

Bioresponsive Dextrin-colistin Conjugates as Antimicrobial Agents for the Treatment of Gram-negative Infection

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"For the best prize that life offers is the chance to work hard at work worth doing" (T. Roosevelt)

Abstract

Multidrug-resistant Gram-negative infection is an important cause of mortality and morbidity. Management of these infections is often dependent upon “treatment of last resort” “small molecule” antibiotics which suffer from significant toxicity and an indiscriminate volume of distribution. The aim of this study was to develop a prototype polymer-antibiotic conjugate that may be customised by polymer modification and binding chemistry to afford selective, controlled release at an infected site. These studies employed the biodegradable, naturally-occurring polymer, dextrin, and a polymyxin antibiotic, colistin, as the first model combination.

Physicochemical characterisation of a library of succinoylated dextrans and dextrin-colistin conjugates demonstrated that conjugation of dextrin to colistin was feasible and reproducible, resulting in masking of colistin's amino groups through incorporation in peptide bonds. Exposure to physiological α -amylase activity resulted in controlled degradation of the dextrin component, leading to sustained colistin release. Following exposure of the conjugates to physiological concentrations of α -amylase, minimally-modified, low molecular weight dextrin, conjugated to colistin, demonstrated significantly earlier, maximal release of colistin and subsequent reinstatement of antimicrobial activity. At maximum unmasking, the lead conjugate reported equivalent antimicrobial activity to the current clinical formulation of colistin (Colimycin[®]) against a range of MDR organisms including: *A. baumannii*, *K. pneumoniae* and *E. coli*. A static two-compartment dialysis bag model was developed under infinite sink conditions, which demonstrated that the conjugates were able to suppress bacterial growth over a significantly greater duration than colistin sulfate. *Ex vivo* studies of infected human wound fluid samples confirmed that colistin could be readily liberated from conjugate in infected sites. Significantly higher amylase activity in these wound fluid samples supported the notion of locally-triggered, enzymatically-mediated unmasking. An *in vivo* intravenous, pharmacokinetic model in rats demonstrated the increased half-life associated with conjugation and succinoylation. Moreover, the dextrin-colistin conjugates were better tolerated than colistin sulfate at higher concentrations.

These studies have demonstrated the feasibility of developing this new class of “nanoantibiotics” and highlighted their potential usefulness as bioresponsive nanomedicines for the treatment of MDR Gram-negative infection.

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List of Abbreviations

ABA	American Burns Association
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ANOVA	Analysis of variance
ATCC	American type culture collection
AU	Arbitrary units
AUC	Area under the curve
BCA	Bicinchoninic acid
BK	Bradykinin
BKR	Bradykinin receptor
BSA	Bovine serum albumin
CAMHB	Cation adjusted Mueller Hinton broth
CFU	Colony forming units
C _{last}	Last quantifiable concentration
CMS	Colistin methanesulphonate
COX	Cyclooxygenase
ddH ₂ O	Double distilled water
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
EGF	Epithelial growth factor
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial Nitric Oxide Synthase

EPR	Enhanced permeability and retention
FCS	Fetal calf serum
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
FMOC-Cl	9-fluorenylmethyl chloroformate
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared
GPC	Gel permeation chromatography
HCl	Hydrochloric acid
HES	Hydroxyethyl starch
HF	Hagemann factor
HMW	High molecular weight
HMWK	High molecular weight kininogen
HPLC	High performance liquid chromatography
IC	Inner compartment
ICJ	Intercellular junction
IEJ	Interendothelial Junction
IFN- γ	Interferon Gamma
IgM	Immunoglobulin M
IL-1	Interleukin 1
IM	Inner membrane
iNOS	Inducible nitric oxide synthase
IU	International units
IV	Intravenous
LMW	Low molecular weight
LPS	Lipopolysaccharide

LTA	Lipoteichoic acid
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MLCK	Myosin Light Chain Kinase
MMP	Matrix metalloproteinase
MMW	Medium molecular weight
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
MWCO	Molecular weight cut-off
Mw _{24h}	Molecular weight at 24 h incubation time in α -amylase
NaOH	Sodium hydroxide
NCTC	National collection of type culture
\cdot NO	Nitric oxide
OC	Outer compartment
OM	Outer membrane
\cdot ONOO \cdot	Peroxynitrite
PAP	Pseudomonas alkaline phosphatase
PBS	Phosphate buffered saline
PD	Pharmacodynamic
PEG	Poly(ethyl glycol)
PG	Prostaglandin
PGE ₂	Prostaglandin E ₂
PGI ₂	Prostaglandin I ₂
PI	Phosphatidyl inositol
PK	Pharmacokinetic
PLA ₂	Phospholipase A ₂

PUMPT	Polymer masking unmasking protein therapy principle
PVDF	Polyvinylidene fluoride
RI	Refractive index
RNA	Ribonucleic acid
RP-HPLC	Reverse phase high performance liquid chromatography
RPM	revolutions per minute
SD	Standard deviation
SERRPIN	Serine protease inhibitor
SLPI	Secretory leucocyte protease inhibitor
SPE	Solid phase extraction
$t_{1/2}$	Half life
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLR	Toll-like receptor
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TTK	Time to kill
UV	Ultraviolet
VD	Volume of distribution
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPE	Vascular permeability enhancement
XDR	Extremely drug resistant

Chapter One

Introduction

1.1 Introduction

Multidrug-resistant (MDR) infection is a global health concern accounting for over 27,000 deaths, considerable healthcare costs estimated at €1.5 billion per year in Europe and \$14-22 billion per year in the USA (2010). The incidence of microbial resistance is increasing and the emergence of MDR pathogens has been followed by extended and pan-drug resistant species (Boucher 2010; Maltezou 2009; Michalopoulos and Falagas 2010). A sustained reduction in the development of novel antibiotics by the pharmaceutical industry has aggravated this situation further (Boucher *et al.* 2009; Bradley *et al.* 2007; Spellberg *et al.* 2008). Gram-negative infection has consequently been identified as an imminent global health threat due to the limited current therapeutic options and the virtual absence of drugs in advanced stages of development (European Medicines Agency 2009; Horton 2009; Morel and Mossialos 2010; Talbot 2008). The purpose of this study is to develop and optimise a prototype, bio-responsive dextrin-colistin conjugate for use in acute infective disease processes where Gram-negative antimicrobial resistance presents a major clinical challenge.

1.2 Gram-negative infection

1.2.1 Gram-negative bacterial structure and antimicrobial targets

Several structural features of the Gram-negative bacterial cell envelope play a significant role in determining the success of antimicrobial therapy. A common denominator for effective activity of several antimicrobials in current clinical use is access to the bacterial cell, which is limited by a formidable barrier: the Gram-negative bacterial envelope. Topoisomerase-catalysed DNA strand breakage and rejoining is essential for DNA synthesis, mRNA transcription, and cell division (Espeli and Marians 2004; Gellert *et al.* 1976). Fluoroquinolone class antibiotics have been markedly successful in the clinical treatment of Gram-negative bacteria through targeting DNA gyrase and topoisomerase IV (Blondeau 2004). Messenger RNA translation has also provided fertile grounds for antimicrobial targeting. For example, macrolides and amphenicols are 50S ribosome inhibitors whilst tetracyclins and

aminoglycosides are 30S ribosome inhibitors (Bulkley *et al.* 2010). Additionally, rifamycins are semi-synthetic antibiotics which inhibit RNA synthesis and affect nucleic acid metabolism (Floss and Yu 2005). Recent evidence suggests a common pathway for antimicrobial-mediated cell death through altered tricarboxylic acid cycle and iron metabolism, resulting in production of lethal hydroxyl radical concentrations (Kohanski *et al.* 2010).

The bacterial periplasm is limited by a thin elastic cytoplasmic membrane mainly composed of phospholipids and proteins. The bacterial cell wall lies on the outer aspect of the cell membrane conferring strength. The rigid peptidoglycan polymer of this inner membrane (IM) prevents osmotic lysis (Silhavy *et al.* 2010). Gram-negative bacteria are distinguished by a second outer membrane (OM) external to the peptidoglycan layer (Kamio and Nikaido 1976). Lipopolysaccharide (LPS) is a major component of this OM and is a glucosamine disaccharide with 6-7 acyl chains, a polysaccharide core and an additional polysaccharide chain termed the O-antigen (Raetz and Whitfield 2002). LPS is responsible for endotoxic shock and is crucial to OM barrier function. The OM plays an important function in impeding the ingress of many antibiotics and access to their periplasmic targets (Figure 1.1). Peptidoglycan biosynthesis is a target employed by antibiotic classes including β lactams and glycopeptides resulting in changes to cell shape and size, induction of cellular stress responses, and ultimately, death by lysis (Tomasz 1979). Additionally, antimicrobial peptides are a class of widely occurring natural antibiotics that exert a lytic, detergent effect on the Gram-negative bacterial cell envelope (Zasloff 2002).

1.2.2 Clinical course of Gram-negative infection

Current therapeutic options for the "ESKAPE" pathogen group identified by the Infectious Diseases Society of North America are limited (Rice 2008). Four of these pathogens are Gram-negative bacteria: *Escherichia coli* and *Klebsiella* species; *Acinetobacter baumannii*; *Pseudomonas aeruginosa*; and

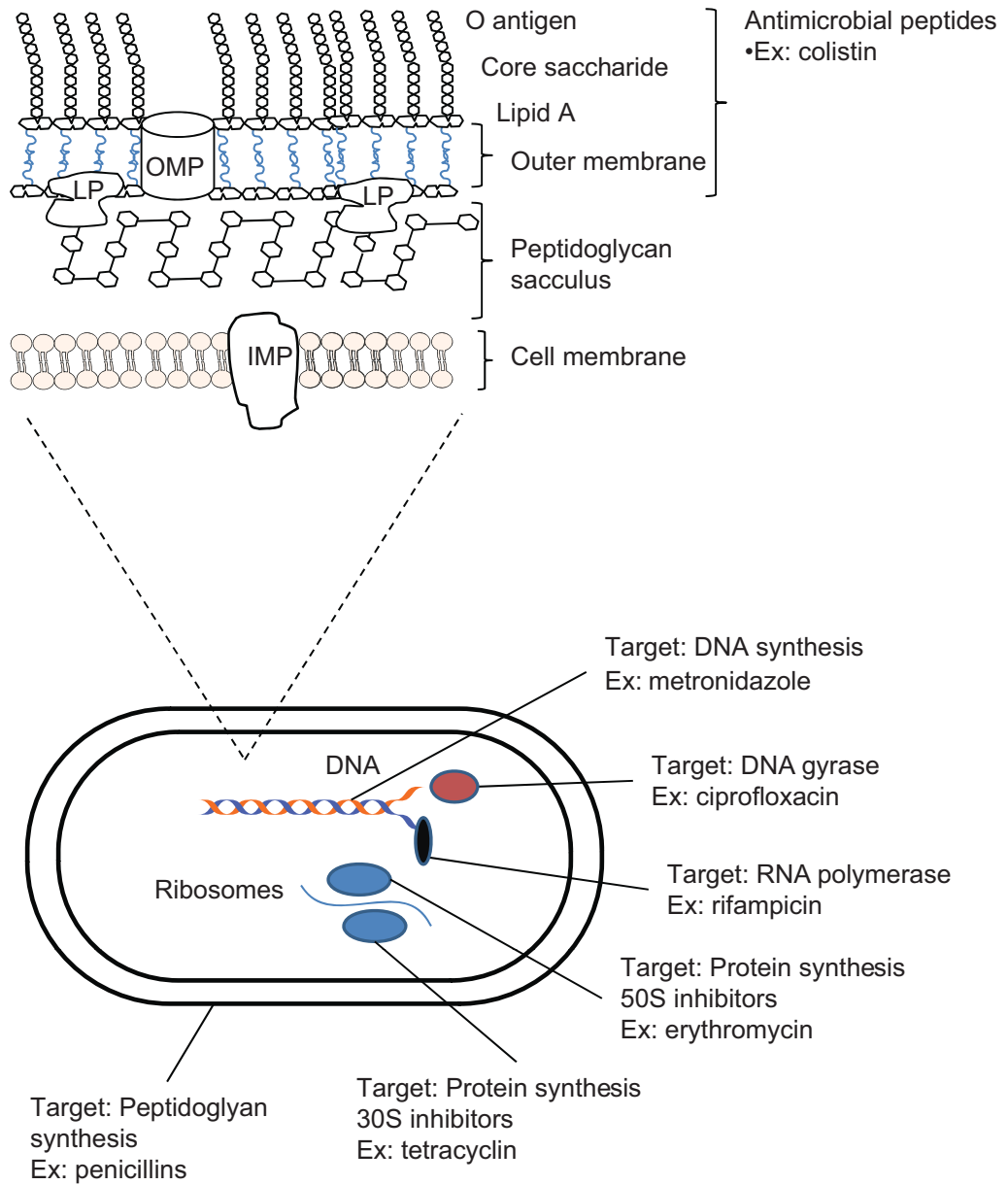


Figure 1.1: Gram-negative bacterial structure. Panel (a) (inset) illustrates the Gram-negative bacterial envelope; Panel (b) illustrates common targets for antimicrobials whose access to periplasmic targets is impeded by the outer membrane. OMP: outer membrane protein; IMP: integral membrane protein.

Enterobacter species (Boucher *et al.* 2009). These pathogens require urgent attention in terms of novel antibiotic discovery (Rice 2008). These infections often complicate treatment of older patients with multiple co-morbidities. This now represents an increasingly common patient profile in western societies. These patients are prone to infection and less tolerant to the adverse, toxic effects of antimicrobial therapy (High *et al.* 2009). The challenge posed by the increasing frequency of susceptible patients is exacerbated by an increasingly common occurrence of organisms with multiple drug resistance (Raetz and Whitfield 2002).

1.2.3 Multidrug resistance in Gram-negative bacteria

Motivated by the demand for better bioactivity and lower toxicity, antibiotic research and development has passed through several phases. Most early discoveries were reported through natural product screening (e.g. macrolides, aminoglycosides, cephalosporins and glycopeptides), followed by semi-synthetic chemistry programs (e.g. clavulanic acid, tobramycin), through discovery of the fluoroquinolones and their analogs (Davies and Davies 2010; Fernandes 2006). Recent approaches such as bacterial genomics have had little notable success (Shlaes and Projan 2009).

The literature presents various definitions to characterise the resistance patterns encountered in healthcare-associated antibiotic-resistant bacteria. Resistance to three or more different antibiotic classes has been frequently adopted in the literature as a working definition for MDR bacteria (D'Agata 2004; Lockhart *et al.* 2007; Zhanel *et al.* 2008). More recently, a joint initiative by the major European and US Centres for Disease Control proposed to define multidrug resistance with regard to *Staphylococcus aureus*, *Enterococcus spp.*, Enterobacteriaceae (other than *Salmonella* and *Shigella*), *P. aeruginosa* and *Acinetobacter spp.* as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al.* 2011).

Multiple mechanisms may give rise to antimicrobial resistance including chromosomal mutations or horizontal gene transfer (Khan *et al.* 2012).

Resistance may occur by several mechanisms including antibiotic degradation (Kumarasamy *et al.* 2010); mutations in specific antibiotic target sites such as DNA gyrase or RNA polymerase (Lambert 2005); limitation of local antibiotic concentration or porin modification (Delcour 2009); specific pump efflux mechanisms (Nehme and Poole 2007) or even antibiotic sequestration within bacterial biofilm (Mah *et al.* 2003). In addition to multidrug resistance, extensively drug resistant (XDR) bacteria have been described where effective antibiotic options are extremely limited (Conly and Johnston 2006; Magiorakos *et al.* 2011).

There are currently only 6 compounds in clinical trials active against Gram-negative infection (Rennie 2012). Moreover, it has been estimated that only 75% of those compounds which complete phase III trials will be marketed (Rennie 2012). In the period between 1940-1962 more than 20 new antibiotic classes reached clinical use. Since then, only two new classes entered clinical use. Of these novel antibiotics only one candidate (leucyl-tRNA synthetase inhibitor) exhibited a novel mechanism of action, leaving a considerable shortfall in the demand for novel effective antibiotics against Gram-negative bacteria (Coates and Halls 2012).

1.2.4 Current therapeutic approaches to Gram-negative infection

In the absence of effective therapeutic alternatives, re-screening and chemical modification of natural antibiotic libraries is a recent approach in antimicrobial research and development (Fernandes 2006). Antimicrobial peptides are a widely distributed class of naturally occurring antibiotics that function through a detergent effect on the bacterial cell envelope (Zaslhoff 2002). The polymyxins are prime examples of this antibiotic class and were amongst the first classes of antimicrobial agents to be discovered (Stansly *et al.* 1947). Colistin is an example of an effective antimicrobial peptide with extensive antimicrobial activity. This resulted in widespread use after its discovery (Stansly *et al.* 1947). Subsequent semi-synthetic modifications of effective antibiotics yielded successive generations of antibiotics with more favourable activity/toxicity profiles (Fernandes 2006). Interest in colistin

subsequently decreased, presumably in favour of newer, less toxic antibiotics (Arnold *et al.* 2007; Falagas and Kasiakou 2006; Ma *et al.* 2009). It is possible that this prolonged period of disuse reduced those selection pressures favouring resistance, allowing colistin to retain antimicrobial activity when efficacy of other antimicrobial classes is failing. Because of toxicity concerns, colistin currently occupies a clinical niche as a "treatment of last resort" (Levy and Marshall 2004). Literature trends demonstrated a marked increase in interest within the medical and academic community in colistin which mirror that observed for MDR bacteria (Figure 1.2).

1.2.5 Colistin redevelopment

Recent interest in colistin addresses two challenges for its effective and safe use. Firstly, research is directed at re-engineering the molecule to reduce the toxicity of the colistin towards a favourable activity/toxicity profile (Table 1.1). The colistin molecule is made up of (a) hydrophilic cycloheptapeptide ring with 3 positively charged amine groups (b) a tail tripeptide moiety with 2 positively charged amine groups (c) a hydrophobic acyl chain tail (Figure 1.3). The first theme reported within this trend reports the "deconstruction" of the colistin molecule by analysing the properties of derivatives substituting or lacking the different structural parts or charges (Figure 1.3 a-c). Colistin was determined to exert a bactericidal detergent action by way of its amphiphilic (part hydrophilic - part hydrophobic) structure on the bacterial membrane (Vaara 1992). The amino groups mediate both the extensive bactericidal effect and toxicity to human cells (Clasell *et al.* 2007; Mares *et al.* 2009; Vaara *et al.* 2008). Therefore, modification of these groups has been the focus of extensive study (Vaara *et al.* 2008). Early studies attempted sulfomethylation of the amino groups forming an uncharged prodrug, which hydrolyzes *in vivo* to colistin (Barnett *et al.* 1964). The result is colistimethate sodium or colomycin, which is the predominant, systemic form of colistin administered in contemporary practice (Beveridge and Martin 1967).

Table 1.1: Research and development trends on colistin: re-engineering the colistin molecule towards a favourable activity/toxicity profile.

	Intent	Bioactivity vs parent compound	Key studies
N-Terminal Fatty Acyl Analogues	OM penetration and disrupt packing of the lipid A fatty acyl chains	Both colistin and PMB nonapeptides do not possess direct antimicrobial activity. Specifically bind to LPS and disrupt OM. Efficiently sensitise bacteria to hydrophobic antibiotics.	Nakajima <i>et al.</i> (1967) Bhattacharjya <i>et al.</i> (1997).
	Analogues of the D-Phe6-L-Leu7 hydrophobic domain	Hydrophobic interactions of the fatty-acyl chains of lipid A and the hydrophobic side chains of D-Phe6-L-Leu7 motif stabilise the LPS-polymyxin complex. This domain is highly conserved among polymyxins, but not imperative for activity.	Pristovtec and Kidric (2004, 1999) Kanazawa <i>et al.</i> (2009)
	Study variation in fatty acyl chain composition on antimicrobial effect	Octanoyl N-terminal fatty acyl chains displayed optimal activity. Activity was reduced with longer/shorter chains. Poor antimicrobial activity against <i>E. coli</i> , and <i>S. enterica</i> but potent activity against <i>P. aeruginosa</i> .	Chihara <i>et al.</i> (1974) Katsuma <i>et al.</i> (1974) Orwa <i>et al.</i> (2001) Sakura <i>et al.</i> (2004)
DAB side-chain	Dab N ^Y -formyl PMB derivatives	Dab N ^Y -Triformyl derivative was equally effective to parent compound Dab N ^Y -diformyl derivative (105) was 70% more active.	Teuber <i>et al.</i> (1970) Srinivasa and Ramachandran (1978) Weinstein <i>et al.</i> (1998)
	Serial DAB substitutions to alanine to determine the most significant contribution to activity.	Loss of the positive charges on the tripeptide linker moiety all displayed effective antimicrobial activity. However, loss of cationic charge in the cyclic heptapeptide moiety resulted in complete loss of antimicrobial activity. DAB in position 5 has an important bactericidal role.	Kanazawa <i>et al.</i> (2009) Vaara <i>et al.</i> (2012, 2008)
	Serial DAB→ amino acid substitutions	Length of the amino acid side-chain, as well as the cationic nature of the amino acid plays an important role in antimicrobial activity	Tsubery <i>et al.</i> (2002)
	Sulphomethylation	Blocking the free amino groups by sulfomethylation yields an uncharged prodrug which hydrolyses to the active compound. CMS is the current clinically employed form of colistin, but hydrolysis is unpredictable.	Barnett <i>et al.</i> (1964) Beveridge and Martin (1967) Molina <i>et al.</i> (2009)
	Polymer therapeutics	Conjugation to large, biodegradable water soluble polymers such as dextran for use as an anti-endotoxin. No bioactivity data available.	Bucklin <i>et al.</i> (1995)
	Acyclic analogs of colistin peptides	The lariat structure is essential for antimicrobial activity. Both branched and linear analogs showed no antimicrobial activity.	Kline <i>et al.</i> (2001)

PMB: polymyxin B, OM: outer membrane

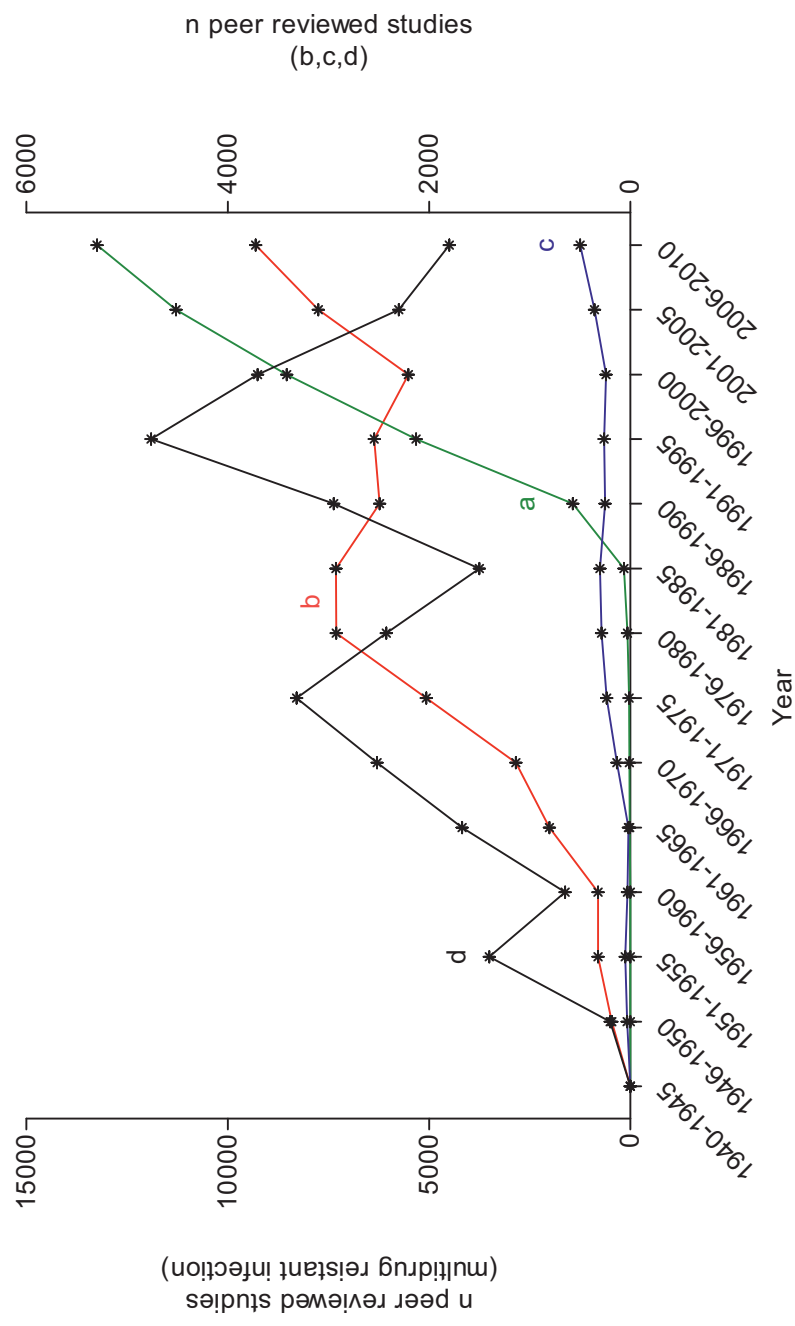


Figure 1.2: Literature trends on multidrug resistant infection (A), colistin (B), and its adverse events (C). Literature on chloramphenicol (D) was plotted as control.

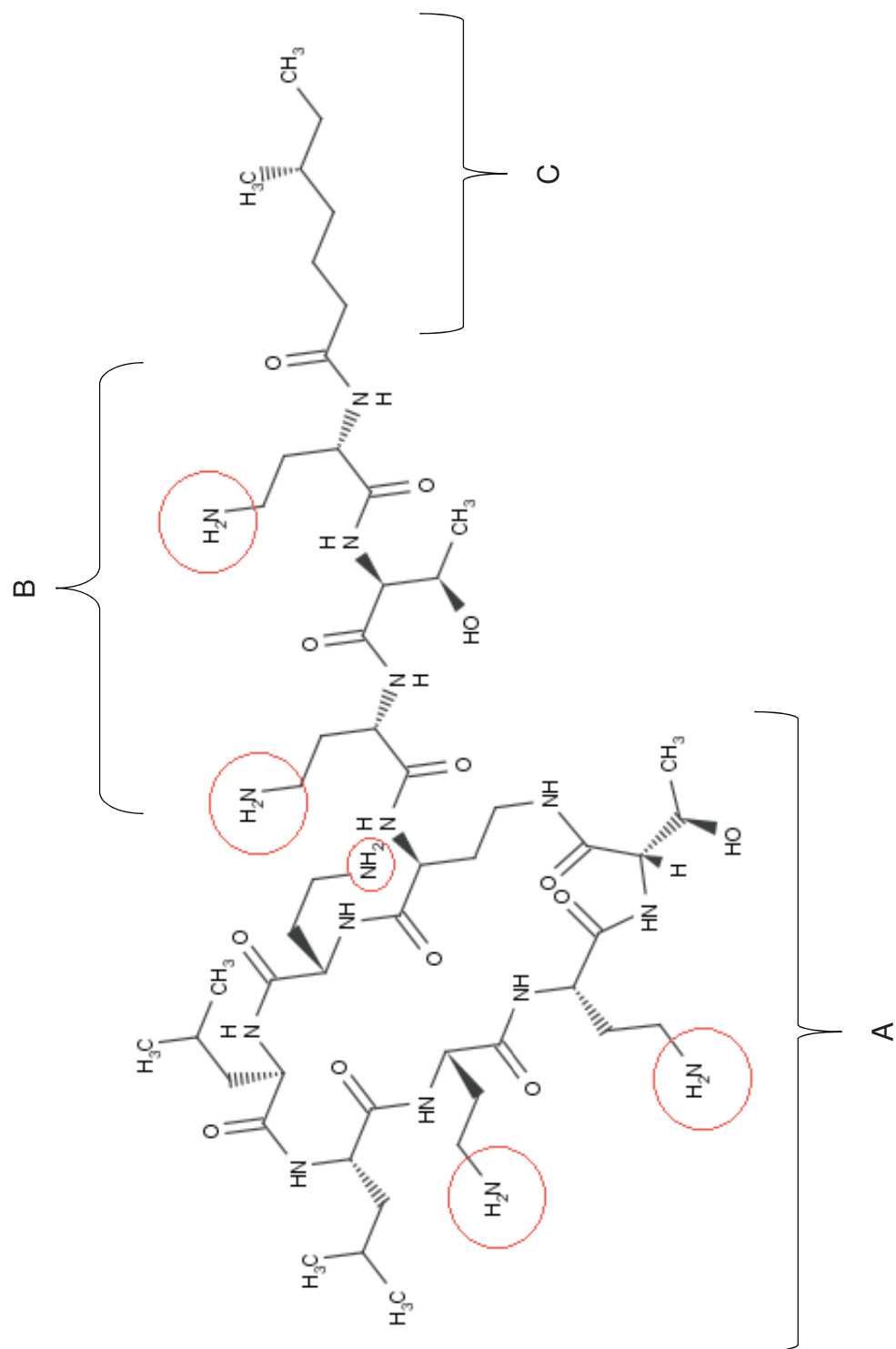


Figure 1.3: Structure of the colistin molecule illustrating the tripartite structure: cyclic heptapeptide ring (A), linear tripeptide segment (B), and hydrophobic acyl tail (C). The reactive amino groups are encircled.

However, hydrolysis into the active drug is concentration- and temperature-dependant. This may result in unpredictable colistin formation and toxicity (Healy *et al.* 2011b; Wallace *et al.* 2008). The second area of research addresses optimisation of the current formulation, its characterisation, purity, structure-activity relationship, PK/PD relationships, dosing, stability, analytical methods and resistance surveillance (Table 1.2). Colistin was developed before the inception of current standardised dosage and pharmacokinetic / pharmacodynamic (PK/PD) requirements (Marchand *et al.* 2010). *In vivo* critical care PK/PD studies have only recently been reported (Healy *et al.* 2011b). Interestingly, dosage regimens vary widely between countries, from once daily to four divided daily doses (Conly and Johnston 2006).

Like other conventional small molecule antibiotics, colistin does not preferentially localise to the intended (infected) area. Distribution to unintended areas away from the intended site of infection may facilitate toxicity (Arnold *et al.* 2007; Falagas and Kasiakou 2006; Falagas *et al.* 2009; Ganapathy *et al.* 2009; Ma *et al.* 2009). A redevelopment strategy to afford selective, controlled release at the site of infection would, therefore, considerably improve patient treatment.

1.3 Nanomedicine approaches to the treatment of infection

Nanomedicines represent nano-scaled tools for the diagnosis, prevention and treatment of disease (Duncan and Gaspar 2011). Over the last decade, nanomedicine has been increasingly employed as a means of drug targeting and improving drug delivery to reduce toxic side effects, in particular cancer therapy (Duncan and Gaspar 2011). A number of studies have also investigated various nanomedicine approaches for the safe and effective delivery of antibiotic drugs (Table 1.3). This growing class of nano-sized antimicrobial therapies includes, amongst others, antimicrobial nanomaterials, nanoparticles, liposomes and polymer therapeutics.

Table 1.2: Research and development trends on colistin: optimisation of current therapy

Theme	Subtheme	Narrative synthesis of major studies
Resistance	Epidemiology	Resistance to polymyxins remains low in most countries (Karageorgopoulos and Falagas 2008) including North America (Sinirtas <i>et al.</i> 2009), the Middle East (Dizbay <i>et al.</i> 2008). However a trend to greater resistance was observed in the Asia-Pacific, Latin American regions (Gales <i>et al.</i> 2011; Hernan <i>et al.</i> 2009) the far-east (Ko <i>et al.</i> 2007; Tan and Ng 2006), and Greece (Gilad <i>et al.</i> 2005; Antoniadou <i>et al.</i> 2007).
	Mechanisms	Early studies reported that membrane structural changes may confer resistance to polymyxins. These included fatty acid alterations and lipopolysaccharide (LPS) binding to polymyxin (Conrad and Galanos 1989), changes in negatively-charged surface lipopolysaccharides induced by the regulatory loci <i>pmrA</i> and <i>phoP</i> , generate resistance to polymyxins (Groisman <i>et al.</i> 1997); different lipid compositions of lipopolysaccharides (Conrad and Galanos 1989; Vaara and Vaara 1983) or substitution of protein OprH for magnesium in the outer membrane (Brown <i>et al.</i> 1990; Nicas and Hancock 1980).
	Genetics	Genetics implicated in polymyxin resistance include those for the 2-component signaling proteins PmrB and PmrA linked by polymyxin resistance protein, PmrD (Adams <i>et al.</i> 2009; Fu <i>et al.</i> 2007; Sabuda <i>et al.</i> 2008) regulatory loci <i>pmrA</i> (Groisman <i>et al.</i> 1997), <i>pho P pho Q</i> , (reviewed in (Velkov <i>et al.</i> 2009); <i>micF</i> and <i>osmY</i> (Oh <i>et al.</i> 2000); chromosomally encoded multidrug efflux systems (Brown <i>et al.</i> 1990; Germ <i>et al.</i> 1999; Li <i>et al.</i> 2000).
Characterization	Formulation	Stability: colistin is susceptible to degradation in solutions of pH ≥ 5 , principally by racemisation; new formulations in solution (Lin <i>et al.</i> 2005); component analysis (Orwa <i>et al.</i> 2001); component <i>de-novo</i> synthesis (Kline <i>et al.</i> 2001).
	Physico-chemical	Initial non-specific microbiological assays (Thomas <i>et al.</i> 1980) were followed by mass spectrometric (MS) (Govaerts <i>et al.</i> 2002a; Govaerts <i>et al.</i> 2002b); liquid chromatography-MS (LC-MS) (Ma <i>et al.</i> 2008); HPLC (Li <i>et al.</i> 2003b); hyphenated liquid chromatography (Decolin <i>et al.</i> 1997) and NMR (Mares <i>et al.</i> 2009; Pristovsek and Kidric 1999) methods.
	Structure	Analysis of <i>de-novo</i> synthesized colistin components and analogs (Kline <i>et al.</i> 2001) and structure analysis (Ikai <i>et al.</i> 1998).
	Pharmacokinetics	Pharmacokinetics (PK) of novel solutions (Lin <i>et al.</i> 2005; Orwa <i>et al.</i> 2002), PK studies in critically ill patients (Healy <i>et al.</i> 2011a; Plachouras <i>et al.</i> 2009).
Toxicity	Clinical studies	Current studies debate the "unacceptable toxicity of colistin", but most studies report significant toxic adverse effects: nephrotoxicity (Cheng <i>et al.</i> 2009; Falagas and Kasiakou 2006; Ganapathy <i>et al.</i> 2009; Hartzell <i>et al.</i> 2009; Pintado <i>et al.</i> 2008), neurotoxicity (Cheng <i>et al.</i> 2009), rhabdomyolysis (Evangelopoulou <i>et al.</i> 2007).

Table 1.3: Nanomedicines that have undergone/are undergoing *in vivo* evaluation for the treatment of infection

Delivery vehicle	Drug	Carrier	Clinical status	Reference
Nanoparticle	Rifampicin	Poly(lactide)-co-glycolide	<i>In vitro</i> , pre-clinical	Esmaeli <i>et al.</i> (2007)
Core-shell nanostructure	Gentamicin	Pluronic™ PEO-PPO block copolymer	<i>In vivo</i> , pre-clinical	Ranjan <i>et al.</i> (2009)
Dendrimer	Erythromycin	PAMAM G4	<i>In vitro</i> , pre-clinical	Bosnjakovic <i>et al.</i> (2011)
Liposome	Amikacin	PC: cholesterol (2:1)	Phase II	Whitehead <i>et al.</i> (1998)
Liposome	Gentamicin	PC: 1,2-DSPE-N-[PEG-2000] (PEG-DSPE) (2.85:0.15)	Phase I	Schiffelers <i>et al.</i> (2001a)
Liposome	Gentamicin and ceftazidime	PC: cholesterol: PEG-DSPE (1.85:1:0.15)	<i>In vivo</i> , pre-clinical	Schiffelers <i>et al.</i> (2001b)
Liposome	Isoniazid and rifampicin	PC: cholesterol: dicetylphosphate: PEG-DSPE (2:1.5:0.2:0.2)	<i>In vivo</i> , pre-clinical	Labana <i>et al.</i> (2002)
Liposome	Vancomycin and ciprofloxacin	PC: stearylamine: cholesterol (7:2:1)	<i>In vivo</i> , pre-clinical	Kadry <i>et al.</i> (2004)
Liposome	Ciprofloxacin	PEG-DSPE: PC: cholesterol (5:50:45)	<i>In vivo</i> , pre-clinical	Bakker-Woundenberg <i>et al.</i> (2002)
Liposome	Colistin	PC: cholesterol (2:1)	<i>In vivo</i> , pre-clinical	Wang <i>et al.</i> (2009)
Liposome	Streptomycin	PC: cholesterol and PG (2:1:0.1)	<i>In vivo</i> , pre-clinical	Fielding <i>et al.</i> (1998)
Polymer-drug conjugate	Ciprofloxacin and norfloxacin	PEG	<i>In vitro</i> , pre-clinical	Pinter <i>et al.</i> (2009)
Polymer-drug conjugate	Peptoid 7	PEG PGA	<i>In vivo</i> , pre-clinical	Vicent <i>et al.</i> (2010)
Polymeric drug	OligoG fragments	Alginate oligomer	Phase I	Ferguson <i>et al.</i> (2012a)
Polymer-drug conjugate	Ubiquitin-PEG	(PEG–N-(N-(3-diphenylphosphinopropionyl)glycyl)-S-tritylcysteine ligand)	<i>In vivo</i> pre-clinical	Melendes-Alafort <i>et al.</i> (2009)

PC: phosphatidylcholine; PEO: poly(ethylene oxide); PPO: poly(propylene oxide); PAMAM: poly(amidoamine); G4: fourth generation; DSPE: distearoyl-sn-glycero-3-phosphoethanolamine; PEG: poly (ethylglycol).

1.3.1 Antibiotic nanomaterials

Nanomaterials exhibit several physicochemical properties that may be advantageously exploited for antimicrobial means including high surface area to volume ratios (Weir *et al.* 2008). Several nanoparticles have been studied for their antimicrobial value including production of reactive oxygen species, interference with energy transduction mechanisms, and DNA synthesis (reviewed in Huh and Kwon 2011). Synergic nanoparticle-antibiotic combinations have been reported potentially reducing toxic antibiotic concentrations necessary to achieve antimicrobial effects (Rai *et al.* 2009; Shahverdi *et al.* 2007). However, non-specific toxicity of the nanomaterials themselves, has been described as a major drawback including mitochondrial adverse effects, nephrotoxicity and myotoxicity (Hussain *et al.* 2005). Insolubility and inherent non-selective toxicity were major concerns for fullerenes and carbon nanotube applications (Jia *et al.* 2005; Tsao *et al.* 2002; Wick *et al.* 2007).

1.3.2 Liposomes as antibiotic delivery systems

Liposomes were among the first clinically viable nanomedicines and are often overlooked in contemporary reviews (Duncan and Gaspar 2011). These vesicles consist of a water space surrounded by single/multiple lipid bilayers. The lipid bilayers may interact with viable cells through absorption, lipid exchange, endocytosis, and fusion (Pinto-Alphandary *et al.* 2000). Since doxorubicin-encapsulating PEG-liposome was first approved in 1995, liposomal antibiotic delivery systems have experienced marked clinical success (Lian and Ho 2001). The ability of their lipid bilayer to fuse with bacteria contributed to their widespread adoption (Zhang *et al.* 2010). Additionally, both the aqueous core and phospholipid bilayers can be used to retain antibiotics of varying hydrophobicity (Lasic 1998; Sosnik *et al.* 2010). Literature reports several attractive properties of liposomes for antibiotic delivery including: improved pharmacokinetics, decreased toxicity, enhanced activity versus both intracellular and extracellular pathogens, and the possibility for target selectivity (extensively reviewed in Drulis-Kawa and Dorotkiewicz-Jach 2010). Liposomes “loaded” with colistin sulfate have been

recently described. Recently, Wang *et al.* (2009), reported colistin sulfate liposomes (phospholipid:cholesterol) exhibiting an initial (40%) burst release (ascribed to unencapsulated colistin), followed by a slower release phase (complete at 24 h) and reduced *in vivo* toxicity. In contrast, colistin and CMS liposomes recently reported by Wallace *et al.* (2012) showed 50% release within 10 min, prompting the authors to comment about their limited utility for long duration controlled-release applications. Short lipid vesicle shelf-life, with consequent drug stability issues, represents a challenge to liposome antibiotic delivery (Drulis-Kawa and Dorotkiewicz-Jach 2010). Short shelf-life is already a concern with current CMS formulations (section 1.2.5). *In vivo*, physical instability may exacerbate drug leakage, resulting in payload transfer to plasma lipoproteins or membranes, (Gregoriadis 1995). Moreover rapid clearance of liposomes (such as the those recently described by Wang *et al.* (2009) and Wallace *et al.* (2012) by the mononuclear phagocytic system has been established, necessitating introduction of various strategies to prolong circulation time, such as polymer conjugation, (Moghimi and Szebeni 2003).

1.4 Polymer therapeutics as antibiotic nanomedicines

Polymer therapeutics represent a class of nano-sized therapeutic agents typically consisting of at least 2 components: a water-soluble polymer covalently attached to an active constituent, such as a drug, protein, gene or peptide (Duncan 2003). This generic term encompasses a number of diverse entities whose common denominator is the possession of a water-soluble polymer. These include: polymeric drugs, polymer–drug conjugates, polymer–protein conjugates, polymeric micelles (to which drug is covalently bound) and multi-component polyplexes (Duncan 2003). The conjugation of relatively toxic drugs to water-soluble polymers has become well-established as a reliable method for delivering proteins, peptides and antibody-based therapeutics. These new chemical entities are distinct from conventional drug delivery systems and offer considerable advantages over them (Duncan and Gaspar 2011; Vicent *et al.* 2009). Polymer conjugation permits the rationalised design of new molecules to alter plasma circulation time, distribution and

bioavailability, decrease immunogenicity, mask protein charge and toxicity (Duncan 2009; Flanagan *et al.* 1990; Greco and Vicent 2008). The nature of the water-soluble polymer can be varied to suit the intended clinical demand.

1.4.1 Polymer designs

Polymers consist of repeating single component units (monomers) to produce a high molecular weight structure with unique physicochemical characteristics. Polymers may be classified into homopolymers (whose chains contain one, repeating monomer unit) or copolymers. Copolymers consist of more than 1 monomer type. The different monomers may take the form of an alternating copolymer chain, a block copolymer or a graft copolymer. Polymers also vary according to structure and may be linear, branched, stellate, or dendrimeric (Duncan *et al.* 2005). Gauthier and Klok (2010) suggest that the influence of flexible linear polymers (e.g. dextrans) on conjugate activity is greater than those polymers displaying branching, folding or bulky side-chains. Conveniently, polymers may also be broadly classified according to their biodegradability (Duncan 2011).

1.4.2 Degradable versus non-degradable polymers

The majority of polymers reaching current clinical practice are non-biodegradable synthetic polymers, including, for example, poly(ethyleneglycol) (PEG) (Pasut and Veronese 2009), and N-(2-hydroxypropyl) methacrylamide (HPMA) co-polymers (Duncan 2009). PEGylated conjugates are clinically well-tolerated and constitute a substantial proportion of the conjugates to have entered clinical practice (Gauthier and Klok 2010). Their lack of biodegradability, however, may have safety implications (Duncan and Gaspar 2011). The need to ensure renal elimination limits the size of non-biodegradable conjugates to below renal threshold (Duncan 2003; Hreczuk-Hirst *et al.* 2001b). Otherwise, non-biodegradable polymers carry a risk of toxic accumulation (Gaspar and Duncan 2009), and lysosomal storage or other metabolic aberrations (Chi *et al.* 2006; Miyasaki 1975). This would be especially relevant where

administration of cumulative doses would be anticipated, such as in infection or the treatment of chronic disease.

Biodegradable polymers offer several advantages that lend themselves to the construction of bioresponsive polymer-antibiotic conjugates intended as therapeutic agents in infection. Hydrolytic or enzymatic degradability is a property of several biodegradable polymers including hydroxyethyl starch (HES) (Besheer *et al.* 2009); polyglutamic acid (PGA) (Santamaría *et al.* 2009) and dextran (Bucklin *et al.* 1995). Biodegradable polymers permit more flexibility for the optimisation of pharmacokinetic profiles since the use of higher molecular weight platforms is possible (Duncan and Gaspar 2011). A number of concerns have been raised with some biodegradable polymers. Dextrans may generate an immunoglobulin-M (IgM) response (Battisto and Pappas 1973), and they are degraded slowly (Vercauteren *et al.* 1990). Specific problems have also been reported with dextrans and HES in the conjugated forms, and with the degradation products of the respective conjugates. For example, HES fractions may cause hypersensitivity and interfere with coagulation processes causing haemorrhage (Bisaccia *et al.* 2007; Treib *et al.* 1997). Dextrans tend to form non-degradable adducts even with low levels of chemical modification, limiting their utility (Vercauteren *et al.* 1990).

1.4.3 Strategies for triggered "payload release" in polymer therapeutics

Many potential mechanisms have been examined for the triggered release of payloads from the respective polymer conjugate, including physicochemical, metabolic and enzymatic (Hoffman 2004; Roy and Gupta 2003). Physical stimulation such as temperature (Shimboji 2001, 2003), and electromagnetic energy (Shimboji 2002) have been used to instigate ligand-receptor recognition. Reversible, steric hindrance has also been employed next to active sites (Hoffman 2004; Shimboji 2001, 2002). Chemically-induced methods such as pH have also been described (Bulmus *et al.* 2003). Polymer-enzyme conjugates were used to instigate the degradation of pre-administered liposomal therapy (Duncan *et al.* 2001). More recently, an

elegant approach for “shielding” the bioactive payload in transit, followed by localised enzymatic controlled release and restitution of bioactivity has been described by Duncan *et al.* (2008).

The polymer masking-unmasking-protein therapy principle (Duncan *et al.* 2008) involves a multi-functional biodegradable polymer to envelope the payload of interest, whilst allowing locally-triggered polymer degradation and re-instatement of the masked bioactive's activity (Figure 1.4). This strategy offers several advantages. The degree of polymer modification and payload coupling can be directly controlled by varying the ratios of reactants (Hreczuk-Hirst *et al.* 2001a). The masked conjugate offers improved biological efficacy, extended plasma circulation time, reduced proteolytic degradation and protein immunogenicity (Werle and Bernkop-Schnürch 2006; Ferguson and Duncan 2009; Roberts *et al.* 2002). Locally triggered unmasking at the intended site allows controlled re-instatement of bioactivity. Proof of concept for the PUMPT principle has been provided in diverse disease states including dextrin-phospholipase A₂ as an anticancer conjugate (Ferguson *et al.* 2006); and dextrin-recombinant human epidermal growth factor (Hardwicke *et al.* 2010). An additional advantage of polymer conjugation is the conversion of a conventional "small molecule" antibiotic into a macromolecule, which may benefit from passive, size-based targeting to an infected locus (Maeda 2012).

1.4.4 The Enhanced permeability and retention effect

The EPR effect relates to the passive, size-dependant accumulation of macromolecules at sites of increased vascular permeability, and their subsequent local retention (Maeda *et al.* 2009; Matsumura and Maeda 1986). The EPR effect has provided a universal and efficient strategy for anticancer drug design, allowing increased selectivity with improved therapeutic efficacy and fewer side-effects (Duncan 2003; Maeda *et al.* 2009).

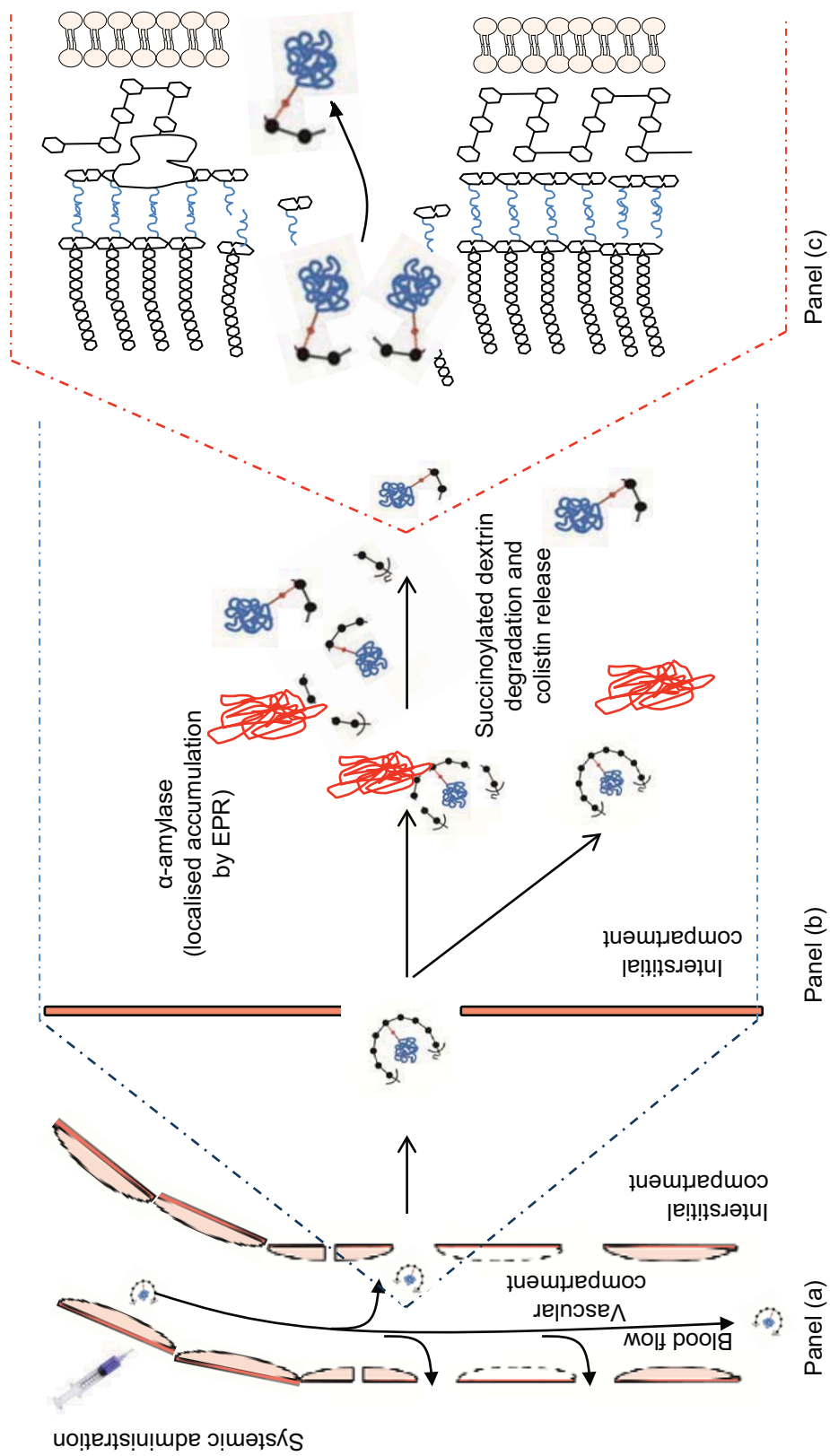


Figure 1.4: The PUMPT effect with reference to infection. Panel (a) shows the masked conjugate in transit from the site of entry into the body, to the target site. Panel (b) shows localised, enzyme mediated unmasking (inset). Panel (c) shows re-instatement of bioactivity at the infected site (inset). VPE: vascular permeability enhancement.

Whilst the EPR effect has been recognised as a breakthrough in anti-tumour targeting, its potential has not yet been fully exploited in infection. The presence of a widespread, significant EPR effect would intimately influence the rationalisation of a novel polymer therapeutic aimed to afford controlled release at the site of infection, in terms of the choice of polymer size, modification, and rate of controlled degradation, and localisation of the enzyme triggering local re-instatement of bioactivity. Before embarking on the construction of a polymer-antibiotic conjugate, it was essential to evaluate the notion of a widespread, clinically significant EPR effect in infection across salient, Gram-negative bacterial pathogens.

1.5 EPR effect as a novel paradigm for antibiotic targeting in infection

EPR in cancer has been attributed to vascular permeability enhancement (VPE) and decreased efflux of macromolecules from the pathological locus (Matsumura and Maeda 1986). Several features of infection-induced inflammation resemble these processes (Figure 1.5). Following an initial insult, rapid vasodilatation recruits additional vessels whose permeability is subsequently enhanced. Vascular permeability has been categorised into an immediate stage (contraction of endothelial cells), a transient response (endothelial injury) and transcytosis (Kumar 2010). Each of these processes has a potential for microbial protease-induced VPE. The ensuing macromolecular extravasation contributes to swelling. Furthermore, inflammation, with protracted angiogenesis (Majno 1998) and ongoing remodelling, provides the opportunity for macromolecular accumulation (Hardwicke *et al.* 2008a) (Figure 1.6).

1.5.1 Abnormal circulation

Tumour vasculature presents characteristics that contribute to the creation of an EPR effect (Maeda 2001; Maeda *et al.* 2009). Angiogenesis and high vascular density also characterise inflammation and infection (Fiedler and Augustin 2006) much like the hypervascularity classically present in solid tumours (Fang *et al.* 2010).

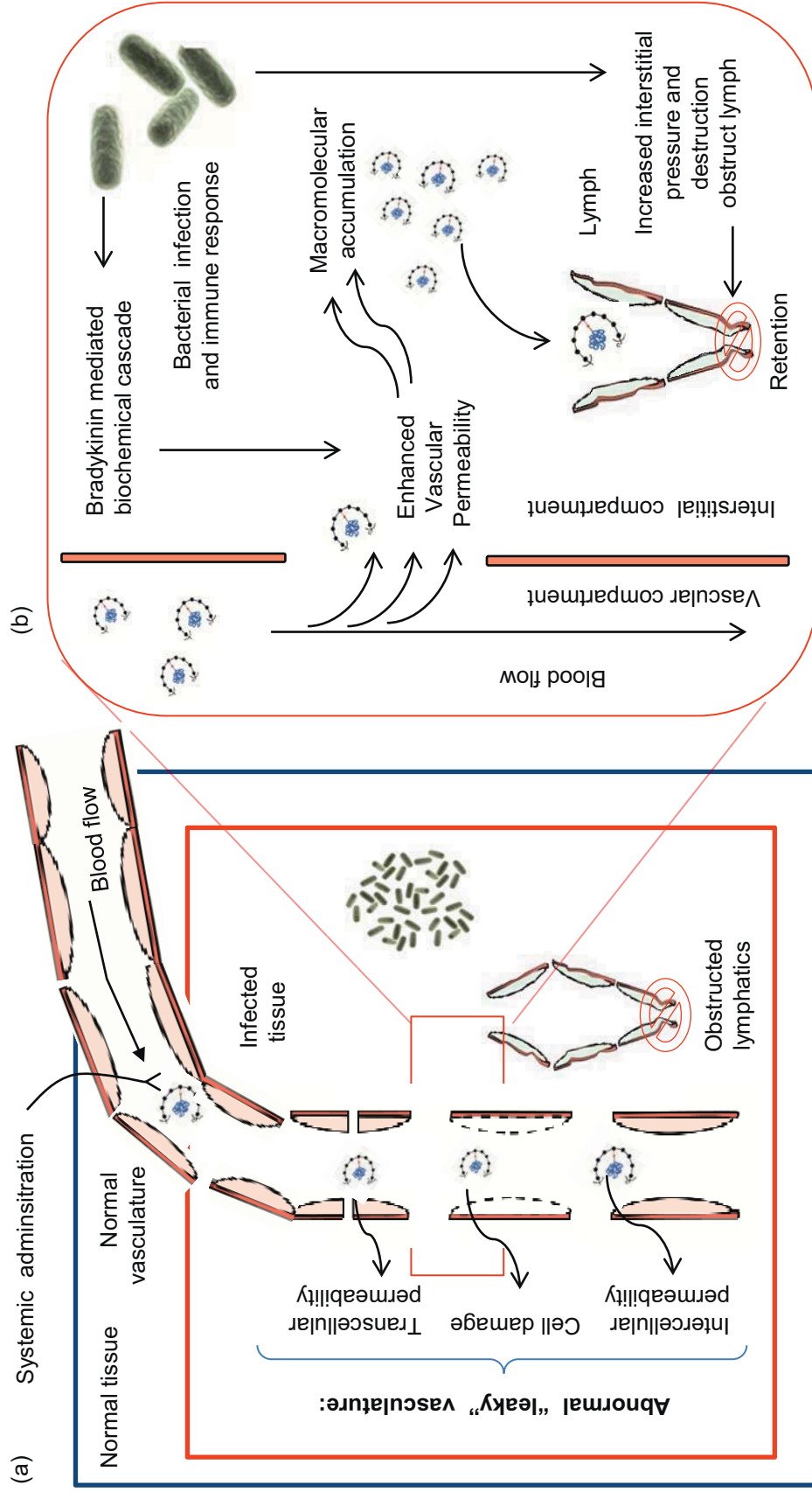


Figure 1.5: The EPR effect in infection. Panel (a) shows the effects of infection in enhancing vascular permeability and obstructing drainage. Panel (b) (Inset) shows the promotion of EPR through a biochemical cascade leading to vascular permeability enhancement, promoting macromolecular retention.

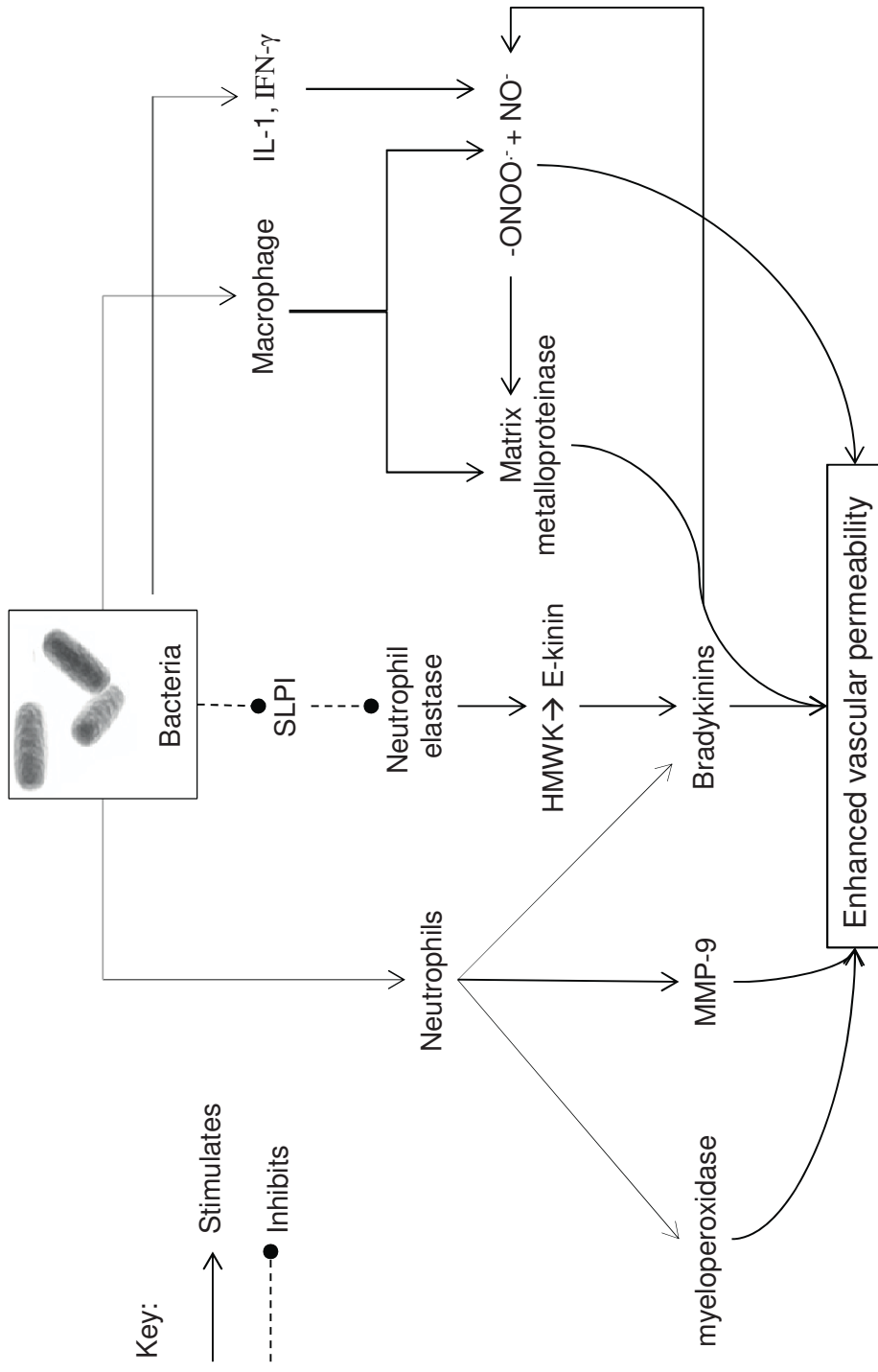


Figure 1.6: Role of the immune system in the enhancement of vascular permeability: contributory functions of macrophages and neutrophils. SLPI: secretory leucocyte protease inhibitor; -ONOO⁻: peroxynitrite; NO⁻: nitric oxide; IL-1: interleukin 1; MPO: myeloperoxidase; HMWK: high molecular weight kininogen.

Moreover, VPE has been attributed to tumour vascular abnormalities, including endothelial fenestrations and lack of smooth muscle (Daruwalla *et al.* 2008; Fang *et al.* 2004; Greish 2007; Suzuki *et al.* 1987). The majority of the VPE contribution to the EPR effect in infection is found to occur at the post-capillary venule region, which resembles the tumoural circulatory compartment (Maeda 2001; Mares *et al.* 2009) in terms of inter-endothelial junctions (Baffert *et al.* 2006; Maruo *et al.* 1998; Mehta and Malik 2006; Spindler *et al.* 2010) and caveolar pathways (Baban and Seymour 1998; Jungmann *et al.* 2008). Maeda *et al.* (2009) hypothesized that retention is the main difference between infection and cancer in the sustenance of an EPR effect, reasoning that a dysfunctional lymphatic system is essential and unique to EPR in cancer. They proposed that an EPR effect may not be tenable in infection due to rapid lymphatic clearance. However, early and significant macromolecular accumulation at sites of infection has been reported (Evans Jr *et al.* 1973; Laverman *et al.* 1999, 2001a, 2001b). Indeed, dysfunctional lymphatic drainage is a feature of infection, by way of increased interstitial pressure and tissue destruction (Swartz 2004).

1.5.2 Indirect mechanisms

The eleven-member toll-like receptor (TLR) family plays a key role in the innate immune recognition of pathogen-associated molecular patterns (Kaisho and Akira 2004; Kielian *et al.* 2005; Kopp and Medzhitov 2003). TLR downstream signalling occurs in both inflammation and cancer (Tsan 2006). LPS, ubiquitously present in Gram-negative bacterial cell walls, binds to TLR-4 and triggers the release of inflammatory mediators, which stimulate vascular permeability (Ianaro *et al.* 2009; Leon *et al.* 2008; Poltorak *et al.* 1998). Lipoteichoic acid (LTA), ubiquitously present in the Gram-positive bacterial cell wall, activates TLR-2 (Gillrie *et al.* 2010; Henneke *et al.* 2008). Both LTA (Herwald *et al.* 1998; Maeda and Yamamoto 1996) and LPS (Imamura *et al.* 2005; Katori *et al.* 1989; Shin *et al.* 1996) stimulate the kallikrein-kinin cascade, increasing vascular permeability by providing a suitably negative surface for Hageman factor (HF) activation (Maeda *et al.* 1993). LPS also strongly upregulates the Bradykinin-1 receptor (BKR-1)

(Leeb-Lundberg *et al.* 2005) and enhances production of vascular endothelial growth factor (VEGF) (Section 1.5.3.4). These observations suggest that enhanced vascular permeability is a common feature across human bacterial pathogens.

