MEMBRANE ACTIVE POLYMER ANTICANCER CONJUGATES

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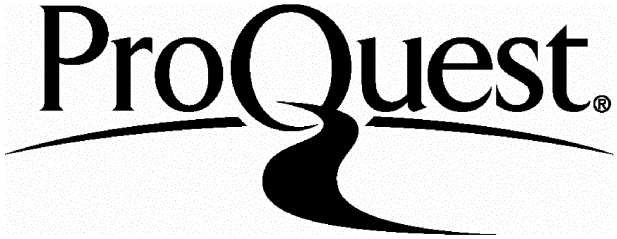

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Abstract

HPMA copolymer-drug anticancer conjugates have been successfully transferred into clinical evaluation. Such macromolecular constructs selectively accumulate within solid tumours by the enhanced permeability and retention (EPR) effect. However, lysosomotropically activated compounds such as HPMA copolymer-doxorubicin (PK1) present limitations in that they require enzymatic activation within lysosomes in order to release the active moiety which in turn exerts its cytotoxic effect.

Hence this study looked at the development of a second generation, membrane active polymeric anticancer conjugates. Using melittin (MLT) as a model peptide, HPMA copolymer-MLT conjugates were synthesised and characterised using standard biochemical techniques (SDS PAGE, BCA protein assay and FPLC). The effect of MLT content using conjugates with high (38.9 ± 2.5 % w/w), medium (25.8 ± 6.2 % w/w) and low (12.1 ± 8.6 % w/w) MLT loading and the effect of peptidyl linker (-Gly-Gly- (GG) and -Gly-Phe-Leu-Gly- (GFLG)) were investigated *in vitro*. The MLT conjugate with medium loading and GG spacer showed reduced haemolytic activity (Hb_{50} 19.9 ± 6.2 μg/ml ($p < 0.05$)) in a rat red blood cell lysis model, and maintained cytotoxic activity against a B16F10 murine melanoma cell line (IC_{50} 7.3 ± 1.5 μg/ml; $p = 0.46$ (NS)) relative to free MLT. This conjugate was chosen to proceed with preliminary *in vivo* studies. The MTD of HPMA copolymer-MLT was found to be 4-fold greater than that of free MLT (10 mg/kg MLT-equivalent) and body distribution of MLT conjugate 3 mg/kg (MLT-equivalent) (i.p.) showed 3 - 4-fold increased circulation time. However, no improved tumour targeting by the EPR effect was established 4 h after i.p. administration. Disappointingly, no antitumour activity was observed *in vivo* following i.p. or i.v. administration. Nevertheless, improved synthetic procedures to prepare more compounds of this new class of anticancer agents is warranted.

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Abbreviations

Acquired Immunodeficiency Syndrome	AIDS
Alpha	α
Antibody Directed Enzyme Prodrug Therapy	ADEPT
Bicinchoninic Acid	BCA
Bovine Serum Albumin	BSA
Becquerel	Bq
Beta	β
Carmustine	BCNU
Circular Dichroism	CD
Counts Per Minute	CPM
Curie	Ci
Daltons	Da
Deoxyribonucleic Acid	DNA
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Divinylether–Maleic Anhydride Copolymer	DIVEMA
Dimethyl Formamide	DMF
Dimethyl Sulphoxide	DMSO
Doxorubicin	DOX
Drug Delivery System	DDS
Elution Volume	*Ve*
Enhanced Permeability and Retention Effect	EPR
Epidermal Growth Factor	EGF
Epsilon	ε
Ethylenediaminetetraacetic acid	EDTA
Fast Performance Liquid Chromatography	FPLC
Foetal Bovine Serum	FBS
Food and Drug Administration	FDA
Fourier Transform Infrared Spectroscopy	FTIR
Forward Scatter	FSC
Galactosylated *N*-(2-Hydroxypropyl)methacrylamide copolymer-Doxorubicin	PK2
Gamma	γ
Gel Permeation Chromatography	GPC

Gene Directed Enzyme Prodrug Therapy	GDEPT
Haemoglobin	Hb
50 % Haemolysis Concentration	Hb_{50}
Hanks Balanced Salt Solution	HBSS
Honey Bee Venom	HBV
Human Immunodeficiency Virus	HIV
Human Serum Albumin	HSA
N-(2-Hydroxypropyl)methacrylamide	HPMA
N-(2-Hydroxypropyl)methacrylamide Copolymer-Doxorubicin	PK1
N-Hydroxy succinimidyl	NHS
50 % Inhibitory Concentration	IC_{50}
Interferon	IFN
Interleukin	IL
Intraperitoneal	i.p.
Intravenous	i.v.
Maximum Tolerated Dose	MTD
Molar Mass	M
Melanocyte Stimulating Hormone	MSH
Melittin	MLT
Minimum Inhibitory Concentration	MIC
Mononuclear Phagocyte System	MPS
Multiple Drug Resistance	MDR
p-Nitroanilide	NAp
p-Nitrophenyl	ONp
Not available	NA
Not determined	ND
Nuclear Magentic Resonance	NMR
Number	No.
Number Average Molar Mass	Mn
Oriented Circular Dichroism	OCD
Phosphate Buffered Saline	PBS
Phosphatidylserine	PS
Poly(ethylene glycol)	PEG
P-glycoprotein	PGP
Poly(ethylenimine)	PEI

Poly(styrene-co-maleic anhydride)-neocarzinostatin	SMANCS
Red Blood Cells	RBC
Reticulo-Endothelial System	RES
Reverse Phase High Performance Liquid Chromatography	RP-HPLC
Room Temperature	RT
Scanning Electron Microscopy	SEM
Sephadex G25	PD-10
Size Exclusion Chromatography	SEC
Sodium Chloride	NaCl
Sodium Dodecyl Sulphate	SDS
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis	SDS PAGE
Sodium Hydroxide	NaOH
Standard Deviation of The Mean	SD
Standard Error of The Mean	SE
Subcutaneous	s.c.
N-Succinimidyl 3-(4-hydroxy-5-^{125}I-di-iodophenyl) propionate	Bolton and Hunter reagent
Sum	Σ
Superoxide Dismutase	SOD
N,N,N',N'-Tetramethylethylenediamine	TEMED
Tris(hydroxymethyl)aminomethane	Tris
United Kingdom Co-ordinating Committee on Cancer Research	UKCCCR
UV-visible	UV-vis
Void Volume	V*o*
Volume for Volume	v/v
Viral Directed Enzyme Prodrug Therapy	VDEPT
Weight Average Molar Mass	Mw
Weight for Weight	w/w

Amino Acid Abbreviations

Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F

Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

Chapter One

General Introduction

1.1 Cancer and its Treatment

Cancer is a broad term which encompasses a vast range of diseases whereby uncontrolled cell proliferation is the common factor (King, 1996). Cancer is the prime cause of mortality in the UK (Mason, 1998). Globally, at present, 10 million people are diagnosed with cancer each year and it is predicted this figure will double by the year 2020 (Sikora, 1999). It is hoped that the determination of the human genome sequence will provide a greater understanding of health and disease, including the molecular basis of cancer, and thus enable the development of appropriate medicines and improve prognosis.

With the currently available treatments, on average an approximately 35% "cure" (defined as long term disease-free survival (reviewed by Frei, 1985)) rate is achieved (Purves, 1996). The means of combating neoplasia include (1) surgical resection of tumours, (2) cytotoxic drug treatment, (3) biological response modifiers (immunotherapy), (4) hormonal therapy and (5) irradiation. These treatments are often given alone or in combination, but even so, are poorly effective. The major draw back of successful treatment by chemotherapy is the selection of tumour cells which possess an acquired or intrinsic resistance to anti-cancer drugs (King, 1996 and Hoffmann *et al*, 1997). The non-selective ubiquitous distribution of cytotoxic compounds equivocally damages rapidly proliferating normal cells as well as neoplasms. This results in haematological side effects e.g. myelosuppresion and consequent neutropenia and thrombocytopenia. Non-haematological side effects include damage of the gastro-intestinal epithelium and hair follicles resulting in emesis and alopecia respectively (Rang *et al*, 1995).

Consequently, a lot of anticancer research has progressed towards identifying new targets, designing novel chemical entities or improvement of existing therapeutics by the development of drug delivery systems (DDS). In the case of DDS, their design seeks to achieve manipulation of the biodistribution of the active drug in order to achieve selective tumour cell kill. The concept of selective drug delivery is the basis of this thesis.

Polymer therapeutics are a class of DDS which have been developed over the last two decades (section 1.5). At present, 8 polymer-drug conjugates are in Phase I / II clinical evaluation (reviewed by Duncan, 2001). These conjugates are activated

intralysosomally to release the active drug (section 1.4.1). The primary aim of this study was to develop (synthesise, physico-chemically and biologically characterise) (Chapters 3, 4 and 5) the first generation of polymer-peptide (melittin, MLT) conjugates which are active at the level of the tumour cell membrane. The secondary aim of this thesis was to improve the understanding of the mode of action of the *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-doxorubicin construct (PK1, FCE 28068) (Chapter 6) which is presently in Phase II clinical trials. In this introduction, the general concepts of tumour targeting, polymer therapeutics and membrane active agents will be reviewed.

1.2 Tumour Targeting

Drug targeting is defined as the selective delivery of active moieties to desired pharmacological sites. Targeted drug delivery to the tumour site offers potential advantages over conventional therapy. These include:

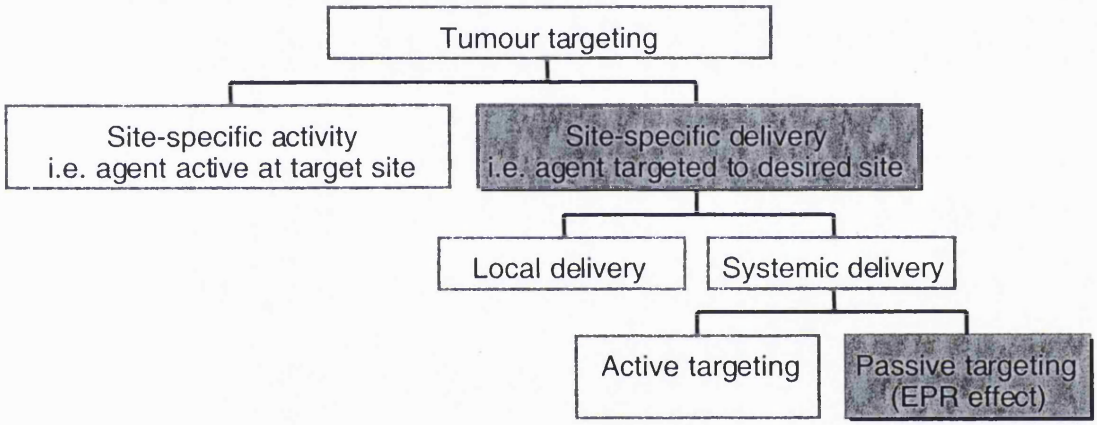
- Increased bioavailabilty of active agent due to prevention of premature metabolism and excretion
- Supressed accumulation in normal tissues and thus reduction in side effects which improves quality of life of the patient
- Decreased frequency of administration and thus improved patient compliance
- Change of route of cellular uptake, hence by-passing cell resistance mechanisms.

In principle, drug targeting may be achieved by 2 mechanisms: site-specific activity and site-specific delivery (summarised in Figure 1.1). These will be discussed below.

Site-specific activity of the agent only at the target site is enabled by local administration. This includes depot preparations such as polyanhydride matrix incorporating carmustine (BCNU) (Gliadel® wafer) for glioma (Brem *et al*, 1995) and direct administration e.g. intra-hepatic poly(styrene-co-maleic anhydride)-modified neocarzinostatin (SMANCS) with lipiodol for hepatoma (Kanematsu *et al*, 1989 and Konno, 1992).

Site-specific delivery of the active agent to the target site may be classified as active and passive targeting (reviewed by Tomlinson, 1987 and Langer, 1998). Examples of delivery systems which capitalise on selectivity following systemic administration include smart polymer systems e.g. photodynamic therapy with meso-

Figure1.1 Summary of tumour targeting strategies



chlorin e6 (Peterson *et al*, 1996), pulsatile drug delivery from polymer matrices by an external magnetic field, ultrasound, electric current or pH (reviewed by Langer, 1998) and prodrug therapy e.g. antibody directed enzyme prodrug therapy (ADEPT) (Bagshawe, 1989 and Knox & Connors, 1995), gene directed enzyme prodrug therapy (GDEPT) and viral directed enzyme prodrug therapy (VDEPT) (Ram, 1999).

Active Targeting

Active targeting (Ehrlich's "magic bullet") (Ehrlich, 1906) involves recognition and localisation of a ligand at the surface of a desired cell and may be defined at three levels; first order (organ-specific), second order (cell-specific) and third order (organelle-specific) (Duncan, 1992; Cassidy *et al*, 1993; Davis & Illum, 1994; Vyas *et al*, 2001). Targeting moieties explored so far for site-specific delivery of anticancer drugs include:

1) Proteins and peptides, such as:

i) Antibodies e.g. the anti-CD33 calicheamicin immunoconjugate, gemtuzumab ozogamicin (Mylotarg®) for $CD33^+$ acute myeloid leukaemia (Sievers *et al*, 1999); the recently FDA-approved engineered (humanised) cancer therapeutic monoclonal antibodies e.g. anti-HER2 trastuzumab (Herceptin®) for $HER2^+$ breast cancer (Molina *et al*, 2001)

ii) Hormones e.g. melanocyte stimulating hormone (MSH)-targeted HPMA copolymer-doxorubicin designed for the treatment of melanoma (O'Hare *et al*, 1993)

iii) Growth factors e.g. epidermal growth factor (EGF)-targeted dextran conjugates (Andersson *et al*, 1991) for certain gliomas, melanomas and squamous carcinomas

iv) Bioconjugate receptor-mediated uptake via serum proteins e.g. transferrin, lipoproteins (reviewed by Kratz & Beyer, 1998)

2) Carbohydrates e.g. galactosylated HPMA copolymer-doxorubicin (PK2, FCE 28069) (Duncan *et al*, 1983a; Kerr *et al*, 1998; Julyan *et al*, 1999) for hepatoma via asialoglycoprotein receptors (Poznansky & Julyan, 1984).

In practice, however, efficient and specific receptor-mediated targeting of therapeutic concentrations of anticancer agent to the tumour site still proves to be a challenge due to factors such as poor affinity of the ligand for the receptor, low density of the target

receptor, poor homogeneity of receptor within a tumour and level of expression of the receptor in normal tissue (reviewed by Ríhová, 1998 and Chari, 1998).

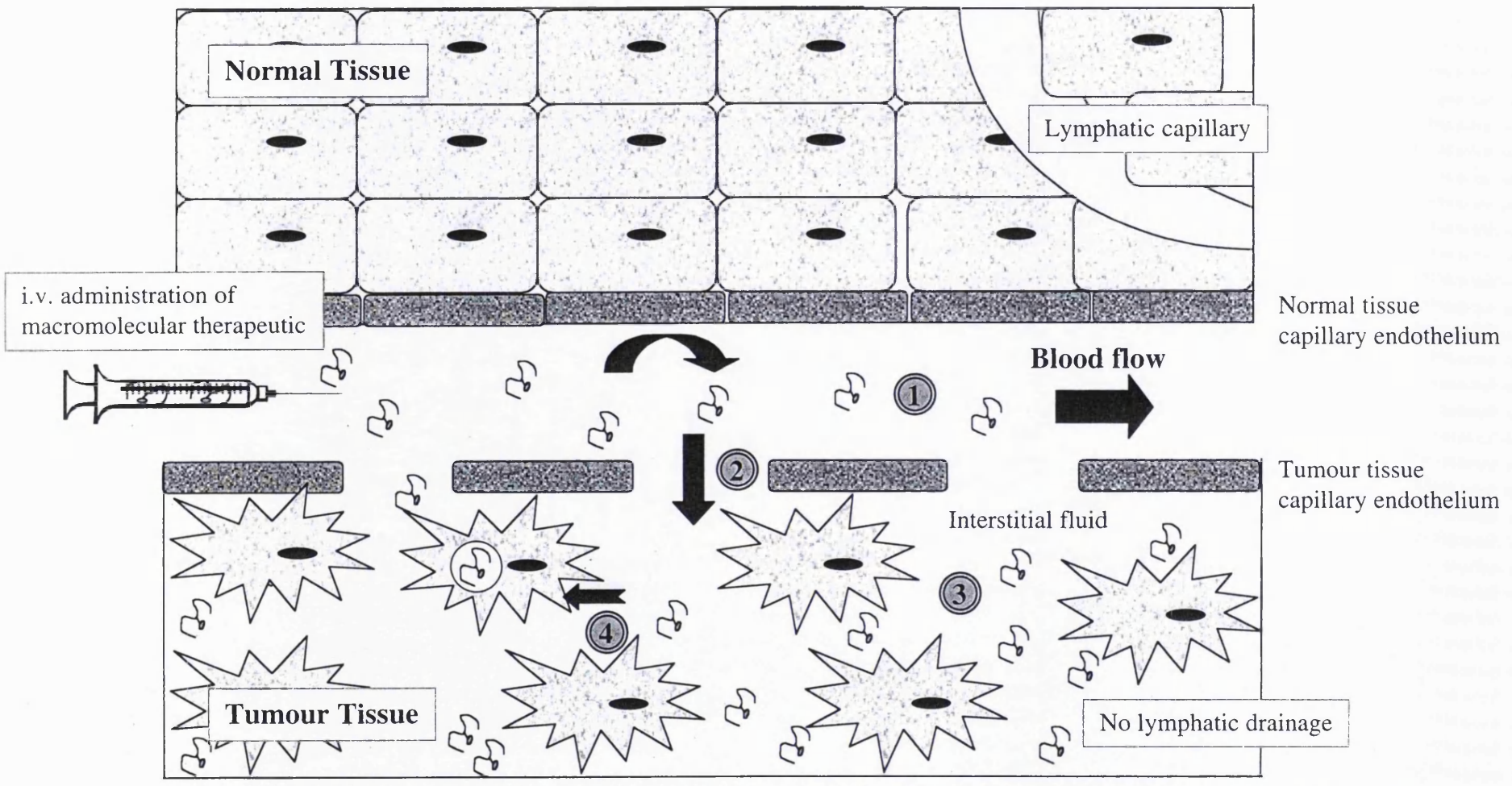
Passive targeting

The limitations of active targeting outlined above stimulated an interest in an alternative methodology of tumour targeting. Colloidal and macromolecular carrier systems have been utilised to target passively low molecular weight anticancer agents to tumours. Passive targeting is therefore fundamental to the concept of polymer therapeutics (defined in section 1.5). The mechanisms that cause passive targeting are generally either: (1) selective extravasation of particles through hyperpermeable capillaries (Matsumura & Maeda, 1986) or (2) reticulo-endothelial / mononuclear phagocyte system (RES / MPS) selective uptake (Davis & Illum, 1994). In contrast to active targeting, passive targeting results from a combination of the physicochemical characteristics of the carrier and the pathophysiological properties of the body, or more specifically of the diseased tissue e.g. the increased permeability of tumour vasculature which causes accumulation of the the carrier (agent) at a specific target site (reviewed by Yokoyama & Okano, 1996 and Maeda *et al*, 2000).

The carrier properties which allow avoidance of RES uptake and thus provide the prolonged plasma half-life necessary for tumour targeting include small particle size. Particles of 100 – 200 nm diameter often escape uptake by the liver and spleen (reviewed by Brannon-Peppas, 1995), whereas microparticles > 7μm in diameter are rapidly filtered out from the lung capillary bed (Davis & Illum, 1986). Particles < 7μm in diameter are usually captured by the RES (Bellanti, 1985). Those polymers which are not captured by the RES but have a molecular weight < 40 kDa (the approximate renal threshold) (Seymour *et al*, 1995) usually display renal clearance by glomerular filtration. The hydrophobicity and charge of water-soluble polymers also influence circulation time and RES localisation (reviewed by Takakura & Hashida, 1996 and Monfardi & Veronese, 1998).

Longer circulating polymer-anticancer conjugates exploit the hyperpermeability of solid tumour vasculature to allow tumour-specific targeting. This process has been termed as the Enhanced Permeability and Retention (EPR) effect (Matsumura & Maeda, 1986; Figure 1.2) and its mechanism of action will now be described in detail.

Figure 1.2 Illustration of the Enhanced Permeability and Retention (EPR) Effect (Adapted from Takakura *et al*, 1998)



Macromolecular compounds in solution are subject to:(1) vascular distribution, (2) penetration across microvascular endothelial barrier, (3) transport through interstitial space and (4) cellular interaction and uptake

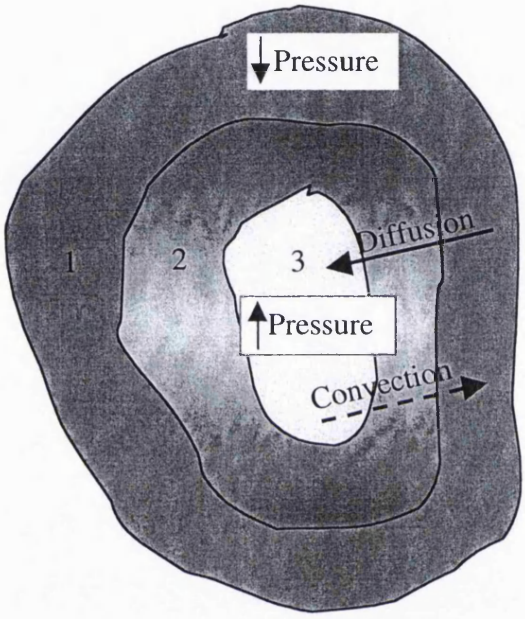
1.3 The EPR Effect for Passive Tumour Targeting

As a tumour develops, it initially obtains its nutrients and oxygen from adjacent blood vessels. However, as growth proceeds, the tumour can not grow beyond ~ 1 - 2 mm in diameter without the need to develop new vasculature to provide oxygen and nutrients and to remove metabolic byproducts (Kerbel, 1990). Characteristically, a high density of angiogenic vessels develop from pre-existing vasculature (reviewed by Hanahan & Folkman, 1996 and Carmeliet & Jain, 2000). Antiangiogenic compounds such as combrestatin (Chaplin *et al*, 1999) are already under clinical investigation and they provide the advantage that in theory, the destruction of one endothelial cell allows the destruction of ~ 100 tumour cells (Chaplin *et al*, 1999). Angiogenic tumour vessels are highly irregular and highly permeable due to their discontinuous endothelium (Jain, 1987a). This flawed architecture allows tumour-selective extravasation of long circulating macromolecules which pass into the tumour interstitial space (reviewed by Takakura *et al*, 1998).

The rate of macromolecular transport in and out of the tumour is regulated by local physiological conditions. Transvascular transport occurs mainly by diffusion (along a concentration gradient), convection (along a pressure gradient) or both (reviewed by Joyner & Kern, 1990). The average vascular surface area per unit tissue weight decreases with tumour growth. It therefore follows that reduced transvascular exchange (extravasation) occurs in large tumours, in comparison with small tumours. In larger solid tumours, the tumour interstitium is characterised by a large interstitial fluid volume and a uniformly high fluid pressure at the tumour core (up to 30 mm Hg) relative to normal tissues (reviewed by Jain, 2001) due to its confined space and absent lymphatic drainage (reviewed by Jain, 1987b). This pressure abruptly drops at the tumour periphery (5 – 15 mm Hg) (Jain & Baxter, 1988 and Baxter & Jain, 1989) and allows adequate extravasation of macromolecules into the tumour site, which then slowly diffuse towards the tumour core. The high intratumoural volume at the core promotes a higher diffusion rate and thus facilitates initial polymer-drug concentration in tumour tissue. On the other hand, high tumoural interstitial pressure may limit convective extravasation of macromolecules and thus compromise tumoural delivery of macromolecules (Jain, 1997; Figure 1.3).

The balance of these events, in combination with absent or ineffectual tumoural lymphatic drainage aid tumoural localisation of macromolecules (Maeda & Matsumura, 1989; Jain, 1994). The phenomenon of EPR may thus be used for targeting

Figure 1.3 Illustration of a solid tumour mass pressure and concentration gradients after administration of a macromolecular therapeutic



1 Well-vascularised periphery
2 Semi-necrotic region
3 Necrotic tumour core

macromolecular anti-cancer drugs (Maeda, 1991). Likewise, macromolecular anti-inflammatory drugs may be targeted to inflammatory sites (Murakami *et al*, 1996) which due to local structural changes, chemical composition (e.g. histamine & bradykinin) and contraction of endothelial cells also display EPR (Maeda *et al*, 1988 and Seymour, 1992).

Since the early observations of Matsumura & Maeda (1986), a number of studies using *in vivo* models have been used to better understand macromolecular targeting to tumours (reviewed by Duncan, 1999). In particular, studies have sought to relate tumour targeting to parameters such as tumour location within the body (Fukumura *et al*, 1997); vascular density (Smith *et al*, 1988); microvessel permeability (reviewed by Jain, 1987a) and extracellular volume fraction (Graff *et al*, 2000).

Using HPMA copolymers of narrow polydispersity (10 – 800 kDa), Seymour *et al* (1995) and Noguchi *et al* (1998) showed tumour targeting in both B16F10 and Sarcoma 180 models respectively did not show molecular weight dependency in early phase (~ 10 min) and long term (72 h) tumoural accumulation. Higher molecular weight copolymers (> 40 kDa) of size above the renal threshold were retained in the circulation. Recent *in vivo* studies by Sat & Duncan (1998) indicate that tumour size and not tumour type or properties of macromolecular DDS examined (albumin-evans blue complex, PK1, dendrimer-DOX conjugate and liposomal DOX) will determine the amount of tumoural localisation following i.v. administration. Using a s.c. B16F10 murine tumour model, PK1 and albumin-evans blue complex showed size-dependent uptake in small tumours with 13.8 ± 2.5 % dose/g tumour and 12.6 ± 4.9 % dose/g tumour respectively as compared to large tumours 1.6 ± 1.1 % dose/g tumour (PK1) and 2.7 % dose/g tumour (albumin-evans blue complex). Similarly, Harrington *et al* (1998) found better uptake of ^{111}In-DTPA-labelled stealth liposomes in small tumours (< 0.1 g) 15.1 ± 10.8 % dose/g tumour in comparison to large tumours (> 1g) with 3 ± 1.3 % dose/g tumour. Using a panel of human tumour xenografts and murine tumour models, tumour type was found not to be of major significance in determining EPR-mediated tumour uptake (Sat, 1999).

1.3.1 Vectors that Utilise EPR for Tumour Targeting

Several types of carrier have been used as DDS for tumour targeting by the EPR effect. These include: particulate delivery vehicles e.g. liposomes (Gabizon *et al*, 1982;

Torchilin *et al*, 1988; Gregoriadis 1988; Allen *et al*, 1995); serum proteins such as human serum albumin (HSA) (reviewed by Kratz & Beyer, 1998), cells e.g erythrocytes (Bax *et al*, 1999); DNA (Deprez-de Campeneere & Trouet, 1980); nanoparticles (10 – 1000 nm) (reviewed by Brannon-Peppas, 1995; Monfardi & Veronese, 1998; Soppimath *et al*, 2001); block copolymers which in aqueous conditions form polymeric micelles (Yokoyama *et al*, 1992 and Kataoka & Kwon 1995); soluble delivery vehicles e.g. natural biodegradable polymers such as the polysaccharides e.g. dextrans (reviewed by Mehvar, 2000); soluble synthetic polymers e.g. PEG (reviewed by Zalipsky, 1995; Greenwald *et al*, 2000; Harris *et al*, 2001); bioresponsive polymers for endosomolytic delivery (Richardson *et al*, 1999 and Murthy *et al*, 1999) and dendrimers (Malik *et al*, 1999). Some advantages and disadvantages of these carriers are listed in Table 1.1.

The liposomal systems were the first to progress into the market. They do, however, display some limitations. For example, palmar-plantar erythrodysesthesia, commonly refererred to as "hand-foot syndrome", characterised by ulcers in the hands and feet displayed by liposomal DOX (Doxil® in USA or Caelyx® in the EU) is due to prolonged circulation time leading to enhanced extravasation through microvessels and subsequent toxicity of DOX on keratinocytes (Gordon *et al*, 1995). Appearance of "hand-foot syndrome" was the dose limiting toxicity that led to the dosing schedule of 50 mg/m^2 every 4 weeks for Caelyx. In contrast, free DOX is administered at a dose of 60 – 75 mg/m^2 every 3 weeks (Martindale, 2002) when used to treat AIDS-related Kaposis's sarcoma.
Disadvantages of the current design of polymer-drug conjugates are discussed in section 1.5.1.

Prior to defining polymer therapeutics, the mechanism of endocytic uptake of macromolecules passively accumulated in the tumour interstitium will be described in detail.

1.4 Endocytic Capture of Polymer Therapeutics

Eukaryotic cells use a system of intracellular organelles which are functionally interconnected by a series of carrier vesicles to remain in constant communication with their environment (reviewed by Mellman, 1996 and Mukherjee *et al*, 1997). They

Table 1.1 Advantages and disadvantages of DDS used for passive tumour-targeting by the EPR effect

Advantages (Reference)	Disadvantages (Reference)
Liposomes[†]	
No degradation of entrapped drug (Monfardi & Veronese, 1998)	Limited drug loading capacity (Langer, 1998)
Altered pharmacokinetics (no RES uptake) by PEGylation (Allen *et al*, 1995)	Possible impairment of macrophages involved in tumour control (Gabizon *et al*, 1982)
Protection of drugs from degradation (Knight, 1981)	Leakage of drug in blood (Soppimath *et al*, 2001)
Decreased toxicity of parent compound (Knight, 1981)	Poor storage stability (Soppimath *et al*, 2001)
HSA[‡]	
Readily available (Kratz & Beyer, 1998)	Polyfunctionality thus heterogeneous products (Peters, 1985)
Good biological stability (Yuan *et al*, 1995)	Structure and properties can not be altered (Drobnik, 1989)
Biodegradable (Kratz & Beyer, 1998)	
Non-toxic & non-immunogenic (Kratz & Beyer, 1998)	
DNA	
Decreased toxicity of parent compound (Deprez-de Campeneere & Trouet, 1980)	Instability of conjugates *in vivo* (non-covalent) (Trouet & Jolles, 1984)
Ease of preparation of complexes (Trouet *et al*, 1980)	Conjugates do not overcome MDR (Atassi *et al*, 1974)
Nanoparticles	
Good stability (Soppimath *et al*, 2001)	Matrix pore size governs size of drug to be used (Monfardi & Veronese, 1998)
Controlled release properties (depot) (Soppimath *et al*, 2001)	Hydrolytically labile compounds damaged by diffusion of water into matrix (Monfardi & Veronese, 1998)
Oral administration of peptides or proteins (Soppimath *et al*, 2001)	Organic solvents may cause denaturation of protein / peptide drugs (Monfardi & Veronese, 1998)

Advantages (Reference)	Disadvantages (Reference)
Polymeric micelles‡	
No renal clearance based on molecular weight (Kwon & Kataoka, 1995)	Control of stability and drug release may be difficult
Good water solubility irrespective of drug loading (Yokoyama *et al*, 1990)	Industrial scale-up may be difficult
Functionality based on distinctive character of constituent polymers (Yokoyama *et al*, 1990)	Industrial characterisation of entrapped and free drug may be difficult
Naturally Occurring Polymers	
Easily available (Monfardi & Veronese, 1998)	Commercial upscale is limited (Duncan & Seymour, 1989)
Biocompatible (Monfardi & Veronese, 1998)	Chemical modification may confer immunogenicity, pyrogenicity and hinder degradation (Drobnik, 1989)
Biodegradable (Brocchini, 2000)	Lack structural uniformity (Brocchini, 2000)
Synthetic Polymers‡	
Biocompatible (Vasey *et al*, 1999)	Drug must possess chemical group for polymer derivatisation (Monfardi & Veronese, 1998)
Decreased metabolism of conjugated drug e.g. proteins (Nucci *et al*, 1991)	Chemical modification of drug may reduce activity of parent compound (Monfardi & Veronese, 1998)
Commercial scale production with control of molecular weight characteristics (Godwin *et al*, 2001)	Internalisation of conjugate necessary for activation (Rejmanová *et al* 1985)
Versatile chemistry (Brocchini, 2000)	Not biodegradable (Brocchini, 2000)
Bioresponsive Polymers	
pH-responsive, thus controlled delivery (Ferruti *et al*, 2000)	Relatively low transfection (Richardson *et al*, 2001)
Non-immunogenic & biocompatible viral-mimetics (Ferruti *et al*, 2000)	

Advantages (Reference)	Disadvantages (Reference)
Dendrimers	
Precisely controlled architecture (Tomalia *et al*, 1985 and Hawker & Fréchet, 1990)	Small size may limit tumour localisation (Malik *et al*, 1999)
Monodisperse (Liu & Fréchet, 1999)	Biocompatibility not fully understood (Roberts *et al*, 1996 and Malik *et al*, 1997)
Large number of chemical functionalities (Liu & Fréchet, 1999)	

† In the market

‡ In Phase I/II clinical trials

import and export molecules by a process that has been termed endocytosis, a general term that encompasses pinocytosis (cell drinking) and phagocytosis (cell eating) (Silverstein *et al*, 1977). The potential value of the endocytic route in drug delivery (so called lysosomotropic delivery) was first proposed by de Duve and co-workers (de Duve *et al*, 1974).

The pinocytic capture of macromolecules and indeed of small water soluble polymer therapeutics (< 0.2 μm diameter vesicles) occurs by either fluid-phase pinocytosis (non-clathrin-mediated internalisation and caveolae) involving the uptake of molecules in solution (i.e. solutes) or alternatively by adsorptive pinocytosis whereby the molecules are internalised in association with cell surface receptors via clathrin-coated vesicles. The latter can be either non-specific receptor-mediated pinocytosis or specific receptor-mediated uptake. Subsequent migration of the newly formed intercellular vesicles (pH 6 – 6.8) (Mellman *et al*, 1986) along microtubules in the cell cytoplasm and fusion with late endosomes in the peri-nuclear region follows to form prelysosomes. These exist at pH 5 – 5.5 as maintained by an intramembranal proton pump (Geisow, 1982) which then mature into or fuse with pre-existing lysosomes.

Lysosomes usually have pH in the range of 4 - 5 and they contain a cocktail of hydrolytic enzymes. Lysosomal enzymes act either in concert or in sequence to liberate low molecular weight degradation products e.g. amino acids and sugars which usually traverse the lysosomal membrane and are used by the cell in synthetic metabolic pathways. After fusion of endosomal and lysosomal vesicles the membrane is often recycled back to the cell membrane (reviewed by Mellman, 1996).

Lysosomal enzymes consist of a variety of hydrolytic enzymes including endo- and exopeptidases (Table 1.2), with diverse functions broadly ranging from the production of metastatic peptides to protein turnover (Bogyo *et al*, 2000). These intracellular proteases are found mainly within lysosomes, but not exclusively. They can also be within the proximity of cells effecting lysosomal enzyme secretion (Duncan, 1986).

Cysteine proteases of the papain superfamily have been found to display a broad substrate-specific activity (reviewed by Michaud & Gour, 1998). The sub-

Table 1.2 Lysosomal enzymes (From Barrett & Heath, 1977 and Duncan, 1986)

Enzyme	Activity	Optimal pH
Endopeptidases (hydrolases which cleave away from ends of polypeptides)		
Acrosin	Cleaves arginyl and lysyl bonds in low MW substrates	8.0
Lysosomal elastase	Cleaves valyl bonds in low MW substrates	8.5
Cathepsin G	Cleaves phenylalanyl and other chymotrypsin bonds	7.5
Cathepsin B	Papain-like specificity	3.5 – 6.0
Cathepsins H & L	Little action on Cathepsin B synthetic substrate	5.0
Cathepsin D	Proteins & peptides of at least 5 residues; preferably bonds between hydrophobic amino acyl residues	2.8 – 5.0
Cathepsin E	Similar to Cathepsin D & pepsin	
Exopeptidases (hydrolases which cleave near ends of polypeptides)		
Lysosomal aminopeptidases	Cleave N-terminal amino acyl residues	various
Lysosomal carboxypeptidase A (Cathepsin A & I)	Cleave C-terminal residues; broad specificity (excluding Arg & Lys)	5.0
Lysosomal carboxypeptidase B	Cleave C-terminal residues; broad specificity (including Arg & Lys)	6.2
Lysosomal carboxypeptidase C	Cleaves C-terminal when subterminal residue is Pro	5.5
Tyrosine acid carboxypeptidase	Cleaves C-terminal residues; preferably hydrophobic	NA
Lysosomal dipeptidase	Cleaves dipeptides; broad specificity	5.5
Dipeptidylpeptidase I (Cathepsin C)	Cleave N-terminal dipeptides from amide, arylamide, ester & peptide substrates; broad specificity; except if terminal residue is Arg or Lys & 2nd or 3rd residue is Pro	5.0 – 6.0
Dipeptidylpeptidase II	Cleave N-terminal dipeptides from amide, arylamine and peptide substrates; preferably Arg or Lys terminal	4.5 – 5.5

classification of these enzymes is defined by the nature of their active site. The presence of a chemical group that exhibits the reactivity to catalyse a reaction and specificity to a substrate which binds sufficiently tightly at the active site for a reaction to occur (Duncan, 1986 and Yan *et al*, 1998). Thus Cathepsin B which is believed to be responsible for the conversion of high molecular weight polymer-drug conjugates to release the active moiety by cleavage of tailor-made peptidyl sequences (Duncan *et al,* 1983b and Duncan, 1986) is a cysteine protease in that it has cysteine in its active site (which confers its nucleophilicity) and a pH optimum which favours acidic conditions and a substrate preference for peptidyl amide bonds.

The selective endocytic uptake and subsequent release of cytotoxic agents within lysosomes of target cells by coupling these agents to a suitable carrier have been investigated and exploited in the fields of cancer and infectious diseases (de Duve *et al*, 1961 and reviewed by Duncan, 1999). Low molecular weight compounds are randomly taken up into the cell by diffusion. Their subsequent conjugation to macromolecular carriers restricts their uptake to the pinocytic route, thus conveying tumour tissue specificity to this new chemical entity (Duncan *et al*, 1983b). The notion of the synthesis of drug conjugates with lysosomally labile linkers ("spacer"), cleaved either by hydrolytic enzymes or the intralysosomal acidic pH which would allow subsequent drug release into the cytosol, where it would exert its pharmacological effect, was first proposed by Trouet and co-workers (Trouet *et al*, 1972 and Trouet, 1978). *In vitro* incubation of a serum albumin-tetrapeptide-daunorubicin conjugate in purified rat lysosomal fractions achieved ~ 80 % drug release (Trouet *et al*, 1982). This conjugate was also found to remain stable in the blood stream *in vivo* (Trouet & Jollès, 1984), a prerequisite for directed delivery.

The limitation of lysosomal delivery is the restriction to active agents which are themselves not inactivated in the lysosomal conditions. In addition, in the case of enzymatic cleavage, the successful release of the active agent is reliant upon the sufficiency of indigenous enzyme required. PK1, currently in Phase II clinical trials in the UK for breast, colon and non small cell lung cancer is such a lysosomotropic agent (Vasey *et al*, 1999). As lysosomes are present in all mammalian cells types, the tumour-selective uptake of such lysosomotropic compounds by the EPR effect is therefore paramount for their clinical success or benefit.

1.5 Polymer Therapeutics

Although biomedical polymers (contact lenses, prostheses, implants, kidney dialysis membranes, etc.) have a long history, the use of water soluble polymers as therapeutics is relatively new.

The term polymer therapeutics, coined by Duncan (1992), may be defined as a family of prodrugs which incorporate polymers as the core constituent, schematically illustrated in Figure 1.4. These comprise of polymer-drug conjugates, polymer-protein or polymer-peptide conjugates, polymeric micelles and and non-viral gene delivery constructs (reviewed by Brocchini & Duncan, 1999 and Duncan, 2000).

It should be noted that polymeric drugs are distinguished from polymer prodrug carriers as they themselves exert a pharmacological effect in their own right. Polymeric carriers, which transport the pharmacophore covalently via pendant side chains or to terminal groups of the polymer to the target site, do not convey this inherent activity. Polymeric drugs with intrinsic activity include glatiramer acetate injection (Copaxone®) which has been marketed for multiple sclerosis (Gilbert, 1998), divenylether–maleic anhydride copolymer (DIVEMA) (Gros *et al*, 1981) and dextrin-2-sulphate which is presently under clinical evaluation as an anti-AIDS therapy (Shaunak *et al*, 1998).

Polymer-Drug Conjugates

Several soluble polymers have been used in macromolecular systems in cancer (reviewed by Duncan, 1992; Duncan *et al*, 1996; Brocchini, 2000) and other diseases e.g leishmaniasis (Nan *et al*, 2001), dyslipidaemias and artherosclerosis (Lovrek *et al*, 2000). Ringsdorf pioneered this model in 1975 (Figure 1.5). He proposed the covalent attachment of drugs to a water soluble polymer backbone by means of a biodegradable spacer with the possible incorporation of a targeting residue e.g. antibodies and sugars. (see section 1.2) to convey receptor-mediated pinocytosis. In limiting the cellular uptake of high molecular weight species to endocytosis (Duncan *et al,* 1981), polymer-drug conjugates provide the opportunity to direct the drug to the particular cell type where drug action is demanded by means of the EPR effect. A wide range of polymers (both biodegradable and non-biodegradable) have been employed in polymeric prodrugs to improve the targeting of anticancer compounds (Table 1.3).

Figure 1.4 Illustration of examples of polymer therapeutics: (A) polymeric drug with intrinsic activity; (B) polymer-drug conjugate; (C) polymer-protein conjugate and (D) polymeric micelle (Adapted from Brocchini & Duncan, 1999)

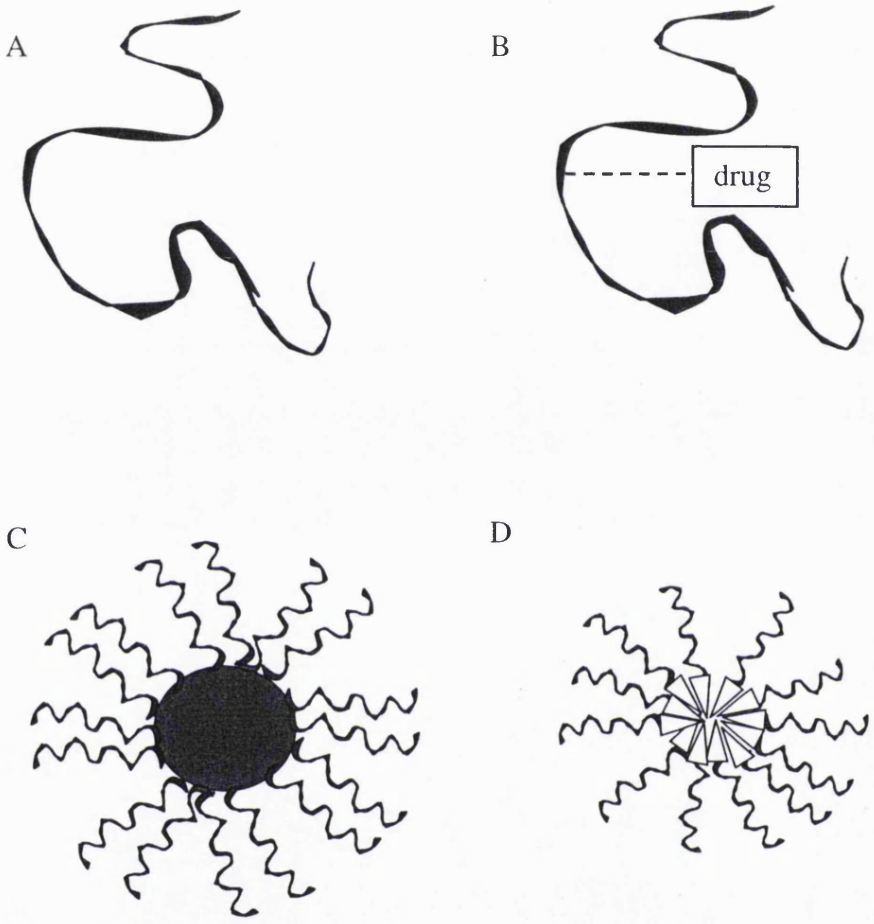


Figure 1.5 Illustration depicting the "Ringsdorf model"

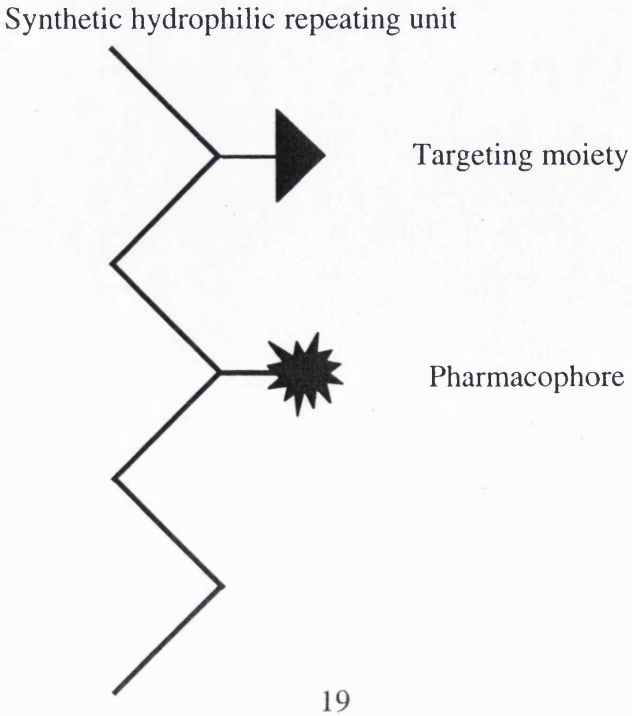


Table 1.3 Representation of examples of soluble polymeric vehicles for parenteral drug delivery in cancer therapy

Polymer	Conjugated Cytotoxic Compound	Antitumour Activity	Reference
Non-Degradable Synthetic Polymers			
PEG	Podophyllotoxin	Similar or improved activity *in vivo* of various analogues compared to free drug	Greenwald *et al*, 1999
	Taxol	Improved solubility and reduction of toxicity, but no improved antitumour activity *in vivo*	Greenwald *et al*, 1996
	Doxorubicin	Reduction on toxicity compared to free drug and increased tumour uptake *in vivo*	Senter *et al*, 1995
HPMA	Doxorubicin (PK1)	Phase II Clinical Trials (CRC / Pharmacia)	Vasey *et al*, 1999
	Doxorubicin & Galactose (PK2)	Phase I / II Clinical Trials (CRC / Pharmacia)	Ferry *et al*, 1999
	Platinate (AP5280)	Phase I Clinical Trials (Access Pharmaceuticals)	Gianasi *et al*, 1999
	Paclitaxel (PNU 166945)	Phase I Clinical Trials (Pharmacia)	Huniunk *et al*, 1998
	Melphalan	Improved activity *in vivo* compared to free drug	Duncan *et al*, 1991
Dendrimers e.g. Poly(amidoamine)	Cisplatin	Improved activity and reduced toxicity *in vivo* compared to free drug	Malik *et al*, 1999
Degradable Natural or Synthetic Polymers			
Naturally occuring Serum proteins e.g. Albumin	Methotrexate	Phase I Clinical Trials (German Association for Medical Oncology)	Hartung *et al*, 1997; Burger *et al*, 2001

Polymer	Conjugated Cytotoxic Compound	Antitumour Activity	Reference
Degradable Natural or Synthetic Polymers (continued)			
Polysaccharides			
e.g. Dextran	Mitomycin	RES uptake *in vivo*	Takakura *et al*, 1987; Bernstein *et al*, 1978
	Daunomycin	Improved activity *in vivo*	
Alginate	Daunomycin	*In vitro* release of free drug in acidic pH only; antitumour activity *in vivo*	Al-Shamkhani & Duncan, 1995
Natural derivatives			
Poly(aminoacids) e.g. Poly(L-lysine)	Methotrexate	Antitumour activity and over-riding of resistance *in vitro*, but polymer related toxicity observed *in vivo*	Ryser & Shen, 1978; Chu *et al*, 1981
Poly(L-glutamic acid)	Doxorubicin	Antitumour activity *in vivo*, but immunogenic	van Heeswijk *et al*, 1984; Hoes *et al*, 1993
	Camptothecin (CPT)	Increased water solubility and antitumour activity *in vivo*	de Vries *et al*, 2000
	Paclitaxel	Phase I Clinical Trials (company)	Li *et al*, 1998
Poly[N^5-(2-hydroxyethyl)-L-glutamine] (PHEG)	Mitomycin C (MMC)	Biocompatibility *in vivo* with reduction in toxicity and improved antitumour activity	Soyez *et al*, 1997; de Marre *et al*, 1995
	Daunorubicin	Hepatotoxic *in vivo*; not suitable for clinical development	Hrdina *et al*, 1991

Polymer	Conjugated Cytotoxic Compound	Antitumour Activity	Reference
Degradable Natural or Synthetic Polymers (continued)			
Polyesters e.g.			
Poly(α-malic acid)	5-Fluorouracil	Slight improvement in antitumour actitivty *in vivo*	Ouchi *et al*, 1990
Polymeric micelles e.g.			
PEG-Aspartate	Doxorubicin	Improved antitumour activity in solid tumours *in vivo*	Yokoyama *et al*, 1990; 1991
Polymers with Inherent Activity			
Divenyl ether - maleic anhydride copolymer (DIVEMA)	Cyclophosphamide	No difference in *in vivo* activity compared to free drug	Hirano *et al*, 1980
	Methotrexate	Improved antitumour activity *in vivo* compared to free drug and adjuvant effect of polymer observed	Przybylski *et al*, 1978
	Doxorubicin	Improved antitumour activity *in vivo* compared to free drug	Pratesi *et al*, 1990
Polymer-Protein Conjugates			
PEG	Interleukin-2	Phase I / II Clinical Trials (Enzon)	Meyers *et al*, 1991
	L-Asparaginase	In Clinical Use for non-Hodgkin's Lymphoma	Park *et al*, 1981
Poly(styrene-co-maleic acid) (SMA)	Neocarzinostatin	In Clinical Use (Japan) for Hepatocarcinoma; Co-administration with Lipiodol	Matsumura & Maeda, 1986; Konno, 1987

Macromolecular polymeric prodrugs enable the attainment of key factors in the principle of drug delivery: (1) the solubility of hydrophobic drugs is enhanced; (2) the pharmacokinetic profile of the pharmacophore is improved and (3) systemic toxicities are minimal as the payload is protected and cytotoxic action is observed after release of the active moiety specifically in the target cells. i.e. pharmacological activity of the active agent remains intact. In addition, the improved therapeutic index of the active agent bound allows a higher dose to be administered. For example, the dose for PK1 in Phase II clinical trials is 280 mg/m^2 (Vasey *et al*, 1999) ~ 4 - 5 times greater than doxorubicin (DOX) which is typically administered at 60 - 80 mg/m^2 (Martindale, 2002).

The ideal characteristics for a polymer used as a drug carrier have been previously reported (reviewed by Duncan *et al*, 1996). These include:

1) Non-immunogenic
2) Possess suitable chemical functionality for covalent linkage to drug
3) Release of drug in required site of action (stability en route to target site)
4) Hydrophilic i.e. water soluble in physiological conditions
5) Biodegradable or excreted
6) Commercially available.

In respect of the polymer-drug conjugate, stability of the payload en route to the desired site of action is paramount for desired non-systemic toxicity.

Design of HPMA Copolymer for Tumour Targeting

HPMA copolymer, in the field of polymer-drug conjugates, has been by far the most systematically designed and extensively studied, and has delivered several conjugates into pre-clinical and clinical development for treatment of solid tumours (Vasey *et al*, 1999; Gianasi *et al*, 1999; reviewed by Duncan, 2000).

Neutral, hydrophilic water soluble linear polymers such as HPMA copolymer display a loose, random flexible coil which is in constant dynamic motion. HPMA homopolymer was originally developed in the Czech Republic as a plasma expander and is non-immunogenic and has been shown to be non-toxic at doses of up to 30 g/kg (Duncan, 1992). Following extensive evaluation of biodistribution (circulating half-life, renal clearance, organ and tumour uptake) of HPMA copolymer (Seymour *et al*, 1987; 1995), due to its non-biodegradable nature, a molecular weight of ~ 30 kDa which is below the

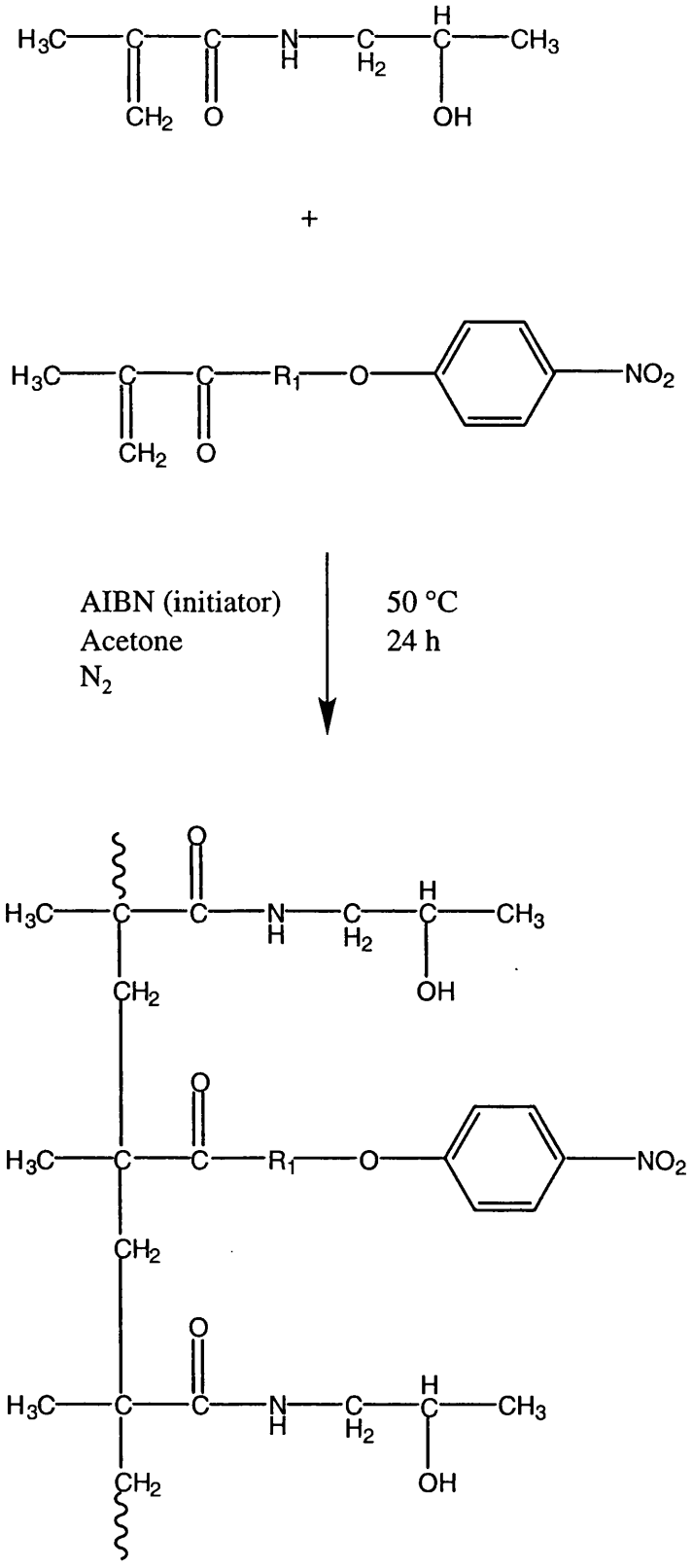
renal threshold molecular weight was chosen as the optimal molecular weight for conjugation to anticancer drugs. In man, no polymer-related toxicities have been observed in ongoing clinical trials (Vasey *et al*, 1999). The advantage of the choice of HPMA copolymer to create a new family of polymer-membrane active agent conjugates is that it has been shown to be "safe" in clinical studies. The reactive *p*-nitrophenyl (ONp) of the HPMA copolymer precursor (Figure 1.6) commonly used as an intermediate allows the conjugation of a various range of pharmacophores which possess pendant amine groups (aminolysis) (see section 3.2.1).

The design of tailor-made lysosomal labile "spacer" oligopeptide sequences in HPMA copolymer conjugates which would be cleaved specifically in the lysosome by thiol-proteases (e.g. cathepsins B, H and L), whilst remaining intact during transit to the chosen site of action stemmed from early work carried out in a collaboration between The Institute of Macromolecular Chemistry, Prague and The University of Keele, UK.

Extensive investigations of a panel of peptidyl linkers (Duncan *et al*, 1980; 1981; 1982; Rejmanova *et al*, 1983) found the glycine-phenylalanine-leucine-glycine (GFLG) linker to be the most suitable with ~ 70 % release of conjugated DOX by rat liver lysosomes which correlated with antitumour activity in a L1210 murine tumour model (i.p.) (Subr *et al*, 1992). The stability of a library of HPMA copolymer-peptidyl linker-*p*-nitroanilide (NAp) conjugates en route to the desired site of action, the tumour cell, was evidenced by Rejmanová *et al* (1985) who found negligible release of drug model NAp in blood or serum from HPMA copolymer, relative to that by lysosomal enzymes. The recent withdrawal of methacrylolated Gly (MAG)-camptothecin from early clinical testing due to side effects such as haemorraghic cystitis, GI toxicity and myelosuppresion (Twelves, 2002) highlights the importance of a systemically stable linker.

Other polymer-drug conjugate linkers which capitalise on the acidic milieu of lysosomes or the typically acidic extracellular environment of the tumour cell have also been developed to combat cancer (reviewed by Soyez *et al*, 1996 and Garnett, 2001). These include daunomycin bound to poly-D-lysine (non-biodegradable backbone) via a *cis*-aconityl spacer which had a half-life of < 3 h at pH 4, as opposed to a half-life of > 96 h at pH 6 (Shen & Ryser, 1981); poly-L-glutamate-paclitaxel (CT-2103) (~ 37 % w/w loading) presently in Phase I clinical trials, whose ester bond liberates free

Figure 1.6 Radical copolymerisation of HPMA with *p*-nitrophenol esters of *N*-methacroylolated oligopeptide (From Kopecek *et al*, 1985)



R_1 = oligopeptide "spacer"

drug (Sabbatini *et al*, 2002).

Due to the difference in cell entry of HPMA copolymer-DOX conjugates (Duncan *et al,* 1981), such polymer-drug systems have been proposed to by-pass resistance mechanisms such as efflux pumps e.g. P-glycoprotein (Pgp) in DOX-resistant human ovarian carcinoma cells *in vitro* (Stastny *et al*, 1999 and Minko *et al*, 1998).

Ideally, polymers used as drug carriers should be biodegradable and subsequently excreted. The HPMA copolymer backbone, however, is not biodegradable and therefore not excreted if a molecular weight exceeding the renal threshold (~ 40 kDa) is used to prepare conjugates. This presents a potential danger of lysosomal storage disease syndrome following drug release (Seymour *et al*, 1987; 1995; Noguchi *et al* 1998). This is more so a threat in chronic administration of a conjugate, resulting in muscle and skin accumulation. This has prompted a search for a new generation of pH dependent (acid labile) biodegradable polymers, with an aim to undergo main chain hydrolytic degradation, in particular at the low pH of the endosome or lysosome after delivering the drug intact to the cell (Clochard *et al*, 2000 and Tomlinson *et al*, 2002).

Some of the HPMA copolymer drug conjugates so far progressed into clinical evaluation (PK1, PK2, HPMA copolymer-paclitaxel) (reviewed by Duncan, 2002) rely on the enzymatic liberation of the active antitumour agent. However, the enzymatic rate of drug liberation from the HPMA copolymer conjugate and thus the efficiency of the conjugated drug at the cellular level is dependent on a number of factors: (1) the rate of endocytic uptake, (2) sufficient thiol protease and enzyme cofactor concentrations in the lysosomal compartment responsible for cleavage of the specific oligopeptide spacer, (3) the design of the spacer to allow a favourable enzymatic active site structure-activity relationship and (4) the concentration of polymeric prodrug within the lysosome i.e. substrate concentration.

In light of the potential limitations of lysosomotropic delivery outlined above, polymer conjugation to compounds with direct membrane action, to synthesise the first generation of HPMA copolymer-membrane active agents was hypothesised. In theory, the targeting of cell surface acting agents specifically to the desired site by EPR, thus

overcoming these barriers may further enhance tumour cell kill (Figure 1.7). The membrane active agents explored as anticancer compounds in their own right will now be briefly reviewed.

1.6 Membrane Active Agents in Cancer Chemotherapy

Pharmacologically active compounds which exert a direct membrane effect are amphiphilic in nature (reviewed by Schreier *et al*, 2000). These compounds range from small molecules such as peptide toxins and venoms to larger molecules such as fatty acid analogues. Phospholipids are thought to exert their cytotoxic effects by causing negative strain curvature on the cell membrane due to the high load of phospholipids attempting to join the cell membrane. In addition, phosphatidylserine has been reported to initiate apoptosis (programmed cell death) (Uchida *et al*, 1998). Synthetic ether lipid analogues of naturally derived phospholipids (reviewed by Berdel, 1991) have been through clinical testing as anticancer agents (Berdel *et al*, 1987 and Herrmann *et al*, 1987). They exert their effect directly by cell membrane lysis, interference with various stages of cell signalling, inhibition of phospholipid metabolism and indirectly by immunomodulatory effects and possibly induction of apoptosis (reviewed by Berdel 1987; 1991; Lohmeyer & Workman, 1995; Arndt *et al*, 1997). However, their toxic effects have delayed their progression into the market. At present, a liposomal preparation of the ether lipid, TLC ELL-12 is in Phase I clinical study in USA (The Liposome Company Inc., USA) following promising results in cancer experimental models (Ahmad *et al*, 1997). Fatty acid analogues e.g. 5,8,11,14-eicosatetraynoic acid (ETYA), an arachidonic acid analogue have also shown potential anticancer activity (Anderson *et al*, 1992).

