Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhanced antibacterial activity of 18β-glycyrrhetinic acid and vancomycin against MRSA

By

Yajna Jaglal

(BSc Medical Science (Hon.) – University of KwaZulu-Natal)

Student number: 216000829

Dissertation submitted in fulfillment of the requirements for the degree of Master of Medical Science in the discipline of Pharmaceutical Sciences of the School of Health Sciences at the University of KwaZulu-Natal

Supervisor: Professor Thirumala Govender

(Ph.D., University of Nottingham, Nottingham, United Kingdom)

Co-supervisor: Dr Calvin Omolo

(Ph.D., University of Kwazulu-Natal, Durban, South Africa)

Date submitted: 30 November 2020

“The only way to do great work is to love what you do.”

- Steve Jobs-

"***This dissertation is dedicated to my grandparents, parents, brother, mentors and best friends for their undivided love, support and great advice.***

I appreciate you all."

Declaration 1 – Plagiarism

I, Miss Yajna Jaglal declare that

1. The research data reported in this dissertation, except where otherwise indicated is my original work.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain data, pictures, graphs, or other information belonging to other people unless specifically acknowledged as being sourced from other people.
4. This dissertation does not contain any other persons' writing unless specifically acknowledged as being sources from other researchers. Where other written sources have been quoted, then:
    a. Their words have been rephrased but the general information attributed to them has been referenced.
    b. Where their exact words have been used, their writing has been placed inside quotation marks and referenced.
5. Where I have reproduced a publication of which I am an author, co-author, or editor, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.
6. This dissertation does not contain any graphics, text or tables copied from the internet unless specifically acknowledged and the source being detailed in the reference sections of the dissertation.

Signed:  Date: 18th November 2020

I, Professor Thirumala Govender as supervisor of the M. Pharm study hereby consent to the submission of this M. Pharm dissertation.

Signed:  Date: 26 November 2020

I, Dr Calvin Omolo as co-supervisor of the M.Pharm study hereby consent to the submission of this M.Pharm dissertation.

Signed:  Date: 18th November 2020

Declaration 2 – Publications

Details of contribution to publications that form part and/or include research presented in this dissertation:

Yajna Jaglal, Nawras Osman, Calvin A. Omolo, Chunderika Mocktar, Nikita Devnarain, Thirumala Govender. Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhancement of antibacterial activity of vancomycin and 18β-glycyrrhetinic acid. SUBMITTED MANUSCRIPT. Reference number: IJP-S-20-04690

Miss Yajna Jaglal contributed to the project design and characterization of the novel nanoformulation. In addition, Miss Jaglal was responsible for the analysis and interpretation of all data, wrote the first draft of the paper and undertook all corrections. Mrs Nawras Osman assisted with formula optimization and characterization and *in vitro* drug release studies. Dr Calvin Omolo contributed to conceptualisation, design and formulation of the drug delivery system and supervision of the characterization and application studies and thesis and abstract editing. Dr Chunderika Mocktar supervised the *in vitro* antibacterial activity studies. Dr Nikita Devnarain conducted the cytotoxicity studies, *in silico* studies and editing. Prof Thirumala Govender served as supervisor and was responsible for project conceptualization, thesis and abstract editing and overall supervision of the study.

Research output from the dissertation

Submitted Manuscript

The following research paper was submitted to the International Journal of Pharmaceutics which has an impact factor of 4.845 from work done during this study.

Yajna Jaglal, Nawras Osman, Calvin A. Omolo, Chunderika Mocktar, Nikita Devnarain, Thirumala Govender. Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhancement of antibacterial activity of vancomycin and 18β-glycyrrhetinic acid. SUBMITTED MANUSCRIPT. Reference number: IJP-S-20-04690

* The submitted manuscript can be found in Chapter 3.

Abstract

Background: Due to the rise in antimicrobial resistance and the challenges accompanied by conventional antibiotic dosage forms, there is a need for developing drug delivery systems that enhance, protect and potentiate the current antibiotics in the market. Furthermore, natural derivatives from plants have proven to be potent antimicrobial agents. Therefore, their combination with antibiotics could be effective in overcoming antimicrobial resistance.

Aim: The aim of this study was to co-deliver vancomycin and 18β-glycyrrhetinic acid via pH-responsive lipid-polymer hybrid nanoparticles (VCM-GAPAH-LPHNPs) formulated from polyallylamine and oleic acid (OA) and to explore its potential for enhanced activity and targeted delivery.

Methods: Molecular dynamics and stability studies were used to determine the stability of the oil and water phases independently as well as VCM-GAPAH-LPHNPs as a complex. VCM-GAPAH-LPHNPs were prepared using the micro-emulsion technique. The size, polydispersity index and zeta potential of VCM-GAPAH-LPHNPs were determined using the dynamic light scattering technique. Transmission electron microscopy analysis was conducted to determine the morphology of VCM-GAPAH-LPHNPs. The entrapment efficiency and drug loading were determined using the ultrafiltration method. Differential scanning calorimetry was used to determine the thermal profiles of VCM-GAPAH-LPHNPs and its components. *In vitro* drug release studies were performed using the dialysis bag technique. Drug release kinetics were analysed using the DDSolver program. Cytotoxicity of VCM-GAPAH-LPHNPs were determined using the MTT assay. Haemolysis of VCM-GAPAH-LPHNPs were performed at different concentrations using sheep blood. *In vitro* antibacterial activity of VCM-GAPAH-LPHNPs were determined against SA and methicillin-resistant *Staphylococcus aureus* (MRSA) at pH 6 and 7.4. Time killing assay was performed using the plate colony count method. MRSA biofilm study was performed using the crystal violet assay.

Results: Molecular dynamics indicated VCM-GAPAH-LPHNPs to be stable. VCM-GAPAH-LPHNPs were successfully prepared using the micro-emulsion technique. VCM-GAPAH-LPHNPs size, polydispersity index, zeta potential and encapsulation

efficiency were found to be 198.4 ± 0.302 nm, 0.255 ± 0.003, - 3.8 ± 0.335 mV and 69.46 ± 2.52 % respectively. Thermal profiles of lyophilized VCM-GAPAH-LPHNPs showed transformation from crystallization to amorphous form. *In vitro* drug release studies revealed that VCM-GAPAH-LPHNPs released 60% of VCM after 24 h whereas bare VCM released 90% of VCM after 24 h hence VCM-GAPAH-LPHNPs showed sustained drug release compared to bare VCM. At pH 6 VCM-GAPAH-LPHNPs released 82% of VCM after 24 h whereas at pH 7.4 VCM-GAPAH-LPHNPs released 60% of VCM after 24 h indicating VCM-GAPAH-LPHNPs had a faster drug release at pH 6 compared to pH 7.4. The Weibull model was considered the best fit model for VCM-GAPAH-LPHNPs. The MTT assay revealed 75% > cell viability which indicated VCM-GAPAH-LPHNPs to be non-cytotoxic. At 0.5 mg/ml VCM-GAPAH-LPHNPs showed < 1% haemolysis. Stability studies at 4 °C and room temperature indicated VCM-GAPAH-LPHNPs to be stable. *In vitro* antibacterial activity against MRSA treated with VCM-GAPAH-LPHNPs demonstrated a 16-fold lower minimum inhibitory concentration than bare VCM at acidic conditions. The time-killing assay study at 12 h revealed that VCM-GAPAH-LPHNPs eliminated 100% of MRSA cells whereas bare VCM eliminated 55% of MRSA cells. The crystal violet assay analysis revealed VCM-GAPAH-LPHNPs ability to eliminate MRSA biofilms.

Conclusion: VCM-GAPAH-LPHNPs could effectively treat MRSA infections at a faster rate as compared to bare VCM. Therefore, this novel pH-responsive LPHNPs may serve as a promising nanocarrier for enhancing antibiotic delivery and antibacterial activity.

Keywords: pH-responsive; lipid-polymer hybrid nanoparticles; vancomycin; methicillin-resistant *Staphylococcus aureus*; 18β- glycyrrhetinic acid

Acknowledgments

I would like to express my sincere gratitude to those without whom the completion of this project would not have been possible.

- To my supervisor, Professor Thirumala Govender, your novelty, understanding, guidance and advice have been an inspiration to me. Pursuing my master's degree under your supervision will brighten me personally, socially and professionally. Thank you for being the best supervisor I could ever have.
- To my mentor, Nawras Osman, it has been a truly wonderful experience working with you and learning from you. Thank you for always being patient and understanding towards me, believing in me and encouraging me to work consistently. I appreciate your guidance and valuable advice. Thank you for being a ray of sunshine on a daily basis. It would not have been possible for me to pursue and complete this work without you.
- To my co-supervisor, Dr Calvin Omolo, thank you for your excellent supervision, problem-solving skills and valuable scientific inputs.
- To Dr Chunderika Mocktar, thank you for your assistance, care, support and guidance.
- To Dr Nikita Devnarain, thank you for your assistance, guidance and valuable advice.
- To my colleagues, Leslie Murugan, Usri Ibrahim and Mohammed Abdeen Salih, it was indeed a wonderful chance to work with you all. Thank you for your assistance and support.
- My sincere thanks go to Electron Microscope Unit and Biomedical Resource Unit at UKZN for technical support.
- I would like to show my profound gratitude to the National Research Foundation, UKZN Nanotechnology Platform and UKZN College of Health Sciences for their support and financial assistance to complete this Master's project successfully and brighten my career.

Table of content

List of acronyms

Ala	Alanine	PAH	Polyallylamine hydrochloride
CACO-2	Human intestinal epithelial cancer cells	PDI	Polydispersity index
CFU	Colony forming units	PGA	Polyglycolides
CO_2	Carbon dioxide	PLA	Polylactides
CV	Crystal violet	PLGA	Polylactide co-glycolides
DL	Drug loading	PNPs	Polymeric nanoparticles
DLS	Dynamic light scattering	PBS	Phosphate-buffered saline
DSC	Differential scanning calorimetry	RBCs	Red blood cells
EE	Encapsulation efficiency	RMSD	Root-mean-square deviation
GA	Glycyrrhetinic acid	RMSE	Root-mean-square error
HepG2	Human liver adenocarcinoma cells	RMSF	Root-mean-square-fluctuation
HPLC	High-performance liquid chromatography	SA	*Staphylococcus aureus*
LPHNPs	Lipid-polymer hybrid nanoparticles	SEM	Scanning electron microscopy
MD	Molecular dynamics	SLNs	Solid lipid nanoparticles
MDR	Multidrug resistance	TB	Tuberculosis
MHA	Mueller Hinton Agar	TEM	Transmission electron microscopy
MHB	Mueller Hinton broth	TFA	Trifluoroacetic acid
MIC	Minimum inhibitory concentration	UA	Uranyl acetate
MRSA	Methicillin-resistant *Staphylococcus aureus*	UV	Ultraviolet
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	VCM	Vancomycin
NDDS	Nano drug delivery systems	XDR	Extreme drug resistance
OA	Oleic acid	ZP	Zeta potential

Table of figures

List of tables

Chapter One

Introduction

Chapter One - Introduction

1.1 Introduction

This chapter provides a brief background of the study including the crisis of infectious diseases, limitations of conventional antibiotic therapy and the threat of a post-antibiotic era. It further discusses the advantages of pH-responsive nanodrug delivery systems and their role in combatting antibiotic resistance. This is followed by the aims, novelty and significance of the study and concludes with an overview of the dissertation.

1.2 Background

Infectious diseases, particularly bacterial infections are considered one of the primary causes of morbidity and mortality worldwide, despite influential research advancements (1). Recent data indicates that by 2050 there will be 10 million annual deaths caused by bacterial infections (2). Antibiotics are designed to inhibit or stop bacterial infections and have revolutionized the treatment of infectious diseases via their bacteriostatic and bactericidal effects (3). However, conventional antibiotic dosage forms need to be administered regularly for a sustained period to maintain adequate concentrations at target sites of infection (4) and to avoid persistent infections due to the development of antibiotic resistance (5). Furthermore, the high dose of antibiotics being administered leads to harmful side effects (6, 7), poor pharmacokinetic properties (6), a burden on the healthcare sector and treatment costs and poor patient compliance (8).

