OVERCOMING BIOLOGICAL BARRIERS IN TREATING INTRACELLULAR INFECTIONS USING LIPID-BASED NANOPARTICLES

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Bachelor of Pharmaceutical Science

Bachelor of Biomedical Research (Honours)

A thesis submitted for the degree of

Doctor of Philosophy

UniSA Clinical and Health Sciences



University of South Australia

Submitted for examination: 7th March 2023

To my dearest parents, Amma and Appa – this is for you

To Uncle Ray, I hope you are smiling down on me from heaven

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Abbreviations

AMP	Antimicrobial peptides
ANOVA	Analysis of variance
BALF	Bronchoalveolar lavage fluid
CFU	Colony forming unit
DDAB	Didodecyldimethylammonium bromide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric substance
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GMO	Glyceryl monooleate
GRAVY	Grand average of hydropathy
LC-MS	Liquid chromatography-mass spectrometry
LCNP	Liquid crystalline nanoparticles
MFI	Mean fluorescence intensity
MIABiE	Minimum Information about a Biofilm Experiment
MIBBI	Minimum Information for Biological and Biomedical Investigations
MIC	Minimum inhibitory concentration
MIRIBEL	Minimum Information Reporting in Bio-Nano Experimental Literature
MOI	Multiplicity of infection
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mW	Molecular weight

PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PDI	Polydispersity index
PEG	Poly (ethylene glycol)
pI	Isoelectric point
SCV	Small colony variant
TSA	Tryptic soy agar
TSB	Tryptic soy broth
WGA	Wheat germ agglutinin

Note: Abbreviations used within published papers are not included in the above list.

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Abstract

Infectious diseases continue to be a major threat to humankind, taking millions of lives and bearing significant costs to the healthcare system globally. Bacteria evolves rapidly, circumventing strategies in place to eradicate them. One such strategy is their ability to invade and persist within host cells causing intracellular infections, thus avoiding the host immune response. Furthermore, antibiotics are ineffective against intracellular bacteria due to their poor penetration into host cells, therefore not reaching the site of infection. This leads to sub-optimal clinical outcomes with patients suffering from persistent and recurrent infections for an extended period. Nanoparticles as carriers for antibiotics, also referred to as "nanoantibiotics", are a promising approach as they facilitate intracellular uptake of antibiotics, thereby leading to improved eradication of bacteria. Despite their significant potential, research limitations and challenges hinder their clinical translation. Thus, the aim of this thesis is to compare the influence of architecture and composition of different lipid-based nanoparticles; liposomes and liquid crystalline nanoparticles (LCNPs) on internalisation in two types of pulmonary host cells and evaluate their subsequent activity against intracellular bacteria. In addition, for the first time, the role of biorelevant proteins on cellular interaction, drug release kinetics and efficacy of nanoantibiotics, was explored.

Firstly, the protein corona formation of two lipid-based nanoparticles, liposomes and LCNPs as examples of lamellar and non-lamellar structures, was determined in presence of fetal bovine serum (FBS). A proteomics approach was utilised to identify major proteins adsorbed to the surface of both nanoparticles. While no significant differences in protein corona composition were observed, their influence on cellular interaction was distinct for each nanoparticle. In RAW264.7 macrophages, a 50% reduction in internalisation was observed for liposomes, with LCNPs achieving 2-fold higher uptake. The trend was similar in A549 alveolar epithelial cells, with internalisation of LCNPs strongly mediated by presence of proteins. The protein corona restricted the release of vancomycin from LCNPs, reaching a

maximum of only 10% release compared to ~40% in absence of FBS. Enhanced cellular uptake of LCNPs with protein corona in macrophages correlated with their improved activity against intracellular small colony variants (SCVs) of *Staphylococcus aureus* (*S. aureus*). This was not observed for A549 epithelial cells, thus confirming a cell-dependent activity of the protein corona on nanoantibiotics.

While FBS is routinely used in cell culture and is the first point of contact for nanoparticles for in vitro assays, their clinical relevance for pulmonary applications is limited. Therefore, the cellular uptake of both nanoparticles, together with their protein corona fingerprint were investigated using murine bronchoalveolar lavage fluid (BALF) as a bio-relevant media for pulmonary applications. A high percentage of relative protein abundance involved in molecular function regulation and binding processes was found on the surface of LCNPs, resulting in enhanced internalisation into both, macrophages and epithelial cells. Albumin was the most abundant protein found in the corona of liposomes, and reduced cellular uptake observed, in line with previous observations using FBS. Interestingly, in presence of BALF, liposomes also demonstrated a rapid endocytosis followed by exocytosis after 6 h, further highlighting the dynamic influence of protein corona on cellular interaction behaviour.

In an attempt to enhance the activity of LCNPs against intracellular bacteria in both cell lines, the cationic lipid, dimethyldioctadecylammonium bromide (DDAB) was incorporated to the lipid matrix. The addition of DDAB strongly enhanced interaction of LCNPs, reaching ~50% uptake in macrophages and ~90% uptake in epithelial cells (corresponding uptake of neutral LCNPs was ~15% and ~1%). Both LCNPs and DDAB-LCNPs were loaded with either vancomycin or tobramycin and their activity evaluated against intracellular SCVs *S. aureus* or *Pseudomonas aeruginosa* (*P. aeruginosa)*. As hypothesized, the enhanced cellular uptake of DDAB-LCNPs resulted in superior performance of nanoantibiotics in both cell lines, although the performance in A549 infected cells with SCVs *S. aureus* remain limited.

Lastly, we have established a guideline and framework for researchers to conduct and report scientific findings using in vitro intracellular infection assays. The guideline aims to improve consistency across experiments, which allows comparison and better judgments to be made, guiding researchers to drive future investigations and clinical translation of experimental findings. Key parameters to consider and disclose in articles are provided for all stages of investigation, i.e. formulation characterisation and synthesis, establishing an in vitro infection model and evaluating performance of formulation against intracellular bacteria.

The findings derived from this thesis provide valuable insights into the influence of nanoparticle composition and structure on their cellular internalisation and activity against Gram-negative and Gram-positive bacteria. Importantly, the influence of bio-relevant media on cellular uptake mechanisms and performance of nanoantibiotics for pulmonary delivery was elucidated. These observations provide a mechanistic insight into the complexity of targeting intracellular infection and addresses key limitations to improve clinical translation of novel therapeutics.

Declaration

I declare that this thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university. To the best of my knowledge, it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.



Santhni Subramaniam

7th March 2023

Publications and Presentations during PhD Candidature

List of Publications in Support of Thesis

Subramaniam S, Joyce P, Prestidge CA. The cellular uptake and in vitro antibacterial activity of nanoantibiotics is influenced by protein corona. *Biomaterials Science* 2023; in preparation.

Subramaniam S, Joyce P, Prestidge CA. Liquid crystalline lipid nanoparticles improve the antibacterial activity of tobramycin and vancomycin against intracellular *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *International Journal of Pharmaceutics* 2023; 639: 122927.

Subramaniam S, Joyce P, Donnellan L, Young C, Wignall A, Hoffmann P, Prestidge CA. Protein adsorption determines pulmonary cell uptake of lipid-based nanoparticles. *Journal of Colloid and Interface Science* 2023; 641: 36-47.

Subramaniam S, Joyce P, Thomas N, Prestidge CA. Bioinspired drug delivery strategies for repurposing conventional antibiotics against intracellular infections. *Advanced Drug Delivery Reviews* 2021; 177:113948.

Other Publications

Joyce P, Ulmefors H, Maghrebi S, **Subramaniam S**, Wignall A, Jõemetsa S, Höök F, Prestidge CA. Enhancing the cellular uptake and antibacterial activity of rifampicin through encapsulation in mesoporous silica nanoparticles. *Nanomaterials* 2020; 10(4):815.

Subramaniam S, Thomas N, Gustafsson H, Jambhrunkar M, Kidd SP, Prestidge CA. Rifampicin-loaded mesoporous silica nanoparticles for the treatment of intracellular infections. *Antibiotics* 2019; 8(2):39.

Scientific Presentations

Subramaniam S, Joyce P, Prestidge CA. Nanoantibiotics against intracellular bacteria: Influence of biological molecules on performance of lipid nanocarriers [Oral]. 2022 Drug Delivery Australia Conference, 24th November: Adelaide, Australia.

Subramaniam S, Joyce P, Prestidge CA. Pulmonary delivery of lipid-based nanoparticles: Identification of proteins that mediate cellular uptake kinetics [Oral]. Health and Biomedical Innovation (HBI) Emerging Researcher Symposium, 23rd September: Adelaide, Australia.

Subramaniam S, Joyce P, Prestidge CA. Pulmonary delivery of lipid-based nanoparticles: Identification of proteins that mediate cellular uptake kinetics [Oral]. 12th International Nanomedicine Conference, 27th – 29th June 2022: Sydney, Australia.

Subramaniam S, Joyce P, Prestidge CA. Addressing current biological limitations in translating nanoantibiotics for intracellular infection [Oral]. Australian Society for Medical Research (ASMR) South Australia Annual Scientific Meeting, 9th June 2022: Adelaide, Australia.

Subramaniam S, Joyce P, Prestidge CA. Architecture and composition of lipid nanoparticles on intracellular delivery: A comparison between macrophages and epithelial cells [Oral]. 33rd Australian Colloid and Surface Science Student Conference, 31st - 2nd February 2022: Virtual.

Subramaniam S, Joyce P, Prestidge CA. Architecture and composition of lipid nanoparticles on intracellular delivery: A comparison between macrophages and epithelial cells [Poster]. Drug Delivery Australia, 18th – 19th November 2021: Virtual.

Subramaniam S, Joyce P, Prestidge CA. Exploring the internalization pattern of lipid-based formulations in cells prone to bacterial infection [Poster]. Drug Delivery Australia, 23rd November 2020: Virtual.

Subramaniam S, Thomas N, Joyce P, Prestidge CA. Nanoantibiotics as a drug delivery strategy for intracellular infections [Oral]. UniSA Clinical & Health Sciences Research Seminar, 17th April 2020: Virtual.

Subramaniam S, Thomas N, Gustafsson H, Jambhrunkar M, Prestidge CA. Rifampicin-loaded mesoporous silica nanoparticles for the treatment of intracellular infections [Poster]. Drug Delivery Australia, 18th – 19th November 2019: Brisbane, Australia

Subramaniam S, Thomas N, Gustafsson H, Jambhrunkar M, Kidd PS, Prestidge CA. Rifampicin-loaded mesoporous silica nanoparticles for the treatment of intracellular infections [Poster]. Controlled Release Society Annual Meeting & Exposition, 21st – 24th July 2019: Valencia, Spain.

Subramaniam S, Thomas N, Gustafsson H, Jambhrunkar M, Kidd PS, Prestidge CA. Mesoporous silica nanoparticles (MSNP) for the treatment of intracellular infections [Poster]. 10th International Nanomedicine Conference, 24th till 26th June 2019: Sydney, Australia.

Acknowledgements

Firstly, I would like to thank my principal supervisor, Prof Clive Prestidge for his unwavering support and guidance throughout this journey. Thank you for believing in me when I first knocked on your door as a naïve undergraduate student seeking a summer research scholarship. Over the years, you have helped me grow far and beyond, provided me with countless opportunities and shaped me into the scientist I am today. To my co-supervisor Dr Paul Joyce, I do not know how I would have made it here without you! Thank you for not only being all ears when I had a scientific idea or question, but also when I needed a brother to talk to. This success would have not been possible without both your encouragement. I would also like to thank Dr Nicky Thomas and Dr Chelsea Thorn for your guidance at the beginning of my journey.

To the Nanostructure and Drug Delivery Group, thank you for elevating my PhD experience with your support, laughter and friendships. To Leah Wright, thank you for sharing this journey with me from the start. You have always been there when I needed a shoulder to lean on and your faith in me has pushed me through the challenging times. I will always treasure our countless memories, travel adventures and the special friendship we share. To Anthony Wignall, it has never been a dull day with you in the lab. Thank you for always being there to share a good laugh and helping me throughout this journey in many ways. Thank you to Carolin Baur and Mitja Pohlen for making the first six months of my PhD a colourful one! I am grateful for our continuing friendship even when we are miles apart. A special thanks to Mirjam von Lucadou, for sharing this journey together from Germany, and for introducing me to your lovely family and country in 2019.

I would also like to thank Dr Abiodun Ogunniyi for reigniting my passion in my research topic towards the last leg of my PhD journey. Albeit a short time, you have taught me valuable skills and built in me a positive attitude towards experiments that did not work! To Jack Blaikie, thank you for your laughter that kept me sane during my long and isolated times in the microbiology lab. A special shout out to my REPL, Dr Lisa Kalisch Ellett for all your support over the last few years. Thank you for being empathetic, and for the catch ups that left me feeling a whole lot better! I would also like to thank Dr Matt Sykes, Dr Sally Plush and Dr Vijay Suppiah for providing me with opportunities to grow beyond a scientist and teaching me skills outside of my PhD. To the international student team, Mr Rishen Shekar and Ms Louise Evershed, thank you for your support and helping me through one of the lowest points of my PhD. I would also like to thank my counsellor Ms Theresa Heim,

for always keeping your door open for me. This accomplishment would not have been possible without your guidance and strategies to be resilient in times of adversity.

Not forgetting my amazing mentors, Dr Jenni Harrison and Dr Jennifer Wong. Thank you for sharing your time and instilling in me your wisdom and drive to learn continuously. To Dr Linda Hong and Dr Sara Codeiro, thank you for believing in me and for your unwavering support during my time in the Young Scientist Committee of Controlled Release Society (CRS). Much of my research work and international conference travels would not have been possible without the financial support from University President's Scholarship, ATA Scientific Encouragement Award, Gould Experimental Grant, CRS Young Scientist and Patrick Couvreur Travel Grant. To Dr Gould and Prof Couvreur, I am grateful for the opportunity to have met a humble and stellar scientist like you during my PhD.

To all my wonderful friends, especially my amazing girlfriends (you know who you are), thank you for being part of this journey all these years. You have been there to support me at my worst and cheer with me at my best. Without all your support and encouragement, I would be nowhere close to achieving this success! A special thanks to Rachel Hibble, for being a wonderful friend and support since day one of my undergraduate. Thank you for always believing in me and reminding me to have a good laugh even when times get tough. To Jayshen Arudkumar, my PhD buddy from another lab, thank you for all the great memories and friendship we have built by sharing our joys and sorrows of research together! My board members of Adelaide City Rotaract Club, thank you for your support in helping me deliver in my role as the club's president while being in the final year of my PhD.

Lastly, the biggest thank you goes to my family for being my number one support and cheerleaders! To Amma and Appa, thank you for enabling me to follow my dreams and passion. I am eternally grateful for the sacrifices you have made since day one in providing me a better future. To Akka, thank you for always being proud of all my successes, no matter how small they were. To all my family back home – thank you for believing in me and for driving me to reach further heights in my academic successes. To Uncle Ramesh and family, thank you for always making me feel at home with your love and care. Thank you to my partner Abilash Sivadasan, for being the Yang to my Yin! Thank you for being my sounding board, always supporting me in the best way possible and for providing me with a loving family who cares for me so well.

Thank you to each and every one of you for walking along this journey with me, for believing in me to reach beyond the stars and encouraging me to pursue my goals.

Introduction and Aims

Clinically resistant bacteria continue to evolve rapidly, costing billions of dollars to the healthcare system. A significant challenge in overcoming this issue is the ability of bacteria to survive within host cells, thereby causing intracellular infections. Antibiotics suffer from poor penetration into host cells, thus delivering suboptimal concentration to the site of infections, rendering current clinical treatments ineffective. Nanotechnology is a promising approach to improve penetration and delivery of antibiotics to the site of infection, leading to enhanced activity against bacteria. An array of nanoparticles, ranging from organic, polymeric and inorganic nanoparticles have been tested for various types of intracellular infections. Despite successful outcomes in pre-clinical studies, the successful translation of these findings to viable clinical formulations is limited. Hence, the search for improved formulations with optimized cellular uptake and drug release kinetics is ongoing.

Of the different types of intracellular infections present, pulmonary infections are a significant clinical challenge and critical target for improvement. Tuberculosis, pneumonia, and cystic fibrosis are examples of the debilitating infections experienced by children and adults, which can be exacerbated in patients with compromised lungs [1-3]. Antibiotics are typically administered orally or intravenously, with reduced bioavailability/biodistribution in the lungs and inhalable antibiotics suffer from not reaching the intracellular site of infection [4]. Nanoparticle systems offer potential here, however there are several practical challenges for pulmonary delivery that have not been well addressed. One of it includes the heterogeneity of cells present as a reservoir for intracellular bacteria to survive and replicate. Surface properties, distinct cellular structure and function of pulmonary cells can alter interaction with nanoparticles [5]. Macrophages are key players of the innate immune response in lungs, specializing in homeostasis regulation, removal of bacteria and foreign materials through phagocytosis and mediating inflammatory responses [6]. Intracellular

bacteria have developed several strategies to escape the phagocytic activity of macrophages, thus are capable of surviving in these cells causing recurrent infections [7]. In recent years, the survival of bacteria in non-phagocytic cells, such as the lung epithelial cells has also been identified [8, 9]. The alveolar region of the cell consists of primarily type I epithelial cells, that closely interacts with pulmonary capillaries and type II epithelial cells, easily identified by their cuboidal structure and play an important role in regulating surface tension of the lung by secreting surfactant lipids and proteins [10]. Lung epithelial cells are commonly known for their passive role in gas exchange processes and mucus secretion, with growing evidence in recent years for their role as mediators of the local immune system [11]. Passive targeting of epithelial cells is difficult to achieve in comparison to macrophages due to the presence of tight junctions often needing additional strategies, such as ligand-functionalized or assisted delivery of nanoparticles. For example, Horsley et al. explored the use of liposome-coated microbubbles to deliver gentamicin to uroepithelial cells using ultrasound technology [12]. While the approach was successful at eradicating intracellular *Enterococcus faecalis* (*E. faecalis*), it is limited in application to treat pulmonary infections, such as cystic fibrosis. Hence, there is a need for improved performance of antibiotics in targeting pathogens residing in both professional and non-professional phagocytes.

Secondly, prior to nanoparticles' interaction with cells, they first encounter and strongly interact with native proteins that adsorb to their surface, forming a protein corona in a biological environment [13]. The presence of the protein corona on the surface of nanoparticles gives them a new and unique identity, referred as the "biological identity", which is distinct from their "synthetic identify" upon fabrication [14]. Protein corona can have a profound impact on intracellular delivery of nanoparticles by altering their interaction with cellular membranes [15]. The interplay of protein adsorption to nanoparticles and their subsequent interaction with biological membranes is a complex process. Thus far, studies have been predominantly focused on the interaction of proteins isolated from plasma for

their implication on systemic delivery of nanoparticles. The interaction of nanoparticles with proteins in the lung and their influence on intracellular delivery of nanoparticles for pulmonary cells remains to be further investigated [16].

To overcome the membrane barrier of two diverse cell types and to explore the influence of nanoparticle-protein complexes, focus will be directed to lipid-based nanoparticles. Specifically, the use of newly emerging non-lamellar systems, i.e. liquid crystalline nanoparticles (LCNPs), is investigated, with comparisons against lamellar systems, i.e. liposomes elucidated. LCNPs are of interest due to their high drug loading capacity and ability to offer better stability of their cargo molecules than liposomes [17]. In the field of antimicrobial delivery, LCNPs thus far have been investigated as carriers for peptides and antibiotics against planktonic bacteria or biofilms [18-20]. Further studies to understand their mechanism of protein interaction and cellular uptake are useful for their application in treating pulmonary intracellular infections.

Therefore, in exploring the above limitations, this thesis addresses the following hypotheses:

Hypothesis 1	The architecture, composition and structure of lipid-based nanoparticles will influence their cellular uptake, drug release and activity against intracellular bacteria. *Chapters 2, 3 and 4*
Hypothesis 2	The use of a biologically relevant media including the presence of proteins will directly impact lipid nanoparticles' behavior, including their cellular interactions, drug release of antibiotics and intracellular killing of bacteria. *Chapters 2 and 3*
Hypothesis 3	Identifying key proteins adsorbed to nanoparticle surfaces will provide further understanding on their cellular uptake kinetics.

Chapters 2 and 3

Hypothesis 4 LCNPs internalisation in phagocytic and non-phagocytic cells can be enhanced using cationic lipid, improving their activity against intracellular bacteria.

Chapter 4

In testing these hypotheses, the research has the following aims:

Aim 1 Synthesise and characterise liposomes and LCNPs of similar size and surface charge for cellular and antimicrobial studies.

Aim 2 Characterise the nanoparticles in presence of a biologically relevant media.

Aim 3 Investigate the interaction of lipid-based nanoparticles with phagocytic and non-phagocytic cells in presence of biologically relevant media.

Aim 4 Determine key proteins present in the nanoparticle corona using a label-free quantitative proteomics approach to improve current understanding on uptake of nanoparticles.

Aim 5 Evaluate the activity of cubosomes encapsulated with vancomycin against intracellular *Staphylococcus aureus* (*S. aureus*) in presence and absence of proteins.

Aim 6 Quantitatively analyse the behavior and activity of cationic LCNPs against intracellular *S. aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*).

Chapter 1 of the thesis provides an in-depth literature review on the current state of the art of nanoparticle strategies employed for intracellular infections. The mechanisms of

intracellular bacterial survival and current treatment challenges are introduced. A detailed overview of the different strategies utilised in drug delivery systems for intracellular infection is provided, particularly highlighting the bioinspired approaches undertaken thus far. The current limitations and challenges in using a nanoparticle approach for intracellular infection is addressed, providing opportunities to improve clinical translation of the technology.

The influence of nanoparticle-protein complexes on nanoparticulate systems for intracellular delivery of antibiotics is first explored in **Chapter 2**. The protein corona of liposomes and LCNPs in presence of fetal bovine serum (FBS) was characterised using proteomics to enhance current understanding of nanoparticle-protein interaction. While the particles were of similar sizes and surface charge, their composition was different, with cubosomes having a complex architecture due to their interconnecting water channels within their lipid matrix. The nanoparticles were further analysed for their cellular interaction in presence and absence of proteins in RAW 264.7 macrophages and A549 epithelial cells. Following higher uptake of LCNPs in presence of a protein corona, the drug release kinetics of vancomycin from the nanoparticles, and subsequent efficacy against intracellular *S. aureus* were evaluated.

Chapter 3 expands on the influence of protein corona on intracellular uptake of the previously developed nanoparticles with relevance to pulmonary delivery. Here, the cellular uptake was investigated in presence of bronchoalveolar lavage fluid (BALF) as a biorelevant media. Significant differences in the protein fingerprint of liposomes and LCNPs were observed, resulting in varied biological responses with the two types of cells used. Identification of the major proteins adsorbed on each nanoparticle type provided further understanding of a pulmonary protein corona on mediating nanoparticle behaviour, providing key criteria to be addressed for the development of an optimised nanocarrier for pulmonary therapeutics.

The potential of LCNPs for intracellular infections was further explored in **Chapter 4**. Utilising a cationic lipid, the internalisation of LCNPs in both phagocytic and non-phagocytic cells was significantly improved. The morphology of the nanoparticles in presence of cationic lipid was characterised. Further, the ability of LCNPs to load both a small and large antibiotic, i.e. tobramycin and vancomycin, respectively, and the in vitro release kinetics were determined. Finally, their antimicrobial activity against two relevant intracellular pathogens, *S. aureus* and *P. aeruginosa* was evaluated.

To address some of the key limitations in translating in vitro findings to preclinical studies and eventually as a viable formulation, **Chapter 5** provides a comprehensive guideline and framework for researchers on conducting in vitro intracellular infection assays. The guideline aims to standardise findings reported in literature and improve cross-comparison of studies performed, ultimately leading to advancements in the field of novel therapeutics against intracellular infections.

Finally, research findings from the thesis are summarized in **Chapter 6**, with future directions of the work explored.

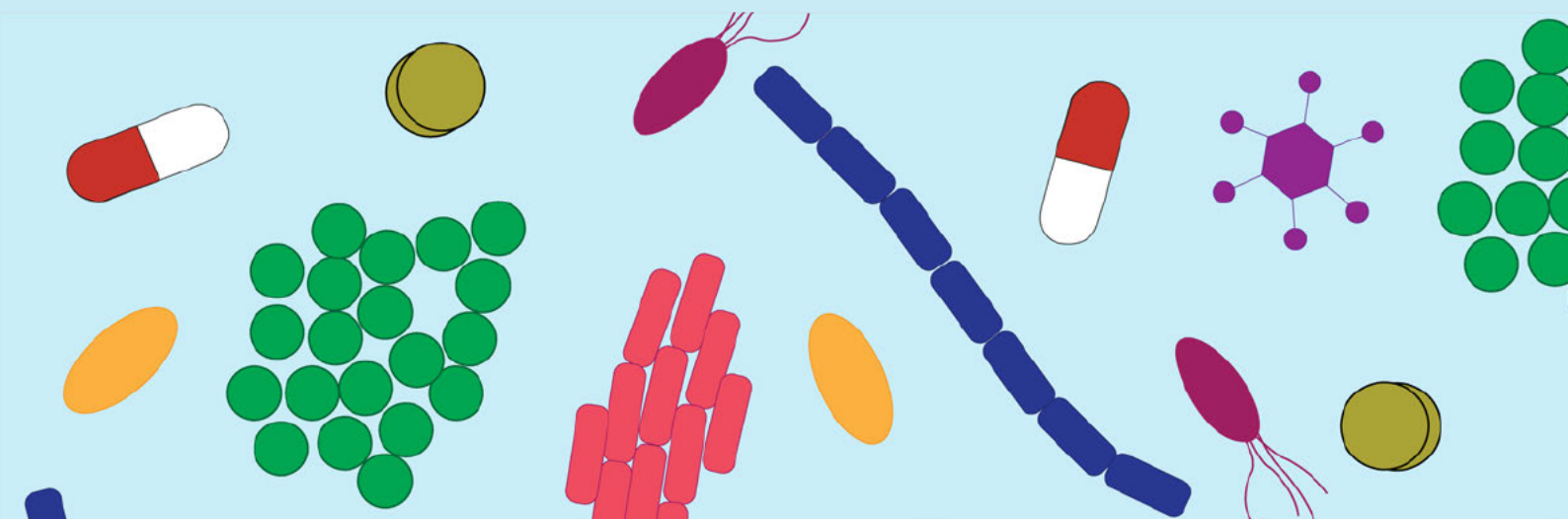
This thesis was submitted to the University of South Australia as a Thesis Containing Published Research in accordance with Appendix AB-58 AD2: Content and Form of the Research Degree Thesis. Santhni Subramaniam was the primary author on all publications and chapters presented in this thesis. The contributions of each author for each publication presented are available in Appendix I. Permission was granted from the respective publisher for all figures and tables which have been reproduced in this thesis.

Thesis Outline

Chapter	Publication Status
1	Bioinspired drug delivery strategies for repurposing conventional antibiotics against intracellular infections **Published.** *Advanced Drug Delivery Reviews* 2021; 177:113948.
2	The cellular uptake and in vitro antibacterial activity of nanoantibiotics is influenced by protein corona **In preparation.** *Biomaterials Science* 2023
3	Protein adsorption determines pulmonary cell uptake of lipid-based nanoparticles **Published.** *Journal of Colloid and Interface Science* 2023; 641: 36-47.
4	Liquid crystalline lipid nanoparticles improve the antibacterial activity of tobramycin and vancomycin against intracellular *Pseudomonas aeruginosa* and *Staphylococcus aureus* **Published.** *International Journal of Pharmaceutics* 2023; 639: 122927.
5	Minimum information for conducting and reporting in vitro intracellular infection assays
6	Thesis Summary

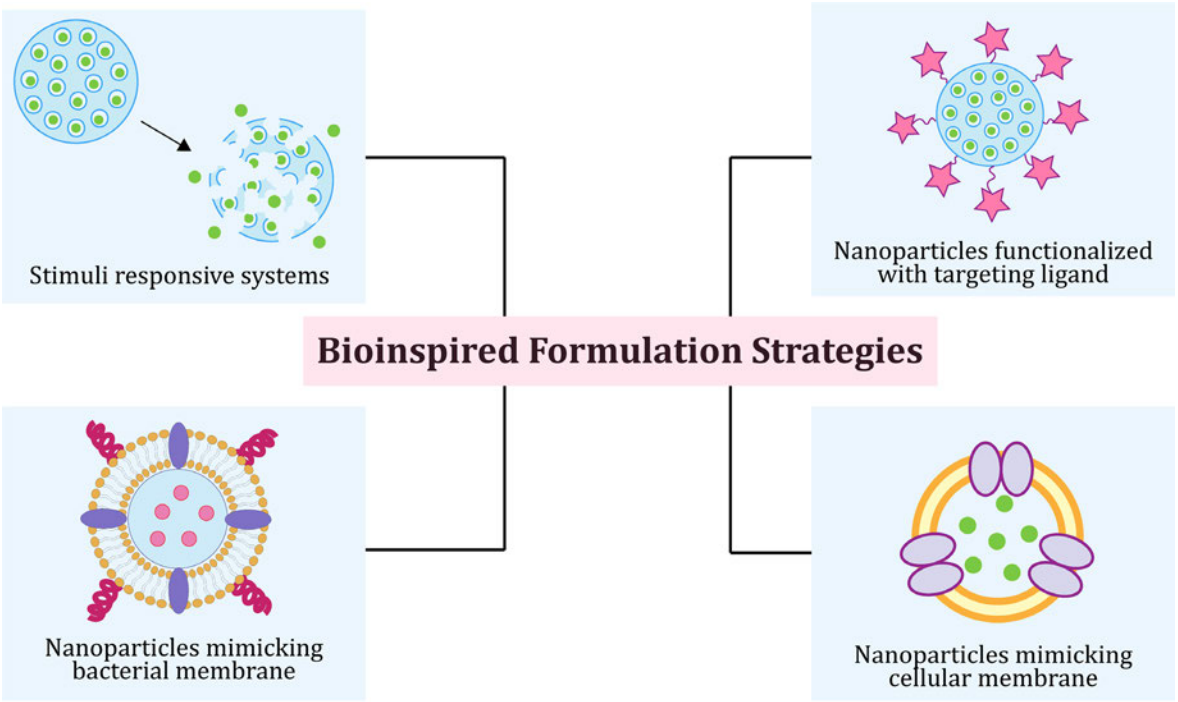
1

BIOINSPIRED DRUG DELIVERY STRATEGIES FOR REPURPOSING CONVENTIONAL ANTIBIOTICS AGAINST INTRACELLULAR INFECTIONS



1 Bioinspired drug delivery strategies for repurposing conventional antibiotics against intracellular infections

This chapter is a literature review that provides a comprehensive overview of challenges and strategies utilised in treating intracellular infections. The bacterial internalization and survival mechanisms are elucidated, together with current challenges with treating intracellular infections discussed. An extensive overview of nano-carrier systems utilized to deliver antibiotics imitating biological mechanisms for cellular uptake and release is appraised, with detailed key case studies illustrated. The limitation in this field is critically addressed and future perspective guiding further development of the nanoantibiotics approach is provided.



Advanced Drug Delivery Reviews 177 (2021) 113948

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Bioinspired drug delivery strategies for repurposing conventional antibiotics against intracellular infections

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ARTICLE INFO

Article history:
Received 15 April 2021
Revised 4 August 2021
Accepted 23 August 2021
Available online 28 August 2021

Keywords:
Infectious diseases
Antibacterial resistance
Nanoparticles
Nanoantibiotics
Bio-formulation
Biomimetics

ABSTRACT

Bacteria have developed a wealth of strategies to avoid and resist the action of antibiotics, one of which involves pathogens invading and forming reservoirs within host cells. Due to the poor cell membrane permeability, stability and retention of conventional antibiotics, this renders current treatments largely ineffective, since achieving a therapeutically relevant antibiotic concentration at the site of intracellular infection is not possible. To overcome such challenges, current antibiotics are 'repurposed' via reformulation using micro- or nano-carrier systems that effectively encapsulate and deliver therapeutics across cellular membranes of infected cells. Bioinspired materials that imitate the uptake of biological particulates and release antibiotics in response to natural stimuli are recently explored to improve the targeting and specificity of this 'nanoantibiotic' approach. In this review, the mechanisms of internalization and survival of intracellular bacteria are elucidated, effectively accentuating the current treatment challenges for intracellular infections and the implications for repurposing conventional antibiotics. Key case studies of nanoantibiotics that have drawn inspiration from natural biological particles and cellular uptake pathways to effectively eradicate intracellular pathogens are detailed, clearly highlighting the rational for harnessing bioinspired drug delivery strategies.

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Abbreviations: AMP, antimicrobial peptides; CM, cecropin melittin; CTCF, corrected total cell fluorescence; DMSO, dimethyl sulfoxide; DCP, dicetylphosphate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPC, dioleoyl phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPG, 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol; EAP, extracellular adherence protein; *E. coli*, *Escherichia coli*; *E. faecalis*, *Enterococcus faecalis*; FDA, Food and Drug Administration; *F. novida*, *Francisella novida*; *F. tularensis*, *Francisella tularensis*; GFP, green fluorescent protein; HPC, hydrogenated phosphatidylcholine; HSPC, hydrogenated soy phosphatidylcholine; InvA, invasin; *L. monocytogenes*, *Listeria monocytogenes*; *M. avium*, *Mycobacterium avium*; MAC, *Mycobacterium avium* complex; MBC, minimum bactericidal concentration; MDV, mitochondria derived vesicles; MIC, minimum inhibitory concentration; MLV, multilamellar vesicles; mPEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; MR, molar ratio; MRSA, methicillin resistant *Staphylococcus aureus*; MSN, mesoporous silica nanoparticle; *M. bovis*, *Mycobacterium bovis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; OMV, outer membrane vesicles; PAL, polymer augmented liposome; PAMAM, poly(amidoamine); PBCA, poly(butyl cyanoacrylate); PDI, polydispersity index; PDPA, poly[2-(diisopropylamino)ethyl methacrylate]; PEG, polyethylene glycol; PEI, polyethyleneimine; PLGA, poly(lactide-co-glycolide); PLH, polymer-lipid hybrid; PMPC, poly[2-(methacryloyloxy)ethyl phosphorylcholine]; RBC, red blood cells; ROS, reactive oxygen species; *S. aureus*, *Staphylococcus aureus*; SCV, small colony variants; *S. enterica*, *Salmonella enterica*; SLN, solid lipid nanoparticle; SPION, superparamagnetic iron oxide nanoparticles; *S. typhimurium*, *Salmonella typhimurium*; SUV, small unilamellar vesicles; UTI, urinary tract infections; WHO, World Health Organization; ZWC, zwitterionic chitosan.

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https://doi.org/10.1016/j.addr.2021.113948
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1. Introduction

The development of antibiotics in the early 20th century witnessed one of history's most significant pharmaceutical breakthroughs, ultimately saving millions of lives from deadly infections. Many medical treatments such as organ transplantation, preterm newborn care, cancer chemotherapy and major surgeries would not be possible without the use of antibacterial agents [1,2]. However, the over prescription and misuse of antibiotics has led to the development of highly resistant infections. The World Health Organization (WHO) has recently released reports demanding immediate action to be taken before we witness another global pandemic of bacterial diseases. Today, approximately 700,000 people die annually due to drug resistant infections, which is expected to increase to 10 million by 2050 if no action is taken [3].

Further to antimicrobial resistance, virulence mechanisms of bacteria pose significant challenges for the successful treatment of infectious diseases. Bacteria have evolved to adapt several survival strategies against their host's defense systems and conventional antimicrobial therapies, including: i) their existence as bacterial biofilms, where communities of bacteria live in complex matrices [4,5], and (ii) their ability to invade, colonize and survive within host cells, causing intracellular infections [6]. Salmonellosis, tuberculosis and urinary tract infections (UTI) are some of the challenging and debilitating infections caused by intracellular bacteria [7]. Readers interested in the pathogenesis of intracellular infections are recommended to read the comprehensive book edited by Schaible & Haas [8]. Currently available antibiotics fail to eradicate these hidden bacteria due to their limited physicochemical properties such as, poor permeability across infected cells, poor intracellular retention, in addition to poor stability inside the cells [9,10]. To overcome such challenges, new antibacterial agents that actively target intracellular bacteria and/or new pharmaceutical devices/formulations that deliver existing antibiotics across cellular membranes to the site of infection, are urgently required. It is estimated that 41 new antibiotics are currently in the development pipeline, with 15 antibiotics in Phase 1 clinical trials, 12 in Phase 2, 13 in Phase 3 and four recently been approved by the US Food and Drug Administration (FDA) [11]. However, none of these antibiotics are designed specifically for intracellular infections. Furthermore, obtaining regulatory approval for new drug entities and receiving clinical validation is a time and resource exhaustive process, with no guarantees that they will overcome issues relating to long term antimicrobial resistance and the treatment of intracellular pathogens.

Alternatively, conventional antibiotics can be encapsulated within micro or nano carriers to promote their transport across cellular membranes. Over the past few decades, nanotechnology derived delivery systems have been extensively researched for therapeutics of varying physicochemical properties and for a wide range of conditions such as cancer, cardiovascular diseases and more recently infectious diseases. Nanoparticles have been explored as carriers for (i) improved delivery of poorly soluble drugs, (ii) penetration of therapeutics across biological membranes, e.g., blood brain barrier and mucus membranes, (iii) targeted therapy, e.g., site specific delivery of therapeutics to cancerous cells, (iv) theragnostic applications and (v) for biotherapeutics, e.g., peptides and vaccines [12]. The rise of antibacterial resistance together with the reluctant development of new antibiotics has resulted in the repurposing of conventional antibiotics through their reformulation as micro or nano particles (referred as nanoantibiotics) [13,14]. These delivery systems facilitate modulated release kinetics and enhanced efficacy that ultimately lowers the required administration frequency and dose of antibiotics [14]. Of particular interest is the ability of nanoparticles to permeate through cell membranes of infected cells through endocytic or phagocytic pathways, providing benefits for the delivery of antibiotics across the plasma membrane of host cells, which are impermeable to many conventional antibiotics [7,15]. Once internalized, the encapsulated antibiotics are released and colocalized with bacterial colonies hidden within intracellular compartments.

Despite the potential of the nanoantibiotics approach, only a small number of pre clinical studies have successfully advanced towards clinical translation for the treatment of infectious diseases [15,16]. Amphotericin B encapsulated in liposomes (e.g., AmBisome®) is currently available for severe fungal infections, an amikacin liposome inhalation suspension was approved by the US FDA for the treatment of *Mycobacterium avium* complex (MAC) in September 2018, and inhaled liposomal ciprofloxacin formulations have been investigated in two phase 3 clinical trials. Due to inconsistency with results across trial groups the latter requires further investigation [17 19]. However, these formulations were designed to reduce the systemic toxicity of antibiotics and there are no clinically approved formulations available for many intracellular infections.

The bench to bedside translation of nanoantibiotics remains poor, largely due to (i) potential toxicity of the nanocarriers (nanotoxicology), (ii) non targeted or off site delivery of nanoantibiotics, both on a tissue and cellular level, and (iii) premature drug release from the carriers prior to reaching the cells of interest, thus limiting their overall efficacy [14,20]. Hence, to further improve the efficacy and to reduce the toxicity of nanoantibiotics, the use of bioinspired materials, such as cellular membrane mimics, peptides and stimuli responsive systems are considered as viable strategies. Bioinspired materials draw inspiration from the interaction of natural particulates with the biological system, such as exosomes used for cell communication as drug delivery carriers [21]. Endogenous stimuli such as pH and redox state can be exploited for stimuli responsive drug delivery systems. In addition, cellular receptors expressed by host or bacterial cells can be targeted to increase the specificity of the nanoantibiotics. By understanding key biological processes of intracellular infection, nanoparticles can be adapted to mimic these processes, therefore be advantageous for the safe and effective delivery of antimicrobial agents.

Intracellular infections are complex, with intricate modulation of the host immune system and bacteria counter defence strategies contributing to persistent infections [22]. It must be noted, the term "intracellular infection" is also used throughout this manuscript and within literature when referring to *in vitro* assays where pathogens are localized and survive within intracellular components [23]. However, *in vitro* models do not accurately mimic the pathophysiology of bacterial infection, as observed in a clinical setting or *in vivo*, and are therefore not a precise model for infection. Rather, they serve as an important preliminary tool to evaluate the effectiveness of nanoantibiotics against intracellular bacteria when appropriately designed and validated. As such, the aim of this review is to explore the invasion strategies of intracellular pathogens and to elucidate the advantages of nanoantibiotics for intracellular infections, specifically highlighting the recent progress made using bioinspired materials in non clinical studies. Insights derived from this review can be harnessed to drive the development of next generation bioinspired nanoantibiotics for the treatment of intracellular infection, to ultimately increase the clinical translatability of this therapeutic approach.

2. Intracellular infections – Evading the immune system

Intracellular bacteria are pathogenic species that have evolved to escape the host immune system and developed strategies to survive and replicate inside their host cells [8,24]. Their exploitation of the intracellular environment and establishment of an intracellular survival behavior results in poor clearance of the bacteria either by prescribed antibiotics or by host defenses, causing chronic and recurrent infections (Table 1). *Salmonella*, *Mycobacterium* and *Listeria* are examples of well known intracellular bacteria. However, many bacteria previously classified as extracellular bacteria, such as *Staphylococcus* and *Enterococcus*, are increasingly known to adapt to the intracellular existence for protection and survival in immune cells (i.e. phagocytes such as macrophages) and non immune cells (i.e. epithelial cells such as urothelial cells), accelerating the severity and complexity of intracellular infections [25]. The ability of pathogens to invade these cells leads to many clinical challenges, including poor diagnosis and infection recurrence. In this section, the internalization and survival mechanisms of bacteria in cells are described, clinical cases of intracellular infections are reviewed, followed by insights on the current treatment approaches and challenges.

2.1. Mechanism of intracellular pathogen localization and survival

The modulation of the host immune system and strategies developed to evade antibiotics by intracellular bacteria is multifaceted. However, understanding the key mechanisms for bacterial survival in phagocytic and non phagocytic cells offers insights for the implications and development of bioinspired drug delivery systems as later discussed in this review. Here, their internalization and survival pathways in phagocytic and non phagocytic cells are explored.

Table 1
Summary of intracellular bacteria and their associated diseases, including survival in different host cells.

Bacterial pathogen	Associated disease	Host cell	Reference
Mycobacterium tuberculosis	Tuberculosis	Macrophages	[26]
Gram-positive bacteria			
Staphylococcus aureus	Rhinosinusitis Osteomyelitis Cystic fibrosis	Epithelial cells of the nasal mucosa, osteocytes, macrophages	[27–29]
Enterococcus faecalis	Urinary tract infections	Urothelial cells	[30]
Listeria monocytogenes	Listeriosis Gastroenteritis	Epithelial cells, macrophages	[31,32]
Gram-negative bacteria			
Escherichia coli	Urinary tract infections	Urinary bladder epithelial cells	[33]
Chlamydia trachomatis	Chlamydia	Genital tract epithelial cells	[34,35]
Salmonella typhimurium	Typhoid fever	Macrophages	[36]
Salmonella enterica	Salmonellosis	Intestinal epithelial cells, macrophages	[37]
Pseudomonas aeruginosa	Cystic fibrosis Pneumonia Keratitis	Macrophages, epithelial cells, primary corneal fibroblasts	[38–41]
Burkholderia cepacia complex	Cystic fibrosis	Macrophages, neutrophils, human respiratory epithelial cells	[42]
Brucella spp.	Brucellosis	Macrophages	[43]

2.1.1. Survival in professional phagocytes: hijacking phagocytosis

Phagocytosis is one of the most important defense mechanisms in humans, where phagocytes are responsible for the ingestion and destruction of foreign particles, including bacteria. The mononu clear phagocyte system comprised of professional phagocytes such as macrophages, neutrophils and monocytes are responsible for the elimination of microbes and presenting microbial antigens for adaptive immune responses [44,45]. The two main processes for effective phagocytosis are (i) pathogen internalization (Fig. 1 (a)) followed by (ii) phagosomal maturation (Fig. 1(b, c)), the key process for bacterial degradation.

Phagocytes display a considerable variety of pattern recognition receptors on their cellular surface to detect pathogens. For example, the scavenger receptor A detects lipopolysaccharides (LPS) of Gram negative bacteria, while mannose receptors recog nize the carbohydrate structures of bacteria [44]. In some cases, opsonins, such as immunoglobulin G from the host, attach to the microbe to initiate phagocytosis [44,45]. Upon recognition of a pathogen, membrane remodeling occurs to form pseudopods that extend from host cell membranes to engulf the pathogen, followed by encapsulation in a membrane bound vesicle (Fig. 1(a)), referred as the phagosome [44]. The binding of the microbial surface to the cellular receptors activates the signaling cascade to further pro mote bacterial internalization.

Once phagosomes are formed, fusion with early and late endo somes is initiated for phagosome maturation, facilitated by a series of protein recruitment processes (Fig. 1(b)). Specifically, V ATPases are found on the phagosome membrane to promote uptake of hydrogen ions into the phagosomes, causing the intra phagosome environment to become slightly acidic (pH 6.1 6.5) [45]. The acidity of phagosomes increases as they mature, and more proteins are recruited, permitting fusion with lysosomes to form phagolysosomes (Fig. 1(c)). Phagolysosomes are the key degradative vesicles, armed with antimicrobial peptides (AMP) and enzymes such as cathelicidin LL 37 and cathepsins that are responsible for destruction of bacteria [44,45]. Phagolysosomes are significantly more acidic in nature (pH 5 5.5) and generate reactive oxygen species (ROS), through the action of NADPH oxi dase, further facilitating bacterial killing.