In this study, we chose the cytolytic peptide derived from the European honey bee venom, MLT (reviewed by Dempsey, 1990; Figures 1.8 & 1.9) as a model membrane active peptide for conjugation to HPMA copolymer.

Cytolytic Peptides

Species transgressing the evolutionary scale, from insects, amphibians, mammals and plants use peptides in cellular physiology processes such as fusion, pore formation and membrane disruption (Andreu & Rivas, 1998; summarised in Table 1.4). These are generally constitutively present or are secreted as a secondary cell-free immune response, as a host-defense mechanism to counter microbial infestation (reviewed by Matsuzaki, 1999). In addition, these peptides are fungicidal, virucidal,

Figure 1.7 Illustration of the different modes of action of lysosomotropic polymer-drug conjugates and polymer-membrane active conjugates

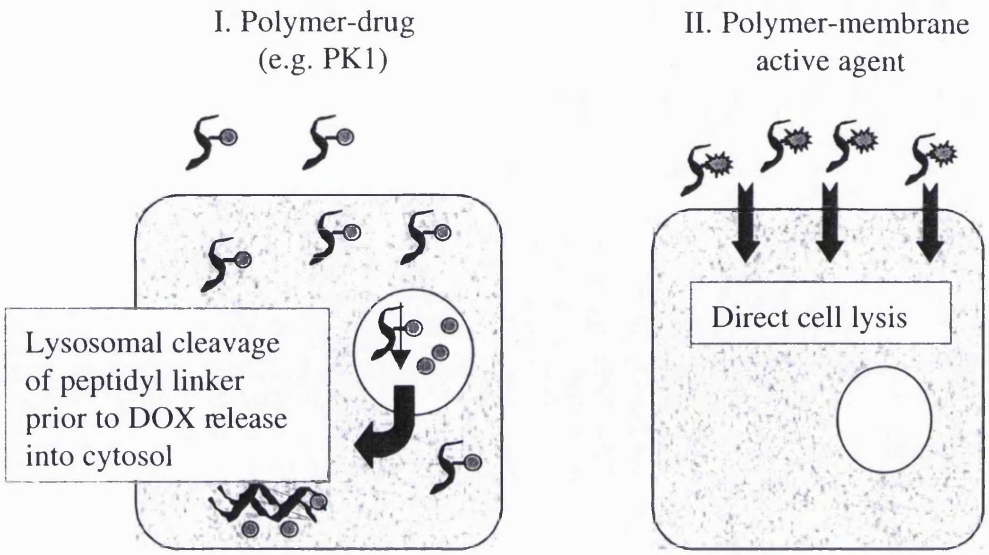


Figure 1.8 European honey bee (*Apis meliffera)* venom constituents

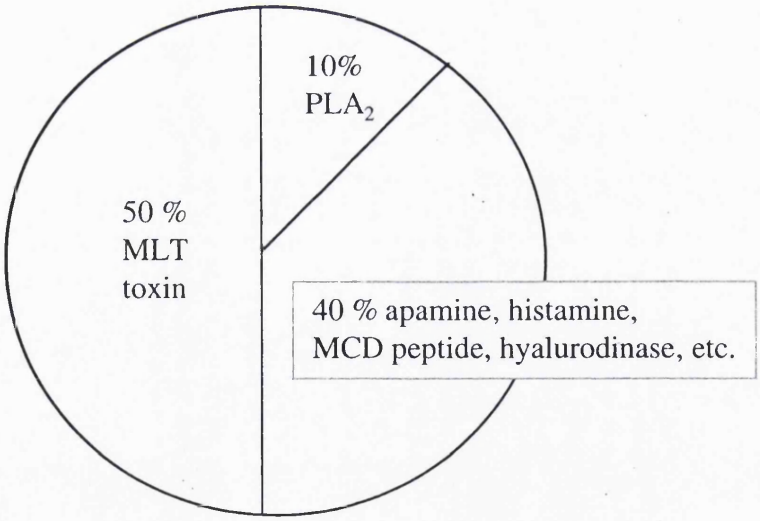


Figure 1.9 MLT amino acid sequence

COOH- Gly (G) - Ile (I) - Gly (G) - Ala (A) -Val (V) - Leu (L) - Lys (K) $^{+}$-Val (V) - Leu (L) - Thr (T) - Thr (T) - Gly (G) - Leu (L) - Pro (P) - Ala (A) - Leu (L) -Ile (I) - Ser (S) - Trp (W) - Ile (I) - Lys (K)$^{+}$ - Arg (R)$^{+}$ - Lys (K)$^{+}$ - Arg (R)$^{+}$ - Gln (Q) - Gln (Q) - $CONH_2$

Table 1.4 Representative cytolytic peptides (From Dathe & Wieprecht, 1999)

Origin	Peptide	No. of Residues; Cationic Charge	Secondary Structure	Cytolytic Activity on Red Blood Cells	Reference
Microorganisms e.g. *Staphylococcus aureus*	δ-Hemolysin	27; linear 0	α-helical	High	Fitton *et al*, 1980
Insects e.g. Honey bee venom *Apis Mellifera*	MLT	26; linear + 6	α-helical	High	Habermann *et al*, 1967
Amphibians e.g. Frog skin *Xenopus laevis*	Magainin 2	23; linear + 4	α-helical	None	Zasloff, 1987
Mammals e.g. Porcine small intestine	Cecropin P1	31; linear + 5	α-helical	None	Lee *et al*, 1989

haemolytic and tumouricidal as a function of membrane interaction (Bechinger, 1997). The number of antimicrobial peptides that have been isolated and characterised exceeds 150 distinct molecules, typically comprising 12 - 45 amino acid residues.

An outstanding feature of membrane active peptides is their structural diversity and broad spectrum of activity. Extensive structure-function studies have shed light on their ability to associate with and modulate membrane lipids. It should be noted that despite laborious efforts, the mode of action of cytolytic and antibacterial peptides remains elusive and the basis for their selectivity towards their respective targets is unclear. These lytic peptides have been subsequently categorised into three groups on the basis of their spectrum of activity as those that show selectivity for eukaryotic or prokaryotic membranes or non-selective cytolysins (reviewed by Shai, 1999).

Most of the antimicrobial peptides are comprised of L-amino acids. They exist as defined α-helix or β-sheet secondary structures as a result of inter-cysteine disulphide bridges which can be accommodated into membranes. In general, direct lysis of the cell membrane is the universal mode of action (reviewed by Shai, 1999). Their common physicochemical properties, namely, the presence of cationic Lys (K) and Arg (R) amino acid residues conveying a net positive charge of + 2 or more gives rise to the amphipathic structure allows for association with membrane lipids has been universally accepted as necessary for membrane binding (reviewed by Sitaram & Nagaraj, 1999). Another feature in this respect is the correlation between net charge and bioactivity. Peptides carrying a low net positive or negative charge along their helix backbone (e.g. MLT and pardaxin) are non cell-selective and lyse both mammalian and bacterial cells. Conversely, peptides with a high positive charge appear to have preferential activity towards bacterial cells (reviewed by Shai, 1999). This brings us to the structure and components of cell membranes.

1.6.1 Eukaryotic and Tumour Cell Membranes

Cell membranes are highly complex systems whose functions are driven by their lipid and protein composition, physical state and organisation. These inter-dependent features affect the conformation of peptides upon binding to biomembranes (reviewed by Blondelle *et al*, 1999). Subsequently, difficulties have arisen in mimicking such complexity in experimental model systems to establish the

conformational states of bioactive peptides. Mainly, small or large unilamellar lipid vesicles with varying lipid compositions have been used in these models.

A number of spectrophotometric techniques have been developed to better understand the molecular mechanisms of peptide-lipid interactions (reviewed by Blondelle *et al*, 1999). The fundamental architecture of cell membranes is the lipid bilayer composed of glycerophospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) in which proteins, carbohydrates, sphingolipids (e.g. sphingomyelin) and sterols such as cholesterol are embedded. It has become increasingly clear that peptide-lipid interactions, as opposed to receptor-mediated interactions, lack stereo-specificity and play a huge role in the mediation of the function of membrane lytic peptides. This has been evidenced with non-selective peptides such as MLT, in which analogues composed of D-amino acids (enantiomers) were shown to be equipotent as their parent peptides, an indication that they interact with achiral components of the cell membrane and that a hydrophobic environment is needed for their action (Shai, 1999).

The driving force of peptide-membrane binding is the hydrophobic interactions between the apolar amino acid residues and the hydrophobic membrane core and electrostatic interactions between positively charged amino acids and anionic lipids. The hydrophobicities of venoms and toxins are quite high (Matsuzaki *et al*, 1995). Tumour cells have an altered membrane composition in comparison to normal cells (Van Hoeven & Emmelot, 1973). The negative surface charge of tumours has been shown to increase during cell proliferation (Uchino, 1981). The overall anionic charge of tumour cell membranes due to higher levels of phosphatidylserine in the outer leaflet of the plasma membrane (Utsugi *et al*, 1991 and Cerbon & Calderon, 1991) suggests the possibility of using positively charged peptides to target neoplasms simply by electrostatic interactions. Various studies have evidenced enhanced MLT-membrane interaction in negatively charged lipid vesicles (Batenburg *et al*, 1988; Lafleur *et al*, 1989; Beschiaschvilli & Seelig, 1990) which suggest selective tumouricidal activity and have conveyed the cytotoxic activity of cationic peptides to gram negative bacteria to their positive charge (Storm *et al*, 1977). Hence MLT was chosen as the model peptide for conjugation to HPMA copolymer.

The apparent loss of architecture of solid tumours may also contribute to their susceptibility to cell surface-activity. Other cationic peptides have also been shown to

be more toxic to tumour cells than to non-malignant cells e.g. the magainins (Cruciani *et al*, 1991), magainin II and its analogues (Baker *et al*, 1993). In a study investigating the cytotoxic and haemolytic nature of short mammalian-derived extended-helical peptides and insect-derived α- helical peptides, the peptides tested were found to be in the range of 2 – 50 times more toxic to tumour cells (murine P388 lymphocytic leukaemia; K562 human chronic mylogenous leukaemia; MCF-7 human breast carcinoma; DOHH-2 human follicular lymphoma) than non-malignant cells (sheep RBCs and human umbilical vein endothelial cells) (Johnstone *et al*, 2000). In addition, this study showed the peptides to overcome MDR. MLT-membrane interaction within these two cell populations would therefore be expected to be tumour cells > red blood cells (RBC).

The relevance of structural conformation, helicity, hydrophobic moment, hydrophobicity and domain size of membrane active peptides is apparent in their interactions with prokaryotic or eukaryotic membranes (Dathe & Wieprecht, 1999). Furthermore, their structural changes upon lipid association is also a function of environmental factors such as ionic strength, levels of hydration, organic co-solvent, pH and temperature (Blondelle, 1999). To reiterate the complexity of these systems, no established correlation between peptide lytic specificity and specific membrane characteristics has been verified in any technique and due to their rapid action, differentiation between primary and secondary effects of toxins is elusive (Blondelle *et al*, 1999). Consequently, the direct correlation between the *in vitro* and *in vivo* settings remains a matter of speculation. An alternative mechanism of cell death that has recently proposed is DNA biosynthesis inhibition (Dathe & Wieprecht, 1999). This emphasises the complexity in the elucidation of their broad spectrum of activity as it anticipates events other than destruction of the membrane barrier.

The interaction of peptides with biomembranes involves a series of steps (White & Wimley, 1998):

1) Initial binding which comprises a balance between hydrophobic and electrostatic interactions
2) Progression to secondary conformation of the peptide
3) Reorientation
4) Membrane insertion into the lipid bilayer
5) Further translocation into the membrane

It should be noted that alteration of membrane integrity does not necessarily constitute cell death (Silversto *et al*, 1997 and Wu *et al*, 1999). This explains the rationale for the use of fusogenic peptides or their analogues for drug delivery into cells (reviewed by Plank *et al*, 1998).

Two distinct mechanisms have been proposed to underlie membrane permeation by membrane lytic peptides (Figure 1.10):

- The "barrel-stave" model
- The "carpet" model

In the "barrel-stave" model, amphipathic α-helical peptides insert into the hydrophobic core and form lipid-peptide pores (Ehrenstein *et al*, 1977). In this model, co-operation is found between progressively recruited monomers to increase the pore size. In the "carpet" model, peptides that are not necessarily in an amphipathic configuration, are in direct electrostatic contact with the polar head group regions entirely during membrane permeation. Furthermore, they do not insert into the hydrophobic membrane core, but rather align themselves on the membrane surface and at a threshold concentration cause membrane disintegration by disrupting the membrane curvature (Pouny *et al*, 1992). Several studies have suggested that such peptides which lie parallel to the membrane surface with no penetration into the hydrophobic core constitute antibacterial peptides, whereas non cell-selective peptides penetrate into the hydrophobic acyl chain region of the biomembranes (Shai, 1999). The mode of action of MLT is discussed below.

1.6.2 MLT

MLT is the principal haemolytic component of the European honey bee (*Apis Meliffera*) venom. It is a small cationic polypeptide (charge +6) consisting of 26 amino acid residues. MLT has an amphipathic character which accounts for its high water solubility (> 250 mg/ml) and spontaneous membrane association (reviewed by Dempsey, 1990). MLT is predominantly hydrophobic, with this segment comprising aminoacid residues 1 - 20, whereas the remaining 6 constitute its hydrophilic segment at the amidated carboxy terminal (Figure 1.11). This detergent-like sequence accounts for its lytic property. MLT's peptide sequence may be divided into four main regions (Blondelle & Houghten 1991):

1) N-terminal α-helix (residues 1-9)

Figure 1.10 Illustration of proposed mode of lytic action of amphipathic α-helical membrane active peptides (Adapted from Shai, 1999)

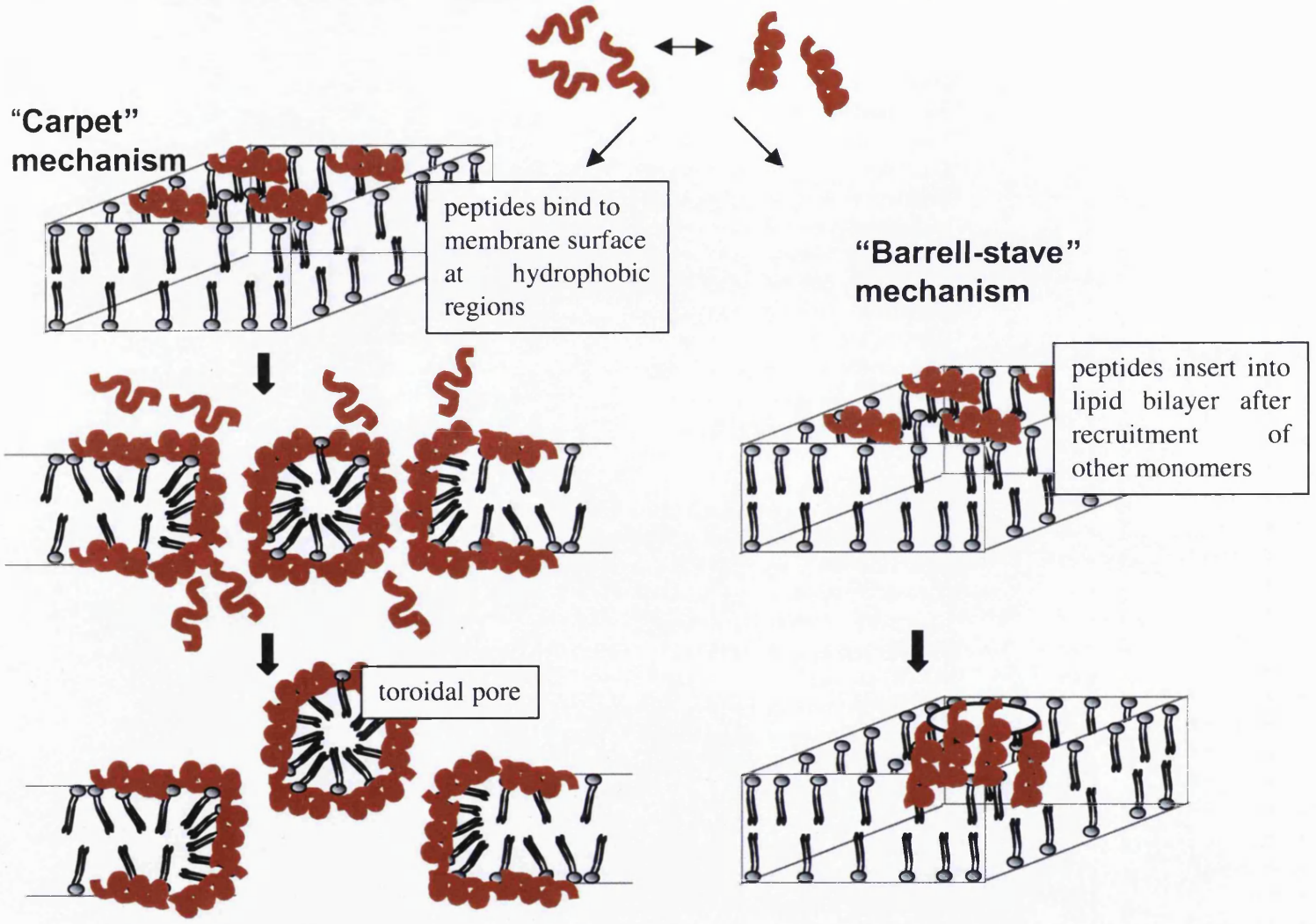
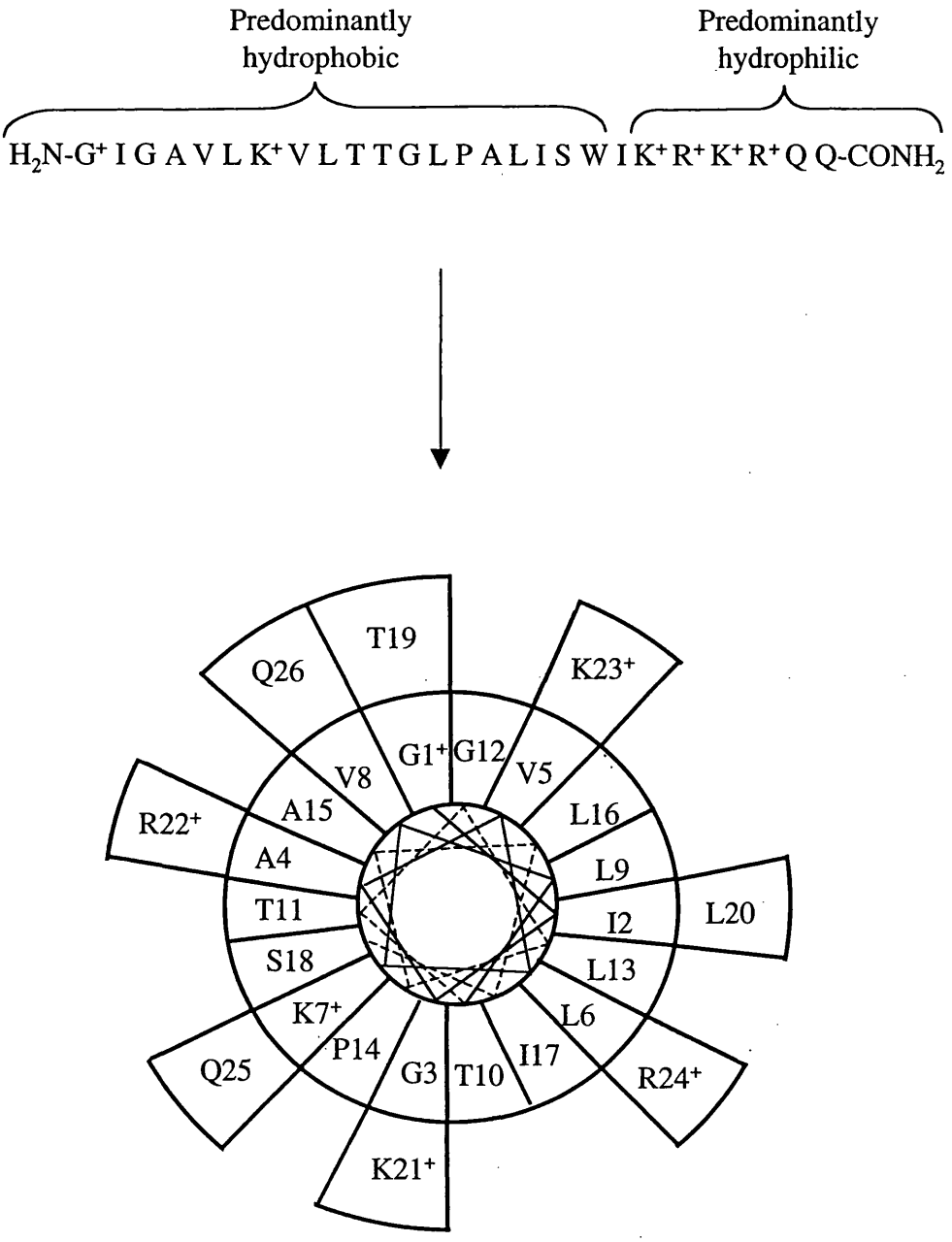


Figure 1.11 Schematic of MLT α-helical wheel (Adapted from Dempsey, 1990)



2) Flexible "hinge" (residues 10-12)
3) Central α-helix (residues 13-20)
4) Cationic C-terminal (residues 21-26)

MLT Mode of Action

MLT is an extensively documented peptide, well known for its characteristically strong and rapid interactions with cell membranes which induce loss of barrier function (reviewed by Dempsey, 1990). Nevertheless, the dynamic processes responsible for change in membrane permeablity properties remain elusive (see section 1.6.1). Various sophisticated technologies have been used extensively to date to characterise structural MLT-membrane interactions. These include circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy and oriented CD (OCD) (reviewed by Sitaram & Nagaraj, 1999), nuclear magnetic resonance (NMR) (reviewed by Bechinger, 1999), reverse phase high performance liquid chromatography (RP-HPLC), surface plasmon resonance (reviewed by Blondelle *et al*, 1999), molecular modelling (reviewed by La Rocca *et al*, 1999) and fluorescence spectroscopy (Kaszycki & Wasylewski, 1990). Quantitative MLT binding studies have also been documented in red blood cells (RBC) (Tosteson *et al*, 1985) and lipid vesicles (Rex & Schwarz, 1998).

MLT's structure has been established as being α-helical in its crystalline state and also forming an aggregate in certain conditions (Terwilliger *et al*, 1982 and Dempsey, 1990). Its conformation within the fluid bilayer has been evidenced to be unlike that of its crystalline one, with a complex equilibrium between the aqueous phase and membrane surface (Dempsey & Butler, 1992; Okada *et al*, 1994; reviewed by Bechinger, 1997; Ladokhin, 1999; Hristova *et al*, 2001; Figure 1.12(a)). It has been shown that at low membrane concentrations, it exists in a monomeric α-helical conformation in a parallel arrangement to the bilayer, with residues 1 - 22 in a α-helical configuration within the fluid bilayer. (Frey & Tamm, 1991; John & Jähnig, 1991; Dempsey & Butler, 1992; Ladokhin & White, 1999; Figure 1.12(b)). The depth within the bilayer, however, remains a matter of debate (Figure 1.13).

The current view on MLT mode of membrane perturbation is that MLT molecules on insertion into the fluid bilayer form multiple aggregates which are

Figure 1.12 (a) General MLT thermodynamic cycle (From Ladokhin & White, 1999)

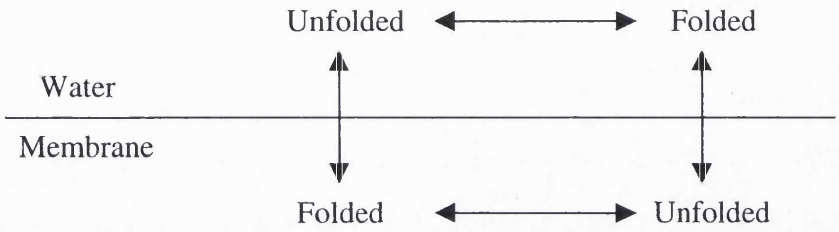


Figure 1.12 (b) MLT-membrane interaction (From La Rocca *et al*, 1999)

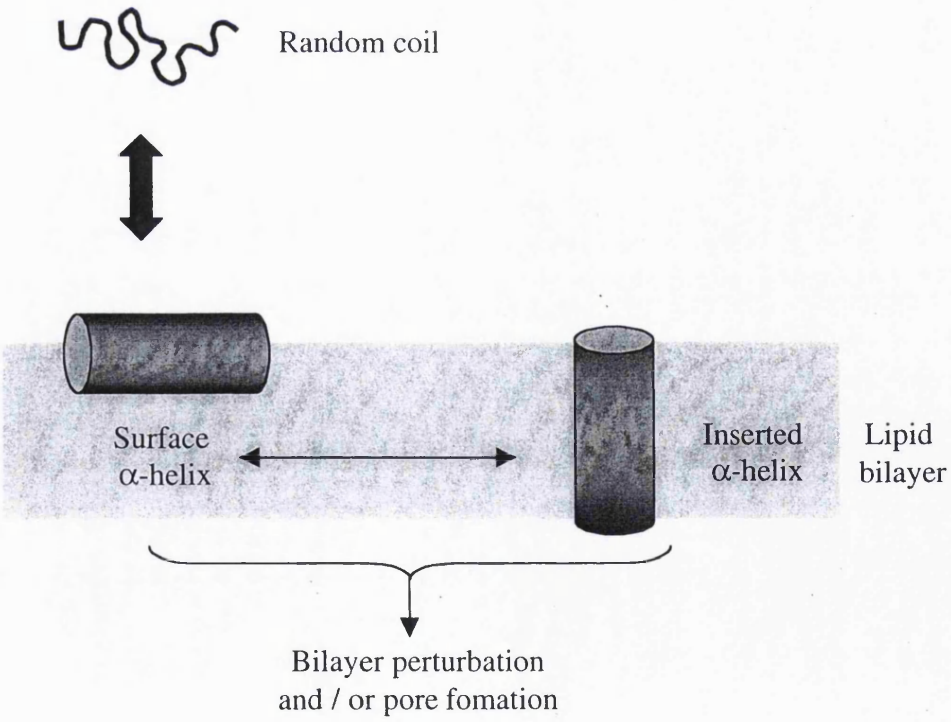
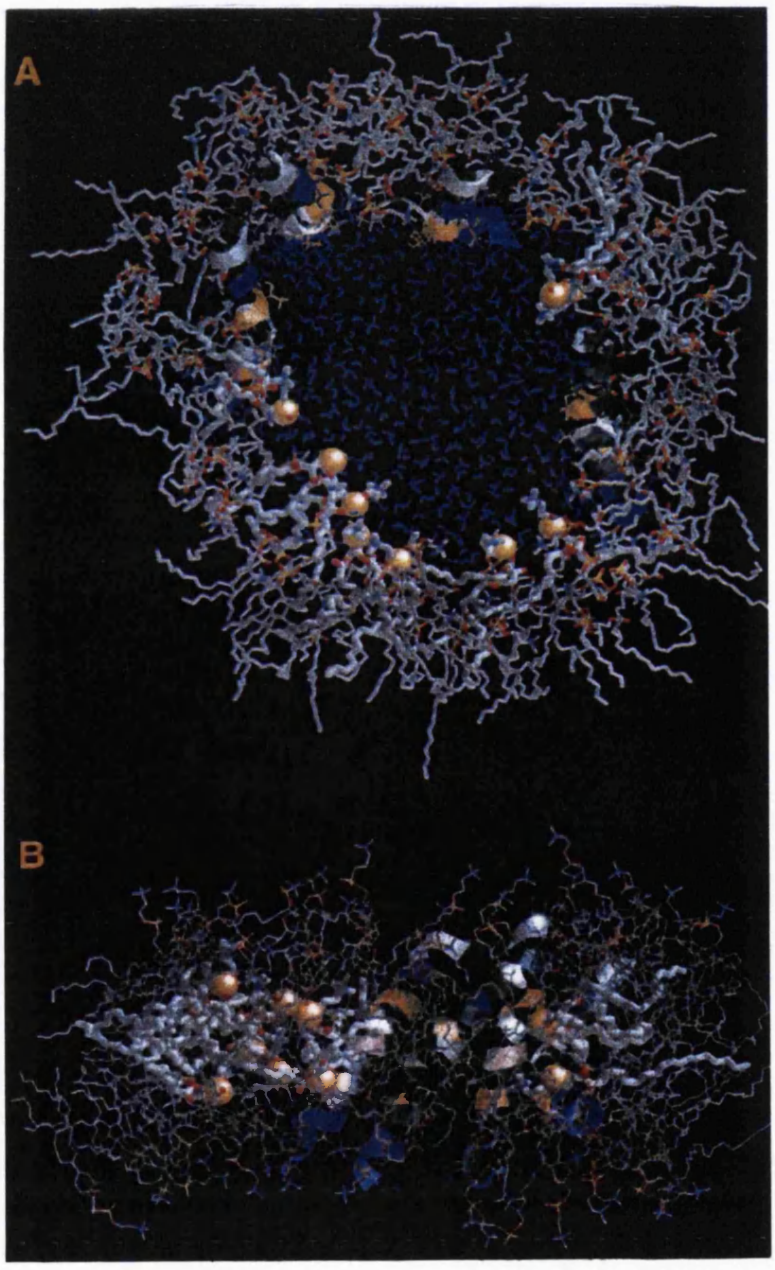


Figure 1.13 Snap shot of 4 MLT α–helices (blue) in bilayer pore, with phospholipid head groups in yellow and water molecules in blue from top view (A) and side view (B). (From Ladokhin & White, 1999).



affected by temperature, pH, ionic strength, lipid composition and lipid : MLT ratio (Lin & Baumgaertner, 2000). The structural and molecular mechanisms of MLT's cytolytic activity, however, remain elusive to date. MLT's position at the interface remains inconclusive, with theories placing its location in the phospholipid headgroup region (Lin & Baumgaertner, 2000) and also within the interface, in various orientations (Bernèche *et al*, 1998 and Bachar & Becker, 1999). The type of aggregate and lytic mechanism, therefore, remain inconclusive.

Two main mechanisms are thought to underlie MLT's cytolytic activity:

1) MLT is proposed to bind parallel to the membrane surface, whereby at high concentrations, upon reorientation perpendicular to the membrane, MLT molecules form cylindrical hydrophilic pores in a "barrell stave" arrangement (section 1.6.1) which results in efflux of fluorescent dyes from unilamellar vesicles or colloidal osmotic lysis of red blood cells (RBC) (Vogel & Jähnig, 1986; Rex, 1996; Ladokhin *et al*, 1997; Matsuzaki *et al*, 1997; Blondelle & Houghten, 1991). Aggregation has been proposed to occur at high salt concentrations and at MLT : peptide > 1 : 200 (Talbot *et al*, 1987 and John & Jähnig, 1991) which is in agreement with NMR (Stanislawski & Ruterjans, 1987) and ERS studies (Altenbach & Hubbell, 1988).
2) An alternative view is MLT co-operative perturbation of fluid bilayer permeability. In this model, MLT is thought to lie parallel to the membrane surface, with the hydrophobic region of the α-helix buried within the membrane. This "wedge" thus results in membrane destabilisation and ultimate lysis (Brown *et al*, 1982; John & Jähnig, 1991; Wall *et al*, 1995; Benachir & Lafleur, 1995).

Both models, however, conclude that vesicle lysis (liposomes / RBCs) is an "all-or-none" event (Benachir & Lafleur, 1995 and Schwarz *et al*, 1992). The size of inner pore diameter induced by MLT activity within lipid bilayers have been documented as 1 – 6 nm (Rex, 1996); 1.3 – 2.4 nm (Matsuzaki *et al*, 1997) and 2.5 – 3 nm (Ladokhin *et al*, 1997). Molecular dynamics studies by Lin & Baumgaertner (2000) propose pore growth and reorientation of MLT within nanoseconds. Kinetic studies by DeGrado *et al* (1982); Schawrz & Beschiaschvili (1998); Scharwz *et al* (1992) and Takei *et al* (1999) have proposed helix association within the lipid bilayer as a prerequisite for pore formation. This has been evidenced by various methods including neutron diffraction (Bradshaw *et al*, 1994), NMR (Smith *et al*, 1994 and Naito *et al*, 2000) and OCD

(Hristova *et al*, 2001). The proposal of dimerisation being the rate limiting step is not in agreement, however, with studies conducted by Rex & Schwarz (1998). MLT helical conformation on association with membranes has also been evidenced to be necessary for membrane fusion (Skehel & Wiley, 1998).

1.7 Aims of This Thesis

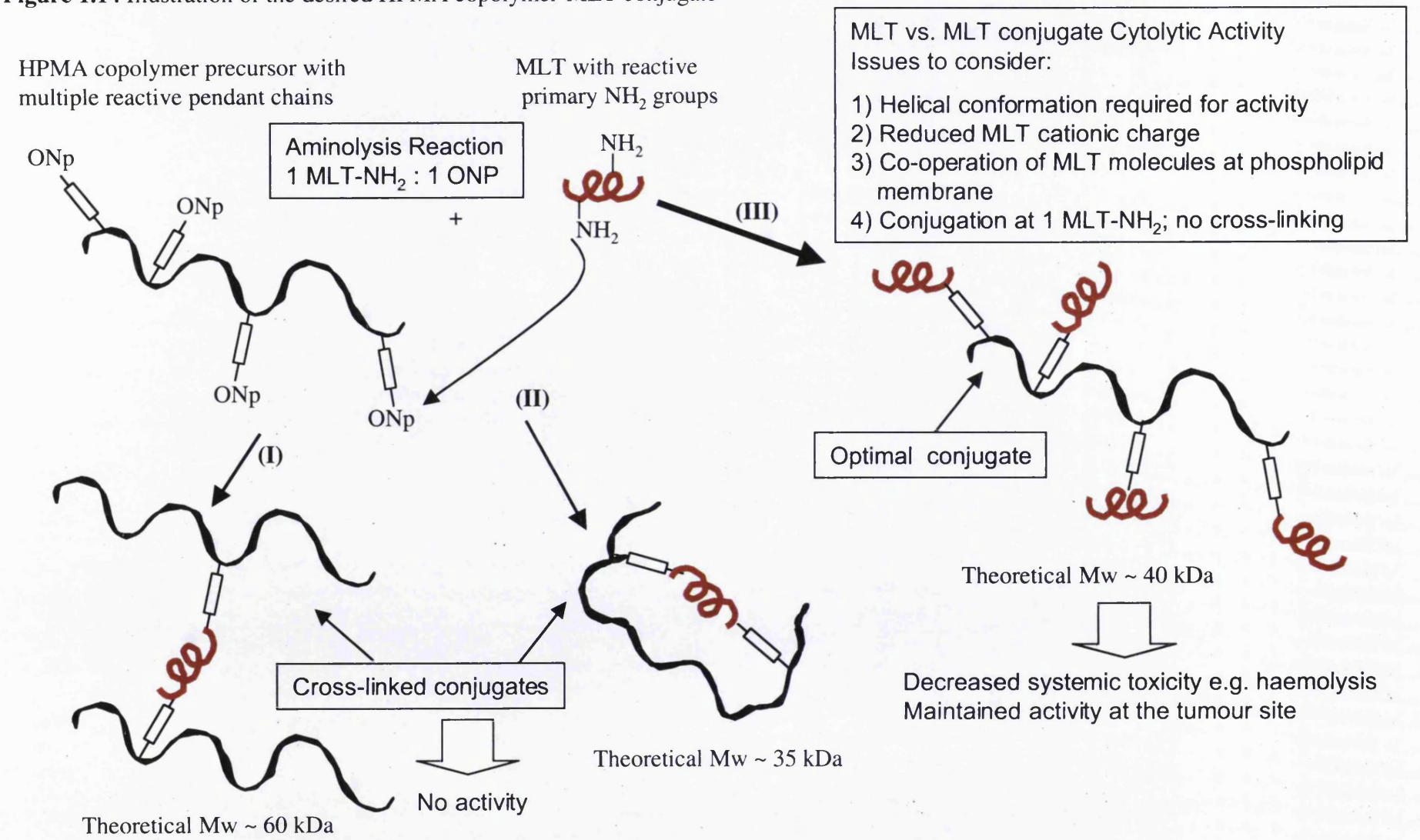
The use of HPMA copolymer anticancer conjugates in the clinic and the EPR effect has already been validated. The pioneering HPMA copolymer anticancer conjugate, PK1, which has recently progressed into Phase II clinical trials in the UK (Vasey *et al*, 1999), as well as other conjugates of this kind so far progressed show no evidence of polymer-related toxicities (Duncan, 2002). Hence HPMA copolymer was the polymer of choice in this study. The potential difficulties of lysosomotropic polymer-drug conjugates (outlined in section 1.5), however, prompted the creation of an alternative system which would exploit the toxicity of surface active peptides.

It was proposed that the reduced haemolytic activity en route to the cancer site by the documented EPR effect, would be feasible due to steric shielding of the relatively small MLT molecules by the hydrophilic polymeric chains. It was hoped that alteration of the MLT structure by conjugation at a single NH_2 group would not greatly affect its cationic charge and α-helical conformation, which are crucial to its membrane lytic activity. Furthermore, it was envisaged that once at the solid tumour site, multiple individual conjugated MLT molecules on single polymer chains in constant dynamic motion would convene and localise on a tumour cell membrane, thereby unrestrictedly interact with membrane phospholipids, and as depicted in the co-operation model of MLT action, cause lytic cell death and thus reduction in tumour mass. This macromolecular cell surface agent would thus by-pass cellular uptake and subsequent intercellular activation that is vital for lysosomotropic agents (Figure 1.14).

The aims of this study were therefore:

1) First, to synthesise HPMA copolymer-MLT conjugates using an activated polymer precursor (HPMA-Gly-Gly-ONp and HPMA-Gly-Phe-Leu-Gly-ONp) and standardise the synthetic procedure (Chapter 3). Also it was considered important to characterise the conjugates using size exclusion chromatography, peptide (MLT) content (BCA assay) and presence of free MLT (gel electrophoresis) (Chapter 3).

Figure 1.14 Illustration of the desired HPMA copolymer-MLT conjugate



2) To establish the membrane activity of the prepared HPMA copolymer-bound MLT and to select an optimal candidate for further investigation *in vivo* (Chapter 4). The isolated red blood cell was chosen as a model as this would be one of the first membranes "seen" by the HPMA copolymer-MLT conjugate after systemic administration. Cytolytic activity was assayed directly by haemoglobin release. In addition, the ability of the conjugates to cause tumour cell kill was establised indirectly using an *in vitro* tetrazolium-based (MTT) assay in a murine melanoma cell line. In this study, HPMA copolymer-MLT conjugates of low, medium and high loading, synthesised using HPMA copolymer-Gly-Phe-Leu-Gly-ONp precursors containing 1, 5 and 10 mol % ONp were assessed to determine the conjugate with optimal loading. In addition, using HPMA copolymer-Gly-Gly-ONp (5 mol % ONp) and HPMA copolymer-Gly-Phe-Leu-Gly-ONp precursors (5 mol % ONp), the effect of length of peptidyl linker was investigated.
3) To investigate the biodistribution and in particular tumour tropism in comparative studies using ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT in C57 mice bearing s.c. murine melanoma. Furthermore, to investigate the biological (antitumour) activity of the HPMA copolymer-MLT conjugate *in vivo* in a subcutaneous (s.c.) tumour model (Chapter 5).

The search for another membrane active anticancer agent was also carried out with a view to propose an alternative lytic agent for conjugation to a water-soluble polymer. This agent would not necessarily be in the form of a cytolytic peptide (Appendix I). A potent cytolytic compound with minimal haemolytic activity was of paramount importance for selection into further studies.

Chapter Two

Materials and Methods

2.1 Materials

General Chemicals

Anhydrous dimethyl sulphoxide (DMSO) was obtained from Aldrich, Dorset, UK. Sodium chloride, acetic acid, ethanol, 3-amino-1-propanol and 3-methoxypropylamine were purchased from BDH, Poole, UK. D,L-1-amino-2-propanol was obtained from Fluka, UK or Acros, UK. Melittin (MLT) 70 % pure (M7391) and 85 % pure (M722), DMSO, poly(ethylenimine) (PEI) (number average molecular weight (Mn) ~ 60 kDa; weight average molecular weight (Mw) 750 kDa; P3143), dextran (average molecular weight 64 - 76 kDa; D4751), Triton X-100, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide pellets, ethidium bromide, tris(hydroxymethyl)aminomethane (Tris), lithium acetate dihydrate, hydrindantin, bicinchoninic acid (BCA), Copper (II) sulphate pentahydrate 4 % (w/v) solution were all purchased from Sigma, Dorset, UK.

Tissue Culture

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or thiazolyl blue tetrazolium bromide were purchased from Sigma and Lancaster, UK respectively. RPMI 1640 with 25 mM HEPES and L-glutamine, foetal bovine serum (FBS), trypsin 0.25 % w/v / EDTA.4Na 1mM in Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (1098895), trypan blue and HBSS were obtained from Gibco BRL, Scotland, UK. Phosphate buffered saline (PBS) (Dulbecco A) (BR14) tablets were from Oxoid, Hampshire, UK. Optical grade DMSO was purchased from Sigma, Dorset, UK.

Electrophoresis

Low melting agarose, normal melting agarose, high molecular weight protein standards (aprotinin 6.5 kDa; lysozyme 14.4 kDa; trypsin inhibitor 21.5 kDa; carbonic anhydrase 31 kDa; ovalbumin 45 kDa; serum albumin 66.2 kDa; phosphorylase b 97.4 kDa; β-galactosidase 116.25 kDa; myosin 200 kDa), low molecular weight protein standards (bacitracin 1.4 kDa; insulin β-chain oxidised 3.5 kDa; aprotinin 6.5 kDa; α-lactalbumin 14.4 kDa; myoglobin 16.9 kDa; triosephosphate isomerase 26.6 kDa), sodium dodecyl sulphate (SDS), 30 % acrylamide monomer / 0.8 % N,N'-methylene-bis-acrylamide (bis-acrylamide) cross-linking agent, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), ready-made Tris-HCl and Tris-tricine gels of specified % polyacrylamide, β-mercaptoethanol, bromophenol blue, and the silver

staining kit were purchased from Bio Rad, UK. Barbital buffer was purchased from Sigma, Dorset, UK.

Radioiodination

N-Succinimidyl 3-(4-hydroxy-5-^{125}I-di-iodophenyl) propionate (Bolton and Hunter reagent) was purchased from Amersham Pharmacia Biotech, Hertfordshire, UK.

HPMA Copolymer Precursors

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers with ~ 1, 5 & 10 mol % oligopeptidyl-*p*-nitrophenol ester (ONp) side chains synthesised by radical precipitation copolymerisation of HPMA monomer with methacrylolated -Gly-Gly- (GG) or -Gly-Phe-Leu-Gly- (GFLG) ONp ester as previously described (Rejmanová *et al*, 1977 and Kopecek, 1985) were from Polymer Labs, Church Stretton, UK (Table 2.1).

Cells

B16F10 cells were a kind gift from Professor Ian Hart, St Thomas' Hospital, London, UK or obtained from The European Collection of Cell Cultures (ECACC), Wiltshire, UK.

2.2 Equipment

General Laboratory Equipment

The Varifuge 3.0 RS centrifuge was obtained from Heraeus Instruments, UK. The Cary and UV-1601 UV-visible (UV-vis) spectrophotometers were purchased from Varian, Australia and Shimadzu, Japan, respectively. The Micro Centaur mini-centrifuge was purchased from Sanyo Gallenkamp, UK. The FTS systems Flexo-Dry freezer drier was obtained VA Howe, UK. The blade homogeniser was purchased from Janke & Kunkel GmbH & Co., Germany. The Cobra II Auto-Gamma γ-counter was purchased from Packard, Berkshire, UK.

Electrophoresis

The Power Pac 300 and EC 135 were from Bio Rad, UK and E-C Apparatus Corporation, St. Petersburg, Florida, USA respectively. The Shandon tank was supplied by SLS, Nottingham, UK.

Table 2.1 Batch analysis of HPMA copolymer precursors (provided by Polymer Laboratories Ltd, UK)

Polymeric Precursor	Batch Number	Peptidyl-ONp Side Chains Content‡	Mw† (kDa)	Polydispersity (Mw/Mn)
HPMA copolymer-Gly-DL-Phe-L-Leu-Gly-ONp	CPT 09	0.93 mol %	64,517	2.99
HPMA copolymer-Gly-Gly-ONp	CPT 02	3.65 mol %	25,500	1.31
HPMA copolymer-Gly-DL-Phe-L-Leu-Gly-ONp	CPT 03	4.85 mol %	ND	ND
HPMA copolymer-Gly-DL-Phe-L-Leu-Gly-ONp	CPT 05	9.09 mol %	ND	ND

† Mw determinations of aminolysed polymer by GPC
‡ Side chain content determined by UV-vis spectroscopy
ND = not determined; however, Mw estimated by Polymer Laboratories to be 25 – 35 kDa

Tissue Culture

The Galaxy S CO_2 incubator was from Wolf Labs, UK. The Class II Type A-B3 microbiological safety cabinet AURA B was purchased from Bio Air s.c.r.l., Milan, Italy. The Sunrise Touchscreen and Titertek Multiskan Plus microtitre plate readers were from Tecan, Reading, UK and Eflab, Finland, respectively.

Gel Permeation Chromatography

The ÄKTA Fast Performance Liquid Chromatography (FPLC) system, the Superdex Peptide 75 HR 10/30 column and the Sephadex G25 (PD-10) columns were all obtained from Amersham Pharmacia Biotech, UK.

2.3 Methods

2.3.1 Analytical Techniques

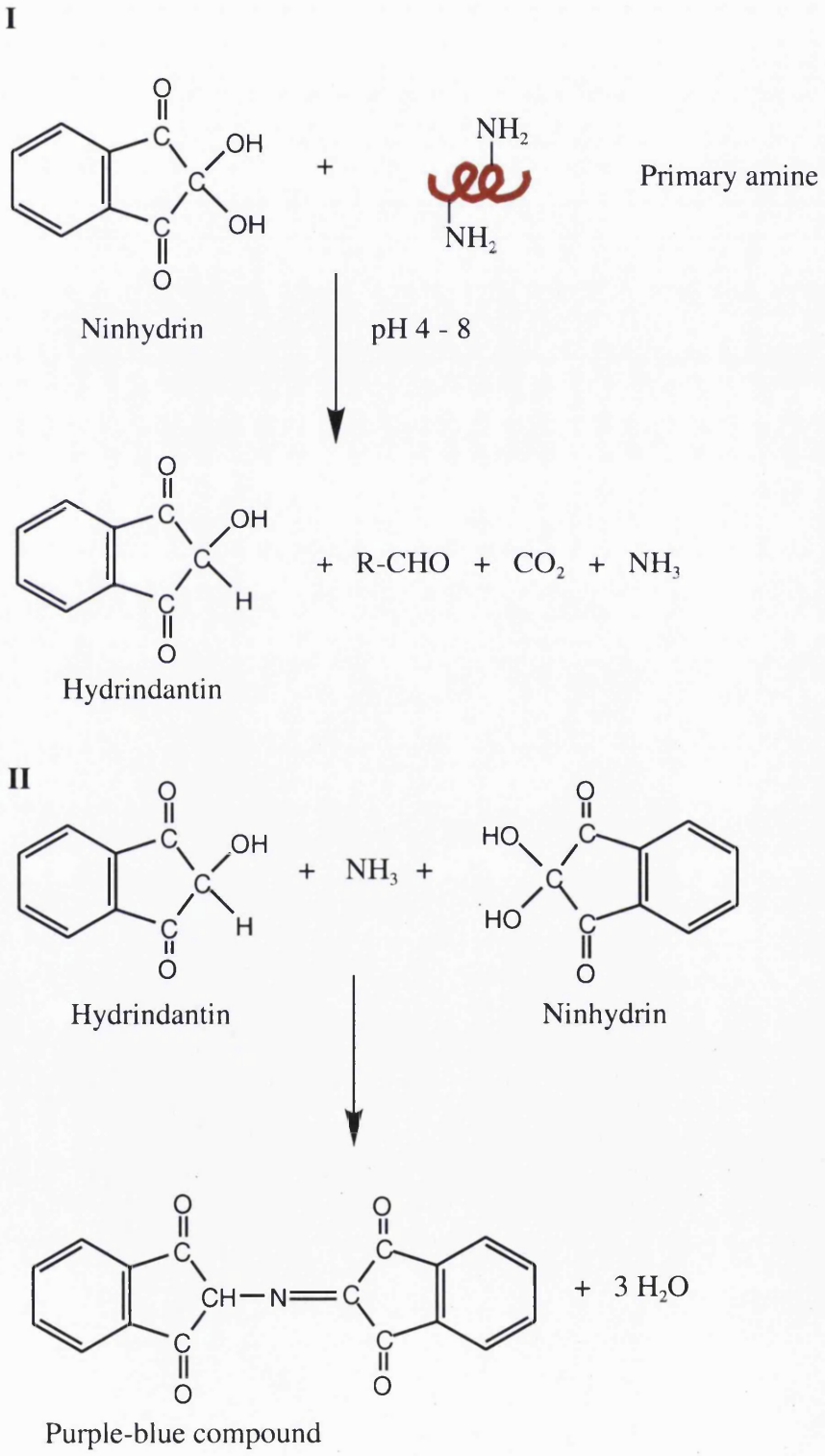
2.3.1.1 Ninhydrin Assay for Amine Quantification

Primary amine quantification may be carried out using colourimetric assays such as ninhydrin and 2,4,6-trinitrobenzenesulfonic acid (TNBSA) or fluorescence e.g. with *o*-phthalaldehyde. Here, the ninhydrin assay was used to determine the number of available pendant (primary) amine groups (provided by Lys, K or Arg, R) on MLT's surface prior to polymer conjugation. This assay is based on the reaction illustrated in Figure 2.1.

Fresh ninhydrin reagent was made for each assay as follows: 0.8 g of ninhydrin and 0.12 g of hydrindantin were dissolved in DMSO (30 ml) and lithium acetate buffer pH 5.2 (4 M; 10 ml). The latter was made by dissolving 102 g lithium acetate dihydrate in 250 ml double distilled water (DDW) and altering the pH to 5.2 with 24 % acetic acid in DDW. A range of concentrations of the monoamine reference standard 3-amino-1-propanol (molecular weight 75; density 0.982 g/ml) ($8 \times 10^{-4} - 2 \times 10^{-5}$ M), test compound MLT (Molecular weight 2,847 Da) (3.5×10^{-4} M) and reference control 3-methoxypropylamine (3×10^{-4} M) (molecular weight 89; density 0.871g/ml) were prepared in DDW.

Buffered ninhydrin reagent (2 ml) was added to samples (2 ml) in triplicate in glass vials. DDW was used as a reference control. The samples were gently mixed and carefully immersed in a boiling water bath (100 °C) for 15 min. The samples were subsequently cooled to room temperature and 50 % ethanol (3 ml) added to each tube.

Figure 2.1 Scheme of the ninhydrin reaction



The absorbance at 570 nm was then read after 10 min using the vial containing DDW as the reference (Plummer, 1978). If absorbance values greater than 1.5 were obtained, the samples were further diluted with 50 % ethanol at a 1:2 ratio. A calibration curve was established each time using 3-amino-1-propanol (Figure 2.2) from which the molarity could be determined and using the number of moles used in the reaction, the number of amines calculated. The monoamine control, 3-methoxypropylamine, was used to validate the reaction.

2.3.1.2 Bicinchoninic Acid (BCA) Assay

The BCA assay is a colourimetric protein assay. It was used to determine the yield of conjugation reactions by quantifying MLT in the HPMA copolymer-MLT conjugates. This assay is based on the reduction of Cu (II) to Cu (I) by proteins in a concentration-dependent manner resulting in formation of a stable purple coloured BCA-Cu(I) complex (Figure 2.3) (Smith *et al*, 1985). The absorbance is thus directly proportional to protein concentration.

Equal volumes (20 μl) of free MLT (15.6 μg/ml - 750 μg/ml), unpurified reaction mixture and purified MLT conjugate samples were added to a flat bottomed 96-well microtitre plate in replicates (n = 4). DDW was used as a blank. A MLT standard calibration curve was created for each assay (Figure 2.4). BCA reagent (20 ml) was prepared by mixing BCA and Cu (II) sulphate at a ratio of 1 ml BCA : 20 μl Cu (II) sulphate. The BCA reagent (200 μl) was then added to each well and the plate gently agitated and left for 20 min. The coloured product was then read spectrophotometrically using a 96-well microtitre plate reader at 550 nm and protein content of the unknown sample estimated using the calibration curve. The percentage yield of a conjugation reaction was then calculated by comparing the protein content in the purified conjugate to that added to the initial reaction.

2.3.1.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE was used as a relatively rapid and sensitive method to assess the degree of purity of the polymer-MLT conjugates and also to determine their approximate molecular weight.

Figure 2.2 Typical ninhydrin assay calibration curve using 3-amino-1-propanol standard. Data represents mean ± SD (n = 3)

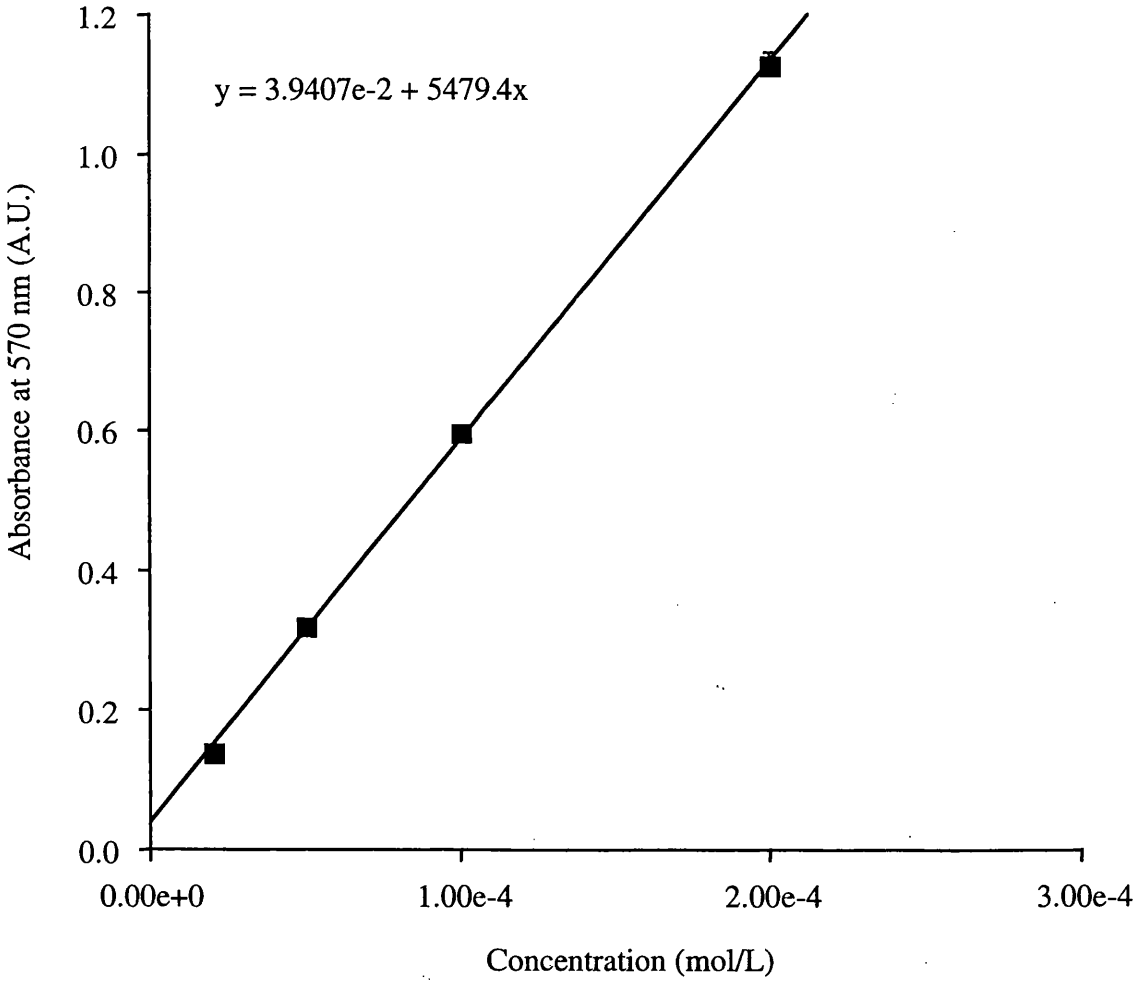


Figure 2.3 Illustration showing the mechanism of protein-mediated reduction of Cu^{2+} and generation of the stable BCA : Cu^{+} chromophore (2 : 1) (From Smith *et al*, 1985)

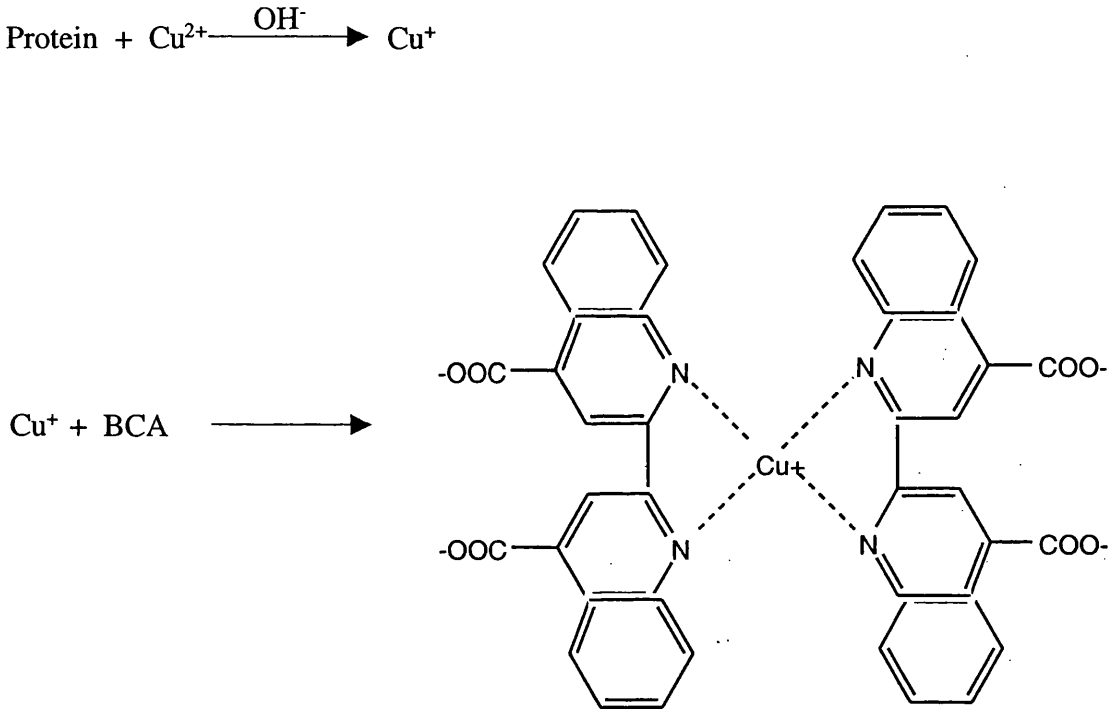
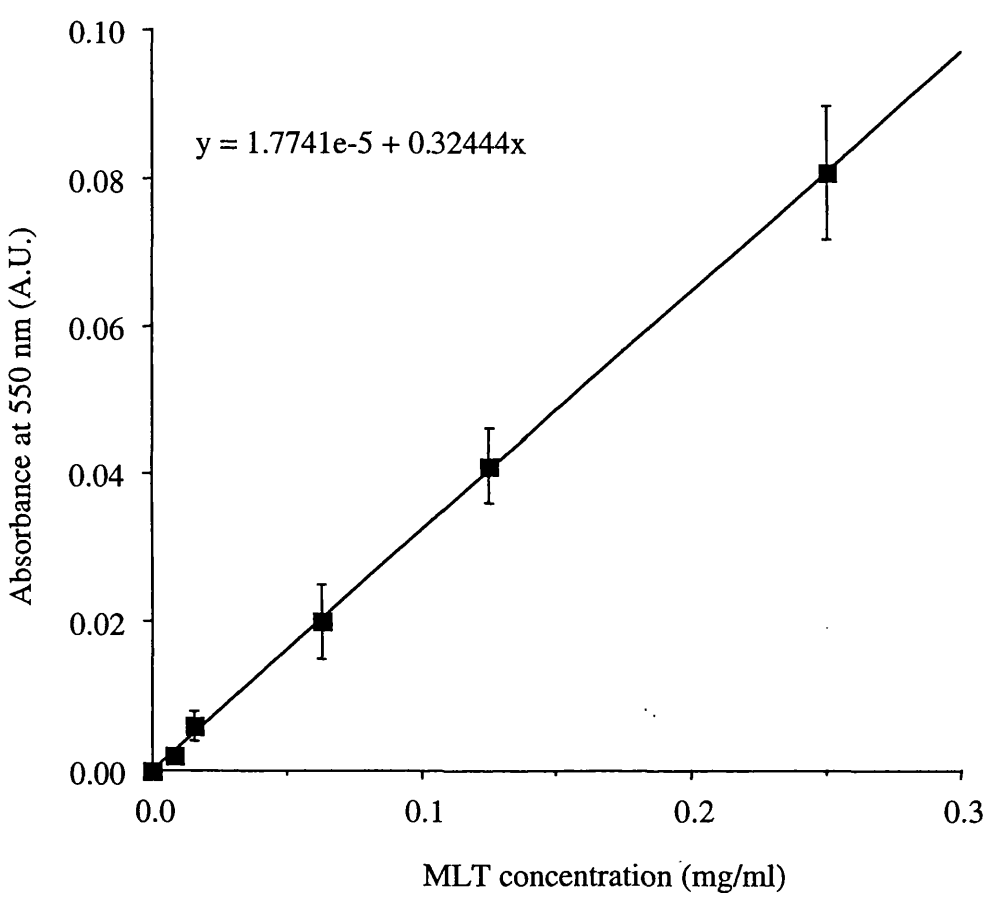


Figure 2.4 Typical MLT BCA calibration curve. Data represents mean ± SD (n = 16)



When placed in an electric field, molecules with a net charge will migrate towards the respective electrode. This is the basis of electrophoresis. In SDS PAGE, the proteins are denatured (i.e. disulphide bonds broken) with a reducing/thiol reagent such as β-mercaptoethanol or dithiothreitol. Treatment with SDS disrupts non-covalent interactions and so unfolds the polypeptide chains and being an anionic detergent, coats the proteins such that they all attain a net negative charge. This is achieved at a constant charge:mass ratio. Thus all proteins applied to the gel migrate towards the anode.

