery systems (Carreno-Gomez and Duncan, 1997), there has been no systematic study of its biodistribution after i.v. administration into animals. As chitosans N1, 2 and 3 displayed a biocompatibility profile suitable for systemic administration, (Chapter 3 (Richardson *et al.*, 1998[b])) the body distribution of ^{125}I-labelled chitosans N1-3 was examined in the rat following i.v. administration.

Studies evaluating the body distribution of many potential polymeric vectors have already been performed. For example, the biodistribution of ^{125}I-labelled alginate, (Al-Shamkhani and Duncan, 1995) ^{125}I-labelled HPMA copolymer tyrosinamide (Duncan *et al.*, 1982), and ^{125}I-labelled HPMA copolymer galactosamine (Duncan *et al.*, 1982; Pimm *et al.*, 1996) and ^{125}I-labelled poly(L-lysine) derivatives (Pimm *et al.*, 1995) have been described.

Pimm *et al.*, (1995) has used several techniques used to label a variety of polymers to high specific activities using [^{125}I]iodide, [^{111}In]indium and [^{51}Cr]Chromium. The most appropriate form of radiolabelling for a particular polymer will depend on the structure of the polymer i.e. its sensitivity to chemical modification by the reaction conditions and also the probability that the method of radiolabelling will not influence the biodistribution properties of the polymer. Radiolabelling using reagents such as Chloramine T can be very effective at attaching [^{125}I]iodide to a phenolic ring, however this technique may lead to

iodination damage and denaturation when used to label proteins such as IgG. As a consequence, alternative methods for labelling proteins with [^{125}I]iodide have been reported (Bolton and Hunter, 1973). As chitosan contains no phenolic rings, two options were considered to facilitate radioiodination. Either, incorporation of a suitable pendant group e.g. tyrosinamide as used previously to allow the radiolabelling of other polymers (Al-Shamkhani and Duncan, 1995) or alternatively labelling the primary amine groups pendant to the chitosan backbone using Bolton and Hunter reagent (Bolton and Hunter, 1973). The latter method required the covalent attachment of either a mono- or di- [^{125}I]iodo-phenolic ring to the primary amine pendant to the polymer backbone and does so in a one-step process (Figure 2.1). The Bolton and Hunter reagent was chosen as only one further characterisation step would be required after the labelling reaction (Hunter and Greenwood, 1962, Greenwood *et al.*, 1963).

As the body distribution of ^{125}I-labelled HPMA copolymer tyrosinamide has already been well characterised, (Duncan *et al.*, 1982; Pimm *et al.*, 1996) it was used as a reference control.

5.2 Methods

The chitosans N1-3 were radiolabelled, characterised and the body distribution experiments performed as detailed (Chapter 2). HPMA copolymer tyrosinamide (Mw. 14 970 Da) was radioiodinated as described in Chapter 2 using the Chloramine T method. The ^{125}I-labelled polymers were then characterised as described in Chapter 2. [^{125}I]Iodide was then assayed for as described previously (Chapter 2) and the experiments repeated at different time points (5 and 60 min) in triplicate.

To evaluate the de-iodination of the ^{125}I-labelled chitosan post injection, urine excreted following the administration of the ^{125}I-labelled N2 was fractionated using a Sephadex G25 PD10 column. Urine (1ml) was then applied to the column and 500µl fractions eluted using distilled water. The fractions were collected in counting tubes each containing 500µl of distilled water and assayed for radioactivity. Radioactivity (cpm) was then plotted against retention volume

and as a reference control, the ^{125}I-labelled chitosan N2 stock solution was also subject to PD10 column chromatography.

5.3 Results

The ^{125}I-labelled HPMA copolymer tyrosinamide preparation had a labelling efficiency of 82% and contained <1% low molecular weight [^{125}I]iodide (Figure 5.1). The paper electrophoresis profiles obtained for the reaction mixtures and purified preparations of ^{125}I-labelled chitosans N1-3 are shown in Figures 5.2-5.4 respectively. The ^{125}I-labelled chitosans had a much lower labelling efficiency, approximately 1% in each case. Following purification, most free [^{125}I]iodide was removed leaving 1.5%, 1.4% and 1% low molecular weight [^{125}I]iodide in ^{125}I-labelled chitosans N1-3 respectively. This was not a percentage of total [^{125}I]iodide but a percentage of the [^{125}I]iodide remaining after dialysis. This was deemed low enough to allow the monitoring of the body distribution of the ^{125}I-labelled chitosans.

Over the first hour little organ accumulation of ^{125}I-labelled HPMA copolymer tyrosinamide was seen and after 5min >80% of the recovered dose was present in the bloodstream (Figure 5.5). Substantial renal excretion was also observed showing >40% of the recovered dose present in the urine at 1h.

In contrast the ^{125}I-labelled chitosans N1-3 show more rapid blood clearance and this clearance was molecular weight-dependent (Figures 5.6-5.8). After 5min approximately 45% of the recovered dose of ^{125}I-labelled N1 is present in the blood, whereas the blood levels of ^{125}I-labelled N2 and N3 were both <15% of the recovered dose. The blood clearance of all of the ^{125}I-labelled chitosan molecules was accompanied by uptake into the liver and lungs and the extent of uptake was again molecular weight dependent. The lung uptake of ^{125}I-labelled N1 is particularly evident as >25% of the recovered dose is evident in the lungs after at 5 min. After 1h the liver associated radioactivity generally remained high but lung levels generally fell. Only the low molecular weight species, ^{125}I-labelled N1 showed appreciable renal elimination after 1h (approximately 15% recovered dose). The ^{125}I-labelled N3 chitosan was evident in the spleen after 5min.

Figure 5.1 Labelling efficiency and purity of ^{125}I-labelled HPMA copolymer tyrosinamide by paper electrophoresis (Electrophoresis standards shown in Appendix 1)

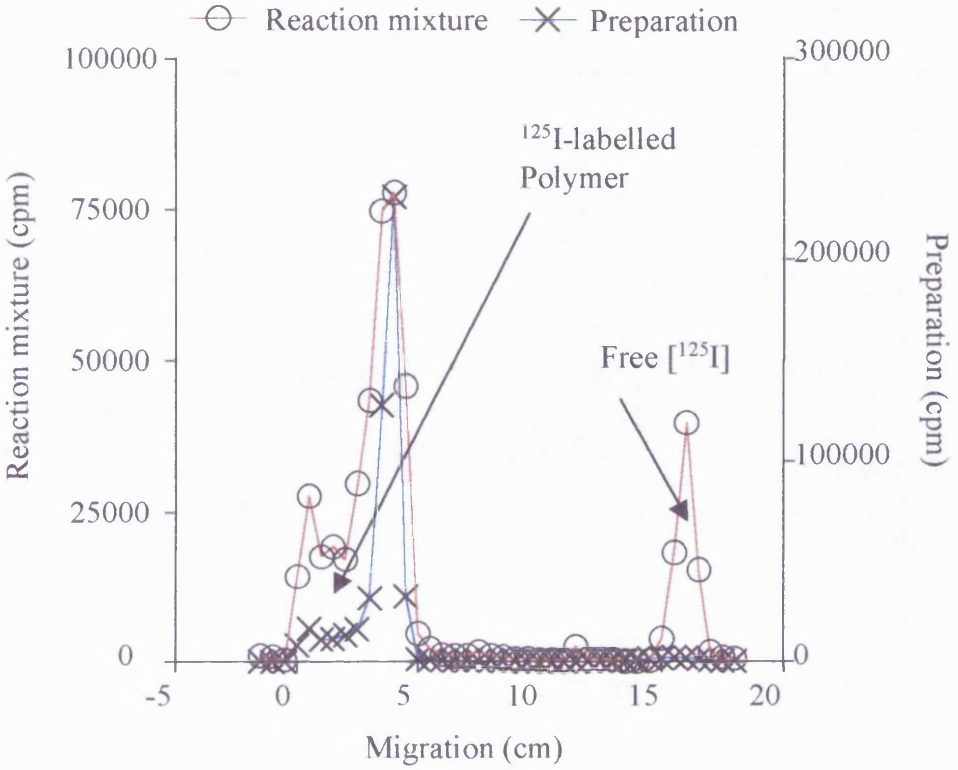


Figure 5.2 Labelling efficiency and purity of ^{125}I-labelled chitosan N1 by paper electrophoresis

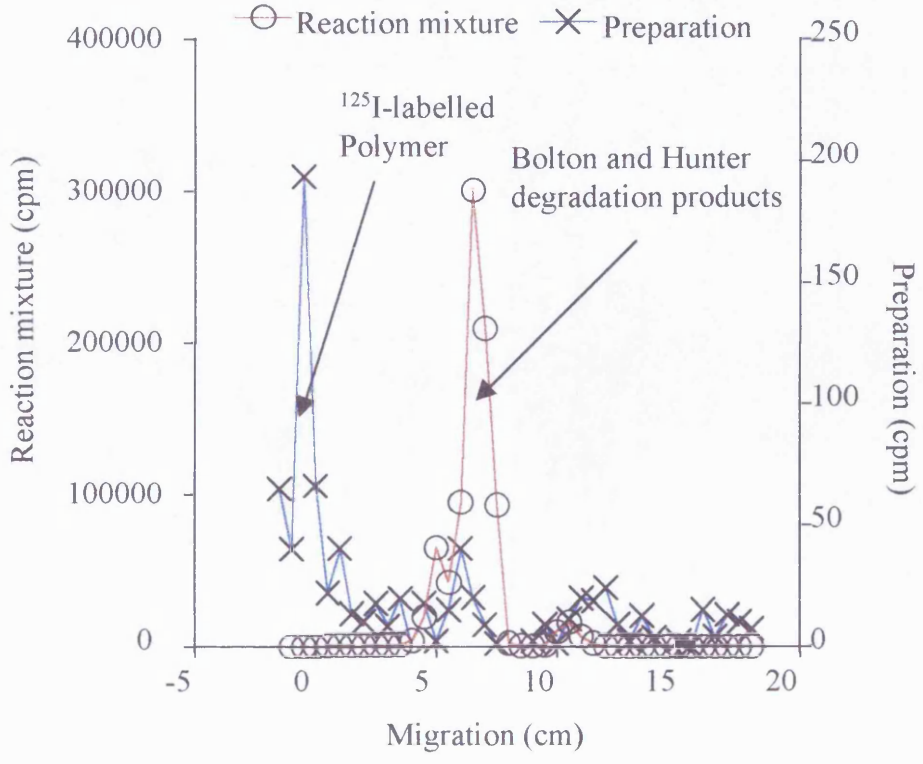


Figure 5.3 Labelling efficiency and purity of ^{125}I-labelled chitosan N2 by paper electrophoresis

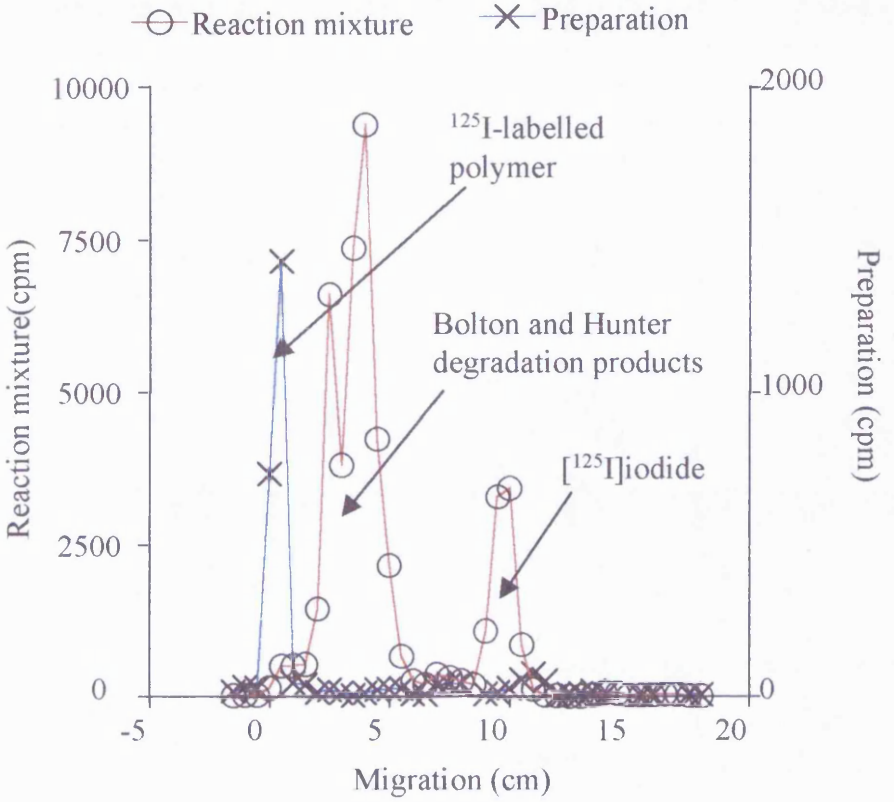


Figure 5.4 Labelling efficiency and purity of ^{125}I-labelled chitosan N3 by paper electrophoresis

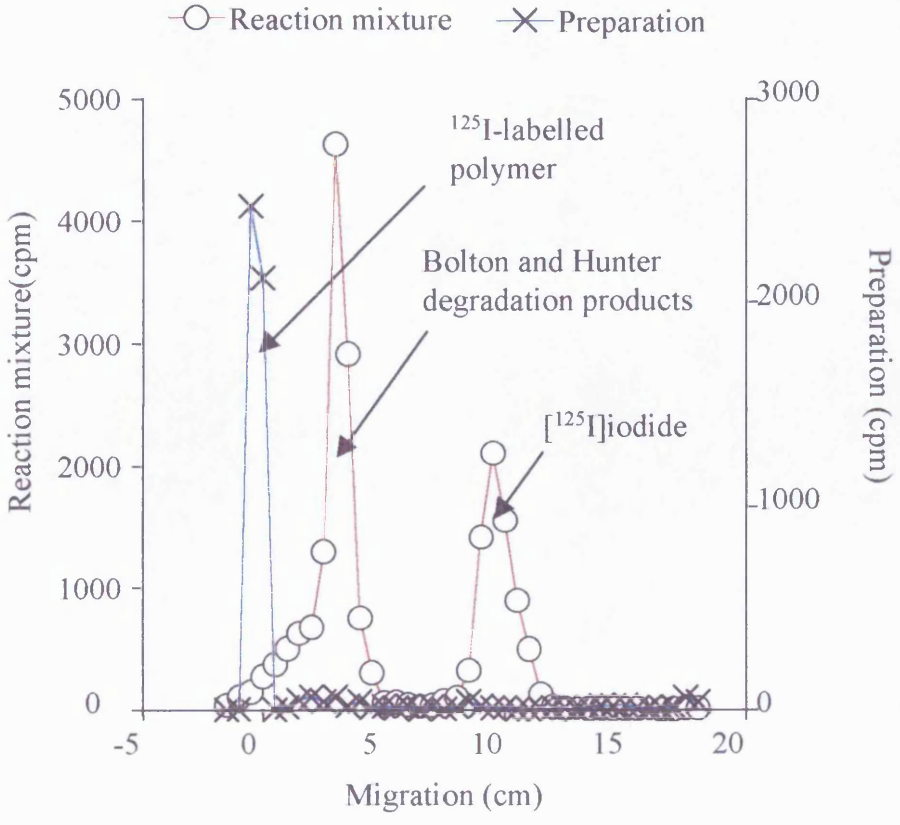


Figure 5.5 Body distribution of ^{125}I-labelled HPMA copolymer tyrosinamide in Wistar rats following i.v. administration (mean ± S.D n=3.)

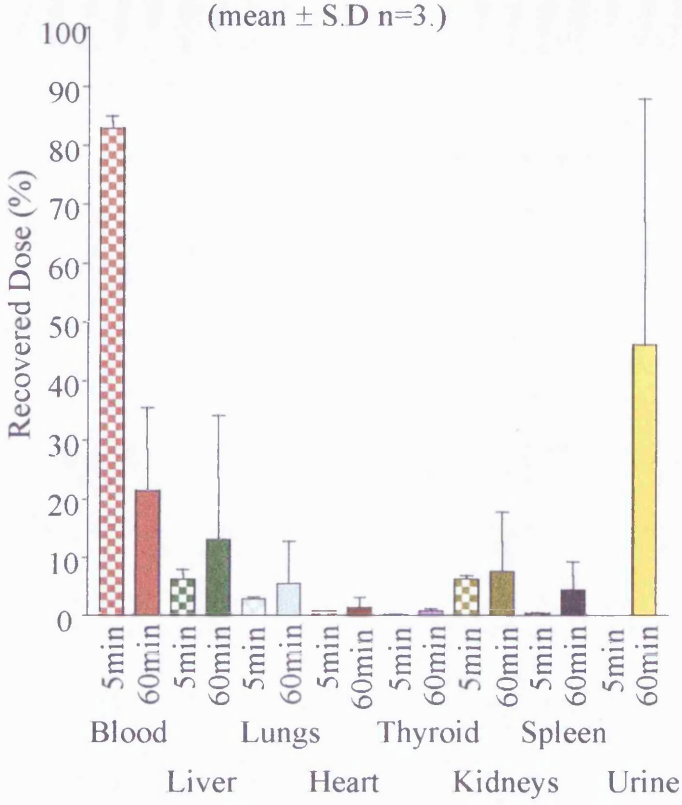


Figure 5.6 Body distribution of ^{125}I-labelled chitosan N1 in Wistar rats following i.v. administration (mean ± S.D n=3.)