2.1.1.1. Bacterial resistance to phagocytosis. Intracellular pathogens have developed a multitude of strategies to reside in professional phagocytes, as depicted in Fig. 1. They can impair biological pro cesses to avoid phagocytosis, build resistance to antimicrobial fac tors of the phagocytosis, or develop specific biological responses to counteract their host defense mechanisms [44,45]. *Mycobacterium tuberculosis* (*M. tuberculosis*) can escape the phagosomal pathway using their specialized protein secretion system, ESX1 [46]. The protein recruitment is impaired by phagosomes containing *M. tuberculosis*, in which they inhibit phagosome maturation and fusion with lysosome. Bacteria can also develop resistance to the AMP in host cells by expressing enzymes, e.g., *Staphylococcus aur eus* (*S. aureus*) diminishes the bactericidal effect of α defensins by staphylokinase mediated inactivation [47]. Some bacteria neutral ize the acidic pH of the phagosomes by expressing urease to hydro lyze urea into ammonia [44]. In addition, bacteria such as *Listeria monocytogenes* (*L. monocytogenes*) permeabilize the phagosome membrane using virulent factor, listeriolysin O to escape the harsh phagosomal conditions and then reside in the cytosol [44]. Some intracellular pathogens (e.g., *S. aureus*) can revert to a metabolically inactive mode of growth, known as small colony variants (SCV), by downregulating virulence factors in host cells to prevent inflam mation signaling pathways, therefore increasing their survival in host cells [48,49]. Elucidating the bacterial survival process in phagocytic cells provides opportunities for novel therapeutic approaches, such as the use of bioinspired nanoparticles.

2.1.2. Survival in non phagocytic cells: hijacking innate defense mechanisms

Bacteria are also capable of invading non phagocytic cells, such as epithelial cells of the lung, intestine and urinary tract, where the bacteria are usually degraded through the explicit defense mechanisms of the cells and/or tissue environment. The internal ization and survival mechanisms of bacteria in non phagocytic cells, e.g., endothelial cells, differ compared to phagocytic cells as elucidated below.

2.1.2.1. Defense mechanisms of non phagocytic cells. Epithelial cells that line the surfaces of organs in human body are constantly exposed to pathogens. In some cases, these pathogens are capable of invading the cells for nutrients and protection from immune defense mechanisms [50 52]. As a protection against bacteria, epithelial cells have developed multiple defense systems including, secretion of mucus and AMPs, rapid epithelial cell layer replace ment and autophagy [50,51]. For example, the gut epithelium is protected by a mucous layer consisting of digestive enzymes and AMPs, including defensins and cathelicidins which are actively involved in bacterial killing [53]. Similarly, epithelial cells secrete mucins in lungs for mucociliary clearance of bacteria [54]. To fur ther prevent bacterial colonization, epithelial cells in the gut and bladder undergo rapid renewal and shedding by upregulating their stem cell proliferation signaling pathways [50,55]. In addition, urothelial cells produce uromodulin, a mannosylated protein to prevent binding of bacteria to urothelial cells and facilitate clear ance of the bacteria in urine [55].

The mechanism of bacteria internalization into non phagocytic cells is complex and remains to be further investigated/elucidated, since the internalization is largely dependent on the bacterial spe cies and cellular receptors present. However, once bacteria suc cessfully breach the epithelial cells, autophagy is a pivotal biological process responsible for degradation of the internalized bacteria. Autophagy is a membrane trafficking pathway, responsi ble for degradation of unwanted organelles and foreign particles in cells by delivering them to the lysosomes [56]. First, bacteria are internalized into epithelial cells using specific bacterial proteins, such as the adhesive filaments of *Escherichia coli* (*E. coli*) (referred to as type 1 pili). The binding of type 1 pili to urothelial cells induces a cascade of signaling pathways promoting bacterial inter nalization into cells [57,58]. It is noteworthy that the protein expressed for internalization in cells differs significantly depending on the bacterial species and host cells of interest. *L. monocytogenes* uses E cadherin for internalization in intestinal epithelial cells and internalins in the form of InIA and InIB for invasion in hepatocytes [59]. Conversely, *S. aureus* uses fibronectin binding proteins (FnBPs) for adhesion to keratinocytes, corneal epithelial cells and embryonic kidney epithelial cells [60]. Once internalized, the bac teria are encapsulated in membrane derived vacuoles, autophago somes, and are degraded upon fusion with lysosomes [50,55].

2.1.2.2. Bacterial escape and survival mechanisms. Despite the highly regulated and multifaceted defense systems of epithelial cells, bac teria have developed strategies to avert the host defense mecha nisms and find protection in non phagocytic cells. *Salmonella typhimurium* (*S. typhimurium*) escape into the cytosol of the gut epithelial cells upon invasion and exploit the intracellular environ ment for rapid bacterial replication [61]. Uropathogenic bacteria such as *E. coli* can survive in the acidic environment of lysosomes [55]. *E. coli* also adapts to the intracellular defense mechanisms in urothelial cells as they replicate very quickly, forming a bacterial community inside their host cells upon internalization [62]. Simi larly, *S. aureus* that invaded cystic fibrosis epithelial cells are not destructed by autophagy once internalized. Instead, the bacteria escape and replicate in the cell's cytosol [63].

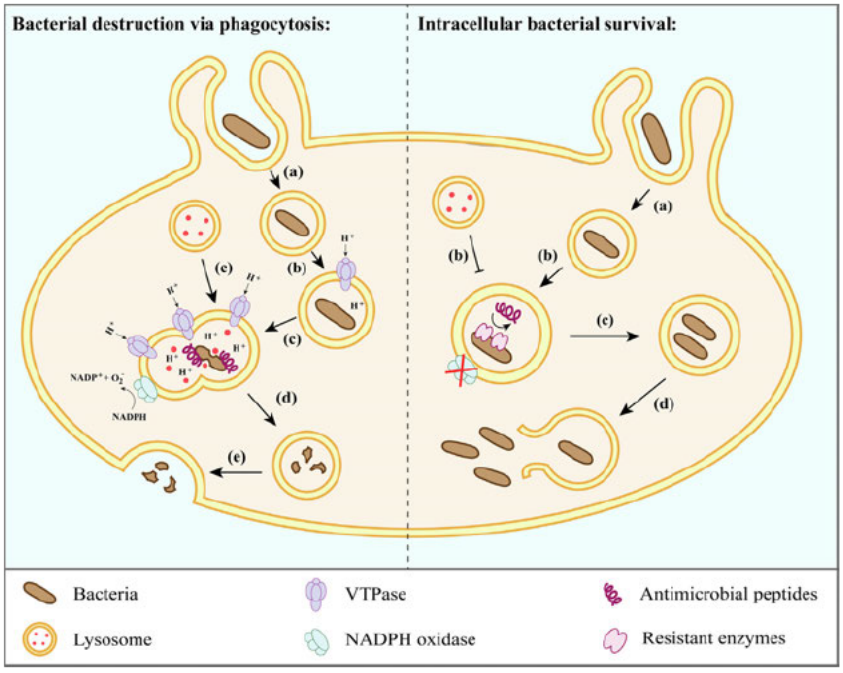


Fig. 1. (Left) Phagocytosis of bacteria in macrophages: (a) Pseudopods engulfment of bacteria and internalization of bacteria in phagosome, (b) maturation and acidification of phagosome by VTPase, (c) fusion of phagosome and lysosome resulting in further acidification of vesicle, with presence of antimicrobial peptides (AMP) and generation of reactive oxygen species (ROS) further facilitating bacterial killing, (d) bacteria are degraded and (e) excreted. (Right) Survival of intracellular bacteria in macrophages: (a) bacteria are internalized into phagosomes, (b) inhibition of fusion with lysosomes, bacteria express enzymes to avoid AMP and stops generation of ROS, (c) bacteria survives and replicates in phagosomes, (d) bacteria escapes and replicates in cytosol of cells. [8]

2.2. Current treatment approaches and challenges of clinical intracellular infections

The underlying challenges for successful treatment of intracel lular infections are the survival and persistence of bacteria in host cells, protected from host defense mechanisms and antibiotic treatments. While surviving in host cells, bacteria can replicate in the cytosol and exit the host cells to initiate another cycle of infec tion [64 66]. Currently, there are no treatment strategies specifi cally targeting intracellular bacteria. In many cases, patients suffer from recurrent infections upon completion of their antibiotic therapy. Hence, patients currently receive a cocktail of antibiotics for an extended period of time (Table 2). This leads to poor patient compliance and increased side effects, such as emesis, diarrhea and nephrotoxicity. When antibiotic therapy fails to disrupt the cycle of recurring infection, surgery is often the only remaining alternative. For example, tonsillectomy is currently the only available option when antimicrobial therapies fail against recurrent tonsillitis [67]. However, it must be noted, that in most intracellular infec tions, surgery is not a viable option, since infections become so widespread or within an organ where surgical procedures are not recommended (e.g., the urinary bladder).

Most antibiotics fail to be effective against intracellular patho gens due to their limited ability to cross the cell membranes of the infected cells, disposition to intracellular removal via cellular efflux, and poor chemical stability within the acidic intracellular environments, such as those within phagosomes [6,68]. As such, the intracellular concentration of antibiotic is often suboptimal and ineffective for bacterial eradication, resulting in patients suf fering from persistent infections and prolonged antibiotic treat ment. Recently, the WHO reported a crisis in the development of new antibiotics since only eight new antibiotics have been approved since 2017. Of which, only two antibiotics represent a new chemical entity, with the other six being derivative of existing antibiotics, which may limit their clinical benefit over time [69,70]. Furthermore, of the 252 antimicrobial agents in preclinical studies, only 2 to 5 products are predicted to be available in 10 years' time, with none designed exclusively for intracellular infections [70]. Although the need for new antimicrobial therapies has been recog nized for many years, diminutive progress has been made so far, necessitating the need for alternative strategies such as encapsula tion of antibiotics into nanoparticles, to improve the efficacy and to reduce the dose of antibiotics required for successful treatment outcomes, in addition to tackling bacterial resistance to antimicro bial agents [7,14,71,72].

3. Repurposing conventional antibiotics using bioinspired strategies

Nano encapsulation offers unprecedented opportunities for the effective delivery of antibiotics, including enhanced cellular pene tration and exposure to bacteria, improved solubility and reduced toxicity and side effects [14]. Furthermore, the surface of nanopar ticles can be functionalized for targeted delivery or triggered release of antibiotics, thereby increasing treatment efficacy [6,80,81]. Further developing on the use of nanoantibiotics, researchers are exploiting the mechanisms of biological particu lates (e.g., exosomes) to increase the specificity of the nano carrier delivery, bringing rise to the field of bioinspired drug deliv

Table 2
Current treatment options for intracellular infections.

Infection	Bacteria	Current treatment option	Commonly prescribed antibiotics	References
Tuberculosis	*Mycobacterium tuberculosis*	Two-phase treatment: Intensive 2-month initial phase with four first-line drugs followed by 4-month treatment with follow up drugs. Drug resistant strains require additional treatment for up to 12 months.	First line drugs: Isoniazid, pyrazinamide, rifampicin, fluoroquinolones, ethionamide Follow up drugs: Isoniazid and rifampicin only	[71,73]
Typhoid fever	*Salmonella (typhi, enterica)*	Antibiotics are given for up to 14 days, with an additional treatment for 28 days if infection persists.	For uncomplicated cases, azithromycin is given, followed by ciprofloxacin if infection continues. Ceftriaxone is prescribed if bacteria are non-susceptible to previous antibiotics.	[74,75]
Brucellosis, Malta fever	*Brucella (melitensis, abortus, suis, canis)*	Antibiotic treatment for up to 6 weeks.	Doxycycline with streptomycin, gentamycin or rifampicin	[76]
Osteomyelitis	*Staphylococcus aureus*	Antibiotic treatment for up to six weeks	Methicillin-resistant: vancomycin or linezolid. Methicillin-sensitive: nafcillin or oxacillin	[77]
Chronic sinusitis	*Streptococcus pneumonia, Hemophilus influenzae, Moraxella catarrhalis, Staphylococcus species*	Broad spectrum antibiotic and topical intranasal steroid for 4–6 weeks.	Amoxicillin-clavulanate, cefpodoxime proxetil, cefuroxime, gatifloxacin, moxifloxacin, or levofloxacin	[78]
Chlamydia	*Chlamydia trachomatis*	Antibiotic treatment for up to 7 days.	Single dose of azithromycin or seven days treatment with either doxycycline, erythromycin, levofloxacin or ofloxacin.	[79]

ery strategies [82]. Examples of bioinspired nanoantibiotics strategies are presented in this section. These are categorized as (i) nanoantibiotics that mimic phagocytosis, (ii) cellular/native membrane mimics, (iii) functionalized nanoparticles for targeted delivery and (iv) stimuli responsive nanoparticles for controlled intracellular drug release (Fig. 2).

3.1. Mimicking the mononuclear phagocyte system

The design of nanoparticles that are rapidly internalized via phagocytosis pathways by the mononuclear phagocyte system are exploited as a promising approach for eradicating bacteria harbored inside macrophages. The uptake of nanoparticles is mediated

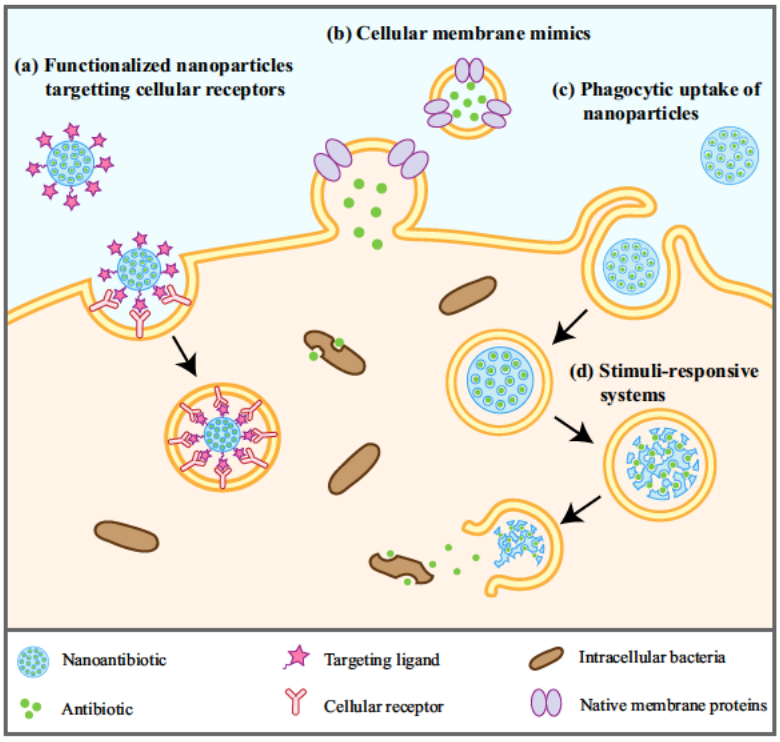


Fig. 2. Bioinspired nanoantibiotics delivery strategies: (a) functionalized nanoparticle targeting cellular receptors for internalization, (b) membrane derived nanoparticles, (c) nanoparticles internalized by phagocytosis and (d) intracellular stimuli responsive delivery systems.

by presence of the protein corona, particularly by adsorption of opsonin proteins to the particle surface that facilitates recognition and phagocytosis [83,84]. Macrophages can also internalize nanoparticles using the endocytic pathway with several uptake pathways simultaneously involved for the internalization of nanoparticles [85 87]. A summary of the various nanoparticles used as antibiotics carrier mimicking the phagocytic or endocytic pathway in macrophages are given in Table 3.

Table 3
Nanoantibiotics mimicking the mononuclear phagocyte system for intracellular delivery of antibiotics.

Nanoparticle	Antibiotic	Intracellular bacteria	Key findings	References
Solid lipid nanoparticles (SLN)	Enrofloxacin	*Salmonella CVCC541*	• 605 nm SLN were more effectively phagocytosed in comparison to 150 and 415 nm. • Surface charge within the range of 24.9 – 7.1 mV had negligible effect on phagocytosis.	[88]
	Doxycycline	*Brucella melitensis*	• 299 ± 34 nm reported as suitable size for phagocytosis. • Increasing dose of doxycycline-SLN from 25 to 100 μg/mL did not increase log CFU reduction, with log CFU reduction capped between 2.4 and 2.8.	[89]
Chitosan	Ceftriaxone	*Salmonella typhimurium*	• Increase in concentration of nanoparticles increased uptake in macrophages, with saturation effect starting at nanoparticle concentration of 0.03 mg/mL.	[90]
	Streptomycin	*Listeria monocytogenes* *Staphylococcus aureus*	• Higher content of streptomycin (22%) conjugated to chitosan increased their antibacterial activity against intracellular *Listeria monocytogenes*. • *N*-deacetylation degrees (DD) and molecular mass of chitosan plays a role in improving efficacy of the system, with 88% DD and 13 kDa exhibiting greater antibacterial activity.	[91]
	Tetracycline	*Staphylococcus aureus*	• Chitosan was chemically modified with *O*-carboxymethyl (*O*-CMC) to improve their solubility.Tetracycline conjugated *O*-CMC permits binding to planktonic bacteria, possibly due to the interaction of positively charged amino group of *O*-CMC with negatively charged bacterial membrane.	[92]
Poly Lactic-co-Glycolic Acid (PLGA)	Cloxacillin	*Staphylococcus aureus*	• Single emulsion solvent evaporation technique resulted in drug encapsulation efficiency of 20.5 ± 1.2 % w/w. • Rapid drug release at pH 7 was observed, with > 60% of drug released within 5 h. • Nanoparticles were distributed in cytoplasm with minimal co-localization with bacteria was observed. • Rapid uptake and internalization of PLGA in chlamydial inclusions were observed within 15 min of nanoparticle addition.	[93]
	Rifampicin Azithromycin	*Chlamydia trachomatis*	• PLGA loaded with both antibiotics were more effective at reducing intracellular *Chlamydia* than individual drugs. • PLGA 50:50 and 75:25 resulted nanoparticles with zeta potential of 77 and 84 mV respectively.	[94]
	Nafcillin	*Staphylococcus aureus*	• Both PLGA systems showed improved efficacy, and the composition of PLGA did not influence their activity against intracellular bacteria. • Non-ionic (-28.2 mV) and cationic (+16.5 mV) nanoparticles (NP) of 94–120 nm were fabricated.	[95]
	Clarithromycin	*Staphylococcus aureus* *Mycobacterium abscessus*	• Cationic NP were better internalized in macrophages and accumulated in the lysosomes. • PLGA nanoparticles remained in acidic phagolysosomes up to 7 days of incubation and did not colocalize with *Mycobacterium bovis* BCG.	[96]
	Rifampicin	*Mycobacterium bovis* BCG	• Rifampicin from PLGA was hypothesized to be permeable across the phagosomal membrane, resulting in up to 100 % CFU reduction.	[97]
Polyisohexylcyanoacrylate	(^{3}H) Ampicillin	*Salmonella typhimurium*	• Autoradiography revealed release of ampicillin from nanoparticles and intracellular redistribution of drug from lysosomes to cytoplasm, allowing for direct antibacterial activity against *Salmonella typhimurium*.	[98]
Polymer-lipid hybrid (PLH)	Rifampicin	*Staphylococcus aureus (SCV)*	• PLH dry powder of 4.56 μm were redispersed into individual lipid and PLGA particles of ~ 1 μm within 20 min in cell culture media. • PLH particles showed significantly higher internalization (>80%) in comparison to 5 μm PLGA particles (>40%).	[99]
	Doxycycline Vancomycin	*Mycobacterium smegmatitis* *Staphylococcus aureus*	• Cationic and zwitterionic based lipid polymer hybrid were investigated, with cationic and non-lipid particles being highly internalized (93% and 80% respectively) in comparison to zwitterionic particles (49%). • Antibiotic encapsulated in non-lipid and cationic formulations had higher intracellular bacterial reduction compared to zwitterionic formulation.	[100]
	Gentamicin	*Listeria monocytogenes* *Pseudomonas aeruginosa*	• Gentamicin loaded in chitosan-phosphatidylcholine was more effective in eradicating intracellular bacteria in comparison to free gentamicin or a mixture of gentamicin and chitosan. • After 2 h of incubation, gentamicin from the formulation was highly localized in the cytoplasm of the cells.	[101]
Mesoporous silica	Rifampicin Isoniazid	*Mycobacterium tuberculosis*	• Mesoporous silica nanoparticles (MSNP) were internalized in late endosomes and lysosomes after 3 h of incubations. • MSNP were coated with polyethyleneimine or beta-cyclodextrin for endosomal responsive drug release at lower pH.	[102]
	Rifampicin	*Staphylococcus aureus (SCV)*	• 100 nm MSNP showed higher internalization in comparison to 40 nm MSNP (~70% and ~30% respectively). • No difference in effectiveness against intracellular bacteria was observed across both nanoparticle sizes.	[103]
	Ciprofloxacin	*Salmonella typhimurium*	• Lipid coating on the MSNP reduced the cytotoxicity of the formulation compared to bare MSNP at particle concentration above 1 mg/mL and promoted sustained drug release with higher drug release in acidic conditions.	[104]

The size and surface chemistry of nanoantibiotics heavily influ ences their phagocytic or endocytic uptake by macrophages. These properties alter the opsonization of nanoparticles, formation of protein corona and cellular recognition processes for internaliza tion in macrophages [105,106]. The protein coating provides the nanoparticles with a "biological identity", different to their "syn thetic identity" of the nanoparticle [107]. The biological identity of the nanoparticle then regulates the internalization and fate of nanoparticles through their interaction with cells and other biolog ical molecules in the body. Nano and micro carriers targeting bacteria harbored inside macrophages have been devised with suc cessful outcomes in reducing the bacterial load. Herein, the influ ence of size and surface chemistry on the uptake of nanoparticles by macrophages and their effect on the efficacy of the drug delivery systems are discussed.

3.1.1. Influence of nanoparticle size on uptake pathways in macrophages

Size is one of the most important factors influencing the uptake and intracellular performance of nanoparticles [105,108]. The pres ence of IgG opsonin influences the size preferential uptake of nanoparticles, highlighting the role of protein adsorption for uptake and internalization. Generally, particles above 500 nm are preferentially internalized via phagocytosis, while smaller particles undergo pinocytosis [109]. Pinocytosis can be further divided into micropinocytosis, clathrin and caveolin mediated endocytosis. The size preferential uptake of nanoparticles further determines their intracellular localization in cells.

In a study by Champion *et al.*, polymeric microspheres of 2 to 3 μm displayed the greatest extent of phagocytosis within an hour of incubation [110]. Interestingly, nanoparticle size only mediated the first part of phagocytosis (particle recognition and attachment) and not the process of internalization [110]. Investigating the size effect of carboxyl polystyrene nanoparticles in the range of 20 to 1000 nm similarly demonstrated higher phagocytic activity for 500 nm and 1000 nm particles [111]. Smaller particles exhibited greater cytotoxicity due to their high surface area that promotes membrane disruption, particularly for suspension cells in compar ison to adherent cells (due to the former having a larger membrane area exposed to particles). Using polystyrene beads of 100 to 1100 nm, the involvement of pinocytosis and phagocytosis was characterized and results showed that increasing particle sizes steadily increased the extent of phagocytosis [112]. As illustrated in a study by Koval *et al.*, phagocytic response to particles can be identified by the formation of an actin driven protrusion of the cell membrane, increased sensitivity to cytochalasin D (inhibitor of actin polymerization) and rapid delivery of particles to the lyso somes in macrophages [113]. Based on these criteria and using polystyrene beads with size range from 200 nm to 3 μm, parti cles>750 nm showed higher phagocytic activity as they were rapidly delivered to lysosomes. Whereas, polystyrene beads of smaller sizes were shown to be internalized via clathrin mediated endocytosis [113].

Small inorganic nanoparticles (≤5 nm) are passively internal ized in macrophages by direct penetration of the cell membrane, enabling localization of particles in the nucleus of the cell [114]. For gold nanoparticles of 5, 20 and 50 nm, the presence of a protein corona significantly reduced their uptake in macrophages [115]. This effect was particularly profound for 50 nm nanoparticles com pared to 5 nm and 20 nm. Furthermore, the presence of a protein corona reduced the attachment of nanoparticles to cell membrane but did not play a role in their internalization pathway. However, it is interesting to note that a difference in the extent of uptake or internalization of nanoparticles was reported to have no effect on the efficacy of the antibiotics delivered against intracellular bacte ria. Despite a significant difference in the internalization, the effi cacy of nanoantibiotics in reducing intracellular bacterial burden was similar across nanoparticles of varying sizes [103,116]. As other factors such as release of drug, degradation of the nanoparti cles and colocalization of nanoparticles with bacteria can also influence their efficacy, and it is difficult to conclude a positive cor relation between size of particles with efficacy of nanoantibiotics against intracellular bacteria.

3.1.2. Influence of surface charge on recognition by macrophages

Surface charge is another important factor affecting particle internalization. In non phagocytic cells, cationic nanoparticles are internalized at a greater rate and extent due to stronger electro static attraction forces that form between the particles and nega tively charged cell membrane [117]. However, influence of surface charge on the extent of phagocytosis is not fully under stood [105,118]. In a study using functionalized albumin micro and nanospheres, a neutral charge reduced the extent of phago cytic uptake in macrophages in comparison to positively and neg atively charged particles [119]. This effect was only observed when nanoparticles were incubated in the presence of serum, mitigating the influence of the biological identity of nanoparticles on phago cytosis. Further supporting this, Liu *et al.*, showed that charged gold particles displayed a similar extent of phagocytosis, regardless of whether they were anionic or cationic [120]. Interestingly, the pos itively charged particles had a negative zeta potential when mea sured in the presence of serum, due to their protein corona; this has also been observed by Jeon *et al.*, using polystyrene nanoparti cles [121]. Hence, it can be speculated that an overall negative charge of the particles in biological environments promotes phago cytosis. As bacterial cells also display a negative charge, this may explain the preferential uptake of anionic formulations by phago cytes [117]. Despite the similar extent of phagocytosis between cationic and anionic particles, cationic particles have been reported to exhibit hemolytic and cytotoxic properties [122]. This can be attributed to their strong attraction, hence disruption to the cell membrane in addition to causing greater lysosomal and mitochon drial damage [117]. However, particle size may play a bigger role than surface charge in mediating phagocytosis. As demonstrated by Maghrebi *et al.*, a negatively charged PLGA lipid hybrid system promoted superior uptake in comparison to a positively charged system [123]. Due to the electrostatic repulsion of the negatively charged PLGA (due to anionic surface stabilizer used) and lipid, the anionic formulation was readily redispersed into their individ ual components of <1 μm in size. Whereas the cationic formulation retained a size of ~4 μm and was less desirable for phagocytosis. The anionic PLGA precursor of ~500 nm was also less phagocy tosed in comparison to the hybrid formulation. Tabata *et al.*, also reported a predominant role of size when comparing surface charge of polymer microspheres for phagocytosis [124].

3.1.3. Influence of hydrophobicity of nanoparticles on macrophage uptake

Hydrophobic nanoparticles allow a higher level of protein adsorption due to their strong interactions, hence, are more rapidly phagocytosed in comparison to hydrophilic nanoparticles [108,125]. Interestingly, upon rapid uptake hydrophobic latex nanoparticles have shown to remain in early endosomes without fusing with lysosomes, possibly due to binding strongly with the serum proteins [80]. Whereas, proteins delivered by hydrophilic polystyrene microspheres were degraded at a faster rate as they mature into phagolysosomes and were found in the acidic environ ment of macrophages [80,126]. In contrast, Saha *et al.* demonstrated gold nanoparticles with less hydrophobic functional groups dis playing higher internalization in macrophages in the presence of serum [127]. The arrangement of the functional groups (composed of tetra(ethylene glycol)) on the nanoparticles played a more

important role for phagocytosis, due to the surface area present for protein adsorption. As such, cyclic and branched functional groups allowed less protein adsorption compared to linear structures with similar hydrophobicity [127]. Zhu *et al.* concluded that surface hydrophobicity in turns decreases cellular uptake of nanoparticles [128]. The reduction in uptake was only observed when 10% and 50% of serum was added, as without the presence of serum, the hydrophobic nanoparticles had higher internalization. As the inter play of serum proteins with formation of protein corona mediating phagocytosis is complex, it is of paramount importance to stan dardize research protocols (e.g., concentration of serum and type of serum proteins) for a deeper understanding of these phenomena. Similarly, the cell culture conditions and phenotypes must be dis closed as the reactivity of macrophages towards nanoparticles can differ depending on their growth conditions [129].

PEGylation of nanoparticles is largely utilized to reduce the hydrophobicity of the nanoparticles, hence altering their biological properties. PEGylation reduces the adsorption of serum proteins by creating a hydration layer, thereby increasing hydrophilicity on the surface of the nanoparticles which reduces macrophage uptake [130]. This is commonly referred to the 'stealth effect'. However, several studies have shown correlation of molecular weight and density of PEG with extent of phagocytosis. For example, nanopar ticles coated with PEG at the highest density (>0.96 PEG/nm^2) per mitted a small amount of serum protein adsorption, but the uptake in macrophages was reduced using less efficient uptake mecha nisms [131]. In a separate study, partially PEGylated nanoparticles were effective in decreasing the internalization as fully PEGylated nanoparticles (<30%) in comparison to non PEGylated nanoparti cles (>70%) [130]. PEGylation also promotes strong interactions with macrophages by displacement of PEG with small thiolated molecules such as cysteine, allowing for serum protein adsorption [132]. However, the effect was reversed when an additional layer of hydrophobic functional (alkyls) groups were added in between the nanoparticle and PEG. Although PEGylation has shown to reduce the extent of internalization in macrophages, other factors such as size, shape and surface charge of the nanoparticles may further mediate the extent of phagocytosis [133].

3.2. Mimicking cellular membranes for enhanced antibiotic internalization

Cells membranes are made of phospholipid bilayers, decorated with proteins, carbohydrates and cholesterol [134]. They act as a barrier for transport of molecules and ions from the extracellular environment, by allowing only small nonpolar and uncharged molecules (e.g., oxygen and water) to diffuse through, hence hin dering the permeability of antibiotics. Membrane bound vesicles are naturally used for transport of biologicals (e.g., mRNA) across cells. Therefore, the use of synthetic and natural nanoparticles with a phospholipid bilayer (e.g., liposomes and exosomes) as drug delivery systems is beneficial since they are highly biocompatible and are readily endocytosed by cells.

3.2.1. Synthetic membrane vesicles to mimic cellular membrane

3.2.1.1. Lamellar systems. Synthetic membrane vesicles and lipo somes are the most frequently investigated nanoparticles in drug delivery research as their lipid bilayers mimic biological mem branes [7,105,135]. Liposomes are composed of biocompatible lipids and are routinely synthesized in the laboratory using thin film hydration method (also referred to as the Bangham method), where the lipid layers self assemble to form multilamellar vesicles (MLV) in aqueous environment and small unilamellar vesicles (SUV) are produced upon sonication or extrusion of MLV [136 138]. Other methods such as reverse phase evaporation tech niques, injection techniques and microfluidic methods can be employed depending on the size of liposomes of interest and scale of production, i.e. laboratory or commercial use [139]. The lipid bilayer encloses an aqueous core that enables encapsulation of hydrophilic antibiotics (e.g., aminoglycosides) and hydrophobic antibiotics (e.g., rifampicin) in the lipid membrane. The compati bility of liposomes with biological membranes facilitate endocyto sis or fusion with the cellular membranes to deliver their cargo intracellularly [140]. They are particularly beneficial for targeting bacteria localized in lysosomes as upon internalization, liposomes are degraded by lysosomal enzymes [141].

The composition of liposomes can significantly influence their chemical properties, such as drug loading and release as well as their biological properties, such as cell fusion, toxicity and intracel lular accumulation in cells. For example, cholesterol is often added to increase the rigidity and stability of liposomes [142]. However, a high percentage of cholesterol can also reduce cellular interaction and uptake by macrophages, reducing the therapeutic effect of the drug [141 143]. The incorporation of negatively charged lipids enhances phagocytosis in comparison to neutral liposomes [118]. Whereas, the incorporation of cationic lipids such as stearylamine induces apoptosis in macrophages through the production of ROS [144]. Liposomes can also be functionalized with PEG for longer cir culation time. Functionalization with specific ligands (e.g., mannose targeting mannose receptors) allows targeted delivery to macro phages. A summary of some of the recent studies exploring the use of liposomes for intracellular infections is provided in Table 4.

As illustrated in Table 4, liposomes have been predominantly investigated for their efficacy in macrophages as they are readily phagocytosed or endocytosed. To enhance their delivery in urothe lial (non phagocytic) cells, Horsley *et al.* explored the use liposomes encapsulated with gentamicin decorated on gas bubbles [155]. The liposome coated microbubbles were transported into the cells by ultrasound technology, for better cellular penetration and targeted treatment. Using this combined technology, the delivery of lipo somes to uroepithelial cells was significantly enhanced as mea sured by corrected total cell fluorescence (CTCF) (control = 425.66, liposomes only = 3533.57 and liposomes delivered using ultra sound = 9544.68), permitting an efficient bacterial killing at very low gentamicin concentrations (0.53 and 1.32 μg/mL, equivalent to bacterial killing at 200 μg/mL of unformulated gentamicin).

To further enhance the drug loading and internalization of syn thetically derived lipid based nanoparticles, Zhang *et al.* chemically conjugated antibiotics to lipids and phospholipids, forming antibiotic derived lipid nanoparticles (Fig. 3(a)) [156]. An alkane based lipid chain (L) and a phospholipid chain were conjugated to penicillin G to form spherical nanoparticles bounded by a lipid membrane (Fig. 3(b)). The penicillin G conjugated phospholipids were endocytosed into lung cells and captured in lysosomes but had no colocalization with the intracellular bacteria (Fig. 3(d)). Sig nificantly, up to 99.9% eradication of bacteria after 8 h of treatment was achieved. The phospholipids were hypothesized to promote a strong binding of the antibiotic to the bacterial membrane, result ing in their antimicrobial activity against intracellular bacteria.

3.2.1.2. Non lamellar systems. The use of non lamellar systems (also referred as liquid crystalline structures) as drug delivery sys tems has gained significant attention in recent years. Amphiphilic lipids self assemble in aqueous environments to form stable 3D mesophases, such as cubosomes and hexasomes with intercon nected water channels [157,158]. Non lamellar structures have higher surface area per volume, permitting solubility of hydropho bic, hydrophilic and amphiphilic molecules within their structures [159]. They also mimic the biological structures and membranes of many organelles in plants and animals, such as the bicontinuous cubic phase, similar to the membrane arrangement of chloroplast [160]. Their high lipid to water interfacial area is attractive for

Table 4
Summary of liposomes explored as carriers for intracellular delivery of antibiotics.

Composition	Antibiotics	Intracellular bacteriaCell line/*in vivo* model	Physicochemical properties of liposomal carriers	Key findings	References
Non-functionalized					
DSPC, DSPG and cholesterol (varying composition)	Ciprofloxacin Azithromycin	*Mycobacterium avium* J774 murine macrophages	Remote-loading procedure increases drug load for ciprofloxacin, 0.52 mol/mol, drug to lipid. Azithromycin was incorporated via lyophilization, ≤ 33 % mol (drug to lipid).	Negatively charged liposomes promoted ↑ levels of ciprofloxacin accumulation in cells and antibacterial effect compared to neutral liposomes and unformulated antibiotics.	[145]
Phosphatidylcholine, cholesterol, dicetylphosphate, stearylamine (varying composition)	Gentamicin	*Brucella abortus* J774 murine macrophages	Higher incorporation of cholesterol increases stability of liposomal formulation in buffer. Addition of negatively charged molecule, dicetylphosphate further improved the liposomal stability.	Positively charged liposomes were more efficient against intracellular bacteria. 30% cholesterol were more effective, 50% cholesterol reduces interaction of liposome with macrophages.	[143]
Phosphatidylglycerol, phosphatidylcholine, cholesterol (MR = 1:9:5)	Streptomycin Ciprofloxacin	*Mycobacterium avium-Mycobacterium intracellulare* complex Human peripheral blood monocyte/macrophages	No information provided	Blank liposomes exhibited an antibacterial effect – possibly activation of macrophage for an immunomodulatory effect. Ciprofloxacin encapsulated in liposomes exhibited a ↑ reduction in CFU than unformulated ciprofloxacin. Liposomes may have protected ciprofloxacin from forming microcrystalline structure at neutral pH of cell media.	[146]
Phosphatidylglycerol, phosphatidylcholine, cholesterol (MR = 1:1:1)	Sparfloxacin	*Mycobacterium avium-Mycobacterium intracellulare* complex J774 murine macrophages	No information provided	Liposomes encapsulated with sparfloxacin had similar antibacterial effect against intracellular bacteria as unformulated sparfloxacin *in vitro*. It is predicted that the liposomes will enhance the antibacterial activity *in vivo* for their ability to localize in phagocytes.	[147]
Phosphatidylcholine, cholesterol (MR = 1:1)	Ciprofloxacin	*Francisella tularensis* BALB/c mice model	100 nm 90 ± 3.5% drug entrapment rate	Ciprofloxacin encapsulated in liposomes delivered via IV injection accumulated in spleen and liver. Aerosolized liposomes increased drug levels in lungs compared to unformulated ciprofloxacin, resulting in 100% survival 14 days post infection.	[148]
DPPC, cholesterol	Amikacin	*Mycobacterium avium, Mycobacterium abscessus* THP-1 human monocyte and C57BL/6 mice model	No information provided	Aerosol delivery of liposomal amikacin reduced the concentration of amikacin required compared to parenteral administration of unformulated amikacin.	[149]
PEGylated Liposomes					
HSPC, cholesterol and mPEG-DSPE (MR 55:40:5)	Mupirocin	*Staphylococcus aureus* C57BL/6 mice model and immortalized murine macrophages (*in vitro*)	~85 nm, PDI = 0.09	Fluorescently labelled bacteria and formulation were internalized similarly in macrophages via phagocytosis. *In vitro* model demonstrated ↑ reduction in intracellular bacteria after 2 hours in comparison to unformulated mupirocin.	[150,151]
DSPC, cholesterol (MR = 3:1) and DSPC, cholesterol, mPEG-DSPE (MR = 3:1:0.02)	Vancomycin	*Staphylococcus aureus* THP-1 human monocyte	254 ± 147 nm and 245 ± 139 nm PEGylated liposomes provided a slightly higher encapsulation efficiency (13 ± 3%) in comparison to non-PEGylated liposomes (9 ± 2%).	All formulations had higher vancomycin intracellular concentration after 1-hour compared to 24-hour post treatment (saturation effect). Unformulated vancomycin had higher intracellular accumulation (53.29 ± 4.2 μg/mL) in comparison to non-PEGylated liposomes (26.0 ± 2.2 μg/mL) and PEGylated liposomes (21.7 ± 4.4 μg/mL) at corresponding vancomycin concentration of 800 μg/mL. These results need to be further validated as the intracellular concentration of vancomycin does not correspond with their efficacy against intracellular *S. aureus*.	[152]

Table 4 (*continued*)

Composition	Antibiotics	Intracellular bacteriaCell line/*in vivo* model	Physicochemical properties of liposomal carriers	Key findings	References
Phosphatidylcholine, phosphatidylglycerol, HPC, DMPC, DMPG, DPPC, DPPG, Cholesterol, PEG (varying compositions and molar ratio)	Rifabutin	*Mycobacterium tuberculosis* BALB/c mice model	All liposomes were of similar size, 100 nm with varying zeta potential between -3 to -33 mV.	Liposomes containing lipids with higher phase transition temperatures (DPPC: DPPG), hence higher stability delivered greater concentration of non-metabolized rifabutin to macrophage rich organs, i.e., spleen, liver and lung.	[153]
Ligand modified liposomes					
HSPC, DOPC, cholesterol, DCP, mannose	Ciprofloxacin	*Mycobacterium, Chlamydia pneumoniae, Listeria, Francisella* NR8383 murine macrophages and Sprague Dawley rats	Unmodified liposomes: 1015 ± 112 nm Mannosylated liposomes: 1005 ± 101 nm Zeta potential of -70 mV for both liposomes	The concentration of ciprofloxacin in plasma and epithelial lining fluid was similar across both liposomes, with free ciprofloxacin achieving higher concentration in plasma. Concentration of ciprofloxacin in macrophages was significantly higher when delivered using mannosylated liposomes.	[154]

Note: DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPG, 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol; MR, molar ratio; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; HSPC, hydrogenated soy phosphatidylcholine; mPEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; PDI, polydispersity index; HPC, hydrogenated phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; PEG, polyethylene glycol; DOPC, dioleoyl phosphatidylcholine; DCP, dicetylphosphate.

the solubilization of biological molecules such as proteins and peptides, while also protecting them from oxidation and proteolysis [161,162]. In addition, Thorn *et al.* have demonstrated the ability to achieve a bacterial lipase triggered release of hydrophobic and macromolecular antibiotics using cubosomes formulated with monoolein, a digestible lipid [163]. As a newly emerging drug delivery system, liquid crystalline nanoparticles have been investigated for the delivery of antibiotics and AMP to treat skin infections, biofilm in cystic fibrosis and endophthalmitis [162,164,165]. Their potential for the delivery of antimicrobial agents for intracellular bacteria is yet to be explored but may be of relevance attributing to their fusogenic properties with cell membrane for intracellular transport [158].

3.2.2. Deriving native membrane vesicles to mimic cellular membrane

Although liposomes provide a synthetic replication of the phospholipid membrane of cells, 'native membrane vesicles' (i.e., nanoparticles derived from cell membranes, rather than synthetic phospholipids) are evolving as the preferred platform for drug delivery. Native membrane vesicles are capable of presenting the functional moieties of a cell membrane in their natural state without the need to identify individual ligands or moieties necessary for cellular internalization [166 168]. The cell membrane is first lysed and extracted from the cells of interest by hypotonic treatment, followed by membrane vesicle formation using sonication and extrusion, which are further extruded with the nanoparticles to form cellular membrane coated nanoparticles [169]. Zhang *et al.* harnessed the potential of cell membranes derived from red blood cells (RBC) as a coating for nanogels encapsulating vancomycin [170]. This unique approach was further enhanced using a redox responsive crosslinker, cystine dimethacrylate for their nanogel, providing the system with an on demand release of vancomycin only in infected cells. The authors also demonstrated the capability of the RBC membrane to neutralize α toxins, thereby increasing proinflammatory cytokines, provoking the immune cells for bacterial clearance.

Another example of native membrane vesicle are extracellular vesicles, also known as exosomes. Exosomes are vesicles secreted into the extracellular environment by cells to mediate intracellular communications, transfer of ligands across cells or as a method to excrete unwanted proteins from cells [171]. Exosomes have been explored as an intracellular drug delivery strategy for linezolid against *S. aureus* harbored in RAW 264.7 murine macrophages [172]. In this study, the exosomes were isolated from the same type of cells, i.e., RAW 264.7 in healthy state. Linezolid in dimethyl sulfoxide (DMSO) was physically mixed with the isolated exosomes and further centrifuged to separate non encapsulated linezolid from the linezolid encapsulated exosomes. The exosomes were found to accumulate in the lysosomes for up to 24 h, which is believed to be the same cellular compartment where the bacteria reside, although this was not directly confirmed in the study. Infected macrophages have shown to induce stress in the endoplasmic reticulum, delivering microbial effectors such as ROS to localized bacteria using mitochondria derived vesicles (MDV) [173]. When successfully isolated, MDVs may be harnessed for their capability to deliver drugs to the specific cellular compartments where bacteria reside.

Native membrane vesicles can also be isolated from bacterial cells as demonstrated by Goes *et al.* where the outer membrane vesicles (OMV) from *Myxobacteria*, a bacteria found abundantly in soil, was isolated to treat intracellular pulmonary infections (Fig. 4) [174]. Two strains, *Cystobacter velatus* (Cbv34) and *Cystobacter ferrugineus* (Cbfe23) exhibit antimicrobial properties in their natural state. Consequently, OMV were isolated and evaluated for their efficacy in killing intracellular *S. aureus* harbored in A549 lung epithelial cells. In comparison to gentamicin and cystobactamid, OMV demonstrated a greater reduction of intracellular bacterial. Although, the immunogenicity of OMV needs to be evaluated in *in vivo*. Gao, *et al.* explored the delivery of PLGA nanoparticles fused with extracellular vesicles (EV) derived from *S. aureus* [175]. The antibiotic EV present themselves as a pathogen to the host cells, which promoted higher internalization (Fig. 4(a)) and higher intracellular concentrations of antibiotic, successfully eliminating intracellular *S. aureus*. Interestingly, rifampicin EV showed higher potency compared to unformulated rifampicin, whereas vancomycin EV showed similar potency to unformulated vancomycin against intracellular bacteria (Fig. 4(b)), confirming the influence of hydrophobicity or hydrophilicity of antibiotics on intracellular accumulation and release kinetics using this nanocarrier. In another study, membrane derived from *E. coli* (bacterial ghosts) loaded with ciprofloxacin demonstrated superior activity against intracellular *S. aureus* in macrophages [176]. The increased number of lysosomes in infected cells promoted greater release of ciprofloxacin from the bacterial ghosts, allowing for a site specific release and enhanced effect of antibiotic. The use of bacterial membranes as nanocarriers is still in their early stages but may provide benefits such as localization in intracellular bacteria rich cellular compartments. However, safety profiles and immunogenicity need

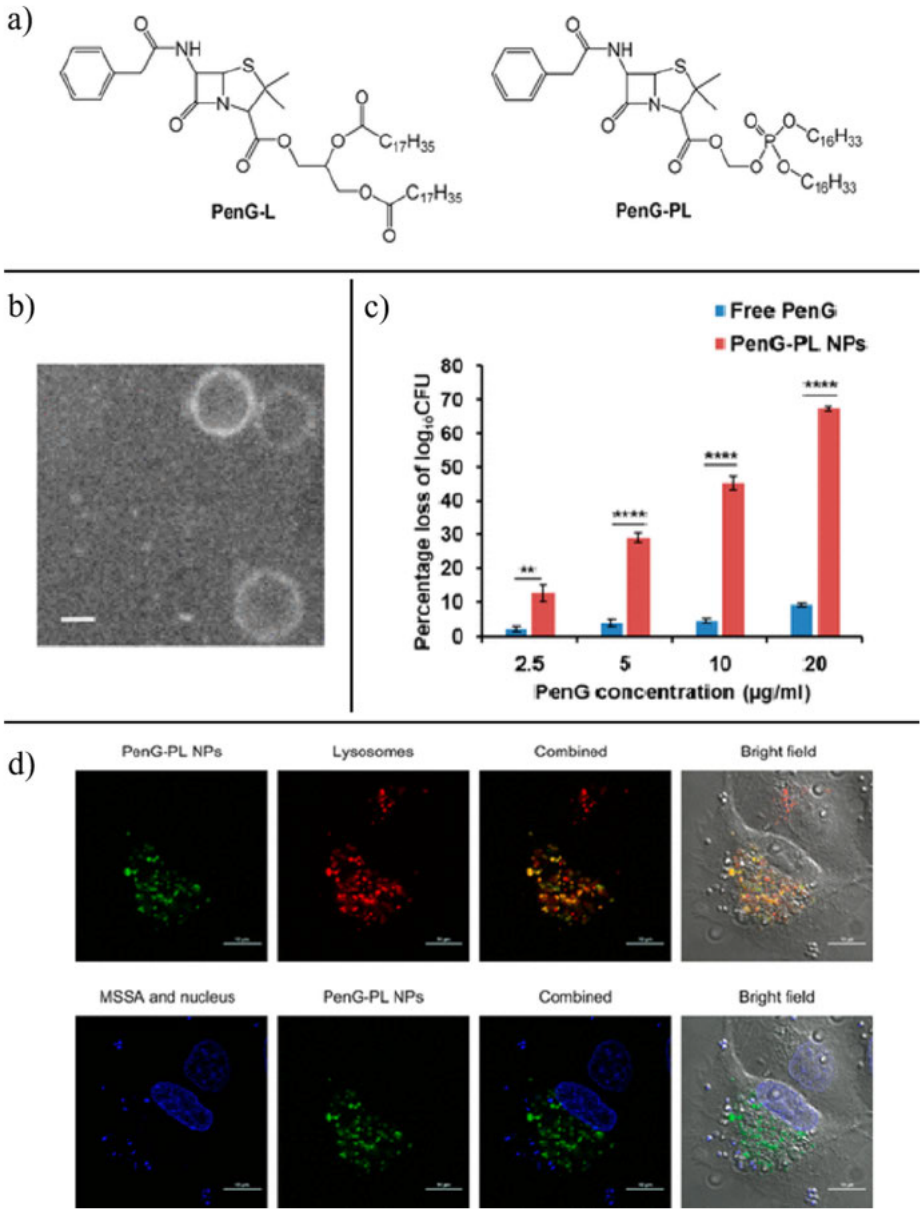


Fig. 3. (a) Chemical structures of penicillin G conjugated with alkane-based lipid (PenG-L) and phospholipid chain (PenG-PL). (b) TEM image of PenG-PL nanoparticles (scale bar = 100 nm). (c) Inhibitory effect of PenG-PL in comparison to unformulated penicillin (free PenG). Mean ± SD (n = 3) (**, $p < 0.01$, ****, $p < 0.0001$). (d) Intracellular trafficking of PenG-PL nanoparticles. Nanoparticles were labelled with FITC, lysosomes stained with Lysotracker and intracellular Staphylococcus aureus stained with Hoechst33342 (scale bar = 10 μm). Reprinted and adapted from [156], copyright © 2019 American Chemical Society.

to be addressed to prevent immune complex disease and autoimmune reactions [82,177,178].

