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Formulation approaches to pulmonary delivery of colistin combination antibiotic therapy

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
from
Monash Institute of Pharmaceutical Sciences
Monash University

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This thesis is dedicated to my father
for his continual support of my academic pursuits

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Abstract

Advanced formulations such as liposomes are increasingly being employed to improve the therapeutic profile of existing drugs, and to facilitate appropriate delivery of combination drug products. This is particularly the case for antibiotic therapies, due to the development of bacterial resistance and consequent need for combination drug approaches.

Multi-drug resistant (MDR) Gram-negative bacteria have become a major medical problem worldwide over the last decade, with rapidly diminishing therapeutic options and very few new agents in the drug development pipeline. This situation has forced the reintroduction of an old antibiotic, colistin, back into clinical use. Colistin inhalation therapy is being increasingly used as a salvage therapy for the treatment of MDR Gram-negative pulmonary infections; however, there is a dearth of information on its pharmacokinetics and pharmacodynamics. Importantly, current inhalable formulations have not been optimised. Combination therapy may be an important approach towards optimising the use of colistin in the treatment of MDR pulmonary infections. Inhalable formulations providing sustained concentrations of the combination of colistin and a second antibiotic within the lungs would offer a more effective inhalation therapy against MDR Gram-negative pulmonary infections. The research undertaken in this thesis investigates the use of colloidal-based drug delivery systems for the co-formulation of colistin and model poorly-water soluble co-drug, azithromycin.

The surfactant-like structure of colistin and its prodrug, colistin methanesulphonate (CMS), has been suggested to impact on their solution behaviour and stability, however their self-assembly in solution has not been well studied. In this thesis dynamic light scattering studies have confirmed the formation of colistin and CMS association

colloids at critical micelle concentrations of 2.1 and 6.1 mg/mL, respectively. The self-assembly of CMS impacted the rate of conversion of CMS to colistin in solution confirming that the concentration-dependent stability of CMS is attributable to micelle formation.

Micelle formation by colistin and CMS in solution enabled solubilization of poorly-water soluble drugs, however the limited capacity of the micelles was deemed insufficient to use micellar solubilization solely as a means to co-formulate a poorly-water soluble antibiotic with either colistin or CMS. Consequently, liposomes were investigated as a drug delivery system with potential to co-formulate colistin and CMS with other antibiotics. Colistin was successfully incorporated into liposomes, and the incorporation of cholesterol into the liposome bilayer enhancing associations between colistin and the bilayer. However, it was found that CMS-loaded liposomes exhibited poor colloidal stability, resulting in particle size growth and changes in particle surface charge over time, and eventual phase separation. The formulation of CMS within a liposomal carrier also accelerated the conversion of the prodrug to colistin. The CMS-loaded liposome approach was therefore abandoned and further studies concentrated on colistin-loaded liposomes.

Co-formulation of azithromycin and colistin in liposomes was consequently investigated. Remote-loading of liposomes using the pH gradient method enabled greater solubilization of azithromycin (azithromycin to lipid ratio 0.20:1) compared to passive loading (azithromycin to lipid ratio 0.16:1). *In vitro* release studies demonstrated that upon dilution, colistin rapidly re-established equilibrium associations with the liposome bilayer. Incorporation of colistin into the remotely-loaded azithromycin formulation accelerated the rate of azithromycin release from 'slow release' liposomes in a concentration-dependent manner, indicating that the association

of colistin with liposomes modified bilayer fluidity. However, the rate of release of azithromycin from liposomes was also reduced by increasing the cholesterol composition in the lipid bilayer.

Liposomes were investigated for their potential to provide co-localisation of colistin and azithromycin within the lungs following pulmonary delivery in a rat model. The temporal aspects of availability of drug in the lungs were investigated under the assumption that local drug availability was reflected by the rate of pulmonary absorption. Pulmonary absorptive drug clearance in turn was assumed to be reflected in plasma drug concentrations. Thus changes in plasma concentration profiles with changes in formulation were used as an indicator of drug availability in the lungs as a consequence of release of drug from the formulation. Liposomal encapsulation resulted in a 47% decrease in maximum plasma concentrations of colistin and a 26% reduction in systemic exposure, compared to unencapsulated colistin. The rate of pulmonary absorption of azithromycin, however, was not impeded by liposomal encapsulation.

This thesis is the first study to investigate the potential of advanced drug delivery systems to provide co-localisation of colistin and azithromycin within the lungs following pulmonary delivery.

Statement of originality

The Faculty Manager

Faculty of Pharmacy and Pharmaceutical Sciences

Monash University

I hereby certify that the work presented in this thesis has not been submitted by me or by any other person for the award of a degree at Monash University of any other institution. To the best of my knowledge, this thesis contains no material previously published or written by another, except where due reference is made in the text of this thesis. Chapter 3, whilst not a direct reproduction of published work, forms the basis of a publication arising from work conducted during this PhD.

Stephanie Wallace

February 2011

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Publications and communications

Publications

1. Wallace S.J, Li J, Prankerd RJ, Velkov T, Boyd B. Self-assembly behaviour of colistin and its prodrug colistin methanesulphonate: implications for solution stability and solubilization. *Journal of Physical Chemistry*, 2010; 114 (14):4836 - 40.
2. Wallace SJ, Li J, Nation RL, Boyd B. Measurement of drug encapsulation and release from nanoparticles, *Journal of Pharmaceutical Science.* (In preparation).
3. Towards understanding the interactions of colistin and colistin methanesulphonate with biomembranes and liposomes. *Journal of Colloid and Interface Science* (in preparation).
4. Wallace SJ, Li J, Nation RL, Boyd B. Azithromycin liposomes: *in vitro* release and pharmacokinetics after pulmonary administration to rats. *Journal of Liposome Research* (in preparation).
5. Wallace SJ, Li J, Nation RL, Boyd B. Pharmacokinetics of colistin liposomes following pulmonary administration to rats. *Antimicrobial Agents and Chemotherapy* (in preparation).

Communications

1. Wallace SJ, Li J, Nation RL, Boyd B. Formulation and characterisation of combination colistin and azithromycin liposomes. Formulation and physicochemical characterisation of colistin liposome formulations. Australian Pharmaceutical Science Association, Canberra, Dec 2008.*
2. Colistin-azithromycin combination liposome inhalation therapy. Royal Australian Chemical Institute Half-Day Symposium, Parkville, Dec 2008.*
3. Wallace SJ, Li J, Nation RL, Boyd B. Formulation and characterisation of combination colistin and azithromycin liposomes. Monash Parkville Postgraduate Symposium, Sept 2009.*
4. Wallace SJ, Li J, Nation RL, Boyd B. Formulation and characterisation of combination colistin and azithromycin liposomes. Formulation and physicochemical characterisation of colistin liposome formulations. 36th Annual Meeting of the Controlled Release Society, Copenhagen, Denmark, July 2009.

*Award winning

List of abbreviations

ACE	acetone
ACN	acetonitrile
A_R	relative atomic mass
BAL	bronchoalveolar lavage
CF	cystic fibrosis
chol	cholesterol
CMC	critical micelle concentration
cP	centipoise
cryo-TEM	cryogenic transmission electron microscopy
% CV	co-efficient of variation
D	translational diffusion co-efficient
Da	daltons
Dab	diaminobutyric acid
DLS	dynamic light scattering
DOPC	dipalmitoylphosphatidylcholine
DOPE	dioloeylphosphatidylethanolamine
DOPG	dioloeylphosphatidylglycerol
DPB	diffuse panbronchiolitis
DPPC	dioloeylphosphatidylcholine
DSC	differential scanning calorimetry
EE	encapsulation efficiency
ELF	epithelial lining fluid
EMA	European Medicines Agency
FDA	Food and Drug Administration
FMCA	formic acid
FMOC-Cl	9-fluorenylmethyl chloroformate
FTIR	fourier transform infra red spectrscopy
GC-MS	gas chromatography-mass spectrometry
h	hour
HAP	hospital acquired pneumonia
HPLC	thin layer chromatography
IT	intratracheal
ITC	isothermal microcalorimetry
IV	intravenous
kDa	kilo Daltons
kcps	kilo counts per second
LC-MS	liquid chromatography coupled to mass spectrometry
LD_{50}	lethal dose 50 %
LPS	lipopolysaccharide
log D	diffusion co-efficient
log P	octanol-water partition coefficient

LUV	large unilamellar vesicle
MDR	multi-drug resistant
MeOH	methanol
MIC	minimum inhibitory concentration
min	minutes
MLV	multi-lamellar vesicle
mOsm	milliosmoles
MS	mass spectrometry
mS/cm	milli seconds per centimetre
mN/m	milli Newtons per metre
M_W	molecular weight
MWCO	molecular weight cut-off
m/*z*	mega hertz
NMR	nuclear magnetic resonance
OLV	oligolamellar vesicle
o/w	oil-in-water
o/w/o	oil-in-water-in-oil
Pa	pascals
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDI	polydispersity index
PDR	pan-drug resistant
PEG	polyethylene glycol
pK_a	acid dissociation constant
PMBN	polymyxin B nonapeptide
psi	pound-force per square inch
PUF	pressure ultrafiltration
QC	quality control
r^2	co-efficient of determination
R_f	retention factor
sec	seconds
SME	submicron emulsion
SPC	soy phosphatidylcholine
SUV	small unilamellar vesicle
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
T_m	phase transition temperature
UV	ultraviolet
µW/sec	micro watts per second
VAP	ventilator associated pneumonia
w/o	water-in-oil
λ_z	terminal half-life

Chapter 1: Introduction

1.1 Statement of the problem

Colistin is an old antibiotic undergoing a resurgence in clinical use owing to its significant activity against multi-drug resistant (MDR) Gram-negative bacteria. It has the status of a last-line of defence antibiotic and is used where other antibiotics are ineffective. Colistin is increasingly being used for the treatment of lung infections caused by MDR Gram-negative bacteria in people with cystic fibrosis and in critically-ill patients with ventilator-associated pneumonia (VAP). Worryingly, however, resistance to this valuable antibiotic has recently begun to emerge, posing a particular problem for the treatment of respiratory infections requiring aggressive acute or chronic antibiotic therapy. As no new antibiotics with activity against important Gram-negative bacteria will be available in the next decade,[1] approaches to intensify and optimise the inhalation use of colistin are urgently needed to minimise the development of resistance and to ensure the clinical utility of colistin for decades to come. Current formulations for the treatment of MDR pulmonary infections via direct delivery to the lungs are not formulated according to a rational formulation approach. Pharmacodynamic evidence suggests that colistin combination therapy may be more efficacious and efficient in preventing the emergence of colistin-resistant bacteria, compared to colistin monotherapy. Therefore, significant scope exists to improve colistin formulations for inhalation by co-formulation with a second antibiotic. Such formulations would have the potential to deliver and localise two active agents in the lungs at the site of infection, thereby intensifying the treatment of MDR Gram-negative pulmonary infections with colistin.

1.2 Background

1.2.1 The emergence of multi-drug resistance

Antimicrobial resistance is an unfortunate but inevitable consequence of antimicrobial use.[2] In fact, bacteria have developed resistance mechanisms against all classes of antibiotics shortly after or even prior to their clinical introduction.[3] Bacteria that develop resistance to two or more antibiotic classes are classified as MDR.[4] These so-called 'superbugs' are now one of the most challenging problems faced by modern medicine[5] and present a major medical problem worldwide.[6] Over the past decade, there has been a dramatic increase in MDR Gram-negative pathogens recovered from nosocomial infections.[7-8] Opportunistic pathogens can cause infections of the bladder, blood stream, brain, respiratory tract and at post-surgical sites. There are numerous respiratory conditions requiring aggressive or chronic antibiotic therapy (section 1.2.3.6), including cystic fibrosis (CF),[9-10] non-CF bronchiectasis,[11-12] VAP,[13-14] and chronic obstructive pulmonary disease (COPD);[15] the emergence of MDR pathogens in patients with such conditions causes a significant burden. Patients with underlying conditions (e.g. acquired immune deficiency syndrome) are particularly prone to hospital-acquired infections caused by MDR bacteria.[16] As a consequence of their astounding ability to rapidly acquire or develop resistance to multiple antibiotics concurrently,[17-18] Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae,* are becoming an increasing problem in the clinical setting and all are associated with high rates of mortality.[18-21]

MDR *P. aeruginosa* is a particular problem for cystic fibrosis (CF) patients, being the major cause of chronic respiratory infection in adult patients[22] and an independent risk factor for mortality.[23] In CF patients, pulmonary disease due to chronic respiratory infection is the cause of death in over 90% of patients.[24-25] The encumbrance of chronic

P. aeruginosa infection is largely due to the ability of *P. aeruginosa* to protect itself from antimicrobial insult by establishing biofilm communities in the airways, as initiated by 'quorum sensing' cell-to-cell communication.[26] *P. aeruginosa* is a common cause of pneumonia in patients with chronic obstructive pulmonary disease and acute respiratory distress syndrome[27-29] and is particularly invasive in intensive care units (ICUs) where it represents one of the leading causes of nosocomial infections,[30-32] frequently causing ventilator associated pneumonia (VAP).[35]

A common pathogen causing infections in critically-ill patients, *A. baumannii* is endemic in ICUs, accounting for up to 10% of all Gram-negative isolates and 10% of all cases of pneumonia in some parts of the world.[33-34] *A. baumannii* has biofilm-forming capabilities and the ability to survive in nutrient-poor environments for extended periods,[35-36] undoubtedly contributing to its tendency to cause respiratory infections associated with the use of mechanical ventilation.[37] Progressively, *A. baumannii* has become increasingly resistant to all major classes of antibiotics including the penicillins, aminoglycosides, cephalosporins, fluoroquinolones, the newer carbapenems and even the polymyxins.[38-46] Its astounding ability to acquire cross resistance[47-49] and rapidly adapt to environmental pressures[50] makes MDR *A. baumannii* a serious global health threat.

Along with its highly virulent nature, the ability of *K. pneumoniae* to rapidly acquire plasmid-mediated resistance from other bacterial species *in vivo*[51] makes it a growing medical concern. Resistance in *K. pneumoniae* is on the rise,[46, 52] reportedly increasing from 1.5 to 20% over a 6-year period in the United States.[53] MDR *K. pneumoniae* is a common cause of life-threatening VAP[38, 46] with mechanical ventilation and ICU stay both risk factors for colonisation and infection with *K. pneumoniae*.[54]

Heightening the issue of multi-drug resistance amongst problematic Gram-negative pathogens is the slow progress in the discovery and development of new antibacterial agents (Figure 1-1);[3, 55-56] since 2000, only two new antibiotics possessing novel pharmacology, and hence potential to overcome current resistance mechanisms, have been introduced.[57]

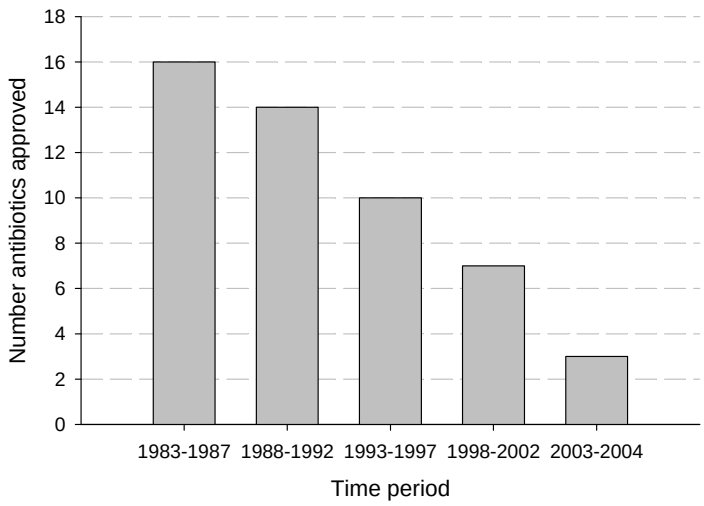


Figure 1-1: New antibiotics approved in US 1983 - 2004 (modified from Spellberg *et al*. 2004).

While progress in antibiotic development is slow, antimicrobial resistance is evolving at an accelerating pace.[58-60] At present, there are no new agents in the drug development pipeline specifically targeting Gram-negative bacteria via a novel mechanism of action.[6] Tigecycline, a novel first-in-class glycyclcycline, was introduced to clinical use in 2005, offering some respite from the burden of MDR *K. pneumoniae* and *A. baumannii*. However, tigecycline is not active against *P. aeruginosa*[61-62] and is already affected by the phenomenon of bacterial resistance.[63-65] Since 2005, no novel antibiotics with activity against Gram-negative bacteria have been introduced, and none is anticipated to be commercially available within the next decade.[66-67] This urgent, unmet medical need has forced the re-introduction of an old class of antibiotics, the polymyxins, which are

frequently used to treat infections when other antibiotic options fail due to multi-drug resistance.

1.3 Colistin

Colistin (Figure 1-2) is a polymyxin antibiotic that is produced as a secondary metabolite by *Bacillus polymyxia*, first identified in 1947 for its significant *in vitro* activity against Gram-negative bacteria.[68-70] There are at least five closely related compounds in the polymyxin class of antibiotics: polymyxins A, B, C, D and E. Of these five, polymyxin E (colistin) and polymyxin B are used therapeutically, but only colistin will be discussed in detail. Polymyxin B is, however, considered a 'model' polymyxin compound and is frequently used for the study of polymyxin chemistry and physical interactions, and hence will be occasionally referred to.

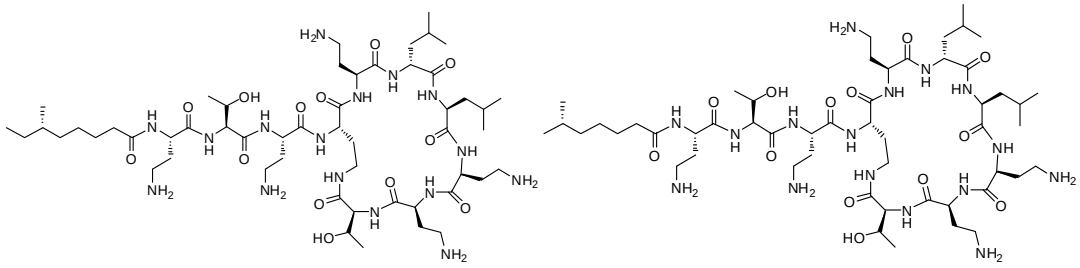


Figure 1-2: Chemical structures of colistin A (left) and colistin B (right).

Commercially, colistin is available in two forms: colistin sulphate and the sodium salt of colistin methanesulphonate (CMS; Figure 1-3). Colistin sulphate is only used orally and topically due to its tendency to cause nephrotoxicity following systemic administration. The prodrug of colistin, colistin methanesulphonate sodium,[71] is the form of colistin most commonly administered intravenously and is also be administered intrathecally and via inhalation. CMS was introduced as a therapeutic agent in 1959.[1, 72-73] However, it fell out of favour in the 1960s due to reports of variable toxicities. The most common toxic manifestation associated with the use of CMS is nephrotoxicity, the

features of which are increased blood urea nitrogen and serum creatinine, and a range of proximal tubular changes.[74-76] Nephrotoxicity may be accompanied by neurotoxicity[74, 77] which can manifest as one or more symptoms including dizziness, numbness, tingling, muscular weakness, peripheral neuropathy or paresthesia.[78]

Early studies reported severe CMS-induced toxicities,[76, 78-80] while others reported only minimal side effects.[81-84] In the 1970s, colistin became superseded by newer agents that were deemed at the time to be 'less toxic'. As such, colistin was relegated to a 'last-line' antibiotic,[72, 85] used only when other therapeutic options failed. More recently however, with the increasing burden of infections caused by MDR bacteria, clinicians have been forced to re-visit the clinical use of colistin due to a lack of alternative therapeutic options. In contrast to earlier clinical studies, the recent use of CMS as salvage therapy has shown it to be a relatively safe and effective therapeutic option.[86-87] Earlier clinical studies, may have exaggerated the severity or frequency of adverse events related to CMS use, perhaps due to inappropriate patient selection, dosage regimens or patient monitoring.[88-92]

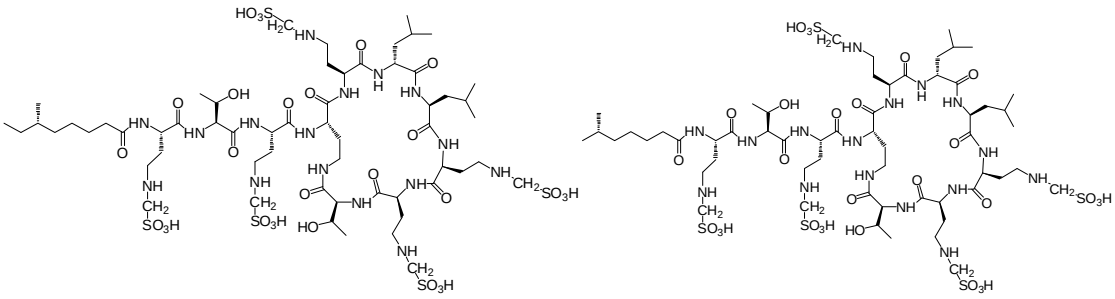


Figure 1-3: Chemical structure of CMS A (left) and CMS B (right).

While the world awaits the commercial release of antibiotics currently undergoing drug development, the prudent use of the polymyxins is absolutely vital in order to preserve their clinical utility. The polymyxins entered clinical use more than half a century ago and so have not been subjected to the typical drug development scrutiny experienced by

modern day drug candidates. The selection of appropriate dosage regimens and dosage form design requires the consideration of pharmacodynamic (PD) and pharmacokinetic (PK) parameters which optimise clinical use by maximising microbiological efficacy, minimising toxicity and minimising the development of bacterial resistance. Unfortunately, the scarcity of information regarding the PK and PD of the polymyxins means that the current clinical use is not informed by modern day PK/PD rationale, and hence, the current clinical use is likely to be sub-optimal. Thus, strategies to optimise and intensify the clinical use of colistin must be urgently implemented.

1.3.1 Chemistry

Colistin ([1264-72-8, CAS registry number], Figure 1-2), is a cyclic, polypeptide, multi-component antimicrobial mixture.[93] The basic chemical structure of colistin consists of a fatty acid side chain linked to a cyclic heptapeptide ring by a tripeptide link. At least 30 components have been isolated from the colistin mixture[94-96] and 13 of these components, differing in the composition of amino acids and the fatty acid,[97] have been structurally identified.[94-96, 98-100] The two major components of colistin are colistin A (polymyxin E_1, [7722-44-3], $C_{53}H_{100}N_{16}O_{13}$, Figure 1-2) and colistin B (polymyxin E_2, [7239-48-7], $C_{52}H_{98}N_{16}O_{13}$, Figure 1-2), comprising approximately 85% of the total antibiotic mixture.[101] Colistin A and colistin B differ only in the composition of the fatty acid tail: colistin A contains 6-methyloctanoic acid while colistin B contains 6-methylheptanoic acid. The relative amounts of colistin A and colistin B contained in the colistin mixture may vary between manufacturers and between batches, but usually range between 4.5:1 and 1:2.5.[102] The five free amino groups of the L-α,γ-diaminobutyric residues impart a positive charge on colistin at physiological pH. By virtue of the hydrophobic fatty acid region and charged amino acid residues colistin is amphipathic and surface active,[103] and is said to distribute between both aqueous and

non-aqueous environments.[104] Colistin A and colistin B have octanol-water partition co-efficient (log P) values of -3.15 and -3.68, respectively (predicted ACD LogD Suite version 9, Toronto, Canada). Despite its surfactant-like nature, the behaviour of colistin at the air-water interface and in bulk solution has not yet been reported.

1.3.1.1 Colistin sulphate

Colistin, usually prepared as the sulphate salt, is a white to cream-colored hygroscopic powder which is stable at room temperature for at least 18 months based on microbiological activity.[105] It is highly water-soluble, slightly soluble in ethanol and practically insoluble in acetone and ether.[106] A 1% solution of colistin sulphate has a pH of 4 to 7 and is stable in water for at least 5 days at 37°C based on microbiological activity.[107] Orwa *et al*. used liquid chromatography coupled with mass spectrometry to study the stability of 0.5 mg/mL colistin A and colistin B at ≥ 37°C across a range of pH values, and found that the stability of both compounds is pH- and temperature-dependent,[100] with an estimated shelf-life of 60 days at pH 3.4.[100]

Colistin, in the form of the sulphate salt, is available as suspensions (Cortisporin® Otic Suspension [Falcon Pharmaceuticals]) and solutions for the treatment of skin, eye and ear infections (Cortisporin® Otic Solution, Colo-Mycin® Syrup [Falcon Pharmaceuticals], Colo-Mycin® S Otic [King Pharmaceuticals]). Solid dosage forms are available for the treatment of infections of the stomach, bowel and digestive tract (Colomycin® tablets [Forest Laboratories]). None of the pharmaceutical formulations mentioned in the preceding two sentences is available in Australia. Colistin sulphate is not orally bioavailable and is degraded by lipase but not by pepsin, trypsin or erepsin.[108] It is believed that colistin sulphate is too toxic to be administered intravenously (LD_{50} = 5.46 mg/kg in mice).

1.3.1.2 Colistin methanesulphonate

For intravenous use, colistin is administered in the prodrug form, colistin methanesulphonate (CMS, Figure 1-3). CMS ([8068-28-8], synonyms colistimethate sodium, pentasodium colistin methanesulphonate, colistin sulphomethate, colistin sulphonyl methate) is the sodium sulphomethyl derivative prodrug form of colistin.[71] CMS is a cream to yellow, amorphous, hygroscopic powder. A 1% solution of CMS has a pH of 6.2-7.5.[109] The chemical structure of CMS, although accepted by official monographs,[106, 110] has yet to be confirmed by spectral analysis. CMS is produced by the sulphomethylation of the five primary amine groups of colistin when colistin is reacted with formaldehyde and then sodium bisulphate according to the simplified reaction schemes in Equation 1.1 and 1.2, respectively, as follows:

$$R - NH_2 + HCHO \leftrightarrow R - N = CH_2 + H_2O \qquad \textbf{Equation 1.1}$$

$$R - N = CH_2 + NaHSO_3 \leftrightarrow R - NH - CH_2 - SO_3^- Na^+ \qquad \textbf{Equation 1.2}$$

Sulphomethylation converts colistin, a basic, cationic compound into the acidic, anionic alkylsulphonic acid, CMS.[111] Derivitisation by sulphomethylation is a strategy that was introduced in the 1950s to improve the properties of drug compounds with less than desirable characteristics, for example poor water solubility, toxicity and therapeutic index.[112-113] Though the conversion of colistin to CMS was successful in reducing toxicity of colistin (LD_{50} = 222.3 mg/kg in mice), CMS itself possesses no antimicrobial activity.[71] As first suggested in the 1960s[111] but not confirmed until recently, to exert the antimicrobial activity CMS must first convert to the active species, colistin.[71, 114] Conversion of CMS to colistin occurs both *in vivo* and *in vitro* (Section 1.6).

1.3.2 Mechanism of action

Many of the studies investigating the mechanism of action of the polymyxin antibiotics have focused on polymyxin B (Figure 1-4) as a 'model' polymyxin. Considering that colistin and polymyxin B differ only by one amino acid in the peptide ring (polymyxin B contains a D-phenylalanine residue on the heptapeptide ring in place of a D-leucine residue), it is believed that they act via very similar mechanisms.[1, 87]

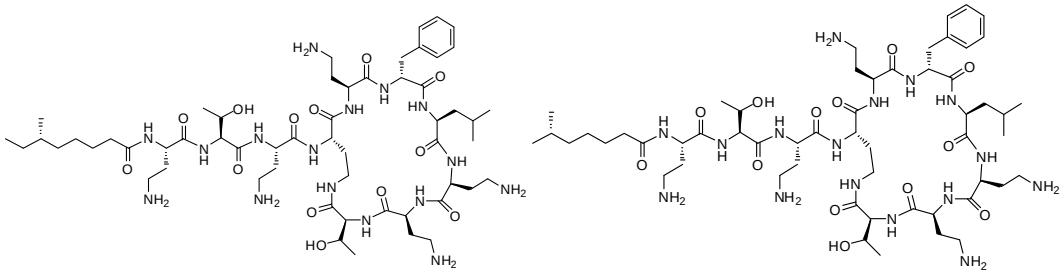


Figure 1-4: Chemical structures of polymyxin B_1 (left) and polymyxin B_2 (right). Polymyxin B contains D-phenylalanine in the cyclic ring structure.

Though there is limited knowledge of the exact mechanism of polymyxin activity towards Gram-negative bacteria at a molecular level,[1] it has been established that the initial interaction between polymyxin B and bacterial cells is via an electrostatic interaction with the lipid A portion of lipopolysaccharide (LPS),[115-117] a key component of the bacterial outer membrane. It is believed that positively charged polymyxin B displaces the divalent cations Ca^{2+} and Mg^{2+} from the negatively charged phosphate groups of lipid A; both cations normally act to stabilise LPS in the outer membrane. This leads to local disturbances in membrane permeability.[118] Once polymyxin B has passed through the outer membrane, it binds to the negatively charged surface of the cytosplasmic membrane. Ultimately, cell death is caused by the leakage of cytoplasmic constituents.[119] Structural features of the polymyxins including the fatty acyl tail, positively charged residues and the lariat ring structure have been shown to be crucial to the interaction with LPS and subsequent membrane permeabilising activity.[120-122]

Hancock and coworkers have proposed the 'self-promoted uptake model' (Figure 1-5) to explain the detailed antibacterial mechanism of the polymyxins.[123]

In addition to antimicrobial activity, colistin also possesses some anti-pathogenic effects. The release of LPS (also termed 'endotoxin') from Gram-negative bacterial cells stimulates a series of inflammatory responses that are a major contributing factor in the onset of bacterial infection[124]. As noted above, the polymyxins have been shown to bind to LPS via a specific interaction with the phosphate groups on the lipid A region of the molecule.[125] The binding of the polymyxins to LPS neutralizes the endotoxic effects of LPS,[126] thereby minimising fever and inflammation associated with the onset of infection.

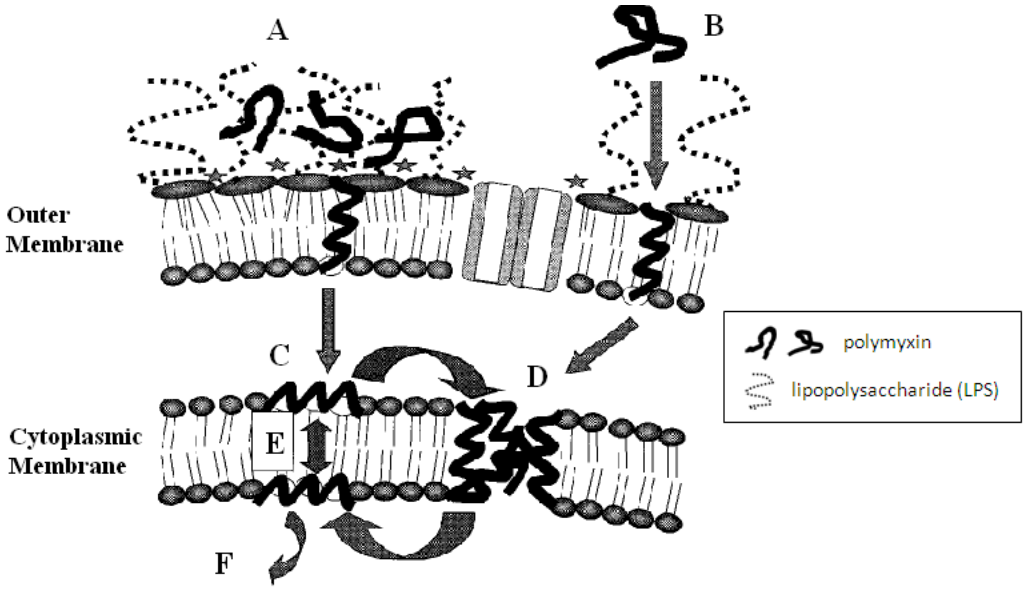


Figure 1-5: Proposed mechanism for the interaction between the polymyxin antibiotics and the cell envelope of Gram-negative bacteria. Polymyxin either (A) associates with the negatively charged surface of the outer membrane to neturalise the local charge, creating cracks in the membrane or (B) binds to the divalent cation binding sites of LPS to disrupt the membrane. The modified permeability allows the peptide to pass through the outer membrane. After passage across the outer membrane, the polymyxins bind to the negatively charged surface of the cytoplasmic membrane. The interaction between the fatty acyl polymyxin tail and the alkyl chains of the phospholipids comprising the cytoplasmic membrane causes the polymyxins to fold into their amphipathic structure (C), though it is not clear at what point the polymyxins take on this amphipathic structure. The polymyxins are believed to insert into the membrane interface and either aggregate in a micelle-like complex (D), or flip-flop across the membrane under the influence of the large transmembrane potential gradient (E). The micelle-like aggregates are proposed to have water associated with them, providing a passage for the leakage of ions and larger water-soluble molecules. Some monomers will be translocated to the cytoplasm to bind to cellular polyanions such as RNA and DNA. (Adapted from Hancock et al., 1999).[123]

1.3.3 Pharmacodynamic (PD) and pharmacokinetic (PK) properties, and PK/PD relationship

Understanding the relationship between *in vivo* drug concentration and effect (i.e. PK/PD relationships) is particularly important in the context of use of CMS/colistin given the advent of increasing colistin-resistance (Section 1.3.5). Since CMS is an inactive prodrug of colistin,[71] PD parameters relating to CMS are only 'apparent' and therefore only the PD of colistin will be described here. Colistin has a narrow spectrum of antimicrobial activity, mostly against important respiratory pathogens including *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. Other species of clinical importance against which colistin is usually active include *E. coli*, *Enterobacter*, *Salmonella*, *Shigella* and *H. influenzae.*[5, 70] Gram-positive bacteria of clinical importance are inherently resistant to colistin. The Clinical and Laboratory Standards Institute defines colistin-resistant *P. aeruginosa*, *A. baumannii*, and *K. pneumonia* as isolates with colistin sulphate minimum inhibitory concentrations (MICs) > 2 mg/L.[127] Although other susceptibility break points also exist, such as that set by the British Society for Antimicrobial Chemotherapy which defines *P.aeruginosa* isolates with MICs > 8 mg/L as colistin-resistant, while isolates with MICs ≤ 2 mg/L are considered susceptible.[104]

In vitro time-kill studies, measuring bacterial growth over time in response to colistin exposure, have shown that colistin exhibits rapid, concentration-dependent kill kinetics against *P. aeruginosa*, *A. baumannii* and *K. pneumonia;*[40, 128-129] a minimal post-antibiotic effect was observed against *P. aeruginosa* only at high concentrations (4 – 16 × MIC).[128]

Despite being in clinical use for over 50 years, only recently has the PK of CMS and the colistin formed from CMS *in vivo* been elucidated.[130-133] Early PK studies relied on the use of microbiological methods for the quantification of colistin and CMS in PK

biological samples. Such methods are incapable of differentiating colistin in samples at time of collection from that formed from CMS due to ongoing conversion to colistin during the incubation period of microbiological assays.[71] The availability of HPLC[134-135] and LC-MS[136-137] methods capable of distinguishing between colistin and CMS has enabled differentiation of the PK of colistin and CMS.

Both CMS and colistin exhibit linear PK after intravenous administration to rats.[138-139] CMS is predominantly cleared by the renal route, undergoing net tubular secretion, with approximately 60% of the dose recoverable in urine within 12 h.[140] Colistin, on the other hand, undergoes very extensive net tubular reabsorption.[141] The small fraction of the administered CMS dose (1 – 12.5%)[139, 141-142] converted to colistin *in vivo* is predominantly cleared via an unknown non-renal pathway.[140] The disposition of CMS and formed colistin, following administration of CMS, is summarised in Figure 1-6**.**

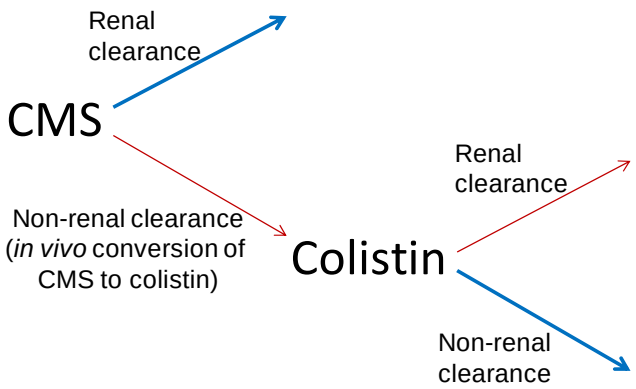


Figure 1-6: Schematic representation of the disposition of CMS and the colistin generated from it in the body, following administration of CMS. Adapted from Li *et al.*, 2006.[87]

In addition to the differences in renal handling, there are other major PK differences between colistin and CMS, evident from studies in both rats and humans. [130-131, 140-141] Colistin exhibits a significantly larger volume of distribution (V_d) (496 mL/kg [rats],) and longer terminal half-life ($t_{1/2}$) (74.6 min [rats]) compared to CMS (299 mL/kg and

23.6 min [rats], respectively).[140-141] Dose ranging studies in rats have demonstrated the linear PKs of intravenously administered CMS and colistin.[138-139]

A PK study of CMS and formed colistin after IV administration to CF patients revealed that the recommended daily dosage regimen of 3 million IU CMS (i.e. 240 mg) every 8 hours achieved plasma concentrations of formed colistin barely comparable to the MIC for the majority of the dosing interval, even without the consideration of plasma protein binding (Section 1.3.3).[130] This finding was later substantiated by a number of other PK studies of IV CMS in critically ill patients.[131-133] In the case of critically ill-patients, it is recommended that the dose of CMS be reduced in consideration diminished renal function.[131, 143]

Even though inhaled CMS has been used to treat pulmonary infections in cystic fibrosis for decades, until recently there has been limited data available regarding the PK of CMS and colistin formed within the lungs following pulmonary delivery of CMS.[144] Plasma and sputum concentration data reported in a study of the PK of inhaled CMS in CF patients may not reflect true plasma colistin concentrations due limitations of the analytical method used to quantify colistin.[144-145] In that study, it was not made clear whether colistin sulphate, colistin A or CMS was used to prepare standard calibration curves. Furthermore, quantification was based on the chromatographic peak of only one of the major biologically active components of colistin, colistin A (polymyxin E_1). Therefore, the results from that study should be interpreted with caution. Studies conducted more recently have utilised more reliable, robust analytical methods.[139, 146-147] PK studies conducted in rat models have shown that following administration of CMS, 100% of the CMS dose reaches the systemic circulation, either as CMS directly absorbed from the lungs (~ 66% of CMS dose) or as colistin formed within the lungs (~33%).[142] Interestingly, the bioavailability (BA) of colistin is four-fold higher after

pulmonary administration of CMS than after IV administration of the same CMS dose.[142] It should be noted that *in vivo* hydrolysis of CMS may occur at a different rate in rats compared to humans and therefore the BA in humans may differ to that reported in rat PK models.

After 1 mg/kg colistin (sulphate) was administered via intratracheal instillation to rats, rapid and extensive pulmonary absorption into the systemic circulation ensued with maximum plasma concentration (C_{max}) of 1.51 µg/mL and time to reach maximum plasma concentration (T_{max}) of 20 min. In addition, the BA of colistin has been shown to be dose-dependent, with BAs of 19 and 52% after pulmonary administration of 0.5 mg/kg and 1 mg/kg, respecitvely.[139]

Colistin is bound to the plasma proteins human serum albumin and α-1-acid glycoprotein.[148] Given that total plasma concentrations of CMS and colistin were measured in the PK studies, reported plasma concentrations which include both the unbound and plasma-protein bound fractions, do not necessarily reflect the concentration that is available for biological activity,[149-150] Moreover, the extent of colistin plasma binding is dependent on the concentration of colistin itself and may also vary according to the concentrations of plasma proteins; for example, α-1-acid glycoprotein may become elevated in infection.[148] Therefore, the plasma binding of colistin should be carefully considered when interpreting information obtained in PK and PK/PD studies.

There are few therapeutic drug classes for which the integration of PK/PD concepts has the potential to contribute so greatly to the design of efficacious dosage regimens; antibiotics are one such class. It is known that the antimicrobial effect correlates with one of the following three so-called PK/PD indices: ratio of the maximum plasma

concentration to MIC (C_{max}/MIC); ratio of area under the plasma concentration – time curve across 24 h to MIC (AUC_{0-24}/MIC); or, the time percentage for which the plasma concentration exceeds the MIC ($\%\,T > MIC$).[151-152] For a given antibiotic, once it is known which of these indices correlates with the antimicrobial effect, and the magnitude of the index required to achieve various magnitudes of microbial killing, it is possible to design rational dosage regimens to maximise the antimicrobial effect. Information gleaned recently from both i*n vitro* PK/PD models[153] and *in vivo* murine infection models,[154-155] which measure bacterial growth response to multiple doses of colistin over time, has enabled identification of the most appropriate PK/PD index to inform the optmial clinical use of colistin.[154-156] These studies have demonstrated that AUC_{0-24}/MIC is the most predictive PK/PD index for colistin against *P. aeruginosa* and *A. baumannii*, and therefore, it is important to maximise time-averaged exposure to colistin *in vivo*.

The integration of information obtained from PK/PD studies has highlighted that optimal AUC_{0-24}/MIC ratios are unlikely to be achieved with the use of the currently recommended intravenous dosage regimens of CMS.[130-132, 157] This is of great concern given that sub-therapeutic doses of colistin are not only ineffective, but may also contribute to the development of bacterial resistance to this very important last-line therapy. Therefore, considerable scope exists to improve clinical use of CMS/colistin and investigations into strategies to intensify its clinical use are warranted. One such approach would involve efforts to achieve more favourable concentrations at the infection site; for example, direct delivery of antibiotic to the lungs for the treatment of pulmonary infections.

1.3.4 Clinical use of colistin in the treatment of MDR pulmonary infections

Colistin has been used in the CF patient population since the 1980s to manage *P. aeruginosa* pulmonary colonisation or infection.[1, 158] As the prevalence of MDR Gram-negative infections has increased more recently the use of colistin has been extended to the treatment of Gram-negative infections,[157, 159-160] and in particular, infections of the respiratory tract.[72, 161-166] Colistin is most commonly administered in the form of its prodrug, CMS, because the latter is less nephro- and neurotoxic than colistin,[111] and is said to cause less irritation to the airways following inhalation.[167, 168-169]

1.3.4.1 Intravenous delivery

CMS is available as a lyophilised powder which is reconstituted prior to administration, by dilution into an infusion solution and subsequent IV infusion over approximately 30 min.[170-172] Commercial formulations of CMS for IV use include Coly-Mycin® M Parenteral (Parkedale Pharmceuticals, Rochester, USA), Colomycin® Injection (Forest Laboratories, Kent, UK) and Promxin for Injection (Profile Pharma, West Sussex, UK). These formulations contain CMS and no other excipients. Commercial CMS products may be labelled as containing 'mg of colistin base activity', though this labelling has no relationship to the chemistry concept of the base form of colistin. Alternatively, in some regions of the world CMS products may be labelled to contain CMS in 'international units' (IU), where there are 12,500 IU in 1 mg of CMS powder. Due to the lack of harmonisation between manufacturers with respect to the labelling of CMS products, there are substantial differences in the daily dosage regimens recommended by the various manufacturers.[87, 173] For a 60 kg patient with normal renal function, the recommended IV daily dose (expressed as mg of CMS) varies from 240 - 480 mg/day to 400 - 800 mg/day, in various global regions.[174]

The outcome and safety of parenteral CMS to treat respiratory infections caused by MDR Gram-negative bacteria has been described in a wide population of patients,[72, 157, 159, 164, 175-177] suggesting that IV CMS is an effective and safe therapeutic option. However, many of these studies are confounded by the fact that CMS was concomitantly administered with other antibiotics, making it difficult to attribute any effect to colistin alone. Moreover, these studies lack power given the small sample sizes. In some cases, poor clinical outcomes have been reported when treating pulmonary infections with IV CMS.[133, 159, 178] Treatment failure, amongst other factors, may be attributed to the poor penetration of colistin into the lungs and epithelial lining fluid (ELF) following IV administration and consequently, sub-optimal exposure at the site of infection. Unfortunately, potential nephro- and neuro-toxicities preclude the use of higher IV doses of CMS to achieve greater lung exposure. The danger of achieving sub-MIC concentrations of colistin at the site of infection is not only lack of efficacy, but also the possibility of selecting for resistant sub-populations of bacteria that can give rise to colistin resistance (Section 1.3.5).[179]

As outlined in Section 1.3.3, PK studies following IV doses of CMS in cystic fibrosis patients and critically-ill patients have shown that the currently recommended IV dosing regimens of CMS result in plasma concentrations of colistin below the MIC for a substantial fraction of the dosing interval in many patients.[130-133] Importantly, however, plasma concentrations of colistin may not necessarily reflect the concentration of colistin achieved within the ELF and lung tissues.[180]A significant proportion of colistin is plasma-bound *in vivo*,[154] therefore, reported colistin plasma concentrations do not necessarily represent the proportion of drug that is available for antibacterial activity at the infection site. Evidence from studies in humans and animal models indicates that the polymyxins penetrate poorly into lung tissues following IV administration.[180-182]

1.3.4.2 Pulmonary delivery

One of the major clinical uses of colistin is the treatment of respiratory infections by administration of CMS directly to the lungs.[183] The delivery of antimicrobials to the lungs to treat pulmonary infections has been utilised since the 1950s[184] and offers a number of advantages over the delivery of antibiotics systemically. Drugs administered to the lungs via the airways are able to circumvent layers of tissues and fluids that separate the bloodstream from the respiratory tract, enabling the drug to concentrate at the interface between the epithelial lining and the air. Direct administration to the lungs via the airways can achieve concentrations of drug within the lungs that are up to a log order of magnitude higher than when delivered intravenously.[185-186] Therefore, the therapeutic effect achieved by pulmonary administration can be far superior to that following systemic drug delivery. Inhalation administration of CMS is becoming increasingly common for the treatment of MDR respiratory infections,[187] despite minimal detailed PK/PD and clinical evidence supporting its superior efficacy compared to IV delivery.[188]

Although pulmonary delivery is not a Food and Drug Administration (FDA) approved route of administration for CMS,[189] it is approved by the European Medicines Agency (EMA) in the UK and various other parts of Europe.[190-191] For pulmonary administration, a IV product (Section 1.3.1.2) is reconstituted and given by aerosolisation, despite the fact that most of these products are not approved or marketed for pulmonary use.[187] Recently, the EMA approved the pulmonary use of Promixin® (Profile Pharma, West Sussex, UK).[190] This product is intended specifically for direct delivery to the lungs and contains lyophilised CMS but no other excipients, to be reconstituted prior to use.[190]

Currently, evidence from randomised controlled clinical trials is insufficient to establish which approach (e.g. IV, inhalation, or both) is most effective for the treatment of pulmonary infections using colistin.[1] The administration of CMS directly to the lungs is a logical approach towards achieving high local concentrations of colistin following CMS inhalation; it can minimise systemic exposure (and thereby minimising potential systemic toxicities) while maximising antibacterial effect. High sputum and tissue concentrations of colistin reported after CMS inhalation suggest that local concentrations of colistin achieved following delivery of CMS directly to the lungs may be in excess of the MIC.[144] This can be contrasted to sub-MIC concentrations achieved within the ELF following CMS delivery by the IV route.[180] The limited studies that have been conducted in humans and animals tend to indicate that IV delivery of CMS is unlikely to achieve concentrations of colistin within the lungs sufficient to eradicate MDR respiratory infections and suppress the emergence of colistin-resistant isolates.[180]

The treatment of respiratory infections caused by *P. aeruginosa* in CF patients has been a major use of aerosolised CMS over the past two decades.[158, 192] Worryingly, CF centres around the world have reported the spread of highly transmissible colistin-resistant *P. aeruginosa* strains.[193-195] While there remains a lack of evidence from well controlled, randomised clinical trials assessing the efficacy of inhaled CMS in the treatment of MDR pulmonary infections, decades of clinical experience with inhaled CMS in the CF patient population indicates that there exists strong *prima facie* evidence of the utility and efficacy of colistin for the treatment of respiratory infections caused MDR pathogens.[192, 196]

The use of inhaled CMS is now becoming increasingly common outside of CF clinics.[187 162-163 159, 161] Inhaled CMS has played a crucial role in ICU in the treatment of pulmonary infections caused by MDR Gram-negative bacteria, e.g. hospital-acquired

pneumonia (HAP) and ventilator-associated pneumonia (VAP).[162-163, 197] HAP, defined as pneumonia occurring within 48 h of hospital admission, is the major cause of morbidity and mortality in ICUs.[198] VAP is a type of HAP which occurs in patients who are mechanically ventilated via an endotracheal tube. The insertion of the endotracheal tube allows the free passage of bacteria into the lungs where they can establish, multiply and cause infection. *P. aeruginosa* is the most commonly isolated pathogen causing VAP[199] and the second most common cause of HAP. Amongst others, *A. baumannii* and *K. pneumoniae* are also other important pathogens causing VAP.[37-38, 46] Appropriate and timely antibiotic treatment is crucial to improve patient outcomes and minimise mortality in VAP.[200] A recent study revealed that better clinical outcomes were achieved when VAP caused by MDR Gram-negative bacteria was treated with IV and inhaled CMS, compared to when IV CMS was used alone.[166] Inhaled CMS has been reported to be successful in the treatment of many cases of HAP[161-163, 201] with one study concluding that the use of inhaled CMS was independently associated with the cure of VAP.[166]

Another condition in which inhaled CMS finds application is chronic obstructive pulmonary disease (COPD), an inflammatory disease caused primarily by long-term cigarette smoking which eventually causes airflow limitation to the lungs.[202] COPD is the fourth leading cause of death in the United States and Europe.[203-204] Patients with COPD are prone to acute pulmonary exacerbations, approximately half of which are bacterial in origin and[205] require aggressive antibiotic therapy. COPD patients often develop VAP as a result of intubation and mechanical ventilation.[206] Inhaled CMS has been successfully used to treat patients suffering COPD-related infective bronchial exacerbations.[207-208]

1.3.5 The emergence of polymyxin resistance

Reports of colistin resistance date back as early as 1972[209] but fortunately since then, resistance to colistin has been a rare occurrence.[4] A surveillance program conducted in 2000 on clinical isolates from 417 CF patients across 17 hospitals in the UK revealed that only 3% of the isolates tested were colistin-resistant.[210] Despite over two decades of use in CF patients, resistance to colistin in this patient population has been uncommon in the past.[91, 192, 196, 211] There may be a number of factors contributing to the low incidence of resistance observed for polymyxin antibiotics to date. Over the last three decades colistin has been relegated to a last-line 'salvage therapy' which has minimised its clinical use.[212] Colistin has a unique mechanism of action and outer membrane targeting (Section 1.3.2) and this may prevent bacteria that are resistant to other classes of anti-pseudomonal agents from acquiring cross-resistance to colistin. Nevertheless, emerging reports of reduced polymyxin susceptibility and polymyxin resistance in isolates from patients around the world,[38, 87, 213-219] including CF patients,[165,163,220] highlights that colistin is not impervious to the phenomenon of bacterial resistance.

Two distinct mechanisms of polymyxin resistance have been identified, both ultimately leading to similar phenotypic alterations in cell surface.[221-222] Firstly, adaptative alterations can occur when bacteria are grown in the presence of colistin.[221] Notably, this kind of resistance is unstable; bacteria developing this kind of polymyxin-resistance revert back to the polymyxin-susceptible phenotype when subsequently grown in the absence of polymyxin.[223] The exact mechanism behind the unstable nature of adaptative resistance is unknown.[221] The second type of polymyxin resistance occurs via genetic mutations.[221] This type of polymyxin-resistance is stable and can be inherited. Cummins *et al*. identified 30 genetic modifications in *P. aeruginosa* following exposure to sub-inhibitory concentrations of colistin.[224] Modifications to genes controlling various

metabolic pathways have been identified, including those that regulate LPS composition and quinolone signalling activities.[224] Phenotypic changes occurring with either type of polymyxin resistance may include structural and chemical alterations to LPS,[225] complete loss of LPS[225-226] or changes in the Mg^{2+}, Ca^{2+}, protein and lipid content of the cell envelope.[227] Such phenotypic changes may suppress the initial electrostatic interaction between colistin and LPS, conferring colistin-resistance.[226] Strains of *P. aeruginosa* isolated from CF patients who have been treated with colistin have modified aminoarabinose in the lipid A portion of LPS, providing evidence that resistance can develop under the conditions of the CF airway.[220, 228]

Evidence from *in vitro* PD studies has highlighted the serious dangers of achieving sub-therapeutic concentrations of colistin; in static and dynamic experimental systems, significant re-growth occurred for *P. aeruginosa*,[156, 229] *A. baumannii*[216, 230] and *K. pneumoniae*[129] after treatment with polymyxin concentrations well above respective MICs (≤ 1 mg/L). Subsequent analysis of bacterial susceptibility profiles revealed growth of sub-populations in the presence of high concentrations (> 4 mg/L) of polymyxins,[71, 129, 216] indicating that the current use of colistin may not be sufficient to suppress growth of polymyxin-resistant sub-populations.

The presence of resistant sub-populations of bacteria within a strain that is susceptible based in MIC values is referred to as 'hetero-resistance'. [216] *In vitro* evidence suggesting the existence of hetero-resistance to colistin appears to be supported by *in vivo* evidence of colistin hetero-resistance; Hawley *et al.* examined the susceptibility of MDR *A. baumannii* isolates from patients with and without previous colistin exposure and found a statistically higher number of isolates exhibiting hetero-resistance from patients with previous colistin exposure.[179] In a separate study, Matthiaou *et al.* found that colistin use was independently and strongly associated with the isolation of colistin-resistant *K.*

pneumoniae, *A. baumannii* and *P. aeruginosa* from patients with VAP.[231] Gilad *et al*., found that the development of colistin-resistance amongst *A. baumannii* isolates in Israel was strongly associated with the clinical use of colistin.[39]

Clinical experience seems to support evidence from *in vitro* studies, indicating that hetero-resistance may be amplified by exposure to colistin.[179] A number of authors have reported significant decreases in polymyxin susceptibility and emergence of polymyxin resistance amongst clinical isolates previously exposed to polymyxins.[38, 219, 232] Given that reduced susceptibility is more likely to be associated with clinical failure, the existence of hetero-resistance and the emergence of reduced susceptibility to colistin is a clear warning of the risks associated with the sub-optimal use of colistin,[129, 233] suggesting that further strategies to intensify colistin treatment and facilitate optimal therapies are urgently needed.

1.3.6 Strategies to intensify the use of colistin

The risks associated with under-dosing colistin should not be taken lightly, given that colistin is frequently the only available option for the treatment of MDR Gram-negative infections. Widespread resistance to colistin would create a situation reminiscent of the pre-antibiotic era, when previously curable infections will become untreatable. Therefore, investigations aimed at optimising the clinical use of colistin are urgently needed. As explained in Section 1.3.4.2, one such approach to the intensification of colistin use is to deliver colistin via the lungs directly to the site of infection.

In general terms, antimicrobial monotherapy has been shown to be a risk factor for the development of bacterial resistance to a number of antimicrobials.[20] The use of colistin in combination with other antibiotics is a possible strategy by which the treatment of MDR pulmonary infections may be intensified. The use of colistin-combination

antibiotic therapy has a number of advantages over monotherapy.[234] Primarily, the use of more than one agent can result in antimicrobial synergy, that is, antimicrobial activity beyond an additive effect.[235] In addition, the use of two antibiotics concomitantly can target more than one bacterial component or biochemical process. Therefore, colistin-combination therapy has the potential to minimise the selection of colistin-resistant mutants or sub-populations[236-237] and give better protection of resistance development compared to monotherapy.[238]

The permeabilisation of the bacterial outer membrane by colistin can allow for the entry of antibiotic compounds that would not otherwise penetrate the bacterial cell wall of Gram-negative bacteria.[239-240] Colistin-resistant clinical isolates have shown increased susceptibility to other antibiotics[241-242] and therefore, an alternative strategy to combination therapy might be to use colistin to eradicate the colistin susceptible sub-population of bacteria, while the second antibiotic targets eradication of the colistin-resistant subpopulation.[1]

In vitro, a large number of antibiotics have been screened for synergy with colistin against *P. aeruginosa* and *A. baumannii* using techniques such as disc diffusion assays, checkerboard methods (measuring fractional inhibitory concentrations) and time-kill experiments. A number of antibiotics, including rifampicin,[243-246] imipenem,[247] azithromycin,[245] meropenem,[248] ceftazidime[249] and minocycline[250] have shown promise as potential candidates for combination therapy with colistin. *In vivo* evidence obtained from a mouse model of pneumonia infection showed that the combination of colistin and rifampicin was synergistic against *P. aeruginosa*, with the intranasal combination proving particularly effective.[124] The combination of colistin and a second antibiotic may even be effective against colistin-resistant bacteria.[251]

The severity of life threatening MDR pulmonary infections has forced clinicians to increasingly turn to the use of colistin combination therapy in order to increase the likelihood of clinical success.[252-253] However, evidence from well controlled clinical trials regarding the efficacy of colistin combination therapy, compared to colistin monotherapy, is scarce. To date, prospective and retrospective clinical studies have not been able to demonstrate significantly greater efficacy of colistin combination therapy over colistin monotherapy against infections caused by *P. aeruginosa* and *A. baumannii*.[88, 176-177, 254-258] However, findings from such studies should be interpreted with care. First, many of these studies lack a control group (monotherapy only),[177, 252, 256, 258] although ethical considerations complicate the use of a monotherapy as a control group in the treatment of life-threatening illnesses. Where control groups have been used, studies lack significance and power due to low patient numbers, heterogeneity of outcome measures and patient population, and variability in dosing regimens, amongst other confounding factors that are difficult to account for.[88, 176, 257, 259] Secondly, and importantly, clinical studies have failed to measure exposure of colistin and combination antibiotics[1] and therefore conclusions regarding the efficacy of antibiotic combinations may be drawn inappropriately. The superior efficacy of colistin-combinations indicated by *in vitro* studies needs to be further investigated in well designed clinical studies. Recent evidence regarding the emergence of colistin hetero-resistance (Section 1.3.5) strongly supports the use of colistin combination therapy.

1.3.6.1 Colistin-combination therapy for delivery directly to the respiratory tract

As introduced in Section 1.3.4, a major application for the use of colistin combination antibiotic therapy is in the treatment of MDR infections of the respiratory tract, e.g. in CF and VAP. Currently, IV administration of combination antibiotics is standard treatment for these difficult-to-treat respiratory infections. CF patients can often harbour

different strains of *P. aeruginosa* in their lungs, which have different antibiotic susceptibilities. Resistant sub-populations can become dominant during antibiotic therapy, necessitating the use of combination therapy as standard treatment.[260] Colistin (as CMS) is routinely administered via inhalation, with a second antibiotic, such as ciprofloxacin, given orally or by IV administration.[158] In the treatment of VAP, implementation of effective antibiotic therapy early in treatment is a major determinant of treatment outcome,[261] and therefore, combination treatments are implemented early in therapy to improve the chances of microbiological eradication.[262]

Combination therapy for respiratory infections may include concomitant administration of IV antibiotics, or a combination of IV, inhalation or oral administration. For the effective treatment of MDR infections of the respiratory tract using combination colistin therapy, sufficient and persistent concentrations of both antibiotics must be achieved locally within the respiratory tract, which may only be achievable by direct administration to the lungs via inhalation. Combination therapy via concomitant administration of two agents to the respiratory tract already plays a crucial role in the treatment of respiratory conditions such as asthma and COPD[263] and offers a strategy by which the treatment of respiratory infections with colistin may be improved. Therefore, scope exists for the development of formulations specifically designed for delivery to the respiratory tract, containing colistin and a second antibiotic agent. Such a formulation, achieving adequate concentrations of both antibiotics within the lungs for extended periods, has the potential to provide a more effective inhalation therapy than the current standard of care does.

Combination therapy for direct pulmonary administration may be provided by the inhalation of each drug separately,[264] or as a combination in a single fixed dose preparation.[265-266] In addition to the aforementioned advantages of combination therapy,

a fixed dose combination formulation may offer significant advantages over current inhalation therapies. The administration of a single dosage form is more convenient than administration of multiple dosage forms, and may require a reduced frequency of dosing which can result in increased patient compliance.[267]

The development of a fixed dose combination formulation containing colistin for delivery to the respiratory tract is not without difficulties. Owing to the physiology of the respiratory tract and its highly efficient mechanisms of drug clearance (Section 1.4.1), achieving adequate local concentrations of two antibiotics simultaneously is a biopharmaceutical challenge. In addition to the potential differences in the pharmacokinetics of co-administered antibiotics, there may be substantial disparities in the physicochemical properties (e.g. solubility) of two drugs, presenting a significant obstacle to successful drug delivery. Overcoming these factors requires the consideration of advanced drug delivery systems having the capacity to solubilise and co-localise colistin together with a second antimicrobial. Such advanced delivery systems may also provide the capacity for sustained or controlled release of both antibiotics *in vivo*, enabling local *in vivo* concentrations to be maintained above respective MICs for an extended period. The investigation of advanced delivery systems for the co-formulation of colistin with a second antibiotic agent is a major focus of this thesis.

1.4 Delivery colistin to the lungs for local activity

The lungs have been extensively investigated as a portal for the delivery of drugs for both local and systemic activity.[268] Due to the huge absorptive area, drugs administered directly to the lungs (in particular small molecule drugs) can be absorbed into the blood stream very rapidly, with bioavailability approaching 100%.[269] Indeed, it has been

shown in a rat pharmacokinetic model that the direct delivery of CMS and colistin to the lungs results in high systemic bioavailability.[139, 142, 146] While this may be desirable from the perspective of drug delivery for systemic activity, the highly efficient absorption and clearance mechanisms of the lungs present a significant biopharmaceutical challenge for the administration of colistin/CMS to the lungs for local activity.

1.4.1 Pulmonary physiology

The human respiratory tract is an anatomically complex network of vessels and passages impeccably designed for the efficient transport and exchange of gases between the blood and air. The respiratory tract (Figure 1-7) can be divided into two distinct regions: the upper respiratory tract (also known as the conducting airway), consisting of the nose, nasal cavity, pharynx, larynx and the trachea, and the lower respiratory tract (also known as the respiratory region) which consists of the bronchi, bronchioles and alveoli. The lungs have an extensive network of progressively smaller branching channels beginning with the trachea, the main portal into the lungs. The trachea divides into two primary bronchi at the bronchial bifurcation, after which point the bronchi divide into smaller diameter bronchi and bronchioles. Terminating the bronchioles are the alveoli, small air sacs of expansive surface area (up to 140 m^2)[270] that are the site of gas exchange between the lungs and air. The alveoli are exposed to an expansive network of capillaries through which the entire cardiac output of blood runs every minute. The alveoli are comprised of many different cell types of varying degrees of thickness which present an extremely thin barrier between the alveolar air space and the pulmonary blood supply. Within the extravascular space between the alveolar epithelium and the capillary endothelium lie the interstitium, connective tissues and fibroblasts which serve as a structural framework for the mounting of lung cells. The capillary endothelia and pulmonary epithelium are connected by a strong fibrous filter

of extracellular fibres called the basement membrane. The cell thickness of the alveolar-capillary area is extremely thin so that the pulmonary capillaries are in intimate contact with the alveolar epithelial cells, allowing for the rapid passage of substances from the alveoli into the blood stream, and *vice versa*.

The entire airway surface, including both the conducting and respiratory region, is covered in a layer of long chain phospholipid surfactants which act to reduce the surface tension of the lung surface liquids.[271] Immediately below the lung surfactant lays a 0.05 – 10 μm thick layer of ELF, the composition of which has not been well studied.[268] Beneath the ELF lies the pulmonary epithelium, a monolayer of cells comprised of two different cell types. The lung interstitium, consisting of fibroblasts, tough connective collagen fibres and interstitial fluid, is the extracellular and extravascular space between cells in the tissue. The alveolar epithelium and capillary endothelium are attached by a two-layer thin matrix of fibres called the basement membrane.[272] Beyond the basement membrane lies the monolayer of cells that make up the capillary endothelium.

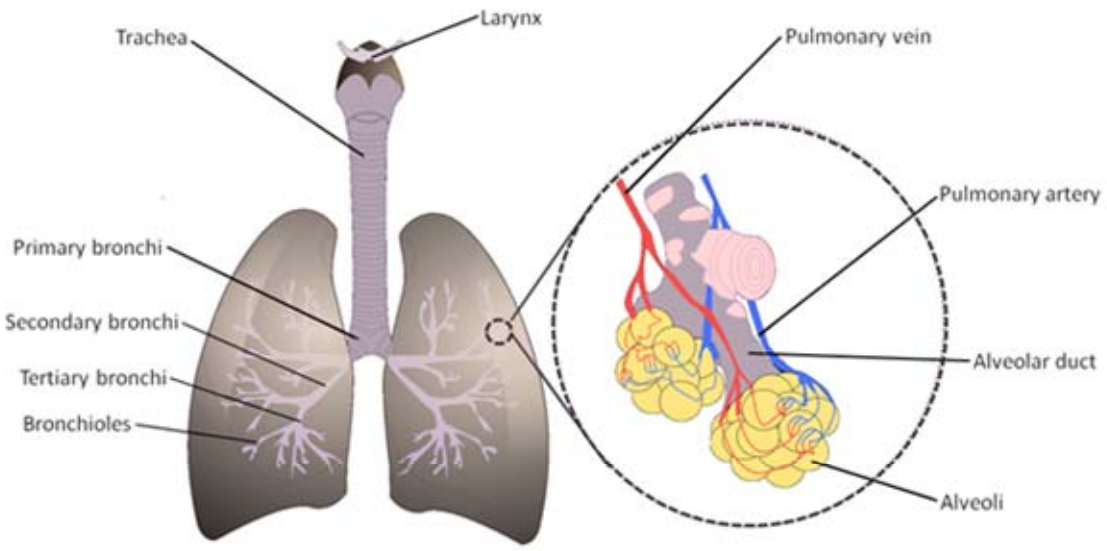


Figure 1-7: Anatomy of the lower respiratory tract (adapted from http://en.wikipedia.org/wiki/Lung).

1.4.2 Pulmonary clearance

Since the respiratory tract has been finely constructed for efficient removal of substances and particles, there are a number of barriers to the successful delivery of drugs to the lungs for local drug activity. Drug clearance from the lungs remains a considerable challenge to delivery for local activity because the rate of clearance is inherently governed by the physicochemical properties of drugs administered.[269, 273] Absorption from the lungs is a complex function of the kinetics of absorptive and non-absorptive processes[274] and therefore depends on the occurrence of multiple simultaneous competing events. Following deposition into the lower respiratory tract, for a molecule or drug particle to be absorbed by the lungs and subsequently appear in the blood stream, it must first dissolve in the lung surfactant and the ELF. After dissolving in lung fluids it must pass through a number of cellular layers including the epithelium, interstitium and the basement membrane, before finally penetrating the vascular endothelium and appearing in the bloodstream. The rate of penetration into and across these cellular layers depends on a number of factors, including the molecular weight, lipophilicity and ionisation state of the drug.[269] For example, very low molecular weight lipophillic compounds can be absorbed across the epithelium membrane in a matter of seconds to minutes,[268, 273, 275-276] while small poorly lipid soluble and cationic molecules may be absorbed at a much slower rate, with pulmonary half lives averaging 60 min.[277-279] It is generally accepted that small lipophilic molecules are absorbed by a non-saturable passive transcellular diffusion,[269] while the passage of small non-lipid soluble molecules occurs via paracellular, non-saturable diffusion.[269, 280] Drugs that are highly water-soluble or cationic can exhibit prolonged absorption.