Such disadvantages, compounded by incorrect usage and exploitation of antibiotics (1, 9) have led to an antibiotic-resistant era (9). Antibiotic-resistant and multidrug-resistant (MDR) bacteria such as MRSA pose a greater threat to humankind than ever imagined (10). MRSA is a common pathogen present in community and hospital-acquired infections (11) and contributes to the development of sepsis (12, 13), peritonitis (13), endocarditis and bacteraemia (14). The prolonged discovery and advancement of novel antibiotics, as well as high production costs, emphasize the need to strategically introduce new dimensions to suppress the rapid increase of bacterial resistance to antibiotics and limitations of conventional antibiotics (15, 16). Nanoengineered antibiotic delivery systems have been reported as a promising approach to overcome the restrictions of conventional antibiotics and resistance of bacteria (17, 18). Nano drug delivery systems (NDDS) are defined as biocompatible, nanosized materials having a large surface area to mass ratio (19). There are several advantages that NDDS have over

conventional dosage forms of antibiotics, these include targeted antibiotic delivery to infection sites, enhanced localisation of the antibiotic within target tissues (20), improved pharmaceutical stability (21), improved antibiotic solubility, improved cellular absorption, sustained antibiotic release (22), improved patient compliance and reduced side effects (23).

Some nanosystems that have been explored as NDDS for antibiotics include liposomes (24), polymeric nanoparticles (PNPs) (25), lipid polymer hybrid nanoparticles (LPHNPs) (26), dendrimers (27), solid lipid nanoparticles (SLNs) (28) and micelles (29). Of these NDDS, lipid-polymer hybrid nanoparticles (LPHNPs) have shown major potential as antibiotic nanocarriers with enhanced antibacterial efficacy. They were first proposed in the late 1970s as a promising antibiotic delivery system (30) and are composed of biocompatible/biodegradable polymers, whereby the antibiotic is solubilized, encased or anchored to the outer surface of nanoparticles (31). There are many advantages of LPHNPs, which include improved concentration of antibiotics at target infection sites thus improving drug safety (32), protection of the antibiotic (23), reduced premature drug release before arrival at the infection site, controlled and sustained antibiotic release, improved cell penetration and solubility (33), cost-effective upscaled production using controlled polymerization techniques (34), improved stability of volatile chemical substances (35), antimicrobial properties (36) and biocompatibility with tissues (31-34, 36). Recently LPHNPs have been employed to deliver the messenger RNA from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for creation of the vaccine against the disease (37). Therefore, LPHNPs are efficient and effective drug delivery nanosystems (38).

Spurred by the advancements in pharmaceutical chemistry and drug delivery, stimuli-responsive NDDS are of recent interest. Researchers are committed to developing stimuli-responsive nanosystems (39) to heighten the efficiency and effectiveness of NDDS. Stimuli-responsive antibiotic delivery systems are designed for optimal response to endogenous stimuli (i.e., enzymes, redox potential, ionic microenvironment and pH levels) and exogenous stimuli (i.e., temperature, ultrasound, electric, mechanical, light and magnetic fields) at an infection site, allowing targeted antibiotic release, improved antibiotic accumulation as well as enhanced bioavailability at the target site (39, 40). Among these stimuli, pH-responsive NDDS have become extremely popular in the literature. The significance of pH-responsive NDDS lies in their ability to deliver antibiotics when minor pH changes according to the pathophysiology properties of the disease are presented, resulting in enhanced therapeutic efficacy and patient compliance (40, 41). Cancer and infectious diseases due to bacterial infection, like tuberculosis,

present with changes towards an acidic pH, which varies from the physiological pH of 7.4 (41, 42). Effective pH-responsive nanosystems such as pH-responsive liposomes (24), polymeric nanoparticles (30) and micelles (29) aim at targeting acidic pH conditions (42). pH-sensitive NDDS have been reported for the delivery of anti-cancer (41-43) and antibacterial (44-46) agents at targeted infection sites. However, limited research has been done on pH-sensitive nano drug delivery to bacterial infection sites, specifically that of MRSA, which has the potential to massively influence infectious disease treatment by improved and targeted drug delivery to acidic infection sites and curb the limitations of conventional antibiotics (47, 48).

Currently, LPHNPs are being employed to efficiently deliver drugs, making them promising nanosystems (49). LPHNPs overcome the above-mentioned problems of conventional antibiotic dosage forms and antimicrobial resistance by combining the structural components of PNPs and liposomes (50). LPHNPs are core-shell self-assembled drug delivery systems fabricated from a hydrophobic lipid core and a polymeric matrix (51). The biofunctional properties of LPHNPs allow the formulation of a system that is stimuli-responsive (52). This is attained by focusing on the properties of the nanoparticle matrix in order to establish targeted drug delivery and enhanced antibacterial activity (53). The development in the field of nanotherapeutics and material sciences has stimulated the advancement of pH-sensitive nanosystems for effective and efficient antibiotic delivery (54, 55). Several pH-responsive LPHNPs have been reported for antibacterial and anticancer studies (56-58). However, there is limited literature on employing pH-sensitive LPHNPs for antibiotic delivery. Therefore, using this approach, systems can be fabricated to have programmable destabilization and drug release due to pH changes that correspond with bacterial infection sites (58, 59).

The bioactive phytochemical composites found in plants and natural plant derivatives possess a broad spectrum of activity, 18β-glycyrrhetinic acid (GA) is such a natural derivative (60). GA is the hydrolyzed product of glycyrrhizic acid which is derived from the *Glycyrrhiza glabra* (liquorice) plant (61).This biologically active compound has been reported to have anti-allergic (62), antibacterial, antiviral, antitumor and anti-inflammatory characteristics (63, 64). Due to the antibacterial activity of GA, it can be explored by co-delivery with antibacterial drugs in the market for synergistic action (63, 65).

Literature reports indicate the combinational therapy of antibiotics in the market and natural plant derivatives that have shown to improve antibacterial activity (66). However, there are no studies of GA being co-delivered with antimicrobial drugs in a NDDS to target bacterial

infections. Therefore, the combined delivery of GA and antibiotics can further be explored by co-loading in a stimuli-responsive nanosystem for targeted and enhanced antimicrobial activity (67). Such a system will also provide scientific advancement to the field of pharmaceutics.

There are numerous reports of GA being co-delivered with cancer and inflammatory drugs in nanosystems for the treatment of cancer diseases (68-70). However, from our search of the literature, such a formulation for co-delivery of GA and antibiotics has not been done before. Thus, in this study, we propose the co-delivery of GA and VCM via polyallylamine hydrochloride (PAH) LPHNPs for enhanced and targeted antibiotic delivery. The pH-responsive LPHNPs matrix consists of PAH and oleic acid (OA). We envisage the pH-response will stem from the ionisable carboxylic acid groups of OA and primary amino groups of PAH. At a basic pH, carboxylic acid groups deprotonate and form electrostatic bonds with the anime groups of PAH. Thus, the system will have an overall negative charge. Consequently, at acidic conditions, both the amine and carboxylic groups will protonate, the carboxylic group will have a slightly positive charge that will repel the highly positive charge of PAH amine groups thus LPHNPs charge shift from negative to positive charge, followed by cleavage of the electrostatic bond, ultimately resulting in swelling of LPHNPs and faster release of drugs. Additionally, the positive charge of PAH polymer may promote attachment to the negatively charged bacterial cell wall, thus enhancing antibacterial activity. Such a system to the best of our knowledge has not been reported before. Moreover, LPHNPs can encapsulate both hydrophilic and hydrophobic drugs, in this system GA is co-delivered with VCM. Such a strategy has not been reported before for delivery of antibiotics with bioactive plant by-products. Therefore, this study will report novel multifunctional LPHNPs that are pH- responsive for co-delivery of GA and VCM.

1.3 Problem statement

Globally, bacterial infections are a significant problem in the healthcare sector. This is due to the several disadvantages of current treatment regimes, including conventional dosage forms of current antibiotics, low drug concentrations at infection sites, frequent high dosages, poor pharmacokinetics, poor patient compliance and adverse side effects. Such restrictions have resulted in the birth of an antibiotic-resistant era that is associated with a rise in mortality and morbidity rates. The advancement of new antimicrobials is deteriorating, urging the design and discovery of novel approaches to improve current antibiotic treatment. The establishment of effective pH-responsive antibiotic co-delivery nanosystems can obtain targeted delivery at

infection sites, thereby enhancing antibiotic therapy. Ultimately, the development of pH-responsive co-delivery systems is required to overcome the challenges associated with current antibiotic dosage forms and antimicrobial resistance.

1.4 Hypothesis

We hypothesize that the co-delivery of GA and VCM via pH-responsive PAH-LPHNPs formulation can enhance its antibacterial activity.

1.5 Aims and objectives

The aim of this study was to identify the potential of the co-delivery of GA and VCM via pH-responsive PAH lipid-polymer hybrid nanoparticles (GAPAH-LPHNPs) for enhancing antibacterial activity.

The objectives of the study were:

1. To synthesize novel pH-responsive GAPAH lipid-polymer hybrid nanoformulation encapsulating VCM and GA.

2. To optimize and characterize VCM-GAPAH-LPHNPs in terms of particle size, polydispersity index (PDI), zeta potential (ZP), pH-responsiveness, morphology, entrapment efficiency and *in vitro* drug release.

3. To assess *in vitro* antibacterial activity of VCM-GAPAH-LPHNPs against SA and MRSA.

4. To identify time-killing kinetics against MRSA.

5. To perform crystal violet assay analysis against MRSA biofilms.

1.6 Novelty of the study

The research conducted in this study is novel for the following reasons:

- This study reports for the first time LPHNPs coined from PAH and OA.
- This study reports the synthesis and characterization of multifunctional LPHNPs designed for pH-responsive co-delivery of VCM and GA, which have not been previously reported in the literature.
- This work also reports for the first time the co-delivery of an antibiotic and bioactive metabolic compound that enhanced the activity of the antibiotic in the market.

1.7 Significance of the study

These reported pH-responsive LPHNPs for co-delivery of GA and VCM present a novel and promising avenue for targeting acidic infection sites, thus enhancing drug localisation within target tissues, reducing concentration of the dosage needed for ideal treatment, improving antibiotic properties and preventing the occurrence of bacterial resistance. Ultimately, this results in minimal side effects and improves patient adherence to treatment. The potential significance of this study is mentioned below:

Advanced nanomedicine

This study proposes the novel pH-responsive LPHNPs for co-delivery of an antibiotic and natural compound formulation as a medicine. This nanomaterial and medication can play a role in pharmaceutical companies in the development of novel pH-responsive drug delivery vehicles that could be more effective than conventional antibiotics.

Advanced patient and disease treatment

This novel nanocarrier has the potential to advance bacterial infection treatment by allowing targeted and controlled release at infection sites, enhancing drug localization and bioavailability at acidic infection site thus, contributing to the improvement of antibacterial properties, reduction of dosage frequency and minimal side effects, which ultimately improves patient adherence and defeats threats against antibacterial resistance.

Creating novel scientific knowledge

This study can identify new scientific advancements in the preparation and characterization of the combinational delivery of antibiotics and natural plant compounds of pH-responsive lipid-polymer hybrid nanoparticles and their potential in the world of nanotechnology. Thereby combinational delivery may contribute to the synthesis of effective and novel nano-drug delivery systems and enhance their capability within pharmaceutical applications.

Stimulation of advanced research

This study can provide novel avenues for the preparation and characterization of pH-responsive LPHNPs for the co-delivery of antibiotics and natural compounds for their potential applications in pH-responsive nanosystems formulation for several antibiotic classes and other natural, bioactive compounds.

1.8 Overview of the dissertation

The research is presented in the following chapters:

Chapter One - Introduction:

This chapter provides a concise background of the study including the burden of infectious diseases on the healthcare sector, limitations of conventional antibiotic therapy and the threat of a post-antibiotic era. It further discusses the advantages of pH-responsive nanodrug delivery systems and their role in combatting antibiotic resistance. This is followed by the aims, novelty and significance of the study and concludes with an overview of the dissertation.

Chapter Two - Literature Review:

This chapter provides an overview of the burden of ID on the healthcare system and antibiotic therapy limitations that have led to the development of bacterial resistance. It also provides an overview of lipid-polymer hybrid nanoparticle characteristics, preparation and characterization technique, outlines the use of pH-responsive NDDS as a strategy to influence the treatment of infectious diseases and concludes with an overview of vancomycin as a model drug.

Chapter Three - Submitted manuscript:

This chapter is a first author article that was submitted to an international ISI journal i.e. International Journal of Pharmaceutics (Impact factor of 4.845). The chapter is presented in the required format of the journal. It describes the synthesis of novel VCM-GAPAH lipid-polymer hybrid nanoparticles. It also highlights the *in vitro* cytotoxicity evaluation, haemolytic study, formulation of the pH-responsive LPHNPs (VCM-GAPAH-LPHNPs) for targeted delivery of VCM and characterization of its physical and antibacterial properties via *in silico* studies and *in vitro* studies.

Chapter Four – Conclusions:

This chapter describes the conclusions reached in achieving the study aim, objectives, outlines the significance of the findings and provides future recommendations for further scientific research into antibiotic and natural compound co-delivery of pH-responsive lipid-polymer hybrid nanoparticles.

