Liposomal antibiotics enhance bacterial killing and ameliorate systemic inflammation

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Declaration page

"I, Dr Naveed Saleem confirm that the work which I have presented in the thesis is my own. Where information has been derived from currently available diverse sources, I confirm that this information has been indicated and acknowledged within the thesis."

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Keywords/Abbreviations

AMR Antimicrobial resistance

CFU Colony-forming units

CHOL Cholesterol

CLSM Confocal laser scanning microscopy

BBB Blood-Brain Barriers

DAMPs Damage-associated molecular patterns

DHP-1 Dehydropeptidase-1

DLS Dynamic light scattering

DSPE-PEG 2000 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine

DOTAP 2,3–dioleyloxypropyl-trimethylammonium chloride

DODAB 2,3–di-methyl dioctadecyl ammonium bromide

DPPC Dipalmitoyl glycerophosphocholine

DMPC Dimyristoyl glycerol phosphocholine

DMPG Dimyristoyl phosphatidylglycerol

E.E. Encapsulation Efficacy

ELISA Enzyme-Linked Immunosorbent Assay

ESBL Extended-spectrum β-lactamase

EUCAST European Committee for Antimicrobial Susceptibility Testing

FACS Flow-assisted cell sorting

HAA Haemolytic assay activity

HBSS Hanks' Buffered Saline Solution

HPLC High-performance liquid chromatography

HGT Horizontal gene transfer

IL Interleukins

IM Intramuscular

IV Intravenous

L.B Lysogeny broth

LPS Lipopolysaccharides

MBC Minimal bactericidal concentration

MIC Minimum inhibitory concentration

MPS Mononuclear phagocyte system

MRSA Methicillin-Resistant

MLV Multilamellar vesicles

PAMPs Pathogen-associated molecular patterns

PBS Phosphate Buffer Saline

PBP Penicillin-binding proteins

PC Phosphatidylcholine

PDI Polydispersity Index

PE Phosphatidylethanolamine

PEG Polyethene glycol

PG Phosphatidylglycerol

RES Reticuloendothelial system

SUV Small unilamellar vesicles

TNF Tumor necrotic factor

TSB Tryptic soy broth

WHO World Health Organization

Z.P Zeta Potential

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- N Saleem, TACS Snow, G Ambler, M Singer, N Arulkumaran. ''Steroids in bacterial pneumonia: A systematic review, meta-analysis, and trial sequential analysis''. DOI: https://doi.org/10.1016/j.chest.2022.08.2229. CHEST. September 2022.
- N Saleem, TAC Snow, N Arulkumaran. ''Corticosteroids in Community-Acquired Pneumonia In or Out? Response''. CHEST Journal, 2023 - ELSEVIER RADARWEG 29, 1043.
- N Saleem, F Ryckaert, TAC Snow, G Satta; M Singer, N Arulkumaran. ''Mortality and clinical cure rates for pneumonia: A systematic review, meta-analysis, and trial sequential analysis of randomized control trials comparing bactericidal and bacteriostatic antibiotic treatments''. DOI: https://doi.org/10.1016/j.cmi.2021.12.021. Clinical Infection and Microbiology. January 2022.
- N Saleem, TAC Snow, G Ambler, E Nastouli, L McCoy, M Singer, N Arulkumaran. ''Convalescent plasma in COVID-19. A meta-analysis, trials sequential analysis, and meta-regression''. DOI: https://doi.org/10.1016/j.bja.2021.07.033. British Journal of Anaesthesia. December 2021 (Joint first author).
- N Saleem, ''Antibiotics Modulate Variable Immunological Responses in Sepsis: A Narrative Review''. African Research Journal of Medical Sciences. January 2024, 1(1), 13-21. DOI: 10.62587/AFRJMS.1.1.2024.13-21.
- TAC Snow, N Saleem, G Ambler, E Nastouli, M Singer, N Arulkumaran. ''Tocilizumab in COVID-19. A meta-analysis, trials sequential analysis, and meta-regression''. DOI: https://doi.org/10.1007/s00134-021-06416-z. Intensive Care Medicine. June 2021.
- TAC Snow, N Saleem, M Singer, N Arulkumaran. ''L'histoire se répète," one size does not fit all''. Author's reply. DOI: https://doi.org/10.1007/s00134-021-06497-w. Intensive Care Medicine. June 2021.
- TAC Snow, A. Serisier, D. Brealey, M. Singer, N. Arulkumaran N.Saleem, C. Antonio, W. Alessia. ''Immunophenotyping patients with sepsis and underlying haematological malignancy reveals defects in monocyte and lymphocyte function''. DOI: https://doi.org/10.1186/s40635-023-00578-4. Intensive Care Medicine Experimental. January 2024.
- TAC Snow, W Alessia, L Richard, F Ryckaert, N.Saleem, C. Antonio, R Rudra, W John, H Ahamd, D. Brealey, M. Singer, N. Arulkumaran.'Early dynamic changes to monocytes following major surgery are associated with subsequent infections". DOI: https://doi.org/10.3389/fimmu.2024.1352556. Frontiers in Immunology. March 2024.
- S Choi, A Cesar, TACS Snow, N Saleem, M Singer, N Arulkumaran: ''Efficacy of Doxycycline for Mild- to moderate Community-acquired Pneumonia in Adults: A Systematic Review and Meta-analysis of Randomized Controlled Trials''. DOI: https://doi.org/10.1093/cid/ciac615. Clinical Infectious Diseases. July 2022.

- S Choi, A Cesar, T Snow, N Saleem, N Arulkumaran, M Singer. ‘’Respiratory Fluoroquinolone Monotherapy Versus ß-Lactam Plus Macrolide Combination Therapy for Hospitalised Adults with Community-Acquired Pneumonia: A Systematic Review, Meta-analysis, and Trial Sequential Analysis’ International Journal of Antimicrobial Agents. DOI: https://doi.org/10.1016/j.ijantimicag.2023.106905. September 2023.

Conference presentation

- Abstract presentation ‘’Cationic nano-liposomal meropenem enhances bacterial killing without adverse effects on immune cells’’. ESICM Lives 2023.
- Abstract presentation "Effects of corticosteroids mortality and clinical cure in community-acquired pneumonia: A systematic review, meta-analysis, and meta-regression of randomized control trials". ESICM Lives 2022.

Contributors

Figures 1 and 2: The figures were completed and finalised with the help of Dr Catherine Wembley.

FACS: The FACS experiments were conducted with the help of Dr Timothy Snow and Dr Jakob Dudziak.

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Summary

The World Health Organization (WHO) has declared antimicrobial resistance a major global health threat. The rise in global antimicrobial resistance (AMR) affects all antibiotic classes, especially those directed against Gram-negative organisms. Despite this, the clinical pipeline of new antimicrobials is sparse. My project aimed to enhance the potency of existing antibiotics to improve bacterial killing, particularly against multi-resistant organisms.

Gram-negative bacterial cell membranes are rich in highly negatively charged phospholipids. I exploited this Achilles heel by encapsulating antibiotics within cationic liposomes to enhance their delivery and potency. The incorporation of fusogenic lipids within the liposomes facilitates fusion with bacterial cells, enhancing antibiotic concentrations within the Gram-negative bacteria. The aim is to overcome antimicrobial resistance.

I have developed unique formulations of meropenem-encapsulated cationic liposomes with enhanced *in-vitro* antimicrobial activity against Gram-negative bacteria compared to non-liposomal (standard) meropenem. Additionally, I have developed unique formulations that demonstrate safety against human erythrocytes and immune cells *ex-vivo*. Future work should refine the encapsulation of different classes of antibiotics within the cationic liposomes to improve efficacy and safety with minimal drug-related adverse events and to develop a therapeutic intervention against AMR.

Abstract

INTRODUCTION

The rising incidence of resistant organisms, particularly Gram-negative, is a global threat. The development of novel antibiotics is limited; hence modification of existing antibiotics is an attractive option. Positively-charged liposomes are preferentially attracted to negatively-charged Gram-negative bacterial membranes via ionic interactions and could be employed to deliver existing antibiotics. This may help to overcome multidrug-resistant bacterial infections by achieving higher target concentrations, as well as facilitating reductions in antibiotic dosing thereby limiting drug toxicity.

METHODS

Meropenem was encapsulated in cationic liposomes by combining phosphatidylcholine (PC), 2,3–di-methyl dioctadecyl ammonium bromide (DODAB) and cholesterol (Chol). Liposome characterization was quantified using Dynamic light scattering (DLS), and high-performance liquid chromatography (HPLC). Encapsulation efficacy and total drug concentrations were calculated for different non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal preparations.

The *in-vitro* antimicrobial activity of free and meropenem-encapsulated cationic liposomes with or without PEG lipids was assessed using 24-hour broth microdilution, through determination of minimum inhibitory concentration (MIC) and plotting growth curves and inhibition kinetics.

To assess liposomal safety, whole blood drawn from healthy volunteers was incubated with standard meropenem and non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes at different therapeutic doses for 6 hours. Leucocyte toxicity was assessed using fluorescence-activated cell sorting (FACS) with staining for reactive oxygen species production and cell viability.

RESULTS

***In-vitro* drug susceptibility findings**

Non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations were associated with enhanced *in-vitro* bactericidal activity. In an *in-vitro* model, up to 30-fold reduction in MIC (minimum inhibitory concentration) was achieved against multiple laboratory and clinical strains of Gram-negative bacteria meropenem-encapsulated cationic liposomes compared to native meropenem.

***In-vitro* biocompatibility findings**

Meropenem-encapsulated liposomes with PEGylation prevented immune cell activation i.e., leukocyte internalization, ROS production, and death cell, pro-inflammatory cytokines in comparison to free standard meropenem irrespective of drug concentrations.

CONCLUSION

I have developed unique formulations of meropenem-encapsulated cationic liposomes with enhanced antimicrobial activity against different laboratory and clinical strains of Gram-negative bacteria, compared to non-liposomal meropenem, *in-vitro.* Additionally, I have found unique formulations that demonstrated safety against human erythrocytes and immune cells *ex-vivo.* Future work should refine the optimization of structurally modified liposomal formulations encapsulating different antibiotics to improve the efficacy and safety with minimal drug-related adverse events, to develop a therapeutic intervention for AMR.

Impact statement

The rise in global antimicrobial resistance (AMR) affects all antibiotic classes, especially those directed against Gram-negative organisms. There is an urgent need for novel approaches to improve the effectiveness of currently available medications due to the growing resistance dilemma and the dearth of newly developed antimicrobials. My project addressed the current issues by developing alternative drug-delivery approaches to enhance the potency of pre-existing antibiotics to improve bacterial killing, particularly against multi-resistant organisms.

Gram-negative bacterial cell membranes are rich in highly negatively charged phospholipids. I exploited this Achilles heel by encapsulating antibiotics within cationic liposomes to enhance their potency and selective and targeted delivery approaches. The incorporation of fusogenic lipids within the liposomes facilitates fusion with bacterial cells, enhancing antibiotic concentrations within the Gram-negative bacteria. The aim is to overcome antimicrobial resistance.

Meropenem-encapsulated cationic liposomes demonstrated enhanced in-vitro antimicrobial activity against different lab, clinical and resistant strains of Gram-negative bacteria compared to non-liposomal (standard) meropenem. Additionally, these formulations demonstrated safety against human erythrocytes and immune cells ex-vivo. The current results highlighted the potential impacts of meropenem-encapsulated cationic liposomes to combat AMR effectively against Gram-negative bacteria, paving their way as an effective and safe alternative therapeutic agent. Future work should refine the encapsulation of different classes of antibiotics within the cationic liposomal formulations to further improve their efficacy and safety with minimal drug-related adverse events, ultimately aiming to mitigate the global impact of antimicrobial resistance and improve patient outcomes in clinical settings.

1. Introduction

1.1 Background

The incidence of antimicrobial resistance (AMR), particularly among Gram-negative organisms, is a global health crisis (Jindal et al., 2015, World Health Organization (WHO), 2021, Murray et al., 2022). Patients with resistant infections incur greater healthcare costs in comparison with those with non-resistant infections due to longer recovery times, additional testing, and treatment with more expensive drugs (Brown and Wright, 2016). The lack of effective treatments against antimicrobial resistance (AMR), compounded by the slow introduction of new antibiotics, has been identified as a global healthcare concern by the 2016 O'Neill Report and the World Health Organization (WHO) (O'Neill, 2016). Novel strategies are urgently needed to combat antimicrobial resistance.

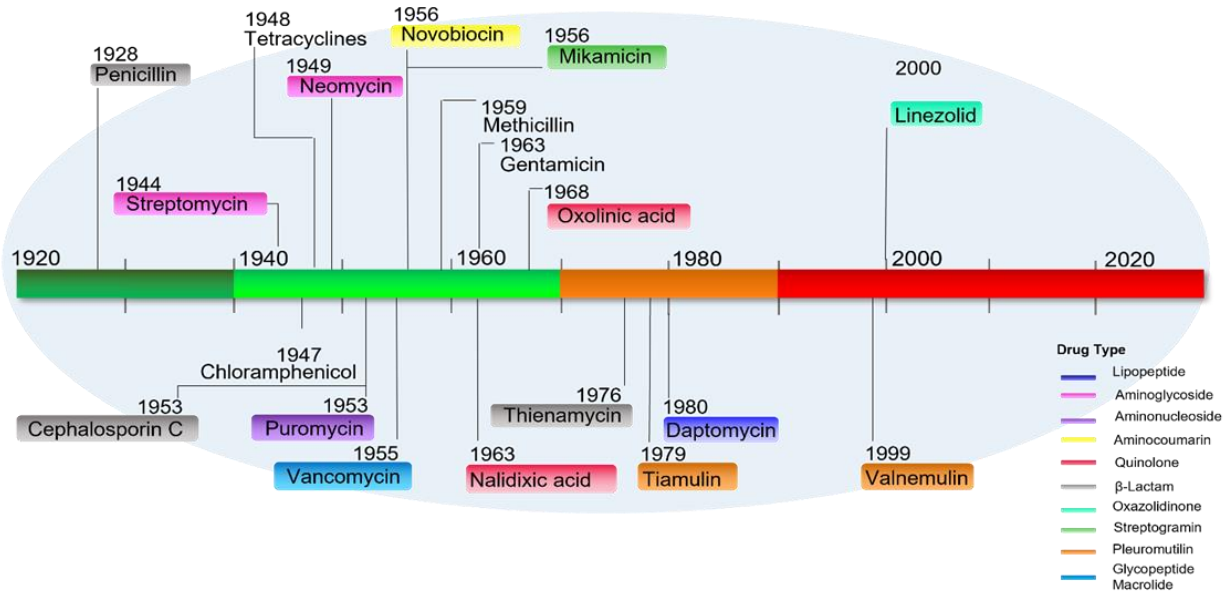

Figure 1: The history of antibiotic discovery. Over the past two decades, the clinical pipeline has been outpaced by the insurgence of antimicrobial resistance.

Antibiotics have been used arbitrarily for decades to treat serious and life-threatening bacterial infections. Bacterial pathogens that were once susceptible to most of the antimicrobial agents in the 20th century are now incurable due to the emergence of resistance (O'Neill, 2016, World Health Organization (WHO), 2021, Abushaheen et al., 2020). To re-establish effective therapeutic options against bacterial infections, the primary focus must be on developing alternative novel drug delivery methods by modifying the pre-existing antimicrobial strategies to combat multidrug-resistant infections. The creation of novel classes of antimicrobial medications is the main strategy used to solve this issue (Marchianò et al., 2020). However, this requires considerable expertise and resources. The development of novel drug-delivery systems to enhance the efficacy and safety of currently available antimicrobial agents could offer an effective alternative. At

present, many drug delivery methods such as nanoparticles, micelles and polymer-based delivery systems are commonly used in biotechnology (Marchianò et al., 2020). Liposome-based delivery (nano) systems are one of the most promising drug carrier routes under investigation (Marchianò et al., 2020) due to their stability, biocompatibility and the ability to achieve slow and sustained drug release with selective and targeting capabilities.

Liposomes are small, synthetic, spherical vesicles with an aqueous core inside surrounded by one or more phospholipid bilayers (Ferreira et al., 2021, Marchianò et al., 2020). They are analogous to human cell membranes. The concept of liposomes as alternative drug delivery systems was first introduced in the 1970s (Juliano and Stamp, 1978, Papahadjopoulos and Watkins, 1967). Because of their special qualities - enhanced bactericidal activity, prolonged circulation-half life and limited local and systematic drug-related adverse events - liposomes have long been used as carriers of specific antimicrobial agents (Juliano and Stamp, 1978, Papahadjopoulos and Watkins, 1967, Ferreira et al., 2021). Liposomes can transport hydrophilic medications in their lipid bilayers or hydrophobic drugs within the core of nanoparticles (Ferreira et al., 2021, Marchianò et al., 2020). They are categorised based on their size, number of bilayers, lipid composition, bilayer fluidity, and surface charge (Ferreira et al., 2021). Liposomal formulations with specific pharmacokinetic and pharmacodynamic qualities can be created by modifying their physicochemical characteristics (Ferreira et al., 2021, Marchianò et al., 2020).

Gram-negative bacterial cell membranes are rich in highly negatively charged phospholipids. As their charge is much greater than that found in human cell membranes (Epand and Epand, 2009) they can be targeted using positively charged moieties incorporated within liposomes to initiate liposomal binding, fusion and controlled drug delivery. The liposome delivery vehicle can be composed of lipids for optimal drug encapsulation efficiency and retention and targeted bacterial action.

Encapsulating antibiotics within liposomes can improve selectivity while limiting adverse side effects (Drulis-Kawa and Dorotkiewicz-Jach, 2010). This can be achieved by selective targeting of bacteria, controlled release of antibiotics at the site of infection, improved bio-distribution and antibiotic half-life, and decreased systemic toxicity of existing antibiotics (Osman et al., 2022, Yang et al., 2018a). Advances within the field of biotechnology also led to the rapid introduction and development of nanoparticles containing RNA vaccines against COVID-19 (Huang et al., 2021). AMR may be effectively addressed by utilizing liposomes that enhance drug delivery and efficacy. (Huang et al., 2021).

Overall, the development of liposomes as controlled delivery systems for antibiotics to sites of action (such as a particular tissue, organ or defined pathogen) is a promising approach to combat infection while preventing resistance and safeguarding the host's native microbiome (Marchianò et al., 2020, Khan et al., 2020). The use of antimicrobial-based liposomes is a potential substitute for traditional antibiotic therapy in an era of rapidly rising bacterial antimicrobial resistance (Marchianò et al., 2020, Khan et al., 2020).

1.2 Major lipids within bacterial membranes

The cell wall of Gram-negative bacteria has two membranes: an outer membrane with outer and inner leaflets and an inner cytoplasmic membrane (**Figure 2**). Gram-positive bacteria have only one membrane that surrounds the cell (**Figure 2**). Both contain a peptidoglycan layer which is present on the outer side of the cytoplasmic membrane and is relatively thicker in Gram-positive bacteria (Epand and Epand, 2009).

Both types of bacteria have different lipid-polysaccharides within the membranes. Gram-negative pathogens contain lipopolysaccharide (LPS) which forms the major lipid component of the outer membrane and consists of three components: lipid A, core saccharides, and polysaccharide side chains (O-antigen) in its outer leaflets, and high-density anionic phospholipids, glucosamine, and fatty acids in the inner leaflets (**Figure 2**). Gram-positive bacteria contain lipoteichoic acid, consisting of teichoic acid and lipids, which covalently bind to either phosphatidyl diglycosyl diglyceride or other related compounds (Epand and Epand, 2009, Shaw, 1974).

Amphipathic lipids are present in bacteria, as they play a pivotal role in maintaining cellular integrity, functionality and viability (Drulis-Kawa and Dorotkiewicz-Jach, 2010, Gonzalez Gomez and Hosseinidoust, 2020). Anionic phospholipids such as phosphatidylglycerol (PG) are present on the outer membranes and facilitate selective and targeted binding of cationic liposomal formulations due to electrostatic attractive forces. Gram-negative bacteria possess a higher content of zwitterionic phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and LPS than Gram-positive bacteria (**Table 1**) (Shaw, 1974, Epand and Epand, 2009). Therefore, they are more negatively charged under physiological pH.

The naturally occurring lipid phosphatidylglycerol (PG) provides a unique therapeutic target against bacteria. Bacterial cell membranes are rich in anionic phospholipids including phosphatidylglycerol (PG) and Phosphatidylethanolamine (PE) (Drulis-Kawa and Dorotkiewicz-Jach, 2010, Gonzalez Gomez and Hosseinidoust, 2020), making them ideal therapeutic targeting options for structurally modified liposomal formulations. My project aims to analyse how effectively and reliably positively charged liposomes encapsulating the beta-lactam antibiotic, meropenem will enhance potency and safety to help overcome antimicrobial resistance.

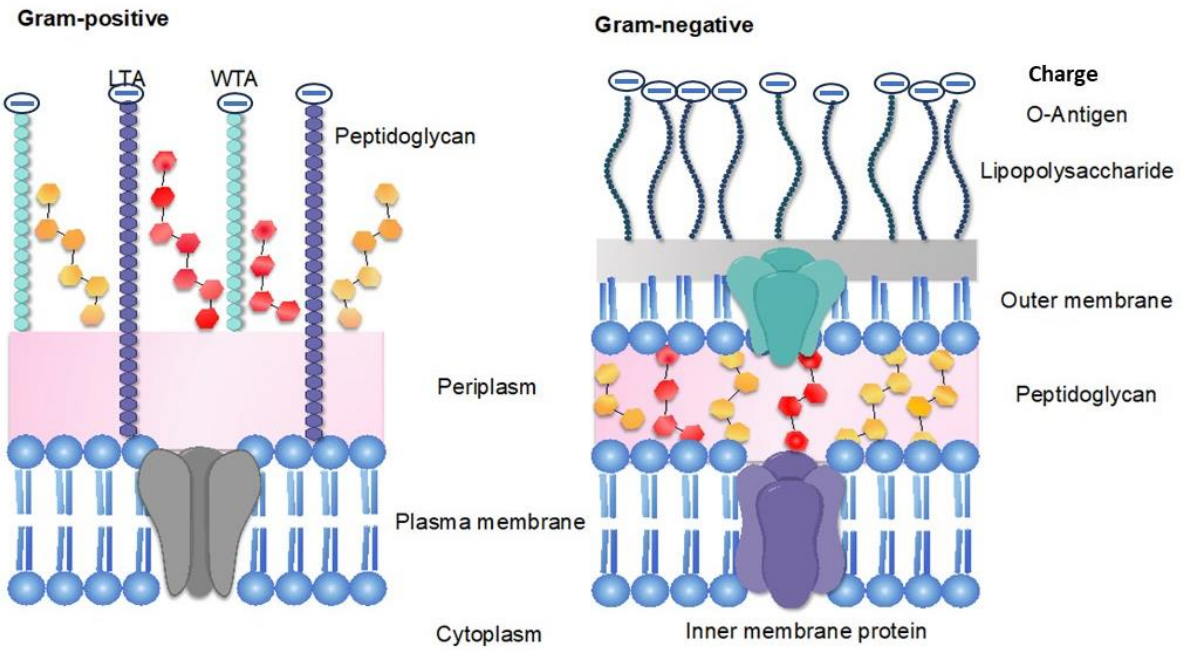

Figure 2: The membranes of Gram-Positive and Gram-Negative bacterial membranes. Infections caused by Gram-negative bacteria pose greater clinical challenges. The double membrane (inner and outer membrane) on Gram-negative bacteria provides an added barrier against drug molecules. LTA = lipoteichoic acids, WTA = wall teichoic acids.

Table 1: Major lipids commonly present within the membranes of Gram-positive and Gram-negative bacteria.

Phospholipids and derivatives	Occurrence	Charge under physiological pH
Phosphatidylglycerol	Found in all bacteria	Net-neutral
Bisphosphatidylglycerol	Co-occurs along with phosphatidylglycerol	Net-neutral
Phosphatidylethanolamine	A major part of all Gram-negative bacteria	Net-neutral
Phosphatidylcholine	Widely distributed among higher organisms, rarely found in bacteria	Net-neutral
Phosphatidylinositol	Commonly present in Corynebacterium, Nocardia	Net-neutral

1.3 Antimicrobial therapy

In recent years, antimicrobial resistance has evolved rapidly. Alarming reports document how the infection burden of both common and diverse bacterial pathogens has developed diverse resistance mechanisms to currently available antimicrobial agents (Codjoe and Donkor, 2017).

Carbapenems are broad-spectrum bactericidal β-lactams that have better efficacy and potency against severe infections caused by extended-spectrum β-lactamase (ESBL)-producing pathogens. Commonly prescribed antimicrobial agents include meropenem, imipenem, and ertapenem. They undergo hepatic metabolism. Side effects include jaundice and hepatotoxicity, therefore dose adjustments are needed for liver failure (Codjoe and Donkor, 2017). Imipenem demonstrates dose-dependent gastrointestinal adverse events. Mostly, they are metabolized by dehydropeptidase-1 (DHP-1) enzymes commonly present within renal tubules. They therefore require co-administration of DHP-1 inhibitors such as cilastatin to prevent enzymatic degradation (Codjoe and Donkor, 2017).

1.3.1 General structure and mechanism of β-lactams

Because of their broad antibacterial spectra, ß-lactam antibiotics are the most commonly prescribed medications for the management of serious and life-threatening bacterial infections (Egorov et al., 2020, Fernandes et al., 2013). Their fundamental chemical structure, the ß-lactam ring, unites them in a common nucleus and protects against β-lactamases such as extended spectrum β-lactamases, and metallo-ß-lactamases (Egorov et al., 2020, Fernandes et al., 2013). Classes include penicillin, cephalosporins, carbapenems and monobactams. These antibiotics are bactericidal in action as they bind to penicillin-binding proteins (PBPs) covalently, thereby preventing the formation of peptidoglycan layers in the bacterial cell wall (Egorov et al., 2020, Fernandes et al., 2013).

Certain autolytic enzymes can halt the terminal transpeptidation process, interfering with peptidoglycan layer cross-linking within Gram-positive and Gram-negative bacterial cell walls (Egorov et al., 2020, Fernandes et al., 2013). Since the cross-linked peptidoglycan layer plays a significant role in maintaining the structural integrity of the cell wall (**Figure 2**), the bactericidal action of the ß-lactam antibiotic makes them less intact. This, in turn, causes the bacterium to lyse due to osmotic pressure (Egorov et al., 2020, Fernandes et al., 2013).

1.4 Antimicrobial resistance

1.4.1 Definition

Clinically significant microorganisms that are resistant to several antimicrobial agents have evolved recently because of the overuse and misuse of antimicrobial agents in human and veterinary medicine (Abushaheen et al., 2020, Holmes et al., 2016). Multidrug-resistant bacteria are those that have developed resistance to three or more types of antimicrobial agents (Abushaheen et al., 2020, Holmes et al., 2016). Ongoing and/or repetitive exposure to antibiotics suppresses micro-organism susceptibility and favours the development of new diverse resistant mechanisms. Resistance genes are subsequently spread to new bacteria because of their increased ability to survive (Abushaheen et al., 2020, Holmes et al., 2016). Due to recent bacterial-resistance trends, infections have become harder to treat with traditional antibiotics, threatening outcomes from medical and surgical procedures.

1.4.2 General mechanisms of antibiotic resistance

Gram-negative bacteria are particularly problematic since they possess an outer membrane (**Figure 2**) that provides innate resistance by enhancing hydrophobicity against most antimicrobial agents (Zaman et al., 2017, Abushaheen et al., 2020). Bacteria may also have acquired resistance to antimicrobial agents (Zaman et al., 2017, Ribeiro da Cunha et al., 2019, Lewis, 2020). There are four main types of acquired resistance 1) structural alteration of drug receptors, 2) upregulation of inactivating enzymes, 3) decreased antibiotic influx (as bacteria can control the rate of antibiotic influx via several mechanisms such as alteration of cell membrane composition (decreasing antibiotic permeability) and downregulation of porins), 4) efflux pump upregulation (**Figures 3 & 4**) (Zaman et al., 2017, Ribeiro da Cunha et al., 2019, Lewis, 2020, Brown and Wright, 2016, Abushaheen et al., 2020, Holmes et al., 2016).

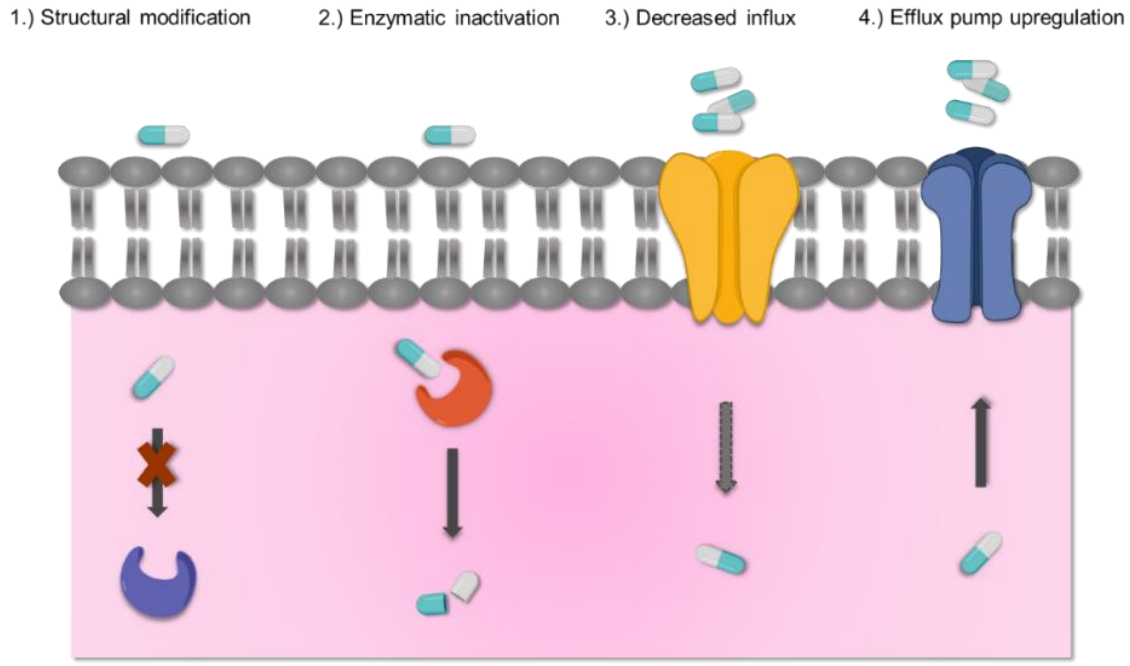

Figure 3: General mechanisms of acquired resistance in Gram-Negative and Gram-Positive bacteria. Both Gram-Negative and Gram-Positive bacteria adopt the following mechanisms of acquired resistance against commonly prescribed antibiotics: 1) structural alteration of drug receptors, 2) upregulation of inactivating enzymes, 3) decreased antibiotic influx and 4) efflux pump upregulation.

a). Enzymatic approaches

Both Gram-positive and Gram-negative bacteria produce enzymes that inactivate drug molecules, the best known involves their synthesis of ß-lactamase enzymes which facilitate the breakdown of the penicillin ß-lactam rings (**Figures 3 & 4**) (Abushaheen et al., 2020, Holmes et al., 2016). The extended-spectrum ß-lactamases (ESBLs) present in Enterobacteriaceae, including K. pneumoniae and E. coli, are particularly clinically significant (Abushaheen et al., 2020, Holmes et al., 2016). These enzymes degrade not only penicillin and first and second-generation cephalosporins but also monobactams and extended-spectrum third-generation cephalosporins (such as cefotaxime, ceftazidime, and ceftriaxone) (Abushaheen et al., 2020, Holmes et al., 2016).

b). Non-enzymatic approaches

Structural alterations at the drug's targeting sites, which are no longer recognised by standard antibiotics, is another common resistance mechanism adopted by bacteria i.e., the production of modified penicillin-binding proteins (PBPs) that have a lower affinity for ß-lactams (**Figures 3 & 4**) (Holmes et al., 2016). By limiting access to the drug's target sites and avoiding antibiotic penetration into the cells, microorganisms may develop resistance (Abushaheen et al., 2020, Holmes et al., 2016).

Porins are transmembrane proteins located in the bacterial outer membrane and are responsible for the passive transport of hydrophilic antimicrobial agents across the bacterial membrane (**Figures 3 & 4**) (Vasconcelos et al., 2018, Darby et al., 2023, Abushaheen et al., 2020). Antimicrobial agents are unable to pass through the bacterial membrane due to reduced outer membrane permeability, either caused by genetic alterations or due to decreased porin production (Abushaheen et al., 2020, Holmes et al., 2016). Fluoroquinolones and ß-lactams can be ineffective due to a lack of porins.

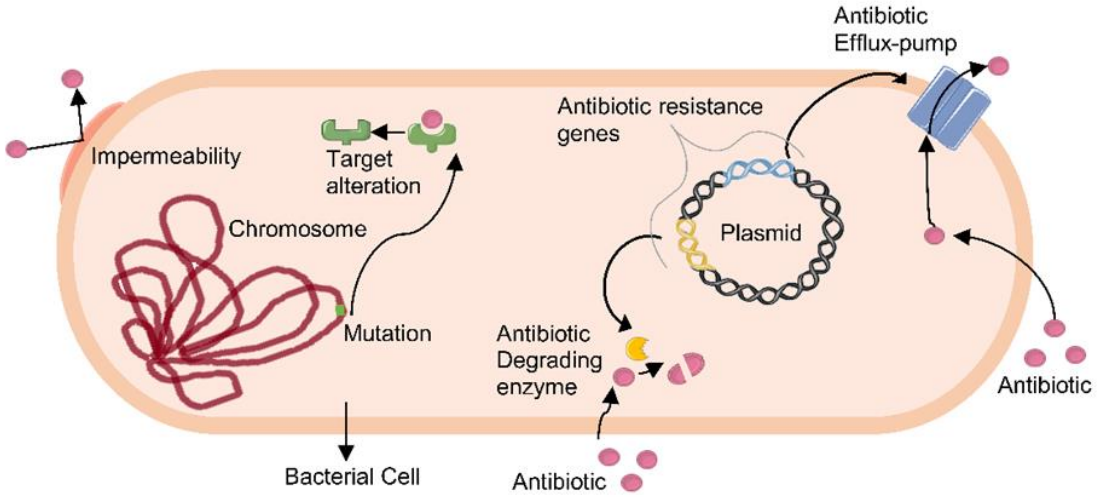

Figure 4: Molecular events associated with acquired resistance in Gram-Negative and Gram-Positive bacteria. Both Gram-Negative and Gram-Positive bacteria develop the following mechanisms of acquired resistance against commonly prescribed antibiotics: 1) structural alterations at drug receptors, 2) upregulation of inactivating enzymes, 3) decreased membrane permeability and antibiotic influx and 4) efflux pump upregulation. Plasmids are tiny, circular, double-stranded, extrachromosomal DNA structures that replicate separately from chromosomes and are the most common cause of acquired resistance among Gram-negative bacteria. (Adapted from Levy and Marshall 2004; Abreu et al. 2011).

Efflux pumps are non-specific transport proteins that facilitate the transport of structurally different molecules (including antibiotics) out of cells. These are usually found in E. Coli, P. aeruginosa, and S. aureus (**Figures 3 & 4**) (Abushaheen et al., 2020, Holmes et al., 2016).

1.4.3 Types of resistance

Antimicrobial resistance is categorised into inherent and acquired resistance. Mechanisms include structural alteration of drug receptors, upregulation of inactivating enzymes, decreased membrane permeability and

antibiotic influx, and efflux pump upregulation (Abushaheen et al., 2020, Holmes et al., 2016). All bacteria belonging to the same species share inherited traits that are generally present in their genomes; this is referred to as ‘’intrinsic resistance’’ (Abushaheen et al., 2020, Holmes et al., 2016). It is not associated with gene transfer nor selective pressure of antibiotic usage (Abushaheen et al., 2020, Holmes et al., 2016). A chromosomal gene that generates a penicillin ß-lactamase is present in all strains of K. pneumoniae and usually confers inherent resistance (Abushaheen et al., 2020, Holmes et al., 2016). Inherent resistant trends within P. aeruginosa also facilitate resistance to certain antibiotics such as ß-lactams due to decreased outer-membrane permeability (Abushaheen et al., 2020, Holmes et al., 2016).

Sensitive bacteria can develop ‘’acquired resistance’’ either due to chromosomal mutations or by picking up mobile genetic elements -‘’ horizontal gene transfer’’ (HGT) - using different mechanisms such as transposons, bacteriophages, bare DNA and plasmids (**Figures 4 & 5**) (Abushaheen et al., 2020, Holmes et al., 2016). Horizontal gene transfer (HGT) indicates that the genetic material is transferred, usually by means other than reproduction, between organisms that are not parents nor progeny. Genetic diversity and evolution are enabled by this mechanism as this allows the exchange of genetic information across various species (Abushaheen et al., 2020). Conjugation (plasmid-mediated transfer) through direct cell-to-cell contact allows the transfer of genetic material between bacteria (Abushaheen et al., 2020, Holmes et al., 2016). A conjugative plasmid that carries genes encoding tools required for DNA transfer must be present for this process to take place. Transduction (phage-mediated transfer) is the process by which bacteriophages transfer bacterial DNA between cells (Abushaheen et al., 2020, Holmes et al., 2016). Bacteriophages may unintentionally encapsulate bacterial DNA in their genetic material during infection, and this can subsequently be transmitted to other bacteria during subsequent infection (Abushaheen et al., 2020, Holmes et al., 2016). Transformation (uptake of free DNA) enables bacteria to absorb free DNA from their surroundings and integrate it into their genomes (Abushaheen et al., 2020, Holmes et al., 2016). Naturally, competent bacteria, who can actively take up and integrate foreign DNA, are frequently seen going through this process.

Bacteria can use conjugation, transduction and transformation as the primary modes of horizontal gene transfer mechanisms (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016). The bacterial cell structure is typically altered by spontaneous chromosomal mutations that can lead to either altered therapeutic targets or decreased membrane cell permeability (Abushaheen et al., 2020, Holmes et al., 2016). Bacteria can exchange chromosomal resistance genes by absorbing bare DNA fragments released from another cell upon cell lysis, or from the environment using ‘’transformation’’. This process involves the uptake and incorporation of extracellular DNA within the recipient cells, where foreign DNA can recombine with the host genome leading to the acquisition of new genetic traits (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016).

Circular, double-stranded, extrachromosomal DNA structures known as ‘’plasmids’’ replicate separately from chromosomes (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016). Through the process of bacterial ‘’conjugation’’, the resistance genes are usually passed from one bacterium to another by direct cell-to-cell contact between a donor cell having a conjugative plasmid and a recipient cell lacking the plasmid. The donor cell transfers the plasmid DNA that carries antibiotic-resistant genes to the recipient cell via specialized complex protein structures called the conjugative pilus. This process facilitates the rapid dissemination of antibiotic-resistance genes between diverse microorganisms (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016).

In bacteriophages or viruses that infect bacteria, transduction facilitates the transfer of genetic material from one bacterium to another. Bacteriophages can encapsulate host DNA rather than viral DNA in their capsids during the lytic stage of viral replication. Injecting the packaged DNA into a newly infected host bacterium allows the phage to either multiply autonomously as a plasmid or to integrate into the recipient cell's genome (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016).

Transposons are tiny, movable segments of DNA that can travel among plasmids as well as into and out of chromosomes (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016). They are linked to resistance genes. Transposons enter bacterial cells by absorbing exposed DNA from the environment or by transduction via bacteriophages (**Figure 5)** (Abushaheen et al., 2020, Holmes et al., 2016). ‘’Transposition’’ facilitates the movement of transposable elements such as transposons, and insertion sequences within and between genomes from one location to another within the genome or between different DNA molecules (Abushaheen et al., 2020, Holmes et al., 2016).

Horizontal gene transfer (HGT) facilitates the rapid spread of beneficial genetic traits associated with antibiotic resistance and plays a pivotal role in the evolution and adaptation of microbial populations. Therefore, an understanding of horizontal gene transfer mechanisms is necessary for developing strategies to combat the emergence and spread of antibiotic-resistant bacteria.

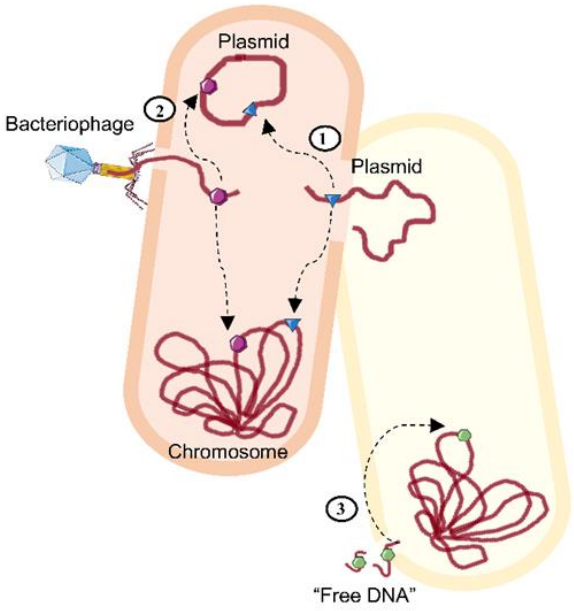

Figure 5: Horizontal gene transfer describes genetic material that is transferred, usually by means other than reproduction, between organisms that are not parents nor progeny. Legends 1: conjugation (plasmid-mediated transfer) through direct cell-to-cell contact, genetic material is transferred between bacteria; 2: transduction (phage-mediated transfer) is the process by which bacteriophages infect bacteria—transfer bacterial DNA between cells. Bacteriophages may unintentionally encapsulate bacterial DNA in their genetic material during infection, which can subsequently be transmitted to other bacteria during subsequent infection; 3: transformation (uptake of free DNA) bacteria absorb free DNA from their surroundings and integrate it into their genomes (Adapted from Levy and Marshall 2004; Willey et al. 2008).

1.5 Liposomal antibiotics

Liposomes are spherical vesicles of variable sizes, composed of amphipathic lipids organized in one or more concentric bilayers having an aqueous phase inside and in between the lipid bilayers (Drulis-Kawa and Dorotkiewicz-Jach, 2010, Sheikholeslami et al., 2022). These particles can cross the blood-brain barrier, and interact with mucosal surfaces, resulting in deeper tissue penetration (Drulis-Kawa and Dorotkiewicz-Jach, 2010, Sheikholeslami et al., 2022). Moreover, they can deliver drugs to the site of infection thereby enhancing local intracellular antibiotic concentrations and limiting the incidence of systemic drug toxicity (Drulis-Kawa and Dorotkiewicz-Jach, 2010, Sheikholeslami et al., 2022).

Liposomes are composed of natural lipids such as phospholipids and cholesterol (Gonzalez Gomez and Hosseinidoust, 2020, Sheikholeslami et al., 2022). Encapsulation of antimicrobial agents within the vesicles improves drug stability, delivers both hydrophilic and hydrophobic therapeutic drugs, and prevents degradation (Gonzalez Gomez and Hosseinidoust, 2020, Sheikholeslami et al., 2022). Liposomal encapsulation of antibiotics may facilitate improved antibiotic pharmacokinetics and biodistribution to the site of infection, with fewer local and systemic adverse events (Gonzalez Gomez and Hosseinidoust, 2020, Sheikholeslami et al., 2022). The size and surface properties of liposomes can be altered to achieve different biochemical functions (Gonzalez Gomez and Hosseinidoust, 2020, Sheikholeslami et al., 2022).

The negatively charged lipid headgroup of phosphatidylglycerol can be targeted using positively charged moieties to initiate targeted liposomal binding, fusion, and controlled drug delivery (**Figure 6**). The liposome delivery vehicle can be composed of different lipids for optimal drug encapsulation efficiency and retention and targeted bacterial action (**Figure 6**). Gel-phase lipids can be employed to prevent undesired drug leakage. A cationic lipid can be used to achieve bacterial cell targeting and fusion (Sheikholeslami et al., 2022).

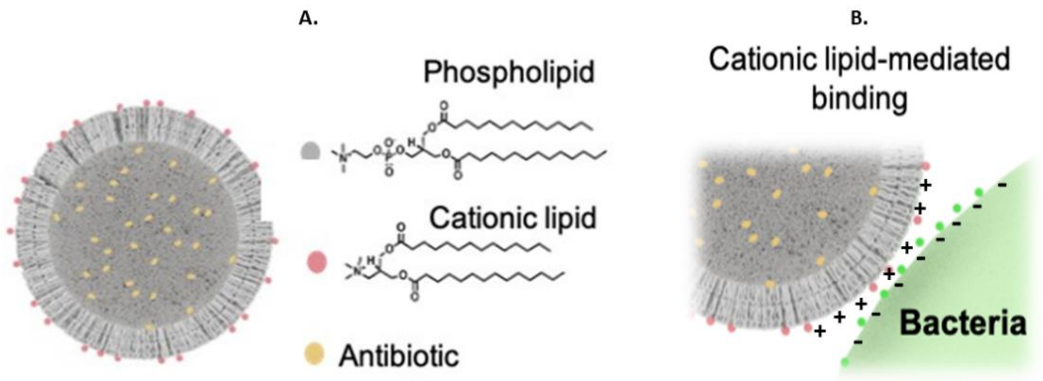

Figure 6: Overview of the proposed research. A) Engineered liposomes with cationic lipids (red) containing small molecule antibiotics (yellow) which specifically target pathogenic bacteria (green). B) Meropenem-encapsulated cationic liposomes are strongly attracted to negatively charged bacterial surfaces due to higher proportions of phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and LPS with their electrostatic attractive forces.

1.5.1 Structure and physiochemical properties of liposomes

Liposomal sizes, ranging from 0.02-10 µm, may alter the physiochemical properties. They are categorised as multilamellar vesicles (MLV) or unilamellar vesicles depending on the numbers and size of bilayers (Sheikholeslami et al., 2022). Unilamellar vesicles are further classified into small unilamellar vesicles (SUVs) (<100 nm) and large unilamellar vesicles (LUVs) (>100 nm) (Sheikholeslami et al., 2022). Smaller liposomes (≤200 nm) have a longer circulation half-life. Therefore, the size of the vesicles affects both the drug's distribution and how long the liposome stays in circulation (Sheikholeslami et al., 2022).

These vesicles can encapsulate and deliver both hydrophilic and hydrophobic compounds which allows them to entrap a wide variety of medicines (Sheikholeslami et al., 2022). Hydrophobic medications are encapsulated within the lipid bilayer whereas hydrophilic drugs settle within aqueous compartments (Sheikholeslami et al., 2022).

Liposomes are usually composed of cholesterol and phospholipids, synthetic or natural, which render them biocompatible, biodegradable and minimally toxic (Sheikholeslami et al., 2022). Liposomal membrane stability and fluidity are determined by its bilayer constituents (Sheikholeslami et al., 2022). For example, cholesterol is frequently added to lipid formulations to control membrane rigidity, thereby improving stability (Sheikholeslami et al., 2022). Liposomes can be classified as neutral, cationic or anionic based on the surface charge of these particles (**Figure 7**) (Sheikholeslami et al., 2022). The type of charge affects both liposome-cell interaction and the physical stability of the liposome (Sheikholeslami et al., 2022).

