PDEPT:

POLYMER DIRECTED ENZYME PRODRUG THERAPY

by

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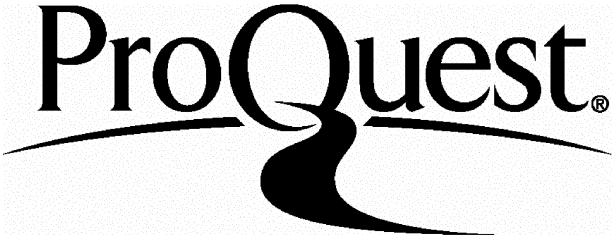

TO MY FATHER

WITH LOVE

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Abstract

Polymer Directed Enzyme Prodrug Therapy (PDEPT) was proposed as a novel two-step antitumour approach. Combination of N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer prodrug and an HPMA copolymer-enzyme conjugate was suggested as a means of generating a potent cytotoxic agent rapidly and selectively at the tumour site. To test the feasibility of PDEPT, two HPMA copolymer-enzyme conjugates were synthesised (containing β-lactamase and cathepsin B as model enzymes) and their *in vitro* and *in vivo* properties were evaluated. HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin (PK1) was used as a substrate for HPMA copolymer-cathepsin B to test the PDEPT combination *in vitro* and *in vivo*.

Following polymer conjugation (yield of 30-35%) both enzymes retained 20-25% of their enzymatic activity. To investigate their pharmacokinetics *in vivo*, ^{125}I-labelled HPMA copolymer-enzyme conjugates were administered intravenously (i.v.) to B16F10 tumour-bearing mice. Due to selective tumour tissue accumulation by the enhanced permeability and retention (EPR) effect, the HPMA copolymer-enzyme conjugates showed a 2-3 fold increase in tumour accumulation compared to the native enzyme, and they also exhibited a longer plasma half-life.

The ability of HPMA copolymer-cathepsin B to access and degrade the prodrug *in vivo* was determined by HPLC evaluation of doxorubicin release. PK1 was injected i.v. to B16F10-bearing mice and after 5 h HPMA copolymer-cathepsin B was administered. This enzyme led to a rapid increase in the rate of doxorubicin release intratumourally (3.6-fold faster than seen for PK1 alone). Moreover, when the antitumour activity (doxorubicin-equivalent dose of 10 mg/Kg) was measured using this tumour model, the combination (PDEPT) had the highest activity (T/C = 168%) compared to that seen for PK1 alone (T/C = 152%) or free doxorubicin (T/C = 144%). No animal weight loss or other toxicity was observed indicating the possibility of dose escalation. As conjugation of proteins to HPMA copolymers is known to reduce their immunogenicity, and antitumour activity has been demonstrated, further development of PDEPT is warranted.

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Abbreviations

ADEPT	Antibody Directed Enzyme Prodrug Therapy
APS	Ammonium persulfate
AUC	Area under the curve
Bz-Phe-Val-Arg-NAp	Benzoyl-phenyl alanine-valine-arginine-p-nitroanilide
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
CFA	Complete Freund's adjuvant
CPG2	Carboxypeptidase G2
CPM	Counts per minute
Da	Daltons
DDW	Double deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNM	Daunomycin
EDTA	Ehylenediaminetetraacetic acid
EGF	Endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
EPR effect	Enhanced permeability and retention effect
FCS	Foetal calf serum
FDA	US Food and Drug Administration
Gly	Glycine
GPC	Gel permeation chromatography
GSH	Reduced glutathione
h	hours
HAD	Highest antibody dilution
HCl	Hydrochloric acid
HPLC	High performace liquid chromatography
HPMA	N-(2-hydroxypropyl)methacrylamide
IgG	Immunoglobulin, class G
IgM	Immunoglobulin, class M
i.p.	Intraperitoneal
i.v.	Intravenous
LDL	Low density lipoprotein
Leu	Leucine
mAb	Monoclonal antibody

MDR	Multidrug resistance
min	minutes
MPS	Mononuclear-phagocyte system
MSH	Melanocyte stimulating hormone
MTD	Maximum tolerated dose
MTT	1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MWM	Molecular weight markers
NaCl	Sodium chloride
Na_2CO_3	Sodium bicarbonate
NaOH	Sodium hydroxide
NCI	National Cancer Institute
ONp	p-nitrophenoxy
o-PDA	*o*-phenylenediamine
PBS	Phosphate buffered saline
PDEPT	Polymer Directed Enzyme Prodrug Therapy
PE	*Pseudomonas* Exotoxin A
PEG	Poly(ethylene)glycol
Phe	Phenylalanine
PHEA	Poly-2-[N-(22-hydroxyethyl)-D,L-aspartamide
PK1	HPMA copolymer-Gly-Phe-Leu-Gly -doxorubicin
PK2	PK1 with addition of galactosamine as a targeting moiety
RES	Reticuloendothelial system
rpm	Revolutions per minute
s.c.	Subcutaneous
SD	Standard deviation of the mean
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error of the mean
SMANCS	Poly(styrene-co-maleic acid)-neocarzinostatin
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol
UV-Vis	Ultraviolet-visible
VEGF	Vascular endothelial growth factor
V/GDEPT	Viral/Gene Directed Enzyme Prodrug Therapy

Chapter One

General Introduction

1.1 Introduction

Today, cancer is one of the most frequent causes of death in the industrialised countries and the number of cancer cases is ever increasing. Some 40 percent of Americans will eventually be stricken with malignant disease and more than one in five will die of it (Rennie and Rusting, 1996). In the UK, cancer is now the leading cause of death, and accounts for one in four of all deaths (Mason, 1998). Globally, the World Health Organisation estimates that cancer kills roughly six million people annually. This is mainly due to the fact that cancer is a disease of older age and also due to the successful treatment of infections allowing more people to reach old age. Despite the progress in the diagnostic and therapeutic fields, the prospects for curing cancer are well below 20% for the most frequent cancers (Mutschler and Derendorf, 1995).

The aim of this study was to develop a two step anticancer system named PDEPT (Polymer Directed Enzyme Prodrug Therapy) to potentially deliver an anticancer drug selectively to the tumour to be activated by a polymer-enzyme conjugate. Hopefully, this approach will overcome some of the problems current anticancer treatments are facing.

1.2 Limitations of current treatments

The main approaches to cancer treatment are: surgery, radiation therapy, immunotherapy, cytotoxic chemotherapy and hormones. Surgery and radiation are especially indicated for the treatment of solid tumours because they can potentially remove all the cancer cells from the body. It is inevitable that healthy tissue is also removed or exposed to radiation and some tumour cells may escape. Common cytotoxic chemotherapeutic agents also lead to destruction or damage of tumour cells. Most antitumour agents used clinically act upon metabolic pathways related to cell growth and high mitotic activity. These effects are usually so non-specific that simultaneous serious damage to healthy cells occurs. Tissues with high cellular division rates are particularly affected (bone marrow, intestinal mucosa, the hair follicle cells) leading to unpleasant dose-limiting side effects and decrease in the quality of life.

Lack of selectivity is only one, albeit major, obstacle hindering the optimisation of drug effectiveness. Others include inaccessibility of target, premature drug metabolism and allergic reactions (Gregoriadis, 1989).

Chemotherapeutic treatment of neoplastic diseases is often restricted by adverse systemic toxicity which limits the dose of drug that can be administered, or by the appearance of drug resistance. Resistance to a cytostatic/cytotoxic agent can be based on many factors such as premature inactivation leading to insufficient concentration at the

target site, formation of inactivating antibodies, increase in the levels of p-glycoprotein that can pump the drug out of the tumour cell, and appearance of DNA repair mechanisms (Mutschler and Derendorf, 1995).

The main conclusion that can be drawn from all these difficulties in achieving effective cancer chemotherapy is that there is a great demand for innovative drug delivery systems that can target better antitumour drugs and that can overcome resistance in its many forms. The question is how to achieve it?

1.3 Drug targeting

Drug targeting is defined very generally as the concept of 'delivering an adequate amount of drug to the target site in the body compartment at an appropriate time' (Kataoka, 1997). At the beginning of this century, Paul Ehrlich was the first to propose the "magic bullet" concept, and he suggested that targeting to affected tissues might be achieved by means of targeting appropriate receptors (Gros *et al.*, 1981). Ehrlich suggested the use of a receptor-binding molecule, he called it a 'haptophore', in order to more selectively deliver the drug, the so-called 'toxophore' (Ehrlich, 1960). As a haptophore, he proposed the use of an antibody. Interest in tackling cancer using immunotherapies has grown constantly due to the increasing understanding of high specifities of cell-cell recognition and the immune response. Since then, various strategies have been explored to improve drug targeting and hopefully increase drug concentration in the tumour to a level that should overcome clinically relevant drug resistance.

The use of drug delivery systems could in theory:

1. Decrease the frequency of administration.
2. Decrease the total dose necessary (preferably in a single bolus thus diminishing both cost and toxicity).
3. Reduce undesirable side effects.
4. Inactivate the drug during transport to the tumour.
5. Prevent drug loss (through premature metabolism or excretion) as a result of carrier-mediated altered pharmacokinetics.

Over the last three decades, intensive efforts have been made to design systems able to deliver the anticancer drugs more efficiently to the target site. A whole range of drug delivery systems have been explored as a means of targeting anticancer drugs, *inter alia,* low molecular weight prodrugs (Waller and George, 1989), macromolecules (Takakura and Hashida, 1995; 1996), liposomes (Gabizon, 1994, Gregoriadis, 1995),

antibodies (Reilly and Sheldon, 1987), natural polymers (reviewed in Sezaki *et al.*, 1989), water soluble synthetic polymers (reviewed by Duncan *et al.*, 1996), microspheres (reviewed by Cummings, 1998; Davis and Illum, 1989), microparticles (reviewed in Kerr and Kaye, 1991) and nanoparticles (reviewed by Roerdink and Kroon, 1989).

Targeting can be achieved either actively by specifically including a recognition moiety into the carrier ("active targeting"), or passively as a result of some physical or chemical characteristics of the carrier ("passive targeting") (reviewed by Illum and Davis, 1985; Duncan, 1992). The active approach relies upon the selective localisation of a ligand at a cell-specific receptor. It refers to a change in the natural distribution pattern of a carrier particle by a deliberate modification of the size or surface characteristics of a carrier particle thereby directing it to specific cells, tissues or organs. Passive targeting refers to the exploitation of the natural (passive) distribution pattern of a drug-carrier *in vivo*. It is based on mechanical entrapment of the carrier by shape or size or uptake by the cells of the reticuloendothelial system (RES). An alternative approach of targeting is the use of physical external means to control localisation, for example use of a magnetic field or locally applied heat to attract a drug carrier to the tumour selectively. These two modalities are widely elaborated in the literature (Widder *et al.*, 1983) and will not be discussed in this thesis. Likewise injection of a drug or drug-carrier directly into the tumour or the feeding artery can be considered "targeting" in a sense, but this has limited value for the treatment of non localised metastatic disease.

There are three different orders of targeting (reviewed in Duncan, 1992); first order targeting is organ specific, second order targeting is cell specific and third order is targeted to a particular compartment within a defined cell.

This thesis has focused on the investigation of a novel concept for tumour targeting called PDEPT. PDEPT relies on the selective delivery of enzymes to solid tumours (using a polymer-enzyme conjugate) to activate selectively a polymer prodrug. First, it is important to review the basic principles of the tumour physiology, 'polymer therapeutics' and prodrugs in order to understand the rationale for design of PDEPT.

1.4 Tumour physiology and the enhanced permeability and retention (EPR) effect

The physiology of solid tumours differs from that of normal tissues in a number of important aspects, the majority of which stem from differences between the two types of vasculature. Compared with the regular, ordered vasculature of normal tissues,

blood vessels in tumours are often highly abnormal, distended capillaries with leaky walls and sluggish flow (Brown and Giaccia, 1998). Tumour growth also requires continuous new vessel growth, or angiogenesis. The physiology of solid tumours at the microenvironmental level is sufficiently different from that of the normal tissues from which they arise to provide a unique and selective target for cancer treatment (Gerlowski and Jain, 1986; Nugent and Jain, 1984). Table 1.1 lists the principal differences in physiology between normal and malignant tissues.

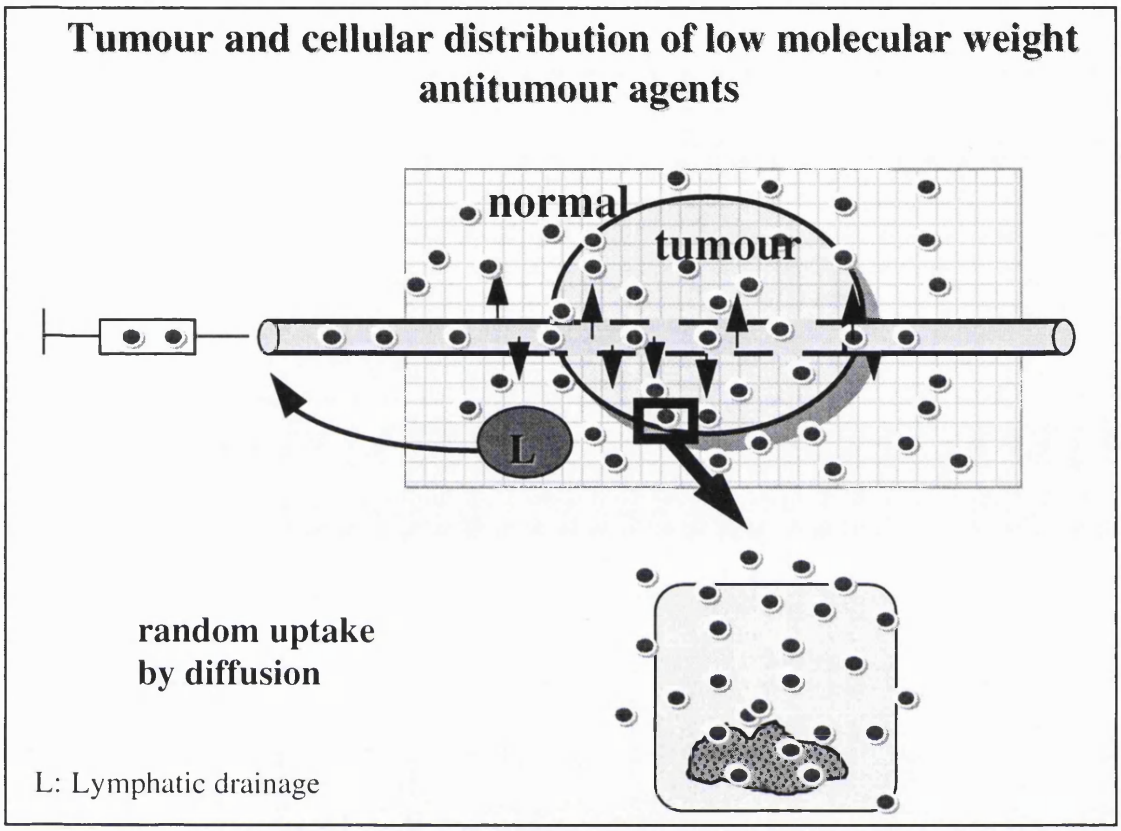
The EPR effect (Figure 1.1) results from enhanced permeability of macromolecules or small particles within the tumour neovasculature due to leakiness of its discontinuous endothelium and this mechanism of tumour targeting of polymeric systems was first described by Matsumura and Maeda (1986). In addition to the tumour angiogenesis (hypervasculature) and irregular and incompleteness of vascular networks, the attendant lack of lymphatic drainage promotes accumulation of macromolecules once they extravasate (Matsumura and Maeda, 1986; Seymour, 1992). This phenomenon of passive diffusion and localisation in the tumour interstitium is observed in many solid tumours for macromolecular agents and lipids (Maeda and Matsumura, 1989; Dvorak *et al.*, 1988). Unless specifically addressed for tumour cell uptake by receptor-mediated endocytosis (elaborated later in section 1.5), macromolecules entering the intratumoural environment are taken up relatively slowly by fluid-phase pinocytosis and then transferred from endosomes to lysosomes (Duncan, 1987). Modified pathways of fluid extravasation and tissue drainage in tumours are thought to be the physiological cause for the passive tumour tropism of macromolecules. Ineffective or absent pathways of lymphatic drainage results in poor convection and elevated interstitial hydrostatic pressures (reviewed by Jain, 1990). This leads to poor oxygenation of the tumour mass, and induces the release of angiogenic and capillary-permeabilising factors such as vascular endothelial growth factor (VEGF) in order to improve the supply of oxygen and nutrients (reviewed by Ferrara *et al.*, 1992). The enhanced vascular permeability will support the great demand of nutrients and oxygen for the rapid growth of the tumour. To realise this demand, tumour tissues recruit blood vessels from the pre-existing network as well as inducing extensive angiogenesis by releasing VEGF (Jain, 1987a; 1987b; Dvorak *et al.*, 1995). Without angiogenesis, the tumours could not increase in size to more than few millimetres (Folkman, 1971; 1985; Folkman and Klagsborn, 1987).

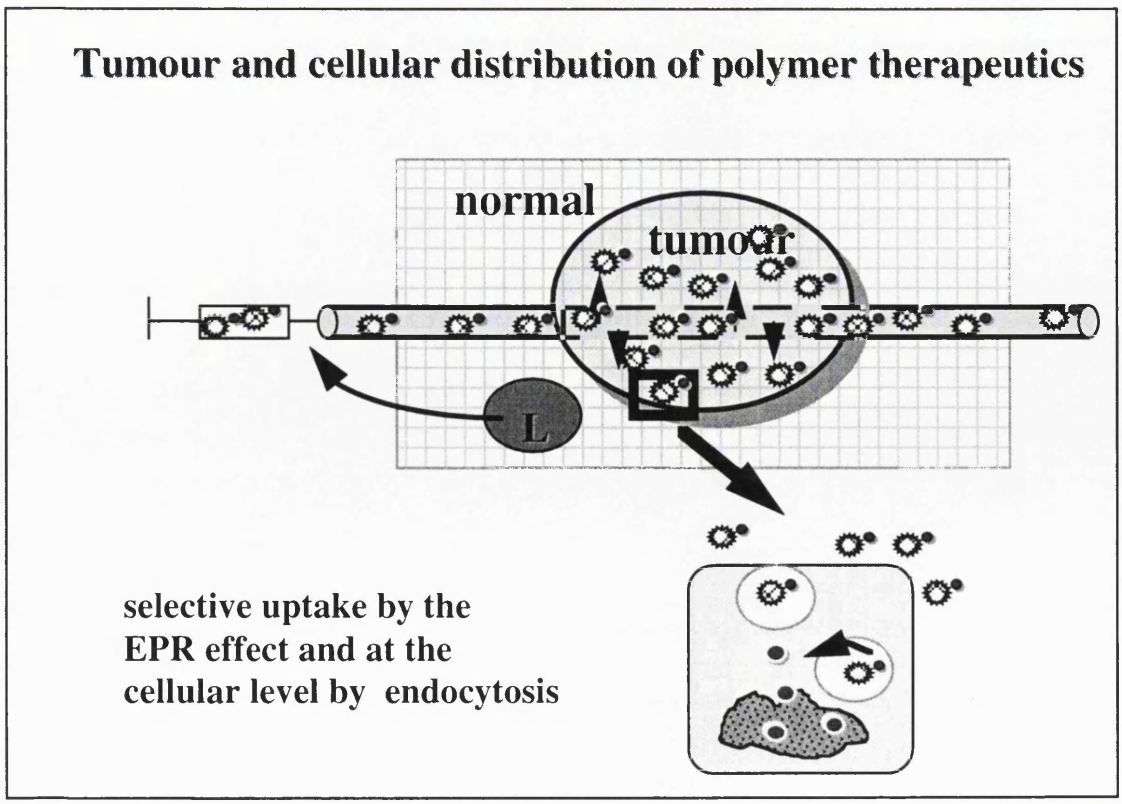
Obviously, when using polymeric drugs the EPR effect is a great advantage as a means for targeting. Low-molecular-weight analogues readily leave the bloodstream as they quickly pass into the tissue or are excreted. In contrast, polymeric drugs can often achieve a prolonged half-life in the bloodstream because of decreased glomerular

Table 1.1 Physiological characteristics of malignant tissues that can potentially be exploited for cancer therapy (from Brown and Giaccia, 1998)

Characteristics	Normal tissue	Tumour	Detrimental aspects for therapy	Method of exploiting for therapy
Microvasculature	Developed with ordered, regulated flow	Constant new vessel growth; leaky and tortuous vessels; often sluggish and irregular flow	Poor delivery of some therapeutic agents due to irregular flow and high interstitial pressure	Antiangeogenic agents, selective extravasation of macromolecules (e.g. polymer conjugates, liposomes)
Oxygenation	Heterogeneous, but rarely hypoxic regions	Highly heterogeneous with common hypoxic regions	Reduces tumour sensitivity to radiation and anticancer drugs; predispose to increased malignancy (e.g. metastasis)	Selective cytotoxins; gene therapy targeted by hypoxia
Necrosis	Not present	present	Not known if any	Gene therapy targeted to necrosis (anaerobic bacteria) and to quiescent cells.

Figure 1.1 The enhanced permeability and retention effect (Duncan, oral presentation, 1998)





excretion (Kataoka, 1997). Extended circulation is a requisite for optimal targeting by the EPR effect. However, an optimum molecular weight exists in terms of achieving the most efficient accumulation by the EPR effect. For example, Seymour *et al.* (1995) showed that for N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers (fractions of molecular weight 22-778 KDa), at 10 min after administration *in vivo*, all fractions were already detectable in the tumour and those of molecular weight greater than the renal threshold showed progressive tumour accumulation up to 20% of dose administered per gram after 72 h in Sarcoma 180 model. HPMA copolymer fractions showed profiles of blood clearance that were strongly dependent on molecular size. For this reason, although the initial tumour levels of all HPMA copolymer fractions were virtually independent of substrate size, their progressive accumulation was different.

Furthermore, macromolecular carriers in the bloodstream may be recognised by scavenger cells, namely the RES. These cells are located within such organs as liver, spleen and lung. RES recognition is particularly serious for colloidal and vesicular carrier systems, including microparticles and liposomes (Oku and Namba, 1994). Thus, it is essential to develop appropriate carrier systems that achieve long circulation in the bloodstream avoiding glomerular excretion and RES recognition (like HPMA copolymer, Seymour *et al.*, 1987), allowing for a significant EPR effect. In a system like PDEPT it is especially important to choose an adequate carrier for both the drug and the enzyme, bearing in mind the possibility of immunogenicity in addition to the above mentioned RE capture.

Having discussed the principles of macromolecular extravasation in tumour tissues and targeting by the EPR effect, it is necessary to elaborate further the mechanism by which macromolecules enter the tumour cell. It has been shown that anticancer drug uptake by cells can be modulated by conjugating the drug to an appropriate polymeric carrier. Polymer conjugates follow a different cellular uptake mechanism compared with low molecular weight analogues which usually enter cells by diffusion through the cell membrane or active transport across the cell membrane. Polymers are internalised into the cell by a process called endocytosis.

1.5 Endocytosis

'Endocytosis' is a comprehensive term which represents those cellular mechanisms responsible for particulate and macromolecular uptake. All eukaryotic cells exhibit one or more forms of endocytosis (reviewed by Mellman, 1996). It is essential to maintain cellular homeostasis controlling the transmission of neuronal, metabolic and proliferative signals. These activities include the uptake of essential nutrients, the regulated interaction of the intracellular compartments with the external world, and the

ability to mount an effective defence against invading micro-organisms. Endocytosis describes a process of vesicle formation at the plasma membrane by a process of invagination, with concomitant internalisation of the extracellular milieu. Although discussion of details and semantics continues, there is a fundamental agreement on the most important features of the endocytic pathway summarised in Figure 1.2.

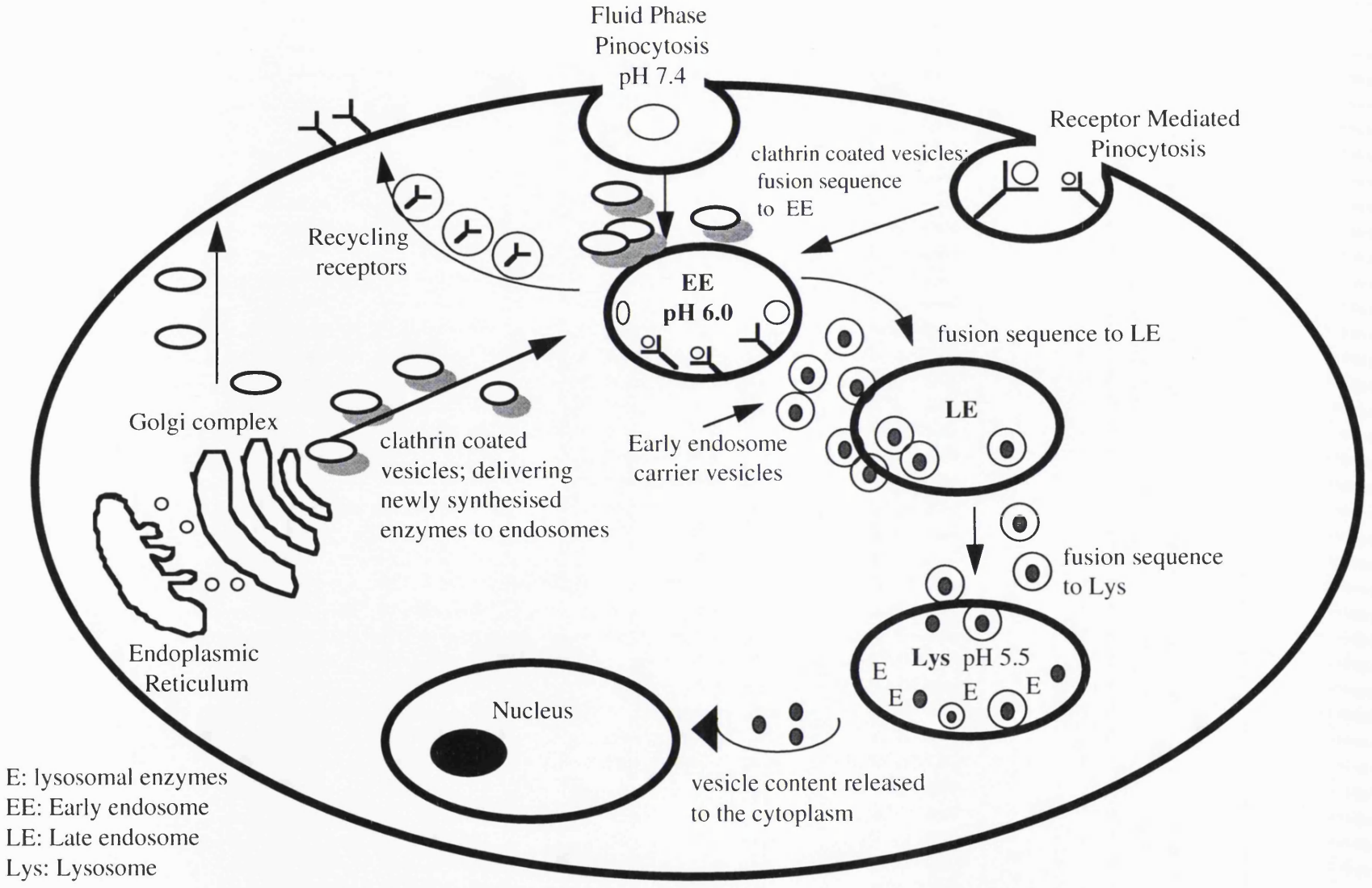
There are at least two types of endocytosis and these have been classified as phagocytosis (cell eating) and pinocytosis (cell drinking). Phagocytosis refers to the ability to internalise large (>0.5 μm diameter) particles (bacteria, latex beads, liposomes) that bind to specific plasma membrane receptors capable of triggering their own uptake, usually by causing the formation of F-actin-driven pseudopods that envelop the bound particle in such a way as to exclude liquid. In mammals, phagocytosis serves as a first line of defence against micro-organisms, as well as providing an important component of the humoral immune response by allowing the processing and presentation of bacterial-derived peptides to antigen-specific T lymphocytes (Harding and Geuze, 1992; Pfeifer *et al.*, 1993). It is a feature of phagocytic protozoa or phagocytic leukocytes of the mammalian immune system (macrophages, neutrophils).

Pinocytosis involves the constitutive formation of smaller (<0.2 μm diameter) vesicles carrying extracellular fluid and macromolecules specifically or non specifically bound to the plasma membrane. These vesicles are usually initiated by the formation of clathrin-coated pits. Unlike phagocytosis, pinocytosis is common to almost all cell types. The pinocytic pathway can be further subdivided into fluid-phase, receptor-mediated and adsorptive pinocytosis (reviewed by Mellman, 1996). Attachment of low molecular drugs to macromolecules, such as polymers, restricts their uptake to the pinocytic pathway.

Non-specific adsorptive pinocytosis is mediated via the binding of a molecule to the cell surface by ionic interaction between the molecule and the plasma membrane or through hydrophobic interactions between non-polar moieties and the lipid bilayer. In the case of polymer conjugates it occurs when a polymeric carrier is inherently hydrophobic or cationic, or modified with pendent hydrophobic or cationic residues (bound drug) (reviewed by Duncan *et al.*, 1996). The rate of uptake for adsorptive pinocytosis is typically 8-80 μl/mg protein/h.

In fluid-phase pinocytosis macromolecules are accumulated simply by virtue of their presence in extracellular fluid. Since this process is indiscriminate, the rate at

Figure 1.2 Organisation of the endocytic pathway in animal Cells



which a given compound is taken up is entirely dependent upon its extracellular concentration, and is thus comparatively slow (0.5-4 µl/mg protein/h).

Receptor-mediated pinocytosis occurs in clathrin coated pits of the plasma membrane, which bud-off to yield clathrin-coated vesicles containing the receptor-ligand complexes. The vesicles rapidly lose their coats, which facilitates fusion with early endosomes, a dynamic array of tubules and vesicles distributed throughout the peripheral and perinuclear cytoplasm. Due to a slightly acidic pH (pH~6.0-6.8) maintained by an ATP-driven proton pump (Al-Awqati, 1986, Mellman *et al.*, 1986, Forgac, 1992a; 1992b), early endosomes host the dissociation of many ligand-receptor complexes in an environment that minimises the risk of damaging receptors intended for reutilisation. Free receptors selectively accumulate in the early endosome's tubular extensions, which bud-off to yield recycling vesicles that transport receptors back to the plasma membrane. Dissociated ligands then pinch off traversing to the perinuclear cytoplasm on microtubule tracks, and fuse with the late endosomes and the lysosomes. These organelles are responsible for accumulating and digesting exogenous and endogenous macromolecules by the low pH (pH~5.0-5.5) and the high concentration of lysosomal enzymes.

The enzyme composition of lysosomes is rather complex: more than 40 enzymes have been identified which are able to break down all major classes of biological important materials (protein, fats, carbohydrates). A list of these lysosomal enzymes taken from Barrett and Heath (1977) is summarised in Table 1.2. Lysosomal enzymes accumulate in the late endosome and the lysosome, delivered by clathrin-coated vesicles originating in the trans-Golgi network (reviewed by Mellman, 1996). The resulting product (e.g. drug released from its polymeric carrier by lysosomal enzymes cleaving the linker) crosses the lysosomal membrane and enters the cytosol by passive diffusion or via interaction with membrane bound porters (Bird and Lloyd, 1991). Biodegradable compounds are digested within lysosomes into their monomeric constituents which can then pass across the lysosomal membrane for reutilisation, in the cell, or excretion. Non degradable materials are sequestered into the lysosomes until they are released by exocytosis or cell lysis. The rate of uptake for receptor mediated pinocytosis is much higher then the former types of pinocytosis (130-700 µl/mg protein/h).

These mechanisms present the potential for site-specific targeting of macromolecules on the basis of the lysosomotropic approach established by De Duve *et al.* (1974) which accounts for both low molecular weight substances and macromolecules which end up in the lysosomal compartment following pinocytic

Table 1.2 Lysosmal enzymes

Enzyme	Reaction
Oxidoreductases	
$NADPH_2$ oxidase	$NADPH_2$ + acceptor $\longrightarrow$ NADP+reduced receptor
Peroxidase	Donor+ H_2O_2 $\longrightarrow$ oxidised donor + $2H_2O$
Carboxylic oxidases	
Arylesterase	Cleaves carboxylic esters of 2-naphtol and other aromatic alcohols.
Triacylglycerol lipase	Cleaves fatty acyl ester linkages of triacylglycerols. Also hydrolyses di- and monoacylglycerols and cholesterol esters.
Phospholipase A_2	Cleaves the fatty acyl ester linkage to carbon 2 of the glyceryl moiety in phosphatidylcholine and other phospholipids.
Cholesterol esterase	Cleaves the fatty acyl esters of cholesterol.
Phospholipase A_1	Cleaves the fatty acyl ester linkage to carbon 1 of the glyceryl moiety in phosphatidylcholine and other phospholipids.
Enzymes acting on bonds involving phosphorus	
Acid phosphatase	Inorganic phosphate is realeased from glycero-2-phosphate, AMP, 4-nitro-phenyl phosphate, 1-naphtol phosphate.
Phosphatidate phosphatase	Liberates phosphate ion from phosphatidic acid.
Phosphoprotein phosphatase	Releases phosphate ion from serine phosphate residues of phosphoproteins.
Deoxyribonuclease II	Endo-cleavage of double-stranded DNA, often both strands at the same point, leaving 3'-phosphate termini.
Sphingomyelin phophodiesterase	Cleaves sphingomyelin to yield phosphocholine and acylsphingosine.
phophodiesterase II	Liberates 3'-phosphomononucleotides from the 5'-hydroxyl end of DNA or RNA fragments.
Ribonuclease II	Endo-cleavage of RNA, leaving 3'-phosphate termini, 2', 3'-cyclic phosphates being intermediates.
Acyl di(glycerophosphoryl) glycerol phophodiesterase	Cleaves acyl di(glycerophosphoryl) glycerol, a deacylation product of cardiolipin, to yield acylglycero-phosphorylglycerol (lysophosphatidyl- glycerol) and glycerophosphate.
Hydrolases acting on sulphuric esters	
Sulphatase A	Liberate sulphate ion from test substrates such as nitrocatechol sulphate. Also act on cerebroside-3-sulphate and ascorbic acid 2-sulphate.
Cerebroside sulphatase	
Sulphatase B	Liberates sulphate ion from test substrates such as nitrocatechol sulphate.
Chondroitin-4-suphatase	Acts on 4-sulphogalactosaminyl residues in chondroitin 4-sulphate and UDP-GalNAc-SO_4.
Chondroitin-6-suphatase	Liberates sulphate ion from 6-sulphogalactosaminyl residues in chondroitin 6-sulphate.
Iduronsulphatase	Liberates sulphate ion from 2-sulphoiduronosyl residues in dermatan sulphate.
Hydrolases acting on glycosides	
β-Galactosidase	Cleaves non-reducing terminal β-galactosyl residues from glycoproteins, glycosaminoglycans, oligosaccharides and glycolipids.
Lysozyme	Cleaves the (1β-4) linkage of N-acetylmuramic acid to N-acetylglucosamine in the polysaccharide component of the cell walls of some bacteria, and the N-acetylglucosaminyl-(1β-4)-N-acetylglucosamine linkage in chitin.

Neuraminidase	Cleaves non-reducing terminal α-glycosidic linkages of N-acetylneuraminic acid in glycoproteins and glycosaccharides.
α-Glucosidase	Cleaves non-reducing terminal α-glucosyl residues from glycogen, maltose and other oligosaccharides.
β-Glucosidase	Cleaves non-reducing terminal β-glucosyl residues from glucosylceramide, glucosylsphingosine and steroid glucosides.
α-Galactosidase	Cleaves non-reducing terminal α-galactosyl residues from oligosaccharides.
α-Mannosidase	Cleaves non-reducing terminal α-mannosyl residues from oligosaccharides and glycopepteides.
β-Mannosidase	Cleaves non-reducing terminal β-mannosyl residues from glycoproteins and oligosaccharides.
β-N-Acetylglucos-aminidase	Cleaves non-reducing terminal β-N-acetylglucosaminyl and β-N-acetylgalactosaminyl residues from glycolipids and glycosaminoglycans.
β- Glucuronidase	Cleaves non-reducing terminal β-glucuronosyl residues from glycosaminoglycans and conjugated steroids, drugs and other xenobiotics.
Hyaluronate endoglucosaminidase	Cleaves (1β-4)-N-acetylglucosaminide linkages in hyaluronate, chondroitin 4-sulphate and chondroitin 6-sulphate.
α-N-Acetylgalactos-aminidase	Cleaves non-reducing terminal α-N-acetylgalactosaminyl residues from desialised glycoproteins and blood group-A-active heterosaccharides.
α-N-Acetylglucos-aminidase	Cleaves non-reducing terminal α-N-acetylglucosaminyl residues from heparan sulphate and UDP-N acetylglucosamine.
α-L-Fucosidase	Cleaves non-reducing terminal α-L-fucosyl residues from glycopeptides, oligosaccharides and polysaccharides.
α-L-Iduronidase	Cleaves non-reducing terminal α-iduronosyl residues from heparan sulphate and dermatan sulphate.
Heparin endoglucuronidase	Cleaves β-glucuronidase linkages in regions of 'macromolecular' heparin proteoglycan close to the protein-polysaccharide junction, releasing the heparin chains normally isolated from tissues.
Glycopeptide endoglucosaminidase	Cleaves the β-N-acetylglucosaminyl-N-acetylglucosamine linkage in glycopeptides.
Heparan sulphate endoglycosidase	Degrades heparan sulphate to large oligosaccharides.
$NAD(P)^+$ nucleosidase	Cleaves the nicotinamide N-riboside linkage in NAD^+, $NADP^+$ and NMN.
Exopeptidases	
Lysosomal aminopeptidase (cathepsin H)	Cleaves N-terminal amino acyl residues from peptides and amino acid naphthoylamides
Lysosomal carboxypeptidase	Cleaves C-terminal residues from peptides with broad specificity (excluding arginine and lysine)
Lysosomal carboxypeptidase B (cathepsin B2)	Cleaves C-terminal residues from peptides with broad specificity (including arginine and lysine)
Dipeptidylpeptidase II	Cleaves N-terminal dipeptides from amide, arylamide and peptide substrates, especially when arginine or lysine is terminal. The bond cleaved may be propyl.
Tyrosine acid carboxypeptidase	Cleaves C-terminal residues (preferably hydrophobic) from peptides.
Dipeptidylpeptidase I	Cleaves N-terminal dipeptides from amide, arylamide, ester and peptide substrates, with broad specificity, but the terminal residue must not be arginyl or lysyl, and the second or third residues must not be propyl. Shows dipeptide transferase activity at higher pH values.

Lysosomal carboxypeptidase C	Liberates a C-terminal residue when the subterminal residue is propyl.
Lysosomal dipeptidase	Cleaves dipeptides with broad specificity.
Hydrolases acting on acid anhydrides	
Nucleoside triphophatase	Cleaves ATP and other nucleoside triphosphates to yield the disphosphate. Also hydrolyses the pyrophosphate bond in FAD, thiamine pyrophosphate and inorganic pyrophosphate, and splits such phosphodiesters as bis(4-nitrophenyl) phosphate and 4-nitrophenyl-5'-phosphotimidine.
Adenylsulphatase	Liberates sulphate ion from adenylyl sulphate.
Phosphoadenylyl-sulphatase	Liberates sulphate ion from 3'-phosphoadenylyl sulphate.
Hydrolases acting on nitrogen-sulphur bonds	
Heparin sulphamatase	Liberates sulphate ion from N-sulphoglucosaminyl residues in heparin and heparan sulphate.

capture. Compounds that are taken up selectively into the lysosomes are called lysosomotropic. Drugs conjugated to polymeric carriers by a bond cleavable by lysosomal enzymes should then be liberated from the carrier in the lysosomal compartment, followed by the penetration of the drug into the cytoplasm through lysosomal membranes. An impermeable drug can be sent into cells through the lysosomal pathway conjugating the drug to a polymeric carrier with lysosomotropic character. Moreover, antitumour agents which cannot enter the cell through the membrane can be transported into the cell via endocytosis when linked to a polymer.

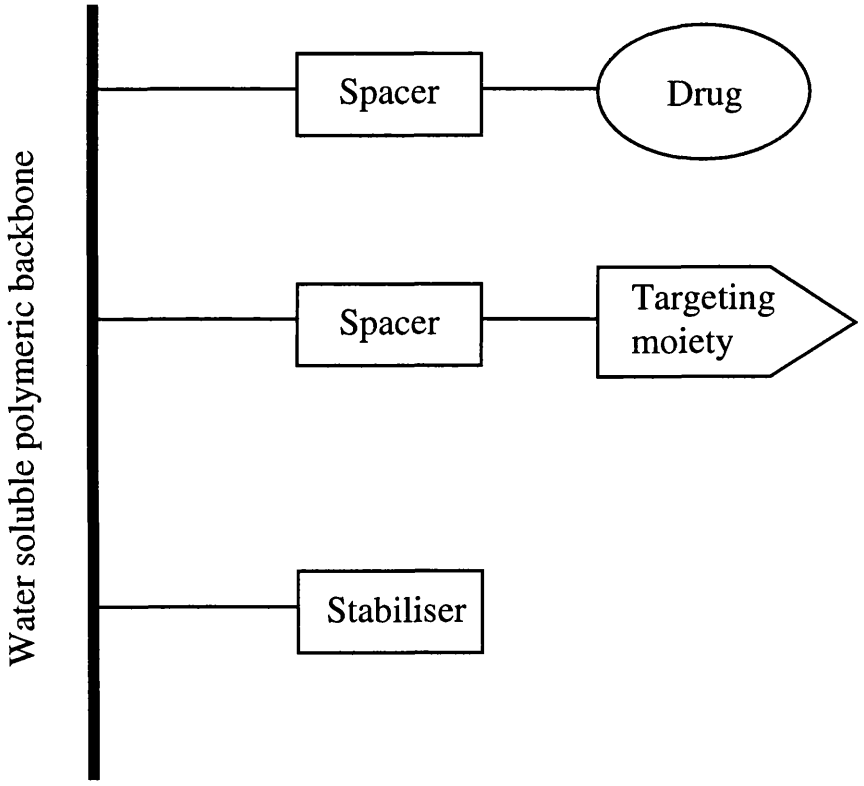
1.6 Polymers as carriers for cancer therapy

In recent years there has been a great deal of investigation of polymers as carriers of anticancer drugs (reviewed in Duncan *et al.*, 1996). The use of water soluble polymers as a means to carry bioactive drugs linked to different domains along a polymeric backbone via a biodegradable spacer and released at the target site was first proposed by Ringsdorf in 1975 (Figure 1.3). To achieve specific localisation in the body a targeting moiety can be additionally incorporated to the polymer to aid cell-specific uptake by receptor-mediated pinocytosis. The basis for much of this work is that attachment of toxic drugs to high molecular weight carriers can lead to reduction in systemic toxicity, longer retention time in the body, alterations in biological distribution, improvements in therapeutic efficacy and site-specific passive capture through the EPR effect. According to the Ringsdorf model one domain of the polymeric carrier may contain units which would increase the solubility of the carrier. A second domain, would contain the drug linked to the polymer chain by a bond, which may be cleaved or may be stable under *in vivo* conditions. A spacer group may be necessary to separate the drug from the main chain in order to make it accessible to enzymes or receptors. Finally, it was proposed that there would be a domain which assures preferential uptake into a target tissue; a targeting moiety was proposed.

Polymers with intrinsic antitumour properties (Seymour, 1991), soluble polymer conjugates and micelles (Ringsdorf, 1975; Gros *et al.*, 1981) and polymer-protein adducts (Nucci *et al.*, 1991; Francis *et al.*, 1992; 1996; Maeda, 1991) have been known for many years. An ideal conjugate would be stable and pharmacologically inactive in the circulation, but after cellular internalisation through endocytosis, would be activated by hydrolytic or enzymatic cleavage in the lysosomes. The ideal polymer conjugates have to fulfil the following requirements:

1. To limit toxicity, the polymer conjugate should be biocompatible, biodegradable, not induce any toxicity nor cause immunogenicity.

Figure 1.3 Schematic diagram of a soluble polymeric drug carrier (modified from Ringsdorf, 1975)



2. The molecular weight should be such that the conjugate has sufficient circulation time and if not degradable is excreted via the kidneys. Also, the uptake should be controlled by the EPR effect, thus ensuring a selective accumulation in the tumour.
3. The polymer must contain functional groups, allowing linkage to the active agent, without inducing toxicity and immunogenicity.
4. The polymer conjugate should have sufficient stability to ensure release of the drug only at the target site.
5. The conjugate should favour the solubility of poorly soluble drugs.
6. The production of the conjugate should be possible on an industrial scale with low manufacturing costs.

Drug delivery systems such as liposomes and polymers, when not bearing any targeting moiety, accumulate passively within solid tumour tissue by the EPR effect (Matsumura and Maeda, 1986). Several examples of polymer-drug conjugates described in the literature are shown in Table 1.3.

1.7 Prodrugs

A polymer-drug conjugate may be considered a macromolecular prodrug. Prodrugs have been used for many years in medicine to treat a variety of disorders (e.g. antidepressant therapy, poisoning with anticholinesterase nerve gases). An ideal prodrug is a derivative of an active molecule which by itself has no intrinsic pharmacological activity, but which can be transformed by a chemical or enzymatic process to yield the pharmacologically active species at the appropriate time and site (Gardner and Alexander, 1985). Prodrugs have been used by the pharmaceutical industry to obtain derivatives which overcome problems of taste, poor water solubility, poor transmembrane penetration, etc. (Roche, 1977). Many of these products have been employed to enhance the oral bioavailability of drugs, but this cannot be considered to offer means of specific drug targeting *per se*. Only when the site of reconversion of the prodrug is itself the target site of drug action, then the prodrug approach has the potential to achieve site-specific delivery.

Usually, in any drug molecule, there are a number of major functional groups which are amenable to derivatisation, and many different derivatisation methods have been used to modify these functional groups. Essentially prodrugs can be divided into two subcategories: (a) those which are chemically unstable and reverse under conditions such as presence of water, change in pH, etc.; and (b) those which require enzymatic action to enhance the rate of reconversion to practical levels (Gardner and Alexander, 1985). This thesis will focus on the latter.

Table 1.3 Polymer-drug conjugates

Polymer	Drug	Reference
HPMA copolymer	melphalan	Duncan *et al.*, 1991
	5-fluorouracil	Putnam and Kopecek, 1995a
	daunorubicin	Duncan *et al.*, 1988
	doxorubicin	Vasey *et al.*, 1999
	taxol	Pesenti *et al.*, 1995
PHEA	chlorambucil	Giammona *et al.*, 1992
	trans-1,2-diaminocyclohexane-platinate	Filipova-Vopralova *et al.*, 1991
Dextran	doxorubicin	Danhauser-Riedl *et al.*, 1993
	Mitomycin C	Takakura *et al.*, 1987
PEG	doxorubicin	Senter *et al.*, 1995
Alginate	doxorubicin	Al-Shamkani and Duncan, 1995

HPMA: N-(2-hydroxypropyl)methacrylamide

PHEA: poly-2-[N-(22-hydroxyethyl)-D,L-aspartamide

PEG: poly(ethylene)glycol

Prodrugs can offer improved solubility, improved pharmacokinetics and tissue distribution, prevention of unfavourable metabolism, selective organ effects and tumour-specific toxicity (Knox and Connors, 1997). These advantages have led to their use in cancer chemotherapy. In order to use prodrugs as anticancer agents the tumour must have a high level of the converting enzyme and no activating enzyme must be present in normal tissues. The prodrug must be innocuous and pharmacodynamically inert and must be a substrate for the enzyme with favourable K_m and Vmax values.

Prodrugs, useful in cancer chemotherapy, would therefore be inert but converted *in vivo* into a highly cytotoxic metabolite either by (1) a unique and enriched enzyme present in the cancer cells (but not in other cells) or (2) by a foreign enzyme delivered selectively to the tumour tissue.

In terms of site selectivity, the most important parameters are the specificity of the enzymatic reaction required for reconversion and the distribution of this enzyme activity. This latter factor is important since even low levels of enzymatic activity in a well-perfused organ may be capable of competing effectively with high levels of the same enzyme in a poorly perfused tissue. The principal factors affecting success of the prodrug approach can be summarised as follows:

1. Adequate accessibility of prodrug to target site.
2. Correct distribution, relative activity and specificity of reconverting enzymes.
3. Parent drug retention at target site.
4. Access of parent drug to target site within target organ.
5. Appropriate pharmacokinetics of prodrug and drug molecules.
6. Adequate pharmacological activity of prodrug or its metabolites.

A list of some of the enzymes and prodrugs that have been proposed for cancer therapy is shown in Table 1.4.

There are three basic approaches making use of prodrugs and enzymes in cancer therapy. The first uses endogenous enzymes in the body, hopefully present selectively in the target site and thus able to activate the prodrug. The second approach is simply to administer an enzyme which in itself acts as an antitumour therapy. The third uses a combination of mammalian or non-mammalian enzymes administered parenterally to activate a prodrug which is also administered to the patient.

Table 1.4 Enzymes and prodrugs proposed for cancer therapy

Enzyme	Prodrug	Drug	Applications
Carboxypeptidase G2	Benzoic acid and mustard glutamates	Benzoic acid mustards (various)	ADEPT * GDEPT **
Thymidine kinase (viral)	Gancyclovir Adenine arabinonucleoside (araM)	Gancyclovir triphosphate Adenine arabinonucleoside triphosphate (araATP)	GDEPT
Glucose oxidase	Glucose	Hydrogen peroxide	radical generating system
Xanthine oxidase	Hypoxanthine	Superoxide, hydrogen peroxide	radical generating system
Cytosine deaminase	5-fluorocytosine	5-fluorouracil	GDEPT ADEPT
Carboxypeptidase A/B	Methotrexate-alanine α-linked derivatives of TS inhibitors	Methotrexate TS inhibitors	ADEPT
α-Galactosidase	N-[4-(a-D-galactopyranosyl)-benyloxycarbonyl]-daunorubicin	Daunorubicin	ADEPT
β-Glucosidase	Amygdalin	Cyanide	ADEPT
Plasmin	Peptidyl-p-phenylenediamine-mustard	Phenylenediamine-mustard	Prodrug therapy
Azoreductase	Azobenzenemustards	Phenylenediamine-mustards (various)	Prodrug therapy
γ-Glutamyl transferase	γ-Glutamyl p-phenylenediamine-mustard	Phenylenediamine-mustard	Prodrug therapy
β-Glucuronidase	Phenolmustard-glucuronide Epirubicin-glucuronide	Phenolmustard Epirubicin	Prodrug therapy ADEPT
β-Lactamase	Vinca-cephalosporin Phenylenediamine-mustard-cephalosporin Nitrogen-mustard-cephalosporin PEG-7-aminocephalosporin-doxorubicin	4-Desacetylvinblastine-3-carboxyhydrazide Phenylenediamine-mustard Nitrogen-mustards (various) Doxorubicin	ADEPT
Alkaline phosphatase	Phenolmustard phosphate Doxorubicin phosphate Mitomycin phosphate Etoposide phosphate	Phenolmustard Doxorubicin Mitomycin Etoposide	ADEPT
Penicillin amidase	Palytoxin-4-hydroxyphenyl-acetamide Doxorubicin-phenoxyacetamide Melphalan-phenoxyacetamide	Palytoxin Doxorubicin Melphalan	ADEPT

Table 1.4 Enzymes and prodrugs proposed for cancer therapy (continued)

Enzyme	Prodrug	Drug	Applications
Nitroreductase	5-(Aziridine-1-yl)-2,4-dinitrobenzamide (CB 1954) 4-Nitrobenzyloxycarbonyl derivatives	5-(Aziridine-1-yl)-4-hydroxylamino-2-nitrobenzamide e.g. Actinomycin D, mitomycin C	ADEPT GDEPT
DT-diaphorase	CB 1954	5-(Aziridine-1-yl)-4-hydroxylamino-2-nitrobenzamide	Prodrug therapy
Aminopeptidase	2-L-Pyroglutamyl-methotrexate	Methotrexate	ADEPT
Thymidine phosphorylase	5'-Deoxy-5-fluorouridine 5'-DFUR	5-FU	GDEPT
Deoxycytidine kinase	Ara-C	Adenine arabinonucleoside triphosphate (araATP)	GDEPT
Cytochrome-P-450	Cyclophosphamide Ifosfamide	Phosphamidate mustard (+acrolein?)	GDEPT

* ADEPT: Antibody Directed Enzyme Prodrug Therapy

** GDEPT: Gene Directed Enzyme Prodrug Therapy

Both approaches described later in section 1.8.

1.7.1 Prodrugs activated by endogenous enzymes

The use of native enzymes already present in the tumour, especially the "cocktail" of lysosomal enzymes, to activate polymeric prodrugs is rapidly expanding. In particular the thiol-dependent proteases, cathepsin B, H and L, have been used to cleave a peptidyl spacer between drug and its polymeric carrier and releasing the free species. This will be described fully later (section 1.9.2).

In the case of prodrugs designed to be activated by tumour hypoxia, the molecular trigger usually undergoes reduction by endogenous reducing enzymes present in all cells, but the transient intermediate formed is only capable of being oxidised to an inactive form by molecular oxygen, thus restricting activation of the prodrug to the hypoxic cells in tumour tissue. In the case of prodrugs designed to be activated by tumour-specific endogenous enzymes (such as DT diaphorase) the main criteria controlling selectivity are enzyme-substrate specificity and turnover, and the relative patterns of enzyme expression in tumour and normal cells (Denny, 1996).

A number of groups have tried to make use of the native enzyme β-glucuronidase to activate glucuronide prodrugs. However, it was found (Roffler *et al.*, 1991) that high levels of endogenous β-glucuronidase did not appear to cause marked activation of hydroxyaniline mustard glucuronide, suggesting that the prodrug does not gain access to native β-glucuronidase *in vivo*. To overcome this obstacle Roffler and co-workers (Wang *et al.*, 1992) used targeted extracellular β-glucuronidase from *Escherichia coli* to activate a glucuronide-hydroxyaniline mustard prodrug by removing the glucuronide. This system also takes advantage of the fact that circulating hydroxyaniline mustard is rapidly detoxified by hepatic enzymes by conversion to the glucuronide form (Connors and Whisson, 1966; Connors *et al.*, 1973). In this system the endogenous enzymes were finally used to deactivate released drug which had escaped its site of selective activation, not to activate the prodrug in the target site as originally planned.

1.7.2 Administration of enzymes as an anticancer therapy

Enzyme therapy using asparaginase has already been used in the treatment of certain leukaemias. Leukaemic cells are unable to produce asparagine due to lack of asparagine synthetase and need an extracellular asparagine supply. Asparaginase used as a therapeutic agent degrades asparagine and thus deprives the cells of a substance which is essential for protein biosynthesis. Soluble polymer-protein conjugates are now also in routine clinical use as anticancer treatments. Oncaspar™ is an mPEG-L-asparaginase conjugate that was approved by the FDA for use in the USA in 1994 for the treatment of patients displaying hypersensitivity to the antitumour enzyme L-

asparaginase (Kurzberg *et al.*, 1988, Muss *et al.*, 1990). It is used mainly to treat children with acute lymphocytic leukaemia. PEGylation of L-asparaginase results in a product with reduced immunogenicity and a longer plasma half-life.

1.7.3 Prodrugs activated by administered enzymes

The enzymes typically used to activate prodrugs, such as microbial enzymes, have a relatively brief persistence in mammalian circulation. Their plasma half-life ($t_{1/2}$) is almost always less than 10 h (Sherwood *et al.*, 1977) and this is a major limitation to their wider therapeutic use. Several methods of prolonging their persistence have been explored such as chemical modification, polymer-conjugation, enclosure of the enzyme within liposomes (Neerunjun and Gregoriadis, 1976) and also entrapment within erythrocytes (Updike *et al.*, 1976). Vegarud and Christensen (1975) showed that various glycosidases and proteinases had increased stability towards thermal and proteolytic inactivation *in vitro* when bound with polysaccharides, and heparin was shown to have a distinctly longer half-life *in vivo* when coupled to the sucrose polymer Ficoll (Pharmacia, Uppsala, Sweden) (Teien *et al.*, 1975). Holcenberg *et al.* (1975) also demonstrated the increased $t_{1/2}$ of *Acinetobacter* glutaminase-asparaginase in mice and rats when the enzyme was modified by glycosylation with glycopeptides prepared from human fibrin and γ-globulin. Furthermore, Sherwood *et al.* (1977) had investigated the persistence of carboxypeptidase G and arginase, two enzymes of therapeutic interest, coupled to a soluble dextran which is used as a blood-volume expander and persists in the circulation for long periods ($t_{1/2}$=20-50 h) (Wilson *et al.*, 1952).

Prodrugs activated by enzymes expressed at high levels in tumours can deliver at least 50-fold the normal dose of drug and can cure animals with tumours normally resistant to chemotherapy (Knox and Connors, 1997). However, this approach has not yet proved to be practicable clinically because of the rarity of human tumours expressing a high level of an activating enzyme. To overcome this limitation it was proposed that exogenous enzyme might be targeted to tumour tissue by i.v. administration or using viral vectors to increase enzyme expression intratumourally.

1.8 ADEPT and G/VDEPT

With the advent of monoclonal antibody technology in the 1970s it was suggested that improved tumour targeting might be facilitated by conjugation of a cytotoxic to an antibody that could specifically bind to an antigen or receptor site that was overexpressed by the target tumour cell. However, a number of limitations became apparent using this approach for the treatment of solid tumours in humans. For example, poor tumour penetration of the immunoconjugate, lack of intratumoural

accumulation of the cytotoxic component and also poor tumour targeting due to the heterogeneity of tumour-associated antigens (reviewed in Shockley *et al.*, 1992; Wawrzynczak, 1991; Pimm, 1988).

However, a few years later Bagshawe suggested a means of harnessing better the potential of antibodies for targeting. He proposed the conjugation of activating enzymes (instead of drugs) to monoclonal antibodies, thus providing the opportunity to localise enzyme specifically in tumour tissue, with activation of a subsequently administered prodrug (Bagshawe, 1987; Bagshawe *et al.*, 1988). This concept was called Antibody Directed Enzyme Prodrug Therapy (ADEPT).

The concepts of ADEPT which uses an antibody-enzyme conjugates to deliver enzyme to tumour (Bagshawe, 1987; Senter, 1990) and more recently Gene/Viral Directed Enzyme Prodrug Therapy (G/VDEPT) which uses a retroviral vector to deliver a DNA sequence to express an enzyme in a tumour (Ram *et al.*, 1993; McNeish *et al.*, 1997) are now well established.

In ADEPT (Figure 1.4), a foreign enzyme which metabolises substrates not normally metabolised by mammalian cells is linked chemically to a tumour-specific or tumour-associated antibody, then the antibody conjugate is injected intravenously and binds strongly to the tumour by recognising the tumour-associated antigen. In GDEPT (Figure 1.5), the retroviral vector is designed to carry a gene expressing a chosen enzyme that can be delivered selectively to tumour or be under the control of a tumour-specific promoter. The foreign enzyme is then used to activate a carefully designed low molecular weight prodrug. For the therapy to be effective it is essential to have local spread of active species from cells, that express the enzyme. This will kill adjacent, untransduced cells in GDEPT and allow cytotoxic effects on cells that have not bound the antibody-enzyme conjugate in ADEPT (Denny and Wilson, 1993). This has been called the "bystander effect" (Freeman *et al.*, 1993; Pope, 1997). Animal models and pilot human studies have proven that it is possible to deliver selectively an activating enzyme such as carboxypeptidase G2 (Blakey *et al.*, 1996); penicillin amidase (Vrudhula *et al.*, 1993a); β-lactamase (Vrudhula *et al.*, 1993b; Meyer *et al.*, 1993; 1995; Mikolajczyk *et al.*, 1996); β-glucuronidase (Bosslet *et al.*, 1994); cytosine deaminase (Wallace *et al.*, 1994); nitroreductase (reviewed in Knox and Connors, 1995; Melton *et al.*, 1996) and alkaline phosphatase (Senter *et al.*, 1988) to a solid tumour mass. However, both approaches have a number of inherent limitations.

In ADEPT, for example, most antibody-enzyme conjugates will be highly immunogenic (Bagshawe *et al.*, 1995; Sharma *et al.*, 1992; reviewed by Bagshawe and

Figure 1.4 The generation of a cytotoxic drug by ADEPT

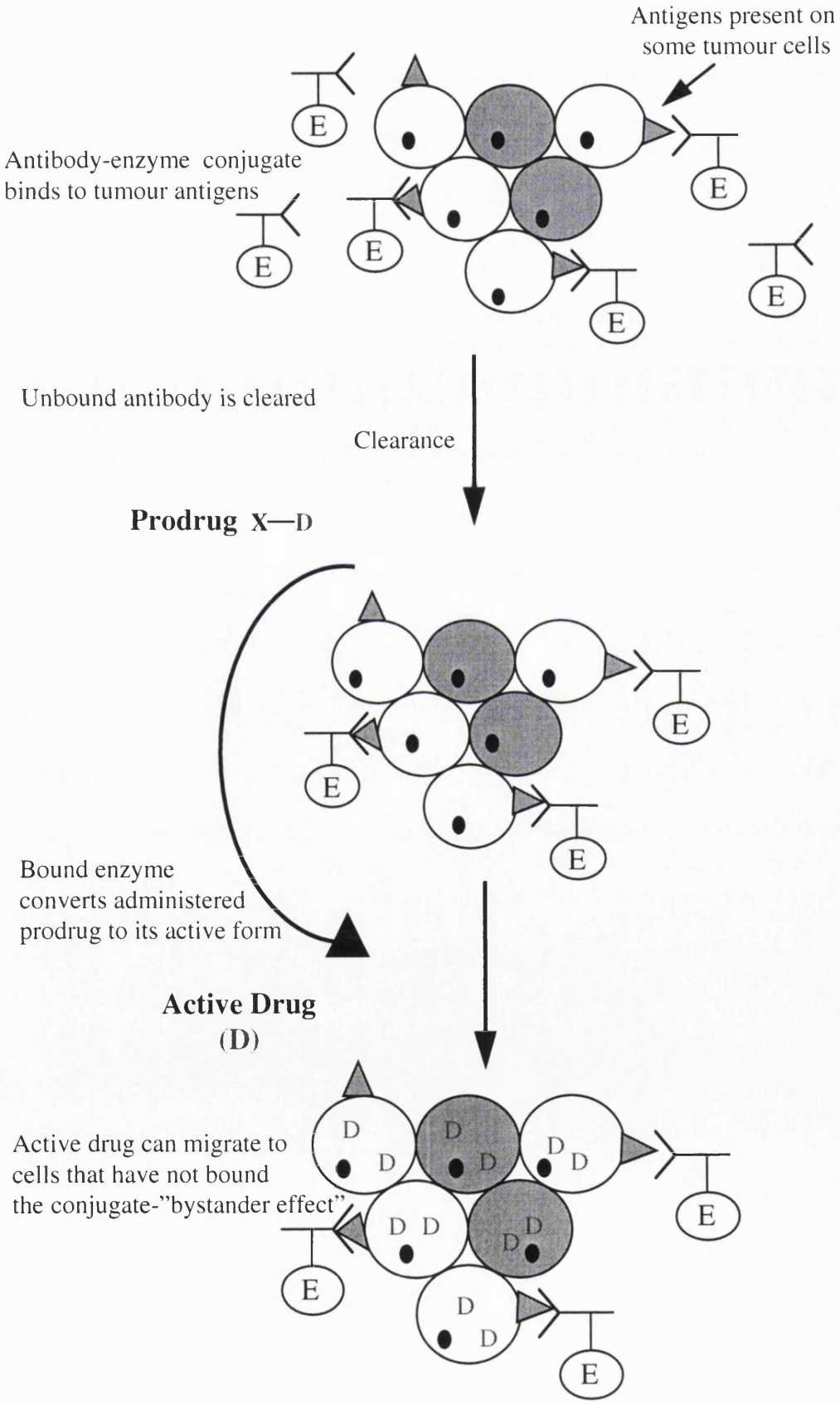
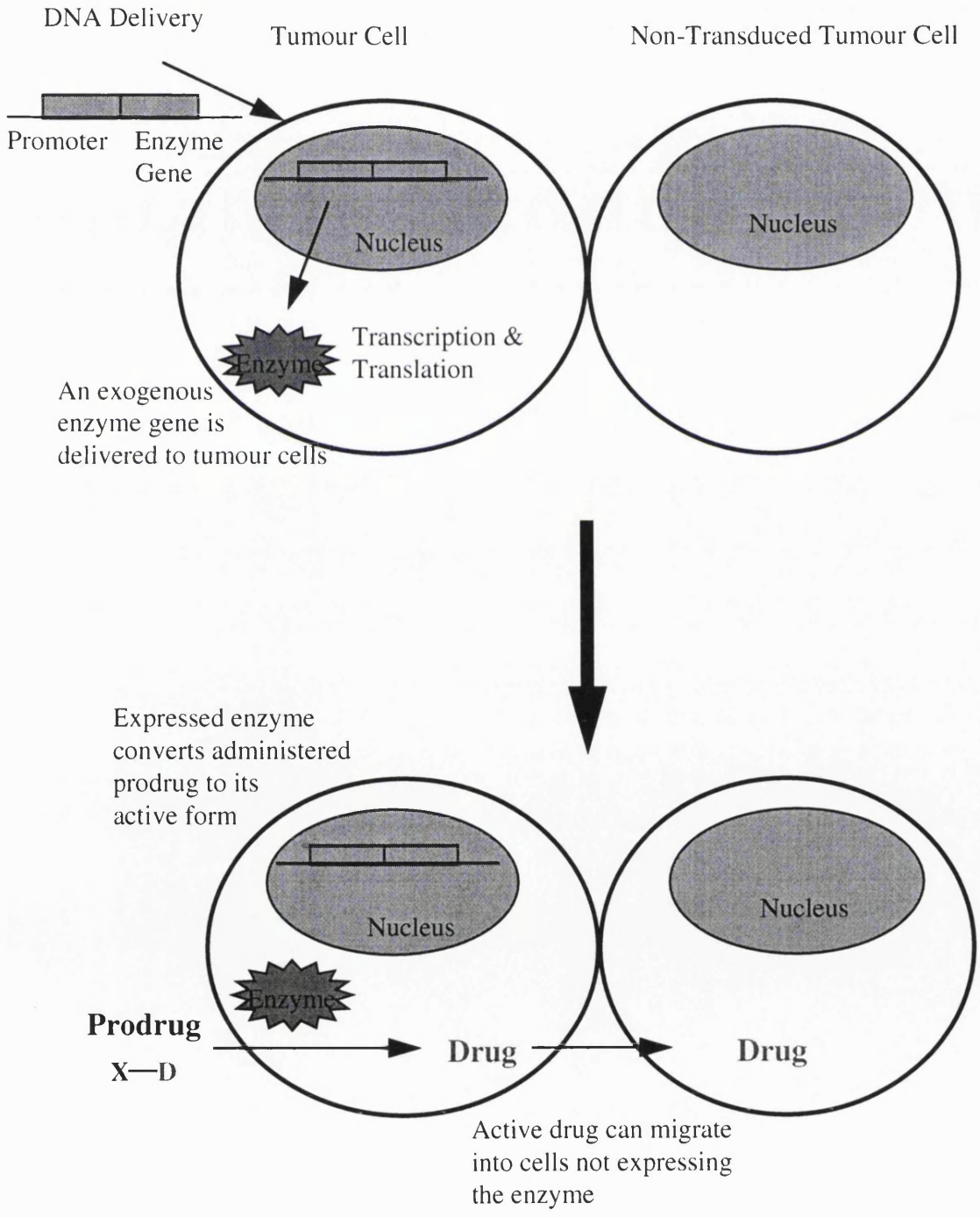


Figure 1.5 The generation of a cytotoxic drug by GDEPT



Begent, 1996; Sharma, 1996b) and several antibody-enzyme constructs may be needed to treat tumour types expressing different antigens. In addition, the long plasma half-life of the antibody-enzyme conjugate given before the prodrug leads to difficulties in optimisation of the dosing schedule and may require a second clearing antibody to prevent non-specific activation of the prodrug (Sharma *et al.*, 1990; reviewed by Sharma, 1996a).