If a drug particle does not dissolve into the lung surfactant and ELF layers, it will be subjected to non-absorptive clearance via a number of possible processes. Exogenous substances may be subjected to enzymatic degradation. While the lung possesses a number of metabolising enzymes including cytochrome P450, monoamine oxidase and aldehyde dehydrogenase,[281] the level of enzymatic clearance in the lungs is low compared to other routes of administration.[277] By the co-ordinated beating of ciliated epithelial cells, the mucociliary escalator moves insoluble particles trapped in mucous upwards from deeper airways towards the larynx where they are swallowed or expectorated. In addition to the mucociliary escalator, insoluble particles may be removed by macrophage endo- or phagocytosis,[282] the rate of which depends on particle size and particle composition.[283-284] The huge absorptive surface area, low surface fluid volume, extremely thin absorptive barrier, and large volume of blood passing through the pulmonary capillaries creates a constant concentration gradient for the diffusion and removal of drug substances administered to the lungs. The use of drug-loaded colloidal carriers has been proposed as a strategy to overcome the difficulties associated with the rapid clearance of drugs from the lungs.[285-286]

1.4.3 Colloidal delivery systems for pulmonary delivery of combination antibiotics

For the treatment of pulmonary infections using combination antibiotics, the intention of drug delivery directly to the lungs is to achieve sufficient local concentrations of antibiotic within the lungs for a period of time that maximises antibacterial activity. The sophisticated and rapid clearance mechanisms by which exogenous substances are removed from the lungs (Section 1.4.2) present barriers to efficient pulmonary drug delivery, and even more so to the delivery of drug combinations for local activity. An approach towards extending the residence time of drugs within the lungs is via loading

of drug into a colloidal drug carrier, or nanoparticle. While delivery directly to the lungs can achieve high local concentrations, such concentrations can be transient, necessitating frequent drug administration. Drug associated with or loaded into a nano-sized colloid can exhibit significantly altered absorption and biodistribution from the lungs.[287-288] When loaded into a colloid, a drug no longer comes into intimate contact with alveolar absorptive surfaces in the lungs, and in this state the drug can take on the pharmacokinetic and biodistribution characteristics of the carrier particle. Particles smaller than 260 nm can evade ingestion by alveolar macrophages to remain in the respiratory tract for extended periods.[289]

The composition of drug carrying colloids can be engineered to manipulate and optimise drug release to provide the slow or sustained delivery of drug into the local environment of the lungs.[283] When deposited in the respiratory tract, nanoparticles can act as a reservoir for the sustained release of drug. The slow release of drug from the carrier matrix may therefore achieve superior efficacy to drug administered in the free form.[288, 290-291] The delivery of drug loaded into nanoparticles may be associated with a lower systemic bioavailability of drug (but higher local availability), and therefore fewer systemic side effects. To the same effect, local irritation or toxicity to the airways can also be significantly reduced.[288, 292 293]

The co-formulation of drugs of vastly differing physicochemical properties can be a significant drug delivery challenge. Aqueous solubility, for example, may be a limiting factor to the dose that can be delivered via inhalation of nebulised solutions. The issue of limited aqueous solubility can be overcome by the loading of drugs into colloidal carriers well beyond their respective aqueous solubility,[294] allowing the attainment of high drug loading and the potential for greatly improved pulmonary delivery.[295]

1.4.3.1 Micelles

Micelles (Figure 1-8) form when amphiphilic molecules self-assemble in an aqueous environment into spherical structures comprising of a hydrophilic shell and hydrophobic core.[296] At low concentrations surfactant molecules exist as monomers in solution. Above a critical concentration, termed the 'critical micelle concentration' (CMC), monomers spontaneously self-assemble with hydrophobic regions associated internally and hydrophilic regions interacting with the surrounding aqueous environment. The lipophilic interior can increase the solubility of drugs with limited aqueous solubility.[297] Micelles have been successfully used as colloidal carriers for poorly-water soluble drugs and offer several advantages as a drug delivery system: they are small in size (typically 5 - 100 nm) and are thermodynamically stable and hence are not subject to colloidal instability such as aggregation.[298] Finally, they can be sterilised by filtration and can be manufactured with relative ease.[299]

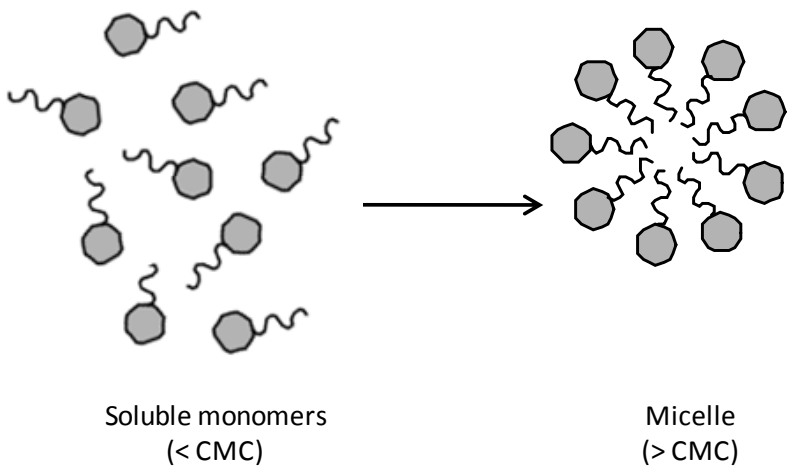


Figure 1-8: Schematic representation of micelle formation by surfactant monomers above the CMC.

Micellar delivery systems have been extensively investigated since the 1980s for their ability to solubilise and deliver poorly-water soluble agents.[300] While micelles are thermodynamically stable, they dissociate at concentrations below the CMC. Therefore, upon dilution in body fluids, solubilised drug payload can undergo precipitation.[298] Typically 'burst' release systems, micelles have a very limited capacity to provide controlled release of drug payload under sink conditions.[301] Micelles also have a limited encapsulation capacity given that they possess a small hydrophobic volume within the lipophilic core.[297] Importantly, as micelles have the potential to solubilise cell membrane and lung surfactant components, they have the potential to cause local irritation to the airways.

A number of marketed commercial products based on micellar technology are approved for IV use. A formulation of diazepam containing mixed micelles of bile salts and phosphatidylcholine was one of the earliest micellar systems to be administered to humans.[300] Since then, there have been significant advances in the development of materials used in micellar delivery systems. Cremophor EL®, a polyethyleneglycol-conjugated castor oil, produces micelles with a large solubilising capacity. Cremophor EL® has been extensively utilised in the formulation of poorly-water soluble compounds including vitamin K (Konakion MM® [Roche]), miconazole (Daktarin® [Jenssen]), ixabepilone (Ixempra® [Bristol Myers Squibb]), cyclosporin (Sandimmune® [Novartis]), nelfinavir (Viracept® [Roche]) and paclitaxel (Taxol [Bristol Myers Squibb]. However, Cremophor EL® itself is often more toxic than the solubilised drug,[297] thereby limiting the dose that can be administered. Polymeric micelles present the most recent advance in micelle formulation technology.[302] Owing to their lower CMC values, polymeric micelles have superior *in vivo* stability compared to conventional PEG-based

micelles.[299] Polymeric micelle-based formulations of doxorubicin and paclitaxel are currently undergoing clinical development.[302]

Micelles have been extensively investigated for IV use. However, the design of micellar carriers for pulmonary delivery is mainly limited to lung cancer therapy. Doxorubicin and image contrast agents have been successfully incorporated into multi-functional polymeric micelles for active targeting to lung cancer cells.[303] However, the biocompatibility of surfactant- and polymeric micelle-based delivery platforms has yet to be established following pulmonary delivery.

1.4.3.2 Sub-micron emulsions

Emulsions are composed of a heterogenous mixture of two or more immiscible liquids with stabilisers to aid dispersion and stabilisation of emulsion droplets.[304] Submicron emulsions (SMEs), as the name suggests, possess droplets of the dispersed phase within the size range 10 - 100 nm.[305] To date, SMEs have been successfully commercialised as drug delivery systems for IV usage. Intralipid® (Glaxo Smith Kline) is a first-generation lipid emulsion approved for clinical use in parenteral nutrition and is made with egg phospholipids and soybean oil. Intralipid® has also been used as a vehicle for the solubilisation and delivery of poorly water-soluble compounds including amphotericin B, propofol, etomidate and prostaglandin E_1.[304] Diazepam (Diazemuls® [Pfizer]) and propofol (Diprivan® [Astra Zeneca]) are lipid emulsion formulations based on soybean oil, acetylated monoglycerides, egg phospholipids and glycerol. Vitalipid® (Kabi), a form of parenteral nutrition, is an emulsion based on retinol palmitate, ergocalciferol, α-tocopherol, soybean oil, phosphatidylcholine and glycerol.

While SMEs have been extensively researched for IV use, very little work has been published in the area of the pulmonary delivery of SMEs. The safety of pulmonary

administration of emulsion surfactants and oils is not known and hence SMEs may represent an underexploited delivery system for pulmonary drug delivery. A number of authors have evaluated the used of DNA-loaded cationic emulsions for gene therapy, with promising results. Liu *et al*., achieved successful DNA transfection into murine melanoma cells using a SME comprised of dioloeylphosphatidylethanolamine (DOPE) and various non-ionic surfactants.[306] Similarly, Kim *et al*. loaded DNA into a cationic emulsion comprised of dioleoylglycero trimethylammonium-propane, DOPE, PEG surfactants and glycerol and found that transfection from an emulsion formulation was superior to transfection from a commercial liposome preparation.[307] Bivas-Bentas and co-workers were able to transfect a human bronchial cell line with a DNA vaccine against *Mycobacterium tuberculosis* using a DNA-loaded cationic emulsion comprised of phosphatidylcholine, phosphatidylehtanolamine, medium chain triglycerides and poloxamer surfactant.[308] These limited studies provide preliminary evidence of the potential use of SMEs in pulmonary drug delivery.

SMEs have a number of advantages over micelles as a drug delivery system: first, they can provide a high solubilising capacity for lipophilic drugs, where the amount of drug solubilised is dependent upon the solubility of the drug in the lipid phase and the volume of the lipid phase used. Secondly, SMEs can be produced on an industrial scale with relative ease. Thirdly, depending on the selected formulation excipients, SMEs can be highly biocompatible and may reduce systemic side effects.[305, 309] Finally, SMEs can stabilise drugs that are susceptible to hydrolysis by incorporating them into the lipid phase.[305]

The major disadvantage of emulsions is that they are not thermodynamically stable and are subject to various mechanisms of colloidal instability including aggregation, flocculation, and coalescence and ultimately phase separation.[309] Emulsions are also not

amenable to filter sterilisation and therefore must be prepared aseptically.[310] As in the case of micelles, controlling the rate of release from SMEs may be difficult as drug release will depend upon the partitioning of the incorporated drug out the lipid phase of and into the surrounding medium, which is highly dependent upon the extent of dilution.[311-312]

1.4.3.3 Liposomes

Liposomes, as first described in the 1960s by Bangham *et al*.,[313-315] are now one of the most extensively investigated drug delivery systems for controlled drug delivery to the lungs.[316] Liposomes consist of spherical vesicles (Figure 1-9) that are formed when certain phospholipids (e.g. phosphatidylcholine) are dispersed in excess water.[317] Liposomes, usually ranging from 50 – 1000 nm, exhibit a number of distinct vesicular morphologies (Figure 1-9).The phospholipid tails provide a lipophilic region within the bilayer, while the phospholipid head groups and encapsulated aqueous volume comprise the hydrophilic regions. Liposomes are therefore a useful colloidal carrier for drugs of varying physicochemical properties. The utility of the liposomal drug delivery system can be demonstrated through its commercial success; to date, at least ten liposome drug products are commercially available (Table 1-1) and many more are currently undergoing pre-clinical and clinical development.[318-319]

Liposomes have a number of advantages which make them a useful colloidal delivery system compared to other alternatives. Liposomes have the capacity to solubilise poorly-water soluble drugs and present a useful vehicle by which drugs of opposing chemical properties may be incorporated in combination formulations.[320-321] Modification of the liposome properties by the addition of lipidic modifiers, such as cholesterol, polyethylene glycol (PEG) and charged lipids, can achieve controlled or

modified release of drug from liposomes, leading to higher drug concentrations in targeted tissues.[322] Liposomes also have the capacity to modify the therapeutic index of drugs with systemic toxicity; delivery and localisation directly at the targeted site can result in lower systemic concentrations of free drug, thereby minimising toxic side-effects.[323-325] In addition, the antimicrobial activity of antibiotics may be enhanced by liposomal encapsulation,[326-332] while local irritation or toxicity of encapsulated materials may be attenuated.[288, 292] Finally, as liposomes are composed mainly of endogenous phospholipids they are highly biocompatible and biodegradable.[333] Following pulmonary administration exogenous phospholipids can be taken up by alveolar cells, catabolised, and stored in lamellar bodies for recycling.[334]

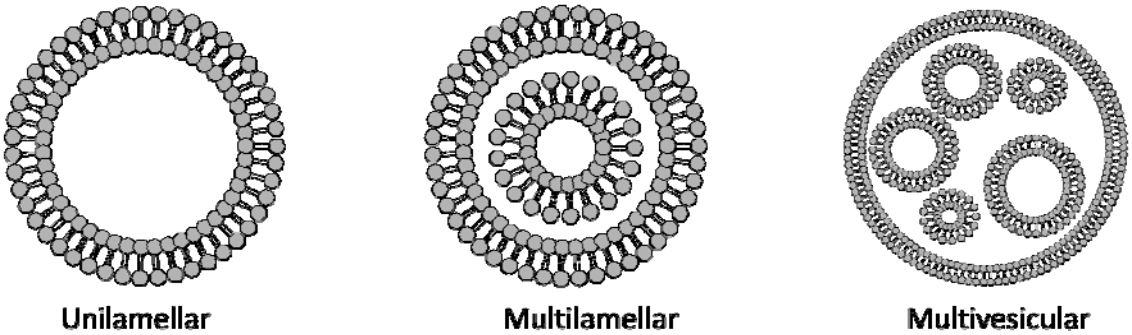


Figure 1-9: Schematic two-dimensional representation of a unilamellar, multilamellar and multi vesicular liposomes. Adapted from Talsma *et al*. [335]

The greatest advance in liposome technology was the discovery that modification of the liposome surface can result in liposomes with long circulation times. This facilitates their accumulation at bodily sites where vasculature is compromised, such as at sites of inflammation and in tumour tissues.[336-337] By grafting the hydrophilic polymer PEG to phospholipids to produce liposomes with a surface coating of PEG, systemic removal of liposomes by the reticuloendothelial system can be avoided[336, 338-339] increasing liposome circulation time from 0.5 h to over 24 h.[322, 336, 340] PEG-liposomes, the so called “stealth’ liposomes, are the basis for the superior performance of the commercial

liposomal doxorubicin formulations, Doxil® (Ortho Biotech) and Caelyx® (Schering Plough). Indeed, different routes of administration impose different requirements on the physical and chemical characteristics of the liposome carrier.[341] For IV administration, the use of PEG-liposomes is desirable where increased circulation times are required.

When administered intravenously, liposomes do not accumulate significantly in the lungs[342] and therefore liposomes must be administered directly to the lungs via inhalation for delivery of drug payload. The utility of liposomes in the delivery of therapeutic compounds directly to the lungs for the local treatment of lung disease is now being realized; liposome formulations of ciprofloxacin (ARD-3100 and ARD-3150 [Aradigm Corp]), tobramycin (AX-TOBRA™ [Axentis Pharma]) and amikacin (Arikace™ [Transave]) are currently under investigation in clinical trials,[343-346] with many more undergoing pre-clinical development.[347]

Table 1-1: Commercially available liposomal drug products.

Trade name	Active agent	Route of administration	Indication
Ambisome®, Abelcet®, Amphotech®	amphotericin B	IV	fungal infections
DaunoXome®	daunorubicin	VI	Kaposi's sarcoma
Doxil®, Caelyx®, Evacet™	doxorubicin	IV	cancer (various)
Myocet®	doxorubicin	IV	metastatic breast cancer
ARD-3100/3150†	ciprofloxacin	pulmonary	bacterial respiratory infections
Verteporfin®, Visudyne®	benzoporphyrin	IV	macular degeneration
Depot Dur®	morphine sulphate	epidural	anaesthesia
Junovan®	muramyltripeptide	IV	osteosarcoma
ThermoDox®§	doxorubicin	IV	cancer (various)
AX-TOBRA™†	tobramycin	pulmonary	bacterial infections
Depocyt®	cytabarabine	intrathecal	meningitis
Lipoplatin®§	cis-platin	IV	cancer (various)
Nyotran®	nystatin	IV	fungal infections
Allovectin-7†	HLA-B7 plasmid	intra-tumoral	Metastatic cancer

†approved with orphan drug status, §anticipated 2011approval.

Adequate loading of drug liposomal carriers is crucial in order to deliver a therapeutically useful dose of drug.[295] A number of approaches are available for the incorporation of drugs into liposome carriers; the most suitable approach will be largely determined by the physicochemical properties of the drug under investigation.[348] Drugs can be amenable to remote-loading techniques utilising a pH or ion gradient if they possess ionisable functional groups, such as amino or carboxyl groups.[348] The commercial formulation of doxorubicin, Doxyl®, is produced by remotely loading doxorubicin into hydrogenated soy phosphatidylcholine and phosphatidylethanolamine-PEG liposomes using an ammonium sulphate gradient.[349] Remote-loading techniques

can result in drug concentrations within the liposome interior at concentrations well beyond drug aqueous solubility,[349] achieving high drug:lipid ratios.[349] Drugs that are lipophilic may be loaded into the lipidic region of the phospholipid bilayer. Alternatively, an ion-pair interaction can be created between ionic drug species and charged phospholipids, such as is utilised in the commercial formulation of amphotericin B, AmBisome®.[350]

The co-encapsulation of drugs within a common liposomal carrier is currently at the forefront of emerging liposome technologies, offering the possibility to amplify the therapeutic potential of drug combinations.[320] The difficulty in the use of liposomes as a vehicle for controlled release of a combination of drugs is in achieving the desired release rate of each agent individually, to accommodate potentially vastly different physicochemical, pharmacodynamic and pharmacokinetic properties. In general, the pharmacokinetics of drugs loaded within a liposomal carrier will be dictated by the pharmacokinetics of the liposome carrier itself, while release rates will be dictated by the tendency of the drugs to partition across the liposomal bilayer.[348, 351]

The opportunity exists for liposomal co-encapsulation to achieve coordinated release, elimination and biodistribution of co-encapsulated agents. The simultaneous delivery of vincristine and quercertin by co-encapsulation into liposomes has been proposed as a strategy by which the poorly water-soluble drug quercertin can be co-formulated with the highly water soluble drug vincristine.[352] Wong and colleagues demonstrated that by co-encapsulation into a common liposome carrier, both vincristine and quercertin could be incorporated with high (> 75%) encapsulation efficiency (EE) and the rate of release of each drug could be independently controlled to co-ordinate release, optimising the *in vivo* drug ratio.[352]

Multi-compartment liposomes have also been proposed as an approach towards tailoring liposome formulations for encapsulation and co-ordinated release of drugs of varying physicochemical and biopharmaceutical properties.[353] While combination liposome formulations have yet to translate into commercial products, the use of liposomes as an approach to co-formulation has been proven to achieve high loading efficiencies of more than one agent and can result in the simultaneous and coordinated release of encapsulated agents.[320-321, 352, 354-355]

1.4.3.4 Other nanoparticles

A range of other nanoparticle based drug delivery systems have been investigated for delivery of poorly-water soluble and water soluble drugs, including solid drug nanoparticles,[356] solid-lipid nanoparticles,[357] polymeric nanoparticles,[358-359] dendrimers[360] and nanotubes.[361] Each of these emerging technologies, while receiving a great deal of attention in the literature, has respective advantages and shortcomings. For example, polymeric nanoparticles can be easily manufactured using biodegradable, inexpensive materials but they are often extremely limited in drug loading capacity, thus limiting their therapeutic utility.[299] Moreover, the toxicological profiles associated with the chronic use of many of these systems are essentially unknown, creating a significant barrier of resistance from a regulatory perspective. Consequently, there has been little translation of these more advanced systems into commercial pharmaceutical products.

1.5 Colistin/CMS formulations for pulmonary delivery

The co-encapsulation, and therefore co-localisation, of colistin or CMS and a second antibiotic within a common colloidal carrier offers scope to improve current colistin inhalation therapy. The development of an aqueous formulation of CMS or colistin for inhalation, however, requires a certain level of knowledge of the chemical stability and

physicochemical behaviour of colistin and CMS. As introduced in Section 1.3.1.2, CMS has the potential to convert back to colistin both *in vitro* and *in vivo*. In a study of the stability of CMS in commercially available solution formulations, it has been reported that the conversion of CMS to colistin in aqueous environments is concentration-dependent.[362] The mechanism governing the stabilisation of CMS at high concentrations raises questions about the physicochemical behaviour of CMS in solutions, and is worthy of further investigation. In addition, the *in vitro* conversion of CMS to colistin within a colloid-based formulation may have implications for the physical stability of the colloidal system, given that both CMS and the colistin formed *in situ* from degradation of CMS, are highly (and oppositely) charged chemical species.

1.6 Stability of CMS in solution

Conversion of CMS to colistin (Equation 1.3) occurs by the removal of the sulphomethyl groups to expose the primary amino groups of all five γ-diaminobutyric acid residues.[71] Although never confirmed experimentally, this is presumed to be a hydrolytic reaction.[111, 363-364]

$$-RNHCH_2SO_3 + H_2O \leftrightarrow RNH_2 + CH_2OH + HSO_3^- \qquad \textbf{Equation 1.3}$$

The complexity introduced by the presence of five reactive groups makes it difficult to determine the precise mechanism of CMS instability. In an attempt to simplify this system, McMillan *et al*. studied the instability of simpler model compounds, n-butylaminomethanesulphonate and phenethylaminomethanesulphonic acid, possessing only one methanesulphonated amine.[363] In that study, it was noted that the conversion of these compounds to the free amines appeared to involve more than simple hydrolysis.[363] Presumably, the cleavage of the sulphomethyl groups of CMS occurs in a step-wise fashion, meaning that in an aqueous environment at any one time, CMS could

exist as a heterogeneous mixture of fully derivitised CMS, a series of partially sulphomethylated derivatives substituted at various positions to varying degrees, and also free colistin.

The presence of free colistin in a dry powder preparation of CMS would depend on the extent of sulphomethylation during the process of manufacture. Electrophoresis has been used to visualise the migration of colistin, various partial derivatives of colistin and CMS.[363-364] McMillan *et al*. investigated the likelihood of free colistin remaining after the sulphomethylation of colistin with increasing molar ratios of colistin:sodium bisulfate and formaldehyde, and concluded that the statistical likelihood of the presence of underivitised colistin, with five unreacted amino groups, is extremely small.[363]

Using electrophoretic analysis, Barnett *et al*. studied the existence of colistin in a dilute, freshly prepared solution of CMS and compared this to the presence of colistin in CMS solutions that had been incubated (exact CMS concentrations not specified).[111] In the electrophoretogram, no 'spot' corresponding to colistin was observed in freshly prepared solutions of CMS, while the presence of a colistin 'spot' was observed after incubation of CMS solutions at 37°C.[364] In the same study, the antimicrobial activity of freshly prepared, dilute CMS solutions was compared with those incubated at 37°C; the incubated solutions exhibited far greater antimicrobial activity than those that were freshly prepared. In that study it was concluded that only partial conversion, yielding partially sulphomethylated CMS, was required for antimicrobial activity. However, the activity of CMS and its partial derivatives has since been questioned.[71]

By incubating *P. aeruginosa* with 8 or 32 μg/mL CMS, Bergen *et al*. first, quantified the amount of free colistin formed from CMS during incubation and, secondly, measured the extent of bacterial kill.[71] By this, Bergen and co-workers were able to

replicate the degree of bacterial kill with equivalent concentrations of colistin (using colistin sulphate) that were formed when CMS was incubated, showing that the killing kinetics of CMS could be attributed to the appearance of free colistin, providing conclusive evidence that CMS is an inactive pro-drug of colistin.[71]

A number of studies have examined the rates of colistin formation from CMS in solution. Using validated analytical methods to quantify colistin, CMS has been shown to rapidly convert to free colistin in dilute environments (20 – 100 µg/mL) *in vitro*[71, 107, 362] and at biologically relevant concentrations *in vivo*.[75, 131, 140] At CMS concentrations of 100 µg/mL, up to 50% and 80% CMS was converted to colistin after 24 h in water and pH 7.4 buffer, respectively, at 37°C.[107] Rapid hydrolysis occurs *in vivo* with colistin appearing systemically within 5 min of the IV administration of CMS to rats[75, 140] and shortly after administration to humans.[130-131]

In contrast to the rapid conversion of CMS to colistin that occurs at low CMS concentrations, pharmaceutical formulations containing high concentrations of CMS have been shown to be stable for extended periods with respect to colistin formation. An inhalation solution containing 77.5 mg/mL CMS was shown to be stable for up to 12 months at 25°C.[362] When Coly-Mycin M® Parenteral was reconstituted with water and further diluted to 4 mg/mL in an infusion solution, after 48 h incubation, less than 4% of the CMS had converted to colistin at 25°C, and 0.3% had converted at 4°C. Like many chemical reactions, the conversion is accelerated at higher temperatures.[365] Importantly, though, the rate of conversion appears to depend on the initial CMS concentration in the system, i.e. conversion is concentration-dependent.

A number of small molecule drugs have been shown to self-assemble into micelles and micellar-like aggregates,[366-369] leading to enhanced stability at high concentrations.[370-372]

It has been proposed that the concentration-dependent stability of CMS observed may be attributable to a self-assembly phenomenon.[362] The formation of micelles in solutions of colistin has been proposed, but the concentrations at which micelles form has not been reported.[373] Owing to limited knowledge regarding the physicochemical behaviour of colistin and CMS in solution, there is currently a lack of information on the interfacial and bulk solution behaviour of colistin and CMS, including their propensity to self-assemble.

The formation of micelles in solutions may provide a physicochemical basis for the 'apparent' concentration-dependent stability of CMS. but is also of interest from a formulation perspective. Some micelle-forming species have the capacity to solubilise poorly-water soluble drugs,[374] so the micelle forming tendencies of colistin and CMS could be utilised as an approach towards the co-formulation of colistin or CMS and other poorly water-soluble antibiotics in combination antibiotic formulations. An appreciation for the behaviour of colistin and CMS in bulk solution will also be important to an understanding of the physicochemical behaviour of colistin and CMS at colloidal interfaces.

1.7 Summary

Colistin, an old antibiotic, has largely retained its activity against MDR Gram-negative bacteria. However, increasing numbers of reports of resistance serve as a warning that the current clinical use of colistin is likely to be sub-optimal. The emergence of colistin-resistant Gram-negative bacteria poses a particular problem in the treatment of pulmonary infections, for which colistin is often used as a last-line therapy.

Consequently, strategies to intensify the use of inhalation treatment are urgently needed to ensure the clinical efficacy of colistin for decades to come. Combination antibiotic therapy using colistin is a potentially powerful tool that can be used in the fight against emerging MDR infections. Current approaches to colistin inhalation therapy may be sub-optimal and significant scope remains to improve formulations of colistin for direct delivery to the lungs. This project aims to investigate new formulations incorporating colistin and a second antibiotic by co-localisation within a colloidal carrier, for direct delivery to the respiratory tract. The selection of the appropriate type of colloidal drug carrier for colistin combinations must take into account the requirements of the intrapulmonary route of administration, the physicochemical properties of colistin and CMS, and should contain excipients that are compatible with pulmonary delivery. Finally, the appropriate delivery system should be manufactured with relative ease at a reasonable cost.

1.8 Hypothesis and aims

The principal hypothesis of this thesis is that colistin or CMS may be loaded into a colloidal drug delivery system, together with a second antibiotic, to produce a formulation exhibiting modified release when administered to the respiratory tract. Specifically, that:

1. the concentration-dependent stability of CMS in solution is attributable to a self-assembly phenomenon;
2. micelles of colistin and CMS have the capacity to solubilise poorly water-soluble drugs;
3. that colistin or CMS can be successfully incorporated into a colloidal carrier to produce a physically and chemically stable formulation;
4. controlled release of colistin or CMS can be achieved *in vitro* by loading into colloidal carrier;
5. the presence of colistin or CMS can modify the release of an antibiotic co-encapsulated into a common colloidal carrier; and that
6. the incorporation of colistin or CMS into a colloidal-based drug delivery system will modify the rate of pulmonary absorption when administered to the lungs in a rat pharmacokinetic model

In addressing these hypotheses, the thesis aims to:

1. characterise the physicochemical behaviour of colistin and CMS in solution (Chapter 3)
2. examine the interactions between CMS and a colloidal carrier (Chapter 4)
3. characterise the physicochemical properties of colistin/CMS loaded into a colloidal carrier (Chapter 4)

4. investigate the potential for colistin or CMS to be co-localised with a second antibiotic by co-formulation into a common colloidal carrier (Chapter 5); and
5. examine the *in vivo* pharmacokinetics of colistin/CMS loaded colloids following pulmonary delivery to the rat (Chapter 6).

Chapter 2: Methods

This chapter deals with two discrete aspects of experimental work. The first part, Section 2.1, deals with the chemical characterisation of materials used in determining the physicochemical behaviour of colistin and CMS in solution (Chapter 3) and for investigations into the interactions of colistin and CMS with liposomes (Chapters 4 and 5). The second part, Section 2.2, provides details of the development and validation of analytical methods crucial to the quantification of drug concentrations in stability, formulation and pharmacokinetic studies (Chapters 3 to 6).

2.1 Materials characterisation

Due to the fact that colistin and CMS were essentially 'shelved' as antimicrobial agents between the 1960s and 1990s, they have not been subjected to the scrutiny typical of modern drug development candidates. As a result, there is currently a significant void in the understanding of the chemical properties of colistin and CMS. Studies of basic chemical properties, usually coined 'pre-formulation' studies, are an integral part of the development of new formulations for any new active pharmaceutical ingredient. The information provided by pre-formulation studies is crucial to the interpretation of data obtained in the characterisation of physicochemical properties. In formulation development, pre-formulation studies are fundamental to elucidating the relationships between underlying variables and formulation performance. Therefore, it was deemed necessary to undertake characterisation of the materials to be used in studies of the physicochemical behaviour of colistin and CMS in solution (Chapter 3) and in formulation studies (Chapter 4, 5 and 6).

Colistin sulphate

Colistin sulphate from Sigma-Aldrich (Castle Hill, Australia) in 1 g pack size is routinely obtained by our laboratories for use in various microbiological and pharmacokinetic studies. Such studies usually only require a small amount of material. Physicochemical characterisation and formulation studies, however, can require a much larger amount of material and therefore impose a greater expense. It was therefore necessary to obtain colistin sulphate from an alternative source, in a larger quantity. Due to the variation in composition that can exist between batches of colistin sulphate from different manufacturers, and between batches from the same manufacturer (as introduced in Section 1.3.1.1), it was considered preferable to carry out all physicochemical characterisation (Chapter 3) and formulation studies (Chapters 4 and 5) on one batch of colistin sulphate. With this in mind, a bulk sample of colistin sulphate (1 kg) was sourced from Zhejiang Shenghua Biok Biology Co. Ltd (Zhejiang, China). Prior to commencing physicochemical characterisation and formulation studies using this new batch of colistin sulphate it was necessary to assess the comparative quality, in terms of chemical composition and antimicrobial activity, of colistin sulphate from Sigma-Aldrich and Zhejiang Shenghua Biok Biology Co.

As described in Section 1.3.1.1 colistin is a mixture of colistin A and B, which differ in chemical structure by only one carbon in the fatty acyl chain length (Figure 1-2), and other minor components.[95] This slight difference in the hydrophobic region of the molecule is likely to confer different interfacial and bulk solution behaviour between colistin A and B.[296] The ratio of colistin A and B in commercially available formulations is said vary between 4.5 and 1:2.5.[102, 134] The interfacial behaviour of colistin may therefore vary between batches, depending on the ratios present in a given batch.

Ideally, physicochemical characterisation studies should be carried out on pure chemical entities, rather chemical mixtures such as colistin. Unfortunately, colistin A and B are not available commercially in the pure form and complete synthesis is labour intensive and time consuming, often resulting in low yields.[375-376] Separation of colistin A and colistin B on a preparative scale would enable purification of colistin A and B for individual physicochemical characterisation. However, the resources required for preparative scale separations were not available at the time these studies were carried out. As a minimum, an estimation of the relative contributions of colistin A and B to the overall mixture would facilitate interpretation of physicochemical characterisation data. The relative amounts of colistin A and colistin B may also help to explain any differences observed between measured and theoretical elemental contributions in the elemental analysis of colistin (Section 2.1.1.4).

Colistin methanesulphonate

The proposed chemical structure of CMS (Figure 1-3), although accepted by official monographs,[106, 110] has never been confirmed by spectroscopic methods. As introduced in Section 1.3.1.2, CMS is produced by the sulphomethylation of colistin, which itself is a mixture of colistin A, colistin B and other minor components. Depending on the degree of sulphomethylation of colistin during CMS manufacture, CMS can potentially contain up to 32 (2^5) different fully and partially sulphomethylated intermediates, plus colistin.[107] The degree of sulphomethylation may affect interfacial behaviour and also the rate at which CMS converts to colistin in solution (Section 1.3.1.2). Confirmation of chemical structure can be carried out to estimate the degree of CMS sulphomethylation. Methods including nuclear magnetic resonance (NMR), mass spectrometry (MS) or elemental analysis may be utilised for this purpose. The use of NMR and MS in the context of CMS is complicated by the fact that CMS is potentially a heterogenous

mixture of numerous closely related chemical entities; interpretation of chemical spectra obtained from analysis of CMS can therefore be difficult. Elemental analysis has been used by other authors to estimate the degree of sulphomethylation of a commercially available lyophilised CMS product (Colomycin, Pharmax Ltd).[111] In that study, Barnett *et al*. suggested that CMS was close to fully derivitised; however, these data were not published or discussed in any detail.[111] The degree of sulphomethylation of the CMS to be used in physicochemical characterisation and formulation studies (Chapters 3 and 4) should be estimated by elemental analysis to aid interpretation of data from physicochemical characterisation, stability and formulation studies.

The presence of excipients in the CMS used in the characterisation of the solution-behaviour of CMS may complicate the interpretation of the results, and may also have implications if included unwittingly in formulation studies. The certificate of analysis for the sample of colistin sulphate obtained for Zhejiang Shenghua Biok Biology Co. states that the product contains no added excipients. However, the certificate of analysis for the sample of CMS obtained from Alpharma (Copenhagen, Denmark) is not so explicit. Moreover, a New Animal Drug Application published on the Food and Drug Administration website referring to the veterinary use of a commercial CMS product made by the same manufacturer as the CMS to be used in the present studies, states that mannitol is added to the final sterile powder to adjust for potency.[377] As such, it would be prudent to ascertain whether or not mannitol is present in the CMS obtained from Alpharma prior to conducting physicochemical characterisation studies.

2.1.1 Methods

2.1.1.1 Materials

Colistin sulphate was obtained from Sigma-Aldrich (Castle Hill, Australia [batch number 095K1048]) and Zhejiang Shenghua Biok Biology Co. (Zhejiang, China [batch number EP5 20061220]). CMS sodium was obtained from Alpharma (Copenhagen, Denmark [batch number A1680552]). Mannitol, ammonium molybdate and sulphuric acid were from Sigma-Aldrich (St Louis, MO, USA). All solvents (HPLC grade) were from Merck (Damstadt, Germany). The batches of colistin and CMS used for material characterisation studies are outlined in Table 2-1.

Table 2-1: Colistin and CMS batches used in materials characterisation.

Material	Supplier	Batch number	Analysis			
			Melting point	Elemental analysis	Antimicrobial activity	Manntiol TLC
Colistin sulphate	Sigma	095K1048	✓	✓	✓	
Colistin sulphate	Zhejiang Shenghua	EP520061220		✓	✓	
CMS sodium	Alpharma	A1680552	✓	✓		✓

2.1.1.2 Differential scanning calorimetry

Approximate melting points for colistin sulphate and CMS sodium were required in order to set suitable combustion conditions for elemental analysis (Section 2.1.1.4). A melting point for CMS sodium is not reported in the literature, while the melting point of colistin sulphate is reported to be within the range 200 - 220°C.[106] Differential scanning calorimetry (DSC) measures the heat fluxes that occur during chemical reactions and phase transitions.[378] Together with visual observations from thermo microscopy (Section 2.1.1.3), DSC can provide the required information about the

thermal properties of colistin sulphate and CMS sodium. DSC was carried out with a Perkin-Elmer DCS7 calorimeter (Perkin-Elmer, Norwalk, USA). The instrument was set to heat from 30 to 270°C at a scanning rate of 5°C/min under nitrogen. A 5.46 mg sample of colistin sulphate (Sigma Aldrich) or 7.49 mg of CMS sodium was introduced into an aluminium pans (50 µL, Perkin Elmer) and an empty pan was used as a reference. Data acquisition and analysis were performed using the software provided with the instrument (PYRIS v3.81, Perkin Elmer).

2.1.1.3 Thermo microscopy

Thermo microscopy was used in conjunction with DSC to identify the melting points of colistin sulphate and CMS sodium. Using an ENKAM TP 93 controlled temperature unit (Linkam Scientific Instruments, Tadworth, UK), a small amount of colistin sulphate or CMS sodium powder was placed on a glass slide and heated from room temperature to 250°C at a gradient of 1°C every 5 sec. The powder was observed under an Axiolab A light reflected microscope (Carl Zeiss Pty Ltd, North Ryde, Australia) to identify the temperature at which a solid to liquid transition occurred. In order to record the nature of any physical changes, digital images were taken prior to and after heating. The temperature at which physical changes occurred was recorded.

2.1.1.4 Elemental analysis

Elemental analysis, described in Section 2.1, can be used to determine the elemental composition of a compound by determining the contribution of carbon, hydrogen, nitrogen and sulphur to the overall composition. Elemental analysis was contracted out to Chemical and Analytical Services Pty Ltd (Belmont, Australia). Briefly, a sample (< 1 g) was burned in a quartz tube with oxygen and injected into a helium carrier gas flow. Combustion was completed over copper oxide and excess oxygen was removed.

Nitrogen oxides were reduced to nitrogen and sulphur trioxide to sulphur dioxide in a layer of metallic copper. The remaining combustion gases – nitrogen, carbon dioxide, water and sulphur dioxide – were separated by gas chromatography and measured with a hot-wire detector and an integrator.[379] The measured elemental contributions to colistin sulphate obtained from Sigma-Aldrich and Zhejiang Shenghua Biok Biology Co. were compared to the theoretical elemental composition, based on the weighted average molecular weight of colistin sulphate ($C_{52.5}H_{99}N_{16}O_{23}S_{2.5}$, M_W 1403 Da). The average molecular weight of colistin sulphate was calculated as an average of the molecular weights of colistin A sulphate ($C_{53}H_{100}N_{16}O_{23}S_{2.5}$, M_W 1410 Da) and colistin B sulphate ($C_{52}H_{98}N_{16}O_{23}S_{2.5}$, M_W 1396 Da). The ratios of colistin A and colistin B were assumed to be 1:1 for the purposes of elemental analysis. The use of the average molecular weight in place of weighted average molecular weights would have little impact on the final results of elemental analysis given that the difference in the molecular weights of colistin A sulphate and colistin B sulphate is only 14 Da.

The average molecular weights of fully derivatised CMS sodium, and partially derivatised intermediates containing one, two, three or four sulphomethyl groups were calculated based on the theoretical average molecular weight of fully derivitised CMS sodium and removal of each of the sulphomethyl groups. The average molecular weights, and corresponding molecular formulas, were used to calculate the percentage contribution (% by weight) of each element to molecular weight of each of the series of fully derivatised CMS, partially derivatised CMS and un-sulphomethylated CMS (i.e. colistin base average M_W [M_W 1163], the average of colistin A [M_W 1,170] and colistin B [M_W 1,156]). The calculated theoretical elemental contributions were then compared to the experimentally determined values.

2.1.1.5 Antimicrobial activity of colistin sulphate

In addition to ascertaining whether the chemical composition of colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. and Sigma-Aldrich were comparable, it was necessary to also compare their antimicrobial activity. Two strains of bacteria were used for susceptibility testing: *A. baumannii* ATCC 19606 and *P. aeruginosa* ATCC 27853. *MIC*s were measured in triplicate by microdilution in Mueller-Hinton broth (Oxoid Australia, West Heidelberg, Australia), according to Clinical and Laboratory Standards Institute protocol.[380]

2.1.1.6 High-performance liquid chromatography to determine proportions of colistin A and colistin B in colistin sulphate

A 1 mg/mL solution of colistin sulphate (Zhejiang Shenghua) was prepared in Milli-Q® water. Colistin A and B were separated using high- performance liquid chromatography (HPLC) according to 2.2.3. The chromatographic peak areas of colistin A and B were calculated and used to determine the relative ratios of colistin A and B to the overall colistin mixture.

2.1.1.7 Thin-layer chromatography to detect mannitol in CMS sodium

Thin layer chromatography (TLC) was used for the qualitative analysis of mannitol in CMS sodium. Aliquots (1 µL) of solutions containing 25, 50 and 100 mg/mL CMS sodium were spotted (approximately 1 mm diameter, spots 1 cm apart) onto a silica gel TLC plate (Analtech TLC uniplate, 250 µm thickness). Standard solutions containing 0.5, 1 and 2 mg/mL mannitol were similarly spotted. The TLC plates were dried in an oven at 60°C for 15 min. Subsequently, the bottom edge of the plate was immersed (to approximately 0.5 cm depth) in a mobile phase of 10:20:70 ethanol/ethyl acetate/water, inside a screw-capped jar, until the solvent front reached 1.5 cm from the top of the

plate. Plates were then dried flat for. After drying TLC plates at 60°C for 30 min, a hand-held spray bottle was used to finely and evenly cover the plates with developing solution of sulphuric acid 10% v/v and ammonium molybdate 10% w/v. Plates were then placed flat in a 100°C oven for 60 min to allow colour to develop.

2.1.2 Results

2.1.2.1 Differential scanning calorimetry

Colistin sulphate

The DSC thermogram of colistin sulphate (Figure 2-1) indicates an endothermic event at approximately 235°C and a second more minor endothermic peak at ~250°C. Confirmation of the endotherm at 235°C by thermo microscopy (Section 2.1.1.3) was required to verify the nature of the endotherm observed.

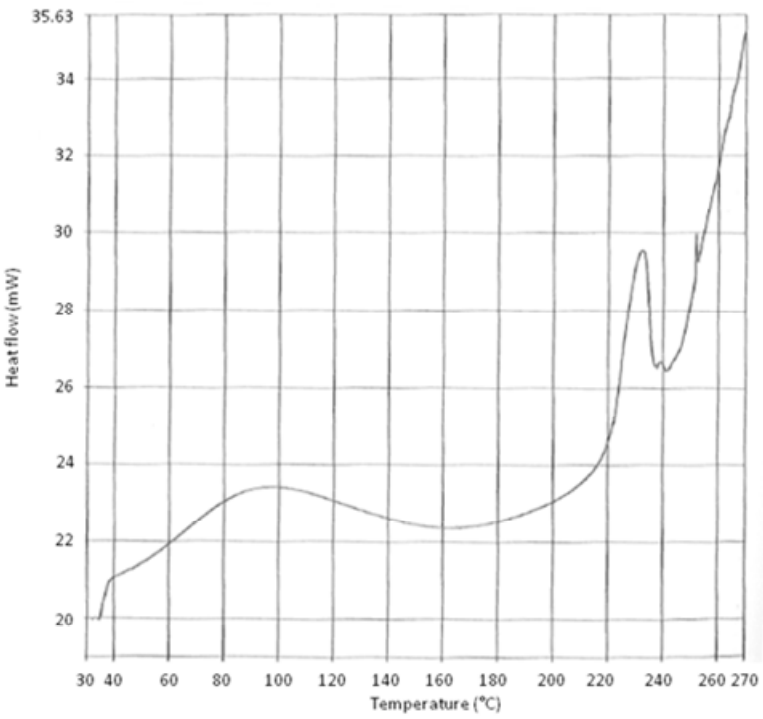


Figure 2-1: DSC thermogram for colistin sulphate when heated at 5°C/min.

Colistin methanesulphonate sodium

The DSC thermogram for CMS sodium shown in Figure 2-2 indicates minor and major endothermic peaks at approximately 250 and 265°C, respectively. To determine whether the endotherm observed was due to melting of the material, thermo microscopy was carried out (Section 2.1.1.3).

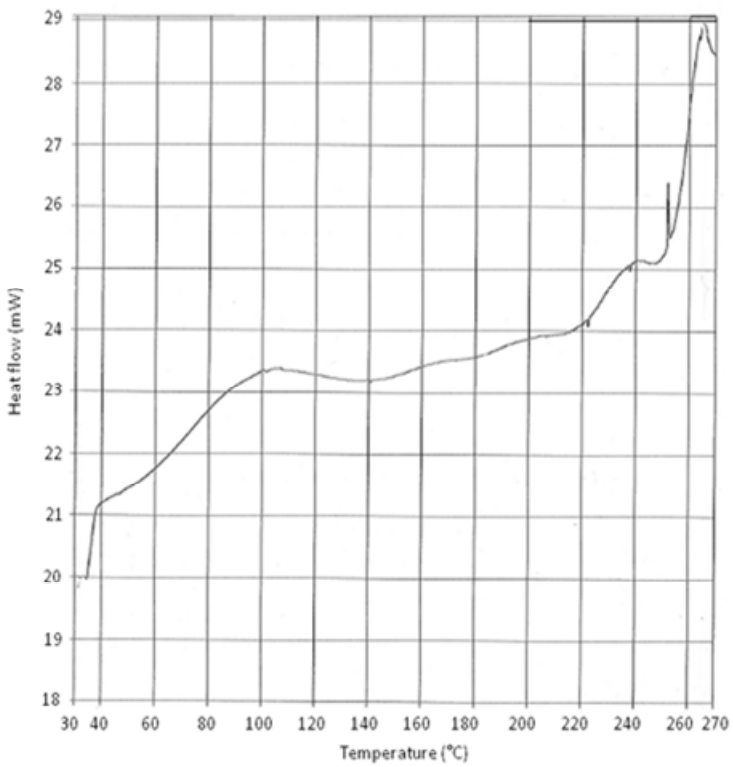


Figure 2-2: DSC thermogram for CMS when heated at 5°C/min.

2.1.2.2 Thermo microscopy

When heated from room temperature to 250°C, both colistin sulphate and CMS sodium underwent a colour change from pale yellow to dark brown, as depicted by Figure 2-3A and B and Figure 2-4A and B. The samples did not appear to undergo a melting across the temperature range examined and birefringence was not observed when viewed under cross polarised light.

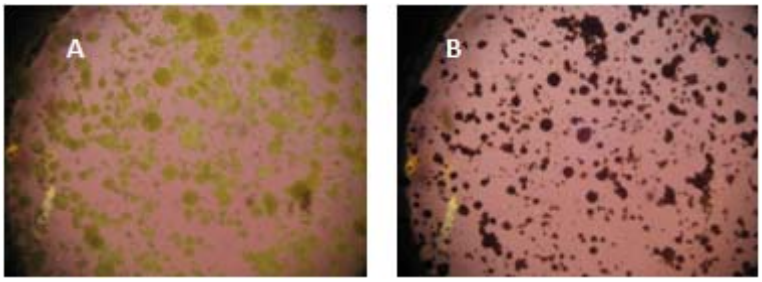


Figure 2-3: Images of colistin sulphate at 210°C (A) and 240°C (B) viewed under × 17.5 magnification.

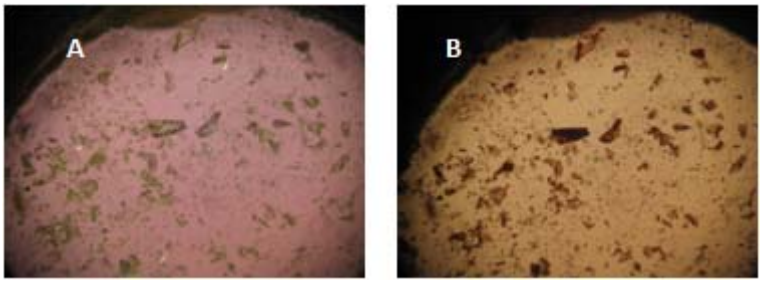


Figure 2-4: Images of colistin methanesulphonate sodium at 200°C (A) and 260°C (B) viewed under × 17.5 magnification.

2.1.2.3 Elemental analysis

Colistin sulphate

The results of the elemental analysis of colistin sulphate obtained from two different sources, to an accuracy of ± 0.3%, are shown in Table 2-2. When the theoretical and measured elemental contributions are compared, colistin sulphate from Zhejiang Shenghua Biok Biology Co. and Sigma-Aldrich are seen to be within 4.45 and 3.57% of the theoretical elemental contributions, respectively. Colistin sulphate from Sigma-Aldrich was closer to the theoretical elemental contributions for carbon, hydrogen and nitrogen, while colistin from Zhejiang Shenghua Biok Biology Co. closer to the theoretical sulphur content.

Colistin methanesulphonate sodium

The elemental analysis results for CMS sodium, to an accuracy of ± 0.3%, are given in Table 2-3 to Table 2-8. By comparison of the theoretical elemental and measured elemental contributions, it can be seen that the composition of CMS sodium from Alpharma was closest to the pentasulphomethyl derivative of colistin (fully derivatised), with the measured contributions of each element deviating from the theoretical contributions of the pentasulphomethyl derivative by no more than 4.23%.

Table 2-2: Theoretical and the experimentally determined elemental contributions of colistin sulphate from two different manufacturers.

Element	**Number of atoms in molecular formula**	**Atomic mass (Da)**	**Total mass contributed by element**	**Theoretical % of M_W given by element**	**Experimental % element by weight**	**Experimental % element by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(colistin\ sulphate)}$	$Colistin\ sulphate$ $(Sigma\ Aldrich)$	$Colistin\ sulphate$ $(Zhejiang\ Shengbiok)$
C	52.5	12.01	631	44.96	41.39	40.51
H	99	1.01	100	7.13	7.23	7.87
N	16	14.01	224	15.97	14.42	14.29
O	23	15.99	368	26.24	Not reported	Not reported
S	2.5	32	80	5.70	3.33	5.34
Total M_W			**1403**			

Table 2-3: Theoretical elemental contribution for fully derivatised CMS sodium (5 methanesulphonate groups) versus the experimentally determined elemental analysis.

Pentasulphomethyl CMS					
Element	**Number atoms in molecular formula**	**Atomic mass (Da)**	**Total atomic mass given by element**	**Theoretical % of M_W given by element**	**Experimental element % by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(CMS\ sodium)}$	
C	57.5	12.01	690.62	39.63	35.38
H	104	1.01	104.83	6.01	6.13
N	16	14.01	224.11	12.86	11.16
Na	5	22.9	114.95	6.60	Not reported
O	28	15.99	448.00	25.70	Not reported
S	5	32.01	160.05	9.18	11.82
Total M_w			**1743.55**		

Table 2-4: Theoretical elemental contribution for partially derivatised CMS (4 methanesulphonate groups) versus experimentally determined by elemental analysis.

Tetrasulphomethyl CMS					
Element	**Number atoms in molecular formula**	**Atomic mass (Da)**	**Total atomic mass given by element**	**Theoretical % of M_W given by element**	**Experimental element % by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(CMS\ sodium)}$	
C	56.5	12.01	678.60	41.70	35.38
H	103	1.01	103.82	6.44	6.13
N	16	14.01	224.11	13.77	11.16
Na	4	22.9	91.96	5.65	Not reported
O	25	15.99	400.00	24.58	Not reported
S	4	32.01	128.04	7.87	11.82
Total M_W			**1627.53**		

Table 2-5: Theoretical elemental contribution for partially derivatised CMS (3 methanesulphonate groups) versus experimentally determined by elemental analysis.

Trisulphomethyl CMS					
Element	**Number atoms in molecular formula**	**Atomic mass (Da)**	**Total atomic mass given by element**	**Theoretical % of M_W given by element**	**Experimental element % by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(CMS\ sodium)}$	
C	56.5	12.01	666.59	44.10	35.38
H	102	1.01	102.81	6.87	6.13
N	16	14.01	224.11	14.83	11.16
Na	3	22.9	68.97	4.56	Not reported
O	25	15.99	352.00	23.29	Not reported
S	3	32.01	96.03	6.35	11.82
Total M_W			**1511.49**		

Table 2-6: Theoretical elemental contribution for partially derivatised CMS (2 methanesulphonate groups) versus experimentally determined by elemental analysis.

Disulphomethyl CMS					
Element	**Number atoms in molecular formula**	**Atomic mass (Da)**	**Total atomic mass given by element**	**Theoretical % of M_W given by element**	**Experimental element % by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(CMS\ sodium)}$	
C	56.5	12.01	654.58	46.90	35.38
H	101	1.01	101.80	7.30	6.13
N	16	14.01	224.11	16.07	11.16
Na	2	22.9	45.98	3.29	Not reported
O	25	15.99	256.00	21.80	Not reported
S	2	32.01	64.02	4.59	11.82
Total M_W			**1395.48**		

Table 2-7: Theoretical elemental contribution for partially derivatised CMS (1 methanesulphonate group) versus experimentally determined by elemental analysis.

Monosulphomethyl CMS

Element	**Number atoms in molecular formula**	**Atomic mass (Da)**	**Total atomic mass given by element**	**Theoretical % of M_W given by element**	**Experimental element % by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(CMS\ sodium)}$	
C	56.5	12.01	642.57	50.22	35.38
H	100	1.01	100.79	7.96	6.13
N	16	14.01	224.11	17.52	11.16
Na	1	22.9	22.99	1.80	Not reported
O	25	15.99	256.00	20.01	Not reported
S	1	32.01	32.01	2.50	11.82
Total M_W			**1279.47**		

Table 2-8: The use of the molecular weight average of colistin A and B assumes that colistin A and colistin B predominated in the samples (over other minor components) and that colistin A and colistin B were present in equal proportions.

Underivitised CMS (no methanesulphonate groups)

Element	**Number atoms in molecular formula**	**Atomic mass (Da)**	**Total atomic mass given by element**	**Theoretical % of M_W given by element**	**Experimental element % by weight**
	N	A_R	$N \times A_R$	$\frac{N \times A_R}{MW(CMS\ sodium)}$	
C	52.5	12.01	630.56	54.24	35.38
H	99	1.01	100.79	8.58	6.13
N	16	14.01	224.10	19.27	11.16
Na	0	22.9	0	0	Not reported
O	13	15.99	207.99	17.89	Not reported
S	0	32.01	0	0	11.82
Total M_W			**1162.45**		

2.1.2.4 Antimicrobial activity of colistin

The antimicrobial susceptibilities of reference strains *A. baumannii* ATCC 19606 and *P. aeruginosa* ATCC 27853 to colistin (sulphate) obtained from the Sigma-Aldrich and Zhejiang Shenghua Biok Biology Co. are displayed in Table 2-9.

Table 2-9: Antimicrobial activities of colistin sulphate obtained from two different suppliers

Supplier	**MIC (µg/mL)**	
	A. baumannii ATCC 19606	*P. aeruginosa* ATCC 27853
Sigma-Aldrich	0.5	0.5
Zhejiang Shenghua	0.5	1

2.1.2.5 Chromatographic determination of colistin A and colistin B ratios in colistin

Based on the relative peak areas of colistin A and colistin B following chromatographic separation, as shown in Figure 2-5, the ratio of colistin A:colistin B in the colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. was found to be 1.5:1.

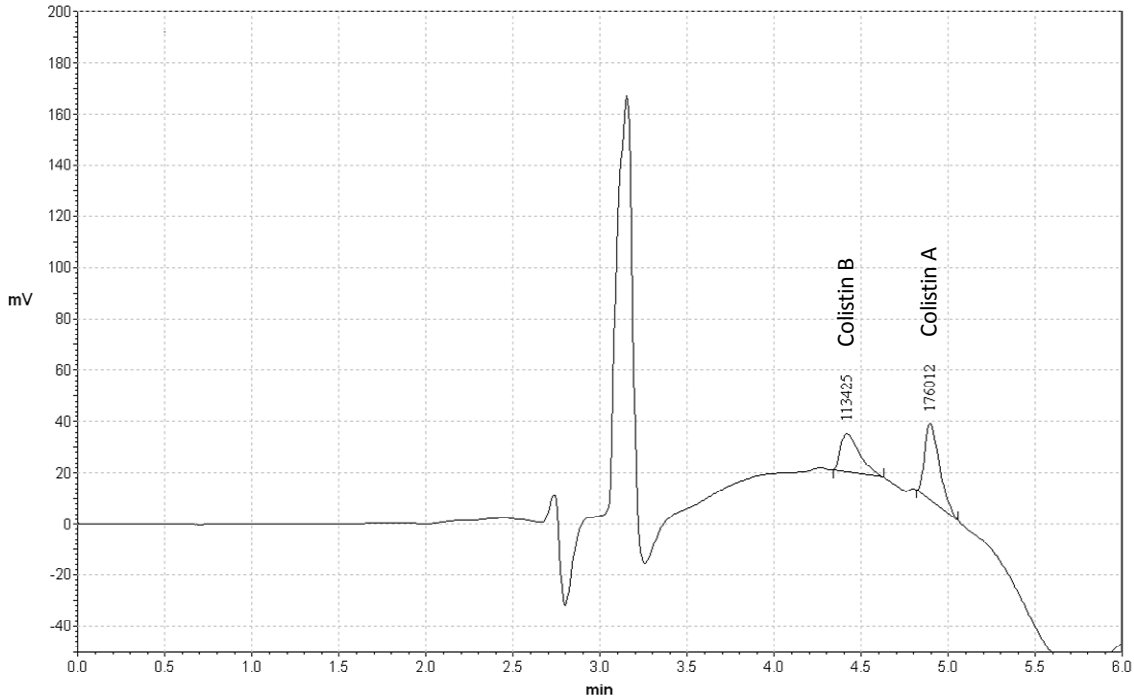


Figure 2-5: Chromatogram showing the relative peak areas of colistin A (second peak) and colistin B (first peak) in colistin obtained from Zhejiang Shenghua Biok Biology Co.

2.1.2.6 Thin- layer chromatography to detect mannitol in CMS sodium

Figure 2-6 shows three dark blue reference spots, each corresponding to mannitol reference standards. The calculated retention factor for mannitol (R_f, the distance travelled by the solvent/distance travelled by mannitol standard) was 0.46. No spot with an equivalent R_f to mannitol could be observed in the CMS sodium lane, even at the highest concentration of CMS sodium tested (100 mg/mL, shown below). Based on the fact that the lowest mannitol concentration detected was 0.5 mg/mL, it was calculated that the CMS sodium from Alpharma contained less than 0.5% w/v mannitol.

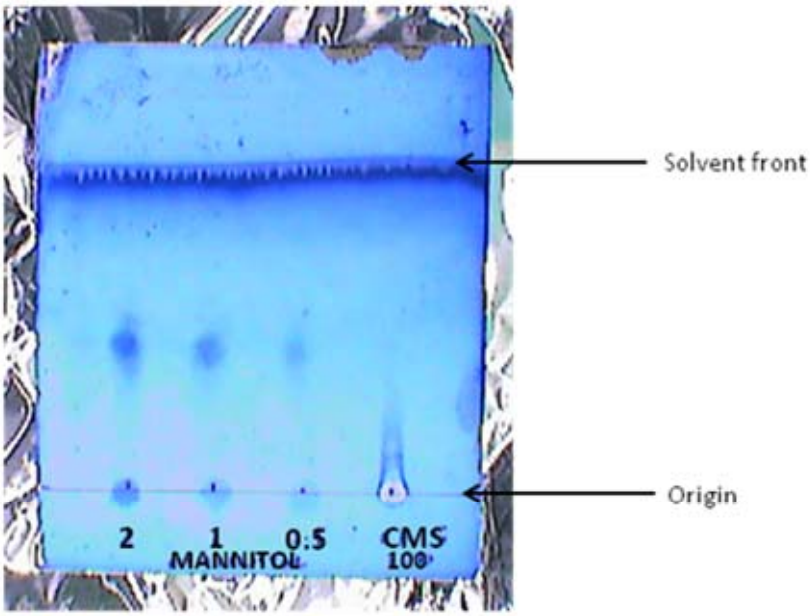


Figure 2-6: Representative TLC plate showing mannitol reference standards and a sample of CMS sodium.

2.1.3 Discussion

2.1.3.1 Differential scanning calorimetry and thermo microscopy

DSC thermograms indicated endothermic events for colistin sulphate and CMS sodium at approximately 235°C and 250°C, respectively. Information from DSC alone was not sufficient to determine the nature of this endothermic event, therefore thermo microscopy was carried out. That no melting process was observed when both solids were heated from room temperature to 250°C suggested that the endothermic events identified by DSC were due to chemical degradation, rather than transition in the physical state of these solids. That CMS and colistin did not exhibit birefringence under cross polarised light indicated that they are not crystalline solids but rather are amorphous in nature. This finding is perhaps not surprising given that both are heterogeneous mixtures of subtly different chemical species.[95] That neither colistin sulphate nor CMS sodium underwent a melting process is consistent with the non-crystalline nature these materials. The information obtained using DSC and thermo microscopy regarding the solid state behaviour of colistin sulphate and CMS sodium was used to optimise the combustion conditions during elemental analysis of these materials.

The ratios of colistin A:colistin B determined by chromatographic separation showed that there was a slightly greater proportion of colistin A than colistin B in the colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. This batch of colistin sulphate is used in subsequent physicochemical characterisation studies. The slightly greater proportion of colistin A has important implications for physicochemical characterisation studies in Chapter 3 given that colistin A, possessing the octanoyl fatty acyl chain, should have a greater capacity than its heptanoyl fatty acyl chain-containing counterpart, colistin B, to interact at interfaces, reduce surface tension and interact with

other hydrophobic molecules, such as phospholipids.[296] This should be kept in mind when interpreting information from physicochemical characterisation (Chapter 3) and formulation studies (Chapter 4).

It should be noted that the use the HPLC-UV method to determine the ratios of colistin A and B in colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. is based on the assumption that both chemical species have identical molar extinction co-efficients (ε). In reality, it is unlikely that the ε of these two species is identical, though the difference between them is likely to be minor given that the structural difference between the two compounds is only one carbon in the hydrophobic tail (Figure 1-2).

Elemental analysis of colistin sulphate from Sigma Aldrich and Zhejiang Shenghua Biok Biology Co. has shown that the two sources of colistin are comparable in terms of chemical composition. The slight deviations of experimentally determined elemental contributions from the theoretical elemental contributions (Table 2-2), though, may be due the presence of other minor components of the antibiotic mixture.[95] The slight difference between the elemental compositions of colistin from the two different sources does not appear to confer any appreciable differences in antimicrobial activity, based on minimum inhibitory concentration measurements (Section 2.1.2.4). This instills some confidence in the use of colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. in future physicochemical characterisation (Chapter 3), formulation (Chapters 4 and 5) and pharmacokinetic studies (Chapter 6).

The chemical heterogeneity of CMS has been demonstrated by a number of authors. Barnett *et al*. and Beveridge *et al*. showed, using electrophoresis,[111, 364] that freshly prepared solutions of CMS contained at least four different chemical fractions. Given that the basis for separation of compounds using electrophoresis is molecular charge,[381]

both groups concluded that the different chemical fractions corresponded to the mono- through penta-substituted partial derivatives of CMS. In contrast to this, Barnett *et al*. stated that the CMS material used in electrophoretic studies was fully substituted, based on elemental analysis (data not published).[111]

The degree of substitution of CMS has the potential to influence the rate at which it converts to colistin in solution (Section 1.3.1.2), therefore, an investigation into the chemical composition of the CMS sodium from Alpharma (to be used in subsequent stability studies, Section 3.4.1.4) was deemed necessary. By comparing the theoretical elemental contributions of CMS of varying degrees of substitution with experimentally determined elemental contributions, CMS sodium obtained from Alpharma is shown to be closer to fully derivatised than partially derivatised. The slight discrepancy that was found between the theoretical and experimentally determined elemental contributions may be attributed to the presence of very small amounts of the partial derivatives, or other minor components contained in the 'precursor' antibiotic mixture.

As shown by TLC of the CMS sodium obtained from Alpharma, it is highly unlikely that mannitol is added as an excipient. This finding further contributes to understanding of the chemical composition of the material to be used in subsequent studies.

2.1.4 Conclusion

Based on the assessment of elemental composition and antimicrobial activity, the colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. appears to be of comparable quality to the colistin sulphate obtained from Sigma-Aldrich. Therefore, colistin sulphate obtained from Zhejiang Shenghua Biok Biology Co. was used in physicochemical characterisation and formulation studies described in subsequent Chapters.

The relative proportions of colistin A and colistin B in colistin sulphate, is an important consideration for the study of the physicochemical properties and behaviour of colistin and CMS in solutions and their behaviour at interfaces. The information obtained from characterisation of colistin sulphate and CMS sodium described in the present chapter will be taken into consideration in the interpretation of data obtained in the study of physicochemical behaviour of colistin and CMS, the stability of CMS in solution (Chapter 3) and the interaction of colistin and CMS with phospholipids (Chapter 4).

Understanding the composition of the starting material in a stability study is crucial to stability data interpretation. In that context, it was an important finding that CMS sodium obtained from Alpharma was determined to be closest to fully derivatised, based on analysis of elemental content. In addition, it was important to confirm the absence of any excipients to the CMS sodium raw material.

2.2 Analytical methods

The *in vitro* and *in vivo* assessment of drug formulation characteristics requires analytical methods for the quantification of drug concentrations. For *in vitro* formulation studies, drug concentrations in samples obtained are typically orders of magnitudes greater than the concentrations encountered during *in vivo* formulation assessment. Therefore, various analytical methods suited to the quantification of drug at low and high concentrations, in a number of different of matrices, were required for *in vitro* and *in vivo* formulation assessments. The analytical methods described in this Chapter were utilised in Chapters 3, 4, 5 and 6 of this thesis.

2.2.1 Materials

Colistin sulphate was obtained from Zhejiang Shenghua Biok Biology Co. (Zhejiang, China; batch number EP5 20061220). Citric acid (anhydrous), sodium hydroxide, disodium hydrogen phosphate dihydrate, derivatisation grade 9-fluorenylmethyl chloroformate (FMOC-Cl), pentanesulphonic acid, erythromycin, polymyxin B and LC/MS grade trifluoroacetic acid (TFA), acetic acid and formic acid (FMCA) were obtained from Sigma-Aldrich (Castle Hill, Australia). HPLC-grade acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF) and acetone were obtained from Merck (Damstadt, Germany). Diazepam was from Alphapharm (Glebe, Australia, batch number 890004-696). European Pharmacopoeial grade azithromycin dihydrate was obtained from Kopran Pty Ltd (Mumbai, India, batch number AZ/P0611851) and clarithyromycin was obtained from Novachem Pty Ltd (Collingwood, Australia). Sep-Pak® solid-phase extraction (SPE) C_{18} cartridges were from Waters (CT, USA).

2.2.2 Apparatus

2.2.2.1 High performance liquid chromatography

The HPLC system (Shimadzu, Kyoto, Japan) comprised of a SIL-10A or CBM-20A controller, two LC-10AD pumps, a SIL-10AD auto-injector fitted with a sample cooling tray held at 4°C, a CTO-2A or CTO-AJ column oven, a DCH-14A degasser, a SPD-10A UV detector or a RF-20A scanning fluorescence detector connected to a multi-instrument data acquisition and data processing system (Class-VP v6.14, Shimadzu, Kyoto, Japan).

2.2.2.2 Liquid chromatography-mass spectrometry (LC-MS)

The chromatography was performed in a liquid chromatography (LC) system (Shimadzu, Japan) comprising of a LC-20AD SP pump, a SIL-10AD auto-injector fitted with a sample cooling tray held at 4°C and a CTO-2A column oven. The MS system, an LCMS-2016 EV single quadrupole mass spectrometer coupled with an electrospray ionization (ESI) interface, was controlled by LCsolution software v3.4 (Shimadzu, Kyoto, Japan). The nitrogen gas flow rate was 1.5 L/min and 10 L/min for both nebuliser and drying gas. The heat block and CDL temperatures were set at 200°C.

2.2.3 HPLC assay for the determination of colistin in formulation samples

2.2.3.1 Method

To separate the major components of colistin, colistin A and B, colistin sulphate dissolved in 50:50 water/ACN and 20 µL was injected onto a C_{18} Phenosphere Next column, (250 mm x 4.60 mm; 5 µm, Phenomonex, CA, USA), at 25°C. The column was protected by a 5.00 x 4.60 mm C_{18} guard column (Phenomonex). The mobile phase of 0.1% (v/v) TFA in Milli-Q® water (phase A) and 0.1% TFA in ACN (phase B) was run at a flow rate of 1 mL/min. The gradient was 26% B between 0 and 2 min, then ramped to 33% B between 2 and 2.5 min and finally decreased back to 26% B between 2.5 and 6 min. Colistin A and B were detected by UV absorbance at a wavelength of 210 nm with peaks eluting at approximately 5.0 and 4.5 min, respectively (Figure 2-7).