3.3. Nanoparticle functionalization for targeted antibiotic delivery to infected cells

3.3.1. Harnessing cellular receptors for entry into infected cells

The uptake of nanoparticles can be further enhanced by actively targeting the receptors present on the cellular surface. To achieve this, the nanoparticles must be functionalized with specific targeting ligands, depending on the receptors of interest. Scavenger receptors can be found on the surface of macrophages and endothelial cells and have been explored to enhance the intracellular delivery of ciprofloxacin using fucoidan [179]. Ciprofloxacin was initially encapsulated in chitosan nanoparticles coated with fucoidan, a natural polysaccharide with antimicrobial properties for active targeting to macrophages. The enhanced uptake of fucoidan coated chitosan nanoparticles resulted in a significant decrease

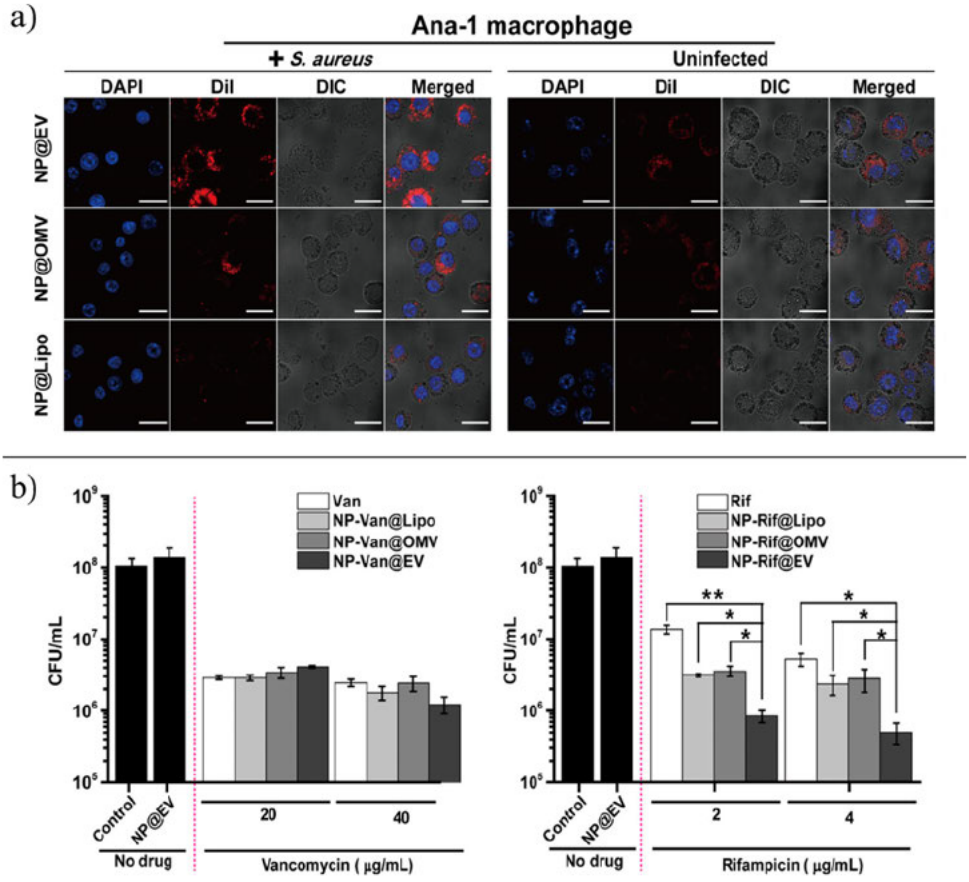


Fig. 4. (a) Fluorescence microscopy images of DiI-labelled EV uptake after 8 h in S. aureus infected and non-infected murine Ana-1 macrophages. (b) Efficacy of vancomycin-EV and rifampicin-EV against intracellular S. aureus in Ana-1 macrophages. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. Reprinted and adapted from [175], copyright © 2018 American Chemical Society.

of intracellular *Salmonella paratyphi A* as compared to unformu lated and chitosan loaded ciprofloxacin (bacterial survival of 20% for fucoidan coated chitosan nanoparticles *vs* 30% for non coated chitosan nanoparticles and 75% for unformulated ciprofloxacin after 12 h).

Salmonella infections increase the expression of cationic amino acid transporters (mCAT1 and mCAT2B) to facilitate uptake of argi nine into the cells, as arginine strongly regulates the host innate immune response [180]. Mudakavi *et al.* demonstrated an increase in uptake and colocalization of mesoporous silica nanoparticles (MSN) in *Salmonella* infected macrophages after 2 h by functional izing the surface of MSN with arginine (colocalization coefficient of 0.6 for arginine MSN compared to 0.1 for MSN) [181]. The arginine coated MSN, loaded with ciprofloxacin showed co localization with intracellular *Salmonella* after 2 h, with an antibacterial activ ity at 0.1 μg/mL of ciprofloxacin encapsulated in arginine MSN compared to 1 μg/mL for unformulated ciprofloxacin.

3.3.2. Mimicking bacterial proteins for internalization into infected cells

Bacteria possess several membrane proteins that allow for their adherence and internalization within host cells, including M pro teins, fibrinogen or fibronectin binding proteins and extracellular adherence protein (Eap) [182,183]. Nanoparticles functionalized with bacterial proteins improve internalization and delivery of antibiotics to infected cells. For example, invasin (InvA) is an outer membrane protein utilized by *Yersinia* species to invade and colo nize intestinal cells and tissues [184]. To enhance the delivery of gentamicin in HEp 2 epithelial cells, liposomes functionalized with InvA were successfully fabricated and demonstrated higher cellu lar adhesion and internalization, with ~ 30% killing of intracellular *Yersinia pseudotuberculosis* compared to ~5% by non functionalized liposomes [185]. InvA functionalized liposomes and PLGA nanopar ticles with encapsulated gentamicin also facilitated killing against intracellular *Salmonella enterica* (*S. enterica*) and *Shigella flexneri* respectively in HEp 2 cells, demonstrating the feasibility of func tionalized delivery systems against different intracellular patho gens [186,187]. In another study, Menina *et al.* demonstrated superior killing of intracellular *S. enterica* in HEp 2 and Caco 2 cells by delivering colistin, an antibiotic reserved as a last resort treat ment option, using Eap functionalized liposomes. Eap derived from *S. aureus* was incorporated in the liposomes by physical adsorption, yielding a functionalization efficiency of up to 75% [188]. Eap liposomes showed higher internalization (~100%) in both cell lines as compared to non functionalized liposomes (~40%). As a result, Eap liposomes reduced intracellular *S. enterica* in both cells lines by ~30% in comparison to non functionalized liposomes and unformulated colistin.

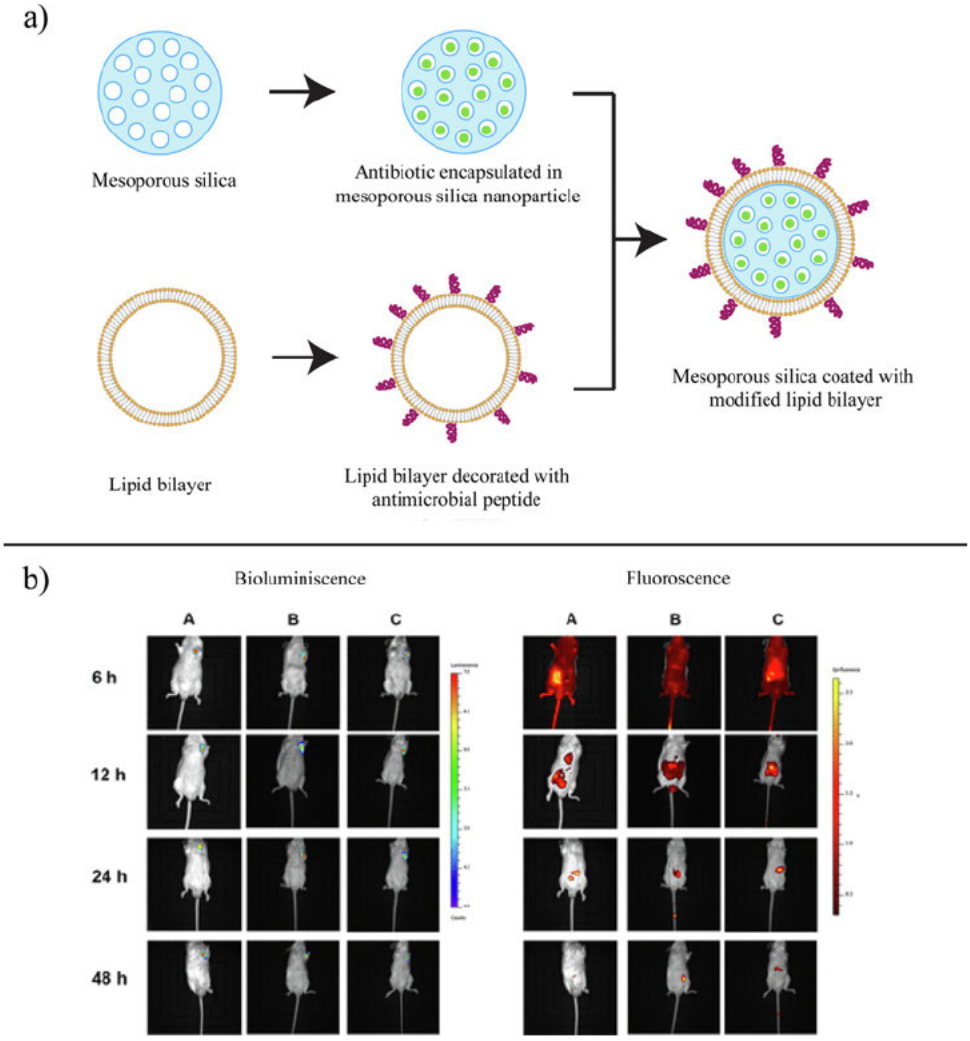


Fig. 5. (a) Schematic of the synthesis of lipid-bilayer coated mesoporous silica nanoparticle (MSN) decorated with bacteria targeting peptide. (b) (Left) In vivo antibacterial activity of gentamicin encapsulated in $UBI_{29\ 41}$ decorated MSN by monitoring bacterial burden using bioluminescence S. aureus and (right) biodistribution of fluorescently labelled MSN. Reprinted and adapted from [190], copyright © 2018 American Chemical Society.

3.3.3. *Antimicrobial peptides for targeted intracellular delivery*

Antimicrobial peptides (AMP) have gained substantial interest recently for their capabilities to fight infections by modulating the host immune response. Cationic peptides interact with the negatively charged bacterial membrane, provoking its disruption [189]. The intracellular delivery of gentamicin encapsulated in MSN to MC3T3 E1 (pre osteoblast cell line) and RAW 264.7 murine macrophages was significantly enhanced by the bacteria targeting peptide, ubiquicidin ($UBI_{29\ 41}$) which was immobilized on the lipid bilayer shell coating of the MSN (Fig. 5(a)) [190]. In this bacterial targeted delivery system, $UBI_{29\ 41}$ was capable of internalizing into both osteoblasts and macrophages, delivering gentamicin at a higher concentration than non functionalized MSN with a trig gered release in the presence of bacterial lipase that degraded the lipid bilayer. The intracellular efficacy of the system was fur ther evaluated in an *in vivo* mouse model (Fig. 5(b)). Although *in vivo* models better resemble the pathogenesis of an infectious disease, the model needs to be further validated as it is difficult to monitor intracellular bacteria in animal models.

Similarly, LL 37, a human cathelicidin AMP has been used as a targeting ligand for the delivery of colistin (a cationic polypeptide) against intracellular *P. aeruginosa* in A549 lung epithelial cells [191]. The core MSN particle was capable of encapsulating colistin electrostatically due to opposing charges, followed by coating of MSN with a liposomal layer modified with LL 37. The liposomal layer provided attachment of the AMP to the silica, in addition to preventing premature release of colistin prior to degradation by bacterial lipase, similar to the $UBI_{29\ 41}$ functionalized MSN dis cussed previously. The modified liposomal layer provided improved internalization and delivery of colistin in comparison to uncoated MSN. This enabled AMP functionalized nanoantibiotics to kill 99% of the intracellular bacteria, although only at a very high colistin concentration of 1 mg/mL, which may not be clinically translatable.

As an alternate carrier to MSN, superparamagnetic iron oxide nanoparticles (SPION) have been functionalized with the AMP cecropin melittin (CM) via covalent bonding [192]. SPION themselves exhibited antibacterial properties at a concentration >100 μg/mL due to generation of ROS. The covalent linking of CM to SPION improved their affinity and permeability across *E. coli* membranes without exhibiting cytotoxicity. Although the efficacy of CM attached to SPION was not investigated against intracellular infections, cellular uptake studies demonstrated a high percentage of nanoparticles localized in the endolysosomes of macrophages potentially offering a suitable system for site specific delivery of antibacterial agents.

3.4. Harnessing intracellular environment for stimuli responsive antibiotic delivery

Nanocarriers internalized within infected host cells are exposed to several different intracellular environments, which vary in respect to pH, enzyme concentration and amount of ROS. Thus, the uptake pathway and intracellular environment can be exploited for the development of stimuli responsive antibiotic delivery systems, where the nanoantibiotic is protected from undesired, premature antibiotic release and when internalized by non infected cells. In this section, examples of pH , cellular enzyme , bacterial enzyme , and redox responsive formulation strategies are provided.

3.4.1. pH as a stimulus for intracellular drug release

Upon internalization of bacteria, the phagosomal pH of host cells decreases to pH 4.0 5.0 due to the upregulation of hydrogen ions by V ATPase to facilitate destruction of bacteria (Fig. 6(a)) [193]. In one of the earliest studies investigating pH dependent antibiotic release in infected cells, Lutwyche *et al.* fabricated pH sensitive liposomes using fusogenic lipids, *N* succinyl DOPE and *N* glutaryl DOPE [194]. The pH sensitive liposomes were capable of releasing gentamicin in the endosomal compartment of the cell at a higher concentration, resulting in efficient killing of intracellular *L. monocytogenes* and *S. typhimurium* in macrophages. Pei *et al.* demonstrated intracellular release of vancomycin at pH 5 using polymeric nanoparticles synthesized from PEGylated PLGA, Eudragit 100 and zwitterionic chitosan (ZWC) [195]. Eudragit 100 is soluble at <pH 5, while ZWC exhibits a positive charge at pH 7.4 and a negative charge at <pH 7.4. ZWC neutralizes the cationic charge of Eudragit 100 at physiological pH while promoting drug release under acidic conditions. The nanoparticles exhibited >60% release of vancomycin at pH 7.4 and >80% release at pH 5. The premature release of vancomycin at pH 7.4, alongside their poor intracellular release in macrophages compared to results obtained using buffer solutions resulted only in one log fold reduction of intracellular methicillin resistant *Staphylococcus aureus* (MRSA) as compared to unformulated vancomycin.

Polymer augmented liposomes (PAL), prepared using the pH sensitive diblock copolymer, poly[(DEAEMA co BMA) b ManEMA] synthesized via RAFT polymerization with a mannose targeting ligand enhanced the efficacy of streptomycin against intracellular *Francisella novicida* (*F. novicida*) (as a surrogate for *Francisella tularensis*) [196]. The mannose targeting ligands improved the intracellular uptake of the nanoparticles compared to PEGylated liposomes (as control) and displayed endosomal release of streptomycin in acidic environment. This permitted higher reduction of intracellular *F. novicida* compared to unformulated streptomycin and PEGylated liposomes at a streptomycin concentration of ~15 μg/mL. pH sensitive polymersomes consisting of the copolymer, poly(2 (methacryloyloxy)ethylphosphorylcholine) co poly(2 (diisopropylamino)ethyl methacrylate) (PMPC PDPA) were used to enhance the activity of antibiotics for intracellular infections, as evaluated using a zebrafish embryo model [197]. The efficacies of five different antibiotics, lysostaphin, vancomycin, gentamicin, rifampicin and isoniazid were evaluated against intracellular *Mycobacterium bovis* (*M. bovis*), *Mycobacterium marinum*, *Mycobacterium tuberculosis* and *S. aureus*. The drug loaded polymersomes displayed varying results, with no enhanced effect observed for vancomycin and lysostaphin. In contrast, significant enhancement of rifampicin activity was observed, resulting in complete eradication of intracellular *M. bovis*, particularly when the polymersomes were co loaded with isoniazid. The alteration of antibiotic behavior in endosomal and cytosolic environment of cells may have impacted their mode of action against bacteria, causing varying results across the five types of antibiotic investigated.

pH sensitive drug release for oral delivery to epithelial cells infected with *Porphyromonas gingivalis* has been investigated as a treatment option for periodontitis [198]. The antimicrobial effects of metronidazole, doxycycline and gentamicin encapsulated in polymersomes, composed of amphiphilic poly[2 (methacryloyloxy)ethyl phosphorylcholine] (PMPC) and poly[2 (diisopropylamino)ethyl methacrylate] (PDPA) were investigated in normal oral keratinocytes (NOKs) and oral squamous carcinoma cell lines, H357 and TR146. Gentamicin encapsulated in polymersomes showed no significant differences in reduction of intracellular bacterial load across all three cell lines. Metronidazole encapsulated polymersomes significantly reduced the bacterial load in all cell lines, while doxycycline encapsulated polymersomes were effective only in TR146.

Maji *et al.* evaluated pH responsive lipid dendrimer hybrid nanoparticles encapsulated with vancomycin as a strategy against intracellular *S. aureus* in HEK 293 embryonic kidney cells [199]. The hybrid nanoparticles were composed of oleylamine and poly(amidoamine) (PAMAM) as the lipid and polymer components, respectively. A sustained drug release profile was achieved at pH 6, and the nanoparticles demonstrated complete eradication of intracellular bacteria after 22 h of incubation. The blank nanoparticles exhibited antibacterial activity by their own, providing synergistic effects against the intracellular bacteria when encapsulated with vancomycin.

Abed *et al.* conjugated penicillin G with geranyl, a natural terpene allowing self assembly of the conjugate into nanoparticles, yielding a drug load of 61 63% [200]. To further improve the release profile of penicillin, it was linked to geranyl by an acylal linker, which enabled selective release under the acidic condition of RAW 264.7 murine macrophages, where *S. aureus* was localized intracellularly. Using a similar approach, penicillin was conjugated with squalene, either using a pH sensitive or pH insensitive chemical bond [201]. Interestingly, although being able to efficiently kill intracellular bacteria compared to unformulated penicillin, it was demonstrated that the squalene conjugated penicillin nanoparticles did not co localize with the intracellular bacteria in macrophages. Further studies indicated the possibility of the drug crossing the intracellular vesicle membrane upon protonation after pH dependent release in the cells, although this needs to be further confirmed. This highlights the complexity in understanding uptake and internalization of nanoparticles, followed by release kinetics of antibiotics to target intracellular pathogens [202,203]. Furthermore, a pH responsive system needs further consideration as some intracellular bacteria, such as *Mycobacterium* are capable of inhibiting the upregulation of V ATPase, therefore preventing phagosomal acidification which may limit pH triggered drug release, and subsequently, the translation of this approach [204].

3.4.2. Cellular enzyme as a trigger for antibiotic release

Lysosomes are organelles containing enzymes that play a key role in digesting bacteria (Fig. 6(a)). Therefore, they are present

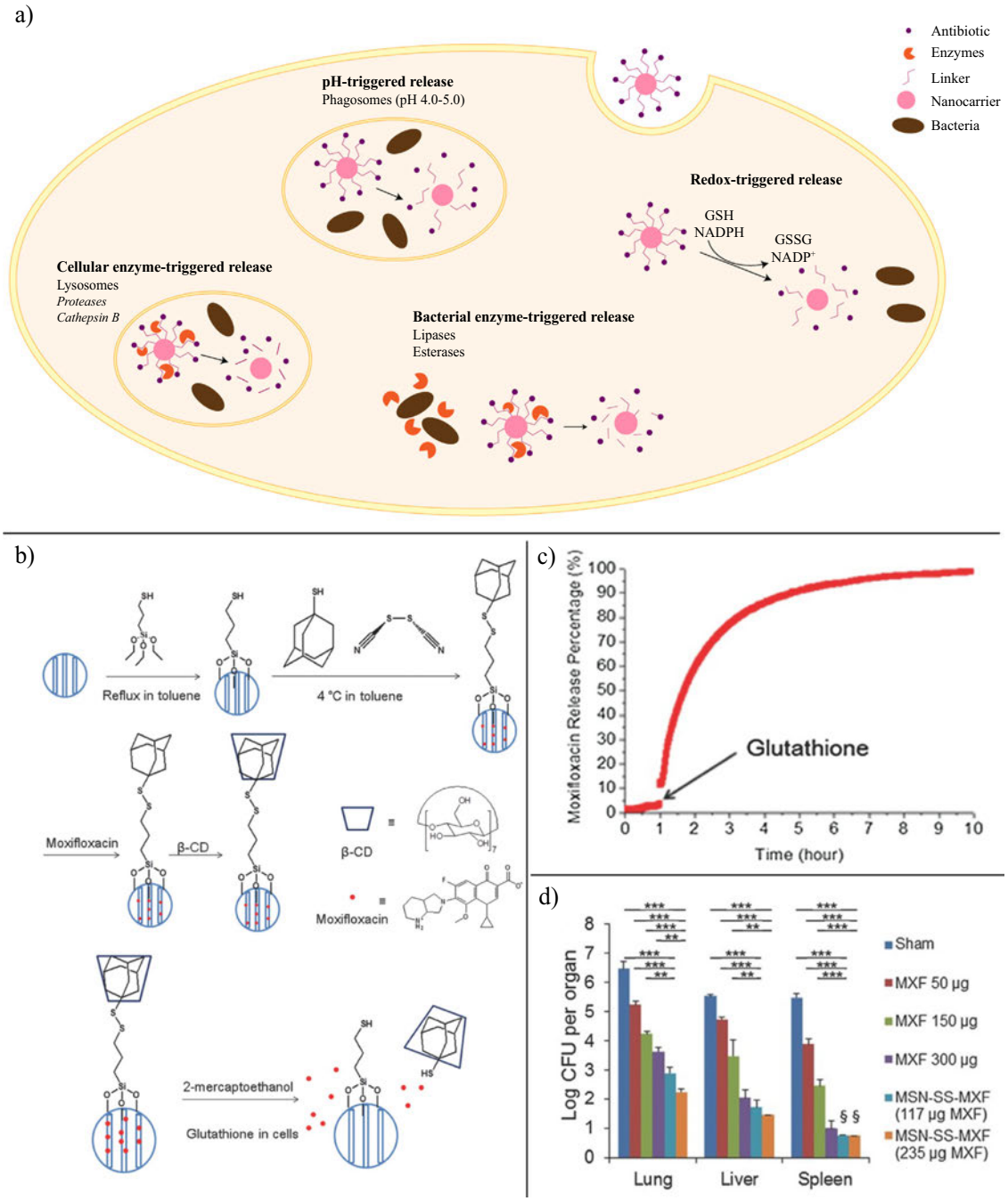


Fig. 6. (a) An overview of stimuli-responsive drug delivery systems to eradicate intracellular bacteria. (b) Disulfide snap-top MSN (MSN-SS-MFX) synthesis and release mechanism. (c) Redox-responsive release of moxifloxacin in presence of glutathione. (d) In vivo efficacy of MSN-SS-MFX against intracellular F. tularensis. **p < 0.01, ***p < 0.001, §bacterial CFU below limit of detection. Error bars represent standard errors. Reprinted and adapted from [212], copyright © 2016 John Wiley & Sons.

abundantly in infected cells. A macromolecular polymeric prodrug referred as drugamer, with enzyme cleavable chains was synthesized for the intracellular release of ciprofloxacin [205]. The drugamer comprised of a valine citrulline linker cleavable by lysosomal protease, cathepsin B and mannose ligands for enhanced internalization in macrophages. Despite exhibiting similar antibacterial

activity in comparison to unformulated ciprofloxacin against intra cellular *F. novicida*, the drugamer provided a longer retention time and sustained drug release with reduced toxicity *in vivo*, which appeals as an alternative to standard treatment with unformulated ciprofloxacin. Peng *et al.* fabricated mannosylated pillar[5]arene self assembled vesicles, cleavable by the activity of cathepsin B in lysosomes and acidic pH for the intracellular release of van comycin [206]. This approach enabled an increased delivery of van comycin, with an intracellular concentration of ~8 μg/mL as compared to only ~2 μg/mL of unformulated vancomycin when dosed at 20 μg/mL, improving bacterial reduction of *S. aureus* in RAW 264.7 murine macrophages.

3.4.3. Bacterial enzyme inducing intracellular antibiotic release

Bacterial enzymes such as lipases, proteases and esterases are found in abundance at an infection site (Fig. 6(a)) as these viru lence factors are excreted by bacteria to exert damage in host cells and to increase bacterial survival. Thus, bacterial enzymes can be exploited for the development of responsive drug delivery systems for intracellular infection [163,207]. The bacterial lipase sensitive polymer, poly(ε caprolactone) was used to control the release of vancomycin from a polymeric nanogel comprising a PEG shell and polyphosphoester core [208]. The triple layered nanogel pro tected vancomycin from premature burst release up to 48 h but exerted a rapid release in the presence of *Pseudomonas* and *S. aur eus* lipase, demonstrating bacterial lipase concentration dependent drug release. The nanogels were well internalized in RAW 264.7 murine macrophages, achieving a 4 fold increase in reduction of intracellular *S. aureus* when incubated for 24 h at vancomycin con centration of 20 μg/mL as compared to unformulated vancomycin. Xiong *et al.* investigated mannose labelled nanogels, using a cross link of PEG and polyphosphoester. The crosslinks were degraded by active phosphatase or phospholipase, triggering the release of van comycin, following active targeting of macrophages via mannose receptors [209]. The intracellular bacterial inhibition of *S. aureus* was confirmed in an *in vivo* zebrafish embryo model, in which the vancomycin loaded mannosylated nanogel successfully reduced the fluorescence intensity of green fluorescent protein (GFP) expressing *S. aureus*, compared to unformulated vancomycin and vancomycin loaded nanogel (without mannose) observed using real time imaging. In another study, polymeric carriers com posed of unsaturated monomer ethyl bis[2 (acryloxy) ethyl] phos phate (EPE acr) and 2 propenoic acid tetraphenylethylene ester (TPE acr) were utilized for bacterial lipase triggered release of deferoxamine ciprofloxacin conjugates in intracellular RAW 264.7 murine macrophages [210]. Deferoxamine was added as a chelating agent and was conjugated with iron, while the polymers were grafted with mannose using PEG spacers. Upon internaliza tion in macrophages, a triggered release of ciprofloxacin was obtained following a two step process, (i) degradation of polymer by bacterial lipase and (ii) ingestion of iron (natural source of food) conjugated with deferoxamine by intracellular bacteria. A signifi cant reduction of intracellular bacteria was observed for the man nose labelled polymeric carriers carrying deferoxamine ciprofloxacin at 24 h compared to 6 h, due to the higher intracellu lar concentration of nanoantibiotics.

3.4.4. Redox as an intracellular drug release stimulus

The regulation of intracellular redox is a complex process and is highly regulated by different chemical species such as, NADPH/ $NADP^+$, glutathione GSH/GSSG, cysteine/cystine and more (Fig. 6 (a)), depending on the cellular compartmentalization [211]. The GSH/GSSG species are mostly found intracellularly and have been utilized to trigger the intracellular release of moxifloxacin from disulfide snap top of MSN (Fig. 6(b)) [212]. The disulfide snap tops act as valves that traps moxifloxacin within the mesopores of MSN and releases moxifloxacin when GSH concentration is equivalent to the intracellular environment ($\sim 10^{-2}$ M) (Fig. 6(c)). The efficacy of moxifloxacin MSN was comparable to unformulated moxifloxacin against intracellular *F. tularensis* in macrophages *in vitro*. However, in a mouse model of pneumonic tularemia, the moxifloxacin MSN exhibited superior efficacy compared to unfor mulated drug by reducing bacterial burdens in organs rich in macrophages, i.e., the lung, liver and spleen (Fig. 6(d)).

4. Limitations and future perspectives

Despite the evolving research on bioinspired nanoantibiotics strategies, there are several limitations that restrict further advancement and translation of these formulations into clinical applications. These limitations and challenges are present at differ ent stages of research, from *in vitro* assessments through to pre clinical translation. Key limitations and future considerations at each of these stages are addressed in this section, categorized as (i) limitations with current *in vitro* assessments for antibacterial efficacy, (ii) limitations with *in vivo* models and (iii) challenges associated with translating pre clinical investigation into clinically relevant nanoantibiotics.

4.1. Limitations of current in vitro assessments for antibacterial efficacy

Cell culture models have provided meaningful insights into the interaction of bacteria with host cells and opportunities to draw key signaling pathways for bacterial internalization and survival [213]. *In vitro* studies are also conducted as preliminary steps to evaluate the effectiveness of a new antibacterial formulation. How ever, key limitations and critical considerations as discussed below need to be addressed in future studies for successful translation of *in vitro* results to *in vivo* studies.

4.1.1. Limitations of in vitro models for bacterial infection

Although *in vitro* models are widely used as a pre screening method for antibacterial efficacy, they fail to mimic the pathophys iology of a bacterial infection that is observed in the clinic [214]. The complex microenvironment of intracellular infections is diffi cult to replicate in a 2D cell culture, in addition to the absence of host immune response [215]. Moreover, the compartmentalization of nanoparticles in cells and colocalization of nanoparticles with intracellular bacteria has often not been well characterized which may limit the antibacterial effect exerted. Utilization of intracellu lar localization sensors to track and quantify the intracellular pres ence of nanoantibiotics and pathogens may offer valuable insights on the fate of nanoparticles in infected cells [216].

In many cases, complete eradication of intracellular bacteria has not been reported. This may be attributed to the lack of research in understanding the fate of nanoantibiotics and release profiles of antibiotics once internalized into cells. Antibiotic release studies have been largely performed in simple buffer solutions and may not reflect the complex intracellular composition of cells. Release studies should be performed in simulated biological fluids to offer better predictability of their efficacy against intracellular bacteria [217]. Performing intracellular release studies using cell culture may also provide useful observations to validate efficacy results [218]. It is also important to note that antibacterial efficacy as mea sured by the minimum inhibitory concentration or minimum bac tericidal concentration (MIC or MBC) are poorly translatable to clinical applications. As described by Li *et al.*, MIC and MBC mea surements do not provide insights on the time dependent activity of the antibiotic and results are heavily dependent on the following factors: (i) size of inoculum tested, (ii) extent of bacterial killing

and (iii) regrowth of resistant subpopulations [219]. Therefore, *in vitro* models only assess the drug pathogen interactions, neglecting many other parameters such as pharmacokinetic and biodistribution, contributing to poor translation of drug dosage regimen for preclinical studies [220].

4.1.2. The primitive nature of in vitro models as a limitation

Many studies thus far have demonstrated superior uptake of nanoantibiotics using a single cell monolayer which is an overly simplified model and may not be translatable into clinical studies. For example, in cystic fibrosis, the presence of the viscous sputum limits the penetration of nanoantibiotics, thereby restricting their internalization into infected cells [221]. Nanoantibiotics can interact with the mucus layer via hydrophobic interactions and nanoparticles entrapped by mucus can be cleared prior to reaching cells of interest [222]. Further advanced experiments are necessary to better understand the permeation properties of nanoantibiotics using mucus permeation assays [223,224].

Furthermore, a large proportion of studies performed thus far have focused on professional phagocytes such as macrophages. However, in most cases, immortalized or secondary cell lines of macrophages, e.g., RAW 264.7 and J774A.1 are used. Secondary macrophages, depending on their culture type and time, can exhibit different responses to biomaterials, in addition to having different cytokine secretion compared to primary macrophages [225]. This can serve as a major limitation in predicting and translating the efficacy of nanoantibiotics to *in vivo* and clinical applications. Besides, there is also an increasing need to explore delivery strategies of nanoantibiotics into non professional phagocytes for diseases such osteomyelitis, rhinosinusitis, cystic fibrosis, and urinary tract infections. It is important to also note that addition of nanoparticles to *in vitro* models can dysregulate cellular function, resulting in poor efficacy results [226,227]. For example, addition of cationic liposomes and polymer based vectors upregulated autophagosomes, resulting in poor transfer of DNA to the nucleus of the cell [227].

4.1.3. Necessity for standardization of parameters in in vitro studies

Standardization in experiments, including nanoparticles characterization, biological characterization and experimental details are also necessary to avoid variations in results and in depth comparison of studies conducted to understand the role of nanoparticles for intracellular infections [228]. Researchers must also disclose important information that can cause variability in results produced. Selection of bacterial species and strain that are clinically relevant, choice of *in vivo* species that can correctly replicate the disease in human and identifying key virulence factors are of crucial importance for validation of any antibacterial results obtained [229]. With regard to the selection of clinically relevant bacterial species, it is important to disclose all important virulence markers and antibiotic resistance determinants as suggested by Campoccia, *et al* [230]. Adding to the complexity of the problem is the existence of bacteria in their dormant state when surviving inside host cells [231 233]. Dormancy refers to the non replicating cellular state of the bacteria as a result of decreased metabolic activity, allowing them to endure defense mechanisms of their host cells, which has been associated with persistent intracellular infections [231,234]. Considering dormancy of bacteria increases their survival in host cells, studies in dormant bacteria such as small colony variants (SCV) are necessary.

4.2. Limitations with in vivo models

To overcome the above challenges, *in vivo* models are next employed to further understand the safety profile and efficacy of the formulation in a more complex environment that aims to mimic intracellular infection as observed in humans in clinical settings. In vivo models are necessary to provide meaningful observations on the pathogenesis of the disease and understanding the interactions between host and pathogen [214]; i.e. key interactions that currently cannot be modulated in an *in vitro* model. However, these models also possess certain limitations, such as (i) the use of young and syngeneic animals that are resistant to infections, (ii) high dosing of bacterial inoculum to replicate an infection that does not resemble clinical exposures and importantly, (iii) the use of antimicrobial agents that are not well adjusted for translation in human use, which may further exaggerate the results in either a positive or negative manner [229,235]. Similarly, discrepancies in biodistribution of the nanoantibiotics *in vivo* may lead to accumulation of nanoparticles at off target sites and provoke stronger immunological responses in humans, for which biodistribution studies and nanotoxicology screening in larger *in vivo* models (e.g., porcine models) are highly recommended [236]. Thus, caution has to be exerted when extrapolating results from *in vivo* studies as the immunological response and antibacterial efficacy can differ significantly, especially when compared to humans due to the differences in immune systems and functions between mouse models and human [237].

In order to overcome such challenges, the development of advanced *in vitro* models are explored, such as the development of an urine dependent human urothelial organoid as a model for intracellular UTI [238]. The organoid model derived from human progenitor cells mimics the human urothelium, expressing key biomarkers, topographical and ultrastructural features. The organoid serves as an advanced *in vitro* model with similar morphological and physiological structures to human urinary bladder to study intracellular *Enterococcus* infection and/or understanding the fate of nanoantibiotics for intracellular UTI. The development of such sophisticated organoid models for other examples of intracellular infection (e.g., tuberculosis) is beneficial for meaningful observations and understanding of nanoantibiotic's efficacy. As they are derived from human cells and resemble conditions of the human organs, organoid models can further validate results and observations made while bridging the gap between 2D *in vitro* and *in vivo* studies [239]. It is, however, important to note that such advanced *in vitro* tools do not eradicate the need for *in vivo* studies and thus, the continual advancement of more relevant animal models for intracellular infection is required.

4.3. Challenges associated with translating pre clinical investigation into clinically relevant nanoantibiotics

The strategies employed for improving the effectiveness of nanoantibiotics are remarkable, unfortunately many fail to replicate *in vitro* successes in *in vivo* studies. Some of the key challenges in translating these results include (i) biological barriers, including changes in biodistribution, stability and protein adsorption *in vivo* and humans in comparison to *in vitro* models, (ii) microenvironmental barriers at target site, (iii) off target toxicity whereby nanoparticles release their antimicrobial payload at sites other than the target site, and (iv) cellular and intracellular barriers, including cellular heterogeneity that can influence intracellular uptake profiles of nanoparticles [236,240]. To overcome such challenges, nanoantibiotics can be formulated with targeting moieties for site specific drug release; however, previous studies utilizing cancer targeting nanoparticles have shown that <5% of the nanocarriers were found to reach the tumour site [241]. In addition, the release of drug at the site of interest, pharmacokinetics and pharmacodynamics of the formulation can vary drastically from *in vitro* to *in vivo* and can differ even more in humans [236,242].

For successful translation into clinical products, the administration route and scalability of the specific nanoantibiotic should be

carefully considered during early phase development. Formulations requiring multi step synthesis may be costly, plus their stability in the delivery medium must be characterized [242]. For example, to target pulmonary infections, aerosol delivery may serve as an attractive platform. However, the size of particles can impact the deposition and efficacy of the system, with particles smaller than 1 μm reported to be exhaled out of the lungs, while particles between 1 and 5 μm deposited in the lower respiratory tract [243]. Similarly, if nanoantibiotics are intended for systemic administration, their stability under physiological conditions, including serum stability, needs to be addressed. Kaczmarek *et al.* have demonstrated a positive correlation between serum stable formulations and efficacy *in vivo*, illustrating the importance of similar studies for successful clinical translation.

Bioinspired nanoantibiotics have shown significant benefits over conventional antibiotics for eradication of intracellular bacteria attributing to the capability of nanoparticles to permeate through infected cells, therefore delivering higher concentration of antibiotics intracellularly. Despite this, further work is required to translate current preclinical studies into clinically relevant formulations. Development of complex and validated models for intracellular infection together with deeper understanding on the fate of bioinspired nanoantibiotics in infected cells will allow development of optimal nanoantibiotics to treat intracellular infection.

5. Conclusion

Limited development of new antimicrobial agents exacerbates the need to tackle virulence mechanisms of bacteria and antimicrobial resistance by repurposing conventional antibiotics using nanotechnology. Bioinspired nanoantibiotics offer great potential to overcome current treatment challenges for intracellular infections by delivering a high load of antibiotics across the host cell membrane to target the hidden bacterial reservoir in cells. However, despite great discoveries and advancement in preclinical animal models, there is still an unmet need that hinders the successful translation of these formulations into clinical practice. Key limitations in understanding the intracellular fate of bacteria and nanoantibiotics may lead to the incomplete eradication of bacteria from host cells, reducing their clinical efficacy. Moreover, while the discovery of new formulation strategies remains necessary, there is a dire need for the development of complex *in vitro* and *in vivo* models for better understanding of the complexity of infected cells and translatable dosing regimens to treat humans which may provide further understanding on the interaction of nanoantibiotics with intracellular pathogens.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Australian Government Research Traineeship Program and Australian Research Council Centre of Excellence in Convergent Bio Nano Science and Technology.

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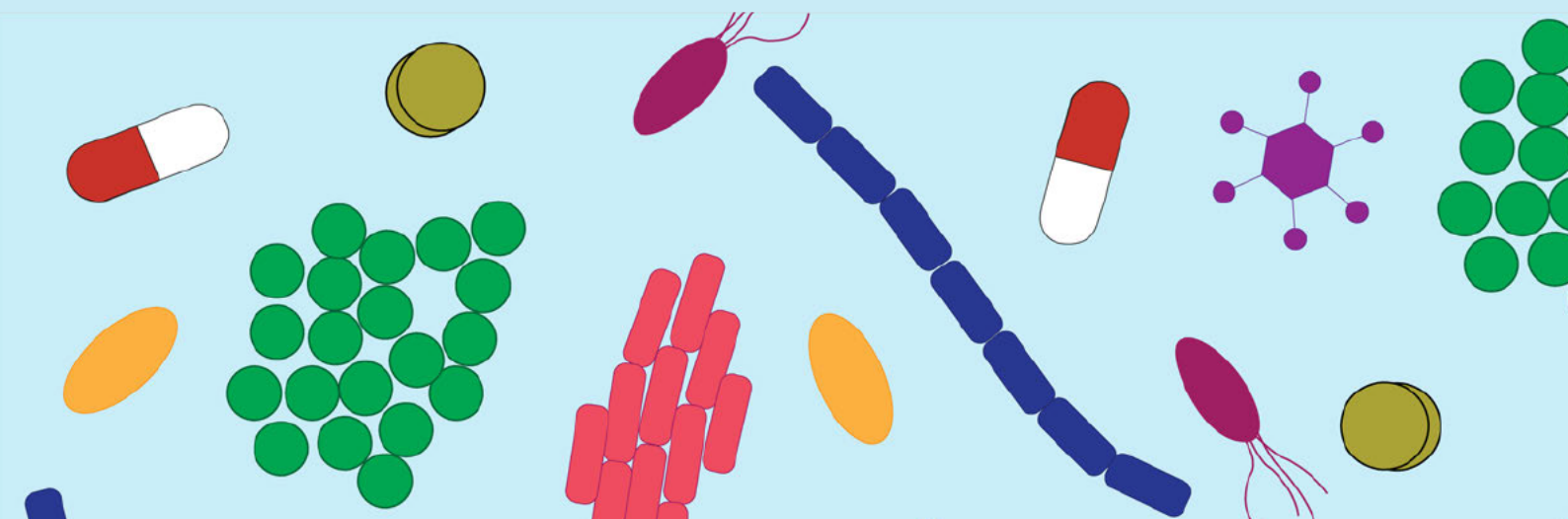
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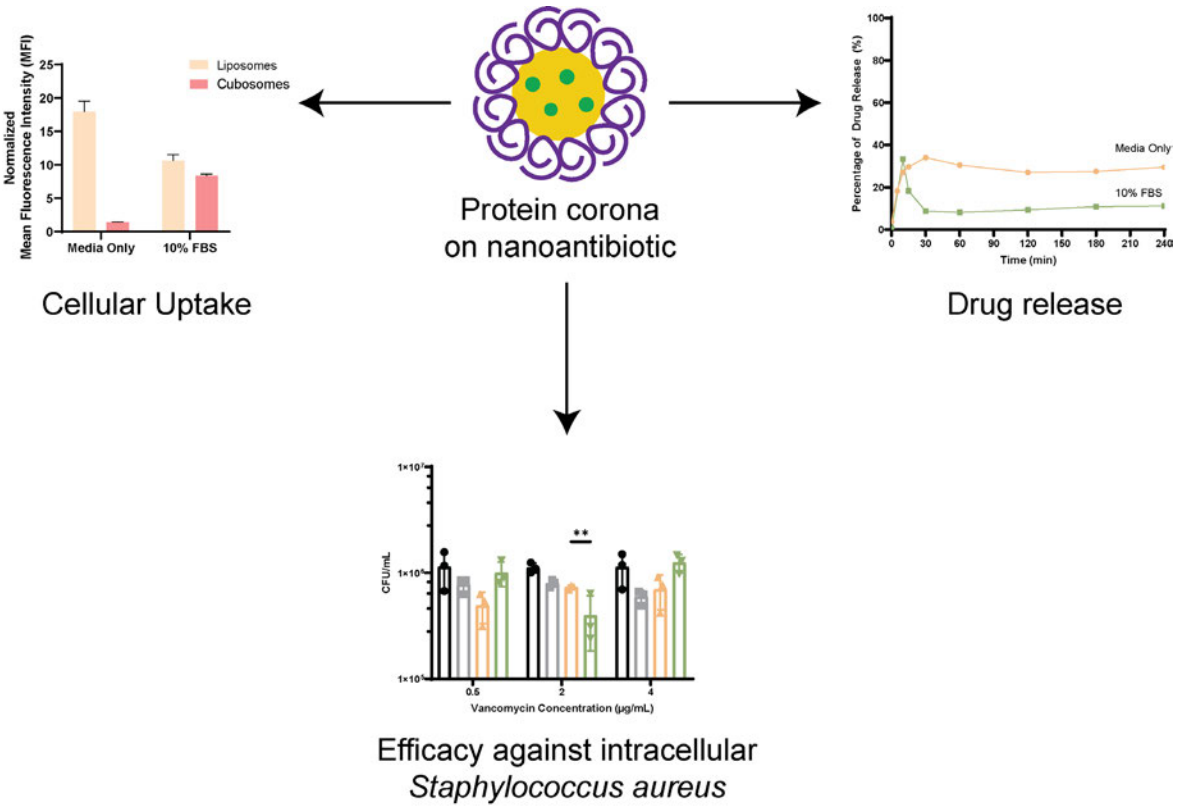
2

THE CELLULAR UPTAKE AND IN VITRO ANTIBACTERIAL ACTIVITY OF NANOANTIBIOTICS IS INFLUENCED BY PROTEIN CORONA



2 The cellular uptake and in vitro antibacterial activity of nanoantibiotics is influenced by protein corona

In this chapter, the cellular uptake of liposomes and cubosomes as a comparison of lamellar and non-lamellar systems is investigated in a phagocytic and non-phagocytic cell, i.e. macrophages and lung epithelial cells, respectively. The influence of a protein corona formed upon incubation of nanoparticles in fetal bovine serum (FBS) were evaluated, and key proteins identified using a proteomics approach. It was hypothesised that the structure and composition of the two nanoparticles will directly impact the protein corona formed and their subsequent cellular interaction. Further, the protein corona influence on release of antibiotic from the carrier and their performance against intracellular bacteria was explored.



