Body distribution of dextrin and D-2-S and evaluation of their potential as novel polymeric-drug carriers

by

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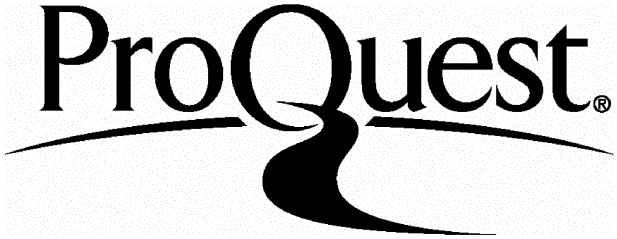

This Thesis is Dedicated To
My Parents

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Abstract

Water soluble polymers, including natural polymers (e.g. polyamino acids and polysaccharides) and synthetic polymers (e.g. polyethyleneglycol (PEG)) and N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers) are finding increasing use as polymer therapeutics. Dextrin and dextrin-2-Sulphate (D-2-S) are poly(α1-4 glucose) polymers that have entered into clinical use: dextrin as a peritoneal dialysis solution and D-2-S as an anti-HIV treatment. The aim of this study was (1) to quantitate the biodistribution of dextrin and D-2-S and (2) to evaluate the potential of these polymers for use as drug carriers.

First, pendant groups were introduced by succinoylation (1- 60 mol %). To study biodistribution (after s.c., i.v. or i.p. administration) probes were then synthesised containing either -TyrNH_2 or –DTPA (~1 mol %) to allow labelling with [^{125}I]iodine or [^{111}In]indium respectively. After i.p. administration ^{125}I-labelled D-2-S remained longer in the peritoneal cavity (~27 times) than ^{125}I-labelled dextrin ($t_{1/2}$ = 2.3 h and 5 min respectively). Maximum tissue accumulation was seen in the liver, for ^{125}I-labelled dextrin approximately 20 % administered dose (2 min) and for ^{125}I-labelled D-2-S (15.3 % administered dose (24 h). Gamma camera images obtained using ^{111}In-labelled polymers were consistent with the data obtained using ^{125}I-labelled compounds.

Using the succinoylated intermediate, dextrin- and D-2-S-amphotericin B conjugates (AmpB) were prepared containing 0.01-3.50 wt % and 0.01–16.0 wt % AmpB respectively. Conjugation increased drug solubility approximately 10 fold. Preliminary *in vitro* testing showed IC_{50} values for AmpB, dextrin- and D-2-S-AmpB (IC_{50} of 11.0 >50, and >50 μg/ml respectively) and haemolytic activity at 24 h (Hb_{50} of 0.06 mg/ml, >50 μg/ml and 8.0 μg/ml respectively).

Additionally, experiments were carried out with dextrin-doxorubicin (Dox) (9 wt %, Dox-equiv) to ascertain its tumour targeting potential and antitumour activity. Whereas free Dox was inactive (T/C= 105 %) in a s.c. B16F10 tumour model dextrin-Dox had a T/C = 144 %.

Dextrin and D-2-S have shown that they have potential for further development as water soluble drug carriers.

Thesis Index

List of Figures

List of Tables

Abbreviations

Alpha	α
Acquired Immunodeficiency Syndrome	AIDS
Amphotericin B	AmpB
Beta	β
Carbodiimidazole	CDI
Continuous ambulatory peritoneal dialysis	CAPD
Counts per minute	cpm
Counts per second	cps
Daltons	Da
Degree Celsius	°C
Dextrin-2-Sulphate	D-2-S
Dextrin-Doxorubicin	Dextrin-Dox
Dihydrogen orthophosphate	NaH_2PO_4
Dimethylaminopyridine	DMAP
Dimethyl formamide	DMF
Dimethyl sulphoxide	DMSO
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide	MTT
Diethyltrianime pentaacetic acid	DTPA
Disodium orthophosphate	Na_2HPO_4
Doxorubicin	Dox
Enhanced permeability and retention effect	"EPR" effect
Ethylenediamine tetraacetate	EDTA
Foetal Calf Serum	FCS
Fourier Transfer Infra Red spectroscopy	FTIR spectroscopy
Gel permeation chromatography	GPC

High Performance Liquid Chromatography	HPLC
Human Immunodeficiency Virus	HIV
Intraperitoneal	i.p.
Intravenous	i.v.
Mega Bequerel	MBq
Maximum tolerated dose	MTD
Minimum Inhibitory Concentration	MIC
Nucleur Magnetic Resonance	NMR
Number average molecular weight	Mn
Phosphate buffered saline	PBS
Sodium hydroxide	NaOH
Red blood cells	RBC
Reticuloendothelial system	RES
Sodium chloride	NaCl
Standard Deviation	S.D.
Standard Error	S.E.
Subcutaneous	s.c.
United Kingdom Co-ordinating Committee on Cancer Research	UKCCCR
UV visible spectroscopy	UV-vis spectroscopy
Volume for volume	v/v
Weight average molecular weight	Mw
Weight for volume	w/v
Weight for weight	w/w

Chapter 1

General Introduction

1.0 Introduction

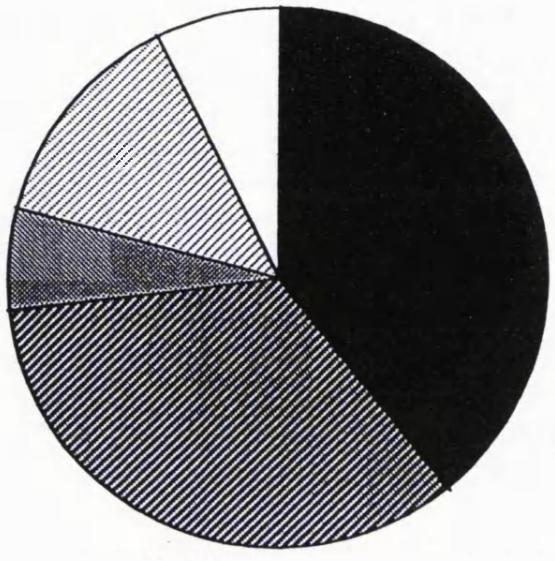
The major causes of mortality worldwide are cancer, cardiovascular diseases, infectious e.g. Acquired Immunodeficiency Syndrome (AIDS) and parasitic diseases. The major causes of death in 1999 can be seen in Figure 1.1. It can be seen that infections and parasitic diseases were responsible for the majority of deaths.

In the developed countries cancer is beginning to take over from cardiovascular disease as the major cause of death. The latest cancer statistics show that by the year 2020 there will be 20 million new cases of cancer each year (Sikora, 1999) and "cancers" are becoming amongst the most feared diseases known to man. In the developed countries the most common cancers are prostate cancer in men and breast cancer in women whilst the major causes of mortality worldwide are lung and liver cancers. Indeed in the UK, since 1973 the dramatic increase in the number of women who smoke has lead to a 124 % increase in the number of women being diagnosed with lung cancer. For men this increase is 10 % (NCI Report, 1998).

Human Immunodeficiency Virus (HIV) is caused by the HIV retrovirus gradually destroying the immune system leading to the development of AIDS (Blanter *et al.*, 1988). At first the immune system responds effectively against the HIV infection but is eventually overwhelmed by the accumulation of more resistant variants. AIDS is the name given to the last stage of the disease and is defined by a specified reduction in the number of T cells and the appearance of secondary infections (Figure 1.2). Progression of the HIV infection to AIDS takes approximately 10 years and during this time the patient only exhibits moderate hints of illness.

The Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) have estimated that the number of people worldwide infected with HIV is over 30 million. Already 11.8 million (UNAIDS Report, June 1998) have died due to HIV infection. In the US alone in 1997 there were 650, 000 HIV infected individuals of which 275 000 have AIDS (Nathanson, 1998). There is also a significant difference in the number of people with HIV living in developed (e.g. Western Europe) and developing (e.g. sub-Sahara Africa and Asia) countries (Figure 1.3).

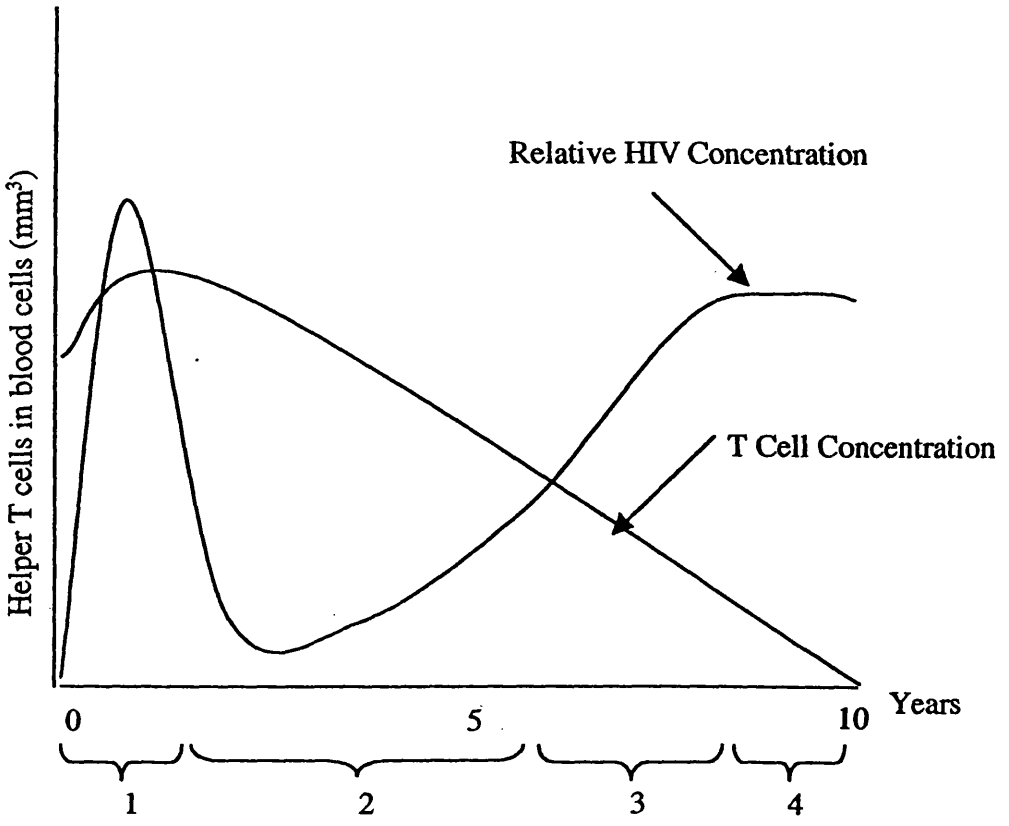
Figure 1.1 Major causes of death in 1999 (taken from World Health Organisation report, 1999)



Key

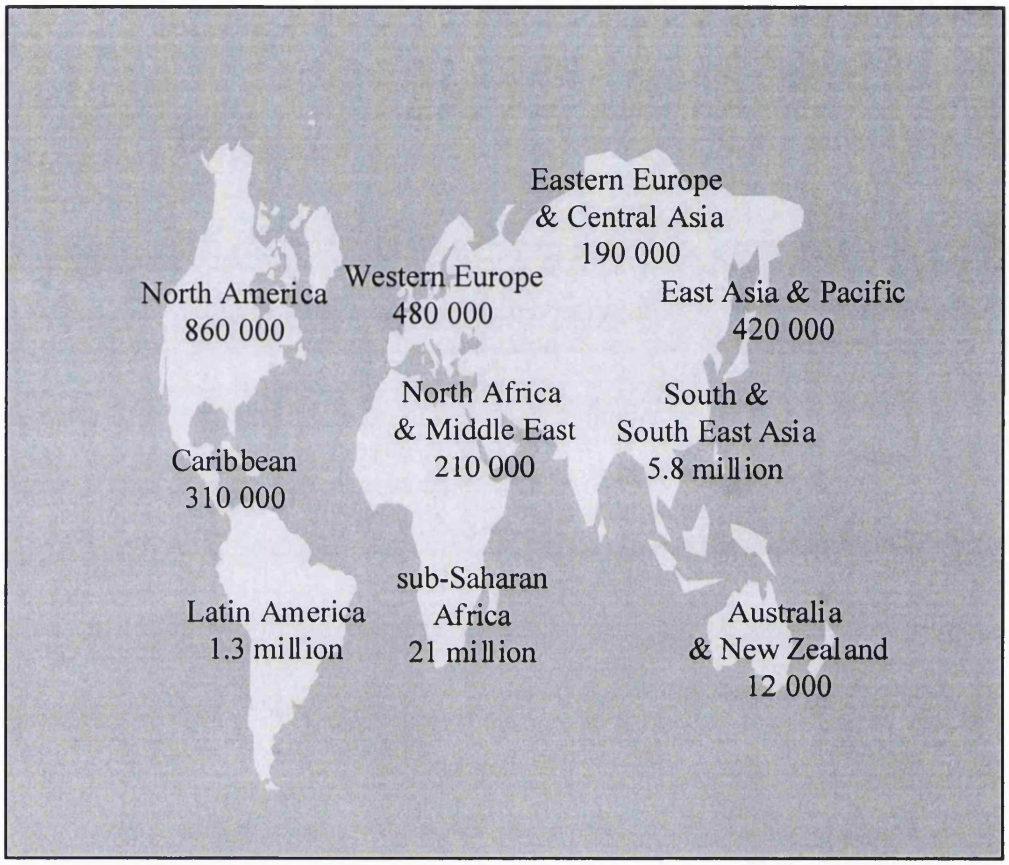
- Infectious and Parasitic Diseases 38% (17.3 million)
- Circulatory Diseases 33% (15.3 million)
- Respiratory Diseases 6% (2.9 million)
- Cancer 13% (6.2 million)
- Perinatal Condition 7% (3.6 million)

Figure 1.2 Progression from HIV to AIDS (taken from Cambell, 1993)



1- Infection: immune system eliminates most of HIV
2- Few symptoms, swollen lymph glands
3- Loss of immune function more apparent appearance of characteristic diseases such as yeast infections
4- Total loss of cellular immunity; AIDS

Figure 1.3 Distribution of people living with HIV or AIDS in the developed and the developing countries (adapted from UNAIDS report, 1998)



Unless new and improved treatments can be found that can prevent, effectively control or cure cancer and infectious diseases such as AIDS, the number of mortalities caused by these life threatening diseases will continue to increase.

Two main approaches are traditionally used to identify new medicines with improved efficacy.

e) Synthesis (or selection by screening) of new chemical entities with inherent pharmacological activity e.g. low molecular weight drugs or prodrugs.

f) Alternatively, the design of new drug delivery systems that can help to improve the therapeutic index of existing or new drugs by targeting or controlled release.

At the beginning of the 20th century the famous German scientist P. Ehrlich was the first person to use a totally synthetic compound as a drug. He also introduced the idea that therapeutic drugs could be conceived of as a "magic bullet" (Ehrlich, 1906); thus he proposed the possibility of targeting a chemical selectively to a particular disease.

Over the last century there has been considerable development of the field of drug targeting and today a number of drug delivery systems are either in routine clinical use or under preclinical investigation. These include prodrugs (Hoes *et al.*, 1985), liposomes (reviewed by Gregoriadis, 1995; Gabizon, 1989, 1990; Allen, 1997; Hillery, 1998), microspheres (reviewed by Cummings, 1998), nanoparticles (Kreuter, 1994; Couvreur *et al.*, 1995), antibody-drug conjugates (Chari, 1998) synthetic polymers (reviewed by Brocchini, 1999; reviewed by Duncan, 1992; Putnam and Kopecek, 1995 and Kwon and Kataoka, 1995), drugs bound to macromolecular carries containing targeting moieties such as saccharides or antibodies (Kopecek and Duncan, 1987, Duncan and Spreafico, 1996) and natural polymers (reviewed by Sezaki *et al.*, 1989). Both synthetic and natural polymers are beginning to find use as polymeric drugs, polymeric micelles, polymer-proteins and polymer-drug conjugates. This emerging field has been termed "Polymer Therapeutics" (Duncan, 1997). In this study, two natural biodegradable polymers, dextrin and dextrin-2-sulphate (D-2-S) were selected for investigation. These carbohydrate polymers are already in routine clinical use.

Figure 1.4a Structure of dextrin

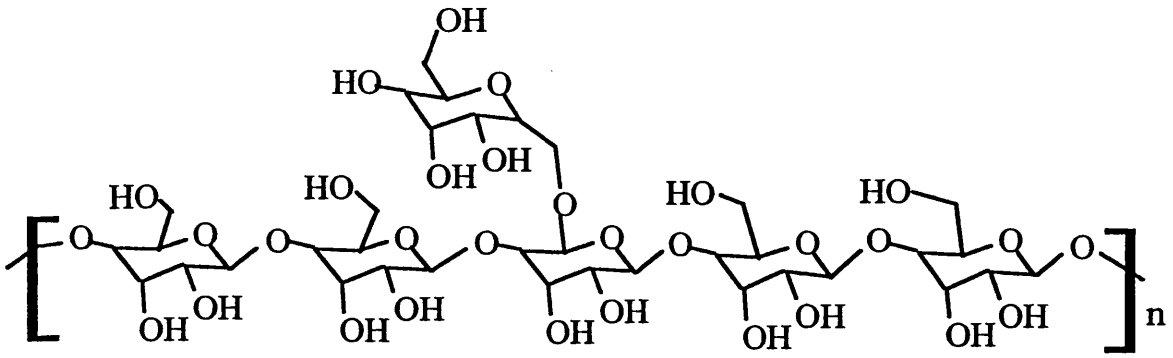
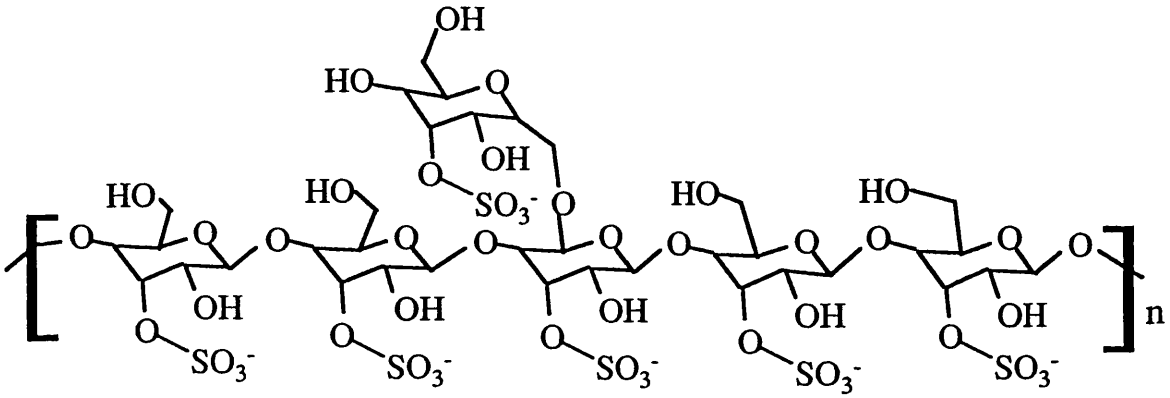


Figure 1.4b Structure of D-2-S



Dextrin (Figure 1.4a) is a α1-4 polyglucose used as a peritoneal dialysis solution to treat >50 % of patients with end stage renal failure (ESRF) (Peers and Gokal, 1997) and continuous ambulatory peritoneal dialysis patients (CAPD) with ultrafiltration failure (Peers *et al.*, 1995).

A sulphated form of this polymer, D-2-S (Figure 1.4b) is in Phase III clinical trials as an anti-AIDS drug. D-2-S has been shown to reduce the replication of HIV-1 in patients with AIDS (Shaunak *et al.*, 1998) and has also been developed as an intravaginal gel that displays activity as a vaginal virucide (Stafford *et al.*, 1997).

To complement the emerging clinical data, it was considered important to quantitate the biodistribution these two polymers (this had never been done before) and to investigate further the factors that might affect polymer biodistribution e.g. dose and volume of administration.

Secondly, this study wished to explore whether dextrin or D-2-S might be useful polymers to develop as polymer-drug conjugates for drug targeting. Dextrin-doxorubicin (Dextrin-Dox) and dextrin- and D-2-S-amphotericin B (Dextrin-AmpB and D-2-S- AmpB) conjugates were studied in respect of their pharmacokinetics and pharmacology.

To introduce this research project the concepts of drug targeting, polymer therapeutics, polysaccharides as drug-carriers and dextrin and D-2-S are described in more detail.

1.1 Drug delivery

Drug delivery systems seek to both optimise drug pharmacokinetics (controlled release) and improve localisation of a specific drug at a specific site (targeting) (Langer, 1998).

Targeting is defined as delivering an adequate amount of drug to a target site in the body at an appropriate time (Mrsny, 1997; Kataoka, 1997). Targeting of drug carriers can take place by active or passive targeting :-

Active targeting: Active targeting relies on the selective localisation of a ligand via interaction with a cell-specific receptor. Active targeting can be achieved at three levels (Duncan, 1992). First order targeting is organ specific i.e. to the lungs or liver. Second

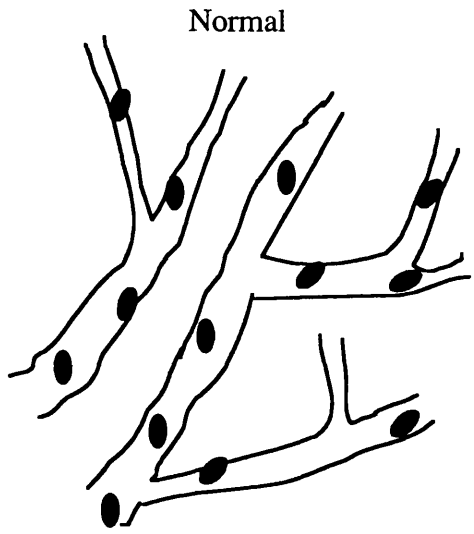
Active targeting: Active targeting relies on the selective localisation of a ligand via interaction with a cell-specific receptor. Active targeting can be achieved at three levels (Duncan, 1992). First order targeting is organ specific i.e. to the lungs or liver. Second order targeting is cell specific and the drug is localised within specific cells of the organ e.g. to hepatocytes or Kupffer cells in the liver. Third order targeting involves delivery of a drug to a specific compartment inside a cell e.g. nucleus in the case of gene delivery.

Passive targeting: Passive targeting relies on the natural distribution pattern of a drug carrier *in vivo* to deliver the drug to a particular tissue or organ (Illum and Davies, 1985). It occurs as a result of the physical or chemical characteristics of the carrier. Examples of passive targeting include particles (>7 μm) trapped in the lung after i.v. administration and tumour targeting of the polymers and liposomes by the enhanced permeability and retention effect (EPR effect).

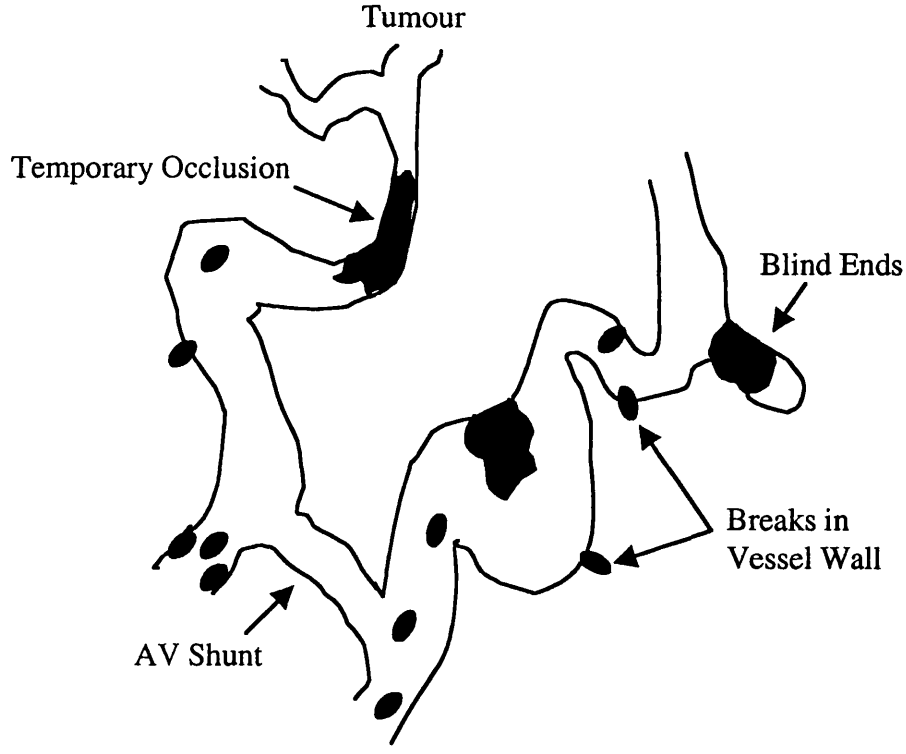
1.2 Passive tumour by the EPR effect

As tumours develop they grow new blood vessels by the process of angiogenesis (reviewed by Folkman and D'Amore, 1996). Tumour vasculature differs from that found in normal tissues as the vessels are disorganised and the vessel wall is incomplete (Kuruppu *et al.*, 1997; Skinner *et al.*, 1990) (Figure 1.5). It is accepted that the leaky vasculature of many solid tumours preferentially allows macromolecules and particulate drug carriers (including liposomes) to escape and this leads to a significant increase in targeting to tumours (Brown and Giacca, 1998) called the passive accumulation of macromolecules in tumours; the 'EPR' effect (Figure 1.6) (Matsumura and Maeda, 1986). The EPR effect concept can be explained by comparing the distribution of low molecular weight anticancer drugs and that of polymer-drug conjugates. After administration, low molecular weight drugs rapidly distribute randomly into both normal and tumour tissues. In contrast, higher molecular weight polymer-drug conjugates can only enter by endocytosis and thus circulate until they enter the leaky tumour vasculature. There they extravasate and they are retained in the tumour tissue due to the absence of lymphatic drainage in the tumour.

Figure 1.5 Differences in normal and tumour vasculature (adapted from Brown and Giaccia, 1998)



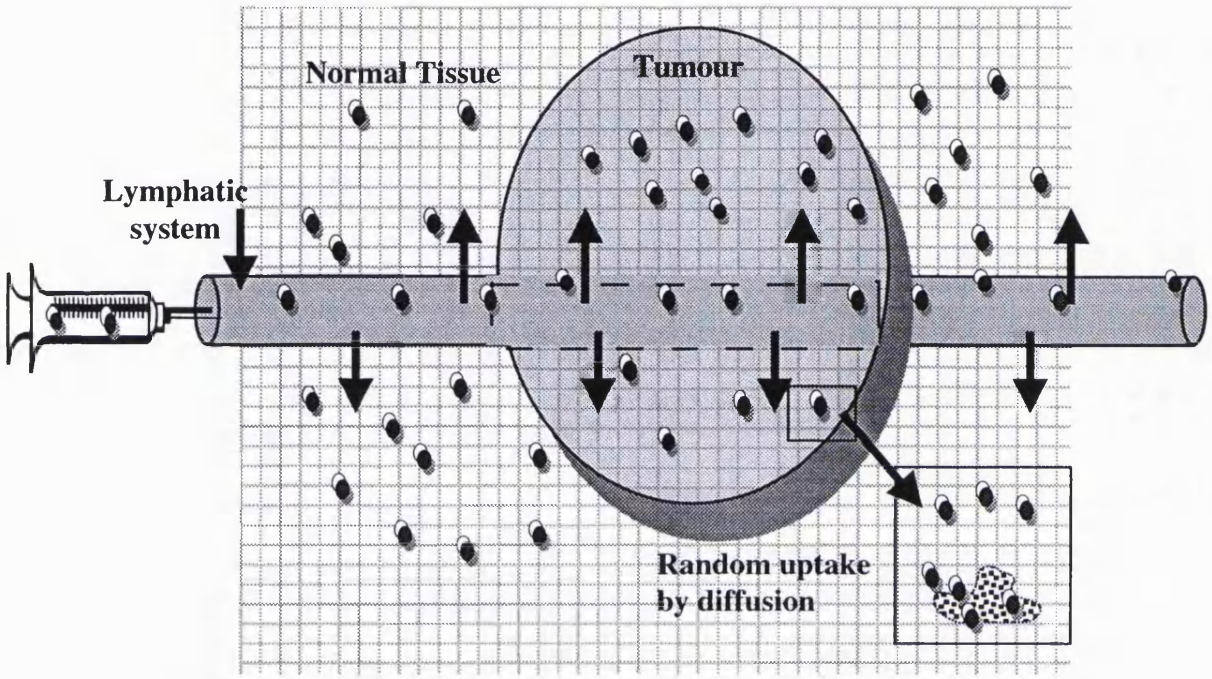
Well ordered blood flow is seen in the normal vasculature with uniform vessels with are close together to allow oxygenation of all tissues.



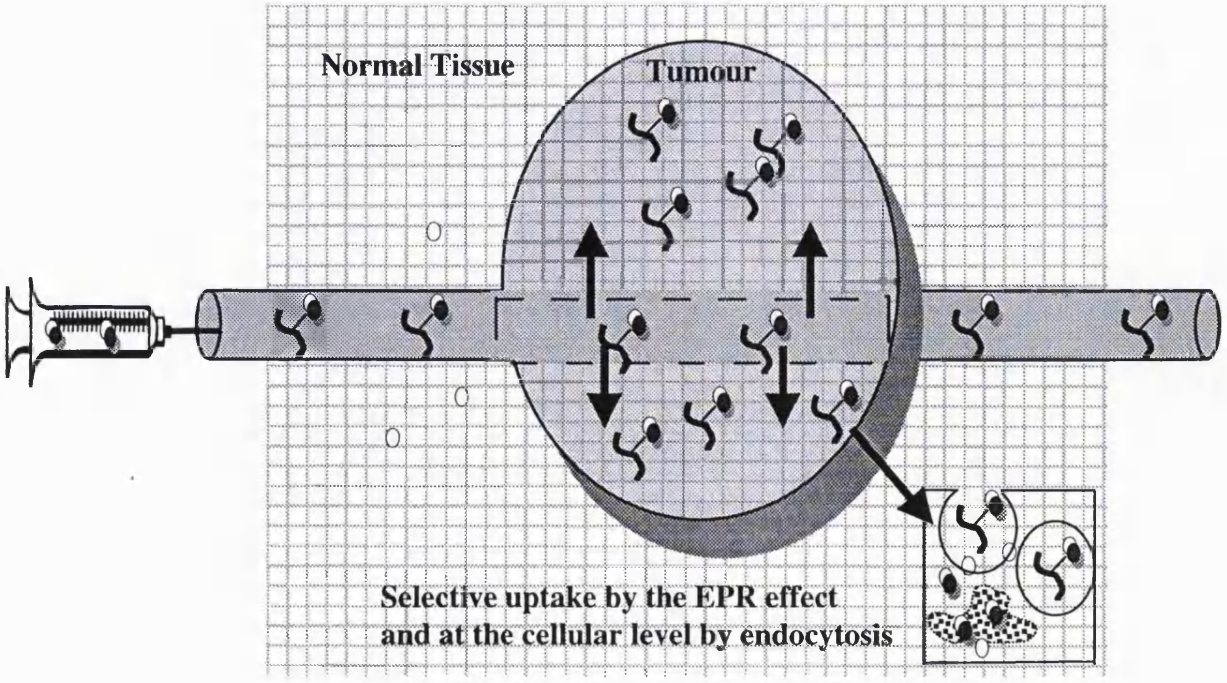
Tortous blood vessels are seen with incomplete Vessel walls and sluggish irregualr blod flow there are regions of hypoxia between the vessels.

Figure 1.6 Schematic representation of the enhanced permeability and retention (EPR) effect (adapted from Duncan, 1999)

A) Tumour and cell distribution of low molecular weight antitumour agents



B) Tumour and cell distribution of high molecular weight antitumour agents



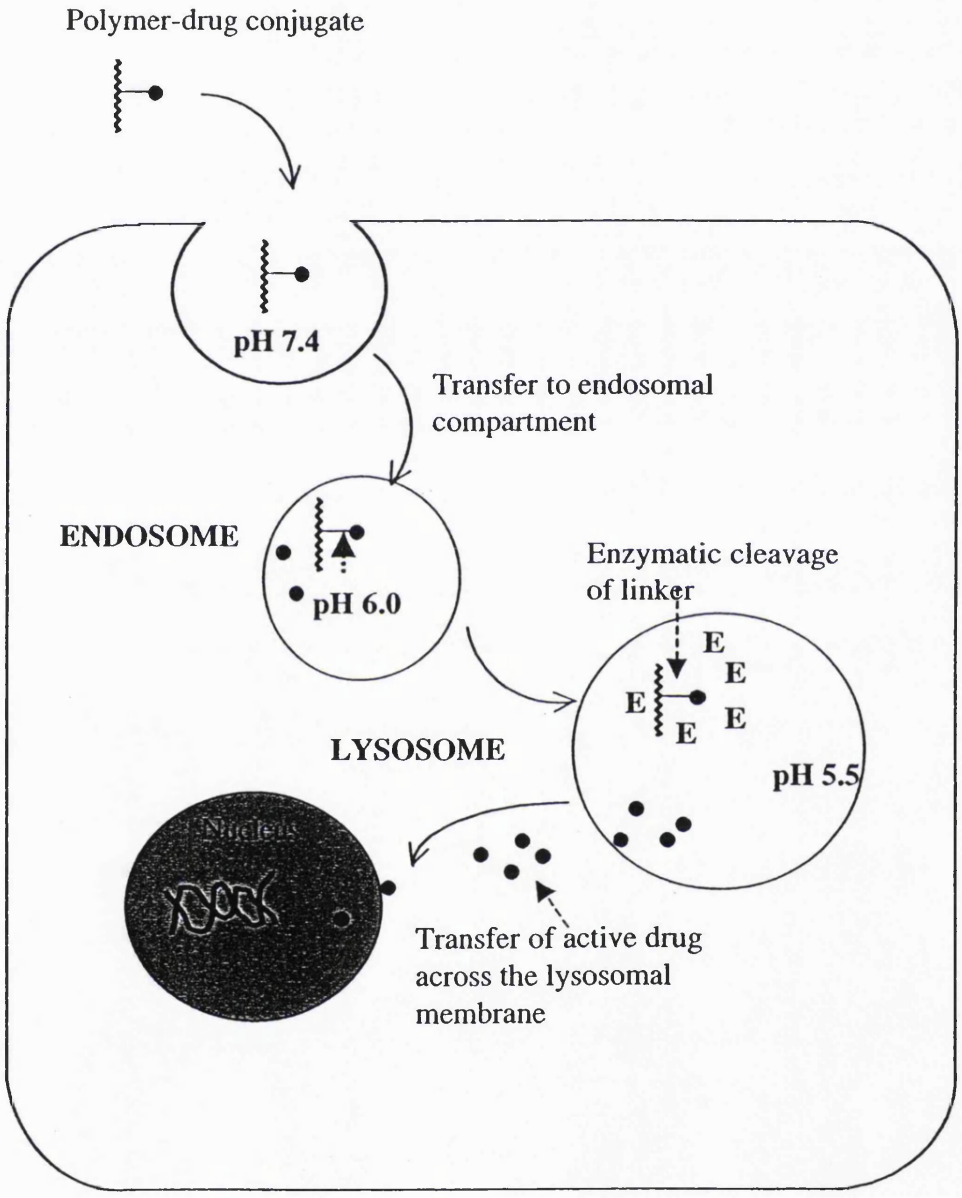
Many factors have now been studied which govern tumour targeting by the EPR effect. Polymer architecture is important (Malik *et al.*, 2000) and the tumour size is also important. Sat and Duncan, (1999) showed that polymer conjugates accumulation is greater in smaller tumours. Seymour *et al.*, (1994) showed that HPMA copolymers of Mw of 10, 000–800, 000 Da accumulate equally well in B16F10 and sarcome 180 tumours. Blood clearance (polymer concentration in the blood stream) was shown to be the most important factor driving tumour uptake. Hyperpermeability of tumour vasculature in different tumour types has been described (reviewed Maeda, 1991; Maeda, 1994).

1.3 Endocytosis

Once present in the extracellular space drug delivery systems must often gain access to the cells' interior. This is achieved by the process known as endocytosis. Endocytosis is the process by which extracellular material is internalised into the cell (Okamoto, 1998) and all eucaryotic cells exhibit one or more forms of endocytosis (reviewed in Mellman, 1996). The term 'endocytosis' has been used to encompass two different mechanisms of uptake. They are phagocytosis and pinocytosis. Phagocytosis (cell eating) involves engulfment of particulate matter (> 0.5 μm diameter) triggered by interaction of the particle with the surface of specialised cells known as phagocytes (Greenberg *et al.*, 1990, 1991).

Pinocytosis (cell drinking) is an uptake pathway common to all cell types and it involves the continuous internalisation of the plasma membrane, extracellular fluid and the solutes dissolved there in. Macromolecular drug conjugates cannot pass through cell membranes and therefore can only enter the cell by pinocytosis (Figure 1.7). During pinocytosis the cell membrane invaginates forming a membrane-bounded vesicle that entraps extracellular fluid, substances in solution and those adhering to the cell surface. After pinching off, the pinocytic vesicle migrates into the cytoplasm fusing with other vesicles to enter the endosomal compartment. Finally, fusion with the lysosomes occurs to enter the secondary lysosome. Once inside secondary lysosomes, the

Figure 1.7 Schematic representation of endocytosis (adapted from Duncan, 1992)



polymeric backbone or the polymer-drug linker can be cleaved if they are biodegradable so that the drug, which is released, passes across the lysosomal membrane and enters the cell. Drug conjugates that enter the cell via this pathway are termed lysosomotropic.

1.4 Polymeric drug carriers

Polymers that have been used as drug carriers include natural polymers (e.g. dextrans), synthetic polymers (e.g. polyethylene glycol (PEG), N-(2-hydroxypropyl methacrylamide (HPMA) copolymers) and pseudosynthetic polymers like polyaminoacids. Synthetic polymers have the advantage that their structure can be tailor-made in respect of size and charge and they can be synthesised to introduce appropriate functional groups for drug conjugation. In addition, synthetic polymers can be robust and therefore they are stable during storage, they can be less immunogenic, and they are often easier and cheaper to produce in large quantities with high purity.

However it is essential that the biocompatibility of novel synthetic polymer carrier is proven. The main disadvantage of those synthetic polymers in routine clinical use e.g. PEG and HPMA copolymers is the lack of biodegradability of the polymer backbone which limits their use to molecular weights lower than the renal threshold (approximately 40, 000Da). To be selected as a drug carrier any polymer must have the following characteristics:-

a) Water soluble and easy to synthesise and have a narrow molecular weight distribution
b) Functionality for drug attachment by linkages that are stable in transit but release drug at the target site
c) Ability to target the required cell types
d) Biocompatible within the biological environment i.e. non toxic and non antigenic
e) Preferably, the polymer should be biodegradable or it must be eliminated from the organism after it has fulfilled its function

Many biological properties of a polymer-drug conjugate are governed by its

Table 1.1 Definition of Mw and Mn

	Definition	Alternative Form	Methods used for measurements
Average Number (Mn)	$\frac{\Sigma_i N_i M_i}{\Sigma_i N_i}$	$\frac{\Sigma_i W_i}{\Sigma_i (W_i M_i)}$	Osmotic pressure and other colligative properties. End group analysis
AverageWeight (Mw)	$\frac{\Sigma_i N_i M_i^2}{\Sigma_i N_i M_i}$	$\frac{\Sigma_i W_i M_i}{\Sigma_i W_i}$	Light scattering sedimentation velocity
Polydispersity	$\frac{Mw}{Mn}$		Gel permeation chromatography

molecular weight. Polymers are characterised by a molar mass distribution identified as the number average molecular mass (Mn) and the weight average molecular mass is defined as (Mw). Mw is always greater than or equal to Mn (Table 1.1). The spread of molar mass in the distribution is described by the polydispersity. This is determined by dividing the Mw by Mn. If Mw/Mn = 1 then the polymer is monodisperse in terms of Mw. Typically PEG has a polydispersity of approximately 1, HPMA copolymer conjugates have a polydispersity of 1.2–1.5 and natural polymers (e.g. dextran) typically display broad polydispersity of 2-5.

1.5 Polymeric drug delivery systems

Over the last two decades a number of polymer-based drug delivery systems have been developed for clinical use. Two distinct technologies have been used. Polymeric implants made from biodegradable polymeric rods, discs and microparticles designed to allow controlled drug release. In contrast, water soluble polymers have been used as drugs and drug-conjugates and as polymer-proteins conjugates. In this case the polymers or conjugates are administered parenterally by injection usually by the i.p. or i.v. routes, and this family of polymeric compounds have been called "Polymer Therapeutics" by Ruth Duncan (Duncan *et al.*, 1996, Duncan, 1997).

Implants: Following implantation, these insoluble depot formulations remain in the tissue (outside the cell) allowing the drug to be released over time (typically over 1-3 months). They have the advantage of delivering a high dose locally or allowing controlled release over several months. The use of polymer implants however, has some disadvantages. The implant maybe large requiring implantation by operation. Once implanted or injected, dose adjustment of the drug is not possible. After completion of the treatment non-biodegradable polymeric implants have to be removed Examples of depot formulations include the biodegradable polyanhydride polymer matrix, Gliadel®, containing carmustine formulation which is implanted in the brain and used to treat malignant glioma (Vattonen *et al.*, 1997; Weingart and Brem, 1996). Another example is Zoladex® a polylactic-co-glycolic acid rod implanted s.c. beneath

the abdominal wall to provide a 28 day controlled release which is widely used to treat prostate and breast cancer.

Polymeric drugs and drug-conjugates: A number of polymeric drugs have been progressing through the various stages of clinical trials and some have already been approved by Regulatory Authorities as drugs in their own right. For example, the polypeptide Copaxone® is approved for the treatment of multiple schlerosis (Comi *et al.*, 1998, 1999). As mentioned above, D-2-S is showing potential as an anti-HIV-1 treatment and dextrin is used as a peritoneal dialysis solution (see Table 1.2).

The concept of using water soluble polymers as drug carriers was first proposed in the mid 1970's by Helmut Ringsdorf (Ringsdorf, 1975). Ringsdorf proposed a model (Figure 1.8) which combined the concepts of the magic bullet, polymeric carriers and lysomotropic drug delivery. He suggested that a water soluble polymer could be covalently bound to a drug via a biodegradable linker that would be able to deliver the drug to the desired site at the optimal rate. Since the 1970's, interest in the use of the water soluble polymers as drug carriers has grown and many polymer conjugates have been developed to achieve longer circulation times (protein conjugates), drug targeting and local controlled release (Monfardini and Veronese, 1998). Examples of polymeric-drug conjugates developed as anticancer agents are given in Table 1.3.

1.6 Polymer Therapeutics

As already mentioned earlier the term "Polymer Therapeutics" (Duncan *et al.*, 1996) includes polymeric drugs (in which the polymeric backbone shows biological activity), polymeric micelles (Kabanov *et al.*, 1994; Kataoka *et al.*, 1997), polymer-drug conjugates and polymer-protein conjugates (Figure 1.9). The styrene-co-maleic-anhydride-neocarzinostatin conjugate called SMANCS and the (Maeda, 1991: reviewed in Maeda 1994) PEG-L-asparaginase conjugate (reviewed Nucci *et al.*, 1991; Duncan and Spreafico, 1994) were the first polymer-conjugates to be approved for routine clinical use as anticancer natural polymer conjugates. This study focuses on two natural

Table 1.2 Examples of polymers in the clinic

Polymeric drug	Treatment	References
Copaxone® (synthetic copolymer glatiramer acetate	Multiple sclerosis	Comi *et al.*, 1998,1999 Arnon , 1996 Blumhort, 2000
Extraneal® (7.5 % Icodextrin)	Alternative to glucose in peritoneal dialysis in chronic renal failure	Hosie *et al.*, 1999 Joel *et al.*, 1999
Dexemel® (4 % Icodextrin)	Alternative to glucose in peritoneal dialysis in chronic renal failure	Gilbert, 1999

Figure 1.8 Schematic diagram of a soluble polymeric drug carrier (modified from Ringsdorf, 1975)

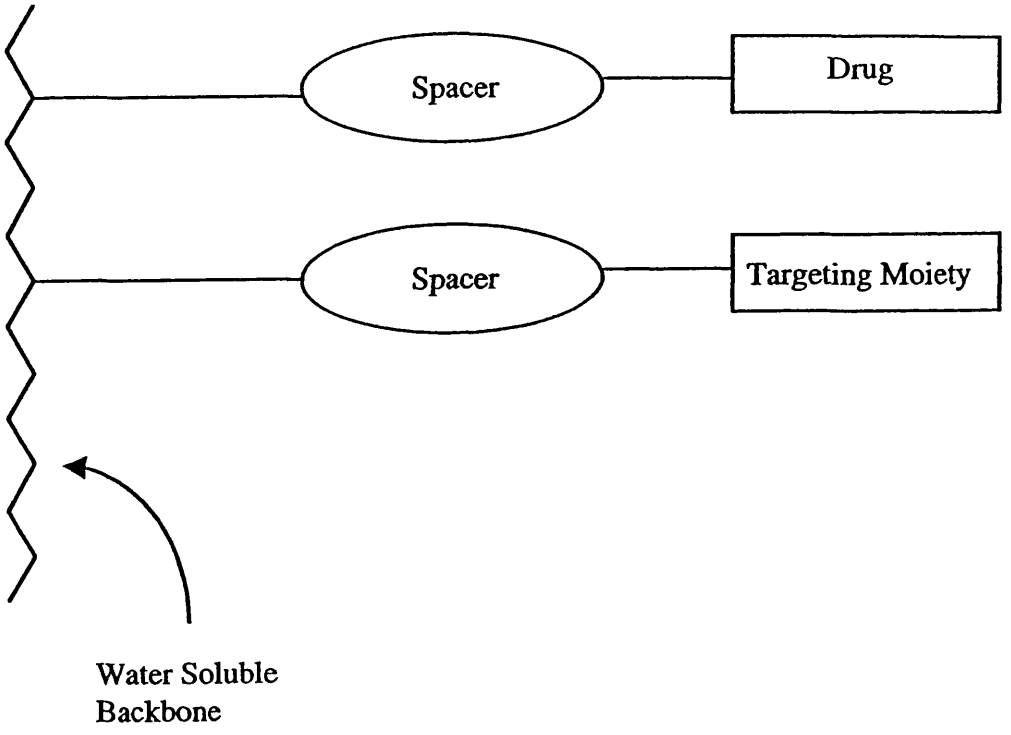


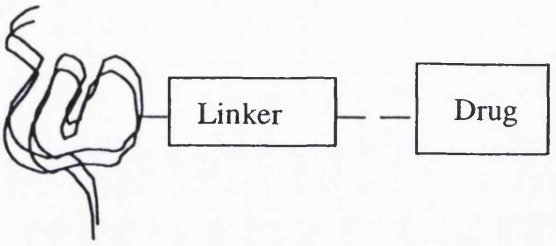
Table 1.3 Examples of polymer conjugates developed as anticancer agents

Polymer	Drug	Reference
HPMA copolymer	Taxol	Mongelli *et al.*, 1994
		Pesenti *et al.*, 1995
	5-Flourouracil	Putman and Kopecek, 1995
	Melphalan	Duncan *et al.*, 1991
	Doxorubicin	Cassidy, 2000
		Vasey *et al.*, 1999
	Daunorubicin	Duncan *et al.*, 1988
PEG	Doxorubicin	Senter *et al.*, 1995
	5 - Fluorouracil	Nichifor *et al.*, 1996, 1997a, 1997b
Alginates	Doxorubicin	Al-Shamkani and Duncan, 1995
		Nomura *et al.*, 1998
Chitin	Cisplatin	Suzuki et al., 1993
Dextran	Mitomycin C	Takakura *et al.*, 1987
	Doxorubicin	Danhauser-Rield *et al.*, 1993
	Naproxen	Azori *et al.*, 1996
	Inulin	Baudys *et al.*, 1998
	Tacrolimus	Yura *et al.*, 1999
	Uricase	Fujita *et al.*, 1990

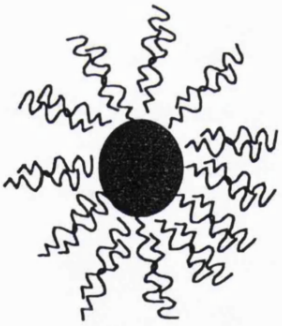
Figure 1.9 Schematic representation of "Polymer Therapeutics" (modified from Duncan, 1999)

Polymeric drug -
e.g. Copaxone, D-2-S

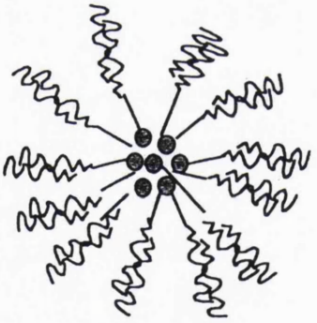
Polymer-drug conjugate
e.g. PK1 and PK2



Polymer-protein conjugate
e.g. PEG-enzymes



Polymeric micelle



polymers, D-2-S and dextrin. Both are currently being used as inherently active polymers but they too have potential for further development as polymer-drug carriers. Dextrin and D-2-S are polysaccharides so this is the family of polymers will be reviewed in more detail.

1.7 Polysaccharides

Polysaccharides are naturally occurring polymers which differ in terms of the repeating monosaccharide units, the nature of the O-glycosidic bond and also the degree of branching (Figure 1.10).

Most polysaccharides are:

a) Water soluble

b) Biodegradable by various enzymes

c) Biocompatible

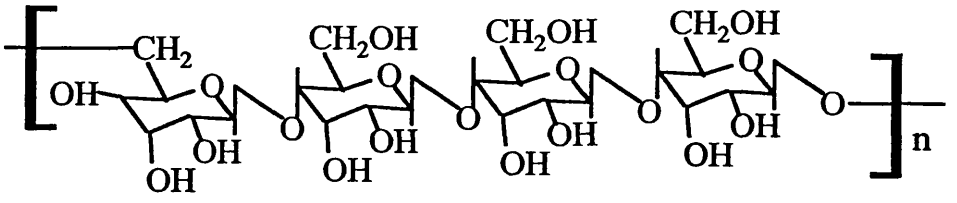
d) Stable over a wide range of temperatures and pH's (although acid hydrolysis is frequently seen).

Most polysaccharides are water soluble or can be easily transformed to make them so (Schacht *et al.*, 1987). Polysaccharides can be chemically modified by alkylation, acylation and phosphorylation and this versatility means that they are ideal candidates for use as drug carriers.

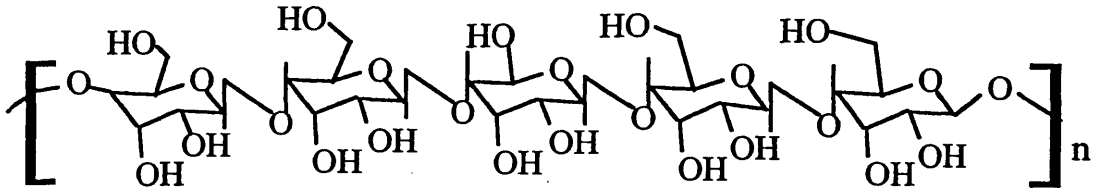
Chitosan and Chitin – Chitin is found in crustacean skeletons (crabs and shrimps), zooplankton and filamentous fungi (cell walls). However, chitin is insoluble in water and most ordinary solvents which has restricted its use to biomaterial applications (Chandy and Sharma, 1990; Muzzarelli *et al.*, 1992). Chitin shows wound healing properties (Kifune, 1992) and this property has lead to its development for use in wound dressings and absorbable suture threads. Chitosan in contrast is rarely found in nature, but commercially is easily derived from crustacean chitin by deacylation with strong alkali. Chitosan is used in a wide range of applications; adhesives, additives in food processing, paper and textiles additives, wound healing additives, waster-water treatment, and to form films and matrices for controlled release delivery (Golomb *et al.*,

Figure 1.10 Structures of various polysaccharides

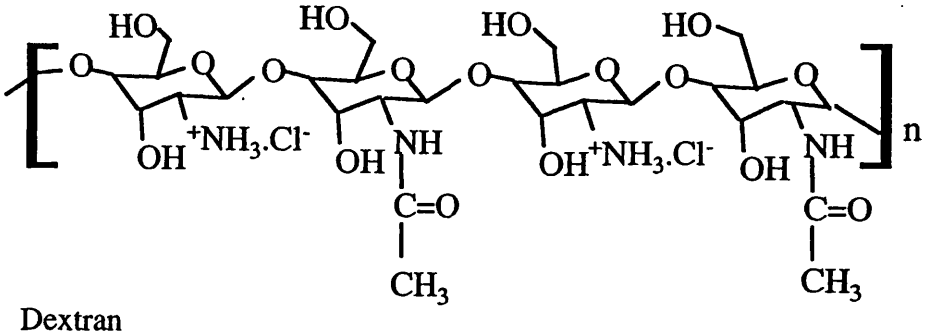
Pullulan



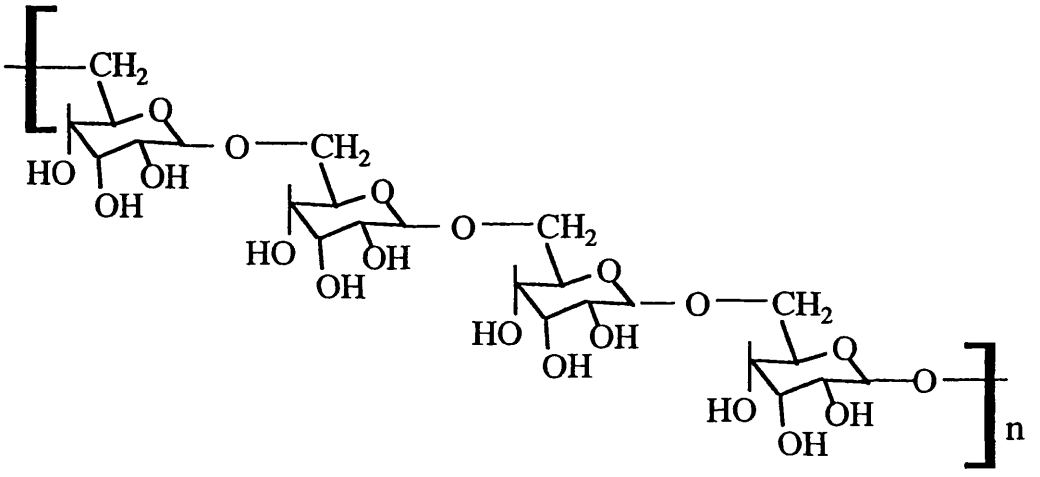
Cellulose



Chitosan



Dextran



1992).

Pullulan:- This polysaccharide is derived from a fungus that is responsible for the deterioration of paint and wood. Pullulan production has been commercialised by Hayashibara Biochemical Laboratories in Japan using batch culture with starch as a carbon source. The polymers produced vary in Mw from under 100, 000 Da to above 5 million Da depending on the culture conditions used. In Japan, pullulan is used as a food additive and a coating material. Due to its biocompatibility and hydrophilic nature pullulan has also been used to prepare the controlled release systems and water soluble conjugates (Tabata *et al.*, 1996).

Xanthan Gum- Xanthan gum has general approval in the USA and European Community for use as a food additive (E-415) and as an excipient in oral controlled release formulations. It produces a viscous layer around tablets through which drugs diffuse. Matrix systems have also been produced (Dumoulin *et al.*, 1993).

Dextran:- Dextran is a bacterial polysaccharide that is degraded very slowly by mammalian enzymes. It became commercially available in 1959 and it is produced by Pharmacia Fine Chemicals, Uppsala, Sweden. Low molecular weight dextrans can be obtained by hydrolysis of the natural dextran product. Native dextrans typically have molecular weight range of 50,000 Da to 5 x 10^8 Da, but the dextran used as a blood expander has a molecular weight of between 50, 000 and 100, 000 Da.

Dextran has been used as a drug carrier in many different ways. It can act as a shield protecting the active moiety against chemical or biological degradation and after chemical modification (Schacht *et al.*, 1987; Mocanu and Carpov, 1996) it can be developed as a drug carrier. Dextran has the advantage that the polymer does not accumulate within the body after systemic administration as it is biodegradable. However, it degrades slowly and after chemical modification its' degradation is even slower (Vercauteren *et al.*, 1992).

The polysaccharides described in Section 1.9 have been used in a variety of applications (Table 1.4).

1.8 Peritoneal Dialysis

Peritoneal dialysis (the word comes from die- through, lucie – to loosen) is the commonest form of support therapy in the UK for patients with chronic renal failure (Goldsmith *et al.*, 2000). CAPD is an alternative to haemodialysis. The number of patients was 35, 000 patients in 1987 and 11 % were receiving dialysis (Gokal, 1994). Glucose remains the only osmotic agent that is universally available in patients undergoing peritoneal dialysis therapy (Ho-Dac-Pannekeet *et al.*, 1997). However, fructose, sorbitol, xylitol, glycerol and amino acids have also been studied as substitutes for glucose. Use of fructose produced hypertriglyceridemia, sorbitol gave rise to hyperosmolarity and fluid retention (Yutue *et al.*, 1967) and use of xylitol was limited by side-effects such as lactic acidoses (Bazzato *et al*, 1982). Only glycerol (Heaten *et al.*, 1986) and amino acids (Goodship *et al.*, 1987) have had long term clinical use in patients with CAPD. Anatomical and functional derrangement of the peritoneum often occur with the use of dialysis solutions (Chaimovitz *et al.*, 1994) and there has been increasing research into alternative agents. Many compounds of different molecular weight have been examined with very disappointing results (Gokal *et al.*, 1996, 1997). Some of the problems associated with these approaches are listed in Table 1.5. The success of a peritoneal dialysis lies in the peritoneum acting as a dialysing membrane (de Fizter *et al.*, 1994). Small molecular size agents are rapidly absorbed through the visceral peritoneum membrane, this very short ultrafiltration (UF) time. UF is maximal at the onset of dialysis and falls in response to a declining gradient. Studies have shown improved ultrafiltration when using compounds of higher molecular weight derived from corn starch for example dextrin (Mistry *et al.*, 1985 and Mistry *et al.*, 1987).