A polyacrylamide gel matrix of suitable porosity is selected to enable separation according to protein molecular weight (Hames, 1990). Thus under these conditions the mobility of proteins (Rf) is linearly proportional to the $\log_{10}$ of their mass. Free MLT and HPMA copolymer-MLT conjugates were compared to a series of protein standards with defined molecular weight. However, it is not possible to assign a specific molecular weight to the polymer-peptide conjugate (see section 3.1).

The Laemmli protocol (1970) for gel electrophoresis was used to analyse high molecular weight MLT conjugates. In this case, Tris-HCl gels were used in combination with a series of broad range molecular weight markers (6 - 200 kDa). For better resolution and thus detection of free MLT, the Tris-tricine method (Schägger & von Jagow, 1987) was also used.

In the Laemmli system, small peptides or proteins (< 10 kDa) form complexes with SDS which are of the same size and charge as the SDS micelles themselves, thus creating peptide or protein stacking within the gel. Tricine (pK 8.15) migrates faster than glycine (pK 9.6) despite its higher molecular mass due to more of it being in the anionic state at the gel pH (6.8 – 8.8). The Tris-tricine gels were run each time with low molecular weight protein standards (1,423 - 26,625 Da). All gels were run concurrently with the polymer-MLT conjugates on 14 % Tris-HCl polyacrylamide gels and 4 – 20 % gradient gels or 16.5 % Tris-tricine polyacrylamide separating gels. Free MLT (2,847 Da) and aminolysed HPMA copolymer precursor (~ 5 mol % side chains) were also run on the gel for the sake of comparison each time. The latter was prepared by reacting a HPMA copolymer-ONp precursor with excess 1-amino-2-propanol to react with and thus inactivate all the active easter (ONp) groups on the polymer precursor side chains.

Apparatus

The gel holding apparatus (Mini-PROTEAN II, Bio Rad, UK) was thoroughly cleaned with Decon and rinsed sequentially with methanol and DDW. Silver staining apparatus was further treated with nitric acid and rinsed with DDW. Once the apparatus was assembled it was tested for leaks using DDW prior to preparing the gels (Figure 2.5).

Preparation of Gels

Mini-gels (8 x 10 cm; ~ 1.5 mm thick) of specified final percentage of polyacrylamide were either prepared in-house or purchased (Bio Rad, UK). The separating or resolving gel and stacking gels were prepared sequentially in side-arm flasks as follows:

Separating Gel 0.375 M Tris; pH 8.8

Stock Solution	14 % Separating Gel
DDW	2.68 ml
Tris HCl pH 8.8 (1.5 M)	2.5 ml
10 % (w/v) SDS	100 µl
30% Acrylamide/bis	4.67 ml

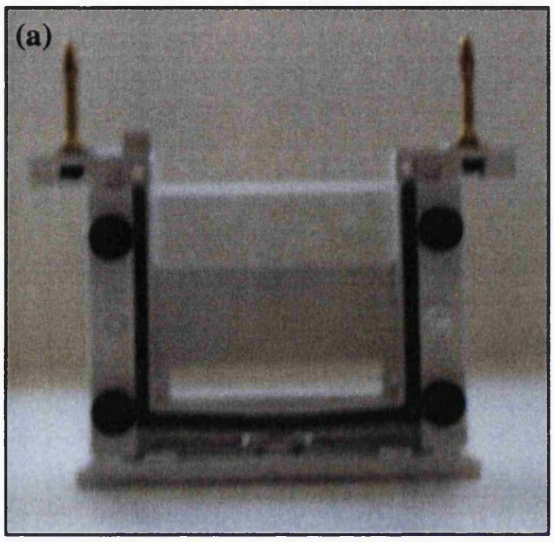
This mixture was degassed for a minimum period of 15 min under vacuum to exclude oxygen which would inhibit the polymerisation process. Subsequently, freshly made 10 % (w/v) ammonium persulfate (50 µl) and TEMED (5 µl) (polymerisation initiators – TEMED catalyses free radical production from persulfate) were added and the mixture gently swirled to mix whilst preventing production of bubbles. This was then immediately carefully introduced into a glass plate sandwich within the gel holder apparatus using a Pasteur pipette and any air bubbles excluded. DDW was immediately layered over the top of the gel to prevent dehydration of the mixture and allowed to polymerise over a 45 min time period.

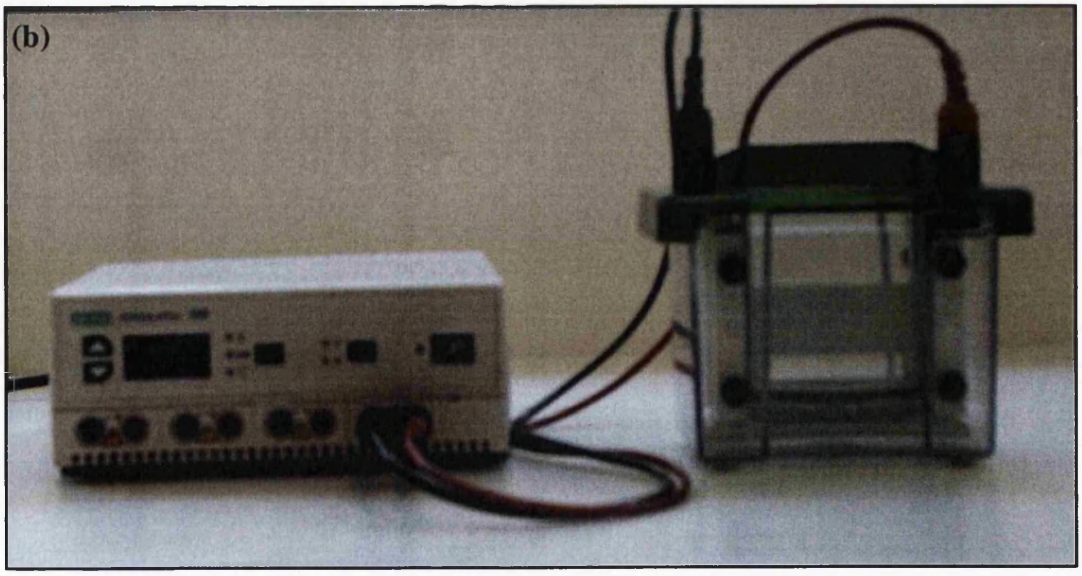
During this time, the stacking gel (4 %) was prepared as follows:

Stacking Gel 0.125 M Tris; pH 6.8

Stock Solution	4 % Stacking Gel
DDW	6.1 ml
Tris HCl pH 6.8 (0.5 M)	2.5 ml
10 % (w/v) SDS	100 µl
30% Acrylamide/bis	1.33 ml

Figure 2.5 (a) Gel holder and **(b)** Gel electrophoresis kit (Bio Rad, UK)





Following degassing over a minimum of 15 min, freshly made 10 % (w/v) ammonium persulfate (50 µl) and TEMED (10 µl) were added and gently mixed as before. This mixture was then layered on top of the polymerised separating gel after drying the top layer of DDW using filter paper and a 10 well comb positioned at the top of the stacking gel. This was allowed to polymerise for 45 min, after which time the comb was cautiously removed and the wells (~ 7 mm wide) carefully rinsed with electrode running buffer.

Running the Gels

This assembly was immersed in the respective electrode running buffer.

	Tris-HCl Buffer (5X) (pH 8.3)	*Tris-tricine Buffer (5X) (pH 8.25)*
Tris base	15.0 g/l	60.55 g/l
Glycine	72.0 g/l	-
Tricine	-	89.60 g/l
SDS	5.0 g/l	5.0 g/l

The electrode running buffers were stored at 4 °C and diluted with DDW at a 1:4 ratio immediately prior to use. The sample reducing buffers were prepared as follows:

	Tris-HCl Gels	*Tris-Tricine Gels*
DDW	3.8 ml	4.0 ml
Tris-HCl (pH 6.8; 0.5 M)	1 ml	2.0 ml
Glycerol	0.8 ml	2.4 ml
SDS 10 %(w/v)	1.6 ml	1.0 ml
β-Mercaptoethanol	0.4 ml	0.2 ml
Bromophenol blue 1 % (w/v)	0.4 ml	-
Coomassie G-250 (0.5%)	-	0.4 ml

These were stored at room temperature. Samples (0.5 - 1 mg/ml) were mixed with reducing buffer at a 1:4 ratio respectively (30 µl). These samples were then centrifuged to ensure adequate mixing and heated for 5 min in a boiling water bath. The molecular weight markers (0.2 mg/ml in sample buffer; 5 µl) were loaded in the outer lanes and the cooled samples loaded in the remaining lanes.

The gels were then run at 100 V until the dye was visible at the bottom end of the gel. The gels were then fixed in fixative enhancer solution: methanol (50 % v/v), acetic acid (10 % v/v), fixative enhancer concentrate (Bio Rad, UK) (10 % v/v) and DDW (30 % v/v) to immobolize the peptides and remove any substances that may intefere with staining (SDS and buffer components), after which they were silver stained by the reduction of silver nitrate to silver (ng sensitivity) (Gottlieb & Chavko, 1987) (silver staining kit, Bio Rad, UK).

The staining and developing solution was prepared by adding the following to 35 ml DDW: silver complex solution (containing NH_4NO_3 and $AgNO_3$; 5 ml), reduction moderator solution (containing tungstosilicic acid; 5ml) and image development solution (containing formaldehyde; 5ml) in that order, to which development accelerator solution (containing Na_2CO_3; 50 ml) was added immediately before use. Once the desired staining was reached, the reaction was stopped with acetic acid solution (5 % v/v), after which the gels were repeatedly rinsed with DDW and dried overnight between cellulose sheets.

2.3.1.4 Fast Performance Liquid Chromatography (FPLC)

FPLC was used for purification and analysis of HPMA copolymer-MLT conjugates. An ÄKTA FPLC (Amersham Pharmacia Biotech, UK) (Table 2.2) system was used which separates proteins and peptides on the basis of their molecular weight (size exclusion chromatography, SEC). A precise determination of molecular weight of HPMA copolymer conjugates is also not possible using this method due to the different characteristics of the peptide standards and conformation of free polymer standards and conjugated peptide (see section 3.1).

A pre-packed Superdex 75 HR 10/30 column was washed through with 2 column volumes (~ 48 ml) of filtered (0.2 μm; Millipore) and sonicated DDW. The column was then equilibrated with 2 - 3 column volumes of filtered and sonicated mobile phase, PBS (0.1 M) with NaCl (0.5 M) (pH 6.8) prior to each use.

First, the column was calibrated (0.25 ml/min flow rate) using proteins of varying molecular weights: aprotinin from horse lung 6.5 kDa; cytochrome c from horse heart 12.4 kDa; carbonic anhydrase from bovine erythrocytes 29 kDa; albumin from bovine serum 66 kDa (Sigma, UK) and vitamin B12 1.35 kDa, myoglobin 17

Table 2.2 ÄKTA FPLC System (Amersham Pharmacia Biotech) used to characterise and purify HPMA copolymer-MLT conjugates

Column	Superdex 75 HR 10/30 (cross-linked agarose and dextran; bead size 13 μm; bed dimensions 10 x 300 - 310 mm; bed volume 24 ml; separation range 3,000 – 70,000 Da)
Pump	P-920
Detector	UPC-900 (UV absorbance at 280 nm)
Fraction collector	Frac-950
Software	Unicorn 3.20
Running conditions	
Mobile phase	PBS (0.1 M) + NaCl (0.5M)
Flow rate	0.25 ml/min

kDa, ovalbumin 44 kDa, gamma globulin 158 kDa, thyroglobulin 670 kDa (Bio Rad GPC standards, UK) and blue dextran (Molecular weight 2 x 10^6 Da) (Amersham Pharmacia Biotech, UK) to mark the void volume (V*o*).

Samples of peptide standards, MLT, aminolysed HPMA copolymer precursors (~ 1, 5 and 10 mol % side chains) or HPMA copolymer-MLT (1 – 5 mg/ml; 500 μl or 3 ml) were injected into a 100 μl or 2 ml loop respectively and then allowed to run at a flow rate of 0.25 ml/min. When HPMA copolymer-MLT conjugates were analysed, conjugate fractions (0.5 ml) were collected and assayed for protein content (BCA assay) (section 2.3.1.2).

The UV data was collected using Unicorn 3.20 software (Amersham Pharmacia Biotech, UK). After use the column was washed thoroughly with eluent and then DDW and for periods of over 2 days without use was stored in 20 % v/v ethanol.

2.4 Cell Culture: Maintenance of Cells and Aseptic Technique

All cell culture procedures were carried out in a class II laminar flow cabinet, where strict aseptic conditions were employed. The cabinet was subject to UV irradiation for a minimum of 30 min prior to use. It was then wiped down sequentially with Klericide® and 70 % v/v ethanol. Equipment and materials used were always sterile and non-sterile materials were autoclaved prior to use. Where possible, filter sterilisation of solutions was carried out using a 0.2 μm pore filter (Acrodisc). Alternatively, UV irradiation of polymer solutions for 30 min was employed. All items were sprayed with 70 % v/v ethanol before introduction into the laminar flow cabinet.

B16F10 cells were maintained in RPMI 1640 medium which was supplemented with 10 % FBS in an atmosphere of 37 °C, 5 % CO_2. All materials added to the cultured cells were sterile and warmed to 37 °C in a water bath. The cells were kept in 75 cm^2 cantered neck flasks with vented tops (0.2 μm). The flasks were kept in an incubator with a humid environment and a small amount of copper sulphate crystals. B16F10 cells were allowed to reach confluence over 4 days and then they were subcultured to ensure an exponential growth phase throughout.

2.4.1. Cell Culture: Recovery from Cell Bank

Frozen vials of B16F10 cells were kept in a liquid nitrogen cell bank. Before use they were rapidly thawed out in the 37 °C water bath. Immediately, 9 ml of 10 % FBS supplemented medium (37 °C) was pipetted into a 75 cm^2 flask, to which ~ 1 ml of thawed cells was added. Flasks were placed in the incubator (5 % CO_2, 37 °C) to allow adherence and cell growth.

Cell Passage

The culture medium was aspirated and the cells washed twice with 10 ml aliquots of PBS in order to remove cell adhesion proteins which would hinder the enzymatic action of trypsin (0.25 %)/EDTA (1mM). An aliquot of trypsin/EDTA (1 ml) was added into the flasks and after gentle agitation, the flasks were incubated (37 °C, 5 % CO_2) for 5 min to optimise the action of the trypsin.

After confirming the cells had been enzymatically harvested, 10% FBS (9 ml) supplemented medium was added to the cells to dilute the trypsin. The obtained cell suspension was then carefully passed through a 20 gauge needle attached to a syringe at least 4 times so as to obtain a fine suspension, and break up any aggregates. Aliquots (1 ml) were then used to seed further 75 cm^2 flasks containing 9 ml of supplemented medium (1:10 split ratio). Unused cells were discarded appropriately (autoclaved). The flasks were then placed back into the incubator and cells grown to confluence or used in an experimental procedure.

2.4.2 Evaluation of Cell Viability using Trypan Blue Exclusion

It was necessary to assess cell viability prior to their use in cytotoxicity experiments, uptake studies or when used for subcutaneous (s.c.) inoculation to grow tumours *in vivo*. In addition, assessment of cell viability was important prior to seeding cells in flat bottomed 96-well microtitre or 6-well plates.

A single cell suspension (20 μl) was mixed with an equal volume of 2 % v/v trypan blue dye (supplied as 4 % v/v and diluted in PBS at a 1:1 ratio). Cells were counted using a Neubauer haemacytometer slide. Dead cells stained blue and were thus excluded from the cell count. Viable (clear) cells were counted in a quadrant of a known volume (0.1 mm^3).

The number of viable cells was then determined using the following formula:
cells/ml = mean number of cells per quadrant x dilution factor (2) x 10^4.

2.4.3 Evaluation of Cell Viability using the Colourimetric Tetrazolium-based (MTT) Assay

The MTT assay was used as a means to determine the cytotoxicty of HPMA copolymer-MLT conjugates, free MLT and other reference polymers. This assay is based on the ability of viable cells to metabolise a water soluble tetrazolium dye, MTT, by mitochondrial dehydrogenase enzymes into a water insoluble formazan salt (Mossman 1983; illustrated in Figure 2.6).

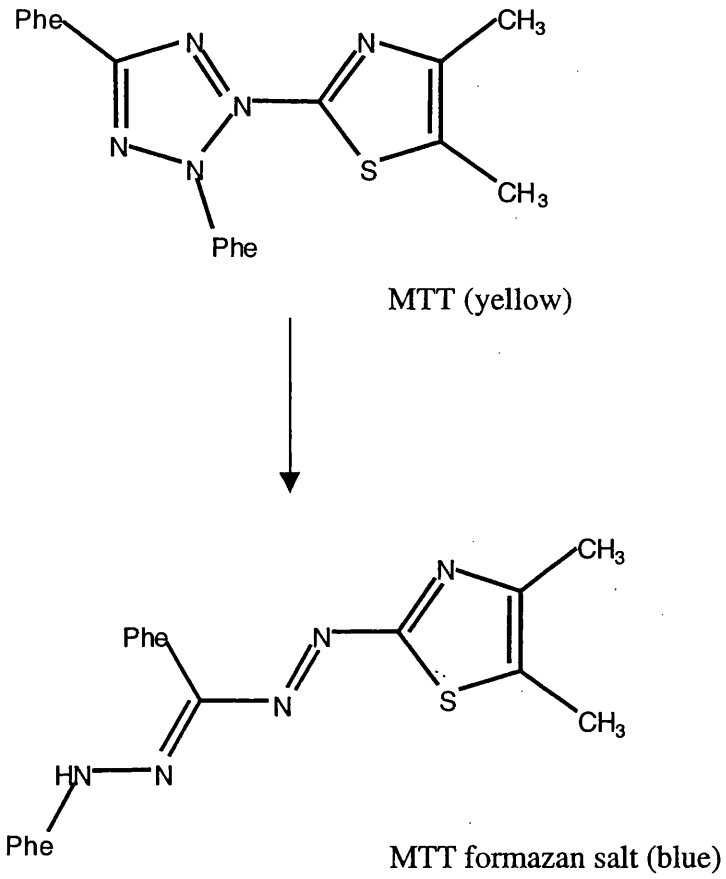
Sgouras & Duncan (1990) standardised this assay for testing the cytotoxicity of polymers. The MTT assay was also used to establish the growth curve for B16F10 cells.

For all cytotoxicity assays, a seeding density of 5 x 10^3 B16F10 cells/well (100 μl) in flat bottomed 96-well microtitre plates was used. After 24 h the cells had become adherent and entered their logarithmic phase of growth and could be used for cytotoxicity assays. The experimental compounds to be tested were dissolved in 10 % FBS-supplemented medium in the range of concentrations required. The cell culture medium was removed and replaced by media containing the test compounds and then incubated at 37 °C in 5 % CO_2 for 67 h.

MTT (20 μl of a 5 mg/ml solution in PBS) was then added to each well and the plate incubated for a further 5 h. After this time, the MTT/medium mixture was aspirated, taking care not to disrupt the formazan crystals at the base of the wells. Spectrophotometric grade DMSO (100 μl) was added to each well and the formazan crystals dissolved over a 30 min period. The plates were then read spectrophotometrically at 550 nm using the microtitre plate reader. DMSO alone was used as a blank. It should be noted that the MTT product is only stable for 2 h (Sgouras & Duncan, 1990). Cells incubated in cell culture medium alone (free of test compound) were used as a control to assess 100 % viability. The results obtained were expressed as:

$$\text{Percentage Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

Figure 2.6 Scheme showing mitochondrial MTT reduction to its insoluble formazan salt



The significance of any differences seen was assessed using a students' t-test for small sample size.

To establish the growth curve of B16F10 cells, the MTT assay (as described above) was repeated at 24 h intervals for 7 days. The growth curve was constructed as a function of optical density (OD) against time (Figure 2.7) and the B16F10 cell doubling time determined to be ~ 24 h.

2.5 The Rat Red Blood Cell Lysis Assay

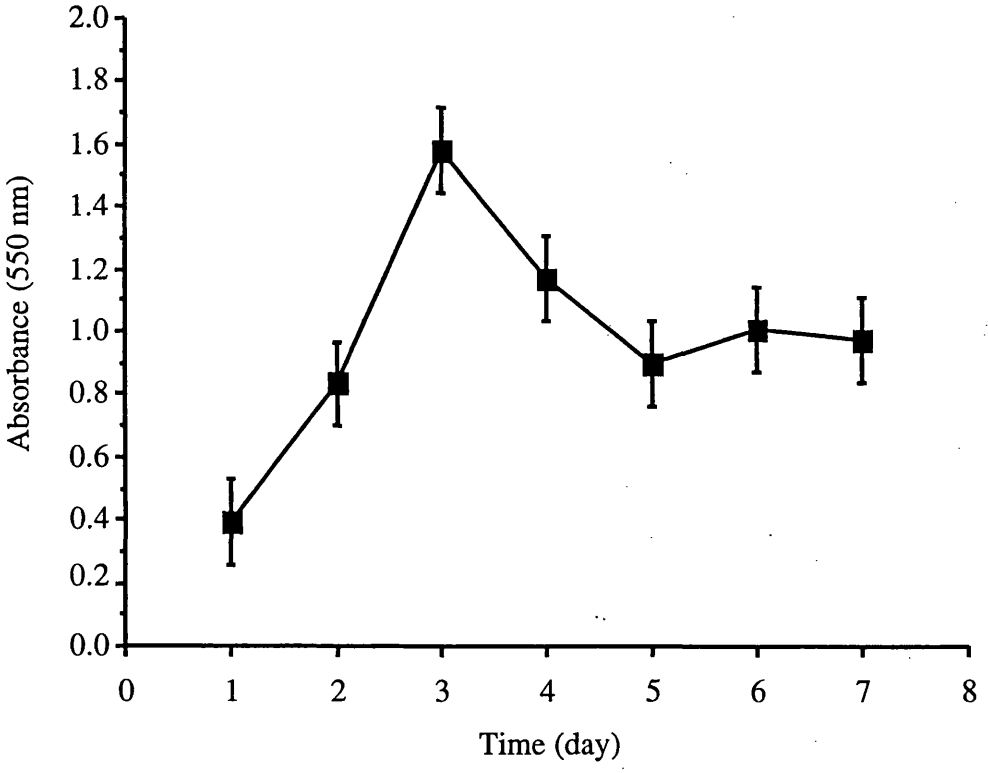
This assay was used as a model to assess membrane damage caused by the surface action of polymer-MLT conjugates and free MLT (Duncan *et al*, 1991).

Fresh rat blood was obtained from male Wistar rats (~ 250 g) by cardiac puncture using a 21 gauge needle attached to a 2 ml syringe. Blood was placed into tubes containing heparin beads and the cells repeatedly washed in PBS (0.1 M; pH 7.4) by centrifugation at 1,500 x g for 10 min at 4 °C (3 - 4 times). On each occasion, the supernatant and upper frothy layer (top few mm) was discarded prior to repetition of the washing process. The erythrocyte pellet was then weighed and the red blood cells (RBC) re-suspended in ice-cold PBS to a concentration of 2 % w/v.

Double strength concentrations of test compounds in PBS (100 μl) were pipetted into flat bottomed 96-well microtitre plates to which the RBC suspension (100 μl) added. The RBC/test compounds mixture was then incubated for the specified time points at 37 °C in a thermostat controlled oven (Jencons-PLS, Bedfordshire, UK). PEI and 1% triton X-100 in PBS were used to provide the positive reference controls. Triton X-100 was used to obtain a value for 100 % haemoglobin release. Dextran and HPMA copolymer aminolysed with 1-amino-2-propanol were used as the negative reference controls.

Following incubation for the desired time, the plates were centrifuged at 1,500 x g for 10 min at RT. The OD of the supernatant was then determined spectrophotometrically at 550 nm using the microtitre plate reader against PBS as the blank. The results were expressed as a percentage of haemoglobin release seen using 1 % triton X-100.

Figure 2.7 B16F10 growth curve. Data represents mean ± SD (n = 6)



2.6 Scanning Electron Microscopy (SEM)

All SEM preparations were carried out in the EM Suite under the direction of Dr. A. Hann (School of Biosciences, Cardiff University). RBC and B16F10 cells exposed to MLT or MLT conjugate were visualised using SEM to assess their effect on membrane morphology.

Protocol for SEM of RBC

A fresh 2 % w/v RBC suspension was prepared as described in section 2.5. Test compounds including free MLT, MLT conjugates RM 5.3 (1 mol %), RM 3.13 (5 mol %) and RM 6.3 (10 mol %), HPMA copolymer precursor aminolysed with 1-amino-2-propanol and PBS (control) were aliquoted into glass vials at their respective Hb_{50} concentrations (500 μl) to which an equal volume of RBC suspension (500 μl) was added. After incubation for 10 min (37 °C), the cells were fixed with glutaraldehyde (2.5 % v/v in 0.2 M cacodylate buffer, pH 7.4) (1 ml) for 3 min (RT). The cells were then centrifuged (300 x g) for 2 min, after which the supernatant was discarded and cells re-suspended in fresh fixative solution and left for 1 h (4 °C). The cells were then centrifuged (300 x g) for 30 s and washed twice (5 min) in 0.1 M cacodylate buffer (pH 7.4) (2 ml). After the final wash, osmium tetroxide (1 % v/v in 0.2 M cacodylate buffer, pH 7.4) (500 μl) was added to the cells and they were left for 1 h (4°C). Cells were then centrifuged and washed in 0.1 M cacodylate buffer (pH 7.4).

Then 2 - 3 drops of the cell suspension was instilled into holding chambers made from beem capsules with Micropore® filter paper (polycarbonate cyclopore track etched membrane) (Whatman International, Maidstone, UK) at the base of the chamber and placed on petri dishes containing Whatman® filter paper soaked in 50 % v/v ethanol. They were then centrifuged (300 x g) for 10 s and immediately dehydrated sequentially with 50 %, 70 %, 80 %, 95 % ethanol (each for 5 min) and 100 % ethanol (6 x 5 min).

The cells were then dried at the critical point temperature (40°C, 1,300 psi) for 1 h (Samdri®-780, Tousimis, Maryland, USA), mounted on 0.5 inch aluminium specimen stubs (Agar Scientific, Essex, UK) and gold-plated (Sputter Coater S150B, Edwards High Vacuum International, West Sussex, UK) by Mr. Mike Turner (EM Suite, School of Biosciences, Cardiff University).

The samples were then viewed using a microscope (SEM XL 20, Philips Eindhoven, The Netherlands).

Protocol for SEM of B16F10 Cells

B16F10 cells (1 x 10^5/well) (2 ml) were seeded in 6-well plates containing sterile plastic Thermanox® cover slips (15 mm diameter) (Emitech Ltd, Kent, UK) and allowed to adhere and reach their exponential phase of growth for 24 h. Test compounds including free MLT, MLT conjugates RM 5.3 (1 mol %), RM 3.13 (5 mol %) and RM 6.3 (10 mol %) and HPMA copolymer precursor aminolysed with 1-amino-2-propanol made up in cell culture medium at their IC_{50} concentrations were used to replace the existing cell culture medium. The cells were then incubated for 72 h at 37 °C, 5 % CO_2. Cells grown in cell culture medium alone were used as a reference control.

At the end of the incubation period the medium/test compounds mixture was removed and immediately fixative solution (2.5 % v/v glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) (2ml) was added to each well and left for 30 min (RT). Subsequently, the cells were then washed twice (5 min) using 0.1 M cacodylate buffer (pH 7.4) (2ml) and then stained with osmium tetroxide (1 % v/v in 0.1 M cacodylate buffer, pH 7.4) (1 ml) for 1 h at 4 °C. The cells were then washed twice (5 min) in 0.1 M cacodylate buffer (pH 7.4) and sequentially dehydrated with 50 %, 70 %, 80 %, 95 % ethanol (each for 5 min) and then 100 % ethanol (6 x 5 min).

Then the cells were dried at the critical point temperature (40°C, 1,300 psi) for 1 h (Samdri®-780, Tousimis, Maryland, USA), mounted on 0.5 inch aluminium specimen stubs (Agar Scientific, Essex, UK) and gold-plated (Sputter Coater S150B, Edwards High Vacuum International, West Sussex, UK) by Mr. Mike Turner (School of Biosciences, Cardiff University).

The samples were then viewed using a microscope (SEM XL 20, Philips, Eindhoven, The Netherlands).

2.7 Radioiodination of MLT and HPMA Copolymer-MLT using the Bolton and Hunter Reagent

The Bolton and Hunter reagent (diiodo derivative) was chosen to radioiodinate MLT and HPMA copolymer-MLT conjugate. This was necessary in order to evaluate their biodistribution in tumour bearing mice. The *N*-hydroxy succinimidyl (NHS) ester functional group of the Bolton and Hunter reagent reacts with free ε- (terminal) amine groups of peptides or proteins to form a stable amide bond (Figure 2.8).

Free MLT (5 mg) and HPMA copolymer-MLT (5 mg MLT-equivalent) (batch RM 3.4) were dissolved in borate buffer pH 8 (0.5 ml) in glass vials. In a fume cupboard, di-iodo Bolton and Hunter reagent (1 mCi ≡ 37 MBq) supplied in benzene with 2 % (v/v) dimethylformamide (DMF) was used under a gentle stream of nitrogen in its original glass vial and 0.5 mCi (100 μl) carefully added to each compound and mixed for 15 min on ice, with periodic careful agitation.

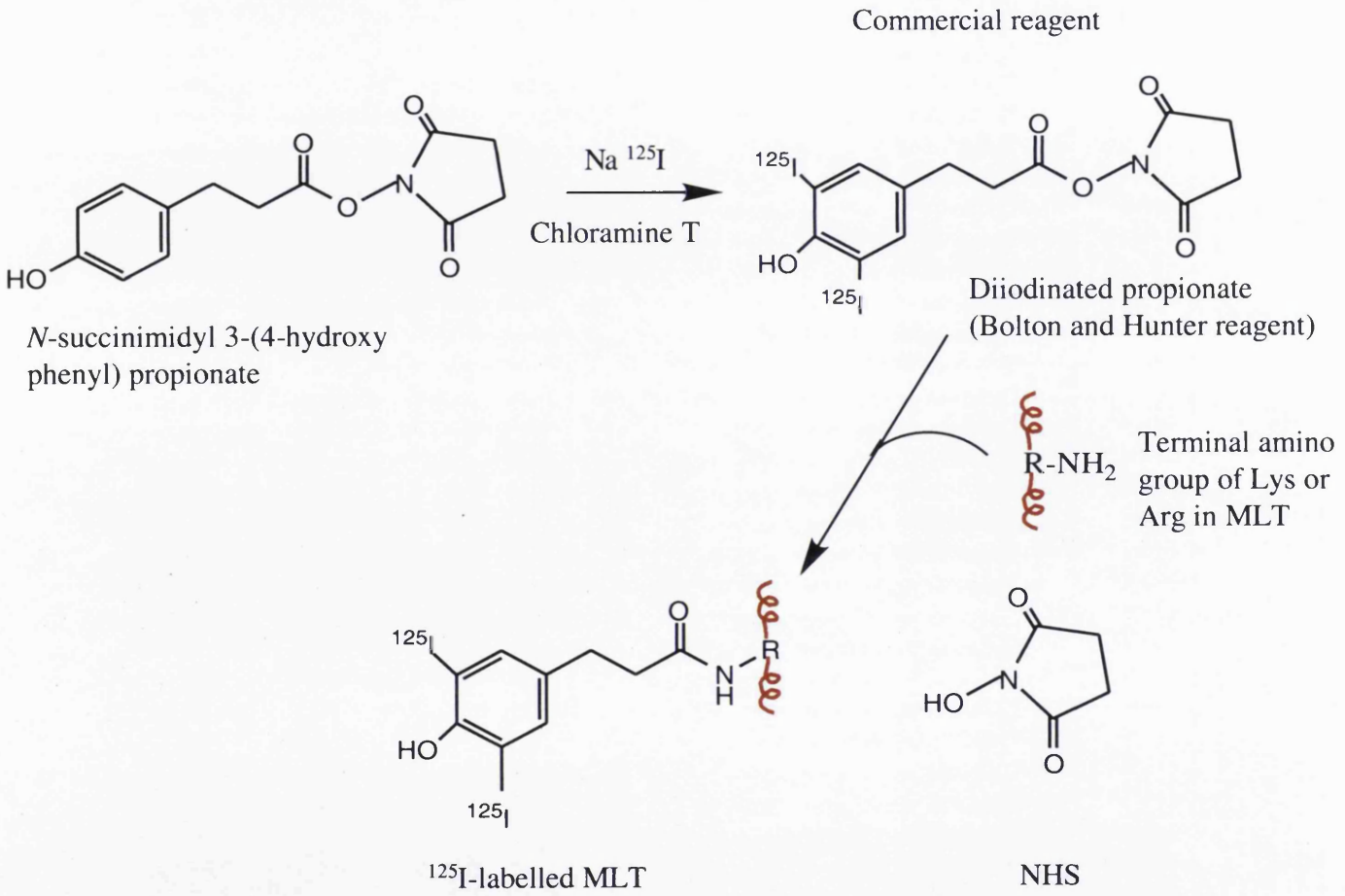
A sample of the crude reaction mixture (10 μl) was kept to determine labelling efficiency (section 2.7.1). Both the ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT conjugate were purified by GPC using pre-packed disposable Sephadex G25 (PD-10) columns (9.1 ml bed volume; 5 cm bed height; 1 – 5 kDa separation range).

First, the PD-10 columns were drained of the storage buffer (DDW with 0.15% Kathon® CG/ICP Biocide preservative) and equilibrated with PBS (3 x bed volume) (30 ml). The crude reaction mixture containing the ^{125}I-labelled MLT or ^{125}I-labelled HPMA copolymer-MLT conjugate preparations (~ 490 μl) were then pipetted onto the top of the column bed and eluted with PBS (3.5 ml). A sample (1 ml) of the primary elute was kept and the remainder (2.5 ml) was eluted with PBS (3.5 ml) using a new PD-10 column to obtain the secondary elute. These samples were appropriately stored at – 20 °C prior to characterisation.

Calibration of PD-10 Column

Elution of blue dextran (1 mg/ml in PBS) (0.5 ml fractions) was used to determine the V*o* of the PD-10 column. A 100 μl sample of each fraction was assessed spectrophotomerically in triplicate (550 nm) using a microtitre plate reader. The data

Figure 2.8 Illustration of diiodination of Bolton and Hunter reagent at its phenolic ring and reaction with an amine-containing molecule (MLT) to form an amide bond



was then plotted as a function of OD (550 nm) versus elution volume (ml) (Figure 2.9).

To determine the elution profile of MLT, a sample in 0.9 % saline (1 mg/ml) was applied to the column and fractions (0.5 ml) collected. The BCA assay (section 2.3.1.2) was used to determine the protein content of each fraction and % recovery of MLT calculated (Figure 2.10 and Table 2.3).

2.7.1 Determination of Labelling Efficiency of ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT using Paper Electrophoresis

Paper electrophoresis was used to determine the labelling efficiency and purity of the ^{125}I-labelled preparations. The electrophoresis tank was prepared by adding freshly made barbital buffer (50 mM sodium barbital + 10 mM barbital) in DDW (1 litre). Whatman chromatography paper was cut into strips (5 x 30 cm). Starting 5 cm from one end, each strip was marked with a pencil with vertical lines, 0.5 cm apart. The fifth line was marked as the loading point. The chromatography paper was soaked in the barbital buffer and excess solution removed by blotting between two pieces of tissue.

The paper was then placed across the electrodes of the electrophoresis tank, with each end in the buffer solution so as to complete the circuit. Crude reaction mixture, purified preparations or Bolton and Hunter reagent reference control (4 μl) were loaded onto the chromatography paper at the marked loading point, at the cathode end of the tank. The electrophoresis was run at 400 V for 20 min.

The chromatography paper was then dried using a hot air gun (Bosch, Germany) and cut into the pre-divided 0.5 cm strips. These were then placed in Luckham tubes containing DDW (1 ml) and assayed for radioactivity using a γ-counter (Packard, Beckshire, UK). The labelling efficiency (% Bolton and Hunter reagent bound) and specific activity could then be calculated (μCi/mg). The results were then used to calculate administered radioactivity doses in biodistribution studies (Chapter 5).

2.8 *In Vivo* Studies

All *in vivo* studies were carried out according to the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (1998) and the Home Office regulations for project

Figure 2.9 Void volume determination of a PD-10 column with blue dextran. Data represents mean ± SD (n = 3)

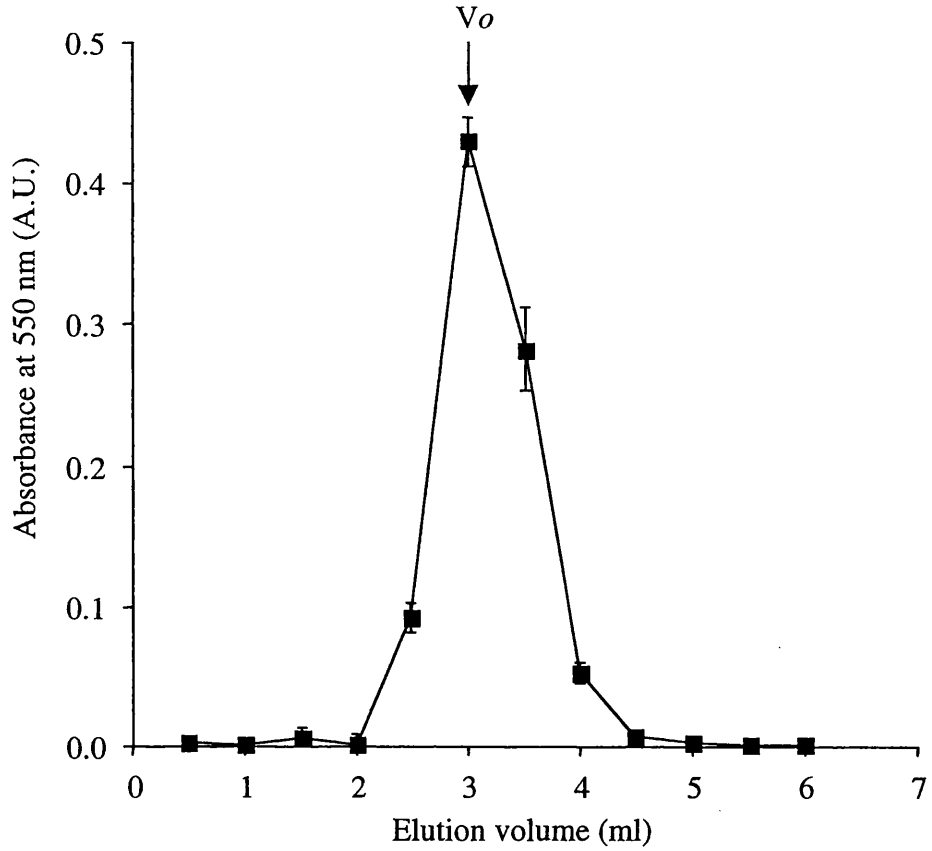


Figure 2.10 Typical elution profile of MLT (1mg/ml) with 0.9% NaCl in a PD-10 column. Data represents mean ± SD (n = 4)

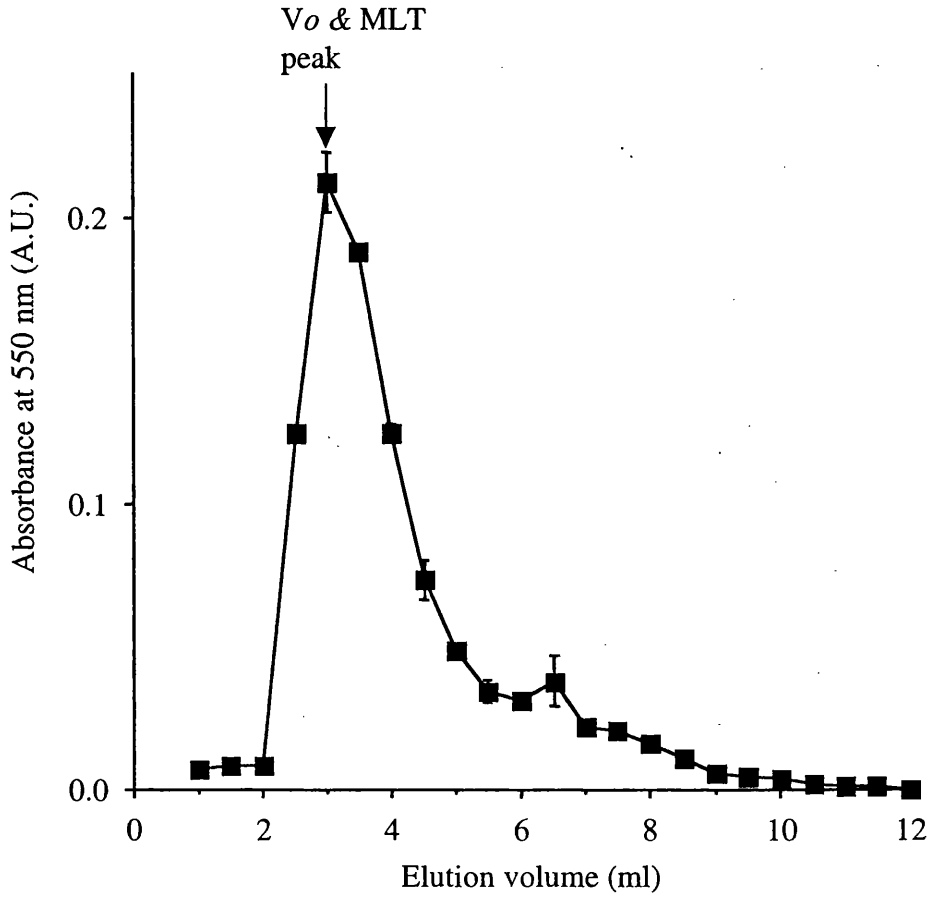


Table 2.3 Data to show elution profile of MLT in a PD-10 column

$$x = \frac{(y - 6.8670 \times 10^{-3})}{1.7320 \times 10^{-2}}$$ BCA calibration with MLT

Fraction number	Elution volume (ml)	Absorbance (550nm)(y)	μg/20μl (*x*)	μg/500μl fraction
1 + 2	1	0.007	0.008	0.384*
3	1.5	0.009	0.094	2.357
4	2	0.009	0.109	2.716
5	2.5	0.125	6.792	169.793
6	3	0.212	11.858	296.453
7	3.5	0.188	10.472	261.81
8	4	0.125	6.806	170.153
9	4.5	0.074	3.862	96.540
10	5	0.049	2.418	60.453
11	5.5	0.035	1.610	40.245
12	6	0.032	1.422	35.555
13	6.5	0.038	1.798	44.938
14	7	0.022	0.874	21.843
15	7.5	0.021	0.802	20.036
16	8	0.017	0.556	13.903
17	8.5	0.011	0.239	5.965
18	9	0.006	0.239	5.965
19	9.5	0.004	-	-
20	10	0.004	-	-
21	10.5	0.002	-	-
22	11	0.002	-	-
23	11.5	0.001	-	-
24	12	0.000	-	-
25	12.5	0.001	-	-
26	13	0.000	-	-
27	13.5	0.000	-	-
				Σ = 961.576 μg

* 1 ml fraction

MLT in:1 mg; MLT out: 961.576 μg
i.e. 96.2 % recovery

and personal license holders. The animals were allowed to acclimatise to their new environment over a period of 5 days prior to studies. During the course of the studies, animals were allowed food and water *ad libitum*.

Cell Preparation for s.c. Inoculation

B16F10 cells were harvested when growing in the exponential phase and viability determined using trypan blue exclusion (section 2.4.2). The harvested cells in 10% FBS-supplemented medium were subject to centrifugation at 1,000 x g for 5 min at room temperature and re-suspended in sterilised 0.9 % saline to a concentration of 1 x 10^6 cells/ml.

Establishment of s.c. Solid Tumours in Mice

Animals were lightly anaesthetised using a 2 % v/v isoflurane and 4 % v/v O_2 mixture. Re-suspended B16F10 cells (1 x 10^5; 100 μl) were then immediately inoculated into 6 – 8 week old (~ 25 g) male C57 BL/6 mice into the back of the neck via the s.c. route. *In vivo* studies began once tumours were palpable (~ 0.5 cm x 0.5 cm), usually on day 10 – 12 following tumoural inoculation.

2.8.1 Evaluation of Body Distribution of ^{125}I-labelled HPMA Copolymer-MLT

Pharmacokinetic studies were carried out in male C57 BL/6 mice (6 – 8 weeks old, ~ 25 g) bearing a s.c. B16F10 murine melanoma (section 2.6). 5 x 10^5 counts per minute (CPM) (100 μl) of ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT in 0.9 % saline were administered i.p. (n = 3). Where different doses of MLT were needed, the radiolabelled compounds were "spiked" with cold (i.e. non-radiolabelled) MLT or cold HPMA copolymer-MLT in 0.9 % saline.

Animals were killed by CO_2 asphyxiation at the pre-determined time points (0.5, 2, 4 and 6 h). Blood was immediately collected by cardiac puncture into heparinised tubes and all the major organs (heart, liver, kidneys, lungs, spleen, thyroid) and tumours isolated, rinsed in 0.9 % saline and final weights obtained. Organs were homogenised using a blade homogeniser in DDW to a known final volume. Blood (100 μl) was made up to 1 ml and 1 ml of homogenised organs assayed for radioactivity using a γ-counter.

Results were then expressed as % dose administered/g of tissue or /ml of blood. The total radioactivity recovered was expressed as % dose administered. The administered HPMA copolymer-MLT conjugate was always expressed as a MLT-equivalent dose.

The total blood volume for each mouse was taken to be 5.77 ml/100 g (Dreyer & Ray, 1910).

2.8.2 Evaluation of Antitumour Activity of MLT and HPMA Copolymer-MLT

Specified doses of MLT or HPMA copolymer-MLT were administered via the i.p. or i.v. route to tumour-bearing male C57 BL/6 mice (6 - 8 weeks old; ~ 25 g) (n = 5) (section 2.8).

Animals were monitored daily and their weight, tumour size and any signs of toxicity noted. Animals were killed by CO_2 asphyxiation once tumours reached a maximum of 289 mm^2 or if physical signs of toxicity were observed. Notably animal appearance, posture, behaviour, response to touch, assessment of food and water intake, body weight loss of more than 20 % and ulceration at the tumour site as stated in the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia (UKCCCR, 1998). By law it is a requirement that all such experiments are overseen by the Project License Holder (Professor R. Duncan) and Deputy (Ms S. Kneller). The designated Veterinary officer is on-call if the animals are unwell. The survival of animals was expressed as % T/C value, whereby:

$$T/C\ (\%) = \frac{\text{mean survival time of treated group}}{\text{mean survival time of control group.}} \times 100$$

In general, a % T/C value of ≥ 125 % is often considered to show an indication of antitumour activity, however, the significance of the results obtained was determined statistically using a student's t-test (normal distribution) (section 2.10).

2.9 Data Analysis

All data analysis was performed using Microsoft Excel 98 software for Macintosh. This package was used to obtain standard deviation of the mean (SD), standard error of the mean (SE) and statistical analysis by two-tailed student's t-test.

Chapter Three

Synthesis and Characterisation of HPMA Copolymer-MLT Conjugates

3.1 Introduction

First, it was necessary to identify suitable methods for reproducible synthesis and characterisation of HPMA copolymer-MLT conjugates. Covalent conjugation of polymers to peptides and proteins has been used extensively to improve their biological efficacy by minimising proteolytic degradation, prolonging plasma circulation time and also by reducing protein immunogenicity (reviewed by Illum & Davis, 1991 and Duncan & Spreafico, 1994). The aim of this study was to develop a polymer conjugate that would be able to selectively target MLT to solid tumours by the EPR effect (see section 1.3).

In the case of polymer-peptide conjugation chemistry parameters, of noteworthy interest is the site of conjugation on both the free polymer and free MLT. Conjugation depends on the availability of functional groups in the protein or peptide chain as well as the nature of reactive groups available in the polymer. Typically conjugation involves either :

1) Activation of the polymer (terminal group or main chain) by incorporation of functional groups and direct reaction of the peptide or protein with functional groups present in the polymer main chain.
2) Polymer analogous reaction using a polymer precursor synthesised to contain a reactive leaving group suitable for displacement by the peptide or protein.

The use of HPMA copolymer precursors containing reactive *p*-nitrophenoxy (ONp) ester groups as an intermediate is a typical example of the latter (Ríhová & Kopecek, 1985). Polymer-peptide or protein conjugation may be carried out in two manners. Polymers may either be:

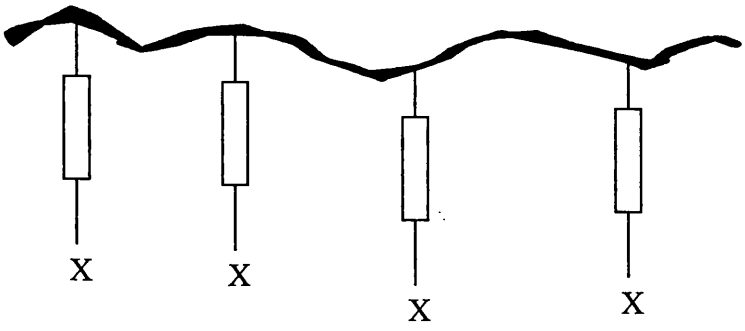
1) Monofunctionalised e.g. monomethoxy Poly(ethylene glycol) (mPEG) (Abuchowski *et al*, 1977 and Davis *et al*, 1991) and semitelechelic HPMA (Lu *et al*, 1998 and Oupicky *et al*, 1999) thus providing single end-terminal group conjugation
2) Bifunctionalised e.g. PEG (reviewed by Greenwald *et al*, 2000)
3) Multivalent, with pendant reactive groups which provide multiple sites for conjugation on the polymer backbone as with HPMA copolymer (reviewed by Brocchini & Duncan, 1999) (Figure 3.1).

Figure 3.1 Illustration of (a) monovalent, (b) bivalent and (c) multivalent reactive polymers

(a) Polymer with single end terminal reactive sites

(b) Polymer with end terminal reactive sites

(c) Polymer with multiple reactive sites



Where X is a functional group attached to the polymer backbone by a chemical linker

The reactive groups typically used for conjugation present in a peptide or protein are the N- (pKa 7.6 – 8.0) and C- (pKa 2.1 – 2.4) terminals (α-amino and α-carboxylate groups respectively) and the side chains of amino acids which possess carboxylate (COOH) (aspartic acid and glutamic acid), sulphydryl (R-SH) (cysteine), thioether ($S-CH_3$) (methionine), amino (NH_2) (lysine, arginine and histidine) and hydroxyl (OH) (tyrosine) groups (Hermanson, 1996; Table 3.1). The stability of derivatised bonds can be listed as a function of how potent a nucleophile as: $R-S^- > R-NH_2 > R-COO^- = R-O^-$ (Hermanson, 1996).

When selecting the polymer and a method for peptide or protein conjugation, it is important to consider the desired characteristics of the resulting conjugate in terms of stability, pharmacokinetic profile and pharmacological activity. These properties are governed by 3 main factors:

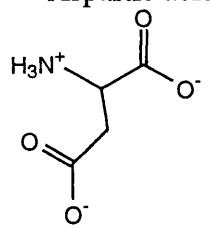
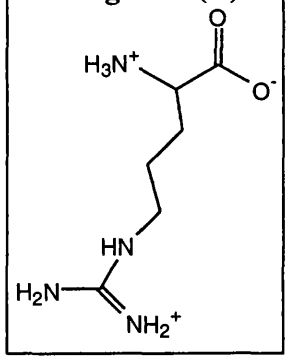
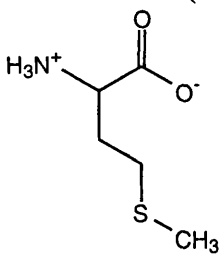
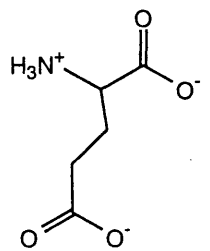
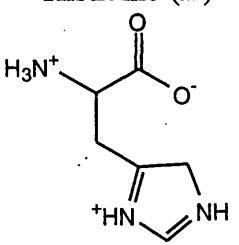
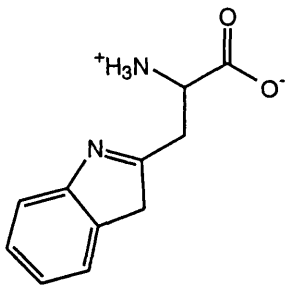
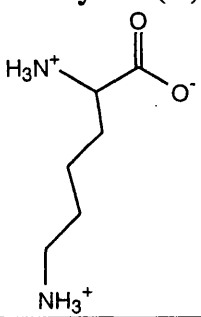
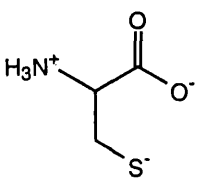
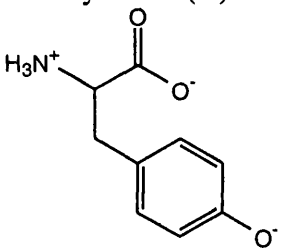
1) The choice of coupling agent and thus nature of the covalent linker formed
2) The molecular weight of the polymer used
3) The number of reactive groups in both the polymer and peptide or protein to be modified.

A frequently observed disadvantage of peptide and protein (antibodies, hormones and enzymes) conjugation is the loss of biological activity (reviewed by Nucci *et al*, 1991). In the case of enzymes, this can be due to a general steric hindrance caused by the polymer chain which may be as a result of covalent attachment of the polymer close to or at the active site or at more than one reactive chemical group. Ideally, it has been suggested that a 1:1 polymer-peptide or polymer-protein conjugation might be best to minimise loss of biological activity (Morgan, 1993; Melton, 1996; Satchi, 1999). As HPMA copolymer-ONp consists of multiple chains (1, 5 or 10 mol %) terminating in reactive ONp ester functionalities, a 1 ONp : 1 MLT molecule (via reactive primary NH_2 groups) conjugation was therefore sought for HPMA copolymer-MLT conjugates. This type of conjugation would ideally avoid potential cross-linking due to MLT's multivalency which would compromise MLT activity.

Choice of Polymer

At the beginning of this study it was necessary to choose an "optimal" polymer for MLT modification. PEG has been extensively used for protein modification (enzymes, antibodies, cytokines, etc.) (reviewed by Fuerteges & Abuchowski, 1990;

Table 3.1 Reactive amino acids and their sub-classifications (boxed amino acids are specific to MLT)

Amino acid	Functional group	Amino acid	Functional group	Amino acid	Functional group
Aspartic acid (D) 	γ-COOH pKa 3.7 - 4 Ionisable amino acid	**Arginine (R)** 	Guanidinyl NH_2 pKa > 12 Ionisable amino acid	Methionine (M) 	$S\text{-}CH_3$ Aliphatic amino acid
Glutamic acid (E) 	γ-COOH pKa 4.2 - 4.5 Ionisable amino acid	Histidine (H) 	Imidazoyl NH_2 pKa 6.7 - 7.1 Ionisable amino acid	**Tryptophan (W)** 	Indole ring Non-polar aromatic amino acid
Lysine (K) 	$\varepsilon\text{-}NH_2$ pKa 9.3 - 9.5 Ionisable amino acid	Cysteine (C) 	R-SH pKa 8.8. - 9.1 Ionisable amino acid	Tyrosine (Y) 	Phenolic OH pKa 9.7 - 10.1 Ionisable amino acid

Delgado *et al*, 1992; Nucci *et al*, 1991, Katre, 1993; Zalipsky & Lee, 1992; Greenwald *et al*, 2000). Some examples of PEG conjugates and conjugates synthesised using other polymers are shown in Table 3.2.

The advantage of PEG for conjugation is its monofunctionality. In this study, however, HPMA copolymers were chosen to prepare MLT conjugates because:

1) HPMA copolymer–anticancer drug conjugates have already been transferred to clinical trials
2) HPMA copolymers have been successfully conjugated to proteins and peptides (Table 3.2)
3) The use of an HPMA copolymer-ONp precursor allows attachment of MLT via pendant NH_2 groups
4) The use of HPMA copolymers with different content of reactive ONp side chains creates the possibility of creating a library of conjugates with different MLT loading

The HPMA copolymer-ONp intermediate readily reacts with primary NH_2 groups present in MLT by an aminolysis reaction to form a stable amide bond (Rejmanová *et al*, 1977; Lääne *et al*, 1983; Figure 3.2). The disadvantages of this method, however, include:

1) The potential for cross linking where more than one primary NH_2 group is available for the reaction
2) Competing hydrolysis of the ONp which results in unwanted side products
3) HPMA is a random copolymer resulting in uneven drug distribution on polymer chains as well as between different polymer chains

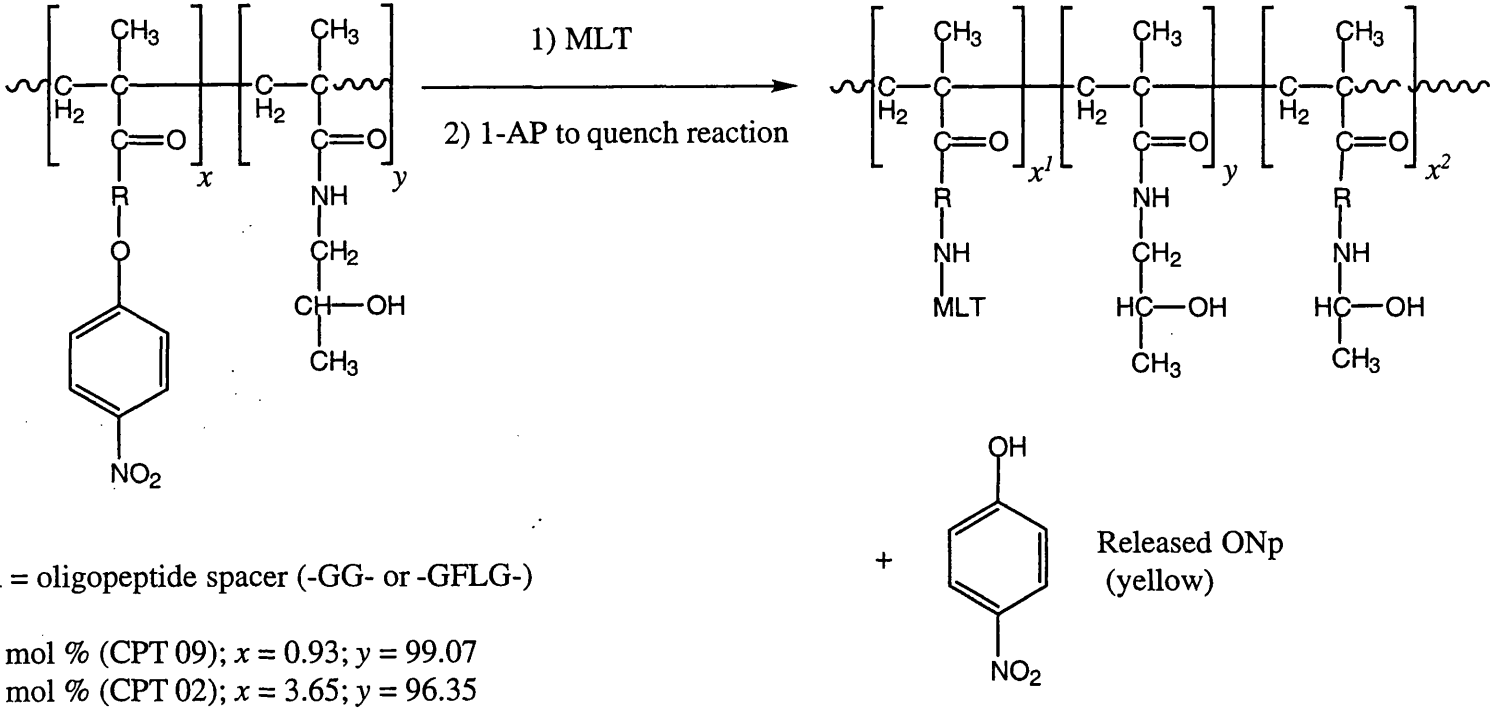
The peptidyl linker -Gly-Phe-Leu-Gly- (GFLG) was originally designed for polymer-drug conjugation to allow cleavage by lysosomal thiol-dependent proteases following pinocytic uptake of the conjugates (see section 1.5). As it was hoped the conjugated MLT would act at the cell surface, a lysosomal degradable linker was not considered essential and so a -Gly-Gly- (GG) spacer was chosen to prepare most conjugates. However, some batches were synthesised using the longer tetrapeptide GFLG spacer to allow comparison of the biological activity of HPMA copolymer-GG-MLT and HPMA copolymer-GFLG-MLT. It should be noted that although the comparision of HPMA copolymer-GGGG-MLT with HPMA copolymer-GG-MLT

Table 3.2 Examples of polymer-peptide and polymer-protein conjugates that have been studied as therapeutics

Polymer / Peptide or Protein	References
HPMA	
Insulin	Chytry *et al*, 1978
Gramicidin-S	Solovskij & Panarin, 1999
Transferrin	Flanagan *et al*, 1992
Insulin β-chain	Morgan *et al*, 1996
RNase A	Soucek *et al*, 2001
Anti-Thy 1.2 antibody	Ríhová *et al*, 2000
Anti-EL4 antibody	Ríhová *et al*, 2000
OV-TL 16 antibody	Omelyanenko *et al*, 1996
Acetylcholinesterase	Lääne *et al*, 1983
Chymotrypsin	Lääne *et al*, 1981
MSH	O'Hare *et al*, 1993
mPEG	
Adenosine Deaminase (Adagen®)	Hershfield *et al*, 1987
L-asparaginase (Oncaspar®)	Ho *et al*, 1986
Interferon (IFN) α (PEG-α-intron®)	Trepo *et al*, 2000
Uricase	Chua *et al*, 1988
MLT	Ahlstedt *et al*, 1983
Batroboxin	Nishimura *et al*, 1985
Interleukin (IL)-2	Meyers *et al*, 1991
Lysozyme	Zalipsky *et al*, 2001
SMA	
Neocarzinostatin (NCS)	Konno & Maeda, 1987
Superoxide dismutase (SOD)	Ogino *et al*, 1988
Dextran	
Glutathione	Kaneo *et al*, 1989
Insulin	Suzuki *et al*, 1972
SOD	Fujita *et al*, 1992
Uricase	Yasuda *et al*, 1990
Trichosanthin	Ko *et al*, 1991

Polymer / Peptide or Protein	References
PVP	
β-D-N-Acetylhexosaminidase A	Geiger *et al*, 1977
Kallikrein	von Sprecht *et al*, 1977
DIVEMA	
NCS	Yamamoto *et al*, 1990
SOD	Maeda *et al*, 1988

Figure 3.2 Scheme to show aminolysis of HPMA copolymer precursor by MLT primary amine groups



R = oligopeptide spacer (-GG- or -GFLG-)

1 mol % (CPT 09); $x = 0.93$; $y = 99.07$
5 mol % (CPT 02); $x = 3.65$; $y = 96.35$
10 mol % (CPT 05); $x = 9.09$; $y = 90.91$

$x = x^1 + x^2$

1-AP = 1-amino-2-propanol

would have been ideal, this polymer precursor was not available. MLT conjugates prepared with the GFLG linker, however, would be useful for direct comparisons with the pioneering polymer-drug conjugate, HPMA copolymer-GFLG-doxorubicin (PK1).