Abstract

Bacteria have evolved survival mechanisms that enable them to invade and live within host cells, triggering persistent intracellular infections that present significant clinical challenges due to the inability for conventional antibiotics to permeate cell membranes. In recent years, nanoparticles or 'nanoantibiotics' have presented a promising strategy for overcoming intracellular infections by facilitating the cellular uptake of antibiotics, and thus improved targeting of the bacteria. However, prior to reaching host cells, nanoparticles experience interactions with proteins that form a corona and alter their physiological response. The influence of this protein corona on the cellular uptake, drug release and efficacy of nanoantibiotics for intracellular infections is poorly understood and commonly overlooked in preclinical studies. In this study, protein corona influence on cellular uptake was investigated for two nanoparticles; liposomes and cubosomes in macrophage and epithelial cells that are commonly infected with pathogens. All studies were undertaken in the presence of fetal bovine serum (FBS) to ensure the lipid nanoparticle systems formed a biologically relevant protein corona. Importantly, the protein corona impact on cellular uptake was shown to be nanoparticle-dependent, where reduced internalization was observed for liposomes, while cellular internalization of cubosomes increased by up to 80% in both cells. Subsequently, vancomycin-loaded cubosomes were explored for their drug delivery performance against intracellular small colony variants of *Staphylococcus aureus*. Vancomycin release from cubosomes was sustained in presence of protein corona. Further, we demonstrated improved bacterial killing in macrophages, with 65% reduction in bacterial viability upon internalization of cubosomes mediated by the protein corona. However, no differences in efficacy were observed in epithelial cells. Thus, this study provides insights and evidence to the role of protein corona in modulating the performance of nanoparticles in a dynamic manner, which findings will facilitate improved understanding and translation of future investigations from in vitro to in vivo.

2.1 Introduction

Bacteria continue to evolve against antibiotics, causing a rapid rise in the global case numbers of antibacterial resistance. Current estimates suggest that antimicrobial resistance will cause 10 million deaths per year by 2050 if left unaddressed [21]. Fueling this clinically unmet challenge is the ability for bacteria to find a survival niche within host cells, thereby causing persistent intracellular infections [22]. This adds to the complexity in treating bacterial infections as most antibiotics are (i) poorly permeable across host cell membranes, (ii) exhibit low intracellular retention, and/or (iii) have poor stability in the harsh intracellular environment of host cell [23].

Nanoparticles as carriers are a promising strategy to overcome the challenges in treating intracellular infections due to their ability to transport conventional antibiotics across host cells, thus facilitating higher payload and improved activity against pathogens [24, 25]. Also referred as nanoantibiotics, they can be engineered for passive targeting of host cells or functionalized with ligands for active targeting. A plethora of particles, ranging from organic-based lipid and polymer nanoparticles to inorganic particles such as silica have been investigated for encapsulation and delivery of antibiotics against intracellular infection [26-29]. However, the clinical translation of nanoantibiotics remains poor. Currently, only one liposomal formulation, Arikayce® has been approved by the US Food and Drug Administration (FDA) for treatment of intracellular *Mycobacterium avium* complex infections [30]. Thus, there is an urgent demand for new formulations as treatments against other intracellular infections, such as cystic fibrosis and osteomyelitis.

One challenge in translating the findings from in vitro to in vivo and ultimately clinical investigations is the poor understanding of physiological factors that can influence the activity of nanoantibiotics. One such factor is the presence of proteins in biological fluids, which strongly interacts and forms a corona around nanoparticles. It is established

that the protein corona provides nanoparticles with a new biological identity, which mediates their physiological behavior and cellular interaction [14]. The in depth understanding of protein corona formed in plasma has resulted in the approval of Abraxane^{TM}, an albumin bound nanoparticle encapsulating paclitaxel for cancer therapy, with enhanced tumor penetrating properties [31]. The presence of albumin potentiates cellular internalization of the nanoparticle by activation of key cellular membrane proteins, resulting in higher accumulation of nanoparticles compared to free paclitaxel [32]. Tang et al. recently demonstrated the inhibitory action of serum albumin on the activity of antimicrobial peptides (AMP), where albumin binds to AMP in the presence of α1-antitrypsin to form a complex, significantly reducing their antibacterial activity [33]. Further investigation is warranted to understand the role of proteins in facilitating cellular uptake and activity of nanoantibiotics against intracellular bacteria.

Therefore, the aim of this study is to investigate the influence of protein corona on cellular interaction and activity of liposomes and cubosomes against intracellular small colony variant of *Staphylococcus aureus* (SCV *S. aureus*). The corona of nanoparticles following incubation in fetal bovine serum (FBS) was identified and characterized to provide an understanding of nanoparticle composition and chemistry on protein adsorption. Interaction of nanoparticles in RAW 264.7 macrophages and A549 lung epithelial cells were investigated in presence and absence of FBS. Attributing to a high serum-dependent internalization across both cell lines, the drug release and efficacy of vancomycin-loaded cubosomes was further investigated. The findings derived provide new insights into developing efficient nanoparticle systems against intracellular bacteria, with a first in time report of the protein corona influence on nanoantibiotic performance, thereby providing further understanding for improved in vitro to in vivo translation of future developments.

2.2 Experimental Section

2.2.1 Formulation preparation and characterization

2.2.1.1 Liposome preparation

Liposomes composed of dimethyldioctadecylammonium (bromide salt) (DDAB) (Avanti Polar Lipids Inc., Alabaster, AL, US) and phosphatidylcholine (PC) (Sigma-Aldrich, Castle Hill, NSW, Australia) were fabricated using a specialized microfluidic device, NanoAssemblr® Benchtop (Precision NanoSystems Inc., Vancouver). The lipids were dissolved at a 1: 1 molar ratio (total lipid concentration 800 ng/mL) in methanol and mixed with phosphate buffer saline (PBS) (3: 1 lipid: aqueous ratio) at a total flow rate of 12 mL/min. The liposomal mixture was left to stir overnight to remove residual methanol.

Fluorescently labelled liposomes were synthesized by incorporating either 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 7) (Cy7-PE) or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss Rhod PE) (Avanti Polar Lipids Inc., Alabaster, AL, US) at a dye to lipid ratio of 1: 2000.

2.2.1.2 Liquid crystalline nanoparticles (Cubosomes)

Cubosomes were synthesized via the hydrotrope dilution method as previously described by Thorn et al., [19]. Briefly, 100 mg of monoolein (Myverol 18-99K) (Kerry Ingredients and Flavours Egham, Surrey, UK), 270 mg propylene glycol and 15 mg Pluronic® F-127 (Sigma-Aldrich, Castle Hill, NSW, Australia) were dissolved in chloroform (Merck Life Science, Bayswater, VIC, Australia). The chloroform was evaporated using a stream of nitrogen gas to form a lipid film. A white emulsion was formed by dissolving the lipid film with 100 µL of PBS and 5 mL of Milli-Q and vortexed for 3 min.

To form fluorescently labelled cubosomes, Cy7-PE or Liss-Rhod PE was introduced to the lipid-chloroform mixture at a ratio of 1: 2000 (dye to lipid).

2.2.1.3 Particle size and zeta potential

Nanoparticles were diluted in Milli-Q and their hydrodynamic diameter was determined via dynamic light scattering using Zetasizer Nano ZS (Malvern, Worcestershire, UK) at 25 ºC. Zeta potential was measured using 10 mM NaCl as the diluent using phase analysis scattering. The size and zeta potential of each nanoparticle was also investigated in the presence of complete cell culture media (with 10% fetal bovine serum). The size measurements and distribution, reported as z-average and polydispersity index (PDI) respectively, were determined using the built-in software, ZetaSizer Software v7.12.

2.2.1.4 Vancomycin loading and release studies

Vancomycin-loaded cubosomes were fabricated using the method described previously, with the incorporation of PBS doped with 50 mg/mL vancomycin (as free base) (Merck Life Science, Bayswater, VIC, Australia). A pressure ultrafiltration stirred cell, Amicon® (Merck Life Science, Bayswater, VIC, Australia) was employed for drug loading and release studies. 2.5 mL of vancomycin-loaded cubosomes were transferred to the cell containing a pre-soaked ultrafiltration membrane disc, 5 kDa NMWL (Merck Life Science). The filtrate containing free drug were collected by applying 100 kPa of nitrogen gas and was quantified for vancomycin concentration.

The formulation was diluted to 10 mL of cell media (without FBS) or complete cell media (with FBS) to determine the release of vancomycin from cubosomes. The mixture was continuously stirred magnetically, and 0.5 mL filtrate was collected at specific time points by applying 100 kPa of nitrogen gas. The mixture was immediately replaced with 0.5 mL of fresh media.

Vancomycin concentrations were quantified using SCIEX ExionLC™ AD ultra-high performance liquid chromatography (UHPLC) coupled with SCIEX Triple Quad™ 4500 LC-MS/MS System (SCIEX, Framingham, MA, US). Samples were diluted in mobile phase made of 50: 50 methanol and Milli-Q, supplemented with 0.1% formic acid. Teicoplanin was used as an internal standard and sample separation was conducted using Gemini-NX 5u C18 column, 50 x 4.60 mm (Phenomenex, Lane Cove, NSW, Australia). Samples were run at a flow rate of 0.3 mL/min.

2.2.2 Proteomic analysis

2.2.2.1 Protein corona formation

Liposomes and cubosomes were diluted at 1: 1 v/v with fetal bovine serum and incubated for an hour at 37 ºC, followed by centrifugation at 15, 300g for 20 min at 4 °C to remove unbound proteins, following modifications from previous protocol [34]. The supernatant was discarded, and the pellet was washed thrice with PBS. 100 µL of lysis buffer was added to the pellet collected after final washing and incubated at 95 ºC for 5 min. The eluted proteins were centrifuged for 15 min at 15, 300g at room temperature and the supernatant was diluted with 4 parts ice-cold acetone and left overnight for protein precipitation. The mixture was centrifuged at 20, 000g for 10 min and the precipitated proteins were washed twice with acetone. Residual acetone was removed by evaporation and redissolved in 8 M urea/ 50 mM ammonium bicarbonate.

2.2.2.2 Protein digestion and quantification

The extracted proteins were quantified using the Coomassie Plus (Bradford) Protein Assay (ThermoFischer Scientific) according to manufacturer's protocol. Following this, 100 µg of proteins were reduced with 10 mM dithiothreitol (DTT) for 1 h at room temperature, protected from light. Samples were further diluted in ammonium bicarbonate to reduce urea concentration to 0.8 M. Protein digestion was performed by overnight incubation with 2 µg

Trypsin Fold (Promega) at 37 ºC followed by acidification to stop the process. Sample clean via solid phase extraction using Sep-Pak C18 cartridges (Waters, Milford, MA) were preformed, and eluted proteins were dried down in vacuum centrifuge. Samples were stored at -20 °C until ready for LC-MS analysis.

2.2.2.3 Protein identification using liquid chromatography-mass spectrometry (LC-MS)

Protein samples were analyzed using an EASY-nLC 1200 system coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, Bremen, Germany). Peptides (150 ng) were reconstituted in 0.1% formic acid and injected onto a C18 column, packed with 25 cm fused silica with an inner diameter of 75 µm. The column was packed with 1.9 µm ReproSil-Pur 120 C18 AQ particles (Dr Maisch, Ammerbuch, Germany) and was heated to 50 ºC. Analysis was conducted over a 70-min linear gradient (3 to 20% acetonitrile in 0.1% formic acid), at a flow rate of 300 nL/min. Compensation voltages at -50 and -70 V were generated by a FAIMS Pro Interface (Thermo Scientific) to regulate entry of ionized peptides into the instrument. MS scans (m/z 300 to 1500) were acquired at a resolution of 60, 000 (m/z 200) using a positive ion mode. Multiple charged precursors, with a minimum threshold of 10, 000 ions were fragmented with 27.5% normalized HCD collision energy and resulting MS/MS scans were measured at resolution 15, 000. Cycle time and dynamic exclusion duration were set to 1.5 s and 40 s, respectively. Post analysis was performed using Proteome Discoverer 2.4 (Thermo Scientific) software with standard settings using the UniProt bovine database.

2.2.3 In vitro cellular studies

2.2.3.1 Cellular toxicity

The cellular viability of RAW264.7 murine macrophages and A549 cell lines in presence of nanoparticles were determined via MTT assay. RAW 264.7 were grown in

Dulbecco's Modified Eagle Medium (DMEM) while A549 were cultured in F-12K Medium (ThermoFisher Scientific, Scoresby, VIC, Australia), both supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin at 37 ºC in 5% CO_2. Cells were seeded in a 96 well plate at a seeding density of 1 x 10^4 cells per well and incubated for 24 h. Following incubation, cells were washed twice and treated with nanoparticles diluted in either media only (i.e., without FBS) or in complete media (i.e., with 10% FBS). Cells treated with media only and in 1% Triton-X were used as maximum and minimum growth. After 24 h, cells were washed several times and incubated with MTT solution (5 mg/mL in PBS) for 4 h. The MTT formazan formed were dissolved in DMSO and absorbance reading was measures at a wavelength of 540 nm. Cellular viability of treated cells was determined based on relative absorbance measurement of cells treated with media only.

2.2.3.2 Fluorescence activated cell sorting

Cellular internalization of nanoparticles was quantified using flow cytometry based on shift in fluorescence intensity. Briefly, cells were seeded at a density of 1 x 10^5 cells per well in a 12 well plate and incubated for 24 h. Following day, the cells were washed several times and incubated with fluorescently labelled formulation at a lipid concentration of 50 µg/mL, diluted in media only or pre-incubated in media supplemented with 10% FBS. After 4 h, cells were washed with PBS and detached via trypsinization for A549, and gentle scrapping for RAW264.7. Cells were fixed in 4% paraformaldehyde solution for 15 min and diluted in FACS buffer for analysis. The BD LSRFortessa™ (San Jose, CA, USA) was used to measure cellular internalization of nanoparticles with a minimum recording of 10, 000 events for each sample.

2.2.3.3 Confocal laser scanning microscopy

For qualitative assessment of nanoparticles internalization, cells were grown in a 6 well plate at a seeding density of 3 x 10^5 cells per well. After 24 h of incubation, cells were

washed and treated with fluorescently labelled nanoparticles, diluted in media only or supplemented with 10% FBS. Following 4 h incubation, cells were washed and attached to a glass slide using Cytospin™ Centrifuge at 300g for 5 minutes to form a uniform cell layer. The cells were fixed in paraformaldehyde and labelled with wheat-germ agglutin (WGA), Alexa Fluor® 488 conjugate (Invitrogen, Carlsbad, CA, USA) and mounted to cover slip using ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher Scientific, Scoresby, VIC, Australia). LMS 710 Carl Zeiss confocal microscopy (Oberkochen, Germany) and Zen Lite 2.6 software was used for imaging and processing respectively.

2.2.4 In vitro bacterial studies

2.2.4.1 Minimum inhibitory concentration determination

A small colony variant of *Staphylococcus aureus* (SCV *S. aureus*) WCH-SK2 (kindly gifted by Dr Stephen Kidd, University of Adelaide) was selected as a relevant intracellular pathogen [35]. The minimum inhibitory concentration (MIC) of vancomycin was determined using broth dilution method. Briefly, an overnight culture of the bacteria was diluted in 0.9% saline to yield 1 x 10^8 colony forming units (equivalent to an absorbance reading of 0.08 - 0.12 at 600 nm). The suspension was further diluted 1: 100 in tryptic soy broth (TSB) and added to the wells of a 96 well plate. Vancomycin was serially diluted at a desired range of concentration and incubated for 24 h at 37 ºC. The lowest concentration of vancomycin with no bacterial growth was visually assessed and determined as the MIC.

2.2.4.2 In vitro intracellular infection assay

To establish an intracellular infection assay, cells were seeded at a density of 3 x 10^5 cells/mL in a 12 well plate for 24 h. Following this, an overnight culture of bacteria in TSB was diluted in cell media only to a multiplicity of infection (MOI) of 10: 1 for RAW 264.7 cells and 100: 1 for A549. The diluted bacterial suspension was added to cells and incubated for 2 h. The cells were washed twice and incubated with 10 µg/mL of lysostaphin for 1 h to

eradicate extracellular and cellular membrane-bound bacteria. After repeated washing, the cells were incubated with vancomycin-loaded cubosomes, diluted in media only or in presence of 10% FBS for 4 h at vancomycin concentration equivalent to 0.5, 2.0 and 4.0 μg/mL. After 4 h, the cells were washed and incubated in 0.1% Triton-X for 10 min to lyse the cells for intracellular bacteria extraction. The solution was serially diluted in 0.9% saline and 1 mL of sample was added to a sterile plate followed by dilution in 20 to 25 mL of tryptic soy agar (TSA). The plates were incubated for 18-24 h, and bacterial colonies were counted.

2.2.5 Statistical analysis

Data are reported as mean ± standard deviation and experiments were conducted in triplicates. Results were analyzed using one or two-way analysis of variance (ANOVA) and Tukey's or Šídák's multiple comparison test at significance level of $\alpha = 0.05$. All tests were performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California, USA).

2.3 Results and Discussion

2.3.1 Fabrication and characterization of lipid nanoparticles

Lipid-based nanoparticles (liposomes and cubosomes) were systematically synthesized to have comparable mean particle sizes and zeta potentials, in the range of 180 to 204 nm and -3 to -9 mV, respectively (Table 1). This facilitates investigations to be made based on their structure and composition, independent of size and surface charge for subsequent cellular studies. The incorporation of phosphatidylcholine (PC), a zwitterionic lipid results in near neutral charge of liposomes. Cubosomes were fabricated using glyceryl monooleate (GMO) with Pluronic F127 incorporated as a stabilizer. The presence of Pluronic results in adsorption of hydroxide ions at the water and lipid interface of cubosomes, resulting in an overall neutral or negative charge [36].

Vancomycin-loaded cubosomes were also fabricated for drug release and antimicrobial studies (see Table 1). The size and zeta potential of vancomycin-loaded cubosomes were comparable to the blank cubosomes. An antibiotic loading level of 4.96 ± 0.02% (w/w) with an encapsulation efficiency of 99.2 ± 0.34% was achieved for vancomycin, similar to previous results demonstrated by Thorn et al., for other hydrophilic antibiotics [37].

Table 1. Particle size, polydispersity index (PDI) and zeta potential of liposomes and cubosomes, measured in protein-free cell media and cell media supplemented with 10% fetal bovine serum (FBS). The particle size and zeta potential of vancomycin loaded cubosomes are also presented.

	Size (nm)	PDI	Zeta potential (mV)
Liposomes			
Protein-free media	203 ± 0.2	0.22 ± 0.01	-8.7 ± 2.3
10% FBS	358 ± 45	0.71 ± 0.06	-12.7 ± 0.9
Cubosomes			
Protein-free media	186 ± 15	0.32 ± 0.02	-3.1 ± 0.2
10% FBS	236 ± 17	0.27 ± 0.02	-10.3 ± 1.2
Vancomycin-loaded	231 ± 9	0.44 ± 0.02	-7.2 ± 0.2

2.3.2 Influence of nanoparticle composition on protein corona formation

The presence of FBS in media increased the size of both nanoparticles by up to 150 nm, confirming protein adsorption on the surface of the nanoparticles or aggregation (Table 1). An increase in negative value for zeta potential was observed; -12.7 mV and -10.30 mV for liposomes and cubosomes, respectively, further confirming the adsorption of proteins to nanoparticles. Nanoparticle-protein complexes often carry a negative charge due to the net charge of proteins thus, results are in comparison with previous observations [38].

Protein distribution within the corona of nanoparticles were compared by first categorizing the proteins according to their molecular weight, isoelectric point, and grand average of hydropathy (GRAVY) score (Figure 1). Across all three properties, the protein fingerprint of FBS (serum only) was different compared to both nanoparticles, in agreement with previous observations as selective protein enrichment can occur on the surface of nanoparticles [39]. When comparing the protein distribution across both nanoparticles, no adsorption patterns could be identified. The largest proportion of proteins on both nanoparticles had a molecular weight between 60-80 kDa, with an isoelectric point of 6 to 7 and GRAVY score of -0.6 to -0.4 (Figure 1). A similar observation was reported by Giulimondi et al., showing liposomes of different surface functionality having a tendency to adsorb proteins of similar size and isoelectric points as described here [34].

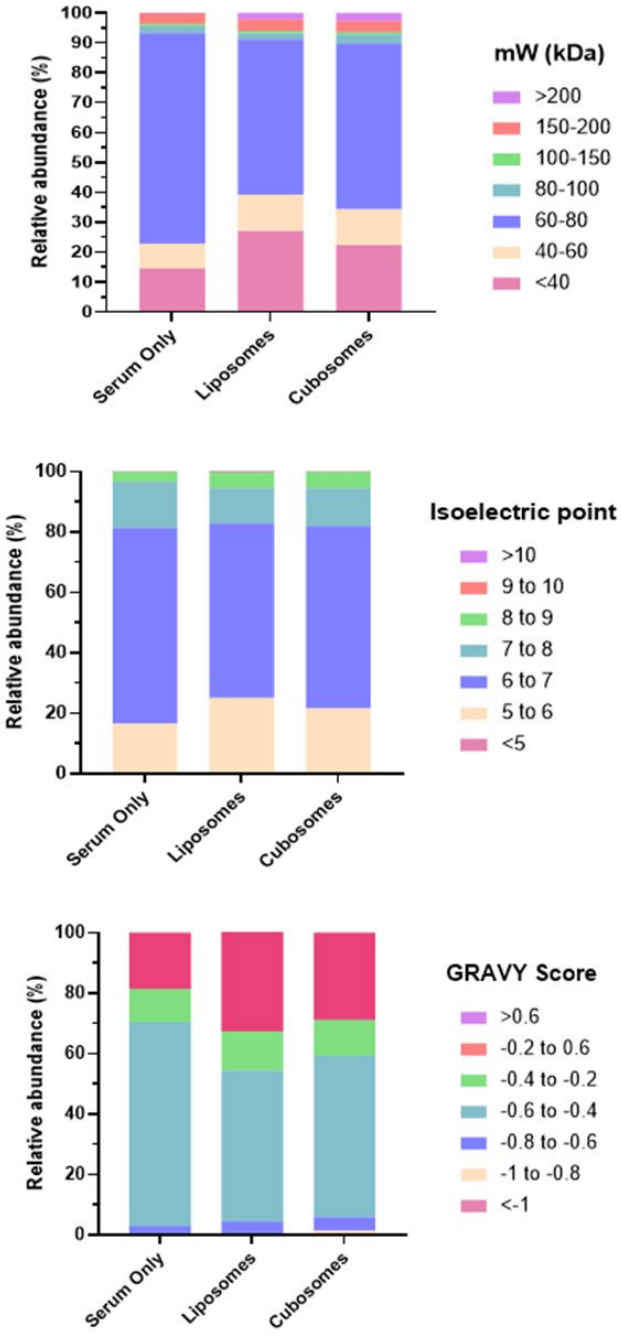


Figure 1. Distribution of proteins found in fetal bovine serum (serum only) versus the corona of liposome and cubosomes, categorized according to their (a) molecular weight (mW), (b) isoelectric point and (c) GRAVY score.

Similarly, when comparing the top 10 proteins isolated from the corona of the nanoparticles, the majority of proteins found were similar, with differences observed in their relative abundance as illustrated in Table 2. Albumin is the most abundant protein in fetal

bovine serum, constituting ~60 to 70% of total proteins [40]. Thus, high adsorption of albumin on the surface of nanoparticle is expected and consistent with previous studies [41, 42]. Similarly, alpha-2-HS-glycoprotein, serotransferrin and alpha-1-antiproteinase are highly abundant in FBS [43]. The similarities in protein corona can be explained using the Vroman effect, where protein adsorption to the surface of nanoparticle is mediated by their relative abundances in biological media [44].

Protein interaction with nanoparticles is controlled by various factors, including size, shape, hydrophobicity, and surface charge of particles [45]. The composition of nanoparticles was initially hypothesized to alter their protein corona, especially due to the hydrophilic layer resulting from Pluronic F127 in cubosomes. However, no differences were observed in this study, with albumin constituting 40% of total proteins found in the corona of cubosomes. As illustrated by Walkey et al., and Du et al., the density and conformation of polymer on nanoparticles are also important factors mediating protein interactions, with higher PEG grafting in mushroom conformation leading to decreased protein adsorption [46, 47]. It is also important to note that protein-protein interactions can occur, which ultimately determines the protein corona composition. Thus, the high adsorption of albumin can lead to recruitment of similar proteins resulting in formation of almost identical corona for both nanoparticles [48].

Table 2. The top 10 most abundant proteins found within the corona of liposomes and cubosomes.

Liposomes		Cubosomes	
Proteins	Relative abundance (%)	Proteins	Relative abundance (%)
Albumin	39.74%	Albumin	41.93%
Alpha-2-HS-glycoprotein	17.38%	Alpha-2-HS-glycoprotein	13.19%
Serotransferrin	8.24%	Serotransferrin	9.21%
Alpha-1-antiproteinase	7.36%	Alpha-1-antiproteinase	7.29%
Alpha-2-macroglobulin	2.30%	Alpha-2-macroglobulin	2.45%
Apolipoprotein A-1	1.88%	Hemoglobin	2.20%
Hemoglobin	1.77%	Apolipoprotein A-I	1.97%
Apolipoprotein A-II	1.76%	Alpha-fetoprotein	1.67%
Apolipoprotein B	1.69%	Fetuin-B	1.41%
Alpha-fetoprotein	1.67%	Desmoplakin	1.28%

2.3.3 Cellular interaction of nanoparticles influenced by their synthetic and biological identity

2.3.3.1 Cytotoxicity profile of nanoparticles

Prior to conducting cellular studies, the nanoparticles were screened for their cytotoxicity across both RAW264.7 macrophages and A549 epithelial cell lines. The nanoparticles were tested at a concentration range of 12.5 to 100 µg/mL, in presence and absence of 10% FBS. Across both cell lines, the liposomes and cubosomes maintained their viability above 80% following a 24 h incubation period (Figure 2). Hence, they were considered non-toxic for subsequent cellular studies within the concentration range of interest.

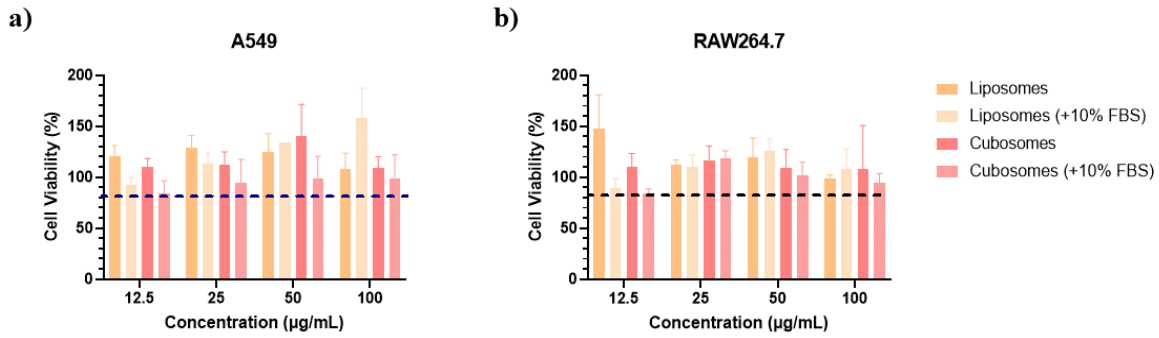


Figure 2. Cellular toxicity profile of liposomes and cubosomes following 24 h incubation in cell media only or supplemented with 10% FBS.

2.3.3.2 Cellular uptake profile of nanoparticles

Following a 4 h incubation, the nanoparticle-cell association as a measure of particle internalization was quantified using flow cytometry, with measurements normalized to the autofluorescence of cells only (untreated control) (Figure 3). Interestingly, regardless of their similarities in protein corona, the nanoparticles demonstrated significant differences in their cellular uptake. In both cell lines, the presence of FBS reduced liposomes' internalization by ~2-fold (Figure 3). On the contrary, uptake of cubosomes in macrophages doubled in presence of serum. Further, serum strongly mediated the interaction of cubosomes with A549, resulting in ~10-fold increase in mean fluorescence intensity (MFI) (equivalent to 80% percentage positive cells) (Figure 3b). This is in line with observations reported by Fleischer et al. in which, similar protein corona formed on cationic and anionic polystyrene nanoparticles interacted with different cell receptors [49]. Their interaction was driven by the structure and orientation of proteins adsorbed, resulting in varied biological response of the nanoparticles.

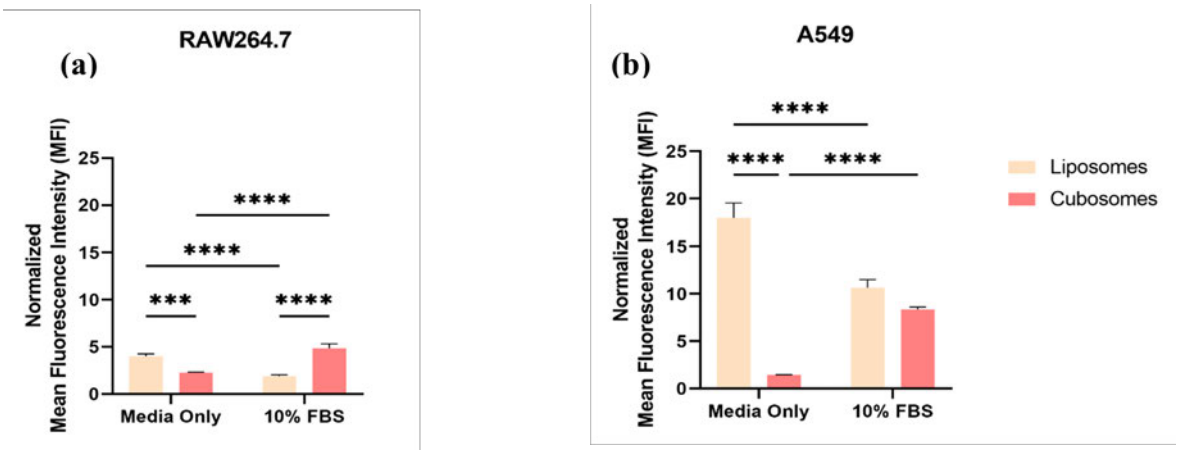


Figure 3. Cellular internalization assessed by comparing mean fluorescence intensity of nanoparticles, normalized to untreated cells, measured using flow cytometry in (a) RAW264.7 macrophages and (b) A549 epithelial cells.

The internalization of liposomes and cubosomes was qualitatively confirmed by visualizing rhodamine-labelled nanoparticles using confocal microscopy. As illustrated in Figure 4, the fluorescence intensity of the liposomes was reduced in cell media supplemented with 10% FBS in comparison to media only across both cell lines. For cubosomes, an enhancement in fluorescence intensity was observed across both macrophages and epithelial cells. Importantly, A549 treated with cubosomes in media only had no fluorescent signal, while the greatest fluorescence intensity was observed when pre-incubated with FBS, similar to flow cytometry results in Figure 3. Across all treatments, the fluorescence signal of nanoparticles was present in the cytoplasm of the cells, surrounding the nucleus, confirming their intracellular localization in cells.

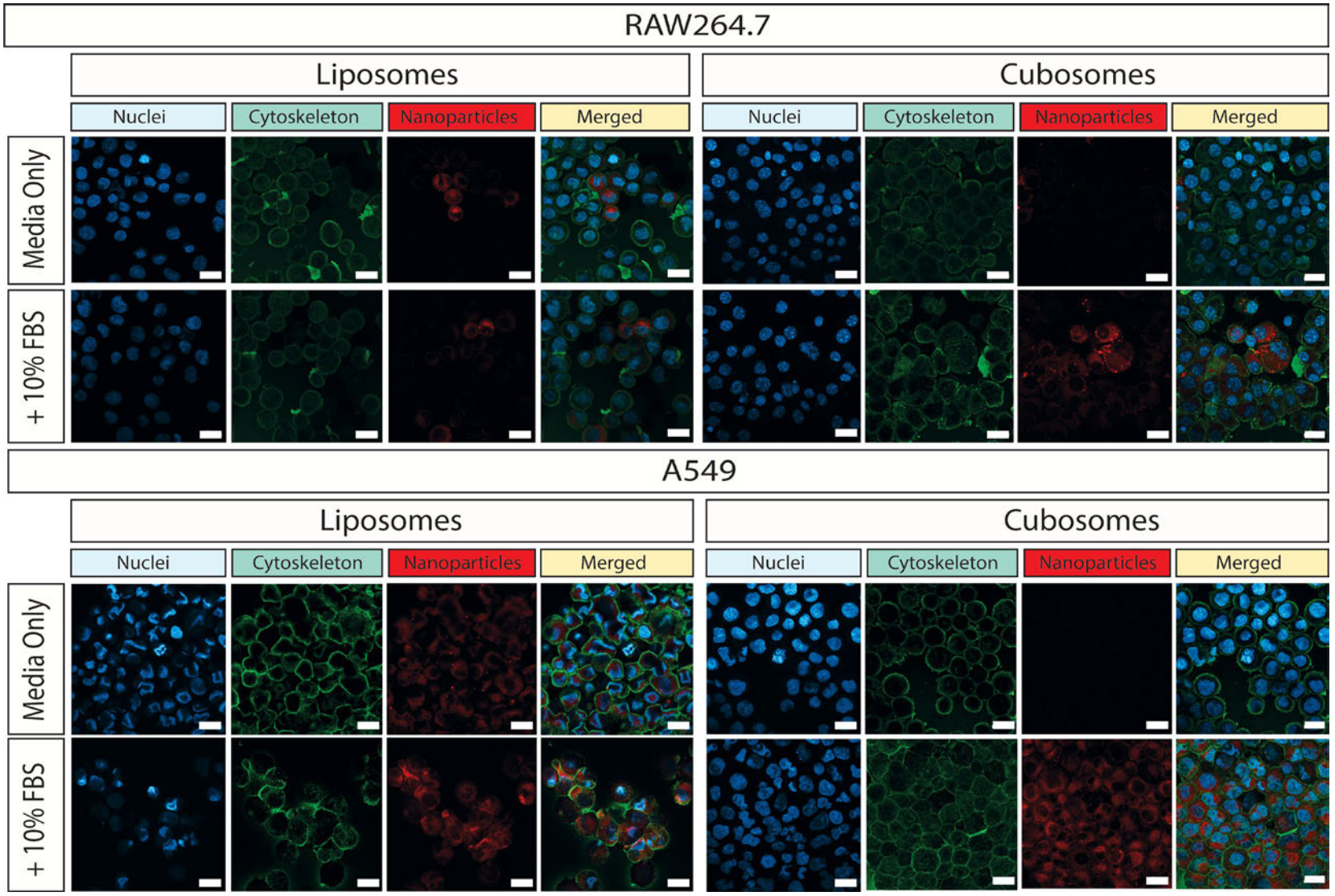
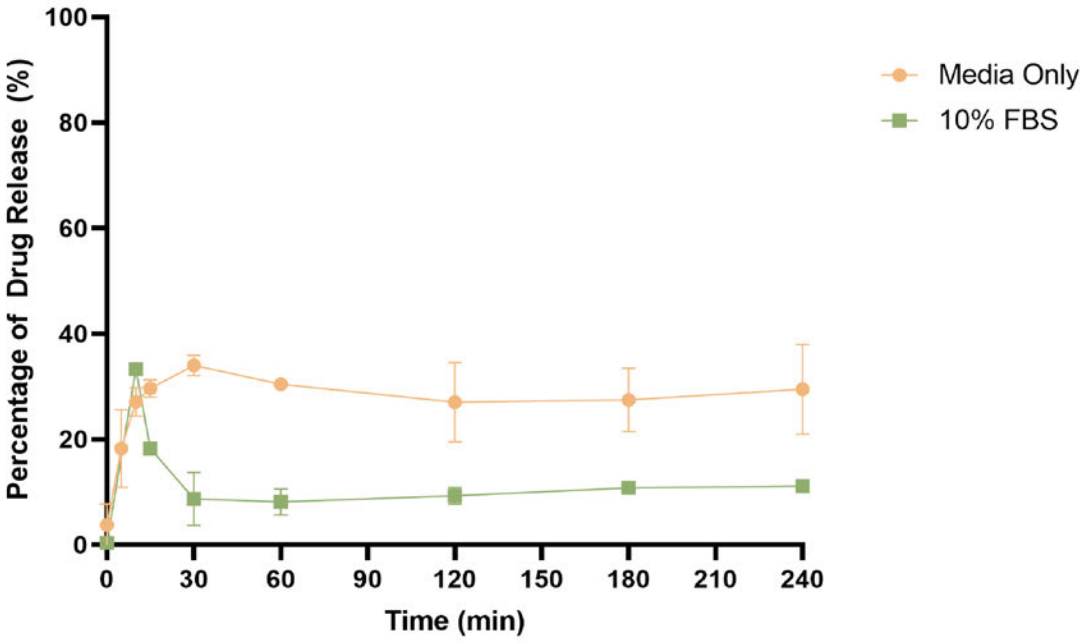


Figure 4. Confocal microscopy images of nanoparticle uptake in RAW264.7 (top) and A549 (bottom) cell lines, in cell media only or cell media supplemented with 10% FBS following 4 h incubation. Nanoparticles are labelled with Liss-Rhod PE (red), cellular cytoskeleton stained with wheat-germ agglutin (WGA), Alexa Fluor™ 488 conjugate (green)and nucleus stained with DAPI (blue) Scale bar represents 20 µm.

2.3.4 In vitro vancomycin release kinetics in presence of protein corona

Cubosomes were chosen as the nanocarrier of choice for subsequent drug release and efficacy studies attributing to their high internalization across both cell lines in presence of protein. The release kinetics of vancomycin in cell media only (serum-free media) showed a rapid release of the drug, with a maximum release of 33.9 ± 1.4 % within 30 minutes and plateaued thereafter (Figure 5a). Hydrophilic molecules are hypothesized to be incorporated within the aqueous pores of the cubosomes and their release is controlled by diffusion through the water channels into the environment [50]. Thorn et al., previously demonstrated a rapid and complete release of the hydrophilic antibiotics, ciprofloxacin and tobramycin, from cubosomes within 2 hours [37, 51]. However, both ciprofloxacin and tobramycin are small molecules with a molecular weight of 368 and 468 Da, respectively. In contrast, vancomycin has a molecular weight of 1486 Da and thus its diffusion across the water channels is expected to be limited in comparison to smaller drug molecules. Furthermore, in evaluating the release kinetics of antimicrobial peptides from cubosomes, Boge et al., reported the release to be significantly lower in presence of physiological salt concentration compared to buffer only, due the electrostatic interaction of peptides and cubosomes [52]. Cell media composition have ionic salts at physiologically relevant concentration, thus the limited diffusion of vancomycin, a glycopeptide reported herewith are in line with previous justifications.

(a)



(b)

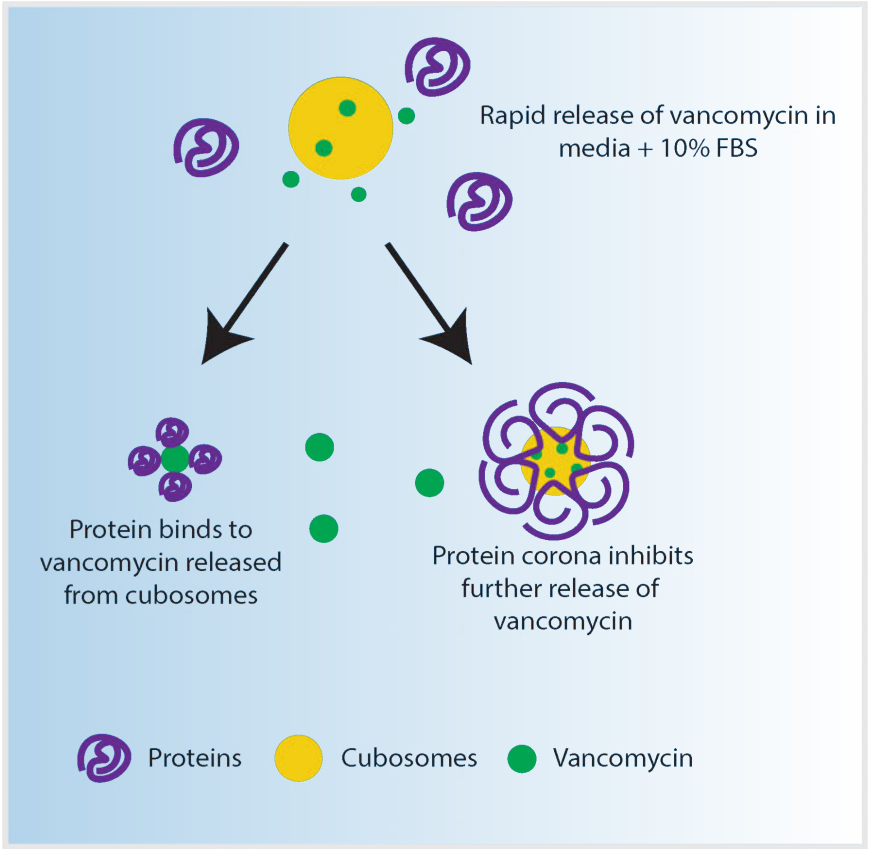


Figure 5.(a) Percentage release of vancomycin from cubosomes in cell media only (peach ●) and in cell media with 10% FBS (green ■) across 4 h. Data are represented as mean ± SD (n = 3). (b) Schematic illustration of proposed drug release mechanisms of vancomycin in presence of FBS.

Vancomycin release was also investigated in complete media, supplemented with 10% FBS to simulate biorelevant conditions upon exposure in vivo. A rapid burst release was observed within the first ten min, reaching maximum release similar to serum-free media, followed by a reduction to approximately 10% after 30 min. The two-phase release kinetics observed for vancomycin in presence of proteins can be explained by mechanisms illustrated in Figure 5b. First, a rapid diffusion of vancomycin closer to the surface of the cubosomes occurred, which was restricted upon adsorption of proteins to the surface of cubosomes, thus providing a protective effect to the cargo molecule. Secondly, vancomycin protein binding is commonly reported in clinical settings, with 24.3 to 64.0% vancomycin reported to be protein-bound [53]. Therefore, it is possible that the drug release from the cubosomes strongly binds to the proteins present in the dissolution media and does not elute through the ultrafiltration membrane, with a 5 kDa MW cut off.

2.3.5 Antibacterial activity of vancomycin-loaded cubosomes in presence of serum

An in vitro efficacy study was conducted using intracellular small colony variants of *Staphylococcus aureus* (SCVs *S. aureus*) in macrophages and epithelial cells. SCVs of *S. aureus* are increasingly found in recurrent and persistent airway infections, thus a relevant strain of intracellular bacteria for this study [54]. Vancomycin alone has minimal activity against intracellular SCVs and their activity when encapsulated in nanoparticle has not been investigated thus far [55]. The minimum inhibitory concentration (MIC) was experimentally determined to be 0.5 µg/mL. Based on this, the efficacy of vancomycin loaded cubosomes was evaluated at a concentration range of 0.5 to 4 µg/mL, at 1x, 4x and 8x MIC.

Following a 4 h incubation, infected macrophages treated with a solution of vancomycin showed a minimal reduction in number of colony forming units (CFU/mL) (Figure 6a) and was determined to be statistically insignificant. This confirms the suboptimal treatment achieved clinically due to poor penetration of antibiotics in host cells and the need

for efficient carrier systems to improve antibiotic sensitivity against intracellular pathogens [56]. At 1x MIC, the cubosomes in serum free and 10% FBS media showed no bacterial reduction, in agreement with previous observation, in which minimal efficacy against intracellular bacteria was obtained [27]. At 4x MIC, cubosomes in serum-free media had similar reduction in bacterial burden as vancomycin treated (free drug). However, cubosomes supplemented with 10% serum showed ~65% reduction in intracellular bacteria viability compared to untreated control (Figure 6a and b). An increase in cubosomes uptake in presence of serum correlated linearly with greater reduction of bacteria (Figure 6c). Previous studies have also shown improved killing of SCV *S. aureus* as a result of higher intracellular accumulation of antibiotic using nanoparticles [26-28]. It is also important to note that 4x MIC has been hypothesized as the optimal concentration for maximal efficacy while reducing dose-dependent toxicity for intracellular infections [55, 57].