Higher molecular weight glucose polymers produce sustained ultrafiltration by a mechanism resembling colloidal osmosis even when the dialysate osmolarity is retained within physiological range, (Mistry, 1987).

Table 1.4 Polysaccharides used in drug delivery

Polymer	Use	Reference
Chitin and derivatives	Drug delivery matrices as a filling for bone defects	Maeda *et al.*, 1995
	Artificial skin in the form of non woven fabric. Wound healing effects	Kifune *et al.*, 1992
	Partially deacetylated chitin in self regulated drug delivery systems	Pangburn *et al.*, 1984
Dextran	Blood plasma substitute	Kaplan *et al.*, 1994
	Antithrombolytic agent	Thoren, 1981
Inulin	Nasal delivery of insulin	Aspenden *et al.*, 1996
Chitosan	Excipient in controlling drug release in oral formulations	Kristh *et al.*, 1993
	Film coating materials	Miyazokuet *et al.*, 1990
Alginates	Used as immunomodulators	Otterlei *et al.*, 1991
	Alginates posses antitumour activity *in vivo*	Espevik *et al.*, 1993 Wright and Suden, 1993
	Alginate-dox conjugates have shown such antitumour activity	Al-Shamkhani and Duncan, 1995

Dextran was found to be unsuitable for peritoneal dialysis due to its insolubility, allergenicity and peritoneal toxicity (Gokal *et al.*, 1994). Polyanions, polycations and gelatin all display problems associated with allergenicity and peritoneal toxicity. In contrast, Icodextrin has been shown to be safe and effective as a peritoneal dialysis agent (Mistry, 1994a and c, Wilkie, 1997 and Mistry and Gokal, 1994b). Human albumin might be the ideal osmotic agent but it is not used due to its very high manufacturing costs.

1.9 Icodextrin (Extraneal ™, Baxter Healthcare Inc: ML Laboratories PLC)

Icodextrin a high molecular weight (Mw of 20, 000 Da and Mn of 6, 500 Da). The starch-derived dextrin (α,1-4 glucose polymer) is degraded by α-amylase in the body to maltose (Alsop, 1994). The fate of Icodextrin following i.p. administration depends on the following factors (Davies, 1994):-

a) The rate of degradation of the polymer in peritoneal fluid

b) The degree of absorption into the systemic circulation

c) Renal and metabolic elimination from the systemic circulation

Extraneal ™ is a 7.5 % w/v solution of Icodextrin now available in 24 European countries and it is used by approximately 20 % of dialysis patients (Gilbert, 2000) for the treatment of chronic renal failure. When used in continuous ambulatory peritoneal dialysis (CAPD) patients Extraneal produces ultrafiltration for 12 h (Mistry *et al.*, 1994a) by the process of colloidal osmosis (Mistry *et al.*, 1997). In contrast, glucose solutions maintain ultrafiltration for much shorter periods of time and act by the mechanism of crystalloid osmosis.

Icodextrin use has been extended to include patients who have lost ultrafiltration (Stein *et al.*, 1994) and in this case Icodextrin peritoneal dialysis displays increase in solute clearances and fluid recovery (Posthuma *et al.*, 1997). Retention of Icodextrin in the peritoneal cavity occurs due to the absence of amylase from the peritoneal cavity.

Icodextrin (7.5 % w/v solution) with nitruprusside (a nitric oxide donor) has also been used to increase ultrafiltration and peritoneal transport during long CAPD dwells

Table 1.5 Osmotic agents studied in CAPD patients (adapted from Gokal, 1996;De Fijter *et al.*, 1994)

Agent	Mw (Da)	Disadvantage
Low molecular weight agents		
Dextrose	182	Hyperlididaemic, obesity and hyperinsulinaemia
Fructose	182	Hyperosmolarity and hypertriglyceridemia
Sorbitol	182	Hyperosmolarity and retention
Xylitol	152	Lactic acidosis and hyperosmolarity
Amino acids	100 - 120	Short lived ultrafiltration
Glycerol	92	Short lived ultrafiltration
Higher molecular weight agents		
Dextran	60 000 - 90 000	Ultrafiltration and peritoneal bleeding in rats
Polycations and anions	40 000 - 90 000	Cardiovasculature instability and peritoneal bleeding
Gelatin	29 000 – 390 000	Prolonged half life and immunogenicity
Polypeptides	2000 – 20 000	Immunogenicity

Douma *et al.*, 1997). The adequacy of the combination therefore is increased with the nitric oxide donor causing vasodilation. The i.p. administration of nitroprusside during a 4 h dwell with glucose (1l) increased the effective peritoneal surface area leading to an increase in glucose adsorption. The study has been repeated using Icodextrin (7.5% w/v solution) with an 8 h exchange time and Icodextrin was well tolerated by CAPD patients with improved peritoneal when nitroprusside was added to the 7.5 w/v% Icodextrin (Douma *et al.*, 1997).

Although it has been suggested that Icodextrin can cause hypersensitivity reactions (Fletcher *et al.*, 1998) the epitope responsible in this has not as yet been identified. In less than 5 % of patients Icodextrin may cause allergic reactions with exfoliation and/or blistering (Goldsmith *et al.*, 2000). This is a rare event and is probably due to a Type 1 hypersensitivity reaction.

1.10 Icodextrin - Based Products

There are currently three other Icodextrin-based products being used in different clinical applications (ML Laboratories).

Dexemel® is a 4 % w/v solution of Icodextrin which produces a long lasting reservoir of fluid for local drug delivery in the peritoneal cavity or for drug delivery to the lymphatic system or liver. It is administered into the peritoneal cavity where it maintains a relatively constant fluid reservoir for 24-36 h (Gilbert *et al.*, 1999). Clinically, Dexemel® has been used to deliver anticancer agents locally for the treatment of cancers such as ovarian and colorectal carcinoma (Joel *et al.*,1999).

Two Phase 1/11 trials using Extraneal™ as a carrier vehicle after i.p. administration have been described in these studies the anticancer drug 5-Fluorouracil (5-FU) was administered to patients with advanced abdominal malignancies but with normal renal function (Kerr *et al.*, 1996). The delivery of a chemotherapeutic agent via the i.p. route offers potential advantages over intravenous (i.v.) administration as exposure to ascites and other peritoneal diseases is maintained over longer periods of time and local drug delivery to the tumour could be improved.

Adept™ is also a 4 % w/v solution of Icodextrin which is increasingly being used in abdominal operations. It is administered in conjugation with standard surgical incision (laparotomy) or key hole surgery (laparoscopy) to prevent post-operative adhesion formation. Adept™ remains resident in the peritoneal cavity for periods of 96 h and works by drawing fluid into the peritoneal cavity over time. The solution is ultimately absorbed and metabolised by the body like all other Icodextrin solutions.

Adept™ has been shown to inhibit the formation of post-surgical adhesions when compared to the buffered saline solutions commonly used in surgical procedures. It is now in the market. A solution of Icodextrin is currently being evaluated for the prevention of postoperative adhesions. Preclinical studies have shown reduced adhesion formation at both surgical and non-surgical sites (Verco *et al.*, 1999a, 1999b).

1.11 D-2-S

There has been considerable interest in the anti-viral activity of sulphated polysaccharides since it was found that dextran sulphate and heparin block infection of HIV-1 *in vitro* (Baba *et al.*, 1988). Shaunak *et al.* (1994) also showed that sulphated polysaccharides such as D-2-S block infection of T-cell lines by laboratory adapted strains of HIV-1. Intraperitoneal administration of D-2-S was designed to allow targeting to abdominal lymph nodes (AIDS virus resides and replicates there) and studies have been undertaken to evaluate this theory (Shaunak *et al.*, 1998; Shaunak *et al.*, 1999). D-2-S was the first sulphated polysaccharide to be used clinically via the peritoneal route in an attempt to target an antiretroviral agent directly to the lymphatic system.

D-2-S is synthesised from dextrin, and sulphation at the 2 or 6 position results in much better anti-HIV-1 activity compared to sulphation at the 3 position. (Shaunak *et al.*, 1994). In the first clinical trial D-2-S was administered to AIDS patients in a carrier solution of 7.5 w/v % Icodextrin. D-2-S was administered daily for 28 days via a catheter into the peritoneal cavity. The treatment was well tolerated up to the maximal daily dose of 150 mg tested. The total cumulative dose administered to patients was 3.4-4.3 g and the total dose absorbed was approximately 1.7-2.0 g. It was found that

intraperitoneal administration of D-2-S reduced the replication of HIV-1 in patients with AIDS (Shaunak *et al.*, 1998). Observations in these clinical trials have also shown that D-2-S has the ability to treat Kaposi's sarcoma tumours (Shaunak *et al*, 1999). A gradual regression of the Kaposi's sarcoma lesions being observed after D-2-S treatment.

More recently, D-2-S has also been formulated as a gel, Emmelle™ for intravaginal application as a potential vaginal virucide (Stafford *et al.*, 1997). A Phase II clinical trial of Emmelle™ is planned to be conducted in South Africa and it will commence in October 2000 (ML Annual Report, 1999)

1.12 Doxorubicin (Dox) and Amphotericin B (AmpB)

In this study Dox and AmpB were chosen as model drugs to attach to evaluate the potential of the polymers as drug carriers. Dox was conjugated to attach to dextrin with the aim of developing a new anticancer drug-conjugate. AmpB was chosen as an antifungal drug for conjugation to both dextrin and D-2-S.

The next sections describe the basic rationale behind the development of these two new polymeric-conjugates. The conjugates are also described in greater detail in Chapter 6 (Dox) and Chapter 7 (AmpB).

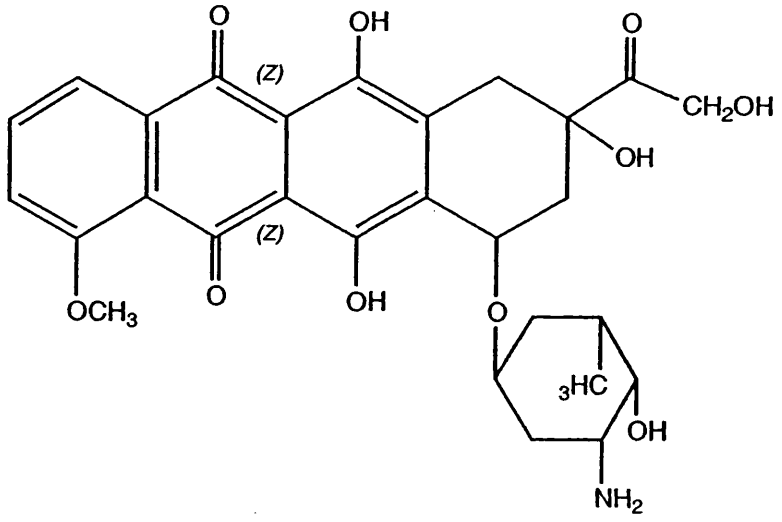
Dox:- The anticancer agents Dox (Mw of 580 Da) belongs to the family of compounds known as anthracyline antibiotics (Mazue *et al.*, 1995) which also includes daunorubicin and epirubicin (Figure 1.11). It is obtained from cultures of *Streptomyces peucetios var caesius.* It consists of a water soluble basic reducing amino sugar linked via a glycosidic bond to the water insoluble tetracylic moiety. This glycosidic bond is sensitive to pH the within range 3-7. Dox-HCl is freely soluble in water, and partially soluble in alcohol and normal saline. Dox has shown a wide range of activity against solid tumours such as sarcomas, carcinomas and melanomas (Silverstric *et al.*, 1970; DiMarco, 1972). In humans wide ranging activity has been shown against both haematological and solid tumour malignancies (Blum and Carter, 1974).

Polymer-Dox conjugates:- Clinical use of Dox is often limited by cardiotoxicity and bone marrow toxicity. In an attempt to improve tumour targeting, Dox has been conjugated to a number of polymers with the aim of reducing toxicity and improving efficacy (reviewed by Brocchini and Duncan, 1999).

PK1 (FCE 28068) is a HPMA copolymer conjugate containing covalently bound Dox (8 wt %) linked via a Gly-Phe-Leu-Gly side chain (Mw 30,000 Da). It is currently undergoing Phase II clinical trials. The HPMA homopolymer was initially developed as a plasma expander and the hydrophilic polymer is captured by cells via pinocytosis (Section 1.3). The peptidyl linker of PK1 was designed to be cleaved by the lysosomal cysteine proteases following cellular uptake (Duncan *et al.*, 1998). Preclinical evaluation of PK1 showed impressive anti-cancer activity against a wide range of tumours and improved solid tumour targeting of Dox due to conjugate capture by the EPR effect (Duncan *et al.*, 1992). PK1 also showed a reduced toxicity compared to Dox alone. Imaging studies using ^{131}I-labelled PK1 were performed in mice at the Queen's Medical Centre in Nottingham (Pimms *et al.*, 1993). Phase I clinical evaluation of PK1 showed some evidence of antitumour activity in chemotherapy refractory patients and a maximum tolerated dose of 320 mg/m^2 equivalence of Dox. PK1 displayed a prolonged plasma retention time compared to Dox, and the PK1 t1/2 α was 1.8 h and t1/2β was 93 h (Vasey *et al.*, 1999). Imaging studies were carried out in patients with ^{131}I-labelled PK1. Uptake of the polymeric-drug conjugate was seen in 6 out of 21 patients and radioactivity was identifiable in head and neck cancers (Vasey *et al.*, 1999; Cassidy, 2000). A Phase II programme is now under way, however, a study in colorectal cancer failed to demonstrate any tumour activity (Cassidy, 2000). In an ongoing study patients with breast and non-small cell lung cancers are receiving PK1 at a Dox-equivalent dose of 280mg/m^2 given every 3 weeks. This is three times higher than the usual clinical dose of Dox alone (Vasey *et al.*, 1999).

A second HPMA copolymer-Dox conjugate PK2 has progressed to clinical testing. PK2 is also an HPMA copolymer-Dox conjugate but also contains galactosamine (2 mol/wt%) as a targeting moiety to promote liver capture (Duncan *et al.*, 1986; Julyan *et al.*, 1999). In preclinical studies administration of PK2 gave rise to

Figure 1.11 Structure of Dox

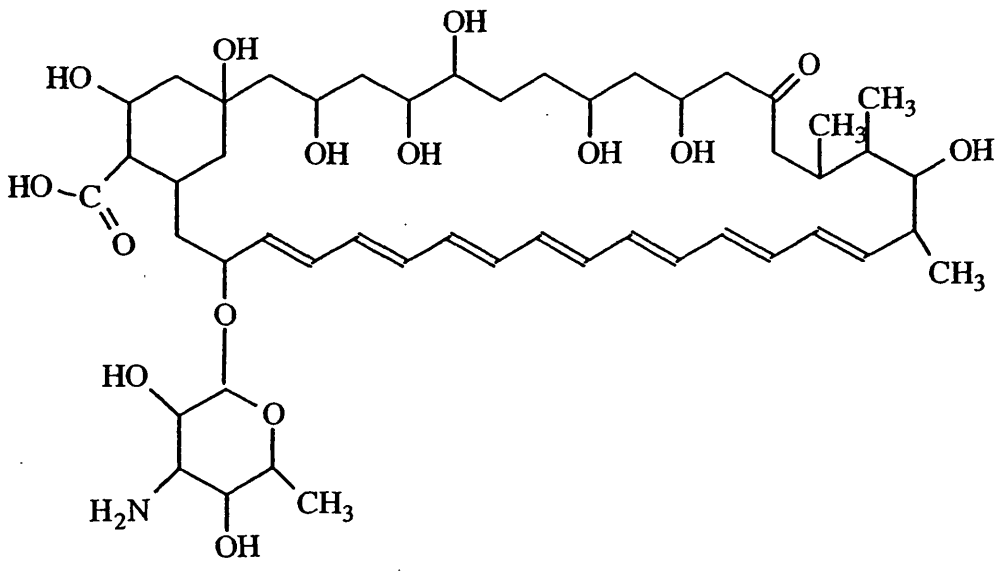


80 % of the administered dose targeting to the liver (Seymour *et al.*, 1991). A ^{123}I-labelled PK2 analogue was used to visualise biodistribution in patients by gamma camera imaging (Julyan *et al.*, 1999). The Phase II clinical study is being carried out in patients with hepatoma and PK2 is administered by short infusion every 3 weeks in a dose of 120 mg/m^2.

Amphotericin B:- Gold and coworkers (1956) were studying a strain of *Streptomyces nodosus,* an aerobic actinomycete from the Orinoco River Valley of Venezuela, when they discovered AmpB. The antibiotic was isolated by Vanderputte and associates in 1956 (Dutcher, 1968). AmpB (Figure 1.12) is a member of polyene macrolide antibiotic family and its structure was determined by Hamilton-Miller (1973) and Kolter-Braztburg *et al.* (1979). AmpB is a heptene macrolide with seven conjugated double bonds in the trans position. The drug has been available for > 30 years and still remains the best choice for the treatment of many severe life threatening fungal infections. These include systemic infections (often caused by aggressive cancer or the antibacterial chemotherapy and long term use of immuno-suppressive agents after organ transplantation) or disseminiated life threatening infections in AIDS patients.

However, AmpB does present problems when used to treat such diseases (Table 1.6). AmpB often induces adverse side effects such as flu-like symptoms (occurs in 50-90% of patients) due to the induction of prostaglandis E2 production by mononuclear cells (Gigliatti *et al.*, 1987). In addition, AmpB causes other toxic effects that can prevent its clinical use (Maddux and Barrier, 1980). Premedication with hydrocortisone can reduce the side effects arising from AmpB infusions (Tynes *et al.*, 1963). AmpB nephrotoxicity has been widely studied and it appears that the drug has a direct vasoconstriction effect on afferent renal arterioles; thus it reduces renal blood flow and consequently glomerular blood flow. A second important problem of AmpB use is the many problems with drug administration. It is virtually insoluble in all aqueous solutions. This causes many problems with the administration of the drug.

Figure 1.12 Structure of AmpB



AmpB is poorly absorbed after oral administration and displays poor availability when given intramuscularly. To improve bioavailabilty and to avoid some of these side-effects (nephrotoxicity, chills, fevers) AmpB is typically given by slow infusion i.v. in a 5 % dextrose solution. Recently, AmpB formulations have been developed that can be administered by other routes. Aerosol administration has been used in patients following bone marrow transplantation to prevent invasive aspergillosis (Beyer *et al.*, 1993). Topical AmpB lotions, creams and ointments have been used to treat cutaneous candidal infections and Amp B lozengers to treat oral candidiasis. To overcome the problems of systemic toxicity, AmpB has been incorporated into liposomes or mixed with lipids. This decreases the risk of both acute and delayed toxicity. It is also interesting to note that AmpB has been added to peritoneal dialysis solutions to assist in the prevention of peritoneal candida infection (discussed in more detail in Chapter 7).

1.13 Aims of this Thesis

The two main objectives of this study were (1) to quantitate the biodistribution of dextrin and D-2-S and (2) to evaluate the potential of these two polymers as drug carriers.

To prepare the probes needed to monitor biodistribution and also to synthesise polymer-drug conjugates it was necessary to add pendant groups to both polymers and standardise the chemistry used. For this purpose the method of succinoylation was chosen (Chapter 3). Subsequently, dextrin- and D-2-S-tyrosinamide conjugates were prepared in order to follow the biodistribution after ^{125}I-labelling of both polymers.

In order to visualise the fate of dextrin and D-2-S using gamma scintigraphy DTPA conjugates were prepared to [^{111}In]Indium labeling. In addition to monitor the fate of both polymers intracellulary biotin was conjugated to the succinoylated intermediate (Chapter 3).

The biodistribution of each ^{125}I-labelled polymer was studied by dissection analysis after s.c., i.v. and i.p. administration to rats (Chapter 4).

Table 1.6 Toxic Effect of AmpB

Type	Effects	References
Infusion Related	Headaches, chills, fevers, muscle joint pain, vomiting, mild insomnia, hypotension and thrombophlebitics	Bennet, 1979; Gigliotti *et al.*, 1987; Sander *et al.*, 1991; Kan *et al.*, 1991.
Nephrotoxicity	Reduces glomerular filtration rates by approximately 40 % after start of therapy. Rise in blood urea and creatinine levels. Tubular lesions. Hypomagnesemia	Medoff and Kabayashia, 1980; Forgan-Smith and Darrell, 1994; Maddus and Barriere, 1980, Barton *et al.*, 1984.
Haemotoxicity	Normocytic anemia, reversible thrombocytopenia and leukopenia	Hoeprick *et al.*, 1992; MacGregor *et al.*, 1978; Chan *et al.*, 1982 Stamm *et al.*, 1987.
Neurotoxicity	This is very rare but leukoencephalopathy, encephalopathy and parkinsonism	Haber and Joseph, 1962; Devensky *et al.*, 1987; Walker and Rosenblum, 1992
Cardiovasculature	Cardiac arrest with rapid infusion. Hypokalemia and ventricular fibrillation	Hildrick –Smith *et al.*, 1964; Craven and Gremiltion,1985 Aguado *et al.*, 1993

In parallel the biodistribution in the rat was visualised by gamma scintography using ^{111}In-labelled polymers (Chapter 4). As D-2-S is administered i.p. clinically this route was chosen to investigate further the parameters (dose, volume of administration, repeated administration) that might influence the biodistribution of D-2-S *in vivo* (Chapter 5).

Dextrin-Dox was examined as a potential anticancer conjugate. The B16F10 murine melanoma tumour model was chosen to study antitumour activity and tumour targeting. This model had been previously shown to demonstrate the EPR effect (Seymour *et al.*, 1994). First, the biodistribution with ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S was examined in tumour-bearing mice to determine whether there was any evidence of tumour accumulation of the polymers alone. Then dextrin-Dox conjugates (synthesised by D.Hirst; Hreczuk-Hirst *et al.*, 1999) were used to study the pharmacological activity and tumour targeting of an antitumour conjugate (Chapter 6).

Finally, dextrin and D-2-S conjugates containing the antifungal agent AmpB were synthesised and the solubility of free AmpB and the conjugates were compared, and preliminary studies were undertaken (haemolysis and cytotoxic assays) to evaluate biological activity (Chapter 7).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Equipment

The UV visible (UV-vis) spectrometer (UV 1601) was supplied by Shimadzu (Japan). PD10 columns were supplied by Amersham Pharmaceutical Biotech. Scientific Laboratory Supplies (Nottingham, UK) supplied the Ultra-Turrax T20 homogeniser and the Shandon paper electrophoresis tank used for paper electrophoresis. The 96 well spectrophotometer (micro-titre plate reader) was Titertek Multiscan Plus supplied by EFLAB (Finland). The Varifuge 3.0 RS centrifuge was supplied by Heraeus Instruments, (UK). Dialysis membrane was supplied by Medicell Int. Ltd (London, UK) Molecular weight cut off 12-15 kDa. The Cobra™ II Auto-gamma counter was supplied by Packard (Berks, UK).

2.1.2 Animals and Cells

Animals: Bantin and Kingman Ltd. (Hull, UK) supplied the Wistar rats and the C57 black male mice. The rats used in Nottingham were bred on site.

Cells: B16F10 murine melanoma cells were kindly donated by Prof. I. Hart (St. Thomas's Hospital, London, UK). Tissue culture grade dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue and optical grade DMSO were from Sigma (Dorset, UK). Trypsin, foetal calf serum (FCS) and RPMI 1640, were from Gibco BRL Life Technologies (Paisley, UK). The growth media was RPMI 1640 containing 10 % FCS.

2.2 Cell Culture

2.2.1 Cell Bank

Vials containing cells were removed from the –80 °C freezer and placed in a 30 ml sterile universal bottle and left to thaw slowly in the 37 °C incubator. Cells (1 ml) were placed in a 15 cm^3 flask and media (5 ml) was added and left for 24 h. After this the media was removed, the cells were washed with phosphate buffered saline (PBS) and fresh media (5 ml) was added and left for a further 24 h. The cells were then

trypsinised (1 ml trypsin-EDTA) made upto 5 ml in growth media. 1 ml of this was added to media (9 ml, 1:5 split) and placed in a 75 cm^3 flask.

2.2.2 Maintainance of Cell Lines

All cell culture procedures were performed in a Class II laminar flow cabinet. Cells were routinely maintained in a carbon dioxide (CO_2) culture incubator at 5 % v/v CO_2 at 37°C. All the materials used for cell culture were sterile, osmotically balanced and were warmed to 37°C prior to use. B16F10 cells were grown in fresh growth media and they were maintained in 75 cm^2 tissue-culture treated cantered neck flasks with vented (0.2 μm) tops. These cells were passaged twice weekly (1:10 split) in order to keep them in the exponential phase of growth.

2.2.3 Splitting of Cells

B16F10 cells were split as in Section 2.2.1 except for twice washing with PBS (10 ml) and 1 ml of trypsinised cells were taken and added to growth media (9 ml). A 1:10 split was then carried out by taking 1 ml of this cell suspension and adding it to growth media (9 ml) in a new flask.

2.2.4 Evaluation of cell viability/ density using Trypan blue

Cell viability was routinely assessed before use for cell suspensions in specific experiments. Trypan blue (0.4 % w/v in PBS, 20 μl) was added to cell suspension (20 μl) and using a pasteur pipette this mixture was transfered to a Neubauer haemocytometer slide and a glass coverslip added before viewing under the light microscope. Cell density was assayed by counting the number of viable cells (blue (dead) cells and clear (viable) cells) within the counting area. The cell density (number of cells per mL) was determined by multiplying by a factor of 2 x 10^4. The percentage viability was calculated and all cultures used for studies were >98 % viable.

2.3 Polymers and reagents

Polymers: The Dextrin and D-2-S polymers were all were supplied by ML Laboratories

(Liverpool, UK.) and their characteristics are shown in Table 2.1.

Reagents: AmpB, solubilized AmpB (fungizone), biotin hydrazide, dimethylamino pyridine (DMAP), piperidine, L-tyrosinamide, sodium chloride, diethyl triamine pentaacetic acid (DTPA), sodium hydroxide (NaOH) and barbitione buffer (B6632) were purchased from Sigma (Dorset, UK). F-moc, sulpho-NHS were purchased from Pierce (Chester, UK). Succinic anhydride, bromothymol blue, dimethyl formamide (DMF) and carbodiimidazole (CDI) were obtained from Aldrich (Dorset, UK). All general reagents were from BDH (Ontario, Canada) or Sigma (Dorset, UK) and were of analytical grade.

Medical grade oxygen, nitrogen and CO_2 (all 95%v/v) and liquid nitrogen were supplied by BOC (Surrey, UK). Isofluorane was supplied by Abbott Labs (Kent, UK) and Amersham Pharmacia Biotech (Hartfordshire, UK) supplied all of the [^{125}I] iodine and [^{111}In] indium.

2.4 Synthesis and chemical characterisation

2.4.1 Succinoylation of dextrin

In these studies (Chapter 3) succinoylated dextrins were routinely used for conjugation to model compound or drugs. Typically the theoretical modification of dextrin sought was 10 mol % (i.e. 10 % of monomer units to be modified). For example:

Dextrin (1 g, MD15/73, 1.96 x 10^{-5} mol) was dissolved in DMF (10 ml). Succinic anhydride (62 mg, 6.2 x 10^{-4} mol, 10 mol %) was added to DMAP (28.5 mg) which is an agent used to promote acylation reactions. The mixture was purged with nitrogen, sealed and left to stir at 50°C overnight (Figure 2.1a). The reaction mixture was poured onto rapidly stirring diethyl ether (250 ml) and magnetically stirred overnight. The ether was removed by filtration under vacuum and the remaining solid was dissolved in the minimum amount of distilled water and poured into a dialysis membrane (visking tubing, molecular weight cut-off 12-14 kDa). Dialysis was carried out against 2 x 2 l of distilled water. The dialysed product was then freeze-dried. To determine the degree of succinoylation, titration against a standard of NaOH (4.925 x

Table 2.1 Characterisation of dextrin and D-2-S batches

Polymer	Product Code	Molecular weight (Mw) (kDa)	Mn (kDa)	Mw/Mn
Dextrin	MLD5/29	5	6	0.83
Dextrin	DX04/OG	15	6	2.74
Dextrin	MD15/73	51	28	1.84
D-2-S	MLD95/50	25	NA*	NA*
D-2-S	MLD56/74	25	NA*	NA*

NA: Not available

* Molecular weight and values Mn were supplied by ML Laboratories and were estimated by gel permeation chromatography using dextran as a standard.

Figure 2.1a Succinoylation of Dextrin

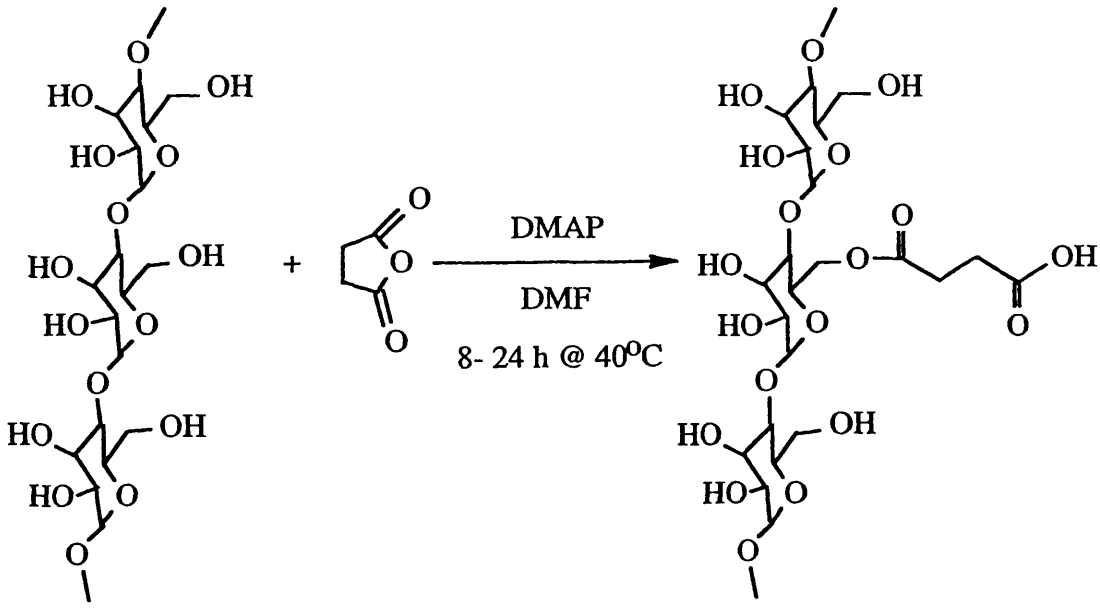
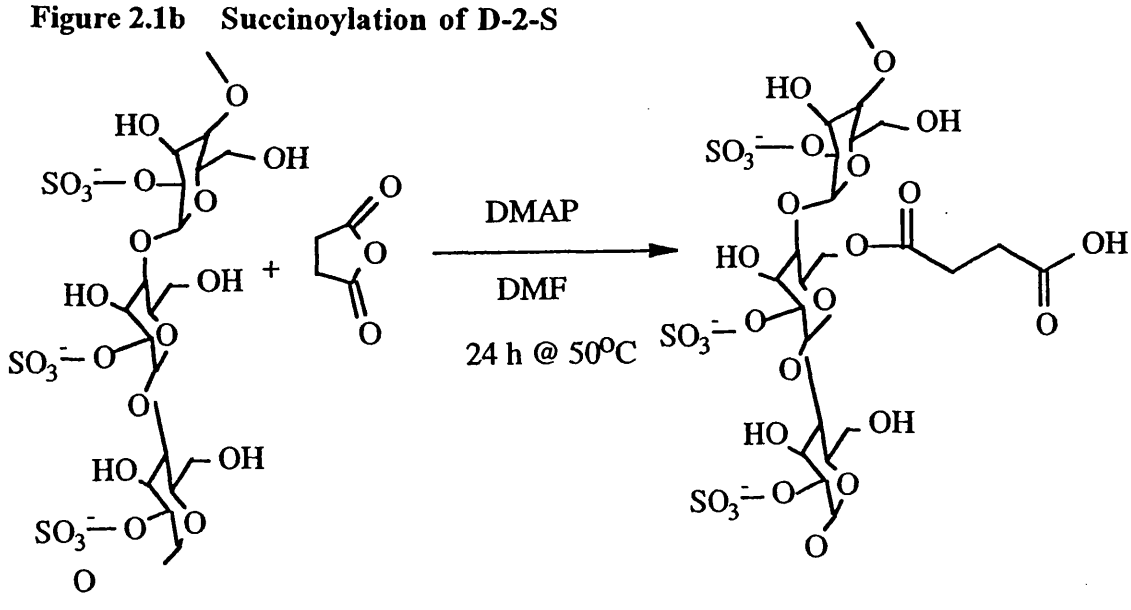


Figure 2.1b Succinoylation of D-2-S



10^{-5} mol) using bromothymol blue as a indicator. Succinoylated dextrin was also characterised by FTIR and ^{13}C or ^{1}H NMR.

2.4.2 Succinoylation of D-2-S

When preparing succinoylated D-2-S typically the aim was to produce a theoretical modification of 1 mol % (1 % of monomer units to be modified). In the case of D-2-S reaction condition used had to be altered from those used with dextrin due to the poor insolubility of D-2-S.

D-2-S (0.5 g, MLD56/74, 2 x 10^{-5} mol) was dissolved in DMF (10 ml). Succinic anhydride (62 mg, 6.2 x 10^{-4} mol) was added followed by DMAP (28.5 mg). The reaction mixture was purged with nitrogen, sealed and left to stir at 50 °C for 48 h. The reaction mixture was then poured onto rapidly stirring diethyl ether (250 ml) and magnetically stirred overnight. (Figure 2.1b). The ether was removed by filtration under vacuum and the remaining solid was dissolved in the minimum amount of distilled water and poured into a dialysis membrane (visking tubing, molecular weight cut-off 12-14 kDa) and purified by dialysis against 2 l of distilled water.

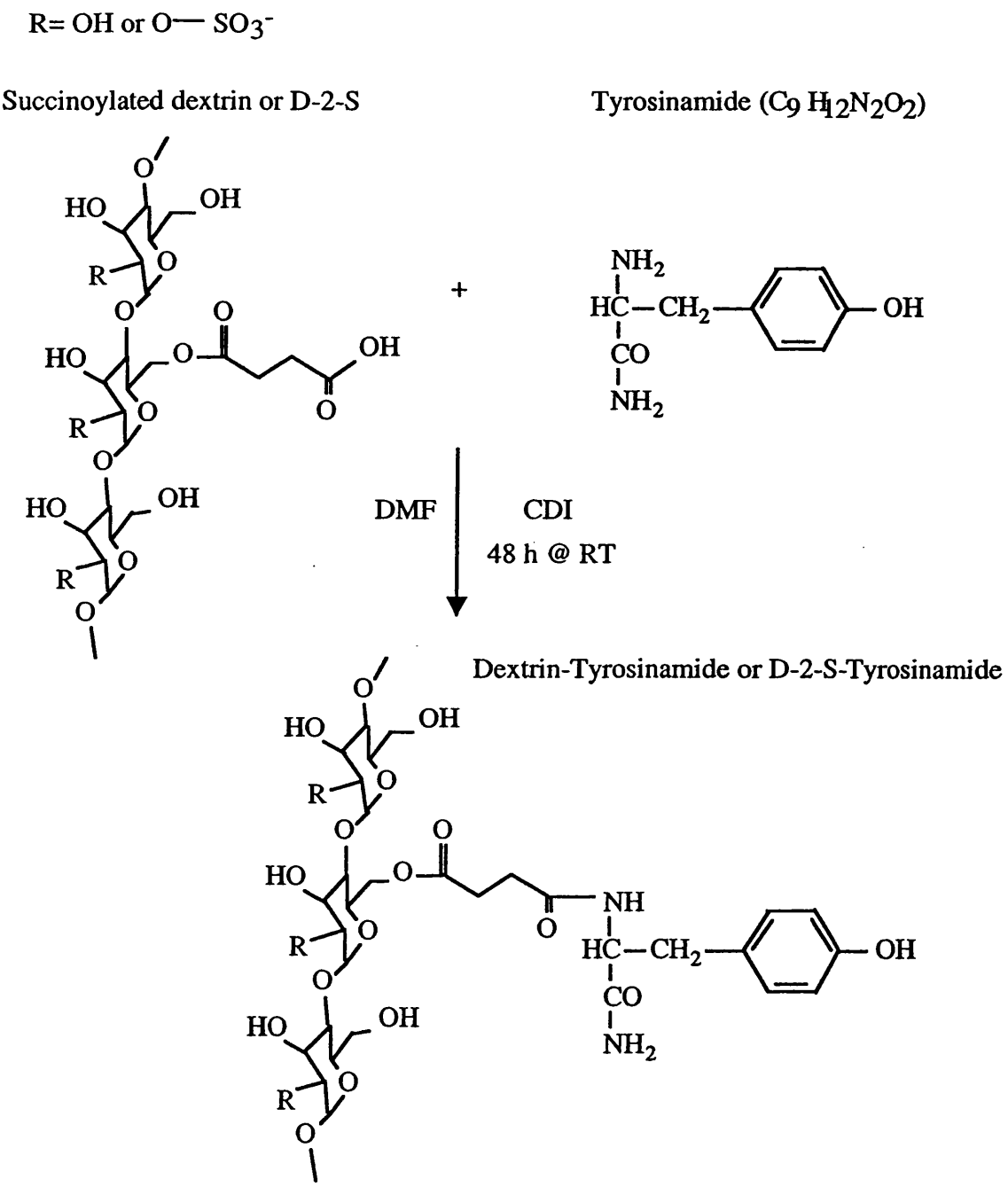
The resulting solution was freeze-dried and the degree of succinoylation was determined by titration against a standard of NaOH (4.925 x 10^{-5} mol).

2.4.3 Modification of succinolyated dextrin and D-2-S with tyrosinamide

In order to radiolabel the products with [^{125}I] iodine (see Section 2.6) it was first necessary to indroduce an aromatic group such as tyrosinamide.

Succinoylated dextrin (57.7 mg, MD15/73, 1 mol %) and succinoylated D-2-S, (57.7 mg, MLD56/74, 1 mol%) were dissolved in DMF (3 ml). To each reaction vessel CDI (10.4 mg, 6.4 x 10^{-5} mol) dissolved in DMF (1 ml) was added. The reaction was allowed to proceed at 25°C under stirring for 1 h. Tyrosinamide (97 mg, 8.96 x 10^{-4} mol) dissolved in DMF (1 ml) was then added and the reaction mixture left stirring at 25°C for 48 h (Figure 2.2). The product was isolated by removal of the DMF using the rotor evaporator. The remaining solid was dissolved in the minimum amount of distilled water and poured into a dialysis membrane (visking tubing, Mw cut-off 12 –14 kDa)

Figure 2.2 Modification of succinoylated dextrin and D-2-S with tyrosinamide



and purified by dialysis against 2 l of distilled water. The product was freeze dried until constant weight was obtained. Incorporation of tyrosinamide was determined by UV-vis spectroscopy and FTIR.

2.4.4 Conjugation of biotin to dextrin and D-2-S

To create probes to allow visualisation of the polymers within cells biotin was conjugated to both polymers.

Succinoylated dextrin (57.7 mg, MD15/73, 1 mol %) or succinoylated D-2-S (57.7 mg, MLD56/74, 1 mol %) was dissolved in DMF (3 ml). To this solution CDI (10.4 mg, 6.4 x 10^{-5} mol) dissolved in DMF (1 ml) was added. The reaction was allowed to proceed at 25°C under stirring for 1 h. Then biotin hydrazide (97 mg, 8.96 x 10^{-4} mol) was dissolved in DMF (1 ml) was added to the reaction, and left stirring at 25°C for 48 h (Figure 2.3). The product was isolated by removal of the DMF using the rotor evaporator. The remaining solid was dissolved in the minimum amount of distilled water and poured into a dialysis membrane (visking tubing, molecular weight cut-off 12-14 kDa) and purified by dialysis against 2 l of distilled water. The product was freeze dried until constant weight was maintained. Incorporation of biotin was determined by the HABA/Avidin assay.

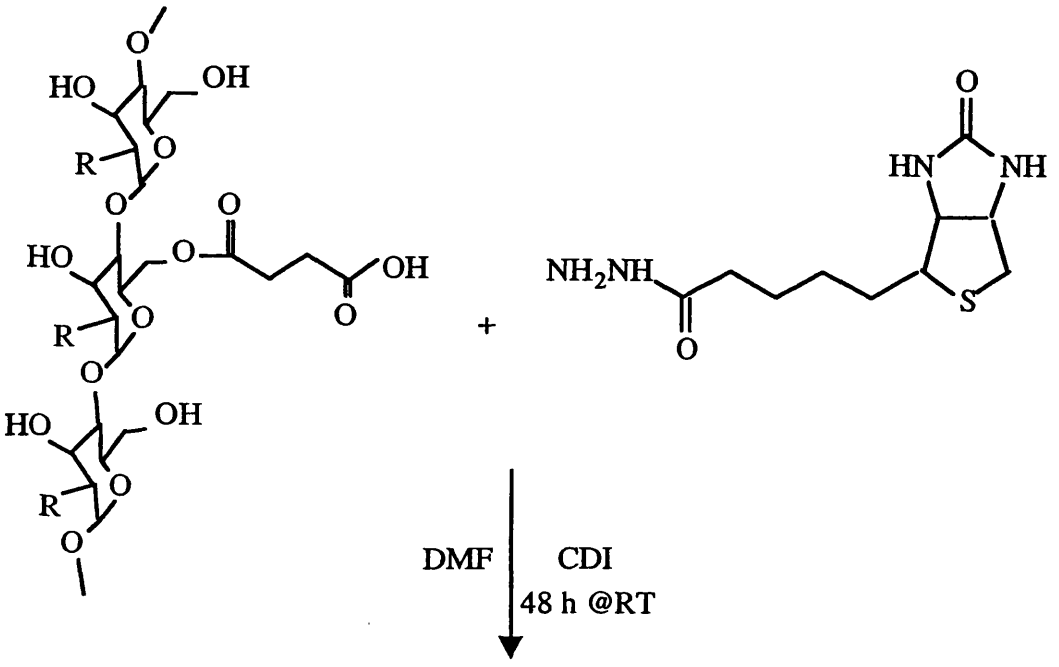
A range of biotin standards from 0 to 2.5 mg ml $^{-1}$ in distilled water were prepared in de-ionised water. A vial of HABA/Avidin reagent was reconstituted with of deionised water (10 ml). In triplicate HABA/Avidin reagent (900 µl) was pipetted into a 1 ml cuvette. To this 100 µl of either standard or sample was added and the cuvette mixed by inversion. Absorbance at 500 nm was measured against a blank of distilled water. A standard curve (Figure 2.4) was constructed and the amount of biotin in the samples was determined from this curve. Finally the weight percent of biotin covalently attached to the polymer was determined.

Figure 2.3 Modification of succinoylated dextrin and D-2-S with biotin hydrazide

R= OH or O— SO_3^-

Succinoylated dextrin or D-2-S

Biotin Hydrazide ($C_{10}H_{18}N_4O_2$)



Biotinoylated dextrin or biotinoylated D-2-S

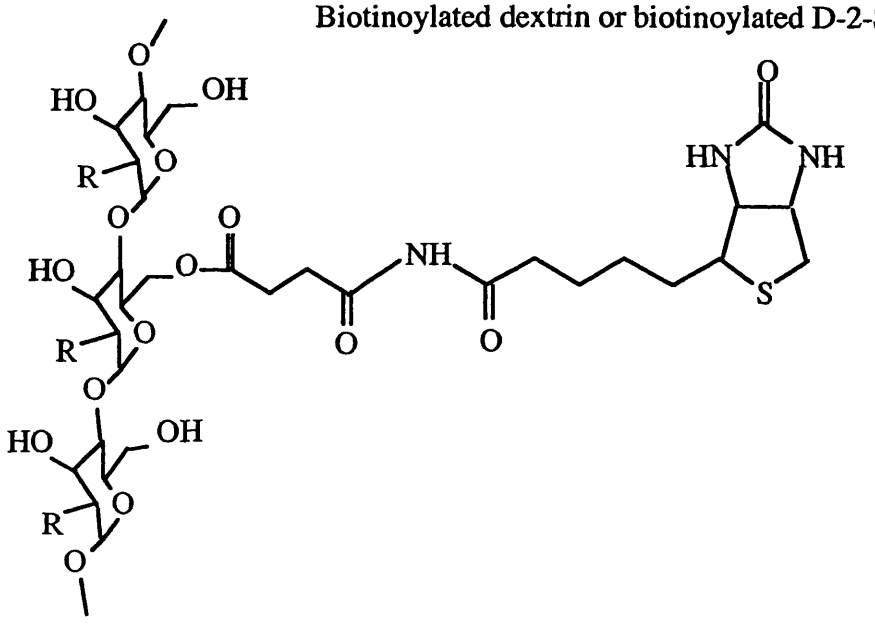
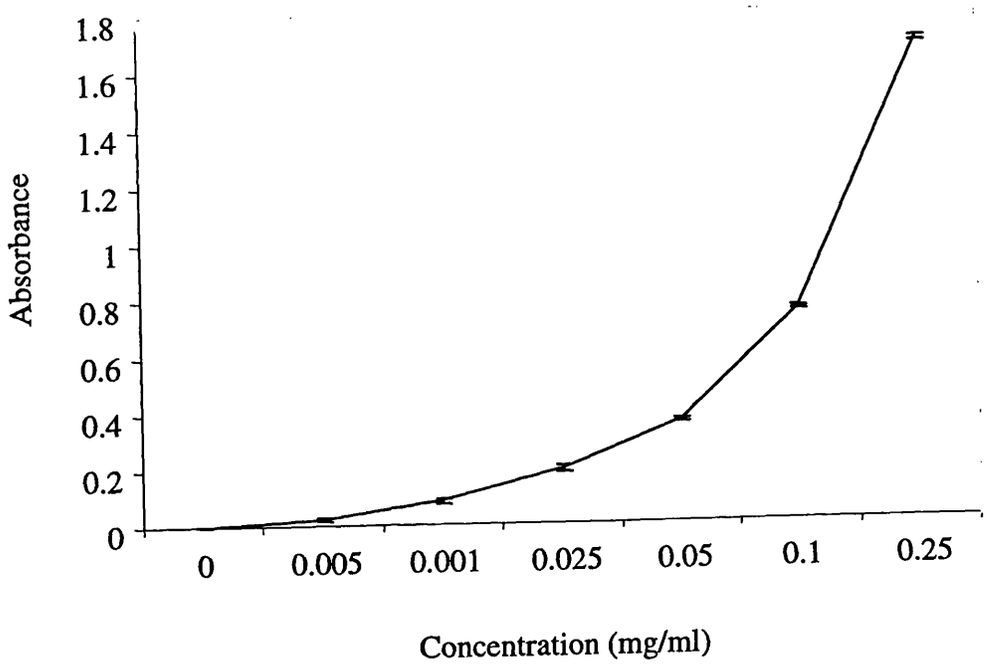


Figure 2.4 Calibration curve for biotin using HABA/Avidin Assay



Concentration	Absorbance	± SD
0.005	0.025	0.005
0.001	0.083	0.007
0.025	0.194	0.010
0.050	0.361	0.004
0.100	0.749	0.004
0.250	1.683	0.006

2.4.5 Conjugation of Amphotericin B to succinoylated dextrin and D-2-S

Succinoylated dextrin (51 mg, 1 x 10^{-6} mol) or D-2-S (2.04 x 10^{-6} mol) was dissolved in 10 ml of DMF. CDI (10.4 mg) was then added and left to react with stirring for 1 h at room temperature. Then AmpB (3 mg) was added, and left at room temperature while gently stirring for 30 min. The product was isolated by removal of the DMF using the rotor evaporator. The remaining solid was dissolved in the minimum amount of distilled water and poured into a dialysis membrane (visking tubing, molecular weight cut-off 12-14 kDa) and purified by dialysis against 2 l of distilled water. The product was freeze dried to constant weight. The reaction is shown in Figure 2.5. The amount of AmpB conjugated to the polymers was determined by UV-vis spectroscopy and FTIR. The UV standard curve for AmpB is shown in Figure 2.6.

2.4.6 Conjugation of diethylenetriaminepentaacetic acid anhydride to succinoylated dextrin and D-2-S

To enable gamma camera visualisation of the pattern of distribution of dextrin and D-2-S it was necessary to conjugate diethylenetriaminepentaacetic acid anhydride (DTPA) to enable chelatin of indium. A typical reaction (addition of DTPA to 1 mol % succinoylated dextrin and 1 mol % succinoylated D-2-S) is described below.

Dextrin: Succinoylated dextrin (204 mg) was dissolved in DMF (10 ml). EDC (4.81 mg, 2 mol) and Sulpho-NHS (3mg, 1.1 mol) were added and the reaction was allowed to proceed at room temperature with gentle stirring for 30 min. Ethylenediamine was added (31 mg, 40 mol) and the reaction mixture then stirred overnight at room temperature. The product was isolated by removal of the DMF using the rotor evaporator. The remaining solid was dissolved in the minimum amount of distilled water and poured into a dialysis membrane (visking tubing, molecular weight cut-off 12-14 kDa) and purified by dialysis against 2 l of distilled water. The product was freeze dried until constant weight was maintained. A solution of DTPA (8.99 mg, 2 mol) in DMF (10 ml) was added to the freeze dried product and left stirring for 1 h (Figure 2.7). The excess DMF was removed and freeze dried as described above.

Figure 2.5 Modification of succinoylated dextrin and D-2-S with AmpB

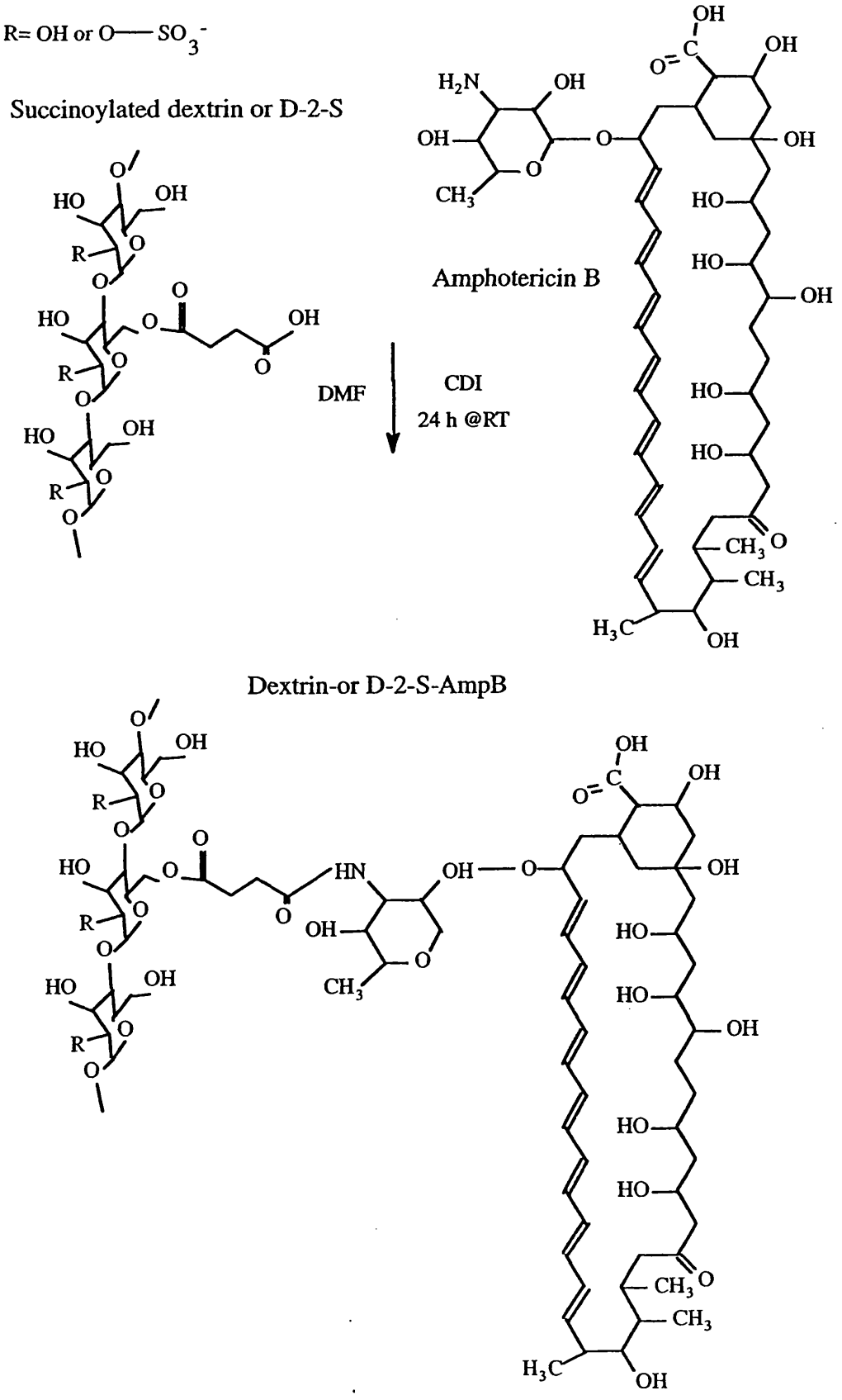
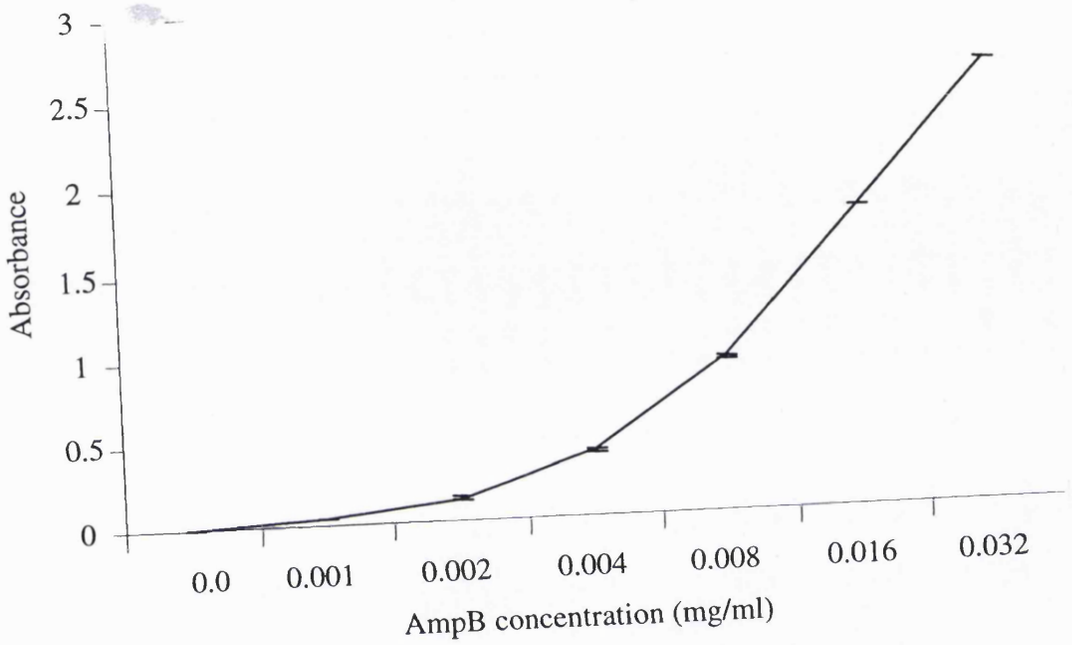
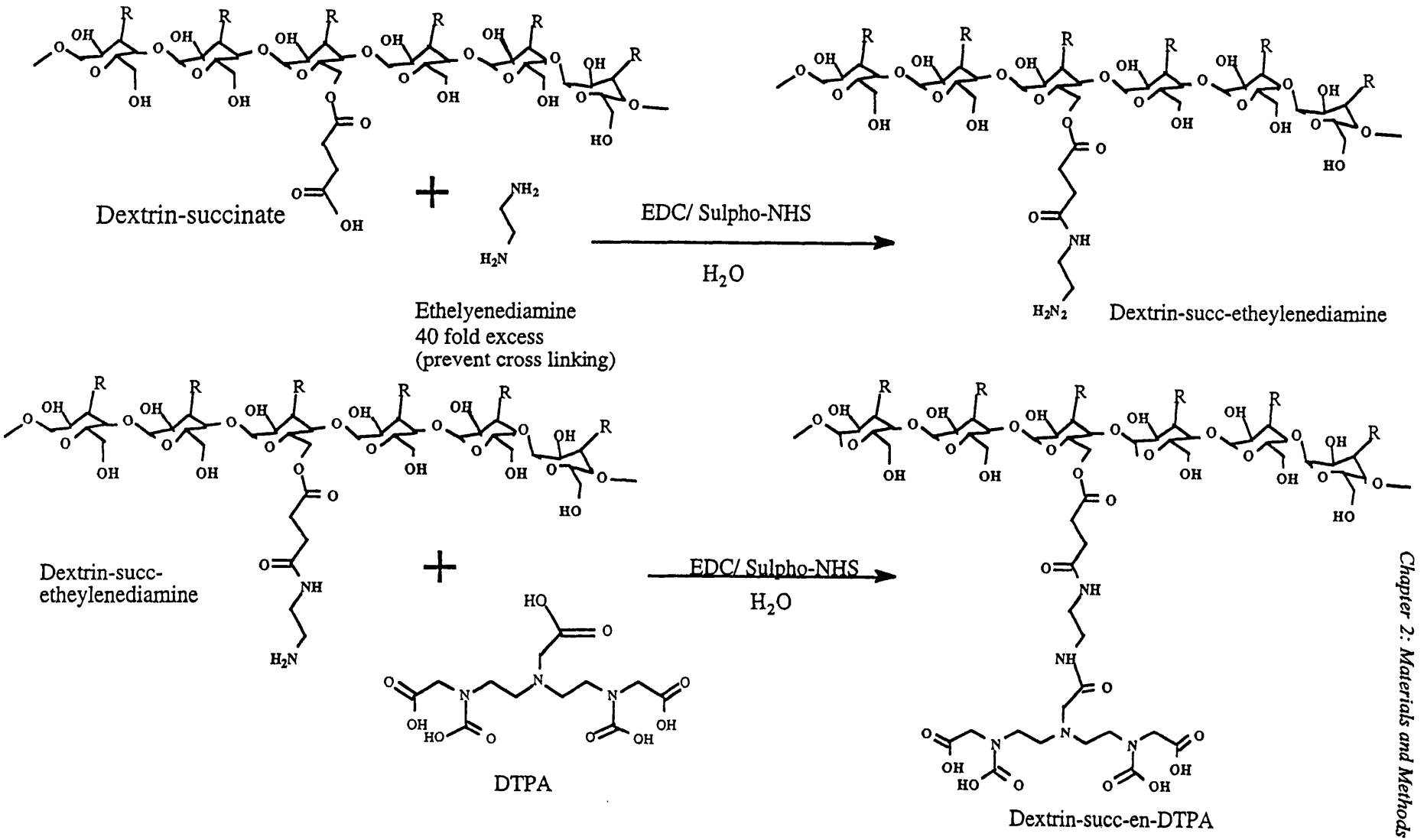


Figure 2.6 Calibration Curve for Amp B using UV-vis spectrometry



Concentration	Absorbance	± SD
0.001	0.042	0.001
0.002	0.134	0.002
0.004	0.385	0.005
0.008	0.905	0.006
0.016	1.751	0.001
0.032	2.581	0.001

Figure 2.7 Reaction mechanism for the modification of dextrin and D-2-S with DTPA



***D-2-S*:** Succinoylated D-2-S (200 mg) was dissolved in DMF (10 ml). As above EDC (9.65 mg, 2 mol) and Sulpho-NHS (6 mg, 1.1 mol) were added and the reaction allowed to proceed at room temperature with gently stirring for 30 min. Then ethylenediamine was added (62 mg, 40 mol) and the reaction stirred overnight at room temperature. The mixture was freeze dried as outlined above. A solution of DTPA (18 mg, 2 mol) in DMF (10 ml) was added to the freeze-dried product and left stirring for 1 h. The excess DMF was removed and the product was dialysed and then freeze-dried. In both cases dextrin-DTPA and D-2-S-DTPA were characterised using FTIR and UV-vis spectroscopy.