1.9 References

1. Kalhapure RS, Suleman N, Mocktar C, Seedat N, Govender T. Nanoengineered drug delivery systems for enhancing antibiotic therapy. Journal of Pharmaceutical Sciences. 2015;104(3):872-905.
2. Gupta A, Mumtaz S, Li C-H, Hussain I, Rotello VM. Combatting antibiotic-resistant bacteria using nanomaterials. Chemical Society Reviews. 2019;48(2):415-27.
3. Grenni P, Ancona V, Caracciolo AB. Ecological effects of antibiotics on natural ecosystems: A review. Microchemical Journal. 2018;136:25-39.
4. Santos R, Ruza D, Cunha E, Tavares L, Oliveira M. Diabetic foot infections: Application of a nisin-biogel to complement the activity of conventional antibiotics and antiseptics against Staphylococcus aureus biofilms. PloS one. 2019;14(7):e0220000.
5. Hall CW, Mah T-F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiology Reviews. 2017;41(3):276-301.
6. Martinez MN, Papich MG, Drusano GL. Dosing regimen matters: the importance of early intervention and rapid attainment of the pharmacokinetic/pharmacodynamic target. Antimicrobial Agents and Chemotherapy. 2012;56(6):2795-805.
7. Rehman K, Kamran SH, Akash MSH. Toxicity of antibiotics. Antibiotics and antimicrobial resistance genes in the environment: Elsevier; 2020. p. 234-52.
8. Boateng J. Drug delivery innovations to address global health challenges for pediatric and geriatric populations (through improvements in patient compliance). Journal of Pharmaceutical Sciences. 2017;106(11):3188-98.
9. Denny KJ, De Wale J, Laupland KB, Harris PN, Lipman J. When not to start antibiotics: avoiding antibiotic overuse in the intensive care unit. Clinical Microbiology and Infection 2020;26(1):35-40.
10. Bassetti M, Poulakou G, Ruppe E, Bouza E, Van Hal SJ, Brink A. Antimicrobial resistance in the next 30 years, humankind, bugs and drugs: a visionary approach. Intensive Care Medicine. 2017;43(10):1464-75.
11. Kavanagh KT. Control of MSSA and MRSA in the United States: protocols, policies, risk adjustment and excuses. Antimicrobial Resistance and Infection Control. 2019;8(1):1-8.
12. Hos NJ, Jazmati N, Stefanik D, Hellmich M, AlSael H, Kern WV, et al. Determining vancomycin Etest MICs in patients with MRSA bloodstream infection does not support switching antimicrobials. Journal of Infection. 2017;74(3):248-59.
13. Jiang X, Wang Y, Qin Y, He W, Benlahrech A, Zhang Q, et al. Micheliolide provides protection of mice against Staphylococcus aureus and MRSA infection by down-regulating inflammatory response. Scientific reports. 2017;7:41964.
14. Tianshui L, Ying Q, Li D, Fayong L, Guoguang X. High-dose teicoplanin combined with daptomycin cured infective endocarditis and septic pulmonary embolism caused by methicillin-resistant Staphylococcus aureus: A case report and literature review. Burns Open. 2019;3(3):126-8.
15. Durand GA, Raoult D, Dubourg G. Antibiotic discovery: History, methods and perspectives. International Journal of Antimicrobial Agents. 2019;53(4):371-82.
16. York A. New drugs for the antibacterial pipeline? Nature Reviews Microbiology. 2020;18(2):61.
17. Choi M, Hasan N, Cao J, Lee J, Hlaing SP, Yoo J-W. Chitosan-based nitric oxide-releasing dressing for anti-biofilm and in vivo healing activities in MRSA biofilm-infected wounds. International Journal of Biological Macromolecules. 2020;142:680-92.

18. Hasan N, Cao J, Lee J, Hlaing SP, Oshi MA, Naeem M, et al. Bacteria-targeted clindamycin loaded polymeric nanoparticles: effect of surface charge on nanoparticle adhesion to MRSA, antibacterial activity, and wound healing. Pharmaceutics. 2019;11(5):236.
19. Jamkhande PG, Ghule NW, Bamer AH, Kalaskar MG. Metal nanoparticles synthesis: An overview on methods of preparation, advantages and disadvantages, and applications. Journal of Drug Delivery Science and Technology. 2019;53:101174.
20. Zhu YJ, Chen F. pH-responsive drug-delivery systems. Chemistry–An Asian Journal. 2015;10(2):284-305.
21. Rai VK, Mishra N, Yadav KS, Yadav NP. Nanoemulsion as pharmaceutical carrier for dermal and transdermal drug delivery: Formulation development, stability issues, basic considerations and applications. Journal of Controlled Release. 2018;270:203-25.
22. Son G-H, Lee B-J, Cho C-W. Mechanisms of drug release from advanced drug formulations such as polymeric-based drug-delivery systems and lipid nanoparticles. Journal of Pharmaceutical Investigation. 2017;47(4):287-96.
23. Sur S, Rathore A, Dave V, Reddy KR, Chouhan RS, Sadhu V. Recent developments in functionalized polymer nanoparticles for efficient drug delivery system. Nano-Structures and Nano-Objects. 2019;20:100397.
24. Alshamsan A, Aleanizy FS, Badran M, Alqahtani FY, Alfassam H, Almalik A, et al. Exploring anti-MRSA activity of chitosan-coated liposomal dicloxacillin. Journal of microbiological methods. 2019;156:23-8.
25. Norouz Dizaji A, Ding D, Kutsal T, Turk M, Kong D, Piskin E. In vivo imaging/detection of MRSA bacterial infections in mice using fluorescence labelled polymeric nanoparticles carrying vancomycin as the targeting agent. Journal of Biomaterials Science, Polymer Edition. 2020;31(3):293-309.
26. Thakur K, Sharma G, Singh B, Chhibber S, Katare OP. Nano-engineered lipid-polymer hybrid nanoparticles of fusidic acid: an investigative study on dermatokinetics profile and MRSA-infected burn wound model. Drug Delivery and Translational Research. 2019;9(4):748-63.
27. Holmes AM, Heylings JR, Wan K-W, Moss GP. Antimicrobial efficacy and mechanism of action of poly (amidoamine)(PAMAM) dendrimers against opportunistic pathogens. International Journal of Antimicrobial Agents. 2019;53(4):500-7.
28. Casadei MA, Cerreto F, Cesa S, Giannuzzo M, Feeney M, Marianecci C, et al. Solid lipid nanoparticles incorporated in dextran hydrogels: a new drug delivery system for oral formulations. International Journal of Pharmaceutics. 2006;325(1-2):140-6.
29. Groo A-C, Matougui N, Umerska A, Saulnier P. Reverse micelle-lipid nanocapsules: a novel strategy for drug delivery of the plectasin derivate AP138 antimicrobial peptide. International Journal of Nanomedicine. 2018;13:7565.
30. Bolhassani A, Javanzad S, Saleh T, Hashemi M, Aghasadeghi MR, Sadat SM. Polymeric nanoparticles: potent vectors for vaccine delivery targeting cancer and infectious diseases. Human Vaccines and Immunotherapeutics. 2014;10(2):321-32.
31. Nagavarma B, Yadav HK, Ayaz A, Vasudha L, Shivakumar H. Different techniques for preparation of polymeric nanoparticles-a review. Asian Journal of Pharmaceutical and Clinical Research. 2012;5(3):16-23.
32. Ahmad N, Ahmad R, Alam MA, Ahmad FJ, Amir M, Pottoo FH, et al. Daunorubicin oral bioavailability enhancement by surface coated natural biodegradable macromolecule chitosan based polymeric nanoparticles. International Journal of Biological Macromolecules. 2019;128:825-38.
33. Mehraban N, Musich PR, Freeman HS. Synthesis and encapsulation of a new zinc Phthalocyanine photosensitizer into polymeric nanoparticles to enhance cell uptake and Phototoxicity. Applied Sciences. 2019;9(3):401.

34. Jurov A, Popović D, Rakić IŠ, Marion ID, Filipič G, Kovač J, et al. Atmospheric pressure plasma jet–assisted impregnation of gold nanoparticles into PVC polymer for various applications. The International Journal of Advanced Manufacturing Technology. 2019;101(1-4):927-38.
35. Jayawardhana AM, Qiu Z, Kempf S, Wang H, Miterko M, Bowers DJ, et al. Dual-action organoplatinum polymeric nanoparticles overcoming drug resistance in ovarian cancer. Dalton Transactions. 2019;48(33):12451-8.
36. Álvarez-Paino M, Muñoz-Bonilla A, Fernández-García M. Antimicrobial polymers in the nano-world. Nanomaterials. 2017;7(2):48.
37. Ullah A, Qazi J, Rahman L, Kanaras AG, Khan WS, Hussain I, et al. Nanoparticles-assisted delivery of antiviral-siRNA as inhalable treatment for human respiratory viruses: A candidate approach against SARS-COV-2. Nano Select. 2020.
38. Choudhury H, Gorain B, Pandey M, Khurana RK, Kesharwani P. Strategizing biodegradable polymeric nanoparticles to cross the biological barriers for cancer targeting. International Journal of Pharmaceutics. 2019;565:509-22.
39. Oshiro-Júnior JA, Rodero C, Hanck-Silva G, Sato MR, Alves RC, Eloy JO, et al. Stimuli-responsive Drug Delivery Nanocarriers in the Treatment of Breast Cancer. Current Medicinal Chemistry. 2020;27(15):2494-513.
40. Canaparo R, Foglietta F, Giuntini F, Della Pepa C, Dosio F, Serpe L. Recent developments in antibacterial therapy: focus on stimuli-responsive drug-delivery systems and therapeutic nanoparticles. Molecules. 2019;24(10):1991.
41. Thambi T, Lee DS. Stimuli-responsive polymersomes for cancer therapy. Stimuli Responsive Polymeric Nanocarriers for Drug Delivery Applications: Elsevier; 2019. p. 413-38.
42. Sadhukhan P, Kundu M, Chatterjee S, Ghosh N, Manna P, Das J, et al. Targeted delivery of quercetin via pH-responsive zinc oxide nanoparticles for breast cancer therapy. Materials Science and Engineering: C. 2019;100:129-40.
43. Liu J, Huang Y, Kumar A, Tan A, Jin S, Mozhi A, et al. pH-sensitive nano-systems for drug delivery in cancer therapy. Biotechnology Advances. 2014;32(4):693-710.
44. Ding X, Wang A, Tong W, Xu FJ. Biodegradable Antibacterial Polymeric Nanosystems: A New Hope to Cope with Multidrug-Resistant Bacteria. Small 2019;15(20):1900999.
45. Hassan D, Omolo CA, Fasiku VO, Mocktar C, Govender T. Novel chitosan-based pH-responsive lipid-polymer hybrid nanovesicles (OLA-LPHVs) for delivery of vancomycin against methicillin-resistant Staphylococcus aureus infections. International Journal of Biological Macromolecules. 2020;147:385-98.
46. Makhathini SS, Omolo CA, Gannimani R, Mocktar C, Govender T. pH-Responsive Micelles from an Oleic Acid Tail and Propionic Acid Heads Dendritic Amphiphile for the Delivery of Antibiotics. Journal of Pharmaceutical Sciences. 2020;109(8):2594-606.
47. Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Singh S, Renukuntla J, et al. pH-responsive chitosan nanoparticles from a novel twin-chain anionic amphiphile for controlled and targeted delivery of vancomycin. Colloids and Surfaces B: Biointerfaces. 2017;158:650-7.
48. Kalhapure RS, Sikwal DR, Rambharose S, Mocktar C, Singh S, Bester L, et al. Enhancing targeted antibiotic therapy via pH responsive solid lipid nanoparticles from an acid cleavable lipid. Nanomedicine: Nanotechnology, Biology and Medicine. 2017;13(6):2067-77.
49. Yan J, Wang Y, Zhang X, Liu S, Tian C, Wang H. Targeted nanomedicine for prostate cancer therapy: docetaxel and curcumin co-encapsulated lipid–polymer hybrid nanoparticles for the enhanced anti-tumor activity in vitro and in vivo. Drug Delivery. 2016;23(5):1757-62.