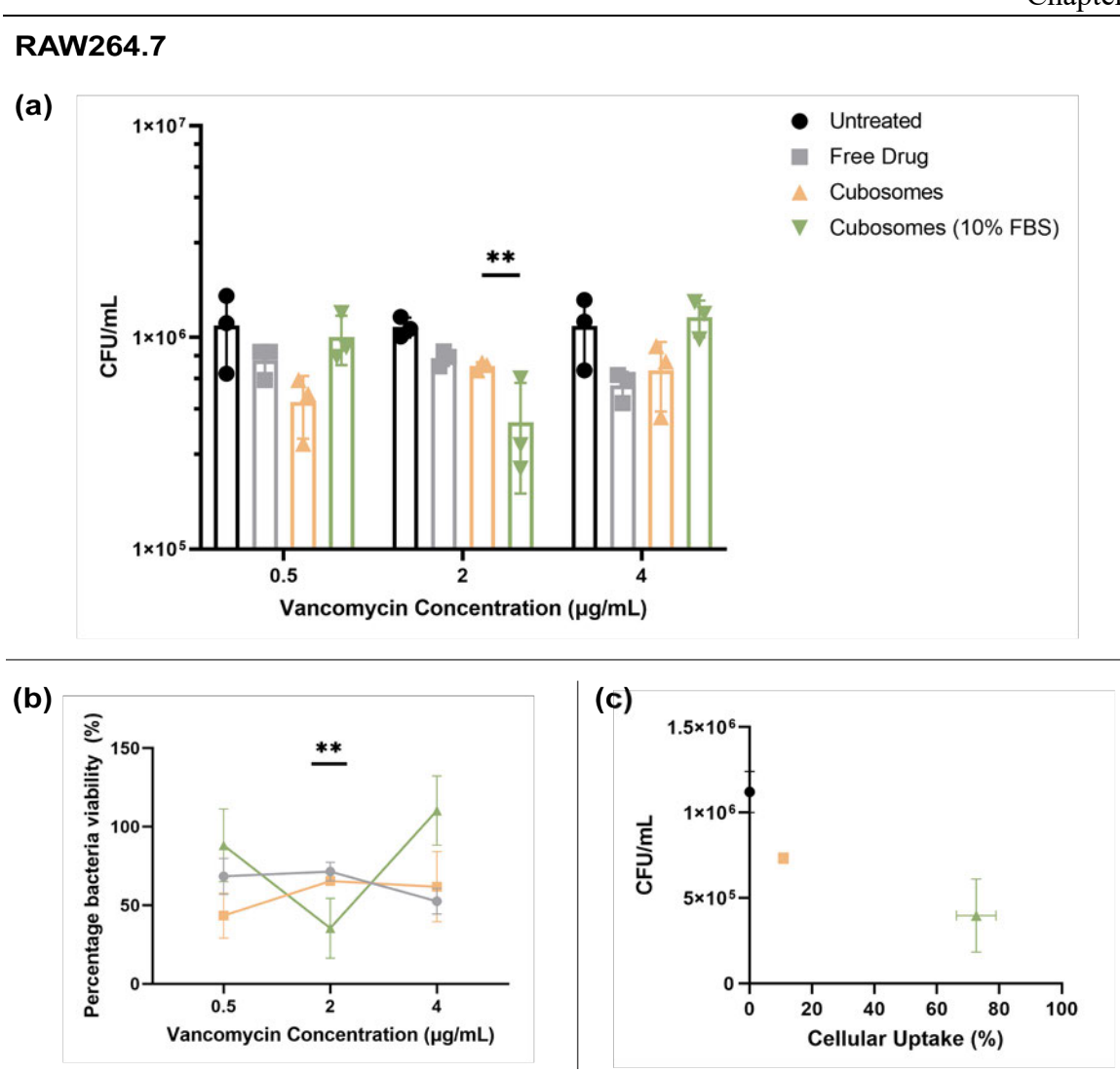


Figure 6. (a) Concentration-dependent effect of vancomycin-loaded cubosomes influenced by presence of serum in macrophages. (b) Percentage bacteria viability relative to untreated control as a function of vancomycin dose concentration. (c) Correlation between colony forming unit of bacteria after treatment at 4x MIC and percentage cellular uptake of cubosomes (from previously presented data).

At 8x MIC, no significant eradication of intracellular bacteria was observed across all treatments. A concentration-dependent effect of vancomycin-loaded nanoparticles against intracellular bacteria have been previously reported [58]. However, a saturation effect for vancomycin has also been observed. In a study by Barcia-Macay et al., the intracellular bacterial killing plateaued when dosed at 3-10x MIC of vancomycin against three different strains [59]. Similarly, Pumerantz et al., reported reduced efficacy of vancomycin-loaded liposomes after a 24 h treatment when dosed at vancomycin concentration of 800 µg/mL.

Conversely, a similar result was not achieved with epithelial cells (Figure 7a and b). It was initially postulated that the 10-fold increase in cellular uptake mediated by serum will result in greater bacterial killing, although no correlation was observed (Figure 7c). Zhang et al., previously demonstrated improved activity of penicillin-derived lipid nanoparticles against intracellular *S. aureus* in A549 [29]. Interestingly, no co-localization was observed between nanoparticles and intracellular bacteria in this study, with bacteria residing in the cytoplasm of the cell and nanoparticles found in the lysosomes. This suggests the drug released from nanoparticle diffuses across cellular organelles to exert their activity against bacteria following internalization. Similarly, diffusion of ampicillin from nanoparticles across lysosomes to reach intracellular site of infection has been proposed as the mechanism for activity of nanoantibiotics [60]. Based on these observations and the limited release of vancomycin from cubosomes in presence of FBS (Figure 5), it is hypothesized that the protein corona limits diffusion of antibiotic from the nanoparticles in A549, thus inhibiting their activity against the bacteria.

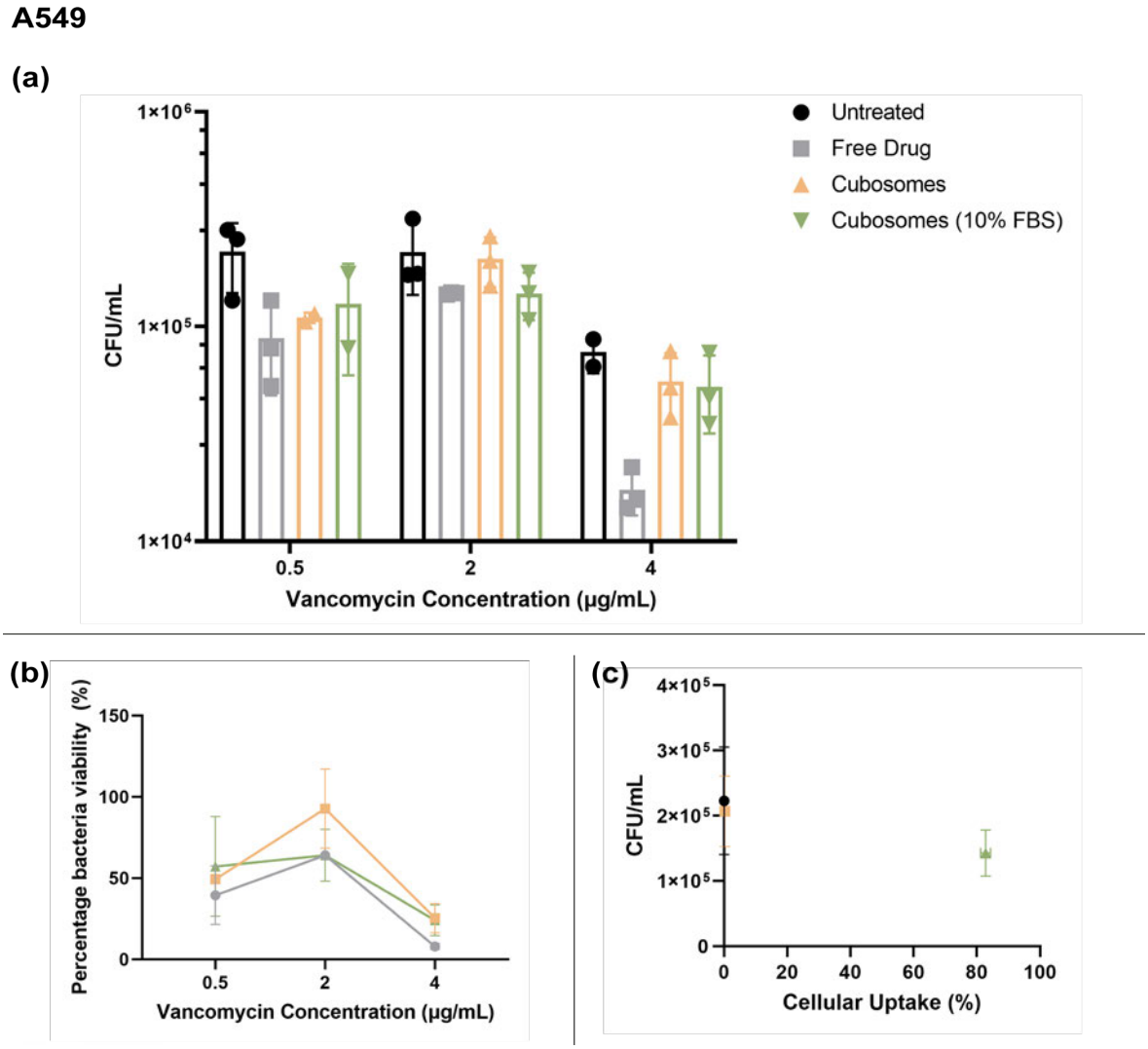


Figure 7. (a) Concentration-dependent killing of intracellular bacteria with vancomycin-loaded cubosomes in A549. (b) Percentage bacteria viability relative to untreated control as a function of vancomycin dose. (c) Correlation between colony forming unit of bacteria after treatment at 4x MIC and cellular uptake of cubosomes (from previously presented data).

Findings derived from this study provides preclinical proof of concept on the influence of biological molecules, predominantly protein corona formation on the activity of nanoantibiotics. For the first time, we have demonstrated the influence of protein corona on efficacy of nanoantibiotics, by modulating (i) cellular uptake of nanoparticles (ii) drug release of antibiotic from nanoparticles and (iii) influencing their activity against intracellular bacteria. Cell-dependent uptake of lipid nanoparticles and their subsequent activity against bacteria was demonstrated, warranting further investigation on understanding the mechanism in eradicating intracellular bacteria in non-phagocytic cells. A

major limitation in this study is the inability for epithelial cells and macrophages to survive beyond 4 h in the absence of serum (data not shown). Therefore, their activity could only be examined following a short incubation time which limits investigation of time-dependent killing of intracellular bacteria. It is expected that the antibacterial activity is improved over time, although saturation of antibiotic can hamper their performance. Understanding the interaction of nanoparticles in biorelevant media is crucial to facilitate translation of results obtained from in vitro to vivo studies and subsequently, for application in clinical setting.

2.4 Conclusion

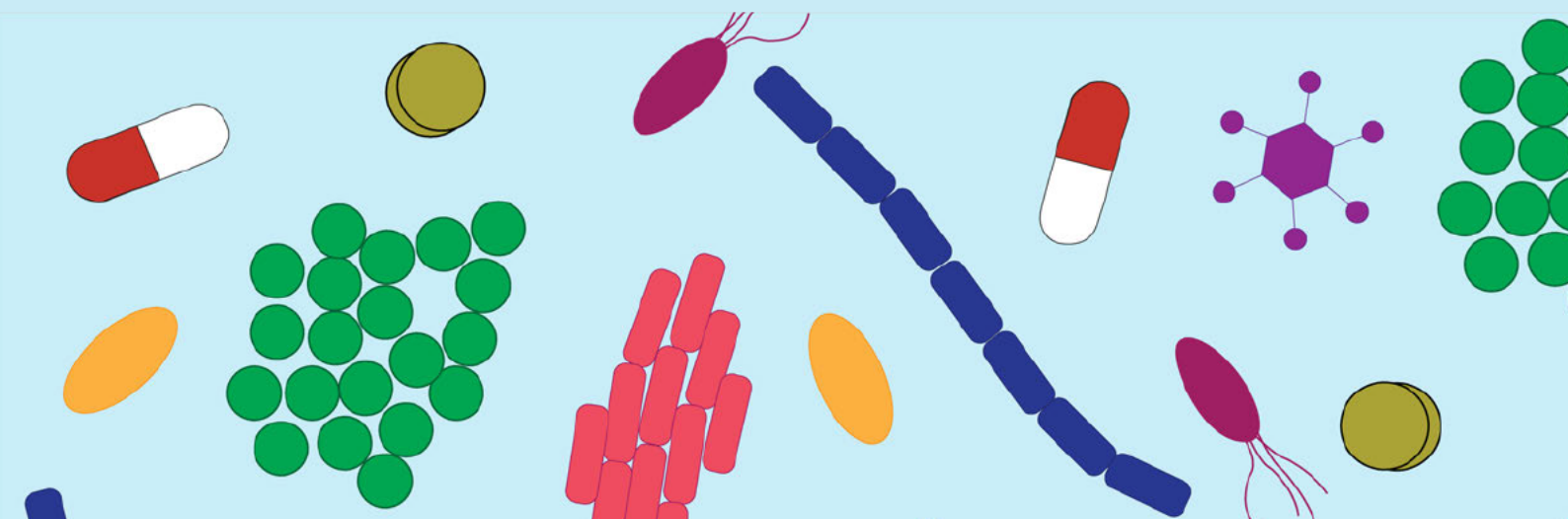
Rapid absorbance of proteins to nanoparticles in biological media, forming a protein corona is extensively reported in the field of nanomedicine. Despite their implications on the performance of nanoparticles, it is often not introduced in in vitro studies and remains poorly understood for their applications towards intracellular infections. Findings derived from this study demonstrates the ability of protein corona to modulate release kinetics of antibiotics encapsulated within the particles, subsequently affecting their performance against intracellular bacteria. A nanoparticle and cell- dependent influence was also observed, with reduced uptake of liposome and an increased internalization of cubosome across both cells. While the majority of proteins isolated from the corona of both nanoparticles were similar, it is stipulated that the conformation of proteins adsorbed are varied, thus exhibiting differences in cellular interactions. A two-phase release kinetics was observed for vancomycin from cubosomes in presence of serum, with a rapid burst release followed by a decrease in drug release over a 4 h dissolution. The enhanced uptake of cubosomes in serum correlated with improved performance against intracellular SCV *S. aureus* in macrophages at 4x MIC, resulting in ~65% reduction in bacterial viability. However, no improvement was observed in epithelial cells, thus illustrating a cell-dependent behavior of the protein corona. This observation necessitates further investigation in understanding the mechanism of intracellular infection exploiting protein corona for delivery of nanoantibiotics.

Acknowledgements

Mr Leigh Donnellan, Dr Clifford Young and Professor Peter Hoffmann (University of South Australia) are kindly acknowledged for their technical assistance in conducting proteomic analysis. Dr Stephen Kidd (University of Adelaide) is also acknowledged for gifting the SCV *S. aureus* strain.

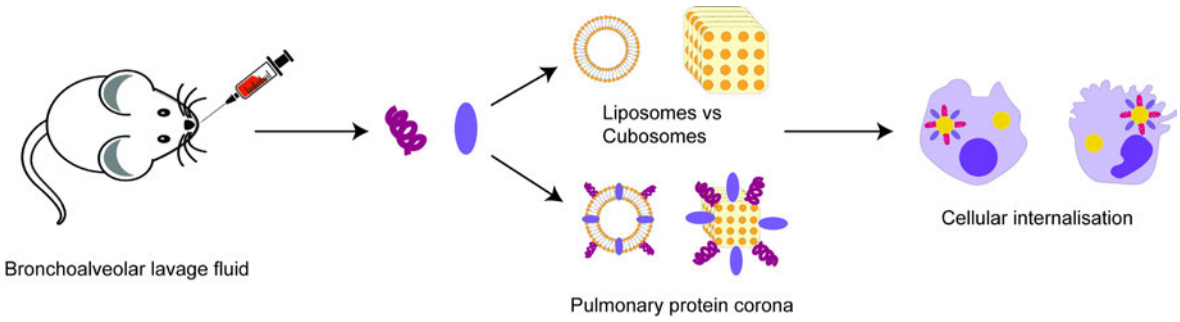
3

PROTEIN ADSORPTION DETERMINES PULMONARY CELL UPTAKE OF LIPID-BASED NANOPARTICLES



3 Protein adsorption determines pulmonary cell uptake of lipid-based nanoparticles

Following initial assessment to decipher the role of protein corona in mediating activity of nanoantibiotics in the previous chapter, protein adsorption pattern and cellular uptake kinetics was further evaluated in a highly biorelevant fluid for pulmonary delivery. Murine bronchoalveolar fluid (BALF) containing native proteins and surfactant molecules in the lung was used as the incubation media to form a biomimetic protein corona on liposomes and cubosomes. Key proteins were isolated and characterized using proteomics, and their mechanisms for mediating cellular interaction patterns of nanoparticles deciphered.



Journal of Colloid and Interface Science 641 (2023) 36–47

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

journal homepage: www.elsevier.com/locate/jcis

Protein adsorption determines pulmonary cell uptake of lipid based nanoparticles

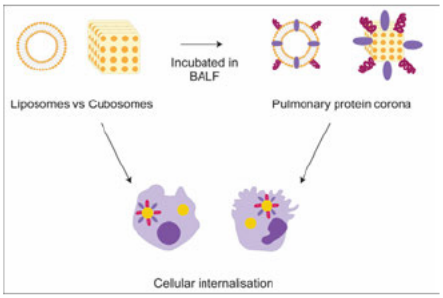
Santhni Subramaniam, Paul Joyce, Leigh Donnellan, Clifford Young, Anthony Wignall, Peter Hoffmann, Clive A. Prestidge *

University of South Australia, UniSA Clinical and Health Sciences, SA 5000, Australia

HIGHLIGHTS

- Liposomes and cubosomes show specific and discrete interaction behavior with macrophages and lung epithelial cells in presence of bronchoalveolar lavage fluid (BALF).
- Label-free quantitative proteomics revealed differences in the protein corona formed on BALF treated liposomes versus cubosomes.
- Albumin rich corona altered cellular uptake kinetics of liposomes, while Apolipoprotein H present on cubosomes enhanced their uptake.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 January 2023
Revised 5 March 2023
Accepted 7 March 2023
Available online 11 March 2023

Keywords:
Lipid-based nanoparticle
Protein corona
Pulmonary delivery
Protein interaction
Cellular uptake

ABSTRACT

The inhalable administration of lipid nanoparticles is an effective strategy for localised delivery of therapeutics against various lung diseases. Of this, improved intracellular delivery of pharmaceuticals for infectious disease and cancer management is of high significance. However, the influence of lipid nanoparticle composition and structure on uptake in pulmonary cell lines, especially in the presence of biologically relevant media is poorly understood. Here, the uptake of lamellar (liposomes) versus non-lamellar (cubosomes) lipid nanoparticles in macrophages and lung epithelial cells was quantified and the influence of bronchoalveolar lavage fluid (BALF), containing native pulmonary protein and surfactant molecules is determined. Cubosome uptake in both macrophages and epithelial cells was strongly mediated by a high percentage of molecular function regulatory and binding proteins present within the protein corona. In contrast, the protein corona did not influence the uptake of liposomes in epithelial cells. In macrophages, the proteins mediated a rapid internalisation, followed by exocytosis of liposomes after 6 h incubation. These findings on the influence of biological fluid in regulating lipid nanoparticle uptake mechanisms may guide future development of optimal intracellular delivery systems for therapeutics via the pulmonary route.

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Abbreviations: ApoH, apolipoprotein H; COPD, chronic obstructive pulmonary diseases; Cy7-PE, 1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine-N-(Cyanine 7); DDAB, dimethyldioctadecylammonium bromide; DMEM, Dulbecco's Modified Eagle Medium; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; GMO, glycerol monooleate; GRAVY, grand average of hydropathicity index; LC-MS, liquid chromatography-mass spectrometry; Liss Rhod PE, 1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)(ammonium salt); MW, molecular weight' PBS, phosphate buffer saline; PC, 1,2-diacyl-*sn-glycero*-3-phosphocholine; PEO-PPO-PEO, poly (ethylene oxide) poly (propylene oxide)-poly (ethylene oxide); pI, isoelectric point; SAXS, small angle X-ray scattering; SP-A, surfactant protein A.

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https://doi.org/10.1016/j.jcis.2023.03.048
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1. Introduction

Pulmonary delivery of therapeutics provides non invasive and localised treatments for lung diseases such as, asthma, chronic obstructive pulmonary diseases (COPD) and respiratory infections. Of recent and growing interest is the intracellular delivery of ther apeutics (e.g. mRNA, peptides and antimicrobials) to targeted cells for their therapeutic action in the cytoplasm or organelles [1]. To overcome the barriers for intracellular delivery of biologics, nanoparticles are employed as carriers to efficiently deliver thera peutics to the sub cellular sites of interest, thus allowing mole cules to better exert their activity. However, the clinical translation of nanoparticles enabling intracellular drug delivery to the lung remains challenging due to limited understanding of nanoparticle interactions with pulmonary cells in a biologically relevant environment (i.e. the alveolar barrier, presence of pro teins, mucus and air flow).

In harnessing the potential of nanocarriers for intracellular drug delivery, lipid based nanoparticles are of high interest due to their resemblance to the structure of cellular membranes. Liposomes are one of the most versatile drug delivery systems explored due to their biodegradable and biocompatible properties [2]. Liquid crys talline lipid nanoparticles with non lamellar structures, have emerged in recent years as a promising alternative to liposomes due to their high drug loading capacity and ability to offer better stability of cargo molecules [3,4]. Nanocarriers composed of the bicontinuous cubic mesophase (cubosomes), are synthesised by dispersion of bulk lipid phases and as a newly emerging platform for drug delivery, have so far been investigated for the delivery of antimicrobial agents and chemotherapeutics [5 7]. While pre liminary findings are promising, further mechanistic investigations on their interaction with biological systems are necessary for their optimal use as drug delivery carriers.

Post dosing and prior to exposure to cells, nanoparticles first encounter and strongly interact with native proteins that adsorb to their surface, forming a protein corona in a biological environ ment [8]. The presence of protein corona on the surface of nanoparticles gives them a new and unique identity and can have a profound impact on their cellular interaction and intracellular delivery [9,10]. Tedja et al. demonstrated an increase in overall nanoparticle uptake and two phase uptake kinetics for serum trea ted titanium dioxide nanoparticles compared to non serum treated counterparts, suggesting clear implications of biological compo nents on internalisation [11]. A study by Raesch et al. explored the proteomic and lipidomic profile of polyethylene glycol (PEG), poly (lactic *co* glycolic acid) (PLGA) and lipid nanoparticles in native porcine lung surfactant which showed the protein corona to be significantly different across the particle types and was more important for regulating cellular interactions than the lipid corona [12].

The influence of protein corona on lipid nanoparticle interac tions with pulmonary cells has not been widely investigated. Alve olar macrophages are crucial for regulating homeostasis and immune response in lungs, and can exist as host cells for patho genic bacteria, therefore presenting as a target for treatment of infectious diseases (e.g. tuberculosis) [13]. They are also a target for other diseases such as COPD and pulmonary inflammation, such as silicosis [14]. Due to the inherent capabilities of macro phages to scavenge nanoparticles, strategies to avoid their recogni tion are also desired when targeting other types of cells, such as epithelial cells for pulmonary cancer. Attributing to the different function and structure of pulmonary cells (macrophages and epithelial cells), their interaction and subsequent internalisation of nanoparticles can be varied. Conversely, the structure of nanoparticles can be engineered to target cells of interest by alter ing their protein adsorption behavior.

In this study, we aim to investigate the dynamics of interactions of liposomes and cubosomes in two cell lines relevant to pul monary delivery: RAW264.7 murine macrophages and A549 basal epithelial cells. We further evaluated the influence of protein cor ona from murine bronchoalveolar lavage fluid (BALF) on nanopar ticle uptake. The proteins adsorbed on the surface of the nanoparticles were characterized to improve the current under standing on nanoparticle protein mediated interaction in pul monary cells. We hypothesised that differences in lipid nanoparticle composition and structure would influence their binding affinity to native proteins and surfactant molecules, thus altering their interaction across different cell lines. The findings presented in this study provide new insights into facilitating effi cient nanoparticle uptake and can help drive the development and optimization of lipid based nanoparticles for intracellular delivery of therapeutics for pulmonary applications.

2. Materials and method

2.1. Materials

1,2 dioleoyl *sn glycero* 3 phosphoethanolamine N (Cyanine 7) (Cy7 PE), 1,2 dioleoyl *sn glycero* 3 phosphoethanolamine N (lissa mine rhodamine B sulfonyl) (ammonium salt) (Liss Rhod PE) and dimethyldioctadecylammonium bromide (DDAB) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 1,2 diacyl *sn glycero* 3 phosphocholine (PC), tri block copolymer, poly (ethylene oxide) poly (propylene oxide) poly (ethylene oxide) (PEO PPO PEO), (Pluronic F 127), propylene glycol and phosphate buffer sal ine tablets were purchased from Sigma Aldrich (Castle Hill, Aus tralia). Glyceryl monooleate (Myverol 18 99 K, part number: 5D01253) was purchased from Kerry Ingredients and Flavors (Egham, UK). Methanol and chloroform were obtained from Merck Millipore (Bayswater, Australia).

2.2. Synthesis of lipid based nanoparticles

2.2.1. Liposomes

DDAB and PC were utilized at a 1: 1 M ratio with total lipid con centration of 0.8 μg/mL and fabricated using the microfluidics device NanoAssemblr® Benchtop (Precision NanoSystems Inc., Vancouver). Briefly, lipids were dissolved in methanol and pumped as the organic phase through the first inlet of a microfluidic chip, while phosphate buffer saline (PBS) (10 mM, pH 7.4) was used as the aqueous phase and injected through the second inlet. Lipo somes were synthesised at a total flow rate of 12 mL/min and the ratio of organic to aqueous phase was 1:3, with mixing process facilitated by herringbone structures of the microfluidic cartridge. The collected liposomes were stirred overnight to remove any residual methanol from the mixture. To synthesise fluorescent liposomes, Cy7 PE was incorporated in the lipid stock at a ratio of 1:2000 (dye to lipid).

2.2.2. Liquid crystalline nanoparticles (cubosomes)

Cubosomes were formed via hydrotrope dilution method as previously described by Thorn et al. [5]. Glyceryl monooleate (100 mg), Pluronic F 127 (F127) (15 mg) and propylene glycol (270 mg) (equivalent to molar ratio (%) of 24.5: 73.6: 1.9), were dissolved in 10 mL of chloroform. A stream of nitrogen gas was introduced to evaporate the chloroform to form a lipid layer. 100 μL of PBS was added to the lipid layer, which was further diluted to 5 mL with Milli Q water and vortexed for 3 min to form

a white emulsion. Cy7 PE at a ratio of 1:2000 (dye to lipid) was added to the chloroform mixture to synthesise fluorescently labelled cubosomes.

2.3. Nanoparticle characterisation

The hydrodynamic diameter of the nanoparticles was deter mined via dynamic light scattering using Zetasizer Nano ZS (Mal vern, Worcestershire, UK). Nanoparticles were diluted in Milli Q prior to measurements at 25 °C. Analysis was also conducted for nanoparticles diluted in media with and without proteins at 37 °C. To measure zeta potential, particles were diluted in 10 mM NaCl and measured accordingly via phase analysis scatter ing. Data acquisition and processing were performed using the built in software, Zetasizer Software v7.12.

2.4. Bronchoalveolar lavage fluid (BALF) collection

The collection of BALF from healthy euthanized scavenged rat were collected using a modified protocol as described previously [15]. Briefly, the trachea was cannulated with a catheter and both lungs were lavaged using 0.9% sodium chloride containing 0.6 mM EDTA. The BALF were retrieved after a 30 s dwell time and the pro cedure was repeated three times. BALF collected from 10 rats were pooled and centrifuged at 300g for 10 min. The supernatant was collected and stored at −80 °C till further use.

2.5. In vitro cellular uptake and toxicity studies

2.5.1. Cell culture

RAW 264.7 murine macrophages from American Type Cell Cul ture (ATCC TIB 71™) were grown in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific, Scoresby, VIC, Aus tralia), supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin streptomycin (both purchased from Thermo Fisher) at 37 °C in 5% CO_2. A549 lung epithelial cells (ATCC CCL 185™) were cultured in F 12 K Medium (also from ThermoFisher), supplemented with 10% FBS and 1% v/v penicillin streptomycin at 37 °C in 5% CO_2. Both cells were passaged at 80 90% confluency and following experimental details are in accordance with MIRIBEL guidelines [16].

2.5.2. Cellular toxicity

The cellular toxicity of liposomes and cubosomes in presence and absence of BALF was evaluated in RAW 264.7 and A549 cell lines via MTT assay. Cells were seeded in a 96 well plate at a den sity of 1×10^4 cells per well and incubated overnight at 37 °C in 5% CO_2. On the next day, cells were washed with PBS and the nanopar ticles, dispersed in either DMEM or F 12 K only, i.e. without FBS were added at a lipid concentration range of 12.5 to 100 μg/mL. Nanoparticles pre incubated in 10% or 25% BALF for an hour at 37 °C were also added to the cells at a similar concentration range. Cells treated with nanoparticle free media and 1% Triton X were used as negative and positive controls respectively. After 24 h of incubation, the cells were washed thrice with PBS and were incu bated with MTT stock solution (5 mg/mL in PBS) for another 4 h. DMSO was added to all wells to dissolve the MTT formazan using an orbital shaker for 15 min. The absorbance reading was then measured at 540 nm. The relative absorbance of treated cells to negative controls (untreated cells) was used as a relative measure of cellular viability.

2.5.3. Flow cytometric analysis

Quantification of cellular uptake was performed via fluores cence activated cell sorting (FACS) analysis. Cells were seeded at a density of $1x10^5$ cells/well in a 12 well plate and incubated for 24 h at 37 °C in 5% CO_2. The cells were washed with PBS and 1 mL of nanoparticles diluted in media (without FBS) were added at a lipid concentration of 50 μg/mL. After incubation for the desired time, the cells were washed thrice with PBS. A549 cells were detached using Trypsin EDTA, whereas RAW 264.7 cells were harvested by gently scraping the wells. The cells were fixed with 4% paraformaldehyde and diluted in FACS buffer prior to analysis. Nanoparticle uptake was quantified using BD LSRFortessaTM (San Jose, CA, USA) with a minimum of 10, 000 events recorded for each sample. The internalisation of nanoparticles in both cell lines, RAW 264.7 and A549 were quantitatively measured based on the shift in fluorescence signal of Cy7 relative to untreated cells.

For BALF dependent uptake, nanoparticles were incubated in media without FBS, supplemented with either 10% or 25% murine BALF for an hour at 37 °C prior to exposure to cells.

2.5.4. Confocal microscopy

To visually analyse nanoparticle internalisation in cells, lipo somes and cubosomes were fluorescently labelled using Liss Rhod PE. Cells were seeded in a 6 well plate at a seeding density of $3x10^5$ cells/mL and incubated at conditions mentioned previ ously for 24 h. Cells were washed and treated with fluorescently labelled nanoparticles for 4 h, diluted in media only. After 4 h, cells were washed and detached, followed by thin layer cell attachment to a glass slide using Cytospin™ 4 Centrifuge at 300g for 5 min. Cells were fixed in 4% paraformaldehyde for 15 min and washed thrice with PBS. WGA, Alexa Fluor® 633 conjugate (Invitrogen, Carlsbad, CA, USA) diluted in PBS was added to stain the cytoskele ton of cells followed by mounting of cover slip onto the stained cells using ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Scoresby, Australia). Once the sides were dry, cells were imaged using LMS 710 Carl Zeiss confocal microscopy (Ober kochen, Germany) and ZEN Lite 2.6 software was used for image processing.

2.6. Proteomic analysis

2.6.1. Nanoparticle corona formation

To elute adsorbed proteins to nanoparticles from biological fluid, the method described by Docter et al. and Giulimondi et al. was performed with some modifications [17,18]. Liposomes and cubosomes were diluted in PBS to yield a concentration of 100 μg/mL. Nanoparticles were incubated with BALF at an equal volume of nanoparticle to biological fluid for an hour at 37 °C. The mixture was then centrifuged for 20 min at 15, 300g, with temperature maintained at 4 °C to remove unbound proteins, also referred as the "soft corona". Unbound proteins in supernatant were discarded and the pellet containing nanoparticle protein complex were washed thrice with PBS. After washing, 100 μL of lysis buffer was added to the pellet and incubated at 95 °C for 5 min for protein elution from nanoparticles. The mixture was cen trifuged for 15 min at 15, 300g at room temperature. Supernatant was collected and proteins were precipitated by adding four vol umes of ice cold acetone and left overnight at −20 °C followed by centrifugation at 20, 000g for 10 min. Precipitated proteins were washed twice with acetone and centrifuged for 10 min at 15, 000g. After the acetone was removed and the air dried protein pellet was redissolved in 8 M urea/ 50 mM ammonium bicarbonate.

2.6.2. Protein quantification and trypsin digestion

Protein quantification was performed using the Coomassie Plus (Bradford) Protein Assay according to manufacturer's protocol (ThermoFischer Scientific). Protein samples (100 μg) were reduced with 10 mM dithiothreitol in the dark for one hour at room tem perature, followed by alkylation with 15 mM 2 chloroacetamide for 30 min in the dark at room temperature. The samples were fur

ther diluted with 50 mM ammonium bicarbonate to reduce the urea concentration to 0.8 M. Proteins were incubated overnight with 2 μg Trypsin Gold (Promega) at 37 °C and the digestion was stopped by acidification. Sep Pak C18 cartridges (Waters, Milford, MA) were used for sample clean up and the eluted peptides were dried down in a vacuum centrifuge before storage at −20 °C.

2.6.3. LC MS analysis

LC MS analysis was conducted on an EASY nLC 1200 system coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, Bremen, Germany). Peptides (100 ng for nanoparticles and BALF only) were reconstituted in 0.1% formic acid and injected onto a 25 cm fused silica C18 column (75 μm inner diameter) heated to 50 °C. The column was packed with 1.9 μm ReproSil Pur 120 C18 AQ particles (Dr Maisch, Ammerbuch, Germany). Pep tide chromatography was conducted over a 70 minute linear gra dient (3 to 20% acetonitrile in 0.1% formic acid) at a flow rate of 300 nL/min. A FAIMS Pro interface (Thermo Scientific) generated compensation voltages at −50 and −70 V to regulate the entry of ionized peptides into the mass spectrometer. MS scans (m/z 300 to 1500) were acquired at a resolution of 60, 000 (m/z 200) in pos itive ion mode. Multiply charged precursors (minimum threshold of 10, 000 ions) were fragmented with 27.5% normalized higher energy collision dissociation collision energy, with the resulting MS/MS scans measured at resolution of 15, 000. Cycle time were limited to 1.5 s, while the dynamic exclusion duration was set at 40 s.

Raw MS files were analysed using Proteome Discoverer 2.4 (Thermo Scientific). Data were processed with the Sequest HT search engine against UniProt rat database (FASTA file downloaded on 23rd February 2022). Precursor and fragment mass tolerances were 10 ppm and 0.02 Da, respectively. Methionine oxidation (+15.994915), *N* terminal acetylation (+42.010565), *N* terminal methionine loss (131.040485) and *N* terminal methionine loss and acetylation (89.02992) were set as dynamic modifications, with cysteine carbamidomethylation (+57.021464) designated a static modification. Protein and peptide false discovery rates were both set at 1%.

2.6.4. Proteomic analysis

The abundances of proteins were automatically calculated within each sample following precursor ions quantification approach. The molecular weight (MW) and isoelectric point (pI) of proteins were obtained from Proteome Discoverer 2.4. Grand average of hydropathicity index (GRAVY) score of individual pro teins were calculated using the ProtParam tool on Expasy server [19]. The molecular function and biological process of proteins were classified using PANTHER classification system [20,21].

2.7. Statistical analysis

All data are presented as mean ± standard deviation and exper iments were conducted in triplicate (n = 3). Results were statisti cally analysed using two way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test with significance level of α = 0.05 using GraphPad Prism (version 9.0.0) (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Synthesis and characterisation of lipid nanoparticles

Liposomes and cubosomes of similar size and zeta potential (Table 1) were systematically synthesised to investigate the impact of lipid composition and structure on cellular uptake. The mean hydrodynamic diameter of both formulations measured using dynamic light scattering were in the range of 200 230 nm. Lipo somes composed of phosphatidylcholine (PC) with the incorpora tion of cationic lipid dimethyldioctadecylammonium bromide (DDAB) were successfully fabricated using a microfluidic tech nique, which allows uniform production of formulation in a highly controllable manner compared to conventional methods [22]. The zeta potential of liposomes was near neutral (7.9 ± 0.5 mV) despite the inclusion of DDAB, attributed either to the structural organisation of the lipid or their conductivity in the diluent of mea surement, i.e.10 mM sodium chloride. Previous studies have demonstrated negatively charged liposomes with inclusion of cationic lipids due to either shielding of charge by a neutral lipid bilayer or the presence of the cationic lipid within the liposomal structure instead of at the surface of the particle [23,24]. Addition ally, liposomes composed of DDAB and PC alone had a zeta poten tial of −3.5 mV and −26.1 mV respectively, thus resulting in an overall near neutral charge of the liposomes (Table S1). Further more, this suggests the liposomes can avoid any charge dependent toxicity effect as previously reported with cationic lipids [25]. Cubosomes of similar size and zeta potential were fabricated from glycerol monooleate (GMO) with the incorporation of Pluronic F127 as a stabilizer and propylene glycol as a co solvent. The expert scientific community (including the authors) have exten sively investigated the structure of liquid crystal lipid nanoparti cles composed of GMO and stabilized by Pluronic F127; and identified the formation of bicontinuous cubic phase in excess water [26 28]. Of direct relevance for the current report, a study by Thorn et al. demonstrated the inversion of the hexagonal liquid crystal phase structure of cubosomes with no drug to a bicontinu ous cubic phase structure upon dilution in media for in vitro stud ies using small angle X ray scattering (SAXS) [29,30]. These cubosomes were identical to those used in this study, hence their structure is justifiably considered to be of bicontinuous cubic phase in subsequent cellular studies. Additionally, Spicer et al. have illustrated the formation of cubic phase particles using the hydrotrope approach as utilised in this study[31].

Table 1
Physicochemical properties of lipid-based nanoparticles in cell media only, or in presence of BALF. Samples were diluted in phosphate buffer saline for size measurement and 10 mM NaCl for zeta potential measurement. Data is represented as mean ± S.D for three individual measurements.

	Particle size (nm) (Z-average)	PDI	Zeta potential (mV)
Liposomes (DDAB: PC)			
Protein-free media	203 ± 10	0.27 ± 0.01	−7.9 ± 0.5
10% BALF	247 ± 7	0.36 ± 0.05	−11.6 ± 3.7
25% BALF	347 ± 21	0.36 ± 0.02	−16.5 ± 1.6
Cubosomes			
Protein-free media	223 ± 4	0.11 ± 0.05	−7.4 ± 1.4
10% BALF	285 ± 13	0.31 ± 0.05	−16.4 ± 3.5
25% BALF	398 ± 21	0.42 ± 0.02	−18.7 ± 2.1

3.1.1. Influence of bio media proteins on physicochemical properties of nanoparticles

Proteins from biorelevant media are known to interact with nanoparticles, giving them a new biological identity, different to their synthetic identity upon fabrication [9]. The size and zeta potential of the liposomes and cubosomes in the presence of 10% or 25% BALF are summarized in Table 1. The size of the liposomes increased from ∼ 200 nm to 350 nm in the presence of BALF. Sim ilarly, cubosomes increased from ∼ 220 nm to 400 nm in media containing a higher concentration of BALF. The zeta potential also increased in negativity from approximately −7 mV to −16 and −18 mV for the liposomes and cubosomes respectively. The

nanoparticles' increase in size and surface potential is indicative of a protein corona present on their surface. The majority of proteins are negatively charged at physiological pH, hence the observed increase in negative zeta potential is in line with previous observations [32].

3.2. Cytotoxicity of nanoparticles

The cytotoxicity of the lipid formulations was analysed using MTT assay for both, RAW 264.7 murine macrophages and A549 alveolar epithelial cells prior to conducting uptake studies. Both cell types retained their viability above 80% following a 24 h incubation in presence of nanoparticles over the concentration range of 12.5 100 µg/mL (Fig. 1). Thus, the nanoparticles were classified non cytotoxic at the concentration of interest for subsequent studies. At similar concentrations, the nanoparticles were also shown to be non cytotoxic in presence of 10% or 25% BALF (Figure S1).

3.3. Cellular uptake behavior of lipid nanoparticles in protein free media

Cellular uptake studies in protein free media (following 2 and 4 h incubation periods), showed liposomes with the highest internalisation in epithelial cells, i.e. ~ 9 times greater internalisation than cubosomes (Fig. 2). Confocal microscopy (Fig. 3) qualitatively confirmed the flow cytometry results obtained, with rhodamine labelled liposomes (red) localised in the cytoplasm of the epithelial cells after 4 h and significantly less fluorescence observed for cubosomes. This may be attributed to the electrostatic attraction between the liposomes and negatively charged cell surface, resulting from the incorporation of the cationic lipid, DDAB [33]. The incorporation of cationic lipid to facilitate intracellular delivery of siRNA, microRNA or chemotherapeutic agents in A549 has been previously demonstrated [34 36], thus correlating to the findings here.

Interestingly, cubosomes showed negligible uptake by epithelial cells, demonstrating significant influence of nanoparticle composition and structure on cellular internalisation. A study by Patil et al. reported high internalisation of cubosomes, thus improving the therapeutic effect of encapsulated bedaquiline against tumor growth using A549 [37]. However, this may have been facilitated by the positively charged zeta potential of cubosomes, reported as 35.4 ± 2.3 mV, potentially from the encapsulation of bedaquiline, a drug molecule with an amine functional group with pKa of 8.91 [38]. The authors did not report the zeta potential of cubosomes without drug, which limits direct comparison with the current study. In addition, A549 epithelial cells produce a large amount of lecithin which may alter their membrane structure and fluidity, possibly limiting the internalisation of cubosomes via fusion dynamics as previously reported in a lipid bilayer model cell membrane and in a fibroblast cell line [27,39,40].

A converse trend was observed for nanoparticle internalisation in RAW264.7 macrophages (Fig. 2b), i.e. cubosomes displayed a 3 fold higher internalisation (at 4 h) in comparison to liposomes. Macrophages are generally capable of phagocytosing or "scavenging" nanoparticles in a non specific manner [41]. However, recognition and uptake are based on several factors, including (i) opsonization of nanoparticles by proteins for recognition, (ii) presence of inflammatory signals enabling phagocytic activity of nanoparticles and (iii) recognition of nanoparticles by receptors present on macrophages. The absence of proteins in this experimental condition thus, diminishing the opsonization process is considered to reduce the uptake of liposomes in macrophages [42]. Furthermore, liposomes with a neutral lipid (PC) and charge have previously shown low cellular uptake in macrophages [43]. This is in line with observations from other studies, whereby the ratio of lipid mix, including cationic lipids can influence the interaction of the liposomes with macrophages, resulting in a lower uptake [44]. In addition, previous studies have shown the targeting of macrophage receptors to influence the time dependent uptake of nanoparticles, with nanoparticles internalised via scavenger receptors requiring significantly longer time than nanoparticles internalised via mannose or Fc receptors [45]. Incubation of liposomes in RAW264.7 over a 24 h period showed a gradual increase in internalisation of nanoparticles (Figure S2), perhaps suggesting their recognition and uptake was through the scavenger receptors.

3.4. Influence of protein interactions on cellular internalisation of nanoparticles

Since the interaction of nanoparticles with cells is influenced by the presence of biomolecules on their surface, further uptake studies were undertaken in the presence of BALF which contains native proteins and surfactant molecules present within the lung environment and is a relevant biological fluid for understanding pulmonary delivery of nanoparticles.

The influence of proteins in BALF on cellular internalisation of liposomes and cubosomes after a 4 h incubation is illustrated in Fig. 4. In epithelial cells, the presence of proteins significantly promoted cubosome uptake after a 4 h incubation, with approximately 40% cellular uptake in presence of 10% BALF, further reaching 90% of uptake at 25% BALF concentration (Fig. 4a). The internalisation of liposomes in epithelial cells (~80%) was also enhanced to above 90% at 25% BALF concentration, although no sig

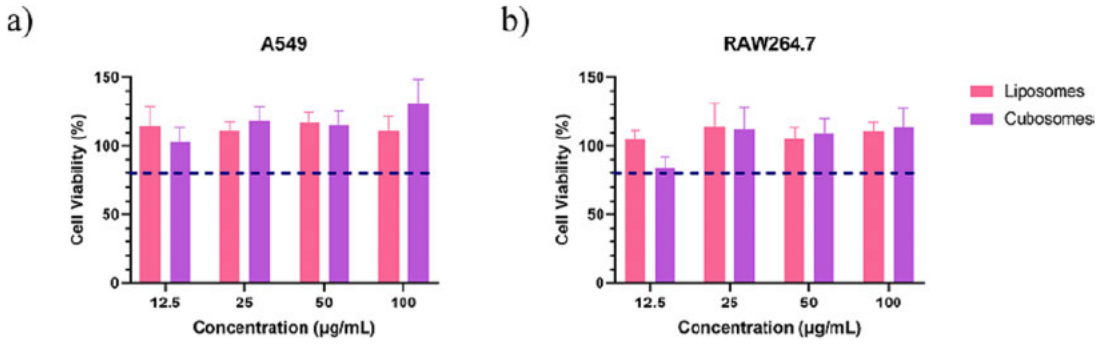


Fig. 1. Cell viability profiles of liposomes and cubosomes in (a) A549 epithelial cells and (b) RAW264.7 macrophages at lipid concentration in the range of 12.5–100 µg/mL over a 24 h incubation period. (mean ± SD, n = 3).