To have an optimal HPMA copolymer-MLT conjugate, it was considered necessary to investigate the optimal MLT loading. Therefore, conjugates of low, medium and high loading were prepared using HPMA copolymer precursors with different ONp content (1, 5 and 10 mol %). In addition, a product was synthesised by aminolysis of HPMA copolymer-ONp (5 mol %) using 1-amino-2-propanol to produce a control HPMA copolymer material for use as a reference polymer in *in vitro* studies (Chapter 4).

3.1.1 Polymer Characterisation

Before progressing, it is important to discuss some of the important features of the HPMA copolymer samples used. Generally, polymer samples consist of many molecules with chains of varying length and thus varying molecular weight. i.e. they are polydisperse in respect of molecular weight.

Polymer characterisation may be defined in terms of weight average molar mass (Mw) and number average molar mass (Mn) (Duncan, 1983 and Young & Lovell, 1991; Figure 3.3).

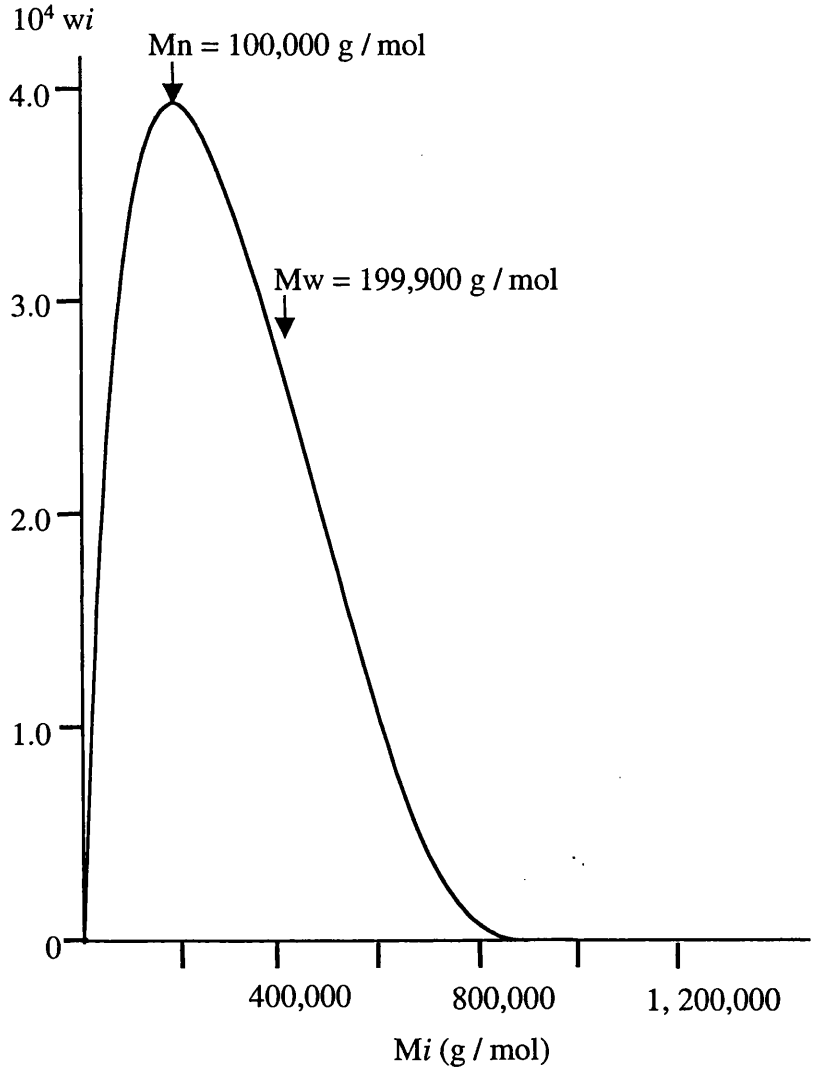
Mw = sum of products of the molar mass of each fraction x mole fraction

Mn = sum of products of the molar mass of each fraction x weight fraction

The molar mass (M) of a homopolymer (e.g. HPMA homopolymer) may be defined by the simple equation $M = xMo$, whereby x = degree of polymerisation (i.e. number of repeat units) and Mo is the molar mass of each repeat unit. For copolymers (e.g. HPMA copolymer), the sum of the products of each type of repeat unit defines the molar mass (i.e. $M = xMo_{(a)} + xMo_{(b)}$). It should be noted that molar mass, M and molecular weight are used interchangeably, although M = molecular weight x g/mol.

The Mw and Mn can be determined using various methods including size exclusion chromatography (SEC) and viscosity (Campbell & White, 1989). These values can be related to give an indication of the molecular weight distribution of the polymer preparation. The heterogeneity index or polydispersity is defined as Mw/Mn.

Figure 3.3 A typical polymeric molar mass distribution curve (From Young & Lovell, 1991)



When Mw/Mn is 1, this indicates a perfectly monodisperse polymer. Typically, polydispersity ranges from 1.5 – 2.0 for synthetic polymers (Young & Lovell, 1991). The HPMA copolymer conjugates taken into clinical trial have a polydispersity of 1.3 – 1.5 due to their preparation by free radical precipitation polymerisation.

Characterisation of Polymer-Peptide Conjugates

Careful characterisation of HPMA copolymer-MLT conjugates was very important prior to their biological evaluation. However, it is not easy to characterise polymer-protein or polymer-peptide conjugates. Peptide (MLT) content was determined by the BCA protein assay (section 2.3.1.2). The purity of HPMA copolymer-MLT conjugates in respect of free MLT was analysed by electrophoresis (SDS PAGE) and SEC which is the basis of the Fast Performance Liquid Chromatography (FPLC) system used in this study.

Although SEC is widely used to characterise polymers in terms of Mw and polydispersity (Campbell & White, 1989), meaningful characterisation of polymer-peptide or polymer-protein conjugates is difficult without appropriate standards and also such conjugates may form micelles (Ulbrich *et al*, 1987).

SEC usually displays a linear relationship between the log molecular weight and retention time of polymer or protein standards. Their elution time is governed by the hydrodynamic volume of the molecule (i.e. shape and size) (Flanagan, 1987; Seymour *et al*, 1987; Welling & Welling-Wester, 1989). Large molecules that can not enter the pores of a given gel are excluded in the void volume (V*o*). The bed volume within the stationary phase (column) (V*i*) is differentially accessible to smaller molecules depending on the pore size. The elution of any polymer, protein or conjugate will be between V*o* and V*i* (V*o* + V*i* = V*t* (total accessible volume)) (Baudys & Kim, 2000).

It is crucial that the hydrodynamic properties of calibration standards are similar to that of the unknown test sample. Characterisation of polymer-peptide conjugates is thus difficult because neither polymer nor protein standards account for hybrid polymer-protein conjugates. In this case, HPMA copolymers are loosely coiled flexible chains and MLT exists in solution as a random coil in equilibrium with its α-helical primary structure. The solution conformation of HPMA copolymer-MLT conjugates is not known, so SDS PAGE was used with reference to free MLT and a range of peptide standards to confirm the high molecular weight properties of prepared MLT conjugates.

Both these methods gave qualitative evidence of the MLT conjugate, therefore the MLT content of the prepared conjugates was quantified using a protein assay (BCA) (Satchi, 1999).

The aim of this study was to synthesise reproducibly HPMA copolymer-MLT conjugates with minimal cross-linking that would retain biological activity. The methods used and further testing of the conjugates is shown in Figure 3.4.

3.2 Methods

Before conjugate synthesis it was necessary to quantify the number of MLT-NH_2 groups available that would contribute to the aminolysis reaction. A colourimetric ninhydrin method was used and this is described in detail in section 2.3.1.1. Although there are 6 NH_2 groups present in the MLT structure, the ninhydrin assay detected only 1.4 (on average). Thus potentially only these groups would be available for reaction with the polymer precursor. The limitation of this assay (section 2.3.1.1) and others used to determine reactive terminal amine groups is that they do not represent the same conditions (pH and ionic strength) as in the conjugation reactions (section 3.2.1) whereby MLT and reactive polymer precursors were dissolved in organic solvent (DMSO). The ninhydrin assay was therefore only a rough estimate of the presence of reactive amine groups (see Table 3.3). To calculate molar ratios for the conjugation reactions, the available number of NH_2 groups as detected by the ninhydrin assay rather than the theoretical "actual" number of NH_2 groups within the known MLT structure was used.

3.2.1 Synthesis of HPMA Copolymer-MLT Conjugates

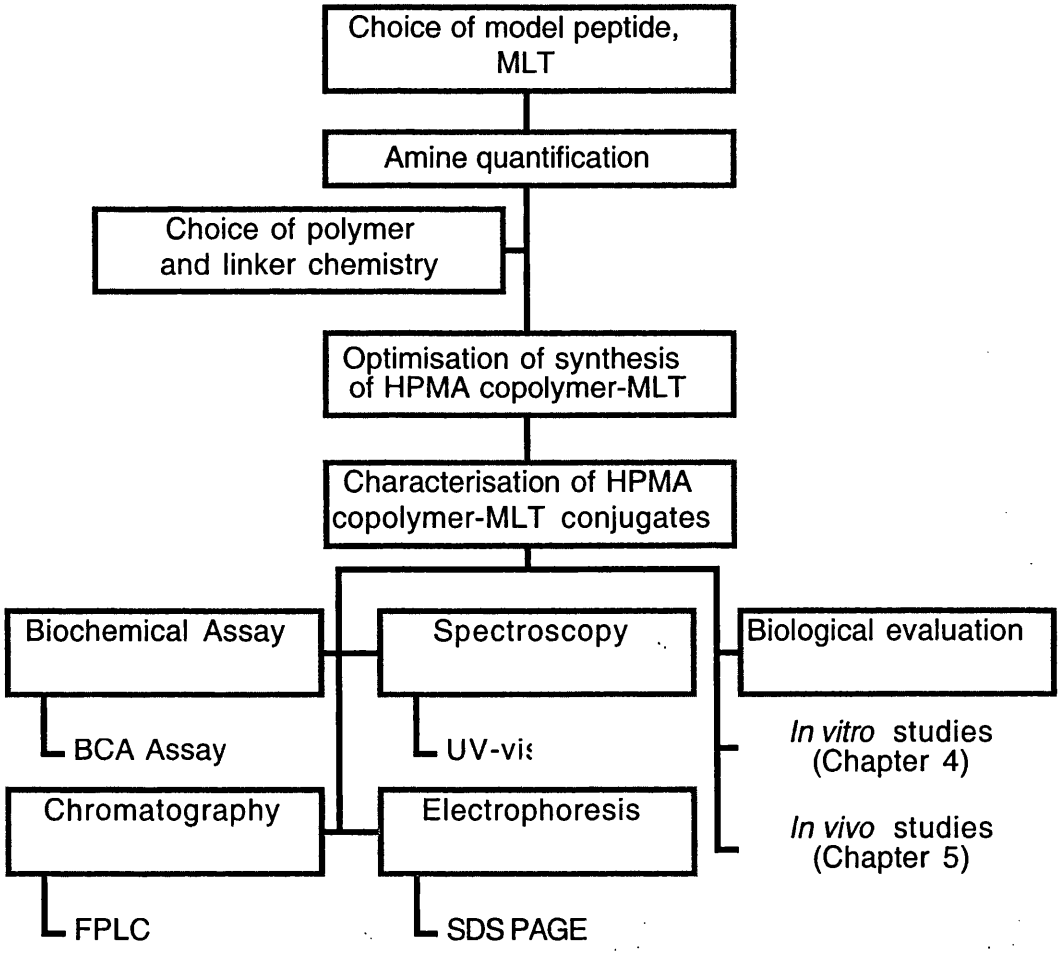
Initial studies synthesised HPMA copolymer-MLT conjugates by aminolysis of an HPMA copolymer-GG-ONp precursor containing 5 mol % side chains (CPT 02).

Optimisation of the Reaction Conditions for Conjugation

A conjugation method with a high yield and minimal cross-linking was sought. To optimise the conjugation method the following parameters were systematically altered:

1) The solvent (DMSO or DDW/borate buffer mixture)
2) The NH_2:ONp molar ratio
3) The reaction time

Figure 3.4 Flow chart summarising synthesis and characterisation of HPMA copolymer-MLT conjugates



The reaction conditions, products arising from these studies and their characteristics are summarised in Table 3.3.

When conjugation reactions were carried out in aqueous conditions (DDW/borate buffer), aminolysis and simple hydrolysis of the reactive ONp ester occurred as competing reactions (Figure 3.5). As the concentration of the nucleophiles (OH^- and NH_2 groups) depends on the pH of the reaction, the pH was raised to 8.5 which is closer to the theoretical pKa value of pendant MLT-NH_2 (provided by lysine (K) and arginine (R)) in order increase their relative nucleophilicity. In theory this should maximise the yield of amide over acid formation (Ríhová & Kopecek, 1985 and Satchi, 1999).

A Typical Reaction in Aqueous Solution

HPMA copolymer-ONp (1 mg/ml in DDW) was added drop-wise to MLT (1 mg/ml in 0.1 M PBS) under careful stirring at 4 °C. The pH of the reaction was raised to pH 8.5 using saturated borate buffer (pH 9) either over 4 h (batch RM 1 and RM 2b) or at the start of the reaction (batch RM 2a). After 4 h the reaction was terminated with the addition of an excess of 1-amino-2-propanol (1 M in DDW; 1ml) which aminolysed any remaining ONp groups from the HPMA copolymer intermediate pendant chains.

When conjugation reactions were carried out in organic solvent, the choice of anhydrous DMSO in order to hinder hydrolysis of the readily reactive ONp ester was possible due to the relatively high solubility (> 5 mg/ml) of both the HPMA copolymer-ONp precursor and free MLT in this solvent. HPMA copolymer-ONp has been previously shown to be stable in DMSO with no detectable ONp release (UV-vis spectroscopy) overnight (Satchi, 1999).

A Typical Reaction in Organic Solvent

To ensure it was free of water, it was important to use anhydrous DMSO stored under nitrogen. HPMA copolymer-ONp (5 mol %) (1 mg/ml in DMSO) was added to MLT (1 mg/ml in DMSO) at varying NH_2:ONp ratios (1:3, 2:3 or 1:5 for batches RM 3 or RM 3a and RM 3b or RM 4a and RM 4b respectively) in a drop-wise manner with gentle stirring at RT for 2 min (batch RM 3a and RM 4a) or 10 min (batch RM 3, RM 3b and RM 4b) so as to minimise potential cross-linking. The reaction was terminated with an excess of freshly prepared 1-amino-2-propanol (1 M in DDW; 1 ml) which

Table 3.3 Summary of optmisation of HPMA copolymer-GG-MLT (5 mol %) conjugation

Batch Number	Reaction Scale† (ONp : NH_2 molar ratio) *	**	Reaction Conditions	Yield‡ (%)	MLT Content (w/w %)	Free MLT¶ (w/w %)
RM 1	1 : 2	1 : 12	Increase pH to 8.5 (borate buffer) over 4 h Reaction time = 4 h at 4 °C	24	19.4	< 0.1
RM 2a	1 : 5	1 : 24	Increase pH to 8.5 (borate buffer) at the start of the reaction Reaction time = 4 h at 4 °C	13.7	27.7	< 0.1
RM 2b	1 : 5	1 : 24	Increase pH to 8.5 (borate buffer) over 4 h Reaction time = 4 h at 4 °C	3.5	8.9	< 0.1
RM 3	1 : 3	1 : 12	DMSO solvent Reaction time = 10 min at RT	40.3	21.8	< 0.1
RM 3a	2 : 3	1 : 6	DMSO solvent Reaction time = 2 min at RT	29.5	22.8	< 0.1
RM 3b	2 : 3	1 : 6	DMSO solvent Reaction time = 10 min at RT	34.8	25.8	< 0.1
RM 4a	1 : 5	1 : 21	DMSO solvent Reaction time = 2 min at RT	16	13.8	< 0.1
RM 4b	1 : 5	1 : 21	DMSO solvent Reaction time = 10 min at RT	15.6	13.5	< 0.1

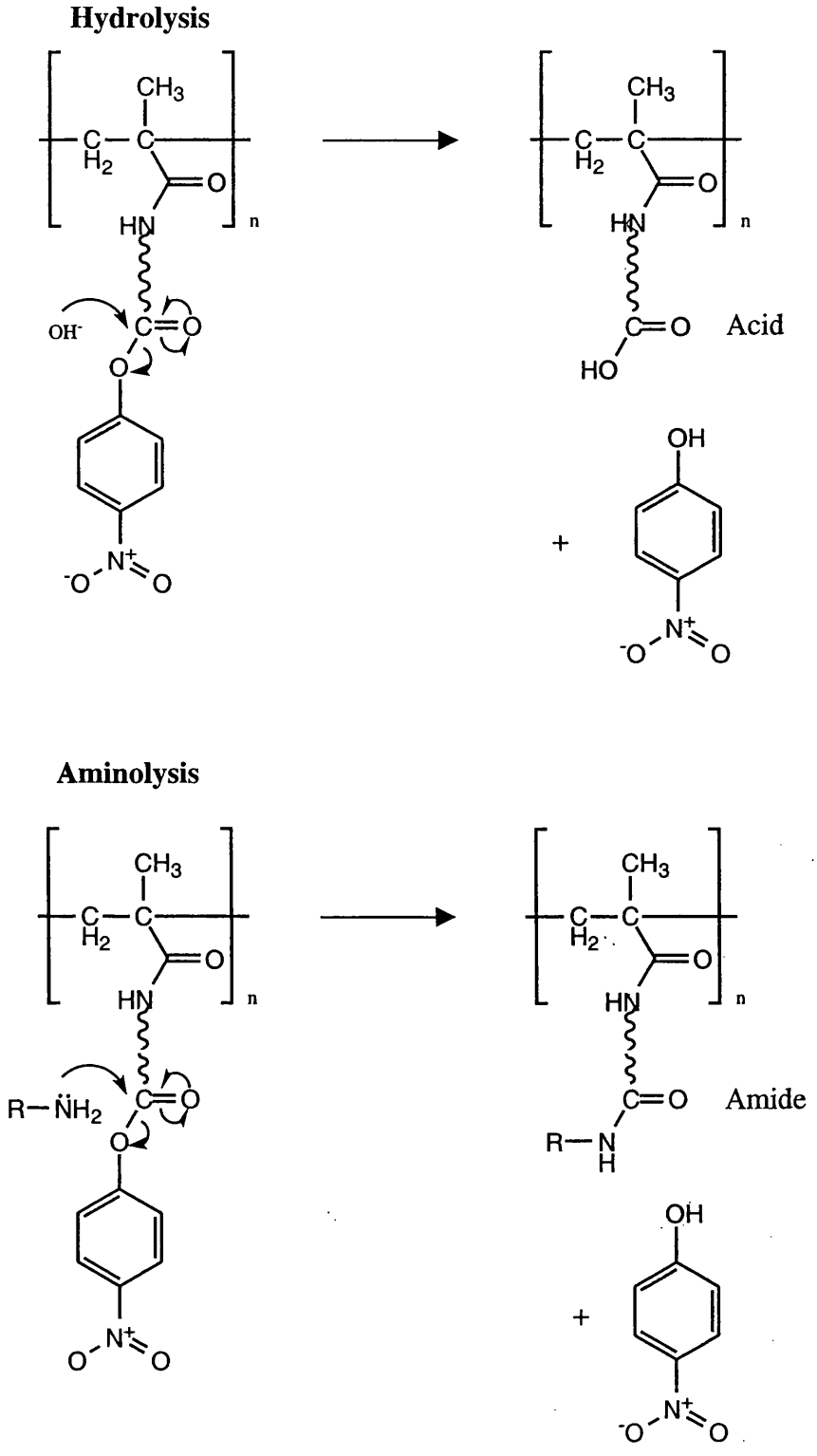
† Reactions carried out with HPMA copolymer-GG-ONp (5 mol %) (CPT 02) (1 mg/ml) and MLT (1 mg/ml), except RM 3 (5 mg/ml)

‡ The yield was expressed as % of MLT bound determined using the BCA assay (Satchi, 1999)

¶ Free MLT was estimated using SDS PAGE

* Number of NH_2 groups available by ninhydrin assay or ** theoretical total number of NH_2 groups from MLT structure

Figure 3.5 Illustration of nucleophilic attack on the ONp ester of HPMA copolymer precursor



R-NH_2 represents MLT

HN ~~~~C=O represents peptidyl spacer (-GG- or -GFLG-)

aminolysed any remaining ONp groups from the HPMA copolymer intermediate pendant chains.

All conjugation reactions (batch RM 1 - RM 4) were carried out with an excess of NH_2 groups relative to ONp groups to minimise potential cross-linking. Conjugation reactions were followed using UV-vis spectrophotometry by monitoring the difference in absorbance between HPMA copolymer-bound ONp (270 nm) and free ONp (400 nm). MLT binding to HPMA copolymer could thus be monitored qualitatively as a function of released ONp.

Standard Conjugation Protocol

From these early experiments it was found that the most effective conjugation method was a modification of Chytry *et al* (1978) (batch RM 3) (Table 3.3). This method was used subsequently to synthesise all HPMA copolymer-MLT conjugates and it is outlined in detail below.

HPMA copolymer-ONp (5 mol %) (5 mg/ml in DMSO) was added to MLT (5 mg/ml in DMSO) in a drop-wise manner with gentle stirring at a molar ratio of 3 NH_2: 1 ONp. A short reaction time of 10 min was used so as to minimise potential cross-linking. The reaction was terminated with an excess of freshly prepared 1-amino-2-propanol (1 M in DDW; 2 ml) which aminolysed any remaining ONp groups from the HPMA copolymer intermediate pendant chains.

All conjugates were purified as described below. As a reference control for biological experiments, it was necessary to prepare a batch of free MLT that was subjected to the same conditions so as to test for possible denaturation of the peptide by DMSO.

3.2.2 Purification of HPMA Copolymer-MLT Conjugates

Removal of free MLT and other low molecular weight compounds such as ONp from the reaction mixture was achieved using either dialysis or ultrafiltration.

Dialysis: First, DMSO was evaporated in a round-bottomed glass flask heated to 40 °C using a paraffin bath attached to a high vacuum pump with a liquid nitrogen trap to retain evaporated solvent. The residual film was then reconstituted in a known volume

of DDW (10 ml) and added into a pre-soaked cellulose-ester membrane of molecular weight cut-off 12 – 14 kDa (Medicell International, UK). This was dialysed against DDW (~ 5 l) for a minimal time period of 72 h with frequent water changes (2 – 3 times a day).

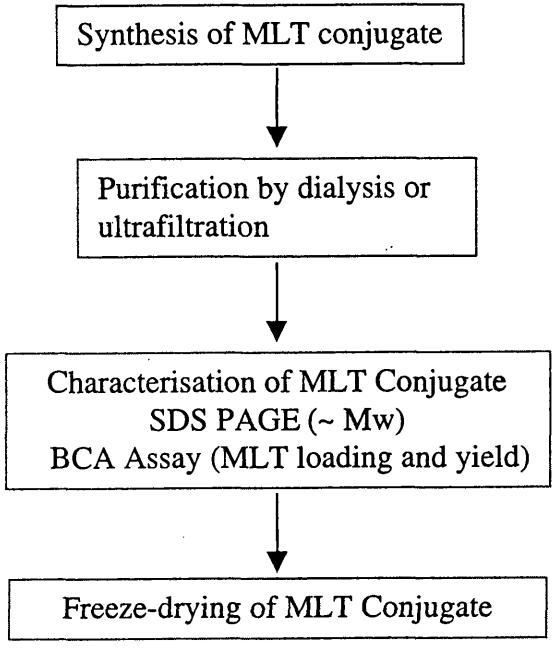
Ultrafiltration: This method used Vivaspin® purification tubes (Viva Science, UK) containing a polyethersufurone membrane of molecular weight cut-off 10 kDa and allowed simultaneous solvent exchange (DMSO with DDW) and removal of low molecular weight contaminants. To avoid membrane denaturation, it was only possible to purify solutions containing a maximum DMSO concentration of 5 % v/v. The crude reaction mixture (1 ml) was placed in the ultrafiltration tubes containing DDW (19 ml) and carefully mixed with a Pasteur pipette and then they were subject to centrifugation (2,500 x g; 30 min). DDW (up to 20 ml) was added to the ultrafiltration tubes and carefully mixed and the centrifugation (2,500 x g; 30 min) repeated until no waste products were detected. The purified sample and filtered low molecular weight products were collected and kept for later analysis by UV-vis spectroscopy to confirm the absence of free MLT or ONp. The resultant purified conjugate was then characterised (SDS PAGE and BCA assay), freeze-dried to a constant mass and stored at – 20 °C.

3.2.3 Characterisation of HPMA Copolymer-MLT Conjugates

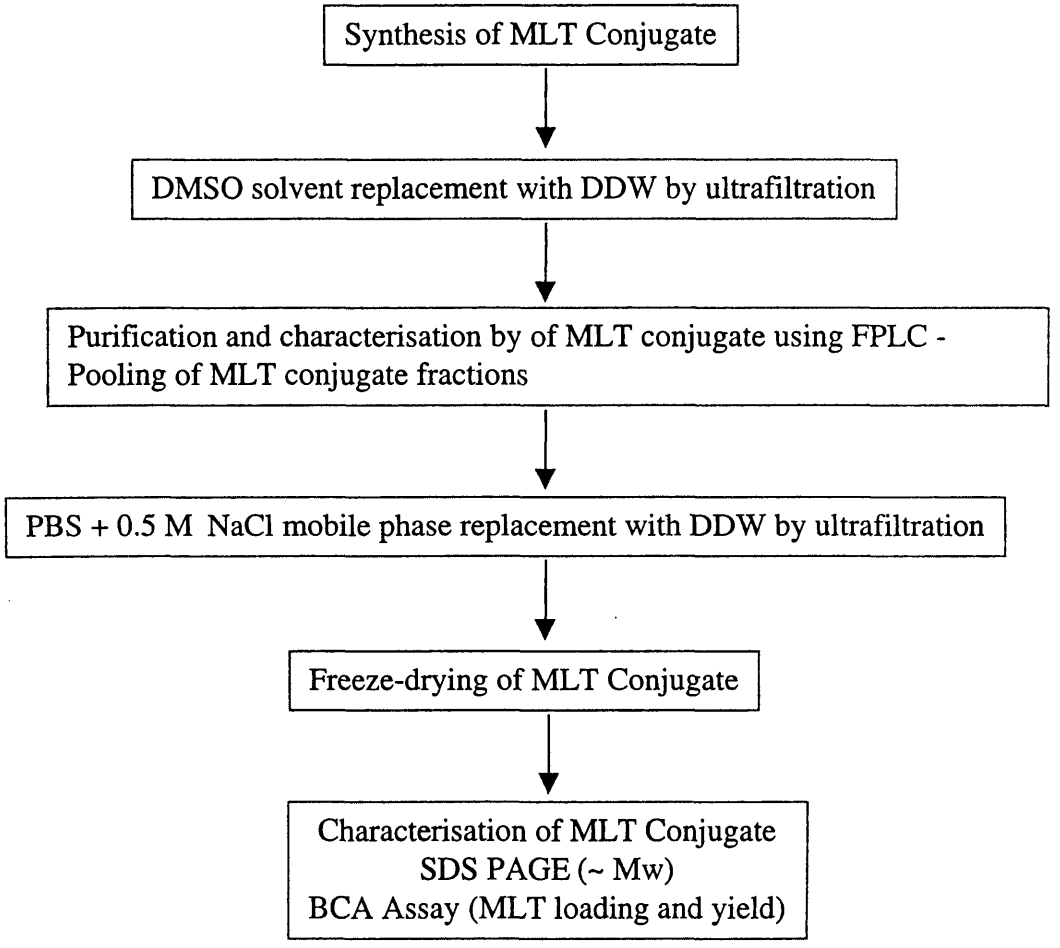
Prior to biological evaluation, HPMA copolymer-MLT conjugates were routinely characterised using the BCA assay and SDS PAGE which are described in detail in section 2.3.1. This protocol was later improved by incorporation of an FPLC step to purify the HPMA copolymer-MLT conjugates (RM 3.14 and RM 7.1). In this case, high molecular weight fractions containing HPMA copolymer-MLT were collected. Salt from the mobile phase (PBS + 0.5 M NaCl) was removed by ultrafiltration and the sample was freeze dried. The final product was then assayed for MLT content using the BCA assay which was calibrated with MLT and aminolysed HPMA copolymer to show that the polymer does not significantly interfere with the assay. A summary of the initial protocol for purification and the current, improved protocol are shown in Figure 3.6.

Figure 3.6 Summary of HPMA copolymer-MLT conjugation methods

Protocol for HPMA copolymer-MLT conjugation



Current protocol for HPMA copolymer-MLT conjugation



3.2.3.1 Purity and Mw Assessment using SDS PAGE and FPLC

Determination of the purity and molecular weight of the prepared conjugates was routinely carried out using a FPLC system (ÄKTA FPLC, Amersham Pharmacia Biotech) and SDS PAGE (see sections 2.3.1.4 and 2.3.1.3 respectively). The FPLC column and SDS PAGE gels were calibrated with a range of peptide standards with known molecular weight.

In the case of FPLC, the V*o* of the column was first determined using blue dextran (5 mg/ml in mobile phase (0.1 M PBS + 0.5 M NaCl)). Then a series of protein standards (5 mg/ml in mobile phase) were used to create a calibration curve expressing log molecular weight against elution volume (V*e*) (Figure 3.7). This was used to estimate the molecular weight of the conjugates.

Free MLT (0.3 - 5 mg/ml), aminolysed HPMA copolymers of different side chain content (1, 5 and 10 mol %) (0.6 - 5 mg/ml) and the purified conjugates (3 - 5 mg/ml MLT-equivalent assuming a maximum % w/w loading) were dissolved in 0.1 M PBS + 0.5 M NaCl buffer and eluted at a flow rate of 0.25 ml/min.

In some cases the FPLC elution profile gave an indication of the molecular weight of the HPMA copolymer-MLT conjugate and its purity (batches RM 3.14 and RM 7.1). Fractions (0.5 ml) containing conjugates were pooled to allow further purification. In this case it was necessary to use ultrafiltration in DDW to remove buffer salt contained in the eluent prior to freeze drying.

3.2.3.2 Determination of the Reaction Yield and MLT Loading using the BCA Assay

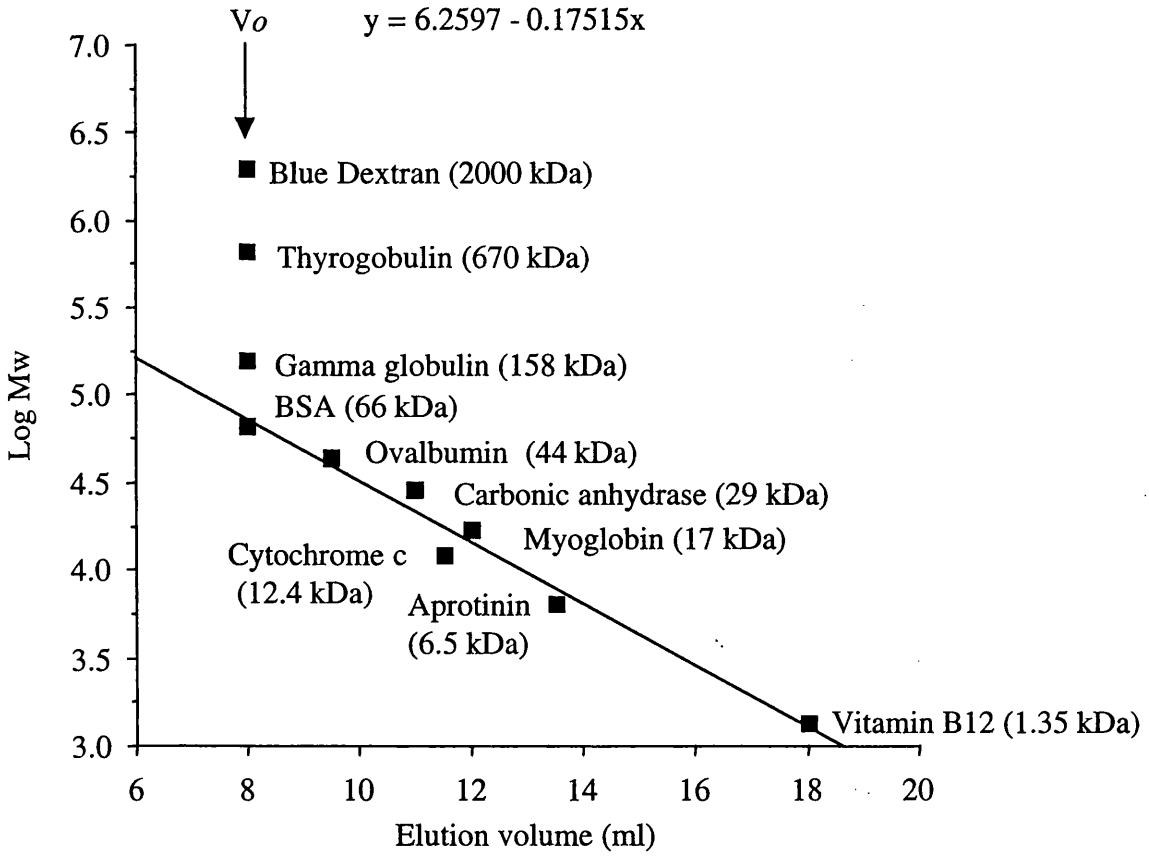
The colourimetric BCA protein assay is described in detail in section 2.3.1.2. It was used routinely to quantify the amount of MLT in the reaction mixture and in the purified conjugate. A MLT calibration curve (see Figure 2.4) was used to calculate the % yield of the reaction. MLT content of the conjugate (% w/w) could then be calculated (n = 2).

3.3 Results

3.3.1 Preparation of HPMA Copolymer-MLT Conjugates

Preliminary experiments with the ninhydrin assay indicated that MLT has 1.40 ± 0.05 (SD) NH_2 groups available for conjugation. The "available" number of NH_2 groups were used in all calculations of NH_2:ONp for all conjugation reactions.

Figure 3.7 Calibration of FPLC column with gel filtration standards (5 mg/ml)



The release of ONp in either aqueous or organic solvent (DMSO) during aminolysis of HPMA copolymer precursor as monitored by UV-vis spectroscopy is shown in Figure 3.8. It can be seen that bound ONp (270 nm) decreased during the course of both reactions, with simultaneous increase of released ONp (400 nm), reaching a peak value with the addition of 1-amino-2-propanol which reacted with any remaining ONp groups.

A UV-vis spectrum for MLT is shown in Figure 3.9. Purification of the reaction mixture proved to be laborious due to the need to replace the organic solvent with DDW as well as the excess MLT in the unpurified reaction mixture. Figure 3.10 shows the UV-vis spectrum of the filtered low molecular weight compounds after ultrafiltration of the reaction mixture. It is evident that the initial absorbance from free MLT (280 nm) and released ONp (400 nm) decreased, indicating progressive purification of the reaction mixture from low molecular weight contaminants.

3.3.2 Characterisation of HPMA Copolymer-MLT Conjugates

FPLC and SDS PAGE were used to assess the purity of the HPMA copolymer-MLT conjugates produced and also to get some indication of their molecular weight.

The FPLC calibration curve obtained with protein standards is shown in Figure 3.7. Here, typical elution profiles obtained for free MLT (Figure 3.11), the aminolysed HPMA copolymer precursors (~ 1, 5 and 10 mol %) (Figure 3.12) and the HPMA copolymer-MLT conjugates (RM 3.14 and RM 7.1) (Figure 3.13) are shown.

It can be seen that the elution profile for free MLT was complicated by a concentration-dependent change in elution time, indicative of aggregation (Table 3.4). However, all MLT concentrations showed separation from either the aminolysed HPMA copolymers and MLT conjugate of various concentrations. The retention time of the aminolysed HPMA copolymers (~ 1, 5 and 10 mol %) and the HPMA copolymer-MLT conjugates corresponded to the V*o* (8 ml). Serial dilutions of prepared MLT conjugates did not show aggregation (data not shown). Thus it was possible to distinguish between free and bound MLT using this FPLC method. The elution of the aminolysed HPMA copolymers (1, 5 and 10 mol %) did not show any concentration dependence (1.25 – 5.00 mg/ml). However, their elution profiles showed slight differences in Mw (see Table 2.1). This method was therefore used to determine the

Figure 3.8 UV-vis spectrum of a HPMA copolymer-MLT reaction (a) in aqueous solvent (RM 1) and (b) in DMSO (RM 6.3)

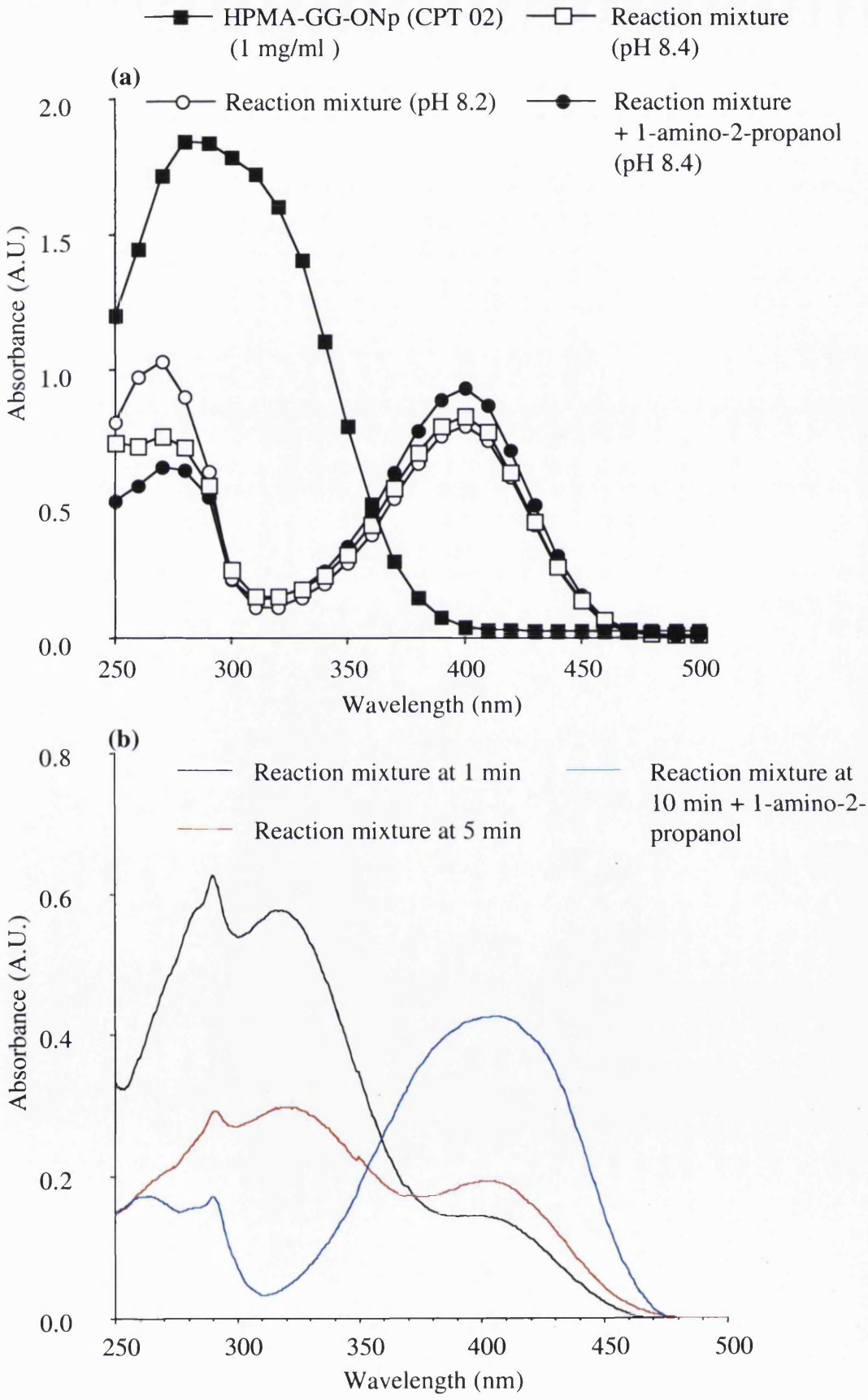


Figure 3.9 MLT UV-vis spectrum. Data represents mean ± SD (n = 3)

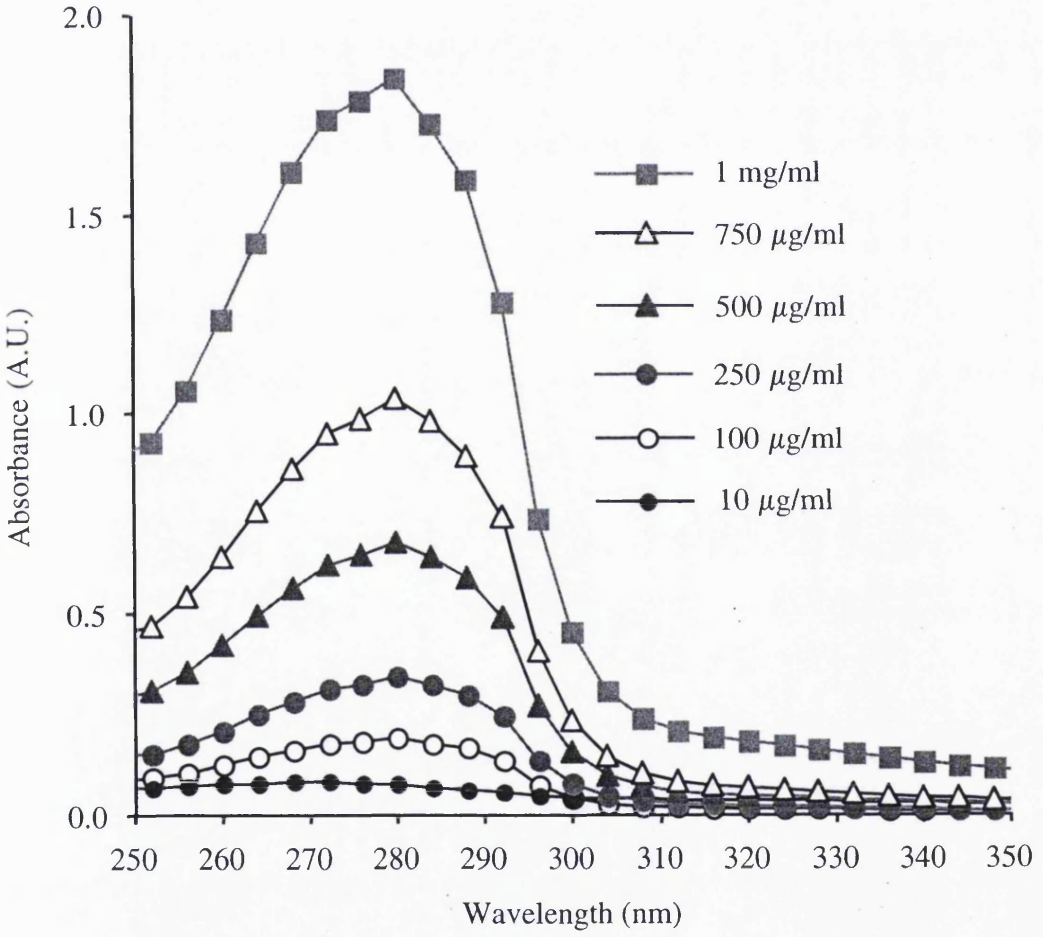


Figure 3.10 UV-vis spectrum of ultrafiltrate of HPMA copolymer-MLT (RM 5.3) reaction mixture showing purification of MLT conjugate with time

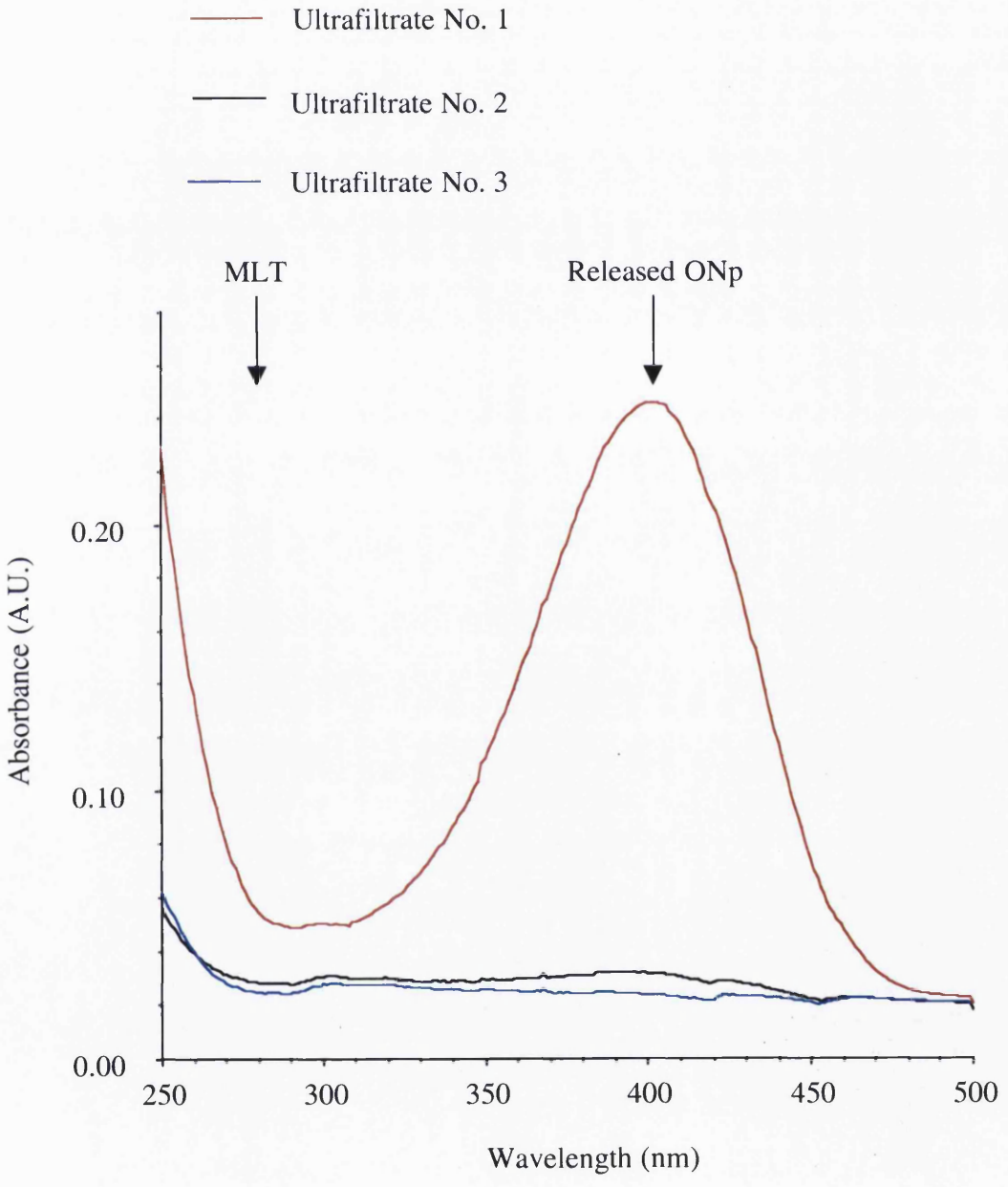


Figure 3.11 Concentration-dependent FPLC elution of free MLT (a) 5mg/ml and (b) 0.3 to 5 mg/ml

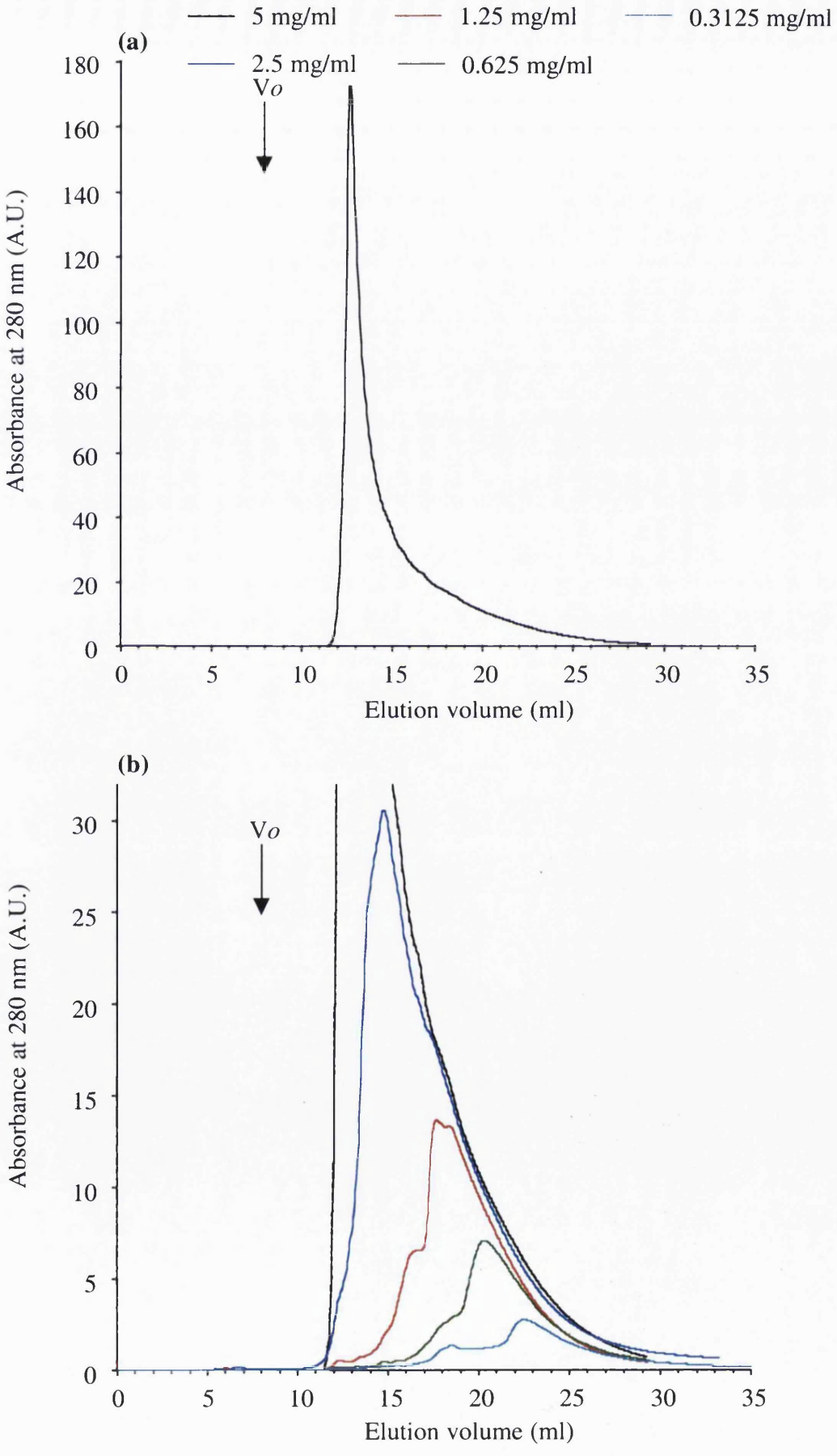
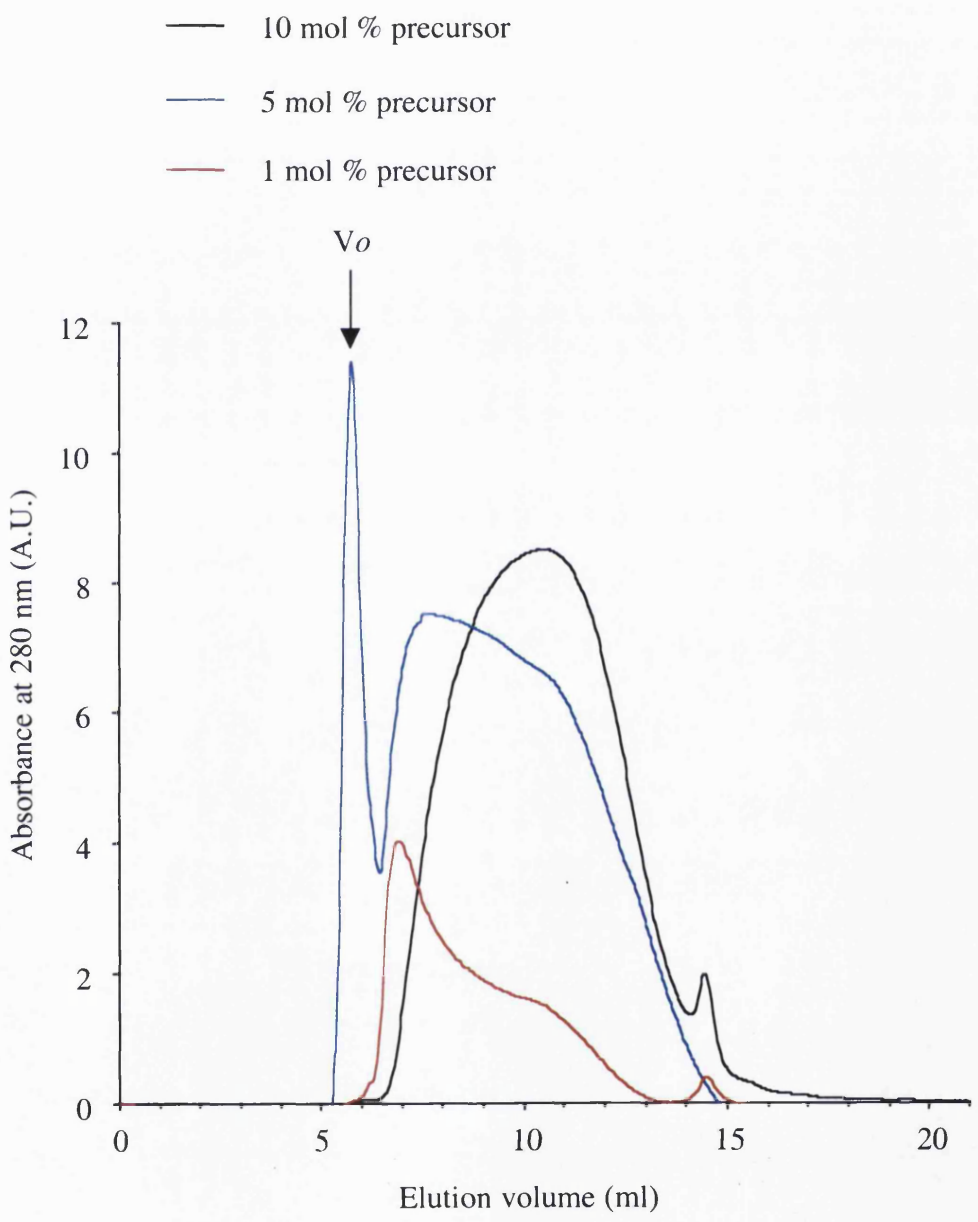
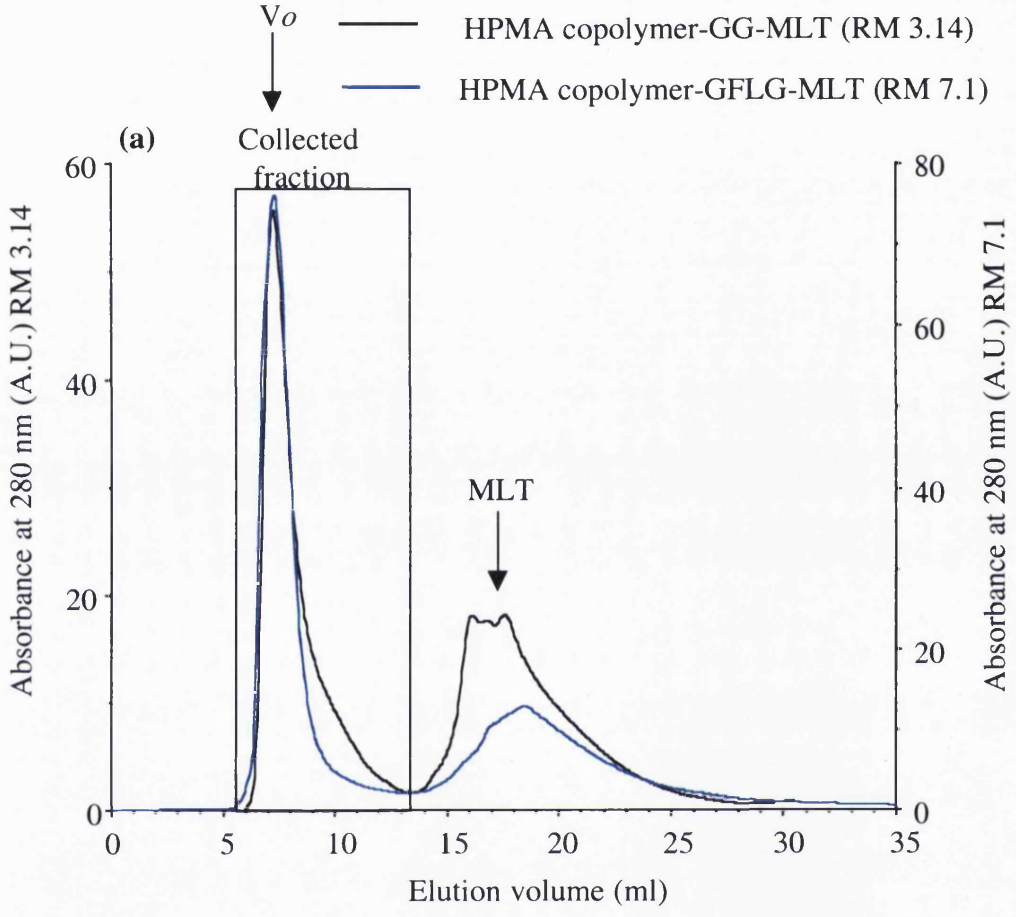


Figure 3.12 FPLC elution of aminolysed HPMA copolymers (5 mg/ml)



Note: The wide elution profiles indicate the high polydispersity of polymer precursors

Figure 3.13 FPLC elution of (a) unpurified HPMA copolymer-MLT (RM 3.14; GG spacer and RM 7.1; GFLG spacer) and (b) purified HPMA copolymer-GG-MLT (RM 3.14)



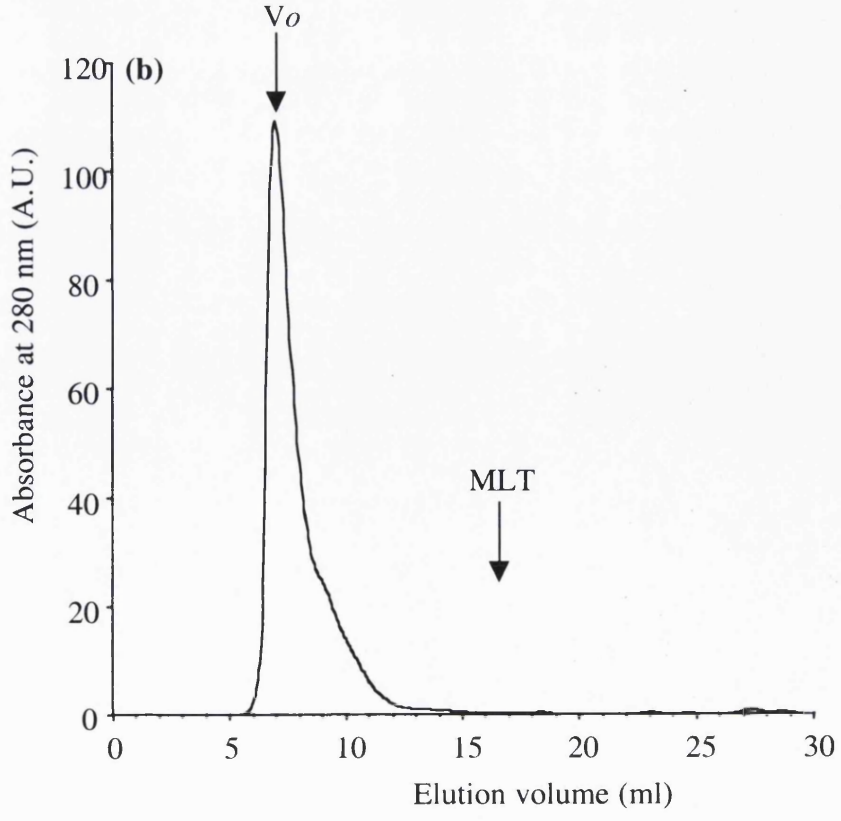


Table 3.4 Estimation of MLT molecular weight after FPLC elution

MLT Concentration (mg/ml)	Calculated Molecular Weight† (kDa)
1.25	1.3
2.5	4.3
5	9.6

† Calculated from the calibration curve equation in Figure 3.7

presence of free MLT in the HPMA copolymer-MLT product and allowed pooling of conjugate fractions, and thus purification of the MLT conjugate.