In the case of GDEPT there are inherent dangers associated with a viral vector (reviewed by McNeish *et al.*, 1997) and generation of an immune response directed against transgene products or the caspid proteins (Yang *et al.*, 1996). The potential lack of specificity of enzyme expression in the tumour due to problems of delivering the gene specifically to cancer cells and the difficulty of evaluating the duration and reproducibility of enzyme expression on a patient basis lead to difficulties in optimising the schedule of prodrug follow-up (reviewed in Springer and Niculescu-Duvaz, 1996).

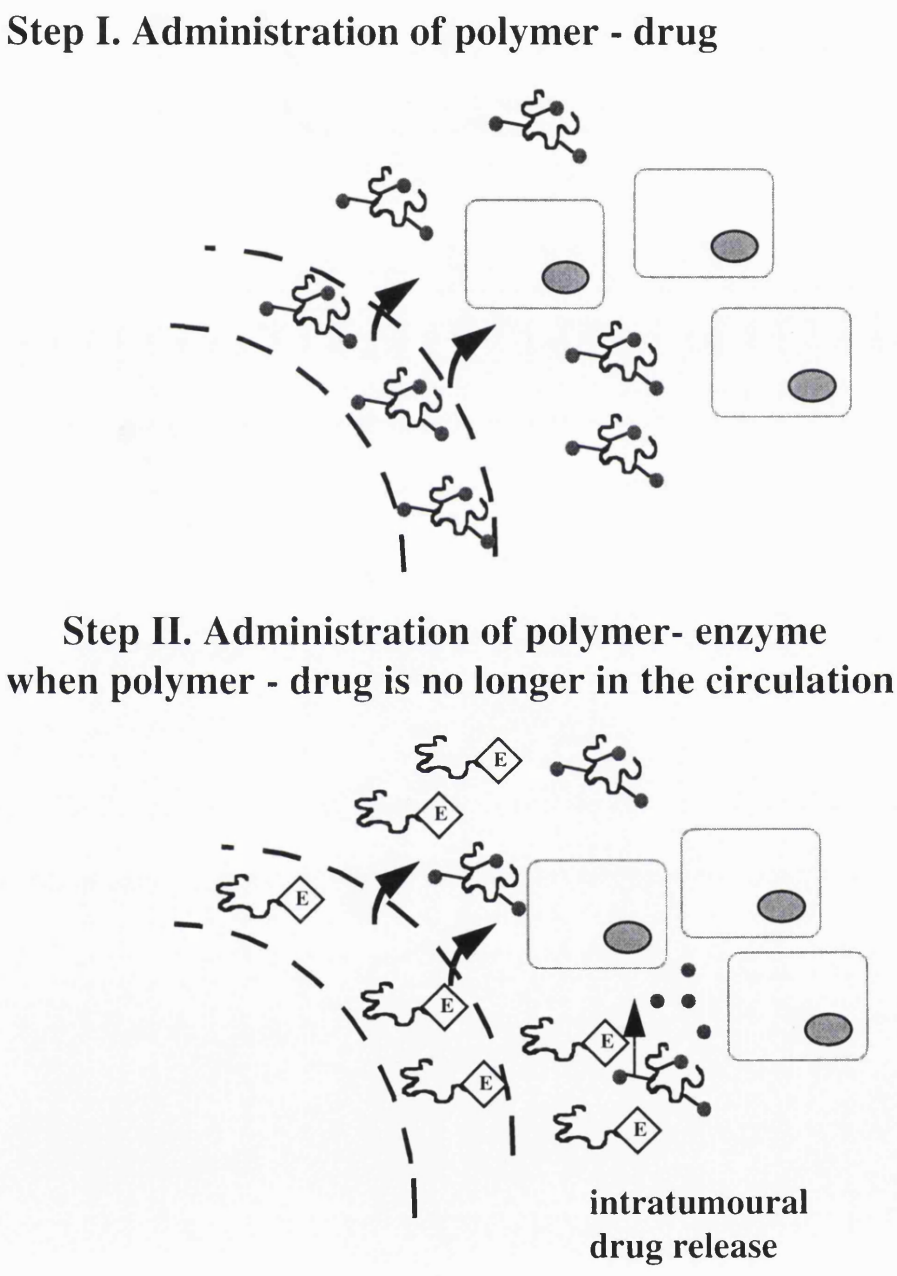
ADEPT and VDEPT are two examples of the "active targeting" concept. There are other ways to achieve active targeting of drugs. For example with the use of proteins such as insulin (Huckett *et al.*, 1990; Burfeind *et al.*, 1996), low density lipoproteins (Firestone, 1994; Mankertz *et al.*, 1997) and transferrin (De Smidt and van Berkel, 1990; Flanagan *et al.*, 1992; Tanaka *et al.*, 1998) as drug-carriers or the use of carbohydrates such as galactose (O'Hare *et al.*, 1989; Seymour *et al.*, 1991b; 1991c), peptides such as melanocyte stimulating hormone (O'Hare *et al.*, 1993; Ghanem *et al.*, 1988, Duncan, 1992) and other targeting moieties such as folic acid (Citro *et al.*, 1994; Wiener *et al.*, 1997) to facilitate the targeting of a drug-carrier by binding to specific receptors present on the target site.

An understanding of polymer therapeutics, together with ADEPT and GDEPT gives the basis for the PDEPT concept.

1.9 PDEPT: Polymer Directed Enzyme Prodrug Therapy

The PDEPT concept proposed in this thesis is designed to make use of the EPR effect to localise both a polymer-drug and a polymer-enzyme conjugate in solid tumour tissue to more selectively limit the activation of the polymer-drug and to maximise the release rate of drug liberation intratumourally. PDEPT has the potential to overcome some of the problems described for ADEPT and GDEPT mentioned above by using a polymer-prodrug which accumulates selectively in the tumour by the EPR effect that is subsequently activated by a polymer-enzyme that likewise accumulates in the tumour specifically (Figure 1.6).

Figure 1.6 Release of anticancer drug from its polymeric conjugae by PDEPT



PDEPT relies on two components, a polymer-enzyme and a polymer-drug conjugate. The design of both components must be optimised. There are different requirements for the polymer-drug and for the polymer-enzyme conjugates, which can be summarised as follows.

The polymer-drug conjugate should:

1. be inactive in the bloodstream
2. accumulate selectively in the tumour by the EPR effect
3. be excreted through the kidneys within 5 h after administration
4. contain a linker which is degraded by the enzyme present in the polymer-enzyme conjugate with effective and favourable activation kinetics
5. release an active drug which would induce a bystander effect
6. ideally release a drug effective in both cycling and non-cycling cells

The polymer-enzyme conjugate should:

1. display enzymatic activity in the polymer conjugate with a favourable K_m towards the polymer-drug conjugate as a substrate *in vivo*
2. ideally contain a non degradable linker *in vivo*
3. accumulate selectively in the tumour by the EPR effect
4. display a long circulation time in the body
5. be non-immunogenic

1.9.1 Choice of polymer model for PDEPT

Polymers selected as carriers for drugs or enzymes should display certain criteria such as: adequate chemical composition (availability of suitable functional groups to permit covalent linkage to drug or enzyme), water solubility, biodegradability (hydrolytically or enzymatically degradable and/or excreted from the body) and biocompatibilty (non toxic and non-immunogenic). The toxicity of low molecular weight compounds can be abrogated by fixing them to a polymer chain (Hurwitz *et al.*, 1978) and this ensures the drug is inactive in the circulation prior to reaching the target. However, release of the drug from the polymeric backbone in the tumour is essential for activity and ideally it should be mediated by the activating enzyme in the tumour.

Several polymer based anticancer agents have now entered the clinic or are passing through clinical trials. Each has proven the basic concept of polymer therapeutics compared to the native drug. At the moment, the most extensively studied polymeric carrier for anticancer agents is the HPMA copolymer (reviewed in Duncan 1992; Duncan *et al.*, 1996). HPMA homopolymer is a hydrophilic, biocompatible polymer originally developed in Czechoslovakia as a plasma expander (Kopecek and

Bazilova, 1973). It is known to be non-toxic in the rat up to a dose of 30 g/Kg. HPMA copolymers are neutral, loosely coiled macromolecules which do not have natural affinity for the plasma membrane, and thus are captured slowly by cells via the mechanism of fluid-phase pinocytosis (Duncan, 1987). HPMA copolymers containing doxorubicin (PK1, FCE 28068), doxorubicin and galactosamine (PK2, FCE 28069) and paclitaxel (PNU 166945) are currently in Phase I/II clinical trial (Vasey *et al.*, 1999, Kerr *et al.*, 1998, ten Bokkel Hunink *et al.*, 1998).

PK1 has already shown promise in early clinical trials and a phase II programme is planned (Vasey *et al.*, 1999). This compound was chosen following a systematic evolution of an optimum molecular weight of carrier to allow tumour selective doxorubicin delivery, and optimisation of linker design (Gly-Phe-Leu-Gly) to mediate controlled release of the drug intratumourally (intracellularly) by the lysosomal thiol dependent proteases (reviewed in Duncan, 1992). PK1 has a molecular weight of 30 KDa and a doxorubicin content of ~ 8 weight% (~2 mol%). The conjugate displays a longer plasma half-life than free doxorubicin ($t_{1/2}$ approximately 1 h). Moreover, it shows significantly increased tumour deposition of doxorubicin (in comparison with free doxorubicin) due to EPR effect (Seymour *et al.*, 1994). Covalent conjugation of doxorubicin via the peptidyl spacer ensures that no significant liberation of free drug in circulation takes place, thus leading to a marked increase in the therapeutic index compared with free doxorubicin. HPMA copolymer-protein conjugates showed little or no immunogenicity (Flanagan *et al.*, 1990). HPMA copolymers are known to display selective accumulation in solid tumours (Cassidy *et al.*, 1989; Seymour *et al.*, 1994) and via recent clinical trial Phase I of HPMA copolymer-doxorubicin are known to be biocompatible in man and amenable to clinical development (Vasey *et al.*, 1999).

In summary, HPMA copolymer conjugate (1) entered clinical trials (Vasey *et al.*, 1999) and (2) has fulfilled *in vitro* and *in vivo* in different tumour models the requirements mentioned in section 1.6 (Kopecek, 1981; Kopecek, 1984; Rihova *et al.*, 1985; Hoes *et al.*, 1993; Rihova, 1996) and (3) it was found that residual HPMA copolymer conjugates which do not accumulate in the tumour are rapidly excreted giving a high tumour:blood ratio (Seymour *et al.*, 1995). Therefore, in this study it was decided to use this copolymer with molecular weight of 30 KDa as the carrier for both the drug and the enzyme, each conjugate with a different linker.

1.9.2 Choice of enzyme model for PDEPT

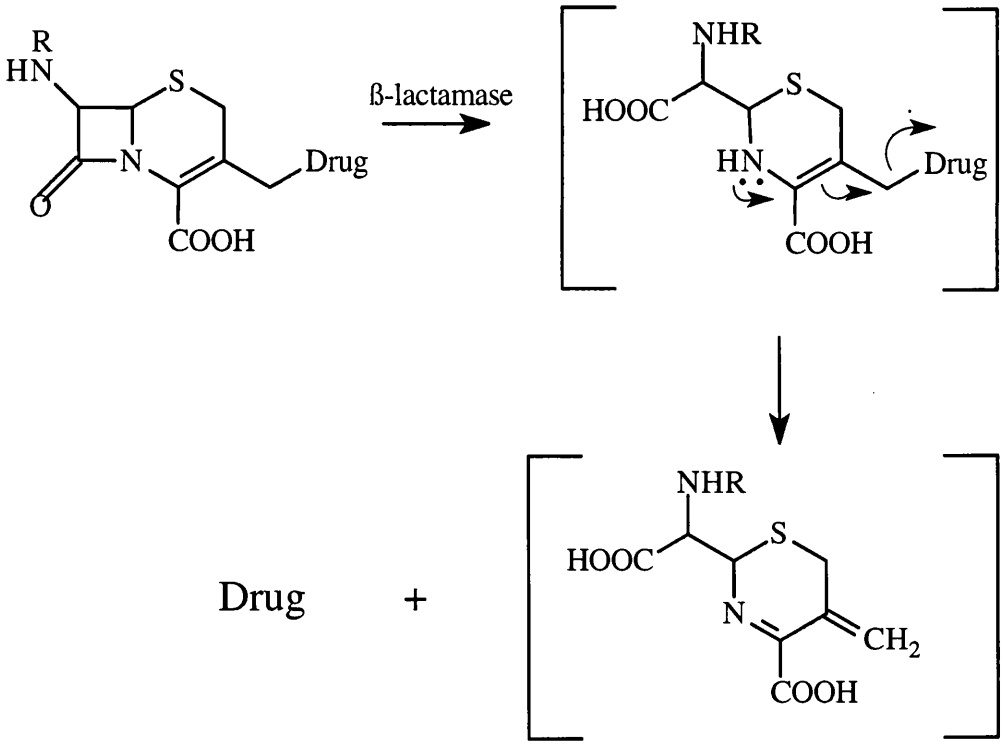
Several polymer-enzyme conjugates have been reported in the literature and are detailed later in Table 3.1. Conjugation of water-soluble polymers to pharmacologically active proteins like enzymes, toxins, immunoglobulins, cytokines or allergens can be

used to reduce the proteolytic degradation of such proteins, improve their biological efficacy and prolong plasma elimination, as well as reduce protein immunogenicity (Abuchowski *et al.*, 1977a; 1977b; 1981; Abuchowski and Davis, 1981). For instance, the conjugation of the enzyme asparaginase to the polymer dextran has been shown to increase the circulation time of the enzyme (Wileman *et al.*, 1983), where its activity in depriving leukaemia cells of asparaginase is useful (Keating *et al.*, 1993). Binding of haptens (low molecular weight molecules which, when attached to certain macromolecular carriers, elicit an immune response) to synthetic polymers leads in almost every case to substantially reduced immune response or even blocks it totally (Vidal-Gomez *et al.*, 1978). The issue of reduction of immunogenicity of a protein following conjugation to a polymer will be described in more detail in Chapter 7.

In selecting the enzyme, one would look for activity under physiological conditions, low immunogenicity, and little or no equivalent endogenous enzyme in humans. If a human enzyme is selected in an attempt to develop a non-immunogenic conjugate, it will probably need to be an intracellular enzyme so that endogenous enzyme does not give rise to activation of the prodrug in the blood. The polymer-enzyme conjugate should include functional enzyme. The level of enzyme activity may be reduced as compared to the native enzyme, provided that significant useful activity is retained. The considerations to be taken into account when conjugating an enzyme to a polymer such as method of conjugation, optimal pH and acceptable range of activity retained by the bound enzyme, are discussed in more detail in Chapter 3.1.

β-Lactamase was selected as the first model enzyme for investigation as a component of the PDEPT system. The β-lactamases have been extensively studied in the ADEPT approach. The β-lactamases are a group of enzymes of varying specificity but all are capable of hydrolysing β-lactams to a substituted β-amino acid. Some act more readily on penicillins, while others have greater activity against cephalosporins. β-Lactamase activity is not endogenous to the mammalian systems and it is therefore subject to minimal interference from inhibitors, enzyme substrates or endogenous enzyme systems. β-Lactamases are small (~30 KDa), soluble monomeric enzymes without a mammalian counterpart. They hydrolyse both penicillins and cephalosporins via a mechanism involving expulsion of a 3' leaving group and the enzyme is particularly tolerant to a wide variety of substituents at this position. β-Lactamase cleaves the lactam ring and after a chemical rearrangement (shown in Figure 1.7) the terminal group, being the anticancer drug, is ejected. Many cytotoxic agents can be attached provided they have NH_2 or OH groups that can be substituted and thus act as prodrugs. The rate of the hydrolysis is measured *in vitro* by the change in absorbance.

Figure 1.7 Mechanism of drug release by ß-lactamase



R= Polymer

The synthesis of a range of cephalosporin derivatives of various mustards (Svensson *et al.*, 1992) and doxorubicin (Senter *et al.*, 1995) have already been described as low molecular weight prodrugs for the ADEPT approach for β-lactamase and these are relatively good substrates for the enzyme. In the context of PDEPT, the spacer between the polymer and the anticancer drug would have to contain a β-lactam ring (in the form of a cephalosporin) if the polymeric-drug conjugate were to be a substrate. Unfortunately, the β-lactamase prodrug was not synthesised during the study due to lack of time.

The second enzyme model selected to explore the PDEPT concept was cathepsin B, a mammalian lysosomal thiol-dependent enzyme which functions in the normal turnover of proteins in mammalian cells. Cathepsin B is a cysteine proteinase which plays an important role in lysosomal proteolysis (Mordier *et al.*, 1995). It is secreted as a pre-pro-enzyme that requires two proteolytic events for activation. Once activated, cathepsin B degrades type IV collagen, fibronectin and lamini, both at acidic and neutral pH (Buck *et al.*, 1992). This enzyme was a convenient choice as it is the native activating enzyme for PK1. This gave the possibility to combine well with PK1 to study aspects of the PDEPT combination. Use of cathepsin B might be considered disadvantageous as the *in vivo* activation of PK1 by HPMA copolymer-cathepsin B is more complicated than by the native enzyme, but in fact use of the enzyme conjugate enables an interesting insight into the intracellular and extracellular cleavage of PK1.

Cathepsin B is known to be present at higher levels in tumour tissues, in particular, its levels are enhanced in human breast cancer cells where they are implicated in metastatic spread (Sameni *et al.*, 1995), in lung, colorectal, and ovarian carcinoma (Werle *et al.*; 1997; Milburn Jessup, 1994; Campo *et al.*; 1994; Ohta *et al.*, 1994) and also present in the lysosome in the cell (Barrett and Heath, 1977). It has been identified in rat, rabbit, bovine and human tissue, being found in liver, spleen and human leukocytes. The enzyme is very labile above pH 7, but has good stability at mildly acidic pH values, particularly when the essential thiol group is reversibly blocked. The optimum pH for cathepsin B is about 6.0 (Nichifor *et al.*, 1996). Its molecular weight is about 27 KDa. The isoelectric point is in the region of pH 5.

Cathepsin B is of major importance in the intralysosomal digestion of proteins (Barrett and Heath, 1977). Its most specific synthesised substrate is benzoyl-Phe-Val-Arg-p-nitroanilide (Bz-Phe-Val-Arg-NAp). The importance of the lysosomal thiol-dependent proteases in the degradation of peptidyl side-chains in HPMA copolymers was discovered by Duncan *et al.* (1981). They found that incubation of HPMA copolymer-pepidyl-p-nitroanilide substrates with rat liver lysosomal enzymes in the

absence of reducing agent gave poor rates of hydrolysis (Duncan *et al.*, 1980). Addition of reduced glutathione (GSH) activated the thiol-dependent proteases and extensive cleavage resulted (Duncan *et al.*, 1983a). Later Rejmanova and colleagues examined the degradation of HPMA copolymer peptidyl-4-nitroanilides by bovine spleen cathepsin B (Rejmanova *et al.*, 1983). The results obtained with 4-nitroanilide as model compound were used to design the tetrapeptide spacer (Gly-Phe-Leu-Gly) in order to bind doxorubicin to HPMA copolymer to produce PK1 and thus control the release of anthracyclines more effectively than tripeptide spacers, while the sequence Gly-Gly was virtually undegradable (Subr *et al.*, 1992). Many substrates contain arginine in the P_1 position, but the most important for efficient cleavage seem to be the amino acids in the P_2 and P_3 position (Barrett and Kirschke, 1981). The presence of Phe at the P_2 position provides an excellent substrate for cathepsin B (Kobayashi *et al.*, 1991).

Although important progress has been made in the design of lysosomotropic drug delivery systems, it should be pointed out that lysosomal enzymes are not specific to the tumour cells or their environment. Lysosomes are present in all type of mammalian cells, even if the composition and specific activity of the proteinases may vary widely from tissue to tissue (De Duve *et al.*, 1974). In order to restrict the activation of polymeric derivatives to the target cells, the conjugate should be provided with either a targeting moiety, or be followed by the delivery of the activating enzyme selectively to the tumour tissue by the EPR effect to ensure activation of the polymeric prodrug intratumourally. The choice of the linker between the drug and the polymer in the PDEPT system will be determined by the enzyme chosen and its activity.

The spacer chosen between the HPMA copolymer and both model enzymes (β-lactamase and cathepsin B) was a glycine-glycine linker, due to the fact that is non degradable in the body. In contrast, the linker between the polymer and the drug may be either a cephalosporin linker, which is cleaved by β-lactamase or a peptide moiety (Gly-Phe-Leu-Gly) when the enzyme is a peptidase (cathepsin B) for which the peptide linker is a substrate.

Enzymatic hydrolysis can occur for conjugates containing oligopeptide spacers. Peptidyl spacers have been developed for the attachment of several antitumour drugs to polymers and proteins and their amino acid sequence can be specifically chosen to facilitate intracellular degradation by the lysosomal thiol proteases. There is an increasing amount of evidence that in the vicinity of solid tumours highly elevated levels of proteases are present, which are absent or present in much more reduced amounts in healthy tissue. Use of cathepsin B in PDEPT enabled the cleavage of the Gly-Phe-Leu-Gly spacer, between the HPMA backbone and the antitumour agent, to occur

intercellulary as well as intracellularly (by endogenous cathepsin B present in the lysosome). This was tested on HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin (PK1) as the prodrug model.

1.9.3 Choice of drug to be conjugated to the polymer for the PDEPT combination

The prodrug is an integral component of PDEPT systems and requires careful design in its own right. An ideal prodrug would be one with a large differential in cytotoxicity between drug and prodrug, which must be a good substrate for the enzyme under physiologic conditions. The enzyme can be one for which there is no mammalian homologue capable of performing the same reaction (in the case of the β-lactamase prodrug) or there is no similar enzyme present in the circulation (in the case of the cathepsin B prodrug). Equal cytotoxicity of the released active drug toward proliferating and quiescent cells is also desirable if residual deposits of viable but non-proliferating cells with the potential for outgrowth are to be eradicated. The chosen drug should be potent enough to ensure the use of the therapeutic advantages this drug delivery system can offer. Once formed, it would be desirable for the drug to have a very short half-life, limiting the possibility of escape of active drug back to the circulation and access to healthy tissue. The drug should be resistant to lysosomal enzymes and pH.

The most commonly used anticancer agents are low molecular weight compounds which readily gain access to all cells by rapid passage across the cell membrane. After i.v. administration, a large percentage of the injected dose leaves the circulation in the first few minutes, resulting in a ubiquitous body distribution of drug and little selective concentration in tumour tissue (normal/tumour tissue ratio is usually 1/1-2) (Cassidy *et al.*, 1993). The creation of a macromolecular polymer-anticancer drug conjugate provides an opportunity to improve tumour specific targeting, minimise drug entry into sites of toxicity, control precisely the rate of drug liberation at the target site and deliver the active principle intratumouraly and maybe intracellularly providing a means to overcome p-glycoprotein related multidrug resistance (Wedge, 1990). If the drug is only released inside the cell, past studies have shown that this method can bypass the development of drug resistance (Cuvier *et al.*, 1992).

A full discussion of cancer chemotherapy is beyond the scope of this thesis. A number of classes of anticancer agents would not, for different reasons, be first choice for the animal models used to evaluate the PDEPT system, e.g. inducers of differentiation, antimetastatic drugs, hypoxic tumour stem cell-specific agents, tumour radiosensitising and normal tissue radioprotecting drugs and biologic response modifiers.

Information on cell and population kinetics of cancer cells in part explains the limited effectiveness of most available anticancer drugs. A schematic summary of cell cycle kinetics is presented in Figure 1.8. This information is relevant to the mode of action, indications, and scheduling of cell cycle-specific and cell cycle-non-specific drugs. Agents falling in these two major classes are summarised in Table 1.5. In general, cell cycle-specific drugs have proved most effective in haematologic malignancies and other tumours in which a relatively large proportion of the cells are proliferating or are in the growth fraction. Cell cycle-non-specific drugs (e.g. alkylating agents) are useful in low-growth-fraction "solid tumours" as well as high-growth-fraction tumours.

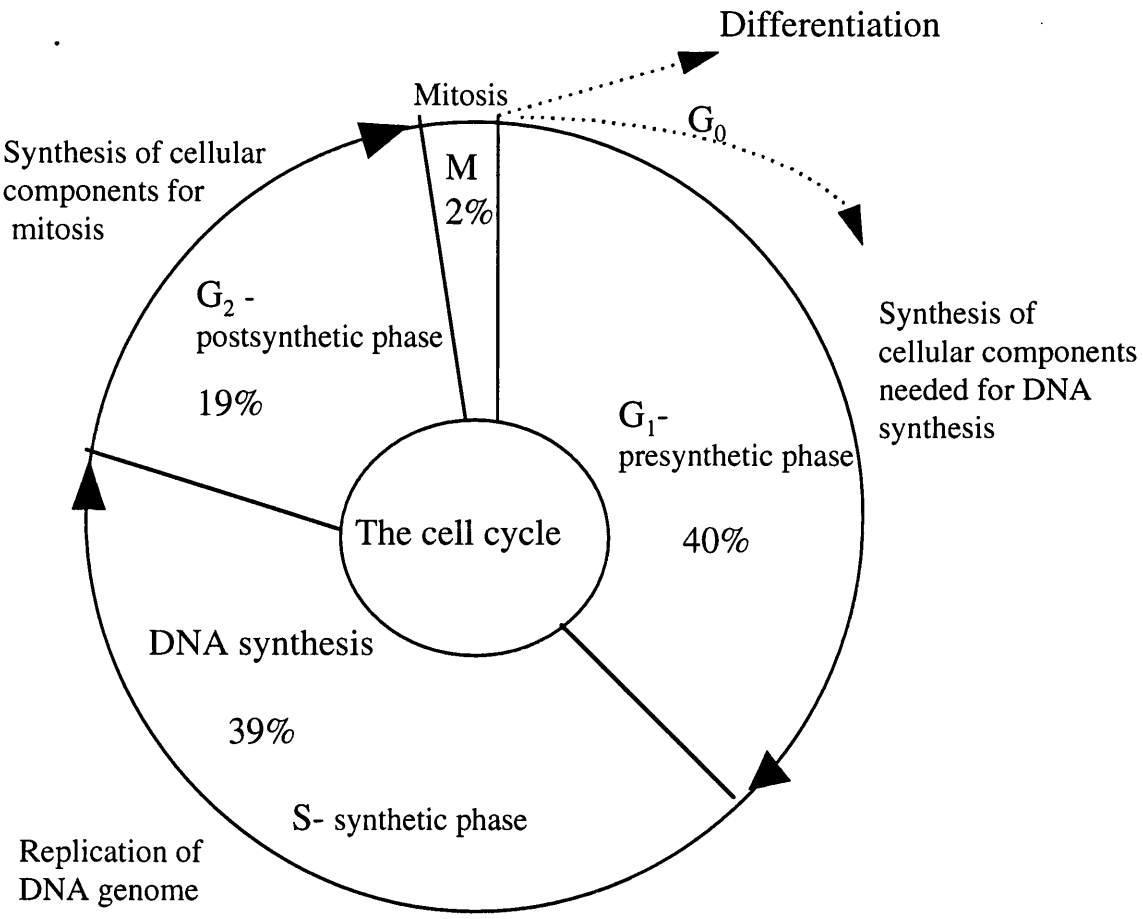
Although cell-cycle specific anticancer drugs have their place in chemotherapy, the drug release from the polymer in the PDEPT system anticipated, suggests choosing a cell-cycle non-specific agent. This led to the choice of an anthracycline.

The anthracycline antibiotics, isolated from *streptomyces peucetius var caesius*, are among the most useful anticancer drugs. Two congeners doxorubicin and daunorubicin are FDA-approved and in general use. Their structures are shown in Figure 1.9. Daunorubicin was first isolated in the early 1960s (Di Marco *et al.*, 1964), shortly followed by the discovery of doxorubicin from a similar mutant strain (Di Marco *et al.*, 1969). Several major actions have been documented for the organ and tumour toxicities of the anthracyclines. These include (1) high-affinity binding to DNA through intercalation with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission (Holbrook *et al.*, 1988; Ross, 1985); * (3) binding to membranes to alter fluidity and ion transport (Murphree *et al.*, 1981; Dasdia *et al.*, 1979); and (4) generation of the semiquinone free radical and oxygen radicals through a cytochrome P-450 mediated reductive process (Lone *et al.*, 1982). This later action may be responsible for the cardiac toxicity through oxygen-radical-mediated damage to membranes. Escrib'a *et al.* (1995) has found recently more information about the mechanism of action of anthracyclines by disrupting cellular signalling pathways through destabilisation of nonlamellar membrane structures. In this way anthracyclines can exert their cytocidal action without entering the cell. In order to prove the concept of PDEPT, doxorubicin was evaluated as a model compound.

Doxorubicin administered as a single treatment exhibits greatest therapeutic effect against acute leukaemias and breast neoplasia (Gale *et al.*, 1981), sarcomas (Pinedo *et al.*, 1984), lymphomas (Bonadonna, 1982; Golomb, 1980) and carcinomas to the breast (Bonadonna, 1984), thyroid (Gottlieb and Hill, 1974) and ovary (Bonadonna *et al.*, 1987). However, as mentioned before, most therapeutic regimes in

* (2) binding to topoisomerase II/DNA complex which may induce apoptosis

Figure 1.8 The cell-cycle and cancer

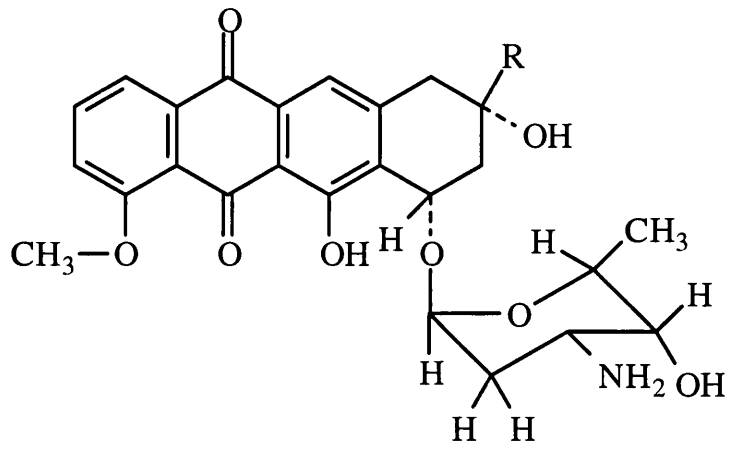


The percentages given represent the approximate percentage of time spent in each phase by a typical malignant cell; the duration of G1, can vary markedly.

Table 1.5 Cell cycle relationships of major classes of drugs

Cell Cycle-Specific Agents	Cell Cycle-Nonspecific Agents
Antimetabolites (azacitydine, cytarabine, fluorouracil, mercaptopurine, methotrexate, thioguanine)	Alkylating agents (busulfan, cyclophosphamide, mechlorethamine, melphalan, thiotepa)
Bleomycin peptide antibiotics	Antibiotics (dactinomycin, daunorubicin, doxorubicin, plicamycin, mitomycin)
Podophyllin alkaloids (etoposide, VP-16, teniposide, VM-26)	Cisplatin
Vinca alkaloids (vincristine, vinblastine, vindesine)	Nitrosoureas (BCNU, CCNU, methyl-CCNU)

Figure 1.9 Structures of doxorubicin and daunorubicin



R is: —C(=O)—CH_3

Daunorubicin

R is: —C(=O)—CH_2OH

Doxorubicin

modern chemotherapy involve the simultaneous administration of a number of antineoplastic agents, and so the clinical utility of doxorubicin is predominantly in combination chemotherapy (Bonadonna *et al.*, 1974). It has been used in cycling regimens with cyclophosphamide, vincristine and procarbazine to treat Hodgkin's disease and non-Hodgkin's lymphomas. For the treatment against ovarian carcinoma it has been used in combination with cyclophosphamide and cisplatin. When used in combination it often synergises, yielding longer remissions than are observed when it is used as a single agent.

As all anticancer drugs, doxorubicin has many deleterious side-effects, including leukopenia, thrombocytopenia, nausea, stomatitis, alopecia and cardiotoxicity (Henderson and Frei, 1980; Blum and Carter, 1974). Cardiotoxicity is the major dose-limiting factor in the administration of doxorubicin, since congestive cardiac failure eventually results from degenerative cardiomyopathy at a total dose exceeding 550 mg/m^2 (Praga *et al.*, 1979). Hundreds of derivatives and related compounds have been synthesised to hopefully reduce the side-effects. Several have reached clinical trials and show promising results, such as idarubicin (approved for use in acute myeloid leukaemia), epirubicin and mitoxantrone (Arlin *et al.*, 1990; Feldman *et al.*, 1993; Wiernik *et al.*, 1992). Numerous attempts have been made to improve the theraeutic index of doxorubicin by modifying its mode of delivery, including attempts to optimise kinetics of drug administration (Robert, 1987) or alternatively by using a variety of drug delivery systems [liposomes (Rahman *et al.*, 1986; Gabizon *et al.*, 1996), microspheres (Willmott *et al.*, 1986), antibodies (Dillman *et al.*, 1986), poly amino acids (van Heeswijk *et al.*, 1985; Pratesi *et al.*, 1985) and soluble synthetic polymers (Zunino *et al.*, 1989; reviewed by Duncan *et al.*, 1996)].

The recommended dose of doxorubicin hydrochloride is 60-80 mg/m^2 administered as a single rapid i.v. injection once every three weeks. Conjugated to HPMA copolymer (PK1) it showed a maximum tolerated dose of 320 mg/m^2 (doxorubicin equivalent) and antitumour activity has been observed in clinical trials phase I/II (Vasey *et al.*, 1999). These clinical results together with the fact that cathepsin B is the endogenous activating enzyme of PK1, were a good basis to choose the PK1 as the model polymer-drug conjugate in the PDEPT approach followed by the administration of HPMA copolymer-cathepsin B.

1.10 Aims of this thesis

The purpose of this study was to examine, for the first time, the feasibility of the PDEPT concept using well-defined HPMA copolymer-enzyme conjugates (HPMA

copolymer-β-lactamase and HPMA copolymer-cathepsin B) and the HPMA copolymer-doxorubicin conjugate (PK1) currently undergoing clinical development.

First, a method was established for the synthesis of HPMA copolymer-Gly-Gly-β-lactamase and HPMA copolymer-Gly-Gly-cathepsin B. The polymer-enzyme conjugation reaction was optimised to give the highest yield whilst ensuring that catalytic activity of the enzyme was maintained (Chapter 3). Activity of free and bound β-lactamase and cathepsin B was first tested using these low molecular weight substrates, benzylpenicillin and Bz-Phe-Val-Arg-NAp respectively. To establish that the polymer-enzyme could degrade a polymeric drug conjugate (as required by the PDEPT combination) the activity of HPMA copolymer-cathepsin B was tested on the high molecular weight substrate, PK1 (Chapter 3).

For PDEPT to be effective both components must have an appropriate body distribution. The pharmacokinetics of PK1 in animals (Seymour *et al.*, 1990; 1994) and man (Vasey *et al.*, 1999) is already well established but it was necessary to determine the biodistribution of the polymer-enzyme conjugates used here. ^{125}I-Labelled HPMA copolymer-β-lactamase and ^{125}I-labelled HPMA copolymer-cathepsin B were used to establish the pharmacokinetics of the conjugated enzymes. Therefore, the body determination of the half-life of the polymeric constructs and their accumulation in the tumour tissue are described in Chapter 4. Subsequently, the kinetics of drug liberation from PK1 by HPMA copolymer-cathepsin B was determined *in vivo* in C57 mice bearing s.c. B16F10 murine melanoma (Chapter 5).

In order to examine the effect of the pharmacokinetic results of the PDEPT combination on its efficacy, the antitumour activity was compared with other systems. *In vivo* pharmacology studies administrating free doxorubicin, PK1 or PK1 followed by HPMA copolymer-cathepsin B were conducted in two different cell lines, B16F10 murine melanoma and COR-L23 non-small cell lung carcinoma, and the results are described in Chapter 6.

Finally, the prevention of development of antibodies to the enzyme, which can contribute to its rapid clearance on prolonged therapy and therefore constitute one mechanism of drug resistance, is beneficial. Therefore, a preliminary immunogenicity study was performed in order to investigate the effect of the conjugation of the enzyme to the polymer compared to the free enzyme (Chapter 7).

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Cell Culture

The cell line B16F10 murine melanoma was kindly donated by Prof. Ian Hart (St. Thomas' Hospital, London, UK). COR-L23 non-small cell lung carcinoma human xenograft cell line was purchased from ECCAC (European Collection of Cell Cultures, Centre for Applied Biology, Microbiology and Research, Salisbury, Wiltshire, UK). Tissue culture grade dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypan blue and optical grade DMSO were from SIGMA (Dorset, UK). Foetal calf serum (FCS), Trypsin and RPMI 1640 medium were all obtained from Gibco BRL Life Technologies (Paisley, UK). Phosphate buffered saline for in vitro diagnostic use from Oxoid Unipath Ltd. (Hampshire, UK). 0.9% NaCl was made up using sodium chloride (BDH, UK) and DDW and autoclaved.

2.1.2 *In vivo*

Bantin and Kingman Ltd. (Hull, UK) supplied the C57 black male mice, Balb/C male mice and nu/nu male mice.

2.1.3 Chemicals

β-Lactamase (penicillinase) from *Bacillus cereus*, cathepsin B (EC 3.4.22.1) from bovine spleen, reduced glutathione, N-benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (Bz-Phe-Val-Arg-NAp), EDTA, bovine serum albumin (BSA), Cu(II) sulphate pentahydrate 4% w/v solution, bicinchoninic acid solution, barbitone buffer (B6632), Triton X-100 and *o*-phenylenediamine (*o*-PDA) were all purchased from Sigma (Dorset, UK). HPMA copolymer-Gly-Gly-p-nitrophenol (HPMA copolymer-Gly-Gly-ONp) (approximately 5 mol% peptidyl side chains and molecular weight of 25-27 KDa) was prepared as previously reported (Rihova *et al.*, 1989) and supplied by Polymer Laboratories (Church Stratton, UK). HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin (PK1) was synthesised as previously described (Rihova *et al.*, 1989) and had the following characteristics: M_w=30 KDa; M_w /M_n=1.3; 6-8% w/w of doxorubicin content. Doxorubicin hydrochloride was obtained from Aldrich Chem. (UK). Daunomycin hydrochloride was kindly donated by Rhone Poulenc (France). Medical grade oxygen, nitrogen and carbon dioxide (all 95% v/v) and liquid nitrogen were supplied by BOC (Surrey, UK). Isoflurane was supplied by Abbott Labs (Kent, UK). Amersham Pharmacia Biotech (Hertfordshire, UK) supplied all radioisotopes. Tris-HCl 0.5 M, pH 6.8, Tris-HCl 1.5 M, pH 8.8, sodium dodecyl sulphate (SDS) 10% (w/v), TEMED, 10% (w/v) ammonium persulfate, mercaptoethanol, bromophenol blue and 30% (w/v) 19:1 acrylamide/bis-acrylamide were

purchased from Bio-Rad (UK). Citric acid, sodium hydrogen orthophosphate dodecahydrate and hydrogen peroxide were supplied by Merck (UK). All other chemicals were of analytical grade.

2.2 Equipment

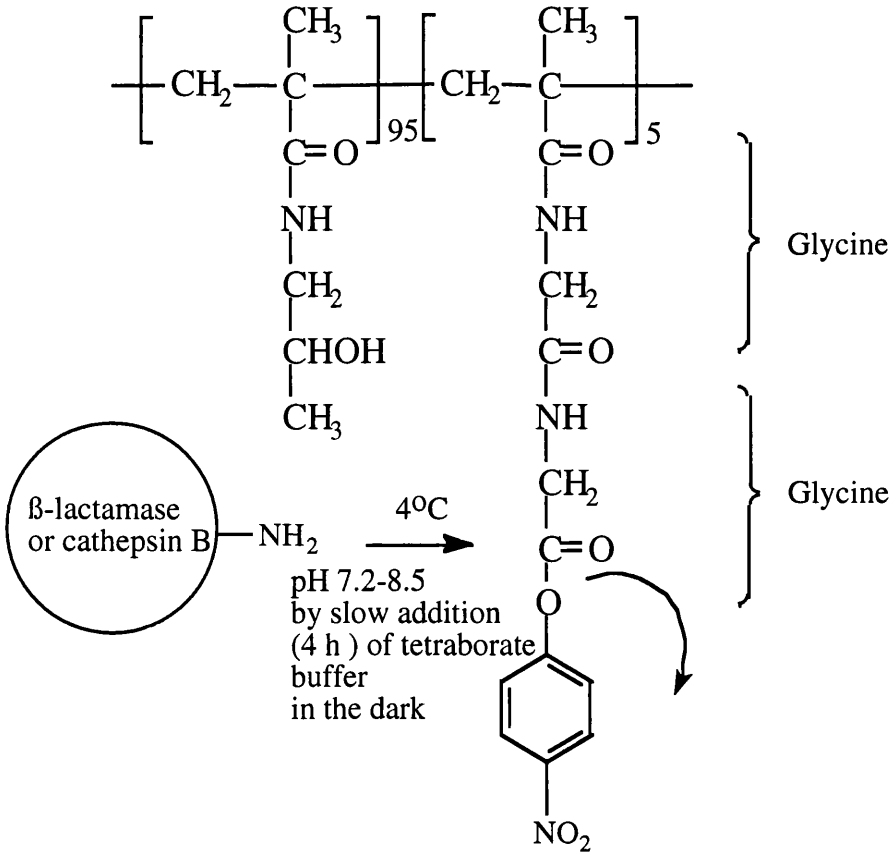
UV/vis spectrophotometer (UV-1601) was from Shimadzu (Japan). Rio-γ-counter 1274 and software were supplied by LKB-Wallack (London, UK). COBRA™ II Auto Gamma® was supplied by PACKARD (Berkshire, UK). Isolator type 12162 (M20) for the nu/nu mice was purchased from Harlan-Isotec (UK). PD10 columns from Amersham Pharmacia Bioteck (Hertfordshire, UK). Paper electrophoresis was performed in a Shandon paper electrophoresis tank supplied by SLS (Nottingham, UK). The 96 well spectrophotometer (micro-titre plate reader) was a Titertek® Multiskan Plus supplied by EFLAB (Finland). Varifuge 3.0 RS (centrifuge) was from Heraeus Instruments (UK). L.I.P. services and equipment Ltd. (West Yorkshire, UK) supplied the heparin/lithium blood tubes. Ultra membrane separator centriprep-50 were from Amicon (US). Dialysis membranes Spectra/POR" CE (Cellulose Ester) sterile DispoDialyzer were from Pierce & Warriner (UK) MW cut-off 50 KDa. Dialysis tubing 3 and 14 KDa were from Spectropor (UK). 717plus Autosampler was from Waters (UK). μBondapak™ C_{18} reversed phase column, 39 x 150 mm attached to Waters Sentry™ Guard Column μBondapak™ C_{18} 125Å 10 μm 3.9 x 20 mm was from Waters (UK). FluoroMonitor™ III fluorescence detector fitted with fluorescence lamp (excitation 480 nm, emission 560 nm) was from LDC/Milton Roy (UK). All data from liquid chromatography experiments were analysed by PowerChrom programme (version 2.0.7). All statistical calculations were performed using GraphPad Prism (GraphPad Software, version 2.0a for PowerPC Macintosh, 1997).

2.3 Methods

2.3.1 Synthesis of conjugates

The selected model enzymes, β-lactamase and cathepsin B, were attached to the polymeric carrier, HPMA copolymer-Gly-Gly-ONp, via the non-specific aminolytic reaction shown in Figure 2.1 (Ulbrich *et al.*, 1996). HPMA copolymer-Gly-Gly-ONp was dissolved in double dionised water (DDW) and the solution of β-lactamase or cathepsin B in 0.05 M phosphate buffer, pH 7.2, was added dropwise at 4^0C under stirring. The reaction mixture was stirred in dark at pH 7.2 for 30 min. Then the pH was carefully raised during 4 h by adding saturated sodium tetraborate buffer up to pH 8.5 (in order to allow competition and to prevent enzyme denaturation). The mixture was stirred for another 4 h

Figure 2.1 Aminolysis reaction between HPMA copolymer-Gly-Gly-ONp and model enzyme



and the reaction was completed by adding 1-amino-2-propanol (1/2 the equivalent related to the original ONp groups) in order to remove unreacted ONp groups. The final yellow solution was acidified by adding diluted HCl solution to pH 7.2.

The conjugation reaction was followed by UV spectrophotometry showing the release of ONp groups from the HPMA copolymers. ONp bound to HPMA absorbs at λmax 270 nm while free ONp at 400 nm.

2.3.2 Purification

To remove free polymer, free enzyme and other low molecular weight compounds, the conjugate was purified at first by centriprep-50 (cut-off 50 KDa the conjugate being 60 KDa at least, the free enzyme ~30 KDa and free HPMA copolymer ~28 KDa), but the enzyme has the potential to stick to the filter. Alternatively, separation was achieved with dialysis membrane Spectra/POR® CE (Cellulose Ester) sterile DispoDialyzer, molecular weight cut-off 50 KDa for separation and it showed better results in terms of yield and purity.

2.3.3 Characterisation of polymer-enzyme conjugates

There was a need to check the stability of HPMA copolymer-Gly-Gly-ONp in different solvents (DDW, sodium phosphate buffer at different pH, DMSO, saturated tetraborate buffer) in order to choose the right solvent so that the release of the ONp groups is due to aminolysis and not hydrolysis during dissolution. Therefore, solutions of HPMA copolymer-Gly-Gly-ONp (1 mg/ml) in each of these solvents were prepared and followed by UV spectrophotometer to observe if there is any change in its spectra with time. After selecting DDW the conjugate was prepared and characterised as follows:

2.3.3.1 Ninhydrin Assay

The ninhydrin assay was used to determine the number of amine groups present on the surface of the enzymes available for conjugation. Ninhydrin reagent and hydrindantin were prepared in lithium acetate buffer with DMSO. 82 g of sodium acetate (CH_3CO_2Na, molecular weight =82.03) was dissolved in 200 ml DDW in order to prepare 4 mol/l acetate buffer. The pH was adjusted from 9.7 to 5.5 with concentrated HCl. The solution was made up quantitatively to 250 ml with DDW. Buffered ninhydrin reagent (1 ml) (0.8 g of ninhydrin and 0.12 g of hydrindantin were dissolved in 15 ml of DMSO and 5 ml of 4 M lithium acetate buffer giving a red-brown colour) was added to 1 ml of the enzyme sample solution of unknown amine content or standard (1-amino-2-propanol or L-alanine). The

latter was used to create a calibration curve. The mixture was placed in a glass test tube and heated in a water bath for 15 min at 75^{0}C. The solution was allowed to cool to room temperature and 3 ml of 50% v/v ethanol solution were added to the solution before it was analysed spectrophotometrically at λ max 570 nm (Plummer, 1978). The ninhydrin reaction is shown in Figure 2.2. A typical calibration curve is shown in Figure 2.3.

2.3.3.2 Bicinchoninic acid (BCA) assay

This method to determine protein content in a sample was based on that of Smith *et al.* (1985). Proteins reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance at λ max 562 nm. The absorbance is directly proportional to protein concentration as shown in the calibration curve in Figure 2.4. A 96 well micro-titre plate was used. In each well 20 µl of sample and 200 µl of BCA assay reagents mixture (30 ml BCA and 600 µl Cu(I) sulfate). The plate was left for 20 min to react and then read in the spectrophotometer. Non-purified and purified samples of polymer-enzyme conjugates were tested to determine the yield of the reaction as well as the protein content in the pure conjugate.

2.3.3.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) Preparation

Due to their amphoteric character, proteins migrate and can be separated in an applied electric field. This phenomenon is known as electrophoresis. SDS-PAGE is the most common form of electrophoresis of proteins, used to assess purity and determine apparent molecular weight. When denatured by heating in the presence of excess SDS and a reducing agent (usually β-mercaptoethanol), most proteins bind the negatively charged SDS in a constant weight ratio so that they acquire identical charge densities and migrate in polyacrylamide gels of adequate porosity according to their size (Hames, 1990). Thus, this system gives a linear relationship between a protein's relative mobility (R_f) and the $\log_{10}$ of its molecular weight. In the experiments performed here the unknown sample (polymer-enzyme conjugate) was referred to a series of molecular weight protein standards.

The SDS PAGE protocol of Laemmli (1970) for the preparation of a discontinuous gel was followed with the following alterations. Free and conjugated enzymes were run on a 10 % acrylamide prepared as follows. First, the gel holding apparatus was cleaned with detergent followed by methanol. The bottom of the casting slot was then evenly coated with

Figure 2.2 ninhydrin reaction

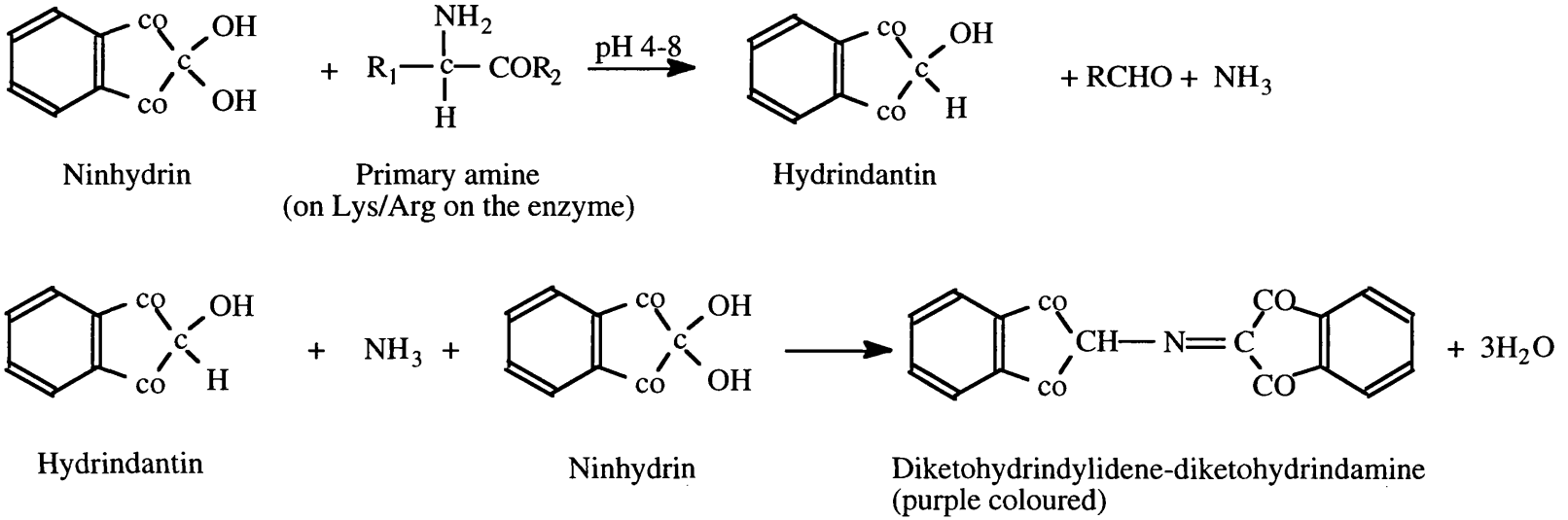


Figure 2.3 Calibration curve for ninhydrin assay using 3-amino-1-propanol standards

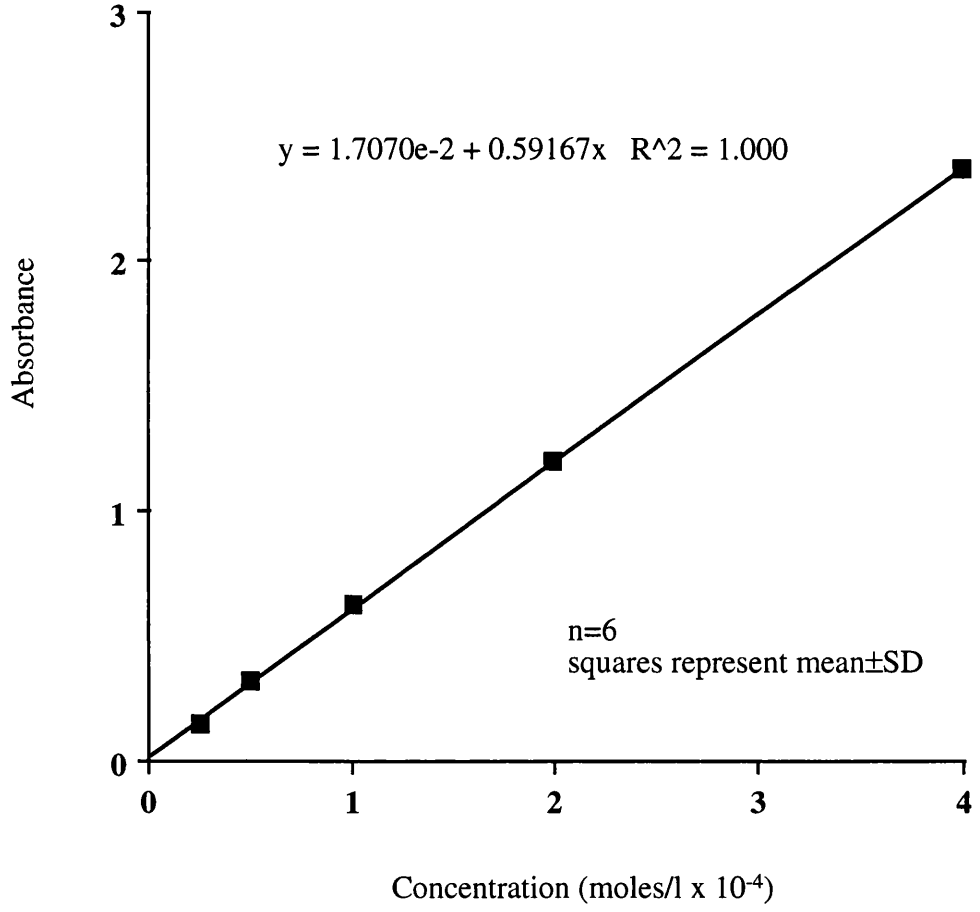
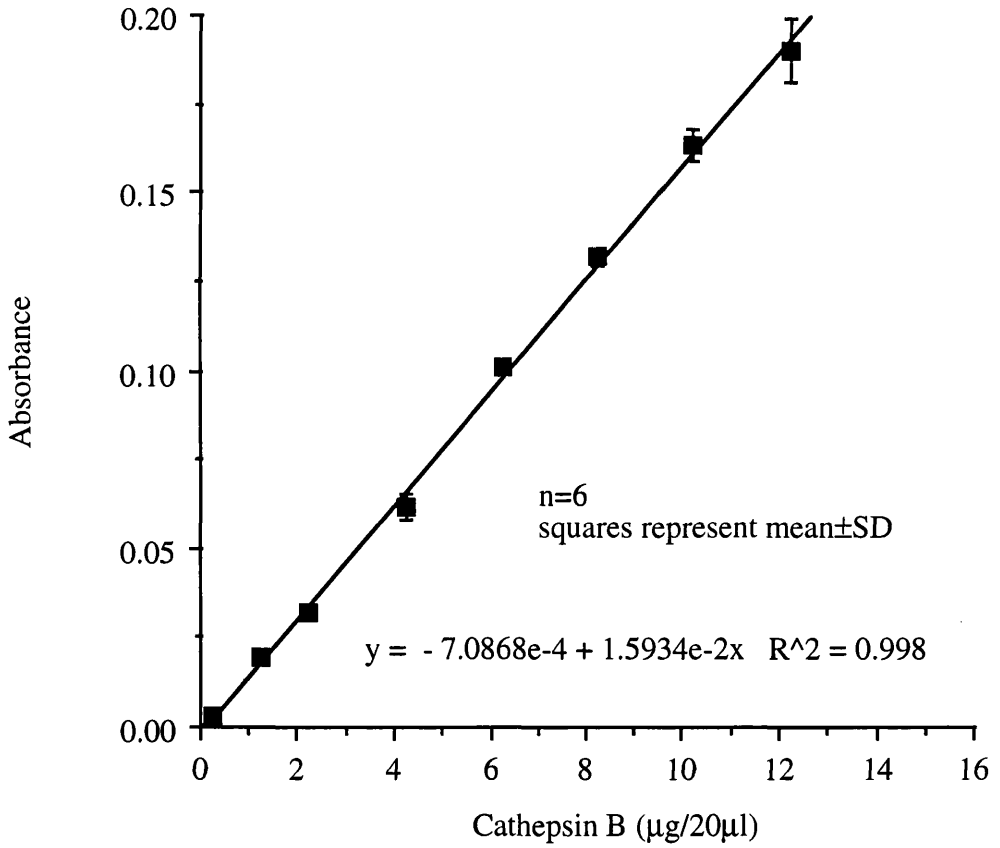


Figure 2.4 Calibration curve to determine cathepsin B concentration using BCA assay



vacuum grease, the apparatus was assembled and tested for leaks. Following this the separating gel was prepared. First, the following reagents were prepared:
1.5 M Tris-HCl, pH 8.8 (Stored at 4^0C), 10% (w/v) SDS (stored at Room Temperature), 30% (w/v)19:1 acrylamide bis acrylamide (photosensitive-stored at 4^0C in dark), 10% (w/v) ammonium persulfate (APS) (prepared fresh daily).

To 4.15 ml DDW, the following reagents were added: 2.5 ml 1.5M Tris-HCl pH 8.8, 100 µl 10% SDS Stock and 3.33 ml 30% (w/v)19:1 acrylamide/bis-acrylamide. This solution was then left to degas for 15 min at a minimum negative pressure of 125 torr. Following this 50 µl of fresh 10% APS and 5 µl of TEMED were added. The mixture was then subject to gentle mixing to avoid the generation of bubbles and poured into the gel holder, leaving room for the stacking gel. Air bubbles were then excluded from the gel and a small quantity of water-saturated isobutanol layered over the top of the gel. The separating gel was then left to polymerise for 30-45 min during which time the stacking gel was prepared and this was done as follows.

To 6.1 ml DDW the following were added: 2.5 ml 0.5 M Tris-HCl pH 6.8, 100 µl 10% (w/v) SDS, 1.33 ml 30% (w/v) acrylamide/bis-acrylamide. This was then degassed as before and the isobutanol flushed out with several charges of DDW. Following this, 50 µl of 10% w/v APS and 10 µl TEMED were then added. This preparation was then gently agitated and poured onto the separating gel. The 10 well comb was then positioned and the gel left to polymerise for 45 min. Following this the comb was removed and the electrolyte buffer added. Stock solution of 5x electrode running buffer consisted of Tris base 15 g, glycine 72 g and SDS 5 g. The pH was then adjusted to 8.3 and the final volume made up to 1 l DDW. This stock solution was then stored at 4^0C and diluted 1:5 prior to use.

Sample preparation: DDW (3.8 ml), 0.5 M Tris-HCl (1 ml) pH 6.8, Glycerol (800 µl), 10% (w/v) SDS (1.6 ml), 2-mercaptoethanol (400 µl) and 1% (w/v) bromophenol blue (400 µl) were mixed. To 10 µl of sample 15 µl of sample reducing buffer were added and then added to the sample well, the gel was then run at 300 V for 1 h. The first well was always loaded with 5 µl molecular weight markers (MWM 1 mg/ml).

2.3.4 Enzyme activity assays

Free or conjugated β–lactamase (10 units/ml) in phosphate buffer 0.1 M, pH 7.0 were added to benzylpenicillin solution 1 mM and the decreasing UV absorbance at λ max 240 nm, due to the cleavage of the β-lactamic ring, was followed with time.

Activity of free and conjugated cathepsin B on a low molecular weight substrate was assayed by measuring the release of p-nitroanilide (NAp) at λ max 410 nm from the tripeptide Bz-Phe-Val-Arg-NAp during incubation at 37^{0}C in citrate phosphate buffer (0.2 M, pH 5.5) containing EDTA (10 mM) and reduced glutathione (50 mM).

The activity of cathepsin B (free and conjugated) against the high molecular weight substrate PK1 was assessed by HPLC (Configliacchi *et al.*, 1996; Fraier *et al.*, 1995). Incubation of PK1 with cathepsin B was carried out at 37^{0}C in a final volume of 1 ml consisting of 400 µl 1 mg/ml of PK1 in citrate buffer (pH 5.5, 0.2 M), 100 µl EDTA solution in buffer (10 mM), 100 µl reduced glutathione (GSH 50 mM) and 400 µl HPMA copolymer-cathepsin B or free cathepsin B in citrate buffer (1 mg/ml) or tritosomes (isolation of tritosomes is described in section 2.3.4.1). Tubes containing PK1 without enzymes were also prepared to assess the levels of hydrolysis as controls. Samples (100 µl) were taken at various times, immediately frozen in liquid nitrogen and stored frozen in the dark until processed by HPLC. Free doxorubicin must first be extracted prior to chromatographic analysis followed by the method summarised in Figure 2.5. Then the samples were mixed with 100 ng daunomycin (DNM), as an internal standard, ammonium formate buffer (pH 8.5) and extraction mixture (chloroform:propan-2-ol). Tubes were centrifuged, the aqueous layer was carefully removed and the organic fraction evaporated to dryness using the Techne nitrogen system operated at no more than 2 lbf/in^2 of $N_{2(g)}$. Evaporated samples were redissolved in methanol prior to analysis. HPLC conditions are defined in Table 2.1. A typical HPLC trace of free doxorubicin and daunomycin as internal standard is shown in Figure 2.6.

2.3.4.1 Isolation of lysosomal enzyme preparations (tritosomes) from rat liver

Lysosomal enzymes were isolated from rat liver in the form of tritosomes (Trouet, 1974). Tritosomes are a purified and enriched form of lysosomal enzymes which due to the presence of a detergent Triton WR-1339 have a reduced density that facilitates separation from other subcellular organelles such as mitochondria. Male rats were injected i.p. with

Figure 2.5 Schematic diagram of method for the determination of free doxorubicin content in tumour or in in vitro samples.