50. Mandal B, Bhattacharjee H, Mittal N, Sah H, Balabathula P, Thoma LA, et al. Core–shell-type lipid–polymer hybrid nanoparticles as a drug delivery platform. Nanomedicine: Nanotechnology, Biology and Medicine. 2013;9(4):474-91.
51. Zhang L, Zhu D, Dong X, Sun H, Song C, Wang C, et al. Folate-modified lipid–polymer hybrid nanoparticles for targeted paclitaxel delivery. International Journal of Nanomedicine. 2015;10:2101.
52. Bose RJ, Ravikumar R, Karuppagounder V, Bennet D, Rangasamy S, Thandavarayan RA. Lipid–polymer hybrid nanoparticle-mediated therapeutics delivery: advances and challenges. Drug Discovery Today. 2017;22(8):1258-65.
53. Crucho CI. Stimuli-responsive polymeric nanoparticles for nanomedicine. ChemMedChem. 2015;10(1):24-38.
54. Joglekar M, Trewyn BG. Polymer-based stimuli-responsive nanosystems for biomedical applications. Biotechnology. 2013;8(8):931-45.
55. Ling D, Li H, Xi W, Wang Z, Bednarkiewicz A, Dibaba ST, et al. Heterodimers made of metal–organic frameworks and upconversion nanoparticles for bioimaging and pH-responsive dual-drug delivery. Journal of Materials Chemistry B. 2020;8(6):1316-25.
56. Fraix A, Conte C, Gazzano E, Riganti C, Quaglia F, Sortino S. Overcoming Doxorubicin Resistance with Lipid–Polymer Hybrid Nanoparticles Photoreleasing Nitric Oxide. Molecular Pharmaceutics. 2020;17(6):2135-44.
57. Radovic-Moreno AF, Lu TK, Puscasu VA, Yoon CJ, Langer R, Farokhzad OC. Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics. ACS nano. 2012;6(5):4279-87.
58. Seedat N, Kalhapure RS, Mocktar C, Vepuri S, Jadhav M, Soliman M, et al. Co-encapsulation of multi-lipids and polymers enhances the performance of vancomycin in lipid–polymer hybrid nanoparticles: In vitro and in silico studies. Material Science and Engineering C. 2016;61:616-30.
59. Jutooru I, Chadalapaka G, Chintharlapalli S, Papineni S, Safe S. Induction of apoptosis and nonsteroidal anti-inflammatory drug-activated gene 1 in pancreatic cancer cells by a glycyrrhetinic acid derivative. Molecular Carcinogenesis. 2009;48(8):692-702.
60. Lefaki M, Papaevgeniou N, Tur JA, Vorgias CE, Sykiotis GP, Chondrogianni N. The dietary triterpenoid 18α–Glycyrrhetinic acid protects from MMC-induced genotoxicity through the ERK/Nrf2 pathway. Redox Biology. 2020;28:101317.
61. Ming LJ, Yin ACY. Therapeutic effects of glycyrrhizic acid. Natural Product Communications. 2013;8(3):415-8.
62. Kowalska A, Kalinowska-Lis U. 18β-Glycyrrhetinic acid: its core biological properties and dermatological applications. International Journal of Cosmetic Science. 2019;41(4):325-31.
63. Wang L, Yang R, Yuan B, Liu Y, Liu C. The antiviral and antimicrobial activities of licorice, a widely-used Chinese herb. Acta Pharmaceutica Sinica B. 2015;5(4):310-5.
64. Wang L-J, Geng C-A, Ma Y-B, Huang X-Y, Luo J, Chen H, et al. Synthesis, biological evaluation and structure–activity relationships of glycyrrhetinic acid derivatives as novel anti-hepatitis B virus agents. Bioorganic & Medicinal Chemistry Letters. 2012;22(10):3473-9.
65. Oyama K, Kawada-Matsuo M, Oogai Y, Hayashi T, Nakamura N, Komatsuzawa H. Antibacterial effects of glycyrrhetinic acid and its derivatives on Staphylococcus aureus. PLoS One. 2016;11(11):e0165831.
66. Madhumathi K, Rubaiya Y, Doble M, Venkateswari R, Kumar TS. Antibacterial, anti-inflammatory, and bone-regenerative dual-drug-loaded calcium phosphate nanocarriers—in vitro and in vivo studies. Drug Delivery and Translational Research. 2018;8(5):1066-77.

67. Wu J-L, Tian G-X, Yu W-J, Jia G-T, Sun T-Y, Gao Z-Q. pH-responsive hyaluronic acid-based mixed micelles for the hepatoma-targeting delivery of doxorubicin. International Journal of Molecular Sciences. 2016;17(4):364.
68. Hefnawy A, Khalil IH, Arafa K, Emara M, El-Sherbiny IM. Dual-Ligand Functionalized Core-Shell Chitosan-Based Nanocarrier for Hepatocellular Carcinoma-Targeted Drug Delivery. International Journal of Nanomedicine. 2020;15:821-37.
69. Sun Y, Lu J, Yan D, Shen L, Hu H, Chen D. Cellular uptake mechanism and clearance kinetics of fluorescence-labeled glycyrrhetinic acid and glycyrrhetinic acid–modified liposome in hepatocellular carcinoma cells. Environmental Toxicology and Pharmacology. 2017;53:46-56.
70. Wang C-Y, Kao T-C, Lo W-H, Yen G-C. Glycyrrhizic acid and 18β-glycyrrhetinic acid modulate lipopolysaccharide-induced inflammatory response by suppression of NF-κB through PI3K p110δ and p110γ inhibitions. Journal of Agricultural and Food Chemistry. 2011;59(14):7726-33.

Chapter Two

Literature review

Chapter Two – Literature review

2.1 Introduction

This chapter provides an overview of the burden of infectious diseases on the healthcare system and limitations of current antibiotic therapy that have led to the development of bacterial resistance. It also provides an overview of the characteristics of lipid-polymer hybrid nanoparticles, preparation and characterization techniques, outlines the use of pH-responsive NDDS as a strategy to influence the treatment of infectious diseases and concludes with an overview of VCM as a model drug.

2.2 The burden of infectious diseases on the healthcare sector and the limitations of antibiotics

Worldwide, infectious diseases pose a significant threat despite the advancement of scientific research (1). The burden of infectious diseases on the healthcare system is related to an increase in morbidity and mortality rates (Figure 1) with lower respiratory infections, lung cancer and tuberculosis (TB) being among the top ten foremost causes of death in developing and developed countries (2). Currently, lower respiratory tract infections are the leading cause of death in Africa since 2010, whereas, in South Africa, TB remains the leading cause of death (Figure 2) (3).

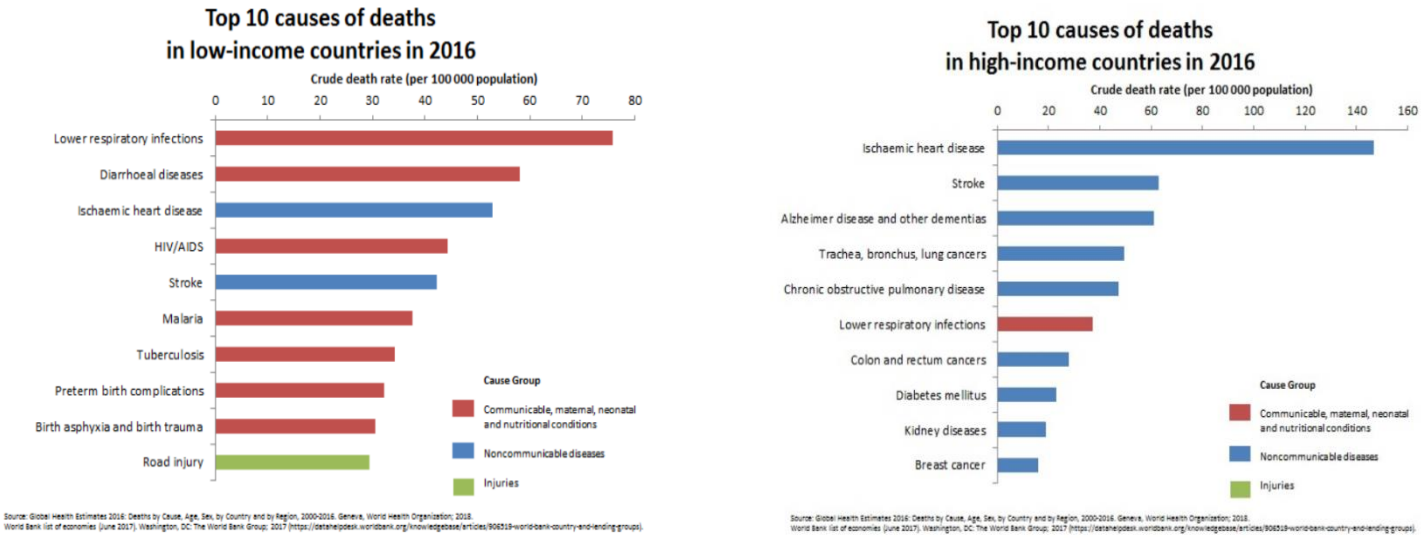


Figure 1. Leading causes of death in developing and developed countries (2).

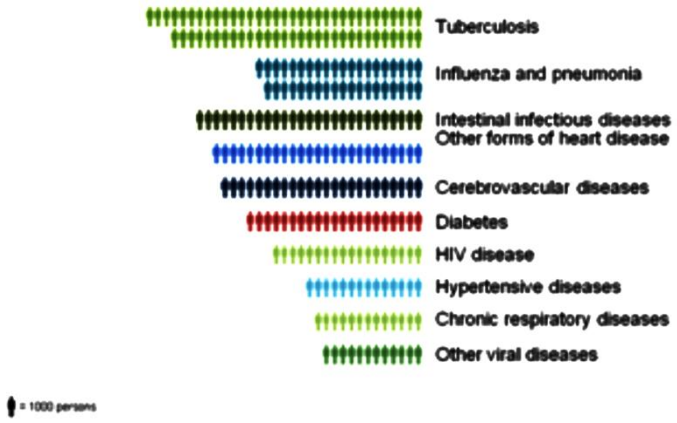


Figure 2. Leading causes of death in South Africa (4).

In 1928, the first antibiotic known as penicillin was discovered by bacteriologist, Alexander Fleming (5). In 1945, penicillin was used to treat bacterial infections (6) and thus marked the beginning of a new era of antibiotics. The discovery of conventional antibiotics contributed to the prevention and treatment of infectious diseases (7). Antibiotics are classified according to their mechanism of actions, such as cell wall synthesis inhibitors, protein synthesis inhibitors, cell membrane permeability inhibitors and nucleic acid synthesis inhibitors (Figure 3) (8). Antibiotics are produced in several dosage forms such as tablets, capsules (9), emulsions, gels (10), suppositories, creams and ointments (11).

Despite the ability to prevent and treat infectious diseases, several limitations are associated with conventional antibiotics, such as inadequate drug concentration at infection sites, harmful side effects, decreased cellular absorption and solubility, non-sustained drug release, poor pharmacokinetic profiles and poor patient compliance (12-14). Such limitations, associated with the incorrect use and unlimited use of antibiotics, has led to the failure of infectious diseases therapy, the emergence of bacterial infections and bacterial resistance (Figure 4) (14).

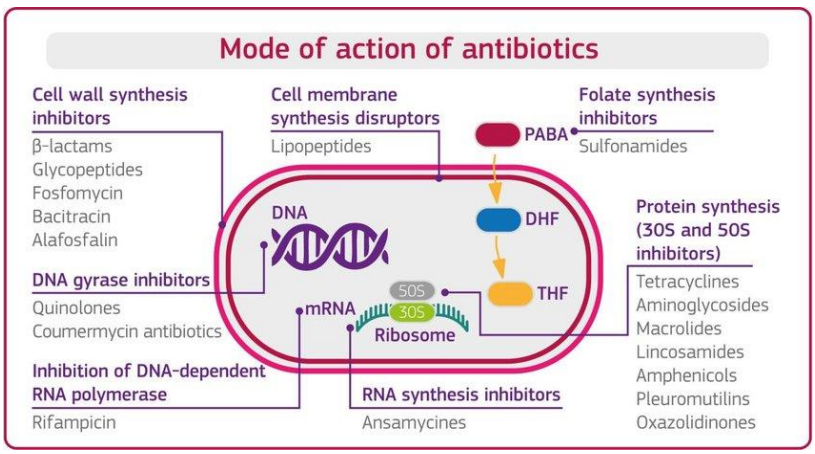


Figure 3. Commonly used antibiotics mechanism of actions (15).