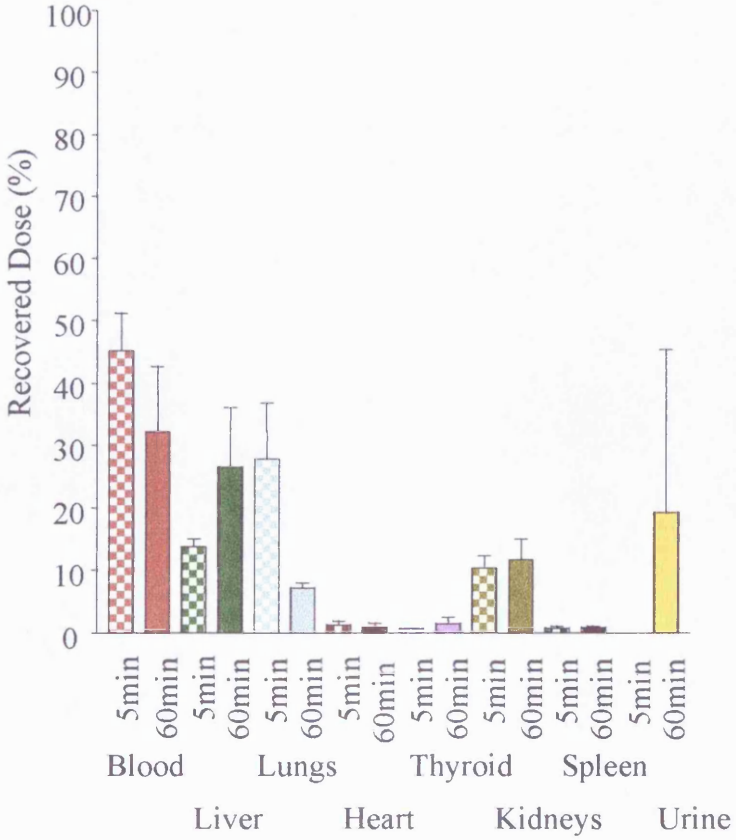


Figure 5.7 Body distribution of ^{125}I-labelled chitosan N2 in Wistar rats following i.v. administration (mean ± S.D. n=3)

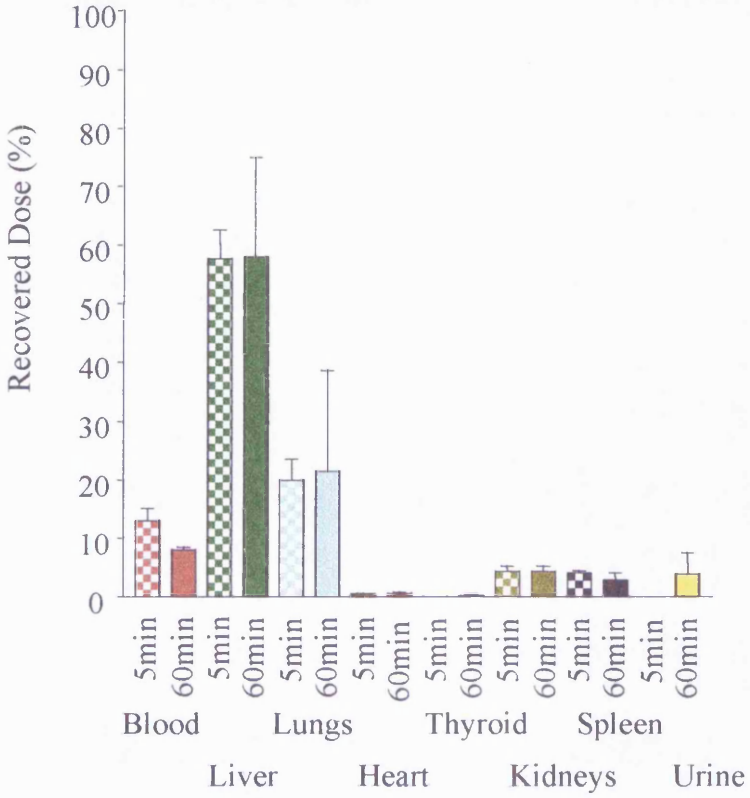


Figure 5.8 Body distribution of ^{125}I-labelled chitosan N3 in Wistar rats following i.v. administration (mean ± S.D. n=3)

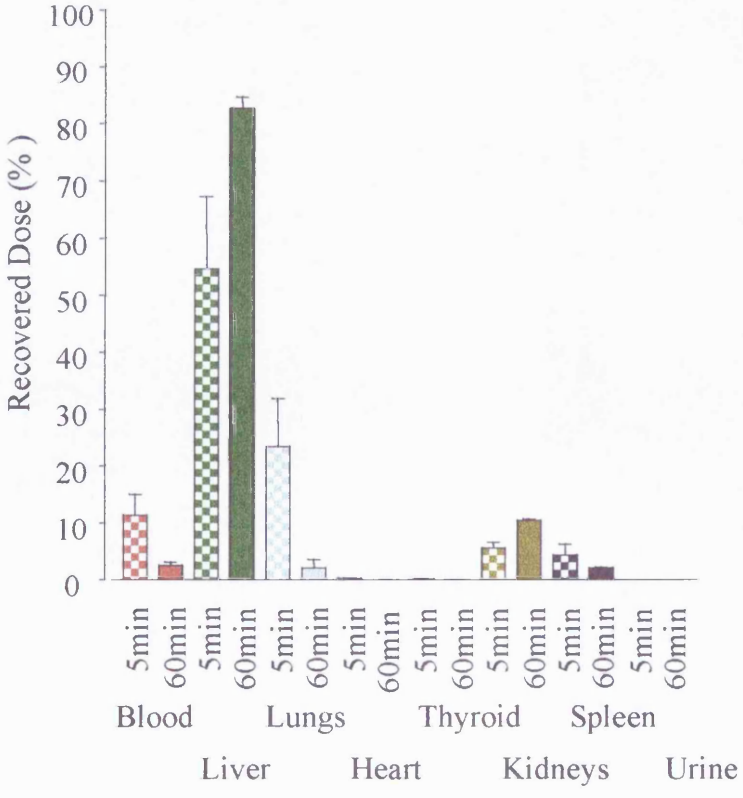


Figure 5.9 shows the appearance of low molecular weight ^{125}I-labelled products in the urine collected 1h post injection of ^{125}I-labelled chitosan N2. A comparison with the ^{125}I-labelled chitosan N2 starting preparation indicated that *in vivo* degradation of ^{125}I-labelled N2 had occurred.

The amount of low molecular weight [^{125}I]iodide present in each sample is summarised (Table 5.1) as well as the amount of radioactivity recovered from the rat. The specific activity of all of the ^{125}I-labelled polymers and the administered dose (mg/kg) of polymer are also summarised (Table 5.1).

5.4 Discussion

The labelling efficiency of ^{125}I-labelled chitosan molecules was very low relative to the labelling efficiency of ^{125}I-labelled HPMA copolymer tyrosinamide. Although similar quantities of [^{125}I]iodide (500μCi) and polymer (5mg) were used in each instance, the methods of conjugation differ. One factor that may have contributed to the reduced labelling efficiency of the chitosan molecules is the limited solubility of chitosan above pH 6.5. Once triethylamine is added to the reaction the chitosan may be seen to precipitate as a function of pH. A consequence of this is that only a limited number of functionalised primary amine groups are available for the condensation reaction that conjugates the [^{125}I]iodide containing phenolic ring to the polymer backbone.

The blood clearance rates of the ^{125}I-labelled chitosans after i.v. administration (Figures 5.6, 5.7 and 5.8) are comparable to those of other cationic polymers such as ^{125}I-labelled poly(L-lysine) (Nishikawa *et al.*, 1996; Pimm *et al.*, 1995) and ^{125}I-labelled DEAE dextran (Yamaoka *et al.*, 1995). The mechanisms of anionic and cationic polymer plasma clearance have been reviewed in Takakura and Hashida, (1996) where it is stated that many physiological mechanisms are responsible for the plasma clearance of macromolecular compounds. Examples of such mechanisms would be the adhesion of cationic

Figure 5.9 GPC of urine collected (60min) after i.v. administration of ^{125}I-labelled N2 to male Wistar rats

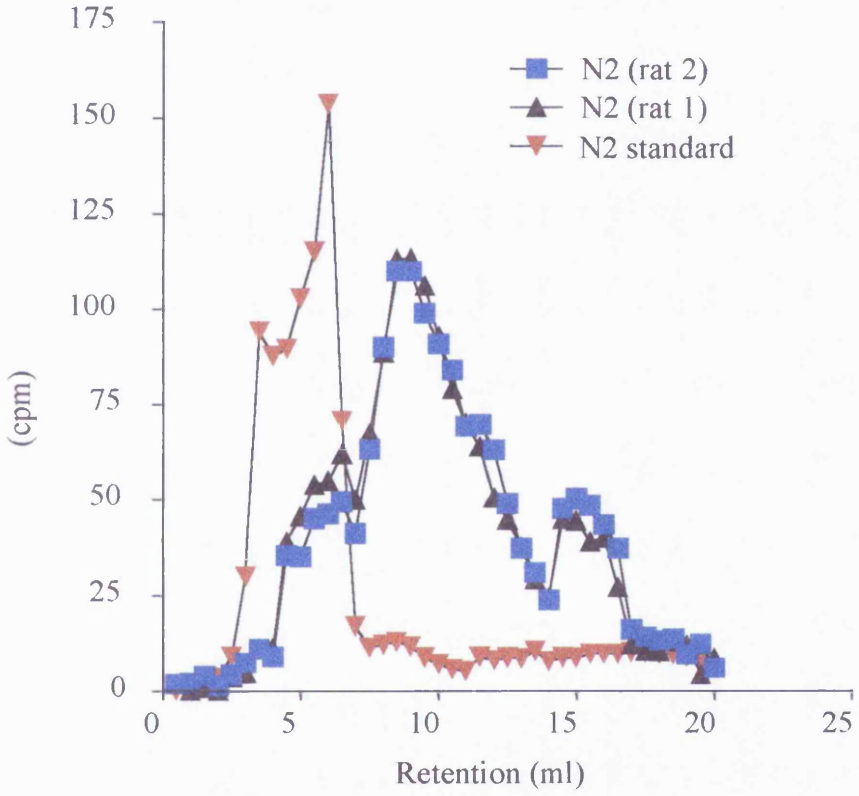


Table 5.1 A summary of the characterisation of the radioiodinated chitosans used for the body distribution experiments

Polymer	Specific Activity (μCi/mg)	Labelling Efficiency (%)	Purity (% free [^{125}I]iodide)	Dose (mg/kg)*	Recovery(%) from animal (5min) ±S.D.	Recovery (%) from animal (60min) ± S.D.
HPMA copolymer tyrosinamide	30.00	82.00	<1.00	0.03	83.10±4.10	56.00±5.80
Chitosan N1	0.3 0	<1.00	1.50	2.52	16.10±8.00	31.80±3.80
Chitosan N2	2.40	<1.00	1.40	0.36	46.00±6.80	55.80±19.20
Chitosan N3	1.80	<1.00	<1.00	0.47	47.10±11.60	36.40±18.50

*Dose (mg/kg ~ $5x10^5$ CPM /animal) calculated assuming no radioactive decay from the time of calculating specific activity to the time of the injection

polymers to negatively charged cell surfaces in the liver and the sequestration of anionic molecules by non-parenchymal liver cells via scavenger receptors.

Pimm *et al.*, (1995) reports the body distribution of radiolabelled cationic poly[lys-(DL-$Ala_{3.10}$)] Mw 45 900 (±5%) Da) poly(amino acid)s designated AK. After the i.v. administration of cationic ^{125}I-labelled AK in mice, less than 10% of the administered dose was evident in the systemic circulation after 15min.

In a separate study, the charge and molecular weight of ^{125}I-labelled dextran derivatives was shown to directly influence their plasma clearance rate (Yamaoka *et al.*, 1995). When the degree of cationic substitution on a DEAE dextran of molecular weight 28 000 – 197 000 Da was 9.8 Mol% or above, the i.v. administration of DEAE dextran proved lethal in BALB/cCrslc mice after 1h (100μg dose/mouse). Death was attributed to blood coagulation as red spots were seen on the lung surface. When DEAE dextran of a lower degree of cationic substitution was used (3.4, 3.8 and 5.3 Mol%) rapid plasma clearance was observed. This study also found dextran of Mw 28 000 Da to have a plasma half-life of approximately 10min regardless of charge following i.v. administration. When the organ distribution of the dextran was considered the anionic, cationic and neutrally charged molecules were found to be very different. The neutrally charged dextran was excreted within 1h (69.0 ± 4.5% administered dose) and did not accumulate in the liver (0.8 ± 0.7% administered dose). In contrast, 7.3 ± 2.2% of the administered dose of the DEAE dextran was excreted after 1h with 39.5 ± 1.3% of the administered dose being located in the liver.

The ^{125}I-labelled DEAE dextran, the ^{125}I-labelled chitosans N1-3, as well as the ^{125}I-labelled cationic polypeptides described by Pimm *et al.*, (1995) all showed rapid plasma clearance relative to ^{125}I-labelled HPMA copolymer tyrosinamide of similar molecular weight. This effect may be attributable to the net positive charge of the macromolecules as described by Takakura and Hashida, (1996).

A large proportion of the administered dose of ^{125}I-labelled N1-3 was not recovered (Table 5.1). The only explanation for this phenomenon is that the

polymer was taken into an organ or body compartment not examined in this study. This may, in the future, provide opportunities for targeting. The DEAE dextran molecules reported by Yamaoka *et al.*, (1995) were also shown to accumulate in the residual carcasses of the mice.

The gel permeation profile of the renally excreted low molecular weight [^{125}I]iodide following the i.v. administration of ^{125}I-labelled chitosan N2, after 1h (Figure 5.14) would suggest that the chitosan was degraded *in vivo*. The nature of the majority of the radioactivity recovered after the administration of ^{125}I-labelled N2 was not in the macromolecular peaks associated with native ^{125}I-labelled chitosan N2. Muzzarelli *et al.*, (1988) has reported that chitin, the fully acetylated precursor of chitosan is degraded by lysozyme. It is possible that the degree of enzymatic degradation associated with chitosan is proportional to the degree of backbone deacetylation. Where 100% deacetylation is observed, no enzymatic degradation may be expected (Carreno-Gomez and Duncan, 1997). Where the degree of deacetylation is reduced, enzymatic degradation may be expected between adjacent β–(1-4)-*N*-acetyl-D-glucosamine monomers as are found in chitin, providing steric factors do not interfere with polymer active site binding. As chitosan N2 has only a 55.2% degree of deacylation, limited degradation may be expected *in vivo*. Exposure to NO has also been reported to degrade chitosan but not chitin and this effect may also contribute to the degradation or de-iodination of the ^{125}I-labelled chitosan observed in Figure 5.9 (Peluso *et al.*, 1994). Pimm *et al.*, (1995) has also reported the release of [^{125}I]iodo-phenol derivatives of polymers labelled by Bolton and Hunter reagent by lysosomal hydrolases.

5.5 Conclusions

This preliminary pharmacokinetic evaluation of the ^{125}I-labelled chitosans N1-3 shows that the cationic chitosan molecules are rapidly cleared from the bloodstream. This clearance is comparable with the plasma clearance rates of

other polycations such as the cationic poly(L-lysine) derivatives reported in Pimm *et al.*, (1995) and also DEAE dextran (Yamaoka *et al.*, 1995).

The low recovery of the ^{125}I-labelled chitosan molecules from the body distribution experiments suggest that further investigations are required to fully understand the tissue localisation of the ^{125}I-labelled chitosan molecules after i.v. administration. Techniques such as whole body autoradiography or gamma camera imaging (Pimm *et al.*, 1996) could be used to further investigate the tissue distribution of the ^{125}I-labelled chitosan molecules.

Chapter 6

Body Distribution and Tumour Accumulation of ^{125}I-Labelled Poly(amidoamine)s and ^{125}I-Labelled Poly(amidoamine)/DNA Complexes

6.1 Introduction

Although the body distribution of ^{125}I-labelled PAMAM-dendrimers has been reported (Malik *et al.*, 1997), the fate of linear poly(amidoamine)s has not yet been investigated. In this study we wished to examine the biodistribution of three poly(amidoamine) ISA 4, 9 and 22. ISA 4 and 9 have similar structures, with the exception that ISA 9 has been synthesised to contain galactosamine in addition to the 2-phenylethylamine monomer added to allow radioiodination (Figure 1.8, Table 1.10 and Table 1.11).

HPMA copolymers, containing galactosamine residues (PK2) accumulate preferentially in the liver following i.v. administration (Pimm *et al.*, 1996; Duncan *et al.*, 1982). This is due to interaction between the polymer-associated galactose moiety and the hepatic asialoglycoprotein receptor (Ashwell and Morell, 1974). Many drug delivery systems have taken advantage of the asialoglycoprotein receptor for liver targeting and it has been shown that >70% of an i.v. bolus dose of PK 2 (Chapter 1) can be directed preferentially to the liver in humans (Seymour *et al.*, 1997). This system has also been used to direct non-viral gene delivery systems to hepatocytes *in vivo* (Wu and Wu, 1998) and also to increase the efficiency of non-viral gene delivery system internalisation *in vitro* (Zanta *et al.*, 1997).

Macromolecular tumour accumulation via the EPR effect (Chapter 1) is also a form of targeting that may be utilised to improve the efficacy of polymer-mediated DNA delivery. If any of the ^{125}I-labelled poly(amidoamine)s demonstrated a suitable plasma residence, passive tumour accumulation would be investigated (Maeda and Matsumura, 1986).