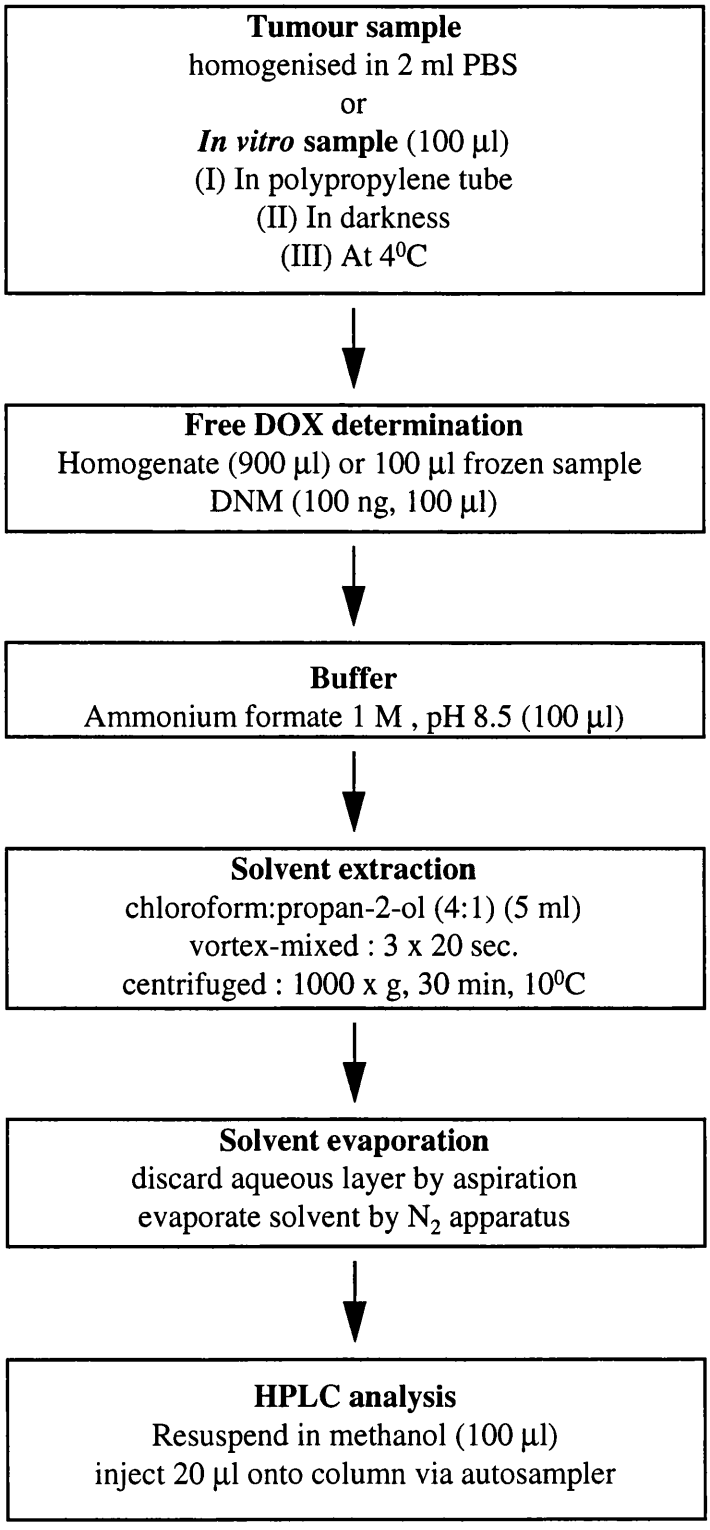
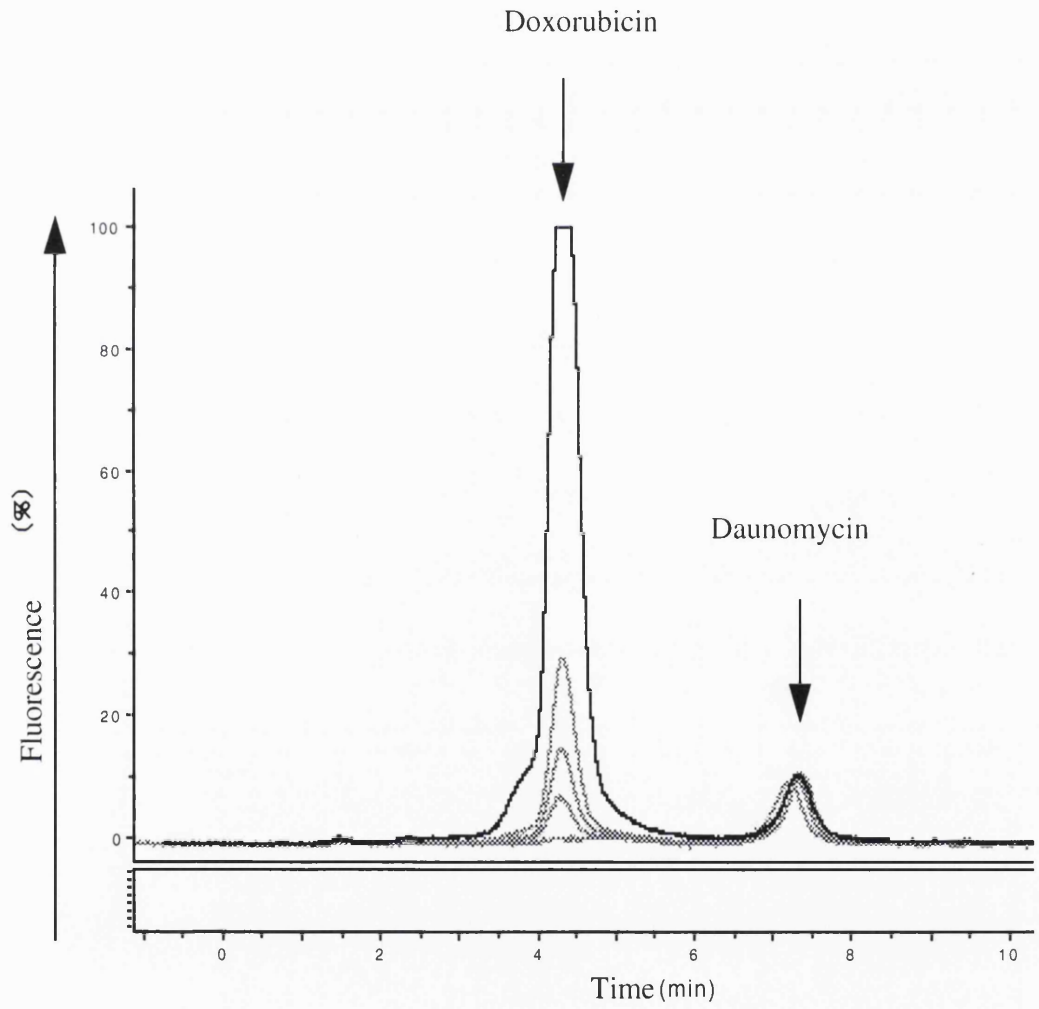


Table 2.1 HPLC conditions for determination of doxorubicin content in samples

Equipment	
Autosampler	Waters 717 plus Autosampler.
Column	Waters μBondapak™ C_{18} reversed phase column, 39 x 150 mm attached to Waters Sentry™ Guard Column μBondapak™ C_{18} 125Å 10 μm 3.9 x 20 mm.
Detector	LDC/Milton Roy fluoroMonitor™ III fluorescence detector fitted with fluorescence lamp (excitation 480 nm, emission 560 nm).
Integrator	PowerChrom AD Instruments attached to Power Macintosh 4400/200 equipped with PowerChrom v2.0.7 software.
Pump	Jasco PU-980 intelligent HPLC pump
Running Conditions	
Mobile phase	29% propan-2-ol in DDW, pH 3.2 (adjusted with orthophosphoric acid)
Flow rate	1 ml/min

Figure 2.6 A typical trace of free doxorubicin and daunomycin as internal standard



Triton WR-1339 Tyloxapol (20% solution, dosed as 1 ml per 100 g body weight) and sacrificed on the fourth day after the injection having starved the night before sacrifice. The liver was then removed and placed in a beaker kept on ice. and pushed through a sieve and the pulp weighed. The pulp was suspended in 0.25 M sucrose (5 ml per 1 g liver) and homogenised to break the hepatocytes. Care should be taken not to overdo this since lysosomes can easily be disrupted. The homogenate was then centrifuged at 1650 rpm at 4^0C for 10 min to separate the cellular membrane components. The supernatant was saved, the pellets were rehomogenised in 0.25 M sucrose (3 ml per 1 g liver) and re-centrifuged at 1400 rpm for 10 min at 4^0C. The two supernatants were pooled and re-centrifuged, this time at 14000 rpm to isolate organelles such as lysosomes, mitochondria, microsomes etc. The supernatant was discarded and the 0.25 M sucrose was drained off by inverting the tubes. The final pellets were resuspended in a small volume of 45% sucrose, pooled and the final volume made up to 30 ml with 45% sucrose. This preparation was then subjected to density gradient centrifugation on a sucrose gradient formed as follows: 9 ml of the preparation in 45% was dispensed in every 25 ml tube. On top of this carefully a layer of 8 ml of 34.5% w/v sucrose was placed. Finally on top a third layer of 14.3% w/v sucrose was laid. Following careful balancing the tubes were centrifuged at 17000 rpm for 2 h at 4^0C. The lysosomal preparation was isolated between the 14.3% and the 34.5% sucrose layers (brown band). the fractions were pooled and the resulting preparation frozen at – 20^0C in individual 1 ml aliquots. The preparations were standardised by measuring the total protein content using the BCA assay and their activity was tested by following spectrophotometrically their ability to release NAp from Bz-Phe-Val-Arg-NAp (the same as in section 2.3.4).

2.3.5 Cell Culture

2.3.5.1 Aseptic technique

All cell culture techniques were carried out in a Class II Laminar flow cabinet using aseptic technique. Equipment and materials used were always sterile. Those items not supplied pre-sterilised were sterilised by either (I) autoclaving (120^0C, 15lb/m^2, 15 min); suitable for glassware, certain plastics, PBS, NaCl 0.9% v/v solution (II) micro-filtration (0.2 μm); suitable for solutions (III) UV irradiation (30 min). The pipetus, suction pump, all associated tubing and the inside of the cabinet were wiped down with methylated spirits (70%) before and after use. The inside of the cabinet was irradiated before each use for 30 min. Sterile solutions were dispensed by means of a pipetus attached to a sterile pipette. Solutions were removed from cell culture by suction via a sterile Pasteur pipette. Whilst

dispensing and removing solutions great care was taken to ensure that neither the pipette, nor solution, touched the neck of any container used.

Cells were maintained in an atmosphere of 5% v/v CO_2 at 37°C in a humidified CO_2 culture incubator. Cells (B16F10 and COR-L23) were maintained in an environment of RPMI 1640 with 10% FCS (11 ml of FCS were added into a new bottle of RPMI using sterile syringe fitted with Acrodisc (0.8 μm, 0.2 μm). All materials added to cell cultures were sterile, osmotically balanced and heated to 37°C (in a 37°C water-bath for 0.5 h prior to use). The cells were kept in 75 cm^2 tissue culture treated, canted neck flasks with vented (0.2 μm) tops and were passaged once every 7 days. The growth medium was replaced every 3-4 days to avoid depletion of essential nutrients. These maintained the cells in the exponential phase of growth.

New/Frozen Cells: Upon arrival, cells were stored in a culture flask which means they are already growing or they arrived in a frozen vial which means they had to be thawed in a water bath (or in the incubator) at 37°C. Media (8 ml) was pipetted into a new sterile culture flask. Then using a new pipette, the defrosted cells (~ 2 ml) were added. The solution was capped and swirled gently. The flask was placed into incubator box with small beaker of PBS (for humidity purposes). The cells were then placed in the 5% CO_2 incubator at 37°C and left to grow until they reached confluence. Cells were checked daily (under light microscope). The media was changed every second day in order to feed the cells.

Changing Media and Trypsination (Cell Splitting): The old media was removed using a 10 ml clean & sterile pipette and the cells washed twice with sterile PBS (10 ml). Once they have reached confluence cells were split to a ratio of 1:5. 1 ml of Trypsin was added to remove the cellular adhesion molecules and the flask was placed in the CO_2 incubator until most of the monolayer had detached. After incubation (approximately 10 min) and gentle agitation, the monolayer was examined microscopically using an inverted microscope. Once the majority of the cells were seen in suspension, media (10 ml) containing 10% FCS was added. Depending on cell density, aliquots of the suspension were used to seed further flasks at a density dictated by the generation time and hence split ratio of the cell line (Table 2.2). Residual cells were discarded autoclaved. The flasks were topped to 10 ml content with fresh media. Then the flasks were placed back into the incubator.

Table 2.2 Cell lines and culture conditions

Tumour origin	Cell line	Culture media	Split	Supplier	Mouse model	No. of viable cells injected s.c.	Average in vivo growth rate (days)
Murine melanoma	B16F10	10% v/v FCS, RPMI 1640 25 mM HEPES 5 mM L-glutamine	1:10	Prof. Ian Hart, St. thomas' Hospital, UK	C57/Black/male	10^5 in 100 µl saline	10
Human non-small cell lung carcinoma	COR-L23	10% v/v FCS, RPMI 1640 25 mM HEPES 5 mM L-glutamine	1:5	ECCAC	nu/nu	10^6 in 100 µl saline	12

Freezing cells: A stock of cells was always stored at -80^{0}C (up to six months) or -196^{0}C in liquid nitrogen (more than six months). 5x10^6 cells/ml were prepared in 90% v/v FCS and 10% v/v DMSO. The cells were then disaggregated. using a sterile 21-gauge needle attached to a sterile 20 ml syringe. 1 ml of the suspension was then transferred into a sterile screw-topped vial, placed in a tissue paper and left at -20^{0}C for 2 h so it was gradually cooled. Then the cells were placed at -80^{0}C overnight and then either transferred to liquid nitrogen vapour or left at -80^{0}C.

2.3.5.2 Evaluation of cell viability by trypan blue exclusion

Before using cells in an experiment, it was necessary to assess their viability. This was achieved by adding 20 µl of 2% v/v trypan blue (supplied as a sterile 4% w/v solution in PBS and diluted 1:1 with PBS) to 20 µl aliquots of the cell suspension. The mixture was shaken and a sample transferred to a haemocytometer slide using a Pasteur pipette. The average number of blue (dead) and clear (viable) cells was estimated using the improved Neubauer haemocytometer under a light microscope. The number of cells in a known volume (0.1 x 0.1 x 0.1 mm) was then recorded. The number of viable cells was then calculated per ml in the following way:
Cells/ml= number of cells in 0.1 x 0.1 x 0.1 mm volume x dilution factor x 10^4. The dilution factor for B16F10 was 2 and for COR-L23 was 8.

2.3.5.3 MTT assay to determine growth curve of cell lines

The MTT assay is used for assessing cell viability for either a growth curve or cytotoxicity (after having exposed the cells to polymers or other chemicals). It is based on the observation that viable cells have the ability to metabolise a water soluble tetrazolium dye MTT, into an insoluble formazan salt (Altman, 1976; Mossman, 1983; Sgouras, 1990; Liu *et al.*, 1997).

Adherent cells (100 µl) in their logarithmic phase of growth were seeded into a sterile, flat-bottomed 96 well micro-titre cell culture plate at a density of 10^3 cells/well. The culture was then placed in the 5% CO_2 incubator at 37^{0}C for 24 h. MTT (5 mg/ml) solution in PBS was prepared and sterilised by filtration. Each day 20 µl of MTT solution were added into each of 8 wells in a row of the micro-titre plate and incubated at 37^{0}C for 5 h in the 5% CO_2 incubator. After 5 h, the MTT media was removed. Care was taken not to disturb the layer of formazan crystals at the bottom. Then 200 µl of DMSO were added in each well using the multi-channel pipette. The absorbance was read spectrophotometrically

at λ max 550 nm using a micro-titre plate reader after blanking with DMSO. These steps were repeated every 24 h for 7 days. The higher the absorbance, the greater the number of viable cells present prior to the addition of MTT. Growth curves were constructed by plotting the absorbance against time. Growth curves were used to determine the cell doubling time by calculating the time taken for the absorbance to double on the linear part of the curve. Growth curves are shown in Figures 2.7 and 2.8 for B16F10 and COR-L23 respectively. The MTT reaction is shown in Figure 2.9.

2.3.6 ^{125}I-Radiolabelling of enzymes and polymer-enzyme conjugates with Chloramine T method

β-Lactamase and HPMA copolymer-β-lactamase were dissolved (10 mg/ml) in 0.1 M phosphate buffer pH 7.4 (0.05 M potassium dihydrogen orthophosphate and 0.05 M disodium orthophosphate) in glass vessels. Na[^{125}I] iodide (500 μCi, 5 μl) was added to each preparation and allowed to stand for 2 min to equilibrate. The stability of the Chloramine T solution (2 mg/ml in 0.1 M phosphate buffer pH 7.4) was tested by placing a drop on a crystal of KI, giving a brown colour change. If this was observed then 75 μl of solution was added to each preparation and left for 15 min. Then 500 μl of sodium metabisulphate (2 mg/ml in 0.1 M phosphate buffer pH 7.5) and a crystal of KI were added and left for further 2 min. 5 μl of the reaction mixture were then removed for the determination of the labelling efficiency of the preparation. The mixture was then placed in dialysis tube (MW 14 KDa cut off) at 4^{0}C and dialysed against 1% NaCl until no radioactivity was found in the dialysate. The purity, labelling efficiency and specific activity (μCi/mg) of the final labelled product were then determined by paper electrophoresis.

2.3.7 ^{125}I-Radiolabelling of enzymes and polymer-enzyme conjugates by Bolton Hunter reagent (Bolton and Hunter, 1973)

Cathepsin B (10 mg) was dissolved in 1.0 ml 0.1 M borate buffer pH 8.5. In a fume-cupboard, 500 μCi (100 μl) of ^{125}I-labelled Bolton Hunter solution (Figure 2.10) in benzene/2% v/v dimethyl formamide, was carefully dried using a stream of nitrogen gas in a glass vial. The enzyme/ polymer-enzyme solution was added to the reagent and was allowed to react for 15 min on ice whilst mixing periodically. The preparation was then saturated with KI and 5 μl of the reaction mixture were removed for the determination of the labelling efficiency. The sample was dialysed at 4^{0}C in a 14 KDa dialysis membrane against 1% NaCl to remove the free Bolton Hunter reagent. The dialysate was changed 3 times a day until no radioactivity was found in the dialysate. The purity, labelling efficiency

Figure 2.7 Growth curve for B16F10 murine melanoma cell line

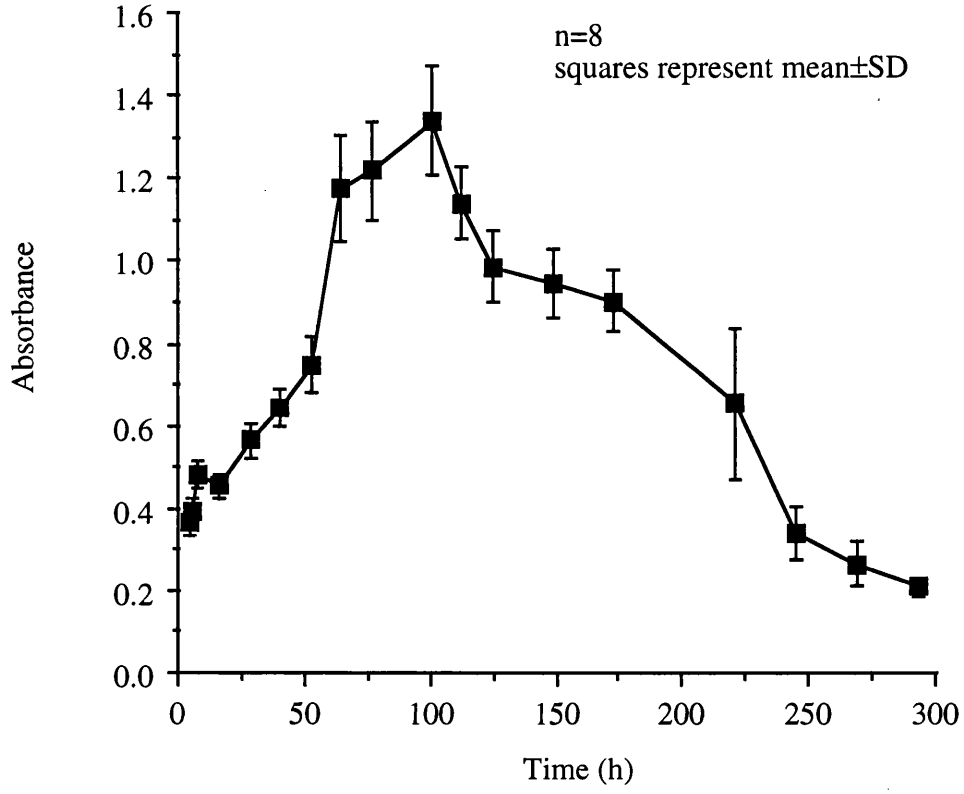


Figure 2.8 Growth curve for COR-L23 non-small cell lung carcinoma cell line

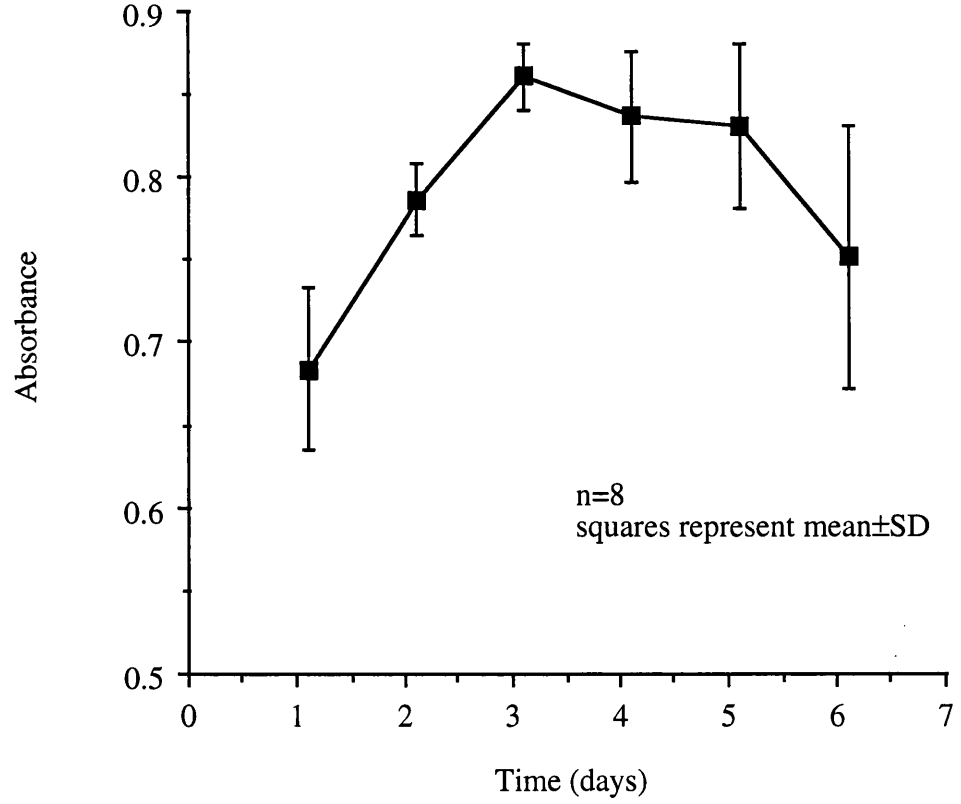
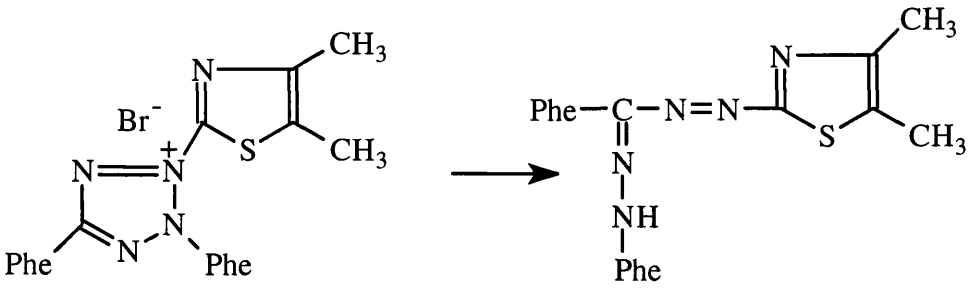


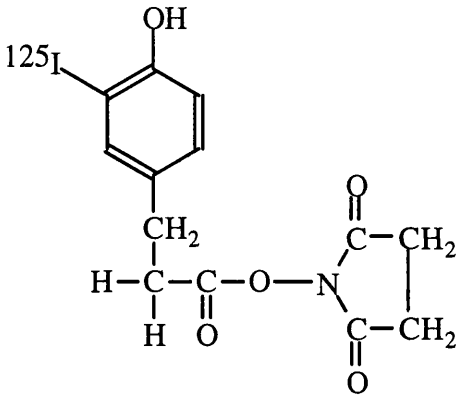
Figure 2.9 MTT reaction



MTT

MTT Formazan
(purple colour)

Figure 2.10 Bolton and Hunter reagent



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

and specific activity (μCi/mg) of the final labelled product were then determined by paper electrophoresis. Before injection into mice the final pH of labelled product was adjusted to 7.4 with diluted Hcl.

2.3.8 Purity assessment of iodinated products

Paper electrophoresis was used to determine the percentage of free [^{125}I]iodide in a ^{125}I-labelled enzyme preparation. This was done using paper electrophoresis. First 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 was used to soak the chromatography paper which was then blotted dry. The same buffer was then placed in a paper electrophoresis tank (Shandon) and the paper strips put in place across the supporting bars. The sample application point was nearest the anode. As a reference control, 4 μl of free Na[^{125}I] iodide was then loaded onto the origin of the first strip. Similarly, 4 μl of each product (crude reaction mixture and the purified preparation) were loaded onto individual paper strips. The Shandon tank was then connected to a power supply and the samples were run at 400 V for 30 min. Subsequently, the chromatography paper strips were removed and the marked 5 mm strips cut out, placed into counting tubes (Luckhams) with 1 ml DDW and assessed for radioactivity using a γ-counter. The results were then plotted as counts per minute (CPM) against distance migrated. The amount of free [^{125}I] iodide present was calculated and a maximum of 1% was allowed for injections *in vivo*. Specific activity was calculated by determining the CPM of the sample in 1 ml of known enzyme concentration and converting to μCi/mg assuming 100% recovery from dialysis (since the molecular weight of the enzyme is 30 KDa and the conjugate 60 KDa).

2.3.9 Body distribution of ^{125}I-labelled free and conjugated enzyme in mice

Male C57BL/6J mice were inoculated with 10^5 viable B16F10 cells subcutaneously (s.c.). The tumour was allowed to establish until the area was approximately 50-70 mm^2 as measured by the product of two orthogonal diameters. 5 x 10^6 counts were diluted into 0.5 ml in 0.9% sterile NaCl. The pH was then neutralised and the final volume was made up to 1.0 ml with 0.9% sterile NaCl. Of this sample, 100 μl were withdrawn using a 1 ml syringe and 12 gauge needle and counted as a measure of injected dose.

100 μl (5x10^5 CPM) of free or conjugated enzyme were injected into the tail vein of C57BL/6J mice (3 replicates per time point). The mice were then placed in a metabolism

cage and sacrificed at different time points up to 48 h. The following tissue samples were dissected: tumour, liver, kidneys, lung, heart, spleen and faeces, urine and blood were collected. Samples were weighed and homogenised in PBS. Samples (1 ml) were then counted in a γ-counter in replicates of 3 (three samples per organ per mouse).

The results were then expressed as % dose injected for each organ or % dose recovered for each organ. The blood volume of the mouse was calculated assuming 5.77 ml blood/100 g mouse (Dreyer and Ray, 1910).

2.3.10 Establishment of s.c. solid tumours from animal/human cell lines

All cells manipulations were carried out in a Class II Laminar flow cabinet using aseptic technique. The cells were washed in PBS and trypsinised as described in the cell manipulation method.

The cells were pipetted into one centrifuge tube. The flask was washed with 5 ml of saline and transferred to polypropylene tube. Then the tube was balanced with a blank and spun in the centrifuge at 1000 rpm for 10 min at 22^{0}C. The supernatant was removed and the cells were resuspended in 10 ml saline.

In order to estimate the viable cell density 20 µl of resuspended cells were withdrawn and mixed with 20 µl of Trypan Blue. 20 µl of sample were withdrawn and placed on the haemocytometer plate and placed under light microscope with a glass plate over it. The cells were counted using the counter for the four quarters and the average number of cells times $2x10^4$ is the amount of the viable cells. The final cell density was brought to 1 x 10^6 cells per 1 ml for the B16F10 model and 1 x 10^7 cells per 1 ml for the COR-L23 model.

The cells were placed into bijou and 0.1 ml of cells loaded into a 1 ml syringe fitted with a 25 gauge needle. (* the tube was always swirled before loading the cells into the syringe and the needle was always placed in the centre of the tube while loading).

Injections of C57 black male mice s.c. Each mouse was lightly anaesthetised. Flurothane was used at 2% with Oxygen at 4% until the animal fell unconscious, then the procedure was carried out immediately. 0.1 ml of cells in suspension were injected via

subcutaneous route into scruff of the mouse. The tumour usually reached palpable size (observable) after 10-12 days post injection.

2.3.11 Evaluation of pharmacokinetics of drug release in the PDEPT combination

Male C57BL/6J mice were inoculated with 10^5 viable B16F10 cells s.c. The tumour was allowed to establish until the area was approximately 50-70 mm^2 as measured by the product of two orthogonal diameters.

Animals were injected i.v. with PK1 (10 mg/Kg equivalent of doxorubicin) or PK1 (10 mg/Kg equivalent of doxorubicin) followed after 5 h by free cathepsin B (3.63 mg/Kg) or HPMA copolymer-Gly-Gly-cathepsin B (3.63 mg/Kg weight equivalent of cathepsin B) and sacrificed at different time points up to 48 h. The following tissue samples were dissected: tumour, liver, kidneys, lung, heart, spleen and urine and blood were collected. Samples were weighed, homogenised in PBS, mixed with 100 ng daunomycin (DNM), as an internal standard, ammonium formate buffer (pH 8.5) and extraction mixture (chloroform:propan-2-ol). Tubes were centrifuged, the aqueous layer was carefully removed and the organic fraction evaporated to dryness using the Techne nitrogen system operated at no more than 2 lbf/in^2 of $N_{2(g)}$. Evaporated samples were redissolved in methanol prior to HPLC analysis. The procedure is described in Figure 2.5.

2.3.12 Evaluation of antitumour activity of the PDEPT combination

Male C57BL/6J or male nu/nu mice were inoculated with 10^5 viable B16F10 cells or 10^6 viable COR-L23 cells s.c. respectively. The tumour was allowed to establish until the area was approximately 50-70 mm^2 as measured by the product of two orthogonal diameters.

Animals were injected i.v. with PK1 (10 mg/Kg equivalent of doxorubicin) or PK1 (10 mg/Kg equivalent of doxorubicin) followed after 5 h by free cathepsin B (3.63 mg/Kg) or HPMA copolymer-Gly-Gly-cathepsin B (3.63 mg/Kg weight equivalent of cathepsin B). 3 additional groups were saline (100 µl) as control, free doxorubicin (10 mg/Kg) and free cathepsin B (3.63 mg/Kg). Each group consisted of 5 mice. Animals were weighed and the tumour was measured daily. Mice were sacrificed when the tumour reached or surpassed the size of 289 mm^2. Animals were monitored for general health, weight loss,

tumour progression and at termination were examined by post-mortem, the tumours were dissected and weighed.

2.3.13 Evaluation of the immunogenicity of the polymer-enzyme conjugate

2.3.13.1 Protocol of immunisation

Free and conjugated enzymes, BSA and saline were administered to six different groups of Balb/C male mice (6 mice per group) to determine if there was a reduction of immunogenicity after conjugation of an enzyme to the HPMA copolymer. BSA was used as a positive control and saline as a negative control. Cathepsin B, β-lactamase and BSA solutions in saline (0.1 mg/ml) were prepared fresh prior to injections. Aliquots of HPMA copolymer-β-lactamase and HPMA copolymer-cathepsin B solutions (0.1 mg/ml enzyme concentration; determined by BCA assay) stored at -20^{0}C were thawed prior to injections. Saline was prepared fresh for the control group. Groups of 6 Balb/C mice were each injected s.c. on day 0 with 100 μl of these solutions, i.e. with the equivalent of 10 μg of enzyme. The procedure was repeated i.p. on days 14 (10 μg enzyme-equivalent) and 30 (50 μg enzyme-equivalent) for the secondary and tertiary responses respectively. The mice were exsanguinated on day 40. The immunisation protocol is shown in Figure 2.11. Blood was collected and immediately centrifuged at 3000 rpm for 10 min to separate the sera from blood cell components. The sera for each mouse was transferred to an eppendorf tube and stored at -70^{0}C until analysed. Control serum was prepared by injecting mice with saline according to the schedules described earlier.

Control sera (used to determine if there were any naturally occurring antibodies to the ligands used in this study) was prepared from unimmunised mice of the same strain.

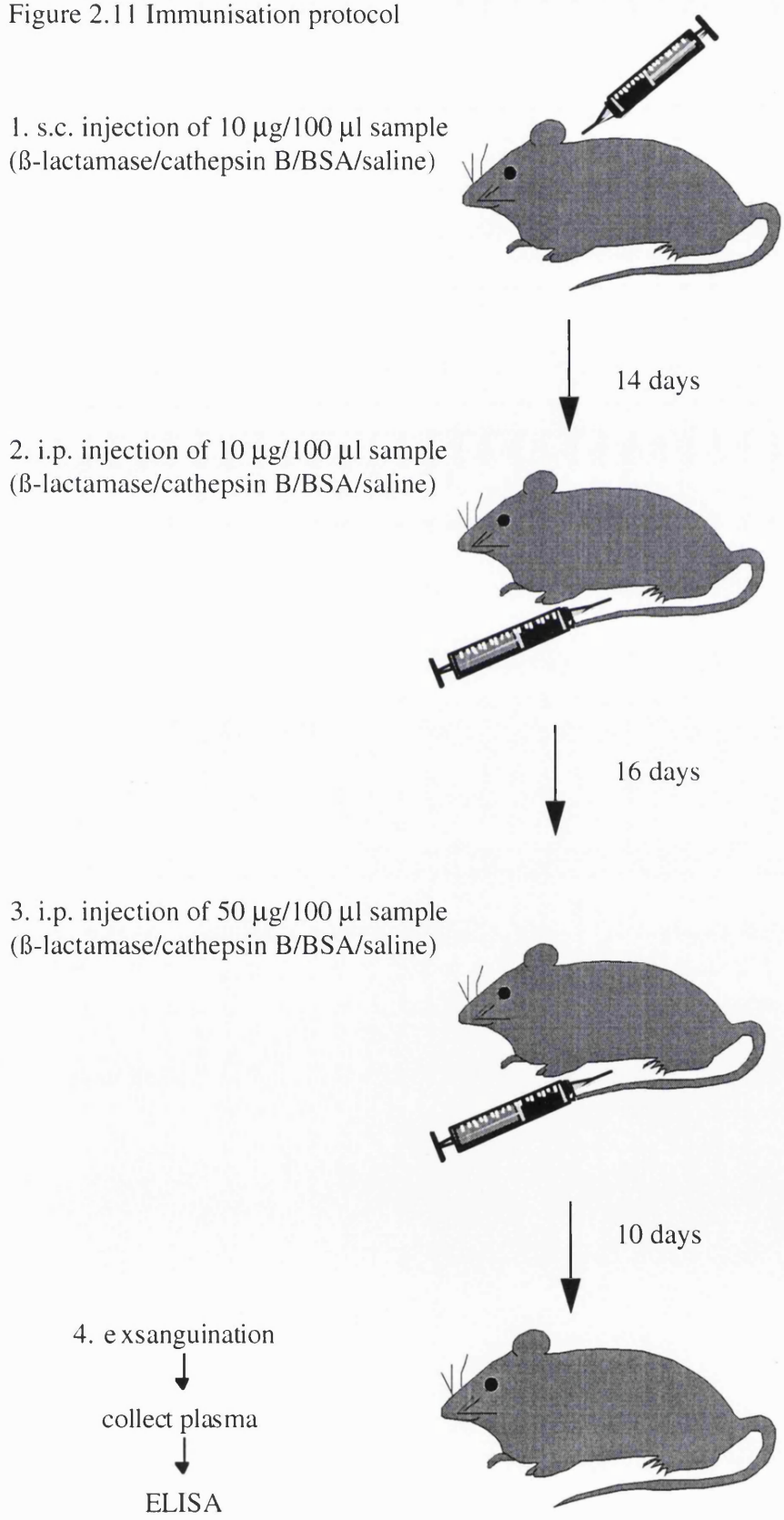
2.3.13.2 Serial dilution of sera

Serial dilution of sera was carried out immediately before use. Sera were serially diluted using PBS containing 0.025% Tween-20 (washing buffer). One volume of serum was mixed thoroughly with one volume of washing buffer. Of this 1:1 dilution one volume was taken and mixed thoroughly with one volume of washing buffer in the next well, and so on.

2.3.13.3 Estimation of antibody titre- ELISA assay

Ligand sample (60 μl, 2 μg/ml) was bound to microtitre 96 well plates overnight at 4^{0}C. Unbound ligand was removed from wells by suction and the plates were thoroughly washed with washing buffer (200 μl, pH 9.2 x 3 times). Blocking solution was added to

Figure 2.11 Immunisation protocol



each well in order to avoid non-specific binding and the plates left to stand at room temperature. This solution was removed after 1 h and again the plates were thoroughly washed as before. Serially diluted serum (2.2.13.2; 60 µl) was added to wells coated with corresponding ligand, starting with the lowest dilution (highest concentration), to produce a series of wells containing the same antigen (ligand) but different concentrations of antibody. The plates were then incubated (37^0C, 90 min) to allow antibody to interact with antigen. Sera was then removed and the plates thoroughly washed as above. Anti-(murine) IgG conjugated to horseradish peroxidase (50 µl, diluted 1:4000 in washing buffer) was then added to each well and the plates incubated again (37^0C, 1 h). Unreacted anti-(murine)IgG-horseradish peroxidase conjugate was removed from the plates, which were thoroughly washed as above. Orthophenylenediamine (substrate for horseradish peroxidase; 200 µl 500 µg/ml in phosphate buffer 0.1 M, pH 6.0 containing H_2O_2, 30%) was added to each well. The reaction was stopped after 1-5 min (depending on the intensity of colour) by addition of H_2SO_4 (25 µl, 1.8 M). The absorbance at λ max 492 nm was read in the Titertek Multiskan. The highest antibody dilution (HAD) at which a positive antibody-antigen reaction could be detected was noted. Each graph was drawn to show the HAD at which a positive antibody-antigen reaction could be detected on the ordinate, against the antigen used to coat the ELISA plate well.

Chapter Three

Synthesis, properties and in vitro characterisation of polymer-enzyme conjugates

3.1. Introduction

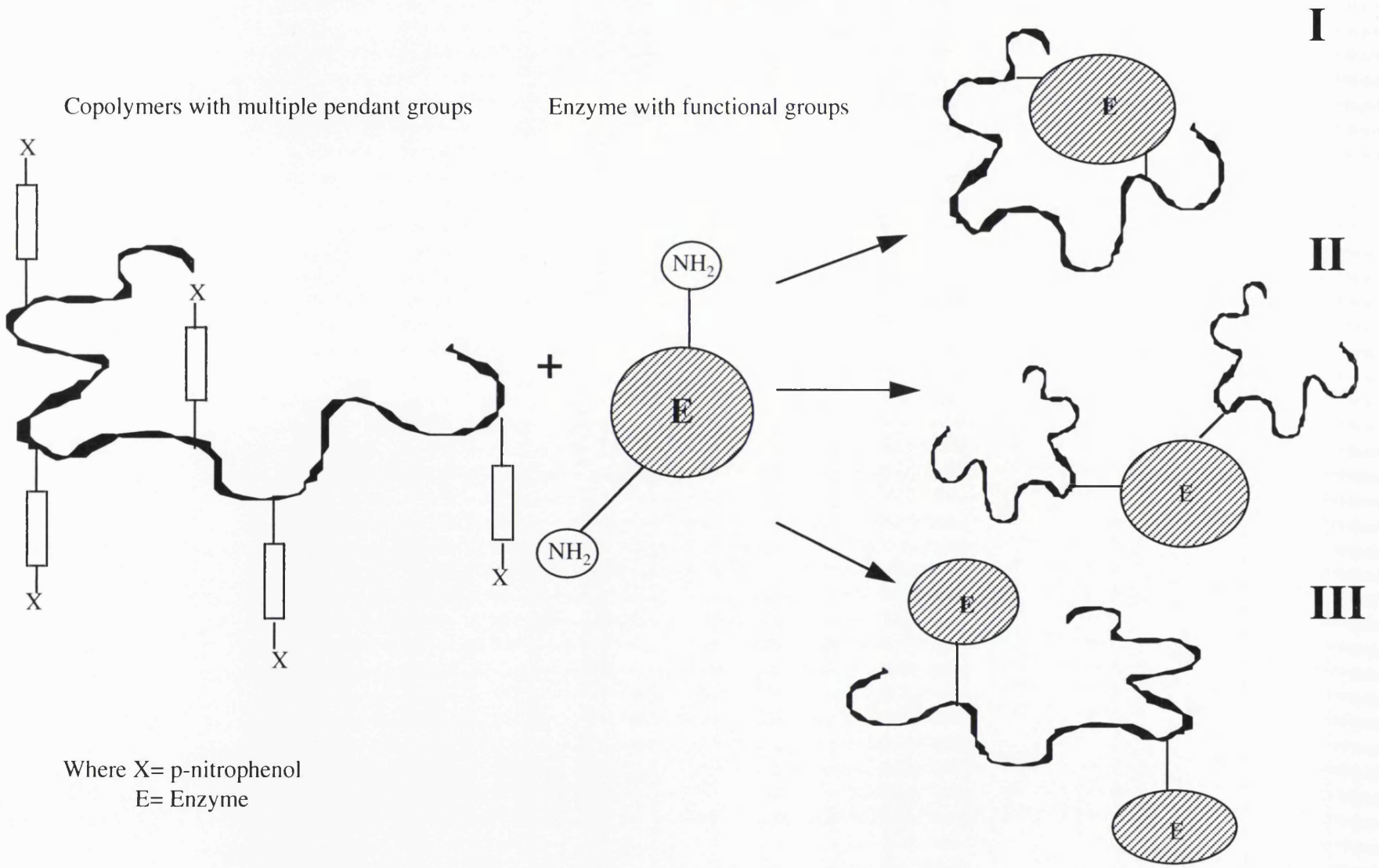
An important factor in the development of a PDEPT system is ability to synthesise polymer-enzyme conjugates which retain enzymatic activity. Many techniques have already been described for polymer-protein conjugation and several can maintain the activity of the bound protein. In addition, a number of different polymers have been used to prepare conjugates. Table 3.1 summarises typical polymer-enzyme conjugates described in the literature. Chemical modification of enzymes with synthetic and natural polymers has resulted in new products in the field of medicine (reviewed in Putnam and Kopecek, 1995b). Basically two methods are used for conjugation. The first is to use a monofunctional polymer, typically with one reactive end group, e.g. monomethoxy poly(ethylene glycol) (PEG) (Kamisaki *et al.*, 1981), and end functionalised semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) (Lu *et al.*, 1998). The second method uses multifunctional polymers which allows conjugation of polymer to protein at several different sites along the polymeric backbone, e.g. HPMA copolymer. Figure 3.1 shows some of the possible products that can arise following conjugation between a multifunctional polymer and a protein.

PEG has been the most widely used for polymer-protein conjugation (reviewed in Fuerteges and Abuchowski, 1990; Duncan and Spreafico, 1994). An important reason for its attractiveness is the fact that PEG is already approved by Regulatory Authorities for use in many pharmaceutical products. Variation in the molecular weight of the PEG used, the chemistry employed for conjugation and the extent of surface modification of the protein all provide a means to tailor-make specific PEG-protein products. Many different techniques have been described for PEG conjugation (reviewed in Delgado *et al.,* 1992) and have broadened the applications of many proteins. Decreased immunoreactivity and/or immunogenicity and increased stability *in vivo* have been described for PEG-modified proteins (Abuchowski *et al.*, 1977a; 1977b). Many of the resultant PEGylated proteins have been shown to have a modified pharmacokinetics profile (reviewed in Katre, 1993), and several are being developed as potential therapeutics as for instance the PEG-L-asparaginase (Kurzberg *et al.*, 1988) for the treatment of acute lymphocytic leukaemia and PEG-adenosine deaminase for the treatment of severe combined immunodeficiency associated with adenosine deaminase deficiency (Hershfield *et al.*, 1987). Using optimised techniques, functionalised PEGs have been conjugated to proteins, forming bioconjugates with altered physicochemical or biological properties without destroying completely the original protein function. Steric hindrance did however, frequently decrease the biological activity of the bound protein. In the case of PDEPT, where the polymer-bound enzyme must cleave a linker between the polymer-drug conjugate which is its substrate, there is considerable

Table 3.1 Examples of polymer-enzyme conjugates

Polymer	Enzyme	References
Polyethyleneglycol		
	Arginase	Savoca *et al.*, 1979
	Asparaginase	Ashihara *et al.*, 1978
	Bilirubin Oxidase	Maeda *et al.*, 1992
	Catalase	Abuchowski *et al.*, 1977b
	Chymotrypsin	Chiu *et al.*, 1993
	Elastase	Koide and Kobayashi, 1983
	β-Galactosidase	Wieder and Davis, 1983
	β-Glucuronidase	Lisi *et al.*, 1982
	Glutaminase	Abuchowski *et al.*, 1981
	Lipase	Baillargeon and Sonnet, 1988
	Phenylalanine ammonia lyase	Wieder *et al.*, 1979
	Purine nucleoside phosphorylase	Hershfield *et al.*, 1991
	Ribonuclease A	Caliceti *et al.*, 1990
	Streptokinase	Rajagopalan *et al.*, 1985
	Superoxide Dismutase	Pyatak *et al.*, 1980
	Trypsin	Abuchowski and Davis, 1979
	Urate oxidase	Chen *et al.*, 1981
	Uricase	Abuchowski *et al.*, 1981 Yasuda *et al.*, 1990
Dextran		
	Asparaginase	Benbough *et al.*, 1979
	Carboxypeptidase G	Sherwood *et al.*, 1977
	Catalase	Marshall, 1978
	Soybean trypsin inhibitor	Takakura *et al.*, 1989
	Uricase	Fujita *et al.*, 1990
Poly(N-vinylpyrrolidone)	β-D-N-acetylhexosaminidase A	Geiger *et al.*, 1977
Styrene-maleic anhydride		
	Neocarzinostatin	Konno *et al.*, 1983
	Superoxide dismutase	Ogino *et al.*, 1988
HPMA copolymers		
	Acetylcholinesterase	Laane *et al.*, 1983
	Chymotrypsin	Laane *et al.*, 1981
Divinylether-maleic acid		
	Superoxide dismutase	Maeda *et al.*, 1988
	Neocarzinostatin	Yamamoto *et al.*, 1990

Figure 3.1 Theoretical conjugation possibilities between HPMA copolymers and enzymes



Where X= p-nitrophenol
E= Enzyme

potential for steric hindrance and it was clear that the HPMA copolymer-enzyme conjugates prepared must maintain as much activity as possible.

HPMA copolymers were chosen for our present study because they are water-soluble and biocompatible polymers already in clinical trial. HPMA copolymer-doxorubicin has completed Phase I clinical trial (Vasey *et al.*, 1999). They were originally developed by Kopecek in the early seventies for use as blood plasma expanders (Kopecek and Bazilova, 1973). Later they were developed as carriers of anticancer agents by attachment of the drug to the polymer chain through a peptidyl spacer pendent to the polymer backbone (Duncan and Kopecek, 1984; Kopecek *et al.*, 1985; Kopecek and Duncan, 1987). Oligopeptide side chains incorporated into HPMA copolymers are also suitable for protein attachment, binding of targeting residues and introducing degradable cross-links that are hydrolysed following pinocytic capture of the polymeric carrier (Duncan *et al.*, 1982, Putnam and Kopecek, 1995b).

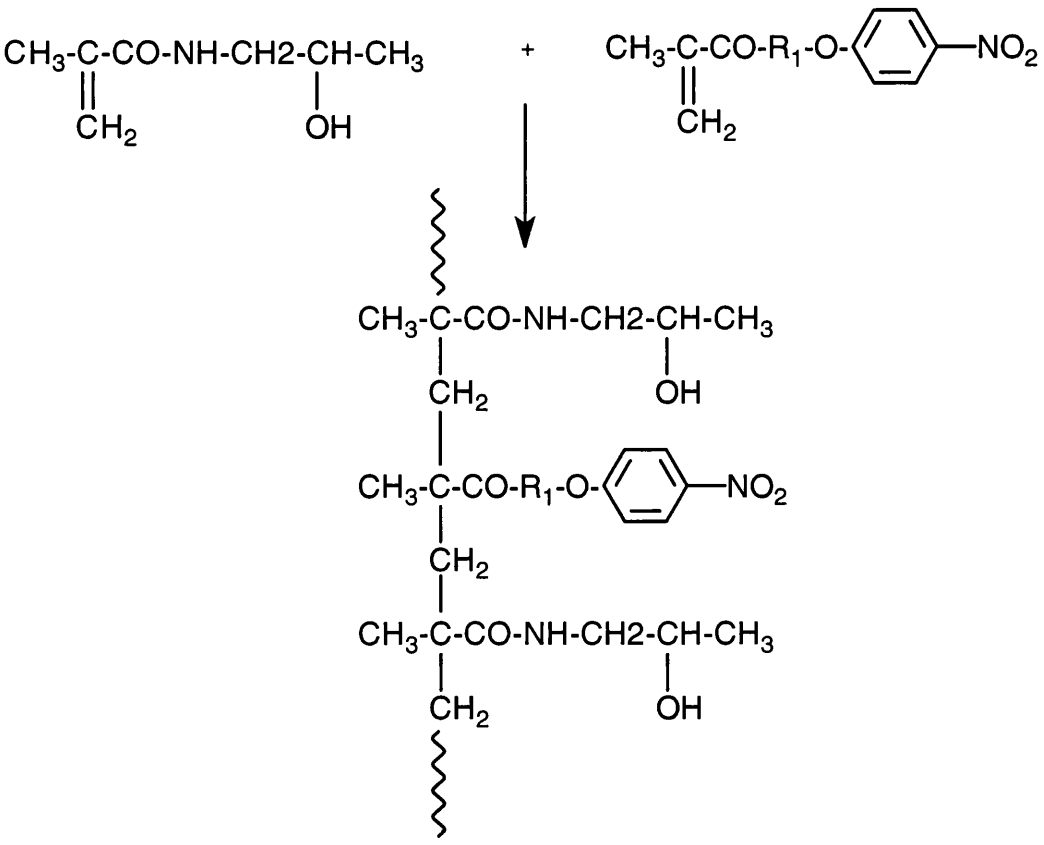
HPMA copolymers have been successfully used for modification of antibodies (Rihova and Kopecek, 1985), transferrin (Flanagan *et al.*, 1992), insulin (Morgan *et al.*, 1996), and chymotrypsin (Ulbrich and Oupicky, 1997). For example, the conjugation of PHPMA to chymotrypsin did not influence significantly the substrate specificity and the enzymatic activity toward low molecular weight substrates (free chymotrypsin against HCl.Gly-Gly-Phe-NAp gave K_2/K_m=335 *vs* PHPMA-chymotrypsin giving K_2/K_m=218) but reduced its activity towards high molecular weight substrate, PEG-Gly-Val-Phe-NAp (23%-43% activity retention compared to the native chymotrypsin depending on the degree of modification). In the case of the high molecular weight substrate BSA, all PHPMA-chymotrypsin conjugates were practically inactive due to steric hindrance. In contrast, conjugation of PHPMA with bovine seminal ribonuclease increased the enzymatic activity and the proteolytic stability of the enzyme.

Design of HPMA copolymer-protein conjugates

In this study HPMA copolymers, prepared by free radical polymerisation to contain N-methacryloylated-Gly-Gly-ONp (Figure 3.2), were used to synthesise HPMA copolymer-Gly-Gly-cathepsin B and HPMA copolymer-Gly-Gly-β-lactamase conjugates.

The aim was to prepare HPMA copolymer-enzyme conjugates with a copolymer:protein ratio of 1:1. This should minimise the conjugate size and so achieve maximum extravasation and penetration of the target tumour. Larger aggregates, even if soluble, would tend to be rapidly taken up by cells of the RES. It is known (Melton,

Figure 3.2 Radical polymerisation of HPMA with p-nitrophenyl esters of N-methacryloylated oligopeptides



1996), that too high levels of substitution tend to decrease enzymatic activity and increase risk of formation of high molecular weight aggregates. Thus it was essential that the correct balance should be found between ensuring linkage, but retaining activity. One potential disadvantage of using HPMA copolymers containing multiple reactive ONp groups is the possibility of multiple attachments to a single enzyme molecule that might interfere with the enzyme active site and could eventually lead to cross-linking and total inactivation of conjugated enzyme (Figure 3.1). For this reason, determination of the optimal conditions for conjugation of a multifunctional HPMA copolymer to a protein is more of a challenge than seen when reacting a protein with a polymer, like PEG or semitelechelic PHPMA, with only one reactive end group.

The inherent structure and properties of a protein are the most important factors in determining the conjugation method selected. The nature of the functional groups in the protein, their availability and reactivity will influence the choice of a suitable polymer derivative. It should be stressed that the reactivity of a functional group ($-NH_2$; -COOH) is influenced by its location within the protein macromolecule. The unique folding of a protein chain can be responsible for masking functional groups, making them less accessible to a small or greater extent. Consequently the variation in the yield of polymer-protein conjugate using identical modification chemistry can be significant when different proteins are used.

One has to consider both the functional groups available for linkage; that on the copolymer and the reactive groups on an enzyme. For the preparation of polymer-modified protein, we used HPMA copolymers with ONp groups linked to the polymer main chain through a dipeptide glycine-glycine spacer. The HPMA copolymers used for enzyme conjugate in these experiments contained 5 mole % of Gly-Gly-ONp groups. The Gly-Gly peptidyl spacer is not enzymatically degraded in the lysosome (Duncan *et al.*, 1981 and reviewed by Soyez *et al.*, 1996). The conjugation reaction was aminolysis of p-nitrophenyl ester (ONp) groups of synthetic polymer molecules by NH_2 groups of cathepsin B or β-lactamase (Chapter 2, Figure 2.1). The effects of various parameters on conjugation reactions were studied including time, pH, ratio between synthetic polymers and the protein and solvent.

The aim of this study was to synthesise HPMA copolymer-enzyme conjugates whilst achieving: high yield of the reaction, retention of activity of the conjugated enzyme and to prepare reproducible batches of material.

3.2 Methods

HPMA copolymer-enzyme conjugates synthesis

The preparation of the HPMA copolymer-enzyme conjugates was carried out in an aqueous medium and thus aminolysis and hydrolysis occur as competing reactions. These two reactions are shown in Figure 3.3. As the concentration of hydroxyl group and deprotonated amino groups, required for hydrolysis and aminolysis respectively, depend on the pH, it was important to determine solvents and pH conditions where the rate of aminolysis greatly exceeded that of hydrolysis and thus maximised the yield of the amide intermediate. However, one should bear in mind that the activity of the enzyme is essential in this conjugation synthesis and extreme and rapid changes in pH might denaturate the enzyme.

The reaction was followed by UV as a difference in absorption was observed between the ONp groups bound to HPMA copolymers (270 nm) and the free ONp groups (400 nm). The reaction products were routinely analysed by SDS electrophoresis. Although attempts were also made to characterise the polymer-enzyme conjugates by Gel Permeation Chromatography (GPC) and mass spectrometry, unfortunately these experiments failed. One can produce a calibration curve for different molecular weight of polymers with good linear correlation and a calibration curve of different enzymes with different molecular weights with good linearity (data not shown), but there is no appropriate standard that can be used for polymer-enzyme conjugates *per se* and it proved impossible to relate the retention time of the final polymer-enzyme to either the polymer or enzyme standard curves so the data is not shown here. Mass spectrometry of HPMA copolymer itself is acknowledged to be very difficult to interpret. The picture obtained for the HPMA copolymer-enzyme conjugate only added to the confusion due to the molecular weight (~ 30 KDa) and polydispersity.

HPMA copolymer-enzyme conjugates were prepared, purified and characterised using the methods described in Chapter 2.3.1, 2.3.2 and 2.3.3.

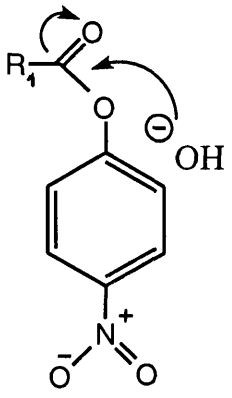
3.3 Results

3.3.1 Stability of HPMA copolymer-Gly-Gly-ONp in different solvents

During HPMA copolymer-protein conjugation using an aminolysis reaction the problem is to achieve aminolysis by the amino groups of the enzyme (necessarily in aqueous solution to preserve enzymatic activity) while minimising hydrolysis of the ONp reactive esters. To study the effects of buffers and solvents on the reaction, several options were tested.

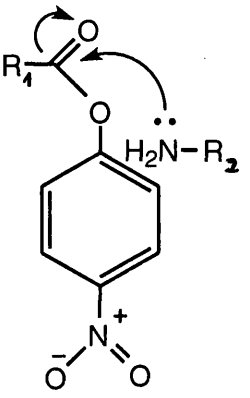
Figure 3.3 Nucleophilic attack: hydrolysis compared with aminolysis

Hydrolysis



R_1=HPMA copolymer-Gly-Gly

Aminolysis



R_2=ß-lactamase or cathepsin B

First, HPMA copolymer-Gly-Gly-p-nitrophenol was dissolved in sodium phosphate buffer pH 7.2 and left in the UV cuvette for 45 min. From the spectra, shown in Figures 3.4 and 3.5, it can be seen that a rapid release of ONp by hydrolysis occurred. This would compromise the aminolysis required to link the enzyme to the copolymer.

A second solvent tested was DMSO and although HPMA copolymer-Gly-Gly-ONp (1 mg/ml) appeared to be stable in these conditions (Figure 3.6), and later a conjugate was obtained (yield 40%), the activity of the enzyme was completely lost.

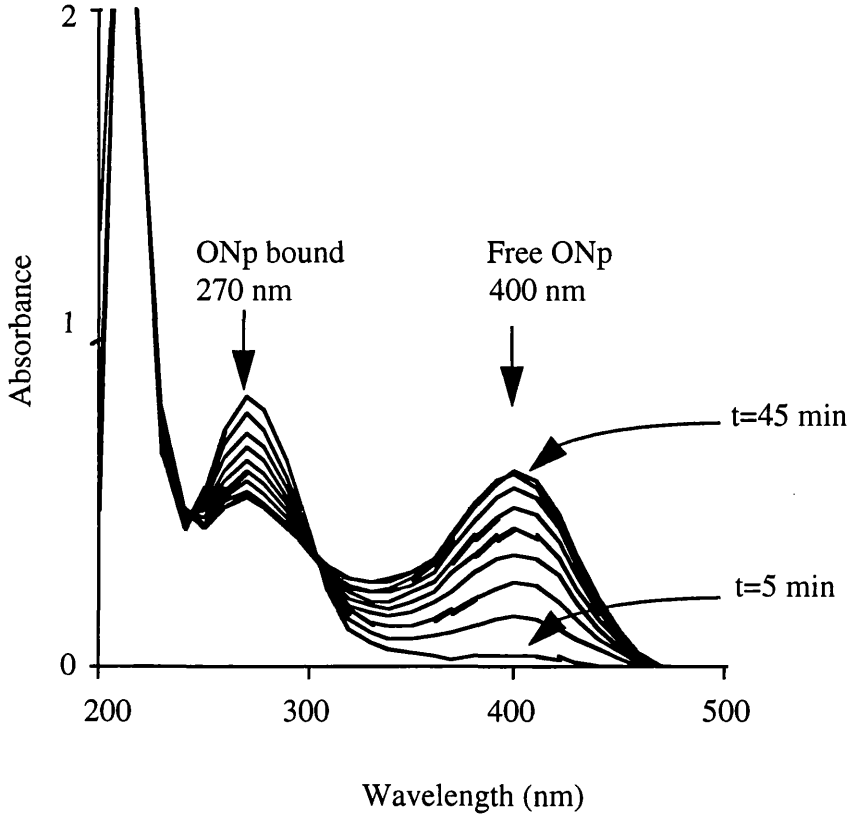
The pH of the reaction mixture had to be raised by addition of saturated borate buffer (as detailed in Chapter 2.3.1). Figure 3.7 demonstrates rapid hydrolysis of HPMA copolymer-Gly-Gly-ONp when dissolved directly in this buffer (1 mg/ml). It was possible, though, to add small, gradual aliquots to bassify the reaction mixture under controlled conditions.

Finally, HPMA copolymer-Gly-Gly-ONp was dissolved in DDW in different concentrations and no release of ONp was observed overnight (Figure 3.8). Both enzymes need a buffered solvent to maintain their activity, and they were dissolved in phosphate buffer, pH 7.2, 0.05 M. The conjugation was finally achieved by mixing aqueous HPMA copolymer solution with slow addition of buffered enzyme solution while raising pH as described in chapter 2.3.1. The conjugation reactions of HPMA copolymer to β-lactamase and cathepsin B were followed by UV/VIS spectrophotometer and are shown in Figure 3.9 and Figure 3.10 respectively. Determination of conjugate present in the mixture was then achieved by the use of SDS PAGE as described in Chapter 2.3.3.3.