Conventional liposomes are comprised of natural phospholipids along with cholesterol; they undergo higher systemic plasma clearance, being rapidly cleared by the mononuclear phagocytic system (MPS) (Gonzalez

Gomez and Hosseinidoust, 2020, Daraee et al., 2016, Sheikholeslami et al., 2022). To avoid MPS uptake, liposomal surfaces can be modified either by hydrophilic polymers or by limiting liposomal diameter (< 200 nm), as larger nanoparticles are more prone to rapid clearance (Daraee et al., 2016, Drulis-Kawa and Dorotkiewicz-Jach, 2010, Gonzalez Gomez and Hosseinidoust, 2020, Sheikholeslami et al., 2022). Polyethene glycol (PEG) and its derivatives are widely used hydrophilic polymers that decrease recognition by the MPS, hinder the adsorption of circulating plasma proteins, and improve the structural stability of the liposomes (**Figure 7**) (Gonzalez Gomez and Hosseinidoust, 2020, Daraee et al., 2016, Sheikholeslami et al., 2022).

In summary, the physiochemical features of lipid content and proportions, surface charge and size directly affect the liposomes' efficacy and dictate their behaviour *in-vivo*. Additional influences on their biological performance are factors including temperature sensitivity, pH and membrane fluidity (Sheikholeslami et al., 2022). The efficacy of the encapsulated medicine can be increased above that of the corresponding free drug by optimising these qualities throughout the production process. Therefore, liposomes are appealing options to consider for usage as drug delivery vehicles.

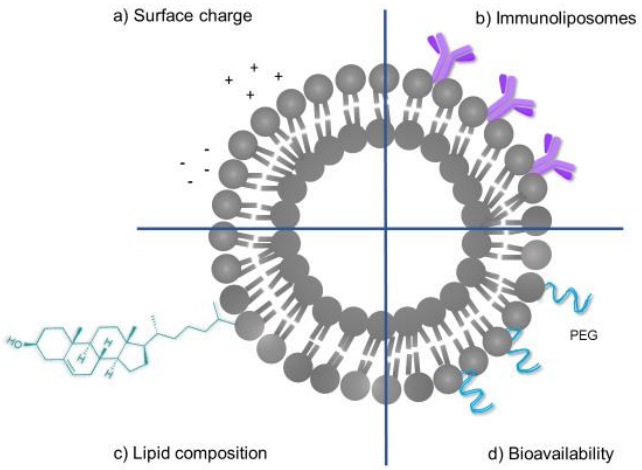

Figure 7: Modifications to the physicochemical properties of liposomes.; a) surface charges, b) immunoliposomes, c) lipid composition and d) improved bioavailability via functionalisation with a polymer such as polyethene glycol.

1.5.2 Surface modification of liposomes

Liposome surface properties can be functionalized to promote binding to bacterial cells (**Figure 7**). Both Gram-negative and Gram-positive bacteria are negatively charged, but Gram-negative bacteria possess stronger anionic charges due to the presence of O-antigens and the lipopolysaccharide (LPS) core (**Figure 2**) (Drulis-Kawa et al., 2006c). Hence, cationic liposomes can be employed to promote electrostatic interactions with bacteria that reduce the minimal inhibitory concentrations (MIC) (thus improving the potency) of existing antibiotics (**Figure 7**) (Brooks and Brooks, 2014, Gao et al., 2018, Drulis-Kawa et al., 2006c, Furneri

et al., 2000, Song et al., 2012, Sheikholeslami et al., 2022). Cationic phospholipids including dimethyl-dioctadecyl ammonium bromide (DODAB), and 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) have been used for these applications (Drulis-Kawa and Dorotkiewicz-Jach, 2010, Drulis-Kawa et al., 2006b, Sheikholeslami et al., 2022).

The pathophysiological events associated with bacterial infections such as increased vascular permeability or endothelial damage can be advantageous for the delivery of liposomal antibiotics using passive targeting routes (Sheikholeslami et al., 2022). All these pathophysiological events favour an accumulation of liposomal antibiotics at infection sites.

Passive transport delivery methods can be potentiated by modifying the liposomal surfaces using specific targeting moieties, e.g. polymers and ligands such as antibodies, peptides, and carbohydrates, which can be covalently attached to different lipid compositions (Sheikholeslami et al., 2022). These approaches have a strong affinity for certain receptors, thereby improving selective and targeted liposomal drug delivery (**Figure 7)** (Sheikholeslami et al., 2022). **Table 2** demonstrates examples of commonly used molecules, peptides and biological membranes that can be covalently attached to the liposomal surfaces to facilitate selective and targeted binding depending upon their site of action.

Table 2: Summary of covalent surface modifications of liposomes.

Saccharide-coated delivery systems for targeted antibiotic therapy			
Saccharide coating	**Benefits**	**Antimicrobial agents**	**References**
Mannose	Target Mannose-binding receptors present on alveolar macrophages.	Streptomycin	(Su et al., 2018)
Mannose	Target Mannose-binding receptors present on alveolar macrophages.	Rifabutin	(Maretti et al., 2017)
Polymer-coated delivery systems for enhancing antibiotic therapy			
Polymers and their derivatives	**Benefits**	**Antimicrobial agents**	**References**
Chitosan	It covalently binds to the outer surfaces of nanoparticles and enhances membrane permeability.	Daptomycin	(Zhu et al., 2016)
PEGylated	Modifies liposomal outer surfaces and enhances drug permeability, stability, and retention time.	Erythromycin	(Pourjavadi and Tehrani, 2014)
Biological membrane and peptide-coated methods for antibiotic enhancement			
Coating material	**Benefits**	**Antimicrobial agents**	**References**
Red blood cell (RBC) membranes	Helps neutralize bacterial toxins and enhance bacterial uptake by phagocytosis.	Vancomycin	(Li et al., 2014)

1.6 Review of studies demonstrating efficacy and safety of liposomal antibiotics

Pre-clinical trials investigating the impact of lipid formulations (including liposomes) on improving bacterial toxin-induced inflammation in sepsis show promising results (Henry et al., 2015a, Alipour and Suntres, 2014, Goldfarb et al., 2003, Gordon et al., 2005, Gordon et al., 2003, Dellinger et al., 2009). Due to the wide diversity of lipid composition and structural modifications, liposomal-encapsulated antibiotics have improved the pharmacodynamic and pharmacokinetic characteristics of current antimicrobial agents (Drulis-Kawa et al., 2006c, Levison and Levison, 2009). They are also capable of neutralizing bacterial toxins.

I conducted a literature search of PubMed, Embase and the Cochrane Library using a Boolean search strategy (MESH); (Liposome or liposomal) AND (Antibiotic, antimicrobial, OR antibacterial) NOT (cancer OR fungal) (**Supplemental Figure 1**). The search strategy identified 46 studies; only 29 were limited to *in-vitro* data involving different laboratory, clinical, and resistant Gram-negative and Gram-positive bacterial strains (Sezer et al., 2004, Furneri et al., 2000, Mugabe et al., 2005, Mugabe et al., 2006a, Rukholm et al., 2006, Drulis-Kawa et al., 2006c, Alipour et al., 2008, Mugabe et al., 2006b, Halwani et al., 2008, Mirzaee et al., 2009, Pumerantz et al., 2011, Torres et al., 2012, Atashbeyk et al., 2014, Serri et al., 2018, Derbali et al., 2019, Savadi et al., 2020, Ebrahimi et al., 2020, Aljihani et al., 2020, Zahra et al., 2017, Nicolosi et al., 2010, Fu et al., 2019, Nicolosi et al., 2015, Ribeiro et al., 2020, Moyá et al., 2019, Vanić et al., 2019, Nacucchio et al., 1985a, Gottesmann et al., 2020, Rukavina et al., 2018, Bartomeu Garcia et al., 2017) (**Supplemental Table 1**). A further 17 studies included *in-vivo* data demonstrating the efficacy of liposomal antibiotics in animal models of infection (Pardue and White, 1996, Webb et al., 1998, Shek et al., 1998, Bakker-Woudenberg et al., 2002, Elmas et al., 2002, Omri et al., 2002, Kadry et al., 2004, Sun et al., 2011, Sande et al., 2012, Gharib et al., 2012, Ong et al., 2014, Liu et al., 2015, Li et al., 2015, Jiang et al., 2016, Yang et al., 2018b, Rani et al., 2022, Henry et al., 2015b) (**Supplemental Tables 2 & 3**).

1.6.1 *In-vitro* studies

In-vitro studies have shown enhanced antimicrobial activity of antibiotics when encapsulated within liposomes compared to non-liposomal formulations (Table 2). As an example, aminoglycoside-loaded liposomes are associated with enhanced delivery of encapsulated drugs into bacterial cells via fusion with the outer membranes of Gram-negative (P. aeruginosa, K. pneumoniae, E. coli and Burkholderia cenocepacia and Gram-positive bacteria (e.g., Staph. aureus) (Mugabe et al., 2005, Mugabe et al., 2006b, Mugabe et al., 2006a, Mirzaee et al., 2009, Halwani et al., 2008).

Liposomes comprised of dipalmitoyl glycerophosphocholine (DPPC) and cholesterol incorporating gentamicin, tobramycin, or amikacin exhibit increased anti-Pseudomonal activity compared to respective non-liposomal formulations (Mugabe et al., 2006a, Mugabe et al., 2005). Liposomal formulations improve killing time and enhance antimicrobial activity up to 16-, 64- and 128-fold with gentamicin, amikacin, and

tobramycin, respectively (Mugabe et al., 2005, Mugabe et al., 2006a). Similar effects were seen with dimyristoyl glycerol phosphocholine (DMPC), liposomal gentamicin and dimyristoyl phosphatidylglycerol (DMPG) vancomycin liposomes (Sande et al., 2012).

1.6.2 *In-vivo* trials

Pre-clinical animal studies have additionally shown the advantages of liposomal antibiotics (**Tables 3 & 4**). Liposome encapsulation of fluoroquinolones is advantageous over non-liposomal formulations in the treatment of both resistant and non-resistant Gram-negative pulmonary infections (Liu et al., 2015, Ong et al., 2014). Compared to systemic administration of ciprofloxacin, pulmonary administration of ciprofloxacin-loaded liposomes with high encapsulation efficacy achieved higher antibiotic concentrations at the site of infection and enhanced the half-life within the pulmonary tissues, therefore reducing dosing frequency (Liu et al., 2015, Ong et al., 2014).

In an animal model of osteomyelitis, contamination of bone tissues resulted in persistent infection attributed in part to rapid clearance of antibiotics by the reticuloendothelial system (RES) (Kadry et al., 2004). Intravenous administration of positively charged liposomal ciprofloxacin and vancomycin for 14 days was associated with a lower side-effect profile while maintaining clinical efficacy compared to the free drug (Kadry et al., 2004).

Liposomal antibiotics can enhance existing antibiotics to overcome antimicrobial resistance (Mugabe et al., 2006b, Atashbeyk et al., 2014, Serri et al., 2018, Pumerantz et al., 2011, Kadry et al., 2004, Drulis-Kawa et al., 2006b). Methicillin-resistant Staphylococcus Aureus (MRSA) infections are a significant healthcare challenge (Moran et al., 2006). Liposomal formulations containing naturally occurring lipids such as oleic acid in combination with gentamicin demonstrated enhanced bactericidal activity due to increased membrane permeability and synergistically lowered MICs against MRSA (Atashbeyk et al., 2014).

In murine systemic MRSA infection, intraperitoneal administration of vancomycin-encapsulated liposomes reduced the bacterial concentration by up to three orders of magnitude within the kidney and spleen (Sande et al., 2012). Improved efficacy was mediated via liposomal fusion with the bacterial cell wall, facilitating intracellular delivery of vancomycin (Sande et al., 2012).

Daptomycin and vancomycin bind to the Gram-positive bacterial cell wall resulting in depolarisation of bacterial cell membranes and cell death (Steenbergen et al., 2005). A compound containing daptomycin conjugated to DSPE via a PEG linker (Dapt-PEG-DSPE) with selectivity for MRSA enhanced targeted delivery and potency of the encapsulated drug in comparison with conventional PEGylated formulations (Jiang et al., 2016). Daptomycin-modified liposomes enhanced targeted delivery and selective binding of encapsulated antibiotics to MRSA, increasing drug accumulation at the infection site with limited side effects (Jiang et al.,

2016). Surface-engineered PEGylated liposomes loaded with vancomycin and daptomycin (VAN-DAPT) increased bacterial permeability resulting in >80% MRS) cytotoxicity in comparison with free drug (Rani et al., 2022).

Encapsulation of polymyxin B in DPPC/cholesterol liposomes enhanced bactericidal activity against resistant strains of Gram-negative bacteria due to fusional interactions between membrane phospholipids of liposomes and bacterial cells, thereby associated with minimal drug-related local and systematic adverse events (Alipour et al., 2008). The MICs of DPPC/cholesterol liposomes against Gram-negative strains were lower than free polymyxin B.

1.7. Therapeutic implications of liposomes

As therapeutic or diagnostic agents, liposomes are now employed in various clinical settings (Sheikholeslami et al., 2022). Compared to their non-liposomal version, several liposomal-formulated medications, including antimicrobials and chemotherapeutics, have been tested for safety and efficacy (Sheikholeslami et al., 2022, Santos Giuberti et al., 2011). However, amphotericin B, used to treat fungal infections, is the only antibiotic approved for use in humans in its liposomal formulation (Aversa et al., 2017, Groll et al., 2019). Liposomal antibiotic formulations are undergoing various phases of pre-clinical and clinical research. (Khan and Chaudary, 2020, van der Weide, 2020). Details of the possible applications of liposomal formulations for antimicrobial drug delivery are discussed below.

1.7.1 Advantages of liposomes as antibiotic carriers

While certain antibiotics have very effective antibacterial activity, they also have several pharmacological drawbacks, including the need for a high therapeutic dose, related toxicity, and poor biodistribution (Sheikholeslami et al., 2022). These problems can be addressed by encapsulation since this changes the drug's pharmacokinetic and pharmacodynamic characteristics, ultimately producing more desired therapeutic effects (Sheikholeslami et al., 2022). As antibiotic carriers, liposomes offer the following benefits: protection against unintended metabolic breakdown; controlled, gradual, and sustained drug release; extended plasma circulation time; target delivery and increased accumulation at the infection site; decreased toxicity and adverse effects; and enhanced antibacterial efficacy (Sheikholeslami et al., 2022).

a). Controlled release of antimicrobial agents

Antibiotic concentration at the infection site is dependent on several factors, including the administration route (e.g., oral, intravenous, intramuscular), protein binding, the volume of distribution, metabolism, and elimination (Levison and Levison, 2009, Olofsson and Cars, 2007). Small-molecule antibiotics are prone to rapid redistribution and elimination, requiring higher and repeated dosing to maintain therapeutic benefit

(Olofsson and Cars, 2007). Certain antimicrobial agents such as aminoglycosides have concentration-dependent activity. Fluoroquinolones are more effective at higher antibiotic dosages (Leekha et al., 2011, Buijk et al., 2002).

A major advantage of encapsulating antibiotics within liposomes is the controlled, sustained release of the entrapped drug, reducing dosing frequency and concomitant systemic toxicity (Levison and Levison, 2009, Olofsson and Cars, 2007). Furthermore, the lipid composition can be carefully controlled. Liposomes can also be engineered to disintegrate and release antimicrobial agents under specific conditions such as changes in pH, temperature or ionic strength (Lian and Ho, 2001b). Controlled release of antibiotics at the site of infection to achieve a higher local concentration with minimal systemic toxicity may be beneficial against resistant bacteria (Levison and Levison, 2009, Olofsson and Cars, 2007).

Increasing the lipophilicity of a drug can increase binding to hydrophobic targets (Silverman and Holladay, 2014). While often increasing potency, it can lead to non-specific interactions with membrane lipid receptors present in mammalian cells, such as the human Ether-a-go-go-related gene (hERG) (Silverman and Holladay, 2014, Jiang et al., 2018). Such off-target interactions are unfavourable. Hence, lipophilicity requires careful control to avoid off-target effects such as cardiac arrhythmias induced via hERG inhibition (Jiang et al., 2018, Silverman and Holladay, 2014).

b). Improved biodistribution at the infection site

The therapeutic level of an antibiotic at the site of infection is influenced by both the local blood supply and concentration within the bloodstream. Antibiotics are small molecules, prone to rapid redistribution, metabolism, and elimination. The use of liposomes as carriers for antibiotics simultaneously decreases protein binding (e.g. to ß-lactam antibiotics) (Zeitlinger Markus et al., 2011) and enzymatic degradation of the antibiotic (Rukholm et al., 2006, Mugabe et al., 2005, Pumerantz et al., 2011, Crommelin et al., 2020). Thus, liposomes may promote prolonged antibiotic circulation. This is potentially advantageous in scenarios where the vascular supply at the site of the infection is compromised, including deep-seated bone infections and abscesses (Santos-Ferreira et al., 2015, Kadry et al., 2004).

Increased vascular permeability often occurs at the site of infection (Azzopardi et al., 2012, Osman et al., 2022), allowing for preferential liposome accumulation. Passive liposome delivery to the infection site can be improved by modifying the liposomal surfaces by targeting moieties that have an affinity for receptors on bacterial surfaces (Osman et al., 2022, Clemons et al., 2018, Wang et al., 2021).

The central nervous system (CNS) poses a challenge for drug delivery; typically, >98% of small-molecule drugs and 98–100% of large-protein drugs penetrate poorly through the blood-brain barrier (BBB) (Brouwer et al., 2010). Bacterial meningitis is a life-threatening infectious disease (Koedel et al., 2002) for which adequate

central nervous system (CNS) antibiotic levels are crucial. Liposomes with unique characteristics to facilitate transport across the BBB have been investigated in the treatment of CNS infections (Brouwer et al., 2010). This can be achieved either by specific or non-specific targeting, the latter accomplished via cationic liposomes that undergo electrostatic interactions with polyanions present at the BBB. This leads to adsorptive-mediated endocytosis (Vieira and Gamarra, 2016). Compared to anionic and neutral liposomes, cationic liposomes have higher uptake into brain parenchymal cells (Joshi et al., 2015). Surface functionalization methods such as PLGA, and c(RGDyK) peptide conjugated with exosomes allow specific targeting across the BBB (Schnyder and Huwyler, 2005, Del Amo et al., 2021, Tian et al., 2018).

c). Prolong plasma circulation

Reducing liposome size and coating its surface with PEG derivatives to prevent MPS uptake, as previously discussed, can lengthen the systemic circulation duration. Well-formulated liposomes can extend circulation time while increasing antibiotic bioavailability because of their resistance to physiological degradation and controlled release capability (Sheikholeslami et al., 2022).

d). Decreased toxicity of antibiotics

Reduction of antibiotic accumulation in non-infected tissues may mitigate side effects. This is particularly relevant where antimicrobials with major side effects are needed for the management of resistant organisms. Polymyxin B is a polycationic peptide that exerts bactericidal action by interacting with LPS and phospholipid bilayers of Gram-negative bacterial membranes (Omri et al., 2002). It has potent antimicrobial activity against a variety of bacterial strains. However, associated toxicity including nephrotoxicity, ototoxicity, and neuromuscular blockade limits its systemic use (Zavascki et al., 2007). The incorporation of polymyxin B into liposomal formulations provides a relatively safer drug delivery route, achieving therapeutic drug levels at the site of infection with minimal toxic effects (Omri et al., 2002). Compared to free polymyxin B, liposomal polymyxin B was associated with enhanced bactericidal activity against E.coli, P. aeruginosa, K. pneumoniae, and Acinetobacter baumannii in vitro (Alipour et al., 2008).

e). Enhanced bactericidal activity

Compared to current medications, the liposome-based antibiotic's increased antibacterial activity is incredibly attractive. Due to their bilayer shape resembling a cell membrane, liposomes possess an exceptional ability to fuse and penetrate most biological membranes throughout the body, including those found in microorganisms (Sheikholeslami et al., 2022, Kube et al., 2017). Antibiotic-encapsulated liposomes are more effective against microbes because of the fusion process with bacterial membranes, delivering a large dose of the drug directly to the cytoplasmic compartment (Sheikholeslami et al., 2022, Kube et al., 2017). The presence of fusogenic moieties at the liposomal surface, such as charged organic compounds, as

well as the characteristics of the bacteria themselves, determine the liposomes' ability to kill bacteria (Sheikholeslami et al., 2022, Kube et al., 2017).

In-vitro susceptibility studies, such as the broth dilution technique, are used to determine the antibacterial activity of a particular antibiotic against a particular disease (Alhariri et al., 2013). These calculate the antimicrobial's minimum inhibitory concentration (MIC), or the lowest concentration of a particular medication that prevents an organism from growing visibly (Alhariri et al., 2013). Numerous studies have shown that the MIC of the equivalent free drug can be lowered by encapsulating antibacterial medications in certain liposome formulations (Alhariri et al., 2013, Mugabe et al., 2005). For instance, liposomal aminoglycosides have far lower minimum inhibitory concentrations (MICs) than free drugs for P. aeruginosa isolates (Khan et al., 2020, Gaspar et al., 2013, Mugabe et al., 2005). The enhanced antibacterial action within these formulations results from their capacity to fuse and penetrate within the bacterial surfaces (Khan et al., 2020, Gaspar et al., 2013, Mugabe et al., 2005). Several *in-vitro* studies show that appropriate liposomal forms can effectively increase antibacterial activity against most common extracellular bacteria, including P. aeruginosa, K. pneumoniae, and E. coli, in comparison to the medication in its free form (Khan et al., 2020, Gaspar et al., 2013, Mugabe et al., 2005).

1.8 Liposomes as an antibiotic carrier to overcome bacterial resistance mechanisms

There is mounting evidence that suggests liposome-incorporated antibiotics may help counteract some bacterial resistance processes by altering interactions between the liposome and the bacteria. The intricate barrier found on the outer membrane of Gram-negative bacteria, for instance, can alter how antibiotics interact with the bacterial wall or restrict their internalisation, a significant cause of emerging resistance (Ferreira et al., 2021). However, as already indicated, liposomes may encourage bacterial membrane fusion leading to structural disruption and reversing the low permeability of the membrane.

a). Improved antibiotic stability by preventing enzymatic degradation

Bacterial resistance associated with enzymatic hydrolysis was avoided by incorporating antibiotics into liposomes. Nacucchio et al. showed that encapsulating piperacillin in liposomes made from cholesterol and phosphatidylcholine could shield the antibiotic from hydrolysis by staphylococcal ß-lactamases and preserve its antibacterial efficacy. Since mechanisms of resistance of enteric rods are typically enzymatic, it would be interesting to investigate whether liposome-encapsulated antibiotics possess unique features to circumvent enzymatic destruction (Ferreira et al., 2021, Nacucchio et al., 1985b). In an S. aureus osteomyelitis model, a liposomal formulation co-loaded with vancomycin and ciprofloxacin permitted total bone sterilisation, demonstrating stronger therapeutic effects against these potentially fatal infections (Kadry et al., 2004).

b). Enhanced drug permeability across bacterial membrane

A potential strategy for overcoming nonenzymatic drug resistance is liposome-bacteria fusion (Ferreira et al., 2021). This has been specifically investigated for P. aeruginosa strains since key factors contributing to their resistance include efflux pump systems and/or low, non-specific permeability of their outer membrane (Ferreira et al., 2021). Mugabe and colleagues found that P. aeruginosa infections produced by resistant clinical strains could be effectively treated with liposomes filled with aminoglycosides (Mugabe et al., 2005). Bacteria exposed to antibiotics-encapsulated liposomes exhibited greater antimicrobial susceptibility compared to free standard drugs (Mugabe et al., 2005).

In contrast to free antibiotics, resistant P. aeruginosa strains treated with a fluid liposome-entrapping polymyxin B had lower minimum inhibitory concentrations (MICs) and higher antibiotic levels inside the bacterial cells (Alipour et al., 2008). One of the most effective impermeable barriers causing bacterial resistance was thus breached by the liposomal formulation (Alipour et al., 2008).

c). Bypassing efflux pump mechanisms

Many bacteria actively use efflux pump mechanisms to pump antibiotics out of the cell. This leads to the development of acquired resistance to antimicrobial agents, particularly among Gram-negative bacteria (Ferreira et al., 2021). Liposomal-encapsulated antibiotics provide shielding effects against efflux pumps by having higher antibiotic concentrations within the bacterial cell, thereby demonstrating stronger bactericidal effects (Ferreira et al., 2021).

d). Synergistic effects

Multiple antibiotics or adjuvants can be co-encapsulated within liposomal formulations to create synergistic interactions that can overcome bacterial resistance (Ferreira et al., 2021). A combination of different classes of antibiotics that can target specific resistance mechanisms within the same liposomes can enhance bactericidal activity by mitigating various enzymatic and non-enzymatic (alteration at drug receptor sites, decreased membrane permeability and efflux pump upregulation) resistance approaches (Ferreira et al., 2021).

1.9 Barriers related to liposomes as antibiotic carriers

Several factors limit the use of liposomes as drug carriers including poor encapsulation efficiency (EE), impaired stability and permeability resulting in drug leakage, short circulation half-life due to chemical and enzymatic degradation, biological interaction with host cells, cost-effectiveness and production (Mullis et al., 2021).

a). Drug-loading efficiency

Of the most crucial factors to manage during the creation of a liposome formulation is its electrochemical potential and encapsulation efficiency (E.E). The E.E. is the proportion of the total amount of medication initially available divided by the percentage of drug integrated into the liposome (Marchianò et al., 2020). Most antibiotics exhibit poor encapsulation efficiency due to poor hydrophilicity, decreased solubility within the lipophilic environment, or interactions with lipids, limiting drug loading capacity within liposomal formulations (Marchianò et al., 2020). Only when the liposome contains a therapeutic dose will it exhibit its maximum pharmacological impact (Marchianò et al., 2020). As a result, the E.E. is the focus of most liposomal preparation methods; it is known to be influenced by lipid composition, surface charge and specific antibacterial characteristics (Marchianò et al., 2020).

b). Liposomal stability

Liposomes are prone to fusion, aggregation, leakage of antibiotics encapsulated within liposomes, and early or premature drug release during storage or circulation within the bloodstream (Jyothi et al., 2022, Sheikholeslami et al., 2022). The drug should ideally be carefully transported and released at the infection site without leaking any content encapsulated within the liposome (Jyothi et al., 2022). Thus, it follows that the liposome's chemical and physical stability is equally important (Jyothi et al., 2022). Lipids utilised to build the vesicle are usually hydrolysed or oxidised, causing chemical instability and a limited shelf life (Jyothi et al., 2022). Lipid composition and storage conditions are the main determining factors (Jyothi et al., 2022).

Liposomes containing short-chain lipids or fluid membranes exhibit the most physical instability (Jyothi et al., 2022, Sheikholeslami et al., 2022). Membranes can be stabilised by some constituents such as cholesterol (Jyothi et al., 2022). Neutral or negatively charged vesicles have improved stability. Drug leakage increases *in-vivo* for positively charged liposomes due to several factors e.g. cationic lipids can cause repulsive interactions among the lipids within the bilayers, which can destabilize the liposomal membrane, interactions with anionic molecules within biological fluids, increased membrane permeability, and interactions with encapsulated drugs (Jyothi et al., 2022, Sheikholeslami et al., 2022). Positively charged liposomes contribute significantly to the increase in liposomal-encapsulated antibiotic bactericidal activity in several *in-vitro* studies because of stronger electrostatic interactions. However, their instability in *in-vivo* models presents a challenge that must be overcome (Jyothi et al., 2022, Sheikholeslami et al., 2022). Liposome size changes may contribute to either liposome fusion or aggregation to generate bigger vesicles, another feature of liposome physical instability, with larger-sized liposomes being removed more quickly (Jyothi et al., 2022, Sheikholeslami et al., 2022), and are often improved by coating them with PEG or negatively charged lipids as these additions reduce self-aggregation.

c). Drug release kinetics

Optimising the therapeutic efficiency of liposomes requires control over the kinetics of antibiotic release. Low-release kinetics may result in insufficient bactericidal activity, whereas rapid drug release following delivery may cause suboptimal drug concentrations at the infection's site (Marchianò et al., 2020). Therefore, it is challenging to achieve continuous, slow, and sustained drug release profiles.

d). Pharmacokinetics and biodistribution

To effectively reach the site of infection, liposome-encapsulated antibiotics need to overcome several biological barriers (Marchianò et al., 2020). Pharmacokinetics and biodistribution can be impacted by several factors that may potentially reduce their therapeutic efficacy, including opsonization by the reticuloendothelial system (RES), rapid clearance from the circulation, and limited tissue penetration (Antimisiaris et al., 2021, Sheikholeslami et al., 2022).

To maximise therapeutic benefits, liposomal antibiotics must be delivered to the infection site with precision while limiting local and systemic drug-related adverse events. For intracellular or deep-seated infections, it remains difficult to enhance local tissue drug concentrations at the infection site (Antimisiaris et al., 2021, Sheikholeslami et al., 2022). To address these barriers, the creation of liposomal drug carriers requires careful consideration of encapsulation and stability techniques (Antimisiaris et al., 2021, Sheikholeslami et al., 2022). Even though liposome drug encapsulation has been the subject of numerous investigations, their clinical applicability has been hampered mostly by problems with stability and low drug entrapment efficiency. By optimizing lipid composition, structurally modifying liposomal surfaces, incorporating targeted ligands, and engineering controlled and sustained drug release mechanisms, these drawbacks can be potentially avoided. If so, this will increase the drug's therapeutic efficacy, paving the way for practical application in patients (Marchianò et al., 2020, Sheikholeslami et al., 2022). Optimization of liposome-encapsulated antibiotics through thorough preclinical evaluations is essential to improve their therapeutic outcomes and clinical transition.

e). Rapid clearance and uptake by the reticuloendothelial system

Liposomal antibiotics administered intravenously are susceptible to clearance by the reticuloendothelial system (RES), particularly by the spleen and liver. Circulating liposomes may be recognized as 'foreign' by immune cells (Harashima et al., 1994, Levison and Levison, 2009). The rapid uptake of liposomes is facilitated via opsonization by circulating serum proteins including immunoglobulins and the complement proteins C3a and C5a (Song et al., 2012, Harashima et al., 1994, Bonté and Juliano, 1986). Minimizing liposomal charge (Lian and Ho, 2001b, Song et al., 2012), or the incorporation of PEG onto the surface of liposomes (Maruyama et al., 1992, Torchilin, 1994, Crommelin et al., 2020) may limit uptake by the RES.

Increasing liposome size is associated with greater liposomal uptake by the RES (Song et al., 2012). Most *in-vivo* studies have used unilamellar vesicles, of ±100 nm in size, for systemic drug delivery (Li et al., 2015, Rani et al., 2022, Shek et al., 1998, Jiang et al., 2016). Larger liposomes are often associated with greater interaction with circulating serum proteins and decreased circulation half-life (Jiang et al., 2016, Rani et al., 2022, Gharib et al., 2012, Pumerantz et al., 2011, Harashima et al., 1994, Sheikholeslami et al., 2022)

In-vivo animal studies have demonstrated that RES uptake of liposomes can be saturated by non-therapeutic liposomes, subsequently limiting the uptake of therapeutic liposomes (Semple et al., 1998, Liu et al., 2015). However, this strategy is not clinically relevant in humans due to associated effects on the RES.

1.10 Approaches to overcome barriers related to liposomes as antibiotic carriers

Lipid composition can be changed in various ways that can increase the effectiveness of liposomal delivery, e.g. using ionizable lipids, fusogenic lipids, PEG lipids and cholesterol (Cavalcanti et al., 2022, Sun and Lu, 2023, Habrant et al., 2016, Lasic and Martin, 2018). Ionizable lipids and PEG lipids have been developed to increase nanoparticle drug-delivery efficiency while minimising negative effects for *in-vivo* applications (Terada et al., 2021, Sun and Lu, 2023, Lasic and Martin, 2018).

a). Ionizable lipids to overcome liposome-related delivery barriers

Lipids with headgroups that exhibit varying charges when protonated or deprotonated in varying ambient pHs (because of the presence of positively charged amino groups) are commonly referred to as ionizable lipids. Examples include 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) (Terada et al., 2021, Sun and Lu, 2023, Habrant et al., 2016). These lipids are designed to formulate nanoparticles that aid in the delivery of gene therapy, mRNA vaccines and nucleic acids (mRNA, siRNA, DNA) into cells (Terada et al., 2021, Sun and Lu, 2023, Habrant et al., 2016). At physiological pH, ionizable lipids are generally neutral or slightly positively charged but, in acidic environments, such as within endosomes, they acquire a positive charge (Habrant et al., 2016, Sun and Lu, 2023, Terada et al., 2021). Ionizable lipids upon entering the cell usually undergo protonation due to the acidic endosomal milieu (Habrant et al., 2016, Sun and Lu, 2023, Terada et al., 2021). This process produces a positive charge that facilitates interaction with the negatively charged endosomal membrane (Habrant et al., 2016, Sun and Lu, 2023, Terada et al., 2021). Nucleic acids that are encapsulated can evade lysosomal breakdown as this interaction destabilises the endosomal membrane (Habrant et al., 2016, Sun and Lu, 2023, Terada et al., 2021). This pH-dependent charge conversion helps nucleic acids to escape from endosomes into the cytoplasm (Habrant et al., 2016, Sun and Lu, 2023, Terada et al., 2021).

b). Fusogenic lipids to overcome liposomes-related delivery barriers

Fusogenic lipids facilitate membrane fusion between the targeted-cell membrane and the lipid-based drug delivery mechanisms by improving intracellular transport and cellular absorption of payload (Kube et al., 2017, Cavalcanti et al., 2022). They are usually composed of lipids having specific hydro group structures along with lipids having higher hydrophobicity such as cholesterol and neutral (dioleoyl phosphatidylethanolamine (DOPE)), cationic 1,2-dioleoyl-3-dimethylammonium-bromide (DODAB), and anionic dioleoyl phosphatidylserine (DOPS) fusogenic lipids (Kube et al., 2017, Cavalcanti et al., 2022). The physicochemical properties of fusogenic lipids facilitate interactions and fusion with targeted cellular membranes by destabilizing the lipid barriers of both cellular membranes and drug-delivery systems (Cavalcanti et al., 2022, Kube et al., 2017). This fusion process allows the direct delivery of payload into the cytoplasm, thereby eluding endocytic pathways and lysosomal destruction (Cavalcanti et al., 2022, Kube et al., 2017).

c). Helper lipids to overcome liposomes-related delivery barriers

Liposome composition can facilitate binding and fusion to bacterial cell membranes, enabling delivery of higher concentrations of antibiotics (Huwaitat et al., 2016, Solleti, 2016, Kolašinac et al., 2018, Mah and O'Toole, 2001). Helper lipids such as phosphatidylcholine and their derivatives (e.g. phosphatidylethanolamine) are usually present in the liquid crystalline phase (Kolašinac et al., 2018, Sheikholeslami et al., 2022). Cationic lipids and helper lipids may decrease the fluidity of lipid bilayers by conversion into an inverted hexagonal lipid phase which serves as an intermediate to facilitate membrane fusion (Kolašinac et al., 2018). Therefore, the incorporation of helper lipids along with a mixture of cationic and neutral lipids (DOTAP/DOPE) enhances liposomal interaction and fusion with mammalian and bacterial cells, thereby facilitating intracellular delivery of payload encapsulated within liposomal formulations (Kolašinac et al., 2018, Sheikholeslami et al., 2022).

d). PEG lipids and stealth liposomes to overcome liposomes-related delivery barriers

Liposomes can be made more stable and capable of targeting by altering their physio-chemical surface properties. This can be done by adding polyethene glycol (PEGylation) (**Figure 7**) which prolongs the bloodstream circulation period by reducing immune system clearance and opsonization. Biocompatible hydrophilic polymers can be used to modify liposome surfaces to minimise the adsorption of circulating proteins and RES uptake (Song et al., 2012). This strategy, known as surface hydration or steric modification, involves conjugating lipids to hygroscopic or hydrophilic polymers such as polyethene glycol (PEG) or its derivatives (Torchilin, 1994). The presence of hydrophilic polymers on liposome surfaces provides a hydration layer that reduces clearance by the RES (Jiang et al., 2016, Maruyama et al., 1992). Polymeric PEG, constituting 5-10 % of total lipid concentrations (Torchilin, 1994, Maruyama et al., 1992) appears to

effectively achieve this (**Figure 5**). Surface modification of liposomes by PEGylation extended the plasma half-life of vancomycin from 30 to >90 minutes with delayed uptake by activated phagocytes; this also promoted bacterial killing due to higher antibiotic concentrations as compared to conventional (non-PEGylated) liposomal vancomycin under similar *in-vitro* experimental conditions (Pumerantz et al., 2011).

Stealth liposomes, also known as long-circulating liposomes, are manufactured to avoid detection and elimination by immune systems such as RES or the mononuclear phagocyte system (MPS) (Deol and Khuller, 1997, Lasic and Martin, 2018). PEGylation is a popular technique for giving liposomes stealth characteristics, enhancing targeted delivery and accumulation within infection sites with longer circulation durations and minimal local and systematic adverse events (**Figure 7**) (Deol and Khuller, 1997, Lasic and Martin, 2018).

To accomplish targeted delivery, extravasation at areas with higher vascular permeability, such as tumours and inflammatory regions, and appropriate biodistribution, the stability of liposomes must first be optimised (Deol and Khuller, 1997, Lasic and Martin, 2018, Sheikholeslami et al., 2022). To prevent opsonization and mononuclear phagocyte system macrophage absorption, the hydrophilic polymer poly (ethylene glycol) (PEG) is an option (Deol and Khuller, 1997, Lasic and Martin, 2018, Sheikholeslami et al., 2022). The liposomes can then pass through to their intended destination (Deol and Khuller, 1997, Lasic and Martin, 2018). Unfortunately, the PEG polymer may prevent liposomes from engaging with the intended cells and dislodging any trapped material, and this could obstruct delivery (Deol and Khuller, 1997, Lasic and Martin, 2018). It is imperative to optimise the PEG lipid percentage to support delivery rather than impede it (Lasic and Martin, 2018).

Summary

Antimicrobial agents, encapsulated within liposomes can improve drug pharmacokinetics and pharmacodynamics, and enhance antimicrobial activity against various bacterial strains. They can deliver hydrophilic and hydrophobic drugs to specific sites of infection, having advantages over free drugs including improved drug stability, biodistribution, reduced toxicity and enhanced bactericidal activity. Altering the structure and physio-chemical properties of liposomes, including lipid composition, size, and surface charge, can influence their efficacy, safety, and interaction with bacterial cells, thereby improving antibacterial activity. The therapeutic advantages of liposome-encapsulated antibiotics include sustained and controlled release of antibiotics, improved biodistribution, and prolonged plasma circulation, with limited drug-related adverse events.

2. Hypothesis, Aims, Objectives and Clinical Impact

2.1 Hypothesis

I hypothesize that encapsulation of meropenem within cationic liposomes will improve the efficacy of meropenem, overcoming meropenem resistance.

2.2 Research aims

- Review literature on current encapsulation strategies.
- Develop different non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations.
- Evaluate the efficacy, stability, and safety of meropenem-encapsulated cationic liposomes against laboratory, clinical and resistant strains of Gram-negative bacteria.
- Assess the *in-vitro* stability (over 24 hours) of meropenem-encapsulated liposomes by measuring the physiochemical properties using DLS and HPLC.
- Assess the *in-vitro* safety of meropenem-encapsulated liposomes at concentrations up to five times those seen in patient blood.
- Assess the efficacy, stability, and safety of the addition of PEG to meropenem-encapsulated cationic liposomes.

2.3 Research objectives

- To develop meropenem encapsulated within cationic liposomes to enhance the efficacy of meropenem in inhibiting bacterial growth.
- To determine if cationic liposome-encapsulated meropenem can overcome bacterial resistance against meropenem.
- To optimize the safety of liposomal meropenem formulations for clinical use.

2.4 Clinical impact

- There is an urgent need to develop new strategies to counter antimicrobial resistance (AMR). This study aims to formulate novel drug delivery methods by modifying pre-existing antibiotics to combat multidrug-resistant bacteria.

- This study aims to prove that the bactericidal activity of liposomal antibiotics provides a significant reduction in antibiotic dosage requirements, thereby improving the therapeutic index of existing antibiotics as well as improving their safety profile.

Summary

Encapsulating meropenem within cationic liposomes could enhance its efficacy and safety, particularly against meropenem-resistant organisms. My research aims to develop different formulations of non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes. I will evaluate their efficacy against different strains of Gram-negative bacteria (laboratory, clinical and resistant) to assess their stability and safety *in-vitro*, and to evaluate the impact of adding PEG on efficacy, stability, and safety.

3 Materials and Methods

In summary, non-PEGylated and PEGylated meropenem-encapsulated liposomes with varying cationic and DSPE-PEG 2000 lipid concentrations were synthesized by the thin-film hydration method (Nwabuife et al., 2021, Drulis-Kawa et al., 2006b, Drulis-Kawa et al., 2006a), and characterized by Dynamic Light Scattering (DLS) and high-performance liquid chromatography (HPLC) with *in-vitro* bactericidal testing using the broth-microdilution method.

Fusion kinetics were determined by incorporating a fluorescent lipid within the liposome (PE) with variable cationic lipids (0%, 10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations. These were co-incubated with (Hoechst-labelled) Gram-negative bacteria and (e450 anti-CD45 labelled) healthy volunteer white blood cells. Flow cytometry was used to measure relative changes in the PE fluorescence of bacteria and immune cells over time compared to baseline values (suggestive of liposome fusion). Confocal laser scanning microscopy (CLSM) was used to assess the fusion of liposomes labelled with Alexa 647 with bacteria and immune cells. Non-PEGylated and PEGylated meropenem-encapsulated liposomes with variable cationic lipids (10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations were co-incubated with (Hoechst- labelled) Gram-negative bacteria and the immune cells stained with dihydroethidium (Thermo) to assess reactive oxygen species), DAPI to show cell nuclei.

To assess safety, *in-vitro* whole blood was incubated with non-PEGylated and PEGylated meropenem-encapsulated liposomes with variable cationic lipids (10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations at different meropenem concentrations, using flow-assisted cell sorting (FACS), Enzyme-Linked Immunosorbent Assay (ELISA), and haemolytic assay activity (HAA).

3.1. Cationic liposomal antibiotic preparation

a). Non-PEGylated meropenem-encapsulated liposomes

The thin-film hydration method was used to prepare non-PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations. Cationic liposomes were prepared using a stock concentration of 100 mM of the following lipids: Egg phosphatidylcholine (PC) (AVANTI), Do-decyl trimethylammonium bromide (DODAB) (AVANTI), and cholesterol (AVANTI). Cationic liposomal formulations with increasing DODAB molar concentrations were prepared. The different ratios of PC: DODAB: Cholesterol was added to prepare non-PEGylated meropenem-encapsulated liposomal formulations with variable cationic lipid contents, namely: 7:0:3, 6:1:3, 5:1:4, 5:2:3, 5:3:2, 3:3:4 and 5:4:1, respectively.

Lipids were dissolved in an organic solvent i.e., chloroform (AVANTI) within a glass specimen vial. Chloroform was evaporated to form a thin dried homogenous lipid layer using a rotary evaporator (**Figure 8**). A vacuum

was applied at 475 mmHg for 15 minutes, followed by 100 mg for 10 minutes, and 0 mmHg for 10 minutes, at a water bath temperature of 50°C (**Figure 8**).

Two different concentrations of meropenem were used for *in-vitro* and *in-vivo* experiments. For *in-vitro* experiments, meropenem (Ranbaxy) was prepared by adding 30 mg into 1 mL of PBS (Gibco) and vortexed for 1 minute. Similarly, 50 mg/mL of meropenem was prepared for *in-vivo* experiments to maximize the drug concentration. One mL of meropenem solution was added to the dried lipid film and sonicated for 15 minutes at room temperature to prepare a homogenous solution.

Liposomes were extruded to achieve specific sizes of 50 nm, 100 nm, and 200 nm (**Figure 8**). Membrane filters with appropriate pore sizes were used to extrude desired-sized liposomal formulations. Liposomes were passed through the membrane a total of 13^{th} times. Following extrusion, NAP-25 columns (Cytiva) were used to remove excess non-encapsulated meropenem (**Figure 8**). NAP-25 columns were equilibrated with 15 mL of PBS, loaded with 1 mL of liposomal suspension, and eluted with 5 mL of PBS.

b). PEGylated meropenem-encapsulated liposomes

In separate experiments, polyethylene glycol (PEG) at molar concentrations of 0.5% and 2.5% were used to synthesize PEGylated meropenem-encapsulated cationic liposomes. DSPE-PEG 2000 (AVANTI) was dissolved in chloroform along with other lipids constituents, and the molar concentrations of cholesterol were reduced in proportion. PEGylated meropenem-encapsulated cationic liposomes were synthesized by following the thin-film hydration as described above.

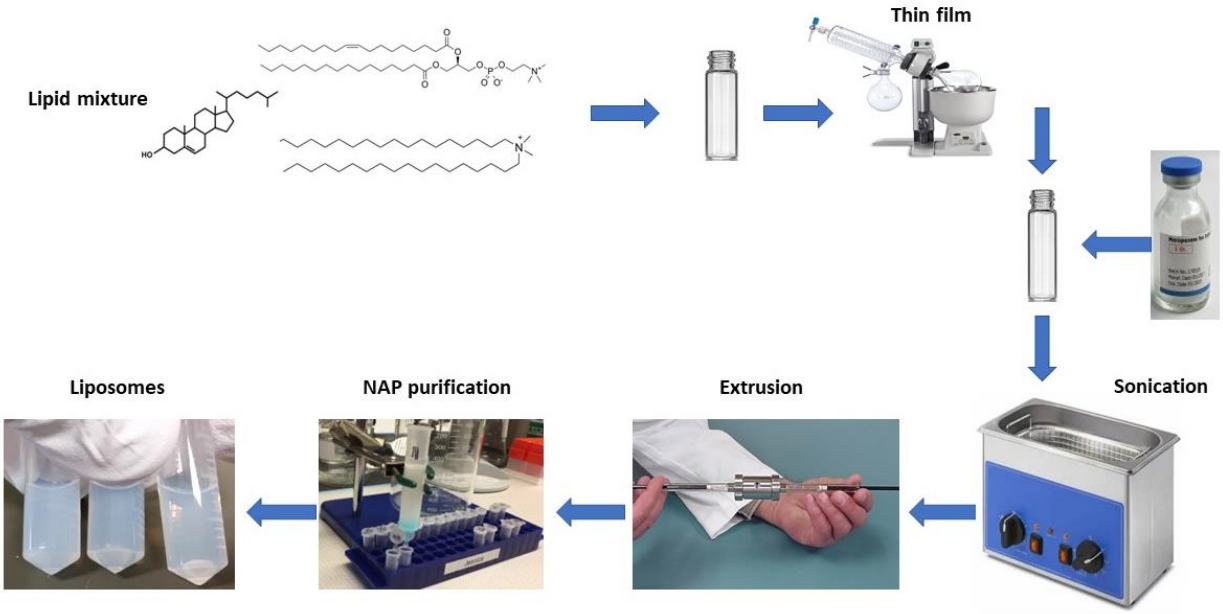

Figure 8: The thin-film hydration method was used to prepare non-PEGylated and PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations. Different lipids were dissolved in chloroform based on molar concentrations. The organic solvent was then evaporated using a rotatory evaporator. Once the thin-dried film was formed, meropenem was dissolved in PBS to hydrate the film. The mechanical dispersion method was used to load the drug within the rehydrated liposomal formulations. Liposomes were extruded using filter membranes to achieve the desired sizes. Following extrusion, NAP columns were used to remove excess drug present over the external liposome surfaces.