Poly(amidoamine)s ISA 4, 9 and 22 are biodegradable, having hydrolytically labile bonds in the main chain (Ferruti *et al.*, 1985; Ranucci *et al.*, 1991[b]). Because of this, the preservation of the integrity of the polymer backbone during radioiodination, storage and use was considered of paramount importance. It was considered prudent to monitor polymer stability in biological fluids over time, as well as in response to a freeze thaw cycle. The radiolabelled polymer

were characterised by assessing the labelling efficiency, specific activity and the proportion of free [^{125}I]iodide in each polymer preparation (Sgouras, 1990).

Assessment of their body distribution of the radiolabelled poly(amidoamine)s following systemic administration in the rat were undertaken. The experiments were repeated using ^{125}I-labelled polymer administered as a ^{125}I-labelled DNA:polymer (Calf thymus DNA) complex (1:10 polymer excess by weight). These preliminary experiments were undertaken to establish whether or not polymer administered as a complex would show an altered body distribution profile compared to the polymer alone.

6.2 Methods

Poly(amidoamine)s were radiolabelled using the chloramine T method and purified as described in Chapter 2, except that after the removal of 5µl of crude reaction mixture, the poly(amidoamine)s were purified by GPC using PD10 columns. This was performed by first passing 3 column volumes of 0.1M phosphate buffer pH 7.4 through the PD10 column. Next, the polymer sample was run into the gel in a volume not exceeding 2.5ml in accordance with the protocol supplied by the manufacturers. The void volume containing the labelled polymer was then collected by elution with 3.5ml of 0.1M phosphate buffer pH 7.4. This process was then repeated using a new PD10 column equilibrated as before. A second aliquot, (5µl) was then removed from the second purification and the proportion of free [^{125}I]iodide in each aliquot assessed using paper electrophoresis (Chapter 2).

The stability of the polymers and their body distribution profiles were assessed as described in Chapter 2 with the following modifications. After the i.v. administration of the ^{125}I-labelled poly(amidoamine)s the animals were left for either 1h or 5h before they were killed. The following organs were removed, washed in PBS and assayed for radioactivity; liver, heart, kidneys, lungs, thyroid and spleen. The tissue samples, in distilled water, were homogenised using a polytron homogeniser and the final volume of the preparation measured. Blood

and urine samples were also taken and assayed for radioactivity. The results were expressed as recovered dose (%)/organ, assuming the blood volume of the rat to be 7.3ml/100g as before. Urine analysis by PD10 GPC was undertaken for all preparations and was performed as mentioned in Chapter 5.

In order to study the body distribution of the ^{125}I-labelled DNA:poly(amidoamine) complexes the IPECs were prepared as follows: Calf thymus DNA was re-hydrated in 0.9%(w/v) sterile sodium chloride at a concentration of 1mg/ml 24h prior to the experiment and stored at 4°C. A volume of ^{125}I-labelled poly(amidoamine) having a total of $5x10^6$ cpm was placed in a sterile container and the quantity of polymer therein calculated, allowing for radioactive decay. Calf thymus DNA was then added to give a DNA:polymer ratio of 1:10 by weight and the complex was left to stand at room temperature for 30min. The final volume of the preparation was adjusted to 2ml using 0.9% sterile sodium chloride and $5x10^5$ cpm of the polymer:DNA preparation (200µl) was injected i.v. into the rat and the body distribution experiments performed as previously described Chapter 2.

To monitor tumour accumulation of ^{125}I-labelled poly(amidoamine)s experiments with tumour bearing animals were conducted according to the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines. First B16 F10 cells were grown to 70% confluence *in vitro* as described in Chapter 2. Cells were then suspended at a density of $1x10^7$ cells/ml in sterile 0.9% sodium chloride. Care was taken to avoid heat shock and shear stress when handling the cells and the suspension was used within 30min of preparation. Male C57 mice were then injected subcutaneously (s.c.) directly between the shoulder blades with $1x10^6$ B16 F10 cells/animal (100µl volume). The B16 F10 tumours were then allowed to grow to palpable size, defined as 30–150mm^3 (about 14 days). Once the tumours had attained palpable size, ^{125}I-labelled ISA poly(amidoamine)s or the DNA:poly(amidoamine) complexes ($5x10^5$ cpm) was injected i.v. in a volume of 100µl. After 1h or 5h the liver, kidneys, blood (0.5ml)

and tumour were removed. The tissues were homogenised and assayed for radioactivity. All experiments were performed in triplicate and the results expressed as dose (%)/g tissue for each organ assuming the blood volume of the mouse to be 5.77 ml/100g (Dreyer and Ray, 1910).

6.3 Results

Paper electrophoresis of the reaction mixture and the purified preparation of ^{125}I-labelled ISA 4, 9 and 22 are shown in Figures 6.1, 6.2 and 6.3. The labelling efficiency and proportion of free [^{125}I]iodide in all of the poly(amidoamine) samples are summarised in Table 6.1 and Table 6.2. The purified ^{125}I-labelled ISA 4, 9 and 22 preparations contain 1.20, 3.1 and 0.28% free [^{125}I]iodide.

PD10 GPC analysis was used to examine the aqueous stability of ^{125}I-labelled ISA 4, (Figure 6.4) ^{125}I-labelled ISA 9 (Figure 6.6) and ^{125}I-labelled ISA 22 (Figure 6.8) incubated in MEM containing 10% FCS. Low molecular weight products containing [^{125}I]iodide started to appear after 24h incubation, however after 5h sufficient stability was seen (<5% release of low molecular weight [^{125}I]iodide or radioiodinated poly(amidoamine) degradation products) for further experimentation. PD10 GPC analysis of the ^{125}I-labelled poly(amidoamine)s, after storage for 30 days at –20°C revealed few (<1%) low molecular weigh products containing [^{125}I]iodide (Figures 6.5, 6.7 and 6.8). In view of this result, the stability of the ^{125}I-labelled poly(amidoamine)s was thought to be sufficient to allow a further evaluation of body distribution *in vivo*.

The *in vivo* body distribution of ^{125}I-labelled ISA 4 and ^{125}I-labelled ISA 9 is shown in Figure 6.10 and Figure 6.11 respectively. After 1h most (>95% recovered dose) of the ^{125}I-labelled ISA 4 and 9 has been cleared from the bloodstream and may be found in the liver (>80% recovered dose). After 5h the amount of ^{125}I-labelled ISA 4 in the kidneys and spleen was < 15% (recovered

Figure 6.1 Paper electrophoresis of ^{125}I-labelled ISA 4

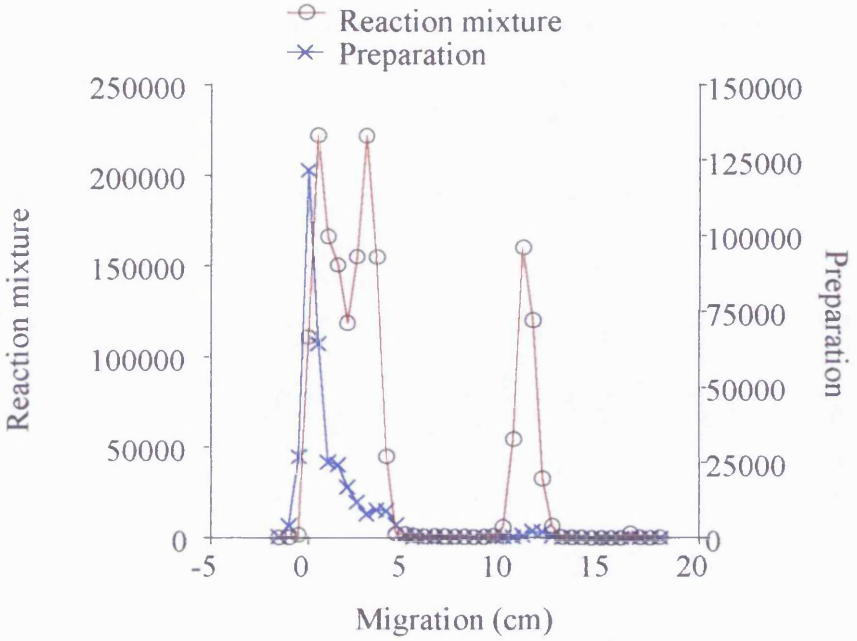


Figure 6.2 Paper electrophoresis of ^{125}I-labelled ISA 9

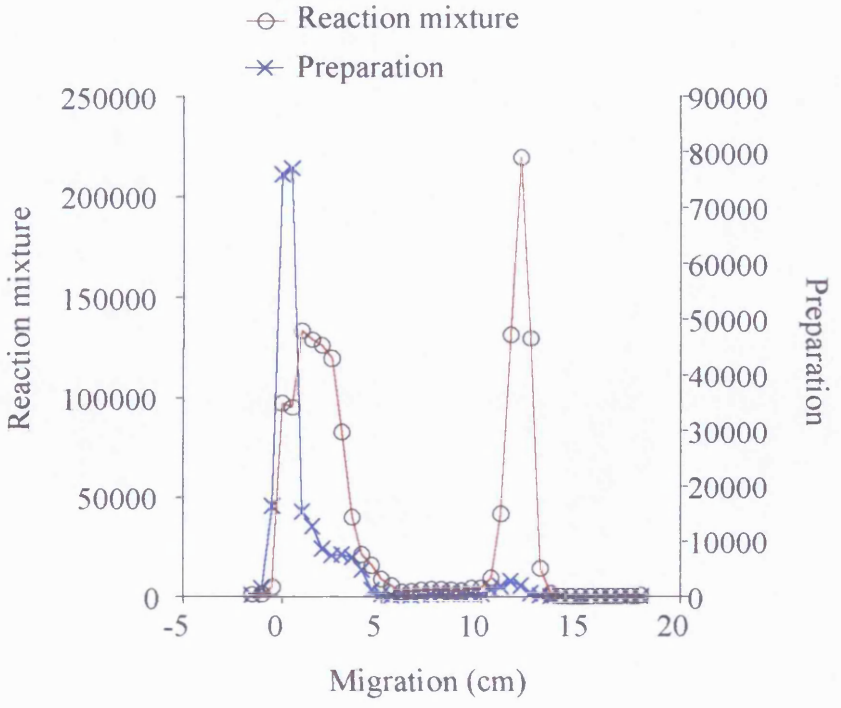


Figure 6.3 Paper electrophoresis of ^{125}I-labelled ISA 22

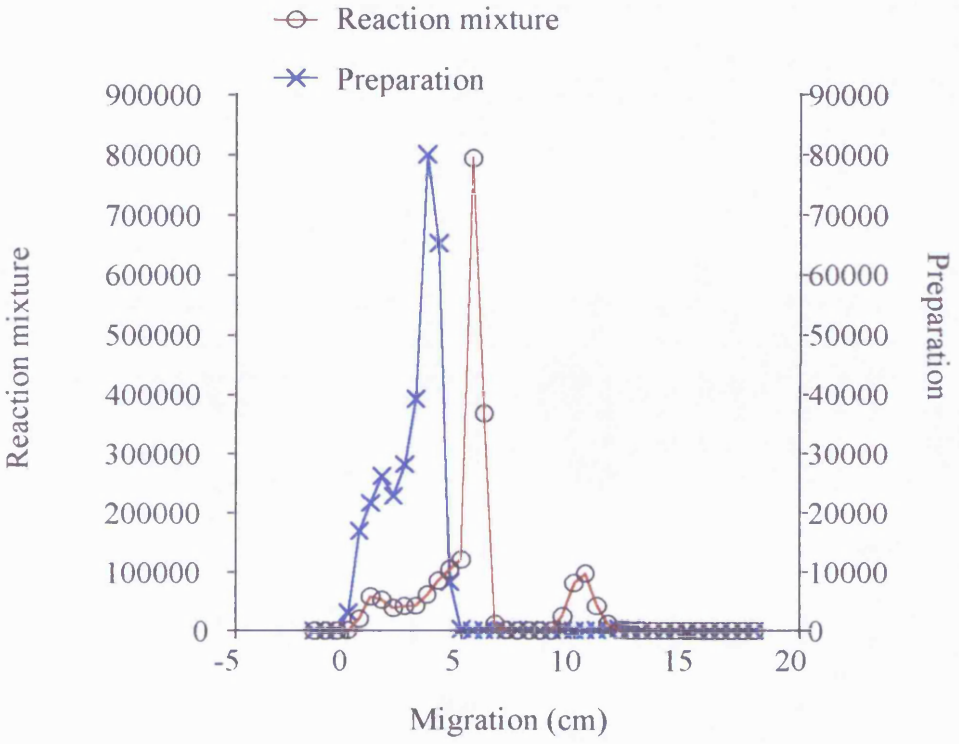


Table 6.1 Summary of the characterisation of ^{125}I-labelled poly(amidoamine)s

Polymer	Labelling efficiency (%)	Purity (free [^{125}I]iodide (%))
ISA4	77.90	1.20
ISA9	61.60	3.10
ISA22	97.40	0.28

Figure 6.4 Stability of ^{125}I-labelled ISA 4 in MEM (10% v/v FCS) over 24h by PD10 fractionation

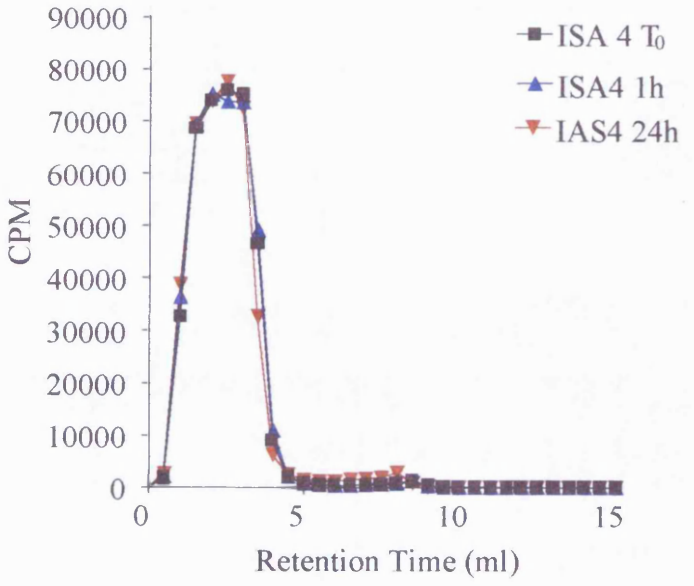


Figure 6.5 Stability of ^{125}I-labelled ISA 4 following 30 days storage at –20°C in 1% w/v sodium chloride by PD10 fractionation

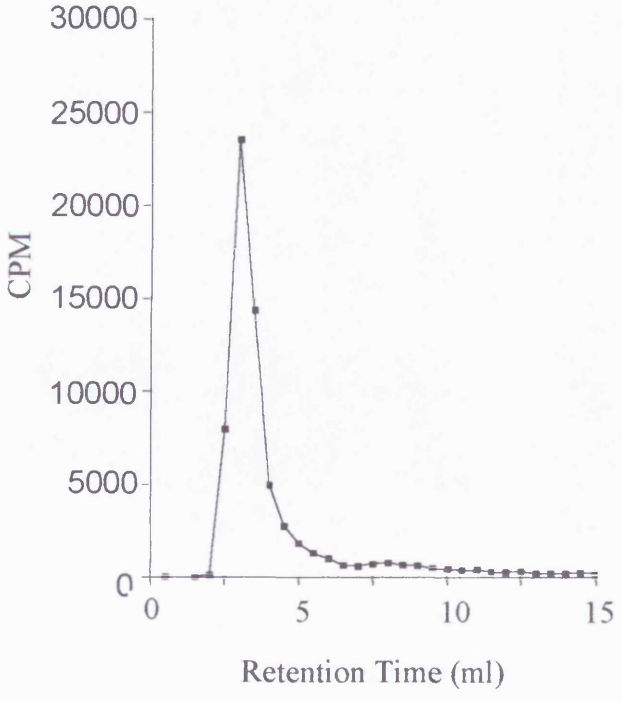


Figure 6.6 Stability of ^{125}I-labelled ISA 9 in MEM (10% v/v FCS) over 24h by PD10 fractionation

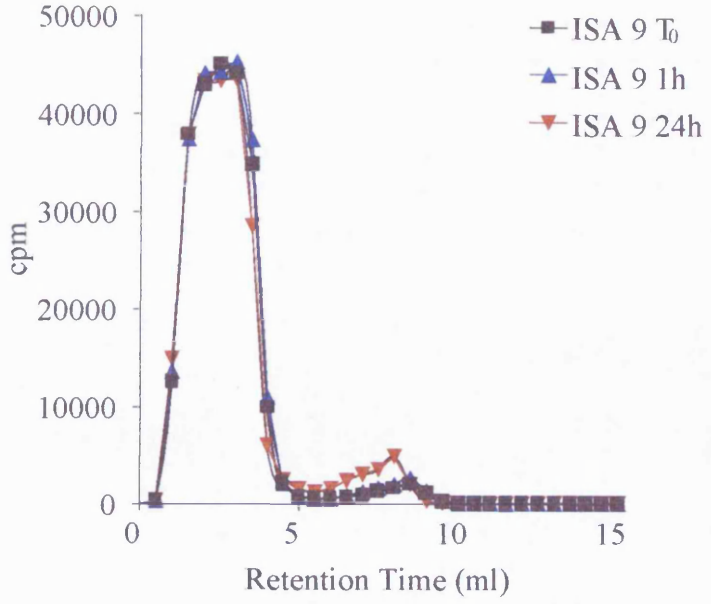


Figure 6.7 Stability of ^{125}I-labelled ISA 9 following 30 days storage at –20°C in 1% w/v sodium chloride by PD10 fractionation