3.3.2 Optimisation of synthesis and purification of polymer-enzyme conjugates

Having selected the reaction buffers it was necessary to optimise the synthesis according to the polymer:enzyme ratio and the reaction time. Different ratios of polymer-enzyme combinations were used and different reaction times were tested. Most of them resulted in no conjugate or in an inactive conjugate as shown in Table 3.2 for HPMA copolymer-Gly-Gly–β-lactamase and Table 3.3 for HPMA copolymer-Gly-Gly-cathepsin B. The ratio chosen for HPMA copolymer: β-lactamase was 1:2 and for cathepsin B 1:1 respectively. As seen from the tables longer reaction time resulted in higher yields, but reduced or no activity. Leaving the mixtures to react overnight would give a yield of 63-82%, but when tested for activity against the low molecular weight

Figure 3.4 Spectra of HPMA copolymer-Gly-Gly-ONp in PBS (1 mg/ml)



* Spectra recorded every 5 min for 45 min

Figure 3.5 Hydrolysis of HPMA-Gly-Gly-ONp in PBS (1 mg/ml)

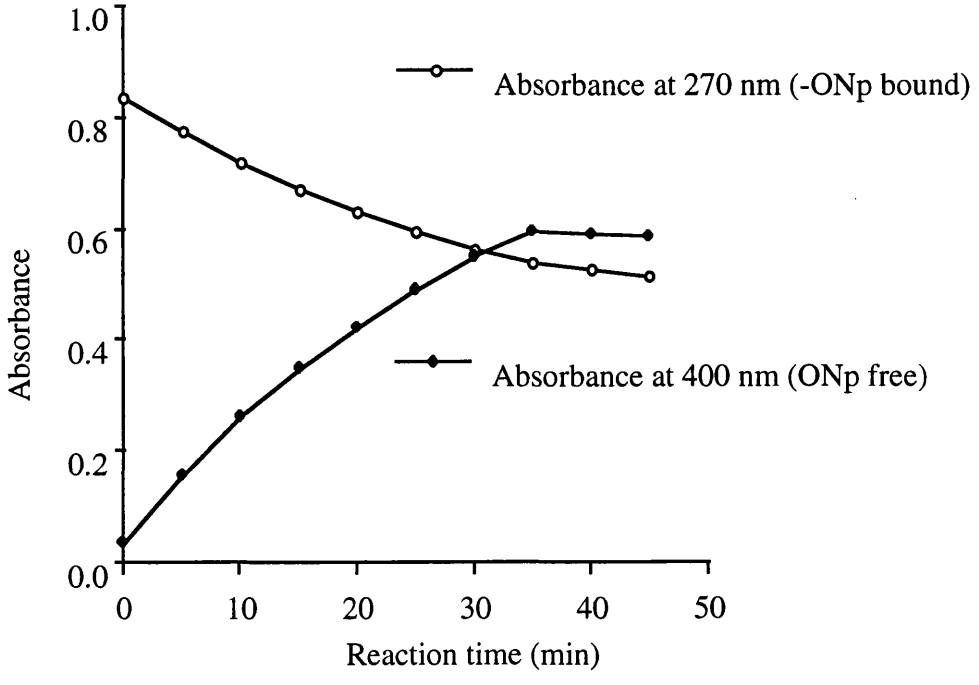
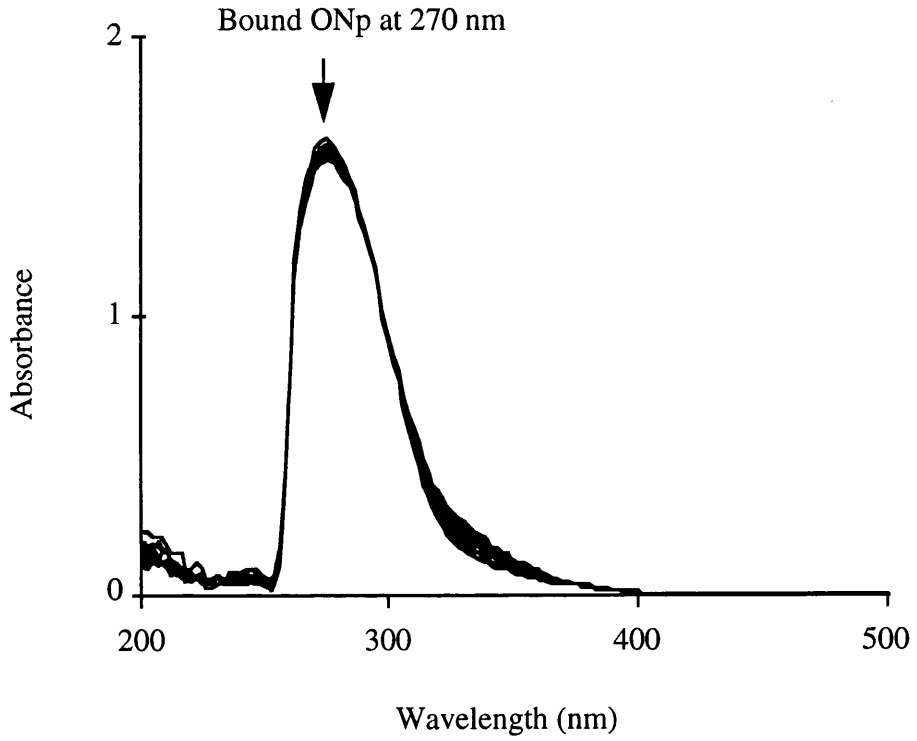
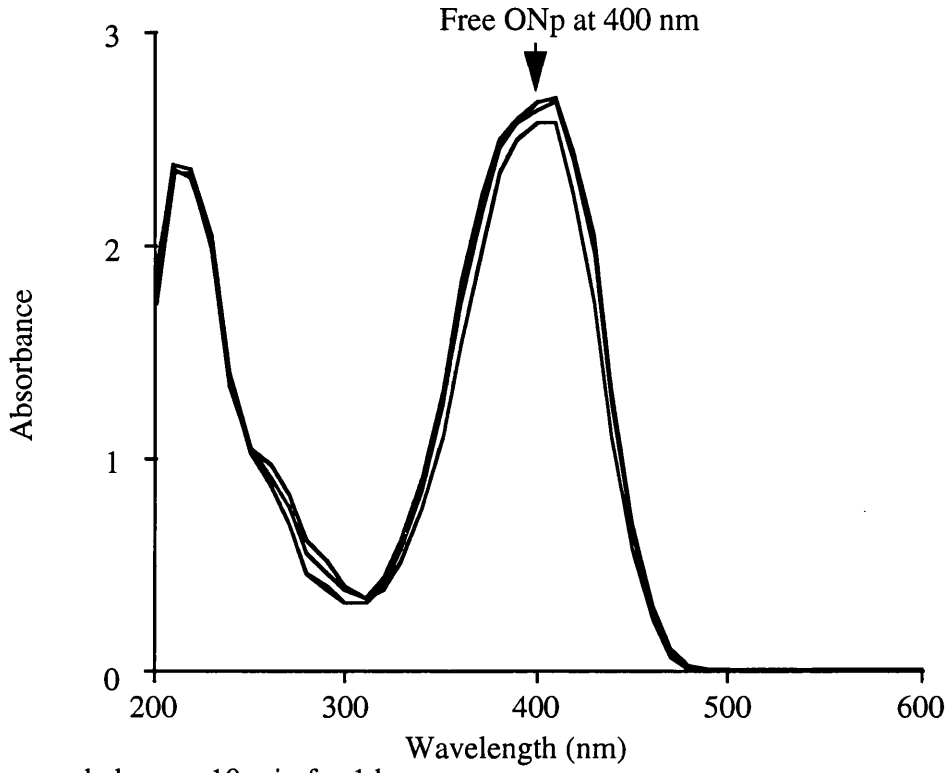


Figure 3.6 Spectra of HPMA copolymer-Gly-Gly-ONp in DMSO (1mg/ml)



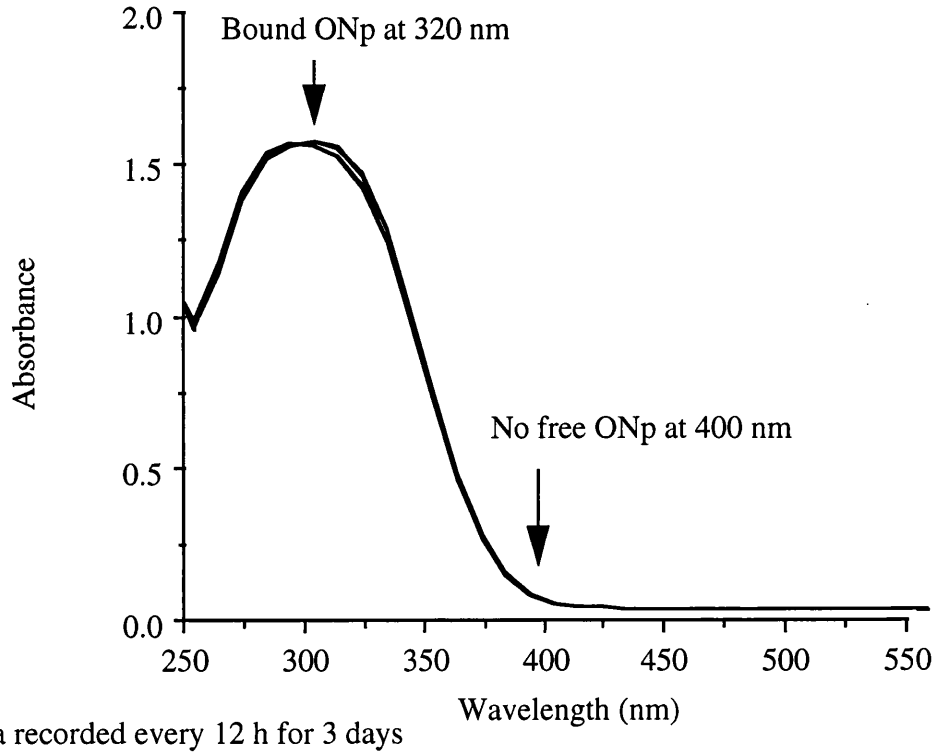
* Spectra recorded every 10 min overnight

Figure 3.7 Spectra of HPMA copolymer-Gly-Gly-ONp in borate buffer, pH 8.5 (1 mg/ml)



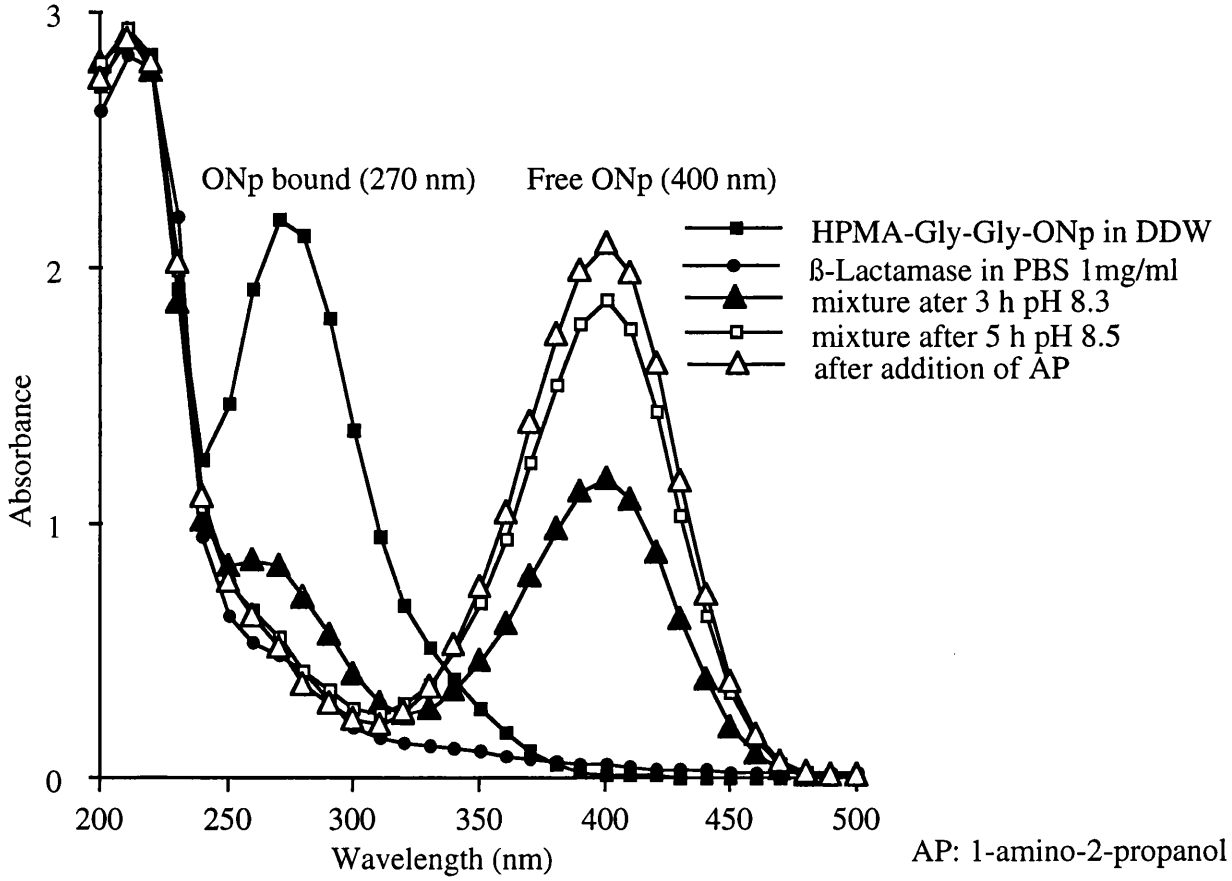
* Spectra recorded every 10 min for 1 h

Figure 3.8 Spectra of HPMA copolymer-Gly-Gly-ONp in DDW (1 mg/ml)



* Spectra recorded every 12 h for 3 days

Figure 3.9 Spectra following the reaction mixture of HPMA-Gly-Gly-ONp and ß-lactamase



AP: 1-amino-2-propanol

Figure 3.10 Spectra following the reaction mixture of HPMA-Gly-Gly-ONp and cathepsin B

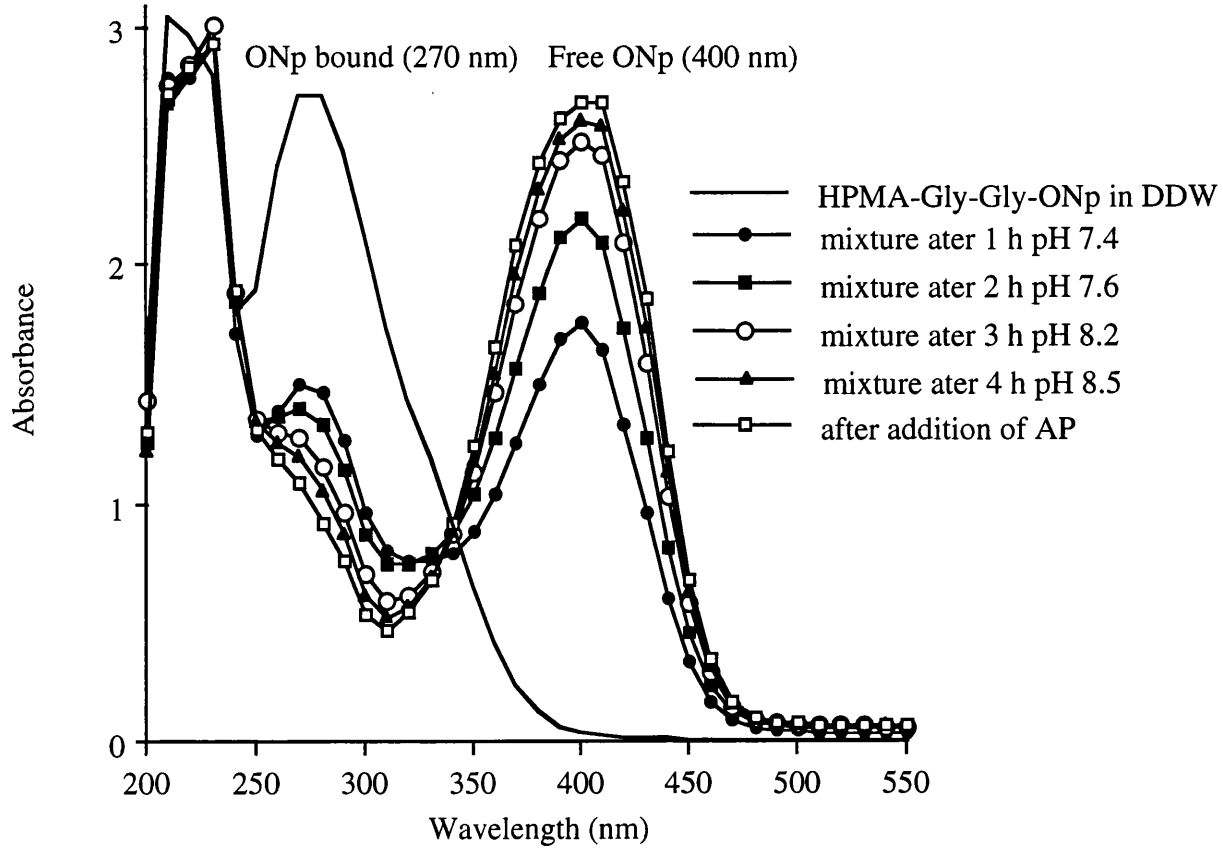


Table 3.2 Examples of variables examined in synthesis of HPMA copolymer-β-lactamase

Batch	HPMA copolymer-Gly-Gly-ONp: β-lactamase ratio	Reaction Time (h)	Conjugate	Yield (%)	% Activity retained against benzylpenicillin
RS-βL-1	1 : 1	12	+	10	40
RS-βL-3	1 : 2	12	+	35	20
RS-βL-5	2 : 1	12	-	-	-
RS-βL-6	1 : 4	12	-	-	-
RS-βL-9	1 : 8	12	-	-	-
RS-βL-15	1 : 2	1 (immediate increase to pH 8.5)	-	-	-
RS-βL-17	1 : 2	4	-	-	-
RS-βL-18	1 : 2	8	+	30	33
RS-βL-21	1 : 2	12	+	63	7
RS-βL-23	1 : 2	24	+	82	-
RS- βL DMSO	1 : 2	8	+	40	-

* All mixtures were prepared in the dark, at 4^0C while stirring.

Table 3.3 Examples of variables examined in synthesis of HPMA copolymer-cathepsin B

Batch	HPMA copolymer-Gly-Gly-ONp: cathepsin B ratio	Reaction Time (h)	Conjugate	Yield (%)	% Activity retained against Bz-Phe-Val-Arg-NAp
RS-cat-1	1 : 1	1 (immediate increase to pH 8.5)	-	-	-
RS-cat-2	1 : 1	4	-	-	-
RS-cat-3	1 : 1	8	+	35	25
RS-cat-4	2 : 1	8	-	-	-
RS-cat-5	1 : 1	12	-	50	-
RS-cat-8	1 : 1	24	+	67	-

* All mixtures were prepared in the dark, at 4^0C while stirring.

substrates, no enzymatic activity was retained. The optimal reaction time for both enzymes was 8 h, divided between 4 h for raising the pH to 8.5 and 4 h to react thereafter at 4^0C with stirring.

The dialysis membrane, chosen for purification of the reaction mixture had a marked effect on the quantity of the recovered HPMA copolymer-enzyme conjugate. Spectra/Por cellulose ester, gave a recovery of 30-35% while the Centriprep-50, used initially, gave a non-reproducible recovery of product typically 5-10% due to the enzyme's potential to stick to the filter. Both membranes had a cut-off size of 50 KDa.

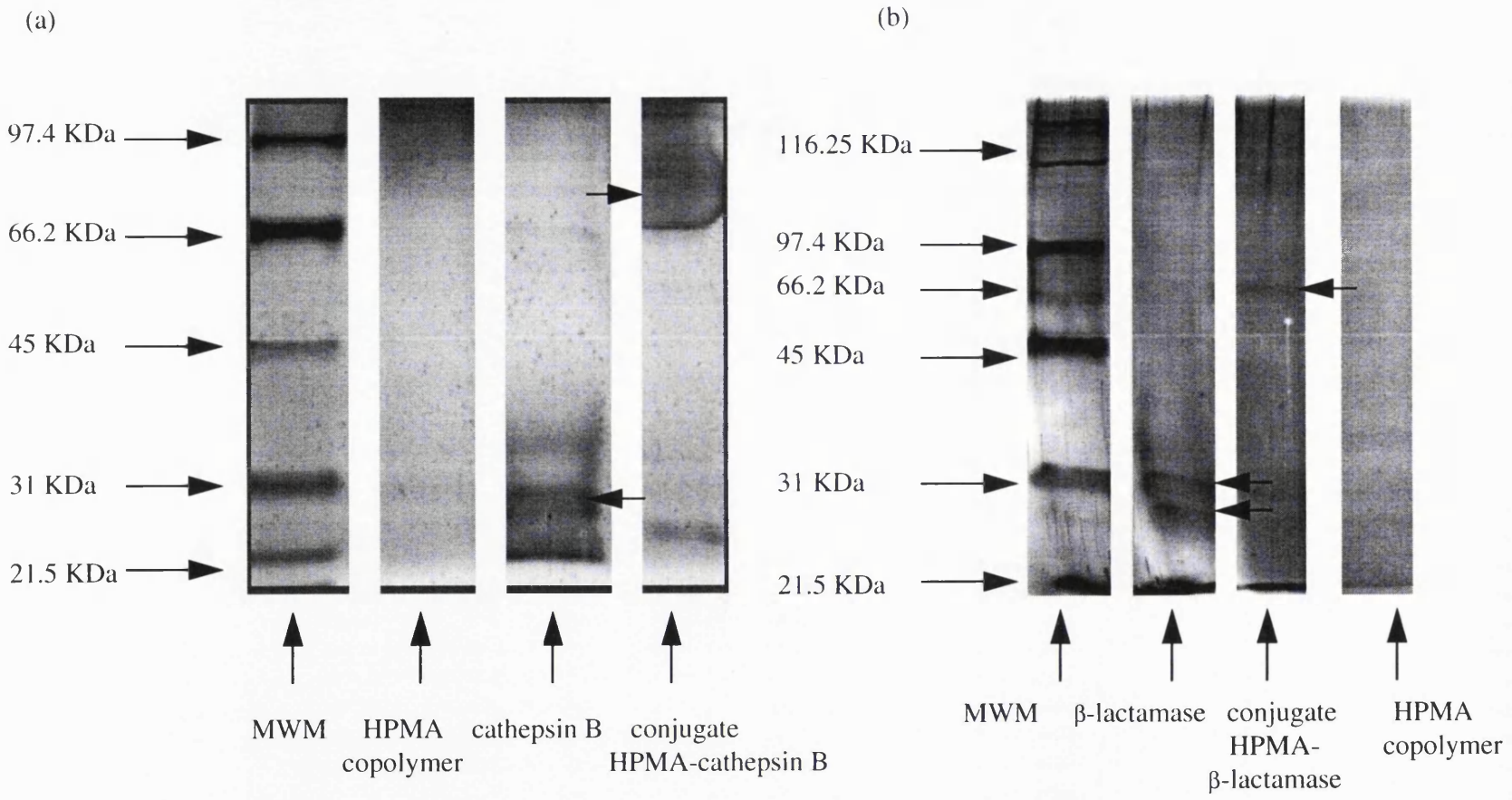
3.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

HPMA copolymer-Gly-Gly-cathepsin B and HPMA copolymer-Gly-Gly-β-lactamase conjugates were prepared using the method described in Chapter 2.3.2. SDS PAGE was undertaken in order to show that the release of ONp observed in the enzyme conjugate preparation (by following with the UV spectrometry) was due to aminolysis with concomitant formation of conjugate and not hydrolysis by the phosphate buffer. Free enzyme was compared to purified product relating both to a series of molecular weight markers used to calibrate a gel. Figure 3.11 shows that unreacted HPMA copolymer, as expected, gave no band with the silver stain. Free β-lactamase gave two bands coincident with the 30 KDa molecular weight marker due to the two types of enzyme present (type I and type II). Free cathepsin B gave a band coincident with 30 KDa. HPMA copolymer-β-lactamase gave a band corresponding 60 KDa. Similarly, HPMA copolymer-cathepsin B conjugate gave a band coincident with ~60 KDa and ~97 KDa (due to cross linking). After purification no free enzyme was detected. If a free enzyme was detected, dialysis was repeated for a longer time.

3.3.4 Amine quantification

To check the yield of aminolysis and hence conjugation levels, an assay for the determination of terminal amines had to be designed. As the spacer is made up of amino acids and thus containing amide bonds, the assay had to be designed such that only primary amines are detected. The colourimetric method using ninhydrin is described in details in chapter 2.3.3.1 and was used to determine the amino groups available on the enzymes for conjugation to the polymer. Relating the results obtained from the assay to the calibration curve shown in Chapter 2, β-lactamase has 18 ± 1 NH_2 functional groups available for conjugation. HPMA copolymer-Gly-Gly-ONp has 15 ONp groups available for conjugation.

Figure 3.11 SDS PAGE analysis of (a) HPMA copolymer-cathepsin B and (b) HPMA copolymer-β-lactamase



MWM: molecular weight markers

3.3.5 BCA assay- yield of conjugates

The results of a typical bicinchoninic acid assay to determine the yield of a polymer-enzyme conjugates are shown in Figure 3.12 for HPMA copolymer-Gly-Gly-cathepsin B, and Figure 3.13 for HPMA copolymer-Gly-Gly-β-lactamase. The yields for an active conjugated cathepsin B were 30-35%. The yields for an active conjugated β-lactamase were 28-32%. It was found inappropriate to use BSA for a standard calibration curve and standard curves were established for the individual enzymes.

3.3.6 Activity assays against low molecular weight substrates

β-Lactamase

The activity assay was performed using the same concentration of enzyme in both the free and conjugated enzyme activity test (2.63 mg/ml). The enzymatic activity was determined using free benzylpenicillin as the substrate being the linker in HPMA-penicillin-drug. Figure 3.14 demonstrates the degradation of the β-lactam ring in benzylpenicillin by free or conjugated β-lactamase. The results showed that the β-lactamase retained enzyme activity after polymer conjugation: reduced but active enough to cleave the spacer in the final form of PDEPT.

Michaelis-Menton kinetic parameters were calculated from the above assay based on the use of free enzyme compared to the conjugated one, in constant concentration (2.63 mg/ml), on different concentrations of benzylpenicillin (1.0 mM, 0.9 mM, 0.75 mM, 0.5 mM, 0.33 mM). The change in absorbance was calculated over time until a plateau was reached. The linear part of the degradation of the different substrate concentrations was plotted and from the slope Vo was calculated and the Lineweaver-Burk graph was plotted (Figure 3.15). β-Lactamase retained enzyme activity after polymer conjugation giving Vmax 0.2 nM/sec/unit and K_m 0.07 mM compared to Vmax 0.77 nM/sec/unit and K_m 0.022 mM.

Cathepsin B

An HPMA copolymer-Gly-Gly-cathepsin B conjugate was prepared with a yield of 35.4% enzyme bound (based on the BCA assay). UV analysis of Bz-Phe-Val-Arg-NAp following incubation with free or conjugated cathepsin B showed increasing release of NAp from the tripeptide over 0.5 h and 3 h respectively (Figure 3.16). The initial velocities were determined from the linear portions obtained from plots of absorbance in 410 nm. Comparison of initial rates of release shows that conjugated cathepsin B retained 24.4% of its activity against the low molecular weight substrate Bz-Phe-Val-Arg-NAp.

Figure 3.12 BCA assay for cathepsin B in HPMA copolymer-cathepsin B conjugate

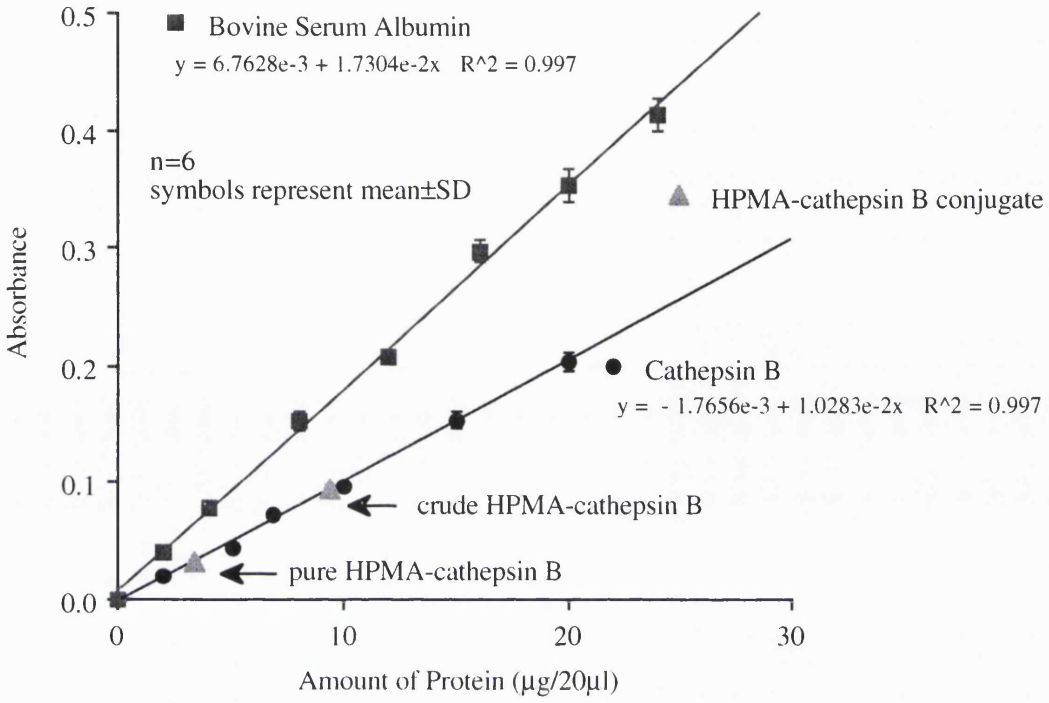


Figure 3.13 BCA assay for ß-lactamase in HPMA copolymer-ß-lactamase conjugate

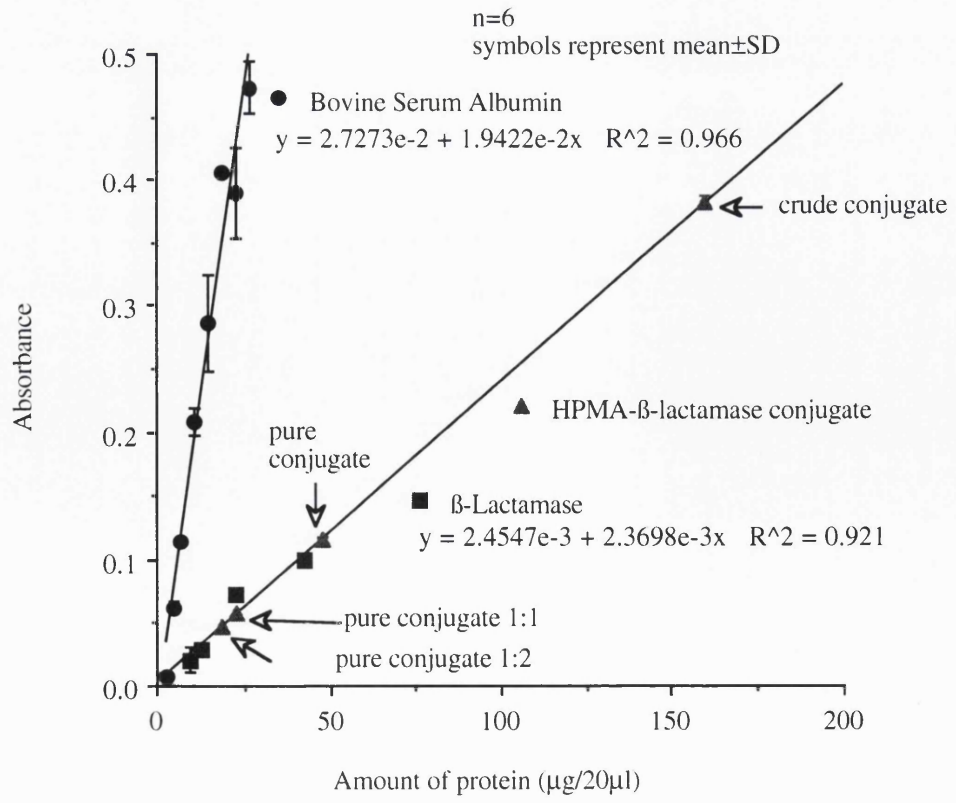


Figure 3.14 Degradation of benzylpenicillin by free and conjugated ß-lactamase

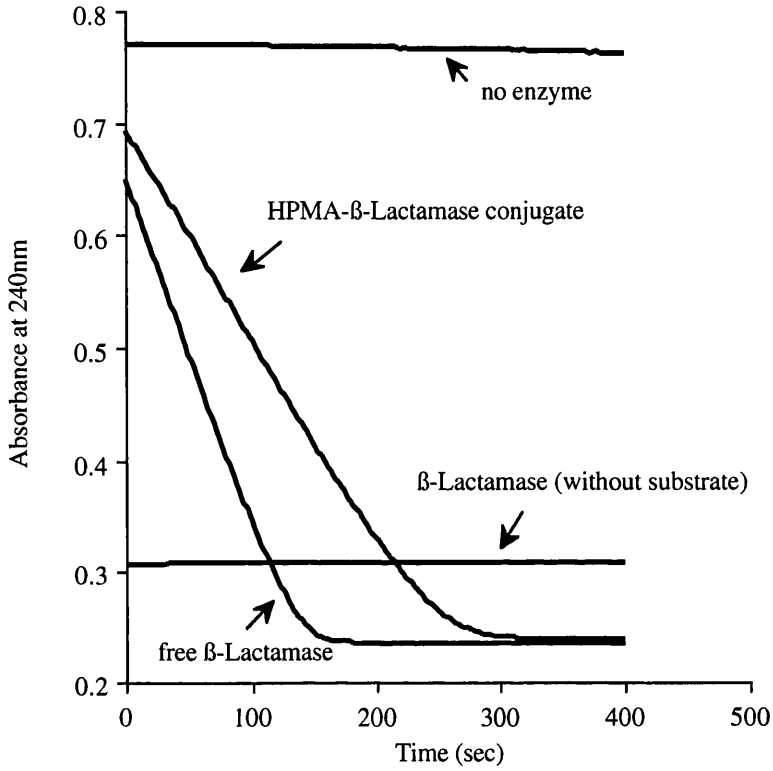


Figure 3.15 Lineweaver-Burk plot [1/Vo] vs [1/S] for degradation of benzylpenicillin by HPMA copolymer-β-lactamase conjugate

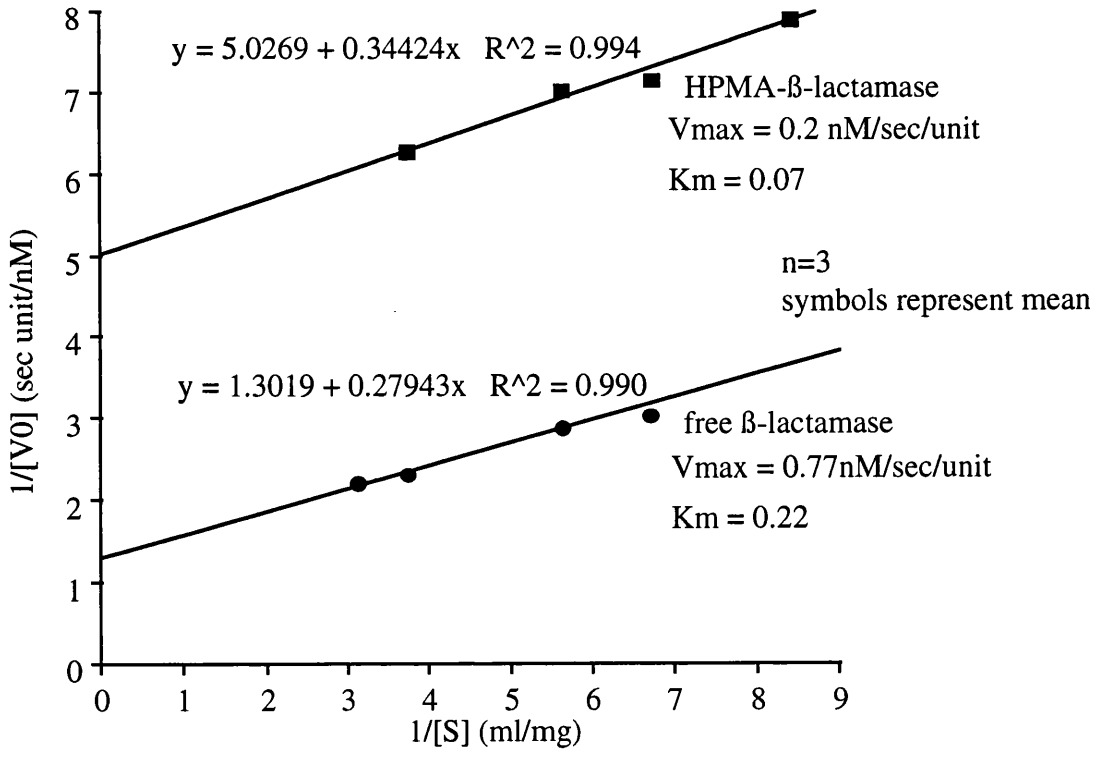


Figure 3.16 NAp release from Bz-Phe-Val-Arg-NAp by free and conjugated cathepsin B

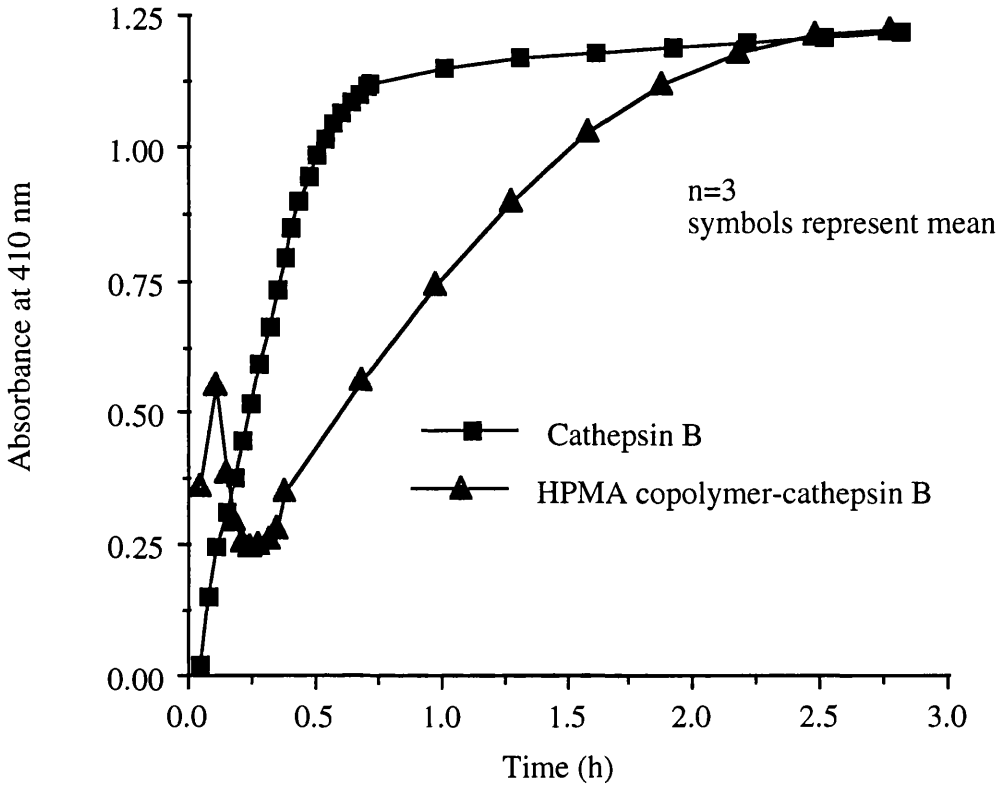
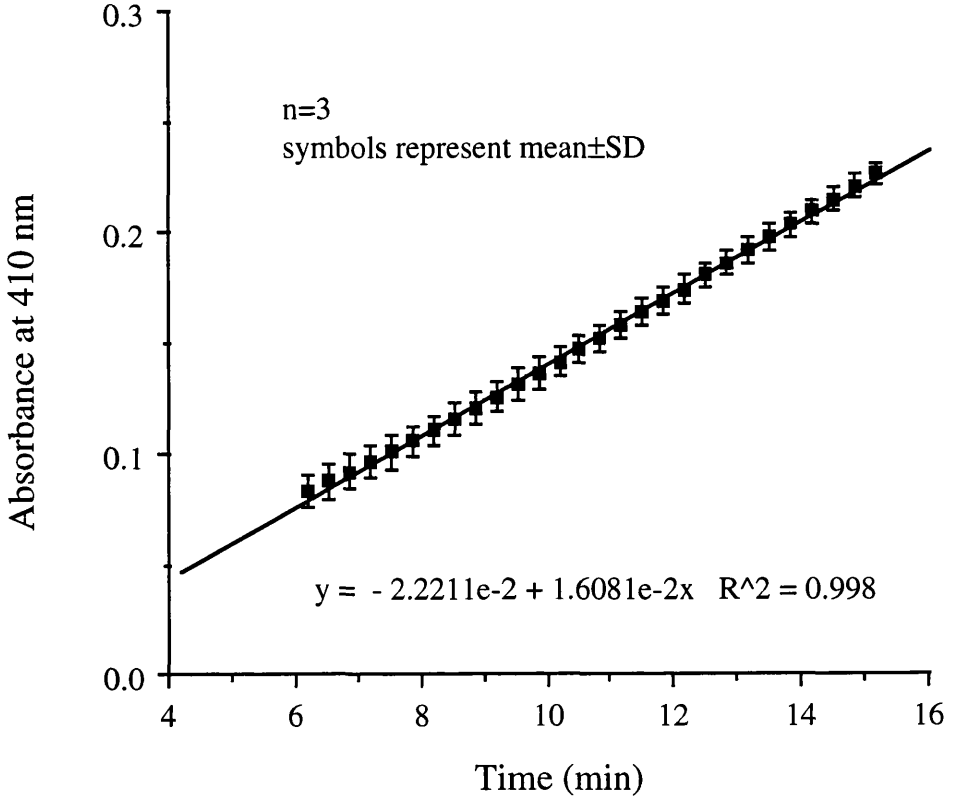


Figure 3.17 Tritosomes Activity - NAp release from Bz-Phe-Val-Arg-NAp by tritosomes



3.3.7 Activity assay of HPMA copolymer-cathepsin B against the high molecular weight substrate PK1

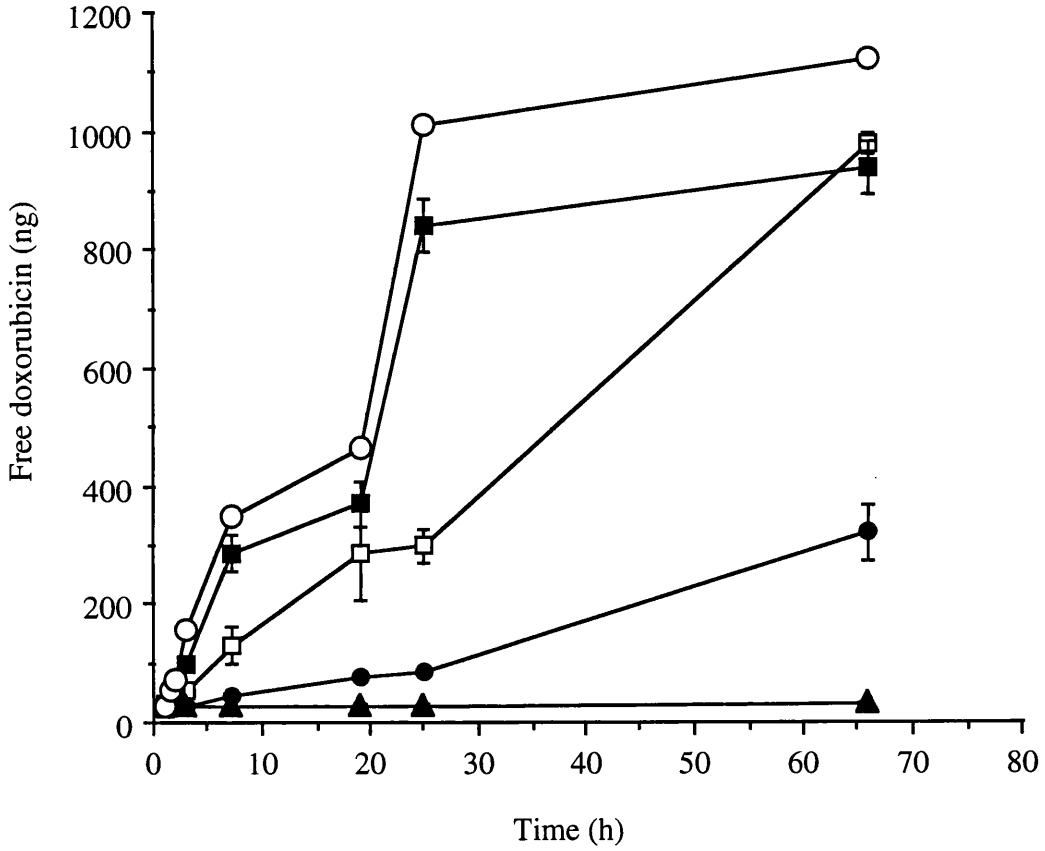
HPLC analysis of HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin (PK1) following incubation with free cathepsin B, HPMA copolymer-Gly-Gly-cathepsin B and tritosomes showed increasing release of free doxorubicin over 70 h (using daunomycin (DNM) as internal standard). In order to determine concentration and activity of tritosomes, Bz-Phe-Val-Arg-NAp was used as substrate (as described in Chapter 2.3.4.1). Calculations from the results shown in Figure 3.17 demonstrated a batch of tritosomes (1.7 mg/ml from BCA assay) with activity of 27.1 nM/min/mg protein. The amount of doxorubicin released (ng/ml) over the experimental period is shown in Figure 3.18. The initial velocity was determined from the linear portion obtained from plots of doxorubicin release by HPLC analysis. From the initial rates of release it can be seen that conjugated cathepsin B retained 19.5% of the enzyme activity of the free cathepsin B when compared at the same protein concentration. Without addition of enzyme, PK1 showed no release of doxorubicin in the assay over the same period. PK1 in the presence of higher concentration of cathepsin B gave a faster release indicating that *in vivo* optimisation of amount of cathepsin B administered will affect the rate of release. *In vitro* release of doxorubicin by tritosomes showed increasing release as well, but the rate *in vivo* will be determined by the pinocytic rate of PK1 to enter the cell and get to the lysosome.

3.4 Discussion

For the PDEPT concept to be effective it is essential that there is successful linkage of the selected enzyme to an HPMA copolymer with retention of enzymatic activity. Secondly it is essential that the polymer linked enzyme should still be able to release drug from the polymer-drug conjugate. Of particular interest are the factors of yield of polymer-enzyme conjugation, the site of conjugation and the retention of activity towards low and high molecular weight substrates.

Both of the HPMA copolymer-enzyme conjugates synthesised with a yield of 30-35%, retained enzymatic activity of 20-25% depending on the substrates. It may seem fortuitous, that HPMA copolymer-cathepsin B could be prepared using the same method used to prepare as HPMA copolymer-β-lactamase, but taking into consideration that both enzymes are of similar molecular weight of 30 KDa, both have approximately 20 NH_2 groups available for conjugation, and both have a PKa around 2.8-3.0, it was not surprising. However, a different enzyme would require an optimisation procedure and might need a totally different conjugation method.

Figure 3.18 Doxorubicin release from PK1 in vitro



PK1+cathepsin-B 176μg/ml
PK1+ HPMA-cathepsin-B 176μg/ml
PK1+ tritosomes 1.7 mg/ml
Control- PK1
PK1+ cathepsin-B 1mg/ml

n=3
symbols represent mean±SD

HPMA copolymer-cathepsin B retained 24.4% enzymatic activity compared to the native enzyme against the low molecular weight substrate Bz-Phe-Val-Arg-NAp. With PK1, its enzymatic activity was reduced to 20%. This product allowed us to explore the PDEPT concept *in vivo*, with the knowledge that the conjugated enzyme activity is lower than that of the native enzyme. From SDS PAGE analysis, there was no sign of free enzyme after purification by dialysis, so the activity retained was due to the bound enzyme present in the activity assay.

Choice of conjugation method

Most proteins have a number of free chemical groups available for substitution reactions along the length of their polypeptide backbone. Methyl, hydroxy, sulphydryl, amino and carboxyl are the most common. The moiety most often used for conjugation is the free ε-amino group of lysine. The conjugation process by aminolysis used here is semi-random, conjugating the NH_2 groups of lysine and arginine present in the enzyme to the diglycine peptidyl side chains present in the HPMA copolymer chain. The controlling factors for conjugation are the reaction time, pH and solvent so active sites may be subject to conjugation. Using this chemistry there is little or no possibility to control the location of the substitution site, so that substitution may occur via any of the available lysine residues. These will not couple equally well as the lysine and arginine - NH_2 have different Pka values.

Several different methods have been routinely used by others for protein conjugation. These include: periodate oxidation, glutaraldehyde activation, glycosylated engineered site-specific coupling and reverse proteolysis. The coupling chemistry chosen for the production of antibody-enzyme conjugates has been based almost exclusively on the use of thioether linkages such as periodate oxidation or glutaraldehyde activation (Boorsma and Streefkerk, 1979; Muzykantov *et al.*, 1990; Stanislawski *et al.*, 1989). These linkages have greater stability *in vivo* compared with the disulphide linkages commonly used for the production of antibody-toxin conjugates (Thorpe and Ross, 1982). Using this type of conjugation chemistry, the modification of the proteins takes place in a semi-random fashion and any exposed modifiable amino acids may be potential coupling sites. There is no homogeneity of the product in the sense that location of modified residues is not controllable. Whilst the yield of the conjugation step is typically about 30-35%, purification is complicated by the changes in the charge properties of the proteins, which occur as a result of heterobifunctional and thiolation agents modifying positively charged lysine residues. The most common method for purifying antibody-enzyme conjugates is size exclusion chromatography.

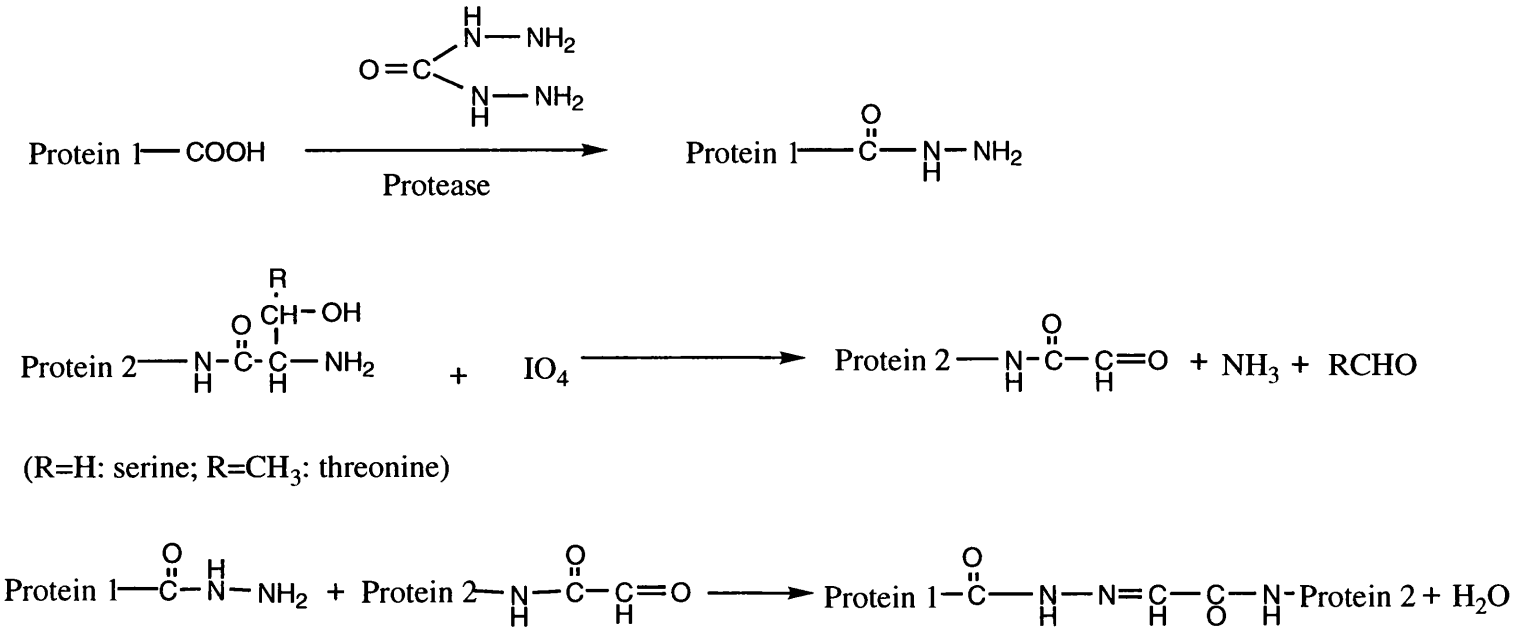
Incomplete separation means that the overall yields of purified product are typically 10-15% at best (Svensson *et al.*, 1994; Melton, 1996).

Use of endogenous sulphydryl residues to facilitate conjugation can ensure specificity of location because these are relatively rare and their position is fixed in the amino acid sequence of the protein. However, modification of amino acids in critical areas of the protein may result in deleterious effects on the enzyme's function. There has been very little effort devoted to devising methods of protecting the active sites of enzymes before conjugation. Such an approach has recently been described for the construction of antibody-hapten conjugates, with the added sophistication of using an engineered unique glycosylation site (Leung *et al.*, 1995). An alternative approach is to bind the enzyme to be modified to an affinity matrix or to carry out the reaction in the presence of substrate or reversible inhibitor in order to protect the active site residues whilst they are undergoing modification (Poznansky, 1986; Cao *et al.*, 1990). Such an approach has been described for the modification of yeast invertase (Pillai and Bachhawat, 1977).

A novel approach for protein conjugation based on reverse proteolysis is illustrated in Figure 3.19. This method offers the production of well defined linkage-sites under mild conditions (Rose *et al.*, 1991). A carbohydrazide residue is specifically attached to the carboxyl terminus of one protein using a proteolytic enzyme under conditions which force it to act as a ligase. This carbohydrazide group is then linked to an oxidised aldehyde or ketone group on the second protein. If the second protein has a suitable amino acid (serine or threonine) as its N-terminus this can be specifically oxidised to provide a second defined linkage site. The technique has been successfully used to conjugate both CPG2 (Werlen *et al.*, 1994) and β-lactamase (Mikolajczyk *et al.*, 1994). The yields obtained were comparable with those achieved by more conventional means (10-15%). However, the yield of 30-35% active HPMA copolymer-enzyme conjugates described here and the ease of conjugation method balanced the semi-random coupling, therefore the aminolysis reaction was used throughout the study for PDEPT.

A new method for protein modification using functionalised semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] (ST-PHPMA) was recently published by Lu *et al.* (1998). The conjugation of chymotrypsin to ST-PHPMA resulted in 20-43% yield and again lower activity of the conjugated enzyme towards a polymeric substrate (P-Gly-Leu-Phe-NAp) was observed. The steric hindrance of the polymer chain of P-Gly-Leu-Phe-NAp renders the formation of the enzyme-polymer substrate complex more difficult, resulting in lower turnover rates, in agreement with the data presented in

Figure 3.19 Coupling of proteins by site-specific reverse proteolytic attachment of carbohydrazide to C-terminal carboxylic acid residues and super-mild oxidation of N-terminal serine or threonine (from Melton, 1996)



this chapter and in the literature (Kopecek *et al.*, 1981). Ulbrich and Oupicky (1997) investigated three PHPMA conjugates of chymotrypsin differing in the length of the PHPMA chain and the degree of enzyme substitution. They found that the higher the degree of chymotrypsin substitution, the lower enzyme activity of the conjugate, probably due to denaturation of the enzyme. The effect of molecular weight of the polymer used for the modification was not very pronounced. In our study we used only one molecular weight of HPMA copolymer so optimisation of different chain lengths will be interesting to investigate. The main advantage of using ST-PHPMA as a monofunctional polymer is the controlled site of conjugation on the polymer which is lacking in our system.

Retention of enzymatic activity

Enzyme kinetics studies revealed an increase in the apparent K_m of the enzyme upon attachment to HPMA copolymer, indicating a reduction of enzyme's affinity for the substrate. Since the first substrates of both enzymes studied were of low molecular weight (Bz-Phe-Val-Arg-NAp for cathepsin B and benzylpenicillin for β-lactamase), it was unlikely that HPMA copolymer hinders their diffusion to the enzyme's active site. A more plausible explanation derives form the change in the enzyme's microenvironment due to the hydrophilicity of the bound HPMA copolymer chains or, possibly, a conformational change of the enzyme on conjugation to the polymer. Further experiments with cathepsin B against the high molecular weight substrate, PK1, showed that the enzyme retained activity in the same range (20-25%) as for the low molecular weight substrate.

Different enzyme conjugates may have different sites of action in the body and therefore different requirements. In ADEPT and PDEPT activation of the prodrug or drug release occurs extracellularly. In the case of GDEPT activation takes place intracellularly. In ADEPT and GDEPT the enzyme is present in the tumour prior to prodrug administration. This contrasts with the proposed concept of PDEPT. In all prodrug approaches, ADEPT, VDEPT and PDEPT, the best activating enzymes would probably be those that activate the prodrug as rapidly as possible (high K_{cat}) and work efficiently at low concentrations of substrate (low K_m). The K_m of β-lactamase from *Enterobacter cloacae* P99 against 7-(phenylacetamido)doxorubicin was shown to be 46±11 μM and against ∂-carboxybutanamido-doxorubicin it was 83±26 μM (Vrudhula *et al.*, 1995). Similarly, the K_m of β-lactamase from *Bacillus Cereus* (type II) was found to be of 38±7.8 μM and 56±12 μM respectively. Also, Sherwood *et al.* (1977) showed that the β-lactamase from *Escherichia coli* TEM-1 had a K_m in the same range of 25±4.4 μM and 73±11 μM. K_m of CPG2 for various folate derivatives are low < 10 μM. Interesting the K_m of β-lactamase when conjugated to HPMA copolymer was 70 μM

which is in the same range as that reported for the other systems, with the added advantage that it may be able to overcome some of their disadvantages *in vivo*.

Conjugation of proteins to dextran and PEG are widely described in the literature and some of the results show different levels of retained enzymatic activity after modification (summarised in Table 3.4). Loss of enzymatic activity is usually concomitant with dextran attachment, the degree of which can be inversely proportional to the molecular weight of the dextran. The activity retention of asparaginase-dextran conjugates did not however, seem dependent on the molecular weight of the carrier. The relationship (if any) between molecular weight of the polymer and the degree of modification of the final construct has not been evaluated.

PEGylation of enzymes is usually accompanied by loss of enzymatic activity as well. An exception to this rule is PEG-trypsin, which exhibited increased esterase activity, probably due to the diminished autodigestive capacity (Abuchowski and Davis, 1979). Ashihara *et al.* (1978) have studied the effect of the molecular weight of PEG on the degree of modification and activity retention by PEG-asparaginase. While the extent of modification did not seem to depend on the size of PEG, enzyme activity decreased drastically with increasing molecular weight of the polymer. The same authors demonstrated that asparaginase activity loss was proportional to the degree of substitution of the amino groups by PEG and Wieder *et al.* (1979) reported similar findings for phenylalanine ammonia-lyase. Moreover, similar to our finding with the HPMA copolymer-enzyme reactions, Wieder *et al.* (1979) reported that the ratio of the reactants and the pH of the reaction influence the degree of modification and consequently can also affect the activity of the PEGylated protein.

The attachment of poly(D,L-alanine), a non-immunogenic (Davis *et al.*, 1991) synthetic amino acid polymer to asparaginase led to a conjugate with increased heat stability and resistance to proteolysis (Uren and Ragin, 1979). Modified asparaginase was completely resistant to tryptic digestion and about 5-fold more resistant to chymotrypsin. Both enzymes suffered considerable loss of activity upon modification (between 35 and 80%) but the K_m remained unchanged. The stability of the modified enzyme was not tested in our system, but would be interesting to compare with the data in the literature showing increased stability for all the constructs obtained and analysed.

Effect of pI

The isoelectric point (pI) of the protein governs charge-charge interactions protein-polymer and protein-protein. Therefore it certainly influences the conjugation of the protein with the polymeric HPMA. Enzymes with a low pI (below 6) are expected

Table 3.4 Effect of polymer modification on protein activity retention

Polymer	Protein	Yield/ (%) Degree of modification	% Remaining activity	Reference
Dextran (60-90 KDa)	α-Amylase	10	43	Marshall *et al.*, 1977
Dextran (40 KDa)	Asparaginase	N/A	50	Wileman *et al.*, 1986
Dextran (70 KDa)	Asparaginase	N/A	50	Wileman *et al.*, 1986
Dextran (250 KDa)	Asparaginase	N/A	50	Wileman *et al.*, 1986
Dextran (110 KDa)	Asparaginase	17	34	Benbough *et al.*, 1979
Dextran (2000 KDa)	Asparaginase	17	36	Benbough *et al.*, 1979
Dextran (70 KDa)	Asparaginase	N/A	50	Wileman *et al.*, 1983
Dextran (60-90 KDa)	Catalase	10	71	Marshall *et al.*, 1977
Dextran (17 KDa)	Catalase	N/A	49-67	Davis *et al.*, 1991
Dextran (40 KDa)	Catalase	N/A	77-82	Davis *et al.*, 1991
Dextran (80 KDa)	Superoxide Dismutase	34	67	Miyata *et al.*, 1988
PEG (5000)	Arginase	53	65	Savoca *et al.*, 1979
PEG (?)	Asparaginase	70	52	Park *et al.*, 1981
PEG (5000)	Asparaginase	55	30	Cao *et al.*, 1990
PEG (5000)	Asparaginase	56	8	Kamisaki *et al.*, 1981
PEG (5000)	Asparaginase	20	50	Ashihara *et al.*, 1978
PEG (5000)	Asparaginase	41	22	Ashihara *et al.*, 1978
PEG (5000)	Asparaginase	79	15	Ashihara *et al.*, 1978
PEG (5000)	Catalase	43	93	Abuchowski *et al.*, 1977
PEG (1900)	Catalase	40	95	Abuchowski *et al.*, 1977
PEG (5000)	Phenylalanine ammonia-lyase	9	71	Wieder *et al.*, 1979
PEG (5000)	Phenylalanine ammonia-lyase	28	41	Wieder *et al.*, 1979
PEG (5000)	Phenylalanine ammonia-lyase	62	7	Wieder *et al.*, 1979
PEG (5000)	Superoxide Dismutase	30-40	80-90	Cao *et al.*, 1990
PEG (5000)	Superoxide Dismutase	24	52	Miyata *et al.*, 1988
PEG (5000)	Trypsin	24	95	Abuchowski and Davis, 1979
PEG (5000)	Trypsin	59	150	Abuchowski and Davis, 1979
PHPMA (2700)	Chymotrypsin	90	65-79 (NAp) 43 (PEG-NAp)	Ulbrich and Oupicky, 1997
PHPMA (5100)	Chymotrypsin	92	67-85 (NAp) NA (PEG-NAp)	Ulbrich and Oupicky, 1997
PHPMA (10900)	Chymotrypsin	67	85-98 (NAp) 23 (PEG-NAp)	Ulbrich and Oupicky, 1997

N/A data not given

to be more available for conjugation with HPMA copolymer-Gly-Gly-ONp in phosphate buffer at pH 7.2 as the amino groups on the lysine residues will have more chance to be deprotonated at this pH. The *in vivo* half-life of enzymes has been related to their pI and generally, the lower the pI the more the enzyme is said to persist in the circulation (Holcenberg *et al.*, 1975). When pI values were between 5.0-6.0 they were found to lead to the highest half-lives but, below these pI values, the rate of removal of the enzymes from the circulation increased again. Similar pI to that of plasma proteins, undergo reduced opsonisation and thus circulate for extended periods (Rutter and Wade, 1971). The supplier of the enzymes used in this study (Sigma) reports a pI of 5.3 for β-lactamase and 5.1 for cathepsin B (Malamud and Drysdale, 1978) and for these reasons it would be interesting to compare these values to the pI of the conjugated enzymes, unfortunately we did not have the equipment to do these experiments.

Route of administration

One of the major problems encountered in the use of enzymes for medical applications is the route of administration. When given orally, enzymes (proteins in general) suffer denaturation and enzymatic hydrolysis in the gastrointestinal tract. Alternative routes of protein administration such as the nasal, pulmonary, rectal, transdermal and vaginal, where epithelial proteolytic activity is diminished, have already been investigated (Lee *et al.*, 1993; Sanders, 1990). However, because of their high molecular weight (specially of the polymer-enzyme conjugate), proteins are generally incapable of crossing physical barriers (e.g. epithelia), leading to poor bioavailability. Strategies to increase the low permeability and reduce cellular proteolytic activity and thus improve the bioavailability of proteins by non-parenteral routes, include the co-administration of penetration enhancers, protease inhibitors or the use of iontophoresis (transdermal route). Although the pulmonary (Lee *et al.*, 1993) and nasal (Breimer, 1991) routes seem to be particularly promising, especially for low molecular mass proteins, problems such as extremely low (with consequent wastage of often expensive proteins, e.g. cathepsin B) and erratic protein absorption (dependent on the patient and their state of health) still have to be resolved. Therefore, proteins are at present usually administered by the parenteral route which, by its invasive nature is not well accepted by patients unless they suffer from life-threatening diseases, such as cancer in this case. Bearing this issue in mind, the aqueous solubility of both the polymer-enzyme conjugates can be considered a major advantage for the following *in vivo* experiments.

The polymer-enzyme conjugates synthesised as described were prepared routinely and characterised before every experiment. The products obtained were reproducible and allowed objective evaluation of the *in vivo* properties of the HPMA

copolymer-cathepsin B and HPMA copolymer-β-lactamase compared to the unbound enzymes.

Chapter Four

In vivo body distribution of ^{125}I-labelled β-lactamase, ^{125}I-labelled cathepsin B and ^{125}I-labelled HPMA copolymer-enzyme conjugates after i.v. administration to mice

4.1 Introduction

Due to their poor absorption through biological membranes and sensitivity to the gastro-intestinal epithelial proteolytic activity, proteins are usually administered parenterally (Breimer, 1991). Once in the blood circulation, they are cleared rapidly and thus, generally exhibit short half-lives. The optimal use of proteins as a part of an anticancer combination therapy is then related to the ability of maintaining high levels in the circulation for extended periods, reducing the frequency of dosing and the probability of undesirable side effects and immune responses (this aspect will be discussed later in Chapter 7). The pharmacokinetic evaluation of a protein therapeutic is particularly important in the understanding of its fate and this subject has been extensively reviewed (Gloff and Benett, 1990).

The body distribution of polymers and the factors that govern their fate (charge, molecular weight and Stokes' radius) has been previously reviewed (Drobnik and Rypacek, 1984). Seymour *et al.* (1995) investigated the effect of polymer molecular size on biodistribution using HPMA copolymers of different molecular weights that were radiolabelled using the Chloramine T method. The higher molecular weight ^{125}I-labelled HPMA copolymer fractions were unable to pass through the kidney glomerulus (24 h urine levels < 15% of dose) and had long circulation times, with $t_{1/2}$ values of 20-24 h in C57 and ddY mice. Noguchi *et al.* (1998) investigated the molecular-weight and time-dependence of the EPR effect in solid tumour by testing the early phase accumulation of macromolecules in tumour and normal tissues and the relationship between blood concentration and tissue clearance. Radioiodinated HPMA copolymers of molecular weights of 4.5-800 KDa were injected to mice bearing sarcoma 180. In 10 min up to 1.5% of injected dose/g tumour were accumulated independent of size of the polymer. However, higher molecular weight copolymers (>50 KDa) showed significantly increased tumour accumulation after 6 h, while the lower molecular weight fractions (<40 KDa) were cleared rapidly from the tumour tissue due to rapid diffusion back to the bloodstream. Both groups (Seymour *et al.*, 1995; Noguchi *et al.*, 1998) showed that blood clearance was also molecular weight- dependent; the lower molecular weight copolymers displayed rapid clearance. These results suggest that the mechanism of the EPR effect in solid tumour, primarily resides in the difference in the clearance rate between the solid tumour and the normal tissues after initial penetration of the polymers into these tissues.