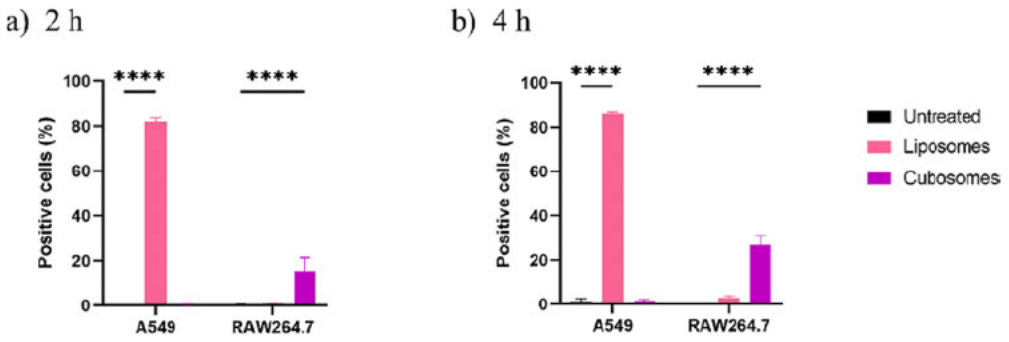


Fig. 2. Uptake of lipid-based nanoparticles quantified by flow cytometry following an incubation period of (a) 2 h and (b) 4 h (mean ± SD, n = 3).

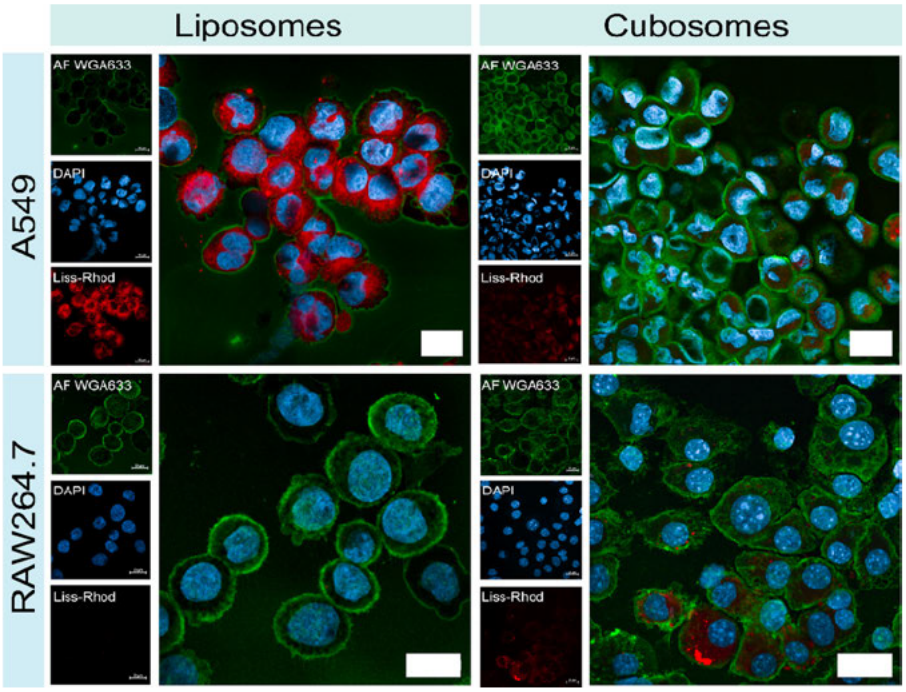


Fig. 3. Laser scanning confocal microscopy (LCMS) images showing internalisation of nanoparticles in A549 (top) and RAW264.7 (bottom), in protein-free media. Nanoparticles were fluorescently labelled with Liss-Rhod (red), cells were stained with Alexa Fluor 633 (green) for cellular cytoskeleton and DAPI (blue) for nucleus. Scale bar represents 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nificant differences was observed in cell media supplemented with 10% BALF (Fig. 4b). Cubosomes exhibited a similar trend in macrophages, demonstrating approximately 2 to 3 fold increase in uptake in presence of 10% and 25% BALF, respectively (Fig. 4b). However, the 10% uptake of liposomes in macrophages as observed in cell media only was diminished in presence of proteins, independent of BALF concentration. Cellular receptors recognize proteins present on nanoparticles, influencing their interaction and subsequent internalisation using different cellular mechanisms [46]. Thus, the proteins on nanoparticles, together with receptors present on cells can enhance or hinder their internalisation.

To further investigate the differences in nanoparticle uptake kinetics in presence of proteins, a time dependent cellular uptake over 6 h incubation, with a 2 h interval was conducted (Fig. 5).

Liposomes were internalized rapidly in epithelial cells, independent of time or presence of proteins (Fig. 5a). In macrophages, liposomes showed a two phase uptake profile in presence of proteins, with a rapid uptake up to ~ 50% within 2 h of incubation, followed by rapid exocytosis at 4 h and 6 h (Fig. 5b). A similar phenomenon has been previously reported for titanium dioxide (TiO_2) nanoparticles in presence of serum proteins, in which, the authors reported nanoparticles localised in recycling endosomes may undergo rapid exocytosis from cells [11]. Exocytosis of nanoparticles in macrophages has also been reported for chitosan nanoparticles within 4 to 24 h, corroborating our findings here [47]. Furthermore, macrophages are reported to metabolise and recycle lipids efficiently and given the close resemblance of liposomes to endogenous lipid vesicles, this may partly explain the rapid uptake and

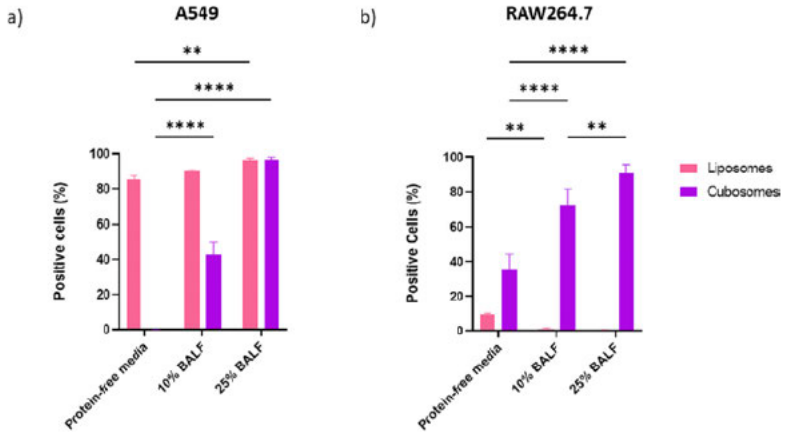


Fig. 4. Cellular internalisation of nanoparticles in presence of proteins present in BALF following 4 h incubation period in two cell types, (a) A549 lung epithelial cells and (b) RAW264.7 macrophages (mean ± SD, n = 3).

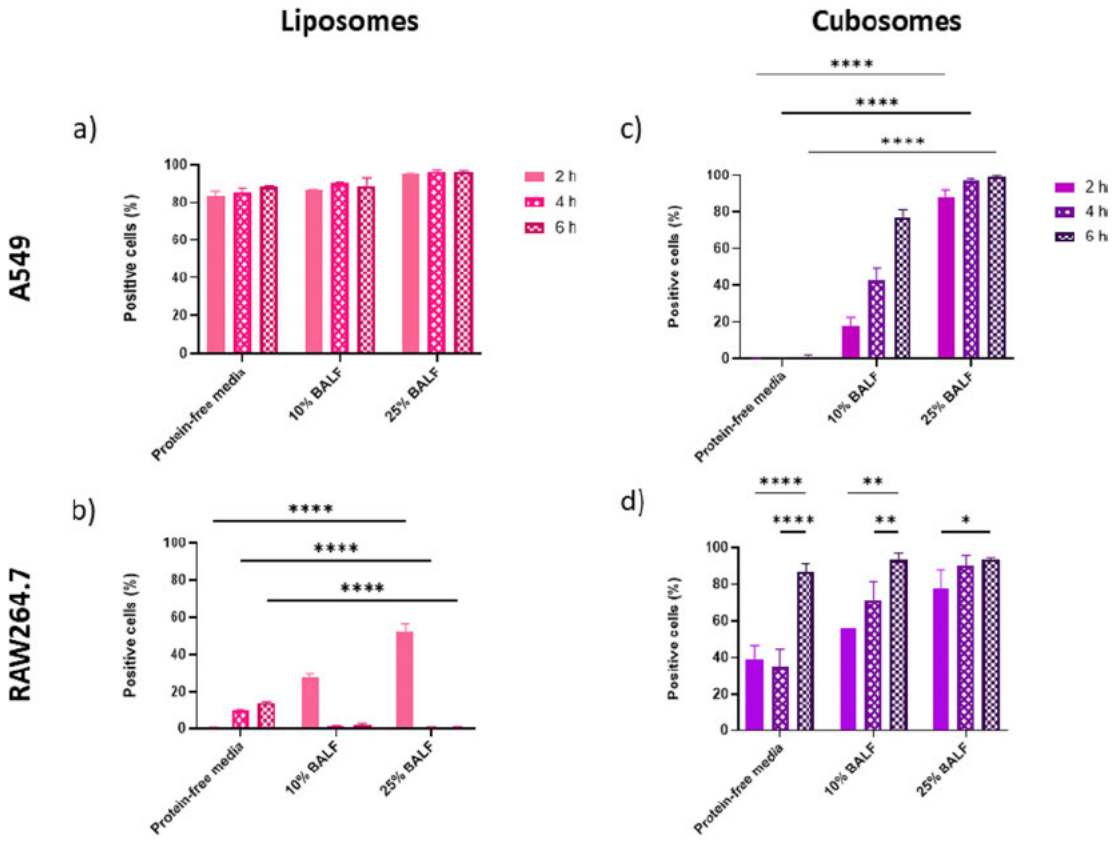


Fig. 5. Time-dependent uptake of (a) liposomes in A549, (b) cubosomes in A549, (c) liposomes in RAW264.7 and (d) cubosomes in RAW264.7 (mean ± SD, n = 3).

exocytosis profile in Fig. 5c [40,48,49]. This phenomenon was not observed for liposomes without BALF treatment, highlighting the role of protein corona in mediating interaction and fate of nanoparticles in cells.

Cubosomes showed a time and concentration dependent uptake in both cell lines as illustrated in Fig. 5c and Fig. 5d. At a 10% BALF concentration, cubosomes had a 2 fold higher uptake at 4 h in comparison to 2 h, reaching 40% internalisation. This

was further increased to 80% after a 6 h incubation, i.e. a 4 fold increase compared to 2 h. A higher concentration of BALF resulted in a rapid uptake of cubosomes within 2 h, i.e. achieving 80% positive cells with minimal changes at 4 h and 6 h. In macrophages, cubosomes also showed a time dependent uptake, most notable for protein free media and 10% BALF. At 6 h, nanoparticles in absence of proteins had an internalisation of 75%, increasing to 90% with presence of BALF. A previous study by Strachan et al. demonstrated a time dependent phagocytosis of cubosomes in macrophages consistent with the above findings [40]. The presence of protein may be further enhancing internalisation of cubosomes in macrophages by activating other internalisation mechanisms such as endocytosis, resulting in a faster internalisation rate compared to bare nanoparticles (without BALF) (see Fig. 8 for illustration).

3.5. Identification and characterisation of proteins from corona of nanoparticles

Since the uptake behavior of nanoparticles was distinctly dependent on cell type and nanoparticle composition in presence of protein, a label free quantitative proteomics analysis using liquid chromatography mass spectrometry (LC MS) was conducted to analyse the individual nanoparticle corona. The nanoparticles were incubated in BALF for 1 h prior to proteomic analysis, ensuring protein corona of nanoparticles was similar as to when performing cellular studies. The top 100 most abundant proteins were classified according to their physical properties, i.e. (a) molecular weight (MW), (b) isoelectric point (pI) and (c) grand average of hydropathicity index (GRAVY) score as a measure of protein hydrophobicity. This enabled investigation of nanoparticle type on bio physical adsorption of proteins (Fig. 6).

The MS data indicates a unique account of protein corona for the cubosomes and liposomes, in comparison to BALF only. A larger proportion of low MW proteins (<40 kDa) were adsorbed to cubosomes in comparison to liposomes (Fig. 6a). The intercalated lipid bilayers with water channels in cubosomes are hypothesised to present a greater surface area for the adsorption of low MW proteins compared to the simple lamellar structure of liposomes [50]. The pI of proteins can be correlated with their subcellular localisation in cells, e.g., acidic proteins are generally found in the cytoplasm and lysosome while basic proteins can be found in the nucleus or mitochondria of cells [51,52]. Interestingly, liposomes had a larger proportion of acidic proteins with pI below 7 than for cubosomes (Fig. 6b). The exocytosis profile of liposomes in macrophages (Fig. 5b), together with the higher adsorption of acidic proteins may imply that liposomes are localised in lysosomes, due to their involvement in regulation and homeostasis of lipids in macrophages [53]. However, this requires further investigation and given the cell dependent internalisation, the same cannot be extrapolated for observations in epithelial cells. A negative GRAVY score is a representation of more hydrophilic proteins and in Fig. 6c, cubosomes showed approximately 10% higher adsorption of hydrophilic proteins than liposomes. Ashby et al. have demonstrated protein adsorption to be largely dependent on hydrophobic forces, with hydrophobic nanoparticles strongly interacting with more hydrophobic proteins to reduce interaction with water molecules in a biological fluid [54]. The presence of F127, providing a hydrophilic coating to the cubosomes is likely to have resulted in the attachment of more hydrophilic proteins in comparison to liposomes. Although, hydrophobic interaction and hydrogen bonding with proteins have also been reported previously and their interaction is dependent on several factors, such as, PEG concentration and structural arrangement of both molecules [55,56].

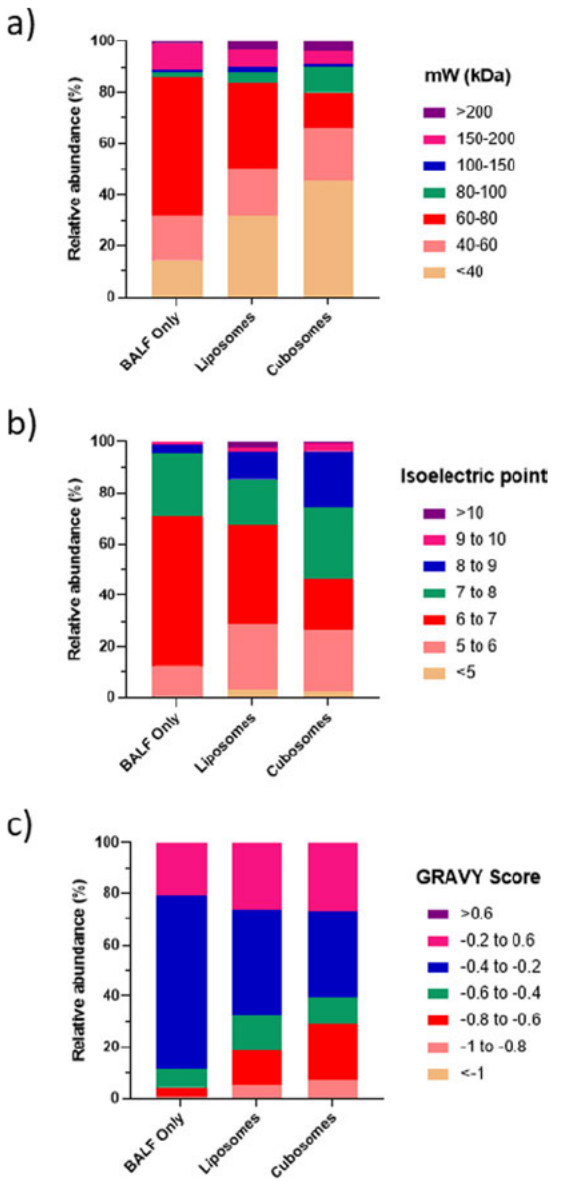


Fig. 6. Relative abundance of proteins classified by their physical characteristics as determined in lipid nanoparticle corona by; (a) molecular weight, (b) isoelectric point and (c) GRAVY score.

The proteins were further categorized based on their molecular function and biological process as depicted in Fig. 7. Briefly, the relative abundance of proteins from nanoparticle corona is not similar to protein fingerprint of BALF. This is consistent with previous literature, as they reflect selective enrichment of proteins on nanoparticle surfaces [57]. By comparing the molecular function by means of relative abundance, cubosomes had a higher adsorption of proteins with molecular function regulator (20.7%), catalytic activity (38.7%) and binding activity (30%) in comparison to liposomes (relative abundance for liposomes: 8.3%, 25.9% and 25% respectively) (Fig. 7a). Similarly, by comparing the biological pro

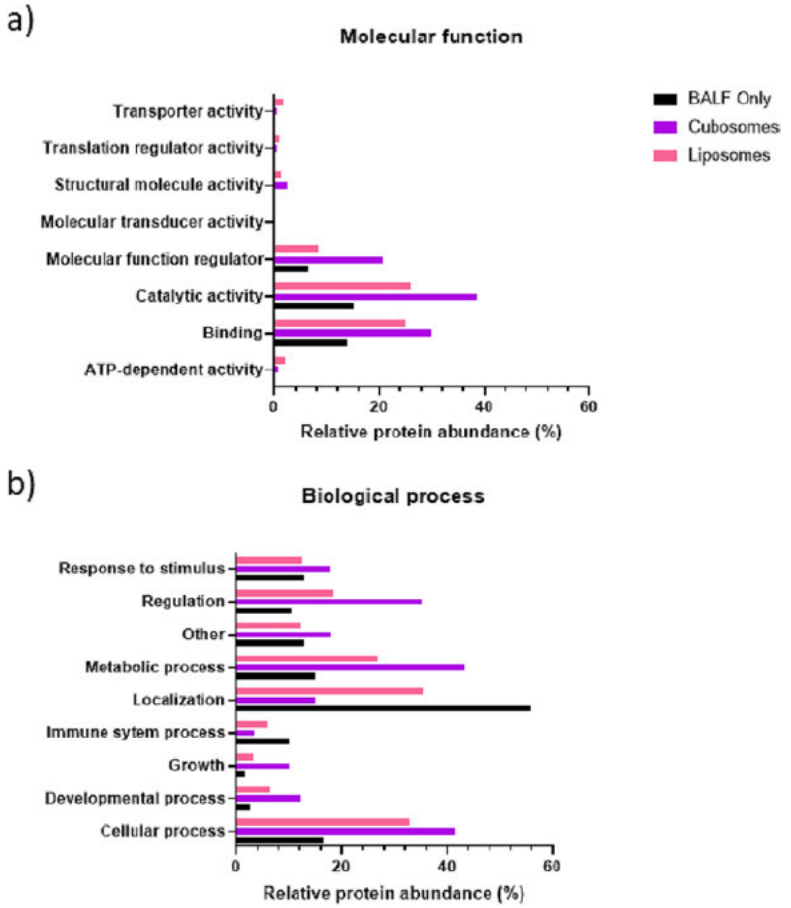


Fig. 7. Comparison of proteins from BALF (serum) only and proteins found on corona of liposomes and cubosomes by means of their relative abundance, classified according to their (a) molecular function and (b) biological process.

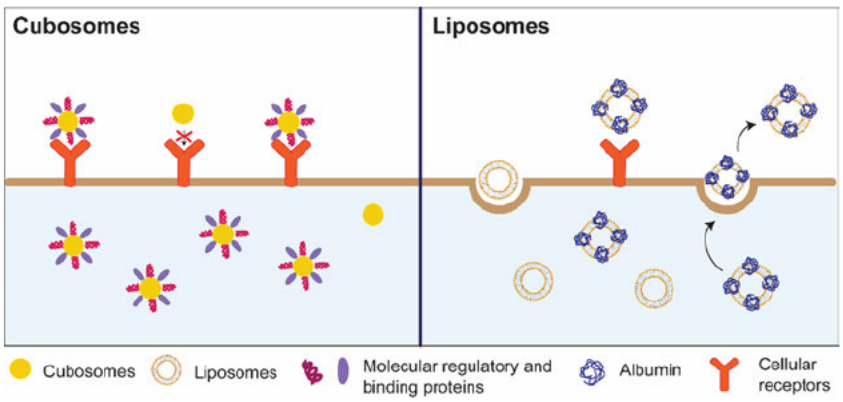


Fig. 8. Schematic illustrating proposed interaction of (left) cubosomes and (right) liposomes with cell membrane. Presence of binding and molecular regulatory proteins on cubosomes increases interaction with cell receptors. Liposomes are rapidly internalized into cells followed by exocytosis in macrophages.

cess of proteins, cubosomes had a higher abundance of proteins belonging to response to stimulus (17.7%), regulation (34.9%), metabolic process (43.3%) and cellular process (41.3%) (respective values for liposomes: 12.4%, 18.5%, 26.8% and 32.6%) (Fig. 7b). Liposomes had a higher percentage of proteins involved in localisation (35.3%) compared to cubosomes (15.0%). The enrichment of binding, cellular process, and regulatory proteins on cubosomes correlates well with higher internalisation in A549 and RAW264.7, as previously observed and illustrated in Fig. 4 and Fig. 5 (see illustration in Fig. 8).

Table 2 summarises the top 10 proteins by abundance isolated from the corona of liposomes and cubosomes. The presence of plasma proteins such as albumin, serotransferrin, hemoglobin and complement C3 may indicate a small percentage of contamination, unavoidable from the invasive procedure for BALF extraction, consistent with previous finding [12]. Interestingly, Bartlett et al. have previously reported similar findings in proteomic analysis of airways fluid and reported some plasma proteins to be normal secretory products by airways epithelia [58]. Although there were similarities in proteins detected for both nanoparticles, their relative abundance were different, further emphasizing the unique protein fingerprints of the specific lipid nanoparticles.

Cubosomes showed a higher enrichment of apolipoproteins than liposomes, with beta 2 glycoprotein, also referred as apolipoprotein H (ApoH) the most abundant protein (13.9%). ApoH is known as a phospholipid binding molecule and their role in respect to cellular internalisation of nanoparticles has been studied extensively [59]. Rapid clearance of liposomes by macrophages has been previously associated with the binding of ApoH and Complement C3 proteins, consistent with findings described here [60,61]. ApoH binding to liposomes was dependent on liposome composition and more prominent to "non self" liposomes, perhaps explaining their selective binding to cubosomes than liposomes. While ApoH has been classified as an opsonin for phagocytic cells, their role in non phagocytic cells needs further investigation [32]. Here, the high abundance of ApoH on cubosomes resulted in their rapid and high internalisation in epithelial cells (Fig. 5c), hence confirming their role as an opsonin. However, the influence of protein corona and cellular internalisation is dynamic, with protein molecular assembly on nanoparticle and protein protein interactions influencing their cellular recognition, hence the findings cannot be based on the involvement of a single protein [62].

On the contrary, albumin was the most abundant surface adsorbed protein for liposomes (Table 2). The preferential attachment of albumin on liposomes can be attributed to the absence of F127, which was incorporated as stabilizer in cubosomes. F127 presence on nanoparticles repels protein interactions and reduces protein fouling, hence reducing albumin adsorption [63]. Albumin adsorption can also reduce non specific adsorption of other proteins, thus improving delivery of nanoparticles to various types of cells, including tumor cells, phagocytes, and epithelial cells [10,64]. While generally categorised as a dysopsonin for macrophages, albumin adsorption to nanoparticles increased the uptake of polymeric nanoparticles in phagocytic cells, showing the influence of albumin conformation, denaturation and folding state as factors regulating macrophage interaction [65]. The high abundance of albumin on liposomes may have resulted in their rapid uptake across both cells lines as observed in Fig. 5. Although, a rapid exocytosis was also observed in macrophages for reasons hypothesised previously. Interestingly, pulmonary surfactant protein A (SP A) was enriched in the protein corona of liposomes compared to cubosomes. SP A interacts with phospholipids and readily binds to lipid nanoparticles [12]. Importantly, SP A is also involved in regulating innate immune response in lungs and involved in opsonization of nanoparticles [66,67]. While experiments in general are conducted with single proteins to decipher their role as opsonin or dysopsonin in cells, these observations albeit useful, may not capture the dynamic of protein corona in whole biological fluid, such as BALF in facilitating cellular interaction. As summarized by Papini et al. conformational modification, binding efficiency, denaturation processes and assembly of other proteins can alter the protein corona interaction in a cell and nanoparticle dependent manner [68].

The observation of differences between interaction of lipid nanoparticles with proteins in a complex biological fluid, subsequently altering their cellular interaction signifies the importance of understanding nanoparticle behavior in biorelevant conditions for their ultimate application as drug delivery carriers. By understanding their interactions using biorelevant media, such as bronchoalveolar fluid and using two distinct cell type, lipid

Table 2
Top 10 most abundant proteins by relative abundance in liposomes and cubosomes. Similar proteins across both nanoparticles are highlighted in bold.

Cubosomes			**Liposomes**		
UniProt Accession	Description (Gene)	Relative abundance (%)	UniProt Accession	Description (Gene)	Relative abundance (%)
P26644	Beta-2-glycoprotein 1 (ApoH)	13.9	P02770	**Albumin (Alb)**	23.7
P24090	**Alpha-2-HS-glycoprotein (Ahsg)**	7.9	P04639	**Apolipoprotein A-I (ApoA1)**	6.8
Q01177	Plasminogen (Plg)	5.1	P12346	Serotransferrin (TF)	3.5
Q99PS8	Histidine-rich glycoprotein (Hrg)	4.5	P01026	**Complement C3 (C3)**	2.8
P04639	**Apolipoprotein A-I (ApoA1)**	4.2	P08427	Pulmonary surfactant-associated protein A (Sftpa)	2.6
P02770	**Albumin (Alb)**	4.0	P12346-2	Isoform 2 of Serotransferrin (Tf)	2.6
P01026	**Complement C3 (C3)**	3.4	P01946	Hemoglobin subunit alpha-1/2 (Hba1)	2.3
D3ZTE0	Coagulation factor XII (F12)	3.1	P11517	**Hemoglobin subunit beta-2**	2.1
P11517	**Hemoglobin subunit beta-2**	3.1	P24090	**Alpha-2-HS-glycoprotein (Ahsg)**	1.9
P05544	Serine protease inhibitor A3L (Serpina3l)	2.3	P02650	Apolipoprotein E (ApoE)	1.8

nanoparticles can be designed for enhanced and targeted intracel lular delivery of drugs and biological molecules in pulmonary cells.

4. Conclusion

This study highlights the influence of lipid nanocarrier type (li posomes versus cubosomes) on interaction with RAW 264.7 macrophages and A549 epithelial cells. Importantly, the influence of protein adsorption in an in vitro lung environment on driving the cellular interaction of lipid based nanoparticles was described. Previous studies have demonstrated the influence of pulmonary surfactant on nanoparticle behaviour and cellular interaction, but mechanistic insight is lacking [12,69]. Here, we synthesised lipo somes and cubosomes of equivalent size and surface charge and quantitatively compared their interaction behaviour with two dif ferent cell types. Cellular uptake was significantly different in pres ence of the protein corona from incubation in bronchoalveolar lavage fluid (BALF). Furthermore, the unique protein fingerprints of liposomes and cubosomes were identified using proteomics, highlighting the role of surface chemistry and composition on reg ulating protein nanoparticle interactions. The presence of proteins, with an enrichment of Apolipoprotein H enhanced uptake of cubo somes in both, RAW264.7 macrophages and A549 epithelial cells in a time and protein concentration dependent manner. An albumin rich corona of liposomes resulted in rapid endocytosis and exocy tosis profile in macrophages, with no changes to their interaction in epithelial cells. This study has advanced knowledge on how nanoparticles can be manipulated for targeted cell delivery under biorelevant conditions and support further design of lipid based nanoparticles for intracellular delivery of therapeutics via the pul monary route.

CRediT authorship contribution statement

Santhni Subramaniam: Conceptualization, Methodology, For mal analysis, Investigation, Data curation, Writing original draft, Visualization. **Paul Joyce:** Writing review & editing, Visualiza tion, Supervision. **Leigh Donnellan:** Investigation, Data curation, Writing review & editing. **Clifford Young:** Investigation, Data curation, Writing review & editing. **Anthony Wignall:** Methodol ogy, Investigation, Writing review & editing. **Peter Hoffmann:** Resources, Writing review & editing, Supervision. **Clive A. Presti-dge:** Resources, Writing review & editing, Visualization, Supervision.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing finan cial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Santhni is grateful for funding support from the Gould Experi mental Science Grant and ATA Scientific Encouragement Award. Dr Nicky Thomas and Dr Chelsea Thorn are kindly acknowledged for their technical guidance and assistance. The authors also acknowledge Bioplatforms Australia, and the State and Federal Governments, which co fund the NCRIS enabled Mass Spectrome try and Proteomics Facility at the University of South Australia.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2023.03.048.

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Supporting Information

Table S1. Surface charge, measured as zeta potential of liposomes composed of DDAB only, PC only and in combination using microfluidic. Measurements were conducted in 10 mM sodium chloride using Zetasizer Nano.

	DDAB only	PC only	DDAB: PC
Zeta potential (mV)	-3.54	-26.1	-7.85

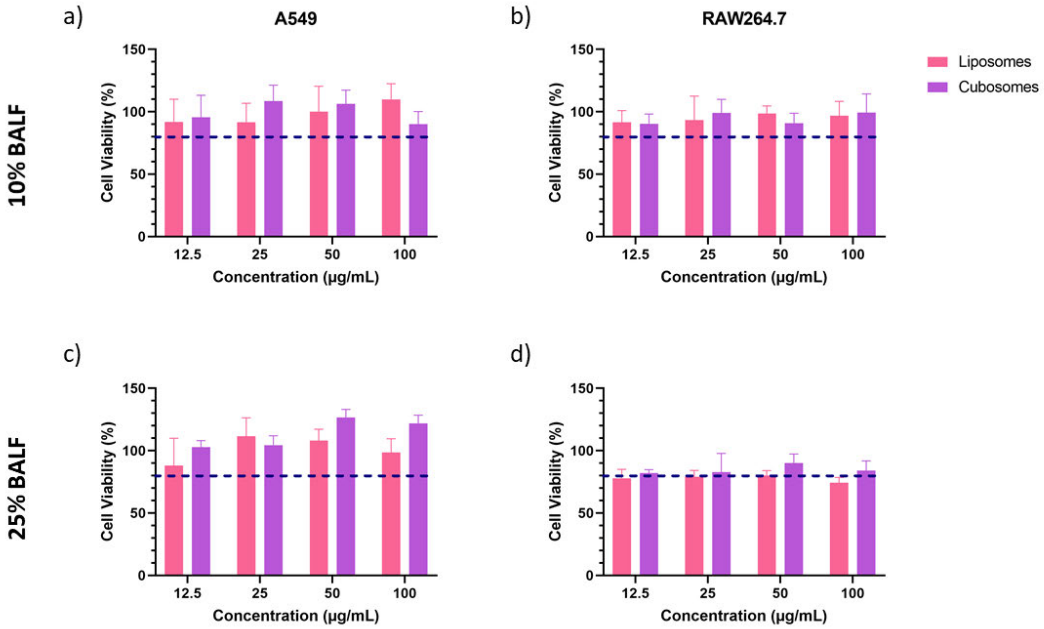


Figure S2. Cytotoxicity of nanoparticles in A549 and RAW264.7, dosed at a concentration range of 12.5-100 µg/mL in cell media supplemented with (top) 10% BALF or (bottom) 25% BALF for 24 h

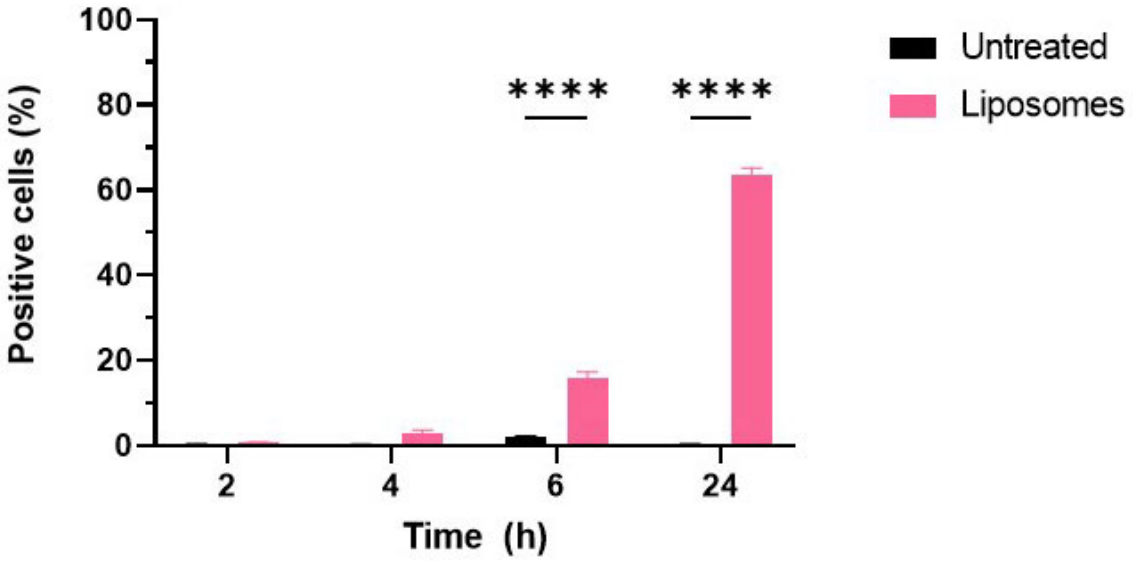
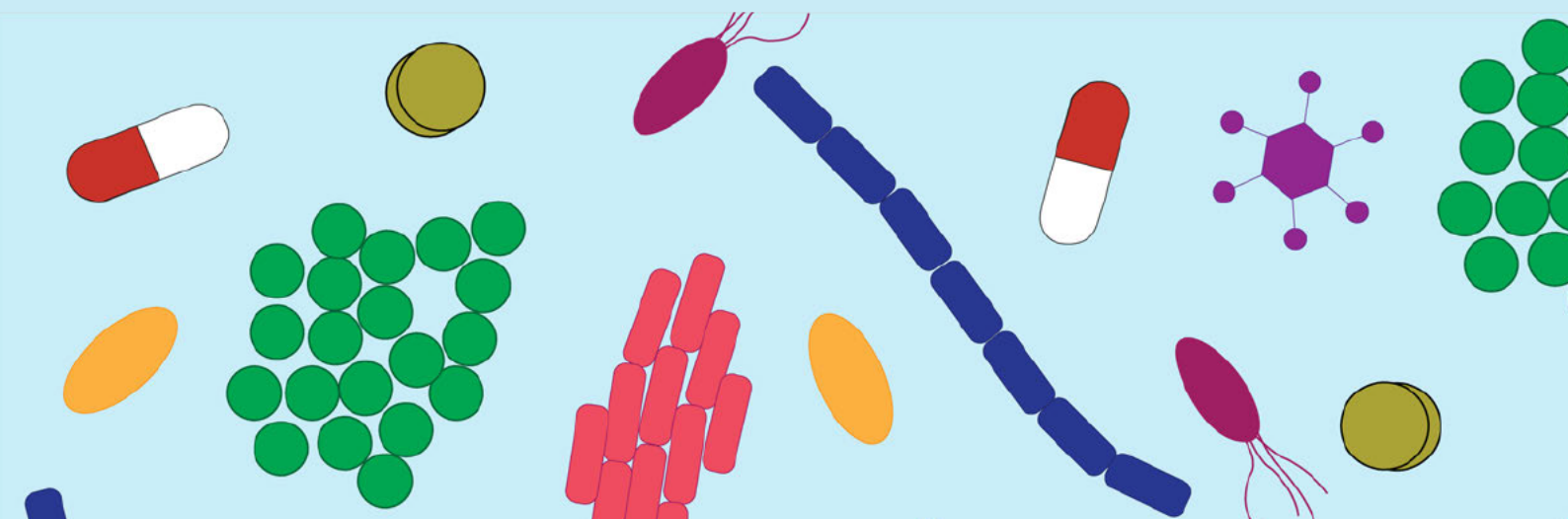


Figure S3. Uptake of nanoparticles, represented as percentage positive cells of DDAB: PC liposomes over 24 h incubation period (mean ± S.D, n = 3).

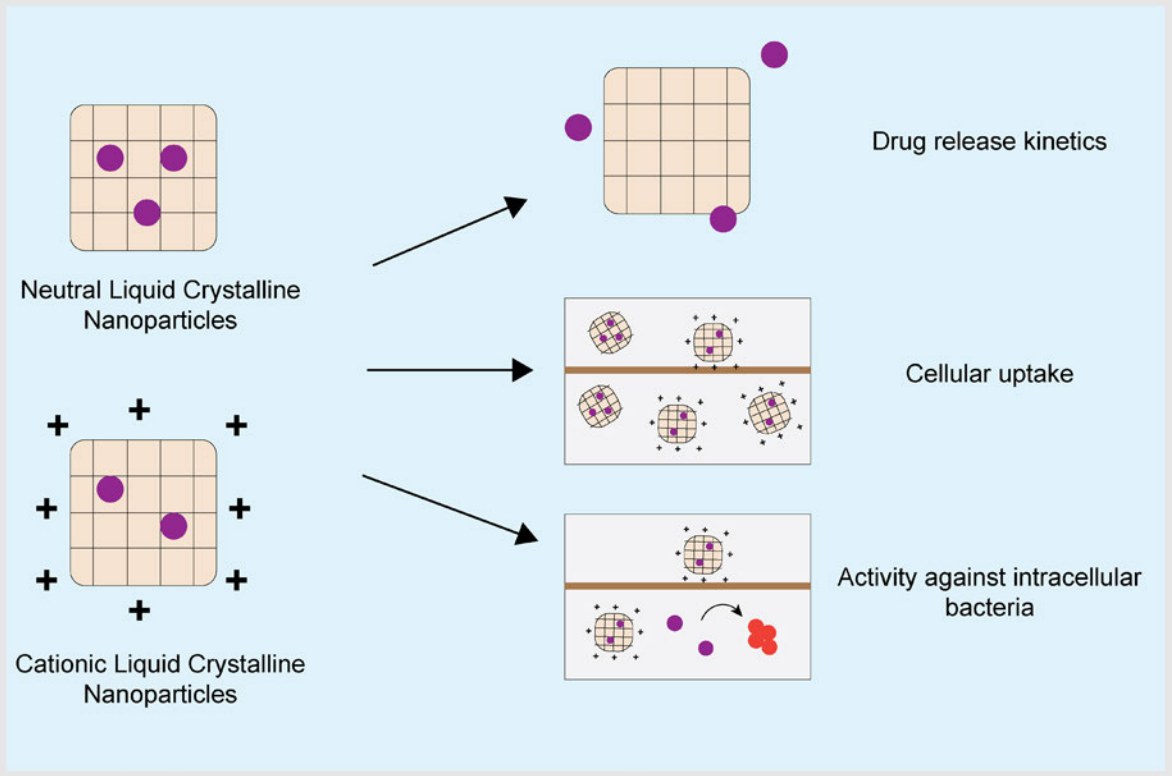
4

LIQUID CRYSTALLINE LIPID NANOPARTICLES IMPROVE THE ANTIBACTERIAL ACTIVITY OF TOBRAMYCIN AND VANCOMYCIN AGAINST INTRACELLULAR *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS*



4 Liquid crystalline lipid nanoparticles improve the antibacterial activity of tobramycin and vancomycin against intracellular *Pseudomonas aeruginosa* and *Staphylococcus aureus*

In this last research chapter, the potential of liquid crystalline nanoparticles (LCNPs) as antibiotic carriers to target two bacterial pathogens in macrophages and lung epithelial cells were further explored. It was hypothesised that the LCNPs can be systemically engineered by incorporating a cationic lipid to their matrix to improve their cellular uptake and antimicrobial performance. The activity of the cationic LCNPs were directly compared to their neutral counterpart for differences in particle morphology, cellular toxicity and uptake, drug release kinetics and antibacterial activity.



International Journal of Pharmaceutics 639 (2023) 122927

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Liquid crystalline lipid nanoparticles improve the antibacterial activity of tobramycin and vancomycin against intracellular *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ARTICLE INFO

Keywords:
Intracellular infection
Liquid crystalline nanoparticles
Macrophages
Epithelial cells
Vancomycin
Tobramycin
S. aureus
P. aeruginosa

ABSTRACT

The intracellular survival of bacteria is a significant challenge in the fight against antimicrobial resistance. Currently available antibiotics suffer from limited penetration across host cell membranes, resulting in suboptimal treatment against the internalised bacteria. Liquid crystalline nanoparticles (LCNP) are gaining significant research interest in promoting the cellular uptake of therapeutics due to their fusogenic properties; however, they have not been reported for targeting intracellular bacteria. Herein, the cellular internalisation of LCNPs in RAW 264.7 macrophages and A549 epithelial cells was investigated and optimized through the incorporation of a cationic lipid, dimethyldioctadecylammonium bromide (DDAB). LCNPs displayed a honeycomb-like structure, while the inclusion of DDAB resulted into an onion-like organisation with larger internal pores. Cationic LCNPs enhanced the cellular uptake in both cells, reaching up to ~ 90% uptake in cells. Further, LCNPs were encapsulated with tobramycin or vancomycin to improve their activity against intracellular gram-negative, *Pseudomonas aeruginosa* (*P. aeruginosa*) and gram-positive, *Staphylococcus aureus* (*S. aureus*) bacteria. The enhanced cellular uptake of cationic LCNP resulted in significant reduction of intracellular bacterial load (up to 90% reduction), compared to antibiotic dosed in its free form; with reduced performance observed for epithelial cells infected with *S. aureus*. Specifically engineered LCNP can re-sensitise antibiotics against both intracellular Gram positive and negative bacteria in diverse cell lines.

1. Introduction

Bacteria have developed a wealth of strategies to succumb activity of current antibiotics, thus rendering clinical therapies ineffective. One such strategy is their ability to survive and persist within the harsh environment of host cells, thereby causing intracellular infections [1–3]. Clinical treatment with antibiotics alone fails to eradicate such bacteria due to their limited penetration across host cells. Thus, even high doses of antibiotics are unable to exert their activity at the site of infection and patients suffer from potential toxicities. In addition, the shortage of new antibiotics creates a bottleneck challenge, urging researchers to repurpose existing antibiotics as an alternative.

A promising approach in overcoming the biological barrier of cellular membranes is by employing nanoparticles to facilitate transport of encapsulated therapeutics across host cell [4,5]. This enables a high antibiotic load to be delivered specifically to the site of infection, increasing their antibacterial activity while minimizing dose-associated side effects. Among the different nanocarriers, lipid-based systems are widely utilized for their biomimetic properties. Of this, liposomes are the most prominent drug delivery systems explored to improve targeting of intracellular bacteria [4]. However, only one liposomal formulation, Arikayce® has been approved by the US Food and Drug administration (FDA) for treatment of intracellular *Mycobacterium avium* complex infections [6]. While liposomes are a promising approach, their simplistic structure limits the ideal antibiotic loading and release kinetics required.

Non-lamellar systems are emerging as the next-generation lipid-based delivery systems, providing benefits over conventional liposomes. Also referred as liquid crystalline nanoparticles (LCNP), they are formed by self-assembly of lipid bilayers in a three-dimensional structure with interconnected water channels [7]. The dispersion of the non-lamellar phases in presence of a colloidal stabilizer forms either the reverse bicontinuous cubic (V_2) or reverse hexagonal (H_2) phases, commonly referred as cubosomes and hexasomes, respectively [8]. The bicontinuous cubic phase can be further categorised based on the symmetrical

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https://doi.org/10.1016/j.ijpharm.2023.122927
Received 23 February 2023; Received in revised form 24 March 2023; Accepted 1 April 2023
Available online 12 April 2023
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arrangement of the lipid membrane and interconnecting water channels, classified as primitive ($lm3m/Q_{II}^{P}$), double diamond ($Pn3m/Q_{II}^{D}$) and gyroid ($la3d/Q_{II}^{G}$) [9]. Their unique structure allows encapsulation of hydrophobic, hydrophilic, and amphiphilic molecules, as well as large therapeutics such as peptides and proteins with a high surface area for improved pay load [10–12]. Furthermore, they are known to fuse with cell membrane, thus allowing better intracellular transport of their cargo [13]. LCNPs have been investigated as carriers for antimicrobial peptides (AMPs) and antibiotics against planktonic bacteria or bacterial biofilm, but to our knowledge there are no reports of LCNP targeting intracellular bacteria [14,15].

In this study, the ability of LCNPs to improve antibacterial activity of two antibiotics; tobramycin and vancomycin against intracellular *Pseudomonas aeruginosa* and *Staphylococcus aureus* are investigated. Both these antibiotics are poorly permeable across eukaryotic cells thus, achieve suboptimal concentration intracellularly [16,17]. To further probe the uptake of LCNPs and antibiotics, the cationic lipid, DDAB is strategically employed to interact with the negatively charged cell membrane [18,19]. Positively-charged nanoparticles have shown to be internalized more than their neutral or negative counterparts in macrophages [20]. Further, cationic LCNPs have been investigated to promote interaction with vaginal mucus membrane, thus improving the delivery curcumin for cervical cancer [21]. Here, the enhanced cellular interaction and improved performance of cationic LCNPs against intracellular bacteria is directly compared to their neutral counterpart.

Therefore, the aim of this study is to engineer a smart delivery system that combines the advantages of liquid crystalline nanostructures and cationic lipids to deliver antibiotics at the site of infection across two diverse host cells, i.e. macrophages and epithelial cells. The ability of LCNPs to deliver a small and large antibiotic, tobramycin and vancomycin respectively, were demonstrated. Furthermore, improved cellular internalisation by incorporation of a cationic lipid was observed for the LCNPs. Subsequently, their efficacy against two opportunistic pathogens, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) were evaluated.

2. Materials and methods

2.1. Formulation preparation and characterization

2.1.1. Liquid crystalline nanoparticles

Liquid crystalline nanoparticles (LCNPs) were fabricated using the hydrotrope dilution method as previously described by Thorn *et al.* [15]. Briefly, 100 mg of glyceryl monooleate (Myverol 18–99 K, Kerry Ingredients and Flavors, Egham, UK), 15 mg of Pluronic F-127 and 270 mg of propylene glycol were dissolved in 10 mL of chloroform. A stream of nitrogen gas was introduced to evaporate the chloroform, leaving behind a thin lipid film. The film was hydrated with 100 μL of phosphate buffer saline (PBS) and diluted to 5 mL with Milli-Q, followed by vortex-mix to form a white suspension.