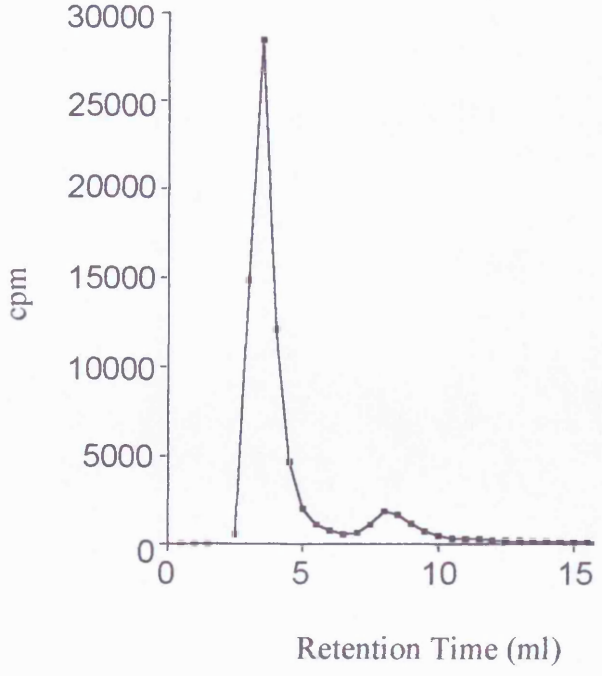


Figure 6.8 Stability of ^{125}I-labelled ISA 22 in MEM (10% v/v FCS) over 24h by PD10 fractionation

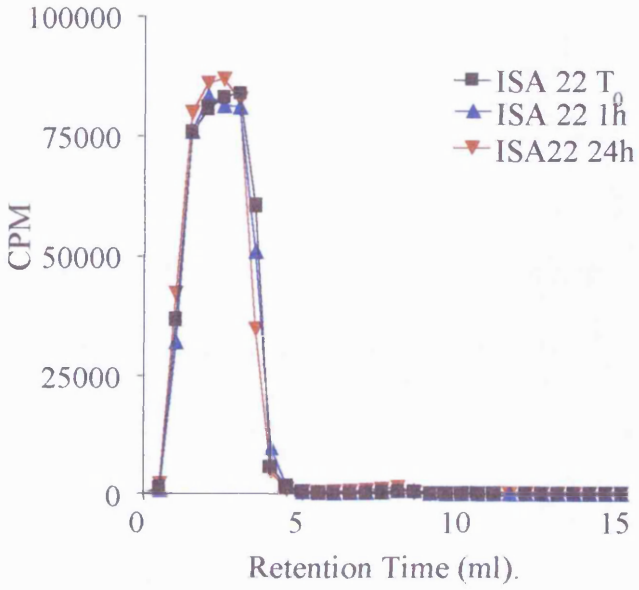


Figure 6.9 Stability of ^{125}I-labelled ISA 22 following 30 days storage at –20°C in 1% w/v sodium chloride by PD10 fractionation

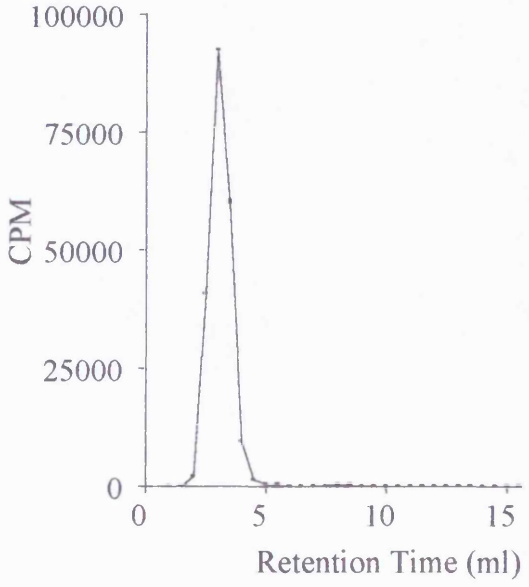


Figure 6.10 Body distribution of ^{125}I-labelled ISA 4 in male Wistar rats after 60min and 300min following i.v administration

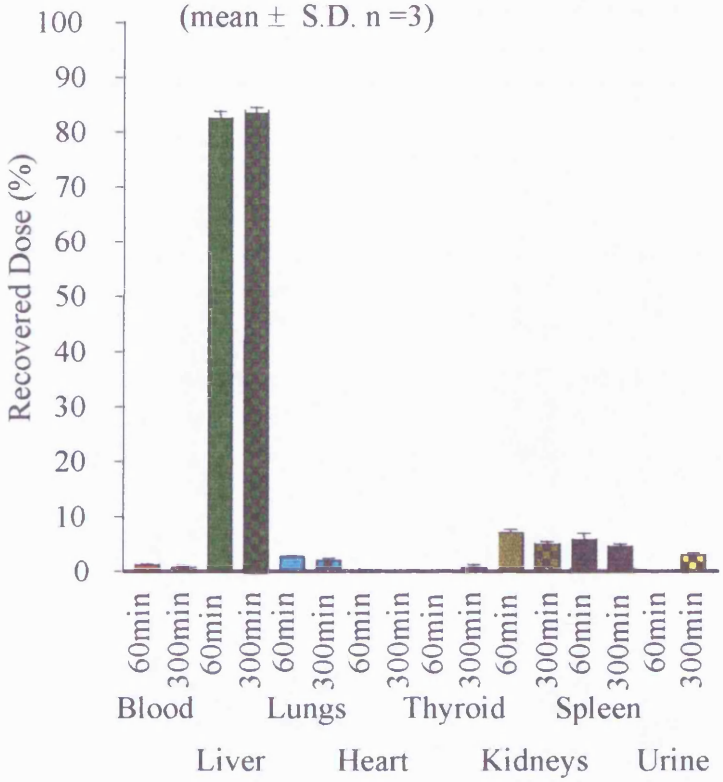


Figure 6.11 Body distribution of ^{125}I-labelled ISA 9 in male Wistar rats after 60min and 300min following i.v. administration

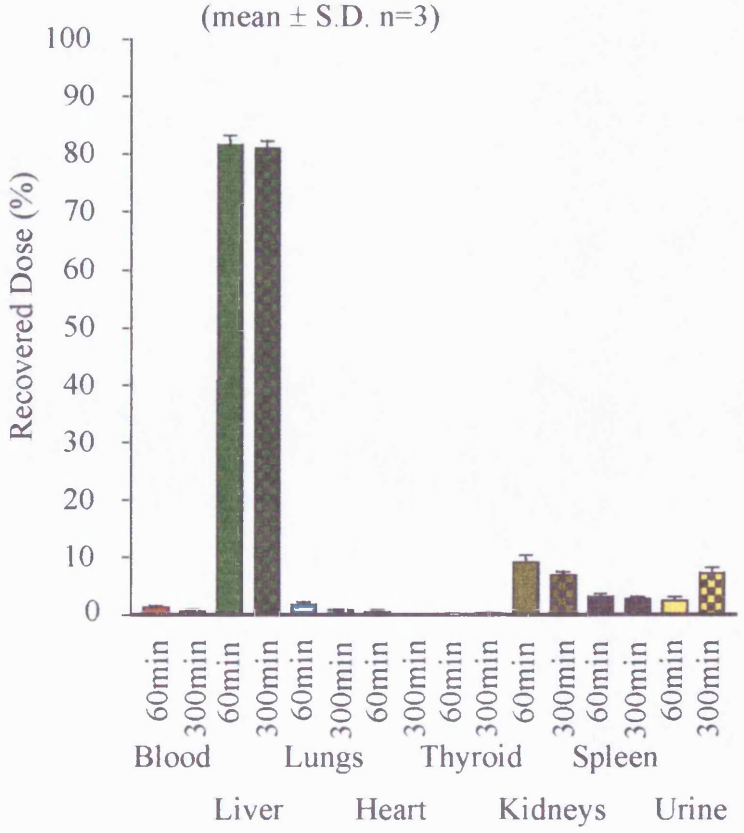


Table 6.2 Summary of the specific activities of final poly(amidoamine) preparations and the recoveries (%) from body distribution experiments

Polymer	Specific activity μCi/mg	Recovered dose (%) ± SD(n=3) 60min No DNA	Recovered dose (%) ± SD (n=3) 300min No DNA	Recovered dose (%) ± SD (n=3) 60min DNA	Recovered dose (%) ± SD(n=3) 300min DNA
ISA4	4.70	62.60 ± 3.80	80.80 ± 5.40	65.50 ± 4.30	89.70 ± 4.80
ISA9	35.00	60.40 ± 11.70	79.60 ± 4.70	68.60 ± 9.20	82.50 ± 4.30
ISA22	47.40	50.00 ± 14.40	67.70 ± 6.60	72.10 ± 10.10	66.80 ± 14.40

dose) and both ^{125}I-labelled polymers are starting to be excreted in the urine (<10% recovered dose).

A very different body distribution pattern was demonstrated by ^{125}I-labelled ISA 22 (Figure 6.12). Over 70% of the recovered dose was found in the blood stream and ~15% of the recovered dose in the urine at 1h. After 5h >20% of the recovered dose was still in the bloodstream and ~65% of the recovered dose had been excreted. Less than 10% of the recovered dose of ^{125}I-labelled ISA 22 was present in the liver after 5h and at this time < 5% recovered dose was found in the spleen. This shows that ^{125}I-labelled ISA 22 has the potential to be a long circulating polymer.

When the urine collected 1h after the administration of ^{125}I-labelled ISA 9 was examined by GPC (Figure 6.13) the elution profile relative to that of the ^{125}I-labelled ISA 9 preparation (Figure 6.6) was altered. The urine GPC profile obtained 5h after the injection of ^{125}I-labelled ISA 4 and 9 (Figure 6.14) was also altered relative to that of ^{125}I-labelled ISA 4 and 9 (Figure 6.4 and 6.6). The GPC profile of urine collected from rats injected with ^{125}I-labelled ISA 22 5h after its i.v. administration (Figure 6.14) was similar to that of ^{125}I-labelled ISA 22 (Figures 6.8). This would suggest that ^{125}I-labelled ISA 22 is stable following excretion 5h after i.v. administration, where as ^{125}I-labelled ISA 4 and 9 were not.

When the ^{125}I-labelled poly(amidoamine)s were administered as a DNA:polymer complex, a body distribution profile similar to that of the parent polymer was demonstrated in each instance. If Figure 6.15 is compared with Figures 6.10, Figures 6.16 with Figures 6.11 and Figures 6.17 with Figures 6.12 the similarities are immediately evident.

When urine was collected following the i.v. administration of ^{125}I-labelled ISA 4, 9 or 22, given as a polymer:DNA complex, (Figures 6.13 and 6.14) and fractionated using PD10 GPC as previously described the results were similar to those seen for the native ^{125}I-labelled polymer (Figures 6.13 and 6.14 respectively). This would indicate that the administration of the polymer as a

Figure 6.12 Body distribution of ^{125}I-labelled ISA 22 in male Wistar rats after 60min and 300min following i.v administration (mean ± S.D. n=3)

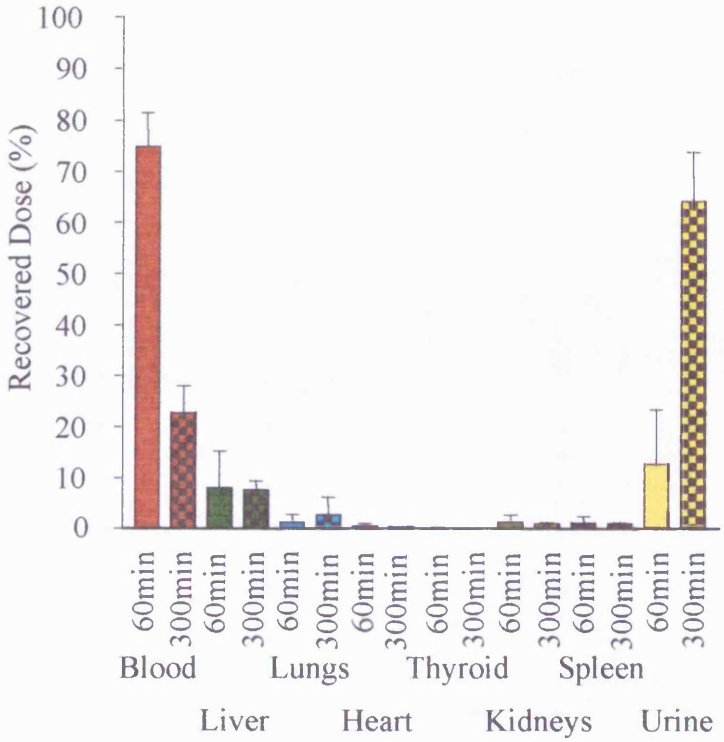


Figure 6.13 GPC of urine collected (60min) after i.v. administration of ^{125}I-labelled ISA 9 and 22 to male Wistar rats (mean ± S.D. n=3)

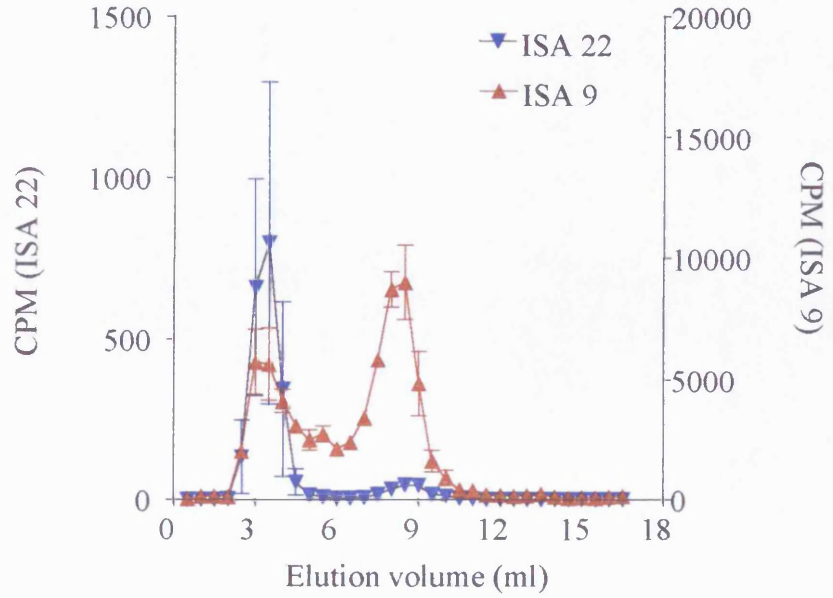


Figure 6.14 GPC of urine collected (300min) after i.v. administration of ^{125}I-labelled ISA 4, 9 and 22 to male Wistar rats (mean ± S.D. n=3)

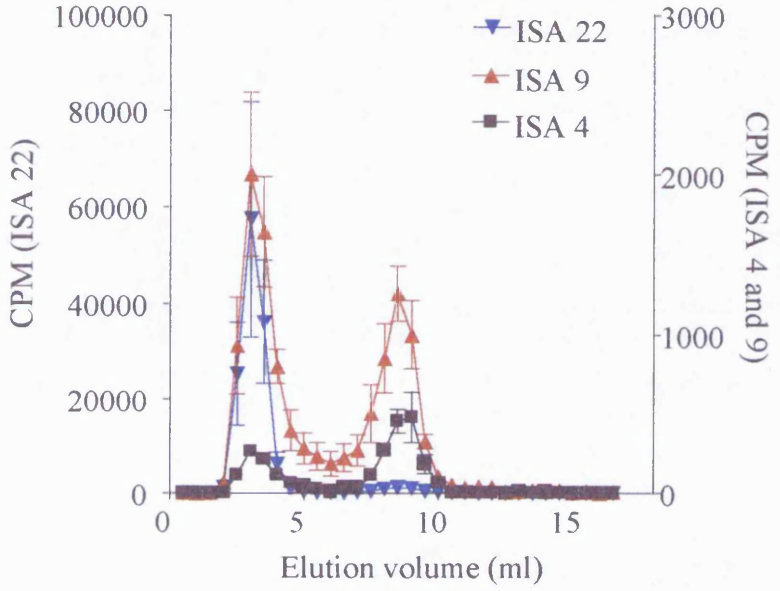


Figure 6.15 Body distribution of ^{125}I-labelled ISA 4:DNA complex in male Wistar rats after 60min and 300min following i.v.administration (1:10) polymer excess by weight) (mean ± S.D. n=3)

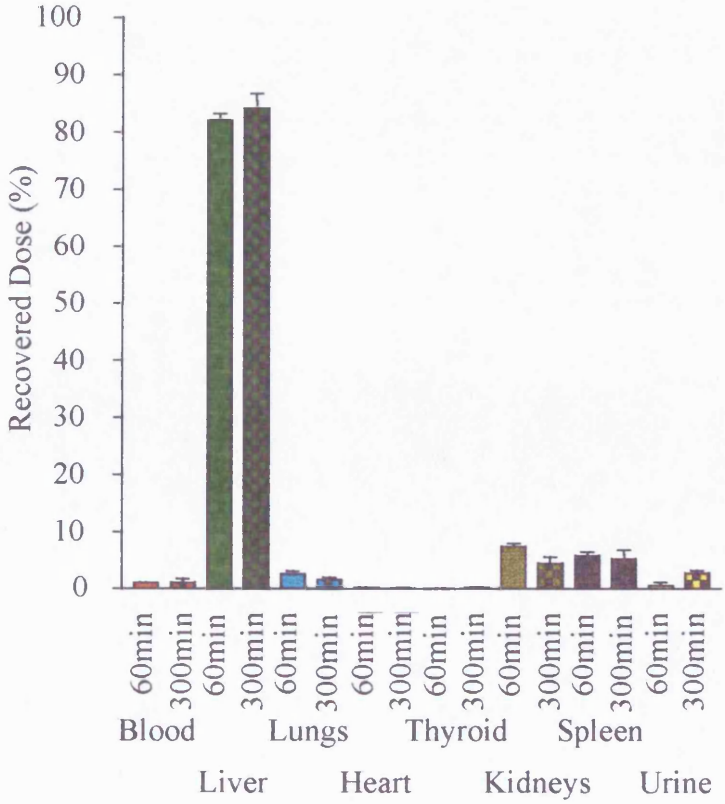


Figure 6.16 Body distribution of ^{125}I-Labelled ISA 9:DNA complex in male Wistar rats after 60min and 300min following i.v.administration (1:10) polymer excess by weight) (mean ± S.D. n=3)