Furthermore, Yamaoka *et al.* (1995) studied the body distribution of ^{125}I-labelled PEG polymers of different molecular weight after i.v. injection to mice. They found that higher molecular weight PEG (190 KDa) remained in the circulation for longer periods than lower molecular weight PEG (6 KDa). The terminal half-life of

PEG in the circulation increased from 18 min to 1 day as the molecular weight increased.

The feasibility of the PDEPT concept is dependent on the ability of HPMA copolymer-enzyme conjugates to accumulate selectively in tumour tissue while circulating for long time in the blood. Therefore, it was considered necessary to examine *in vivo* the distribution of these conjugates compared to the corresponding free enzymes. The pharmacokinetics of conjugated enzymes, β-lactamase and cathepsin B, was examined in this chapter to assess the possibility of using these conjugates to accomplish an altered body distribution of protein.

4.2 Methods

Prior to the animal experiments, free and HPMA copolymer-bound β-lactamase were radioiodinated using Bolton Hunter method as described in Chapter 2.3.7. The major drawback of this radiolabelling method was that although a high labelling efficiency was achieved the specific radioactivity was low (Means and Feeney, 1995) and this method was therefore abandoned. Subsequently free and HPMA copolymer bound cathepsin B were radioiodinated using Chloramine T method (Greenwood *et al.*, 1963; Duncan *et al.*, 1981), which was considered to give higher specific activity. The method is described in Chapter 2.3.6. Dialysis against 1% NaCl was used to remove remaining free [^{125}I]iodide. The purity assessment of iodinated products using paper electrophoresis is described in Chapter 2.3.8.

In pharmacokinetic studies, s.c. B16F10 murine melanoma tumours were allowed to establish to give a size of approximately 100 mm^2 (see Chapter 2.3.9) and radiolabelled products administered i.v. (see Chapter 2.3.10) to C57 black male mice (6-8 weeks old). Radioactivity levels in blood, urine, tumour and different organs were assessed by γ-counter and accumulation expressed as a percentage of the administered dose.

4.3 Results

4.3.1 Labelling efficiency and purity assessment

After ^{125}I-labelling, a 5 μl sample was removed from the reaction mixture to determine the labelling efficiency and 5 μl of the product following dialysis was analysed by paper electrophoresis to determine its purity. Figures 4.1, 4.2, 4.3 and 4.4 provide examples of electrophoresis profiles seen for free and conjugated β-lactamase and free and conjugated cathepsin B respectively, before and after purification. The analysis of final purity with respect to free iodine in preparation was therefore calculated

Figure 4.1 Labelling efficiency of free β-lactamase and HPMA copolymer-β-lactamase with ^{125}I

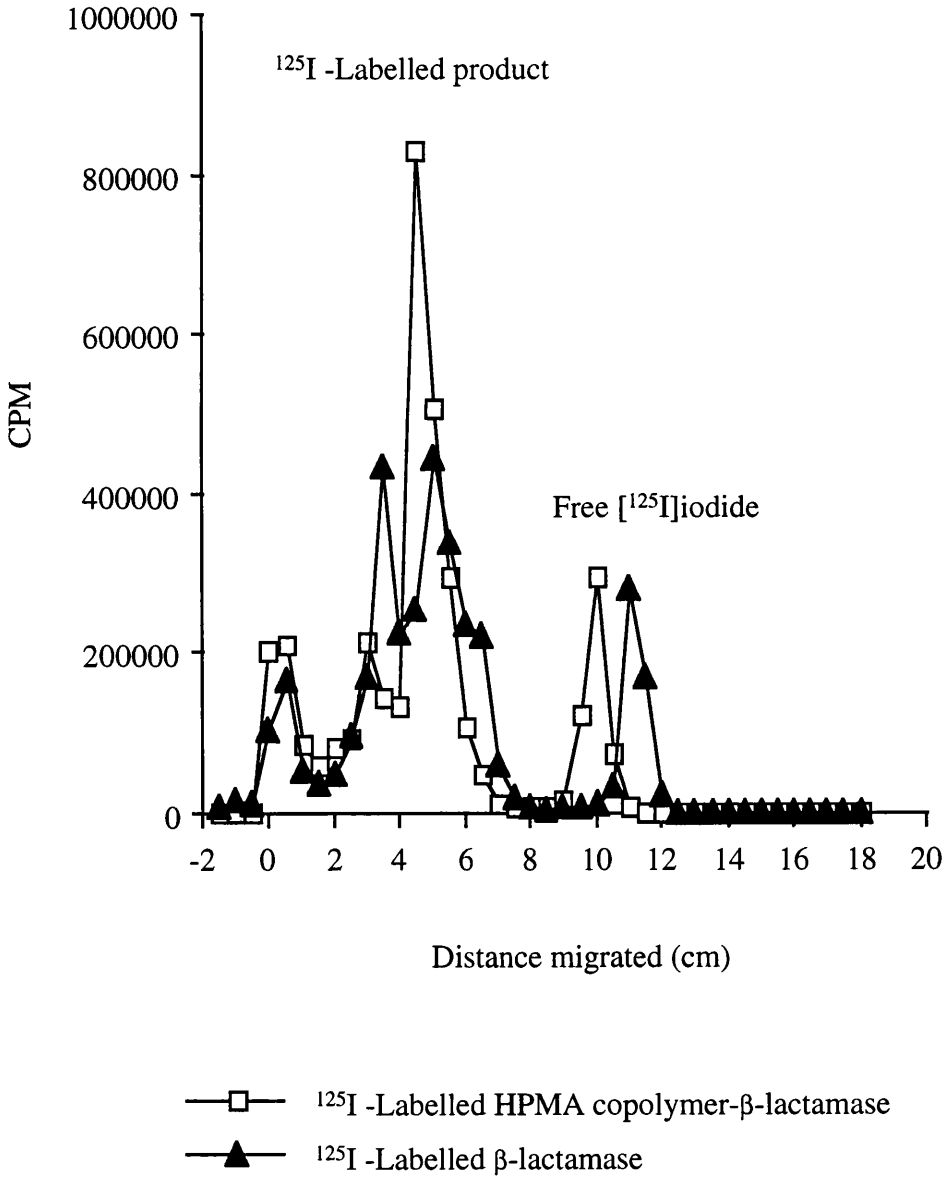


Figure 4.2 Product purity determined by paper electrophoresis following dialysis against NaCl

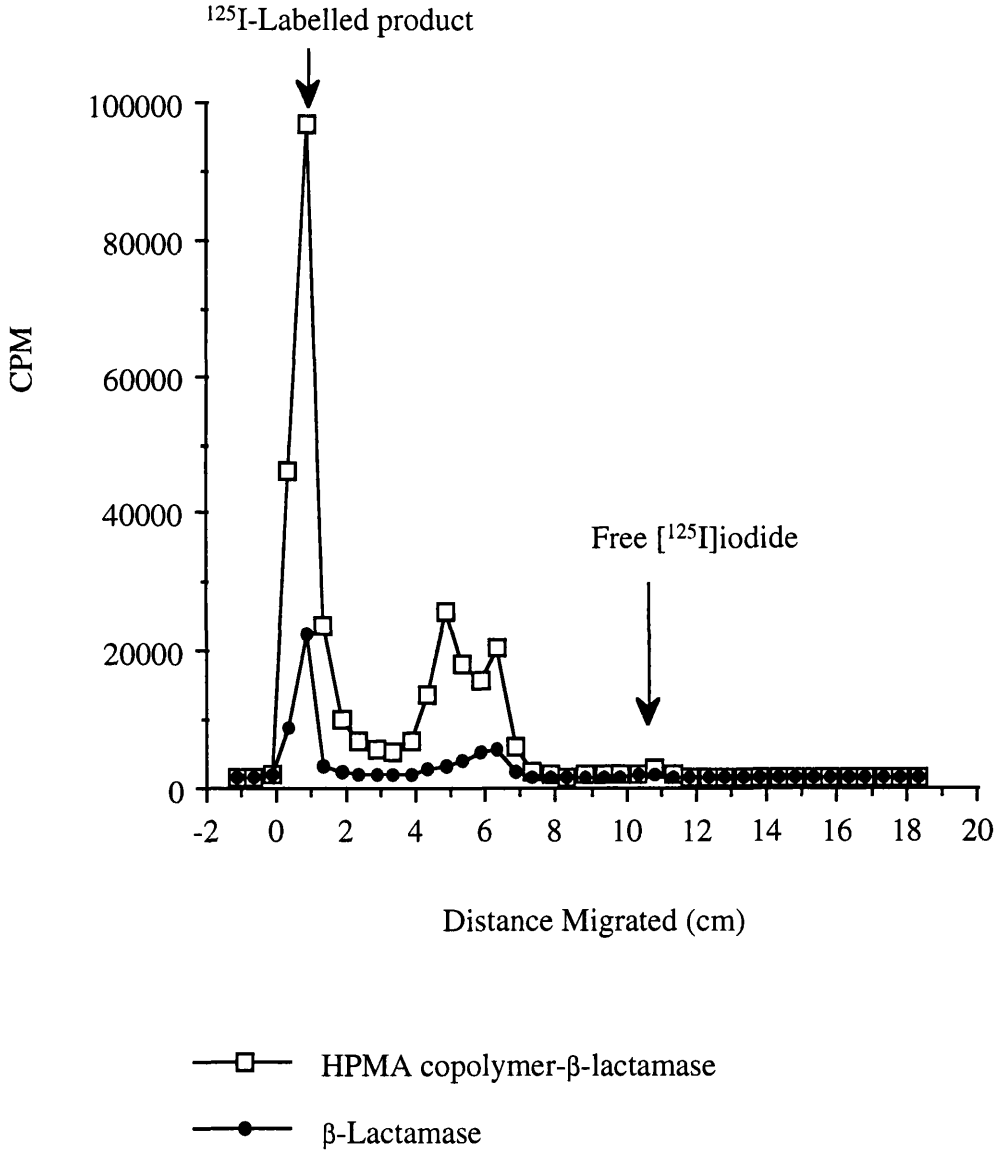


Figure 4.3 Labelling efficiency of free cathepsin B and HPMA copolymer-cathepsin B with ^{125}I

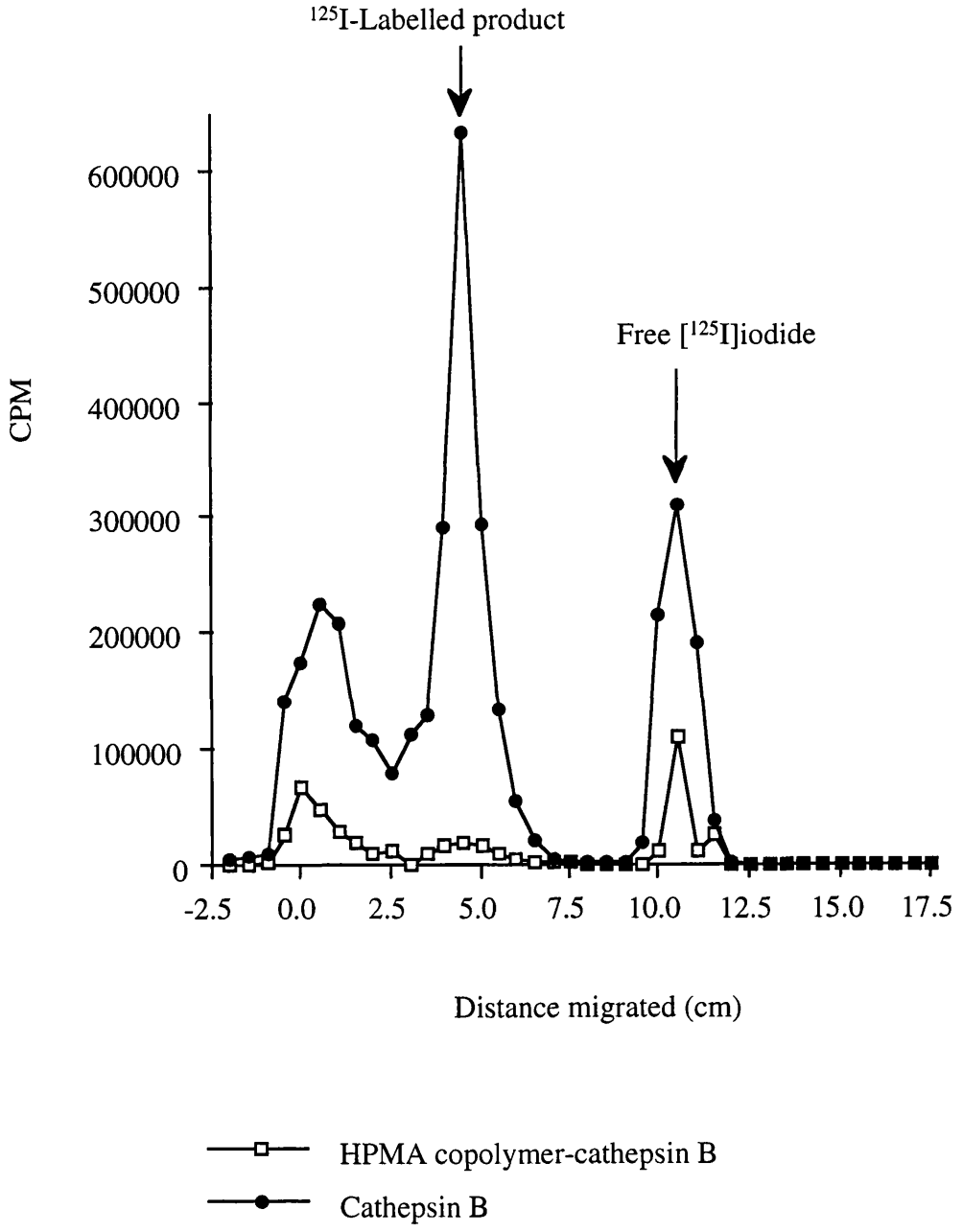
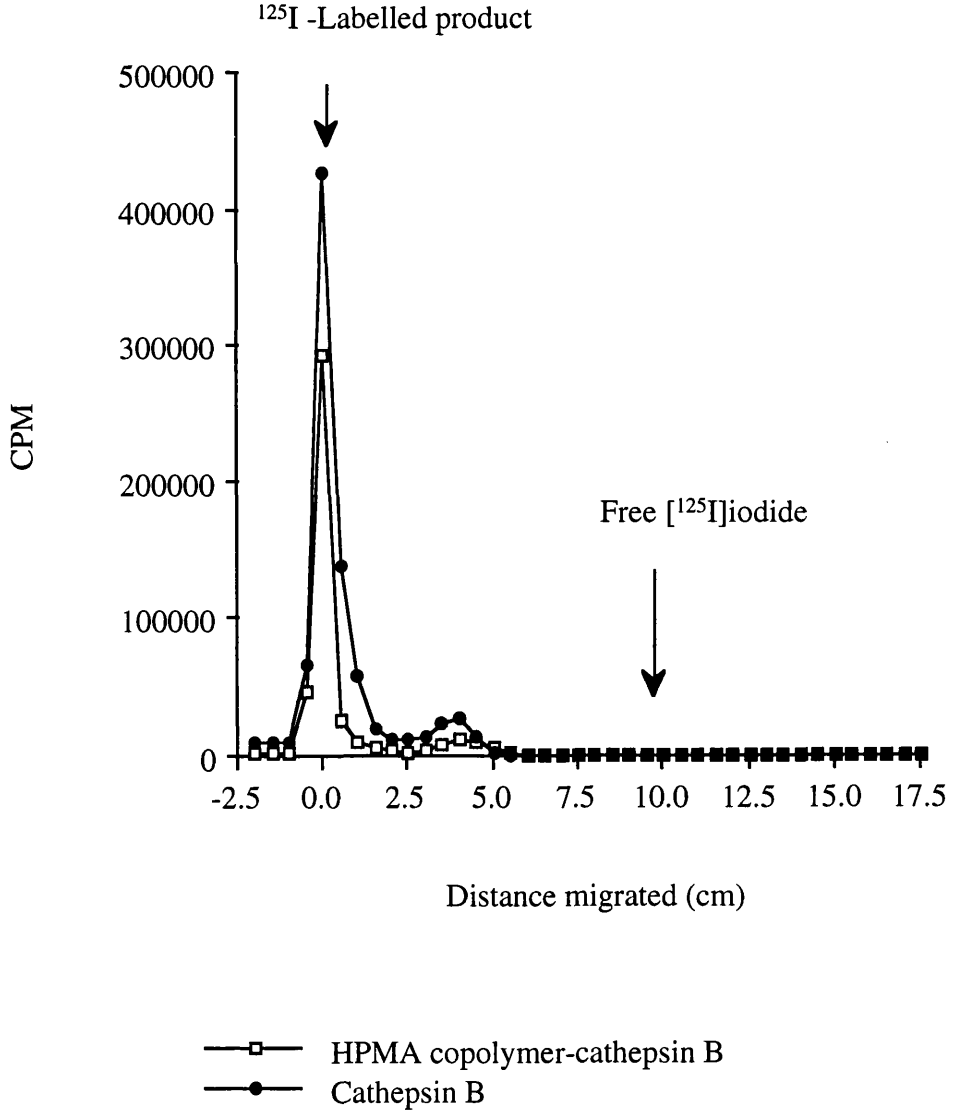


Figure 4.4 Product purity determined by paper electrophoresis following dialysis against NaCl



after paper electrophoresis. Table 4.1 shows the labelling efficiency, degree of final purity with respect to free [^{125}I]iodine, and the specific activity of ^{125}I-labelled constructs. Final purity was assessed using paper electrophoresis. When levels of free [^{125}I]iodine were found to be less than 1%, the products were considered pure. The radioactivity of the preparations was checked daily during each experiment, in order to make sure that the same amount ($5x10^5$ CPM) is injected to each mouse.

4.3.2 Specific activity

The specific activity of the ^{125}I-labelled HPMA copolymer bound enzymes was lower compared to that of the ^{125}I-labelled free enzymes in both cases. This might have been caused by the low availability of amine groups on the β-lactamase after conjugation to HPMA copolymer compared with 18-20 per molecule for the free enzymes. This would have resulted in a lower probability of the Bolton-Hunter reagent to successfully couple to an amine group on the conjugated enzyme. Another plausible explanation is based on differences of surface availability of these lysine or tyrosine residues (in the case of cathepsin B labelled with the Chloramine T method) due to distinct tertiary conformations of the enzymes. Basically, the incorporation of the label in the conjugated enzymes is dependent on the extent of their modification (meaning their yield of conjugation). Although the ^{125}I-labelling efficiency of the Bolton Hunter method was higher than that with the Chloramine T method, the specific activity was higher in the case of cathepsin B when using the Chloramine T method compared to the β-lactamase, which was labelled with Bolton-Hunter reagent.

4.3.3 Biodistribution of free and conjugated ^{125}I-labelled β-lactamase

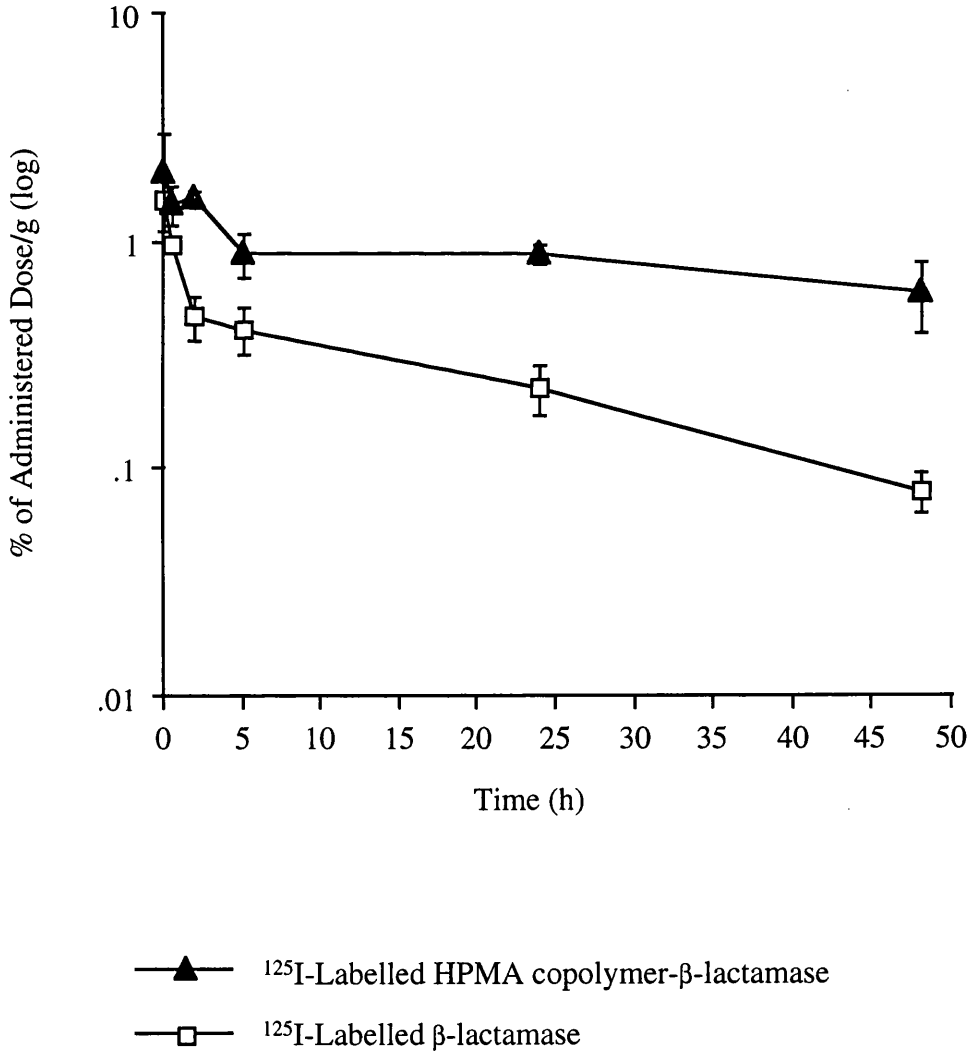
^{125}I-labelled β-lactamase and ^{125}I-labelled HPMA copolymer-Gly-Gly-β-lactamase were administered i.v. to C57 mice bearing s.c. B16F10 melanoma to compare their pharmacokinetics. Analysis of the levels of accumulation of ^{125}I-labelled free and bound β-lactamase in key organs (including tumour, heart, kidney, spleen, liver, thyroid and lungs) and blood were expressed as percent of administered dose, $5x10^5$ CPM/mouse.

Figures 4.5 (tumour), 4.6 (heart), 4.7 (kidney), 4.8 (spleen), 4.9 (liver) and 4.10 (lungs) show the data describing the accumulation of ^{125}I-labelled β-lactamase and ^{125}I-labelled HPMA copolymer-Gly-Gly-β-lactamase in these organs. The polymer-enzyme conjugate showed a 3.2-fold increase in AUC in the tumour compared to free enzyme. Table 4.2 shows the ratio of AUC between free and conjugated β-lactamase in the different organs.

Table 4.1 Characteristics of ^{125}I-labelled free and conjugated β-lactamase and cathepsin B

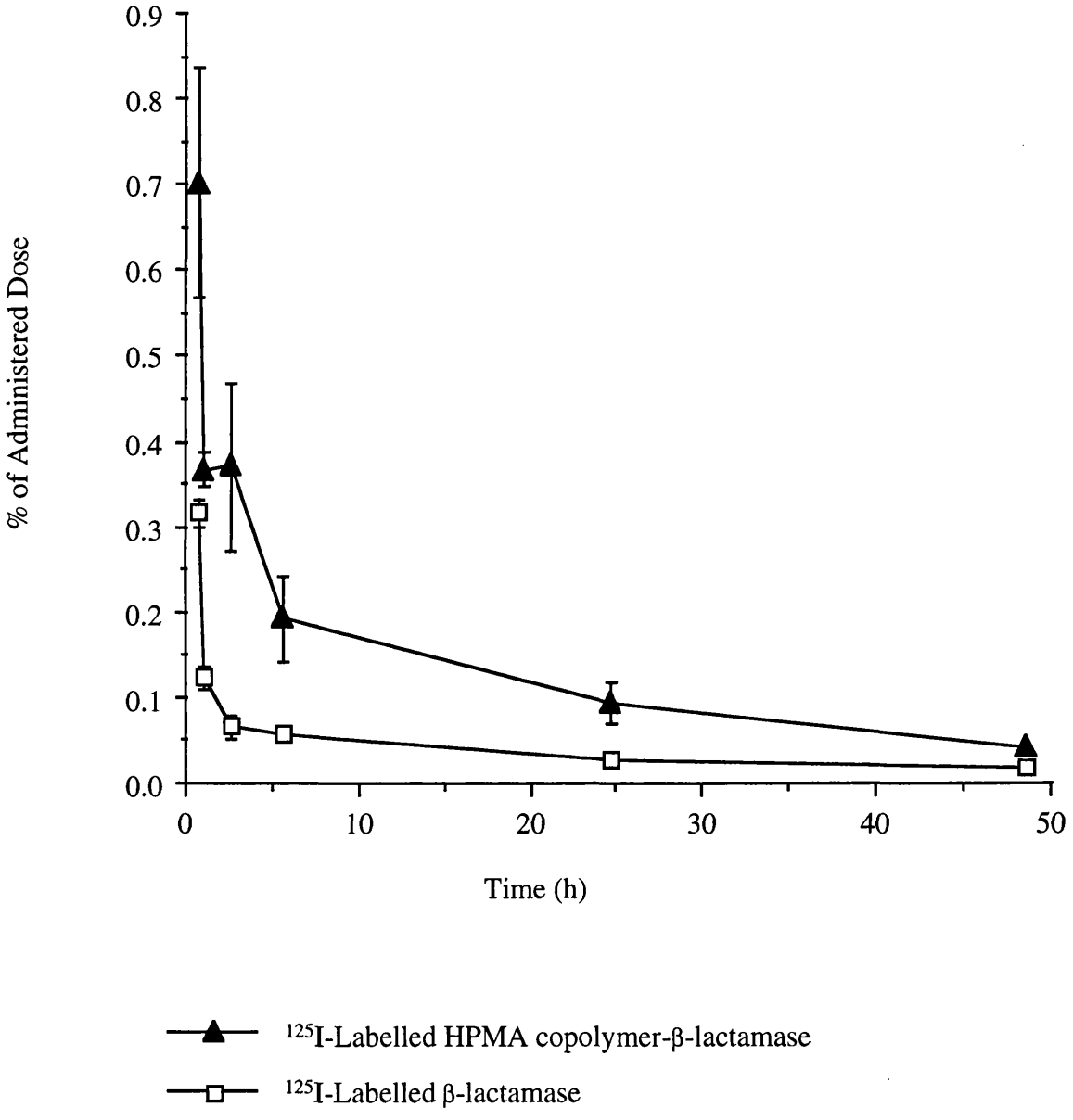
Sample	Radiolabelling Method	Labelling Efficiency (%)	Free [^{125}I]iodide in preparation (%)	Specific Activity (μCi/mg)
β-Lactamase	Bolton-Hunter	85.1	0.72	25.18
HPMA copolymer-Gly-Gly-β-lactamase	Bolton-Hunter	85.6	0.98	6.22
Cathepsin B	Chloramine T	71.3	0.21	86.53
HPMA copolymer-Gly-Gly-cathepsin B	Chloramine T	42.3	0.15	50.37

Figure 4.5 Tumour accumulation of free and conjugated ^{125}I-labelled β-lactamase in C57 black male mice bearing B16F10 melanoma



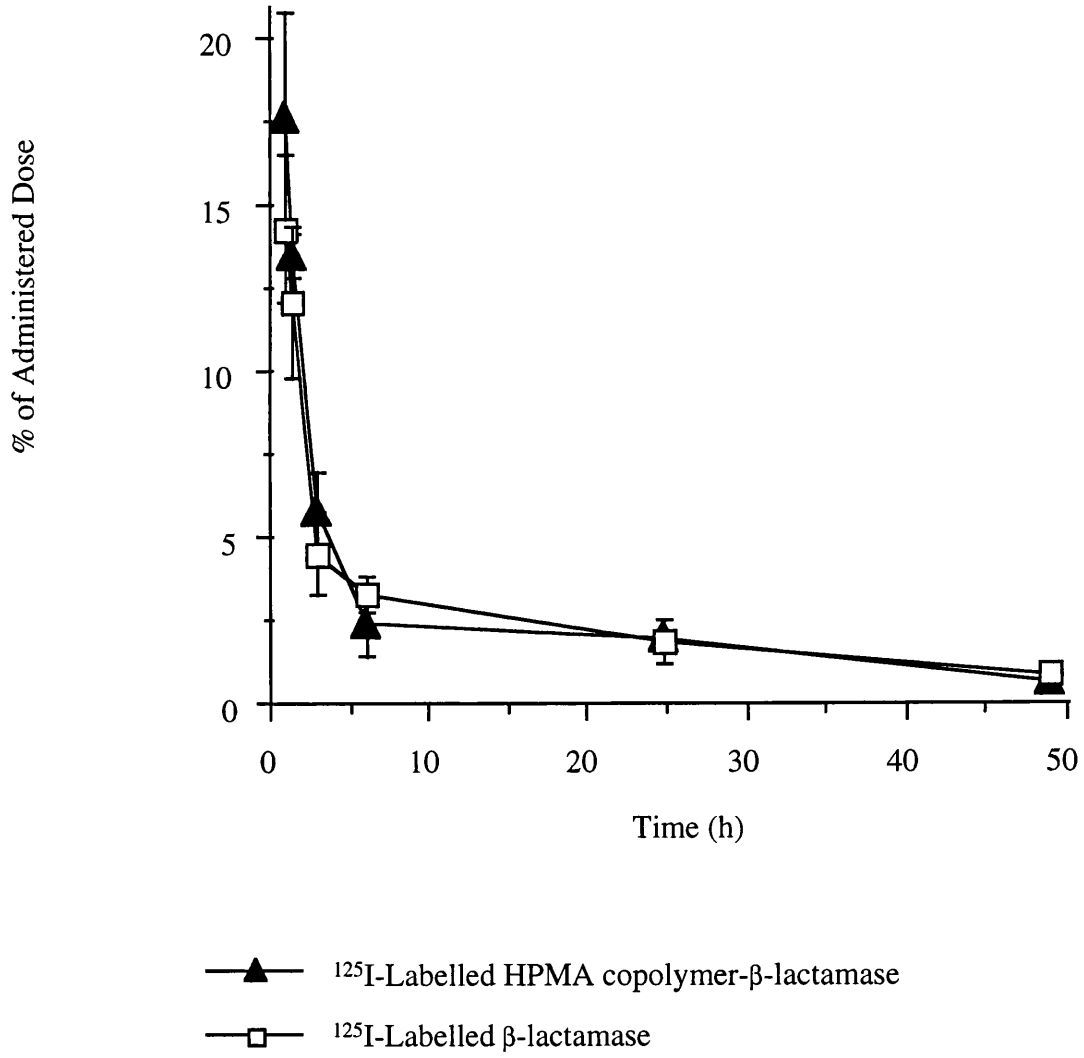
n=3
Symbols represent mean±SE

Figure 4.6 Heart accumulation of free and conjugated ^{125}I-labelled β-lactamase in C57 black male mice bearing B16F10 melanoma



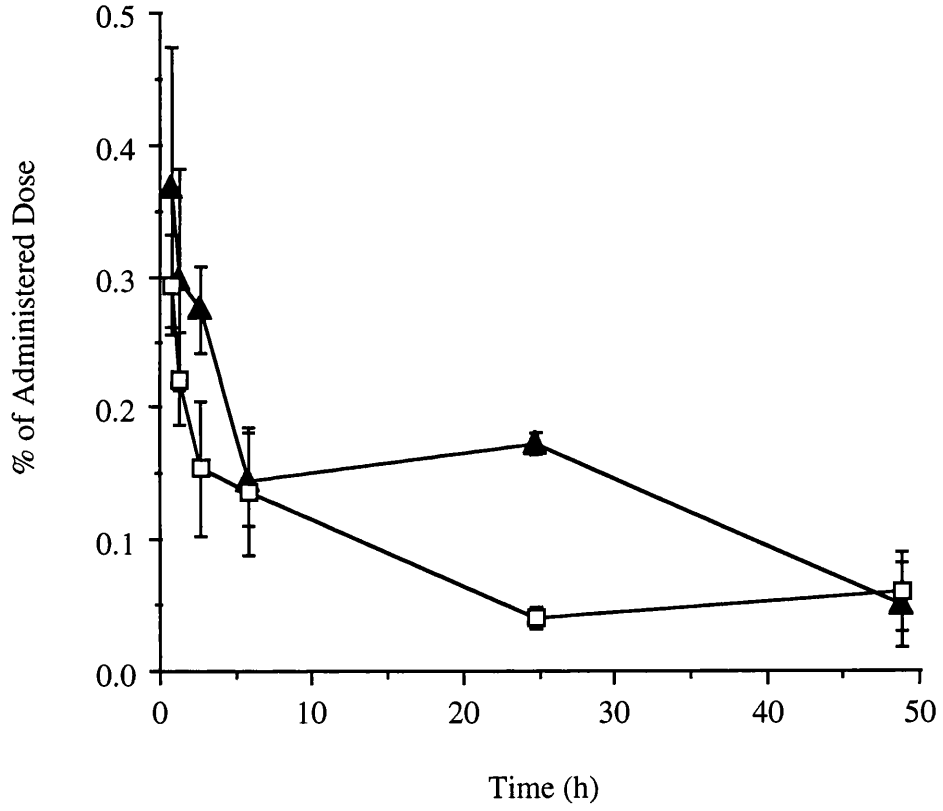
n=3
Symbols represent mean±SE

Figure 4.7 Kidney accumulation of free and conjugated ^{125}I-labelled β-lactamase in C57 black male mice bearing B16F10 melanoma



n=3
Symbols represent mean±SE

Figure 4.8 Spleen accumulation of free and conjugated ^{125}I-labelled β-lactamase in C57 black male mice bearing B16F10 melanoma

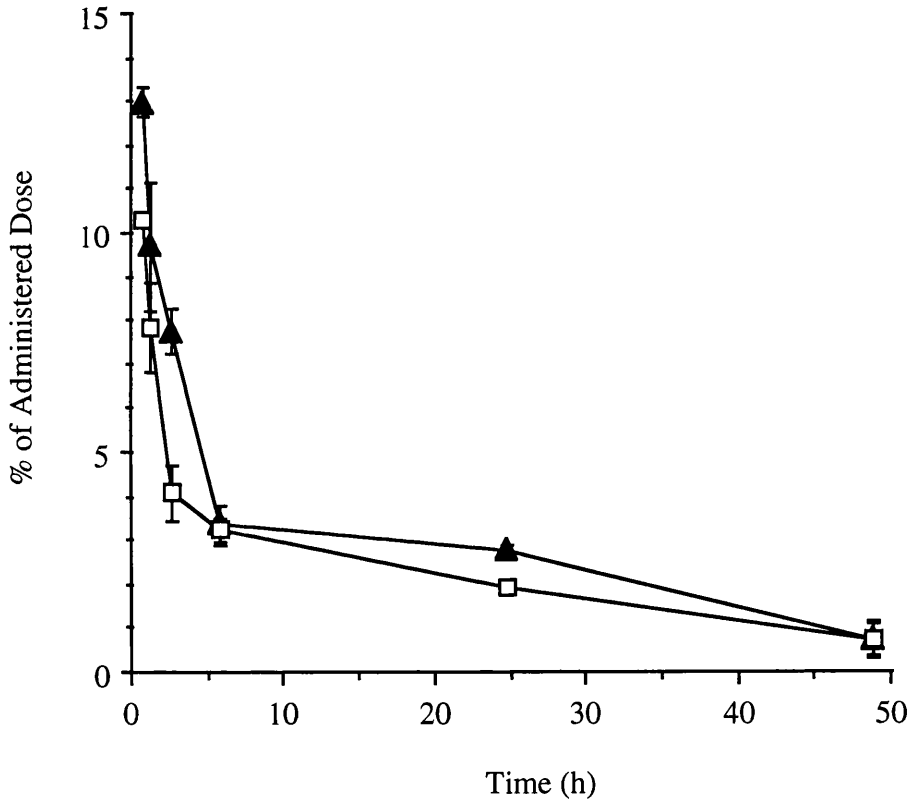


^{125}I-Labelled HPMA copolymer-β-lactamase

^{125}I-Labelled β-lactamase

n=3
Symbols represent mean±SE

Figure 4.9 Liver accumulation of free and conjugated ^{125}I-labelled β-lactamase in C57 black male mice bearing B16F10 melanoma

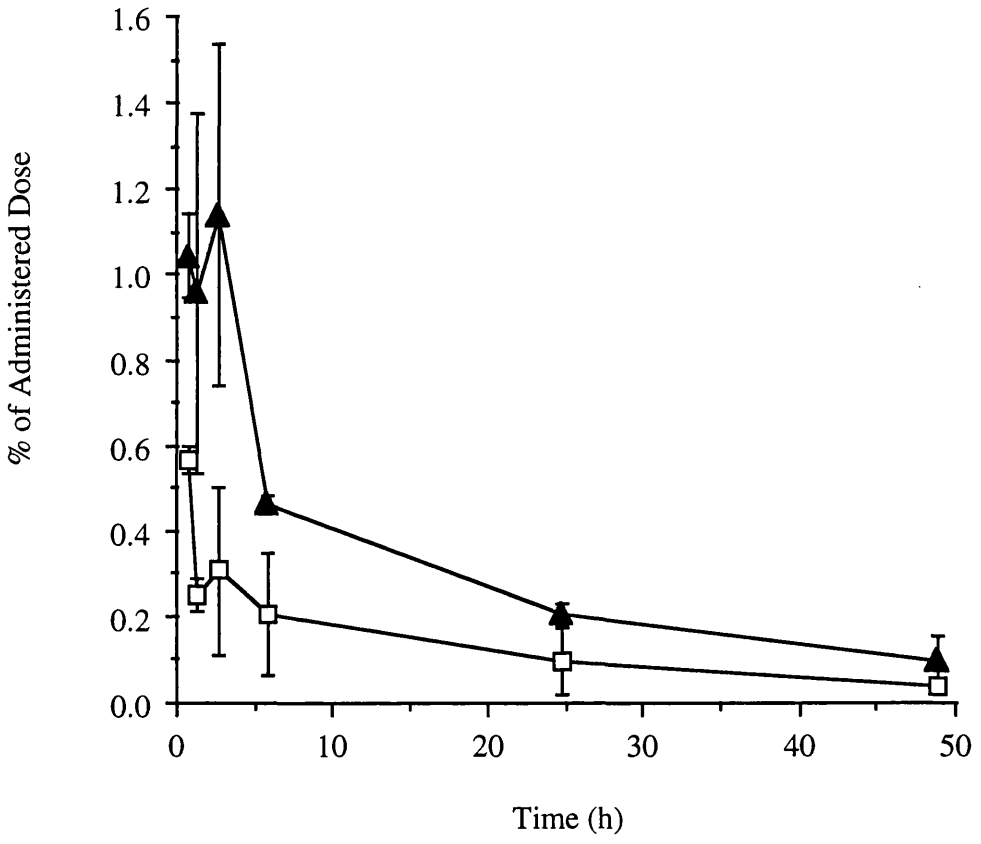


^{125}I-Labelled HPMA copolymer-β-lactamase

^{125}I-Labelled β-lactamase

n=3
Symbols represent mean±SE

Figure 4.10 Lung accumulation of free and conjugated ^{125}I-labelled β-lactamase in C57 black male mice bearing B16F10 melanoma



n=3
Symbols represent mean±SE

Table 4.2 Comparison of AUC value between free and conjugated β-lactamase in different organs

Organ	AUC β-lactamase (%administered dose/g h)	AUC HPMA copolymer-β-lactamase (%administered dose/g h)	AUC bound/ AUC free
Tumour	0.26	0.86	3.24 *
Liver	1.93	2.58	1.33 n.s.
Kidneys	1.87	1.78	0.95 n.s.
Heart	0.03	0.11	4.48*
Spleen	0.07	1.36	1.96 n.s.
Lungs	0.1	0.27	2.82 *
Blood	1.09	4.03	3.7 *

* Significant difference obtained by the Student's t-test $p<0.05$

n.s. No significant difference obtained ($p>0.05$)

Figure 4.11 shows the residence of free and bound ^{125}I-labelled β-lactamase in the circulation. The polymer-enzyme conjugate showed a 3.7 fold increase in AUC in the blood with respect to the free enzyme. Free ^{125}I-labelled-β-lactamase had $t_{1/2\alpha}$ =2.8 min and $t_{1/2\beta}$ =1.3 h. ^{125}I-labelled HPMA copolymer-β-lactamase had $t_{1/2\alpha}$ =3.6 min and $t_{1/2\beta}$ =3.5 h.

The thyroid was routinely taken and the presence of radioactivity used as an indication of free [^{125}I] iodine in the preparation. No radioactivity was detected in any of these samples (results not shown).

Urine was collected from mice kept for 24 h and 48 h in metabolic cages. Collected urine was passed down a Sephadex G25 column (PD-10) and the radioactivity, in the collected fractions assessed using the γ-counter. A clear separation of free and bound radioactivity was observed (Figure 4.12).

The levels of radioactivity recovered from the total organs dissected, blood and urine, expressed as a recovery of dose administered, were between 70-85% for all mice.

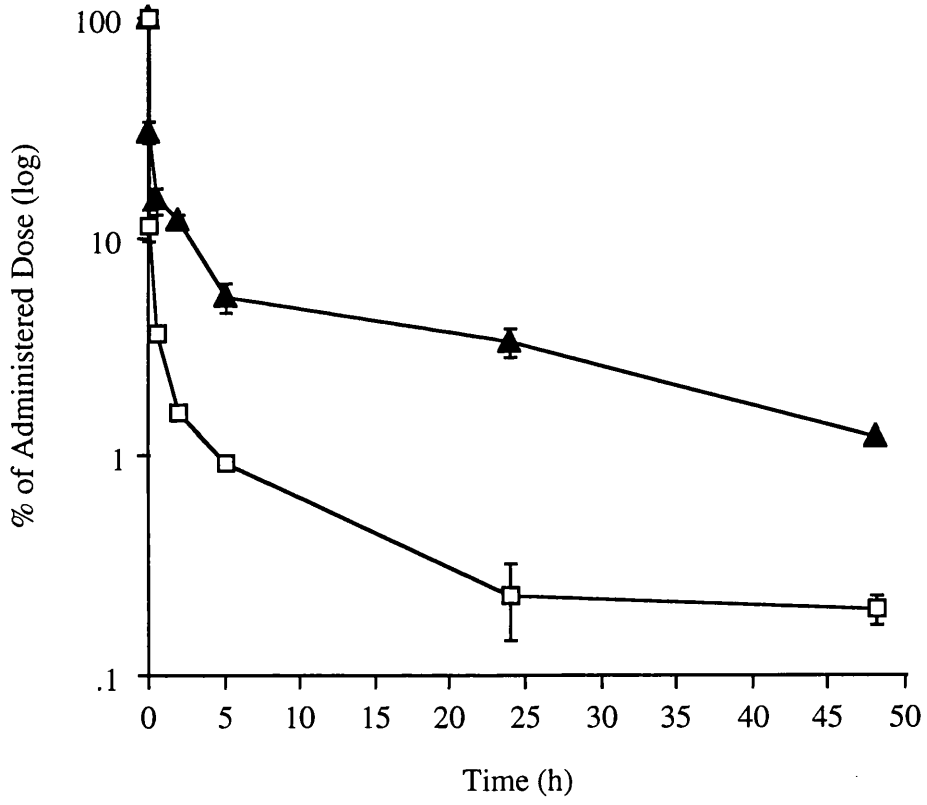
4.3.4 Biodistribution of free and conjugated ^{125}I-labelled cathepsin B

^{125}I-Labelled cathepsin B and ^{125}I-labelled HPMA copolymer-Gly-Gly-cathepsin B were administered i.v. to C57 mice bearing s.c. B16F10 melanoma to compare their pharmacokinetics. Analysis of the levels of accumulation of ^{125}I-labelled free and bound cathepsin B in key organs (including tumour, heart, kidney, spleen, liver, thyroid and lungs) and blood were expressed as percent of the administered dose ($5x10^5$ CPM/mouse).

Figures 4.13 (tumour), 4.14 (heart), 4.15 (kidney), 4.16 (spleen), 4.17 (liver) and 4.18 (lungs) show the data collected for all the organs for ^{125}I-labelled cathepsin B and ^{125}I-labelled HPMA copolymer-Gly-Gly-cathepsin B. The polymer-enzyme conjugate showed a 4.2-fold increase in AUC in the tumour compared to free enzyme. Table 4.3 shows the ratio of AUC between free and conjugated cathepsin B in the different organs.

Figure 4.19 shows the residence of free and bound cathepsin B in the circulation. The polymer-enzyme conjugate showed a 1.4-fold increase in AUC in the blood with respect to the free enzyme. Free cathepsin B had $t_{1/2\alpha}$ =2.8 h and $t_{1/2\beta}$ =8.9 h. HPMA copolymer-cathepsin B had $t_{1/2\alpha}$ =3.2 h and $t_{1/2\beta}$ =9.3 h.

Figure 4.11 Free and conjugated ^{125}I-labelled β-lactamase in blood of C57 black male mice bearing B16F10 melanoma

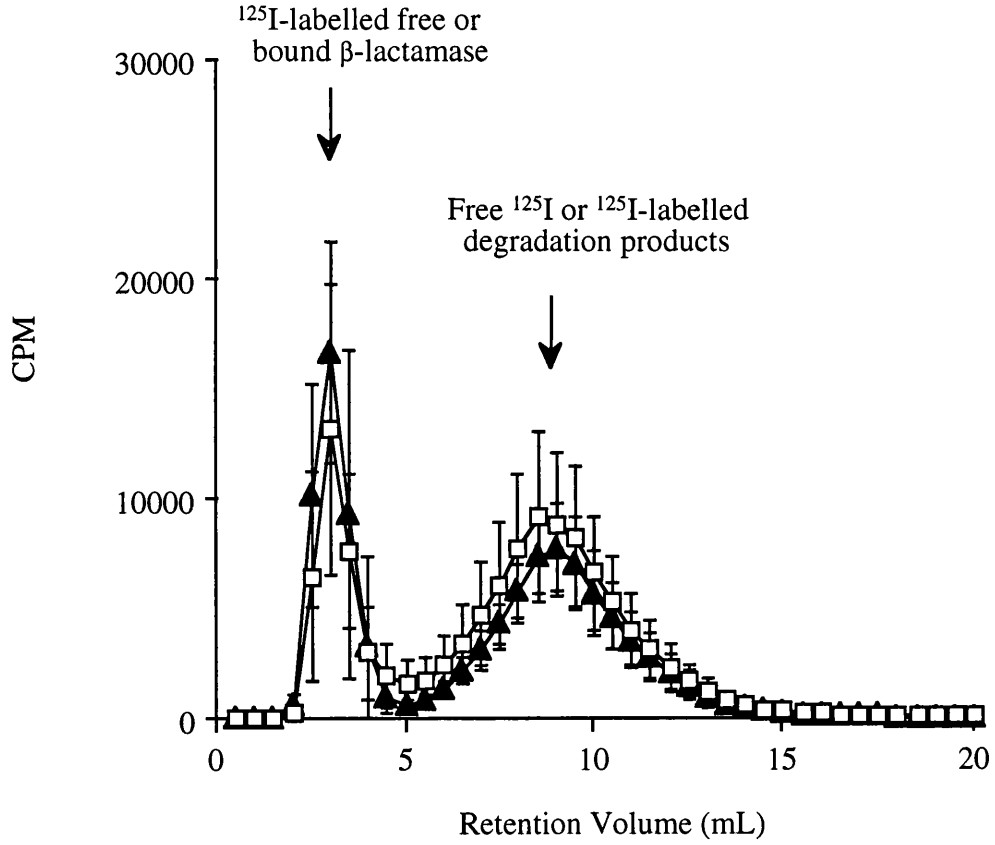


^{125}I-Labelled HPMA copolymer-β-lactamase

^{125}I-Labelled β-lactamase

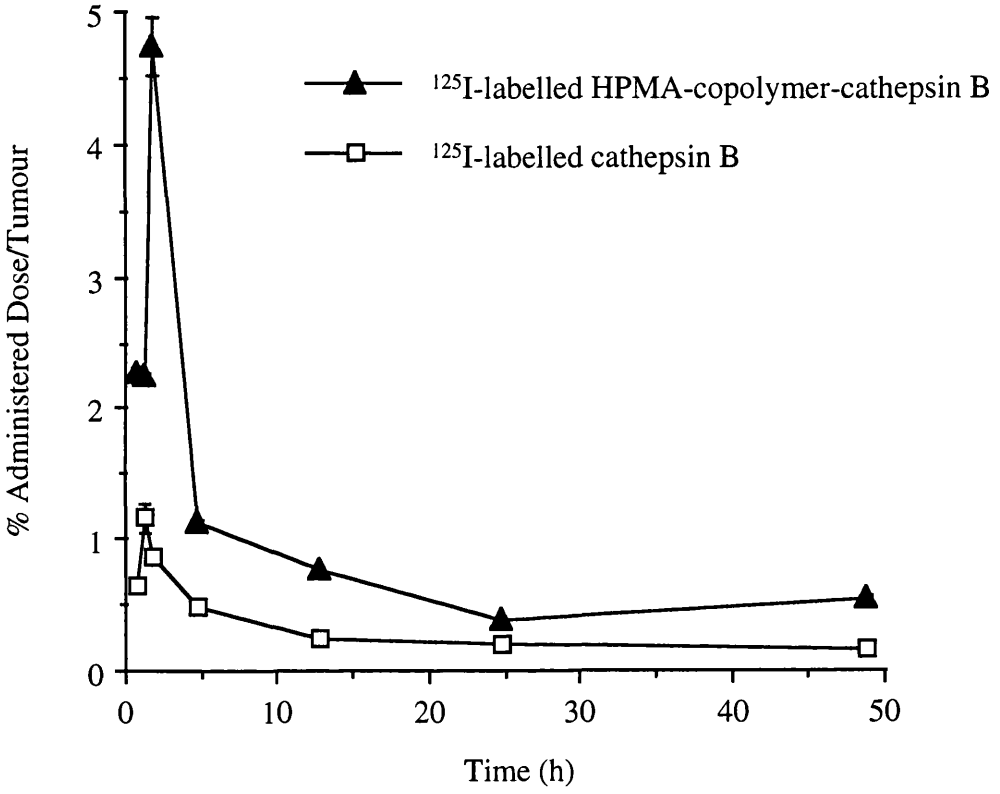
n=3
Symbols represent mean±SE

Figure 4.12 PD-10 column chromatography of urine collected at 24 h after administration of ^{125}I-labelled β-lactamase or ^{125}I-labelled HPMA copolymer-β-lactamase



n=3
Symbols represent mean±SE

Figure 4.13 Tumour accumulation of free and conjugated ^{125}I-labelled cathepsin B in C57 black male mice bearing B16F10 murine melanoma

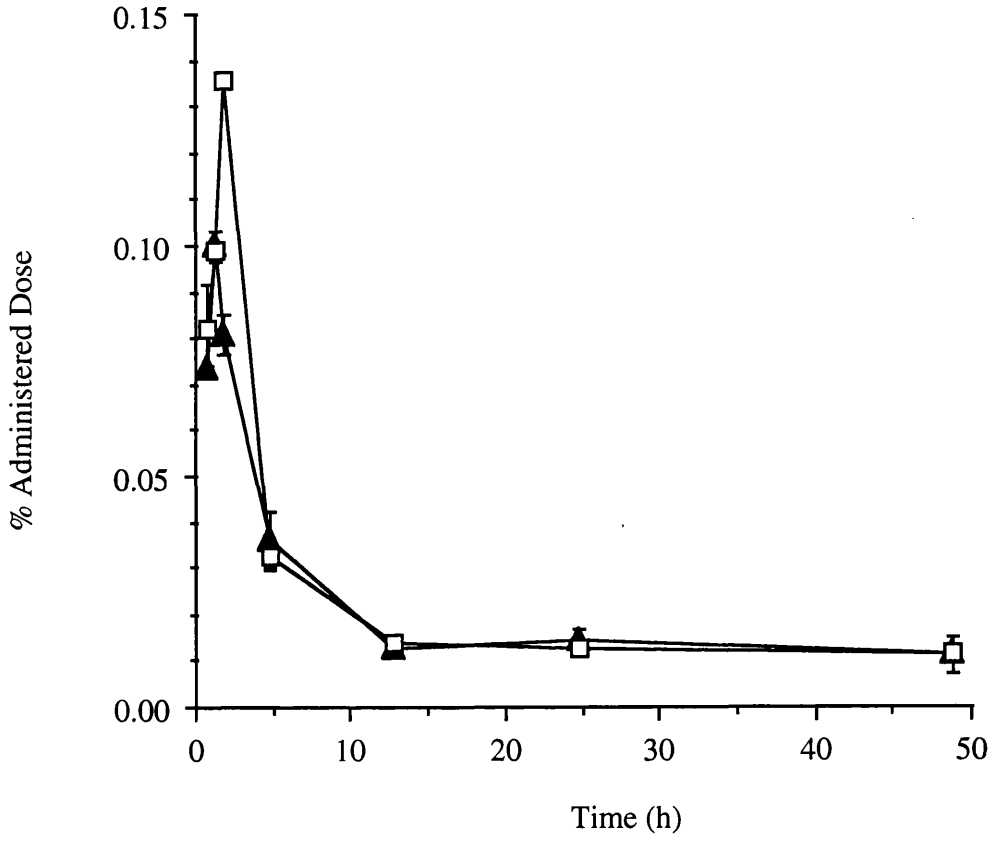


^{125}I-Labelled HPMA copolymer-β-lactamase

^{125}I-Labelled β-lactamase

n=3
Symbols represent mean±SE

Figure 4.14 Heart accumulation of free and conjugated ^{125}I-labelled cathepsin B in C57 black male mice bearing B16F10 murine melanoma

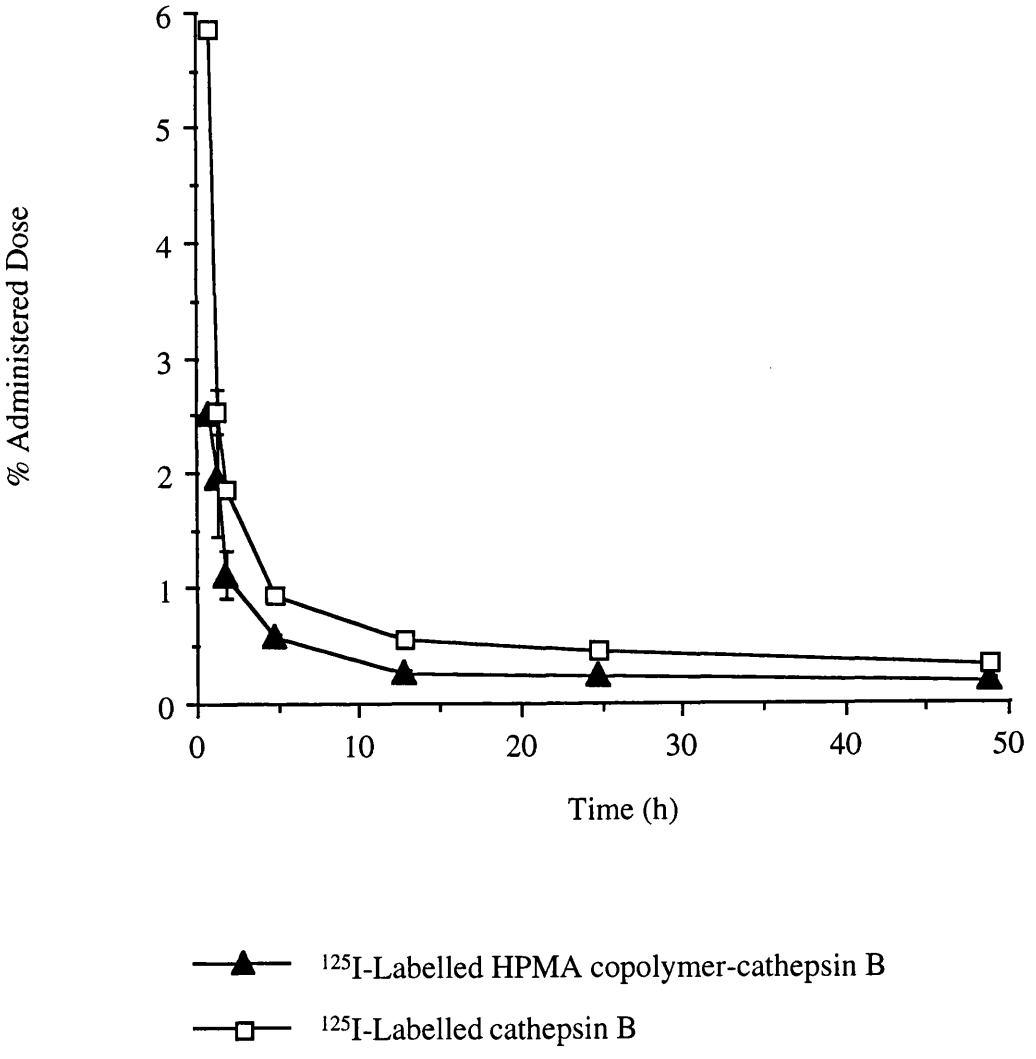


▲— ^{125}I-Labelled HPMA copolymer-cathepsin B

□— ^{125}I-Labelled cathepsin B

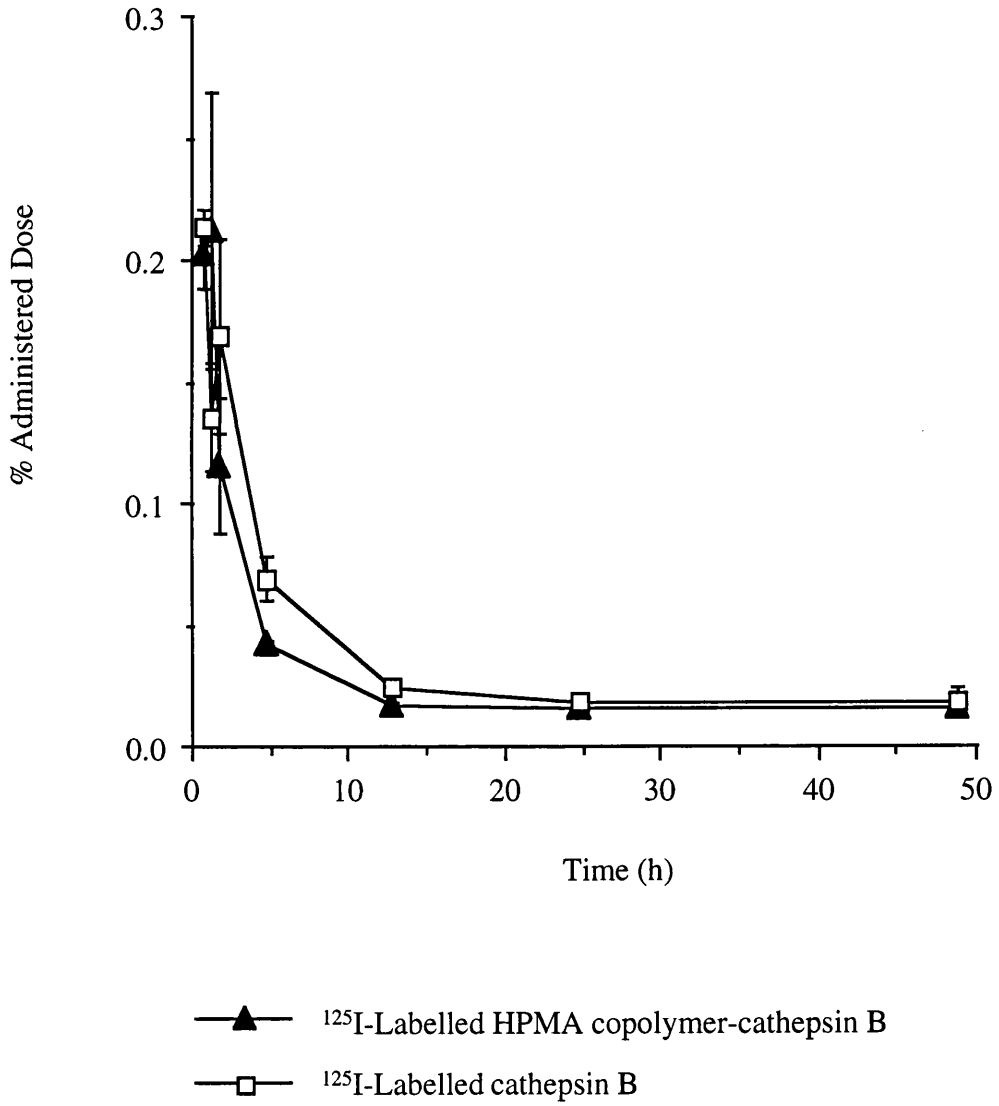
n=3
Symbols represent mean±SE

Figure 4.15 Kidneys accumulation of free and conjugated ^{125}I-labelled cathepsin B in C57 black male mice bearing B16F10 murine melanoma



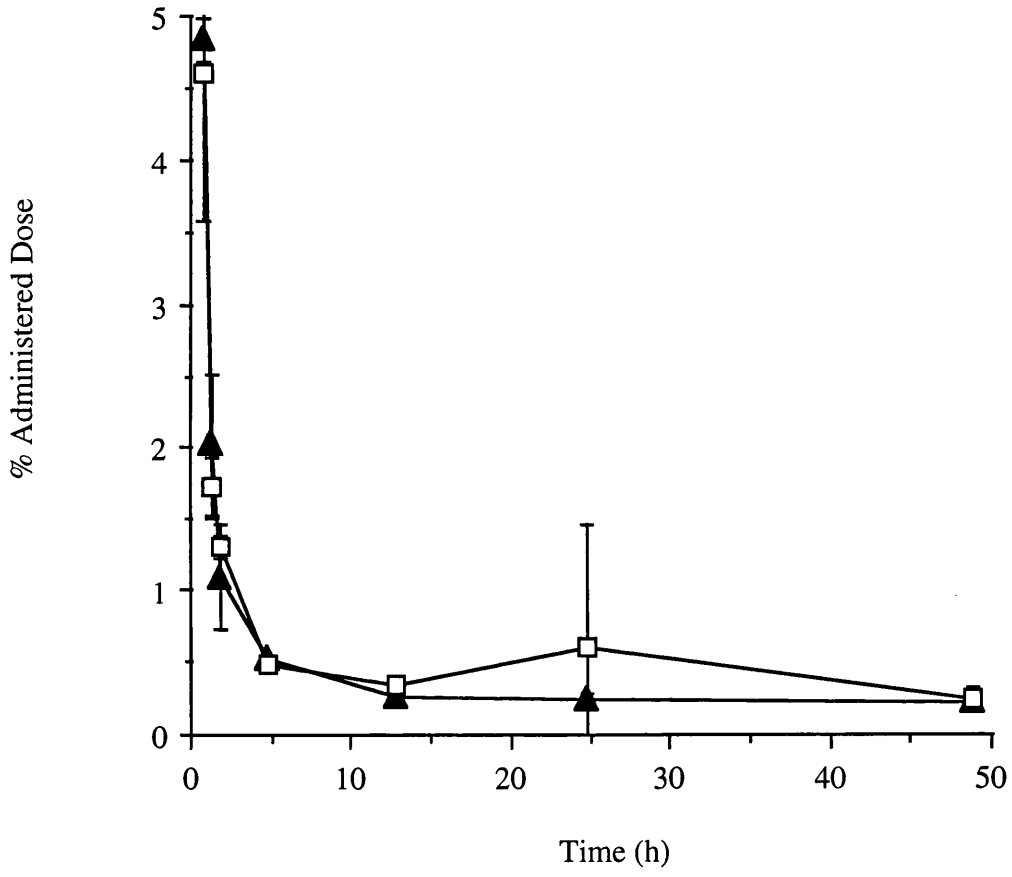
n=3
Symbols represent mean±SE

Figure 4.16 Spleen accumulation of free and conjugated ^{125}I-labelled cathepsin B in C57 black male mice bearing B16F10 murine melanoma



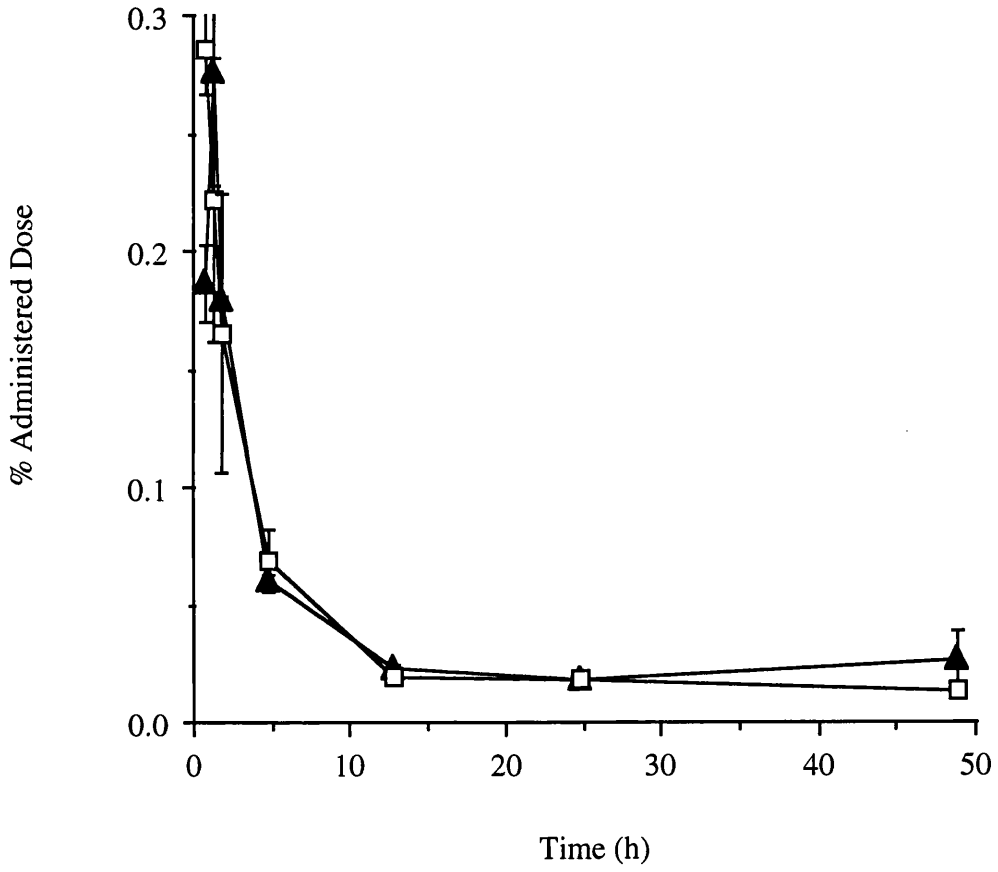
n=3
Symbols represent mean±SE

Figure 4.17 Liver accumulation of free and conjugated ^{125}I-labelled cathepsin B in C57 black male mice bearing B16F10 murine melanoma



n=3
Symbols represent mean±SE

Figure 4.18 Lung accumulation of free and conjugated ^{125}I-labelled cathepsin B in C57 black male mice bearing B16F10 murine melanoma



n=3
Symbols represent mean±SE

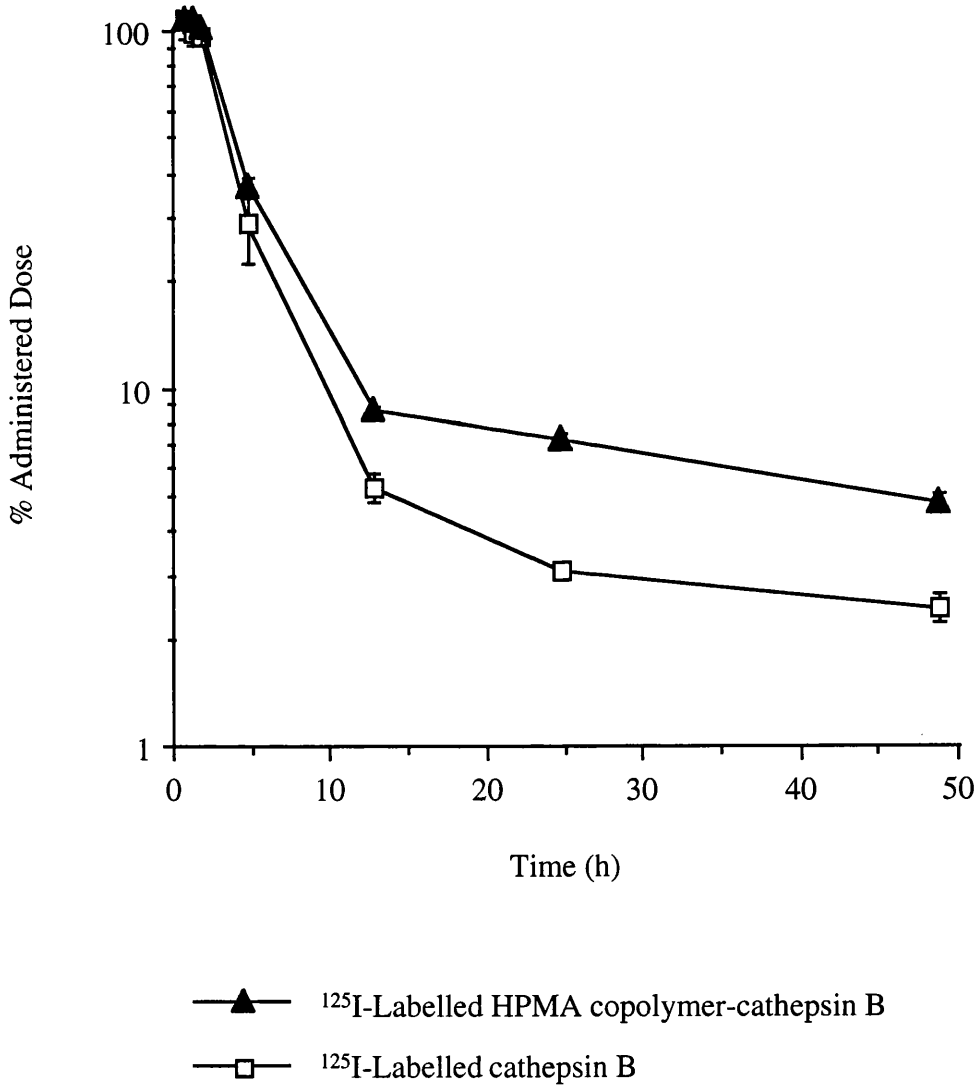
Table 4.3 Comparison of AUC value between free and conjugated cathepsin B in different organs

Organ	AUC cathepsin B (%administered dose/g h)	AUC HPMA copolymer-cathepsin B (%administered dose/g h)	AUC bound/ AUC free
Tumour	0.19	0.79	4.19 *
Liver	0.41	0.26	0.62 [n.s.]
Kidneys	0.5	0.23	0.46 [n.s.]
Heart	0.02	0.02	0.93 [n.s.]
Spleen	0.03	0.02	0.74 [n.s.]
Lungs	0.01	0.03	4.27 *
Blood	10.28	14.19	1.38 *

* Significant difference obtained by the Student's t-test $p<0.05$

[n.s.] No significant difference obtained ($p>0.05$)

Figure 4.19 Free and conjugated ^{125}I-labelled cathepsin B in blood of C57 black male mice bearing B16F10 murine melanoma



n=3
Symbols represent mean±SE

Proteins degrade to ^{125}I-labelled tyrosine moiety and also maybe free [^{125}I]iodine. In order to identify the presence of free iodine and radiolabelled degradation products in urine or excreted ^{125}I-labelled enzymes, free iodine was passed down a PD-10 column (the column does not separate between free [^{125}I]iodide and degraded ^{125}I-labelled tyrosine moieties). Free [^{125}I]iodine was eluted after collection of 9.5 ml. The γ- counter identified a number of peaks after passing the urine collected from the mice down the PD-10.