2.5 Biological evaluation of dextrin and D-2-S *In Vitro*

2.5.1 Assessment of cell viability using the MTT Assay

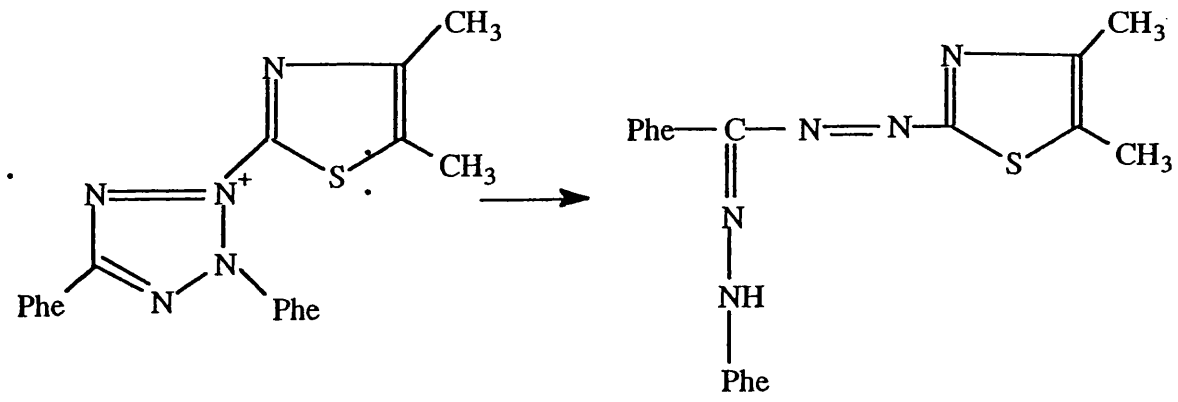
The MTT assay is a rapid colourmetric assay for assessing cell viability. Estimation of cell density also allows determination of a cell growth curve. The basis of this assay is the fact that viable cells are able to reduce the water soluble tetrazonium dye MTT, to a water insoluble coloured formazan salt (Altman, 1976; Sgouras, 1990) (Figure 2.8).

A cell growth curve was constructed for the B16F10 cells (Figure 2.9) by plotting the absorbance against time (days). The growth curve determined the cells in each study were comfined to a exponential phase of cell growth.

Initially, 96 well microtitre plates were seeded with a suspension of B16F10 murine melanoma cells in growth media containing a density of 10^4 cells per well. The cells were then incubated for 24 h. After this time growth media was carefully removed and 100 µl sterile controls and polymers dissolved in growth media (log10 dilutions) were added (n=8) to each plate.

The plates were then incubated for a further 67 h before addition of MTT (20 µl) to each well. After a further 5 h incubation the growth medium was carefully removed and DMSO (100 µl) cell culture grade was added to each well to dissolve the blue crystals of the tetrazolium dye that had formed and the absorbance at 550nm was then read using a microtitre plate reader.

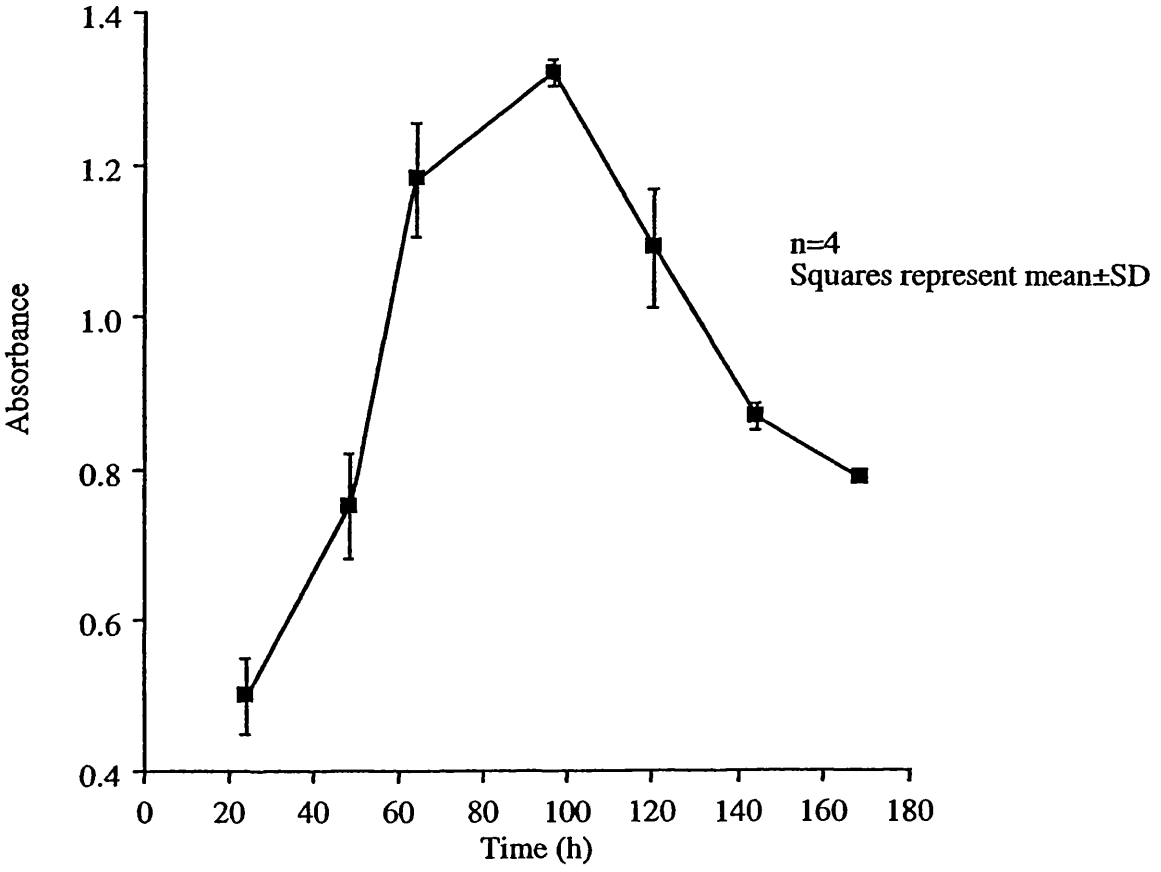
Figure 2.8 Reaction for MTT assay



MTT

MTT Formazan
(purple colouration)

Figure 2.9 Growth curve for the B16F10 murine melanoma cell line



These absorbance values were then converted to % cell viability using the following formula:

$$\frac{A_{550} \text{ treated cells}}{A_{550} \text{ control cells}} \times 100 = \text{Percentage Cell Viability}$$

2.5.2 Red blood cell (RBC) lysis assay

An adult rat (male Wistar) was killed and blood was quickly removed by cardiac puncture. Blood was placed in a lithium/heparinised tube (10 ml) and placed on ice. PBS (2 ml pre-chilled) was added and the diluted blood centrifuged three times (1500 g, 10 min). Each time the supernatant was removed along with the heparin beads. A 2 % w/v solution of RBC was then prepared in PBS. Stock solutions of the polymers or samples to be tested were made in PBS (n=4). To measure 100% haemolysis Triton X-100 (1 % solution) was added. Dextran and polyethyleneimine were used as positive and negative reference polymers respectively. All samples (100 µl) were placed in 96 well micro-titre plate (n=4) and 100 µl of RBC's were added. After the incubation period (1 h or 24 h) the samples were centrifuged (1500 g, 10 min) and the supernatant (100 µl) was pipetted into a 96 well micro-titre plate. The absorbance at 500 nm was measured using a micro-titre plate reader blanked with PBS. The degree of lysis was determined by expression as a percentage of 100% lysis seen using Triton X-100.

2.5.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate changes in cell morphology (B16F10 cells and RBC's) during exposure to the polymer or polymer conjugates.

B16F10 cells (1×10^5 / ml) were grown in a 6 well sterile plate where each well contained a sterile glass coverslips for 24 h. Polymers at various concentrations were incubated over 1 h and 24 h. The cells were examined under the electron microscope after preparation.

Red blood cells isolated as described in Section 2.5.2 were incubated at various concentrations of polymer (0 - 5mg/ml) after 24 h the preparations were transferred

into a microfuge tube and were pelleted by centrifugation at 1500 g for 10 min and the supernatant was removed. Glutaraldehyde (0.25 % v/v, 1 ml) in PBS was added to the cells (to fix them) and were then gently aspirating to re-suspend the cells. After 18 h the cells suspension was centrifuged (1500 g for 10 min) and the supernatant removed. The cells were incubated with osmium tetroxide (1 % w/v, 500 µl) in PBS for 1 h. The cells suspension was again centrifuged (1500 g for 10 min) and the supernatant removed. The dehydration of the cells involved numerous steps each time increasing the amount of ethanol (50 % v/v, 60 % v/v, 70 % v/v, 80 % v/v, 90 % v/v, ethanol and acetone in sterile distilled water for 30 min). After each addition of dehydrating agent the cells suspension was again centrifuged (1500 g for 10 min) and the supernatant removed. After the last drying step hexamethyl disilazane (HMDS) was and cells were then placed on an SEM platform using carbon cement and then gold coated. The samples were examined using SEM.

2.6 Biological evaluation of dextrin and D-2-S and their conjugates *In Vivo*

2.6.1 Body distribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S.

To monitor the fate of dextrin and D-2-S it was necessary to introduce an aromatic residue that would allow radioiodination. The synthesis of dextrin-tyrosinamide and D-2-S-tyrosinamide is described in Section 2.4.3. The products were radioiodinated using the Chloramine T Method (Maniartis *et al.*, 1989). Chloramine T (n-chloro-p-methyl-benzenesulphonamide) is an oxidising agent used to iodinate proteins/polymers that have a tyrosine residue. The Chloramine T oxidises [^{125}I] iodine and generates the cation $^{125}I^{+}$, which is incorporated into the aromatic ring of the tyrosine by electrophilic substitution. This occurs with the aromatic ring at the ortho position of the benzene ring. The reaction is terminated by sodium metabisulphate which reduces the Chloramine T and any free $^{125}I^{+}$ cation.

2.6.2 Radioiodination of polymers using the Chloramine T method

Dextrin-tyrosinamide or D-2-S-tyrosinamide (5 mg) were dissolved in 0.1 M phosphate buffer pH 7.4 (0.5 M disodium orthophosphate (Na_2HPO_4) and 0.5 M

sodium dihydrogen orthophosphate, (NaH_2PO_4, 0.5 ml)). Sodium [^{125}I]iodide (5 µl, 500 µCi) was then added and the mixture left stirring for 2 min to equibrilate. Before use the viability of the Chloramine T reagent (2 mg/ml in phosphate buffer pH 7.4) was tested by placing a drop of the solution a crystal of KI. The Chloramine T reagent turned brown showing oxidising activity. The Chloramine T solution (75 µl) was then added to the reaction mixture and this was left stirring for 15 min. To stop the reaction sodium metabisulphate (500 µl, 2 mg/ml in phosphate buffer pH 7.4) was added and also a crystal of KI to displace free [^{125}I] iodine. The crude reaction mixture was left for 2 min before removal of an aliquot (5 µl) to allow determination of the labelling efficiency of the preparation.

The reaction mixture was placed into dialysis tubing (molecular weight cut-off 12-14 KDa) and dialysed against 1 % NaCl (to remove any free iodine) until no radioactivity (<20 cps) was found in the dialysate. The purity, labelling efficiency and specific activity of the final labelled products were determined by paper electrophoresis.

2.6.3 Determination of the purity of radioiodinated polymers by paper electrophoresis

Three 5 cm x 30 cm strips of Whatman chromatography paper were cut to size and the central portion divided into 5 mm strips by pencil lines (40 strips). The fifth strip was marked as the point for sample application. Barbitone buffer (Sigma B6632) was used to soak the chromatography paper which was then blotted dry. The same buffer was placed in the paper electrophoresis tank (Shandon) and the paper strips put in place across the supporting bars. The sample application was placed nearest the anode. As a reference control, 4 µl of free Na[^{125}I]iodide was loaded onto the first strip. For each polymer the crude and purified preparations 4 µl were loaded onto individual strips. The Shandon tank was switched on and the samples were run at 400 V for 30 min. The paper strips were removed and the marked 5 mm strips were cut out and placed into Luckmans tubes containing 1 ml of water and assessed for radioactivity

using a gamma counter. The results were plotted as counts per minute against distance migrated.

2.6.4 Body distribution of ^{125}I-labelled-polymers in Wistar rats and s.c. B16F10 tumour-bearing C57 mice.

All animal experiments were performed according to Home Office Guidelines, and when involving tumour models, to the UKCCCR guidelines.

Evaluation of body distribution of body distribution in Wistar rats: Male Wistar rats weighing between 200-250 g were lightly anaesthetised using isoflurothane and the polymer (100 µl, 5 x 10^5 counts per minute (cpm)) was injected intraperitoneally (i.p.), subcutaneously (s.c.) or intraveneously (i.v.) via the tail vein. After injection the rats were killed the specified time points (each time point was repeated in triplicate). A blood sample was taken and the rat weighed. The major organs were removed and homogenised to a known volume. Samples of homogenate (1ml) were assayed for radioactivity. The total amount of radioactivity per organ was expressed as a percentage of the injected dose.

Evaluation of body distribution in tumour bearing mice: Mice were lightly anaesthetised using isoflourane (2 %) with oxygen (4 %). Viable B16F10 cells (1 x 10^5 cells) in saline (100 µl) were injected s.c into the back of the neck. When the tumour reached a palpable size usually 10 - 12 days after injection ^{125}I-labelled-polymers (100 µl, 5 x 10^5 cpm) was injected i.p. or i.v. into the tail vein of the C57/BL mice. The mice were killed at various time points (each time point was repeated in triplicate). A blood sample was taken and the mouse weighed. The major organs were removed and homogenised to a known volume in water. Samples (3 x 1 ml) of each tissue were taken and assayed for the radioactivity and the total amount of radioactivity per organ was expressed as the percentage of the injected dose and as radioactivity per gram of organ.

2.6.5 Pharmacology of dextrin-Dox

Twenty four black C57 mice were injected with 10^5 viable B16F10 murine melanoma cells. When the tumours reached palpable size (50 mm^2) measured by the product of two orthogonal diameters they were randomly assigned into groups of six and their tumours measured with a micrometer gauge. The tumour size and the weight of each mouse was recorded. Each group was then injected i.p. with either sterile saline (-ve control), free Dox (5 mg/kg) in sterile saline or dextrin-Dox (11.8 wt% Dox, 34 mol%) at either 5 mg/kg or 10 mg/kg, on days 0, 1 and 2. The mice were monitored daily and tumour size and the body weight was recorded. Once the tumour area exceeded 2.89 cm^2 the mice were culled according to the UKCCCR guidelines. Mouse survival was expressed as % T/C (test/control (saline)).

2.7 *In Vivo* studies using ^{111}Indium-labelled dextrin and D-2-S

2.7.1 Radiolabelling of dextrin and D-2-S using [^{111}In]indium

To label dextrin-DTPA and D-2-S-DTPA the conjugates (10 mg) were dissolved in of citrate buffer (300 µl) at pH 4and [^{111}In]indium chloride (45 µl, 29 MBq) was added. The mixture was left at room temperature for 10 min (Figure 2.10).

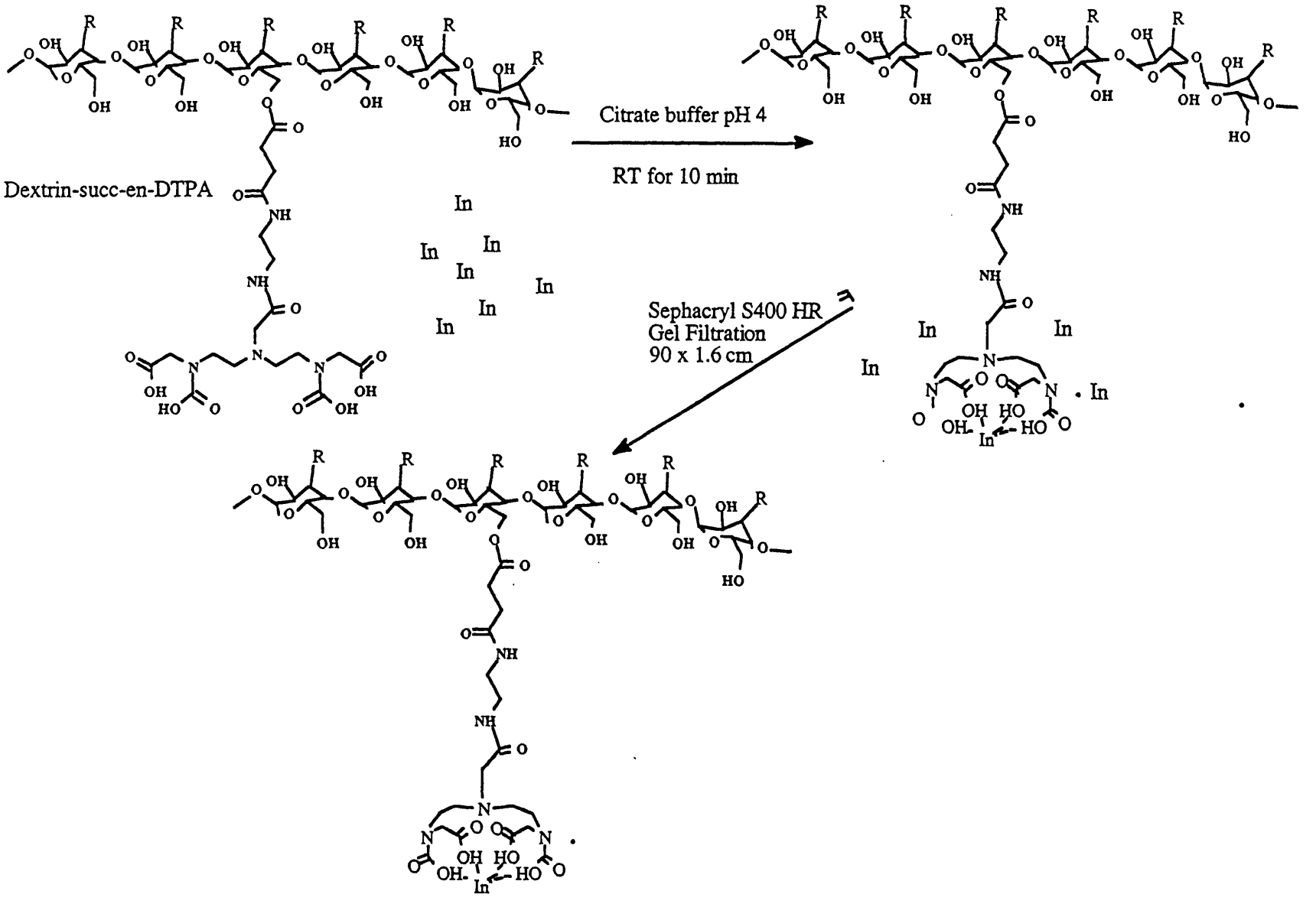
To examine the conjugates and purify them Sephacryl S400 column (90 cm x 1.6 cm) was used. The flow rate was set at 0.5 ml/min with the mobile phase being citrate buffer (pH 4) with NaCl (150 mM). All of the sample (300 µl) was loaded onto the column and fractions were collected (5 ml) and read on a gamma counter. Blue dextran (void volume) and [^{111}In]indium chloride were used to calibrate the column before applying the polymers. Blue dextran was monitored on a Uv/vis spectrometer at 280 nm and [^{111}In]indium chloride using a gamma counter.

To purify the probes [^{111}In]indium chloride was removed by gel filtration over Sephacryl S400 HR and spin fractionated using a membrane (Mw cut off 8 KDa) see Chapter 4 (Section 4.6.3).

2.7.2 Imaging of ^{111}In-labelled-dextrin and ^{111}In-labelled-D-2-S

A tracer dose (140 µl, 0.74 MBq) of ^{111}In-labelled-D-2-S and a tracer dose (300

Figure 2.10 Reaction mechanism for radiolabelling of dextrin and D-2-S with [^{111}In]indium



µl, 0.53 MBq) of ^{111}In-labelled-dextrin was injected i.p. into male Wistar rats and the distribution of radioactivity followed over time by imaging with a Maxi camera 400T gamma camera (Figure 2.11).

Rats injected with free ^{111}Indium (200 µl, 0.95 MBq) were also studied in order to quantify the distribution of the isotope as a reference control. After 24 h the animals were killed, major organs were removed and the presence of radioactivity was assessed in a gamma counter.

2.8 Expression of data and statistical analysis

All *in vivo* and tissue levels of substances are expressed in terms of percentage of administered dose. All error bars shown *in vitro* studies are standard error of the mean (SE) while error bars shown *in vivo* represent standard deviation of the mean (SD). Statistical significance was calculated using a Student's T test (two tailed). Statistical differences of at least $p<0.05$ were considered statistically significant. All calculation were performed by Microsoft Excel version 5.0.

Figure 2.11 Gamma Camera Imager

Chapter 3

Dextrin and D-2-S: Introduction of pendant groups by succinoylation

3.1 Introduction

Icodextrin is a natural polymer used in the clinic for peritoneal dialysis (Peers and Gokal, 1997). The sulphated derivative D-2-S is used as an anti-HIV agent (Shaunak *et al.*, 1994). The properties and use of these two polymers have been described in Chapter 1 (Sections 1.5 and 1.7). The biocompatibility of Icodextrin and D-2-S is proven and therefore they have potential for development as polymeric carriers for drug targeting and/or controlled drug release.

In order to develop dextrin and D-2-S as polymer-conjugates, it was first necessary to introduce pendant groups suitable for the conjugation of model compounds (used to study biodistribution) and to produce polymer-drug conjugates.

Activation of polysaccharides to allow the introduction of pendant groups has been extensively studied. Many different methods have been used including organosulfonyl activation (Nilsson *et al.*, 1981; Tay *et al.*, 1989), triazine activation (Sturgeon, 1988) and CDI activation (Aalmo *et al.*, 1981; Ferruti, 1979). However, today the most commonly used methods are cyanogen bromide activation, chloroformate activation, periodate oxidation and succinoylation (Table 3.1). These methods and their advantages/disadvantages are described briefly below.

Cyanogen bromide activation: Cyanogen bromide activation is one of the most widely used reactions for covalently linking compounds to polysaccharides. The reaction is easily carried out in aqueous solution at room temperature (Hermanson *et al.*, 1992). This method also has the advantage that primary amine groups can be introduced directly into the reactive intermediate. However, this method does have a number of disadvantages. During activation of the hydroxyl group (Figure 3.1) reactive intermediates can be produced that can disrupt the polymer backbone. Additionally it is important to prevent liberation of the hydrogen cyanide so all of the excess reagents must be carefully removed. The linkage produced using this method is not stable and allows slow release of the pendant group. This may or may not be desirable depending on the proposed use of the conjugate.

Table 3.1 Methods used to introduce functional groups into polysaccharides

Polysaccharide	Modification Methods	Reference
Dextran	Succinoylation	Arranz *et al.*, 1992
		Bruneel and Schacht, 1994
	Chloroformate activation	Chaves and Arranz, 1985
		Drobnik *et al.*, 1982
Pullulan	Chloroformate activation	Bruneel and Schacht, 1995a, 1995b
	Succinoylation	Bruneel and Schacht, 1994, 1995b
	Periodate oxidation	Bruneel and Schacht, 1993, 1995b
Inulin	Succinoylation	Vermeersch and Schacht, 1985

Chloroformate activation: Chloroformate activation (Chaves and Arranz, 1985; Bruneel and Schacht, 1992) also involves modification of the hydroxyl groups within the polymer. However, it has the disadvantage of the possibility of the formation of intra- or inter-chain carbonate esters. Moreover, five membered rings can also be produced thus altering the polymer backbone (Figure 3.2). The hydroxyl groups of agarose, cellulose and dextran have all been modified using chloroformate activation (Vandoorne *et al.*, 1985 and Drobnik *et al.*, 1982).

Periodate oxidation: Periodate oxidation of the 1,2-diol groups of polysaccharides leads to the formation of an aldehyde structure (Schacht *et al.*, 1985 and Bruneel and Schacht, 1995). If not completely neutralised these aldehyde groups can cause toxicity (Figure 3.3). In the phase 1 study of dextran-dox this method of modification was used and, maybe the reason why toxicity was observed (Bapot and Boroujerdi, 1993).

Although frequently used, each of the above methods have disadvantages that make them less than ideal for the synthesis of polysaccharide conjugates. Therefore succinoylation was selected as the method of choice for the preparation of all the dextrin and D-2-S conjugates used in this study.

3.1.1 Succinoylation

Polysaccharide modification using succinic anhydride introduces a carboxyl group which enables subsequent derivatisation without disrupting the polymer backbone. Succinoylation of polysaccharides have been extensively studied using different catalysts and solvents to mediate the reaction.

Arranz *et al.* (1992) modified dextran using succinic anhydride with pyridine as the catalyst, DMF/LiCl as the solvent, and a reaction temperature of 90 ^{O}C. However when Vermeersch and Schacht (1985) studied the reaction of inulin with succinic anhydride, the catalyst DMAP gave better results than pyridine when DMF was used as a solvent at a reaction temperature of 40^{O}C. Their choice of DMAP as a catalyst was made following the method of Hifle *et al.* (1978).

The succinoylation of pullulan was studied (Bruneel and Schacht, 1994) using

Figure 3.1 Cyanogen bromide activation

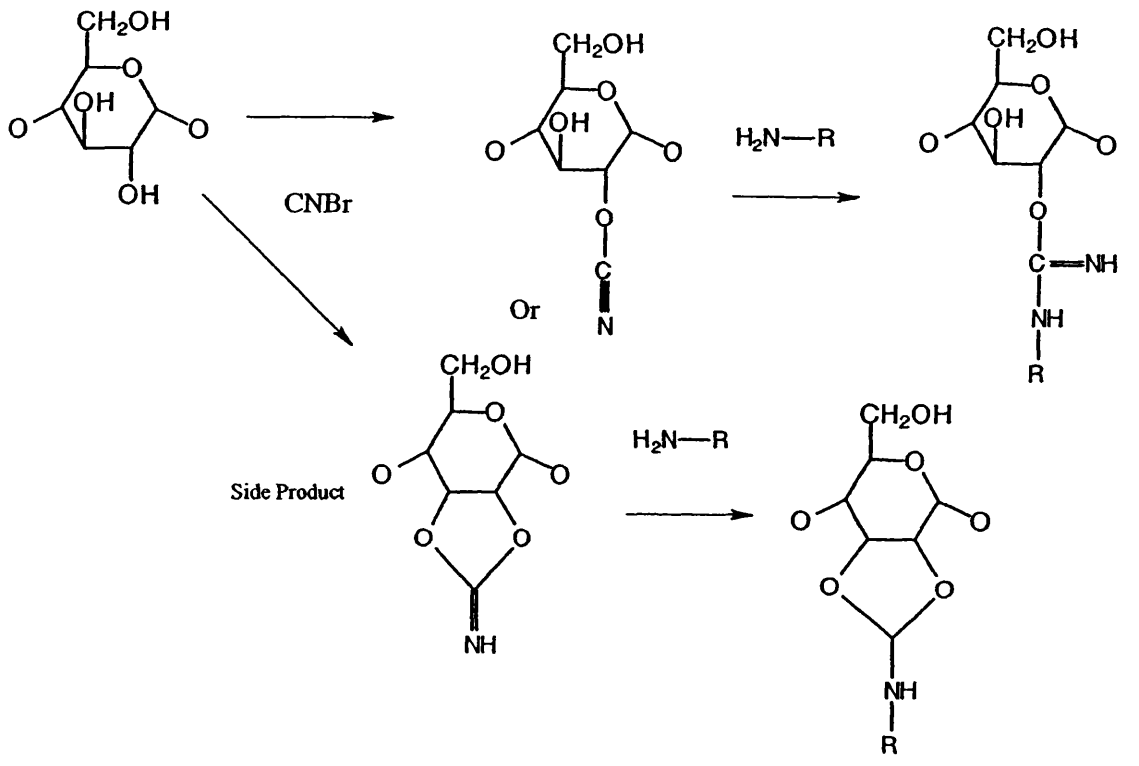


Figure 3.2 Chloroformate activation

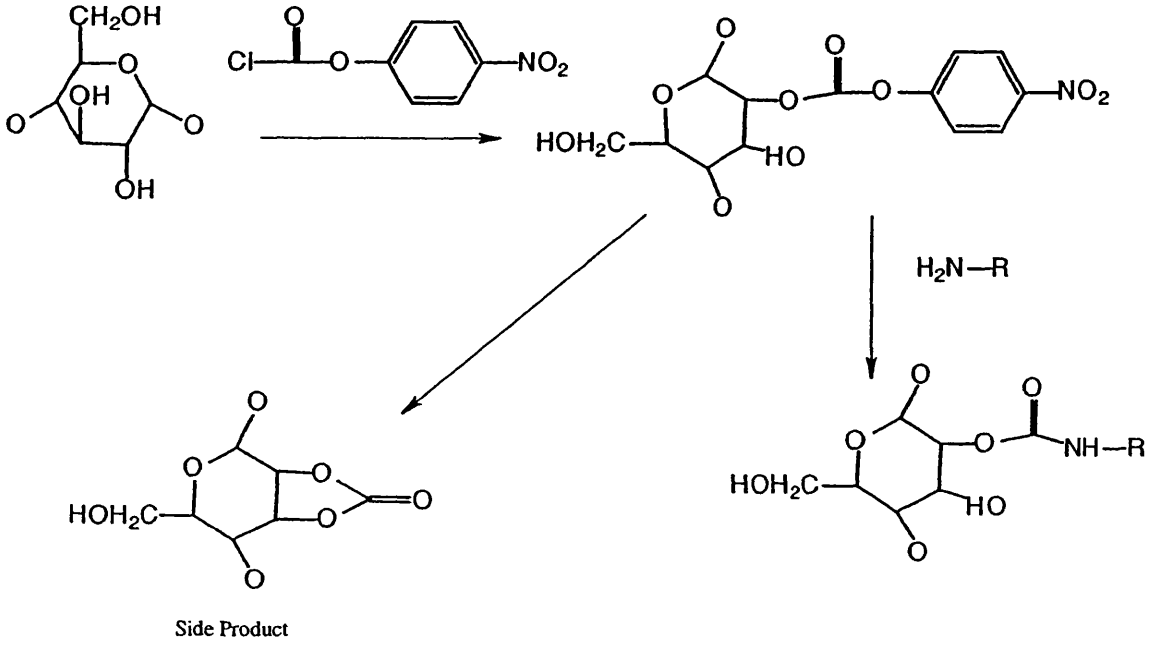
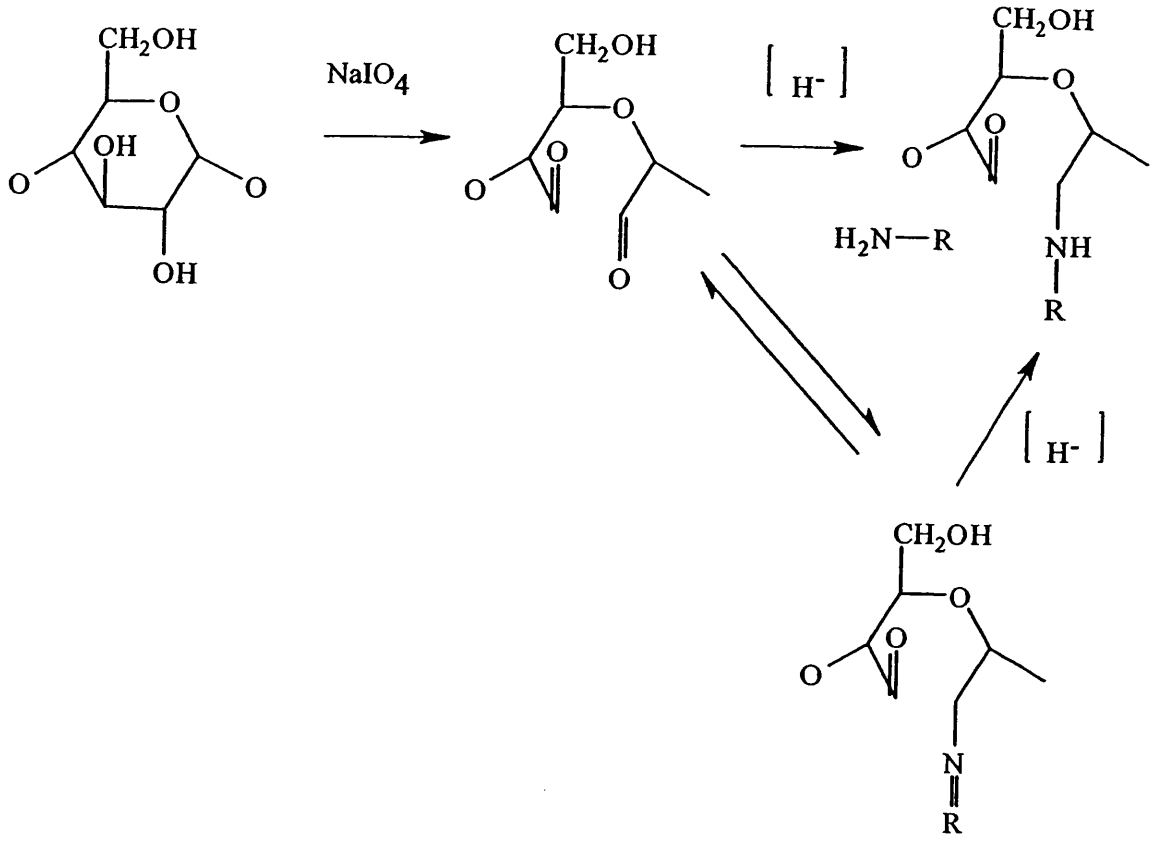


Figure 3.3 Periodate oxidation



DMAP as the catalyst with DMSO as the solvent at 40°C for 24 h. Here, the catalyst DMAP and the solvent DMF were chosen to allow succinoylation of dextrin and D-2-S. Once this intermediate was prepared it was subsequently possible to introduce:

a) Probes to monitor cell (biotin) and whole body pharmacokinetics (tyrosinamide and DTPA) (Chapter 4 and 5)

b) Drugs such as Dox to evaluate the potential of dextrin and D-2-S as anti-cancer agents (Hrezcek-Hirst *et al.*, 1999, 2000) (Chapter 6) or amphotericin B to produce anti-fungal conjugates (Chapter 7).

The structure and properties of Dox are described in Chapter 1 (Sections 1.8). For completeness, the chemical structure and properties of biotin, tyrosinamide and DTPA are briefly outlined here.

3.1.2 Biotin

The dextrin-biotin and D-2-S-biotin conjugates were prepared to allow visualisation of the intracellular fate of these polymers. Biotin (Figure 3.4) is a naturally occurring growth factor found in small amounts within every cell where it plays an important role in carboxylation reactions. It is the co-enzyme that catalyses the incorporation of CO_2 into various compounds. Biotin was originally discovered through symptoms of deficiency caused by eating too many raw egg whites and was found to be complexed and inactivated by the egg-white protein avidin (Boas, 1927; du Vigneaud, 1940).

Biotinoylated molecules retain the ability to bind avidin or streptavidin with high affinity and this interaction has been used to allow preparation of coloured reagents that can be used to visualise tagged molecules. Avidin, a glycoprotein (Mw 66, 000 Da) is found in egg whites (Green, 1975) and streptavidin can be isolated from *Streptomyces avidinii* (Chaiet and Wolf, 1964). Streptavidin has a slightly lower Mw of 60, 000 Da (Bayer *et al.*, 1986,1989). Biotin-hydrazide (Figure 3.4b) has been used previously to biotinoylate antibodies via their carbohydrate residues (O'Shannessy *et*

Figure 3.4 Structure of biotin

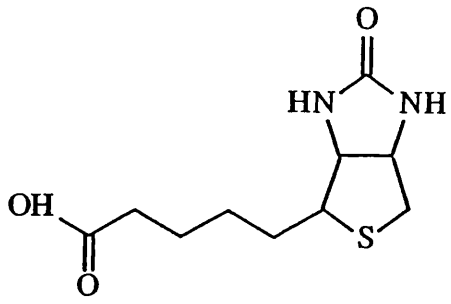
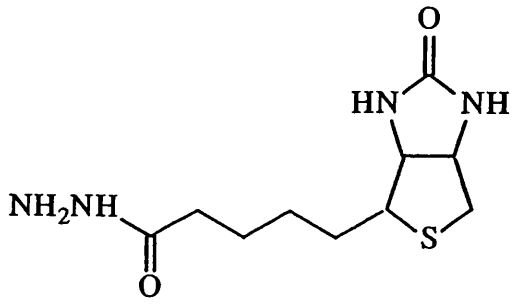


Figure 3.4b Structure of biotin hydrazide



al., 1984, 1987; Hoffman and O'Shannessey, 1988), to modify the low density lipoprotein (LDL) receptor (Wade *et al.*, 1985), to biotinoylate nerve growth factor (NGF) (Rosenberg *et al.*, 1986) and to modify cytosine groups in oligonucleotides to produce probes suitable for hybridization assays (Reisfeld *et al.*, 1987).

3.1.2 Tyrosinamide and DTPA

Tyrosinamide (Figure 3.5) and DTPA (Figure 3.6) were conjugated to dextrin and D-2-S to produce probes that could be radiolabelled with [^{125}I]iodine or [^{111}In]indium chloride to allow monitoring of the fate of these polymers *in vivo*. In both cases the aim was to prepare polymers with a level of substitution which would not alter the biodistribution of the polymers. Therefore a modification of approximately 1 mol % was chosen. The advantage of using radioactive tracers is the ease, rapidity, reliability and precision of the quantitation of tissue localisation.

The fate of the polymers *in vivo* was determined either by analysis of individual organs (dissection analysis) or by visualisation of the polymers using gamma camera imaging using [^{111}In]indium chloride.

DTPA is a bifunctional chelating agent containing two amine reactive anhydride groups used routinely to allow radiolabelling of antibodies. Addition of indium to dextrin and D-2-S creates multiple metal binding sites (Trubetskoy *et al*, 1993). This process involves ring opening to form an amide bond that enables coordination complexes with radionuclides such as [^{111}In] indium chloride.

In this initial study it was considered important to optimise the methods used to succinoylate dextrin and D-2-S. The effect of reaction temperature and time were studied and also, the effect of the degree of succinoylation on yield. Once the reaction conditions were optimised the reproducibility was determined.

Having determined the optimal conditions for the preparation of the succinoylation dextrin and D-2-S intermediates, the compounds containing biotin, tyrosinamide and DTPA as pendant groups were prepared. Synthesis of dextrin-Dox conjugates has been described elsewhere (Hreczuk-Hirst *et al.*, 1999; 2000) and the preparation and characterisation of dextrin-AmpB and D-2-S-AmpB is described in

Figure 3.5 Structure of tyrosinamide

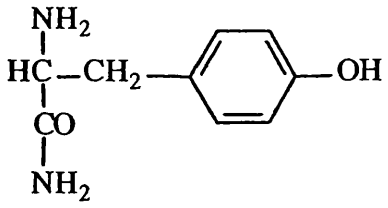
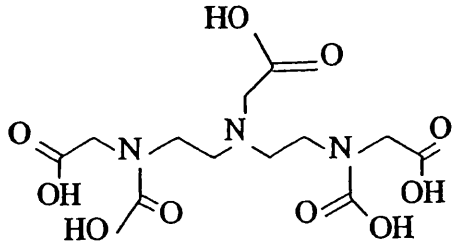


Figure 3.6 Structure of DTPA



detail in Chapter 7.

3. 2 Materials and Methods

A typical synthesis for 10 mol % succinoylation of dextrin has already been described in Chapter 2 (section 2.4.1).

For the purpose of this study the aim was to modify dextrin and D-2-S to a level of 1 mol %. The degree of succinoylation was initially limited to 1 mol % to ensure that the polymer structure was preserved as much as possible. Subsequently, succinoylated polymers were prepared with the aim to modify to a higher level (34 mol %) in order to:

a) Introduce more pendant groups to allow for adequate drug loading
b) To increase polymer stability as the 1 mol % succinoylated dextrin degraded so rapidly that it was not suitable for use as a drug carrier.

3.2.1 Optimisation of succinoylation of dextrin

In order to ascertain the optimum temperature for the succinoylation, reactions were carried out at 20, 30, 40, 50 and 60 ^{0}C (in triplicate) over 24 h and the conditions were set up to give a theoretical maximum succinoylation of 10 mol %. Then using the optimum temperature of 50 °C, the effect of reaction time was studied using reactions of 2, 4, 8 and 24 h. Finally the effect of the degree of succinoylation was investigated using a reaction time of 8 h and a temperature of 50 °C. A series of reactions were set up to give a theoretical maximum succinoylation of 2, 10, 30 and 60 mol %. All the reaction conditions were kept the same except the amounts of succinic anhydride were increased; 12.4, 62, 186 or 372 mg to give the above mentioned degrees of succinoylation.

3.2.2 Succinoylation of D-2-S

The method used for the succinoylation of D-2-S is described in Chapter 2 (section 2.4.2) and the reaction conditions were essentially the same as those used to succinoylate dextrin. Initially the reaction was carried out at 50 °C for either 24 h or 48 h to determine the minimum reaction time. As for dextrin, an experiment was also

carried out to investigate the degree of D-2-S succinoylation where reactions were set up to give theoretical degrees of succinoylation of 2, 10, 30 mol %.

3.2.3 Conjugation of tyrosinamide to dextrin and D-2-S

The method used for the conjugation of tyrosinamide to dextrin and D-2-S is described in Chapter 2 (section 2.4.3). The incorporation of tyrosinamide into the final product was determined by UV-vis spectrometry and FTIR.

3.2.4 Conjugation of biotin to dextrin and D-2-S

The method used to bind biotin hydrazide to dextrin and D-2-S is described in Chapter 2 (section 2.4.4) The extent of biotin incorporation into the product was measured using the HABA/avidin assay and UV-vis spectrometry (Green, 1965). Binding of avidin to biotin is one of the strongest non-covalent affinities that is known. The HABA/Avidin assay is based on the initial binding of the HABA to avidin then measurement of the ability of the biotin to displace the dye in stoichiometric proportions. The latter is accompanied by a change in UV absorbance at 500 nm. The methods and calibration curve for this reaction can be found in Chapter 2 (section 2.4.4).

3.2.5 Conjugation of DTPA to succinoylated dextrin and succinoylated D-2-S

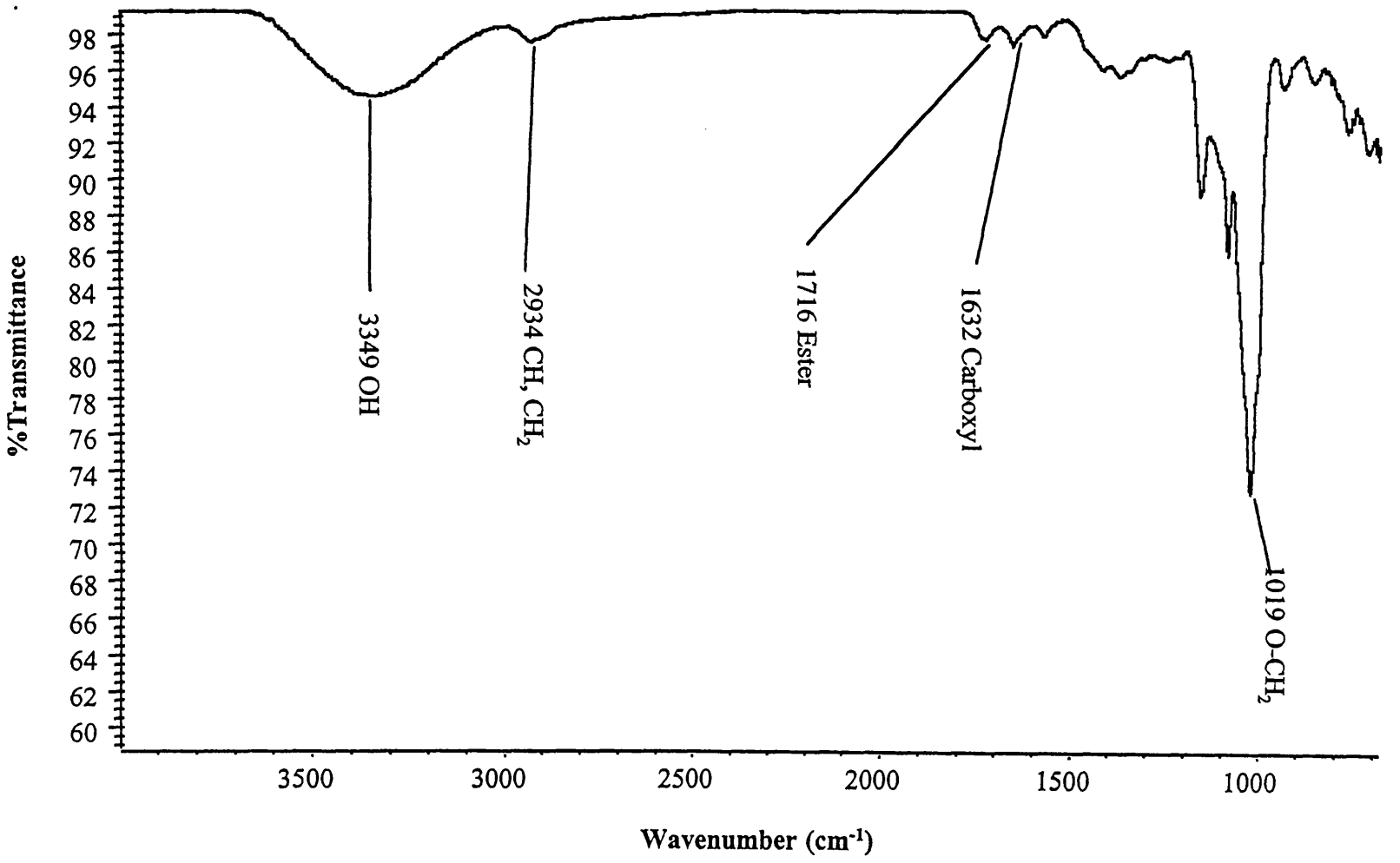
Conjugation of DTPA to succinoylated dextrin and succinoylated-D-2-S was carried out as described in Chapter 2 (section 2.4.6). The products were characterised by measuring their ability to bind [^{111}In] indium. It is not possible to evaluate DTPA incorporation spectrophotometrically (Hekman, 1996).

3.3 Results

3.3.1 Succinoylation of dextrin

First pilot experiments were conducted at 50 °C using a reaction time of 24 h at a theoretical maximum succinoylation of 10 mol %. The FTIR spectrum of the product (Figure 3.7) confirmed the presence of the succinoyl group by ester formation. Although ^{1}H and ^{13}C NMR were not able to confirm the presence of the succinoyl

Figure 3.7 FTIR of 1 mol % confirming the incorporation of the succinoyl group by ester formation



group at a substitute of 1 mol % (due to low pendant group to polymer ratio), the ^{13}C NMR spectrum of the 34 mol % product showed the presence of a carbonyl ester (176.8 ppm, C8,) and an carbonyl acid (179.9 ppm, C11) as well as 2 aliphatic carbons (99 and 66 ppm, C9 and C10). Similary the ^{1}H NMR (Figure 3.8) spectra of this product indicated the presence of aliphatic carbons (2.8 (4 H)) not seen in the spectra for native dextrin.

Experiments were carried out to study the effect of temperature on the succinoylation of dextrin. The results are shown in Table 3.2. At lower temperatures (20 °C and 30 °C) dextrin was poorly soluble and at 20 °C dextrin did not dissolve within 24 h. This poor solubility probably contributes to the high variability in succinoylation seen when the reaction was carried out at 30 °C. There was no statistical difference (students t test) seen in the degree of succinoylation between 40 ^{0}C and 50 ^{0}C. Therefore a temperature of 50 ^{0}C was selected for all subsequent reactions.

When reactions were allowed to progress for different times reaction time was found to be important. Dextrin had not dissolved within 1 h (Table 3.3). There was no significant difference between the degree of succinoylation seen at different times (p= 90 % confidence). A reaction time of 24 h was chosen as standard for convienience due to a higher average and lower variability.

The reproducibility of the synthesis of dextrin batches having different degrees of succinoylation is shown in Table 3.4. The value of 'n' reflects the total number of batches prepared during the course of this study. The introduction of –COOH groups by succinoylation was studied by simple titration.

The FTIR spectra of the dextrin preparations with increasing degrees of succinoylation are shown in Figure 3.9. The peaks at 1632 cm^{-1} (carbonyl) and at 1716 cm^{-1} (ester) show increased signal strength as the degree of succinoylation increases. The degree of succinoylation was reproducible at each level of modification and throughout modification represents approximately 50 % of the maximum theoretical degree of succinoylation possible.

Figure 3.8 **^{1}H NMR spectra for the 34 mol % succinoylated dextrin sample**

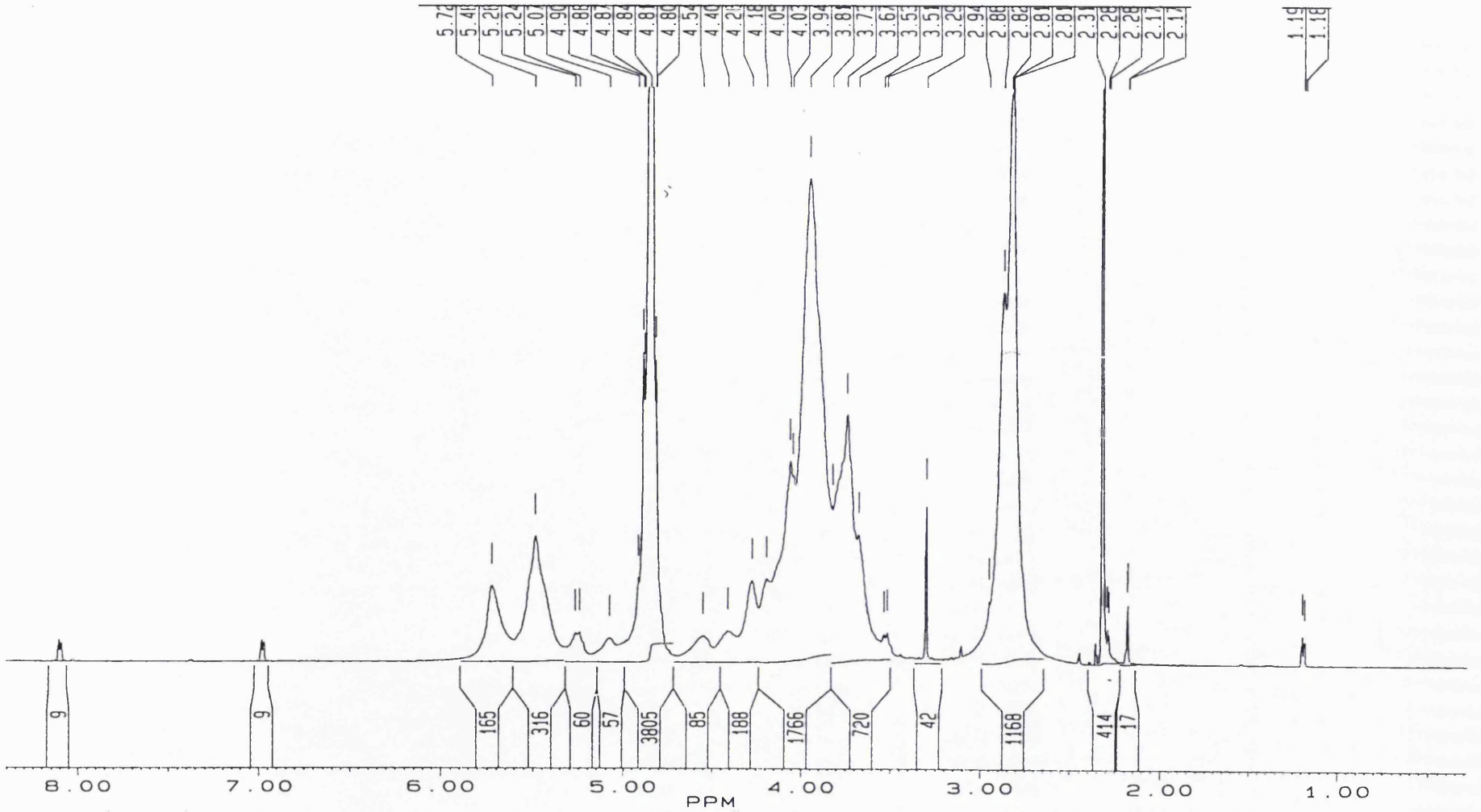


Table 3.2 Effect of temperature on the succinoylation of dextrin (n=3 ±SD)

Temperature (^{O}C)	Mol % Succinoylation†
20	ND
30	6.6 ± 0.7
40	5.5 ± 0.3
50	5.3 ± 0.1

† Theoretical maximum 10 mol %

ND no results due to insolubility

Table 3.3 Effect of time on the succinoylation of dextrin (n = 3, ± SD)

Time (h)	Mol % Succinoylation†
1	6.6 ± 1.7
2	3.1 ± 0.3
4	4.4 ± 0.7
8	5.4 ± 0.3
24	5.2 ± 0.3

† Theoretical maximum 10 mol %

Table 3.4 Effect of the ratio of reactants on the extent of dextrin succinoylated observed

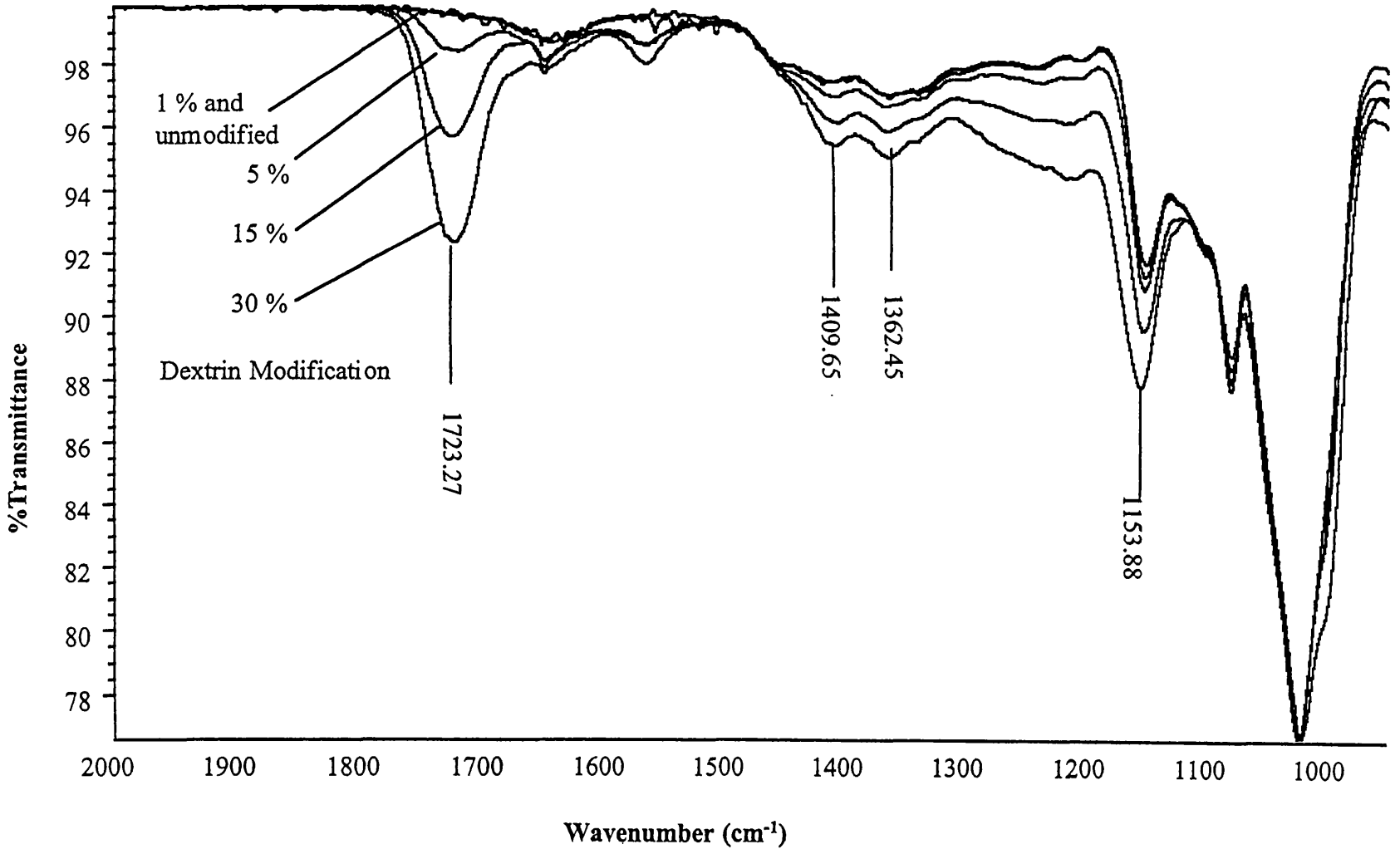
Theoretical Mol %	Actual Mol % (± SD)	Number of replicates	Maximum modification achieved (%)
60	32.3 ± 1.3	7	53.8
30	15.6 ± 1.3	3	52.0
10	5.7 ± 0.5	7	57.0
2	1.0 ± 0.1	13	50.0
1	0.4 ± 0.05	6	40.0

Maximum modification achieved (%) =

Actual mol% of succinoylation that was obtained in the reaction x 100

Theoretical mol % that was expected based on the amount of succinic anhydride added

Figure 3.9 **FTIR spectra of dextrin with an increasing degree of succinoylation**



3.3.2 Succinoylation of D-2-S

The first pilot succinoylation reactions using D-2-S were carried out at a temperature 50 °C over 24 h or 48 h using a ratio of reactants designed to give substitution of 1 mol %. Table 3.5 shows that the 24 h reaction time seemed to give a very high degree of modification, higher than was theoretically possible. Over 48 h a modification of 0.95 ± 0.1 mol % was seen. ^{13}C and ^{1}H NMR spectra of the succinoylated D-2-S proved to be difficult to interpret due to the nature of the polymer including the sulphate groups and showed also due to low incorporation level of succinoyl moiety. However, characterisation by FTIR showed peaks at 3349 (OH), 2934 (CH, CH_2), 1716 (ester), 1632 (carboxyl) and 1019 cm^{-1} (O-CH_2) confirming the incorporation of the succinoyl group by ester formation. Table 3.6 shows the reproducibility of the batches made during the course of this study.