3.2. Liposome characterization

3.2.1 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC; Agilent 1260 II HPLC (Cheshire, UK)) was used to measure concentrations of meropenem encapsulated within cationic liposomes based on the physiochemical properties of the drug. The integrated temperature-controlled column compartment and the autosampler were set at 35°C and 4°C, respectively. An Agilent Porshell 120 EC-C18 4.6 x 150 mm, 4 µm analytical column was used. Data signals were processed and presented using Open LAB CDS LC ChemStation (Agilent, Cheshire, UK).

The mobile phase for the HPLC study for meropenem was prepared by using monobasic sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$), dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) and acetonitrile (all from Sigma); and pH adjusted to around 7.4. A final solution of 10 mM phosphate (buffer: acetonitrile) 90:10 (v/v) was used. Meropenem stock solution was prepared to a concentration of 1 mg/ml by reconstituting 1 mg meropenem with 1 ml HPLC water. The mobile phase flow rate was set at 1.3 ml/min with a retention time set to 4

minutes. The peak absorbance of meropenem was read at 290 and 300 nm. A standard curve was prepared by serial dilutions of the meropenem stock solution with a concentration range from 0.97 to 800 mcg/ml. HPLC gradient water was used as a negative control. 50 µl of each sample was injected by the autosampler.

Both non-PEGylated and PEGylated cationic liposomes (lysed and non-lysed) were used to calculate the encapsulation efficacy (EE) and % internal drug concentrations for meropenem-encapsulated liposomal formulations. Liposomes were lysed with 2% triton solution (1:10 dilution of liposomal suspension to 2% triton). The peak heights were analysed, and the linear regression of the calibration curve was calculated. The coefficient of determination (R^2) was calculated by GraphPad Prism (Version 9 for Windows, GraphPad Software, La Jolla, CA, USA). A standard curve was created to evaluate meropenem concentrations in the liposomal formulations.

The amount of meropenem within liposomes as a percentage of the total meropenem in solution (% internal drug concentration) is calculated as:

% internal drug concentration =

(drug released after liposome lysis - drug concentration without lysis) / (drug released after lysis)

The amount of meropenem entrapped within liposomes as a percentage of the concentration of meropenem used for thin film rehydration (encapsulation efficiency (EE%)) is calculated as:

Encapsulation efficiency (%) =

(meropenem entrapped within liposomes) / (initial concentration of meropenem) ✕100

3.2.2 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) (Zetasizer, Malvern pan analytical with ZS Xplorer software) was used to characterize the liposomal size, charge (zeta potential), polydispersity index (PDI), and particle concentration. Samples were diluted 1:10 in distilled water and placed into a non-disposable Z.P. measuring cuvette for measurements. To assess particle concentration, samples were diluted 1:10 in PBS and placed into the disposable glass cuvettes.

a) Size

The refractive index of the nanoparticles is usually slightly different from the dispersed solvent. Light is primarily scattered by particles suspended within a solution that exhibit Brownian motion (Karmakar, 2019). Fluctuations within the intensity of scattered light are measured and analysed to obtain the diffusion constant and hence the nanoparticles' hydrodynamic radius (Karmakar, 2019). Liposome size was calculated by measuring the diffusion coefficient of the nanoparticles by estimating the hydrodynamic diameter by using the following Stokes-Einstein equation:

$$d\,(H) = kT / 3\,\pi\eta D$$

Where d (H) represents particle size; k is the Boltzmann constant; T is the thermodynamic temperature; η is the viscosity, and D is the diffusion coefficient (Karmakar, 2019).

The Brownian movement of nanoparticles depends on their size, solvent viscosity and temperature (Karmakar, 2019). Smaller particles diffuse very quickly as compared to larger particles, thus intensity fluctuations are very rapid for smaller particles relative to the larger nanoparticles (Karmakar, 2019).

b) Zeta Potential

Zeta potential represents the potential difference between the dispersion medium and the stationary layer of fluid attached to dispersed nanoparticles within a suspending medium (Bhattacharjee, 2016, Karmakar, 2019). Zeta potential provides insight into the behaviour of nanoparticles in a dispersing medium., as it can influence the interactions, stability, and functional characteristics of nanoparticles (Karmakar, 2019). DLS itself does not directly measure the charge present over the liposomal surfaces (Bhattacharjee, 2016). DLS measures the electrostatic potential at the slipping/shear plane (the interface between the diffuse layer of ions in the bulk solution and the tightly bound layer of ions surrounding the liposome surfaces) of a colloidal particle (Bhattacharjee, 2016, Karmakar, 2019).

DLS can however provide indirect information about the zeta potential by measuring the Brownian motion of nanoparticles within the solution (Bhattacharjee, 2016, Karmakar, 2019). When a monochromatic laser beam is passed through a sample containing liposomes, light is scattered by particles due to fluctuations within the refractive index of the medium (Bhattacharjee, 2016, Karmakar, 2019). These fluctuations within the intensity of the scattered light cause random Brownian motion of the particles that is influenced by several factors such as their shape, size and surface charge, as well as the surrounding medium (Bhattacharjee, 2016, Karmakar, 2019). Particles having higher zeta potentials typically have greater

electrostatic repulsion between them, associated with increased stability in the dispersion (Bhattacharjee, 2016).

c) Polydispersity Index (PDI)

The polydispersity index (PDI) defines the size distribution of the unknown particles by quantifying the non-uniformity of nanoparticles within a solution (Karmakar, 2019). The numerical value of PDI ranges from 0 for a perfectly uniform particle size sample to 1.0 for a highly polydisperse sample with multiple particle size populations (Karmakar, 2019).

3.2.3 Stability of liposomal antibiotic in selected liposomes

The stability of the non-PEGylated and PEGylated meropenem-encapsulated liposomes was assessed using Light Scattering (DLS) and High-Performance Liquid Chromatography (HPLC) to measure the physiochemical properties of liposomes stored at 4°C over separate times. Meropenem-encapsulated cationic liposomes were stored at 4°C over 24 hours and the physiochemical properties of liposomes were measured at 0 and 24 hours using DLS and HPLC.

3.3. *In-vitro* bactericidal testing of liposomes

For *in-vitro* susceptibility studies, the broth dilution technique is often used to determine the bactericidal activity of commonly used antibiotics against a particular pathogen. The minimum inhibitory concentration (MIC) is the lowest concentration (usually expressed in µg/mL) of an antimicrobial agent that inhibits the visible growth of bacteria *in-vitro* following 24 hours of incubation. The MICs of free and non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes were determined.

EUropean Committee for Antimicrobial Susceptibility Testing (EUCAST) was used to determine the susceptibility of free and non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes against laboratory strains: Acinetobacter (ATCC 19606), E. coli (ATCC 25322, ATCC 12241, ATCC 12014), K. pneumoniae (ATCC 13882, ATCC 13883), Pseudomonas (ATCC 10125, ATCC 35422), clinical: (E. coli (US142, GS065), P. Aeruginosa (US00G, US115), K. pneumoniae (US131, GS008), Enterobacter cloacae (US055), and resistant: Acinetobacter (48-9043, CS023, RS080), E. coli (11M105778, DH5alpha -pk0X015, 11M212929), P. Aeruginosa (11M57609, 14M124832), P. Aeruginosa (CS008, 48-1997, PA01 pMATTX, 12M174258, 11M369086, 73-12198, TS007, CS029) strains of Gram-negative bacteria. Micro-organisms were considered to be ''susceptible'' if the MICs for meropenem-encapsulated cationic liposomes were ≤2 µg/ml, ''intermediate'' if between 2-8 µg/ml, and ''resistant'' for MICs ≥8 µg/ml (Giske et al., 2022a). An E.coli (ATCC

25922) strain was used as the reference strain. The results were recorded as a binary outcome, i.e., *in-vitro* visible growth of micro-organisms or no growth.

Different cultured media were used to isolate and grow the different strains of Gram-negative bacteria. Clinical and resistant Gram-negative bacterial strains were cultured in cationic-adjusted Mueller Hilton broth (ThermoFisher) with 10.5 g of cationic-adjusted Mueller Hilton broth (ThermoFisher) dissolved in 0.5 litre of distilled water. Laboratory strains were cultured either in lysogeny broth (LB) (ThermoFisher) 2.32 g/50 ml and tryptic soy broth (TSB) (ThermoFisher) 1.50 g/50 ml dissolved in distilled water The reconstituted broth was autoclaved at 121°C for 15 minutes and stored at 2-8°C.

3.3.1 Preparation of bacterial inoculum

MacFarland standards were used as a reference to adjust the turbidity of the bacterial suspension such that the number of bacteria must be within a given concentration for standardized microbial testing. Specific bacteria of interest were cultured on blood agar plates. A single colony of the cultured bacterium was picked using a sterile loop and dissolved in 3 mL sterile PBS. The bacterial suspension was diluted in PBS to obtain absorbance optical densities of 0.5, 0.2 and 0.1 at a wavelength of 600 nm.

3.3.2 Miles and Misra Colony counts

Miles and Misra colony counts were performed at an optical density (OD) of 0.1 to identify specific colony-forming units (CFUs) at a given OD. An inoculum of the bacteria at a specific OD (0.1) was serially diluted ten-fold in broth. All bacterial strains were cultured in cationic-adjusted Muller-Hinton broth. Ten-fold serial dilutions were performed eight times (100 µl of neat suspension added to 900 µl of sterile media). Each agar plate was divided into 3 equal sectors and 20 µL of each bacterial broth dilution was pipetted onto a single plate and the drop was allowed to spontaneously spread and dry. Plates were inverted and left overnight at 37°C. The following day, colonies were counted in the sector with the highest number of full-size discrete colonies. Total CFUs were calculated as follows:

$$\text{CFU per mL} = \text{average number of colonies at a dilution} \times 50 \times \text{dilution factor}$$

Table 3 demonstrates the total number of colony-forming units (CFUs) calculated at an optical density (OD) of 0.1 for different laboratory, clinical, and resistant strains of Gram-negative bacteria used to determine the MICs for different non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes and standard commercially available meropenem.

Table 3: Colony-forming units (CFUs) /ml at specific optical densities (ODs) (0.1).

Lab strains	McFarland OD 0.1	Clinical strains	McFarland OD 0.1
ATCC 19606 Acinetobacter	1.06 ✕ 10^8	US142 E. coli	3.76 ✕ 10^8
ATCC 25322 E. coli	8.3 ✕ 10^8	GS065 E. coli	6.3 ✕ 10^8
ATCC 12241 E. coli	1.65 ✕ 10^8	US00G P. Aeruginosa	8.3 ✕ 10^8
ATCC 12014 E. coli	3.5 ✕ 10^8	US115 P. Aeruginosa	2.4 ✕ 10^8
ATCC 13882 K. pneumoniae	1.2 ✕ 10^8	US131 K. pneumoniae	8.1 ✕ 10^8
ATCC 13883 K. pneumoniae	1.06 ✕ 10^8	GS008 K. pneumoniae	1.65 ✕ 10^8
ATCC 10125 P. Aeruginosa	8.1 ✕ 10^8	US055 Enterobacter cloacae	8.3 ✕ 10^8
ATCC 35422 P. Aeruginosa	1.2 ✕ 10^8		

3.3.3 Minimum inhibitory concentrations (MIC) using the broth microdilution method.

Broth microdilution was performed on a 96-well plate. 297 µl of sterile broth was added to the first well and 143 ul to the next wells. As per EUCAST guidelines 3 µl of meropenem at a final concentration of 1 mg/mL for lab strains, 25 mg/ml for clinical strains, and 80 mg/ml for resistant strains (or equivalent concentration of liposomal encapsulated meropenem) were added to the first well and (2-fold) serial dilutions to subsequent wells. Using 0.1 MacFarland OD with 1 x 10^8 CFUs, 7.5 ul of bacterial inoculum was added to each well. The 96-well plates were sealed and incubated at 35±1 °C for 16–18 hours. The MIC breakpoints were set up by determining the dilution at which a bacterial pellet was visible as per EUCAST recommendations (Giske et al., 2022b).

3.4 Bacterial fusion kinetics

Bacterial fusion kinetics is used to determine the interactions between non-PEGylated or PEGylated meropenem-encapsulated cationic liposomes, bacteria, and healthy volunteer immune cells. Particular interest is paid to the binding or fusion of liposomes with bacterial and immune cells to illustrate the efficiency and dynamics of liposome-bacteria and liposome-immune cell interactions.

Flow cytometry was used for the quantitative assessment of the interactions between fluorescent-labelled liposomes, bacteria, and immune cells by tracking changes in fluorescence intensity over time, showing the extent of liposome-bacteria and liposome-immune cell fusion. Confocal Laser Scanning Microscopy (CLSM) was used to visualize and analyze the interaction and fusion between labelled liposomes, bacteria, and immune cells.

3.4.1 Flow cytometry

Fusion kinetics was determined by incorporating a fluorescent lipid within the liposome (PE) and bacteria stained with the nuclear stain Hoechst. To isolate WBCs from the cell pellet, RBCs were lysed using one times red cell lysis buffer (Beckton Dickinson Biosciences, UK). Cells were washed and re-suspended in Hanks' buffered saline solution (HBSS, 5 ml, Gibco, UK). Immune cells were stained with e450 anti-CD45, a commonly used pan-leukocyte marker, (BD Biosciences) to identify the healthy volunteer white blood cells.

Fluorescent probe (PE)- labelled liposomal meropenem with variable cationic lipids (0%, 10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations were co-incubated with (Hoechst- labelled) Gram-negative bacteria and (e450 anti-CD45 labelled) healthy volunteer white blood cells. Differential fusion between liposomes, healthy volunteer immune cells and Gram-negative bacteria (A. Baumanni, E. Coli, K. Pneumoniae, and P. Aeruginosa were quantitatively assessed. Flow cytometry was used to measure the relative changes in PE-fluorescence of bacteria and immune cells over time compared to baseline values (i.e. suggestive of liposome fusion).

3.4.2 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was used to assess the fusion of liposomes labelled with Alexa 647 with bacteria and immune cells. Aggregation of liposomes is associated with increasing surface charge which can be minimised by PEGylation.

Non-PEGylated meropenem-encapsulated liposomes with variable cationic lipids (10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations were co-incubated with(Hoechst- labelled) Gram-negative bacteria and immune cells. These cells were stained with dihydroethidium (ThermoFisher) to assess reactive oxygen species and DAPI to identify cell nuclei. CLSM images were collected using a 60x oil objective mounted on an FV-1000 Olympus microscope. Solutions were deposited on a fluorodish (World Precision Instruments, Sarasota, FL, USA) and left to settle for 5 min before imaging. All imaging conditions were kept identical. Images were analyzed using ImageJ software. Experiments were conducted with the aid of Dr Arulkumaran.

3.5. Assessment of *in-vitro* biocompatibility of liposomal antibiotics

Experiments with human blood samples were approved by the UCL Research Ethics Committee (Reference: 11963/001). All experiments followed local procedures. Blood was withdrawn from a homogeneous group of healthy male and female volunteers ranging from 25-65 years with no active medical problems.

Whole blood was incubated with non-PEGylated and PEGylated meropenem-encapsulated liposomes with variable cationic lipids (10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations at different meropenem concentrations to assess *in-vitro* compatibility with immune cells.

3.5.1 Whole blood stimulation

10 ml of whole blood was collected from healthy volunteers and placed into heparinized blood tubes to prevent coagulation. The blood was diluted 1:1 with sterile PBS and 300 µL was added to each well within a 96-well plate. Liposomal meropenem or free meropenem was added to the blood to achieve final concentrations of 10 mcg/mL, 30 mcg/mL, or 100 mcg/mL to represent therapeutic and supra-therapeutic levels based on the literature. All *ex-vivo* whole-blood experiments were conducted at 37°C in a cell culture chamber. The blood was then incubated for 6 hours before being centrifuged at 1000 g for 10 minutes at room temperature. The plasma layer was collected for assessment of haemolysis and then stored at -80°C for analysis of released cytokines. The remaining cell pellet was processed for flow cytometry.

3.5.2 Flow cytometry

To isolate WBCs from the cell pellet, RBCs were lysed using 1x red cell lysis buffer (BD Biosciences, UK). Cells were washed and re-suspended in 5 mL Hanks' buffered saline solution while immune cells were stained with anti-CD45 (BD biosciences). DCFDA (final concentration 5 µM; Thermo, UK) was to assess reactive oxygen species, and the Far-red Live/ Dead stain (Thermo Fisher) to assess cell viability. After 30 minutes at 37°C flow cytometry was performed. Heat (65°C for 30 minutes) was used to kill cells as a positive control for cell viability.

Cells were analyzed using flow cytometry on the LSR Fortessa (BD) flow cytometer (BD Biosciences). Identical gates were applied to all samples. A minimum of 5000 events/measurements within the immune cell population were read. All data were collected from 4-8 individual replicates per experiment and the median fluorescence was assessed using FlowJo version 10.0 (Tree Star Inc, USA). Statistical data were analyzed using a non-parametric Mann-Whitney t-test using GraphPad Prism v5 (San Diego, USA). The FACS experiments were conducted by Dr Tim Snow.

3.5.3 ELISA

Commercially available ELISA kits were used to measure extracellular cytokines IL-6 and TNF-α (Human Duoset ELISA kit range, R&D Systems). Experiments were conducted following the manufacturer's protocols. ELISAs were confirmed by running standards and samples in duplicates for each experiment. The data were optimised by running variable dilutions (1:200, 1:150, 1:50 respectively) of serum.

A BMG Labtech plate reader was used to measure the optical density at 450 nm of each sample. MARS data analysis software was used to produce four-parameter logistic curves from the OD values of the standards and samples. Standard curves were constructed for each cytokine by plotting the mean absorbance for each standard on the y-axis against the concentration's OD on the x-axis.

3.5.4 *Ex-vivo* Endotoxin Neutralization

Peripheral blood was collected from healthy volunteers. Ten mL of whole blood was collected within heparinized blood tubes to prevent coagulation. Whole blood was diluted 1:1 with sterile PBS and 300 µL was added to each well within a 96-well plate along with a commercially prepared formulation of LPS (10 ng/mL) (MERCK). LPS and PBS were used as positive and negative controls. Liposomal meropenem and free meropenem were added to whole blood to achieve final concentrations of 10 µg/mL, and 30 µg/mL to represent therapeutic and supra-therapeutic levels. The blood was then incubated for 6 hours. Following 6 hours incubation, the samples were centrifugation at 1000 g for 10 minutes at room temperature. Serum was collected to measure the extracellular pro-inflammatory cytokines IL-6 and TNF-α (Human Duoset ELISA kit range, R&D Systems).

3.5.5 Haemolysis assay

Drug-induced haemolysis was assessed using the haemolytic assay activity (HAA) test by determining the absorbance of serum at 540 nm. Distilled water was added to whole blood (100% haemolysis), or healthy serum (0% haemolysis) in a 1:1 ratio. Serum from 100% haemolysis samples was serially diluted in healthy serum to create a standard curve (e.g., 10 µL serum from 100% haemolysis into 90 µL normal serum to give 10% haemolysis, etc).

3.5.6 Confocal Laser Scanning Microscopy

Effects of PEG on immune cell internalisation and ROS production were qualitatively assessed using CLSM. Cells were stained with dihydroethidium to measure reactive oxygen species, DAPI to identify cell nuclei, and Alexa 647 to identify liposomes. CLSM images were collected using a 60x oil objective mounted on an FV-1000 Olympus microscope as per Section 3.4.2.

3.6 Statistical analysis

Data were recorded in Office Excel (Microsoft, Redmond, WA, USA). Statistical analysis and graphical representation were performed using GraphPad Prism (Version 10 for Windows, GraphPad Software, La Jolla, CA, USA). For DLS or MIC data, where there was little or no variance between samples, parametric testing (t-test for 2 unpaired groups, or ANOVA for >2 groups) was used. For biological data (e.g., immune cell death or ROS production), non-parametric testing was used (Mann- Whitney- U test for 2 unpaired groups, Kruskal-

Wallis test for >2 unpaired groups or Friedman tests for >2 paired groups. Where >2 groups were compared, post-hoc multiplicity testing was not used as it reduced the statistical power of the analysis, making it more challenging to detect true differences between groups.

Summary

Non-PEGylated and PEGylated meropenem-encapsulated liposomes with varying cationic and DSPE-PEG 2000 lipid concentrations were synthesized by thin-film hydration method and characterized by dynamic light scattering (DLS) and high-performance liquid chromatography (HPLC) and *in-vitro* bactericidal testing using broth-microdilution method. To assess safety, *in-vitro* whole blood was incubated with non-PEGylated and PEGylated meropenem-encapsulated liposomes with variable cationic lipids (10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations at different meropenem concentrations, using flow cytometry, proinflammatory cytokines and haemolytic assay. Fusion kinetics was determined by incorporating a fluorescent lipid within the liposome (PE) with variable cationic lipids (0%, 10%, 20% and 30% DODAB) and DSPE-PEG 2000 (0.5%, and 2.5%) concentrations were co-incubated with (Hoechst- labelled) Gram-negative bacteria and (e450 anti-CD45 labelled) healthy volunteer white blood cells using flow cytometry and confocal laser scanning microscopy (CLSM) was used to assess the fusion and internalization of liposomes labelled with Alexa 647 in bacteria and immune cells.

4. Results

4.1 Validation of different experiment techniques (control)

4.1.1 Dynamic Light Scattering (DLS)

Assessment of varied sizes and charges of liposomes was performed using DLS. The thin film hydration method was able to create liposomes of predictable sizes and charges (**Figure 9 a, b, c**).

The desired sizes of meropenem-encapsulated liposomes were slightly bigger than expected, indicating some fusion and aggregation due to interaction between fusogenic lipids (**Figure 9a)**. These measurements typically involved both bilayer lipid contents and the amount of drug encapsulated within liposomes (**Figure 9a)**.

An increasing amount of DODAB incorporated into the liposome was associated with a higher positive charge (zeta potential) of the liposome (**Figure 9b, 9c**).

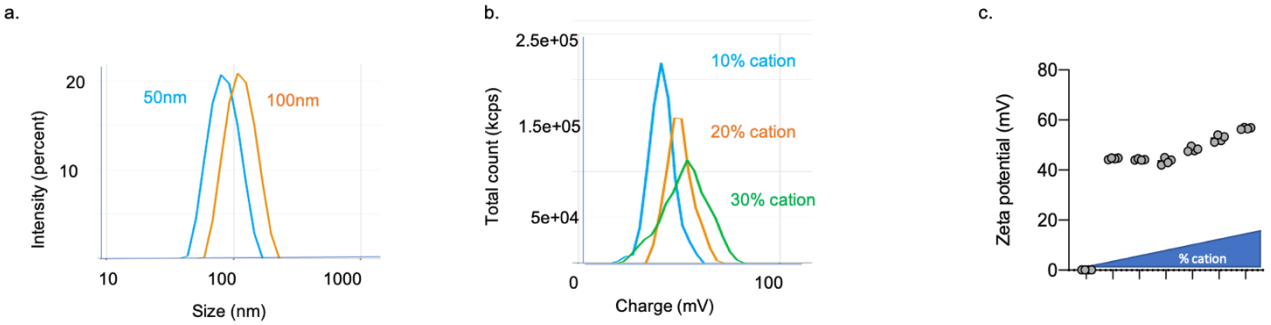

Figure 9: Graphical presentations of non-PEGylated and PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations. Using DLS. (a) the desired sizes were slightly bigger than expected due to fusion and aggregation between helper/fusogenic lipids. (b,c) the zeta potential corresponds with the cationic lipid concentration within the meropenem-encapsulated liposomes.

4.1.2 High-Performance Liquid Chromatography (HPLC)

The standard curve for free meropenem was performed at different peak absorbance values (230, 260, 290, and 300 nm) using HPLC (**Figures 10 a & b**). Values were plotted as area under the curve and area against free drug concentrations, accordingly (**Figure 10b**).

The peak absorbance of meropenem on HPLC was at 300 nm with a retention time of 2.8 ± 0.9 mins when dissolved in PBS (**Figs 10 a, b**). This enabled the creation of a standard curve with free meropenem concentrations ranging from 5-500 µg/mL using either the peak height or the area under the curve.

Meropenem dissolved in PBS was stable for 24 hours at 4°C. Meropenem concentrations fell significantly (up to 42%; p=0.009) when stored at 37°C (**Figure 10c**). Due to degradation of up to 25% of meropenem with sonication periods exceeding 15 mins, sonication times for incorporating meropenem within the liposomes were limited to 15 mins (**Figure 10 di**). Over 1 hour, meropenem remained stable in solution at different temperatures (between 4-50°C (**Figure 7dii**).

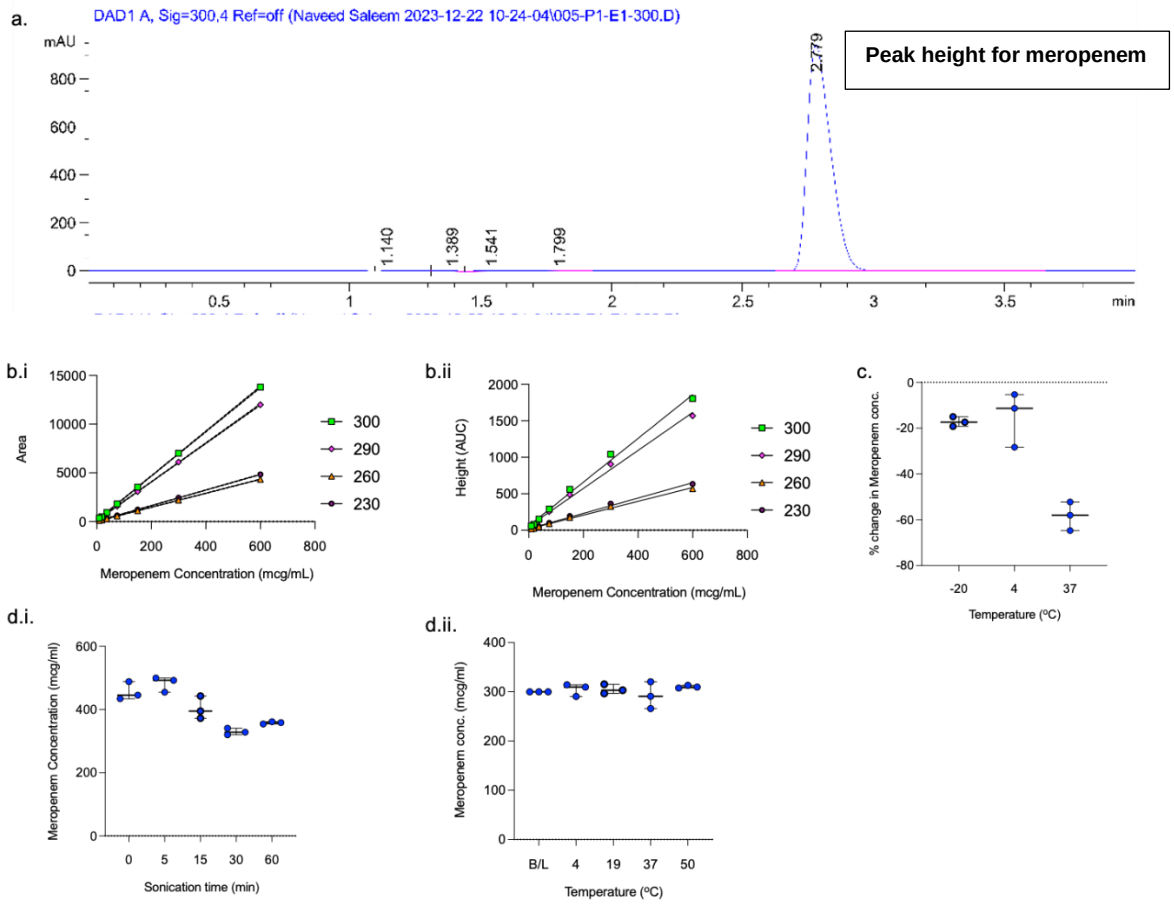

Figure 10: HPLC measurements (a) Meropenem peaked at a wavelength of 300 nm with a retention time of 2.8 mins. (b) Standard curve of meropenem at different wavelengths using the (bi) area under the curve and (bii) peak height of the curve. (c) Stability of meropenem dissolved in PBS at different temperatures over 24 hours. (d). Stability of meropenem with (di) different sonication times at room temperature and (dii) at different temperatures for one hour. Data presented as median for 24 hours meropenem stability and mechanical dispersion (sonication) effects (n=3).

4.1.3 FACS- Gating strategy. Single stains. ROS/ cell death controls. FSC and SSC.

FACS was used to assess the real-time fusion kinetics between liposomes and bacteria or immune cells. Immune cells show significantly higher baseline Mean Fluorescence Intensity (MFI) compared to bacterial cells. Therefore, the changes in MFI were represented as a fold-change compared to baseline Mean Fluorescence Intensity (MFI), rather than a direct comparison of mean fluorescence intensity (MFI) between bacteria and immune cells (**Figure 11**).

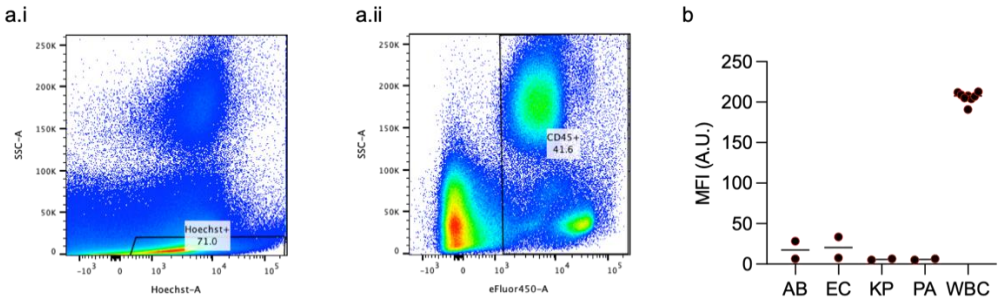

Figure 11: (a) FACS gating for identification of (ai) bacteria stained with Hoechst and (aii) human leukocytes stained with eFluoro 450- labelled CD45 antibody. (b) Background fluorescence (MFI; median fluorescence intensity) of different bacteria and human leukocytes.

4.1.4 ELISA standard curves and Haemolytic assays

Standard curves were generated using included commercially available standards, diluted as per the manufacturer's instructions, and interpolated using GraphPad Prism (**Figures 12 & 13**). Both standards and samples were analysed in duplicate. The coefficient of variation between each sample in the duplicate was <10%.

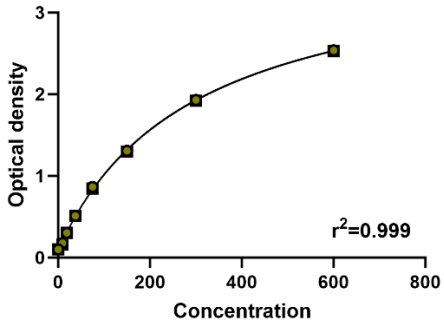

Figure 12: Example standard curve of IL-6 concentration. The x-axis represents concentrations for IL-6 standards and the y-axis is the measured optical density (O.D).

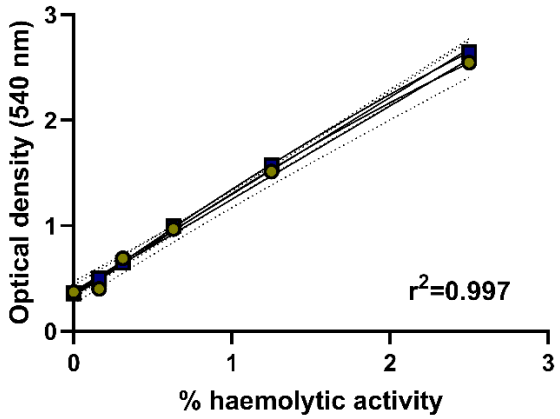

Figure 13: Example of the standard curve for haemolytic assay activity. The x-axis represents the percentage of haemolytic activity, and the y-axis is the measured optical density (O.D) of the serum samples.

4.2 Characterisation of non-PEGylated liposomes

4.2.1 DLS findings

Meropenem-encapsulated liposomal formulations with variable cationic lipid concentrations i.e., ranging from 0-40% DODAB were synthesized by the thin-film hydration method and analysed using DLS and HPLC (**Table 4** & **Figure 14**).

The measured liposome size was consistently higher than expected, showing fusion and aggregation due to interactions between fusogenic lipids. This typically measures both bilayer lipid contents, and the amount of drug encapsulated within liposomes. This ranged from between 55-88 nm for 50 nm liposomes,106-122 nm for 100 nm liposomes, and 216-226 nm for 200 nm liposomes, respectively (**Table 4 & Figure 14)**.

An increasing concentration of the cationic lipid DODAB was associated with a higher zeta potential within the liposomes. i.e., the higher the cationic lipid content, the stronger the positive charge over the liposomal surface. The liposomal formulation without a cationic lipid (PC: DODAB: Chol (7:0:3)) had no charge while liposomes with 40% cationic lipids demonstrated a high positive charge of +58 mV (**Table 4 & Figure 14)**.

The polydispersity index (PDI) determines homogeneity within the size of a particle solution. The calculated PDI values varied between 0.04-0.1 identifying reasonable homogeneity of the cationic liposomal formulations (**Table 2).**

4.2.2 HPLC Findings

The percentage of internal meropenem is the amount of meropenem encapsulated within the liposomes. It varied from 37-81% depending upon the different lipid proportions (**Table 4**). Liposomal formulations with higher cationic lipid contents were associated with lower internal meropenem concentrations, indicating poor stability, fluidity, and membrane permeability.

EE (%) ranged from 0.1 to 3.8% (**Table 4**). Liposomal formulations with higher cationic lipid contents showed lower total drug concentrations as compared to liposomes with either lower or moderate cationic lipid concentrations (**Table 4**).

PC: DODAB: Chol (molar ratio)	Size (nm)	Measured size (nm)	Zeta potential (mV)	Polydispersity index (PDI)	Internal meropenem (%)	E.E (%)	Drug concentration (µg/mL)
7.0.3	50	79 ± 1	0.1 ± 0.0	0.08 ± 0.004	43 ± 1.0	1.9±0.1	467 ± 35
7.0.3	100	119 ± 3	0.5 ± 0.1	0.04 ± 0.01	46 ± 1.7	3.0±0.2	734 ± 57
6.1.3	50	79 ± 1	44 ± 0.2	0.06 ± 0.01	54 ± 1.9	2.1±0.3	554 ± 76
6.1.3	100	114 ± 3	46 ± 0.5	0.05 ± 0.01	56 ± 2.4	3.0±0.1	762 ± 44
5.1.4	50	88 ± 1	44 ± 0.2	0.12 ± 0.01	50 ± 5.3	0.1±0.05	130 ± 15
5.1.4	100	114 ± 3	45 ± 0.4	0.07 ± 0.01	46 ± 2.2	0.5±0.04	132 ± 12
5.1.4	200	211 ± 2	47± 0.9	0.06± 0.01	56 ± 2.2	0.8±0.04	230 ± 13
5.2.3	50	55 ± 9	44 ± 1	0.05 ± 0.01	70 ± 6.5	2.4±0.1	612 ± 41.1
5.2.3	100	106 ± 2	46 ± 1	0.05 ± 0.01	70 ± 6.1	3.7±0.1	740 ± 35
5.2.3	200	216 ± 2	47 ± 2	0.06 ± 0.003	58 ± 13	3.8±0.08	788 ± 9.1
5.3.2	50	81 ± 5	48 ± 1	0.05 ± 0.01	59 ± 4.9	0.4±0.09	135 ± 28.4
5.3.2	100	122 ± 3	52 ± 3	0.07 ± 0.01	58 ± 1.7	1.2±0.2	314 ± 62.2
5.3.2	200	218 ± 2	54± 1	0.06 ± 0.01	60 ± 5.9	2.1±0.2	416 ± 62.4
3.3.4	50	87 ± 2	53 ± 1	0.05 ± 0.02	81 ± 0.6	2.5±0.2	625 ± 70.4
3.3.4	100	115 ± 3	54 ± 3	0.06 ± 0.01	74 ± 3.3	3.3±0.1	831 ± 47
3.3.4	200	218 ± 3	54 ± 1	0.06 ± 0.004	77 ± 6.3	3.4±0.3	883 ± 24
5.4.1	50	76 ± 1	57 ± 0.3	0.09 ± 0.01	37 ± 0.9	0.3±0.1	173 ± 26.2
5.4.1	100	117 ± 1	58 ± 0.4	0.05 ± 0.01	67 ± 1.1	0.5±0.04	134 ± 12.3
5.4.1	200	226 ± 1	59 ± 0.4	0.06 ± 0.001	69 ± 1.2	0.3±0.06	287 ± 58

Table 4: Synthesis and characterisation of twenty different non-PEGylated meropenem-encapsulated cationic liposomal formulations (DLS and HPLC). Cationic liposomal formulations (PC: DODAB: CHOL) encapsulating meropenem had positive charges ranging from no charge (0 mV) to highly positive (+58 mV). The intended sizes were slightly larger than the intended sizes i.e., 50, 100, 200 nm. The Polydispersity Index (PDI) quantifies the non-uniformity of the size distribution of particles ranging from 0.04 to 0.1 for a perfectly uniform sample regarding particle size). Percentage (%) internal meropenem is the amount of meropenem within liposomes. This ranged from 37- 81% with total drug concentrations dependent upon the physicochemical properties of the cationic liposomal formulations. Data presented as median ± SD (n=4).

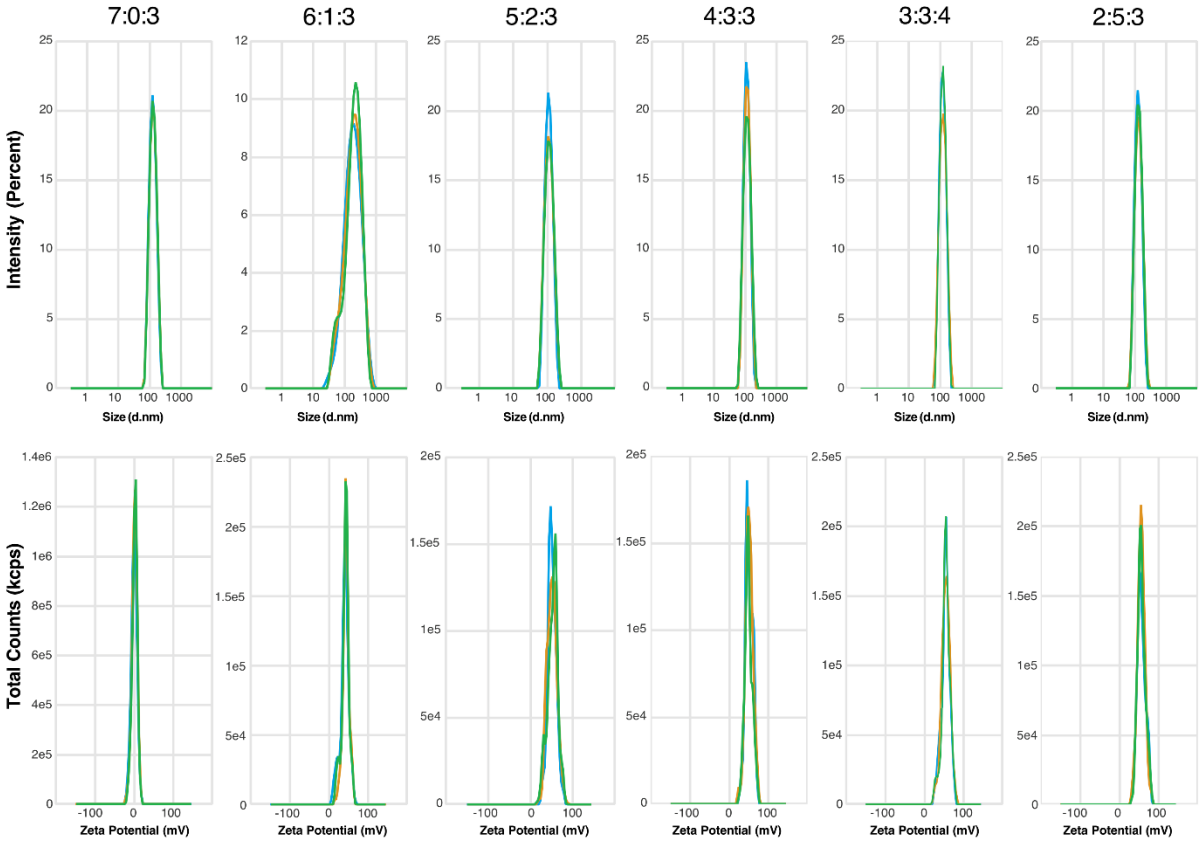

Figure 14: Graphical presentations of the size and charge of different non-PEGylated meropenem-encapsulated cationic liposome formulations containing variable cationic lipid concentrations measured using DLS. The desired sizes were slightly bigger than the intended sizes i.e., 100 nm due to high flexibility and interactions between fusogenic lipids. The zeta potential corresponded to the cationic lipid concentration present within the meropenem-encapsulated liposomes. The liposomal formulations PC: DODAB: Chol (7:0:3) without cationic lipids showed no charge, while liposomes with 40% cationic lipids PC: DODAB: Chol (5:4:1) had a high charge (+58 mV) over the liposomal surface. Three measurements were calculated for each sample to ensure reproducibility, indicated by different colours within the graphs.

4.3 Efficacy of non-PEGylated cationic liposome antibiotic

Specific formulations of non-PEGylated meropenem-encapsulated liposomes with variable cationic lipid contents have higher bactericidal activity than the corresponding free antibiotic against different laboratory strains of Gram-negative bacteria.

4.3.1 *In-vitro* bactericidal activity against lab gram-negative strains

The *in-vitro* bactericidal activity of non-PEGylated meropenem-encapsulated cationic liposomal formulations was assessed against laboratory strains of Gram-negative bacteria. Controls included free meropenem, liposomes without encapsulated meropenem (empty liposomes), and non-encapsulated liposomes (empty liposomes) with free external meropenem.

Liposomal formulations with different cationic lipid concentrations i.e., 10%, 20% and 30% DODAB demonstrated enhanced bactericidal activity against laboratory strains of Gram-negative bacteria (**Table 5, Figure 15**). Liposomal formulations (PC: DODAB: Chol) with variable cationic lipid concentrations such as (6:1:3), (5:2:3) and (3:3:4) illustrated a 2-30-fold reduction in MICs in comparison to standard free meropenem (**Table 5, Figure 15**).

Several liposomal meropenem formulations reduced the MIC of Gram-negative bacteria compared to free meropenem (**Table 5, Supplemental Figures 1-7**). A smaller liposome size (50 nm) was associated with lower MIC (**Table 5, Supplemental Figures 1-7**). There was no clear association between increasing the percentage of cationic DODAB and the MIC (**Table 5, Supplemental Figures 1-7**). Based on these data, three liposomal formulations with increasing DODAB concentration (6.1.3; 5.2.3; 3.3.4) were taken forward for further testing.

Formulation	Size (nm)	AB 19605	EC 12014	EC 12241	KP 13882	KP 13883	PA 10145	PA 35422
Free meropenem		0.63	0.63	0.07	0.63	0.63	0.63	2.5
7.0.3	**50**	0.11	0.1225	0.12	0.12	0.68	0.73	0.68
7.0.3	**100**	0.93	0.2	0.2	0.2	0.2	0.22	0.44
6.1.3	**50**	0.35	0.02	0.02	0.02	0.02	0.02	0.02
6.1.3	**100**	0.3	0.06	0.06	0.1	0.08	0.06	0.06
5.1.4	**50**	0.35	0.35	0.035	0.295	0.295	0.685	0.675
5.1.4	**100**	0.37	0.4	0.0395	0.385	0.375	0.86	0.925
5.1.4	**200**	0.4	0.45	0.36	0.4	0.46	1.1	1.1
5.2.3	**50**	0.05	0.0225	0.01	0.0375	0.05	0.05	0.075
5.2.3	**100**	0.1	0.1	0.02	0.1	0.07	0.07	0.5
5.2.3	**200**	0.3	0.49	0.0315	0.525	0.465	0.555	1.53
5.3.2	**50**	0.1	0.03	0.01	0.09	0.015	0.04	0.45
5.3.2	**100**	0.225	0.1125	0.023	0.27	0.08	0.09	0.7
5.3.2	**200**	0.5	0.475	0.07	0.325	0.475	0.45	0.78
3.3.4	**50**	0.08	0.02	0.02	0.03	0.02	0.02	0.04
3.3.4	**100**	0.09	0.04	0.03	0.04	0.03	0.06	0.06
3.3.4	**200**	0.35	0.4	0.06	0.3	0.4	0.4	1.25
5.4.1	**50**	0.31	0.31	0.032	0.22	0.23	0.1	0.36
5.4.1	**100**	0.41	0.43	0.04	0.37	0.335	0.25	0.625
5.4.1	**200**	0.47	0.47	0.375	0.43	0.43	0.485	1.5

Table 5: Heatmap of MICs of free meropenem (top row) and non-PEGylated meropenem-encapsulated cationic liposomes against different lab strains of Gram-negative bacteria i.e., AB Acinetobacter, EC E coli, K.P Klebsiella pneumoniae, PA Pseudomonas aeruginosa. Horizontal blue bars denote the amount of free and cationic liposomal encapsulated meropenem required to inhibit visible in-vitro growth of Gram-negative micro-organisms. Sizes of blue horizontal bars are proportional to the amount of antibiotic needed for MIC i.e., cationic liposomes showing smaller blue horizontal bars demonstrate lower MICs in comparison to free meropenem. Data presented as median (n=4).

Empty liposomes did not inhibit bacterial growth. The combination of empty liposomes with external meropenem had marginally lower MIC values compared to free meropenem although this depended on the liposomal formulation and bacteria assessed (**Table 6, Supplemental Figure 10**).