Thyroid was removed to monitor the presence of free [^{125}I]iodine in the preparation. This confirmed together with the paper electrophoresis of the ^{125}I-labelled constructs, that the levels of free [^{125}I]iodine in the preparation itself were extremely low.

The levels of radioactivity recovered from the organs dissected, blood and urine, expressed as a recovery of dose administered were between 70-85% for all mice.

4.4 Discussion

4.4.1 Blood clearance

The first consequence of the grafting of polymer chains to the protein is the increase in its apparent molecular weight and consequent decrease of the glomerular filtration rate and prolonged time of residence in circulation. This holds particularly true for small proteins and peptides that are smaller than the renal threshold for renal clearance (67-68 KDa; Francis *et al.*, 1992). However, for larger proteins (and if molecular weight alone is to be taken into consideration) the opposite would be expected due to the increased probability for the conjugate to be taken by the mononuclear-phagocyte system (MPS) leading to rapid blood clearance.

The liver and the kidney are the major sites where the catabolism of foreign proteins occurs (Bocci, 1990; Kompella and Lee, 1991; Ferraiolo and Mohler, 1992). The liver, for instance, exhibits a particularly rich vascularisation, possesses underlining discontinuous endothelia as well as several different cell surface receptors (Kompella and Lee, 1991) which explains its significant contribution to the catabolism of protein pharmaceuticals. However, since proteins are administered parenterally, liver first pass elimination does not occur (Bocci, 1990) and the kidney is thus mainly responsible for the clearance of small proteins that are within the range of glomerular filtration. Once in the ultrafiltrate, the protein is reabsorbed by endocytosis in the proximal tubule and digested in the lysosomes (Kompella and Lee, 1991; Meijer, 1994). Proteolysis in body fluids such as plasma (Bocci, 1990) also plays an important role.

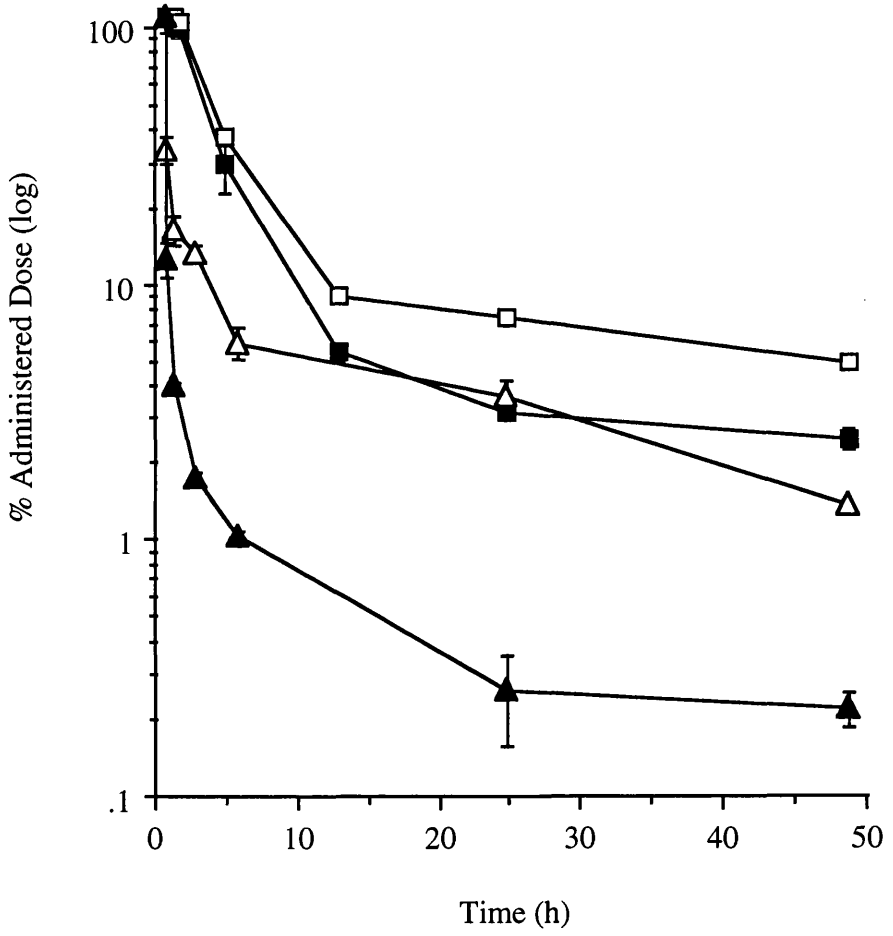
Molecular weight, charge and the presence of specific cellular receptors for the protein are factors likely to influence its clearance from plasma. Molecular weight, shape and charge for instance, determine the extent to which macromolecules undergo transcapillary passage or renal filtration (Bocci, 1990). In the latter case the molecular weight cut-off is 67-68 KDa (Francis *et al.*, 1992) i.e. around the molecular weight of the polymer-enzyme conjugates described here. In fact, if only molecular weight was to be taken into account, the opposite results (i.e. reduced $t_{1/2}$) would be expected because of the increased probability for the adduct to be taken by the MPS (Ryser, 1968).

As shown in Figure 4.20 (overlaying data from Figures 4.11 and 4.19) most of the injected labelled enzymes was eliminated from the circulation of mice within 30 min for ^{125}I-labelled β-lactamase and the first 3 h for ^{125}I-labelled cathepsin B. Both free and bound enzymes exhibited a curved log % dose *vs* time profile indicating biphasic disposition kinetics. The first part of the curve is the α phase during which the enzyme is being distributed out of the plasma into tissues or extravascularly. The second, straight line part is the β phase, during which the complete volume of distribution has been attained and enzyme elimination proceeds. However, it is not easy to use classical pharmacokinetics to describe the distribution of macromolecules. It is more likely that the conjugates, and even the free enzymes in both cases (being 30 KDa), might be rapidly excreted through the kidneys in the α phase and then distributed between only few organs in the β phase, due to the low probability to extravasate into normal tissues. The transition between α and β phases occurred at about 4 h after dosing for β-lactamase and 10 h for cathepsin B.

A plausible reason for the longer half-life of cathepsin B compared to β-lactamase (as summarised in Table 4.4) could be the fact that β-lactamase is from a bacterial source and is catabolised faster than cathepsin B, this being a mammalian enzyme and probably displaying high homology between the bovine and the mouse cathepsin B. β-Lactamase might be degraded in the plasma.

On the basis of the data shown in this chapter, it is more likely that the increased residence of the conjugated enzymes, HPMA copolymer-β-lactamase and HPMA copolymer-cathepsin B, in the circulation can be partially explained by the improved resistance to plasma proteases. Here we found that the blood residence time of both cathepsin B and β-lactamase increased on conjugation. In the case of β-lactamase there was a much higher increase of 3.6-fold compared to cathepsin B which displayed only a 1.3-fold increase in AUC of blood residence whilst conjugated to HPMA copolymer. It may be that the effect of conjugation to polymer was much more effective in terms of protection from proteolysis in the case of the β-lactamase than for the cathepsin B.

Figure 4.20 Free and conjugated ^{125}I-labelled enzymes in the circulation of C57 black male mice bearing B16F10 melanoma



n=3
symbols represent mean±SE

Table 4.4 Summary of changes in enzyme plasma half-life after conjugation to HPMA copolymer

Half-life	β-Lactamase	HPMA copolymer-β-lactamase	Cathepsin B	HPMA copolymer-cathepsin B
$t_{1/2\alpha}$	2.8 min	3.6 min	2.8 h	3.2 h
$t_{1/2\beta}$	1.3 h	3.5 h	8.9 h	9.3 h

These observations, showing extended circulation time of the modified enzymes, are consistent with the literature where asparaginase-albumin conjugates were present in the blood circulation of mice for extended periods (11% of the conjugate was still circulating 48 h post-injection, compared to only 3% of native asparaginase). Native asparaginase exhibited a half-life of 3-5 h (depending on strain) when injected i.p. to mice compared to 20-36 h observed for the poly-(D,L-alanine) derivatives. In i.v. injected rats, modified asparaginase displayed a biphasic clearance ($t_{1/2\alpha}$=4 h; $t_{1/2\beta}$=13 h) compared to the monophasic one for the native ($t_{1/2}$=1.5 h) (Uren and Ragin, 1979).

Dextran is routinely used as a plasma expander in man and its low toxicity is well established (Wileman *et al.*, 1983). Table 4.5 shows some of the proteins that have been modified with dextran and the respective circulating half-lives as compared to the native counterparts. In every case (except for uricase), the dextran-enzyme's blood residence was markedly improved by a factor of between 2 and 71. The half-life of the adducts usually increased with increasing size of the dextran. However, dextran of extremely high molecular weight may not be as efficient carrier as one with a lower molecular weight (Wileman *et al.*, 1986). The circulation times of asparaginase-dextran conjugates compared particularly well with the native enzyme in pre-immune mice. Catalase-dextran conjugates attained maximum plasma activity levels 4.5 h after i.p. injection, as compared to the 2 h shown by the native enzyme (Marshall *et al.*, 1977). This probably reflects the decreased ability of the conjugate to extravasate from the peritoneal cavity, as anticipated from the increased size.

Following PEG attachment, both small (e.g. superoxide dismutase) and oligomeric proteins (e.g. catalase, asparaginase and uricase), showed enhanced blood half-lives (Table 4.6). Superoxide dismutase, an enzyme that undergoes glomerular filtration, shows the most dramatic increase in blood residence time by PEGylation (Fuertges and Abuchowski, 1990), supposedly on account of hindered renal clearance. Extended blood residence of higher molecular weight proteins, on first injection, is attributed to reduced cellular clearance and increased resistance to proteolysis (Francis *et al.*, 1992). As a general rule, the more PEG grafted to the protein (Francis *et al.*, 1992) and the higher the molecular weight of the PEG (Abuchowski *et al.*, 1977b; Miyata *et al.*, 1988), the greater the extension of the circulating half-life. The decreased *in vivo* antigenicity of PEG-proteins explains the improved half-life (as compared with the native enzymes) even after repeated injection (Table 4.6). PEG-arginase (Savoca *et al.*, 1979; Savoca *et al.*, 1984) and PEG-albumin (Abuchowski *et al.*, 1977a), for instance, circulated in mice pre-immunised for a period of 90 days with the corresponding native

Table 4.5 Circulating half-lives of proteins modified with dextran of different molecular weights

Protein	Animal	$t_{1/2}$ native	$t_{1/2}$ modified	$t_{1/2}$ fold increase	Reference
α-Amylase	Rat	2 h: 16% *	2 h:75% *	5	Marshall *et al.*, 1977
Asparaginase bound to dextran (70 KDa)	Man	12 h	11 d	22	Wileman *et al.*, 1983
	Rabbit	11 h	190 h	17	Benbough *et al.*, 1979
Asparaginase bound to dextran (40 KDa)	New Zealand white rabbits	8 h	46 h	6	Wileman *et al.*, 1986
Asparaginase bound to dextran (70 KDa)			56 h	7	Wileman *et al.*, 1986
Asparaginase bound to dextran (250 KDa)			36 h	5	Wileman *et al.*, 1986
Asparaginase bound to dextran (40 KDa)		<0.1 h after repeated dose	1.6 h after repeated dose	16	Wileman *et al.*, 1986
Asparaginase bound to dextran (70 KDa)			1.7 h after repeated dose	17	Wileman *et al.*, 1986
Asparaginase bound to dextran (250 KDa)			7.1 h after repeated dose	71	Wileman *et al.*, 1986
Catalase	Acatalasemic mice	17 min (α)	140 min (α)	8	Marshall *et al.*, 1977
Uricase bound to dextran (10 KDa)	ddY mice	25 min (α)	31 min (α)	1.2	Fujita *et al.*, 1990
		3.5 h (β)	2.3 h (β)	<1	Fujita *et al.*, 1990

All $t_{1/2}$ were calculated following i.v. administration
* When $t_{1/2}$ were not reported the % of protein in circulation at a certain time is given instead

Table 4.6 Circulating half-lives of PEGylated proteins of different molecular weights

Protein	Animal	$t_{1/2}$ native	$t_{1/2}$ modified	$t_{1/2}$ fold increase	Reference
Arginase	B6D2 F1/J mice	12 h: 10%	12 h: 52% 72 h: 16%	5	Savoca *et al.*, 1979
		<1 h after repeated dose	12 h after repeated dose	12	Savoca *et al.*, 1979
Asparaginase	Man	7-28 h	16-25 d	26	Park *et al.*, 1981
	Man	20 h	357 h	18	Ho *et al.*, 1986
	Wistar rats *	2.9 h	56 h	19	Kamisaki *et al.*, 1981
	BDF_1 mice *	5 h	3.75 d	18	Abuchowski *et al.*, 1984
Catalase bound to PEG-1900	Acatalasemic mice	12 h: 2%	12 h: 10% 48 h: 3%	5	Abuchowski *et al.*, 1977b
Catalase bound to PEG-5000			12 h: 20% 48 h: 10%	10	Abuchowski *et al.*, 1977b
	Man	2 h after repeated dose	50 h after repeated dose	25	Fuertges and Abuchowski, 1990
Phenylalanine ammonia-lyase	Swiss-Webster mice	6 h	20 h	3	Wieder *et al.*, 1979
		1 h after repeated dose	4 h after repeated dose	4	Wieder *et al.*, 1979
Superoxide dismutase	Man	25 min	4.4 d	253	Fuertges and Abuchowski, 1990
	Rat	1.6 h	17.5 h	11	Miyata *et al.*, 1988

All $t_{1/2}$ were calculated following i.v. administration except * i.p.
When $t_{1/2}$ were not reported the % of protein in circulation at a certain time is given instead

enzyme, as in intact animals. Under the same conditions, native enzyme was rapidly cleared from the circulation.

4.4.2 Tumour capture by the EPR effect

Figure 4.21 (overlaying data from Figures 4.5 and 4.13) shows the accumulation of both ^{125}I-labelled free and bound enzymes in B16F10 melanoma tumours. The higher accumulation in the tumour, is probably due to the combination of size and stability in the circulation. The molecular weight of the free enzymes, β-lactamase and cathepsin B, (30 KDa) is already high enough to allow them to exhibit the EPR effect in tumour tissues, but the higher molecular weight of the conjugate (and maybe resistance to proteolysis) allows it to exhibit a longer half-life and thus higher progressive accumulation in the tumour (as described in Chapter 1.4; Seymour *et al.*, 1995).

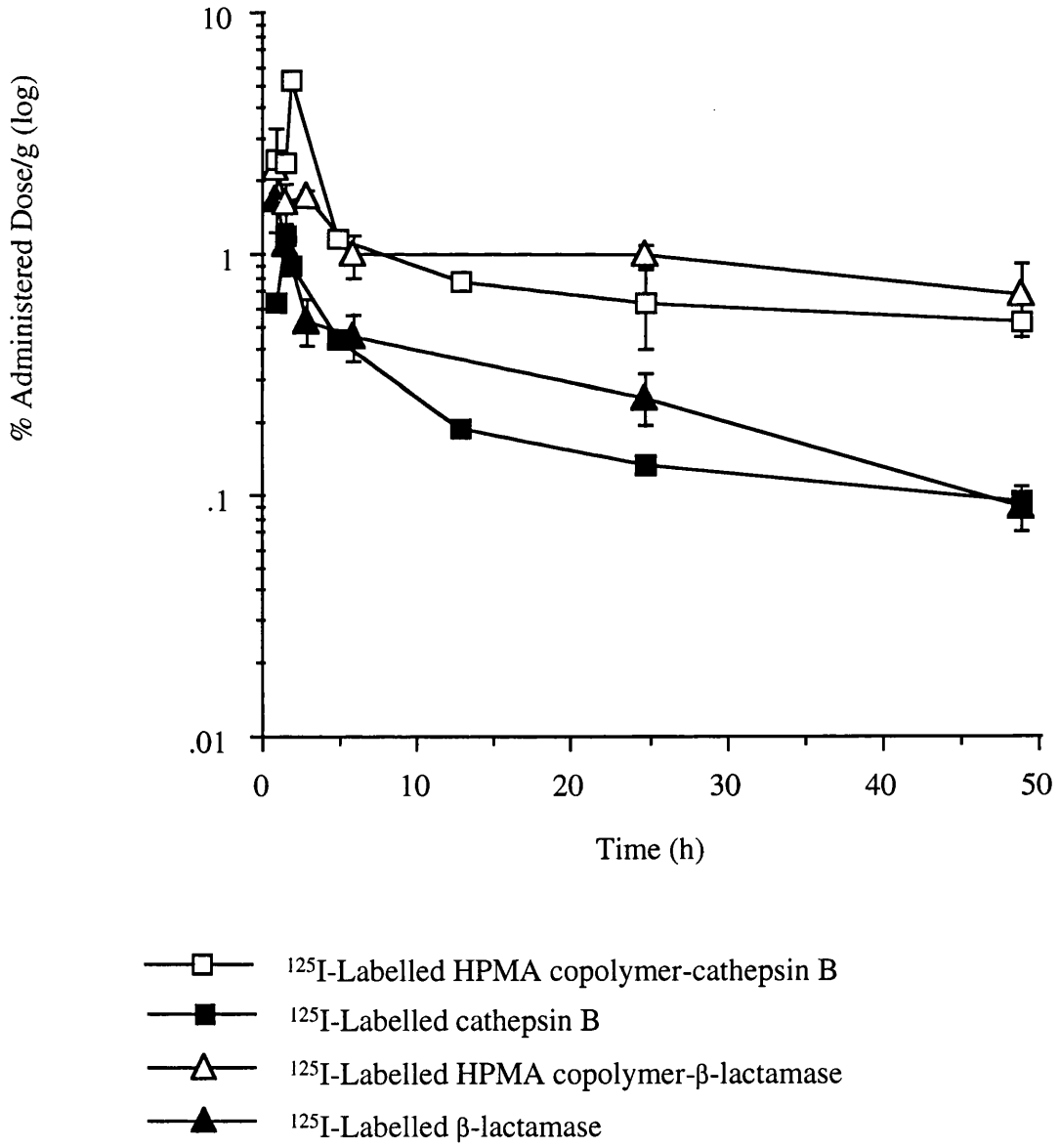
There was, however, a difference between the two systems. ^{125}I-Labelled HPMA copolymer-β-lactamase increased the AUC of the tumour accumulation of free β-lactamase by 2-fold, whilst ^{125}I-labelled HPMA copolymer-cathepsin B increased the AUC by 3.7 compared to free cathepsin B. Comparison of the AUC of tumour accumulation of the two bound enzymes shows that ^{125}I-labelled HPMA copolymer-cathepsin B displayed a 1.6-fold increase in AUC compared to ^{125}I-labelled HPMA copolymer-β-lactamase. A plausible reason for this would be the longer half-life of bound cathepsin B compared to bound β-lactamase allowing progressive accumulation in the tumour by the EPR effect.

4.4.3 General body distribution-potential for toxicity?

In both systems of β-lactamase and cathepsin B there was no significant difference in accumulation in liver, kidneys, heart and spleen (as shown in Tables 4.2 and 4.3 respectively). However, there was a significant difference in lungs which could be explained by the increased molecular size of the conjugates (see Chapter 1.4) and should be taken into consideration when examining the toxicity of the PDEPT combination. There was a significant increase in AUC of tumour accumulation in which the conjugates showed a favourable accumulation implying its advantage in targeting the polymer-enzyme to release a drug from its polymeric backbone selectively in the tumour when applying a PDEPT combination.

Although there was no significant difference in AUC in liver and kidney, both the free and conjugated enzymes displayed a high accumulation in those organs. One should bear in mind that the liver was not perfused prior to analysis, and as it exhibits a particularly rich vascularisation, the radioactivity present in the blood was read as well.

Figure 4.21 Tumour accumulation of free and conjugated ^{125}I-labelled enzymes in C57 black male mice bearing B16F10 murine melanoma



n=3
symbols represent mean±SE

As for the accumulation in the kidneys, macromolecules are excreted via the kidneys (as discussed in the introduction 4.1) so it is reasonable that high radioactivity would be present. ^{125}I-Labelled HPMA copolymer-β-lactamase displayed a 5-6-fold increase in AUC in liver and kidneys compared to ^{125}I-labelled HPMA copolymer-cathepsin B, probably catabolised in the liver due its bacteriologic source and excreted more rapidly that its mammalian counterpart.

Results suggest that since HPMA copolymer-enzyme conjugates have long circulating time and both accumulate preferably in the tumour, they may be efficient as the activating enzyme in the second step of the PDEPT combination. Moreover, since the extent of retention of enzymatic activity of conjugated cathepsin B on PK1 *in vitro* was 20% (as evaluated in Chapter 3.3.7) it was reasonable to follow investigation of *in vivo* pharmacokinetics of drug release with the PDEPT combination. This is reported in the next chapter.

Chapter Five

PK1 and HPMA copolymer-cathepsin B combination: in vivo pharmacokinetics of doxorubicin release

5.1 Introduction

It is universally accepted that pharmacokinetics and toxicokinetics provide useful tools to assist in drug development from preclinical into the clinical phase (Duncan and Spreafico, 1994). Therefore it was considered important to evaluate the pharmacokinetics of doxorubicin release from PK1 by HPMA copolymer-cathepsin B in different tissues and blood. It was important to prove that the polymer enzyme could indeed release doxorubicin from PK1 *in vivo* and knowledge of the time course of this release would help in optimisation of the PDEPT schedule.

The antitumour antibiotic doxorubicin is a small amphipatic molecule that diffuses easily through biological membranes (Brown and Haider, 1984; Chapter 1.9.3). When administered in free form, doxorubicin displays a ubiquitous body distribution confirmed by a study which showed little or no preferential accumulation of doxorubicin in breast tumour tissue (tumour : normal tissue ratio, 1.1-1.8:1.0) (Stallard *et al.*, 1988). Seymour *et al.* (1990; 1994) investigated the pharmacokinetics of PK1. A fluorimetric HPLC method (as described in Chapter 2.3.11) was used to measure levels of free doxorubicin in plasma and tissue samples, and a new technique to quantitate levels of polymer-bound anthracyclines was developed (Wedge, 1990; Wedge *et al.*, 1991). These early studies showed that PK1 is unable to diffuse through cellular membranes and consequently displays a lower volume of distribution and longer plasma half-life. It was found that the circulating half-life of PK1 was approximately 15 times longer than that of free doxorubicin (Seymour *et al.*, 1990). The initial peak of free doxorubicin in the heart was reduced 100-fold following administration of PK1 because of its poor membrane permeability preventing its entry into cardiac tissue (Seymour *et al.*, 1990). These alterations in pharmacokinetics may account for the decreased toxicity and improved efficacy reported for PK1 compared to free doxorubicin both in animal models (Yeung *et al.*, 1991; Duncan *et al.*, 1992; O'Hare *et al.*, 1993; Seymour *et al.*, 1994) and man (Vasey *et al.*, 1999).

It was demonstrated already in Chapter 4 that HPMA copolymer-enzyme conjugates can display specific tumour accumulation by the EPR effect and from the literature it was known that PK1 accumulates preferentially in solid tumours (Seymour *et al.*, 1994). The kinetics of drug liberation from PK1 by endogenous lysosomal proteases is already well established but here it was necessary to determine the kinetics of release of doxorubicin from PK1 following the administration of HPMA copolymer-cathepsin B (as part of the PDEPT combination) *in vivo* in mice. Initially to determine the appropriate experimental conditions an experiment was conducted in the s.c. B16F10 model where PK1 (5 mg/Kg) was first administered i.v. followed after 5 h by the administration of HPMA copolymer-cathepsin B i.v. An HPLC assay was used to

determine the levels of free doxorubicin in tumour and blood at 5 h for PK1 administered alone, 6 h and 10 h for PK1 followed after 5 h by free or bound cathepsin B. After this initial study a more elaborated experimental protocol was developed using a PK1 dose of 10 mg/Kg and analysing other tissues levels of free doxorubicin in addition to tumour levels. The main purpose of this study was to compare the rate or amount of doxorubicin release in the tumour tissue following administration of PK1 alone and in the case of PK1 administration followed by HPMA copolymer-cathepsin B i.e. the PDEPT model. The tumour model used was B16F10 murine melanoma in C57 mice in order to be able to correlate the work to the studies described in Chapter 4 (for polymer-enzyme conjugate) and the literature (for PK1). Free doxorubicin levels were evaluated also in blood, liver and heart as they are potential sites for toxicity. Therefore, the levels of free doxorubicin in these tissues were compared with those present in the tumour.

5.2 Methods

As indicated before, two separate experiments were conducted following basically the same protocol, only differing in the PK1 administered dose. The first preliminary study was performed using PK1 at 5 mg/Kg doxorubicin-equivalent dose compared with PK1 followed by cathepsin B (3.63 mg/Kg) or HPMA copolymer-cathepsin B (3.63 mg/Kg cathepsin B equivalent). The second study was conducted using 10 mg/Kg PK1. C57 black male mice (6-8 weeks old) were inoculated s.c. with B16F10 murine melanoma cells and tumours were allowed to establish, as described in Chapter 2.3.10. The release of doxorubicin with time was assessed after i.v. administration via the lateral vein of a single dose of either PK1 alone (5 or 10 mg/Kg), PK1 (5 or 10 mg/Kg) followed after 5 h by either free cathepsin B or HPMA copolymer-cathepsin B (3.63 mg/Kg equivalent concentration by weight of cathepsin B). Animals were sacrificed at different time points (1 h to 48 h), tumours and other organs immediately isolated, washed in PBS and weighed. Figure 5.1 shows a diagram of experimental protocols for both studies conducted.

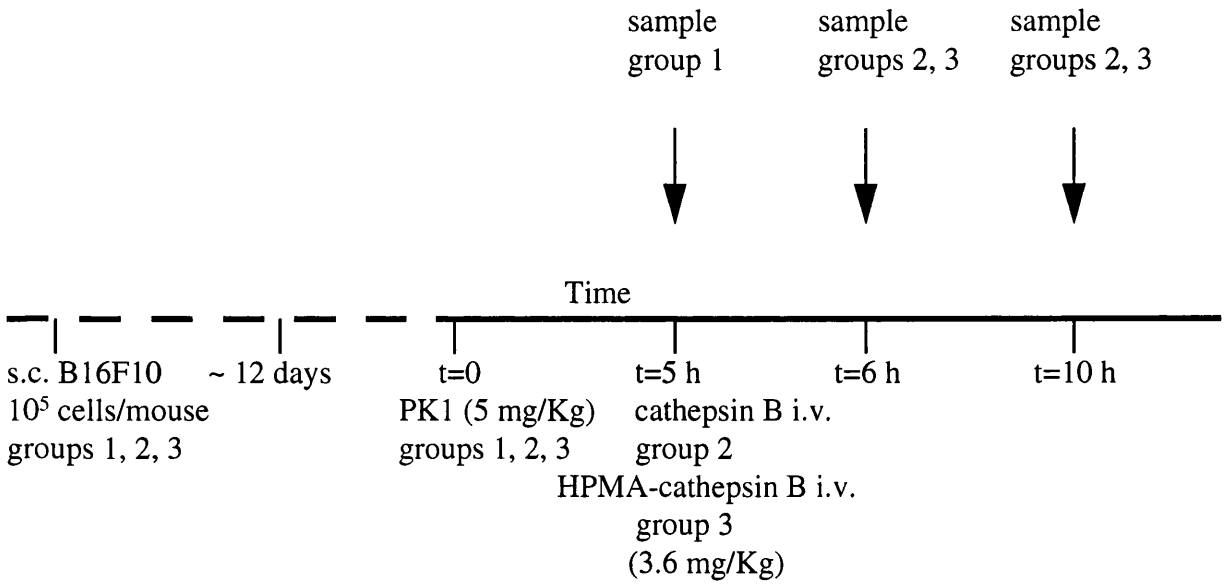
HPLC analysis of homogenised tissues (tumour, liver, heart) and blood was used to determine the levels of free doxorubicin following the protocol described in Chapter 2.3.11. Doxorubicin levels in organs are expressed as ng per g organ. The total blood volume of the mouse was calculated assuming 5.77 ml per 100 g body weight (Dreyer and Ray, 1910).

Statistical analysis

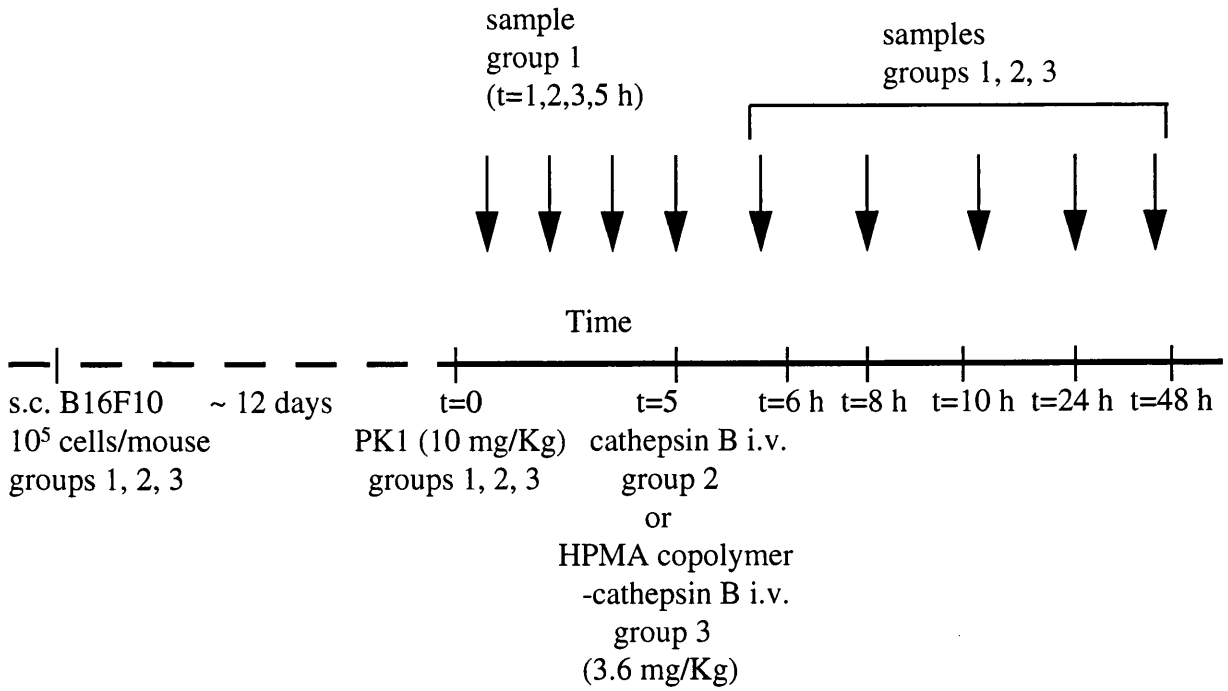
All the *in vivo* data are expressed as the mean ± SE. The areas under the amount of doxorubicin released vs time curves from time zero to 48 h (AUC_{0-48}) or from 5 h to

Figure 5.1 Diagram of experimental pharmacokinetic studies

(a) Pilot study
sample: blood, tumour



(a) Extensive time course study and dose escalation
sample: blood, tumour, liver, heart



48 h (AUC_{5-48}) were estimated by the linear trapezoidal rule. A student t-test was used for unpaired samples. P values of 0.05 or less were considered statistically significant. All statistical calculations were performed using GraphPad Prism (GraphPad Software, version 2.0a for PowerPC Macintosh, 1997).

5.3 Results

The HPLC elution profile typically obtained following extraction of blood and tissue samples displayed two well-defined peaks corresponding to the internal standard, daunomycin (t = 6 min) and the free doxorubicin (t = 3 min) present in the sample (Figure 5.2). A complication for the quantitation of doxorubicin levels in tissues using the above HPLC method was the appearance of a number of additional hydrophilic peaks at elution time 1-2 min (can be seen in Figure 5.2). Such peaks were found to be greatest for liver samples and smallest for blood samples and were clearly tissue-derived (Cummings *et al.*, 1984) since they were present also in tissue samples taken from untreated mice (Seymour *et al.*, 1990). Therefore, these peaks were ignored.

5.3.1 Pilot study (PK1 at 5 mg/Kg doxorubicin equivalent dose)

The level of free doxorubicin detected in the tumour tissue at 5 h, 6 h and 10 h after administration of PK1 (5 mg/Kg doxorubicin-equivalent dose) is shown in Figure 5.3. When PK1 was administered followed by administration of free or HPMA copolymer-bound cathepsin B it can be seen that there was an increase in level of free doxorubicin detected at 6 h and further more at 10 h. Tumour tissue samples from mice administered with PK1 alone were collected after 5 h only. It is known (Seymour *et al.*, 1994) that within that time point doxorubicin release has already reached its peak and collecting samples at later time points were unnecessary at this preliminary stage. Therefore, showing an increase above that level at 6 and 10 h due to the administration of free or HPMA copolymer-bound cathepsin B 5 h after the administration of PK1 was the proof-of-principle to continue more extensive time course study (taking samples from all three groups at all relevant time points).

5.3.2 Extensive time course study (PK1 at 10 mg/Kg doxorubicin equivalent dose)

The results of the pilot study were encouraging and led to a more extensive time course study escalating PK1 dose to 10 mg/Kg doxorubicin-equivalent dose and more time points (up to 48 h).

Figure 5.2 Typical elution chromatograms obtained following HPLC analysis of tissues from mice following intravenous administration of PK1 (a) Tumour; (b) Heart; (c) Liver
T- tissue derived; Dox-doxorubicin; Dnm- daunomycin

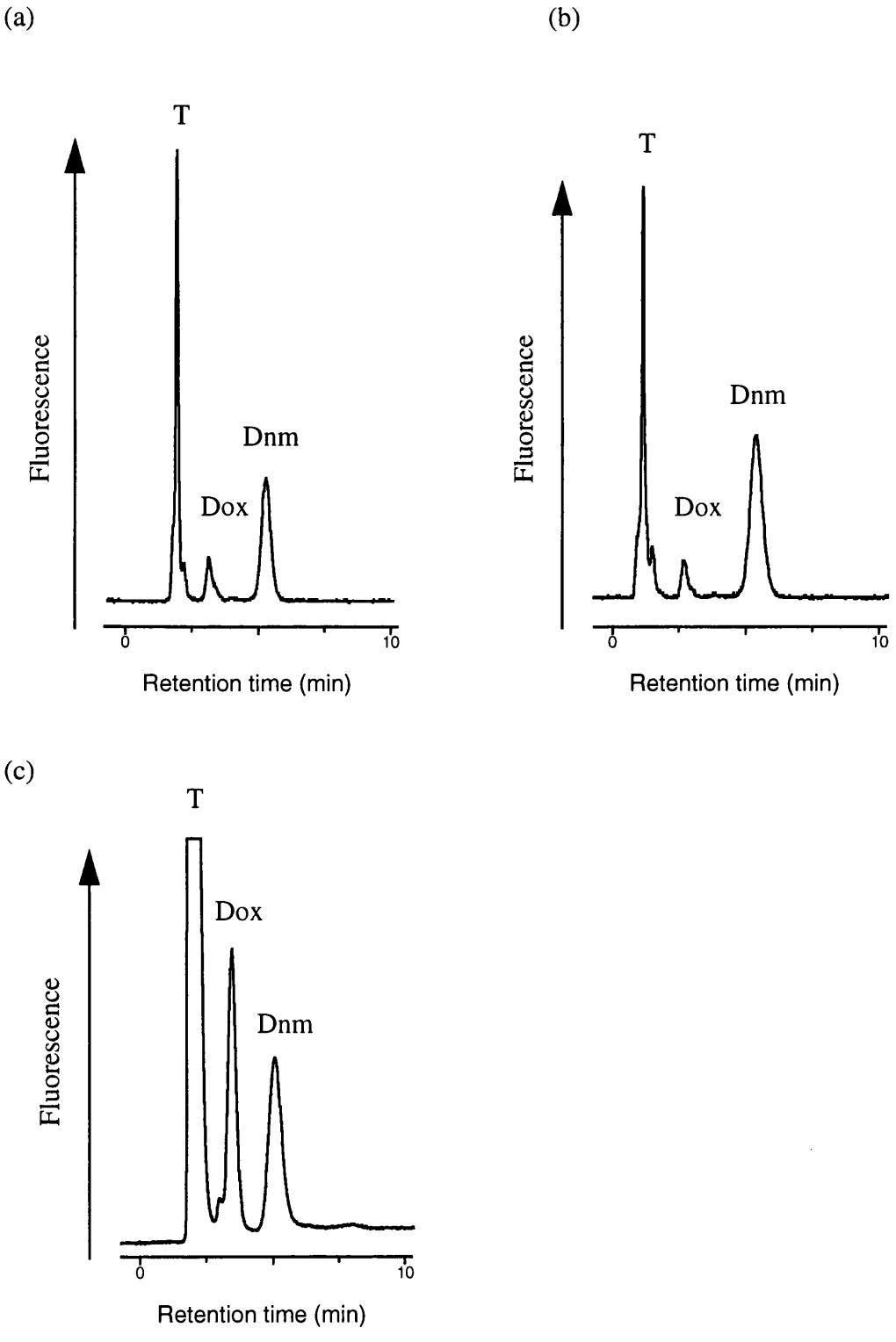
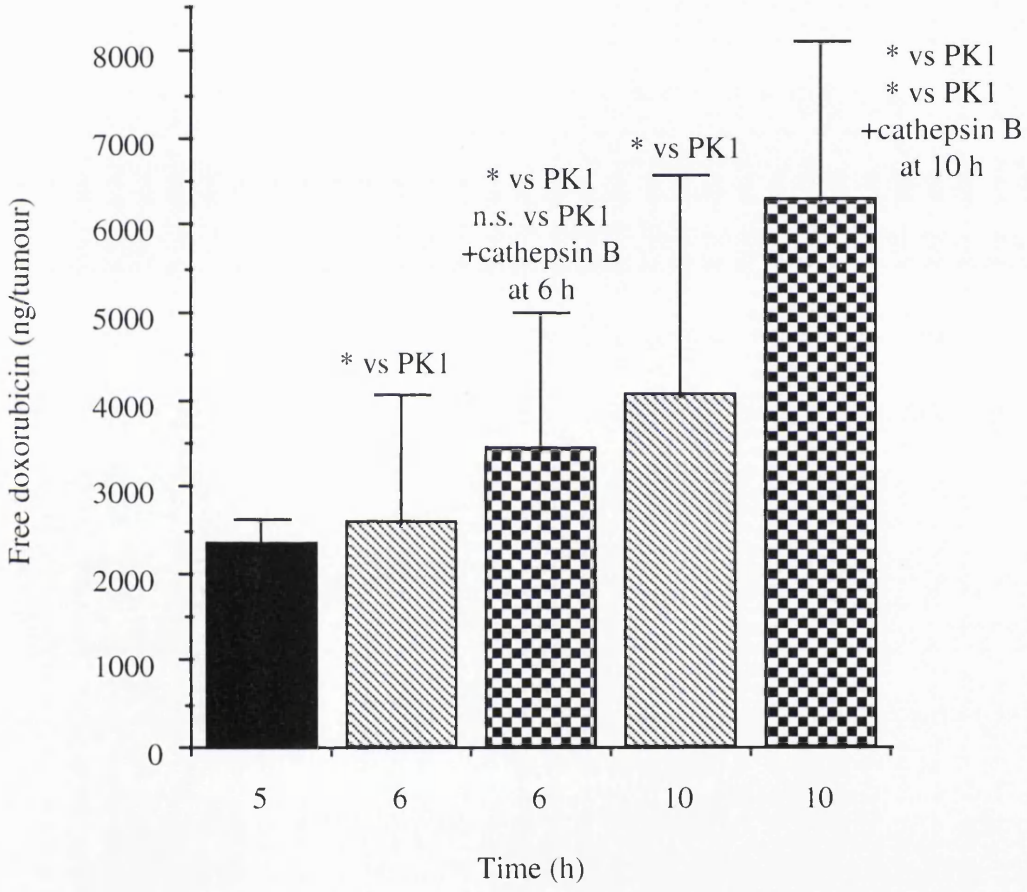


Figure 5.3 Release of doxorubicin from PK1 (5 mg/Kg) in C57 mice bearing B16F10 murine melanoma



PK1 alone (at t= 5h only)

PK1 followed after 5 h by cathepsin B

PK1 followed after 5 h by HPMA copolymer-cathepsin B

n=3
columns represent mean±SE
* $p < 0.05$
n.s. no significant difference

5.3.2.1 Tumour doxorubicin levels

Similar to the previous study, it can be seen (Figure 5.4) that administration of free or HPMA copolymer-bound cathepsin B after the administration of PK1 led to an increase in level of doxorubicin released compared to the administration of PK1 alone. Furthermore, the dose escalation from 5 to 10 mg/Kg (keeping cathepsin B at a constant dose as in pilot study) showed a doubling correlation (1.5-2.4-fold) in free doxorubicin seen at the same time points (6 h and 10 h).

Administration of HPMA copolymer-Gly-Gly-cathepsin B produced a 2.6-fold increase in AUC of free doxorubicin detected in the tumour compared to that seen for PK1 alone (at 10 mg/Kg doxorubicin-equivalent dose) which is degraded by endogenous lysosomal cathepsin B. This result is produced if we compare total AUC of PK1 (1-48 h) with AUC calculated from the PDEPT combination (5-48 h). However, ratio of the AUC values for both treatments (PK1 followed by free cathepsin B and PK1 followed by HPMA copolymer-cathepsin B) over the same time period 5-48 h gave a 3.6-fold increase and this is the correct value to be considered, since both systems should release the same amount of free doxorubicin in the first 5 h.

Although administration of free and conjugated cathepsin B increased the release of doxorubicin from PK1 to a similar extent (not significantly different), it should be noted that, in terms of activity units, the amount of enzyme activity given with HPMA copolymer-cathepsin B injected represented only 19.5% of the enzymatic activity of the injected dose of the free cathepsin B owing to the diminished specific activity of conjugated enzyme as shown in the *in vitro* drug release studies (see Chapter 3.3.7).

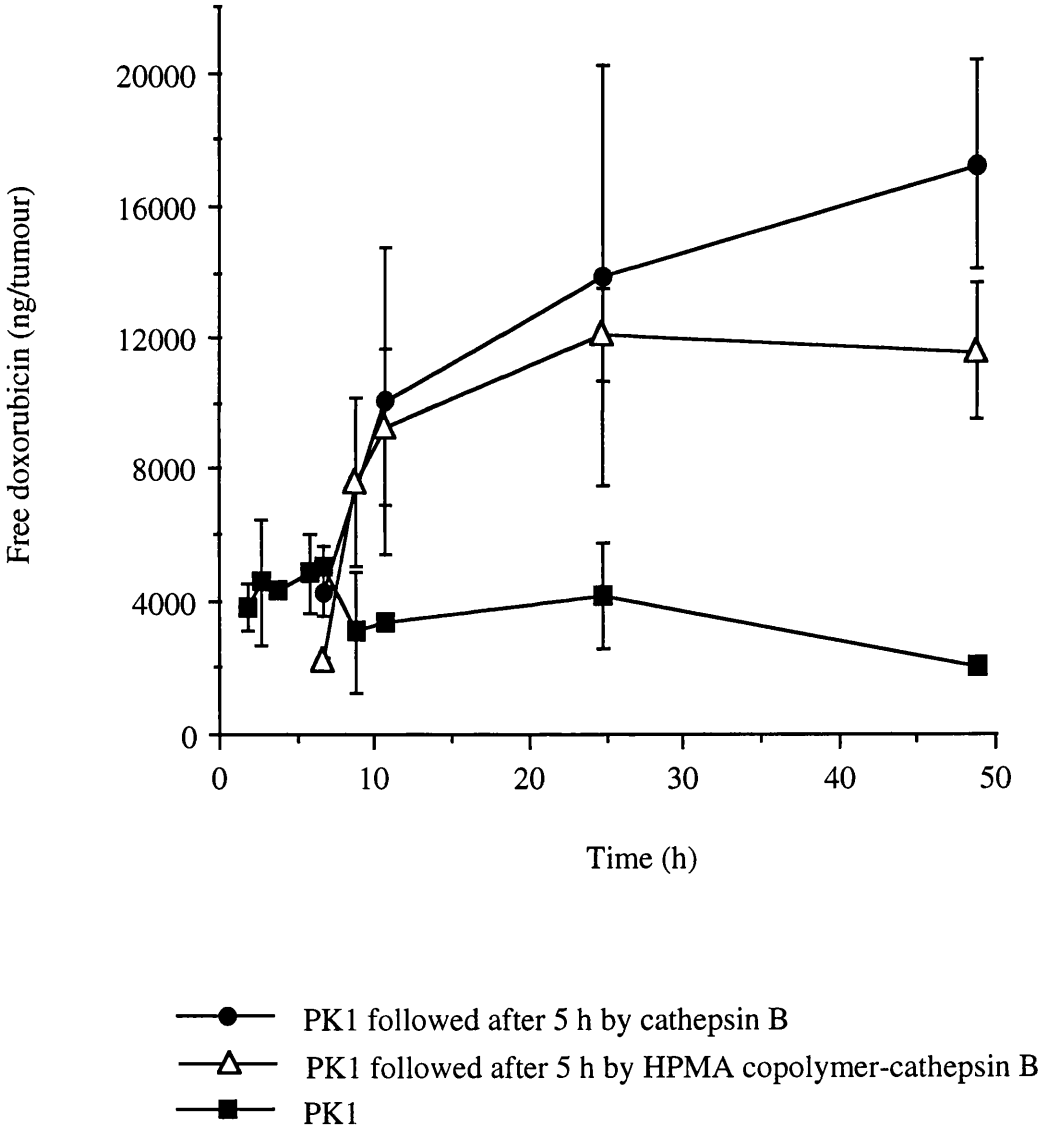
5.3.2.2 Blood doxorubicin levels

There was virtually no trace of free doxorubicin detected in the blood at any time in any of the three groups (results not shown) showing that 5 h after administration, there is no PK1 left in the circulation.

5.3.2.3 Liver and heart doxorubicin levels

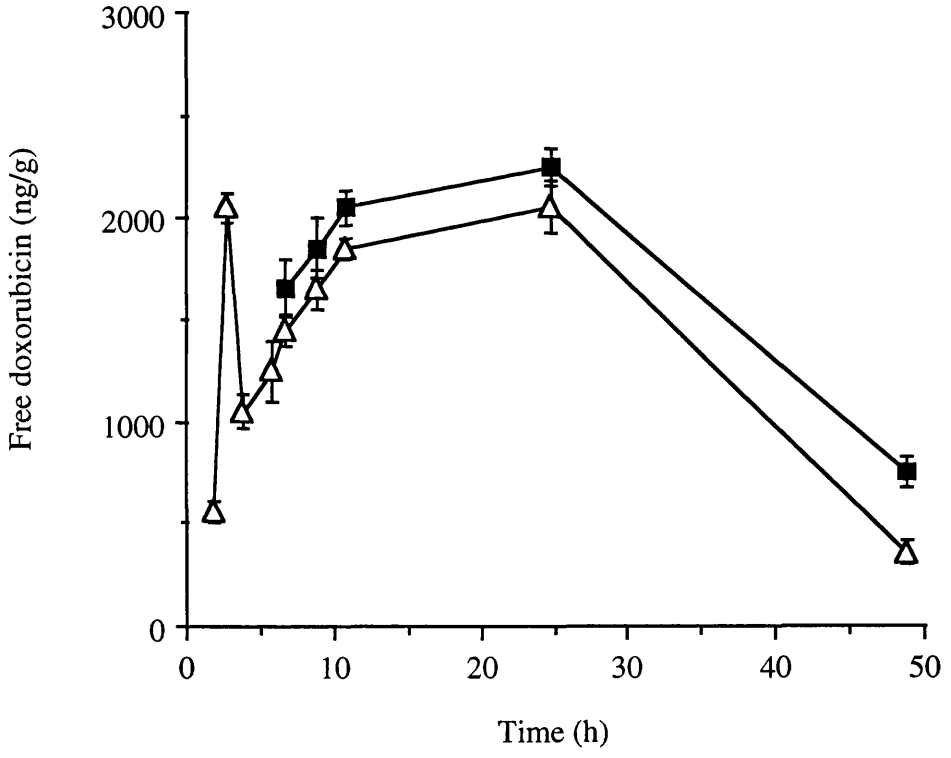
The results of the HPLC analysis showing the concentration of free doxorubicin in liver and heart are presented in Figures 5.5 and 5.6 respectively. Liver was not perfused prior to isolation, but from the analysis of the blood we know that there was no free doxorubicin present at any time, so we can conclude that the free doxorubicin detected in the liver was present in the tissue, and not in occluded blood. In both groups the absolute concentrations of free doxorubicin were higher in liver than in heart. Maximum levels of free doxorubicin present in the liver were 2000 ng/g and the maximum levels of free doxorubicin present in the heart were 300 ng/g and obtained for

Figure 5.4 Free doxorubicin released from PK1 (10 mg/Kg) in the tumours of C57 mice bearing B16F10 murine melanoma



n=3
symbols represent mean ± SE

Figure 5.5 Levels of free doxorubicin released in liver of C57 mice bearing B16F10 melanoma following administration of PK1

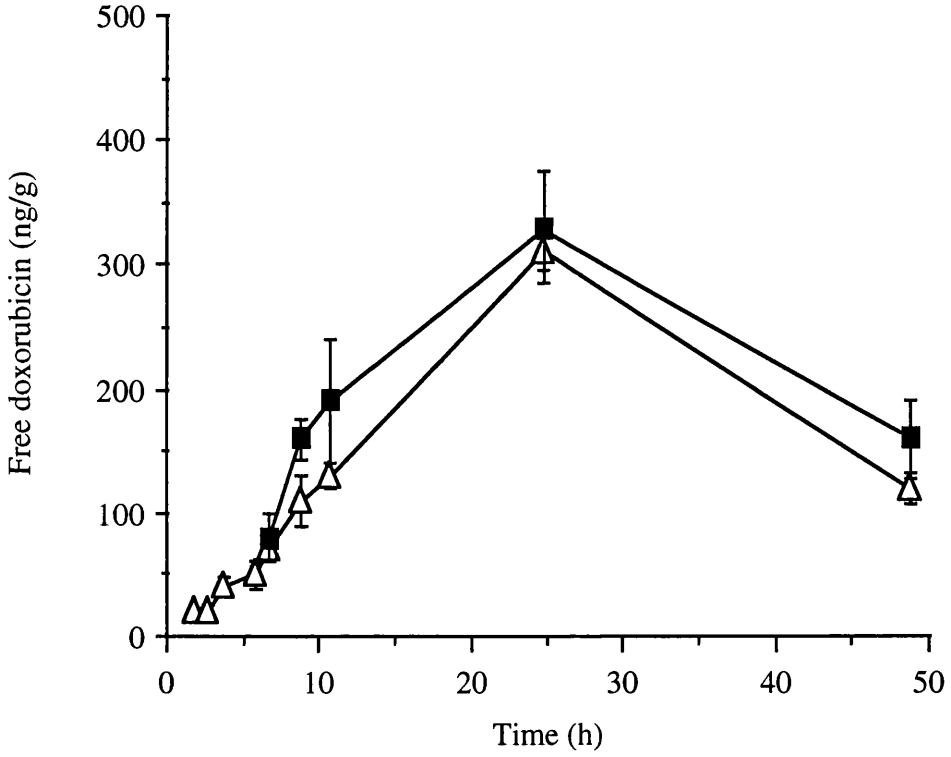


PK1

PK1 followed after 5 h by HPMA copolymer-cathepsin B

n=3
symbols represent mean±SE

Figure 5.6 Levels of free doxorubicin released in heart of C57 mice bearing B16F10 melanoma following administration of PK1



PK1

PK1 followed after 5 h by HPMA copolymer-cathepsin B

n=3
symbols represent mean±SE

both organs 24 h after PK1 administration. AUC values over the experimental period (t=0-48 h) for liver and heart tissues were similar (not significantly different) between the two treated groups (PK1 alone and the PDEPT model combination).

To summarise the data Figure 5.7 presents the release of doxorubicin in tumour, heart and liver expressed as AUC (5 h to 48 h) and it can be seen that there was a preferential release of doxorubicin in tumour compared to the normal tissues examined (Table 5.1 shows statistical analysis of AUC ratios between different tissues and different treatments).

5.4 Discussion

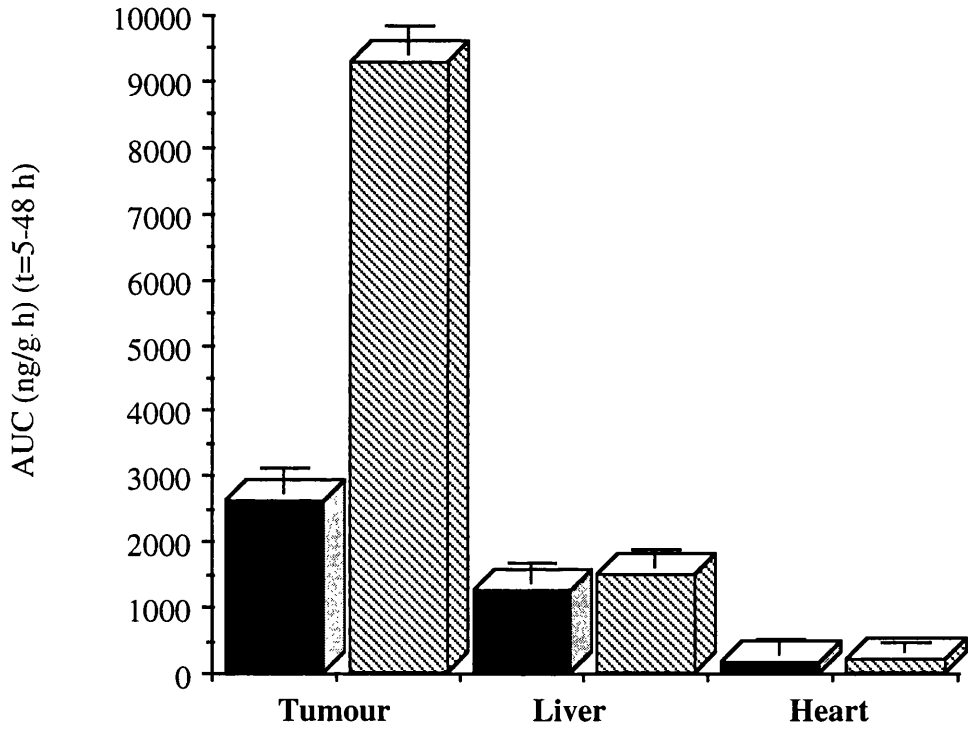
5.4.1 Tumour

HPMA copolymer-cathepsin B showed *in vivo* accumulation in the tumour and produced a significant increase in the rate of doxorubicin release from PK1 compared to PK1 alone which is degraded by endogenous lysosomal cathepsin B. A diagram of the pharmacokinetics of doxorubicin release from PK1 in the tumour tissue is shown in Figure 5.8.

Duncan and Sat (1998) showed that in the murine s.c. implanted tumour model, B16F10 melanoma displayed tumour size dependent accumulation of PK1. The level of PK1 was highest (18.3±3.6% dose/g tumour) when tumours were small (20-30 mg). As tumours increased in size (0.9-1.0 g), the accumulation decreased (1.1±0.9% dose/g tumour). This observation may explain why, if tumour size is not carefully standardised, the higher variability in the tumour accumulation of PK1 other than seen for normal organs where levels of PK1 observed are controlled by enzyme levels and not EPR effect.

A theoretical calculation of a ubiquitous distribution of PK1 administered i.v. at 10 mg/Kg doxorubicin equivalent dose to an average 25 g mouse would release 1000 ng doxorubicin in every 100 mg tissue. Since the average tumour weight in this study was 100 mg we can assume that average of 1000 ng doxorubicin should have been detected theoretically in tumour samples after HPLC analysis. The reality was that due to the EPR effect allowing PK1 to extravasate in the tumour tissue and accumulate there, and due to the presence of cathepsin B (endogenous or exogenous) there was a release of 4640 ng (1.9 %/administered dose/tumour) at the maximum release time point of PK1 alone (4.6-fold increase in release compared to the theory), 16,911 ng (6.8 %/administered dose/tumour) doxorubicin in the case of PK1 followed by cathepsin B (16.9-fold) and 11,742 ng (4.7 %/administered dose/tumour) when administering the PDEPT combination (11.7-fold).