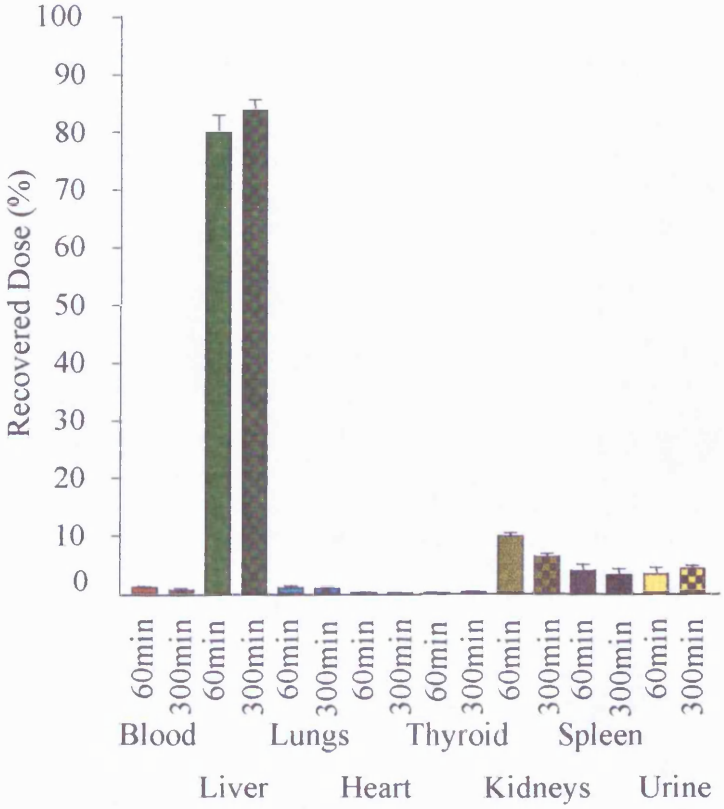


Figure 6.17 Body distribution of ^{125}I-labelled ISA 22:DNA complex in male Wistar rats after 60min and 300min following i.v.administration (1:10) polymer excess by weight) (mean ± S.D. n=3)

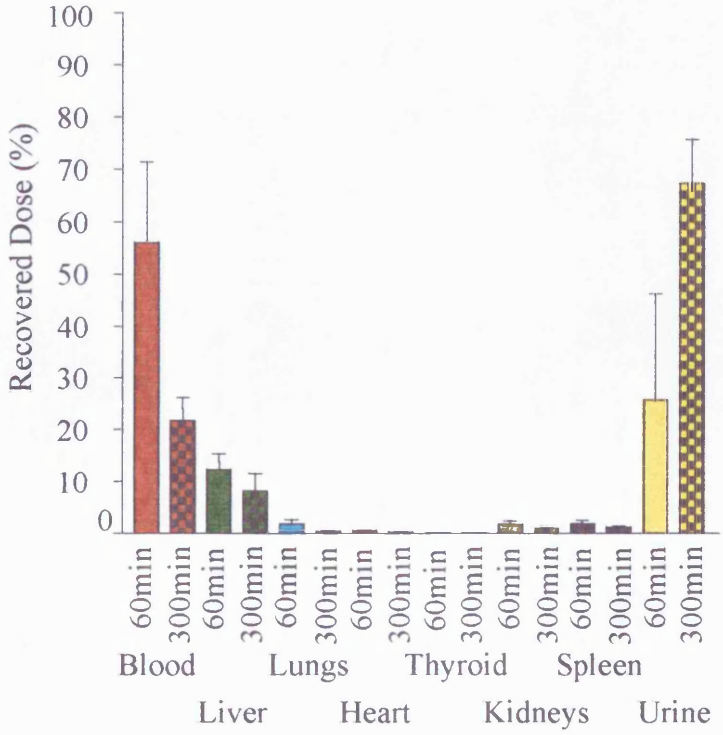


Figure 6.18 GPC of urine collected (60min) after i.v. administration of ^{125}I-labelled ISA 4, 9 and 22:DNA complexes to male Wistar rats (mean ± S.D. n=3)

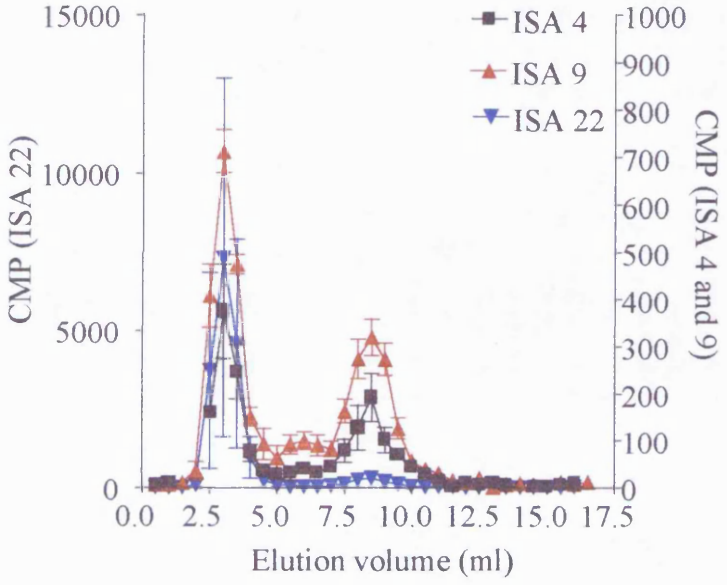
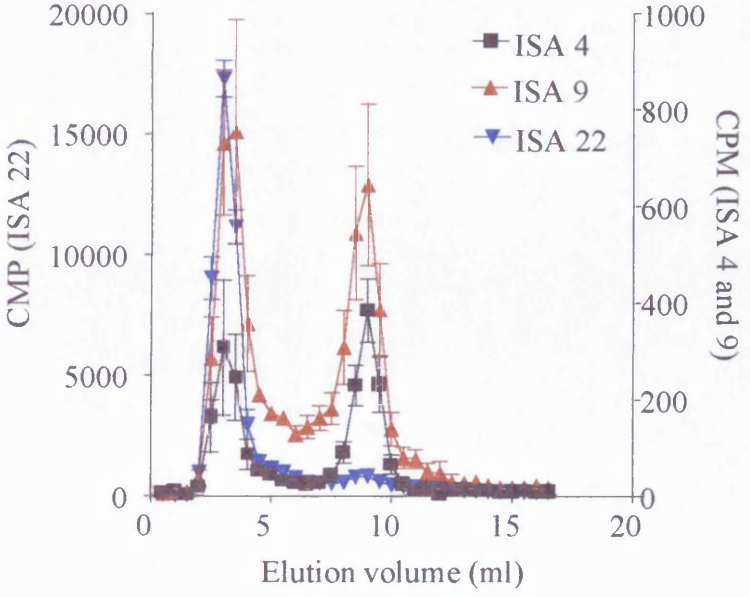


Figure 6.19 GPC of urine collected (300min) after i.v. administration of ^{125}I-labelled ISA 4, 9 and 22:DNA complexes to male Wistar rats (mean ± S.D. n=3)



complex did not alter the stability of the polymer following renal excretion up to 5h after i.v. administration.

The B16 F10 tumour accumulation of ^{125}I-labelled ISA 22 and^{125}I-labelled ISA 22 administered as a polymer:DNA complex is shown in Figures 6.20 and 6.21 respectively. Time-dependent tumour accumulation of macromolecular ^{125}I-labelled ISA 22 is evident (~2.5% dose/gram tissue after 5h in both instances) regardless of whether the polymer is administered as the native ^{125}I-labelled polymer or as a DNA complex.

6.4 Discussion

Differences between the *in vitro* and *in vivo* PD10 GPC profiles of ^{125}I-labelled ISA 4 and ISA 9 were observed over identical time courses. This suggests the *in vivo* degradation of the polymer by mechanisms other than hydrolysis. The GPC profile of ^{125}I-labelled ISA 22 dose not alter following its i.v. administration and subsequent excretion from the rat suggesting polymer stability *in vivo*. The lack of thyroid accumulation of [^{125}I]iodide also indicates that [^{125}I]iodide is not released from the polymer *in vivo*.

The body distributions of ^{125}I-labelled ISA 4 and 9 appear to be very similar as both poly(amidoamine)s showed substantial liver capture. HPMA copolymer galactosamine (Duncan *et al.*, 1982) has been shown to target the liver by interaction with the hepatocyte asialoglycoproteoin receptor. It cannot be concluded that it is the galactosamine that is responsible for the liver capture of the ^{125}I-labelled ISA 9 or the poly(L-lysine) galactose constructs described in Wu and Wu, (1998) as both ^{125}I-labelled ISA 4 and ^{125}I-labelled poly(L-lysine) are themselves subject to rapid plasma clearance and liver capture (reported in Figure 6.10 and Pimm *et al.*, (1994) respectively). ^{125}I-labelled ISA 4, 9 and the poly(L-lysine) derivatives described in Pimm *et al.*, (1995) have a net positive charge at pH 7.4 and this may be responsible for their rapid blood clearance and liver

Figure 6.20 Tumor accumulation of ^{125}I-labelled ISA 22 in male B16 F10 bearing C57 black mice 60min and 300min after i.v. Administration (mean ± S.D. n=3)

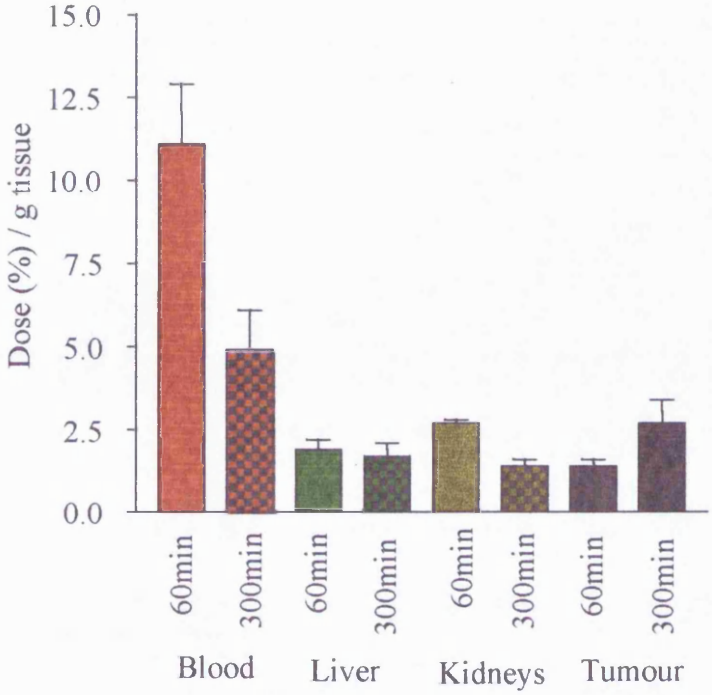


Figure 6.21 Tumor accumulation of ^{125}I-labelled ISA 22: DNA complex in male B16 F10 bearing C57 black mice 60min and 300min after i.v. administration (mean ± S.D. n=3)

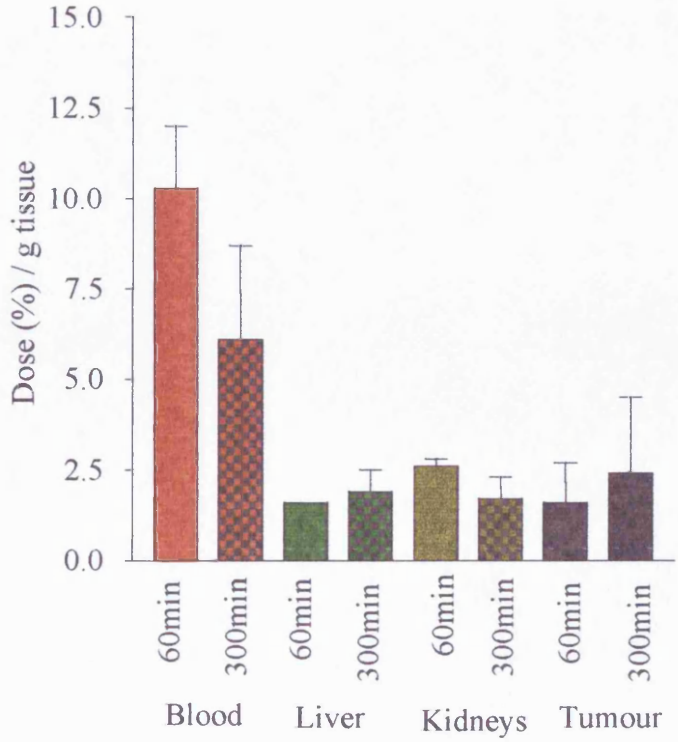


Table 6.3 Poly(amido amine) doses (μg/kg) administered *in vivo*

Animal	Polymer ISA4	ISA9	ISA22
Wistar rat	146.70	19.80	14.60
C57 black Mouse	--	--	197.00

All doses calculated assuming 100% polymer recovery from dialysis.

accumulation. A similar phenomenon was found for chitosan of increasing molecular weight and is described in Chapter 5.

^{125}I-labelled ISA 22 does not accumulate rapidly in the liver (Figure 6.12) and as has been mentioned previously (Chapter 1) is much less cationic that ISA 9 and ISA 4 at neutral pH (Table 1.10 and 1.11). This may explain the extended plasma residence time of ^{125}I- labelled ISA 22 relative to ^{125}I-labelled IS 4 and 9 as well as the ^{125}I-labelled chitosans (Chapter 5).

The body distribution profiles of the polymer and DNA:polymer complexes were essentially identical (Figures 6.10-12 and 6.15-17). This study is preliminary and must be considered inconclusive as doubt may be casts upon the stability of the DNA:polymer complex in the systemic circulation. If the liver accumulation of ^{125}I-labelled ISA 4 was indeed related to charge, the liver capture of ^{125}I-labelled ISA 4 would be reduced if the DNA was still associated with the polymer. The effect of charge neutralisation upon the liver capture of ^{125}I-labelled ISA 4 would have to be examined systematically before this hypothesis can be addressed.

Wu and Wu, (1998) reported gene expression *in vivo* following the i.v. administration of a poly(L-lysine)-galactose:plasmid complex. This was performed in the rat model after the animal had undergone partial hepatectomy to stimulate hepatocyte division. In this instance a much more highly charged polybases was used (poly(L-lysine)) relative to the poly(amidoamine)s used here. *In vitro* assays have been proposed to monitor the stability of plasmid:polymer complexes in the presence of various biological materials (MacLaughlin *et al.*, 1998). Before any further *in vivo* work is performed using these complexes, it may be prudent to address the question of complex stability *in vitro*.

As ^{125}I-labelled ISA 22 displayed a relatively high plasma residence time, it was considered interesting to examine whether this poly(amidoamine) could demonstrate tumour accumulation via the EPR effect (Maeda and Matsumura, 1986). This preliminary experiment showed that tumour

accumulation of ^{125}I-labelled ISA 22 and ^{125}I-labelled ISA 22 administered as a DNA:polymer complex was observed over time. Again it must be stressed that this experiment dose not confirm that any of the IPEC associated DNA has localised to the tumour mass.

In relation to other polymers that have been reported to accumulate in B16 F10 tumour mass *in vivo* such as PK1 (13.7 ± 3.6 dose(%)/g tissue/1h for small tumours and 1.1 ± 1.0 dose(%)/g tissue/1h for large tumours) (Duncan and Sat, 1998) ^{125}I-labelled ISA 22 compares favourably (1.4 ± 0.2 dose(%)/gtissue/1h mean ± S.D. of 3 tumours of varied size).

Future experiments should assess the integrity of a non-mammalian transgene sequence following delivery *in vivo*. Tumour accumulation could be monitored using the polymerase chain reaction (PCR), reverse transcriptase PCR, fluorescent *in situ* hybridisation, Southern or Northern analysis probing for the presence of transgene specific sequences in *ex vivo* tissue or homogenates following construct administration *in vivo* could also be undertaken.

6.4 Conclusions

The body distribution profiles of ^{125}I-labelled ISA 4 and ISA 9 would suggest that these polymers may not be suitable for non-liver targeting. ^{125}I-labelled ISA 22 demonstrated a longer plasma circulaation profile and did not localise to the liver and this may afford the ability to target non-liver tissue. This polymer also accumulates in tumour mass over time and this may facilitate tumour targeting via the EPR effect.

No problems were encountered with the stability of ^{125}I-labelled ISA 22, however its limited ability to complex DNA (chapter 4) may limit its usefulness as a gene delivery vehicle via IPEC formation. This does not detract from the potential use of ISA 22 as a component of a drug delivery vehicle though the synthesis of covalent constructs containing ISA 22, possibly exploiting the endosomolytic potential of this polymer (Chapter 3) to achieve cytosolic delivery.