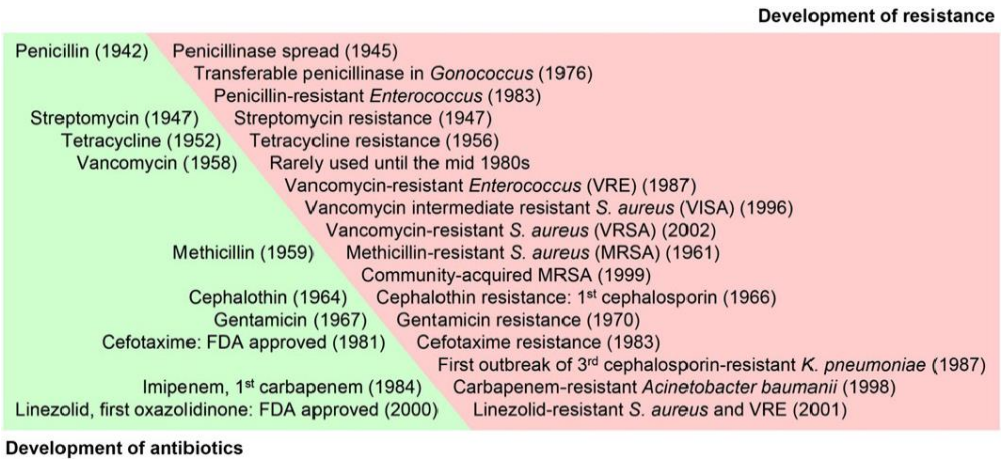


Figure 4. History of antimicrobial development and resistance of bacteria (16).

Bacteria acquire antibiotic resistance intrinsically or by horizontal gene transfer (17). Multidrug-resistant and extreme drug-resistant (XDR) bacterial strains are resistant to multiple antibiotic classes. Such untreatable bacterial infections are associated with ineffective and highly toxic antibiotic therapy (18, 19). Among universal bacterial pathogens, MRSA has developed resistance to methicillin and is responsible for skin and soft skin infections resulting in increased morbidity and mortality rates (19, 20).

Despite the increase in antimicrobial resistance, the rate of development of new antimicrobials against MDR organisms is deteriorating (Figure 5) (20). The primary reasons include the massive cost involved in the production of new chemical entities, low return on investment and lengthy drug approval procedures. Thus, novel approaches have been investigated in order to enhance the delivery of conventional drug dosage forms and to restore efficiency and effectiveness (21, 22), such as individualizing antibiotic treatment, therapeutic drug monitoring and sustained and targeted drug delivery (14, 23, 24).

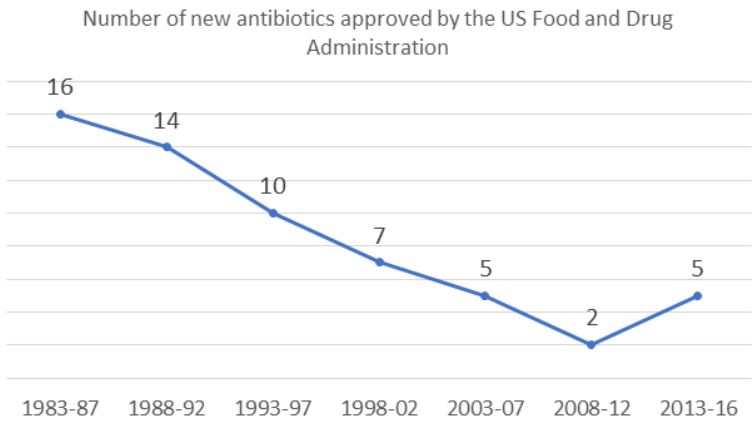


Figure 5. Declining number of new antibiotics (25).

2.3 Nanoengineered drug delivery systems

Nanotechnology is defined as the manipulation of atoms and molecules conducted on a nanoscale, used within scientific fields (26, 27). Nanotechnology within the field of medicine involves the synthesis and application of nanosized substances or compounds designed to maximize the outcome of therapy (28). Nanoparticles possess unique chemico-physical properties including, their subcellular size, large surface area to mass ratio, enhanced interactions between pathogen and host cells/tissues and ability to be modified structurally and functionally (29, 30).

Nano drug delivery systems possess the ability to improve drug stability, increase drug absorption within target tissues and enhance localization, thus enhancing drug efficacy (31). Examples of NDDS, illustrated in Figure 6, encapsulating the various drug classes include liposomes (32), PNPs (33), dendrimers (34), micelles (35), lipid polymer hybrids (36), solid lipid nanoparticles (37) and nanostructured lipid carriers (38). Table 1 illustrates several types of nanocarriers entrapping antibiotics for the treatment of different bacterial infections. Nanosystems are applied in several routes for antibiotic administration such as oral, intravenous, inhalation, topical and transcutaneous (39).

NDDS have several advantages over conventional antibiotics, which include targeted and sustained drug release at infection sites (40), selective targeting of tissues and cells (41, 42), improved cellular absorption and solubility (29, 42), improved drug stability, synergistic effects via co-delivery of antimicrobials (41, 43), enhanced patient compliance (42), minimized side effects (44) and potentiation of antibacterial activity (32). Due to the emergence of antibiotic resistant bacteria possessing evolved resistance mechanisms, innovative NDDS need to be developed to overcome antimicrobial resistance (45, 46). The various mechanisms by which nanosystems have the potential to overcome antimicrobial resistance include, increased concentration at the target site of infection, high entrapment efficiency of hydrophilic or lipophilic drugs, decreased dosage, protection of encapsulated drugs from bacterial enzymatic inactivation, increased uptake or decreased efflux, physical damage of the plasma membrane, increased removal of cytoplasmic fluid and anti-biofilm efficacy (47-49). Hence, NDDS can

overcome antibiotic limitations and offer a promising approach to combatting bacterial resistant pathogens (14).

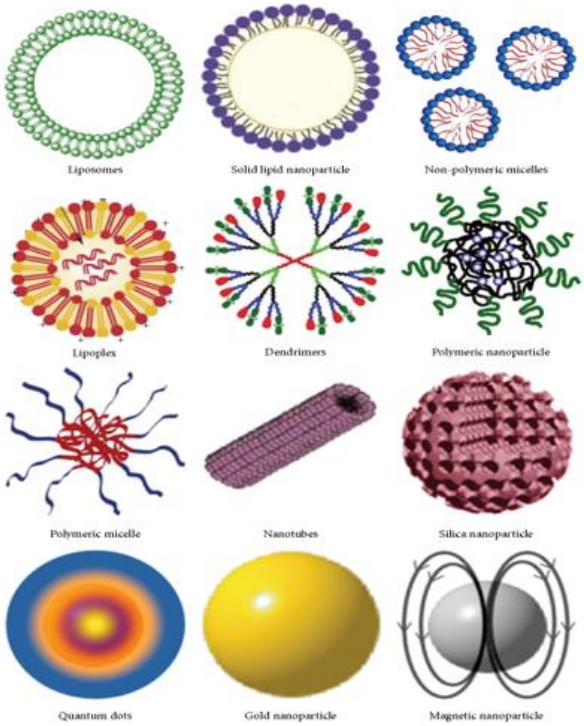


Figure 6. Examples of nanosystems reported for drug delivery (50).

Table 1. Examples of nanosystems reported for antibiotics.

Nanosystem	Encapsulated antibiotic	Targeted bacteria	Main findings	Reference
Polymeric nanoparticles	Clindamycin	MRSA	- Enhanced antibacterial activity - Enhanced wound healing	(51)
Liposomes	VCM	SA and MRSA	- Sustained release - Enhanced *in vitro* antibacterial activity	(32)
Dendrimers	Ciprofloxacin	SA and *Escherichia coli*	- Co-administration of dendrimers reduced the required effective dose of the drug - Synergistic antibacterial activity	(52)
Micelles	VCM	SA and MRSA	- Sustained drug release - Enhanced *in vitro* and *in vivo* antibacterial activity	(14)
Solid lipid nanoparticles	Meropenem	*Escherichia coli*	- Sustained release - Enhanced antibacterial activity	(13)
Nano emulsions	Amoxicillin	*Helicobacter pylori*	- Sustained drug release - Enhanced drug localization at the site of infection.	(53)
Carbon nanotubes	Ciprofloxacin	*Escherichia coli*	- Enhanced antibacterial activity	(54)
Polymersomes	VCM	SA and MRSA	- Sustained drug release - Enhanced *in vitro* and *in vivo* antibacterial activity	(55)

2.4 Lipid-polymer hybrid nanoparticles

Merging the structural components of liposomes and PNPs formulates LPHNPs (56). LPHNPs combine the potential advantages of liposomes and PNPs (56). Hybrid nanoparticles may be produced in different morphologies such as core shell and matrix LPHNPs (57). The core may be entrapped in single/multiple layers of the lipid on the polymer core material that may provide the site for surface functionalization with different targeting ligands and receptors to potentiate desired characteristics of LPHNPs (58). Natural or synthetic lipids such as, glycerol and its derivatives are amphiphilic molecules as well as convenient and inexpensive materials (59). Natural or synthetic polymers are stable, convenient and inexpensive materials for the production of numerous unique nanoparticle constructs with great potential applications in the medical field (60, 61). Additionally, polymers can also show antimicrobial properties (60, 62,

63). Such polymers demonstrate enhanced efficacy, reduced toxicity, minimal environmental hazards and provide higher resistance against MDR bacteria. Hence, the development of dynamic and non-toxic antimicrobial lipid-polymer hybrid nanoparticles are recommended for the treatment infectious diseases (64, 65). Well known synthetic polymers include polylactides (PLA), polyglycolides (PGA), an example of a copolymer is polylactide co-glycolides (PLGA) (58) and natural polymers include chitosan (66-68), alginate, albumin and gelatin (69). Figure 7 depicts the structure of LPHNPs.

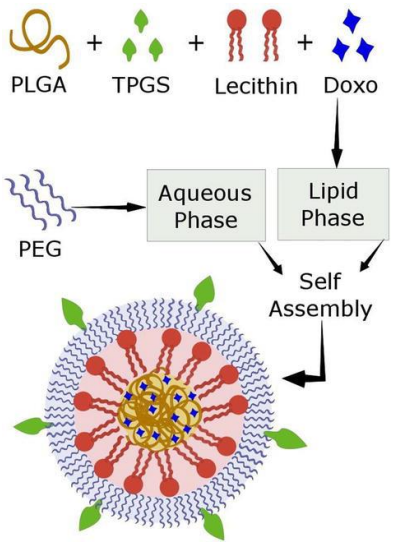


Figure 7. Lipid-polymer hybrid nanoparticle structure (70)**.**

2.4.1 Main types of lipid-polymer hybrid nanoparticles

2.4.1.1 Monolithic lipid-polymer hybrid nanoparticles

Monolithic or mixed lipid polymer hybrids are made up of a polymeric matrix in which the lipid molecules are dispersed throughout (69). The monolithic LPHNPs are structurally comprised of copolymers and lipids as shown in Figure 8 (69). The applications include localized drug delivery, tissue engineering and cancer immunotherapy (70).

2.4.1.2 Biomimetic lipid-polymer nanoparticles

These nanoparticles are coated with red blood cell and also known as erythrocyte membrane-camouflaged PNPs (71). Lipid bilayer-coated nanoparticles are prepared by extrusion of erythrocytes and drug entrapment is within PNPs (71). These lipid-polymer-based

nanoparticles are widely applied in the fields of bioimaging, gene therapy and tissue engineering (72).

2.4.1.3 Polymer-caged liposomal nanoparticles

These are stable systems formed when polymers are anchored on the surface of liposomes (73). Their applications in the medical field include bioimaging and tissue engineering (74).

2.4.1.4 Core shell lipid-polymer hybrid nanoparticles

There are two types of core shell LPHNPs namely, polymer core-lipid shell nanoparticles (75) and hollow core-lipid-polymer-lipid nanoparticles (76). Such systems have a multi layered structure comprising of a polymeric nucleus, lipid-PEG and lipids in the outermost layers acting as the shell (77). Their applications include immunology kits and biosensors for magnifying biomolecular identification (78). Figure 8 depicts the structural image of the four main types of lipid-polymer hybrid nanoparticles.

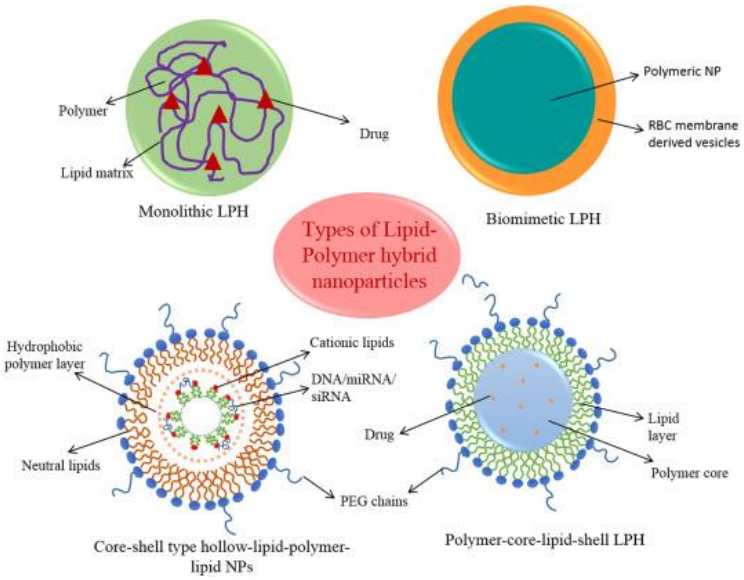


Figure 8. Four main types of LPHNPs (79).