Figure 5.7 AUC values of doxorubicin release following administration of PK1 (10 mg/Kg) or PK1 (10 mg/Kg) followed by HPMA copolymer-cathepsin B



PK1

PK1 (10 mg/Kg) followed after 5 h by HPMA copolymer-cathepsin B (PDEPT)

n=3 at each time point
columns represent mean AUC±SE

Table 5.1 Comparison of AUC values of doxorubicin released in tissues between treatment by PK1 (10 mg/Kg) or PK1 (10 mg/Kg) followed by HPMA copolymer-cathepsin B (PDEPT)

	PK1	PDEPT	PDEPT : PK1
Tumour : Liver	2.07* (p=0.0169)	6.25* (p=0.0068)	
Tumour : Heart	15.27* (p=0.0003)	46.03* (p=0.002)	
Tumour : Tumour			3.55* (p=0.0264)
Liver : Liver			1.18[n.s.] (p=0.5603)
Heart : Heart			1.18[n.s.] (p=0.5547)

* significant difference $p<0.05$

[n.s.] no significant difference

Figure 5.8 Diagram showing pharmacokinetics of doxorubicin released from PK1 in the tumour tissue

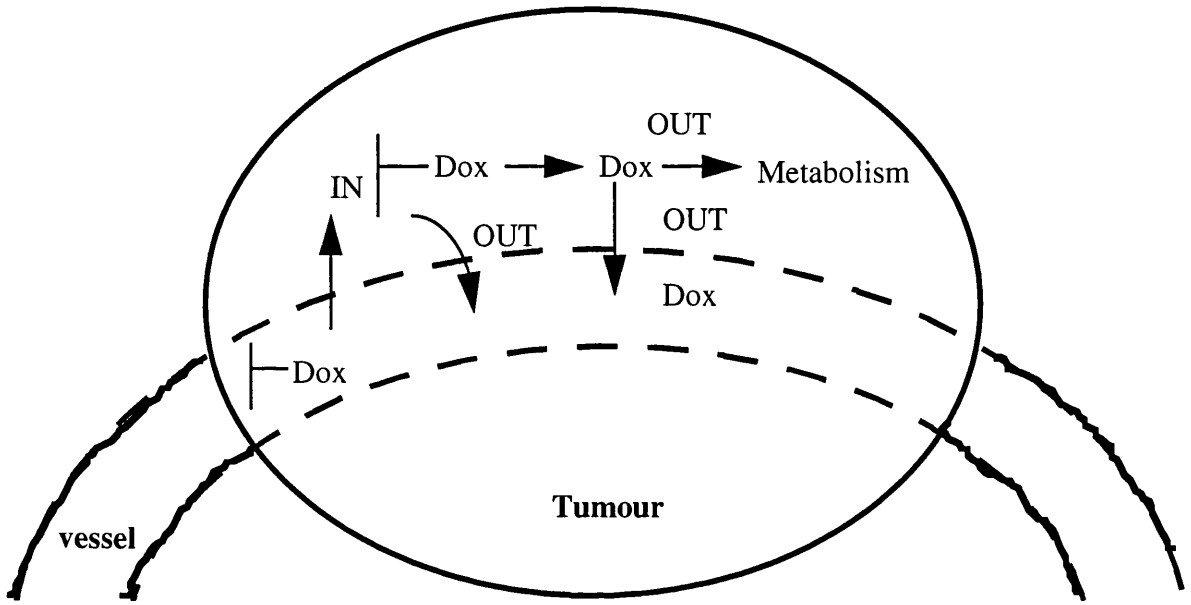
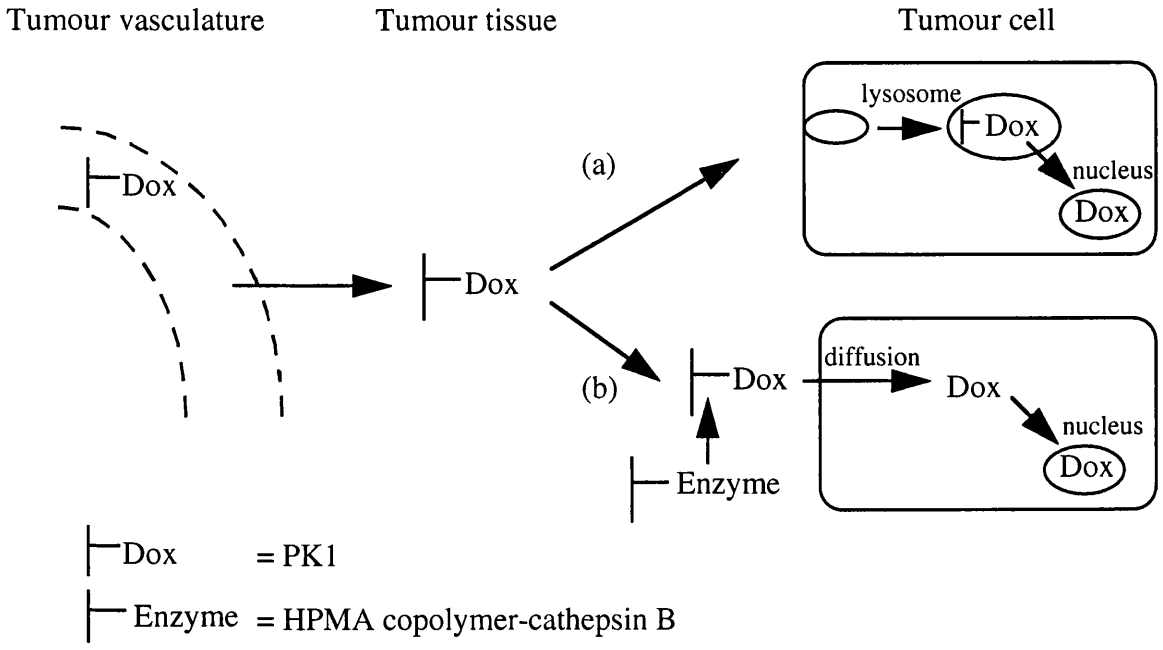


Figure 5.9 Doxorubicin released intracellularly from PK1 alone (entering the cell via pinocytosis) (a) and extracellularly by HPMAcopolymer-cathepsin B as a model PDEPT combination (b)



PK1 displayed passive tumour tropism previously recorded for a series of doses (Seymour *et al.*, 1994) and a series of HPMA copolymers of different molecular weights (Seymour *et al.*, 1995). This resulted in tumour localisation up to 10-15 times more than that achieved using free doxorubicin administered at equal doses (5 mg/Kg). Here, followed by bound cathepsin B, localisation of free doxorubicin in tumour rose to 2.6-fold in AUC value compared to PK1 alone. Previous studies using radiolabelled doxorubicin-HPMA copolymer conjugate (Seymour *et al.*, 1994) have shown that in the period 6-12 h following injection the rate of doxorubicin disappearance from the tumour were faster than the rate of accumulation. Disappearance of doxorubicin from the tumour may result from its metabolism to undetected forms, or from its drainage out of the tumour, in either free or copolymer-conjugated form. The rate of proteolytic release of free doxorubicin from the copolymer conjugate is likely also to influence rates of disappearance. In the same study Seymour *et al.* (1994) showed that improved activity of PK1 compared to free doxorubicin was in proportion to the increased tumour AUC values for free doxorubicin (elevated 1.7- to 4.6-fold) than for total doxorubicin (17.1- to 77.0-fold), confirming that therapeutic activity of the PK1 was dependent on proteolytic release of the free anthracycline. Hence, it was suggested that the therapeutic activity of high doses of PK1 within this model system might have been limited by rates of proteolytic activation of the polymeric prodrug within the solid tumour. The results of these studies might explain the higher absolute amount of doxorubicin released in the PDEPT treatment compared to PK1 alone. The faster the release occurred (by the addition of HPMA copolymer-cathepsin B) the more doxorubicin was able to be released.

Looking carefully at the results from the pharmacokinetic study, it can be seen that after PK1 accumulation in the tumour by the EPR effect, and subsequent administration of the activating enzyme cathepsin B in either free or conjugated form, release of doxorubicin from its polymeric backbone occurred in the tumour tissue rapidly and probably extracellularly. In contrast, release of doxorubicin from PK1 alone relatively slowly and its rate of liberation is consistent with intracellular release in the lysosome by endogenous lysosomal thiol-dependent proteases, after uptake by fluid-phase endocytosis (see Chapter 1.5). This can be shown by the slow release of free doxorubicin in the tumour compared to that seen after addition of the conjugated enzyme. Figure 5.9 clarifies the difference in doxorubicin release from PK1 intracellularly and extracellularly. This is another part of the jigsaw in understanding the mechanism of the EPR effect. Both the polymeric prodrug and the polymer-enzyme extravasate to the tumour tissue by the EPR effect due to their high molecular weight. Drug liberation occurs intratumourally much faster than intracellularly, because both

constructs will meet first extracellularly and interact immediately. Then the free drug will diffuse into the tumour cells.

5.4.2 Blood

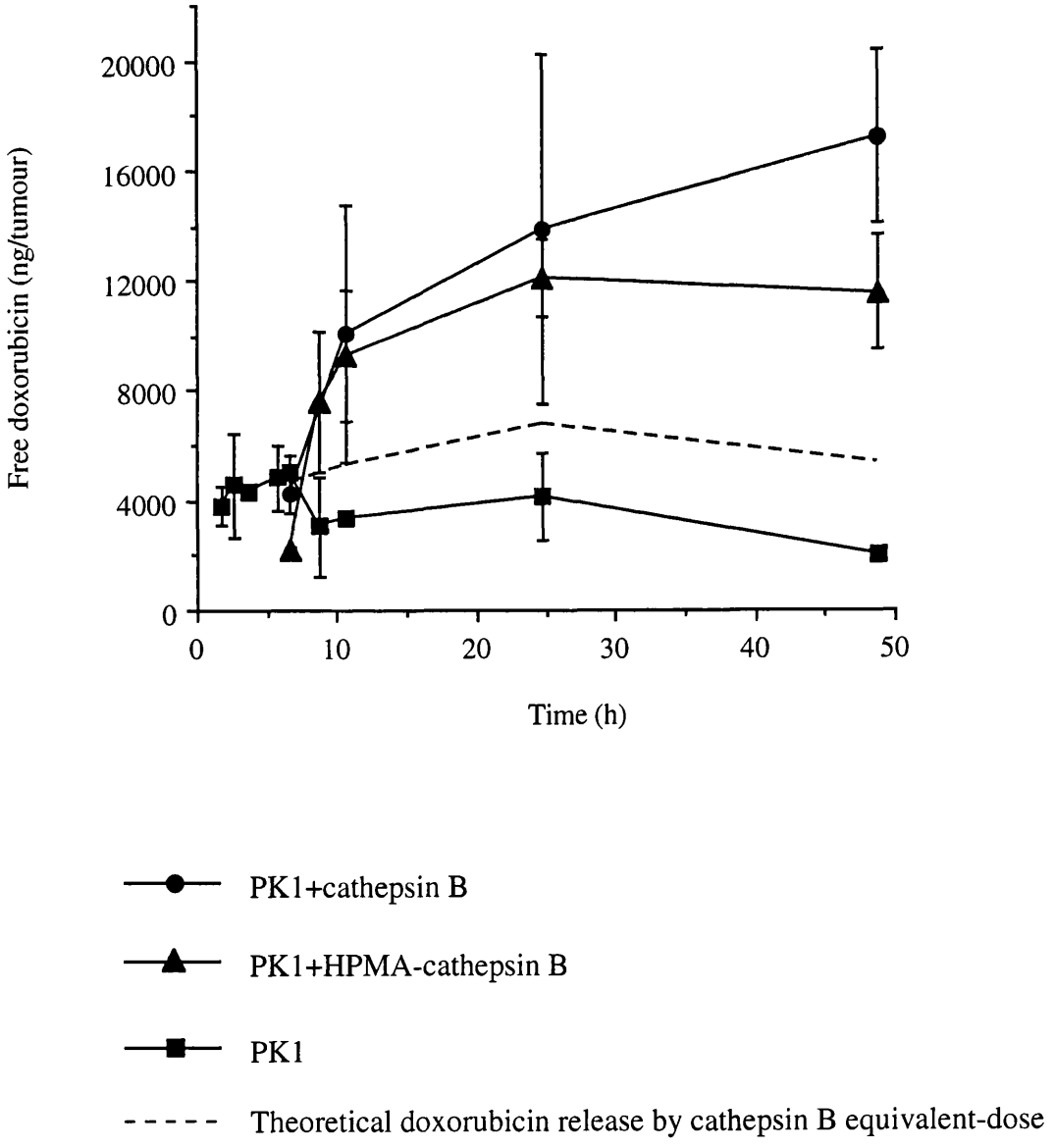
It was already known that most of the polymeric prodrug PK1 is already excreted from the circulation within 5 h (Seymour *et al.*, 1990) and results obtained here confirmed no release of free doxorubicin in the bloodstream due to administration of free or bound cathepsin B at 5 h. Although the levels of polymer-bound doxorubicin present in blood and tissues were not analysed throughout this study, previous work (Seymour *et al.*, 1990) showed that there was 5 μg/ml bound-doxorubicin detected in the blood 24 h after administration of PK1 (5 mg/Kg). Therefore it was surprising that after administration of HPMA copolymer-cathepsin B 5 h after administration of PK1 (10 mg/Kg) no free doxorubicin was released. A plausible explanation could be the fact that doxorubicin, being a small molecule, diffuses into tissues so quickly after being released from the polymeric backbone that this step was missed. To examine this issue further experiments evaluating the free and total doxorubicin present in blood should be performed.

5.4.3 Theory

Chapter 3.3.7 evaluates the activity retained by cathepsin B following conjugation to HPMA copolymer compared to the free enzyme. It was shown that it retained ~20% of its activity on PK1 *in vitro*. Based on these experiments we can conclude that although the amount of cathepsin B injected to mice was similar (present in polymer-bound as free), the actual activity in terms of enzyme units was only ~20%. Therefore, a calculation of theoretical release of doxorubicin was performed assuming the same activity units of cathepsin B were injected and HPMA copolymer-cathepsin B appeared to be >5 fold more effective in releasing doxorubicin over 50 h when compared with administration of the equivalent enzyme activity of cathepsin B alone. Figure 5.10 shows the release in tumour tissue of doxorubicin from PK1 after administration of bound cathepsin B compared to the theoretical release of free doxorubicin if the equivalent activity of free cathepsin B was administered. Although it seems tempting to use free cathepsin B instead of polymer-bound, one should bear in mind that the main reason for conjugating the enzyme to the polymer was the increased half-life and the known reduced immunogenicity of proteins when attached to HPMA copolymer. This aspect will be examined in Chapter 7.

Providing that a specific relationship exists between toxicity or efficacy and the concentration of a drug within accessible body fluids, or in specific body compartments, pharmacokinetics may be used to assist in the design and optimisation of

Figure 5.10 Free doxorubicin theoretically released from PK1 (10 mg/Kg) in the tumours of C57 mice bearing B16F10 murine melanoma



clinical dosage regimens (Duncan and Spreafico, 1994). From this study it can be concluded that 5 h interval between the administration of the prodrug and the polymer-enzyme conjugate is a reasonable schedule to be followed. Also, the fact that there was a linear dose response in the tumour tissue at 6 h and 10 h between 5 mg/Kg and 10 mg/Kg PK1 dose. Therefore, although further pharmacokinetic studies are required in order to determine (1) the optimal dose of conjugated cathepsin B administered following the administration of PK1 and (2) the MTD of PK1 when followed by administration of bound cathepsin B, it was interesting to investigate the effect of this combination (following the same dosage schedule) on the efficacy of PK1. The antitumour activity of this PDEPT combination is described in the next chapter.

Chapter Six

Antitumour activity of doxorubicin, PK1 and PK1 followed by HPMA copolymer-cathepsin B in vivo

6.1 Introduction

As the PDEPT combination relies on pharmacokinetics to facilitate targeting, the assessment of a PDEPT combination must be conducted *in vivo* . Whereas the polymeric prodrug PK1 has already been designed for activation by endogenous cathepsin B, it was possible to compare the antitumour activity of the PDEPT combination with that of PK1 alone. It was shown (Chapter 5) that HPMA copolymer-cathepsin B retained the ability to increase the rate of release of doxorubicin from PK1 after i.v. administration. It was therefore considered essential to test the system and see if it is feasible to achieve improved antitumour activity.

As a general rule, tumours selected for pharmacological activity studies should be non-immunogenic, otherwise the host immune system might eradicate the tumour. The NCI has used murine tumours from inbred strains to circumvent this problem (Van Putten, 1987). The use of nude mice (nu/nu), deficient in T lymphocytes, allows pharmacological testing of conjugates against human xenografts tumours.

HPMA copolymer-anthracycline conjugates, in particular PK1, have already shown significant antitumour activity *in vivo*, and drug conjugation found to afford a substantial reduction in doxorubicin toxicity (Cassidy *et al.*, 1989; Duncan *et al.*, 1989; Duncan *et al.*, 1998). Preclinical studies using different tumour models (Walker sarcoma (Cassidy *et al.*, 1989, M5076 (Duncan *et al.*, 1992), B16F10 melanoma (O'Hare *et al.*, 1993), L1210 (Duncan *et al.*, 1992; Duncan *et al.*, 1989), P388 leukaemia (Wedge, 1990) and the human colon xenograft LS174T (Duncan *et al.*, 1992)) confirmed PK1 antitumour activity, in all cases equal to or significantly better than that seen for free doxorubicin. In addition, PK1 displays decreased general toxicity (reviewed in Duncan, 1995), with a marked reduction in cardiotoxicity (Yeung *et al.*, 1991) and bone marrow toxicity (Rihova *et al.*, 1989). However, no studies have previously been reported using PK1 against COR-L23 non-small cell lung carcinoma and no PDEPT combinations using PK1 were ever tested before.

Development of melanoma-specific targetable drug delivery systems has already received considerable attention, both because of the poor prognosis for metastatic disease and because of the specific biochemical and immunological properties of melanoma (O'Hare *et al.*, 1993). However, no outstanding progress has been made (Spitler *et al.*, 1987; Liu *et al.*, 1988). Since HPMA copolymers covalently bound to antitumour agents but not specifically targeted to tumour cells have already shown their ability to improve the drug therapeutic index against a number of tumour models (reviewed in Duncan, 1992), it was considered important to investigate the possibility of further potentiating the activity of such conjugates by active tumour-specific

targeting. The attachment of targeting moieties such as MSH (O'Hare *et al.*, 1993), galactose (Duncan *et al.*, 1983b, Seymour *et al.*, 1991a), transferrin (Flanagan *et al.*, 1992) and polyclonal and monoclonal antibodies (anti-Thy-1.2, anti-lak, B3/25, anti-CD3) (Ulbrich *et al.*, 1996; Omelyaneko *et al.*; 1996, Flanagan *et al.*, 1992; Rihova *et al.*, 1992) are described in the literature.

The first tumour model used in this study to compare the antitumour activity of PK1 and PDEPT, PK1 followed by HPMA copolymer-cathepsin B, was s.c. B16F10 murine melanoma. This model has been used previously as a model for establishing a solid tumour prior to i.v. treatment. It is often aggressive, thereby screening only those conjugates with real preclinical potential (Duncan *et al.*, 1992). The reason for choosing this cell line as the first model was due to the fact that if we see any improvement in survival in such an aggressive model the concept of PDEPT will be proven as not only feasible but effective as well. O'Hare *et al.* (1993) used B16F10 first to compare PK1 and a PK1-like conjugate containing also melanocyte stimulating hormone (MSH) as a means to facilitate receptor-mediated targeting of the conjugate. Seymour *et al.* (1987; 1990; 1994; 1995) used B16F10 to follow pharmacokinetics and the EPR targeting of PK1 and also, together with sarcoma 180, as a model to examine the effect of molecular weight of polymer on the EPR effect. The B16F10 model has been a standard screen in our laboratory to compare the EPR effect of polymer-conjugates e.g. HPMA copolymer-platinates (Gianasi *et al.*, 1999) and dendrimer-platinates (Malik and Duncan, 1998). This cell line was widely used as a model to test the pharmacological activity of such polymer-drug conjugates, thus supplying a sound basis for comparison.

The second tumour model used here to test PK1 and the PDEPT combination was a human xenograft COR-L23 a non-small cell lung carcinoma. The COR-L23 tumour exhibits a much slower growth rate *in vitro* (3 days doubling time compared to 1.5 days for B16F10, as seen in Chapter 2.3.5.3) and also *in vivo* it grows more slowly than the B16F10 line. The COR-L23 cell line was originally established from a pleural effusion from a giant cell anaplastic carcinoma and was grown as an adherent monolayer of very large cells, often multinucleate (Baillie-Johnson *et al.*, 1985). Lung carcinomas are usually resistant to doxorubicin. This was another reason COR-L23 was selected as a model for these studies.

6.2 Methods

All animal experiments were carried out according to the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (UKCCCR guidelines, 1998). All animals were

checked twice a day to monitor their health and observe any change in behaviour as a sign of toxicity. Animals displaying either signs of distress or tumour ulceration were culled. Nu/nu mice were kept in separate cages for each group in the isolator. Bedding was changed three times a week. Tumour measurement and i.v. injections were performed in a negative pressure Class I Laminar Flow cabinet to avoid contamination.

In short, B16F10 murine melanoma and COR-L23 non-small cell lung carcinoma cell lines were used. The B16F10 model forms a semi-solid, difficult to cure tumour in C57 mice. The COR-L23 model forms a solid tumour in nu/nu mice and it readily metastasises to liver and lung. Mice (6-8 weeks) were inoculated s.c. with tumour cells (10^5 B16F10 cells for C57, 10^6 COR-L23 cells for nu/nu). When tumours became palpable (25 mm^2) treatment was initiated. All groups consisted of 5 mice per group and the treatment was injected i.v. to the tail vein of the mice. Drug doses expressed in mg/Kg drug were calculated based on the weight percent of the drug bound to the copolymers. The treatment day was considered to be day zero, typically this was approximately 10 days after inoculation of B16F10 cells to C57 mice and 11 days after inoculation of COR-L23 cells to nu/nu mice.

Synthesis of HPMA copolymer-cathepsin B

Polymer-enzyme conjugate was synthesised and characterised, according to the protocols described in Chapter 2, before each one of the studies.

Statistical analysis

All the *in vivo* data are expressed as the mean ± standard error (SE). Statistical analysis of the mice survival time data and tumour volume data were determined using Student t-test for small sample sizes. P values of 0.05 or less were considered statistically significant.

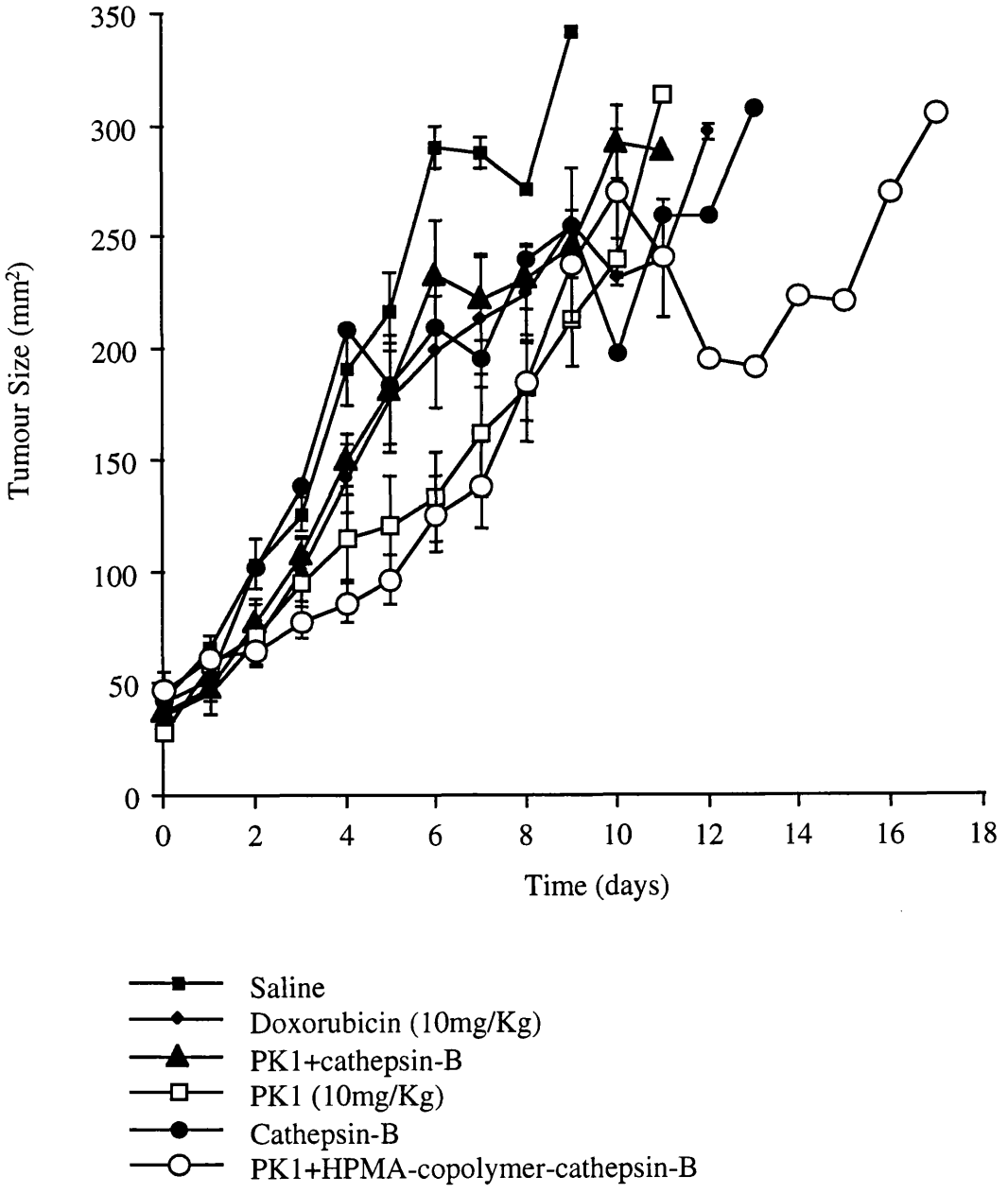
6.3 Results

6.3.1 Antitumour activity in the B16F10 murine melanoma model

Figure 6.1 shows the increase in tumour area with time. It can be seen that a significant decrease (compared with control mice group) in tumour growth rate was observed after treatment of animals with free doxorubicin, PK1, and PK1 followed after 5 h by HPMA copolymer-cathepsin B (PDEPT). In each case animals were treated with 10 mg/Kg doxorubicin-equivalent.

The ratio (T/C) expressed as a percentage of the mean survival time for a treated group of animals (T) compared with the mean survival time of the control group (C) value is typically used to define antitumour activity. A drug is usually considered

Figure 6.1 Effect of treatment on tumour size of C57 mice bearing B16F10 murine melanoma



n=5
symbols represent mean±SE

efficacious when the survival time is above that of the control group by more than 25% (i.e. a T/C > 125%). The T/C value was significantly increased when the B16F10 bearing mice were treated with the PDEPT combination, T/C=168% (p=0.018 *vs* control group) (Figure 6.2). When animals were treated with PK1 the T/C was 152% (p=0.0004 *vs* control group) and when treated with doxorubicin the T/C was 144% (p=0.03 *vs* control group). The results are summarised in Table 6.1.

PK1 followed after 5 h by the administration of free cathepsin B gave a T/C value of 135.3% (p=0.0497 *vs* control group). No significant antitumour activity was seen when cathepsin B was injected alone giving a T/C value of 123.5%, p=0.367 *vs* control. The above results demonstrate that the use of PDEPT combination enhances the survival time compared with doxorubicin and PK1 at the same dose of doxorubicin-equivalent by slowing down the increase in growth.

There were neither toxic deaths nor animal weight loss in this study (Figure 6.3) and from the post-mortem analyses there was no sign of toxicity in any of the 6 groups. Treatment did not give rise to any measurable toxicity and animals were eventually put down owing to extensive tumour growth.

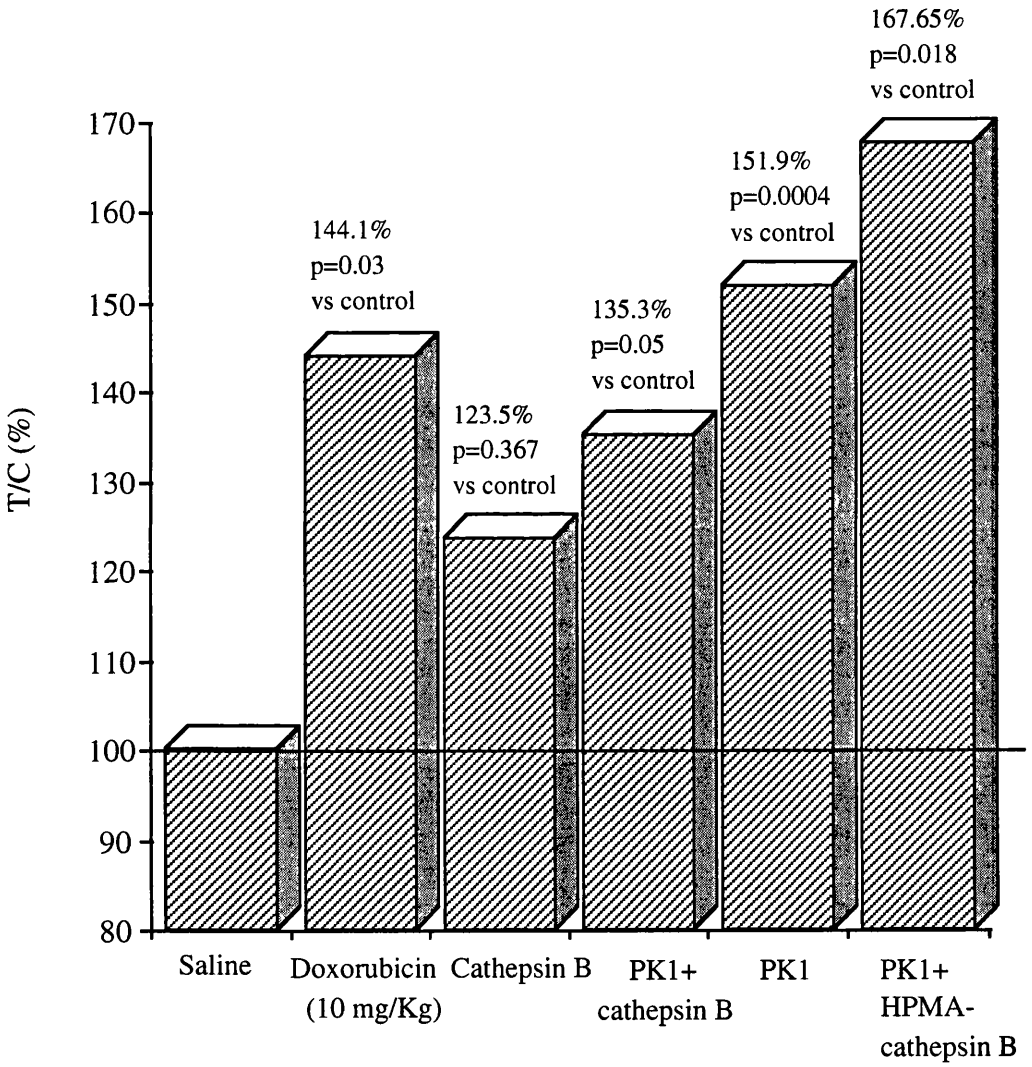
6.3.2 Antitumour activity in the COR-L23 non-small cell lung carcinoma model

Figure 6.4 shows the increase in tumour area with time after treatment of nu/nu mice bearing COR-L23. A significant decrease (compared with control mice group) of tumour growth rate was observed after treatment with free doxorubicin or the PDEPT combination; PK1 followed after 5 h by HPMA copolymer-cathepsin B.

The T/C value was significantly increased when the COR-L23 bearing mice were treated with doxorubicin, T/C=129.6% (p=0.03 *vs* control group) (Figure 6.5). When animals were treated with the PDEPT combination the T/C value was 126.5% (p=0.02 *vs* control group). The results are summarised in Table 6.2. Although PK1 caused some increase in survival this was not significant, T/C was 115.3% (p=0.2 *vs* control group), which is considered not active.

Analysis of the final tumour weights (Figure 6.6) (using the student t-test for small sample sizes), after post-mortem for each group of mice bearing the COR-L23 tumours s.c., demonstrated that the tumour weights of the mice treated with the PDEPT combination were significantly lower from the 3 other groups: Saline (control group) mean tumour weight 0.98±0.5 g; free doxorubicin 0.48±0.3 (p=0.09 *vs* control group-

Figure 6.2 Activity of PDEPT, PK1 and doxorubicin (10mg/Kg) after single i.v. injection in C57 black male mice bearing B16F10 murine melanoma

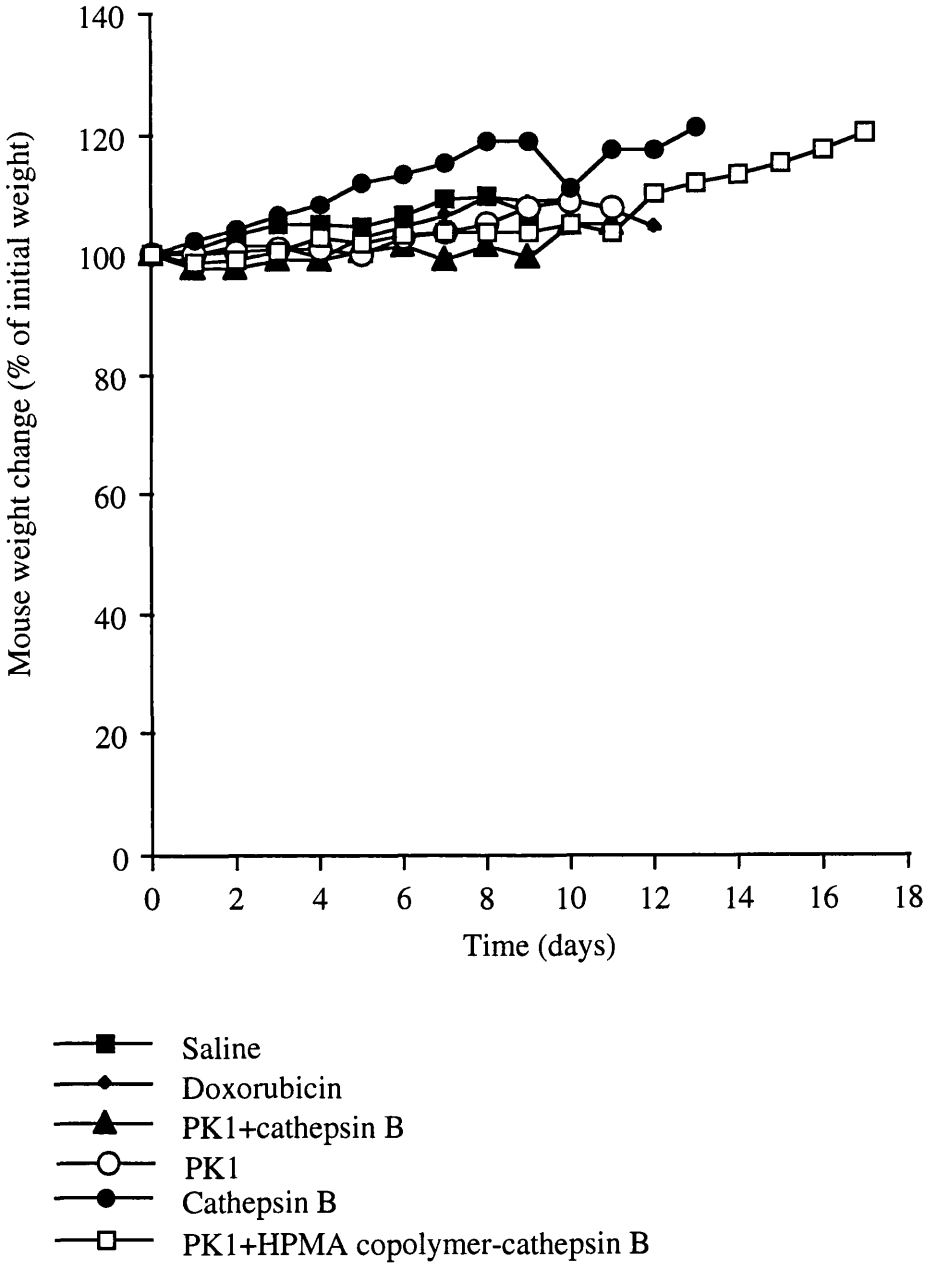


n=5
columns represent mean

Table 6.1 Effect of single i.v. administration of saline, doxorubicin, cathepsin B, PK1, PK1+cathepsin B or PK1+HPMA copolymer-cathepsin B on the survival and tumour development in C57 black male mice bearing s.c. B16F10 murine melanoma.

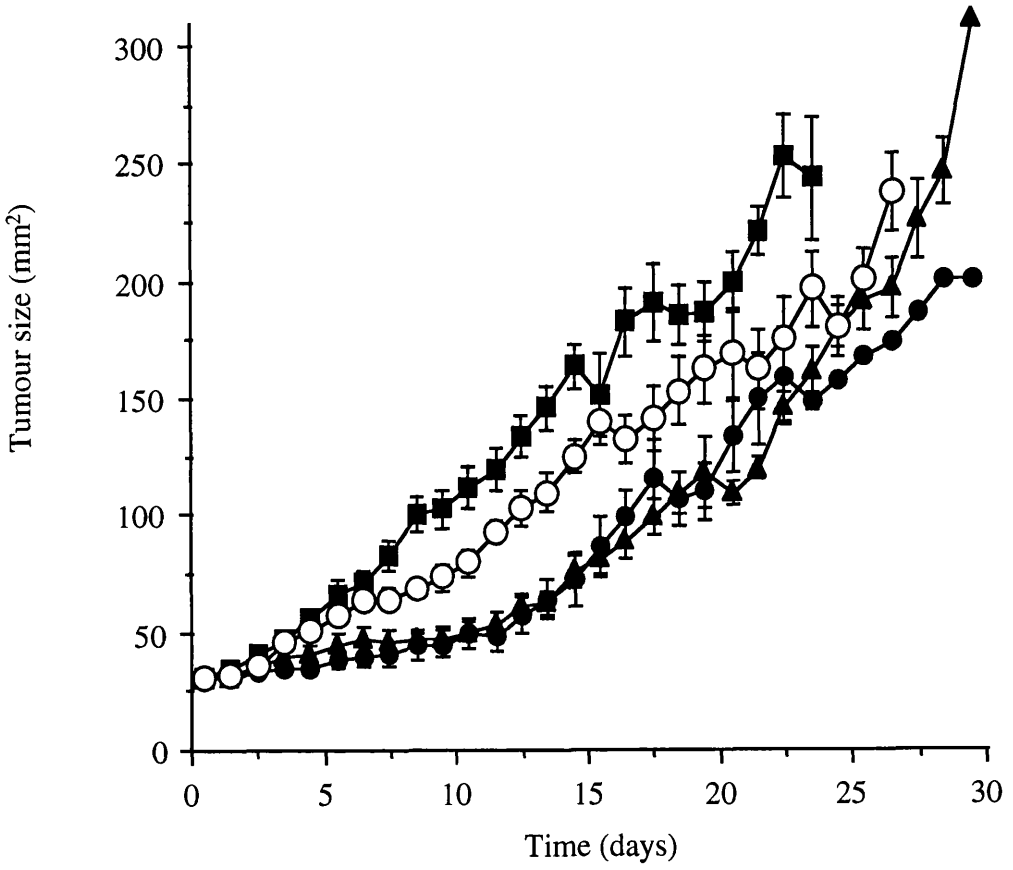
Group	**Compound (Dose-see text)**	**Day treated (mean±SE)**	**Day sacrificed (mean±SE)**	**Days survived after treatmeant (mean±SE)**	**Initial tumour size (mm^2±SE)**	**T/C (%)**	**No. toxic deaths**
1	Saline	8,8,8,11,11 (9.2±0.66)	14,17,15,17,17 (16±0.57)	6,9,7,6,6 (6.8±0.52)	48,56,36,36,42 (43.6±3.41)	100	0/5
2	Doxorubicin	8,8,8,9,9 (8.4±0.22)	15,17,17,21,21 (18.2±1.07)	7,9,9,12,12 (9.8±0.87)	64,42,36,20,20 (36.4±7.3)	144.1 (p=0.03)	0/5
3	Cathepsin B	8,8,8,10,11 (9±0.57)	18,17,14,14,24 (17.4±1.64)	10,9,6,4,13 (8.4±1.4)	56,42,48,42,25 (42.6±4.56)	123.5 NS	0/5
4	PK1+cathepsin B	9,9,9,9,9 (9±0)	20,19,19,18,15 (18.2±0.77)	11,10,10,10,9,6 (9.2±0.77)	30,36,25,56,42 (37.8±4.8)	135.3 (p=0.05)	0/5
5	PK1	9,9,9,9,9,12 (9.5±0.5)	20,19,20,19,20,21 (19.83±0.31)	11,10,11,10,11,9 (10.33±0.33)	36,30,30,25,25,25 (28.5±1.8)	151.9 (p=0.0004)	0/6
6	PK1+HPMA-cathepsin B	10,10,10,10,10 (10±0)	19,20,20,27,21 (21.4±1.28)	9,10,10,17,11 (11.4±1.28)	78,56,42,30,30 (47.2±8.11)	167.7 (p=0.018)	0/5

Figure 6.3 Effect of treatment on weight of C57 black male mice bearing B16F10 murine melanoma



n=5
symbols represent mean±SE

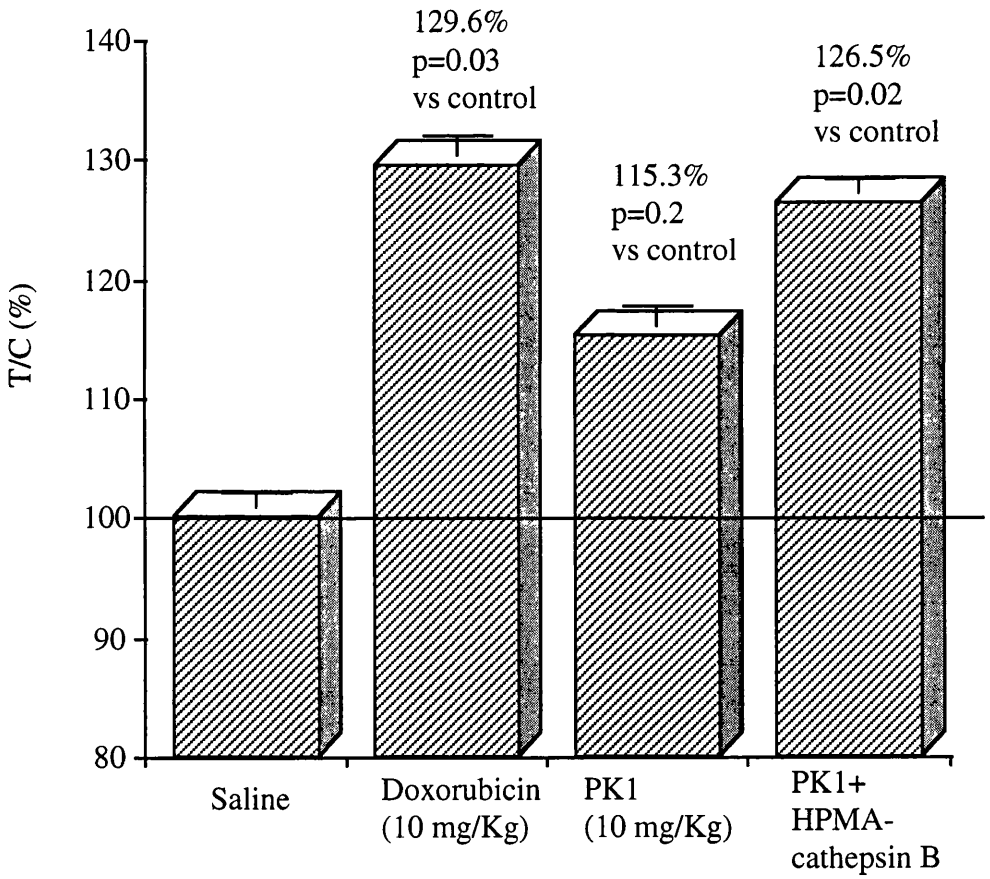
Figure 6.4 Effect of treatment on tumour size of nu/nu mice bearing COR-L23 non-small cell lung carcinoma



PK1+HPMA copolymer-cathepsin B
Doxorubicin (10 mg/Kg)
control
PK1 (10 mg/Kg)

n=5
symbols represent mean±SE

Figure 6.5 Activity of PDEPT, PK1 and doxorubicin (10 mg/Kg) after single i.v. injection in nu/nu male mice bearing COR-L23 non-small cell lung carcinoma

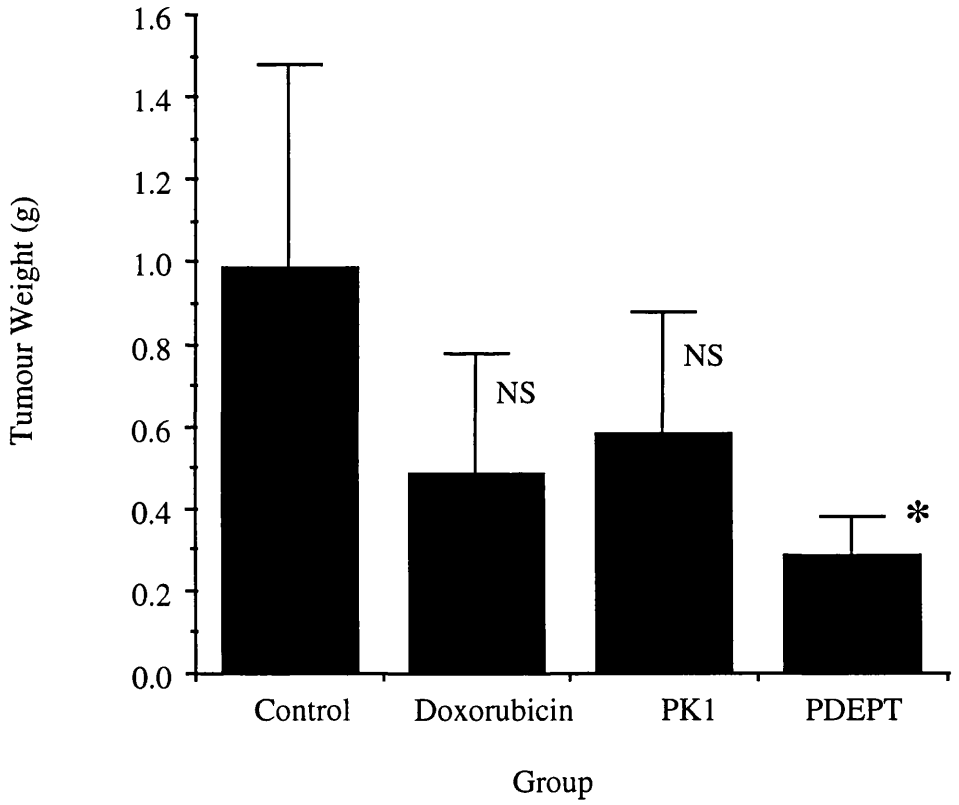


n=5
columns represent mean±SE

Table 6.2 Effect of single i.v. administration of saline, doxorubicin, PK1 or PK1 + HPMA copolymer-cathepsin B on the survival and tumour development in nu/nu male mice bearing s.c. COR-L23 non-small cell lung carcinoma.

Group	Compound (Dose-see text)	Day treated (mean±SE)	Day sacrificed (mean±SE)	Days survived after treatmeant (mean±SE)	Initial tumour size (mm^2±SE)	T/C (%)	No. toxic deaths
1	Saline	8,8,9,9,16 (10±1.35)	31,25,32,26,34 (19.6±1.25)	23,17,23,17,18 (19.6±1.25)	25,25,25,25,25 (25±0)	100	0/5
2	Doxorubicin	8,8,9,13,18 (11.2±1.72)	36,35,33,42,37 (36.6±1.34)	28,27,24,29,19 (25.4±1.61)	25,25,25,25,25 (25±0)	129.6 (p=0.03)	0/5
3	PK1	8,9,11,12,12 (10.4±0.73)	34,32,31,38,30 (33±1.26)	26,23,20,26,18 (22.6±1.43)	25,25,25,25,25 (25±0)	115.3 NS	0/5
4	PK1+HPMA-cathepsin B	11,11,11,14,16 (12.6±0.92)	33,35,35,41,43 (37.4±1.73)	22,24,23,29,26 (24.8±1.1)	25,25,25,25,25 (25±0)	126.5 (p=0.02)	0/5

Figure 6.6 Effect of treatment on tumour weight of nu/nu mice bearing COR-L23 non-small cell lung carcinoma at end point



n=3
columns represent mean±SE
NS No significant difference compared to control ($p>0.05$)
* $p<0.05$

no significant difference); PK1 0.58g±0.3g (p=0.21 *vs* control group; no significant difference); PDEPT 0.28±0.1 (p=0.02 *vs* control group).

There were neither toxic deaths nor animal weight loss in this study (Figure 6.7) and from the post-mortem analyses there was no sign of toxicity in any of the 4 groups.

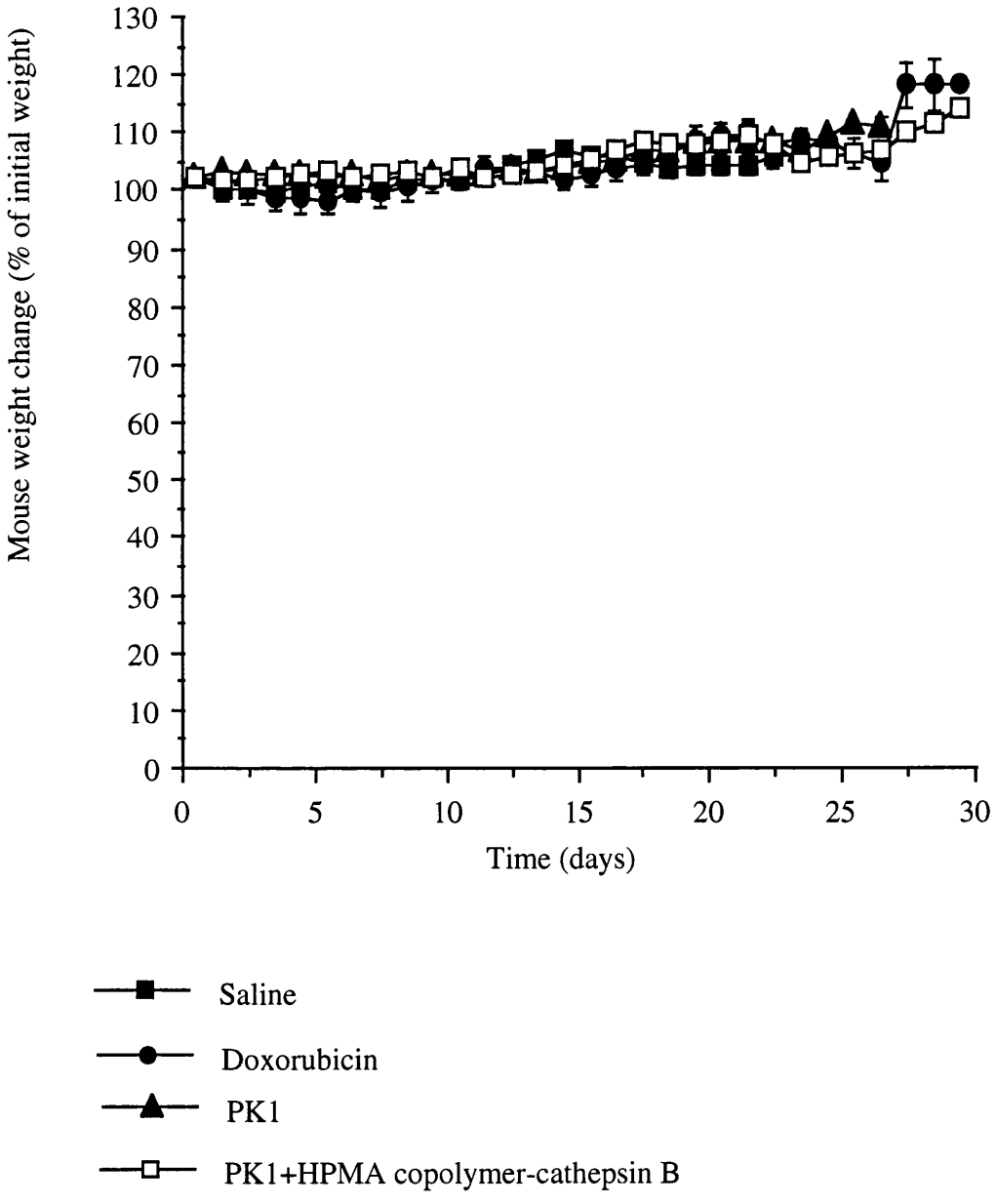
6.4 Discussion

6.4.1 B16F10 tumour model

The antitumour activity study demonstrated that PDEPT decreased the growth rate of B16F10 murine melanoma. This result is very encouraging due to the fact that B16F10 tumour model is considered to be very aggressive and any improvement in prognosis is important. The PDEPT combination proved to be better than PK1 in both tumour models in terms of efficacy after single injection. Comparison of the PDEPT combination with free doxorubicin showed a 25% increase in T/C value in the B16F10 model. One should bear in mind that optimisation of dose and schedule of treatment are necessary. It was shown (Duncan *et al.*, 1992) that PK1 has a maximum tolerated dose (MTD) of 90 mg/Kg, so further experiments following administration of higher doses comparing both systems (PK1 and PDEPT) should be interesting. Free doxorubicin cannot reach those doses due to its toxicity, as previously detailed in Chapter 1.9.3.

HPMA copolymer-cathepsin B was able to accelerate release of doxorubicin from PK1 in B16F10 tumour tissue, as shown in chapter 5, and this led to superior antitumour activity in the B16F10 murine melanoma model at doxorubicin-equivalent dose of 10 mg/Kg. No toxicity was observed in any of the studies indicating dose escalation to be possible. O'Hare *et al.* (1993) showed that even when given at a doxorubicin equivalent dose of 20 mg/Kg none of the polymeric formulations (PK1 and HPMA copolymer-doxorubicin containing melanocyte-stimulating hormone moiety) induced weight loss, whereas free doxorubicin clearly reduced weight gain or caused weight loss. Furthermore, Duncan *et al.* (1992) showed that doxorubicin doses up to 90 mg/Kg have been given to mice in the form of PK1 without any overt sign of toxicity. The underlying importance of the EPR effect in determining PK1 antitumour activity has been supported by the observation that PK1 causes regression in a transplantable syngeneic murine adenocarcinoma model MAC 26, which displays a discontinuous vasculature and is resistance to doxorubicin (Bibby *et al.*, 1996). In contrast, PK1 was equiactive compared to doxorubicin in the MAC 16 model, which has a more normal vascular architecture and displays a tight endothelial barrier (Bibby *et al.*, 1996).

Figure 6.7 Effect of treatment on weight of nu/nu male mice bearing COR-L23 non-small cell lung carcinoma



n=5
symbols represent mean±SE

Phase I clinical trial of PK1 performed by Vasey *et al.* (1999) investigated the maximum tolerated dose, toxicity profile, and pharmacokinetics of PK1 as an i.v. infusion every 3 weeks to patients with refractory or resistant cancers. Altogether, 100 cycles in the range of 20-320 mg/m^2 (doxorubicin-equivalent) were administered to 36 patients (20 males and 16 females) with a mean age of 58.3 years (age range 34-72). The MTD was 320 mg/m^2, and the dose-limiting toxicities were febrile neutropenia and mucositis. No congestive cardiac failure was seen despite individual cumulative doses up to 1680 mg/m^2. Responses (two partial and two minor responses) were seen in four patients with non-small cell lung carcinoma, colorectal cancer and anthracycline-resistant breast cancer. PK1 demonstrated antitumour activity in refractory cancers, no polymer-related toxicity, and proof of principle that polymer-drug conjugation decreases doxorubicin dose-limiting toxicities. The recommended Phase II dose is 280 mg/m^2 every 3 weeks. Hopefully, the improved antitumour effects achieved with the PDEPT combination in our study compared to PK1 alone could make polymer-drug conjugates such as PK1 a better drug in the clinic.

Different routes of treatment (i.p., i.v.) and different sites of tumour implantation can affect the antitumour activity results seen with any drug. The closest schedule found in the literature to that tested in this study was demonstrated by O'Hare *et al.* (1993). Their results for PK1 administered i.p. as a single injection (at 10 mg/Kg doxorubicin-equivalent dose) to C57 black male mice bearing s.c. B16F10 melanoma showed T/C value of 148% corresponding with the result shown here for PK1 administered i.v. (152%). In the same study dose escalation to 20 mg/Kg increased the T/C value to 175%. In the case of tumour implanted i.p. and PK1 (10 mg/Kg) administered i.v., T/C increased to >218%, but free doxorubicin at the same dose gave T/C of >254%, so i.p. was not considered a comparable model to the one used in the study above. From a pharmacokinetic viewpoint PK1 would be more efficacious compared with free doxorubicin in solid tumour compared with ascitic model because of the EPR effect responsible for the accumulation and retention of macromolecules in solid tumours.

6.4.2 COR-L23 tumour model

The case of the human xenograft tumour, COR-L23, is different and interesting indicating a different tumour physiology or different enzyme content. The fact that the PDEPT combination proved to be more effective than PK1 alone, but displayed almost the same efficacy as free doxorubicin needs explanation. As discussed earlier, lung carcinomas are usually resistant to doxorubicin, but this specific cell line, COR-L23, was not tested before with any of the treatments examined in this study. Having a very different morphology (as discussed in the introduction) from other lung carcinoma cell

lines could affect its sensitivity to different antitumour therapies and this might be the reason for its improved T/C after treatment with free doxorubicin, PK1 or PDEPT.

6.4.3 Effect of tumour lysosomal enzyme content on the antitumour activity

Duncan and Sat (1998) showed that accumulation of PK1 in B16F10 tumours is in the range of 2-18% dose/g tumour depending on tumour size at the time of treatment and in COR-L23 approximately 8% dose/g tumour (independent of size). Furthermore, release of doxorubicin from PK1 was much faster (22.9±1.7%/h) in B16F10 tumours compared with that of COR-L23 (2.3±0.6%/h). These observations are indicative of different levels of PK1 activating enzyme in the different tumour types. The observed differences in the extent of polymer capture by the EPR effect and the differences in intratumoural enzyme activity can be important for the interpretation of antitumour activity seen in these models. The improvement in antitumour activity of PK1 in the COR-L23 model when given as the PDEPT combination due to the administration of the exogenous HPMA copolymer-cathepsin B may be attributable to the low enzyme content of this tumour. A higher dose of cathepsin B administered after PK1 would have probably released more doxorubicin. Pharmacokinetic studies using a constant dose of PK1 and different doses of HPMA copolymer-cathepsin B would help to optimise the PDEPT system.

6.4.4 Choice of drug

Anthracycline antibiotics are still amongst the most effective antitumour agents known today and they display activity against a wide range of solid tumours, leukaemias and lymphomas (Young *et al.*, 1981). Routine clinical use is hampered, however, by haematological toxicity (Freidman and Carter, 1978) and cardiotoxicity (Launchbury and Hobboubi, 1993). The latter is cumulative and becomes dose-limiting at doses in excess of 450-550 mg/m^2 (Praga *et al.*, 1979). HPMA copolymer conjugates containing doxorubicin have already been shown to be significantly less cardiotoxic than doxorubicin (Yeung *et al.*, 1991; Hopewell *et al.*, 1996). It was found that a PK1 (doxorubicin-equivalent dose of 4 mg/Kg) caused no change in cardiac function over a 20 week experimental period in contrast with free doxorubicin at the same dose which induces a reduction in cardiac function within 6-8 weeks and this ultimately causes animal death (Yeung *et al.*, 1991). A recent dose escalation study using HPMA copolymer-doxorubicin with a galactose moiety, named PK2, has shown that conjugated anthracycline displays a ~ 5-fold reduction in cardiotoxicity relative to free doxorubicin (Hopewell *et al.*, 1996).

The post-mortem analysis of the animals used in the two studies included visualisation of the different organs in respect of change in size and/or colour. None was observed. Additionally, neither study showed a change in behaviour of mice in any of the treated groups, indicating no major toxicities. One should bear in mind that in both studies the single dose of 10 mg/Kg doxorubicin-equivalent that was used is a relatively low dosage. The rationale design of those experiments was to look for increase in survival time and signs of major toxicities. Having obtained these initial encouraging results, this paves the way to continue pharmacological experiments using the PDEPT system. A dose escalation should be used to determine the maximum tolerated dose (MTD) of doxorubicin-equivalent for PDEPT and there should be a thorough analysis of dose limiting toxicity of PDEPT. Additionally a multiple dose schedule of polymer-drug followed by polymer-enzyme must be examined to identify the optimum dosing schedule.

6.4.5 Comparison with other two step systems

It is interesting to compare the antitumour activity of PDEPT (even though these are first preliminary experiments at non optimised doses) with ADEPT combinations. Table 6.3 present some of the results obtained with ADEPT system tested on a CC3 human choriocarcinoma xenograft in male nude mice (Springer *et al.*, 1991). It can be seen clearly that the antitumour activity of ADEPT improved dramatically following optimisation of the drug schedule and the dose of both the antibody-enzyme conjugate, anti-human chorionic gonadotropin antibody-CPG2 (anti-HCG Ab-CPG2) conjugate, and the prodrug. Only after having optimised the schedule were improved responses, including long term survival of treated mice obtained. Therefore, it is important to stress that the PDEPT antitumour activity seen here is only the first step. The results obtained in the two studies reported here are very encouraging concerning the fact that the mice were treated with only a single i.v. injection of the polymeric prodrug PK1 at a low doxorubicin dose and only one dose of HPMA copolymer-cathepsin B was given as a follow up.

Svensson *et al.* (1995) worked on a different and very interesting ADEPT combination using cephalosporin derivative of doxorubicin (C-Dox) as the prodrug and β-lactamase conjugated to F(ab') fragments of mAbsL6 antibody (L6-βL). Again, the antitumour effect in mice bearing H2981 tumours was dependent on the enzyme and the prodrug dose. A 10-day delay in tumour growth to 500 mm^2 (compared to control group) was found with L6-βL in combination with C-Dox at the MTD (120 mg/Kg). Increasing the L6-βL by 10-fold (1.5 mg/Kg injection) and using three rounds of treatment resulted in a 60-day growth delay to 500 mm^2. The same schedule using C-Dox below the MTD (40 mg/Kg/injection) resulted in a 30-day delay of tumour growth.

Table 6.3 Survival of CC3-bearing nude mice in response to anti-HCG Ab-CPG2 conjugate followed by different prodrugs in different schedules

Tumour (s.c.)	Treatment (i.v./i.p.)	Dose	Schedule	p vs control group	Long term survival	Reference
CC3 human choriocarcinoma xenograft in male nude mice	anti-human chorionic gonadotropin Ab-CPG2 (i.v.) 4-[bis(2-mesyloxy-ethyl)amino]benzoyl-L-glutamic acid (i.v.)	10 U enzyme 3 x 5.6 mg	0, 22, 27 h. the first injection was after 24 h after the conjugate injection.	no significant improvement compared to control group p=0.636 (S10 conjugate)	0/6	Springer *et al.*, 1991
CC3 human choriocarcinoma xenograft in male nude mice	anti-human chorionic gonadotropin Ab-CPG2 (i.v.) 4-[2-chloroethyl)(2-mesyloxy-ethyl)amino]benzoyl-L-glutamic acid (i.v.)	10 U enzyme 3 x 5 mg	0, 22, 27 h. the first injection was after 24 h after the conjugate injection.	no significant improvement compared to control group p=0.105 (S10 conjugate)	0/6	Springer *et al.*, 1991
		50 U enzyme 3 x 10 mg	0, 22, 27 h. the first injection was after 72 h after the conjugate injection.	p=0.0009 (S10 conjugate) p=0.00006 (W14 conjugate)	4/6 5/6	Springer *et al.*, 1991
CC3 human choriocarcinoma xenograft in male nude mice	anti-human chorionic gonadotropin Ab-CPG2 (i.v.) 4-[bis(2-chloroethyl)amino]benzoyl-L-glutamic acid (i.v.)	10 U enzyme 3 x 5 mg	0, 22, 27 h. the first injection was after 24 h after the conjugate injection.	no significant improvement compared to control group p=0.101	0/4	Springer *et al.*, 1991

Table 6.3 continued

Tumour (s.c.)	Treatment (i.v./i.p.)	Dose	Schedule	p vs control group	Long term survival	Reference
CC3 human choriocarcinoma xenograft in male nude mice	anti-human chorionic gonadotropin Ab-CPG2 (i.v.) 4-[bis(2-chloroethyl)amino]benzoyl-L-glutamic acid (i.v.)	25 U enzyme 3 x 5 mg	0, 22, 27 h. the first injection was after 48 h after the conjugate injection.	no significant improvement compared to control group p=0.298	0/4	Springer *et al.*, 1991
		50 U enzyme 3 x 10 mg	0, 22, 27 h. the first injection was after 56 h after the conjugate injection	no significant improvement compared to control group p=0.101	0/4	Springer *et al.*, 1991
		50 U enzyme 3 x 10 mg	0, 22, 27 h. the first injection was after 72 h after the conjugate injection	p=0.027	1/4	Springer *et al.*, 1991

Once the polymeric prodrug of cephalosporin derivative of doxorubicin will be fully synthesised, it will be interesting to compare its antitumour effect, using the HPMA copolymer-β-lactamase as the activating enzyme, to the ADEPT system described here.

6.4.6 Experimental design

There is another issue which should be addressed with thought, concerning the experimental design of the pharmacological study in the COR-L23 cell line. The mice included in the PDEPT group showed a decreased tumour growth rate compared to the other groups, but not as high a T/C value as expected. Most of the mice had to be culled before reaching the tumour size of 289 mm^2, due to bursting or bleeding of the tumour. If the limit size of tumour would have been lower, the T/C values would have been very much different.