The SDS PAGE system was calibrated by running different concentrations of free MLT. It was therefore possible to estimate the amount of free MLT in the purified conjugate knowing the limit of detection using silver stain. A typical SDS PAGE trace is shown in Figure 3.14. The Tris-HCl gels showed a HPMA copolymer-MLT band at a molecular weight of 67 – 97.4 kDa when the purified conjugate was applied. No free MLT band was detectable in the purified product with silver stain. As expected, the aminolysed HPMA copolymer gave no visible band with silver stain using the Tris-HCl gels. Using the Tris-tricine gels, free MLT (2.8 kDa) and the unpurified conjugation mixture had a distinct band at 1.4 – 3.5 kDa (data not shown).

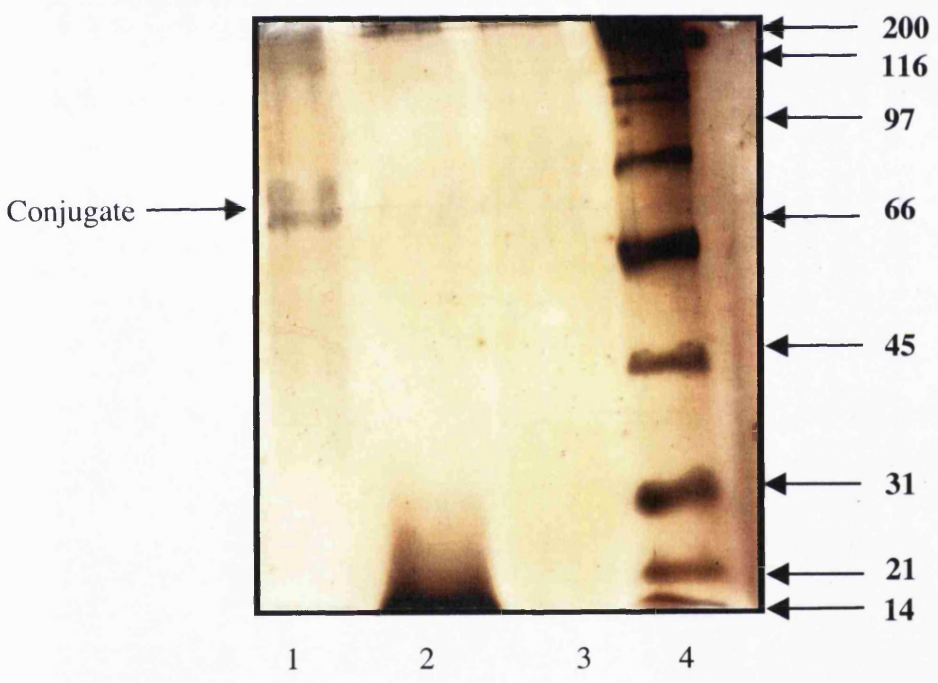
The BCA assay was calibrated with free MLT (see Figure 2.4). It was possible to determine the approximate yield of the reaction by comparing the crude (unpurified) and purified conjugates. The presence of aminolysed HPMA copolymer showed minimal intereference with the assay with a relative contribution of absorbance of not more than 7 % (Figure 3.15). MLT content in a HPMA copolymer-MLT conjugate was calculated assuming no loss of polymer. Using this approach, the calculated maximum content (% w/w) of MLT in the HPMA copolymer-MLT conjugates prepared using HPMA copolymer-ONp (1, 5 and 10 mol %) can be estimated and are shown in Table 3.5.

A summary of all the HPMA copolymer-MLT conjugates prepared in this study is given in Table 3.6. The conjugates prepared with 5 mol % polymer precursor (CPT 02) had an average MLT content of 25.8 ± 6.2 % w/w. As expected, the MLT conjugates prepared with 1 mol % polymer precursor (CPT 09) had a lower loading, with an average MLT content of 12.1 ± 8.6 % w/w. The MLT conjugates synthesised using the 10 mol % polymer precursor (CPT 05) had a higher loading with an average MLT content of 38.9 ± 2.5 % w/w.

3.4 Discussion

Here it was shown that it is possible to synthesise reproducibly HPMA copolymer-MLT conjugates of MLT content approximately 10 – 40 % depending on the precursor used. It is interesting to consider the structure of these HPMA copolymer-MLT

Figure 3.14 SDS PAGE (14 % Tris-HCl) gel to show: Lane (1) MLT conjugate (RM 3), (2) Free MLT, (3) Aminolysed HPMA copolymer precursor (4) MWM



MWM = High molecular weight protein standards (see section 2.1)

Figure 3.15 Calibration curve for the BCA protein assay using either free MLT or aminolysed HPMA copolymer precursor standards. Data represents mean ± SD (n = 4)

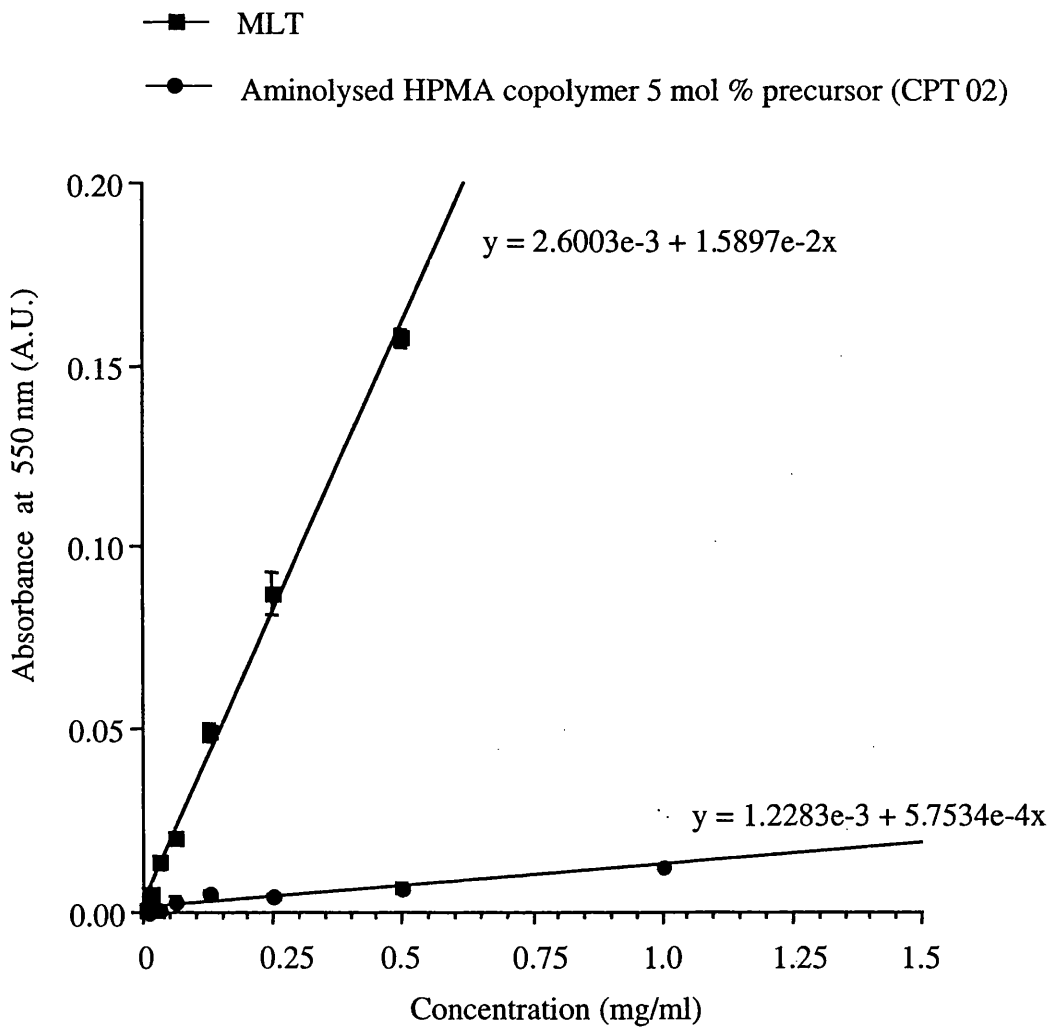


Table 3.5 Maximum theoretical loading of MLT onto HPMA copolymer intermediates

HPMA Copolymer Precursor Reference No.	Side Chain Content (mol %)	No. of ONp per Chain	HPMA Copolymer-MLT Mw[§] (kDa)	MLT Content (% w/w)[§]
CPT 09[†]	1	~ 2	35.7	15.6
CPT 02	5	~ 7	45.4	43.9
CPT 05[†]	10	~ 19	84.1	64.3

[†] Assuming a Mw of 30 kDa
[§] Calculated assuming a 1 : 1 (ONp : NH_2) reaction

Table 3.6 Summary of the characteristics of all HPMA copolymer-MLT conjugates synthesised

Batch Number	HPMA Copolymer Precursor (Linker)	Purification Method	Yield[†] (%)	MLT Loading (% w/w)	Free MLT[‡] (% w/w)
RM 5	1 mol % (GFLG)	Dialysis	39.1	13.9	< 0.1
RM 5.2	1 mol % (GFLG)	Dialysis	41.0	19.6	< 0.1
RM 5.3	1 mol % (GFLG)	Dialysis	14.3	2.7	< 0.1
RM 3.2	5 mol % (GG)	Dialysis	40.3	21.8	< 0.1
RM 3.3	5 mol % (GG)	Dialysis	45.8	31.6	< 0.1
RM 3.4	5 mol % (GG)	Dialysis	46.4	24.3	< 0.1
RM 3.6	5 mol % (GG)	Ultrafiltration	29.9	17.1	< 0.1
RM 3.7	5 mol % (GG)	Ultrafiltration	57.2	31.7	< 0.1
RM 3.8	5 mol % (GG)	Ultrafiltration	56.3	30.1	< 0.1

Batch Number	HPMA Copolymer Precursor (Linker)	Purification Method	Yield† (%)	MLT Loading (% w/w)	Free MLT‡ (% w/w)
RM 3.9	5 mol % (GG)	Ultrafiltration	44.3	33.9	< 0.1
RM 3.10	5 mol % (GG)	Ultrafiltration	33.1	22.3	< 0.1
RM 3.11	5 mol % (GG)	Ultrafiltration	28.5	18.8	< 0.1
RM 3.12	5 mol % (GG)	Ultrafiltration	46.5	41.0	< 0.1
RM 3.13	5 mol % (GG)	Ultrafiltration	39.7	21.5	< 0.1
RM 3.14	5 mol % (GG)	Ultrafiltration and FPLC	37.9	21.7	< 0.1
RM 7.1	5 mol % (GFLG)	Ultrafiltration and FPLC	40.5	34.6	< 0.1
RM 6.2	10 mol % (GFLG)	Ultrafiltration	45.4	37.1	< 0.1
RM 6.3	10 mol % (GFLG)	Ultrafiltration	48.1	40.6	< 0.1

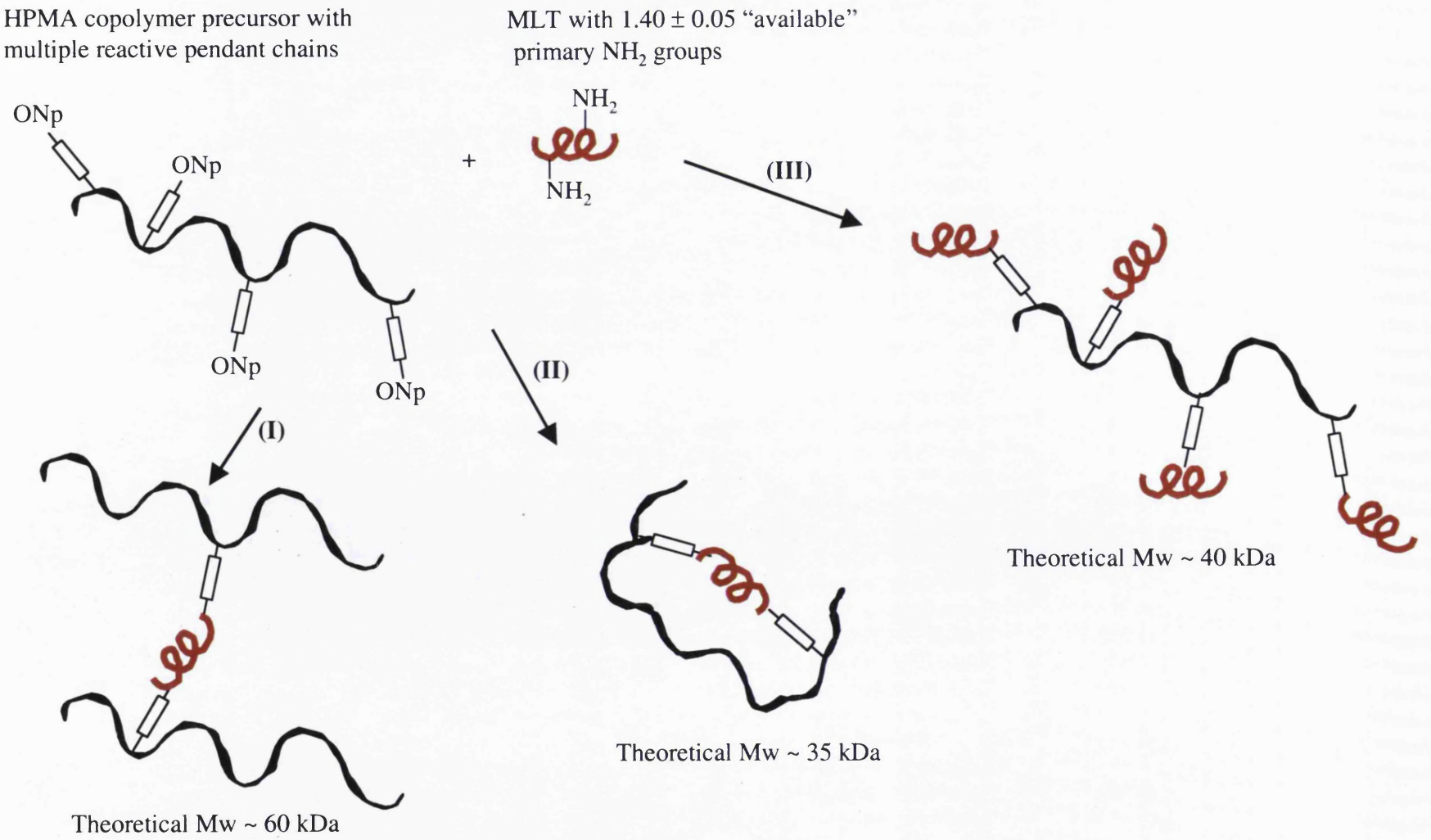
† The yield was expressed as % of MLT bound determined using the BCA assay
‡ Free MLT was estimated using SDS PAGE

conjugates. HPMA copolymer-ONp precursors contain multiple pendant side chains that can potentially react with MLT. Therefore the products may have different theoretical structures as shown in Figure 3.16. The MLT-NH_2 groups present in the peptide chain (ε-NH_2 of K and R) and the terminal α-NH_2 should be potent nucleophiles in their non-ionised state (Hermanson, 1996). However, in general, it is known that the reactivity of NH_2 groups within a peptide or protein is highly dependent on the individual pKa values (Hermanson, 1996) and the conformation. The location of NH_2-carrying residues within the polypeptide α-chain will determine the accessibility to a reactive group. This is particularly important when binding to a high molecular weight reactive polypeptide.

The ninhydrin assay indicated the availability of 1 - 2 NH_2 groups per molecule of MLT. This was not surprising considering the α-helical conformation of this relatively small peptide (26 residues) that has hydrophobic residues buried in the core and hydrophilic ones on the surface. The pKa in turn, determines the charge and thus the reactivity of any surface pendant NH_2 groups. When incubated at a pH < pKa amines exist in their protonated state (HN^{3+}), whereas at a pH > pKa they exist as in their "electron rich" unionised state (NH_2). It is of noteworthy interest, however, that the reagent conditions in the ninhydrin assay provided a different environment to that in the conjugation reactions and thus altered MLT conformation. However, this was the only means of assessing the reactive terminal groups. Table 3.3 therefore documents optimisation of the synthesis of the HPMA copolymer-MLT conjugates using different molar ratios of MLT as determined by the ninhydrin assay and also from the theoretical structure.

The reactive ONp ester of the HPMA copolymer intermediates provided a site for aminolysis by MLT's reactive pendant NH_2 groups whose unshared electrons impart their potent nucleophilic character. The ONp ester is a good leaving group and has been widely used in polymer-peptide or polymer-protein conjugation reactions (Chytry *et al*, 1978; Morgan, 1993; Satchi, 1999; Zalipsky *et al*, 1999; Ulbrich *et al*, 2000). Aminolysis is a random reaction, leading to the potential for multiple derivatisation and thus heterogeneity of the end product. This is discussed further in Chapter 7.

Figure 3.16 Illustration of the theoretical structures of HPMA copolymer-MLT



As Chytry *et al* (1978) had shown, the molecular weight of HPMA copolymer-GG-insulin conjugates and degree of cross-linking could be controlled by synthesis in DMSO and by controlling the ratio of NH_2:ONp ester and reaction time. During the synthesis of HPMA copolymer-MLT a similar procedure was used here. An excess of MLT-NH_2 groups (3 NH_2: 1 ONp) and a short reaction time (10 min) was used to restrict conjugation to the more reactive MLT-NH_2 groups and minimise cross linking.

Importantly, the chosen conjugation method had been shown to conserve activity of the parent protein, insulin (Chytry *et al*, 1978). This was later found to be true for HPMA copolymer-MLT (Chapters 4 and 5). Other methods for conjugation of HPMA copolymers to peptides and proteins have not been so successful. Flanagan (1987) bound HPMA copolymer chains to transferrin and the antitransferrin receptor antibody McAb B3/25 and found that the 10 - 20 polymer molecules bound per protein decreased binding to the transferrin receptor. Similarly, when Satchi *et al* (2001) synthesised HPMA copolymer conjugates of the enzymes β-lactamase or cathepsin B, reactions with a high yield (63 - 82 % of protein bound) diminished enzyme activity, whilst reactions with a lower yield (30 - 35 % of protein bound) retained enzyme activity (20 - 25 %). Morgan (1993) used a molar excess of ONp : NH_2 (2 : 1) when conjugating a HPMA copolymer precursor containing side chains 4.5 mol % ONp to peptides including peptide 525 and insulin β-chain. These conditions were chosen with the hope of achieving 100 % coupling to avoid the need for further purification. Although this method achieved a good loading (24 - 36 % w/w peptide incorporation), the degree of cross-linking was increased.

In these experiments, HPMA copolymer-MLT conjugates bearing peptidyl –GG- or –GFLG- linkers were prepared for comparison of *in vitro* activity of conjugates (Chapter 4). In future it would be interesting to synthesise and investigate the structure-activity relationships of a series of peptidyl (e.g –G-, -GG-, -GGG-, -GGGG-) and carbonyl linkers.

Characterisation of the HPMA copolymer-MLT conjugates was not easy. SDS PAGE and FPLC proved to be useful tools to provide evidence of MLT conjugation and also showed free MLT remaining in the conjugate. FPLC was an additionally useful purification method. SDS PAGE and FPLC suggested the presence of multiple species in each batch of conjugate. This was probably due to the multivalency of both

the HPMA copolymer-ONp precursor and MLT itself. It is clear that this could lead to a variety of products.

As mentioned earlier, it is impossible to obtain absolute molecular weight values for HPMA copolymer-MLT using polymer or protein standards. A dimerisation of such conjugates could give the higher molecular weights seen on both SDS PAGE and FPLC. The precise structure of HPMA copolymer-MLT is therefore not known. However, a conjugate molecular weight of ~ 60 kDa could represent two HPMA copolymer chains (each ~ 30 kDa) bound to a single MLT molecule (2.8 kDa). The ~ 20 % w/w MLT content of conjugate frequently seen is suggestive of a single polymeric chain (~ 30 kDa) bound to four MLT molecules. Further resolution of the purified conjugates could be obtained by a sequential SEC run through a column of narrower range of molecular weights and thus fractionation of conjugates into a less polydisperse mixture.

MLT content of the conjugates as expected, reflected the side chain content of the respective precursors used. Conjugates bearing 1 mol % peptidyl side chains had a lower loading (12 % w/w), whereas conjugates with 10 mol % side chains had a higher loading (39 % w/w) relative to the standard conjugates with 5 mol % side chains (26 % w/w). The HPMA copolymer-GFLG-MLT (RM 7.1) had the highest MLT loading (34.6 % w/w) of the conjugates prepared with HPMA copolymers containing 5 mol % reactive side chains. It is not clear, however, if this was an effect of the longer peptidyl spacer as only one batch of this kind was made. It would be of interest therefore to investigate this further.

Using FPLC, it was evident that increasing concentrations of MLT (but not of aminolysed copolymers) had decreasing retention time (Figure 3.11). This might be explained as MLT in solution exists as an equilibrium of the monomer and a tetramer. The equilibrium is known to shift towards the tetramer with increasing concentration, pH and ionic strength (reviewed by Dempsey, 1990).

It should also be noted that it was not possible to use the FPLC column described here to distinguish between aminolysed HPMA copolymer (a by-product of the conjugation reaction) and HPMA copolymer-MLT conjugate due to the poor resolution of the column. However, during biological evaluation of the MLT conjugates, to check for the effects of any aminolysed polymer present, experiments

were carried out with the aminolysed polymer present in the final conjugate (Chapter 4).

Wen *et al* (1996) and Ulbrich *et al* (2000) have used SEC together with light scattering, UV-vis and refractive index (RI) detectors in an attempt to get a more accurate measurement of molecular weight and stoicheiometry of polymer-protein conjugates. Unfortunately this technique was not available in our laboratory. As an alternative to the BCA assay, amino acid analysis of the conjugates has been used to quantify protein content (Oupicky *et al*, 1999). This may be achieved by hydrolysis of the protein or peptide conjugate by heating with 6 M HCl for 24 h at 110 °C *in vacuo*. The resultant amino acid mixture may then be identified chromatographically (e.g. ion-exchange chromatography or reverse phase-HPLC (RP-HPLC)) and quantified using a post-column derivitisation (e.g. using ninhydrin to obtain coloured products or fluorescamine to yield fluorescent products) (Mathews & van Holde, 1990).

Novel conjugation chemistry technologies which convey site-specific coupling of macromolecular substrates to peptides and proteins are emerging with the aim to generate a homogeneous conjugate of well-defined composition which is of paramount importance for their application as therapies. This topic is addressed in detail in Chapter 7.

It was considered important to establish if the methods used to prepare HPMA copolymer-MLT conjugates resulted in compounds with retained biological activity. Providing the HPMA copolymer-MLT conjugates were pure, they could be used for *in vitro* (Chapter 4) and *in vivo* (Chapter 5) studies (Table 3.7).

Table 3.7 Summary of the characteristics of all HPMA copolymer-MLT conjugates that were subsequently used in biological experiments

Batch number	HPMA Copolymer Precursor	Peptidyl Linker	Yield (%)	MLT Content[†] (% w/w)	Study Conducted
RM 5.3	1 mol % (CPT 09)	GFLG	14.3	2.7	*In vitro* haemolysis and cytotoxicity assays - effect of loading (Chapter 4)
RM 6.3	10 mol % (CPT 05)	GFLG	45.5	40.6	*In vitro* haemolysis and cytotoxicity assays - effect of loading (Chapter 4)
RM 3.2	5 mol % (CPT 02)	GG	40.3	31.6	*In vitro* haemolysis and cytotoxicity assays (Chapter 4)
RM 3.13	5 mol % (CPT 02)	GG	39.7	21.5	*In vitro* haemolysis and cytotoxicity assays - effect of loading (Chapter 4)
RM 7.1	5 mol % (CPT 03)	GFLG	40.5	34.6	*In vitro* haemolysis assays – effect of peptidyl linker (Chapter 4)
RM 3.4	5 mol % (CPT 02)	GG	46.4	24.3	*In vivo* determination of MTD (Chapter 5) *In vivo* biodistribution study (i.p.) (Chapter 5)
RM 3.7	5 mol % (CPT 02)	GG	67.2	31.7	*In vivo* single dose antitumour study (i.p.) (Chapter 5)
RM 3.9	5 mol % (CPT 02)	GG	74.3	33.9	*In vivo* biodistribution study (i.p.) (Chapter 5) *In vivo* multidose antitumour study (i.p.) (Chapter 5)
RM 3.14	5 mol % (CPT 02)	GG	37.9	21.7	*In vivo* antitumour study (i.v.) (Chapter 5)

[†] All conjugations were carried out with with MLT (M 7391) except RM 3.13, RM 3.14, RM 7.1, RM 5.3 & RM 6.3 (M 2272)
[‡] Determined using the BCA protein assay.

Chapter Four

Biological Evaluation of HPMA Copolymer-MLT In Vitro

4.1 Introduction

As described in Chapter 1 (sections 1.6.1 and 1.6.2), MLT is a potent non-specific membrane active agent with antitumour activity. Therefore prior to evaluation of the HPMA copolymer-MLT conjugates as potential anticancer agents *in vivo*, it was necessary to establish whether the conjugate cytolytic activity was retained and also to determine if it was non-specifically toxic. A secondary aim of this study was to investigate the effect of MLT loading on the haemolytic and cytotoxic properties of the HPMA copolymer-MLT conjugates to allow optimisation of a candidate for subsequent *in vivo* experiments (Chapter 5).

The desired biological and physicochemical properties of an ideal polymeric carrier have already been described in detail (Chapters 1 and 3). If HPMA copolymer-MLT is to be administered intravenously as a antitumour therapeutic which will target tumours by the EPR effect, it is essential that it displays minimal haematoxicity en route to its site of action i.e. the tumour. However, once in the tumour, the HPMA copolymer-MLT conjugate must be cytotoxic. In this study, the haemolytic activity of HPMA copolymer-MLT conjugates compared to free MLT and the aminolysed HPMA copolymer precursor was evaluated using a RBC lysis assay. The cytotoxicity of the MLT conjugates and the same reference controls was assessed using the B16F10 murine melanoma cell line.

It should be noted that these *in vitro* model systems represent a gross simplification of the *in vivo* situation. They lack the complexity of multicomponent physiological processes and thus the data obtained will only give a possible indication of the *in vivo* situation. Nonetheless, *in vitro* evaluation was still an essential preliminary study before carrying out *in vivo* evaluation of the prepared MLT conjugates (Chapter 5).

In addition to haemolysis, blood compatibility is of paramount importance for effective systemic administration of any DDS (Duncan *et al*, 1991). Other complex bio-interactions can occur after administration of a DDS such as complement activation, coagulation, platelet aggregation or stimulation of an immunogenic response. This topic has been reviewed by Ríhová (1996) and Janatova (2000).

For these studies, a simple haemolysis assay was chosen because MLT is very haemolytic. Haemolysis of intact RBCs may be assayed as a function of K^+ or haemoglobin (Hb) release (Domurado *et al*, 2001). The RBC lysis (RBCL) assay (Hb release) has been standardised in our research group as a screening tool to study polymer-membrane interactions with many different polymers and polymer-drug conjugates (Malik, 1999; Richardson, 1999; German, 2001; Keane, 2002). The pH-dependance of lytic activity of polymeric compounds has also been reported (Duncan *et al*, 1994 and Richardson, 1999). The RBCL assay (Hb release) has also been widely used to investigate MLT-membrane interactions (DeGrado *et al*, 1982; Tosteson *et al*, 1985; Katsu *et al*, 1988).

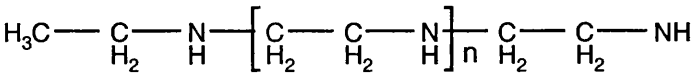
Traditionally, when the RBCL assay has been used to assess haemolytic activity of polymers and polymer-drug conjugates it has been standardised using poly(ethyleneimine) (PEI) as a positive control. Due to its cationic nature, PEI is extremely membrane active (Nevo *et al*, 1955; Katchalsky *et al*, 1959; Domurado *et al*, 2001; Figure 4.1 a). Dextran has been used as the polymer of choice for a negative reference control polymer in this model (Duncan *et al*, 1991) (Figure 4.1 b). In addition, the aminolysed HPMA copolymer (with 1-amino-2-propanol), a by-product of HPMA copolymer-MLT conjugation reactions (Chapter 3) was also used as a reference control to confirm any membrane damage to be solely due to conjugated MLT.

Various methods are routinely used to assess cell viability. These include the ability of cells to incorporate radioactive precursors of DNA or proteins e.g. [^{3}H]-thymidine (Eli *et al*, 2001) or [^{14}C]-leucine (Kubo *et al*, 1999) and cell exclusion of dyes as an indication of membrane integrity e.g. alamar blue (Gloeckner *et al*, 2001) and trypan blue (Kubo *et al*, 1999). The antibacterial activity of MLT has also been widely assessed using bacterial cultures and minimum inhibitory concentration values (MIC) determined (Blondelle & Houghten, 1991).

In this study, the MTT assay was chosen as a preliminary screen for tumour cell kill induced by free MLT and the MLT conjugates. This assay has been used to determine the cytotoxicity of many polymer-drug conjugates and potential polymeric carriers (Sgouras & Duncan, 1990). Advantages of the MTT assay include its simplicity, reproducibility and the assay does not use radioisotopes (Sgouras & Duncan, 1990).

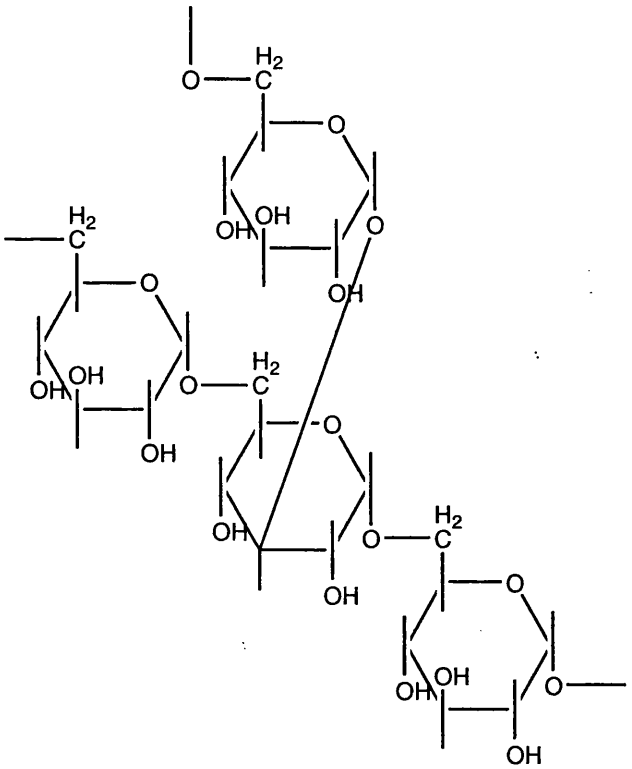
Figure 4.1 Structures of (a) PEI and (b) dextran

(a)



Mw 750 KDa

(b)



Mw 74 KDa

In parallel with the above-mentioned RBCL and MTT assays, it was considered important to visualise any changes in cell morphology. Scanning electron microscopy (SEM) of RBC and B16F10 cells exposed to free MLT or the HPMA copolymer-MLT conjugates was undertaken (Katchalasky *et al*, 1959; Malik, 1999; Richardson, 1999).

4.2 Methods

A detailed description of the RBCL protocol is documented in section 2.5. The first objective of the studies was to compare the activity of free MLT and its conjugate. It should be noted that MLT exposed to the reaction conditions and experimental conditions of the MLT conjugate was used as a reference in these studies. The haemolytic activity of HPMA copolymer-MLT conjugate (~ 5 mol % side chains; GG linker) (RM 3.2, 31.6 % w/w) (up to 110 μg/ml MLT-equivalent) and the reference compounds free MLT and the HPMA copolymer precursor aminolysed with 1-amino-2-propanol was determined. In some cases, dextran and PEI were also used as reference compounds.

A second series of experiments set out to investigate:

1) The effect of MLT content on the haemolytic activity of HPMA copolymer-MLT conjugates
2) The effect of HPMA copolymer-MLT linker (GG or GFLG) on haemolytic activity of the conjugates

The conjugates (up to 100 μg/ml MLT-equivalent):

- HPMA copolymer-MLT (1 mol %; GFLG linker) (2.7 % w/w) (RM 5.3)
- HPMA copolymer-MLT (5 mol %; GFLG linker) (34.6 % w/w) (RM 7.1)
- HPMA copolymer-MLT (5 mol %; GG linker) (21.5 % w/w) (RM 3.13)
- HPMA copolymer-MLT (10 mol %; GFLG linker) (40.6 % w/w) (RM 6.3)

and reference compounds: HPMA copolymer aminolysed with 1-amino-2-propanol and free MLT were used in these studies. In this case, the incubation time was 10 min.

Experiments were also undertaken to visualise RBC by SEM after incubation of RBC with free MLT, MLT conjugate and aminolysed polymer at concentrations equivalent to their Hb_{50} values as determined in the RBCL studies. The protocol is described in detail in section 2.6.

An MTT assay was used to determine the cytotoxicity of the MLT conjugates and free MLT. The general protocol is described in section 2.4.3. Again, the first objective was simply to compare the cytotoxicity of MLT and the conjugate. The test compounds used included HPMA copolymer-MLT (~ 5 mol % side chains; GG linker) (RM 3.2, 31.6 % w/w) (up to 110 μg/ml), and as reference compounds free MLT (exposed to the reaction and purification conditions) and HPMA copolymer aminolysed with 1-amino-2-propanol.

In addition, a second set of studies were carried out to investigate the effect of MLT content of HPMA copolymer-MLT conjugates on cytotoxic activity. The conjugates and reference compounds used for RBCL assay were also used for these cytotoxicity studies.

The B16F10 cells were again visualised using SEM. In this case, MLT, HPMA copolymer-MLT conjugates and aminolysed polymer were incubated with cells at their respective IC_{50} values. The SEM protocol used for B16F10 cells is given in section 2.6.

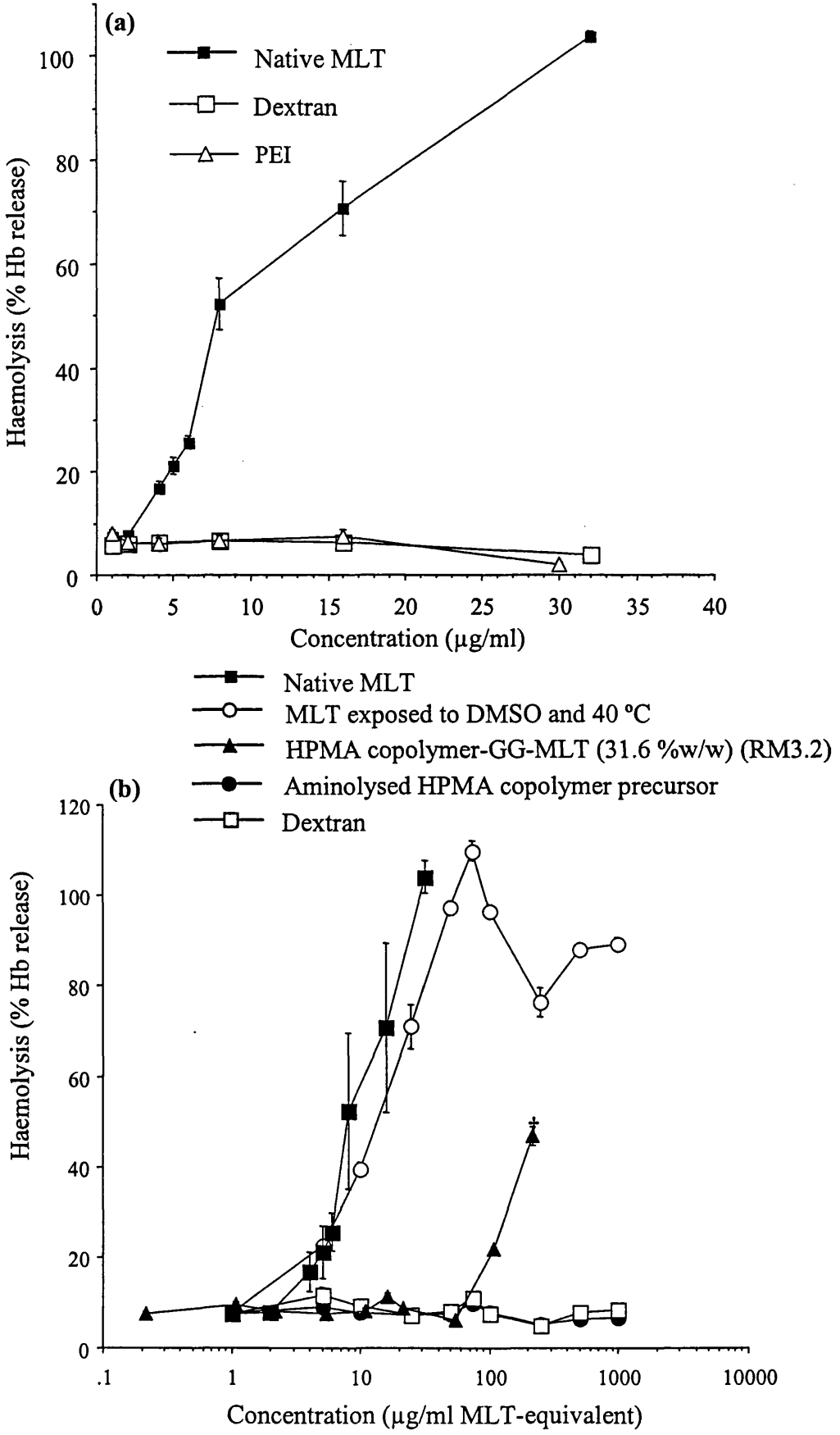
4.3 Results

4.3.1 Haemolytic Activity of MLT and HPMA Copolymer-MLT Conjugates

Free native MLT was found to be very haemolytic. It had a Hb_{50} value at 1 h of 8.0 ± 4.5 μg/ml (Figure 4.2 a). When MLT was exposed to the reaction and purification conditions of the MLT conjugate i.e. DMSO and high temperature (40 °C), the haemolytic activity was similar (*p* value 0.5), with a Hb_{50} value (1 h) of 10.8 ± 3.8 μg/ml (Figure 4.2 b). At MLT concentrations above 60 μg/ml, haemolysis was found to decrease to ~ 75 %. This was resultant of Hb precipitation, and thus a disadvantage of this type of assay. It should be noted that PEI was found to be non-toxic at concentrations up to 30 μg/ml (Hb_{50} 1 mg/ml; Malik, 1999). The MLT Hb_{50} value at 10 min (9.8 ± 0.8 μg/ml) was found to be similar to that at 1 h (*p* value 0.5) (Figure 4.3). In contrast, the MLT conjugate RM 3.2 (31.6 % w/w) displayed a greatly reduced haemolytic activity (*p* value < 0.0005) with a Hb_{50} value (1 h) of 192.3 ± 29.2 μg/ml (Figure 4.2 b). The aminolysed HPMA copolymer, like dextran, as would be expected, was found to be non-lytic to RBCs at concentrations up to 1 mg/ml (Figure 4.2 b).

The Hb_{50} values (10 min) obtained when RBC were incubated with the HPMA copolymer-MLT conjugates of different MLT content and different peptidyl spacers are shown in Table 4.1. When HPMA copolymer-MLT conjugates with the same GFLG

Figure 4.2 Haemolytic activity of (a) MLT and (b) HPMA copolymer-MLT (RM 3.2) after 1 h incubation. Data represents mean ± SE (n = 48 and n = 12 respectively)



†Note: Not enough conjugate (batch RM 3.2) to go beyond 200 μg/ml MLT-equivalent

Table 4.1 Haemolysis caused by MLT and HPMA copolymer-MLT conjugates of different MLT content. The data represents mean ± SD (n = 18), except RM 3.2 (n = 12).

Compound	MLT content (% w/w)	Peptidyl Linker	Batch	Hb_{50} value (μg/ml)†
MLT	-	-	Native	8.0 ± 4.5§
MLT*	-	-	Exposed to DMSO and 40 °C	10.8 ± 3.8§
MLT*	-	-	Exposed to DMSO and 40 °C	9.8 ± 0.8‡
PEI¶	-	-	See section 2.1	1,000
HPMA copolymer-MLT	2.7	GFLG	RM 5.3	39.9 ± 15.8‡
HPMA copolymer-MLT	31.6	GG	RM 3.2	192.3 ± 29.2§
HPMA copolymer-MLT	21.5	GG	RM 3.13	19.9 ± 6.2‡
HPMA copolymer-MLT	34.6	GFLG	RM 7.1	12.0 ± 1.3‡
HPMA copolymer-MLT	40.6	GFLG	RM 6.3	13.1 ± 4.5‡

Haemolysis was assessed after a 10 min‡ or 1 h§ incubation with RBC (2 % w/v).

† All concentrations are presented in MLT-equivalent

¶ Taken from Malik, 1999

linker but different MLT loadings were compared it was found that the conjugate with the lowest MLT loading (2.7 wt % bound to ~ 1 mol % side chains) was less haemolytic than those conjugates with a higher MLT content (Table 4.1 and Figure 4.3 a). The MLT conjugates studied were haemolytic in the order RM 7.1^{NS} and RM 6.3^{NS} > RM 3.13* and RM 5.3* (* p value < 0.05, in comparison to MLT). HPMA copolymer-GFLG-MLT (RM 7.1) and HPMA copolymer-GG-MLT (RM 3.13), both made from polymer precursors with 5 mol % side chain content, however, did not show a clear effect of peptidyl linker (p value 0.1). The Hb_{50} values at 10 min were 12.0 ± 1.3 μg/ml and 19.9 ± 6.2 μg/ml respectively (Figure 4.3 b).

Additionally, it was found that MLT induced haemolysis at 5 μg/ml showed a pH dependent effect over the range tested, with greatest haemolytic activity at pH 6.5 and the lowest at pH 5.5 (Figure 4.4).

SEM observation of RBC after exposure to MLT or the conjugates at their respective Hb_{50} values showed mainly cell debris. RBC exposed to aminolysed HPMA copolymer (1 mg/ml) were comparable with the control RBC which were intact biconcave discs (see Figure 4.5).

4.3.2 Cytotoxicity of MLT and HPMA copolymer-MLT Towards B16F10 Cells

Native MLT was cytotoxic toward B16F10 cells with an IC_{50} value of 5.8 ± 1.2 μg/ml (data not shown). When exposed to the reaction and purification conditions of the MLT conjugate (DMSO solvent and 40 °C) a similar (p value 0.2) IC_{50} value of 7.4 ± 1.5 μg/ml was found (Figure 4.6). In contrast, the HPMA copolymer-GG-MLT conjugate (RM 3.2) (21.8 % w/w) was less toxic (p value < 0.0005) in comparison to MLT subject to the same experimental conditions, with an IC_{50} value of 31.3 ± 8.8 μg/ml. As expected, the aminolysed HPMA copolymer did not show toxicity toward the B16F10 cells over the concentration range tested (up to 1 mg/ml) (Figure 4.6). It should be noted that at concentrations above 100 μg/ml, the relatively high polymer concentration appeared to have an effect on cell growth, with cell viability dropping to ~ 80 %. However, cell viability at 0.5 and 1 mg/ml was not significantly different to that at 100 μg/ml (p = 0.2 and 0.3 respectively).

Experiments to investigate the effect of MLT content on cytotoxicity showed no effect of MLT loading (Figure 4.7 and Table 4.2). In these experiments there were no

Figure 4.4 Effect of pH on haemolytic activity of MLT after 1 h incubation. Data represents mean ± SE (n = 4)

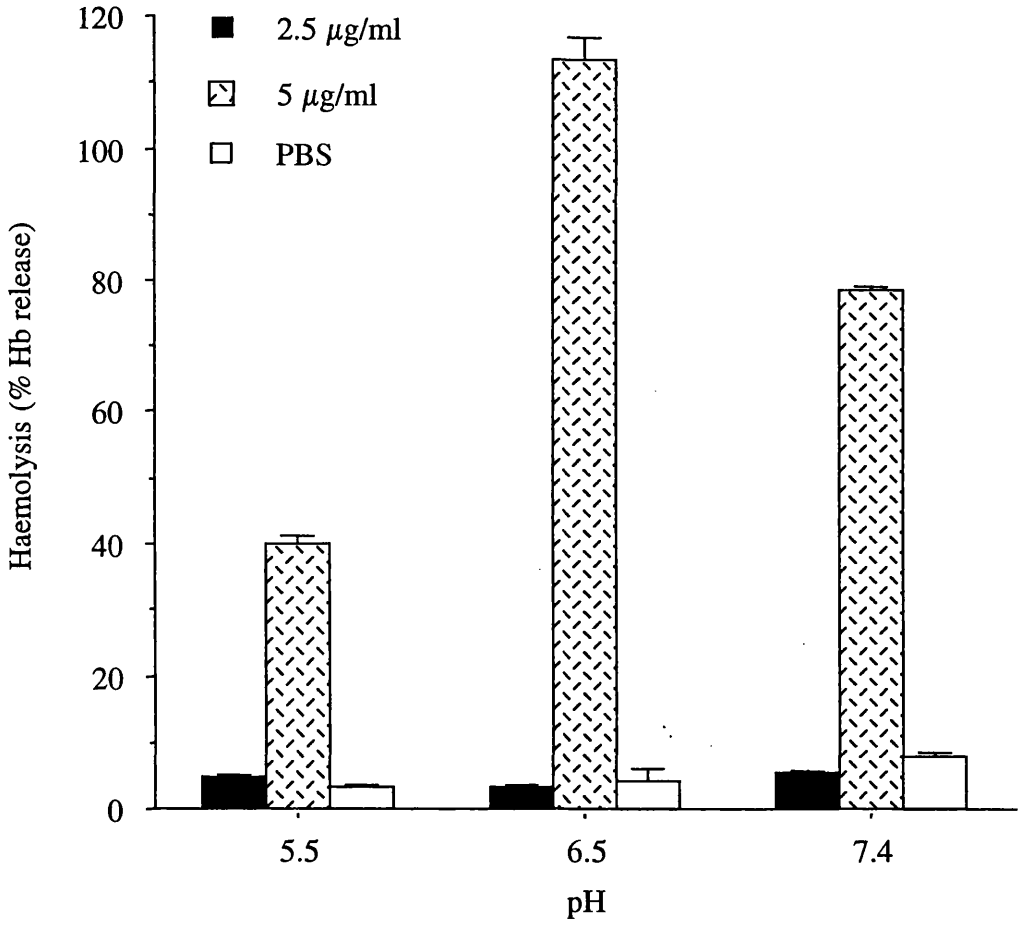


Figure 4.5 SEM pictures of RBCs exposed to (A) control, (B) aminolysed HPMA copolymer, (C) HPMA copolymer-MLT (RM 5.3), (D) HPMA copolymer-MLT (RM 3.13), (E) HPMA copolymer-MLT (RM 6.3) and (F) free MLT at their IC_{50} values for 10 min (X 4,000)

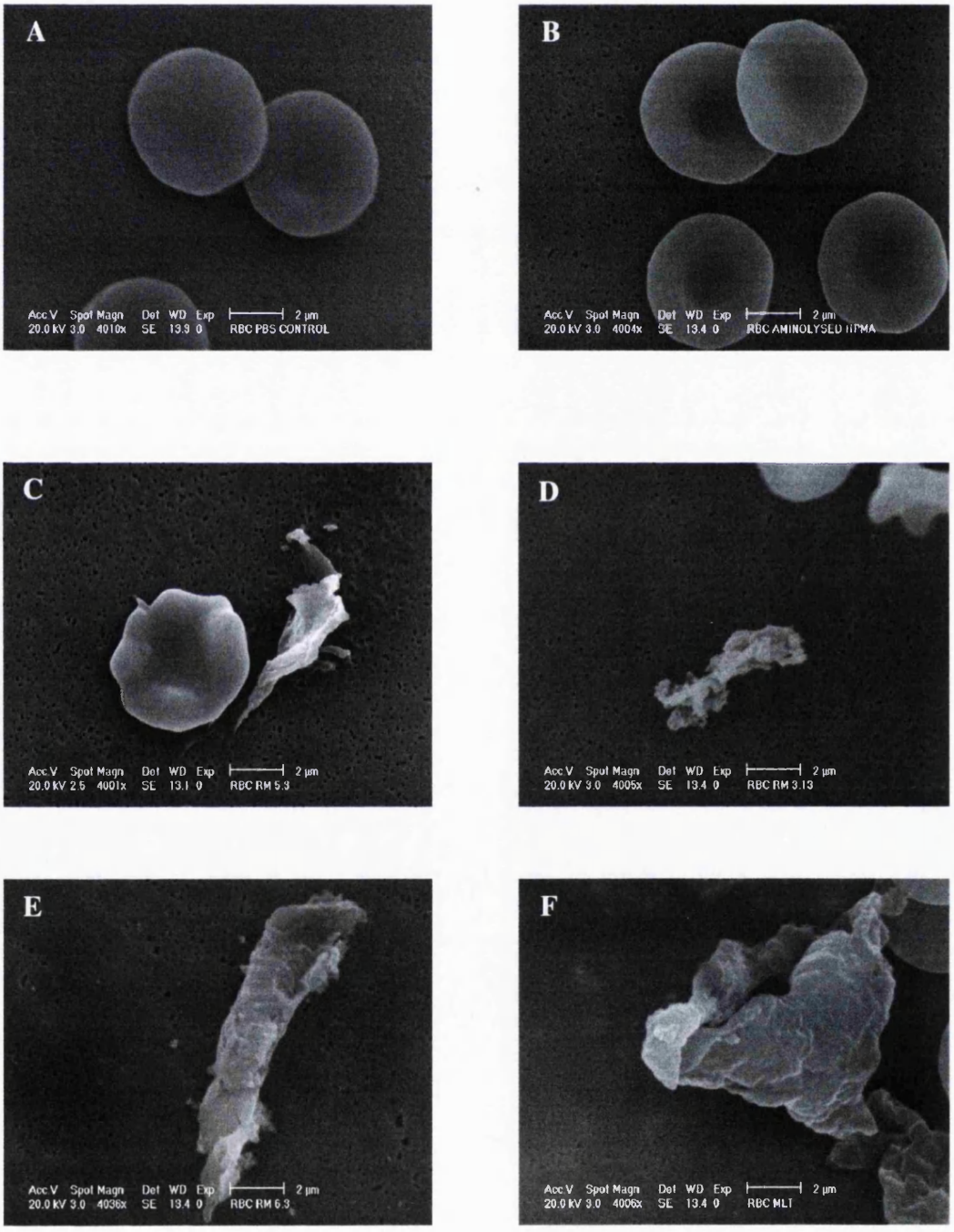


Figure 4.6 Cytotoxicity of HPMA copolymer-MLT (RM 3.2) against B16F10 cells after 72 h incubation. Data represents mean ± SE (n = 12)

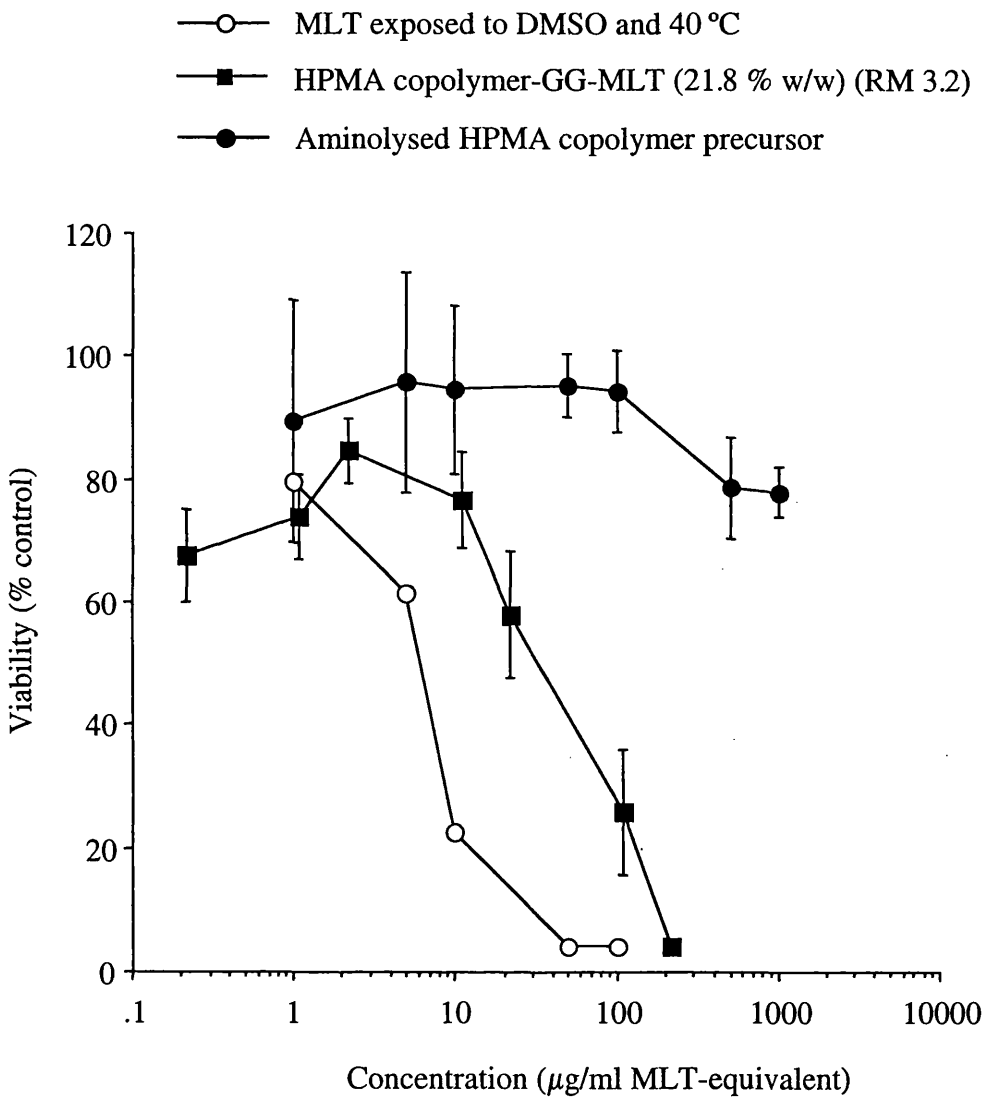


Figure 4.7 Effect of MLT content of HPMA copolymer-MLT conjugates on cytotoxicity against B16F10 cells after 72 h incubation. Data represents mean ± SE (n = 18), except RM 3.2 (n = 12)

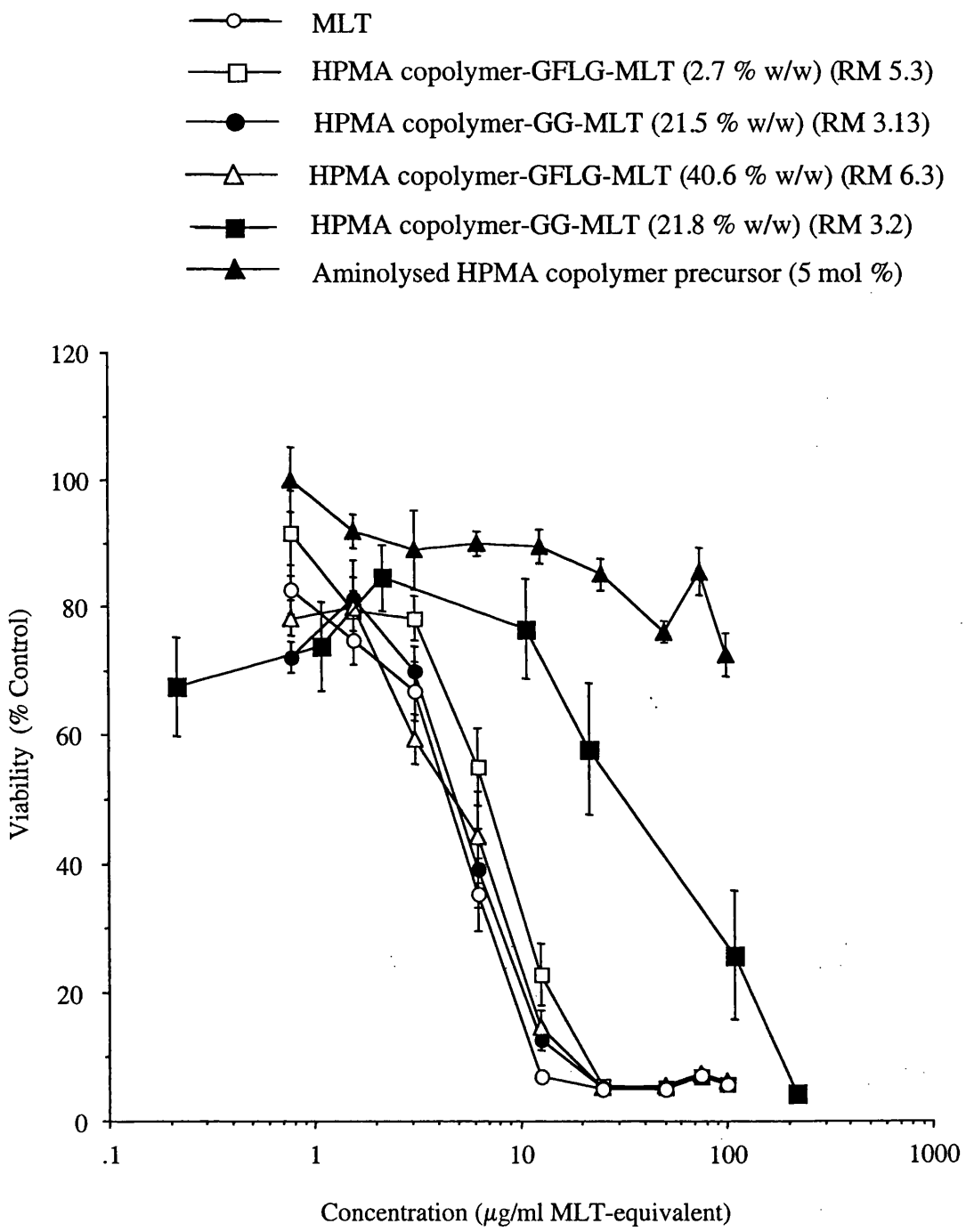


Table 4.2 Cytotoxicity of MLT and HPMA copolymer-MLT conjugates against B16F10 cells. The data represents mean ± SD (n = 18), except RM 3.2 (n = 12)

Compound	MLT content (% w/w)	Peptidyl Linker	Batch	IC_{50} value (μg/ml)[†]
MLT	-	-	Native	5.8 ± 1.2
MLT*	-	-	Exposed to DMSO and 40 °C	7.4 ± 1.5
HPMA copolymer-MLT	21.8	GG	RM 3.2	31.3 ± 8.8
HPMA copolymer-MLT	2.7	GFLG	RM 5.3	11.0 ± 5.2
HPMA copolymer-MLT	21.5	GG	RM 3.13	7.3 ± 1.5
HPMA copolymer-MLT	34.6	GFLG	RM 7.1	ND
HPMA copolymer-MLT	40.6	GFLG	RM 6.3	7.7 ± 3.1

Note: Cells were incubated with test compounds for 72 h and cytotoxicity assessed using the MTT assay

[†] All concentrations are presented in MLT-equivalent

ND = not determined

significant differences between the conjugates and free MLT, except for batch RM 3.2.

SEM visualisation of B16F10 cells after exposure to MLT or the conjugates showed the cells were generally no longer identifiable but amorphous masses of debris. As expected, however, after exposure to aminolysed HPMA copolymer (1 mg/ml) the B16F10 cells were identical to the control cells (see Figure 4.8).