VPE may be induced by the immune system itself (Figure 1.6). Neutrophils predominate in acute inflammation. They promote vascular permeability by releasing neutrophil elastase, which cleaves high molecular weight kininogens (HMWK) to E-kinin (Imamura *et al.* 2002). Secretory leukocyte protease inhibitor (SLPI), which normally suppresses neutrophil elastase activity, may also be inactivated by bacterial proteases leading to potentiated, elastase-induced permeability (Into *et al.* 2006). Peroxynitrite (ONOO^-) and nitric oxide ($\cdot\text{NO}$) radicals (Section 1.5.3.2) may activate matrix metalloproteinases (MMPs) secreted by macrophages which, alongside neutrophil-produced elastase, increase vascular permeability. MMPs, activated by ONOO^- and $\cdot\text{NO}$, are involved in the proteolytic degradation and inactivation of α 1-proteinase inhibitor, facilitating kallikrein-kinin-mediated permeability as well as neutrophil elastase activity (Maeda *et al.* 1999; Moreno and Pryor 1992; Okamoto *et al.* 2001). Neutrophil lysis releases myeloperoxidase, elastase and MMP-9, which contribute to nitration of albumin and enhanced vascular permeability (Mirastschijski *et al.* 2002; Trengove *et al.* 1999). BKR-1-induced endothelial inducible nitric oxide synthase (iNOS) may be augmented by the inflammatory cytokines interleukin-1 (IL-1) and interferon- γ (IFN- γ), resulting in prolonged $\cdot\text{NO}$ release (Ignjatovic *et al.* 2004).

1.5.3 Direct mechanisms

The kallikrein-kinin cascade is a major effector of systemic inflammatory responses and a key contributor to the EPR effect in tumours mediated by upregulation of kallikrein gene expression and receptor upregulation in target cell populations (Bhoola *et al.* 2001; Maeda *et al.* 1999; Wu *et al.* 2002). BK plays a central role in directly activating VPE (Filipovich-Rimon and Fleisher-Berkovich 2010; Maeda *et al.* 2003; Maeda *et al.* 1999) which may be

amplified by several effector cascades (NO, MMP, VEGF and eicosanoids) (Colman 2006; Morrissey *et al.* 2008; Zhang *et al.* 2008b) (Table 1.4).

1.5.3.1 The Kallikrein Kinin system

Characterisation of the two main BK receptors and their cognate ligands suggest temporally and pathophysiologically distinct (but complimentary) functions (Figures 1.7 and 1.8). BK and kallidin (lys1-BK) are first generation BKs which preferentially activate the BKR-2 receptor (Leeb-Lundberg *et al.* 2005). BK has a half-life of only 27 seconds, since it is rapidly degraded by the angiotensin-converting enzyme (ACE) and dipeptidyl carboxypeptidase kininase II, on the endothelial cell surface (Yong *et al.* 1992). Despite its short half-life, the effects of BK and the kallikrein-kinin cascade extend into each stage of VPE by the resulting products: des-Arg⁹-BK, Lys-des-Arg⁹-BK (Mehta and Malik 2006) and des-Arg¹⁰-kallidin, (McLean *et al.* 2000) which preferentially activate BKR-1. BKR-2 is ubiquitous, constitutively expressed, rapidly sequestered and internalised, (Blaukat *et al.* 1996; Blaukat *et al.* 2001; Leeb-Lundberg *et al.* 2005; Oehmcke and Herwald 2010). BKR-1 is however only induced during an infective insult, and is less susceptible to internalisation and desensitisation (Bascands *et al.* 1993; Mathis *et al.* 1996; Smith *et al.* 1995). In fact, BKR-2 has been shown to activate early phase oedema whereas later phase oedema is dependent on stimulation of BKR-1 (Todorov *et al.* 2002). BKR-1 has also been implicated in animal models of persistent inflammation, including infection (Leeb-Lundberg *et al.* 2005). These findings support BKR-2 mediation of the initial response and BKR-1 mediation of the delayed, but more prolonged, reaction. A prolonged effect for BK-mediated VPE is also suggested by other recently reported mechanisms (Figures 1.7 and 1.8). Activation of HF or HMWK by microbial proteases results in the production of HKa (a kinin-free derivative of HMWK). Formation of HKa results in exposure of the D5 domain of HMWK. Both HKa and D5 inhibit *in vitro* endothelial adhesion to vitronectin and fibrinogen, leading to apoptosis (Colman 2006). Endothelial injury, cell necrosis, cell

Table 1.4: Factors responsible for enhanced vascular permeability in Gram-negative bacterial infection

Organism	Active protease or inducer	VPE	Substrate	References
<i>Pseudomonas aeruginosa</i>	Protease elastase	HF	HMWK	Hochstraber <i>et al.</i> (1973); Morihara <i>et al.</i> (1979); Maeda and Molla (1989); Shibuya <i>et al.</i> (1991); Khan <i>et al.</i> (1993); Maeda <i>et al.</i> (1993); Kaminishi <i>et al.</i> (1994); Maeda and Morihara (1995); Ridings <i>et al.</i> (1995); Shin <i>et al.</i> (1996); Machado <i>et al.</i> (Machado <i>et al.</i> 2010)
<i>Escherichia coli</i> and <i>Salmonella</i> spp.	Curlii Fibers Endotoxin	Pre-Kallikrein HMW		Ben Nasr <i>et al.</i> (1996); Herwald <i>et al.</i> (1998); Persson <i>et al.</i> (2000); Frick <i>et al.</i> (2007)
<i>Serratia manascens</i>	Cysteine protease Metalloproteinase	HMWK LMWK		Kamata <i>et al.</i> (1985); Kamata <i>et al.</i> (1985); Maruo <i>et al.</i> (1993); Matsumoto (2004).
<i>P. gingivalis</i>	Gingipains R & (K) (cysteine protease)	Pre-kallikrein HMWK (RGP releases from PK) RGP +KGP release BK from HMWK		Imamura (2001); Kadowaki <i>et al.</i> (2004); Into <i>et al.</i> (2006); Matsushita <i>et al.</i> (2006); Hinode <i>et al.</i> (1992)
<i>Vibrio vulnificus</i>	Vibrio vulnificus protease	HF PK		Molla <i>et al.</i> (1988); Chowdhury <i>et al.</i> (1991); Maeda <i>et al.</i> (1993); Maruo <i>et al.</i> (1998); Kawase <i>et al.</i> (2004); Miyoshi <i>et al.</i> (2004)
<i>Acinetobacter</i> spp.	VPE-Like factor HMWK	HMWK		Soares <i>et al.</i> (2009)

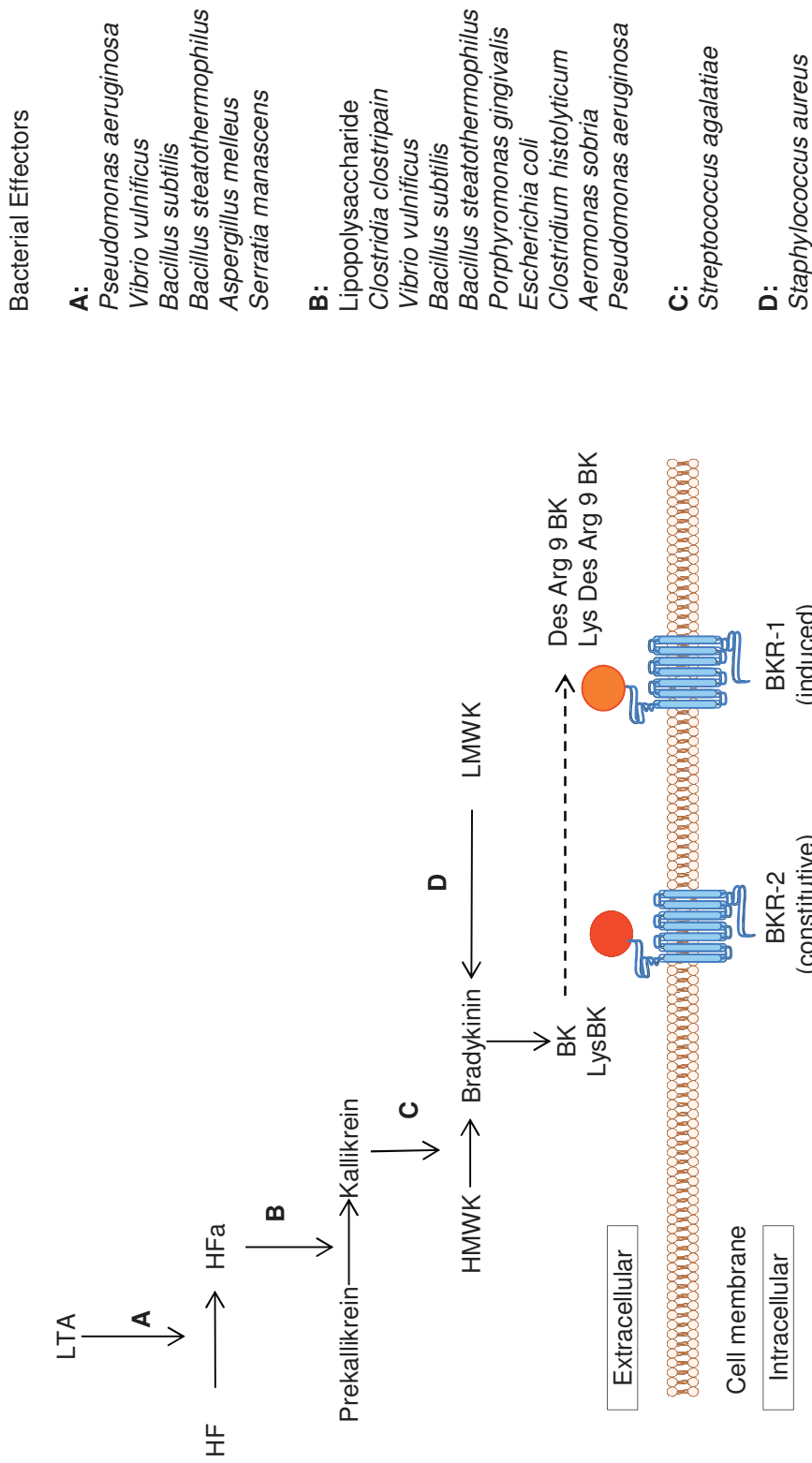


Figure 1.7: Action of specific bacterial proteases and mediators on the kallikrein-kinin cascade and receptors, in the mediation of enhanced vascular permeability. Key \longrightarrow stimulates, \dashrightarrow degrades into.

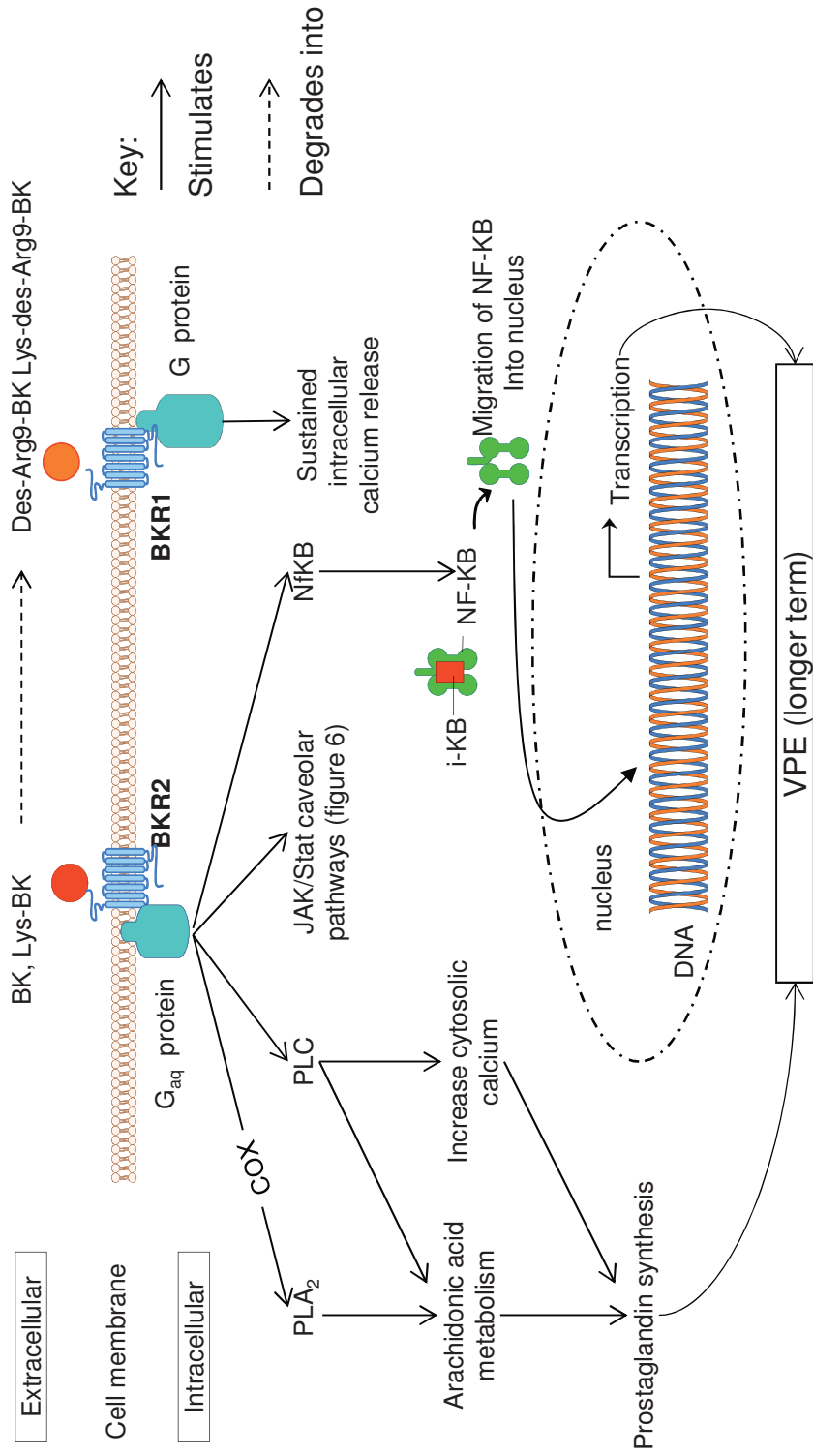


Figure 1.8: Sub-cellular pathways involved in Kallikrein-Kinin enhanced vascular permeability. PLC: phospholipase C; PKC: protein kinase C; GTPase: guanidine triphosphatase; NF-KB: nuclear factor kappa beta; EGFR: epidermal growth factor; MAPK: mitogen activated protein kinase; COX: cyclo-oxygenase, VPE vascular permeability enhancement.

detachment and apoptosis have been proposed as mechanisms for prolonged vascular leakage (Lentsch and Ward 2000). BK contributes to VPE through both a transcellular and an intercellular route (Figure 1.9), although the precise mechanisms are still contended (Baffert *et al.* 2006; Jungmann *et al.* 2008). BK-induced VPE is apparently independent of the guanidine triphosphatases (GTPases), Rho and Rac, two key regulators of endothelial barrier function (Adamson *et al.* 2002; Wojciak-Stothard and Ridley 2002). BK may directly influence adherens junctions via production of NO and indirectly, through filamin-mediated Rho-induced adherens junction control (Mehta and Malik 2006). An ability to widen endothelial gaps may explain why the presence of an EPR effect significantly increases cancer metastasis (Maeda *et al.* 1996) and infection dissemination in pathogenic species such as *Vibrio* spp. and *Pseudomonas* spp., (Jones and Oliver 2009; Maeda *et al.* 1999; Maeda and Yamamoto 1996) (Quinones-Ramirez *et al.* 2010). Continuous production of first generation BKs is further enhanced by downstream activation/release of second generation products and simultaneous BKR-1 upregulation (Komarova and Malik 2010; Marceau and Regoli 2004). BKR also contributes to delayed processes such as nuclear transcription and translation via nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κB) and caveolins.

1.5.3.2 Nitric Oxide

NO is synthesised from L-arginine by nitric oxide synthase (NOS), which exists in 3 different forms: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). eNOS and nNOS are constitutively expressed at low levels but may be rapidly activated by increased cytoplasmic calcium. iNOS is induced when macrophages and other inflammatory cells are activated by cytokines (Kumar 2010). Prolonged generation of NO by BKR-1 may contribute to sustain VPE. An association between the NO pathway, BK, and VPE has been reported (Maeda *et al.* 2000; Palmer *et al.* 1988). It has since been shown that BK, despite its ability to activate the G_i/G_q pathway, cannot directly activate

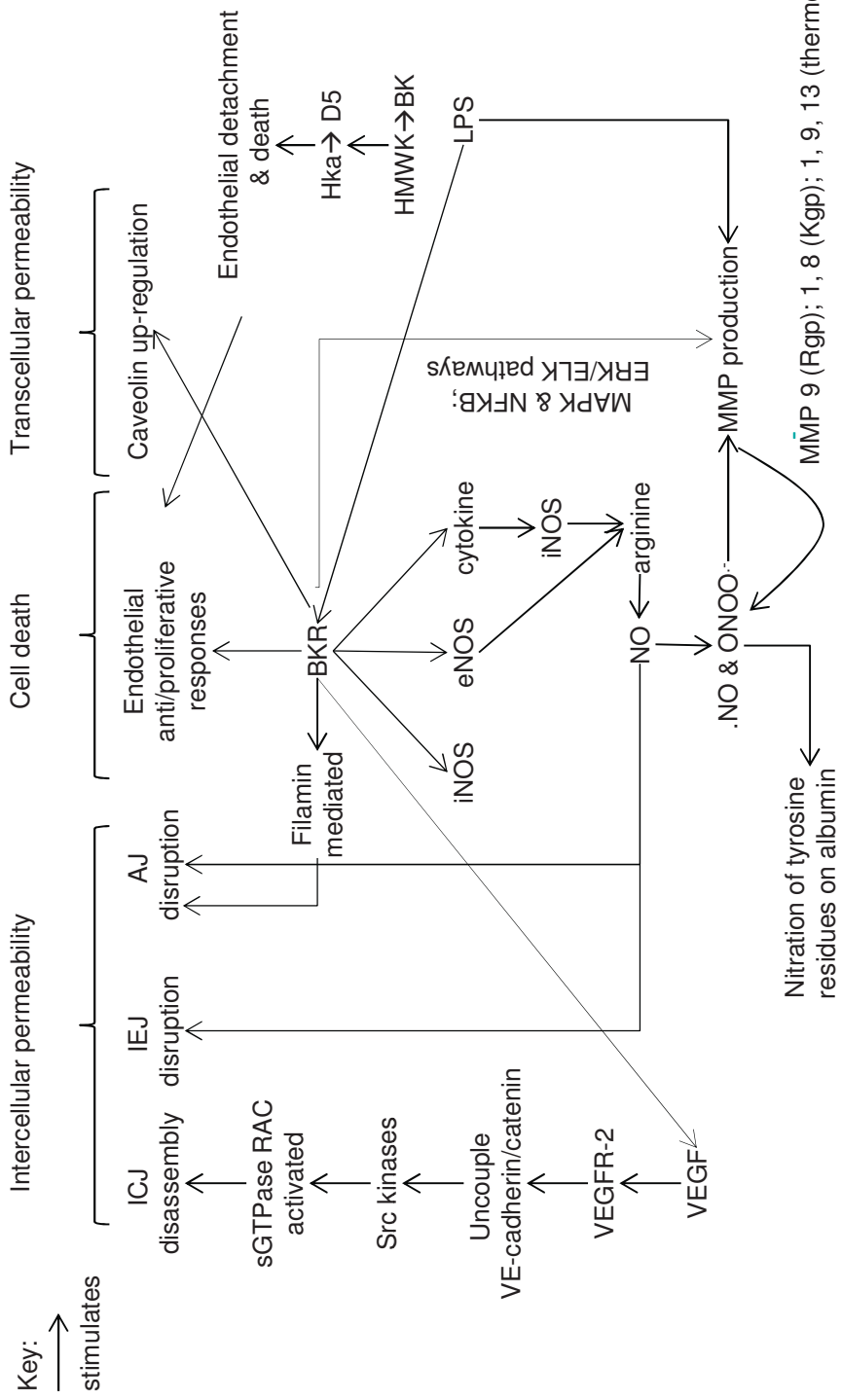


Figure 1.9: The central role of bradykinin in enhancing vascular permeability. BK: Bradykinin; iNOS: inducible nitric oxide synthase; eNOS: endothelial Nitric Oxide Synthase; sGTPase: small GTP-ase; Hka: kinin-free derivative of HMWK; D5 domain 5.

myosin light chain kinase (MLCK) or Rho (Mehta and Malik 2006). G_i/G_q activation, however, increases NO synthesis (as well as phosphatidyl inositol (PI), cytosolic calcium and prostaglandins (PG) (Bhoola *et al.* 1992; Conklin *et al.* 1988). Ignatovic *et al.* (2004) demonstrated that BKR-1 may stimulate human endothelial iNOS, with prolonged NO release. This NO release is important in inter-endothelial junction integrity (Predescu *et al.* 2005). In cancer, generation of NO through NOS activation significantly increases vascular permeability in a tumour size-dependent manner (Doi *et al.* 1998; Maeda *et al.* 1994; Wu *et al.* 1998). Within solid tumours, and in parallel to infection, $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ induce extremely reactive $\cdot\text{ONOO}^-$. Moreover, $\cdot\text{NO}$ and $\cdot\text{ONOO}^-$ may also activate pro-MMP (1, 8, and 9) (Okamoto *et al.* 2001). Downstream targets of $\cdot\text{ONOO}^-$ in infection and cancer include activation of MMPs and nitration of tyrosine residues in albumin (Maeda *et al.* 2003; Okamoto *et al.* 2001; Predescu *et al.* 2002).

NO-releasing polymers for use in wound healing, and ischaemic stroke have been described in the literature, including cross-linked polyethylenimine (PEI) (Shabani *et al.* 1996; Smith *et al.* 1996), ethylene-vinyl acetate (EVAc) (Gabikian *et al.* 2002) and poly(vinyl alcohol) (Masters *et al.* 2002). NO-releasing monofilament polypropylene (Engelsman *et al.* 2009) has been investigated for use in infection. NO is a promising antimicrobial alternative since reactive oxygen species (ROS) possess significant antimicrobial activity (Huh and Kwon 2011). Since NO is a mediator of VPE in infection, such polymers would theoretically benefit from NO-promoted EPR, whilst exerting an antimicrobial effect.

1.5.3.3 Matrix metalloproteinases

The endothelium is supported by an extracellular matrix (ECM), which contributes to the endothelium's size and charge barrier (Berrier and Yamada 2007; Hassell *et al.* 2002; Kalluri 2003; Paulsson 1992). Permeabilisation of this barrier may be enhanced by BK-mediated induction of constitutively expressed host MMP (Webb *et al.* 2006), in part mediated through induction of pro-MMP-9 expression via mitogen activated protein kinase (MAPK), and

the nuclear factor kappa beta (NfKB) and ERK/Elk-1 pathways (Hsieh *et al.* 2008). Several bacterial proteases are capable of inducing human MMP (DeCarlo *et al.* 1998; Imamura 2003; Matsushita *et al.* 2006). Moreover, LPS induces the formation of BK (through HMWK) while upregulating BKR_s (Aasen and Wang 2006; Seguin *et al.* 2008) simultaneously inducing cytokine formation and upregulating MMP-9 expression (Aasen and Wang 2006). Activation of MMP may increase VPE in normal skin through a variety of pathways, including 'ONOO' generation (Okamoto *et al.* 2001; Wu *et al.* 2001) This evidence suggests that during infection, MMP's contribute to VPE directly and by acting as downstream effectors of the Kallikein-Kinin system, prolonging and amplifying its effect.

1.5.3.4 Vascular Endothelial Growth Factor

VEGF may potently enhance vascular permeability in bacterial infection (Hippenstiel *et al.* 1998; Maeda *et al.* 2000) as it does in solid tumours (Hostacka 2000; Maeda *et al.* 2003; Maeda *et al.* 1999; Senger *et al.* 1983; Tomaras *et al.* 2008). Activated VEGF receptor-2 (VEGFR-2) may "uncouple" VE-cadherin- β -catenin, which are essential for endothelial junction integrity (Weis *et al.* 2004). Activated small GTPase Rac promotes intercellular junction (ICJ) disassembly (Gavard and Gutkind 2006). Bradykinin activation of VEGF (Colman 2006; Johnson *et al.* 1999) may, therefore, provide an indirect route for BK-induction of Rho/Rac, and ICJ control. Furthermore, endothelial NO, which determines IEJ integrity, is up-regulated by VEGF,(Leung *et al.* 1989; Maeda *et al.* 2000; Murohara *et al.* 1998; Papapetropoulos *et al.* 1997) through Src (Duval 2007). Since a similar VPE effect is observed by picomolar VEGF concentrations and nanomolar (nM) BK concentrations, (Maeda *et al.* 2003) significant and sustained modification of the paracellular route may result from BK-VEGF-NO crosstalk.

1.5.3.5 Eicosanoid pathways

The eicosanoid pathway in inflammation may contribute to the infection-induced VPE effect, in a similar manner to cancer. It is dependent on various regulatory elements, which may be BK-dependent or BK-independent, and these responses may be organ- or tissue- specific. BK induces cyclooxygenase (COX) expression in various human cell types, including endothelial cells, cerebral arterioles, human airway epithelial cells, smooth muscle cells and fibroblasts (Conklin *et al.* 1988) which are responsible for upregulating prostaglandin (PG) synthesis in solid tumours (Cahill *et al.* 1988; Maeda *et al.* 1999). Prostaglandin I₂ (PGI₂) and prostaglandin E₁ (PGE₁) may induce VPE in a similar fashion to NO (Maeda *et al.* 2000) and this has been recently applied *in vivo* in Beraprost[®], a synthetic prostacyclin analog (Maeda *et al.* 2003). BK results in endothelial production of PGE₂ (Jose *et al.* 1981), activation of phospholipase A₂ (PLA₂) (Burch and Axelrod 1987) and phospholipase C (Gilroy *et al.* 1999), both of which metabolise arachidonic acid for PGE₂ synthesis. BK receptors play distinct roles within this pathway. Within human umbilical vein endothelial cells (HUVEC) BKR-2, but not BKR-1, induces increased synthesis and activation of cytosolic PLA₂ in a Ca²⁺-dependent manner which, in turn, induces the synthesis of PGI₂ (Yamasaki *et al.* 2000). In these models, BK stimulation of COX-1 is the main pathway by which PGE is released. COX-2 stimulation has an initial pro-VPE effect (production of PGE₂), later exerting an anti-VPE effect through 15-deoxy,12,14-PGJ₂ (Gilroy *et al.* 1999).

Microbial-induced VPE may also be induced by BK-independent mechanisms, such as *P. aeruginosa* Exotoxin U (ExoU) (Hauser *et al.* 1998; Holder *et al.* 2001; Sato and Frank 2004; Sawa *et al.* 1998; Shaver and Hauser 2004).

1.5.4 Relevance across human pathogenic bacteria

EPR-enhancing mechanisms appear to be widespread among human bacterial pathogens. Successful exploitation of this phenomenon for targeted antimicrobial delivery is dependent on the extent of vascular permeability, its

ubiquitous occurrence in pathogenic bacteria and, importantly, the magnitude of this effect. The close evolutionary conservation of the kallikrein-kinin cascade across several kingdoms and species (Marcondes and Antunes 2005), and the presence of multiple activators of an enzymatic cascade suggests that BK-induced VPE may be common feature of human microbial pathogens.

1.5.4.1 Direct bacterial protein activation

Several major pathogens, including the ESKAPE organisms are known to enhance vascular permeability. This may occur via direct protease activation or through inactivation of natural inhibitors for major effector systems (Tables 1.3 and 1.4). Targets for *P. aeruginosa* alkaline phosphatase and elastase stimulation of BK production, include HF (Tanaka *et al.* 1992) mediated via MMPs (alkaline protease and elastase) (Machado *et al.* 2010; Maeda *et al.* 1993; Maeda and Molla 1989; Maeda and Morihara 1995; Molla *et al.* 1989). Human pre-kallikrein (PK) may be activated *in vitro* by *P. aeruginosa*-derived elastase and protease (Molla *et al.* 1989). Effects on VPE are also likely to be localised to the pathophysiological locus or in the interstitial fluid, rather than in blood, due to the presence of α 2-macroglobulin in the latter (Shibuya *et al.* 1991). *P. aeruginosa* expression of alkaline phosphatase and elastase are widespread, therefore it is expected that induction of EPR may be common.

In a comparative study of sepsis patients and healthy controls, Soares *et al.* (2009) showed that components of the kallikrein-kinin system, including HMWK, are significantly depleted in infection. *Acinetobacter* spp. secretes a vascular permeability factor (VPF), which significantly increases vascular permeability *in vitro* (Hostacka 2000; Tomaras *et al.* 2008). However, *Acinetobacter* spp. and *Klebsiella* spp. also significantly activate the kallikrein-kinin cascade via LPS (Section 1.5.2). The presence of multiple activators of an enzymatic cascade suggests a significant EPR effect is evident.

A study performed in healthy volunteers has demonstrated that *E. coli* induces significant VPE by LPS-induction of VEGF (Mittermayer *et al.* 2003).

Evans (1973) has also investigated the VPE properties of *E. coli* enterotoxin, and these studies demonstrated that accumulation of Evans blue dye in rabbit models was linearly related to dermal concentrations of enterotoxin. They hypothesised that Evans blue dye binds tightly to albumin, creating a macromolecule that facilitated permeability and retention. Furthermore, curli fibres expressed by *E. coli* can bind and assemble contact factors resulting in BK release (Ben Nasr *et al.* 1996; Frick *et al.* 2007; Herwald *et al.* 1998; Persson *et al.* 2000). The enzymatic activity of *Enterobacter* spp. includes several MMPs, including gelatinase (secreted by *E. aerogenes*, *E. cloacae* and *Citrobacter* spp.) and elastase (Goncalves *et al.* 2007) which may contribute to an EPR effect through increasing vascular permeability (Section 1.5.1).

1.5.4.2 Inactivation of regulatory protease inhibitors

Inhibition of regulatory host protease inhibitors may also play a significant contributory role in microbial VPE (Table 1.5). The human kallikrein-kinin cascade is normally controlled by a well-characterised system of inhibitors. Pseudomonal proteases (PAP and elastase), and serratial proteases may effectively inactivate serine protease inhibitors (SERPINS), such α 1-protease inhibitor (Kress 1986; Molla *et al.* 1988; Morihara *et al.* 1979), α 2-antiplasmin and α 2-macroglobulin (Molla *et al.* 1986; Virca *et al.* 1982). A separate function is the ability of the inhibitor- α 2-macroglobulin complex to be internalised via α 2-macroglobulin receptors. After internalization, regeneration of the free protease may cause cell death (Maeda and Molla 1989). However, unless α 2-macroglobulin was significantly depleted systemically, the effect would most likely be localised (Shibuya *et al.* 1991).

Besides ubiquitous bacterial components capable of low-level VPE activation (Section 1.5.2), there are several salient examples of the evolutionary conservation of EPR mechanisms. *Porphyromonas gingivalis* Gingipains R and K, whose role in VPE production are widely acknowledged have evolutionarily well-conserved active sites (Imamura 2003). Their catalytic dyad is common to caspases, clostripain and mammalian legumain,

Table 1.5: Bacterial inhibition of specific protease inhibitors

Protease Inhibitor	Species/Protease	Reference
SERPINS	<i>Pseudomonas aeruginosa</i>	Morihara <i>et al.</i> (1979)
	<i>Serratia manascens</i>	Kress <i>et al.</i> (1986)
	<i>Staphylococcus aureus</i>	Molla <i>et al.</i> (1988, 1986)
α 1-protease inhibitor	<i>Pseudomonas aeruginosa</i>	Morihara <i>et al.</i> (1979)
	<i>Serratia manascens</i>	Kress <i>et al.</i> (1986)
	<i>Porphyromonas gingivalis</i>	Potempa <i>et al.</i> (1998)
		Molla <i>et al.</i> (1988) Nelson <i>et al.</i> (1998)
α 2-antiplasmin	<i>Pseudomonas aeruginosa</i>	Virca <i>et al.</i> (1982)
	<i>Serratia manascens</i>	Molla <i>et al.</i> (1986)
α 2-antiplasmin	<i>Pseudomonas aeruginosa</i>	Virca <i>et al.</i> (1982)
	<i>Serratia manascens</i>	Molla <i>et al.</i> (1986)
	<i>Porphyromonas gingivalis</i>	
α 2-macroglobulin	<i>Pseudomonas aeruginosa</i>	Virca <i>et al.</i> (1982)
	<i>Serratia manascens</i>	Molla <i>et al.</i> (1986)
		Shibuya <i>et al.</i> (1991)

suggesting they are probably evolutionarily-related (Chen *et al.* 1998). Some of these VPE-generating proteases been reported as members of a core of key factors responsible for colonisation and infection, by many infectious strains (Dubin 2003). These findings all suggest a common, evolved system for sustaining VPE at sites of bacterial infection.

1.6 Targeting potential of the EPR effect in infection

Infected patients are prone to rapid deterioration (Section 1.2.2). The successful clinical exploitation of the EPR effect in the treatment of infection will, ultimately, depend upon an expeditious establishment of an EPR effect (Opal and Cohen 1999). Several studies report that rapid, passive, size-based accumulation around infected foci is feasible *in vivo*. ⁹⁹Tc-labelled poly(ethylene glycol)-coated liposomes (^{99m}Tc-PEG-liposomes) accumulate around an infected locus within 2 h (Dams *et al.* 1999), and gallium-transferrin can clearly delineate intra-abdominal abscesses within 4 h of injection (Dams *et al.* 1999). Radiolabelling studies have demonstrated that radiolabelled PEG-coated liposomes preferentially permeate into, and are specifically and selectively retained at sites of infection (Laverman *et al.* 1999; Laverman *et al.* 2001a; Oyen *et al.* 1996). Sikkink and co-workers later demonstrated a significant quantitative correlation between uptake of these particles and the size of intra-abdominal abscesses (Sikkink *et al.* 2009). Komarek *et al.* (2005) reported that ^{99m}Tc- labelled proteins including aprotinin (6,512 g/mol) allow rapid localisation around infected foci induced *in vivo* animal models and their concentration was up to 6.5 times higher than control tissue. More recently, Tectentium⁹⁹ labelled antimicrobial peptide ubiquicidin (UBI), conjugated to low molecular weight PEG (poly(ethyleneglycol)-N-(N-(3-diphenylphosphinopropionyl)glycyl)-S-tritylcysteine ligand), (conceived as radiological marker) has been shown to selectively and specifically accumulate around infected sites in an *in vivo* (animal) model of infection, within 2 h (Melendez-Alafort *et al.* 2009). This situation contrasts with anti-cancer drug design where a long-circulating "stealth" formulation approach is preferred (Duncan and Gaspar 2011; Gaspar and Duncan 2009).

1.7 Rationale for the design of a polymer-antibiotic conjugate

The value of polymer therapeutics in treating life-threatening disorders such as neoplasia, hematological and immune disease (Duncan *et al.* 2005) has been well-established. More recently, the scope of polymer therapeutics has widened to include wound healing, and viral disease (Hardwicke 2009; Wannachaiyasit *et al.* 2008). However, there are currently no antibiotic products aimed at Gram-negative infection designed on the concept of a polymer therapeutic using PUMPT and EPR. Rationalised construction of an antibiotic polymer therapeutic may offer an interesting and innovative solution to the treatment of infective disease. It is evident that the literature entertains the notion of a widespread, harnessable EPR effect in infection. Conjugation offers the possibility of masking the bioactive in transit, shielding the payload itself from biofouling and reducing toxicity to the body. It may also be hypothesised that a macromolecular enzyme usually present in limited quantities in the blood (e.g. plasma amylase) may accumulate at the target site, to stimulate localised, re-instatement of the bioactive. This would favour enzymatically mediated, locally-triggered unmasking (Duncan *et al.* 2008). Additionally, evidence presented in this section supports the notion that the macromolecular size bestowed on a "small molecule" antibiotic through polymer conjugation offers the possibility of passive targeting to an infected focus where the respective enzyme provides local re-instatement of bioactivity (Section 1.4.3). Classical design of anticancer polymer-drug conjugates requires polymers that may be used at the highest possible molecular weights/modifications to promote the greatest possible EPR and "stealth" characteristics as a paramount concern (Duncan *et al.* 2005). However, application of polymer therapeutics for the treatment of patients who have acute life-threatening infection (Section 1.2.1) requires a construct capable of affording masking during an expeditious transit to an infected site, as well as early and sustained unmasking.

1.8 Aims and experimental hypotheses

The aim of this Study is to develop a prototype polymer-antibiotic conjugate based on colistin that may be customised by polymer modification and

binding chemistry to afford selective, controlled release at the site of infection. The specific objectives of this study were:

1. To establish and physicochemically characterise a library of bioresponsive dextrin-colistin conjugates from a range of succinoylated intermediates, geared towards use in acute Gram-negative infection
2. To characterise the activity of candidate dextrin-colistin conjugates against a range of multidrug-resistant Gram-negative bacteria
3. To evaluate the pharmacokinetic / pharmacodynamic (PK/PD) profile of colistin, dextrin-colistin and the colistin/dextrin-colistin conjugates in terms of unmasking/drug release and antibacterial activity using *in vitro* model systems
4. To perform an *in vivo* pharmacokinetic evaluation of the lead agent through an ascending dose study

Chapter Two

General Methods

2.1 Chemicals

2.1.1 General chemicals and reagents

Aprotinin (from bovine lung), cytochrome C (from equine heart), carbonic anhydrase (from bovine erythrocytes), bovine serum albumin (BSA), and alcohol dehydrogenase (from *Saccharomyces cerevisiae*), blue dextran; colistin sulfate (Batch C4461, $\geq 15,000$ U/mg activity), ethanol, acetic acid (glacial), dimethylsulfoxide (DMSO), hydrochloric acid, human salivary α -amylase, ninhydrin, hydrindantin, lithium acetate dihydrate, ethanolamine, 4-dimethylaminopyridine (DMAP), anhydrous N,N-dimethylformamide (DMF), succinic anhydride, diethyl ether, Lugol's iodine, type 1 dextrans from corn starch, acetonitrile (ACN)*, boric acid*, 9-fluorenylmethyl chloroformate (Fmoc-Cl)*, tetrahydrofuran (THF)*, methanol*, sodium bicarbonate*, and bromothymol blue were purchased from Sigma Aldrich (Poole, UK). Sodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, potassium chloride, and sodium hydroxide (NaOH), copper (II) sulphate pentahydrate, bicinchoninic acid solution (BCA) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) were purchased from Thermo Fisher Scientific (Loughborough, UK). Pullulan standards were purchased Polymer Laboratories (Church Stretton, UK). Sterile 0.9% sodium chloride solution was obtained from Baxter (Berkshire, UK). Foetal calf serum was purchased from Invitrogen (Paisley, UK). *N*-hydroxysulfosuccinimide (sulfo-NHS), dextrin type 1 (13,900 g/mol) from potato starch, was purchased from Fluka (Buchs, Switzerland). Strata[®] C₁₈ solid phase extraction (SPE) cartridges (100 mg sorbent/mL, 55 μ m particle size, 70 Å pore size) were purchased from Phenomenex (Torrance, CA, USA). Sep-pak C₁₈ SPE cartridges (100 mg sorbent/mL, 55-105 μ m particle size) were purchased from Waters (Mass, USA).

Trypticase soy agar (TSA), trypticase soy broth (TSB) and cation adjusted Mueller Hinton broth (CAMHB, lot 43145) were purchased from Oxoid (Basingstoke, UK). Polyvinylidene fluoride (PVDF) sterile membrane syringe filters (0.22 μ m) were from Elkay (Basingstoke, UK). Tegaderm[®] semi-

permeable dressings were purchased from 3M Healthcare (Neuss, Germany). Sterile graduated plastic syringes were from BD plastipak (Madrid, Spain). Phadebas[®] assay kits were from Magle Life Sciences (Lund, Sweden). MaxSignal[®] enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bio Scientific Corp. (Austin, USA). Unless marked (*) to indicate HPLC grade, all chemicals were of analytical grade.

2.2 Equipment

2.2.1 General equipment for conjugate synthesis

100 mL and 10 mL round-bottomed flasks, 25 mL flat-bottomed beakers and rubber stoppers, were purchased from Sigma Aldrich (Poole, UK). Spectra/POR 7[™] regenerated cellulose dialysis membrane molecular weight cut-off (MWCO) 2,000 and 10,000 g/mol were purchased from Spectrum Laboratories Inc. (California, USA). Filter paper, (5-13 μ m pore size) was purchased from VWR International (Lutterworth, UK). Vivaspin[®] 6 centrifugal concentrators were obtained from Sartorius Stedim Biotech (Goettingen, Germany).

2.2.2 General equipment for microbiology

Sterile flat-bottomed 96-well plates, sterile universal containers, 15 mL and centrifuge tubes were purchased from Starstedt (Leicester, UK). Microbank vials were purchased from Prolab (Wirral, UK). The microbiological incubator was from LTE Scientific (Greenfield, UK). The orbital shaking incubator was from Stuart Ltd (Stone, UK). The microflow cabinet was from Bassaire (Southampton, UK). The Class II, laminar flow Astec Microflow 2[®] cabinet was from Bioquell (Andover, UK).

2.2.3 Analytical equipment

2.2.3.1 Spectrophotometry: Ultraviolet-visible (UV-vis)

A Fluostar Optima microplate reader (BMG Labtech, Aylesbury, UK), equipped with 540 nm and 570 nm absorption filters, was used, with BMG Optima[™] software version 22.10.r2 and firmware v.1.23. A Dupont 800

UV/Vis Spectrophotometer was purchased from VWR International (Lutterworth, UK).

2.2.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

A Nicolet 380 FTIR spectrophotometer, fitted with a Nicolet Smart-Arc diffuse reflectance accessory, was from Thermo Fisher Scientific (Loughborough, UK). Nicolet E-Z Omnic software version 7.4.127 was used for data collection.

2.2.3.3 Gel permeation chromatography (GPC)

The aqueous GPC, equipped with a Jasco[®] HPLC pump and two TSK-gel columns in series (4000 PW, followed by 3000 PW) and a guard column (progel PW_{XL}), was purchased from Polymer Laboratories (Church Stretton, UK). The eluate was monitored using a differential refractometer (Gilson 153, Gilson, Inc., Middleton, USA). Data was collected and analysed using Cirrus GPC software version 3.2.

2.2.3.4 Fast protein liquid chromatography (FPLC)

An ÄKTA FPLC system, from GE Healthcare (Amersham, UK), was used. The unit was connected to a pre-packed Superdex 75 10/300 GL column and a Frac-950 fraction collector (GE Healthcare). The UV detector data was collected and analysed using Unicorn software version 5.20.

2.2.3.5 High pressure liquid chromatography (HPLC)

The HPLC system from Thermo Fisher Scientific (Hemel Hempstead, UK) consisted of a Dionex ICS-3000 suite connected to a Dionex AS autosampler and a Dionex RF 2000 fluorescence detector, with a data processing unit. Data was collected and analysed using Chromeleon[®] software suite version 6.8.3. An Onyx[®] monolithic C₁₈ guard (4.6 mm internal diameter) was connected to Phenomenex C₁₈ (4.6 mm internal diameter) reverse-phase HPLC columns of various lengths from 50-210 mm (Phenomenex, Torrance,

USA). Detection by monitoring for fluorescence was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm.

2.2.3.6 Miscellaneous equipment

A Hanna 209 pH meter was purchased from Hanna Instruments (Bedfordshire, UK). Needles and syringes were purchased from Tyco Healthcare (Gosport, UK). An ALC-PK 120-R refrigerated centrifuge purchased from ALC™ SpA (Cologno Monzeze, Italy) was used for centrifugation. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). A freeze-dryer (Edwards Benchtop Modulyo) connected to a high vacuum pump was purchased from Edwards High Vacuum (Sussex, UK).

2.3 Bacterial isolates

Clinical bacteriological isolates, *Pseudomonas aeruginosa* PA01 American Type Culture Collection (ATCC) 15692 and *Escherichia coli* National Collection of Type Cultures (NCTC) 10418 were donated by Dr Robin Howe (Public Health Wales Microbiology Laboratory, University Hospital of Wales, Cardiff, UK) and Dr. Ann Lewis (Public Health Wales Microbiology Laboratory, Singleton Hospital, Swansea, UK). *Bacillus subtilis* ATCC 6633 was purchased from Thermo Fisher Scientific, Basingstoke, UK. Multiple drug resistance was confirmed from the referring laboratories.

2.4 Animals

Thirty-five male Sprague-Dawley® rats were purchased from Charles River Laboratories (Edinburgh, UK). These were 8-10 weeks old at dosing. Animals were fed a standard laboratory diet of known formulation, which did not contain colistin bacitracin or polymyxins (SDS Rat and Mouse maintenance diet no 1, Special Diet Services Ltd, Essex, UK).

2.5 Methods

This section details general methods used in these studies. Specific methods are described within the chapters where they are used.

2.5.1 Purification of dextrin-colistin conjugates

FPLC was used to purify dextrin-colistin conjugates to remove unwanted protein and cross-linking agents by separation according to their molecular weights. A prepacked Superdex[®] 75 10/300 GL column was washed with 2 column volumes (~50 mL) of filtered (0.2 µm) and degassed double-distilled water (ddH₂O). Next, the column was equilibrated with 2 column volumes of filtered and degassed mobile phase, phosphate buffered saline (PBS, pH 7.4) before use. This was repeated periodically after storage, service and maintenance. First the column was calibrated (0.5 mL/min flow rate) using standardised proteins of various molecular weights: aprotinin from bovine lung 6,500 g/mol; cytochrome c from bovine heart 12,400 g/mol; carbonic anhydrase from bovine erythrocytes 29,000 g/mol; albumin from bovine serum 66,000 g/mol; and alcohol dehydrogenase 150,000 g/mol. The calibration curve is reported in Figure 2.1. The vortexed reaction mixture was centrifuged (5 min, 1,200 g, 10 °C) and supernatant (500 µL) was injected (complete fill method) with PBS (pH 7.4) as moving phase (0.5 mL/ min). The elution profile was monitored over 30 mL and fractions corresponding to the dextrin-colistin conjugate (7-13 mL) were collected, pooled, desalted and concentrated by ultrafiltration. Samples were transferred to Vivaspin[®] centrifugal concentrators (MWCO 5,000 g/mol) and centrifuged at 4000 *g*. The final product was washed in three times its volume with ddH₂O. Finally the dextrin-colistin conjugate was lyophilised to constant weight and stored at -20 °C until required. After use, the column was thoroughly washed with two column volumes of eluant, followed by two column volumes of ddH₂O, and finally stored in 20 % v/v ethanol.

2.5.2 Characterisation of dextrin-colistin conjugates

2.5.2.1 Characterisation by GPC

Dextrin-colistin conjugates were analysed using aqueous phase GPC to determine molecular weight. Polysaccharide (pullulan) standards (molecular weight 738-788,000 g/mol) were used to produce a calibration curve, from which the weight average molecular weight and polydispersity could be

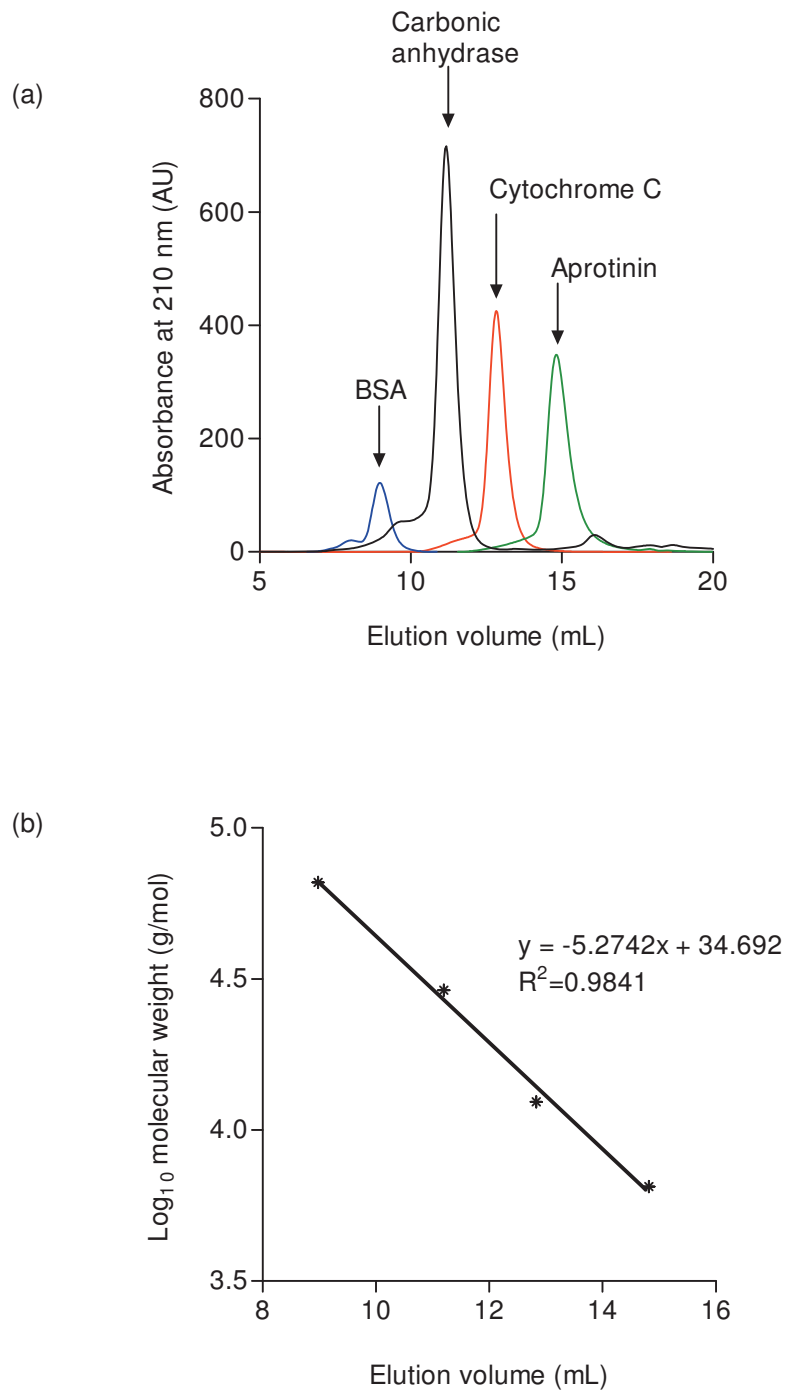


Figure 2.1: FPLC calibration using various protein molecular weight standards. Panel (a) shows the chromatogram of the protein standards (6,500 to 66,000 g/mol). Panel (b) shows a typical FPLC molecular weight calibration curve for estimation of molecular weight.

calculated (Figure 2.2). All samples were prepared in PBS (3 mg/mL, pH 7.4) and approximately 60 μ L of each solution was injected into a 20 μ L injection loop in turn. Filtered, degassed PBS was used as a mobile phase (flow rate 1.0 mL/min). Refractive index (RI) detection was used and the data was analysed using PL Caliber™ instrument software.

2.5.2.2 Characterisation by FPLC

FPLC (as described previously) was also used to estimate the ratio of free and bound colistin. After appropriate equilibration and calibration, samples of purified conjugate (150 μ L, 3 mg/mL) were injected into a 100 μ L loop and allowed to run at a flow rate of 0.5 mL/min. Area of the peaks was used to determine the ratios of area under the curve (AUC).

2.5.2.3 BCA protein assay

A BCA assay was used to determine the total protein content of the dextrin-colistin conjugates (Smith *et al.* 1985). This reaction involves two steps. The Biuret reaction is the first step whereby copper chelates with protein in an alkaline environment. In the second step of the reaction, BCA reacts with the reduced (cuprous) cation. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance with increasing protein concentrations.

The BCA assay was conducted in a 96-well plate using colistin sulfate as a calibration standard. BCA reagent was prepared by mixing 1 mL BCA: 20 μ L Cu(II)SO₄. Reference wells were prepared to contain 20 μ L colistin standard (0-1 mg/mL, n=3) or dextrin-colistin conjugate (1 and 3 mg/mL, n=4), and BCA reagent (200 μ L). The microtitre plate was gently agitated and then left in the dark (37 °C for 20 min). Absorbance was then measured spectrophotometrically at 544 nm. A typical calibration curve is illustrated in Figure 2.3.

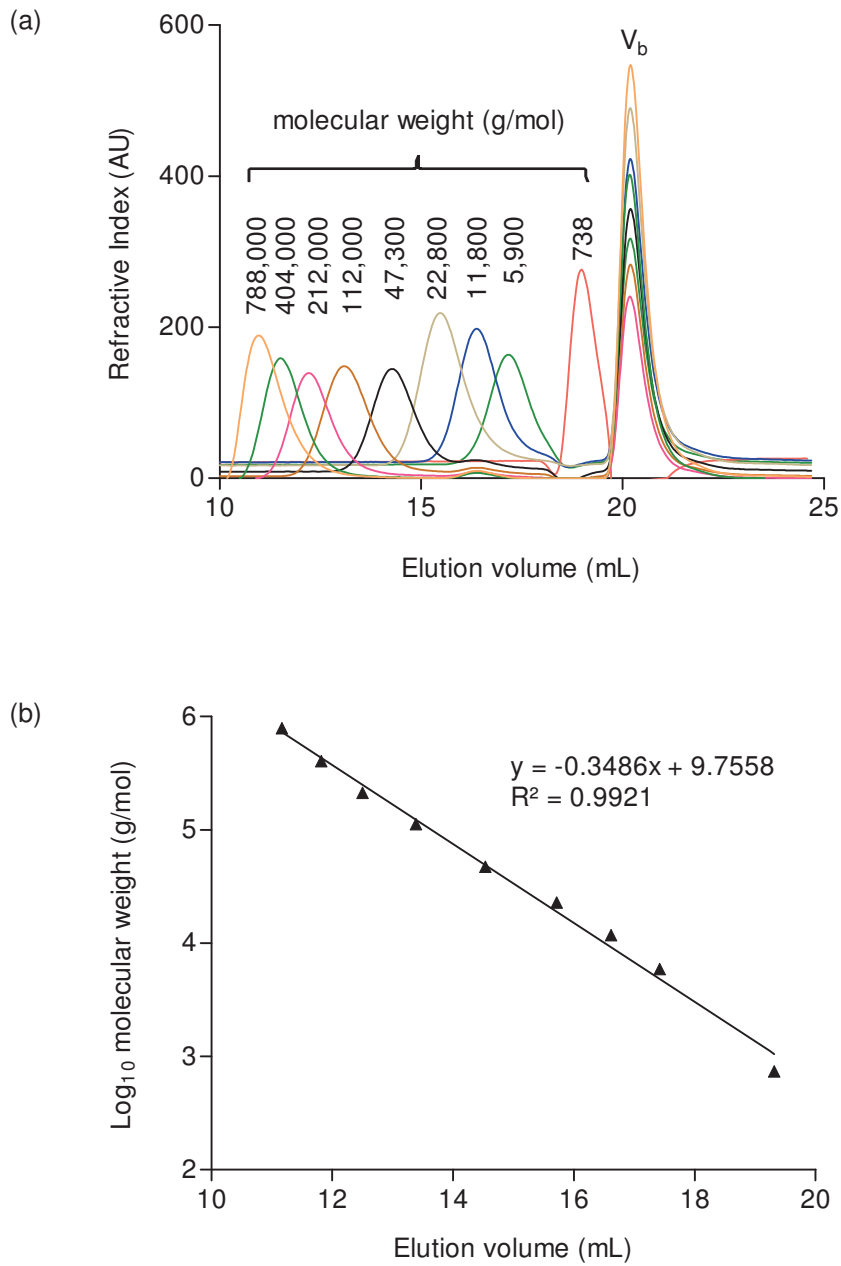


Figure 2.2: GPC calibration using pullulan molecular weight standards. Panel (a) shows a chromatogram of the pullulan standards (738-788,000 g/mol). V_b (20 mL) represents the bed volume. Panel (b) represents a typical calibration curve for estimation of sample molecular weight.

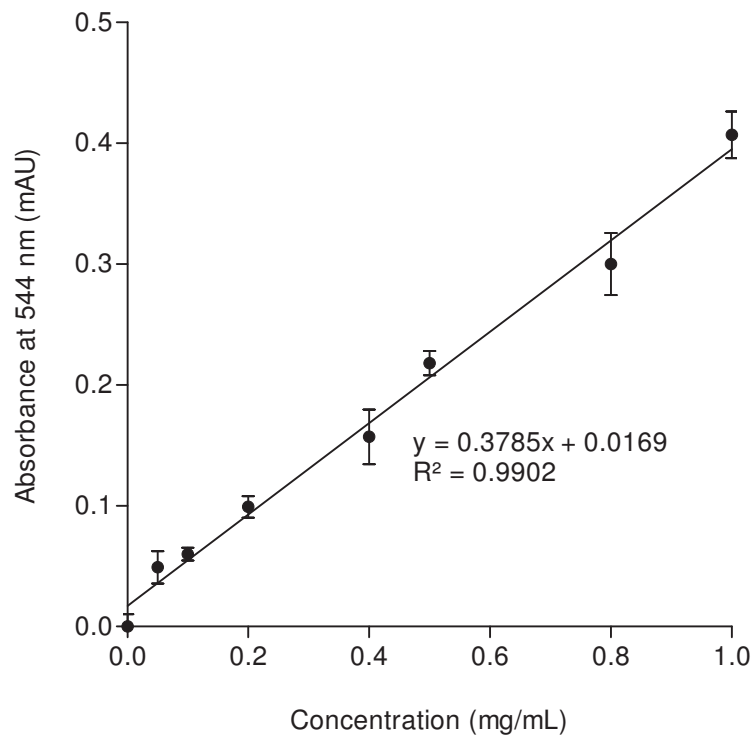


Figure 2.3: A typical BCA assay calibration curve obtained using colistin sulfate. Data shown represents mean \pm SD (n=3).

2.5.2.4 Ninhydrin assay

Ninhydrin, a potent oxidising agent, reacts with primary amines (pH 4-8) to produce Ruhemann's purple pigment enabling spectrophotometric quantification of amine content (Plummer 1978). First, lithium acetate buffer solution (4 M) was prepared by dissolving lithium acetate dihydrate (40.81 g) in ddH₂O (60 mL). Glacial acetic acid was added until the solution reached pH 5.2 and the solution was then made up to a final volume of 100 mL with double distilled water (ddH₂O). Ninhydrin (200 mg) and hydrindantin (30 mg) were next dissolved in DMSO (7.5 mL) and lithium acetate buffer (2.5 mL). Buffered ninhydrin reagent (86 µL) was added to an equal quantity of sample / calibration standard solution and heated in a waterbath for 15 min at 100 °C. After allowing the solutions to cool to room temperature, ethanol (50 % v/v in ddH₂O, 130 µL) was added and mixed thoroughly. An aliquot of each sample/standard solution (200 µL) was added to a well in a 96-well plate in triplicate and analysed spectrophotometrically at 570 nm. Standard samples were prepared by diluting ethanolamine (0 - 1.1158 mM) in PBS (pH 7.4). A typical calibration curve is illustrated in Figure 2.4.

2.5.3 Microbiological characterisation of dextrin-colistin conjugates

2.5.3.1 Equipment sterilisation

In order to ensure sterility, microbiology incubators, safety cabinets, equipment and all working surfaces were sprayed with 70 % v/v ethanol in aqueous solution. Those items not supplied pre-sterilised were sterilised by either autoclaving (120 °C, 15 lb/m², 15 min) for glassware, certain plastics, PBS and ddH₂O or microfiltration (0.2 µm) for solutions.

2.5.3.2 Agar preparation

TSA was prepared as per manufacturer instructions (Oxoid Limited 2011a). Briefly, TSA (40 g) was added to 1 L ddH₂O and stirred vigorously until fully dissolved. This was sterilised by autoclaving at 121 °C for 15 min. Once cooled to 56 °C in a sterile waterbath, agar aliquots was poured into sterile

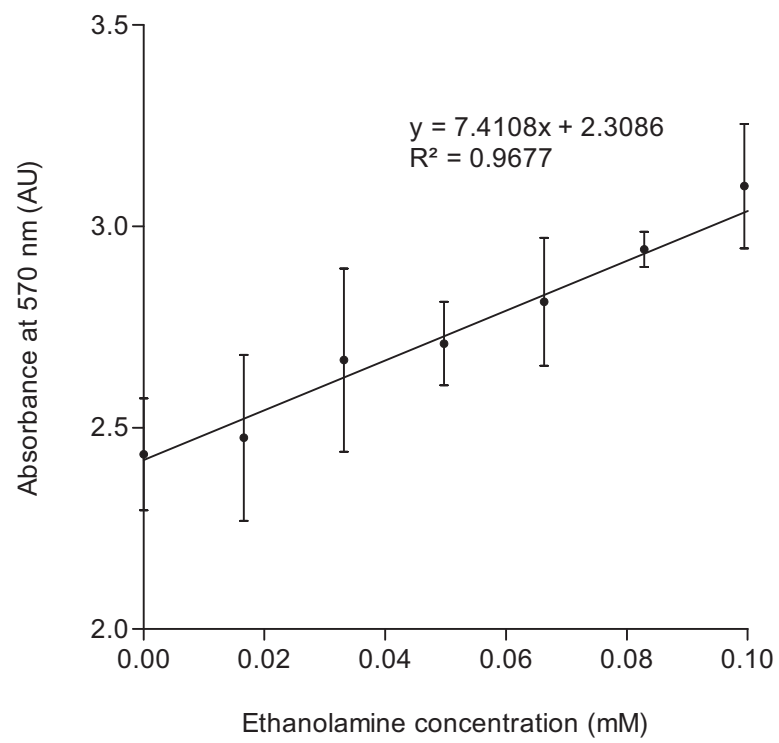


Figure 2.4: A typical calibration curve for the ninhydrin assay using ethanolamine standards. Data shown represents mean \pm SD (n=3).

Petri dishes in a microflow cabinet and left to cool until solid. The prepared plates were stored inverted at 2-8 °C in a refrigerator, until required. Fresh TSA plates were produced every 15 days.

2.5.3.3 Preparation of microbiological broths

TSB was prepared as per manufacturer's instructions (Oxoid Limited 2011c). Briefly, TSB (30 g) was added to ddH₂O (1 L) and stirred thoroughly until fully dissolved. Aliquots (~200 mL) were placed into glass bottles, which were sealed and sterilised by autoclaving at 121 °C for 15 min.

MHB was prepared as per manufacturer's instructions (Oxoid Limited 2011b). Briefly, MHB (21 g) was added to ddH₂O (1 L) and stirred thoroughly until fully dissolved. Aliquots were placed into final containers and these were sterilised by autoclaving at 121 °C for 15 minutes.

2.5.3.4 Preparation of microbiological cultures

Bacterial isolates were grown from frozen stock (-80 °C). After warming to room temperature, each isolate was streaked on a TSA plate, and incubated overnight (35 °C with ambient air).

2.5.3.5 Preparation of overnight cultures

All overnight broth cultures were produced in TSB and were inoculated as follows. Four morphologically similar colonies were touched with a sterile loop and used to inoculate sterile TSB (10 mL) in a sterile 25 mL universal container. This preparation was then incubated overnight (35 °C with ambient air) prior to use.

2.5.3.6 Minimum Inhibitory Concentration (MIC) assay

MIC assay with serial broth microdilution was used to determine antimicrobial susceptibility, in sterile 96-well plates. In the first column, a starting concentration of test compound was prepared (1,024 µg/mL) in CAMHB (200 µL) in triplicate. Serial log₂ dilutions were performed for each test compound in subsequent columns. Overnight cultures were diluted in sterile PBS to an

optical density (OD_{625}) of between 0.08 and 0.10 (McFarland standard). The bacterial cultures were then diluted ten-fold in CAMHB and 5 μ L of the final solution was added to each well of the 96-well plate within 30 min of inoculum preparation. The microtitre plates were then wrapped in parafilm and incubated at 35-37 °C in ambient air for 18-20 h (Andrews 2006). The lowest concentration at which there was no visible growth (the first clear well), was taken as the MIC of the test compound. The modal result (n=3) was reported. A significant difference in two MIC readings was accepted if they differed by more than a two-fold dilution (Andrews 2006). A typical plate layout for MIC determination is reported in Figure 2.5.