2.4.2 Main techniques of lipid-polymer hybrid nanoparticle preparation

Several methods have been applied to produce LPHNPs from lipids and preformed polymers, including solvent evaporation (80), nanoprecipitation (81), emulsification (82), dialysis and salting out. Also, several methods have been applied to produce PNPs of LPHNPs from the

polymerization of monomers, such as emulsion, micro-emulsion, mini-emulsion and interfacial polymerization (58, 83).

Solvent evaporation was the first method developed to prepare LPHNPs (84). Using the method, shown in Figure 9, emulsions can be formulated by firstly, dissolving in volatile organic solvents. Then the emulsion is transformed into a nanoparticle suspension via evaporation of the solvent from the polymer. Two processes have been applied for the formulation of emulsions: 1) oil-in-water (single emulsions) and 2) (water-in-oil)-in-water (double emulsions). High-speed homogenization or ultra-sonication takes place followed by solvent evaporation and thereafter magnetic stirring at room temperature. LPHNPs are centrifuged and washed to remove unwanted substances (84-86).

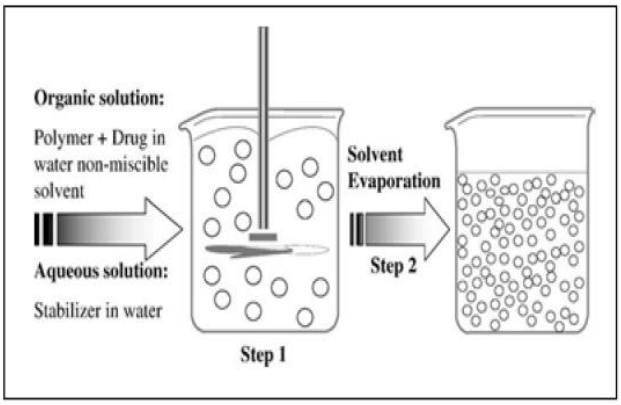


Figure 9. Solvent evaporation technique (58).

Micro-emulsion polymerization is a novel and effective approach for nanoparticle preparation (87). Particle size and the number of chains per particle are considerably lower in micro-emulsion polymerization as compared to emulsion polymerization (58, 88, 89). A water-miscible initiator is immersed into the aqueous phase of a thermodynamic micro-emulsion. Thereafter, the surfactant and all initiator molecules within the system are used up by the polymer particles. The reaction mixture consisting of swollen polymer micelles with dissolved monomers result in formed microdroplets (90, 91). Figure 10 shows the micro-emulsion technique.

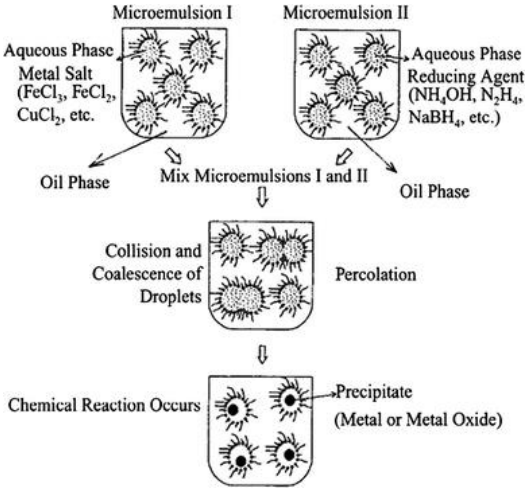


Figure 10. Micro-emulsion technique (92).

2.4.3 Lipid-polymer hybrid nanoparticle characterization

Several methods are applied for the characterization of LPHNPs in order to explore their potential application, stability and kinetic profiles. LPHNPs are characterized in terms of particle size, PDI, surface charge/ZP, morphology and other parameters depending on LPHNPs applications (93, 94).

2.4.3.1 Particle size and size distribution

Dynamic light scattering (DLS) is a technique used to determine size distribution of submicron particles or polymers in a formulation. The DLS technique is most frequently used for determining particle size and PDI as it is quick, practical and manageable (95-98). The instrument used to measure particle size and PDI is a Zetasizer. The rate at which the intensity of the scattered light fluctuates depends on the size of the particles (95).

2.4.3.2 Surface charge

The ZP of nanoparticles is a crucial indicator of the stability of LPHNPs during storage. A high positive or negative surface charge indicates high electrostatic repulsion, thus, avoiding the formation of LPHNP agglomerates; hence improved stability of the nanosystem is maintained. Analytical instruments based on the DLS technique is used for ZP measurement (99-102).

2.4.3.3 Morphology

Scanning electron microscopy (SEM) produces images of a sample by scanning the surface of a sample using a beam of electrons. The instrument used is a scanning electron microscope.

Electrons interact with the sample providing data about surface morphology and sample composition. SEM technique is advantageous for nanoparticle size and shape characterization due to easy preparation and fast image acquisition (99, 102). Similarly, transmission electron microscopy (TEM) produces images of a sample whereby an accelerated beam of electrons penetrates through a sample. The instrument used is a transmission electron microscope. Electrons interact with the sample providing data on structure and morphology. TEM is a standard method for measuring nanoparticle size, size distribution and morphology (101). Amorphous particles usually demonstrate a spherical shape, small nanoparticles form clusters and various shapes like rods and fibers can also be identified on the range of 1-1000 nm (100, 103).

2.4.3.4 Entrapment efficiency

Drug entrapment efficiency (EE) is the concentration of drug entrapped within the matrix of LPHNPs. The unentrapped or free drug is separated from the LPHNPs via ultra-centrifugation and ultra-filtration methods, followed by quantification of the free drug using UV or HPLC analysis (104-106).

2.4.3.5 Thermal profiles

Analytical instruments based on differential scanning calorimetry (DSC) techniques are used to analyze the crystallization form and thermal profile of LPHNPs (107). This technique uses a differential scanning calorimeter instrument. DSC analysis can measure melting temperature, glass transition temperature, reaction energy, crystallinity, precipitation energy and temperature (105).

2.4.3.6 In vitro drug release

Drug release studies are commonly carried out using a dynamic dialysis bag method to investigate the release behaviour of drug loaded LPHNPs, followed by quantification of the drug concentration using a reliable analytical method, such as UV or HPLC (108). Factors such as particle size (surface area) of LPHNPs can influence drug release behaviour. Smaller particles have a larger surface area to volume ratio, hence the drug associated with small nanoparticles would be situated at/near the surface resulting in a faster drug release (107, 108). Several other characterization analyses can be done, such as antibacterial studies (98) and haemolysis assays (109) for antibacterial research, cytotoxicity studies (84) for anticancer research, as well as histological evaluations and radiographic examinations for bone and tissue

engineering studies (110). Hence the type of characterization assessment depends on the application of the LPHNPs.

2.4.4 Lipid-polymer hybrid nanoparticles for antibiotic delivery

Antibiotic-loaded LPHNPs have emerged as one of the promising formulations in antibiotic therapy against infections (111). Advantages associated with LPHNPs used for antibiotic delivery are their stability during storage, easy preparation techniques and functionalization, controlled antibiotic release, improved biocompatibility and enhanced circulation time (112). The subcellular size of LPHNPs allows them to effectively penetrate the target site and release the antibiotic locally, enhancing localization and antibiotic concentration at the infection site (113). Different antibiotics can be incorporated into LPHNPs for different routes of administration such as, oral, topical, transdermal, ocular and intravenous (114, 115).

2.4.5 Responsive lipid-polymer hybrid nanoparticles for antibiotic delivery

First generation nanoparticles were developed and approved more than 15 years ago. Second generation nanocarriers advanced this field by achieving long blood circulation and passive targeting (116, 117). While third generation nanocarriers aimed at achieving molecular recognition and active targeting, hence the development of stimuli-responsive nanoparticles (116, 117). Recent scientific advancements focus on developing stimuli-responsive nanosystems that release the drugs only when triggered by pH, enzymes, temperature, electric field and magnetic field (118-122). The stimuli-responsive drug delivery strategy can provide targeted drug release, improved tissue and cellular internalization and drug accumulation at targeted sites (123). This can result in enhanced drug stability, bioavailability and therapeutic efficacy (124).

Despite the advancements of LPHNPs application incorporating different drug classes via different routes of administration such as oral (125-127), topical (128, 129), intravenous (130, 131), few studies have been reported for drug delivery via responsive LPHNPs, but with great potential. Li *et al*. reported enzyme-responsive lipid-polymer hybrid vesicles for bacterial-strain-selective delivery system for antibiotics (128), Moreno *et al*. reported pH-responsive LPHNPs for bacterial cell wall targeted delivery of antibiotics (129) and Michalak *et al*. reported antibiotic-loaded temperature-responsive LPHNPs against SA (130). Advantages of responsive LPHNPs include enhanced systemic release, enhanced internalization, improved bioavailability, minimal side effects, stabilization and protection of the antibiotic (114).

2.5 pH-Responsive LPHNPs

Current investigations of pH-responsive drug delivery systems have received much attention (132). As certain organs, cellular and tissue compartments exhibit different pH values, such as the blood, lysosomes, endosomes and gastrointestinal tract (mouth, stomach, duodenum, colon) (132). Diseases such as cancer (120), TB (133), H1N1 influenza virus (134), Alzheimer's disease (135), chronic lung diseases (136) and infections (bacterial, viral, fungal and parasitic) present with an acidic pH change different from the physiological pH of 7.4.

The human body maintains a slightly alkaline pH of 7.2, but certain conditions such as hypoxemia, whereby there is low oxygen in the blood leading to an oxygen deficiency in the tissues, otherwise known as hypoxia, can ultimately result in bodily acidosis and inflammation. Decreased oxygen intake/decreased pH (acidic pH) allows bacteria, viruses and cancer cells to thrive (137, 138). During an infection, serum lactate dehydrogenase enzyme level increases, anaerobic conditions develop and lactate production increases from pyruvate (139). Increased lactate levels elevate the production and accumulation of proinflammatory cytokines and oxygen reactive species (ROS) resulting in oxidative stress (139, 140). Lactate production continues to increment as the acidic environment increases (139, 140). This indicator can potentially trigger the design of pH-responsive nanosystems for targeted drug release (140-142). Hence, stimuli-responsive nanosystems, specifically pH-responsive drug delivery systems, can be more promising drug delivery systems than conventional NDDS. Table 2 summarizes the studies reported on pH-responsive drug delivery of LPHNPs.

At acidic conditions, pH-responsive nanoformulations operate via two mechanisms of action:

1) Protonation: pH-Responsive materials possess ionizable groups that remain unprotonated, while in acidic pH, these groups undergo protonation and a reversal of surface charge, leading to conformational variations followed by drug release.
2) Hydrolysis of labile acid bonds: pH-responsive materials can form an acid-labile bond with a drug that undergoes hydrolysis at acidic conditions, resulting in a targeted antibiotic release (121, 141, 143).

Some bacterial infections, such as SA and *E. coli*, are acidic and produce acetic acid and lactic acid under oxygen deprived conditions. Therefore, the pH-responsive approach is advantageous in reduced exposure of the antibiotic to non-infected sites, improving targeted drug delivery, controlled drug release, enhancing antibiotic localization at acidic infection sites,

which can enhance the antibacterial activity, ultimately preventing the development of bacterial resistance (129). Thus, pH-sensitive nanocarriers can impact infectious disease therapy by improving antibiotic protection at the acidic infection site, improving biofilm penetration, enhancing antibacterial activity and enhancing targeted release, thus enhancing the antibacterial activity and preventing bacterial resistance development (143-145).

Table 2. Examples of pH-responsive lipid-polymer hybrid nanoparticle delivery systems that have been tested against different target pathogens/diseases.