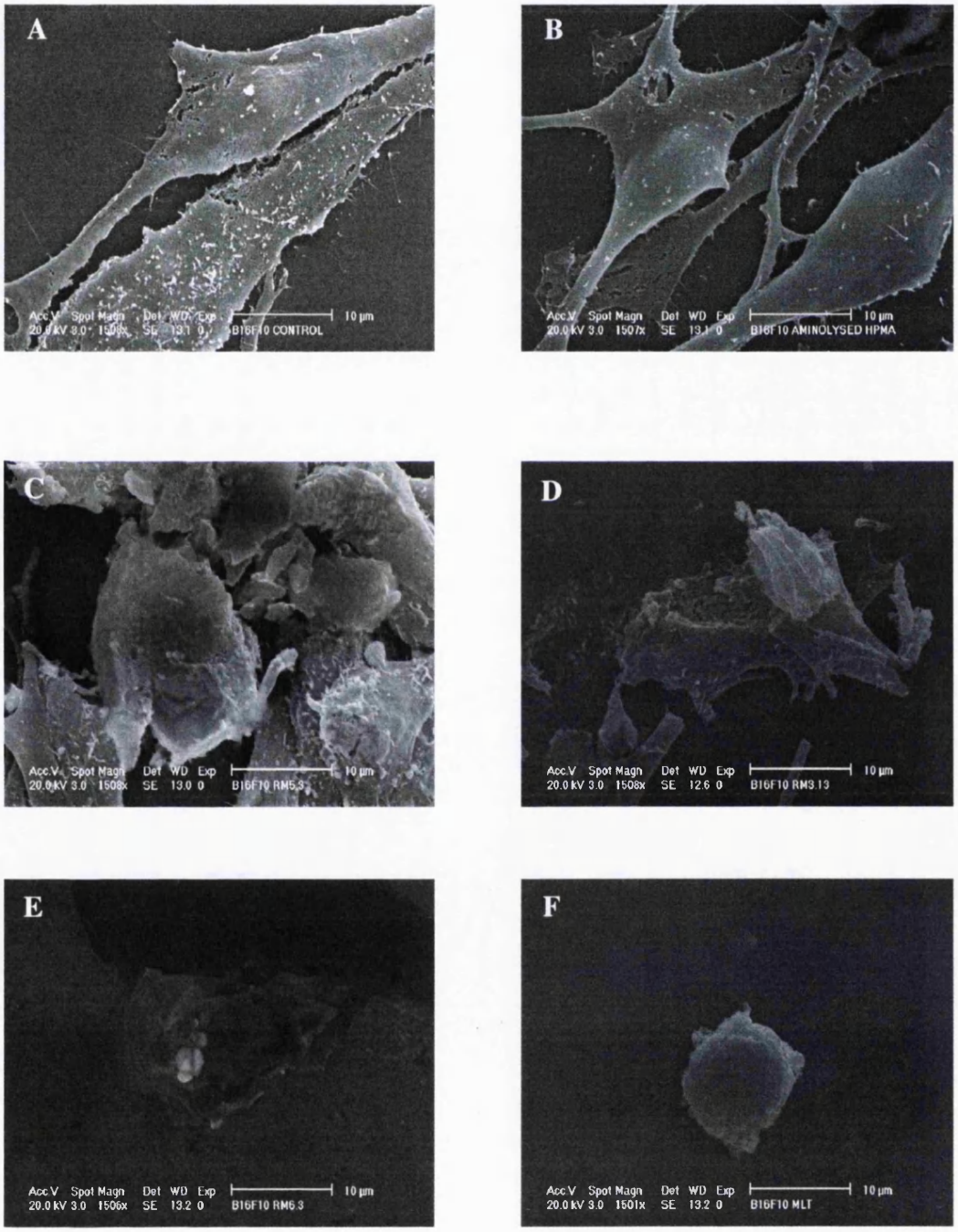
4.4 Discussion

In this study, it was found that incubation of RBCs with free MLT (both native or exposed to DMSO and 40 °C) for 10 min or 1 h gave similar Hb_{50} values. This is not surprising as kinetic studies of MLT-membrane binding have indicated rapid membrane interaction in the region of milliseconds (Sekharam *et al*, 1991) to nanoseconds (Lin & Baumgärtner, 2000). Although some other peptides e.g. α-haemolysin seem to act via inter-vesicular migration (Ostolaza *et al*, 1993), the fast cytolytic action displayed by MLT is not thought to rely on migration of MLT molecules but rather aggregation (section 1.6.2, Dufourcq & Faucon, 1977 and DeGrado *et al*, 1982). Previous studies using the RBCL model (DeGrado *et al*, 1982 and Tosteson *et al*, 1985) and liposome leakage (Schwarz *et al*, 1992 and Benachir & Lafleur, 1995) as a measure of MLT activity have also shown that a plateau of membrane perturbation is reached within 30 min.

The haemolytic activity of native MLT observed here using 0.1 M PBS as a diluent (Hb_{50} = 8.0 ± 4.5 μg/ml) was found to be relatively low. Dempsey (1990) used 0.15 M NaCl, 20 mM Tris, pH 7.6 as a diluent and measured a Hb_{50} value of 0.77 μg/ml after 1 h incubation. Pérez-Payá *et al* (1997) used 20 mM Tris-HCl, 130 mM NaCl, 1mM EDTA, pH 7 as a diluent and incubated RBC with MLT for 1 h and found an even lower Hb_{50} value of 0.45 μg/ml. The observed differences may reflect an effect of varying pH or ionic strength on MLT conformation and hence surface activity (reviewed by Dempsey, 1990).

In future it would be interesting to investigate the effects of buffer on MLT haemolytic activity more systematically. Here, preliminary experiments were conducted to examine the effect of pH on haemolysis caused when MLT was incubated with RBC for 1 h at a concentration of 5 μg/ml. The haemolysis observed was in the

Figure 4.8 SEM pictures of B16F10 cells exposed to (A) control, (B) aminolysed HPMA copolymer, (C) HPMA copolymer-MLT (RM 5.3), (D) HPMA copolymer-MLT (RM 3.13), (E) HPMA copolymer-MLT (RM 6.3) and (F) free MLT at their IC_{50} values for 72 h (X 4,000)



order of pH 6.5 > pH 7.4 > pH 5.5 (Figure 4.4). This is possibly as a result of MLT's conformational changes at different pH values, which has a knock-on effect on membrane binding and thus cytolytic activity. This observation suggests MLT conformation for peptide-lipid interaction was optimal at pH 6.5. It would have been of interest to perform circular dichroism (CD) studies to elucidate MLT helical structure and relate it to these results. It should be noted that the MLT concentrations used in all the studies were low enough to ensure MLT would be in the monomeric form (Tosteson *et al*, 1985). Additionally and importantly, the treatment of MLT with DMSO and high temperature (as occured during conjugate purification) was found not to have an effect on its Hb_{50} value (10.8 ± 3.8 μg/ml), in comparison to native MLT (8.0 ± 4.4 μg/ml).

Haemocompatibility of the HPMA copolymer-MLT conjugate is essential for its safe systemic administration and i.v. injection is in turn of paramount importance if tumour targeting is to be achieved by the EPR effect (Chapter 1). Here it was found that HPMA copolymer-GG-MLT conjugate (RM 3.2) had a Hb_{50} value that was more than 10-fold greater than free MLT. i.e. the conjugate was at least 10 times less lytic. It may be hypothesised that the relatively large HPMA copolymer chains (Mw ~ 30 kDa) are constantly in dynamic motion and they surround MLT's cationic surface charge (~ 3 kDa), thus shielding it from membrane interaction. These observations were consistent with observations made using a poly(amidoamine) (MBI)-Triton X-100 conjugate where conjugation was found to reduce the well known haemolytic effect of Triton X-100 (Duncan *et al*, 1994). In another study, conjugation of HPMA copolymer to an aminoellipticine derivative (APE) (2.3 – 7 % w/w) was shown to cause a > 10-fold reduction in haemolytic activity of APE at 1 h. HPMA copolymer-APE caused no haemolysis up to a concentration of 300 μg/ml (APE-equivalent) whereas the Hb_{50} value for free APE was 35 μg/ml (Searle *et al*, 2001). This observation aided the progression of HPMA copolymer-APE into *in vivo* studies. Likewise, the reduction in haemolytic activity of HPMA copolymer-MLT suggests the possibility of *in vivo* application. Notably however, the effect of the MLT conjugate on other cell types would require extensive investigation.

This study also looked at the effect of peptidyl linker on the haemolytic activity of HPMA copolymer-MLT prepared with HPMA copolymer precursors with ~ 5 mol % side chains and thus similar conjugate structures. RBCL studies comparing HPMA

copolymer-GG-MLT (RM 3.13) (21.5 % w/w) with HPMA copolymer-GFLG-MLT (RM 7.1) (34.6 % w/w) indicated that MLT-membrane interactions are not affected by the length of peptidyl linkers used (*p* value 0.1). However, whereas HPMA copolymer-GFLG-MLT (RM 7.1) was not less haemolytic relative to free MLT (Hb_{50} value = 12.0 ± 1.3 μg/ml), HPMA copolymer-GG-MLT (RM 3.13) was significantly (*p* value 0.05) less haemolytic (Hb_{50} value = 19.9 ± 6.2 μg/ml) (Table 4.1 and Figure 4.3). This observation suggested that a MLT conjugate with the GG linker may be more suitable for *in vivo* use. Interestingly, the haemolytic activity of HPMA copolymer-GFLG-MLT (RM 7.1) was similar (*p* value 0.1) to that of HPMA copolymer-GFLG-MLT (RM 6.3). It may be speculated that the longer peptidyl linker allowed the conjugated MLT molecules to more freely associate with the phospholipid membranes.

The reduced haemolytic activity of HPMA copolymer-GFLG-MLT conjugates with low MLT content (RM 5.3, 2.7 % w/w) (Hb_{50} value = 39.9 ± 15.8 μg/ml) is consistent with the proposed mode of action of MLT, whereby co-operation of MLT molecules to reach a threshold concentration on the cell surface is necessary to cause cell membrane lysis (see section 1.6.1). The number of "binding sites" on a RBC has been reported to be ~ 1.8×10^7 (De Grado *et al*, 1982 and Tosteson *et al*, 1985). In a separate study, Blaskó & Schagina (1996) reported a Hb_{50} value of 4×10^6 molecules of MLT.

It should be noted there was a significant difference in the haemolytic activity of two different batches of HPMA copolymer-GG-MLT (~ 5 mol % side chains). RM 3.2 (31.6 % w/w) gave an Hb_{50} value of 192.3 ± 29.2 μg/ml whereas RM 3.13 (21.5 % w/w) gave an Hb_{50} value of 19.9 ± 6.2 μg/ml (*p* value < 0.005). This finding raises the issue of inter-batch variation in activity, despite identical physico-chemical characteristics (Chapter 3). This observation may be explained by the semi-random aminolysis of HPMA copolymer-ONp precursors to MLT, resulting in uneven MLT distribution on polymer chains not only at different pendant groups along the length of the polymer, but also at different MLT sites (NH_2 groups), which adds to the complexity in dissecting the activity of the prepared MLT conjugates. MLT's positive charge which is provided by K and R residues has been shown to be crucial to its membrane lytic activity. It is therefore likely that this charge is altered following chemical modification with HPMA copolymer, which thus has implications for MLT configuration and thus membrane perturbation activity. This finding therefore reiterates

the disadvantage of uncontrolled or random aminolysis of HPMA copolymer precursors by MLT and resultant heterogeneity of prepared MLT conjugates which may result in an overall different pattern of activity. Chemical modification of MLT's surface NH_2 groups and its relation to its activity has been widely investigated. In a study conducted by Blondelle & Houghten (1991) which compared the cell surface activity of MLT with 24 MLT analogues derived from the omission of individual amino acid residues, K-7's positive charge was shown to have a role in MLT haemolytic activity. In a similar fashion, conversion of ε-NH_2 groups provided by K into guanidino groups (homoarginine) retained the positive charge and therefore maintained haemolytic activity despite MLT chemical modification. This observation is in agreement with studies whereby a MLT analogue was succinylated at K residues (Habermann & Kowallek, 1970; Hider *et al*, 1983; Manjunatha-Kini & Evans, 1989). This issue of inter-batch variation is addressed further in Chapter 7.

Other methodologies which have been used to assess MLT-induced membrane perturbation include lactate dehydrogenase (LDH) release (Choksakulnimitir *et al*, 1995), ^{51}Cr release (Kubo *et al*, 1999) and leakage of fluorescent dyes from lipid vesicles (Schwarz *et al*, 1992; Benachir & Lafleur, 1995; Rex 1996; Ladokhin *et al*, 1997). It would have therefore been of interest to see if the findings in the RBCL were comparable to these assays.

If the HPMA copolymer-MLT conjugates are to be useful anticancer agents, however, they must be cytotoxic once they reach the tumour. MLT's cytotoxic activity (IC_{50} value 5.8 ± 1.2 μg/ml) was not reduced by conjugation (RM 5.3, RM 3.13 and RM 6.3; IC_{50} values 7.3 ± 1.5 to 11.0 ± 5.2 μg/ml). (Table 4.2 and Figure 4.7).

It was found that the cytotoxic activity of HPMA copolymer-GFLG-MLT conjugates with low MLT content (RM 5.3, 2.7 % w/w) was similar (*p* value 0.4) to that caused by MLT conjugate with a high MLT content (RM 6.3, 40.6 % w/w) (Table 4.2). This would therefore also be expected for the MLT conjugate with an intermediate loading (RM 7.1, 34.6 % w/w). The IC_{50} value found with the HPMA copolymer-GG-MLT conjugate (RM 3.13, 21.5 % w/w) was also similar to that of the HPMA-GFLG-MLT conjugates (Table 4.2).

Again, the different batches of HPMA copolymer-GG-MLT (RM 3.2, 31.6 % w/w and RM 3.13, 21.5 % w/w) which were prepared using HPMA copolymer

precursor with ~ 5 % ONp side chains demonstrated significantly different IC_{50} values (p value < 0.05) after incubation with B16F10 cells. This observation reiterated the issue of batch to batch variation.

A reduction in haemolytic activity relative to the cytotoxicity of MLT conjugates would be beneficial for their proposed use as a novel i.v. administered high molecular weight anticancer therapeutic (summarised in Table 4.3). It should be noted that these assays are conducted at different time points (72 h cytotoxicity and 1 h RBCL) and therefore direct comparisons can not be made. However, time-dependence is not the simple explanation for the observed differences as MLT interacts rapidly with cells. Kinetic studies have shown that MLT binds to RBC at a rate of 7.1/μM/min (Pérez-Payá *et al*, 1997). In addition, using 5 different cytotoxicity assays (trypan blue uptake, PI uptake, incorporation of [^{14}C]-leucine, [^{51}Cr] or LDH release) in a human leukaemia cell line (K562) or a promyelocytic cell line (HL-60), Kubo *et al* (1999) showed that MLT at a low concentration (14 μg/ml) damages cells in culture within 1 h. Unfortunately, this was not investigated for the MLT conjugate. The MTT assay for *in vitro* screening of cytotoxicity of polymer-drug conjugates was standardised to 72 h (Sgouras & Duncan, 1990). It was therefore thought useful to carry out cytotoxicity assays with the MLT conjugate at this time point, to allow for direct comparison with other polymer anticancer conjugates. It is suggested that the clearance from the solid tumour site is minimal due to lack of or ineffectual lymphatic drainage (Maeda & Matsumura, 1989 and reviewed by Jain, 1994) (section 1.3), thus aiding localisation of the macromolecular entity. Pharmacokinetic studies conducted with PK1 (5 and 18 mg/kg) and DOX (5 mg/kg) administered i.v. evidenced sustained tumour levels of only the conjugate up to 48 h. The 5 mg/kg PK1 dose led to 3 % injected dose/g in tumour at 48 h (Seymour *et al*, 1994).

However, in retrospect, it would have been useful to carry out additional cytotoxicity assays at the same time point as the red blood cell lysis experiments (1 h) to allow direct comparison between these assays. It would have been interesting to investigate if the difference in the membrane composition of RBC and B16F10 tumour cells may have had an effect on the cell surface activity of MLT and prepared MLT conjugates. As stated earlier, MLT's + 5/+ 6 charge plays a role in MLT-lipid electrostatic interactions. RBCs have an overall zwitterionic charge and thus the transmembrane potential across the RBC membrane is relatively small (Rink & Tsien, 1982). It has been proposed, however, that negatively charged sialic acid molecules in

Table 4.3 Summary of effect of MLT and HPMA copolymer-MLT on haemolysis of RBC after incubation for 10 min or 1 h and cytotoxicity against B16F10 cells after 72 h

Compound	Peptidyl Linker	MLT Content (% w/w)	Batch	Hb_{50} (μg/ml)	IC_{50} (μg/ml)
MLT*	-	-	Exposed to DMSO and 40 °C	10.8 ± 3.8[§]	7.4 ± 1.5
HPMA copolymer-MLT	GG	31.6	RM 3.2	↑↑↑[§]	↑↑↑
MLT*	-	-	Exposed to DMSO and 40 °C	9.8 ± 0.8[†]	7.4 ± 1.5
HPMA copolymer-MLT	GFLG	2.7	RM 5.3	↑↑↑[†]	↑↑
HPMA copolymer-MLT	GG	21.5	RM 3.13	↑↑[†]	↑
HPMA copolymer-MLT	GFLG	34.6	RM 7.1	↑[†]	ND
HPMA copolymer-MLT	GFLG	40.6	RM 6.3	↑[†]	↑

Note: Test compounds were incubated in RBC (2 % w/v) for [†]10 min or [§]1 h
ND = Not determined

RBC membranes which project out of the RBC membrane facilitate cationic peptide-lipid interactions (Viitala & Järneflt, 1985). Additionally, RBC membrane constituents include sphingomyelin and cholesterol which convey a protective effect due to the tighter lipid packing, thicker bilayer and reduced lipid surface area (Dufourc *et al*, 1984 and Nezil & Bloom, 1992). This protective effect has been further evidenced in studies whereby MLT-induced leakage of liposomes was reduced in the presence of cholesterol (Benachir *et al*, 1997). It may also be noteworthy to point out that the protective effect of plasma proteins *in vivo* (Moreau *et al*, 2000) would probably contribute to a further increase in the Hb_{50} value of the MLT conjugate.

The cytotoxic and haemolytic activity of a library of peptidyl toxins was also investigated as part of this study (Appendix 1). Additionally, the search for a potent marine-derived membrane active compound was also undertaken in a collaboration with Dr. J. L. Fernandes (Biomar S.A., León, Spain) (Appendix I). However, none of the compounds investigated were more potent than MLT.

To conclude, some MLT conjugates (RM 5.3, RM 3.2 and RM 3.13) showed reduced haematotoxicity relative to free MLT, whereas all conjugates retained cytotoxic activity at their respective time points. In order to verify the potential of HPMA copolymer-MLT as an anticancer agent, it was necessary to move *in vivo* and investigate, on a small scale, the maximum tolerated dose of this compound, and if successful, its body distribution and antitumour activity. The HPMA copolymer-GG-MLT (~ 5 mol % side chains) conjugate was selected for further *in vivo* studies (Chapter 5).

Chapter Five

In Vivo Pharmacokinetics and Pharmacology of HPMA Copolymer-MLT

5.1 Introduction

It has been shown that HPMA copolymer-GG-/-GFLG-MLT conjugates from 5 mol % polymer precursors can be synthesised (Chapter 3) and the batches that were used in experiments in this thesis (RM 3.2, RM 3.4, RM 3.7, RM 3.9, RM 3.13, RM 3.14 and RM 7.1), were equivalent in terms of physico-chemical characteristics, with an average loading of 28.47 ± 5.76 % w/w. The HPMA copolymer-GG-MLT conjugates (RM 3.2 and RM 3.13) showed a decrease in haemolysis (Hb_{50} value ~ 20 - 200 μg/ml) but not in cytotoxicity towards B16F10 cells (IC_{50} value ~ 5 - 30 μg/ml) relative to MLT at the respective time points (Chapter 4). The primary concern was to establish, first, the maximum tolerated dose (MTD) of HPMA copolymer-GG-MLT made from 5 mol% precursor. This allowed to study first, the biodistribution of HPMA copolymer-GG-MLT in mice bearing s.c. B16F10 tumours and secondly, its antitumour efficacy against a s.c. B16F10 murine melanoma model. The antitumour activity of numerous polymer-drug conjugates has been studied in many pre-clinical models (summarised in Table 1.3). The general observation with polymer-drug conjugates is that *in vitro* studies are not usually a good representation of their *in vivo* activity profile, which makes analysis of conjugates difficult. For instance, the first compound to progress into clinical evaluation, HPMA copolymer-doxorubicin (PK1), does not show any antitumour activity *in vitro* (Wedge, 1991). The B16F10 murine melanoma model was chosen for this study as it has been particularly well characterised for both biodistribution and antitumour studies of polymer-drug conjugates. Additionally, the accumulation of HPMA copolymer-doxorubicin (PK1) in B16F10 tumours of varying sizes has been characterised (see section 1.3). Importantly, the panel of mouse tumour models used for evaluation of PK1 (Duncan *et al*, 1992) have been valuable as predictors for observed antitumour activity and reduction in toxicity in clinical evaluation (Vasey *et al*, 1999).

In general, the biodistribution of polymers is determined by their physical characteristics, namely molecular weight, charge and Stoke's radius (which in turn determines the hydrodynamic volume). The biodistribution of HPMA copolymers is well documented. Seymour *et al* (1987) studied the effect of HPMA copolymer molecular weight (12 – 778 kDa) on biodistribution following s.c., i.p. and i.v. administration. The renal threshold for the HPMA copolymers was ~ 45 kDa. The study also showed that there was no effect of molecular weight (over this range) on transfer from the peritoneal cavity to the circulation. Additionally, it has been shown

that tumour targeting of HPMA copolymers via the EPR effect was also independent of molecular weight (4.5 - 800 kDa) (Seymour *et al,* 1995 and Noguchi *et al,* 1998). In these studies, radioactivity (^{125}I-labelling) was used to monitor the fate of HPMA copolymers by incorporation of methacryolylated tyrosinamide into the HPMA copolymer backbone. Here, radioiodination was also chosen to study the biodistribution of HPMA copolymer-GG-MLT following i.p. administration. Radioiodination was chosen due to the relative high specific activity of [^{125}I]iodide (2.18 kCi / 80.5 TBq), relative long half-life (60 days) and lack of toxicity (Amersham Life Science hand book). The potential disadvantage of the use of radioiodination, however, is the fact that the label is not an integral part of the molecule of interest. Essentially, radiolabelling results in the production of a new compound which may interact biologically in a different manner to the initial compound of interest. In addition, the stability of the radiolabelled compound is vital for meaningful evaluation. De-iodination is easily detected by large amounts of [^{125}I]iodide in the thyroid gland or [^{125}I]iodotyrosine in the urine. Degradation of the radiolabelled parent peptide may therefore result in detection of MLT degradation products. It should be noted though that two radioiodinated HPMA copolymer-doxorubicin conjugates ([^{123}I]iodide and [^{131}I]iodide) have been successfully used as clinical gamma camera imaging agents (Vasey *et al*, 1999 and Julyan *et al*, 1999). Both conjugates, however, unlike the ones prepared in this chapter, are radiolabelled on the polymer backbone by initial reaction of the HPMA copolymer precursor with tyrosinamide, prior to conjugation with the drug of choice, doxorubicin. This provides the advantage of comparisons of free polymer and polymer-drug conjugate. Other methods which have been used to visualise the fate of polymer-drug conjugates *in vivo* include the use of high pressure liquid chromatography (HPLC) after extraction from homogenates of major organs (Wedge, 1991; Sat, 1999; Keane, 2002) and fluorescent-labelled compounds (Duncan *et al*, 1992 and Steyger *et al*, 1996).

In this study, Bolton and Hunter reagent and Chloramine T were considered as techniques to radiolabel MLT and HPMA copolymer-MLT. Bolton and Hunter reagent was specifically developed for protein radioiodination via reaction with NH_2 groups along the polypeptide chains (Bolton & Hunter, 1973). Alternatively, Chloramine T labelling can be used and this oxidising agent allows iodination via a phenolic ring, a technique that has been applied to HPMA copolymer-tyrosinamide (Duncan *et al*, 1982; Seymour *et al*, 1987; 1995) and MLT (Ferraiolo & Mohler, 1992). The MLT

product prepared by Chloramine T oxidation, however, was highly unstable so the Bolton and Hunter method was used to prepare ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-GG-MLT (RM 3.4, 24.3 % w/w) for all biodistribution studies.

Initially, both biodistribution and antitumour activity comparative studies used i.p. injection of MLT and HPMA copolymer-GG-MLT due to MLT's toxicity. However, ultimately it would be anticipated that clinical use involves i.v. administration, therefore the final antitumour activity study using HPMA copolymer-GG-MLT (RM 3.14, 21.7 % w/w) was undertaken using i.v. administration of the conjugate.

5.2 Methods

The methods used are described in detail in section 2.8. All *in vivo* studies were carried out according to The United Kingdom Coordinating Committee on Cancer Research (UKCCCR) (1998) and The Home Office Guidelines (Animals Scientific Procedures Act, 1986).

The biodistribution studies were conducted using ^{125}I-labelled-MLT and ^{125}I-labelled-HPMA copolymer-GG-MLT (RM 3.4, 24.3 % w/w) with < 2 % free [^{125}I]iodide as described in detail in section 2.7. The specific activity of the radiolabelled test compounds used was calculated on the day of the study (see section 2.7.1 and Table 5.1).

In antitumour activity studies (see section 2.8.2), once tumours were palpable (~ 0.5 cm x 0.5 cm) this was assigned as day 0 of the study and treatment commenced. Animals were treated, monitored for general well being and weighed daily at the same time of day. Animals presenting signs of distress or > 20 % loss of body weight were killed by CO_2 asphyxiation and major organs dissected for observation. These were quoted as toxic deaths.

All HPMA copolymer-GG-MLT doses were expressed as MLT-equivalent.

5.2.1 Evaluation of Body Distribution of ^{125}I-labelled-MLT and ^{125}I-labelled-HPMA copolymer-MLT

To study the time-dependent (30 min, 2, 4 and 6 h) body distribution of MLT and its conjugate, ^{125}I-Labelled MLT (15.18 μCi/mg or 0.56 MBq/mg) or ^{125}I-labelled HPMA

Table 5.1 Summary of the protocols and characteristics of test compounds used in the biodistribution studies

Biodistribution Study	Specific Activity (μCi/mg)		Dose Administered (mg/kg)[¥]	
	MLT[§]	HPMA copolymer-MLT[†]	MLT	HPMA copolymer-MLT*
Time-Dependent (0.5, 2, 4 & 6 h)	15.2	68.4	0.5	0.1[‡]
Dose-Dependent (4 h)	4.5	20.1	2, 2.5 or 3	1, 1.5 or 3[¶]

MLT-equivalent ([‡]RM 3.4 (24.3 % w/w) to 5 x 10^5 CPM; [¶]supplemented with RM 3.9 (33.9 % w/w) to the required dose in the dose-dependent study)
[¥] 5 x 10^5 CPM or 0.2 μCi
[§] 2 % free [^{125}I]iodide
[†] 0.3 % free [^{125}I]iodide

copolymer-MLT (68.42 μCi/mg or 2.53 MBq/mg) made up in 0.9 % sterile saline were injected i.p. (100 μl) to C57BL/6 mice bearing palpable (~ 0.5 cm x 0.5 cm) s.c. B16F10 tumours (n = 3 at each time point) (see section 2.8.1). The samples contained 5 x 10^5 CPM i.e. 0.2 μCi or 0.08 MBq. Radioactivity in each organ was then calculated as follows assuming the blood volume for a mouse to be 5.77 ml/100 g (Dreyer & Ray, 1910).

$$(1)\ \text{Percentage injected dose} = \frac{\text{CPM (1 ml homogenate)} \times \text{Total homogenate volume}}{\text{Injected dose (CPM)}} \times 100$$

$$(2)\ \text{Percentage injected dose/g of organ or /ml of blood} = \frac{\{\text{CPM (1 ml)} \times \text{Total volume}\} / \text{Weight of organ (or total blood volume)}}{\text{Injected dose (CPM)}} \times 100$$

A second study was carried out to investigate the dose dependency of MLT and HPMA copolymer-MLT biodistribution. In this study, the ^{125}I-labelled MLT (4.5 μCi/mg or 0.17 MBq/mg) and ^{125}I-labelled HPMA copolymer-MLT (20.1 μCi/mg or 0.74 MBq/mg MLT-equivalent) solutions for injection in sterile 0.9 % saline were supplemented with non-radioactive MLT or conjugate (RM 3.9, 33.9 % w/w) respectively to obtain solutions for injection that contained 5 x 10^5 CPM (equivalent to 0.2 μCi or 8.3 MBq) and various doses of MLT (2, 2.5 or 3 mg/kg) or HPMA copolymer-MLT (1, 1.5 or 3 mg/kg MLT-equivalent).

Again, these compounds were administered i.p. (100 μl) to C57BL/6 mice bearing palpable (~ 0.5 cm x 0.5 cm) s.c. B16F10 tumours (n = 3 for each dose). It was decided to measure biodistribution at 4 h because this time point showed peak levels of radioactivity in the circulation for both MLT and the conjugate in the earlier time-dependent study. Results were expressed as described above.

5.2.2 Evaluation of Antitumour Activity of MLT and HPMA Copolymer-MLT

First experiments were designed to identify the maximum tolerated dose (MTD) of MLT and HPMA copolymer-MLT. A single dose of MLT or HPMA copolymer-MLT (RM 3.4, 24.3 % w/w) was administered to pairs of non-tumour bearing C57BL/6 mice via the i.p. route. Initially a dose of 5 mg/kg was given and then the subsequent doses escalated according to the animal well being. The range of doses given for free MLT were 1.75 – 5 mg/kg and for HPMA copolymer-MLT, 5 – 20 mg/kg (MLT-equivalent).

Animals were continuously monitored at regular intervals throughout the day for signs of toxicity (see section 2.8.2) and weights taken daily.

The MTD was defined as the dose whereby no physical signs of toxicity were evident 10 days following administration (Duncan *et al*, 1998). After this time all animals were then killed by CO_2 asphyxiation and the major organs (heart, lungs, kidneys, spleen, liver and thyroid) dissected and examined visually for changes in morphology.

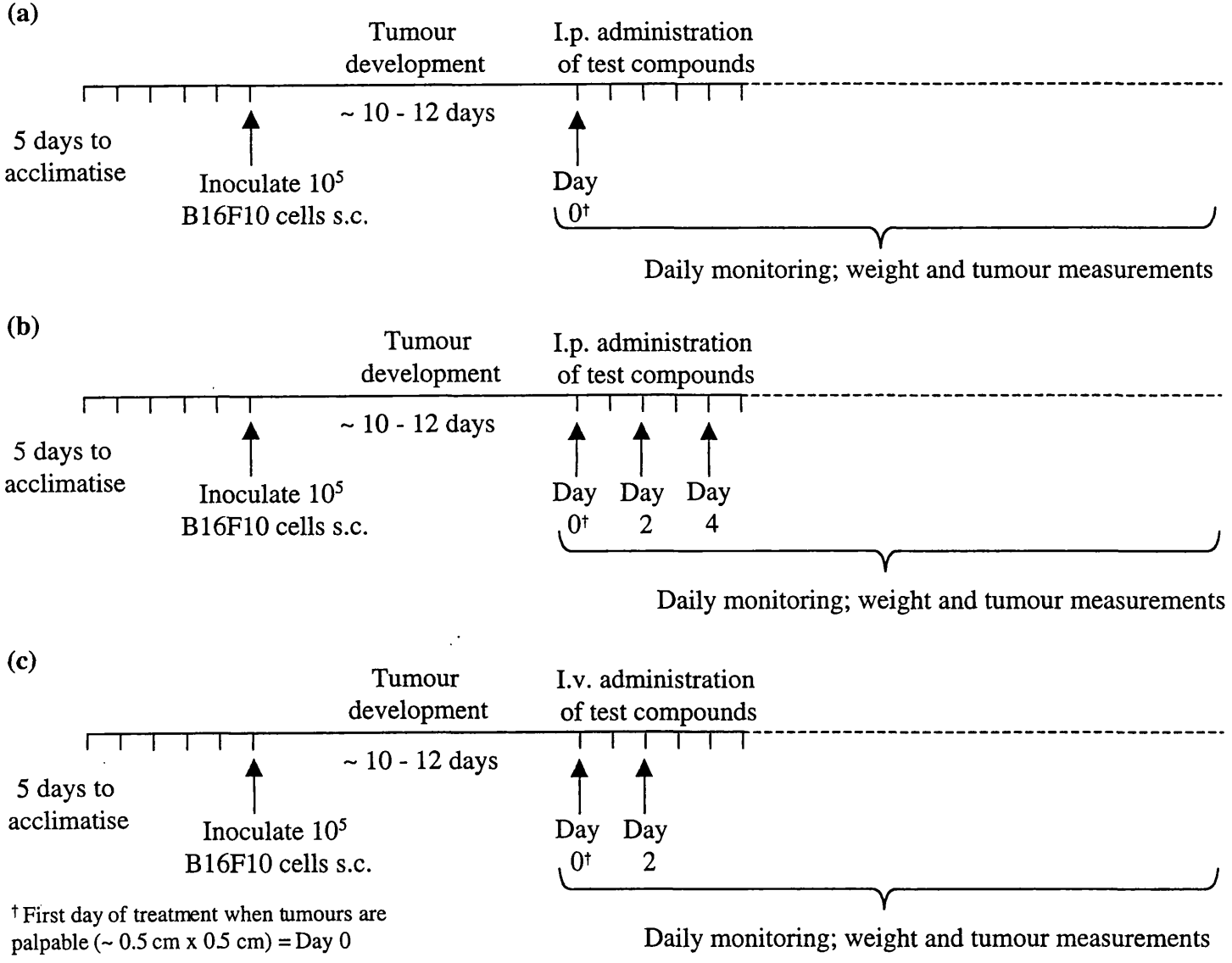
To determine the antitumour activity of MLT and HPMA copolymer-MLT, first experiments were carried out with a single i.p. dose to C57 BL/6 mice bearing palpable s.c. B16F10 tumours at their MTD doses. i.e. 2.5 mg/kg MLT and 10 mg/kg (MLT-equivalent) HPMA copolymer-MLT (RM 3.7, 31.7 %w/w) made up in 0.9 % sterile saline (n = 5 for each dose). The injection volume was adjusted to 100 μl/25 g mouse. The protocol for this single dose study is summarised in Figure 5.1 a. Animals were monitored daily for possible signs of toxicity (see section 2.8.2), weighed and tumours measured (orthogonal diameters) at the same time each day. Antitumour activity was expressed as a T/C value (see section 2.8.2)

$$T/C = \frac{\text{Mean survival time treatment (T) (days)}}{\text{Mean survival time control (C) (days)}} \times 100$$

The National Cancer Institute (USA) has usually considered a compound with a T/C value of > 125 to show antitumour activity.

Following this study, a second study was undertaken using multiple doses of MLT or HPMA copolymer-MLT given i.p. on days 0, 2 and 4. The protocol for this study is shown in Figure 5.1 b. In this case, three doses of MLT (1, 0.5 or 0.1 mg/kg) and HPMA copolymer-MLT (RM 3.9, 33.9 % w/w) (2.5, 1 or 0.5 mg/kg MLT-equivalent) in sterile 0.9 % saline were administered i.p. (n = 5 for each dose) and again each time the injection volume being 100 μl/25 g mouse. Animals were monitored, weighed and tumours measured (orthogonal diameters) daily and again results expressed as T/C as described above.

Figure 5.1 Protocols for the antitumour activity studies (a) single dose i.p., (b) multidose i.p. and (c) multidose i.v.



† First day of treatment when tumours are palpable (~ 0.5 cm x 0.5 cm) = Day 0

A final antitumour activity study was carried out using multiple doses of HPMA copolymer-MLT given i.v. to capitalise on the EPR effect on days 0 and 2. The protocol for this study is shown in Figure 5.1 c. In this case, four doses of HPMA copolymer-MLT (RM 3.14, 21.7 % w/w) (2, 1, 0.5 or 0.1 mg/kg MLT-equivalent) in sterile 0.9 % saline were administered i.v. (n = 5 for each dose) and again each time the injection volume being 100 μl/25 g mouse. Animals were monitored, weighed and tumours measured (orthogonal diameters) daily and again results expressed as T/C as described above.

5.3 Results

5.3.1 Body Distribution of ^{125}I-labelled MLT and ^{125}I-labelled HPMA Copolymer-MLT

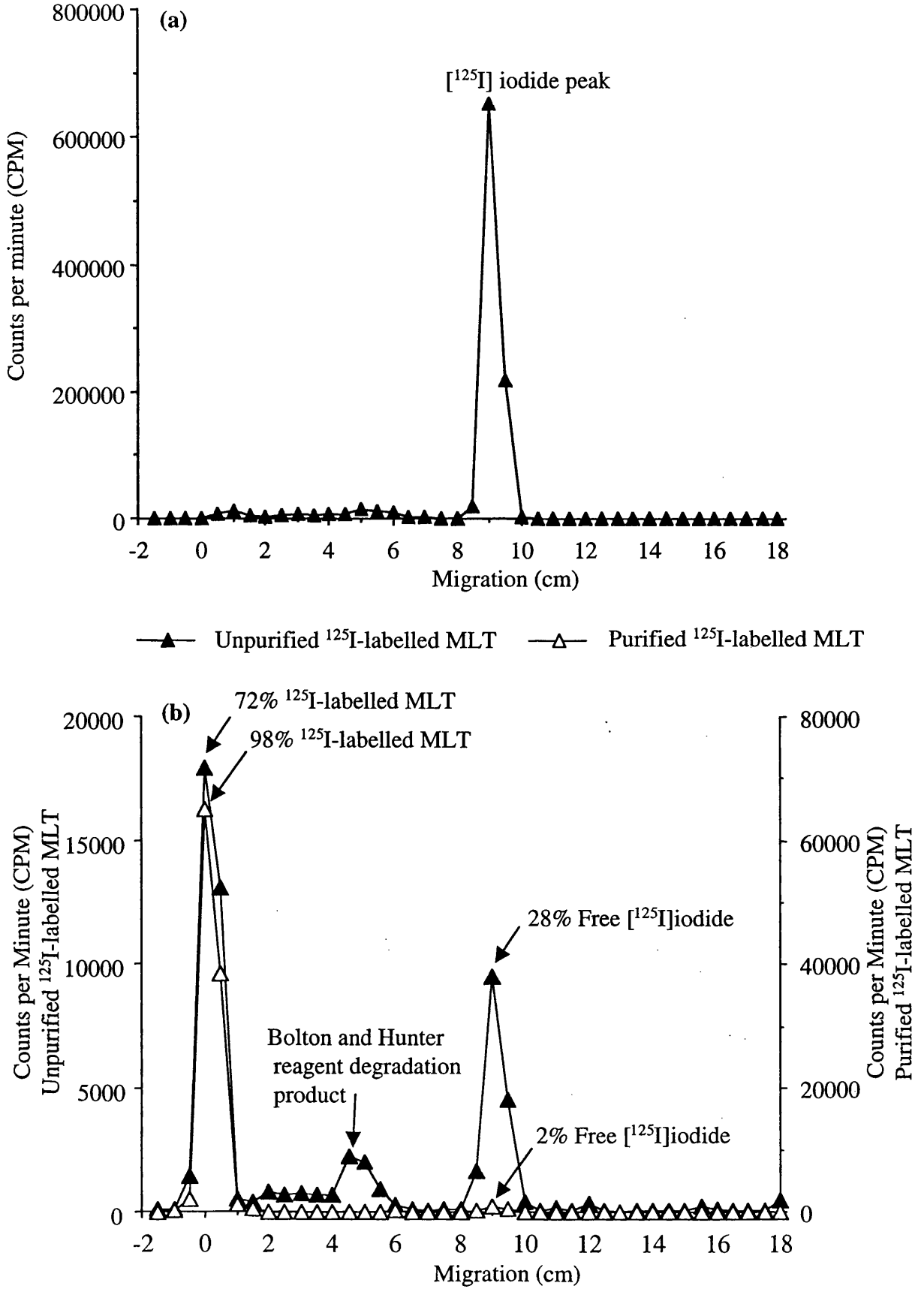
Paper electrophoresis of the reaction mixture and the purified ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT are shown in Figure 5.2. It can be seen the labelling efficiency of MLT and HPMA copolymer-MLT was 72 % and 97 % respectively. After purification, the free [^{125}I]iodide content was only 0.3 - 2 % so they were considered suitable for the biodistribution studies. The characteristics of the ^{125}I-labelled probes are shown in Table 5.1.

Time-Dependent Biodistribution Study

First, time-dependent body distribution after i.p. administration was examined and these data are summarised in Figure 5.3. The data obtained which show the distribution of radioactivity in each organ are summarised in Figure 5.4 - 5.11.

^{125}I-Labelled MLT and the ^{125}I-labelled HPMA copolymer-MLT conjugate passed relatively quickly out of the peritoneal cavity and into the circulation (Figure 5.4). Blood levels of radioactivity showed similar peak levels of 37.95 ± 0.55 % injected dose/ml for ^{125}I-labelled MLT and 43.57 ± 6.56 % injected dose/ml for ^{125}I-labelled HPMA copolymer-MLT. These peak levels occurred at 2 h and 4 h respectively. This is mirrored by the peak radioactivity recovery of ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT at 2 h and 4 h respectively (Table 5.2). A significantly greater amount of radioactivity was seen in the circulation at 6 h (19.91 ± 1.99 % injected dose/ml) after administration of ^{125}I-labelled HPMA copolymer-MLT than that seen after injection of ^{125}I-labelled MLT (1.27 ± 0.81 % injected dose/ml) (p = 0.001).

Figure 5.2 Paper electrophoresis of (a) Bolton and Hunter diiodo reagent (b) the ^{125}I-labelled MLT reaction mixture and purified product and (c) the ^{125}I-labelled HPMA copolymer-MLT (RM 3.4) reaction mixture and purified product



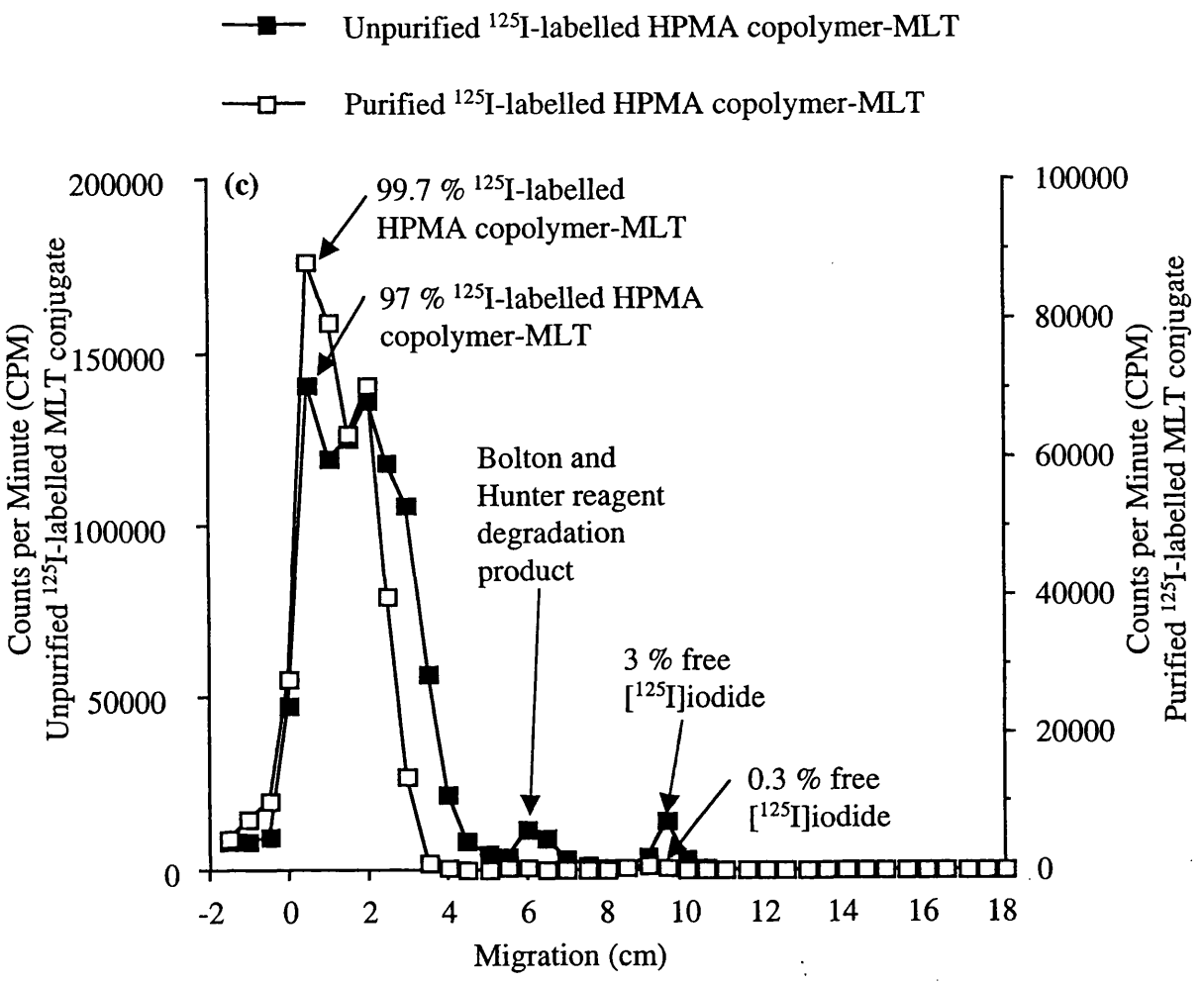

Unpurified ^{125}I-labelled HPMA copolymer-MLT
Purified ^{125}I-labelled HPMA copolymer-MLT
(c)
99.7 % ^{125}I-labelled HPMA copolymer-MLT
97 % ^{125}I-labelled HPMA copolymer-MLT
Bolton and Hunter reagent degradation product
3 % free [^{125}I]iodide
0.3 % free [^{125}I]iodide
Counts per Minute (CPM) Unpurified ^{125}I-labelled MLT conjugate
Counts per Minute (CPM) Purified ^{125}I-labelled MLT conjugate
200000
150000
100000
50000
0
100000
80000
60000
40000
20000
0
-2
0
2
4
6
8
10
12
14
16
18
Migration (cm)

Figure 5.3 Biodistribution of (a) ^{125}I-labelled MLT and (b) ^{125}I-labelled HPMA copolymer-MLT (RM 3.4) administered as a single dose (i.p.). Data represents mean ± SE (n=3)

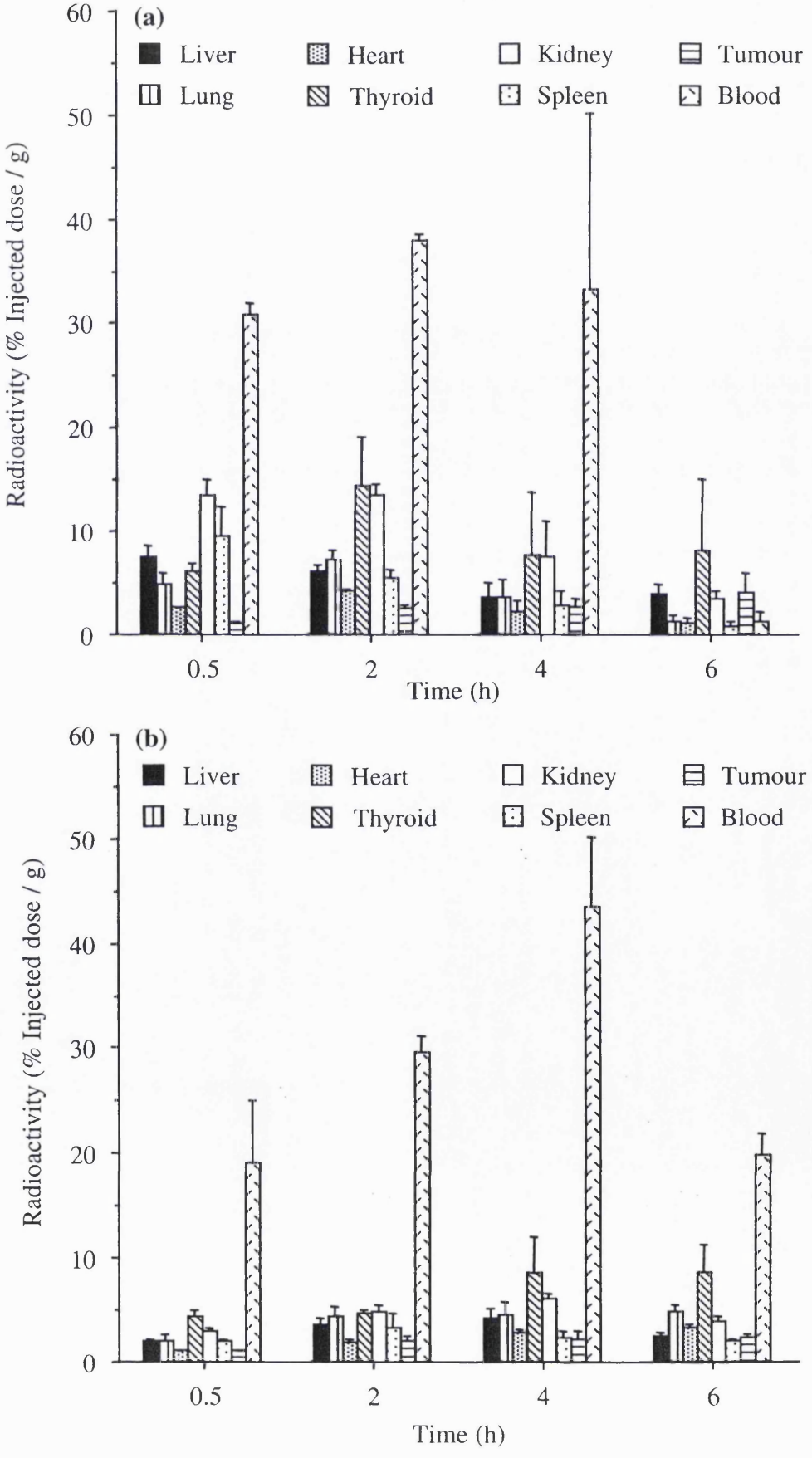


Figure 5.4 Radioactivity in the blood after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value *** < 0.001; ^{NS} = not significant

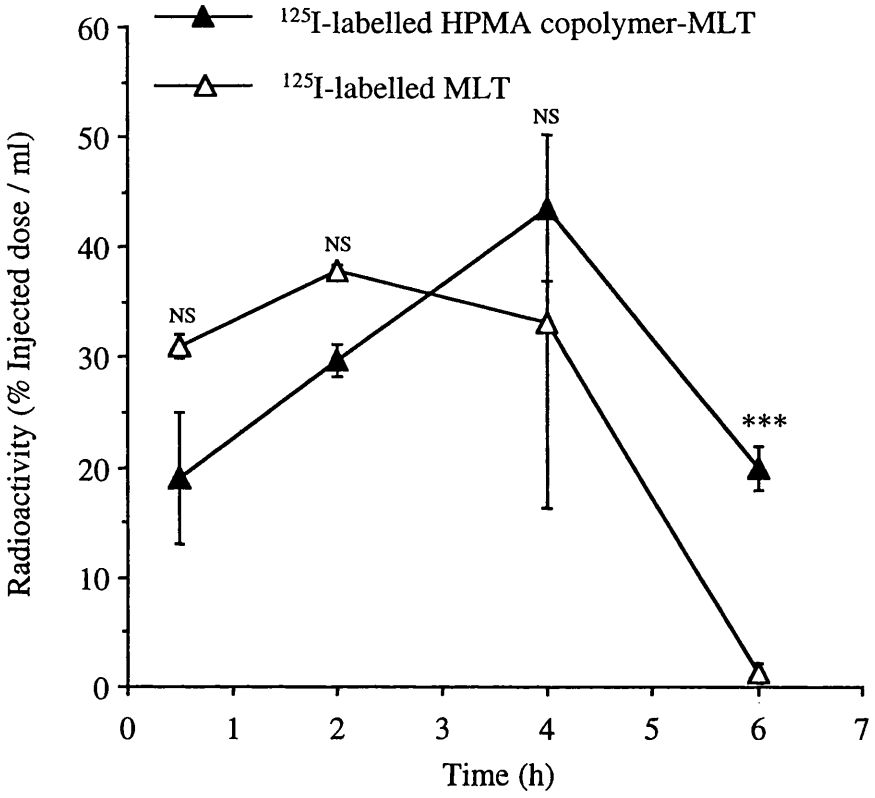


Figure 5.5 Radioactivity in the kidneys after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value ** < 0.005; ^{NS} = not significant

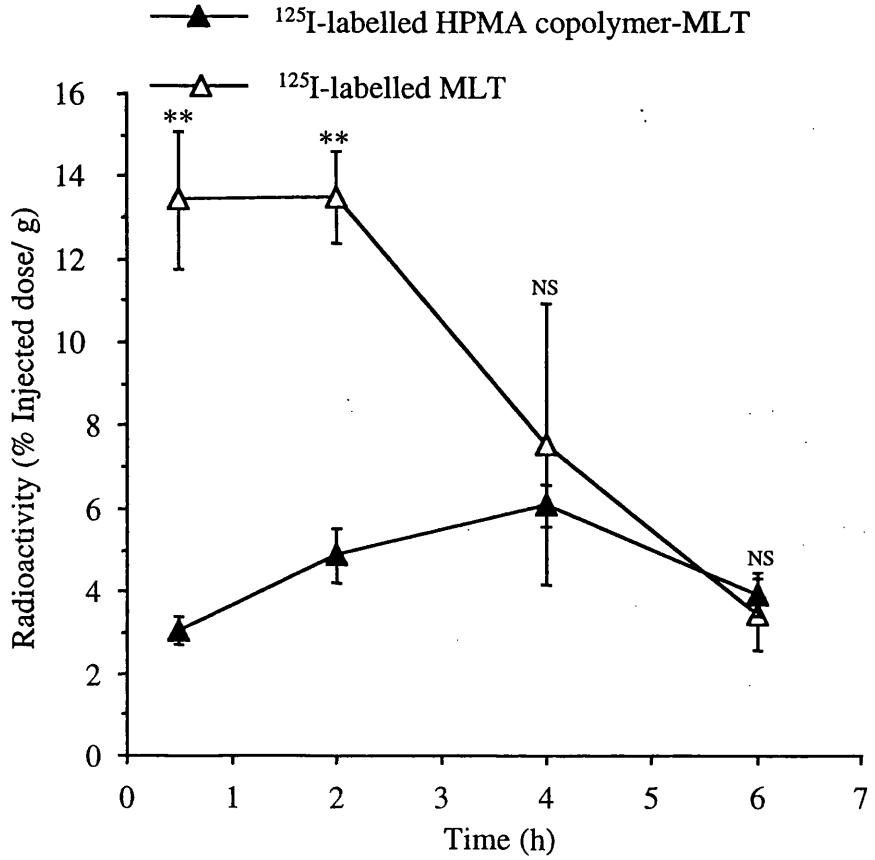


Figure 5.6 Radioactivity in the heart after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value *** < 0.001; ** < 0.005; ^{NS} = not significant

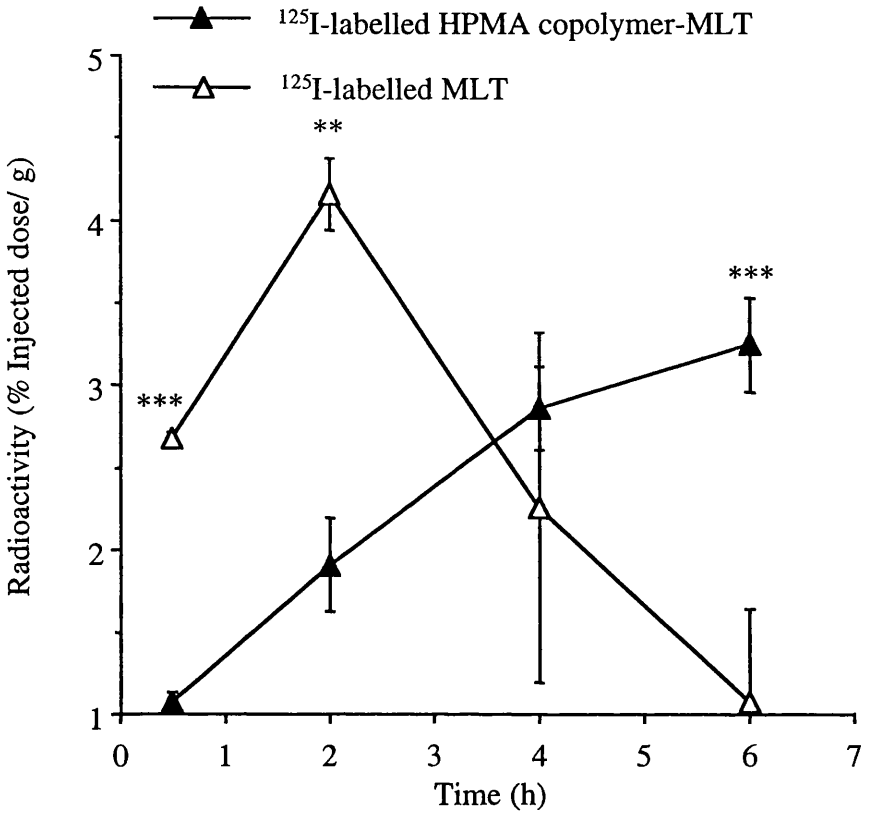


Figure 5.7 Radioactivity in the liver after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value * < 0.05; ^{NS} = not significant

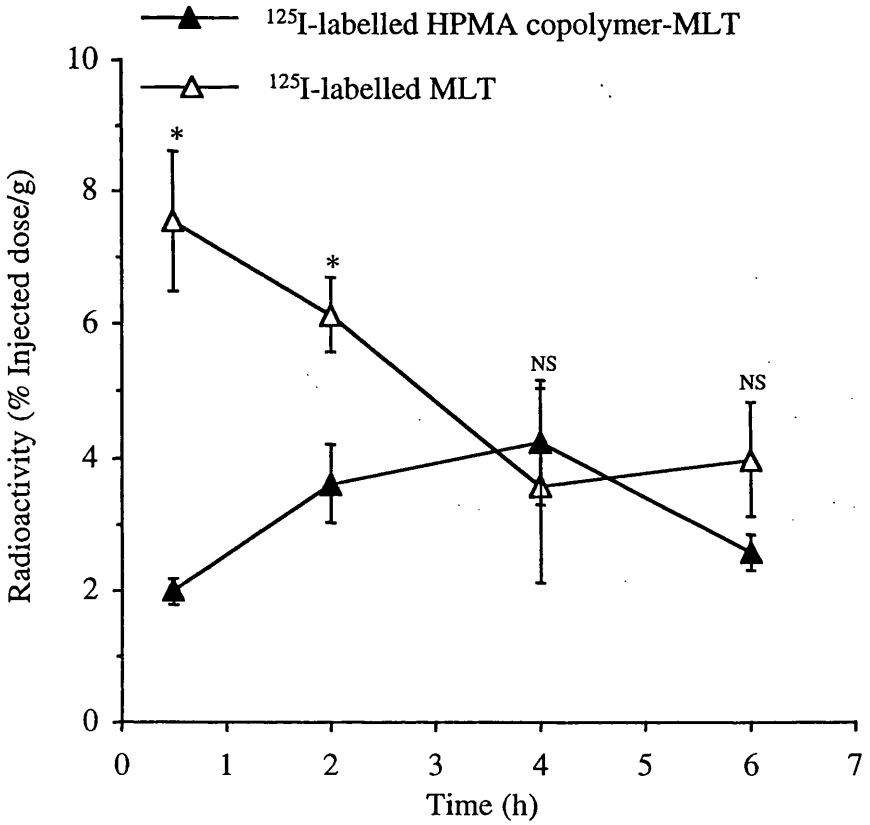


Figure 5.8 Radioactivity in the lungs after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value * < 0.05; ^{NS} = not significant

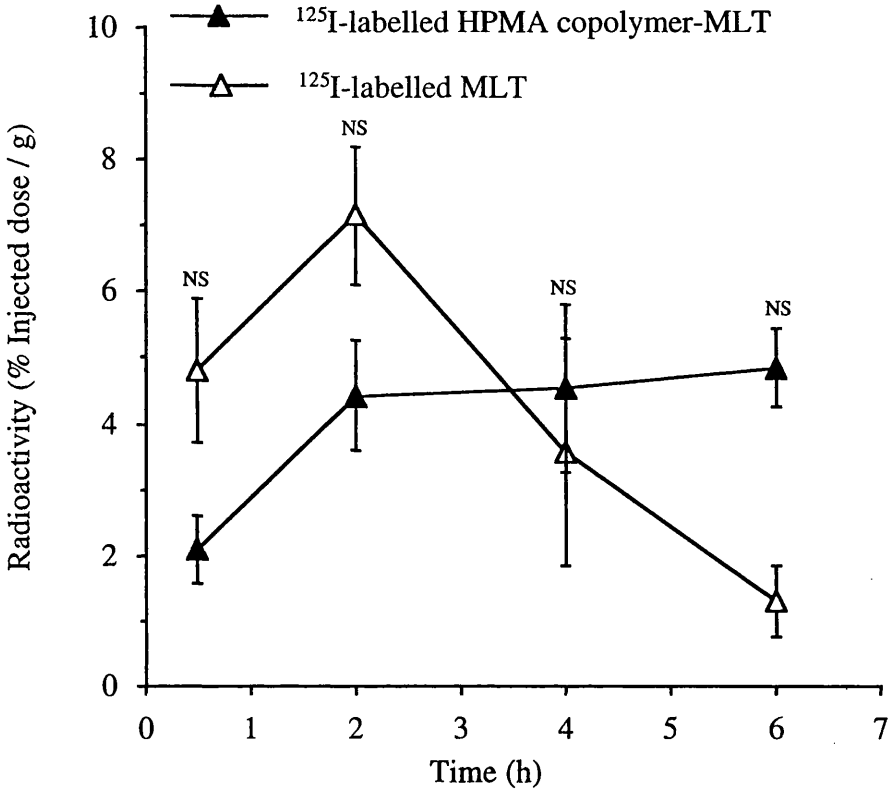


Figure 5.9 Radioactivity in the spleen after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value * < 0.05; ^{NS} = not significant

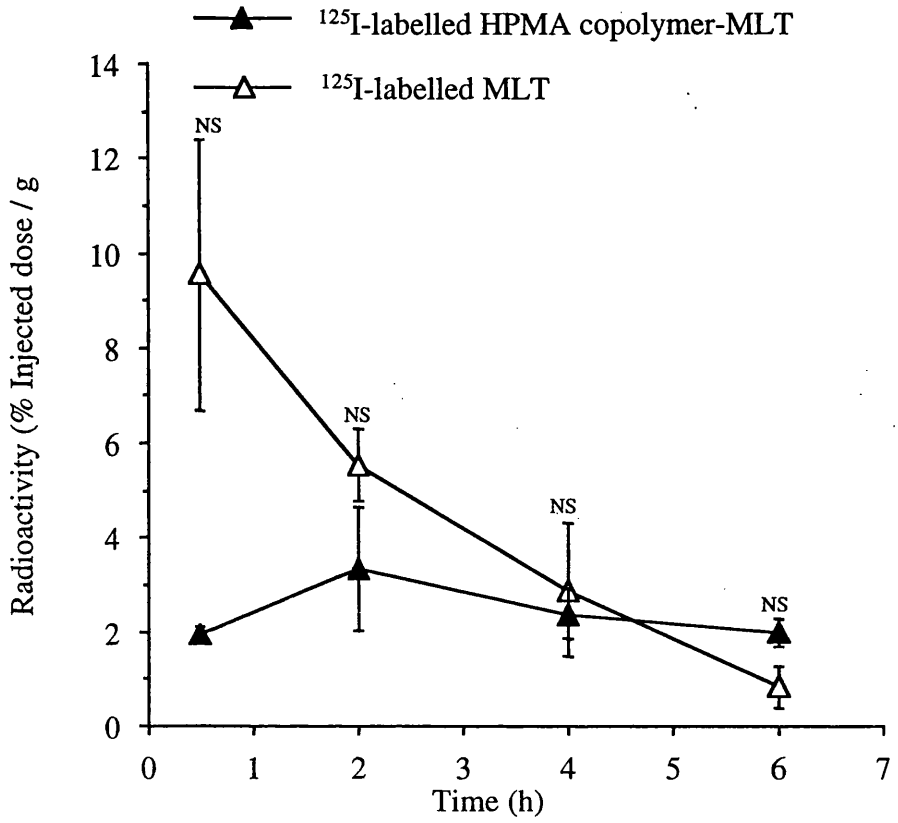


Figure 5.10 Radioactivity in the thyroid after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value ^{NS} = not significant

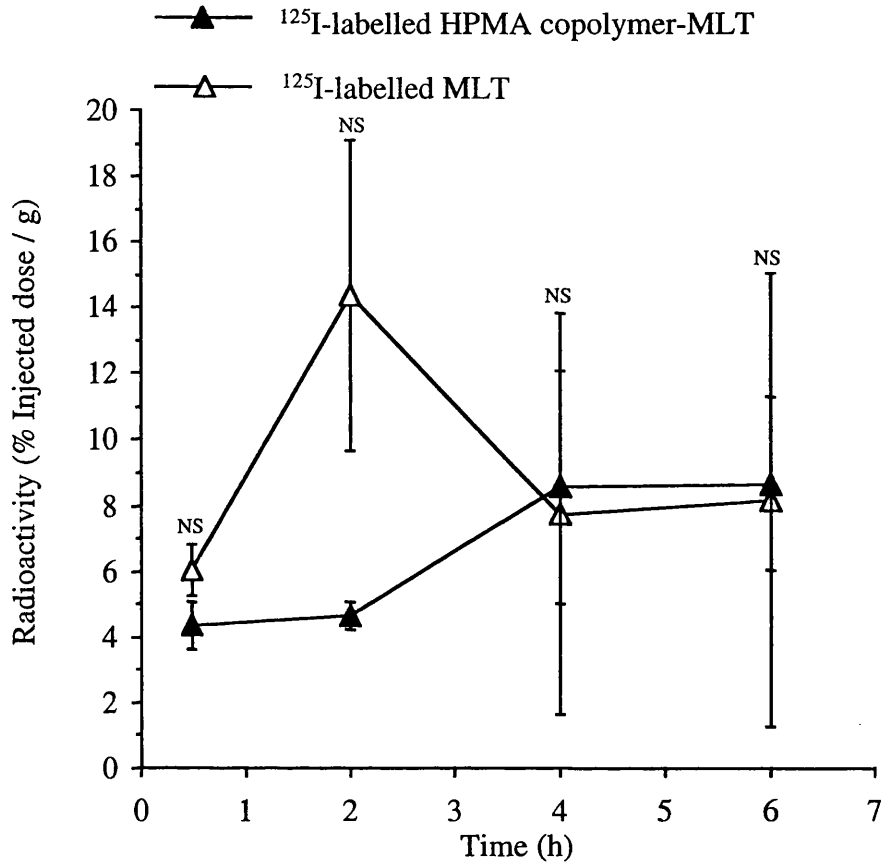


Figure 5.11 Radioactivity in the tumour after administration of ^{125}I-labelled MLT or HPMA copolymer-MLT (RM 3.4). Data represents mean ± SE (n=3). *p* value ^{NS} = not significant

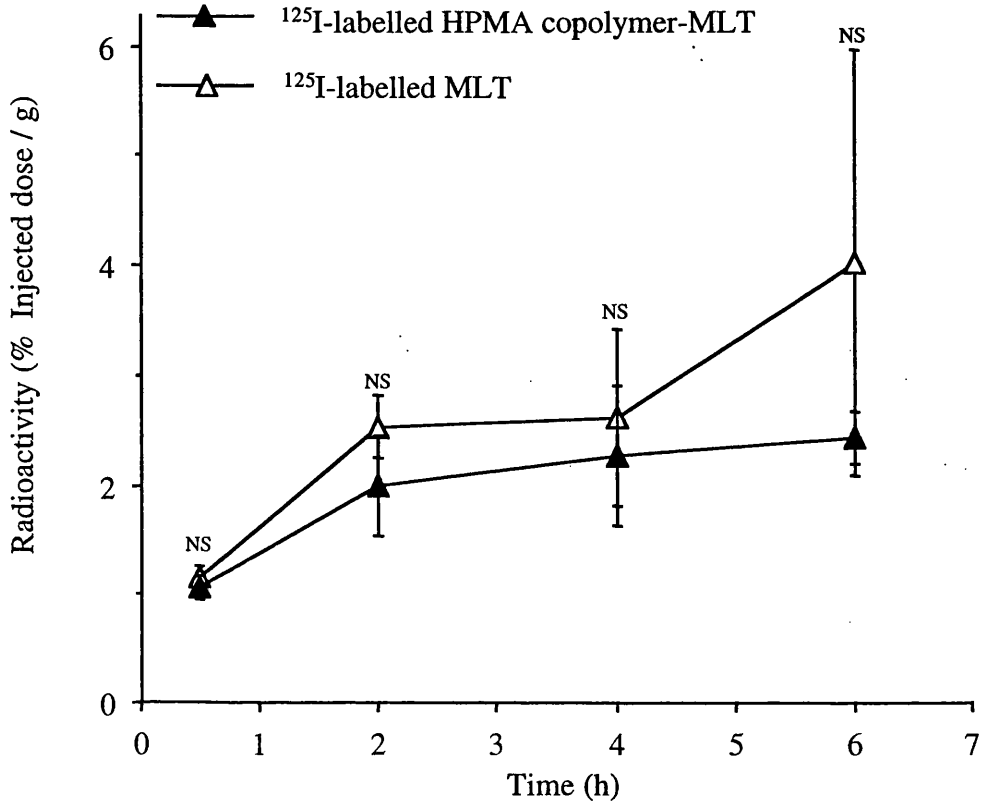


Table 5.2 Total radioactivity recovery following administration (i.p.) of ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT (RM 3.4) to tumour bearing mice (n = 3). Data represents mean ± SE

Time (h)	MLT Total Radioactivity Recovered (% Injected Dose)	HPMA Copolymer-MLT Total Radioactivity Recovered (% Injected Dose)
0.5	64.42 ± 3.09	32.56 ± 9.15
2	71.60 ± 9.83	51.15 ± 4.83
4	61.00 ± 29.91	73.99 ± 10.95
6	16.57 ± 3.67	41.33 ± 5.70

The relatively high blood levels of radioactivity seen after injection of ^{125}I-labelled MLT correlated with the greater levels of radioactivity seen in the kidneys at the early time points (30 min and 2 h) (13.43 ± 1.67 and 13.49 ± 1.11 % injected dose/g respectively). Kidney radioactivity seen after injection of ^{125}I-labelled HPMA copolymer-MLT peaked at 4 h (6.07 ± 0.52 % injected dose/g). In contrast, the highest kidney level seen for the conjugate was approximately 2-fold less than the MLT peak at 2 h (Figure 5.5).