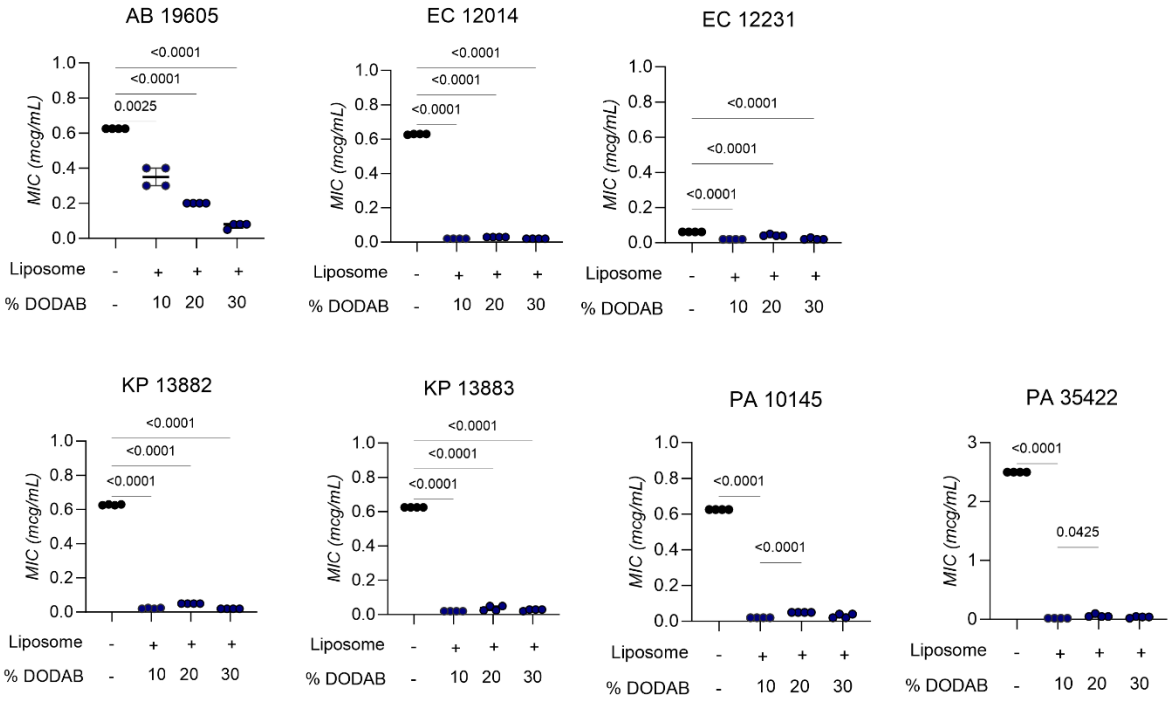

Figure 15: MICs of free and non-PEGylated meropenem encapsulated cationic liposomes against different lab strains of Gram-negative bacteria i.e., AB Acinetobacter, EC E. coli, K.P Klebsiella pneumoniae, PA Pseudomonas aeruginosa. ANOVA with post-hoc pairwise comparisons was used to compare differences between liposomal formulations and free meropenem. Data presented as median (n=4).

	AB 19605	EC12014	EC 12241	KP 13882	KP138833	PA 10145	PA 35422
Free meropenem	0.63	0.63	0.07	0.63	0.63	0.63	2.5
6.1.3	0.315	0.315	0.03	0.63	0.63	0.63	2.5
5.2.3	0.315	0.315	0.07	0.315	0.63	0.63	2.5
3.3.4	0.315	0.315	0.07	0.315	2.5	0.63	2.5

Table 6: MICs heatmap of free meropenem (top row) and external meropenem along with non-encapsulated cationic liposomes against different lab strains of Gram-negative bacteria i.e., AB Acinetobacter, EC = E coli, K.P = Klebsiella pneumoniae, PA Pseudomonas aeruginosa. The size of the horizontal blue bars is proportional to the MIC. Data presented as median (n=4).

4.4 Stability over 24hrs for non-PEGylated liposomes

Non-PEGylated meropenem-encapsulated liposomes prepared by the thin-film hydration method showed variable physiochemical properties at different time points at 4°C over 24 hours, as compared to free standard meropenem.

4.4.1 DLS findings

Meropenem-encapsulated liposomes with variable cationic lipid concentrations were stored at 4°C for 24 hours to assess the physiochemical properties of the cationic liposomes using DLS and HPLC (**Table 7, Supplemental Figures 8 & 9).** The measured vesicle size for 50 nm liposomes varied from 50-89 nm and 107-120 nm for 100 nm liposomes ($p>0.05$ for all) (**Table 7 & Supplemental Figures 8 & 9).** Over 24 hours, there was a gradual drop in mean charge depending on the DODAB concentration, though this was not statistically significant ($p>0.05$ for all) **Table 7 & Supplemental Figures 8 & 9).** Calculated PDI values varied from 0.057-0.075 after 24 hours showing homogeneity of the cationic liposomal formulations at each size.

4.4.2 HPLC Findings

There was a slight reduction in (%) internal meropenem for 6.1.3 (50 nm) vesicles of up to 7%, and up to 14% for 5.2.3 (100 nm) respectively over 24 hours. This demonstrates some charge-dependent effects, but these differences were not statistically significant ($p>0.05$ for all) (**Table 7, Supplemental Figures 8 & 9).**

Over 24 hours, EE% dropped from 4.8% to 2.3% for 50 nm, and from 4.8% to 2.7% for 100 nm, respectively. However, the drop within meropenem concentrations encapsulated within the liposomes was statistically insignificant ($p>0.05$ for all), but consistent with the literature in comparison to free meropenem (**Table 7, Supplemental Figures 8 & 9).**

Liposomes with a stronger positive charge (3:3:4) had higher total meropenem concentrations -1691 µg/mL with 50 nm liposomes and 1730 µg/mL with 100 nm liposomes - as compared to formulations with a lower charge ($p=0.03$) (**Table 6**). Total drug concentrations fell (1620 and 1641 µg/mL) for the cationic liposomal formulations with 30% DODAB over 24 hours (**Table 7, Supplemental Figures 8 & 9**).

There were slight differences in the measured size, charge, PDI and internal meropenem concentration over 24 hours, but these findings were not statistically significant ($p>0.05$ for all). Although total meropenem concentration was consistently lower at 24 hours, it did not reach statistical significance (**Table 7, Supplemental Figures 8 & 9).**

PC: DODAB: Chol	Size	Measured size (nm)	Zeta potential (mV)	PDI	Internal meropenem (%)	EE (%)	Drug concentration (mcg/mL)
6:1:3	**50**						
0 hr		79 ± 4	45 ± 0.3	0.060 ± 0.008	75 ± 5	2.4±1.2	615 ± 18.8
24 hr		80 ± 4	46 ± 0.3	0.065 ± 0.005	68 ± 5	2.3±1.1	569 ± 24.2
6:1:3	**100**						
0 hr		111 ± 2	46 ± 0.5	0.070 ± 0.005	55 ± 6	2.8±0.1	724 ± 31
24 hr		112 ± 2	47 ± 0.9	0.075 ± 0.005	60 ± 7	2.7±0.05	692 ± 13
5:2:3	**50**						
0 hr		50 ± 9	44 ± 1.6	0.052 ± 0.019	74 ± 9	3.1±0.4	543 ± 48
24 hr		59 ± 14	40 ± 2.4	0.057 ± 0.019	78 ± 8	2.9±0.3	490 ± 36
5:2:3	**100**						
0 hr		107 ± 2	46 ± 0.7	0.044 ± 0.026	74 ± 6	3.7±0.1	941 ± 26
24 hr		107 ± 2	41 ± 0.8	0.058 ± 0.010	58 ± 8	3.5±0.1	875 ± 49
3:3:4	**50**						
0 hr		87 ± 2	53 ± 3.8	0.074 ± 0.021	81 ± 10	4.8±0.8	1691 ± 294
24 hr		89 ± 1	51 ± 2.7	0.065 ± 0.018	82 ± 11	4.6±0.8	1620 ± 300
3:3:4	**100**						
0 hr		117 ± 3	53 ± 3.5	0.060 ± 0.011	69 ± 10	4.9±0.4	1730 ± 158
24 hr		120 ± 4	51 ± 4.8	0.065 ± 0.020	77 ± 6	4.6±0.8	1641 ± 380

Table 7: 24-hour stability study for non-PEGylated meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) stored at 4°C. Size, charge, PDI, % internal meropenem and total drug concentration were measured at 0 and 24 hours to assess physiochemical properties. Paired t-test was used to assess differences between baseline and 24 hours. Data presented as mean ± SD (n=4).

4.5 Binding kinetics studies

Non-PEGylated meropenem-encapsulated liposomes with higher cationic lipid concentrations demonstrated rapid fusion between cationic liposomes, and bacterial and immune cells as compared to liposomes with either no or minimal cationic lipid content.

A qualitative assessment of the effect of liposomal charge on the real-time fusion between healthy volunteer immune cells or bacteria with non-PEGylated liposomes *in-vitro* was performed using flow cytometry (**Figures 16 & 17**). There was no fusion of uncharged liposomes with bacteria or WBC. (**Figures 16 & 17**). The fusion of cationic liposomes with healthy volunteer immune cells or bacteria occurred within seconds. (**Figures 16 & 17**). A modest degree of binding was evident between liposomes containing 10% DODAB, bacteria and WBCs (**Figure 16**). Fusion between bacteria, WBCs and liposomes increased with increasing liposomal charge (liposomes containing 20% or 30% DODAB) (**Figure 16**).

Using the same dataset, I assessed whether the binding between liposomes was greater with immune cells or bacteria (Figure **17**). Liposomes having 20% or 30% DODAB demonstrated similar or greater binding to immune cells compared to bacteria. In contrast, liposomes containing 10% DODAB demonstrated similar or less avid binding to immune cells compared to bacteria.

Fusion between liposomes and bacteria varied by the type of bacteria. Fusion between A Baumanni and E coli was more apparent with liposomes containing 30% DODAB compared to other liposomes. Up to a 5-fold relative increase in fusion was evident with liposomes containing 30% DODAB to A Baumanni and E coli compared to other liposomes. In contrast, with K Pneumoniae and P. Aeruginosa, liposome fusion was similar between liposomes containing 20% or 30% DODAB; and lower compared to A Baumanni and E coli.

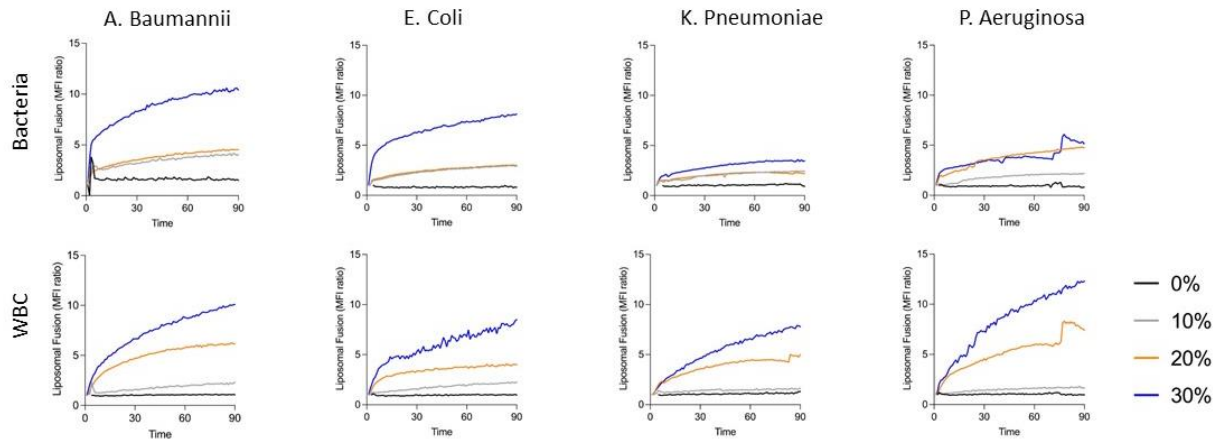

Figure 16: Fluorescent probe (PE)-labelled non-PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations (0%, 10%, 20% and 30% DODAB) were co-incubated with (a) (Hoechst-labelled) Gram-negative bacteria (A Baumanni, E coli, K pneumoniae, and P aeruginosa) and (b). (e450 anti-CD45 labelled) healthy volunteer white blood cells (WBCs). Fusion between liposomes with 0% DODAB (black line), 10% DODAB (grey line), 20% DODAB (orange line), and 30% DODAB (blue line) was assessed. Datapoints indicate the relative change in PE-fluorescence of bacteria or immune cells over time compared to baseline values (suggestive of liposome fusion). A total of four replicates were performed with the line representing the median.

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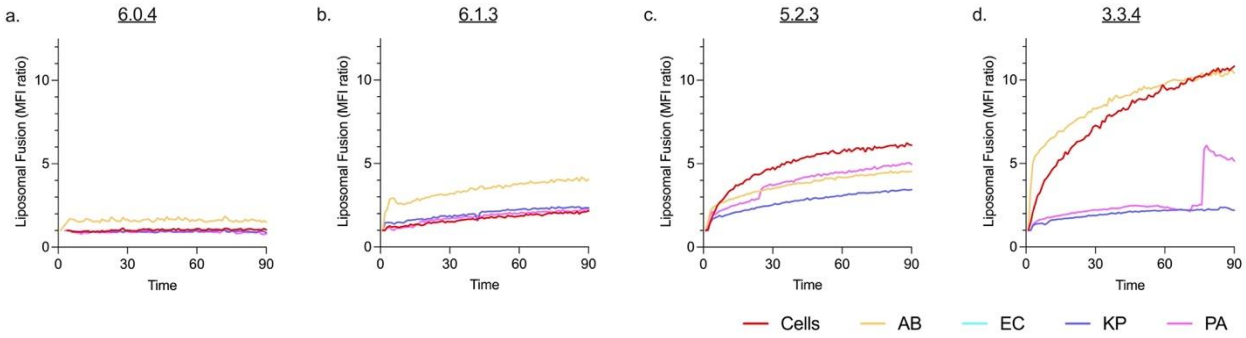


Figure 17: Fluorescent probe (PE)-labelled non-PEGylated meropenem encapsulated with variable cationic lipid concentrations (0%, 10%, 20% and 30% DODAB) were co-incubated with (Hoechst-labelled) Gram-negative bacteria and (e450 anti-CD45 labelled) healthy volunteer white blood cells. Differential fusion between liposomes and healthy volunteer immune cells (red line), A Baumanni (yellow line), E. Coli (blue line), K pneumoniae (purple line), and P. aeruginosa (pink line) were quantitatively assessed. Datapoints reveal the relative change in PE-fluorescence of bacteria or immune cells over time compared to baseline values (suggestive of liposome fusion). A total of four replicates were performed with the line representing the median value.

4.6 *In-vitro* biocompatibility of non-PEGylated liposomes (FACS, ELISA, Haemolytic assay)

Host cytotoxicity and inflammation resulting from bacterial death will be lower for non-PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations than with free antibiotic treatment because these liposomal formulations bind and neutralize bacterial toxins.

Healthy volunteer whole blood was incubated *in-vitro* with liposomal meropenem or equivalent doses of free meropenem for 6 hours at 37°C. The effects of non-PEGylated meropenem-encapsulated cationic liposomal formulations on healthy volunteer immune cell fusion, ROS production, and death were assessed using flow cytometry. Released cytokines were measured by ELISA. Safety against erythrocytes was assessed using a haemolysis assay. (**Figure 18**)

Minimal immune cell fusion occurred between liposomes with 10% DODAB, even at supratherapeutic (100 mcg/mL) concentrations of meropenem (**Figure 18 aii**). With increasing dose and/or charge, the fusion between immune cells increased significantly ($p<0.001$) (**Figure 18 aii**). At low, medium, and high doses, fusion was significantly lower between 6:1:3 liposomes compared to 5:2:3 or 3:3:4 liposomes ($p<0.01$ for all) (**Figure 18 aii**). There was no statistically significant difference in immune cell fusion between 5:2:3 and 3:3:4 liposomes at low, medium, or high doses ($p>0.05$ for all) (**Figure 18 aii**).

There was a significant effect of liposome formulation ($p=0.006$) and a strong trend towards a dose effect ($p=0.057$) on immune cell ROS production (**Figure 18 b**). At high dose (100 mcg/mL), 3:3:4 liposomes were associated with increased immune cell ROS production compared to equivalent doses of free meropenem ($p=0.001$), 6:1:3 liposome ($p<0.001$), and 5:2:3 liposome ($p=0.005$).

There was a significant effect of liposome formulation ($p<0.001$) and dose ($p=0.002$) on immune cell death (**Figure 18 c**). This was not evident at a low dose (10 mcg/mL) of meropenem. At medium ($p<0.05$) and high ($p<0.001$) dose meropenem, 3:3:4 liposomes were associated with greater immune cell death compared to free meropenem, 6:1:3 and 5:2:3 liposomes.

Incubation of liposomes in whole blood did not elicit any significant pro-inflammatory cytokine (IL-6 or TNF-a) release, nor haemolysis (**Figure 18 di, dii, e**)

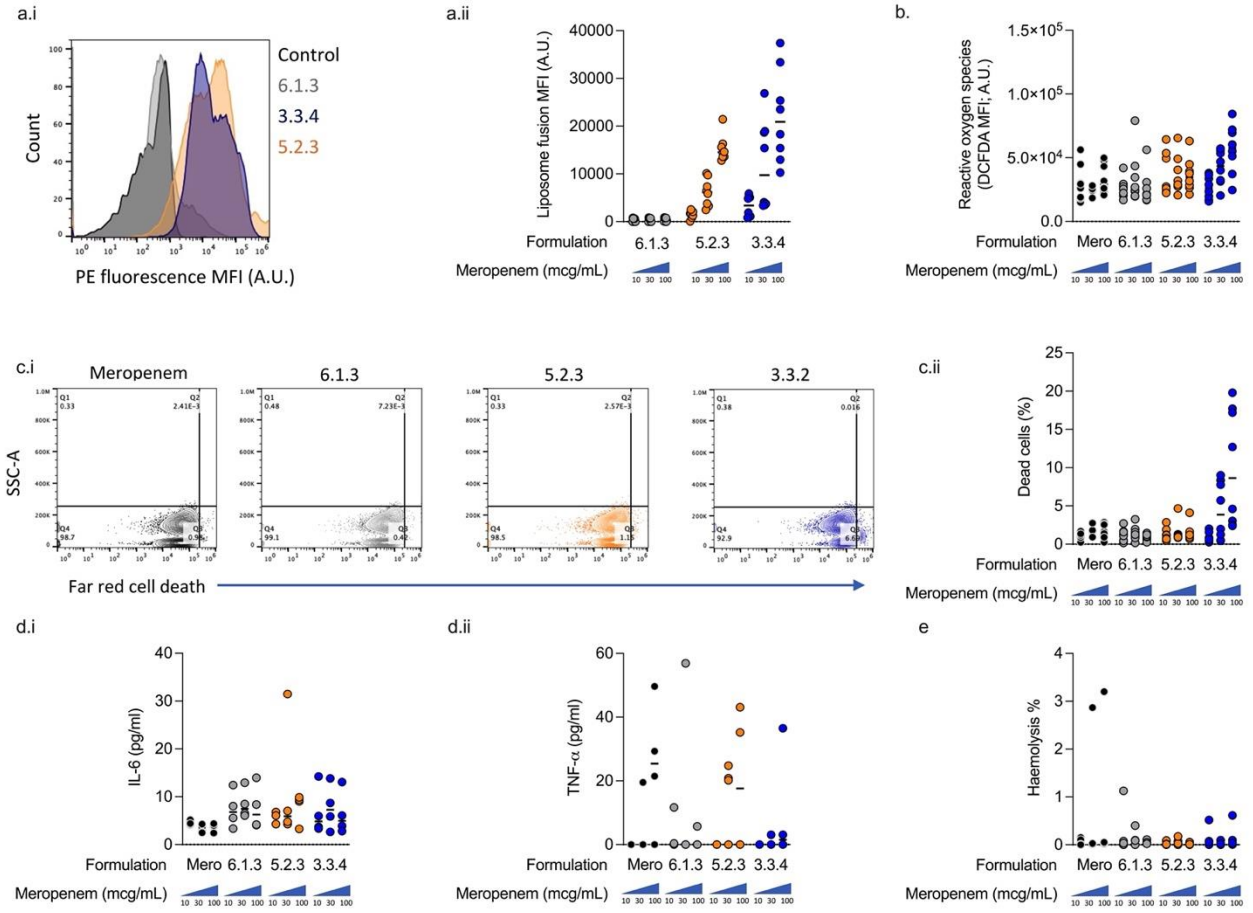


Figure 18: In-vitro safety assessment of non-PEGylated meropenem-encapsulated cationic liposomes at therapeutic (10 and 30 µg/mL) and supra-therapeutic (100 µg/ml) meropenem concentrations were co-incubated for 6 hrs with healthy volunteer whole blood at 37°C. Comparisons were made to free meropenem at equivalent concentrations. (ai-ii). Liposome fusion to immune cells is associated with higher surface charge and increasing doses. (b) Immune cell reactive oxygen species associated with liposome charge and dose. (ci-ii). The liposome with the highest charge (3:3:4) was associated with more immune cell death, especially at higher doses. (di-ii). Pro-inflammatory cytokine production associated with liposomes was minimal, as was (e) haemolysis. Experiments represent data from 4-8 biological replicates. The Friedman test was used to assess differences between doses and formulations.

5. Effect of PEGylation on liposome safety and efficacy

5.1 Characterisation of PEGylated liposomes

PEGylated meropenem-encapsulated liposomes formulated using the thin-film hydration method will show variable physiochemical properties depending upon cationic and DSPE-PEG 2000 lipid concentrations as compared to free standard meropenem.

5.1.1 DLS findings

As immune cell fusion and toxicity were seen with increasing liposomal charge, 50 nm liposomes were incorporated with 0.5% or 2.5% surface DSPE-PEG 2000. Meropenem-encapsulated liposomal formulations with variable cationic and PEGylated lipid concentrations were analysed using DLS to assess the effects of pegylation on the physiochemical properties of the liposomes.

With all three liposomal formulations, there was a significant reduction in surface charge associated with increasing amounts of surface PEGylation ($p<0.001$ for all 3 liposome formulations) (**Table 8, Supplemental Figure 11**). There were differences in the measured size of 6:1:3 and 5:2:3 liposomes with the addition of 0.5% or 2.5% PEG compared to non-PEGylated liposomes ($p<0.05$), but not 3:3:4 liposomes (**Table 8 & Supplemental Figure 11**). In contrast, PDI increased with increasing amounts of surface PEGylation for 3:3:4 liposomes ($p<0.001$), but not for 6:1:3 ($p=0.529$) or 5:2:3 liposomes ($p=0.63$) (**Table 8 & Supplemental Figure 11**).

5.1.2 HPLC Findings

Increasing PEGylation was associated with a reduction in the percentage of internal meropenem ($p<0.01$ for all 3 liposome formulations) and encapsulation efficacy ($p<0.05$ for all 3 liposome formulations) (**Table 8 & Supplemental Figure 11**). Meropenem drug concentration was significantly lower with 2.5% PEGylated liposomes compared to non-PEGylated 6:1:3 liposomes ($p=0.015$), thus showing poor drug penetration (**Table 8 & Supplemental Figure 11**). In contrast, 0.5% ($p=0.009$) and 2.5% ($p=0.017$) PEGylated 5:2:3 liposomes had higher drug concentrations compared to non-PEGylated liposomes) (**Table 8, Supplemental Figure 11**). PEGylation did not affect the drug concentration of 3:3:4 liposomes) (**Table 8, Supplemental Figure 11**).

PC: DODAB: Chol	PEG %	Measured size (nm)	Zeta potential (mV)	PDI	Particle concentration	Internal meropenem (%)	E.E (%)	Drug concentration (µg/mL)
6:1:3	**0%**	79 ± 1	44 ± 0.2	0.060 ± 0.008	$8.4 * 10^{11}$	75 ± 5	2.4 ± 0.09	568 ± 129
	0.5%	69 ± 2	35 ± 0.5	0.055 ± 0.007	$1.39 * 10^{12}$	53 ± 3	2.2 ± 0.2	558 ± 69
	2.5%	60 ± 0	8 ± 0.1	0.098 ± 0.039	$4.7 * 10^{12}$	48 ± 6	2.6 ± 0.02	539 ± 44
5:2:3	**0%**	50 ± 9	44 ± 2.0	0.052 ± 0.019	$7.28 * 10^{11}$	74 ± 6	2.8 ± 0.07	658 ± 29
	0.5%	73 ± 5	33 ± 0.3	0.100 ± 0.021	$5.3 * 10^{11}$	48 ± 2	2.5 ± 0.1	639 ± 45
	2.5%	67± 2	21 ± 0.2	0.100 ± 0.070	$1.68 * 10^{12}$	44 ± 5	2.4 ± 0.1	630 ± 42
3:3:4	**0%**	87 ± 2	53 ± 4	0.074 ± 0.021	$2.5 * 10^{11}$	81 ± 10	5.9 ± 1.2	1492 ± 303
	0.5%	85 ± 0	40 ± 0.4	0.075 ± 0.007	$2.4 * 10^{11}$	68 ± 1	5.8 ± 1.9	1457 ± 477
	2.5%	83 ± 2	15 ± 0.2	0.100 ± 0.900	$6.2 * 10^{11}$	48 ± 1	5.3±1.8	1350 ± 453

Table 8: Physiochemical properties of non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations prepared by the thin-film hydration method with variable DSPE-2000 concentrations i.e., 0.5% and 2.5%, respectively measured by DLS, and HPLC. ANOVA with post-hoc pairwise comparisons was used to assess differences between PEGylated and non-PEGylated liposomes. Data presented as median ± SD (n=4).

5.2 Stability over 24hrs for PEGylated liposomes

PEGylated meropenem-encapsulated liposomes prepared by the thin-film hydration method showed variable physiochemical properties at different time points at 4°C over 24 hours, as compared to free standard meropenem.

5.2.1 DLS findings

PEGylated meropenem-encapsulated liposomes with variable cationic and DSPE-PEG 2000 lipid concentrations were stored at 4° over 24 hours to assess the physiochemical properties of the cationic liposomes using DLS and HPLC (**Table 9).**

Compared to non-PEGylated liposomes there were differences in the measured size of 6:1:3 and 5.2.3 liposomes with the addition of 0.5% or 2.5% PEG ($p<0.05$), but not 3:3:4 liposomes (**Table 9, Supplemental Figures 12-14**). The measured size of liposomal formulations was slightly higher than expected (**Table 9, Supplemental Figures 12-14).**

With all three liposomal formulations, there was a significant reduction in surface charge associated with increasing amounts of surface PEGylation ($p<0.001$ for all 3 liposome formulations) (**Table 9 & Supplemental Figures 12-14**). The zeta potential corresponded with the cationic lipids present within non-PEGylated as opposed to PEGylated liposomal formulations. Over 24 hours, there was a gradual drop in the mean charge of up to 5-6% over the liposomal surfaces.

In contrast, PDI increased with increasing amounts of surface PEGylation for 5:2:3 liposomes ($p<0.001$) and 3:3:4 liposomes ($p<0.001$), but not for 6:1:3 ($p=0.529$) or (**Table 9, Supplemental Figures 12-14).**

5.2.2 HPLC Findings

Higher concentrations of DSPE-PEG 2000 were associated with lower total drug concentrations (1670 µg/ml) as compared to non-PEGylated formulations (1705 µg/ml), but these differences were not statistically significant **(Table 9, Supplemental Figures 12-14).** Increasing PEGylation was associated with a reduction in % internal meropenem ($p<0.01$ for all 3 liposome formulations) and encapsulation efficacy ($p<0.05$ for all 3 liposome formulations) (**Table 9, Supplemental Figures 12-14**).

The EE% was significantly lower with 2.5% PEGylated liposomes compared to non-PEGylated or PEGylated liposomes with 0.5% DSPE-PEG 2000 ($p=0.01$) (**Table 9, Supplemental Figures 12-14**).

In summary, there were differences in the measured size, charge, PDI and internal meropenem concentration over 24 hours, but these differences were not statistically significant ($p>0.05$ for all). Although total meropenem concentrations were consistently lower at 24 hours, they did not reach statistical significance (**Table 9, Supplemental Figures 12-14).**

PC: DODAB: Chol PEG %	Time	Measured size (nm)	Zeta potential (mV)	PDI	Internal meropenem (%)	E.E (%)	Drug concentration (mcg/mL)
6:1:3							
0%	**0 hr**	79 ± 1	44 ± 0.2	0.060 ± 0.008	75 ± 5	2.4 ± 0.09	568 ± 129
	24 hrs	80 ± 4	41 ± 0.8	0.065 ± 0.005	71 ± 1	2.2 ± 0.1	534 ± 93
0.5%	**0 hr**	69 ± 2	35 ± 0.5	0.075 ± 0.007	53 ± 3	2.2 ± 0.2	558 ± 69
	24 hrs	73 ± 3	30 ± 0.1	0.05 ± 0.00	45 ± 6	2.1 ± 0.2	474 ± 27
2.5%	**0 hr**	60 ± 0	8 ± 0.1	0.098 ± 0.039	48 ± 6	2.6 ± 0.02	539 ± 44
	24 hrs	66 ± 1	6 ± 0.07	0.08 ± 0.01	45 ± 5	2.4 ± 0.02	501 ± 19
5:2:3							
0%	**0 hr**	50 ± 9	44 ± 2.0	0.052 ± 0.019	74 ± 6	2.8 ± 0.07	689 ± 29
	24 hrs	59 ± 14	40 ± 0.1	0.057 ± 0.019	70 ± 4	2.6 ± 0.08	645 ± 17
0.5%	**0 hr**	73 ± 5	33 ± 0.3	0.100 ± 0.021	48 ± 2	2.5 ± 0.1	608 ± 45
	24 hrs	79 ± 4	27 ± 0.3	0.09 ± 0.02	46 ± 2	2.4 ± 0.1	578 ± 30
2.5%	**0 hr**	67 ± 2	21 ± 0.2	0.100 ± 0.070	44 ± 5	2.5 ± 0.1	609 ± 42
	24 hrs	76 ± 5	15 ± 0.1	0.1 ± 0.09	40 ± 7	2.4 ± 0.1	562 ± 32
3:3:4							
0%	**0 hr**	87 ± 2	53 ± 4	0.074 ± 0.021	81 ± 10	5.9 ± 1.2	1705 ± 303
		89 ± 1	48 ± 0.9	0.065 ± 0.018	77 ± 7	5.7 ± 1.9	1661 ± 187
0.5%	**0 hr**	85 ± 0	40 ± 0.4	0.085 ± 0.007	68 ± 1	5.8±1.9	1694 ± 477
		89 ± 1	35 ± 0.1	0.061 ± 0.01	66 ± 2	5.6 ± 1.9	1664 ± 306
2.5%	**0 hr**	83 ± 2	15 ± 0.2	0.100 ± 0.900	48 ± 1	5.3±1.8	1670 ± 453
		90 ± 4	10 ± 0.5	0.7 ± 0.4	46 ± 5	5.2 ± 1.8	1622 ± 327

Table 9: 24-hour stability study for non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) with variable DSPE-PEG 2000 concentrations (0%, 0.5% & 2.5%). Size, charge, PDI, % internal meropenem, % E.E, and total drug concentration were measured at 0 and 24 hours to illustrate the physiochemical properties using DLS, and HPLC. Paired t-test was used to assess differences between baseline and 24 hours. Data presented as median ± SD (n=3).

5.3 Efficacy of PEGylated cationic liposomal meropenem against lab strains

Specific formulations of PEGylated meropenem-encapsulated liposomes with variable DSPE-PEG 2000 lipid contents have higher bactericidal activity than corresponding free antibiotic and non-PEGylated meropenem-encapsulated cationic liposomes against different laboratory strains of Gram-negative bacteria.

5.3.1 *In-vitro* bactericidal activity against lab gram-negative strains

The *in-vitro* bactericidal activity of PEGylated meropenem-encapsulated liposomal formulations was assessed against different laboratory strains of Gram-negative bacteria using serial two-fold dilutions. This was compared with free meropenem alone and non-PEGylated meropenem-encapsulated (empty) liposomes as positive and negative controls. Following PEGylation, the MIC of liposomal meropenem was assessed against various laboratory strains of Gram-negative bacteria (**Table 9**). The addition of 2.5% PEG to 6:1:3 liposomes was associated with higher MICs compared to non-PEGylated liposomes ($p<0.05$ against all bacteria assessed), apart from AB. 0.5% PEGylated liposomes had similar MIC values to non-PEGylated liposomes (**Table 9, Supplemental Figure 12**).

PEGylation of 5:2:3 liposomes had variable effects on MIC values. Addition of 0.5% or 2.5% PEG to 5:2:3 liposomes was associated with higher MIC values against 3 of 7 bacterial strains assessed (AB, EC21014 and EC12241; $p<0.05$) (**Table 9, Supplemental Figure 12**). Addition of 2.5% PEG to 5:2:3 liposomes was associated with higher MIC values against KP13882 ($p<0.001$).

Addition of 0.5% PEG to 3:3:4 liposomes was associated with higher MICs compared to non-PEGylated liposomes against two bacteria (KP13882, PA 35422) (**Table 9, Supplemental Figure 12**). Addition of 2.5% PEG to 3:3:4 liposomes was associated with higher MICs compared to non-PEGylated liposomes ($p<0.05$ against all bacteria assessed) (**Table 9 & Supplemental Figure 12**).

Compared to free meropenem, all but two PEGylated formulations maintained better efficacy. 6:1:3 and 3:3:4 liposomes were no longer associated with lower MIC values against EC12214 compared to free meropenem on the addition of 2.5% PEG (**Table 9 & Supplemental Figure 12**). All other non-PEGylated, 0.5% and 2.5% PEGylated liposomes had lower MIC values compared to free meropenem across all bacterial strains ($p<0.05$ for all).

	PEG	AB 19605	EC 12014	EC 12241	KP 13882	KP 13883	PA 10145	PA 35422
Free mero		0.63	0.63	0.06	0.63	0.63	0.63	2.5
06:01:03	0%	0.4	0.02	0.02	0.02	0.02	0.02	0.02
	0.50%	0.1	0.02	0.02	0.02	0.02	0.02	0.02
	2.50%	0.2	0.06	0.06	0.06	0.06	0.06	0.1
05:02:03	0%	0.4	0.02	0.02	0.02	0.02	0.02	0.02
	0.50%	0.2	0.05	0.05	0.05	0.05	0.02	0.05
	2.50%	0.1	0.04	0.04	0.04	0.04	0.04	0.04
03:03:04	0%	0.4	0.02	0.02	0.02	0.02	0.02	0.02
	0.50%	0.2	0.03	0.03	0.03	0.03	0.03	0.07
	2.50%	0.5	0.06	0.06	0.06	0.06	0.06	0.06

Table 9: MICs of free meropenem (top row), non-PEGylated and PEGylated liposomal meropenem with incremental amounts of PEG (0.5% and 2.5%) against different lab strains of Gram-negative bacteria i.e., AB: Acinetobacter, EC: E coli, K.P: Klebsiella pneumoniae, PA: Pseudomonas aeruginosa. The size of blue horizontal bars within cells is proportional to the MIC. Data presented as median (n=4).

5.4 Binding kinetics studies

PEGylated meropenem-encapsulated liposomes with higher DSPE-PEG 2000 concentrations demonstrated no or minimal fusion between cationic liposomes and the bacterial and immune cells as compared to liposomes with no or minimal DSPE-PEG 2000 lipid contents, due to ''steric effects''.

Meropenem-encapsulated liposomal formulations with incremental PEGylated lipid concentrations (0.5% and 2.5%, respectively) were co-incubated with Gram-negative bacteria and healthy volunteer immune cells. The differences in binding of the liposome to the bacteria and immune cells (fusion kinetics) measured using flow cytometry. Non-PEGylated liposomes added to Gram-negative bacteria and healthy volunteer immune cells acted as the positive control.

Using flow cytometry, a qualitative assessment was performed of the effect of liposomal PEGylation on real-time fusion between healthy volunteer immune cells, bacteria, and liposomes *in-vitro* (**Figure 19**). There was a reduction in fusion between liposomes and either bacteria or immune cells with the addition of increasing amounts of PEG. The addition of 2.5% PEG abrogated all fusion between liposomes and both bacteria and immune cells.

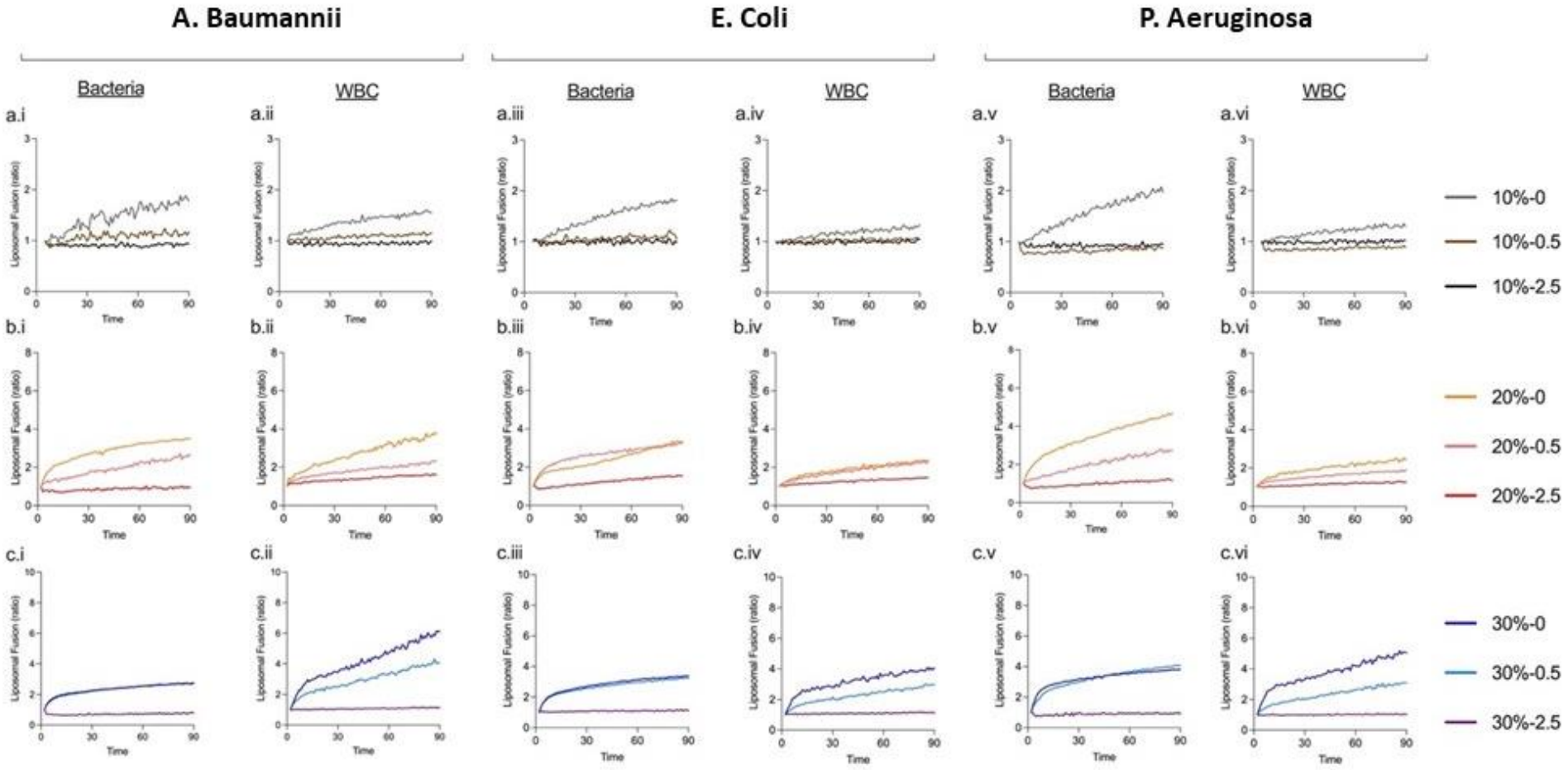


Figure 19: Fluorescent probe (PE)-labelled non-PEGylated and PEGylated liposomal meropenem with (a) 10%, (b) 20% and (c) 30% DODAB and different PEG concentrations (0%, 0.5%, 2.5%) were co-incubated with Gram-negative bacteria (A Baumanni, E coli, P aeruginosa) and healthy volunteer white blood cells. Non-PEGylated liposomes with higher DODAB concentrations had more rapid and avid binding. Datapoints reveal the relative change in PE-fluorescence (suggestive of liposome fusion) over time compared to baseline values. Four replicates were performed with the line representing the median value.

5.5 *In-vitro* biocompatibility of PEGylated liposomes (FACS, ELISA, Haemolytic assay)

Host cytotoxicity and inflammation resulting from bacterial death are lower for PEGylated meropenem-encapsulated liposomes with variable cationic and DSPE-PEG 2000 lipid concentrations than free antibiotic treatment as these liposomal formulations bind and neutralize bacterial toxins. Surface modification of cationic liposomes by PEGylation reduces neutrophil interactions and uptake of meropenem-encapsulated liposomes.

6:1:3 liposomes demonstrated liposomal fusion to immune cells. This was dose-dependent ($p<0.001$) and minimised by adding 0.5% or 2.5% PEG from low to high doses ($p<0.001$) (**Figure 20**). Adding 2.5% PEG did not confer additional benefit in preventing fusion to immune cells compared to 0.5% PEG ($p>0.05$ at low, medium, and high doses). At medium and high doses, 2.5% PEGylated 6:1:3 liposomes induced an increase in IL-6 production compared to free meropenem, non-PEGylated and 0.5% PEGylated 6:1:3 liposomes ($p<0.05$ for all). 2.5% PEGylated 6:1:3 liposomes induced increased TNF-alpha production compared to non-PEGylated 6:1:3 liposomes or 0.5% PEGylated liposomes at high doses only. Neither dose nor PEGylation of 6:1:3 liposomes was associated with immune cell ROS production, cell death or haemolysis ($p>0.05$ for all).

5:2:3 liposomes demonstrated liposomal fusion to immune cells. This too was dose-dependent ($p=0.001$) and minimised by adding 0.5% or 2.5% PEG from low to high doses ($p<0.001$) (**Figure 20**). At low ($p=0.04$) and medium ($p=0.06$) doses, liposomal fusion was minimised by 2.5% PEGylation only. At high doses, where liposomal fusion was most evident, both 0.5% ($p<0.001$) and 2.5% ($p<0.001$) PEGylation reduced liposomal fusion to immune cells. Additionally, 2.5% PEG conferred less liposomal fusion to immune cells compared to 0.5% PEGylation ($p=0.014$).

5:2:3 liposomes induced immune cell ROS production in a dose-dependent manner ($p=0.003$) though evident only at high doses (**Figure 20**). Compared to free meropenem, non-PEGylated liposomes ($p<0.001$) and 0.5% PEGylated liposomes ($p=0.0135$) increased immune cell ROS production, but not 2.5% PEGylated liposomes ($p=0.154$). Compared to non-PEGylated liposomes, 2.5% PEGylation ($p<0.001$) but not 0.5% PEGylation ($p=0.1644$) significantly reduced immune cell ROS production.

5:2:3 liposomes were associated with increased IL-6 release compared to free meropenem; this was both dose-dependent ($p<0.001$) and associated with more PEGylation ($p<0.001$) (**Figure 20**). This was seen for non-PEGylated liposomes ($p=0.013$), 0.5% PEGylated liposomes ($p=0.019$), and 2.5% PEGylated liposomes ($p=0.039$). 2.5% PEGylated liposomes were associated with greater IL-6 release compared to non-PEGylated liposomes and 0.5% PEGylated liposomes ($p<0.001$ for both). Similar trends were observed for TNF-alpha release, especially at higher doses. Non-PEGylated liposomes ($p=0.010$), 0.5% PEGylated liposomes ($p=0.013$), and 2.5% PEGylated liposomes ($p=0.030$) were associated with greater TNF-alpha release at high doses.

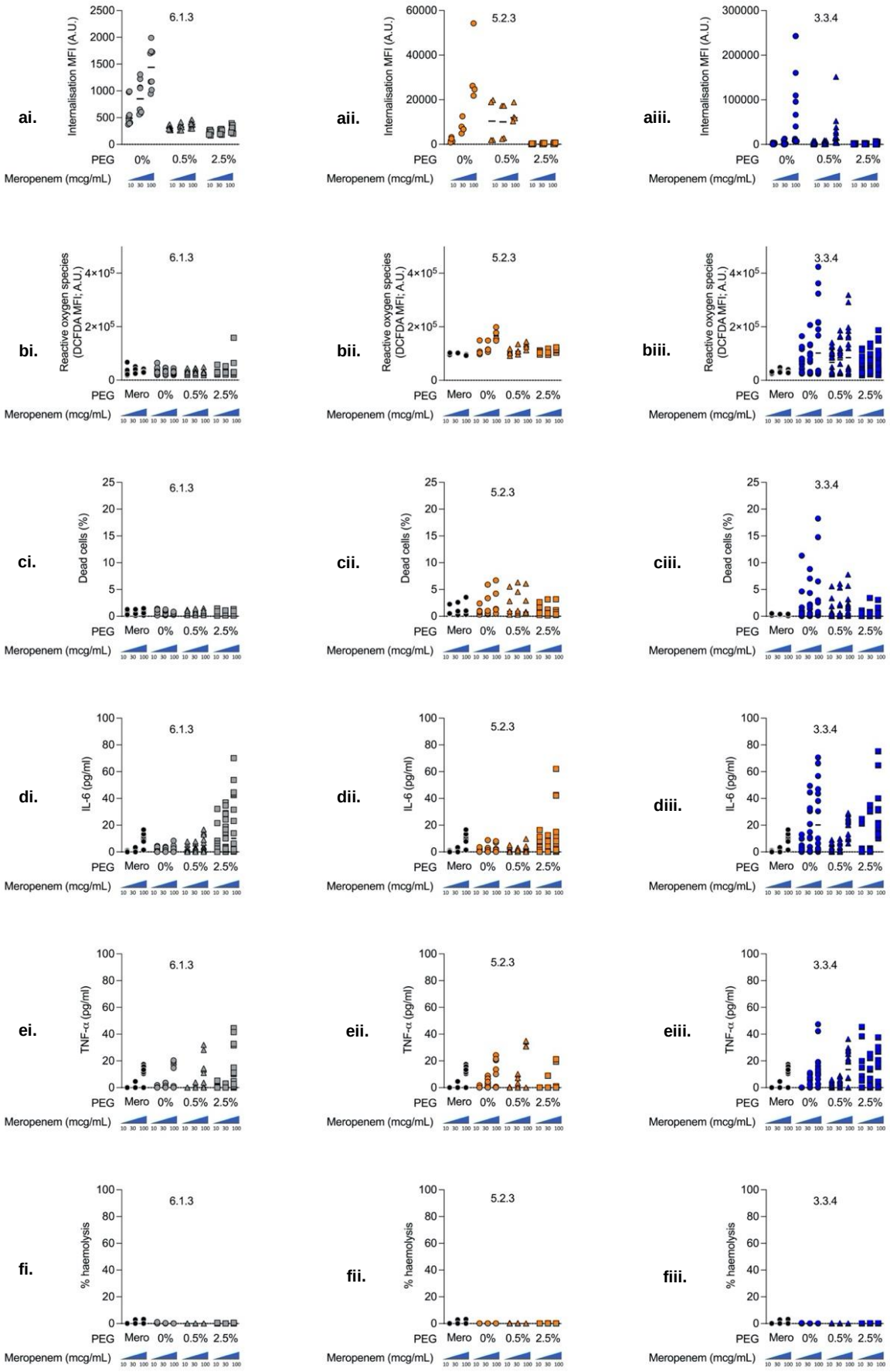
2.5% PEGylated liposomes were associated with greater immune cell ROS production compared to free meropenem, non-PEGylated liposomes and 0.5% PEGylated liposomes ($p<0.05$ for all).