2.5.3.6.1 Quality control procedures

Each MIC determination was accompanied by growth, sterility and quality controls. Growth controls (no antibiotic) were considered acceptable if they showed heavy turbidity. Sterility controls were considered acceptable if they were free from visible bacterial growth. After reading the MIC, a purity control was performed by subculturing growth controls and the first turbid well of each test onto TSA plates. Each TSA plate was then incubated for a further 18-20 h (35 °C with ambient air). Plates were then visually inspected to confirm homogeneity of colonies. Antimicrobial susceptibility of *E. coli* NCTC 10418 to colistin (or colistin and ciprofloxacin for checkerboard assays) determined with each batch of MIC determinations (in triplicate) was considered acceptable if within one serial dilution from published standards (Andrews 2006).

2.5.4 ELISA studies

Colistin content was also estimated using an ELISA kit according to the manufacturer's instructions. Typically, sample (40 μ L) was mixed with ddH₂O (40 μ L) in an Eppendorff tube. One part colistin extraction buffer (diluted ten-fold in ddH₂O) was added to four parts 20% v/v acetonitrile. Aliquots of the resulting solution (240 μ L) were added to an Eppendorff tube followed by cleanup buffer I (16 μ L) and vortexed for 10 s. After addition of cleanup buffer II (16 μ L) the resulting solution was centrifuged (10 min, 4000 *g*, 21 °C).

Supernatant (75 μ L) was extracted, diluted 100-fold in ddH₂O, re-vortexed and assayed using the ELISA kit. After addition of stop buffer, the absorbance was measured at 450 nm. A standard calibration curve (range 0.5 to 50 μ g/L) was constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration on a logarithmic curve (Figure 2.6). Sample concentrations of colistin were derived by interpolation.

2.6 Statistical analysis

Unless otherwise stated, data was expressed as mean \pm the error, expressed as 1 standard deviation (SD). Statistical significance was set at $p < 0.05$ (indicated by *). Where only two groups were compared, Student's t-test for small sample sizes was used (Swinscow and Campbell 2002). When more than two groups were compared, significance was evaluated using analysis of variance (ANOVA). Goodness-of-fit was investigated with coefficients of determination (R^2) using least-squares regression. Unknown values were directly interpolated from the respective best-fit calibration curve. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows, (San Diego USA). Specific statistical techniques are described in the chapters where they are used.

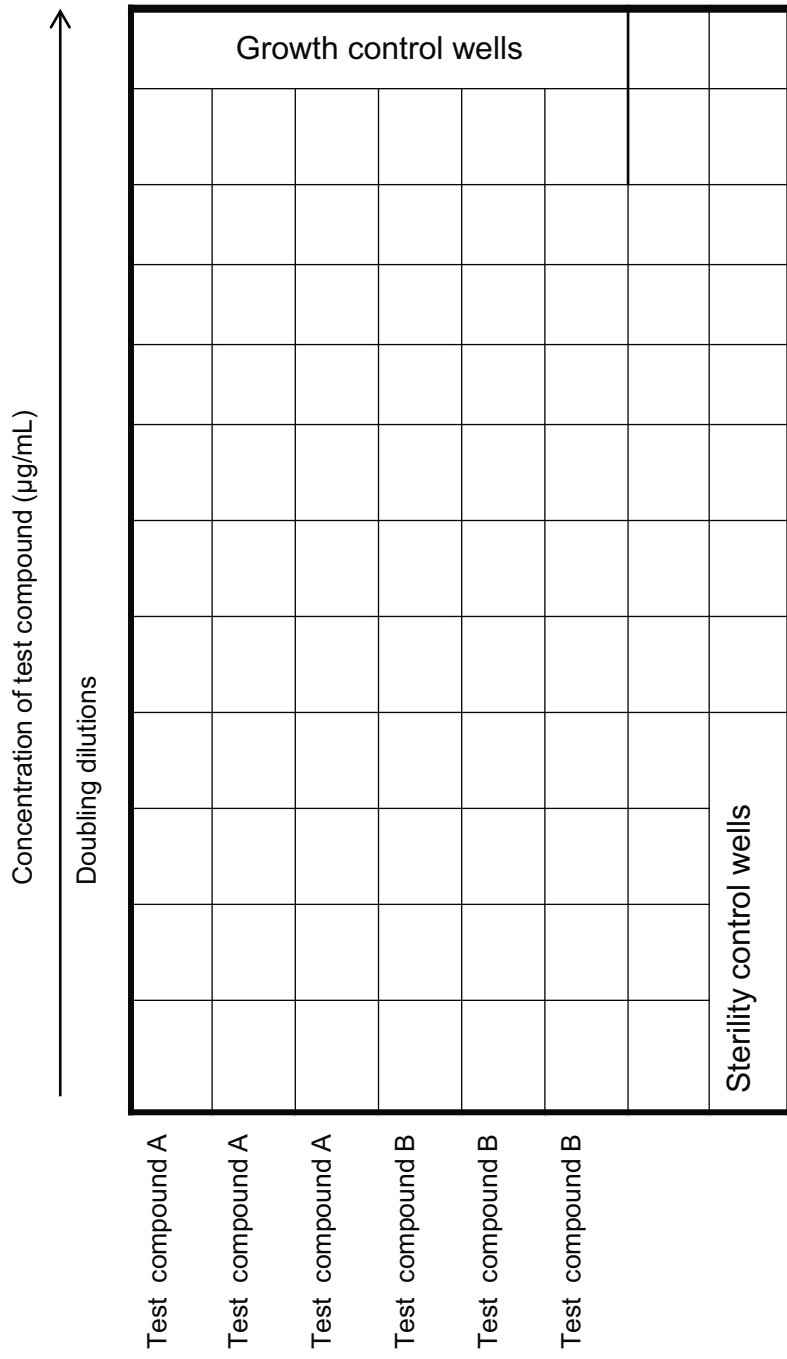


Figure 2.5: Typical plate layout for MIC determination, showing serial double dilutions of the test compound. Growth and sterility controls were included with each plate.

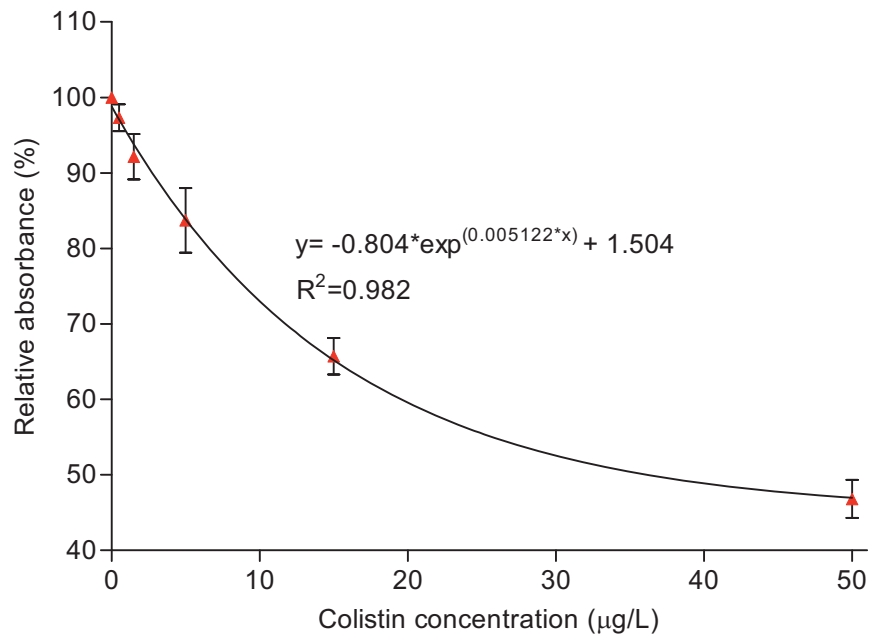


Figure 2.6: The ELISA calibration curve using colistin sulfate standards. Data shown represents mean \pm SD (n=6).

Chapter Three

Synthesis and Optimisation of Dextrin-colistin Conjugates

3.1 Introduction

This chapter aims to synthesise and characterise a rationally designed library of dextrin-colistin conjugates for subsequent biological evaluation. Polymer-protein conjugates classically exhibit a tripartite structure, consisting of a polymer, linker and the bioactive payload (Duncan 2003). In this study, the polymer is intended to be a covalently bound structural component of the construct without intrinsic activity. Ideally the polymer should possess functional groups or permit introduction of functional groups that activate the polymer and allow conjugation to the bioactive payload, generating a stable masked conjugate. It should also lend itself to enzyme-triggered degradation, unmasking the bioactive payload without generating toxic by-products (Chapter 1). To design a polymer conjugate that would have the required stability, pharmacokinetic profile and pharmacological activity, features such as polymer molecular weight, conjugation chemistry and number of reactive groups on the polymer were initially considered.

3.1.1 Dextrin

Dextrin is a α -1, 4 poly (D-glucose) polymer obtained from starch hydrolysis. Dextrins are largely linear polymers with limited (< 5%) branching in the α -1, 6 position and an established safety profile (Federal Drug Agency 2010; Guo *et al.* 2002). Dextrin's versatility to several clinical applications has been well-established. It is used as a supplement for renal and hepatic failure (Woolfson *et al.* 1976) as a carrier for intraperitoneal 5-fluorouracil (Davies 2006; Kerr *et al.* 1996) and as a component of peritoneal dialysis solutions (Peers and Gokal 1998).

The enzyme-triggered degradation of dextrin offers several advantages, not least that degradation results in non-toxic metabolites. Amylase triggers hydrolysis of α -1,4 glucosidic linkages forming maltose and iso-maltose which are subsequently metabolised to glucose by tissue maltases, and/or excreted into urine (Burkart 2004; Davies 1993; Roberts and Whelan 1960). Dextrin also lends itself well to masking of protein activity by conjugation and subsequent α -amylase-triggered reinstatement. The PUMPT concept was

first characterised using dextrin as the model polymer (Duncan *et al.* 2008). Since then, several bioresponsive dextrin conjugates have been reported (Table 3.1). This body of evidence provides a robust rationale for dextrin to be chosen as the model polymer.

3.1.2 Linker and activation chemistry

A key requirement for the optimised synthesis of a polymer-protein conjugate is the introduction of a linker through a reproducible reaction that allows targeted modification, while generating the least toxic or immunogenic by-products (Duncan 2003). However, dextrin does not bear convenient carboxyl or amino groups to which the bioactive may be coupled and requires functionalisation. In contrast to most other methods used to functionalise dextrin (Table 3.2), succinylation involves relatively non-toxic reagents and by-products (Hreczuk-Hirst *et al.* 2001b). Any residual succinate would be naturally metabolised to fumarate by succinate dehydrogenase, and non-toxic metabolites (Bruice 1998; Olson 1997). The number of reactive groups can be controlled by altering the reactant ratios (Hreczuk-Hirst *et al.* 2001a).

Previous studies have also reported the use of succinylation as a means of controlling the rate of dextrin enzymolysis and regeneration of protein/peptide activity through succinylation (Duncan *et al.* 2008; Hreczuk-Hirst *et al.* 2001a; Treetharnmathurot *et al.* 2009). Therefore, the use of optimally succinoylated dextrans would introduce the opportunity of rationalising conjugate design to its specific application in infection. For these reasons, succinylation was employed to functionalise dextrin.

3.1.3 The bioactive payload

Several considerations governed the selection of the antibiotic payload. First, the choice antibiotic should be structurally characterised and permit conjugation via accessible amine groups. It should also possess established efficacy against Gram-negative MDR bacteria, but be limited by a significant toxicity profile and an indiscriminate volume of distribution to a "last treatment resort" niche.

Table 3.1 : Prior studies using succinoylated dextrin in bioresponsive conjugates in polymer therapeutics

Bioactive component	Coupling agents	Study
Trypsin	EDC, sulfo-NHS	Duncan <i>et al.</i> (2008)
Melanocyte stimulating hormone		
PLA ₂	EDC, sulfo-NHS	Ferguson and Duncan (2009)
Recombinant Human Epidermal Growth Factor (rhEGF)	EDC, sulfo-NHS	Hardwicke <i>et al.</i> (2008a)
Doxorubicin	EDC, sulfo-NHS	Hreckzuk-Hirst <i>et al.</i> (2001a)
Tyrosinamide	Carbodiimide	Hreckzuk-Hirst <i>et al.</i> (2001a)
Biotin	Carbodiimide	Hreckzuk-Hirst <i>et al.</i> (2001a)
Trypsin	EDC, sulfo-NHS	Treetharnmathurot <i>et al.</i> (2009)

Table 3.2: Alternative methods for activation and functionalisation of polysaccharides

Method	Activation	Drawback	Reference
Cyanogen bromide activation	Primary amine groups are introduced directly into the reactive intermediate	A reactive intermediate is formed that may disrupt the polymer backbone In the presence of acid, hydrogen cyanide may be formed	Hermanson <i>et al.</i> (1991)
Chloroformate activation	Introduces hydroxyl groups into the polymer	Possibility of formation of intra- or inter-chain carbonate esters exists. Formation of five-member rings can interfere with polymer backbone.	Bruneel and Schacht (1993b)
Periodate oxidation	Aldehyde groups may be formed	Aldehyde groups can lead to toxicity if not completely neutralised.	Bruneel and Schacht (1993a)

Colistin is an antimicrobial peptide that retains excellent activity against Gram-negative MDR bacteria (Gales *et al.* 2011). Small molecules such as colistin (molecular weight 1,155 g/mol) readily traverse blood vessels to distribute indiscriminately along their volume of distribution, facilitating their toxicity (Chapter 1). Colistin's significant toxicity has been well-established (Conly and Johnston 2006; Vaara 2010; Wunsch *et al.* 2012). Colistin is an amphiphilic lipodecapeptide composed of a polycationic hydrophilic cyclic heptapeptide moiety attached to a tri-peptide side-chain. Attached to the tripeptide side-chain's N-terminus is a hydrophobic fatty-acyl tail (Orwa *et al.* 2002; Suzuki *et al.* 1963). It consists of at least 30 closely related components (Govaerts *et al.* 2003; Ikai *et al.* 1998; Orwa *et al.* 2002) of which colistin A (polymyxin E₁) and colistin B (polymyxin E₂) are the major constituents (Orwa *et al.* 2002). They differ in that they are acylated by (S)-6-methyloctanoic acid and (S)-6-methylheptanoic acid, respectively (Velkov *et al.* 2009).

Colistin shows an intimate relationship between structure and activity which needs to be considered when selecting the conjugation process. Colistin's structural integrity is essential for its bactericidal function. Colistin's polar and hydrophobic domains form two distinct three-dimensional faces, conferring structural amphipaticity (Velkov *et al.* 2009). The polar face of colistin interacts with the polar lipid-A head, while the hydrophobic face inserts into the LPS fatty-acyl layer (Meredith *et al.* 2009; Pristovsek and Kidric 1999, 2004). Loss of the tripeptide tail moiety, fatty acyl chain and N-terminal amino acid results in a compound that does not bear antimicrobial activity (Nakajima 1967; Vaara 1992). Acyclic polymyxin analogs also lack antimicrobial activity (Kline *et al.* 2001). Colistin's 5 D-amino groups per molecule mediate bactericidal activity and exert significant toxicity (Perazella 2009; Vaara *et al.* 2008; Velkov *et al.* 2009). These accessible amino residues are cationic under physiological conditions (pKa = 10) and available for conjugation (Ma *et al.* 2009). Literature reports numerous attempts to decrease colistin's toxicity/efficacy balance through chemical manipulation of these amino groups (Chapter 1). Sulphomethylation produces an uncharged prodrug

which hydrolyses to colistin (Barnett *et al.* 1964; Beveridge and Martin 1967), and remains the only technique to yield a clinically viable product and the commonest parenterally administered formulation of colistin (colistimethate sodium, CMS, Colomycin[®]).

3.1.4 The conjugation procedure

Polymer conjugation may reduce a bioactive's toxicity and immunogenicity, extend plasma circulation time, and increase stability against proteolytic degradation (Duncan *et al.* 2008). Furthermore, creation of macromolecular constructs may achieve passive targeting through the EPR effect, which would limit colistin's volume of distribution and consequent toxicity (Chapter 1). Hreczuk-Hirst *et al.* (2001b) popularised the coupling of amino group-containing compounds to succinoylated dextrin using zero-length cross-linking agents, namely EDC and sulfo-NHS (Chapter 2). This technique was successfully applied for several bioactives including doxorubicin, PLA₂, trypsin, and epidermal growth factor. In these studies, Treetharnmathurot *et al.* (2009), Hardwicke *et al.* (2008a), Ferguson and Duncan (2009), generated by-products that were relatively easily removed by chromatographic protocols. A zero-length cross-linking reaction using EDC and sulfo-NHS was therefore chosen to form amide bonds between succinoylated dextrin's carboxylic acid groups and colistin's amine residues. Next, it was necessary to establish an experimental process for synthesis, characterisation and optimisation, leading to an optimal dextrin-colistin conjugate-library for *in vitro* testing.

3.2 Experimental aims

The overall aim of this study was to establish a library of bioresponsive dextrin-colistin conjugates from a range of succinoylated intermediates. The specific aims of this chapter were:

1. To select a library of succinoylated dextrin intermediates, through physicochemical and biological characterisation for conjugation to colistin

2. To synthesise a dextrin-colistin conjugate library and establish methods for conjugate physicochemical characterisation with respect to molecular weight, polydispersity index, protein content, dextrin: colistin molar ratio, and free amine groups per molecule after conjugation
3. To evaluate the effect of dextrin molecular weight and the degree of succinylation on the unmasking of colistin after exposure to human α -amylase
4. To identify and optimise a dextrin-colistin conjugate library for which masking/unmasking would be feasible in infection

3.3 Methods

A number of general methods (Chapter 2) were used for characterisation of succinoylated dextrans and dextrin-colistin conjugates, including the BCA protein assay (Section 2.5.2.3), ninhydrin assay (Section 2.5.2.4), GPC (Section 2.5.2.1) and FPLC (Section 2.5.2.2).

3.3.1 Succinylation of dextrin

A low molecular weight (LMW) dextrin (8,100 g/mol), a high molecular weight (HMW) dextrin (28,900 g/mol) from corn starch, a medium molecular weight (MMW) dextrin from potato starch (13,900 g/mol) were used to synthesise a library of succinoylated dextrin intermediates. Succinylation of each of these dextrans was based on the method of Hreczuck-Hirst *et al.* (2001b) to create three succinoylated dextrin libraries. In each case, increasing degrees of succinylation were produced by adjusting the molar ratio of dextrin to succinic anhydride. Typical reaction conditions used to derive 2 mol% theoretical succinylation of LMW dextrin are described below.

Dextrin (1g, 1.23×10^{-4} mol), succinic anhydride (12.32 mg, 1.2×10^{-4} mol) and DMAP (5.7 mg, 4.6×10^{-5} mol) were added to a round-bottomed flask, which was then purged with liquid nitrogen. Anhydrous DMF (10 mL) was withdrawn under nitrogen gas and added to the flask. The mixture was stirred

continuously at 50 °C for 14 h. The product was added dropwise to diethyl ether (~800 mL) and vortex-stirred overnight. Residual solid was extracted by filtration and dissolved in ddH₂O (10 mL). Dialysis (MWCO 2,000 g/mol) against ddH₂O (6 x 1 L) over 6 h, was used to remove water-soluble impurities. Finally, the succinoylated dextrin solution was lyophilised to a constant weight, and stored at 4 °C. The reaction scheme is summarised in Figure 3.1.

3.3.2 Confirmation and quantification of dextrin functionalisation

Molecular weight and polydispersity were determined by GPC (Section 2.5.2.1).

3.3.2.1 Titrimetric analysis

The degree of succinoylation was determined by titration against a standard solution of NaOH (5×10^{-4} M) with bromothymol blue as colorimetric indicator. Acid-base titrimetric analysis was used to quantify the degree of carboxyl groups incorporated into dextrin polymer. Succinoylated dextrin was dissolved in ddH₂O (1.5 mg/mL, 2 mL) and titrated against 5×10^{-4} M NaOH using bromothymol blue indicator (1% w/v in ethanol, pH range 6-7.6). The endpoint of the titration reaction was indicated by a colour change to bright green. The final titer volume was subtracted from the volume required to titrate ddH₂O (2 mL). All titrations were performed in triplicate and the amount of carboxyl groups incorporated to the dextrin by succinoylation (mol %) was calculated.

3.3.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to confirm incorporation of C=O bonds with succinoylation. FTIR of dextrin prior to succinoylation was used as a negative control. Succinoylated dextrans were analysed over the mid-infrared (400-4,000 cm⁻¹) and near-infrared (>4,000 cm⁻¹) regions, with background interferences being subtracted. Qualitative analysis of the FTIR spectra was conducted by interpretation of the peaks in the double-bond region, 2,000-1,500 cm⁻¹.

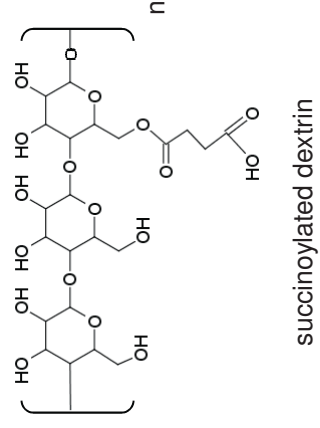
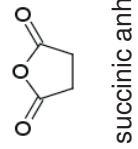
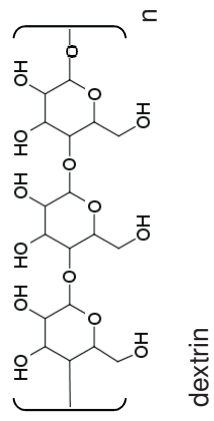


Figure 3.1: Reaction scheme for functionalisation of dextrin by succinylation

The resulting spectra were examined for peaks in the $1,750\text{ cm}^{-1}$ region to confirm qualitatively the incorporation of carboxyl functional groups by succinylation.

3.3.3 Synthesis of the dextrin-colistin conjugates

Succinoylated dextrin was conjugated to colistin using EDC and sulfo-NHS as zero-length cross-linking agents (Hermanson 2008). Typical reaction conditions used to conjugate 1 mol% succinoylated dextrin to colistin were as follows. Succinoylated dextrin (200 mg, 1.28×10^{-5} mol) EDC (2.22 mg, 1.16×10^{-5} mol) and sulfo-NHS (2.50 mg, 1.16×10^{-5} mol) were dissolved in ddH₂O (2 mL) in a round-bottomed flask and stirred for 20 min. Next, colistin sulphate (35 mg, 0.0133 mol) was added followed by dropwise addition of aqueous NaOH (0.5 M) until the reaction mixture reached pH 7 and stirred for 2 h at 21 °C. After 2 h, the product was centrifuged (4000 rpm, 5 min at 10 °C) to remove undissolved sediment. The supernatant was immediately frozen at -20 °C until required for purification by FPLC. The reaction scheme is summarised in Figure 3.2.

3.3.4 Conjugate purification and characterisation

Dextrin-colistin conjugates were purified from the reaction mixture by FPLC. Fractions (1 mL) corresponding to the conjugate were pooled, desalted, lyophilised to constant weight and stored at -20°C (Section 2.5.1). Dextrin-colistin conjugates were then characterised by GPC for molecular weight and polydispersity (Section 2.5.2.1), FPLC for % free colistin (Section 2.5.2.2), BCA protein assay for total protein (Section 2.5.2.3) and ninhydrin assay for free amine groups (Section 2.5.2.4).

3.3.5 Degradation of dextrin, succinoylated dextrin, and dextrin-colistin conjugate

To study the rate of α -amylase degradation of dextrin, and succinoylated dextrin, each polymer (3 mg/mL, in PBS pH 7.4) was incubated in the presence / absence of α -amylase (100 IU/L) at 37 °C for up to 24 h (n=3). Sample aliquots (300 μ L) were taken at various time-points.

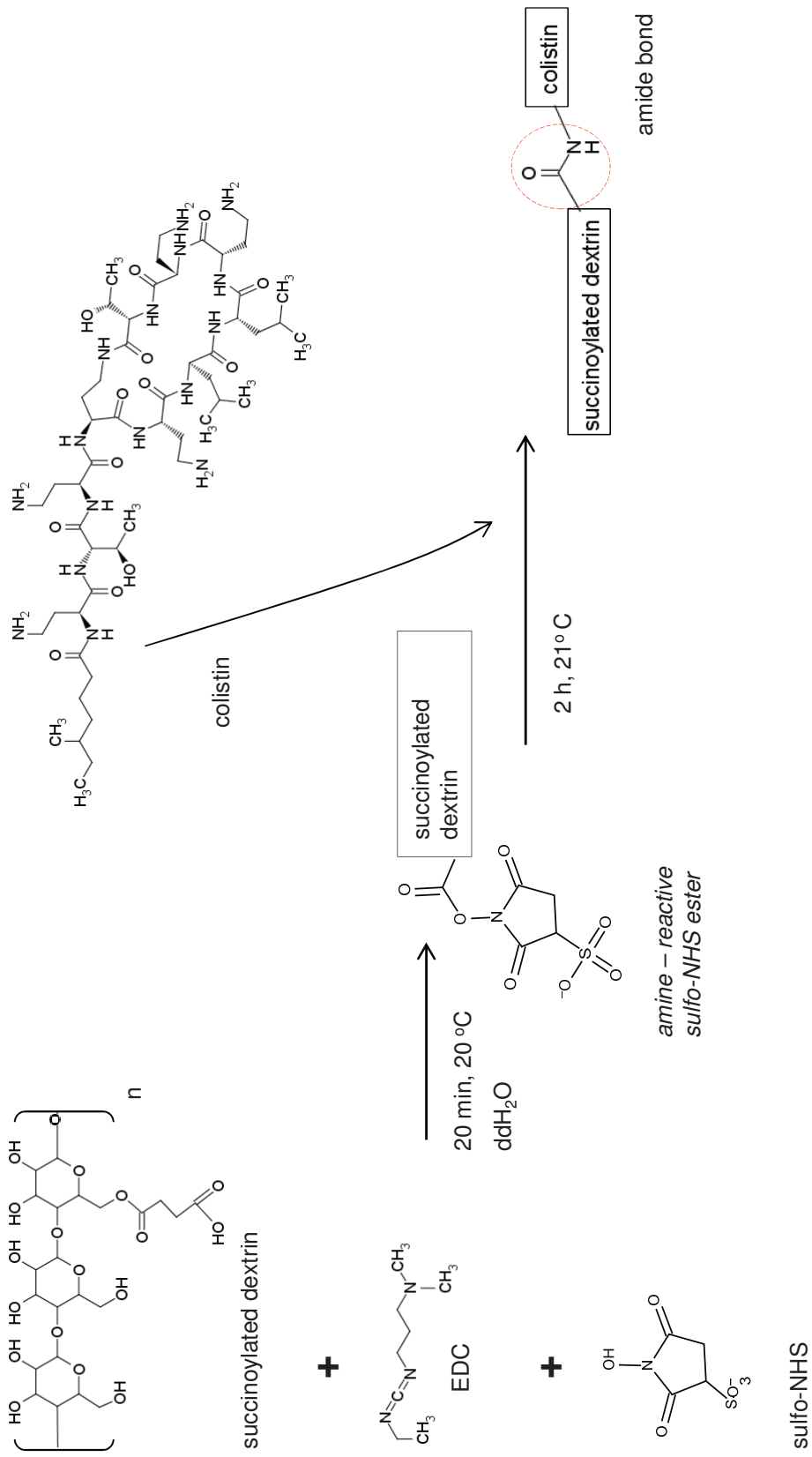


Figure 3.2: Reaction scheme for conjugation of succinoylated dextrin to colistin

These were immediately snap-frozen on dry ice to stop the reaction, and then stored at -20 °C prior to GPC analysis. To study the effects of α -amylase on the dextrin-colistin conjugates, conjugates (3 mg/mL) were incubated in the presence / absence of α -amylase (100 IU/L in PBS, pH 7.4) for up to 48 h at 37 °C (n=3). Samples (300 μ L) were removed at various time-points and snap-frozen on dry ice prior to analysis by GPC and FPLC (n=3). The % apparent free colistin was estimated from the ratio of area under the curve peaks (mAU/mL) corresponding to conjugated and free colistin.

3.4 Results

3.4.1 Synthesis, purification and characterisation of succinoylated dextrin libraries

Functionalisation of dextrin by succinylation resulted in a library of products (Table 3.3). Yield efficiency ranged from 46.5 to 54.6%. However, the variance in yield efficiency between the three parent-dextrin libraries was not significantly different (univariate ANOVA, $p = 0.471$) (Figure 3.3a).

FTIR of dextrin showed characteristic peaks at 3,350 cm^{-1} (OH), 2,800 cm^{-1} (-CH stretching vibrations) and 1,450 cm^{-1} (O-CH₂). However, analysis of succinoylated dextrans reported an additional C=O peak (1,720 cm^{-1}) which increased in strength with increasing mol% succinylation. Figure 3.4 represents typical absorption spectra for the LMW parent-dextrin library. Similar spectra were obtained for MMW and HMW parent-dextrin libraries (Appendix 2.1).

Overall % conversion efficiency was 49.4% \pm 2.7 (mean \pm SD) (range 48.2% to 53.6%). Mean conversion efficiency was 49.0 % (LMW parent-dextrin library), 50.2% (MMW parent-dextrin library) and 51.2 mol% (HMW parent-dextrin library). The conversion efficiency did not vary significantly between the three parent-dextrin libraries or between succinoylations (Bonferroni multiple comparison tests, $p > 0.05$, Figure 3.5).

Table 3.3: Physicochemical characteristics of succinoylated dextrans

	Succinoylation (mol %)		Batch n	Conversion efficiency (%)*	Yield efficiency (%)*	Mw (g/mol) [‡]	PDI -
	Theoretical	Actual*					
LMW parent-dextrin library	30	14.2 (0.7)	3	48.1 (0.8)	54.4 (2.6)	14,800 (400)	1.9 (0.2)
	15	7.2 (0.2)	4	47.9 (1.1)	52.5 (2.8)	11,900 (300)	1.8 (0.2)
	10	4.8 (0.6)	6	50.1 (3.4)	52.7 (2.8)	10,900 (600)	2.0 (0.3)
	5	2.5 (0.2)	6	49.1 (3.0)	52.0 (3.0)	8,900 (500)	1.9 (0.2)
	2	1.0 (0.1)	7	49.7 (3.6)	51.0 (7.0)	8,600 (500)	1.9 (0.1)
	0	n/a ¹ (control)	n/a	n/a	n/a	8,100 (200)	1.7 (0.1)
MMW parent-dextrin library	30	14.4 (0.4)	3	48.2 (1.3)	50.6 (1.7)	24,500 (800)	2.1 (0.2)
	15	7.5 (0.7)	3	50.3 (4.6)	49.2 (5.8)	18,000 (300)	2.0 (0.2)
	10	5.0 (0.3)	3	50.6 (3.1)	53.1 (4.7)	16,400 (400)	2.2 (0.3)
	5	2.6 (0.1)	3	51.1 (0.6)	49.0 (7.1)	14,300 (400)	2.0 (0.2)
	0	n/a ¹ (control)	n/a	n/a	n/a	13,900 (300)	1.8 (0.2)
	HMW parent-dextrin library	30	14.8 (0.9)	3	49.4 (2.9)	46.5 (4.7)	51,700 (500)
15		7.6 (0.4)	3	50.6 (3.1)	53.9 (14.8)	46,300 (700)	1.9 (0.1)
10		5.4 (0.3)	3	53.6 (1.3)	51.6 (3.7)	41,700 (500)	2.0 (0.2)
5		2.6 (0.1)	3	51.1 (0.6)	54.6 (8.6)	33,500 (1000)	1.9 (0.1)
0		n/a ¹ (control)	n/a	n/a	n/a	28,900 (400)	1.9 (0.2)

* Mean (\pm SD); values reported to 1 d.p.

[‡] Mean (\pm SD) relative to pullulan standards, to nearest 100 g/mol

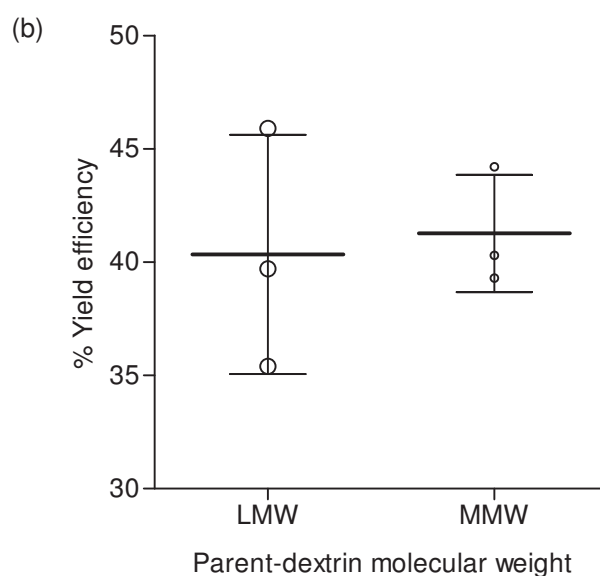
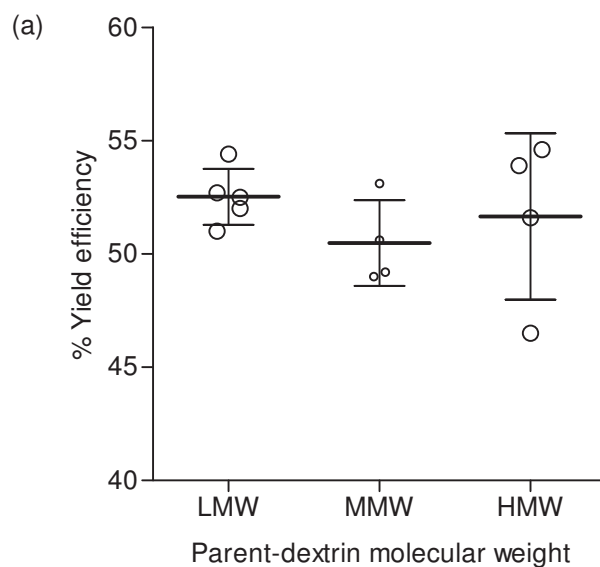


Figure 3.3: Comparison of % yield efficiency for succinoylated dextrans and dextrin-colistin conjugates. Panel (a) shows variance in % yield efficiency between the three parent dextrin libraries for the succinoylated dextrin intermediates. (Non significant: univariate ANOVA: $F(2, 10) = 0.814$, $p = 0.471$).

Panel (b) shows variance in % yield efficiency between the LMW and MMW parent-dextrin libraries for the dextrin-colistin conjugates (Non significant: unpaired t test, $p = 0.7537$, $t = 0.33$ degrees of freedom=5; F test to compare variance: $F(3, 2) = 2.771$, $p = 0.553$). Horizontal lines and error bars represent mean \pm SD. NS: non-significant.

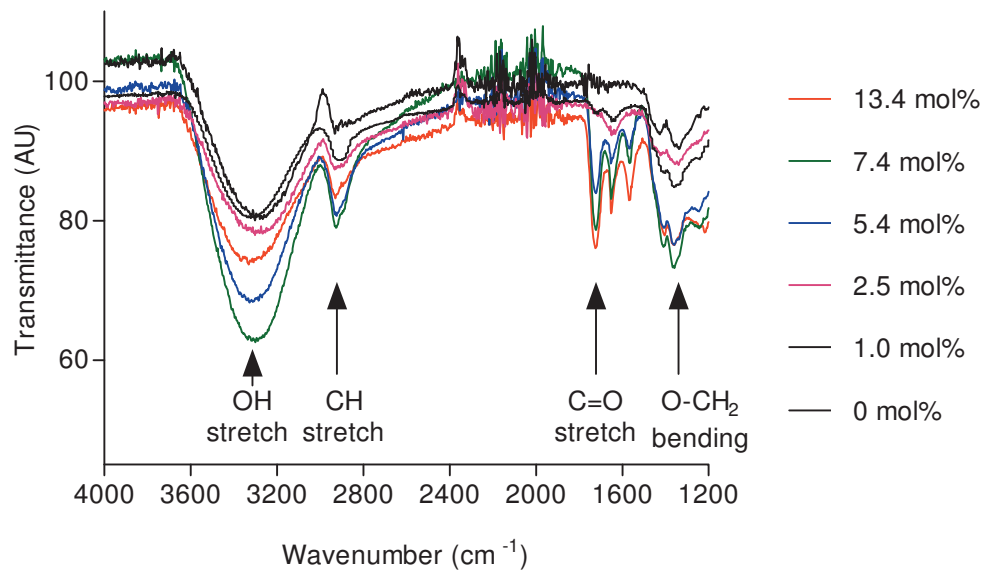
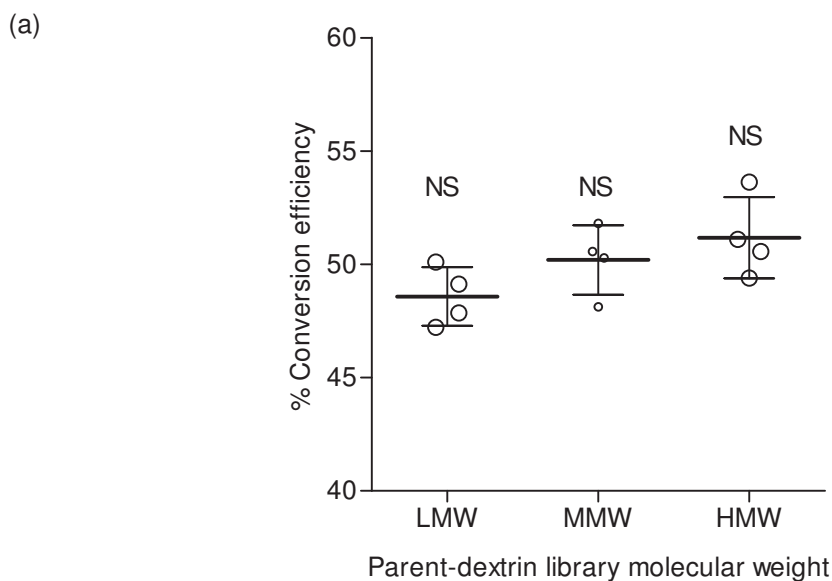


Figure 3.4: Typical characterisation of succinoylated dextrans by FTIR (LMW parent-dextrin family), showing the increase in peak intensity at 1720 cm⁻¹ caused by succinylation.



(b)

Comparison	Theoretical degree of succinylation (mol%)	Difference	t statistic
LMW vs MMW parent-dextrin library	30	0.9022	0.3991
	15	2.418	1.070
	10	0.4667	0.2384
	5	2.670	1.364
LMW vs HMW parent-dextrin library	30	2.169	0.9595
	15	2.707	1.197
	10	3.533	1.805
MMW vs HMW parent-dextrin library	5	1.970	1.006
	30	1.267	0.5604
	15	0.2889	0.1278
	10	3.067	1.357
	5	-0.7000	0.3097

Figure 3.5: Comparison of mol% conversion efficiency between the three parent-dextrin libraries (LMW, MMW, HMW parent-dextrin). Panel (a) reports two-way unweighted means ANOVA for mol% conversion efficiency versus parent-dextrin library Mw and mol% succinylation. The effect of parent dextrin on mol% conversion efficiency was not statistically significant ($F(2, 30) = 2.93$, $p = 0.0687$).

Panel (b) reports Bonferroni post hoc multiple comparison tests comparing mol% conversion efficiency between parent-dextrin libraries for each succinylation. In each case, this was non significant ($p > 0.05$).

In all cases, it was observed that apparent molecular weight (derived from GPC analysis using pullulan standards) increased in consistence with mol% succinylation (Table 3.4): the variance in apparent molecular weight was associated to both mol% succinylation and parent-dextrin Mw (two-way ANOVA, $p < 0.05$) (Figure 3.6).

3.4.2 Synthesis and characterisation of the conjugate library

Preliminary investigations reported that optimum reaction conditions for dextrin-colistin conjugation were RT (21° C) in ddH₂O, at pH 7 over 2 h reaction time. It was hypothesised that conjugation results in the covalent reaction of free amino groups on colistin to carboxyl groups on the succinoylated dextrin, producing peptide (amide) bonds. Consequently, the number of available amine groups on the colistin molecule before and after conjugation needed to be quantified. The ninhydrin assay indicated that colistin possessed 5 ± 0.4 (mean \pm SD, $n = 6$) amine groups per molecule. After conjugation, the ninhydrin assay reported 1.7 ± 1.0 to 3.1 ± 0.6 (mean \pm SD) unconjugated amino groups per colistin molecule.

When succinoylated dextrin was conjugated to colistin using EDC and sulfo NHS, FPLC confirmed the presence of a high molecular weight conjugate (7-13 mL) with additional peaks at 15-17 mL (colistin, sulfo-NHS) and 19-21 mL (DMAP) for unreacted products. Figure 3.7a reports a typical elution of the reaction mixture. Peaks corresponding to the various reactants are inset. After purification, these reactants and unbound colistin were below the limit of detection (Figures 3.7b, 3.8).

Colistin content of the conjugates ranged from 9.6 to 26.4% w/w, as determined by the BCA protein assay, corresponding to incorporation of 0.7 to 1.7 moles of colistin per mole of succinoylated dextrin. Neither dextrin nor succinoylated dextrin interfered with the BCA protein assay (Table 3.4). Overall mean % yield efficiency was $40.6\% \pm 3.2\%$ (mean \pm SD), and ranged from 35.4% to 45.0%. However, variance in % yield efficiency between the two dextrin-colistin conjugate libraries was not significantly different (Figure 3.3b).

Table 3.4: Physicochemical characterisation of the dextrin-colistin conjugate library

Designation	Degree of succinylation	n	Yield ^a (%)	Mw ^b (g/mol)	PDI ^a	Protein content (% w/w) ^a	Molar ratio (colistin: dextrin) ^a	Free amino groups/molecule ^a
EA-1	LMW parent- dextrin family	4	35.4 (5.3)	18,200 (900)	1.7 (0.1)	19.2 (4.0)	1.7 (0.5) :1	2.3 (1.2)
EA-2		4	39.7 (6.0)	14,700 (600)	1.6 (0.1)	18.4 (3.3)	1.3 (0.3) :1	1.7 (0.9)
EA-3		5	45.0 (7.5)	12,800 (600)	1.6 (0.2)	15.5 (3.1)	1.0 (0.3) :1	3.1 (0.6)
EA-4		6	40.3 (3.9)	9,600 (500)	1.7 (0.1)	9.6 (2.3)	0.7 (0.1) :1	2.1 (0.8)
EA-5	MMW parent- dextrin family	3	40.3 (3.9)	21,300 (600)	1.9 (0.3)	23.0 (3.6)	1.7 (0.4) :1	2.5 (0.7)
EA-6		3	39.9 (4.4)	17,800 (400)	1.9 (0.4)	16.3 (2.3)	1.1 (0.2) :1	1.9 (1.0)
EA-7		3	44.2 (14.3)	15,000 (400)	1.8 (0.3)	11.6 (1.0)	0.7 (0.7) :1	2.6 (1.1)

a: Mean (±SD) reported to 1 d.p;

b: relative to pullulan standards, to the nearest 100 g/mol

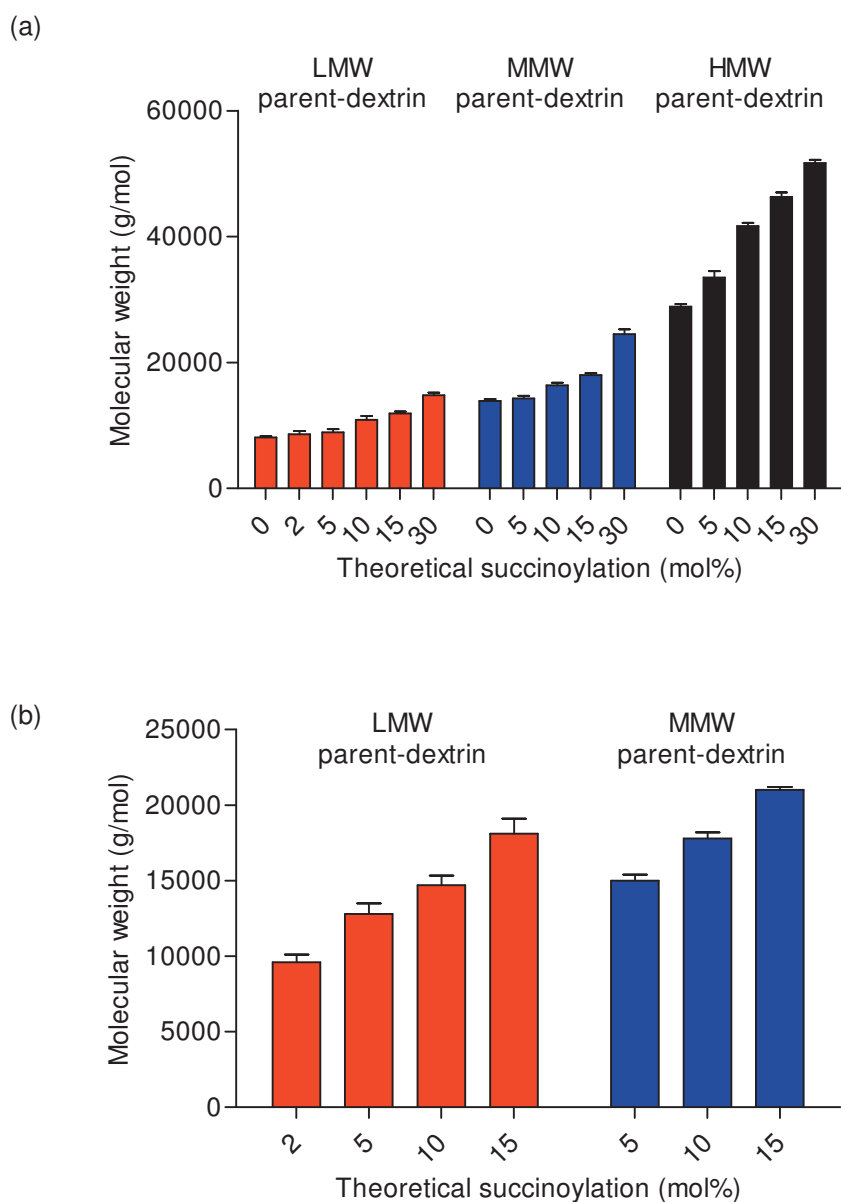


Figure 3.6: Effect of theoretical mol% succinylation and parent-dextrin on apparent molecular weight for (a) succinoylated dextrans (LMW, MMW and HMW parent-dextrin libraries) and (b) dextrin-colistin conjugates (LMW and MMW parent-dextrin libraries).

Panel (a) shows two-way ANOVA reporting that variance in apparent molecular weight was associated to both mol% succinylation ($F(4, 38) = 1023.72, p < 0.0001$), parent-dextrin molecular weight ($F(2, 38) = 14,546.69, p < 0.0001$) and interaction between the two variables ($F(8, 38) = 162.83, p < 0.0001$). Panel (b) shows two way ANOVA reporting that variance in apparent molecular weight was associated to both mol% succinylation ($F(2, 16) = 131.69, p < 0.0001$); and parent-dextrin molecular weight ($F(1, 16) = 91.60, p < 0.0001$).

Data shown represents mean \pm SD (n=3). Molecular weight (GPC) is given relative to pullulan standards.

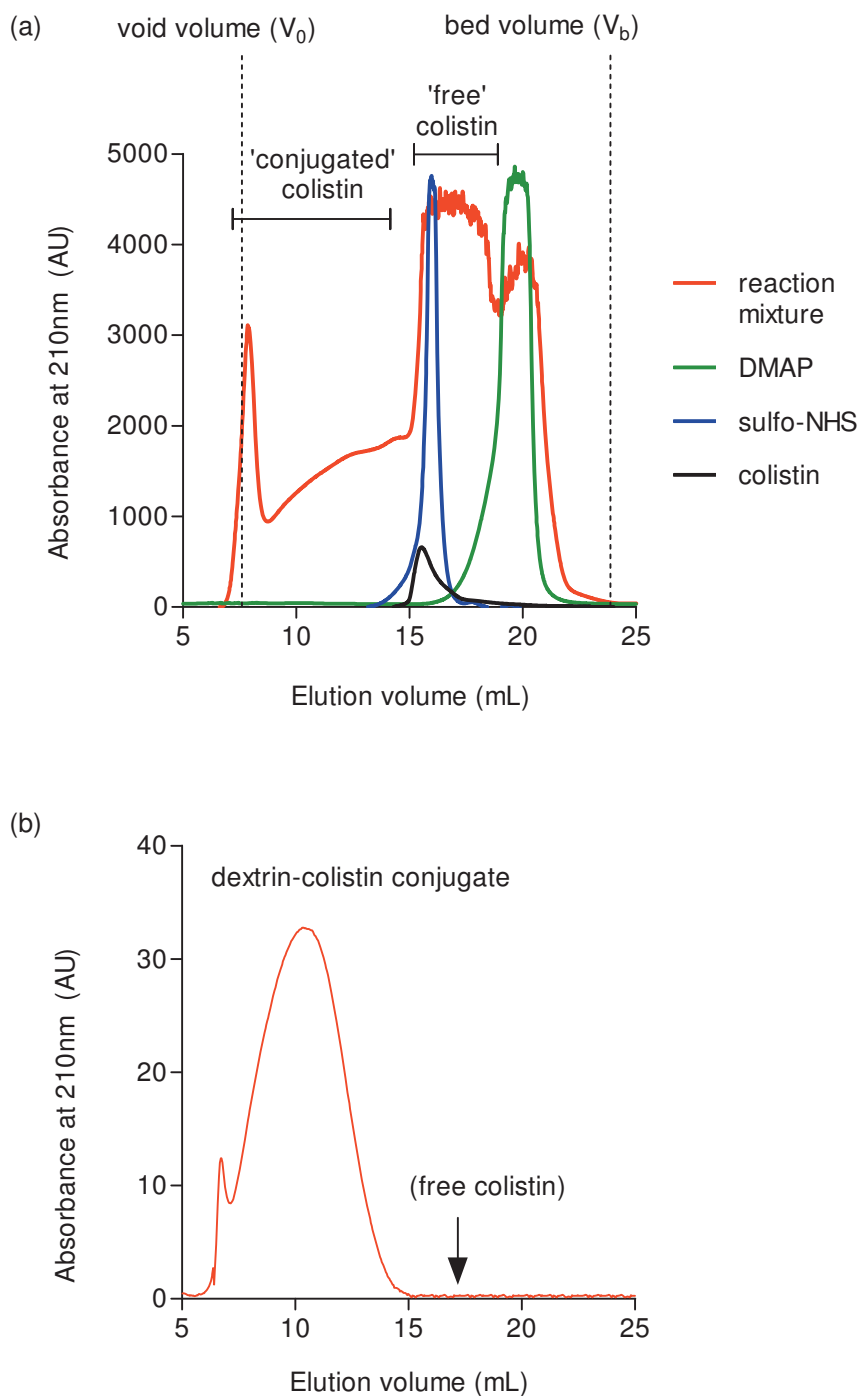


Figure 3.7: Typical FPLC analysis of the conjugation reaction. Panel (a) shows a typical elution profile of the reaction mixture and reactants for EA-4 conjugate. Panel (b) shows a typical FPLC chromatogram of the conjugate after purification. Void volume (V_0) = 7.7mL; Bed volume (V_b) = 24 mL.

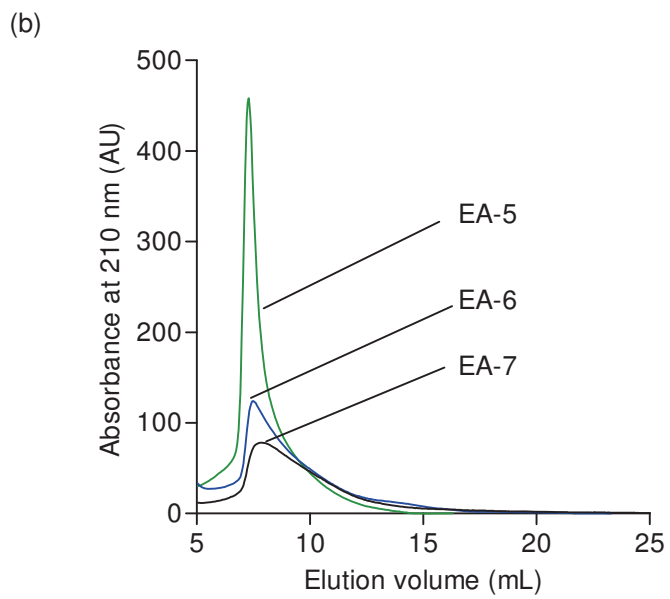
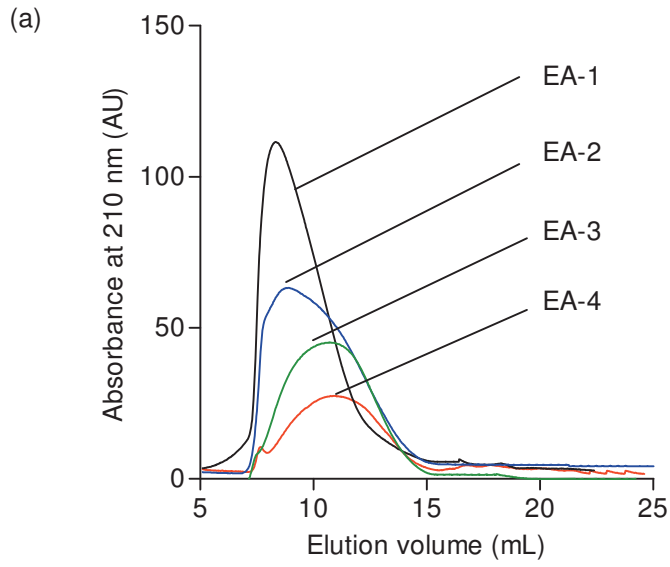


Figure 3.8: Typical FPLC chromatograms for purified dextrin-colistin conjugates showing (a) EA-1 to EA-4 dextrin-colistin conjugates (LMW parent-dextrin library) and (b): EA-5 TO EA-7 dextrin-colistin conjugates (MMW parent-dextrin library).

Final confirmation of successful conjugation was achieved using GPC, where retention time was decreased for dextrin-colistin conjugates compared to free colistin. An increase in succinylation of dextrin-colistin conjugates led to an increased apparent molecular weight but the change in polydispersity was limited. Average molecular weight and polydispersity for each dextrin-colistin conjugate are reported in Table 3.4. Variance in apparent molecular weight was associated to both mol% succinylation and parent-dextrin molecular weight (two-way ANOVA, $p < 0.05$).

3.4.3 Degradation studies of dextrin, succinoylated dextrin by α -amylase

When dextrin and succinoylated dextrin were exposed to α -amylase, a decrease in molecular weight was reported over time (Figure 3.9-3.12) that followed a biphasic pattern, with an earlier rapid and a latter slower phase. Degradation of succinoylated dextrans by α -amylase was best fit by logarithmic regression. Some degradation also occurred in the absence of α -amylase, but did not attain statistical significance ($p > 0.05$, Appendix 2.2).

In the presence of α -amylase, varying parent-dextrin molecular weight (at constant succinylation) resulted in a significantly increased degradation rate in all cases ($p < 0.05$, Figure 3.13). However, final molecular weight at 24 h (Mw_{24}) was still associated with increasing parent-dextrin molecular weight (LMW < MMW < HMW) (Figure 3.14). In the presence of α -amylase succinylation resulted in a significantly increased variance in degradation rate despite constant parent-dextrin Mw. However, this was not the case in the absence of amylase (Figure 3.15; Appendix 2.2).

The contribution of both independent factors (parent-dextrin molecular weight and mol% succinylation) to the variance in degradation rate was highly statistically significant (Figure 3.16). Succinylation accounted for a greater contribution to the variance in degradation rate with decreasing parent-dextrin Mw (LMW > MMW > HMW). Overall the greatest contribution to variance in degradation rate was accounted for by the parent-dextrin molecular weight.

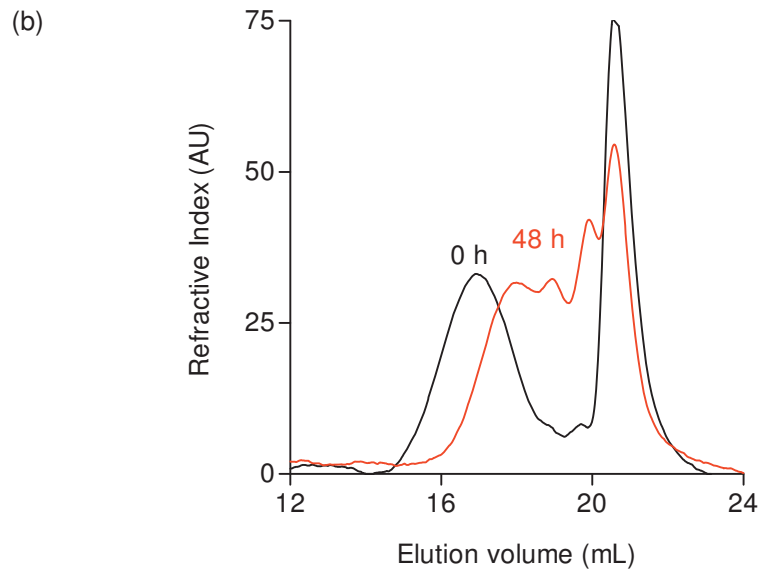
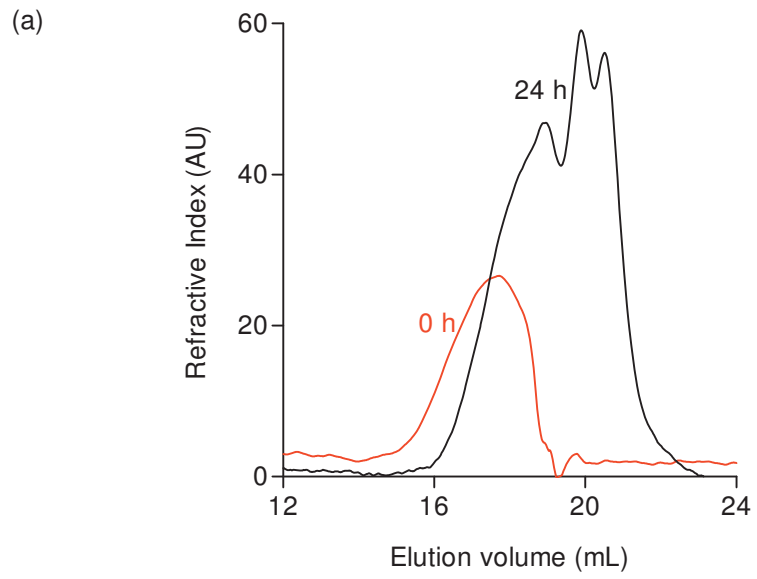


Figure 3.9: Typical time-dependant change in the GPC elution profile during incubation with α -amylase for (a) 1 mol% succinoylated dextrin (LMW parent dextrin library) and (b) EA-3 dextrin-colistin conjugate.

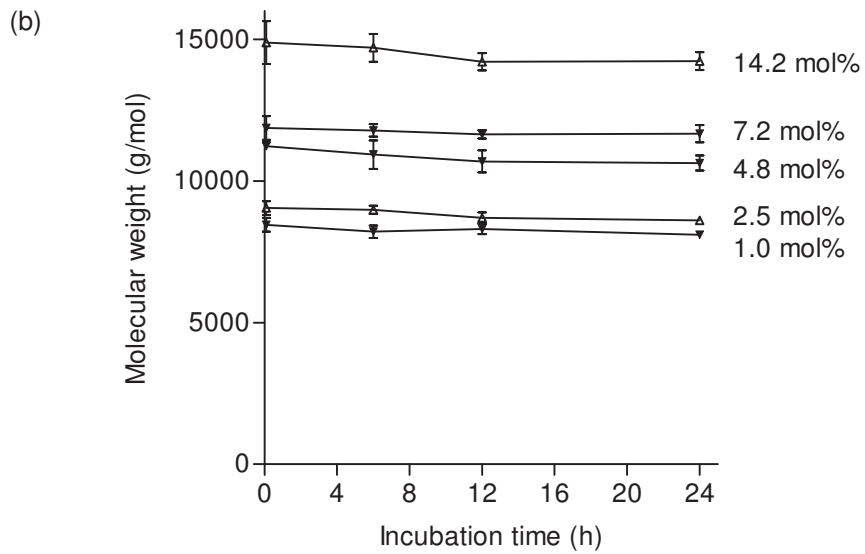
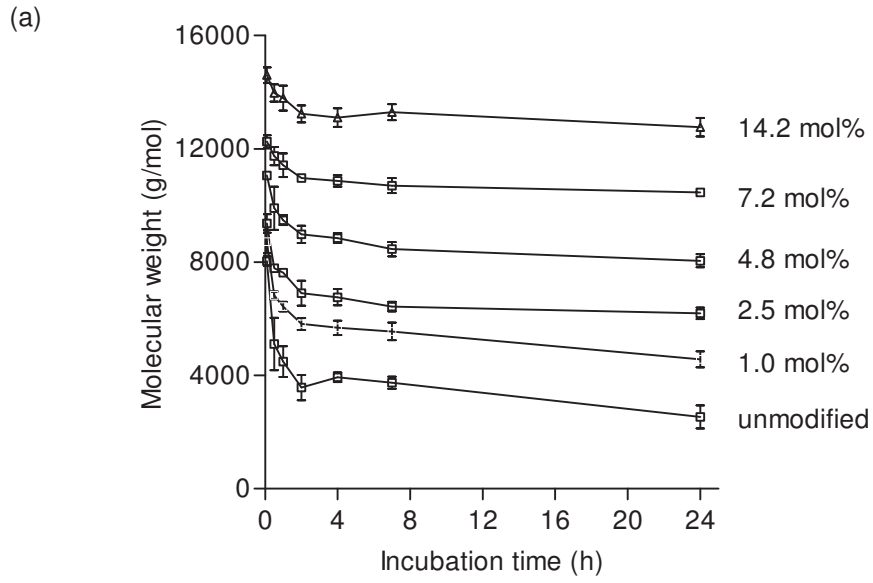


Figure 3.10: Degradation of succinoylated dextrans (LMW parent-dextrin library) measured by GPC during (a) incubation with α -amylase and (b) controls (no amylase). Data shown represents mean \pm SD ($n=3$). Molecular weight is given relative to pullulan standards.