Heart levels of radioactivity after injection of ^{125}I-labelled MLT peaked at 2 h (4.15 ± 0.21 % injected dose/g) which correlated with peak radioactivity levels in the circulation. In contrast, the heart associated radioactivity steadily rose up to 3.24 ± 0.28 % injected dose/g 6 h after administration of ^{125}I-labelled HPMA copolymer-MLT (Figure 5.6).

Radioactivity levels of ^{125}I-labelled MLT in the liver were initially higher than with the ^{125}I-labelled MLT conjugate at the early time points (30 min and 2 h) (Figure 5.7). Peak radioactivity levels of ^{125}I-labelled HPMA copolymer-MLT were observed 4 h after administration.

A similar profile for radioactivity accumulated with time for both ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT was observed in the lungs (Figure 5.8) and spleen (Figure 5.9).

Disappointingly, the radioactivity seen in the B16F10 tumour were similar after 6 h (< 4 % injected dose/g) following injection of free ^{125}I-labelled MLT or the ^{125}I-labelled conjugate (Figure 5.11).

Dose-Dependent Biodistribution Study

A 4 h time point was chosen to study the dose-dependency of body distribution. Table 5.3 shows the recovery (% total injected dose) of ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT at the doses investigated. Again, recoveries were generally poor and < 100 % of radioactivity was recovered in this study. It can be seen a dose-dependent recovery was not evident for either ^{125}I-labelled MLT or ^{125}I-labelled HPMA copolymer-MLT. However, a greater recovery of the ^{125}I-labelled conjugate (39.41 ± 4.51 % injected dose) relative to free ^{125}I-labelled MLT (14.68 ± 10.94 % injected dose) was achieved after administration of 3 mg/kg (MLT-equivalent).

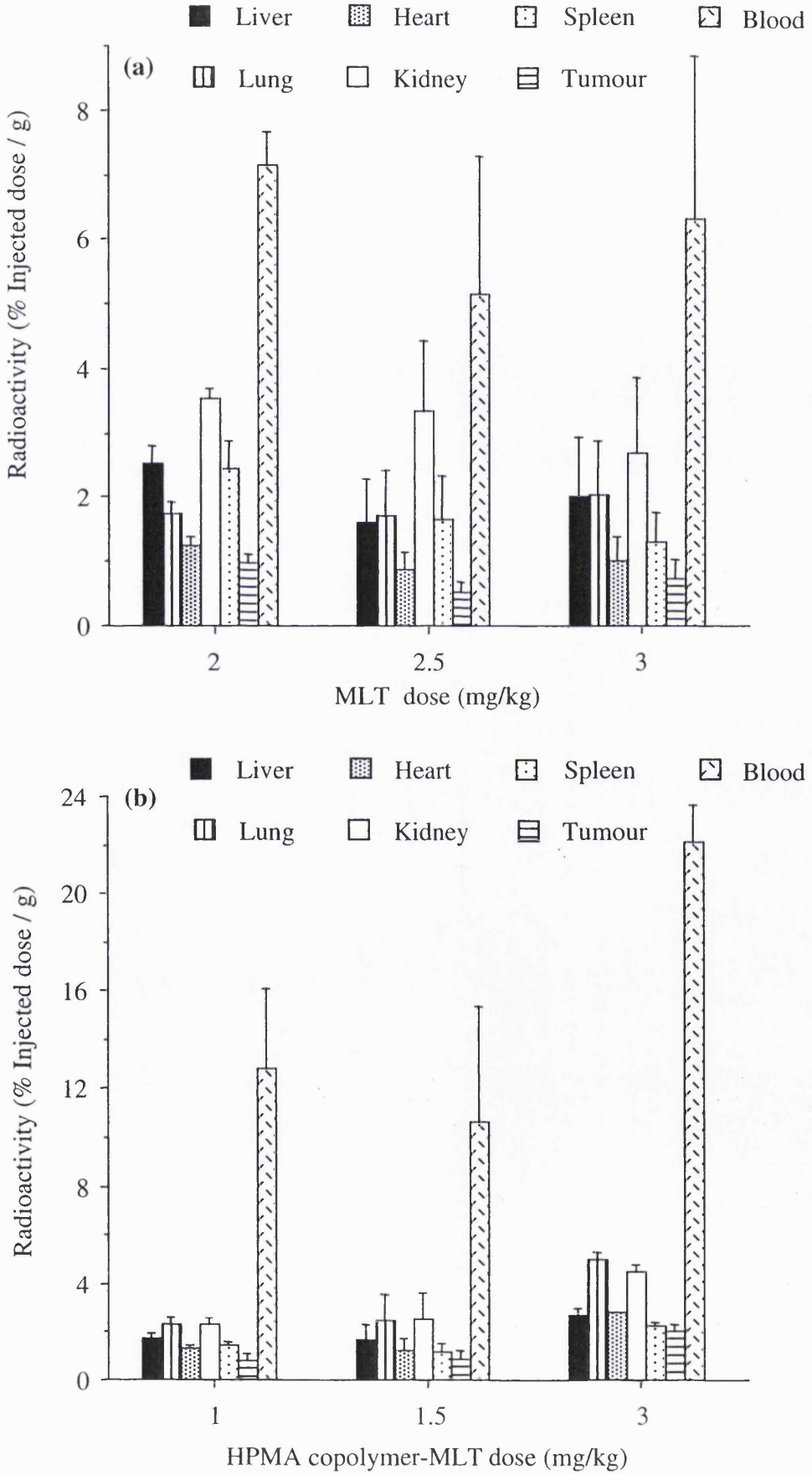
Dose administered did not show a clear effect on distribution of ^{125}I-labelled HPMA copolymer-MLT or ^{125}I-labelled MLT (Figure 5.12). Direct comparison of biodistribution of ^{125}I-labelled MLT with the ^{125}I-labelled MLT conjugate was possible

Table 5.3 Total radioactivity recovery at 4 h following administration (i.p.) of ^{125}I labelled MLT and ^{125}I-HPMA copolymer-MLT (RM 3.4 "spiked" with RM 3.9) to tumour-bearing mice (n = 3). Data represents mean ± SE

MLT		HPMA Copolymer-MLT	
Dose Injected (mg/kg)	**Radioactivity Recovered (% Injected Dose)**	**Dose Injected (mg/kg[§])**	**Radioactivity Recovered (% Injected Dose)**
2	17.33 ± 3.77	1	21.9 ± 7.40
2.5	11.49 ± 8.90	1.5	19.70 ± 8.58
3	14.68 ± 10.94	3	39.41 ± 4.51

[§]MLT-equivalent

Figure 5.12 Dose-dependent biodistribution of (a) ^{125}I-labelled MLT and (b) ^{125}I-labelled HPMA copolymer-MLT 4 h following i.p. administration. Data represents mean ± SE (n = 3).



at the 3 mg/kg (MLT-equivalent) dose (Figure 5.13). ^{125}I-labelled HPMA copolymer-MLT had significantly higher levels (approximately 3 – 4-fold) in the circulation. Additionally, significantly higher levels of the ^{125}I-labelled conjugate were observed in the lungs (~ 2-fold), heart (~ 3-fold) and tumour (~ 2-fold) at this time point relative to free ^{125}I-labelled MLT.

5.3.2 Antitumour Activity of MLT and HPMA copolymer-MLT

MTDs of MLT and HPMA copolymer-MLT administered as a single dose via the i.p. route were found to be 2.5 mg/kg and 10 mg/kg (MLT-equivalent) respectively. In both cases, animals did not exceed 10 % loss of body weight (Figure 5.14). Although no significant loss of body weight was seen for HPMA copolymer-MLT at 20 mg/kg (MLT-equivalent), animals showed initial signs of distress after administration and so the MTD was defined to be 10 mg/kg (MLT-equivalent).

Single Dose i.p.

The first experiment examined the antitumour activity of MLT and the HPMA copolymer-MLT conjugate given by single dose i.p to treat a palpable B16F10 s.c. tumour. A summary of the results obtained is given in Table 5.4. Once s.c. B16F10 tumours reached a size of 289 mm^2 (~ 0.7 cm x 0.7 cm), the control (untreated group) had an average survival time of 6.4 ± 0.9 days. At the doses chosen for this study (based on the earlier work in non-tumour bearing mice) both MLT and HPMA copolymer-MLT did not show an improvement in survival (4.8 ± 1.79 and 3.2 ± 1.0 days respectively). There were no significant differences in tumour sizes of animals treated with free MLT or the MLT conjugate compared with animals in the control group up to day 4 of the study (Figure 5.15). Daily measurements of weight showed inter-animal variation thus individual animal data rather than a mean value were plotted (Figure 5.16). A similar trend in animal weights was observed for test compounds and control groups. Although weight monitoring did not indicate toxicity in the MLT treatment group, four animals showed signs of distress on day 4 and were killed. Although this was not also observed for animals treated with the conjugate, all tumour sizes exceeded Home Office restrictions (289 mm^2) by day 4 of the study.

Multiple Dose i.p.

In order to try and improve activity, a study using lower multiple doses of MLT and HPMA copolymer-MLT was undertaken. In this experiment, a treatment schedule

Figure 5.13 Biodistribution of ^{125}I-labelled MLT and HPMA copolymer-MLT 4 h following administration (i.p.) of 3 mg/kg MLT-equivalent. Data represents mean ± SE (n = 3). *p* value * < 0.05; ** < 0.005; ^{NS} = not significant

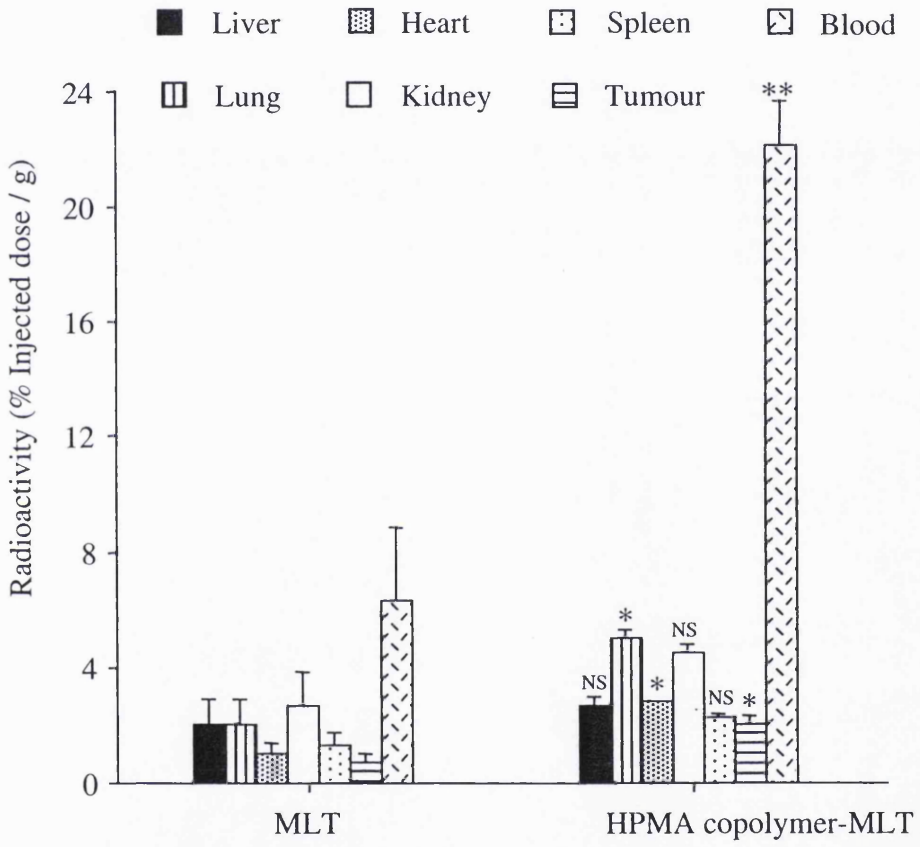


Figure 5.14 Effect of (a) MLT and (b) HPMA copolymer-MLT (RM 3.4) on animal weight after single dose i.p. (n = 2)

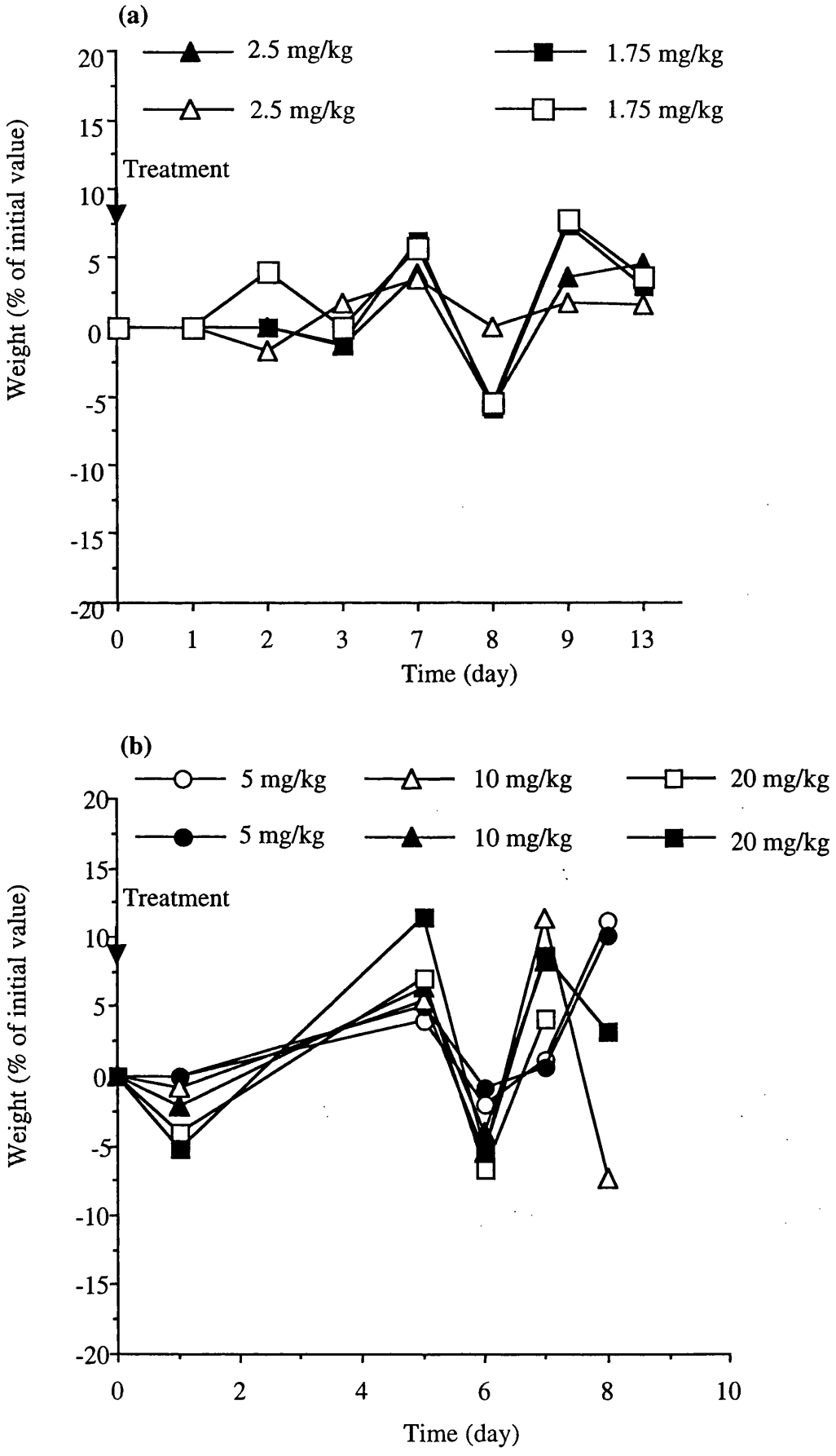
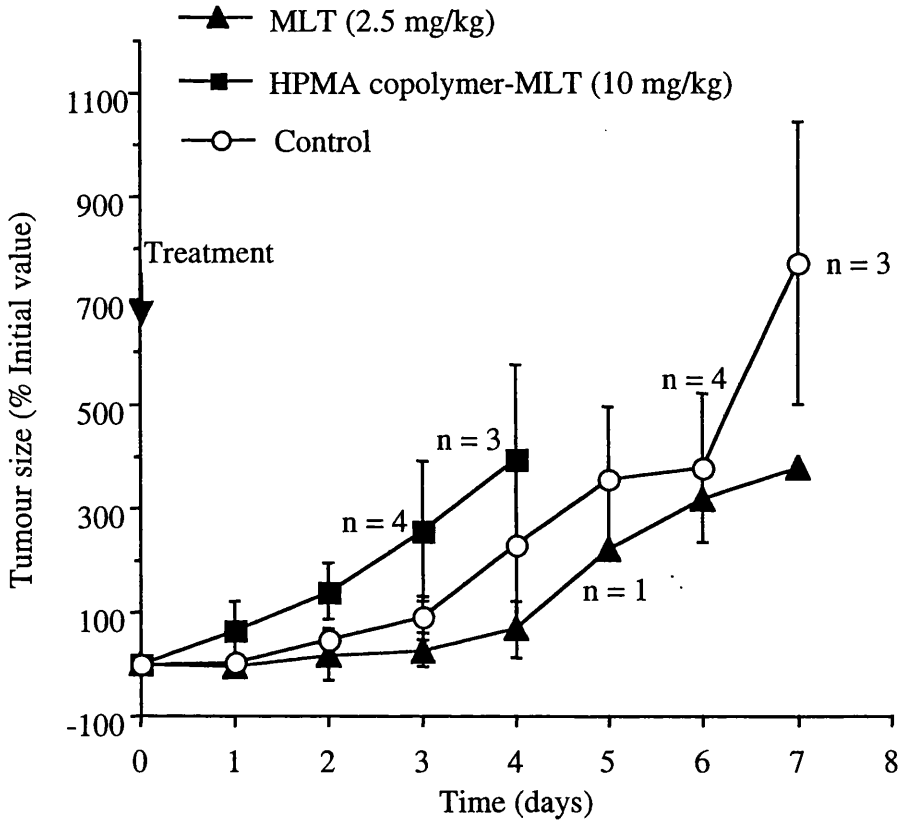


Table 5.4 Antitumour activity of MLT and HPMA copolymer-MLT (RM 3.7) against s.c. B16F10 after administration by single dose (i.p.). *p* value [NS] = not significant

Treatment	Dose (mg/kg[§])	Day of Treatment	Survival (days)	Toxic deaths	Mean Survival[‡] (days)	T/C[‡] (%)
Control	-	0	7, 5, 6, 7, 7	0/5	6.4 ± 0.9	100
MLT	2.5	0	4[†], 4[†], 4[†], 4[†], 8	4/5	4.8 ± 1.8 [NS]	75.0 ± 28.0
HPMA copolymer-GG-MLT[¥]	10	0	4, 2, 4, 2, 3, 4	0/5	3.2 ± 1.0 [NS]	49.5 ± 15.4

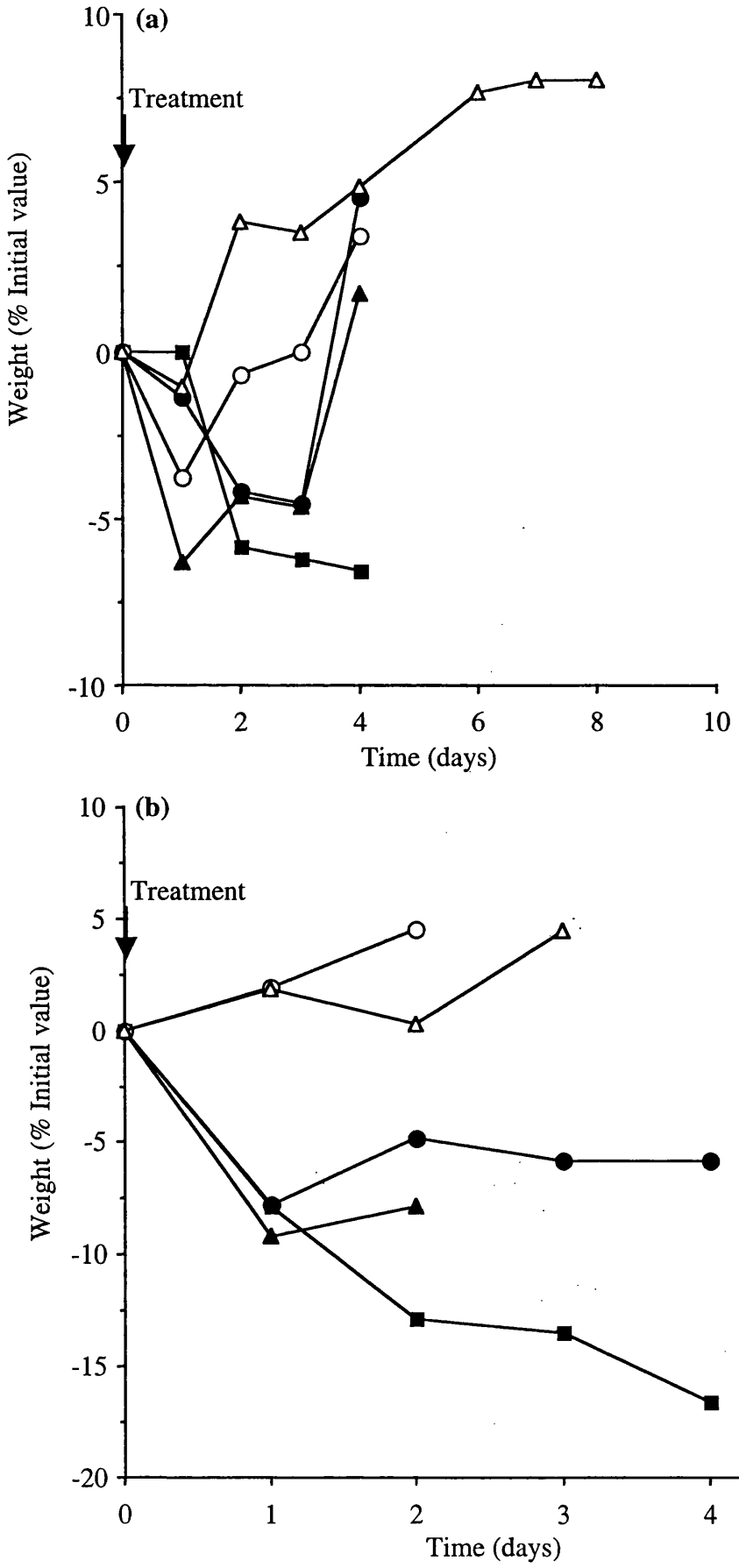
[‡]Results are expressed as mean ± SD
[§] MLT-equivalent
[†] Toxic deaths
[¥] Batch RM 3.7 (31.7 % w/w)

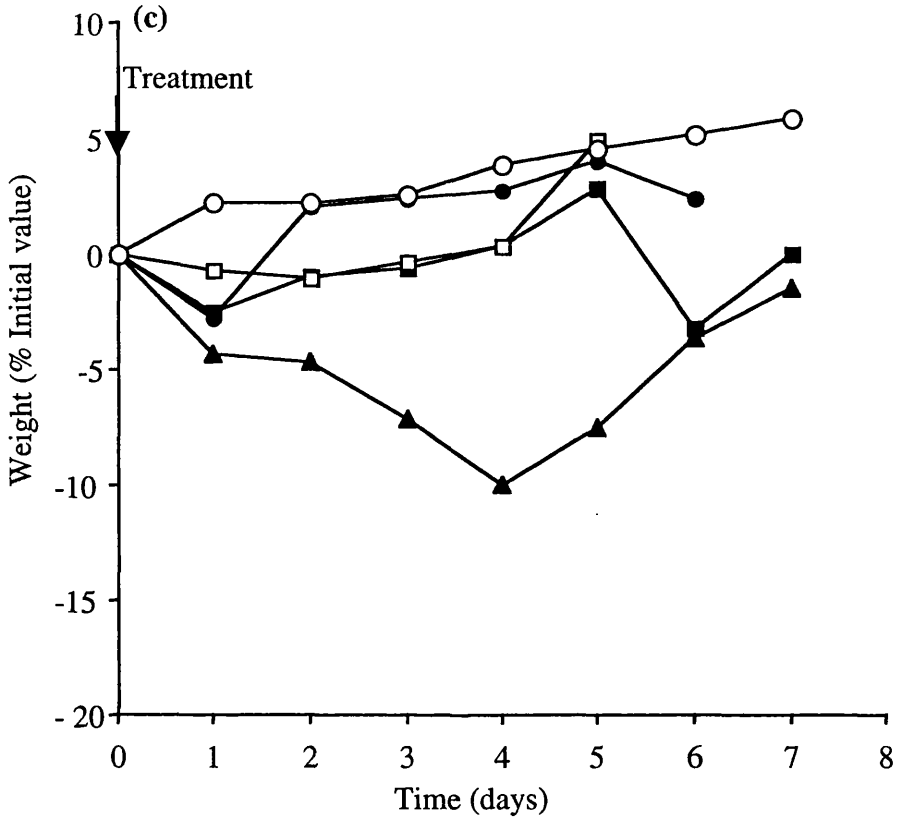
Figure 5.15 Effect of a single dose (i.p.) of HPMA copolymer-MLT (RM 3.7) (10 mg/kg) and MLT (2.5 mg/kg) on s.c. B16F10 tumour size. Data represents mean ± SD (n = 5 unless otherwise stated).



Note: Decrease in numbers due to toxic deaths or if tumour size ≥ 0.7 cm x 0.7 cm or 289 mm^2

Figure 5.16 Effect of (a) MLT (2.5 mg/kg); (b) HPMA copolymer-MLT (RM 3.7) (10 mg/kg) and (c) control (i.p.) on animal weight. In each case the weight change of each mouse in the group is shown




(c)
Treatment
10
5
0
-5
-10
-15
- 20
Weight (% Initial value)
0
1
2
3
4
5
6
7
8
Time (days)

of day 0, 2 and 4 was chosen because increased frequency of dosing of PK1 has shown better uptake by the EPR effect (Seymour *et al*, 1994). The results are summarised in Table 5.5. Similar to the previous study above, in this case, the untreated control group survival was 7.0 ± 0.7 days after the start of the experiment. At the doses chosen for this study, MLT (1, 0.5 and 0.1 mg/kg) did not improve animal survival time (6.0 ± 1.9 - 6.8 ± 1.6 days). Similarly, for animals in the HPMA copolymer-MLT treated groups, no increase in survival time was observed (6.4 ± 1.7 - 7.2 ± 2.3 days).

In both cases, MLT and HPMA copolymer-MLT at these doses had no effect on tumour size (Figure 5.17). Additionally, there was no evident dose-dependency of antitumour activity. Again, daily measurements of weight showed inter-animal variation thus individual data were plotted (Figures 5.18 – 5.20). A similar trend in animal weights was observed for all test compounds in comparison to the control group indicating none of the compounds had reached their MTD. 2/5 animals each in two MLT treatment groups (1 and 0.1 mg/kg), however, showed signs of distress and were killed. No toxic deaths were observed for animals treated with the HPMA copolymer-MLT at this dose range.

Multiple dose i.v.

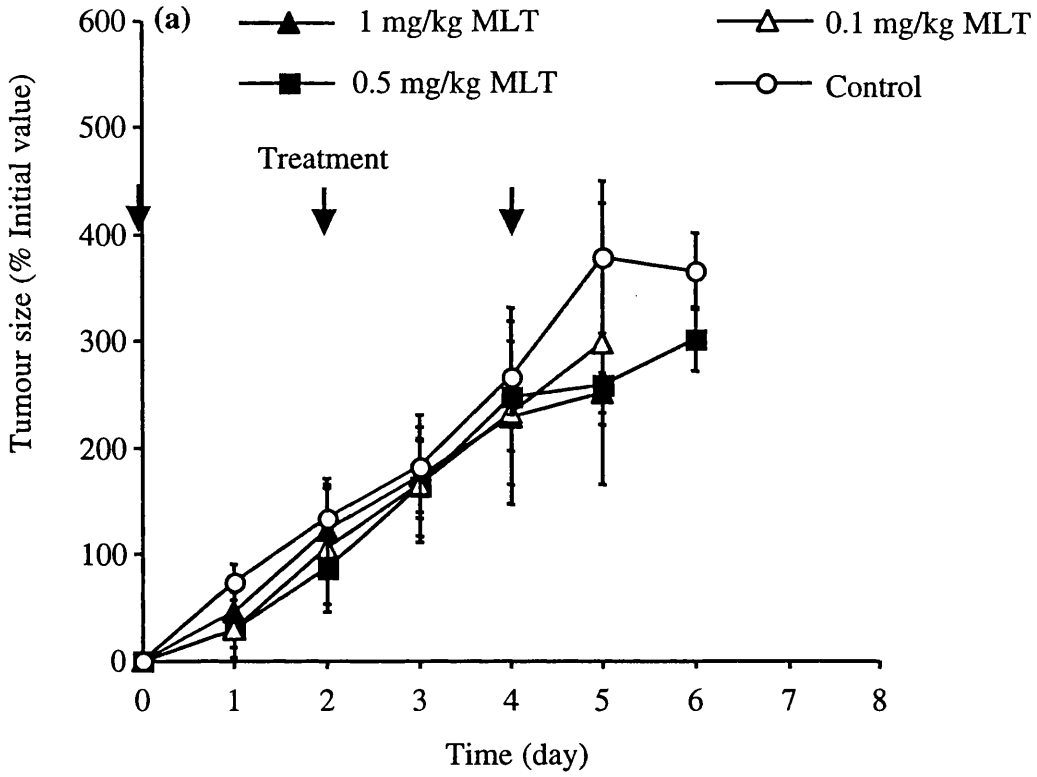
As the antitumour experiments conducted with i.p. administration of MLT and HPMA copolymer-MLT did not show beneficial antitumour activity, an experiment was carried out using a double dose schedule i.v. (days 0 and 2). In this case it was not ethically possible to attempt control experiments with MLT as it is extremely haemolytic so i.v. administration would not be possible. Therefore only the HPMA copolymer-MLT conjugate was used at a dose range of 0.1 - 2.0 mg/kg (MLT-equivalent). The results obtained in this study are documented in Table 5.6. In this experiment the untreated control group survived 7.6 ± 1.1 days. Following treatment with HPMA copolymer-MLT, only the dose of 0.5 mg/kg (MLT-equivalent) showed toxic deaths. This was surprising as the higher and lower doses did have this effect. The T/C values in the MLT conjugate treated group were 129, 126, 100 and 113 for 2, 1, 0.5 and 0.1 mg/kg (MLT-equivalent) doses respectively (Figure 5.21). None of the MLT conjugate treated groups showed an improvement in survival in comparison to the control group. Again, animal weights of conjugate treated animals were similar to the untreated control groups (Figure 5.22 and 5.23), indicating the MTD dose had not been reached.

Table 5.5 Antitumour activity of MLT and HPMA copolymer-MLT (RM 3.7) against s.c. B16F10 after administration by multiple doses (i.p.). *p* value [NS] = not significant

Treatment	Dose (mg/kg[§])	Day of Treatment	Survival (days)	Toxic deaths	Mean Survival[‡] (days)	T/C[‡] (%)
Control	-	0, 2 & 4	7, 7, 6, 7, 8	0/5	7.0 ± 0.7	100
MLT	1	0, 2 & 4	9[†], 6[†], 6, 5, 4	2/5	6.0 ± 1.9 [NS]	85.7 ± 23.9
MLT	0.5	0, 2 & 4	7, 7, 5, 4, 8	0/5	6.2 ± 1.6 [NS]	88.6 ± 23.5
MLT	0.1	0, 2 & 4	6[†], 6, 5[†], 9, 8	2/5	6.8 ± 1.6 [NS]	97.1 ± 21.0
HPMA copolymer-GG-MLT[¥]	2.5	0, 2 & 4	7, 6, 5, 5, 9	0/5	6.4 ± 1.7 [NS]	91.4 ± 23.9
HPMA copolymer-GG-MLT[¥]	1	0, 2 & 4	9, 7, 6, 5, 7	0/5	6.8 ± 1.5 [NS]	97.1 ± 21.2
HPMA copolymer-GG-MLT[¥]	0.5	0, 2 & 4	7, 7, 11, 5, 6	0/5	7.2 ± 2.3 [NS]	102.9 ± 32.6

[‡]Results are expressed as mean ± SD
[§]MLT-equivalent
[†]Toxic deaths
[¥]Batch RM 3.7 (31.7 % w/w)

Figure 5.17 Effect of multiple doses (i.p.) of (a) MLT or (b) HPMA copolymer-MLT (RM 3.9) administered on days 0, 2 and 4 on s.c. B16F10 tumour size. Data represents mean ± SE (n = 5)



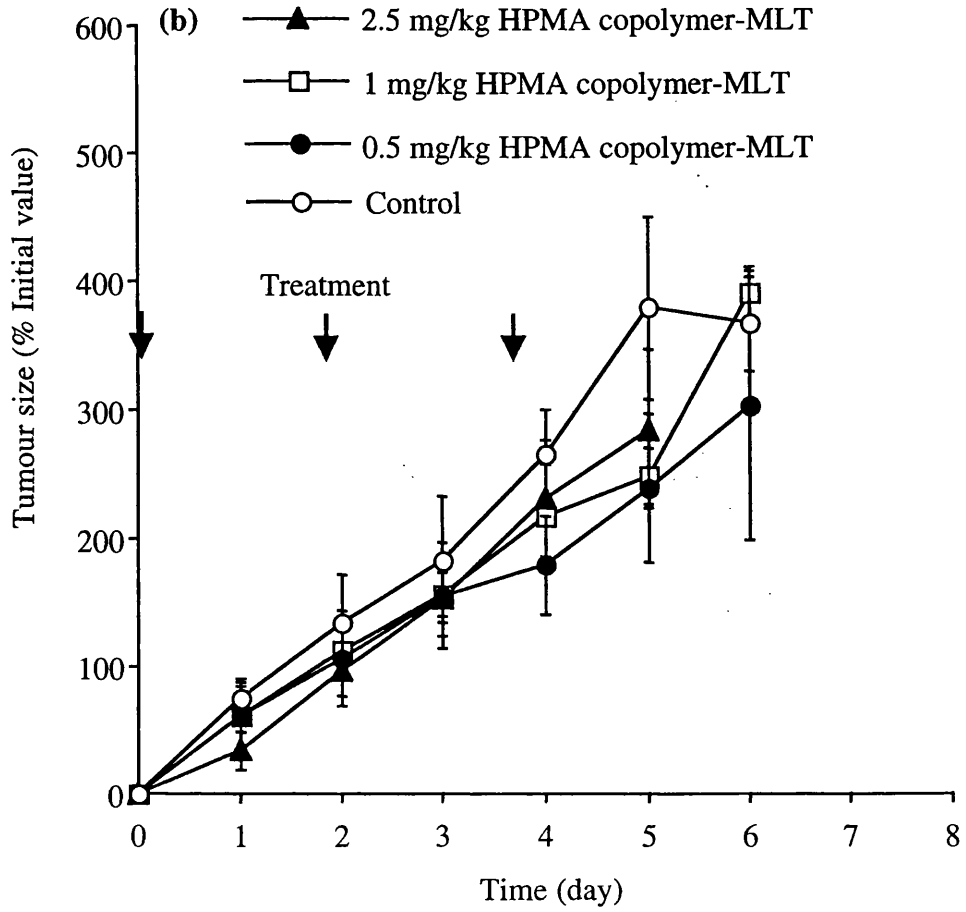
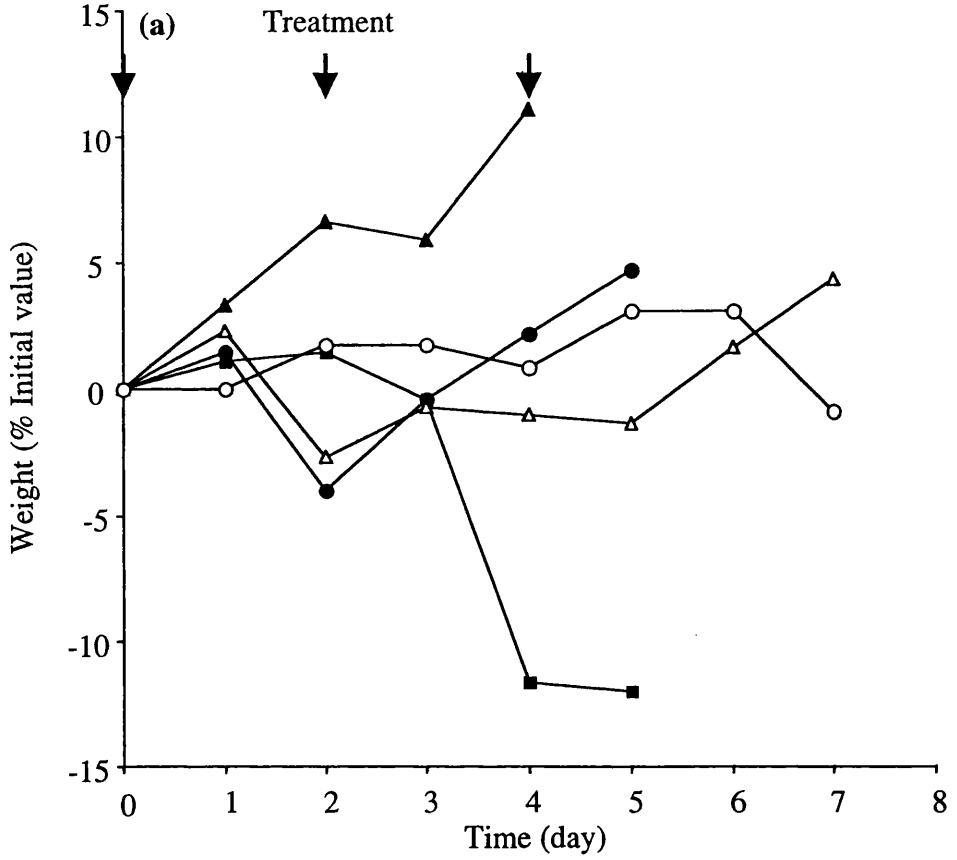
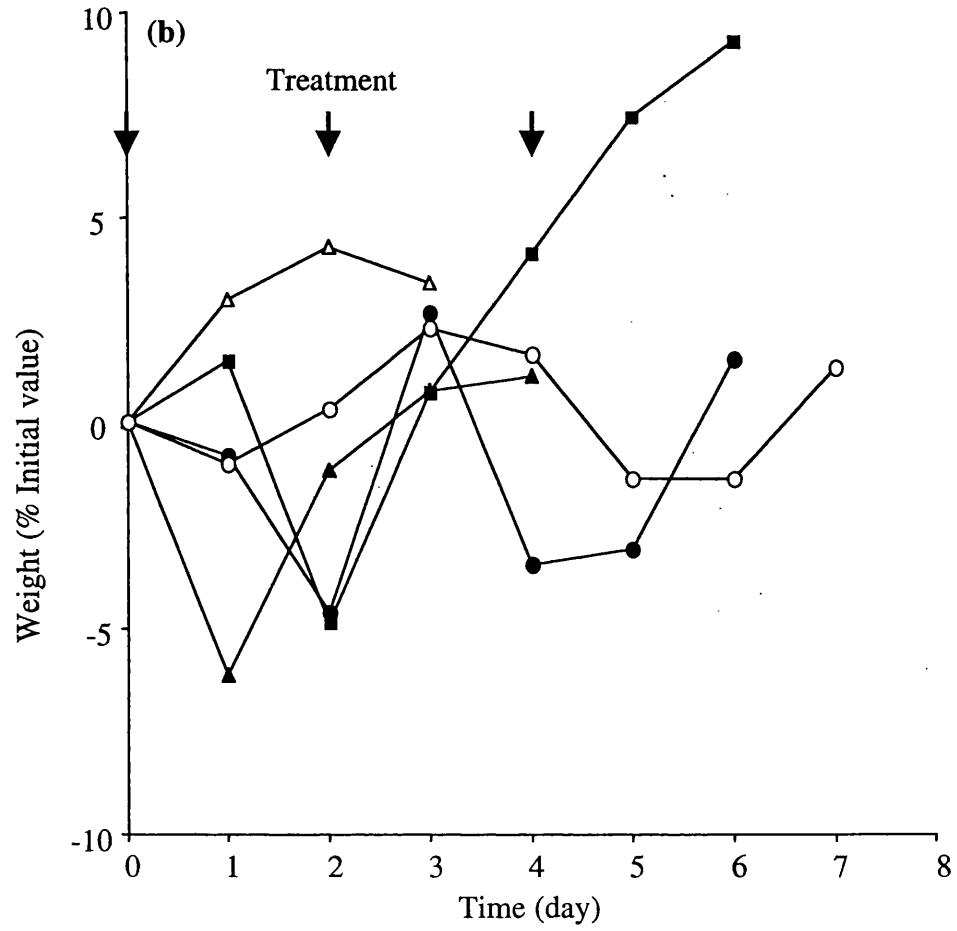


Figure 5.18 Effect of MLT (a) 0.1 mg/kg; (b) 0.5 mg/kg or (c) 1 mg/kg (i.p.) on animal weight. In each case the weight change of each mouse in the group is shown





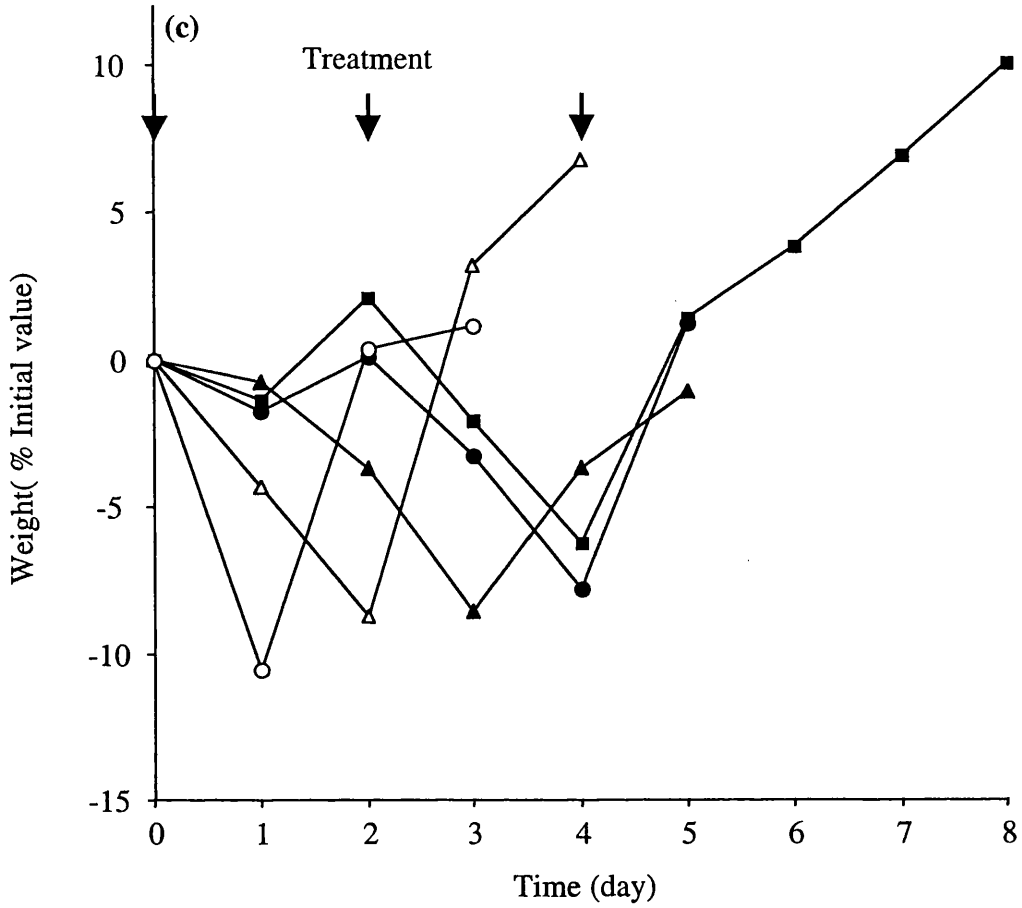
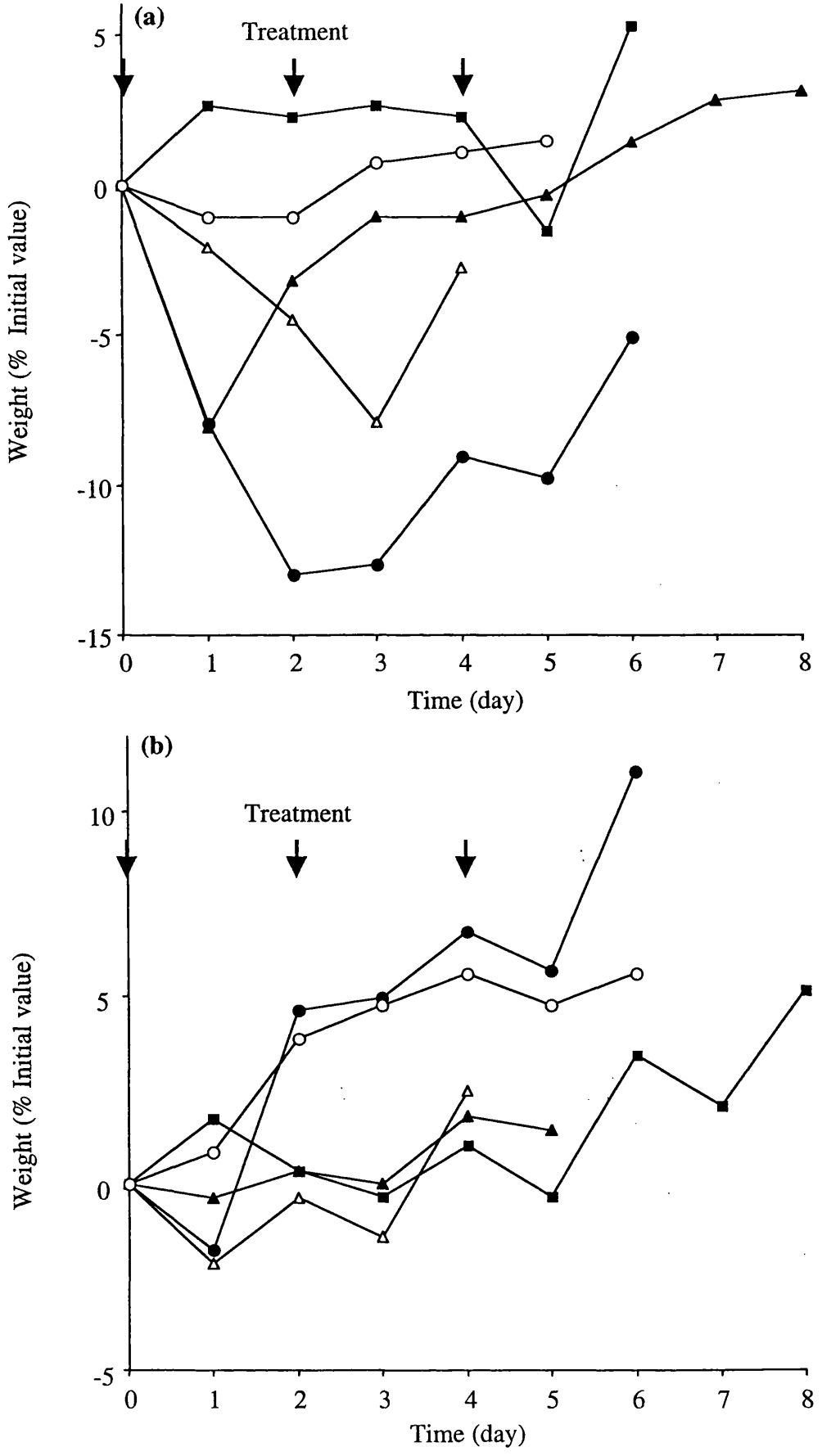

(c)
Treatment
10
5
0
-5
-10
-15
Weight(% Initial value)
0
1
2
3
4
5
6
7
8
Time (day)

Figure 5.19 Effect of HPMA copolymer-MLT (RM 3.9) (a) 0.5 mg/kg; (b) 1 mg/kg or (c) 2.5 mg/kg (i.p.) on animal weight. In each case the weight change of each mouse in the group is shown



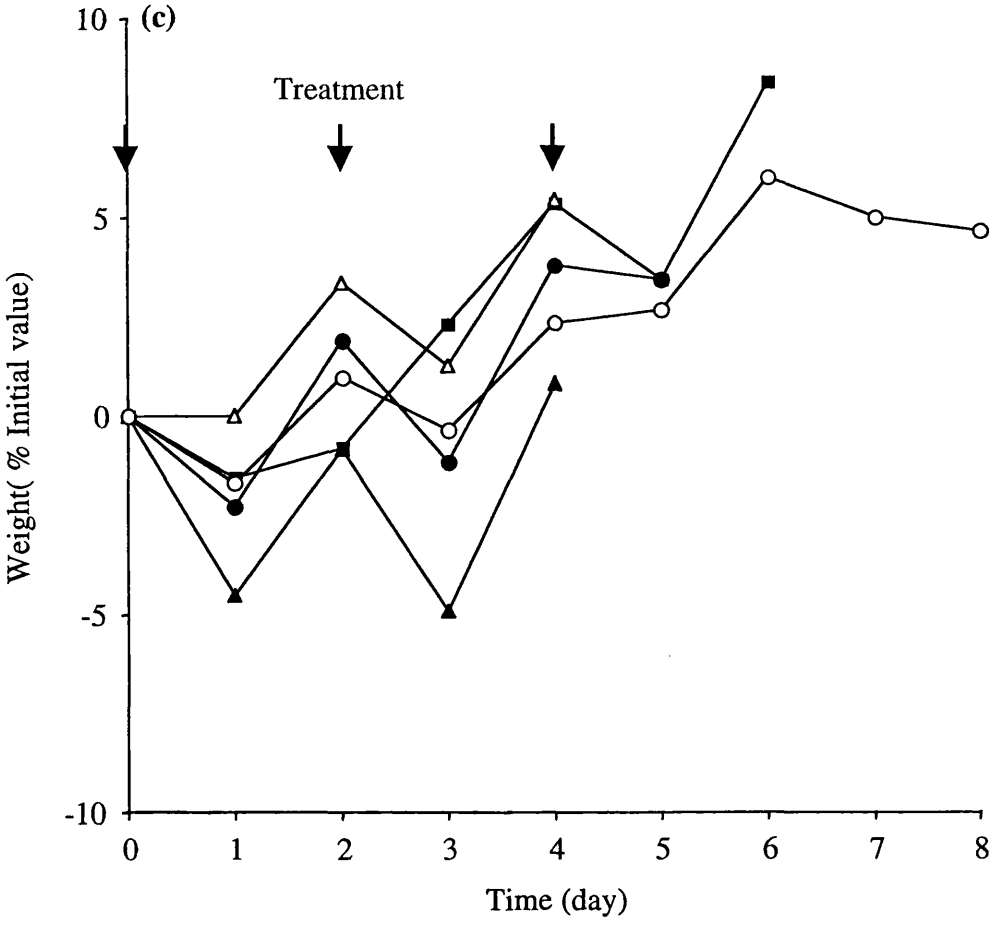

(c)
Treatment
10
5
0
-5
-10
Weight(% Initial value)
0
1
2
3
4
5
6
7
8
Time (day)

Figure 5.20 Effect of control (i.p.) on animal weight. In each case the weight change of each mouse in the group is shown

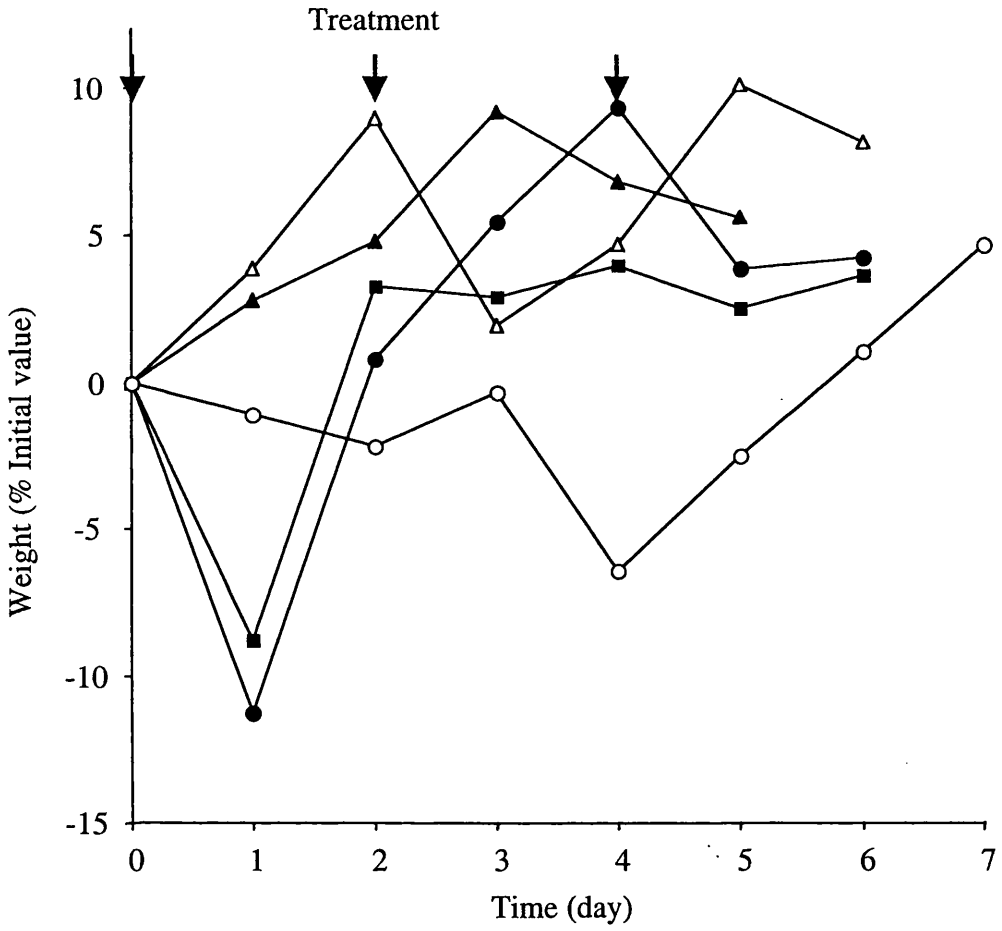


Table 5.6 Antitumour activity of MLT and HPMA copolymer-MLT (RM 3.14) against s.c. B16F10 after administration by multiple doses (i.v.). *p* value[NS] = not significant