3.3.3 Conjugation of dextrin-tyrosinamide and D-2-S-tyrosinamide

UV-Vis spectroscopy was used to determine the presence of tyrosinamide in the conjugate. Figure 3.10 shows the tyrosinamide calibration curve determined by measuring absorption at 254 nm and Table 3.7 gives the tyrosinamide incorporation into the different dextrin-tyrosinamide and D-2-S-tyrosinamide conjugates prepared. For the biodistribution studies these tyrosinamide conjugates were radiolabelled with [^{125}I]iodine.

3.3.4 Conjugation of dextrin-biotin and D-2-S-biotin

Although dextrin does not display any UV absorption, biotin hydrazide displays UV absorption at 217nm. Therefore the UV spectrum was used to get a qualitative indication of biotin incorporation, furthermore modification can be seen by FTIR by monitoring the acid leaving the ester. The HABA/avidin assay was used to determine quantitatively biotin incorporation, and the calibration curve for this assay is shown in Chapter 2 (Figure 2.4). The biotin incorporation into the dextrin and D-2-S conjugate was 6.8 and 9.7 wt % respectively (Table 3.8). These probes were used to visualise the cellular uptake of the polymers.

Table 3.5 Effect of reaction time on the succinoylation of D-2-S

(n = 3, ± SD)

Time (h)	Mol % Succinoylation
24	1.81 ± 0.2
48	0.95 ± 0.1

Table 3.6 Reproducibility of the synthesis of different mol % succinoylation of D-2-S

Theoretical Mol %	Actual Mol % (± SD)	Number of replicates	Theoretical maximum modification (%)
30	23.9 ± 1.4	3	79.7
20	14.5 ± 0.2	3	72.5
4	1.8 ± 0.2	3	45.0
2	0.9 ± 0.08	8	45.0
1	0.5 ± 0.05	5	50.0

Figure 3.10 Concentration dependence of UV absorption of tyrosinamide (at 275 nm)

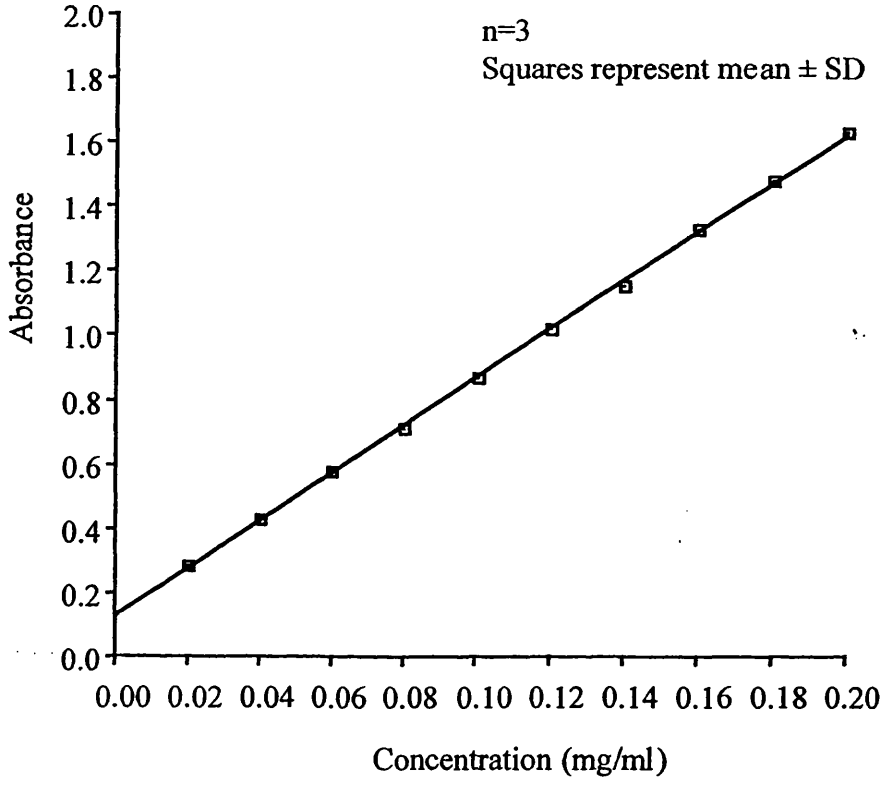


Table 3.7 Amount of tyrosinamide incorporated into dextrin and D-2-S conjugates

Polymer	Reaction 1 (mol %)	Reaction 2 (mol %)	Reaction 3 (mol %)	Average± SD (mol %)
Dextrin	0.9	0.8	0.7	0.8±0.1
D-2-S	0.7	0.7	0.8	0.7±0.1

Table 3.8 Amount of biotin incorporated into dextrin and D-2-S conjugates

Polymer	Reaction 1 (wt %)	Reaction 2 (wt %)	Reaction 3 (wt %)	Average± SD (wt %)
Dextrin	6.7	7.4	6.9	7.0 ± 0.3
D-2-S	9.7	10.6	9.8	10.0 ± 0.4

3.3.5 Conjugation of DTPA to succinoylated dextrin and succinoylated D-2-S

None of the analytical techniques that were carried out including NMR, FTIR and UV-vis could positively determine the incorporation or the amount of DTPA conjugated to either dextrin or D-2-S probably due to the very low loading (less than 1 mol %). A spectrophotometric method for the quantitative determination of the purity of DTPA was carried out according to the methods described by Hekman (1996) but the method outlined did not give enough details so our results were inconclusive. For the imaging studies these DTPA conjugates were radiolabelled with [^{111}In]indium chloride, as described in Chapter 4. Effective radiolabelling provides further indirect evidence of the presence of DTPA in the polymer.

3.4 Discussion

These studies confirmed that the biodegradable polysaccharides dextrin and D-2-S can be derivatised reproducibily by succinoylation to introduce a carboxylic acid group. The degree of succinoylation of this reaction was always approximately 50 % of the maximum degree of succinoylation that could be achieved.

The reasons for this could be:

c) Water associated with the dextrin or D-2-S

b) Competing reactions

c) Inefficient DMAP-based catalysis

Using a two-fold excess of succinic anhydride allowed relatively precise control of the number of functional groups introduced. For dextrin derivatives modification was achieved between 1 and 60 mol % and for D-2-S derivatives modification between 1 and 5 mol %.

It is well known that the C-2, the secondary hydroxyl group is more reactive than the C-3 and C-4 hydroxyl groups. Bruneel and Schacht (1994) demonstrated using pullulan and ^{1}H NMR that succinic anhydride preferably reacts with the C-6 hydroxyl group confirming that in this case the primary hydroxyl groups is more reactive than secondary hydroxyl groups. In these studies succinoylated dextrin and D-2-S samples did not give data that clearly indicated the site of derivitisation. Throughout the thesis these derivatives are drawn showing a pendant group at position C-2. However, it should be noted the modification may occur at other or multiple sites along the polymer

did not give data that clearly indicated the site of derivitisation. Throughout the thesis these derivatives are drawn showing a pendant group at position C-2. However, it should be noted the modification may occur at other or multiple sites along the polymer chain.

Arranz *et al.* (1992) found that succinic anhydride modification of dextran gave a maximum succinoylation degree of 60 mol % after 10 h. Similarly, here it was found that maximum succinoylation was 5.4 mol % at 8 h (theoretical yield of 10 mol %). In contrast, the reaction between inulin and succinic anhydride (using DMAP as a catalyst) gave approximately 90 mol % succinoylation after 2 h (Vermeersch and Schacht, 1985) although it should be noted that no other time points were included in this study other than 24 h. At that time inulin displayed 100 mol % succinoylation. Further studies increasing the duration of the reaction showed that once 100 mol % succinoylation had been achieved no further modification was possible.

When comparing the succinoylation of dextran with and without pyridine as catalyst, in the presence of pyridine 60 mol % after 10 h was achieved. Without the catalyst only 30 mol % of succinoylation of dextran was achieved. However, when modifying pullulan with succinic anhydrides (Bruneel and Schacht, 1995) they found they obtained only 25 mol % of the maximum theoretical yield.

Further evidence that DMAP is a better catalyst than pyridine was obtained by Hifle *et al.* (1978). For this reason DMAP was selected as catalyst for all the synthesis described here.

The main technical problems encountered during dextrin and D-2-S succinoylation was the optimisation used for the purification of the final product. Earlier studies have shown that inulin could be precipitated with ether and pullulan using an ethanol and ether (1:1, v/v) mixture. Both approaches proved unsuitable for precipitation of dextrin and D-2-S due to poor reproducibility. Use of diethylether under rapid stirring for precipitation of the reaction mixture overcame these problems.

Otherwise the succinoylation reaction was found to be very reproducible. Once purified the –COOH containing intermediates could be used to allow addition of a variety of pendant groups.

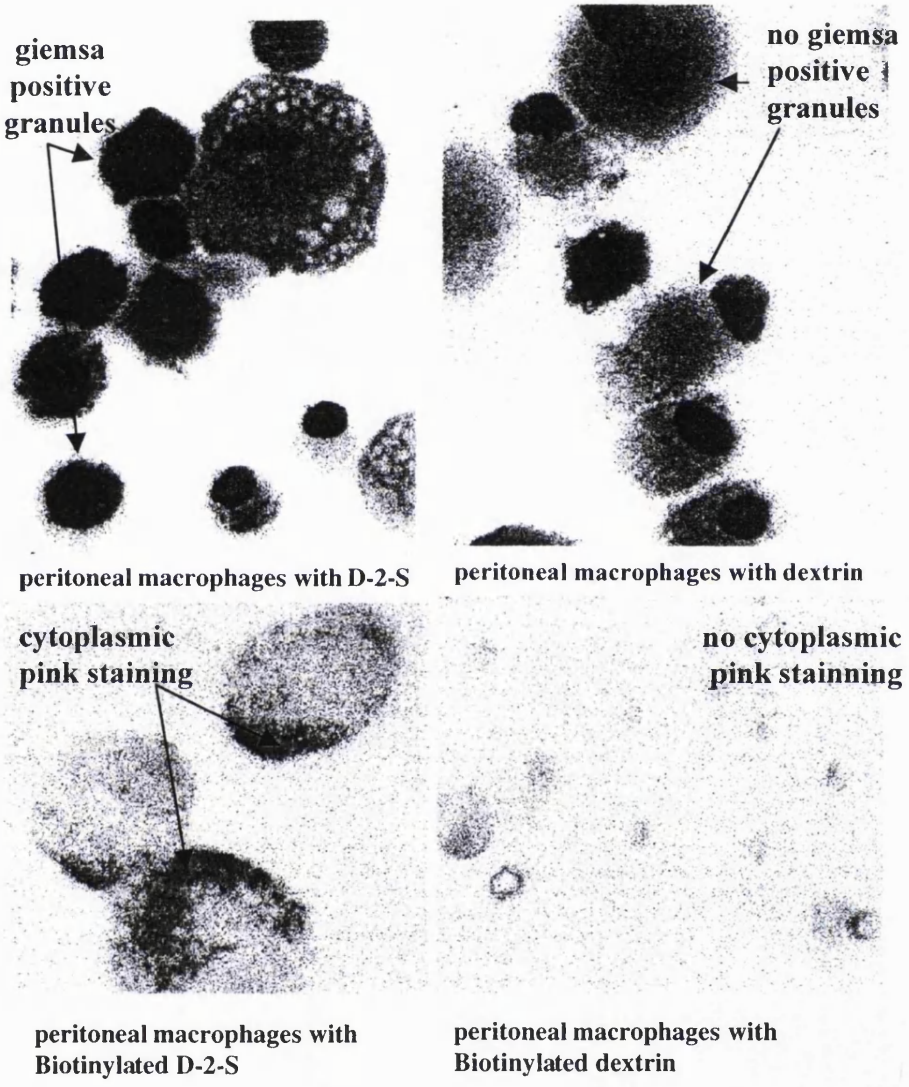
The dextrin and D-2-S tyrosinamide conjugates were prepared to allow radioiodination and then measurement of fate *in vivo*. These studies are described in detail in Chapter 4. The DTPA conjugates were synthesised to allow gamma camera studies after labelling with [^{111}In] indium. These experiments are described in Chapter 4.

Experiments were carried out in collaboration with M. Thornton and S. Shaunak (Hammersmith Hospital) to monitor the cellular fate of these biotin conjugates. Cultured human peritoneal macrophages were incubated with the biotinoylated probes and the cells were stained. Peritoneal macrophages cultured with D-2-S showed dark Giesma-positive granules intracellularly (Figure 3.11) and biotinoylated D-2-S showed pink cytoplasmic staining. After incubation of monocyte-derived-macrophages with biotinoylated-dextrin, no Giesma-positive intracellular granules were observed. Although it had been hoped to follow up on these initial studies and study more systematically the mechanism of capture of dextrin and D-2-S in human peritoneal macrophages and other cell types, time did not allow these studies. Thus no further investigation of intracellular trafficking is reported in this thesis.

Conjugates containing the model drugs Dox and AmpB which were also prepared from the succinoylated dextrin and D-2-S intermediates are described later in this thesis (Chapter 6 and 7).

Using the succinoylated dextrin intermediate described here dextrin-Dox conjugates were prepared by Dr. Dale Hirst in our group as potential anticancer conjugates (Hrezcuk-Hirst *et al.*, 1999, 2000). Tumour targeting of these was examined after i.p. administration to mice bearing the B16F10 murine melanoma cell line and is described in Chapter 6.

Figure 3.11 Appearance of human macrophages with biotinoylated-dextrin and biotinoylated-D-2-S



Synthesis of conjugates containing AmpB using the succinoylated dextrin and D-2-S intermediates reported here and their preliminary biological evaluation described in Chapter 7.

Chapter 4

Biodistribution of dextrin and D-2-S

4.1 Introduction

In order to evaluate the potential of targetable drug carriers, it is first important to understand the tissue distribution of the carrier after administration *in vivo*. As there had been no previous investigation into the biodistribution of dextrin and D-2-S it was considered important to establish their fate. Here ^{125}I-labelled or ^{111}In-labelled probes (from Chapter 3) were used to quantitate and visualise biodistribution.

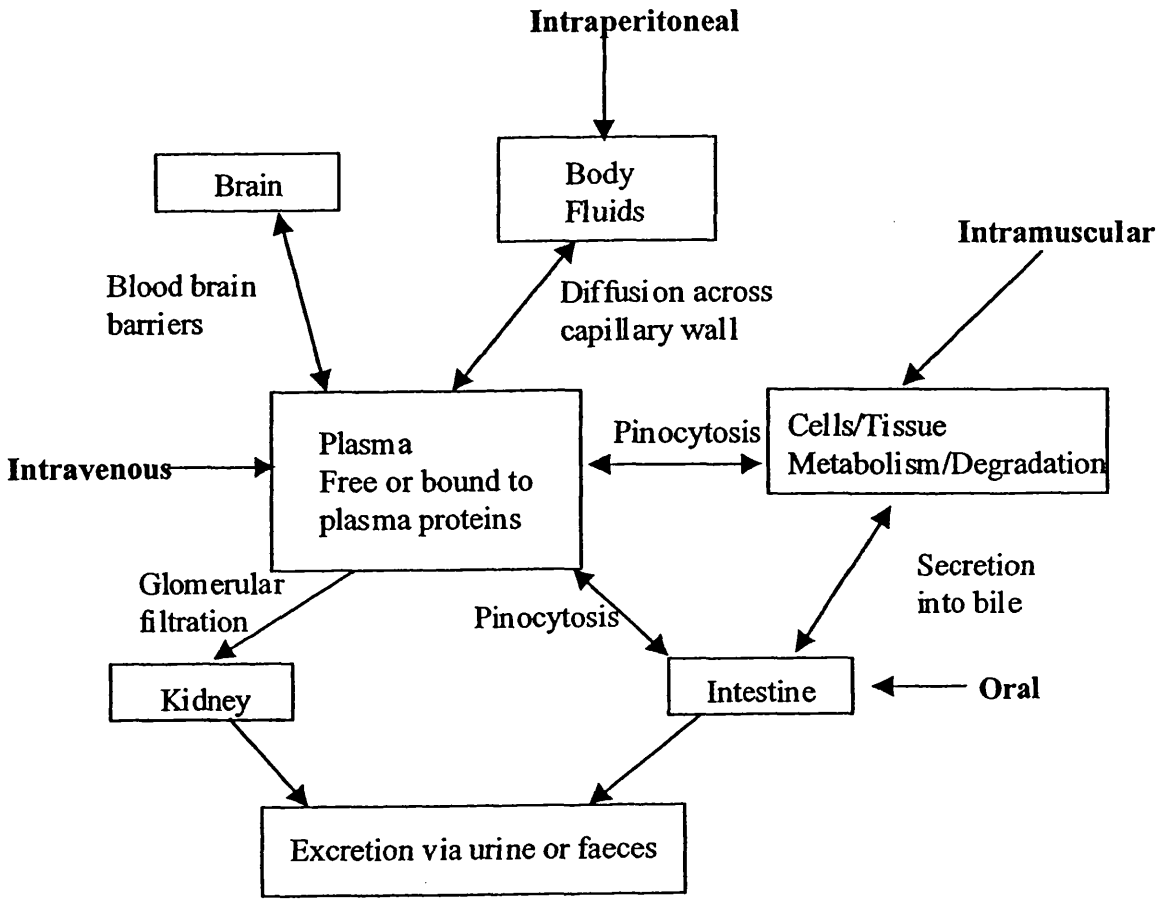
As already discussed in Chapter 1, dextrin and D-2-S have been administered to patients via the i.p. route (Davies, 1994). D-2-S has also been used as a vaginal gel (Stafford *et al.*, 1997). However, if used as a drug carrier, it is possible that the conjugates may be administered by other routes; e.g. i.v. or s.c. Therefore, the biodistribution of dextrin and D-2-S was assessed after administration i.p., i.v. and s.c. The potential fate of polymers injected into the body via various routes is summarised in Figure 4.1.

Intravenous administration is the most commonly used method for administration of water soluble polymers or polymer-conjugates. It has the advantage that polymers enter the circulation directly and also this route can be used to administer polymers that are not absorbed well from tissue deposits or from the gastrointestinal tract. Polymer-conjugates that would be painful if administered s.c. or i.p. maybe injected slowly into a vein without causing discomfort. I.v. administration does have some disadvantages. Once injected the drug cannot be recalled, and too rapid an injection may create a bolus of drug in the circulation and elicit hypersensitivity reactions.

The general factors that influence the biodistribution of polymeric carriers have been studied widely. Most important are polymer Mw and chemical characteristics such as charge and conformation (Suindquist *et al.*, 1988).

It is accepted that the pharmacokinetics of a polymer-drug conjugate are largely governed by its polymer carrier moiety. Many studies have investigated the fate of dextran *in vivo*. When studying the biodistribution of ^{125}I-labelled dextran, Artursson *et al.*, (1964) showed that polymers of Mw 50, 000 Da were able to leave the body within 48 h. Higher molecular weight dextrans were retained for greater periods of time (Hint, 1968). Early biodistribution studies using ^{125}I-labelled dextran showed tissue specific accumulation mainly in the intestinal mucosa, spleen, liver and kidneys (Fisher and Stein, 1960). Increasing the dextran dose caused problems associated with

Figure 4.1 **Fate of polymers in the body after administration by different routes**



intracellular storage as the polymer is not readily degraded in the body (Labride and Vigneron, 1975). Synthesis of dextrans carrying different charge showed that modification influenced dextran fate (Yamaoka *et al.*, 1995). The biodisposition of fluorescein-labelled dextran with different molecular weights have been studied after i.v. administration (Kaneo *et al.*, 1997) showing that with increased molecular weight the blood circulation increases causing higher hepatic circulation.

Chitin and chitosan have been used as drug carriers because they are biocompatible and biodegradable (Pangburn *et al.*, 1982: Yomato *et al.*, 1990). Studies have shown that due to very slow and incomplete degradation chitosan should accumulates in the body (Nakaamura *et al.*, 1992). A study examining the distribution of fluorescein isothiocyanate labelled in mice (i.p. administration) showed that the polymer moved quickly to the kidneys and was found in the urine after 14 h. Distribution to any other organs was scarce (Onishi and Machida, 1999).

^{125}I-labelled chitin (Richardson *et al.*, 1999) and ^{125}I-labelled alginates (Al-Shamkhani and Duncan, 1995) have also been studied. Throughout there was always a tendency for polyglucose polymers to localise in macrophages in the liver and spleen.

Most of the studies investigating polysaccharide biodistribution have used radiolabelling techniques to monitor the fate of the polymer or polymer-drug conjugates. The probes most commonly used to label macromolecules include [^{125}I]iodine, [^{14}C] carbon and [^{3}H] hydrogen by labelling of pendant groups which can be incorporated into the main chain.

In this study radioiodination was chosen for dextrin and D-2-S labelling. ^{125}I-Labelled polymers have the advantage that the radioactivity is easily found during the experiment, the presence of isotope can be followed with a hand monitor and also at the end of the experiment quantitated by dissection analysis. However this technique has the disadvantage that [^{125}I]iodine is tagged onto the molecule (it is not an integral part) and thus it must be established that the labelled conjugate is stable.

An ideal radionucleotide should be readily available at a reasonable cost. It must have a high enough specific activity that the required dose can be injected without toxicity. The isotope emission should allow adequate quantitation and the $t_{1/2}$ for decay must allow both sufficient time to produce the labelled probe in a form suitable for use, and be long enough to allow biodistribution to be followed over the desired time period.

In this study the biodistribution of ^{125}I-labelled dextrin and ^{125}I labelled D-2-S was followed after s.c., i.v. and i.p. administration to rats. To visualise the biodistribution in real time ^{111}In-labelled dextrin and ^{111}In-labelled D-2-S probes were prepared and administered to rats for gamma camera imaging.

4.2 Materials and Methods

Dextrin-tyrosinamide (0.7 mol %), D-2-S-tyrosinamide (0.8 mol %) and the related conjugates containing DTPA were synthesised and characterised as described in Chapter 2 (Section 2.4.3) and Chapter 3 (Sections 3.3.7) respectively.

4.2.1 ^{125}I-Labelled probes: Labelling efficiency and purity assessment

The tyrosinamide-containing conjugates were radiolabelled using the Chloramine T method as described in Chapter 2 (Section 2.6.1.1) except that a second aliquot (5 µl) was removed after purification. The presence of free [^{125}I]iodine in the reaction mixture and the purified product was assessed using paper electrophoresis as described in Chapter 2 (Section 2.6.1.2).

4.2.2 Body distribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after administration s.c., i.v. and i.p.

Male Wistar rats (200-250 g) were injected i.v., i.p. and s.c. with 100 µl of the polymer in saline (5 x 10^5 cpm, ~ 150 µg/kg of ^{125}I-labelled dextrin and ~300 µg/kg of ^{125}I-labelled D-2-S). At various times after administration (Table 4.1) the animals were killed, and a blood sample was taken. The following organs were removed and washed in PBS before being assayed for radioactivity; liver, heart, kidneys, lung, thyroid and spleen. The organs were weighed and then homogenised in distilled water using a polytron homogeniser. The final volume of the homogenate was recorded. Radioactivity recovered in the blood and organs was expressed in terms of radioactivity (% administered dose). The blood volume of the rat was taken to be 7.2 ml/100 g (Dreyer and Ray, 1910).

Intraperitoneal administration:- Following i.p. administration, the radioactivity remaining in the peritoneal cavity was also determined by performing an i.p. wash. Once the animal had been killed, 10 ml of saline was injected into the peritoneal cavity

and the peritoneal region was given a gentle massage to ensure that the saline passed over all the organs. A small incision was made into the region and as much fluid as possible was withdrawn; this being the peritoneal wash. The volume withdrawn was noted but in the calculations was corrected to the 10 ml that was administered.

4.2.3 Urine analysis using a PD10 column

To determine the nature of the radioactivity found in the urine, samples were subjected to gel permeation chromatography using a PD10 column (prepacked disposable column containing Sephadex G25 swollen in distilled water). A sample of urine (100 μl) was applied to the column and the eluant collected in Luckhams tubes. The column was eluted by the addition of 0.5 ml aliquots of distilled water and the fractions (0.5 ml) running off the column were collected and assayed for radioactivity.

4.2.4 ^{111}In-labelled probes: Labelling efficiency and purity assessment

Dextrin-DTPA and D-2-S-DTPA were radiolabelled with [^{111}In]indium as described in Chapter 2 (Section 2.4.6). The polymers were analysed for the presence of free [^{111}In] indium using GPC and spin centrifugation. The samples (100 μl) were passed down a column (90 cm x 1.6 cm) containing Sephacryl 400HR at a flow rate of 0.5 ml/min in citrate buffer pH 4 containing NaCl (150 mM). The column was calibrated with blue dextran (void volume) and [^{111}In]indium. Fractions (5 ml) were collected and assayed for radioactivity and the polymer peaks were collected and placed in a centriprep (Mw cut-off 8 kDa) and were centrifuged at 500 rpm for 5 min. Repeated centrifugation and purification continued until there was no more [^{111}In]indium being released.

4.2.5 Body distribution of ^{111}In-labelled dextrin and ^{111}In-labelled D-2-S: Evaluation using gamma camera imaging and dissection analysis

Male Wistar rats (200-250 g) were injected i.p. with a tracer dose of ^{111}In –labelled dextrin (0.7 MBq in 140 μl) or ^{111}In-labelled D-2-S (0.55 MBq in 300 μl) and lightly held on the gamma camera table for the 3 min scan. Scans were conducted at various times (0 h, 2 h, 4 h, 8 h and 24 h). At the end of the study the rats were killed and the following organs removed and assayed for radioactivity: brain and thyroid,

Table 4.4 The time course used to follow the biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after administration i.v., i.p. or s.c.

Polymer	Time Course of the Experiment		
	i.v.	i.p.	s.c.
^{125}I-labelled Dextrin	2 min	2 min	2 min
	5 min	5 min	5 min
	15 min	15 min	ND
	30 min	30 min	30 min
	45 min	45 min	ND
	1 h	1 h	1 h
^{125}I-labelled D-2-S	2 min	2 min	2 min
	1 h	1 h	1 h
	ND	2.5 h	ND
	ND	5 h	ND
	ND	10 h	ND
	ND	24 h	ND

ND – Not determined

heart and lung, liver, spleen, kidney and bladder, gut, carcass and i.p. wash. The results were expressed as radioactivity (% recovered dose).

4.3 Results

4.3.1 ^{125}I-Labelled probes: Labelling efficiency and purity assessment

Paper electrophoresis of the reaction mixture and the purified ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S preparations are shown in Figures 4.2 and 4.3. The labelling efficiency, specific activity and purity of the probes are shown in Table 4.2, and it can be seen that the levels of free [^{125}I]iodine were always less than 1 %.

4.3.2 Biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after s.c. administration

When ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S were administered s.c. the overall recovery of radioactivity in the organs (as a % of the administered dose) was very low (Figure 4.4, Figure 4.5 and Appendix 1, Table 1). Recovery of ^{125}I-labelled dextrin increased over time from 4 % of the administered dose at 2 min to 9 % of the administered dose at 30 min. This was due to retention of radioactivity in the carcass. When the radioactivity present in the carcass was taken into account the overall recovery at 2 min was 88 % of the administered dose. Similarly, recovery of the radioactivity after injection of ^{125}I-labelled D-2-S increased from 5 % of the administered dose at 2 min to 20 % of the administered dose at 1 h. In both cases the majority of the dose remained localised to the injection site. No further time points were examined after s.c. administration of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S.

4.3.3 Biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after i.v. administration

After i.v. administration of ^{125}I-labelled dextrin (Figure 4.6) only 29 % of the injected dose was recovered in the bloodstream at 2 min. Rapid blood clearance continued over 1 h. Most of the radioactivity recovered was found in the liver; 18 % of the administered dose after 2 min falling to 8 % of the administered dose after 1 h. There was a marked increase in the amount of radioactivity found in the bladder at 1 h showing that the polymer was being rapidly degraded and/or excreted from the body.

Similarly, administration of ^{125}I-labelled D-2-S (Figure 4.7) led to most of the radioactivity being found in the liver. After 2 min 25 % of the administered dose was

Figure 4.2 Paper electrophoresis of ^{125}I-labelled dextrin: Reaction mixture and preparation

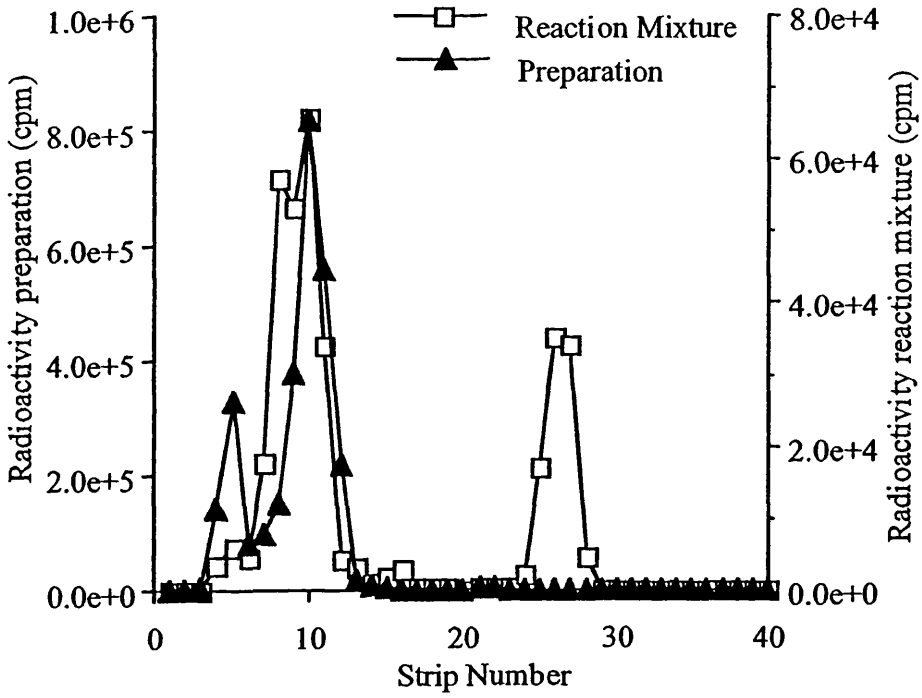


Figure 4.3 Paper electrophoresis of ^{125}I-labelled D-2-S: Reaction mixture and preparation

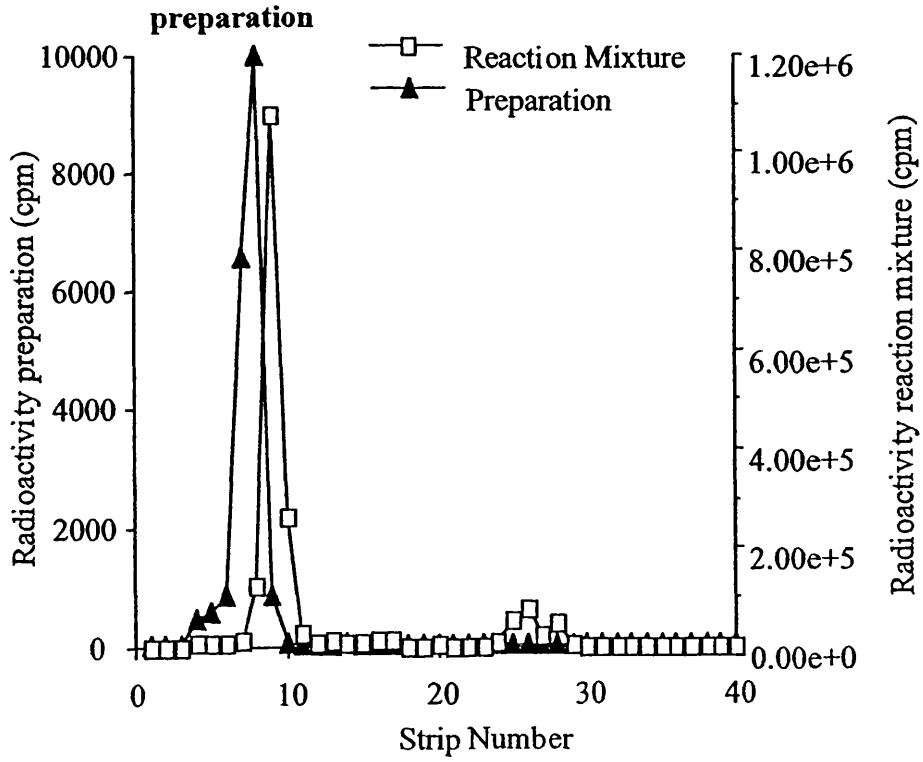


Table 4.2 Characteristics of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S

Sample	Labelling efficiency (%)	Free [^{125}I] iodine preparation (%)	Specific Activity (μCi/mg)
Dextrin	79.6	1.0	12.2
	80.1	1.0	11.8
	76.4	0.8	9.4
D-2-S	89.3	1.0	1.15
	86.1	0.9	1.2
	82.9	0.9	1.2

Figure 4.4 Body distribution of ^{125}I-labelled dextrin after s.c. administration to Wistar rats (n=3 ± SD)

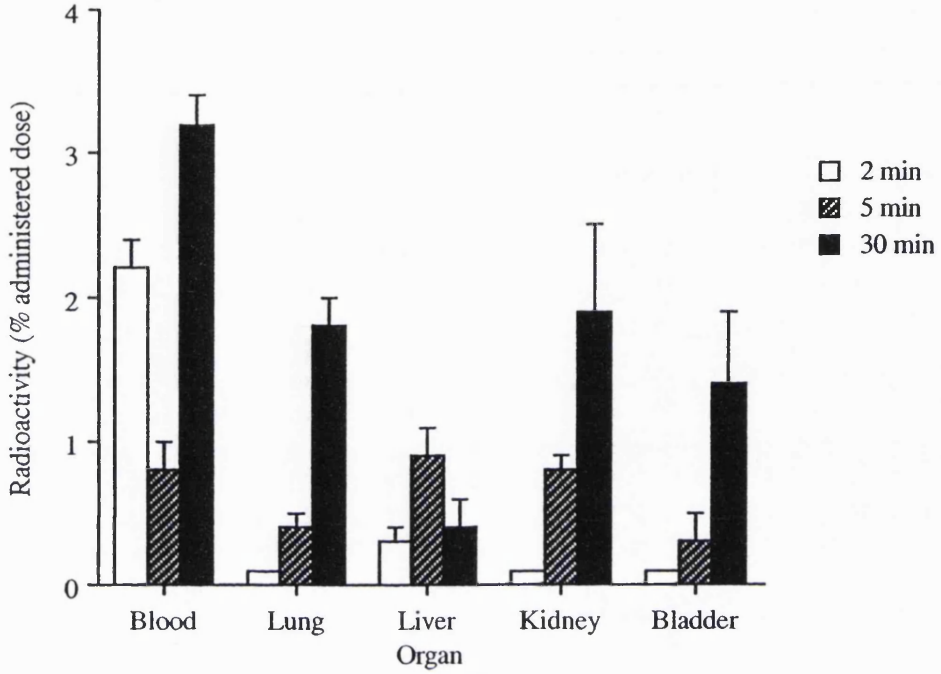


Figure 4.5 Body distribution of ^{125}I-labelled D-2-S after s.c. administration to Wistar rats (n=3 ± SD)

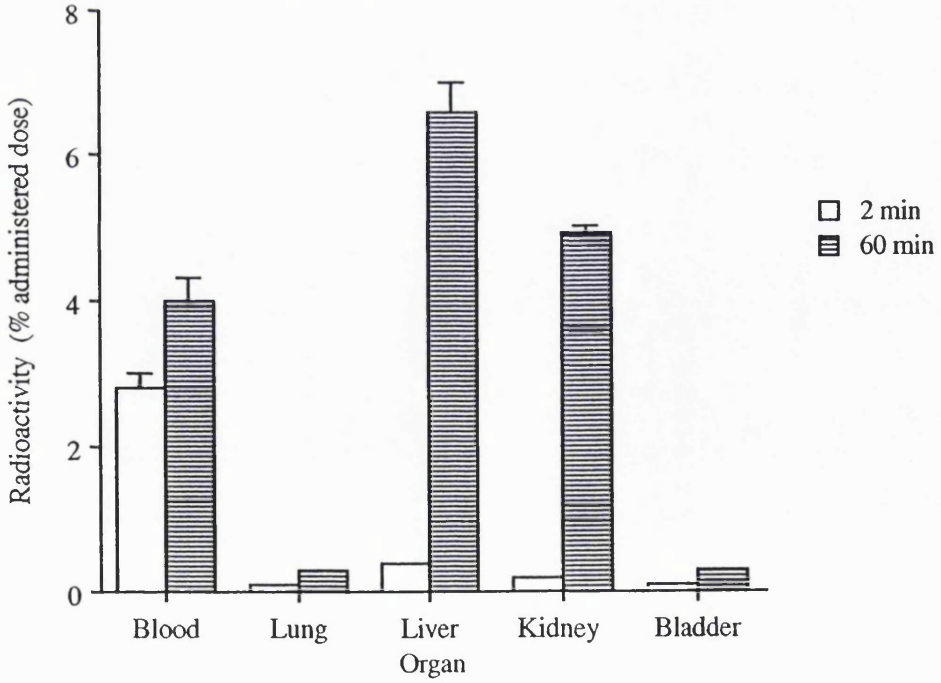


Figure 4.6 **Body distribution of ^{125}I-labelled dextrin after i.v. administration to Wistar rats (n=3 ± SD)**

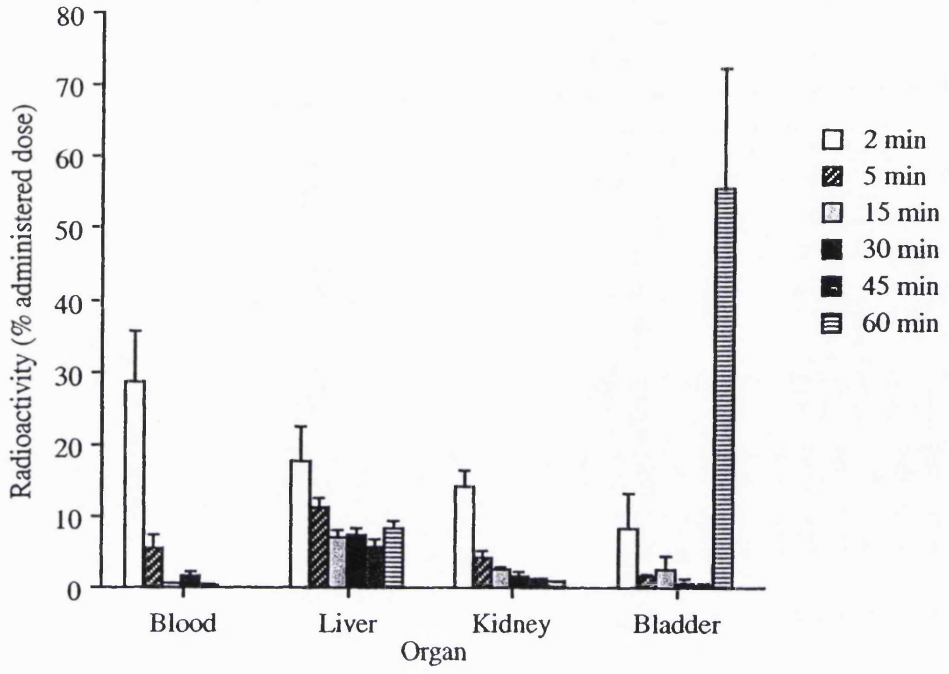
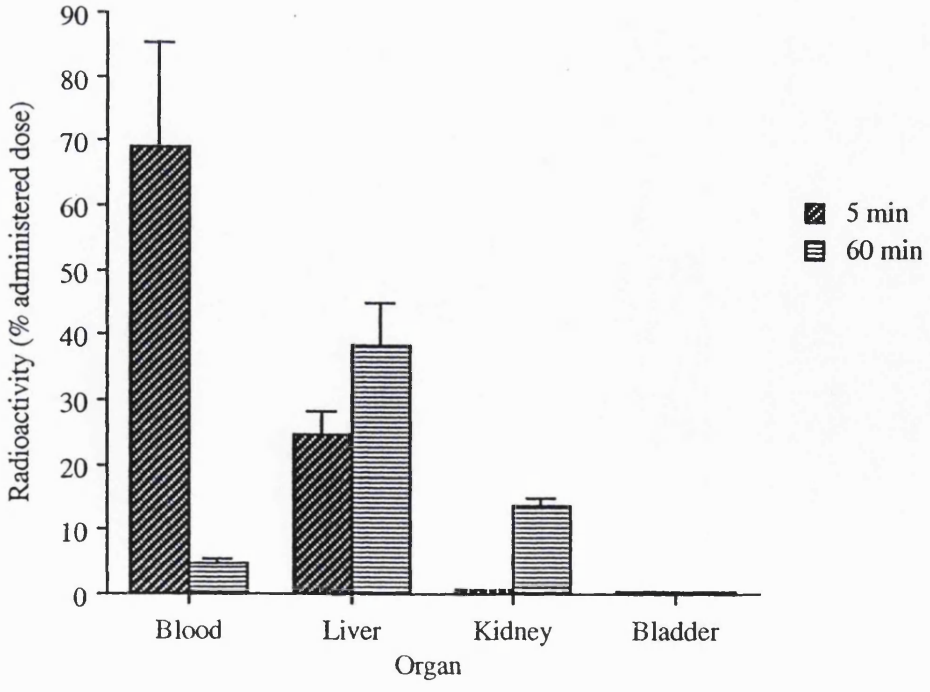


Figure 4.7 **Body distribution of ^{125}I-labelled D-2-S after i.v. administration to Wistar rats (n=3 ± SD)**



found in the liver with the value rising to 38 % of the administered dose after 1 h. ^{125}I-Labelled D-2-S quickly left the bloodstream, 69 % of the administered dose present after 2 min and only 5 % of the administered dose found in the blood after 1 h. Administration of ^{125}I-labelled D-2-S i.v. led to signs of toxicity (even at the low dose used). The animals appeared stressed, hunched-up with fur standing on end. Therefore no further time points were investigated using i.v. route for administration of ^{125}I-labelled D-2-S. All results are summarised in Appendix 1, Table 2 and Table 3.

4.3.4 Biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after i.p. administration

After i.p. administration of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S to male Wistar rats there were obvious differences in their pattern of distribution. All results are summarised in Appendix 1, Table 4 and Table 5 and Figure 4.8 – 4.21.

During the first hour ^{125}I-labelled dextrin was very quickly lost from the peritoneal cavity. Only 43 % of the administered dose remained after 30 min and only 5 % of the administered dose after 1 h (Figure 4.8). In contrast ^{125}I-labelled D-2-S was retained in the peritoneal cavity for much longer with 57 % of the administered dose remaining after 1 h and 6 % of the administered dose after 10 h (Figure 4.9).

Very small amounts of both polymers entered the bloodstream (Figure 4.10 and 4.11). The maximum amount of ^{125}I-labelled dextrin in the blood was 8 % of the administered dose at 45 min. ^{125}I-Labelled D-2-S was transferred into the bloodstream over the first 1 – 4 h. The maximum blood level was slightly higher than that seen for dextrin being approximately 9 % of the administered dose at 1 h and 2.5 h. Both ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S showed progressive accumulation over time in the liver and lung (Figures 4.12 – 4.15). The ^{125}I-labelled D-2-S recovery in the liver was much higher than that seen for ^{125}I-labelled dextrin at 60 min.

The thyroid was routinely taken but very little radioactivity was detected there in any of the samples indicating that deiodination or presence of free [^{125}I]iodine was not influencing the results.

4.3.5 Analysis of the radioactivity in urine after biodistribution

Urine samples were collected after the i.p. administration of ^{125}I-labelled polymers. Urine obtained from an animal given ^{125}I-labelled dextrin (1 h time point)

Figure 4.8 **Recovery of ^{125}I-labelled dextrin over time after i.p. administration (n=3 ± SD)**

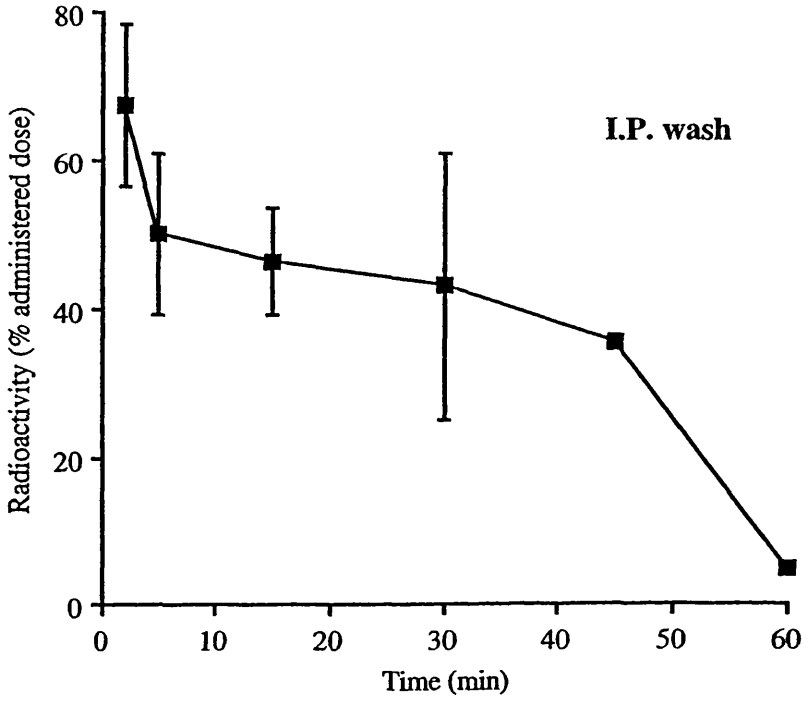


Figure 4.9 **Recovery of ^{125}I-labelled D-2-S over time after i.p. administration (n=3 ± SD)**

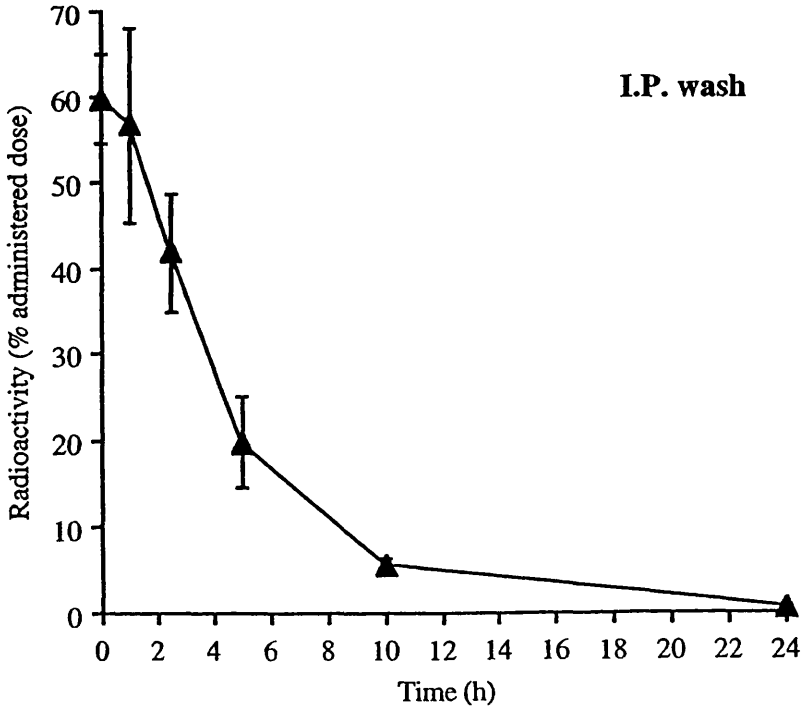


Figure 4.10 Recovery of ^{125}I-labelled dextrin over time after i.p. administration (n=3 ± SD)

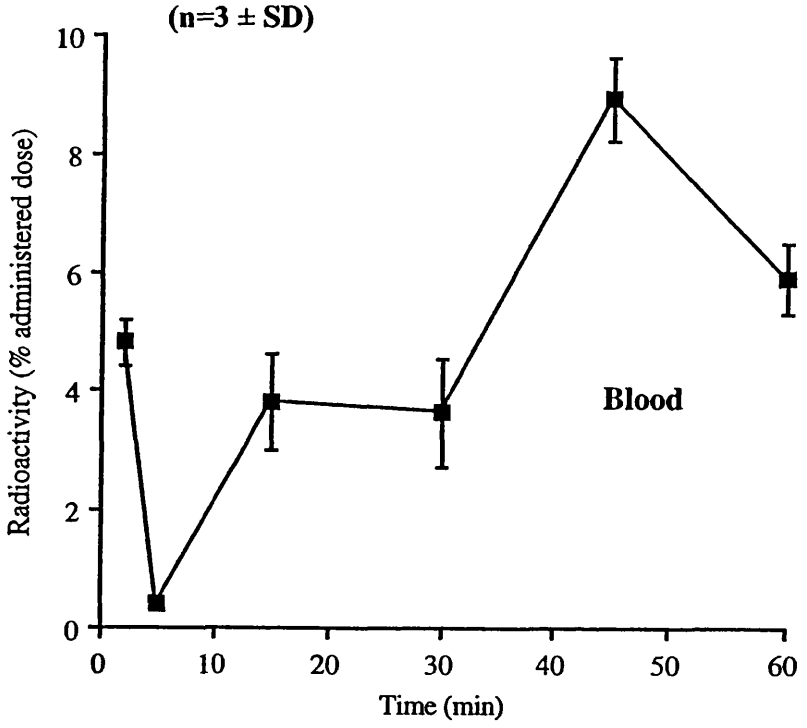


Figure 4.11 Recovery of ^{125}I-labelled D-2-S over time after i.p. administration (n=3 ± SD)

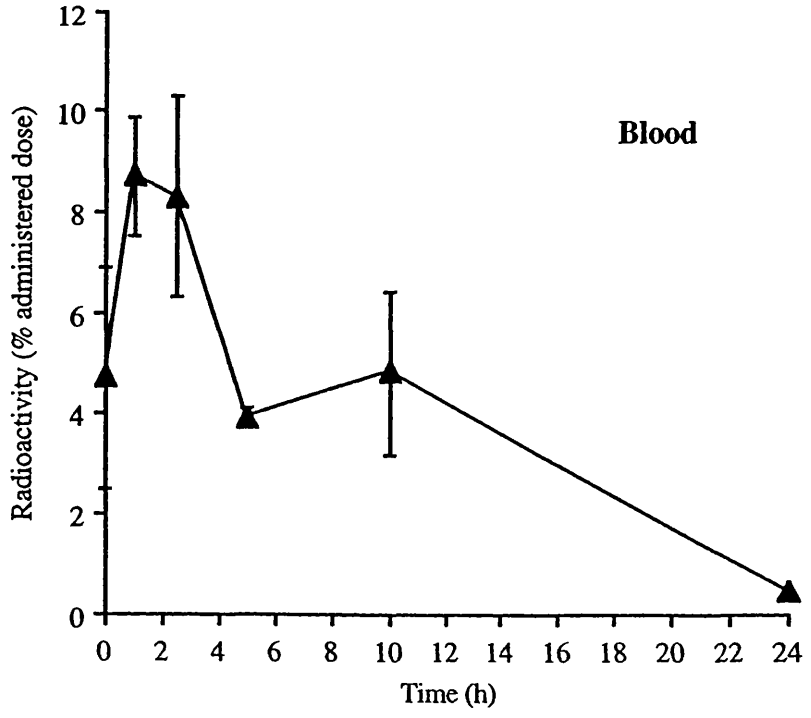


Figure 4.12 Recovery of ^{125}I-labelled dextrin over time after i.p. administration (n=3 ± SD)

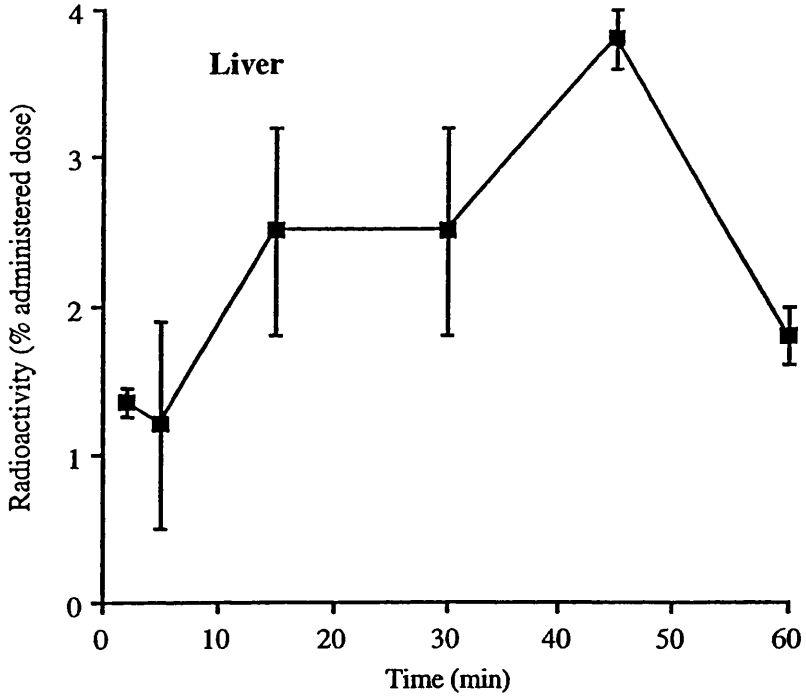


Figure 4.13 Recovery of ^{125}I-labelled D-2-S over time after i.p. administration (n=3 ± SD)

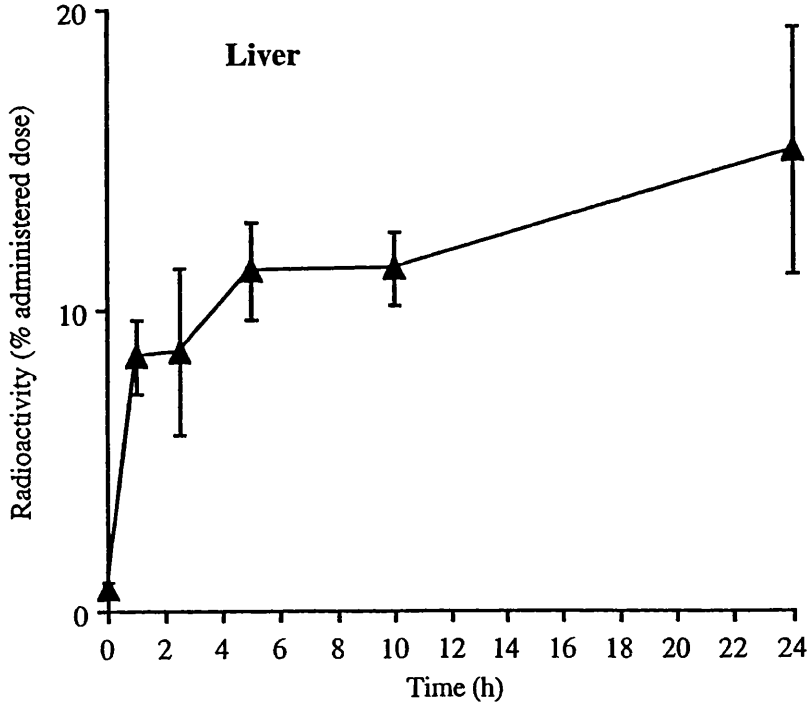


Figure 4.14 **Recovery of ^{125}I-labelled dextrin over time i.p. after administration (n=3 ± SD)**