PK1 followed after 5 h by the administration of free cathepsin B gave an increased T/C value of 135.3%. The reason for testing the antitumour activity of this combination was to check if there was a difference between the free and conjugated enzyme administered in terms of ability to activate PK1. HPMA copolymer-cathepsin B has the ability to circulate for longer time in the bloodstream due to its higher molecular weight, as seen in Chapter 4, and this allows increased accumulation in the tumour. Still, free cathepsin B having a molecular weight of ~30 KDa has had a relating long circulation time and this led to some tumour accumulation due to the EPR effect, but the uptake was not as high as seen for the conjugated cathepsin B. Free cathepsin B is more likely to be excreted through the renal pathway being filtered by glomerular excretion. As mentioned previously, Seymour *et al.* (1995) studied the effect of molecular weight of ^{125}I-labelled HPMA copolymer (22-778 KDa) against the B16F10 model and demonstrated that the HPMA copolymers of molecular weight less than 40 KDa were subject to rapid renal clearance while higher molecular weight HPMA copolymers were retained in the body by avoiding renal clearance and persisted in the circulation for long periods. The blood concentration of HPMA copolymer with time indicated that circulation time played an important role in the level of accumulation in the tumour tissue. The same probably occurs with cathepsin B (30 KDa) and HPMA copolymer-cathepsin B (~60 KDa) in this study.

HPMA copolymer-cathepsin B was found to be a significantly better activating system than free cathepsin B. As previously discussed in Chapter 5, free cathepsin B would have theoretically released less doxorubicin from PK1, if given at the same activity units dose (and not the same concentration). This accounts for longer circulation time and thus higher accumulation in the tumour allowing better antitumour effect. It is important to note that free cathepsin B was not an effective therapy in its own right.

Although free cathepsin B was able to release doxorubicin from PK1 and gave an increased T/C value compared to control group in the B16F10 model, it was not tested on the COR-L23 model. It was decided not to pursue free cathepsin B further because of the expected disadvantage in the long term of free enzyme being immunogenic. The next chapter describes studies examining the immunogenicity of free enzymes (β-lactamase and cathepsin B) compared with HPMA copolymer-enzyme conjugates.

Chapter Seven

Evaluation of immunogenicity of HPMA copolymer-enzyme conjugates; preliminary results

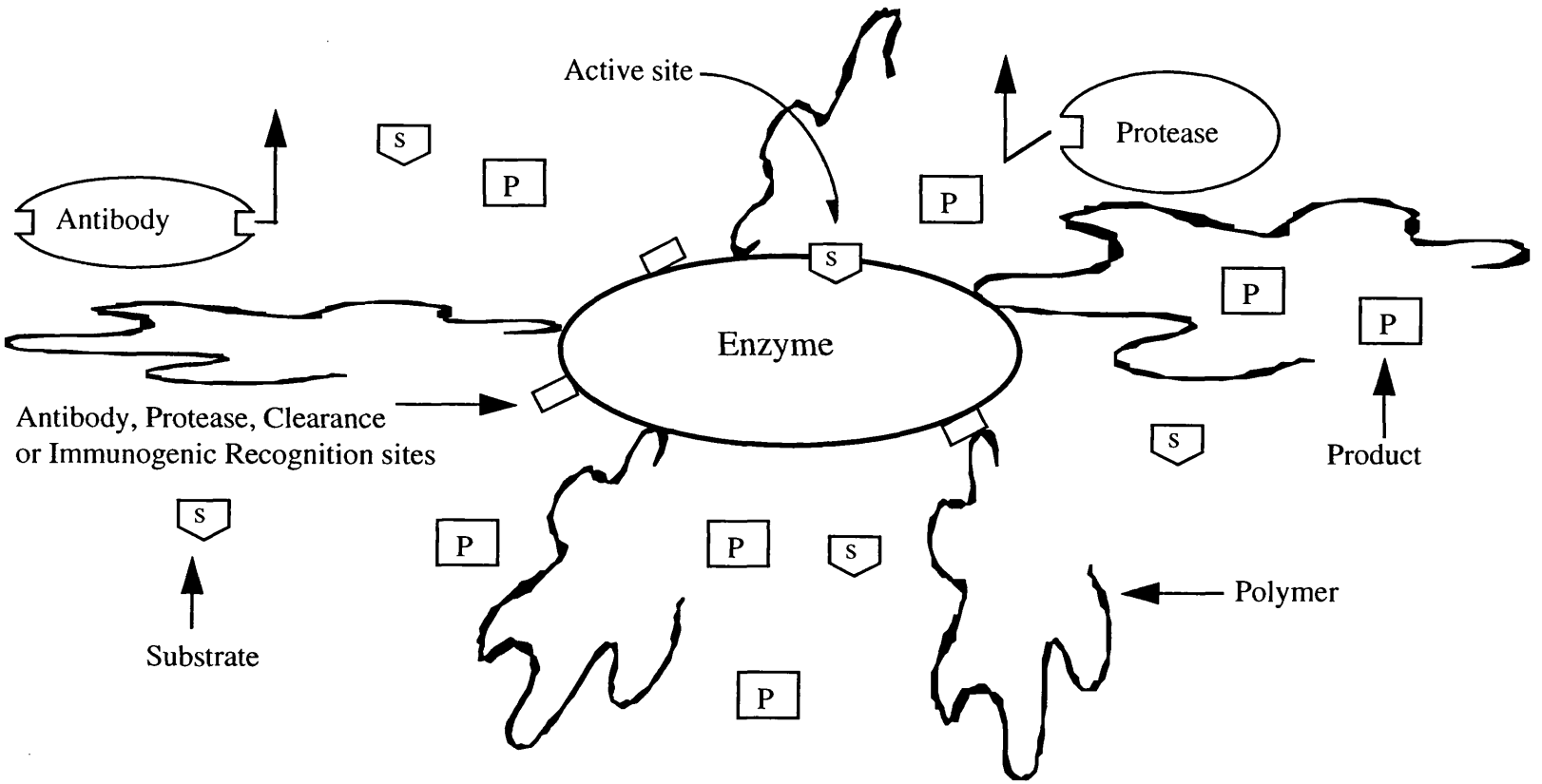
7.1. Introduction

Immunogenicity is already known to be a major problem associated with administration of antibodies and immunoconjugates to patients, either for diagnostic purposes (Courtenay-Luck *et al.*, 1986), radiotherapy (Klein *et al.*, 1986), or in the form of antibody conjugates for chemotherapy (Harkonen *et al.*, 1987). It is well known that foreign, high molecular weight protein drugs such as the enzymes asparaginase and catalase are usually immunogenic (Roitt, 1994) and that this is one major limitation to their use in human therapy. In addition to the inherent problem of immunogenicity, once a patient (or animal) has developed antibodies against a particular protein, it is likely that, on subsequent injections the protein will be rapidly removed from the circulation with the consequent need of increasing the therapeutic dose and/or the frequency of protein administration if desired pharmacological activity is to be maintained.

The attachment of bovine serum albumin and bovine liver catalase to polyethylene glycol was shown to render them nonimmunogenic (Abuchowski *et al.*, 1977a; 1977b), possibly by sterically hindering the antigenic determinants from immune surveillance. Sehon (1991) reviewed some studies which demonstrate the down regulation of the host's immune response to antigens coupled to monomethoxypoly(ethylene glycol) (mPEG). There is evidence to support the view that the specific immunosuppression induced by antigen-mPEG conjugates involves the activation of antigen-specific suppressor T cells (Sehon, 1982; Wilkinson *et al.*, 1987; Mokashi *et al.*, 1989; Takata *et al.*, 1990). Flanagan and colleagues (1990) reported that the immunogenicity of a protein was altered by covalent coupling to HPMA copolymer. They found a 250-fold reduction in the titre measured against IgG (antigen) after conjugation to HPMA copolymer. An explanation provided for this result was based on the structure of the conjugate, with up to 20 molecules of the polymer coupled to IgG (antigen) (Flanagan, 1987) possibly masking the antigenic determinants, thus rendering the protein nonimmunogenic. The factors believed to be responsible for the improved biological properties of proteins after the attachment of hydrophilic polymers are summarised in Figure 7.1. Not only does polymer conjugation increase circulation time (see Chapter 4) but the polymer side chains may also help to mask immunogenic determinants that would otherwise elicit antibody formation. Polymers may also sterically hinder the approach of pre-formed antibodies to the antigenic sites. The result of polymer conjugation is therefore decreased (or abrogated) immunogenicity and reduced or abolished immune clearance of the conjugate.

The problem of immunogenicity has been documented extensively and little serious effect has been reported for those polymers recently developed for

Figure 7.1 Proposed model to explain the biological properties and increased half-life of soluble polymer-enzyme adducts (adapted from Uren and Ragin, 1979)



* Polymer modification may: (a) mask antigenic determinants
(b) mask immunogenic recognition sites
(c) mask protease succeptible sites
(d) mask clearance recognition signals
(e) allow free access to low molecular weight substrates
(f) maintain systemic injectability

pharmaceutical use (Maeda *et al.*, 1992; Konno *et al.*, 1983, Konno *et al.*, 1984). It could be understood as biocompatibility (Sgouras, 1990; Maeda, 1994). Many polymers are known to exhibit very high compatibility, particularly, polyethylene glycol (Milton Harris, 1992). Extensive clinical applications have been performed in Japan using poly(styrene-co-maleic acid anhydride)neocarzinostatin (SMANCS) (Maeda *et al.*, 1992; Konno *et al.*, 1983; Maeda and Konno, 1997) which also seem to be without serious clinical or immunological problems. Considerable attention has already been devoted to testing the immunogenicity of the HPMA copolymers and it was found that HPMA copolymers do not only fail to induce an immune response against themselves, but they even have the capacity to decrease the antibody response against immunoglobulins or other proteins bound to them as targetable residues (Flanagan, 1987).

In this study it was expected that conjugation of the enzymes used to HPMA copolymer would mask the enzyme's antigenic determinants as previously described in the literature. The immunogenicity of HPMA homopolymer and a number of HPMA copolymers including protein conjugates has previously been investigated (Rihova *et al.*, 1983; 1984; 1985, 1989; Rihova and Riha, 1985; Flanagan, 1987; Flanagan *et al.*, 1993; Morgan, 1993). A variety of techniques have been used in these studies:

1. Determination of the number of antibody-producing cells in the spleen of immunised mice by extracting the bone marrow and subsequently measuring the number of colony-forming unit-spleens in recipient irradiated mice.
2. Demonstration of serum IgG and IgM antibody levels using either a passive haemagglutination assay or an ELISA test.

It was shown that HPMA homopolymer does not induce a measurable antibody response (Rihova *et al.*, 1983), and that modification of the homopolymer to incorporate oligopeptide side-chains (or side-chains terminating in model drugs such as fluorescein isothiocyanate; 2,4-dinitrophenol; arsanilic acid) results only in a weak immune response. The magnitude of the response was dependent on the side-chain structure (Rihova *et al.*, 1984). Using side-chains composed of different amino acids, the relative order of immunogenicity was found to be a Gly-Gly-OH > aminocaproyl-Leu-hexamethylene diamine > aminocaproyl-Phe-Ala-OH > Gly-Phe-Leu-Gly-OH. IgG antibodies were raised mainly against the oligopeptide side-chains and model drugs. The magnitude of the antibody response was not affected by the number of side-chains per HPMA copolymer molecule. Rihova *et al.* (1989) (and later repeated in a slightly different way by Flanagan *et al.*, 1993) found that the attachment of doxorubicin to HPMA copolymers (PK1) and in addition, the targeting residue

galactosamine (PK2) did not cause significant IgG antibodies production against the conjugate. On average, the titres of the antibodies against PK1 were lower by four orders of magnitude than those of antibodies raised against the bovine gamma globulin (Rihova *et al.*, 1984), which indicates a very low immunogenicity of the tested compounds. Following immunisation with soluble or complete Freund's adjuvant incorporated HPMA copolymers, different inbred strains of mice responded with a low IgM antibody response (1/600) (Flanagan *et al.*, 1993). For comparison, the response measured following administration of a protein antigen, such as serum albumin, using the same protocol in this strain of mouse is a titre of approximately $1/10^5$. HPMA copolymers activate neither the classic nor the alternative complement activation pathway (Simeckova *et al.*, 1986). If the molecular weight is below 45 KDa, they are eliminated from the organism within hours by urinary excretion (Seymour *et al.*, 1990) and do not accumulate in any tissue.

The immune response elicited by HPMA copolymers was dependent upon the mean molecular weight of the polymer. After injection of HPMA copolymer of different mean molecular weight to mice, the number of antibody-producing cells detected in the spleen was 2-5 fold greater after injection of HPMA copolymers with a mean molecular weight 150-200 KDa, than after injection of similar HPMA copolymer conjugates of mean molecular weight 5 KDa. Also, the immune response was dependent on the dose of antigen given. Using a dose range of 1-100 μg Rihova *et al.* (1985) found that the optimal dose of polymer was 10 μg. Higher doses were thought to be tolerogenic. In the same study they also found that the genetic background of the mice was very important in determining the immune response. It has been shown by numerous studies (Fink and Quinn, 1953; Rihova and Riha, 1985) that antibody response is under genetic control. Moreover the magnitude of the response can be quite different and characteristic for each inbred strain. Consequently one inbred strain of mice is not sufficient and at least two strains should be tested simultaneously for evaluation of new antigens (Anderson and Hughes, 1986). However, the strain is usually chosen in view of availability, cost and the facilities available (Hurn, 1980). In the present study, Balb/c mice were used which are known to produce a good humoral immune response (Rihova, 1998). Future development of the evaluation should be the incorporation of a second strain. Flanagan *et al.* (1990) have used both high (female A/J mice) and low (B/10 mice) IgG responders for the evaluation of immunogenicity of protein-HPMA copolymer conjugates.

It was important to ensure that conjugation of HPMA copolymer to β-lactamase or cathepsin B would reduce the immunogenicity of β-lactamase or cathepsin B. Here a

preliminary study is described which measured the IgG titre 6 weeks after administration of free and conjugated enzymes.

7.2 Methods

7.2.1 Preparation of antigens

HPMA copolymer-enzyme conjugates were synthesised and characterised as previously described in Chapter 2.3.1.

7.2.2 Immunisation protocol

The immunisation protocol was followed according to the procedure described in Chapter 2.3.13.1 to 6 groups of Balb/C mice (6 mice per group).

7.2.3 Determination of antibody titers

IgG antibody titers were measured by an indirect enzyme-linked immunosorbent assay (ELISA) (Engvall, 1980). In short, ELISA plates were coated with the antigen, the serum to be tested then applied and the bound specific antibodies revealed by an enzyme-labelled anti-immunoglobulin raised in a different animal species. Addition of the enzyme's substrate, yields a coloured product whose absorbance can ultimately be related to the serum antibody titer. A detailed description of the ELISA procedure is in Chapter 2.3.13.3. The use of BSA as a blocking step could not be applied here, since BSA is used in this experiment, as a positive control for the induction of immune response. An alternative solution to the problem was to use 3% skimmed milk in PBS-Tween-20 as a blocking agent as was done previously by Aramaki *et al.* (1994). The ELISA procedure includes 6 steps as follows:

Step 1: Antigen coating of the plates

Immunoassay plates were coated with either cathepsin B, β-lactamase or BSA by placing into each well 60 μl of 2 μg antigen per ml coating buffer (0.31 g Na_2CO_3 and 0.58 g $NaHCO_3$ up to 200 ml with DDW, pH 9.6). Plates were incubated overnight at 4^0C whereby antigen absorption to the plates occurred.

Step 2: Addition of blocking solution

Unbound antigen was aspirated and the plates washed three times with PBS (pH 7.4) containing 0.025% Tween-20 (washing buffer). To avoid non specific binding, plates were treated with 0.2 ml per well of 3% skimmed milk in washing buffer for 2 h at 37^0C and washed three times with washing buffer.

Step 3: Addition of serum samples
After washing the plates, 60 µl of washing buffer was added to each well, 60 µl duplicate serum samples added to the first well, were serially diluted. The plates were then incubated for 1 h at 37^{0}C.

Step 4: Addition of sheep anti-mouse serum
After washing the plates as previously, 50 µl per well of horseradish peroxidase-conjugated sheep anti-mouse immunoglobullins (IgG) were added at 1:4000 dilutions in washing buffer containing 3% skimmed milk, and incubated at 37^{0}C for 1 h.

Step 5: Substrate reaction
Plates were washed as above and 200 µl of freshly prepared *o*-PDA substrate solution added to each well. The reaction was allowed to proceed for 25 min and then stopped with 25 µl of 1.8 M sulfuric acid.

Step 6: Determination of optical density
The colorimetric end point was measured spectrophotometrically at 492 nm on a Titerteck Multiskan ELISA Reader (Flow Laboratories, Herts, UK). A known positive serum and pooled normal mouse sera collected from untreated mice were employed as positive and negative controls respectively.

The serum IgG antibody titers against the antigen were expressed as the reciprocal of the highest sera dilution at which a positive antibody-antigen reaction was detected (absorbance reading of 0.2 at 492 mm in the microplate reader), when compared with the reactivity of control serum with the same antigen, using the ELISA technique.

It is important to note that Balb/C mice were not challenged with the free and bound enzymes i.v. (although this is the usual route of administration of proteins) due to the fact that a higher immunological response is to be expected from s.c. or i.p. injection. Also injections were given in saline since it is no longer legal to use complete Freund's adjuvant (as was used in similar studies in the past), to elicit the highest response possible. However saline is more relevant to a likely pharmaceutical formulation.

7.3 Results

When carrying out an ELISA assay, serum samples are diluted serially into microtiter plates. It is important to understand the consequences of this ongoing serial

dilution of antibody. Table 7.1 shows the different dilutions used in this study for β-lactamase and cathepsin B.

7.3.1 IgG response to free β-lactamase and HPMA copolymer-β-lactamase

Serum antibody titers against β-lactamase, as measured by ELISA are presented in Figure 7.2. It can be seen that there was a 4-fold reduction in immunogenicity after conjugation of β-lactamase to HPMA copolymer. It is apparent that although the immunogenicity was clearly not eliminated by the conjugation of β-lactamase to HPMA copolymer, the conjugated enzyme elicited significantly ($p < 0.02$) less antibody than the free enzyme.

7.3.2 IgG response to free cathepsin B and HPMA copolymer-cathepsin B

Due to the different efficiencies of antigen binding, the coating concentration used varied. At the beginning the same concentration as the one used for β-lactamase was used (2 μg/ml; meaning 0.12 μg per well), but no results were obtained. Therefore the concentration was increased to 1 μg per well and a serial dilution curve was obtained from the ELISA analysis.

IgG titers raised against free and conjugated cathepsin B are shown in Figure 7.3. Antibodies against HPMA copolymer-cathepsin B were not detectable and the antibody titre towards free cathepsin B was also very low giving a titre of 10 (i.e. 10-fold sera dilution at the end point).

7.4 Discussion

It is perhaps not surprising that there was only a 4-fold reduction in the immunogenicity of the conjugated β-lactamase and it was not abrogated, considering that (a) it is a bacterial enzyme and (b) that there are free amino groups that are not linked to the HPMA copolymer (see Chapter 3), therefore acting as antigenic determinants.

The low immune response raised against the free cathepsin B might indicate that there is some degree of species homology and the bovine cathepsin B used in this study may not be presented as a totally foreign antigen to the mouse. The same phenoma was observed by Flanagan *et al.* (1990) who investigated the effect of HPMA copolymer conjugation to transferrin as compared to free transferrin. Free transferrin showed a low immune response, due to some degree of species homology between the human transferrin used in that study and the murine transferrin (Metz-Boutigue *et al.*, 1984;

Table 7.1 Serial dilution of serum

Well row	β-Lactamase antibody dilution	Cathepsin B antibody dilution
A	20	20
B	40	40
C	80	80
D	160	160
E	320	320
F	640	640
G	1280	1280
H	2560	2560
A *	140 *	5 *
B	280	10
C	560	20
D	1120	40
E	2240	80
F	4480	160
G	8960	320
H	17920	6400

* New serial dilutions were examined since the first set gave results, readings, which were too high for the sensitivity of the counter for β-lactamase and too low for cathepsin B)

Figure 7.2 Immunogenicity of free and bound ß-lactamase

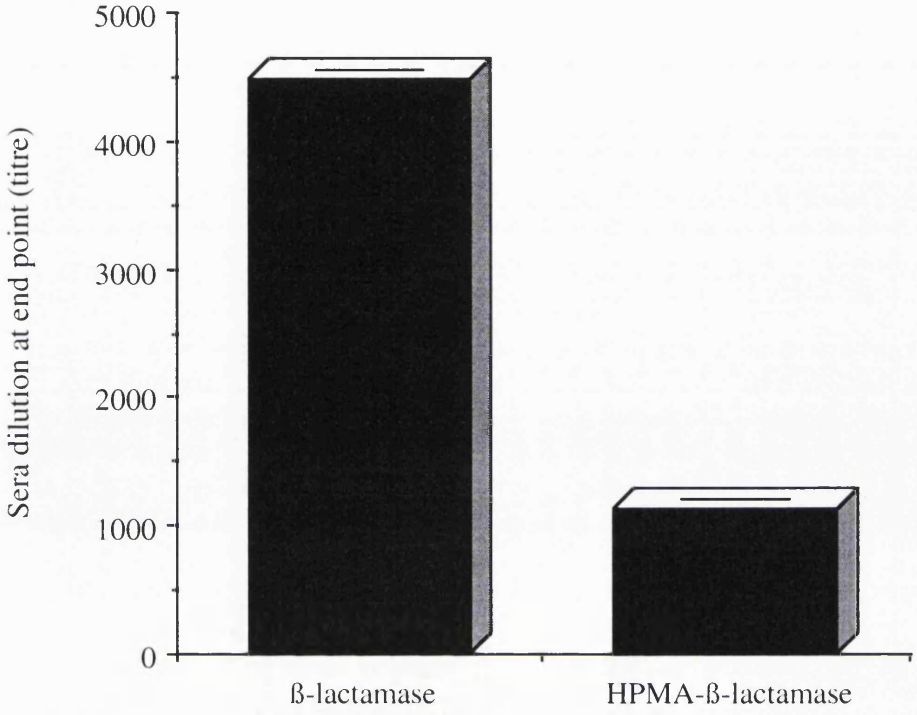
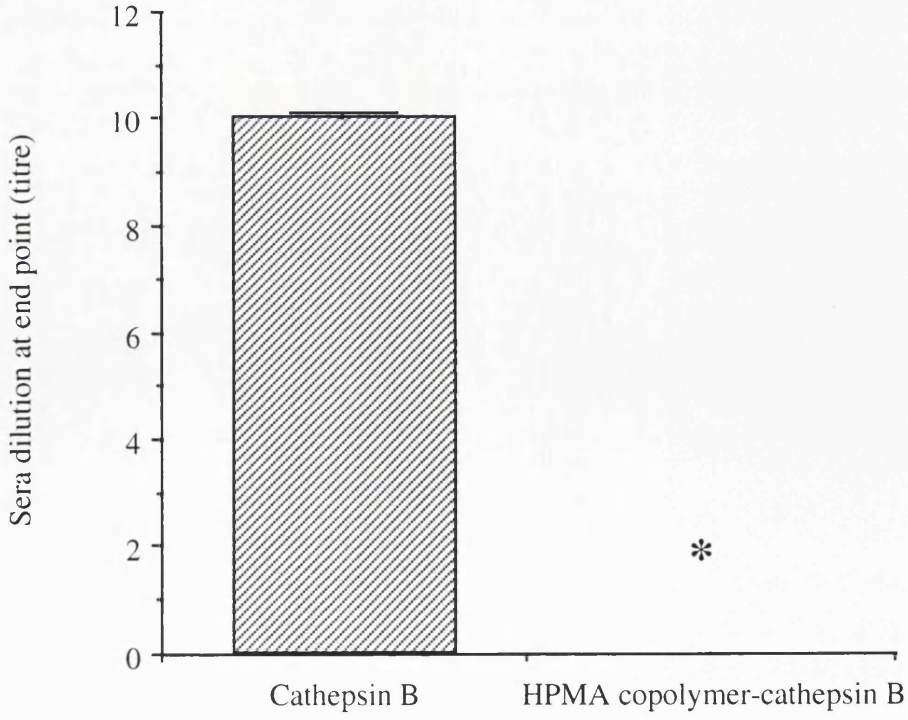


Figure 7.3 Immunogenicity of free and bound cathepsin B



Tsavaler *et al.*, 1986), so that the "human transferrin" may not be presented as a totally foreign antigen.

As mentioned in the introduction to this chapter, IgG conjugated to HPMA copolymer showed a 250-fold decrease in the antibody titer against the conjugated IgG compared to free IgG (Flanagan, 1987). A lower reduction was observed when conjugating transferrin to HPMA copolymer (4-fold decrease). It should be noted though, that this study was performed in the presence of complete Freund's adjuvant (CFA). When the study was performed in PBS solution in the absence of CFA, there was a 4-16-fold reduction in immunogenicity (depending on the mouse strain AJ or B10) when conjugating IgG to HPMA copolymer compared to free IgG, and 2-4-fold reduction when conjugating transferrin to HPMA copolymer compared to free transferrin (Flanagan, 1987). It can be seen that the preliminary results shown here are in agreement with previous work conjugating proteins to HPMA copolymer, but how does it compare to conjugation of proteins to other polymers, such as PEG and dextran?

Polyethylene glycols have stimulated a lot of research into their immunological properties, more specifically due to their ability to reduce or eliminate the immunogenicity of several enzymes covalently conjugated to them (Abuchowski *et al.*, 1977b). The mechanism for reduced antigenicity and immunogenicity of strong antigens following conjugation to PEG, could partly be explained due to masking of antigenic determinants by the PEG molecules, but could also be due to a tolerance mechanism induced by activation of suppressor cells by PEG (Lee and Sehon, 1977, Sehon, 1988). The immunogenicity of PEG-proteins is usually either reduced or abolished depending on the degree of PEGylation and on the molecular weight and type of PEG. The reported ability of PEG to disguise the antigenic determinants of catalase (Abuchowski *et al.*, 1977b) and other proteins such as phenylalanine ammonia-lyase (Wieder *et al.*, 1979), albumin (Sasaki *et al.*, 1993) or superoxide dismutase (Miyata *et al.*, 1988) is dependent on the degree of PEGylation as seen in Table 7.2. For instance, PEG-catalase modified to different extents (13,19,37 and 43% of the amino groups available) when injected intravenously in rabbits, showed a decrease in antibody production directly related to the number of PEG chains attached to the enzyme. Total suppression of immunological response was however, only achieved with the preparation most PEGylated. It is thus conceivable that more extensive degree of aminolysis while conjugating the β-lactamase could further reduce its immunogenicity (although not recommended due to decrease in the enzymatic activity, see Chapter 3.3.2), but additional experiments are required.

Table 7.2 Reduced immunogenicity of PEGylated proteins as related to the degree of modification and type of PEG.

PEG-protein	Degree of modification (%)	PEG type	Route of administration	Animal	Immunogenicity	Reference
Arginase	53	PEG 1	i.v.	Mice	Abolished	Savoca *et al.*, 1979
Asparaginase	56	PEG 2	i.v.	Balb/C mice	Reduced	Kamisaki *et al.*, 1981
	?	PEG 2	i.v.	Mice	Reduced	Wada *et al.*, 1990
Bovine albumin	90	PEG 1	i.v. + i.m.	Rabbits	Abolished	Abuchowski *et al.*, 1977a
Catalase	93-95	PEG 1	i.v. + i.m.	Rabbits	Reduced *	Abuchowski *et al.*, 1977b
Phenylalanine ammonia-lyase	48	PEG 1	i.m.	New Zealand white rabbits	Reduced	Wieder *et al.*, 1979
Superoxide dismutase	?	?	i.p. + i.v.	Mice	Reduced	Fuertges and Abuchowski, 1990
		PEG 2	i.p.	Mice	Reduced	Miyata *et al.*, 1988

* PEG 1900-catalase was immunogenic i.m. but not i.v. PEG 5000-catalase was not immunogenic by either route

The route of PEG-protein administration seems to have little effect on the antibody titer for enzymes such as asparaginase (Abuchowski *et al.*, 1984) and superoxide dismutase (Fuertges and Abuchowski, 1990). The immunogenicity of PEG-1900-catalase administered by the i.v. route was on the other hand, different than that obtained by the i.m. route (Abuchowski *et al.*, 1977b) (Table 7.2). The inability to reduce the immunogenicity of phenylalanine ammonia-lyase to the same extent observed with other enzymes (Wieder *et al.*, 1979), was attributed to the enzyme's dissociation into subunits following injection and simultaneous exposure of antigenic determinants. Abuchowski and colleagues (1984) reported that PEG-asparaginase was a poor immunogen when evaluated by immunodiffusion but definitely immunogenic if assessed by ELISA (a method that is about a thousand-fold more sensitive than immunodiffusion). These results cast some doubts on the validity of the immunogenic evaluation of PEG-proteins, considering that immunodiffusion and the precipin assay have been the most commonly used techniques (Table 7.2). Human antibodies (mainly of the IgM type) raised against PEG occur naturally in 0.2% of the population and are deemed to be of no clinical significance (Richter and Akerblom, 1984).

Dextrans were extensively studied for their immunogenic properties due to their use as plasma expanders (Maurer *et al.*, 1953; Kabat and Berg, 1953). Their immunogenicity depends on the form of presentation, molecular size, resistance to degradation and injected dose (Bishop and Jennings, 1982). In man and mouse dextrans were shown to be immunogenic only if injected in a low dose, whereas in higher doses they induce immunological tolerance. The ability to respond to dextran immunisation appears late and bears the characteristics of a thymic dependent antigen (Schlossman and Kabat, 1962). The immunological properties of dextran conjugates have not been extensively investigated but seem to vary with the protein. Asparaginase modified with dextran (70 KDa), for example, was non-immunogenic in rabbits (Table 7.3). After repeated injections with the dextran conjugate, free enzyme circulated as in intact animals (Wileman *et al.*, 1986). No hypersensitivity reactions were observed when the conjugate was given as a single injection to patients suffering from lymphoblastic leukaemia (Wileman *et al.*, 1983). On the other hand, dextran modification of superoxide dismutase increased the enzyme's immunogenicity (Table 7.3). Other enzymes such as catalase and α-amilase showed reduced but not abrogated immunogenicity (Davis *et al.*, 1991). Concerns about the use of dextran as a protein modifier derive from its reported immunogenicity in humans (Torii *et al.*, 1976) and non-biodegradability in the lysosomes.

Specific antibodies developed against the enzyme might play a role in the enzyme's blood clearance. This investigation may offer a means of prediction of

Table 7.3 Immunogenicity of dextran conjugated enzymes

Dextran-protein	Animal (route)	Immunogenicity	Reference
Asparaginase	Mice (i.p.)	Increased	Wileman *et al.*, 1986
Superoxide dismutase	New Zealand white rabbits (i.m.)	Abolished *	Miyata *et al.*, 1988

* No antibodies were detected either against the enzyme or the dextran moieties.

eventual problems that may arise from the prolonged use of conjugated enzymes in cancer therapy. The data presented indicate that it may be possible to give multiple doses of HPMA copolymer-enzyme conjugates before an immune response becomes limiting. Further immunisation experiments are therefore warranted to determine:

1. an appropriate dose regimen of immunisation.
2. relationship between molecular weight of antigen and the amount needed to coat an ELISA plate well with a monolayer.
3. antibody titer, in terms of moles of antibody produced.
4. if other antibodies were produced as IgM, by taking blood samples after each injection. In this way, we can detect at what stage each antibody is produced

However, the limited control of stoichiometry and orientation of the enzyme in these conjugates (see Chapter 3) means that no two preparations of a single conjugate will ever be identical. Necessarily, further experiments will therefore be able to characterise only single batches of antigens with integral controls.

Chapter Eight

General Discussion

The work described in this thesis represents the first attempt to use the combination of a polymer-drug and a polymer-enzyme conjugates as a means to improve anticancer therapeutic efficacy and therapeutic index. A new concept called PDEPT (Satchi and Duncan, 1998) was developed and tested *in vitro* and *in vivo*.

It was hoped that attachment of the linear, flexible and hydrophilic chains of the HPMA copolymer to the enzyme would elevate its molecular weight and cover antigenic determinants on the enzyme's surface thus diminishing interaction with tissue and blood components. After conjugation, it was predicted that the reduced enzyme clearance, and therefore increased plasma half life would allow tumour targeting by the EPR effect as had been shown when the low molecular weight anticancer drug, doxorubicin, was previously attached to HPMA copolymer (Seymour *et al.*, 1994). It was hoped that combining an HPMA copolymer-enzyme and the appropriate HPMA copolymer-drug conjugate as substrate in the form of the PDEPT schedule would enable selective accumulation of both components in the tumour by the EPR effect and subsequent drug release selectively within the tumour tissue. The main findings of this study and the major recommendations for the application of PDEPT and required future work, are presented here.

PDEPT- a comparison with ADEPT and GDEPT

At the start of this study the two step anticancer approaches called ADEPT and GDEPT were already well known (reviewed in Bagshawe and Begent, 1996; Springer and Niculescu-Duvaz, 1996). Although there is still a long way to go with PDEPT (the system is yet not optimised) it is possible to compare the results obtained to date with the more established concepts of ADEPT and GDEPT.

In the course of this study, β-lactamase was attached to HPMA copolymer as a model polymer-enzyme conjugate. Having established, with β-lactamase, that it was possible to achieve enzyme conjugation by aminolysis whilst retaining enzymatic activity, a second enzyme cathepsin B was subsequently conjugated to HPMA copolymer using the same method. It was shown (Chapter 3) that both β-lactamase and cathepsin B retained 20-25% of their activity, and the conjugates displayed a similar K_m and V_{max} to those achieved by enzyme-antibody conjugates: The difference between PDEPT and ADEPT and GDEPT is the fact that bound enzyme was selected to cleave a high molecular weight polymeric substrate (PK1 in this case). In the case of ADEPT and GDEPT prodrug activation has been limited to low molecular weight prodrugs (Niculescu-Duvaz *et al.*, 1998; Haisma *et al.*, 1998; Blakey, 1997; Connors, 1995; Connors and Knox, 1995).

The next challenge for PDEPT design was the optimisation of the coupling method so as to obtain the highest yield of the polymer-enzyme conjugate, whilst still retaining enzymatic activity. The extent to which the β-lactamase and cathepsin B were bound to HPMA copolymer was directly related to the duration of the conjugation reaction. In Chapter 3 it can be seen that the content of enzyme in the conjugate was amenable to manipulation by changing the ratio of enzyme:HPMA copolymer in the reaction mixture, and also the pH at which the reaction was held. It is interesting to note that enzyme activity retained was dependent on degree of enzyme modification.

Study of the *in vivo* pharmacokinetics of enzyme and conjugate revealed an increase in blood residence of the conjugated enzyme compared to that seen for the free enzyme (Chapter 4). The increase in half-life could be attributed to the decrease in the number of amino groups of the conjugated enzyme and/or the increase in molecular weight (from 30 KDa, free enzyme to 60 KDa, bound enzyme). The observed decreased (Chapter 7) immunogenicity of β-lactamase and cathepsin B after conjugation to HPMA copolymers was encouraging. Since both half-life in the circulation and immunogenicity were related to the degree of conjugation, it is reasonable to suggest that a greater degree of enzyme conjugation will further increase the former and decrease the latter. This could, however, prove particularly difficult due to possible denaturation of the enzyme (see Tables 3.2 and 3.3). Additional immunologic and pharmacokinetic evaluation in different animal species using other parenteral routes and different dosages, is required.

Figure 8.1 proposes the next pharmacokinetic studies that I believe would be needed to optimise the dosing schedule of both the components, polymeric-prodrug and polymer-enzyme conjugate. Measurement of the pharmacokinetics of the polymeric prodrug at 90 mg/Kg (the MTD of PK1 in the B16F10 model (Duncan *et al.*, 1992)) with a constant dose of polymeric enzyme and *vise versa*, would allow to optimise the dosing combination and achieve the highest possible therapeutic dose (MTD) for it. Following this, a multiple dose at days 0, 5 and 10 with the optimised combination schedule would be needed to examine its pharmacokinetics. A five day interval between repeated injections is necessary for the neovasculature to recover, since the polymeric prodrug and the polymer-enzyme need the leaky vessels to be able to extravasate into the tumour. The reason for selecting these intervals is that the increase in B16F10 tumour size (Figure 6.1) is much slower until day 5 compared to the following days (due to the fast recovery of neovasculature in this aggressive model). In the case of the COR-L23 tumour model the interval would probably need to be 10 days due to the slower tumour growth rate (Figure 6.4). In every tumour model examined, a single dose treatment would enable us to predict the optimised multiple dosing schedule.

Figure 8.1 Pharmacokinetics experimental design

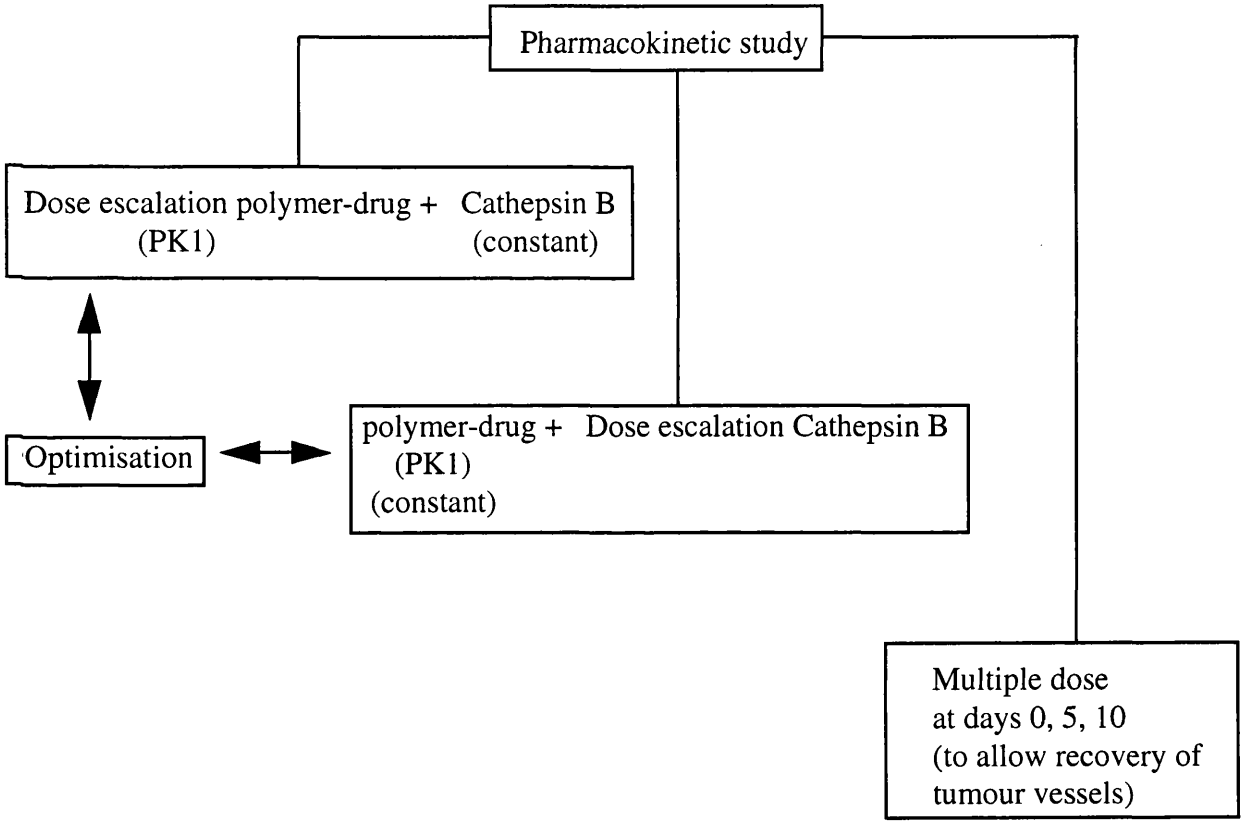
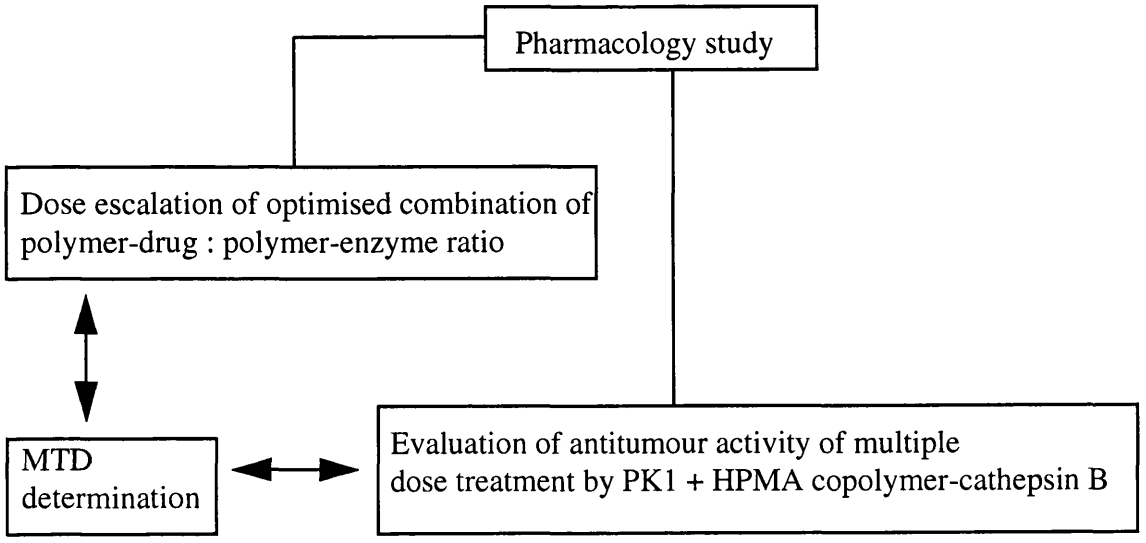


Figure 8.2 Pharmacology experimental design



It is interesting to note that HPMA copolymer-enzymes may display better passive tumour targeting than receptor-mediated targeting of antibody-enzyme conjugates. Stribbling *et al.* (1997) examined the biodistribution of the antibody-enzyme conjugate, CPG2 conjugated to anti-carcinoembryonic antigen (anti-CEA) antibody A5B7, and found that optimised dose and timing of administration of A5B7-CPG2 conjugate resulted in 0.08% of dose administered/g tumour. In contrast, in this study it was shown that HPMA copolymer-β-lactamase and HPMA copolymer-cathepsin B produced tumour levels of 3 and 5%/g respectively in a non optimised schedule (Chapter 4). Furthermore, for best ADEPT antitumour activity Stribbling *et al.* (1997) had to wait at least 72 h to administer the prodrug in order to achieve tumour:blood ratio of conjugate activity of 5.5. This ratio is very impressive, but one has to remember that after 72 h there is very little antibody-enzyme remaining in the tumour.

The data presented here relating to HPMA copolymer-cathepsin B and HPMA copolymer-β-lactamase conjugates show that attachment of other enzymes to HPMA copolymer may be a suitable means to overcome problems of immunogenicity or inappropriate pharmacokinetics therapy. Enzymes which exhibit either short circulating half-life or immunological side-effects (e.g. bacterial enzymes such as β-lactamase) would gain the most from conjugation to the polymer. However, extrapolation of the observations made here to other proteins should be made systematically and each combination would need to be fully evaluated.

In this study conjugation of enzymes to HPMA copolymers yielded complex heterogeneous mixture of molecular species which proved difficult to characterise by conventional methods (e.g. GPC, Mass Spectrometry; Chapter 3.2). The production of defined and well characterised polymer-enzyme conjugates (in terms of reproducible binding characteristics, conformation and spatial orientation of the polymer-enzyme conjugate) will be needed for scale-up manufacture and regulatory approval of this kind of constructs. Nonetheless, it is important to stress that the problem of polymer-enzyme heterogeneity is also shared by the PEGylated enzyme conjugates and this has not prevented their approval by licensing authorities (e.g. FDA) for human use (e.g. PEG-L-asparaginase for treatment of leukaemia). Indeed a random copolymer of 4 amino acids called Copaxone® has been approved for the treatment of multiple sclerosis.

When administered 5 h after PK1, HPMA copolymer-cathepsin B also showed *in vivo* accumulation in the tumour and produced a significant increase in the rate of doxorubicin release from PK1 compared to the rate of drug release when PK1 is

degraded by endogenous cathepsin B (Chapter 5). After 5 h PK1 is already lost from the circulation due to renal elimination so there was no risk of free doxorubicin being released systemically when administering the HPMA copolymer-enzyme conjugate at this time point. In contrast, when using the ADEPT system the optimum schedule described involved administration of antibody-enzyme conjugate 72 h prior to administration of the prodrug to ensure removal of the antibody enzyme from the bloodstream. The antitumour activity studies found that the PDEPT combination of PK1 followed by HPMA copolymer-cathepsin B decreased the progression of both B16F10 murine melanoma and the human xenograft COR-L23 (non-small cell lung carcinoma) compared to that seen in the control group and also when animals were treated with PK1 alone (Chapter 6).

Many other aspects of PDEPT antitumour activity have yet to be optimised. These include, the effect of repeated cycles of administration of polymer-drug and polymer-enzyme and the dosage optimum. Although ADEPT showed much more impressive antitumour results in animal models (with long term survivors) than those described here for PDEPT (see Table 6.3, Chapter 6.4.5), PDEPT must in future be evaluated against a number of tumour models with high and low EPR targeting using the optimised PDEPT dosing schedule and multiple dose injections as proposed schematically in Figure 8.2. This experimental design of pharmacological studies would enable establishment of the MTD for PDEPT.

PDEPT- contribution to science?

The choice of PK1 as model polymeric prodrug for PDEPT combination was a convenient one. Many studies describing animal pharmacology and pharmacokinetics (Duncan *et al.*, 1989; Duncan *et al.*, 1992; Flanagan *et al.*, 1993; O'Hare *et al.*, 1993; Pimm *et al.*, 1996; Rihova *et al.*, 1989; Seymour *et al.*, 1987; Seymour *et al.*, 1990; Seymour *et al.*, 1994; Seymour *et al.*, 1995) had been described already when this study was initiated. The novelty was the possible use of PK1 in combination with a polymer-enzyme to amplify doxorubicin release and therefore improve its activity. This study also describes for the first time HPMA copolymer-cathepsin B and HPMA copolymer-β-lactamase conjugates. Amplification of activating enzyme present in tumour (in the case of mammalian enzymes as cathepsin B) or delivery of foreign enzyme to the tumour (Chapter 4), while reducing its immunogenicity (Chapter 7), were achieved.

It has been shown in the past that HPMA copolymer conjugation is able to reduce the immunogenicity of a bound protein (reviewed in Chapter 7), but this polymer has never been used to reduce the immunogenicity of a bacterial enzyme.

Quantitatively, the reduction in immunogenicity of β-lactamase shown here (a 4-fold decrease) is in accordance with the literature (2-16-fold decrease, Flanagan *et al.*, 1993; Morgan, 1993). However, unexpectedly free cathepsin B induced a very low IgG response. As an alternative to PDEPT it might be suggested that cathepsin B alone could be used as a means to increase the rate of doxorubicin release from PK1. However, there is an obvious risk that repeated injections of cathepsin B might produce an antibody response. The HPMA copolymer-cathepsin B conjugate also showed better tumour accumulation making it a superior choice from that viewpoint too.

Furthermore, this study is the first time that a polymer-drug has been used in combination with a polymer-enzyme conjugate. Prior to PDEPT, the polymeric combinations used included:

- the incorporation of a targeting residue into the polymeric drug (e.g. PK2 using galactosamine conjugated to the same HPMA copolymer chain to target hepatocytes (Duncan *et al.*, 1983b; Seymour *et al.*, 1997); the use of peptide hormone such as MSH on the same HPMA copolymer chain containing doxorubicin (O'Hare *et al.*, 1993) or the use of proteins as transferrin-containing daunomycin conjugates (Flanagan *et al.*, 1989; Flanagan *et al.*, 1992).

- simultaneous administration of HPMA copolymer-doxorubicin in conjunction with an HPMA copolymer-photosensitiser, meso-chlorin e_6 monoethylene diamine disodium salt, to be activated by light (Krinick *et al.*, 1994).

Neither of these approaches employ an administered enzyme to activate the release of the drug from its polymeric backbone.

During the three years since this study started there have been no major breakthroughs in cancer therapy. Awareness of the importance of polymeric anticancer agents and the opportunities for tumour targeting have however increased dramatically and this will move cancer therapy forward in the next millennium. The first paper describing a Phase I clinical trial involving a polymer therapeutics (PK1) has recently been published (Vasey *et al.*, 1999). After preclinical studies (Springer *et al.*, 1991; 1995) and an exploratory Phase I trial (Bagshawe *et al.*, 1995), a controlled clinical trial of ADEPT was undertaken in 10 patients with colorectal carcinoma using anti-CEA antibody conjugated to CPG2 (Begent *et al.*, 1998). In this study a galactosylated antibody directed against the active site of CPG2 was used to clear, and inactivate, circulating antibody-enzyme. Only when plasma enzyme levels had fallen to a pre-determined safe level was a benzoic acid-mustard-glutamate prodrug given and this was

converted by CPG2 in the tumour into a benzoic acid mustard. Only one patient showed partial tumour response and 6 had stable disease for a median of 4 months.

Another clinical trial of ADEPT using a prodrug which is converted by CPG2 to a di-iodophenylenediamine mustard is ongoing. As this drug has superior properties compared to the benzoic acid mustard it will be interesting to see the results.

The therapeutic possibilities of gene therapy in general (reviewed in Richardson, 1999), and GDEPT in particular (Niculescu-Duvaz *et al.*, 1998), have also come to the for greatly in the last few years. Although, GDEPT has not reached yet the clinical stage due to problems in targeting the gene to the tumour tissue specifically, pilot trials are ongoing to evaluate GDEPT against ovarian carcinoma where an adenocarcinoma adenovirus containing genetically modified nitroreductase is administered i.p. to activate CB1954 as the prodrug. Another proposed trial involves injection of transcription activation factors intratumourally to colon metastasis in the liver. At the moment, the gene has been injected intratumourally to animals (Springer, 1999). Targeting a gene to the tumour to express the activating enzyme by conjugating it to a polymer (Richardson, 1999) or a liposome might be the answer for GDEPT problems of tumour selectivity.

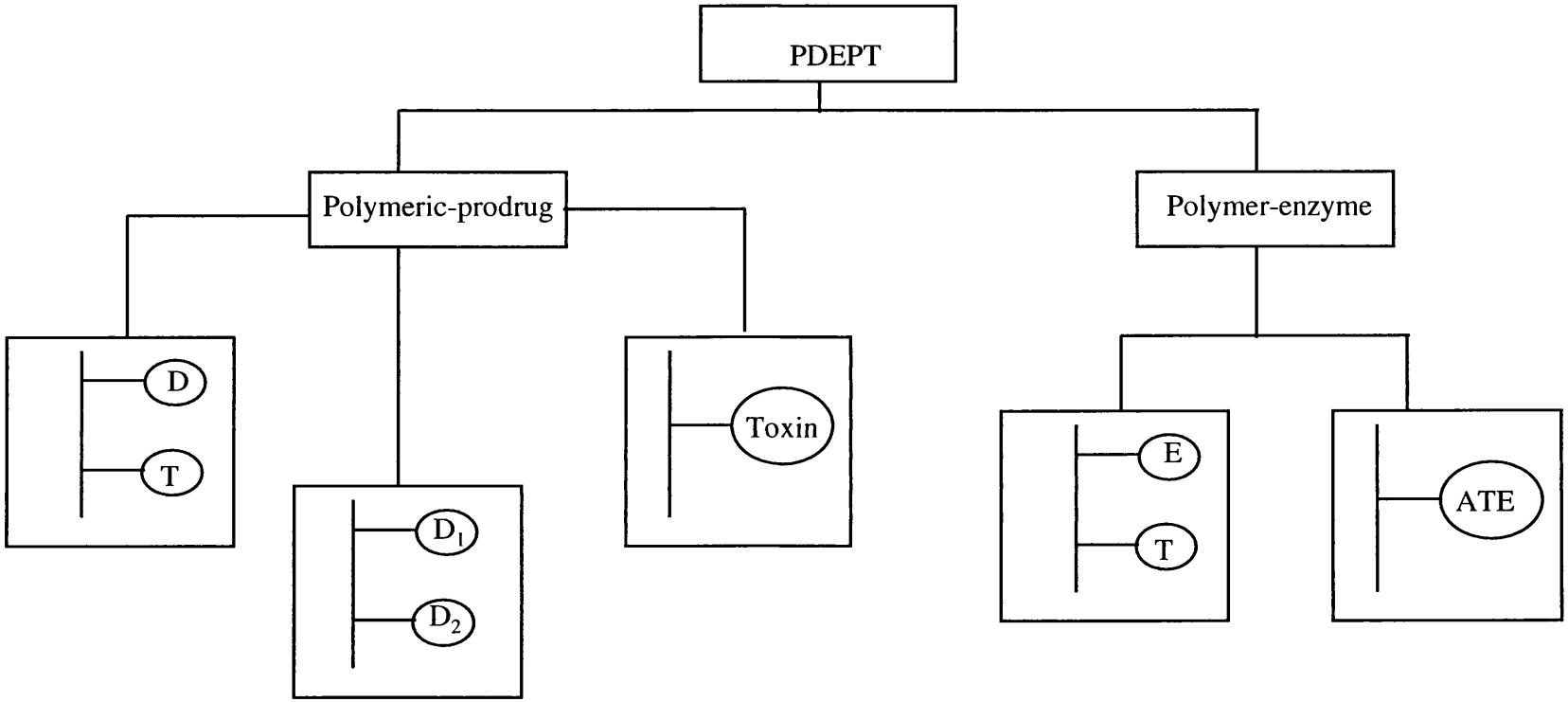
In the last few years the development of humanised antibodies and fusion proteins have started to overcome the problem of protein/antibody immunogenicity (reviewed in Chester and Hawkins, 1996). Rituximab (Rituxan; IDEC Pharmaceuticals, San Diego, CA) is the first monoclonal antibody approved by the US Food and Drug Administration (FDA) for the treatment of cancer. It is a genetically engineered chimeric (murine-human) monoclonal antibody (mAb) directed against the CD20 antigen found on the surface of normal and malignant B cells. Multicenter studies have demonstrated its efficacy against relapsed low-grade and follicular non-Hodgkin's lymphoma. Because of its human component, rituximab has low immunogenicity and should not significantly hinder future retreatment (Leget and Czuczman, 1998). Smith *et al.* (1997) amongst others have used crystallography, molecular modelling, shuffling PCR and site-directed mutagenesis to develop a human enzyme that is capable of catalysing a reaction that is otherwise not carried out in the human body. This would allow use of prodrugs that are otherwise stable *in vivo* but that are substrates for a tumour-targeted mutant human enzyme and hopefully avoid the immunogenicity problems.

Options for future development of PDEPT

Drug combinations

PDEPT opens a whole new field of two-step process and combination therapies using polymers as carriers for both the drugs and the enzymes. Figure 8.3 shows

Figure 8.3 Options for future PDEPT combinations



T- Targeting moiety
D- Drug
E-activating enzyme
ATE-Antitumour enzyme

several ways of applying polymer combinations in the future. As mentioned in Chapter 1.9.3, a combination of two different anticancer drugs (e.g. doxorubicin and cisplatin) would have the advantage of synergistic antitumour activity and reduced toxicity. Therefore, conjugation of two drugs on the same polymeric chain with the same linker as the polymeric-prodrug in the first step of PDEPT would be interesting. This could be followed by the administration of a polymer-enzyme conjugate directed to cleave the linker present in the polymeric prodrug.

Use of anticancer enzymes or toxins

Use of an antitumour enzyme (e.g. anti-tumour necrosis factor) or a toxin (e.g. Diphtheria toxin (Chiron *et al.*, 1994), *Pseudomonas* Exotoxin A (PE) Mutants (Benhar *et al.*, 1994)) or even a viral vector (to improve the specificity of GDEPT) conjugated to a polymer could be another form of novel combinations of PDEPT. PE is cleaved within mammalian cells between Arg^{279} and Gly^{280} to generate an enzymatically active toxin. Chiron *et al.* (1994) have prepared an enriched and soluble form of protease, with PE cleaving activity, as a furin-like enzyme. It cleaves PE on the COOH-terminal side of the selected sequence producing the same toxic fragments as those generated within cells. Attaching this furin-like enzyme to HPMA copolymer can achieve tumour targeting to cleave a polymeric prodrug of HPMA copolymer-PE specifically as a PDEPT combination.

Alternative HPMA copolymer-drug conjugates as components of PDEPT

Another important point to consider is the drug used. Doxorubicin was chosen here as a model drug but in future other drugs could become the agents of choice. A more potent drug such as the topoisomerase I inhibitor camptothecin (Liu *et al.*, 1998; Rowinsky *et al.*, 1998) might be a better more potent candidate. Agents interacting with signal transduction pathways, in particular Tyrosine Kinase Inhibitors (Matter, 1998) are another important group to consider as candidates for PDEPT combinations. Kinases are being used as cancer targets since they are overexpressed or mutated in cancer (e.g. EGF receptor, erbB2), translocated in lymphocytic leukaemia (bcr-abl translocation), activated in colorectal and other cancers (src) and mainly involved in cell-cycle kinetics (cyclin-dependent kinases) (Jackson, 1999). Conjugation of kinase inhibitors to a polymer and their release specifically in tumours can improve their poor pharmacokinetics (Jackson, 1999).

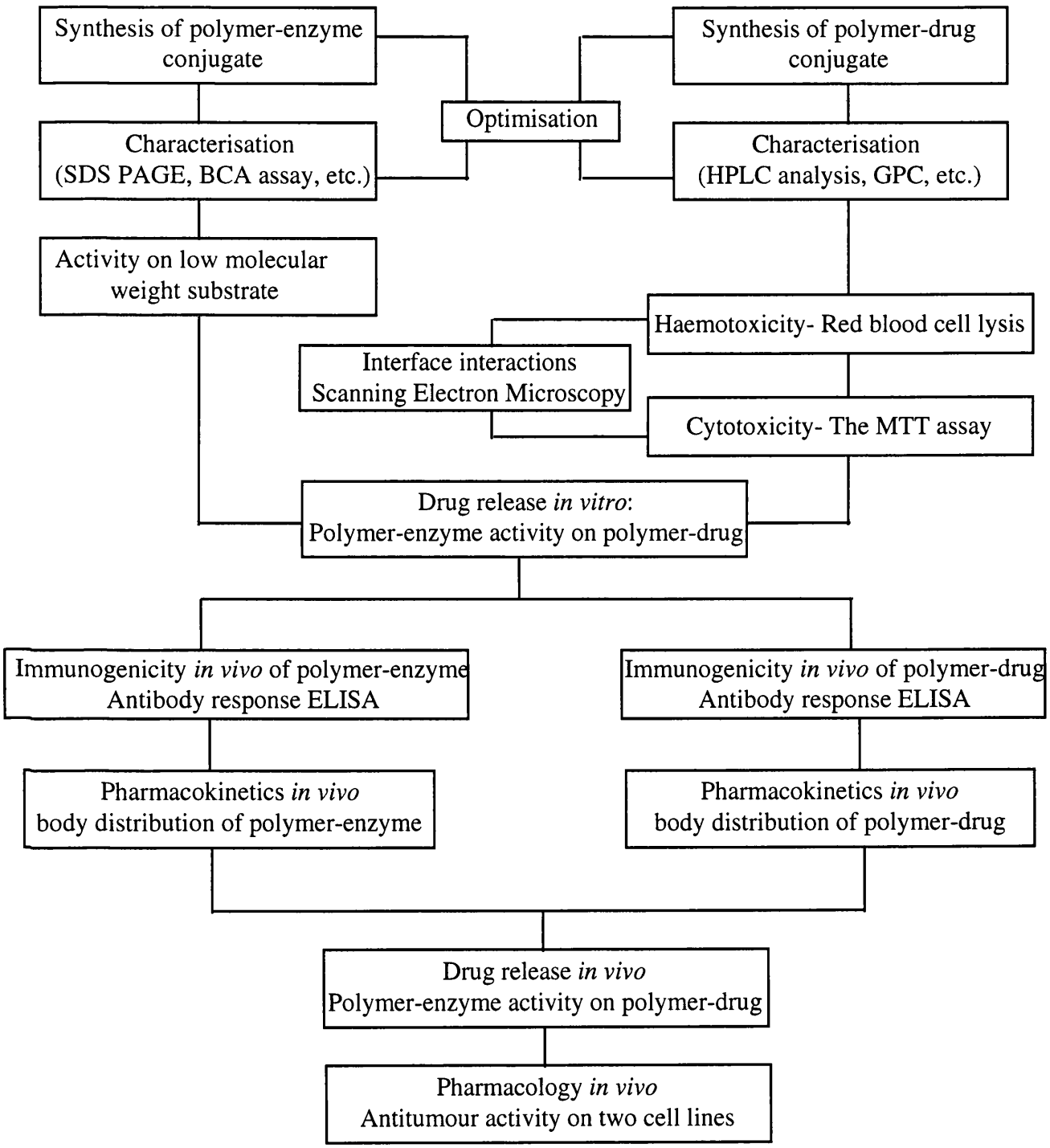
Alkylating agents might theoretically be good candidates for PDEPT since their cytotoxicity is dose-related and they can be given repeatedly with less induced resistance than other classes of anticancer agents (Teicher and Frei, 1988). The problem with alkylating agents is that they will not be detoxified enough, even when conjugated

to polymers, because of their chemical instability. The azo mustards are the more likely to be chosen from this family since they would be more stable and could be activated by azoreductases present in high levels in the tumour and also by administered polymer-azoreductase conjugate as the second step of PDEPT.

β-Lactamase as the cleaving enzyme might be used in conjunction with Protax (a taxol molecule attached by a short linking group to cephem sulphoxide produced by Genentech (Rodrigues *et al.*, 1995)). The taxol will be released when the β-lactamic ring reacts with β-lactamase. Again, both constructs will be conjugated to polymers in order to allow selective accumulation in the tumour. This combination might solve the problems taxol exhibits such as insolubility and toxicity to bone marrow, nerve fibres and mucus membranes and take advantage of its high potency.

All these different options are very nice in theory, but each combination has to be tested and fully optimised individually. Figure 8.4 shows the development process that every new PDEPT combination would have to pass through. As stated before (Connors, 1996), the results of testing of hundreds of thousands of chemicals against test systems ranging from a wide number of different tumour cell lines is generally a waste of time. Evaluation of two cell lines (murine tumour and human xenograft tumour models) should be enough to continue further preclinical testing. In the end, as with any new and exciting set of findings, we are left with more questions than answers. What is clear from the results to date is that a proof of principle of the strategy of PDEPT was achieved and a more critical research is required before its true potential is known.

Figure 8.4 Evaluation of PDEPT combination



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Zunino F, Pratesi G and Micheloni A, Poly(carboxylic acid) polymers as carriers for anthracyclines, J. Controlled Rel., **10**, 65-74 (1989).

Appendix I

Publications

List of Publications

1. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy, Proceedings of the International Symposium on Controlled Release of Bioactive Materials, Stockholm, Sweden, **24**, 773-774 (1997).
2. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy, Joint meeting of the British Association of Surgical Oncology and British Association for Cancer Research; Cancer Cure: Drugs, Genes or Surgery ? London, U.K., P16 (1997).
3. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy, Proceedings of the International Symposium on Polymer Therapeutics- From Laboratory to Clinical Practice, London, UK, **3**, 58 (1998).
4. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy- *in vitro* and *in vivo* characterisation, Proceedings of the NCI-EORTC Symposium on New Drugs in Cancer Therapy, Amsterdam, The Netherlands, **10**, 312 (1998) .
5. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy, British Journal of Cancer, **78**(2), 149-150 (1998).
6. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy- in vitro and in vivo characterisation, Annals of Oncology **9** (S2), 312 (1998).
7. Duncan R, Sat YN, Satchi R, Gianasi E, Malik N and Richardson S, Tumour targeting of anticancer drugs and oligonucleotides using polymeric carriers, British Association for Cancer Research/ Royal Society of Medicine (Oncology Section) Joint meeting on "Carcinogenesis and Chemoprevention", London, U.K., P2.10 (1998).
8. Satchi R and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy- from synthesis to pharmacokinetics and pharmacology, United Kingdom & Ireland Controlled Release Society Symposium on: "Polymeric Drug Delivery into the New Millenium", Aston, U.K., **5** (1999).
9. Satchi R Connors TA and Duncan R, PDEPT: *in vivo* pharmacokinetics and pharmacology, Proceedings of the International Symposium on Controlled Release of Bioactive Materials Boston, USA, **26** (1999).
10. Satchi R Connors TA and Duncan R, PDEPT: Polymer directed enzyme prodrug therapy I. HPMA copolymer-cathepsin B and PK1 as a model combination, British Journal of Cancer, in preparation (1999).

Patent

Satchi R and Duncan R, PDEPT, Enzyme conjugates and their therpaeutic use with prodrugs, International Patent No 97304070.2 (1998).