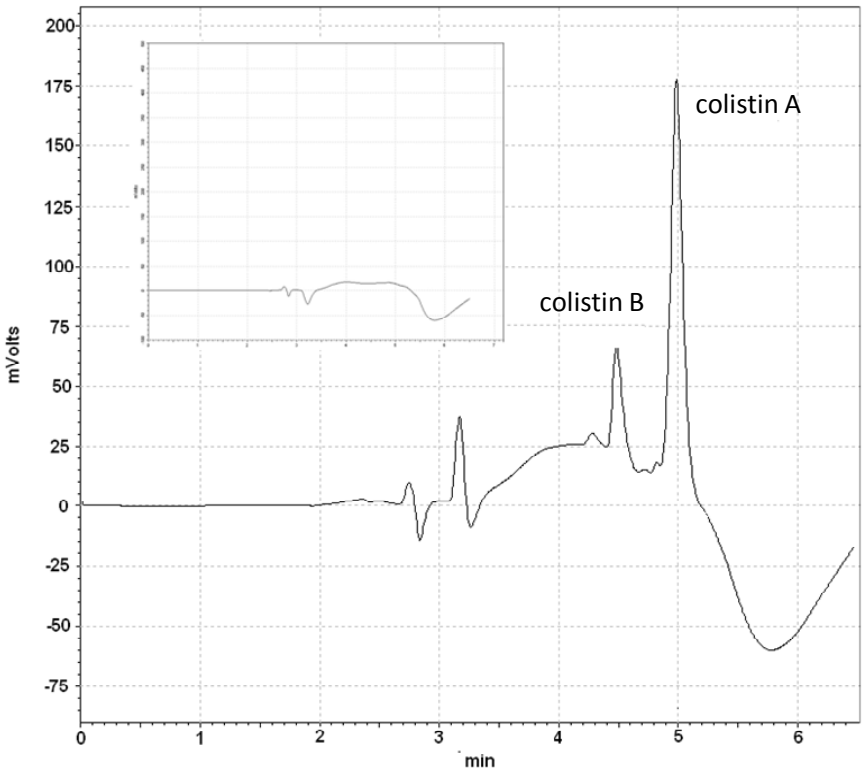


Figure 2-7: Typical chromatogram obtained for 1000 µg/mL colistin with UV detection at 210 nm. The insert represents the chromatogram of a blank sample.

2.2.3.2 Preparation of calibration standards and quality control samples

For aqueous samples, calibration and quality control stock solutions of colistin sulphate (1 mg/mL) were prepared by dissolving colistin sulphate (from independent weighings) in water. Calibration working solutions were prepared from the stock solution by diluting an appropriate volume of stock solution with water in a 1.5 mL polypropylene tube. From the working standards, six calibration standard solutions (50 µg/mL - 1000 µg/mL) were prepared. Quality control (QC) samples containing 150 µg/mL and 900 µg/mL were prepared in triplicate by dilution of the QC stock solution with water. All calibration and QC standards were mixed 1:1 with ACN before being injected onto the HPLC system.

For samples containing phospholipid, calibration and QC stock solutions were prepared by mixing a solution of colistin sulphate (2 mg/mL) prepared in water (independent weighings) with a 10% w/v phospholipid dispersion (containing Dioleoylphosphatidylcholine (DOPC) and in some cases cholesterol) in a 1:1 ratio to produce a stock solution of 1 mg/mL colistin sulphate in 5% w/v DOPC. Calibration standards, ranging between 200 and 2000 µg/mL were prepared by dilution of the appropriate volume of stock with drug-free (i.e. blank) 5% DOPC dispersion. QC samples containing 300 µg/mL and 1500 or 1800 µg/mL were prepared by dilution of the QC stock solution with 5% DOPC. A 200 µL aliquot of each calibration and QC standard was then mixed with 200 µL of ACN and vortex mixed to precipitate the phospholipid. The samples were then centrifuged for 10 min at 9,300 *g* and 20 µL of the supernatant was injected onto the HPLC system. All stock, calibration and QC standards were freshly prepared. Calibration curves were constructed using the summed peak areas of colistin A and B and the corresponding concentrations of colistin sulphate.

2.2.3.3 Sample pre-treatment

For aqueous samples (e.g. filtrate samples containing only colistin and water, Section 4.3.6) an aliquot of sample (200 µL) was diluted with 200 µL ACN and vortex mixed before being loaded directly onto the HPLC system. Six calibration standards (100 - 1000 µg/mL), three replicates of QCs at each level (150 and 900 µg/mL) and one blank containing 50:50 water/ACN, together with unknown samples from experiments conducted in Chapters 4 through 6, were used in each analytical run.

For formulation samples (containing phospholipid, cholesterol, water), an aliquot of ACN (200 µL) was added to an aliquot of unknown sample (200 µL) and vortex mixed to precipitate phospholipid. Samples were centrifuged at 9,300 *g* for 10 min and the supernatant was injected directly onto the HPLC system. At least six calibration standards (200 - 2000 µg/mL), three replicates of QCs at each level (300 and 1500 or 1800 µg/mL) and one blank, together with unknown samples from experiments conducted in Chapters 4 through 6, were used in each analytical run.

2.2.3.4 Method validation

Chromatographic peak areas of colistin A and B were summed to obtain the total peak area for colistin. Linear calibration curves were constructed as described in Section 2.2.3.2. The calibration slope, intercept and co-efficient of determination (r^2) were obtained by unweighted linear least-squares regression analysis of the summated peak areas and corresponding nominal colistin concentrations. Reproducibility was assessed by (1) the intra-day assay with six consecutive analyses of independently prepared QC samples dissolved in 50:50 water:ACN and (2) the inter-day assay with analysis of QC samples on three consecutive days. Accuracy was calculated according to Equation 2.1. Precision was calculated by the coefficient of variation (% CV) of the estimated

concentrations for each QC standard according to Equation 2.2. The limit of quantification (LOQ) was 150 µg/mL for aqueous samples and 300 µg/mL for formulation samples. The performance characteristics of this assay are shown in Table 2-10 and Table 2-11.

$$Accuracy\ \% = \frac{mean\ estimated\ concentration - nominal\ concentration}{nominal\ concentration} \times 100$$

Equation 2.1

$$Precision\ \% = \frac{standard\ deviation\ of\ estimated\ concentations}{mean\ estimated\ concentration} \times 100$$

Equation 2.2

Table 2-10: Summary of standard curve performance parameters for the HPLC-UV assay for colistin.

	Mean	**% CV**	**n**
Slope	149.89	10.77	3
Intercept	8298.8		
r^2	0.99		

Table 2-11: Intra- and inter-day QC samples for the assay of colistin.

		Intra-day (n = 6)		**Inter-day (n = 3)**	
		150 µg/mL	900 µg/mL	150 µg/mL	900 µg/mL
Aqueous samples	**Mean concentration (µg/mL)**	154.5	923.4	157.0	919.3
	Accuracy (%)	3.89	2.60	4.64	1.49
	Precision (%)	3.55	5.76	7.41	11.62
		Intra-day (n = 6)		**Inter-day (n = 3)**	
		300 µg/mL	1800 µg/mL	300 µg/mL	1500 µg/mL
Formulation samples	**Mean concentration (µg/mL)**	307.0	1839.5	329.4	1501.0
	Accuracy (%)	2.47	2.20	9.81	0.06
	Precision (%)	2.63	2.31	14.61	8.19

For intra-day assays, six replicates were assayed. For inter-day assays, three replicates were assayed on each of the three days.

The specificity of the assay was investigated by analysing blank samples of 50:50 water/ACN to check for the presence of interfering peaks. No significant interfering peaks were observed. With a view to using this assay to quantify colistin in the presence of other compounds in solubilisation (Chapter 3) and co-formulation (Chapter 5) studies, samples containing combinations of colistin (500 µg/mL) and diazepam (50 µg/mL) or azithromycin (500 µg/mL) were prepared in 50:50 water/ACN were injected onto the column and the retention time of colistin was monitored. Neither diazepam nor azithromycin interfered with the chromatographic behaviour or detection of colistin A and B.

2.2.3.5 Summary

This simple HPLC assay for the determination of colistin in formulation samples using UV detection is valid based upon the performance data shown in Table 2-8 and Table 2-10. The assay is accurate and reproducible over the relevant concentration range 100 - 1000 µg/mL (aqueous samples) and 200 - 2000 µg/mL (formulation samples). This analytical method has been successfully employed to quantify colistin concentrations in the formulation studies conducted in Chapter 4.

2.2.4 HPLC assay for the determination of diazepam in formulation samples

Diazepam was selected as a model poorly-water soluble drug to probe the solubilising capacity of colistin and CMS solutions in solubilisation studies (Section 3.5.2). A method for the quantification of diazepam was available in the literature[382] and this method was validated for use in solubilisation studies.

2.2.4.1 HPLC Method

For the separation of diazepam, an aliquot (20 µL) of diazepam dissolved in MeOH was injected onto a C_8 Symmetry® HPLC column (3.90 mm × 150 mm; 5 µm, Waters, CT, USA), protected by a C_{18} Symmetry® guard column (3.90 × 20 mm, Waters). The mobile phase, consisting of 50:50 v/v water/MeOH, was eluted isocratically at a flow rate of 1 mL/min. Chromatographic analysis was carried out at 25°C. Diazepam was detected by UV absorbance at a wavelength of 230 nm with the diazepam peak eluting at approximately 4.6 min (Figure 2-8).

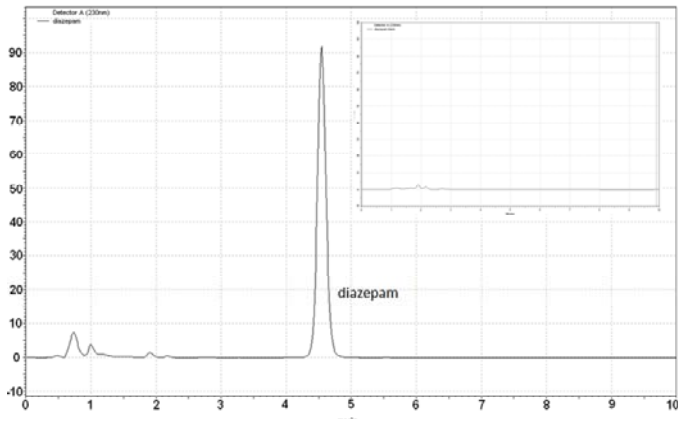


Figure 2-8: Typical chromatogram of 75 µg/mL diazepam obtained with UV detection at 230 nm. The insert represents the chromatogram of a blank sample.

2.2.4.2 Preparation of calibration standards and quality control samples

Calibration and QC stock solutions of diazepam (1 mg/mL) were prepared by dissolving diazepam in MeOH. Six calibration standards, ranging from 10 µg/mL - 150 µg/mL, were prepared by spiking an appropriate volume of calibration stock solution into 90:10 water/MeOH. QC samples containing 20 and 100 µg/mL diazepam were prepared in triplicate by spiking the appropriate volumes QC working stock solution into 90:10 water:MeOH.

2.2.4.3 Sample pre-treatment

Samples (100 µL) of filtrate obtained after filtration of drug suspensions in solubilisation studies (Section 3.4.1.2), calibration and QC samples were mixed with a 100 µL aliquot of MeOH. The purpose of the dilution step was to re-dissolve any diazepam that might have precipitated out of solution following the filtration of diazepam saturated solutions. Samples were vortex mixed and 10 µL was injected onto the HPLC system for analysis.

2.2.4.4 Method validation

Linear calibration curves were constructed as described in Section 2.2.4.2 using the relationship between diazepam peak area and the nominal concentration of calibration standards. Reproducibility was assessed by (1) the intra-day assay with six consecutive analyses of independently prepared QC samples containing dissolved in 50:50 water-MeOH, and (2) the inter-day assay with analysis of QC samples three times consecutively. Accuracy and precision were calculated using Equations 2-1 and 2-2, respectively. The assay LOQ was 20 µg/mL. The performance characteristics, calculated as described in Section 2.2.3.4, are shown in Table 2-12 and Table 2-13.

Table 2-12: Summary of standard curve performance parameters for diazepam HPLC-UV method.

	Mean	% CV	n
Slope	69216.67	0.02	3
Intercept	433507		
r^2	> 0.99		

Table 2-13: Intra- and inter-day QC samples for diazepam HPLC-UV method.

		Intra-day (n = 6)		Inter-day (n = 3)	
		20 µg/mL	100 µg/mL	20 µg/mL	100 µg/mL
	Mean concentration (µg/mL)	17.9	107.5	19.7	103.6
Filtrate	**Accuracy (%)**	10.7	7.5	1.4	3.6
	Precision (%)	6.1	1.6	8.4	2.2

For intra-day assays, six replicates were assayed. For inter-day assays, three replicates were assayed on each of the three days.

The specificity of the assay was investigated by analysing a blank sample of 50:50 water/MeOH to check for the presence of interfering peaks. No interfering peaks were observed. In view of the fact that diazepam was to be quantified in the presence of high concentrations of colistin or CMS in solubilisation studies (Section 3.4.1.2), potential interference due to the presence of CMS and colistin was screened by running samples containing up to 50 mg/mL colistin or CMS and 20 µg/mL diazepam. No interference with the diazepam peak was observed.

2.2.4.5 Summary

A method for the determination of diazepam in formulation solubilisation samples has been validated. The assay was linear over the relevant concentration range of 10 - 150 µg/mL and was successfully used in Chapter 3 for the quantification of diazepam in solubilisation experiments.

2.2.5 HPLC assay for the determination of azithromycin in formulation samples

Azithromycin was selected as a model poorly-water soluble drug for co-formulation with colistin/CMS in the investigation of colistin-combination formulations (Chapter 5). Characterisation of formulation parameters required an analytical method for the determination of azithromycin in formulation samples. Therefore, the objective of this work was to develop and validate an analytical method for the determination of azithromycin in formulation samples. Azithromycin is most commonly detected by electrochemical detection[383-387] or mass spectrometry.[388-391] At the time of assay development, neither of these detection methods were available in our laboratories. Derivatisation with fluorescent compounds can be used to improve the sensitivity to poorly UV absorbing compounds, such as azithromycin,[392] however sample pre-treatment is a time consuming process and therefore a method utilising UV detection of azithromycin was investigated.

2.2.5.1 Method development

A number of methods are available in the literature for the determination of azithromycin in formulation samples using HPLC-UV.[393-398] Ideally, a method utilising an available column, with a run time of less than 10 min for the rapid turnover of high sample numbers should be developed. The first of a number of chromatographic conditions to be investigated was mobile phase containing 0.02 M ammonium acetate using an Alltech Altima C_{18} HPLC column (150 × 4.5 mm, Alltech Associates, IL, USA). Mobile phases containing various proportions of ACN and 0.02 M ammonium acetate with pH values ranging between 3 and 7 were tested for chromatographic performance. Under these conditions, the chromatographic behaviour of azithromycin was found to be highly pH dependent. Ratios of 0.02 M ammonium acetate and ACN ranging from 50:50 to 30:70 were tested on the C_{18} column but did not achieve

acceptable retention or acceptable peak symmetry, with a run time > 10 min and substantial peak tailing was observed. Peak symmetry was difficult to obtain using the C_{18} column, presumably due to the highly lipophilic nature of azithromycin and its subsequent relatively strong interaction with the hydrophobic C_{18} phase.

Given the strong retention of azithromycin to the C_{18} column a C_8 Waters Symmetry® HPLC column (3.90 mm × 150 mm, 5 µm, Waters, CT, USA) was investigated as an alternative column. After screening a number of buffers on the C_8 column, including acetate and phosphate, it was decided that phosphate buffers had the most potential to provide a method with acceptable retention and peak symmetry. The ratios of the buffer and organic component of the mobile phase were varied from 50:50 to 20:80 0.01 M KH_2PO_4 (adjusted to pH 7 with 10% NaOH)/ACN. A pH of 7 was selected for the buffer component due to the instability of azithromycin outside the neutral pH region.[399] The inclusion of MeOH in the organic component of the mobile phase was observed to improve peak shape, and in this case, the complete replacement of ACN with MeOH, using a mobile phase of 20:80 (0.01 M KH_2PO_4 adjusted to pH 7 with 10% NaOH)-MeOH improved peak shape substantially. The robustness of this method to withstand strong buffers of sample matrices was tested by injecting azithromycin dissolved in 0.3 M citric acid onto the system. Increased azithromycin peak tailing was observed when citric acid was injected, however this effect was diminished by increasing the phosphate buffer strength to 0.02 M. Finally, peak broadening was reduced by increasing the column temperature to 40°C.

HPLC methods described in the literature used UV detection at 210 - 215 nm to detect azithromycin. In order to ascertain that the optimal wavelength was selected for maximum azithromycin sensitivity, the UV spectrum of azithromycin dissolved in a mobile phase of 20% 0.02 M KH_2PO_4 (adjusted to pH 7 with 10% NaOH) and 80%

MeOH was obtained, showing a wavelength of maximum UV absorption of 212 nm, as shown in Figure 2-9 below.

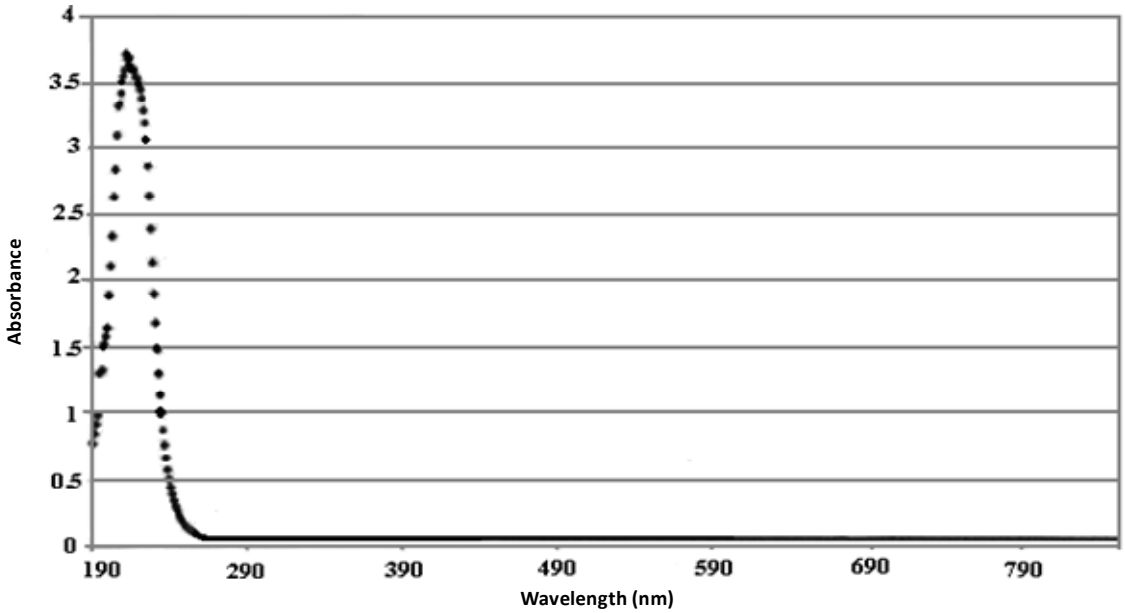


Figure 2-9: UV-spectra of azithromycin dissolved in mobile phase of 20:80 v/v 0.02 M KH_2PO_4/MeOH.

The final conditions selected for the optimal separation of azithromycin consisted of a mobile phase of 20:80 v/v 0.02 M KH_2PO_4 (adjusted to pH 7 with 10% NaOH)/MeOH run through a Waters C_8 Symmetry HPLC column (3.90 mm × 150 mm, 5 µm Waters, CT, USA), protected by a 3.90 × 20 mm C_{18} Symmetry guard column (Waters, CT, USA) at a flow rate of 1 mL/min. Chromatographic analysis was carried out at 40°C. Azithromycin was detected at a wavelength of 212 nm, with the azithromycin peak eluting at 6.3 min (Figure 2-10).

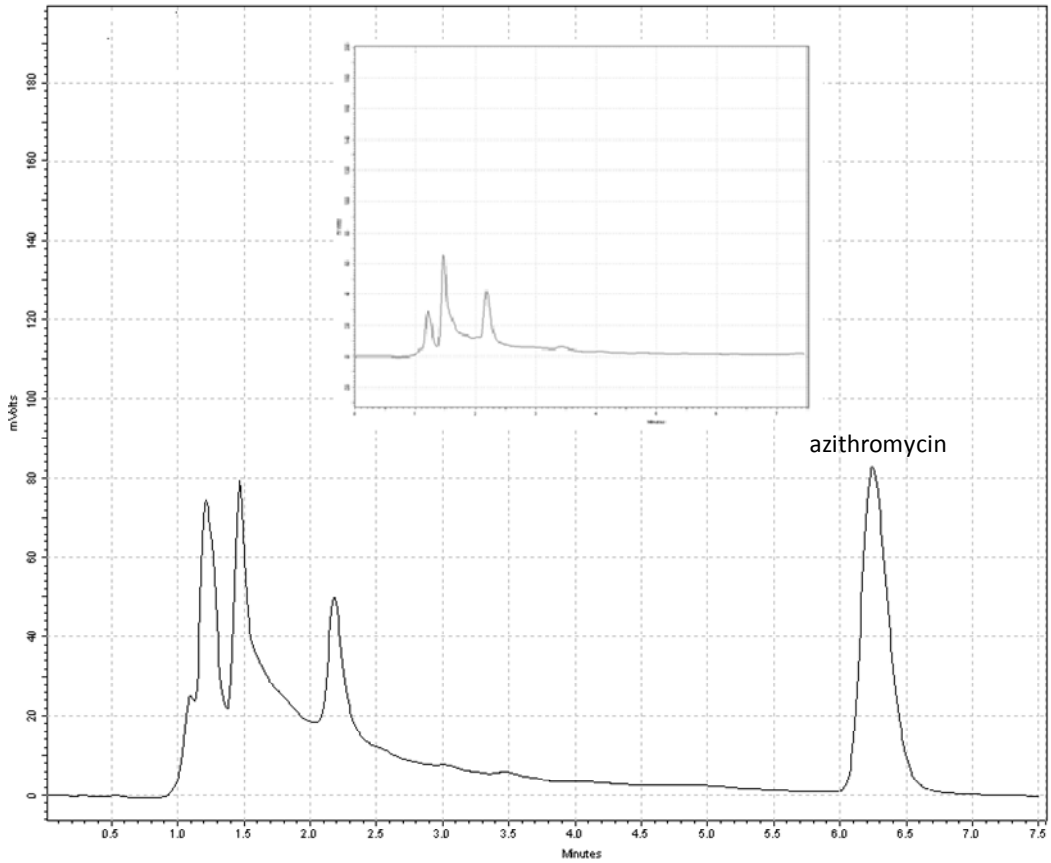


Figure 2-10: Typical chromatogram obtained for 500 µg/mL azithromycin dissolved in a blank aqueous/organic solvent matrix with UV detection at 212 nm. The insert represents the chromatogram of a blank sample.

2.2.5.2 Preparation of calibration and quality control samples

For aqueous samples, calibration and QC stock solutions of azithromycin (4 mg/mL) were prepared by dissolving azithromycin (from independent weighings) in MeOH. Working stock solutions containing 2 mg/mL were prepared by a 1:1 dilution of stock solutions with the appropriate matrix of water or phosphate buffered saline. Calibration and QC standard solutions were prepared by dilution of the respective working stock solution with 50:50 water/MeOH. Six calibration standard solutions ranging between 100 µg/mL - 1000 µg/mL were prepared. QC samples containing 150 µg/mL and 900 µg/mL azithromycin were prepared in triplicate by dilution of the QC stock solution with 50:50 water/MeOH.

Vitamin E acetate-based emulsions were investigated in Chapter 5 as a potential solubilising drug delivery system of azithromycin. For analysis of azithromycin in formulation samples containing vitamin E acetate and/or phospholipid, calibration and QC stock solutions of 20 mg/mL azithromycin were prepared in MeOH from independent weighings. Stock solutions were then diluted 1:1 with water to produce working stock solutions containing 10 mg/mL azithromycin in 50:50 water/MeOH. Working stock solutions were then mixed 1:1 with a DOPC dispersion (and in some cases cholesterol) or DOPC/vitamin E emulsion (containing DOPC) to produce a formulation stock solution of 5 mg/mL azithromycin in 5% w/v DOPC. Calibration standards were prepared by dilution of the appropriate volume of formulation stock solution with blank 5% DOPC dispersion to 1000 µL. The concentrations of calibration standards ranged from 200 µg/mL - 2000 µg/mL. QC samples containing 300 µg/mL and 1800 µg/mL or 200 µg/mL and 1500 µg/mL were prepared by dilution of aliquots of QC formulation stock solutions to 1000 µL with 5% DOPC. All stock, calibration and QC standards were prepared freshly. A 200 µL aliquot of each calibration and QC standard was then mixed with 200 µL of ACN and vortex mixed to precipitate the phospholipid. Insoluble phospholipid was then removed by centrifugation for 10 min at 9,300 *g*. Subsequently, 20 µL of the supernatant was injected onto the HPLC system.

2.2.5.3 Sample pre-treatment

For aqueous samples of unknown azithromycin concentration (e.g. filtrate samples, see Sections 4.3.6 and 5.4.3), a 200 µL aliquot of sample was diluted with 200 µL of MeOH and vortex mixed before 10 µL was injected into the HPLC system. Six calibration standards, (100 - 1000 µg/mL), three replicates of QCs at each level (150 and 900 µg/mL) and one blank containing 50:50 water/MeOH, together with unknown samples from experiments conducted in Chapters 3, 5 and 6 were used in each analytical run.

For formulation samples containing vitamin E and/or phospholipid, 200 µL of sample was mixed with 200 µL of ACN to precipitate the phospholipid. Samples were centrifuged at 9,300 *g* for 10 min and 20 µL supernatant collected was injected directly onto the HPLC system.

2.2.5.4 Method validation

Calibration standards and QC samples were prepared and pre-treated according to Section 2.2.5.2 and Section 2.2.5.3, respectively. Linear calibration curves for azithromycin were constructed using the relationship between azithromycin peak area and the nominal concentration of the calibration standards. Reproducibility was assessed by (1) the intra-day assay with six consecutive analyses of independently prepared QC samples dissolved in 50:50 water/MeOH, and (2) the inter-day assay with analysis of QC samples on three consecutive days. Accuracy and precision were calculated using Equations 2.1 and 2.2, respectively. The LOQ of the assay was 150 µg/mL for filtrate samples and 200 µg/mL for formulation samples. The performance characteristics of this assay, as calculated by equations described in Section 2.3.6.1, are shown in Table 2-14 and 2-15.

Table 2-14: Summary of standard curve performance parameters for azithromycin HPLC-UV method.

	Mean	% CV	n
Slope	532.43	1.93	3
Intercept	14757.33		
r^2	> 0.99		

Table 2-15: Intra- and inter-day quality control samples for azithromycin HPLC-UV method.

		Intra-day (n = 6)		Inter-day (n = 3)	
		150 µg/mL	900 µg/mL	150 µg/mL	900 µg/mL
Filtrate	**Mean concentration (µg/mL)**	152.9	923.4	157.0	919.4
	Accuracy (%)	1.93	2.60	4.64	1.49
	Precision (%)	5.11	5.76	7.41	11.62
		Intra-day (n = 6)		**Inter-day (n = 3)**	
		300 µg/mL	1800 µg/mL	200 µg/mL	1500 µg/mL
Formulation	**Mean concentration (µg/mL)**	307.0	1839.5	229.4	1501.0
	Accuracy (%)	2.47	2.20	14.72	0.06
	Precision (%)	2.63	2.31	14.61	8.19

For intra-day assays, six replicates were assayed. For inter-day assays, three replicates were assayed on each of the three days.

The specificity of the assay was investigated by analysing blank samples of 50:50 water/MeOH and 50:50 water/ACN to check for the presence of interfering peaks. No interfering peaks were observed.

In view of the fact that azithromycin was to be quantified in the presence of high concentrations of colistin or CMS in solubilisation studies (Section 3.4.1.2), and high concentrations of buffer species in formulation studies (Section 5.5.4.1), potential interference due to the presence of CMS, colistin and buffer was assessed by running

samples containing up to 100 mg/mL colistin and CMS, and 0.15 M citric acid with azithromycin spiked at 500 μg/mL. No interference with the azithromycin peak was observed in the presence of CMS or citric acid. However, the presence of high concentrations of colistin caused a tailing peak to elute over the azithromycin peak. In the case of samples containing concentrations of colistin > 25 mg/mL, samples were diluted appropriately to reduce the effect of the colistin peak. In that case, appropriate dilution QC samples were included. Formulation components including phospholipids, cholesterol and vitamin E were also screened, with no interfering peaks observed.

2.2.5.5 Summary

An HPLC-UV method for the determination of azithromycin in formulation samples has been developed and validated. The assay was accurate and reproducible over the relevant concentration ranges required for the quantification of azithromycin in samples obtained from formulation studies. This analytical method was successfully used in Chapters 3, 5 and 6 for the quantification of azithromycin in formulation samples.

2.2.6 HPLC assay for the determination of colistin in biological and stability samples

A previously developed and validated method for the quantification of colistin in biological samples was used for the determination of colistin A and B in formulation samples produced in Chapter 3 (Section 3.4.1.4) and 6 (Section 6.5.2).[134] The method involves a derivitisation of colistin to fluorescent colistin derivatives and utilises fluorescence detection.

2.2.6.1 Method

An Onyx monolithic C_{18} HPLC column (50 mm x 4.6 mm, Phenomenex, CA, USA) was used to separate the fluorescent FMOC derivatives of colistin A and colistin B. All analyses were carried out at 30°C. The mobile phase of 35:39:24 MeOH/THF/water v/v/v was set at a flow rate of 1 mL/min and the run time was 8 min with the FMOC-Cl derivatives of colistin A and colistin B eluting at approximately 6.15 and 5.20 min, respectively, as shown in Figure 2-11. The peak areas of colistin A and colistin B derivatives were summed to calculate the total colistin concentration.

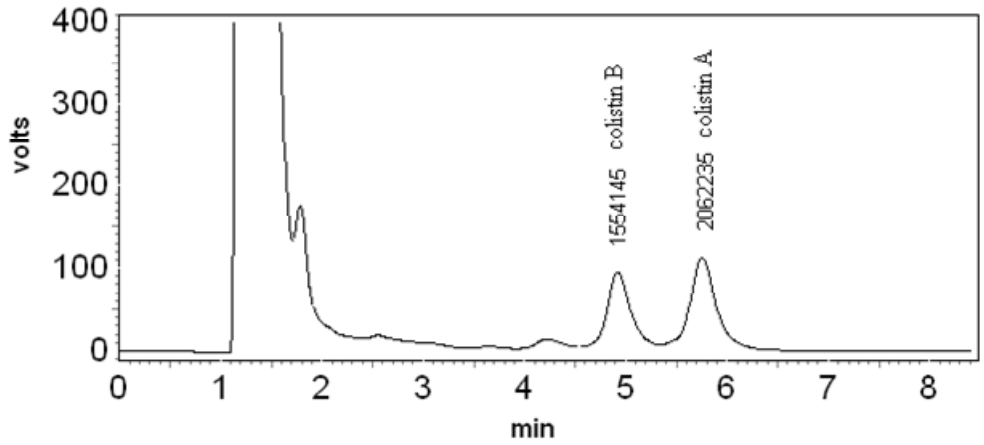


Figure 2-11: Typical chromatogram of colistin FMOC-Cl fluorescent derivatives in a rat plasma matrix.

2.2.6.2 Preparation of calibration and QC samples

Two independently prepared stock solutions containing 1 mg/mL colistin sulphate in water were used to prepare a series of calibration and QC working standards.

Rat plasma samples

Calibration working standards ranging between 1.56 and 200 µg/mL colistin sulphate were prepared by serial dilution of the stock solution with an appropriate volume of water. QC stock solutions were diluted appropriately with water to produce working QC solutions of 2 and 160 µg/mL colistin. A 10 µL aliquot of each calibration or QC working standard was spiked into 190 µL blank rat plasma to produce a calibration curve ranging between 0.078 and 10 µg/mL and QC samples containing 0.1 and 8 µg/mL colistin sulphate. An aliquot (100 µL) of each calibration standard and QC sample in rat plasma was mixed with 100 µL of ACN and vortex mixed. The entire supernatant was transferred to an SPE cartridge to be treated as per Section 2.2.6.3.

Formulation samples

Samples obtained from studies of the stability of CMS in solution (Section 3.4.1.4) and in liposome formulations (Section 4.3.7) contained higher concentrations of colistin than biological samples. A calibration stock solution of 4 mg/mL colistin sulphate was used to prepare working calibration standards. A 50 µL aliquot of stock solution was spiked into 950 µL of 5% DOPC dispersion to produce a calibration standard containing 200 µg/mL colistin sulphate. The calibration curve ranging between 3.125 and 200 µg/mL was prepared by serial dilution of the 200 µg/mL standard with 5% DOPC dispersion. QC samples containing 4 and 200 µg/mL were prepared by spiking stock solutions, or diluted stock solutions, into the 5% DOPC dispersion. A 200 µg/mL aliquot of each calibration standard and QC sample was then mixed with 200 µL ACN

to precipitate phospholipids. All standards were centrifuged for 10 min at 9,300 *g* and 200 µL of supernatant was transferred to an SPE cartridge to be treated as per Section 2.2.6.3.

2.2.6.3 Sample pre-treatment

Rat plasma samples

The fluorescent derivatives of colistin A and B were produced by reacting standards and QC and unknown samples with FMOC-Cl on pre-conditioned SPE cartridges, according to the method described by Li *et al.*[134] The entire sample supernatant was transferred to a SPE cartridge that had been pre-conditioned with 1 mL acetone, 1 mL of MeOH and 1 mL of 10% w/v $NaHCO_3$ buffer (adjusted to pH 10 with 10% (w/v) NaOH). After slowly washing the sample through the SPE cartridge using pH 10 $NaHCO_3$ buffer and subsequently allowing the sample to dry by drawing air through the cartridge using a vacuum, 30 µL of 100 mM FMOC-Cl in ACN was mixed with 80 µL MeOH and was added to the cartridge. Following a 10-min reaction time on the cartridge, 900 µL of acetone was used to elute the FMOC derivatives of colistin A and B. The eluent was collected in a 10-mL polypropylene tube containing 500 µL ACN and 600 µL 0.2 M boric acid. After vortex mixing and equilibrating samples at room temperature for at least 3 h, samples were centrifuged for 10 min at 3,220 *g* and 20 µL of the supernatant was injected onto the HLPC column.

Formulation and stability samples containing CMS

For formulation samples (containing phospholipid) a 200 µL of sample was mixed with 200 µL ACN before vortex mixing and centrifugation at 9,300 *g* for 10 min. A 200 µL aliquot of supernatant was transferred to the SPE cartridge and the sample was treated with FMOC-Cl as described in Section 2.2.6.3. For aqueous stability samples, a 200 µL aliquot of sample was mixed with 200 µL ACN and vortex mixed before loading onto a pre-conditioned SPE cartridge.

2.2.6.4 Validation

Calibration standards and QC samples were prepared as described in Section 2.2.6.2. Linear calibration curves were constructed using the summated peak areas of colistin A and B and the nominal concentrations of colistin. Weighted ($1/y^2$) least squares regression analysis was used to determine the slope, intercept and r^2. Reproducibility was assessed by (1) the intra-day assay with six consecutive analyses of independently prepared QC samples containing colistin sulphate in blank rat plasma and (2) the inter-day assay with analysis of QC samples on three consecutive days. Accuracy and precision were calculated using Equations 2.1 and 2.2, respectively. The LOQ for the assay of plasma samples was 0.1 µg/mL while the LOQ for the assay of formulation samples was 0.2 µg/mL.The performance characteristics of this assay are shown in Tables 2-16 and 2-17. The specificity of the assay was investigated by analysing samples of blank rat plasma or phospholipid dispersion following the sample pre-treatment procedure described in Section 2.2.6.3. No peaks interfering with peaks of colistin A and B were observed.

Table 2-16: Summary of standard curve performance parameters for the colistin HPLC-derivatisation method.

		Mean	% CV	n
Plasma	**Slope**	301586	0.98	3
	Intercept	-23061		
	r^2	> 0.99		
Formulation	**Slope**	25820	6.94	3
	Intercept	-66272		
	r^2	> 0.99		

Table 2-17: Intra- and inter-day quality control samples for the colistin HPLC method using FMOC-Cl derivitisation.

		Intra-day (n = 6)		**Inter-day (n = 3)**	
		0.10 µg/mL	8.0 µg/mL	0.10 µg/mL	8.0 µg/mL
Plasma	**Mean concentration (µg/mL)**	0.10	7.78	0.12	8.19
	Accuracy (%)	1.52	2.76	15.27	2.42
	Precision (%)	6.71	3.19	5.27	1.74
		Intra-day (n = 6)		**Inter-day (n = 3)**	
		0.20 µg/mL	3.0 µg/mL	0.20 µg/mL	3.0 µg/mL
Formulation	**Mean concentration (µg/mL)**	4.01	197.97	4.07	194.07
	Accuracy (%)	0.14	1.02	1.75	2.96
	Precision (%)	1.81	1.03	4.75	2.76

2.2.6.5 Summary

Methods for the determination of colistin at low concentrations in various biological matrices and in formulation samples containing CMS have been validated. The assays were accurate over the relevant concentration ranges. These analytical methods were successfully used for the quantification of colistin in CMS formulation samples (Chapters 3 and 4) and rat plasma (Chapter 6).

2.2.7 LC-MS assay for the determination of colistin in aqueous samples

Samples obtained from filter adsorption studies (Section 4.4.6.3) were to contain up to 100 µg/mL colistin sulphate. Access to an LC-MS method became available for the simple and rapid determination of colistin in aqueous samples, therefore this method was validated for the quantification of colistin in samples from filter adsorption experiments.

2.2.7.1 MS method

The ESI probe was set to positive ion mode and the ionization products of colistin A and colistin B were monitored in selective ion monitoring (SIM) mode at 390.8 *m/z* $[M+3H]^{3+}$ and 386.05 *m/z* $[M+3H]^{3+}$. The ionization product of polymyxin B_2 at 401.80 *m/z* $[M+3H]^{3+}$ was monitored as the internal standard. The Q-array, CDL and interface voltages were set to the tuning conditions and the detector voltage was set at 1.5 kV. The nitrogen gas flow rate was 1.5 L/min and 10 L/min for both nebuliser and drying gas. The heat block and CDL temperatures were set at 200°C. Figure 2-12 depicts the mass spectral analysis of colisitin, showing the main product ions for colistin A and colistin B.

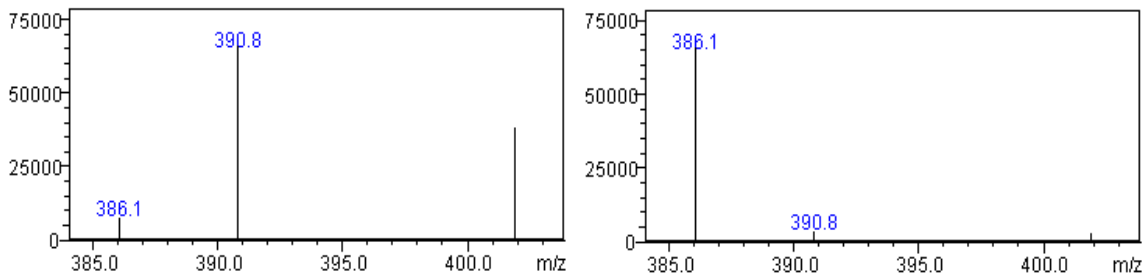


Figure 2-12: MS product ion spectra of colistin A (left) and colistin B (right).

2.2.7.2 LC conditions

A Gemini-NX 5µm 110A column (5 x 2.00 mm, Phenomonex, Lane Cove, Australia) was used to separate colistin A and B. The mobile phase, consisting of 0.1% TFA (Phase A) and 0.1% TFA in ACN (Phase B) was run through the column under a gradient elution program: 5% B between 0 and 6 min, increased to 90% B between 6 and 6.3 min, held at 90% B between 6.3 and 9 min, then finally decreased back to 5% B between 9 and 10 min, at a flow rate of 0.4 mL/min. The column temperature was set at 30°C and the sample cooling tray was held at 4°C. Chromatographic peaks corresponding to colistin A and colistin B eluted at approximately 3.46 and 3.29 min, respectively, with the internal standard peak for polymyxin B_2 eluting at approximately 3.58 min.

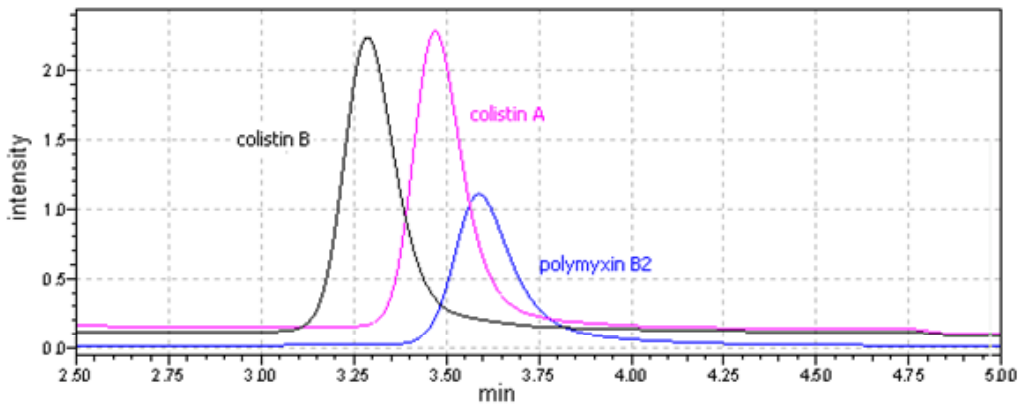


Figure 2-13: Typical SIM mode chromatogram of colistin A, colistin B and polymyxin B_2 (internal standard).

2.2.7.3 Preparation of calibration standards and quality control samples

Calibration standards and QC samples were prepared from the dilution of a 1 mg/mL stock solution of colistin sulphate in water. Calibration standards were prepared between 5 and 100 µg/mL and QC samples were prepared in triplicate at 10 and 90 µg/mL from an independently weighed stock solution. The internal standard working solution containing 60 µg/mL polymyxin B was prepared by dilution of a 1 mg/mL solution in water.

2.2.7.4 Sample pre-treatment

To a 100 µL aliquot of unknown sample, 100 µL of ACN and 100 µL of internal standard solution was added. The sample was vortex mixed and 5 µL was injected directly onto the LC-MS system.

2.2.7.5 Validation

Calibration standards and QC samples were prepared as described in Section 2.2.7.3. Linear calibration curves were constructed based on the summated peak areas of colistin A and colistin B and nominal colistin concentrations. The peak area of the internal standard was monitored for any changes or trends in fluctuating peak areas. The calibration slope, intercept and r^2 were obtained by unweighted linear least-squares regression analysis. Reproducibility was assessed by (1) the intra-day assay with six consecutive analyses of independently prepared QC samples and (2) the inter-day assay with analysis of QC samples on three consecutive days. Accuracy and precision were determined as described in Equations 2.1 and 2.2, respectively. The LOQ of the assay was 10 µg/mL. The performance characteristics of this assay are shown in Table 2-18 and Table 2-19. The selectivity of the assay was investigated by analysing samples of

water containing ACN and the internal standard. No peaks were observed to interfere with colistin A and colistin B peaks.

Table 2-18: Summary of standard curve performance parameters for the colistin LC-MS method.

	Mean	% CV	n
Slope	860150	9.28	3
Intercept	79832		
r^2	> 0.99		

Table 2-19: Intra- and inter-day quality control samples for the colistin LC-MS method.

		Intra-day (n = 6)		Inter-day (n = 3)	
		10 µg/mL	90 µg/mL	10 µg/mL	90 µg/mL
	Mean concentration (µg/mL)	10.98	94.28	11.10	97.77
Filtrate	**Accuracy (%)**	9.81	4.76	11.02	8.63
	Precision (%)	4.17	2.46	12.63	2.58

2.2.7.6 Summary

An LC-MS assay for the determination of colistin in aqueous matrices has been validated. The assay was accurate and reproducible over the appropriate sample concentration range (5 - 100 µg/mL). This analytical method has been successfully used to quantify concentrations of colistin in filter validation experiments conducted in Section 4.3.6.

2.2.8 LC-MS assay for the determination of azithromycin in plasma

2.2.8.1 Method development

The main products of molecular ionisation of azithromycin and clarithromycin were obtained by flow injection analysis of samples containing 5 µg/mL of each compound, operating under full-scan mode covering the range 100 – 1000 *m*/*z*. Ions identified at 375.4 *m*/*z* $[M+ 2H]^{2+}$ and 748.4 *m*/*z* $[M+ H]^{+}$, as shown in Figure 2-14, were determined to be the most abundant products of the molecular ionization of azithromycin and clarithromycin, respectively. Both ESI and atmospheric pressure ionisation methods were tested for relative sensitivity towards the products of azithromycin ionisation, with ESI in positive-ion mode determined to be the most sensitive.

To determine the optimal interface voltage settings for maximum MS detector response, the CDL, Q-array and interface voltages were then systematically varied while monitoring the intensity of main ion products in SIM mode. The tuning conditions were determined to be the optimal conditions for the interface, CDL and Q-array voltages, while the detector voltage was set at 1.5 kV.

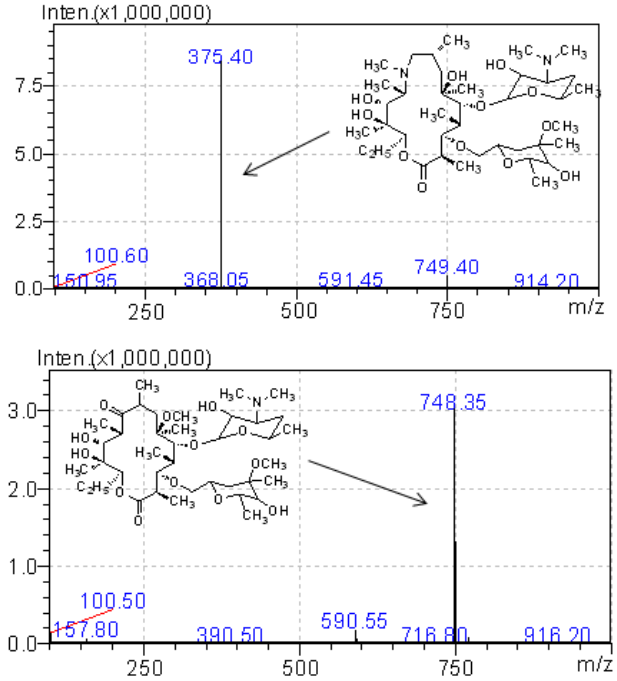


Figure 2-14: MS product ion spectra of azithromycin (top) and clarithromycin (bottom). Arrows indicate main ion in each case.

Given the successful separation of macrolide antibiotics by reversed-phase chromatography utilising mobile phases containing acetate buffer and a combination of MeOH-ACN reported in the literature,[389-391, 400-402] this was considered a logical starting point for the development of a new method for the quantification of azithromycin using LC-MS. The separation of azithromycin was initially investigated using a Gemini-NX 5 µm 110A column (5 x 2.00 mm, Phenomonex, Lane Cove, Australia). Gradient time programs utilising various compositions of 0.05 M ammonium acetate (adjusted to pH 5 with acetic acid) and ACN were explored. The selection of mobile phase pH was guided by previously reported methods.[389, 400, 403] A retention time of approximately 5.15 min with acceptable peak symmetry could be produced using a flow rate of 0.25 mL/min. The gradient elution program used was: held at 30% ACN between 0 and 0.5 min, increased to 50% ACN between 0.5 and 1 min, increased further to 70% ACN between 1 and 2 min, held at 70% ACN between 2 and 6 mins, then returned to 30% between 6 and 8 min. Upon validation of this method, however, it became clear that it could not be

used for the accurate quantification of azithromycin at low concentrations given that calibration curves ranging between 0.125 and 5 µg/mL were not linear (Figure 2-15), with the accuracy of low QC samples exceeding 15%.

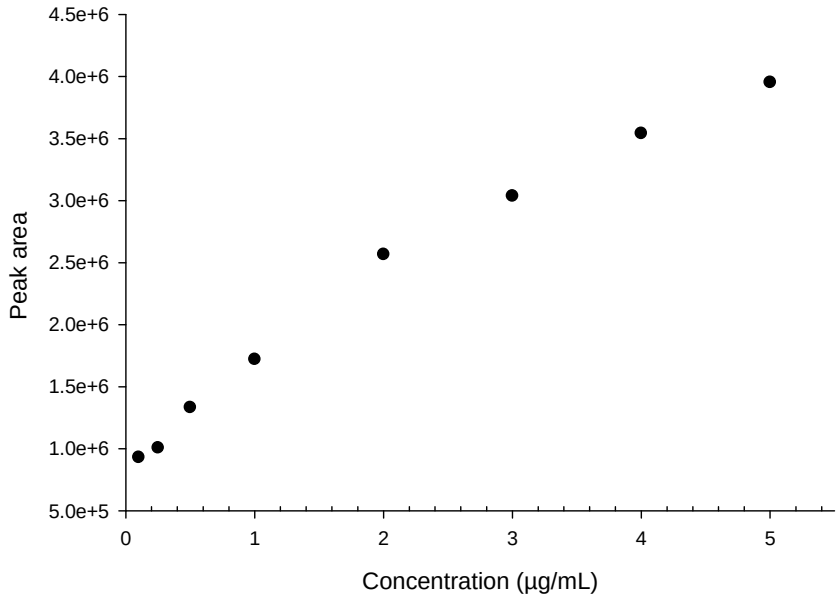


Figure 2-15: Typical calibration curve of LC-MS analysis of azithromycin using Gemini C18 column and ammonium acetate mobile phase under gradient elution.

The availability of a new Synergi Hydro 80Å C_{18} column (50 x 2.0 mm; 4 µm) (Phenomonex, Lane Cove, Australia) allowed for the further development of the azithromycin LC-MS method, utilising different chromatographic conditions. The use of mobile phases containing formic acid has been reported for erythromcyin[404-408] and a number of other macrolide antibiotics,[409-410] and was thus investigated for potential separation of azithromycin and clarithromycin. Due to the world-wide shortage of ACN at the time, MeOH was used alone as the organic component of the mobile phase. Various compositions of 0.1% FMCA v/v in water (Phase A) and 0.1% FMCA v/v in MeOH (Phase B) were tested under gradient elution at a flow rate of 0.25 mL/min. Finally, the following gradient time program produced symmetrical peaks for both azithromycin and clarithromycin: held at 20% B between 0 and 2.74 min, increased to

30% B from 2.75 to 2.99 min; increased to 95% B between 3 and 5 min and returned to 20% B between 5 and 5.5 min, the re-equilbrated at 20% B between 5.5 and 8 min. Azithromycin and clarithromycin eluted at 4.52 and 4.73 min, respectively (Figure 2-16).

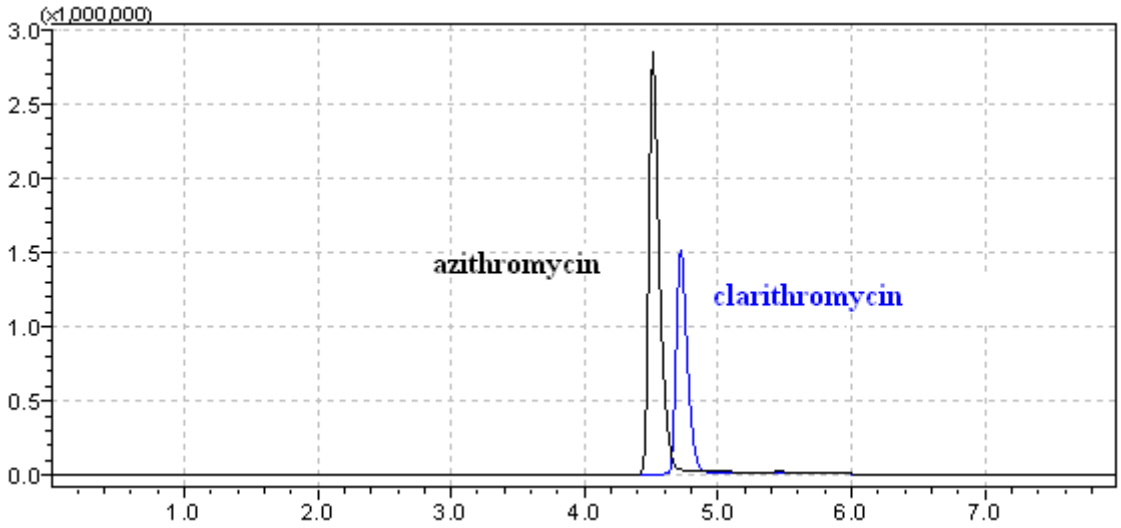


Figure 2-16: SIM mode chromatograms of azithromycin and clarithromycin (internal standard) using the formic acid/MeOH mobile phase run under gradient elution.

2.2.8.2 Preparation of calibration standards and quality control samples

Standard stock solutions containing 1 mg/mL azithromycin were prepared in MeOH. The internal standard stock solution of 2 mg/mL clarithromycin was prepared in dimethyl sulphoxide and was stored at 4°C, protected from light. On the day of analysis, an appropriate volume of the internal standard stock solution was spiked into 100 mL ACN for use in the protein precipitation step. Subsequent sample pretreatment is described in Section 2.2.8.3.

A working calibration stock solution containing 80 µg/mL azithromycin was prepared from the stock solution by dilution into 50:50 water/MeOH. A series of working stock solutions ranging in concentration between 0.313 and 80 µg/mL were then prepared by serial dilution of the 80 µg/mL working stock solution in 50:50 water/MeOH. An

aliquot (10 µL) of each working calibration standard solution was then spiked into 190 µL of plasma and vortex mixed to produce the calibration standards ranging between 0.0156 and 4.0 µg/mL azithromycin. QC samples were prepared from an independently made stock solution of 1 mg/mL azithromycin in MeOH. Working QC solutions containing 0.6, 10 and 60 µg/mL azithromycin were prepared by dilution of the QC stock solution with 50:50 water-MeOH. QC plasma samples containing 0.03, 0.5 and 3 µg/mL azithromycin were prepared (in triplicate) by spiking 10 µL of each QC working solution into 190 µL of blank rat plasma followed by vortex mixing. Subsequent sample pre-treatment is described in Section 2.2.8.3.

2.2.8.3 Sample pre-treatment

To a 100 µL aliquot of an unknown sample, calibration standard or QC sample of plasma, a 900 µL aliquot of ACN (containing 0.05 µg/mL internal standard) was added. The sample was vortex mixed and centrifuged at 9,300 *g* for 10 min. A 900 µL aliquot of the supernatant was transferred to a clean 1.5 mL polypropylene tube and placed at 40°C on a dry-block heater under a continuous stream of nitrogen gas until the sample was completely dry. The dried sample was then reconstituted in 200 µL mobile phase and vortex mixed before centrifugation at 9,300 g for 10 min to remove any insoluble material. A 5 µL sample was injected onto the LC-MS system.

2.2.8.4 Method validation

Calibration standards and QC samples were prepared according to Section 2.2.8.2 and treated according to Section 2.2.8.3. Calibration curves were constructed based on the chromatographic peak area of azithromycin and corresponding nominal azithromycin concentrations, while the internal standard area was monitored for fluctuations or trends in changing peak area. The calibration slope, intercept and r^2 were obtained by

unweighted linear least-squares regression analysis of the peak areas and corresponding nominal azithromycin concentrations. Reproducibility was assessed by (1) the intra-day assay with six consecutive analyses of independently prepared QC samples containing colistin sulphate in water, and (2) the inter-day analysis of the QC samples on three consecutive days. The limit of quantification was 0.05 µg/mL. Accuracy and precision were calculated by Equations 2.1 and 2.2, respectively. The LOQ of the assay was 0.05 µg/mL. The assay performance characteristics of the azithromycin LC-MS method are shown in Table 2-20 and Table 2-21. The selectivity of the assay was examined by analysing blank samples of rat plasma. No peaks interfering with the peaks of azithromycin or clarithromycin were observed.

Table 2-20: Summary of standard curve performance parameters for azithromycin in plasma using the LC-MS method.

	Mean	**% CV**	**n**
Slope	3451114	21.8	3
Intercept	741456	3.57	
r^2	> 0.99		

Table 2-21: Intra- and inter-day quality control samples from the azithromycin LC-MS method

	Intra-day (n = 6)		**Inter-day (n = 3)**	
	0.05 µg/mL	3 µg/mL	0.05 µg/mL	3 µg/mL
Mean concentration (µg/mL)	0.054	2.61	0.052	2.93
Accuracy (%)	8.11	13.00	4.00	2.27
Precision (%)	5.83	5.10	5.27	1.43

2.2.8.5 Summary

A LC-MS assay for the determination of azithromycin in biological samples has been developed and validated. The assay was accurate and reproducible over the sample concentration range of 0.05 - 4 µg/mL. This analytical method has been successfully used to quantify concentrations of azithromycin in biological samples obtained from *in vivo* formulation assessment experiments described in Section 6.5.3.

2.2.9 Conclusion

Methods for the quantification of colistin, azithromycin and diazepam in formulation and biological samples of various matrices have been developed and validated. The assays were accurate and precise over the required concentration ranges. These analytical methods have been utilised to analyse drug concentrations in samples obtained in studies described in Chapters 3 through 6.

Chapter 3: Self-assembly behaviour of colistin and its prodrug colistin methanesulphonate

3.1 Introduction

The self-assembly of pharmacologically active compounds in solution is an interesting, important and frequently understated phenomenon. Aggregation of small drug molecules in an aqueous medium follows the same principles as for classical surfactants:[411] above a critical concentration, attractive forces drive intramolecular associations between the lipophilic regions of the monomers, while hydrogen bonding between the hydrophilic regions and surrounding water molecules facilitates the orientation of hydrophilic regions towards the external aqueous environment (Figure 1-8).[412] The result is the re-organisation of monomers into an energetically favourable arrangement of clustered monomers in which the hydrophobic regions of the monomers are shielded from the polar aqueous external environment.[296]

While the chemical structure of self-assembling small drug molecules can vary greatly, the common structural feature is amphiphilicity.[367, 370, 413-426] Amphiphilicity is often a prerequisite for pharmacological activity, and can be key to the interaction between drug molecules and biological membranes.[411] Both colistin and CMS are structurally amphipathic, by virtue of the octanoic and heptanoic acyl residues and the charged unmasked (colistin) or masked (CMS) diaminobutyric acid residues situated around the heptapeptide cyclic ring and on the tripeptide side chain (Figure 1-2 and Figure 1-3, respectively). The amphipilic structure of polymyxins has been shown to be crucial to the interaction with its biological target, lipid A of lipopolysaccharide (LPS), a major component of Gram-negative bacterial outer membrane.[121, 427] In addition, amphiphilicity has been shown to be crucial to the membrane permeabilising,

bactericidal action of polymyxins against Gram-negative bacteria; structural analogues of polymyxins which lack the hydrophobic fatty acyl region, such as polymyxin B nonapeptide (PMBN, Figure 3-1), are not bactericidal.[428-429]

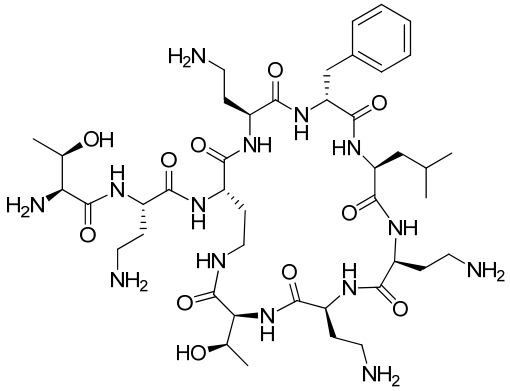


Figure 3-1: PMBN chemical structure.

During the decades in which the clinical use of polymyxin B and colistin fell out of favour, the process of drug development advanced significantly and the regulatory requirements for drug approval became far more rigorous. A full assessment of physicochemical properties is considered a basic expectation of modern-day drug candidates. The recent, urgent re-introduction of the polymyxins into clinical use means that a full physicochemical assessment has not been carried out on this class of antibiotics. More recently, investigations into the chemical and physical properties of polymyxins, and how they interact with biological targets, have been instigated. It has been suggested in the literature that colistin (sulphate),[373] CMS (sodium)[430] and polymyxin B (sulphate)[331, 430] form association colloids in solution, however, no direct evidence has been published to confirm this phenomenon.[373, 431]

The potential for colistin and CMS to self-assemble in solution is of interest from a number of perspectives. As introduced in Section 1.3.6, antibiotic combination therapy is becoming an important approach in the treatment of MDR infections. Micellar solutions formed by self-assembling monomers can dissolve substances of low aqueous

solubility by incorporation of the poorly water-soluble compound into the hydrophobic microenvironment of micelles.[432] If indeed colistin and CMS self-assemble in solution, micellar solutions of colistin and CMS could potentially be exploited as a formulation approach to the co-localisation of colistin and other antibiotics for combination inhalation therapy, by providing micellar solubilisation of poorly water-soluble antibiotics.[239, 433-434]

The formation of self-assembling colloids in solutions of CMS is also of interest from the perspective of CMS formulation stability. As reviewed in Section 1.3.1.2, CMS converts to the pharmacologically active compound, colistin, in aqueous solutions[71, 107, 362] by cleavage of all five of the sulphomethyl groups.[71] The presence of appreciable amounts of colistin in solutions of CMS for administration to patients can be of serious consequence because colistin is significantly more toxic than CMS.[111, 435] Therefore, it is important to understand the rate of conversion of CMS to colistin in aqueous environments from the perspective of the stability of pharmaceutical formulations.

In the past, the lack of a specific HPLC method to quantify colistin has limited investigations into the stability of CMS in pharmaceutical formulations. Early studies relied on the use of microbiological methods to quantify colistin, which required extended periods of incubation at 37°C during which significant conversion of CMS to colistin was likely to occur. Only recently has the stability of CMS in solution been investigated using a specific HPLC method.[107] At low CMS concentrations, substantial conversion to colistin has been shown to occur both *in vitro* and *in vivo*. Li *et al*. showed that up to 80% of the original CMS present was converted to colistin in 48 h when 100 μg/mL CMS in water was incubated at 37°C.[107] Similar conversion was observed in 24 h at when the same CMS concentration was incubated in pH 7.4 phosphate buffer and plasma at 37°C.[107] Bergen *et al*. showed that 17% of CMS was

converted to colistin over 4 h when 32 μg/mL CMS was incubated microbiological broth at 37°C. In contrast, solutions containing high concentrations of CMS (> 70 mg/mL) have been shown to be stable with respect to colistin formation for one year or more.[362] Based on the previous studies of CMS stability in solution, it appears that the conversion of CMS to colistin is dependent on CMS concentration.

Given that previous studies of CMS stability were conducted across the concentration range 8 μg/mL to 70 mg/mL in different experimental media (phosphate buffer, water, saline, plasma, urine, microbiological broth), and under different conditions of temperature, the speculation of the dependence of CMS stability on CMS concentration cannot be absolutely confirmed based on these studies. Matrix and temperature effects obscure the comparison of CMS conversion to colistin at the different concentrations studied.[71, 107, 362] The rate of colistin formation in solutions of CMS across a wide concentration range has not yet been investigated under standardised conditions of temperature and sample matrix, and therefore further investigation of the apparent concentration-dependent stability is warranted. CMS appears to be more stable at high concentrations, however, the mechanism underlying stabilisation is currently not understood. For a number of drugs, including penicillins,[296, 366, 368, 370, 421, 436] enhanced drug stability in solution at high concentrations can be attributed to the aggregation of monomers into self-assembling colloids at the critical micelle concentration. The self-assembly of CMS in solution offers a potential explanation for the apparent concentration-dependent stability of CMS.[362]

In summary, characterisation of the bulk solution behaviour of colistin and CMS is of interest from a formulation perspective. Firstly, micellar solutions of colistin and CMS may be used to solubilise poorly water-soluble drugs, which may be an important approach to the co-formulation of antibiotics with colistin in combination inhalation therapy (Chapter 5). Secondly, the formation of micelles in solutions of CMS may provide an explanation for the solution stability of CMS at high concentrations (Chapter 3). Finally, the self-assembly of CMS and colistin in solution may affect the interactions of these peptide antibiotics with colloidal based drug delivery systems (Chapter 4).

3.2 Hypotheses and Aims

Bearing in mind the aforementioned voids in the literature, the hypotheses that will be addressed in this chapter are that:

1. colistin and CMS self-assemble into association colloids in aqueous solution;
2. poorly water-soluble drugs can be solubilised in solutions of colistin/CMS, above a critical concentration; and that
3. the stability of CMS in solution is concentration-dependent and this is attributable to a self-assembly phenomenon.

In addressing these hypotheses, the aims of this chapter are to:

1. characterise the physicochemical properties of colistin/CMS solutions
2. assess the solubilising capacity of colistin/CMS for poorly water-soluble drugs, and
3. measure the conversion of CMS to colistin over a range of CMS concentrations.

3.3 Materials

Triton® X-100, polymyxin B nonapeptide hydrochloride (PMBN), sodium chloride, potassium chloride, sodium phosphate, sodium hydroxide, potassium dihydrogen phosphate and 9-fluorenylmethyl chloroformate (FMOC-Cl) were obtained from Sigma (St Louis, MO). Colistin sulphate was from Zhejiang Shenghua Biok Biology Co. Ltd (Huzhou, China [EP5 200612202]), CMS sodium was from Alpharma (Copenhagen, Denmark [batch numbers A1680552 and A1680557]), azithromycin dihydrate EP was obtained from Kopran Pty Ltd (Mumbai, India [batch number AZ/P0611851]). Diazepam was from Alphapharm (Glebe, Australia [batch number 890004-696]). Water was purified using a Milli-Q® water purification system (Millipore Corp., Bedford, MA). All analytical reagents were of HPLC grade and all chemicals were used as received. Solutions of CMS sodium and colistin sulphate were freshly prepared.

3.4 Methods

3.4.1 Characterisation of colistin and CMS self-assembly

3.4.1.1 Surface tension

Surface tension measurements were carried out on solutions of colistin sulphate and CMS sodium (batch number A1680552) using a NIMA DST 9005 automatic tensiometer (Nima Technology Ltd, UK) fitted with a platinum Du Nouy ring (ring diameter 20.6 mm, wire diameter 500 μm). Solutions were allowed to equilibrate to 25°C before the ring was immersed 5 mm below the surface of the solution. The tensiometer was automatically retracted from the solution at a rate of 5 mm/min. Between measurements, the ring was rinsed with Milli-Q® water and ethanol, and further cleaned by flaming. All solutions were prepared and measured in triplicate.

3.4.1.2 Solubilisation

To probe the capacity of colistin and CMS (batch number A1680557) micelles to solubilise poorly-water soluble drugs, the solubility of diazepam and azithromycin (Figure 3-2) (aqueous solubility ~ 50 μg/mL and < 200 μg/mL, respectively)[437-438] in solutions of CMS sodium and colistin sulphate was investigated, and compared to that in water and pH 7.4 phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4). Excess diazepam or azithromycin was added to the dissolution medium (CMS or colistin solutions) contained in 50 mL polypropylene tubes and was incubated at 25°C in a shaking water bath (100 shakes/min). Each saturated solution was prepared in three replicates. Samples were collected at various time points over 48 h to determine the time at which no further dissolution of poorly water-soluble drug occurred, deemed to be the equilibrium solubility. At a time when the equilibrium had been established, drug suspensions were filtered through a 0.2 μm regenerated cellulose Minisart® RC4 filter (Sartorius, Hanover, Germany) to remove any undissolved drug.

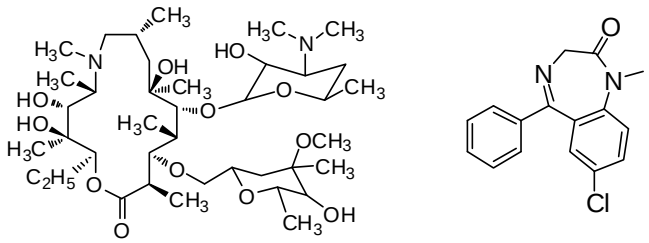


Figure 3-2: Chemical structures of azithromycin (left) and diazepam (right).