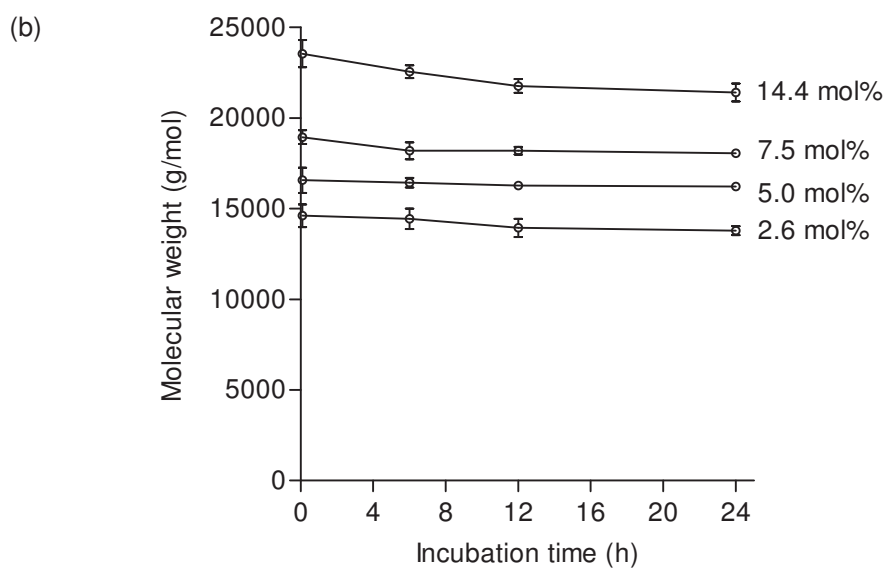
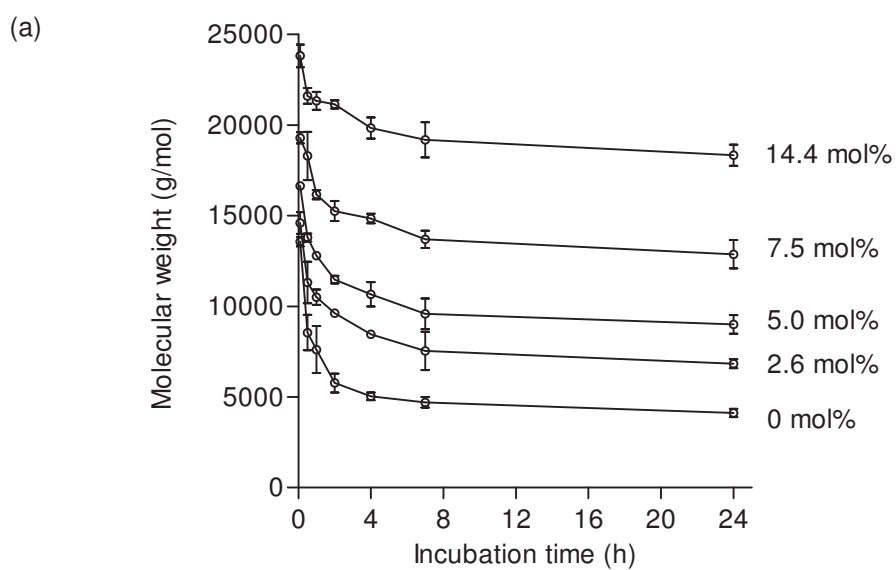


Figure 3.11: Degradation of succinylated dextrans (MMW parent-dextrin library) measured by GPC during (a) incubation with α -amylase and (b) controls (no amylase) for increasing mol% succinylations. Data shown represents mean \pm SD (n=3). Molecular weight is given relative to pullulan standards.

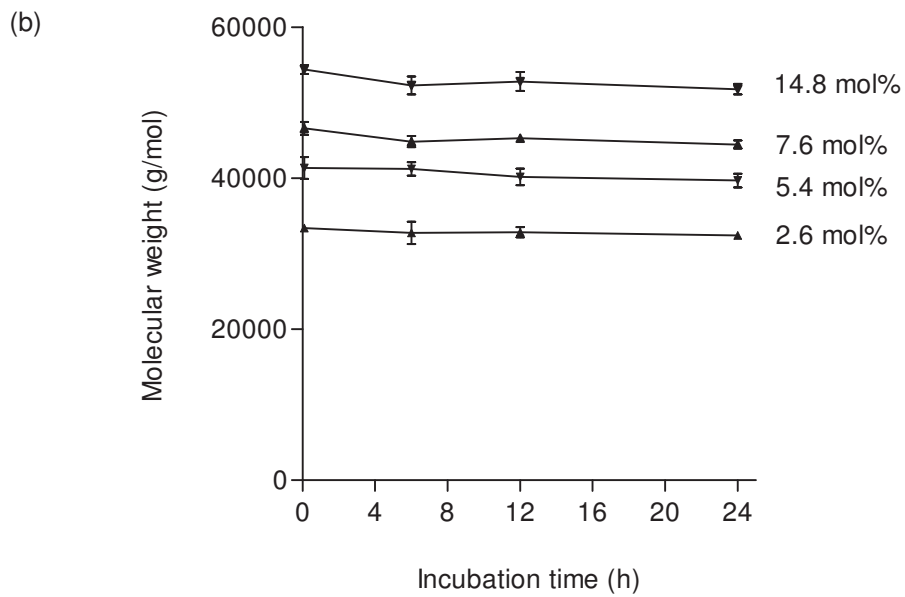
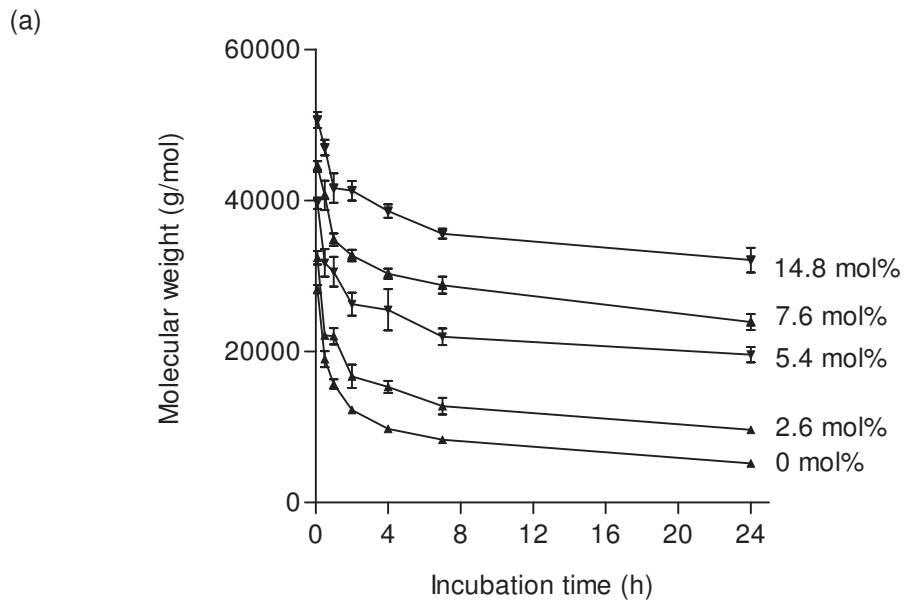


Figure 3.12: Degradation of succinylated dextrans (HMW parent-dextrin library) measured by GPC during (a) incubation with α -amylase and (b) controls (no amylase) for increasing mol% succinylations. Data shown represents mean \pm SD, (n=3). Molecular weight is given relative to pullulan standards.

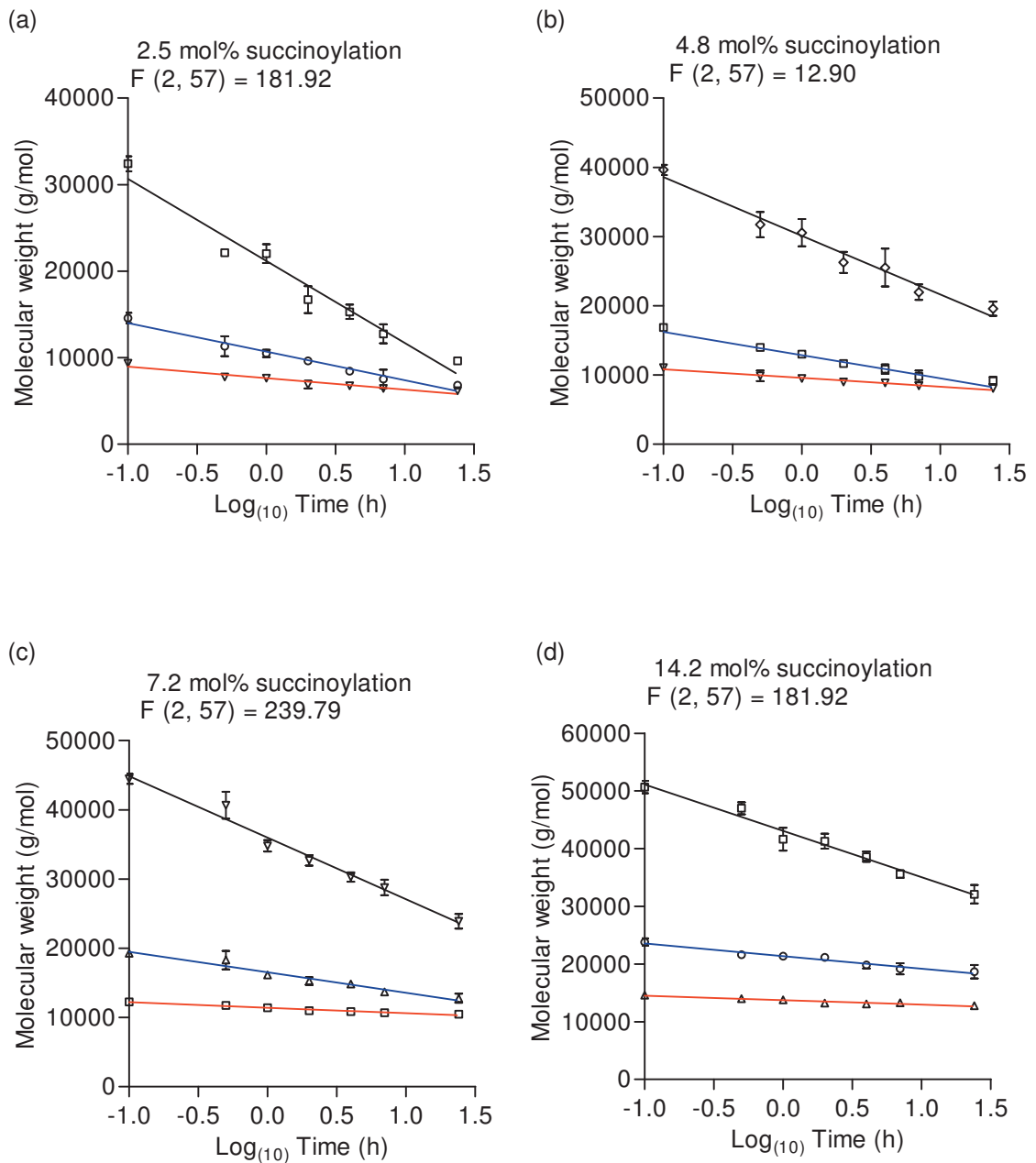
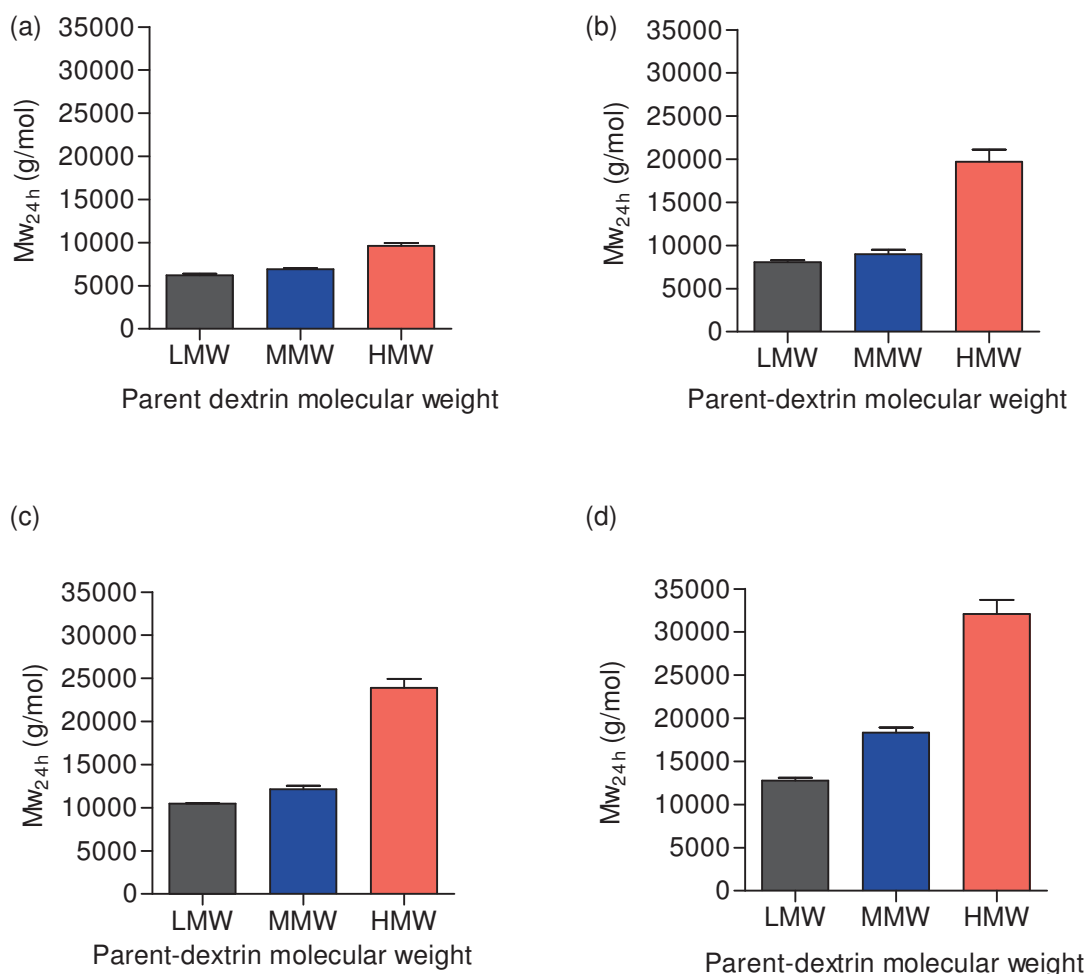


Figure 3.13: Change in degradation rate of dextrin-colistin conjugates with increasing parent-dextrin molecular weight at constant succinylation in the presence of α -amylase for (a) 2.5 mol%; (b) 5.0 mol% (c): 7.5 mol% (d): 14.5 mol% succinylation. ANCOVA statistics for the corresponding figures are reported for each data set. In all cases p was < 0.05 . Data shown represents mean \pm SD ($n=3$). Molecular weight is given relative to pullulan standards. HMW succinylated dextrans are shown in black, MMW succinylated dextrans in blue and LMW succinylated dextrans in red.



		Library comparison	Mean Difference	T statistic
Degree of succinylation (mol %)	2.5 mol%	LMW vs HMW	-3.42	18.76
		MMW vs HMW	-2.72	14.87
	4.8 mol%	LMW vs HMW	-11.65	17.59
		MMW vs HMW	-10.69	16.14
	7.2 mol%	LMW vs HMW	-13.43	25.38
		MMW vs HMW	-11.76	22.22
14.2 mol%	LMW vs HMW	-19.34	23.50	
	MMW vs HMW	-13.76	16.70	

Figure 3.14: One-way ANOVA of Mw_{24h} for each succinylation versus parent-dextrin library, for succinoylated dextrin intermediates, showing that variance in Mw_{24h} was significantly associated to parent-dextrin molecular weight, in all cases ($p < 0.05$). Panel (e) reports the Bonferroni post hoc multiple comparison tests, showing that in all cases, Mw_{24h} for the HMW library was significantly higher. All comparisons were statistically significant ($p < 0.05$). Data shown represents mean \pm SD ($n=3$). Molecular weight by GPC analysis is given relative to pullulan standards.

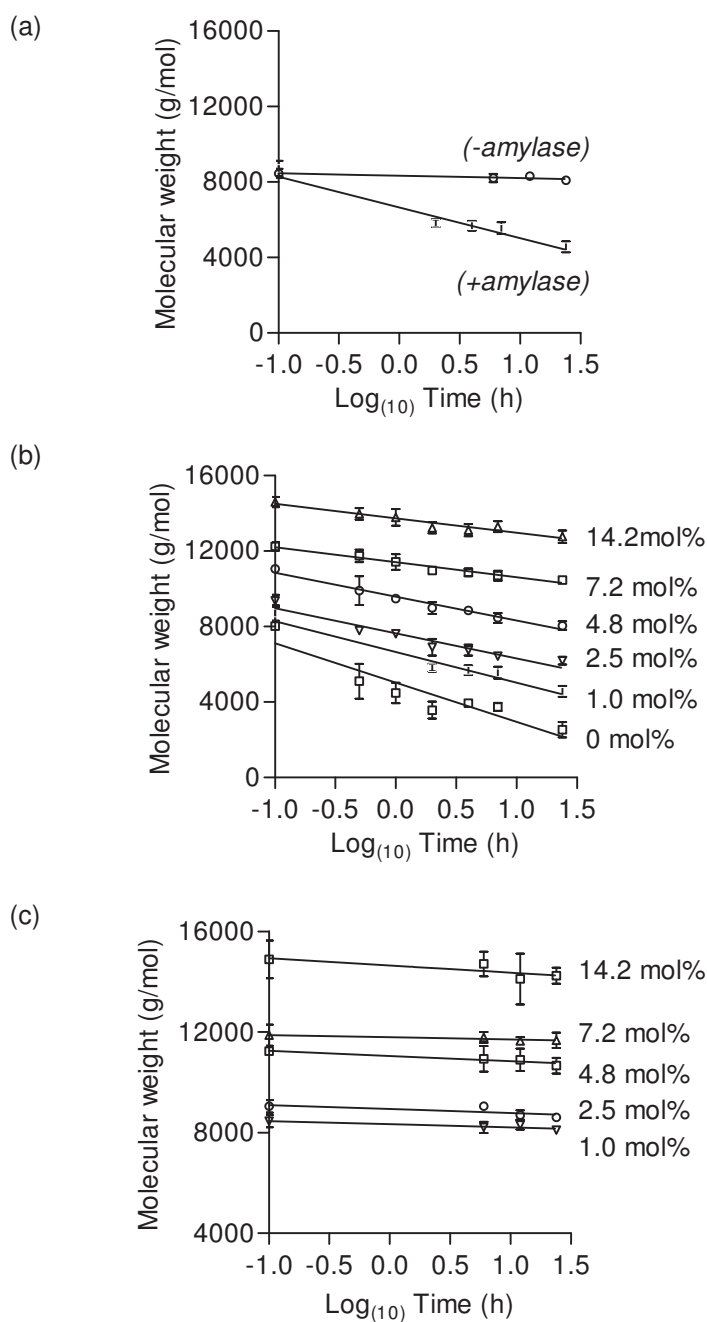
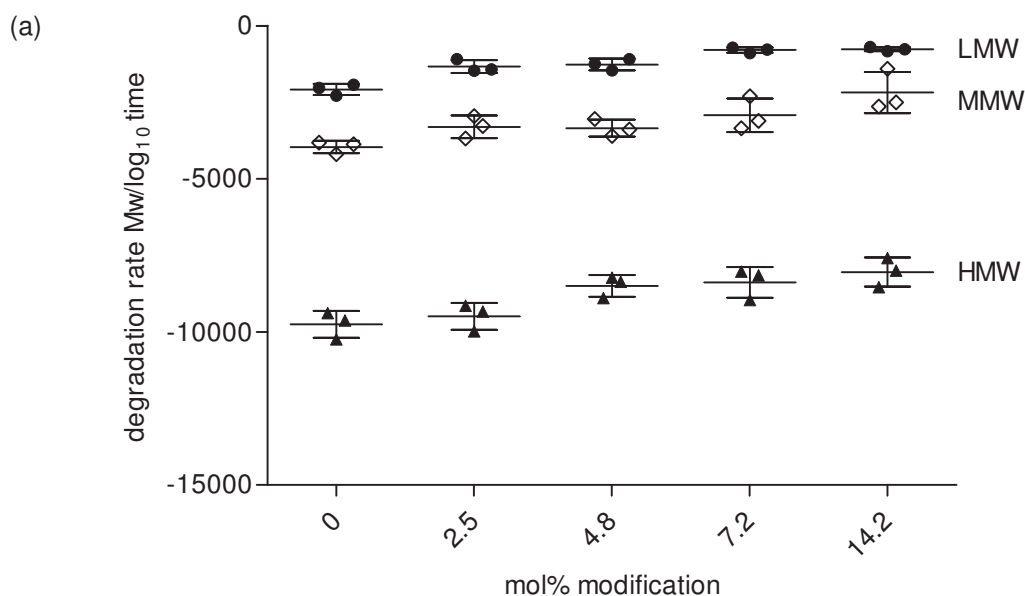


Figure 3.15: Typical analysis of the effect of polymer functionalisation on degradation rate of succinoylated dextrin intermediates. Panel (a) shows the typically increased degradation rate in the presence of amylase for 1 mol% succinoylated LMW dextrin (ANCOVA $F(1, 28) = 116.9$, $p < 0.0001$).

Panel (b) shows that degradation increased significantly with increasing mol% succinoylation (ANCOVA: $F(5, 113) = 14.88$, $p < 0.0001$).

Panel (c) shows that in the absence of amylase degradation rate did not differ significantly with increasing succinoylation (ANCOVA: $F(4, 49) = 0.452$, $p = 0.77$).

Data shown represents mean \pm SD ($n=3$). Molecular weight (by GPC analysis) is given relative to pullulan standards. LMW parent dextrin library: A: 0 mol%; B: 1.0 mol%; C: 2.5 mol%; D: 4.8 mol%; E: 7.2 mol%; F: 14.2 mol% succinoylation.



(b)

	Contribution of parent-dextrin Mw to variance in degradation rate (%)	Contribution of mol% succinylation to variance in degradation rate (%)
LMW versus MMW parent dextrin library	71.36 % F (1, 20) = 236.76, p < 0.05	21.33 % F (4, 20) = 17.69, p < 0.05
MMW versus HMW parent dextrin library	93.9 % F (1, 20) = 1212.6, p < 0.05	4.19 % F (4, 20) = 13.51, p < 0.05

Overall contribution of parent-dextrin molecular weight: $F(2, 30) = 1647.90, p < 0.0001, r^2 = 96.01$

Figure 3.16: Contribution of mol% succinylation and parent-dextrin Mw to variance in degradation rate (two-way ANOVA) for succinoylated dextrin intermediates. Values are reported as mean and standard deviation. Panel (b) reports the contribution (%) of succinylation and parent-dextrin molecular weight to the degradation in molecular weight. Points on the graph represent mean degradation rate for each degree of succinylation \pm SD.

Since the HMW parent-dextrin family and 14.6 mol% succinoylated dextrans reported very slow degradation these intermediates were not tested further.

3.4.3.2 Degradation of dextrin-colistin conjugates

α -Amylase degradation of dextrin-colistin conjugates was similar to succinoylated dextrin intermediates, displaying a pronounced biphasic pattern of degradation (Figures 3.17-3.18) and increasing succinoylation (at constant parent-dextrin Mw) was inversely related to the degradation rate (Appendix 2.2). In the absence of α -amylase, some decrease in molecular weight was observed but this was not statistically significant ($p > 0.05$) (Appendix 2.2).

3.4.3.3 Unmasking of colistin

When unmasking of dextrin-colistin conjugates was analysed using FPLC, a peak corresponding to free colistin appeared (15-17 mL), which increased in intensity during incubation, while the peak corresponding to dextrin-colistin conjugate (7-13 mL) decreased reciprocally (Figure 3.19). This corresponded to an increasing concentration of "unmasked" colistin, mirrored by decreasing concentration of dextrin-colistin conjugate and indicative of release of colistin from conjugate due to dextrin degradation by α -amylase. Unmasking in the presence of α -amylase was greater than controls for both LMW and MMW parent-dextrin families (Figures 3.20 and 3.21 respectively). In these experiments, dextrin-colistin conjugates of LMW dextrin with low degrees of succinoylation released most free colistin after 48 h incubation time. In fact, unmasking at 48 h was most complete in EA-4 conjugate in the presence of α -amylase (70.3 ± 2.3 mol%, mean \pm SD), when compared to the respective controls (57.0 ± 3.8 mol%, mean \pm SD). Figure 3.22 demonstrates that for both LMW and MMW parent-dextrin families, the variance in % unmasking was significantly associated to mol% succinoylation and time (two-way ANOVA, $p < 0.05$). When compared to the respective MMW conjugates, unmasking occurred consistently earlier in the LMW dextrin-colistin conjugates, and was more complete (Bonferroni multiple comparison tests, $p < 0.05$).

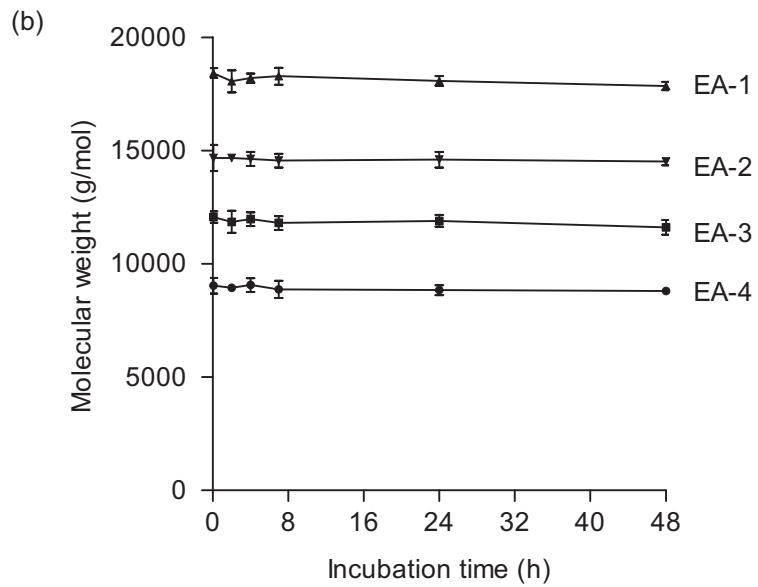
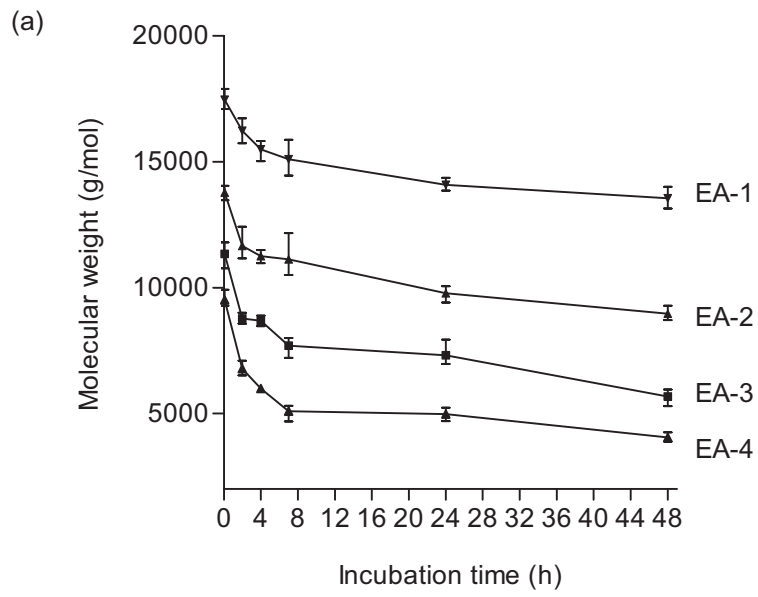


Figure 3.17: Degradation of dextrin-colistin conjugates (LMW parent-dextrin library) measured by GPC during (a) incubation with α -amylase and (b) controls (no amylase). Data shown represents mean \pm SD, (n=3). Molecular weight is given relative to pullulan standards.

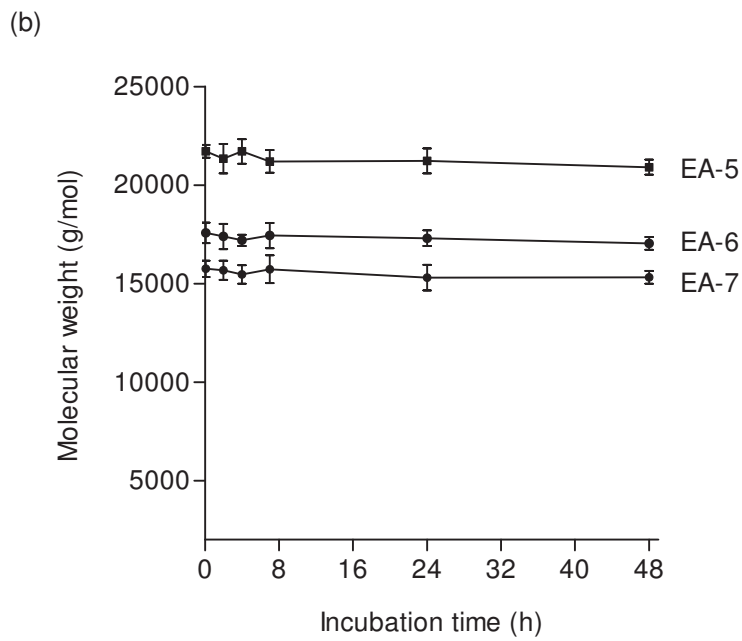
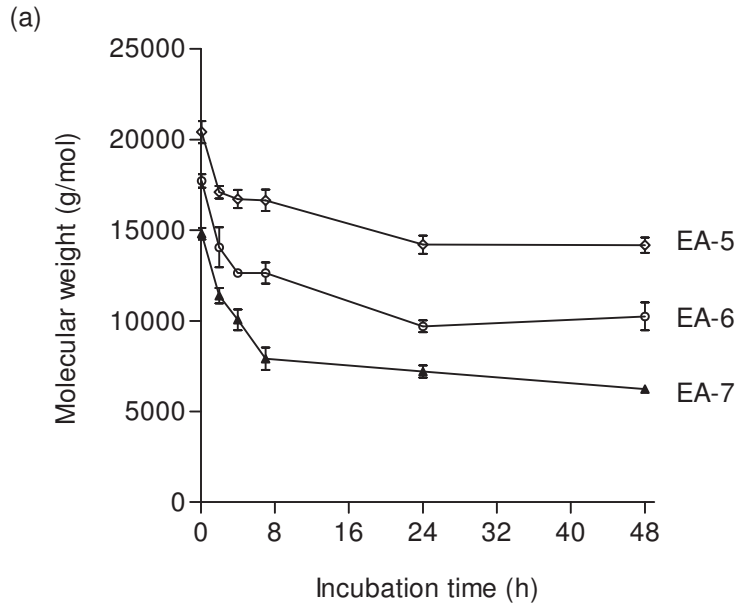


Figure 3.18: Degradation of dextrin-colistin conjugate (MMW parent-dextrin library) measured by GPC during (a) incubation with α -amylase and (b) controls (no amylase). Data shown represents mean \pm SD (n=3). Mw is given relative to pullulan standards.

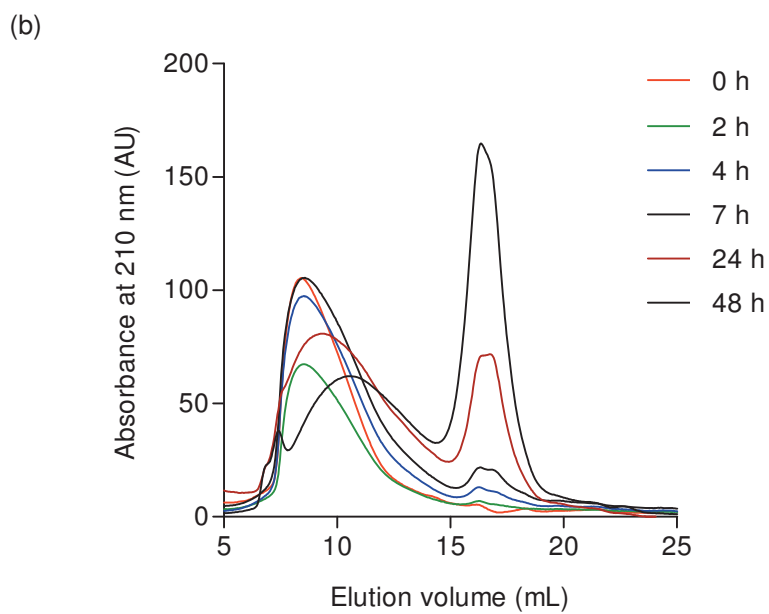
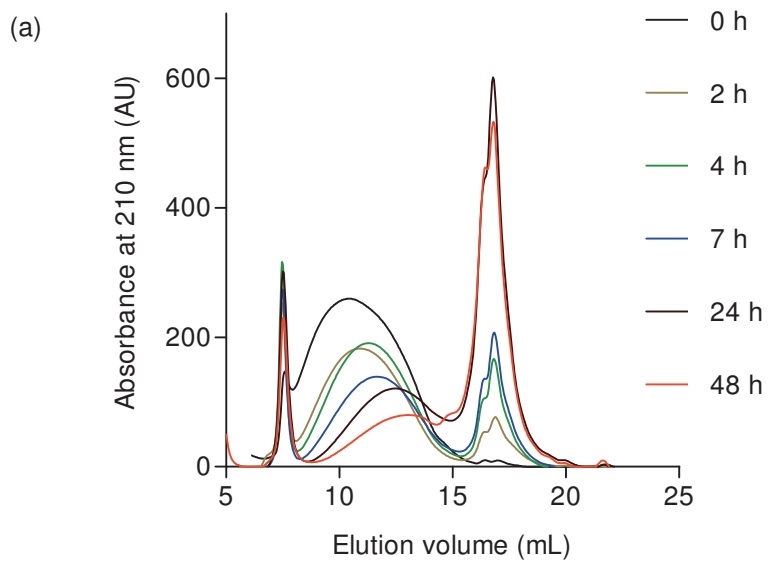


Figure 3.19: Typical change in dextrin-colistin elution by FPLC during unmasking of dextrin-colistin conjugates, by α -amylase for (a) EA-4; (b) EA-1 dextrin-colistin conjugates.

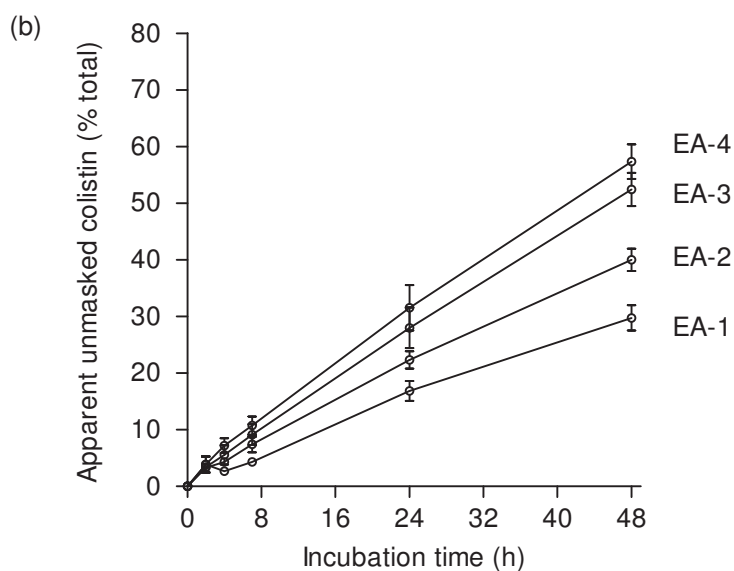
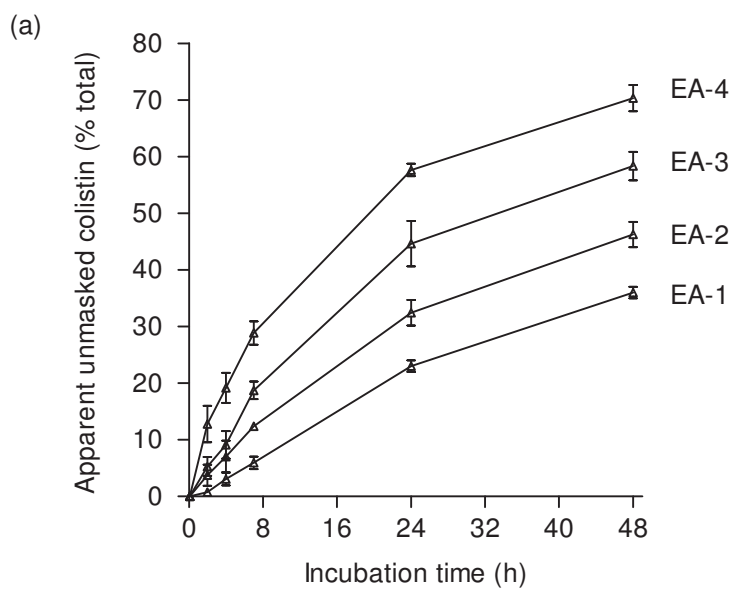


Figure 3.20: Release of colistin from dextrin-colistin conjugates (LMW parent-dextrin library) measured by FPLC, during (a) incubation with human α -amylase and (b) control (no amylase). Data shown represents mean \pm SD (n=3).

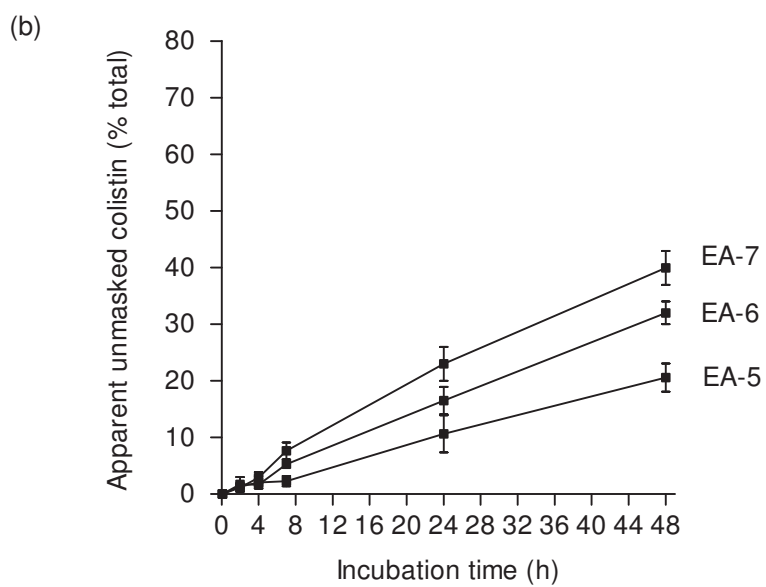
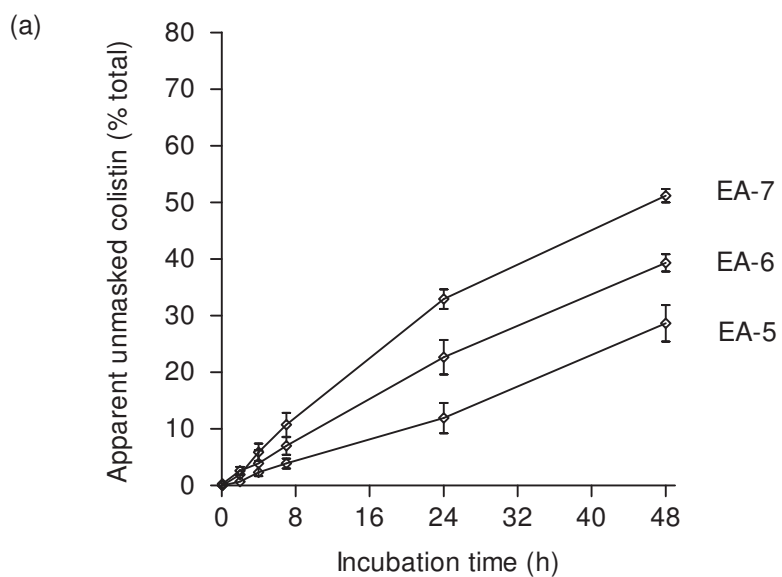


Figure 3.21: Release of colistin from dextrin-colistin conjugates (MMW parent-dextrin library) measured by FPLC, during (a) incubation with α amylase and (b) control (no amylase). Data shown represents mean \pm SD, n=3.

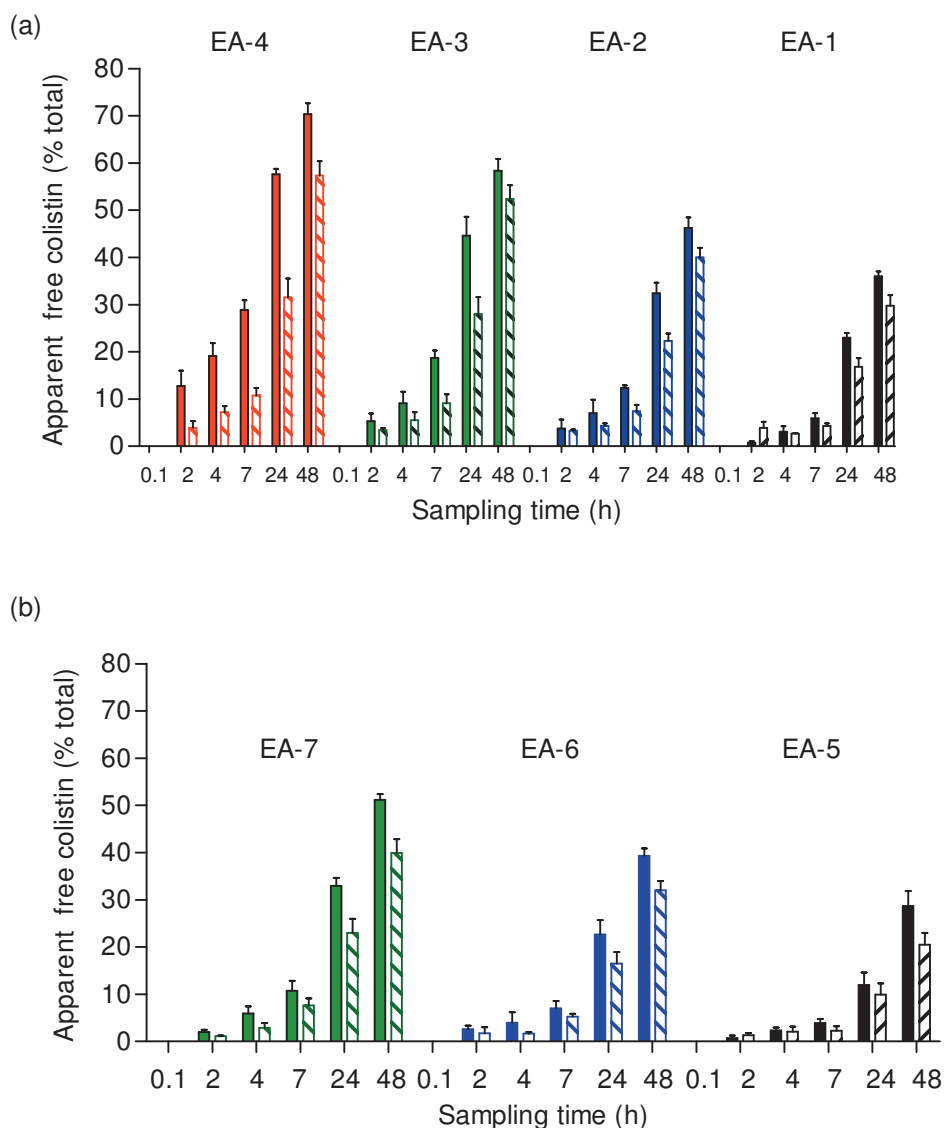


Figure 3.22: Two-way time-series analysis of variance (ANOVA) for apparent unmasked colistin versus degree of succinylation and time: (a) 1.0-7.2 mol% succinoylated conjugates (LMW parent-dextrin library) and (b) 2.6 to 7.5 mol% succinoylated conjugates (MMW parent-dextrin library).

(a) Variance in % apparent colistin unmasking was significantly associated to mol% succinylation ($F(35, 80) = 121.57, p < 0.0001$), time $F(5, 80) = 3117.90, p < 0.0001$) and interaction between the two variables ($F(35, 80) = 36.58, p < 0.0001$); Matching was effective ($F(6, 80) = 2.69, p < 0.0019$).

(b) Variance in % apparent colistin unmasking was significantly associated to: mol% succinylation ($F(25, 60) = 26.60, p < 0.0001$); time ($F(5, 12) = 60.84, p < 0.0001$), and the interaction between these two independent variables ($F(12, 60) = 2.0, p < 0.0396$). Matching was effective ($F(12, 60) = 2.00, p < 0.05$).

Bonferroni replicate means tests are reported in Appendix 2.3. Data shown represents mean \pm SD, $n=3$.

In fact, in the presence of α -amylase, EA-4 conjugate reported that % unmasked conjugate was significantly different from the respective control as early as 2 h post incubation time. Detailed statistical analyses are reported in Appendix 2.3.

3.5 Discussion

This chapter aimed to establish a library of dextrin-colistin conjugates from a range of succinoylated intermediates, optimised towards use in infection, and to characterise the rate and extent of colistin release by a physiologically relevant α -amylase concentration.

3.5.1 Characterisation of the succinoylated dextrin libraries

In concordance to pivotal studies in this field (Hreczuk-Hirst *et al.* 2001b), overall yield of the succinoylation reaction did not vary significantly between parent-dextrin libraries (Section 3.4.1). This suggests that under the reaction conditions employed, reaction yield is reproducible. Moreover, the overall % conversion efficiency, ($49.4\% \pm 2.71$, mean \pm SD) was similar to that reported by Hreczuk-Hirst *et al.* (2001b) and Hardwicke *et al.* (2008a). Since conversion efficiency did not vary significantly between the three different parent-dextrin molecular weights (8,100 - 28,900 g/mol) and range of succinoylations (1 - 14.5 mol %) used, it was possible to compare the behaviour of similar mol% succinoylated dextrans from different parent-dextrans in later experiments.

The literature reports varying conversion efficiencies (50 to 90%) across a range of succinoylations (2-32 mol %) and polymer molecular weights (7,000 to 51,000 g/mol) (Duncan *et al.* 2008; Hreczuk-Hirst *et al.* 2001b). In contrast, using NMR, Bruneel and Schacht (1994) reported consistent conversion efficiency for pullulan succinoylation. Parent-dextrans (α -1,4 poly (D-glucose)) used in this experiment contained less than 5% α -1, 6 links (Ferguson and Duncan 2009). However, earlier studies by Marchal *et al.* (1999) and

White *et al.* (2003) reported that depending on the starch source, manufacture and hydrolysis process, apparently similar dextrans may exhibit varying degrees of bioavailability in solution. All the source dextrans cited in these studies are of food grade quality (Chapter 1) and this may explain the slight difference in conversion efficiency since the dextrans used in these studies were from corn (LMW, HMW dextrin) and potato (MMW dextrin).

GPC was used to infer molecular weight from elution time, based on hydrodynamic radius in solution, using pullulan calibration standards. Determining an exact molecular weight was challenging, because both dextrin and pullulan exhibit a random coil structure in aqueous solution and consist of a polydisperse mix rather than a single molecular species (Alvani *et al.* 2009; Shingel 2004). However, by maintaining identical analysis conditions, it was possible to keep any confounding variables constant throughout the analyses (including analysis of pullulan standards), making it possible to characterise trends in the behaviour of apparent molecular weight after succinylation and after exposure to α -amylase. Increasing dextrin succinylation resulted in an increased molecular weight relative to the parent-dextrin which could not be attributed to the absolute mass alone. This discrepancy may be explained by the incorporation of charged carboxyl groups. It is possible that their resulting negative charges contribute to opening up the random polymer coil conformation, increase hydrodynamic radius in solution, elution time, and therefore apparent molecular weight (Duncan *et al.* 2008; Hardwicke *et al.* 2008a; Treetharmathurot *et al.* 2009). In fact, variance in apparent molecular weight after succinylation was significantly associated to both mol% succinylation and parent-dextrin Mw (Figure 3.7), confirming previous studies' observations (Duncan *et al.* 2008; Hreczuk-Hirst *et al.* 2001a).

Predictability and reproducibility of drug release are critical issues in the design of controlled release medicines (Deshpande *et al.* 1996; Gaspar and Duncan 2009; Lin and Metters 2006; Youan 2010). Succinylation did not alter degradation behaviour (curve shape) which remained best-predictable by logarithmic regression in keeping with "biphasic" degradation patterns

described in earlier studies (Hardwicke *et al.* 2008a; Hreczuk-Hirst *et al.* 2001a). Both independent factors (parent-dextrin Mw and mol% succinoylated) significantly contributed to the variance in degradation rate mediated by α -amylase. Parent-dextrin displayed a dominant contribution to variance in degradation rate. In classical applications of polymer therapeutics requiring prolonged release such as cancer HMW dextrans have been preferred (Duncan 2011). However, application of polymer therapeutics for "acute diseases" demanded exploration of lower Mw dextrans, that provide sufficient masking during transit to the target site, but expeditious unmasking and re-instatement of bioactivity thereafter (Chapter 1).

In this study, parent-dextrin displayed a dominant contribution to variance in degradation rate but the contribution of succinoylation to variance in degradation rate progressively and substantially increased with decreasing initial parent-dextrin Mw. One possible explanation is that the carboxylate groups introduced through succinoylation effectively limit the rate/site of amylase-catalysed hydrolysis. These results suggest that in higher molecular weight polymers, succinoylation may provide a mechanism for "fine-tuning" degradation rates (parent-dextrin Mw is the major determinant of variance in degradation rate), but in smaller dextrans, the degradation rate may be significantly delayed by increasing the degree of polymer functionalisation. These results demonstrated that the HMW parent-dextrin family and > 15 mol% succinoylated dextrans exhibited degradation rates that were deemed not suitable for use in acute infection (Chapter 1). Therefore 1.0 to 7.2 mol% succinoylated LMW and MMW dextrans were selected for conjugation to colistin for further studies (Figure 3.23).

In agreement with Hreczuk-Hirst *et al.* (2001a) limited non-statistically significant degradation of succinoylated dextrin occurred in the absence of α -amylase therefore subsequent degradation rate changes could be attributed to presence of this enzyme (Figure 3.15).

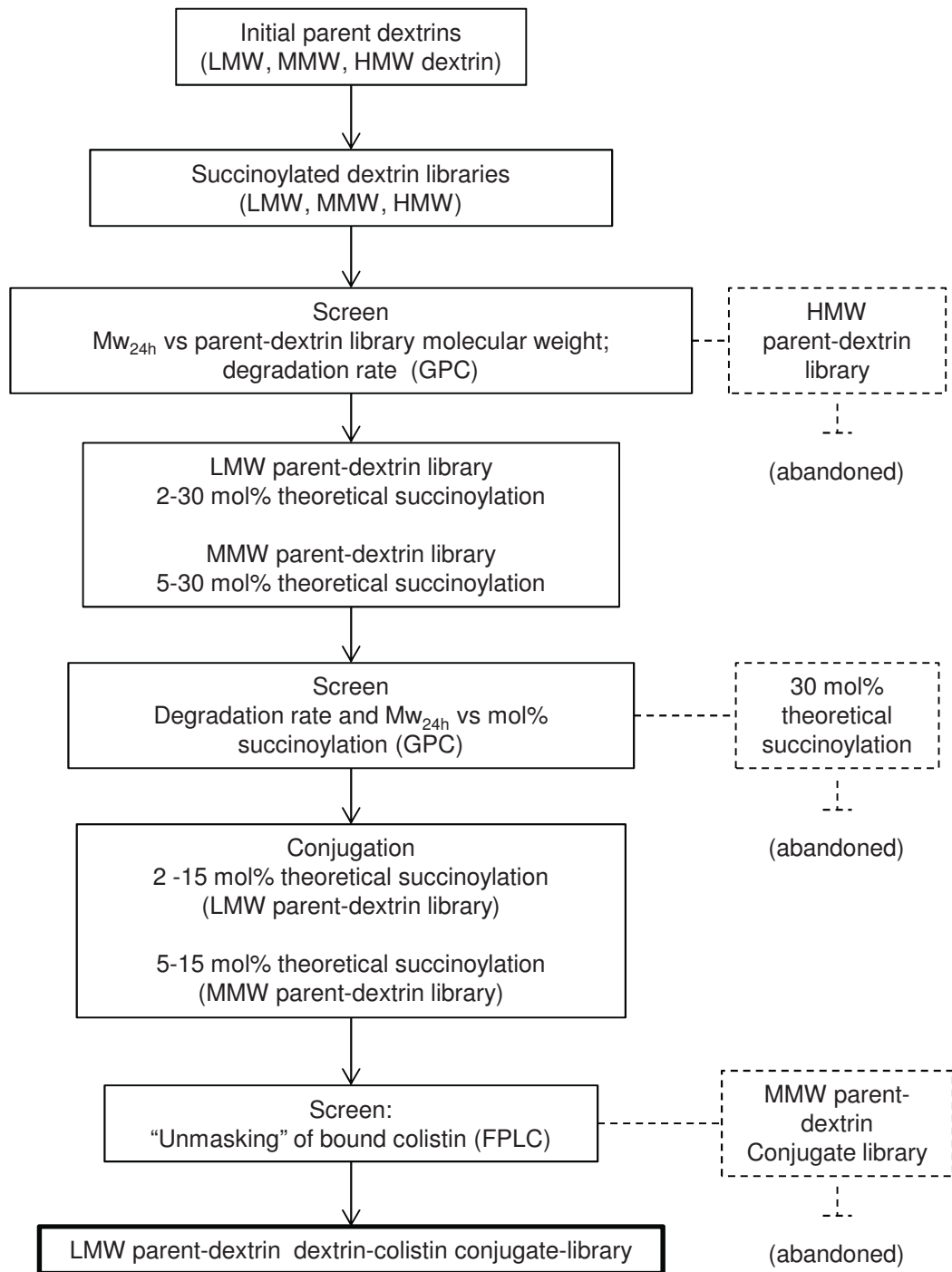


Figure 3.23: Scheme for selection of the optimal dextrin-colistin library for further investigation. Mw_{24h} = molecular weight at 24 h.
 Legend: \longrightarrow = carried forward; $- - - - |$ = abandoned

3.5.2 Characterisation of dextrin-colistin conjugates

Successful, reproducible conjugation of succinoylated dextrans' functional groups to the amino groups on colistin was evident from the resultant conjugate size, protein content and decrease in amino groups per molecule. BCA assay also confirmed reproducible incorporation of colistin in the conjugate ($SD \leq \pm 0.5$), and suggested an increase in colistin incorporation with increasing mol% succinoylation (Table 3.4). Moreover, ninhydrin assay confirmed that after conjugation, there was a consistent reduction in the number of primary amines on the colistin molecule, from which it was possible to determine that approximately 2-3 amino groups were consistently incorporated in peptide bonds, and therefore "masked" by conjugation. Final confirmation of successful conjugation was achieved using GPC where retention time was decreased for dextrin-colistin conjugates compared to free colistin and free succinoylated dextrin. Therefore, using reliable methods that detected both free- and conjugated colistin, conjugation was evident, feasible and reproducible.

Contamination of the final purified product with residual reactants or by-products was below the limit of FPLC detection (Figures 3.7 and 3.8). However, a similar study reported that some residual free dextrin remained detectable by NMR spectroscopy after purification (Duncan *et al.* 2008). Since dextrin is non-immunogenic and clinically well-tolerated, (Section 3.1.1) its presence would not be concerning in these proof of concept studies. FPLC reported that these conjugates were a polydisperse mixture of species, which may be accounted for by the multivalency of both dextrin and bioactive component (Ferguson 2008a). Since increasing mol% succinoylation of dextrin resulted in an increased hydrodynamic radius in solution (Section 3.4.2), it may be postulated that some degree of "steric" masking was possible in accordance to previous studies (Duncan *et al.* 2008; Hardwicke *et al.* 2008b). Variance of % yield efficiency was not significantly different between the two dextrin-colistin conjugate-libraries, suggesting consistent % yield efficiency between parent-dextrin libraries (Figure 3.5b).

GPC was used to estimate dextrin-colistin conjugate molecular weight (Ferguson and Duncan 2009; Hardwicke *et al.* 2008a; Hreczuk-Hirst *et al.* 2001a). Increasing degrees of succinylation resulted in a corresponding increase in apparent conjugate molecular weight. Determining an exact polymer-protein conjugate molecular weight by this method was not straightforward since behaviour of polymer-protein conjugates is not identical to that of either individual component (Duncan *et al.* 2008). Heterogeneity of the product was unavoidable, and attributable to the polydisperse dextrin fraction used for conjugation (Hardwicke *et al.* 2008b). The conjugated protein's charge, molecular weight, and tertiary structure, including the presence of hydrophobic groups within the colistin molecule (Section 3.1.3), may have further distorted the polymer coil, influenced hydrodynamic radius, elution time, (Hreczuk-Hirst *et al.* 2001a) and apparent molecular weight. Consequently, FPLC could not be used to derive molecular weight since the dextrin-colistin conjugates largely eluted in the column's void volume.

3.5.3 Degradation and unmasking of dextrin-colistin conjugates

Dextrin-colistin conjugates exhibited similar behaviour to the respective succinoylated dextrin intermediates in the presence and absence of amylase, in agreement with Hardwicke *et al.* (2008a). The dextrin-colistin conjugate degradation rates determined by GPC were mirrored by the rate of colistin unmasking determined by FPLC, indicating that in response to a physiological α -amylase concentration, bioresponsive unmasking of colistin was feasible according to the PUMPT hypothesis (Chapter 1).

When FPLC was used to study colistin release, it reported a slower, smaller and more linear shift from masked to apparent unmasked conjugate under control conditions. However GPC analysis of conjugate degradation under control conditions reported only limited degradation over time which did not attain statistical significance. These results suggest that limited hydrolytic degradation occurring under control conditions produces smaller dextrin chains attached to colistin which would not result in complete unmasking.

In this study, 70 % of conjugate unmasking was achieved at 48 h incubation by EA-4. Since these dextrin-colistin conjugates were designed for relatively rapid drug release, the optimisation required use of a shorter range of molecular weight parent-dextrins and degree of succinylation. Therefore direct comparisons to other dextrin-conjugates described in the literature in terms of reinstated activity after unmasking are not straightforward. The vast majority of dextrin conjugates described in prior literature were designed for chronic disease processes such as wound healing or cancer, and were therefore optimised for prologed release, using markedly higher parent polymer molecular weights and / or modification (Maeda 2012). However, these findings are in keeping with prior reports investigating succinoylated dextrin conjugates of trypsin, melanocyte stimulating hormone, recombinant human epidermal growth factor and PLA₂, showing that 20-100% reinstatement of activity can be through α -amylase mediated unmasking (Duncan *et al.* 2008; Ferguson *et al.* 2006; Hardwicke *et al.* 2008a; Treetharnmathurot *et al.* 2009).

The seminal studies that originally described this relationship used significantly higher concentrations of rat plasma amylase (Hreczuk-Hirst *et al.* 2001a) and these levels may indeed reflect the higher α -amylase levels that may exist at target sites such as cancer or wounds which may permit preferential unmasking (Hardwicke *et al.* 2010). However, plasma α -amylase concentrations provide a baseline level that the succinoylated dextrans need to contend with, whilst in transit to the target site, and such physiological levels have been reported during rationalised conjugate planning and optimisation studies (Ferguson and Duncan 2009; Hardwicke *et al.* 2008a). The experiments in this chapter were conducted using salivary amylase. Although circulating plasma amylase would likely be of pancreatic in origin,

behaviour of these isoenzymes would be likely to yield similar results (Hardwicke 2009).

3.5.4 Determination of an optimal dextrin-colistin library

LMW conjugates presented optimal physicochemical characteristics for which masking/unmasking would be feasible in infection. Unmasking of LMW conjugates occurred earlier than the respective MMW conjugates (Figure 3.22) and within a time-frame compatible with an EPR effect, which occurs early in infection (Chapter 1). It was also evident that unmasking was significantly more complete and occurred earlier with decreasing succinylation. These considerations provided a robust rationale for selecting the LMW dextrin-colistin conjugate family for *in vitro* antimicrobial susceptibility studies (Figure 3.23). The physicochemical data presented here suggests that EA-4 may provide the quickest and most complete release of colistin that would permit sufficient "masking" of the bioactive payload whilst in transit to the target site, but that would also enable suitably rapid, efficient and maximal "unmasking" in concordance to the PUMPT principle.

3.6 Conclusion

These studies demonstrated that the degradation rate of succinoylated dextrin intermediates depended on mol% succinylation and parent-dextrin molecular weight permitting the refinement of a library of succinoylated dextrin intermediates. Conjugation of dextrin to colistin was a feasible and reproducible reaction that resulted in masking of amino groups through incorporation in peptide bonds. Moreover the LMW conjugate family presented optimal physicochemical and degradation characteristics, for which "masking/unmasking" was a feasible concept with potential for use in infection. These dextrin-colistin conjugates were chosen for further investigation. The bioactivity of the LMW dextrin-colistin conjugate family will now be established through *in vitro* bacteriological assays, both in the presence of α -amylase and in an amylase-free environment.

Chapter Four

***In Vitro* Antimicrobial Activity of Dextrin-colistin Conjugates**

4.1 Introduction

Colistin is a potent antibiotic with a detergent-like action on the bacterial cell membrane. The LMW dextrin-colistin conjugates exhibited optimal characteristics for which 'masking/unmasking' was physicochemically feasible (Chapter 3). Prior to testing the nanoantibiotic *in vivo*, it was deemed important to demonstrate that the conjugate retained antimicrobial activity towards Gram-negative bacteria. Therefore, in these studies, the *in vitro* antimicrobial activity of dextrin-colistin conjugates, as well as colistin sulfate and CMS (as reference controls) were evaluated against a panel of clinically important Gram-negative bacteria. In addition, approximately 59-74% of total colistin is bound to albumin (Mohamed *et al.* 2012). This study also sought to investigate whether dextrin conjugation could reduce colistin's protein binding. Finally, since combination therapy is being increasingly used to combat antibiotic resistance and enhance antimicrobial activity (Fischbach 2011), the activity of dextrin-colistin conjugate in combination with a commonly used antibiotic, ciprofloxacin, was considered.

4.1.1 Gram-negative pathogens investigated in this study

P. aeruginosa, *A. baumannii*, *K. pneumoniae* and *E. coli* (Table 4.1) have emerged as Gram-negative pathogens of global concern, and high annual mortality rates have been reported (Dellit *et al.* 2007; Obritsch *et al.* 2005; Parkins *et al.* 2010; Smolinski *et al.* 2003; Sunenshine *et al.* 2007). These organisms also have a propensity to rapidly acquire multiple drug resistance (Keen *et al.* 2010a; Parkins *et al.* 2010). MDR *A. baumannii* infection, in particular, is associated with significant healthcare costs and a high mortality, whilst its incidence in military casualties has risen to epidemic proportions (Lee *et al.* 2011; Towner 2009; Visca *et al.* 2011). Consequently, colistin is often favoured as the therapeutic option of last resort against these pathogens since resistance to this antibiotic is low (Boucher *et al.* 2009; Gales *et al.* 2011). The global health concern caused by *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *A. baumannii* due to their MDR status, and their continued susceptibility to colistin underscores the

Table 4.1 Characteristics of Gram-negative pathogens investigated in this Chapter, and isolates used in this study

Species	Description	Annual mortality rates	Incidence of MDR isolates	Isolate	Strain
<i>P. aeruginosa</i>	Rod-shaped, non-sporing,	34-38% (Aliaga <i>et al.</i> 2002; Wisplinghoff <i>et al.</i> 2004)	37-46% (Hanberger 1999)	PAO1	Reference Strain
	non-capsulate aerobic,			R22	China
	Gram-negative bacterium (Greenwood <i>et al.</i> 2002).			301 MW513232	Poland UK
<i>A. baumannii</i>	Oxidase negative Gram-negative rod. (Greenwood <i>et al.</i> 2002).	17-68% crude mortality (Cisneros and Rodriguez-Baño 2002; Eliopoulos <i>et al.</i> 2008)	15-fold increase in last decade (Doi <i>et al.</i> 2009; Higgins <i>et al.</i> 2010; Perez <i>et al.</i> 2007)	22222 7789 MW52385 MW523685	Libya UK UK UK
	Gram negative rod-shaped facultative anaerobe (Greenwood <i>et al.</i> 2002).	22 - 46% crude mortality (Kang <i>et al.</i> 2006; Wang <i>et al.</i> 1990; Yinnon <i>et al.</i> 1996).	13 -30% (Ashour and El-Sharif 2009; Hawkey and Jones 2009)	5725	UK
				05506	India
<i>E. coli</i>	Lactose fermenting, facultatively anaerobic non-motile Gram-negative rod (Ryan and Ray 2004).	22.4% (Wisplinghoff <i>et al.</i> 2004)	8-25% (Ashour and El-Sharif 2009; Hawkey and Jones 2009)	K3 NCTC 10418 7273 AIM-1 MW513311	India Reference Strain UK UK UK

rationale for including these organisms in the assessment of the dextrin-colistin conjugates' antimicrobial activity.