Cationic-LCNPs were synthesized by addition of 1% w/w of dimethyldioctadecylammonium bromide (DDAB) (Avanti Polar Lipids Inc., Alabaster, AL, US) to the chloroform mix. Fluorescently-labelled particles were produced by the inclusion of either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(Cyanine 7) (Cy7-PE) or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss-Rhod PE) (Avanti Polar Lipids Inc.) to the lipid matrix.

Vancomycin and tobramycin loaded LCNPs and cationic-LCNPs were also prepared using the same method as described above, but with the addition of 100 μL PBS doped with antibiotic (50 mg/mL).

2.1.2. Particle size and zeta potential

The hydrodynamic diameter of LCNPs as a measure of particle size and polydispersity index (PDI) were determined via dynamic light scattering using Zetasizer Nano ZS (Malvern, Worcestershire, UK). Analysis was conducted for particles diluted in Milli-Q at 25 °C. The zeta-potential was measured via phase analysis scattering in 10 mM NaCl. Data acquisition and processing were performed using the built-in software, Zetasizer Software v7.12.

2.1.3. Cryo-transmission electron microscopy (Cryo-TEM)

The structure of LCNPs were captured using Glacios 200 kV Cryo-TEM (ThermoFisher Scientific). 5 μL of LCNPs was deposited onto a lacey copper grid and plunged into a mixture of liquid ethane and propane for vitrification. Samples were maintained at 180 °C and imaged using NANOSPRT15 camera (ThermoFisher Scientific) operating microscope at 120 kV. Fast fourier transform (FFT) analysis of the LCNPs were performed using ImageJ software v1.53 t [22].

2.2. Quantification of antibiotic

2.2.1. Antibiotic loading and release

The antibiotic loading was determined using a pressure ultrafiltration stirred cell, Amicon® (Merck Life Science, Bayswater, Australia). Briefly, 2.5 mL of the formulation was transferred to the cell containing a pre-soaked filter membrane (500 kDa) (Merck Life Science). 0.5 mL of filtrate containing free drug was collected by applying 100 kPa of nitrogen gas and was quantified for tobramycin or vancomycin concentration.

The formulation was then diluted to 10 mL of PBS, pH 7.4 and continuously stirred to measure release of antibiotic from LCNPs. At predetermined time points, 0.5 mL filtrate was collected, and the mixture was replaced immediately with fresh buffer.

2.2.2. Tobramycin quantification

Recovered tobramycin was quantified using a modified method as previously described by Horstmann, *et al.* [23] Briefly, a modified ion-pairing method was utilized for quantification using an ultra-HPLC system (Shimadzu, Kyoto, Japan) coupled with SCIEX Triple Quad 6500 + LC-MS/MS System (SCIEX, Framingham, MA, US). Analytes were separated using a Kinetex® 2.6 μm C18 column, 100 × 3 mm (Phenomenex) attached to a C18 column guard, by gradient elution using water as mobile phase A and acetonitrile as mobile phase B, both supplemented with 0.2% trifluoracetic acid. The gradient relating to mobile phase A was as follows: 20: 80 for 1 min, 70: 30 from 1 to 3.5 min and restored to 20: 80 from 3.5 to 6.0 min, with a total flow rate of 0.3 mL/min. Injection volume was set at 5 μL and samples were quantified using positive ionisation mode and selected reaction monitoring (SRM) of ion at 468.2/324.1.

2.2.3. Vancomycin quantification

Vancomycin concentrations were quantified using SCIEX ExionLCTM AD ultra-high performance liquid chromatography (UHPLC) coupled with SCIEX Triple QuadTM 4500 LC-MS/MS System (SCIEX, Framingham, MA, US). Prior to measurements, samples were diluted in mobile phase containing methanol and Milli-Q (50: 50 v/v), with 0.1% formic acid. Teicoplanin was used as an internal standard and Gemini-NX 5 μm C18 column, 50 × 4.60 mm (Phenomenex, Lane Cove, Australia) was used for separation. Each sample (injection volume of 5 μL) was analysed for 6 min at a flow rate of 0.3 mL/min in positive ionisation mode. Mass to charge ratios (m/z) in multiple reaction monitoring (MRM) for vancomycin were 725.6/1306.8, 725.6/144 and 725.6/100.1 while teicoplanin was monitored at 940.5/316.3.

2.3. In vitro cellular studies

2.3.1. Cell culture

RAW 264.7 murine macrophages (ATCC TIB-71^{TM}) and A549 lung epithelial cells (ATCC CCL-185^{TM}) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific, Scoresby, Australia) and F-12 K Medium (ThermoFischer) respectively, supplemented with

Table 1
Physicochemical properties of liquid crystalline nanoparticles, blank or encapsulated with vancomycin or tobramycin.

Formulation	z-average (nm)	PDI	z-potential (mV)	Drug load (% w/w)
Blank				
LCNP	199.9 ± 3.07	0.23 ± 0.01	7.17 ± 0.49	–
LCNP-DDAB	196.4 ± 4.46	0.28 ± 0.03	13.03 ± 0.57	–
Tobramycin				
LCNP	209.0 ± 2.7	0.28 ± 0.04	5.64 ± 0.09	4.80 ± 0.06
LCNP-DDAB	178.7 ± 6.3	0.25 ± 0.05	11.03 ± 0.57	4.78 ± 0.07
Vancomycin				
LCNP	208.5 ± 3.8	0.09 ± 0.02	7.73 ± 1.52	4.91 ± 0.01
LCNP-DDAB	197.4 ± 3.1	0.12 ± 0.02	10.01 ± 0.27	4.28 ± 0.84

10% v/v fetal bovine serum (FBS) and 1% v/v penicillin–streptomycin. Both cell lines were passaged at 80–90% confluency and maintained at 37 °C and 5% CO_2.

2.3.2. In vitro cytotoxicity of formulation

Cytotoxicity of nanoparticles were assessed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay for both cell lines. Cells were seeded in a 96 well plate at a density of 1×10^4 cells per well and incubated for 24 h at 37 °C and 5% CO_2. Following incubation, the cells were washed and treated with nanoparticles at a lipid concentration of 25 – 200 μg/mL. Cells treated with media only and 1% Triton-X were included as negative and positive controls respectively. Following 24 h of incubation, cells were washed several times and treated with MTT dissolved in PBS (5 mg/mL) for another 4 h. The formazan crystals formed were dissolved by addition of dimethyl sulfoxide (DMS0) and absorbance was measured at 540 nm.

2.3.3. Fluorescence activated cell sorting

RAW 264.7 or A549 at a seeding density of 1×10^5 cells per well were cultured in a 24 well plate for 24 h. The cells were washed and dosed with Cy7 PE labelled LCNPs at a lipid concentration of 50 μg/mL. After 4 h, the cells were detached following washing and fixed in 4% paraformaldehyde for 15 min. Cells were diluted in FACS buffer prior to analysis. Shift in fluorescence intensity relative to untreated cells were recorded as a measure of nanoparticle internalisation using BD LSRFortessa™ (BD Biosciences San Jose, California, USA), with a minimum recording of 10, 000 events per sample.

2.3.4. Fluorescence live cell imaging

To qualitatively assess nanoparticle internalisation, RAW 264.7 and A549 were grown in a 96 well plate at a seeding density of 1×10^4 cells per well. Following incubation for 24 h, the cells were washed and Liss-Rhod labelled LCNPs were introduced at a lipid concentration of 50 μg/mL. After 4 h, the cells were washed and treated with Hoechst 33,342 (ThermoFisher) for nuclei staining according to manufacturer's protocol, followed by imaging using Cytation 5 Cell Imaging Multimode Reader (Biotex Inc., Vermont, USA). The built-in Gen5 software was

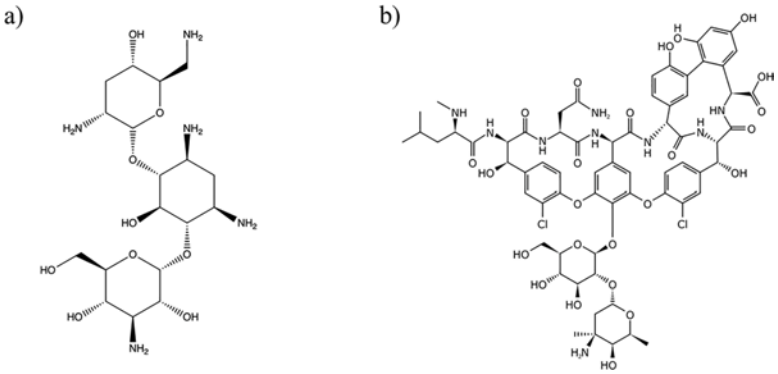


Fig. 1. Chemical structures of (a) tobramycin and (b) vancomycin.

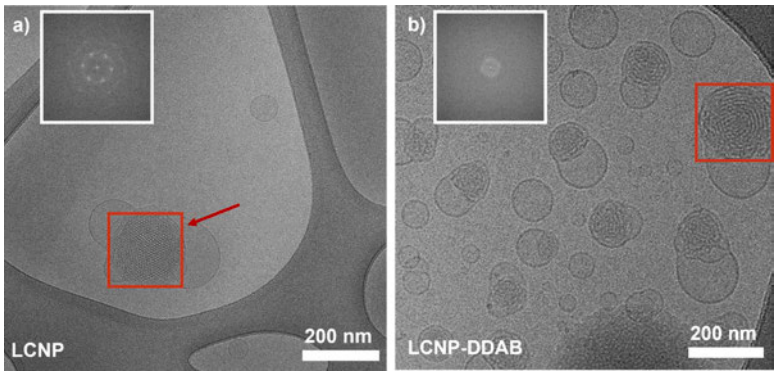


Fig. 2. Cryo-TEM images of blank (a) LCNP and (b) LCNP-DDAB, highlighted using red arrows with their corresponding fast Fourier transform (FFT) (insets).

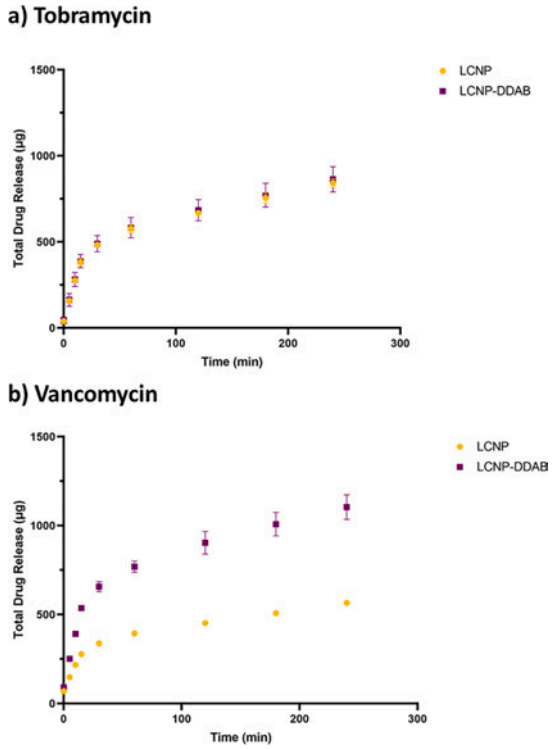


Fig. 3. Total release of (a) tobramycin and (b) vancomycin over a period of 4 h.

used for image processing.

2.4. In vitro bacterial studies

2.4.1. Minimum inhibitory concentration determination

Pseudomonas aeruginosa (*P. aeruginosa*), PA-01 and small colony variant (SCV) of *Staphylococcus aureus* WCH-SK2 [24] were selected as relevant strains for intracellular infection in this study. The lowest concentration at which the antibiotic inhibits bacterial growth, referred as the minimum inhibitory concentration (MIC) was determined to guide dosing of formulations for efficacy studies. The log-phase of both bacteria were diluted to an absorbance of 0.10 ± 0.02 measured at OD_{600}. The bacterial suspension was further diluted 1:100 in Luria-Bertani (LB) broth *P. aeruginosa* and tryptic soy broth (TSB) for *S. aureus*. 100 µL of the suspension was added to a 96 well plate and serially diluted with 100 µL of tobramycin (for *P. aeruginosa*) or vancomycin (for *S. aureus*) dissolved in broth. The plates were incubated at 37 °C for 24 h. The lowest concentration of antibiotic with no bacterial growth was determined as the MIC.

2.4.2. In vitro efficacy of liquid crystalline nanoparticles against intracellular bacteria

An intracellular infection assay was established by first seeding cells at a density of 3×10^5 cells/mL in a 12 well plate. Following 24 h incubation, an overnight culture of bacteria diluted in cell media (serum free) was introduced at a multiplicity of infection (MOI) of 100: 1 for both cells. A MOI of 10: 1 was used for SCV *S. aureus* and RAW 264.7. The cells were incubated for 2 h and washed repeatedly to remove extracellular bacteria. Infected cells were incubated with 200 µg/mL gentamicin and 10 µg/mL of lysostaphin for *P. aeruginosa* and *S. aureus*, respectively to kill remaining extracellular bacteria. After 1 h, the cells were washed thrice and incubated with free (unformulated) antibiotic or antibiotic loaded LCNPs at 1x, 4x and 8x MIC concentrations. Following 4 h incubation, the cells were washed and lysed with 0.1% Triton-X to extract intracellular bacteria. The bacterial suspension was serially diluted and plated in LB agar or tryptic soy agar for 18–24 h at 37 °C. Single colonies of bacteria for each sample were calculated and reported as colony forming unit (CFU)/mL.

2.5. Statistical analysis

Experiments were conducted in triplicates and data reported as mean ± standard deviation. Results were analyzed using one or two-way analysis of variance (ANOVA) followed by either Tukey's or Šídák's multiple comparison test at a significance level of α 0.05. Analysis was performed using GraphPad Prism (version 9.0.0 for Windows, GraphPad Software, California, USA).

3. Results and discussion

3.1. Synthesis and characterization of liquid crystalline lipid nanoparticles

LCNPs in the size range of 190–210 nm were successfully synthesized from glyceryl monooleate, stabilized with Pluronic F-127 and incorporated with either vancomycin or tobramycin (Table 1). Tobramycin is a small molecule with a size of 467.5 Da while vancomycin represents a large molecule of 1485.7 Da (Fig. 1). A drug load of ~ 5% w/w, with > 95% encapsulation efficiency was achieved for both antibiotics, in line with previous observations in which the large water channels of LCNPs allows loading of molecules ranging from < 1 to 174 kDa [9]. The LCNPs bear a near neutral zeta-potential (~-7 mV), due to the adsorption of hydroxide ions from Pluronic on the water and lipid interface of the particles (Table 1) [25]. The inclusion of the cationic lipid, DDAB (hereafter referred as LCNP-DDAB) resulted in a positive increase of zeta-potential to + 13 mV for the blank formulation (Table 1). No significant differences in size and zeta-potential were observed for both blank and antibiotic-loaded formulation.

Previously, Sarkar *et al.* demonstrated no changes to the cubic

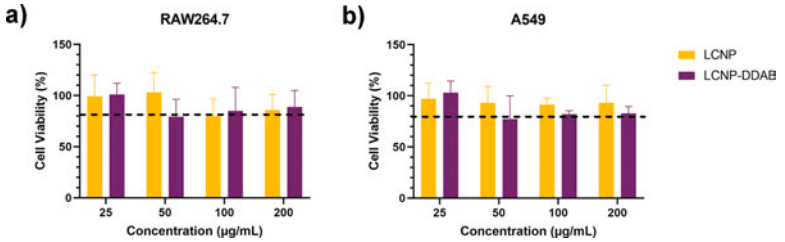


Fig. 4. Cell viability of RAW 264.7 macrophages and A549 epithelial cells incubated with LCNP and LCNP-DDAB for 24 h at a range of lipid concentration from 25 to 200 µg/mL.

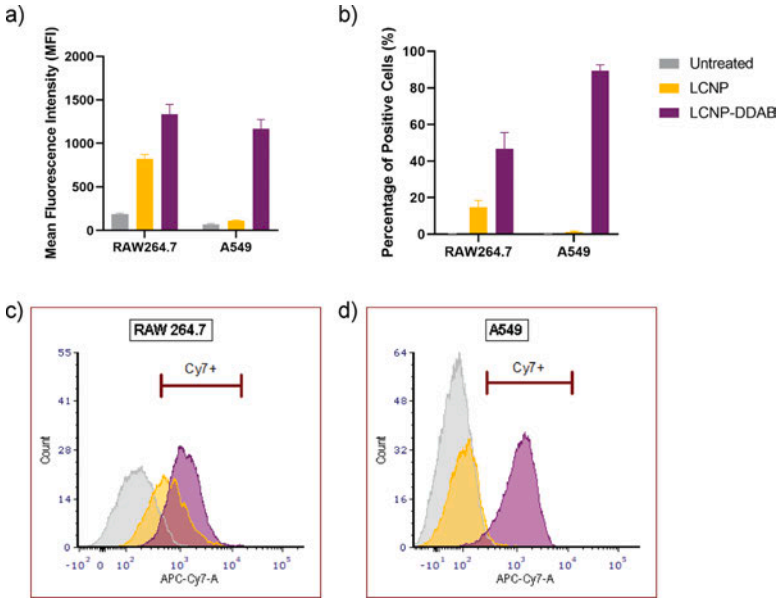


Fig. 5. Cellular uptake of LCNP and LCNP-DDAB expressed as (a) mean fluorescence intensity, (b) percentage of positive cells and (c-d) histogram illustrating the shift in fluorescence signal of nanoparticles compared to untreated cells.

nanostructure of LCNPs with the incorporation of up to 2 mol % of cationic lipids [26]. Here, the morphology of blank LCNP and LCNP-DDAB were assessed using Cryo-TEM (Fig. 2). For both formulations, the presence of LCNPs and vesicular structures formed from the dispersion of lipids were observed, consistent with previous findings [27,28]. A large pore size for the LCNP-DDAB was identified, with an onion-like organisation compared to the honeycomb structure as typically seen in LCNP. Although, similar morphology and organisation for cationic cubosomes have been reported previously, corroborating findings here [29]. The larger pore size is due to formation of a bicontinuous water-swollen cubic membranes, resulting from electrostatic interactions and steric stabilization of the lipid membrane interfaces [29].

3.2. Release of antibiotics form liquid crystalline nanoparticles

The release of both antibiotics from LCNP and LCNP-DDAB were investigated in phosphate buffer saline (PBS) at pH 7.4. For tobramycin, the differences in molecular structure of the two LCNPs did not influence their rate of release over 4 h. The release of hydrophilics from LCNPs is controlled by diffusion of the molecules across the water channels of the lipid matrix [9].Previous studies by Thorn *et al.*, demonstrated tobramycin to be rapidly released within 2 h [15,30]. Here, a lower release rate was achieved by reducing the antibiotic load, thus preventing rapid diffusion of tobramycin closer to the surface of the particle.

Contrarily, the release differed significantly for vancomycin from LCNP and LCNP-DDAB, resulting in faster release with the inclusion of cationic lipid. The larger molecular size of vancomycin in comparison to tobramycin is expected to limit their diffusion across the LCNPs. Further, their diffusion kinetics is also controlled by the pore size of water channels, thus the increased release of vancomycin from LCNP-DDAB is expected [31]. It must also be noted that the electrostatic interaction of the antibiotic can mediate their diffusion across LCNPs. A study by Negrini *et al.*, have shown the changes in release profile of charged molecules across negative and positively charged LCNPs [32]. At pH 7, the oppositely charged molecule and LCNPs demonstrated a slower release. In this study, tobramycin is positively charged while vancomycin remains neutral at pH 7 Purdy Drew, 2009; [57]. Based on observations in Fig. 3, pH influence is hypothesised to be at a lesser extent in comparison to the effect of pore size of LCNPs.

3.3. Cytotoxicity screening of nanoparticles

Charge-dependent toxicity is often reported with the use cationic nanoparticles due to their strong interaction with the negatively charged cell membrane [35]. Therefore, the toxicity of LCNP and LCNP-DDAB were investigated in RAW 264.7 murine macrophages and A549 epithelial cells (Fig. 4). Over the concentration range tested, the cells retained a viability above 80% and thus, the formulations were considered non-toxic for subsequent cellular studies [36]. The low amount of cationic lipid used in this study (1% w/w) enabled the LCNPs to retain their biocompatibility profile. Strachan *et al.* showed LCNP combined with different cationic lipids at 20 mol % to be non-toxic up to 125 μg/mL, with a steep decline in viability observed at 200 μg/mL in fibroblast cell lines [37]. However, the toxicity profile of nanoparticles is influenced by various factors including, dose administered, time of incubation and cell lines used thus, limiting extrapolation of results from previous studies.

3.4. Cellular uptake of liquid crystalline lipid nanoparticles

The *in vitro* cellular uptake of LCNPs were measured via flow cytometry, based on the shift in fluorescence intensity relative to untreated cells. As illustrated in Fig. 5a, a higher mean fluorescence intensity (MFI) was observed for both nanoparticles compared to untreated macrophages and epithelial cells following a 4 h incubation. In macrophages, LCNP had a 4-fold increase in fluorescence intensity compared to untreated cells, corresponding to ~ 15% internalisation (Fig. 5a and Fig. 5b). Strachan *et al.* previously demonstrated the internalisation of LCNPs in macrophages to be facilitated via endocytosis [37]. As immune cells, macrophages are responsible for rapid internalisation and metabolism of lipids, thus LCNPs are postulated to be ingested following a similar mechanism [38]. The LCNP-DDAB demonstrated > 50% internalisation in macrophages, ~3 times greater than their neutral counterpart. A greater magnitude of

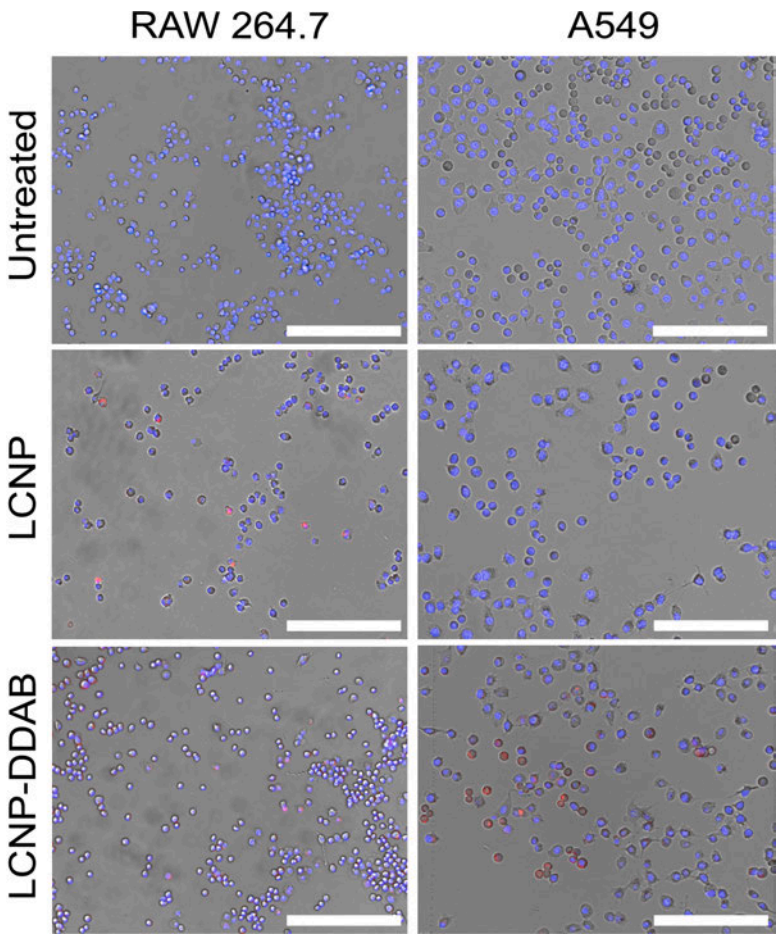


Fig. 6. Cellular uptake of LCNP and LCNP-DDAB in RAW 264.7 macrophages and A549 epithelial cells. Nanoparticles are labelled with Liss-Rhod PE (red) and nucleus stained with Hoechst 33,342 (blue. Scale bar represents 200 μm.

internalisation for cationic particles, compared to neutral and anionic particles of comparable size and hydrophobicity has been reported previously [39,40]. Cationic particles have longer residence times on cell membrane due to their electrostatic interactions and can be internalized via endocytosis or fusion mechanisms [13,41]. Their uptake was visually confirmed using fluorescence imaging and as depicted in Fig. 6, brighter red fluorescence signal was observed for the LCNP-DDAB in macrophages.

Interestingly, no shift in fluorescence intensity and fluorescence signal was observed for LCNPs in epithelial cells, resulting in $< 1\%$ uptake (Fig. 5 and Fig. 6). Generally, LCNPs demonstrate high internalisation in epithelial cells, although cell-dependent association has been reported [42,43]. For example, Prange at al. showed cubosome uptake in two intestinal epithelial cells, HK2 and mPTCs, but not in OK cells, possibly due to differences in cellular structure and receptors present [44]. Furthermore, A549 cells synthesize lecithin which may alter their membrane interaction with LCNPs [45]. On the contrary, LCNP-DDAB had the highest shift in fluorescence intensity, achieving $>$ 80% uptake in epithelial cells. Similarly, the brightest fluorescence was observed for LCNP-DDAB in epithelial cells (Fig. 6). Bedaquiline-loaded cubosomes with a zeta-potential of $+ 35.4 \pm 2.3$ mV was highly internalized in A549 cells, improving tumour eradication in comparison to unformulated drug [46]. Thus, the charge-dependent uptake is in comparison to results reported previously.

3.5. Antibacterial activity of liquid crystalline lipid nanoparticles against intracellular pathogens

The minimum inhibitory concentration (MIC) for tobramycin and vancomycin against *P. aeruginosa* and SCVs *S. aureus*, respectively, were experimentally determined to be 1 μg/mL and 0.5 μg/mL. Based on their MIC value, the infected cells were treated at an equivalent antibiotic concentration at 1x, 4x and 8x of MIC. No effect was observed for free tobramycin and tobramycin encapsulated in LCNPs against *P. aeruginosa* in macrophages (Fig. 7 a, b). Tobramycin, an aminoglycoside is poorly permeable in cells and is ineffective by themselves against intracellular bacteria, similar to observations reported here [47,48]. At 4x MIC (4 μg/mL), LCNP-DDAB demonstrated improved killing of the bacteria attributing to a higher payload of antibiotic delivered to the site of infection across cell membrane. Previously, Qiu *et al*, showed improved activity of gentamicin (also an aminoglycoside) against intracellular *P. aeruginosa* when delivered using phosphatidylcholine-chitosan nanoparticles, showing a correlation between nanoparticle uptake and improved efficacy as shown here [49]. At a higher dose, equivalent to tobramycin concentration of 8 μg/mL, a saturation effect was observed. Similar phenomena have been reported for several drug delivery

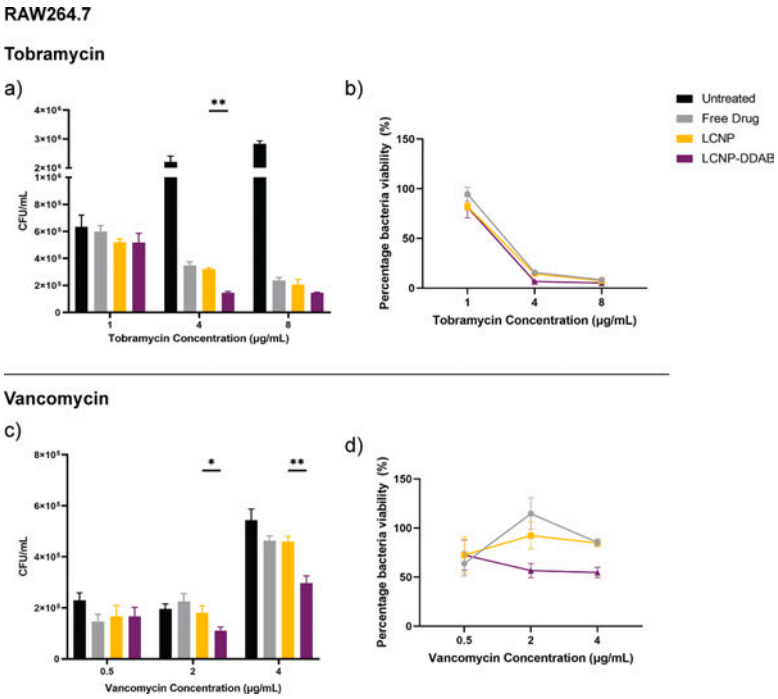


Fig. 7. Concentration-dependent effect of LCNP and LCNP-DDAB encapsulated with (top) tobramycin and (bottom) vancomycin, plotted as (a and c) reduction of intracellular bacteria as CFU/mL and (b and d) percentage bacteria viability (%) relative to untreated bacterial count (* $P < 0.01$ and ** $P < 0.001$).

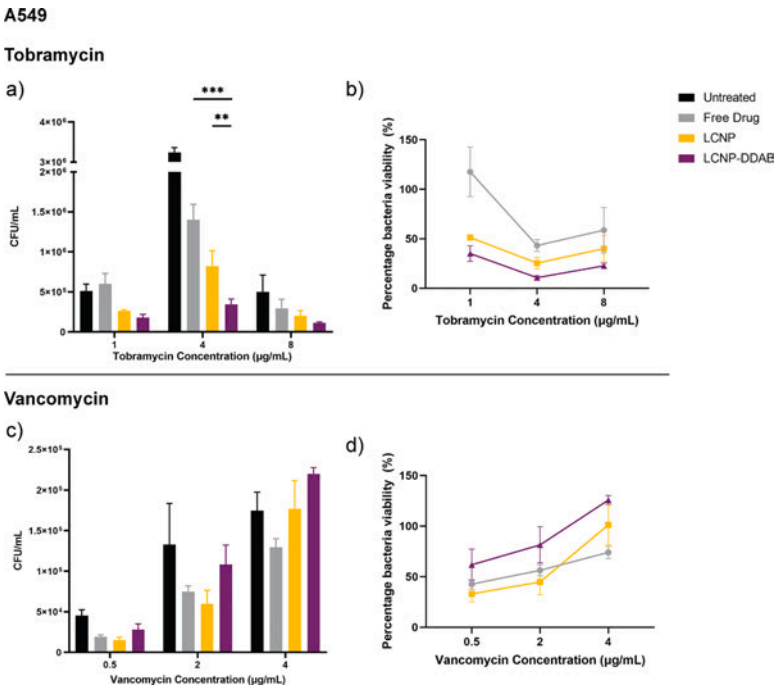


Fig. 8. Activity of LCNP and LCNP-DDAB encapsulated with (top) tobramycin or (bottom) vancomycin against intracellular bacteria in A549 epithelial cells (** $P < 0.001$ and *** $P < 0.0001$).

systems and their effect is highly dependent on the bacterial strain, cell type and antibiotic used [50,51]. Furthermore, 4x MIC of unformulated antibiotic has been suggested to be the optimal concentration for intracellular pathogen, reaching maximum efficacy without signs of toxicity, thus in line with findings here [52].

In SCV *S. aureus* infected macrophages, treatment with vancomycin alone at all concentrations showed no significant reduction in CFU/mL (Fig. 7 c, d). Vancomycin LCNP also did not reduce the bacterial burden, attributing to their minimal internalisation in macrophages. On the contrary, treatment with LCNP-DDAB showed significant reduction in bacterial viability at 4x (2 μg/mL) and 8x (4 μg/mL) MIC, resulting in over ~ 55% bacterial killing. This is in agreement with previous studies, where improved delivery of rifampicin using polymer-lipid hybrid or mesoporous silica particles resulted in improved killing of intracellular SCV *S. aureus* [53–55].

The efficacy of LCNP and LCNP-DDAB was also investigated in A549 epithelial cells, against both pathogens. Tobramycin delivered as a solution had no effect against the intracellular bacteria, correlating with poor internalisation of aminoglycoside as discussed previously (Fig. 8). Similarly, the inadequate uptake of LCNP in A549 (as observed in Fig. 5), resulted in their limited ability to target the intracellular bacteria. In contrast, tobramycin encapsulated in LCNP-DDAB resulted in significant reduction of bacterial count at 4x MIC against *P. aeruginosa* (Fig. 8). A saturation effect was also observed at 8x MIC, similar to observations in macrophages.

Conversely, no reduction in bacteria was observed across all treatments against SCV *S. aureus* in epithelial cells (Fig. 8c, d). Several factors could have contributed to the outcome, including different subcellular localisation of bacteria and nanoparticles, the ability of SCV *S. aureus* to switch into a dormant phenotype when exposed to high antibiotic concentrations or limited diffusivity of antibiotic to site of infection [56,57]. This is the first time a nanoantibiotic approach has been utilized to eradicate SCV *S. aureus* in non-phagocytic cell line, limiting comparison with prior investigations.

Despite the decrease in bacterial viability, complete eradication of intracellular bacteria, for both *P. aeruginosa* and *S. aureus* was not achieved. Further optimization of the nanocarrier system to improve their subcellular localisation and drug release kinetics, thereby improving the intracellular antibacterial payload may enhance their performance. Further studies to evaluate their activity following longer treatment times and repeated dosing are also necessary to achieve the desired outcome.

Based on these findings, we have demonstrated the ability of cationic LCNPs to target intracellular bacteria in phagocytic and non-phagocytic cells. Using a cationic lipid, the cellular uptake profile in both cells was improved, although the high surface area of LCNP and larger pore size of their cationic counterpart resulted in faster release for vancomycin. It is anticipated that further optimization of the nanocarrier can provide a stimuli-responsive release of the drug, thus improving their overall activity against intracellular bacteria. Their limitation in treating SCV *S. aureus* in A549 warrants further investigations to improve mechanistic understanding in targeting bacteria present in non-phagocytic cells.

4. Conclusion

The ability of a cationic LCNP to improve the performance of antibiotics against intracellular bacteria present in macrophage and epithelial cells was determined. The presence of DDAB altered the morphology of LCNPs into an onion-ring like structure. The inclusion of a cationic lipid enhanced cellular uptake, >50% and > 90% in macrophages and epithelial cells, respectively. In macrophages, LCNP-DDAB reduced intracellular *P. aeruginosa* and *S. aureus* viability by ~ 90% and ~ 55% at 4x MIC of antibiotics. Limitation in targeting *S. aureus* in epithelial cells was observed, however LCNP-DDAB enhanced the activity of tobramycin against intracellular *P. aeruginosa*. In summary, the potential of LCNP-DDAB to target intracellular bacteria has been demonstrated, which will enable improved treatment outcomes for intracellular infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Dr Stephen Kidd (University of Adelaide, South Australia) is kindly acknowledged for gifting the SCV *S. aureus* strain. Mr Anthony Wignall and Mrs Mitra Hosseini are kindly thanked for their technical assistance. Dr Abiodun Ogunniyi and Associate Professor Rietie Venter are also acknowledged for their technical assistance and guidance in using the Cytation5 Cell Imagine Multimode Reader.

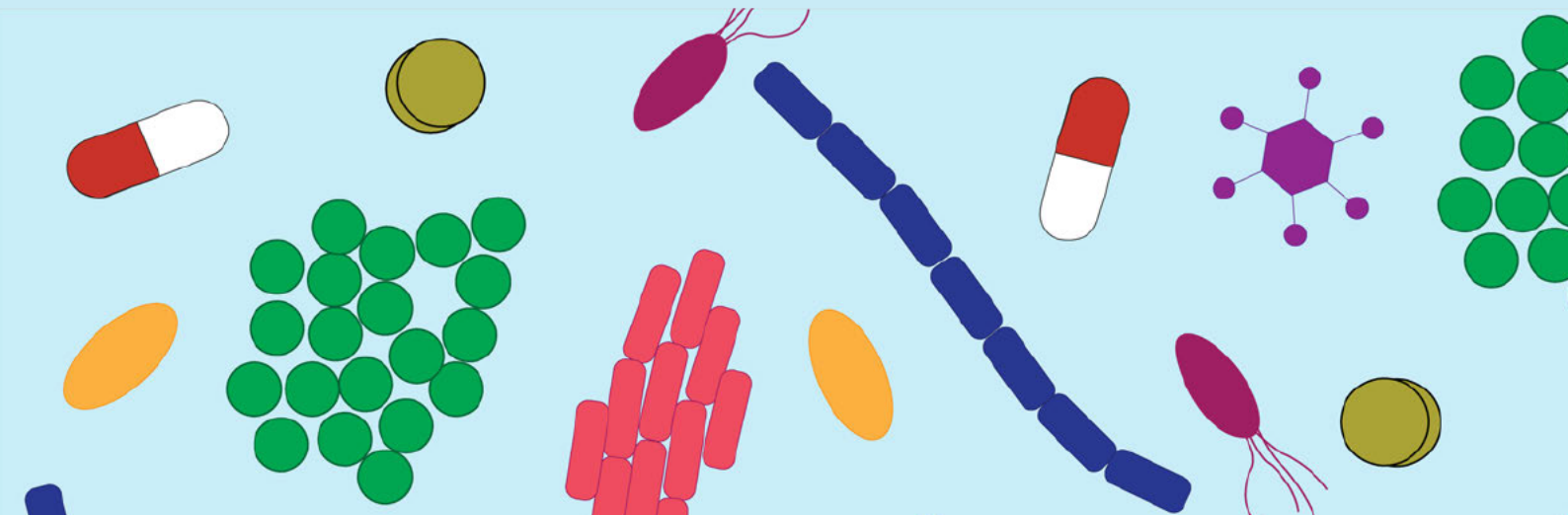
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5

MINIMUM INFORMATION FOR CONDUCTING AND REPORTING IN VITRO INTRACELLULAR INFECTION ASSAYS



5 Minimum information for conducting and reporting in vitro intracellular infection assays

In this chapter, a guideline and framework is provided to assist the scientific community when evaluating performance of novel therapeutics using in vitro intracellular infection assays. By following the set of recommendations provided, reproducibility of reported studies can be improved in addition to comparisons made across studies for scientific judgment. Together, this will facilitate translation of experimental findings into meaningful clinical outcomes.



Abstract

Bacterial pathogens are constantly evolving to outsmart the host immune system and antibiotics developed to eradicate them. One key strategy involves the ability for bacteria to survive and replicate within host cells, thereby causing intracellular infections. To tackle this unmet clinical need, researchers are adopting new approaches, such as the development of novel molecules that can penetrate host cells, thus exerting their antimicrobial activity intracellularly, or repurposing existing antibiotics using nanocarriers (i.e., nanoantibiotics) for site-specific delivery. However, inconsistency in information reported across published studies makes it challenging for scientific comparison and judgement of experiments for future direction by researchers. Together with the lack of reproducibility of experiments, these inconsistencies limit the translation of experimental results beyond preclinical evaluation. Minimum information guidelines have been instrumental in addressing such challenges in other fields of biomedical research. Guidelines and recommendations provided herein have been designed for researchers as essential parameters to be disclosed when publishing their methodology and results, divided into three main categories: (i) experimental design in establishing an in vitro model, (ii) assessment of efficacy of novel therapeutics, and (iii) statistical assessment. This guideline has been designed with the intention to improve reproducibility and rigor of future studies while enabling quantitative comparisons of published studies, ultimately facilitating translation of emerging antimicrobial technologies into clinically viable therapies that safely and effectively treat intracellular infections.

5.1 Introduction

Infectious diseases caused by bacteria continue to be a global threat to humankind due to the rise in antimicrobial resistance, where current antibiotics suffer from suboptimal efficacies and resistance development in treating challenging infections. Bacterial pathogens have evolved three main mechanisms to evade the biological immune response and antibiotic treatment, including (i) the formation of a bacterial biofilm, where communities of bacteria survive within an extracellular polymeric substance (EPS) matrix; (ii) intracellular infection caused by bacteria capable of invading, surviving and proliferating within host cells; and, (iii) acquisition and dissemination of antimicrobial resistance through *de novo* mutations and horizontal gene transfer [24, 61-63]. Together, they contribute to persistent and recurrent debilitating clinical infections such as chronic skin infections, pneumonia, tuberculosis, and urinary tract infections [3, 23]. While a breadth of research has focused on targeting the different resistance mechanisms employed by bacteria for the development of new therapies, this review will specifically highlight the challenges associated with intracellular infection.

In context of intracellular infections, new drug entities and molecules, such as antimicrobial peptides and oligonucleotides that target either the bacteria or virulence mechanism employed for intracellular survival is a promising approach [64-66]. However, the drug discovery pipeline is time and cost intensive. Another challenge for successful treatment is the inability of current and new antibacterial agents to overcome biological barriers such as, penetration across the biofilm matrix and lack of permeation across host cells, together with poor intracellular stability and retention of antibiotics to target the hidden bacteria [67, 68]. To overcome these challenges, the use of nanocarriers to deliver currently available antibiotics, also referred to as nanoantibiotics is a promising approach [69]. Researchers have utilized various types of nanoparticles, ranging from inorganic silica nanoparticles to organic lipid-based nanoparticles as vehicles to deliver antibiotics, overcoming the aforementioned challenges. In particular, the use of nanoantibiotics for

intracellular infection is gaining wide range of interest among current researchers. However, while preliminary findings are promising, the translation of these bench-side discoveries to clinical products remains poor and without a clear regulatory pathway.

The nature of intracellular infection is complex, with intricate interactions of bacteria and the host immune system, which are difficult to replicate in a laboratory setting. Nevertheless, *in vitro* studies in this field are crucial, often conducted as a preliminary step to evaluate the activity of new molecules and formulations against intracellular pathogens. Despite inadequately mimicking the pathophysiology of a clinical infection, they serve as a screening tool and provide mechanistic understanding on the use of nanoantibiotics. However, challenges remain in advancing these pre-clinical investigations into therapeutic application. One such challenge is the lack of reproducibility between published research findings, including insufficient reporting of important experimental parameters and outcomes that can guide the scientific community. A recent meta-analysis on drug delivery approaches for intracellular infection highlighted the inadequate methodological quality of studies published, impeding successful clinical translation of new and emerging therapeutics [70]. Examples identified include insufficient evidence to confirm intracellular delivery of nanoparticles and unsatisfactory interpretation and comparison of efficacy results without inclusion of appropriate study controls (e.g., lacking comparison of antibacterial activity of novel formulation versus free antibiotic).

Minimum reporting guidelines and checklist are strategies successfully employed by the scientific community to address disparities in reporting standards. FAIRsharing.org, a website with curated resources on data standards currently has 39 reports under the overarching collection of Minimum Information for Biological and Biomedical Investigations (MIBBI) [71-73]. The MIBBI serves as a tool for researchers in various fields of research including toxicology, neuroscience, and proteomics for standardized reporting of methodologies. Within the field of microbiology, Minimum Information about a Biofilm

Experiment (MIABiE) was published in 2014 for consistent reporting of biofilm data [74]. This was preceded by the Minimum Information Guideline for Spectrophotometric and Fluorometric Methods to assess Biofilm Formation in Microplates in 2020, a comprehensive recommendation to improve quality and reproducibility of biofilm microplate assays [75]. In the field of nanomedicine, the Minimum Information Reporting in Bio-Nano Experimental Literature (MIRIBEL) was published in 2018, specifically detailing information required in research articles to produce robust, reliable and comparison data in the field of bio-nano science [76].

While standards exist as a guideline for researchers within the biofilm and bio-nano field of research, no guidelines have been published thus far for reporting of methods and data for intracellular infection. Hence, to promote scientific rigor and quality of studies reported in this upcoming and growing area of interest, this guideline aims to assist researchers on planning, conducting, and reporting experiments involving the use of nanoantibiotics for intracellular infections. By providing a detailed set of experimental considerations and recommendations, this guideline serves to promote the quality of research articles published while guiding the scientific community to accelerate the clinical translation of future investigations. It is important to note that this guideline serves as a guide only and not as a specific rule for researchers to comply on how these studies should be performed.

5.2 Experimental considerations and reporting recommendations for intracellular infection: in vitro assays

This guideline focusses on the parameters that need to be considered when planning and conducting studies on nanoantibiotics to target intracellular bacteria. While viral, fungal and parasitic intracellular infections exist, bacterial infections have been chosen as the case study given their high prevalence in studies conducted (i.e. 62.6% of intracellular infection studies), and similar principles can be applied to other pathogens [70]. When evaluating the

effectiveness of a novel antibacterial molecule or formulation, it is first important to establish a robust intracellular infection assay. There are several factors to be considered when developing and reporting such assays, starting from selection of cell strain and bacterial strain through to the assessment of efficacy of formulations. Table 3 illustrates the differences in experimental methods across five published studies, illustrating the differences in multiplicity of infection (MOI), methods of removing extracellular bacteria and treatment times in each study. The differences make it difficult for consensus to be reached when comparing scientific observations, forming the basis of this guideline for standardization in methods. This guideline is divided into four main sections: (i) experimental design, (ii) establishing an intracellular infection assay, (iii) assessment of nanoantibiotic efficacy and (iv) statistical assessment and data presentation, as illustrated in Figure 8.

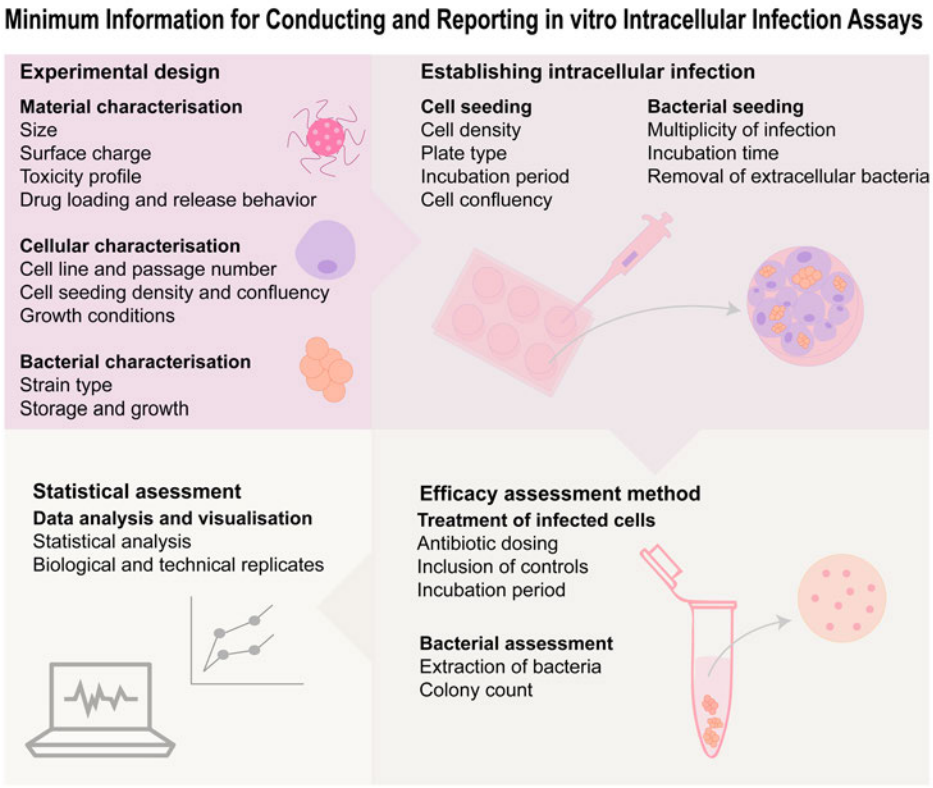


Figure 8. Schematic diagram of guideline and critical parameters to consider for assessment nanoantibiotic efficacy using an intracellular infection assay in vitro, divided into four categories: (i) experimental design, (ii) establishing an intracellular infection, (iii) efficacy assessment method and (iv) statistical assessment for data analysis.