Filter adsorption studies were carried out to eliminate the possibility of non-specific adsorption of diazepam and azithromycin to the filter membranes. Solutions of diazepam (saturated solution, approximately 50 μg/mL) or azithromycin (70 μg/mL) in water were pushed through a regenerated cellulose syringe filter. The process was repeated with three separate syringe filters for each drug. The filtrate was collected in

fractions of approximately 100 μL and the concentrations of diazepam and azithromycin in each fraction were analysed by HPLC (Section 2.2.4 and Section 2.2.3, respectively). Filtrate samples were diluted 1:1 with MeOH after collection to prevent drug precipitation.

Upon solubilisation of a poorly water-soluble compound, the structure of the micelles can be perturbed and this may be detected as a change in micelle size or shape.[439] Therefore, DLS was used to measure the size of colistin micelles in which azithromycin was solubilised. Size measurements were carried out on a 40 mg/mL (28.5 mM) solution of colistin sulphate saturated with azithromycin using the Zetasizer Nano ZS. The size of micelles in the solubilising solution (free of azithromycin) was also measured for comparison.

3.4.1.3 Dynamic light scattering

A critical parameter to be determined in surfactant characterisation is the CMC. Dynamic light scattering (DLS) is a technique that has been used since the 1940s to measure particle size and can also be used in the determination of the CMC of self-assembling species.[421, 440-441] DLS relies on the Faraday-Tyndall effect, whereby colloidal particles scatter light in a visible cone, which can be detected at an angle to the plane of observation.[442] In the absence of self-assembling aggregates, the intensity of back-scattered light is comparable to that of the solvent. The intensity of back-scattered light increases when aggregates form, and increases linearly thereafter as the concentration of aggregates increases. Except at concentrations close to the CMC, scattering by micelles dominates over the scattering by monomers. The concentration at which back-scattered light begins to increase above that of baseline solvent scattering can be identified as the CMC.[419] Older instruments used in studies of micelle formation by small drug molecules typically detect light scattered at an angle of 90°.[368, 421] At this

detection angle, light scattering plots enabling identification of CMCs could be produced, however, no information regarding size of the micellar aggregates could be obtained.[418-419, 421]

The DLS instrument used in this study (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK)[443-444] detects light scattered at 173°. This angle of detection confers substantial sensitivity, enabling information regarding the size of colloidal aggregates to be derived, even for particles in the low nanometer range. This Zetasizer Nano ZS uses a 4 mW He-Ne laser (λ = 632.8 nm) as a light source. The viscosity and refractive index of water at 25°C, 0.8937 cP and 1.333,[445] respectively, were used for all measurements. The thermostatted sample chamber was set to 25°C. Solutions of colistin sulphate, CMS sodium, PMBN and Triton® X-100 were prepared in Milli-Q® water by dilution from stock solutions and were filtered through a 0.02 μm Anotop® filter (Whatman, Maidstone, UK) to remove dust prior to DLS measurements in a low-volume polystyrene cuvette (Sigma, Castle Hill, Australia). Triton® X-100 served as a 'positive control' to show that the CMC of a well characterised surfactant could be identified using this method. The CMC of Triton® X-100 is reported in the literature as 0.25 - 0.3 mM.[446-447] Polymyxin B nonapeptide (PMBN, Figure 3-1), which is similar in structure to colistin but lacks the fatty acyl chain and is therefore not amphiphilic, was used as a 'negative control' to demonstrate the scattering pattern of a compound not expected to form colloidal aggregates in solution.

Size measurements were carried out at attenuator setting 11 and the measurement range was limited between 0.3 and 100 nm. At the end of each measurement, the sample scattering (derived count rate) in kilo counts per second (kcps), was recorded. The scattering of the solvent ($S173_{solvent}$) was subtracted from that of each sample (S173) and was plotted as a function of concentration. Each sample was prepared in triplicate

and was measured three times to ensure reproducibility. Fluctuation in the correlation functions were monitored throughout measurements to ensure similarity between signals, and therefore the reliability of DLS data.

Size information was obtained from the correlation function by two means on the Zetasizer Nano ZS using the software package DTS Nano v5.10. For z-average diameters, a single exponential was fitted to the correlation function to yield a cumulant analysis. To obtain the distribution of particle sizes, a multiple exponential was fitted to the correlation function.

3.4.1.4 Stability of CMS in solution

The degradation of CMS in solution over time was assessed by measuring the extent of colistin formation in solutions of CMS sodium by HPLC.[134] Solutions of CMS sodium were prepared at 0.1, 1, 10 and 100 mg/mL (0.052, 0.52, 5.2 and 52 mM, respectively) in 0.9% (154 mM) NaCl. A 50 mL aliquot of each solution was dispensed into 50 mL polypropylene tubes and stored in the dark at 4°C or 25°C for 120 h (n = 3 for each concentration at each temperature). Samples (1 mL) were removed from each tube at 0, 2, 4, 8, 12, 24, 48 and 120 h and stored at -20°C (for no longer than 1 week[448]) pending analysis for colistin concentration by a validated HPLC assay (Section 2.2.6).

It is not possible to monitor the disappearance of CMS in solution directly because currently there is no available HPLC method to specifically quantify CMS at high concentrations. Therefore, the appearance of colistin, rather than the disappearance of CMS, was used to measure the rate of CMS conversion to colistin. The amount of CMS remaining was calculated as the difference between the initial CMS concentration and the measured colistin concentration.

Concentrations of colistin (sulphate) formed, were then used to calculate (on a molar basis) the equivalent amount of colistin base, according to Equation 3.1 below. The amount of CMS remaining in solution was calculated (on a molar basis) from colistin base concentrations, according to Equation 3-2:

$$[colistin\ base] = \frac{M_W\ (colistin\ base)}{M_W\ (colistin\ sulphate)} \times [colistin\ sulphate] \qquad \textbf{Equation 3.1}$$

$$\%\ CMS\ remaining =$$

$$intial\ [CMS] - [colistin\ base] \times \frac{M_W CMS}{M_W colistin\ base} \qquad \textbf{Equation 3.2}$$

The average M_W of 1,163 Da was used for colistin (base) (colistin A M_W = 1,170 Da and colistin B M_W = 1,156 Da) and 1,408 Da was used for colistin sulphate. The average molecular weight of CMS (sodium) was set at 1,743 Da (CMS A M_W = 1,750 Da and CMS B M_W = 1,736 Da) for the conversion calculation. The degradation kinetics of CMS was explored by plotting data according to the general rate law equations: concentration of CMS remaining versus time (zero order kinetics), the log of CMS concentration versus time (first order kinetics) and the reciprocal of CMS concentration versus time (second order kinetics). The linearity of data plotted according to the general kinetic rate order laws was examined.

3.4.1.5 Data analysis

Statistical analysis was conducted using SPSS for Windows v17 (SPSS Inc., Chicago, USA).

3.5 Results

3.5.1 Surface tension

Figure 3-3 represents the change in surface tension of colistin and CMS solutions with increasing concentrations. The surface tension measurements clearly show that both colistin and CMS are surface active, reducing the surface tension to approximately 35-40 mN/m at 10 mM, however, a definitive CMC could not be deduced from these data.

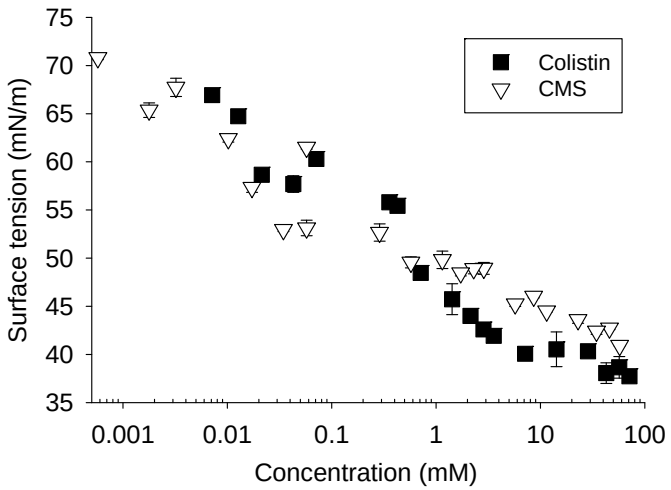


Figure 3-3: Surface tension of aqueous solutions of colistin and CMS (n = 3).

3.5.2 Solubilisation of diazepam and azithromycin by colistin and CMS micellar solutions

Figure 3-4 shows the results of the filter validation experiment conducted to determine the extent of adsorption of drugs used in the solubilisation studies to the membranes of the filters used to remove insoluble drug particles. It can be seen that very little azithromycin or diazepam adsorbed to the regenerated cellulose filters, validating their use in solubilisation studies.

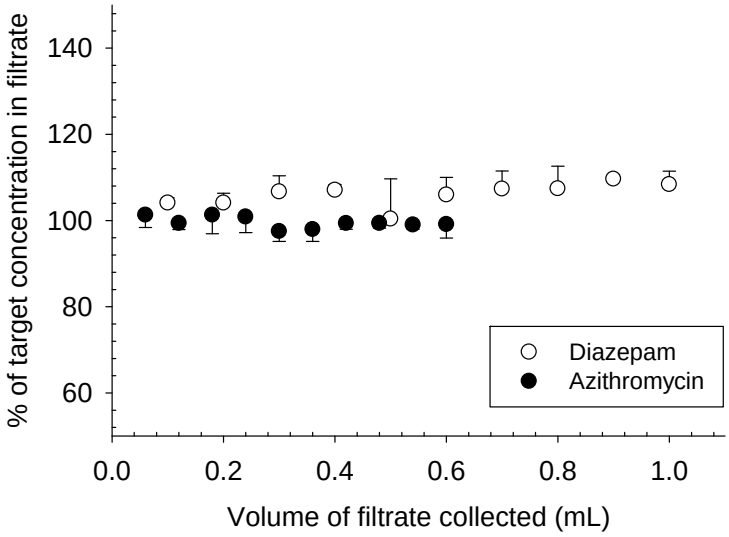


Figure 3-4: Concentration of azithromycin and diazepam in the filtrate collected, expressed as % of target concentrations, after filtering solutions of each drug (70 µg/mL and 50 µg/mL, respectively) through regenerated cellulose filters (n = 3).

Figure 3-5 shows that the solubility of the poorly water-soluble drug diazepam was enhanced with increasing concentrations colistin and CMS in solution. A one-way repeated measures ANOVA with a Tukey post-hoc comparison revealed that the concentration of diazepam solubilised by colistin and CMS solutions was significantly higher than that solubilised by the control solution ($p < 0.05$). A significant linear trend (increasing) between peptide concentration and the concentration of diazepam solubilised was also identified by the statistical analysis.

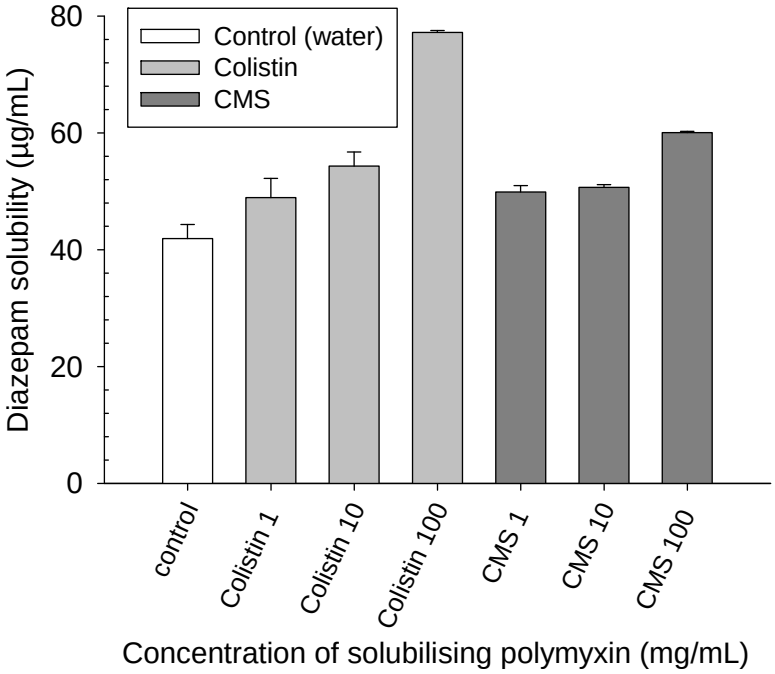


Figure 3-5: Solubilisation of diazepam in colistin and CMS solutions.

The data presented in Figure 3-6 show the capacity of colistin and CMS solutions to solubilise azithromycin, an antibiotic with limited aqueous solubility. The solubility of azithromycin increased approximately linearly with colistin or CMS concentrations above the respective CMCs identified by DLS measurements (Section 3.5.3). A one-way repeated measures ANOVA with a Tukey post-hoc test showed that the concentration of azithromycin solubilised by colistin and CMS solutions was significantly higher than that solubilised by both water and buffer control solutions ($p <$

0.05). A significant increasing linear trend was identified between peptide concentration and the concentration of azithromycin solubilised.

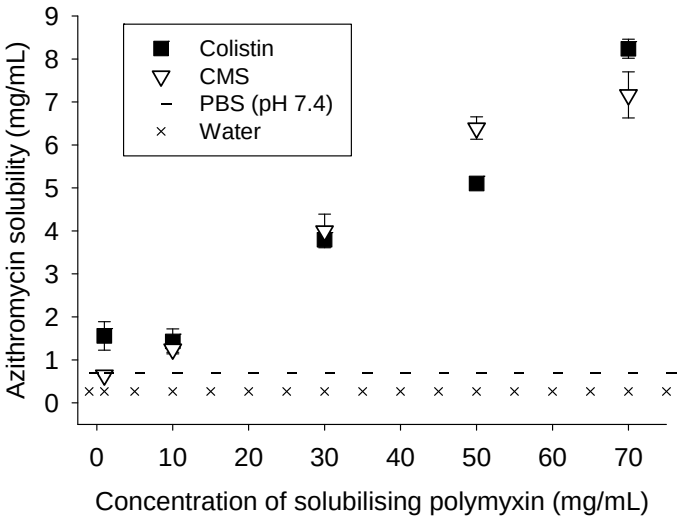


Figure 3-6: Solubilisation of azithromycin in colistin and CMS solutions. The broken bold line represents the solubility of azithromycin in phosphate buffered saline. The cross hashed markers indicate the aqueous solubility of azithromycin in the absence of any buffer species.

Size measurements of the z-average diameter of colistin micelles with or without solubilised azithromycin revealed that colistin micelles containing azithromycin were significantly larger than micelles free of azithromycin (Figure 3-7). Interestingly, empty colistin micelles appear to be more monodisperse than those containing solubilised azithromycin, as indicated by the broader particle size distribution.

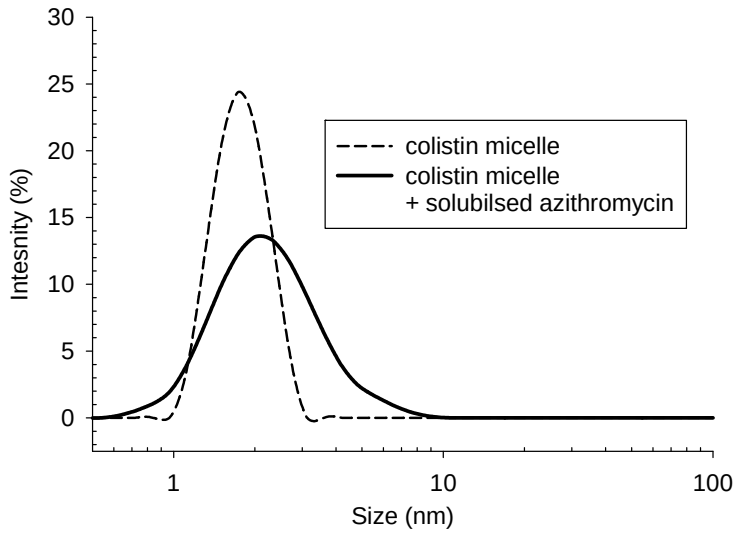


Figure 3-7: Particle size distribution for colistin micelles (broken line) and colistin micelles containing solubilised azithromycin (continuous line). Data are expressed as the mean of three separate determinations.

3.5.3 Dynamic light scattering

The light scattering plot of Triton® X-100 shown in (Figure 3-8) demonstrates that the CMC of a well characterised surfactant, known to form micelles at 0.25 - 0.3 mM, [446-447] could be clearly identified from the inflection in the scattering pattern obtained using the DLS method. This plot serves as a 'positive control' for the validity of the DLS method to identify surfactant CMC.

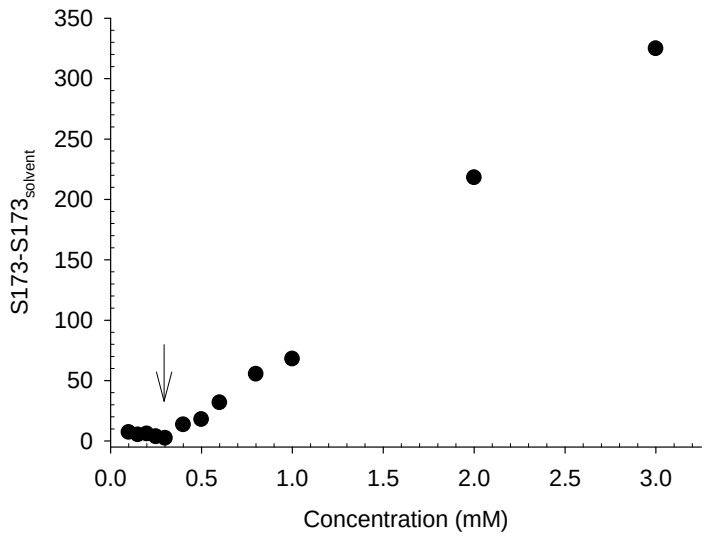


Figure 3-8: Dynamic light scattering of aqueous solutions of Triton® X-100 versus concentration. The scattered light is expressed as the intensity of light scattered at an angle of detection of 173° (S173) minus the intensity of light scattered by the solvent (S173solvent). The arrow indicates the point of inflection identified as the CMC.

Figure 3-9 shows the intensity of light scattered by solutions of CMS sodium, colistin sulphate and PMBN, as a function of concentration. Inflections in the scattering patterns are observed for colistin and CMS at approximately 2.1 and 6.1 mg/mL, respectively, where the scattering pattern increases above that of the solvent approximately linearly with increasing concentration. These scattering patterns indicate the formation of self-assembling structures in solution. Conventionally, the point of inflection is identified as the CMC.[296] Solutions of PMBN up to 20 mg/mL showed negligible scattering above that of the solvent, water (Figure 3-9).

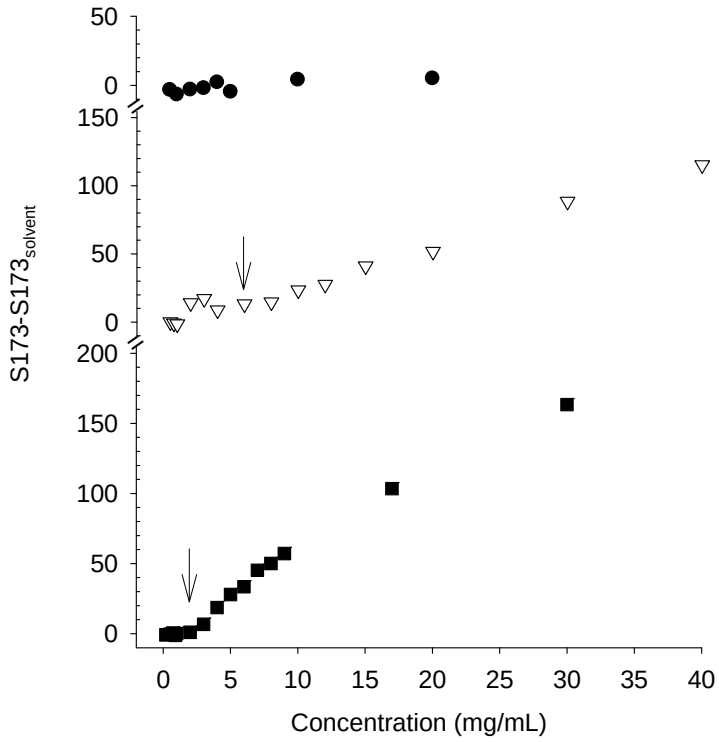


Figure 3-9: Dynamic light scattering of aqueous solutions of colistin sulphate, CMS sodium and PMBN versus concentration of the respective species. The dynamic light scattering is expressed as the intensity of light scattered at 173° (S173) for solutions of the species minus the intensity of light scattered by the solvent ($S173_{solvent}$). Arrows indicate points of inflection (CMC).

Particle size distributions obtained from size measurements of solutions containing colistin, CMS or Triton® X-100 micelles are shown in Figure 3-10. Colistin and CMS micelles had approximately comparable z-average particle size diameters of 2.07± 0.30 and 1.98 ± 0.36 nm (mean ± SD), respectively, at concentrations above the CMC. Triton® X-100 micelles, not surprisingly, had a much larger particle size with a z-average of 8.17 ± 0.59 nm. Z-average sizes measured with increasing concentration of surfactant (Figure 3-11) indicated no change in micelle size with increasing concentration for colistin, CMS and Triton® X-100 solutions. No reliable size data could be obtained from DLS measurements of PMBN solutions up to 20 mg/mL, consistent with a lack of self-assembly.

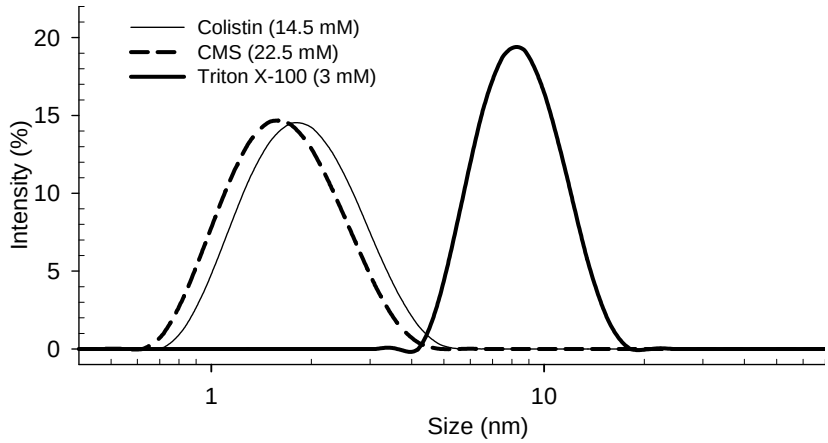


Figure 3-10: Size distribution profiles for colistin, CMS and Triton® X-100 micelles.

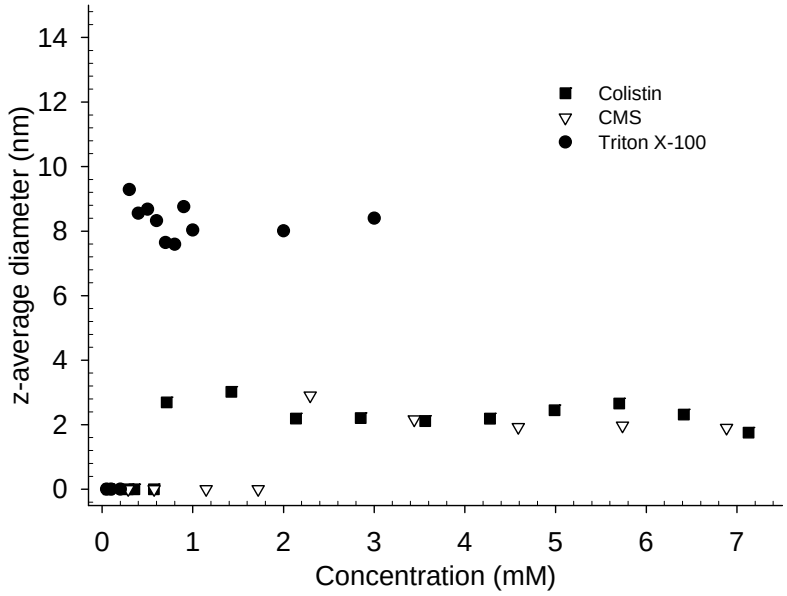


Figure 3-11: The z-average diameters of colistin, CMS and Triton® X-100 micelles plotted as a function of concentration.

3.5.4 Stability of CMS

Figure 3-12 shows the stability of CMS presented as the percentage of CMS remaining over 120 h at various initial concentrations of CMS. Rapid conversion of CMS to colistin occurred in the CMS solution with an initial concentration of 0.1 mg/mL. Slower conversion to colistin occurred in the CMS solution with an initial concentration of 1 mg/mL, while degradation of CMS at 10 and 100 mg/mL was negligible.

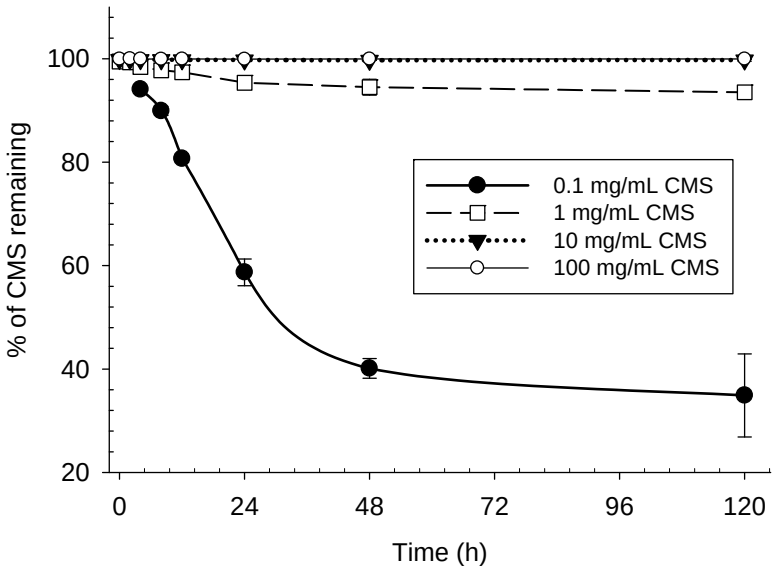


Figure 3-12: Stability of CMS at increasing concentrations in 0.9% saline at 25°C, presented as the percentage of CMS remaining versus time (n = 3). The percentage (± SD) of CMS remaining was determined as the difference between the measured molar colistin concentration and the initial molar CMS concentration.

Figure 3-13 shows the concentrations of colistin measured in CMS solutions, which were used to calculate the percentages of CMS remaining in solution (Figure 3-12). A two-way ANOVA showed that there was a significant main effect of CMS concentration on the rate of colistin formation in CMS solutions. Interestingly, the ANOVA showed significant main effects of both time and CMS concentration on the rate of colistin formation in 0.1 and 1 mg/mL CMS solutions ($p < 0.01$). For 10 and 100 mg/mL CMS solutions, on the other hand, there was no significant effect of time on the rate colistin formation ($p > 0.05$).

The higher concentrations of colistin identified at the initial time point in the 10 and 100 mg/mL CMS solutions, compared to that present in 0.1 and 1 mg/mL solutions, may be explained by the trace levels of colistin (completely unsulphomethylated) present in the CMS dry powder material. The levels of colistin observed in the raw material are comparable to that reported in a previous study.[362]

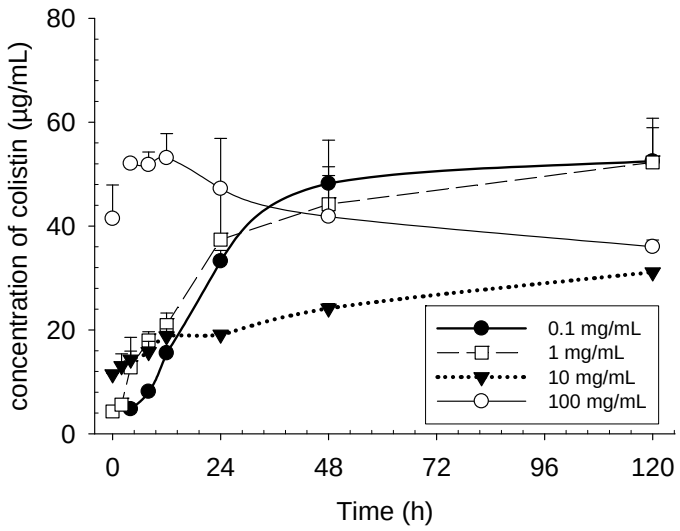


Figure 3-13: The formation of colistin is solutions of CMS over time at increasing concentrations of CMS in 0.9% saline (n = 3). Lines are intended to guide the eye.

3.6 Discussion

There have been a limited number of reports suggesting the self-assembly of colistin in aqueous solution. While studying the interaction of colistin with phospholipids using two probes that fluoresce in a hydrophobic environment (e.g. within the interior of a micelle), Mestres *et al*. found that both probes exhibited moderate fluorescence intensity when incubated in colistin solutions in the absence of phospholipids.[373] It was concluded from this observation that the fluorescence indicated the existence of micelles or aggregates of colistin. However, neither the fluorescence data nor the colistin concentrations at which these experiments were conducted were reported. In another study, the interactions between polymyxin B and liposomes were investigated. The authors conveyed that they had identified a CMC for polymyxin B at 6% w/v (equivalent to 60 mg/mL), however these data and the methods used to determine the CMC were not reported.[431]

The physicochemical characterisation of CMS is complicated by the possibility that rapid conversion to colistin occurs at low concentrations of CMS (< 10 mg/mL), changing the surface active behaviour of the solutions with time. The identification of the CMC from surface tension measurements in this system is complicated by the aforementioned polydisperse nature of colistin and CMS structures, and potentially trace concentrations of other structurally related surface active compounds (Section 1.3.1.1). Nevertheless, the interfacial activity of the colistin amphiphiles is also evident in the surface tension measurements shown in Figure 3-3. For compounds exhibiting atypical self-assembly behaviour, such as the bile salts, surface tension measurements may fail to identify a precise CMC.[449] The interfacial tension for colistin solutions above the CMC (approximately 40 dynes/cm) was comparable to that observed for other cationic octyl-chain surfactants.[450]

Poorly-water soluble drugs can be solubilised by the hydrophobic interior of micelles and the amount of drug solubilised generally increases linearly with increasing surfactant concentration above the CMC.[434, 451] While a statistically significant amount of diazepam was solubilised by colistin and CMS solutions at concentrations well above respective CMCs (Figure 3-6), the magnitude of the solubilisation effect was minimal. At the highest concentration of colistin studied, the solubility of diazepam was increased by only 35 μg/mL, not even doubling the aqueous solubility of 50 - 60 μg/mL.[437]

A linear relationship was shown between the amount of azithromycin solubilised and the concentration of colistin and CMS solutions above the respective CMCs (Figure 3-6). Both colistin and CMS solutions solubilised azithromycin to a much greater extent than diazepam, increasing the solubility of azithromycin by up to 55 times the measured aqueous solubility at 100 mg/mL (42 mM) colistin. That colistin and CMS micelles can solubilise diazepam and azithromycin strongly suggests the association of the fatty acid tails of colistin and CMS, providing a hydrophobic solubilising microenvironment within the micelle interior. The structural conformation of the polymyxins in solution has been examined in NMR studies. In solution, the polymyxins have been shown to exhibit structural flexibility and may change conformation according to the surrounding local environment.[19] Colistin has been shown to possess an envelope-like bend conformation in the cyclic ring structure, which separates the hydrophobic and hydrophilic amino acid residues, conferring structural amphiphilicity to the molecule[427, 430, 452] It is therefore possible, that the tendency of colistin to micellise in solution is not only attributed to the presence and association of the fatty acyl tail; the hydrophobic residues situated around the ring structure may contribute to the forces driving the self-assembly of colistin and CMS.

That azithromycin is more extensively solubilised than diazepam could be a reflection of the relative lipophilicity of these two compounds; diazepam is very poorly water-soluble, while azithromycin would be considered of intermediate water solubility. Moreover, the micelle interior, comprising of small fatty acyl chains, may not be lipophillic enough to extensively solubilise diazepam. An ion-pair interaction between ionised azithromycin (pK_{a1} 8.74, pK_{a2} 9.45) and anionic CMS within micelles was considered as a possible explanation for the solubilisation of azithromycin in CMS; however, this kind of interaction cannot explain the solubilisation of azithromycin in colistin solutions.

In the present study, DLS measurements have established the existence of aggregates in solutions of colistin and CMS, but not those of the non-amphiphilic PMBN. The slight deviation from linear scattering intensity *versus* concentration for both colistin and CMS (Figure 3-9) is typical of charged compounds and has been observed in light scattering studies of other charged, micelle-forming drug molecules, specifically methadone, chlorpromazine, promethazine, trifluoperazine and some penicillins.[413, 421, 453-454]

The inflection points in the curves shown in Figure 3-9 indicate a CMC for colistin at 2.1 mg/mL and for CMS at 6.1 mg/m. Pristovek *et al*. used NMR to deduce the structural conformation of polymyxin B and CMS in solution.[430] They observed concentration-dependent chemical shifts in the NMR spectra of solutions of polymyxin B and CMS between 1 and 5 mM, and suggested that this was due to the aggregation of the peptides. This is consistent with the CMCs determined by DLS in the present study. Lawrence *et al*. reported a substantially higher value for the CMC of polymyxin B of 60 mg/mL.[431] However, the methodology used to arrive at that CMC was not explicitly stated, nor was any experimental data presented in support of that claim.[431]

The potential for batch-to-batch variation in the ratios of colistin A:colistin B, and CMS A: CMS B in colistin and CMS raw materials should be kept in mind when interpreting any physicochemical data from studies of colistin or CMS. As demonstrated in Section 2.1.2.5, the colistin batch used in the present studies contained a slightly greater proportion of colistin A than colistin B. Theoretically colistin A, possessing an octanoyl fatty acyl chain, should have a greater tendency towards micellisation than colistin B which possesses a slightly shorter heptanoyl fatty acyl chain.[296] Due to the different contributions to hydrophobic interactions of the different acyl chain lengths, the CMC of colistin obtained from other batches or alternative manufacturers may vary slightly according to the proportions of the octanoyl and heptanoyl fatty acyl tails. This should be kept in mind when discussing the results from the present study in the context of results reported by other authors.

CMS micelles are apparently slightly smaller than colistin micelles as shown in the particle size distributions in Figure 3-10. CMS is a larger molecule because of the bulkier sulphomethylated head group. However, it is not unreasonable to expect colistin sulphate to form slightly larger micelles than CMS sodium by virtue of the divalent sulphate counter ion, which would have a greater capacity to reduce head group electrostatic repulsion, allowing closer packing of monomers and a greater number of monomers per micelle.[296] Other membrane permeabilising peptides have been observed to form micelles within a similar size range to those reported in this study;[455] for example, gramicidin, a membrane-permeabilising peptide comprising 15 amino acids has been shown to form micelles of approximately 3.1 nm.[411, 456]

The size of colistin and CMS micelles remained the same with increasing concentration (Figure 5). Based on this observation, it is likely that colistin and CMS micelles follow a ‘closed association’ model, having a discrete number of monomers per micelle, rather

than associating following the 'open' or 'step-wise' growth model.[296] Polymyxin B has been shown to exhibit flexibility in the hydrophobic tail region,[452] which is a structural prerequisite for the closed association model.[369]

The formation of colistin in CMS pharmaceutical formulations is of concern because colistin is much more toxic than CMS (LD_{50} values: colistin 217.7 mg/kg, CMS 5.43 mg/kg).[435] Recently, the death of a cystic fibrosis patient following the inhalation of a solution of CMS was purportedly due to the formation of colistin in the CMS formulation prior to use,[457] however, the direct link to stability in that case has been questioned.[362] Nevertheless, the instability of CMS at low concentrations has potential to impact on the clinical use of CMS. While CMS for parenteral use is presented in freeze dried form, it is reconstituted and diluted substantially in intravenous fluids prior to administration, which has been shown to accelerate the conversion of CMS to colistin.[362]

Organisation of drug molecules into self-assembling colloids has been shown to affect rates of drug degradation,[370-372] and has been proposed as the mechanism for the increased stability of CMS at high concentrations.[362] The rate of colistin formation was observed to markedly decrease in the concentration range 1 - 10 mg/mL CMS. The CMC of CMS (6.1 mg/mL), as measured by DLS (Figure 3-9), was identified to occur precisely within that concentration range. This provides strong evidence that the stability of CMS at high (> 10 mg/mL) concentrations is attributable to a self-assembly phenomenon.

Theoretically, it is possible that micellisation of CMS affords some protection of the labile sulphomethyl groups from hydrolytic cleavage. Assuming that micellisation is largely driven by attractions between the fatty acid tails of CMS, the anionic,

susceptible sulphomethyl groups would be orientated towards the exterior surface of the micelle. The activity of water at micelle surfaces has been shown to be different from that in the bulk;[458] high concentrations of counter ions immobilised in the Stern layer can cause the depletion and reduced mobility of water at the micellar interface.[459] As a result, the sulphomethyl groups of monomers dispersed in the bulk may be greater than that of sulphomethyl groups which are oriented towards a micellar surface. It is also possible that arrangement into a micellar structure could result in the steric shielding of sulphomethyl groups from collision with hydrolytic reactants. Steric hindrance of even one sulphomethyl group would prevent the formation of colistin, the species quantified in this study of CMS stability.

Compared to solutions containing 0.1 and 1 mg/mL CMS, which contained very little colistin at the initial time point, solutions of 10 and 100 mg/mL CMS contained a relatively high concentration of free colistin at the initial testing time point (Figure 3-12). This is likely to be attributed to the presence of trace amounts of underivitised colistin in the raw material CMS powder used in these studies. Solutions containing high concentrations of CMS therefore contain relatively high concentrations of free colistin at the initial time point. Despite the higher concentration of colistin present at the initial time point in the 100 mg/mL CMS solution, no significant change in colistin concentration of this solution was observed to occur over the course of the stability study. On the other hand, a small increase in colistin concentration was observed over 120 h in the solution containing 10 mg/mL CMS. The 10 mg/mL CMS concentration corresponds to two times the CMC. Formation of colistin at CMS concentrations above the CMC can be explained by the fact that monomers aggregating within a micelle are in dynamic state[296] and will constantly distribute between micellar aggregates and the

bulk. CMS conversion to colistin is expected to occur for CMS monomers existing in the bulk at any given time.

It does not appear that the degradation kinetics of CMS can be neatly categorised as following any one particular kinetic rate order. This is perhaps not surprising, given the highly complex chemistry of CMS. The CMS molecule possesses of five sulphomethylated amino residues, all of which have the potential to be cleaved to produce a free amino group. Assuming that cleavage of the five sulphomethyl groups is not a step-wise process and occurs in a random order, there could be up to 32 (2^5) different chemical species arising from the hydrolysis of CMS present at any one time.[107] Therefore, the kinetics of conversion of CMS to colistin are likely to be extremely complex. Adding a further layer of complexity to the elucidation of CMS degradation kinetics is the self-assembly phenomenon, which introduces the possibility that different rate order processes occur above and below the CMC. In an attempt to simplify the kinetics of CMS hydrolysis, McMillan *et al*.[363] studied the hydrolysis of a model compound, n-butylamino methanesulphonic acid, possessing only one methanesulphonate group. In that study, NMR, infra-red spectroscopy and electrophoresis were used to demonstrate that the degradation kinetics of even one amino methanesulphonate group are highly complex and cannot be easily elucidated.[363]

Unfortunately, no analytical methods are currently available for the quantification of the numerous, individual partially sulphomethylated derivatives. All methods require that partially sulphomethylated derivatives be converted to colistin for quantification of CMS. Therefore, partial derivatives of colistin could not be measured and intermediates possessing one or more sulphomethyl derivatives could not be differentiated in this study.

3.7 Conclusion

The self-association of the polymyxin antibiotic peptides, colistin and CMS, has been demonstrated in aqueous solution using DLS. CMCs for colistin and CMS have been identified at 2.1 and 6.1 mg/mL, respectively. The CMC identified for CMS correlated well with the concentration range of 1 - 10 mg/mL in which CMS was observed to become more stable in solution. The formation of micelles of CMS in aqueous solutions is therefore proposed to be the mechanism for the greater stability of CMS observed at high concentrations.

Although the CMC of colistin and CMS could not be identified by the measurement of surface tension, solubilisation studies (which correlated well with stability and DLS data) showed that colistin and CMS micelles have the ability to solubilise poorly water-soluble drugs. The solubilising capacity of colistin and CMS, however, did not appear to be substantial enough to be useful from a drug delivery perspective. In order to solubilise 8 mg/mL azithromycin, solutions of 100 mg/mL colistin or CMS would need to be administered to patients by nebulisation. This is in excess of the concentrations currently used in inhalation solutions and is unlikely to be feasible because high concentrations of colistin and CMS can cause irritation to airways.[167, 460]

Given that the solubilisation approach is limited for the formulation of colistin or CMS combination antibiotic therapies, alternative approaches to the co-formulation of colistin and a second poorly water-soluble antibiotic need to be investigated. It is believed that greater co-localisation of poorly water-soluble antibiotics could be achieved by co-encapsulation of colistin into a colloidal drug delivery system, and this is the subject of investigation in subsequent chapters of this thesis.

Chapter 4: Investigation of the interactions of colistin and CMS with liposomes

4.1 Introduction

4.1.1 Colistin and CMS formulations for pulmonary delivery

During the period between the 1950s and 1980s, in which the clinical use of colistin remained somewhat dormant,[87] significant advances were made in the rigour and scrutiny of drug registration and approval processes.[461] Recently, the increasing prevalence of multi-drug resistance amongst Gram-negative bacteria has forced re-introduction of colistin back into clinical use.[1] The early clinical use of colistin was based on less stringent regulatory requirements, which did not require the consideration of important pharmacokinetic and pharmacodynamic concepts (Section 1.3.3) in the design of optimal dosing schedules. The current clinical use of colistin has not been optimised in the context of modern drug development strategies which work towards maximising efficacy while minimising antibiotic resistance.[1]

For the treatment of pulmonary infections caused by MDR Gram-negative bacteria, colistin is commonly administered via inhalation in the form of its pro-drug, CMS.[183] Until recently, the absence of widely available CMS formulations designed specifically for inhalation forced the 'off-label' pulmonary administration of products intended only for intravenous (IV) delivery.[167-168, 189, 192] Though, CMS IV products are not approved for pulmonary use.[170-172] For pulmonary delivery, IV products containing only lyophilised CMS, such as Coly-Mycin M Parenteral (JHP Pharmaceuticals, MI, USA), Colistin Link® Injection (Link Medical Products, Mosman, Australia), and Colomycin® Injection (Forest Laboratories, Kent, UK), are reconstituted and subsequently administered into the lungs via nebulisation or instillation.[162-163, 462] Only recently has

the European Medicines Agency approved inhalational use of Promixin® (Profile Pharma, West Sussex, UK). Intended specifically for pulmonary delivery, this product contains lyophilised CMS, but no other excipients to optimise delivery to the respiratory tract.[190]

As reviewed in Section 1.3.3, recent pharmacodynamic evidence draws attention to the risk of colistin-resistance associated with the use of colistin monotherapy.[156, 230] Combination antibiotic therapy may be a more appropriate approach to the clinical use of colistin with regards to both the efficacy of colistin and preventing the development of colistin-resistance. This warrants investigations into colistin combination formulations designed specifically for inhalation, with the capability to co-localise colistin and a second antibiotic agent within the lungs.[1]

A number of antibiotic candidates for colistin combination therapy have the potential to provide more effective antibiotic therapy than colistin monotherapy (Section 1.3.6). Many of these combination therapy candidates will differ vastly from colistin with respect to physicochemical and biopharmaceutical properties, including aqueous solubility, log P, and biodistribution following delivery to the lungs.[273] Therefore, in the investigation of a suitable approach to the formulation of colistin combinations as inhalable formulations, an advanced drug delivery system capable of accommodating these differences should be considered. Liposomes represent one such drug delivery system.

4.1.2 Liposomes

Liposomes are a drug delivery system with the potential to provide improved colistin formulations for inhalation. Consisting of one or more bilayers of self-assembled phospholipids, liposomes are vesicles that form when certain lamellar-phase

phospholipids are dispersed in excess water above the phase transition temperature (T_m) of the phospholipid.[315, 463] Liposomes are a useful drug delivery system because they are able to encapsulate drugs of a wide range of physicochemical properties. The lipidic phospholipid bilayer encloses within it an aqueous core in which water soluble drugs may be encapsulated, while lipophilic drugs can associate with the lipidic region of the phospholipid bilayer.[341] Amphiphilic drugs may occupy the interface between the lipid bilayer and internal and/or external aqueous media.

Liposomes provide a further benefit for drug incorporation in addition to their capacity to incorporate both water soluble and poorly-water soluble drugs; while associated with or encapsulated within the liposome, the encapsulated drug displays the pharmacokinetic behaviour of the liposome.[325] Therefore, liposomes have the capacity to modify the pharmacokinetics and biodistribution of the encapsulated or associated materials.[323, 464] These key features make liposomes an attractive delivery system for the reformulation of colistin or its prodrug, CMS. Moreover, liposomes represent a drug delivery system that can combine colistin or CMS with a second antibiotic. In view of this potential application of liposomes, the next sections review the use of liposomes in pulmonary drug delivery, drug-liposome interactions and the necessary characterisation aspects of liposomes.

4.1.2.1 Liposomes for pulmonary delivery in the treatment of respiratory infections

Since the first description of liposomes in 1965,[315] and the discovery of their ability to entrap solutes as a drug delivery carrier in the early 1970s,[465-466] liposomes have advanced to become one of the most successful drug delivery platforms on the market.[467] While the commercial success of liposomes is exemplified mainly by products for IV use,[349-350] there is increasing interest in the delivery of liposomes directly to the respiratory tract for the treatment of airway disesases.[468 286, 318] The utility

of liposomes for pulmonary delivery is now being realised; antibiotic liposome formulations, in particular, are emerging as efficacious therapies for the treatment of pulmonary infections.[469] For example, liposome formulations of ciprofloxacin (ARD-3100 [Aradigm Corp]), tobramycin (AX-TOBRA™ [Axentis Pharma]) and amikacin (Arikace™ [Transave]) have shown promise in clinical trial investigations.[343-346] Drug-loaded liposomes for pulmonary delivery have advantages over and above administration of the free drug form. Such advantages include confinement of the drug within the lungs,[470] decreased extent of systemic drug absorption[470] and provision of a slow drug release within the lungs, resulting in a sustained local pharmacological effect.[470-475]

4.1.2.2 Liposome composition and drug loading

For targeted pulmonary delivery of encapsulated materials, the selection of liposomal phospholipid composition is critical as lipid composition may affect drug release rate and hence residence time of drug within the lungs.[283] Moreover, the phospholipid composition is critical because it can determine the nature of the associations between the encapsulated drug and its liposome carrier. Phospholipid composition can therefore bears on the drug loading capacity. Drug loading refers to the amount of drug that can be encapsulated into a liposome and is usually expressed as a ratio of the drug encapsulated:amount of lipid used in the formulation, on a molar basis (i.e. drug : lipid mol ratio). The amount of drug associated with a liposome carrier may also be described by the EE, defined as the proportion of the drug associated with the liposome, as a fraction or percentage of the drug added in the process of manufacture. Drug loading and EE are essential formulation characteristics to be measured before proceeding to *in vitro* and *in vivo* studies of the behaviour of the liposomal formulation of a drug. Low drug loading requires high doses of lipid to be administered in order to achieve an

adequate dose of drug, while low EE means that most of the drug is acting as unencapsulated drug. Therefore, reasonably high drug:lipid ratios, and/or EE, is usually required for the liposomal carrier to be of therapeutic benefit.[295]

4.1.2.3 Drug loading techniques and the nature of the drug-liposome interaction

The nature of the interaction between a drug and its liposomal carrier can greatly affect the *in vivo* properties of the liposome formulation, and can be heavily influenced by the drug loading technique employed.[340, 476] Drug loading techniques can be classified as passive or active (also known as 'remote').[477] Passive loading techniques rely on the non-specific interactions between the drug and lipidic liposome components, or upon the encapsulation of the drug within the interior aqueous volume. Such techniques usually result in low EE and therefore require the removal of free drug after manufacture by techniques such as dialysis, ultracentrifugation and washing or chromatographic column separation.[478] Remote-loading techniques, such as that used in the preparation of the liposomal formulation of doxorubicin DOXIL®, involves the generation of a transmembrane electrochemical gradient to drive drug(s) into the liposomal aqueous interior.[349, 479] Remote-loading can achieve very high drug:lipid ratios.[341] The details of remote-loading methods are described in Chapter 5.

Passive encapsulation techniques are generally used to encapsulate drugs, due to their physicochemical properties, are not suitable for remote-loading. Water-soluble drugs that are not solubilised by the lipids of the bilayer and do not interact with the bilayer to any great extent become encapsulated predominantly within the aqueous core of the liposome (Figure 4-1); for example, amikacin in the commercial liposome formulations MiKasome® and Arikace™.[480] EE in the case of water-soluble drugs depends on the volume of water encapsulated, which in turn depends on the liposome size and the lipid concentration.[335] The EE achieved by this technique is variable between drug

compounds and is difficult to predict solely on the basis of drug chemical structure.[481] The drug:lipid ratio of water soluble drugs may be improved by increasing the concentration of drug in the solution used to disperse the lipid, increasing the concentration of lipid used and or by increasing liposome size.[482]

An alternative basis for passive encapsulation is to harness non-specific interactions between the drug and phospholipid bilayer. As drug physicochemical properties are inherent, passive encapsulation techniques rely on the strategic selection of phospholipids and lipidic modifiers to manipulate and maximise the interactions of the drug with the liposomal bilayer (Figure 4-1). For example, electrostatic interactions may be harnessed by encapsulation of charged drug species into liposomes composed of oppositely charged phospholipids. Where the encapsulation of drug results from electrostatic interactions, the availability of electrostatic binding sites and the density of the charge at the liposome surface will limit the amount of drug associated with the liposome.[335] Lipophilic drugs with limited aqueous solubility can reside within the lipidic region of the phospholipid bilayer (Figure 4-1).[483] In this case, the capacity of the lipid bilayer to solubilise a poorly-water soluble drug depends on the solubility of the drug in the chosen lipid or lipid mixture, the amount of lipid used and the state of the phospholipids in the bilayer (i.e. gel or crystalline).[335]

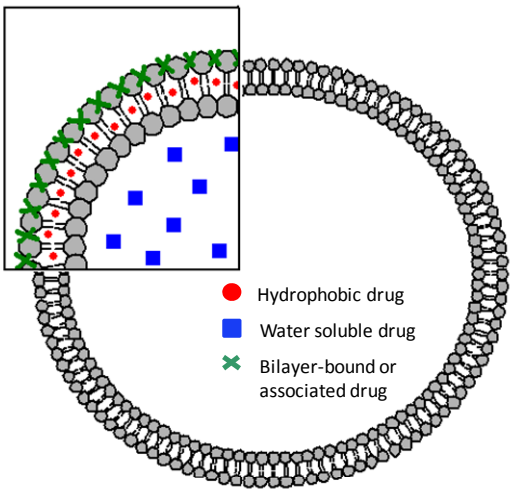


Figure 4-1: Schematic representation of the possible locations of liposome-associated drug. Modified from Maurer *et al*.[341]

Information regarding the location of the drug within the liposome carrier and the nature of the drug-liposome interaction can be obtained by a variety of methods including fourier transform infrared spectroscopy,[484] fluorescence spectroscopy,[485-487] NMR[488-489] and cryo-transmission electron microscopy (cryo-TEM). Other methods, such as atomic force microscopy,[486] differential scanning calorimetry,[488] and UV spectroscopy[487] have been successfully used to identify the effect of the drug on the integrity and fluidity of the phospholipid bilayer. Isothermal microcalorimetry (ITC) is a technique reported in the literature to be very useful to deduce the thermodynamic parameters that govern interactions between drug and phospholipids in liposome bilayers.[373, 485, 490]

4.1.2.4 Methods of manufacture

Though several methods of liposome manufacture are available, the most commonly used method on the laboratory scale is the hydration of a dry lipid film[491] (hereafter referred to as the 'dry film method'). The dry film method involves dissolution of the lipid and other hydrophobic components in an organic solvent, removal of the organic solvent by rotary evaporation, with subsequent hydration of the lipid film at a

temperature above T_m using aqueous solution containing hydrophilic drug, buffers, tonicity agents or other additives as required.

Liposomes can also be prepared by detergent removal,[492] reverse phase evaporation,[493] solvent injection[494] and freeze drying of lipid-solvent solutions.[495] Further processing by extrusion,[496] French pressure cell,[497] sonication[478] and microemulsification[478] can refine the particle size by physical means to the desired size range. The method of manufacture and subsequent processing may influence EE; cycles of freeze-thawing or dehydration-rehydration are said to improve EE in some cases.[498-500] Another approach offering promise for the convenient production of liposomes by freeze drying is the method presented by Cui *et al*, involving the freeze drying of liposomes from a monophase t-butanol co-solvent system.[501] This method has been shown to improve the EE of a number of compounds including ketoprofen and propranolol.[501-502]

4.1.3 Liposome characterisation

The development of liposomal drug products is a complex process which requires the consideration and control of many variables that can influence formulation attributes, and hence, formulation performance. Properties of liposomes such as drug loading/EE, surface charge, particle size, morphology, location of drug, colloidal stability, chemical stability of the encapsulated drug and drug release rate, just to name a few, are important factors affecting the performance of the formulation *in vivo*. Physicochemical characterisation studies are crucial early in development to enable relationships between the physical and chemical properties of liposomes and *in vitro*/*in vivo* performance to be established. Understanding such relationships can enable the manipulation of the formulation properties to achieve improved formulation performance, ultimately working towards achieving the desired *in vivo* effect.

4.1.3.1 Size

Liposome size is a fundamental variable to be considered when studying liposome formulations for inhalation because particle size greatly influences biodistribution and clearance rates following delivery to the lungs.[503] Alveolar macrophages readily engulf particles within the range of 0.5 - 3 μm, while liposomes smaller than 0.26 μm can evade phagocytosis and remain in the lungs for extended periods.[504-505] In addition to affecting clearance rates from the lung, liposome size may also influence the rate of drug release in some cases.[506] Smaller liposomes, having a larger surface area to volume ratio, may release their contents more rapidly than larger liposomes. Liposome size is largely dependent on the method of manufacture[478] (Section 4.1.2.4). Measurement of liposome size can be carried out by techniques such as DLS and microscopy.[478]

4.1.3.2 Colloidal stability

The colloidal stability of liposomes is of paramount importance given that many of the features which make liposomes therapeutically useful relate to their sub-micron size. Particle size growth, aggregation or sedimentation occurring over time can therefore affect liposome performance *in vivo*. Colloidal stability can be assessed by monitoring time-dependent changes in particle size, polydispersity index (PDI) and surface charge. Colloidal stability may be improved by increasing the electrostatic repulsion between particles, which can be achieved by the addition of charged lipidic modifiers or the incorporation of charged drug molecules that reside in the bilayer. Zeta potential, the potential difference between a dispersion medium and the stationary layer of fluid at the surface of a dispersed particle, can be used as an indicator of the degree of electrostatic repulsion between particles. Zeta potential is obtained by the measurement of the electrokinetic mobility of a colloid in an external electric field.[507] Using the Helmholtz-Smoluchowski equation, the electrokinetic mobility can be used to estimate the

electrostatic potential at the surface of a liposome,[508] which can be predictive of long term colloidal stability.[509] Changes in zeta potential over time can therefore indicate colloidal and/or chemical instability in a dispersed system.

4.1.3.3 Morphology

Phospholipids spontaneously form liposomes of a variety of morphologies when dispersed in water (Figure 4-2). Multi-lamellar vesicles (MLV) are liposomes with more than one phospholipid bilayer. Oligo-lamellar vesicles (OLV) are vesicles of various sizes contained within a larger vesicle. Small uni-lamellar vesicles (SUV) and large uni-lamellar vesicles (LUV) consist of one phospholipid bilayer only and are differentiated by their relative size. As the bilayer presents a physical barrier to drug release, drugs may be released at different rates from liposomes of different morphologies. It is thought that MLVs may provide a more sustained release of drug than ULVs,[478] because the drug has to traverse numerous lipid bilayers before being released into the external aqueous environment.

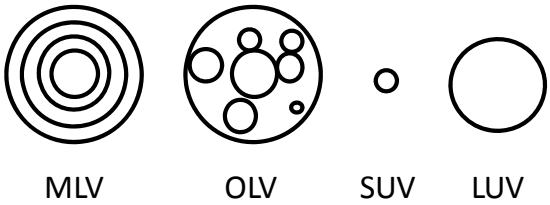


Figure 4-2: Schematic representation of liposome morphologies.

Electron microscopy techniques are commonly used to characterise liposome morphology. One such method, cryo-TEM, involves the rapid freezing of a sample at a rate that prevents the formation of ice crystals, protecting and preserving the hydrated particles in their true shape and form. Other imaging techniques requiring the dehydration of the sample can distort or flatten particles, introducing artifacts and misrepresenting the true morphology of particles.[510] The morphology of liposomes

(Figure 4-2) is largely influenced by the method of manufacture (Section 4.1.2.4), with each manufacturing method producing liposomes of characteristic sizes and morphologies. For example, liposomes produced by mechanical dispersion of a dry lipid film are typically polydisperse and multi-lamellar.[478]

4.1.4 Liposomal drug retention and release

Knowledge of the *in vitro* release of drug from a colloidal carrier is useful where it is suitable to enable prediction of the *in vivo* behaviour of the encapsulated drug.[511-512] The environment in which liposomes are used *in vivo* is most often, but not always, a sink condition. Hence, *in vitro* release studies should most often be conducted under sink conditions. When performed in an appropriate manner *in vitro* release studies can provide fundamental information about the availability of free drug following administration *in vivo*.

Though, drug retention *in vivo* must be balanced with drug release so that the drug becomes available for pharmacological activity at the intended site of action. If the drug is released too rapidly, the purpose of encapsulation within the liposome is defeated. If the drug is too well retained, the liposome may be cleared before the drug becomes available, or the temporal aspects of therapy become difficult to control. Hence, information about the drug release rate obtained from *in vitro* studies can provide guidance for manipulation of the formulation to achieve the optimal release properties. *In vitro* release studies should be carried out prior to *in vivo* assessments to inform the optimisation of drug release from the liposomal carrier, to maximise the potential for *in vivo* success.

The mechanism of drug release depends largely on the drug lipophilicity. The release of hydrophobic bilayer-associated drugs will depend on the propensity for the drug to

partition out of the bilayer on dilution.[295] In contrast, the efflux of water soluble drugs located within the aqueous core of the liposome is dictated by the permeability of the bilayer[513] which, in turn, depends on the phospholipids selected and their transition temperature (T_m) relative to physiological temperature. Below the T_m, in the gel state, the hydrocarbon tails of the phospholipids arrange into a well-aligned and ordered structure.[514-515] Above the T_m, phospholipids exist in a liquid crystalline state in which the hydrocarbon tails are more disordered.[335] Encapsulated materials leak out of liposomes more rapidly when the phospholipids are in the liquid crystalline state.[335] However, the fluidity of the bilayer can be altered by the addition of bilayer modifying additives, such as cholesterol. Cholesterol is a well known lipidic bilayer modifier.[516] It inserts into the bilayer and interacts with the hydrophobic chains of the phospholipids leading to a more rigidly packed bilayer, thereby slowing drug release.[517]

Because the rate of drug release from liposomes can be dictated by the physical form and location of the drug within the liposome, it is imperative to understand the location of the drug within the liposome and the nature of the physicochemical interaction between drug and phospholipid. For example, doxorubicin is known to precipitate on the interior of liposomes, which subsequently exhibit a slow release of doxorubicin.[294] The *in vitro* release profile may therefore provide information about the behaviour of the liposomal drug at a molecular level, specifically with regard to the possible interactions between the drug and liposomes and the mechanism for release.[511]

4.1.4.1 Methods for the measurement of encapsulation efficiency and in vitro release

The measurement of EE and *in vitro* drug release from liposomes requires a method for the rapid physical separation of particles from their surrounding dispersing medium. This can be a challenge due to the small size of liposomes.[518] For measurement of EE,

formulations are usually assessed in an undiluted form. The study of the *in vitro* release of drug from carrier particles over time, on the other hand, usually requires that the formulation is diluted into a release medium to simulate introduction into a relevant physiological fluid.

A major problem associated with any method applied to the separation of liposomes from the surrounding dispersant is incomplete or inefficient separation. The presence of liposomes in the ultrafiltrate or supernatant after separation can be difficult to detect visually and can lead to inaccurate estimations of EE or drug release. The presence of liposomes in separated samples may lead to an underestimation of EE and overestimations of *in vitro* drug release over time. The efficiency of separation using the various methods described depends largely on the properties of the nanoparticle and formulation in question. There are a number of available methods described in the literature for the separation of nanoparticles and their surrounding dispersion medium. The important ones are briefly discussed below, with particular reference to the separation of particles from free drug solution.

Ultracentrifugation

Ultracentrifugation (Figure 4-3) forces the sedimentation or creaming of liposomes into a separated layer. It therefore requires that there be a difference in density between the liposomes and the surrounding aqueous dispersion medium. Following sedimentation, the concentration of the drug in the supernatant is then assayed to determine the amount of unencapsulated drug, while the liposome layer may be analysed for the determination of encapsulated drug. In most cases, SUVs can be sedimented by ultracentrifugation at 200,000 *g* for 10 to 20 h using a preparative ultracentrifuge.[478]

The advantage of ultracentrifugation is that it is not labour intensive. However, the long centrifugation times required means that it is impossible to get a 'snapshot' of drug distribution between liposomes and the dispersing medium at any one time as the distribution may change during the centrifugation period.[310] Therefore it may not be possible to accurately determine the profile of drug release over time using ultracentrifugation. Furthermore, the high centrifugal forces required can cause considerable disruption and deformation to liposome particles, altering the distribution of drug. Finally, if the density difference between the liposome particles and the dispersing medium is not sufficiently large, it will not possible to sediment all the particles.[478] Formulations may therefore need to be diluted prior to ultracentrifugation. However, dilution of samples is not ideal in measurements of EE and *in vitro* drug release given that dilution can alter the distribution of drug between the liposomal carrier and the dispersion medium.[519]

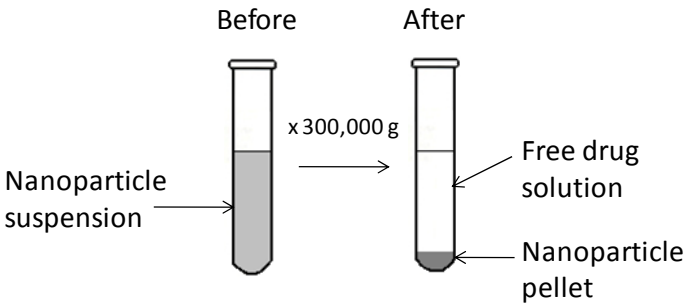


Figure 4-3: Schematic representation of nanoparticle separation from unencapsulated drug by ultracentrifugation.

Dialysis methods

Dialysis methods, including equilibrium dialysis (Figure 4-4), reverse dialysis and sac dialysis, achieve physical separation of liposome loaded drug particles from free drug by the use of a semi-permeable membrane of an appropriate molecular weight cut-off (MWCO).[520] These methods are frequently reported in the literature for the

measurement of *in vitro* drug release.[521-523] Most commonly, the undiluted nanoparticle formulation is loaded into a 'donor' compartment which is physically separated by a membrane from a sink compartment containing a receptor solution. The released drug crosses the size selective membrane, while liposome-bound drug is unable to traverse the membrane.

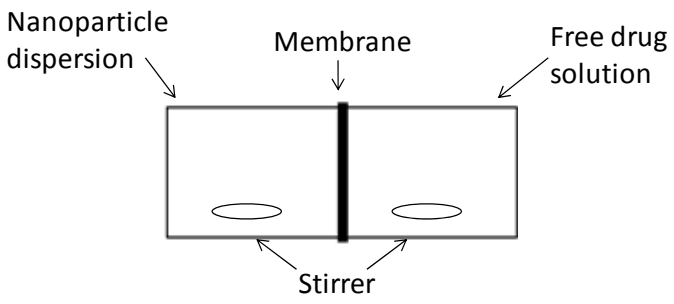


Figure 4-4: Schematic representation of nanoparticle separation from unencapsulated drug by equilibrium dialysis.

In theory, quantification of drug in the receptor compartment of the dialysis cell over time allows for the determination of drug release from liposomes. However, the concentration of drug in the receptor compartment may not always reflect the true free concentration of drug in the donor compartment, so the appropriateness of dialysis in measuring release of drug from nanoparticles has come under question in the literature.[310, 511, 524] Release of drug can often be dictated by membrane transport effects, masking the true release rate of drug from nanoparticles.[310] Liposomes loaded with doxorubicin have been shown to exhibit apparent slow release properties when assessed by equilibrium dialysis, but are seen to rapidly release doxorubicin as determined by ultarcentrifugation.[525] Slow membrane partitioning can often be wrongly attributed to the slow release of drug from a nanoparticle. The true release rate, therefore, must be deconvoluted from the kinetics of drug diffusion across the membrane,[511] which is not always as straightforward as observing diffusion of the drug across the membrane when free in solution. If the time required to reach equilibrium concentrations on either side

of the membrane takes hours, drug release profiles are unlikely to reflect the true rate of drug release from a colloid.

Centrifuge ultrafiltration

Centrifuge ultrafiltration (Figure 4-5) has been proposed as a rapid method of determining the proportion of free drug molecules within liquid samples.[526] Centrifuge ultrafiltration uses commercially available units which consist of two reservoirs separated by a semi-permeable membrane of a specific MWCO. The formulation is placed into the upper reservoir and the unit is centrifuged, forcing the movement of the dispersion medium containing unencapsulated drug into the lower reservoir while the liposomes are retained in the upper compartment. The proportion of liposome-bound drug can then be calculated from the total concentration of drug in the formulation less the concentration of drug in the filtrate.

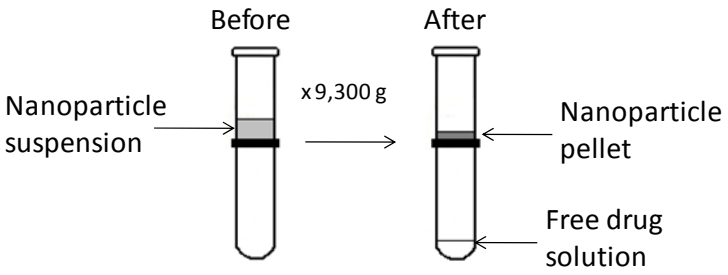


Figure 4-5: Schematic representation of centrifuge ultrafiltration apparatus.

For liposome dispersions of high phospholipid content, separation of free drug can be difficult using centrifuge ultrafiltration. The high volume fraction of nanoparticles can clog the membrane filter and hence filtrate yield can be very low. Low yield is a significant issue for the quantification of drug if there is substantial adsorption of the drug of interest to the membrane of the filter device. The potential for liposomes to penetrate the pores of the ultrafiltration membrane also needs to be considered.

On the other hand, the centrifuge ultrafiltration method has several advantages: separation is quick taking only minutes. Also, lower centrifuge speeds can be used compared to ultracentrifugation,[527] so there is less compromise to liposome integrity due to deformation under centrifugal forces.[528]

Pressure ultrafiltration

Pressure ultrafiltration was first described by Magenheim *et al.*[518] as a method for the rapid and efficient separation of drug from colloidal carriers. Pressure ultrafiltration uses a semi-permeable membrane of a particular MWCO to separate free and nanoparticle encapsulated drug in a similar fashion to centrifuge ultrafiltration. The pressure ultrafiltration apparatus (illustrated in Figure 4-6) consists of a cell of a given capacity mounted on a base that is lined with an ultrafiltration membrane. The formulation is added to the cell and pressure is then applied using nitrogen gas. The gas creates a gentle hydrostatic pressure which forces the drug in solution through the membrane, after which it can be collected and analysed for drug concentration. The advantage of pressure ultrafiltration over centrifuge ultrafiltration is that the pressure ultrafiltration cell can be continuously stirred to prevent particles from clogging the filter pores. Therefore, reasonable ultrafiltrate volumes can be collected (> 1 mL). Separation is quick, taking between 5 and 10 min depending on the viscosity of the formulation.