Minimal (<2%) haemolysis was seen with high-dose free meropenem and this was significantly reduced by liposomal meropenem ($p<0.01$ for all) (**Figure 20**). Neither dose nor PEGylation of 5.2.3 liposomes was associated with immune cell death.

3:3:4 liposomes showed liposomal fusion to immune cells which was dose-dependent ($p<0.001$) and minimised by adding 0.5% or 2.5% PEG at high doses ($p<0.001$) (**Figure 20**). At high doses, 2.5% PEGylation was associated with less immune cell fusion compared to 0.5% PEGylation ($p=0.046$). At low and medium doses, liposomal fusion to immune cells was unaffected by liposome PEGylation. At high doses, liposomal meropenem induced increased immune cell ROS production compared to free meropenem ($p=0.002$). Increased immune cell ROS production was also associated with 0.5% PEGylated liposomes ($p=0.023$), but not 2.5% PEGylation ($p=0.2$).

Increased immune cell death was associated with high dose non-PEGylated liposomes compared to free meropenem ($p=0.025$). This effect was not seen with either 0.5% PEGylation ($p=0.20$) or 2.5% PEGylation ($p=0.71$) (**Figure 20**). Addition of 2.5% PEG conferred no benefit over 0.5% PEG ($p=0.157$); although 2.5% PEGylated liposomes minimised immune cell death compared to non-PEGylated liposomes ($p=0.003$). Minimal (<2%) haemolysis was seen with high-dose free meropenem; this effect was significantly reduced by liposomal meropenem ($p<0.01$ for all).

3:3:4 liposome-induced IL-6 release was dose-dependent ($p<0.001$) and associated with PEGylation of liposomes (**Figure 20**). At low doses, liposomes were not associated with IL-6 production. At high doses, however, non-PEGylated ($p<0.001$), 0.5% PEGylated ($p=0.056$) and 2.5% PEGylated ($p<0.001$) liposomes induced increased IL-6 production compared to free meropenem. 0.5% PEGylated liposomes induced less IL-6 production compared to non-PEGylated ($p=0.066$) and 2.5% PEGylated ($p=0.045$) liposomes. TNF-alpha release was not dose-related. At low doses, 2.5% PEGylation was associated with increased TNF-alpha compared to free meropenem, 0% and 0.5% PEGylation ($p<0.01$ for all). At medium and high doses, PEGylation of liposomes did not influence TNF-alpha release compared to free meropenem or non-PEGylated liposomes. Minimal (<2%) haemolysis was seen with high-dose free meropenem and this was significantly reduced by liposomal meropenem ($p<0.01$ for all) (**Figure 20**).


ai.
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eiii.
fi.
fii.
fiii.
6.1.3
5.2.3
3.3.4
Internalisation MFI (A.U.)
Reactive oxygen species (DCFDA MFI; A.U.)
Dead cells (%)
IL-6 (pg/ml)
TNF-α (pg/ml)
% haemolysis
PEG
Mero
0%
0.5%
2.5%
Meropenem (mcg/mL)
10 30 100

Figure 20: In vitro safety of non-PEGylated and PEGylated cationic liposomal meropenem. Variable concentrations of PEG at therapeutic (10 and 30 µg/mL) and supra-therapeutic (100 µg/ml) meropenem concentrations were co-incubated for 6 hours with healthy volunteer whole blood at 37°C. Comparisons were made against free meropenem at equivalent concentrations. Liposome fusion to immune cells was associated with higher surface charge and increasing doses (ai-aiii). Immune cell reactive oxygen species production was associated with higher liposome charge and doses but reduced by the addition of PEG (bii-biii). The liposome with the highest charge (3:3:4) was associated with more immune cell death, especially at higher doses (cii-ciii). This was ameliorated by the addition of PEG. Pro-inflammatory cytokine (IL-6 and TNF-alpha) production associated with liposomes was minimal but increased with higher concentrations of PEG (d-e). Haemolysis at baseline was minimal and reduced further in the presence of liposomes (f). Experiments represent data from 4-8 biological replicates.

5.6 Effect of PEGylation on immune cell activation and binding

Host cytotoxicity and inflammation resulting from bacterial death were lower with PEGylated meropenem-encapsulated liposomes with variable cationic and DSPE-PEG 2000 lipid concentrations than free antibiotic treatment as surface modification of cationic liposomes by PEGylation reduces neutrophil uptake of meropenem-encapsulated cationic liposomes.

In summary, increasing amounts of DSPE-2000 PEGylation of liposomes were associated with significant changes to liposomal physicochemical properties (including size, charge, PDI, and % internal meropenem concentration) (**Figure 21**). Increasing PEGylation was associated with decreased fusion of bacteria and immune cells. Confocal microscopy showed significant aggregation of liposomes associated with increasing surface charge that was minimised by PEGylation. 3:3:4 liposomes were associated with immune cell activation and death, and this too was minimised by PEGylation. However, the addition of higher concentrations of PEG (2.5%) was associated with increasing amounts of cytokine release in whole blood assays. Based on these findings, two liposomal formulations were selected, and their efficacy was assessed against clinical strains of bacteria (MIC).

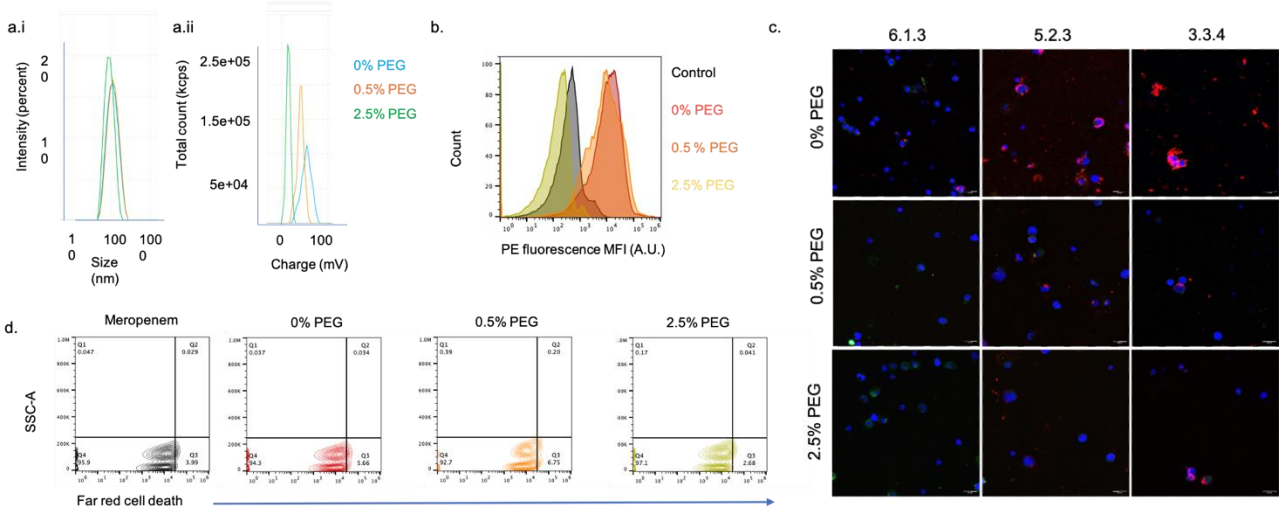


Figure 21: The effect of PEGylation on (ai) liposome size and (aii) charge as assessed by DLS. (b) The addition of increasing concentrations of PEG to 3:3:4 liposomes were associated with decreased immune cell fusion as assessed by flow cytometry. (c) Increasing liposome charge was associated with increased fusion of liposomes (PE-labelled; pink channel) to immune cells (DAPI labelled; blue channel). The addition of increasing concentrations of PEG to liposomes was associated with decreased immune cell fusion. (d). The addition of increasing concentrations of PEG to 3:3:4 liposomes was associated with decreased immune cell death.

5.7 *Ex-vivo* Endotoxin Neutralization

Host cytotoxicity and inflammation resulting from bacterial toxin (LPS) were lower for meropenem-encapsulated liposomes with variable cationic and DSPE-PEG 2000 lipid concentrations compared to free antibiotics as the liposomal formulations bind and neutralize bacterial toxins.

At lower concentrations, 6:1:3 (p=0.014) non-PEGylated liposomes lowered IL-6 production, while 6:1:3 (p=0.006) and 3:3:4 (p=0.008) non-PEGylated liposomes were associated with minimal TNF-α production in comparison to free standard meropenem and different PEGylated meropenem-encapsulated liposomes (**Figure 22**).

At higher concentrations, 3:3:4 non-PEGylated liposomes were associated with lower pro-inflammatory cytokine production i.e., IL-6 (p=0.02) and TNF-α (p=0.02) respectively, whereas 6:1:3 (p=0.01) non-PEGylated formulations lowered TNF-α production as compared to free meropenem, non-PEGylated and PEGylated meropenem-encapsulated liposomal formulations (**Figure 23**).

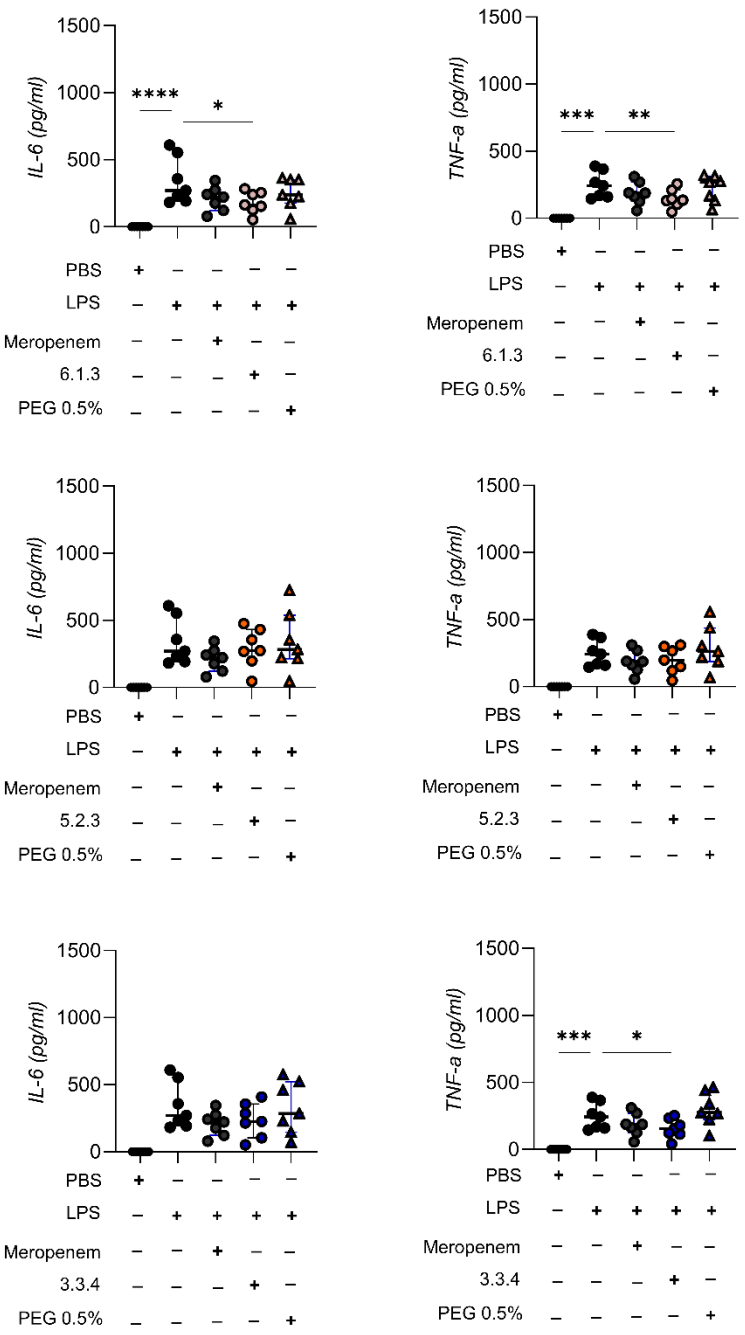


Figure 22: The in vitro safety assessment of non-PEGylated and PEGylated cationic liposomal formulations at therapeutic (10 µg/ml)) meropenem concentrations were co-incubated for 6 hours with healthy volunteer whole blood at 37°C. Comparisons were made against free meropenem at equivalent concentrations. ELISA measured pro-inflammatory cytokine production. Data from 4-8 biological replicates.

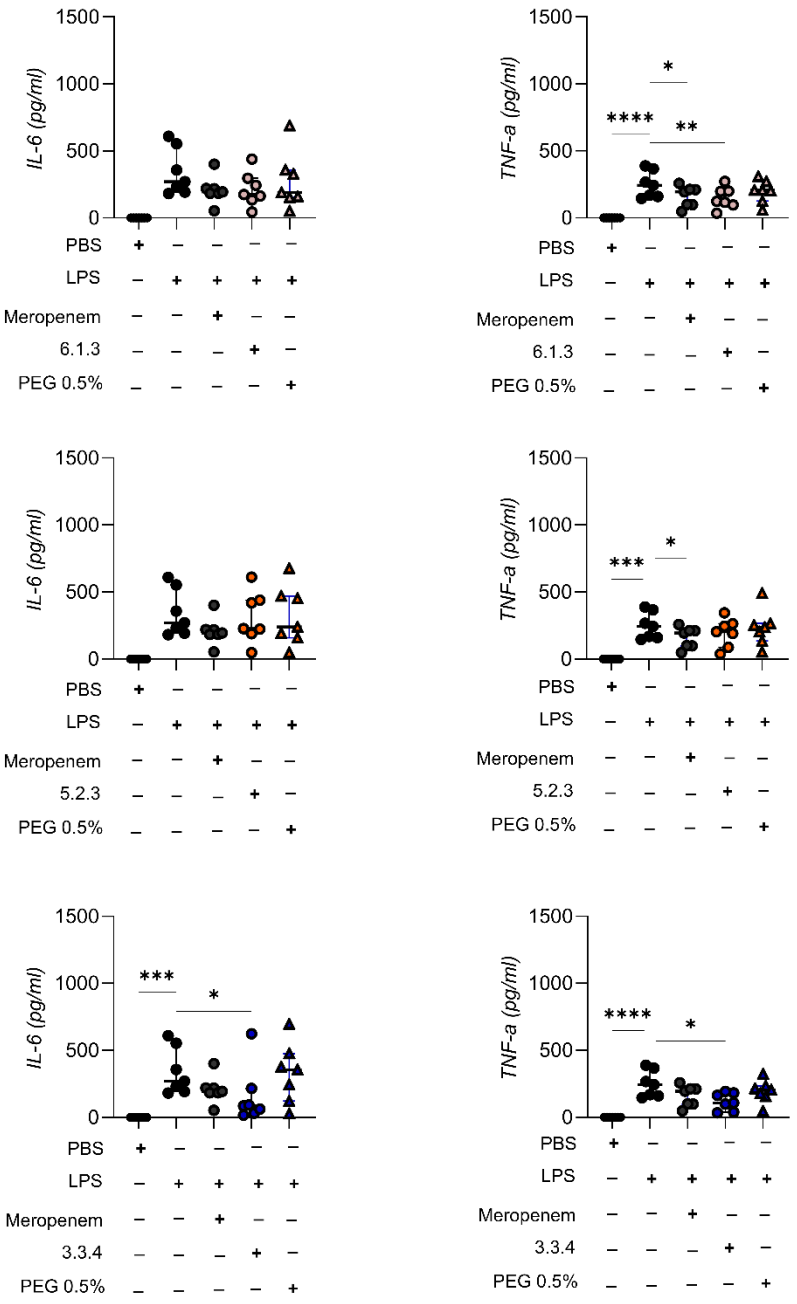


Figure 23: In vitro safety assessment of non-PEGylated and PEGylated cationic liposomal formulations at supra-therapeutic (30 µg/ml)) meropenem concentrations after co-incubation for 6 hours with healthy volunteer whole blood at 37°C. Comparisons were made against free meropenem at equivalent concentrations. ELISA measured pro-inflammatory cytokine production. Data from 4-8 biological replicates.

5.8 Efficacy of PEGylated cationic liposomal meropenem against clinical and resistant strains

The specific formulations of PEGylated meropenem-encapsulated liposomes with variable DSPE-PEG 2000 lipid contents have higher bactericidal activity than corresponding free antibiotic and non-PEGylated

meropenem-encapsulated cationic liposomes against different clinical and resistant strains of Gram-negative bacteria.

5.8.1 *In-vitro* bactericidal activity against clinical gram-negative strains

The *in-vitro* bactericidal activity of PEGylated meropenem-encapsulated liposomal formulations such as 6:1:3 and 3:3:4 liposomes (± PEGylation) were assessed against different clinical strains of Gram-negative bacteria using serial two-fold dilutions. MIC values were compared with free meropenem alone, liposomes without encapsulated meropenem (empty liposomes), and non-encapsulated liposomes (empty) with free external meropenem as positive and negative controls.

Both PEGylated and non-PEGylated meropenem-encapsulated liposomes demonstrated a 12-46-fold reduction in MICs compared to standard free meropenem (**Table 10, Figures 23 & 24**). Non-encapsulated liposomes enhanced the effect of free external meropenem with an up to 2-fold reduction in MIC. Liposomal formulations without encapsulated meropenem (empty liposomes) did not prevent bacterial growth.

	E.C ATCC 25922	E.C US142	EC GS065	KP US131	KP GS008	PA US005	PA US115	Ecl US055
Free meropenem	0.244	0.244	0.244	0.48	0.244	1.9	1.9	0.244
6.1.3 empty + external mero	0.07	0.1	0.1	0.1	0.1	0.2	0.2	0.07
6.1.3 0% PEG	0.01	0.02	0.01	0.03	0.01	0.1	0.1	0.01
6.1.3 0.5% PEG	0.01	0.02	0.01	0.02	0.02	0.05	0.05	0.01
6.1.3 2.5% PEG	0.02	0.02	0.02	0.02	0.02	0.06	0.07	0.04
3.3.4 empty + external mero	0.15	0.3	0.3	0.3	0.3	0.6	0.6	0.1
3.3.4 0% PEG	0.01	0.01	0.01	0.02	0.01	0.08	0.1	0.01
3.3.4 0.5% PEG	0.01	0.02	0.01	0.02	0.02	0.06	0.06	0.02
3.3.4 2.5% PEG	0.02	0.03	0.02	0.04	0.03	0.09	0.09	0.03

Table 10: MICs of free meropenem alone, non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes, liposomes without encapsulated meropenem (empty liposomes), and non-encapsulated liposomes with free external meropenem against reference and different clinical strains of Gram-negative bacteria. AB: Acinetobacter, EC: E coli, K.P: K pneumoniae, PA: P aeruginosa, EC: E Cloacae. Sizes of blue horizontal bars within cells are proportional to MIC. Data presented as median (n=3).

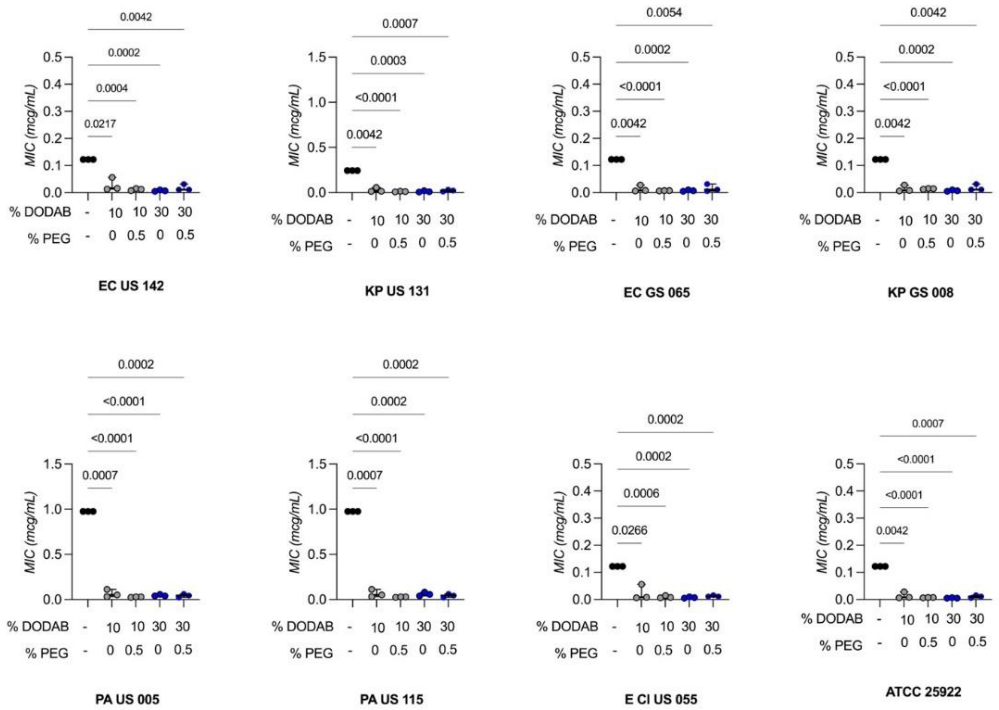


Figure 24: MIC of free and non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes with 10% DODAB ± addition of PEG concentrations (0.5% and 2.5%) against different clinical strains of Gram-negative bacteria. (AB: Acinetobacter, EC: E coli, K.P: K pneumoniae, PA: P aeruginosa, EC: E Cloacae). Data presented as median (n=3).

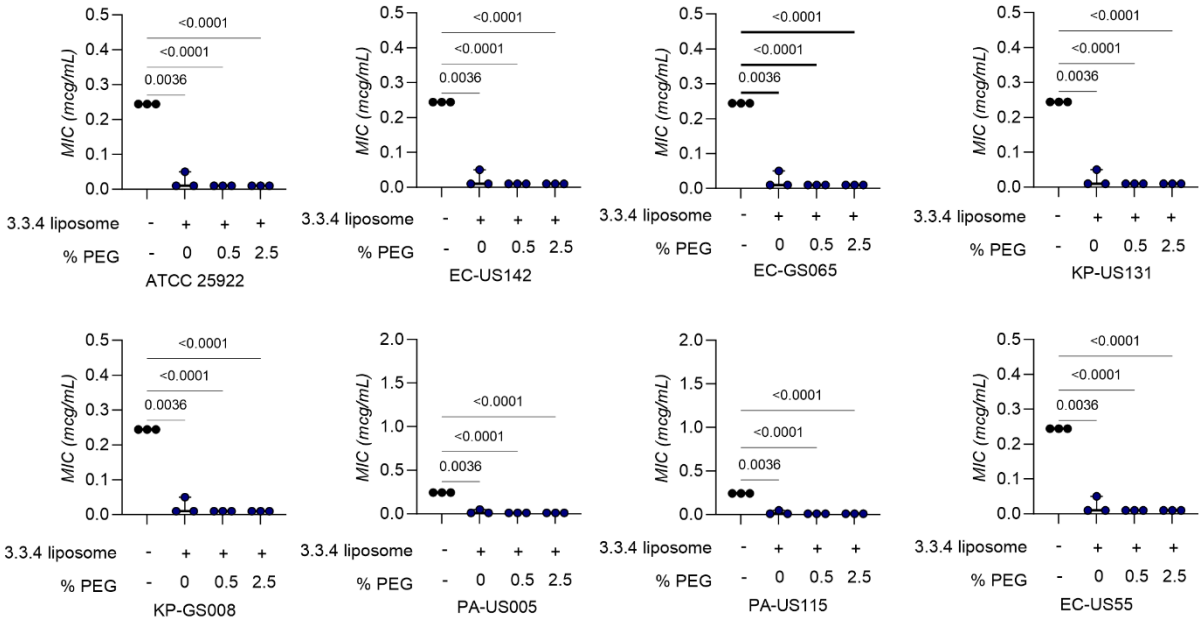


Figure 25: MIC of free and non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes with 30% DODAB ± addition of concentrations of PEG (0.5% and 2.5%) against different clinical strains of Gram-negative bacteria. (AB: Acinetobacter, EC: E coli, K.P: K pneumoniae, PA: P aeruginosa, EC: E Cloacae . Data presented as median (n=3).

5.8.2 *In-vitro* bactericidal activity against resistant Gram-negative strains

The *in-vitro* bactericidal activity of meropenem-encapsulated liposomal formulation such as 6:1:3 (± PEGylation) was assessed against four different meropenem-resistant bacteria (16 strains) isolated from patients (**Supplemental Table 4**) using serial two-fold dilutions. MIC values were compared with free meropenem alone, liposomes without encapsulated meropenem (empty liposomes), and non-encapsulated liposomes (empty) with free external meropenem as positive and negative controls. Compared to free meropenem, liposomal meropenem was associated with lower MICs ($p<0.05$) for different bacterial strains.

Within the sixteen strains that I evaluated, 6:1:3 liposomes demonstrated statistically significant reductions in MIC of meropenem against some carbapenem-resistant strains. However, 6.1.3 (with and without PEGylation) reduced MIC to the susceptible range for only 1 of 16 resistant strains (**Table 11, Figure 25**). This reduction is unlikely to have any clinical significance. Trends (albeit not statistically significant with different biological replicates) were also seen towards lower MICs associated with different bacterial strains, but these were not within the susceptible ranges proposed by EUCAST guidelines (**Table 11, Figure 25**).

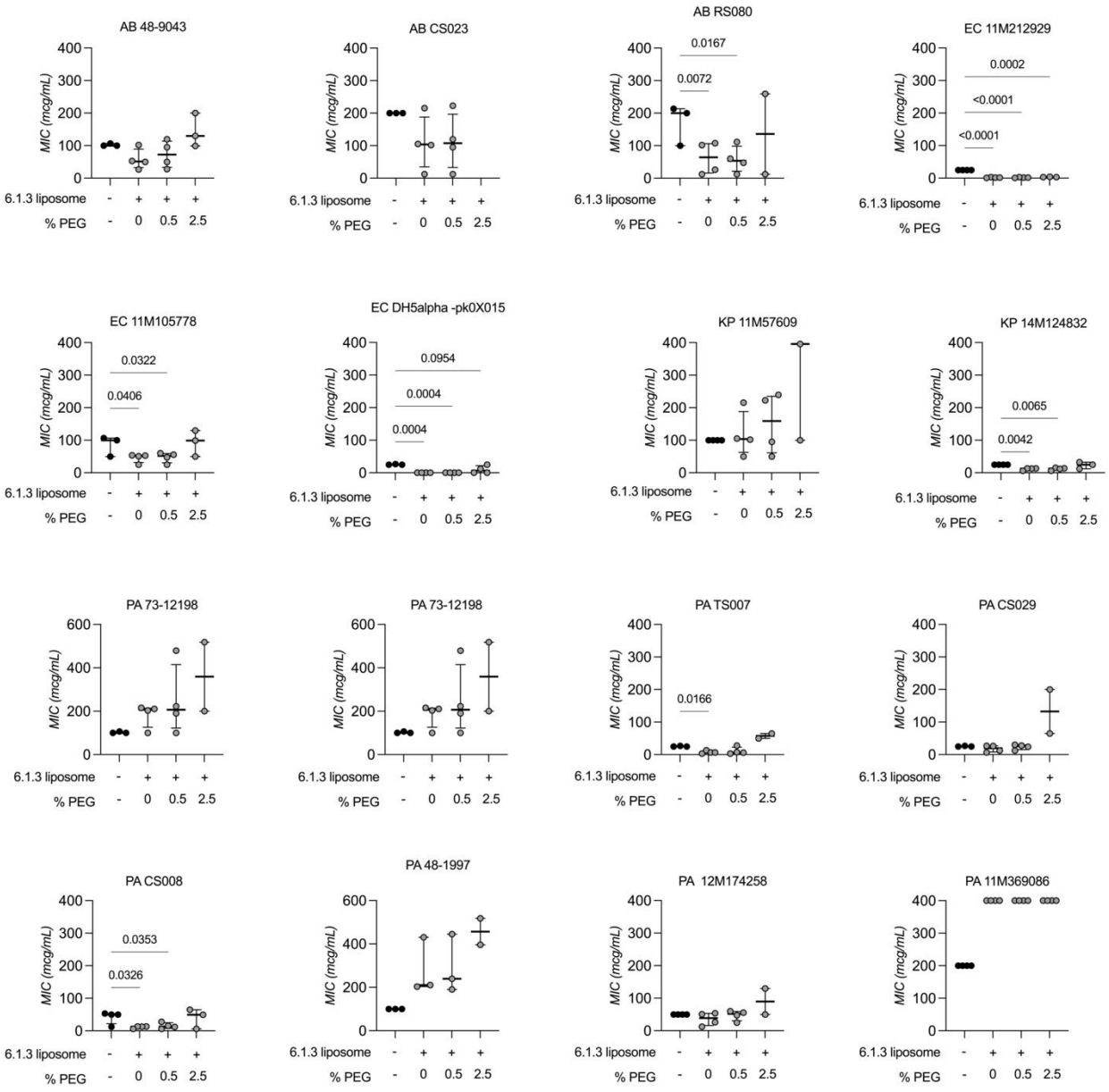


Figure 26: MIC of non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes with 10% DODAB ± addition of PEG (0.5% and 2.5%) against different resistant strains of Gram-negative bacteria i.e., AB: Acinetobacter, EC: E. coli, K.P: K pneumoniae, PA: P aeruginosa. Data presented as median (n=4).

	Resistance Mechanism	Free meropenem	6.1.3 0% PEG	6.1.3 0.5% PEG	6.1.3 2.5% PEG	Empty liposome + free meropenem	Empty Liposome
ATCC 25922	(Reference strain)	0.05	0.01 *	0.01 *	0.02 *	0.07	Growth
AB 48-9043	IMP-1	100	50	75	125	200	Growth
EC 11M105778	OXA-48	100	50	50	100	200	Growth
EC DH5alpha -pk0X015	VIM-1	25	0.2	0.2	0.2	12.5	Growth
EC 11M212929	NDM	25	1.6 *	1.6 *	3.2 *	25	Growth
KP 11M57609	KPC	100	100	150	400 *	200	Growth
KP 14M124832	NDM	25	12.5 *	12.5 *	25	50	Growth
PA CS008	Unknown	50	12.5	12.5	50	50	Growth
PA 48-1997	SPM	100	200	225	450 *	50	Growth
PA PA01 pMATTX	VIM-7	50	6.6 *	6.6 *	22	100	Growth
PA 11M369086	IMP	200	400 *	400 *	400 *	100	Growth
PA 73-12198	GIM-1	100	207	207	360	Growth	Growth
AB CS023	OXA-23	200	103	107	518	200	Growth
AB RS080	OXA-23	100	64	55	135	200	Growth
PA TS007	Unknown	25	6.5	6.5	57	100	Growth
PA CS029	VEB	100	19	25	132	50	Growth
PA 12M174258	VIM-1	50	38	52	90	200	Growth

*Table 11: MICs of free meropenem alone, non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes, liposomes without encapsulated meropenem (empty liposomes), and non-encapsulated liposomes with free external meropenem against reference and different resistant strains of Gram-negative bacteria i.e., AB: Acinetobacter, EC: E. coli, K.P: K pneumoniae, PA: P aeruginosa. * represents p values, indicating either increase or decrease in MICs against different resistant Gram-negative bacteria in comparison to free meropenem but these statistical values were not within the susceptible ranges proposed by EUCAST guidelines. Data presented as median (n=4).*

Summary

Non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations were associated with enhanced *in vitro* bactericidal activity. Up to a 12-30-fold reduction in MIC (minimum inhibitory concentration) was achieved against multiple laboratory and clinical strains of Gram-negative bacteria using non-PEGylated and PEGylated meropenem-encapsulated cationic liposomes compared to native meropenem. Meropenem-encapsulated meropenem with lower concentrations of DSPE-2000 PEG prevented immune cell activation i.e., leukocyte internalization, ROS production, cell death and pro-inflammatory cytokine production in comparison to free standard meropenem irrespective of the drug concentration.

6. Discussion

Antimicrobial resistance (AMR) is a major global health threat, yet novel antimicrobials remain elusive (O'Neill, 2016). Improving the efficacy of pre-existing antibiotics is one solution. This could be achieved by targeting delivery to bacteria using cationic liposomes and incorporating antibiotics to enhance selective bacterial binding and killing. This approach will enable resistant bacteria to be killed at normal (or even subnormal) current dosing regimens with no harm to the human host. The highly negatively charged property of Gram-negative bacterial membranes enhances stronger fusion and interactions along with increased payload release using positively charged cationic liposomes. Biocompatible hydrophilic polymers can modify liposome surfaces to minimise the adsorption of circulating proteins and reticuloendothelial system (RES) uptake (Song et al., 2012). Surface pegylation minimises toxicity and off-target effects.

Antibiotic-encapsulated liposomes can provide an alternative drug delivery route because of their unique inherent bactericidal properties that distinguish them from conventional commercially available antibiotics (Haeri et al., 2017, Moyá et al., 2019). They can serve as inert drug delivery vehicles, thereby facilitating encapsulation, stability (physically and chemically) and delivery of antibiotics (Haeri et al., 2017). The physiochemical properties of liposomes, such as size, charge, and pegylation can also influence their therapeutic impact by prolonging the circulation half-life and enabling slow, sustained drug release at infection sites with minimal unwanted drug-related events (Haeri et al., 2017, Moyá et al., 2019, Serri et al., 2018).

Summary of the findings

6.1 Characterization of non-PEGylated cationic liposomes

Non-PEGylated meropenem-encapsulated liposomes were formulated with variable cationic lipid concentrations ranging from 0% to 40% DODAB using the thin-film hydration method. Techniques such as DLS and HPLC were used to assess the physicochemical properties of meropenem-encapsulated cationic liposomes.

Size

Measured liposome sizes were consistently higher than expected, indicating fusion and aggregation due to interactions between fusogenic lipids (Ferreira et al., 2021, Lechanteur et al., 2018, Smith et al., 2017). Measured sizes ranged from 55-88 nm for 50 nm liposomes and 106-122 nm for 100 nm liposomes. Several factors such as liposomal formulation aggregation within the concentrated solutions and exposure to certain environmental conditions i.e., changes in pH, ionic strength, temperature, changes in internal osmotic pressure, and hydration of lipid bilayers may overestimate liposomal size as measured by DLS (Ferreira et al.,

2021, Lechanteur et al., 2018). Careful consideration should be given to the above-mentioned factors while preparing and characterizing antibiotic-encapsulated liposomal formulation, and understanding factors associated with discrepancies between expected and measured size.

Zeta potential

Several factors, such as the proportion of cationic lipids within the liposomal formulations, can influence the zeta potential, a measure of the surface charge of the particles (Moyá et al., 2019, Lechanteur et al., 2018, Smith et al., 2017). Cationic lipids possess positively charged head groups (e.g., ammonium groups), a key factor responsible for the overall positive charge of the liposome surface. Higher zeta potential was associated with increasing concentrations of cationic lipids (DODAB) incorporated within liposomes, reflecting the increased density of positively charged groups over the liposomal surface, resulting in an overall stronger positive charge. Liposomal formulations without cationic lipids (PC: DODAB: Chol (7:0:3)) had no charge, whereas liposomes with 40% cationic lipids (PC: DODAB: Chol (5:4:1)) had a positive charge of +58 mV (Moyá et al., 2019). There is no linear association between zeta potential and the cationic lipid concentrations. Other factors such as lipid bilayer composition, presence of other lipid contents (cholesterol, PEGylated lipids), pH, and ionic strength of the surrounding medium may have a potential impact on the zeta potential (Moyá et al., 2019, Smith et al., 2017).

Polydispersity Index

The polydispersity index (PDI) of meropenem-encapsulated liposomes indicates the uniformity or heterogeneity of unknown particles by measuring liposomal size distribution within the solution (Moyá et al., 2019, Lechanteur et al., 2018). The measured PDI values for different cationic liposomes varied between 0.04 and 0.1, demonstrating the homogeneity of liposomal formulations per size, contributing to consistent and effective drug delivery and therapeutic efficacy.

% internal meropenem

The percentage (%) of internal meropenem indicates the amount of meropenem encapsulated within the liposomes. Higher percentages of internal meropenem encapsulated within the liposomes indicate effective drug-loading methods, thereby maximizing the therapeutic potential of the liposomal formulations and ensuring that higher meropenem concentrations are available at target sites (Moyá et al., 2019, Lechanteur et al., 2018, Shaaban et al., 2021). Using HPLC, the calculated % internal meropenem of cationic liposomes varied from 37-81% depending upon the different lipid proportions. Therefore, the liposomal formulations with higher cationic lipid contents were associated with lower internal meropenem concentrations, indicating less stability and membrane permeability.

Encapsulation efficacy and total meropenem concentrations encapsulated within liposomes

Encapsulation efficacy (E.E) is the amount of meropenem encapsulated within the liposomes as compared to the total meropenem added at the initial steps of thin-film hydration. A higher encapsulation efficacy indicates that a greater proportion of the drug is successfully trapped within the liposomes, thereby maximizing meropenem concentration availability at the target site. This will potentially lead to enhanced therapeutic efficacy and limited drug-related local and systemic side effects (Moyá et al., 2019, Shaaban et al., 2021).

Cationic lipids can enhance the stability and membrane permeability of liposomes, thereby facilitating the encapsulation of hydrophilic drugs (Moyá et al., 2019, Lechanteur et al., 2018). However, higher cationic lipid contents may lead to aggregation or destabilization of liposomes with negative impacts on encapsulation efficacy (Moyá et al., 2019, Lechanteur et al., 2018, Shaaban et al., 2021). Optimization of cationic lipid content is critical in achieving the desired encapsulation efficacy without affecting the stability or integrity of the meropenem-encapsulated cationic liposomes (Moyá et al., 2019, Lechanteur et al., 2018). The calculated EE ranged from 0.1-3.8%, consistent with published literature, and demonstrates the drug's encapsulation efficiency depends upon several factors such as lipid content, drug-to-lipid ratio, physiochemical properties of the liposomes, liposomal size, and preparation techniques. By addressing these factors, the efficiency of drug encapsulating within the liposomes can be improved significantly.

Total meropenem concentrations encapsulated within cationic liposomes corresponded with the lipid contents and the physicochemical properties of cationic liposomes. Liposomal formulations with higher cationic lipid contents had lower total drug concentrations as compared to liposomes either with lower or moderate cationic lipid concentrations. This is due to poor membrane permeability as the higher DODAB content alters liposome fluidity and stability.

Effect of temperature on liposomal stability

Temperature can influence the physiochemical properties, stability, and integrity of liposomal formulations, leading to fusion, leakage of encapsulated drugs and, eventually, degradation due to lipid oxidation or hydrolysis. Non-PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations were stored at 4°C over 24 hours to assess the physiochemical properties of the cationic liposomes. No significant differences were seen in measured size, charge, PDI or internal meropenem concentration. Although total meropenem concentration was consistently lower at 24 hours, this did not reach statistical significance, indicating minimal molecular mobility and temperature-induced degradation of meropenem-encapsulated liposomes.

6.2 Non-PEGylated cationic liposomal antibiotics to enhance bactericidal activity of pre-existing antibiotics

The extent of the interaction between bacterial cells and the antibiotic-encapsulated positively charged liposomes can be enhanced by incorporating different lipid formulations having variable physicochemical properties (Gubernator et al., 2007). There are increased electrostatic interactions between cationic liposomes and anionic phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and lipopolysaccharide present on the outer surfaces of Gram-negative bacterial cell membranes (Gubernator et al., 2007). These fusion interactions between antibiotic-encapsulated cationic liposomes and bacterial membrane phospholipids improve the delivery of antibiotics to bacterial cells and enhance the effectiveness of pre-existing antibiotics by overcoming non-enzymatic drug resistance mechanisms such as decreased drug permeability and upregulation of efflux pumps (Drulis-Kawa et al., 2006b).

In an *in-vitro* drug efficacy study, encapsulation of meropenem within liposomal formulations with variable cationic lipid contents was associated with enhanced bactericidal activity i.e., a 12-30-fold reduction in MIC (minimum inhibitory concentration) against multiple laboratory or clinical strains of Gram-negative bacteria compared to both native meropenem and empty liposomes. Similar trends were reported when meropenem, gentamicin and ciprofloxacin were encapsulated within non-PEGylated cationic liposomal formulations i.e., 2-4 fold reduction in MIC against clinical isolates of Gram-negative and Gram-positive bacteria compared to neutral and anionic liposomes, and free commercially available antimicrobial drugs (Gubernator et al., 2007, Drulis-Kawa et al., 2006b, Drulis-Kawa et al., 2006a).

Gram-negative bacteria may have varying resistance mechanisms, either non-enzymatic approaches (efflux pumps, decreased bacterial membrane permeability, structural modifications at drug receptor sites) or enzymatic associated with antibiotic degradation (Drulis-Kawa et al., 2006b). Meropenem-encapsulated liposomes may avoid these different resistance approaches by facilitating enhanced drug delivery directly into the bacterial cells, while others (free standard antibiotics) may not, depending on the physiochemical properties of liposomes and the mechanism of action of the encapsulated antibiotics (Gubernator et al., 2007). On the contrary, meropenem-encapsulated cationic liposomes had no bactericidal activity against resistant Gram-negative isolates (Drulis-Kawa et al., 2006b). Increased uptake of liposomal particles by bacterial cells ensures selective and targeted delivery of antibiotics by increasing local drug concentrations at the infection site (Gubernator et al., 2007).

Several *in-vitro* studies have shown enhanced bactericidal activity of antibiotics when encapsulated within liposomes compared to non-liposomal formulations or free standard antibiotics. The broth dilution technique was used to determine the antibacterial activity of an antibiotic against some pathogens (Alhariri et al., 2013). The MIC of the equivalent free drug can be lowered by encapsulating antibacterial medications within certain

liposome formulations (Alhariri et al., 2013, Mugabe et al., 2005). For instance, liposomal aminoglycosides had lower MICs than free drugs against P. aeruginosa isolates (Khan et al., 2020, Gaspar et al., 2013, Mugabe et al., 2005). The enhanced antibacterial action results from the enhanced capacity to fuse and penetrate within the bacterial surfaces (Khan et al., 2020, Gaspar et al., 2013, Mugabe et al., 2005). Several published *in-vitro* studies showed that appropriate liposomal formulations can effectively increase antibacterial activity against most common extracellular bacteria, including P. aeruginosa, K. pneumoniae, and E. coli, in comparison to the medication in its free form (Khan et al., 2020, Gaspar et al., 2013, Mugabe et al., 2005).

6.3 Non-PEGylated cationic lipid encapsulation enhanced *in-vitro* safety of pre-existing antibiotics

The incorporation of antibiotics within the cationic liposomes can also enhance the *in-vitro* safety of pre-existing antibiotics. Cationic lipids interact with negatively charged bacterial cell membranes, thereby facilitating the drug's delivery within the bacterial cells (Gubernator et al., 2007). Some antibiotics have a short-circulation life due to enzymatic degradation, limiting the effectiveness of the drugs. Encapsulation of antibiotics within the fusogenic lipids can protect antibiotics from non-enzymatic and enzymatic degradation, thereby increasing stability and circulation half-life (Kolašinac et al., 2018). Moreover, encapsulation can enhance solubility and drug bioavailability by improving its delivery to specific target cells.

Encapsulating pre-existing antibiotics within the liposomes reduces their systemic toxicity by enhancing antibiotic accumulation specifically at the infection site, thereby minimizing exposure to healthy cells. In *in-vitro* studies, I could demonstrate that meropenem-encapsulated liposomes with 10% DODAB were not associated with any dose-dependent effect on immune cell activation i.e., liposomal internalization within the neutrophils, ROS production, immune cell viability, pro-inflammatory cytokine production or haemolysis, as compared to standard free meropenem at different therapeutic (10 µg/ml), sub-therapeutic (30 µg/ml), and supra-therapeutic (100 µg/ml) concentrations. By contrast, meropenem-encapsulated liposomes with higher cationic concentrations (20%, 30%) had dose-dependent adverse cytotoxic events on healthy human immune cells, with liposomal fusion and internalization by neutrophils, immune cell death, ROS production and increased pro-inflammatory cytokine production.

Circulating liposomes may be recognized as foreign by immune cells (Harashima et al., 1994, Levison and Levison, 2009). Rapid uptake of liposomes is facilitated via opsonization by circulating serum proteins including immunoglobulins and the activated complement proteins, C3a and C5a (Song et al., 2012, Harashima et al., 1994, Bonté and Juliano, 1986). Minimizing liposomal charge (Lian and Ho, 2001b, Song et al., 2012), or incorporating PEG onto the liposome surface (Maruyama et al., 1992, Torchilin, 1994, Crommelin et al., 2020) may limit uptake by the reticuloendothelial system (RES). Following systemic administration, uptake by the RES was lower in liposomes with a neutral surface charge compared to charged liposomes, although the former had a higher tendency to aggregate (Allen et al., 1991, Lian and Ho, 2001a).

RES uptake was mediated via interactions between cationic liposomes and serum proteins (Scheule et al., 1997, Drulis-Kawa et al., 2006c, Furneri et al., 2000), and was associated with dose-dependent proinflammatory responses (Scheule et al., 1997). Interaction (and associated toxicity) between liposomes and mammalian cells was greater with cationic liposomes due to the negative charge on mammalian cells (Derbali et al., 2019, Furneri et al., 2000, Dokka et al., 2000).

In an animal model of osteomyelitis, contamination of bone tissues resulted in persistent infection which was attributed to rapid clearance of antibiotics by the RES (Kadry et al., 2004). Intravenous ciprofloxacin encapsulated within cationic liposomes and vancomycin for 14 days was associated with a lower side effect profile while maintaining clinical efficacy compared to standard free antibiotics (Kadry et al., 2004).