4.1.2 The mechanism of action of colistin

Colistin's broad Gram-negative spectrum of activity has been attributed to membrane disruption (Rustici *et al.* 1993). In Gram-negative bacteria, lipid-A anchors the LPS component of the outer membrane, whose key role is to control permeability (Velkov *et al.* 2009) (Figure 4.1). Colistin's positively charged amino groups are thought to interact with negatively charged Lipid-A to displace divalent ions, destabilising the bacterial membrane (Clauzell *et al.* 2007; Melo *et al.* 2009; Powers and Hancock 2003). This is aided by fatty acyl chain and hydrophobic amino acid domains weakening the packing of bacterial fatty acyl lipid-A chains, leading to outer membrane expansion (Velkov *et al.* 2009). Colistin straddles the hydrophobic tails and hydrophilic heads of the phospholipid inner-membrane bilayer further disrupting its integrity (Clauzell *et al.* 2007; Hancock 1997; Hancock and Lehrer 1998).

Colistin's structural integrity is essential for antimicrobial function (Chapter 3). In these studies, successful conjugation of colistin to succinoylated dextrin was achieved through incorporation of at least one amino group into an amide bond. However, previous studies investigating the effect of conjugation on enzyme and growth factor activity demonstrated different extents to which payload function was reinstated after unmasking, varying from 20-100% (Duncan *et al.* 2008; Ferguson *et al.* 2006; Treetharnmathurot *et al.* 2009). It was uncertain how polymer conjugation would affect colistin's antimicrobial activity following its amylase-triggered release. Therefore, it was necessary to determine the unmasked conjugates' antimicrobial activity and compare it to colistin sulfate and CMS, in order to assess the feasibility of reinstating dextrin-colistin conjugates' antimicrobial activity by exposure to α -amylase within the context of the PUMPT principle (Chapter 1).

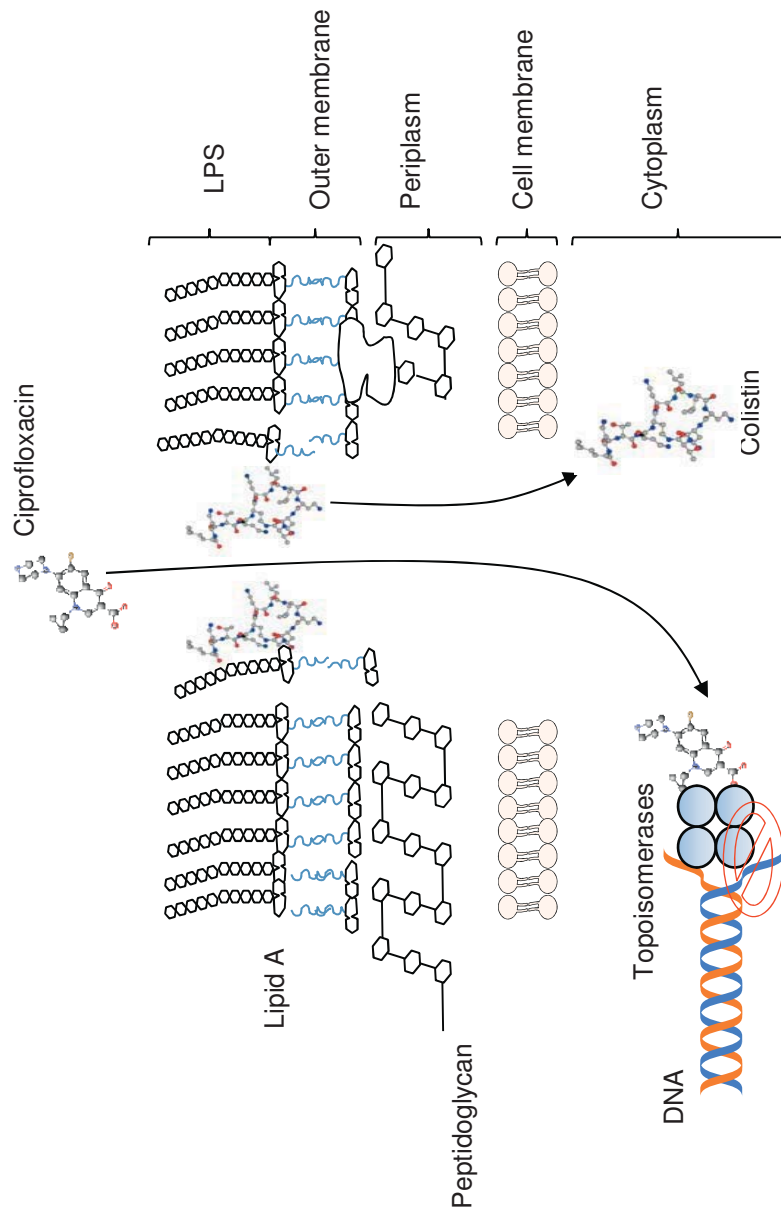


Figure 4.1: Postulated mechanism of action of colistin by detergent action and self-promoted uptake; ciprofloxacin requires entry into the bacterial cytoplasm to inactivate DNA topoisomerases. LP, lipoprotein; LPS, lipopolysaccharide

4.1.3 Assessment of antimicrobial susceptibility

Antimicrobial susceptibility may be assayed using a variety of different methods (Table 4.2). However, colistin diffuses poorly in agar (Galani *et al.* 2008), and the reliability of diffusion methods for colistin susceptibility testing and their ability to detect low-level resistance to colistin is contended (Andrews and Howe 2011; Lo-Ten-Foe *et al.* 2007; van der Heijden *et al.* 2007). The MIC assay is widely accepted as a 'gold standard' for investigating the susceptibility of organisms to antimicrobials (Andrews 2001; 2006) It has been specifically recommended for determining colistin susceptibility (Andrews and Howe 2011; European Committee on Antimicrobial Susceptibility Testing 2011; Gales *et al.* 2001), and it allows simultaneous testing of several concentrations. The serial broth microdilution technique presents an established, convenient and reproducible application for MIC testing (Andrews 2006; Barry *et al.* 1978). Conjugation is known to reduce biofouling and binding to albumin (Zhang *et al.* 2007). Since colistin binds readily to serum albumin, limiting its antibiotic activity (Craig and Welling 1977), the MIC assay also provided a suitable method to investigate how dextrin conjugation may reduce colistin's binding to serum proteins. It was hypothesised that the antimicrobial activity of conjugates would be less affected by the addition of serum than the free drug.

4.1.4 Combination antimicrobial therapy

The antimicrobial activity of dextrin-colistin conjugates in combination with a conventional antibiotic was also studied in these systems. Preserving colistin's antimicrobial efficacy is important due to its status as a "last line antibiotic" (Nation and Li 2009). Worldwide, the incidence of resistance to colistin remains low, but several resistance mechanisms have been identified (Gales *et al.* 2011). Combination antimicrobial therapy, such as amoxicillin and clavulanic acid has resulted in wide clinical success (Silver and Bostian 1993). The use of colistin in combination therapy has been extensively reported (Michalopoulos and Karatza 2010; Petrosillo *et al.* 2008; White *et al.* 2010). Moreover, synergism between polymyxin derivatives and agents normally excluded by the Gram-negative outer membrane has been

Table 4.2: Comparison of antimicrobial susceptibility testing methods for single and combination therapies

Method	Principle	Advantages	Disadvantages
VITEK-2	Repetitive turbidimetric monitoring of bacterial growth	Rapid, automated	Low sensitivity in the detection of heteroresistant subpopulations (Lo-Ten-Foe <i>et al.</i> 2007).
E-test	Agar diffusion method for the quantitative determination of susceptibility.	Simple, rapid, scalar reading	Poor concordance to MIC testing in organisms with higher MICs (Arroyo <i>et al.</i> 2005). Relies on agar diffusion (Galani <i>et al.</i> 2008).
Serial broth microdilution	Serial broth dilution of antibiotic, assayed for the minimal concentration producing visible inhibition of microbial growth.	Does not rely on diffusion of colistin through agar medium, allows simultaneous testing of several antibiotic concentrations.	Susceptibility values only available in categorical (\log_2 -fold) intervals. Labour-intensive. Measures inhibitory effect not bacterial killing (Andrews 2006).
Checkerboard MIC	Two-dimensional array of serial concentrations of test compounds is used to investigate their interaction (Odds 2003).	Direct comparability to single antibiotic determination by serial broth microdilution MIC .	Provides only categorical values in \log_2 -fold dilutions. Measures inhibition not killing.
Time-to kill	Bacterial cell viability in response to an antibiotic dose.	Direct observation of bacterial killing in response to an antibiotic dose	Poor agreement to MIC determinations (Cappelletty and Rybak 1996). Labour-intensive.
Epsilometer	Antibiotic-impregnated agar strips serially positioned onto prior antibiotic gradient on the test agar plate. Effect determined from the reading where the inhibition zone intersects the E strip scale.	Less labour intensive (Bonapace <i>et al.</i> 2000).	Poor concordance of the Epsilometer test method to the MIC method was reported when testing antibiotic combinations of colistin (Pankey and Ashcraft 2005), especially in organisms with higher MICs (Arroyo <i>et al.</i> 2005). Antagonism may be more difficult to detect (Bonapace <i>et al.</i> 2000).

reported (Vaara *et al.* 2010). Ciprofloxacin is a second-generation fluoroquinolone, which is widely used against Gram-negative pathogens (Akter *et al.* 2012; Patel *et al.* 2009; Tong *et al.* 2011). At lower concentrations, ciprofloxacin complexes bacterial DNA inhibiting bacterial growth, but at higher concentrations ciprofloxacin also exerts a bactericidal effect attributed to DNA cleavage (Hawkey 2003; Figure 4.1).

Since colistin exerts a detergent effect on the bacterial membrane (Section 4.1.2), and since ciprofloxacin needs ingress through the cell wall to exert its action, there is potential for colistin to facilitate ciprofloxacin's entry to the bacteria. Despite this seemingly cooperative mechanism, studies have reported that the ciprofloxacin + colistin combination was additive, but not synergistic (Petrosillo *et al.* 2008). In contrast to colistin sulfate, dextrin-colistin conjugates offer the promise of both sustained release (through unmasking) and passive targeting (through the EPR effect), which could enhance the entry of ciprofloxacin into the bacteria. Moreover, long-term clinical randomised control studies have shown that colistin + ciprofloxacin combination therapy successfully prevented emergence of resistance, in contrast to other antibiotic/colistin combinations (Prentice *et al.* 2001). Investigating the combination of ciprofloxacin with colistin, CMS or dextrin-colistin conjugate was therefore relevant to this study.

Several methods have been described to investigate antimicrobial combinations including: i) time-kill curves, ii) Epsilometer test and iii) MIC checkerboard technique. The checkerboard technique, first described by Elion *et al.* (1954), is commonly used to screen potential antibiotic combinations. It enables direct comparison of combination antibiotic activity to individual antibiotic susceptibilities determined by the MIC assay, minimising the risk of methodological bias (Foweraker *et al.* 2009; Pankey and Ashcraft 2011). Checkerboard assays allow testing of a range of concentration combinations, which is appropriate for simultaneously studying several concentrations to reflect the distribution of the antibiotic in different tissues (Matson *et al.* 2009; Varela *et al.* 2000). The fractional inhibitory concentration index (FICI) (Eliopoulos and Moellering 1996; Hall *et al.* 1983)

is the commonest method used to report the results of checkerboard tests (Lorian 2005; Odds 2003). Since variation in a single result places an MIC in a 3-dilution range (mode $\pm 1 \log_2$ dilution) (Odds 2003) the conservative approach preferred by Odds to interpreting the FICI was adopted in these experiments. Therefore, synergy was defined as an FICI of ≤ 0.5 ; 'no interaction' was defined as an FICI of 0.5 to 4; and antagonism was defined as an FICI of ≥ 4 .

4.2 Aims

In summary, the aims of this study were to:

1. Determine the *in vitro* antimicrobial activity of dextrin-colistin conjugates, compared to colistin sulfate and CMS, towards a panel of Gram-negative organisms.
2. Assess the feasibility of reinstating/enhancing dextrin-colistin conjugates' antimicrobial activity by incubation with α - amylase.
3. Identify a lead compound for further investigation.
4. Investigate the antimicrobial activity of dextrin-colistin lead candidate, colistin sulphate and CMS in the presence of foetal calf serum.
5. Compare the antimicrobial activity of ciprofloxacin in the presence of EA-4 (24 h-unmasked in α -amylase), colistin sulfate and CMS as possible combination therapy.

4.3 Methods

General methods, including preparation of agar, broths, and overnight cultures were reported in Section 2.5.3. Dextrin-colistin conjugate was prepared as detailed in sections 2.5.1 and 3.3.2, (Table 3.4).

4.3.1 Bacterial strains

The Gram-negative bacterial strains used in this chapter and their origins are reported in Table 4.1. Bacterial identification codes represent internal

laboratory reference numbers. *P. aeruginosa* PAO1 (ATCC 15692), *K. pneumoniae* 5725, *E. coli* 7273 and *E. coli* NCTC 10418 constituted an initial screening panel (test panel A).

4.3.2 Dextrin-colistin conjugate unmasking by incubation with α -amylase

To study the effect of α -amylase on conjugate antimicrobial activity, dextrin-colistin conjugates (3 mg/mL colistin equivalent) were first incubated with α -amylase (100 IU/L) for up to 24 h at 37 °C in PBS buffer (pH 7.4). Equal volumes (1.5 mL) were collected at defined intervals (0, 3, 6, 24 h), snap-frozen in dry ice to stop the reaction and then stored at -20 °C until analysed by MIC.

4.3.3 Minimum inhibitory concentration (MIC) assays

Bacterial susceptibility to dextrin-colistin conjugates, colistin sulfate and CMS controls was determined using the MIC serial broth microdilution assay as detailed in Section 2.5.3.

To study the effect of increasing degrees of succinoylation and time in the presence of α -amylase, a dextrin-colistin conjugate (for example EA-1) was pre-incubated in α -amylase, as detailed in Section 4.3.2. For each time point, a solution of test antibiotic (1,024 μ g/mL, 200 μ L) was produced in CAMHB in triplicate within wells of the first column of a 96-well plate, then serially double diluted in CAMHB. MIC against *P. aeruginosa* PAO1 was determined by serial broth microdilution (Section 2.5.3). The procedure was repeated for all candidate dextrin-colistin conjugates (EA-1, EA-2, EA-3, EA-4) against *K. pneumoniae* 5725, *E. coli* 7273, and *E. coli* 10418.

To study the antimicrobial activity of the lead candidate (EA-4) against an extended range of Gram-negative strains, EA-4 was incubated in α -amylase (Section 4.3.2) and in the absence of amylase. For each time-point, antimicrobial susceptibility of all Gram-negative bacterial strains (Table 4.1) was determined using an MIC assay (Section 2.5.3).

To investigate the antimicrobial activity of the 24-hour-unmasked EA-4, colistin sulphate and CMS in the presence of serum proteins, MIC determination was performed against *K. pneumoniae* 5725, *E.coli* 7273 and *E.coli* NCTC 10418 as detailed in section 2.5.3.6, but using CAMHB enriched with 10% v/v foetal calf serum.

4.3.4 Checkerboard experiments

The susceptibility of *A. baumannii* 7789, and *A. baumannii* 22222, *E.coli* 5702, and *E. coli* 10418 to the combination of ciprofloxacin with either EA-4 (after 24 h incubation in α -amylase), colistin or CMS was determined through a series of 2-directional checkerboard experiments. Within the checkerboard panel, the MIC of each antibiotic was in the middle of the range of concentrations tested with three double dilutions on either side (Cappelletty and Rybak 1996; Champney 2008). A typical two-directional checkerboard experiment for ciprofloxacin + EA-4 (after 24 h incubation in α -amylase) versus *E. coli* NCTC 10418 is described below. Figure 4.2 represents a typical checkerboard layout including quality controls.

The MIC for each antibiotic against *E. coli* 10418 strain was determined (Section 2.5.3.6). Ciprofloxacin was diluted in CAMHB to achieve a stock solution (2 mL) at a concentration 8 times greater than the MIC of ciprofloxacin against the test organism. Serial two-fold dilutions were then produced in sterile universal containers in CAMHB. Equal volumes (100 μ L) of the highest antibiotic concentration were pipetted into wells (row 1 of a 96-well plate). The procedure was repeated in subsequent rows, resulting in serial two-fold dilutions of ciprofloxacin down the plate's rows. Next, EA-4 (24 h-incubated in α -amylase as detailed in Section 4.3.2) was diluted in PBS in a universal container (2 mL) to produce a concentration 16-fold that of the previously determined MIC). A 100 μ L volume was then pipetted into each well of the first column in the 96-well plate. Two-fold dilution of EA-4 in each column was achieved by serial transfer of 100 μ L. The plate was then inoculated with *E. coli* 10418 strain (Section 2.5.3.6) prepared as detailed in Section 2.5.3. The plate was wrapped in parafilm and incubated for 18-20 h at 35 ° C in ambient air. The lowest concentration at which there was no

→ Colistin serial double dilutions

Ciprofloxacin serial double dilutions ↓	0.1248 16	8	4	2	1	0.5	0.25	Colistin control ↑
	0.0624							
	0.0312							
	0.0156							
	0.0078							
	0.0039							
	0.00195							GC
	← Ciprofloxacin control →						SC	

MIC of ciprofloxacin to *E.coli* NCTC 10418 = 0.0156 µg/mL
 MIC of 24 h- unmasked EA-4 to *E.coli* 10418 = 2 µg/mL

Figure 4.2: A typical two-dimensional checkerboard assay layout for colistin + ciprofloxacin versus *E.coli* NCTC 10418. MICs to the individual antibiotics were also quantified on each plate as quality controls. MIC values within the checkerboard are reported in µg/mL. GC: growth control; SC: sterility control.

visible growth was taken as the MIC of the antibiotic combination at that particular concentration. The procedure was repeated for *A. baumannii* 22222 *E. coli* 5702 and *E. coli* NCTC 10418. For each antibiotic, the Fractional Inhibitory Concentration (FIC) of each first non-turbid well along the turbid/non-turbid interface was calculated (MIC of each drug in combination divided by the MIC of the individual drug). The mean FIC index of all non-turbid wells along the turbidity/non-turbidity interface was then calculated (Bonapace *et al.* 2000). The average of the mean FIC indices from triplicate microtiter trays was used to categorise results as synergy / antagonism / no interaction, according to Odds (2003). Quality control procedures are described in section 2.5.3.6.1.

4.4 Results

4.4.1 Antimicrobial activity, effect of bacterial strain and succinylation

P. aeruginosa demonstrated several differences in the susceptibility behaviour to the dextrin-colistin conjugates (Tables 4.3 - 4.4). Compared to *A. baumannii*, *K. pneumoniae* and *E. coli* all conjugates exhibited substantially higher MICs for *P. aeruginosa*. The lowest drop in MIC with incubation time occurred with *P. aeruginosa*. The lowest MIC drop between conjugates lying at the extremes of the succinylation range (EA-1 and EA-4) was also seen with *P. aeruginosa* at 24 h pre-incubation time.

At 0 h, all dextrin-colistin conjugates (with the exception of EA-1, the conjugate with the highest degree of succinylation) had limited antimicrobial activity against "test-panel A" organisms. Antimicrobial susceptibility at 0 h also increased with decreasing succinylation. Bacterial susceptibility generally increased with pre-incubation in α -amylase over 24 h (Table 4.5). The greatest increase in antimicrobial susceptibility with pre-incubation time was observed in minimally modified conjugate (EA-4). Bacterial susceptibility following 24 h incubation with α -amylase steadily increased with decreasing succinylation: again, EA-4 reported the lowest MIC after 24 h unmasking in α -amylase. EA-4 showed the most marked antimicrobial activity in the preliminary screening, as it exhibited higher initial antimicrobial activity and

marked potentiation of this activity was evident following amylase exposure. EA-4 was designated 'lead conjugate' and selected for further investigation.

4.4.2 Antimicrobial activity of the lead conjugate

Similar MICs for colistin and CMS were evident against the extended Gram-negative screening panel (test panel B, Table 4.4) to those in test panel A (Table 4.3). The exceptions were two MDR strains (*P. aeruginosa* MDR 301 and *A. baumannii* MDR 22222, for which higher MICs (2 µg/mL) to colistin were apparent. EA-4 exhibited a time-dependant increase in antimicrobial susceptibility after pre-incubation with α-amylase. The MIC of EA-4 after pre-incubation with α-amylase was generally within 1 two-fold dilution of the MIC for CMS against *A. baumannii*, *K. pneumoniae* and *E. coli*. In keeping with the initial test-panel screen, only one *P. aeruginosa* strain (513232) showed time-potentiation in response to pre-incubation in α-amylase. Limited intraspecies variation was also noted: whilst susceptibility of one *K. pneumoniae* strain (05506) was higher than CMS, *E. coli* AIM-1 had a susceptibility equivalent to colistin (0.125 µg/mL) and two orders of magnitude lower than CMS. In the absence of α-amylase, after being incubated in PBS alone, there was no significant change in MIC over time. Moreover, when MIC assays were performed after pre-incubation in α-amylase, antimicrobial activity increased as the duration of pre-incubation with α-amylase increased.

In the presence of 10% v/v FCS, colistin sulfate exhibited a substantial increase in MIC (range: 3-5 log₂-fold increase, Table 4.4). In contrast, the change in antimicrobial susceptibility was lower for both CMS and 24 h-unmasked EA-4 (range: 1-2 log₂-fold increase Table 4.5).

4.4.3 Checkerboard assays with ciprofloxacin

Combination testing of ciprofloxacin with either colistin, CMS or EA-4 after 24 h preincubation in α- amylase resulted in average FICI values that ranged from 0.59 to 2.9, which were within the 'no-interaction' category (Table 4.6). The ciprofloxacin / EA-4 (24 h pre-incubation time) combination reported

Table 4.3: MIC determination of the LMW conjugate library compared to colistin and CMS against an initial bacterial screening panel after pre-incubation with amylase: test panel "A"

Test antibiotic	Pre-incubation time (h)	<i>P. aeruginosa</i> * PAO1	<i>K. pneumoniae</i> 5725 *	<i>E. coli</i> 7273 *	<i>E. coli</i> NCTC 10418 *
Colistin CMS	0	0.25	0.5	0.25	0.5
	0	8	8	8	2
EA-1 (7.2 mol% succinoylation)	0	1024	256	256	128
	3	512 (1)	256 (0)	256 (0)	128 (0)
	6	256 (2)	128 (1)	128 (1)	64 (1)
	24	256 (2)	64 (2)	128 (1)	32 (2)
EA-2 (4.7 mol% succinoylation)	0	512	128	128	16
	3	256 (1)	64 (1)	128 (0)	8 (1)
	6	256 (1)	64 (1)	128 (0)	8 (1)
	24	128 (2)	32 (2)	64 (1)	8 (1)
EA-3 (2.4 mol% succinoylation)	0	256	32	64	16
	3	128 (1)	32 (0)	32 (1)	16 (0)
	6	128 (1)	16 (1)	32 (1)	8 (1)
	24	64 (2)	16 (1)	32 (1)	4 (2)
EA-4 (1.0 mol% succinoylation)	0	128	32	64	8
	3	64 (1)	16 (1)	32 (1)	4 (1)
	6	64 (1)	8 (2)	16 (2)	4 (1)
	24	32 (2)	4 (3)	4 (4)	2 (2)

* All MIC data represents mode MIC ($\mu\text{g}/\text{mL}$, n=3).
Values in parentheses indicate \log_2 -fold decrease in MIC as a measure of time-potential compared to 0 h control.

Table 4.4: MIC determination of colistin, CMS and EA-4 after 24 h pre-incubation in the presence and absence of α -amylase against an extended bacterial panel: test panel "B"

Strain	Pre-incubation time (h)	Colistin*	CMS*	EA-4 + α -amylase *				EA-4, No amylase *			
				0	6	3	6	24	6	3	6
<i>P. aeruginosa</i> R 22	0.5	0	8	128	64 (1)	128 (0)	64 (1)	64 (1)	128 (0)	64 (1)	128 (0)
<i>P. aeruginosa</i> MDR 301	2	0	8	128	64 (1)	128 (0)	64 (1)	64 (1)	128 (0)	64 (1)	128 (0)
<i>P. aeruginosa</i> PAO1	0.25	0	8	128	64 (1)	64 (1)	64 (1)	64 (1)	64 (0)	64 (1)	64 (0)
<i>P. aeruginosa</i> 513232	0.25	0	2	256	64 (2)	128 (1)	32 (3)	32 (3)	256 (0)	32 (3)	256 (0)
<i>A. baumannii</i> 22222	2	0	8	64	16 (2)	32 (1)	8 (3)	8 (3)	32 (1)	8 (3)	32 (1)
<i>A. baumannii</i> 7789	0.0625	0	8	32	8 (2)	16 (1)	8 (2)	8 (2)	32 (0)	8 (2)	32 (0)
<i>A. baumannii</i> 52385	0.0625	0	4	8	2 (2)	4 (1)	2 (2)	2 (2)	8 (0)	2 (2)	8 (0)
<i>A. baumannii</i> 523685	0.0625	0	8	16	8 (1)	16 (0)	4 (2)	4 (2)	16 (0)	4 (2)	16 (0)
<i>E. coli</i> AIM-1	0.125	0	0.5	2	0.5 (2)	1 (1)	0.125 (4)	0.125 (4)	1 (1)	0.125 (4)	1 (1)
<i>E. coli</i> 5702	0.5	0	8	32	8 (2)	16 (1)	8 (2)	8 (2)	32 (0)	8 (2)	32 (0)
<i>E. coli</i> 7273	0.25	0	8	32	16 (1)	32 (0)	4 (3)	4 (3)	32 (0)	4 (3)	32 (0)
<i>E. coli</i> NCTC 10418	0.5	0	2	8	4 (1)	4 (1)	2 (2)	2 (2)	8 (0)	2 (2)	8 (0)
<i>E. coli</i> 513311	0.125	0	2	16	8 (1)	16 (0)	4 (2)	4 (2)	16 (0)	4 (2)	16 (0)
<i>K. pneumoniae</i> 05506	0.5	0	4	64	32 (1)	32 (1)	32 (1)	32 (1)	32 (1)	32 (1)	32 (1)
<i>K. pneumoniae</i> K3	0.5	0	4	32	16 (1)	32 (0)	8 (1)	8 (1)	32 (0)	8 (1)	32 (0)
<i>K. pneumoniae</i> 5725	0.5	0	8	32	8 (2)	16 (1)	4 (3)	4 (3)	32 (0)	4 (3)	32 (0)

* All MIC data represents mode MIC ($\mu\text{g/mL}$, n=3). Values in parentheses indicate \log_2 -fold decrease in MIC as a measure of time-potential compared to 0 h control.

Table 4.5: MIC determination of colistin, CMS and the lead conjugate after pre-incubation in the presence of 10% fetal calf serum

	Colistin	CMS	EA-4 (24 h-unmasked)
<i>K. pneumoniae</i> 5725	4 (3)	16 (1)	8 (1)
<i>E. coli</i> 7273	8 (5)	16 (1)	16 (2)
<i>E. coli</i> NCTC 10418	4 (3)	8 (2)	8 (2)

* All MIC data represents mode MIC ($\mu\text{g}/\text{mL}$, n=3). Values in parentheses indicate \log_2 fold increase in MIC from test repeated in CAMHB only).

Table 4.6 (a): Checkerboard assay results: Individual MIC values for colistin, CMS or EA-4 (24-h unmasked) against 4 bacterial test strains

Test Strain	MIC for the Individual Antibiotic *			Ciprofloxacin
	Colistin	CMS	EA-4 (24 h-unmasked)	
<i>A. baumannii</i> 7789	0.0625	8	8	128
<i>A. baumannii</i> 22222	2	8	8	64
<i>E. coli</i> 5702	0.5	8	8	128
<i>E. coli</i> NCTC 10418	0.5	2	2	0.0156

* All MIC data represents mode MIC ($\mu\text{g/mL}$, n=3).

Table 4.6 (b): Checkerboard assay results: average FIC index values for ciprofloxacin in combination with colistin, CMS, or EA-4 (24h-unmasked)

Test Strain	Ciprofloxacin + Colistin *	Ciprofloxacin + CMS *	Ciprofloxacin + EA-4 (24h-unmasked) *
<i>A. baumannii</i> 7789	2.90	1.22	0.818
<i>A. baumannii</i> 22222	1.59	2.86	1.43
<i>E. coli</i> 5702	2.22	0.83	1.61
<i>E. coli</i> NCTC 10418	0.59	2.11	1.76

* All data represents the fractional inhibitory concentration index for the respective combination (mean, n=3).

lower FICI values for both *A. baumannii* strains than for the *E. coli*.

4.4.4 Quality control

Each experiment satisfied the quality control criteria (Section 2.5.3.6.1). Antimicrobial susceptibility of *E. coli* NCTC 10418 to colistin and ciprofloxacin, determined with each experimental batch, were within one two-fold dilution of the published standard (Andrews 2006), for each experiment. Amylase activity in MHB was below the limit of detection of the Phadebas[®] assay (data not shown). Purity controls showed homogenous growth.

4.5 Discussion

The focus of this chapter was to investigate the *in vitro* antimicrobial susceptibility of a range of clinically relevant Gram-negative bacteria to dextrin-colistin conjugates and identify a lead compound for further studies. Due to the inherent toxicity of colistin sulfate, CMS is currently the predominant formulation administered intravenously (Falagas and Kasiakou 2005; Lim *et al.* 2011). CMS, rather than colistin sulfate was therefore chosen as the comparator for the novel dextrin-colistin conjugates. As there are no current susceptibility standards for CMS, evaluating antimicrobial susceptibility to colistin sulfate additionally allowed comparison to published cut-offs validating MIC reproducibility and quality control (Andrews 2006; Giamarellou and Poulakou 2009). Whilst all the conjugates tested in the initial screen exhibited antimicrobial activity, the degree of antimicrobial activity varied with bacterial species, degree of succinoylation, exposure to α -amylase and the presence of serum protein.

In this study, MIC's for 24 h-unmasked EA-4 were within a two-fold dilution of CMS for *A. baumannii*, *E. coli* and *K. pneumoniae* species. Some intra-species variation in conjugate sensitivity was also evident from *K. pneumoniae* 05506, and *E. coli* AIM-1. Since variation in a single result places MICs in a three dilution range (Odds 2003), it may be argued that antimicrobial susceptibility to maximally unmasked conjugate was generally not inferior to CMS in most isolates from three of the four bacterial species

tested. Antimicrobial susceptibility results for 24 h- unmasked EA-4 compared favourably with prior literature detailing PEGylation of other antimicrobial peptides, which led to 5 to 30-fold reductions in antimicrobial activity when tested against non-spore forming bacteria (Guiotto *et al.* 2003; Imura *et al.* 2007; Zhang *et al.* 2008a). With one exception (*E. coli* AIM-1 versus EA-4), all conjugates were less active than colistin sulfate. The importance of cationic charges for interaction with the polar lipid A head and consequent mediation of colistin's antimicrobial effect is well-established (Section 3.4.2). An overall reduction in cationic amine groups resulting from the conjugation process may have contributed to this relative reduction in activity (Clauzell *et al.* 2007; Velkov *et al.* 2009). Literature reports that loss of cationic charge in the cyclic heptapeptide moiety (in contrast to those on the tripeptide linker moiety, Figure 1.3) resulted in complete loss of polymyxin antimicrobial activity (Vaara *et al.* 2008; Vaara *et al.* 2012). It is therefore possible that incorporation of amine groups residing on the heptapeptide moiety into peptide bonds by conjugation to succinoylated dextrin may have contributed to the reduction in activity of unmasked conjugate when compared to colistin sulfate.

The reduction in overall cationic charge with conjugation may have also contributed to the reduced sensitivity of *P. aeruginosa* to 24-unmasked EA-4 compared with the other Gram-negative species. Like dextrin-colistin conjugates, a polymyxin B derivative carrying reduced (only 3 out of 5) positive charges retained more activity against *A. baumannii*, *K. pneumoniae*, and *E. coli* than *P. aeruginosa* in a large scale serial broth dilution MIC determination study (Vaara *et al.* 2012). The same compound sensitised *A. baumannii* and *K. pneumoniae* to other antibiotics more effectively than *P. aeruginosa* (Vaara *et al.* 2008). Moreover, Piers *et al.* (1994) reported that decreasing cationic content of antimicrobial peptides significantly decreases *P. aeruginosa* LPS-binding and outer membrane permeabilisation.

The decrease in bioactivity prior to unmasking may be attributed to increasing steric hindrance. In prior experiments, it was argued that increasing succinylation resulted in increased hydrodynamic radius.

Therefore, conjugation to succinoylated dextrin may have contributed to steric hindrance as evidenced by increasing MIC's at 0 h with increasing succinoylation, in all strains of test panel A. The plausibility of this notion is also affirmed by NMR studies which suggest that bulky polymyxin derivatives may hinder insertion of the resultant molecule into the bacterial outer membrane reducing antimicrobial efficacy (Mares *et al.* 2009; Pristovsek and Kidric 1999, 2004). In another study, defensin conjugated to functionalised cellulose or agarose also reported reduced antimicrobial activity against Gram-negative bacteria in serial broth microdilution assay compared to the original antimicrobial peptide (Bishop 2006). In control experiments conducted in the absence of α -amylase (Table 4.4), antimicrobial activity did not vary by more than one two-fold dilution, and it may be argued that this change was not significant, suggesting that in the conjugated form, masking of activity was evident, followed by α -amylase-mediated unmasking and reinstatement of bioactivity.

The subsequent reinstatement of antimicrobial activity on exposure to α -amylase compared well with unmasking studies, governance of succinoylated dextrin degradation rates reported in the physicochemical characterisation experiments and with key preceding studies (Hreczuk-Hirst *et al.* 2001a). These results confirm that enzymatically-triggered unmasking resulted in reinstatement of antibiotic activity, in keeping with the PUMPT principle (Duncan *et al.* 2008) and underscored the importance of α -amylase in the unmasking process.

Pre-incubation in α -amylase reduced methodological bias. Whilst CAMHB did not possess detectable α -amylase activity, divalent cations contained in CAMHB may interfere with α -amylase. CAMHB may also contain starch which is a substrate for α -amylase (Deshpande and Cheryan 1984; Oxoid Limited 2011b). Variation in divalent cations contained in CAMHB may interfere with colistin function (Conly and Johnston 2006). However, the aim of these experiments was comparative (colistin to CMS to dextrin-colistin conjugate). The use of the same batch of CAMHB (Chapter 2) reduced this potential source of bias.

Antimicrobial activity of colistin dropped substantially in the presence of FCS compared to 24 h-unmasked EA-4 and CMS. In agreement with these findings it has been reported that 59-74% of total colistin is bound to albumin (Mohamed *et al.* 2012), but only the unbound colistin exerts an antibiotic effect (Dudhani *et al.* 2009). This suggests that sulphomethylation and polymer conjugation may reduce albumin binding, which could increase *in vivo* bioavailability.

Determining the 'true' antimicrobial potential of macromolecular antibiotics is challenging. Susceptibility results in the presence of FCS suggest that conjugation would impart the conjugate an advantage over colistin *in vivo*. Moreover, standard antimicrobial susceptibility analysis by MIC does not take into account the potential for passive accumulation of the conjugate by the EPR effect (Chapter 1). It is, therefore, likely that the true antimicrobial potential of these conjugates is underestimated *in vitro*. Nonetheless, MIC determination provided a standard, accepted and robust methodology whereby the activity of different candidate conjugates could be compared to each other and to CMS and colistin.

Checkerboard assays suggest that masking/unmasking did not alter the interaction behaviour of the resulting unmasked product, and the conjugation process did not result in antagonistic activity. The checkerboard assay provided an objective method to compare the antimicrobial effect of the different ciprofloxacin combinations. It is possible that the ciprofloxacin + dextrin colistin conjugate combination could benefit from prolonged *in vivo* bioactivity owing to its controlled release nature (hence prolonged detergent effect on the bacterial membrane) and, potentially, passive accumulation by EPR. Moreover, recent studies have shown that addition of colistin to fluoroquinolone antibiotics effectively lowered the concentration necessary to prevent the emergence of resistant mutants *in vitro* as well as in retrospective clinical studies (Cai *et al.* 2012; Prentice *et al.* 2001; Zhanel *et al.* 2006). The potential *in vivo* benefits of dextrin-colistin combination to ciprofloxacin may, consequently, have been underestimated by this assay. Through controlled, prolonged release (by the PUMPT effect), and passive accumulation (by the

EPR effect), use of dextrin-colistin conjugates may serve to achieve greater efficacy in preventing the emergence of resistant strains.

4.6 Conclusion

These studies demonstrated that incubation of dextrin-colistin conjugates in the presence of α -amylase resulted in re-instatement of antimicrobial activity, against *A. baumannii*, *K. pneumoniae* and *E. coli*, whilst *P. aeruginosa* was resistant. EA-4 showed the most marked antimicrobial activity, which after 24 h incubation with α -amylase was generally not inferior as an antimicrobial agent to the current clinically used formulation (CMS) using well-established *in vitro* tests. There was a general agreement between polymer modification and conjugate activity with conjugates containing lower degrees of succinylation displaying more antimicrobial activity. Based on these studies it was also possible to determine a lead candidate, EA-4, for which masking/unmasking, and reinstatement of antimicrobial activity in the presence of α -amylase was a feasible concept *in vitro*, in the context of PUMPT. Whereas the antimicrobial activity of colistin was decreased by the addition of FCS, CMS and 24 h-unmasked EA-4 activity was largely unaffected. This was presumably due to the altered affinity to serum protein. Addition of ciprofloxacin to bacteria incubated with dextrin-colistin conjugate resulted in 'no interaction' by the checkerboard method. To investigate further the pharmacological / pharmacodynamic profile of dextrin-colistin conjugates, an *in vitro*, static two-compartment model under infinite sink conditions was constructed to measure unmasking and bacterial killing and will be characterised in Chapter 5.

Chapter Five

Pharmacokinetic/Pharmacodynamic and *Ex Vivo* Studies

5.1 Introduction

This study has determined a "lead" conjugate (EA-4) for which controlled unmasking by α -amylase led to reinstatement of antibiotic activity and whose antibacterial activity was generally equivalent to CMS against *A. baumannii*, *K. pneumoniae*, and *E. coli*. Establishing the α -amylase activity within target tissue, and viability of conjugate unmasking at these sites is critically important in supporting the concept of PUMPT (Duncan *et al.* 2008) for dextrin-colistin conjugates. In this study, α -amylase activity in *ex vivo* infected wound fluid samples, collected from a series of burn patients, and bacterial amylase production were investigated. Evaluation of *in vitro* antibacterial activity during unmasking of dextrin-colistin conjugates is an important step in determining optimal dosing strategies. Therefore, a two-compartment, static, dialysis bag model under infinite sink conditions was validated. Using this model, the pharmacokinetic/pharmacodynamic (PK/PD) profile of colistin sulfate, EA-4 and the colistin sulfate + EA-4 combination was investigated in a modified time-kill model against a MDR *A. baumannii* strain. The PK/PD model was then used to evaluate real-time unmasking of EA-4 in infected human wound exudates. *A. baumannii* infection is associated with significant mortality and multidrug resistance (Dijkshoorn *et al.* 2007). In military casualties *A. baumannii* infection has reached epidemic proportions (Keen *et al.* 2010b). The clinical importance of *A. baumannii* infection provided a clinically valid rationale for selecting an *A. baumannii* strain to investigate the PK/PD profile of the EA-4 dextrin-colistin conjugate.

5.1.1 Amylase

Several physicochemical characteristics of α -amylase provide a rationale for investigating its use in harnessing PUMPT in infection. The α -amylases randomly hydrolyse α -1, 4-glycosidic bonds along the polysaccharide chain (Burkart 2004). The by-products of this reaction (intermediate length dextrans, shorter limit dextrans, maltotrioses, and the disaccharides maltose and isomaltose, (Figure 5.1) are non-toxic and easily assimilated in normal physiological metabolic processes. Human production of α -

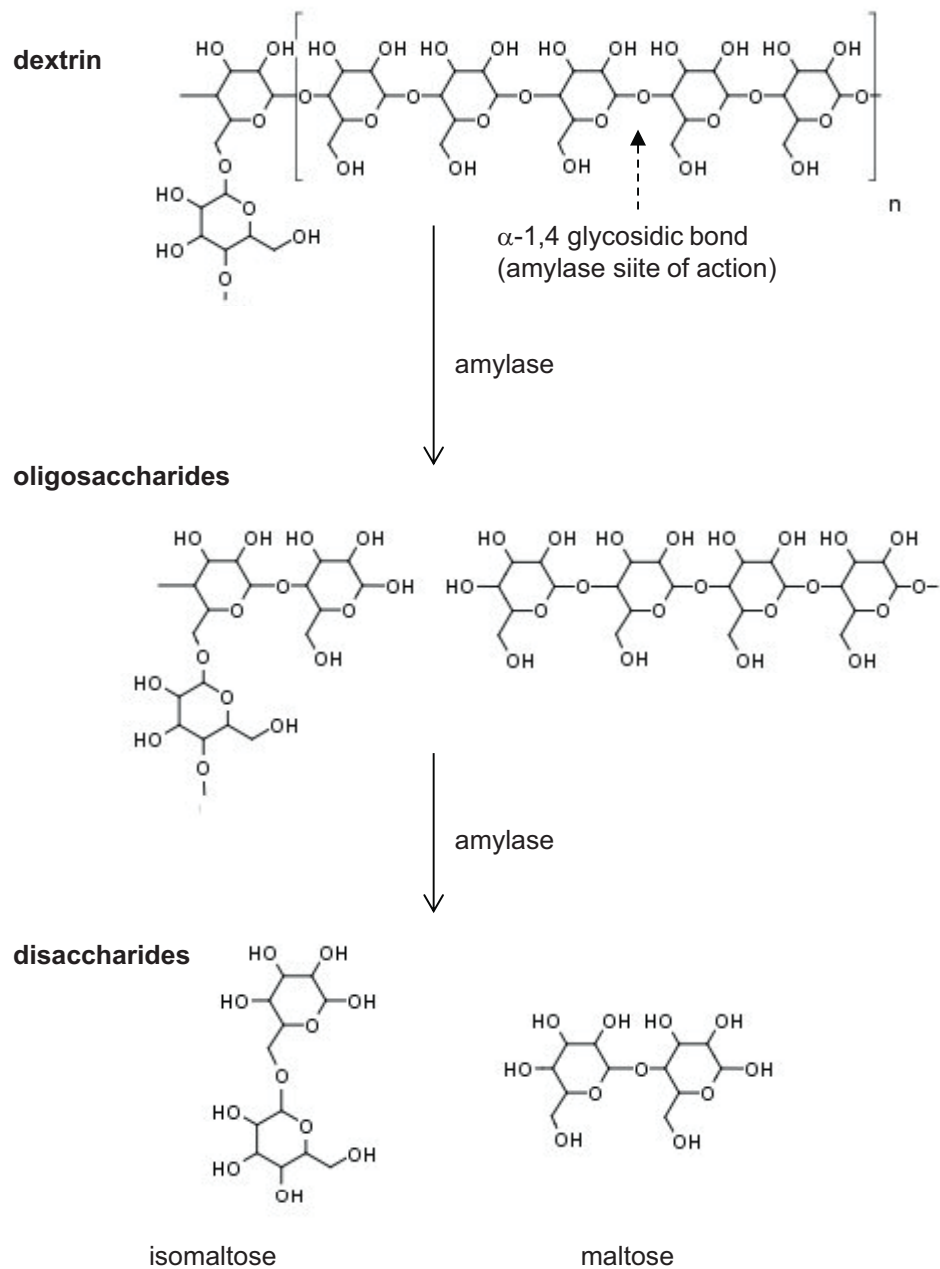


Figure 5.1: Schematic representation of dextrin degradation by amylase. First, random hydrolysis at α -1,4 glycosidic bonds, produces medium length dextrans ($n = 200\text{--}300$ monomers) and subsequent hydrolysis produces oligosaccharides and disaccharides.

amylase accounts for 5-6% of total protein secretion (Whitcomb and Lowe 2007), resulting in limited α -amylase activity in plasma under physiological conditions. However, its high molecular weight (~57,000 g/mol) is compatible with passive targeting and retention at an infected site, by EPR (Section 1.7). Moreover, this high molecular weight would conveniently facilitate partitioning in a two-compartment model using dialysis membranes. The optimal pH of α -amylase is 6.7-7 (Abrams *et al.* 1987), which is compatible with the acidic pH expected in and around infected foci (Kumar 2010).

Clinical application of α -amylase has so far been limited to diagnostics, for example, of salivary and pancreatic disease (Longmore *et al.* 2010). Previous studies have demonstrated the presence of α -amylase activity within acute and chronic wounds (Hardwicke *et al.* 2010). To date, however, no studies have investigated α -amylase activity in infected wounds or its therapeutic potential within this context. Successful application of dextrin-colistin conjugates is dependant on locally triggered polymer degradation to permit time-dependant protein 'unmasking', and controlled reinstatement of bioactivity at the infected site (Duncan 2011). It was, therefore, essential to investigate α -amylase activity in *ex vivo* infected wound fluid to substantiate a proof of concept regarding the viability of dextrin-colistin conjugates in infection.

The two human amylase isoforms (pancreatic and salivary) have the same mechanism of action and their predicted amino acid sequences differ by only 6% (Lott and Lu 1991; Ramasubbu *et al.* 1996). Amylases are, however, not unique to humans (Pandey *et al.* 2000). In particular, bacterial production of amylases has been extensively explored in the food industry (Reddy *et al.* 2004). It was, therefore, also considered interesting to investigate whether the pathogenic strains used in this study produced amylase.

5.1.2 Infected wound fluid collection

Wound fluid is a valid representation of the biochemical status in the wound environment (Tregrove *et al.* 1996) and has been used to monitor biochemical events in the dermis in acute and chronic wounds (Faria *et al.*

1997; Staiano-Coico *et al.* 2000; Trengove *et al.* 2001). Moreover, wound fluid is a commonly used *ex vivo* model for analytical and experimental purposes (Bowler *et al.* 2012; Staiano-Coico *et al.* 2000). α -Amylase activity and its unmasking potential has been successfully assessed in wound fluids from healing wounds (Hardwicke *et al.* 2010). The literature suggested that collection of infected wound fluids was appropriate for conducting these experiments.

A variety of validated non-invasive techniques (Table 5.1) has been described for collection of wound fluids. Indirect collection methods involve collection of wound fluid into absorbent media or dressings followed by wound fluid elution. However, such methods entail significant time (minimum of 1–4 h), rendering them unsuitable for use in unstable, critical care patients. In contrast, direct collection of infected fluid from the wound bed presents a simple, rapid and effective direct sampling technique, with minimal interference to the wound bed (Moseley *et al.* 2004).

Infected burn wounds present a potentially large, "weeping" surface area which necessitates multiple dressing changes and operative interventions. This lends itself well to direct collection of wound fluid samples, whilst avoiding sampling bias that may arise from indirect fluid collection methods, such as variance in the volume of wound fluid eluted, fluid protein levels and adsorption of proteins to dressing materials (Cook *et al.* 2000; Moseley *et al.* 2004). The care-costs of these patients are markedly higher than for the general in-patient population (Sanchez *et al.* 2008). Burn wound infection remains a primary cause of morbidity and mortality in these patients, for whom novel and effective antibiotic agents are needed (Ansermino and Hemsley 2004; Church *et al.* 2006; Sharma *et al.* 2006).

5.1.3 PK/PD models

PK/PD models offer the possibility of linking the concentration-timecourse (unmasking at the target site) to the drug effect (bacterial killing). Antimicrobial activity has been traditionally explored with time-to-kill (TTK) assays, where a fixed concentration of both antibiotic and bacteria is

Table 5.1 : Wound fluid sampling methods reported in the literature, and considered for infected burn wound fluid sampling

Sampling method	References
Aspiration of fluids from beneath occlusive dressing	James <i>et al.</i> (2000); Drinkwater <i>et al.</i> (2002); Wysoki <i>et al.</i> (1999); Yager <i>et al.</i> (1997); Tengrove <i>et al.</i> (2001)
Micro-capillary fluid collection	Wreckroth <i>et al.</i> (1996)
Tissue harvesting with homogenization	Lobmann <i>et al.</i> (2002)
Absorption onto dressing	Fivenson <i>et al.</i> (1997)
Viscose cellulose sponge attached to a rubber tube, followed by wound elution	Pajulo <i>et al.</i> (1999, 2001)
Silicone rubber tube with a viscose cellulose sponge at the end, inserted subcutaneously between the wound edges, followed by wound elution	Pajulo <i>et al.</i> (1999, 2001)

added to an enclosed container and colony counts are determined over time (Manduru *et al.* 1997). In a classical TTK study (static, one compartment model), the growth medium remains unchanged (Bernaerts *et al.* 2004; Murakawa *et al.* 1980). Single compartment static models have been well-established for studying the effect of a fixed antibiotic dose on a fixed bacterial load (Tan and Ng 2006). However, selecting an appropriate PK/PD model to investigate drug-release and real-time antimicrobial effect for a single dose of a controlled-release, bioresponsive conjugate (whose unmasking depends on a local, enzymatically-triggered reaction) presented specific challenges in comparison to conventional antibiotic *in vitro* models.

Continuous removal of peripheral compartment fluid is a classical method used to establish a concentration gradient from a central compartment containing the drug, to a peripheral compartment containing the bacteria (Gloede *et al.* 2010). However, such methods require specialist equipment and interpretative software. In their extensive review, Gloede *et al.* (2010) proposed a novel perspective into methods harnessing "infinite sink" conditions which use a concentration gradient as a driving force from one compartment to another. This method has been successfully used to study *in vitro* drug release from a variety of drug delivery systems, including drug-eluting stents (Schwartz *et al.* 2002) and anaesthetic-containing microparticles (Colombo *et al.* 2004). Therefore, a static two-compartment dialysis bag model under infinite sink conditions offered a simple and cost-effective model that was appropriate for an initial study exploring the antimicrobial effect of a single conjugate dose whose unmasked proportion changed over time. Use of a two-compartment model would also allow content of each compartment to be varied and assayed separately. This model would further permit flexibility to introduce increasing levels of complexity, from model validation, to a modified TTK assay. Moreover, partitioning α -amylase to the central compartment would minimise interference from α -amylase inhibitors potentially present in artificial media such as TSB (used for overnight bacterial growth) and MHB used for sustaining bacterial growth (Oishi *et al.* 1991; Ryan 1990).

5.2: Aims

The aims of these studies were to:

1. Determine α -amylase activity in *ex vivo* infected wound fluid collected from a series of burn patients
2. Establish the bacterial contribution to α -amylase activity
3. Investigate EA-4 unmasking directly in *ex vivo* infected wound fluid
4. Develop a two-compartment, static, dialysis bag PK/PD model under infinite sink conditions to study re-instatement of EA-4 activity by α -amylase
5. Describe the PK/PD profiles of colistin sulfate, EA-4, and a combination of colistin sulfate + EA-4 against *A. baumannii* strain 7789, in terms of drug release and antimicrobial activity

5.3 Methods

General methods used, including BCA assay (Section 2.5.2.3), FPLC (section 2.5.2.2) and MIC assay (section 2.5.3) were previously described. Dextrin-colistin conjugates EA-4 (1.0 mol% succinoylation, 11.2 % w/w colistin content, Mw ~10, 300 g/mol) was prepared as detailed in Sections 2.5.1 and 3.3.2. Unless otherwise specified, the concentration of the doses described in this study refers to colistin component of the conjugate.

5.3.1 *Ex vivo* sample collection

Informed consent was obtained for all procedures, which were performed under Research Ethics Committee approval (11/WA/0252, Appendix 1). Wound fluid (up to 3 mL) was collected from infected burn wounds of six adult civilian patients treated at the Welsh Burns Centre (Swansea, UK). Burn wound infection was diagnosed according to the American Burn Association diagnostic criteria (Greenhalgh *et al.* 2007). Patients with recorded pancreatic or salivary disease, multiple trauma, or other concurrent foci of infection were excluded. Infected wounds originating from within 1% total body surface area of body orifices or *in situ* catheters/endotracheal tubes were also excluded.

Wound fluid collection was performed intra-operatively or during dressing change, under standard sterile operating theatre conditions. After dressing removal, wounds were lightly washed with sterile medical grade 0.9% sodium chloride solution. After 15 min, a sterile Teak[®] graft board with a smooth bevelled edge was gently passed over the wound surface to collect the exudate bathing the infected wound in a galley pot. Using this method, the widest infected area was sampled. Infected wounds surrounding or within 1% total body surface area of body orifices, arterial lines, endotracheal tubes or suspected urinary/faecal contamination were avoided. Each sample was transferred to a sterile syringe, sealed, then frozen on dry ice and stored at -80 °C until required. Samples were clarified by centrifugation (15,000g x 5 min at 4 °C) prior to use (Hardwicke *et al.* 2010).

5.3.1.1 α -Amylase activity assay

The α -amylase content of infected wound fluid samples was determined using a Phadebas[®] starch-dye assay (Akiko *et al.* 1972). Briefly, wound sample (200 μ L) was added to ddH₂O (4 mL) and pre-warmed (37 °C, 5 min). One Phadebas[®] tablet was added to each sample, vortexed for 10 s and incubated (37 °C, 15 min). The reaction was terminated by the addition of NaOH (0.5 M, 1 mL), before centrifugation (1,500 g, 5 min). Supernatant absorbance at 620 nm was measured in triplicate using distilled water as blank using a 1 cm path-length cuvette. The entire procedure was repeated in triplicate for each sample. Mean α -amylase activity in the samples was derived from the batch-specific calibration curve provided with the reagent.

5.3.2 Bacterial production of amylase

Amylase secretion by bacteria was explored using a modified procedure previously described by Wróblewska *et al.* (2011), whereby LMW dextrin was used instead of starch. Dextrin-agar was produced by dissolving TSA agar (40 g) and LMW dextrin (10 g) in ddH₂O (1 L). Then, it was autoclaved (121 °C for 15 min). Once cooled to 56 °C in a waterbath, aliquots (20-30 mL) were poured into sterile Petri dishes in a microflow cabinet and left to cool until solid. The prepared plates were stored inverted at 2-8 °C until

required. Bacterial isolates were inoculated onto the dextrin-agar medium plates and incubated for 24 h. Amylase production was determined by the appearance of a colourless halo after Lugol's solution (1% iodine in 2% potassium iodide) was added to each plate. *Bacillus subtilis* ATCC 6633 was used as a positive control. Blank plates were flooded with Lugol's iodine and the resulting red-brown colour was used as a negative control. The procedure was repeated in triplicate for all clinical strains used in this study.

5.3.3 Construction of a static two-compartment PK/PD model under infinite sink conditions

Unmasking of dextrin-colistin conjugates was simulated using a two-compartment static, 'dialysis bag' model under sink conditions (total volume: 20 mL, inner compartment volume: 5 mL), as shown schematically in Figure 5.2. Dialysis membrane (10,000 g/mol MWCO) was pre-soaked for 15-30 min in ddH₂O, secured with dialysis clips and suspended from an injection port to separate the inner compartment (IC) from the outer compartment (OC) in a sterilised 25 mL beaker sealed with sterile medical grade polyurethane membrane. The model system was established under aseptic conditions in a class 2 laminar air flow cabinet, and transferred to a shaking incubator set at 37° C in ambient air and constant orbital agitation at 70 RPM for 48 h.

5.3.3.1 Method validation

First, the integrity of the membrane to the masked conjugate was assessed. Here, the IC contained dextrin-colistin conjugate EA-4 diluted in PBS (10 mg/mL colistin equivalent), in absence of α -amylase. The OC contained PBS (15 mL). The sealed beaker was incubated as described previously for 17 h. After this time, FPLC was used to estimate the proportion of masked conjugate in both compartments (n=3).

To validate the PK/PD model, the rate of change in total protein content in the OC and IC was measured over time. The OC contained sterile PBS (pH 7.4, 37 °C) while the IC contained EA-4 (10 mg colistin equivalent) and α -amylase (100 IU/L) in sterile PBS (pH 7.4, 37 °C) (Figure 5.2).

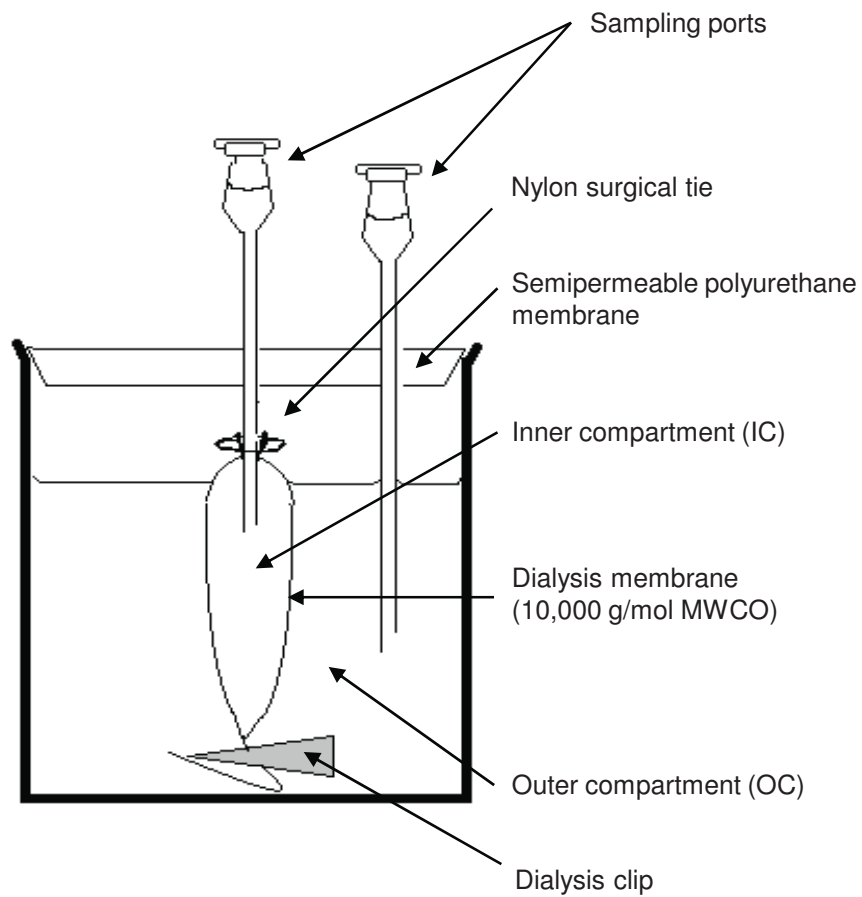


Figure 5.2: Schematic illustration of the two-compartment 'static' dialysis bag PK/PD model under infinite sink conditions. The model system was incubated at 37 °C with 70 RPM orbital shaking agitation.

Samples (150 μ L) were extracted from each compartment at various intervals (0, 4, 8, 12, 24, 36, 48 h) using sterile, medical-grade single-use vascular catheters, and immediately frozen in dry ice. Samples were analysed for total protein content using BCA assay, and for antimicrobial susceptibility using an MIC assay. At 48 h, the % unmasked and masked colistin in both compartments was confirmed by FPLC using area under the respective curve. At the end of the experiment, integrity of the dialysis membrane to amylase was confirmed using carbonic anhydrase as a molecular weight surrogate. Contents of both IC and OC were removed. The dialysis membrane was flushed with several volumes of sterile ddH₂O and then resuspended in sterile PBS as described previously. The IC was spiked with carbonic anhydrase (29,000 g/mol, 2 mg/mL) in PBS. After a further 17 h incubation against PBS within the OC under identical conditions, the ratio of carbonic anhydrase in both compartments was quantified by UV/vis spectrophotometry (n=3).

5.3.3.2 Modified *A. baumannii* TTK model

The model was converted to a modified, TTK study for investigating the effect of a single dose of colistin, EA-4, and the colistin sulfate/ EA-4 combination against *A. baumannii* 7789 strain in terms of drug release and antimicrobial activity, over 48 h. Here, the OC contained a 0.5 Mcfarland concentration of *A. baumannii* 7789 strain in MHB at 0 h. In separate experiments, the IC contained α -amylase (100 IU/L) in sterile PBS in combination with either:

- a. Colistin sulfate (1.3 μ g colistin equivalent, 1 x MIC)
- b. EA-4 (160 μ g colistin equivalent, 1 x MIC)
- c. EA-4 (320 μ g colistin equivalent, 2 x MIC)
- d. A mixture of colistin sulfate (1.3 μ g colistin equivalent, 1x MIC) and EA-4 (160 μ g colistin equivalent, 1 x MIC)

The model was incubated and aliquots from the OC were sampled as described previously (Section 5.3.2.1). Colony counts (CFU/mL) were

determined according to the method of Miles *et al.* (1938). The procedure was repeated in triplicate. Freshly prepared TSA plates were dried inverted in sterile conditions in a laminar flow cabinet (20-30 min). OC aliquots (50 μ L) were diluted 10-fold in PBS and centrifuged. Pellets were re-suspended in an equivalent volume of fresh PBS. Serial 10-fold dilutions were performed in triplicate across a 96-well plate (columns 2-12). Samples (5 μ L) from each dilution were dropped onto the surface of the dried TSA plates in triplicate. TSA plates were left undisturbed for 30 min, inverted, and incubated for 18-20 h (35-37 °C in ambient air) then visually inspected for growth. The drop-position displaying the highest number of full-size discrete colonies, (range 2-20 colonies) was quantified and multiplied by the dilution factor. The resulting colony count (CFU/mL) was plotted versus time to produce a time-kill model. Controls were included with each experimental procedure (Table 5.2.)

5.3.3.3 Unmasking of dextrin-colistin conjugate in infected wound fluid

The same model was used to evaluate the feasibility of dextrin-colistin conjugate unmasking in infected burn wound samples. Equal aliquots of each wound fluid sample (clarified as detailed in Section 5.3.1) was diluted in PBS to make up the IC volume (5 mL) and EA-4 (320 μ g colistin equivalent) was added. The OC contained sterile PBS as described previously. OC samples (150 μ L) were extracted (0, 3, 6, 12, 24, 48 h), and frozen on dry ice until required. For each wound sample the procedure was repeated in triplicate. The total colistin content in each sample was determined in triplicate using the MaxSignal[®] ELISA assay as detailed in section 2.5.4.

5.4 Results

5.4.1 Wound fluid collection and determination of α -amylase activity

Wound fluid was collected from a total of 6 patients (Table 5.3). Typical patients were middle aged adults with partial thickness skin burns. Dry heat was the commonest cause of injury. Total protein content in the supernatant was 50.7 ± 15.1 mg/mL (mean \pm SD). Infected wound fluid contained significantly higher α -amylase activity (408.4 ± 168.3 IU/L, mean \pm SD) than

Table 5.2: Control procedures used in validation of the two-compartment dialysis bag model under infinite sink conditions

Control	Procedure
Sterility	Sterile PBS (IC, 5 mL) was dialysed against sterile freshly prepared MHB in the OC (15 mL) for 48 h. At 48 h, samples from both compartments were subcultured on sterile TSA plates and incubated for 18-20 h at 37 °C in ambient air.
Contamination	At the end of each experiment, an aliquot from both IC and OC was subcultured onto TSA and incubated for a further 18-20 h. Results were only considered acceptable in the presence of homogenous cultures.
Growth	Sterile PBS was dialysed against 5×10^5 CFU/mL <i>A. baumannii</i> in MHB in the OC (Section 5.3.3.1) for 48 h.
Growth controls on colony counting plates	3 x 5 µl drops of neat washed bacterial cells were used as growth controls with each determination of viable bacterial cells.
Reporting controls	Colony counts confirmed by a blinded observer.

Table 5.3: Basic demographic characteristics of patients included in this study

Parameter	Data
Aetiology (n)	Domestic scald (2); Flame (4)
Age mean (range)	50.6 (22-65)
Total body surface area % burnt (range)	5-90
Weight on admission in kg, mean, (range)	80.6 (65-105.4)
Predominant burn depth (n)	Partial thickness (4); full thickness (2)
Time of sample collection post burn (days) (mean \pm SD)	17 \pm 6

140

serum (60.0 ± 25.3 IU/L, mean \pm SD) concurrently reported from clinical charts on the same patient cohort ($p < 0.05$) (Figure 5.3).

5.4.1.1 Bacterial production of amylase

None of the bacterial strains produced amylase. In contrast *B. subtilis* strain ATCC 6633 reported a clear zone surrounding the colonies (Figure 5.4).