The advantages of the pressure ultrafiltration method are that rapid separation of drug from drug carrier can be achieved. The disadvantage of this method is that a reasonably large volume of formulation is required for separation (~ 5 mL). The large volume required may not be an issue for *in vitro* release studies where the formulation is substantially diluted prior to separation using pressure ultrafiltration, but may be more of a problem for EE measurements where formulation volume might be limited. Like

centrifuge ultrafiltration, the potential for liposomes to penetrate the ultrafiltration membrane should not be overlooked. As this method relies on quantification of the unencapsulated proportion of drug, the extent of drug binding to the filter membrane must be measured before this technique can be used to assess EE or *in vitro* drug release.

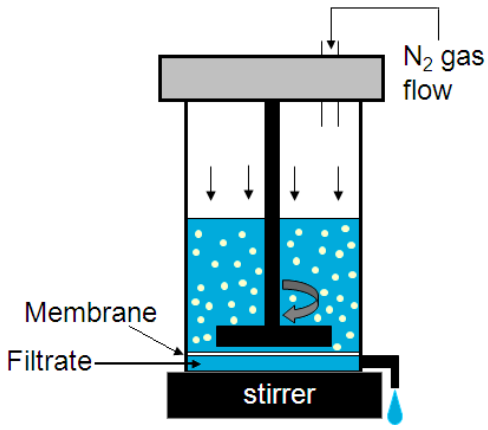


Figure 4-6: schematic representation of the stirred pressure ultrafiltration cell apparatus.

4.1.5 Liposomes as an approach to the formulation of colistin and CMS combinations for pulmonary delivery

As established in Chapter 3, the solubilisation of poorly-water soluble co-drugs by colistin and CMS micelles in unlikely to present a useful approach to co-formulation of colistin or CMS together with a second antibiotic ('co-drug') (Section 3.5.2). High concentrations of colistin or CMS were required to achieve modest solubilisation of a model co-drug, azithromycin. A promising alternative approach to the co-formulation of colistin or CMS with an antibiotic co-drug is to encapsulate both agents within a common liposomal carrier. The key features that present liposomes as an attractive drug delivery carrier for this application are their capacity to encapsulate drugs of differing physicochemical properties, and the scope for modification of the absorption and biodistribution of colistin or CMS following pulmonary administration.[472, 529] Based on the current understanding of colistin pharmacodynamics, attainment of both of these

performance characteristics by co-loading into a liposomal carrier should lead to a more efficacious colistin inhalation therapy.[154]

The chemistry of CMS and colistin do not lend towards encapsulation via remote-loading techniques,[348] so the encapsulation of CMS and colistin into a liposomal carrier would rely on passive encapsulation techniques (Section 4.1.2.3). Both colistin and CMS are highly water soluble compounds, so the efficiency of liposomal encapsulation is anticipated to be predominantly determined by the encapsulated aqueous volume. However, given that both colistin and CMS exhibit a level of amphilicity and surface activity (Section 3.5.1), a degree of association with the phospholipid bilayer is anticipated (Figure 4-7). In some cases, drug-bilayer interactions (Section 4.1.2.3) can be detrimental to the integrity of the liposome formulation, while in other cases interactions with the bilayer do not affect the release of the encapsulated contents. The effect of encapsulated materials on bilayer fluidity and integrity is an important consideration, especially in the context of colistin- and CMS-combination liposome formulations given that colistin is a known membrane permeabilising agent (Section 1.3.2).

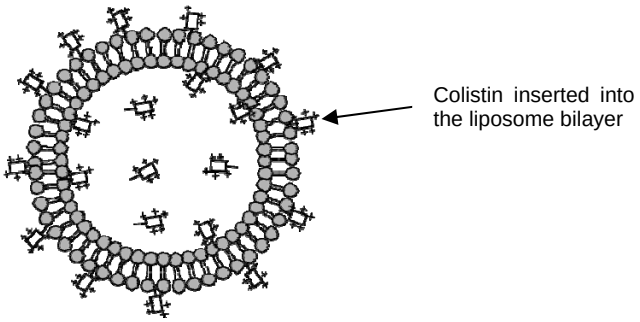


Figure 4-7: Proposed interaction of colistin with liposomes.

The biological activity of colistin against Gram-negative bacteria is initiated by an electrostatic interaction between the charged diaminobutyric acid residues of the colistin molecule and an anionic component of the bacterial outer membrane (Section 1.3.2). While electrostatic interactions can sometimes be harnessed to encourage drug-liposome bilayer binding and increase EE (Section 4.1.2.2), the electrostatic mediated membrane permeabilising activity of colistin has the potential to compromise liposome bilayer integrity and cause the uncontrolled release of encapsulated contents. Therefore, neutral phospholipids may provide the most stable encapsulation of colistin within the liposome aqueous interior. Moreover, neutral liposomes have been shown to slow the rate of pulmonary absorption of encapsulated materials[288, 530] and have been established as non-toxic following delivery via the pulmonary route.[531-532] Anionic phospholipids such as dipalmitoylphosphatidyl glycerol, on the other hand, are thought to increase pulmonary alveolar uptake of liposomes,[533] and therefore may increase the pulmonary clearance of liposome encapsulated materials.

In Chapter 3 it was shown that CMS converts to colistin in dilute aqueous environments, but is very stable at concentrations above the CMC (Section 3.5.4). For a liposome formulation of CMS to present a useful dosage form, the interaction of CMS with neutral phospholipid bilayers should not disrupt the micellar intermolecular associations which contribute towards the chemical stabilisation of CMS at high concentrations. Disruption of the self-assembling arrangement of CMS in solution may lead to accelerated conversion of CMS to colistin. A physically stable liposome formulation containing appreciable amounts of both CMS and colistin, bearing multiple opposite electrostatic charges, is difficult to envisage.

Preparation of colistin and CMS liposomes by the dry film method is considered to be the most practical way to produce liposomes for physicochemical characterisation

studies. An alternative method of freeze drying, as reported by Li *et al*.[502] may provide an alternative method by which CMS- and colistin-loaded liposomes can be efficiently produced. Liposome manufacture by this method has been shown to produce high EE for a number of drugs.[501]

In summary, there are many variables that will ultimately affect the performance of liposome drug formulations. The characterisation of these variables is crucial in anticipating and understanding the *in vitro* and *in vivo* performance of liposome products. Not all drug candidates are suited to formulation within a liposome carrier.[467] Therefore, preliminary characterisation studies are important to assess the suitability of a drug candidate to loading in liposomes. Characterisation studies can provide valuable information that enables the optimisation of formulations to maximise the chances of *in vivo* success. With a view to producing improved formulations of CMS or colistin for administration to the respiratory tract, the work detailed in this Chapter assesses the suitability of liposomes as a colloidal carrier for colistin or CMS.

4.2 Hypothesis and aims

The overall objective of this study was to understand the interactions of colistin and CMS with liposomes, in order to assess the suitability of liposomes as a colloidal carrier for colistin or CMS in formulations for pulmonary delivery. Specifically, it was hypothesised that:

1. colistin incorporates into the phospholipid bilayer, imparting a surface charge and colloidal stability;
2. CMS incorporates into the phospholipid bilayer imparting charge upon the liposomes, but degradation of CMS over time will lead to modification of the surface properties of liposomes, impacting upon colloidal stability;
3. the EE of colistin in liposomes will depend on the method of manufacture; and that
4. a sustained release of colistin and CMS from liposomes can be achieved *in vitro*.

In addressing these hypotheses, the aims of the studies constituting Chapter 4 are to:

1. assess the chemical and colloidal stability of CMS and colistin when formulated within a liposomal carrier. More specifically, the rate of CMS degradation in liposomes will be examined. The effect of colistin and CMS on liposome charge, and the impact of CMS degradation on liposome properties will be studied
2. determine the most appropriate method to measure the efficiency of encapsulation and release of colistin from liposomes
3. characterise the formulation performance characteristics including encapsulation efficiency and *in vitro* release

4. determine the impact of preparation method (dry film vs. freeze drying of a monophase solution) on encapsulation efficiency and physicochemical characteristics of colistin-containing liposomes.

4.3 Methods

4.3.1 Materials

Sep-Pak® C_{18} solid phase extraction (SPE) cartridges (100 mg) were supplied by Waters (Milford, MA, USA). The Visiprep 24™ vacuum manifold was made by Supelco (Bellefonate, PA, USA). Water was purified by a Milli-Q® water system (Millipore, Milford, MA). Saline 0.9% (provided in 100 ml ViaFlex™ bags) was from Baxter (Old Toongabbie, NSW) and 1 mL syringes were from Terumo Corporation (Tokyo, Japan). Soy dioleoylphosphatidylcholine (DOPC) (stored at -20°C) was obtained as the commercial preparation Phospholipon® 90G from Phospholipid GmbH (Cologne, Germany). Cholesterol, sodium chloride, potassium chloride, sodium phosphate dibasic, sodium hydroxide and potassium dihydrogen orthophosphate were obtained from Sigma (St Louis, MO). Colistin sulphate was obtained from Zhejiang Shenghua Biok Biology Co. Ltd (Huzhou, China), CMS was from Alpharma (Copenhagen, Denmark, batch number A1680557). All HPLC reagents were of analytical grade. Nitrogen gas was from BOC gases (Sydney, Australia). All reagents and chemicals used for HPLC analysis were of HPLC grade.

4.3.2 Preparation of liposomes by the dry film method

DOPC (Figure 4-8) was selected as the main constituent phospholipid for the formulation of CMS and colistin liposomes. DOPC (Figure 4-8) is a zwitterionic phospholipid containing two 18-carbon alkenyl tails. It has a transition temperature of ~19°C[534] and is inexpensive to obtain in large quantities. Although the transition temperature for DOPC alone is below body temperature, the incorporation of cholesterol (Figure 4-8) into the bilayer can raise T_m above body temperature.[535]

Liposomes were produced by the dry film method.[313] Unless stated otherwise, 500 g soy dioleoylphosphatidylcholine (DOPC) was weighed into a 100 mL round-bottomed flask and was dissolved with 1.5 mL of chloroform/methanol (2:1 v/v), with or without the addition of cholesterol. The solvent was evaporated under vacuum at 40°C using a rotary evaporator (Buchi Labortechnik, Switzerland). The resulting dry lipid film was flushed with a continuous stream of nitrogen gas for 5 min to remove trace solvent and was stored at -20°C until required. When required the dry lipid film was dispersed in 10 mL of a solution of colistin or CMS (1, 5, 10 or 20 mg/mL) dissolved in Milli-Q® water (final DOPC concentration 64.5 mM). Empty liposomes were prepared by hydration of the lipid film with 10 mL of Milli-Q® water. On an ice water bath, liposomes were ultrasonicated using a probe ultrasonicator with a 3-mm titanium probe (Misonix Incorporated, Farmingdale, NY, USA) for 15 min on pulse mode (1 sec pulses interrupted by 1 sec breaks, total process time 30 min, total sonication time 15 min).

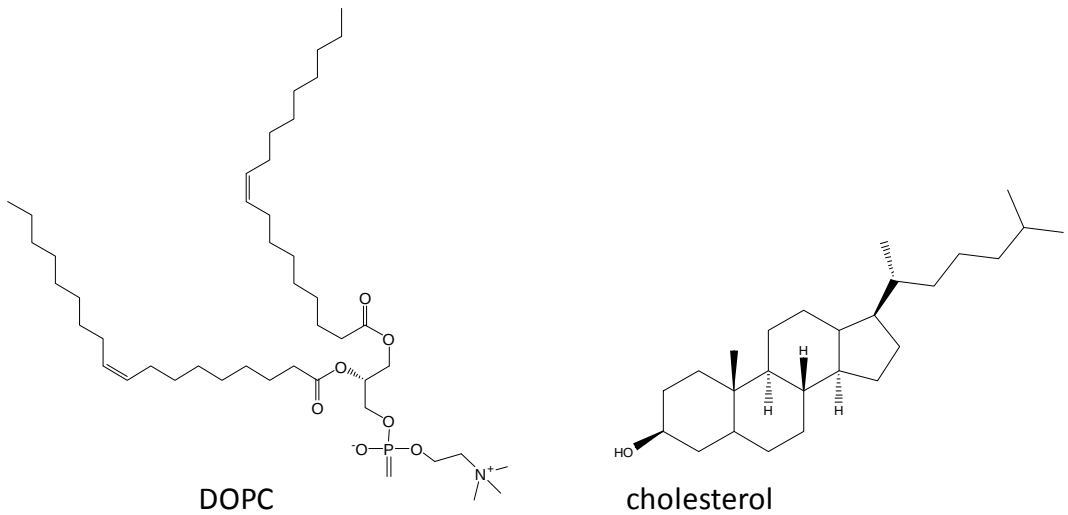


Figure 4-8: The chemical structures of DOPC and cholesterol.

4.3.3 Preparation of liposomes by freeze drying from t-butanol

Liposomes containing colistin were prepared by an alternative method to the dry film method involving the freeze drying of a t-butanol-based single phase solution, as described by Li *et al.*[501] DOPC (100 mg) was dissolved in 2.5 mL t-butanol to produce a lipid solution (phase A). Colistin was dissolved in 15 mL water containing 100 mg/mL sucrose (phase B) to produce solutions of varying colistin concentration. A 5-mL aliquot of phase B was then added to 2.5 mL of phase A and was mixed by shaking until clear. The lipid:sucrose mole ratio in the final co-solvent mixture was 1:5. The freeze drying process was carried out in a laboratory freeze drier (VirTis Advantage 2.0 bench top freeze dryer, SP Scientific, PA, USA) as follows: freezing -80°C for 8 h, then primary drying at -40°C for 48 h with a second drying step at 25°C for 24 h. The chamber pressure was kept below 20 Pa during the drying process. The lyophilisate was reconstituted in 5-mL Milli-Q® water to produce liposomes containing between 1 to 20 mg/mL colistin. The final lipid concentration in the reconstituted lyophilisate was identical to that of liposomes produced by the dry film method (64.5 mM).

4.3.3.1 Analysis of residual t-butanol by gas chromatography-mass spectrometry (GC-MS/MS)

Residual t-butanol was determined using GC-MS, conducted by the Department of Chemistry, Monash University (Clayton, Australia). The GC system comprised of an Agilent 6890 GC coupled to a HP7694E headspace sampler (Agilent Technologies, Forest Hills, Australia). Helium gas was run through the HP-5MS 5% phenyl methyl siloxane column (30 m x 250 µm, 0.25 µm) with a maximum column temperature of 325°C. The system back inlet was run in split mode (ratio 5:1) at a temperature of 220°C and pressure of 7.06 psi, with a maximum helium flow rate of 9 mL/min. An Agilent N5973 MS was used in EI mode, scanning between 30 and 400 *m*/*z* with source

and quad temperatures of 200 and 150°C, respectively. A series of calibration and QC standards containing known concentrations of t-butanol ranging from 0 - 1% were prepared in a matrix of 5% DOPC which was prepared by the dry film method. Samples of 1 µL were injected onto the column. The peak areas of standard samples were used to create a calibration curve from which the concentrations of t-butanol were determined in unknown samples.

4.3.4 Liposome characterisation

4.3.4.1 Particle size and polydispersity index

The particle size and PDI of undiluted liposome formulations was determined by DLS (Section 3.4.1.3). For each sample, three measurements consisting of 10 autocorrelation functions were analysed using cumulant analysis.[536] The temperature was maintained at 25°C by an in-built thermostat and the viscosity was set to that of water (0.8937 cP).[445] From this analysis, the z-averages were obtained to provide an approximation of the hydrodynamic diameter of the liposomes. Data are reported as the mean z-average diameter of the three determinations. The PDIs of the particle size distributions were obtained from the same measurement to indicate the width of the distribution of particle size around the z-average mean.

4.3.4.2 Zeta potential

Undiluted liposome samples were transferred to a disposable capillary cell (DTS1060, Malvern Instruments, Worcestershire, UK) for measurement using a Zetasizer Nano ZS (Malvern Instruments,Worcestershire, UK) at 25°C. Zeta potentials were calculated automatically by the instrument software (Dispersion Technology Software version 5.10, Malvern Instruments, UK). The measurement duration, repetitions, voltage and attenuator were determined automatically by the instrument software and conductivity

was between 0 and 3.9 mS/cm. The data are reported as the mean zeta potential of three separate determinations with standard deviation.

4.3.4.3 Optimisation of liposome manufacture method

As described in Section 4.1.3.1, liposome size is a critical factor to be controlled for with liposomal formulations intended for pulmonary delivery. As explained in Section 4.1.3.1, the optimum liposome size for targeted pulmonary delivery is believed to be between 50 and 260 nm.[504-505] To determine the required sonication time to consistently produce liposomes within this size range, the effect of sonication time on liposome size was studied. Liposome suspensions (n = 3) (10 mL) containing 10% DOPC w/v were prepared by the dry film method (Section 4.1.2.4) and transferred into a 20 mL borosilicate glass scintillation vial for ultrasonication. The formulations were ultrasonicated for 5 min at a time on pulse mode (1 sec pulses interrupted by 1 sec breaks, 10 min total process time for 5 min sonication) either on an ice bath (to minimise the heat generated by the sonication process, thereby reducing degradation of phospholipids during processing) or without temperature control. At the end of each 5 min sonication interval, a 1 mL sample was removed from the vial and was mixed with 1 mL of Milli-Q® water to produce 2 mL of a 5% DOPC dispersion. The particle size, PDI (Section 4.3.4.1) and zeta potential (Section 4.3.4.2) of the each sample was measured. The process was repeated four times until the formulation had been sonicated for a total of 20 min (in pulse mode, 40 min total process time).

4.3.4.4 Interactions of colistin and CMS with DOPC liposomes

To investigate the association of colistin and CMS with DOPC liposomes, the effect of increasing concentrations of colistin or CMS on the surface charge of DOPC liposomes was measured by zeta potential. DOPC forms neutral liposomes (zeta potential ≈ 0).

Adoption of a surface charge upon the addition of colistin or CMS to the liposomes can indicate an association of the drug with liposomes (as illustrated schematically in Figure 4-7 above). The effect of increasing concentrations of colistin or CMS on the zeta potential of 5% DOPC w/v liposomes was examined by preparing liposomes containing 1, 5, 10 and 20 mg/mL colistin or CMS, by the dry film method (Section 4.3.2). Zeta potential measurements were made according to Section 4.3.4.2. Zeta potentials were plotted against colistin or CMS concentration to observe any changes or trends in zeta potential with composition.

ITC was employed to further probe the interaction of colistin with DOPC liposomes. Titrations were carried out on a CSC4200 isothermal titration calorimeter (Calorimetry Sciences Corp, UT, USA) and data processed using the software Bindworks™ version 3.0.78 (TA Instruments, UT, USA). A 100 mg/mL colistin sulphate solution (71.24 mM colistin) was prepared in phosphate buffered saline (PBS) (pH 7.4) and was titrated into a 1275 µL reaction cell. The cell was pre-equilibrated (baseline drift < 0.001 µW/sec) containing 31 mM DOPC (2.5% w/v) with or without cholesterol (lipid: cholesterol mole ratio 2:1) prepared in phosphate buffered saline (pH 7.4), using 25 injections of 10 µL each. The cell was maintained at 25.00°C and stirred at a rate of 297 rpm. The pre-titration equilibration time was 500 sec, with a 500 sec interval between injections. The concentration range studied was selected to represent the concentrations of colistin, DOPC and cholesterol used in EE and *in vitro* release experiments.

4.3.4.5 Colloidal stability of CMS and colistin in DOPC liposomes

Liposomes containing 1, 5 and 10 mg/mL colistin or CMS were prepared with 5% DOPC by the dry film method (Section 4.3.2). Briefly, 10% DOPC dispersions were prepared then mixed 1:1 with CMS solutions to produce a liposome formulation of the desired concentration. A 15 mL sample of each liposome formulation was filled into a

20 mL borosilicate glass vial and allowed to equilibrate for 1 h at room temperature before being stored at 25°C in a temperature controlled laboratory incubator (Thermoline, Eltham, Australia) for 7 days. At 0, 4, 8, 12, 24, 48, 72, 96, 120 and 168 h of storage, an 800 μL sample was removed from each vial using a 1 mL syringe. Zeta potential (Section 4.3.4.2), particle size and PDI (Section 4.3.4.1) were immediately measured. In separate 20 mL glass scintillation vials, 5 mL of each CMS and colistin liposome formulation was stored for observations of formulation physical stability at 25°C over time. Digital photographs were taken of these formulations to record any changes in physical appearance over time.

After it was observed that CMS-loaded liposomes phase separated upon storage in glass vials, the storage stability in polypropylene vials was tested to investigate for the effects of storage container. Aliquots (10 mL) of empty liposomes and liposomes containing 10 or 20 mg/mL CMS were dispensed into 20 mL polypropylene tubes and the physical stability was observed by visual observation and digital photography over 7 days.

It was also observed that vials containing smaller volumes of CMS-loaded liposomes exhibited phase separation more rapidly than vials containing larger volumes. Hence, in addition to the influence of storage container type on the physical stability of CMS liposomes, the effect of variations in container fill volume was also investigated. Liposomes containing 10 mg/mL CMS were prepared by the dry film method. Aliquots of 2, 5, 10 and 15 mL of CMS liposomes were filled into borosilicate glass vials and stored in the temperature controlled laboratory incubator at 25°C for 7 days. Digital images were taken throughout the 7-day period to record any changes to the physical appearance of liposome dispersions over time.

4.3.4.6 Chemical stability of CMS in DOPC liposomes

Given the chemical instability of CMS at low concentrations in biological fluids and in saline[71, 107] (refer to Section 1.6), the chemical stability of CMS when loaded into liposomes was assessed. The chemical stability of CMS formulated in liposome suspensions across a range of concentrations was compared to the stability of CMS at the same concentrations, when free in solution. Liposomes containing 1, 5 and 10 mg/mL CMS were prepared with 5% DOPC using the dry film method described previously (Section 4.3.2) A 10 mL aliquot of each liposome formulation or CMS solution was dispensed into a 20 mL glass scintillation vial, and the vial was stored at 25°C in a temperature controlled laboratory incubator for 24 h. At 0, 4, 8, 12 and 24 h a 1 mL sample was removed from each vial using a 1 mL syringe and was frozen at -20°C for no longer than 1 week[448] pending HPLC analysis of colistin (Section 2.2.6).[107, 448]

4.3.4.7 Chemical stability of colistin in solution

The formation of colistin was used as an indicator of the degradation of CMS in solution and in liposome formulations. Consequently, the stability of colistin itself needed to be established under the conditions of the CMS stability studies described in section 4.3.4.6. Solutions containing 1 and 50 µg/mL colistin sulphate were prepared in water in triplicate by independent weighings. An aliquot of 2 mL of each solution containing 1 or 50 µg/mL colistin were dispensed into 12 x 10 mL polypropylene tubes and the tubes were stored at 25 ± 2°C in a temperature controlled oven for 72 h. At 0, 24, 48 and 72 h three tubes at each concentration (3 x 1 µg/mL and 3 x 50 µg/mL) were removed and stored at -20°C for no longer than 1 week pending HPLC analysis for colistin content.[448] Prior to analysis, 2 mL of acetonitrile was added to each tube to remove any colistin bound to the polypropylene tube via non-specific adsorption. Samples were loaded directly onto the SPE cartridge without further dilution and

assayed for colistin concentration using the validated HPLC method described in Section 2.2.6.

4.3.4.8 Cryo-Transmission Electron Microscopy

Cryo-TEM was selected as the most appropriate method for studying the morphology of colistin-loaded liposomes for the reasons detailed in Section 4.1.3.3. DOPC liposomes were prepared by the dry film (Section 4.3.2) and freeze dry (Section 4.3.3) methods. Empty liposomes and colistin-loaded liposomes were compared to assess the effect of colistin on the structural morphology and integrity of the liposomes. The procedure for sample preparation and cryo-TEM imaging is described in Section 4.3.4.8.

4.3.5 Assessment of methods for measuring encapsulation efficiency and *in vitro* release of colistin liposomes

A number of the methods were compared for the separation of liposomes in the assessment of EE and *in vitro* release, namely, centrifuge ultrafiltration, ultracentrifugation and pressure ultrafiltration.

The presence of liposomes in the ultrafiltrate or ultracentrifuge supernatant following separation of liposome samples was measured by DLS using a similar method to that described in Section 3.4.1.3. In the absence of liposomes, samples of ultrafiltrate or ultracentrifuge supernatant should only scatter light at a level comparable to that of the formulation dispersant, i.e. free drug solution. On the other hand, filtrate or supernatant samples containing liposomes, will scatter light in proportion to the concentration of liposomes present, which can be monitored using DLS particle size measurements. Because the extent of light scattering in a sample depends on the size and number of particles present, the derived count rate (which takes into account the automatic adjustment of the instrument attenuator setting), or degree of light scattered by a

sample, can be used to assess the efficiency of the separation by indicating the relative amount of liposomes in the sample. The measurement of light scattered by samples obtained by various separation methods enabled a comparison of the efficiency of the different separation methods.

A suspension of 1% w/v DOPC was prepared by the dry film method described previously. A series of standards, containing between 0 and 0.125% w/v DOPC were prepared by serial dilution of the 1% DOPC stock. The degree of light scattering, expressed as the derived count rate in kilo counts per second (kcps) was obtained using DLS. The calibration curve established using highly diluted liposomes showed that a count rate of less than 500 kcps meant that less than 0.005% of the liposomes had passed through the membrane. A count rate of less than 500 kcps was thus adopted as the criteria for effective absence of liposomes from the filtrate. Any filtrate samples obtained in subsequent EE and *in vitro* release studies with scattering greater than 500 kcps were rejected and separations were repeated using a new membrane.

4.3.5.1 Ultracentrifugation

Ultracentrifugation of liposome suspensions containing 1 mg/mL colistin sulphate prepared by the dry film method, without cholesterol, was carried out using a Beckman Optima L-90 K preparative ultracentrifuge fitted with a SW60Ti rotor (Beckman Coulter, Gladesville, Australia). Formulations were ultracentrifuged at speeds of up to 300,000 *g* for up to 8 h at 25°C. The efficiency of the separation of liposomes from the dispersion medium was assessed by visual inspection and also by DLS measurements of the supernatant, as described in Section 4.3.5.

4.3.5.2 Centrifuge ultrafiltration

Centrifuge ultrafiltration was performed using Nanosep 300K (300 kDa MWCO) and 100K modified polyethersulphone (100 kDa MWCO) (Pall Life Sciences, NY, USA) and Microcon YM50 (50 kDa MWCO) and Microcon YM30 (30 kDa MWCO) (regenerated cellulose) (Millipore Corp., MS, USA) ultrafiltration devices. Liposome formulation (up to 0.5 mL) was loaded into centrifuge ultrafiltration units and centrifuged for 15 min at 9,300 *g*. The filtrate was collected and the back scattering of the filtrate was measured using DLS.

4.3.5.3 Pressure ultrafiltration

The pressure ultrafiltration apparatus (Figure 4-6) used an Amicon 8010 10 mL pressure ultrafiltration cell fitted with a 25 mm regenerated cellulose YM10 ultrafiltration membrane (10,000 kDa nominal MWCO) (Millipore Corp., Bedford, MA). The membranes were stored in 10% ethanol and were rinsed and soaked in Milli-Q® water prior to use. A minimum volume of 5 mL of the liposome dispersion was loaded into the ultrafiltration cell. Gentle hydrostatic pressure was applied using nitrogen gas (350 kPa) while the cell was stirred using the inbuilt magnetic stirrer. The filtrate was collected from the nozzle of the ultrafiltration cell and the back scattering of the filtrate was measured using DLS.

Both encapsulation and release measurements involved placing 5 - 10 mL of liposome suspension in the ultrafiltration cell. Approximately 1 mL of ultrafiltrate was collected in two separate 500 µL fractions. The first fraction of ultrafiltrate was used to measure the efficiency of separation (Section 4.3.5). The second 500 µL fraction of ultrafiltrate was retained for determination of drug concentration in the ultrafiltrate (free drug concentration) by HPLC (Section 2.2.3).

4.3.6 Separation of liposomes from dispersion medium using pressure ultrafiltration for encapsulation efficiency and *in vitro* release studies

Prior to using the pressure ultrafiltration technique to determine EE and *in vitro* release of colistin from liposomes, adsorption of colistin to the ultrafiltration membrane was quantified. This procedure ensures that the concentrations of drug obtained in the filtrate accurately reflect the concentration of drug in the dispersion medium. The adsorption of colistin was measured by filtering solutions containing 100 µg/mL colistin sulphate and quantifying the recovery of colistin in the subsequent filtrate. The filtrate obtained was collected in fractions of five drops at a time and the volume of each fraction was determined by weighing (assuming a solution density of 1 $g.mL^{-1}$). The concentration of colistin in each fraction of the filtrate was determined by LC-MS (Section 2.2.7). The process was repeated using three different YM10 ultrafiltration membranes. The volume of solution that was required to pass through the membrane such that the concentration of drug in the ultrafiltrate was within 95% of that inside the cell was determined.

Pressure ultrafiltration was used as described in Section 4.3.5.3. A 5 mL sample of liposome formulation was added to the pressure ultrafiltration cell and approximately 1 mL of ultrafiltrate was collected in two separate 500 µL fractions. The first 500 µL fraction of ultrafiltrate was retained for the measurement of separation efficiency (Section 4.3.5). Samples exhibiting > 500 kcps light scattering were discarded and the separation was repeated. The second 500 µL fraction of ultrafiltrate was retained for determination of drug concentration in the ultrafiltrate (unencapsulated drug concentration) by HPLC (Section 2.2.3).

4.3.7 Encapsulation efficiency of colistin in liposomes

The EE of liposomes made by the dry film (Section 4.3.2) and freeze dry (Section 4.3.3) methods were compared using pressure ultrafiltration. Dry film DOPC and

DOPC/cholesterol (2:1) liposomes containing 1, 2, 5, 10 and 20 mg/mL colistin, and freeze dried DOPC liposomes containing 1, 2 5 and 20 mg/mL colistin were prepared. A 200 µL aliquot of each formulation was retained for analysis of total colistin concentration by HPLC (Section 2.2.3). PUF was used to separate liposomes from the dispersion medium (containing unencapsulated drug) as described in Section 4.3.6. The first fraction of filtrate (500 µL) was retained for measurement of backscattering (Section 4.3.5). The second fraction of ultrafiltrate (500 µL) was collected for the determination of unencapsulated colistin by HPLC (Section 2.2.3). The EE was calculated according to Equation 4.1:

$$EE\ \% = 1 - ([colistin\ in\ ultrafiltrate] \div total\ liposome\ [colistin]) \times 100$$

Equation 4.1

4.3.8 *In vitro* release of colistin from liposomes

Liposomes (DOPC/cholesterol (2:1)) containing 2.5, 10, or 20 mg/mL colistin (n = 3 for each concentration) were prepared by the dry film method. The study of *in vitro* release requires the dilution of the liposomal dispersion into a release medium and subsequent measurement of released drug. In this study, a 10 mL aliquot of each liposome suspension was diluted into 40 mL phosphate buffered saline (1:5 dilution) in a 50 mL polypropylene tube and the tubes was placed in a shaking water bath at 37°C for 72 h. The dilution of the formulation by one fifth into PBS was selected to represent the dilution into lung fluids following pulmonary delivery, based on the human ELF volume of 15 - 70 mL.[537] In addition, the dilution was required to produce a sufficient concentration gradient of colistin across the liposome bilayer. At 0, 1, 2, 4, 8, 24, 48 and 72 h, a 5 mL sample was transferred to the PUF cell for separation of liposome-bound colistin and unencapsulated colistin. The filtrate was collected in two fractions of 500 µL. The first fraction was retained for measurement of light scattering. As described in

Section 4.3.7, filtrate samples measuring greater than 500 kcps using DLS were discarded and the measurement was repeated. The second fraction was retained for HPLC analysis of colistin concentration by HPLC (Section 2.2.3). The extremely high water solubility of colistin allowed sink conditions to be maintained throughout the duration of the *in vitro* release experiment. Colistin has previously been shown to be stable in phosphate buffered saline under the conditions used in these *in vitro* release experiments.[107]

4.4 Results

4.4.1 Optimisation of liposome manufacture

4.4.1.1 Particle size and polydispersity

The effect of sonication time on the particle size and PDI of DOPC liposomes is shown in Figure 4-9. The average particle sizes of the liposome formulations were reproducible at the end of each sonication period. Each 5 min sonication period resulted in a reduction in z-average particle size, though the PDI of liposomes did not change substantially with increasing sonication time. Liposomes that were sonicated without temperature control exhibited the greatest reduction in particle size with increasing sonication time. It can be seen from the particle size distribution shown in Figure 4-10 that 5 min sonication time was not sufficient to break up particles larger than 1000 nm. After 10 min sonication time, however, particles greater than 1000 nm were not identified. Increasing sonication time reduced the intensity of the second peak (~ 200 nm) while increasing the intensity of the first peak (~ 30 nm), as shown in Figure 4-10.

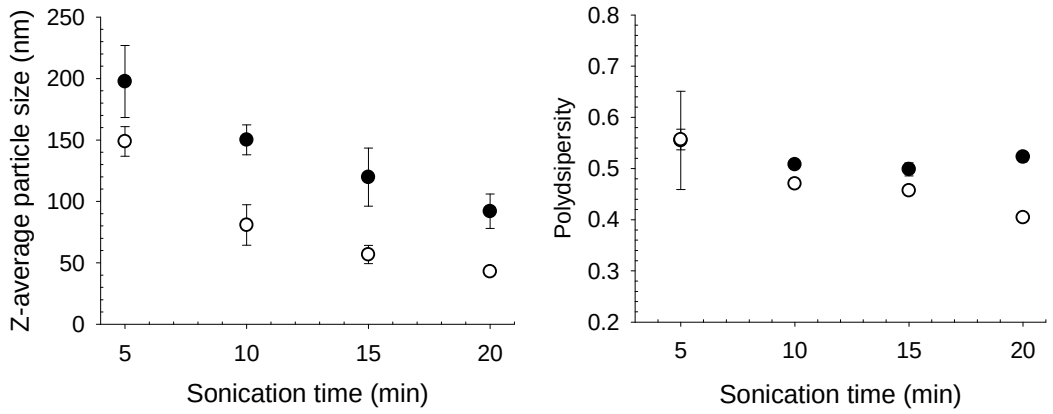


Figure 4-9: The effect of sonication time on the particle size (left) and PDI (right) of empty DOPC liposomes when sonicated on ice (●) or without temperature control (○).

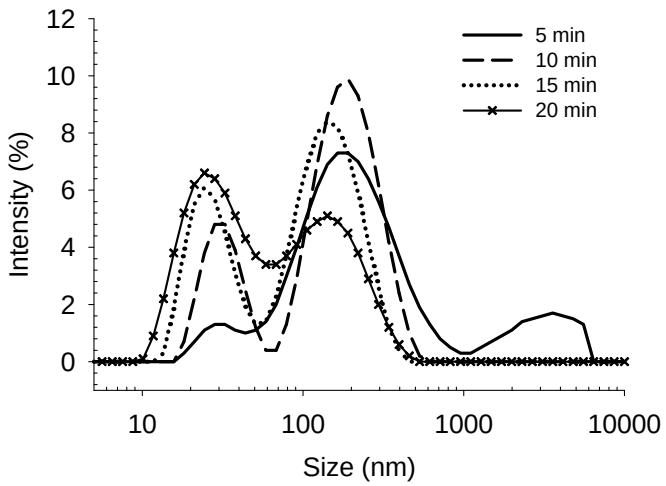


Figure 4-10: Particle size distributions of empty DOPC liposomes sonicated for increasing periods of time.

A final sonication time of 15 min (pulse mode: 1 sec on, 1 sec off, total sonication time 15 min, total process time 30 min) was selected as a compromise. This sonication time consistently produced liposomes with z-averages ranging between 90 and 150 nm and was deemed sufficient to reduce the fraction of larger liposomes (> 1000 nm) without substantially reducing the intensity of the peak occurring at 150 - 200 nm.

4.4.1.2 Zeta potential

As displayed in Figure 4-11, the effect of sonication time on the zeta potential of liposomes was minimal. Though there was a trend from a slightly negative to a slightly positive zeta potential with increasing sonication time, a repeated measures ANOVA revealed that this change in time was not statistically significant ($p > 0.05$). The repeated measures ANOVA also showed no significant difference in the zeta potential of the two conditions of sonication (on ice or without temperature control) ($p > 0.05$).

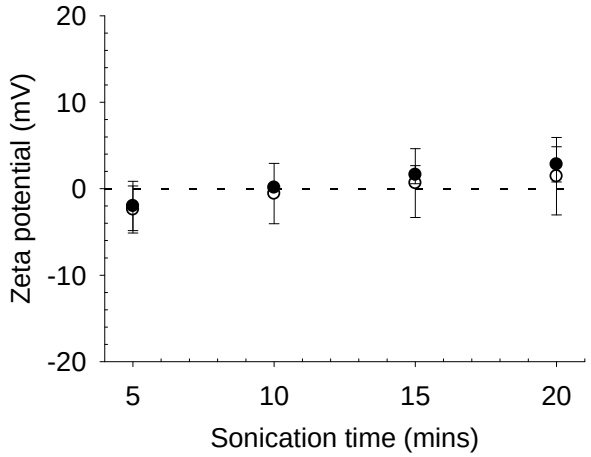


Figure 4-11: The effect of sonication time on the zeta potential of empty DOPC liposomes sonicated without temperature control (○) and on ice (●). Dashed line represents zeta potential = 0 mV.

4.4.2 CMS and colistin DOPC liposomes made by the dry film method

4.4.2.1 Association of CMS and colistin with the DOPC bilayer measured by zeta potential

Figure 4-12 shows the zeta-potential of DOPC liposomes with increasing concentrations of incorporated colistin or CMS. The liposomes adopted the anticipated charge from the anionic CMS or cationic colistin, indicating a direct association of the compounds with the bilayer structure in each case. A one-way ANOVA revealed a highly significant influence of drug concentration on zeta potential ($p < 0.01$), with a Tukey's post-hoc test revealing highly significant differences ($p < 0.01$) and in zeta potential at the different drug concentrations.

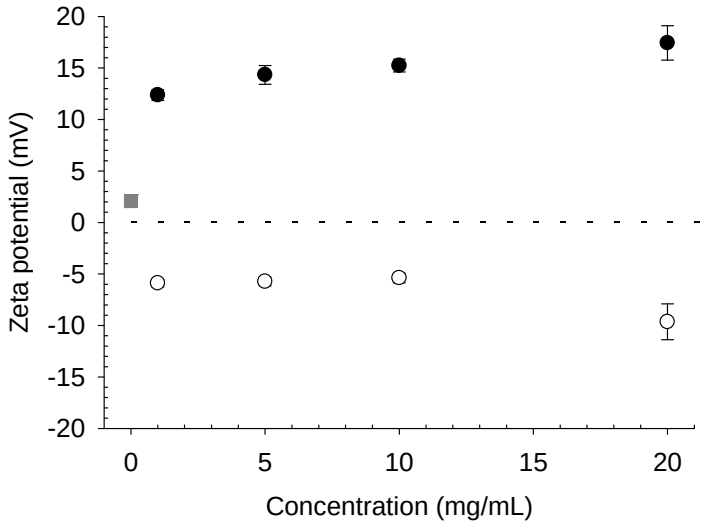


Figure 4-12: Zeta potential of empty DOPC liposomes (■) with increasing concentrations of colistin (●) and CMS (○). Dashed line represents zeta potential = 0 mV.

4.4.2.2 Interactions of colistin with DOPC and DOPC/cholesterol liposomes measured by isothermal microcalorimetry

Figure 4-13 shows the results of titrations of 100 mg/mL (71.24 mM) colistin into DOPC liposomes (2.5% w/v) with or without cholesterol. On titration of colistin into both DOPC and DOPC/cholesterol (2:1) liposomes, each injection initially resulted in an endothermic heat flux, as can be seen from the signals in the heat flow versus colistin concentration diagram. After 3 aliquots (without cholesterol) or 8 aliquots (with cholesterol), the heat flux became exothermic. From this it can be seen that the incorporation of cholesterol into DOPC liposomes resulted in significantly more endothermic heat flux. Both titrations ended with a heat flux plateau, where the exothermic heat flux on further injections of colistin resulted from dilution only. This plateau was observed at a much lower colistin concentration for the titration of colistin into liposomes without cholesterol, compared to those containing cholesterol.

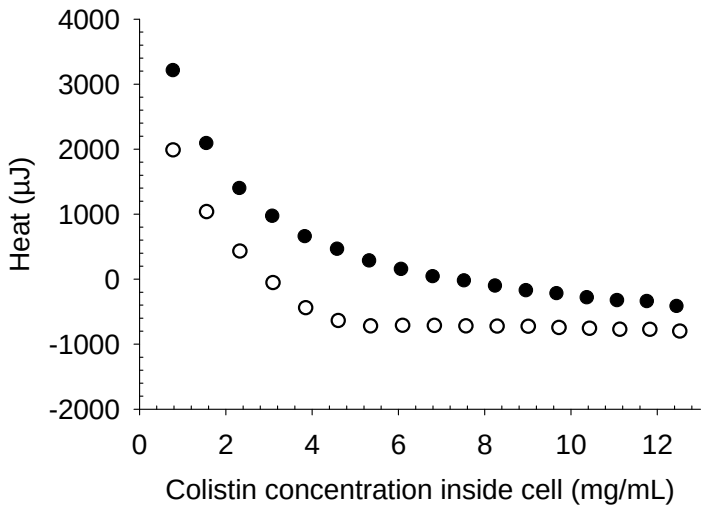


Figure 4-13: Titration of colistin into DOPC (●) and DOPC/cholesterol (2:1) (○) liposomes, after subtracting the baseline heat flux of the matrix titrated into DOPC or DOPC/cholesterol liposomes.

4.4.2.3 Colloidal stability of CMS and colistin liposomes

Particle size

The particle size distribution of a typical colistin liposome sample is shown in Figure 4-14. The average particle size of liposomes loaded with colistin was remarkably stable over time, with no significant change in z-average size observed over the 7-day storage period (Figure 4-15). Based on the z-average particle size, CMS-loaded liposomes exhibited colloidal stability for up to 48 h. However, in contrast to the behaviour of colistin-loaded liposomes, after storage longer than 48 h particle size growth was evident for CMS-loaded liposomes (Figure 4-15) and the corresponding PDI increased substantially (Figure 4-16).

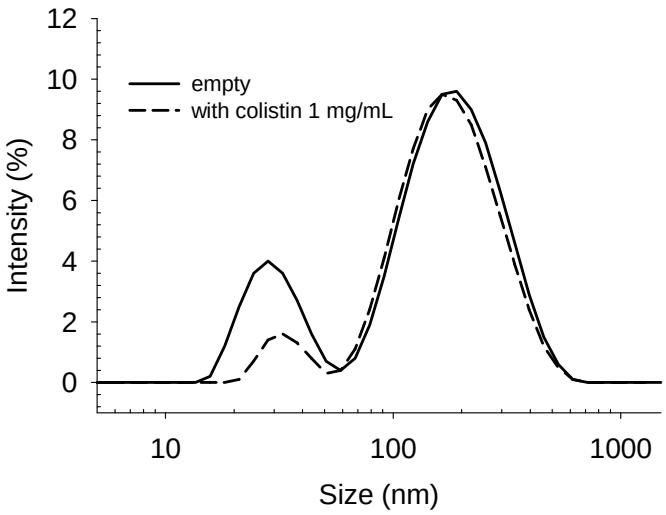


Figure 4-14: Particle size distributions of empty and colistin loaded DOPC liposomes.

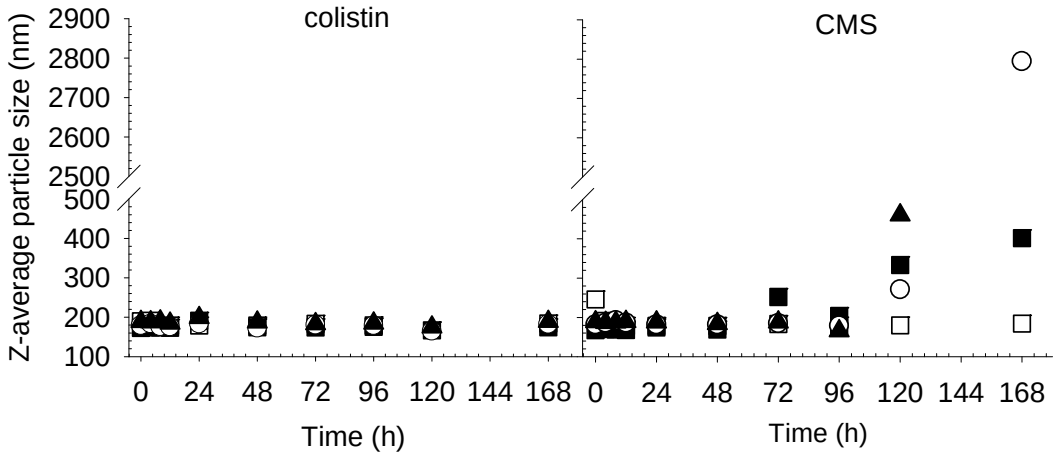


Figure 4-15: Particle sizes of empty DOPC liposomes (□) and DOPC liposomes containing 1 (■), 5 (○) and 10 (▲) mg/mL colistin (left) or CMS (right) (n = 1) over 1 week.

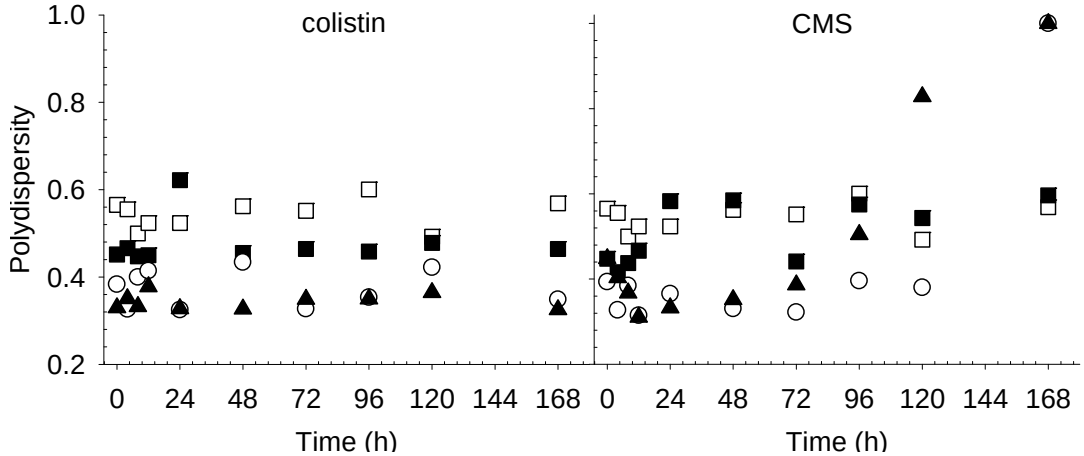


Figure 4-16: PDI of empty DOPC liposomes (□) and DOPC liposomes containing 1 (■), 5 (○) and 10 (▲) mg/mL colistin (left) or CMS (right) (n = 1) over 1 week.

Physical appearance

After approximately 72 h, the presence of separate phases in the CMS-loaded liposome formulations became visible to the naked eye (Figure 4-17); it was evident that sedimentation of the CMS-loaded liposome particles had begun to occur. After approximately 120 h, it became obvious that there were not only two separate phases present (i.e. an oily phase containing phospholipid and an aqueous phase containing water-soluble drug), but rather, there were three phases present; an aqueous phase, an oily phase and a third phase consisting of an off-white flocculated powder settled on top of the oily layer (Figure 4-18). The precipitated off-white material was not soluble in acetonitrile, methanol or water.

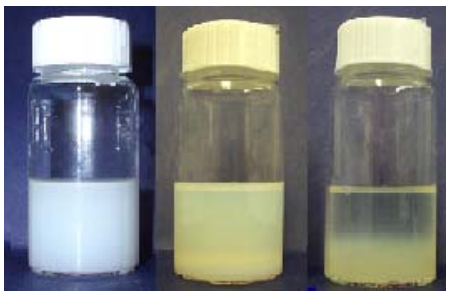


Figure 4-17: The phase separation of 10 mg/mL CMS liposomes over 72 h at 25°C. Left hand vial is at 0 h, middle vial after 48 h, right hand vial after 72 h.

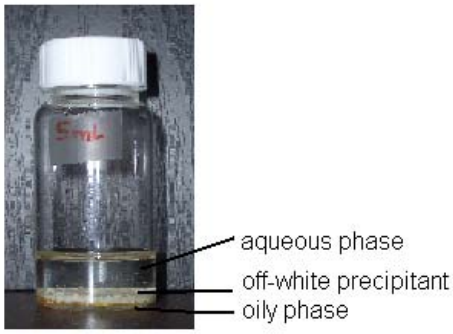


Figure 4-18: Complete phase separation of 10 mg/mL CMS liposomes after168 h storage at 25°C.

Figure 4-18 illustrates the appearance of CMS liposomes after the complete sedimentation of the liposome particles, as indicated by the clarity of the aqueous layer. The appearance of control samples (empty liposomes) and colistin-loaded liposomes, in contrast, were markedly different from CMS-loaded liposomes, both remaining as milky white dispersions after storage under identical conditions for the same duration.

Zeta potential

The zeta potential of colistin-loaded liposomes was consistent at approximately +10 - +15 mV over the week storage period (Figure 4-19). Conversely, the zeta potential of CMS-loaded liposomes markedly changed over the storage period (Figure 4-19) trending from negative to positive, indicating a charge reversal phenomenon.

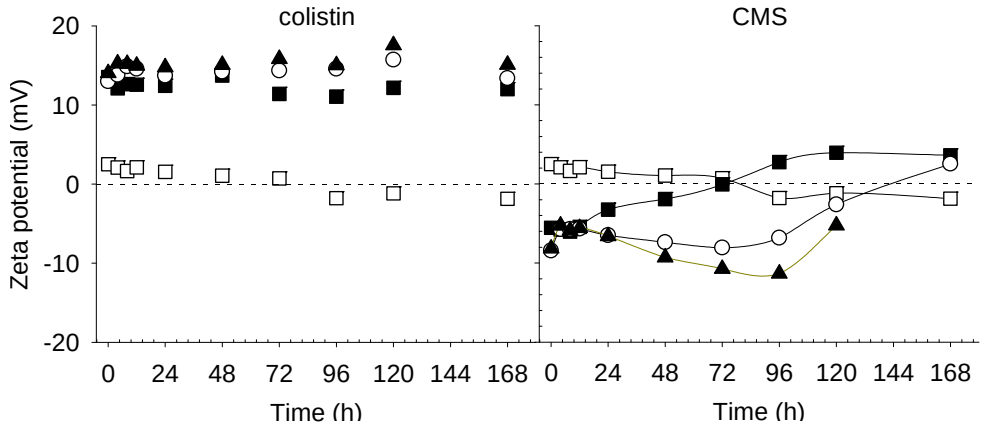


Figure 4-19: The colloidal stability of DOPC liposomes passively loaded with colistin (left) or CMS (right), as indicated by liposome zeta potential over 168 h. Blank (□), 1 mg/mL (■), 5 mg/mL (○)and 10 mg/mL (▲) CMS (n = 1).

To rule out the possibility that the colloidal instability of CMS in liposomes was due to an interaction with the glass storage container, the colloidal stability of CMS liposomes in polypropylene containers was also screened. Phase separation of CMS liposomes also occurred in polypropylene vials (Figure 4-20) within a similar timeframe to that which occurred in glass vials.

Figure 4-20: Colloidal instability of liposome formulations stored in polypropylene vials for 1 week. Left to right: empty liposomes, 10 mg/mL CMS liposomes, 20 mg/mL CMS liposomes.

A further experimental anomaly was observed while studying the stability of CMS liposome formulations. The rate of phase separation appeared to depend of the volume of liposome formulation dispensed into the storage container, with smaller volumes reaching phase separation more rapidly than larger volumes. This is illustrated visually in Figure 4-21, where the 15 mL samples were visually more stable than the other three fill volumes, and the rate of phase separation was more rapid for the 2 mL compared to the 5 mL fill volume (comparing the images for day 3 and day 7 at those volumes).

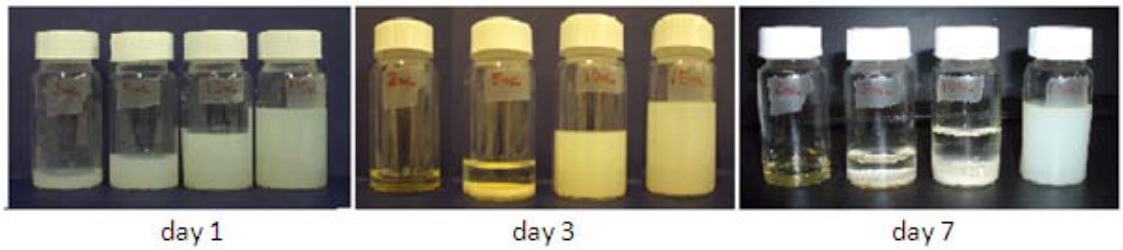


Figure 4-21: The effect of formulation volume on colloidal stability of liposomes containing 10 mg/mL CMS over 7 days. Vials contain (left to right) 2, 5, 10 and 15 mL of liposome suspension.

4.4.2.4 Chemical stability of CMS in DOPC liposomes

A potential explanation for the colloidal instability of CMS liposomes, as indicated by the particle size growth (Figure 4-15), particle sedimentation Figure 4-18 and surface charge reversal phenomenon (Figure 4-19) is the time-dependent conversion of CMS to colistin (Section 3.5.4).[362] Figure 4-22 illustrates the rates of formation of colistin in solution in the presence and absence of liposomes. In the absence of liposomes there was no substantial conversion of CMS to colistin, while the presence of liposomes greatly accelerated the formation of colistin.

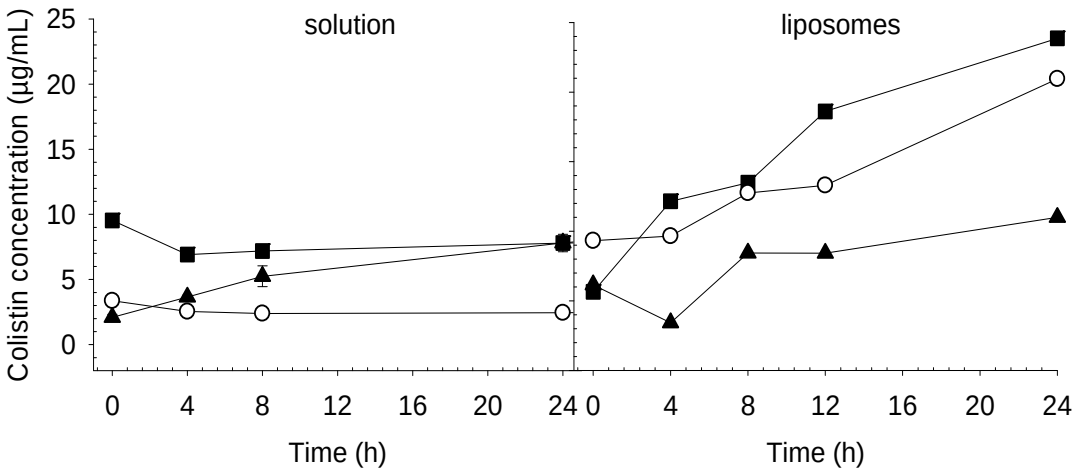


Figure 4-22: Colistin formed from CMS solutions (left) and DOPC liposomes (right) at 1 (■), 5 (○) and 10 (▲) mg/mL CMS (n = 1).

4.4.3 Chemical stability of colistin in solution

In order to use the formation of colistin as a measure of CMS instability in formulations, the stability of colistin itself had to be confirmed in solution under the conditions of CMS stability testing. The stability of colistin at 1 and 50 µg/mL when stored in solutions at 25°C is shown in Figure 4-23. Approximately 90% of the original amount of colistin present remains after 24 h, the duration of CMS liposome and solution stabilities studies shown in Figure 4-22. Therefore, the amount of colistin formed in CMS solutions at 25°C can be considered representative of the amount of CMS converted to colistin.

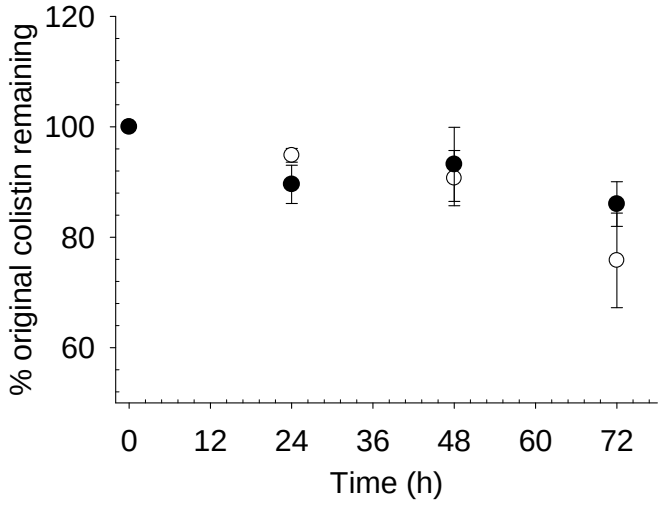


Figure 4-23: The stability of 1 (○) and 50 (●) µg /mL colistin in water at 25°C. Data are presented as mean ± SD (n = 3).

In light of the chemical and colloidal instability of CMS formulated into liposomes, and with the overall aim of this chapter being to provide an understanding of the interactions of colistin and CMS with liposomes for further formulation development, CMS-loaded liposomes were not further investigated. Remaining studies focus on colistin-loaded liposomes only.

4.4.4 Cryo-TEM

Cryo-TEM images of empty DOPC liposomes prepared by the dry film method are shown in Figure 4-24A and B. In these images, MLVs, OLVs and SUVs can be easily identified. On some grids, a number of LUVs were present. Images of colistin-loaded liposomes prepared by the dry film method, as pictured in Figure 4-25A and B, revealed rigid, intact, spherical vesicles that appeared to be mainly SUVs (although a small number of MLVs were observed on some grids). Unlike the empty liposomes, colistin-loaded liposomes appeared to contain fewer OLVs and MLVs. Very few LUVs were identified in colistin liposome samples, despite the fact that empty and colistin-loaded liposomes had very similar z-average particle sizes and exhibited similar particle size distributions (Figure 4-14). Furthermore, the colistin-loaded liposomes appeared to contain a larger population of SUVs, which is consistent with the wider polydispersity measured.

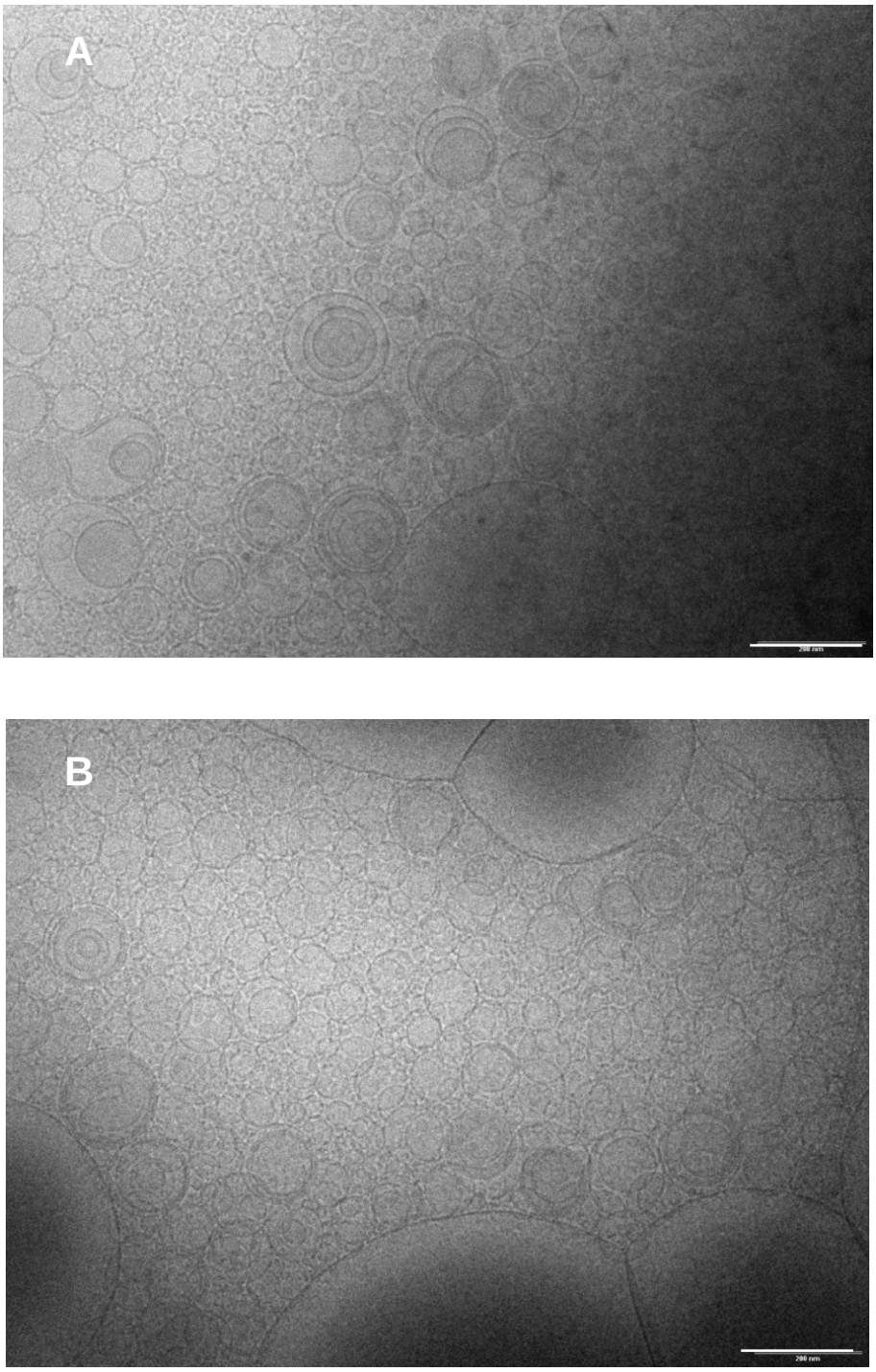


Figure 4-24: Cryo-TEM images of empty 5% DOPC liposomes prepared by the dry film method. Scale bars represent 200 nm.

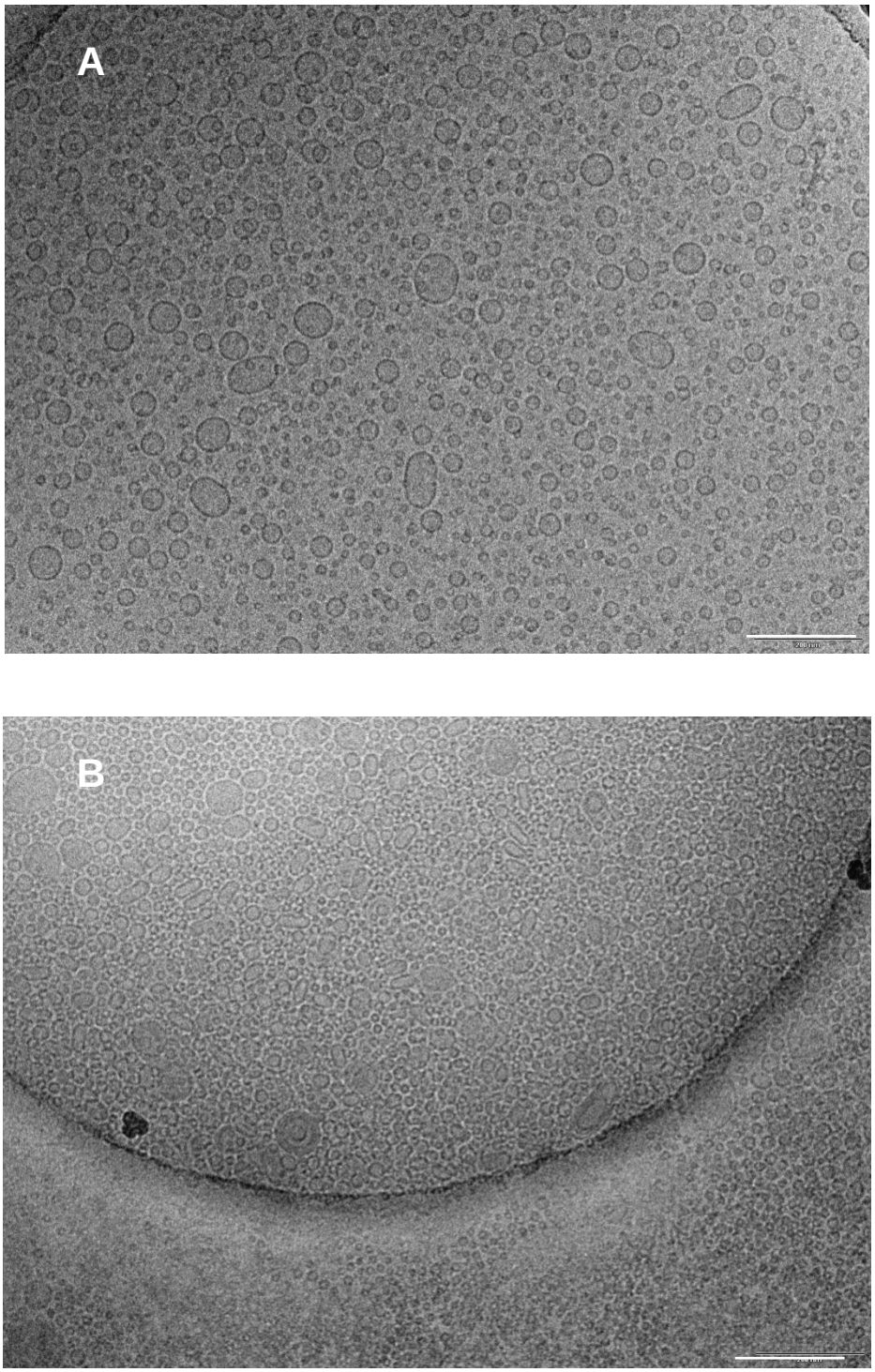


Figure 4-25: Cryo-TEM images of 5% DOPC liposomes containing 5 mg/mL colistin. Scale bars represent 200 nm.

4.4.5 Colistin liposomes produced by the freeze dry method

4.4.5.1 Particle size

The particle size properties of empty and colistin-loaded liposomes prepared by the freeze dry method were compared. Both methods produced liposomes of similar particle size distributions (Figure 4-26) with the addition of colistin resulting in slightly smaller z-average particle size and PDI (Table 4-1). The presence of a population of larger liposomes, ranging 3 - 6 µm, was detected (Figure 4-26) for both empty and colistin-loaded liposomes prepared by the freeze dry method.

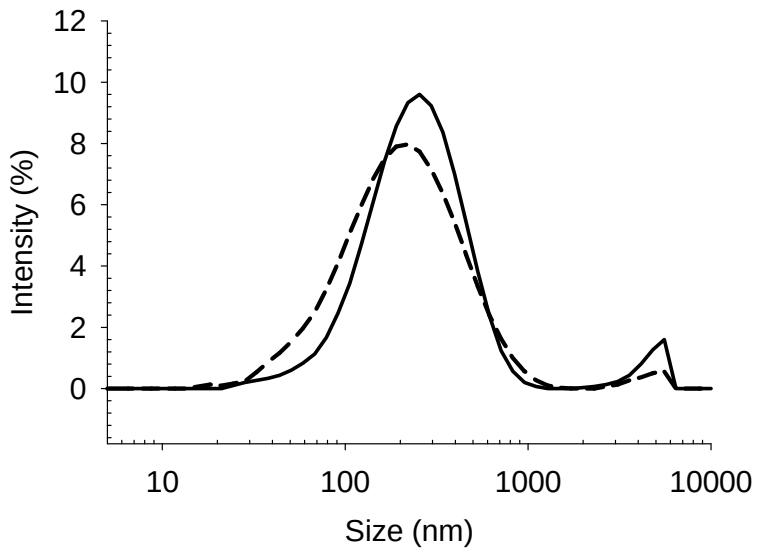


Figure 4-26: Particle size distributions of 1 mg/mL colistin liposomes prepared by the freeze dry method without colistin (solid line) and with 1 mg/mL colistin (dotted line).

Table 4-1: The z-average diameters and PDI of liposomes prepared with or without 1 mg/mL colistin by the freeze dry method.