6.4 Association between non-PEGylated cationic lipid concentrations and bacterial fusion kinetics

Fusogenic liposomes facilitate disruption of bacterial cell membranes through different mechanisms such as pore formation, lipid mixing and membrane permeabilization. This facilitates effective fusion and delivery of payload, leading to increased intracellular drug concentrations and improved bactericidal activity (Kolašinac et al., 2018). Cationic liposomes can facilitate the binding of antibiotic-encapsulated liposomal formulations to bacterial outer membrane phospholipids because of a stronger electrostatic interaction between positively charged liposomes and negatively charged bacterial cell surfaces (Kolašinac et al., 2018). A higher content of cationic lipids within liposomes can thus lead to increased binding of liposomal formulations to bacteria, improving the delivery of payload within the bacterial cells (Gubernator et al., 2007). I demonstrated that non-PEGylated meropenem-encapsulated liposomes with higher cationic lipid concentrations rapidly fused with bacterial and immune cells as compared to liposomes with no or minimal cationic lipid contents.

Flow cytometry was used for qualitative assessment of the effects of liposomal charges on real-time fusion between healthy volunteer immune cells or bacteria with non-PEGylated cationic liposomes *in vitro*. Uncharged liposomes did not fuse either with bacteria or white blood cells. However, fusion of cationic liposomes with healthy volunteer immune cells or bacteria occurred within seconds. A modest degree of binding was seen between liposomes containing 10% DODAB and bacteria and white cells. Fusion between liposomes and bacteria or WBCs increased with increasing charges over the liposomal surface (cationic liposomes having 20% and 30% DODAB, respectively).

Fusion between cationic liposomes and bacteria varied both by the type of bacteria and the cationic lipids content i.e., higher concentrations may promote faster fusion kinetics by facilitating closer interactions between liposomes and bacteria, thereby enhancing membrane destabilization (Laune et al., 2022). I found fusion rates to A Baumanni, and E coli were much more avid with liposomes having 30% DODAB compared to other liposomes. Up to a 5-fold relative increase in fusion was evident with liposomes containing 30% DODAB and A Baumanni and E coli compared to other liposomes. In contrast, liposome fusion to K

pneumoniae and P aeruginosa was similar between liposomes containing 20% or 30% DODAB, and lower compared to A Baumanni and E coli. Higher concentrations of anionic lipids such as phosphatidylglycerol and phosphatidylethanolamine are usually present on the outer bacterial surface (Laune et al., 2022). Therefore, direct interactions between antibiotic-encapsulated liposomes and bacterial cells strongly depend upon global charges on liposomal surfaces and bacterial surface patterns such as anionic lipid content, LPS structure and hydrophobic properties (Gubernator et al., 2007).

Immune cells possess negatively charged cell surfaces due to the presence of sialic acid residues on glycolipids and glycoproteins (Smith Korsholm et al., 2007). Immune cells also express receptors that can recognize and internalize cationic nanoparticles because of their scavenging and phagocytic properties (Smith Korsholm et al., 2007, Kedmi et al., 2010). Liposomes having higher cationic lipids can be recognized by these receptors, leading to rapid binding and uptake by immune cells. Using flow cytometry, liposomes containing 10% DODAB demonstrated similar or less avid binding to healthy immune cells compared to bacteria. By contrast, liposomes containing 20% or 30% DODAB demonstrated similar or greater binding to immune cells compared to bacteria, highlighting the complex interplay between cellular interactions and liposomal physiochemical properties; this can be used for selective and targeted drug delivery.

7. PEGylation to enhance the liposome's safety and efficacy

Liposomes can be made more stable and capable of targeting by altering their surface. This can be accomplished by adding polyethene glycol (PEGylation) which lengthens the circulation half-life by reducing immune system clearance (Haeri et al., 2017). Biocompatible hydrophilic polymers can be used to modify liposome surfaces to minimise the adsorption of circulating proteins and RES uptake (Song et al., 2012). This strategy, known as surface hydration or steric modification, involves conjugating lipids to hygroscopic or hydrophilic polymers such as polyethene glycol (PEG) or its derivatives (Haeri et al., 2017). The presence of hydrophilic polymers on liposome surfaces provides a hydration layer which reduces clearance by the RES (Jiang et al., 2016, Maruyama et al., 1992, Haeri et al., 2017). Stealth liposomes avoid detection and elimination by the RES. PEGylation is a popular technique to give liposomes stealth characteristics which helps them accumulate in specific areas and have a longer circulation time (**Figure 7**) (Deol and Khuller, 1997, Lasic and Martin, 2018, Haeri et al., 2017).

To accomplish targeted delivery, extravasation (at areas with higher vascular permeability, such as tumours and inflammatory regions) and appropriate biodistribution, liposomes must be stable within the bloodstream (Deol and Khuller, 1997, Lasic and Martin, 2018, Sheikholeslami et al., 2022). To prevent opsonization and phagocytosis, the hydrophilic polymer, polyethylene glycol (PEG) can protect and modify liposomes (Deol and Khuller, 1997, Lasic and Martin, 2018, Sheikholeslami et al., 2022). Polymeric PEG, constituting 5-10 % of total lipid concentrations (Maruyama et al., 1992, Haeri et al., 2017), can effectively modify liposomal surfaces, allowing them to pass through their intended destination unharmed (Deol and Khuller, 1997, Lasic and Martin, 2018). Surface modification of liposomes by PEGylation extended vancomycin plasma half-life from 30 min to >90 min and delayed uptake by activated phagocytes as compared to conventional (non-PEGylated) liposomal vancomycin under similar *in vitro* experimental conditions (Pumerantz et al., 2011).

PEG polymers may however prevent liposomes from engaging with the intended cells and dislodging any trapped material; this could, in turn, obstruct delivery (Deol and Khuller, 1997, Lasic and Martin, 2018). It is imperative to optimise the lipid percentages of PEG within the liposomal formulations to support rather than impede delivery (Lasic and Martin, 2018).

Summary of findings

7.1 Characterization of PEGylated cationic liposomes

As immune cell fusion and toxicity were seen with increasing liposomal charge, I incorporated 50 nm liposomes with 0.5% or 2.5% surface DSPE-PEG 2000. Meropenem-encapsulated liposomal formulations with variable cationic and PEGylated lipid concentrations were analysed using DLS and HPLC to assess the effects of pegylation on the physiochemical properties of the liposomes.

Size

Measured liposome sizes were consistently higher than expected, indicating fusion and aggregation due to interactions between fusogenic/helper lipids (Ferreira et al., 2021, Lechanteur et al., 2018, Haeri et al., 2017, Smith et al., 2017). Differences were seen in the measured size of 6:1:3 and 5:2:3 liposomes with the addition of 0.5% or 2.5% PEG compared to non-PEGylated liposomes. This was not seen with 3:3:4 liposomes, where increasing PEG lipid content resulted in smaller sizes due to steric hindrance and hydration effects, preventing liposomal aggregation and thereby reducing between-particle interaction.

Zeta potential

A significant reduction was seen in surface charge with increasing amounts of surface PEGylation ($p<0.001$ for all 3 liposome formulations). Higher concentrations of PEG lipids can reduce the overall liposomal surface charge by shielding the positive charges because of their hydrophilic chains. The reduction in surface charge can lead to decreased electrostatic repulsion between cationic liposomes, resulting in lower zeta potential (surface charge) and smaller aggregate sizes (Ferreira et al., 2021, Lechanteur et al., 2018, Haeri et al., 2017).

Polydispersity Index

The polydispersity index (PDI) increased with increasing amounts of surface PEGylation for 3:3:4 liposomes with higher DODAB, but not for 6:1:3 or 5:2:3 liposomes due to their heterogeneous surface properties and PEG chain mobility leading to fluctuations in liposomal size distribution and higher PDI values.

% internal meropenem, and total meropenem concentrations encapsulated within liposomes

Incorporation of PEG lipids within cationic liposomes can create a hydrostatic steric barrier that prevents effective encapsulation of meropenem within the liposome cores. Greater density of PEG chains on the liposomal surfaces led to greater steric hindrance, resulting in poor penetration and encapsulation of meropenem within the liposomal core (Ferreira et al., 2021, Lechanteur et al., 2018, Haeri et al., 2017). Increasing PEGylation was associated with reduced % internal meropenem ($p<0.01$ for all 3 liposome formulations) and encapsulation efficacy ($p<0.05$ for all 3 formulations).

Increased PEGylation results in denser PEG layers over the liposomal surfaces, so there are fewer available spaces within the liposomal core resulting in poor entrapment of meropenem (Haeri et al., 2017). Competition for available spaces between meropenem molecules and PEG chains can reduce the amount of meropenem that can be encapsulated within liposomes (Haeri et al., 2017, Bakker-Woudenberg et al., 1995). Using HPLC, I found that the drug concentration was significantly lower with 2.5% PEGylated liposomes compared to non-PEGylated 6:1:3 liposomes indicating poor drug penetration due to shielding effects. In contrast, PEGylated 5:2:3 liposomes 0.5% ($p=0.009$) and 2.5% ($p=0.017$) had higher drug concentrations

compared to non-PEGylated liposomes. PEGylation did not affect the drug concentration of 3:3:4 liposomes. Higher PEG concentrations enhance colloidal stability by providing a steric barrier, thereby preventing premature drug leakage due to degradation and aggregation.

24 hours liposomal stability

Over 24 hours at 4^{o}C, no significant differences were seen in measured size, charge, PDI or internal meropenem concentration. Although total meropenem concentration was consistently lower at 24 hours, showing minimal drug leakage and degradation of meropenem-encapsulated liposomes, this did not reach statistical significance.

7.2 PEGylated cationic liposomal antibiotics enhance the bactericidal activity of antibiotics

Incorporating biocompatible hydrophilic polymers within liposomes provides stealth properties, minimising adsorption by circulating proteins and RES uptake, thereby enhancing the circulation half-life (Rani et al., 2022, Song et al., 2012, Abu Lila et al., 2014). This will also facilitate the slow and sustained release of encapsulated antibiotics at the infection site, maximizing drug exposure to bacterial cells with enhanced bactericidal activity (Song et al., 2012). Non-PEGylated liposomes are rapidly cleared by the mononuclear phagocyte system, reducing circulation time and limiting opportunities for liposomes to reach the targeted sites (Song et al., 2012).

Incorporating PEGylated and cationic lipids within liposomes may create complementary effects by mitigating the toxicity associated with cationic lipids due to non-specific interactions and by preventing premature degradation., thereby enhancing the bactericidal activity of encapsulated antibiotics (Song et al., 2012, Abu Lila et al., 2014). Cationic lipids facilitate interaction and fusion with bacterial membranes, whereas PEGylated lipids improve biocompatibility, resulting in enhanced efficacy and safety against Gram-negative bacteria (Song et al., 2012, Abu Lila et al., 2014). PEGylation also enhances the physicochemical stability of liposomes by preventing aggregation and premature leakage of encapsulated drugs (Song et al., 2012, Abu Lila et al., 2014). This thereby preserves therapeutic payload until it reaches the target site.

Non-PEGylated liposomes with increasing DODAB had more rapid binding because of stronger electrostatic interactions between the cationic liposomal formulations and the negatively charged bacterial cell surface. However, the addition of incremental amounts of PEG lipids resulted in minimal binding with Gram-negative bacterial cells due to reduced electrostatic attractive forces.

Both PEGylated meropenem-encapsulated liposomes with variable cationic lipid concentrations (10%, and 30% DODAB) had enhanced bactericidal activity against clinical strains of Gram-negative bacteria i.e., a 12 to 46-fold reduction in MIC compared to standard free meropenem or liposomal formulations without encapsulated meropenem (empty liposomes). Meropenem, gentamicin and ciprofloxacin, when

encapsulated within non-PEGylated cationic liposomal formulations demonstrated a 2 to 4-fold reduction in MIC against clinical isolates of Gram-negative and Gram-positive bacteria in comparison to anionic and neutral liposomes, and commercially available free antimicrobial drugs (Gubernator et al., 2007, Drulis-Kawa et al., 2006b, Drulis-Kawa et al., 2006a).

PEGylated meropenem-encapsulated cationic liposomes improved drug delivery by preventing enzymatic degradation and enhancing local drug concentrations. This may help to mitigate different resistance mechanisms. I used the broth-microdilution method to assess the MIC for 6:1:3 liposomes (with and without PEGylation) against four different meropenem-resistant bacteria (16 strains) isolated from patients. Among these sixteen strains, statistically significant reductions in MIC for meropenem were seen for some carbapenem-resistant strains. 6.1.3 (with and without PEGylation) only brought MIC down to within the susceptible range for 1 of 16 resistant strains. This reduction in MIC is therefore unlikely to have any clinical significance, suggesting the incorporation of different classes of antibiotics or a combination of different classes of antibiotics that can target specific resistance mechanisms within the same liposomes can enhance bactericidal activity by mitigating various enzymatic and non-enzymatic (alteration at drug receptor sites, decreased membrane permeability and efflux pump upregulation) resistance approaches (Ferreira et al., 2021).

Gram-negative bacteria may have varying resistance mechanisms, such as non-enzymatic (efflux pumps, decreased bacterial membrane permeability and structural modifications at drug receptor sites), or enzymatic approaches associated with antibiotic degradation (Drulis-Kawa et al., 2006b). Meropenem-encapsulated liposomes may avoid different resistance approaches by facilitating enhanced drug delivery directly into the bacterial cells. This depends on the physiochemical properties of liposomes and the mechanism of action of the encapsulated antibiotics (Gubernator et al., 2007). PEGylated cationic liposomes thus help to overcome different resistance mechanisms, thereby enhancing the effectiveness and safety of antibiotics against resistant Gram-negative bacterial strains.

7.3 PEGylation enhances *in-vitro* safety of cationic liposomes

PEGylation of meropenem-encapsulated liposomes can reduce cytotoxicity associated with higher positively charged liposomes (Song et al., 2012, Abu Lila et al., 2014). Without PEGylation the liposomes may interact with negatively charged cell membranes, leading to immune cell activation and cytotoxic effects (Song et al., 2012, Abu Lila et al., 2014). PEGylation creates a steric hydrostatic barrier around liposomal surfaces, thereby shielding the cationic charges and reducing nonspecific interactions with circulating cells and proteins; this limits the immunogenicity of liposomes (Song et al., 2012, Abu Lila et al., 2014). PEGylation also enhances the stability of encapsulated antibiotics by preventing aggregation, opsonization and recognition by the RES. This helps to modulate the drug's release kinetics from liposomes, resulting in the sustained release of

antibiotics, enhancing the circulation half-life of meropenem-encapsulated cationic liposomes, and reducing the potential for systemic toxicity (Song et al., 2012, Abu Lila et al., 2014).

In the *in-vitro* model, I demonstrated that meropenem-encapsulated liposomes with 10% and 20% DODAB with an incremental increase of PEG (0.5% and 2.5% DSPE-2000) did not show dose-dependent immune cell activation i.e., liposomal internalization, ROS production, immune cell viability, increased pro-inflammatory cytokine production, and haemolysis as compared to non-PEGylated and standard free meropenem at both therapeutic (10 μg/ml) and supra-therapeutic levels (100 μg/ml). Non-PEGylated meropenem-encapsulated liposomes with higher cationic concentrations (20% and 30%) had dose-dependent cytotoxic adverse events on immune cells.

Incorporating PEG can impair the release kinetics of drugs encapsulated within liposomes (Abu Lila et al., 2014). Surface-engineered PEGylated liposomes containing Dapt-PEG-DSPE (daptomycin conjugated to DSPE via a PEG linker) with selectivity for Methicillin-Resistant Staphylococcus Aureus (MRSA) enhanced the targeted delivery and potency of the encapsulated drug in comparison with conventional PEGylated formulations. (Jiang et al., 2016). Daptomycin-modified liposomes enhanced targeted delivery and selective binding of encapsulated antibiotics to MRSA, increasing drug accumulation at the infection site with limited side effects (Jiang et al., 2016).

7.4 Association between PEGylated cationic lipid concentrations and bacterial fusion kinetics

Polymeric PEG, constituting 5–10 % of total lipid concentrations (Maruyama et al., 1992, Haeri et al., 2017), introduces hydrophilic PEG chains over the surface of liposomes, creating steric hydrostatic barriers and thereby hinders interactions between cationic liposomal formulation and bacterial membranes. PEGylation enhances cationic liposomal stability by reducing fusion, aggregation and opsonization (Haeri et al., 2017). However, higher PEG concentrations can alter the physiochemical properties of liposomes such as curvature and membrane fluidity due to increased steric hindrances, and this may influence their interactions with bacterial membranes (Haeri et al., 2017). Higher PEG concentrations had stronger steric hindrances over the liposomal surface, thereby limiting the efficiency of cationic liposome-bacterial fusion kinetics (Haeri et al., 2017). Optimal PEG concentrations are required to facilitate the balance between steric repulsive forces provided by PEG chains and the desired interactions between liposomes and bacteria.

Increasing amounts of DSPE-2000 PEGylation of liposomes were associated with significant changes to liposomal physicochemical properties (including size, charge, PDI and % internal meropenem concentration). PEGylated meropenem-encapsulated liposomes with higher DSPE-PEG 2000 concentrations showed no or minimal fusion between cationic liposomes irrespective of higher DODAB concentrations (20%, 30%) and bacterial and immune cells as compared to cationic liposomes with no or minimal DSPE-PEG 2000 lipid contents. Moreover, non-PEGylated liposomes with increasing DODAB were associated with more rapid and

avid binding because of stronger electrostatic interactions between cationic liposomes and the negatively charged bacterial cell surface. The addition of incremental amounts of PEG (0.5% and 2.5%) resulted in minimal or no binding with Gram-negative bacteria due to hydrostatic steric barriers providing stronger electrostatic repulsive forces.

Host cytotoxicity and inflammation resulting from bacterial death was lower for PEGylated meropenem-encapsulated than non-pegylated liposomes as surface modification by PEGylation reduces neutrophil uptake of the cationic liposomes. Non-pegylated liposomes with higher positively charged lipids, and PEGylated cationic liposomes with lower DSPE-PEG 2000 concentrations, bound more avidly to immune cells. Increasing amounts of PEGylation showed decreased fusion to bacteria and immune cells. Confocal imaging also demonstrated significant aggregation of liposomes with an increase in surface charge which was reduced by PEGylation. 3:3:4 liposomes caused increased immune cell activation and cell death, but this too was reduced by PEGylation. Higher concentrations of PEG (2.5%) resulted in increasing amounts of cytokine release on *ex-vivo* whole blood assays. Interactions between higher concentrations of PEG (2.5%) and increased pro-inflammatory cytokine production depend on multiple factors e.g. higher PEG concentrations altering cell membrane permeability. This results in immune cell activation due to osmotic stress and the triggering of inflammatory pathways (Zhang et al., 2007). Higher PEG concentrations may facilitate cellular PEG uptake by endocytosis; this may induce cellular stress and subsequent pro-inflammatory cytokine production as a defensive response (Zhang et al., 2007).

Understanding these mechanisms is crucial for optimizing the use of PEG in biomedical applications, ensuring that it is used at concentrations that do not inadvertently trigger unwanted immune responses. Further research into specific pathways and cellular interactions could offer more detailed insights into how PEG influences cytokine release at different concentrations.

Surface modification of liposomes by PEGylation extended the vancomycin plasma half-life from 30 min to >90 min and delayed uptake by activated phagocytes as compared to conventional (non-PEGylated) liposomal vancomycin under similar *in-vitro* experimental conditions (Pumerantz et al., 2011). Therefore, the incorporation of optimal concentrations of PEG lipids within liposomal formulations will reduce attractive forces due to stronger steric repulsive forces between PEGylated cationic liposomal formulations, and human immune and bacterial cells.

7.5 Lipid emulsions ameliorate inflammatory responses associated with LPS

Cell membranes of both Gram-positive and Gram-negative bacteria are rich in acidic phospholipids including phosphatidylglycerol (PG) and LPS. (Harm et al., 2021b) Anionic phospholipids present within the outer bacterial cell membrane possess a greater negative charge compared to mammalian cells (Silhavy et al., 2010). Different lipid emulsions may facilitate unique therapeutic targets against bacteria and are pivotal for

the binding and neutralization of LPS (Zasloff, 2002). Lipopolysaccharide (LPS) is a major constituent of the outer cell wall of Gram-negative bacteria and is proinflammatory (Harm et al., 2021b). Microbial lysis releases LPS, increasing the host inflammatory response and worsening organ dysfunction (Peng et al., 2012, Skorup et al., 2020). Different lipid emulsions bind to and neutralize LPS (Harm et al., 2021a).

I found meropenem-encapsulated liposomes with variable cationic and DSPE-PEG 2000 lipid concentrations had lower host cytotoxicity and inflammation resulting from bacterial toxin (LPS) compared to free standard meropenem. At lower concentrations non-PEGylated 6:1:3 liposomes reduced IL-6 production, while non-PEGylated 6:1:3 and 3:4:4 liposomes induced minimal TNF-α production compared to free meropenem and different meropenem-encapsulated liposomes. At higher meropenem concentrations, non-PEGylated 3:3:4 liposomes lowered the production of pro-inflammatory cytokines, while non-PEGylated 6:1:3 liposomes reduced TNF-α production only (compared to free meropenem and various non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations).

Numerous *ex-vivo* and preclinical studies have demonstrated that lipoproteins neutralize endotoxins dependent on the phospholipid composition of liposomes (Goldfarb et al., 2003). Protein-free, phospholipid emulsions in healthy individuals receiving endotoxin resulted in a marked reduction in circulating cytokines and an attenuated physiological response (Gordon et al., 2005). Hypolipidaemia, particularly circulating levels of serum lipoproteins, was associated with an increased risk of life-threatening infections in critically ill patients (SIRS) (Goldfarb et al., 2003, Dellinger et al., 2009). This may relate to their endotoxin binding and neutralization capabilities (Goldfarb et al., 2003, Dellinger et al., 2009). The LIPOS study assessed the dose-dependent efficacy and safety benefits of phospholipid emulsions in sepsis but this failed to demonstrate any benefit in all-cause mortality or prevention of new-onset organ dysfunction (Dellinger et al., 2009). A more recent clinical trial investigated the safety profile and efficacy of CAL-02, a toxin-binding phospholipid, at variable concentrations in ICU patients with severe community-acquired pneumonia. It improved inflammatory and clinical outcomes with fewer adverse events as compared to antibiotics alone (Laterre et al., 2019).

8. Conclusion

The rising incidence of antimicrobial resistance (AMR) is one of the greatest modern healthcare challenges. The use of liposomes to improve antibiotic delivery, specificity, bioavailability, and efficacy should be explored to enhance existing antibiotics and overcome antimicrobial resistance (AMR).

Non-PEGylated and PEGylated meropenem-encapsulated cationic liposomal formulations had enhanced in-vitro bactericidal activity. A 12 to 30-fold reduction in MIC was achieved against multiple laboratory and clinical strains of Gram-negative bacteria using meropenem-encapsulated liposomes compared to native meropenem in-vitro. PEGylation further improved biocompatibility. Incorporation of antibiotics into the liposomes using different approaches, and/or the addition of other antimicrobial agents such as antimicrobial peptides (AMPs), that can target specific resistance mechanisms, may enhance bactericidal activity by mitigating various enzymatic and non-enzymatic resistance approaches. Future work should aim to refine and optimise drug encapsulation and delivery methods for antimicrobial agents to develop novel therapeutic approaches, particularly for the treatment of antimicrobial resistance (AMR).

Further investigations in different *in-vitro* and animal-based infection models and clinical studies are needed to ascertain if antibiotic-encapsulated liposomes could achieve adequate drug concentrations within the infected tissues. Hence, the use of liposomes as alternative drug carriers to enhance bactericidal activity and the safety of pre-existing antibiotics are worth exploring.

9. Future research directions

9.1 *In-vitro* work plan

a) Encapsulation and optimization of different liposomal antibiotics

Plan:

- The liposomal properties screen will include vesicle diameter, surface modifications and small molecule antibiotic drug cargo. Broad-spectrum antibiotics with different mechanistic approaches against Gram-Negative and Gram-Positive pathogens will be encapsulated within structurally modified liposome formulations and the physiochemical properties will be assessed using DLS and HPLC techniques.

b) Establishing the optimal formulation of different liposomal antibiotics, with the best efficacy and safety

Plan:

- Various permutations of PEG at variable concentrations, with different lipid compositions, and sizes will be assessed to demonstrate the efficacy and safety profile of different pre-existing antibiotics. Variable concentrations of PEG-cholesterol will be incorporated into the liposome to decrease immunogenicity. The optimal PEG ratio to minimize immune activation whilst maintaining antimicrobial activity will be determined.

c) In-vitro bactericidal activity of the liposomal antibiotics

Plan:

- Various lab strains (Gram-Negative and Gram-Positive bacteria) will be incubated with a range of different liposomal antibiotics overnight to evaluate the MIC values using the broth microdilution method and bacterial viability will be decided by overnight growth on agar plates to ascertain minimum bactericidal concentrations (MBC) following EUCAST guidelines.

d) Testing optimal liposomal formulations against clinical strains of antibiotics including clinical and resistant strains

Plan:

- Cationic liposomal formulations encapsulating different classes of antibiotics will be evaluated against different clinical and resistant strains of Gram-Negative and Gram-Positive microbes to demonstrate the *in-vitro* bactericidal activity.

e) Time to kill assay

This study will be performed to understand the interactions between different antibiotics encapsulated within the cationic liposomes and microorganisms to demonstrate time or concentration-dependent effects. This study will help determine the antimicrobial activity of cationic liposomal formulations against Gram-Negative and Gram-Positive bacterial strains.

Plan:

Culture Preparation:

- Gram-negative bacteria will be inoculated into Mueller-Hinton broth medium and incubated overnight to reach the exponential growth phase. The culture will be then adjusted to a specific optical density (0.1) to standardize the inoculum.

Inoculation:

- A series of sterile tubes containing the broth medium with the standardized microbial culture will be inoculated. Antibiotics encapsulated cationic liposomes and free standard antimicrobial agents will be added to each tube to achieve the desired concentrations. A culture containing the microorganisms without an antimicrobial agent to monitor natural growth will be used as a positive control, whereas a sterile medium with the antimicrobial agent will be used as a negative control to monitor the contamination. Aliquots will be removed from each culture at predetermined time points (e.g., 0, 1, 2, 4, 6, 8, 24 hours) and serially diluted in sterile saline to achieve countable colonies (CFUs). The agar plates will be incubated at the appropriate temperature until colonies are visible and the number of colonies will be counted on each plate to determine the number of viable cells (CFU/mL) at different predetermined time points.

f). Evaluating the safety of liposomal antibiotics against mammalian cell lines and primary cells

Plan:

- Whole blood isolated from healthy volunteers will be incubated with various formulations (liposomal antibiotics ± phospholipids ± PEG) / doses of liposomes to assess effects on immune cell activation, using flow cytometry to assess cell viability, ROS production, and ELISA for cytokine release.
- Human cell lines (e.g., HepG2 hepatocytes) will be incubated with different liposomal antibiotics at increasing concentrations and different durations. Inflammation and cell viability assays will be assessed. Incubate fluorophore (Alexa 647)-tagged different liposomal antibiotics for 6 hours. Perform CLSM and flow cytometry studies to reveal the internalization of liposomes with reactive oxygen species (ROS) formation, and cell viability. Increasing doses of PEG-cholesterol will be incorporated into the liposome to decrease immunogenicity.

10. References

https://www.WHO.int/news/item/15-04-2021-global-shortage-of-innovative-antibiotics-fuels-emergence-and-spread-of-drug-resistance.

ABU LILA, A. S., UEHARA, Y., ISHIDA, T. & KIWADA, H. 2014. Application of polyglycerol coating to plasmid DNA lipoplex for the evasion of the accelerated blood clearance phenomenon in nucleic acid delivery. J Pharm Sci, 103, 557-66.

ABUSHAHEEN, M. A., FATANI, A. J., ALOSAIMI, M., MANSY, W., GEORGE, M., ACHARYA, S., RATHOD, S., DIVAKAR, D. D., JHUGROO, C. & VELLAPPALLY, S. 2020. Antimicrobial resistance, mechanisms, and its clinical significance. Disease-a-Month, 66, 100971.

ALHARIRI, M., AZGHANI, A. & OMRI, A. 2013. Liposomal antibiotics for the treatment of infectious diseases. Expert opinion on drug delivery, 10, 1515-1532.

ALIPOUR, M., HALWANI, M., OMRI, A. & SUNTRES, Z. E. 2008. Antimicrobial effectiveness of liposomal polymyxin B against resistant Gram-negative bacterial strains. International Journal of Pharmaceutics, 355, 293-298.

ALIPOUR, M. & SUNTRES, Z. E. 2014. Liposomal antibiotic formulations for targeting the lungs in the treatment of Pseudomonas aeruginosa. Therapeutic delivery, 5, 409-427.

ALJIHANI, S. A., ALEHAIDEB, Z., ALARFAJ, R. E., ALGHORIBI, M. F., AKIEL, M. A., ALENAZI, T. H., AL-FAHAD, A. J., AL TAMIMI, S. M., ALBAKR, T. M. & ALSHEHRI, A. 2020. Enhancing azithromycin antibacterial activity by encapsulation in liposomes/liposomal-N-acetylcysteine formulations against resistant clinical strains of Escherichia coli. Saudi Journal of Biological Sciences, 27, 3065-3071.

ALLEN, T., HANSEN, C., MARTIN, F., REDEMANN, C. & YAU-YOUNG, A. 1991. Liposomes containing synthetic lipid derivatives of poly (ethylene glycol) show prolonged circulation half-lives in vivo. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1066, 29-36.

ANGSANTIKUL, P., THAMPHIWATANA, S., ZHANG, Q., SPIEKERMANN, K., ZHUANG, J., FANG, R. H., GAO, W., OBONYO, M. & ZHANG, L. 2018. Coating Nanoparticles with Gastric Epithelial Cell Membrane for Targeted Antibiotic Delivery against Helicobacter pylori Infection. Advanced Therapeutics, 1, 1800016.

ANTIMISIARIS, S., MARAZIOTI, A., KANNAVOU, M., NATSARIDIS, E., GKARTZIOU, F., KOGKOS, G. & MOURTAS, S. 2021. Overcoming barriers by local drug delivery with liposomes. Advanced drug delivery reviews, 174, 53-86.

ATASHBEYK, D. G., KHAMENEH, B., TAFAGHODI, M. & FAZLY BAZZAZ, B. S. 2014. Eradication of methicillin-resistant Staphylococcus aureus infection by nanoliposomes loaded with gentamicin and oleic acid. Pharmaceutical Biology, 52, 1423-1428.

AVERSA, F., BUSCA, A., CANDONI, A., CESARO, S., GIRMENIA, C., LUPPI, M., NOSARI, A. M., PAGANO, L., ROMANI, L. & ROSSI, G. 2017. Liposomal amphotericin B (AmBisome®) at the beginning of its third decade of clinical use. Journal of Chemotherapy, 29, 131-143.

AZZOPARDI, E. A., FERGUSON, E. L. & THOMAS, D. W. 2012. The enhanced permeability retention effect: a new paradigm for drug targeting in infection. Journal of Antimicrobial Chemotherapy, 68, 257-274.

BAKKER-WOUDENBERG, I. A., TEN KATE, M., STEARNE-CULLEN, L. & WOODLE, M. 1995. Efficacy of gentamicin or ceftazidime entrapped in liposomes with prolonged blood circulation and enhanced localization in Klebsiella pneumoniae-infected lung tissue. Journal of Infectious Diseases, 171, 938-947.

BAKKER-WOUDENBERG, I. A. J. M., KATE, M. T. T., GUO, L., WORKING, P. & MOUTON, J. W. 2002. Ciprofloxacin in Polyethylene Glycol-Coated Liposomes: Efficacy in Rat Models of Acute or Chronic Pseudomonas aeruginosa Infection. Antimicrobial Agents and Chemotherapy, 46, 2575-2581.

BARTOMEU GARCIA, C., SHI, D. & WEBSTER, T. J. 2017. Tat-functionalized liposomes for the treatment of meningitis: an in vitro study. Int J Nanomedicine, 12, 3009-3021.

BAXA, U. 2018. Imaging of Liposomes by Transmission Electron Microscopy. In: MCNEIL, S. E. (ed.) Characterization of Nanoparticles Intended for Drug Delivery. New York, NY: Springer New York.

BHATTACHARJEE, S. 2016. DLS and zeta potential – What they are and what they are not? Journal of Controlled Release, 235, 337-351.

BONTÉ, F. & JULIANO, R. L. 1986. Interactions of liposomes with serum proteins. Chemistry and physics of lipids, 40, 359-372.

BROOKS, B. D. & BROOKS, A. E. 2014. Therapeutic strategies to combat antibiotic resistance. Adv Drug Deliv Rev, 78, 14-27.

BROUWER, M. C., TUNKEL, A. R. & VAN DE BEEK, D. 2010. Epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis. Clinical microbiology reviews, 23, 467-492.

BROWN, E. D. & WRIGHT, G. D. 2016. Antibacterial drug discovery in the resistance era. Nature, 529, 336-343.

BUIJK, S., MOUTON, J., GYSSENS, I., VERBRUGH, H. & BRUINING, H. 2002. Experience with a once-daily dosing program of aminoglycosides in critically ill patients. Intensive care medicine, 28, 936-942.

CAVALCANTI, R. R., LIRA, R. B. & RISKE, K. A. 2022. Membrane fusion biophysical analysis of fusogenic liposomes. Langmuir, 38, 10430-10441.

CLEMONS, T. D., SINGH, R., SOROLLA, A., CHAUDHARI, N., HUBBARD, A. & IYER, K. S. 2018. The distinction between active and passive targeting of nanoparticles dictates their overall therapeutic efficacy. Langmuir, 34, 15343-15349.

CODJOE, F. S. & DONKOR, E. S. 2017. Carbapenem resistance: a review. Medical Sciences, 6, 1.

CROMMELIN, D. J. A., VAN HOOGEVEST, P. & STORM, G. 2020. The role of liposomes in clinical nanomedicine development. What now? Now what? Journal of Controlled Release, 318, 256-263.

DARAEE, H., ETEMADI, A., KOUHI, M., ALIMIRZALU, S. & AKBARZADEH, A. 2016. Application of liposomes in medicine and drug delivery. Artificial cells, nanomedicine, and biotechnology, 44, 381-391.

DARBY, E. M., TRAMPARI, E., SIASAT, P., GAYA, M. S., ALAV, I., WEBBER, M. A. & BLAIR, J. M. 2023. Molecular mechanisms of antibiotic resistance revisited. Nature Reviews Microbiology, 21, 280-295.

DEL AMO, L., CANO, A., ETTCHETO, M., SOUTO, E. B., ESPINA, M., CAMINS, A., GARCÍA, M. L. & SÁNCHEZ-LÓPEZ, E. 2021. Surface Functionalization of PLGA Nanoparticles to Increase Transport across the BBB for Alzheimer's Disease. Applied Sciences, 11, 4305.

DELLINGER, R. P., TOMAYKO, J. F., ANGUS, D. C., OPAL, S., CUPO, M. A., MCDERMOTT, S., DUCHER, A., CALANDRA, T. & COHEN, J. 2009. Efficacy and safety of a phospholipid emulsion (GR270773) in Gram-negative severe sepsis: results of a phase II multicenter, randomized, placebo-controlled, dose-finding clinical trial. Critical care medicine, 37, 2929-2938.

DEOL, P. & KHULLER, G. 1997. Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. Biochimica et Biophysica Acta (BBA)-General Subjects, 1334, 161-172.

DERBALI, R. M., AOUN, V., MOUSSA, G., FREI, G., TEHRANI, S. F., DEL'ORTO, J. C., HILDGEN, P., ROULLIN, V. G. & CHAIN, J. L. 2019. Tailored nanocarriers for the pulmonary delivery of levofloxacin against Pseudomonas aeruginosa: a comparative study. Molecular Pharmaceutics, 16, 1906-1916.

DOKKA, S., TOLEDO, D., SHI, X., CASTRANOVA, V. & ROJANASAKUL, Y. 2000. Oxygen Radical-Mediated Pulmonary Toxicity Induced by Some Cationic Liposomes. Pharmaceutical Research, 17, 521-525.

DRULIS-KAWA, Z. & DOROTKIEWICZ-JACH, A. 2010. Liposomes as delivery systems for antibiotics. International journal of pharmaceutics, 387, 187-198.

DRULIS-KAWA, Z., GUBERNATOR, J., DOROTKIEWICZ-JACH, A., DOROSZKIEWICZ, W. & KOZUBEK, A. 2006a. A comparison of the in vitro antimicrobial activity of liposomes containing meropenem and gentamicin. Cellular and Molecular Biology Letters, 11, 360-375.

DRULIS-KAWA, Z., GUBERNATOR, J., DOROTKIEWICZ-JACH, A., DOROSZKIEWICZ, W. & KOZUBEK, A. 2006b. In vitro antimicrobial activity of liposomal meropenem against Pseudomonas aeruginosa strains. International Journal of Pharmaceutics, 315, 59-66.

DRULIS-KAWA, Z., GUBERNATOR, J., DOROTKIEWICZ-JACH, A., DOROSZKIEWICZ, W. & KOZUBEK, A. 2006c. In vitro antimicrobial activity of liposomal meropenem against Pseudomonas aeruginosa strains. International journal of pharmaceutics, 315, 59-66.

EBRAHIMI, S., FARHADIAN, N., KARIMI, M. & EBRAHIMI, M. 2020. Enhanced bactericidal effect of ceftriaxone drug encapsulated in nanostructured lipid carrier against gram-negative Escherichia coli bacteria: drug formulation, optimization, and cell culture study. Antimicrobial Resistance & Infection Control, 9, 1-12.

EGOROV, A. M., ULYASHOVA, M. M. & RUBTSOVA, M. Y. 2020. Inhibitors of β-Lactamases. New Life of β-Lactam Antibiotics. Biochemistry (Moscow), 85, 1292-1309.

ELMAS, M., YAZAR, E., BAŞ, A., TRAŞ, B., BAYEZIT, M. & YAPAR, K. 2002. Comparative Pharmacokinetics of Enrofloxacin and Tissue Concentrations of Parent Drug and Ciprofloxacin after Intramuscular Administrations of Free and Liposome-Encapsulated Enrofloxacin in Rabbits. Journal of Veterinary Medicine, Series B, 49, 507-512.

EPAND, R. M. & EPAND, R. F. 2009. Domains in bacterial membranes and the action of antimicrobial agents. Molecular BioSystems, 5, 580-587.

FERNANDES, R., AMADOR, P. & PRUDÊNCIO, C. 2013. β-Lactams: chemical structure, mode of action and mechanisms of resistance. Reviews and Research in Medical Microbiology, 24, 7-17.

FERREIRA, M., OGREN, M., DIAS, J. N., SILVA, M., GIL, S., TAVARES, L., AIRES-DA-SILVA, F., GASPAR, M. M. & AGUIAR, S. I. 2021. Liposomes as antibiotic delivery systems: a promising nanotechnological strategy against antimicrobial resistance. Molecules, 26, 2047.

FU, Y.-Y., ZHANG, L., YANG, Y., LIU, C.-W., HE, Y.-N., LI, P. & YU, X. 2019. Synergistic antibacterial effect of ultrasound microbubbles combined with chitosan-modified polymyxin B-loaded liposomes on biofilm-producing Acinetobacter baumannii. International journal of nanomedicine, 1805-1815.

FURNERI, P. M., FRESTA, M., PUGLISI, G. & TEMPERA, G. 2000. Ofloxacin-loaded liposomes: in vitro activity and drug accumulation in bacteria. Antimicrobial agents and chemotherapy, 44, 2458-2464.

GAO, W., CHEN, Y., ZHANG, Y., ZHANG, Q. & ZHANG, L. 2018. Nanoparticle-based local antimicrobial drug delivery. Advanced Drug Delivery Reviews, 127, 46-57.

GASPAR, M., COUET, W., OLIVIER, J.-C., PAIS, A. & SOUSA, J. 2013. Pseudomonas aeruginosa infection in cystic fibrosis lung disease and new perspectives of treatment: a review. European Journal of Clinical Microbiology & Infectious Diseases, 32, 1231-1252.

GHARIB, A., FAEZIZADEH, Z. & GODARZEE, M. 2012. In vitro and in vivo activities of ticarcillin-loaded nanoliposomes with different surface charges against Pseudomonas aeruginosa (ATCC 29248). DARU Journal of Pharmaceutical Sciences, 20, 1-7.

GISKE, C. G., TURNIDGE, J., CANTÓN, R. & KAHLMETER, G. 2022a. Update from the European Committee on antimicrobial susceptibility testing (EUCAST). Journal of clinical microbiology, 60, e00276-21.

GISKE, C. G., TURNIDGE, J., CANTÓN, R. & KAHLMETER, G. 2022b. Update from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). J Clin Microbiol, 60, e0027621.

GOLDFARB, R. D., PARKER, T. S., LEVINE, D. M., GLOCK, D., AKHTER, I., ALKHUDARI, A., MCCARTHY, R. J., DAVID, E. M., GORDON, B. R. & SAAL, S. D. 2003. Protein-free phospholipid emulsion treatment improved cardiopulmonary function and survival in porcine sepsis. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 284, R550-R557.

GONZALEZ GOMEZ, A. & HOSSEINIDOUST, Z. 2020. Liposomes for Antibiotic Encapsulation and Delivery. ACS Infectious Diseases, 6, 896-908.

GORDON, B. R., PARKER, T. S., LEVINE, D. M., FEUERBACH, F., SAAL, S. D., SLOAN, B.-J., CHU, C., STENZEL, K. H., PARRILLO, J. E. & RUBIN, A. L. 2005. Neutralization of endotoxin by a phospholipid emulsion in healthy volunteers. The Journal of Infectious Diseases, 191, 1515-1522.

GORDON, B. R., PARKER, T. S., LEVINE, D. M., SAAL, S. D., HUDGINS, L. C., SLOAN, B.-J., CHU, C., STENZEL, K. H. & RUBIN, A. L. 2003. Safety and pharmacokinetics of an endotoxin-binding phospholipid emulsion. Annals of Pharmacotherapy, 37, 943-950.

GOTTESMANN, M., GOYCOOLEA, F. M., STEINBACHER, T., MENOGNI, T. & HENSEL, A. 2020. Smart drug delivery against Helicobacter pylori: pectin-coated, mucoadhesive liposomes with antiadhesive activity and antibiotic cargo. Applied Microbiology and Biotechnology, 104, 5943-5957.

GROLL, A. H., RIJNDERS, B. J., WALSH, T. J., ADLER-MOORE, J., LEWIS, R. E. & BRÜGGEMANN, R. J. 2019. Clinical pharmacokinetics, pharmacodynamics, safety, and efficacy of liposomal amphotericin B. Clinical Infectious Diseases, 68, S260-S274.

GUBERNATOR, J., DRULIS-KAWA, Z., DOROTKIEWICZ-JACH, A., DOROSZKIEWICZ, W. & KOZUBEK, A. 2007. In vitro antimicrobial activity of liposomes containing ciprofloxacin, meropenem and gentamicin against gram-negative clinical bacterial strains. Letters in Drug Design & Discovery, 4, 297-304.

HABRANT, D., PEUZIAT, P., COLOMBANI, T., DALLET, L., GEHIN, J., GOUDEAU, E., EVRARD, B., LAMBERT, O., HAUDEBOURG, T. & PITARD, B. 2016. Design of ionizable lipids to overcome the limiting step of endosomal escape: application in the intracellular delivery of mRNA, DNA, and siRNA. Journal of medicinal chemistry, 59, 3046-3062.

HAERI, A., SADEGHIAN, S., RABBANI, S., SHIRANI, S., ANVARI, M. S. & DADASHZADEH, S. 2017. Physicochemical characteristics of liposomes are decisive for their anti-restenosis efficacy following local delivery. Nanomedicine, 12, 131-145.

HALWANI, M., BLOMME, S., SUNTRES, Z. E., ALIPOUR, M., AZGHANI, A. O., KUMAR, A. & OMRI, A. 2008. Liposomal bismuth-ethanedithiol formulation enhances the antimicrobial activity of tobramycin. International journal of pharmaceutics, 358, 278-284.

HARASHIMA, H., SAKATA, K., FUNATO, K. & KIWADA, H. 1994. Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. Pharmaceutical research, 11, 402-406.

HARM, S., SCHILDBÖCK, C., STROBL, K. & HARTMANN, J. 2021a. An in vitro study on factors affecting endotoxin neutralization in human plasma using the Limulus amebocyte lysate test. Sci Rep, 11, 4192.

HARM, S., SCHILDBÖCK, C., STROBL, K. & HARTMANN, J. 2021b. An in vitro study on factors affecting endotoxin neutralization in human plasma using the Limulus amebocyte lysate test. Scientific Reports, 11, 1-23.

HENRY, B. D., NEILL, D. R., BECKER, K. A., GORE, S., BRICIO-MORENO, L., ZIOBRO, R., EDWARDS, M. J., MÜHLEMANN, K., STEINMANN, J. & KLEUSER, B. 2015a. Engineered liposomes sequester bacterial exotoxins and protect them from severe invasive infections in mice. Nature Biotechnology, 33, 81-88.

HENRY, B. D., NEILL, D. R., BECKER, K. A., GORE, S., BRICIO-MORENO, L., ZIOBRO, R., EDWARDS, M. J., MÜHLEMANN, K., STEINMANN, J., KLEUSER, B., JAPTOK, L., LUGINBÜHL, M., WOLFMEIER, H., SCHERAG, A., GULBINS, E., KADIOGLU, A., DRAEGER, A. & BABIYCHUK, E. B. 2015b. Engineered liposomes sequester bacterial exotoxins and protect them from severe invasive infections in mice. Nature Biotechnology, 33, 81-88.

HOLMES, A. H., MOORE, L. S., SUNDSFJORD, A., STEINBAKK, M., REGMI, S., KARKEY, A., GUERIN, P. J. & PIDDOCK, L. J. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. The Lancet, 387, 176-187.

HUANG, Q., ZENG, J. & YAN, J. 2021. COVID-19 mRNA vaccines. Journal of Genetics and Genomics, 48, 107-114.

HUWAITAT, R., MCCLOSKEY, A. P., GILMORE, B. F. & LAVERTY, G. 2016. Potential strategies for the eradication of multidrug-resistant Gram-negative bacterial infections. Future microbiology, 11, 955-972.

JIANG, H., XIONG, M., BI, Q., WANG, Y. & LI, C. 2016. Self-enhanced targeted delivery of a cell wall– and membrane-active antibiotics, daptomycin, against staphylococcal pneumonia. Acta Pharmaceutica Sinica B, 6, 319-328.

JIANG, S.-Y., LI, H., TANG, J.-J., WANG, J., LUO, J., LIU, B., WANG, J.-K., SHI, X.-J., CUI, H.-W. & TANG, J. 2018. Discovery of a potent HMG-CoA reductase degrader that eliminates statin-induced reductase accumulation and lowers cholesterol. Nature Communications, 9, 1-13.

JINDAL, A., PANDYA, K. & KHAN, I. 2015. Antimicrobial resistance: A public health challenge. Medical Journal Armed Forces India, 71, 178-181.