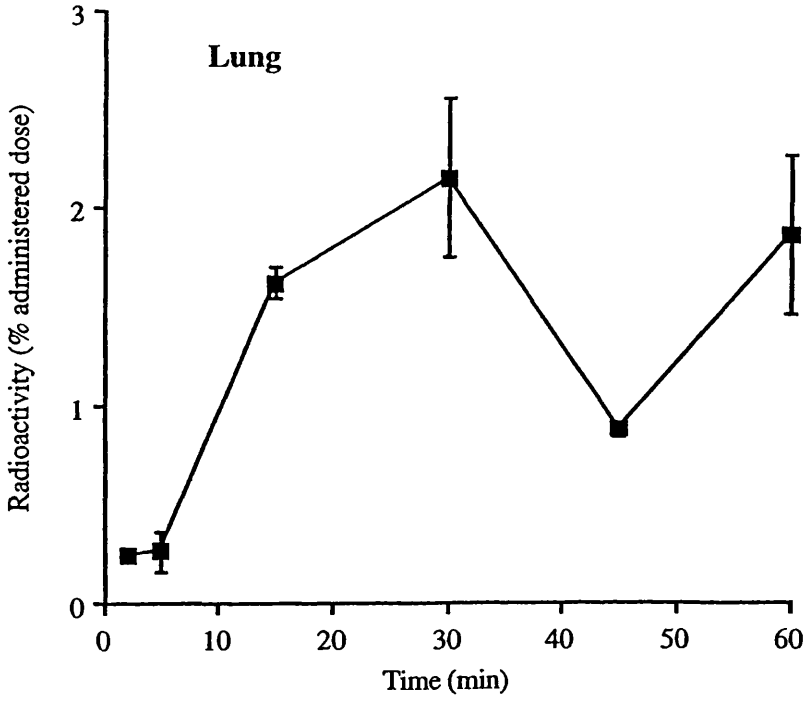


Figure 4.15 **Recovery of ^{125}I-labelled D-2-S over time i.p. after administration (n=3 ± SD)**

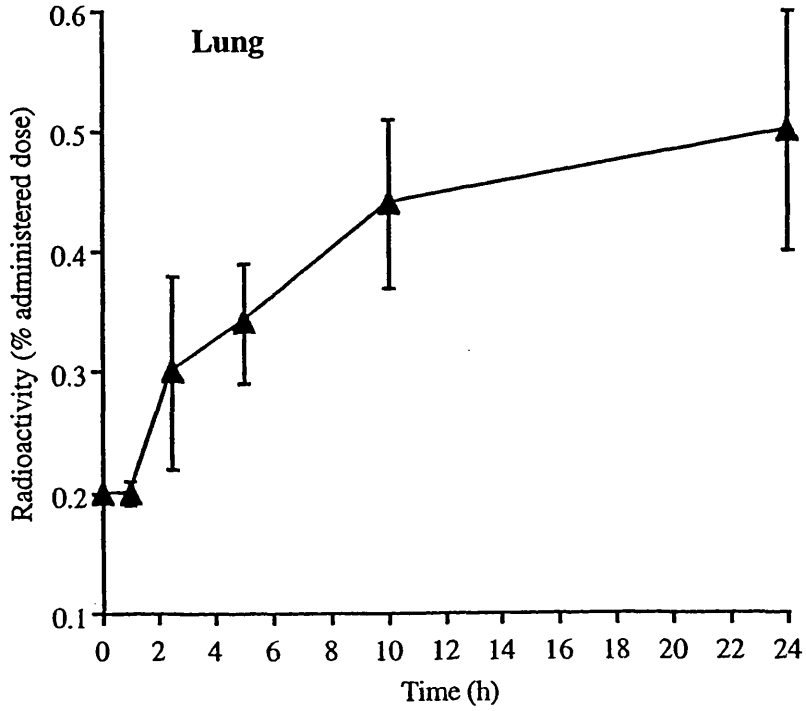


Figure 4.16 Recovery of ^{125}I-labelled dextrin over time after i.p. administration (n=3 ± SD)

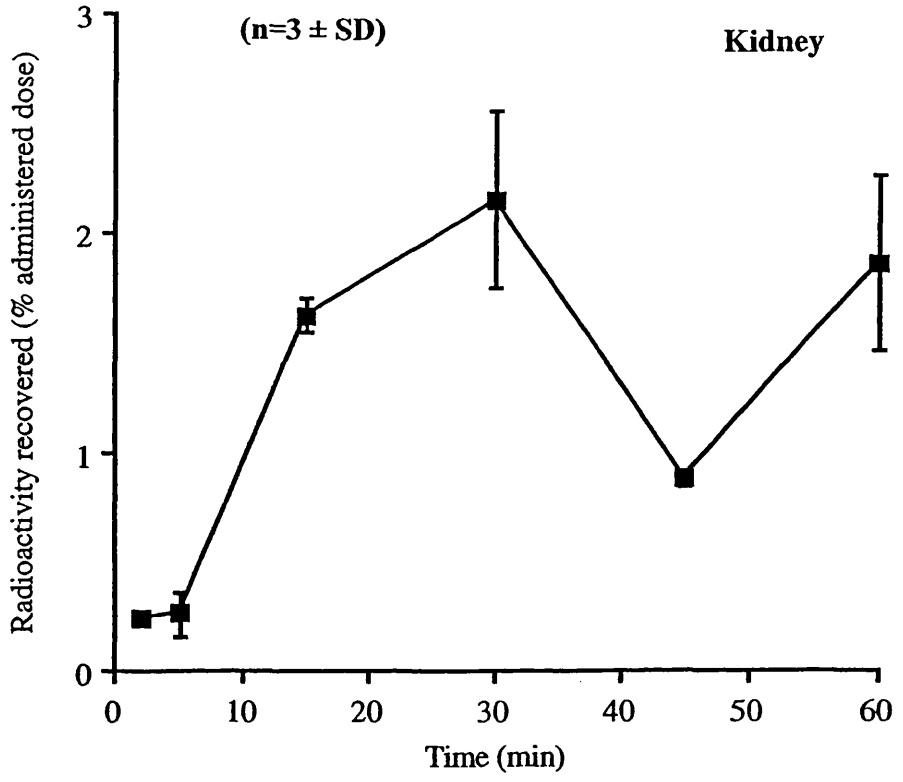


Figure 4.17 Recovery of ^{125}I-labelled D-2-S over time after i.p. administration (n=3 ± SD)

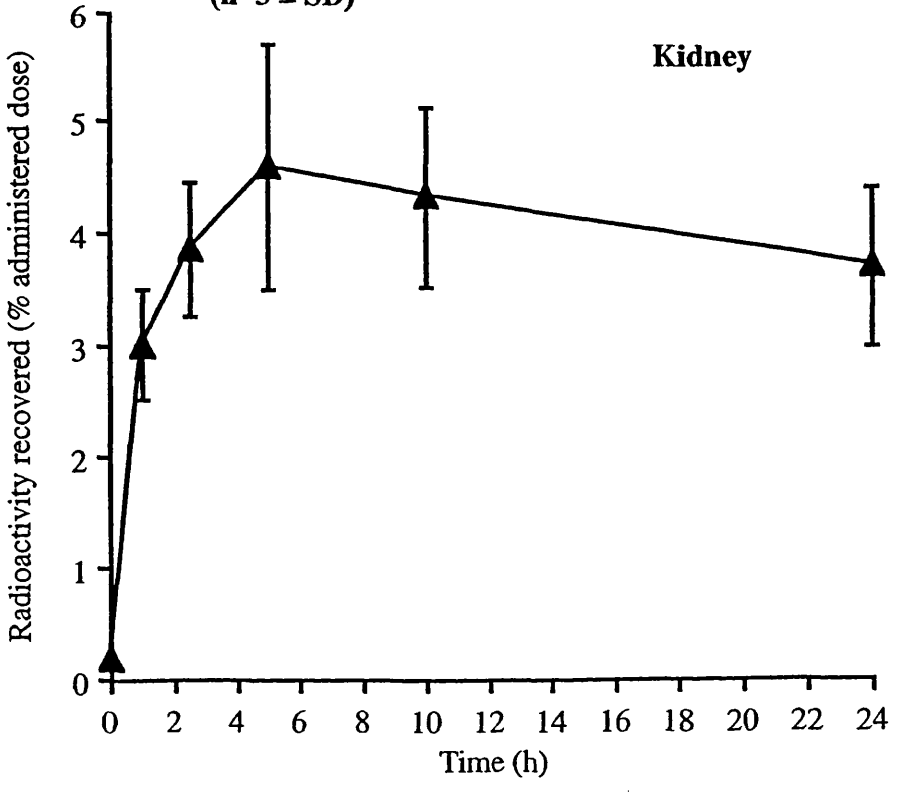


Figure 4.18 Recovery of ^{125}I-labelled dextrin over time after i.p. administration (n=3 ± SD)

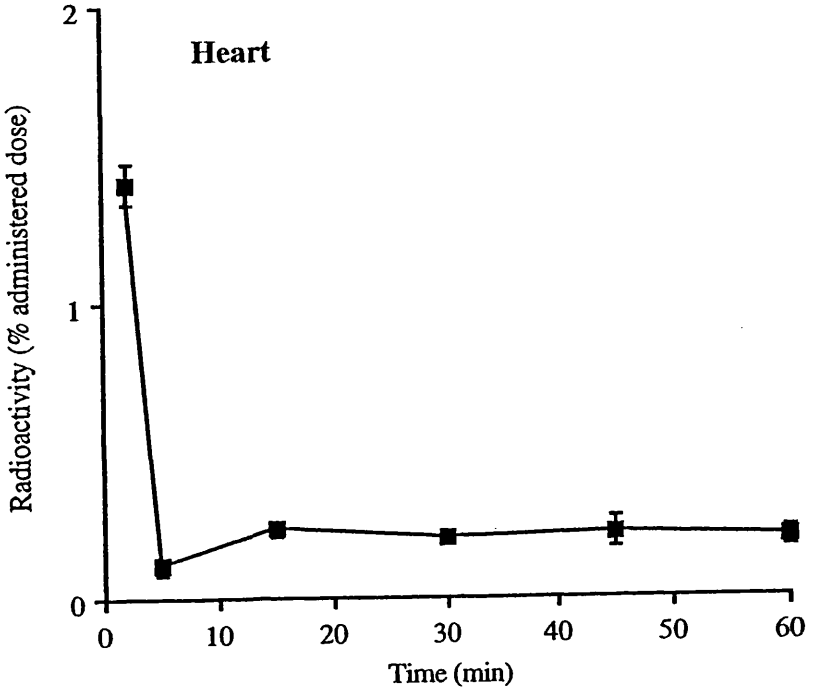


Figure 4.19 Recovery of ^{125}I-labelled D-2-S over time after i.p. administration (n=3 ± SD)

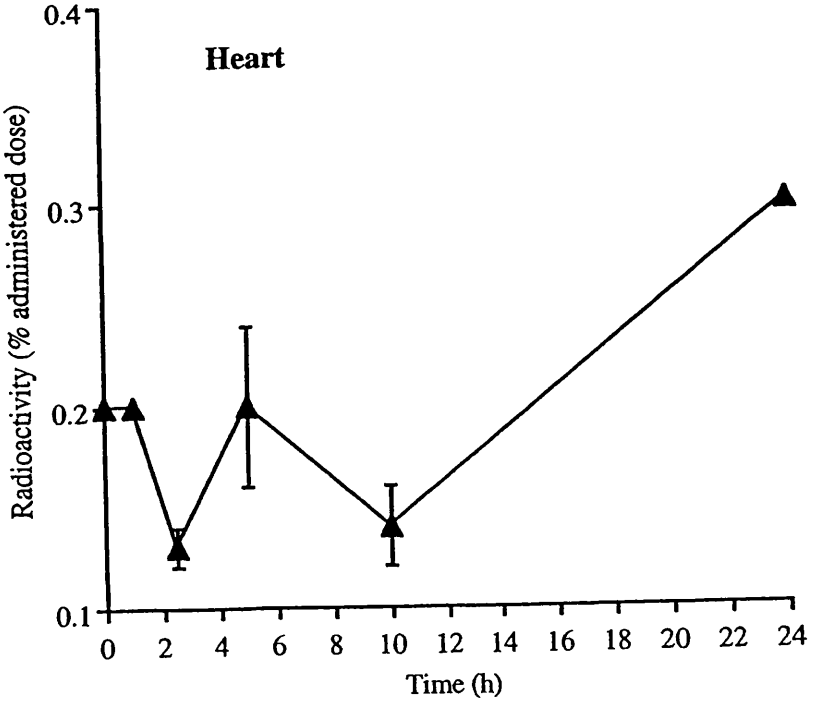


Figure 4.20 **Recovery of ^{125}I-labelled dextrin over time after i.p. administration (n=3 ± SD)**

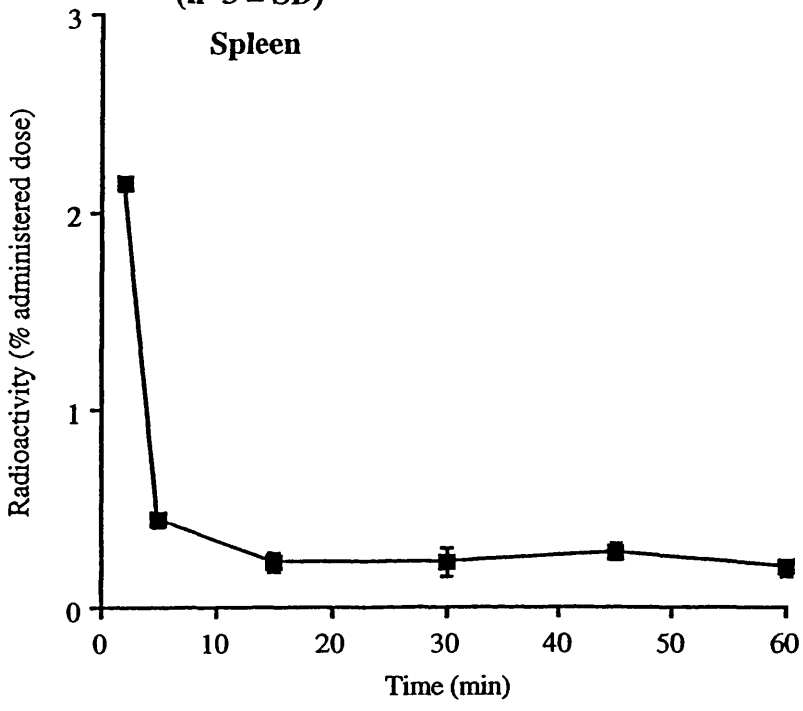
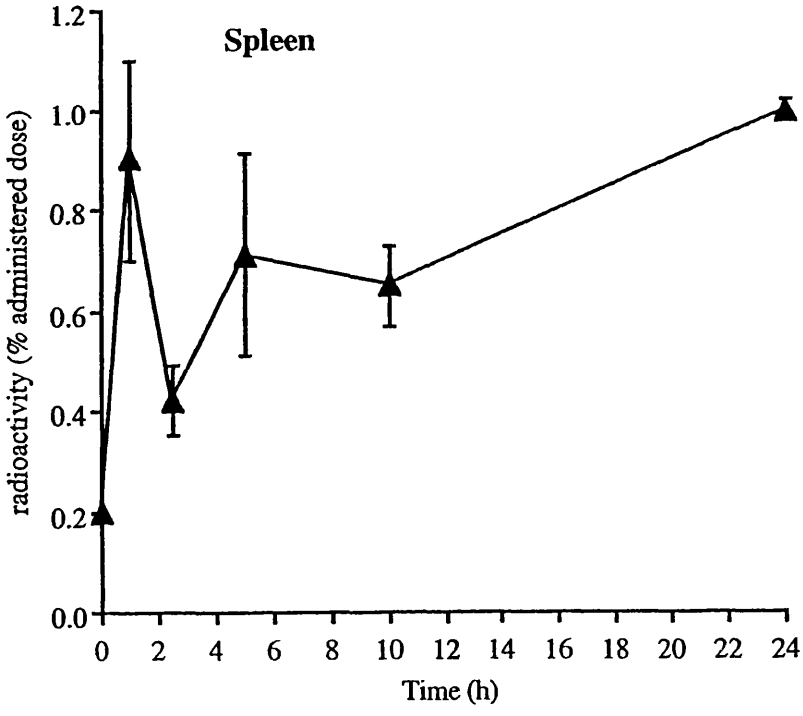


Figure 4.21 **Recovery of ^{125}I-labelled D-2-S over time after i.p. administration (n=3 ± SD)**



contained a peak eluting at 6 ml and a second peak eluting at 13 ml (Figure 4.22). This would suggest that the polymer has partially degraded in the body. The peak for the [^{125}I]iodine eluted at 17 ml. Urine obtained from an animal given ^{125}I-labelled D-2-S (24 h time point) contained a peak which elutes at 14 ml while the urine collected after 2.5 h gave peaks at 6 ml and 19 ml (Figure 4.23).

4.3.6 ^{111}In-labelled probes: Labelling efficiency and purity assessment

The GPC elution profile of the reaction mixture and the purified preparations of ^{111}In-labelled dextrin and ^{111}In-labelled D-2-S from the column are shown in Figures 4.24 and 4.25. Both of the ^{111}In-labelled polymers showed two distinct peaks with the second peak being free [^{111}In]indium. As free [^{111}In]indium passed down the column can interact with the column or aggregate in part, it elutes very close to the polymer peak. Thus the polymer fractions collected were purified carefully by using a spin centrifugation before use. The ^{111}In-labelled polymers used in the imaging contained less than 1 % free [^{111}In]indium.

4.3.7 Biodistribution of ^{111}In-labelled dextrin and ^{111}In-labelled D-2-S after i.p. administration

The images of rats injected with ^{111}In-labelled dextrin and ^{111}In-labelled D-2-S and free [^{111}In]indium are shown in Figure 4.26 and Figure 4.27 and Figure 4.28. The images seen were consistent with the results obtained using ^{125}I-labelled polymers. ^{111}In-Labelled dextrin quickly left the peritoneal cavity with very little of the radioactivity remaining after 24 h. In contrast, ^{111}In-labelled D-2-S remained in the body for much longer with visible accumulation in the liver. Free [^{111}In]indium showed a different distribution with the majority of the dose accumulating in the liver.

Following gamma camera studies it was also possible to carry out dissection analysis to quantitate the amount of radioactivity in each organ, and the distribution of ^{111}In-labelled dextrin (Figure 4.29), ^{111}In-labelled D-2-S (Figure 4.30) and free [^{111}In]indium (Figure 4.31) at 24 h. The results are summarised in Appendix 1, Table 6.

4.4 Discussion

Dextrin and D-2-S are administered to patients via the i.p. route as Icodextrin or in an Icodextrin solution. Although the subsequent fate of these polymers has been

Figure 4.22 Analysis of the urine collected 1 h after i.p. administration of ^{125}I-labelled dextrin

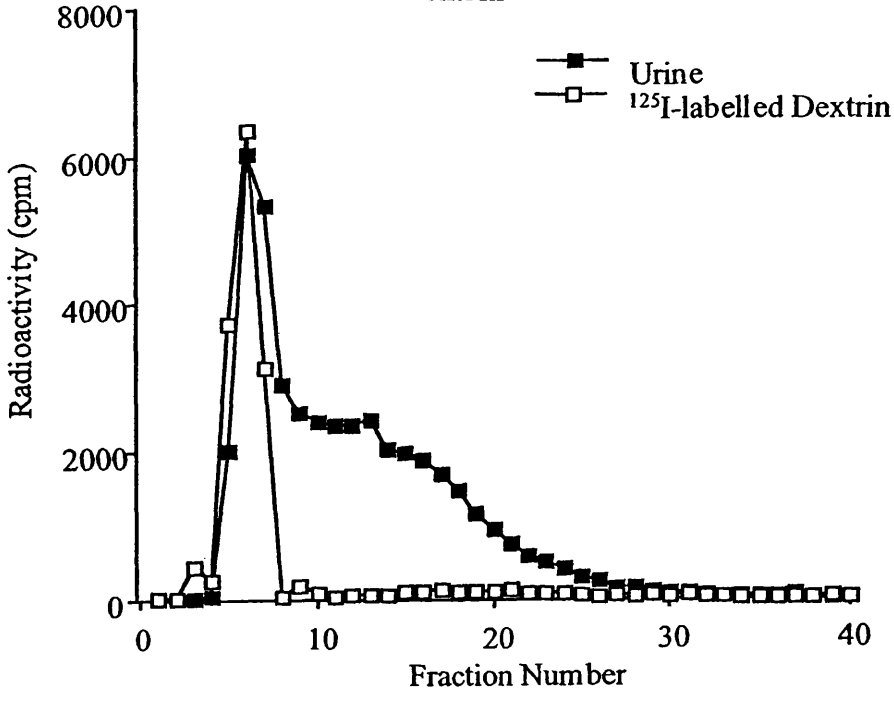


Figure 4.23 Analysis of the urine collected 2.5 h after i.p. administration of ^{125}I-labelled D-2-S

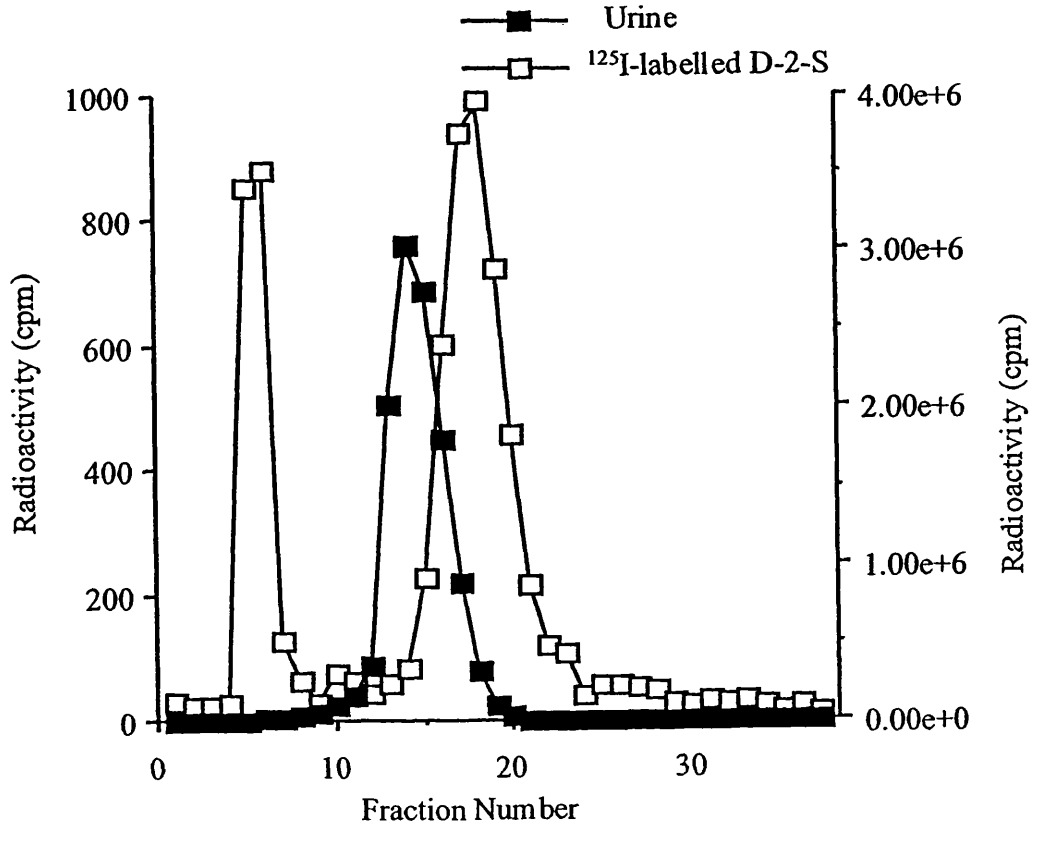


Figure 4.24 S400HR GPC elution profile of ^{111}In-labelled dextrin

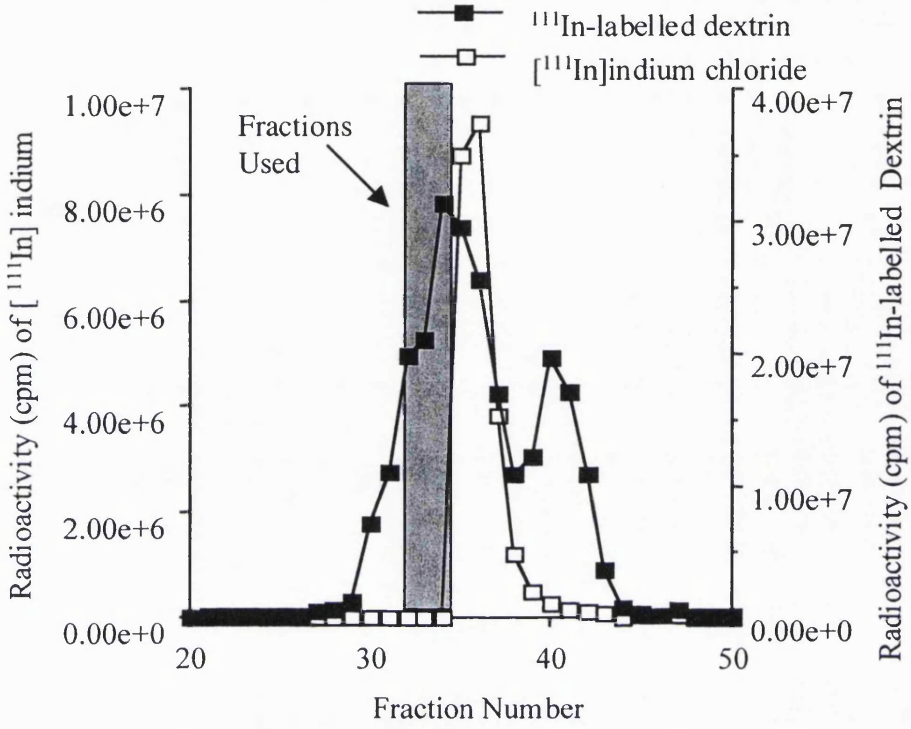


Figure 4.25 S400HR GPC elution profile of ^{111}In-labelled D-2-S

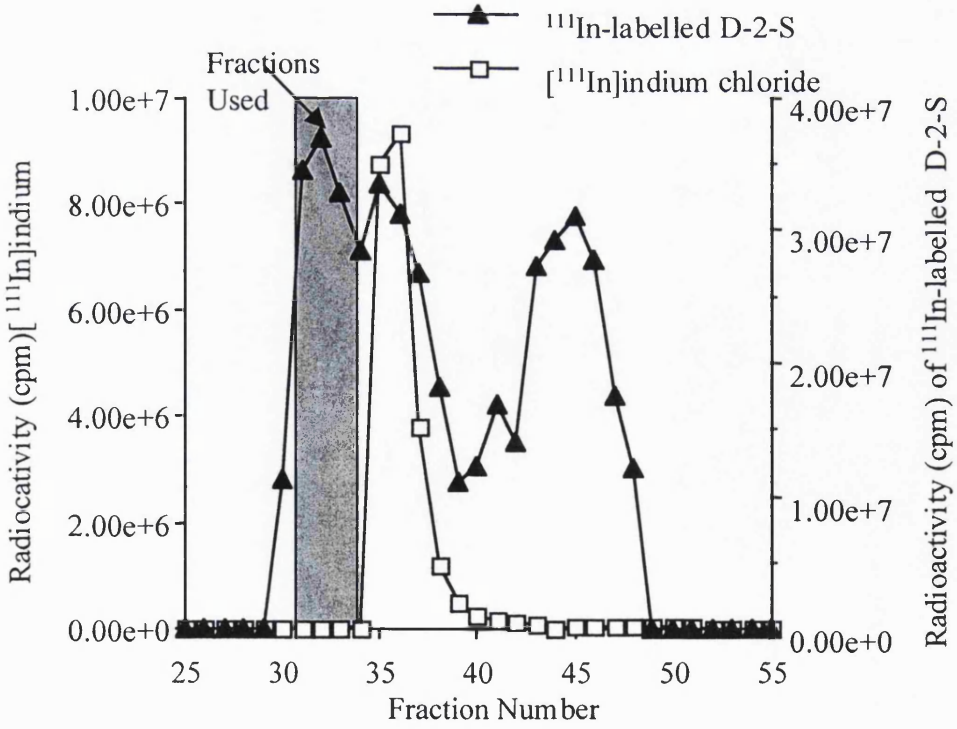


Figure 4.26 Gamma camera images of rats injected with ^{111}In-labelled dextrin (100µl) after i.p. administration

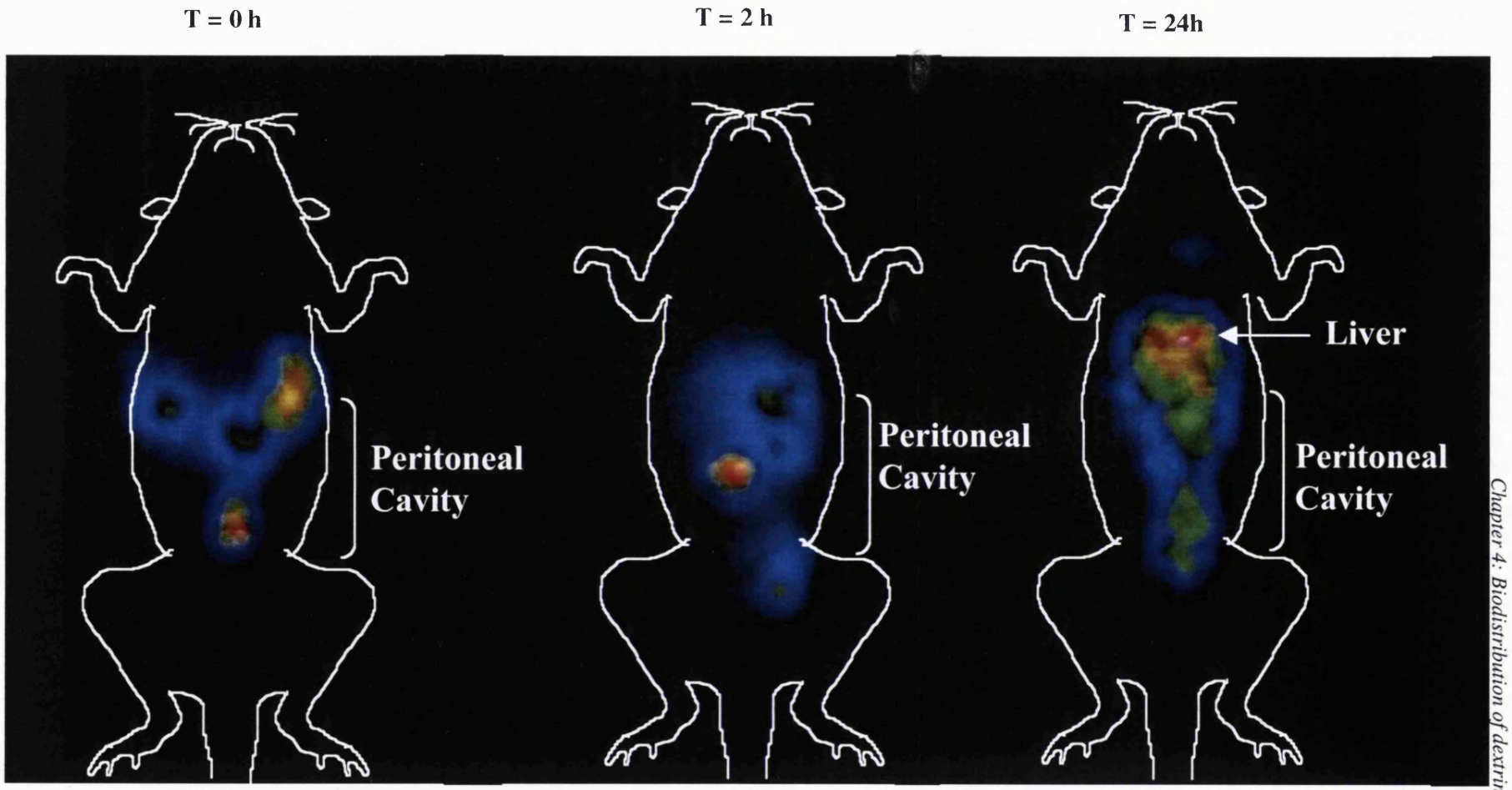


Figure 4.27 Gamma camera images of rats injected with ^{111}In-labelled D-2-S (100 µl) after i.p. administration

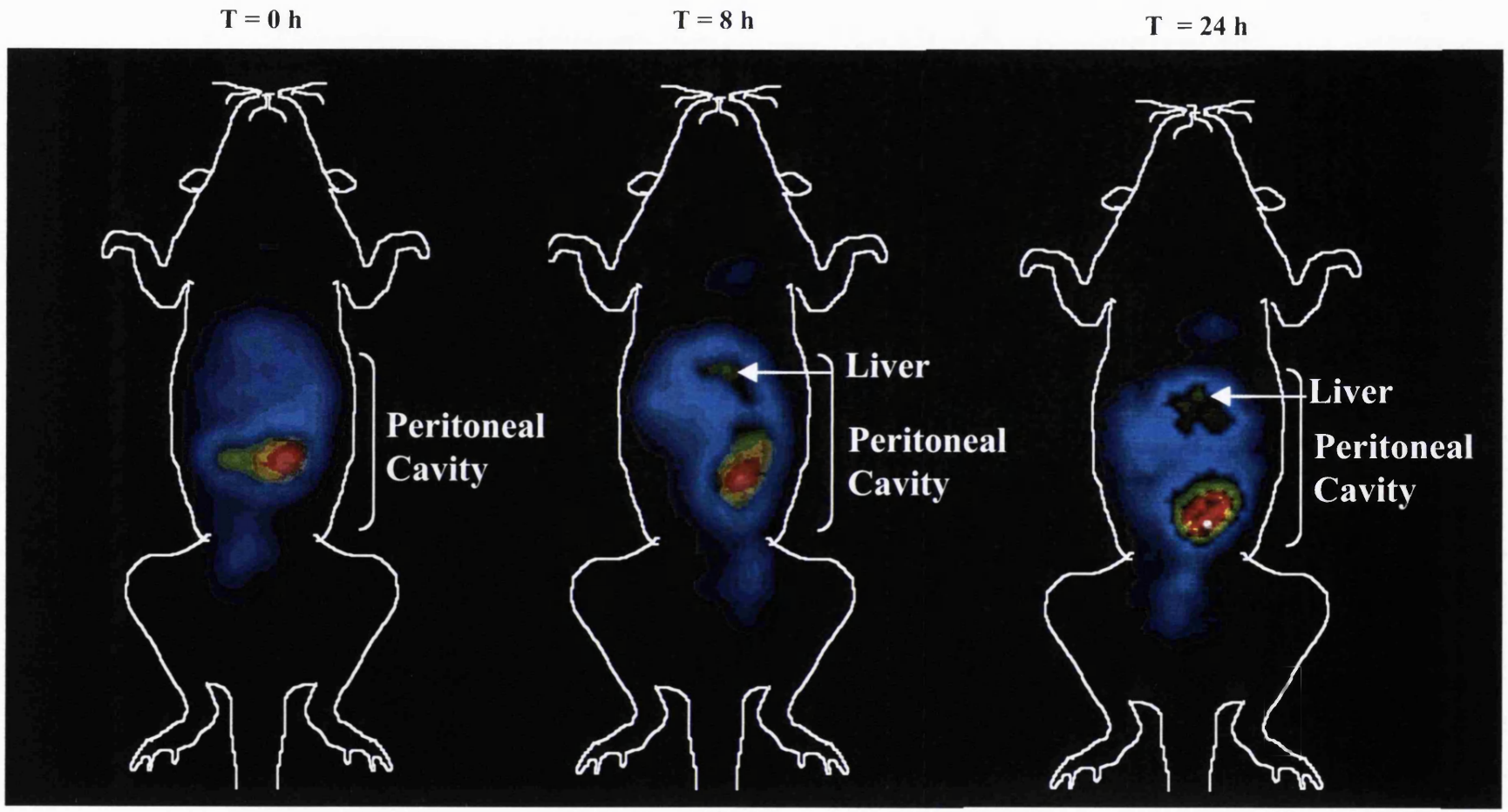


Figure 4.28 Gamma camera images of rats injected with free [^{111}In]indium chloride after i.p. administration

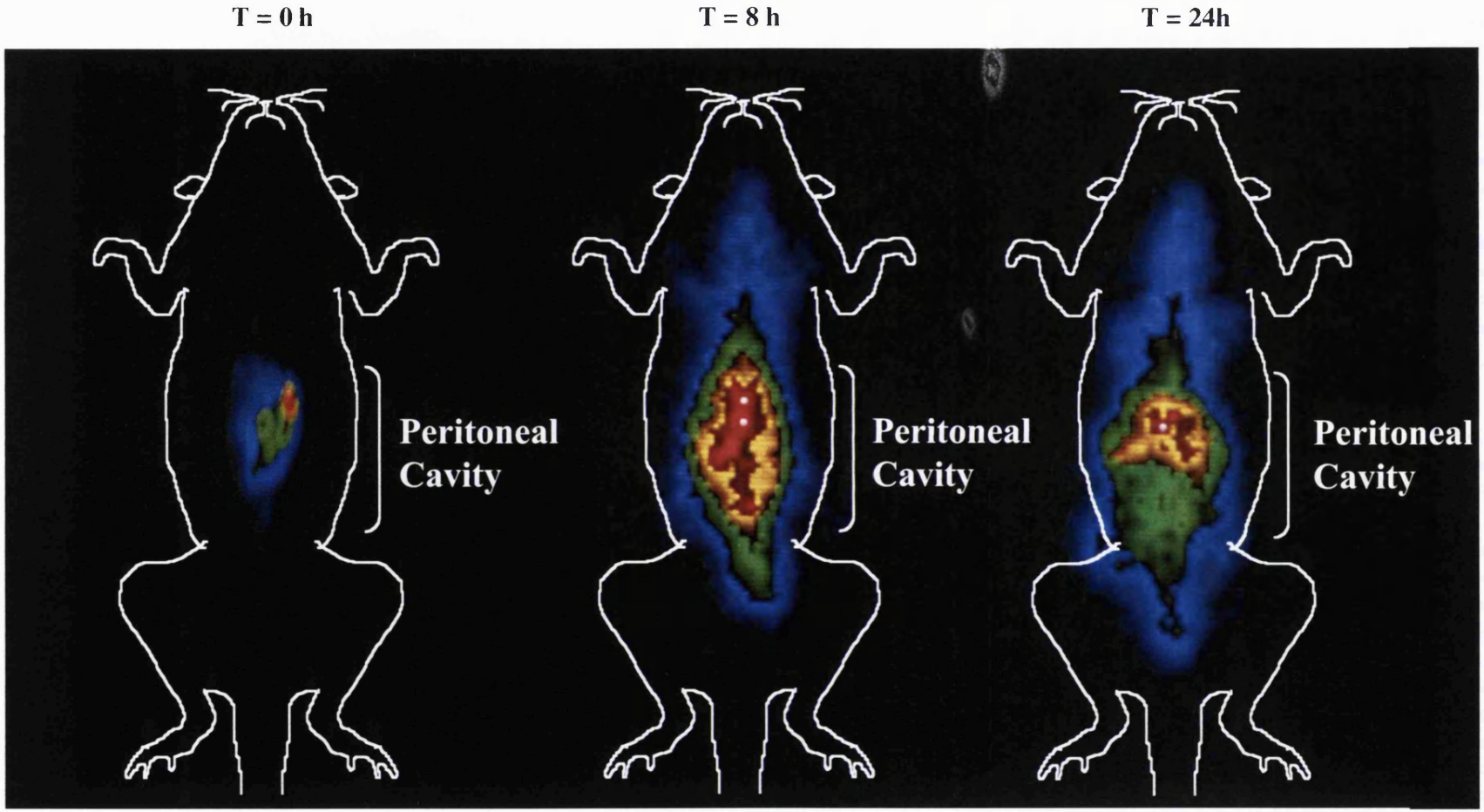


Figure 4.29 Biodistribution (24 h) of ^{111}In-labelled dextrin after i.p. administration (n=2)

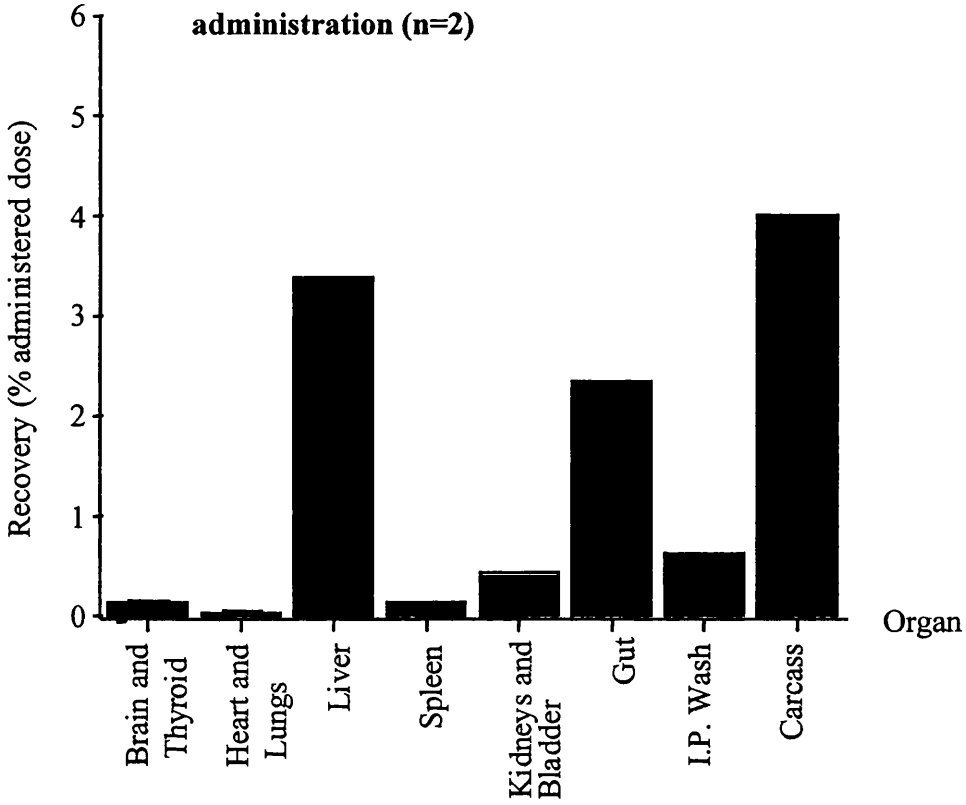


Figure 4.30 Biodistribution (24 h) of ^{111}In-labelled D-2-S after i.p. administration (n=2)

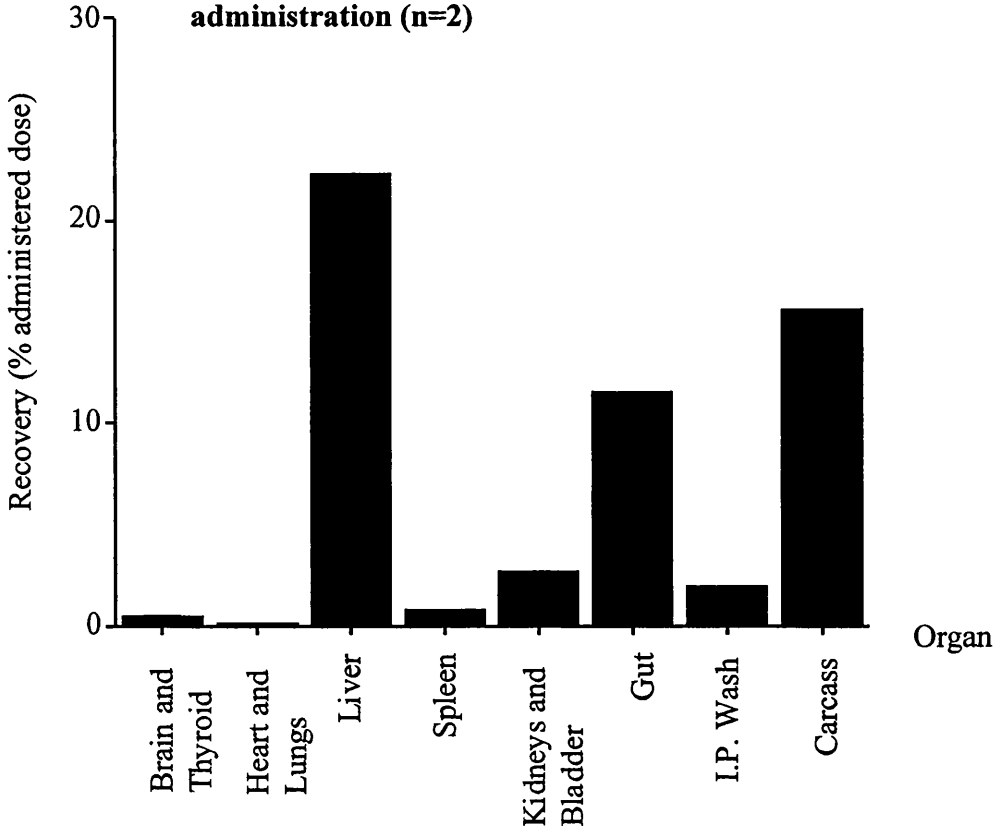
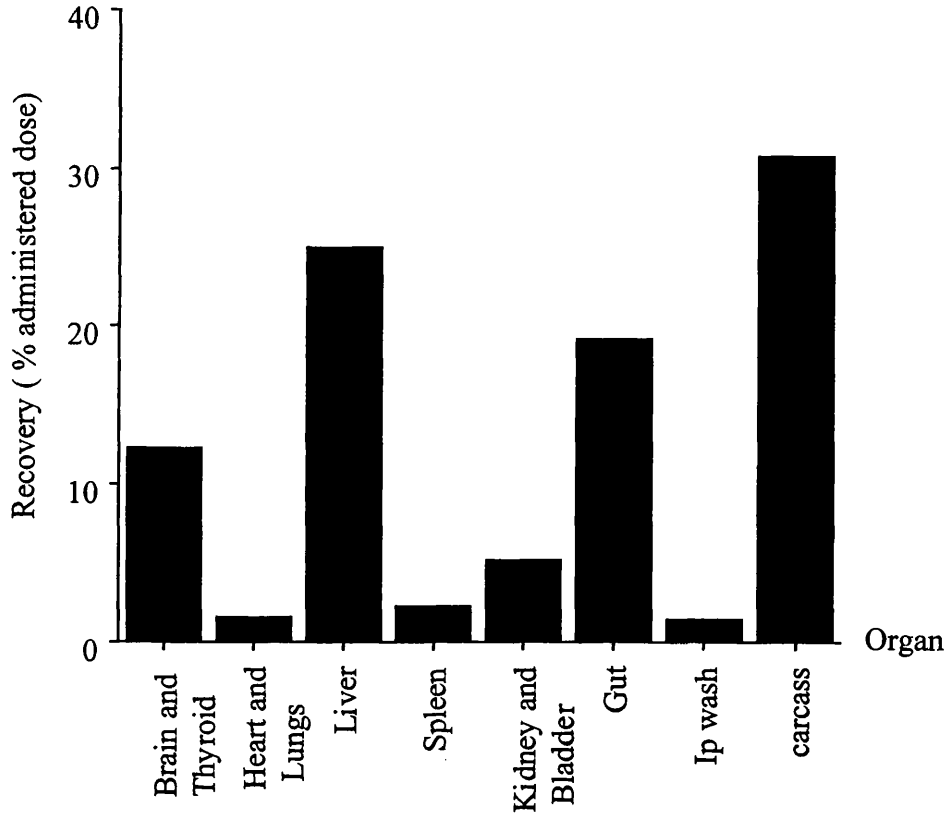


Figure 4.31 Biodistribution (24 h) of [^{111}In]indium chloride after i.p. administration (n=2)



investigated in the clinic there has never been any systematic study of their biodistribution in animals or man. This study was carried out (1) to quantitate the fate of dextrin and D-2-S and (2) to develop a gamma camera imaging agent that could provide a non invasive method for studying biodistribution in future clinical studies.

After i.p. administration, polymers can theoretically either adhere to the peritoneal wall or rapidly leave the peritoneal cavity. Exit can be via the lymphatic system or by passing directly into blood vessels lining the peritoneal cavity. Here, it was found that ^{125}I-labelled D-2-S remained in the peritoneal cavity for up to ten times longer than the ^{125}I-labelled dextrin (Figures 4.8 and 4.9). Studies with radiodinated probes and gamma camera imaging both supported this conclusion. The low total recovery of the radioactivity of the injected dose seen following administration of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S might indicate that these polymers were distributing to other tissues. This was clear when carcass levels of radioactivity were assessed. It is possible that polymers transferred via the lymphatics are retained there rather than continuing on into the bloodstream. It should be note that the rapid removal of the ^{125}I-labelled dextrin from the peritoneal cavity could also be due to the fact that dextrin is degraded very quickly by plasma α-amylase which is present in peritoneal fluid. Studies in our laboratory (Hrezcuk-Hirst *et al.*, 1999) have shown that the rate of dextrin degradation by procine pancreatic α-amylase was dependant upon the degree (mol%) of succinoylation. Unmodified dextrin was degraded within minutes (Figure 4.32) as would be the 1 mol% succinoylated probe here.

Studies from other laboratories have shown that dextran accumulates in the liver and spleen (Yamaoka *et al.*, 1995). It was thus considered interesting to make a direct comparison of the biodistribution of dextran and dextrin (Table 4.3). It can be seen that dextran remained longer in the circulation than dextrin, but it should be noted that the Mw of dextran studied here was 197 000 Da; i.e. double that of the dextrin used here. This may in part be an explanation for the difference in their biodistribution. Dextrin is excreted from the body much quicker than dextran. Once again this could be due to the difference in polymer molecular weight and/or the rate of degradation of the backbone. The differences in structure (dextrin containing more α1-4 linkages) may also be important.

Table 4.3 Comparison of the biodistribution (1 h) of ^{125}I-labelled dextrin and ^{125}I-labelled dextran after i.v. administration to rats .

Organ	Dextran†	Dextrin††
Blood	49.8 ± 5.1	0.1 ± ND
Heart	0.2 ± 0.1	0.1 ± ND
Lung	0.1 ± 0.1	0.7 ± 0.1
Liver	12.7 ± 0.9	8.3 ± 1.1
Spleen	1.0 ± 0.2	0.5 ± ND
Kidney	0.5 ± 0.1	0.9 ± 0.1
Spleen	1.0 ± 0.2	0.5 ± ND
GI Tract	2.8 ± 1.4	*
Thyroid	0.4 ± 0.1	0.1 ± ND
Carcass	4.1 ± 1.8	*
Urine and Faeces	28.4 ± 1.7	55.6 ± 16.8
Total Recovery	83.5 ± 2.1	65.0± 18.1

Results shown are expressed as % administered dose

† from Yamaoka *et al.*, 1995

†† from this study

* not determined

As mentioned in the introduction, radioiodination adds a pendant group to polymers which might in theory influence biodistribution. Therefore it was pleasing that the biodistribution seen following administration of ^{125}I-labelled polymers was confirmed by the gamma camera images seen following i.p. administration of ^{111}In-labelled polymers. From the images (and also the dissection analysis) we found that dextrin and D-2-S showed uptake in the liver. This may occur for a number of reasons. Firstly, it is known that macrophages have cell surface receptors that recognise glucose. Secondly, it is known that have shown that the free [^{111}In]indium shows high uptake in the liver (Figure 4.31). This can be due to the transchelation of [^{111}In]indium onto transferrin in plasma (6-10 % per day).

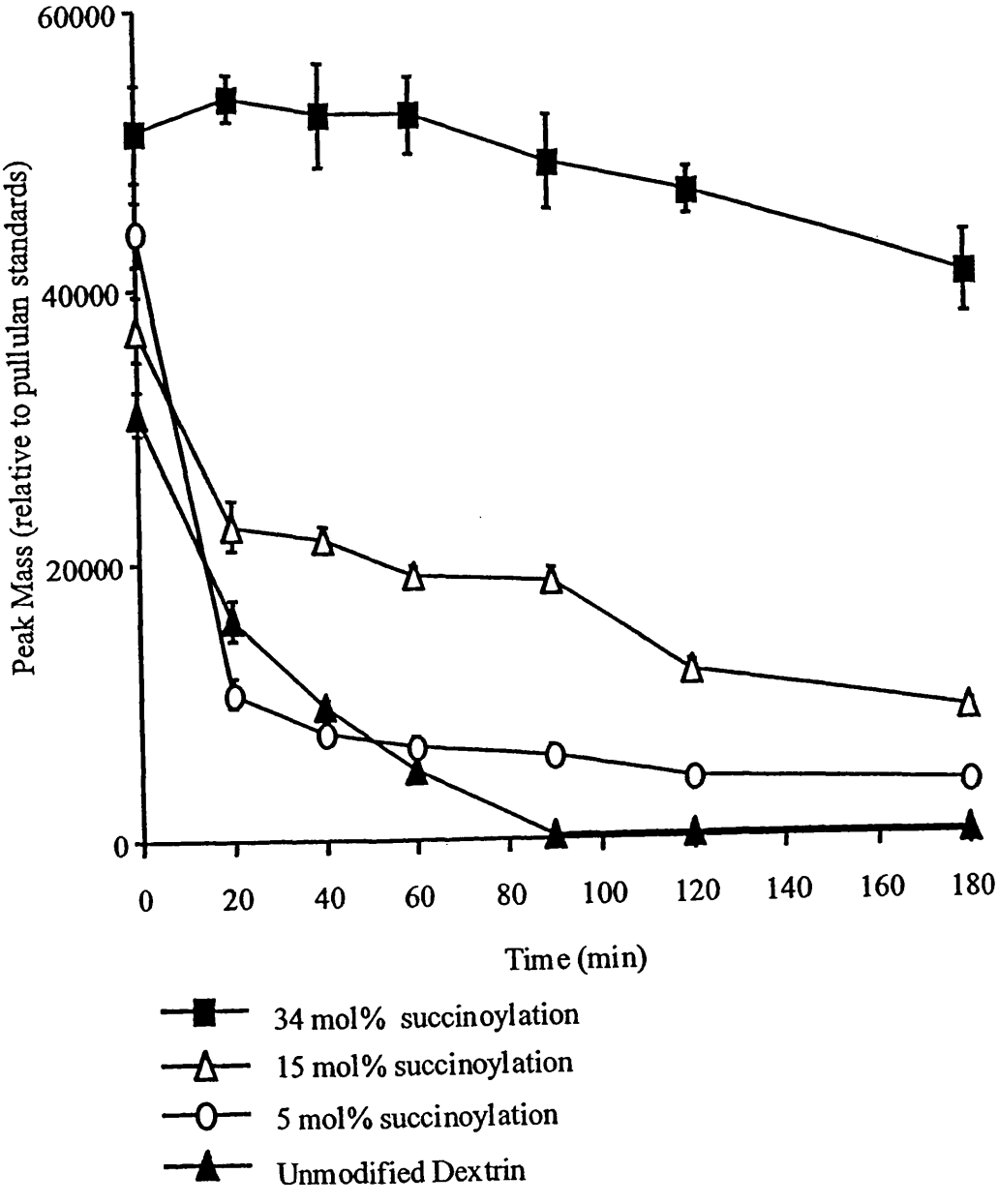
Observations made during the imaging study showed that the 100 µl injection volume may cause localisation of radioactivity at the site of injection in the peritoneal cavity. It was felt that increasing the volume injected may alter this pattern of distribution (this has been studied further in the next Chapter).

The differences in the D-2-S biodistribution seen when compared to dextrin could be due to the fact that D-2-S is a sulphated polysaccharide. Sulphated conjugates tend to be excreted into the general circulation and the 'SAINT clinical trial' suggested that 2.5 g of D-2-S was absorbed by patients (Shaunak *et al.*, 1997), although it was never detectable in the blood and no anticoagulant effect was seen.

^{125}I-Labelled dextrin showed no toxicity after i.v. administrationMost of the radioactivity recovered (1 h) 55.6 % was found in the urine At this time only 8.3 % of the dose was recovered in the liver. After i.v. administration of ^{125}I-labelled alginate (Mw 48 000 Da), 70 % of the recovered dose was excreted in the urine (1 h). Very little radioactivity was recovered in any of the other organs. The exceptions were the liver which contained 7.8 % of the administered dose and the small intestine 2.8 % of the administered dose after 1 h. The biodistribution of ^{125}I-labelled dextrin (51 000 Da) and ^{125}I-labelled alginate (48 000 Da) was very similar. Intravenous administration of ^{125}I-labelled D-2-S caused toxicity in the animals when administered at a maximum time of 1 h so no further studies were continued.

Subcutaneous administration of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S resulted in localisation of almost all of the radioactivity at the injected site probably due

Figure 4.32 Degradation of succinoylated dextrin modified to different mol % with α-amylase (Hrezcuk-Hirst *et al.*, 1999)



to the slow movement of the macromolecule into the bloodstream from the s.c. site via the lymphatic vessels (Flessner *et al.*, 1985). Injection of other polysaccharides s.c. also showed local retention. For example when ^{125}I-labelled alginate was injected s.c. to rats the overall recovery at 1 h was only 30 % (Al-Shamkani and Duncan, 1995).

In the studies reported here all radiolabelled polymers were administered as tracer doses given in small volumes. The next study (Chapter 5) investigates the effect of various parameters on biodistribution which might be important in the clinical setting:- volume of injected dose, increasing dose and repeated administration.

Chapter 5

Effect of injection volume, dose and repeated administration on the biodistribution of D-2-S

5.1 Introduction

The previous Chapter (Chapter 4) described the body distribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after i.p. administration. It should be noted that these studies were carried out using a tracer dose of the polymer given in small volumes (100 µl). During clinical administration however, there are many factors which might be expected to influence biodistribution. These include the volume of administration, polymer dose and also the frequency of polymer administration.

As the clinical studies reported to date have varied these parameters, it was thought important to investigate their effect on D-2-S biodistribution here.

For example, when Icodextrin was used as an i.p. infusion for the cytotoxic drug 5-FU (Kerr *et al.*, 1996) a dose of 5-FU (300 mg/ml) of given. The volume administered to patients was increased from 900 ml to 1750 ml depending on the patient size.

Three clinical trials have been conducted to evaluate D-2-S administered as a treatment for HIV. The polymer was administered in the carrier solution. These trials used different D-2-S doses and also different volumes of the carrier fluid Icodextrin. The Phase I /II dose escalation study called the 'SAINT trial' (Shaunak *et al.*, 1998) used a standard volume of 1.5 L of 7.5 % Icodextrin solution (Extraneal) for administration of D-2-S. Patients received seven courses of D-2-S administered over 28 d. Six patients were divided into three groups with each group receiving a different dose escalation course. Patients A and B were given D-2-S as follows 37.5 mg (5 d), 75 mg (5 d), and 150 mg (18 d). Patients C and D were given 37.5 mg (2 d), 75 mg (2 d) 112 mg (2 d) and 150 mg (22 d) of D-2-S and patients E and F were given 150 mg (28 d) of D-2-S (Shaunak *et al.*, 1998).