Before this can be achieved the internalisation and subcellular trafficking of the poly(amidoamine) will need to be studied. In a preliminary effort to understand the potential of poly(amidoamine)s to mediate endosomal escape, ISA 22 and 23 were used as *in vitro* transfection agents (Chapter 7).

Chapter 7

Preliminary Experiments to Determine the Ability of Poly(amidoamine)s to Mediate Transfection In Vitro

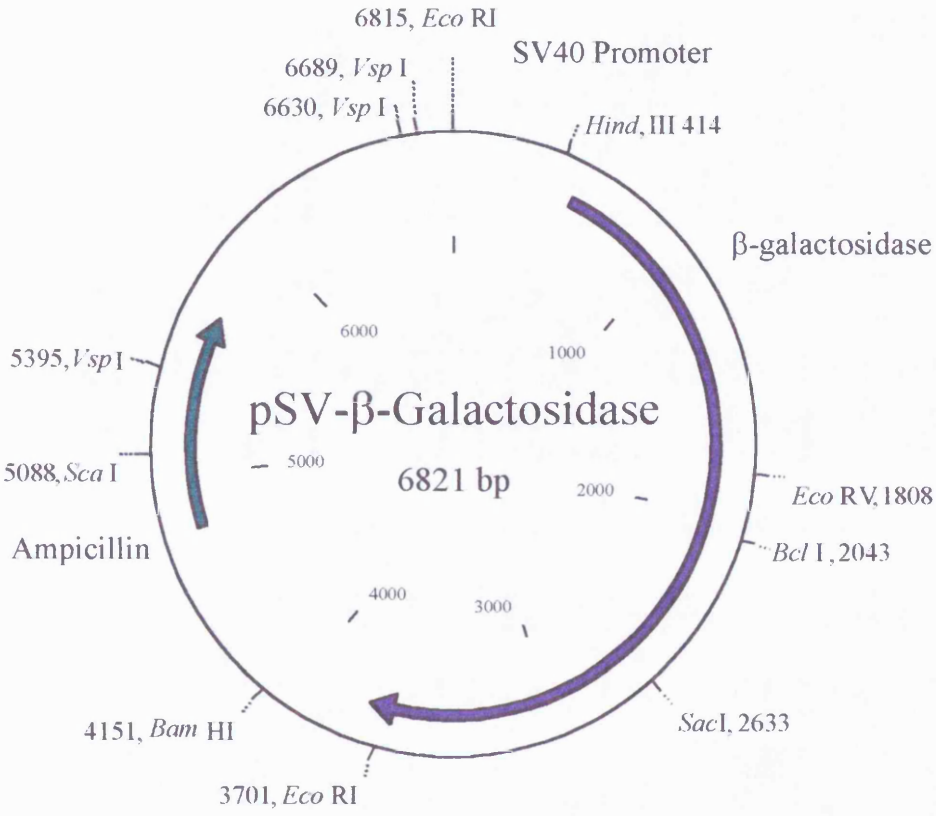
7.1 Introduction

Much interest has been shown in the ability of polycationic and neutral polymers to mediate transfection both *in vitro* and *in vivo* (Chapter 1). Many different polycations have been explored as transfection agents and these include PAMAM dendrimers (Tang *et al.*, 1996), poly(ethylenimine) (Zanta *et al.*, 1997), poly(L-lysine) (Wu and Wu, 1998) and DEAE dextran (McCutchan and Pagano, 1969). All of these polymers are toxic and probably have limited application as systemically administered delivery vehicles (Chapter 1, 3 and 5).

In this preliminary study, the ability of ISA 22 and ISA 23 to mediate pSV-β-galactosidase transfection of Hep G2 cells was investigated *in vitro*. The plasmid pSV-β-galactosidase (Figure 7.1) is often used as a positive control for monitoring transfection in mammalian cells (Promega, 1996). In addition to the ampicillin resistance gene, it contains a bacterial *LacZ* gene, encoding β-galactosidase. The *LacZ* gene is regulated by an SV40 promoter system which allows the expression of bacterial β-galactosidase in mammalian cells (Promega, 1996). The β-galactosidase assay system allows the direct quantitation of β-galactosidase activity within a cell population by lysing the transfected cells in the presence of O-nitrophenyl-β-D-galactopyranoside. The level of β-galactosidase activity can then be quantitated by measuring the rate of O-nitrophenol release from O-nitrophenyl-β-D-galactopyranoside spectrophotometrically at OD_{420}. As a reference control a liposomal formulation (1:2.5) of the cationic lipids dioctadecylammoniumbromide and DOPE was used. This preparation is sold under the brand name LipofectACE by Gibco BRL and has been reported to successfully mediate transient transfection *in vitro* (Shaw *et al.*, 1986; Crise *et al.*, 1990).

As a large quantity of plasmid was required, first pSV-β-galactosidase was used to transform competent *E. coli* DH5α cells which were then cultured (Chapter 2). Plasmid recovery and characterisation was then performed using standard protocols (Maniatis *et al.*, 1986).

Figure 7.1 Restriction map of pSV-β-galactosidase



pSV-β-galactosidase sequence from Promega

It has been reported that there is a relationship between polycation-mediated transfection efficiency and cell cytotoxicity (Van de Wetering *et al.*, 1997). This may due to membrane destabilisation by either the polybase or the IPEC (Chapter 1 and Chapter 3). In view of this, transfection-mediated cytotoxicity was examined by monitoring the ability of the cells to exclude the dye trypan blue, a cellular function indicative of cell membrane integrity (Chapter 3).

7.2 Methods

Competent *E.coli* DH5α cells were transformed with pSV-β-galactosidase, cultured and the plasmid isolated as detailed (Chapter 2). The plasmid was then characterised by restriction analysis and the transfection and cytotoxicity experiments performed as detailed (Chapter 2).

The β-galactosidase assay was performed as described (Chapter 2) and the standardisation for this assay was performed as follows. The velocity of o-nitrophenol release mediated by the β-galactosidase standards was measured and plotted against the amount of enzyme present. The amount of β-galactosidase in an unknown sample could be established by comparing the rate of o-nitrophenol release in the unknown sample with that of the standards.

The integrity of the plasmid stock used during the transfection experiments was also monitored by agarose electrophoresis after the transfection experiments were performed. This was done as follows: First a 0.8% (w/v) agarose gel was prepared containing 1xTAE and 0.25μg/ml ethidiumbromide. The following samples was loaded into separate lanes: 1) 500ng λ *Hind* III digest, 2) 500ng original plasmid (pre-transformation), 3) 500ng plasmid used for transfection and 4) 250ng plasmid used for transfection. The samples were then subject to electrophoretic separation at 80V for 60min and photographed using a gel documenting system.

7.3 Results

The results of the bacterial transformation can be seen (Figure 7.2). The misting of the ampicillin containing agar plates (outlined in red) inoculated with the transformed plasmid is indicative of bacterial cell growth. Bacterial growth is made possible by the ampicillin resistance gene found in pSV-β-galactosidase, when non-transformed (control) cells are used to inoculate a similar plate no growth results.

Following the amplification and isolation of the plasmid, further characterisation was performed by restriction analysis. The results of this assay are shown (Figure 7.3) and the recovered plasmid is of approximately the same molecular weight as the original plasmid. Both the covalent closed circular (CCC) form and the open circular (OC) forms of plasmid are visible in each instance. Following digestion with *Vsp* 1 two fragment are visible indicating the plasmid has been cut 2 times. If the restriction map of the plasmid is examined (Figure 7.1) *Vsp* 1 should cut pSV-β-galactosidase in 3 positions generating 3 restriction fragments. As one of the three fragments generated is only 59 base pairs (bp), it is too small to be resolved on a 0.8% agarose gel, consequently the result seen (Figure 7.3) is indicative of plasmid purity. The result of the digestion with *Bam* H1 shows a linear plasmid. When the size of the linear plasmid is compared with the molecular weight marker (λ *Hind* III) it is seen to be ~6.8KBp. This would indicate that the plasmid isolated from the cultured cells demonstrates all the characteristics of pSV-β-galactosidase.

After the characterisation of the recovered plasmid a standard curve denoting β-galactosidase activity (o-nitrophenol release) over a series of known concentrations was established in triplicate. The results of these experiments are shown in Figure 7.3 and 7.4.

The results of the preliminary *in vitro* transfection experiments are shown (Figure 7.6). The poly(amidoamine)s show some ability to mediate transfection in

Figure 7.2 Transformed *E.coli* DH5α cells (pSV-β-galactosidase) growing on ampicilllin containing media

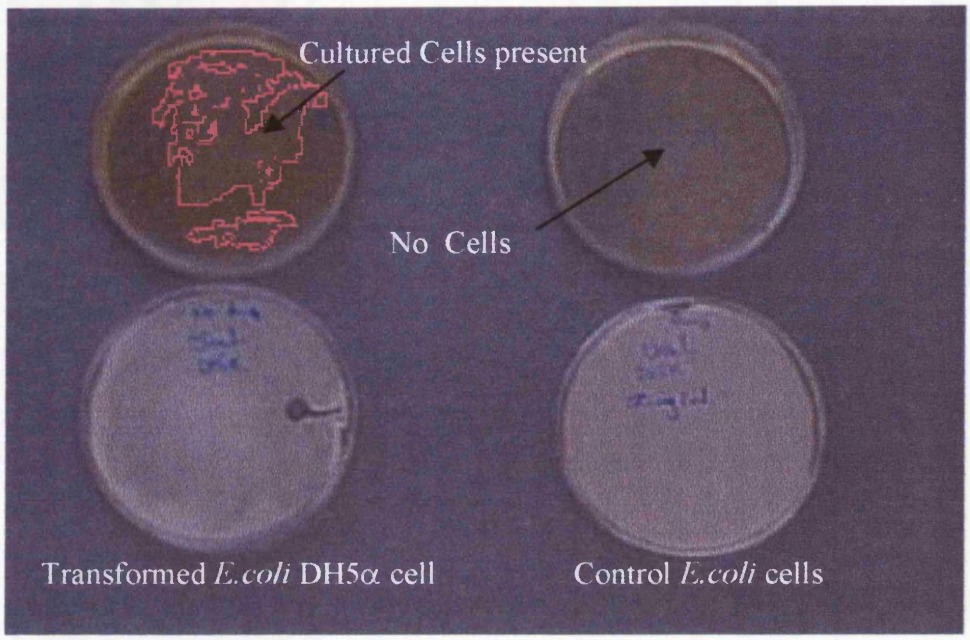


Figure 7.3 Restriction analysis and agarose electrophoresis of plasmid recovered from transformed, cultured *E.coli* DH5α.

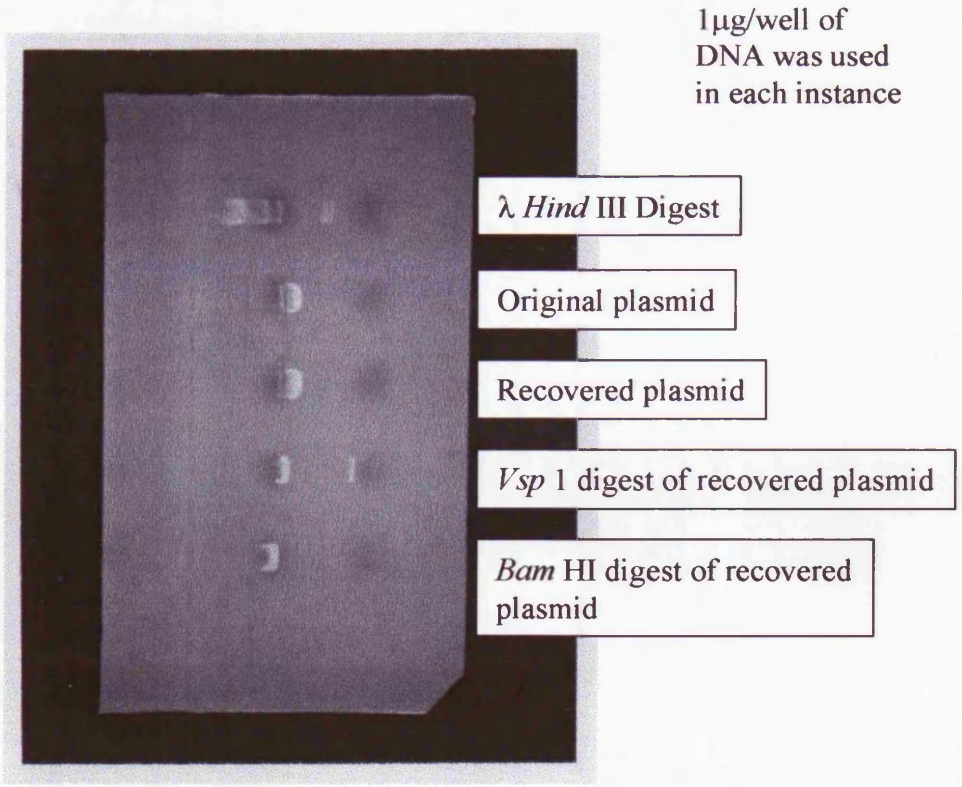


Figure 7.4 Example of β-galactosidase standard activity

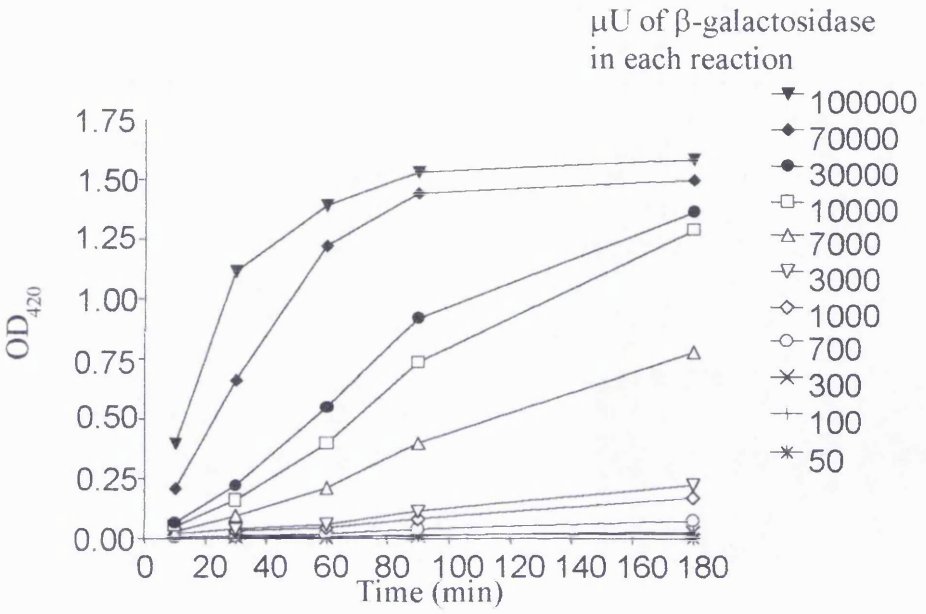


Figure 7.5 Standard curve denoting rate of substrate hydrolysis in relation to quantity of β-galactosidase used (n=3)

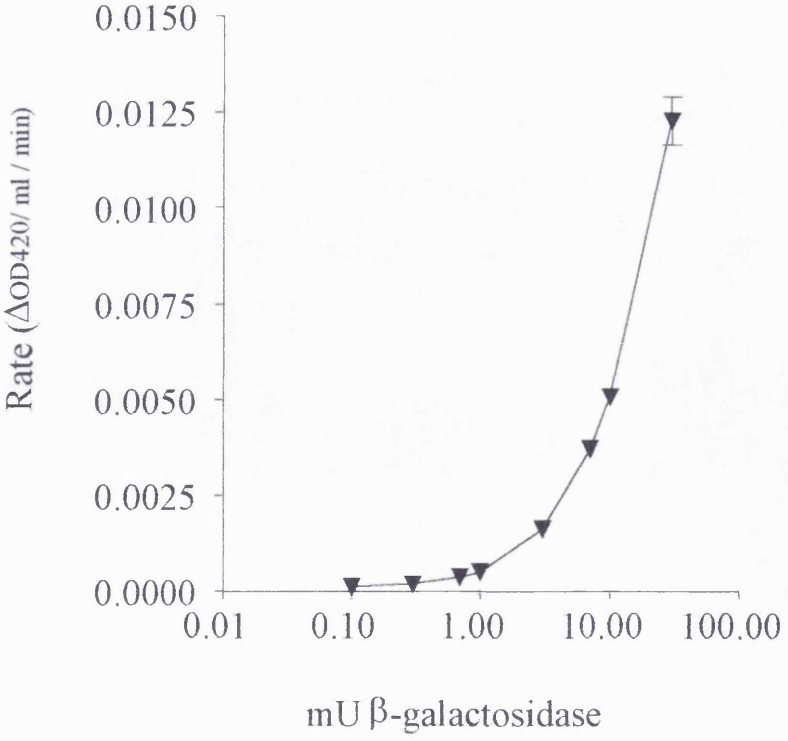


Figure 7.6 *In vitro* transfection potential of ISA 22 and 23 using Hep G2 cells and a β-galactosidease marker gene (mean ± S.D. n =3)