JOSHI, S., SINGH-MOON, R. P., ELLIS, J. A., CHAUDHURI, D. B., WANG, M., REIF, R., BRUCE, J. N., BIGIO, I. J. & STRAUBINGER, R. M. 2015. Cerebral hypoperfusion-assisted intra-arterial deposition of liposomes in normal and glioma-bearing rats. Neurosurgery, 76, 92-100.

JULIANO, R. L. & STAMP, D. 1978. Pharmacokinetics of liposome-encapsulated anti-tumor drugs: Studies with vinblastine, actinomycin D, cytosine arabinoside, and daunomycin. Biochemical Pharmacology, 27, 21-27.

JYOTHI, V. G. S., BULUSU, R., RAO, B. V. K., PRANOTHI, M., BANDA, S., BOLLA, P. K. & KOMMINENI, N. 2022. Stability characterization for pharmaceutical liposome product development with a focus on regulatory considerations: An update. International Journal of Pharmaceutics, 624, 122022.

KADRY, A. A., AL-SUWAYEH, S. A., ABD-ALLAH, A. R. & BAYOMI, M. A. 2004. Treatment of experimental osteomyelitis by liposomal antibiotics. Journal of Antimicrobial Chemotherapy, 54, 1103-1108.

KARMAKAR, S. 2019. Particle size distribution and zeta potential based on dynamic light scattering: Techniques to characterize stability and surface charge distribution of charged colloids. Recent Trends Mater. Phys. Chem, 28, 117-159.

KEDMI, R., BEN-ARIE, N. & PEER, D. 2010. The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. Biomaterials, 31, 6867-6875.

KHAN, F., PHAM, D. T. N. & KIM, Y.-M. 2020. Alternative strategies for the application of aminoglycoside antibiotics against the biofilm-forming human pathogenic bacteria. Applied microbiology and biotechnology, 104, 1955-1976.

KHAN, O. & CHAUDARY, N. 2020. The Use of Amikacin Liposome Inhalation Suspension (Arikayce) in the Treatment of Refractory Nontuberculous Mycobacterial Lung Disease in Adults. Drug Des Devel Ther, 14, 2287-2294.

KOEDEL, U., SCHELD, W. M. & PFISTER, H.-W. 2002. Pathogenesis and pathophysiology of pneumococcal meningitis. The Lancet Infectious Diseases, 2, 721-736.

KOLAŠINAC, R., KLEUSCH, C., BRAUN, T., MERKEL, R. & CSISZÁR, A. 2018. Deciphering the Functional Composition of Fusogenic Liposomes. International Journal of Molecular Sciences, 19, 346.

KUBE, S., HERSCH, N., NAUMOVSKA, E., GENSCH, T., HENDRIKS, J., FRANZEN, A., LANDVOGT, L., SIEBRASSE, J.-P., KUBITSCHECK, U. & HOFFMANN, B. 2017. Fusogenic liposomes as nanocarriers for the delivery of intracellular proteins. Langmuir, 33, 1051-1059.

LASIC, D. D. & MARTIN, F. J. 2018. Stealth liposomes, CRC press.

LATERRE, P.-F., COLIN, G., DEQUIN, P.-F., DUGERNIER, T., BOULAIN, T., DA SILVEIRA, S. A., LAJAUNIAS, F., PEREZ, A. & FRANÇOIS, B. 2019. CAL02, a novel antitoxin liposomal agent, in severe pneumococcal pneumonia: a first-in-human, double-blind, placebo-controlled, randomised trial. The Lancet Infectious Diseases, 19, 620-630.

LAUNE, M. A., ZAHIDI, S. A., WIEMANN, J. T. & YU, Y. 2022. Distinct antibacterial activities of nanosized cationic liposomes against gram-negative bacteria correlate with their heterogeneous fusion interactions. ACS Applied Nano Materials, 5, 15201-15210.

LECHANTEUR, A., SANNA, V., DUCHEMIN, A., EVRARD, B., MOTTET, D. & PIEL, G. 2018. Cationic liposomes carrying siRNA: impact of lipid composition on physicochemical properties, cytotoxicity, and endosomal escape. Nanomaterials, 8, 270.

LEEKHA, S., TERRELL, C. L. & EDSON, R. S. 2011. General principles of antimicrobial therapy. Mayo Clin Proc, 86, 156-67.

LEVISON, M. E. & LEVISON, J. H. 2009. Pharmacokinetics and pharmacodynamics of antibacterial agents. Infect Dis Clin North Am, 23, 791-815, vii.

LEWIS, K. 2020. The science of antibiotic discovery. Cell, 181, 29-45.

LI, L.-L., XU, J.-H., QI, G.-B., ZHAO, X., YU, F. & WANG, H. 2014. Core-Shell Supramolecular Gelatin Nanoparticles for Adaptive and "On-Demand" Antibiotic Delivery. ACS Nano, 8, 4975-4983.

LI, Y., SU, T., ZHANG, Y., HUANG, X., LI, J. & LI, C. 2015. Liposomal co-delivery of daptomycin and clarithromycin at an optimized ratio for treatment of methicillin-resistant Staphylococcus aureus infection. Drug Delivery, 22, 627-637.

LIAN, T. & HO, R. J. 2001a. Trends and developments in liposome drug delivery systems. Journal of Pharmaceutical Sciences, 90, 667-680.

LIAN, T. & HO, R. J. 2001b. Trends and developments in liposome drug delivery systems. J Pharm Sci, 90, 667-80.

LIU, C., SHI, J., DAI, Q., YIN, X., ZHANG, X. & ZHENG, A. 2015. In-vitro and in-vivo evaluation of ciprofloxacin liposomes for pulmonary administration. Drug Development and Industrial Pharmacy, 41, 272-278.

MAH, T.-F. C. & O'TOOLE, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in microbiology, 9, 34-39.

MARCHIANÒ, V., MATOS, M., SERRANO-PERTIERRA, E., GUTIÉRREZ, G. & BLANCO-LÓPEZ, M. D. C. 2020. Vesicles as an antibiotic carrier: State of art. International Journal of Pharmaceutics, 585, 119478.

MARETTI, E., COSTANTINO, L., RUSTICHELLI, C., LEO, E., CROCE, M. A., BUTTINI, F., TRUZZI, E. & IANNUCCELLI, V. 2017. Surface engineering of Solid Lipid Nanoparticle assemblies by methyl α-d-mannopyranoside for the active targeting of macrophages in anti-tuberculosis inhalation therapy. International journal of pharmaceutics, 528, 440-451.

MARUYAMA, K., YUDA, T., OKAMOTO, A., KOJIMA, S., SUGINAKA, A. & IWATSURU, M. 1992. Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine

and cholesterol-containing amphipathic poly (ethylene glycol). Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1128, 44-49.

MIRZAEE, M., OWLIA, P. & MEHRABI, M. 2009. Comparison of the bactericidal activity of amikacin in free and liposomal formulation against gram-negative and gram-positive bacteria. Jundishapur Journal of Natural Pharmaceutical Products, 4, 1-7.

MORAN, G. J., KRISHNADASAN, A., GORWITZ, R. J., FOSHEIM, G. E., MCDOUGAL, L. K., CAREY, R. B. & TALAN, D. A. 2006. Methicillin-resistant S. aureus infections among patients in the emergency department. New England Journal of Medicine, 355, 666-674.

MOYÁ, M. L., LÓPEZ-LÓPEZ, M., LEBRÓN, J. A., OSTOS, F. J., PÉREZ, D., CAMACHO, V., BECK, I., MERINO-BOHÓRQUEZ, V., CAMEAN, M. & MADINABEITIA, N. 2019. Preparation and characterization of new liposomes. Bactericidal activity of cefepime encapsulated into cationic liposomes. Pharmaceutics, 11, 69.

MUGABE, C., AZGHANI, A. O. & OMRI, A. 2005. Liposome-mediated gentamicin delivery: development and activity against resistant strains of Pseudomonas aeruginosa isolated from cystic fibrosis patients. Journal of Antimicrobial Chemotherapy, 55, 269-271.

MUGABE, C., AZGHANI, A. O. & OMRI, A. 2006a. Preparation and characterization of dehydration–rehydration vesicles loaded with aminoglycoside and macrolide antibiotics. International journal of pharmaceutics, 307, 244-250.

MUGABE, C., HALWANI, M., AZGHANI, A. O., LAFRENIE, R. M. & OMRI, A. 2006b. Mechanism of enhanced activity of liposome-entrapped aminoglycosides against resistant strains of Pseudomonas aeruginosa. Antimicrob Agents Chemother, 50, 2016-22.

MULLIS, A. S., PEROUTKA-BIGUS, N., PHADKE, K. S., BELLAIRE, B. H. & NARASIMHAN, B. 2021. Nanomedicines to counter microbial barriers and antimicrobial resistance. Current Opinion in Chemical Engineering, 31, 100672.

MURRAY, C. J. L., IKUTA, K. S., SHARARA, F., SWETSCHINSKI, L., ROBLES AGUILAR, G., GRAY, A., HAN, C., BISIGNANO, C., RAO, P., WOOL, E., JOHNSON, S. C., BROWNE, A. J., CHIPETA, M. G., FELL, F., HACKETT, S., HAINES-WOODHOUSE, G., KASHEF HAMADANI, B. H., KUMARAN, E. A. P., MCMANIGAL, B., AGARWAL, R., AKECH, S., ALBERTSON, S., AMUASI, J., ANDREWS, J., ARAVKIN, A., ASHLEY, E., BAILEY, F., BAKER, S., BASNYAT, B., BEKKER, A., BENDER, R., BETHOU, A., BIELICKI, J., BOONKASIDECHA, S., BUKOSIA, J., CARVALHEIRO, C., CASTAÑEDA-ORJUELA, C., CHANSAMOUTH, V., CHAURASIA, S., CHIURCHIÙ, S., CHOWDHURY, F., COOK, A. J., COOPER, B., CRESSEY, T. R., CRIOLLO-MORA, E.,

CUNNINGHAM, M., DARBOE, S., DAY, N. P. J., DE LUCA, M., DOKOVA, K., DRAMOWSKI, A., DUNACHIE, S. J., ECKMANNS, T., EIBACH, D., EMAMI, A., FEASEY, N., FISHER-PEARSON, N., FORREST, K., GARRETT, D., GASTMEIER, P., GIREF, A. Z., GREER, R. C., GUPTA, V., HALLER, S., HASELBECK, A., HAY, S. I., HOLM, M., HOPKINS, S., IREGBU, K. C., JACOBS, J., JAROVSKY, D., JAVANMARDI, F., KHORANA, M., KISSOON, N., KOBEISSI, E., KOSTYANEV, T., KRAPP, F., KRUMKAMP, R., KUMAR, A., KYU, H. H., LIM, C., LIMMATHUROTSAKUL, D., LOFTUS, M. J., LUNN, M., MA, J., MTURI, N., MUNERA-HUERTAS, T., MUSICHA, P., MUSSI-PINHATA, M. M., NAKAMURA, T., NANAVATI, R., NANGIA, S., NEWTON, P., NGOUN, C., NOVOTNEY, A., NWAKANMA, D., OBIERO, C. W., OLIVAS-MARTINEZ, A., OLLIARO, P., OOKO, E., et al. 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet, 399, 629-655.

NACUCCHIO, M., BELLORA, M., SORDELLI, D. & D'AQUINO, M. 1985a. Enhanced liposome-mediated activity of piperacillin against staphylococci. Antimicrobial agents and chemotherapy, 27, 137-139.

NACUCCHIO, M. C., BELLORA, M. J., SORDELLI, D. O. & D'AQUINO, M. 1985b. Enhanced liposome-mediated activity of piperacillin against staphylococci. Antimicrobial Agents and Chemotherapy, 27, 137-139.

NICOLOSI, D., CUPRI, S., GENOVESE, C., TEMPERA, G., MATTINA, R. & PIGNATELLO, R. 2015. Nanotechnology approaches for antibacterial drug delivery: Preparation and microbiological evaluation of fusogenic liposomes carrying fusidic acid. International Journal of Antimicrobial Agents, 45, 622-626.

NICOLOSI, D., SCALIA, M., NICOLOSI, V. M. & PIGNATELLO, R. 2010. Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria. International Journal of Antimicrobial Agents, 35, 553-558.

NWABUIFE, J. C., PANT, A. M. & GOVENDER, T. 2021. Liposomal delivery systems and their applications against Staphylococcus aureus and methicillin-resistant Staphylococcus aureus. Advanced Drug Delivery Reviews, 178, 113861.

O'NEILL, J. 2016. Tackling drug-resistant infections globally: final report and recommendations.

OLOFSSON, S. K. & CARS, O. 2007. Optimizing Drug Exposure to Minimize Selection of Antibiotic Resistance. Clinical Infectious Diseases, 45, S129-S136.

OMRI, A., SUNTRES, Z. E. & SHEK, P. N. 2002. Enhanced activity of liposomal polymyxin B against Pseudomonas aeruginosa in a rat model of lung infection. Biochem Pharmacol, 64, 1407-13.

ONG, H. X., BENAOUDA, F., TRAINI, D., CIPOLLA, D., GONDA, I., BEBAWY, M., FORBES, B. & YOUNG, P. M. 2014. In vitro and ex vivo methods predict the enhanced lung residence time of liposomal

ciprofloxacin formulations for nebulisation. European Journal of Pharmaceutics and Biopharmaceutics, 86, 83-89.

ORGANIZATION, W. H. 2021. Global antimicrobial resistance and use surveillance system (GLASS) report: 2021.

OSMAN, N., DEVNARAIN, N., OMOLO, C. A., FASIKU, V., JAGLAL, Y. & GOVENDER, T. 2022. Surface modification of nano-drug delivery systems for enhancing antibiotic delivery and activity. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 14, e1758.

PAPAHADJOPOULOS, D. & WATKINS, J. C. 1967. Phospholipid model membranes. II. Permeability properties of hydrated liquid crystals. Biochimica et Biophysica Acta (BBA) - Biomembranes, 135, 639-652.

PARDUE, R. L. & WHITE, C. A. 1996. Chronokinetics of liposomal encapsulated ampicillin in fasting and nonfasting rats. Journal of Pharmaceutical Sciences, 85, 309-312.

PENG, Z. Y., WANG, H. Z., SRISAWAT, N., WEN, X., RIMMELE, T., BISHOP, J., SINGBARTL, K., MURUGAN, R. & KELLUM, J. A. 2012. Bactericidal antibiotics temporarily increase inflammation and worsen acute kidney injury in experimental sepsis. Crit Care Med, 40, 538-43.

POURJAVADI, A. & TEHRANI, Z. M. 2014. Mesoporous Silica Nanoparticles (MCM-41) Coated PEGylated Chitosan as a pH-responsive Nanocarrier for Triggered Release of Erythromycin. International Journal of Polymeric Materials and Polymeric Biomaterials, 63, 692-697.

PUMERANTZ, A., MUPPIDI, K., AGNIHOTRI, S., GUERRA, C., VENKETARAMAN, V., WANG, J. & BETAGERI, G. 2011. Preparation of liposomal vancomycin and intracellular killing of methicillin-resistant Staphylococcus aureus (MRSA). International journal of antimicrobial agents, 37, 140-144.

RANI, N. N. I. M., CHEN, X. Y., AL-ZUBAIDI, Z. M., AZHARI, H., KHAITIR, T. M. N., NG, P. Y., BUANG, F., TAN, G. C., WONG, Y. P. & SAID, M. M. 2022. Surface-engineered liposomes for dual-drug delivery targeting strategy against methicillin-resistant Staphylococcus aureus (MRSA). Asian journal of pharmaceutical sciences, 17, 102-119.

RIBEIRO DA CUNHA, B., FONSECA, L. P. & CALADO, C. R. 2019. Antibiotic discovery: where have we come from, where do we go? Antibiotics, 8, 45.

RIBEIRO, L. N. D. M., DE PAULA, E., ROSSI, D. A., MONTEIRO, G. P., JÚNIOR, E. C. V., SILVA, R. R., FRANCO, R. R., ESPÍNDOLA, F. S., GOULART, L. R. & FONSECA, B. B. 2020. Hybrid pectin-liposome formulation against multi-resistant bacterial strains. Pharmaceutics, 12, 769.

RUKAVINA, Z., ŠEGVIĆ KLARIĆ, M., FILIPOVIĆ-GRČIĆ, J., LOVRIĆ, J. & VANIĆ, Ž. 2018. Azithromycin-loaded liposomes for enhanced topical treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections. International Journal of Pharmaceutics, 553, 109-119.

RUKHOLM, G., MUGABE, C., AZGHANI, A. O. & OMRI, A. 2006. Antibacterial activity of liposomal gentamicin against Pseudomonas aeruginosa: a time–kill study. International journal of antimicrobial agents, 27, 247-252.

SANDE, L., SANCHEZ, M., MONTES, J., WOLF, A. J., MORGAN, M. A., OMRI, A. & LIU, G. Y. 2012. Liposomal encapsulation of vancomycin improves the killing of methicillin-resistant Staphylococcus aureus in a murine infection model. Journal of Antimicrobial Chemotherapy, 67, 2191-2194.

SANTOS-FERREIRA, I., BETTENCOURT, A. & ALMEIDA, A. J. 2015. Nanoparticulate platforms for targeting bone infections: meeting a major therapeutic challenge. Nanomedicine (Lond), 10, 3131-45.

SANTOS GIUBERTI, C. D., DE OLIVEIRA REIS, E. C., RIBEIRO ROCHA, T. G., LEITE, E. A., LACERDA, R. G., RAMALDES, G. A. & DE OLIVEIRA, M. C. 2011. Study of the pilot production process of long-circulating and pH-sensitive liposomes containing cisplatin. Journal of liposome research, 21, 60-69.

SAVADI, P., TAGHAVI-FARD, T., MILANI, M., HASHEMZADEH, N., PANAHI, V., MCMILLAN, N. A. & HALLAJ-NEZHADI, S. 2020. Piperacillin encapsulation in nanoliposomes using modified freeze-drying of a monophase solution method: preparation, characterization, and in vitro antibacterial activity. Current Microbiology, 77, 2356-2364.

SCHEULE, R. K., GEORGE, J. A. S., BAGLEY, R. G., MARSHALL, J., KAPLAN, J. M., AKITA, G. Y., WANG, K. X., LEE, E. R., HARRIS, D. J. & JIANG, C. 1997. Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. Human gene therapy, 8, 689-707.

SCHNYDER, A. & HUWYLER, J. 2005. Drug transport to the brain with targeted liposomes. NeuroRx, 2, 99-107.

SEMPLE, S. C., CHONN, A. & CULLIS, P. R. 1998. Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo. Advanced drug delivery reviews, 32, 3-17.

SERRI, A., MAHBOUBI, A., ZARGHI, A. & MOGHIMI, H. 2018. Investigating the Antimicrobial Efficacy of Liposomal Vancomycin in Gram-positive and Gram-negative bacteria-A Preliminary Mechanistic Study. Iranian Journal of Pharmaceutical Sciences, 14, 13-24.

SEZER, A. D., BAŞ, A. L. & AKBUĞA, J. 2004. Encapsulation of enrofloxacin in liposomes I: preparation and in vitro characterization of LUV. Journal of liposome research, 14, 77-86.

SHAABAN, M. I., SHAKER, M. A. & MADY, F. M. 2021. Liposomal Delivery: A Powerful Tool to Promote the Efficacy of Antimicrobial Agents. Interfaces Between Nanomaterials and Microbes. CRC Press.

SHAW, N. 1974. Lipid composition as a guide to the classification of bacteria. Advances in applied microbiology, 17, 63-108.

SHEIKHOLESLAMI, B., LAM, N. W., DUA, K. & HAGHI, M. 2022. Exploring the impact of physicochemical properties of liposomal formulations on their in vivo fate. Life Sciences, 300, 120574.

SHEK, P., TIKUISIS, P., ZAMECNIK, J., SOLTES, S., MUSTARD, R. & MITTELMAN, M. 1998. Distribution of free and liposomal cefoxitin in plasma and peritoneal fluid in a porcine intra-abdominal sepsis model. Journal of drug targeting, 5, 353-364.

SILHAVY, T. J., KAHNE, D. & WALKER, S. 2010. The bacterial cell envelope. Cold Spring Harbor perspectives in biology, 2, a000414.

SILVERMAN, R. B. & HOLLADAY, M. W. 2014. The organic chemistry of drug design and drug action, Academic press.

SKORUP, P., MAUDSDOTTER, L., LIPCSEY, M., LARSSON, A. & SJÖLIN, J. 2020. Mode of bacterial killing affects the inflammatory response and associated organ dysfunctions in a porcine E. coli intensive care sepsis model. Crit Care, 24, 646.

SMITH KORSHOLM, K., AGGER, E. M., FOGED, C., CHRISTENSEN, D., DIETRICH, J., ANDERSEN, C. S., GEISLER, C. & ANDERSEN, P. 2007. The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. Immunology, 121, 216-226.

SMITH, M. C., CRIST, R. M., CLOGSTON, J. D. & MCNEIL, S. E. 2017. Zeta potential: a case study of cationic, anionic, and neutral liposomes. Analytical and bioanalytical chemistry, 409, 5779-5787.

SOLLETI, V. S. T. 2016. Formulation and efficacy of liposome-encapsulated azithromycin for pulmonary infection due to Pseudomonas Aeruginosa. Laurentian University of Sudbury.

SONG, G., WU, H., YOSHINO, K. & ZAMBONI, W. C. 2012. Factors affecting the pharmacokinetics and pharmacodynamics of liposomal drugs. Journal of liposome research, 22, 177-192.

STEENBERGEN, J. N., ALDER, J., THORNE, G. M. & TALLY, F. P. 2005. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. Journal of Antimicrobial Chemotherapy, 55, 283-288.

SU, F.-Y., CHEN, J., SON, H.-N., KELLY, A. M., CONVERTINE, A. J., WEST, T. E., SKERRETT, S. J., RATNER, D. M. & STAYTON, P. S. 2018. Polymer-augmented liposomes enhancing antibiotic delivery against intracellular infections. Biomaterials Science, 6, 1976-1985.

SUN, D. & LU, Z.-R. 2023. Structure and function of cationic and ionizable lipids for nucleic acid delivery. Pharmaceutical Research, 40, 27-46.

SUN, Q., SHI, M., SHAO, W., SHI, Y., XI, Y. & HUANG, G. 2011. Tissue distribution and pulmonary targeting studies of cefpiramide sodium-loaded liposomes. Journal of Drug Targeting, 19, 49-55.

TERADA, T., KULKARNI, J. A., HUYNH, A., CHEN, S., VAN DER MEEL, R., TAM, Y. Y. C. & CULLIS, P. R. 2021. Characterization of lipid nanoparticles containing ionizable cationic lipids using design-of-experiments approach. Langmuir, 37, 1120-1128.

TIAN, T., ZHANG, H.-X., HE, C.-P., FAN, S., ZHU, Y.-L., QI, C., HUANG, N.-P., XIAO, Z.-D., LU, Z.-H., TANNOUS, B. A. & GAO, J. 2018. Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. Biomaterials, 150, 137-149.

TORCHILIN, V. P. 1994. Immunoliposomes and PEGylated immunoliposomes: used for targeted delivery of imaging agents. Immunomethods, 4, 244-258.

TORRES, I. M. S., BENTO, E. B., ALMEIDA, L. D. C., SÁ, L. Z. C. M. D. & LIMA, E. M. 2012. Preparation, characterization, and in vitro antimicrobial activity of liposomal ceftazidime and cefepime against Pseudomonas aeruginosa strains. Brazilian journal of microbiology, 43, 984-992.

VAN DER WEIDE, H. 2020. Development of novel nanomedicines based on antimicrobial peptides for the treatment of multidrug-resistant Gram-negative pneumonia.

VANIĆ, Ž., RUKAVINA, Z., MANNER, S., FALLARERO, A., UZELAC, L., KRALJ, M., AMIDŽIĆ KLARIĆ, D., BOGDANOV, A., RAFFAI, T. & VIROK, D. P. 2019. Azithromycin-liposomes as a novel approach for localized therapy of cervicovaginal bacterial infections. International Journal of Nanomedicine, 5957-5976.

VASCONCELOS, N., CRODA, J. & SIMIONATTO, S. 2018. Antibacterial mechanisms of cinnamon and its constituents: A review. Microbial pathogenesis, 120, 198-203.

VIEIRA, D. B. & GAMARRA, L. F. 2016. Getting into the brain: liposome-based strategies for effective drug delivery across the blood-brain barrier. International journal of nanomedicine, 11, 5381.

WANG, T., RONG, F., TANG, Y., LI, M., FENG, T., ZHOU, Q., LI, P. & HUANG, W. 2021. Targeted polymer-based antibiotic delivery system: A promising option for treating bacterial infections via macromolecular approaches. Progress in Polymer Science, 116, 101389.

WEBB, M. S., BOMAN, N. L., WISEMAN, D. J., SAXON, D., SUTTON, K., WONG, K. F., LOGAN, P. & HOPE, M. J. 1998. Antibacterial Efficacy against an In Vivo Salmonella typhimurium Infection Model and Pharmacokinetics of a Liposomal Ciprofloxacin Formulation. Antimicrobial Agents and Chemotherapy, 42, 45-52.

YANG, S., HAN, X., YANG, Y., QIAO, H., YU, Z., LIU, Y., WANG, J. & TANG, T. 2018a. Bacteria-Targeting Nanoparticles with Microenvironment-Responsive Antibiotic Release To Eliminate Intracellular Staphylococcus aureus and Associated Infection. ACS Applied Materials & Interfaces, 10, 14299-14311.

YANG, Z., TIAN, L., LIU, J. & HUANG, G. 2018b. Construction and evaluation in vitro and in vivo of tedizolid phosphate-loaded cationic liposomes. Journal of Liposome Research, 28, 322-330.

ZAHRA, M. J., HAMED, H., MOHAMMAD, R. Y., NOSRATOLLAH, Z., AKBARZADEH, A. & MORTEZA, M. 2017. Evaluation and study of antimicrobial activity of nano liposomal meropenem against Pseudomonas aeruginosa isolates. Artif Cells Nanomed Biotechnology, 45, 975-980.

ZAMAN, S. B., HUSSAIN, M. A., NYE, R., MEHTA, V., MAMUN, K. T. & HOSSAIN, N. 2017. A review on antibiotic resistance: alarm bells are ringing. Cureus, 9.

ZASLOFF, M. 2002. Antimicrobial peptides of multicellular organisms. Nature, 415, 389-95.

ZAVASCKI, A. P., GOLDANI, L. Z., LI, J. & NATION, R. L. 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. Journal of antimicrobial chemotherapy, 60, 1206-1215.

ZEITLINGER MARKUS, A., DERENDORF, H., MOUTON JOHAN, W., CARS, O., CRAIG WILLIAM, A., ANDES, D. & THEURETZBACHER, U. 2011. Protein Binding: Do We Ever Learn? Antimicrobial Agents and Chemotherapy, 55, 3067-3074.

ZHANG, X., KIMURA, Y., FANG, C., ZHOU, L., SFYROERA, G., LAMBRIS, J. D., WETSEL, R. A., MIWA, T. & SONG, W.-C. 2007. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood, The Journal of the American Society of Haematology, 110, 228-236.

ZHU, J., SUN, L., DING, B., YANG, Y., XU, X., LIU, W., ZHU, D., YANG, F., ZHANG, H. & HU, F. 2016. Outbreak of NDM-1-producing Klebsiella pneumoniae ST76 and ST37 isolates in neonates. European Journal of Clinical Microbiology & Infectious Diseases, 35, 611-618.

11. Supplemental material

Supplemental Table 1: Characteristics of *in-vitro* studies comparing different liposomal encapsulated antibiotics and free standard antibiotics.

Study **n=(29)** **Year** **Country** **Funding**	**Lipid formulation** **Size & Charge**	**Antibiotic(s)**	**Microbiology**	**Reported outcomes**
Nacucchio et al. 1985 (Nacucchio et al., 1985a).	PC: Chol	Piperacillin	**Gram (+) :** S. Aureus	Fifty per cent reduction in bacterial growth with liposomal formulations. Adsorption of piperacillin onto liposomal surfaces protected against enzymatic hydrolysis, thereby enhancing anti-staphylococcal activity.
Furneri et al. 2000 (Furneri et al., 2000). Italy	**(-) Charged**: DMPC:Chol: DPP DMPC:Chol: DPPS **(+) Charged**: DMPC:Chol: DPPE DMPC:Chol: DPPA ±190 nm	Ofloxacin	**Gram (+) :** S. Aureus **Gram (-) :** E. coli E. faecalis Pseudomonas	Encapsulated Ofloxacin yielded MICs two-fold lower than the standard drug, along with higher intracellular concentrations.
Sezer et al. 2004 (Sezer et al., 2004).	**Neutral:** DPPC: Chol 3.12–4.95 µm	Enrofloxacin	NA	Higher CHOL contents within liposomes are associated with better encapsulation efficacy.
Mugabe et al. 2004 (Mugabe et al., 2005).	**(-) Charged** DMPC: Chol **Neutral:** DPPC: Chol DSPC: Chol 408 ± 28 to 418 ± 21 nm	Gentamicin	**Gram (+):** S. Aureus **Gram (-):** Pseudomonas clinical strain	MICs of liposomal gentamicin for were 2-4 folds lower than the non-liposomal gentamicin.
Mugabe et al. 2005 (Mugabe et al., 2006a).	**Neutral:** DPPC: Chol 163.37 ± 38.44 to 259.83 ± 11.80 nm	Aminoglycosides (Gentamicin, Tobramycin, Amikacin) Macrolide	**Gram (+):** Lab strains of Bacillus subtilis S. Aureus	Small vesicles prepared by the modified DRV method yielded high entrapment for aminoglycoside and macrolides & released more drugs in the plasma.
Rukholm et al. 2005	**(-) Charged** : DMPC: Chol	Gentamicin	**Gram (-):** Lab & clinical strains of Pseudomonas	liposomal gentamicin had MICs 16 folds lower than the standard drug. **Time-kill study**

(Rukholm et al., 2006).	426.25 ± 13.56 nm		**Gram (+) :** Lab strain S. Aureus	Both free and liposomal gentamicin had a large initial drop (4 logs CFU/mL) in CFU within 6 h, which was followed by re-growth to initial concentrations after 24 h.
Drulis-Kawa et al. 2006 (Drulis-Kawa et al., 2006c).	PC : DOTAP PC : DOTAP PC : Chol : SA DPPC : Chol : SA DSPC : Chol : SA 107-152 nm	Meropenem	**Gram (-):** Pseudomonas	MICs of cationic liposomal meropenem were 2-4-fold lower than free meropenem.
Alipour et al. 2008 (Alipour et al., 2008).	**Neutral:** DPPC: Chol POPC: Chol	Polymyxin B	**Gram (-):** Pseudomonas E.coli K. pneumoniae Bordetella bronchiseptica	DPPC/Chol liposomal formulation was associated with lower MICs as compared to free polymyxin B. A combination of free polymyxin B and empty liposomes had similar activity to free polymyxin B alone.
Mugabe et al. 2006 (Mugabe et al., 2006b).	**Neutral:** DPPC: Chol 210 ± 25 nm	Aminoglycosides (Gentamicin, Tobramycin, Amikacin)	**Gram (-):** Mucoid & non-mucoid strains Pseudomonas **Gram (+):** Lab strain of S. Aureus	Not available.
Halwani et al. 2008 (Halwani et al., 2008).	DSPC: Chol 908.0 ± 42.7 nm	Tobramycin	**Gram (-):** Non-mucoid & mucoid strains of Burkholde-ria cenocepacia & Pseudomonas	The liposomal formulation showed lower MICs than the free drug (0.25 mg/L vs. 1024 mg/L) and was equally effective against the resistant strain of Pseudomonas (PA-48913) at extremely low concentrations.
Mirzaee et al. 2009 (Mirzaee et al., 2009).	DDPC: Chol	Amikacin	**Gram (-):** E.coli Pseudomonas. **Gram (+):** Strep. Faecalis S. Aureus	MICs of liposomal amikacin against all bacterial strains were 2-3 folds lower than free amikacin.
Nicolosi et al. 2010 (Nicolosi et al., 2010).	DOPE: DPPC: Chol: Chol hemisuccinate (CHEMS)	Vancomycin	**Gram (-):** E.coli, Actinobacter	MICs of liposomal vancomycin were as low as 6 mg/L.
Pumerantz et al. 2010 (Pumerantz et al., 2011).	**Conventional:** DSPC: Chol **Pegylated**: DSPC Chol: MPEG-2000-DSPE 254 ± 147 nm	Vancomycin	MRSA	MRSA-infected macrophages treated with liposomal vancomycin formulation associated with a significant intracellular reduction in viability of MRSA.

Torres et al. 2012 (Torres et al., 2012).	PC: Chol 131.88 nm	Ceftazidime & Cefepime	**Gram (-):** Pseudomonas	MIC for liposomal antibiotics was 50% lower than the free drug.
Atashbeyk et al. 2014 (Atashbeyk et al., 2014).	PC: Chol 148.7 ± 1.61	Gentamicin	MRSA	MIC values were 15- 27-fold lower for liposomal formulation as compared to standard drugs.
Nicolosi et al. 2015 (Nicolosi et al., 2015)	DOPE: DPPC: Chol: Chol hemisuccinate (CHEMS) 100 nm	Fusidic Acid	**Gram (-):** Actinobacter **Gram (+):** S.Aureus, *Staph epidermidis*	MICs against clinical strains of *Staph epidermidis* (≤0.15 µg/mL) & *Acinetobacter* (37.5 µg/mL).
Zahra et al. 2016 (Zahra et al., 2017).	PC: Chol 100.4 ± 0.344	Meropenem	**Gram (-):** Pseudomonas	liposomal meropenem were associated with lower MICs f against clinical & lab strains of Pseudomonas than free drug (6.25 mcg/ml vs 100 mcg/ml).
Bartomeu et al. 2017 (Bartomeu Garcia et al., 2017).	DOPE: CHS: DPPC: DSPE-DSPE-PEG2000-MAL 100 nm	Vancomycin Methicillin Ampicillin	**Gram (+):** MRSA, Staph. pneumoniae **Gram (-):** E.coli	The bacteria population decreased by 76% with free drug, while liposomal formulations were associated with up to 96% reduction.
Serri et al. 2018 (Serri et al., 2018).	**Formulation A:** DPPC: DOPE: Chol **Formulation B:** DPPC:DOPE: CHEMS Formulation A: Extrusion:198 nm, Sonication: 125 nm Formulation B: Extrusion:144 nm, Sonication: 97 nm	Vancomycin	**Gram (-):** E.coli K pneumonia Salmonella typhimurium Pseudomonas **Gram (+):** Aureus, MRSA	Probe sonicated liposomes showed a smaller size (125nm vs 198nm) than those prepared by extrusion.
Rukavina et al. 2018 (Rukavina et al., 2018).	**PC**: DPPC: PG: DODAB Non-extruded: 972-1628 nm Extruded: 132-217 nm	Azithromycin	**Gram (+):** MRSA	Liposomal formulations prevented biofilms by inhibiting MRSA growth, exhibiting MICs up to 32-fold lower than the free drug.
Derbali et al. 2019 (Derbali et al., 2019).	**Anionic**: DSPC: Chol: DSPE-PEG2000 **Cationic**: DOTAP: Chol ± 200 nm	Levofloxacin	**Gram (-):** Pseudomonas	Anionic liposomes are associated with sustained release and better bactericidal activity than free levofloxacin.
Zhang et al. 2019 (Fu et al., 2019).	DPPC: DSPE: Chol 225.17±17.85 nm	Polymyxin B	**Gram (-):** Actinobacter	CLPs had MBICs on the biofilm was 8±2 µg/mL as compared to polymyxin B.

Luisa Moyá et al. 2019 (Moyá et al., 2019).	**Neutral**: PC: Chol **(+) Charged**: PC:CH: 12NBr **(-) Charged**: DOPE:12NBr 100 nm	Cefepime	**Gram (-):** E.coli	Cationic liposomes showed good internalization into cells No improvement in MICs as compared to free drugs.
Vanic et al. 2019 (Vanić et al., 2019).	EPC: EPG: SPC-3: SLPC-80 189 – 341 nm	Azithromycin	**Gram (-):** E.coli *C. trachomatis*	AZI-liposomes had MICs 3-fold lower than the free AZI against *E. coli* with MIC50 values.
Savadi et al. 2020 (Savadi et al., 2020).	PC: Chol 100.9-444.13 nm	Piperacillin	**Gram (-):** Bacillus Subtilis Pseudomonas	liposomal Piperacillin had MIC against Pseudomonas was one-half of the MIC (21.25 µg/ml) of free Piperacillin.
Ebrahim et al. 2020 (Ebrahimi et al., 2020).	GMS (solid lipid): 0.09 g of oleic acid (liquid lipid): Soy lecithin (oil phase). Average= 86 nm	Ceftriaxone	**Gram (-):** E.coli	The drug dosage of Ceftriaxone in nanostructured form was reduced to half, as compared to the free drug.
Aljihani et al. 2020 (Aljihani et al., 2020).	DOPE: Chol LA : 484.5 – 34.7 nm LAN : 451.6 – 21.16 nm	Azithromycin	**Gram (-):** E.coli **Gram (+):** S. Aeurus	MIC of the E. coli SA10 strain was reduced to three µg/ml and 2.5 µg/ml respectively as compared to the free drug.
Morais Ribeiro et al. 2020 (Ribeiro et al., 2020)	EPC : Chol: α-tocopherol, pectin 253–425 nm	Norfloxacin	**Gram (-):** Salmonella sp., Pseudomonas, E.coli, Campylobacter jejuni,	MIC ranged from 2 to 208 µg/mL for NOR, while the modified formulations, varied from 0.2 to 80 µg/mL.
Gottesmann et al. 2020 (Gottesmann et al., 2020)	PC: Chol: D'DAB 178 ± 44	Amoxicillin	H. pylori	Liposomal formulations had direct interaction & binding with the H. pylori. However, they did not influence the viability of HT29-MTX and AGS cells i.e., one hundred µg/mL but exert cytotoxicity at 10 µg/mL against H. pylori.

CHOL Cholesterol, **DMPC** Dimyristoylphosphatidylcholine, **DMPS** dipalmitoyl phosphatidylserine, **DOTAP** Dioleoyl-3-trimethylammonium propane, **DOPE** dioleoylphosphatidylethanolamine, **DPPC** Dipalmitoylphosphatidylcholine, **DPP** di hexadecyl phosphate **DSPE-PEG2000** 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino(polyethene glycol)-2000), **DSPC** Distearoylphosphylcatidholine, **PC** Phosphatidylcholine, **POPC** 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.

Supplemental Table 2: Characteristics of *in-vivo* PK/PD studies using different liposomal antibiotics and commercially available free antibiotics.

Study N=(7) **Year** **Country** **Funding**	**Lipid formulations** **Size, Charge & EE%**	**Antibiotic**	**Source of infection ± microbiology**	**Route and Dosage**	**Biodistribution/ PK/PD outcomes**
Roseline et al. 1996 (Pardue and White, 1996). Athens Yes	SM: Chol: DCP MUV (1600-200 nm) SUV (30-50nm) **EE %:** At 12:00 (22.7± 4.0 to 46.8 ± 6.9 mins) At 24:00 (12.3 ± 1.0 to 22.3 ± 3.0 mins)	Ampicillin	Surgery, followed by fasting & non-fasting. Not reported	IV 24-27 mg/animal	During fasting liposomal drugs resulted in a 50% reduction in mean residence time (MRT) and systemic clearance (Cltotal) was increased by 20%, reflected by an increase in biliary & renal clearance after 24 hrs. There were no differences in pharmacokinetic parameters in fasting animals at 12 & 24 hrs.
Shek et al. 1998 (Shek et al., 1998). Canada NA	POPC: Chol 103±30	Cefoxitin	IP injection of a faecal inoculum E.coli	IV 30 mg/kg	liposomal cefoxitin had a half-life of 3-4 times longer than a free drug. Cumulative plasma drug levels for liposomal Cipro were 3-4 folds higher than free cefoxitin.
Elmas et al. 2002 (Elmas et al., 2002). Türkiye Yes	PC: Chol 4.27 ± 1.91 μm **(EE) %:** 44.3%	Enrofloxacin	NA	IM 5 mg/kg	peak conc was higher, absorption rate was slower, and time to peak conc (tmax 1.5 h) was longer for liposomal enrofloxacin as compared to free drug. Mean residence time (MRT¼17.6 h) and half-life(t1/2b¼12.9 h) of enrofloxacin encapsulated liposomes were longer (P< 0.05).
Sun et al. 2011 (Sun et al., 2011). China Yes	PC: Chol 7.146±0.29 μm **(EE) %:**	Cefpiramide sodium-	Not mentioned	IV 152 mg/kg	Mean residence times (MRT) and the bioavailability of Liposomes were 2.8- and 4.5-fold higher than CPMS-Sol. It had a significant difference in the tissue distribution in animals as compared to free drugs.

	82.10±4.21 %				
Xin Ong et al. 2013 (Ong et al., 2014). Australia Yes	DPPC: Chol DSPC: Chol SM: Chol	Ciprofloxacin	Pseudomonas	Intra-tracheal 5 mg/mL.	The cumulative amount of ciprofloxacin being transported across the membrane was slower 12.3 ± 1.8 than the free drug i.e., 82 ± 5.5% over 25 mins.
Liu et al. 2013 (Liu et al., 2015). China Yes	DMPC/Chol 349.6 nm **(EE) %:** 93.96%	Ciprofloxacin	Not mentioned	Intra-tracheal 20 mg/kg	The AUC lung ratio between liposomal and free Cipro was 288.33 & the bioavailability was 72.42%.
Yang et al. 2017 (Yang et al., 2018b). China NA	Soy lecithin: Chol 194.9 ± 2.93 nm **(EE) %:** 53.52 ± 2.18%	Tedizolid phosphate	Not mentioned	IV 12.5 mg/kg	Half-life increased by 0.74 times and 0.51 times higher than that of the TDZA-Inj group and TDZA-Lips group. MRT of SA-TDZA-Lips was 1.30 and 1.09 times higher than that of the TDA-Inj group and TDZA-Lips group. Tissue distribution showed the uptake rate (Re) of TDZA in the lung was 1.527.

Supplemental Table 3: Characteristics of *in-vivo* studies reporting clinical outcomes using different free standard and liposomal antibiotics.

Study **N=(10)** **Year** **Country** **Funding**	**Lipid formulations** **Size, Charge & EE%**	**Antibiotic**	**Source of infection** **± microbiology**	**Route and** **Dosage**	**Clinical outcomes** **(MICs, CFUs, survival, toxicity)**
(Webb et al., 1998). Canada NA	DPPC: Chol DSPC: Chol SM: Chol 130-nm	Ciprofloxacin	IV injection of Salmonella typhimurium into the tail vein	IV 15 mg/kg of body weight	**CFUs:** At 1 mg/kg, CFUs in the liver and spleen < 10- to 100-fold. At 20 mg/kg, the viable CFUs were < 10^3 - to 10^4-fold in the livers and spleens of infected mice treated with liposomal formulations.
(Bakker-Woudenberg et al., 2002). Netherland Yes	PEG 2000: HSPC: Chol 126 ± 11 nm	Ciprofloxacin	Inoculation of the lung with twenty µl of a saline suspension of Pseudomonas	IV 20-160 mg	In the acute model, the survival rate was 100% with pegylated liposomal ciprofloxacin either at low or high dosages in combination with free ciprofloxacin on the first day of treatment. **CFUs:** 10^2 to 10^7
(Omri et al., 2002). Canada NA	DPPC: Chol POPC: Chol 165± 20 nm **(EE) ٪:** 3.7 ± 0.5%	Polymyxin B	Intra-tracheal administration of bacteria Pseudomonas	Intra-tracheal route 500 mg in ninety mmol lipid	**CFUs:** (3.7 ± 0.4 logs CFU/paired lungs)

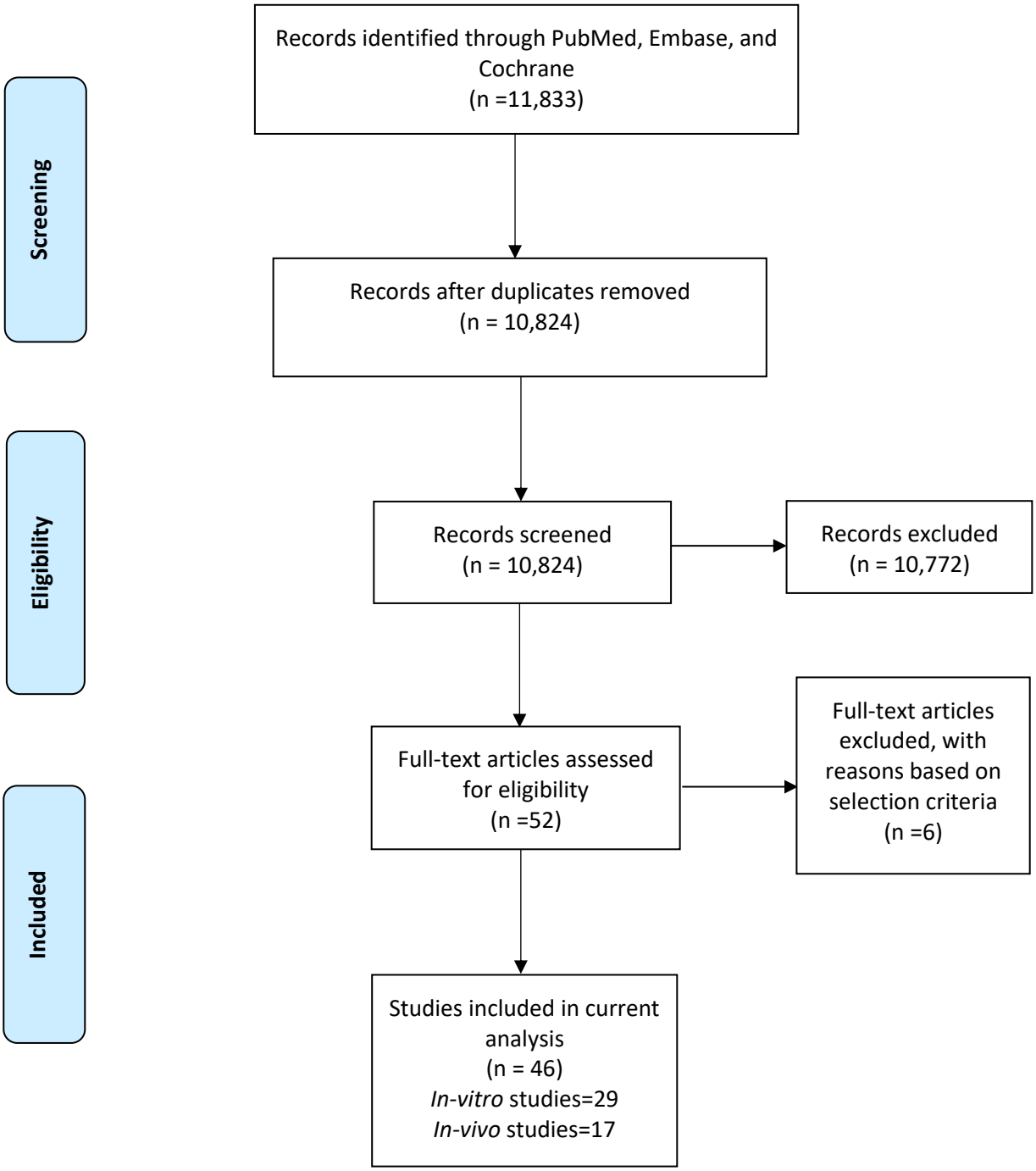
(Kadry et al., 2004). KSA Yes	**Cationic:** PC: Chol: stearyl-amine **Anionic:** PC: Chol: PG **Neutral:** PC: Chol <100 nm **(EE) ٪:** **Cationic:** 8.65% **Anionic:** 7.84% **Neutral:** 3.9%	Ciprofloxacin Vancomycin	Staph. Aureus injection into the bone marrow	IV 10-15 mg/kg	Liposomal formulations showed lower drug-related adverse events i.e., nephrotoxicity, and severe diarrhoea than free drugs.
(Sande et al., 2012). USA Yes	DCP liposomes: DSPC : DCP: Chol DMPG liposomes: DSPC : DMPG: Chol	Vancomycin	Intra-peritoneal inoculation of MRSA	IM 50 mg/kg	liposomal formulation showed a 2-fold reduction in MICs. **CFUs:** <2–3 logs within the kidneys and spleen
(Gharib et al., 2012). Iran Yes	**Neutral:** PC: Chol **Cationic:** Stearyl-amine **Anionic:** DCP **(EE) ٪:** **Cationic:** 76% ± 0.17 **Anionic:** 55% ± 0.21 **Neutral:** 43% ± 0.14	Ticarcillin	Inoculum administration into the wound site Pseudomonas	Topical 75 mg/ kg	The MIC values for free, cationic, neutral, and anionic liposomal ticarcillin were 24, 3, 6 and 48 mg/L, with higher killing rates. **Survival rate** Positive 100% Negative 60% Neutral twenty%
(Li et al., 2015). China Yes	HSPC: Chol: mPEG2000-DSPE 98.2 ± 2.21 nm **(EE) ٪:** 94.71 ± 1.37% (Dapto) and 92.94 ± 1.21% (clari)	Daptomycin Clarithromycin	Inoculum of MRSA into the tail vein	IV PL[C] (51.8 mg/kg clarithromycin, equivalent to 4.2 mg/kg in humans); PL[D] (49.3 mg/kg daptomycin, equivalent to 4 mg/kg in humans).	PL[CD] inhibited bacterial growth at 1 ✕MIC, then PL[D], while PL[C], demonstrated a significant reduction in bacterial growth within 6 h after treatment at 1 or 2 ✕ MIC. **Survival rate** After 4 days, the survival rate was 80% with PL[D] and 90% with PL[CD].
(Henry et al., 2015b). Germany	Chol: SM (66 mol/% cholesterol)	Penicillin Vancomycin	Inoculum into the tail vein	300 µg 600 µg	6/8 (75%) mice received Chol:SM + SM -only liposomal mixture and 3/8 (38%0 mice received Chol: SM liposomes survived.