The TITAN trial (Leen *et al.*, 2000) used a D-2-S dose of 150 mg for each infusion which was given 3 times weekly for 8 or 12 weeks in a standard volume of 1 L of a 4 % Icodextrin solution (Dexemel).

The ATLAS trial (Leen *et al.*, 2000) used a D-2-S dose of 225 mg for each infusion which was given 3 times weekly for 8 weeks in a standard volume of 1.5 L of a 4 % Icodextrin solution (Dexemel).

In all of these clinical trials D-2-S was given by the i.p. route. This route seems logical as most HIV-1 replication occurs in the lymphatic system. Therefore by

administering D-2-S via the peritoneal cavity the drug can be delivered directly into the lymphatic circulation. (Shaunak *et al.*, 1999).

In this study, ^{125}I-labelled D-2-S and ^{111}In-labelled D-2-S were used as probes to examine the factors that might affect the biodistribution of D-2-S after i.p. administration to Wistar rats. The parameters studied were:

a) Injection volume (100 µl to 2.5 ml),

b) Dose (0.1 mg/kg to 10 mg/kg)

c) Effect of repeated dosing.

5.2 Methods

Earlier studies (Chapter 4, Figure 4.8 - 4.12) examined the biodistribution of ^{125}I-labelled D-2-S and showed that 41.8 % of the of the administered dose was found in the peritoneal cavity 2.5 h after i.p. administration. Therefore a time point of 2.5 h was chosen to study the effect of injection volume, dose and repeated administration on biodistribution of D-2-S.

5.2.1 Probes: Labelling efficiency and purity assessment

^{125}I-Labelled D-2-S and the ^{111}In-labelled D-2-S used in this study were prepared and characterised as described in Chapter 2 (Section 2.6.1.2) and Chapter 4 (Section 4.2.1 and 4.2.4). The ^{125}I-labelled D-2-S batch used here contained 0.9 % free [^{125}I]iodine and it had a specific activity of 1.2 µCi/mg. A new batch of D-2-S-DTPA was labelled with [^{111}In]indium for these studies (due to the half-life of [^{111}In]indium being 67.5 h). The ^{111}In-labelled D-2-S had a specific activity of 0.9 µCi/mg and contained 0.4 % of free [^{111}In]indium.

5.2.2 Effect of administration volume on the body distribution of ^{125}I-labelled D-2-S at 2.5 h after i.p. administration.

In all of the experiments Male Wistar rats (200-250 g) were injected i.p. with ^{125}I-labelled D-2-S (5×10^5 cpm, ~300 µg/kg) and placed in metabolic cages. The administration volume was varied (100 µl, 500 µl, 1 ml, 1.75 ml or 2.5 ml) but dose given was kept the same (~300 µg/kg). The animals were killed after 2.5 h and the following organs were removed, washed in PBS and assayed for radioactivity; liver,

heart, kidneys, lung, thyroid and spleen. In addition all of the gastrointestinal organs were removed and their contents were collected. The organs were weighed before homogenisation in distilled water using a polytron homogeniser. Radioactivity remaining in the peritoneal cavity was also assessed by an i.p. wash as described in Chapter 4 (Section 4.2.2). The final volume of each tissue homogenate was recorded. Radioactivity in the blood and organs was expressed as % of the administered dose.

5.2.3 Effect of D-2-S dose on the body distribution (2.5 h) after i.p. administration

Male Wistar rats (200-250 g) were injected i.p with ^{125}I-labelled D-2-S (5 x 10^5 cpm, - 300 μg/kg) containing also non-radiolabelled D-2-S to give doses of 0.025, 0.25, 0.5, 1.25 and 2.5 mg/kg. After injection animals were placed in their housing cages rather than metabolic cages. After 2.5 h animals were killed and the following organs were removed and washed in PBS before being assayed for radioactivity:- liver, heart, kidneys, lung, thyroid and spleen. In addition all of the gastrointestinal organs were removed and their contents were collected. The organs were weighed before homogenisation in distilled water using a polytron homogeniser. Radioactivity remaining in the peritoneal cavity was also assessed by an i.p. wash as described in Chapter 4 (Section 4.2.2). The final volume of each tissue homogenate was recorded. Radioactivity in the blood and organs was expressed as % of the administered dose.

5.2.4 Effect of repeated dosing on the body distribution of ^{125}I-labelled D-2-S after i.p. administration

Male Wistar rats (200-250 g) were injected i.p. with ^{125}I-labelled D-2-S (5 x10^5 cpm, ~300 μg/kg) in 1 ml of saline. Injections were given i.p. on days 0, 2, 4, 7 and 9 and animals were killed on day 11. The following organs were removed and washed in PBS and assayed for radioactivity as described above; liver, heart, kidneys, lung, thyroid and spleen. In addition all of the gastrointestinal organs were removed and their contents collected. The organs were weighed before homogenisation in distilled water using a polytron homogeniser. Radioactivity remaining in the peritoneal cavity was also assessed by an i.p. wash as described in Chapter 4 (Section 4.2.2). The final volume of each tissue homogenate was recorded. Radioactivity in the blood and organs

were expressed as % of the administered dose.

5.2.5 Effect of administration volume on the body distribution of ^{111}In-labelled D-2-S visualised by gamma camera imaging after i.p. administration

MaleWistar rats (200-250 g) were injected i.p. with a tracer dose of ^{111}In-labelled D-2-S (0.44 MBq in 100 µl, 1.5 ml or 2.5 ml) and lightly held on the gamma camera during 3 min scans. Images were taken over various time periods (0 h, 2 h, 6 h, 24 h). At the end of the study the rats were killed and the following organs were removed and assayed for the presence of radioactivity:- brain and thyroid, heart and lung, liver, spleen, kidney and bladder, gut, carcass and i.p. wash. The results were expressed as % of the administered dose.

5.3 Results

5.3.1 Effect of administration volume on the biodistribution of ^{125}I-labelled D-2-S after i.p administration

The injection volume influenced the radioactivity recovered in some organs (Figure 5.1 and Appendix 2, Table 1). For example, radioactivity detected in the liver went from 8.6 % to 15.6 % of the administered dose as the volume injected increased from 100 µl to 1 ml respectively ($p < 0.001$). At the same, time radioactivity detected in the spleen went from 0.4 % to 1.5 % of the administered dose ($p < 0.001$). This pattern of increased recovery was seen in other organs including the kidneys and the gastrointestinal tract ($p < 0.001$). However and surprisingly, the radioactivity recovered in the i.p. wash did not alter significantly with changing injection volume with similar peritoneal levels being found for all of the administration volumes. As the overall recovery was the greatest (89.9 % of the administered dose) at the 1 ml injection volume, this volume was chosen for studies to examine the effect of dose and repeated administration.

5.3.2 Effect of administration volume on the biodistribution of ^{111}In-labelled D-2-S after i.p. administration seen by gamma camera imaging

The injection volume also influence the radioactivity recovered in some of the organs (Figure 5.2a - 5.2e and Appendix 2, Table 2). There is a visible difference in the pattern of distribution with increasing administration volume. At 0 h, the 100 µl was

Figure 5.1 Effect of volume on the biodistribution of ^{125}I-labelled D-2-S (2.5 h) after i.p. administration (n=3 ± SD)

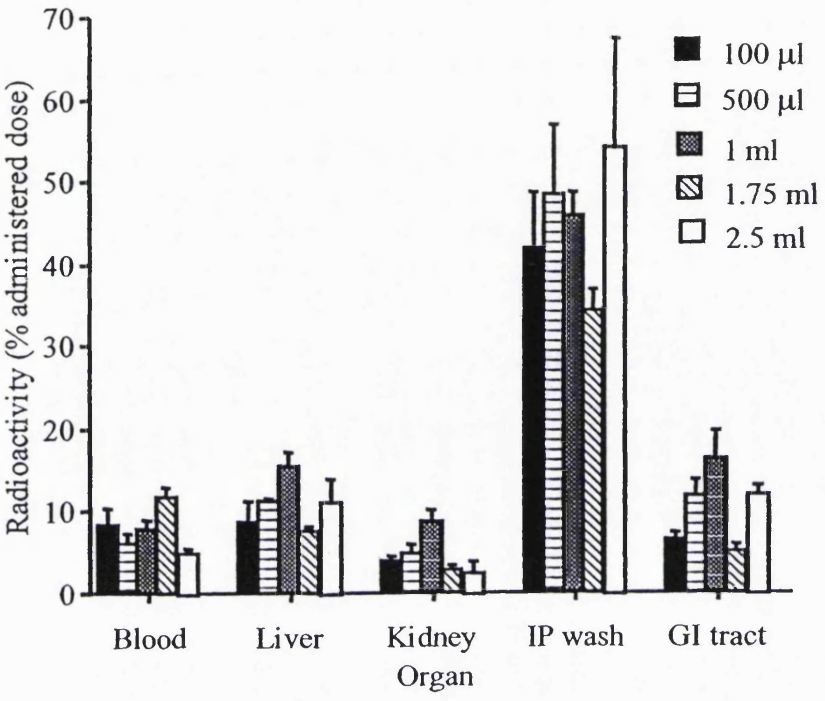


Figure 5.2a Gamma camera images of rats injected with ^{111}In-labelled D-2-S after i.p. administration with increasing the volume (t = 0 h)

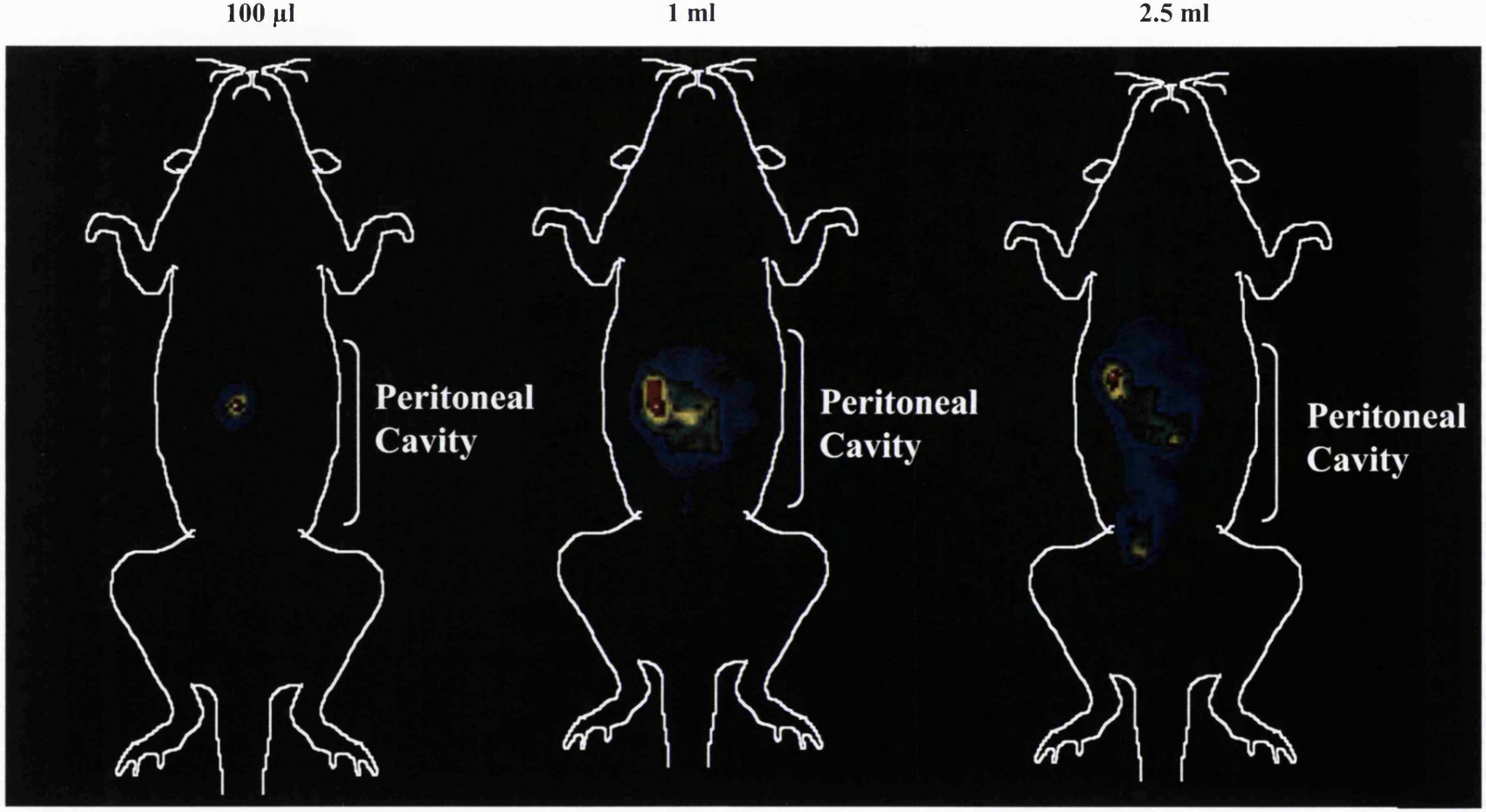
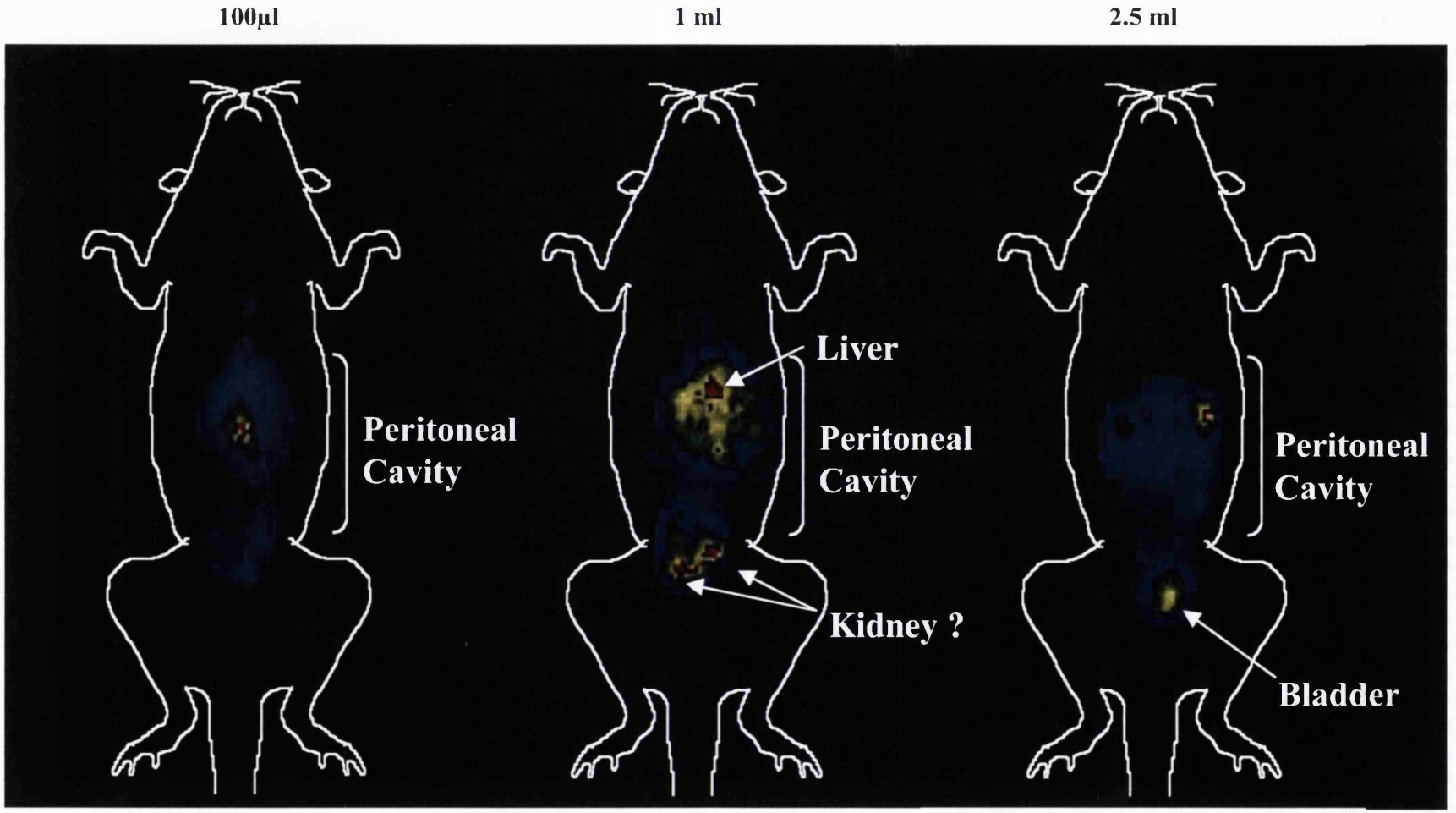
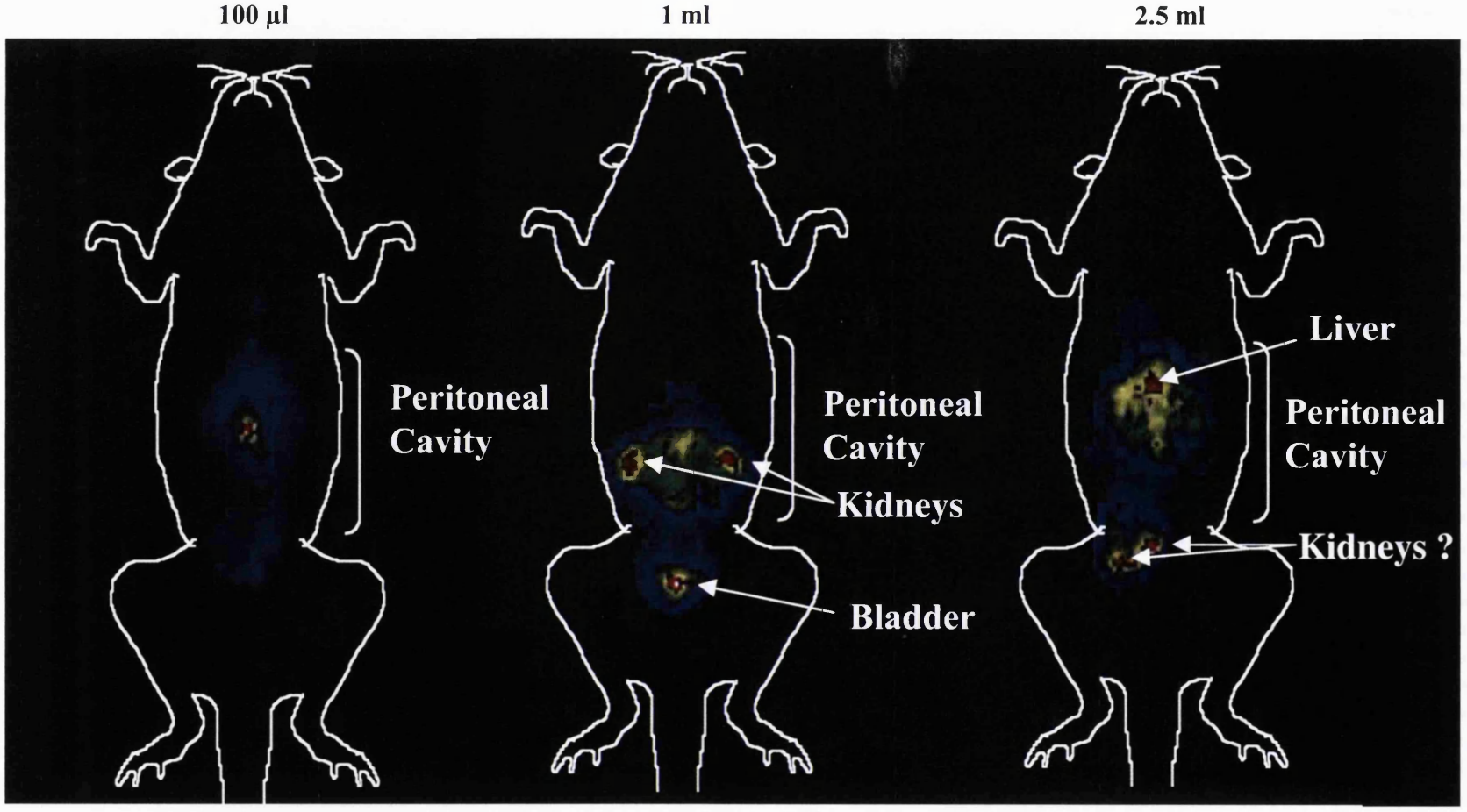


Figure 5.2b Gamma camera images of rats injected with ^{111}In-labelled D-2-S after i.p. administration with increasing the volume (t = 2 h)



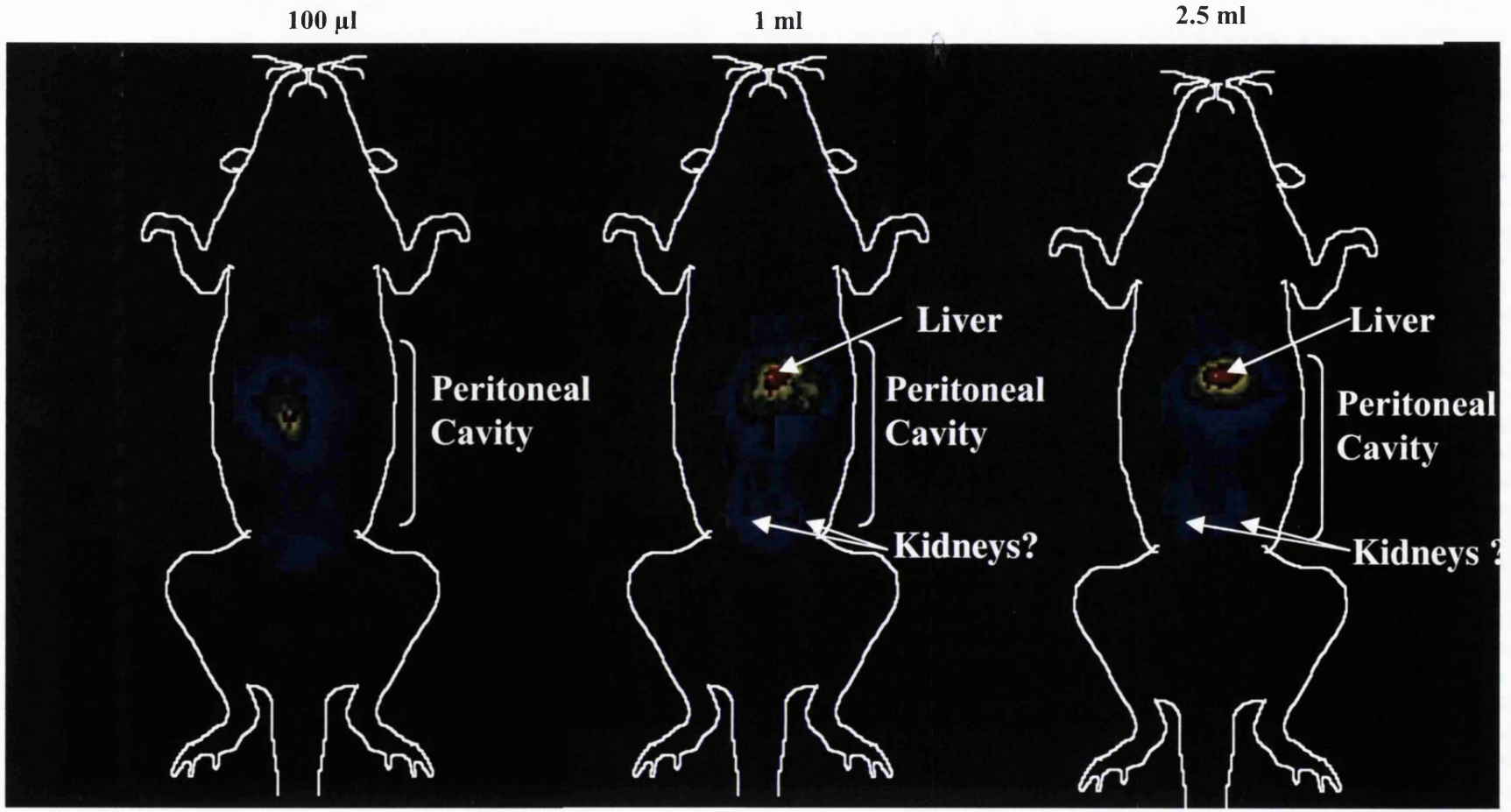
Kidneys? -these appear very low down the body and there could be some discrepancy that these could be another organ

Figure 5.2c Gamma camera images of rats injected with ^{111}In-labelled D-2-S after i.p. administration with increasing the volume (t = 4 h)



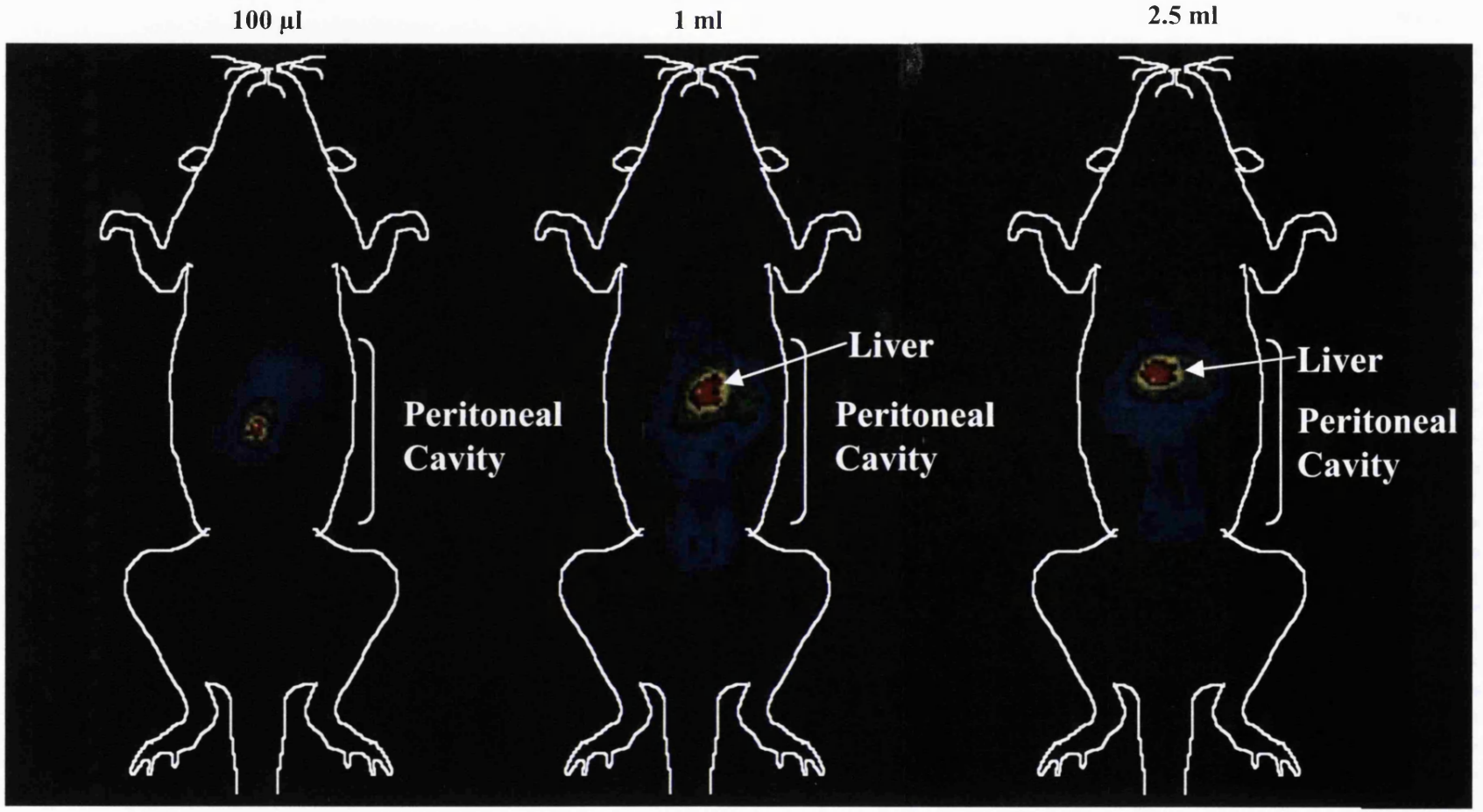
Kidneys? -these appear very low down the body and there could be some discrepancy that these could be another organ

Figure 5.2d Gamma camera images of rats injected with ^{111}In-labelled D-2-S after i.p. administration with increasing the volume (t = 6 h)



Kidneys? -these appear very low down the body and there could be some discrepancy that these could be another organ

Figure 5.2e Gamma camera images of rats injected with ^{111}In-labelled D-2-S after i.p. administration with increasing the volume (t = 24 h)



localised at immediately spread throughout the peritoneal region and when the injection volume the injection site. In contrast at t = 0 h the probe given in a volume of 1 ml was seen to was raised to 2.5 ml the dose of ^{111}In-labelled D-2-S was seen to be spreading to the lower abdomen. At all time points the increase in volume does show a greater distribution of ^{111}In-labelled D-2-S throughout the body.

Dissection analysis of different volumes of administration after 24 h (Figure 5.3) shows very little of the administered dose remaining in the body with 14.7 % of the administered dose at 100 µl, 15.8 % of the administered dose at 1 ml and 5.5 % of the administered dose at 2.5 ml.

The results from the biodistribution of studies examining ^{125}I-labelled D-2-S and ^{111}In-labelled D-2-S cannot be compared directly because of different time scales used in each experiment.

5.3.3 Effect of increasing dose on the body distribution of ^{125}I-labelled D-2-S after i.p. administration

Increasing doses of ^{125}I-labelled D-2-S were administered i.p. in 1 ml of saline (Figure 5.4). The overall recovery increased with increasing dose from 14.7 % of the administered dose at 100 mg/kg to 31.7 % of the administered dose at 10 mg/kg. The overall recovery seen here is very much lower than that observed in all of the other experiments because animals were replaced in their homing cages after i.p. injection rather than in metabolic cages. This shows that mobility does have an effect on the biodistribution and the % of the administered dose found in the organs.

There was a marked increase in the levels of radioactivity recovered in the peritoneal wash from 6.1 % of the administered dose at 0.1 mg/kg to 16.4 % of the administered dose at 10 mg/kg ($p < 0.001$). The recovery in the kidneys increased from 0.8 % of the administered dose at 0.1 mg/kg to 2.5 % of the administered dose at 10 mg/kg ($p < 0.001$). The liver also showed an increase in recovery with increasing dose from 2.3 % of the administered dose at 0.1 mg/kg to 4.2 % of the administered dose at 10 mg/kg. The recovery in the blood also doubled from 2.0 % of the administered dose at 0.1 mg/kg to 4.0 % of the administered dose at 10 mg/kg (Appendix 2, Table 3).

Figure 5.3 Effect of volume on the biodistribution of ^{111}In-labelled D-2-S (24 h) after i.p. administration (n= 2)

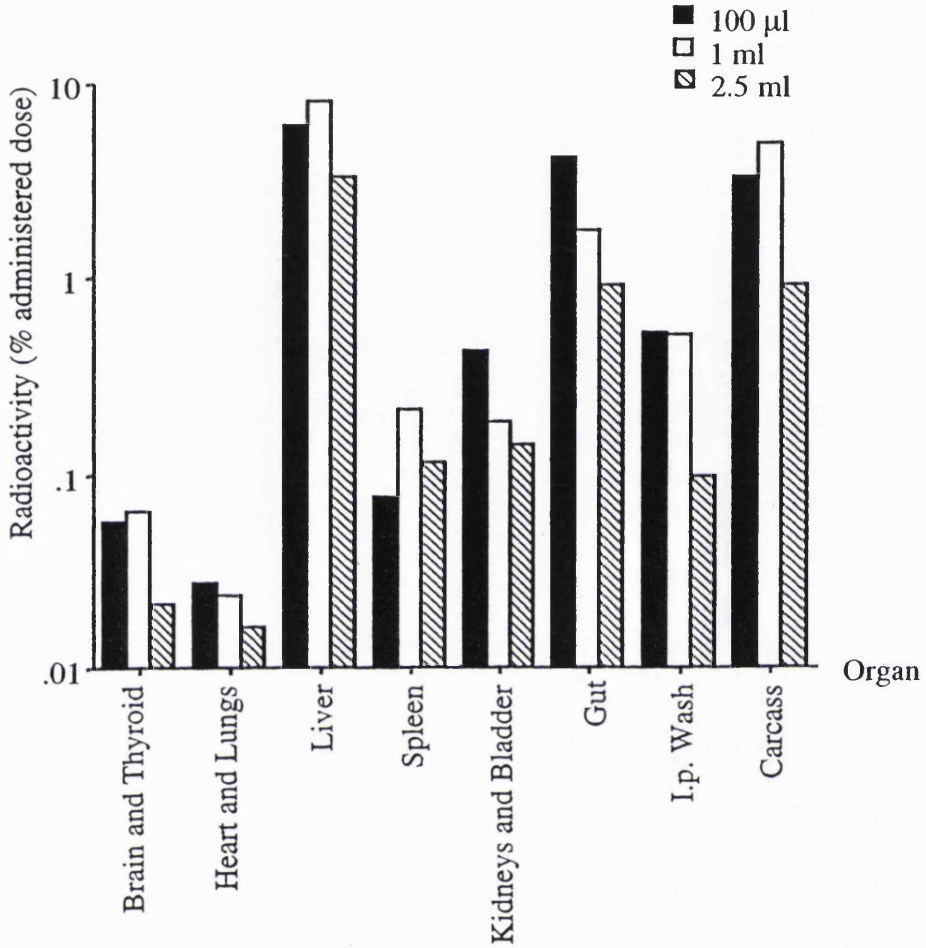
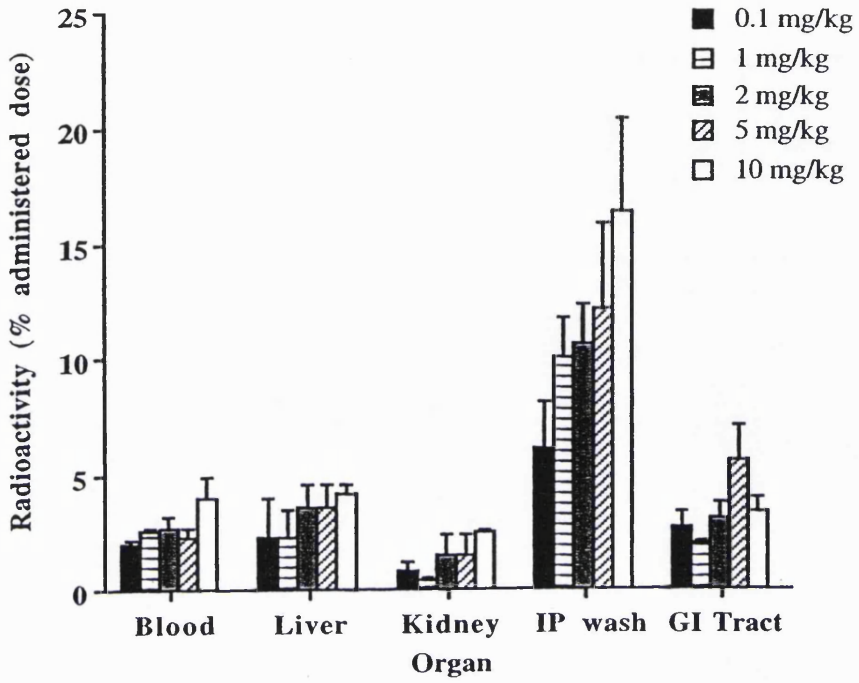


Figure 5.4 Effect of increasing dose on the biodistribution of ^{125}I-labelled D-2-S (2.5 h) after i.p. administration (n=3 ±SD)



5.3.4 Effect of repeated dose on the biodistribution of ^{125}I-labelled D-2-S after i.p. administration

By the end of the study (after 5 injections of 5 x 10^5 cpm, ~300 μg/kg, 1 ml volume) only 0.2 % of the administered dose remained in the peritoneal cavity. (Figure 5.5, Appendix 2, Table 4). The overall recovery showed that 19.5 % of the administered dose was recovered in all of the organs examined at the end of the study. Most of the recovered dose was found in the liver (9.5 % of the administered dose). It is presumed that the remainder of radioactivity was excreted (as the rats were placed back into homing cages and the urine/ faeces were not collected) although this could not be confirmed. However, there was no evidence of ^{125}I-labelled D-2-S accumulation in any of the organs analysed. There was also no sign of any polymer-related toxicity due to repeated administration.

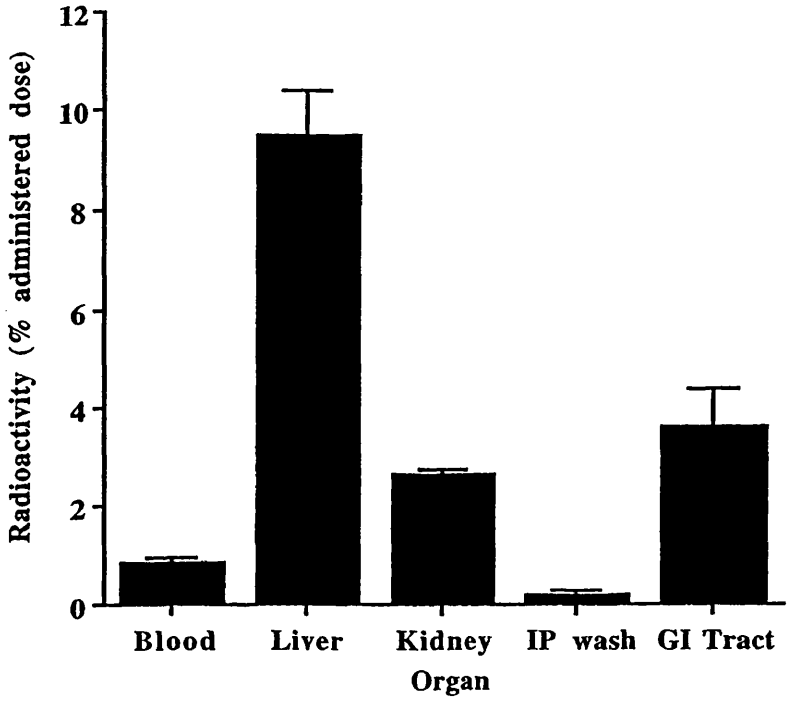
5.4 Discussion

The parameters studied here (dose, repeated administration and volume) all had an effect on the biodistribution of ^{125}I-labelled D-2-S after i.p. administration. Before discussing this in more detail, it is important to point out the differences in distribution seen when animals were kept in metabolic cages after administration of ^{125}I-labelled D-2-S rather than being replaced in their homing cages. ^{125}I-Labelled D-2-S retention in the peritoneal cavity was much greater (45.7 % of the administered dose) when animals were confined to metabolic cages (with very little movement) than seen in animals allowed greater mobility (6.1 % of the administered dose) (Figures 5.2 and 4.8). These observations suggest that the lymphatic circulation must be important in determining biodistribution of D-2-S.

Further experiments could be undertaken to investigate this phenomenon in more detail for example by following the transport of ^{125}I-labelled D-2-S through the lymphatic system by lymphatic cannulation. These preliminary observations might have clinical implications and it could be advisable to encourage patients treated with D-2-S to be as mobile as possible after peritoneal administration to ensure that D-2-S is well distributed around the body.

In the clinic D-2-S has been administered i.p. in an Icodextrin solution using volumes ranging from 1 - 1.5 L. It was therefore interesting to study whether volume itself would have an effect on D-2-S biodistribution. The results obtained from both dissection analysis of ^{125}I-labelled D-2-S and the gamma camera images of ^{111}In-

Figure 5.5 Effect of repeated dose on the biodistribution of ^{125}I-labelled D-2-S after i.p. administration (n=3 ±SD)



Doses were given on days 0,2,4,7, and 9 and the body distribution was assessed on day 11

labelled D-2-S showed that the volume of administration is very important (Figure 5.2).

Images produced by the gamma camera showed clearly that the localisation of D-2-S at the injection site seen after i.p. injection in 100 µl (Chapter 4) disappears when the probe is administered in a large volume. The radioactivity becomes much more rapidly distributed into the whole peritoneal cavity when the larger volumes were used and this also promotes more rapid dispersion around the body. Unfortunately it is not possible to make a direct comparison between the dissection analysis seen following administration of ^{125}I-labelled and ^{111}In-labelled D-2-S because of the different times of termination of the experiments (2.5 h and 24 h).

To be effective, drugs are usually administered on a repetitive basis over a period of time. The duration of treatment is determined by the illness, its response and the toxicity profile of the drug. D-2-S has been administered by repetitive cycles in the clinic. In the first clinical trial (Shaunak *et al.*, 1998), D-2-S (7.5 % w/v of Icodextrin) was administered every day for 28 days in the hope that the amount of the drug given on each occasion would be the same as its rate of elimination; i.e. a steady state would be achieved. It was therefore considered important to establish whether repeated i.p. doses of ^{125}I-labelled D-2-S would lead to the accumulation of the drug in any particular organ.

The results shown here (Figure 5.5) suggest that no cumulative organ deposition of D-2-S occurs after repeated injection. This treatment schedule also caused no obvious signs of toxicity. At the end of the 11 day cycle, dissection analysis showed that only 19.5 % of the administered dose was present in the organs studied, and that there was little radioactivity remaining in the peritoneal cavity. We could speculate that either the D-2-S had been

1) excreted due to relatively low Mw,
2) some D-2-S was deposited in the tissues not examined (e.g. lymphatics)
3) alternatively the D-2-S could have adhered to the peritoneal wall. Further experiments are needed to determine the fate of D-2-S.

In the dose escalation study, increasing the dose of D-2-S up to 10 mg/kg (Figure 5.4) caused no signs of toxicity. As the animals in this study were not placed in metabolic cages i.e. no collection of urine and faeces there seemed to be a lower overall recovery of radioactivity. Dose only seemed to influence the recovery of radioactivity

radioactivity seen in the i.p. wash. More D-2-S remained in the cavity with increasing dose, but the reason for this is not clear. Patients receiving increasing doses of D-2-S in the clinic have showed a decrease in viral load of HIV-1 as measured by plasma viraemia, cellular viraemia and p24 antigenaemia (Shaunak *et al.*, 1998).

The studies described here suggest that for experiments in Wistar rats, the optimum conditions for D-2-S administered i.p. are an injection volume of 1 ml at a dose of 10 mg/kg because at these parameters the maximal delivery of the drug from the peritoneal compartment is achieved.

In the clinic, short term i.p. administration of D-2-S has led to a significant fall in the viral load in patients with AIDS (Shaunak *et al.*, 1998). It is still unclear how the parameters studied here could be used in the clinic to optimise the biodistribution of D-2-S in patients. Having demonstrated that ^{111}In-labelled D-2-S is a good probe for gamma camera imaging it would be interesting to use this probe for clinical imaging of patients given D-2-S in the therapeutic trials particularly when injection volume or dose was altered.

An important observation that might be related to the biodistribution of D-2-S comes from Thornton *et al.* (1999) who found that after treatment with D-2-S (in an Icodextrin solution) AIDS patients with Kaposi's sarcoma showed a tumour response. Thus in the following chapter (Chapter 6), the biodistribution of D-2-S and dextrin have been examined *in vivo* in mice bearing s.c. tumours to determine whether there was any evidence of tumour targeting.

Chapter 6

Dextrin Dox: Determination of *In vivo* pharmacokinetics and pharmacology

6.1 Introduction

The data presented in Chapters 4 and 5 showed that dextrin and D-2-S administered i.p. are not toxic and are able to leave the peritoneal cavity over time. Therefore, studies were carried out here to determine whether there would be any specific tumour accumulation of either polymer in mice bearing the s.c. B16F10 murine melanoma.

Dextrin (in the form of Icodextrin) has already been used as an i.p. delivery system for the anticancer agent 5-FU (McArdle *et al.*, 1994: Kerr *et al.*, 1996). This formulation was developed as a means to increase 5-FU peritoneal residence time, localising drug near to the colon cancer cells which it was used to treat. In our laboratory dextrin has also been developed as a water soluble polymer-drug carrier for delivery of the anticancer agent Dox (Hrezcuk-Hirst *et al.*, 1999). In this context it was considered interesting to see whether dextrin would leave the peritoneal cavity and localise in the tumour by the EPR effect.

Some sulphated polysaccharides have been shown to display antiangiogenic properties *in vitro* (Zugmaier *et al.*, 1992: Murata *et al.*, 1991) and two sulphated polysaccharides-peptidoglycan compounds have previously shown to inhibit antiangiogenesis using cells derived from Kaposi's sarcoma lesions (Nakamura *et al.*, 1992: Baba *et al.*, 1994). As of yet however, none has shown any clinical benefit in patients suffering from Kaposi's sarcoma.

In contrast, in clinical studies D-2-S was found to display antitumour activity against Kaposi's sarcoma in AIDS patients (Thornton *et al.*, 1999). A gradual sign of regression of their Kaposi's sarcoma (reduction in the level of tumour associated aedema) after treatment i.p. with D-2-S was seen. *In vitro* D-2-S is not taken up by the Kaposi's Sarcoma cell line (KSY-1) suggesting that D-2-S was acting at the level of cell surface. It has been previously shown using lymphocytes and monocyte-derived macrophages (Javan *et al.*, 1997) that D-2-S binds to a cell surface protein which is not CD4 (Shaunak *et al.*, 1994: Beddows *et al.*, 1993). The specificity of the membrane interaction could possibly be related to the position of the negatively charged sulphate groups along the polymer backbone.

In these studies, it was considered interesting to determine whether either dextrin or D-2-S could show tumour accumulation by the EPR effect and secondly to

determine whether a dextrin-Dox conjugate could display either tumour-specific targeting or improved antitumour activity.

Dox was chosen as a candidate to conjugate to dextrin (Hrezcuk-Hirst *et al.*, 1999) as anthracycline antibiotics are widely used in cancer therapy. Their clinical use however is limited because of their non-specific toxicity. To overcome these problems macromolecular conjugates have been developed which, due to their large size, tend to accumulate within tumours by the EPR effect as discussed in Chapter 1 (Section 1.3). Two HPMA copolymer conjugates containing Dox, (PK1 and PK2) have progressed into clinical trials (Vasey *et al.*, 1999). Polysaccharides have also been investigated as potential carriers for anthracyclines and other anticancer agents (Table 6.1).

Many polysaccharides (with and without drugs attached) have also been studied as antitumour agents. Dextran is one of the most widely studied carriers largely due to its acceptance as a plasma expander, and it has shown tumour targeting which is molecular weight-dependant (Mehvar *et al.*, 1994). [^{14}C]Dextran of molecular weight 64 400 Da given i.v. to mice bearing sarcoma 180 cell line showed greater tumour accumulation (1.05 % of the administered dose) than dextran of molecular weight 9 900 Da (0.48 % of the administered dose) (Takakura *et al.*, 1990).

Mitomycin conjugated to anionic [^{14}C] dextran conjugate (Mw 70 000 Da) injected i.v. accumulated within the sarcoma 180 tumour (1.77 % administered dose) after 24 h and subsequently showed inhibition of tumour growth (Takakura *et al.*, 1987). The slightly anionic dextran had a prolonged plasma retention time in the circulation and larger accumulation in target organs. The cationic dextran-mitomycin conjugate showed only 0.15 % administered dose in the tumour. [^{14}C]dextran (Mw 70 000 Da) administered i.v. showed 1.05 % administered dose in the tumour (Takakura *et al.*, 1987a, 1987b and 1990).

The pharmacokinetics of a dextran-[^{14}C] Dox conjugate have also been studied in Lewis lung carcinoma (LLC) in mice and Walker 256 ascites in the rat (Munechika *et al.*, 1994). In the mice, the dextran-[^{14}C] Dox conjugate showed an increased plasma half- life and gave a higher plasma concentration time curve (AUC) than seen for [^{14}C] Dox. AUC values in the tumour tissues were 1.6 and 1.9 times higher than for [^{14}C] Dox in mice bearing LLC and rats bearing Walker 256 ascites respectively. When the antitumour activity of dextrin-Dox was examined growth inhibition observed in various tumour models including LLC, MethA sarcoma and Walker sarcoma 256 (Ueda *et al.*,

Table 6.1 Polysaccharide-conjugates examined as anticancer agents

Polysaccharides	Drug	Tumour model	Reference
Dextran	Adriamycin	Walker 254 Lewis lung carcinoma	Munechika *et al.*, 1994
	Dox	B16F10	Munechika *et al.*, 1994
	Mitomycin C	Walker 254	Takakura *et al.*, 1987a, 1987b and 1990
Pullulan	Dox	Walker 256 Yoshida sarcoma	Nogusa *et al.*, 1997
	Adriamycin	AH66 hepatoma	Ichinose *et al.*, 1998
Alginate	Daunorubicin	B16F10	Al Shamkhani and Duncan, 1995
Polygalactosamine	Dox	MH134Y hepatoma	Ouchi *et al.*, 1998
Chitin	Dox	P388 lymphocytic leukemia,	Ohya *et al.*, 1995
Chitosan	Mitoxantrone	Ehrlich ascites carcinoma	Jameela *et al.*, 1996

1989a, 1989b).

The passive targeting of anticancer drugs using dextrans is based on the increased plasma half-life of drugs conjugated to the polymer, an increased permeability of the tumour vasculature, and retention of macromolecules in tumour tissue (Maeda *et al.*, 1992).

B16F10 murine melanoma is used as a standard screen in our laboratory to test the pharmacokinetics and pharmacology of polymer conjugates. This model has been well characterised by studying the effect of PK1 on B16F10 tumours of different size (Sat *et al.*, 1998). B16F10 has previously been used to study the EPR effect of PK1 (Seymour *et al.*, 1987 and 1994), HPMA copolymer-platinates (Gianasi *et al.*, 1999) and dendrimer-platinates (Malik and Duncan, 1999). This model was therefore chosen to investigate the pharmacokinetics of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S, and the antitumour activity of dextrin-Dox.

As Hrezcuk-Hirst *et al.*, (1999) showed that dextrin with a 1 mol% degree of succinoylated is degraded rapidly it was decided to study the effect of different degrees of succinoylation (1 mol% and 34 mol%) on the biodistribution of ^{125}I-labelled dextrin.

Studies reported here investigate the biodistribution of ^{125}I-labelled dextrin (1 mol% and 34 mol%, i.v. and i.p. administration) and ^{125}I-labelled D-2-S (i.p. administration) to mice bearing s.c B16F10 melanoma. The i.p. administration of both ^{125}I-labelled dextrin (1 mol% and 34 mol%) and ^{125}I-labelled D-2-S was also studied with increasing injection volume (100 µl to 500 µl) to determine whether volume would influence tumour accumulation. ^{125}I-Labelled dextrin (1 mol% and 34 mol%) was studied after i.v. administration (100 µl) at time points of 5 min and 1 h.

Finally, a dextrin-Dox conjugate (34 mol% succinoylation, 9 wt% Dox loading) was used to determine pharmacokinetics (by HPLC analysis of drug) and pharmacological activity after i.p. administration to mice bearing s.c. B16F10.

6.2 Materials and Methods

All the animal experiments were conducted according to the UK Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals with experimental neoplasia (UKCCCR guidelines, 1998). In all experiments B16F10 cells (10^5) were injected into the back of the neck of C57 black mice. After 10-12 days the tumours reached a palpable size (approximately 25 - 50 mm^2). After injection of

conjugates all animals were checked twice a day to ensure they appeared well and showed no signs of toxicity. Animals that lost > 20 % body weight or showed other signs of distress were humanely killed.

^{125}I-labelled dextrin and ^{125}I-labelled D-2-S: Dextrin-tyrosinamide containing (0.7 mol% tyrosinamide) and D-2-S-tyrosinamide containing (0.8 mol% tyrosinamide) were synthesised as described in Chapter 3 (Section 3.3.7) and were radiolabelled and characterised as described in Chapter 2 (Section 2.6.1 and 2.6.2).

Dextrin-Dox: The dextrin-Dox conjugate used in these studies were prepared and characterised by Dale Hrezcuk-Hirst as described by Hrezcuk-Hirst *et al.* (1999). Dextrin was succinoylated to a degree of 34 mol % and the conjugate contained 9 wt% Dox loading which was determined by HPLC and UV-vis spectrometry.

6.2.1 Biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S in tumour bearing mice

In all of the experiments mice (25-30 g) were injected i.p. with either ^{125}I-labelled dextrin (1 mol % or 34 mol%, 5 x 10^5 cpm, ~300 μg/kg) or ^{125}I-labelled D-2-S (5 x 10^5 cpm, ~150 μg/kg). Once the tumours had reached palpable size polymers were administered in saline. The administration volume varied (100, 200, 300, 400 or 500 μl) but the doses were kept the same. Whereas ^{125}I-labelled dextrin was administered i.p and i.v., ^{125}I-labelled D-2-S was administered i.p. (previously it was shown to be toxic after i.v. administration, Chapter 4).

At various times after administration the animals were killed and a blood sample was taken. The following organs were removed and washed in PBS before being assayed for radioactivity; tumour, liver, lung, thyroid and spleen. The organs were weighed, then homogenised in distilled water using a polytron homogeniser. Radioactivity in the blood and organs was expressed in terms of radioactivity (% administered dose). The volume of blood of the mouse was taken to be 5.77 ml/kg (Dreyer and Ray, 1910).

Intraperitoneal administration: ^{125}I-Labelled D-2-S and ^{125}I-labelled dextrin (1 and 34 mol%) were administered in increasing volumes (100 to 500 μl). The same amount of

radioactivity (5 x 10^5 cpm) was used in all experiments. In the case of ^{125}I-labelled dextrin, the animals were killed at 1 h and ^{125}I-labelled D-2-S treated animals were killed at 2.5 h. These times were chosen as previous experiments in Chapter 4 had shown that approximately 50 % of the injected dose was remained in the peritoneal compartment at these times.

In the case of i.p. administration, a peritoneal wash was also carried out at the end of the experiments. Once the animal had been killed and the blood removed, 5 ml of saline was injected into the peritoneal cavity and the abdomen was given a gentle massage. A small incision was made and as much fluid as possible withdrawn (peritoneal wash). The volume withdrawn was noted but in the calculations was corrected to the 5 ml that was administered.

Intravenous administration: ^{125}I-Labelled dextrin (1 mol % and 34 mol %: 5 x 10^5 cpm) were administered i.v. in a volume of 100 µl. The animals were killed at time points 5 min and 1 h.

6.2.2 Determination of the maximum tolerated dose of dextrin-Dox

Pairs of normal (no tumour) C57 black mice were injected i.p. with increasing doses of dextrin-Dox (100 µl, 20 – 40 mg/kg) in sterile saline, and their behaviour and weight checked daily over a period of 9 days. After this time, the mice were culled and major organs examined for changes in morphology.

6.2.3 Antitumour activity of dextrin-Dox in mice bearing s.c. B16F10 tumours

C57 black mice were injected s.c. with 100 µl of saline containing B16F10 murine melanoma cells (10^5) and were monitored daily for the appearance of tumours. When the tumours were approximately 25 – 50 mm^2, the mice were randomly assigned into treatment groups of 5. These groups were then treated i.p. with sterile saline (control group), Dox.HCl, dextrin-Dox or PK1. Injections were given on days 0, 1 and 2 in all cases except for the dextrin-Dox dose of 25 mg/kg (which was given on day 0 because we wanted to observe whether a large dose given on one day would show the same results as an accumulated dose given over 3 days). The mice were monitored daily, and tumour size and body weight recorded. Once their tumour area exceeded

2.98 cm^2 the mice were culled- as designated by the UKCCCR guidelines. The mouse survival was then expressed as:-

$$T/C\ \% = \frac{\text{mean time to tumour progression of test group}}{\text{mean time to tumour progression of control group}} \times 100$$

6.3 Results

6.3.1 Biodistribution of ^{125}I-labelled dextrin after i.v. administration in C57 mice bearing s.c. B16F10 murine melanoma

At 5 min after i.v. administration of ^{125}I-labelled dextrin (1 mol %), (Figure 6.1, Appendix 4) only 42.4 % of the administered dose was recovered in the tissues examined. After 1 h this recovery had decreased to 11.7 % of the administered dose. The error bars seen for the bladder values may appear large this can be accounted for by the volume of urine removed from the bladder before the bladder was removed form the body. Some animals had more bladder content than others. In contrast when ^{125}I-labelled dextrin (34 mol%) was administered (Figure 6.2, Appendix 3, Table 1) 56.4 % and 64.0 % of the administered dose was recovered at 5 min and 1 h respectively. This shows that ^{125}I-labelled dextrin (34 mol%) remained longer in the body.

However, the blood levels of both polymers were low. Dextrin (1 mol%) blood levels were 12.1 % and 0.9 % of the administered dose at 5 min and 1 h respectively. In the case of the higher modified dextrin (34 mol%) 2.0 % and 7.3 % of the administered dose remained in the blood stream at 5 min and 1 h respectively.

In the kidneys the recovered dose followed the same pattern for both modifications of dextrin. For dextrin (1 mol%) 7.2 % of the administered dose at 5 min decreased to a level of 2.4 % of the administered dose at 1 h and dextrin (34 mol%) with 11.5 % of the administered dose at 5 min decreased to a level of 3.0 % of the administered dose in the kidneys at 1 h.

At 5 min 14.1 % of the administered dose of ^{125}I-labelled dextrin (1 mol%) was recovered in the bladder compared to ^{125}I-labelled dextrin (34 mol%) 7.0 % of the administered dose. At 1 h this pattern altered with more radioactivity being found in the bladder (21.0 % of the administered dose) for dextrin (34 mol%) compared to 5.6 % of the administered dose for dextrin (1 mol%) at 1 h.

These results confirm that the dextrin (1 mol%) is being excreted from the body much more rapidly with a larger amount of the administered dose being found in the bladder and kidneys at a much quicker time that that seen for dextrin (34 mol%).