Treatment	Dose[§] (mg/kg)	Day of Treatment	Survival (days)	Toxic deaths	Mean survival[‡] (days)	T/C[‡] (%)
Control	-	0, 2	6, 7, 8, 9, 8	0/5	7.6 ± 1.1	100
HPMA copolymer-GG-MLT[¥]	2	0, 2	8, 10, 7, 10, 14	0/5	9.8 ± 2.7[NS]	128.9 ± 20.4
HPMA copolymer-GG-MLT[¥]	1	0, 2	12, 8, 6, 10, 12	0/5	9.6 ± 2.6[NS]	126.3 ± 19.8
HPMA copolymer-GG-MLT[¥]	0.5	0, 2	[†]3, 4, 13, [†]6, 12	2/5	7.6 ± 4.6[NS]	99.9 ± 42.9
HPMA copolymer-GG-MLT[¥]	0.1	0, 2	8, 10, 7, 8, 10	0/5	8.6 ± 1.3[NS]	113.2 ± 12.5

[‡]Results are expressed as mean ± SD
[§] MLT-equivalent
[†] Toxic deaths
[¥] Batch RM 3.14 (21.7 % w/w)

Figure 5.21 Effect of multiple doses (i.v.) of HPMA copolymer-MLT (RM 3.14) administered on days 0 and 2 on s.c. B16F10 tumour size. Data represents mean ± SE (n = 5 unless otherwise stated)

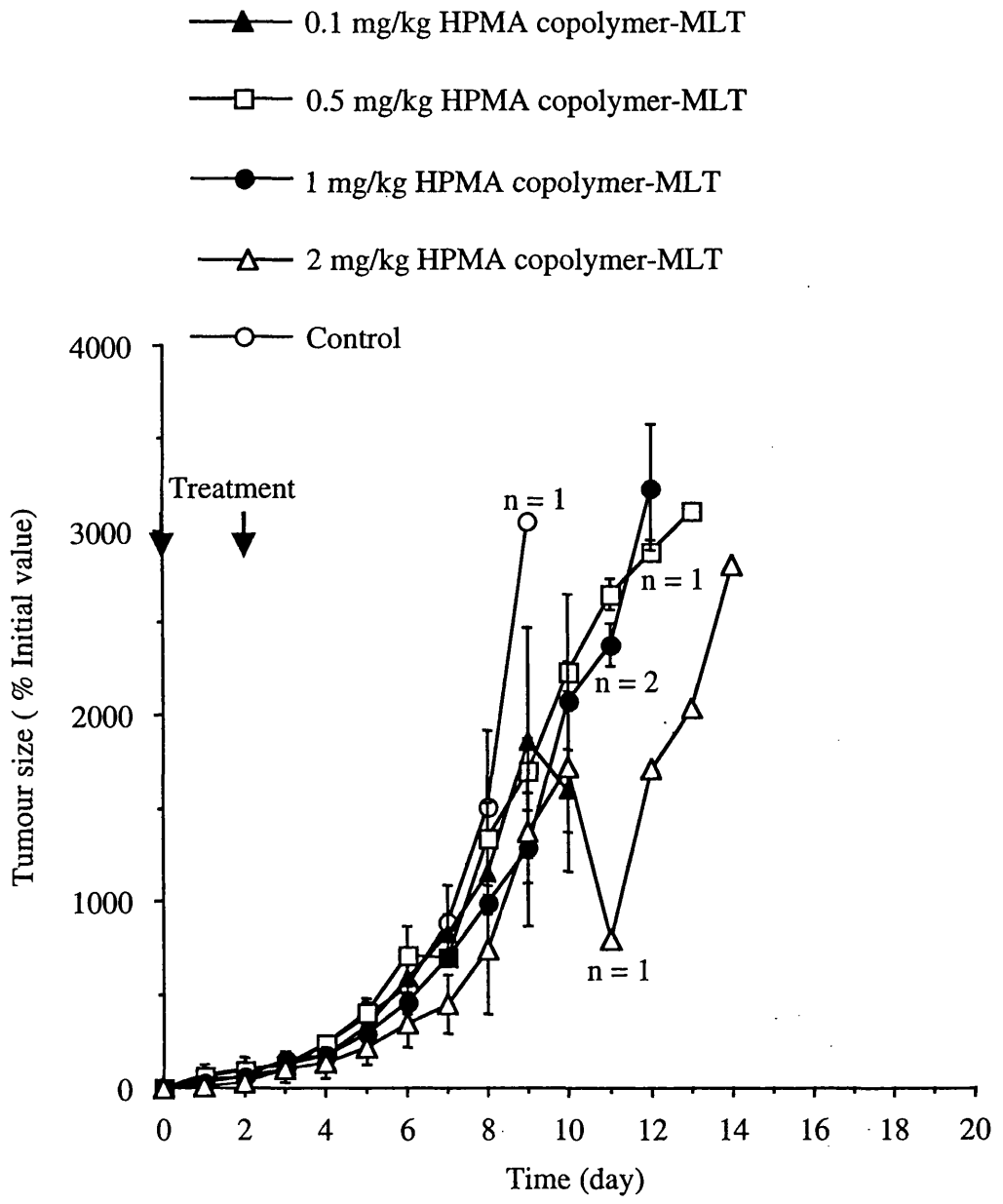
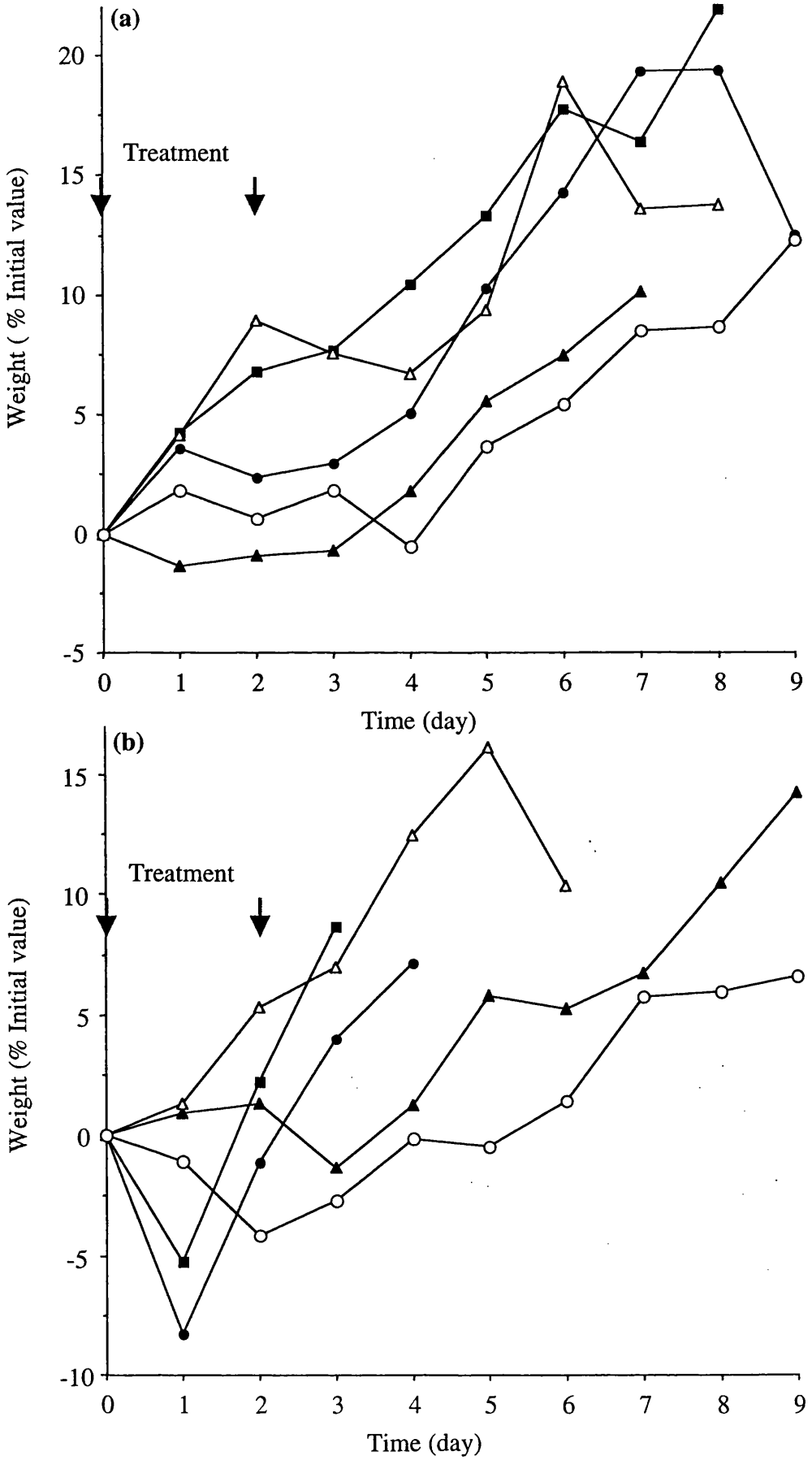
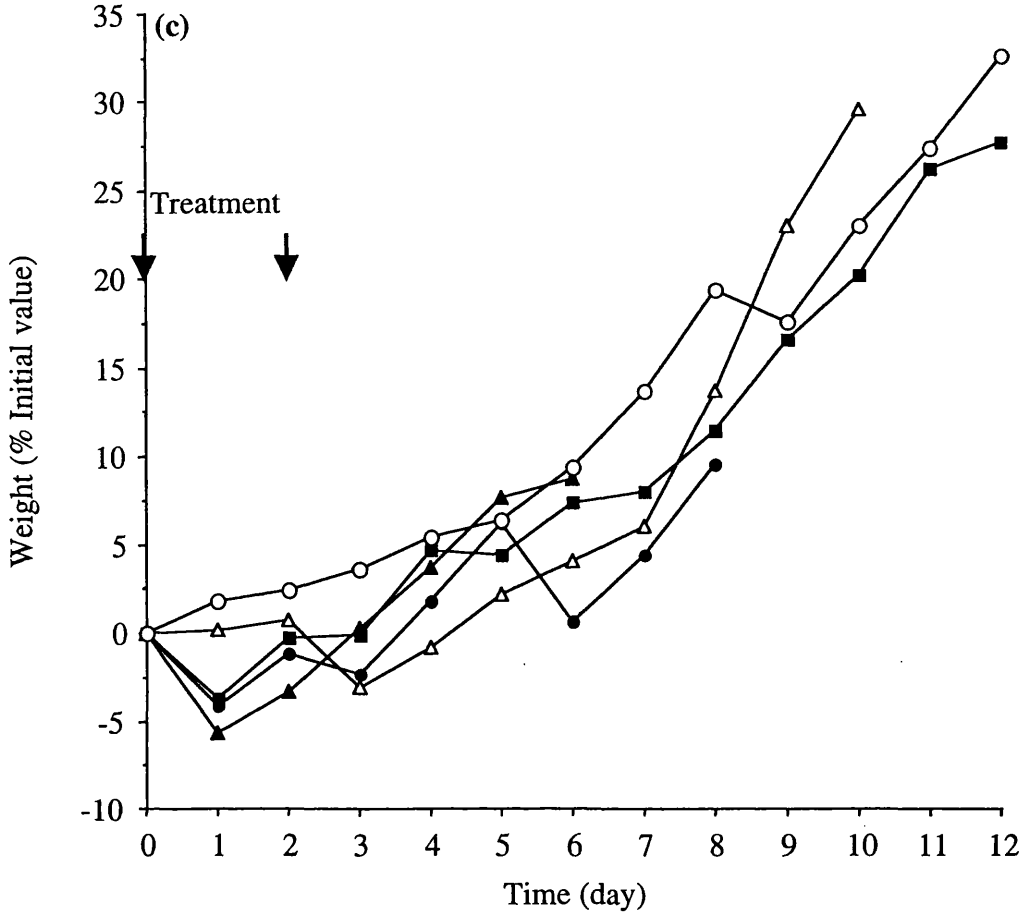


Figure 5.22 Effect of HPMA copolymer-MLT (a) 0.1 mg/kg; (b) 0.5 mg/kg; (c) 1 mg/kg or (d) 2 mg/kg (i.v.) on animal weight. In each case the weight change of each mouse in the group is shown




(c)
Treatment
Weight (% Initial value)
Time (day)
35
30
25
20
15
10
5
0
-5
-10
0 1 2 3 4 5 6 7 8 9 10 11 12

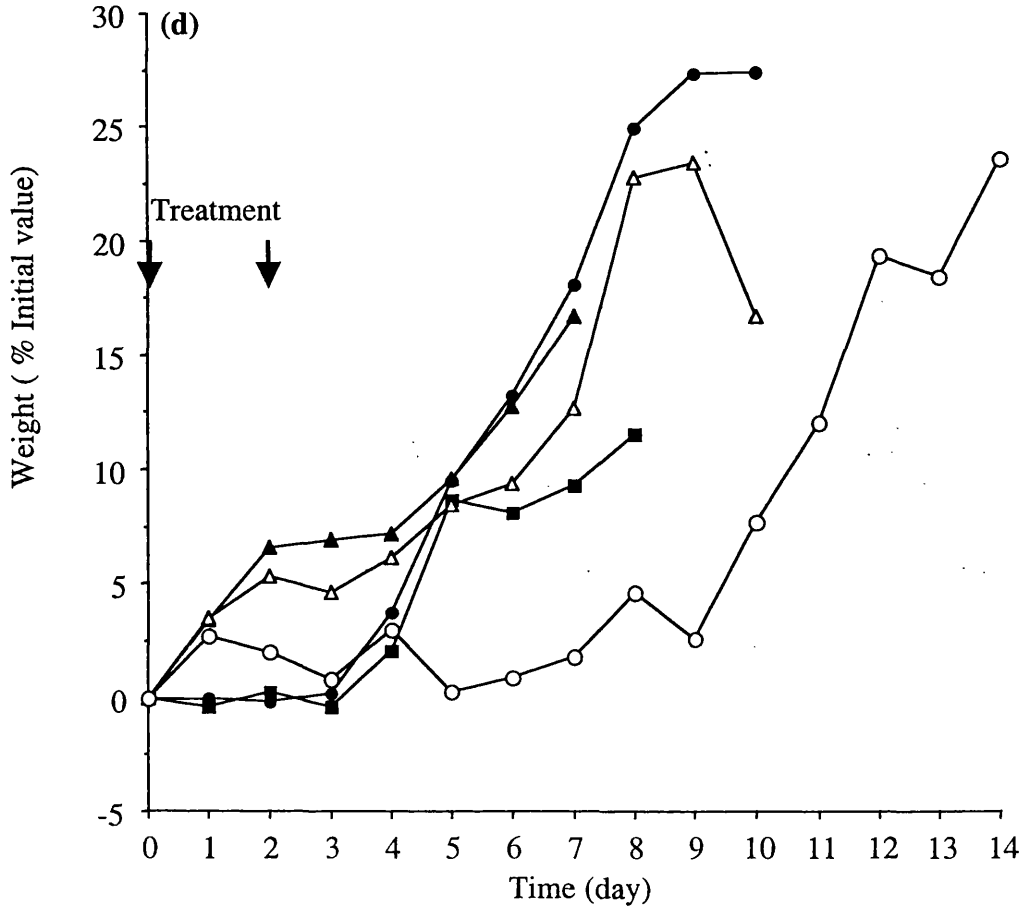
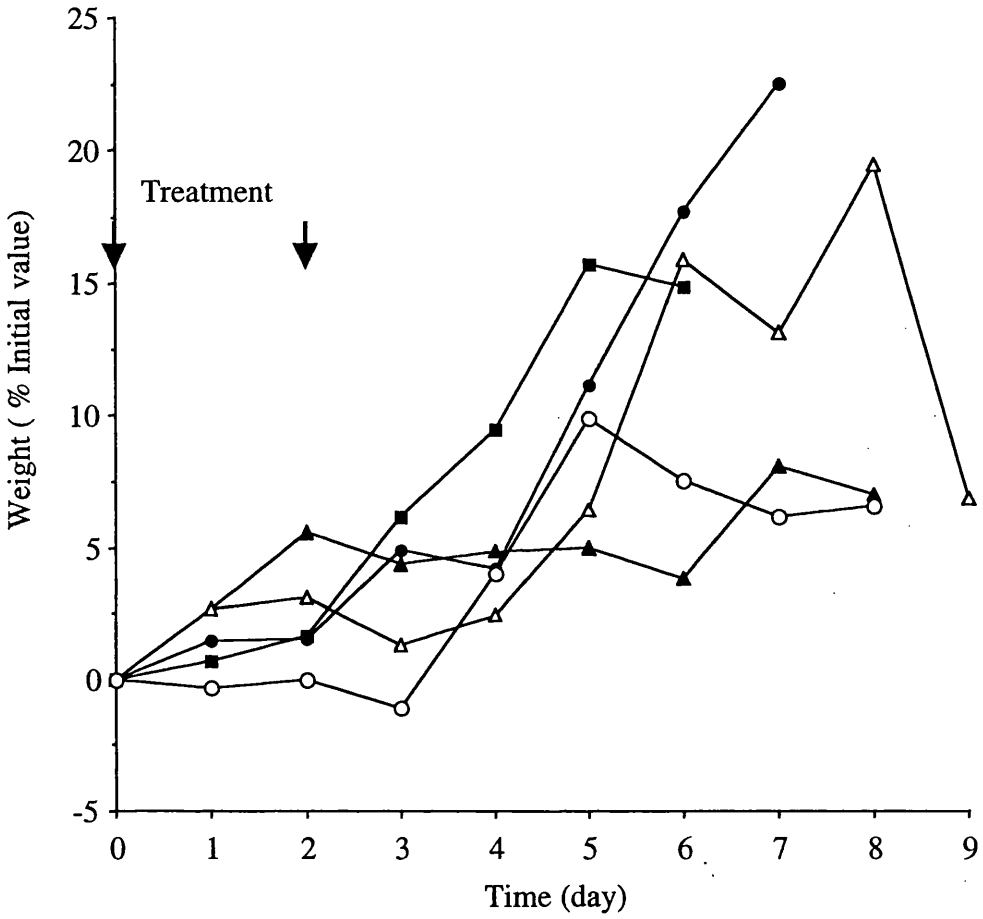

(d)
Treatment
Weight (% Initial value)
Time (day)
30
25
20
15
10
5
0
-5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 5.23 Effect of control (i.v) on animal weight. In each case the weight change of each mouse in the group is shown



5.4 Discussion

The aim of this work was to design a HPMA copolymer-MLT conjugate that showed reduced toxicity due to polymer conjugation and tumour targeting by the EPR effect and thus selective antitumour activity. As the HPMA copolymer-MLT conjugate was found to be 4-fold less toxic than MLT in the MTD study, it was possible to examine biodistribution and antitumour activity in mice bearing s.c. B16F10 tumours.

To compare the tumour targeting of MLT and HPMA copolymer-GG-MLT conjugate it was first necessary to radiolabel the compounds using the Bolton and Hunter reagent. This reaction was efficient (72 - 97 %) and it proved possible to remove free [^{125}I]iodide. It should be noted, however, that since it is MLT that is labelled with the Bolton and Hunter reagent, physiological degradation of the peptide would result in the tracking of the ^{125}I-labelled degradation products and not MLT or the conjugate.

It is expected that free MLT would be more easily accessible to enzymatic hydrolysis as the HPMA copolymer chains would protect the conjugated MLT. This may explain the lower recovery seen with free ^{125}I-labelled MLT in comparison to the ^{125}I-labelled conjugate in both the time-dependent and dose-dependent distribution studies. The low recovery seen could also be attributed to glomerular filtration and passing into the urine.

Ideally, animals in metabolic cages would have given an indication of radioactivity present in urine and faeces and thus aided to account for < 100 % recovery of radioactivity in both of the biodistribution studies. In addition, in retrospect, 0.9 % saline washes of the peritoneal cavity would have aided to account for < 100 % recovery. Interestingly, German (2001) showed that biodistribution profiles after i.p. administration vary greatly according to whether animals can freely move about (homing cages) as opposed to limited movement in metabolic cages.

Improved circulation time is of paramount importance to achieve targeted delivery (reviewed by Boddy & Aarons, 1989). The EPR effect in the B16F10 model is known to rely on plasma concentrations (Seymour *et al*, 1994) and therefore i.v. administration would have been ideal for the biodistribution studies. However, i.p. administration was necessary to allow comparison of body distribution of MLT and the conjugate (Sigma Data Sheet). Once administered i.p., polymers may either adhere to

the peritoneal wall or leave the peritoneal cavity via diffusion into blood vessels lining the peritoneal cavity or the lymphatic system. I.p. administration of HPMA copolymers resulted in 50 % of the injected dose in the blood circulation within 2 h, reaching a plateau within 5 – 6 h (Seymour *et al*, 1987). Proteins and peptides < 6 kDa are thought to be transferred equally as well via capillaries and lymphatics into the blood compartment (Fincher *et al,* 1989). It should be noted that due to MLT's membrane active nature, adherence of MLT conjugate to the peritoneal cavity and/or incomplete diffusion into the capillaries or lymphatic system would be a cause for toxicity to the animals.

As expected, it was shown that the long circulating feature of HPMA copolymer (Seymour *et al*, 1987 and Cartlidge *et al*, 1987) was imparted to MLT by conjugation. ^{125}I-labelled HPMA copolymer-MLT circulation levels were approximately 20-fold greater at 6 h. In the dose-dependent study, comparison of equi-doses (3 mg/kg) of free ^{125}I-labelled MLT and ^{125}I-labelled MLT conjugate (MLT-equivalent) found ~ 3 - 4–fold augmentation in circulating levels of the ^{125}I-labelled MLT conjugate at 4 h. This was shown to be due to faster excretion of free MLT through the kidneys. This observation was not surprising as the renal threshold for small peptides or proteins has been reported to be < 20 kDa and cationic molecules such as MLT are filtered more efficiently due to the negative charge of the glomerular filter (McMartin, 1994).

Comparison of 3 mg/kg (MLT-equivalent) ^{125}I-labelled MLT and the ^{125}I-labelled conjugate showed that polymer conjugation of the MLT toxin improved tumour targeting with 0.72 ± 0.30 % injected dose/g and 2.04 ± 0.29 % injected dose/g respectively. In this study, standardisation of tumour sizes prior to the start of the study was as far as described in section 2.8 (i.e. 0.5 cm x 0.5 cm). However, experiments were not conducted to look at the effect of tumour size which has been shown by Sat and Duncan (1998) to have an effect on accumulation of polymer-drug conjugates by the EPR effect (section 1.3).

Administration of PK1 (5 mg/kg DOX-equivalent) i.v. showed significantly greater tumour targeting with 10 % injected dose/g at 1 h in s.c. B16F10 tumours of various sizes (< 200 – 500 mg) (Sat, 1999). Seymour *et al* (1994) found i.v. administration of PK1 (5 mg/kg DOX-equivalent) resulted in peak radioactivity levels of 5 % injected dose/g in s.c. B16F10 tumours which was 10-fold greater than free DOX (5 mg/kg) at 12 h and maintained a plateau up to 24 h after administration.

Additionally, AUC values of 8.7 μg h/g of free DOX (5 mg/kg) and 149.1 μg h/g of PK1 (5 mg/kg) 1 h after administration were seen.

At the end of the study (48 h), radioactivity levels had dropped to ~ 3 % injected dose/g. It was hoped that the significantly improved circulation time of the i.p. administered MLT conjugate at 4 h (Figure 5.13) would result in EPR-mediated tumour uptake. It would have therefore been useful to carry out this study over longer time points. It should be noted that although comparison of equal doses of ^{125}I-labelled MLT and the ^{125}I-labelled conjugate (3 mg/kg) showed higher circulation levels (~ 3 - 4-fold) and improved tumour targeting (~ double) at 4 h with ^{125}I-labelled HPMA copolymer-MLT conjugate, there were also increased radioactivity levels in normal tissues, specifically the lungs and heart. The reason for these observations is unclear. It would have been interesting to investigate later time points because the lungs are an important extrahepatic route of elimination (Chaturvedi, 1999) which would possibly contribute to diminished MLT conjugate circulating time. In addition, a worry for increased heart levels is that cardiovascular abnormalities arising from administration of honey bee venom to rats (Marsh and Whaler, 1980) has been attributed to MLT which has been shown to cause cardiotoxicity in cultured foetal mouse cardiac myocytes (Okamoto *et al*, 1995). It is hoped that this is diminished by polymer conjugation as seen with PK1 (Yeung *et al*, 1991). This, however, needs to be investigated further. It may therefore be concluded that there was no evidence of the EPR-effect in these experiments.

Anti-tumour Activity

These experiments were disappointing. In the first study, both MLT and HPMA copolymer-MLT appeared to be toxic and not anticancer agents. Although significantly improved targeting of the MLT conjugate was evidenced via the i.p. route, both i.p. antitumour studies (single dose 10 mg/kg and multiple-dose 0.5, 1 or 2.5 mg/kg MLT-equivalent) showed no antitumour activity. This observation highlights the complexity in the relationship between biodistribution and pharmacodynamics (effects of the administered drug) (illustrated in Figure 5.24) and was disappointing in comparison to other polymer-drug conjugates tested using this model.

I.p. administration of multiple dose schedule PK1 (18, 27 or 36 mg/kg DOX-equivalent) to animals bearing a B16F10 tumour (s.c.) on days 5, 9 and 15 showed improved antitumour activity relative to free DOX (5 mg/kg) with T/C values of 209, 293 and > 320 % respectively (Duncan *et al,* 1992). I.p. administration of single dose HPMA copolymer-GFLG-APE (1, 5 and 10 mg/kg) in mice bearing B16F10 s.c.

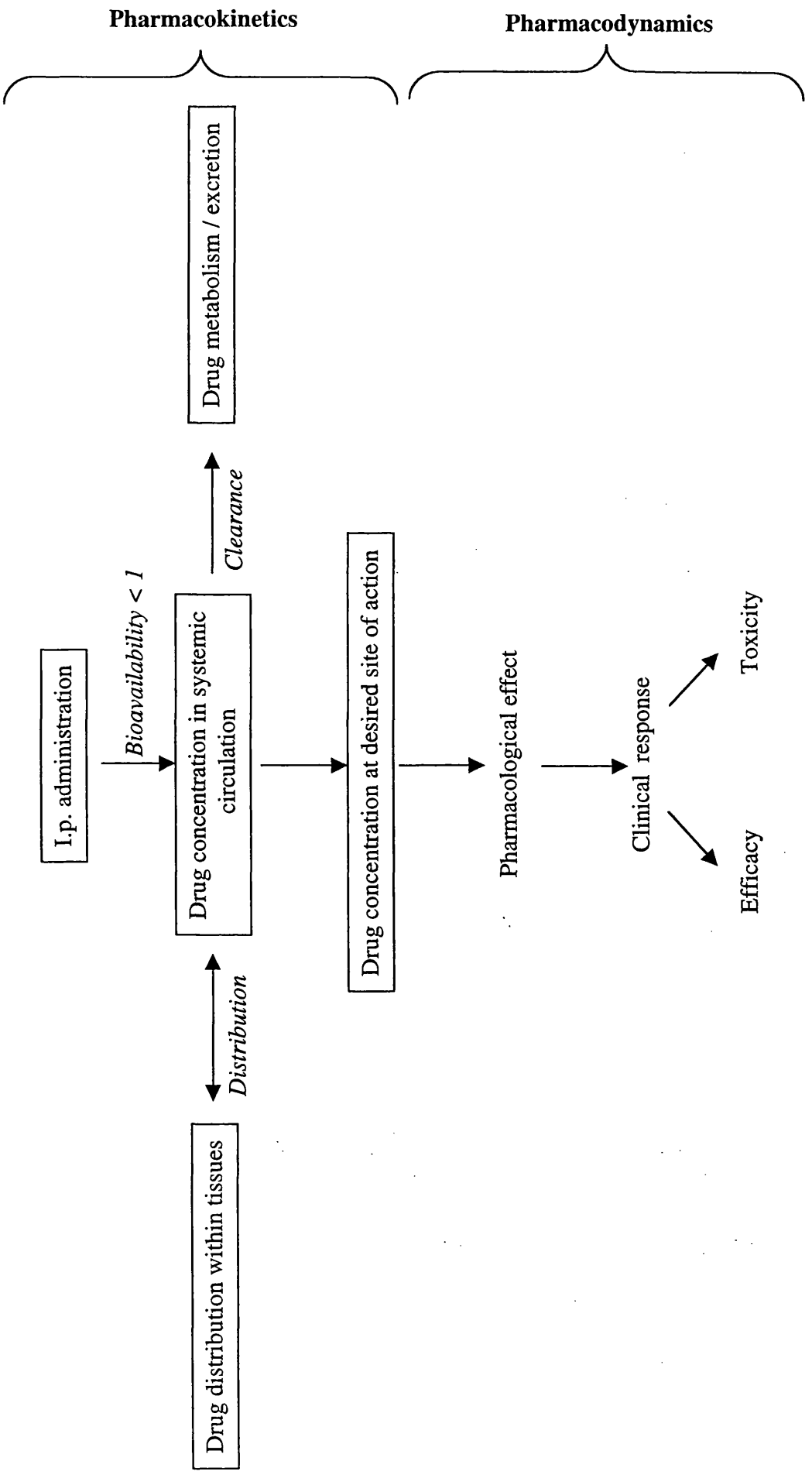


Figure 5.24 Schematic illustration of dose-response relationships of a drug (Adapted from Gloff & Benet, 1990)

tumours gave T/C values of 143 ($p < 0.001$), 128 (NS) and 110 (NS) (Searle *et al*, 2001).

O'Hare *et al*, 1993 investigated the route of injection on the antitumour activity of PK1 and MSH-targeted PK1 administered on the first day of the study to mice bearing B16F10 i.p. tumours. They found i.p. administered PK1 (5, 10 or 20 mg/kg DOX-equivalent) to improve survival of the animals with T/C values of > 183 ($p < 0.001$), 171 ($p < 0.001$) and > 237 ($p < 0.001$) % respectively. I.v. administration also improved animal survival with T/C values of 174 ($p < 0.01$), > 218 ($p < 0.001$) and 200 ($p < 0.001$) % respectively. However, it should be noted that DOX (5, 10 or 20 mg/kg i.p. or 5 and 10 mg/kg i.v.) also significantly improved animal survival when administered i.p. (T/C values > 231 ($p < 0.01$), 188 ($p < 0.01$) and > 246 ($p < 0.001$) respectively) or i.v. (>210 ($p < 0.001$) and > 254 ($p < 0.001$)).

It should be noted, however, that doses of HPMA copolymer-MLT administered were not optimised and that in both antitumour studies conducted via the i.p. route, unlike with free MLT, there were no toxic deaths observed for animals treated with the HPMA copolymer-MLT conjugate. This apparent reduction in toxicity warranted further investigation. It should be noted that German (2001) found that there is an effect of dose, repeated administration and injection volume on the body distribution of i.p. administered ^{125}I-labelled dextrin-2-sulphate. This would indicate that such studies would be useful in dose optimisation studies of HPMA copolymer-MLT by this route.

In order to capitalise on the EPR effect, an i.v. antitumour study of HPMA copolymer-MLT was carried out, in the hope that the greater bioavailability of circulating conjugate would contribute to greater tumour uptake. However, administration of multiple dose HPMA copolymer-MLT (0.1, 0.5, 1 or 2 mg/kg MLT-equivalent) on days 0 and 2 was not found to significantly improve animal survival. Surprisingly 2/5 toxic deaths occurred in the lower dose (0.5 mg/kg MLT-equivalent) treatment group. Again, this observation was disappointing in comparison to other polymer-drug conjugates tested using this model. When administered i.v. as a single dose against B16F10 s.c. tumours, HPMA copolymer-GFLG-platinate (5, 10 and 15 mg/kg platinate-equivalent) showed antitumour activity with T/C values of 118 (NS), 134 ($p < 0.05$) and 124 ($p < 0.05$) % respectively (Gianasi *et al*, 1999). Likewise, i.v. administration of single dose PAMAM dendrimer-platinate (5, 10 and 15 mg/kg

platinate-equivalent) against B16F10 s.c. tumours showed antitumour activity with T/C values of 120 ($p < 0.05$), 140 ($p < 0.05$), and 140 ($p < 0.05$) % respectively (Malik *et al*, 1999). I.v. administration of dextrin-DOX (5, 15, 20 and 25 mg/kg DOX-equivalent) to mice bearing B16F10 s.c. tumours on days 0,1 and 2 showed antitumour activity with T/C values of 130 ($p < 0.01$), 143 ($p < 0.001$), 127 ($p < 0.01$) and 110 (NS) % (German, 2001).

These observations highlight the apparent lack of dose-dependency on the antitumour activity of polymer-drug conjugates and thus the difficulty in determining the optimal dose for these type of compounds. This is an interesting feature true to polymer-drug conjugates and needs to be investigated.

To conclude, in this preliminary investigation, MLT conjugation to HPMA copolymer showed alteration in free MLT disposition characteristics, including prolonged circulation time. Disappointingly, however, the EPR-effect was not evidenced and the MLT conjugate did not fulfil an antitumour effect in single and multiple dose administration of the MLT conjugate by either the i.p. or i.v. route. Nevertheless, the reduced toxicity of free MLT imparted by polymer conjugation, as shown in the MTD study and the reduced toxic deaths in antitumour studies, warrants further investigations to optimise MLT loading, doses and dosing schedule before ruling out HPMA copolymer-MLT as a potential antitumour agent. It would be of value to repeat experiments where a trend is observed using a larger group of animals e.g. n = 20. As the B16F10 is a very aggressive tumour, the antitumour activity observed in the i.v. study may be more evident in a less aggressive mouse model e.g. CORL23 non-small cell lung cancer which has already been used to investigate the antitumour activity of polymer-drug conjugates including the polymer directed enzyme prodrug therapy (PDEPT) concept (Satchi *et al*, 2001) and HPMA copolymer-APE (Searle *et al*, 2001). It would also be of interest to perform further studies (both biodistribution and antitumour) in animals bearing small tumours of standardised size. However, perhaps it is more reasonable to consider first ways to improve the conjugate structure and this is addressed in Chapter 6.

Chapter Six

General Discussion

The field of polymer therapeutics in cancer and other diseases is now becoming well established with polymer-protein conjugates such as PEG-L-asparaginase (Oncaspar®), PEG-α-interferon (PEG-α-INTRON®), PEG-adenosine deaminase (ADAGEN®) and SMANCS (Zinostatin stimaler®) in the market and the emergence of a number of anticancer polymer-drug conjugates in Phase I/II clinical trials (reviewed by Duncan, 2001).

Before I started this study, MLT had already been explored in various ways as an anticancer agent. Dunn *et al* (1996) described the construction and expression of a recombinant MLT immunotoxin (scFv-mel) in *Escherichia coli*. Like the native K121 antibody, scFv-mel showed antigen (KMA) binding (albeit 2-fold lower) in a human lymphoblastoid cell line, HMy2 but not in a non-KMA expressing cell line, K562. Additionally, the observed specific antigen binding properties of KI21 and scFv-Mel were inhibited after pre-incubation with soluble antigen Bence Jones Protein (BJP) by 90.6 % and 95.3 % respectively. Evaluation of the cytotoxic activity (18 h) in the same cell lines found native MLT to be equally (non-specifically) lytic to both cell lines (IC_{50} ~ 3 μM ≡ 8.5 μg/ml) and scFv-mel to be specifically toxic to the Hmy2 antigen bearing cells, 70-fold more cytolytic than unmodified MLT. Again, this activity was inhibited when scFv-mel was preincubated with BJP. The preparation of a MLT analogue immunotoxin which has been modified to diminish its immunogenicity has also been reported by the Commonwealth Science and Industry Research Organisation (CSIRO), Australia (Whitehouse, 1999).

Using a different approach, Winder *et al* (1998) tried to avoid generalised toxicity of MLT by production of clones of a EJ human bladder carcinoma cell line which were genetically engineered to express two MLT precursor constructs (preMLT and preproMLT), which would then be constitutively (enzymatically) activated. After s.c. implantation in nude mice, the EJ cell clones were analysed for tumourigenicity and rate of tumour growth. Expression of preMLT and preproMLT reduced tumour formation (5 % and 20 % respectively) and significantly reduced the rate of tumour growth relative to the parental non-microbial peptide expressing EJ cells ($p < 0.05$; two-tailed unpaired t test).

To date, no further studies on the above approaches have been reported. As MLT is a cationic membrane active peptide, this activity has also been exploited in gene

therapy (reviewed by Plank *et al*, 1998). Recently, a dimerised MLT analogue with two amino acid substitutions (P14A and G1C) (CP36) which showed increased (> 1 log) lytic activity in RBC at pH 5 (1 h at RT) but diminished cytotoxic activity in human umbilical vein endothelial cells (HUVECs) (~ 100 % viability at 5 μM ≡ 14.2 μg/ml) was reported to show DNA condensation and subsequently superior transfection of EGFP in HeLa (91 %), HUVECs (57 %), NSO (24 %) and HepG2 (19 %) cell lines. Using a luciferase reporter, CP36 gave the best transfection (~ 50 %) in HUVECs when compared to 11 commercially available transfection agents including ExGen 500 (PEI), Superfect, Lipofectin, Lipofectamine, and DOTAP. Additionally, 18 h after i.v. administration of CP36/DNA complexes to CD1 mice, luciferase expression was detected in the lungs and to a less extent the spleen, whereas poly-L-lysine gave negligible transfection (Haines *et al*, 2002).

Thus it is clear that MLT is considered an important membrane active peptide with interesting properties suitable for development as a "drug" or part of a delivery system. This thesis presents for the first time, the synthesis of a model polymer-membrane active conjugate, HPMA copolymer-MLT, and the assessment for its potential use as an anticancer agent on the assumption that it could target tumours by the EPR effect (Matsumura & Maeda, 1986) like other polymer anticancer conjugates (reviewed by Duncan, 1999).

A procedure was developed for the reproducible synthesis of HPMA copolymer-MLT and the conjugates contained < 0.1 % free MLT (estimated by SDS PAGE). The use of an FPLC system allowed best separation of free MLT to give pure MLT conjugates (Chapter 3). However, this study reiterated the need for polymer-peptide SEC standards to allow improved characterisation of the prepared conjugates, which is vital to its application as a therapy in man. It would have been interesting to further fractionate the obtained heterogeneous conjugate mixture based on the molecular weight to obtain a more homogeneous and monodisperse end product (Flanagan *et al*, 1992).

HPMA copolymer-MLT conjugates of high (38.9 ± 2.5 % w/w; GFLG spacer), intermediate (25.8 ± 6.2 % w/w; GG or GFLG spacer) and low (12.1 ± 8.6 % w/w; GFLG spacer) MLT loading were prepared in order to optimise the conjugate structure. An ideal conjugate would have relatively low haemolytic activity whilst maintaining

antitumour cytotoxicity. The medium loading conjugate of ~ 25 % w/w with the shorter GG peptidyl linker (RM 3.13) was significantly less lytic than free MLT (Hb_{50} 19.9 ± 6.2 μg/ml; $p < 0.05$) and retained tumouricidal activity relative to free MLT (IC_{50} 7.3 ± 1.5 μg/ml; $p = 0.5$) (Chapter 4). This conjugate was selected for further preliminary investigations *in vivo*. Interestingly, the *in vitro* experiments (Chapter 4) showed apparent batch to batch (RM 3.2 and RM 3.13) variation in haemolysis (Hb_{50} value) and cytotoxicity (IC_{50} value) despite reproducible synthesis (reaction yield 40.3 % and 39.7 % respectively) and physico-chemical characterisatics. Batch RM 3.2 was less active in both haemolysis ($p < 0.005$) and cytotoxicity ($p < 0.05$), relative to batch RM 3.13. It may therefore be concluded that this was probably as a result of the heterogeneity of the prepared samples due to the multivalency of both HPMA copolymer and free MLT. This problem will be addressed later in this chapter.

The *in vivo* MTD of HPMA copolymer-MLT conjugate was 4-fold greater than seen for free MLT after single dose administration (i.p.), which indicated a reduction in toxicity due to MLT conjugation. However, additional in depth biochemical investigations would have been useful. In a study to compare the body distribution of equidose (3 mg/kg MLT-equivalent; i.p.) ^{125}I-labelled MLT and ^{125}I-labelled HPMA copolymer-MLT 4 h after administration, as expected, the ^{125}I-labelled conjugate showed ~ 3 - 4-fold longer circulation time (22.1 ± 1.49 % injected dose/ml) than free ^{125}I-labelled MLT. However, the EPR effect was not established following i.p. administration of the ^{125}I-labelled MLT conjugate. ^{125}I-labelled HPMA copolymer-MLT showed ~ 2-fold greater tumour uptake (2.04 ± 0.29 % injected dose/g) than free ^{125}I-labelled MLT. However, the levels of ^{125}I-labelled HPMA copolymer-MLT in the heart and lungs were 2.8 ± 0.1 % injected dose/g and 5.0 ± 0.3 % injected dose/g respectively, which is ~ 3-fold and 2-fold greater than seen for free ^{125}I-labelled MLT. Importantly, for further progression of this work, an investigation into the pattern of heart and lung levels at longer time points needs to be addressed due to the reported cardiotoxicity of honey bee venom (HBV) (Marsh & Whaler, 1980) and in particular MLT (Okamoto *et al*, 1995). The documented elimination of peptides or proteins via the lungs (Chatuverdi, 1999) could imply faster clearance of the MLT conjugate via this route.

Disappointingly, no antitumour activity was observed in antitumour studies following either i.p. (single 10 mg/kg MLT-equivalent on day 0 or multidose 0.5, 1 or 2.5 mg/kg MLT-equivalent on day 0, 2 and 4) or i.v (multidose 0.1, 0.5, 1 or 2 mg/kg

MLT-equivalent on day 0 and 2) administration of the MLT conjugate (Chapter 5). Nevertheless, the antitumour studies generally reiterated the reduced toxicity of HPMA copolymer-MLT relative to free MLT. It should also be noted that dose optimisation was not carried out and therefore the potential of HPMA copolymer-MLT as an antitumour agent should not be immediately ruled out. However, it is impossible to make further progress without first optimising the structure of HPMA copolymer-MLT.

Optimised Synthesis of HPMA Copolymer-MLT Conjugate

The need to generate a homogeneous conjugate of well-defined composition is paramount for its progression and approval by the regulatory authorities for its application as an anticancer therapy. To improve the synthesis there are a number of facts to be considered. First, the source of MLT. Natural products often have problems with their purity and the MLT from Sigma used here is no exception. The main contaminants of this commercially available MLT from HBV are phospholipase A_2 (PLA_2) and an N-terminally formylated form of MLT (2 – 10 %) (Maulet *et al*, 1982). The potential effects of these impurities are discussed here.

Formylation of MLT's N-terminal produces a 20 % reduction in haemolytic activity, indicating the terminal α-NH_2 group is not crucial for MLT membrane activity (Lubke *et al*, 1971). PLA_2 is known to cleave the β ester bond of membrane glycerophospholipids of intact cells to release arachidonic acid and lysophospholipids, which in turn also lyse cell membranes (reviewed by Mathews & Holde, 1990 and Murakami & Kudo, 2002). However, the possible influence of PLA_2 contamination on MLT induced cell death is unclear. A study investigating the effects of synthetic MLT on the activity of PLA_2 derived from various sources (HBV, snake venom, bovine pancreas and synovial fluid of arthritic patients) found MLT to be a non-competitive inhibitor of HBV-derived PLA_2 (Saini *et al*, 1997). Naturally derived MLT has also been reported to enhance endogenous PLA_2 activity (Mollay *et al*, 1976; Sharma, 1993; Signor *et al*, 1994). This is supported by studies conducted with synthetic MLT, which also showed enhanced endogenous PLA_2 activity. (Rao, 1992 and Choi *et al*, 1992). Contrary to these findings, PLA_2 contamination in commercially obtained MLT has been reported to be responsible for activation of 75 % endogenous PLA_2 activity (Fletcher *et al*, 1990). If this is the case, this latter observation has implications for the activity of the HPMA copolymer-MLT conjugate.

It is possible that the lytic activity of the conjugated MLT is partially attributed to any contaminating PLA_2. Additionally, and importantly, as PLA_2 has multiple NH_2 groups, these could also react with the HPMA copolymer-ONp precursor. It should be noted that PLA_2 (14.5 kDa) was not detected using FPLC or SDS PAGE. Therefore I believe the conclusions drawn from this work are sound. The use of a synthetic MLT prepared by solid phase synthesis (Tosteson *et al*, 1987) would have been much better, but this was not available for these experiments and it was hoped that native MLT would allow synthesis of conjugates for the proof-of-principle studies.

MLT was bound not only as randomly pendant groups to the polymer chain, but also via different MLT-NH_2 groups. As a result, the conformation and hence activity of polymer-bound MLT molecules was not identical, thus contributing to the complexity in dissecting the activity of the prepared HPMA copolymer-MLT conjugates. It is established that MLT charge and α-helical conformation are crucial to its lytic activity (reviewed by Dempsey, 1990) and chemical modification of K and R residues which provided the pendant NH_2 groups for conjugation to HPMA copolymer has been shown to have an effect on MLT conformation and thus activity (Habermann & Kowallek 1970; Hider *et al*, 1983; Manjunatha-Kini & Evans 1989; Blondelle & Houghten 1991; Werkmeister *et al*, 1993; Juvvadi *et al*, 1996). Regio-specific conjugation of a fatty acid to MLT at K7 maintained cytolytic activity, but conjugation to K21 or K23 diminished MLT's lytic acitivity. However, conjugation to MLT at the N-terminal showed increased activity relative to native MLT (Dr. H. Aojula, University of Manchester, UK, personal communication). It would be interesting to investigate if this is true for HPMA copolymer-MLT.

The inter-batch variation in activity of HPMA copolymer-MLT (RM 3.2 and RM 3.13) despite identical physico-chemical characteristics as seen in terms of haemolytic activity and cytotoxicity (Chapter 4) underlines the disadvantage of semi-random aminolysis and the resultant heterogeneous macromolecular mixture (Chapter 3). The preparation of homogeneous MLT conjugates may be addressed by regio-specific coupling, for example:

1) The use of a monovalent polymer e.g. semitelechelic HPMA homopolymer (Lu *et al*, 1998) or monomethoxy poly(ethylene glycol) (mPEG) (Davis *et al*, 1991). This however, may not provide adequate "protection" of the bound MLT, unless the conjugates underwent micellar formation.

2) Chemical protection of reactive NH_2 groups along the MLT peptide chain as carried out during peptide synthesis (reviewed by Bodanzsky & Ondetti, 1966 and Schelhaas & Waldmann, 1996). Suitable (mild) conditions would be necessary to ensure the activity of native MLT is not compromised.
3) Novel conjugation chemistry technologies, which convey site-specific coupling of macromolecular substrates to peptides and proteins, are emerging. These methods in turn give rise to different chemical linkers by derivatisation of peptide and protein functional groups. These include:

i) Thioether linkages by alkylation of endogenous sulphydryl (R-SH) moieties (Wang *et al*, 1992 and Melton & Sherwood, 1996)
ii) Glycosylated site-specific coupling at Asp (*N*-glycosidic linkage), Thr (*O*-glycosidic linkage) or Ser residues (*O*-glycosidic linkage) (Rodwell *et al*, 1986; Hermanson, 1996; reviewed by Seitz *et al*, 2001)
iii) Regio-specific coupling of proteins at the COOH terminus with the use of a 2-step approach (Rose *et al*, 1991; Fisch *et al*, 1992 and Vilaseca *et al*, 1993).

Investigation of Mode of Action of HPMA Copolymer-MLT

The experiments investigating the effect of MLT content in the HPMA copolymer-MLT conjugates (Chapter 4) indicated that like free MLT, the MLT conjugate lysed cells only after a "threshold" number of MLT molecules was reached on the cell surface. In order to better understand the mechanism of action of MLT, a great number of studies have looked into MLT-membrane interactions (Tosteson *et al*, 1985; Kaszycki & Wasylewski, 1990; Rex & Schwarz, 1998; reviewed by Sitaram & Nagaraj, 1999; Bechinger, 1999; Blondelle *et al*, 1999; La Rocca *et al*, 1999). It would have also been interesting to investigate HPMA copolymer-MLT-membrane interactions in comparison to free MLT.

Assessment of Immunogenicity of HPMA Copolymer-MLT

If time had allowed, it would have been interesting to investigate whether HPMA copolymer-MLT is immunogenic. HBV is highly immunogenic and can cause anaphylaxis. It has been long known that protein conjugation to polymers reduces their immunogenicity. This has been evidenced for a plethora of peptide and protein conjugates e.g. PEG conjugates (reviewed by Nucci *et al*, 1991) and HPMA copolymer conjugates including HPMA copolymer-IgG and HPMA copolymer-transferrin (Flanagan *et al*, 1992), HPMA copolymer-β lactamase and HPMA copolymer-cathepsin

B (Satchi, 1999). Although conjugation of HBV to PEG suppressed IgE antibody responses, IgG antibody responses, however, were unchanged or enhanced (Ahlstedt *et al*, 1983). One reason for the choice of HPMA copolymer in this study is its lack of immunogenicity *in vivo* (Simeckova *et al*, 1986 and Rihová *et al*, 1989) and lack of toxicity in humans (Vasey *et al*, 1999). It is thus likely that polymer conjugation to MLT would also decrease its immunogenicity and therefore allow administration of multiple doses *in vivo*.

The Future of HPMA Copolymer-MLT in Cancer Chemotherapy

Although the antitumour activity studies did not show significant antitumour activity, a dose optimisation study (i.v.) would have been of value to this work.

It is known that MLT at sub-lethal concentrations causes an increase in membrane permeability without causing lysis (reviewed by Shai, 1999) and it has been documented that this property may be utilised by co-administering MLT with conventional anticancer compounds which are subject to MDR (Sharom *et al*, 1995 and Johnstone *et al*, 2000). It may therefore be feasible to administer the long circulating MLT conjugate in combination with cytotoxic agents that may be subject to MDR, which like free MLT will non-specifically inhibit Pgp.

Other disease states that display EPR e.g. at inflammation sites may also be a target for therapy with HPMA copolymer-MLT. Antimicrobial peptides such as MLT (Oren & Shai, 1997) have also been reported to act against fungi (Ahmad *et al*, 1995), protozoa (Bevins & Zasloff, 1990) and viruses e.g. HIV (Wachinger *et al*, 1998). A recently prepared HPMA copolymer-gramicidin S conjugate which has shown antimicrobial activity *in vitro* (Solovskij & Panarin, 1999) implies that HPMA copolymer-MLT may also be used in this application.

Choice of Polymer

The use of HPMA copolymer in this study is justified by its non toxic and non immunogenic nature. Furthermore, it has progressed to clinical trials as the component of a number of polymer-drug conjugates (reviewed by Duncan, 2001). However, HPMA is non-biodegradable, thus limiting the molecular weight at which it can be used to that above the renal threshold (Seymour *et al*, 1987). In addition, HPMA copolymer is prepared by the uncontrolled process of radical precipitation polymerisation which

influences its polydispersity (~ 1.5). Controlled synthesis of HPMA copolymer is currently under investigation (Godwin *et al*, 2001). With the progression of anticancer conjugates of degradable polymers into clinical evaluation e.g. poly(glutamic acid)-paclitaxel (Li *et al*, 1998) it may be of interest to explore other polymers, e.g. the polyacetal based pH-labile polymers which capitalise on the lower intracellular pH of the cell and the tumour milieu (Tomlinson *et al*, 2002).

Choice of Toxin

Disappointingly, both the search for a more potent naturally derived membrane active peptide and for a novel marine-derived cytolytic peptide in a collaboration with Dr. J. L. Fernandes were unsuccessful (Appendix 1). Since I began this study, the use of polymer therapeutics to target marine derived toxins e.g. halichondrin B that have already shown in vivo antitumour activity has been reported (Munro *et al*, 1999).

The comparison of a recently documented antimicrobial HPMA copolymer-gramicidin S conjugate (Solovskij & Panarin, 1999) with HPMA copolymer-MLT would be of interest as gramicidin S is reported to have the same membrane lytic effect as MLT (Portlock *et al*, 1990).

The preparation of anticancer synthetic membrane active peptides has been reported (Robertson *et al*, 1998 and Arlotti *et al*, 2001). An actively targeted lytic peptide (Phor14) by conjugating to luteinizing hormone (LH) showed promising activity against prostate PC-3 xenografts in nude mice after i.v. administration (Leuschner *et al*, 2000). Lee & Oh (2000) have prepared a synthetic antimicrobial peptide analogue with enhanced α-helicity and thus enhanced antibacterial and cytotoxic activity, but with low haemolytic activity has been recently reported. The synthesis of a MLT hybrid with another membrane active peptide, MLT-cecropin A (CA(1-8)-ME(1-12)) with reduced haemolytic activity and potential use as an antifungal agent has also been documented (Lee *et al*, 1997). The use of engineered pseudo peptides would thus be of value as conjugates with greater loading, relative to a conjugate prepared with the native non-selective peptide, could be safely administered systemically and target tumour cells by the EPR effect.

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Appendix I

Haemolysis and Cytotoxicity of Membrane Active Agents

Figure 1 Haemolytic activity of peptide toxins after 1 h incubation with RBC (2 % w/w) at 37 °C. Data represents mean ± SEM (n = 12)

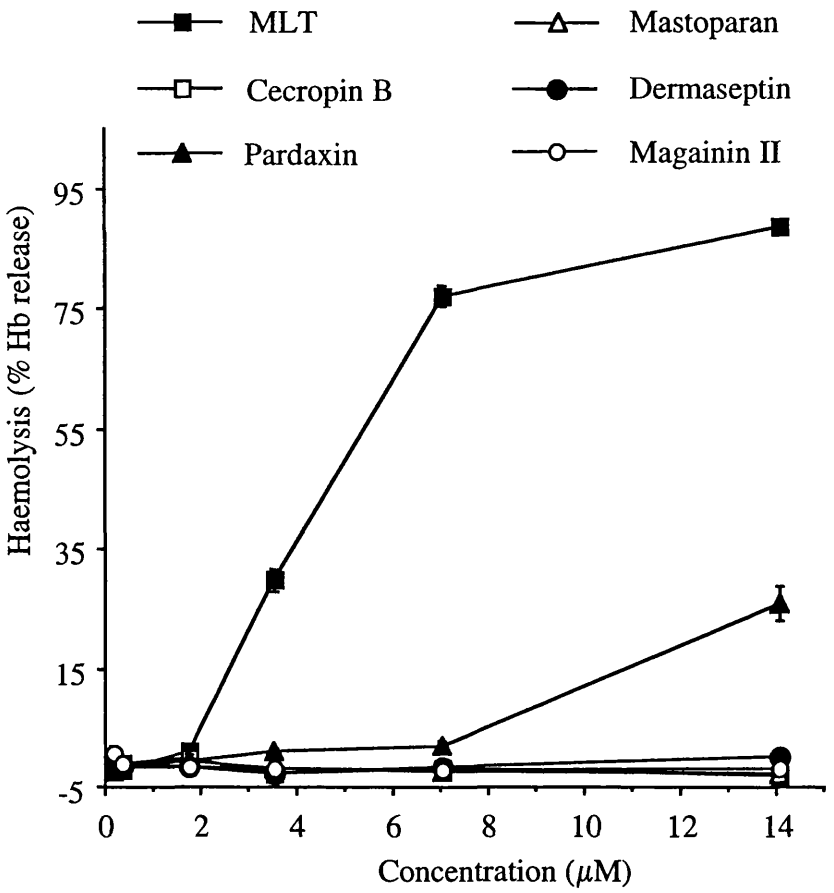


Figure 2 Haemolytic activity of ikarugamycin after 1 h incubation with RBC (2 % w/w) at 37 °C. Data represents mean ± SEM (n = 4)

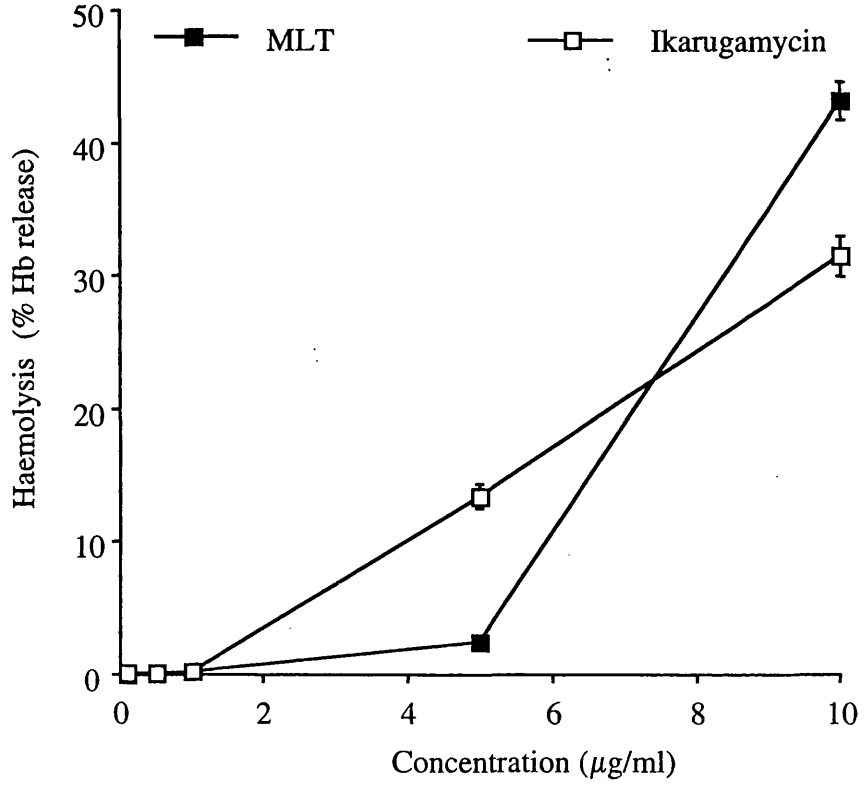
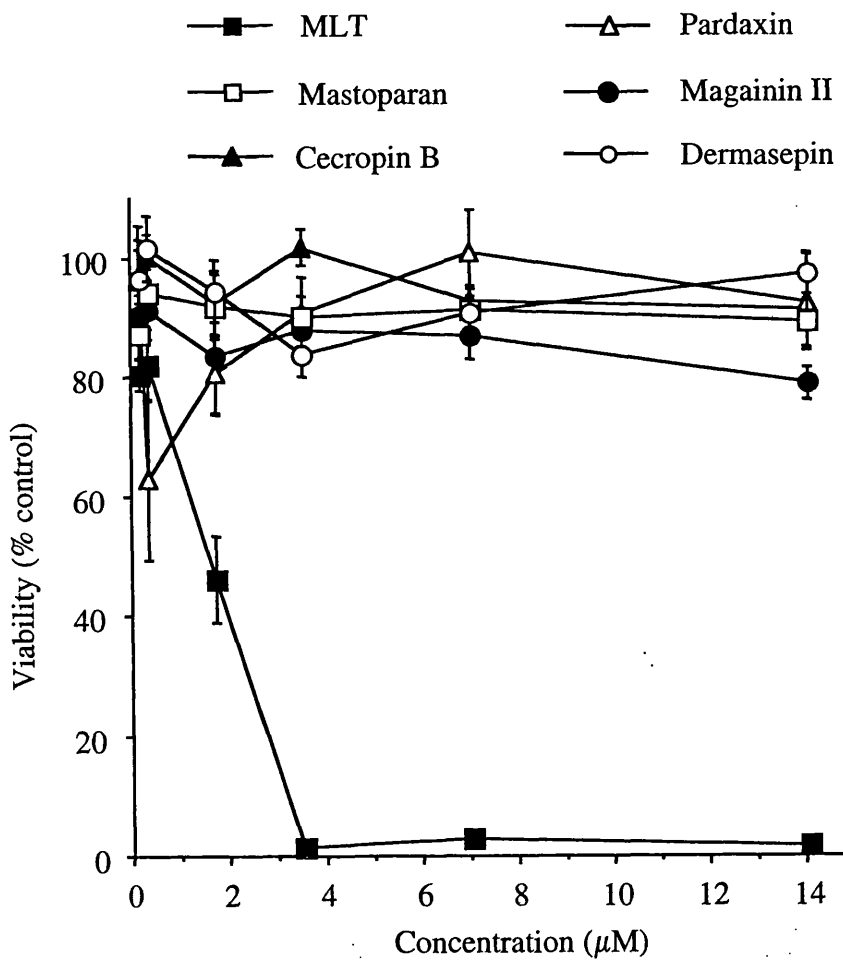


Figure 3 Cytotoxicity of peptide toxins against B16F10 cells after 72 h incubation. Data represents mean ± SEM (n = 18).



Appendix II

Publications

R. Musila & R. Duncan

Synthesis and Evaluation of *N*-(2-hydroxypropyl)methacrylamide (HPMA) Copolymer-Melittin; A Potential Novel Anticancer Approach, *Proceedings of the 4th International Symposium on Polymer Therapeutics: From Laboratory to Clinical Practice*, London, UK, January 2000, pp. 78

R. Musila & R. Duncan

Synthesis and Evaluation of HPMA copolymer-Melittin as a Potential Anticancer Agent - *In Vivo* Studies in the Mouse, *Journal of Pharmacy and Pharmacology* (2000), **52** (supplement), pp. 51

R. Duncan, S. Gac-Breton, R. Keane, R. Musila, Y-N. Sat, R. Satchi & F. Searle

Polymer-Drug Conjugates, PDEPT and PELT: Basic Principles for Design and Transfer from the Laboratory to the Clinic, *Journal of Controlled Release* (2001), **74**, 135 - 146

R. Musila, N. Quarcoo, A. Kortenkamp & R. Duncan

In Vivo Assessment of Time-Dependent DNA Damage induced by HPMA Copolymer-Doxorubicin (PK1) Using the Alkaline Single Cell Electrophoresis (Comet) Assay, *Proceedings of the 28th International Symposium on Controlled Release of Bioactive Materials*, June 23 – 27, 2001, No. 7131