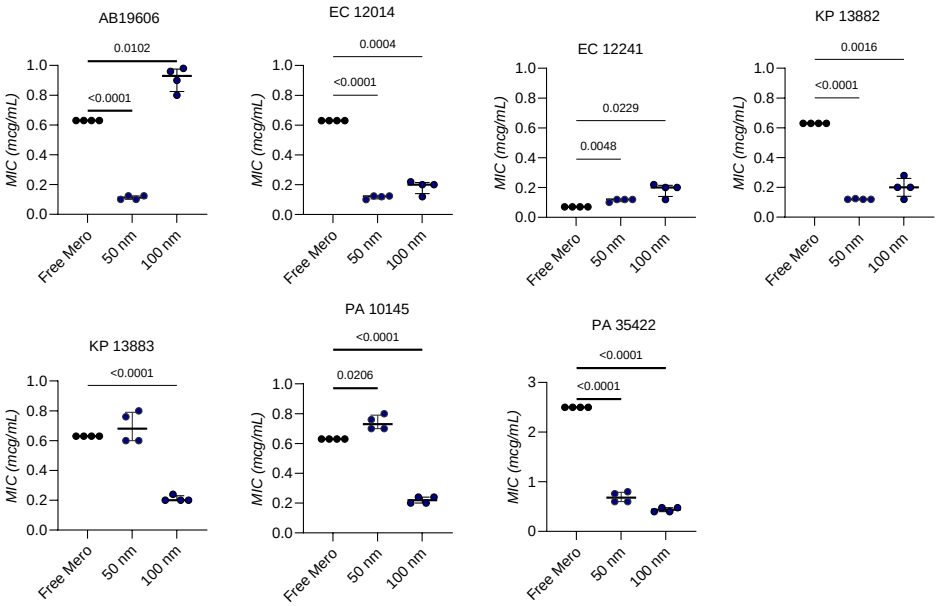
Yes	NA		S. Aureus S.Pneumoniae		
(Jiang et al., 2016). China Yes	SPC: Chol: mPEG2000–DSPE: Dapt–PEG–DSPE ±100 nm **(EE) % :** DPD-L[D] 91.85 ± 2.16 mPEG-L[D] 93.73 ± 1.47	Daptomycin	IV injection into the tail vein MRSA	IV 25 mg/kg	**Survival rate** DPD-L[D]= 60% mPEG-L[D]= 40%
(Rani et al., 2022). Malaysia Yes	PC: Chol: mPEG2000-DSPE ±100 nm **(EE) % :** VAN-L 5.36±4.17 DAPT-L 90.53±3.90	Vancomycin daptomycin	Not mentioned MRSA	IV 92.5 mg/kg	VAN liposomes reduced the MIC 6-8-fold with enhanced permeability i.e., > 80% bacterial death within 4 h.

Supplemental Table 4: Genetics factors associated with resistance mechanisms against four different meropenem-resistant bacteria (16 strains) isolated from patients at Royal Free Hospital. The following table shows different genes associated with different resistance mechanisms against commercially available standard meropenem.

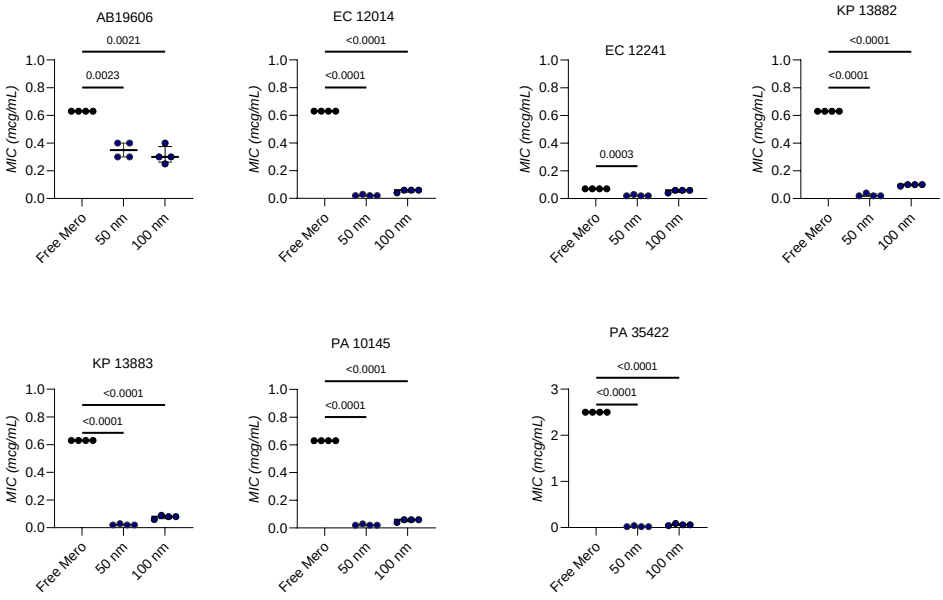
Bacteria	Genes	Mechanism	Function
AB 48-9043	IMP-1	Plasmid expression	Class B, or metallo-**β**-lactamases
EC 11M105778	OXA-48	Plasmid expression	Class D oxacillinase of OXA-48-type
EC DH5alpha - pk0X015	VIM-1	Plasmid expression	Class B, or metallo-**β**-lactamases
EC 11M212929	NDM	Plasmid expression	Class B, or metallo-**β**-lactamases
KP 11M57609	KPC	Plasmid expression	Class A
KP 14M124832	NDM	Plasmid expression	Class B, or metallo-**β**-lactamases
PA CS008	Unknown	-----	-----
PA 48-1997	SPM	Plasmid expression	Class B, or metallo-**β**-lactamases
PA PA01 pMATTX	VIM-7	Plasmid expression	Class B, or metallo-**β**-lactamases
PA 12M174258	VIM-1	Plasmid expression	Ambler class B, or metallo-**β**-lactamases
PA 11M369086	IMP-1	Plasmid expression	Class B, or metallo-**β**-lactamases
PA 73-12198	GIM-1	Plasmid expression	Class B, or metallo-**β**-lactamases
AB CS023	OXA-23	Plasmid expression	Class D, oxacillinase of OXA-48-type
AB RS080	OXA-23	Plasmid expression	Class D, oxacillinase of OXA-48-type
PA TS007	Unknown	----	----
PA CS029	VEB-1	Plasmid expression	Class A, ESBL

Supplemental Figure 1: PRISMA flow chart

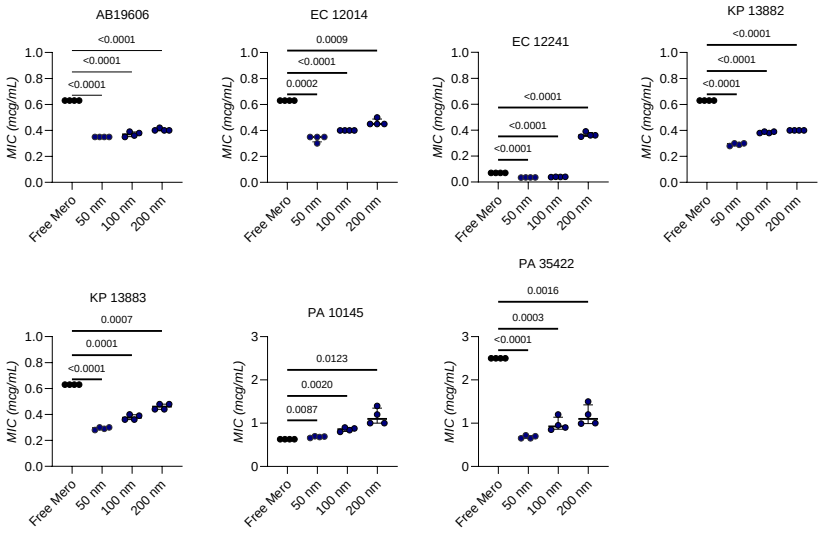




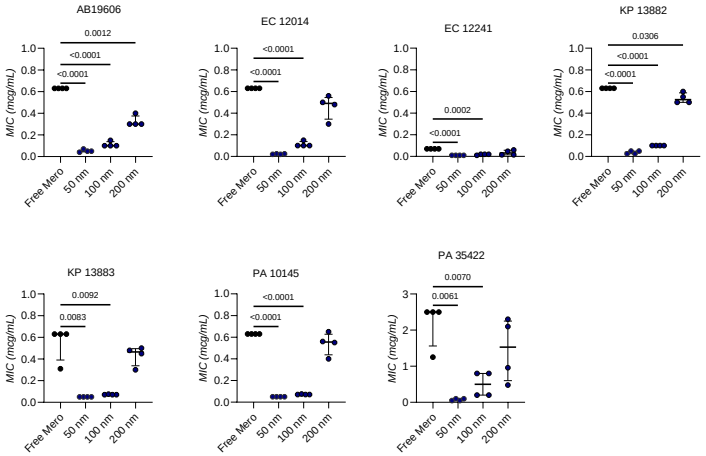
Supplemental Figure 2: MICs of free meropenem and non-PEGylated meropenem-encapsulated liposomes (7.0.3) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



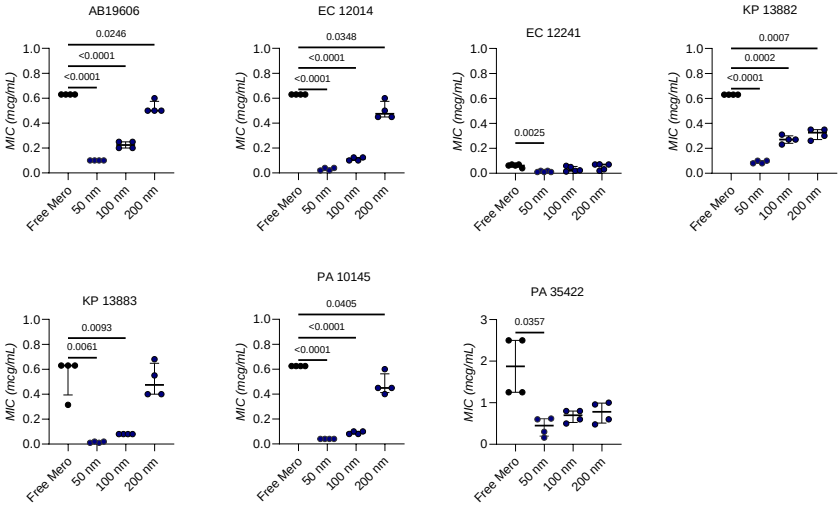
Supplemental Figure 3: MICs of free meropenem and non-PEGylated meropenem-encapsulated cationic liposomes (6.1.3) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



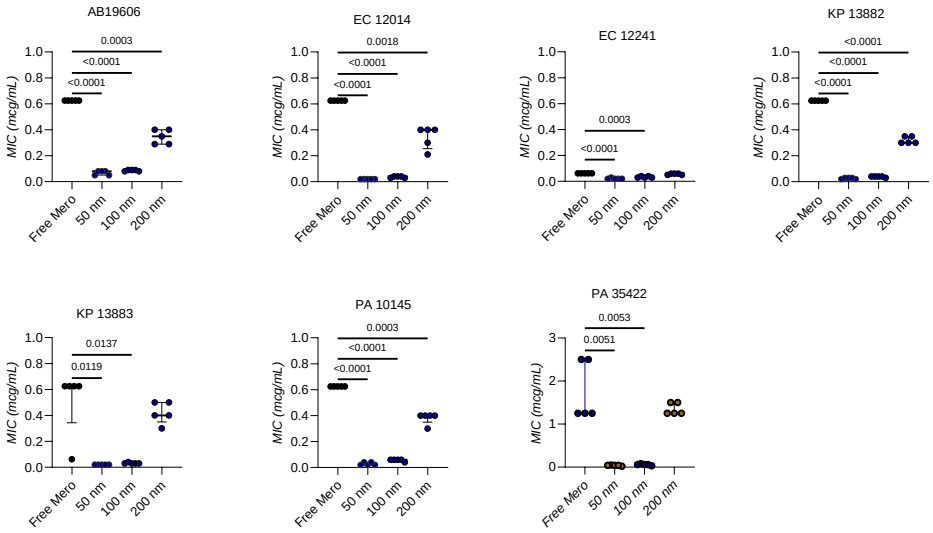
Supplemental Figure 4: MICs of free meropenem and non-PEGylated meropenem-encapsulated cationic liposomes (5.1.4) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



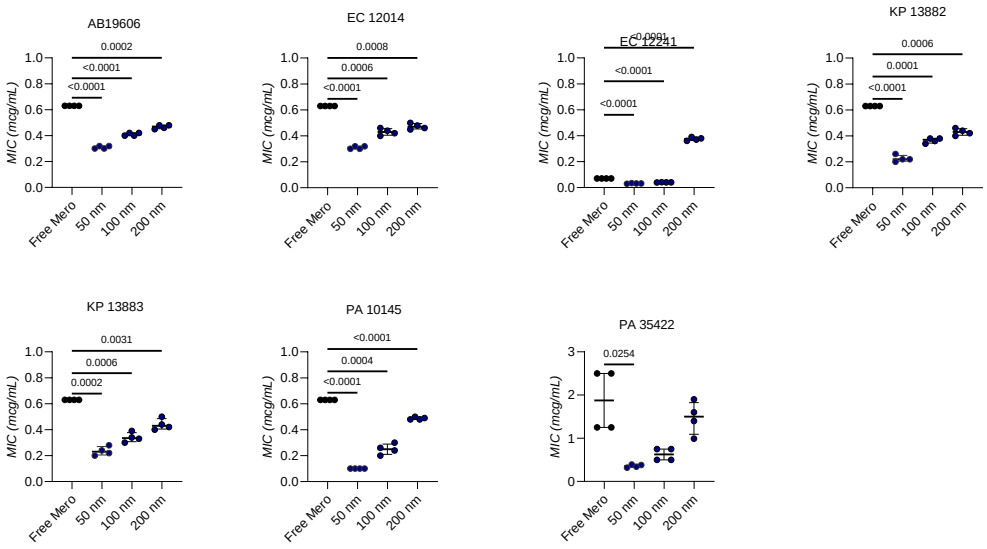
Supplemental Figure 5: MICs of free meropenem and non-PEGylated meropenem-encapsulated cationic liposomes (5.2.3) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



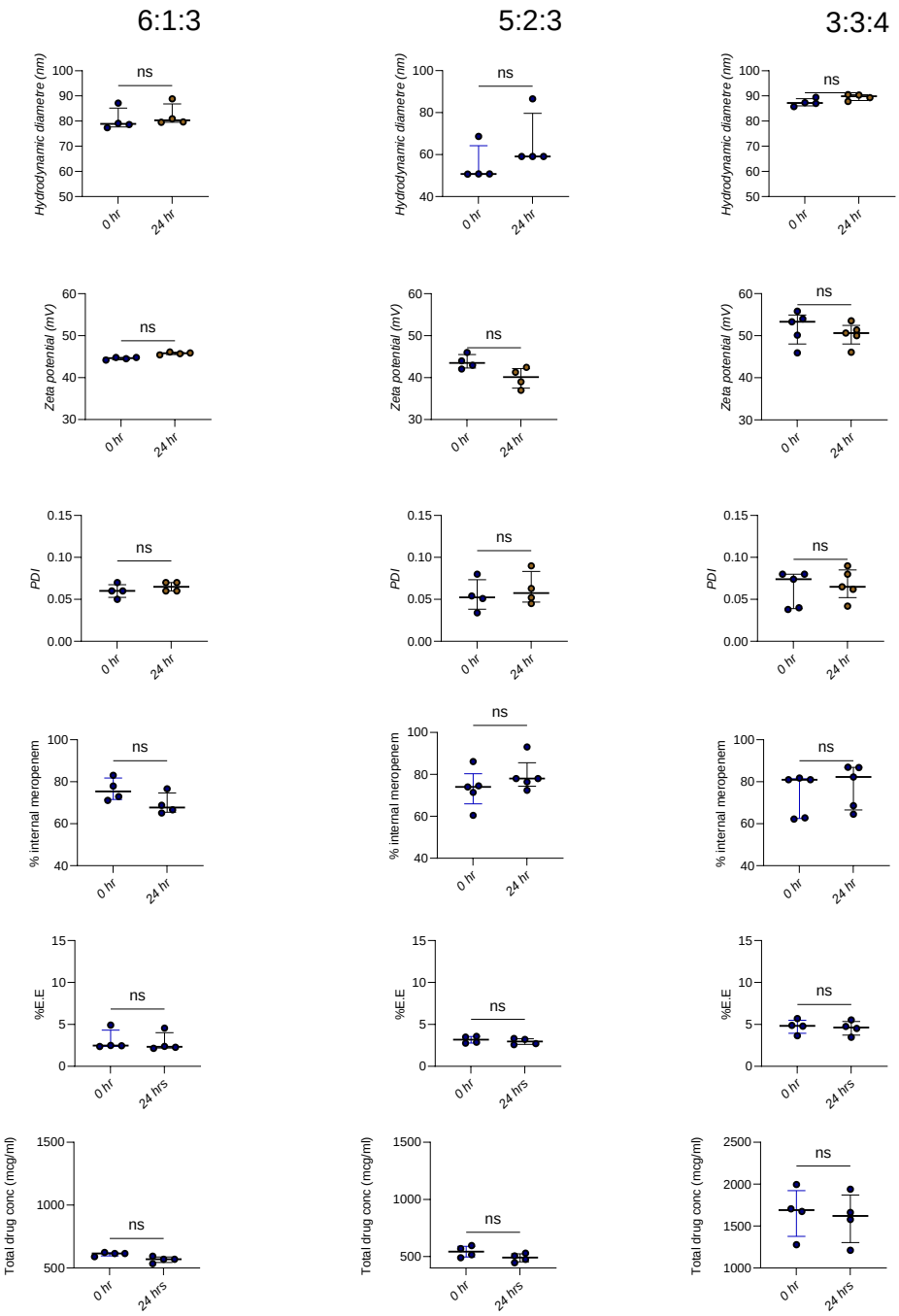
Supplemental Figure 6: MICs of free meropenem and non-PEGylated meropenem-encapsulated cationic liposomes (5.3.2) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



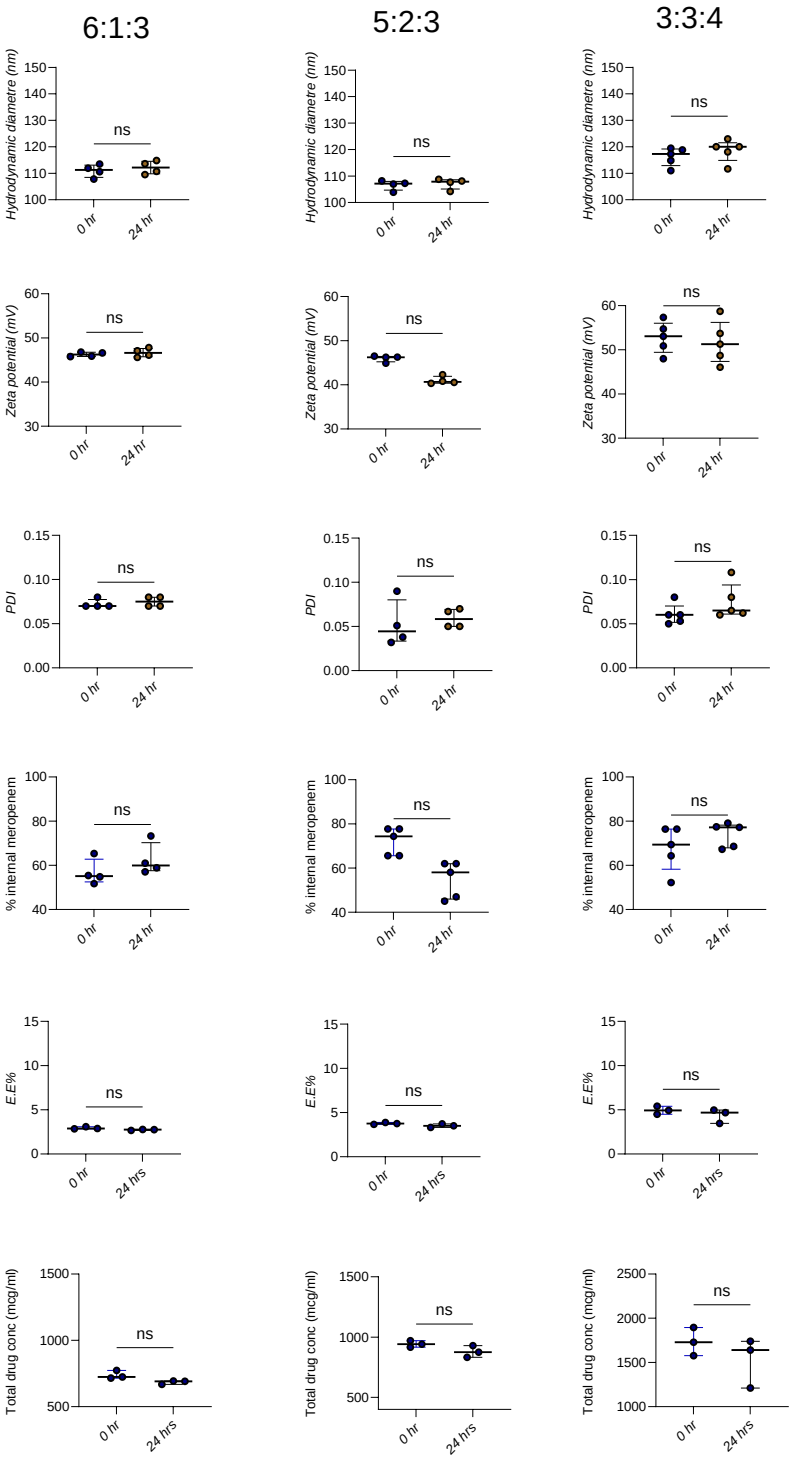
Supplemental Figure 7: MICs of free meropenem and non-PEGylated meropenem-encapsulated cationic liposomes (3.3.4) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



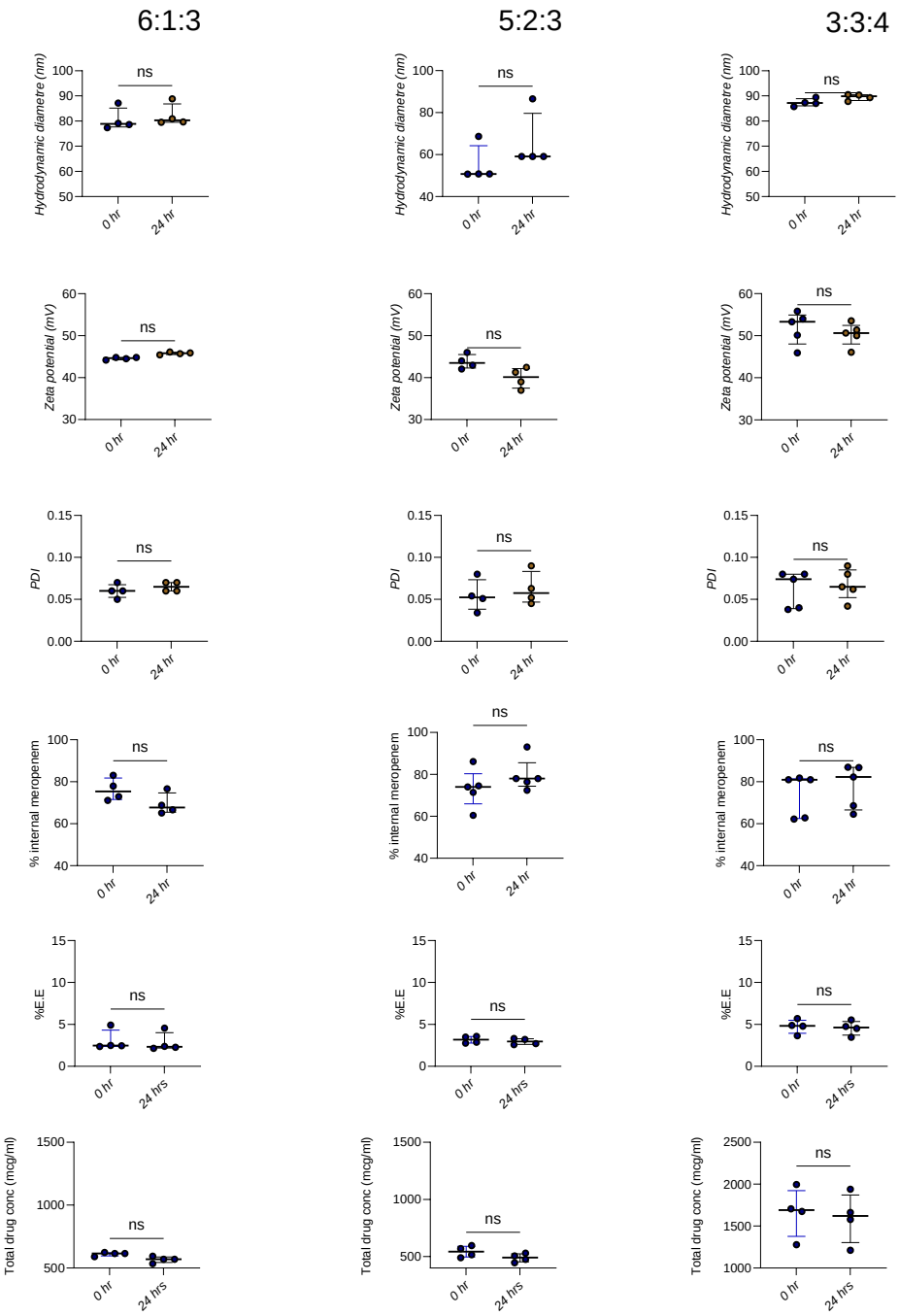
Supplemental Figure 8: MICs of free meropenem and non-PEGylated meropenem-encapsulated cationic liposomes (5.4.1) against different lab strains of Gram-negative bacteria. Data presented as median values (n= 4).



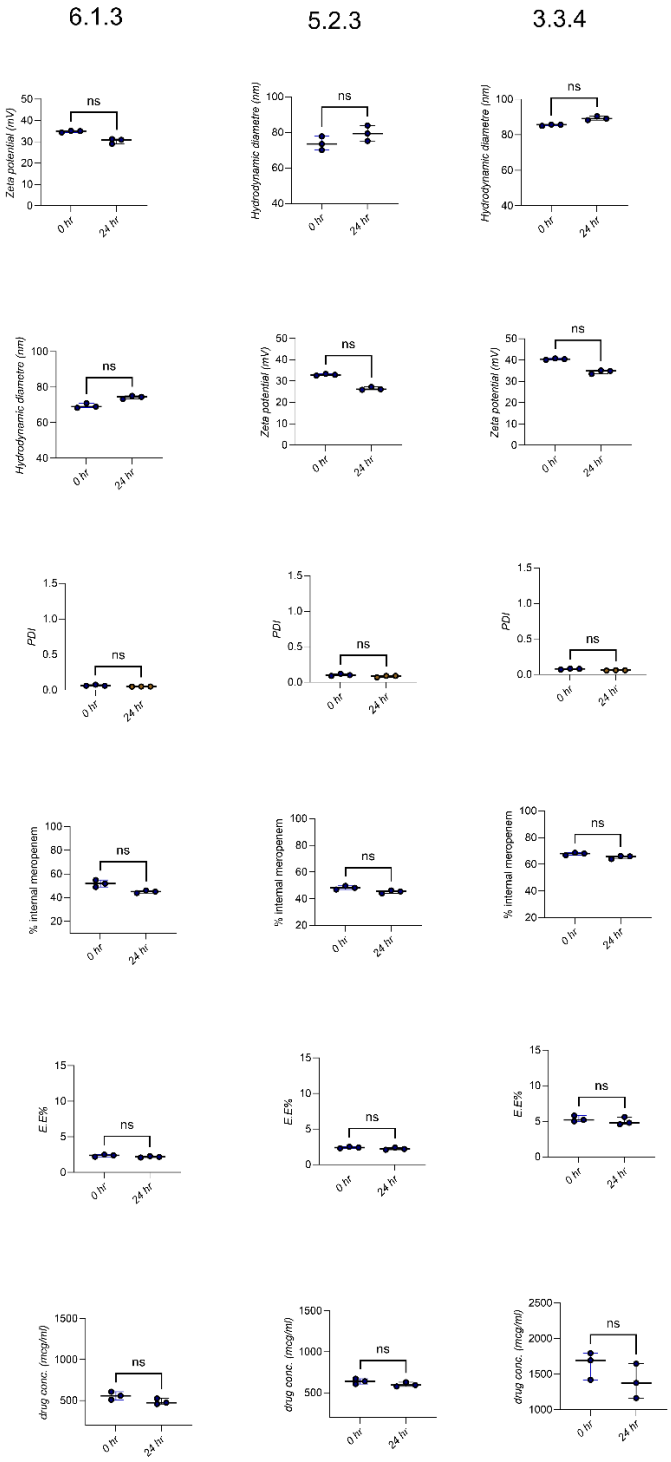
Supplemental Figure 9: 24-hour stability study for non-PEGylated meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) having size around 50 nm prepared by thin-film hydration method stored at 4°C using DLS. Size, charge, PDI, % internal meropenem and total drug concentration were measured at 0 and 24 hours to illustrate the physiochemical properties of the meropenem-encapsulated cationic liposomes. Size, charge, % internal meropenem, E. E and total drug concentrations were variable across different formulations, depending upon the physicochemical properties of cationic liposomal formulations. There was no significant difference in the measured size, charge, PDI, internal meropenem concentration, and E.E% over 24 hours. There was an expected decrease in total meropenem concentration over 24 hours, which was consistent with free standard meropenem. Data presented as median values (n= 4).



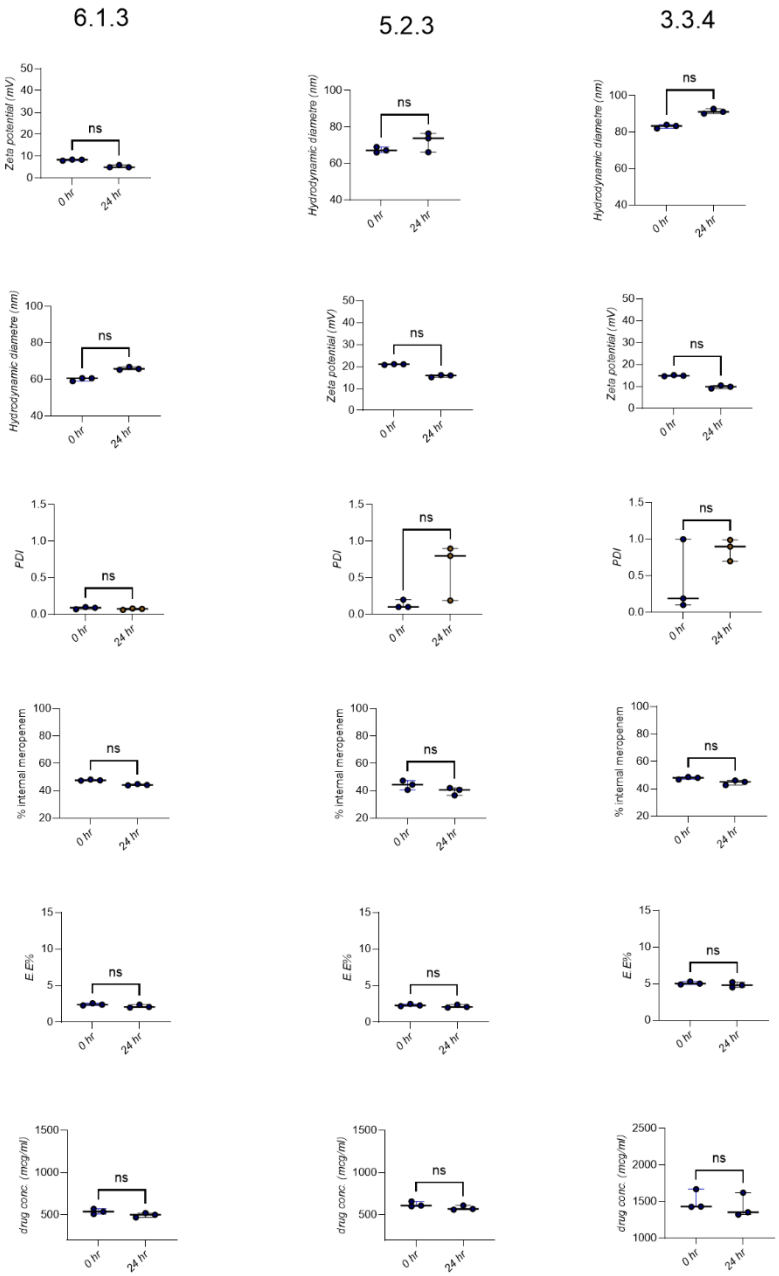
Supplemental Figure 10: 24-hour stability study for non-PEGylated meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) having size around 100 nm prepared by thin-film hydration method stored at 4°C using DLS. Size, charge, PDI, % internal meropenem and total drug concentration were measured at 0 and 24 hours to illustrate the physiochemical properties of meropenem-encapsulated liposomes with variable cationic lipids. Size, charge, % internal meropenem, E. E and total drug concentrations were variable across different formulations, depending upon the physicochemical properties of cationic liposomal formulations. There was no significant difference in the measured size, charge, PDI, internal meropenem concentration, and E.E% over 24 hours. There was an expected decrease in total meropenem concentration over 24 hours, which was consistent with free meropenem. Data presented as median values (n= 4).



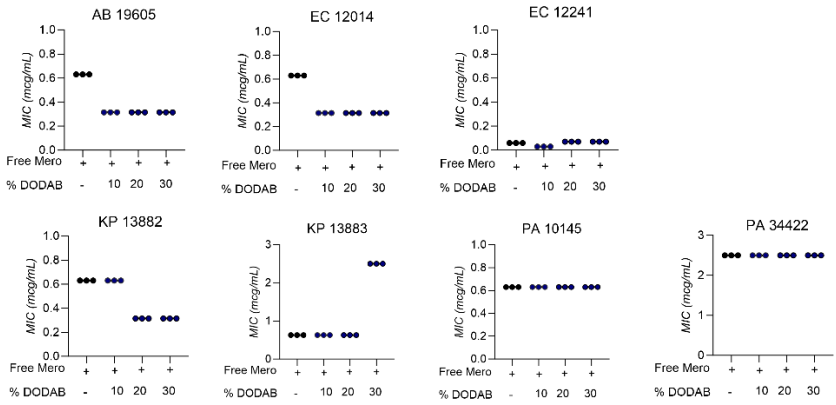
Supplemental Figure 11: 24-hour stability study for meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) with variable cationic and DSPE-PEG 2000 concentrations (0%, 0.5%, and 2.5%) prepared by thin-film hydration method stored at 4°C using DLS. Size, charge, PDI, % internal meropenem and total drug concentration were measured at 0 and 24 hours to illustrate the physiochemical properties of PEGylated meropenem-encapsulated cationic liposomal formulations. Size, charge, % internal meropenem, E. E and total drug concentrations were variable across different formulations, depending upon the physicochemical properties of cationic liposomal formulations. There was no significant difference in the measured size, charge, PDI, internal meropenem concentration, and E.E% over 24 hours. There was an expected decrease in total meropenem concentration over 24 hours, which was consistent with free meropenem. Data presented as median values (n= 3).



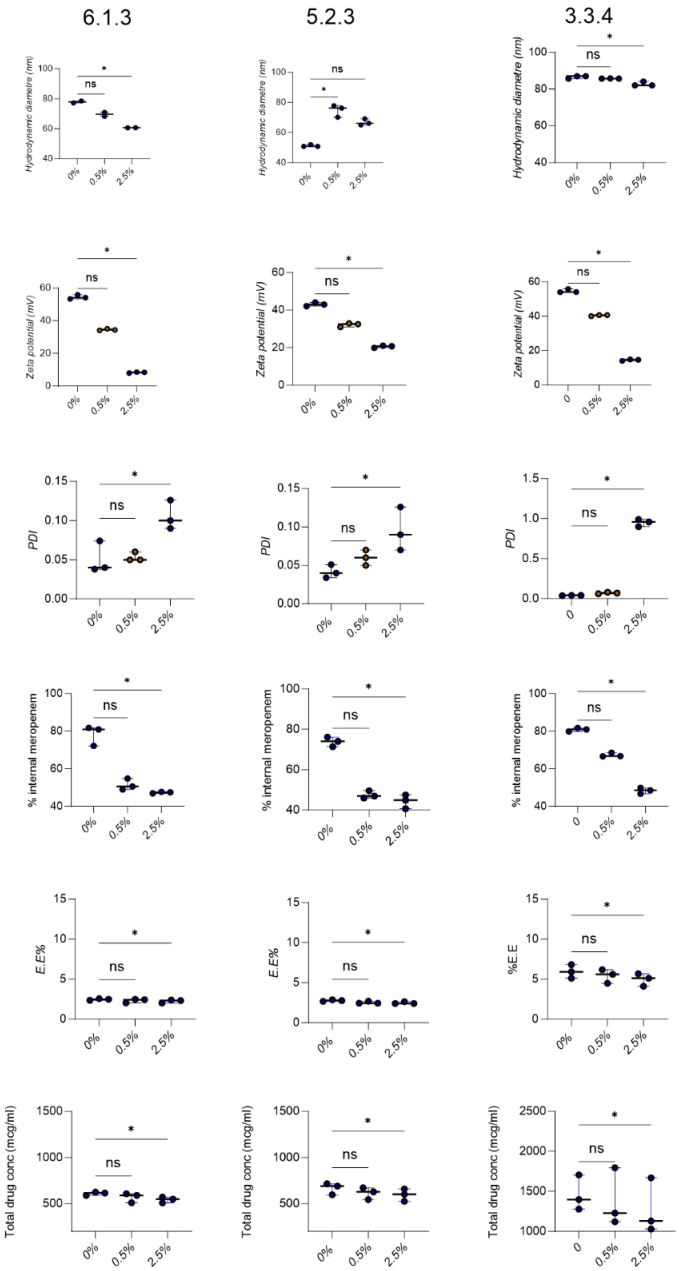
Supplemental Figure 12: 24-hour stability study for meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) with variable cationic and DSPE-PEG 2000 concentrations (0%, 0.5%, and 2.5%) prepared by thin-film hydration method stored at 4°C using DLS. Size, charge, PDI, % internal meropenem and total drug concentration were measured at 0 and 24 hours to illustrate the physiochemical properties of PEGylated meropenem-encapsulated cationic liposomes. Size, charge, % internal meropenem, E. E and total drug concentrations were variable across different formulations, depending upon the physicochemical properties of cationic liposomal formulations. There was no significant difference in the measured size, charge, PDI, internal meropenem concentration, and E.E% over 24 hours. There was an expected decrease in total meropenem concentration over 24 hours, which was consistent with free meropenem. Data presented as median values (n= 3).



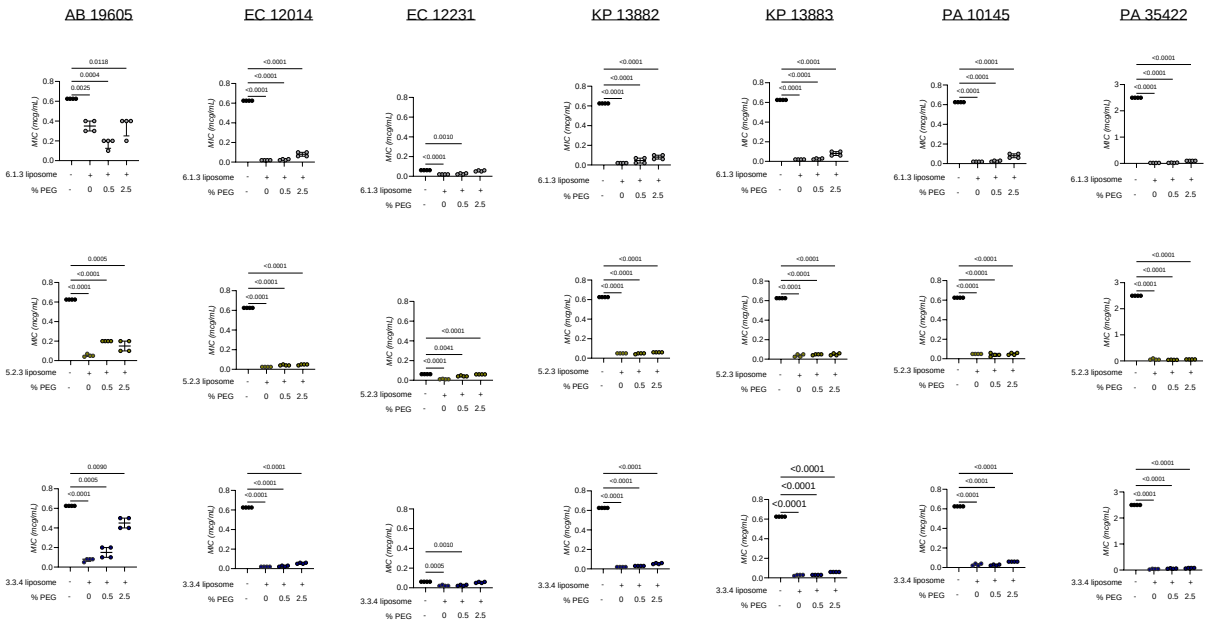
Supplemental Figure 13: 24-hour stability study for meropenem-encapsulated cationic liposomal formulations (PC: DODAB: CHOL) with variable cationic and DSPE-PEG 2000 concentrations (0%, 0.5%, and 2.5%) prepared by thin-film hydration method stored at 4°C using DLS. Size, charge, PDI, % internal meropenem and total drug concentration were measured at 0 and 24 hours to illustrate the physiochemical properties of the PEGylated meropenem-encapsulated liposomal formulations. Size, charge, % internal meropenem, E. E and total drug concentrations were variable across different formulations, depending upon the physicochemical properties of cationic liposomal formulations. There was no significant difference in the measured size, charge, PDI, internal meropenem concentration, and E.E% over 24 hours. There was an expected decrease in total meropenem concentration over 24 hours, which was consistent with free meropenem. Data presented as median values (n= 3).



Supplemental Figure 14: MICs of free meropenem and empty liposomes against different lab strains of Gram-negative bacteria (n=3).



Supplemental Figure 15: Characteristics of meropenem-encapsulated liposomes along with the addition of 0.5% or 2.5% DSPE-PEG2000. Cationic liposomal formulations (PC: DODAB: CHOL) encapsulating meropenem prepared by thin-film hydration method had positive charges ranging from no charge i.e., + 0 mV to highly positive charges + 58 mV liposomes. The desired sizes are slightly bigger than the expected sizes i.e., 50, and 100 nm due to the high flexibility of lipids. The Polydispersity Index (PDI) quantifies the non-uniformity of the size distribution of particles ranging from 0.04 to 0.1 (for a perfectly uniform sample concerning the particle size). % internal meropenem is the amount of meropenem within liposomes ranging from 44% to 81% and the total drug concentrations and E.E% depend upon the physicochemical properties of cationic liposomal formulations. Data presented as median (n=3).



Supplemental Figure 16: Graphical representation for antimicrobial efficacy of liposomal meropenem following the addition of PEG. MICs of free meropenem), non-PEGylated and PEGylated liposomal meropenem with incremental amounts of PEG (0.5% and 2.5%) against different lab strains gram-negative bacteria i.e., Acinetobacter, E.C = E. coli, K.P = Klebsiella pneumoniae, P.A = Pseudomonas aeruginosa. The addition of incremental amounts of PEG (0.5% and 2.5%) resulted in a small decrease in the antimicrobial efficacy of liposomal meropenem as compared to non-PEGylated formulations but more efficacious than free standard meropenem. Data presented as median (n=3).