Figure 6.1 **Biodistribution of ^{125}I-labelled dextrin (1 mol %) after i.v. administration in mice bearing the B16F10 tumour model at 5 min and 1 h (n=3 ±SD)**

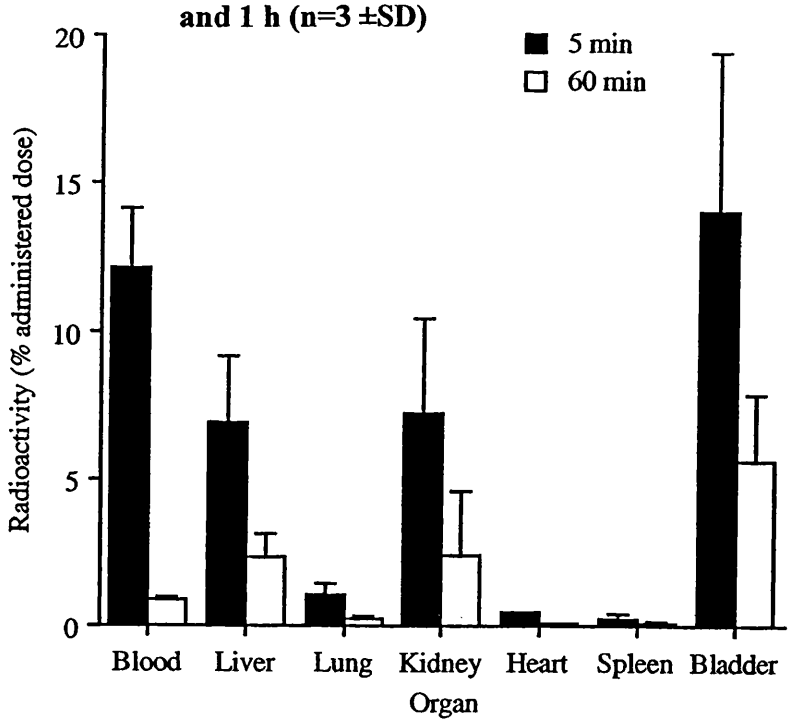
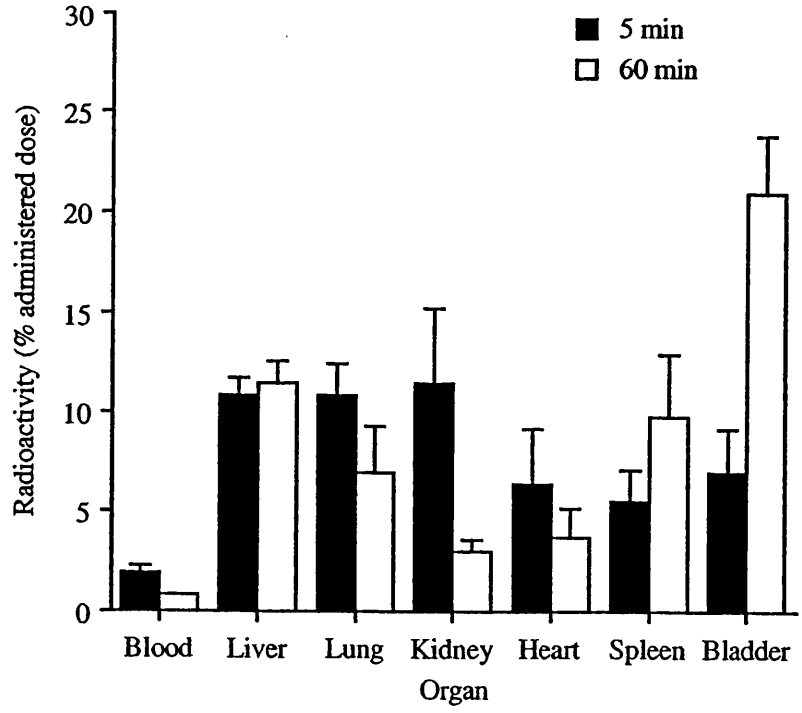


Figure 6.2 **Biodistribution of ^{125}I-labelled dextrin (34 mol %) after i.v. administration in mice bearing the B16F10 tumour model at 5 min and 1 h (n=3 ±SD)**



After i.v. administration, accumulation of ^{125}I-labelled dextrin (1 mol%) (Figure 6.3) in the tumour decreased over time from 0.3 % and 0.1 % of the administered dose at 5 min and 1h respectively. In contrast, tumour levels seen following i.v. administration of ^{125}I-labelled dextrin (34 mol%) increased from 2.5 % to 7.3 % of the administered dose at 5 min and 1 h respectively. The dextrin (34 mol%) therefore shows a greater accumulation than the dextrin (1 mol%).

6.3.2 Biodistribution of ^{125}I-labelled dextrin (1 and 34 mol%) and ^{125}I-labelled D-2-S after i.p. administration in C57 mice bearing s.c. B16F10 murine melanoma tumours

^{125}I-labelled dextrin (1 mol%): After i.p. administration of ^{125}I-labelled dextrin (1 mol%) in increasing volumes (100 µl to 500 µl), the greatest overall recovery (1 h) was when given in 300 µl (Figure 6.4). Recovery was significantly greater ($p < 0.001$) than that seen when injection volumes of 100 µl or 500 µl were used. The overall recovery after administration in 100 µl was 16.4 % of the administered dose and this increased to 37.1 % of the administered dose for 300 µl before decreasing to 15.4 % of the administered dose for 500 µl administration volume.

The blood level increased from 2.2 % of the administered dose for a 100 µl injection volume to 3.4 % of the administered dose for 300 µl and then decreased to 2.1 % of the administered dose for 500 µl. The heart, liver and kidney radioactivity levels all showed the same trend (Appendix 4).

^{125}I-labelled dextrin (34 mol%): After i.p. administration of ^{125}I-labelled dextrin (34 mol%) the greatest recovery (1 h) was in contrast seen after injection of 500 µl (Figure 6.5). Recovery was significantly greater ($p < 0.01$) than seen when injection volumes of 100 µl or 300 µl were used. The overall recovery after administration in 100 µl was 69 % of the administered dose and this decreased to 61.6 % of the administered dose for 300 µl and then increased to 82.1 % of the administered dose for 500 µl. The lung, liver and spleen showed a similar trend (Appendix 4).

The blood level increased from 3.7 % of the administered dose at 100 µl, to 6.7 % of the administered dose at 400 µl and then decreased to 5.9 % of the administered dose at 500 µl. The recovery in the blood was higher than that seen for dextrin

Figure 6.3 **Accumulation in the tumour after i.v. administration of ^{125}I-labelled dextrin (modified to a level of 1 mol % or 34 mol %) at 5 min and 1 h (n=3 ±SD)**

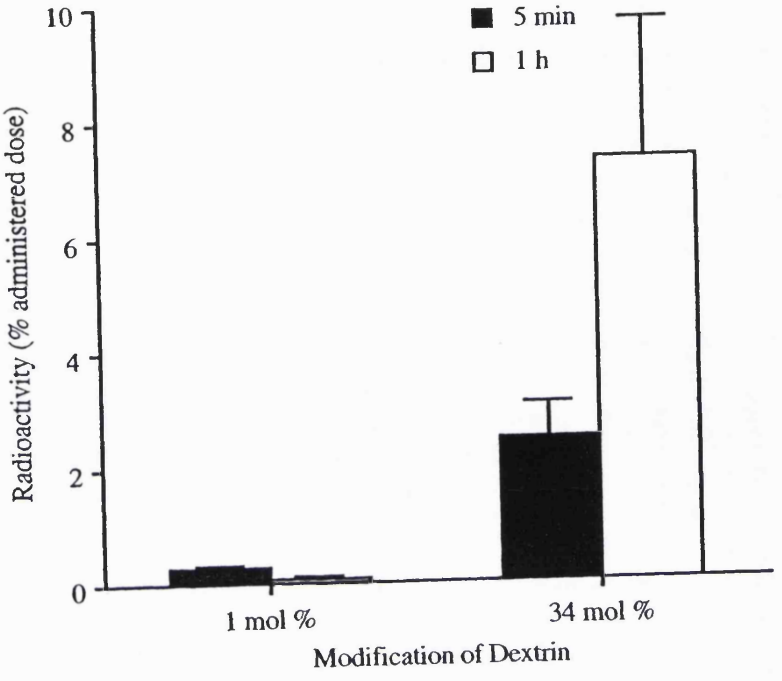


Figure 6.4 **Effect of increasing injection volume on the biodistribution of ^{125}I-labelled dextrin (1 mol%) administered i.p. to mice bearing s.c.B16F10 (1 h) (n=3 ± SD)**

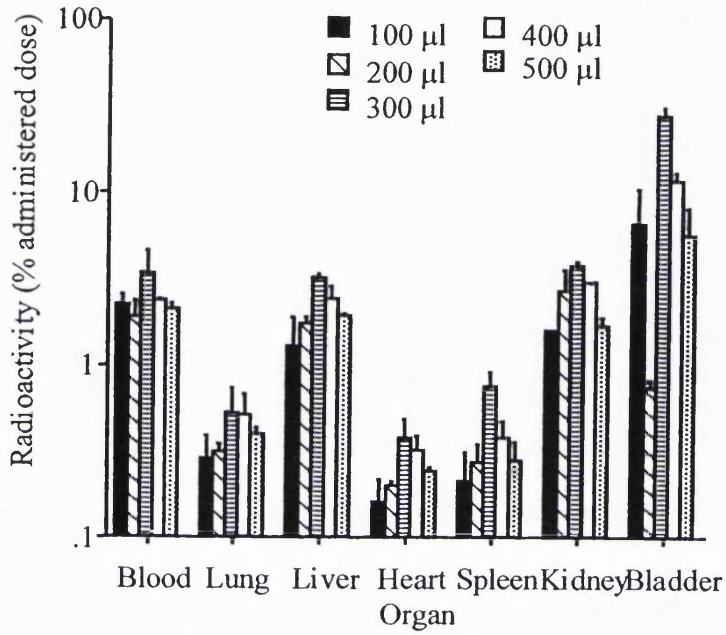
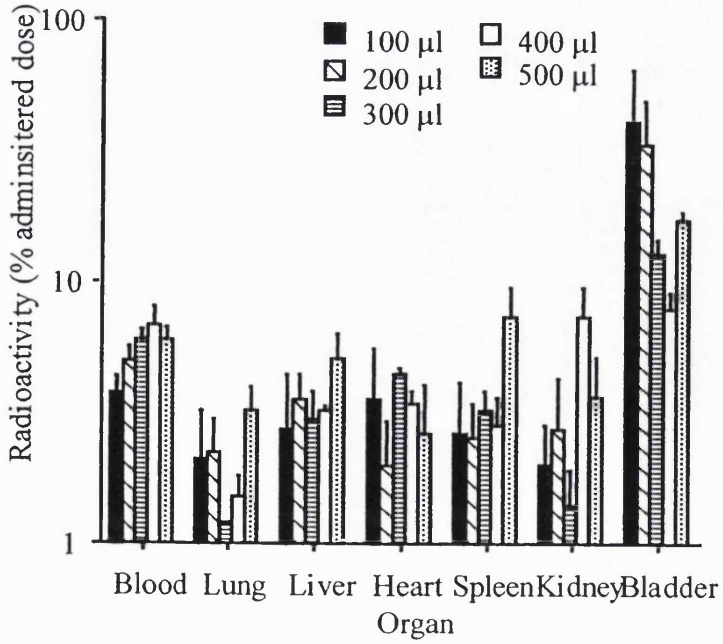


Figure 6.5 **Effect of increasing injection volume on the biodistribution of ^{125}I-labelled dextrin (34 mol%) administered i.p. to mice bearing s.c.B16F10 (1 h) (n=3 ± SD)**



(1 mol%).

^{125}I-labelled D-2-S: In contrast, after i.p. administration of ^{125}I-labelled D-2-S the overall recovery was greatest when the injection volume was 200 µl (Figure 6.6). Recovery was significantly greater ($p < 0.001$) than that seen at all other administration volumes. The recovery after injection in 100 µl was 18.4 % of the administered dose and this increased to 47.2 % at 200 µl and decreasing to 25.4 % of the administered dose at 500 µl.

The recovery in the liver increased from 8.1 % of the administered dose after injection in 100 µl to 20.3 % of the administered dose for 200 µl and decreased to 16.4 % of the administered dose when the injection was 500 µl.

Heart and spleen radioactivity showed the same trend (Appendix 4) with increasing % administered dose being found up to 200 µl and then decreasing to 500 µl.

Peritoneal retention of ^{125}I-labelled dextrin (1 mol% and 34 mol%) and ^{125}I-labelled D-2-S: The effect of injection volume on the peritoneal retention of ^{125}I-labelled dextrin (1 mol% and 34 mol%) and ^{125}I-labelled D-2-S is shown in Figure 6.7. ^{125}I-Labelled dextrin (34 mol%) showed greater peritoneal retention as injection volume increased (11.3 % of the administered dose at 100 µl to 36.3 % of the administered dose at 500 µl). In contrast, ^{125}I-labelled dextrin (1 mol%) showed less retention in the peritoneal cavity as injection volume increased, peritoneal radioactivity falling from 10.3 % of the administered dose for the 100 µl injection to 0.2 % of the administered dose at 500 µl.

For ^{125}I-labelled D-2-S, the peritoneal radioactivity levels showed an unusual biphasic patterm. Peritoneal levels increased from 5.0 % of the administered dose at 100 µl to 15.5 % of the administered dose at 200 µl and then decreased to 2.0 % of the administered dose at an injection volume of 500 µl (Figure 6.7).

Tumour accumulation of ^{125}I-labelled dextrin (1 mol% and 34 mol%) and ^{125}I-labelled D-2-S (1 mol%): The tumour accumulation of ^{125}I-labelled dextrin (1 mol%) mol%) was maximum after injection in 400 µl (0.36 % of the administered dose). This was also true for ^{125}I-labelled dextrin (34 mol%) which showed 0.9 % of the

Figure 6.6 **Effect of increasing injection volume on the biodistribution of ^{125}I-labelled D-2-S administered to mice bearing s.c. B16F10 (2.5 h) (n=3 ±SD)**

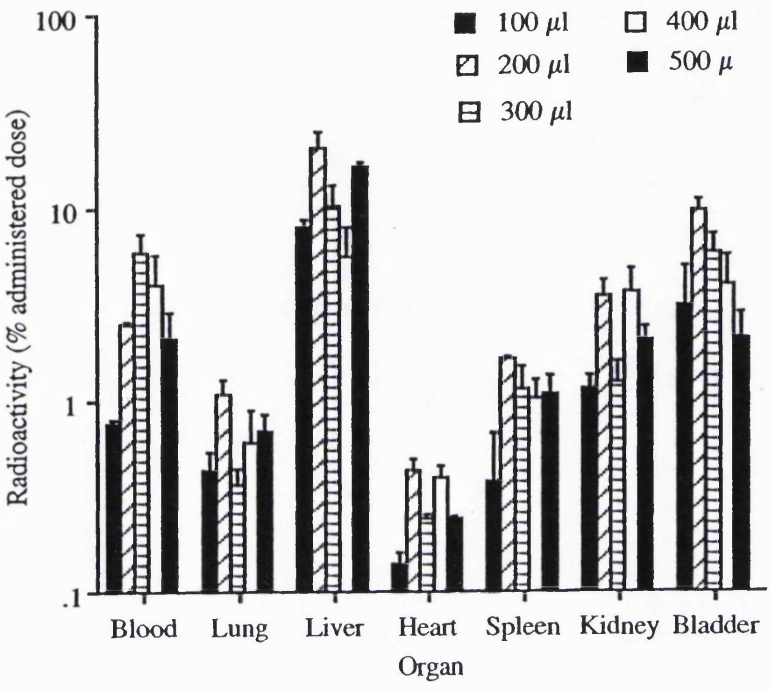


Figure 6.7 **Recovery of ^{125}I-labelled dextrin (1 mol % and 34 mol %) at 1 h and ^{125}I-labelled D-2-S (1 mol%) at 2.5 h in the peritoneal wash after i.p. administration to mice bearing s.c. B16F10 (n=3 ±SD)**

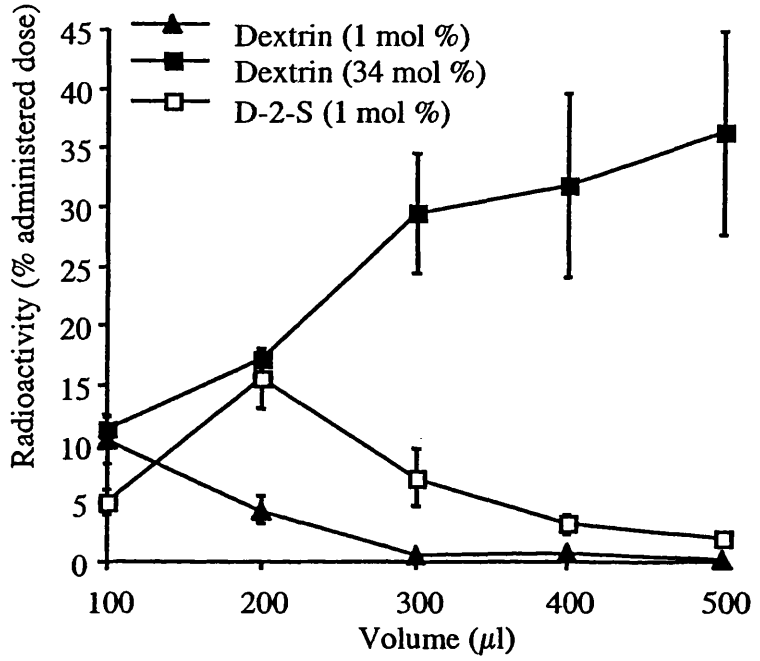
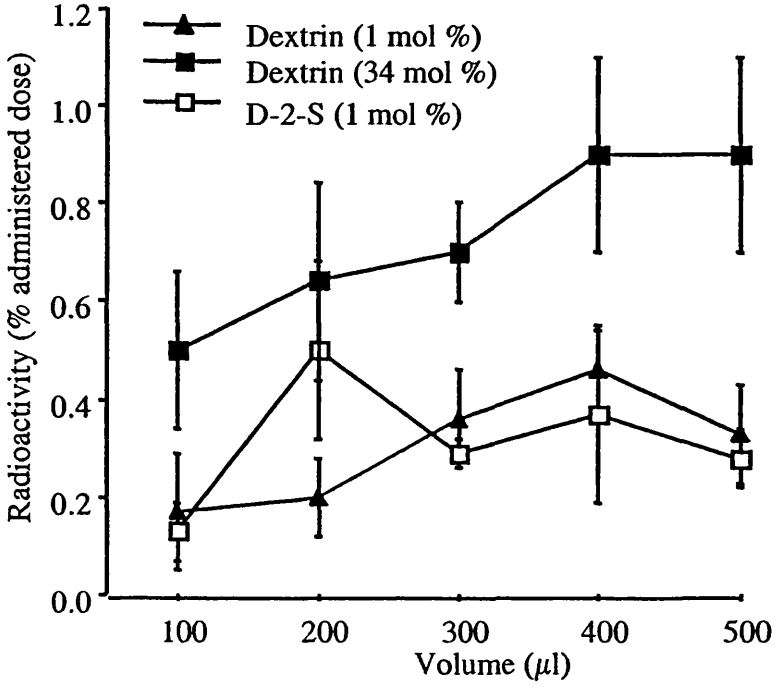


Figure 6.8 **Recovery of ^{125}I-labelled dextrin (1 mol% and 34 mol%) at 1 h and ^{125}I-labelled D-2-S (1 mol%) at 2.5 h in the tumour after i.p. administration to mice bearing s.c. B16F10 (n=3 ±SD)**



administered dose at 400 µl.

Injection volume did not clearly influence tumour accumulation of ^{125}I-labelled D-2-S (Figure 6.8). The most radioactivity was found at the injection volume of 200 µl (0.5 % of the administered dose).

It should be noted that a direct comparison between dextrin and D-2-S is not really appropriate as different times (1 h or 2.5 h) were chosen for these studies. However it should also be noted that the times selected did have a rational basis as they were the times when 50 % of the polymer was remaining in the peritoneal cavity after i.p. injections to Wistar rats (Chapter 4).

6.3.3 Maximum tolerated dose

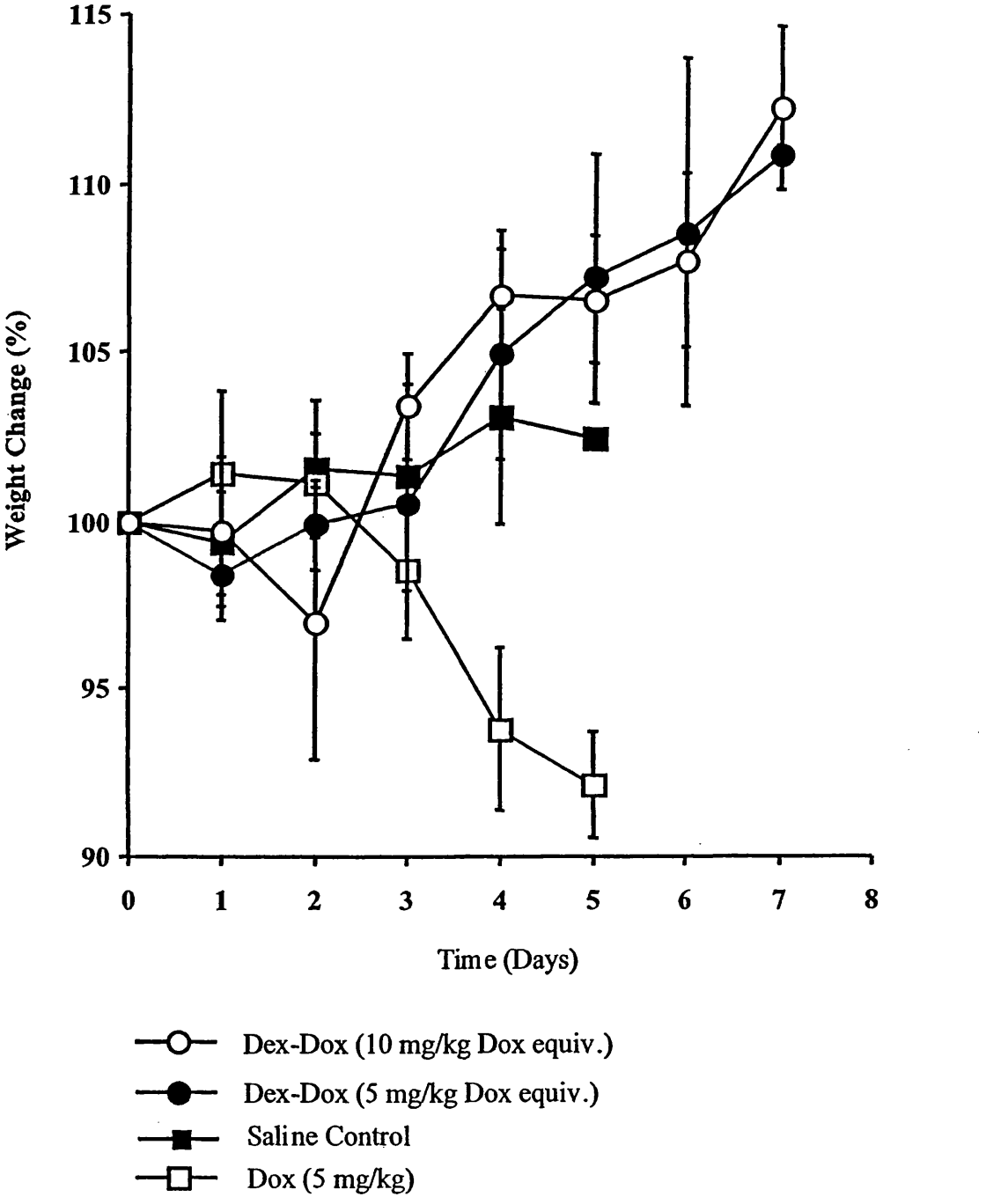
After injection with dextrin-Dox none of the animals showed any weight loss. The group treated with the highest dose (40 mg/kg Dox equiv.) did show mild transient changes in behaviour for the first 30 min after injection. Higher doses could not be administered because larger injection volumes are not permitted according to the Home Office Guidelines on Animal Experimentation and more concentrated samples, while soluble, were too viscous.

6.3.4 Antitumour activity in the B16F10 murine melanoma model

Firstly a preliminary investigation was carrier out to determine the antitumour activity of dextrin-Dox at 5 and 10 mg/kg in comparison to controls of saline and Dox (5 mg/kg).

The increased area of the tumour was measured daily (Figure 6.9) and it can be seen that a decrease in tumour growth rate was observed after treatment of animals with free Dox when compared with the control mice group. The groups injected with dextrin-Dox (5 mg/kg or 10 mg/kg) showed the tumour growth to be slower and the animals survived an extra 2 days showing the growth to be slightly inhibited by the polymer-drug conjugate. Typically used to define antitumour activity is the ratio (T/C) expressed as a percentage of the mean survival time for a treated group of animals (T) compared to the mean survival time of the control group (C). A drug is usually considered efficacious when the survival time is above that of the control by more than 25 % (i.e. a T/C >125 %).

Figure 6.9 Effect of dextrin-Dox on the tumour size of mice bearing s.c.B16F10 (n=5 ±SE)



When the B16F10 tumour bearing mice were treated with the dextrin-Dox at both 5 mg/kg and 10 mg/kg the T/C value increased from 144.2 % at 5 mg/kg (p=< 0.001 vs control group) to 138 % at 10 mg/kg (p= <0.01 vs control group). The results are summarised in Table 6.2. All of the mice treated with Dox (5 mg/kg) displayed a loss in body weight consistent with toxicity (Figure 6.10). The mice injected with dextrin-Dox (5 and 10 mg/kg) showed a gradual increase in the body weight indicating that the mice were showing no signs of toxicity.

A further study was then carried out using a greater concentration range of dextrin-Dox conjugates and also included PK1 so that a direct comparison could be made with a conjugate that is currently under clinical evaluation.

Figure 6.11 shows the change in the recorded tumour size when different doses were administered to the mice. When dextrin-Dox was administered as a single dose at 25 mg/kg to C57 mice bearing B16F10 tumours the anti-tumour activity was not as high as seen after administration of lower doses given consecutively over three days.

T/C values compared to the control showed that all of the dose gave an increased T/C value. Dextrin-Dox gave a T/C value of 130 % (p=0.01 vs control group) for 5 mg/kg injected, 143.3 % (p= <0.001 vs control group) for 15 mg/kg, 126.7 % for 20 mg/kg (p=0.01) and 110 % (not significant) for the single dose of 25 mg/kg. No dose gave a T/C value as high as that for PK1, 173 % (p= <0.001). It is interesting to note here that the repeated doses of dextrin-Dox (5 mg/kg) gave a T/C value higher than that of a single dose. All of the results have been summarised in Table 6.3.

Figure 6.12 shows the change in body weight of C57 mice after treatment. Dox (5 mg/kg) and PK1 (20 mg/kg) generally caused a decrease in body weight, which was not observed for dextrin-Dox where no weight loss was shown indicating maximum tolerated dose had not been reached.

6.4 Discussion

Pharmacokinetics in tumour bearing animals: The success of a targeted drug delivery approach for the treatment of cancer relies to a great extent on the tumour-specificity. After i.v. administration, dextrin (34 mol %) showed greater recovery (65.0 % of the administered dose) than dextrin (1 mol%) (12.1 % of the administered dose). The results are consistent with those arising from studies that examined the tumour

Table 6.2 Antitumour activity of Dox and dextrin-Dox in C57 male mice bearing s.c. B16F10 murine melanoma tumour

Treatment	Dose (mg/kg)	Day of Treatment	Survival (days)	Mean survival (days ± SD)	Mean T/C (%)	Toxic Deaths
Control		0,1,2	4, 4, 4, 4, 5, 5	4.3 ± 0.5	100	0/6
Dox	5	0,1,2	4, 4, 4, 5, 5, 5	4.5 ± 0.5	103 ^{NS}	0/6
Dextrin-Dox	5	0,1,2	5, 6, 6, 6, 7, 7	6.2 ± 0.8	142 ***	0/6
Dextrin-Dox	10	0,1,2	4, 6, 6, 6, 7, 7	6 ± 1.0	138 **	0/6

NS- Not significant

** p= <0.010 *** p= <0.001

Figure 6.10 Effect of dextrin-Dox on the weight of mice bearing s.c. B16F10 (n=5 ± SE)

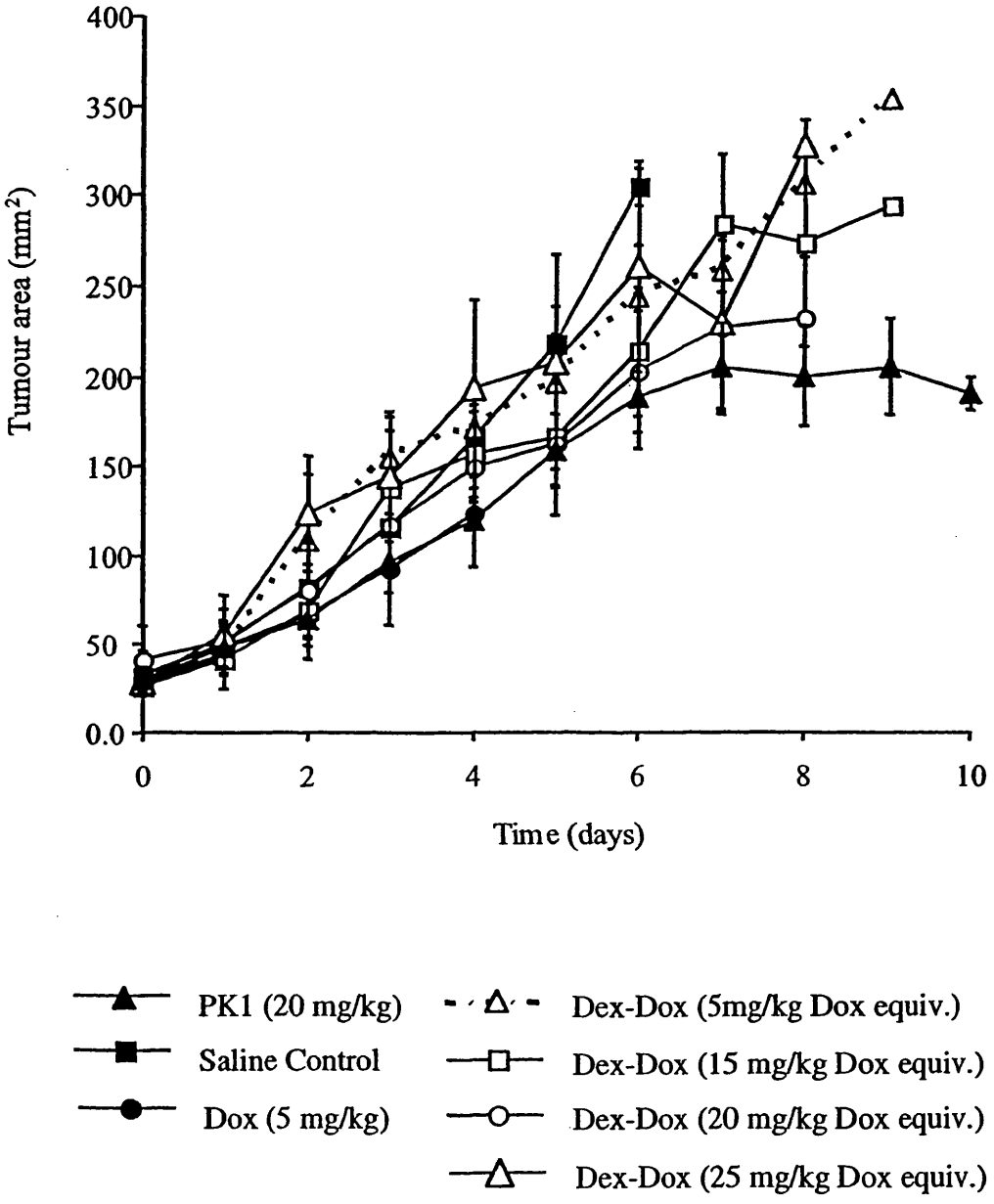


Figure 6.11 Effect of dextrin-Dox dose on the tumour size in mice bearing s.c. B16F10 (n=5 ± SE)

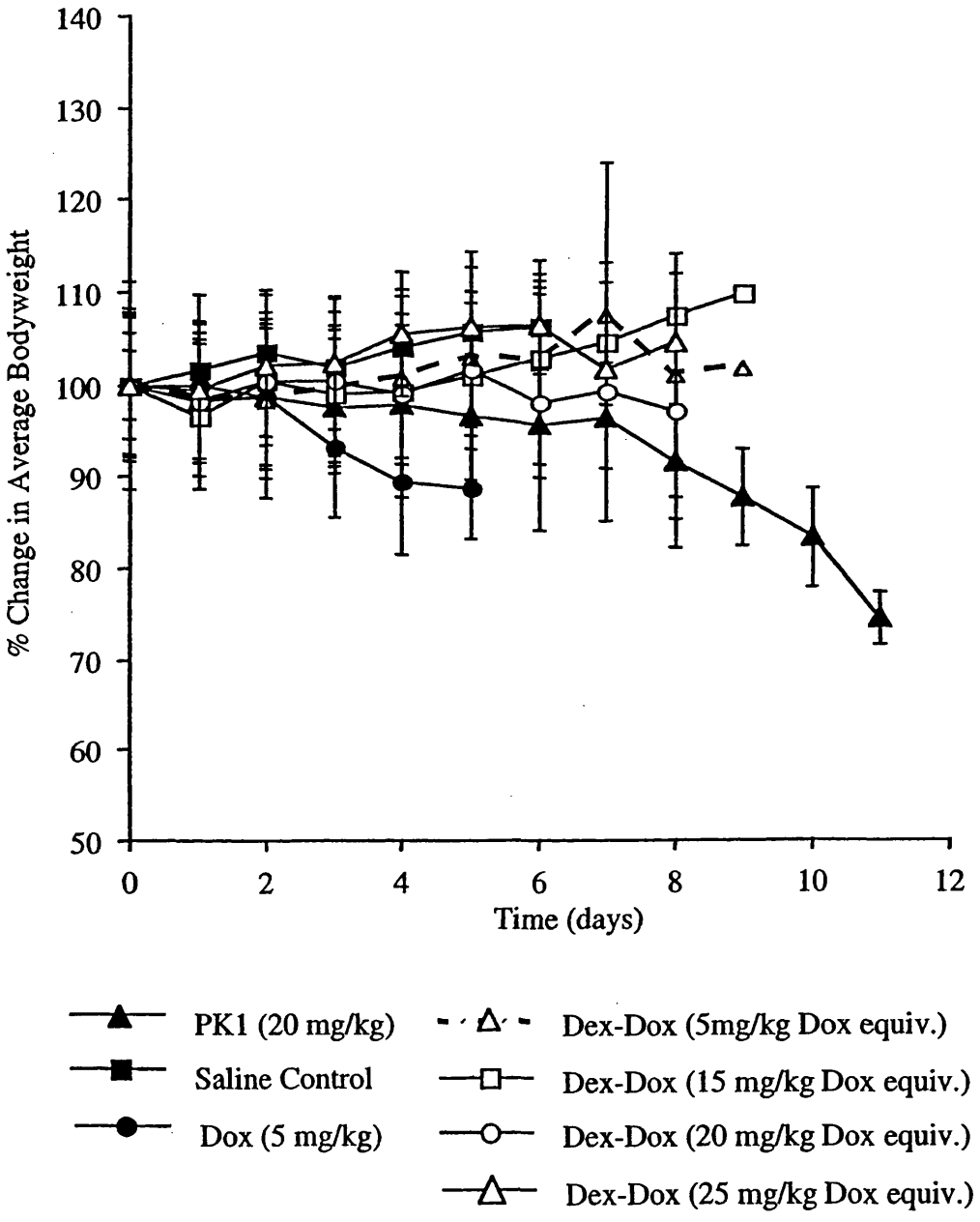


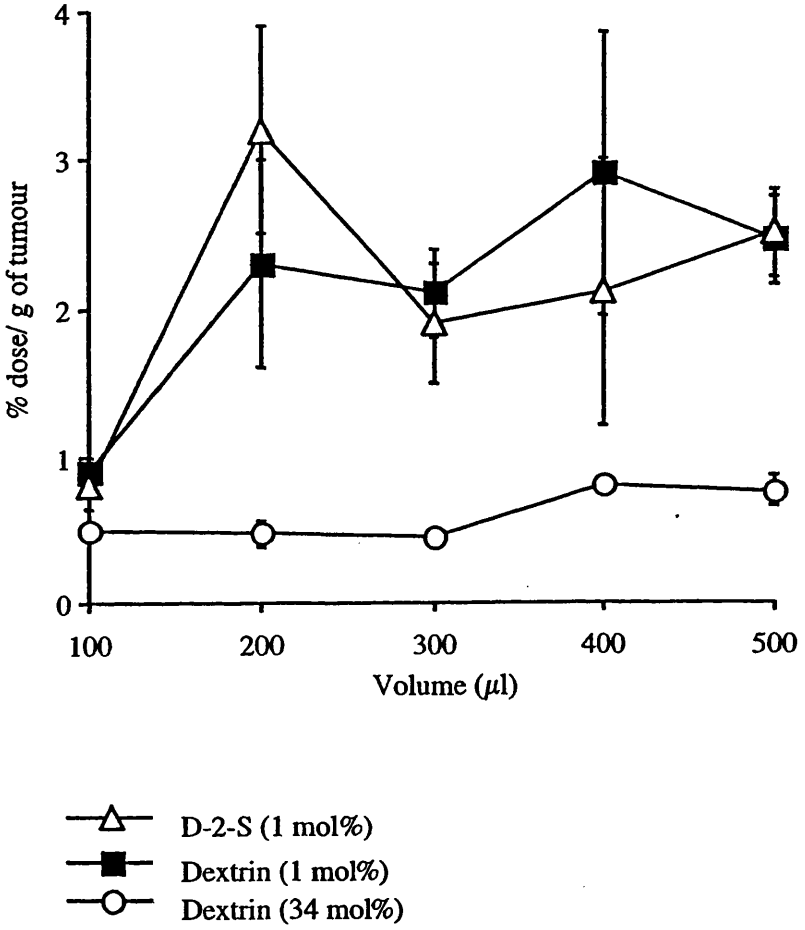
Table 6.3 **Effect of dose and schedule on the antitumour activity of Dox and dextrin-Dox in C57 male mice bearing B16F10 murine melanoma tumour**

Treatment	Dose (mg/kg)	Day of Treatment	Survival / (days)	Mean survival (days ± SD)	Mean T/C (%)	Toxic Deaths
Control		0,1,2	6, 6, 6, 6, 6	6.0 ± 0	100	0/5
Dox	5	0,1,2	5, 5, 5, 5, 5	5.0 ± 0	83.3 (NS)	5/5
PK1	20	0,1,2	10, 10, 11, 11, 10	10.4 ± 0.5	173.3 (***)	1/5
Dextrin-Dox	5	0,1,2	8, 8, 7, 7, 9	8.6 ± 0.9	130 (**)	0/5
Dextrin-Dox	15	0,1,2	10, 8, 9, 8, 8	8.6 ± 0.9	143.3 (***)	0/5
Dextrin-Dox	20	0,1,2	8, 8, 8, 6, 8	7.6 ± 0.9	126.7 (**)	0/5
Dextrin-Dox	25	1	6, 6, 7, 6, 8	6.6 ± 0.9	110 (NS)	0/5

NS- Not significant

** p= <0.010 *** p= <0.001

Figure 6.12 Effect of dextrin-Dox dose on the weight in mice bearing s.c. B16F10 (n=5 ± SE)



targeting of dextran. When [^{14}C] dextran was administered to rats bearing S-180 tumours tumour levels were 1.5 % administered dose for a dextran of Mw 10 kDa and 4 % administered dose for a dextran of 70 kDa (Takakura *et al.*, 1990). This study also showed that the lower Mw was rapidly eliminated from the body and that higher Mw dextran localised in the liver and lymph. after i.v. administration.

The variability in tumour accumulation seen here (Figure 6.3 and 6.8) for ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S generally was higher than seen in the other organs. Sat and Duncan (1999) showed that in the s.c. B16F10 tumour model displays a clear tumour size dependency in the amount of EPR-mediated targeting of PK1 with the accumulation of PK1 in the tumours between 2-18 % dose/g tumour. This could explain the higher variability, and further experiments in this model must standardise tumour size as far as possible. It should be noted that the ^{125}I-labelled dextrin (34 mol%) tumour levels were significantly greater than seen for either [^{14}C]dextran or ^{125}I-labelled dextrin (1 mol%). This result was not surprising as we have shown the rapid degradation of dextrin (1 mol%).

In Chapter 5 it was found that injection volume had a profound effect on the subsequent body distribution of the probe injected. Therefore it was considered important to see if injection volume had an effect on ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S biodistribution. The overall recovery of radioactivity was again dependant on the volume injected. The maximum recovery was seen when using injection volume of 300 µl for ^{125}I-labelled dextrin and 200 µl for ^{125}I-labelled D-2-S although in both cases the recovery was low after i.p. administration. The polymer did however eventually appear in the blood possibly by travelling through the lymphatic system.

After administration i.p. into mice ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S leave the peritoneal cavity more rapidly than they do seem to in the rats (Chapter 4). For example for ^{125}I-labelled D-2-S (tracer dose at 2.5 h) 41.8 % of the administered dose remained in the peritoneum of the rat, while in the mice the value is between 2.0 % and 14.5 % of the administered dose depending on the administration volume.

While ^{125}I-labelled dextrin (1mol%, tracer dose at 1 h) 3.2 % of the administered dose remained in the peritoneal of the rat while in the mice the value is between 4.4 % and 20.2 % of the administered dose depending on the volume of administration.

The tumour accumulation of dextrin-Dox has been studied with the results of the accumulation of Dox being determined using HPLC. The results have shown that there is a greater accumulation of dextrin-Dox in the tumour than Dox alone (Figure 6.13) accumulation in the tumour increases at a concentration of 5 mg/kg Dox/equiv. The results cannot be compared to those observed using dextrin or D-2-S alone as only tracer doses were administered.

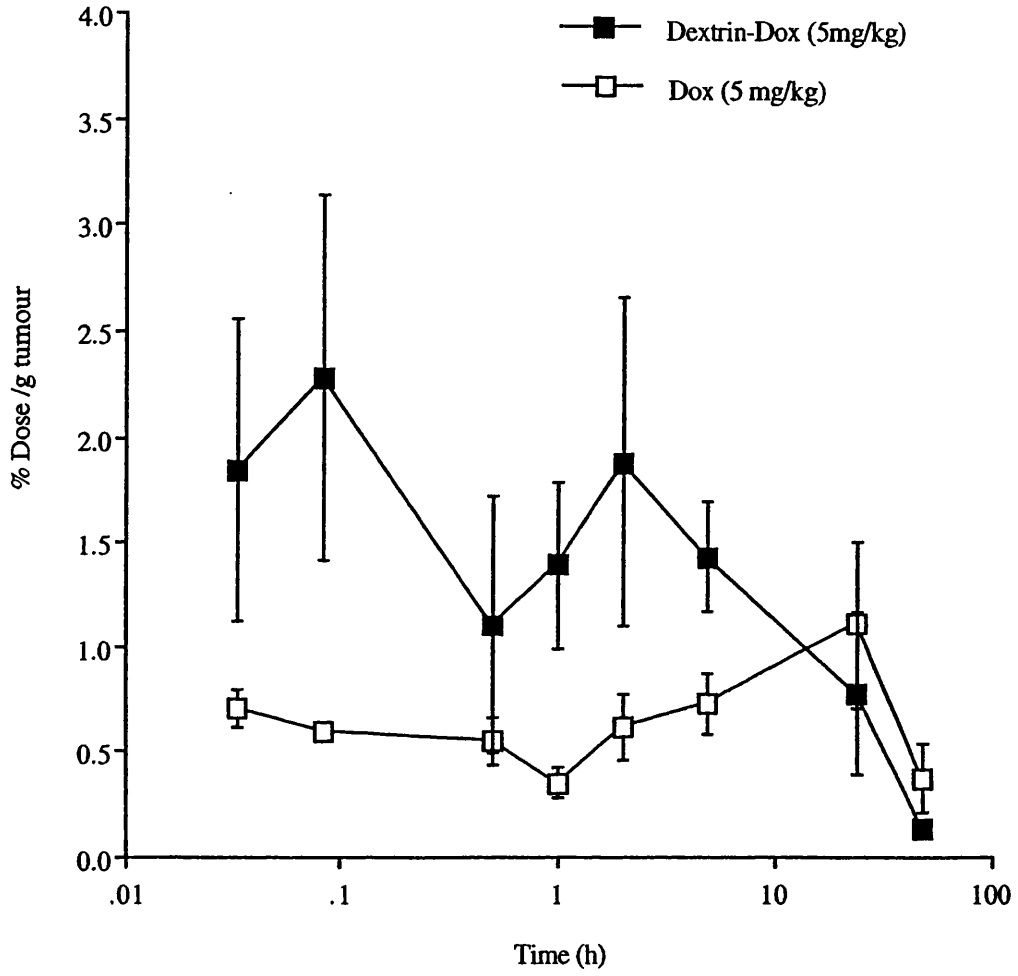
Antitumour activity in the B16F10 murine melanoma model

Dextrin-Dox demonstrated an ability to decrease the growth rate of B16F10 murine melanoma. Any improvement in tumour accumulation is important as the tumour model B16F10 is considered to be very aggressive. It should be noted that here we have not optimised the dextrin-Dox dose and the schedule of treatment which is necessary for future experiments. When comparing PK1 (at its MTD is 90 mg/kg), with free Dox past experiments have shown that PK1 can produce a significant increase in the T/C in the B16F10 model. However, here the MTD of dextrin-Dox could not be reached because of the volumes of product that could be legally administered. Higher doses could not be administered due to the poor solubility of dextrin-Dox when dissolved in PBS at higher concentrations. Therefore, if dextrin-Dox was to be used at higher concentrations the dose regimen would have to be altered to involve continuous infusions.

The maximum administered dose (MAD) of dextrin-Dox was 40 mg/kg for i.p. administration. After administration on days 0, 1 and 2, the highest accumulated dose of 60 mg/kg of dextrin-Dox was given. We have given higher doses than free Dox showing an increase in the life span of the mice. The conjugation of Dox to dextrin has also decreased the toxicity when compared to the drug on its own. As no toxicity was observed in any of the studies a dose escalation study should be performed in the future.

Alginate-daunorubicin has been studied for the tumour accumulation in B16F10 murine melanoma in C57 mice. Injection was given as a single i.p. bolus injection of 5 mg/kg. daunorubicin equivalence. Antitumour activity of the conjugate was shown with disappointing results and there was a small but significant ($p < 0.01$) delay in the growth of the tumour compared to the control group.

Figure 6 .13 Tumour uptake of Dextrin-Dox and Dox in the C57/Bl mice bearing B16F10 tumours after i.p. administration (n=6 ± SD)



In this study we have only used i.p. as a route of administration. Different routes of treatment (i.v. and i.p.) and different sizes of tumours implant can affect the antitumour activity of any drug or drug-conjugate.

O'Hare *et al.* (1993) investigated the effect of different routes of administration of PK1 on antitumour activity in the s.c. B16F10 tumour model. When PK1 was administered i.v. as a single bolus injection (10 mg/kg Dox equivalent), a T/C value of 148 % was observed. In the same study a dose escalation to 20 mg/kg increased the T/C value to 175 %.

Dextrin-Dox administered i.p. in a single bolus injection of 25 mg/kg the T/C value was 110 %.

O'Hare *et al.* (1993) also showed that when the B16F10 tumour was inoculated i.p. and PK1 administered i.v. (10 mg/kg), the T/C value rose to 218 %. However, in the experiment Dox given at the same dose produced a T/C value of 254 % so the activity of PK1 was not significantly different.

It has been suggested that the extent of targeting by the EPR effect in different tumour models may control the activity of PK1 observed. For example, Mac 26 (synergistic murine adenocarcinoma) displays a discontinuous epithelium in the vasculature and this tumour is resistant to Dox but caused tumour regression to PK1 (Bibby *et al.*, 1996). In contrast in the Mac 16 model, which has a more normal vasculature architecture, PK1 was equi-active when compared to Dox (Bibby *et al.*, 1996).

In summary, this study showed that ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S do show some tumour accumulation after i.p. administration. Although the extent of targeting was very small (1 – 4 % administered dose), it is still five-fold greater than would be seen for a cytotoxic agent such as Dox. D-2-S tumour targeting might explain why this polymer displayed antitumour activity in Kaposi's sarcoma.

Although the dextrin-Dox conjugate was not as effective as PK1 in these studies, it did show promising antitumour activity as well as tumour targeting. Further experiments using i.v. administration are essential to test fully the potential of these new conjugates. Dextrin, when compared with PK1 and alginates is notable because it

is the only polymer that has a biodegradable backbone.

Chapter 7

Dextrin-Amphotericin B conjugates: Synthesis and preliminary *in vitro* characterisation

7.0 Introduction

There are many problems associated with the use of the antifungal agent AmpB including its poor solubility and the inherent toxicity of the drug. AmpB has been known to cause many different types of toxicity, which include thrombocytopenia (Chan *et al.*, 1982), myelopathy (Carnvale *et al.*, 1980), chills and fevers (Gigliotti *et al.*, 1982), renal toxicity (Forgan-Smith and Darrell, 1974), neurological manifestations (Haber and Joseph, 1962), anaemia (MacGregor *et al.*, 1975) and leukoencepathy (Walker and Noserubin, 1992). In-order to overcome these problems pharmaceutical companies have developed several new AmpB formulations.

Encapsulation of AmpB within lipidic or liposomal systems has led to a major reduction in AmpB toxicity (reviewed in de Marie *et al*, 1994: Juliano *et al*, 1987). Currently, there are three liposomal/lipidic formulations available on the market. AmBisome (Nexstar Inc.), AmpB-lipid complex ABLC (Liposome Co.) and Amphocil (AmpB colliodal dispersion, ABCD) (Sequus Pharmaceuticals). All have shown an increased therapeutic index resulting in higher dose-efficacy rates in animal studies. Even though these new formulations are an improvement it must be noted that they cause mild side effects when given by slow i.v. infusion and they are very expensive. Liposome- or lipid-AmpB toxicity is greatly reduced (Juliano *et al.*, 1987) compared to that seen for Fungizone. Table 7.1 summarises the major characteristics of AmpB formulations and they are each discussed below.

AmpB deoxycholate (AmBdoc, Fungizone): AmBdoc is the treatment of choice for many invasive fungal infections. It does however have many problems including considerable toxicity. Side effects include chills, fever, also anaemia and nephrotoxicity (Gallis *et al.*, 1990). The clinical dose given is between 0.7 - 1.5 mg/kg.

Albecet (ABLC): ABLC is ten-fold less toxic than Fungizone in animals (Janoff *et al.*, 1993). Studies in AIDS patients suffering from cryptoccocal meningitis has shown

Table 7.1 Characterisation of different formulations of AmpB. (Modified from Bekersky *et al.*, 1999; De Marie *et al.*, 1994)

	Fungizone (AmB_{DOC})	ABLC (Abelcet)	AmBisome	Amphotec
Manufacturer	Bristol-Myers Squibb	Liposome Co	Nexstar	Sequus Pharmaceutics
Structure		Ribbon-like	Unilamellar liposome	Disc-shaped
Size (μm)	<0.4	1-10	<0.1	0.12
Lipid Composition	NA	Fluid phospholipds	Rigid phospholipids	Cholesterol sulphate
AmpB (wt%)	12.5	50	12.5	65
AmpB (mol%)	34	35	10	50
Dose (mg/kg)	0.25	5 daily infusion given over 12 h	3-4 daily given over 60-90 min.	5 daily over 30 – 60 min
Clearance (ml. kg/h)	10	211	960 ml/min	112
Cmax[a] (μg/ml)		1.7	83	3.1
Distribution	Liver, spleen, lung and kidney	RES, spleen, liver and lung	RES, liver, spleen, lung and kidney	Liver

[a]Maximum plasma concentration of total AmpB achieved after a dose of 5 mg/kg in man.

ABLC to be clinically safe (Graybill *et al.*, 1982, 1995), but this formulation was not any more efficacious than AmpB.

Amphotec (Amphocil, ABCD): Similary to ABLC, ABCD was tolerated at higher doses than conventional AmpB. Similar side-effects still occurred such as chills and fevers, but there was less renal toxicity (Sanders *et al.*, 1991). ABCD is currently approved in the USA for second line treatment of aspergillosis. In Europe, it can be used as first- or second-line treatment. The recommended daily dose is 3-4 mg/kg. ABCD is also effective against *Candida* at a dose of 3.4 mg/kg (Anaissia *et al.*, 1998). Preliminary results from a dose escalation study showed that ABCD is also effective in the treatment of systemic mycoses (Guo and Working, 1994).

***AmBisome*:** AmBisome is a liposomal preparation of AmpB which overcomes many of the problems associated with AmpB. AmBisome has been registered in a number of European countries as a salvaging agent for patients who have experienced toxicity with Fungizone. The lipid vesicles encapsulate the drug and enclose it in a bilayer which enhances delivery whilst reducing drug toxicity. Once again, administration of Ambisome led to an increase in the tolerated dose compared to conventional AmpB (Proffitt *et al.*, 1991). Lower incidences of acute, infusion-related toxicity and nephrotoxicity were observed. In order to maintain liposomal structure only 10 wt % AmpB was encapsulated (Bekersky *et al.*, 1999). AmBisome like Albecet and ABCD retains the AmpB activity against a variety of fungal diseases.

AmpB has been widely used to treat various fungal diseases. It is often used in conjunction with flucytosine in patients with cryptococcal meningitis (Bennett *et al.*, 1979) and aspergillosis (Denning, 1994: Denning and Stevens, 1990). AmpB is also a powerful antileishmanial drug but it remains second line therapy because of its toxic side-effects. AmpB is highly lipophilic, and its effect in leishmania arises from its selective high affinity for the episterol that are precursors of ergosterol present in the parasitic cell membrane rather than for cholesterol in mammalian cell membranes.

The cost of all of these treatments is considerably more than that of Fungizone. On a weight basis, AmBisome is approximately 12-fold more expensive than Fungizone. However, as the daily dose is also 2-5 times higher, the daily cost of treatment is approximately 24–60 times higher. Even bearing these costs in mind, treatment of patients with systemic mycoses following liver or bone marrow transplantation is still essential as it results in a much higher survival rate (Persson *et al.*, 1992).

The aim of this study was to prepare dextrin- and D-2-S-AmpB conjugates with the hope of decreasing AmpB toxicity and increasing the solubility. The D-2-S-AmpB conjugates might, in addition, have potential use in immunosuppressed patients with chronic fungal infections. Preliminary studies were carried out to determine the biological activity of both conjugates.

7.2 Materials and Methods

7.2.1 Conjugation of AmpB to succinoylated dextrin and D-2-S

AmpB was conjugated to dextrin (0.9 mol%) and D-2-S (1 mol%). The method is described in Chapter 2 (Section 2.4.5). Initially there was concern that high AmpB loading might lead to insoluble conjugates. Therefore, the first aim was to modify dextrin and D-2-S to a level of 1 mol%.

Conjugation to dextrin: Firstly succinoylated dextrin (51 mg, 1 x 10^{-6} mol was dissolved in DMF (10 ml). To this solution CDI (10.4 mg, 6.4 x 10^{-5} mol) was added. The reaction was allowed to proceed at 25°C under stirring for 1 h. Then AmpB (1.2 mg, 3.1 x 10^{-6} mol) was dissolved in DMF (2 ml) and was added to the reaction, and left stirring at 25°C for 24 h. The product was then purified and the incorporation of AmpB was determined by UV-vis, and FTIR spectrometry.

Conjugation to D-2-S: Succinoylated D-2-S (51 mg, 2.04 x 10^{-6} mol) was dissolved in DMF (10 ml). To this solution CDI (10.4 mg, 6.4 x 10^{-5} mol) was added. The reaction

was allowed to proceed at 25°C under stirring for 1 h. Then AmpB (1.2 mg, 4.6 x 10^{-5} mol) was dissolved in DMF (2 ml) and was added to the reaction and left stirring at 25°C for 24 h. The product was then purified and the incorporation of AmpB was determined by UV-vis, and FTIR spectrometry.

***Purification*:** The AmpB conjugates were purified by dialysis (Mw cut-off 12-14 kDa) againist DI water. Every 4 h the dialysate was changed and a sample (5 ml) was collected for UV-vis (415 nm) analysis of released AmpB (yellow) until no absorbance was seen. At this time, it was presumed that all free AmpB had been removed from the conjugate.

Characterisation: Dextrin-and D-2-S-AmpB conjugates were characterised by using FTIR to indicate the presence of conjugated AmpB onto the conjugates and UV-vis (415 nm) spectroscopy (Chapter 2, Figure 2.6) to determine total AmpB content.

After purification and characterisation it was shown that these first products had very low loading. Conjugation was later repeated using dextrin and D-2-S with higher degrees of succinoylation with the aim of increasing AmpB content. Dextrin was succinoylated to levels of 10 and 34 mol% and D-2-S succinoylated to levels of 14 and 24 mol%. Chapter 2 (Sections 2.4.1 and 2.4.2).

All the parameters were kept the same as described above except that larger batches were prepared therefore the quantities of the chemicals used were scaled-up accordingly.

Conjugation to dextrin: Firstly, dextrin (10 mol%) (204 mg, 4 x 10^{-6} mol) was dissolved in DMF (10 ml). To this solution CDI (40.2 mg) was added. The reaction was allowed to proceed at 25 °C under stirring for 1 h. Then AmpB (12 mg) was dissolved in DMF (2 ml) and was added the reaction and left stirring at 25°C for 24 h. The product was then purified and the incorporation of AmpB was determined by UV-vis, and FTIR spectrometry.

Secondly, keeping the reaction condition the same as above, AmpB (34 mg) was added to dextrin (30 mol%).