Lipid used	**Polymer/s used**	**Encapsulated drug**	**Mechanism of action of the drug**	**Targeted pathogen or disease**	**Main findings**	**Reference**
Bovine serum albumin	PLGA	Doxorubicin	Nucleic acid synthesis inhibitor	Multi drug resistant cancer	Enhanced drug intracellular bioavailability, improved cytotoxic activity.	(146)
Soya lecithin	PLA	Norfloxacin	Nucleic acid synthesis inhibitor	SA and *Pseudomonas aeruginosa*	Enhanced drug delivery, in vitro antibacterial activity.	(147)
Glyceryl tripalmitate	Eudragit RS100 and chitosan	Vancomycin	Cell wall synthesis inhibitor	SA and MRSA	Sustained drug release, in vitro antibacterial studies, showed better activity compared to the free drug.	(148)
Bovine serum albumin	Chitosan	Thymol	Nucleic acid synthesis inhibitor	*Escherichia coli*	Enhanced antibacterial activity, sustained drug release in simulated gastro-intestinal pH condition.	(149)
Oleic acid	Polyacrylic acid	Fusidic acid	Protein synthesis inhibitor	SA and MRSA	Controlled drug release of the drug at acidic pH conditions.	(150)

2.6. Vancomycin as a model drug for antibiotic delivery

Vancomycin is a tricyclic glycopeptide antibiotic (Figure 14), which is the 'last resort' drug against MRSA infections. Vancomycin functions by forming a complex with the D-Ala-D-Ala terminals, thereby inhibiting the function of peptidoglycan synthetase enzyme and subsequently preventing elongation of peptidoglycan matrix and cell wall synthesis (151-153). Intravenous administration of VCM is associated with severe side effects, such as thrombophlebitis (154), neutropenia, nephrotoxicity (155), ototoxicity, thrombocytopenia and most commonly red man or red neck syndrome (156).

2.6.1 Vancomycin nano delivery systems

Vancomycin is a large hydrophilic molecule, which accounts for its low bioavailability, cellular absorption and penetration (157). The bactericidal activity of VCM is aimed at attaching to the bacterial cell wall rather than to a protein target, initially VCM was immune to resistance (158). However, the emergence of two complex resistance mechanisms involving a multi-enzyme pathway compromised the efficacy of the drug (159). Resistance to the drug involves breakdown of D-Ala-D-Ala terminals and its replacement with D-Ala-D-lac or D-Ala-D-Ser regions to which VCM has low affinity (158, 160). Vancomycin resistance is a progressing healthcare issue that has led to the failure of VCM treatment and increased minimum inhibitory concentration (MIC) (161). Vancomycin NDDS has shown to be highly effective as compared to VCM conventional dosage forms. The liposomal-VCM formulation had enhanced antibacterial performance and sustained circulation time (162, 163), while VCM-PNPs had enhanced antibacterial activity and biocompatibility with tissues (14). VCM-LPHNs showed sustained drug release and antimicrobial activity (36). Omolo *et al*. reported VCM-loaded polymersomes that enhanced its anti-MRSA activity (55).

On the other hand, pH-responsive VCM-PNPs have been reported for enhancing VCM targeted delivery (129, 164). Kalhapure *et al*. (2017) (164) reported PNPs with size of 220.57 ± 5.9 nm and ZP of 21.9 ± 0.9 mV showed higher antibacterial activity, compared to Moreno *et al*. (2012) (129) who reported PNPs with size of 196.0 ± 7.8 nm and ZP of 2.3 ± 1.0 mV. Hence, a more positive ZP could lead to improved antibacterial activity. Kalhapure *et al*. (2017) also reported acid cleavable lipids for pH-responsive VCM-SLNs formulation; their results showed enhanced antimicrobial activity against MRSA and SA (165). Makhathini *et al*. (2020) (166) and Sonawane *et al*. (2020) (14) reported pH-responsive VCM-micelles with results showing enhanced antibacterial activity against MRSA and a faster drug release profile at pH 6.0

compared to physiological pH of 7.4. Sonawane *et al*. (2020) (14) reported micelles with size of 130.33 ± 7.36 nm and ZP of -4.33 ± 0.55 mV, which showed superior antibacterial activity than the VCM-micelles reported by Makhathini *et al*. (2020) (166) with size of 84.16 ± 0.184 nm and ZP of -42.6 ± 1.98 mV. Hence smaller particle size and a less negative ZP could lead to better antibacterial activity.

Wu *et al*. (2020) reported pH-responsive VCM-carbon dots against staphylococcal biofilms, which showed enhanced VCM penetration and killing of non-extracellular-polymeric-substance producing staphylococcal strains (167). Osman *et al*. (2019) reported pH-responsive VCM-nanostructured lipid carriers against SA and MRSA; their results indicated enhanced antibacterial activity (38). Zhang *et al*. (2020) (168) reported VCM-hybrid magnetic nanoparticles formulation; which resulted in enhanced antimicrobial activity against SA and *E. coli*. Xie *et al*. (2020) (169) reported pH-responsive VCM-silver nanoparticles formulation; their results showed enhanced antibacterial activity against MRSA and *E. coli*. Salih *et al*. (2020) (170) reported pH-responsive VCM-nanovesicles formulation; which demonstrated enhanced antibacterial activity against SA and MRSA . Thus, pH-responsive nanosystems for VCM targeted delivery can enhance VCM activity, overcome the limitations of VCM conventional dosage forms and combat bacterial resistance.

2.7 Conclusion

This chapter highlighted the potential of nano delivery systems to improve the treatment of bacterial infections and combat antimicrobial resistance. Nanocarriers that are pH responsive are emerging as a siginificant strategy to potentiate the performance of nanocarriers for overcoming the restrictions of antibiotic therapy and curbing the evolution of a post-antibiotic era. Furthermore, the chapter also demonstrated that vancomycin is an ideal model drug being widely used to develop novel nanosystems to treat bacterial infections. Novel pH-responsive systems of vancomycin will make a significant impact in the field of nanoantibiotics.

2.8 References

1. Al Amiry A. A call for constructing a metric for monitoring CA-MRSA and other infectious diseases. Archives of Public Health. 2020;78(1):1-2.
2. WHO, The top 10 causes of death. http://www.who.int/mediacentre/factsheets/fs310/en/, 2018 (accessed 21.03.2020).
3. STATSSA, Leading causes of death. https://www.africacheck.org/factsheets, 2018 (accessed 21.03.2020).
4. STATSSA,What are South Africans dying of ?.http://www.statssa.gov.za/?p=1023, 2018 (accessed 21.04.2020).
5. Palmer C. Fighting The Dangerous Increase of Multidrug-Resistant Microbes: Attacking the problem from multiple avenues to find a new path. Genetic Engineering and Biotechnology News. 2019;39(6):27-30.
6. Sillankorva S, Pereira MO, Henriques M. Antibiotic alternatives and combinational therapies for bacterial infections. Frontiers in Microbiology. 2019;9:3359.
7. Jeong D, Nguyen HNT, Tyndall M, Schreiber YS. Antibiotic use among twelve Canadian First Nations communities: a retrospective chart review of skin and soft tissue infections. BMC Infectious Diseases. 2020;20(1):118.
8. Kapoor G, Saigal S, Elongavan A. Action and resistance mechanisms of antibiotics: A guide for clinicians. Journal of anaesthesiology, Clinical Pharmacology. 2017;33(3):300.
9. Nasution ES. Prescribing patterns of antibiotics outpatients received by pharmacies in Medan city, Indonesia in 2017. Physiology and Pharmacology. 2020;24(1):28-33.
10. Siddig A, Ramirex R, Wood M, Sun X, Smith G. Formulation and Comparative Characterization of Methylcellulose, Carbomer and Petrolatum Gels for Topical and Transdermal Delivery of Zosyn (Piperacillin/Tazobactam) Antibiotics. Journal of Pharmacy Practice and Pharmaceutical Sciences. 2020;1:96-114.
11. Alam MS, Sharma A, Dubey K, Annamalai P, Kaliaperumal J, Kumar M, et al. Antibiotic Use Evaluation in Genitourinary Tract Infections in Female Patients at a Tertiary Care Hospital. Journal of Evolution of Medical Dental Sciences. 2020;9(8):532-9.
12. Cote CK, Blanco II, Hunter M, Shoe JL, Klimko CP, Panchal RG, et al. Combinations of early generation antibiotics and antimicrobial peptides are effective against a broad spectrum of bacterial biothreat agents. Microbial Pathogenesis. 2020;142:104050.
13. Mhango EK, Kalhapure RS, Jadhav M, Sonawane SJ, Mocktar C, Vepuri S, et al. Preparation and optimization of meropenem-loaded solid lipid nanoparticles: in vitro evaluation and molecular modeling. AAPS PharmSciTech. 2017;18(6):2011-25.
14. Sonawane SJ, Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Govender T. AB2-type amphiphilic block copolymer containing a pH-cleavable hydrazone linkage for targeted antibiotic delivery. International Journal of Pharmaceutics. 2020;575:118948.
15. Sanseverino I, Navarro Cuenca A, Loos R, Marinov D, Lettieri T. State of the Art on the Contribution of Water to Antimicrobial Resistance. Brussels: European Union. 2018:29592.
16. Huh AJ, Kwon YJ. "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. Journal of Controlled Release. 2011;156(2):128-45.
17. Guo Y, Song G, Sun M, Wang J, Wang Y. Prevalence and Therapies of Antibiotic-Resistance in Staphylococcus aureus. Frontiers in Cellular and Infection Microbiology. 2020;10:107.
18. Yin C, Xie L, Guo Y. Phytochemical analysis and antibacterial activity of Gentiana macrophylla extract against bacteria isolated from burn wound infections. Microbial Pathogenesis. 2018;114:25-8.

19. Zhou Y-F, Li L, Tao M-T, Sun J, Liao X-P, Liu Y-H, et al. Linezolid and Rifampicin Combination to Combat cfr-Positive Multidrug-Resistant MRSA in Murine Models of Bacteremia and Skin and Skin Structure Infection. Frontiers in Microbiology. 2020;10:3080.
20. Leonard A, Möhlis K, Schlüter R, Taylor E, Lalk M, Methling K. Exploring metabolic adaptation of Streptococcus pneumoniae to antibiotics. The Journal of Antibiotics. 2020:1-14.
21. Brooks BD, Brooks AE. Therapeutic strategies to combat antibiotic resistance. Advanced Drug Delivery Reviews. 2014;78:14-27.
22. Kalhapure RS, Mocktar C, Sikwal DR, Sonawane SJ, Kathiravan MK, Skelton A, et al. Ion pairing with linoleic acid simultaneously enhances encapsulation efficiency and antibacterial activity of vancomycin in solid lipid nanoparticles. Colloids and Surfaces B: Biointerfaces. 2014;117:303-11.
23. Annoni F, Grimaldi D, Taccone FS. Individualized antibiotic therapy in the treatment of severe infections. Expert Review of Anti-infective Therapy. 2020;18(1):27-35.
24. Vinks AA. The application of population pharmacokinetic modeling to individualized antibiotic therapy. International Journal of Antimicrobial Agents. 2002;19(4):313-22.
25. OECD, WHO, FAO, OIE. Tackling antimicrobial resistance, ensuring sustainable R&D. 2017.
26. Adams FC, Barbante C. Nanoscience, nanotechnology and spectrometry. Spectrochimica Acta Part B: Atomic Spectroscopy. 2013;86:3-13.
27. Sweeney AE, Seal S, Vaidyanathan P. The promises and perils of nanoscience and nanotechnology: Exploring emerging social and ethical issues. Bulletin of Science, Technology and Society. 2003;23(4):236-45.
28. Boisseau P, Loubaton B. Nanomedicine, nanotechnology in medicine. Comptes Rendus Physique. 2011;12(7):620-36.
29. Kalhapure RS, Suleman N, Mocktar C, Seedat N, Govender T. Nanoengineered drug delivery systems for enhancing antibiotic therapy. Journal of Pharmaceutical Sciences. 2015;104(3):872-905.
30. Şen Karaman D, Ercan UK, Bakay E, Topaloğlu N, Rosenholm JM. Evolving Technologies and Strategies for Combating Antibacterial Resistance in the Advent of the Postantibiotic Era. Advanced Functional Materials. 2020;30(15):1908783.
31. Gupta P, Garcia E, Sarkar A, Kapoor S, Rafiq K, Chand HS, et al. Nanoparticle based treatment for cardiovascular diseases. Cardiovascular and Haematological Disorders-Drug Targets. 2019;19(1):33-44.
32. Makhathini SS, Kalhapure RS, Jadhav M, Waddad AY, Gannimani R, Omolo CA, et al. Novel two-chain fatty acid-based lipids for development of vancomycin pH-responsive liposomes against Staphylococcus aureus and methicillin-resistant Staphylococcus aureus (MRSA). Journal of Drug Targeting. 2019;27(10):1094-107.
33. Shaaban MI, Shaker MA, Mady FM. Imipenem/cilastatin encapsulated polymeric nanoparticles for destroying carbapenem-resistant bacterial isolates. Journal of Nanobiotechnology. 2017;15(1):29.
34. Arshad P, Dineshkumar P, Jyothi KN, Karthik M, Saravanan G. Dendrimers as a Novel Carrier in Anti-HIV Therapy. Journal of Drug Delivery and Therapeutics. 2019;9(5-s):195-200.
35. Gao M, Yang Y, Bergfel A, Huang L, Zheng L, Bowden TM. Self-assembly of cholesterol end-capped polymer micelles for controlled drug delivery. Journal of Nanobiotechnology. 2020;18(1):1-10.
36. Hassan D, Omolo CA, Fasiku VO, Mocktar C, Govender T. Novel chitosan-based pH-responsive lipid-polymer hybrid nanovesicles (OLA-LPHVs) for delivery of vancomycin against methicillin-resistant Staphylococcus aureus infections. International journal of Biological Macromolecules. 2020;147:385-98.