Table 3. Differences in experimental methods for assessment of nanoantibiotics against intracellular pathogens [27, 29, 77-79].

	Case study 1 (Yang *et al.*)	**Case study 2 (Mu *et al.)***	**Case study 3 (Gao *et al.*)**	**Case study 4 (Maghrebi *et al.*)**	**Case study 5 (Zhang, *et al.*)**
Cell line	MC3T3-E1 (preosteoblast) and RAW264.7 (murine macrophages)	RAW264.7 (murine macrophages)	Ana-1 (murine macrophages)	RAW264.7 (murine macrophages)	A549 (ATCC 6538, lung epithelial cancer cell line)
Bacterial strain	*Staphylococcus aureus* (ATTC 25923)	*Listeria monocytogenes* (ATCC 19114) and *Pseudomonas aeruginosa* (PAO1)	*Staphylococcus aureus* (ATTC 25923)	*Staphylococcus aureus* (small colony strain, SCV WCH SK2)	*Staphylococcus aureus* (MSSA, ATCC 6538)
Multiplicity of infection (MOI)	30: 1	10: 1	10: 1	10: 1	10: 1
Incubation time of bacteria with cells	30 min	1 h	1 h	1 h	2 h
Removal of extracellular bacteria	30 µg/mL of gentamicin	50 µg/mL of gentamicin	1 µg/mL of lysostaphin	Repeated washing with phosphate buffer saline	50 µg/mL of gentamicin
Confirmation of intracellular bacteria	Confocal imaging	No	Fluorescence imaging	Leishman's staining	Confocal imaging
Concentration of antibiotic used (encapsulated in formulation)	1 to 8 µg/mL of gentamicin	0.116 mg/mL of gentamicin	2 or 4 µg/mL of rifampicin, 20 or 40 µg/mL of vancomycin	0.125, 0.5. 2.5 and 5 µg/mL of rifampicin	2.5, 5, 10 and 20 µg/mL of penicillin G
Incubation time for nanoantibiotics	24 h	2 h, followed by washing and further incubation for 12 h	24 h	4 h	1 h, followed by washing and further incubation for 18 h
Control treatments	Gentamicin at equivalent concentration	Gentamicin at equivalent concentration	Vancomycin or rifampicin at equivalent concentration	Rifampicin at equivalent concentration	Penicillin G at equivalent concentration

5.2.1 Experimental design for an intracellular infection assay

In general, an intracellular infection assay is established by first growing cells of interest until they reached confluency, followed by incubation with the bacterial species of interest for internalization, performing an extracellular bacterial killing assay, and lastly assessment of nanoantibiotic efficacy against intracellular bacteria. Detailed characterisation of biological specimens, materials and methods used are crucial for the transparency and reproducibility of the assay. Key considerations, suggestions, and parameters to report at each stage of the characterisation are described in this section, divided into three parts: (i) formulation characterisation, (ii) cellular characterisation and (iii) bacterial characterisation.

5.2.1.1 Formulation characterisation

Before cellular studies are conducted, the formulation, either a novel molecule or nanoparticle needs to be well characterized given that their properties significantly influence biological interactions. Readers are highly encouraged to refer to the perspective article on reporting bio-nano experimental literature MIRIBEL) for detailed description of this section if using a nanomaterial [76]. Briefly, materials and methods for fabrication of the compound, antibiotic used, and formulation must be provided in detail, including any steps conducted for purification. The size and zeta-potential, as a measure of surface charge are parameters commonly reported for nanoparticles. For organic nanomaterials, researchers should be aware of the differences between their geometric size, when measured in dry state (e.g., using electron microscopy) compared to the hydrodynamic size when measurement is conducted in a wet state (e.g., using dynamic light scattering) [80]. As the size and zeta potential of the nanomaterials are influenced by the medium of measurement, details of solution used during measurement should also be reported [81].

The amount of active drug loaded or conjugated in the system should be quantified and the drug release kinetics needs to be measured. Physiologically relevant media should

be used when conducting drug release studies, depending on the route of administration of the formulation. Since the intracellular environment of cells is acidic, researchers should also consider conducting release studies of their formulation at a low pH. A comprehensive literature review detailing the composition and preparation of stimulated biological fluids by Marques, *et al*, serves as a useful tool to guide researchers on the relevant media to use for their experiments [82]. Consideration should also be given to the short and long-term stability of the antibacterial agent to ensure bioactivity of the formulation is retained during experiments and for further optimization during drug development [83, 84]. Lastly, the toxicity profile of the synthesized material against cell lines of interest should be reported. Tetrazolium reduction, resazurin reduction, protease markers and ATP detection are commonly used assay methods to measure cellular cytotoxicity [85]. When conducting these studies, researchers should be aware of any interference of the materials with the assays [86].

5.2.1.2 Cellular characterisation

Cellular studies are invariably employed as a preliminary justification prior to pursuing in vivo studies which are costly and time-consuming. They provide crucial information on the cellular toxicity and efficacy profile of newly fabricated formulations. Cellular studies are often conducted with either primary cells, derived from clinical samples or secondary cell lines, in which the cells have been immortalized for repeated passaging and maintenance. Appropriate selection of cell lines, relevant to the clinical disease of interest is important, together with awareness of the cellular characteristics that can influence their interaction with the formulation tested.

Secondary cell lines are commonly used as they represent a pure population of cells providing consistency in results, easier to handle and does not require ethical approval for isolation [87]. However, researchers should be aware that the use of secondary cell lines do not replace primary cell lines due to their functional differences. Moreover, while secondary

cell lines are long lasting compared to primary cells, regular passaging can alter their genotypes and phenotypes of cell lines, which can lead to a difference in response to biomaterials [88]. Furthermore, cellular senescence or cellular aging, can result in cells being resistant to apoptosis and have a different gene expression and chromatin organisation. A study using young and senescent fibroblast and epithelial cells demonstrate higher internalisation of nanoparticles in young cells [89]. The young cells also had greater resistance to the toxicity profile of the nanoparticles in comparison to the senescent cells when dosed at equal concentration of nanomaterials. As such, maintaining cells within a certain age and including the passage number of cells used for experiments is crucial for reproducibility of results.

Similarly, cell seeding density, together with the confluency of cellular monolayer during experiment should also be specified and determined empirically. A confluent monolayer verses a sparse monolayer of cells resulting from different seeding density can alter the toxicity profiles of nanomaterials [90]. In addition, the type of tissue culture plate and volume of media used for seeding needs to be included in the experimental section. Additional information on conditions used for cellular growth and maintenance, such as the type of growth medium, growth factors, antibiotics supplemented in media, temperature and humidity should be reported. If using a primary cell line, detailed methods for isolation and list of all chemicals used for growth and maintenance must be specified.

5.2.1.3 Bacterial characterisation

Choosing the appropriate bacterial strain of clinical relevance is critical when conducting an intracellular infection assay, further facilitating clinical translation of experimental results obtained. *Staphylococcus aureus* (*S. aureus*) and *Mycobacterium tuberculosis* (*M. tuberculosis*) are the two of the most commonly used organisms for intracellular infection studies [70]. The choice of bacterial strain needs careful consideration

as their genetic composition and virulence factors can have an impact on their activity against the antibiotics used.

The overexposure to antibiotics has led to resistance development in *S. aureus*, including methicillin-resistance, and recently, vancomycin-resistance. *S. aureus* can also switch into a dormant phenotype to avert host immune response, existing as small colony variants (SCVs) that survive persistently in host cells [91]. Clinical SCVs have been isolated from various type of chronic infections, including bone and lung-related infections [92, 93]. SCVs rely on different metabolic pathways for their survival, therefore also altering their susceptibility to antibiotics [94]. Their slow growth, small size of colony and altered antibiotic susceptibility profile makes it challenging to work with. However, given their contribution to chronic and persistent intracellular infections, researchers should consider using SCVs and optimize conditions for their intracellular infection assays.

When reporting, researchers should describe the species of bacterial strain used, including strain number or reference with details on strain type used, and the bacterial identity confirmation by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectroscopy under the Methods section. If using a clinical isolate, all available information should be included, including method of isolation and media used for maintenance. Information regarding the storage and growth of the bacterial strain, including inoculum preparation method should be provided. Further details, such as solution used for maintenance of stock in liquid nitrogen, type of culture medium used for propagation, temperature and growth conditions of bacterial culture, i.e., statically or with agitation under aerobic, microaerophilic or anaerobic conditions should be indicated. If using a genetically modified organism (GMO) for visualization purpose, any reagents added as gene promoters must be specified. For further information on the growth and maintenance of *S. aureus*, authors are recommended to read the article written by Missiakas and Schneewind [95].

5.2.2 Establishing bacterial invasion as an in vitro model of intracellular infection

The establishment of a robust intracellular infection assay is paramount to the success of the efficacy study conducted. Many important details for the development of the assay are often omitted, which causes discrepancies in techniques used by researchers. Once cells have achieved the desired confluency, cells are generally resuspended in and washed with phosphate buffer solution (PBS) prior to incubation with bacteria diluted in cell culture media. Time of cell incubation and confluency prior to infection should be provided, including details of solution used to wash the cells, number of times cells were washed, and method (e.g. centrifugal speed and time) used for washing. Repeated washing can cause loss of cells and researchers should be cautious during this step and are advised to assess the viability visually using a light microscope or other validated measures of viability (e.g. using live-dead staining with propidium iodide or alamarBlue).

The concentration of bacteria, termed colony-forming units (CFU) is commonly measured using absorbance (optical density) or McFarland unit. This information, together with the logarithmic phase of bacterial growth should be provided. If any washing step is done prior to diluting the bacterial solution, the washing agent used and method performed, e.g., centrifugation should be provided. The number of bacteria cells added in ratio to number of cells, referred as the multiplicity of infection (MOI) used in each experiment must be disclosed, ideally determined empirically by researchers for the cell type and bacterial strain used. Additionally, the type of media used for bacterial incubation in cells, presence of serum or other growth factors, together with time, temperature and presence of air for incubation needs to be specified.

Next, any extracellular bacteria remaining must be removed prior to conducting intracellular bacterial uptake and drug efficacy studies. This ensures reporting of efficacy results is based on the formulations' activity against intracellular bacteria and not planktonic

bacteria present outside of the cells or adhered to cell membrane. Several methods have been employed by researchers, including repeated washing, or using a gentamicin protection assay. Washing alone may not be effective to remove extracellular bacteria hence, the use of an antibiotic solution may be better suited. The type and concentration of antibacterial agent used, together with the time of incubation to kill extracellular bacteria must be disclosed. For *S. aureus* intracellular infection assay, gentamicin is commonly used to kill any presence of extracellular bacteria. However, internalization of gentamicin into host cells and alteration of the host physiology has been reported [96]. Alternatively, an enzyme protection assay using lysostaphin, a bacteriolytic enzyme is suggested for efficient killing of extracellular and host cell membrane bound bacteria. Washing methods after killing of extracellular bacteria should be described and the viability of host and bacterial cells should also be determined after establishing an intracellular infection.

5.2.3 Nanoantibiotic efficacy assessment method

Once an intracellular infection assay has been established following the above guideline, researchers can use this assay to conduct efficacy studies for their newly synthesized formulation. In this section, key recommendations are provided for treatment of infected cells and bacterial colony assessment method as a measure of efficacy.

5.2.3.1 Treatment of infected cells

When introducing an antibiotic or formulation to the cells, the suspending solution used for formulation (most often cell culture media free of antibiotic), concentration of formulation added together with dose of antibiotic delivered and time of incubation are important parameters to include. If the formulation is first dispersed using external force, such as sonication or vortex, this should be described in detail, including the time and frequency of such measurements used. Appropriate treatment controls should be introduced, including untreated cells, where only suspending solution, e.g., media or PBS were added to

the cells. Importantly, the infected cells should be treated with the non-formulated antibiotic (free drug), dosed at an equivalent concentration as the formulated antibiotic. If a hydrophobic antibiotic is used, authors should describe the solvent used for solubilization of the free drug (e.g., dimethyl sulfoxide) and provide final concentration of solvent introduced to cells. In this case, a separate control where cells are treated with only solvent should be considered, to ensure any decrease in bacterial count is not due to solvent effect.

After incubation of formulation for the desired time, cells are washed several times to remove any remaining formulation. The washing agent used, washing method and number of washes should be described clearly. Following this, cells are detached from wells usually with trypsin-EDTA solution and then exposed to conditions inducing cell lysis for extraction of remaining intracellular bacteria. Several methods have been previously described in literature, including the use of ice-cold Milli-Q water, cell lysis buffer or Triton-X. The cell lysis method, including concentration of reagent used (if any) must be described in detail. The extracted bacteria are then transferred to Eppendorf tubes and serially diluted for bacterial colony count.

5.2.3.2 Bacterial assessment method

Once the intracellular bacteria have been harvested and transferred, they are diluted to a desirable CFU for retrospective colony counting. Several methods can be used for assessing colony growth, including spot, or spread plating in an agar plate or pour plate method, where the sample is added to a plate followed by addition of liquid agar. Spot plating uses a small amount of the sample (usually 20-50 µL) which are susceptible to systemic errors. Hence, pour plate method using a lager sample volume (generally 500 µL to 1 mL) may be better suitable. For a detailed protocol of the different plating methods, readers are encouraged to read the article by Sanders [97]. The method used for bacterial colony count, type of agar plate, incubation time and conditions of agar plates should be reported. It is

important to note that some bacteria in the viable but nonculturable state may not be identified on agar plates. Therefore, macromolecular and cellular techniques such as real-time PCR (DNA), transcriptomic (RNA), metabolic activity (protein, lipid, luminescence) measurements, fluorescence-based imaging flow cytometry, and morphometric analyses (transmission and scanning electron microscopy) might be required in such cases [93, 98, 99]. As such, researchers should consider alternative methods to determine bacterial survival for a true representation of results obtained.

5.2.4 Statistical assessment and data presentation

When analyzing and reporting results, information on statistical and data analysis performed should be well described. Any methods used to process, normalize, or transform raw data should also be mentioned. The number of replicates, including technical and biological replicates, must be provided. Results should be displayed using appropriate visual illustrations, with mean and standard deviation, standard error or confidence interval provided in tandem. Researchers are encouraged to illustrate their results using scatter plots (for small samples) or box-and-whisker plot (for large samples), displaying individual data points for transparency and accurate representation of results [100]. Appropriate selection of statistical test and interpretation of results is important for valuable analysis of data obtained. The guideline by Greenland et al., provides detailed explanation and common misinterpretations of statistical test and analyses [101]. Finally, details of statistical package and version used for data analysis must be provided, including appropriate links and references of open-source platforms used for analysis.

5.3 Future directions

In vitro assays for intracellular infection studies are important for screening of suitable formulations that can enhance the site-specific delivery of antibiotics prior to further testing in in vivo models for clinical translation. However, these assays are difficult to

reproduce, given the intricate nature of cellular experiments. While the field is growing at an exponential rate, without standardization in methods and reproducibility of data, scientific conclusions across different experiments are hard to be drawn. Standardization of experiments provides direction for future studies within the field and adds to the quality and scientific rigor of the studies performed. Together, this facilitates advancement of in vitro studies conducted into meaningful clinical observations.

This guideline aims to help the scientific community address current gaps in literature, thus allowing observations across studies to be made, leading to either development of clinical products or advancement of current in vitro model or therapeutics in the field. While variation in results is inevitable across experiments, standardization in methods and results detailed helps reduces discrepancy across experiments conducted. For a standardization in method to be adopted by the community, authors should consider key parameters early on during the experimental design phase. Journal editors and reviewers also play a part in checking the thoroughness of experimental methods detailed in articles. To assist both authors and reviewers, a checklist of the guideline has been developed as illustrated in Table 4.

It is hopeful that this guideline serves as a discussion tool for researchers to help establish a consensus within the community. As the field continues to grow and advancement in technologies are made, we expect amendments and refinements in this guideline to help us grow our current understanding and knowledge together.

Table 4. Checklist for minimum information for conducting and reporting in vitro intracellular infection assays.

Experimental design
Material characterisation
Synthesis and purification
Size and zeta-potential
Drug loading and release
Toxicity profile
Cellular characterisation
Cell line
Passage number
Growth requirements o Media o Temperature o Aerobic, anaerobic or microaerophilic incubation o Humidity
Isolation method (for primary cells)
Bacterial characterisation
Bacterial strain reference
Growth conditions o Media o Temperature o Aerobic, anaerobic or microaerophilic incubation o Broth or agar used
Isolation method (for clinical isolates)
Intracellular infection *in vitro* model
Cell culture
Density of cells and confluency achieved
Plate dimension
Volume of growth media
Time of incubation
Bacterial culture
Inoculum preparation
Multiplicity of infection (MOI)
Media for incubation
Incubation time
Temperature and humidity
Aerobic, anaerobic or microaerophilic incubation
Removal of extracellular bacteria
Method used for removal of extracellular bacteria (e.g., washing only)
Concentration of antibiotic used (if any)
Time of incubation
Treatment of infected cells
Inclusion of controls

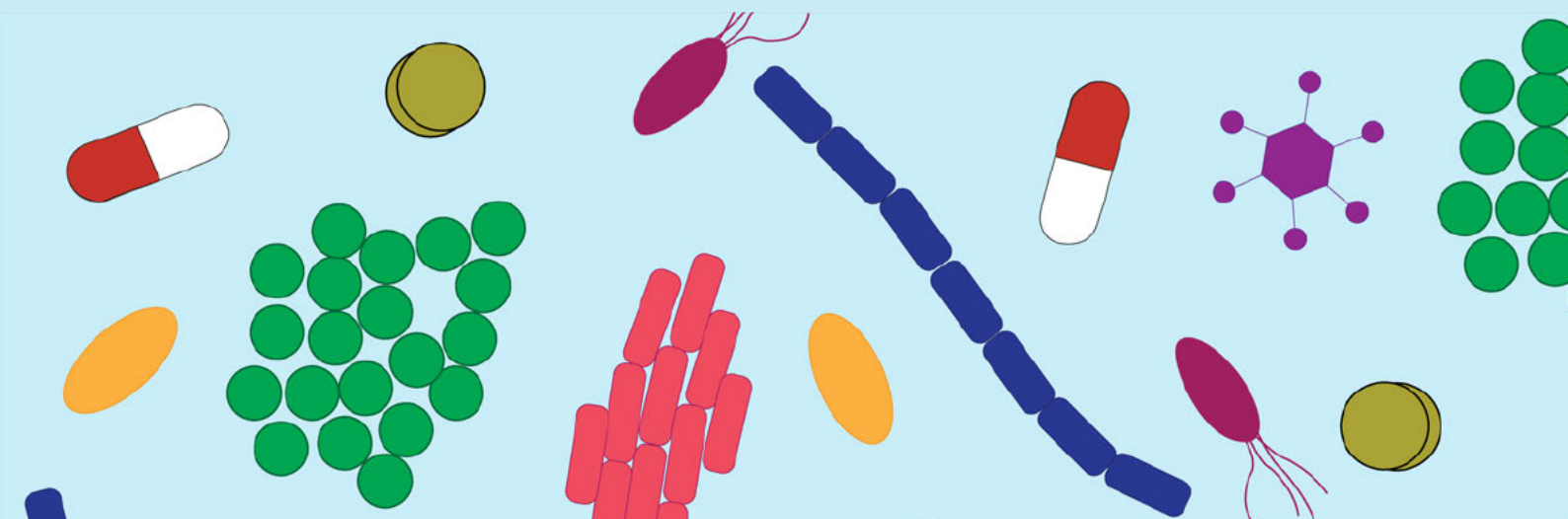
○ Untreated infected cells ○ Unformulated antibiotic at equivalent dose
Concentration of formulation or drug
Time of incubation
Solubilization agent (if any) or media used
Efficacy assessment method
Method for extraction of intracellular bacteria ○ Reagent and concentration used for cell lysis ○ Mechanical disruptors used (i.e., vortex)
Bacterial viability assessment ○ Colony count technique (i.e., spot plating, pour plate, etc.)
Statistical assessment
Data presentation
Biological and technical replicates
Statistical program and test used

5.4 Conclusions

A set of recommendations and guideline have been provided to assist the scientific community when conducting in vitro intracellular infection assays. Key parameters to disclose across each stage of the experiment, including formulation design, establishing an in vitro assay, evaluating performance of novel formulation and statistical assessment of results was provided. By adhering to this guideline, it is hopeful that discrepancies in future studies can be reduced, reported experiments are robust and scientific judgements across published studies can be made. Together, this will facilitate translation of current investigations into development of viable clinical therapeutics against intracellular infections.

6

SUMMARY AND CONCLUSIONS



6 Summary and Conclusions

Lamellar and non-lamellar lipid-based nanocarrier systems, i.e. liposomes and LCNPs, were investigated for intracellular delivery of antibiotics in two distinct pulmonary host cells: RAW 264.7 macrophages and A549 epithelial cells. Specifically, the influence of protein adsorption on nanoparticles and their subsequent consequence on cellular interaction, drug release behaviour and performance against intracellular bacteria was addressed. Differences in protein corona of the nanoparticles when utilising a highly biorelevant media (BALF vs FBS) were observed by utilising a proteomic approach, promoting current understanding of nanoparticle-protein adsorption behaviour. The potential of a cationic LCNPs as a delivery platform for antibiotics against intracellular infections was also elucidated.

It was hypothesized that an improved understanding of the nanoparticle's composition on cellular uptake, together with their behaviour and performance under biorelevant conditions would improve current understanding of nanoantibiotic delivery strategies for intracellular infections. Furthermore, it was hypothesized that the LCNPs (i.e. cubosomes) can be strategically engineered to improve their cellular uptake and subsequent activity against bacteria in a non-phagocytic cell line.

Summary of research findings

Chapter 1 provides a comprehensive literature review of into delivery strategies employed for intracellular infections. The survival mechanisms utilised by bacterial pathogens in phagocytic and non-phagocytic cell lines was reviewed, providing opportunities for bioinspired nanoantibiotic strategies that mimic the bacterial pathway for intracellular delivery. Accordingly, the current strategies utilised were critically appraised and limitations in advancing this field of research were elucidated. From this literature review, it was evident that the majority of investigations has been focused on phagocytic cell lines, with application for epithelial cells significantly less explored. Further, while liposomes have been widely

utilised as a delivery strategy, the use of LCNPs has not been explored for intracellular infections. In addition, the primitive nature of current in vitro assays was highlighted, including the limitation in understanding the role of protein corona in governing fate of nanoantibiotics, which hinders translation of preclinical investigations into meaningful outcomes.

Chapter 2 explored the interaction of liposome and LCNPs in RAW 264.7 murine macrophages and A549 lung epithelial cells, providing a comparison across phagocytic and non-phagocytic cell lines respectively. The influence of nanoparticle structure and composition on protein interaction using FBS present in cell media was investigated using a proteomics approach. Interestingly, the protein fingerprints of both nanoparticles were similar, attributed to the Vroman effect. However, their biological interaction differed significantly. The presence of a protein corona reduced cellular internalisation of liposomes by 2-fold in both cell lines. On the contrary, LCNPs uptake increased significantly in both cells in presence of proteins. Their differences in biological interaction despite similarities in protein corona composition suggest the structure and orientation of proteins adsorbed to nanoparticle surface play a critical role in interacting with cellular receptors for internalization. As a result of their serum-dependent uptake, the LCNPs were further investigated for their drug release behaviour and activity against intracellular SCV *S. aureus* in presence of a protein corona. LCNPs demonstrated a two-phase release kinetics for vancomycin in media supplemented with FBS, possibly due to restriction of vancomycin diffusion in presence of protein corona or binding of proteins to vancomycin released from LCNPs. Lastly, the enhanced cellular uptake correlated with improved antibacterial performance against SCV *S. aureus* in macrophages, reducing bacterial burden by 65%. However, a similar observation was not obtained in the epithelial cells and warrants further mechanistic studies to improve current knowledge on targeting bacteria in non-phagocytic cell.

Following confirmation that the presence of a protein corona significantly influences cellular interaction of nanoparticles, **Chapter 3** sets to further explore this phenomenon using murine BALF as a highly biorelevant media to mimic the fate of nanoparticles in pulmonary delivery. The presence of BALF did not affect internalisation of liposomes in epithelial cells, although uptake was diminished in macrophages. Similarly to previous observation in Chapter 2, LCNPs internalisation was positively influenced by BALF, reaching >90% uptake after 4 h. A time-dependent cellular uptake study was performed to further investigate nanoparticle uptake kinetics in presence of BALF. Liposomes demonstrated a rapid uptake, followed by exocytosis after 6 h incubation. A time and BALF concentration-dependent uptake was observed for LCNPs in both cells, suggesting activation of different internalisation mechanisms when a protein corona is present. Proteins isolated from the individual corona of liposomes and LCNPs were identified using a label-free quantitative proteomics approach. The proteins were characterized based on their physical characteristics, molecular function and biological process to further improve understanding of lipid nanoparticle interaction with biological molecules. LCNPs had a higher enrichment of proteins involved in cellular binding and regulatory process, thus correlating with their enhanced cellular uptake. Apolipoprotein H was highly present on LCNPs and is hypothesized to facilitate the cellular internalisation observed in macrophages and epithelial cells. Liposomes displayed an albumin-rich protein corona, resulting in rapid uptake across both cells, followed by exocytosis in macrophages. The insights derived from this chapter can improve development of therapeutics for a wide range of pulmonary diseases, including intracellular infections.

In **Chapter 4**, the potential of LCNPs as a drug delivery strategy against intracellular infections was further explored in both cells. Their internalisation in RAW264.7 and A549 was enhanced by strategically incorporating a cationic lipid, DDAB to their lipid matrix. The presence of DDAB (LCNP-DDAB) modified the distinctive honeycomb structure of LCNPs

into an onion-like organisation, with larger pore size due to membrane swelling from electrostatic interactions and steric stabilization of the lipid interfaces. Further, LCNPs as a platform to deliver a small and large antibiotic was demonstrated using tobramycin and vancomycin. The tobramycin release rate was independent of the two different LCNPs structures. On the other hand, vancomycin displayed a slower release from LCNP compared to tobramycin as their large molecular size limits diffusion across the interconnected water channels. However, the larger pore size of LCNP-DDAB accelerated their release kinetics to a greater extent. As hypothesized, LCNP-DDAB demonstrated increased cellular uptake, i.e. >50% and >80% in macrophages and epithelial cells, respectively. The presence of DDAB promotes stronger electrostatic interaction with the negatively charged cell membrane, thus promoting higher internalisation compared to their neutral counterpart. Following this, tobramycin and vancomycin encapsulated LCNPs were investigated for their antibacterial performance against intracellular *P. aeruginosa* and SCV *S. aureus*. In macrophages, LCNP-DDAB demonstrated the highest bacterial killing, 90% and 50% for *P. aeruginosa* and *S. aureus*, respectively at 4x MIC concentration. Similarly, tobramycin loaded LCNP-DDAB significantly reduced intracellular bacterial load compared to antibiotic only or delivered using LCNP. However, the performance of LCNP-DDAB against intracellular *S. aureus* in epithelial cells was suboptimal. Further investigations to determine the subcellular localization of nanoparticles and bacteria in epithelial cells can assist targeting using nanoantibiotics.

Lastly, **Chapter 5** sets to promote standardization in information reported by providing guidelines and recommendations for researchers when conducting in vitro assays for intracellular infections. This chapter sets to address one of the current limitations in advancing pre-clinical findings of nanoantibiotics as previously discussed in Chapter 1. It is hopeful that by following the sets of recommendations provided and disclosing important parameters in future investigations, the reproducibility of experiments will improve and

enable systematic comparison and better scientific judgement across studies. Together, it is hopeful that by adopting this guideline, the quality of studies performed will improve, leading to advancement in lab-to-bedside translation of future discoveries of therapeutics for intracellular infections.

Future directions

This thesis explored the biological limitations present in enhancing the performance of nanoantibiotics against intracellular infections. LCNPs as a promising carrier for multiple antibiotics to treat pulmonary infections was also investigated. Future directions of this research are outlined below.

Cellular entry pathway of nanoantibiotics

Investigating protein corona influence on cellular internalisation mechanism of nanoparticles, e.g., endocytic uptake via caveolin or clathrin-dependent pathways will be extremely valuable in deciphering the fate of nanoparticles for delivery of nanoantibiotics. The entry mechanism of nanoparticle dictates their ultimate destination in cells or their exocytosis profile into the extracellular milieu [102, 103]. Thus, identifying key proteins that regulate the cellular uptake pathways may hold benefits in ensuring efficient uptake and delivery of the antibiotics to site of infection.

Similarly, the fusogenic properties of neutral and cationic LCNP in presence of a protein corona needs further investigation. Previous study by Dyett et al., demonstrated the ability of LCNPs to fuse with model cell membrane in presence of cationic lipid and a protein corona [104]. Therefore, evaluating their fusion dynamics in a range of cell lines using different biorelevant media is necessary for future application of LCNPs.

Subcellular trafficking and localization

Following understanding of the uptake mechanism of lipid nanoparticles containing a protein corona, subcellular trafficking and localization of both, nanoparticles and bacteria in different cell types should be performed. As described in Chapter 2 and 4, the enhanced uptake of nanoparticles in A549 epithelial cells (up to 90%) did not translate to improved antibacterial activity against SCV *S. aureus*. In Chapter 1, the different strategies employed by bacterial pathogens for survival in phagocytic and non-phagocytic cell lines were discussed. The bacterial species and cell-type regulate their subcellular localization, including their presence in a membrane-bound vesicles.

FitzGerald et al., have previously demonstrated the ability to track and quantify biologics (DNA and protein) and nanoparticles in cells using a molecular sensor [105]. Utilising this approach to improve current understanding of bacterial survival and nanoantibiotic delivery will significantly advance development of future therapeutics, particularly for infections in non-phagocytic cells. Nanoparticles engineered for targeted site-specific delivery to the subcellular compartments with bacteria can be synthesised, improving treatment outcomes and reducing the dose-related side effects.

Time-dependent studies and survival kinetics

A limitation in the research presented is the inability to conduct time-dependent efficacy studies using the current in vitro models. Further, containment of bacteria intracellularly was difficult to achieve, limiting time-dependent investigations. As such, performance studies of nanoantibiotics were limited to 4 h, which prevent investigations on bacterial relapse and survival of host cells. Advancements in the development of a robust in vitro infection model is necessary and can guide optimization of nanoantibiotic dose for subsequent in vivo studies. Initial attempts to understand long-term cellular viability of infected cells under different treatments were conducted using the NanoLuc® luciferase enzyme (Figure 9).

LCNP-DDAB showed the brightest luminescence, indicating improved cellular viability, correlating linearly with their enhanced bacterial killing. However, further optimization of this method is necessary, as a steep decline in cellular viability was observed at ~18 h and a similar assay was not replicable in A549.

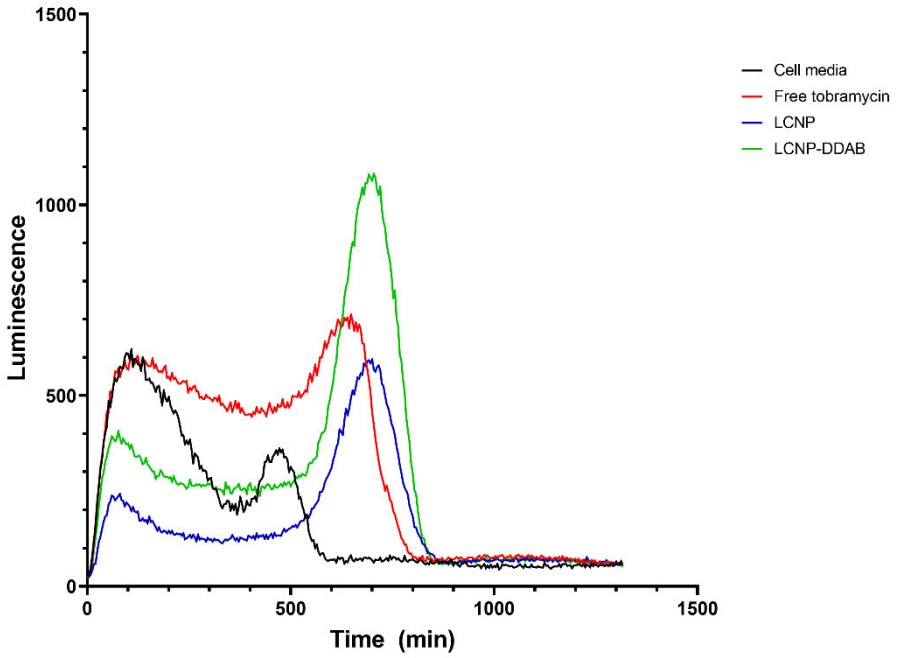


Figure 9. Cellular viability of RAW 264.7 infected with PA-01 over time under different treatments.

Penetration of nanoparticle through airway mucus and implication on intracellular delivery

Besides the protein corona, presence of airway mucus also presents as a barrier for intracellular uptake of nanoantibiotics. This is of a significant challenge in cystic fibrosis, where production of viscous mucus is upregulated. Mucus layers can negatively interact with inhaled particles, thus inhibiting the delivery of antibiotics to the site of interest. Hence, to understand the intracellular transport of lipid nanoparticles upon inhalation, their interaction with mucus must also be investigated. The composition of nanoparticles can be altered to improve particle penetration through mucus for intracellular delivery. For example, polymer nanoparticles were engineered with a lipid outer shell encapsulating siRNA to improve mucus penetration and cellular uptake in an in vitro

model [106]. The potential of LCNPs as a drug delivery system against pulmonary intracellular infections should also be evaluated in a similar manner.

Evaluating nanoantibiotic performance using a nebulization approach at air-liquid interface

While the intracellular infection assay performed in this thesis is useful for the screening of nanoparticle activity in a monoculture system, more sophisticated models are necessary to fully comprehend their behaviour in a clinically relevant setting. One such approach is by utilising the air-liquid interface (ALI) model, in which the nanoparticle deposition following aerosolization can be better represented. Juntke et al., have recently developed an ALI model using a cystic fibrosis cell line, CFBE41o- with the presence of mucus to evaluate the activity of ciprofloxacin encapsulated nanoparticles delivered via nebulization against PA-01 biofilm [107]. The development of such model for pulmonary intracellular infections, together with the presence of biorelevant lung fluid and mucus will validate the results obtained here under a highly biomimetic environment and provide further understanding on the use of nanoantibiotics.

Conclusion

Nanoantibiotics represent a promising strategy to improve the activity of currently available therapeutics against intracellular infections. However, translation of current technologies to clinical products remains challenging due to the complex biological barriers present prior to nanoparticle interaction with cells. This thesis explored some of the current biological limitations in using nanoparticles for intracellular infections by (i) providing a direct comparison of nanoparticle uptake in two different cell types with varied structure and physiological function, (ii) an understanding of the influence of protein corona on cellular uptake and activity of nanoantibiotics, (iii) the importance of using a biorelevant media to understand nanoparticle-protein interaction and (iv) employing a cationic lipid to enhance performance of LCNPs against intracellular bacteria. The insights derived from this thesis can be utilised to guide the development of an optimized drug delivery system for intracellular infection, including exploration of the pulmonary protein corona as a biological barrier.

Lessons learned

- The lipid nanoparticle structure and composition significantly influence cellular interaction. The physiological function of a cell can also directly influence fate of nanoparticles. While LCNPs were internalised in a phagocytic cell (macrophages), their uptake in epithelial cells was not achieved.

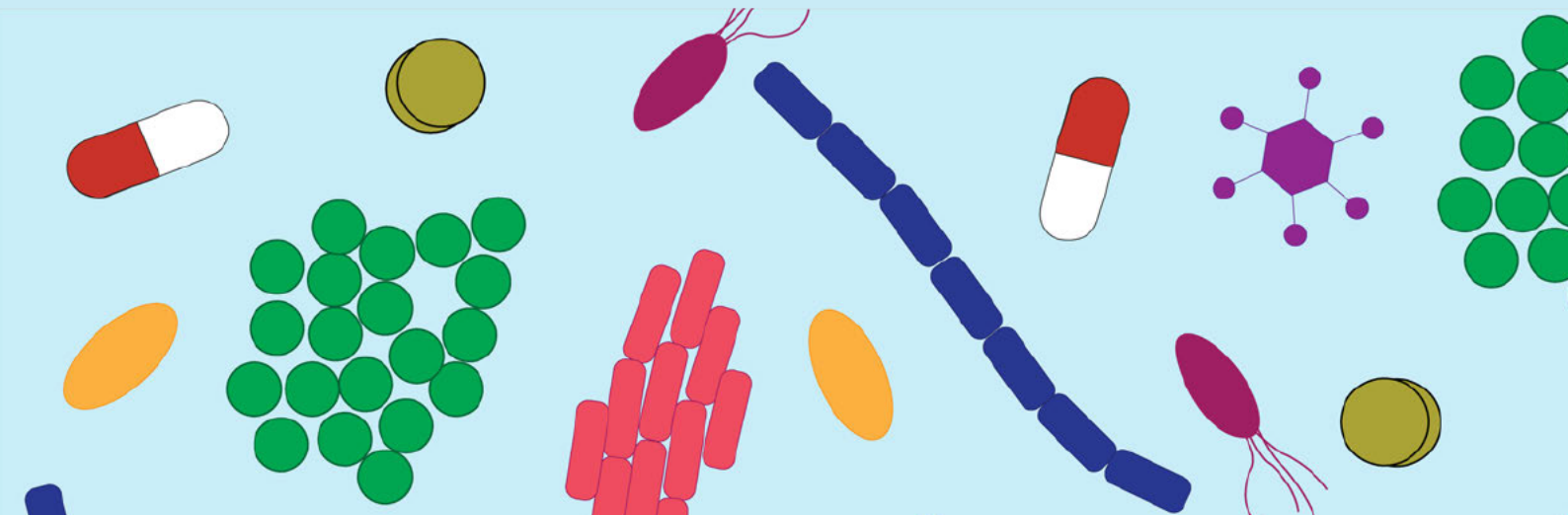
- Composition of lipid nanoparticle influences protein adsorption, which is inherently driven by the relative abundance of protein present in media. In FBS, the adsorption was driven by the Vroman effect resulting in a similar protein corona for both nanoparticle types. However, when incubated in BALF, the protein corona of nanoparticles differed significantly, with an enrichment of albumin and Apolipoprotein H observed in liposomes and LCNPs, respectively.

- Similarities in protein corona do not fully correlate with cellular uptake. The complexity of protein corona and nanoparticle behaviour with cells is yet to be fully understood. While protein corona composition can be similar, their configuration and molecular arrangement can mediate subsequent protein-protein and/or cellular receptor interaction, thus ultimately leading to a different and unpredictable biological response.

- Enhanced cellular uptake of lipid nanocarriers alone does not explicitly translate to improved antimicrobial activity against intracellular bacteria. As previously discussed, the localisation of bacteria in non-phagocytic cell lines needs further investigation to improve future development of nanoantibiotics.

- LCNPs present as a promising strategy for the delivery of antibiotics and their lipid composition can be modified for improved performance against intracellular bacterial pathogens.

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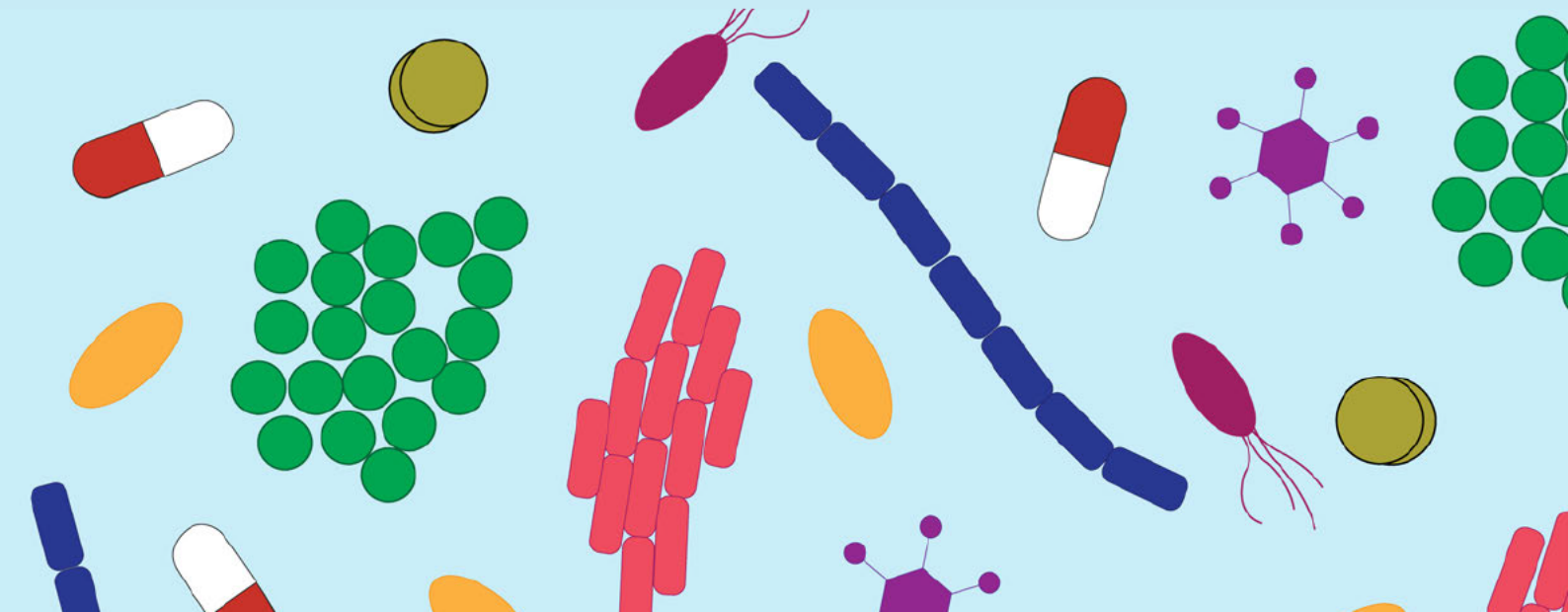
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Note: References contained within published papers are not included in the above list.

APPENDIX 1:

PUBLICATION CONTRIBUTIONS



Thesis Chapter 1

Subramaniam S, Joyce P, Thomas N, Prestidge CA. Bioinspired drug delivery strategies for repurposing conventional antibiotics against intracellular infections. *Advanced Drug Delivery Reviews* 2021; 177:113948.

The published version is available online at: https://doi.org/10.1016/j.addr.2021.113948

Author Contributions:

Author	Designed Research	Performed Research	Analysis of Data	Data Interpretation	Wrote Manuscript	Revised Manuscript
S Subramaniam	✓	✓	✓	✓	✓	✓
P Joyce						✓
N Thomas						✓
CA Prestidge						✓



Santhni Subramaniam _____



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Thesis Chapter 2

Subramaniam S, Joyce P, Prestidge CA. The cellular uptake and in vitro antibacterial activity of nanoantibiotics is influenced by protein corona. *Biomaterials Science* 2023; in preparation.

Author Contributions:

Author	Designed Research	Performed Research	Analysis of Data	Data Interpretation	Wrote Manuscript	Revised Manuscript
S Subramaniam	✓	✓	✓	✓	✓	✓
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Thesis Chapter 3

Subramaniam S, Joyce P, Donnellan L, Young C, Wignall A, Hoffmann P, Prestidge CA. Protein adsorption determines pulmonary cell uptake of lipid-based nanoparticles. *Journal of Colloid and Interface Science* 2023; 641: 36-47.

The published version is available online at: https://doi.org/10.1016/j.jcis.2023.03.048

Author Contributions:

Author	Designed Research	Performed Research	Analysis of Data	Data Interpretation	Wrote Manuscript	Revised Manuscript
S Subramaniam	✓	✓	✓	✓	✓	✓
P Joyce						✓
L Donnellan		✓	✓			✓
C Young		✓	✓			✓
A Wignall		✓				✓
P Hoffmann						✓
CA Prestidge						✓

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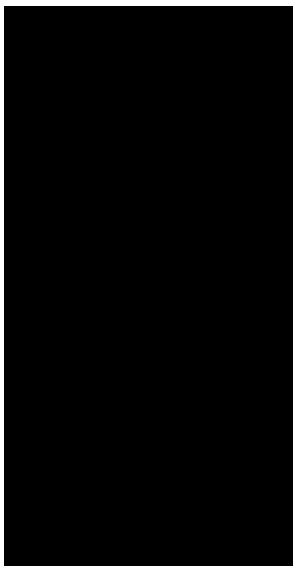
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Thesis Chapter 4

Subramaniam S, Joyce P, Prestidge CA. Liquid crystalline lipid nanoparticles improve the antibacterial activity of tobramycin and vancomycin against intracellular *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *International Journal of Pharmaceutics* 2023; 639: 122927.

The published version is available online at: https://doi.org/10.1016/j.ijpharm.2023.122927

Author Contributions:

Author	Designed Research	Performed Research	Analysis of Data	Data Interpretation	Wrote Manuscript	Revised Manuscript
S Subramaniam	✓	✓	✓	✓	✓	✓
P Joyce						✓
CA Prestidge						✓

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