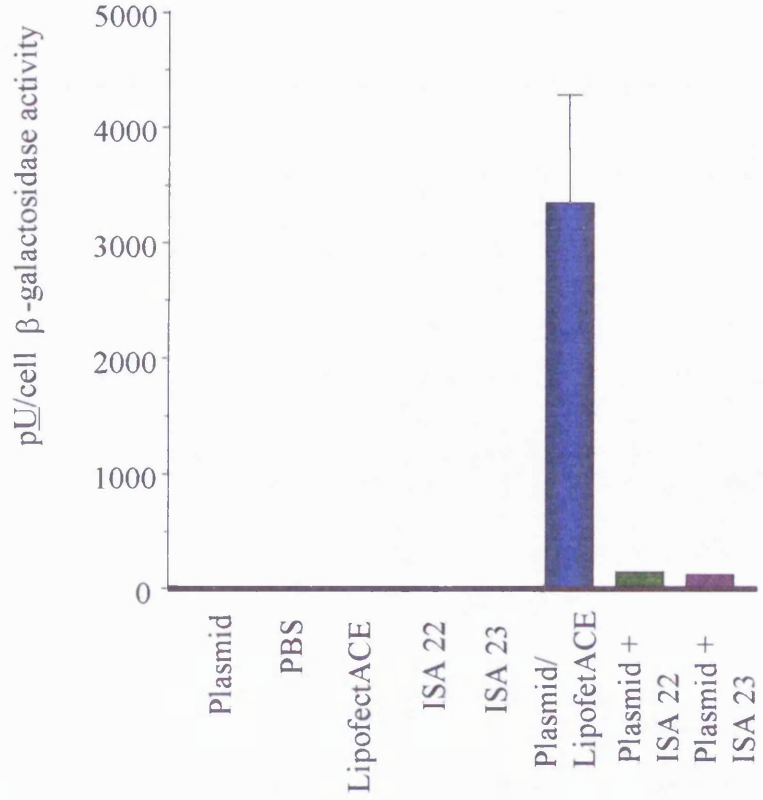
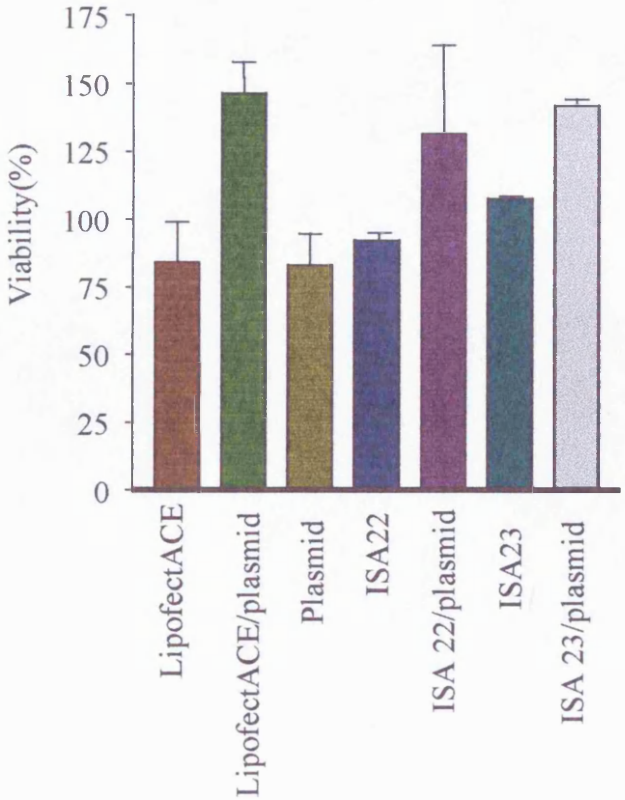


Figure 7.7 Transfection-mediated cytotoxicity assayed by trypan blue exclusion (mean ± S.D. n=3)



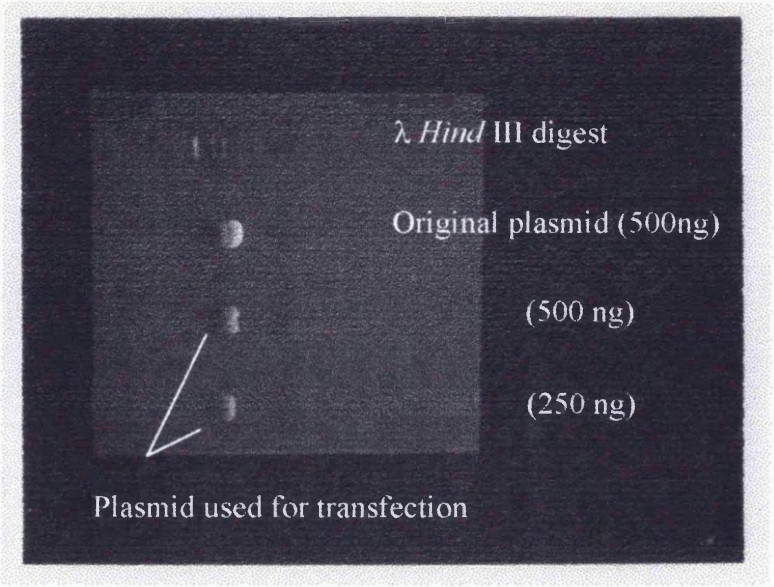
Hep G2 cells but the level of transfection is <10 times that of LipofectACE when the polymers are mixed with the plasmid. When naked plasmid was added to the Hep G2 cultures no transfection was evident and the control cultures have shown that the levels of β-galactosidase indigenous to Hep G2 cells are below the sensitivity of the assay. Figure 7.7 shows the effect of each transfection agent on cellular viability. It may be observed that no cytotoxicity is evident at the concentrations examined here in (10ng plasmid and 100ng vector/2.5ml culture media).

Following the transfection and cytotoxicity experiments the plasmid stock that was used was re-characterised again by agarose electrophoresis. Figure 7.8 shows that the integrity of the plasmid has been maintained throughout the experiment.

7.4 Discussion

Many cationic polymers have been shown to mediate transfection. All show a varying degrees of efficiency (Remy *et al.*, 1998) and utilise a variety of possible mechanisms of action (Chapter 1). When cationic lipids and liposomes are also considered the possible mechanisms of action responsible for transfection widen further (Remy *et al.*, 1998; Kichler *et al.*, 1997). On account of this, the term transfection efficiency should be used with caution. This is because no universally applied gold standard exists that can be used to compare the transfection vehicles such as polymers or lipids. This is because many factors may contribute to the overall apparent transfection efficiency of a given system. Factors such as the strength of a promoter driving the marker gene, the confluence of the cells to be transfected, the quantity of DNA used relative to the cell number as well as the manner in which the vehicle is mixed with the DNA. In addition to the above, the proportions of the vehicle relative to the quantity of DNA used and the physicochemical properties of the delivery vehicle will also effect the apparent transfection efficiency of a system. If lipidic and polymeric transfection systems are to be compared, it should be remembered that the physical

Figure 7.7 Characterisation of pSV-β-galactosidase used for the transfection of Hep G2 cells following 30 days storage at 4°C



characteristics of the two systems and consequently their interactions with biological systems are very different. This is exemplified when the passage of the plasmid out of the endosome is considered. Kichler *et al.*, (1997) demonstrated that the endosomal escape of plasmid DNA was a rate limiting step during polymer (poly(L-lysine)) mediated transfection though not during cationic lipid mediated transfection. For this reason a comparison between the poly(amidoamine)s and the positive, lipidic control should be avoided. Optimal transfection conditions have not been investigated or attained for either vector and this also is likely to add a bias to the perceived transfection efficiencies obtained herein. It is possible that the addition of more poly(amidoamine) relitave to the DNA concentration may offer a significantly increased degree of nuclease protection (Chapter 4) or may further facilitate the movement of DNA out of the endosome, though this is conjecture. It is equally possible that an increased quantity of DNA may redress the transfection efficiencies between the two systems. What can be concluded is that specific cellular events are taking place in order for gene expression to be seen and this is what is of interest herein. It is possible that this event is being facilitated by the pH dependent lytic properties of ISA 22 and 23 as detailed in Chapter 3, though again this needs further substantiation not warranted in a preliminary pilot study.

There is a fundamental problem with this experimental system as it is not conclusive that the results obtained herein are due to plasmid β-galactosidase expression and not the up-regulation of the indigenous mammalian β-galactosidase found in the lysosome (Chapter 1). It may be possible to answer this question by finding regions of non-homologous bacterial and mammalian β-galactosidase nucleotide sequence and using these regions as either a probe for Northern analysis or primers for the PCR. Both of these techniques can then be used to identify bacterial sequences of β-galactosidase mRNA within a mammalian cell type. This would be very costly as well as time consuming and as this is a pilot study, this level of precision was not deemed appropriate. There are other commercially available markers genes such as luciferase encoding plasmids,

however the detection and quantification of the gene products is much more expensive than with this system and as this is a pilot study, the added expense was not warranted.

It is interesting that no cytotoxicity was evident during this study. When well-characterised systems are examined, typically 40-80% cell death is seen (Van de Wetering *et al*, 1997) where optimal conditions for transfection have been achieved. It is possible that the optimal conditions have not been used no cytotoxic cell death is evident where transfection has been induced.

7.5 Conclusions

It was shown that *in vitro* ISA 22 and ISA 23 can mediate the transfection of pSV-β-galactosidase into Hep G2 cells cultured in the presence of serum nucleases. No cytotoxicity was observed over the time frame investigated herein though optimum conditions for transfection were not established.

Chapter 8

General Discussion

General Discussion

In contrast to lipidic gene delivery systems, no polymer-mediated gene delivery systems have gone into clinical trial to date (reviewed in Hersh and Stopeck, 1998). However, in the last 5 years many papers investigating the cellular mechanisms responsible for transfection mediated by polymeric non-viral vectors have been published.

Attention has been directed towards understanding the intracellular molecular events responsible for transfection. These include studies dissecting the release of DNA from cationic lipid:DNA complexes following endocytosis, (Xu and Szoka, 1996) the release of DNA from the endosome mediated by both lipidic and polymeric delivery systems (Kichler *et al.*, 1997) and also the influence of particle charge upon transfection efficiency (Remy *et al.*, 1998; Zanta *et al.*, 1998; Ogris *et al.*, 1998; Wolfert and Seymour, 1996). Further, the intracellular trafficking of non-viral delivery systems has been studied (Zelphati and Szoka, 1996[b]; Coonrod and Horwitz, 1997) as well as the possible mechanisms by which the polymer systems may enter membrane limited intracellular compartment (Zanta *et al.*, 1997). Boussif *et al.*, (1995) proposed a mechanism describing the endosomal exit of plasmid DNA mediated by poly(ethylenimine). Here endosomal rupture was reported to be mediated by poly(ethylenimine) buffering the endosomal pH. A subsequent increase in intra-vesicular hydrostatic pressure as a result of osmosis and "polymer swelling" was thought to facilitate the rupture of the endosomal limiting membrane. This is apparently in contradiction to more conservative theories concerning mechanisms of polycation (poly(lysine)) mediated membrane rupture which are driven by a random coil to α helix transition upon the association of the polymer with a negatively charged membrane (Chapter 1 and Chapter 3; Hartmann and Galla, 1978).

Improvements in understanding the biological and physicochemical properties of IPECs have also been aided by imaging techniques such as atomic force microscopy (Wolfert and Seymour, 1996). The role of factors such as IPEC

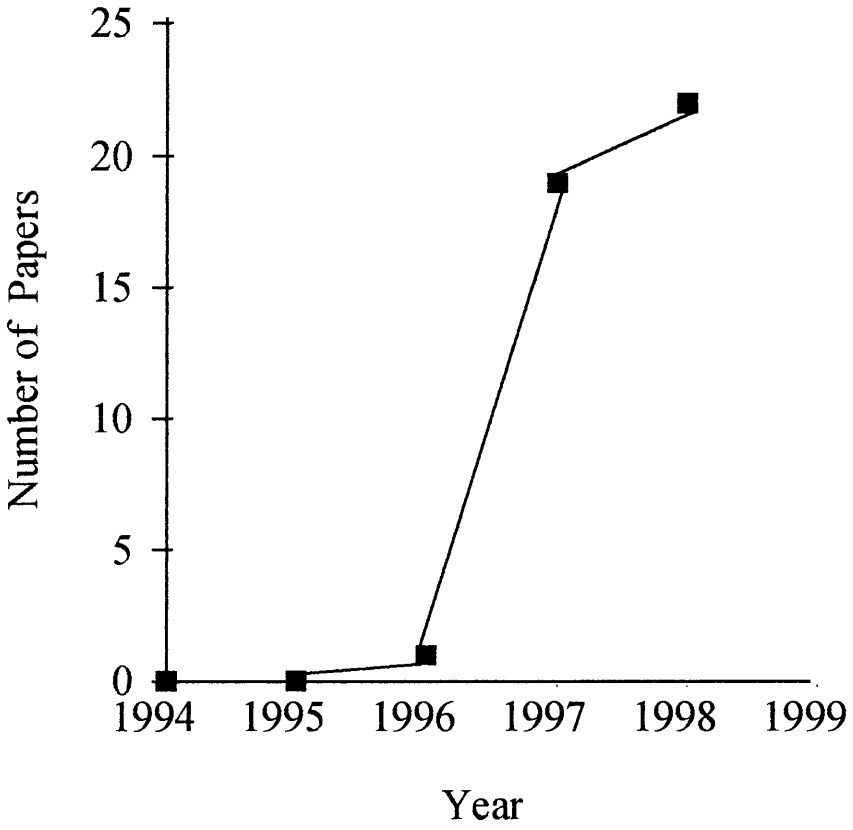
size in relation to transfection efficacy has also been recently addressed (Ogris *et al.*, 1998).

Figure 8.1 shows the number of publications listed in BIDS containing the key words "non-viral" and "gene". It is evident that research in this field has grown over the last 10 years. The poly(lysine) and lipidic DNA delivery systems are starting to be understood and one of the current challenges it to identify novel polymers capable of mediating transfection *in vivo*.

All of the work contained herein may be viewed as novel as the polymers evaluated herein are themselves novel. Previous work has identified high molecular weight chitosans as toxic (Carreno-Gomez and Duncan, 1997; Chapter 3) and the discovery that the cytotoxic profile of the chitosans N1-3 could be reduced relative to the polymers investigated in Carreno-Gomez and Duncan (1997) is encouraging. The body distribution of linear ^{125}I-labelled chitosan molecules has not been previously reported. Previously, branched galactosolated chitosan molecules were investigated by Murata *et al.*, (1997) though these molecules were designed to demonstrate a liver tropic body distribution profile as dictated by the galactose moiety (Chapter 6). Chitosan molecules effect transfection *in vitro* though this process has been reported to be inefficient (MacLaughlin *et al.*, 1998). This fact in conjunction with the pharmacokinetic profile associated with these molecules i.e. rapid liver clearance may detract from the usefulness of these molecules as systemically administered DNA delivery vehicles.

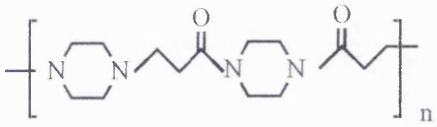
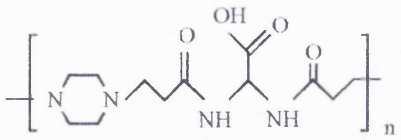
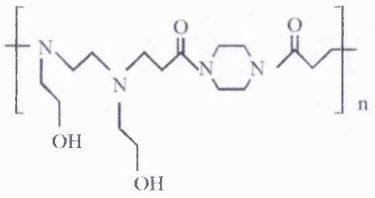
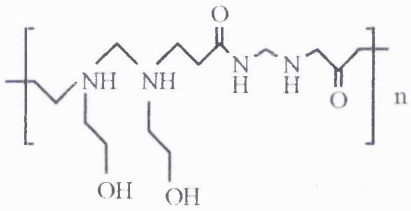
Previously, the biocompatibility of several different poly(amidoamine)s has been investigated and the results of previous work are summarised in Table 8.1. It was reported in Richardson *et al.*, (1998), for the first time, that not only are two families of poly(amidoamine) suited to systemic administration (Chapter 3) but also show potential as pH-dependent endosomolytic delivery systems. These have a potential advantage over the peptide derived pH lytic systems (Fattal *et al.*, 1994; Paul *et al.*, 1997; Thomas *et al.*, 1995) as it is likely to be non-immunogenic (Ferruti *et al.*, 1998).

Figure 8.1 Number of papers listed in the BIDS ISIS Science citation index containing the key words "Non-viral" and "Gene"



Data correct November 1998

Table 8.1 Characteristics of poly(amidoamine)s

Structure	Name	IC_{50}
	BPP Mn 6300	> 0.5 mg/ml (Hep G2 and CCRF-CEM)
	BACP Mn 5300	>2mg/ml (Hep G2 and CCRF-CEM)
	BPI Mn 6200	>1mg/ml (Hep G2) 50µg/ml (CCRF-CEM)
	MBI Mn 3000	0.6 mg/ml (CCRF-CEM)

Adapted from Sgouras, (1990)

As stated in Chapter 6 this was the first time the body distribution of the linear poly(amidoamine) had been undertaken. This was interesting as the opportunity was presented to evaluate poly(amidoamine)s with a varying pKa's as dictated by monomeric composition (Chapter 1 and Richardson *et al.*, 1998). Further, this was the first time poly(amidoamine)s had been used as transfection vehicles *in vitro*.

Concluding comments

As described in Chapter 1, a nucleic acid delivery system that is to be administered systemically must perform a number of specific functions (Table 1.5). Once vehicle biocompatibility has been established, (Chapter 3) the vehicle must protect the DNA from nucleases (Chapter 4). Organ or tissue targeting may then be required, raising the local concentration of the IPEC and consequentially potentially raising transfection efficiency (Chapters 5 and 6). Following this the IPEC must mediate the transfer of functional DNA from the external surface of the cell to the nucleus where the transgene may be expressed at an appropriate level (Chapter 7).