5.4.2 PK/PD experiments using the static two-compartment model under infinite sink conditions

5.4.2.1 Model validation

Protein concentration in the IC decreased in a logarithmic fashion over time, while protein concentration in the OC increased reciprocally (Figure 5.5). A corresponding increase in antimicrobial activity of the OC over time was also observed (Figure 5.5). FPLC demonstrated that, at 48 h, significant unmasking occurred ($p < 0.05$) and unmasked conjugate accounted for ~68% of total colistin content in the system (Figure 5.6). At 48 h there was no significant difference in the distribution of unmasked conjugate between the two compartments ($p > 0.05$). On the other hand, the system reported a significant difference in the masked/minimally unmasked dextrin-colistin conjugate between OC and IC ($p < 0.05$), with ~80% being retained in the IC (Figure 5.5). Membrane integrity testing confirmed the presence of carbonic anhydrase in the OC was always below the lower limit of quantification (Figure 5.7). When the experiment was repeated using masked dextrin-colistin conjugate in the absence of α -amylase, the OC protein content was <10% after 17 h satisfying quality control criteria (Spectrum Labs Inc 2012).

5.4.2.2 PK/PD modelling against MDR *A. baumannii* isolates

Colistin sulfate, at its previously determined MIC (Table 4.4) showed rapid initial killing, and viable bacterial counts were below the assay's lower limit of quantification at 4 h. However, early and significant bacterial re-growth following a colistin sulfate dose virtually nullified the gains registered by the rapid reduction in bacterial counts, within 8 h thereafter (Figure 5.8). In

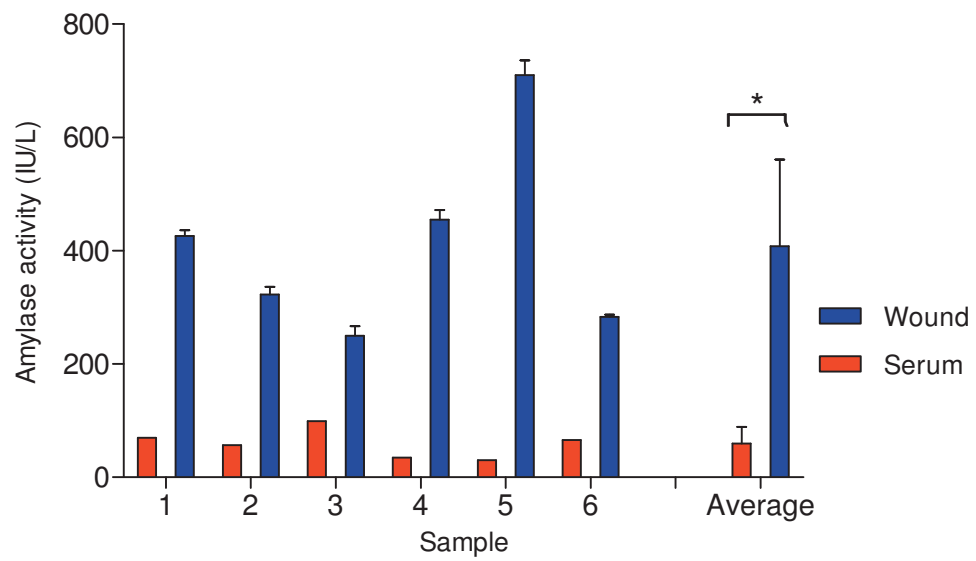


Figure 5.3: Estimation of α -amylase activity by Phadebas[®] assay in infected wound fluid (n=3, mean \pm SD) or from clinical reports (n=1, serum). The average data set represents mean amylase activity in wound versus plasma samples (mean \pm SD, n=6). * Indicates a statistically significant difference (t-test, $p < 0.05$).

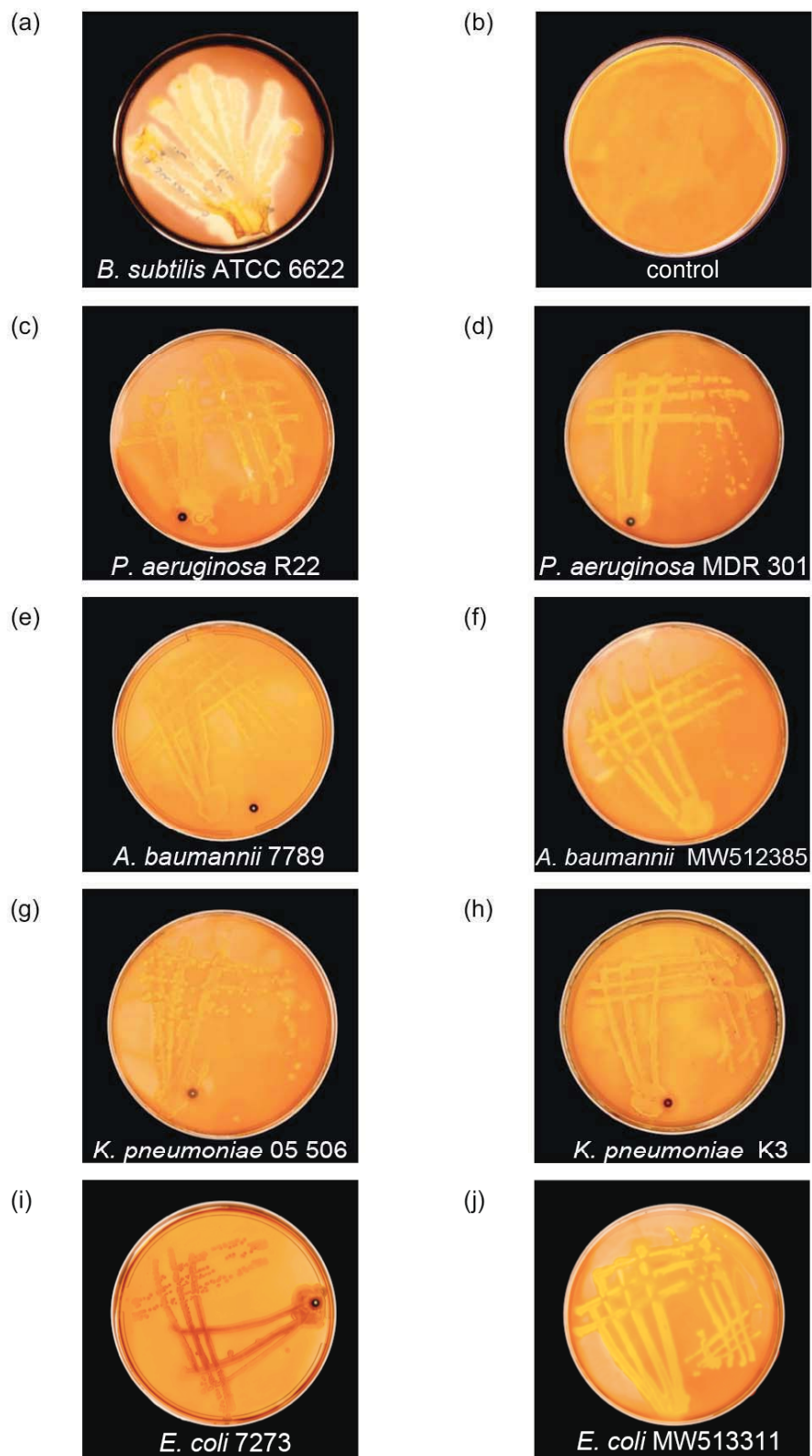


Figure 5.4: Typical images of bacteria grown on dextrin-agar plates tested with Lugol's iodine. Panel (a) shows a positive control (*Bacillus subtilis*); panel (b) shows a negative control (no bacteria); subsequent panels (c-j) show two strains from each bacterial species tested.

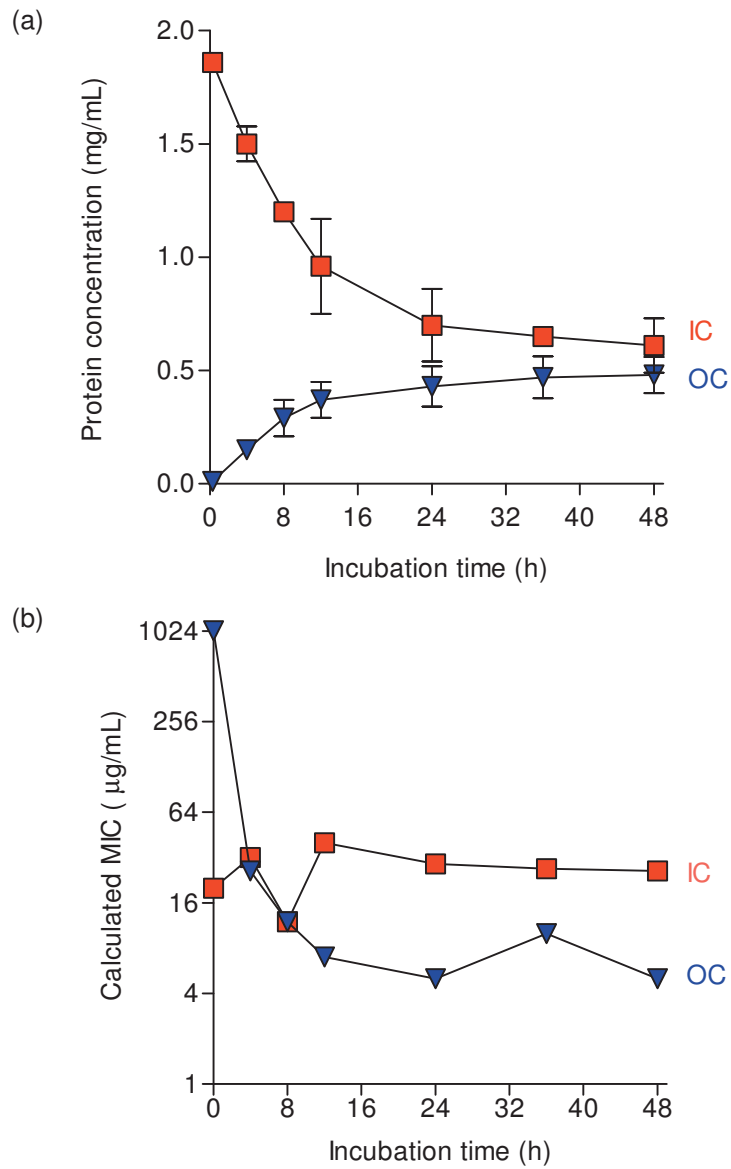


Figure 5.5: Distribution and antimicrobial activity of dextrin-colistin conjugates in a two-compartment dialysis bag model under infinite sink conditions. Panel (a) shows protein concentration in the IC and OC over time (mean \pm SD, $n=3$); panel (b) shows the corresponding MIC for each time point as a measure of antimicrobial susceptibility ($n=3$).

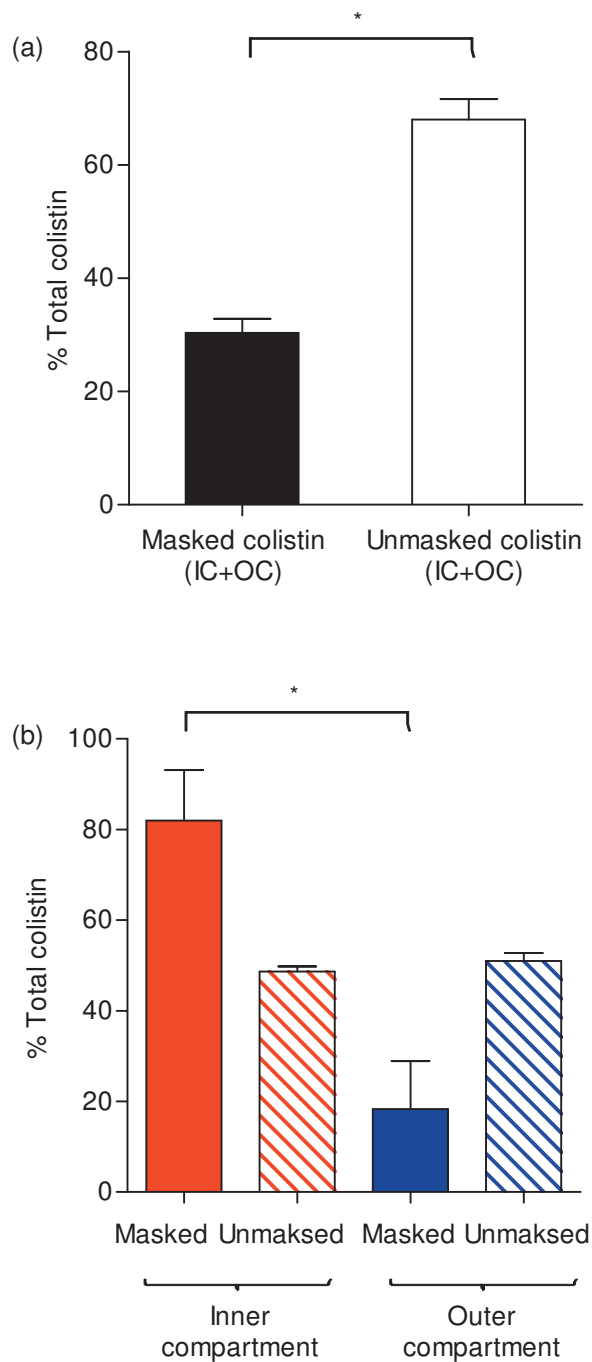


Figure 5.6: Distribution of masked and unmasked dextrin-colistin conjugate at 48 h in a two-compartment dialysis bag model under infinite sink conditions. Panel (a) shows the relative amounts of masked and unmasked dextrin-colistin conjugates in the system at 48 h; (b) shows the distribution of masked and unmasked dextrin-colistin conjugate in each compartment at 48 h. Data shown represents mean \pm SD (n=3). * Indicates statistical significance ($p < 0.05$).

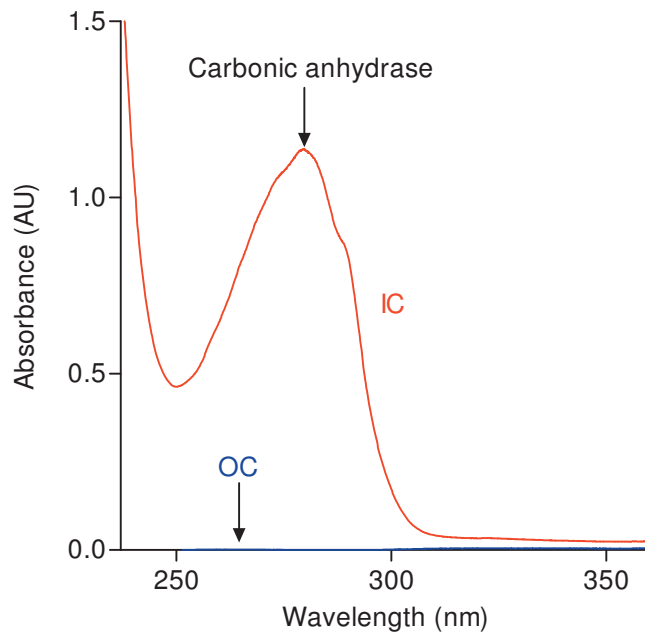


Figure 5.7: Detection of carbonic anhydrase by UV/vis spectroscopy, in IC and OC after integrity testing.

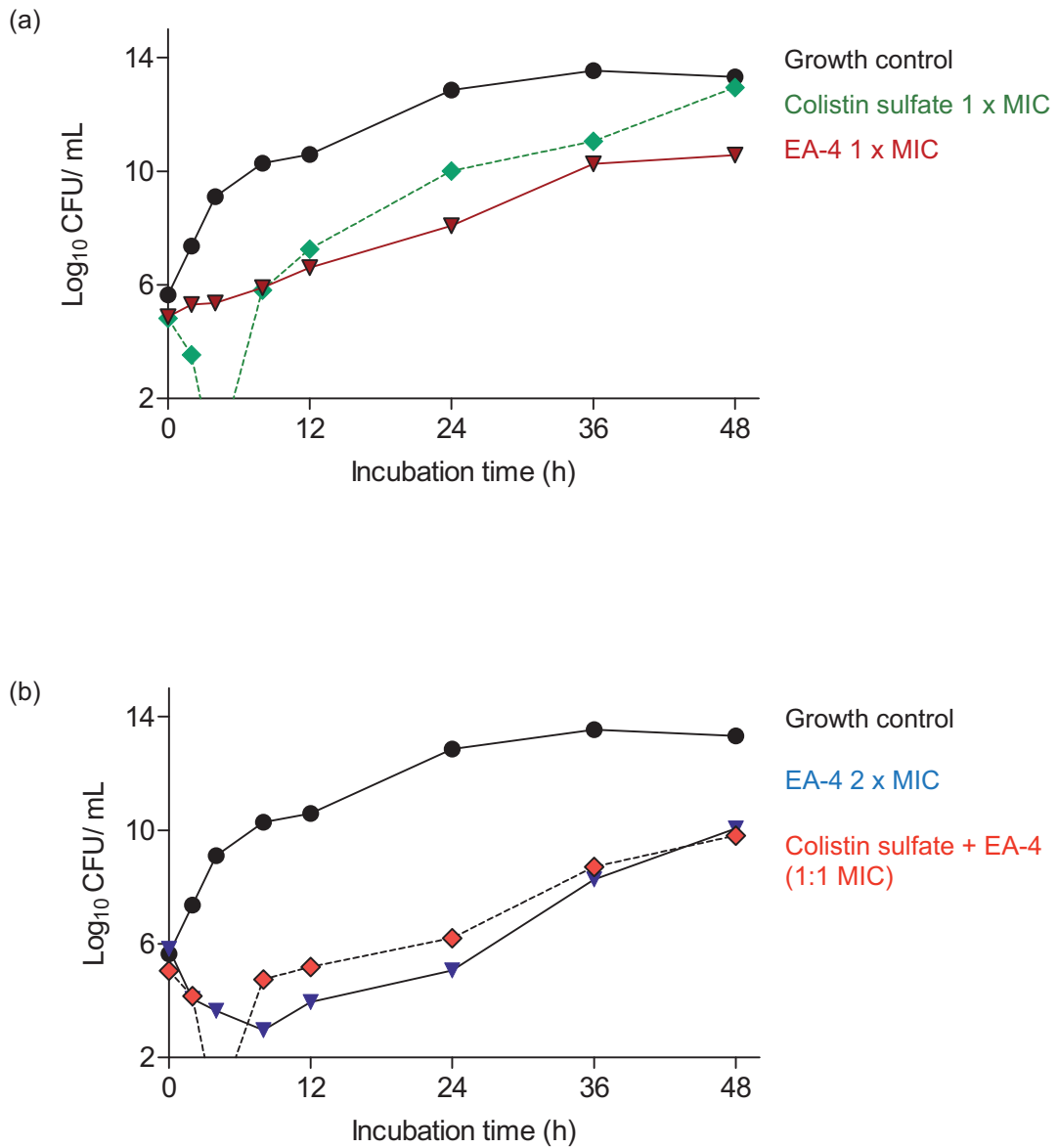


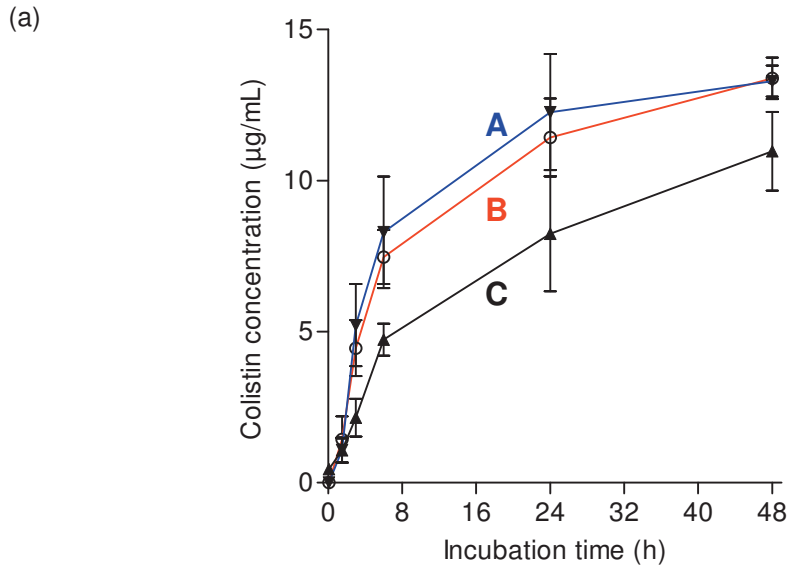
Figure 5.8: TTK curves against the *A. baumannii* clinical isolate. Panel (a) shows bacterial viability count following a challenge with colistin sulfate or EA-4 in the presence of α -amylase (100 IU/L). Panel (b) reports bacterial viability count after a challenge with colistin sulfate+EA-4 in the presence of α -amylase (100 IU/L), or EA-4 at 2 x MIC. Data shown represents mean CFU values (n=3). Lower limit of quantification: 200 CFU/mL.

contrast, antimicrobial activity of the EA-4 conjugate was gradual and sustained. Maximum reduction in viable bacterial counts ($\sim 10^4$ CFU/mL) occurred at 24 h compared to growth controls. Dextrin-colistin conjugate was bactericidal in a concentration-dependant manner (1 x MIC versus 2 x MIC). In contrast, the colistin sulfate + EA-4 combination retained optimal characteristics of both components, with rapid initial bacterial killing, maximum reduction in viable bacterial counts at 4 h and sustained reduction of viable bacteria throughout the experiment, compared to control and colistin. The control growth curves reported that bacterial growth could be maintained under the experimental conditions, while sterility controls confirmed that sterility conditions were maintained throughout. Culture onto TSA plates at the end of each experiment confirmed the presence of a homogenous culture.

5.4.2.3 *Ex vivo* unmasking

After sample clarification only three wound samples contained sufficient supernatant volume to enable assessment of direct dextrin-colistin unmasking in triplicate. The resulting α -amylase activity in the system (after dilution needed to make up three aliquots of each clarified, infected wound fluid sample to the required IC volume), is reported in Figure 5.9.

An increasing colistin concentration in the OC was observed in all samples over time (Figure 5.9). As concentration of α -amylase in wound fluid increased (25.6 to 71 IU/L) the total concentration of colistin in the OC also increased correspondingly. At 48 h this equated to a mean total OC colistin content of 68.1 to 86.2 % of the theoretical concentration of the original dose concentration at equilibrium (32 μ g/mL). Unmasked colistin in this compartment at 48 h was derived from the total (measured by ELISA) by subtracting the % of masked colistin reported in this compartment at the same timepoint. The resulting values for unmasked colistin and respective 95% confidence intervals are detailed in Figure 5.9. In the system with the highest α -amylase activity, OC colistin concentration plateaued earliest, starting at 24 h.



(b)

Sample	Amylase activity in undiluted wound fluid (IU/L)	Amylase activity in IC (IU/L)	Total OC Colistin at 48 h (µg/mL)	Calculated unmasked EA-4 in OC (%) *
A	710 (26)	71	13.8 (0.7) a	34.3 (28.6 to 39.4)
B	322 (13)	64	13.2 (0.5) b	32.5 (26.9 to 32.6)
C	256 (3.9)	25.6	10.9 (1.3) c	25.1 (20.3 to 30.4)

Values represent mean \pm SD (n=3). *After application of a correction factor for masked EA-4 content, values represent mean unmasked EA-4 (95% confidence interval).

Figure 5.9: Estimation of unmasked colistin release after incubation of masked conjugate in infected burn wound fluid. Panel (a) reports OC colistin content over time. Data shown represents mean \pm SD, n=3. Panel (b) shows amylase concentration determined by Phadebas assay and the final amylase activity in the IC after dilution.

5.5 Discussion

5.5.1 Determination of α -amylase activity in infected wounds

The notion of a prototype polymer-antibiotic conjugate which affords selective release at an infected site was critically dependent on locally triggered polymer degradation to allow time-dependant "unmasking" and controlled reinstatement of activity (Duncan 2011). Establishing the presence of viable α -amylase activity within sites of infection was important to this study. Analysis of infected burn wound fluids allowed quantification of α -amylase activity within these fluids as well as providing further evidence that unmasking would be clinically feasible.

Significantly increased wound α -amylase activity, compared to serum, suggests that α -amylase-triggered local re-instatement of nanoantibiotic activity is feasible, and supports a novel, therapeutic role for α -amylase in infection. A similar therapeutic role for α -amylase has been suggested in the management of chronic wounds (Hardwicke *et al.* 2008a) and cancer (Ferguson and Duncan 2009) in relation to PUMPT facilitation (Duncan *et al.* 2008).

Despite the pathological insult presented by burn surgery and infection, in this study, serum α -amylase activity was within normal physiological range of 30-110 IU/L (Branca *et al.* 2001). Physiological plasma α -amylase activity provides a baseline level that conjugates need to contend with in transit from the site of administration to the target site. Several studies have adopted these levels during the initial physicochemical characterisation and optimisation of dextrin-colistin conjugates (Ferguson and Duncan 2009; Hardwicke *et al.* 2008a). Literature suggests that α -amylase-mediated degradation of succinoylated dextrans is compatible with unmasking over a wide range of α -amylase activity, from 90 to 8,000 IU/L (Hardwicke *et al.* 2010; Hreczuk-Hirst *et al.* 2001a). In this study local α -amylase concentration was markedly higher than plasma, in keeping with findings in other disease states such as ovarian, lung and breast cancer (Grove 1994; Weitzel *et al.* 1988; Zakowski *et al.* 1984). Increased α -amylase activity at the infected site

would favour locally triggered polymer degradation and re-instatement of bioactivity specifically at the target site (Duncan 2011), in keeping with the tenets of PUMPT (Duncan *et al.* 2008).

In this study, mean time of sample collection was more than 2 weeks post-burn, when vascular permeability, interstitial fluid leakage and fluid shift (classically reported to occur in the first 24 h post-injury) have subsided (Pham *et al.* 2008; Ward and Till 1990). Moreover, these burn injury-related phenomena principally occur after major burns (> 25 % total body surface area) (Warden 2007). In this study, significant differences in wound versus plasma α -amylase activity were observed throughout a range of body surface areas involved in thermal injury. The protein concentration reported in these samples confirmed the presence of an exudate, which is typical of infected wounds (Longmore *et al.* 2010).

Few studies have investigated α -amylase production in Gram-negative pathogenic species (Momma 2000). Investigating the bacterial contribution to the significantly higher local α -amylase concentration showed that no bacterial strains used in this study produced amylase (Figure 5.4). It could, therefore, be argued that that local amylase accumulation occurs in response to an infection, rather than due to production by specific pathogens. These findings suggest that α -amylase activity reported in infected wound fluid is of human origin and imply that accumulation of endogenous α -amylase at an infected focus may occur by EPR (Sections 1.4.4 and 1.7). Consequently, locally-controlled, enzymatically-triggered re-instatement of bioactivity may depend on the generic production of an EPR effect in infection, independent of the actual infecting strain, suggesting this paradigm may be harnessed in a wide variety of infections.

5.5.2 Model evaluation

Several *in vitro* models have been used to study antibiotic PK/PD profiles, offering a choice from various levels of complexity to emulate *in vivo* conditions (Gloede *et al.* 2010). Although traditional TTK models have been extensively used to describe the effects of different antibiotics on a bacterial

pathogen (viable bacterial cells) in response to a fixed antibiotic challenge these models presented several drawbacks (Section 5.1.3).

Time-kill models reported in the literature typically describe bacterial counts up to 24 h post-dose (for example Liang *et al.* 2011; Owen *et al.* 2007; Tang *et al.* 2009a). This approach was not appropriate for a controlled release formulation whose maximal unmasking was observed between 24-48 h. These models were also unable to prevent the potential alteration of α -amylase activity by artificial media used to sustain bacterial growth (Section 5.1.3). In contrast, the static, two-compartment dialysis bag model under infinite sink conditions offered a simple and cost-effective method for a PK/PD evaluation of locally triggered, α -amylase-mediated unmasking of dextrin-colistin conjugate.

Several considerations were essential to the viability and reproducibility of this model. Selection of Spectra/POR 7[®] membrane avoided extensive, non-specific colistin binding to other commonly used membranes, such as Amycon[®], and reduced the risk of heavy metal contamination that could interfere with enzyme activity (Dudhani *et al.* 2010b; Li *et al.* 2003b). An IC:OC volumes ratio of 1:4 and total volume of 20 mL in the system maintained infinite sink conditions (Caffrey, 2006), provided a concentration gradient as a driving force, and ensured that saturation of the OC with unmasked conjugate would not occur, whilst minimising antibiotic use. An infinite sink strategy presented a convenient means to achieve changing drug concentrations (Gloede *et al.* 2010). Consistent and reproducible results were achieved during validation studies. Colistin concentration in the OC increased over the time-course of the experiment, and this was mirrored by an increase in antimicrobial activity in the same compartment. Final distribution of masked and unmasked dextrin-colistin conjugate upheld the initial physicochemical characterisation studies of colistin unmasking (section 3.5.3). It also confirmed that unmasked conjugate was equally distributed between compartments whilst intact or minimally unmasked colistin was largely retained in the IC. Membrane integrity to α -amylase and to masked conjugate was also confirmed.

Considering the controlled release nature of the dextrin-colistin conjugate, it was essential to provide an incubation environment that emulated *in vivo* conditions, whilst ensuring bacterial viability throughout. Therefore, 37 °C was selected to mimic *in vivo* conditions and constant agitation (70 RPM) reduced the risk of boundary layer effects at the dialysis membrane (Schwartz *et al.* 2002). Growth control curves were in keeping with other TTK studies performed under similar conditions, with regard to curve shape and *A. baumannii* bacterial counts (Liang *et al.* 2011; Tan *et al.* 2007).

In contrast to traditional TTK studies, this versatile model allowed increasing levels of complexity to be constructed, to emulate *in vivo* conditions around a focus of infection. Outer compartment modification by introduction of a quantifiable *A. baumannii* inoculum allowed measurement of the effect of bacterial viability over time in response to colistin, EA-4, or a colistin sulfate+EA-4 combination in the presence of α -amylase. Modification of the IC to incubate EA-4 directly in *ex vivo* infected wound fluid further allowed the viability of endogenous α -amylase mediated dextrin-colistin unmasking in an *ex vivo* setting to be investigated.

5.5.3 PK/PD modelling against MDR *A. baumannii* isolates

Colistin is a concentration-dependent antibiotic which exhibits a limited post-antibiotic effect only at high concentrations (Li *et al.* 2006, 2005c; Owen *et al.* 2007). In keeping with previous studies (Giamarellos-Bourboulis *et al.* 2001; Tan *et al.* 2007), colistin sulfate produced rapid bacterial killing followed by substantial regrowth (Figure 5.8). Indeed, Owen *et al.*'s (2007) TTK studies reported substantial *A. baumannii* regrowth at 24 h after a colistin challenge even at 64 x MIC. Current clinical dosing regimes vary substantially, and are disputed by the limited *in vivo* PK/PD studies available (Section 1.2.5). Moreover, several studies have reported that bacterial recovery occurs earlier than the shortest currently recommended dosing interval for colistin (Ganapathy *et al.* 2009; Landman *et al.* 2008).

In contrast, dextrin-colistin conjugate activity was more sustained, reflecting the need for unmasking, although bacterial recovery was substantially

suppressed over a prolonged time-span. These results demonstrated that the EA-4 dextrin-colistin conjugate suppressed bacterial growth for up to 6 times longer than an equivalent dose of colistin sulfate. The controlled, sustained release and consequent bacterial killing reported by α -amylase-triggered EA-4 over 48 h compared to colistin sulfate may, therefore, present several advantages over the traditional formulation. Such advantages reported in the literature for extended release dosage formulations included maximised therapeutic effects, minimised antibiotic resistance and improved patient compliance (reviewed in Gao *et al.* 2011). Based on these principles, several concentration-dependent antibiotics have been incorporated into extended release mechanisms: for example ciprofloxacin in a bilayer matrix (Talan *et al.* 2004), azithromycin microspheres (Blasi *et al.* 2007) and liposomal amikacin (Meers *et al.* 2008). Recently, release kinetics of colistin-laden liposomes have been described (Wang *et al.* 2009). However, rapid release (< 10 min) limited their effectiveness for controlled release. In contrast, covalent bonding of polymer to payload confers dextrin-colistin conjugates additional advantages to liposomes, including stability (Section 1.3.2) and localised, enzymatically triggered unmasking. Moreover, dextrin-colistin conjugate is bactericidal in a concentration-dependant manner (Figure 5.8b), suggesting that accumulation at infected sites by EPR (Chapter 3) could further enhance its antibacterial efficacy. Additionally, the colistin sulfate+EA-4 combination exhibited optimal characteristics of both rapid bacterial killing and slower regrowth up to 48 h. Combination therapy would, therefore, present the additional advantage of a rapid decrease in bacterial load, followed by sustained suppression of regrowth.

5.5.4 Colistin unmasking in infected wound exudates

Incubation of dextrin-colistin conjugates with patient-derived burn wound exudates revealed an increasing concentration of colistin in the OC over time, demonstrating that colistin would be readily liberated from the conjugate within the infected sites due to local α -amylase activity within the wound environment. Unmasked colistin content at 48 h was apparently higher than the unmasking predicted from previous experiments (Section 3.4.3.3).

However, the ELISA kit employed in this system measured total colistin (masked and unmasked). Masked conjugate constituted $18.3\% \pm 10.6$ (mean \pm SD) of total OC colistin. Therefore, adjusting for this data, the unmasking at 48 h reported in Figure 5.6b was in keeping with physicochemical characterisation studies reported in section 3.4.3.3. Prior experiments determined that bioresponsive unmasking of dextrin-colistin conjugate in the presence of α -amylase was feasible. However, under control conditions, only limited degradation occurred over time (Section 3.5.3). In keeping with these findings, IC α -amylase-mediated unmasking resulted in decreasing molecular weight of the dextrin-colistin conjugate, allowing diffusion through the dialysis membrane, and consequently, increasing OC colistin concentration. The corrected unmasking values compare favourably to published values for CMS, for which conversion to colistin in *ex vivo* human plasma samples after 48 h varied from 60.3% to 62.8% (Li *et al.* 2003a). Modification of the two-compartment, static, dialysis bag model under infinite sink conditions to an *ex vivo* model, emulated *in vivo* conditions around a focus of infection, to demonstrate the viability of local, endogenous α -amylase-mediated unmasking. In conformity with these findings, a recent study reported that incubation of a dextrin-EGF conjugate in chronic wound fluid also led to sustained, endogenous α -amylase-mediated EGF release over 48 h (Hardwicke *et al.* 2010).

5.6. Conclusion

In conclusion these studies demonstrated significantly increased endogenous α -amylase activity in *ex vivo* infected wound samples, which supported the concept of locally triggered dextrin-colistin unmasking. A static two-compartment dialysis bag model under infinite sink condition was also validated for evaluating the relationship between drug release, bacterial killing, and the viability of endogenously mediated dextrin-colistin unmasking directly in *ex vivo* infected wound fluid samples. This versatile model allowed increasing levels of complexity to be constructed.

Sustained killing of *A. baumannii* by dextrin-colistin conjugate was observed. The combination of the dextrin-colistin conjugate with colistin sulfate demonstrated rapid bacterial killing followed by significantly delayed bacterial regrowth. Incubation of EA-4 in *ex vivo* samples confirmed that colistin could be readily liberated from the conjugate in infected sites due to local α -amylase activity, supporting the notion of elevated, local reinstatement of activity. The encouraging results reported in this study justified further investigation of dextrin-colistin conjugates using animal models to evaluate *in vivo* pharmacokinetics and tolerance.

Chapter Six

In Vivo Evaluation of Dextrin-colistin Conjugates

6.1 Introduction

Previous experiments have identified a lead dextrin-colistin conjugate for which masking / unmasking in the acute infection environment was viable, *in vitro* and *ex vivo*. Antimicrobial activity at maximum unmasking was generally equivalent to the current clinical alternative (CMS), and demonstrated both rapid and prolonged killing in a modified TTK model. Following these encouraging studies, a preliminary investigation into its *in vivo* pharmacokinetics and clinical toxicity was the next essential step, prior to further pre-clinical development.

6.1.1 *In vivo* colistin pharmacokinetic studies

The development of colistin for clinical use pre-dated requirements for PK optimisation that modern drugs must satisfy. There is, therefore, limited data in this field (Li *et al.* 2006; Nation and Li 2007). Moreover, many early studies employed microbiological assays, whose accuracy has been questioned (Section 6.1.3). Colistin's clinical toxicity has been partly attributed to the lack of suitable PK information (reviewed in Azzopardi *et al.* 2012). PK evaluation of the dextrin-colistin conjugate would therefore be advantageous.

In their seminal work, Li *et al.* (2003b) used pre-column derivatisation and RP-HPLC to measure colistin concentration in plasma and urine. In this study, bolus IV administration of 1 mg colistin sulfate/kg to Sprague Dawley rats resulted in a short distribution phase followed by a log-linear decline, with an elimination $t_{1/2}$ of 1.2 h. Colistin clearance was estimated to be 5.2 ± 0.4 mL/min/kg, with <1% of colistin being excreted unchanged in urine. These results were repeated in later publications investigating novel polymyxin derivatives (Ali *et al.* 2009) and the co-administration of melatonin or ascorbic acid with colistin as nephroprotective agents (Yousef *et al.* 2011, 2012).

Several PK parameters have been used to define colistin's efficacy, impeding straightforward comparisons (Falagas and Kasiakou 2005). However, common trends could be discerned from these *in vivo* PK analyses. Following a rapid distribution phase, which ranged from ≤ 5 min (IV

administration of colistin sulfate) to 20 min (colistin released after IV administration of CMS) (Li *et al.* 2004; Marchand *et al.* 2010), studies concur that plasma colistin concentration follows a log-linear decline (Table 6.1). The last quantifiable concentration (C_{last}) recorded in these studies varied from 180-240 min and colistin's $t_{1/2}$ varied from 0.6 to 1.2 h (Table 6.1). However, these variations could be related to dissimilar sampling intervals and lower limits of quantification reported in these studies. Pharmacokinetics of colistin released from CMS remained linear over a range of IV doses (Li *et al.* 2004; Marchand *et al.* 2010).

6.1.2 Study design considerations

The Sprague Dawley rat is an outbred animal model which has found extensive favour as a cost-effective model in drug development and pharmacokinetic studies (Morgan *et al.* 2012), including the development of colistin and its derivatives (Table 6.1). Moreover, the IV route of administration has been applied in the vast majority of polymer therapeutics entering *in vivo* trials (reviewed in Duncan and Gaspar 2011; 2009) and the majority of *in vivo* PK colistin studies conducted in animals have used this route (Table 6.2). In these colistin PK studies, plasma harvesting was performed by centrifugation (Table 6.2).

Antibiotic administration and sampling procedures differed between studies (Table 6.2). Dose administration and sample collection through the same vascular catheter required washing of the cannula, which complicated the experimental procedure (Li *et al.* 2003b). On the other hand, tail vein injection for IV antibiotic administration is well-established and may reduce the potential complications of multiple *in situ* IV catheters such as thrombogenicity (Jin *et al.* 2009; Kaushal and Shao 2010; Lagas *et al.* 2008; Turner *et al.* 2011). Several colistin dosing regimes were encountered in the literature, depending on the studies' terms of reference (Couet *et al.* 2012). Marchand *et al.* (2010) administered bolus IV CMS to Sprague Dawley rats at doses up to 120 mg/kg, with 16.7 mg/kg colistin appearing in the plasma ≤ 5 min post-dose. Therefore, tail vein administration of an

Table 6.1: Colistin *in vivo* pharmacokinetic data on studies performed on Sprague Dawley rats available in the literature (2003-present)

Study	Main terms of reference	Dose and Formulation	Plasma concentration-time curve	$t_{1/2}$ (h)	C_{last} (min)	Reference
1	PK of colistin after bolus IV dose	Colistin sulfate, (1 mg/kg in 300 μ L bolus IV)	Short initial distribution phase then a slower log-linear decline	1.2 (\pm 0.2)	240	Li <i>et al.</i> (2003b)
2	CMS + colistin disposition after IV CMS dose	CMS (15 mg/kg bolus IV)	Short initial distribution phase for both CMS and colistin, 20 min) then log-linear decline	0.9 (\pm 0.3)	NR	Li <i>et al.</i> (2004)
3	PK of novel antimicrobial cationic derivatives of colistin	Colistin sulfate (1 mg/kg) IV	Short initial distribution phase followed by log-linear decline over 2 orders of magnitude	1.2 (\pm 0.2)	NR	Ali <i>et al.</i> (2009)
4	Nephroprotective effect of melatonin against colistin	Colistin sulfate (0.5 mg/kg IV bolus)	Short initial distribution phase followed by slower log-linear decline	1.1 (\pm 0.1)	180	Yousef <i>et al.</i> (2011)
5	PK linearity in a range of doses	CMS IV bolus at escalating doses (5-120 mg/kg)	Short initial distribution phase (<5 min). Log-linear decline	0.6 (\pm 0.1)	180	Marchand <i>et al.</i> (2010)
6	Effects of ascorbic acid on colistin induced nephrotoxicity	Colistin sulfate IV bolus (0.5 mg/kg)	Short initial distribution phase followed by log-linear decline	1.2 (\pm 0.2)	180	Yousef <i>et al.</i> (2012)

NR, not reported. All values are reported as mean \pm SD, to 1 d.p.

Table 6.2: Methodological comparisons of prior *in vivo* colistin PK studies in Sprague Dawley rats

Weight at dosing (g)	Cannulation and administration	Sample (μL)	Sample processing	Sampling route and interval	Reference
300-350	Colistin sulfate jugular vein collection	400	Plasma harvested by heparin anticoagulation and centrifugation.	Via jugular vein: 0, 10, 20, 30, 60, 90, 120, 180, and 240 min	Li <i>et al.</i> (2003b)
300-350	CMS administered in 300 μL sterile saline through jugular vein cannula.	500	Plasma harvested by centrifugation	Via jugular vein: 0, 5, 10, 20, 30, 60, 90, 120 and 180 min	Li <i>et al.</i> (2004)
Adult rats, weight not reported	Colistin (1 mg/kg) in 300 mL of sterile saline as a bolus into the jugular vein.	200	Plasma harvested by centrifugation	Via jugular vein: 0, 10, 20 and 30 min, and 1, 1.5, 2, 3 and 4 h	Ali <i>et al.</i> (2009)
284 \pm 22 *	Colistin sulfate, via jugular vein. Ascending (cumulative) dose regimen from: 0.5 mg/kg on day 1, to 4.0 mg/kg on day 7	300	Plasma harvested by centrifugation	Via carotid artery: 0, 30 min post-dose on days 1, 3, and 6, and 7	Yousef <i>et al.</i> (2011)
265-335	CMS, doses of 5, 15, 30, 60 or 120 mg/kg via femoral vein;	300-400	Plasma harvested by centrifugation	Via femoral artery: 0, 5, 15, 30, 60, 90, 120, 150 and 180 min	Marchand <i>et al.</i> (2010)
272 \pm 10 *	Colistin sulfate, via jugular vein, twice daily 8h apart for 7 days.	300	Plasma samples harvested by centrifugation	Via carotid artery: 0 h, 10, 30, 60, 90, 120, 180 and 360 min	Yousef <i>et al.</i> (2012)

*(mean \pm SD). All studies were performed on Sprague Dawley rats

equivalent dose was selected to facilitate comparison of colistin sulfate and dextrin-colistin conjugates in terms of clinical toxicity and colistin concentration in plasma samples.

6.1.3 Colistin assay methods for *in vivo* studies

A consideration of the currently available quantification methods was essential in determining an accurate strategy for analysing plasma samples. Quantification of colistin in biological media presents a formidable challenge since it has no inherent fluorescence and poor UV absorption (Dotsikas *et al.* 2011; Govaerts *et al.* 2003; Li *et al.* 2001a). Microbiological bioassays using *Bordetella bronchiseptica* as the indicator organism are commonly used as they are inexpensive and avoid use of specialist equipment (David and Gill 2008; Healy *et al.* 2011a; Li *et al.* 2005a). However, this method lacks the sensitivity required for these *in vivo* studies and the microorganism and its growth medium are difficult to obtain. More recently, an *E. coli* microbiological bioassay for colistin was described (Wootton *et al.* 2005). In this method, colistin concentration is derived from plotting mean diameter of a zone of inhibition after diffusion through agar, versus log concentration. However, assay precision varied from 3.5–18.8% and the lower limit of quantification was only 0.5 mg/L. The reliability of diffusion methods has been questioned (Section 4.1.3). Moreover, previous experiments have shown that dextrin-colistin conjugates exhibited different antimicrobial activity to colistin sulfate, making interpretation of this bioassay difficult (Section 4.4.2).

More recently, HPLC-based methods have been preferred for colistin quantification in biological fluids (Table 6.3). Typically, samples are initially purified by ACN or TCA extraction, then analysed by RP-HPLC, coupled with either pre-column derivatisation or post-column mass spectrometry analysis (Table 6.3). Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) reported lower quantification limits (10.4 µg/L, Table 6.3). However, it requires specialist equipment which is not widely available. Protein purification methods, on which LC-MS/MS techniques still rely for purification from the biological sample, also vary in efficiency, and co-elution

Table 6.3: Comparison of the various methods for detection of colistin in biological samples described in the literature

Matrix	Sample preparation; extraction; Concentration	Derivatisation and assay method	Lower Limit of Detection	Study
Feeds	Sonication in HCl; SPE (C ₁₈); MeOH/HCl; sample concentration 1:20	OPA-Merc; RP-HPLC using Ultracarb 5 µm column in ACN/sodium sulfate	1000 mg/kg	Cancho-grande <i>et al.</i> (2001)
Human plasma	MeOH-TCA; Extraction by centrifugation and MeOH-HCl; sample concentration 1:6	FMOC-Cl derivatisation (C ₁₈ SPE). RP-HPLC using ultrasphere C ₁₈ column in ACN:THF:Water	0.1 mg/L	Li <i>et al.</i> (2001a)
Serum (rat, dog)	N/A; 96-well extraction disk-plate extraction; sample concentration 2:1	Dansyl chloride derivatisation; RP-HPLC (Zorbax eclipse column in MeOH-acetic acid)	0.05 mg/L	Gmur <i>et al.</i> (2003)
Rat plasma	MeOH-TCA; extraction by centrifugation* (4 °C) MeOH-HCl; sample concentration 1:6	FMOC-Cl (C ₁₈ SPE); C ₁₈ column in ACN/THF/H ₂ O (50:30:20 v:v:v)	0.1 mg/L	Li <i>et al.</i> (2003b)
Rat plasma	MeOH-TCA; centrifugation *(4 °C), MeOH-HCl; sample concentration 1:6	FMOC-Cl (C ₁₈ SPE); RP-HPLC on Ultrasphere C ₁₈ column in ACN/THF/water (50:30:20 v:v:v)	0.1 mg/L	Li <i>et al.</i> (2004)
Raw milk	Oxalic acid, TCA-HCl; SPE (sulfonic acid /silane) MeOH, ACN, HCl, TEA, potassium acetate; sample concentration 2:1	Derivatisation with OPA-Merc; RP-HPLC with cromolith column in ACN/phosphate	0.0143 mg/kg	Suhren and Knapstein (2005)
Human plasma	ACN; Centrifugation* (RT °C)	FMOC-Cl derivatisation; RP-HPLC in ACN/water/acetic acid (50:25:25 v:v:v)	0.125 mg/L	Cao <i>et al.</i> (2008)
Human plasma, urine	MeOH-TCA; centrifugation* (4 °C); SPE (Oasis HLB); elution with MeOH/H ₂ O/acetic acid (80:90:1 v:v:v)	RP-HPLC in ACN, water and acetic acid (80:19:1, v:v:v); LC-MS/MS	0.028 mg/L	Ma <i>et al.</i> (2008)
Mouse brain	TCA; centrifugation (16,000 g x 15 min, RT)	FMOC-Cl derivatisation; RP-HPLC in ACN/water/acetic acid (80:19:1, v:v:v)	93 µg/kg	Jin <i>et al.</i> (2009)
Human plasma	0.1% TFA in ACN (200 µL)	RP-HPLC (Bondapak C ₁₈) in 25% ACN in 0.03% TFA; LC-MS/MS	10.5 µg/L	Jansson <i>et al.</i> (2009)
Human plasma	ACN; centrifugation *(RT)	FMOC-Cl derivatisation; RP-HPLC in ACN:water:acetic acid (50:25:25 v:v:v)	0.125 mg/L	Dudhani <i>et al.</i> (2010a)

MeOH methanol, HCl hydrochloric acid, LOQ, limit of quantification, RT room temperature. * centrifugation performed at 1000g for 10 min,

of contaminants is a valid concern (Dotsikas *et al.* 2011). Pre-column derivatisation with FMOC-Cl has gained considerable popularity over ortho-phthalaldehyde (OPA) and dansyl chloride labelling because of the increased stability of FMOC-colistin derivatives (Gmur *et al.* 2003; Le Brun *et al.* 2000; 2001a). Successive methodological refinements in this technique resulted in consistently lower limits of quantification (range 0.125 - 4 mg/L), and shortened analytical times (Cao *et al.* 2008; Dudhani *et al.* 2010a; Li *et al.* 2003b), favouring selection of this method to evaluate sample colistin content in this study.

A common limitation in colistin PK studies conducted in animals is that the lower limit of colistin quantification (0.125 mg/L) was reached before the end of the experiment (Li *et al.* 2004; Yousef *et al.* 2011, 2012). ELISA determination of colistin concentrations was first reported by Kitagawa *et al.* (1985). Traditionally, preparation of colistin immunogen for immunochemical determination in biological samples was complex and time-consuming (Hammer 1998; Suhren and Knappstein 2005). More recently a proprietary, competitive ELISA kit for colistin extraction and quantification from animal samples, with quantification limits from 0.5 to 50 µg/L has become commercially available (BioO Corp 2012). This method was successfully used to investigate *ex vivo* pharmacokinetics (Chapter 5).

6.2 Experimental aims

The aims of this chapter were:

1. To establish a PK profile of colistin sulfate, EA-4 and EA-1 dextrin-colistin conjugates in an *in vivo* Sprague Dawley rat model
2. To compare the toxicity of colistin sulfate to dextrin-colistin conjugates through observation of *in vivo* clinical adverse events

6.3 Methods

General methods used, including ELISA (Section 2.5.4) were previously described.

6.3.1 Animal husbandry

Thirty Sprague Dawley rats aged 8-10 weeks at dosing were used in this study. The mice were housed in groups of 4 to 5 animals, according to Home Office regulations. All animals were acclimatised to the experimental unit for 1 week prior to the study. During the acclimatisation period the animals were closely observed to ensure that they were in good health and suitable for inclusion in the study. During the pre-trial period, the rats were housed in solid floored polycarbonate and stainless steel caging with bedding material. Animals were fed a standard laboratory diet of known formulation which did not contain colistin, polymyxin or bacitracin (Section 2.4). Domestic tap water was available *ad libitum*. Holding and study areas had automatic control of light cycles and temperature. Light hours were 0700 to 1900 h. Ranges of temperature and humidity measured during the study were 20 - 24 °C and 50-73% respectively. All animal procedures were carried out in a Home Office Licensed Establishment (experiment no. 195183). For phases 1 and 2, six male Sprague Dawley rats were used. In phase 3, twenty-four Sprague Dawley rats were used (Appendix 2.4). All animals were weighed prior to dose administration.

Animals were monitored for clinical signs at regular intervals throughout the study in order to assess any reaction to treatment. To compare the *in vivo* "single dose" toxicity data of colistin to dextrin-colistin conjugates the rats were constantly monitored for the presence of adverse events throughout the study.

6.3.2 Dose preparation

Dextrin-colistin conjugates EA-4 (1.0 mol% succinylation, 10.75 % w/w colistin content) and EA-1 (7.1 mol% succinylation, 15.2 % w/w colistin content) were used. Unless otherwise specified, the concentration of the doses described in this study refers to colistin component of the conjugate. Formulations were prepared on the morning of dose administration (Table 6.4). Doses for phases 1 and 2 were prepared as detailed in Table 6.5. In phase 3, each pre-weighed test item was dissolved to achieve a stock

concentration of 0.5 mg/mL. An appropriate volume of each stock (where required) was made up to volume with 0.9% sterile saline to achieve a target dose concentration of 0.01, 0.05, 0.1, or 0.5 mg/mL. Each formulation was filtered (0.22 μ m) prior to administration and stored at -20 °C. For example, for a 0.1 mg/kg colistin sulfate dose (phase 3), 7.0 mg was dissolved in 14.2 mL sterile saline to produce 0.49 mg/mL stock solution. This was then diluted by a factor of 5 to produce a final solution of 0.1 mg/mL.

6.3.3 Dose administration

Antibiotic administration was achieved via the tail vein in each phase of the study. In phase 1, colistin sulfate was administered IV to a single animal at a target dose of 15 mg/mL. In phase 2, five rats received a bolus IV administration of colistin at a target dose of 0.5 mg/mL. In phase 3, an ascending dose approach was used. Four groups of six rats each received a single dose of 0.01, 0.05, 0.1 and 0.5 mg/kg of each test item. A bolus IV dose was administered over ~ 30 s at a target dose volume of 1 mL/kg (Table 6.5). Each group was dosed on consecutive days to ensure the previous dose level was suitable for the continuation of the study. For each test item and dose level, 2 rats were dosed IV via the tail vein, as a bolus over ~30 s, at a target volume of 1 ml/kg. Typically, for a 0.1 mg/mL dose in a rat with body weight 0.41 kg, 0.04 mg was dissolved in 0.9% saline (0.41 mL) to produce a final concentration of 0.1 mg/kg.

6.3.4 Sample collection

In phases 1 and 2, animals were heated to facilitate sample extraction by the tail vein. In phase 3, each rat was supplied with jugular vein cannulation to facilitate blood sampling. Following tail vein administration, blood samples (0.3 mL) were harvested from the jugular vein cannula into tubes containing lithium heparin at: 5 min, 30 min, 1 h, 4 h, 8 h, and 24 h post-dose. Plasma was generated by centrifugation (3,000 rpm for 10 min at 4 °C) and immediately stored at -20 °C until analysed. Immediately prior to analysis samples were warmed to room temperature and vortexed for 3 min.

Table 6.4: Preparation and formulation of the doses administered to Sprague Dawley rats.

Phase	Rats (n)	Target dose (mg/kg)	Test antibiotic	Weight of colistin (mg)	Sterile 0.9% saline (mL)	Stock (mg/mL)	Volume of stock (mL)	Volume of sterile saline (mL)	Calculated concentration (mg/mL)
1	1	15	Colistin	-	-	-	-	-	15
2	5	0.5	Colistin	3.4	-	-	-	6.8	0.5
3	2	0.01	Colistin	4.3	8.6	0.5	0.2	9.8	0.01
	2		EA-1	3.3	6.6	0.5	0.2	9.8	0.01
	2		EA-4	3.2	6.4	0.5	0.2	9.8	0.01
	2	0.05	Colistin	4	8	0.5	1.0	9.0	0.05
	2		EA-1	2.9	5.8	0.5	1.0	9.0	0.05
	2		EA-4	3.2	6.4	0.5	1.0	9.0	0.05
	2	0.1	Colistin	7	14.2	0.5	2.0	8.0	0.1
	2		EA-1	3.0	6.0	0.5	2.0	8.0	0.1
	2		EA-4	3.1	6.2	0.5	2.0	8.0	0.1
	2	0.5	Colistin	3.4	-	-	-	6.8	0.5
	2		EA-1	2.8	-	-	-	5.6	0.5
	2		EA-4	3.6	-	-	-	7.2	0.5

Table 6.5: Pre-dose body weight and nominal dose administered to male Sprague Dawley Rats following IV administration of test compounds

Phase	(n)	Sprague Dawley Rat ID	Body weight (kg)	Target dose (mg/kg)	Test antibiotic	Weight of administered dose (g)	Dose concentration (mg/mL) *	(mg)	Colistin equivalent (mg/kg)
1	1	001M	0.281	15	Colistin	0.558	7.50	4.185	14.9
2	5	001R	0.329	0.5	Colistin	0.335	0.50	0.168	0.51
		002M	0.303			0.300		0.150	0.5
		003M	0.338			0.342		0.171	0.51
		004M	0.348			0.349		0.175	0.50
		005M	0.329			0.330		0.165	0.50
3	2	016M	0.370	0.01	Colistin	0.370	0.01	0.004	0.01
		017M	0.370			0.380		0.004	0.01
	2	018M	0.390		EA-4	0.400		0.004	0.01
		019M	0.360			0.370		0.004	0.01
	2	020M	0.370		EA-1	0.380		0.004	0.01
		021M	0.360			0.370		0.004	0.01
	2	022M	0.370	0.05	Colistin	0.370	0.05	0.02	0.05
		023M	0.340			0.340		0.02	0.05
	2	024M	0.360		EA-4	0.360		0.02	0.05
		025M	0.360			0.360		0.02	0.05
	2	026M	0.380		EA-1	0.380		0.02	0.05
		027M	0.360			0.330		0.02	0.05
	2	028M	0.410	0.1	Colistin	0.420	0.10	0.04	0.10
		029M	0.470			0.490		0.05	0.10
	2	030M	0.390		EA-4	0.400		0.04	0.10
		031M	0.390			0.400		0.04	0.10
	2	032M	0.390		EA-1	0.400		0.04	0.10
		033M	0.400			0.410		0.04	0.10
	2	034M	0.410	0.5	Colistin	0.420	0.50	0.21	0.51
		035M	0.400			0.410		0.21	0.51
	2	036M	0.310		EA-4	0.320		0.16	0.51
		037M	0.370			0.380		0.19	0.52
	2	038M	0.420		EA-1	0.430		0.21	0.51
		039M	0.420			0.420		0.21	0.50

* calculated to 2 d.p.

6.3.5 Colistin assay

Several methods were explored to select an optimal method for analysing colistin concentration in the rat plasma samples. Determination of a suitable strategy for assaying colistin in the rat plasma samples was investigated in parallel with the animal dosing experiments, using model samples of colistin sulfate in PBS.

6.3.5.1 Evaluation of HPLC analysis for colistin

Pre-column FMOC-Cl derivatisation of colistin followed by RP-HPLC assay was initially performed, based on the method developed by Cao *et al.* (2008). Colistin samples (0.125 to 4 mg/L) were independently prepared in PBS. First, SPE cartridges (C₁₈, 100 mg sorbent 70Å pore size) were washed with acetone (1 mL), and conditioned with methanol (1 mL) followed by carbonate buffer (1 mL, 1% w/w, pH 10). ACN (100 µL) was then mixed with samples of PBS (100 µL) in a 1.5 mL Eppendorff tube, vortex-stirred for 30 s and centrifuged (10,000 g for 10 min at RT). The following procedure was then adopted:

- a) The sample (100 µL) was transferred to a preconditioned cartridge mounted on a vacuum manifold, and then the SPE cartridge was washed with carbonate buffer (1 mL).
- b) This was followed by FMOC-Cl (110 µL, containing 30 µL of 100 mM FMOC-Cl in ACN and 80 µL methanol).
- c) After 10 min of reaction time the FMOC-colistin derivative was eluted with acetone (900 µL) and the eluent was collected in a 2 mL Eppendorff tube.
- d) Boric acid (600 µL, 0.20 M) was added, followed by ACN (500 µL).

After vortex-mixing, an aliquot of the resulting mixture (500 µL) was pipetted into a short thread autosampler vial, loaded and injected onto the HPLC column (30 µL). The HPLC set-up was used as detailed in section 2.2.3.5. A calibration curve was constructed from the area under the chromatogram curve corresponding to colistin A and B. PBS served as control. The isocratic

mobile phase consisted of ACN: THF: water (50:25:25, v:v:v) at a flow rate of 1.0 mL/min.

6.3.5.2 Evaluation of HPLC analysis for dextrin-colistin conjugate

The procedure above was repeated for EA-4 conjugate. Given the lack of a quantifiable trace for the dextrin-colistin conjugates using this method, the RP-HPLC procedure was modified systematically as detailed in Table 6.6. First, flow rate and column length were varied (isocratic method). Then, a gradient method was set up as follows, using a 250 mm C₁₈ column. Mobile phase A was 0.01% TFA in water. Mobile phase B was a mixture of ACN and 0.01% aqueous TFA (9:1, v:v). At 0 - 3 min mobile phase A was fixed at 100% using a flow rate of 0.6 mL/min. From 3 - 28 min mobile phase B was linearly increased to 100%. From 28 - 33 min mobile phase B was held at 100%. From 33 - 38 min mobile phase B was linearly returned to 0% while mobile phase A was reciprocally increased to 100% to re-equilibrate the column. During these experiments, detection by monitoring for fluorescence was maintained at an excitation wavelength of 260 nm and an emission wavelength of 315 nm (Section 2.2.3.5).

Given the lack of a quantifiable trace for the dextrin-colistin conjugates with variation of the HPLC analysis conditions, the precolumn labelling procedure was subsequently investigated. In these experiments, Blank PBS was used as negative control, whilst colistin sulfate in PBS served as a positive control. All experiments were performed at two known concentrations of EA-4 (4 mg/L or 8 mg/L) to enable any relationships between areas under the HPLC curve peaks to be identified. Analyses were performed using the gradient method described above with the 250 mm C₁₈ column:

i) The process for labelling EA-4 with FMOC-Cl was repeated as described above for colistin sulfate. However, effluent from each stage (a-d above) was collected, titrated to pH 7 using 0.01 M HCl solution and analysed for colistin content using the MaxSignal[®] ELISA kit (Section 2.5.4).

Table 6.6: Conditions investigated for synthesis and HPLC analysis of dextrin-colistin-FMOC derivatives

	Parameter	Range of variables explored		
HPLC analysis	Elution time	C ₁₈ column length: 50 mm	↔ C ₁₈ column length: 250 mm	
	Method	Isocratic method	Gradient Method	
	Mobile phase	ACN/THF/H ₂ O (50:25:25 v:v)	TFA to ACN (see text)	
	Flow rate	0.6 mL/min	1 mL/min	
Solid phase synthesis	SPE cartridge pore and particle size	Pore size (70 to 130 Å)	Packing particle size (50 -130 μm)	
	Pressure through SPE cartridge	1 mBar vacuum pressure	↔ Gravity	
Liquid phase synthesis	Liquid phase synthesis	Liquid phase synthesis in organic solvents		Liquid phase synthesis in aqueous phase solvents
	Organic synthesis (reaction time)	30 min	4 h	24 h
	Aqueous synthesis (aqueous volume)	200 μL	500 μL	1,000 μL
	Aqueous synthesis (reaction time)	30 min	4 h	24 h
	Aqueous synthesis (reaction pH)	pH 7	pH 7.4	pH 10
	Aqueous synthesis (temperature)	37 °C	↔	60 °C

ii) Liquid phase synthesis (in solution) was attempted next. From a mixture of ACN (100 μ L) and EA-4 sample (4 mg/L in 100 μ L PBS), an aliquot (100 μ L) was added to a 2 mL Eppendorff tube. Carbonate buffer (1 mL, 1% w/w in aq., pH 10), 110 μ L of Fmoc-Cl (containing 30 μ L of 100 mM Fmoc-Cl in ACN and 80 μ L methanol) were added. After 30 min reaction time, acetone (900 μ L) was added followed by boric acid (600 μ L, 0.20 M) and ACN (500 μ L). The reaction was repeated at different reaction times (30 min, 4 h and 24 h).

iii) Finally, aqueous phase synthesis of EA-4 - Fmoc derivatives was attempted based on the method described by Gawande and Branco (2011). Briefly EA-4 dextrin-colistin conjugate was weighed in a 1 mL Eppendorff tube. Fmoc-Cl (representing 50 x molar excess) was added, followed by ddH₂O (200 μ L). The reaction was terminated by lowering the pH through addition of 0.1 M HCL (1 mL), and boric acid (1:1 v:v, 0.02 M) was added and followed by immediated analysis. Conditions for this reaction were subsequently varied as detailed in Table 6.6. pH was varied by substituting ddH₂O with an equal volume of PBS (pH 7.4) or carbonate buffer (pH 10).

6.3.5.3 Analysis of colistin concentration using ELISA

Plasma from animals dosed at 0.1 mg/kg was analysed using the colistin ELISA kit as detailed in Section 2.5.4.