	Z-average	**PD**
Without colistin	213.3 ± 0.49	0.368 ± 0.0050
With colistin	165.03 ± 3.35	0.355 ± 0.043

As the incorporation of colistin into freeze dried liposomes appeared to reduce the z-average diameter and the PDI of the freeze dried liposomes, the effect of increasing concentrations of colistin on particle size and PDI was investigated. As can be seen in Figure 4-27, the incorporation of colistin into liposomes beyond a final concentration of 2 mg/mL colistin did not have any substantial effect on reducing the z-average particle size or PDI of freeze dried liposomes.

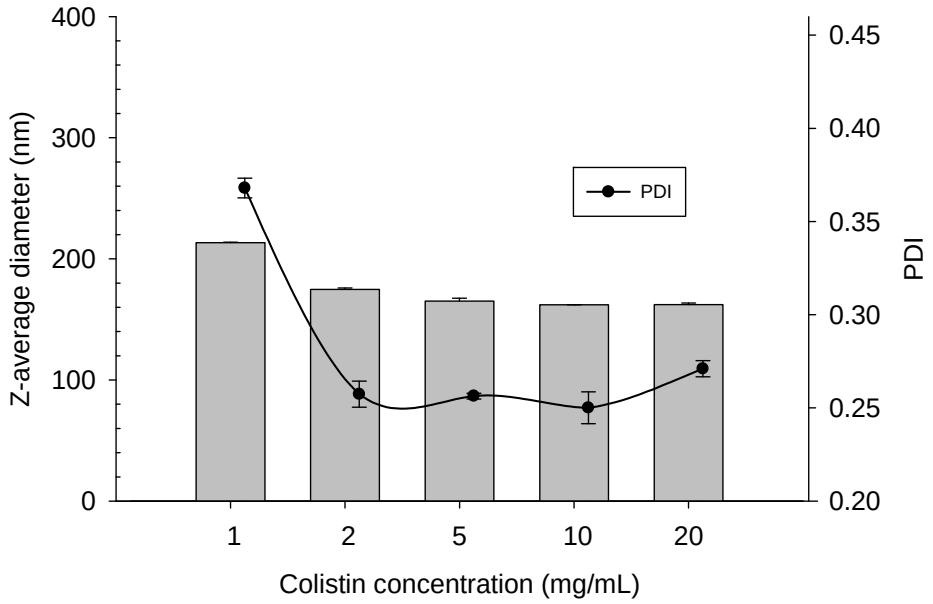


Figure 4-27: Effect of increasing concentrations of colistin on the z-average particle size and PDI of colistin liposomes prepared by the freeze dry method.

4.4.5.2 Cryo-TEM

Liposomes prepared by the freeze dry method proved to be somewhat difficult to analyse under cryo-TEM. The high radiation exposure rapidly degraded the sucrose in the samples, compromising image clarity and contrast. As a result of the high concentration of sucrose present, these particular formulations had to be diluted in water prior to analysis. In general, liposomes prepared without colistin appeared to be larger in size, with a population of large MLVs and OLVs dominating the image landscape. This appears to be consistent with the particle size distributions (Figure 4-26) obtained using DLS. Notably, empty liposomes were irregular in shape, less spherical and appeared to be more deformable than empty liposomes prepared by the dry film method (Figure 4-28). Due to the lack of clarity of the images obtained for freeze dried colistin liposomes (Figure 4-29), the exact morphology and structural the integrity of the freeze dried colistin-containing liposomes could not be clearly distinguished despite dilution in water prior to analysis.

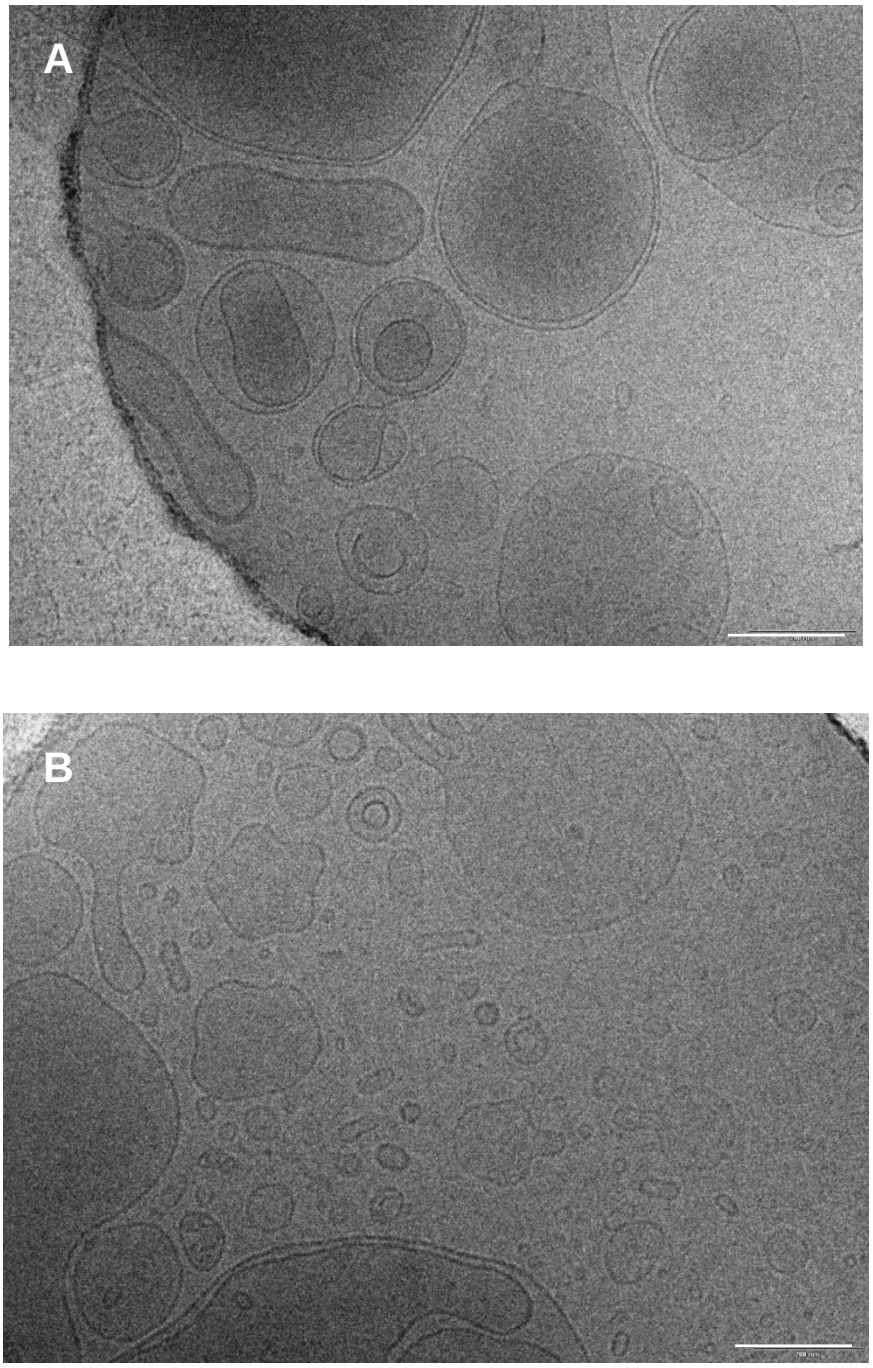


Figure 4-28: Cryo-TEM images of empty 5% DOPC liposomes prepared by the freeze dry method. Scale bars represent 200 nm.

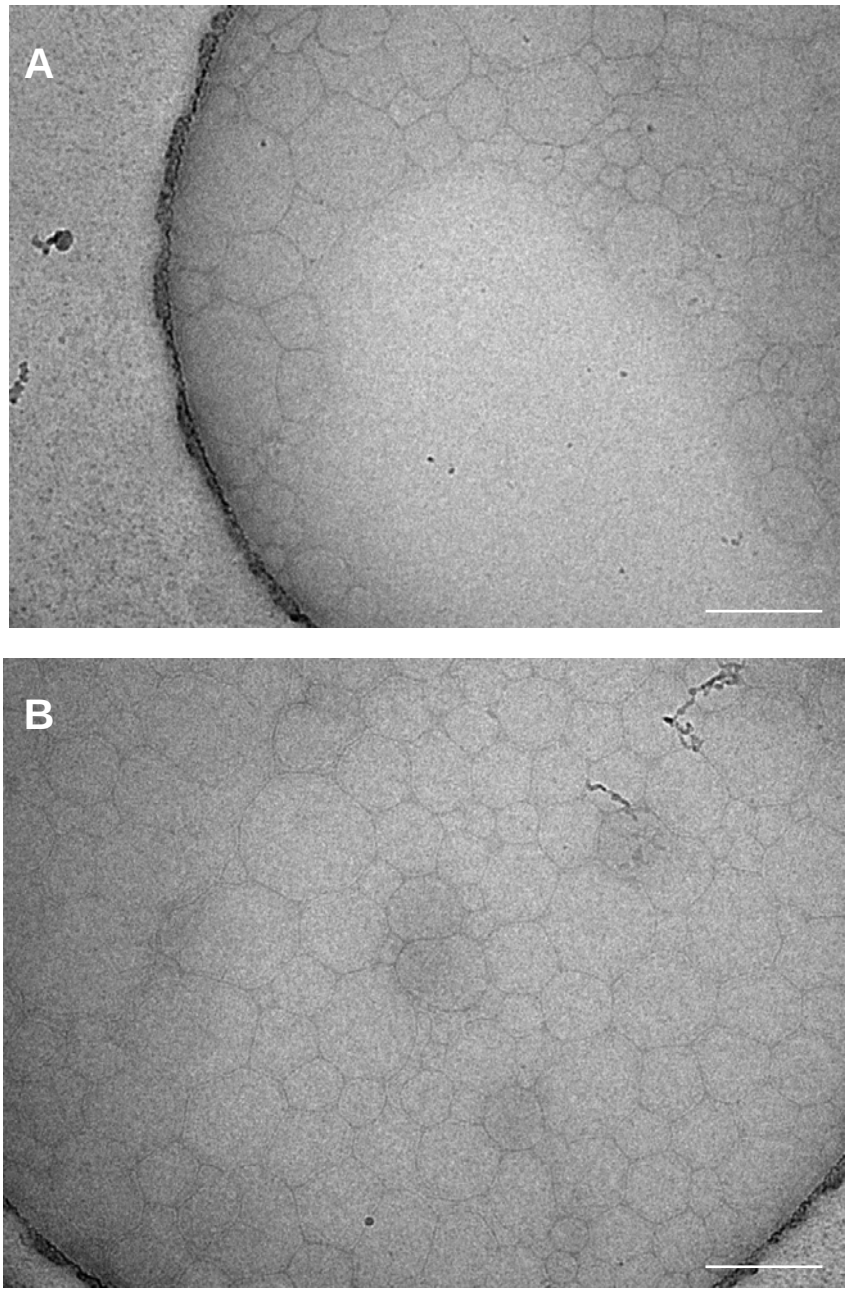
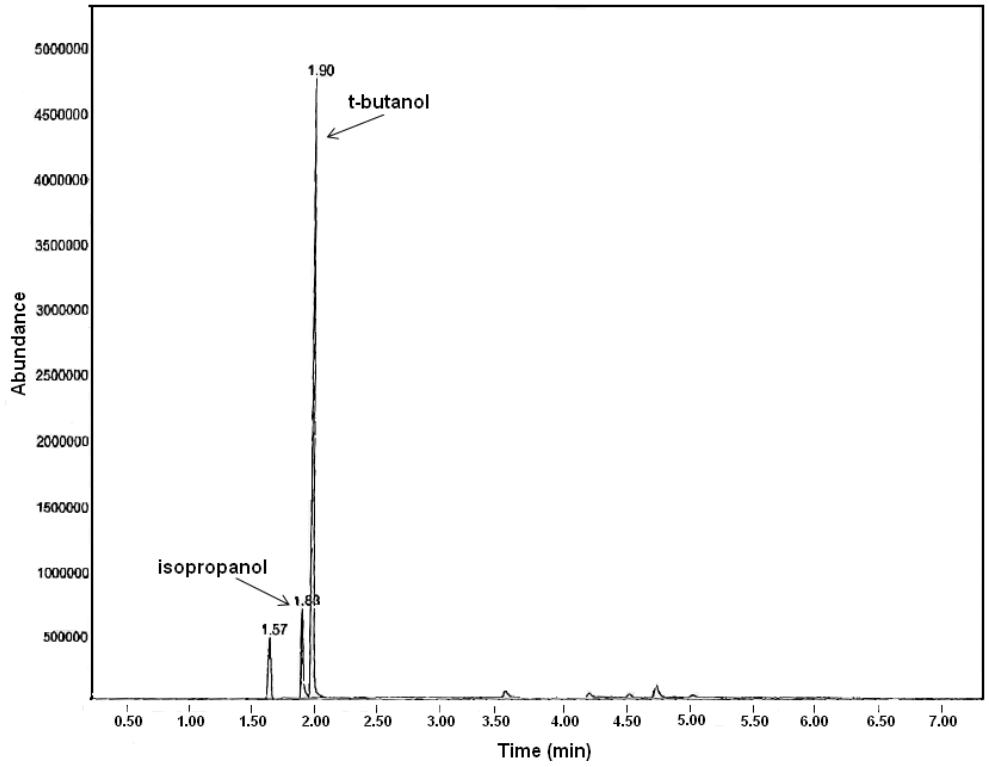


Figure 4-29: Cryo-TEM images of 5% DOPC liposomes containing 5 mg/mL colistin, prepared by the freeze dry method. Scale bars represent 200 nm.

4.4.5.3 Analysis of residual t-butanol in freeze dried liposomes by GC-MS

GC-MS revealed that less than 0.8% t-butanol remained in the reconstituted samples of liposomes prepared by the freeze dry method. The residual levels of t-butanol are consistent with those reported by Cui *et al.*[501] In addition to the quantification of t-butanol, this method also provided some qualitative information about the residual chloroform in liposome samples prepared by the dry film method, which were used as a matrix in which t-butanol standards were prepared. Interestingly, no chloroform could be detected.



4-30: Typical chromatogram obtained from GC-MS analysis of t-butanol in unknown freeze dried liposome samples.

4.4.6 Assessment of methods for measuring the encapsulation efficiency and *in vitro* release of colistin liposomes

Figure 4-31 shows the calibration curve produced by measurement of scattered light by DLS from highly diluted DOPC liposomes with increasing concentrations of DOPC. This calibration plot demonstrates the relationship between the light scattering of a sample and the concentration of DOPC liposomes in the sample. The use of this calibration plot enabled the quantitative comparison of the efficiency of separation of liposomes from free solution using the different techniques.

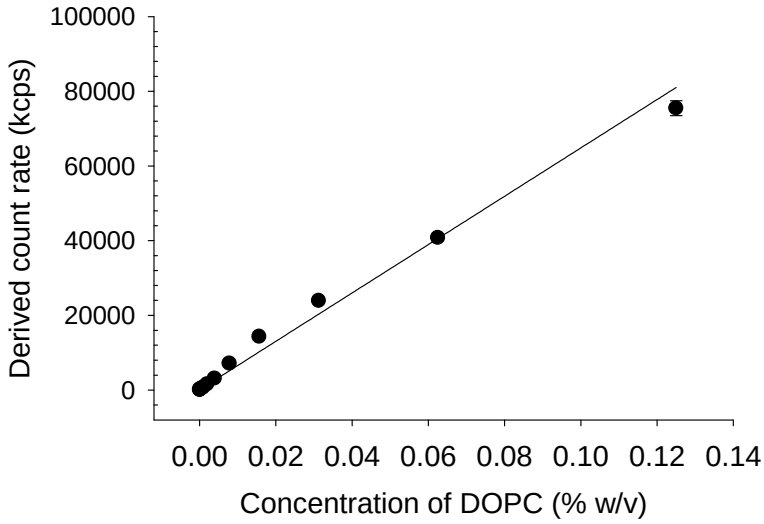


Figure 4-31: The light scattering (derived count rate, kcps) of DOPC liposomes in water with increasing concentration of DOPC. Data are presented as the mean ± SD (n = 3).

4.4.6.1 Ultracentrifugation

Visual observation of the high turbidity of ultracentrifuged liposome samples indicated that a large amount of liposomes remained in the supernatant, despite centrifugation at high *g*-forces for extended periods. Figure 4-32 shows that ultracentrifugation successfully sedimented the population of liposomes greater than 100 nm, however, liposomes between 15 - 100 nm were still present in the supernatant. Although the fraction of liposomes >100 nm could have been isolated by washing and resuspending the liposome pellet, the considerable amount of liposomes still present in the supernatant indicated by the degree of light scattering of the sample (Figure 4-33), meant that ultracentrifugation was an inefficient separation method for DOPC liposomes even at low concentrations of colistin (1 mg/mL). As determined from the calibration plot (Figure 4-31), the scattering of the ultracentrifuge supernatant indicated that 0.06% DOPC was present in the filtrate, equating to ~ 2.26% of the total liposomes in the formulation remaining in the supernatant.

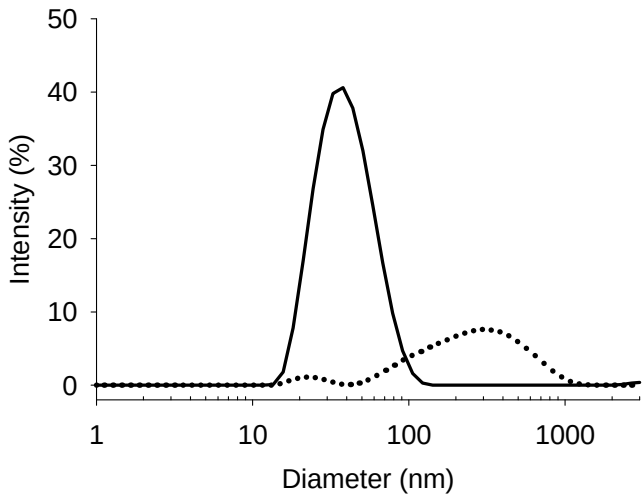


Figure 4-32: The particle size distribution of the supernatant obtained following centrifugation of DOPC liposomes containing 1 mg/mL colistin for 8 h at 300,000 *g*.

4.4.6.2 Centrifuge ultrafiltration

Figure 4-33 shows the relative light back scattering of filtrates obtained from the centrifuge ultrafiltration of 1 mg/mL colistin-loaded liposomes using the centrifuge ultrafiltration devices YM30, YM50 and Nanosep 100 (30, 50 100 kDa MWCO, respectively). As indicated by the decrease in light scattering by the ultrafiltrates, fewer or smaller liposomes penetrated the membrane as the MWCO of the membranes in the devices decreased. While the YM30 device achieved the best separation of liposomes from the surrounding dispersion medium, only an extremely small volume of filtrate (< 20 µL) could be recovered from each separation due to the clogging of the filter by the larger liposome particles. A slightly larger filtrate yield (50 µL) was obtained using the YM50 device, however light scattering measurements revealed a greater penetration of liposomes through the YM50 device, as indicated by the greater degree of light scattered by that sample (Figure 4-33). The light scattering data indicated that approximately 0.03, 0.05 and 2.26% of the total liposomes in the formulation penetrated the YM30, YM50 and Nanosep 100 ultra filters, respectively.

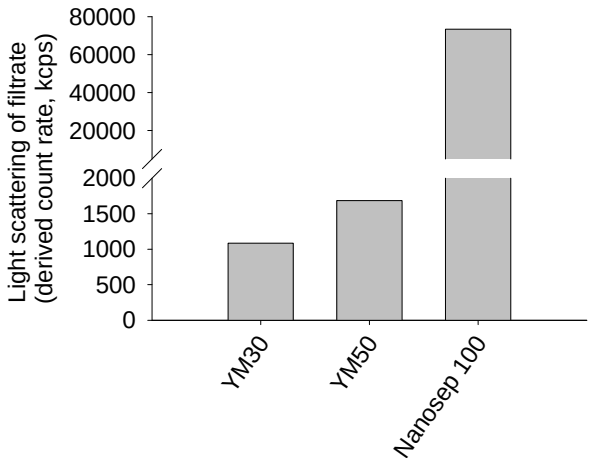


Figure 4-33: Light scattering of filtrates obtained using centrifuge ultrafiltration after separation of 1 mg/mL colistin DOPC liposomes using various ultrafiltration devices.

4.4.6.3 Pressure ultrafiltration

Separation of liposomes from their dispersing solution using the pressure ultrafiltration method consistently produced filtrates with light scattering similar to that of pure water, as shown in Figure 4-34. This clearly demonstrates that pressure ultrafiltration produces filtrates free of liposomes and is by far the most effective method for the separation of liposomes from their surrounding dispersion medium.

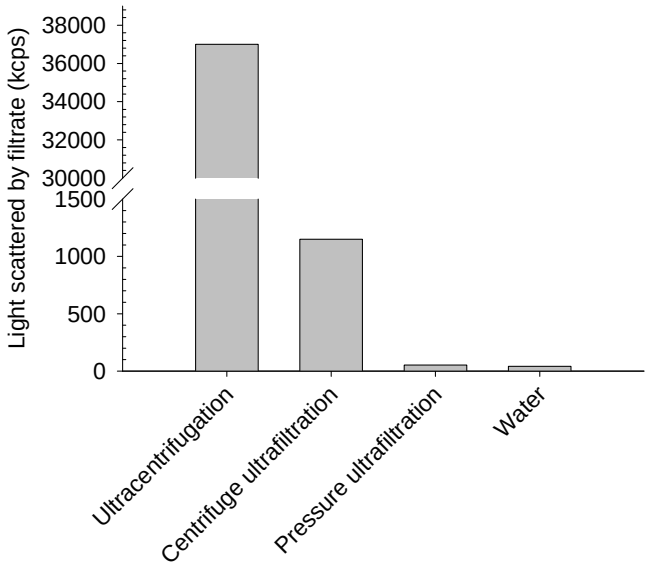


Figure 4-34: The presence of liposomes in the filtrate or supernatant, as measured by DLS, following separation of liposomes from their surrounding aqueous environment by ultracentrifugation, centrifuge ultrafiltration and pressure ultrafiltration. The light scattering of water is shown as a reference to samples completely free of liposomes.

Filter validation

Figure 4-35 shows the recovery of colistin following filtration of a 100 µg/mL colistin solution through the YM-10 pressure ultrafiltration membrane, showing that colistin adsorbed to regenerated cellulose ultrafiltration membrane. It was determined that 500 µL of a 100 µg/mL colistin solution had to be filtered through the membrane before the filter became saturated with colistin. Therefore, the adsorption capacity of the filter for colistin was determined to be approximately 50 µg. This degree of filter adsorption was taken into account when using pressure ultrafiltration for the assessment of EE and *in vitro* release.

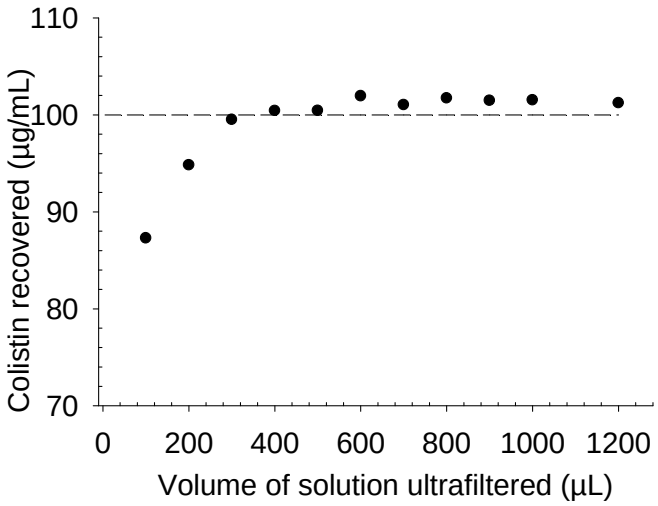


Figure 4-35: Adsorption of colistin to the YM-10 ultrafiltration membrane as measured by colistin recovery in the ultrafiltrate during filtration of colistin sulphate solution (100 µg/mL) (●). Dotted line indicates the target concentration.

4.4.7 Encapsulation efficiency

4.4.7.1 Colistin encapsulation in DOPC liposomes prepared by dry film and freeze dry methods

The EE of colistin in DOPC liposomes produced with increasing colistin concentrations by the two different methods is shown in Figure 4-36 (left panel). The EE was comparable between the two methods for the lower colistin concentrations, while at 20 mg/mL there was a slightly greater encapsulated fraction for the freeze dried preparation. The fraction of total colistin encapsulated was observed to decrease with increasing colistin concentration. Figure 4-36 (right panel) demonstrates there was a linear relationship between the encapsulated colistin:phospholipid mole ratio with increasing concentrations of colistin, for both methods. It also reveals that the encapsulated drug:lipid mole ratio was relatively low, typical of passive encapsulation techniques. It should be noted that concentrations of colistin obtained in the filtrate of liposome samples was five times greater than the concentration tested in filter adsorption studies (Figure 4-35).

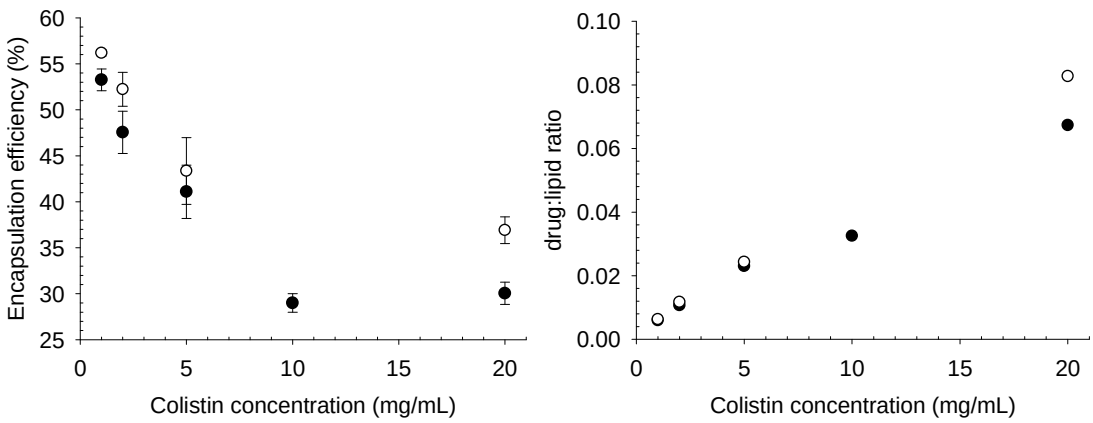


Figure 4-36: Left panel - the EE of colistin-loaded DOPC liposomes prepared by the dry film (●) and freeze dry (○) methods. Right panel - the relationship between the concentration of colistin in the aqueous phase used to disperse the DOPC, and the molar ratio of encapsulated colistin:phospholipid in the resulting liposomes.

4.4.7.2 Colistin encapsulation in DOPC and DOPC/cholesterol (2:1) liposomes prepared by the dry film method

The effect of the incorporation of cholesterol on the EE of colistin in liposomes prepared by the dry film method is shown in Figure 4-37. Cholesterol slightly improved the EE of colistin at all colistin concentrations tested.

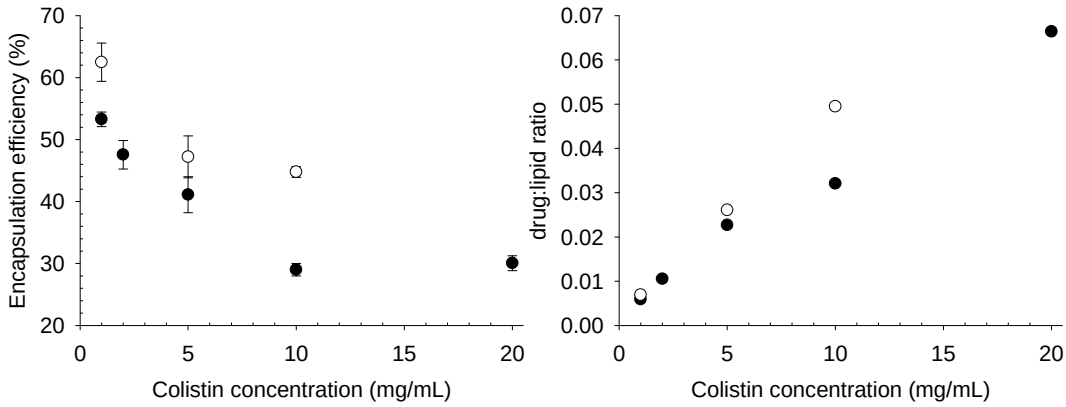


Figure 4-37: The effect of cholesterol on the EE of colistin in DOPC liposomes with (○) and without (●) cholesterol (molar ratio lipid:cholesterol of 2:1). Data are presented as the mean (± SD) of three separate determinations. Data for liposomes 'without cholesterol' are reproduced from Figure 4-36.

4.4.8 *In vitro* release of colistin from DOPC/cholesterol (2:1) liposomes

Figure 4-38 shows the *in vitro* release of colistin from liposomes prepared by the dry film method after the five-fold dilution v/v into phosphate buffered saline (PBS). Interestingly, by the time the first sample was taken (within 10 min post dilution), 50% of the total colistin content was freely available in the *in vitro* release medium. This proportion of unassociated or unencapsulated colistin remained constant throughout the 72 h sampling period, despite the colistin concentration gradient created by dilution into the *in vitro* release media.

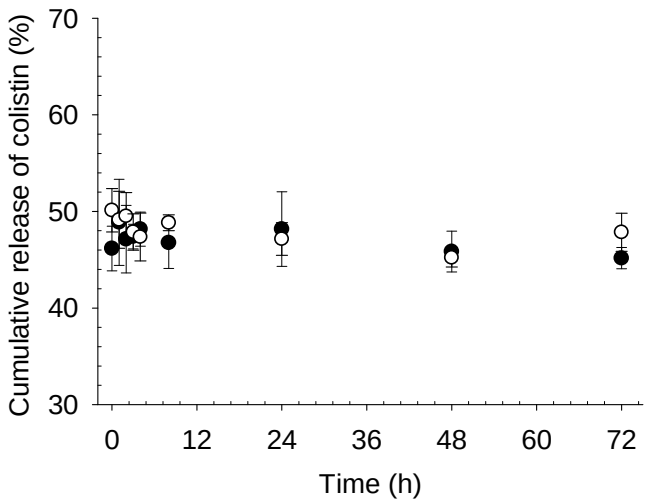


Figure 4-38: Cumulative release of colistin from DOPC/cholesterol (2:1) liposomes made by the dry film method, containing 10 (●) and 20 (○) mg/mL colistin. Data are presented as the mean (± SD) of three separate determinations.

4.5 Discussion

4.5.1 Association of colistin and CMS with DOPC liposomes

The fact that neutral liposomes adopted a surface charge in the presence of CMS or colistin seems to indicate an association between these peptide antibiotics and the DOPC bilayer. Similar observations were reported by McAllister and co-workers who investigated the use of cholesterol containing phosphatidylcholine liposomes for encapsulation of polymyxin B sulphate.[538] Increasing concentrations of CMS or colistin did not result in a linear increase in surface charge (Figure 4-12), suggesting that there may be limited capacity for interactions between the peptides and the bilayer. An alternative explanation for the plateau in zeta potential observed despite increasing concentrations of CMS and colistin is that the association of these antibiotics with the bilayer changes the properties of the bilayer, affecting association of further CMS or colistin molecules. It should be noted, however, that it has been reported that electrophoretic mobility is not always a true reflection of the net charge or electrostatic potential of a suspended colloid.[539-540] Hence, measurement of surface charge alone is not sufficient to fully define the interaction between colistin or CMS with the DOPC liposomes, but does indicate a direct interaction. Therefore, alternative methods to probe the nature of the interactions of colistin or CMS with DOPC liposomes were sought.

ITC was employed as a method to investigate the nature of the interactions between colistin and DOPC bilayers. Because the heat flux associated with CMS degradation was anticipated to complicate interpretation of CMS titration heat flux data, only the interactions of colistin with liposomes were investigated by this technique. The initial substantial endothermic heat flux upon titration of colistin into DOPC (Figure 4-13), beyond that of the titration of colistin into the matrix buffer, provides further evidence of an interaction between colistin and DOPC liposomes. Data from ITC experiments in

this study suggest that hydrophobic forces are the predominant interaction between colistin and DOPC liposomes, as shown by the strongly endothermic heat flux. The presence of the cholesterol (after correcting for all other influences) had a further substantial endothermic effect, raising the free energy of the system. This implies that a large entropic effect was driving the incorporation of the colistin into the liposomes and suggests that the role of ionic interactions between the cationic colistin and any surface charge of the liposomes was secondary, at least for this system. This is consistent with the driving force for micellisation of regular surfactants, due to the entropic gain on release of water molecules ordered around the hydrophobic tail. It is evident then, that the main interaction between colistin and DOPC liposomes occurred between the lipophilic region of the liposome bilayer (C_{18} alkyl chains) and the hydrophobic moiety of colistin (C_7 or C_8 acyl chain). This behaviour would seem consistent with the biological, membrane permeabilising mode of action of the polymyxins in which the fatty acid tail has been shown to play a crucial role.[429, 541] The increased heat flux upon incorporation of cholesterol into DOPC liposomes, indicating stronger interactions with the liposome bilayer, appears to be consistent with the data obtained for EE, whereby the incorporation of cholesterol resulted in greater encapsulation of colistin (Figure 4-37).

4.5.2 Effect of liposomes on chemical stability of CMS

Colistin liposomes exhibited colloidal stability, as indicated by stable z-average particle size, zeta potential and the visual appearance of the formulations over a 7-day storage period. CMS liposomes, on the other hand, exhibited obvious colloidal instability as indicated by growth in z-average particle size, an increase in PDI, surface charge inversion, particle sedimentation and finally, complete phase separation. A comparison of the chemical stability of CMS in water versus in DOPC liposomes indicated that, in the presence of DOPC, the conversion of CMS to colistin appears to be greatly accelerated. In Chapter 3 it was shown that CMS forms micellar aggregates in solution and is stable above the CMC, with micellisation proposed as the mechanism for the concentration-dependant stability of CMS in solution. Even at concentrations above the identified CMC of 6.1 mg/mL, rapid conversion of CMS to colistin occurred in the presence of liposomes. That the presence of liposomes accelerates the conversion of CMS to colistin strengthens the evidence presented in Chapter 3, that the self-assembly of CMS is the mechanism for the stabilisation of CMS at high concentrations. The association of CMS molecules with the liposomal bilayer must disrupt the intermolecular associations between CMS monomers that give rise to the CMS micellar clusters. The disruption of micelle formation would expose the susceptible sulphomethyl groups of CMS to the hydrolytic aqueous environment, thereby increasing the rate of CMS conversion to colistin.

4.5.3 Effect of degradation of CMS on liposome colloidal stability

The observed surface charge inversion and phase separation of CMS liposomes over time may be attributed to the chemical instability of CMS. The formation of appreciable amounts of colistin (a polycation) in the presence of CMS (a polyanion) would result in a complex mixture of oppositely charged ionic species. It is entirely possible that the formation of an insoluble ion pair complex between colistin and CMS instigated the colloidal instability of the system, causing phase separation. This explanation would account for the presence of the distinct off-white precipitant between the water and oil phases shown in Figure 4-18. Interestingly, unlike the conversion of CMS to colistin in water in the absence of liposomes, the phase separation of CMS liposomes did not follow a concentration-dependent pattern. This does not eliminate the possibility that the conversion of CMS to colistin is the underlying mechanism for the colloidal instability of CMS liposomes. It is possible that a certain threshold or ratio of colistin must be formed in the presence of CMS before the onset of phase separation. It must be noted that the phase separation of CMS liposomes made it difficult to obtain homogenous samples after 24 h for the measurement of zeta potential, particle size and colistin concentration.

That the volume of CMS liposomes affected the rate of phase separation was considered an unusual experimental anomaly which demonstrates the complexity of the interfacial behaviour occurring in CMS-loaded liposome formulations. Clearly, CMS liposomes in their simple form do not present a useful dosage form. Further experiments would have been required to elucidate the mechanism underlying the volume effect but this is beyond the scope of this thesis, and was not considered necessary given the failure of CMS liposomes from a stability perspective.

In order to adequately address the remaining hypotheses of this experimental chapter, physicochemical characterisation continued using colistin liposomes only. Even though CMS is the form of colistin currently used clinically to treat pulmonary infections via inhalation, there may be advantages associated with the use of colistin, rather than CMS, for pulmonary administration. First, the administered amount of the prodrug CMS does not necessarily predict therapeutic efficacy because the pharmacological activity of CMS relies on the conversion of CMS to colistin *in vivo*.[71] Concentrations of colistin achieved within the lungs following administration of CMS may be difficult to predict, and may be variable according to the concentration of the CMS solution administered and the extent of dilution within lung fluids. For example, Marchand *et al*., have shown that, following IT administration of a 50 mg/mL CMS solution to rats, only 1/3 of the CMS administered was converted to colistin within the lung fluid.[142] The same study demonstrated that it may take a considerable period of time (up to 2 h) following IT administration for colistin concentrations in lung fluid to reach detectable levels. Given that the conversion of CMS to colistin is concentration-dependent, the rate of conversion within the lungs may also be variable, depending on the concentration of CMS in the solution administered and the extent to which that solution is diluted within lung fluids upon administration.

The systemic bioavailability (BA) of colistin following administration of CMS is higher than that occurring after administration of colistin following intratracheal instillation to rats.[139, 147] Therefore, pulmonary administration of colistin may offer several advantages over delivery of CMS. Administration of colistin, instead of CMS, would result in immediate local availability of colistin and lower systemic BA of colistin. It is possible that administration of colistin would also result in establishing more predictable concentrations of colistin within lung fluid. Although colistin is more irritable to the

airways than CMS,[167] the encapsulation of colistin within a liposomal carrier may act to reduce local airway irritability. Furthermore, lower doses of colistin may be required to achieve comparable *in vivo* concentrations of colistin to those that are currently achieved following pulmonary dosing of CMS.[542-543] Polymyxin B (sulphate) (Figure 1-4) is already given by inhalation for the treatment of respiratory infections.[253, 544]

4.5.4 Differences between liposomes prepared by dry film and freeze dry methods

The differences in morphology observed between empty DOPC liposomes and those loaded with colistin, prepared by the dry film method, may be attributed to the surface activity of colistin (Figure 3-3). The surface active behaviour of colistin, and more specifically the charge that it imparts on the forming liposome particles, may have aided dispersion of the lipid, producing more SUVs and fewer MLVs. The surface activity of colistin may also account for the population of very small vesicles in the order of 10 - 30 nm observed in the images of colistin liposomes. It has been suggested by other authors that mixed micelles can exist in lipid formulations of polymyxin B.[545] The possibility that these smaller vesicles were mixed micelles of colistin and phospholipid should not be overlooked, though cannot be confirmed on the basis of particle size measurements and cryo-TEM images alone.

The manufacture of liposomes by freeze drying a lipid and t-butanol monophase solution, as first reported by Li *et al.*,[502] was investigated as an alternative method for the convenient manufacture of colistin-loaded liposomes. The manufacture of liposomes by this method has been associated with high EEs for a number of drugs,[502] so the potential for this method to improve the EE of colistin above that of the dry film method was worthy of investigation. Physicochemical characterisation of freeze dried liposomes revealed that the liposomes produced by this method had slightly larger z-averages than

those reported previously by other authors.[502] In addition, the PDI of liposomes produced in this study (0.35 - 0.37) were notably larger than those reported by Li *et al*. (0.07 - 014).[502] This may be due to one of a number of factors reported to influence the size of liposomes produced by this method, including the rate of hydration upon reconstitution.[501-502] The incorporation of colistin into the freeze dried liposomes had a minor effect on the z-average particle size and PDIs, but no noteworthy reduction occurred beyond the addition of 2 mg/mL colistin.

The apparent difference in the structural rigidity of liposomes prepared by the dry film and freeze dry methods may be attributed to the presence of residual amounts of t-butanol in the lyophilisate of the freeze-dried liposomes. Cui *et al*.[501] reported that residual t-butanol does not affect the retention of encapsulated materials,[501] however, residual t-butanol is a concern from the perspective of potential toxicity; the presence of appreciable amounts may cause airway irritation. The utility of the freeze dry method in the production of colistin liposomes was therefore dependent upon the relative capacity for the freeze dried liposomes to encapsulate colistin, compared to the dry film method.

4.5.5 Assessment of liposome separation efficiency

To enable reliable measurement of the EEs of liposomes, a method for the efficient and reliable separation of unencapsulated and encapsulated colistin was required. There are numerous methodologies available for the separation of free drug from drug encapsulated within or bound nanoparticles (Section 4.1.4.1), however the validation of the separation rarely describes an independent verification of exclusion of particles from ‘free solution’. In this study DLS was used to compare the efficiency of three separation methods for separation of liposomes from unencapsulated drug by detecting the presence of liposomes in separated solutions using back scattering of light. To the best of the author’s knowledge, this technique has not been reported in the literature

previously. It is believed that this DLS method can be directly applied to many other nanoparticle systems, not just liposomes.

Ultracentrifugation is a commonly reported method for the separation of liposomes and other nanoparticles from a dispersing medium.[478] Separation by ultracentrifugation is impossible to achieve in a situation where the density of the dispersing solution is closely matched to the density of the particles to be separated, as occurred in this study. The only resolution to this problem is to dilute the liposome suspension. For many microdisperse systems, including liposomes, dilution can alter the distribution of drug between the nanoparticle and dispersion medium.[519] The ideal method for separation in the measurement of EE will allow separations to be carried out on the undiluted formulation, as intended to be administered *in vivo*.

Centrifuge ultrafiltration is commonly reported in the literature for the determination of nanoparticle EE. However, this approach was shown to have a number of significant drawbacks. Filtration devices with molecular weight cut-offs ≥100 kDa did not efficiently separate liposomes from the surrounding aqueous medium as indicated by DLS, with 2.26% of the liposomes penetrating the ultrafiltration membrane. The 30 and 50 kDa units achieved efficient separation with <0.05% of the liposomes in the formulation crossing the membrane, though the yield of filtrate obtained with these devices was extremely low due to the clogging of the filter by liposomes. Low yield was not only an issue because a minimum volume is required for HPLC analysis of drug content, but also because colistin has the potential to non-specifically bind to the filter membrane, and plastic of the filter devices, meaning that any errors due to binding are exaggerated by the low yield. Due to non-specific binding, the concentration of colistin in filtrates may not necessarily reflect the concentration of unencapsulated colistin in the liposome dispersion medium.

Pressure ultrafiltration was determined to be a highly efficient method for the separation of liposome drug from free drug in solution. A sufficient volume of filtrate could be obtained within 5 - 10 min for the quantification of drug in the filtrate, taking into consideration the volume that had to be discarded due to the adsorption of colistin to the regenerated cellulose ultrafiltration membrane. This method is therefore highly useful, even for drugs which exhibit a high degree of non-specific adsorption to the membrane filter such as colistin. Pressure ultrafiltration was therefore employed as the method for separations in subsequent EE and *in vitro* drug release experiments.

4.5.6 Encapsulation efficiency of colistin liposomes

The low log P values of -3.15 for colistin A and -3.68 for colistin B (predicted by ACD LogD Suite version 9, Toronto, Canada), respectively, would dictate that colistin be classified as highly water soluble and therefore a drug that is most suitable for loading within the aqueous liposome interior. Maurer *et al*. estimated that the maximum EE of drugs within the liposomal aqueous core on the basis of high water solubility, should be around 25% when 100 mM phospholipid is used.[341] The liposomal encapsulation of colistin was shown to be twice that predicted by Maurer *et al*., even though colistin liposome formulations contained lower lipid concentrations (64.5 mM). This suggests that the encapsulation of colistin may not solely be attributed to the encapsulated volume, but also to association between colistin and the phospholipid bilayer.

Despite the reasonably high EE measured following the passive loading of colistin into DOPC liposomes, it is the drug:lipid ratio which gives the most information about the utility of liposomes as a drug carrier. In this study, the molar ratios of encapsulated colistin to phospholipid were determined to be very low (<0.1:1, Figure 4-36 and Figure 4-37) and this may ultimately limit the practical application of liposomes as a colloidal carrier for colistin. The slightly higher encapsulation achieved by the freeze dry method

is unlikely to translate into a significant advantage over the dry film method from a drug delivery perspective given that the drug:lipid mole ratio is relatively low in either case.

Omri *et al*. previously reported the encapsulated drug:lipid ratio of 5.6:1 for polymyxin B (a compound closely related to colistin, Figure 1-4) passively loaded into dipalmitoyl phoshphatidylcholine (DPPC, a neutral phospholipid) liposomes by the dry film method.[332] Given that encapsulated drug:lipid ratios of that order are extremely unlikely to result from passive encapsulation, and are usually only obtainable using remote-loading techniques, it is difficult to understand how that level of encapsulation was attained.[523] Wang *et al*.[546] used centrifuge ultrafiltration to measure the EE of 1 mg/mL colistin liposomes (lipid:cholesterol 1:3, phospholipid used not specified) and found an EE of 40 - 50%, which is similar to that determined in the present study.

The presence of residual t-butanol would have the potential to introduce confounding factors to the pharmacokinetic evaluation of freeze dried liposomes following pulmonary delivery (Chapter 6). Because of this, and also the fact that the freeze dry method did not confer any significant advantage over the dry film method with respect to drug loading, it was decided that subsequent *in vitro* and *in vivo* assessments of colistin liposomes would be carried out on liposomes prepared by the freeze dry method only.

4.5.7 *In vitro* release of colistin from liposomes

The assessment of *in vitro* liposome performance is crucial in understanding the factors controlling drug release from a liposome carrier. When studying the release of drug from nanoparticles *in vitro*, it is necessary to simulate *in vivo* conditions as closely as possible and this often requires the dilution of the nanoparticle formulation into a release medium. The dilution of colistin liposomes by 1:5 in the assessment of the *in*

vitro release of colistin was selected with a number of considerations in mind. First, because the unencapsulated proportion of colistin was not removed prior to *in vitro* release studies, the dilution into the *in vitro* release medium needed to be sufficiently large to create a transmembrane concentration gradient of colistin, in order to induce leakage of the encapsulated proportion. Secondly, the extent of liposome dilution that would occur in the ELF when liposomes are administered via inhalation to humans needed to be considered. The estimated volume of human ELF is reported to be between 15 - 70 mL.[537] Therefore, the inhaled dose of 2 - 5 mL of liposome formulation[197, 547] may be diluted by a factor anywhere between 1:3 and 1:5. Finally, the limits of the analytical method for quantification of colistin in *in vitro* release experiments had to be considered. After taking all of the aforementioned considerations into account, a dilution factor of 1:5 was selected.

Data from *in vitro* release experiments shown in Figure 4-38 should be interpreted carefully. Prior to dilution into the *in vitro* release media, 50% of the colistin in the liposome formulation was shown to be unencapsulated or not associated with the liposomes. Within minutes of dilution into the *in vitro* release media, the unencapsulated colistin was determined to be approximately 50% and the cumulative release of colistin remained at this level over the entire 72 h period, despite that a colistin concentration gradient had been created across the liposomal bilayer upon dilution. Similar *in vitro* release behaviour was shown by Schreier *et al*. following liposomal encapsulation of triamcinolone acetonide.[548] Two explanations are provided to explain this observation. The first is that colistin is tightly bound within liposomes and does not leak out upon dilution, though this situation would seem unlikely. An alternative explanation is that colistin rapidly redistributes out of the liposome upon dilution, due to the rapid partitioning of colistin across the liposomal bilayer. Given that

colistin is amphipathic and has been shown to interact with biological membranes,[549-550] the prospect of colistin undergoing spontaneous partitioning into and out of liposomes by passage across the lipid bilayer is not out of the question. In addition, the interaction of colistin with the bilayer may affect the permeability of the bilayer, contributing to the rapid partitioning of colistin across the bilayer in response to a concentration gradient.

Wang *et al*.[546] used the sac dialysis method to study *in vitro* release of colistin from liposomes. They reported an initial burst release of 40%, which they attributed to the passage of unencapsulated colistin across the dialysis membrane, followed by an apparent slow release to 100% over the subsequent 24 h period. However, the use of dialysis to study *in vitro* release has significant drawbacks. First, the use of membrane dialysis does not require that the formulation be diluted prior to measuring *in vitro* release. Substantial dilution occurs upon administration *in vivo*, therefore dialysis misrepresents the *in vivo* situation. Further, the passage of drug across the dialysis membrane is often dictated or limited by membrane transport effects,[310] so slow appearance of free drug in the acceptor compartment can incorrectly be attributed to "controlled' or "slow" release, when in actual fact this is not the case. In the studies carried out by Wang *et al*.[546] the time to establish equilibrium concentrations of colistin on either side of the dialysis membrane was reported to be 6 h. Due to this long equilibration time, the concentration of drug in the acceptor chamber may not necessarily reflect the concentrations in the donor chamber at any one time, due to the significant delay in equilibration. Hence, it can be difficult to obtain a true representation of the distribution of drug between the liposomes and surrounding dispersant. Regardless of the time to reach equilibrium, equilibrium dialysis has another major shortcoming in the study of drug release from nanoparticles; frequent sampling from and dilution into the same acceptor compartment causes the system to re-

equilibrate, in effect, forcing the release of drug from the donor compartment by creating a constant concentration gradient. This may account for the apparent 'slow release' observed following the initial "burst" release of 40% in the study carried out by Wang *et al*.[546]

The evidence from EE (Figure 4-36 and Figure 4-37) and *in vitro* release experiments (Figure 4-38) in this study tends to indicate that colistin does interact with the liposomal bilayer *via* non-hydrophobic interactions. Evidence from the literature regarding the nature of the interaction between colistin and neutral phospholipids, on the other hand, seems to be somewhat contradictory. Some authors suggest that interactions occur between charged phospholipids, but not zwitterionic neutral phospholipids,[551] while other authors suggest that there is no substantial difference between the association of the polymyxins with neutral and charged phosopholipids.[373, 485] Mestres *et al*. studied the interaction between colistin and DPPC liposomes based on fluorescence measurements made following the incubation of DPPC liposomes with colistin and two different fluorescent probes.[373] They concluded that the magnitude of fluorescence was modest in comparison to other compounds capable of modifying bilayer fluidity,[373] which is very surprising given the high ratios of colistin:phospholipid used in that study. The ratios used in the present study were much lower in comparison, but still seem to imply that colistin modifies the liposome bilayer enough to affect its own release. Mestres *et al*. noted little difference in the interactions between colistin and neutral or oppositely charged phospholipids, based on compression isotherm studies,[373] suggesting that the contribution of electrostatic interactions is probably not substantial.

At low concentrations of colistin (in the μM range) and phospholipid, but at a high colistin to lipid ratio of 1:1, Colome *et al*., showed that colistin can induce the leakage of a small fluorescent compound, carboxyfluorscein from phosphatidylcholine/

cholesterol liposomes. In agreement with Mestres *et al*., Colome and co-workers observed little difference in CF leakage when anionic phospholipids where incorporated into the system, implying little contribution of electrostatic interactions on the fluidising effect on the bilayer. Pache *et al*., on the other hand, suggested a more significant contribution of electrostatic interactions between polymyxin B and phosphatidylcholine liposomes based on evidence from NMR studies.[551]

4.6 Conclusion

The potential utility of liposomal formulations of CMS or colistin for improved pulmonary delivery of colistin has been screened by physicochemical characterisation of liposome formulations. Due to poor colloidal stability, CMS liposomes in their simple form are unlikely to present a useful dosage form for the delivery of CMS to the respiratory tract. Colistin-loaded liposomes, on the other hand, have the potential for further investigation and development. DOPC and DOPC/cholesterol (2:1) liposomes achieved reasonable EE of colistin, considering the passive basis of the encapsulation process. However, the drug:lipid ratio is quite low in comparison to that achieved in commercial liposome formulations.[349]

This study has highlighted the requirement for efficient, reliable methods for the study of drug release from liposomes, particularly those drugs undergoing complex interactions with the bilayer and exhibiting atypical drug release. Pressure ultrafiltration was determined to be the best method for the separation of liposome encapsulated or associated drug from drug free in solution in the determination of EE and *in vitro* release.

The release of colistin from DOPC liposomes appears to be under partition control. To this end, it seems that the prospect of using liposomes for the controlled release of colistin to the lungs may be limited. However, if the interactions between colistin and the liposomal bilayer are strong enough to be maintained when the formulation is dosed to the lungs, the absorption of colistin may potentially be modified by incorporation of colistin into liposomes. Irrespective of the potential of liposomes to provide a controlled or sustained release of colistin to the lungs, liposomes are still considered to be a useful vehicle for the incorporation and co-localisation of colistin and a second poorly-water

soluble antibiotic and will be the subject of further investigation in the following chapter (Chapter 5).

It has been shown, using ITC, that the interaction between colistin and neutral DOPC liposome bilayers is predominantly driven by hydrophobic forces. Based on *in vitro* release data, the interaction between colistin and DOPC seems to modify the fluidity of the liposomal bilayer enough to affect the release of colistin itself. The consequence of colistin-induced altered bilayer fluidity on the release of co-encapsulated agents was the subject of investigations in Chapter 5.

Chapter 5: Investigation into colloidal carriers for co-formulation of colistin with a model 'co-drug'

5.1 Introduction

Efficient delivery of colistin in combination with a second antibiotic agent directly to the lungs in the treatment of MDR Gram-negative respiratory infections may be an important approach towards minimising the emergence of bacterial resistance to colistin. A major issue that needs to be overcome in the development of combination therapies is that the two agents may differ substantially in their physicochemical and biopharmaceutical properties. This issue may be overcome through co-formulation of the drug combination into a colloidal drug delivery system. This approach may also result in the modification of drug release upon administration, enabling co-ordinated release of both agents within the lungs, thereby optimising local pulmonary concentrations of both antibiotic agents.

Colistin has been shown to interact with liposome bilayers[373] (Chapter 4) and therefore has the potential to affect the loading and release of antibiotics co-formulated into a colloidal-based carrier. Liposomes, as investigated in Chapter 4, may have the capacity to modify the release of colistin following pulmonary delivery and have therefore been selected as a colloidal system to be investigated for loading and release of a model co-drug. Lipid emulsions represent a drug delivery system with the capacity to co-localise drugs of differing physicochemical properties, and have therefore been identified as an alternative drug delivery system to liposomes. Characterisation of the capacity of each system to solubilise and release a selected 'co-drug' will allow investigations into the effect of colistin on the release of a co-encapsulated agent. The colloidal drug delivery system deemed to have the greatest potential to provide efficient delivery of the

antibiotic combination to the lungs will be progressed towards *in vitro* release studies in which the effect of colistin on co-drug release from the colloidal carrier will be examined.

5.1.1 Sub micron emulsions (SMEs)

SMEs are lipid emulsions comprised of lipid droplets stabilised by one or more surfactants in an aqueous phase, having a droplet size range of 100 - 1000 nm.[305] Unlike 'self-nanoemulsifying systems', SMEs do not spontaneously form nanometer sized droplets upon dispersion of lipid and surfactants in water, but require the input of energy for particle size reduction. SMEs are therefore considered to be thermodynamically unstable systems. SMEs provide an alternative formulation strategy to liposomes in the co-formulation of colistin and a second antibiotic, whereby colistin can be contained within the aqueous phase, and/or at the interface between the oil and aqueous phase, and a poorly-water soluble 'co-drug' can be contained within the dispersed lipid phase. The solubilisation capacity of SMEs for a given poorly-water soluble drug depends primarily on the solubility of the drug in the chosen lipid.[552] SMEs, with the exception of multiple emulsions, are typically 'burst' release systems, releasing their drug payload rapidly upon dilution through partition of drug out of the oil into the aqueous phase.[310, 518, 553] Multiple emulsions, whose structure consists of dispersed lipid droplets with water droplets inside (i.e. water-in-oil-in-water emulsions) have the potential to provide a sustained or controlled release of drug due to the existence of the middle oil layer which may act like a liquid membrane (Section 1.4.3.2).[305, 554-555] Extensive research has been carried out into the use of SMEs for the delivery of hydrophobic drugs including testosterone, progesterone and vitamins A, D, E and K via various routes of administration.[552] To date, the investigation of SMEs for pulmonary delivery has primarily been limited to the delivery of prostaglandin E_1[556] and

nucleic acids.[306, 308] Emulsions intended specifically for pulmonary delivery have not received a great deal of attention in the literature[557] and may represent an under-utilised formulation approach for the delivery of drugs to the respiratory tract.

5.1.2 Liposomes

The use of liposomes as an approach to the co-formulation of drug combinations was proposed as early as 1982 by Kirby and Bangham.[558] Although liposomes containing more than one biologically active agent have yet to translate into a commercial pharmaceutical product, excellent drug loading and co-ordinated release of co-encapsulated drugs from liposomes has been demonstrated, giving promise to the future of combination products based on this drug delivery system.[320-321, 352, 354-355, 558] As introduced in Section 4.1.2.3, one approach to the liposomal-loading of poorly-water soluble drugs is via passive loading into the liposomal bilayer. A further advance in liposome technology that has most certainly contributed to the commercial success of liposomes as a drug carrier is the discovery of methods by which drugs may be 'actively' or 'remotely' loaded into liposomes.

Remote-loading of drug into the aqueous interior of liposomes can achieve high drug loading[559] and controlled release of drug from liposomes.[349, 479] A remote-loading technique is utilised in the production of the commercial liposomal formulation of doxorubicin, Doxil®.[560-561] Remote-loading techniques rely on the generation of an electrochemical gradient across the liposomal bilayer using H^+ or other ions,[320, 477, 559, 562] as summarised in Figure 5-1. Importantly, however, high drug loading can be achieved by remote-loading methods only if the drug under investigation exhibits certain physicochemical properties and therefore is only suitable for the loading of many drugs for which a liposomal formulation may provide benefit. To be amenable to remote-loading techniques, the drug should have one or more weakly basic or weakly

acidic ionisable groups.[348] Secondly, the drug should have reasonable aqueous solubility.[348]

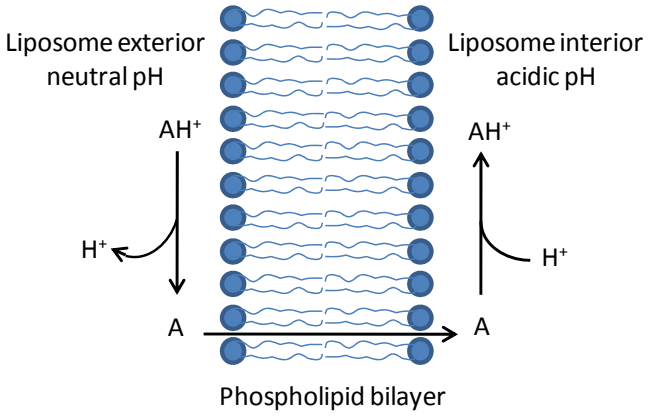


Figure 5-1: Schematic representation of the uptake of drug 'A' in response to the transmembrane gradient. Drug 'A', a weak base, is ionised in an acidic extra-liposomal environment. Increasing the pH of the liposome exterior results in the conversion of drug A into its unionised state. The unionised form rapidly partitions into and across the lipid bilayer into the acidic environment of the intra-liposomal space, where it becomes protonated and trapped within the liposome interior. This process drives the continuous uptake of the unionised form of drug A into the liposome interior. Adapted from Cullis *et al*., 1997.[563]

5.1.3 Selection of a model 'co-drug'

As introduced in Section 1.3.6, *in vitro* and *in vivo* studies have highlighted a number of possible antibiotic candidates that could be used in combination therapy with colistin for inhalation therapy. To demonstrate the effect of colistin on the release of co-encapsulated agents, adequate loading of a suitable co-drug into a colloidal carrier needs to be achieved. Formulations containing only the model co-drug should be characterised before formulations also containing colistin are investigated, so that the effects of colistin on loading and release of the co-drug may be delineated.

A suitable model co-drug for passive loading into the lipid phase of emulsions and into the bilayer of liposomes will have good lipid solubility (as indicated by log P). A suitable model co-drug for remote-loading into the liposome interior requires at least one ionisable basic functional group and reasonable solubility in water.[348] Azithromycin

(Figure 3-2), a macrolide antibiotic, was selected to fulfil these requirements. With a log P of 4.02,[564] azithromycin is suited to passive loading into a SME or liposome system. Given that azithromycin has two ionisable basic functional groups with pK_as of 8.74 and 9.49,[109] azithromycin is also a suitable candidate for remote-loading into liposomes. Although azithromycin has limited intrinsic solubility in water (~ 0.2 mg/mL at 25°C, as shown in Figure 3-6), azithromycin can be highly solubilised in the presence of citric acid. Incidentally, citric acid is a buffer species commonly used for the generation a transmembrane gradient in the remote-loading of liposomes. Azithromycin therefore not only represents a poorly-water soluble drug that can be passively loaded into the lipid regions of SMEs and liposomes, but may also be suitable for remote-loading into the aqueous interior of liposomes. Azithromycin was therefore selected as a model 'co-drug' for investigations into colistin-combination colloid-based formulations.

Azithromycin is also considered an appropriate model co-drug for formulation with colistin due to its biological properties. Conventionally, azithromycin is used to treat infections of soft tissues and the respiratory tract caused by Gram-positive cocci. Exerting its antimicrobial activity by reversibly binding to the 23S ribosomal RNA in the 50S subunit of susceptible organisms, azithromycin inhibits mRNA-directed protein synthesis and is therefore bacteriostatic.[565-567] Gram-negative bacteria are inherently resistant to azithromycin as azithromycin is unable to penetrate the Gram-negative cell wall.[568-569] Treatment sensitisation with a membrane permeabilising agent, such as colistin, may enable azithromycin to gain entry into Gram-negative bacteria cells.[570] As introduced in Section 1.3.6, azithromycin has been observed to exhibit synergistic activity against MDR infections when combined with colistin.[245] Azithromycin also exhibits some anti-inflammatory, mucoregulatory[571-572] and anti-biofilm[573-575] effects

and is used routinely in the treatment of a number of airway diseases including CF, bronchiectasis, COPD, asthma and diffuse panbronchiolitis.[576-578]

5.2 Hypothesis and aims

The studies described in this Chapter aim to investigate colloidal formulations containing the model co-drug, azithromycin, to develop an understanding of the effect of colistin on co-loading and release of a second antibiotic, which in turn will provide further insight into colistin-membrane interaction. Investigation of suitable combination formulations containing colistin and a co-drug for future *in vivo* work would also then be enabled. In this work it was hypothesised that:

1. remotely loaded liposomes will have the greatest capacity to solubilise azithromycin, compared with passively loaded liposomes or a lipid based SME;
2. slow release of azithromycin can be achieved by the remote-loading of azithromycin into the liposome interior; and that
3. the presence of colistin will modify the rate of *in vitro* release of azithromycin from an azithromycin-loaded colloidal based drug delivery system.

To address these hypotheses, the specific aims of the studies in this Chapter were to:

1. characterise the physical morphology of azithromycin-loaded colloids and
2. determine the encapsulation efficiency and loading capacity of azithromycin-loaded colloids and
3. determine the encapsulation efficiency and *in vitro* release profile of the most promising colloidal carrier for azithromycin and
4. determine the effect of colistin on the *in vitro* release of azithromycin from a colloidal carrier.

5.3 Materials

Water was purified by a Milli-Q® water system (Millipore, Milford, MA). Soy dioloeylphosphatidylcholine (DOPC) (stored at -20°C) was obtained as the commercial preparation of Phospholipon® 90G from Phospholipid GmbH (Cologne, Germany). Cholesterol, sodium chloride, potassium chloride, disodium hydrogen phosphate, sodium hydroxide, potassium dihydrogen orthophosphate and vitamin E acetate were obtained from Sigma (St Louis, MO). Azithromycin was obtained from Kopran Pty Ltd (Mumbai, India). Colistin sulphate was obtained from Zhejiang Shenghua Biok Biology Co. Ltd (Huzhou, China). All HPLC reagents were of analytical grade. Nitrogen gas was from BOC gases (Sydney, Australia). All reagents and chemicals used for HPLC analysis were of HPLC grade.

5.4 Methods

5.4.1 Formulation of azithromycin-loaded colloids

5.4.1.1 Submicron emulsion (SME)

DOPC (500 mg) and vitamin E acetate (400 mg) (Figure 5-2) and increasing amounts of azithromycin (80, 100 and 150 mg) were weighed into 100-mL round bottomed flasks. Chloroform/methanol 2:1 v/v (1.5 mL) was used to dissolve the lipid soluble components before being evaporated under vacuum at 40°C on a rotary evaporator (Buchi Labortechnik, Switzerland). The dry lipid/vitamin E film was then dispersed in 10 mL Milli-Q® water. To aid dispersion of the lipids, the formulation was mixed on the rotary evaporator at 40°C. Precipitation or sedimentation of insoluble azithromycin was assessed by visually. Vitamin E acetate was selected as the lipid component of emulsion formulations due to its known capacity to solubilise lipophilic drugs, including the

macrolides.[579-581] Furthermore, the use of vitamin E in aerosol formulations may actually be beneficial to the injured lung.[582]

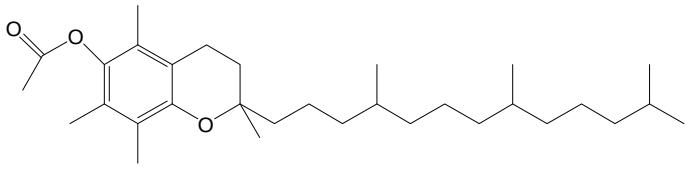


Figure 5-2: Chemical structure of vitamin E acetate.

5.4.1.2 Passively-loaded liposomes

DOPC (500 mg) and increasing amounts of azithromycin (80, 100 and 150 mg) were weighed into a 100-mL round bottomed flask and dissolved in 1.5 mL of 2:1 chloroform/MeOH v/v. The solvent was evaporated to produce a dry film which was dispersed in 10 mL Milli-Q® water by vigorous vortex mixing. The size of the azithromycin-loaded liposomes was then reduced by ultrasonication according to Section 4.3.2.

5.4.1.3 Remote-loaded liposomes

The lipid compositions used in these formulations were DOPC only, DOPC:cholesterol 2:1 mol parts or DOPC/cholesterol 4:1 mol parts. Empty liposomes were prepared by the dry film method as described in Section 4.3.2. The dry lipid film was dispersed in a 5 mL of 300 mM citric acid (adjusted to pH 3.2 with disodium hydrogen phosphate dihydrate) to produce a 10% w/v DOPC dispersion. The 10% w/v DOPC liposome dispersion was then mixed 1:1 with a solution of azithromycin dissolved in 300 mM citric acid buffer (pH 3.2). The concentration of azithromycin was varied to produce 5% DOPC liposomes containing 5, 10, 20 and 30 mg/mL azithromycin. Azithromycin was remotely-loaded into the liposome interior by use of a transmembrane pH gradient. The liposome suspensions were titrated gradually to pH 9 by adding 10% w/v NaOH while

constantly stirring the formulation. The mechanism by which the remote-loading method drives azithromycin into the liposome interior is demonstrated by Figure 5-1.

5.4.2 Characterisation of azithromycin-loaded colloids

5.4.2.1 Particle size and zeta potential measurements

Particle size and zeta potential measurements were performed using DLS according to the procedure described in Sections 4.3.4.1 and 4.3.4.2, respectively.

5.4.2.2 Osmolarity

The required osmolarity range of formulations for pulmonary delivery is between 250 - 350 mOsm.[583] With a view to assessing the pharmacokinetics of prototype formulations in future *in vivo* studies, the osmolarity of the formulations had to be considered. Osmolarity measurements were made using a Fiske One:Ten micro-osmometer (Advanced Instruments, Norwood, USA). A 10 µL sample was dispensed into a disposable sample tube (John Morris Scientific, Balwyn, Australia). Air bubbles were removed by gently tapping on a bench top. The tube was then transferred to the osmometer for automatic measurement of osmolarity by freezing point depression determination.

5.4.2.3 Cross polarised light microscopy (CPLM)

Self-assembling structures formed when phospholipids are in contact with excess water are optically birefringent and exhibit a maltese-cross or cross-hatched appearance under cross polarised light.[584] CPLM was used to determine the phase structure of the phospholipid contained in the SME containing azithromycin**.** The microscope used was a Zeiss Axiolab E (Carl Zeiss Microimaging, Thornwood, NY, USA) fitted with cross polarising filters. A drop of formulation was placed on a glass slide underneath a cover

slip and was viewed under cross polarised light. A digital image was taken to record the appearance of the emulsion under cross-polarised light.