There are also several other critically important steps detailed in Chapter 1 that have not been addressed here. Complex stability in response to biological fluid and membranes, intracellular DNA release, DNA nuclear trafficking and the molecular biology governing transgene expression levels and gene product half life (Chapter 1) all need to be optimised. However, an evaluation of these events is beyond the scope and resources allocated to this thesis.

As the evaluation of poly(amidoamine)s and chitosans N1-3 as potential nucleic acid delivery vehicles has progressed, another slightly more subtle problem has become apparent and this is due to the charge associated with the IPEC particle.

To maintain a biocompatibility profile suitable for systemic administration a net negative charge is preferential to a positive charge, though as reported in Chapter 3 and Chapter 5 a small degree of cationic substitution may be expectable.

If a non-liver tropic body distribution is required, it is important that the IPEC has a net neutral charge in the systemic circulation. It was reported in Chapters 5 and 6 that, with the exception of ISA 22 all of the polymers investigated here in demonstrated a liver tropic body distribution as a function of polymer charge. In contrast, for an IPEC to maintain a regular structure such as a condensed toroid or "doughnut" an excess of polycation is required (Manning, 1980; Ogris *et al.*, 1998) so giving the IPEC an overall positive charge. Excessive positive charge (beyond the stoichiometric point) has also been reported to be required for nuclease protection (Kabanov and Kabanov, 1995) as the DNA:polymer association below this point is fluid (Shapiro *et al.*, 1969). An excess of polycation is also required for optimal transfection efficiency (Remy *et al.*, 1998; Zanta *et al.*, 1997; Ogris *et al.*, 1998; Wolfert and Seymour, 1996).

In summary, it may be seen that if a particle is to be directed to a body compartment other than the liver, it must have a net negative charge however if this is the case it will most likely be unstable and ineffectual at performing transfection.

In view of this, it may be prudent to direct research efforts away from IPEC-mediated gene delivery and more towards the generation of covalent constructs.

Future work

The most immediate area of experimental research is the reaffirmation of the poly(amidoamine) pH-triggered membrane lysis model. Preliminary studies could be undertaken to determine whether polymer concentration-dependent RBC lysis could be observed in response to an alteration in pH. If the delivery of a substance into the cytosol of a specified cell type is to be accomplished *in vivo,* then the quantitation, characterisation and optimisation of pH-triggered membrane lytic event must be investigated. It may be possible to tailor this endosomolytic delivery by altering the chemistry and molecular weight of the poly(amidoamine)s as well by utilising branched poly(amidoamine) scaffolds to increase the local concentration of linear poly(amidoamine) within the endosomal compartment. This

idea is shown conceptually (Figure 8.2). At all times during these studies biocompatibility and toxicity should be monitored.

The potential application of an endosomolytic delivery system is vast. Substances such as low molecular weight, labile, proteinacious drugs, i.e. tyrosine kinase inhibitors (Levitzkie 1994), antisense oligonucleotides, anti gene agents and ribozymes (Chapter 1) have all been reported to require intracytoplasmic delivery before their optimum therapeutic value may be realised.

It is not inconceivable that an antisense delivery system utilising an neutrally charged single stranded nucleic acid analogue (such as PNA, Chapter 1) may benefit therapeutically via its covalent conjugation to an endosomolytic polymer via an acid labile linking moiety (reviewed in Soyez *et al.*, 1996) (Figure 8.3). If this can be achieved, a significant expansion in the range of therapeutic tools available to the clinician may result as well as an increase in the tools available to perform basic research and development, characterising and understanding and treating the molecular basis of disease.

Figure 8.2 Branched endosomolytic polymer

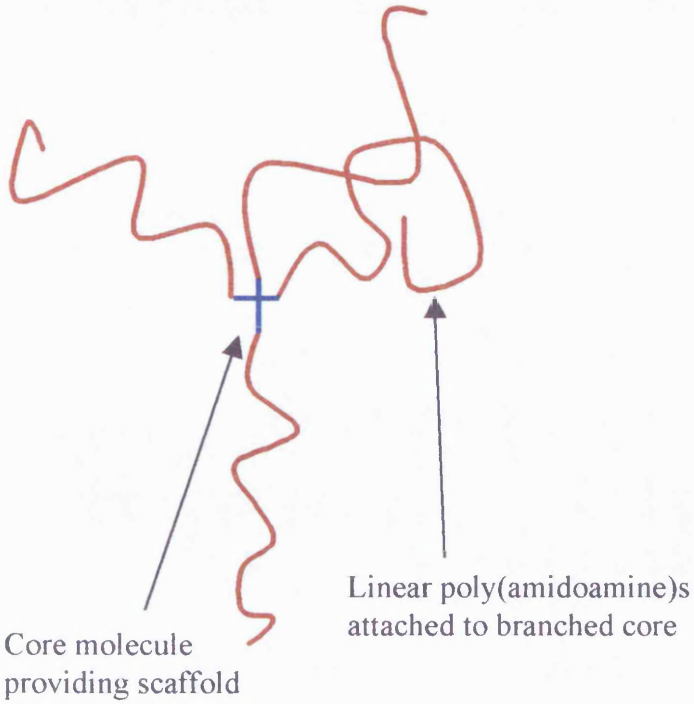
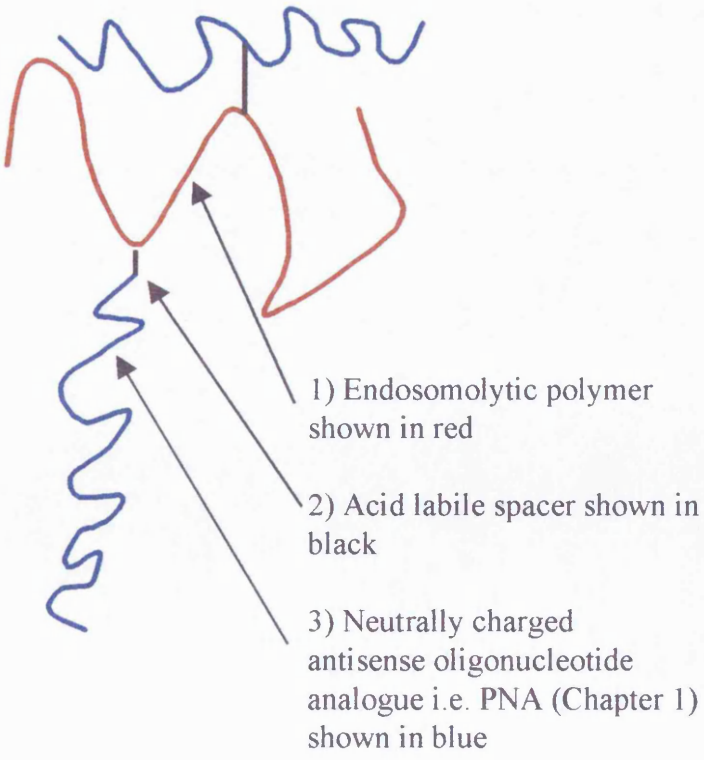


Figure 8.3 Cartoon depicting a possible covalent endosomolytic construct for the delivery of antisense oligonucleotide analogue *in vivo*



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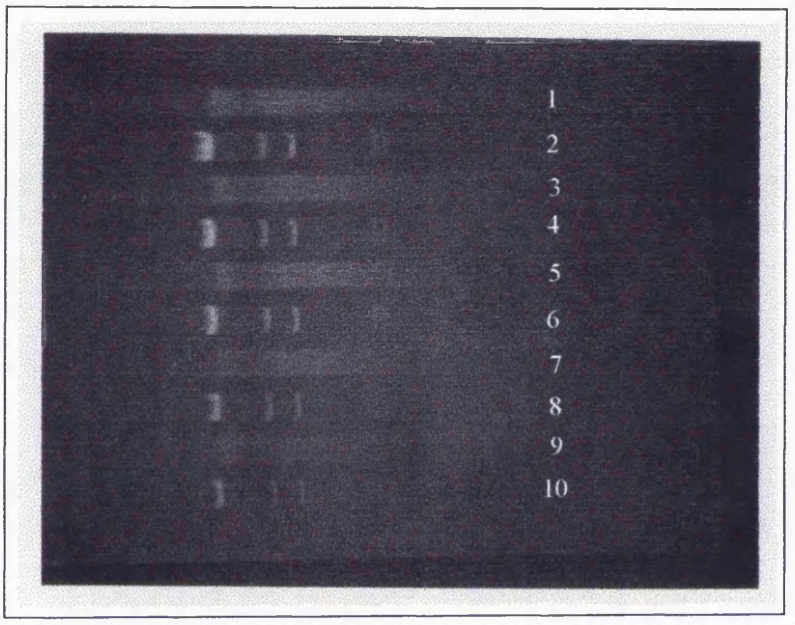
Appendix 1

Electrophoresis Standards

Electrophoresis standards

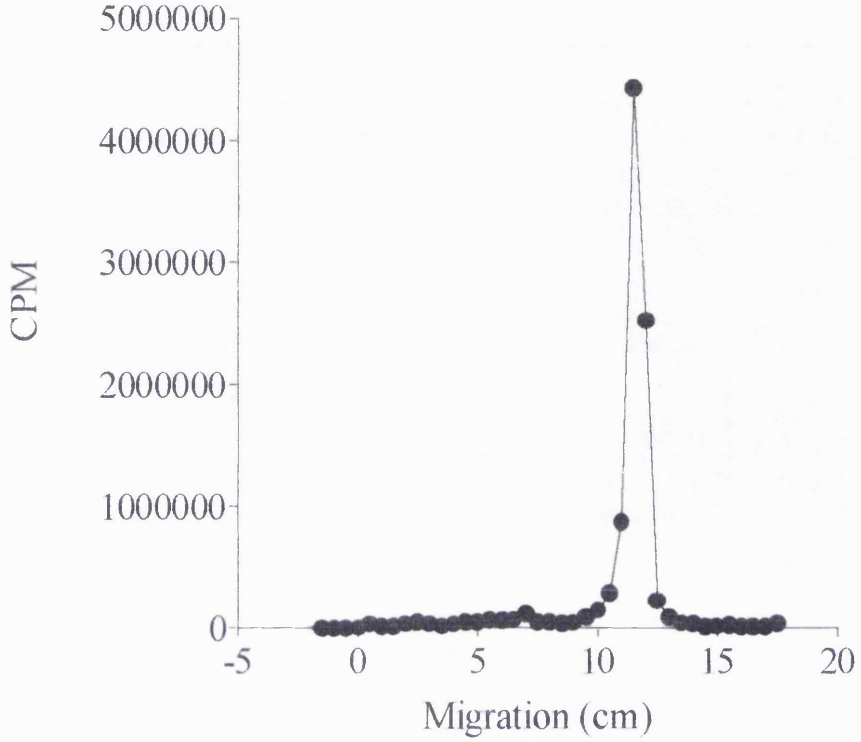
A1.1 Characterisation of calf thymus DNA by agarose electrophoresis



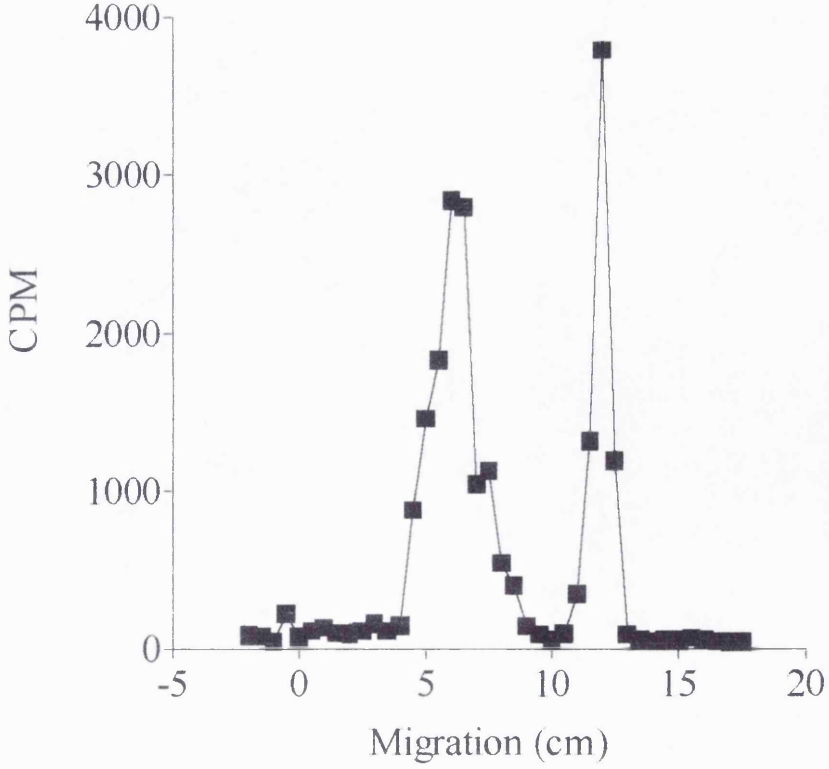
1=1μg Calf Thymus DNA
2=1μg λ *Hind* III DNA
3=800ng Calf Thymus DNA
4=800ng λ *Hind* III DNA
5=600ng Calf Thymus DNA
6=600ng λ *Hind* III DNA
7=400ng Calf Thymus DNA
8=400ng λ *Hind* III DNA
9=200nμg Calf Thymus DNA
10=200ng λ *Hind* III DNA

Characterization performed on a 0.5%(w/w) agarose gel made in 1xTAE containing 0.5% (w/v) ethidium bromide. Electrophoresis performed at 100 V for 60min at room temperature (Chapter 2)

A1.2 Paper alectrophoresis of Na[^{125}I]iodide



A1.3 Paper electrophoresis of Bolton and Hunter degredation products



Appendix II

Publications

Publications

Review Articles

Ferruti P., Duncan R. and Richardson S. (1998). Tailor-made soluble polymer carriers, In *Targeting of Drugs: Stealth Therapeutic Systems,* Eds Ggregoriadis, G. and McCormack B., Pub. Plenum press: New York, (In Press)

Papers

Richardson S., Kolbe H. V. J and Duncan R. (1998). Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA, *International Journal of Pharmaceutics,* (In press)

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Abstracts

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Appendix 3
Explanatory Notes

Table 3.1 contains IC_{50} values derived from, in each instance, three separate graphs (not shown) which were, in turn derived from three separate experiments performed at separate times. In the instance of Figure 3.1 each experiment contained 6 repetitions of each concentration of polymer and the IC_{50} value were determined from each of the three experiments. The mean and standard deviation for the three IC_{50} values are quoted in Table 3.1. The graphs depicting polymer mediated cytotoxicity shown in Chapter 3 (all cytotoxicity graphs) show the mean of the data points from all of the experiments that were performed, that is, in the instance of Figure 3.1 the mean of three experiments each containing 6 data points. The number of experiments and repetitions in each experiment is quoted in the tittle of each graph.

Figures 4.5 and 4.6 depict data derived in the following way: First control experiments were performed to establish, within defined experimental parameters, (Chapter 2) the amount of DNA degradation mediated by a specified amount of DNase II over time using a defined amount of substrate. This was calculated by measuring the area under the curve of a control experiment described by the graph "Control degradation at 60 min (%) verses time" (examples shown in Figure A3.1 and A3.2). Maintaining exactly the same experimental parameters, with the exception of the addition of a pre-defined quantity of polymer, the experiments were repeated. The area under the curve was assessed for each repetition of the experiment (n=3). The area under the curve for the control experiment (no polymer) was then defined as 100% DNase II degradation and the areas under the curve for the polymer containing experiments expressed as:

100% - (AUC of polymer containing experiment /(%AUC of control/100))

DNase II inhibition (%) was calculated for each polymer at each charge ratio (See also Richardson *et al.*, 1998[b] for data).

Figure A3.1 DNase II inhibition mediated by chitosans N1, N2 and N3 mixed with DNA at a charge ration of 1:1 (n=3)

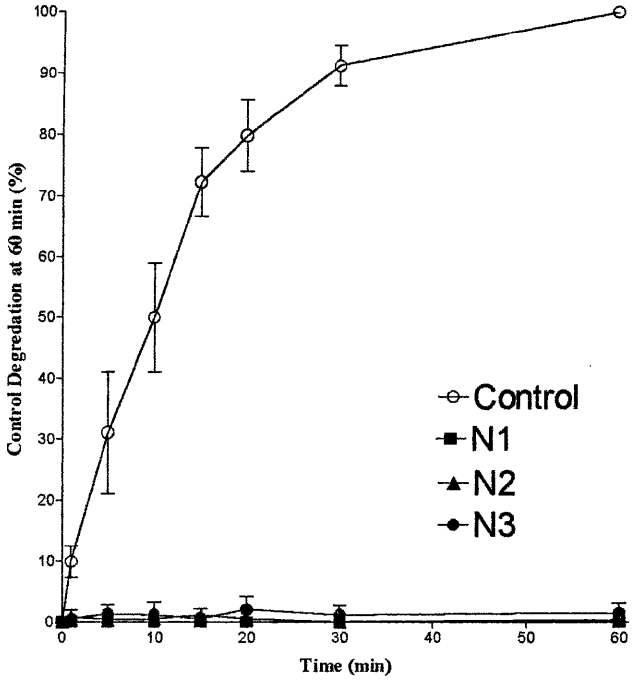

Figure A3.2 DNase II inhibition mediated by chitosans N1, N2 and N3 mixed with DNA at a charge ration of 1:0.01 (n=3)