D-2-S: Firstly, D-2-S (10 mol%) (112 mg, 4.5 x 10^{-6} mol) was dissolved in DMF (10 ml). To this solution CDI (40.2 mg,) was added. The reaction was allowed to proceed at 25°C under stirring for 1 h. Then AmpB (12.8 mg) was dissolved in DMF (2 ml) and was added the reaction and left stirring at 25°C for 24 h. The product was then purified and the incorporation of AmpB was determined by UV-vis, and FTIR spectrometry.

Secondly, keeping the reaction condition the same as above, AmpB (32 mg) was added to dextrin (25 mol%).

Solubility: To test solubility the conjugates were dissolved in water and their solubility compared with that of free AmpB. AmpB solutions in water (1, 0.5, 0.25, 0.125, 0.065 and 0.0313 mg/ml AmpB equiv.) was compared with dextrin-AmpB concentrations of 0.4, 0.16 and 0.10 mg/ml AmpB-equiv. and D-2-S-AmpB concentrations of 0.014 and 0.009 mg/ml AmpB equiv.

7.2.2 RBC Lysis

The detailed method for the RBC lysis is described in Chapter 2 (Section 2.5.2). The 96 well plate was prepared with samples (100 µl: n=4) of the appropriate control, polymer or polymer-conjugate concentrations before the killing of the rat and were pre-chilled. Concentration ranges used for the controls (dextrin, D-2-S, PBS, Fungizone, AmpB, DMSO, dextran and polyethyleneimine) were in the range of 0 - 5 mg/ml.

Dextrin-AmpB, D-2-S-AmpB and AmpB were all dissolved in DMSO (10 µl) before being made up to the appropriate concentration in media. Concentration ranges used for the conjugates varied according to the loading of AmpB. Dextrin-AmpB concentrations were made in the range of 0 - 2.5 µg/ml of AmpB-equiv., D-2-S-AmpB in the range of 0 – 14 µg/ml AmpB equiv. and AmpB concentrations in the range of 0 – 14 µg/ml.

The blood (100 µl: 2 % w/v solution) was added to each well and left to incubate at 35°C for either 30 min, 1 h or 24 h. Samples were centrifuged (1500g, 10 min) and the supernatant (100 µl) was pipetted into a 96 well micro-titre plate. The absorbance was measured and using a micro-titre plate reader blanked with PBS. The degree of lysis was expressed as a percentage of 100 % lysis seen using Triton X-100.

Scanning electron microscopy: SEM was also used to examine the polymer-conjugate interactions with the RBC's cells. A detailed method is described in Chapter 2 (Section 2.5.3). The concentrations used for the SEM are PBS (control), dextrin and D-2-S (5 mg/ml), AmpB at EC_{50} (7.2 µg/ml), dextrin-AmpB (0.5 mg/ml) and D-2-S-AmpB at EC_{50} (9.8 µg/ml). The method was carried out as described in Chapter 2 (Section 2.5.3) but before the end of the centrifuge spin the preparations were transferred to a microfuge tube and were pelleted by centrifugation (1500 g, 10 min). The supernatant was then removed and glutaraldehyde (0.25 % v/v, 1 ml) in PBS was added to the cells to fix them. The cells were then fixed with osmium tetrroxide (1 % w/v, 500 µl) in PBS for 1 h. The cells were dehydrated as described in Chapter 2 (Section 2.5.3) at the last step hexamethyl disilazane (HMDS) was added and the cells were placed on an SEM platform using carbon cement and then gold coated. The samples were examined using the SEM.

7.2.3 Assessment of cell viability using the MTT assay

Standard cell culture protocols as described in Chapter 2 (Section 2.5.1) were used to establish the cytotoxic profiles of dextrin-AmpB, D-2-S-AmpB, AmpB, dextrin and D-2-S. 96 well plates were seeded with a suspension of B16F10 murine melanoma (10^4 cells per well). The cells were incubated for 24 h and after this time the media was carefully removed and 100 µl of controls and polymers dissolved in growth media (n=8) were added to each plate. Dextrin-AmpB concentrations were made in the range of 0 - 2.5 µg/ml of AmpB-equiv., D-2-S-AmpB in the range of 0 – 14 µg/ml AmpB equiv. and AmpB concentrations in the range of 0 – 14 µg/ml. The plates were then incubated for 67 h before the addition of MTT (20 µl). After 5 h incubation the media was removed

and DMSO (100 μl) was added to each well, After 30 min the absorbance was read at 550 nm using a micro-titre plate reader. The absorbance values were converted to % cell viability.

7.3 Results

7.3.1 Dextrin- and D-2-S-AmpB conjugates

***Chemical Characterisation*:** The amount of AmpB incorporated onto each polymer at different mol% made was determined using the AmpB calibration curve using UV-vis (415 nm). From the UV trace the of dextrin- and D-2-S-AmpB the four peaks that are characteristic of AmpB 350, 368, 388 and 412 nm were seen. Also interesting to note is the peak in the 280 nm region which is characteristic of the succinic anhydride linker indicating that not all of the AmpB has been conjugated to all the reaction sites. This is further shown in Table 7.1 which summarises the amount of AmpB incorporated onto each batch of the dextrin and D-2-S conjugates prepared.

Evidence shown by the FTIR spectra (Figure 7.1) showed that AmpB had been successfully conjugated to both dextrin and D-2-S. The peaks clearly visible at 2650 cm^{-1} show the presence of the -NH group confirming the incorporation of AmpB but to further emphasise the point that not all of the succinoyl groups have been conjugated to AmpB, there are still peaks at 1632 cm^{-1} (carbonyl) and 1716 cm^{-1} (ester).

Solubility: AmpB is almost totally insoluble in water. In comparison the polymer conjugates have been shown to have a ten-fold increase in solubility when compared to AmpB at the same concentrations. For all of the biological studies evaluated here dextrin-AmpB had a drug content of 0.006 wt % and the D-2-S-AmpB a drug content of 0.014 wt %.

7.3.2 Effect of free AmpB and the conjugates on RBC cells

The ability of compounds to release haemoglobin (Hb) from rat red blood cells was used to assess the likelyhood of adverse effects upon i.v. administration as the blood is the first compartment in the body that substances will meet. After 30 min, 1h and 24 h incubation times both AmpB and Fungizone produced classic sigmoidal dose-response

Table 7.2 Incorporation of AmpB to dextrin and D-2-S

Polymer	Theoretical (weight %)	Batch Number 1	2	3	Mean weight % (±SD)
Dextrin-AmpB (1 mol%)	2.9	0.0032	0.0061	0.0059	0.005±0.001
Dextrin-AmpB (10 mol%)	29	0.33	0.39	0.34	0.35±0.03
Dextrin-AmpB (34 mol%)	98.6	16.0	17.3	15.6	16.3±0.7
D-2-S-AmpB (1 mol%)	2.73	0.014	0.015	0.013	0.014±0.0
D-2-S-AmpB (14 mol%)	27.3	0.55	0.64	0.44	0.54±0.08
D-2-S-AmpB (24 mol%)	65.5	1.4	1.4	1.3	1.3±0.06

Theoretical weight % - is the amount of of AmpB that would be conjugated if all of the free AmpB conjugated to the polymer.

Mean weight %- the average incorporation of AmpB conjugated to the polymer

Figure 7.2 Effect of AmpB and Fungizone upon the lysis of red blood cells following 30 min and 1 h incubation period

curves. 100 % lysis had been reached by the highest concentration (10 mg/ml) within 30 min. AmpB showed to have approximately a 10-fold increase in the Hb release (Figure 7.2) when compared to Fungizone. The Hb_{50} values (concentrations at which there was 50% Hb release) are can be determined for AmpB, Fungizone and D-2-S-AmpB (Table 7.3).

However, dextrin, dextran, D-2-S, DMSO and polyethylenimine at 1 h incubation time showed no significant lysis upto a concentration of 10 mg/mL After an incubation time period of 24 h there was no difference in the Hb release except polyethylenimine displayed a time dependent factor.

With a 1 h and 24 h time-point the experiment was repeated for the dextrin-AmpB and D-2-S-AmpB polymers and equivalent quantities of AmpB. The ability of dextrin-AmpB to release Hb was approximately equal to that of AmpB alone at both time points, without an Hb_{50} value being obtained (Figure 7.3).

D-2-S-AmpB however, resulted in significant Hb release at a concentration of 0.5 mg/ml, (or 0.007 mg/ml equivalent AmpB) (Figure 7.4). There was little difference between the polymer and AmpB produced release.

Electron microscopy of RBCs incubated with test compounds for 24 h revealed cells to be mainly biconcave discs with some appearing to be more spherical (Figure 7.5). Cells treated with AmpB were no longer identifiable and they appeared as a large amorphous mass. Dextrin and D-2-S treated cells were comparable with the control cells (PBS) which displayed protrusions (crenations) into the surrounding space. It has been suggested that these could be caused by calcium deposits. Dextrin-AmpB treated cells displayed no morphological abnormalities. RBCs incubated with D-2-S-AmpB appeared to have congregated with a slightly sponge-like surface, although not to the same extent as those treated with AmpB alone and some of the cells are visibly dead.

Table 7.3 Hb_{50}'s of AmpB, Fungizone and AmpB at various time points.

	Time		
	30 min	1 h	24 h
Fungizone (μg/ml)	0.011±0.0	0.1±0.005	0.016±0.0
AmpB (μg/ml)	0.129±0.001	0.01±0.0	0.06±0.002
D-2-S-AmpB (μg/ml AmpB equiv)	*	11.0±0.78	8.0±0.64
Dextrin-AmpB (μg/ml AmpB equiv)	ND	ND	ND

ND - Hb_{50} Not Detected (doses given not high enough)

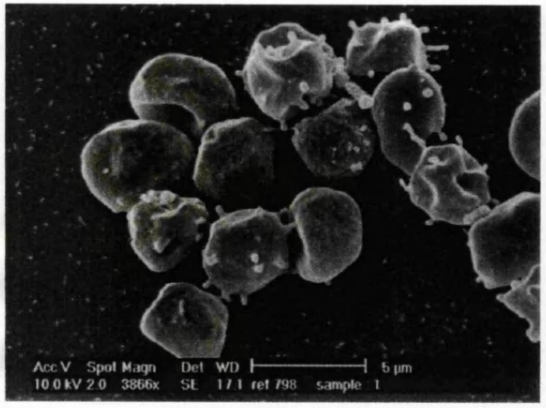
* - Time point not carried out

Figure 7.3 Effect of Dextrin -AmpB upon the lysis of red blood cells following 1 h and 24 h incubation period

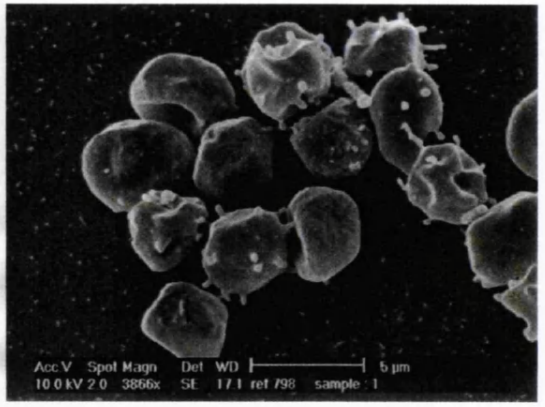
Figure 7.4 Effect of D-2-S -AmpB upon the lysis of red blood cells following 1 h and 24 h incubation period

Figure 7.7 Electron micrographs of RBC's incubated with control, test compounds, dextrin- and D-2-S-AmpB at different concentrations after incubation for 24 h

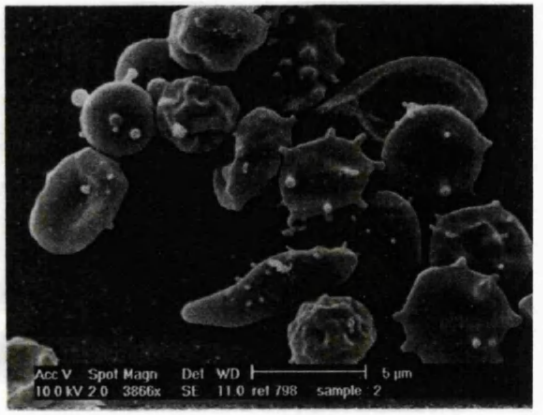
PBS (control)



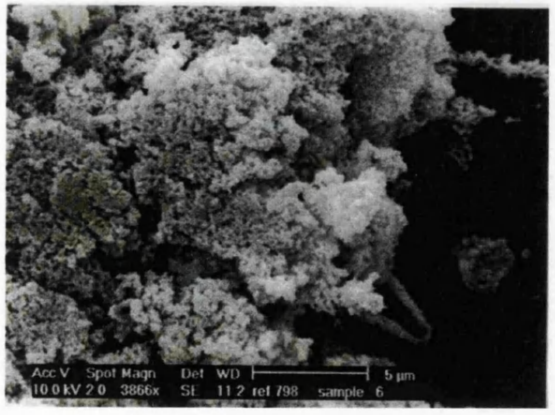
Dextrin at 0.5 mg/ml



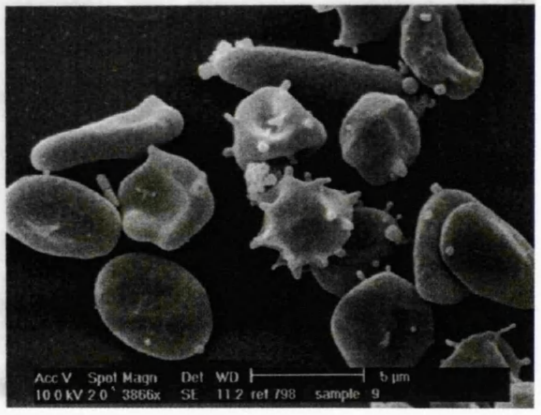
D-2-S at 5 mg/ml



AmpB at EC_{50}



Dextrin-AmpB at 0.5 mg/ml



D-2-S-AmpB at EC_{50}

7.3.3 MTT Assay

Control Compounds: The MTT assay with an incubation period of 72h showed dextran, dextrin and D-2-S caused very little decrease in cell viability at the highest concentrations used (5 mg/ml). Poly-L-lysine however, acted as a positive control with an IC_{50} value (concentration at which there was a 50% decrease in cell viability compared with controls) of approximately 0.04 mg/ml (Figure 7.6). Fungizone and AmpB also caused a significant decrease in cell viability with an exponential pattern.

Conjugates: Both dextrin-AmpB and D-2-S-AmpB caused a greater decrease in cell viability than equivalent quantities of AmpB. The dextrin-AmpB conjugate caused an initial decrease in cell viability to approximately 75 % at a dose of 0.4 mg/ml (0.2 mg/ml AmpB equivalents) which then remained at that level even at the highest concentration used (Figure 7.7).

The D-2-S-AmpB conjugate however, was significantly more toxic to the B16F10 cells than equivalent quantities of AmpB, with an IC_{50} value of approximately 10 mg/ml AmpB equivalents (Figure 7.8), which is roughly 8 times lower than AmpB alone.

7.4 Discussion

It is well known that conjugation of an insoluble drug to water soluble polymers can be used to increase the water solubility, drug circulation time and also targeting diseases tissues. In the case of anticancer agents this has resulted in an improved therapeutic effect and a decrease in toxicity. The earlier biodistribution studies (Chapter 4, 5 and 6) in both Wistar rats and mice showed that dextrin and D-2-S have the tendency to localise in the liver and spleen. This might be predicted as it is known that macrophages contain receptors that recognise glucose/polyglucose. The opportunity described here to use dextrin and D-2-S conjugates of AmpB to encourage liver targeting was seen as a way of increasing AmpB concentrate at the site of infection. It is accepted that the selective damaging action of AmpB originates due to the ergosterol in fungal cells walls. Mammalian cell membranes contain cholesterol.

Figure 7.6 Cytotoxicity of test compounds upon the B16F10 cell line using the MTT Assay at 72 h (n=6 ±SD)

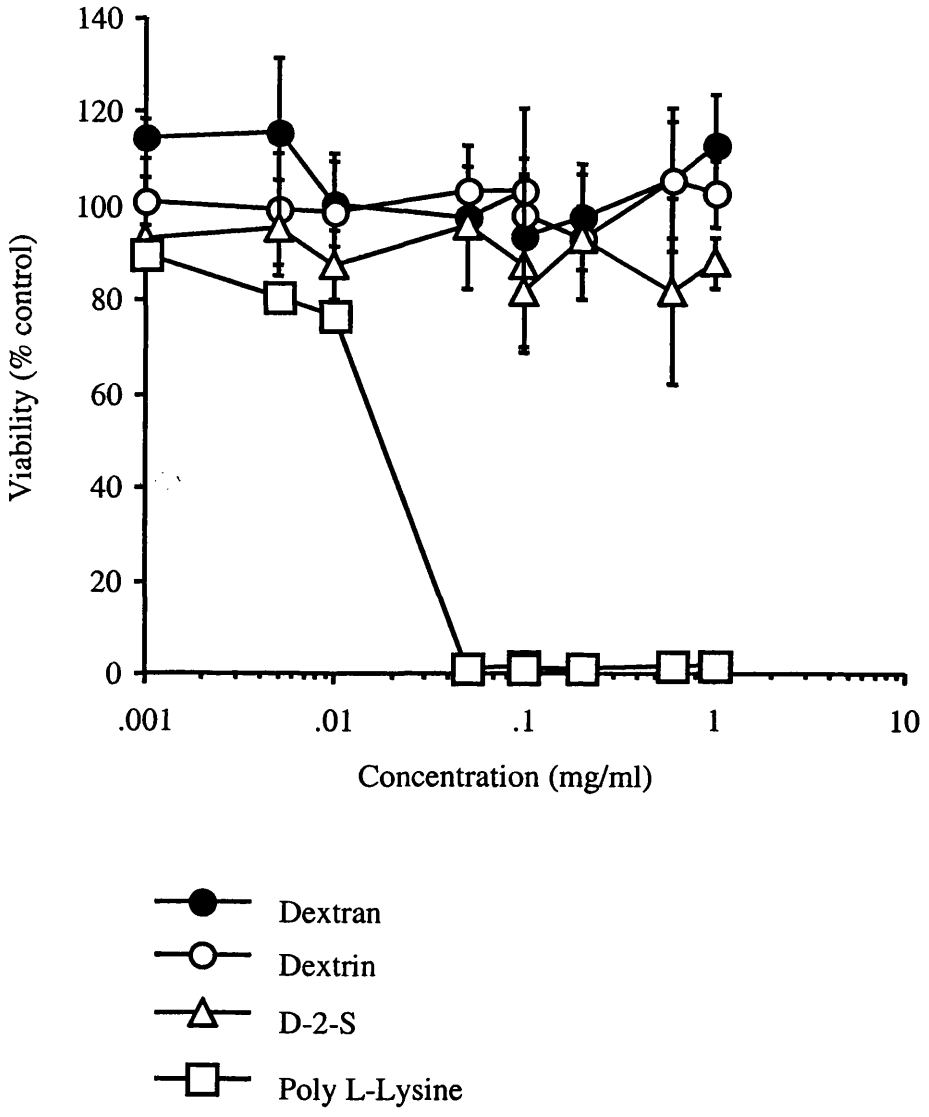


Figure 7.7 Cytotoxicity of dextrin-AmpB compared to AmpB upon B16F10 cell line using the MTT Assay at 72 h (n=6±SD)

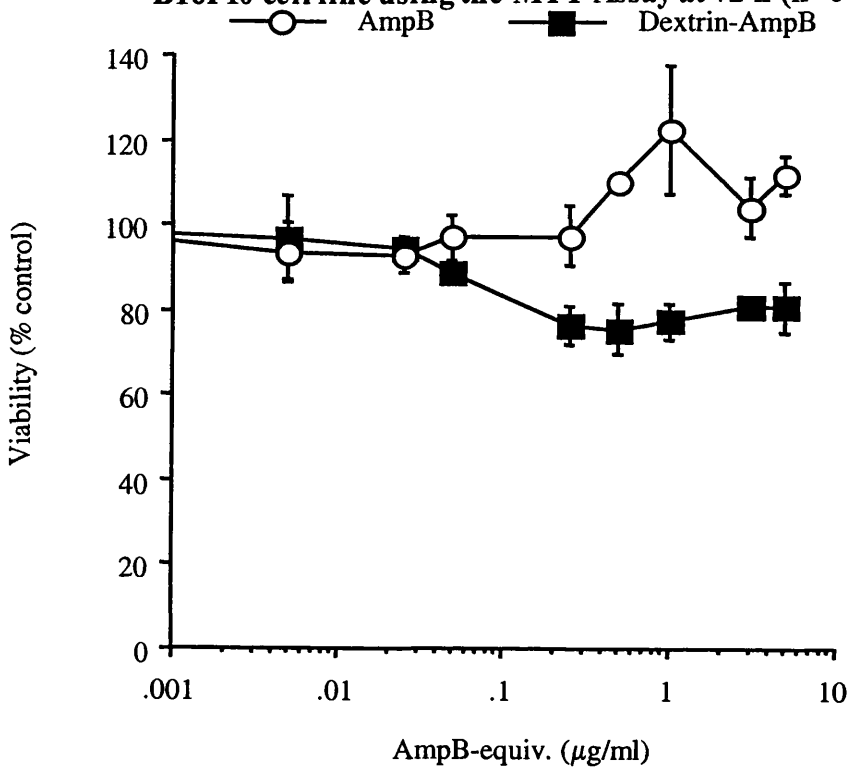
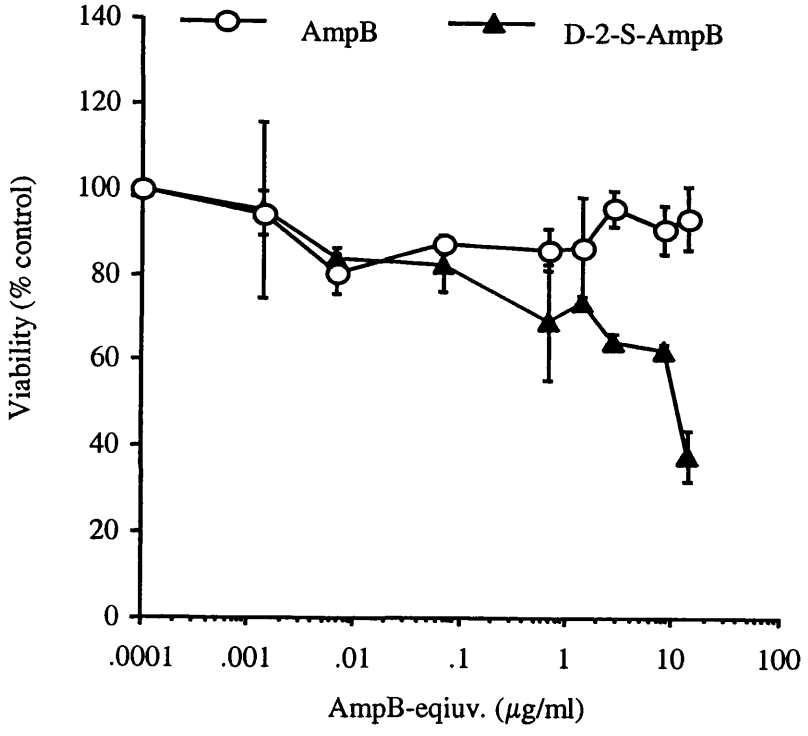


Figure 7.8 Cytotoxicity of D-2-S-AmpB compared to AmpB upon B16F10 cell line using the MTT Assay at 72 h (n=6±SD)



The successful conjugation to AmpB to dextrin and D-2-S was shown by FTIR and UV-vis. When AmpB is solubilised with a small amount of DMF and diluted with water a single broad peak of 328 nm is seen. This represents the high aggregated form of AmpB. In apolar solvents (DMSO or DMF) it exists in a monomeric form characterised by four peaks at 350, 360, 388 and 412 nm and this is known as the B form. Interestingly both AmpB conjugates also suggested AmpB was present in the non aggregated monomeric form. This might suggest, at too low loading AmpB loading the conjugates do not form an intermolecular micelle. The dextrin-and D-2-S-AmpB conjugates had very good water solubility. However the loading was very low (0.06-0.3 wt%). Further experiments are underway to investigate the limit (in terms of loading) of AmpB content that would still give good water solubility. AmpB loading is increased, it will also be necessary to ensure that crosslinking does not occur (it should be noted that AmpB theoretically has two possible sites of conjugation).

In the preliminary biological tests, free AmpB and Fungizone were both very haemolytic after 30 min (Figure 7.4, HB_{50} values of 0.06 μg/ml and 0.016 μg/ml respectively). AmpB was an order of magnitude more haemolytic than Fungizone. Neither dextrin nor D-2-S were haemolytic (Figure 7.3). The dextrin-AmpB conjugate showed the same activity as free AmpB over the AmpB concentration range used. The D-2-S-AmpB conjugate was significantly different less haemolytic than free AmpB. In future experiments it would be important to (1) increase conjugate concentration to obtain the HB50 values and (2) to test conjugates with higher AmpB loading. The observation seen can be compared to those which are already ion the clinic.

AmBisome is stable and was not heamolytic upto drug levels of 100 μg/ml in contrast to free AmpB at the concentrations of between 1μg/ml to 6 μg/ml which caused more than 90% haemolysis (Alder-Moore and Proffitt, 1998). The same study showed Albecet haemolysed red blood cells at concentrations very similar to those of conventional AmpB suggesting that this formula releases drug readily. The unique colloidal formulation of Amphotec has been shown to reduce the heamolysis of AmpB (Guo *et al.*, 1991).

Measurement of haemoglobin release gives a quantitative measurement of gross membrane damage. However, it was considered important to look qualitatively at the RBC structure using SEM. The SEM was carried out at the HB50's values except where there was not one and thus the highest concentration tested was used. The time of exposure chosen was 24 h because this showed the greatest haemolysis of our polymers.

The control RBC's had "smooth" membranes although sometimes there was evidence of crenations. Dextrin did not alter RBC morphology at the concentrations examined. The D-2-S-AmpB conjugate which was moderately haemolytic caused less severe membrane damage that AmpB alone which caused complete lysis of the cells visualised by appearance of a sponge-like mass. This is probably due to AmpB complexing with the membrane sterols (in particular cholesterol) and the subsequent formation of pores (Urbina *et al.*, 1987).

The MTT assay assesses the ability of compounds to decrease cell viability. B16F10 murine melanoma cells were utilized since they are easily cultured and grow rapidly. They are a mammalian cell line against which AmpB is active although to a lesser extent than fungal cells (Vertut-Croquin *et al.*, 1983). In some cases AmpB is used in media to prevent fungal contamination. Neither of the two polysaccharide polymers displayed pronounced cytotoxic effects alone. D-2-S did cause a small decrease in cell viability, which may be interesting in the context of D-2-S antitumour activity seen in Kaposi's sarcoma (Thornton *et al.*, 1999).

Fungizone and AmpB were both able to decrease cell viability at the concentration range used with IC_{50} values of 20 and 80 mg/ml respectively. AmpB was more effective at releasing haemoglobin although, it appears that in melanoma cells Fungizone is the more cytotoxic. This may in part be due to differences in water solubility. The increased solubility of Fungizone may in some way increase its ability to interact with melanocyte membranes.

Dextrin-AmpB was slightly more cytotoxic than AmpB towards B16F10 ells but not as cytotoxic as Fungizone (Figure 7.10) at the same AmpB equiv.

Unfortunately these preliminary experiments did not use a high enough concentration of the conjugate to make a direct comparison with free AmpB. This was also due to a lack of product that was made at the time and also that there was lack of time.

In producing a new therapy it is important to establish that the therapeutic doses of a compound can be easily administered. In the case of the two conjugates the AmpB content is very small. For dextrin-AmpB approximately 200 mg of the compound is required to receive a 1 mg dose of AmpB. As the recommended dose is 1 mg/kg/day for the average 70 kg person approximately 14 g/day of conjugate would be needed to be infused to obtain a therapeutic dose of AmpB. D-2-S-AmpB contains a higher wt % of AmpB than the dextrin conjugate, and only 5 g/day would be required for the average person.

It would be interesting to determine in future experiments, whether the conjugated form of D-2-S retains any activity in the clinic as AIDS patients are highly susceptible to fungal infections, which they are unable to fight effectively.

These experiments show that conjugation to the polysaccharides dextrin and D-2-S did no increase the haemolytic activity of AmpB and the D-2-S-AmpB conjugate was more cytotoxic than AmpB. This may be therapeutically useful since it could results in lower doses being required which in turn would decrease the potential for adverse reactions. However, if D-2-S-AmpB is not selective to fungal cells its potential therapeutic use as an anti-fungal agent is greatly reduced since its toxicity to mammalian cells is high.

To add to the preliminary studies, experiments were carried out in collaboration with Prof. Dave Humber's laboratory at the University of West London. Compounds were tested against a protozoan parasite *Leischmania*. *Leischmani*a is an important opportunist infection in a host that is immunocompromised. This includes AIDS patients. AmpB conjugated to arabinogalactan (highly branched natural polysaccharide) to a weight percent of 20. Results *in vitro* have shown EC_{50} values were obtained for

reduced and unreduced forms of the drug-conjugate. In *Leischmania major* promastigotes this EC_{50} was 0.17 and 0.31 μl/ml for unreduced and reduced forms respectively for amistigotes and 0.19 and 0.34 μl/ml respectively for promastigotes (Golenser *et al.*, 1999). The results (Table 7.3) were compared to dextrin-AmpB (16 wt %) and D-2-S-AmpB (1.3 wt %) the LD_{50} was found to be for both conjugates 5 μg/ml and 0.8 μg/ml respectively. These experiments demonstrated that dextrin-AmpB (for both of the wt % analysed) was more effective than D-2-S-AmpB showing that the sulphation results in lower activity against the parasite. The dextrin-AmpB (0.35 wt%) was more toxic than against *Leischmania* than the conjugate with a higher drug loading (16 wt %) indicating that simply increasing the amount of AmpB loading is not necessary beneficial as needed is a compound that will be toxic against fungal cells.

These studies for the first time describe the use of dextrin and D-2-s as conjugates to treat such diseases. The improvement in solubility, differences in haemolytic activity, cytotoxicity in B16F10 and *Leischmania* suggests the possibility of developing interesting compounds with potential for further investigation. To go further with these conjugates it is necessary first to optimise the loading of AmpB onto both dextrin and D-2-S at different modifications of succinoylation. This would enable determination of an optimum therapeutic index in each of the proposed uses.

Table 7.3 Effect of AmpB and conjugates on *Leischmania aethiopica* promastigote *in vitro*.

Conjugate	AmpB loading (wt %)	LD_{100} (μg/ml)	LD_{50} (μg/ml)		MIC (μg/ml)	
		1 and 24 h	1 h	24 h	1 h	24 h
D-AmpB	0.35	10	ND	ND	0.63	0.63
D-AmpB	16	100	5	50	2.5	2.5
D-2-S-AmpB	0.5	10	1.25	ND	0.63	0.63
D-2-S-AmpB	1.3	>150	5	50	2.5	2.5

LD_{100} – Lethal dose at 100 %

LD_{50} – Lethal dose at 50 %

MIC – Minimum Inhibitory Concentration

Method

L. Aethiopica promastigotes (strain MHOM/ET/72/L100, obtained from the London School of Hygiene and Tropical Medicine) were grown in RPMI 1640 medium (Sigma) supplemented with 10 % foetal calf serum. The parasites were cultured at a concentration of 0.5 x 10^6 parasites per ml and incubated at 22°C. Different concentrations of the polymers (prepared in media) were added and the parasite concentrations were determined (1 h and 24 h) using a Neubauer haemocytometer and a 2 % formal-saline fixative (2 % formalin, BDH chemicals).

Chapter 8

General Discussion

8.1 General Discussion

This thesis represents the first attempt to assess the potential of dextrin and D-2-S as polymeric carriers. Chemical modification of both polymers was needed to allow introduction of a variety of chemical entities (drugs and model compounds).

Succinoylation was chosen as a means to produce conjugates with the first objective being modification of dextrin and D-2-S to 1 mol% (Hrezcuk-Hirst *et al.*, 2000) in order to keep polymer backbone as close to the original structure as possible. The conditions of the reaction were optimised (time and temperature) and all were very reproducible. When compared to other studies using succinoylation as a method to modify other polysaccharides (Bruneel and Schacht, 1995: Arranz *et al.*, 1992) the results were consistent to what was observed in the literature.

As the interest was to develop dextrin and D-2-S as potential polymers in drug conjugates it was decided that initially the biodistribution after s.c., i.v. and i.p. administration should be studied (Chapter 4) (German *et al.*, 1999, 2000). By the incorporation of tyrosinamide, polymers could be labelled using [^{125}I]iodine.

I.p. administration of a tracer dose of ^{125}I-labelled dextrin (1 mol%) to Wistar rats showed the polymer was removed from peritoneal cavity and the body far too quickly. Studies carried out by Hrezcuk-Hirst (2000) indicated that α-amylase degradation of dextrin modified to 1 mol % was extremely fast. Thus, modification was increased to 15 and 34 mol % and this slowed down the rate of α-amylase degradation thus providing us with a much more stable polymer to which drugs could be added.

I.p. administration of a tracer dose of ^{125}I-labelled D-2-S with 1 mol% modification showed the tracer dose remained in the peritoneal cavity for much longer periods of time (German *et al.*, 1999).

It was decided to study further the effect of volume, dose and repeated administration upon the tissue targeting of ^{125}I-labelled D-2-S as these parameters could be of clinical importance (German et al., 1999). All of the parameters studied causes differences in the pattern of biodistribution observed which might be important when choosing the correct volume and dose in the clinic.

Incorporation of DTPA in dextrin and D-2-S allowed labelling using [^{111}In]indium chloride, which enabled the fate of the polymers to be monitored by gamma camera imaging (or dissection analysis).

For the first time in this thesis we have conjugated [^{111}In]indium chloride successfully to dextrin and D-2-S (German *et al.*, 2000). The biodistribution observed with ^{125}I-labelled polymers and ^{111}In-labelled polymers was similar. Even when the study was repeated with increased injection volume the results were consistent for ^{111}In-labelled D-2-S with ^{125}I-labelled D-2-S. These probes could be useful in the clinical studies that are currently underway to enable visualisation of the fate of dextrin of D-2-S in humans.

^{125}I-Labelled dextrin (1 and 34 mol%) were used to study the possibility of tumour targeting in mice bearing B16F10 tumours after i.v. and i.p. administration. Only with between 0.6 - 1 % of the administered dose was recovered in the tumour after i.p. administration of ^{125}I-labelled dextrin (1 mol%). However, recovery in the tumour was much higher when the more stable ^{125}I-labelled dextrin (34 mol %) was injected i.v. (0.4 % to 7 % of the administered dose respectively). As treatment of AIDS patients with D-2-S produced a shrinking of Kaposi's sarcoma s lesions (Thornton *et al.*, 2000) it was interesting to find that administration of ^{125}I-labelled D-2-S to tumour bearing mice gave a relatively small degree of tumour targeting (0.1% to 0.4% of the administered dose) after i.p. administration.

The first two model drug compounds that were chosen for conjugation to dextrin or D-2-S were Dox and AmpB.

Dox it could be argued is now an old-fashioned drug (Cassidy, oral presentation, 2000) but it was chosen here as a model drug because its properties are so well known. By conjugation of Dox (anticancer anthracycline) to dextrin it was hoped that selective accumulation of the polymer-conjugate in the tumour by the EPR effect and subsequent drug release selectivity within the tumour would occur (Chapter 6). In the future however other drugs, which have shown potential, could be used e.g. cisplatin would have the advantage of synergistic antitumour activity and reduced toxicity and is the treatment of choice in many different types of cancers such as various carcinomas. Paclitaxel (taxol) has shown toxicity such as hematological and neurological and is in use in various types of cancer such as breast and ovarian, and

hydrazine sulphate (HS) even though it is carcinogenic is part of the chemotherapeutic regimens of patients with advanced non-small-cell lung cancer and leukemia, advanced colorectal cancer, and with newly diagnosed non-small-cell lung cancer. (Kosty *et al.*, 1994: Loprinzi, 1994 : Loprinzi, 1994b)

Dextrin (34 mol%) Dox (9 wt%) caused improved antitumour activity against s.c. B16F10 (T/C= 130 %) when compared to Dox alone (T/C = 83.3%). There was also an increase in tumour uptake of dextrin-Dox (~ 2.5 % dose/gram tumour) when compared to Dox alone (~ 1 % dose/gram tumour).

Dextrin-Dox must in future be evaluated against a number of different tumour models with high and low EPR targeting using the optimised dextrin-Dox to evaluate its real potential. Dosing schedules, multiple dose injections are needed to be studied and the experimental design must establish of MTD for dextrin-Dox.

In all of the studies the only route of administration used was that of the peritoneal route. This method of administration has shown accumulation within tumour cells but in the theory EPR-mediated targeting would be better after i.v. injection. In future all studies need to compare tumour targeting i.p. or i.v. administration of the polymer-conjugate.

Dextrin and D-2-S were covalently attached to AmpB (antifungal agent) to provide a new polymer drug conjugate in the treatment of fungal diseases (Chapter 7).

In this thesis the results shown are very preliminary but we have shown that the dextrin or D-2-S conjugates can overcome the disadvantages of AmpB poor solubility. Increased loading of the AmpB onto the polymers might, or might not be beneficial. The conjugates with lower loading seemed to give a greater toxicity to the *Leischmania promastigote*. Future work within this area should establish the optimal drug loading to give best therapeutic index.

Even though there is still a long way to go with the development of both dextrin and D-2-S in the field of "polymer therapeutics", I feel both do have potential as either anticancer or antifungal drug conjugates. Further investigations are warranted and maybe one day one or more of these conjugates found useful (or gamma camera imaging agents) in the clinic.

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Appendix 1

Tables to show biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after administration s.c., i.v. and i.p.

Table 1 Body distribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after s.c. administration to rats

Organ	^{125}I-labelled dextrin			^{125}I-labelled D-2-S	
	2 min	5 min	30 min	2 min	60 min
Blood	2.2±0.2	0.8±0.2	3.2±0.2	2.8±0.2	4.0±0.3
Lung	0.1±0.0	0.4±0.1	1.8±0.2	0.1±0.0	0.3±0.0
Liver	0.3±0.1	0.9±0.2	0.4±0.2	0.4±0.0	6.6±0.4
Heart	0.1±0.0	0.6±0.5	0.2±0.0	0.1±0.0	0.2±0.0
Spleen	0.1±0.0	0.4±0.3	0.1±0.0	0.1±0.0	0.5±0.0
Kidney	0.1±0.0	0.8±0.1	1.9±0.6	0.2±0.0	4.9±0.1
Urine	0.1±0.0	0.3±0.2	1.4±0.5	0.1±0.0	0.3±0.0
Thyroid	0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.3±0.1
Stomach	0.1±0.0	*	*	0.1±0.0	0.3±0.1
Stomach wash	0.2±0.0	*	*	0.2±0.0	0.6±0.1
Small intestine	0.2±0.0	*	*	0.1±0.0	0.8±0.1
Small intestine wash	0.1±0.0	*	*	0.1±0.0	0.8±0.2
Caecum	0.1±0.0	*	*	0.1±0.0	0.2±0.0
Caecum wash	0.1±0.0	*	*	0.1±0.0	0.1±0.0
Large intestine	0.1±0.0	*	*	0.1±0.0	0.2±0.0
Large intestine wash	0.1±0.0	*	*	0.1±0.0	0.0
Carcass	83.4±12.9	*	*		
Total Recovery	87.5±13.2	4.6±1.6	9.1±1.7	4.8±0.2	20.1±1.4

* Not analysed

Results are expressed as mean ±SD (n=3) of administered dose (5 x 10^5 cpm, 100µl)

Table 2 Body distribution of ^{125}I-labelled D-2-S after i.v. administration to rats

Organ	^{125}I-labelled D-2-S	
	2 min	60 min
Blood	69.2±4.8	4.8±0.5
Lung	2.7±0.2	0.8±0.3
Liver	24.6±3.5	36.5±0.4
Heart	9.5±2.8	0.3±0.1
Spleen	1.0±0.0	2.5±0.1
Kidney	0.7±0.0	13.6±1.3
Urine	0.25±0.1	0.2±0.0
Thyroid	0.2±0.0	0.2±0.0
Stomach	0.4±0.1	1.0±0.3
Stomach wash	0.2±0.0	1.9±0.6
Small intestine	1.2±0.3	3.5±0.7
Small intestine wash	0.5±0.1	3.2±1.6
Caecum	0.3±0.1	0.1±0.0
Caecum wash	0.1±0.0	0.2±0.0
Large intestine	0.4±0.0	0.5±0.0
Large intestine wash	0.2±0.0	0.0
Total Recovery	87.5±12.0	69.3±5.9

Results are expressed as mean ±SD (n=3) of administered dose (5 x 10^5 cpm, 100µl)

Table 3 Body distribution of ^{125}I-labelled dextrin after i.v. administration to rats

Organ	Time (min)					
	2	5	15	30	45	60
Blood	28.6±7.0	5.4±2.1	0.7±0.0	1.7±0.4	0.2±0.2	0.1±0.0
Lung	2.2±0.5	1.5±0.3	0.7±0.2	1.5±0.0	0.4±0.0	0.7±0.1
Liver	17.8±4.6	11.2±1.4	7.1±1.0	7.3±1.2	5.8±1.1	8.3±1.1
Heart	1.0±0.5	0.4±0.0	0.3±0.0	0.1±0.0	0.2±0.0	0.1±0.0
Spleen	0.7±0.2	0.3±0.2	0.3±0.0	0.4±0.0	0.3±0.1	0.5±0.0
Kidney	14.0±2.5	4.3±0.7	2.6±0.2	1.7±0.5	1.2±0.2	0.9±0.1
Urine	8.2±5.0	1.5±0.4	2.5±2.1	0.8±0.5	0.3±0.0	55.6±16.8
Thyroid	0.2±0.0	0.2±0.0	0.3±0.0	0.1±0.0	0.2±0.0	0.1±0.0
Stomach	0.7±0.1	*	*	*	*	*
Stomach wash	0.2±0.0	*	*	*	*	*
Small intestine	2.4±0.4	*	*	*	*	*
Small Intestine Wash	0.5±0.1	*	*	*	*	*
Caecum	0.5±0.0	*	*	*	*	*
Caecum wash	0.4±0.1	*	*	*	*	*
Large intestine	0.6±0.2	*	*	*	*	*
Large intestine wash	0.2±0.0	*	*	*	*	*
Total recovery	78.2±21.1	24.8±5.1	14.5±3.5	13.6±2.6	8.6±1.6	66.3±18.1

* Not analysed

Results are expressed as mean ±SD (n=3) of administered dose (5 x 10^5 cpm, 100µl)

Table 4 Body distribution of ^{125}I-labelled dextrin after i.p. administration to rats

Organ	Time (min)		
	2	15	60
Blood	4.7±2.2	8.7±1.2	0.5±0.0
Lung	0.2±0.0	0.2±0.1	0.5±0.2
Liver	0.7±0.3	8.4±1.2	15.3±4.1
Heart	0.2±0.0	0.2±0.0	0.3±0.0
Spleen	0.2±0.0	0.9±0.2	1.0±0.5
Kidney	0.2±0.0	3.0±0.5	3.7±0.7
Urine	0.1±0.0	0.2±0.1	0.2±0.0
Thyroid	0.1±0.0	0.1±0.0	1.6±0.8
Stomach	0.3±0.1	0.6±0.1	0.6±0.1
Stomach wash	0.4±0.3	0.5±0.3	0.4±0.0
Small intestine	2.0±0.5	1.4±0.1	1.5±0.6
Small Intestine Wash	1.1±0.3	0.8±0.3	0.5±0.0
Caecum	0.8±0.4	0.2±0.0	0.5±0.1
Caecum wash	0.2±0.0	0.1±0.0	0.4±0.0
Large intestine	1.9±1.6	0.6±0.0	0.5±0.1
Large intestine wash	0.4±0.1	0.2±0.0	0.3±0.0
I.p. Wash	59.7±5.3	56.7±11.4	0.6±0.2
Total recovery	73.2	82.6	28.4

Table 5 Body distribution of ^{125}I-labelled D-2-S after i.p. administration to rats

Organ	Time					
	2 min	1 h	2.5 h	5 h	10 h	24 h
Blood	4.7±2.2	8.7±1.2	8.3±2.0	4.0±0.2	4.8±1.6	0.5±0.0
Lung	0.2±0.0	0.1±0.1	0.3±0.0	0.3±0.0	0.4±0.0	0.5±0.2
Liver	0.7±0.3	8.4±1.2	8.6±2.7	11.3±1.6	11.3±1.2	15.3±4.1
Heart	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.3±0.0
Spleen	0.2±0.0	0.9±0.2	0.4±0.1	0.7±0.2	0.7±0.1	1.0±0.5
Kidney	0.2±0.0	3.0±0.5	3.9±0.6	4.6±1.1	4.3±0.8	3.7±0.7
Urine	0.1±0.0	0.2±0.1	0.4±0.1	0.4±0.0	0.2±0.0	0.2±0.0
Thyroid	0.1±0.0	0.1±0.0	0.3±0.0	1.3±0.1	2.6±0.1	1.6±0.8
I.p. wash	59.7±5.3	56.7±11.4	41.8±6.8	19.7±5.2	5.7±0.7	0.6±0.2
Stomach	0.3±0.1	0.6±0.1	0.6±0.1	1.2±0.1	1.8±0.6	0.6±0.1
Stomach wash	0.4±0.3	0.5±0.3	1.6±0.2	2.3±0.3	1.7±0.5	0.4±0.0
Small intestine	2.0±0.5	1.4±0.1	2.1±0.4	2.0±0.2	2.0±0.5	1.5±0.6
Small intestine Wash	1.1±0.3	0.8±0.3	1.1±0.1	1.5±0.3	0.8±0.1	0.5±0.0
Caecum	0.8±0.4	0.2±0.0	0.5±0.0	0.4±0.0	0.5±0.0	0.5±0.1
Caecum wash	2.0±0.0	0.1±0.0	0.0	0.5±0.0	0.9±0.2	0.4±0.0
Large intestine	1.9±0.6	0.6±0.0	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.1
Large intestine wash	0.4±0.1	0.2±0.0	0.1±0.0	0.3±0.0	0.5±0.3	0.3±0.0
Total recovery	75.0±7.9	82.7±15.6	70.5±13.1	51.1±9.3	38.8±6.7	28.4±7.4

Results are expressed as mean ±SD (n=3) of administered dose (5×10^5 cpm, 100 µl)

Appendix 2

Tables to show factors affecting the biodistribution of ^{125}I-labelled D-2-S after administration i.p.

Table 1 Body distribution of ^{125}I-labelled D-2-S after i.p. administration at 2.5 h with increasing volume

Organ	100µl	500µl	1 ml	1.75 ml	2.5 ml
Blood	8.3±2.0	6.1±1.1	7.8±1.3	11.9±1.0	5.1±0.5
Lung	0.3±0.0	0.6±0.0	0.5±0.0	0.2±0.0	0.4±0.0
Liver	8.6±2.7	11.3±0.2	15.6±1.5	7.5±0.6	11.2±2.8
Heart	0.1±0.0	0.2±0.0	0.3±0.0	0.1±0.0	0.2±0.0
Spleen	0.4±0.0	0.6±0.1	1.2±0.2	0.4±0.0	0.6±0.0
Kidney	3.9±0.6	4.9±0.9	8.7±1.3	2.9±0.0	2.6±1.3
Bladder	5.8±0.9	0.1±0.0	2.3±0.2	2.2±0.3	5.3±0.9
Thyroid	0.3±0.0	0.0±0.0	0.4±0.1	0.4±0.0	0.4±0.1
Stomach	0.6±0.0	2.4±0.0	1.8±0.7	0.6±0.0	0.9±0.1
Stomach wash	1.6±0.2	3.4±0.2	5.0±0.7	1.3±0.3	2.4±0.0
Small intestine	2.0±0.4	2.1±0.8	5.2±0.7	1.2±0.2	1.8±0.2
Small intestine wash	1.1±0.1	1.9±0.7	1.8±0.3	1.0±0.0	1.4±0.3
Caecum	0.5±0.0	0.6±0.1	0.9±0.2	0.3±0.0	0.3±0.0
Caecum wash	0.2±0.0	0.4±0.0	0.5±0.0	0.2±0.0	0.2±0.0
Large intestine	0.4±0.0	0.2±0.0	1.0±0.4	0.5±0.0	0.6±0.1
Large intestine wash	0.1±0.0	0.0±0.0	0.2±0.0	0.1±0.0	0.2±0.0
I.p. wash	41.8±6.8	48.3±8.4	45.7±2.8	34.2±2.8	54.2±13.4
Total Recovery	76.0±13.7	83.1±4.1	89.9±6.9	65.0±5.2	87.8±19.9

Table 2 Body distribution of ^{125}I-labelled D-2-S after i.p. administration at 2.5 h with increasing dose

Organ	0.1mg/kg	1.0 mg/kg	2.5 mg/kg	5 mg/kg	10.0 mg/kg
Blood	2.0±0.2	2.6±0.1	2.7±0.5	2.3±0.4	4.0±0.9
Lung	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Liver	2.3±1.7	2.3±1.2	3.6±0.1	3.6±0.1	4.2±0.4
Heart	0.1±0.0	1.3±0.3	0.1±0.0	0.1±0.0	0.2±0.0
Spleen	0.2±0.0	0.3±0.1	0.2±0.0	0.3±0.0	0.4±0.0
Kidney	0.8±0.4	0.4±0.1	1.5±0.9	1.5±0.9	2.5±0.1
Bladder	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.1
Thyroid	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
Stomach	0.3±0.0	0.6±0.0	0.2±0.0	0.3±0.1	0.4±0.0
Stomach wash	0.5±0.1	0.6±0.0	0.5±0.2	0.6±0.1	0.6±0.2
Small intestine	0.4±0.0	0.3±0.0	0.9±0.1	0.8±0.1	0.9±0.0
Small intestine wash	0.8±0.4	0.2±0.0	0.6±0.1	0.5±0.2	0.5±0.1
Caecum	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.1
Caecum wash	0.3±0.1	0.1±0.0	0.2±0.1	0.3±0.2	0.2±0.1
Large intestine	0.1±0.0	0.1±0.0	0.3±0.1	0.2±0.1	0.3±0.0
Large intestine wash	0.2±0.0	0.1±0.0	0.2±0.1	0.1±0.0	0.2±0.0
I.p. wash	6.1±2.0	10.0±1.7	10.6±1.7	12.1±3.8	16.4±4.0
Total Recovery	14.7±8.5	19.6±3.5	22.3±3.9	23.3±2.2	31.7±6.0

Table 3 Body distribution of ^{125}I-labelled D-2-S after repeated injections administered i.p.

Organ	Tracer
Blood	0.9±0.1
Lung	0.2±0.1
Liver	9.5±0.9
Heart	0.1±0.0
Spleen	0.5±0.1
Kidney	2.7±0.1
Bladder	0.1±0.0
Thyroid	1.7±0.3
Stomach	0.6±0.3
Stomach wash	0.2±0.1
Small intestine	2.0±0.0
Small intestine wash	0.1±0.0
Caecum	0.2±0.0
Caecum wash	0.1±0.0
Large intestine	0.3±0.0
Large intestine wash	0.1±0.0
I.p. wash	0.2±0.1
Total Recovery	19.5±2.1

Table 4 Body distribution of ^{111}I-labelled D-2-S after i.p. administration (24 h) with increasing volume

Organ	100 µl	1 ml	2.5 ml
Brain and Thyroid	0.056	0.064	0.021
Heart and Lungs	0.027	0.024	0.016
Liver	6.10	8.19	3.291
Spleen	0.076	0.215	0.115
Kidneys and Bladder	0.43	0.184	0.142
Gut	4.16	1.768	0.926
I.p. Wash	0.53	0.517	0.098
Carcass	3.30	4.870	0.925
Total Recovery	14.7	15.8	5.5

Appendix 3

Tables to show biodistribution of ^{125}I-labelled dextrin and ^{125}I-labelled D-2-S after administration i.p. to tumour bearing mice

Table 1 **Body distribution of ^{125}I-labelled dextrin at 1 mol % and 34 mol % after i.v. administration over time**
Results expressed as % recovery (% administered dose)

Organ	Dextrin (1 mol %)		Dextrin (34 mol %)	
	5 min	60 min	5 min	60 min
Blood	12.1±2.0	0.7±0.1	1.9±0.4	0.8±0.1
Lung	1.1±0.4	0.2±ND	10.8±1.6	7.0±2.3
Liver	6.9±2.3	2.3±0.8	10.8±0.9	11.5±1.0
Heart	0.5±ND	0.1±ND	6.4±2.7	3.7±1.5
Spleen	0.3±0.1	0.1±ND	5.5±1.6	9.7±3.2
Kidney	7.2±3.2	2.3±2.0	11.5±3.7	3.0±0.6
Thyroid	0.5±0.1	0.7±0.3	1.1±0.3	1.0±0.1
Bladder	14.1±5.4	5.6±2.3	7.0±2.2	21.0±2.8
Tumour	0.3±0.1	0.1±ND	2.5±0.6	7.3±2.4
Total recovery	43.0±17.8	12.1±5.5	57.5±13.0	65.0±12.0

Table 2 **Body distribution of ^{125}I-labelled Dextrin after i.p. administration in mice bearing B16F10 tumours**
Results expressed as % recovery (% administered dose)

Organ	Volume (µl)				
	100	200	300	400	500
Blood	2.2±0.3	1.9±0.5	3.4±1.2	2.3±0.1	2.1±0.2
Lung	0.3±0.1	0.3±ND	0.5±0.2	0.5±0.2	0.4±ND
Liver	1.3±0.6	1.7±0.2	3.2±0.2	2.4±0.5	1.9±0.1
Heart	0.2±0.1	0.2±ND	0.4±0.1	0.3±0.1	0.2±ND
Spleen	0.2±0.1	0.3±0.1	0.8±0.2	0.4±0.1	0.3±0.1
Kidney	1.6±ND	2.7±0.9	3.8±0.2	3.0±0.1	1.7±0.2
Thyroid	0.1±ND	0.2±0.1	0.6±0.1	0.7±0.2	0.2±ND
I.p. wash	10.3±2.0	4.4±1.1	24.0±4.8	20.0±2.9	8.3±0.9
Tumour	0.2±0.1	0.2±0.1	0.4±0.1	0.5±0.1	0.3±0.1
Total recovery	16.4±3.3	11.9±3.0	37.1±7.1	30.1±4.3	15.4±1.6

Table 3 **Body distribution of ^{125}I-labelled D-2-S after i.p. administration in mice bearing B16F10 tumours**
Results expressed as % recovery (% administered dose)

Organ	Volume (µl)				
	100	200	300	400	500
Blood	0.8±0.1	2.5±0.1	5.9±1.5	4.0±1.7	2.1±0.8
Lung	0.4±0.1	1.1±0.2	0.4±0.1	0.6±0.3	0.7±0.2
Liver	8.1±0.7	20.3±4.8	10.2±3.1	5.6±2.5	16.4±1.2
Heart	0.1±ND	0.4±ND	0.2±ND	0.4±ND	0.2±ND
Spleen	0.4±0.3	1.7±ND	1.2±0.4	1.0±0.3	1.1±0.2
Kidney	1.2±0.2	3.5±0.7	1.3±0.3	3.7±1.2	2.1±0.3
Thyroid	1.5±0.2	0.9±0.3	0.4±0.1	1.9±1.1	0.5±0.1
I.p. wash	5.8±1.1	14.5±2.3	7.1±2.4	4.2±0.8	2.0±0.4
Tumour	0.1±ND	0.3±0.1	0.3±ND	0.4±0.2	0.3±0.1
Total recovery	18.4±2.5	47.2±8.5	26.9±7.9	21.8±8.1	25.4±3.3

Table 4 **Body distribution of ^{125}I-labelled Dextrin after i.p. administration in mice bearing B16F10 tumours**
Results expressed as % recovery (% administered dose per gram of organ)

Organ	Volume (µl)				
	100	200	300	400	500
Lung	1.3±0.6	1.7±0.3	2.9±1.7	2.3±0.8	2.2±0.3
Liver	1.2±0.6	1.4±0.1	1.9±0.4	1.4±0.3	1.1±0.2
Heart	1.4±0.2	0.8±0.2	3.6±1.1	1.9±0.6	1.7±0.4
Spleen	1.3±0.4	1.8±0.7	2.5±1.3	2.4±1.1	1.4±0.3
Kidney	5.0±2.6	6.9±2.6	6.4±3.9	7.6±1.6	4.0±0.3
Bladder	79.0±5.6	22.8±4.9	74.4±1.8	42.4±3.0	10.9±0.8
Tumour	1.8±1.1	2.4±0.9	2.0±0.6	2.9±1.5	2.3±0.5
Total Recovery	91.0±11.1	37.8±9.7	93.7±10.8	60.9±8.9	23.6±2.8

Table 5 **Body distribution of ^{125}I-labelled D-2-S after i.p. administration in mice bearing B16F10 tumours**
Results expressed as % recovery (% administered dose per gram of organ)

Organ	Volume (μ)				
	100	200	300	400	500
Lung	2.0±0.6	5.6±1.9	2.7±0.8	1.3±0.1	3.1±1.1
Liver	5.7±0.9	12.6±2.4	7.9±1.0	4.4±2.1	12.4±0.9
Heart	0.9±0.1	3.6±1.0	2.1±0.5	3.4±0.9	2.3±0.3
Spleen	3.6±1.5	9.3±0.7	7.7±2.5	8.0±1.8	9.8±1.0
Kidney	2.9±0.6	10.5±2.3	4.2±1.5	13.5±6.0	5.7±0.6
Bladder	34.2±16.9	38.4±0.5	41.1±7.5	51.6±6.1	27.8±11.2
Tumour	0.8±0.3	3.1±0.6	1.9±0.3	2.0±0.9	2.6±0.4
Total Recovery	50.1±20.9	73.8±9.4	67.6±14.1	84.2±17.9	63.7±15.5

Appendix 4

Patents, Papers and Abstracts

Patents

Duncan R, German L, Hreczuk-Hirst D and Cassidy J. Biologically Active Materials. UK Patent No. PCT/GB98/01567 (1998).

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Papers

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