5.4.2.4 Cryo-TEM

The procedure for sample preparation and cryo-TEM imaging was identical to that described in Section 4.3.4.8. Undiluted samples were loaded directly onto grids.

5.4.2.5 Encapsulation efficiency

EE was measured by pressure ultrafiltration, as described in Section 4.3.6. The adsorption of azithromycin to the membrane filter had to be quantified prior to using pressure ultrafiltration for measurement of EE so that the concentration of azithromycin in the ultrafiltrate reflected that in the free drug solution. In order to determine the volume of solution that was required to saturate the membrane a solution containing 100 µg/mL azithromycin in Milli-Q® water was passed through the membrane, and fractions of five drops at a time were collected and the volume of each fraction was determined by weighing. The concentration of azithromycin in each fraction of the filtrate was determined by LC-MS (Section 2.2.8) and the volume required for the concentration in the ultrafiltrate to reach 95% of the concentration in the cell was determined. This process was repeated using three different YM10 ultrafiltration membranes.

5.4.3 *In vitro* release of remote-loaded azithromycin liposomes

5.4.3.1 Azithromycin liposomes

Remote-loaded azithromycin liposomes containing DOPC only, DOPC/cholesterol 4:1 and DOPC/cholesterol 2:1 were prepared according to Section 5.4.1.3. Following the remote-loading procedure, a 10 mL aliquot of remote-loaded liposomes containing approximately 8 mg/mL azithromycin was spiked into a 50 mL polypropylene tube

containing 40 mL of PBS (pH 7.4), in triplicate. The tube was vortex mixed and a 5 mL sample was immediately withdrawn, of which 0.5 mL was reserved for HPLC determination of the total azithromycin content (Section 2.2.5) and 4.5 mL was used for measurement of free azithromycin by pressure ultrafiltration (Section 4.3.6). A schematic summary of the preparation of liposomes for *in vitro* release studies is shown in Figure 5-3. The remaining 45 mL of diluted liposome suspension was stored at 37°C in a shaking water bath for 72 h. Further 5-mL samples were removed at 1, 2, 4, 8, 24, 48 and 72 h for measurement of the free azithromycin concentration by pressure ultrafiltration. The final pH of the liposomes diluted in PBS (pH 7.4) was 7.8.

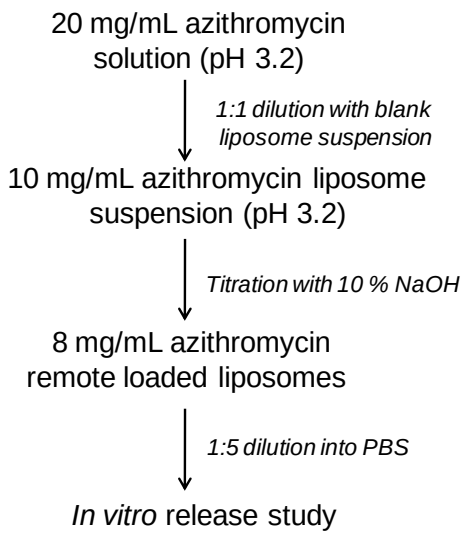


Figure 5-3: Schematic representation of sample workup for remote-loaded azithromycin liposome for *in vitro* release studies.

5.4.3.2 Azithromycin liposomes in the presence of colistin

The effect of colistin on the loading and release of azithromycin from DOPC/cholesterol 2:1 liposomes was studied by measuring the *in vitro* release of azithromycin from liposomes manufactured in the presence of colistin. Remote-loaded azithromycin liposomes containing DOPC/cholesterol 2:1 were prepared as described in Section 5.4.1.3 with a minor modification. The lipid film was dispersed in a 5, 20 or 40 mg/mL

solution of colistin dissolved in pH 3.2 citric acid buffer. The resulting liposome suspension was then diluted 1:1 with a 10 mg/mL solution of azithromycin dissolved in pH 3.2 citric acid buffer to produce 10 mg/mL azithromycin liposomes containing 2.5, 10 and 20 mg/mL colistin. Following 15 min incubation after loading, a 10 mL aliquot of the colistin/azithromycin liposome suspension was then diluted into 40 mL of PBS in a 50-mL polypropylene tube. At designated time points, 5 mL of the *in vitro* release media was sampled, from which 0.5 mL was reserved for HPLC determination of the total azithromycin (Section 2.2.5) and colistin (Section 2.2.3) concentrations and 4.5 mL was used for measurement of free azithromycin by pressure ultrafiltration (Section 4.4.6.3). The *in vitro* release of azithromycin from each formulation was measured in triplicate from independently prepared liposome formulations. Figure 5-4 summarises the concentration of colistin contained in the liposome samples at each step in the preparation of combination colistin-azithromycin liposomes for *in vitro* release studies. Size dependent leakage of drug from liposomes has been demonstrated *in vitro*,[585] therefore the particle size distributions for the liposomes used in the *in vitro* release studies were measured to ensure consistency between replicates.

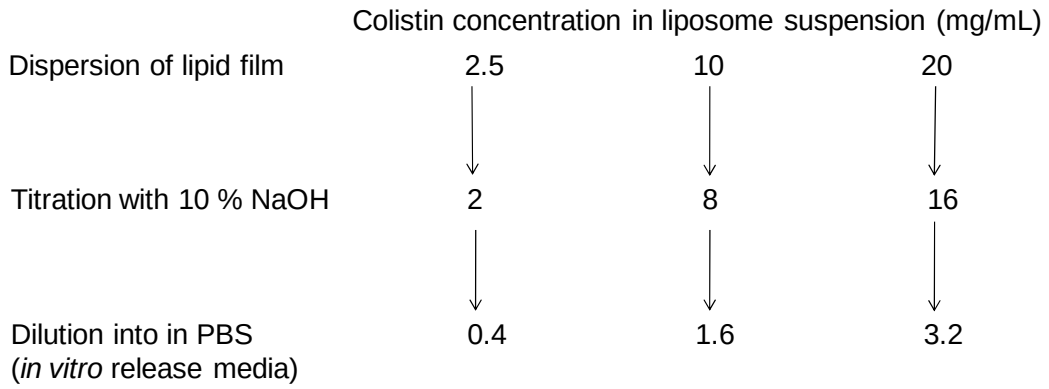


Figure 5-4: Colistin concentration at each step in the preparation of combination liposomes for *in vitro* release studies.

5.4.3.3 Stability of azithromycin under in vitro release study conditions

The stability of azithromycin under the conditions of the *in vitro* experiment was determined to ensure that the observed rate of azithromycin release *in vitro* was not obscured by azithomycin degradation. A solution representing the liposome dispersion medium was prepared by dissolving azithromycin at 1.5 mg/mL in 0.5 M citric acid (adjusted to pH 3.2 with 0.3 M disodium hydrogen phosphate) that had been diluted into PBS by 1:5. An acidic solution, representing the conditions within the interior of the liposomes, containing 1.5 mg/mL azithromycin in 0.5 M citric acid (adjusted to pH 3.2 with 0.5 M disodium hydrogen phosphate) was also prepared. The solutions were stored in triplicate (50 mL in a 50 mL polypropylene tube) in a shaking water bath (100 shakes/min) at 37°C. A 5 mL sample of each solution was removed at 0, 24, 48 and 72 h and the concentration of azithromycin was measured by HPLC as described in Section 2.2.5. The stability of free azithromycin in solution was compared to the stability of azithromycin when encapsulated within the interior of the liposomes. Degradation half lives were calculated according to appropriate rate order models.

5.5 Results

5.5.1 Submicron emulsion

Lipid emulsions containing azithromycin appeared as milky white formulations. The solubility of azithromycin in the emulsion was between 10 and 15 mg/mL; precipitated or sedimented azithromycin could be seen in formulations containing greater than 15 mg/mL azithromycin, but not in formulations containing 10 mg/mL azithromycin.

5.5.1.1 Particle characterisation

The particle size distributions of the emulsion is shown in Figure 5-5; the z-average particle size was approximately 240 nm, with reasonably low polydispersity of approximately 0.2 (Table 5-1). The particle size between 100 - 500 nm confirms that the lipid emulsion produced was a SME. The emulsion has close to neutral zeta potential Table 5-1 which was anticipated when using an overall electrically neutral phospholipid was used to prepare the emulsion. The low osmolarity (Table 5-1) is also not surprising in the absence of osmotically active species in the formulation.

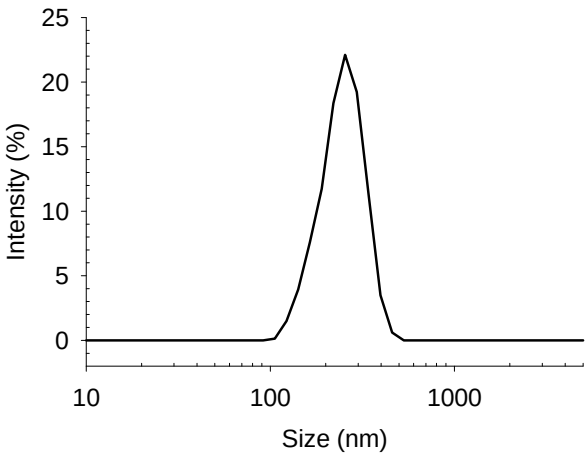


Figure 5-5: Particle size distribution of azithromycin-loaded SME.

Table 5-1: Particle characteristics of azithromycin-loaded vitamin E emulsion.

Z-average particle size	242 ± 45 nm
PDI	0.208 ± 140
Zeta potential	3.5 ± 0.7 mV
Osmolality	52 ± 12 mOsm

5.5.1.2 Cross polarised light microscopy

Morphological examination of the azithromycin SME using CPLM revealed that the emulsion contained a mixture of spherical vitamin E droplets and liposomes. Liposomes, which exhibit birefringence under cross-polarised light, can be identified by the red and blue the Maltese-cross hatched structures.

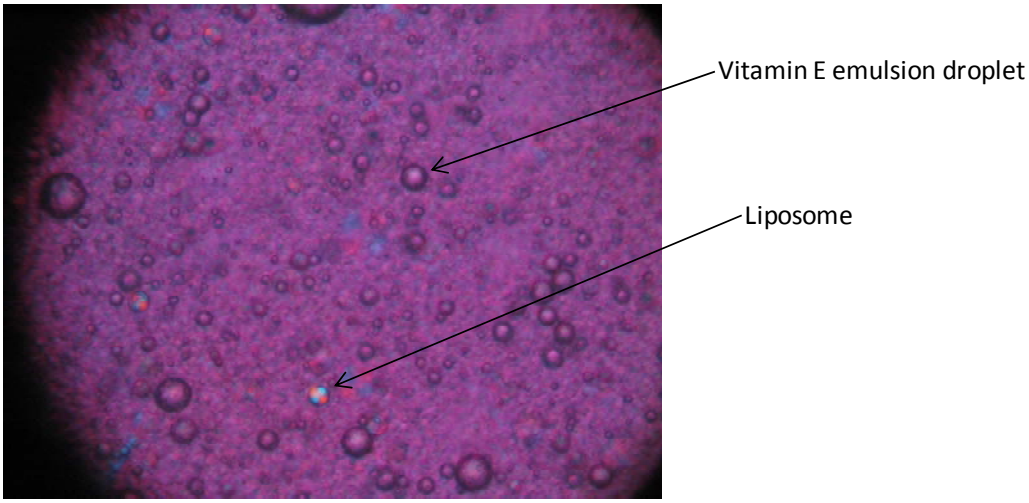


Figure 5-6: CPLM photomicrograph of a phospholipid/vitamin E emulsion containing 8 mg/mL azithromycin.

5.5.1.3 Cryo-TEM

Cryo-TEM proved to be a useful method for investigating the internal structure of the SME droplets, which cannot be characterised using CPLM. The droplet and vesicle sizes indicated in cryo-TEM images (Figure 5-7) supports the data provided by particle size measurements.[442] Multiple layer droplets dominate the cryo-TEM image landscapes in which two distinctly different single layer species can be identified; one species which is lighter in colour and irregularly shaped, and second species which is a darker, more spherical droplet type with a stark white ring at the interface. Each of these distinct species is identified by arrows in Figure 5-7. It is most likely that this formulation represents a mixture of a submicron emulsion droplets and vesicles. There was no evidence of solid azithromycin precipitates in cryo-TEM images.

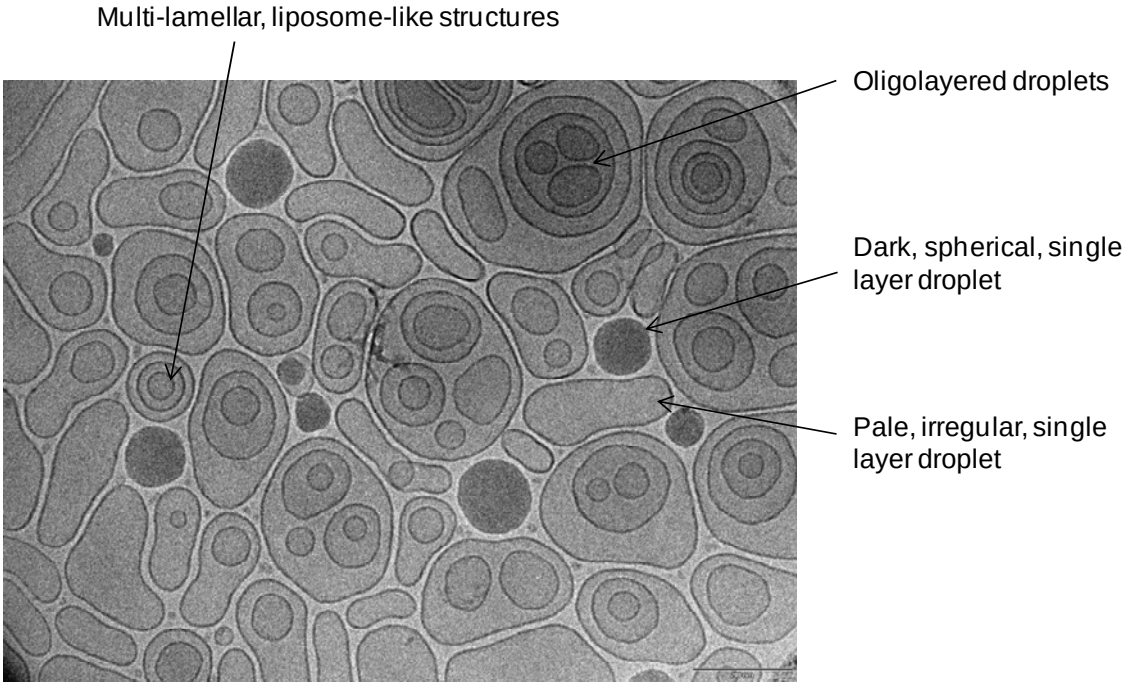


Figure 5-7: Cryo-TEM images of azithromycin DOPC-vitamin E SME. Scale bar represents 200 nm.

5.5.1.4 Encapsulation efficiency

Filter adsorption studies showed very little adsorption of azithromycin to the ultrafiltration membrane filter. As shown in Figure 5-8, the concentration of azithromycin in the ultrafiltrate was within 95% of the target concentration in the second fraction collected. By this it was determined that approximately 20 µg azithromycin was adsorbing to the ultrafiltration membrane. This was taken into consideration during EE measurements of azithromycin colloid-based formulations. The loading efficiency of the emulsion was determined to be 88%.

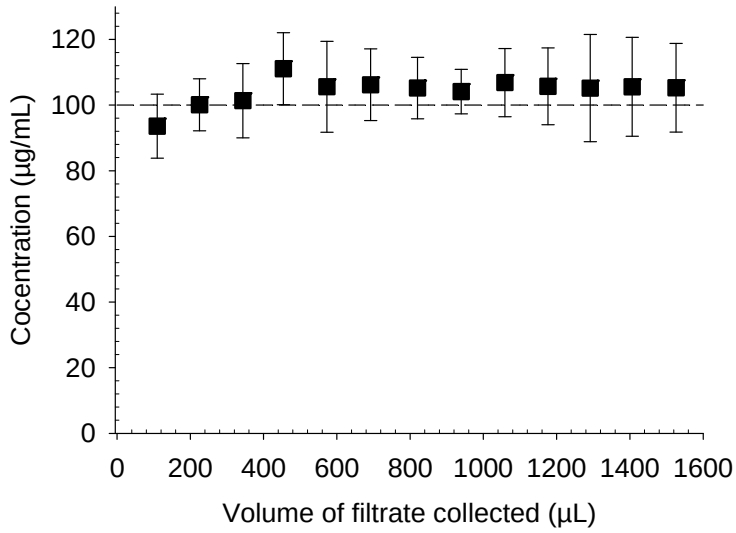


Figure 5-8: Filter adsorption of azithromycin (data are presented as mean ± SD of n=3 separate membranes).

5.5.2 Passively loaded azithromycin liposomes

Passively loaded azithromycin liposomes containing 10 mg/mL azithromycin appeared as opaque-white formulations. Precipitated or sedimented azithromycin could be seen in formulations containing than 10 mg/mL azithromycin.

5.5.2.1 Particle characterisation

The particle size distribution of passively-loaded azithromycin liposomes is shown in Figure 5-9, with the corresponding z-average size and PDI given in Table 5-2. The preparation of passively loaded azithromycin liposomes consistently produced emulsions with acceptable z-average particle sizes (<260 nm) and narrow PDIs. Excellent EE was achieved (Table 5-2) even though a passive loading technique was used. The neutral particle surface charge, as indicated by the zeta potential (Table 5-2), is consistent with that of empty DOPC liposomes (Section 4.4.2.1).

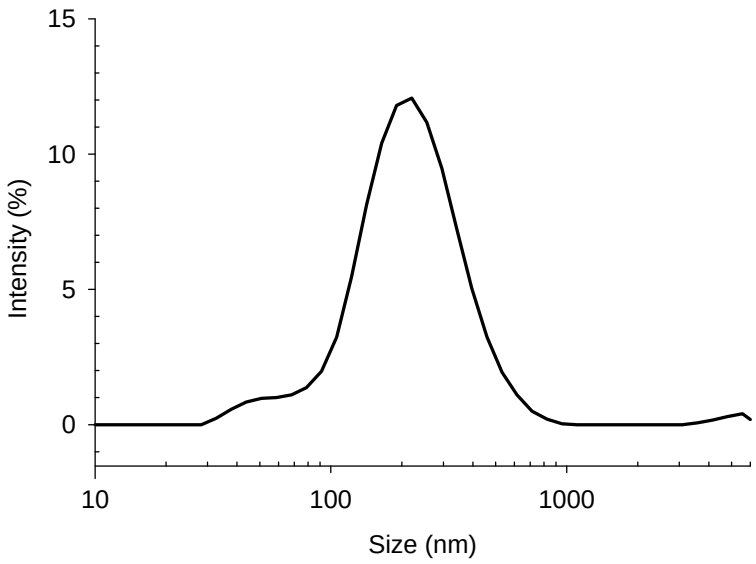


Figure 5-9: Particle size distribution of passively-loaded azithromycin liposomes**.**

Table 5-2: Particle characteristics of passively-loaded azithromycin liposomes. Data are presented as mean ± SD of three separate determinations.

Z-average particle size	184 ± 26 nm
PDI	0.256 ± 0.130
Zeta potential	3.2 ± 0.2 mV
Osmolarity	20 mOsm
Encapsulation efficiency (%)	94.6 ± 1.2

5.5.3 Remote-loaded liposomes

During the remote-loading of azithromycin into liposomes, solid precipitates of azithromycin could be seen in formulations containing 20 and 30 mg/mL azithromycin. Therefore, further characterisation studies of remote-loaded liposomes involved the use of azithromycin liposomes containing a maximum of 10 mg/mL.

5.5.3.1 Particle characterisation

The particle size distributions of remote-loaded liposomes with and without cholesterol are shown in Figure 5-10. There was no appreciable difference in liposome particle size with increasing amounts of cholesterol; the major peak being centred at approximately 200 nm for all three formulations.

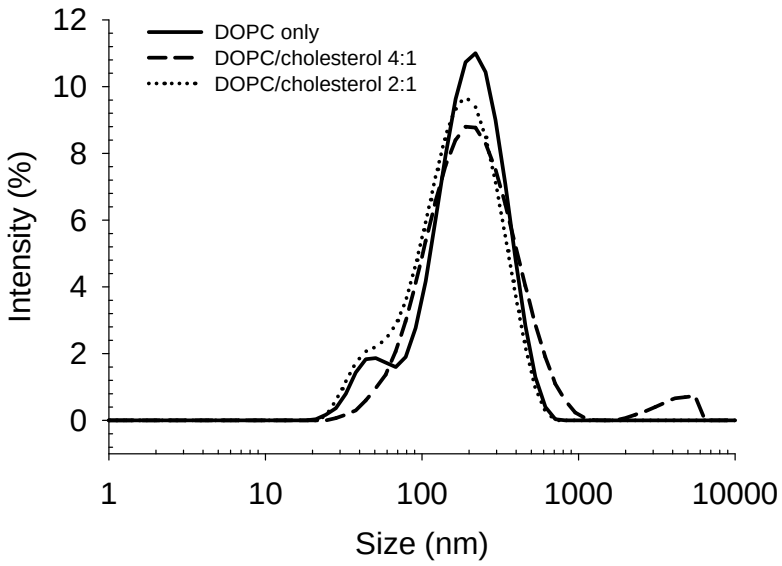


Figure 5-10: Particle size distribution of azithromycin remote-loaded liposomes, with varying cholesterol content.

Due to the effect of the high concentration of ionic species and subsequent high conductivity of the remote-loaded formulation samples, it was difficult to obtain an estimate of zeta potential without adjusting the measurement voltage. An adjustment of the measurement voltage would mean that the measured zeta potential value for remote-loaded liposomes could not be directly compared to the zeta potential measured for other formulations. As a result, zeta potentials of the remote-loaded azithromycin liposomes are not reported. It is expected, however, that the liposomes would exhibit a near-neutral charge due to the lack of charged phospholipids in the formulation.

Table 5-3: Particle characteristics of remote-loaded azithromycin liposomes containing DOPC/cholesterol 2:1.

Z-average particle size	155 ± 18 nm
PDI	0.293 ± 0.036
Osmolarity	1044 ± 32 mOsm

5.5.3.2 Encapsulation efficiency of remote-loaded azithromycin liposomes

Excellent EE of azithromycin was achieved by remote-loading into DOPC liposomes (Table 5-4). The EE was observed to decline at 20 mg/mL azithromycin, with some drug precipitation observed at this concentration during the titration step. Typical of remote-loading methods, high drug: lipid ratios were achieved.

Table 5-4: EE of azithromycin remote-loaded liposomes. Data are presented as mean ± SD (n = 3).

	Concentration (mg/mL)			
	1	5	10	20
Encapsulation efficiency (%)	> 90*	> 98*	> 97 ± 2	> 92 ± 1
Drug:phospholipid ratio	0.02	0.10	0.20	0.37

*True value could be greater, quantification in this case limited by the LOQ of the HPLC assay.

5.5.3.3 Cryo-TEM

Cryo-TEM images presented in Figure 5-11 show the presence of a mixture of SUVs, LUVs, MLVs and MVSs. Importantly there does not appear to be any solid drug precipitated on the interior of the liposomes, which has been reported to occur in liposome formulations containing drug encapsulated by remote-loading techniques.[294]

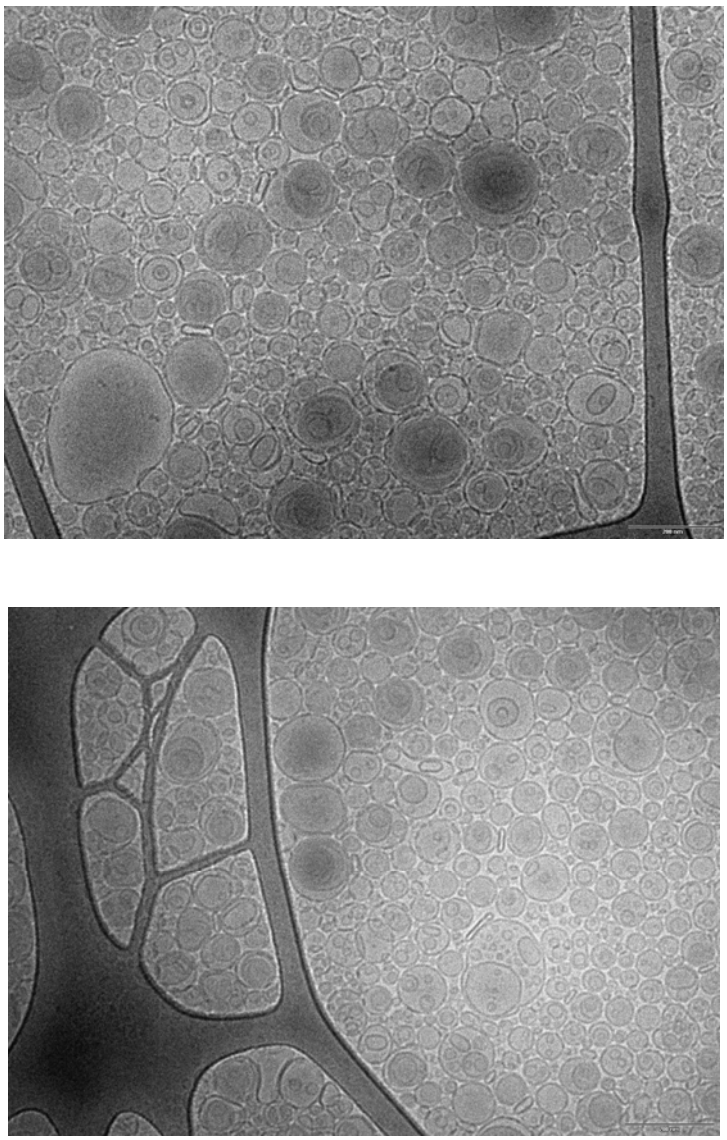


Figure 5-11: Cryo-TEM images of DOPC/cholesterol (2:1) azithromycin liposomes produced by the pH gradient loading method.

5.5.4 *In vitro* release studies

5.5.4.1 Release of azithromycin from remote-loaded liposomes

Figure 5-12 shows the cumulative release of azithromycin from azithromycin remote-loaded liposomes containing varying amounts of cholesterol. As can be seen in Figure 5-12, the release rate for azithromycin decreased with increasing amounts of cholesterol. Liposomes without cholesterol achieved maximum azithromycin release within 24 h, while liposomes containing either 4:1 or 2:1 phospholipid/cholesterol achieved only 53.3 and 12.6% release, respectively, over the course of the 72 h study. Of note is the observed plateau at approximately 70% in the release profile for observed liposomes containing no cholesterol. The cause of the plateau was investigated by assessing the stability of azithromycin in the *in vitro* release media under the same conditions as the *in vitro* release experiment (Section 5.5.4.2).

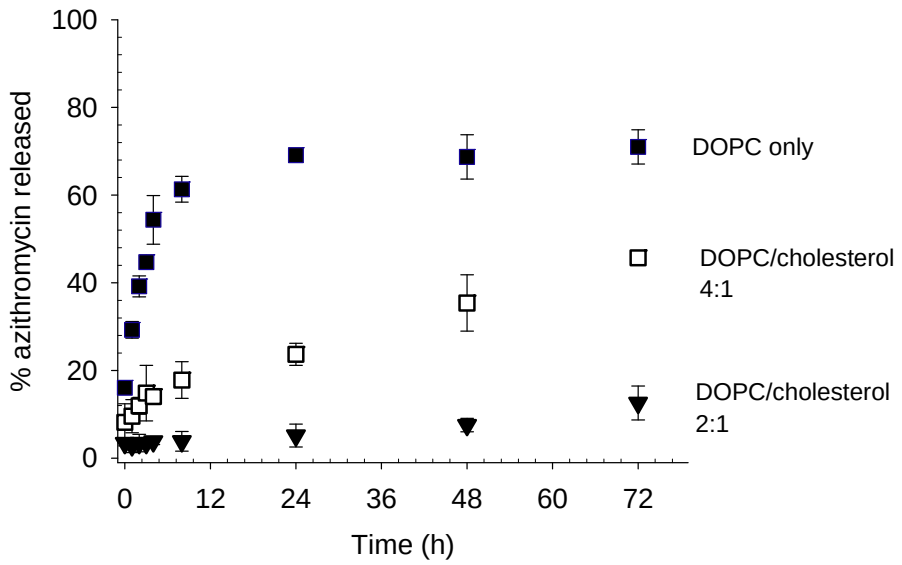


Figure 5-12: *In vitro* release of azithromycin from pH gradient loaded liposomes comprised of DOPC, DOPC/cholesterol 4:1 and DOPC/cholesterol 2:1 in PBS at 37°C. Data are presented as mean ± SD (n = 3).

5.5.4.2 Stability of azithromycin under in vitro release conditions

To investigate the possibility that the plateau observed in the *in vitro* release profile in Figure 5-13 was due to the instability of azithromycin under the conditions of the *in vitro* release experiment, the stability azithromycin was studied in different media at 37°C. Figure 5-13 shows the stability of azithromycin under the conditions tested. Free azithromycin degraded rapidly following first order degradation kinetics when free in citric acid buffer (representing the environment within the interior of the liposome), with a calculated half life of 13.5 h (r^2 = 0.973 when plotted as log of concentration versus time). In a 5:1 mixture of PBS and citric acid buffer at pH 7.8 (representing the *in vitro* release medium), azithromycin degraded apparently following zero order degradation kinetics with a calculated half-life of 46.5 h (r^2 = 0.983 when plotted as concentration versus time). Azithromycin loaded within the liposome interior remained stable for the duration of the study as indicated by the steady concentration of azithromycin in DOPC/cholesterol liposomes.

5.5.4.3 Particle size

Because particle size can affect drug release rates,[506] the particle size of liposomes used in *in vitro* release studies were measured to ensure consistency between batches of liposomes containing different phospholipid/cholesterol compositions. The z-average particle sizes of liposome formulations used in *in vitro* release studies are shown in Figure 5-14. The small differences in z-average particle sizes between formulations are unlikely to be responsible for the vastly different azithromycin release rates from the different formulations, shown in Figure 5-12.

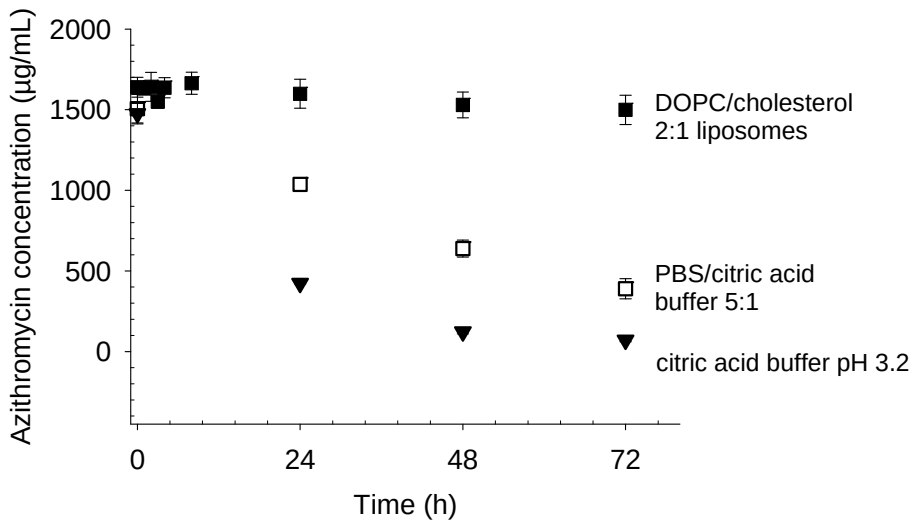


Figure 5-13: Stability of azithromycin in at 37°C: free in citric acid buffer (pH = 3.2), free in PBS (pH = 7.8) and when encapsulated within the interior of DOPC/cholesterol (2:1) liposomes. Data are presented as the mean ± SD (n = 3).

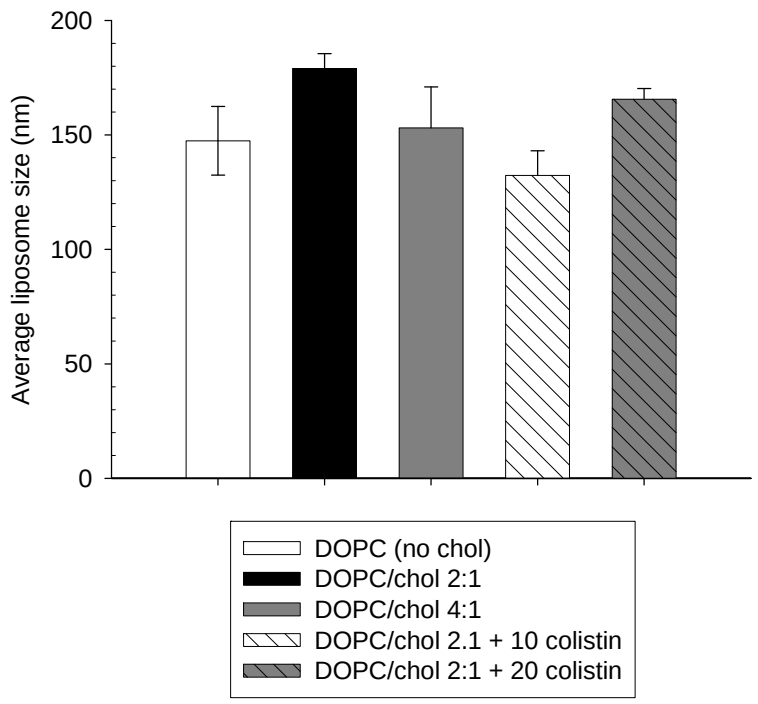


Figure 5-14: Average particle size of liposomes for *in vitro* release studies. Data are presented as the mean ± SD (n = 3).

5.5.4.4 Release of azithromycin from remote-loaded azithromycin liposomes prepared in the presence of colistin

The effect of colistin on the loading into and release of azithromycin from slow release liposomes (containing DOPC/cholesterol 2:1) is shown in Figure 5-15. Colistin significantly accelerated the rate of azithromycin release from DOPC/cholesterol 2:1 liposomes. This effect appeared to be concentration-dependent, with a two-fold increase in concentration resulting in a near two-fold increase in the rate of azithromycin release.

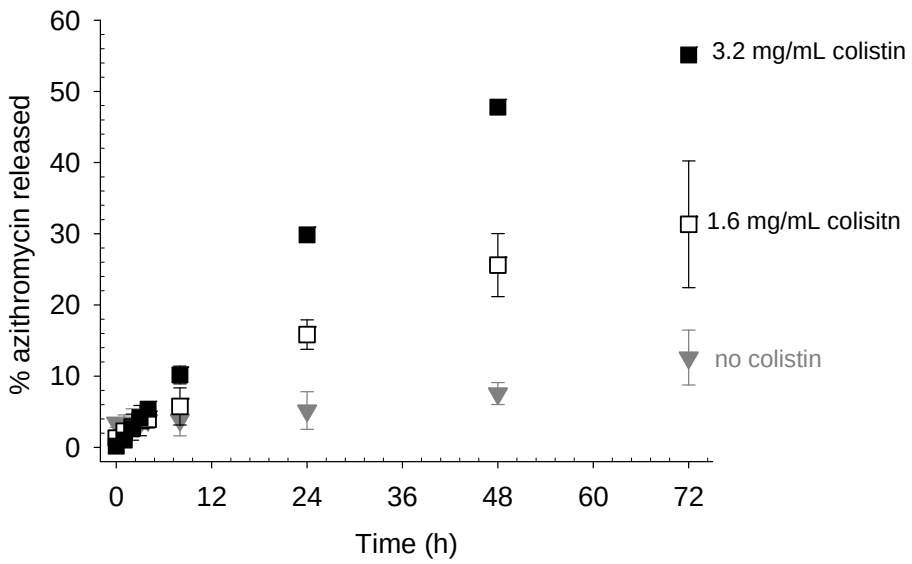


Figure 5-15: *In vitro* release of azithromycin from remote-loaded 'slow release' liposomes comprised of DOPC/cholesterol 2:1 at 37°C in the presence of 3.2 mg/mL colistin, 1.6 mg/mL colistin or no colistin in PBS. Data are presented as mean ± SD (n = 3).

5.6 Discussion

Three approaches to the formulation of azithromycin into colloidal carriers have been investigated: passive loading into a SME, passive loading into liposomes and remote-loading of liposomes. The physicochemical characterisation of these systems was critical for selecting a delivery system which would allow the effect of colistin on azithromycin release to be studied.

It is apparent that DOPC alone was adequate to disperse the vitamin E oil phase within an acceptable size range and PDI without the need for a co-surfactant. The formation of a SME without the use of co-surfactants has been reported previously by Valenta *et al*.[586] DOPC was present in excess as indicated by the existence of the Maltese cross-hatched structures under CPLM; after complete coverage of the oil-water interface, excess DOPC would form liposomes. Therefore, there is potential that azithromycin exists within a number of different localities in the SME formulation: within the vitamin E oil droplets, dissolved in the external aqueous phase (to a maximum of saturated solubility), within the lipidic region of the liposomes and finally, within the aqueous region of liposomes between oligolayers or within the aqueous core. While lipophilic drugs are generally known to be released from SMEs in a burst release fashion by partitioning,[310, 518, 555, 587-588] it is difficult to predict the release behaviour of the azithromycin-loaded SME when one considers that it contains a number of different colloidal species. The overall rate of azithromycin release from the SME would be a consequence of the combination of various rates of drug release from and between the different colloidal phases. Furthermore, the release of lipophilic drug from lipid emulsions has been shown to be dependent upon the dilution factor, amongst other variables.[310-311] Emulsions administered via the pulmonary route would not experience the large dilution factor encountered by parenterally administered emulsion

formulations. Therefore, it would be difficult to anticipate how useful the azithromycin-loaded SME developed in this study would be in localising azithromycin within the lungs following pulmonary delivery.

The high EE of the azithromycin-loaded SME was comparable to that reported of other vitamin E emulsions containing the macrolide antibiotics clarithromycin and azithromycin.[579-580] Though not directly measured, the high solubility of azithromycin in vitamin E is expected to be the reason for the high EE of azithromycin in the current SME. The predicted vitamin E solubility of clarithromycin, a macrolide similar in chemical structure to azithromycin, is reported to be 52.25 mg/mL.[580] The interactions between colistin and the vitamin E SME were not investigated in this study. A nanoemulsion of polymyxin B (a compound structurally similar to colistin, Figure 1-4) has been reported by Pattani *et al*., however, they did not report any details of the nature of the association of polymyxin B with the emulsion system, nor was EE reported.[589]

SMEs are typically observed to be 'burst release' systems,[587] and were considered to have low potential to provide co-localisation of azithromycin and colistin within the lungs following pulmonary delivery of the emulsion formulation. Therefore, subsequent efforts focused on the assessment of liposomes for the co-formulation of colistin and azithromycin. Passively loaded azithromycin liposomes exhibited similar particle size and surface properties to the azithromycin SME. Surprisingly, however, the EE of azithromycin in passively loaded liposomes was greater than that observed for the passively loaded SME, suggesting the greater solubility of azithromycin in DOPC compared to the mixture of vitamin E and DOPC. This is most probably because in the case of liposomes, the azithromycin dissolved within the internal water is included in the encapsulated fraction in the measurement of EE.

Liposomes loaded with azithromycin by the remote-loading method achieved superior EE to the SME and passively loaded liposomes (Table 5-2). The cryo-TEM imaging of remote-loaded liposomes was important in understanding the physical form of azithromycin within the interior of liposomes. While dark shaded regions could be observed within the larger liposomes of cryo-TEM images (Figure 5-11), distinct solid precipitates could not be clearly identified. Azithromycin appeared to remain in a dissolved state within the interior of the liposome. The form and location of a drug within a liposome, which is largely determined by physicochemical behaviour of the drug[341] and the remote-loading conditions,[590] can influence the release rate in response to changes in the external environment including the depletion of the pH gradient.[341] If the intraliposomal concentration of drug exceeds the intrinsic solubility of the drug, complexation with the intraliposomal counter ion can cause the drug to precipitate within the liposome interior.[349] Precipitation of the drug within the interior of the liposomes has been observed in liposomal formulations of doxorubicin,[523, 591] mitoxantrone,[592] topotecan[593] and vincristine.[594] The dissolution of the precipitated drug form can result in slow drug release following zero order kinetics as the dissolved drug partitions across the liposomal bilayer.[351] The physical form of azithromycin within the liposome interior following remote-loading was therefore an important characteristic to be understood in the interpretation of the *in vitro* release data.

The characteristics of the formulations investigated were taken into consideration when selecting a formulation for progression into *in vitro* and *in vivo* (Chapter 6) performance studies. The SME system was not progressed to *in vitro* release studies as it exhibited the lowest EE of the systems studied (drug:lipid mole ratio 0.14:1). Although both passively and remotely loaded liposomes achieved high encapsulation efficiencies (drug lipid mole ratios of 0.16:1 and 0.2:1, respectively), release of passively entrapped agents

has been shown to be faster than those loaded by a remote-loading process.[595-596] Remote-loaded compounds become sequestered within the liposome interior, adopting a charge which limits diffusion across the bilayer and out of the liposome.[595, 597] Passively loaded agents are not under the influence of a transmembrane gradient and therefore can more freely diffuse out of the bilayer upon dilution. Remote-loaded azithromycin liposomes, exhibiting superior EE, were deemed to provide the greatest potential for controlled or slow release of azithromycin and were therefore selected as the delivery system for further investigations of *in vitro* release and the effect of colistin on the rate of drug release.

The rate of *in vitro* drug release is an important consideration and can give some indication of the local availability of drug following pulmonary administration. If the drug is too well retained within the liposome, the drug-loaded liposome may be phagocytosed and destroyed before the drug is released and becomes bioavailable.[511] Conversely, if rapid leakage of drug occurs, the purpose of liposomal encapsulation becomes defeated.

The rapid release of azithromycin from DOPC only liposomes in the *in vitro* release studies (Figure 5-15) tends to support the findings from cryo-TEM imaging that azithromycin remains dissolved within the interior of liposomes and indicates the leaky nature of the DOPC bilayer. Incorporation of cholesterol into DOPC liposomes in molar ratios of DOPC/cholesterol 4:1 and 2:1 clearly modified the rate of azithromycin release *in vitro*. Cholesterol is a well known lipid modifier that alters the lamellar state of phospholipids by increasing the packing order of the DOPC hydrophobic chains, returning them to a pre-transition temperature gel state.[515] Cholesterol has been shown to decrease the leakage of both passively and actively loaded drugs including cytosine arabinoside,[598] doxorubicin[597] and irinotecan.[321] Cholesterol has also been shown to

increase the stiffness of phosphatidylcholine bilayers by up to seven-fold, as demonstrated by atomic force microscopy.[599] The result of the increased rigidity of the bilayer is to impede the free passage of ions (e.g. ionised azithromycin) down the electrochemical gradient and across the phospholipid bilayer. Interestingly, the use of DOPC/cholesterol 4:1 resulted in a much larger degree of variability in rate of release from liposomes (Figure 5-15), suggesting that there is likely to be a finite amount of cholesterol required to create the rigid phospholipid packing structure. The incorporation of cholesterol into DOPC liposomes at a mole ratio of DOPC/cholesterol 2:1 significantly slowed the release of azithromycin compared to liposomes containing no cholesterol and DOPC/cholesterol 4:1. The rigidifying effect of cholesterol on liposomes has multiple influences on the rate drug release; not only does the rigid bilayer impede the passage of azithromycin out of the liposomes, but it also slows the diffusion of ions responsible for creating the transmembrane gradient. As a result the dissipation of the transmembrane pH gradient occurs at a slower rate when cholesterol is incorporated into the liposomes, causing a slower release of azithromycin. Azithromycin itself has been reported to disrupt the molecular packing of phospholipids in liposomal bilayers, causing erosion and disappearance of the phospholipid gel-domains,[486, 600] however, such disruption was not observed in liposomes containing cholesterol.[600]

Close to sink conditions were maintained throughout the course of the *in vitro* release experiment and that the plateau observed in the release profile is unlikely to be attributed to the existence of non-sink conditions. Strictly speaking, to maintain sink conditions, the free concentration of drug should be kept at < 10% of its solubility in the *in vitro* release medium.[511] While the solubility of azithromycin dihydrate in water at 37°C is reported to be approximately 2 mg/mL,[601] the solubility of azithromycin in the

mixture of citric acid and PBS used in the *in vitro* release study is well in excess of the aqueous solubility at 37°C; the presence of citric acid markedly increases the solubility of azithromycin in the *in vitro* release media. This ensured that near-sink conditions were created in the *in vitro* release experiment so that limited solubility did not limit the release of azithromycin from the remote-loaded liposomes.

The plateau observed in the cumulative release of azithromycin *in vitro* release profile for DOPC only liposomes (Figure 5-12) may be attributed to a number of possible factors. The incomplete release of drugs loaded into liposomes via pH gradient loading methods has been reported previously.[602] Loading and stable entrapment of drugs into liposomes by the remote-loading methods requires that a sufficient transmembrane electrochemical gradient be maintained. Conversely, the release of entrapped drug relies on the dissipation of the transmembrane gradient.[602] The rate of drug release from remote-loaded liposomes, therefore, is highly dependent upon the pH of the external release medium.[602-603] An alternative explanation for the apparent incomplete release of azithromycin from liposomes in the *in vitro* release study is chemical decomposition of azithromycin in the *in vitro* release media. Azithromycin was observed to be stable when encapsulated within the interior of liposomes (Figure 5-13), though was not stable when free in the pH 3.2 citric acid solution comprising the intra-liposomal environment. This suggests that the loading of azithromycin into the liposome interior has a stabilising effect on azithromycin. The stark difference in the stability of azithromycin within the two environments strongly suggests that the physical nature or reactivity of azithromycin must be altered when encapsulated within the liposome interior by remote-loading. Encapsulation of other drugs within a liposomal carrier has been shown to stabilise the encapsulated drug compound by affording protection from chemical degradation by mechanisms such as hydrolysis.[604-607]

Colistin is well known to have a permeabilising effect on biological membranes.[608] ITC investigations described in Chapter 4 (Figure 4-13) showed that colistin strongly interacts with DOPC bilayers containing cholesterol, primarily via hydrophobic interactions. The strong interaction between colistin and phospholipid bilayers highlights the potential for colistin to modify the release of co-drugs loaded into colloidal-based carriers including SMEs and liposomes, and therefore, the effect of colistin on the release of co-encapsulated azithromycin was of interest. Establishing the *in vitro* release profile of azithromycin from remote-loaded liposomes enabled the identification of liposomes containing DOPC/cholesterol 2:1 as 'slow-release' liposomes. 'Slow release' remote-loaded liposomes were then selected to study the effect of colistin on the loading and release of azithromycin. While the presence of colistin did not appear to affect the loading of azithromycin into remote-loaded liposomes, as indicated by the low free concentrations of azithromycin in the *in vitro* release profiles at time zero (Figure 5-15), colistin substantially increased the rate of azithromycin release following dilution into the *in vitro* release media. The increase in rate of azithromycin release that occurred as a result of the two-fold increase in colistin concentration indicates the concentration dependency of the permeabilising effect of colistin. This observation strongly supports the finding from Chapter 4 that colistin interacts DOPC/cholesterol liposomes.

It is clear from *in vitro* release studies carried out in the presence of colistin that the interaction of colistin with the DOPC bilayer modifies the permeability and/or the fluidity of the bilayer. This is of interest from a number of perspectives. Firstly, it highlights that colistin can modify membrane permeability even in the absence of a specific binding site. Given the lack of understanding around the precise mechanism of the biological activity of colistin, this is an interesting finding. The relevance of the

permeabilising effect of colistin in the context of liposome-based formulations is that colistin could potentially be used in parallel with cholesterol to optimise co-drug release; cholesterol works create rigidity in the liposome bilayer and slow drug release, and colistin may be incorporated to increase the rate of release of a co-encapsulated agent. For example, azithromycin was extremely well retained by liposomes containing DOPC/cholesterol 2:1, with less than 10% of encapsulated azithromycin released over 48 h in the absence of colistin. The incorporation of colistin increased the rate of azithromycin release to achieve ~50% release after 48 h. The concentration of colistin incorporated into liposome formulations could therefore be optimised to achieve the desired release rate of azithromycin.

5.7 Conclusion

A number of approaches to the formulation of azithromycin in colloidal drug delivery systems have been investigated, including passive encapsulation into a SME and liposomes and active loading into liposomes via pH gradient mediated remote-loading. The SME formulation achieved the lowest EE for azithromycin of the systems investigated. Passively loaded and remote-loaded azithromycin liposomes had similar encapsulation efficiencies. Remote-loaded liposomes were selected as the drug delivery system for further investigation given that they provided the greatest potential for slow or controlled time-dependent release of azithromycin.

In vitro release studies of remote-loaded DOPC liposomes showed that azithromycin rapidly leaked out of the liposomes. However, the release rate of azithromycin could be controlled by the incorporation of cholesterol into the liposome bilayer. The *in vitro* release experiments also confirmed the concentration-dependent permeabilising effect of colistin on the DOPC/cholesterol liposome bilayer.

The physical co-localisation of the two antibiotics, colistin and azithromycin, has been achieved by formulation of both drugs into a common liposomal carrier. In remote-loaded liposomes, azithromycin is predominantly located within the interior of the liposomes, while colistin is thought to be located within the aqueous interior of the liposome and also associated with the phospholipid bilayer (Chapter 4). The benefit of the co-localisation of colistin and a second antibiotic by reformulation into a liposomal carrier in providing an improved treatment for MDR Gram-negative respiratory infections remains to be seen.

Chapter 6: Evaluation of the pharmacokinetics of antibiotic liposomes following pulmonary delivery

6.1 Introduction

Information regarding the pharmacokinetics of CMS following pulmonary administration of CMS in humans, and importantly the colistin formed *in vivo*,[144] is extremely limited despite the increasing use of nebulised CMS in the clinical setting. Therefore, the recommended dosage regimen of inhaled CMS is not based upon solid pharmacokinetic evidence. Recent pharmacokinetic (PK) data obtained in biodistribution studies in rats have shown that pulmonary administration of the prodrug CMS results in extensive systemic absorption of both CMS and the colistin formed from CMS *in vivo*.[142, 147]

Pulmonary administration of the active drug, colistin, may have advantages over the pulmonary administration of the prodrug, CMS. PK data obtained in biodistribution studies in rats have shown that pulmonary administration of the prodrug CMS results in extensive systemic absorption of both CMS and the colistin formed from CMS *in vivo*.[142, 147] In fact, the bioavailability (BA) of formed colistin following intrapulmonary administration of CMS exceeds the BA of formed colistin following IV dosing of CMS.[147] The reason for this is that following delivery of CMS into the aqueous environment of the lungs, substantial conversion to colistin can occur due to the somewhat protracted residence time in the lungs.[146] The colistin so formed is absorbed from the lungs into the systemic circulation.[147] Following an IV dose of CMS, on the other hand, CMS is rapidly cleared from the systemic circulation by the kidneys, limiting the opportunity for any substantial conversion to colistin to occur.[138] Therefore,

following IV administration, only a very small amount of the CMS administered is converted to colistin.[138, 147] Notwithstanding the higher BA of formed colistin after pulmonary, as compared to IV, administration of CMS, the fraction of the CMS dose converted to colistin in the lungs after pulmonary administration of CMS is relatively low.[142, 146] The administration of colistin to the lungs may result in lower systemic exposure to colistin than that occurring following administration of CMS to the lungs.

Yapa *et al*. have reported that following pulmonary instillation of a colistin solution to rats, rapid absorption occurred with maximum plasma concentrations (C_{max}) approximately 20 min after administration of the intrapulmonary dose.[139] PK/PD rationale (Section 1.3.3) dictates that the time-averaged concentration of colistin within the lungs should be maintained (i.e. concentrations above the MIC for an extended period of time) for maximal antimicrobial efficacy and to minimise the emergence of resistance.[153-155] Hence, from a PK/PD perspective, the pulmonary PK of colistin administered in an immediately available form (e.g. solution) is not ideal. Improvement of the PK profile of colistin by reducing the rate and extent of systemic absorption could lead to enhanced efficacy of colistin administered to the lungs for pulmonary infections. Liposomal encapsulation has the potential to modify the absorption of colistin following administration into the lungs, as has been demonstrated for numerous other compounds.[290-291, 470, 555, 609-611]

The interactions between colistin and the liposomal bilayer (Section 4.4.2.1) have indicated the potential of the liposomal carrier to modify the absorption of colistin following pulmonary delivery. The benefit of formulating colistin within a liposomal delivery system extends beyond the possibility of modified PK; liposomes offer the capacity to simultaneously co-localise a second antibiotic agent, together with colistin,

within the lungs. Combination therapy has been highlighted as an important strategy for the intensification of colistin inhalation therapy (Section 1.3.6).[1] Further intensification of colistin inhalation therapy could be achieved if pulmonary retention of colistin was accompanied by the pulmonary retention of a co-administered synergistic antibiotic (Section 1.3.6.1).

Studies described in Chapters 4 and 5 have demonstrated the capacity of liposomes to co-localise colistin and a model antibiotic co-drug, azithromycin, within a common liposomal carrier. *In vitro* release studies of colistin-loaded liposomes (Section 4.4.8), azithromycin-loaded liposomes (Section 5.5.4.1) and liposomes loaded with the combination of colistin and azithromycin (Section 5.5.4.4) have demonstrated the potential of the combination liposome system to provide modified drug release of both agents. However, the absence of physiological factors in the *in vitro* experimental environment (e.g. clearance of drug-loaded liposome by metabolism, macrophage endocytosis, mucociliary escalator etc; see Section 1.4.2 and 1.4.3.3) means that the release profiles determined *in vitro* may not always reflect the *in vivo* situation. Therefore, the true effect of liposomal encapsulation on pharmacokinetics and biodistribution can only be assessed *in vivo*.

The appearance of free drug in the systemic circulation following pulmonary administration of a liposomal dose will be the outcome of a number of simultaneously occurring processes. Figure 6-1 is a diagrammatic representation of the multiple processes occurring following the pulmonary administration of a liposomal dose.

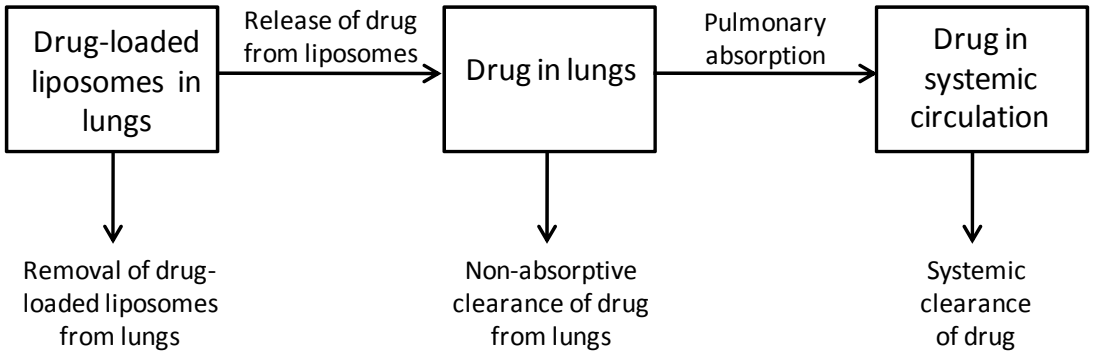


Figure 6-1: Schematic representation of the absorption of drug following pulmonary administration of drug-loaded liposomes.

As is evident from Figure 6-1, the extent of drug absorption will largely be influenced by: 1) the residence time of the liposomal formulation within the lungs and the ability of the retained liposomes to release their drug load; and, 2) the relative rates of non-absorptive clearance *versus* absorption of drug following release from the liposomes. The overall rate of drug absorption into the systemic circulation will depend on either: 1) the rate of release from liposomes that are retained in the lungs; or, 2) the rate of absorption of drug once it has been released from the liposomes. Assuming that the rate of absorption of unencapsulated drug is the same for both solution and liposome formulations, delayed overall absorption following administration of a liposomal formulation most likely results from delayed release from liposomes. Therefore, the effect of liposomal encapsulation on the rate and extent of pulmonary drug absorption can be assessed by comparing the differences in the plasma concentration-time profiles between solution and liposomal drug formulations.

It was shown in Chapter 5 that the rate of azithromycin release from liposomes may be accelerated by the presence of colistin in liposomes loaded with both agents (Section 5.5.4.4). In the present chapter, the PK of colistin and azithromycin following pulmonary administration of liposomes loaded with colistin (Chapter 4) and

azithromycin (Chapter 5) is to be examined. Prior to assessing the effect of liposome encapsulation on the PK of combination colistin/azithromycin liposome formulations, colistin and azithromycin each loaded separately into liposomes will first be assessed.

6.2 Hypotheses and aims

With regard to the pulmonary delivery of colistin- and azithromycin-loaded liposomes, it is hypothesised that:

1. liposomal encapsulation of colistin will result in modified pulmonary absorption of colistin, as indicated by changes in the time-course of plasma colistin concentrations, when compared to that which occurrs following pulmonary instillation of colistin in solution;
2. liposomal encapsulation of azithromycin will result in modified pulmonary absorption of azithromycin, as indicated by plasma azithromycin concentrations, compared to the free form of the drug, following pulmonary instillation to the rat; and that
3. the rate and extent of absorption of azithromycin from a liposomal dose form will be altered by its co-loading with colistin into a common liposomal carrier.

To address these hypotheses, this study aims to compare the systemic PK of colistin and azithromycin when administered to the lungs of rats in respective solutions *versus* liposomal dose forms of each drug alone and combined.

6.3 Materials

Polyethylene tubing (medical grade, single lumen, 0.5 mm ID x 0.8 mm OD) was purchased from Microtube Extrusions (North Rocks, NSW). Hypodermic needles (23G x 1½, single use) were from Terumo Corporation (Tokyo, Japan). Sep-Pak™ C_{18} solid-phase extraction (SPE) cartridges (100 mg) were supplied by Waters (Milford, MA, USA). The Visiprep 24™ vacuum manifold was made by Supelco (Bellefonate, PA, USA). Dysilk® surgical silk (size 3.0 and 4.0, braided, sterile, non-absorbable) was from Dynek (Adelaide, SA) and gauze swabs (sterile) were from Livingstone International Pty Ltd (Rosbery, NSW). Heparin was from Pharmacia and Upjohn (Melbourne, Vic). All surgical tools were purchased from World Precision Instruments (Florida, USA). The Selectatek isofluroane vapouriser was from Datex-Ohmeda (Herts, UK) and the small animal laryngoscope (rat) Model LS-1 and laryngoscope blade were from PennCentury (Philadelphia, USA). The Fiske® One-Ten Osmometer was from Fiske Associates (Norwood, USA). Saline 0.9% (provided in 100 mL Viaflex™ bags) was from Baxter (Old Toongabbie, NSW). Isofluorane was obtained from Abbott (Kurnell, NSW), medical oxygen was from BOC gases (Clayton, Vic) and Betadine® (10% povidone-iodine) was from Faulding Healthcare Pty Ltd (Virginia, QLD).

Soy dioleoylphosphatidylcholine (DOPC) (stored at -20°C) was obtained as the commercial preparation Phospholipon® 90G from Phospholipid GmbH (Cologne, Germany). Cholesterol, sodium chloride, potassium chloride, sodium phosphate dibasic, citric acid anhydrous, sodium hydroxide, potassium dihydrogen orthophosphate, sucrose and cholesterol were obtained from Sigma (St Louis, MO). Colistin sulphate was obtained from Zhejiang Shenghua Biok Biology Co. Ltd (Huzhou, China) and azithromycin dihydrate was from Kopran Pty Ltd (Mumbai, India). Water was purified

using a Milli-Q® water purification system from Millipore Corp., (Bedford, MA). All HPLC reagents were of analytical grade.

6.4 Methods

6.4.1 Animals

Male Sprague Dawley rats (300 - 320 g) were supplied by Monash Animal Services (Clayton, Vic). All experiments and protocols were conducted under the approval of the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee of Monash University. Rats were allowed access to food and water *ad libitum.*

6.4.2 Insertion of carotid artery and jugular vein cannulas

For IV PK studies, both the right carotid artery and right jugular vein were cannulated. A 90 cm length of polypropylene tubing filled with heparinised saline and capped with a 1 mL syringe was prepared for insertion into the blood vessel. For intratracheal (IT) PK studies, only the carotid artery was cannulated. One day prior to the administration of a drug dose, cannulation surgeries were performed. Rats were anaesthetised with 5% isofluorane and medical oxygen and maintained under general anaesthesia for the duration of the surgery. The rat was placed on its back on a heated surgical board (37°C) and the area of the neck was swabbed with Betadine®. A longitudinal incision of approximately 2 cm was made above the right clavicle, over the position of the jugular vein and carotid artery. Using a blunt dissection technique, connective tissue was separated to expose the artery and/or vein. Using a large needle, a subcutaneous tunnel was made from the site of incision to between the two ears of the rat. The cannula tubing was fed through the tunnel. After mobilising the carotid artery or jugular vein, securing it with a suture and occluding the blood flow using a pair of open forceps, a small incision (1 - 2 mm) was made into the vessel. Using fine forceps, the incision was

opened and a length of polyethylene tubing was inserted 2.5 cm into the artery towards the heart. A small clamp was used to secure the tubing while sutures were tied to fix the tubing in place. Heparinised saline (1,000 units/mL) was used to ensure a free flow of blood when vacuum was applied to the cannula via the attached syringe.

The skin was closed using a silk suture and Marcaine 5% (bupivacaine HCl) was applied to the wound. Rats were allowed to recover on the heated surgical board with recovery usually taking about 5 min. Animals were connected to a swivel tether apparatus then placed directly into a wire-bottomed cage. All tools used during the surgery were disinfected with Betadine®. A strictly aseptic technique was not necessary but the surgical area was kept clean.

6.4.3 Preparation of colistin dosing solutions and liposomal formulations for pharmacokinetic experiments

Dosing solutions and liposomal formulations were prepared to deliver target doses outlined in Table 6-1. Rats in each group were to receive the same dose normalised for body weight (mg/kg) but different absolute amounts of drug due to the slight difference in body weight between individuals.

Table 6-1: Target doses for colistin PK experiments.

	IV	IT solution	IT liposomes
Target dose (mg/kg)	0.8	1.5	1.5
Volume administered (µL)	200	100	100
Number of rats (n)	9	6	7

Solutions for IV delivery were prepared in Milli-Q® water while solutions for intrapulmonary delivery were prepared with 0.9% saline. Liposome suspensions were prepared by dispersing a 500 mg DOPC and 126 mg cholesterol dry lipid film in 0.9% saline to produce a 10% w/v DOPC/cholesterol 2:1 (molar ratio) dispersion. A colistin solution was prepared in 0.9% saline and mixed 1:1 with the DOPC/cholesterol 2:1 dispersion to produce colistin liposomes at the desired colistin concentration. To ensure that the incorporation of saline into the colistin liposome formulation did not affect the EE of colistin, the EE of liposomes prepared with 0.9% saline 5 mg/mL colistin sulphate was measured using pressure ultrafiltration (Section 4.3.5.3). Liposomes were ultrasonicated on an ice bath for 20 min (1 sec on, 1 sec off, 40 min total process time). Particle size was measured as described in Section 4.3.4.1.

6.4.4 Preparation of azithromycin dosing solutions and liposomal formulations for pharmacokinetic experiments

Azithromycin dosing solutions and liposomal formulations were prepared to deliver target doses outlined in Table 6-2. Rats in each group were to receive the same dose normalised for body weight (mg/kg) but different absolute amounts of drug due to the slight difference in body weight between individuals.

Table 6-2: Target doses for azithromycin PK experiments.

	IV	IT solution	IT liposomes
Target dose (mg/kg)	10	10	10
Volume administered (µL)	200	100	100
Number of rats (n)	5	8	4

For IV dosing of azithromycin, azithromycin was dissolved in a buffer containing 0.1 M citric acid (adjusted to pH 5 with 0.5 M disodium hydrogen phosphate). For IT administration of azithromycin solution, azithromycin was dissolved in 0.1 M citric acid and then titrated to pH 7 with 10% NaOH. The volume of NaOH added to the solution was ~10% of the total volume.

For azithromycin liposomal formulations, a 10% DOPC/cholesterol 2:1 dispersion was produced by dispersing a 500 mg DOPC and 126 mg cholesterol dry lipid film with 5 mL of 0.1 M citric acid solution. The dispersion was subsequently vortex mixed prior to ultrasonication for 15 min (1 sec on, 1 sec off, total process time 30 min). Following the ultrasonication step, the 10% DOPC/cholesterol 2:1 dispersion was mixed 1:1 with a 20 mg/mL azithromycin dihydrate dissolved in 0.1 M citric acid. While constantly stirring the dispersion, the pH was titrated from pH 3 to 9 using a 10% NaOH solution. After the pH gradient step, the liposomal dispersion was loaded, 1 mL at a time, onto a PD-10

desalting column (GE Healthcare Life Sciences, Rydalmere, NSW, Australia) to remove buffer salts, NaOH and unencapsulated azithromycin from the exterior of the liposomes. The liposomal fraction was collected and 10 mg of sucrose added per mL of liposome loaded on to the desalting column. Sucrose was added as a cryo-protectant/carrier for the freeze-drying process. This ensured ease of re-suspension of the freeze-dried liposome formulation.

The freeze-drying process involved a freezing step at -40°C for 12 h, followed by freeze drying at -40°C for 48 h and a final drying step at 25°C for 24 h. The freeze-dried liposomal powder was assayed for total azithromycin content (% w/w) using the HPLC method described in Section 2.2.5. Liposome dispersions for IT dosing were prepared by weighing the appropriate amount of lyophilised azithromycin liposomes to deliver 10 mg/kg of azithromycin and dissolving it in Milli-Q® water. The osmolarity of the final liposome formulation was adjusted to 250 - 350 mOsm/L by addition of sucrose. The EE of the liposome dosing suspensions was measured using pressure ultrafiltration (Section 4.3.5.3). The particle size of all liposome formulations used in PK experiments was measured using DLS (Section 4.3.4.1). The concentration of azithromycin in dosing solutions and liposome formulations was measured by HPLC analysis (Section 2.2.5).

6.4.5 Pharmacokinetics following intravenous administration

The PK of colistin and azithromycin following IV administration of solutions were determined for the purposes of calculating the relative BA of IT-administered solution and liposome formulations. After overnight recovery from surgery, rats were administered IV target doses of 0.8 mg/kg colistin sulphate or 10 mg/kg azithromycin via the jugular vein cannula. The actual doses administered were calculated by weighing the dosing syringe before and after administration and recording the weight of the

solution administered (target volume 200 µL). The cannula was flushed with two times the cannula tubing volume to ensure the whole dose was administered. Blood samples (200 µL) were collected via the carotid artery cannula prior to dosing and at 1, 3, 5, 10, 20, 30 min and at 1, 1.5, 2, 3 and 4 h following administration. When collecting blood samples, the first 250 µL (void volume of the cannula) was withdrawn and put aside. The actual blood sample was next collected in a separate syringe and the first 250 µL collected was subsequently returned to the rat. Finally, a 200-µL volume of heparinised saline was administered to ensure that a constant intravascular volume was maintained. Blood samples were centrifuged at 9,300 *g* immediately after collection and 100 µL of plasma was stored at -20°C pending HPLC analysis to determine colistin concentrations (Section 2.2.6) or LC-MS to determine azithromycin concentrations (Section 2.2.8) concentrations. Rats were humanely killed at the end of the sampling period by exsanguination via the carotid artery cannula under 5% isofluorane anaesthesia.