6.4 Results

6.4.1 Analysis of colistin assay

Construction of an HPLC calibration curve using colistin sulfate in PBS yielded a linear calibration curve between 0.125 - 4 mg/L (Figure 6.1 a, b). However, attempts at reproducing the same method for EA-4 produced no signal in the RP-HPLC chromatogram, as seen with the negative control run using PBS only (Figure 6.1c).

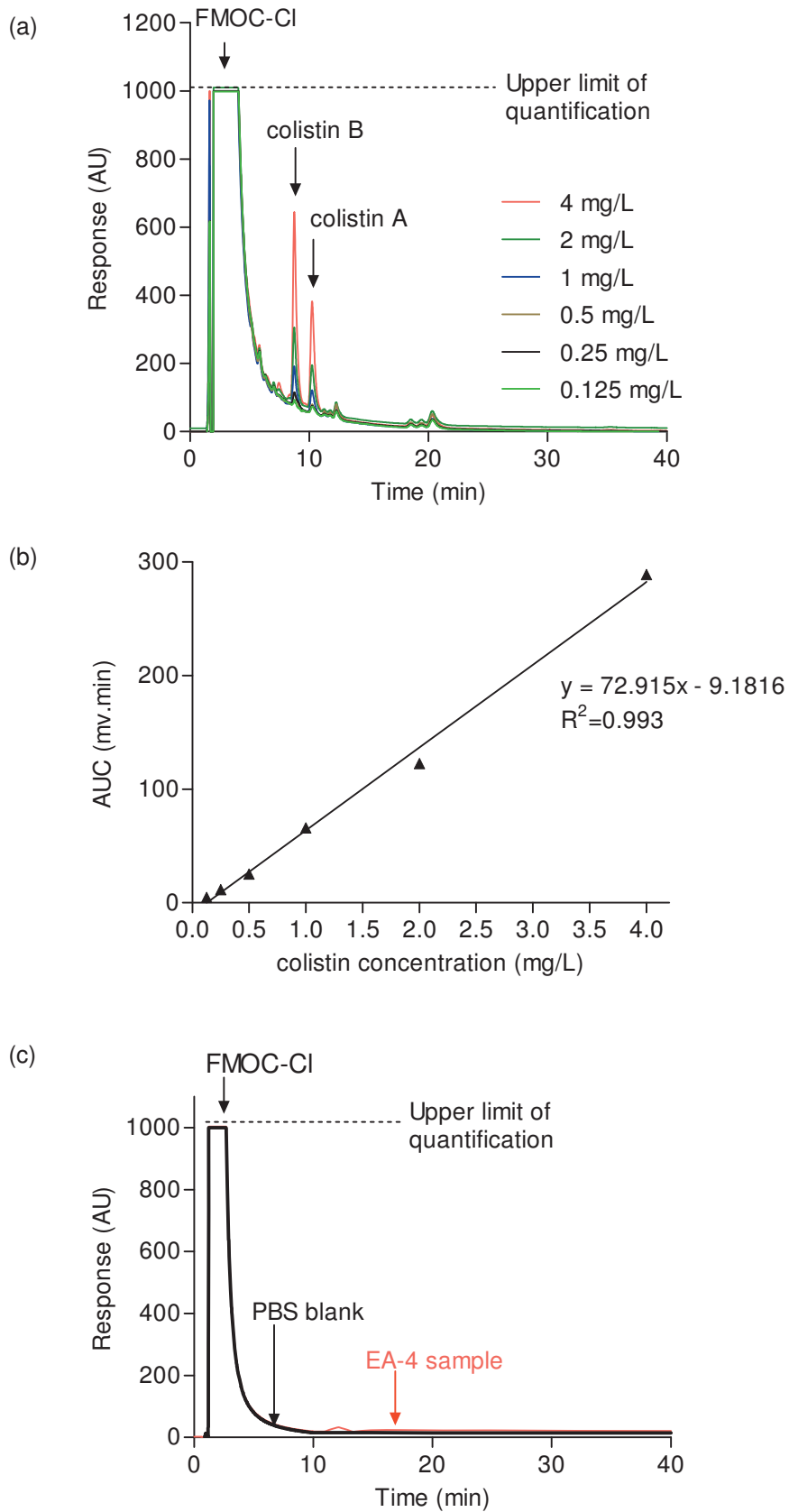


Figure 6.1: Typical RP-HPLC chromatograms from method validation. Panel (a) reports typical HPLC chromatograms for FMOCl-labelled colistin. Panel (b) reports the resulting calibration curve. Panel (c) reports a typical chromatogram for PBS and EA-4 after derivatisation on an SPE cartridge.

Similarly, no signal was noted when a range of different analytical conditions, as detailed in Table 6.6, were used. Increasing the SPE pore size and varying the sorbent particle size still produced no quantifiable trace on HPLC for EA-4. However, eluate analysis after loading the cartridge with EA-4 and washing with aqueous carbonate solution reported a significant concentration of colistin within the eluent, which doubled in concordance to the concentration of EA-4 loaded onto the column (data not shown). Further attempts to use liquid phase derivatisation also did not yield any quantifiable trace for dextrin-colistin conjugates when analysed, in contrast to colistin sulfate controls (Appendix 2.5). ELISA was therefore used as an alternative method for colistin quantification in plasma samples.

6.4.2 Experimental procedure

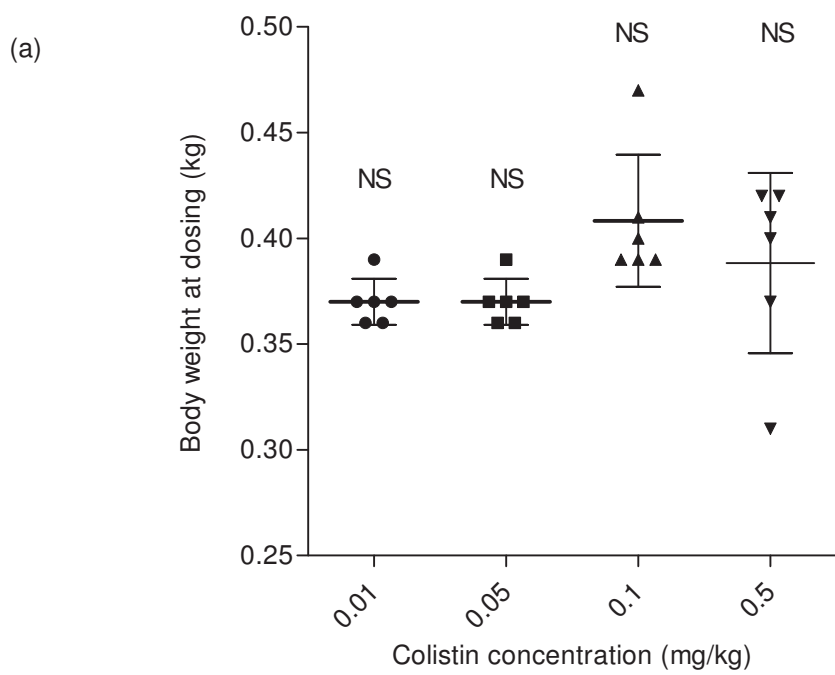
Pre-dose body weight did not vary significantly between the experimental subgroups ($p > 0.05$). Similarly, actual blood sampling times following IV administration did not vary significantly ($p > 0.05$; Appendix 2.6).

6.4.3 *In vivo* PK profiles of colistin and dextrin-colistin conjugates

Figure 6.2 reports the mean (\pm SD) total colistin concentration in plasma for colistin, EA-4 and EA-1, as a function of time. An initial distribution phase was observed for colistin (5 min), EA-4 (30 min) and EA-1 (60 min). In each case this was followed by a log-linear decline in concentration. For colistin sulfate, $t_{1/2}$ was 0.9 h over 2 orders of magnitude. In contrast, $t_{1/2}$, for EA-4 and EA-1 was 1.6 h and 18.6 h respectively (Figure 6.3). The last recorded colistin concentration (C_{last}) was 4 h, but for both EA-4 and EA-1, C_{last} was \geq 24 h. Area under the curve at 24 h (AUC_{24}) increased from 135 $\mu\text{g/L/h}$ (colistin) to 613 $\mu\text{g/L/h}$ (EA-4) and 1605 $\mu\text{g/L/h}$ (EA-1).

6.4.4 Observation of clinical adverse effects

In phase 1, rat 001M was found dead approximately 2 min post-IV dosing. In phase 2 of the study, all rats administered colistin sulfate experienced clinical signs which were outside the remit of the experimental license and were

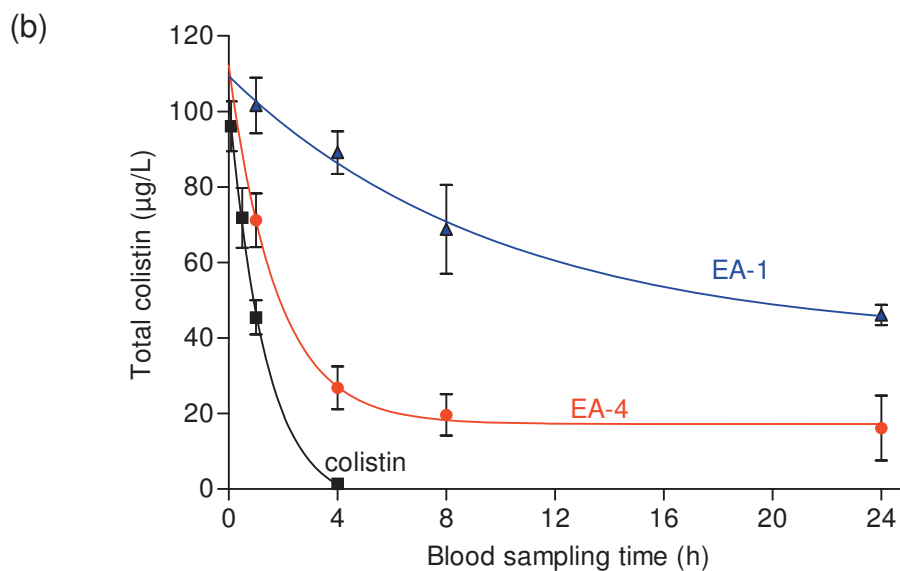
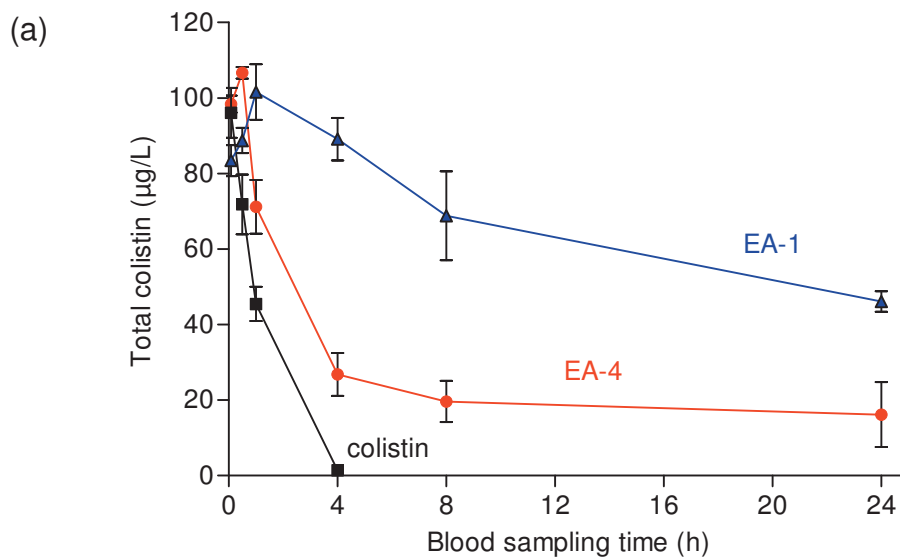


(b)

Dosing Group	"0.01 mg/kg"	"0.05 mg/kg"	"0.1 mg/kg"	"0.5 mg/kg"
	weight (kg)	weight (kg)	weight (kg)	weight (kg)
colistin	0.370	0.370	0.410	0.41
	0.370	0.370	0.470	0.40
EA-4	0.390	0.390	0.390	0.31
	0.360	0.360	0.390	0.37
EA-1	0.370	0.370	0.390	0.42
	0.360	0.360	0.40	0.42
Average weight (kg)*	0.37	0.37	0.41	0.39

* 2 d.p.

Figure 6.2: Two-way ANOVA (unweighted means ANOVA) of rat body weights reporting no statistical significance between different dosing and formulation groups ($p > 0.05$). Panel (b) reports the average weight in each dosing group. NS, not significant.



(c)

Antibiotic	Half-life (h)	95% Confidence interval (h)
Colistin	0.9	0.8 – 1.2
EA-4	1.6	1.2 – 2.1
EA-1	18.6	11.4– 24.6

Figure 6.3: Concentration of colistin in rat plasma over time after a 0.1 mg/kg dose. Panel (a) reports the increase in distribution and disposition phases from colistin through EA-1 to EA-4. Panel (b) reports the curve of best fit for same results from the disposition phase which was used to calculate $t_{1/2}$. Panel (c) reports $t_{1/2}$ and 95% confidence intervals for each antibiotic tested. Plasma from two rats was analysed for each antibiotic tested. Data reported represents mean \pm SD (n=2).

terminated on welfare grounds. In phase 3, EA-4 and EA-1 were well tolerated up to 0.5 mg/kg. However, both rats dosed with colistin sulfate at 0.5 mg/kg exhibited adverse effects precluding sample extraction during the first 2 h period. Animals appeared subdued, had dark muzzles, and their eyes and ears were dark and red. Following close observation, the animals returned to normal ~2 h post-dose and were suitable for continuation of blood sampling.

6.5 Discussion

The main aim of this chapter was to evaluate the PK distribution of dextrin-colistin conjugates in an *in vivo* model. Since the sampling and purification procedure produced low-volume plasma samples, it was essential to establish an optimal strategy with which to accurately determine colistin levels. Given that the majority of prior studies have used RP-HPLC, this working approach was initially adopted and satisfactorily replicated for colistin in PBS (Figure 6.1).

Attempts at replicating this pre-column derivatisation and RP-HPLC method for the dextrin-colistin conjugate did not result in a quantifiable trace. Initially developed by Li *et al.* (2001a), pre-column FMOc derivatisation and RP-HPLC has undergone several modifications, being optimised for maximal specificity to colistin and minimum elution times (Cao *et al.* 2008; Dudhani *et al.* 2009; Li *et al.* 2005b, 2004, 2003b). Consequently, several modifications were attempted to improve this method's sensitivity (Table 6.6) including a gradient method using ACN/TFA. This method has been commonly employed to resolve compounds where elution time was unknown, including novel colistin derivatives (Bai *et al.* 2011; Biswas *et al.* 2009; Carr 2002). This method was, therefore, applied on the C₁₈ column of maximal length (250 mm). Despite these alterations, a signal for FMOc-labelled dextrin-colistin conjugate was not detected on RP-HPLC. Subsequent analyses focussed on the solid phase derivatisation process.

In this study, a conventional "small molecule" antibiotic was custom-engineered into a macromolecule with increased hydrophilicity (Section

3.5.2). Detection of EA-4 by ELISA in the effluent immediately after washing the column with carbonate buffer provided further confirmation of the change in physicochemical properties occurring upon conjugation. Therefore, rapid elution/washout of EA-4 through the SPE cartridge, and its inability to adsorb to C₁₈ afforded a plausible indication as to the lack of a quantifiable trace on RP-HPLC, despite several modifications to this method (Table 6.6). Consequently, the labelling procedure was repeated using liquid phase synthesis (in solution). Prior studies reported successful Fmoc labelling to various molecules bearing free amino groups under similar conditions (Vreeken *et al.* 1998). To mitigate for the lower efficiency of liquid phase synthesis reported in the literature, reaction times were lengthened up to 24 h (Kent 2009). However, this was unsuccessful for EA-4. Finally, derivatisation of colistin's amino groups to Fmoc was also attempted in aqueous conditions, which had been successfully applied to a considerable number of molecules (Gawande and Branco 2011; Ibáñez *et al.* 2005). Notwithstanding several modifications of the reaction conditions, including time and pH (Rebane and Herodes 2012), the reaction remained unfeasible for dextrin-colistin conjugates. The precise reason for failure of this method was not immediately apparent. However, Fmoc-Cl is a bulky and hydrophobic molecule (Kohmura *et al.* 1990) whose reaction to amine groups may be hindered by steric hindrance and hydrophilic-hydrophobic interaction (Ptolemy and Britz-McKibbin 2005). It is, therefore, possible that the hydrophilic, bulky and negatively charged succinoylated dextrin polymer may have contributed to unsuccessful labelling. Moreover, fewer amine groups were available as a result of incorporation into amino groups by conjugation (Section 3.5.2).

Whilst use of post-column analysis methods such as LC-MS/MS would have simplified preparation protocols and lowered limits of quantification (Jansson *et al.* 2009; Ma *et al.* 2008), it would still require substantial (20:1) sample concentration, given the IV dose administered. Therefore, it was deemed that RP-HPLC was not suitable for quantification of dextrin-colistin conjugate in plasma samples for these studies.

In comparison, the running cost of the colistin ELISA technique was considerably more expensive and it could only detect total colistin content. Therefore, the PK observations reported in this study may best be considered as hybrid parameters for dextrin-colistin conjugates at various stages of unmasking. However, the ELISA method provided a simple and rapid assay which had already been successfully used in this study (Chapter 5). This approach provided a lower limit of quantification, minimised the multiple, complex steps required for pre-column derivatisation in RP-HPLC and allowed quantification of total colistin over two orders of magnitude, in agreement with previous studies (Ali *et al.* 2009; Feda *et al.* 2008).

Route of administration was an important decision influencing the PK behaviour of the dextrin-colistin conjugates. Rapid attainment of optimal levels and maximal bioavailability is desirable in acute infection. Orally administered colistin has negligible bioavailability (Kwa *et al.* 2008). The inhalational route is clinically well-established for cystic fibrosis (Foweraker *et al.* 2009). However, serum colistin concentrations peaked at 1.5 h post-inhalation, and subsequent serum levels were markedly below equivalent IV doses (Ratjen *et al.* 2006). In animal models, intramuscularly (IM) administered colistin sulfate plasma levels peaked late (~ 2 h), were prone to increased incidence of local side effects (Michalopoulos *et al.* 2011; Tang *et al.* 2009b). In contrast, IV administration presents several advantages in treating acute disease, including shorter distribution time and the ability to bypass multiple barriers. Moreover, IV administration remains the preferred method of delivery for polymer therapeutics (Markovsky *et al.* 2011) and has been extensively preferred by modern *in vivo* colistin PK studies (Couet *et al.* 2012). Colistin sulfate displayed a short distribution time, in agreement with prior studies (Table 6.1). Distribution time increased on administration of dextrin-colistin conjugates, but this still remained shorter than for colistin sulfate administered by other routes.

These *in vivo* studies demonstrated that conjugation decreased clinical toxicity associated with colistin. Prior experiments demonstrated that succinoylated dextrin forms amide bonds with 2-4 amine groups on colistin

(Sections 3.4.2 and 3.5.2). These amine groups are commonly associated with toxicity (Section 1.2.5). CMS and, more recently, polymyxin derivatives with only three positive charges were also well-tolerated in similar *in vivo* models (Ali *et al.* 2009). However, the markedly longer $t_{1/2}$ reported by dextrin-colistin conjugates when compared to colistin suggest a more stable drug level is possible following single dose IV administration. Therefore, the decreased clinical toxicity observed in this *in vivo* model may also be accounted for by a controlled release effect. Such findings have also been reported with other dextrin conjugates (Wannachaiyasit *et al.* 2008). These findings are also in keeping with the PUMPT concept (Duncan *et al.* 2008) and established literature investigating *in vivo* pharmacokinetics of polymer therapeutics (reviewed in Duncan and Gaspar 2011; Gaspar and Duncan 2009). With appropriate dose adjustment dextrin-colistin conjugates may provide therapeutic levels with less frequent administration. For example, PEGylated interferon α -2a, reported a 70-fold increase in serum half-life, resulting in significantly superior *in vivo* efficacy in human clinical trials despite reduced dosing frequency and similar adverse events (Reddy *et al.* 2001).

Observational findings of clinical adverse events have been reported in previous colistin PK studies (Ali *et al.* 2009) justifying the comparison of the toxicity of colistin sulfate to dextrin-colistin conjugates through observation of *in vivo* clinical adverse events. Classical studies identified hyperthermia as an occasional side effect of colistin (Al-Khayyat and Aronson 1973). To minimise the confounding risk associated with this spurious adverse event, heating the animals to obtain blood samples was not used in phase 3. Furthermore, batch to batch variation in colistin composition has been reported in the literature (Thomas *et al.* 2012). Therefore, use of the same batch of colistin sulfate (Section 2.1.1) to generate the dextrin-colistin conjugate administered in this study facilitated comparison between these antibiotics in terms of toxicity and PK profile. Moreover, as a result of the adverse effects experienced in phases 1 and 2, an ascending dose approach was adopted in phase 3. Therefore, each group was dosed on a different day

to ensure the previous dose level was suitable for continuation of the study. Because of the adverse events observed at 0.5 mg/kg, the highest initial dose for which a full sample set was available was 0.1 mg/kg. This starting dose also justified use of the ELISA method on account of its markedly lower limits of quantification.

Within these operational constraints, colistin sulfate reported a log-linear decrease in concentration and $t_{1/2}$ which was in agreement with prior literature (Table 6.1). Colistin sulfate is a "concentration-dependent" antibiotic (Li *et al.* 2001b) and possesses only a modest post-antibiotic effect (Li *et al.* 2006). Recent studies suggest that time-averaged exposure to colistin was more important than achieving high peak concentrations in terms of antibacterial efficacy both within *in vitro* models and clinical practice (Bergen *et al.* 2008; Dudhani *et al.* 2010b; Michalopoulos and Falagas 2011). These studies, therefore, suggest that the markedly increased circulation time for the dextrin-colistin conjugates may result in greater *in vivo* antimicrobial efficacy.

Similar *in vivo* models comparing PK disposition of colistin A and B and polymyxin derivatives with only three positive charges reported that subtle alterations in the chemical structure of the colistin molecule may lead to marked changes in disposition on plasma concentration-time curves (Ali *et al.* 2009; Feda *et al.* 2008; Li *et al.* 2003b, 2004). Therefore, the marked physicochemical changes seen after colistin was conjugated to succinoylated dextrin may explain the significantly increased circulation time, in keeping with established literature (Section 1.4) and the *in vivo* PK characteristic of polymer therapeutics (reviewed in Duncan 2011; Duncan and Gaspar 2011; Duncan *et al.* 2008). For example, a recent pharmacokinetic studies of dextrin (Mw 6,600 g/mol) conjugated to succinoylated zidovudine (18.8 %w/w) reported a marked rise in $t_{1/2}$ from 1.3 to 19.3 h when administered IV to Sprague Dawley rats when compared to free drug (Wannachaiyasit *et al.* 2008). Interferon- α 2a conjugated to trimer-structured PEG increased terminal $t_{1/2}$ from 1.3 to 47.4 h, its serum activity peaked after 1 h and was retained for 23 h (Jo *et al.* 2006).

Seminal studies by Hreczuk-Hirst *et al.* (2001a) reported that typical plasma amylase concentrations in the Sprague Dawley rat are at least 20-fold higher than for infected skin wounds (Section 5.5.1). These considerations suggest that dextrin-colistin conjugate $t_{1/2}$ in humans would be even longer than was observed in the rat model used in this study. The increased $t_{1/2}$ and AUC_{24} of the dextrin-colistin conjugates may present an advantage compared to colistin sulfate and other polymyxin derivatives described in the literature (Ali *et al.* 2009). Moreover, increased $t_{1/2}$ may also reduce injection frequency, increase convenience and improve patient compliance (Sinclair and Elliott 2005).

6.6 Conclusion

This study reported an initial evaluation of PK behaviour and toxicity of dextrin-colistin conjugates in a well-established animal model. In summary, this study found that plasma $t_{1/2}$, C_{last} , and AUC increased with conjugation and increasing degree of succinylation. These changes support the notion that 'masking' of colistin by conjugation to succinoylated dextrin increased persistence of colistin in plasma in this animal model, which could have beneficial effects on antimicrobial activity by increasing time-averaged exposure to colistin. Clinical observations suggest that dextrin-colistin conjugates are well-tolerated *in vivo*, and that at higher concentrations were better tolerated than colistin sulfate. Ineffectiveness of pre-column derivatisation and RP-HPLC analysis corroborated the substantial changes in physicochemical characteristics produced by the conjugation process. This study suggests that further *in vivo* investigation of dextrin-colistin conjugates is warranted.

Chapter Seven

General Discussion

7.1 Introduction

This study has designed, engineered and tested a novel polymer-antibiotic conjugate for use in the treatment of Gram-negative infectious disease, addressing the unmet clinical demand for novel effective antibiotics against Gram-negative bacteria (see Chapter 1; Coates and Halls 2012), whilst minimising the toxicity of systemically administered colistin (Carlet *et al.* 2012; Karvanen *et al.* 2013).

7.2 Polymer therapeutics in infection

Whilst cancer therapy remains the main focus of research within the field of polymer therapeutics (Chapter 1), infection-associated EPR has also been recognised as a common feature of Gram-negative infection (Azzopardi *et al.* 2013). The concept of PUMPT, and its application in the treatment of infection, is consequently not novel. Researchers first described the potential use of polymer therapeutics as anti-endotoxin / anti-inflammatory therapy against Gram-negative bacteria over 15 years ago (Bucklin *et al.* 1995). Successful research translation of clinical polymer therapies in infectious diseases has, however, been limited principally to anti-viral polymer-conjugated interferons (PEGIntron[®] and PEGasys[®]) (reviewed in Duncan 2011). In the field of Gram-negative bacterial infection, Vicent *et al.* (2010) described PEGylated peptoid 7 conjugates as anti-endotoxin therapy, and Bosnjakovic *et al.* (2011) described dendrimer-erythromycin conjugates for the treatment of infection-induced inflammation. Hypothetical polymer-antibiotic (and polymer-antimicrobial peptide) structures have been described for the treatment of infectious disease (Bishop 2006). This study is the first to exploit the PUMPT concept (Duncan *et al.* 2008) in designing and delivering a prototype polymer-antibiotic conjugate to afford selective controlled release at sites of infection. Whilst previous studies have demonstrated the feasibility of conjugation to antimicrobial peptides, this procedure may reduce their antimicrobial effectiveness by up to 200-fold in some studies (Bishop 2006). Recently, fourth generation PAMAM dendrimers conjugated to azithromycin

have been described for the treatment of *Chlamydia trachomatis* infection with improved *in vitro* drug release, although this occurred indiscriminately through autocatalysis (Mishra *et al.* 2011), negating the potential of locally-targeted enzymatically-mediated release seen with dextrin-colistin conjugates.

Dextrin was employed to provide a polymer which was readily functionalised and conjugated. In the delivery of a novel clinical therapy, ensuring the predictability and reproducibility of controlled drug release is an essential consideration (Deshpande *et al.* 1996; Gaspar and Duncan 2009; Lin and Metters 2006; Youan 2010). In this study, the rate of degradation of the succinoylated dextrin polymer and the rate of colistin release could be conveniently governed by varying the degree of parent-dextrin molecular weight and succinoylation.

In vitro timecourse experiments conducted in the presence of α -amylase clearly showed that the rate of conjugate degradation observed by GPC reflected the colistin release measured by FPLC. However, in control experiments, FPLC analysis demonstrated a slow, linear shift from masked to unmasked conjugate, whilst GPC analysis under the same conditions demonstrated non-significant degradation. These results may not be contradictory. Slow non-statistically significant hydrolytic degradation under control conditions may result in a shift in FPLC elution peaks, colistin remaining attached to smaller dextrin whilst not completely unmasked. The lack of significant time-dependent potentiation of antimicrobial activity in the absence of amylase affirms the plausibility of this hypothesis. Later experiments (Section 5.4) demonstrated that under infinite sink conditions, the amylase-mediated decrease in molecular weight allowed unmasked conjugate to cross a dialysis membrane to an outer compartment, resulting in a corresponding increase in antimicrobial activity over time (Section 5.4) further supporting this notion.

Natural polymers such as the dextrin used in this study tend to exhibit a relatively high degree of polydispersity and contribute to polydispersity of the

resulting conjugate (Duncan 2003). Moreover, colistin sulfate is a multicomponent entity and inter-batch differences in the component composition have been documented (Decolin *et al.* 1997; Govaerts *et al.* 2003; Orwa *et al.* 2002). However, this study employed a single source (i.e. batch) of colistin sulfate, to mitigate the potential confounding effects of varying component compositions. Colistin A is the principal pharmacologically active entity in colistin sulfate (Kline *et al.* 2001). The exact composition of a heterogenous preparation can affect both efficacy and toxicity (Gaspar and Duncan 2009). Therefore, in future 'scale-up' for clinical use, conjugating colistin A to low-polydispersity dextrin may facilitate physicochemical / structural characterisation, determination of bioactivity and toxicity as well as detection of impurities.

Based on *in vitro* physicochemical and initial antimicrobial susceptibility data, EA-4 was selected and methodically taken through a number of investigations. Dextrin-colistin conjugates with lower degrees of succinylation displayed more complete, faster unmasking, and a greater degree of antimicrobial activity against all strains tested *in vitro* (Section 4.4). The unmasked dextrin-colistin conjugates displayed a narrower spectrum of activity, notably against *P. aeruginosa* strains, than colistin sulfate or CMS. However, their antimicrobial spectrum of activity compared favourably with other antibiotics in current clinical development for Gram-negative bacterial infections (Rennie 2012). In most of the strains tested, 24-h unmasked EA-4 reported similar MICs to CMS. This contrasted with the relatively poor reinstatement of antimicrobial activity after conjugation attempts to antimicrobial peptide reported in the wider literature (Bishop 2006).

Given that *in vitro* non-clinical models are usually intended for screening "low molecular weight" drugs (Gaspar and Duncan 2009), a two-compartment static dialysis bag model under infinite sink conditions was developed to test dextrin-colistin conjugates, whose controlled release nature indicated a different PK profile. Under these conditions, the time-dependent change in bacterial viability reflected the unmasking rate reported in earlier experiments. An obvious clinical concern from the results in this experiment

(and in earlier MIC studies) was that production of a “functional” antibiotic from the conjugate was clearly time–dependent therefore conventional antibiotic therapy would be likely required to supplement dextrin-colistin conjugate use in clinical practice, at least during the initial dosing. Within this context, the value of a combination of colistin sulfate and dextrin-colistin conjugate was demonstrated to provide a rapid decline in viable counts, followed by sustained suppression of bacterial growth.

The modified TTK model used in this study also demonstrated the advantages of controlled release. It was evident in this system that administration of EA-4 induced suppression of bacterial growth for a considerably prolonged period compared to colistin sulfate. In contrast, early and significant bacterial re-growth following a colistin sulfate dose virtually nullified the gains registered by the rapid reduction in bacterial counts (Section 5.4.2.2). Moreover, antibiotic-induced bacterial lysis and overwhelming endotoxin release, with exacerbation of septic shock has been described (Lepper *et al.* 2002). Considering the rapid decrease in viable bacterial counts observed with colistin sulfate (Figure 5.8), the sustained activity of dextrin-colistin conjugates could reduce this risk.

The use of well-established, conventional assay techniques such as MIC determination allowed comparison of the dextrin-colistin conjugates' bioactivity to colistin sulfate and CMS, and antimicrobial activity with unmasking. Whilst these remain valuable analytical techniques, it must be recognised that these assays were designed for screening “low-molecular weight” chemical entities, and further model development may be required to truly realise the benefits afforded by the alteration of pharmacodynamic parameters through application of polymer therapeutics (Gaspar and Duncan 2009). Similarly, validation of the two-compartment dialysis bag model provided advantages over traditional TTK studies and was more adept to studying effect of controlled colistin release on bacterial viability. However, an important limitation of such *in vitro* models is an inability to factor in the potential benefits of the EPR effect *in vivo*. Prior studies performed in the setting of anti-cancer therapeutics reported that polymer therapeutics could

achieve tumour/blood ratios of 5-30 in as little as 10 min (Maeda *et al.* 2001; Matsumura and Maeda 1986; Reddy 2005). It is therefore possible that such conventional assays may underestimate the true antimicrobial potential of these conjugates.

Ex vivo studies demonstrated a significant increase in α -amylase activity within infected burn wound fluid. In this study, samples were collected markedly later than the first 24-48 h following major thermal injury where transient increase in vascular permeability and fluid shifts have been documented (Sjöberg 2012). Results are therefore in keeping with the notion that the significantly increased amylase activity detected around the infected foci was due to endogenous plasma amylase accumulating by an infection-induced EPR effect. Since none of the bacterial strains in this study produced amylase it is likely that the increased α -amylase activity observed around infected foci was endogenous in nature. Conjugate unmasking in the presence of wound fluid provided further evidence about the feasibility of the PUMPT concept in acute infection.

The increased amylase activity levels observed in infected wound fluid in the clinical study may represent a challenge to controlled release of colistin. However, these *ex vivo* experiments showed that, irrespective of disease state, serum α -amylase concentrations remained within similar (physiological) levels as those employed during *in vitro* physicochemical, antimicrobial susceptibility and PK/PD characterisation. Such levels could be considered the main challenge to "masking" of the conjugate whilst still in transport to the infected focus. It could also be argued that substantially increased amylase activity observed around an infected focus may be a challenge to controlled release. Locally increased amylase activity may lead to faster degradation and release of colistin. Despite the high amylase activity in Sprague-Dawley rat plasma (8,000-10,000 IU/L), even minimally modified dextrin-colistin conjugate displayed a markedly improved plasma $t_{1/2}$. This parameter suggests that controlled release is possible even in the presence of higher amylase concentrations. Moreover, behaviour of succinoylated dextrin degradation reported in these experiments is mirrored by similar

observations over a wide range of α -amylase levels (Hardwicke *et al.* 2008; Hreczuk-Hirst *et al.* 2001).

In vivo studies (Chapter 6) were essential to establish a working $t_{1/2}$ on which to base the initial doses and dosing intervals in future studies. It is worth noting that the polymer molecular weight and degree of modification was selected on the premise of fulfilling an "acute disease" niche. Traditional applications of polymer therapeutics such as cancer favour use of markedly higher molecular weights and modifications (Maeda *et al.* 2009). Recent studies demonstrated that *in vivo* accumulation of macromolecules around an infected focus may occur with macromolecules of smaller molecular weight such as PEGylated ubiquicidin (5,000 g/mol) and technetium⁹⁹-labelled aprotinin (6,512 g/mol) than traditionally favoured in the design and development of anticancer drugs (Komarek *et al.* 2005; Maeda *et al.* 2009; Melendez-Alafort *et al.* 2009). Given that EA-4 was designed for acute life-threatening infection (Section 1.2.1), it was selected as the lead candidate based on its rapid, maximal release characteristics. However, the final selection of polymer molecular weight / modification / conjugation combination may need further refinement, given the limited initial activity observed during *in vitro* PK/PD studies, its markedly extended *in vivo* half-life, and the surprising, localised increase in amylase activity around infected foci.

In these studies, *ex vivo* release of colistin was successfully characterised by ELISA, and the results yielded by this method were in agreement with the model validation data obtained by BCA and FPLC assays. The choice of ELISA over HPLC, whilst bearing advantages (Section 6.5) limited the *in vivo* studies by being able to detect total colistin without discrimination between bound and free drug (reflecting the detection of an 'exposed' epitope by the polyclonal antibody used in the proprietary ELISA kit). As a result, PK observations reported in this study may best be considered as hybrid parameters for dextrin-colistin conjugates at various stages of unmasking. The use of ELISA to study payload release from polymer-drug conjugates has been previously described (Hardwicke *et al.* 2008a). In contrast to the

present study, Hardwicke *et al.* (2008) reported that their masked conjugate was not detected by their ELISA kit. The significantly higher molecular weight and degree of succinylation used by Hardwicke *et al.* (2008) may have increased steric hindrance to antibody binding. The decrease in residual antimicrobial activity of masked conjugates with increasing dextrin succinylation was in keeping with this observation (Table 4.4). Moreover, it is also possible that the colistin ELISA kit did not distinguish between masked/unmasked colistin because of the presence of carbohydrate lytic reagents in the proprietary cleanup kit which could have contributed to succinoylated dextrin polymer degradation (Bhatt 2012, personal communication, Appendix 2.7).

Future studies would benefit from establishing an analytical technique which would distinguish unmasked and masked conjugate at the required levels of quantification. Literature suggests that urinary free colistin concentrations would be several orders of magnitude below the lower limit of detection of the ELISA kit used in this study (BioO Corp 2012; Li *et al.* 2003b). Development of a novel analytical technique would facilitate the analysis of urinary colistin concentrations in samples collected during the *in vivo* study, and consequently afford a more detailed PK characterisation of *in vivo* dextrin-colistin conjugate pharmacokinetics.

7.3 Future work

The stepwise process of *in vivo* pre-clinical evaluation of novel pharmaceuticals is ultimately aimed at defining a dose which is safe for entry into human trials (Eisenhauer *et al.* 2000; Reigner and Blesch 2002). Following on from the encouraging *in vivo* data (Section 6.4), it is envisaged that the development of dextrin-colistin conjugates towards clinical use will progress to further escalation and repeat dose studies. The *in vivo* experiments in this study were limited by toxicity of colistin sulfate (Section 6.5). However, dextrin-colistin conjugates did not exhibit signs of toxicity at the highest dose tested in this study (0.5 mg/kg). Therefore, further dose escalation studies would be beneficial in determining a maximum tolerated

dose for the lead dextrin-colistin conjugate. Such data would be useful to allow adequate characterisation of potential clinically relevant effects, whilst minimising animals use in later experiments (US Center for Drug Evaluation and Research 2010). Subsequent repeated dose toxicity studies would be based on dose intervals determined from these initial *in vivo* PK studies.

It will also be important to demonstrate *in vivo* the extent of partitioning between the infected target site and normal tissue, antimicrobial efficacy as well as monitoring the metabolic fate of both the succinoylated dextrin and conjugated colistin (Gaspar and Duncan 2009). However, the choice of an appropriate *in vivo* model for studying these aspects presents formidable challenges. Gaspar and Duncan (2009) suggest that such models should closely reflect the specific disease state, including pathophysiological progression, localisation, and immune status for which the nanomedicine is targeted. Tachi *et al.* (2004) validated an *in vivo* experimental model of an infected skin ulcer in Sprague Dawley rats. In this model, inoculation of an induced, gauze-covered full thickness skin wound resulted in infection being induced within 24 h, and maintained for 9 days. This model was originally intended to investigate topical antimicrobial application to infected chronic wounds. However, the possibility of quantifying the bacterial load and nanoantibiotic concentration from wound fluid or tissue biopsy as well as the opportunity of following the course of infection from induction through to 9 days, would support the adoption of this model. Moreover this model would still allow further, simultaneous *in vivo* pharmacokinetic evaluation, such as the extent of nanoantibiotic distribution and partitioning between the infected target site and other tissues. This model may, therefore, facilitate future *in vivo* PK/PD characterisation of a lead compound.

An interesting, additional use of these conjugates may be in the treatment of sepsis. The potential of polymyxins as anti-endotoxin therapy has been extensively investigated, however, its intracorporeal use is limited by extensive toxicity (Bhor *et al.* 2005; Cohen 2009). Whilst dextran 70-polymyxin B conjugates have been described (Bucklin *et al.* 1995), clinical

success in sepsis has been limited to extracorporeal dialysis devices based on polymyxin B immobilised to polystyrene fibers (Shoji 2003). The anti-endotoxin properties of dextrin-colistin conjugates, as well as toxicity studies, are being investigated by this Research Group (Ferguson *et al.* 2012b, 2012c).

7.4 Contribution of this study to the field of nanomedicine

This study presented proof of principle for a novel class of nanoantibiotics effective against multidrug-resistant Gram-negative bacteria, for which limited resources currently exist. It exploited the PUMPT concept, described by Duncan *et al.* (2008), to design and deliver a prototype polymer-antibiotic conjugate that affords selective controlled release at sites of Gram-negative infection. Significantly increased amylase activity around clinically infected foci suggests a therapeutic role for amylase in infection, within the context of PUMPT. These studies underscore the potential usefulness of bioresponsive polymer-antibiotic conjugates as a new class of nanoantibiotics and open a novel and exciting avenue for the application of polymer therapeutics in the treatment of acute infection.

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Appendix 1

1.1 Published Manuscripts

Azzopardi, E. A., Ferguson, E. L. and Thomas, D. W. 2013. The enhanced permeability retention effect: a new paradigm for drug targeting in infection. *Journal of Antimicrobial Chemotherapy* 68(2) 257-274.

Azzopardi, E. A., Boyce, D. E., Thomas, D. W. and Dickson, W. A. 2013. Colistin in burn intensive care: Back to the future? *Burns* 39(1) 7-15.

Azzopardi, E. A., Ferguson, E. L. and Thomas, D. W. 2012. Colistin past and future, a bibliographic analysis. *Journal of Critical Care*. In press, DOI: 10.1016/j.jcrc.2012.04.008.

1.2 Published abstracts

Azzopardi, E. A., Ferguson, E. L. and Thomas, D. W. 2012. *In vitro* Pharmacokinetic/Pharmacodynamic Modelling of Bioresponsive Dextrin-colistin Conjugates. P24. In: International Society of Polymer Therapeutics. *Proceedings of the 9th International Symposium on Polymer Therapeutics*. Valencia, Spain 28-30 May 2012. Valencia: Centro de Investigacion Principe Felipe.

Azzopardi, E. A., Ferguson, E.L., Thomas, D.W. 2012. Targeted antibiotic therapy using polymer therapeutics - a novel paradigm. P15. In: Royal College of Surgeons in Edinburgh. *Proceedings of the Lister Centenary Meeting*. Edinburgh, UK 9-11 February 2012. Edinburgh: The Joint Royal Colleges of Surgery.

Azzopardi, E.A., Ferguson, E.L., Thomas, D.W. 2012. Development of an *in vitro* pharmacokinetic/pharmacodynamic model for studying novel nanoantibiotics: dextrin-colistin conjugates. F-2006. In: American Society for Microbiology, *Proceedings of the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy*. San Francisco, USA 9-12 September 2012. Washington DC: American Society of Microbiology.

Azzopardi, E.A., Ferguson, E.L., Thomas, D.W. 2011. Polymer therapeutics for safe and effective antibiotic targeting: a novel strategy. P0115. In: British Society for Oral and Dental Research. *Proceedings of the 2012 Annual Conference*. Sheffield, UK 13-15 September 2011. London: Royal College of Surgeons.

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1.3 Research Ethics Documentation

Part of the research infrastructure for Wales funded by the National Institute for Social Care and Health Research, Welsh Government.
Yn rhan o sailwaith ymchwil Cymru a ariannir gan y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd, Llywodraeth Cymru



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Ymchwil | **RES** | Research
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Research Ethics Committee (REC) for Wales

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21 November 2012

Dr Ernest Azzopardi
Floor 5 Room 503 Wound Biology Group
Wound Biology Group
School of Dentistry
Cardiff University
Heath Park
Cardiff.
CF14 4 XY

Dear Dr Azzopardi

Study title: Investigating amylase concentration in wound fluid from Gram negative infected burn (skin) wounds in adults
REC reference: 11/WA/0252
Protocol number: 01

This study was given a favourable ethical opinion by the Committee on 04 October 2011.

Research Ethics Committees are required to keep a favourable opinion under review in the light of progress reports and any developments in the study. You should submit a progress report for the study 12 months after the date on which the favourable opinion was given, and then annually thereafter. Our records indicate that a progress report is overdue. It would be appreciated if you could complete and submit the report by no later than one month from the date of this letter.

Guidance on progress reports and a copy of the standard NRES progress report form is available from the National Research Ethics Service website.

The NRES website also provides guidance on declaring the end of the study.

11/WA/0252: Please quote this number on all correspondence

Yours sincerely

Mrs Helen Williams
Assistant Co-ordinator

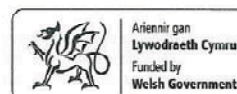
E-mail: helen.williams19@wales.nhs.uk

Copy to: *Professor David Thomas*
Professor Stephen Charles Bain, Swansea University, ABMU NHS Trust

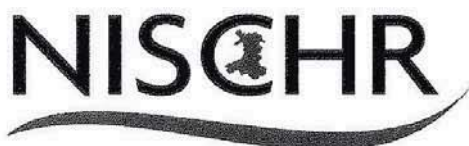


Cynhelir Cydweithrediad Gwyddor Iechyd Academaidd y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd gan Fwrdd Addysgu Iechyd Powys

The National Institute for Social Care and Health Research Academic Health Science
Collaboration is hosted by Powys Teaching Health Board



Part of the research infrastructure for Wales funded by the National Institute for Social Care and Health Research, Welsh Government.
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13 December 2012

Dr Ernest Azzopardi
Office F5/
The University Dental Hospital
Academic Drive
Heath Park
Cardiff.
CF14 4 XY

Dear Dr Azzopardi

Study title: Investigating amylase concentration in wound fluid from Gram negative infected burn (skin) wounds in adults
REC reference: 11/WA/0252
Protocol number: 01
IRAS project ID: 86715

Thank you for sending the declaration of end of study form, notifying the Research Ethics Committee that the above study concluded on 01 November 2012. I will arrange for the Committee to be notified.

A summary of the final research report should be provided to the Committee within 12 months of the conclusion of the study. This should report on whether the study achieved its objectives, summarise the main findings, and confirm arrangements for publication or dissemination of the research including any feedback to participants.

In England, Wales and Northern Ireland, legal authority to hold any human tissue under the ethical approval for this project has now expired. To ensure that any continued storage is lawful, either the tissue must be held on premises with a storage licence from the Human Tissue Authority, or an application made for ethical approval of another project. Otherwise the tissue would need to be destroyed in accordance with the HTA Codes of Practice.

11/WA/0252: Please quote this number on all correspondence

Yours sincerely

Mrs Helen Williams
Assistant Co-ordinator

E-mail: helen.williams19@wales.nhs.uk

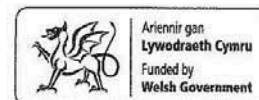
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Professor Stephen Charles Bain, Swansea University, Abertawe Bromorgannwg
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Powys Teaching
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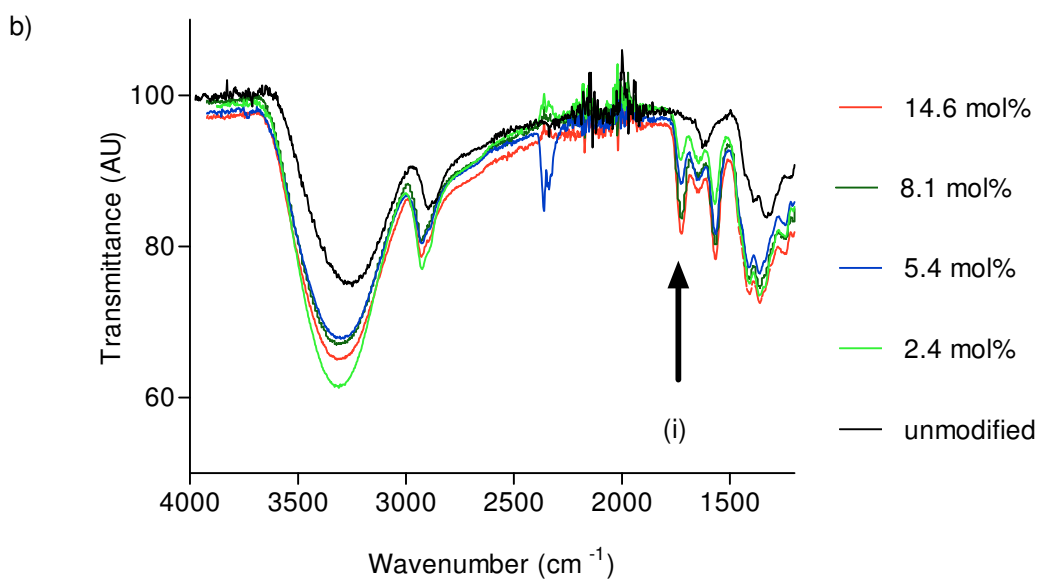
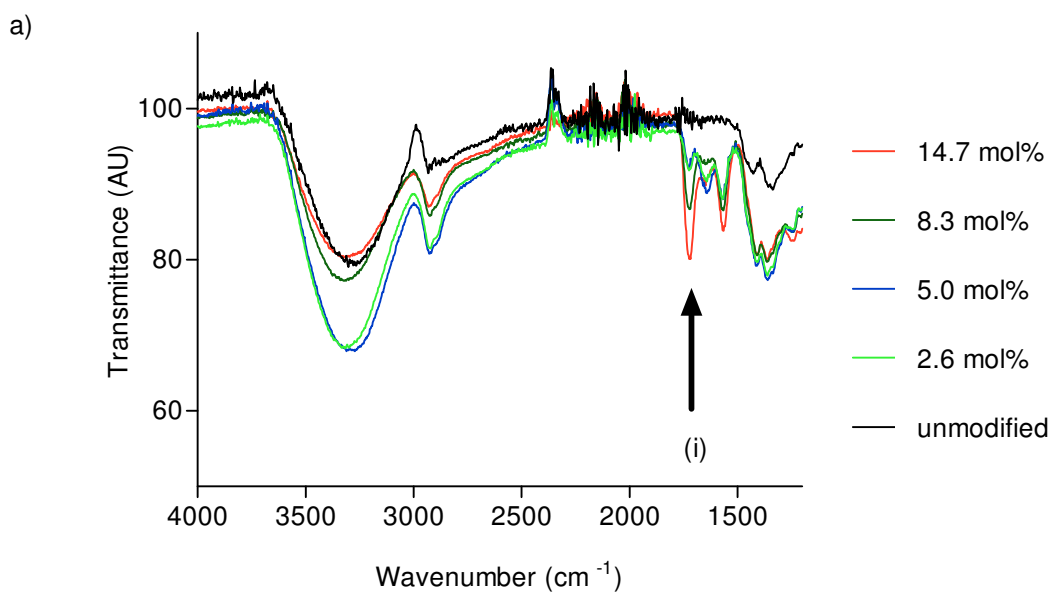
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The National Institute for Social Care and Health Research Academic Health Science
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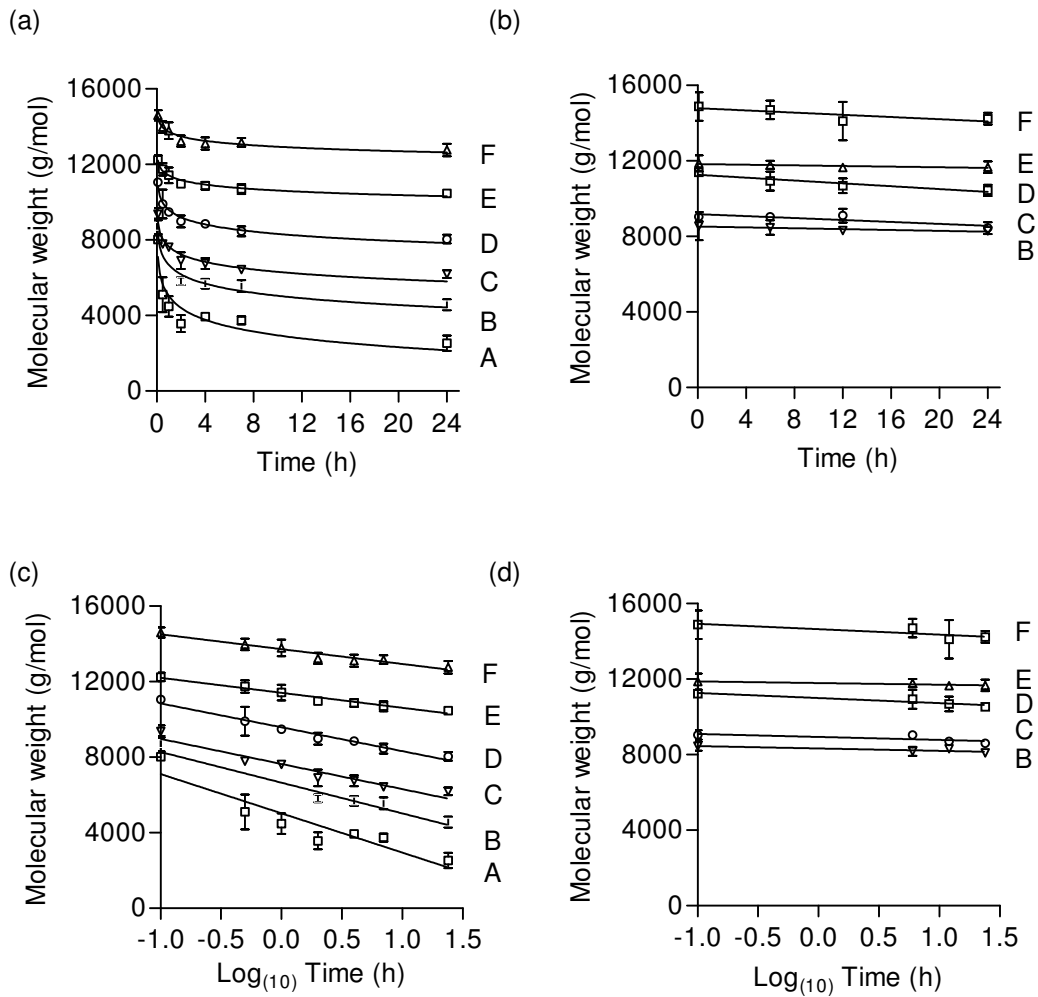


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Lywodraeth Cymru
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Welsh Government

Appendix 2

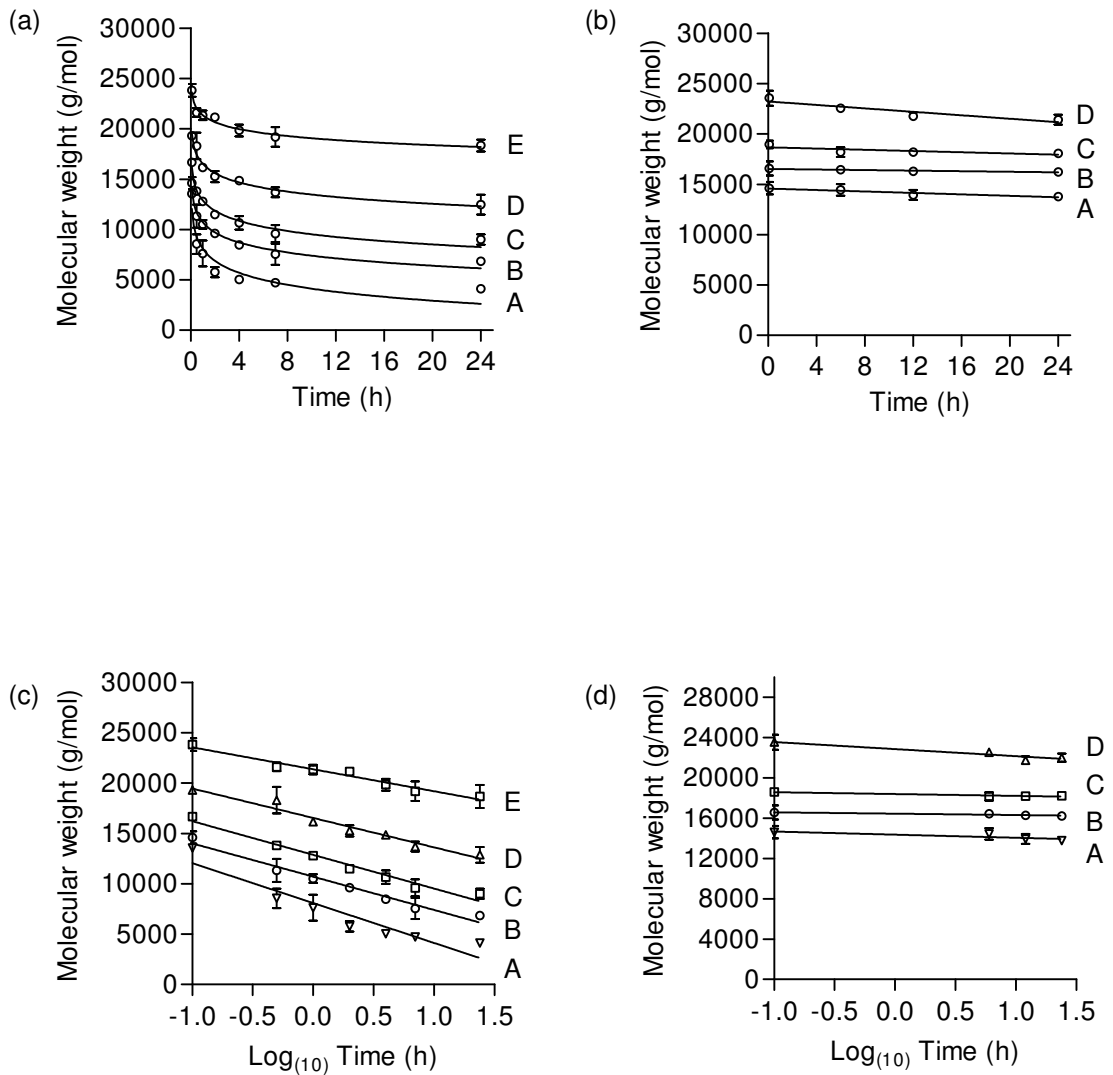


Appendix 2.1: Typical characterisation of succinoylated dextrans by FTIR. Panel (a) shows MMW parent-dextrin family. Panel (b) shows a typical HMW parent-dextrin family. The arrow indicated the increase in peak intensity at 1720 cm^{-1} caused by succinoylation.



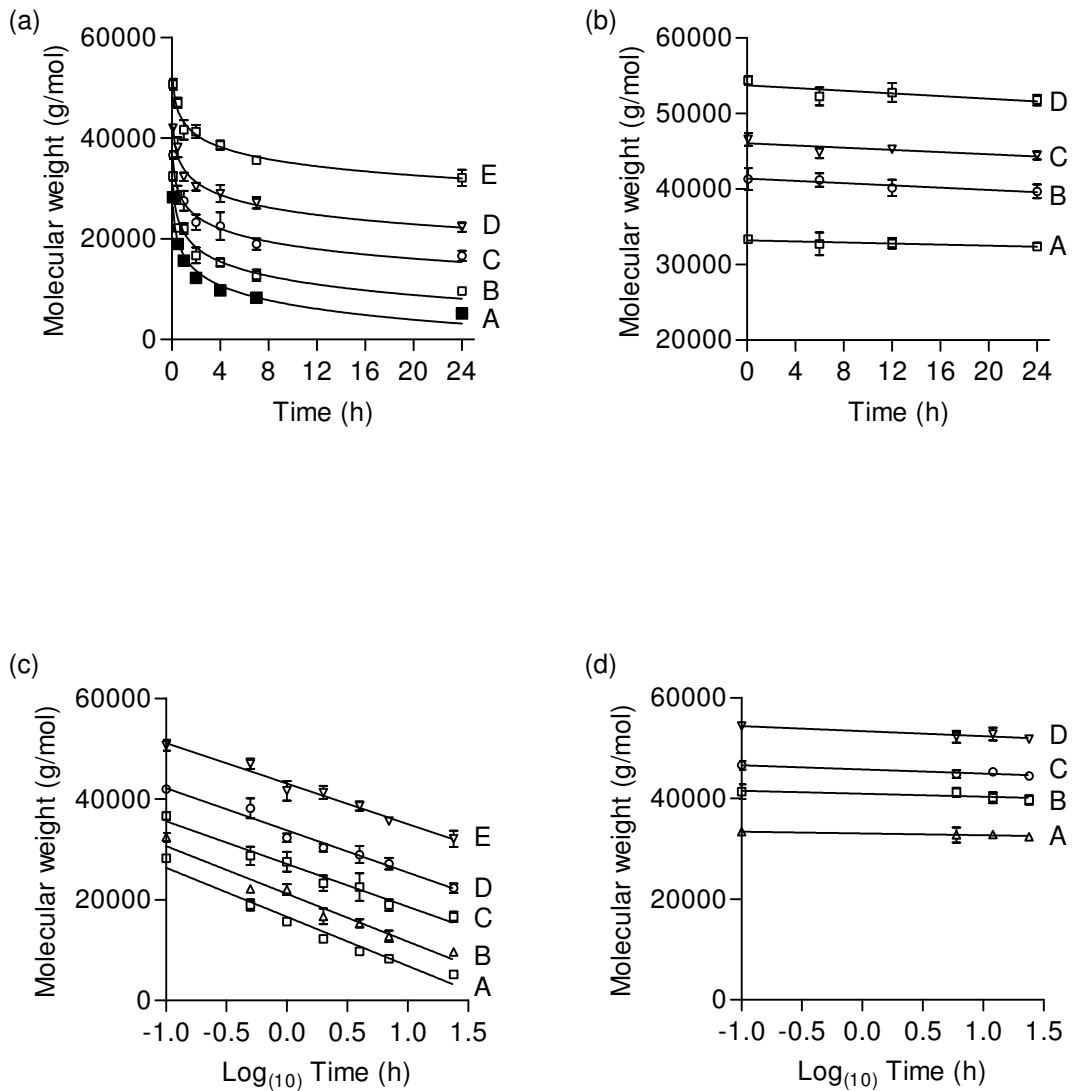
Appendix 2.2a: Best fit models and the respective semilog transformations of Mw versus time for succinoylated dextrans (LMW parent-dextrin library). These graphs show that (a) in the presence of α -amylase degradation of succinoylated dextrin was best fit by logarithmic regression; (b) the respective controls were best fit by linear regression; semilog transformation of both experimental data (c) and control (d) were best fit by linear regression, enabling statistical comparison.

Data shown represents mean \pm SD ($n=3$). Legend: A: unmodified control; B: 1.0 mol% succinoylated C: 2.5 mol% succinoylated; D: 4.8% mol succinoylated; E: 7.2 mol% succinoylated F: 14.2 mol% succinoylated dextrin. Coefficients of determination are reported in Appendix 2.2d - e.



Appendix 2.2b: Best fit models and the respective semilog transformations of Mw versus time for succinoylated dextrans (MMW parent-dextrin library). These graphs show that (a) in the presence of α -amylase degradation of succinoylated dextrin was best fit by logarithmic regression; (b) the respective controls were best fit by linear regression; semilog transformation of both experimental data (c) and control (d) were best fit by linear regression, enabling statistical comparison.

Data shown represents mean \pm SD (n=3). Legend: A: unmodified control; B: 2.6 mol% succinoylated; C: 5.0 mol% succinoylated; D: 7.5% mol succinoylated; E: 14.4 mol% succinoylated dextrin. Coefficients of determination are reported in Appendix 2.2d, e.



Appendix 2.2c: Best fit models and the respective semilog transformations of Mw versus time for succinoylated dextrans (HMW parent-dextrin library). These graphs show that (a) in the presence of α -amylase degradation of succinoylated dextrin was best fit by logarithmic regression; (b) the respective controls were best fit by linear regression; semilog transformation of both experimental data (c) and control (d) were best fit by linear regression, enabling statistical comparison.

Data shown represents mean \pm SD (n=3). Legend: A: unmodified control; B: 2.6 mol% succinoylated (C): 5.4 mol% succinoylated; (D): 7.6 % mol succinoylated; (E): 14.8 mol% succinoylated dextrin. Coefficients of determination are reported in Appendix 2.2d - e.

Appendix 2.2d: Best Fit Data for Enzymatic Degradation of Succinoylated Dextrin, and Controls.