6.4.6 Pharmacokinetics following intratracheal administration

Quantification of drug absorption from the airways following aerosolisation is complicated by the fact that a proportion of the dose is not inhaled but retained in the throat and upper airways.[612] Furthermore, the aerodynamics of inhaled particle deposition in rodents are not applicable to humans because rodents primarily breath through the nose.[613] To obviate the complexities introduced by aerosolisation of the formulation into the airway, in the current studies the dose was introduced to the lungs by instillation. Pulmonary instillation involves the delivery of liquid to the site directly above the bronchial bifurcation, circumventing the aerodynamics of inhalation and particle deposition, enabling an estimate of the dose administered to the lungs. It should be recognised, however, that instillation can result in variability in the regional distribution of the dose within the lungs, which can affect absorption.[614]

After cannulation of the right carotid artery and overnight recovery, rats were lightly anaesthetised using inhaled isofluorane (with medical oxygen). While under anaesthetic, the rats were rested in the supine position against a restraining board angled at 60 - 70° from the horizontal. To deliver the pulmonary dose, the mouth of the rat was opened using forceps and a laryngoscope with a light on the end was used to observe the position of the trachea. The nozzle of the pulmonary delivery device, consisting of a syringe attached to a suitable length of cannula tubing, was manoeuvered into the trachea. The tubing was inserted into the trachea to a depth of 2.5 cm to position the tip of the tubing just above the tracheal bifurication.[280] The target dose of 1.5 mg/kg colistin sulphate or 10 mg/kg azithromycin was administered as a bolus directly into the upper airways. Following delivery of the dose into the lungs, the nozzle of the delivery device was removed from the trachea. The difference in the weight of the dosing syringe before and after the administration event was used to calculate the actual volume of formulation administered (target volume 100 μL).

The rat was returned to a metabolic cage and observed during recovery from anaesthetic. Blood sampling and analysis of drug concentration in plasma samples from IT PK experiments was identical to that described in IV experiments (Section 6.4.5). Rats were humanely killed at the end of the sampling period by exsanguination via the carotid artery cannula under 5% isofluorane anaesthesia.

6.4.7 Pharmacokinetic analysis

Measured plasma concentrations were dose normalised to the targeted doses due to the slight deviation of doses administered from the target doses (Table 6-1 and Table 6-2). Dose-normalised plasma concentrations were calculated from measured plasma concentrations by multiplying the measured plasma concentrations by a dose-normalisation factor (Equation 6.2). The dose normalisation factor was calculated as defined by Equation 6-1.

$$Dose\ normalisation\ factor\ = \frac{target\ dose\ (mg/kg)}{actual\ dose\ administered (mg/kg)}$$

Equation 6.1

$$\begin{aligned} &Dose\ normalised\ plasma\ concentration\ (\mu g/mL) \\ &\qquad = measured\ plasma\ concentration\ \ (\mu g/mL) \\ &\qquad \times\ dose\ normalisation\ factor \end{aligned}$$

Equation 6.2

The time to peak plasma concentration (T_{max}) and peak plasma concentration (C_{max}) were determined from the dose-normalised plasma concentration *versus* time profiles for the IT solution and liposomal formulations. Non-compartmental analysis of the pharmacokinetics of colistin and azithromycin was performed using WinNonlin (Model 201, Version 5.2.1, Pharsight Corp, Cary, NC, USA) using dose-normalised plasma concentrations. The terminal rate constant (λ_z) was calculated following linear least-squares regression analysis using the last four log-transformed plasma concentration-time points. The area under the concentration-time curve to the time of the last sample at 240 min (AUC_0^{240}) was calculated by the linear trapezoidal rule. AUC_0^{240} was used instead of the area to infinity (AUC_0^{∞}) since estimation of AUC_0^{∞} yielded a mean

extrapolated component of greater than 20%. The pharmacokinetic parameters were calculated using the following equations:

$$t_{1/2} = ln2/\lambda_z$$ **Equation 6.3**

$$CL = D/AUC_0^{240}$$ **Equation 6.4**

$$V_{ss} = D \times AUMC_0^{240} / AUC_0^{240^2} = CL \times MRT$$ **Equation 6.5**

where CL is the total body clearance, D is the dose, $AUMC_0^{240}$ is the area under the first moment - time curve to the time of the last sample at 240 min, and V_{ss} is the volume of distribution at steady state. The BA (F %) of the IT solution and liposome doses, relative to the IV dose, was calculated using the mean dose-normalised IV and mean dose-normalised IT AUC_0^{240} values, according to Equation 6.6. Comparison of AUC_0^{240} and calculation of F % values required the assumption that systemic clearance was the same across groups.

$$F\ \% = \frac{AUC_0^{240}{}_{IT}}{AUC_0^{240}{}_{IV}} \times \frac{dose_{IV}}{dose_{IT}}$$ **Equation 6.6**

SPSS for Windows (Version 18.0, Chicago, IL) was used to statistically analyse differences between the PK parameters of solution and liposome groups; $p \leq 0.05$ was regarded as a statistically significant difference.

6.5 Results

6.5.1 Encapsulation of antibiotics in liposome formulations

6.5.1.1 Colistin liposomes

Formulations for pulmonary delivery are required to be made iso-osmotic, hence the EE of colistin liposomes dispersed in 0.9% saline was determined and compared with that in water. Figure 6-2 shows that the dispersion of colistin liposomes in 0.9% saline did not substantially alter the EE.

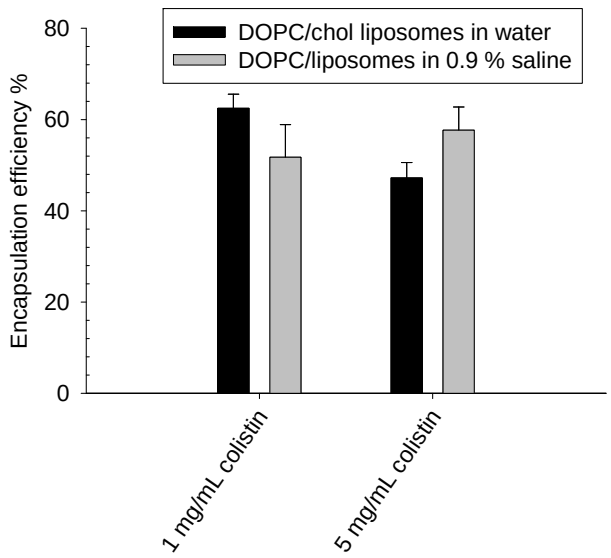


Figure 6-2: EE of colistin in DOPC/cholesterol 2:1 liposomes dispersed in either water or 0.9% saline (mean ± SD, n = 3).

6.5.1.2 Azithromycin liposomes

Using pressure ultrafiltration (Section 4.3.5.1) and the HPLC method described in Section 2.2.5, the EE of azithromycin liposomes following the desalting procedure was determined to be 59 ± 4% (n = 3).

6.5.2 Pharmacokinetics of colistin formulations

6.5.2.1 Intravenous colistin sulphate

Following IV administration of ~0.8 mg/kg colistin sulphate, minor toxicity was observed within the first 10 min following administration of the bolus dose. All rats exhibited a pale discoloration of the mouth and ears and were noticeably inactive. The toxicity appeared to be transient, however, and was not evident 20 min after the dose was administered.

The mean (± SD) dose-normalised concentrations of colistin in plasma as a function of time are shown in Figure 6-3. Dose-normalisation factors used to calculate IV dose-normalised plasma concentrations are shown in Table 6-3. The PK parameters calculated from the plasma concentration *versus* time profiles of Figure 6-3 are shown in Table 6-4.

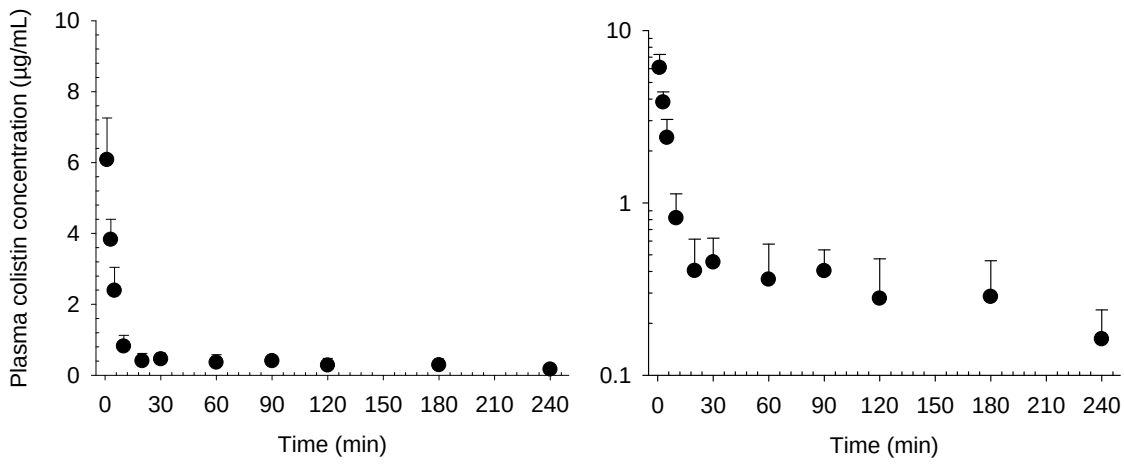


Figure 6-3: Plasma concentration *versus* time profile for colistin following IV administration of the target dose of 0.8 mg/kg colistin sulphate (n = 9). Data are normalised to 0.8 mg/kg and presented as mean ± SD. Linear-linear coordinates in left panel and semi-logarithmic coordinates in right panel. Individual data are presented in Appendix I.

Table 6-3: Actual doses of colistin sulphate administered by the IV route and corresponding dose-normalisation factors for a target dose of 0.80 mg/kg colistin sulphate.

Formulation	Rat	Actual dose (mg/kg)	Dose normalisation factor
IV solution	1	0.81	0.987
	2	0.75	1.066
	3	0.77	1.039
	4	0.80	1.000
	5	0.83	0.963
	6	0.83	0.963
	7	0.79	1.013
	8	0.79	1.013
	9	0.80	1.000

6.5.2.2 Intratracheal colistin solution and liposomal colistin

Following IT administration of ~1.5 mg/kg colistin solution or colistin liposomes there was no observed toxicity. The dose-normalised plasma concentrations of colistin following IT dosing, shown in Figure 6-4 were calculated according to the dose-normalisation factors shown in Table 6-3. Relevant PK parameters are reported in Table 6-5.

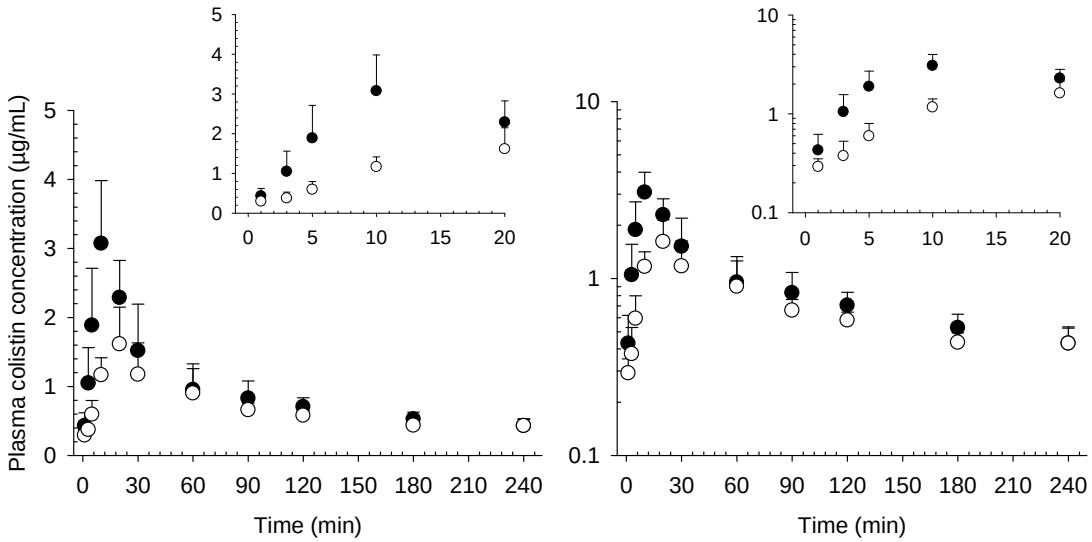


Figure 6-4: Plasma concentration *versus* time profile for colistin following IT administration of the target dose of 1.5 mg/kg colistin sulphate solution (●) (n = 6) or colistin liposomes (n = 7) (○). Data are normalised to 1.5 mg/kg and presented as mean ± SD. Linear-linear coordinates in left panel and semi-logarithmic coordinates in right panel. Insets in the panels contain an expanded time scale. Individual data are presented in Appendix I.

Table 6-4: Pharmacokinetic parameters after IV administration of colistin solution and IT administration of colistin solution or colistin liposomes. Data are expressed as mean ± SD except for $\boldsymbol{T_{max}}$ where the median and range are reported.

Parameter	IV colistin solution (n = 9)	IT colistin solution (n = 6)	IT colistin liposomes (n = 7)
Target dose (mg/kg)	0.80	1.50	1.50
CL (mL/min/kg)	1.30 ± 0.42	1.40 ± 0.32†	2.16 ± 0.37†
V_{ss} (mL/kg)	370 ± 128	353 ± 72†	539 ± 114†
$t_{1/2}$ (min)	126 ± 48	116 ± 23	121 ± 36
T_{max} (min)	-	20 (10 - 20)	20 (10 - 30)
*C_{max} (µg/mL)	-	3.20 ± 0.81	1.70 ± 0.47
*AUC_0^{240}(ug·mL/min)	194 ± 127	224 ± 35	165 ± 25
*F %	-	104	77

†represents apparent CL, (CL/F), and apparent Vss, (Vss/F), where F is the bioavailability. *dose-normalised values presented.

A Mann-Whitney U-test was used to determine whether there was a statistical difference in the median time to reach the maximum plasma concentration (T_{max}) between the IT colistin solution and IT colistin liposome groups. The test showed no statistically significant difference between the two groups (p = 0.171). An independent samples t-test showed a statistically significant difference in the dose-normalised maximum plasma concentrations (C_{max}) and AUC_0^{240} values (p = 0.006 and 0.001, respectively). A one-way ANOVA showed that there was no significant difference in the half-life of colistin between any of the groups tested (p > 0.19).

6.5.3 Pharmacokinetics of azithromycin formulations

6.5.3.1 Intravenous azithromycin

Following IV administration of the target dose of ~10 mg/kg azithromycin, there were no indications of toxicity. The dose-normalised plasma concentrations of azithromycin following IV dosing, shown in Figure 6-5, were calculated according to the dose-normalisation factors shown in Table 6-5. The PK parameters calculated from the plasma concentration *versus* time profiles are presented in Table 6-6.

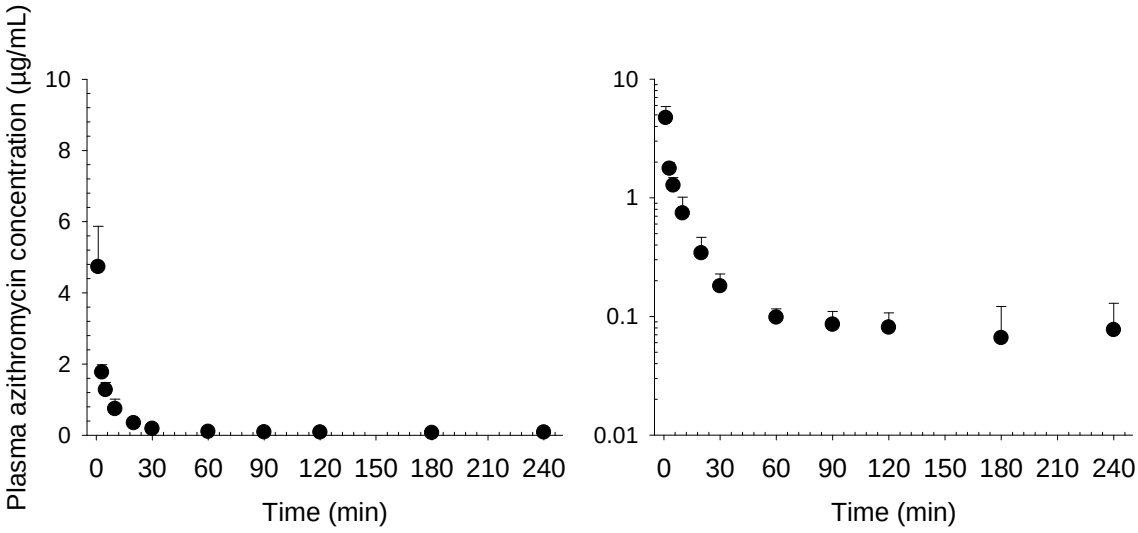


Figure 6-5: Plasma concentration *versus* time profile following IV administration of the target dose of 10.0 mg/kg azithromycin solution (n = 5). Data are dose-normalised to 10.0 mg/kg and presented as mean ± SD. Linear-linear coordinates in left panel and semi-logarithmic coordinates in right panel. Individual data are presented in Appendix 1.

Table 6-5: Actual doses of azithromycin administered by the IV route and corresponding dose-normalisation factors for a target dose of 10.0 mg/kg azithromycin.

Formulation	Rat	Actual dose (mg/kg)	Dose-normalisation factor
IV solution	1	10.90	0.917
	2	10.86	0.921
	3	10.88	0.919
	4	10.96	0.912
	5	10.70	0.935

6.5.3.2 Intratracheal azithromycin solution and liposomal azithromycin

Following IT administration of ~10 mg/kg azithromycin solution or azithromycin liposomes there were no observed toxicities. The dose-normalised plasma concentrations of azithromycin following IT dosing, shown in Figure 6-6, were calculated with the dose-normalisation factors shown in Table 6-6. The most notable feature of the plasma profile resulting from the IT administration of azithromycin liposomes, compared to the azithromycin solution, was the difference in the absorption phase. It appeared that the absorption phase following IT administration of the liposome formulation was so rapid that a 'typical' absorption phase was not observed. The PK parameters calculated from the plasma azithromycin concentration *versus* time profiles in Figure 6-6 are shown in Table 6-7.

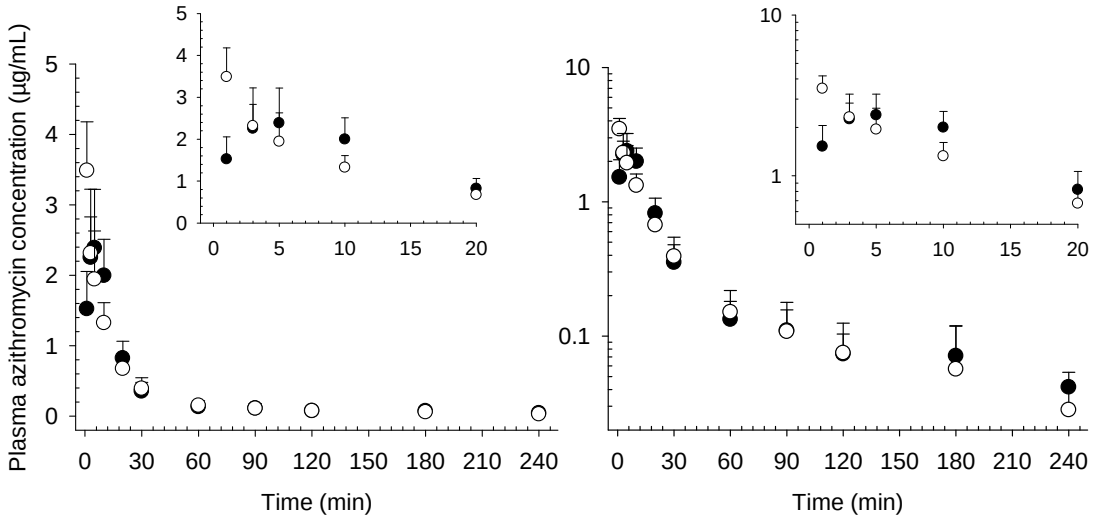


Figure 6-6: Plasma concentration *versus* time profile for azithromycin following IT administration of the target dose of 10 mg/kg azithromycin solution (●) (n = 8) or azithromycin liposomes (○) (n = 4). Data are normalised to 10.0 mg/kg and presented as mean ± SD. Linear-linear coordinates in left panel and semi-logarithmic coordinates in right panel. Insets in the panels contain an expanded time scale. Individual data are presented in Appendix I.

Table 6-6: Actual doses of azithromycin administered by the IT route and corresponding dose-normalisation factors for a target dose of 10.0 mg/kg.

Formulation	Rat	Actual dose (mg/kg)	Dose-normalisation factor
IT solution	1	9.41	1.063
	2	9.52	1.050
	3	7.95	1.257
	4	8.91	1.122
	5	8.25	1.212
	6	9.90	1.010
	7	9.97	1.003
	8	9.73	1.028
IT liposomes	1	6.09	1.642
	2	8.13	1.230
	3	7.81	1.280
	4	7.77	1.287

Table 6-7: Pharmacokinetic parameters after IV administration of azithromycin solution and IT administration of azithromycin solution or azithromycin liposomes. Data are expressed as mean ± SD except for $\boldsymbol{T_{max}}$ where the median and range are reported.

Parameter	**IV azithromycin solution (n = 5)**	**IT azithromycin solution (n = 8)**	**IT azithromycin liposomes (n = 4)**
Target dose (mg/kg)	10.0	10.0	10.0
CL (mL/min/kg)	52.4 ± 19.4	48.6 ± 13.4†	62.7 ± 9.6†
V_{ss} (mL/kg)	9897 ± 3492	10284 ± 2647†	10279 ± 1524†
$t_{1/2}$ (min)	240 ± 92	151 ± 41	101 ± 27
T_{max}(min)	-	5 (3 - 10)	1 (1 - 1)
*C_{max} (µg/mL)	-	2.52 ± 0.59	2.89 ± 0.68
*AUC_0^{240} (µg·min/mL)	44.5 ± 5.9	60.1 ± 16.9	57.1 ± 5.0
*F %	-	135	129

†represents apparent CL (CL/F) and apparent Vss, (Vss/F) where F is the bioavailability. *dose-normalised values presented.

Independent sample *t*-tests were used to test for differences in the dose-normalised C_{max} and AUC_0^{240} achieved following IT dosing of azithromycin solution and azithromycin liposomes, with no significant differences found between the IT azithromycin solution and IT azithromycin liposome groups (p > 0.7 and 0.3, respectively). A one-way ANOVA revealed that the $t_{1/2}$ of azithromycin was statistically different following IV administration of a solution, compared to IT administration of a solution or liposome formulation (p = 0.044 and 0.008, respectively). A Mann-Whitney U-test was used to determine whether there was a statistical difference in the time to reach maximum plasma concentrations (T_{max}) between the IT solution and IT liposome groups. This test showed a significant difference in the T_{max} between the two groups (p = 0.004).

6.6 Discussion

Liposomal encapsulation has been shown to effectively modify the pulmonary PK of numerous drugs following administration directly to the airways.[470, 529, 611] The effectiveness of a liposomal carrier in modifying the absorption of pulmonary-administered drugs depends on its ability to retain and modify the absorption of drug following administration to the lungs. On the other hand, a drug that is extensively retained by liposomes will not be bioavailable within the lungs if the pulmonary clearance of liposomes precedes drug release from the liposomes (Figure 6-1). In the present study, the respective time courses of plasma concentrations following IT administration of solution and liposomal formulations were used as an indicator of relative rates and extents of pulmonary availability of drug.

IV administration of colistin solution was included in this study for the purposes of providing an estimate of the absolute BA (F %) of IT-administered solution and IT-administered liposome formulations. In this study, the initial plasma concentrations following IV administration of colistin were comparable to those reported from previous studies in rats[139, 141] when adjusted for different doses across the studies. The CL of colistin following an IV dose of 0.8 mg/kg colistin sulphate was lower than the values reported by other authors following IV administration of 0.75 mg/kg colistin sulphate (3.02 mL/min/kg)[139] and 1 mg/kg colistin sulphate (5.2 mL/min/kg),[141] consistent with the longer $t_{1/2}$ calculated in this study. The differences may be accounted for by variations arising between different batches of animals and the subtly different doses administered in the different studies (0.75 – 1 mg/kg).

The PK parameters calculated following IT administration of a colistin solution were comparable to those reported in a study by Yapa *et al.*[139] In the present study, a

mean $t_{1/2}$ of 116 min (Table 6-4) was calculated following a dose of 1.5 mg/kg, while Yapa *et al*. reported a $t_{1/2}$ of 102 min following IT administration of 1 mg/kg colistin sulphate in solution.[139] Values for AUC_0^{240}, T_{max} and C_{max} were similar to those reported by Yapa *et al*., taking into consideration the different doses administered between studies.

The AUC_0^{240} of colistin after pulmonary dosing of colistin liposomes (165 ± 25 µg.min/mL) was shown to be significantly different from that of colistin following IT administration of a colistin solution (224 ± 35 µg·min/mL). The mean AUC_0^{240} for the liposomal group was ~26% lower than that for the solution group and these values were consistent with the estimated BA (based upon group data) of 77% and 104% for the liposomal and solution groups, respectively. It should be noted that the use of AUC_0^{240}values in this way and the estimation of BA assumes that the CL was the same between IT solution and IT liposome groups. In this study, different animals were used in each group (i.e. this was not a cross-over study design). Therefore, the comparison of the relative AUC_0^{240} values and estimation of BA must be undertaken with appropriate caution.

Liposomal encapsulation of colistin reduced the C_{max} of colistin by ~47% compared to IT administration of a colistin solution. The magnitude of reduction in C_{max} is comparable to those reported in other studies in which the pulmonary PK of liposomal dose forms of ketotiden,[615] amikacin[287] and benzylpenicillin[616] were assessed following IT administration. The reduction in the C_{max} of colistin from the IT liposomal formulation in the current study may in part be related to a decrease in the relative extent of absorption as reflected in the respective AUC_0^{240} values (discussed above), but may also reflect slower absorption from the liposomal formulation. Although the T_{max}

values (a relatively insensitive indicator of rate of absorption) were not significantly different between the IT solution and liposomal groups, examination of the respective profiles (Figure 6-4, and insets) reveals a clearly slower rate of attainment of peak plasma colistin concentrations for the IT liposomal group as compared with the IT solution group. This is consistent with encapsulation of colistin slowing the overall rate of absorption.

It is recognised that colistin would exist in three different phases of the liposome formulation administered: that which is unencapsulated and not associated with the lipid bilayer, that which is associated or bound to the lipid bilayer and, finally, that which is encapsulated within the interior aqueous space of the liposomes. As described in Section 6.1, and outlined by Figure 6-1, the overall rate (and extent) of pulmonary absorption of colistin into the systemic circulation will depend on the rate of removal of drug-loaded liposomes by non-absorptive clearance, the rate of drug release from the liposomes remaining in the lungs, and the rate of drug transfer across the epithelial membrane and into the blood after the drug is released from the liposomes. As liposomes have been shown to be retained within the lungs for up to 24 h after pulmonary administration to rats,[617] the appearance of colistin in the systemic circulation may reflect the rate at which colistin is released from liposomes within the lungs (assuming that the rate of trans-membrane movement of unencapsulated colistin does not change due to the presence of liposomal material).

That the terminal half life values for colistin were not different between IV, IT solution and IT liposome groups in the present study indicates that the process limiting the overall disposition of colistin after administration to the lungs is elimination of colistin from the body, rather than absorption of colistin from the lungs. Nevertheless, as

discussed above, the slower rate of attainment of the peak concentration of colistin in plasma suggests that the encapsulation of colistin retarded the overall absorption due to a sustained release from the liposomes. The apparent decrease in the extent of absorption after the liposomal formulation may be due to increased clearance of liposomes containing their load of colistin (Figure 6-1) by processes such as mononuclear phagocytosis[305, 618] or clearance by the mucociliary escalator.[619] Alternatively, it is possible that a proportion of the dose was retained within the lungs beyond the time that the last blood sample was taken.

McAllister and co-workers investigated the use of liposomes as a drug delivery system for the pulmonary administration of polymyxin B (Figure 1-4), a compound of similar chemical structure to colistin.[538] In that study, polymyxin B-loaded liposomes were administered to the lungs of rats via IT instillation at a dose of 0.5 mg/kg. Polymyxin B liposomes comprised of phosphatidylcholine and cholesterol (2:1 mole ratio) were prepared by the dehydration-rehydration method and were shown to have an EE of approximately 45%. In that study, plasma concentrations resulting from pulmonary administration of polymyxin B liposomes were not reported, however, local concentrations of polymyxin B within the lungs were measured 4 and 24 h after administration of the IT dose. Interestingly, McAllister *et al.* found that 45 - 65% of the dose administered remained within the lungs 4 h post IT instillation. This is more than would be anticipated to remain in the lungs following IT administration of the colistin liposomes used in the present study, based on the fact that almost 80% of the dose was bioavailable after 4 h, leaving only 20% of the dose unaccounted for. The apparent difference in the BA of polymyxin B and colistin following liposomal delivery to the lungs is difficult to explain given the similarity in chemical structure of the two compounds, and the similar compositions of the liposomes used in the two studies.[538]

A large number of liposomal formulations for which modification of *in vivo* pulmonary PK has been demonstrated have not achieved clinical utility.[470, 620-625] Therefore, demonstration of modifications in the pulmonary PK of drugs due to liposomal encapsulation does not necessarily translate into a clinically useful product. Liposomal amikacin, Arikace™, is an example of a liposomal formulation for which the substantial differences in the pulmonary PK of the solution and liposomal forms resulted in a therapeutically useful inhalation therapy.[626-628] Following IT administration to sheep, the liposomal form of amikacin resulted in a decrease of the C_{max} by 33%, a prolonging of T_{max} by 1.6-fold, and notably, an increase in the half-life of amikacin from 117 min to over 10 h.[287]

By comparison with the liposomal formulation of amikacin, the modification of the pulmonary PK by liposomal encapsulation of colistin, as reflected by the changes in PK parameters, may be considered modest. Notwithstanding, however, if increased pulmonary retention of colistin via liposomal encapsulation was accompanied by prolonged pulmonary residence of a second, synergistic antibiotic, a more efficacious colistin-inhalation therapy may be achieved. The co-localisation of colistin with the model antibiotic 'co-drug', azithromycin, was demonstrated by liposomal encapsulation and modified release *in vitro* (Section 5.5.4.4). Prior to examining the PK properties of the combination colistin-azithromycin liposomal formulation, the PK properties of liposomal azithromycin were assessed after IT administration.

In order to estimate the absolute BA (F %) of IT-administered azithromycin solution and azithromycin liposomes, the PK of azithromycin following IV administration was first examined. The IV PK of azithromycin has been extensively reported in the literature. In both rats and dogs, azithromycin serum concentrations have been shown to

decline in a polyphasic manner, exhibiting a tri-exponential disposition curve following IV administration.[629] In rats, the three phases of disposition are described as a rapid initial distribution phase (0 - 2 h), an intermediate phase (2 - 6 h) and a later terminal phase (6 - 48 h).[629] The $t_{1/2}$ calculated based upon the intermediate disposition phase following IV administration of 20 mg/kg to rats is reported to be 210 min.[629] The true half-life of azithromycin, based on the later terminal phase, is reported to be 30 h in rats.[629] The half-life calculated in the present study should be treated with caution because it was based upon blood samples taken over a limited time frame (240 min following IV administration). Consequently, the PK parameters calculated for azithromycin in the present study should be treated with caution.

In the present study, the $t_{1/2}$ of azithromycin (240 ± 91 min) following IV administration was calculated based upon the plasma concentrations of samples obtained between 90 and 240 min, and is comparable to the 'intermediate' phase $t_{1/2}$ reported by Shepard *et al.*[629] It was not possible in the present study to extend the sampling regimen beyond 240 min to calculate the $t_{1/2}$ of the terminal phase due to sensitivity limits of the analytical assay (Section 2.2.8.4). The difference in the estimates of $t_{1/2}$ across the IV solution, IT solution and IT liposome groups probably results from the estimation of $t_{1/2}$ in a region of the plasma concentration – time profiles other than the terminal phase.

The PK of azithromycin following IT administration have not been extensively examined, with only one report of pulmonary dosing in animal models.[630] Dailey *et al.* compared the PK of azithromycin following pulmonary instillation of an azithromycin solution and an azithromycin suspension to rats.[630] IT instillation of 0.1, 0.3 and 1

mg/kg azithromycin (solubilised in a citrate-based buffer solution) resulted in a dose proportional increase in azithromycin concentrations in all compartments tested including serum, lung tissue and ELF.[630] In that study, the T_{max} and C_{max} occurring in plasma after IT administration were not reported.

It is recognised that a difficulty associated with the use of azithromycin as a 'model' poorly-water soluble 'co-drug' is the unique PK characteristics of azithromycin. Azithromycin tends to concentrate within certain compartments including alveolar macrophages, ELF and lung tissues, explaining the multiple phases of distribution observed following IV administration.[630-631] The ratio of tissue (e.g. alveolar macrophages, lung tissue) to serum concentrations of azithromycin following a single oral dose can be as high as 100:1.[629] Therefore, the concentrations of azithromycin in the serum compartment may not necessarily reflect the concentration of azithromycin within the various compartments of the lungs (ELF, lung tissue, alveolar macrophages). Azithromycin has been shown to persist within alveolar macrophages for up to five days post IV administration, with comparatively low concentrations present in plasma.[632] The large V_{SS} measured can therefore be attributed to high intracellular and tissue concentrations of azithromycin.[633]

Though a striking difference between the plasma concentration *versus* time profiles of IT administered azithromycin solution and liposomal formulations was observed (Figure 6-6), no significant difference was detected between the bioavailability of the two formulations. This indicates that liposomal encapsulation of azithromycin had no effect on the extent of azithromycin absorption from the lungs. Moreover, no significant difference was observed in the maximum plasma concentrations achieved following dosing of the solution and liposome formulations.

Perhaps the most striking difference between the PK properties of azithromycin solution and liposome doses was the time to reach maximum plasma concentrations. Following IT administration of an azithromycin solution, a median T_{max} of 5 min was observed. In contrast, following administration of the liposomal azithromycin dose, maximum plasma concentrations of azithromycin were achieved within 1 min. The 'typical' absorption phase was not obvious following administration of the azithromycin liposome formulation. That liposomal encapsulation of azithromycin resulted in more rapid absorption from the lungs than administration of the solution form of azithromycin was an unusual and unexpected result.

It is well established that pulmonary absorption of drugs is controlled by partitioning- and molecular weight-dependent diffusive processes, in most cases.[269, 278, 280, 634-635] Schanker and co-workers demonstrated that lipid soluble substances are more rapidly absorbed from the lungs than those that are less lipid soluble, and that the log P and molecular weight of a compound may be predictive of the rate of drug absorption from the lungs.[269, 280] Azithromycin, a lipophillic compound with a log P of 4.02[564] and molecular weight (M_w) of 748, may therefore be rapidly absorbed across the alveolar epithelium when administered in its unencapsulated form. Rabinowitz *et al.* demonstrated that after delivery into the alveolar space, small, lipophilic molecules can exhibit 'ultra-fast' pulmonary absorption, appearing in the bloodstream within 10 sec.[636] Such rapid absorption has been demonstrated for prochlorperazine (log 4.6, M_w 374) and alprazolam (log P 4.9, M_w 308).[637] As the alveolar blood supply passes directly to the pulmonary vein, left heart and arterial circulation, high concentrations of drug can be detected in the blood within seconds of administration of a pulmonary dose, when sampled from the heart.[637] This is reflected in the results of the present study, in which samples were obtained from the carotid artery following pulmonary doses and peak

plasma concentrations occurred at 3 - 10 min following pulmonary administration of the solution and within 1 min of administration of the liposomal dose.

Additional studies were undertaken in this thesis to investigate the possible mechanism underlying the more rapid absorption of azithromycin observed from liposomes relative to the solution formulation. The rate of absorption of azithromycin was investigated from a 'control' formulation. This formulation, containing azithromycin solubilised in pH 7 citric acid (equivalent to the solution formulation) together with empty liposomes, represented the vehicles of both solution and liposome formulations. The resulting plasma profiles were similar to those for the liposomal formulation (Figure 6-6), suggesting an impact of lipid on azithromycin absorption. Since it has been demonstrated previously that the rate of carboxyfluorescein absorption from pulmonary administered carboxyfluorescein liposomes is lipid-dose dependent, with higher lipid doses resulting in faster absorption rates,[530] the potential for a similar phenomenon was examined in the current research. The effect of lowering the dose of lipid by 75% was investigated; the resulting absorption rate was equivalent to that of the high lipid dose formulation. Together, these studies suggest that the lipid content modified the rate of azithromycin absorption in a manner that was not lipid dose dependent over the range of lipid doses examined. The threshold dose of lipid to elicit the effect and the precise mechanism was not investigated further as it was deemed to be beyond the scope of the present study.

To the author's knowledge, this is the first report of the PK resulting from the delivery of remotely-loaded liposomes to the lungs. That the remote-loading of azithromycin into the liposome interior resulted in more rapid absorption of azithromycin from the lungs, the liposomal bilayer not presenting any barrier to absorption, was not

anticipated. Owing to the rapid absorption of liposomal azithromycin from the lungs, it was deemed unnecessary to pursue the PK of colistin-azithromycin combination liposomes *in vivo***.**

6.7 Conclusion

Liposomal encapsulation of colistin has been shown to only modestly modify the pulmonary PK of colistin. The statistically significant reductions in maximum plasma concentrations and systemic bioavailability of colistin due to liposomal encapsulation suggest that encapsulation within liposomes can reduce the overall rate and extent of colistin absorption from the lungs. Further studies examining the time-course of the concentrations of colistin remaining within the lungs and ELF would provide a greater insight of the ability of liposomal colistin to modify pulmonary retention of colistin.

Azithromycin proved to be an unsuitable model drug for investigations into the effect of colistin on the rate of *in vivo* release/absorption of a co-encapsulated antibiotic. The current combination liposomal formulation containing colistin and azithromycin, as it stands, would have a limited ability to provide co-localisation of both colistin and azithromycin within the lungs.

Chapter 7: Summary and perspectives

The emergence of multi-drug resistance amongst Gram-negative bacteria is a significant global health issue.[6, 58] Heightening the problem is the lack of novel antibacterial agents in the drug development pipeline.[55-56] Colistin is an old polymyxin antibiotic experiencing a resurgence in clinical use for the treatment of MDR pulmonary infections due to its activity against multi-drug resistant Gram-negative pathogens. Worryingly, resistance to colistin has begun to emerge, highlighting the need to intensify the use of colistin in order to maintain its clinical utility for the future. Colistin combination antibiotic inhalation therapy is one approach towards optimisation of colistin use against pulmonary infections.

The studies described in this thesis have investigated specific aspects of the solution behaviour and self-assembly of colistin and its prodrug, CMS, with a view to their use as a component of colloidal drug carriers for combination therapy. Specifically, their propensity to self-assemble in aqueous solution, their potential to provide micellar solubilisation for poorly-water soluble drugs, their interactions with liposomes and effect on release of a co-loaded antibiotic, and the effect of liposomal encapsulation on pharmacokinetics following pulmonary administration have been addressed.

7.1 Self-assembly of colistin and its prodrug, CMS

Understanding the physicochemical behaviour of colistin and CMS is essential for the rational design of new formulations of these old antibiotics. The first studies described in this thesis therefore addressed characterisation of the self-assembly of colistin and CMS in aqueous solution. Micelle-forming solutions of colistin and CMS were assessed for the capacity to solubilise and thereby co-formulate colistin with poorly-water

soluble antibiotics. This study, now published, confirmed the formation of colistin and CMS self-assembling colloidal aggregates, and is the first study to have identified and reported a CMC for colistin and CMS. Association colloids of colistin and CMS showed a weak solubilising capacity for the model poorly-water soluble drug, diazepam, but increased the solubility of a model poorly-water soluble antibiotic, azithromycin, by up to 40-fold. The magnitude of colistin and CMS micellar solubilisation of azithromycin was not considered sufficiently high to be utilised as an approach to the co-formulation of colistin and a second agent for delivery to the respiratory tract, but nevertheless did support the finding of assembly of these molecules in solution. These data provided important information for the rational formulation of colistin and CMS for delivery to the airways. Further studies, utilising molecular modelling approaches, could provide further insight into the conformational arrangement of colistin molecules in micellar clusters.

The conversion of the pro-drug, CMS, to the biologically active compound, colistin, was shown to be a concentration-dependent process. Moreover, it was shown that the conversion of CMS to colistin is markedly slower at concentrations above the CMC identified by DLS. The self-assembly of CMS provides a rational explanation for the stability of CMS at high CMS concentrations (>10 mg/mL) and rapid degradation at low CMS concentrations (<1 mg/mL). An investigation into the chemical kinetics of this process was limited by the chemical complexity of the CMS molecule; elucidation of the kinetics of CMS conversion to colistin is an area with scope for further investigation. Development of chromatographic or spectroscopic assays able to differentiate between partial derivatives of colistin would aid further investigation of the chemical stability and degradation kinetics of CMS in solution.

7.2 Formulation studies

Liposomes were investigated as an alternative approach to the formulation of colistin in combination with a poorly-water soluble antibiotic, with a view to improving and controlling the co-localization of the combination drugs. The interaction of colistin and CMS with neutral DOPC liposomes was first investigated to assess the utility of liposomes as a colloidal drug delivery system for the polymyxin antibiotics. The interaction of CMS with the DOPC liposomes was shown to accelerate the conversion of CMS to colistin, resulting in a physically and chemically unstable colloidal formulation. Thus, liposomes are not a useful formulation approach for CMS. Colistin-loaded liposomes, on the other hand, exhibited superior colloidal stability and were therefore subjected to further investigation. Interaction between colistin and the bilayer of DOPC liposomes was demonstrated using measurements of zeta potential (changes in surface charge indicating direct association between charged colistin and the bilayer) and association *via* ITC studies. These studies provided evidence that hydrophobic forces are the predominant interaction driving the association between colistin and DOPC liposomes, supporting the notion that the hydrophobic tails of colistin insert into the lipidic region of the DOPC bilayer. The incorporation of cholesterol into the bilayer strengthened the interactions between colistin and the bilayer.

This work also identified the need to sufficiently validate the separation of nanoparticles from their surrounding dispersing medium in the assessment of EE and *in vitro* release. This is an area that has not been carefully considered in the literature. In this thesis, a new approach to the assessment of nanoparticle separation efficiency was presented using DLS to validate separation of colloidal species from free drug in solution. The

method can be applied to separation of liposomes as well as other nanoparticle-based drug delivery systems.

The EE of colistin-loaded liposomes prepared by the dry-film method was approximately 50%, although the EE of colistin was found to be concentration-dependent, decreasing with increasing concentrations of colistin. A low drug:lipid ratio of <0.1:1 for colistin:DOPC was calculated based upon EE measurements. EE of colistin was slightly improved by the incorporation of cholesterol into the formulation, consistent with the findings from ITC studies which showed that incorporation of cholesterol into DOPC liposomes increased the strength of the interactions between colistin and the bilayer.

An alternative freeze-drying method was investigated for potential to improve liposomal encapsulation of colistin. While this method has been previously shown to enhance the EE of a number of drugs, this was not the case for colistin. Colistin liposomes prepared by the freeze-dry method exhibited comparable EE to liposomes prepared by the dry-film method. Future work therefore used colistin liposomes prepared by the dry-film method only.

In vitro release studies were employed to predict the rate of release of colistin from colistin-loaded liposomes upon dilution of the formulation into physiologically relevant fluids. Colistin was not released over time from liposomes, but rather, spontaneous partitioning of colistin out of the liposome bilayer occurred following dilution. The interactions between colistin and the bilayer, however, still provided scope for modification of the rate of pulmonary absorption of colistin, with 50% of the colistin still associated with the liposome bilayer after a 5-fold dilution.

The studies described in Chapter 5 examined the use of colloidal drug delivery systems for the co-formulation of colistin with a model poorly-water soluble 'co-drug', azithromycin. Liposomes and sub-micron emulsions were identified as two formulation strategies by which colistin and a second antimicrobial agent could be co-localised for optimised combination antibiotic delivery to the lungs. Passively-loaded azithromycin liposomes and sub-micron emulsion systems achieved high encapsulation efficiencies of 88 and 95%, respectively. The use of a pH-gradient loading technique achieved superior EE of 98% and a high drug:lipid ratio (0.2:1). To the author's knowledge, this is the first study to investigate azithromycin liposomes produced by pH-gradient remote-loading methods.

In vitro release studies were used as a preliminary measure of the potential for liposomes to co-localise azithromycin and colistin within the lungs following dilution. In the absence of cholesterol, azithromycin leaked rapidly out of liposomes with >50% of the encapsulated contents released over 12 h. The incorporation of cholesterol in a lipid:cholesterol ratio of 2:1 slowed the release of azithromycin, with <10% released after 72 h. The cholesterol-containing formulation therefore presented a potential 'slow release' liposome formulation from which controlled release of azithromycin might be achieved following pulmonary delivery. Additionally, the loading of azithromycin into the liposome interior was shown to significantly reduce the rate of chemical degradation of azithromycin.

Given the known membrane-permeabilising effect of colistin, it was necessary to assess the effect of colistin on the release of azithromycin from liposomes. The presence of colistin during the remote-loading process did not affect the loading of azithromycin. However, colistin caused approximately 40% more azithromycin to be released from the

liposomes over 72 h, consistent with a permeabilising effect of colistin on the liposome bilayer.

7.3 Pharmacokinetic assessment of antibiotic-loaded liposomes

The effect of liposomal encapsulation of colistin on the pulmonary PK of colistin was investigated by administration of the formulations characterised in Chapters 4 and 5 to the lungs of rats. These studies revealed little effect of liposomal encapsulation on the T_{max}, though the C_{max} and overall BA were significantly lower indicating a modified overall rate of absorption due to a modest sustained release effect. These studies suggest that liposomal formulations investigated may have a limited capacity to modify the rate of absorption of colistin following delivery to the lungs. Given that re-formulation into a liposomal carrier increases the cost of the drug preparation, a liposomal formulation must prove to be of significant advantage over standard therapy for the liposomal carrier to be feasible.

For colistin liposomes to offer an advantage over the current standard therapy, further formulation optimisation would be required. Modification of the formulation to enhance the interaction between colistin and the liposomal bilayer may be one strategy by which more substantially delayed pulmonary absorption of colistin might be achieved. Investigations into the capacity of alternative phospholipids (e.g. negatively charged) to enhance the association between colistin and the liposomal bilayer may prove to identify liposome formulations with improved EE and more stable encapsulation of colistin. Methods to achieve remote-loading of colistin within the liposomal interior would be worth investigating as a means to improving the drug:lipid ratio.[348]

PK studies of azithromycin-loaded liposomes following pulmonary delivery indicated, somewhat unexpectedly that liposomal encapsulation of azithromycin resulted in more rapid absorption of azithromycin from the lungs and into the blood stream. While rapid absorption has been demonstrated for other lipophilic drugs following delivery to the lungs, this thesis is the first to report the increased rate of absorption of drug following pulmonary delivery of a remote-loaded liposome formulation.

The poor correlation of *in vitro* release data with *in vivo* data indicates that the inherent biopharmaceutical properties of azithromycin could not be overcome by formulation within a liposomal carrier. The lack of correlation is attributed to the high lipophilicity of azithromycin. The co-formulation of colistin with poorly water-soluble antibiotics in liposomal delivery systems for administration to lungs may therefore be limited to those drug candidates that have lesser to intermediate lipophilicity. Other antibiotics which have shown synergistic activity with colistin, such as rifampicin,[243, 245, 638] may be suitable for liposomal co-encapsulation. In the event that liposomal encapsulation cannot achieve adequate pulmonary retention of colistin and a second antibiotic, formulation approaches to optimising the treatment of MDR pulmonary infections should not be completely abandoned. There are several other available drug delivery systems, including polymeric nanoparticles and solid-lipid nanoparticles, just to name a few, which could be considered as an alternative to liposomal encapsulation.

7.4 Concluding comments

The potential for liposomes to modify the absorption of colistin and a model poorly-water soluble co-drug, azithromycin, has been evaluated in this thesis as an approach to providing efficient delivery of colistin combination antibiotic therapy to the lungs for local activity. The studies described in this thesis have demonstrated that the liposomal

formulations did not offer a substantial advantage in terms of modifying the rate of absorption of colistin *in vivo*. Advanced drug delivery systems are still worth further investigation for the optimisation of colistin inhalation therapy. The development time and cost of reformulation is significantly lower than that for the development of new chemical entities. Until new antibiotics emerge from the drug development pipeline, increased efforts to identify a suitable formulation approach to the improved delivery of colistin and colistin combination therapy to the respiratory tract may prove to be valuable in combating the issue of emerging resistance to colistin.

Appendix I

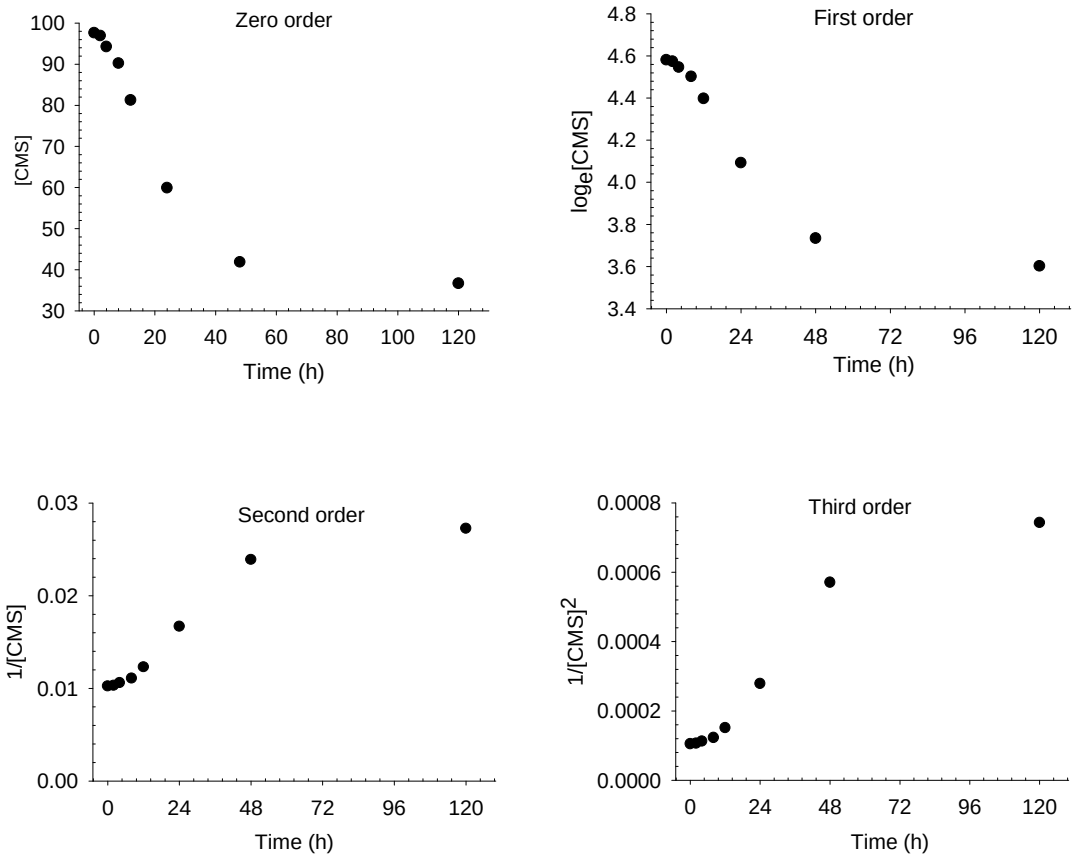


Figure A1-1: Fitting of the degradation kinetics of 0.1 mg/mL CMS to zero, first, second and third order rate laws.

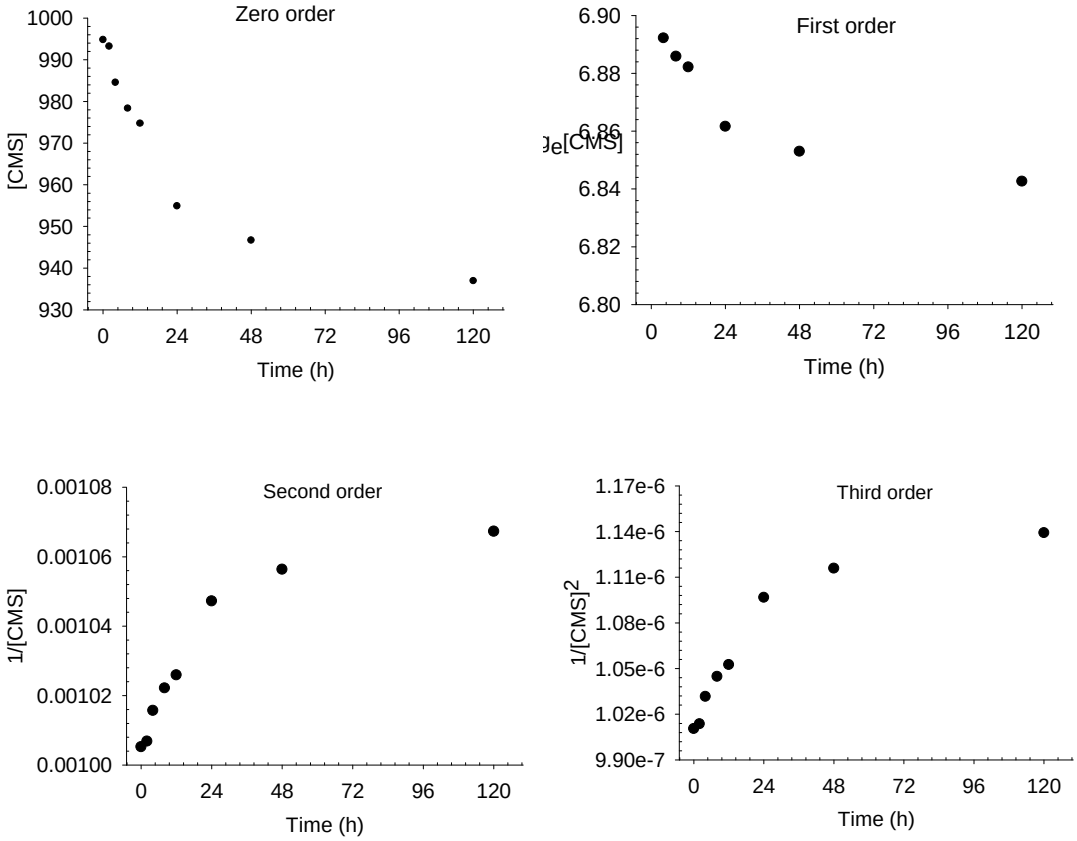


Figure A1-2: Fitting of the degradation kinetics of 1 mg/mL CMS to zero, first, second and third order rate laws.

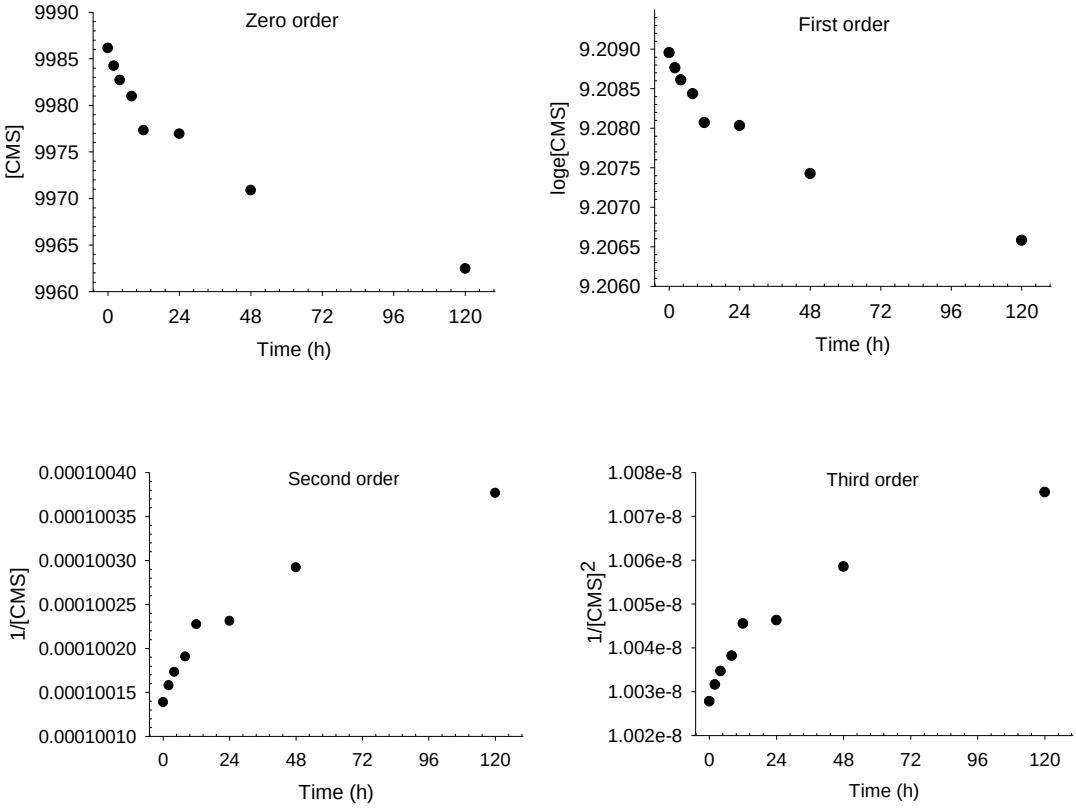


Figure A1-3: Fitting of the degradation kinetics of 10 mg/mL CMS to zero, first, second and third order rate laws.

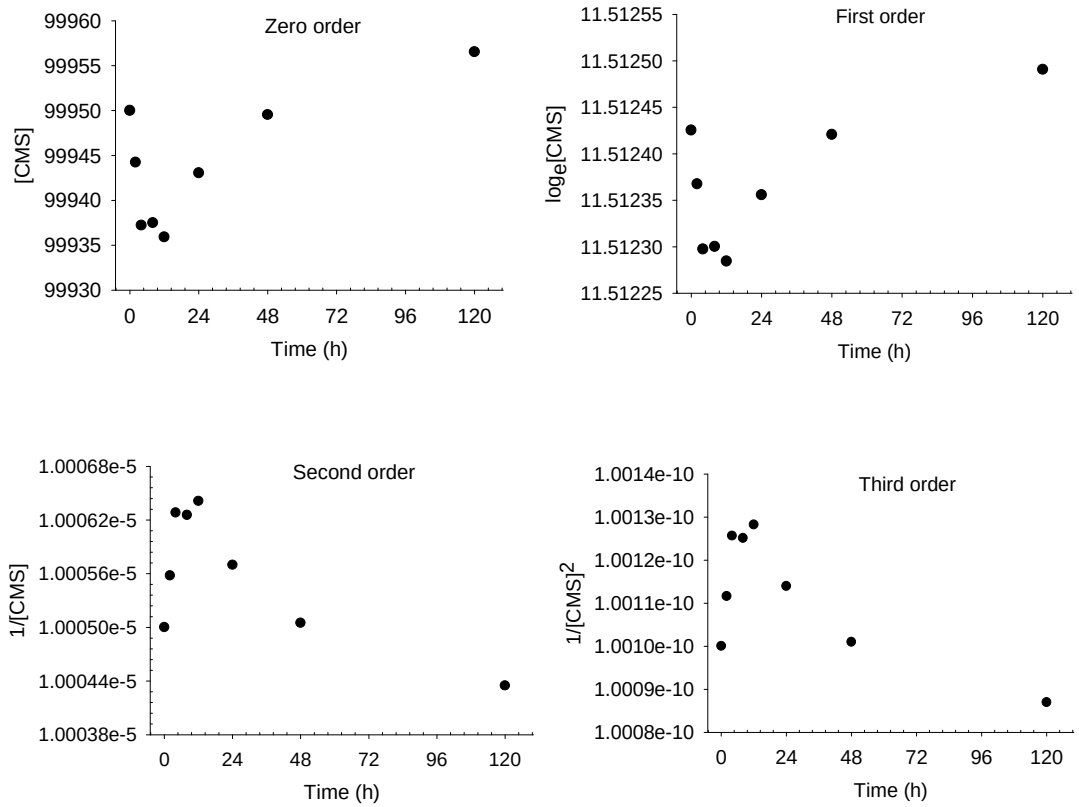


Figure A1-4: Fitting of the degradation kinetics of 100 mg/mL CMS to zero, first, second and third order rate laws.

Appendix II

Data from pharmacokinetic studies described in Chapter 6.

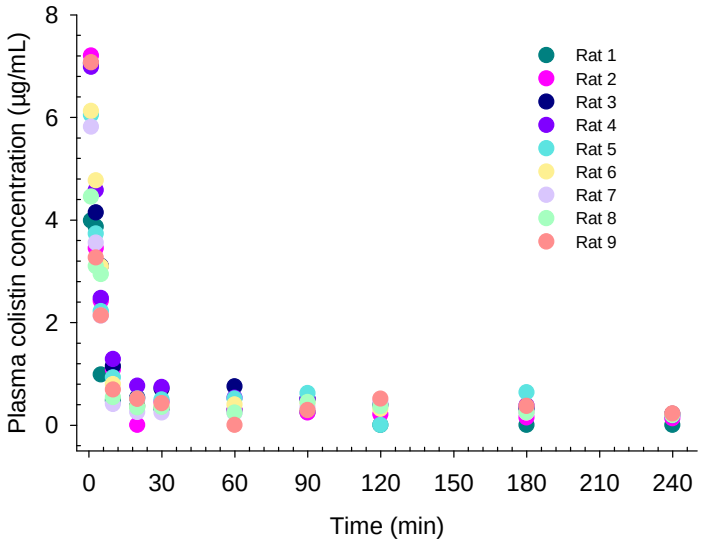


Figure A2-1: Individual plasma concentration versus time profile for colistin following IV administration of the target dose of 0.8 mg/kg colistin sulphate solution (n = 9). Data are normalised to 0.8 mg/kg.

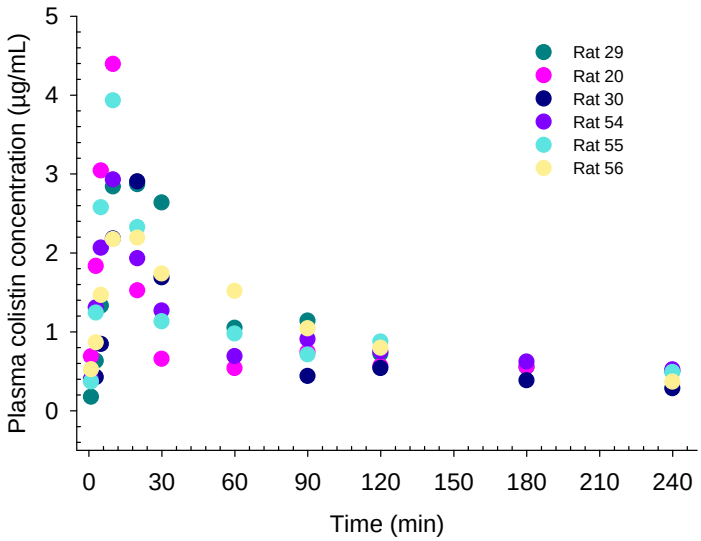


Figure A2-2: Individual plasma concentration versus time profile for colistin following IT administration of the target dose of 1.5 mg/kg colistin sulphate solution (n = 6). Data are normalised to 1.5 mg/kg.

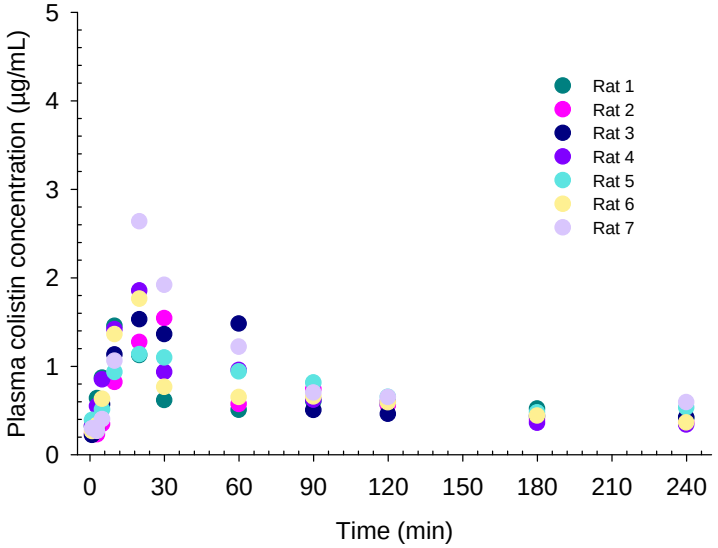


Figure A2-3: Individual plasma concentration versus time profile for colistin following IT administration of the target dose of 1.5 mg/kg colistin sulphate liposomes (n = 7). Data are normalised to 1.5 mg/kg.

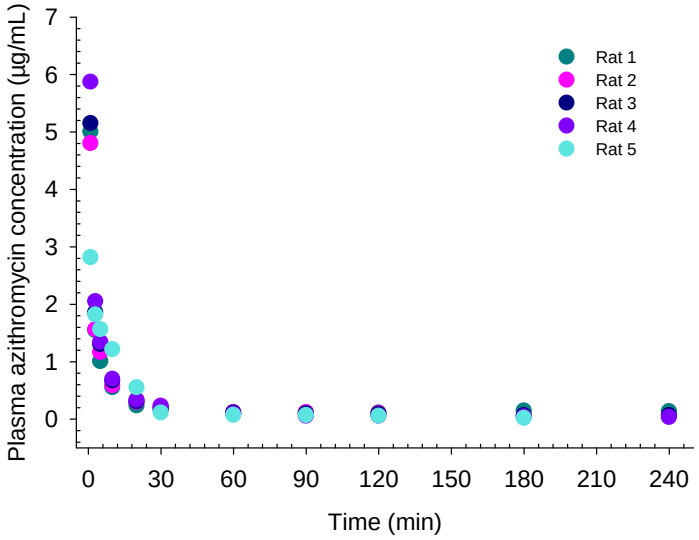


Figure A2-4: Individual plasma concentration versus time profile for azithromycin following IV administration of the target dose of 10 mg/kg azithromycin solution (n = 5). Data are normalised to 10 mg/kg.

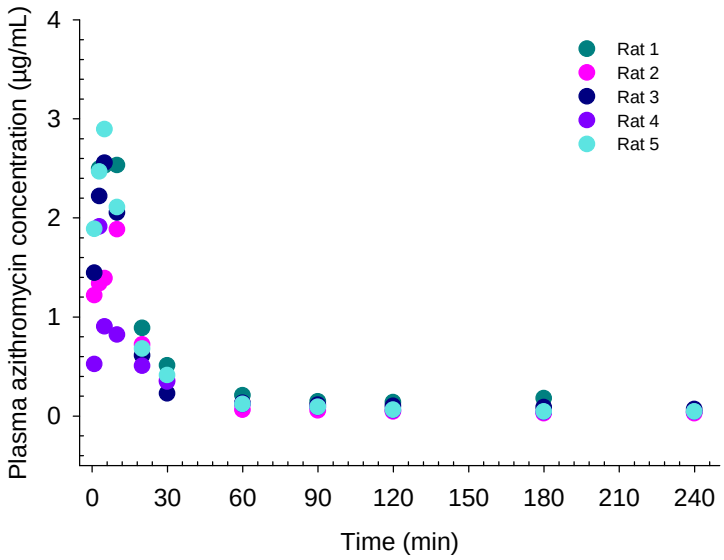


Figure A2-5: Individual plasma concentration versus time profile for azithromycin following IT administration of the target dose of 10 mg/kg azithromycin solution (n = 5). Data are normalised to 10 mg/kg.

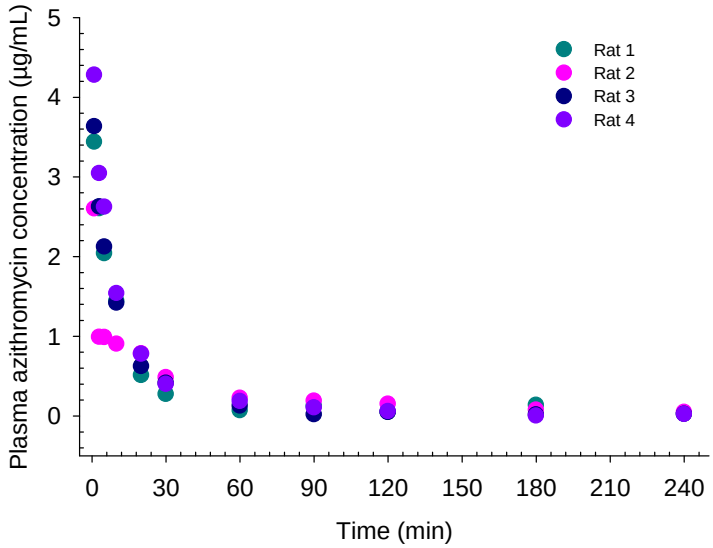


Figure A2-6: Individual plasma concentration versus time profile for azithromycin following IT administration of the target dose of 10 mg/kg azithromycin liposomes (n = 4). Data are normalised to 10 mg/kg.

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