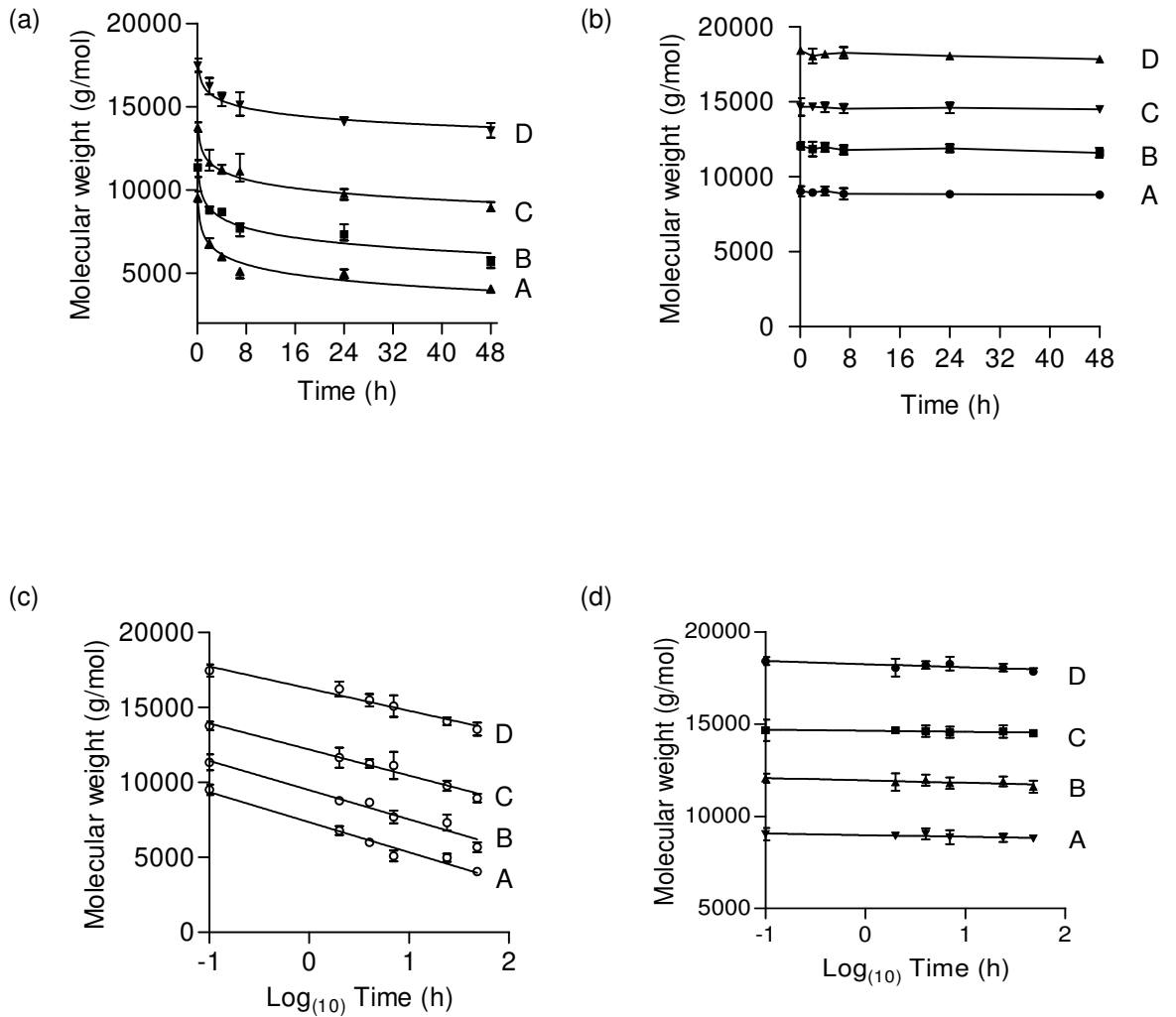
	Theoretical Succinoylation (mol %)	A-amylase degradation Logarithmic regression model ($y=m.\ln x+c$)			Control Linear regression model ($y=m.x+c$)		
		R ²	D.O.F.	95% C.I. (slope)	R ²	D.O.F.	95% C.I. (slope)
LMW dextrin library	30	0.96	19	243 to 420	0.90	7	-33 to 0.54
	15	0.86	19	280 to 400	0.80	7	-40 to 8.3
	10	0.89	19	460 to 640	0.90	7	-77 to 1.6
	5	0.89	19	480 to 680	0.88	7	-76 to 24.5
	2	0.92	18	600 to 800	0.72	7	-28 to 5.2
	0	0.82	19	670 to 1110	-	-	-
MMW dextrin library	30	0.85	19	1,100 to 1,500	0.86	7	-192 to 21
	15	0.91	19	1,290 to 1,600	0.60	7	-110 to 44
	10	0.95	19	1,300 to 1,600	0.85	7	-32 to 4.0
	5	0.93	19	1,240 to 1630	0.88	7	-76 to 5.0
	0	0.86	19	1,390 to 2,000	-	-	-
HMW dextrin library	30	0.98	19	3,600 to 4,300	0.64	7	-290 to 140
	15	0.97	19	3,500 to 4,200	0.64	7	-240 to 94
	10	0.97	19	3,300 to 4,200	0.87	7	-190 to 28
	5	0.96	19	3,700 to 4,600	0.80	7	-95 to 46
	0	0.96	19	3,800 to 4,600	-	-	-

R²: coefficient of determination; DOF: degrees of freedom; 95% CI (slope): 95% confidence interval (slope). All values are reported to 2 significant Figures.

Appendix 2.2 e: Best fit data for the semilogarithmic transformation for enzymatic degradation of succinoylated dextrin, and controls.

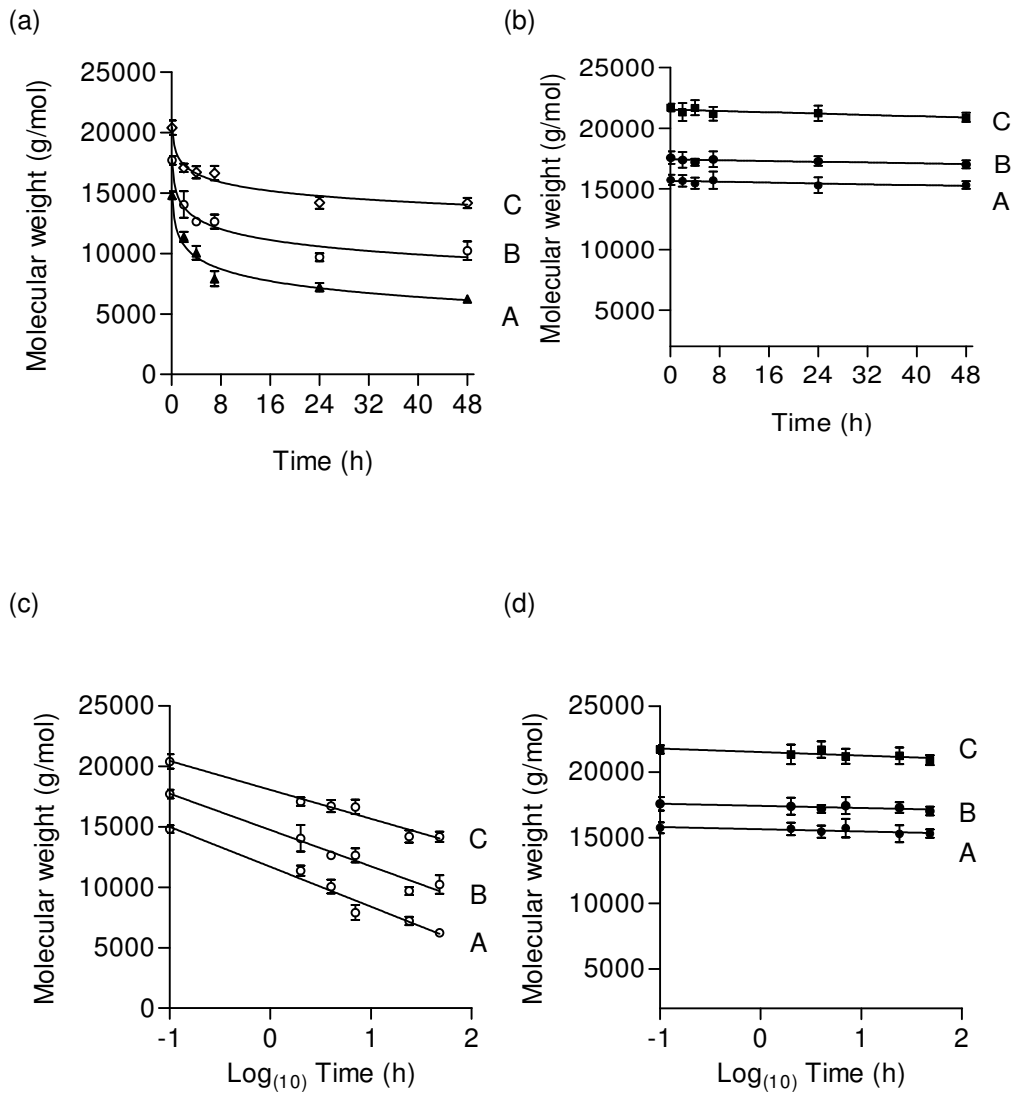
	Theoretical Succinoylation (mol %)	α -amylase degradation			Control		
		R ²	D.O.F.	95% C.I. (slope)	R ²	D.O.F.	95% C.I. (slope)
LMW dextrin library	30	0.91	19	-1221 to -558	0.68	7	-290 to 140
	15	0.97	19	-963 to -624	0.81	7	-87 to 30
	10	0.98	19	-1498 to -1030	0.90	7	-270 to 70
	5	0.93	19	-1753 to -908	0.66	7	-160 to 100
	2	0.95	18	-2078 to -1174	0.76	7	-130 to 53
	0	0.86	19	-3032 to -1127	-	-	-
MMW dextrin library	30	0.96	19	-2682 to -1681	0.90	7	-700 to 170
	15	0.96	19	-3624 to -2215	0.87	7	-180 to 51
	10	0.97	19	-3991 to -2697	0.85	7	-130 to 40
	5	0.97	19	-3963 to -2646	0.71	7	-310 to 140
	0	0.89	19	-5547 to -2371	-	-	-
HMW dextrin library	30	0.98	19	-10,400 to -7,700	0.89	7	-930 to 230
	15	0.98	19	-10,400 to -7,400	0.89	7	-820 to 200
	10	0.98	19	-10,100 to -7,201	0.61	7	-600 to 380
	5	0.97	19	-11,500 to -7,600	0.90	7	-355 to 84
	0	0.96	19	-11,800 to -7,500	-	-	-

R²: coefficient of determination; DOF: degrees of freedom; 95% CI (slope): 95% confidence interval (gradient).



Appendix 2.2f: Best fit models and the respective semilog transformations of Mw versus time for a dextrin-colistin conjugates (LMW parent-dextrin library). These graphs show that (a) in the presence of α -amylase degradation of dextrin-colistin conjugate was best fit by logarithmic regression; (b) the respective controls were best fit by linear regression; semilog transformation of both (c): experimental data and (d): controls were best fit by linear regression, enabling statistical comparison. Data shown represents mean \pm SD (n=3).

Legend: A: 1.0 mol% succinoylated conjugate; B: 2.5 mol% succinoylated conjugate; C: 4.8 mol% succinoylated conjugate; D: 7.2 mol% succinoylated conjugate. Coefficients of determination are reported in Appendix 2.2h - i.



Appendix 2.2g: Best fit models and the respective semi log transformations of Mw versus time for a dextrin-colistin conjugates (MMW parent-dextrin library). These graphs show that (a) in the presence of α -amylase degradation of dextrin-colistin conjugate was best fit by logarithmic regression; (b) the respective controls were best fit by linear regression; semilog transformation of both (c): experimental data and (d): control were best fit by linear regression, enabling statistical comparison. Data shown represents mean \pm SD (n=3).

Legend: A: 2.6 mol% succinoylated (B): 5.0 mol% succinoylated; (C): 7.5 % mol succinoylation. Coefficients of determination are reported in Appendix 2.2h - i.

Appendix 2.2 h: Best fit data for the enzymatic degradation of dextrin-colistin conjugate and the respective controls.

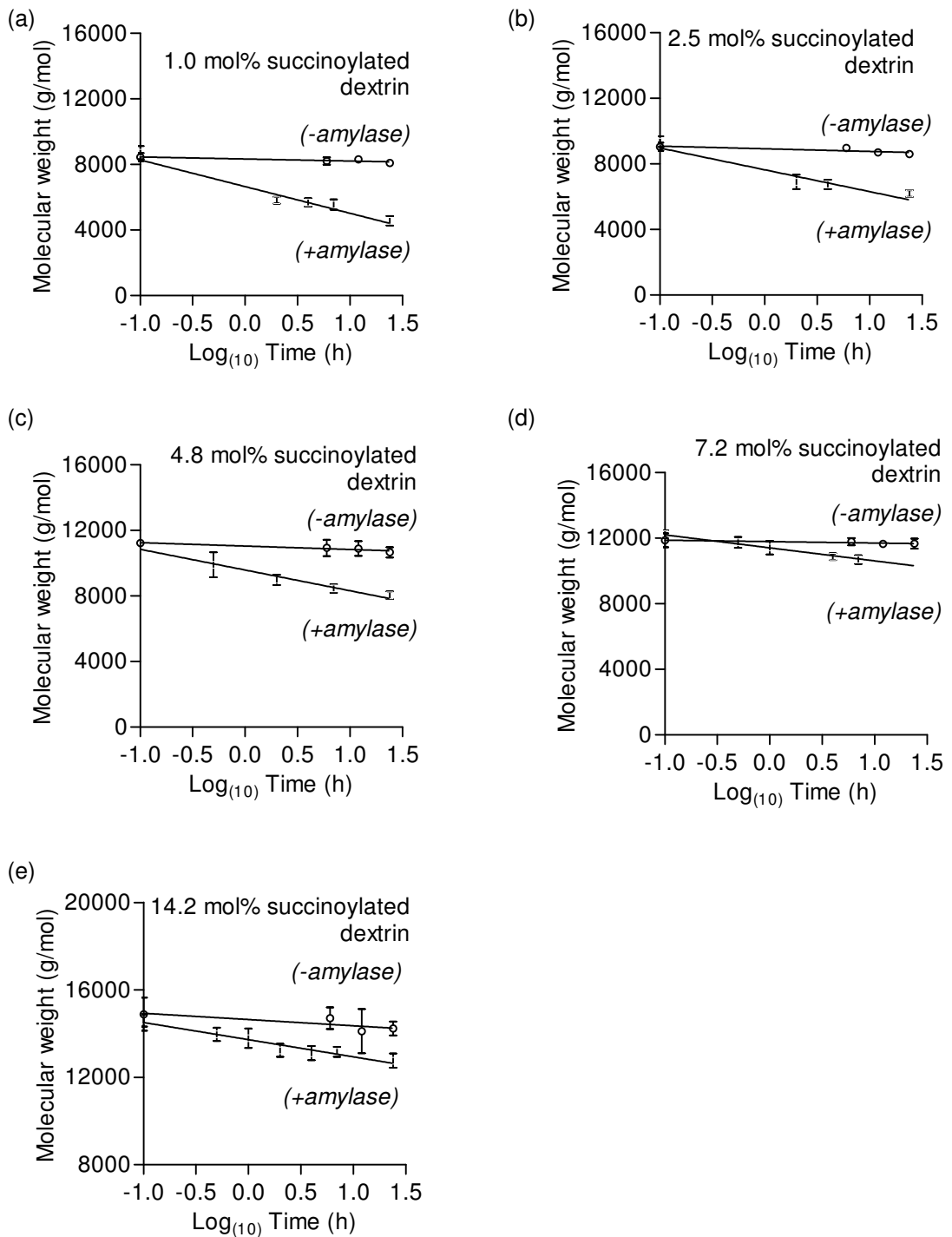
	Theoretical Succinylation (mol %)	α -amylase degradation			control		
		R ²	D.O.F.	95% C.I. (slope)	R ²	D.O.F.	95% C.I. (slope)
LMW parent-dextrin library	15	0.900	16	538 to 769	0.646	16	-17.1 to 0.0110
	10	0.907	16	627 to 883	0.605	16	-5.710 to 0.0959
	5	0.926	16	721 to 975	0.619	16	-13.68 to 0.308
	2	0.956	16	774 to 972	0.621	16	-9.491 to 0.494
MMW parent-dextrin library	15	0.935	16	894.0 to 1,190	0.653	16	-27.5 to 0.162
	10	0.930	16	1,120 to 1,500	0.612	15	-16.9 to 0.846
	5	0.953	16	1,270 to 1,600	0.627	16	-18.0 to 0.605

R²: coefficient of determination; DOF: degrees of freedom; 95% CI (slope): 95% confidence interval (slope). All values are reported to 3 significant Figures.

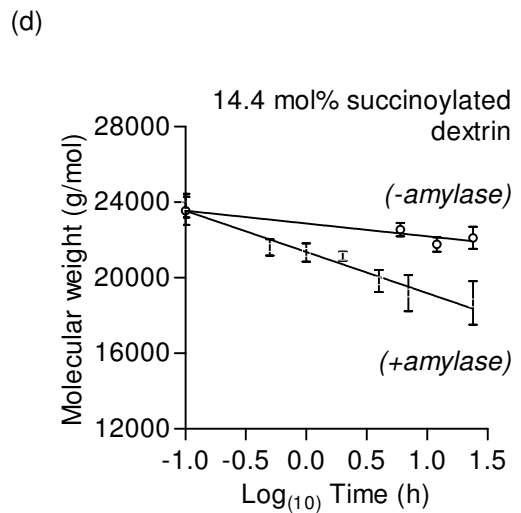
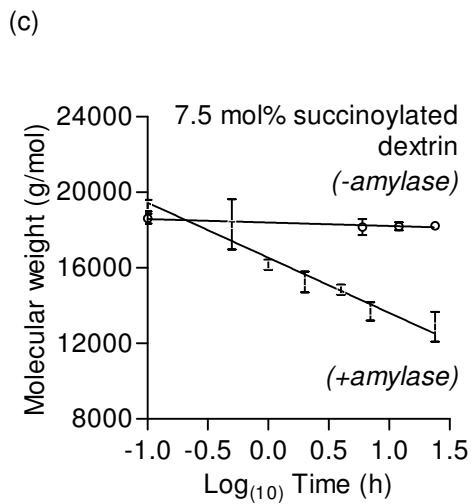
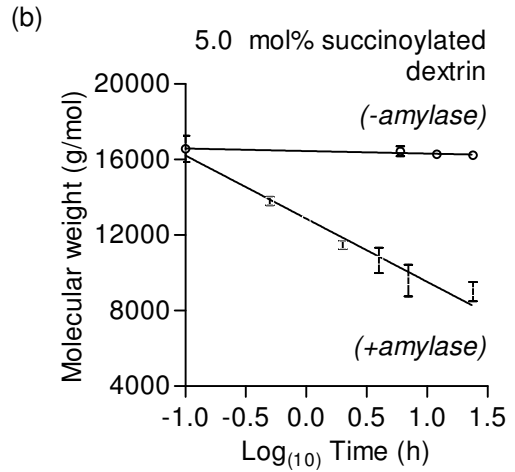
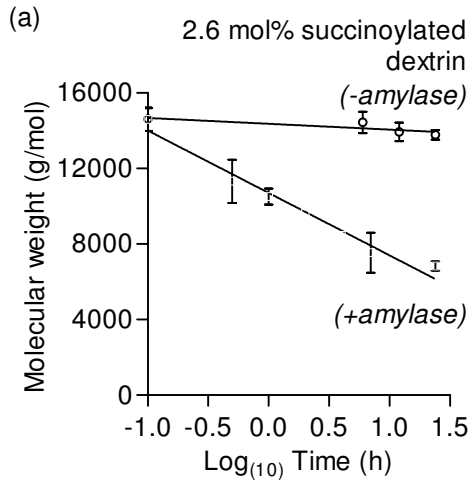
Appendix 2.2i: Best fit data for the enzymatic degradation of dextrin-colistin conjugate and the respective controls (semilog conversion)

	Theoretical Succinoylation (mol %)	A-amylase degradation			Control		
		R ²	D.O.F.	95% C.I. (slope)	R ²	D.O.F.	95% C.I. (slope)
LMW parent-dextrin library	15	0.973	16	-1,851 to -1,159	0.646	16	-338.3 to 4.466
	10	0.978	16	-2,094 to -1,382	0.605	16	-113.9 to 6.490
	5	0.959	16	-2,517 to -1,389	0.619	16	-271.3 to 11.59
	2	0.971	16	-2,485 to -1,535	0.621	16	-186.5 to 7.475
MMW parent-dextrin library	15	0.971	16	-2970 to -1817	0.613	16	-27.5 to 0.162
	10	0.966	16	-3794 to -2224	0.646	15	-16.8 to 0.846
	5	0.968	16	-4,140 to -2,470	0.611	16	-18.0 to 0.605

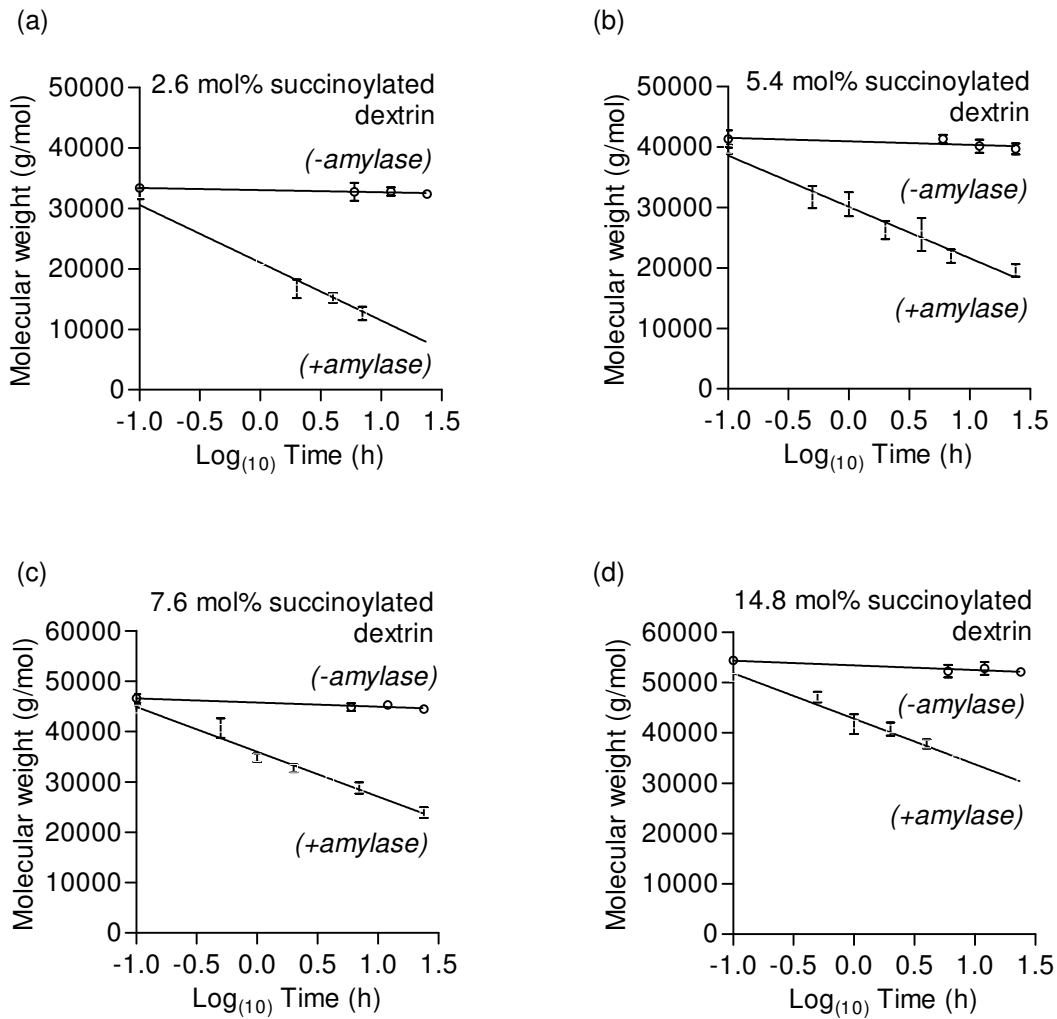
R²: coefficient of determination; DOF: degrees of freedom; 95% CI (slope): 95% confidence interval (slope). All values are reported to 3 significant Figures.



Appendix 2.2j: Degradation rate analysis of succinoylated dextrans in the presence of α -amylase and the respective controls (LMW dextrin library). This graph shows that for all mol% succinoylations studied, slope analysis reported a significantly increased degradation rate in the presence of α -amylase, versus controls: (a):1.0 mol% ($F(1, 28) = 116.88$, $p < 0.0001$); (b) 2.5 mol% ($F(1, 29) = 75.09$, $p < 0.0001$); (c) 4.8 mol% ($F(1, 29) = 50.91$, $p < 0.0001$); (d) 7.2 mol% ($F(1, 29) = 41.71$, $p < 0.0001$); (e) 14.2 mol% ($F(1, 28) = 7.72$, $p < 0.0096$). Data shown represents mean \pm SD ($n=3$). The Shapiro-Wilk test was used to test the normality assumption of the dependent variable and statistical significance was accepted if $p < 0.05$ for this and all subsequent analyses (data not shown).

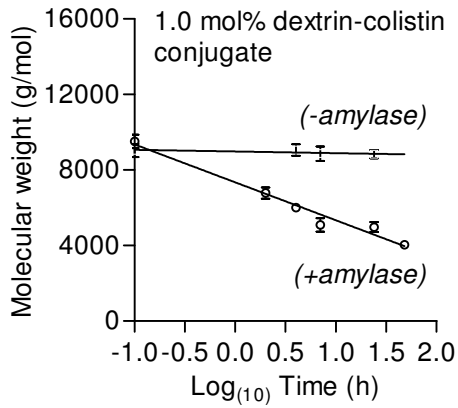


Appendix 2.2k: Degradation rate analysis of succinoylated dextrans in the presence of α -amylase and the respective controls (MMW dextrin library). This graph shows that for all mol% succinoylations studied, slope analysis reported a significantly increased degradation rate in the presence of α -amylase, versus controls: (a) 2.9 mol% ($F(1, 28) = 110.91$, $p < 0.0001$); (b) 5.4 mol% ($F(1, 28) = 194.57$, $p < 0.0001$); (c) 7.7 mol% ($F(1, 29) = 103.8$, $p < 0.0001$); (d) 14.5 mol% ($F(1, 29) = 29.09$, $p < 0.0001$). Data shown represents mean \pm SD ($n=3$).

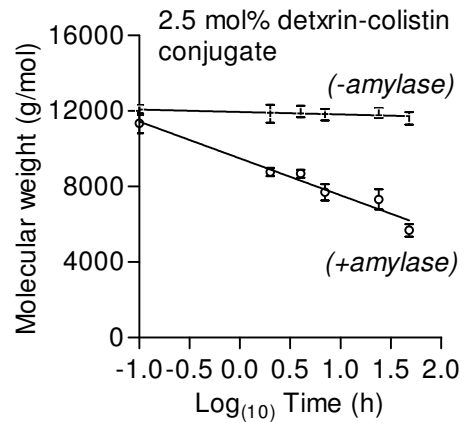


Appendix 2.2I: Degradation rate analysis of succinoylated dextrans in the presence of α -amylase and the respective controls (HMW dextrin library). This graph shows that for all mol% succinoylations studied, slope analysis reported a significantly increased degradation rate in the presence of α -amylase, versus controls: (a) 2.6 mol% ($F(1, 28) = 264.24$, $p < 0.0001$); (b) 5.0 mol% ($F(1, 29) = 136.22$, $p < 0.0001$); (c) 7.8 mol% ($F(1, 29) = 254.07$, $p < 0.0001$); (d) 15.2 mol% ($F(1, 29) = 245.94$, $p < 0.0001$). Data shown represents mean \pm SD ($n=3$).

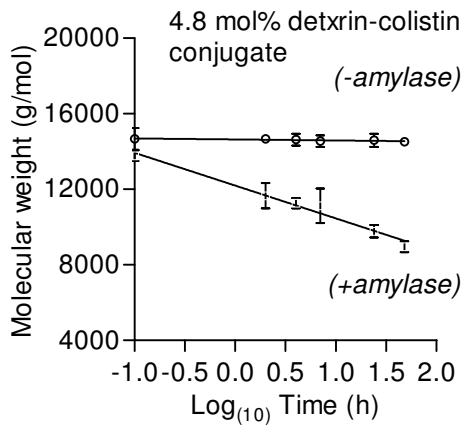
(a)



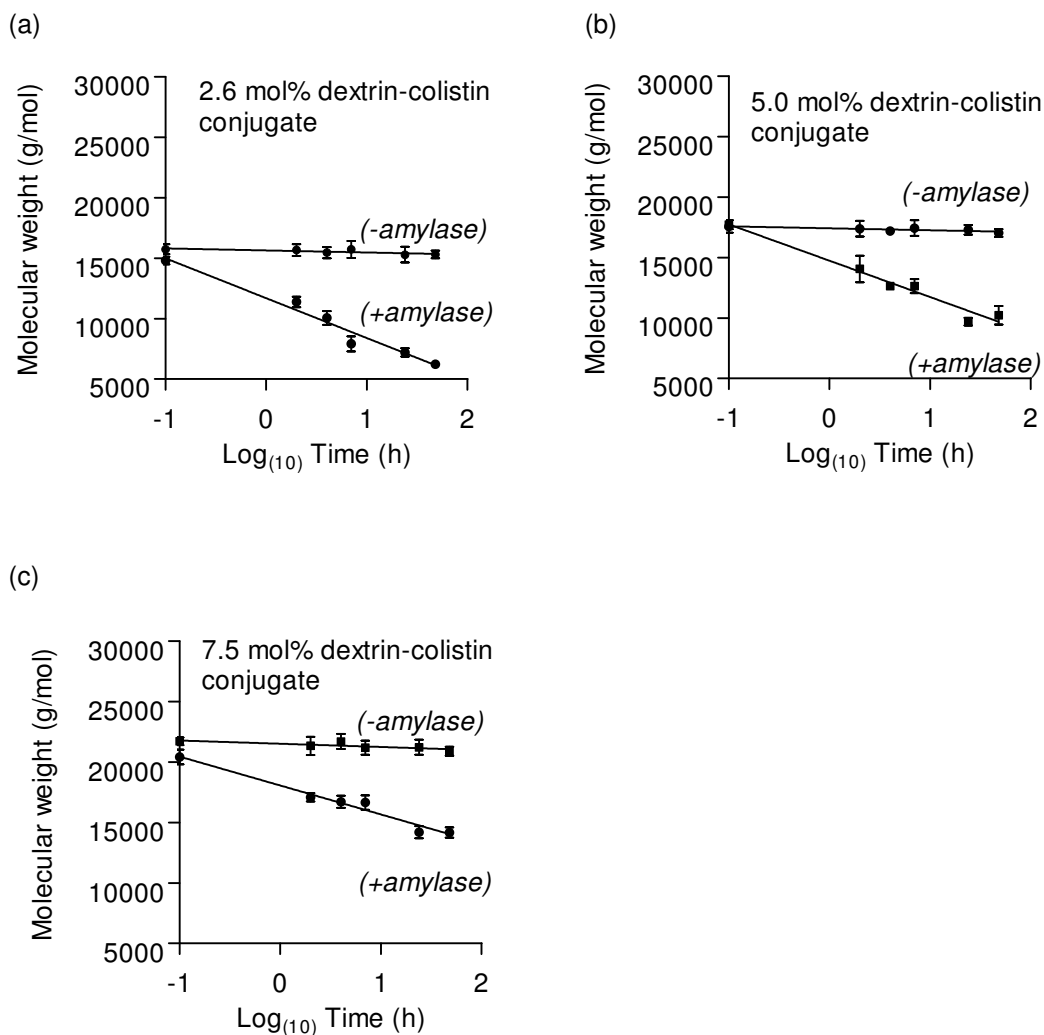
(b)



(c)



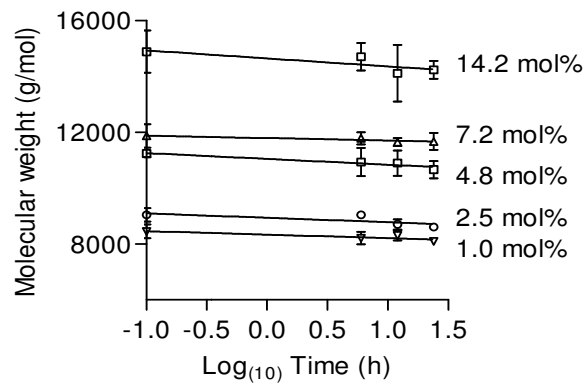
Appendix 2.2m: Degradation rate analysis of dextrin-colistin conjugates in the presence of α -amylase and the respective controls (LMW parent-dextrin library). These graph shows that for all mol% succinoylations reported a significantly increased degradation rate in the presence of α -amylase: (a) 1.0 mol% ($F(1, 32) = 232.33, p < 0.0001$); 2.5 mol% ($F(1, 32) = 128.36, p < 0.0001$); (c) 4.8 mol% ($F(1, 32) = 109.54, p < 0.0001$). Data shown represents mean \pm SD ($n=3$).



Appendix 2.2n: Degradation rate analysis of dextrin-colistin conjugates in the presence of α -amylase and the respective controls (MMW dextrin library). These graphs show that all mol% succinoylations reported a significantly increased degradation rate in the presence of α -amylase.

Using slope analysis of linear regression a significantly increased degradation rates were reported versus controls, for all succinoylations: (a) 2.6 mol% ($F(1, 32) = 198.91, p < 0.0001$); (b) 5.5 mol% ($F(1, 31) = 139.23, p < 0.0001$); (c) 7.7 mol% ($F(1, 32) = 99.53, p < 0.0001$). Data shown represents mean \pm SD ($n=3$).

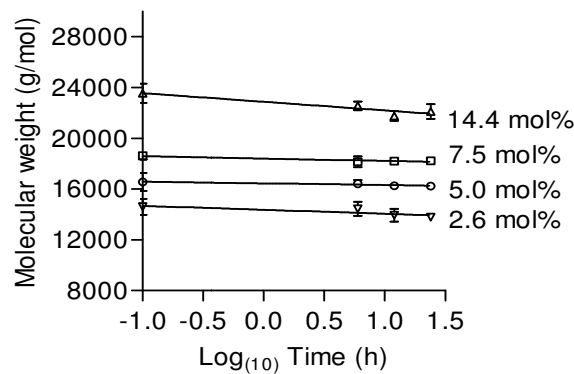
(a)



(b)

Mol%	Slope analysis
14.2	$F(1,2)=4.18$ $p=0.178$
7.2	$F(1,2)=8.46$ $p=0.100$
4.8	$F(1,2)=16.9$ $p=0.054$
2.5	$F(1,2)=2.33$ $p=0.266$
1.0	$F(1,2)=6.38$ $p=0.127$

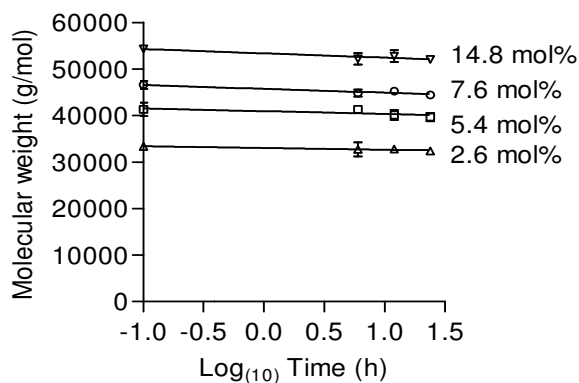
(c)



(d)

Mol%	Slope analysis
14.4	$F(1,2)=14.80$ $p=0.061$
7.5	$F(1,2)=13.0$ $p=0.069$
5.0	$F(1,2)=11.70$ $p=0.762$
2.6	$F(1,2)=4.98$ $p=0.160$

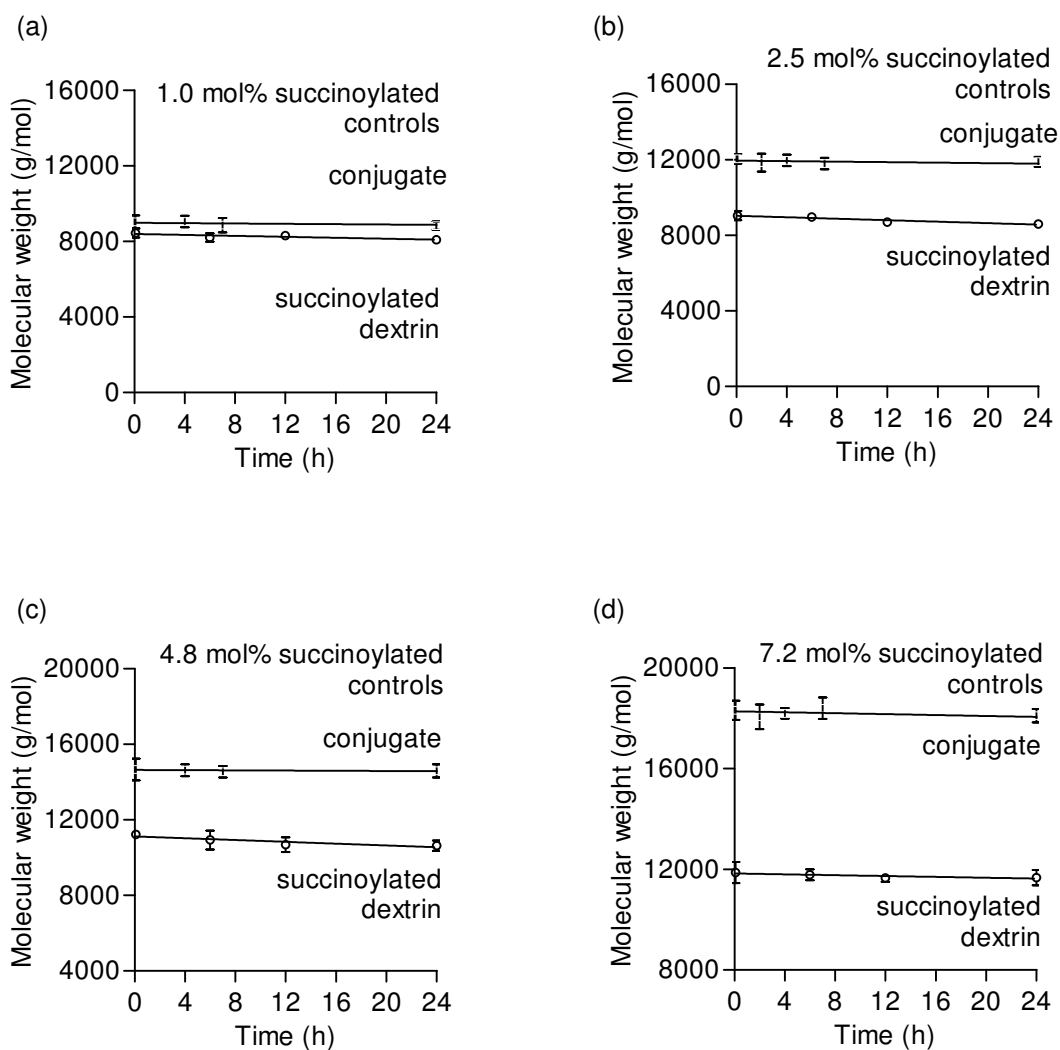
(e)



(f)

Mol%	Slope analysis
14.8	$(F(1,2)=16.50$ $p=0.056$
7.6	$F(1,2)=16.20$ $p=0.056$
5.4	$F(1,2)=2.34$ $p=0.265$
2.6	$F(1,2)=17.90$ $p=0.052$

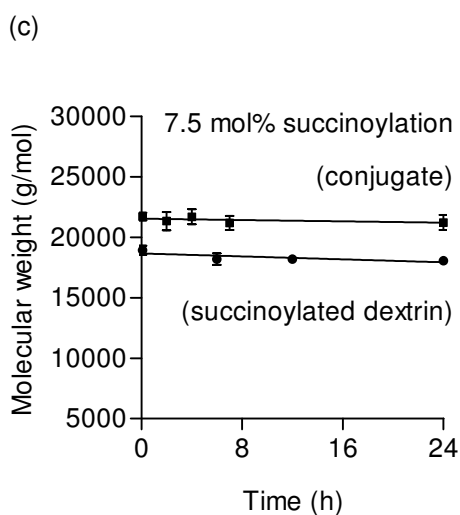
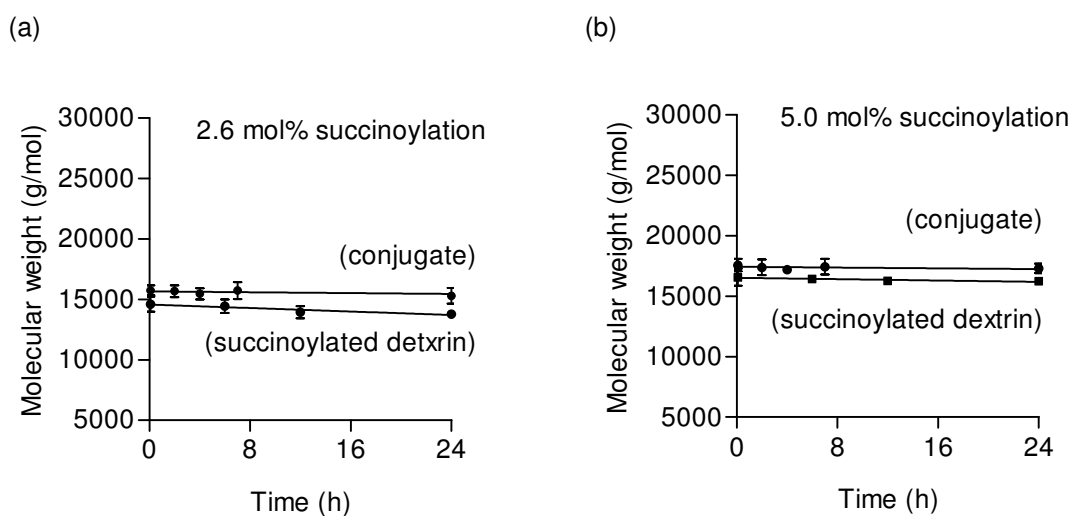
Appendix 2.2o: Degradation rate analysis for succinoylated dextrin controls over time. These graphs report analysis of covariance for all parent-dextrin libraries. Statistical significance of the degradation rate, for each mol% succinoylation is reported in panel (a1): LMW parent-dextrin library; (b1): MMW dextrin library; (c1) HMW parent dextrin library. In all cases some degradation occurred but did not reach statistical significance ($p > 0.05$). Data shown represents mean \pm SD ($n=3$).



(e)

Graph	Mol% succinoylation	ANCOVA statistic	Significance
(a)	1.0	F(1,26)=0.758 p=0.31	p > 0.5
(b)	2.5	F(1,26)=1.95, p=0.17	p > 0.5
(c)	4.8	F(1,25)=3.67, p=0.66	p > 0.5
(d)	7.2	F(1,26) =0.006, p=0.941	p > 0.5

Appendix 2.2p: Comparison of degradation rate of dextrin-colistin conjugates (LMW dextrin library) to their respective succinoylated dextrin precursors. Data shown represents mean \pm SD (n=3). Molecular weight is given relative to pullulan standards. Where error bars are invisible they are within size of data points. Panel (e) reports ANCOVA statistics, showing that in each case, statistical significance was not attained i.e.: degradation rate was not statistically different (p > 0.5).

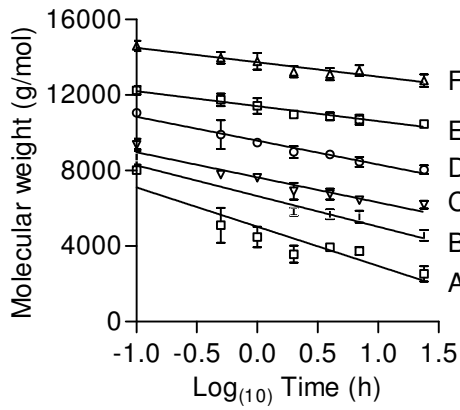


(d)

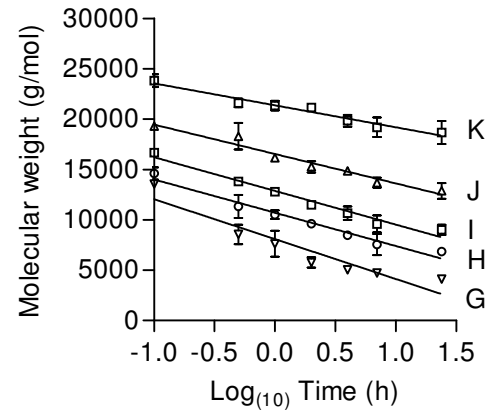
Graph	Mol% succinylation	ANCOVA statistic	Significance
(a)	2.6	F (1, 25) = 2.66, p = 0.12	p > 0.5
(b)	5.0	F (1, 24) = 0.135, p = 0.72	p > 0.5
(c)	7.5	F (1, 26) = 1.01, p = 0.32.	p > 0.5

Appendix 2.2q: Comparison of degradation rate of dextrin-colistin conjugates (MMW dextrin library) to their respective succinoylated dextrin precursors: (a) 2.6 mol% succinylation; (b) 5.0 mol% succinylation; (c) 7.5 mol % succinylation. Data shown represents mean \pm SD (n=3). Where error bars are invisible they are within size of data points. Panel (e) reports ANCOVA statistics, showing that in each case, statistical significance was not attained i.e.: degradation rate was not statistically different (p > 0.5).

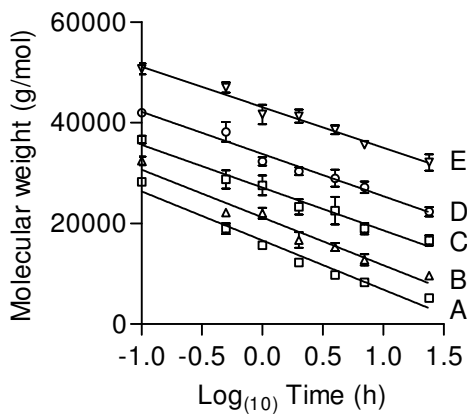
(a)



(b)

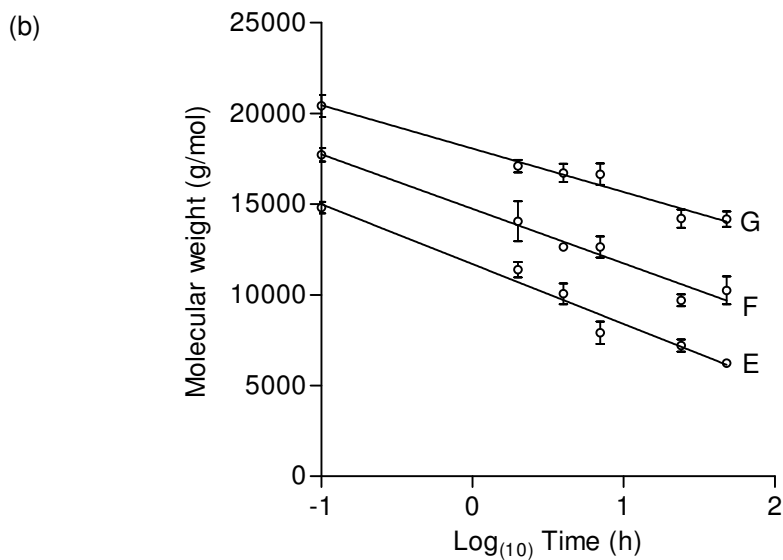
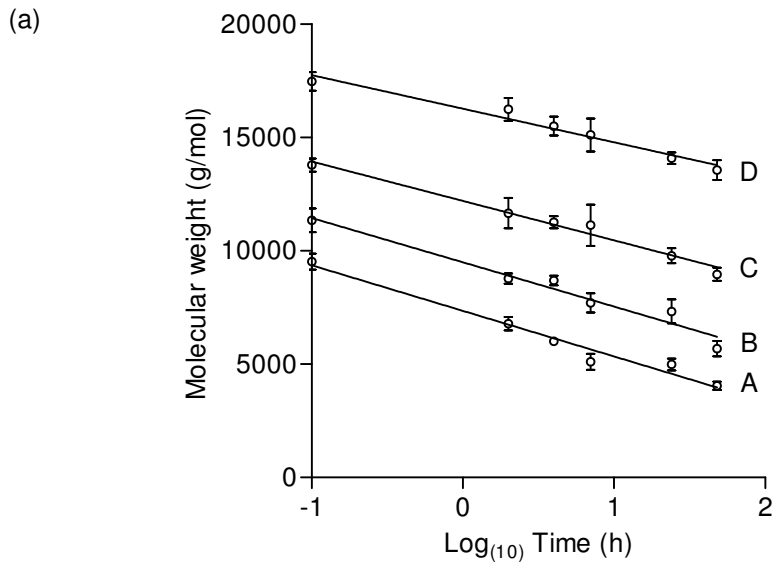


(c)



Appendix 2.2r: Effect of mol% succinoylation on degradation rate. In the presence of α -amylase, degradation rate differed significantly with increasing mol% succinoylation for both (a) LMW dextrans (linear regression, ANCOVA: $F(5, 113) = 14.88$, $p < 0.0001$) and (b) MMW dextrans, ANCOVA: $F(4, 95) = 7.09$, $p < 0.0001$) and (c) HMW dextrans.

Data shown represents mean \pm SD ($n=3$). Legend: A: 0 mol%; B: 1.0 mol%; C: 2.5 mol%; D: 4.8 mol%; E: 7.2 mol%; F: 14.2 mol% succinoylation. MMW dextrin library G: 0 mol%; H: 2.6 mol%; I: 5 mol%; J: 7.5 mol%; K: 14.4 mol% succinoylation.



Appendix 2.2s: Effect of mol% succinylation on degradation rate. Panel (a): in the presence of α -amylase degradation rate differed significantly with increasing mol% succinylation for both (a) LMW conjugates (linear regression, ANCOVA: $F(5, 113) = 14.88$, $p < 0.0001$) and (b) MMW conjugates, ANCOVA: $F(4, 95) = 7.09$, $p < 0.0001$).

Legend: LMW dextrin colistin conjugate family: A: 1.0 mol% succinoylated conjugate; B: 2.5 mol% succinoylated conjugate; C: 4.8 mol% succinoylated conjugate; D: 7.2 mol% succinoylated conjugate. MMW dextrin-colistin conjugate library. Legend: E: 2.6 mol% succinoylated F: 5.0 mol% succinoylated; G: 7.5 % mol succinylation.

Appendix 2.3: Bonferroni multiple comparison tests to compare unmasking between dextrin-colistin conjugates

(i): 2 mol% theoretical succinylation: experiment versus control (LMW parent-dextrin conjugate-library)

Time	Difference	t statistic	p value	Significance (y/n)
0.1 h	0.0	0.0	p > 0.05	N
2 h	-8.93	6.11	p < 0.0001	Y
4 h	-12.0	8.18	p < 0.0001	Y
7 h	-18.1	12.4	p < 0.0001	Y
24 h	-26.1	17.8	p < 0.0001	Y
48 h	-13.0	8.89	p < 0.0001	Y

(ii): 2 mol% (LMW conjugate-library) versus:

Time	5 mol% theoretical succinylation: (LMW parent-dextrin conjugate-library)				5 mol% theoretical succinylation: (MMW parent-dextrin conjugate-library)			
	Diff.*	t statistic	p value	Significance (y/n)	Diff.	T statistic	p value	Significance (y/n)
0.1 h	0.0	0.0	p > 0.05	N	0.0	0.0	p > 0.05	Y
2 h	-7.50	5.13	p < 0.001	Y	-10.8	7.41	p < 0.0001	Y
4 h	-10.0	6.86	p < 0.0001	Y	-13.3	9.07	p < 0.0001	Y
7 h	-10.1	6.93	p < 0.0001	Y	-18.1	12.4	p < 0.0001	Y
24 h	-13.0	8.89	p < 0.0001	Y	-24.7	16.9	p < 0.0001	Y
48 h	-12.0	8.20	p < 0.0001	Y	-19.2	13.1	p < 0.0001	Y

Diff: difference

iii: 5 mol% theoretical succinylation: (LMW parent-dextrin conjugate-library) versus:

5 mol% theoretical succinylation: control (LMW parent-dextrin conjugate-library)					5 mol% theoretical succinylation: experiment (MMW parent-dextrin conjugate-library)			
Time	Diff.*	t statistic	p value	Significance (Y/N)	Diff.	t. statistic	p value	Significance (Y/N)
0.1 h	0.0	0.0	p > 0.05	N	0.0	0.0	p > 0.05	N
2 h	-1.87	1.28	p > 0.05	N	-3.33	2.28	p > 0.05	N
4 h	-3.58	2.45	p > 0.05	N	-3.23	2.21	p > 0.05	N
7 h	-9.60	6.56	p < 0.0001	Y	-8.00	5.47	p < 0.0001	Y
24 h	-16.6	11.4	p < 0.0001	Y	-11.7	8.00	p < 0.0001	Y
48 h	-5.92	4.05	p < 0.05	Y	-7.17	4.90	p < 0.01	Y

Diff: difference

iv: 10 mol% theoretical succinylation: (LMW parent-dextrin conjugate-library) versus:

10 mol% theoretical succinylation: control LMW parent-dextrin conjugate-library)					10 mol% theoretical succinylation: experiment (MMW parent-dextrin conjugate-library)			
Time	Diff.*	t statistic	p value	Significance (Y/N)	Diff.	t statistic	p value	Significance (Y/N)
0.1 h	0.0	0.0	p > 0.05	N	0.333	0.228	p > 0.05	N
2 h	-0.477	0.326	p > 0.05	N	-1.15	0.789	p > 0.05	N
4 h	-2.73	1.87	p > 0.05	N	-3.10	2.12	p > 0.05	N
7 h	-4.97	3.40	p > 0.05	N	-5.36	3.66	p > 0.05	N
24 h	-10.1	6.91	p < 0.0001	Y	-9.77	6.68	p < 0.0001	Y
48 h	-6.24	4.27	p < 0.05	Y	-6.91	4.72	p < 0.01	Y

Diff: difference

v: 15 mol% theoretical succinylation: (LMW parent-dextrin conjugate-library) versus:

15 mol% theoretical succinylation: control (LMW parent-dextrin conjugate-library)					15 mol% theoretical succinylation: experiment (MMW parent-dextrin conjugate-library)			
Time	Diff.*	t statistic	p value	Significance (Y/N)	Diff.	t statistic	p value	Significance (Y/N)
0.1 h	0.0	0.0	p > 0.05	N	0.0	0.0	p > 0.05	N
2 h	-0.028	0.0189	p > 0.05	N	3.12	2.13	p > 0.05	N
4 h	-0.733	0.501	p > 0.05	N	-0.433	0.296	p > 0.05	N
7 h	-2.03	1.39	p > 0.05	N	-1.63	1.12	p > 0.05	N
24 h	-11.1	7.57	p < 0.0001	Y	-6.13	4.19	p < 0.05	Y
48 h	-7.33	5.01	p < 0.001	Y	-6.25	4.27	p < 0.05	Y

Diff: difference

vi: 5 mol% theoretical succinylation: experiment versus control (MMW parent-dextrin conjugate-library)

Time	Difference	t statistic	p value	Significance (Y/N)
0.1 h	0.0	0.0	p > 0.05	N
2 h	-0.833	0.570	p > 0.05	N
4 h	-3.00	2.05	p > 0.05	N
7 h	-3.10	2.12	p > 0.05	N
24 h	-9.93	6.79	p < 0.0001	Y
48 h	-11.2	7.68	p < 0.0001	Y

Diff: difference

vii: 10 mol% theoretical succinylation: experiment versus control (MMW parent-dextrin conjugate-library)

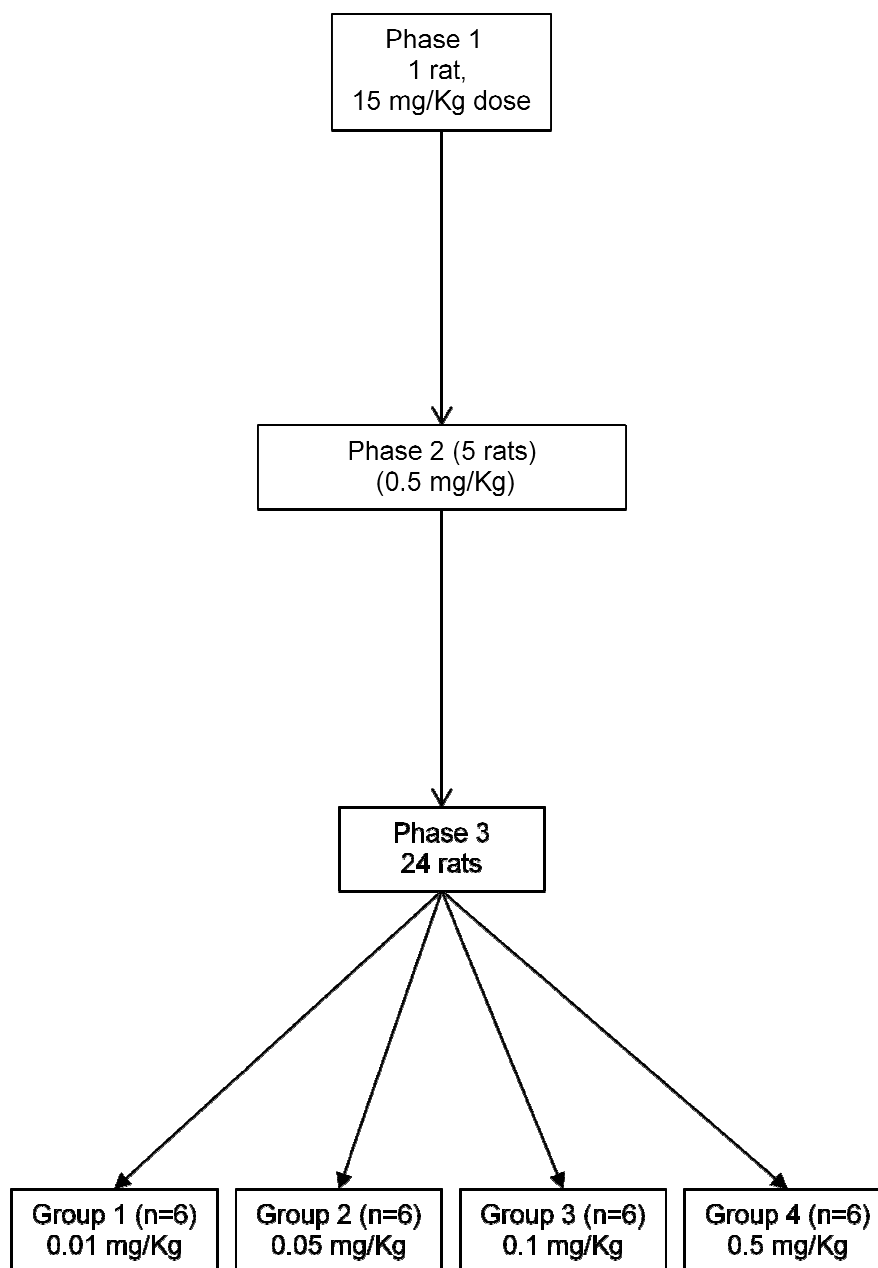
Time	Difference	t statistic	p value	Significance (Y/N)
0.1 h	-0.333	0.228	p > 0.05	N
2 h	-1.88	1.28	p > 0.05	N
4 h	-1.57	1.07	p > 0.05	N
7 h	-3.11	2.13	p > 0.05	N
24 h	-10.7	7.34	p < 0.0001	Y
48 h	-10.7	7.29	p < 0.0001	Y

Diff: difference

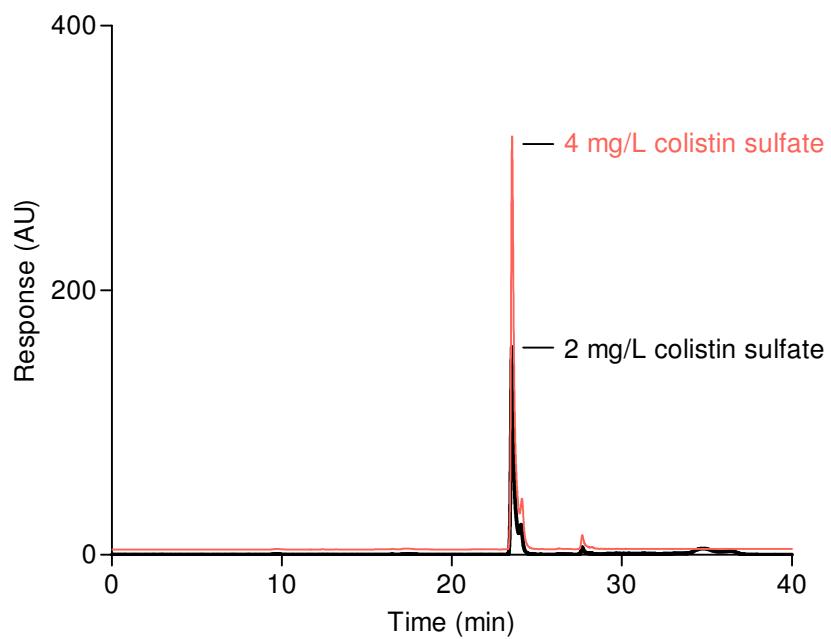
viii: 15 mol% theoretical succinylation: experiment versus control (MMW parent-dextrin conjugate-library)

Time	Difference	t statistic	p value	Significance (Y/N)
0.1 h	0.0	0.0	p > 0.05	N
2 h	0.410	0.280	p > 0.05	N
4 h	0.533	0.365	p > 0.05	N
7 h	3.73	2.55	p > 0.05	N
24 h	11.1	7.57	p < 0.0001	Y
48 h	11.3	7.70	p < 0.0001	Y

Diff: difference



Appendix 2.4: Temporal progress of the *in vivo* study showing the three phases.



Appendix 2.5: Typical RP-HPLC chromatogram for colistin (positive control) using the gradient method on a 250 mm C₁₈ column, when derivatisation was attempted using method (ii) liquid phase synthesis (in solution), showing a peak for colistin which doubled with doubling concentration.

(a)

ID	Test Antibiotic	Nominal sampling time (min)					1440
		5	30	60	240	480	
		Actual sampling time (min)					
028M	EA-4	6	31	60	240	480	1442
029M		6	31	60	240	480	1442
030M	EA-1	6	30	60	240	480	1452
031M		6	31	61	240	480	1441
032M	Colistin	6	31	60	240	480	1441
033M		5	30	60	240	480	1440
<i>Average sampling time*</i>		<i>5.8 (0.4)</i>	<i>30.7 (0.5)</i>	<i>60.2 (0.4)</i>	<i>240.0 (0)</i>	<i>480.0 (0)</i>	<i>1443.0 (4.6)</i>

* (mean ± SD)

(b)

ID	Test Antibiotic	Nominal sampling time (min)					1440
		5	30	60	240	480	
		Actual sampling time (min)					
034M	EA-4	6	31	60	241	480	1440
035M		6	30	60	240	480	1440
036M	EA-1	NS	31	60	240	480	1441
037M		NS	30	60	240	480	1441
038M	Colistin	NS	NS	NS	240	480	1440
039M		NS	NS	NS	240	480	1440
<i>Average sampling time*</i>		<i>6.0 (0)</i>	<i>30.5 (0.6)</i>	<i>60.0 (0)</i>	<i>240.0 (0)</i>	<i>480.0 (0)</i>	<i>1440.0 (0.5)</i>

* mean (± SD); NS: no sample

Appendix 2.6: Group comparisons: (a) blood sampling times following intravenous administration of test-antibiotic at 0.1 mg/kg to Male Sprague Dawley rats (b) blood sampling times following intravenous administration of test-antibiotic at 0.5 mg/Kg to Male Sprague Dawley rats. Actual and nominal sampling times did not vary significantly (two-way ANOVA, $p > 0.05$).



Dear Dr Azzopardi,

Thank you for your interest in our MaxSignal Colistin ELISA Test Kit (Catalog #: 1095-01). The MaxSignal Colistin kit has been successfully used in the food industry for detection of colistin antibiotics in chickens since Jan 2012 to meet Chinese USDA's regulation. Please bear in mind that this is a proprietary information, and therefore the information I am allowed to disclose is limited by Bioo Scientific Corporation's policy. However it is possible for me to assist you on this occasion.

Q1. The antibody used in this case is indeed a polyclonal antibody.

Q2. The Clean Up solutions are intended to extract the maximum amount of colistin present in a sample with reduced amount of interfering substances. The reagents used in the process contain organic solvents which could lead to breakdown of carbohydrates present in the sample.

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Senior Chemist

01/25/2013



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