37. Wissing S, Kayser O, Müller R. Solid lipid nanoparticles for parenteral drug delivery. Advanced Drug Delivery Reviews. 2004;56(9):1257-72.
38. Osman N, Omolo CA, Gannimani R, Waddad AY, Rambharose S, Mocktar C, et al. Novel fatty acid-based pH-responsive nanostructured lipid carriers for enhancing antibacterial delivery. Journal of Drug Delivery Science and Technology. 2019;53:101125.
39. Jijie R, Barras A, Teodorescu F, Boukherroub R, Szunerits S. Advancements on the molecular design of nanoantibiotics: current level of development and future challenges. Molecular Systems Design and Engineering. 2017;2(4):349-69.
40. Bosnjakovic A, Mishra MK, Ren W, Kurtoglu YE, Shi T, Fan D, et al. Poly (amidoamine) dendrimer-erythromycin conjugates for drug delivery to macrophages involved in periprosthetic inflammation. Nanomedicine: Nanotechnology, Biology and Medicine. 2011;7(3):284-94.
41. Qu J, Zhang L, Chen Z, Mao G, Gao Z, Lai X, et al. Nanostructured lipid carriers, solid lipid nanoparticles, and polymeric nanoparticles: which kind of drug delivery system is better for glioblastoma chemotherapy? Drug delivery. 2016;23(9):3408-16.
42. Sonawane SJ, Kalhapure RS, Rambharose S, Mocktar C, Vepuri SB, Soliman M, et al. Ultra-small lipid-dendrimer hybrid nanoparticles as a promising strategy for antibiotic delivery: in vitro and in silico studies. International Journal of Pharmaceutics. 2016;504(1-2):1-10.
43. Panáček A, Smékalová M, Kilianová M, Prucek R, Bogdanová K, Večeřová R, et al. Strong and nonspecific synergistic antibacterial efficiency of antibiotics combined with silver nanoparticles at very low concentrations showing no cytotoxic effect. Molecules. 2016;21(1):26.
44. Kumar M, Sharma HK. TARGETED NANOTECHNOLOGY FOR ANTICANCER DRUG DELIVERY: CURRENT ISSUE AND CHALLENGE. Journal of Drug Delivery and Therapeutics. 2018;8(5):23-7.
45. Brown MG, Balkwill DL. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. Microbial Ecology. 2009;57(3):484.
46. Ding X, Wang A, Tong W, Xu FJ. Biodegradable Antibacterial Polymeric Nanosystems: A New Hope to Cope with Multidrug-Resistant Bacteria. Small. 2019;15(20):1900999.
47. Eleraky NE, Allam A, Hassan SB, Omar MM. Nanomedicine fight against antibacterial resistance: an overview of the recent pharmaceutical innovations. Pharmaceutics. 2020;12(2):142.
48. Valappil SP. Nanosystems and antibacterial applications. Drug Delivery Nanosystems for Biomedical Applications: Elsevier; 2018. p. 75-90.
49. Zhang N, Li M, Sun X, Jia H, Liu W. NIR-responsive cancer cytomembrane-cloaked carrier-free nanosystems for highly efficient and self-targeted tumor drug delivery. Biomaterials. 2018;159:25-36.
50. Masserini M. Nanoparticles for brain drug delivery. ISRN biochemistry. 2013;2013:238428.
51. Hasan N, Cao J, Lee J, Hlaing SP, Oshi MA, Naeem M, et al. Bacteria-targeted clindamycin loaded polymeric nanoparticles: effect of surface charge on nanoparticle adhesion to MRSA, antibacterial activity, and wound healing. Pharmaceutics. 2019;11(5):236.
52. Wrońska N, Felczak A, Zawadzka K, Janaszewska A, Klajnert B, Bryszewska M, et al. The antibacterial effect of the co-administration of poly (propylene imine) dendrimers and ciprofloxacin. New Journal of Chemistry. 2014;38(7):2987-92.
53. Lin Y-H, Chiou S-F, Lai C-H, Tsai S-C, Chou C-W, Peng S-F, et al. Formulation and evaluation of water-in-oil amoxicillin-loaded nanoemulsions using for Helicobacter pylori eradication. Process Biochemistry. 2012;47(10):1469-78.

54. Bains D, Singh G, Bhinder J, Agnihotri PK, Singh N. Ionic Liquid-Functionalized Multiwalled Carbon Nanotube-Based Hydrophobic Coatings for Robust Antibacterial Applications. ACS Applied Bio Materials. 2020;3(4):2092-103.
55. Omolo CA, Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Ndesendo VM, et al. Pegylated oleic acid: A promising amphiphilic polymer for nano-antibiotic delivery. European Journal of Pharmaceutics and Biopharmaceutics. 2017;112:96-108.
56. Bolhassani A, Javanzad S, Saleh T, Hashemi M, Aghasadeghi MR, Sadat SM. Polymeric nanoparticles: potent vectors for vaccine delivery targeting cancer and infectious diseases. Human Vaccines and Immunotherapeutics. 2014;10(2):321-32.
57. Ahmad M, Khan AU. Global economic impact of antibiotic resistance: a review. Journal of Global Antimicrobial Resistance. 2019;19:313-6.
58. Nagavarma B, Yadav HK, Ayaz A, Vasudha L, Shivakumar H. Different techniques for preparation of polymeric nanoparticles-a review. Asian Journal of Pharmaceutical and Clinical Research. 2012;5(3):16-23.
59. Vasile C. Polymeric Nanomaterials in Nanotherapeutics: Elsevier; 2018.
60. Álvarez-Paino M, Muñoz-Bonilla A, Fernández-García M. Antimicrobial polymers in the nano-world. Nanomaterials. 2017;7(2):48.
61. Wurm FR, Weiss CK. Nanoparticles from renewable polymers. Frontiers in Chemistry. 2014;2:49.
62. Huang K-S, Yang C-H, Huang S-L, Chen C-Y, Lu Y-Y, Lin Y-S. Recent advances in antimicrobial polymers: a mini-review. International Journal of Molecular Sciences. 2016;17(9):1578.
63. Kamaruzzaman NF, Tan LP, Hamdan RH, Choong SS, Wong WK, Gibson AJ, et al. Antimicrobial polymers: The potential replacement of existing antibiotics? International Journal of Molecular Sciences. 2019;20(11):2747.
64. Mehraban N, Musich PR, Freeman HS. Synthesis and encapsulation of a new zinc Phthalocyanine photosensitizer into polymeric nanoparticles to enhance cell uptake and Phototoxicity. Applied Sciences. 2019;9(3):401.
65. Mukherjee A, Waters AK, Kalyan P, Achrol AS, Kesari S, Yenugonda VM. Lipid–polymer hybrid nanoparticles as a next-generation drug delivery platform: state of the art, emerging technologies, and perspectives. International Journal of Nanomedicine. 2019;14:1937.
66. Ahmad N, Ahmad R, Alam MA, Ahmad FJ, Amir M, Pottoo FH, et al. Daunorubicin oral bioavailability enhancement by surface coated natural biodegradable macromolecule chitosan based polymeric nanoparticles. International journal of Biological Macromolecules. 2019;128:825-38.
67. Jurov A, Popović D, Rakić IŠ, Marion ID, Filipič G, Kovač J, et al. Atmospheric pressure plasma jet–assisted impregnation of gold nanoparticles into PVC polymer for various applications. The International Journal of Advanced Manufacturing Technology. 2019;101(1-4):927-38.
68. Pund S, Joshi A. Nanoarchitectures for Neglected Tropical Protozoal Diseases: Challenges and State of the Art. Nano-and Microscale Drug Delivery Systems: Elsevier; 2017. p. 439-80.
69. Lin T, Wang H, Wang H, Wang X. The charge effect of cationic surfactants on the elimination of fibre beads in the electrospinning of polystyrene. Nanotechnology. 2004;15(9):1375.
70. Chaudhary Z, Ahmed N, . ur. Rehman A, Khan GM. Lipid polymer hybrid carrier systems for cancer targeting: A review. International Journal of Polymeric Materials and Polymeric Biomaterials. 2018;67(2):86-100.

71. Hu C-MJ, Zhang L. Nanoparticle-based combination therapy toward overcoming drug resistance in cancer. Biochemical Pharmacology. 2012;83(8):1104-11.
72. Tanaka A, Nakashima H, Kashimura Y, Sumitomo K. Electrostatically induced planar lipid membrane formation on a cationic hydrogel array by the fusion of small negatively charged unilamellar vesicles. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2015;477:63-9.
73. Lee S-M, Chen H, Dettmer CM, O'Halloran TV, Nguyen ST. Polymer-caged lipsomes: a pH-responsive delivery system with high stability. Journal of the American Chemical Society. 2007;129(49):15096-7.
74. Lee S-M, Nguyen ST. Smart nanoscale drug delivery platforms from stimuli-responsive polymers and liposomes. Macromolecules. 2013;46(23):9169-80.
75. Mandal B, Bhattacharjee H, Mittal N, Sah H, Balabathula P, Thoma LA, et al. Core–shell-type lipid–polymer hybrid nanoparticles as a drug delivery platform. Nanomedicine: Nanotechnology, Biology and Medicine. 2013;9(4):474-91.
76. Zeng S-Q, Chen Y-Z, Chen Y, Liu H. Lipid–polymer hybrid nanoparticles for synergistic drug delivery to overcome cancer drug resistance. New Journal of Chemistry. 2017;41(4):1518-25.
77. Troutier A-L, Delair T, Pichot C, Ladavière C. Physicochemical and interfacial investigation of lipid/polymer particle assemblies. Langmuir. 2005;21(4):1305-13.
78. Thevenot J, Troutier A-L, David L, Delair T, Ladavière C. Steric stabilization of lipid/polymer particle assemblies by poly (ethylene glycol)-lipids. Biomacromolecules. 2007;8(11):3651-60.
79. Date T, Nimbalkar V, Kamat J, Mittal A, Mahato RI, Chitkara D. Lipid-polymer hybrid nanocarriers for delivering cancer therapeutics. Journal of Controlled Release. 2018;271:60-73.
80. Zhang Y, Kang L, Huang H, Deng JJAAM, Interfaces. Optically Active Janus Particles Constructed by Chiral Helical Polymers through Emulsion Polymerization Combined with Solvent Evaporation-Induced Phase Separation. ACS Applied Materials and Interfaces. 2020;12(5):6319-27.
81. Gérard Yaméogo JB, Mazet R, Wouessidjewe D, Choisnard L, Godin-Ribuot D, Putaux J-L, et al. Pharmacokinetic study of intravenously administered artemisinin-loaded surface-decorated amphiphilic Î³-cyclodextrin nanoparticles. Material Sciences and Engineering C. 2020;106:110281.
82. Poletto FS, Fiel LA, Donida B, Ré MI, Guterres SS, Pohlmann AR. Controlling the size of poly (hydroxybutyrate-co-hydroxyvalerate) nanoparticles prepared by emulsification–diffusion technique using ethanol as surface agent. Colloids and Surfaces A: Physicochemical and Engineering Aspects 2008;324(1-3):105-12.
83. Krauel K, Davies N, Hook S, Rades T. Using different structure types of microemulsions for the preparation of poly (alkylcyanoacrylate) nanoparticles by interfacial polymerization. Journal of Controlled Release. 2005;106(1-2):76-87.
84. Boondireke S, Léonard M, Durand A, Wongsatayanon BT. Encapsulation of monomyristin into polymeric nanoparticles improved its in vitro antiproliferative activity against cervical cancer cells. Colloids and Surfaces B: Biointerfaces. 2019;176:9-17.
85. Ding S, Serra CA, Vandamme TF, Yu W, Anton N. Double emulsions prepared by two–step emulsification: History, state-of-the-art and perspective. Journal of Controlled Release. 2019;295:31-49.
86. Qiu L, Zhao X, Zu Y, Zhang Y, Liu Y, Wu W, et al. Ursolic acid nanoparticles for oral delivery prepared by emulsion solvent evaporation method: characterization, in vitro

evaluation of radical scavenging activity and bioavailability. Artificial cells, nanomedicine, and biotechnology. 2019;47(1):609-20.
87. Demirkurt B, Cakan-Akdogan G, Akdogan Y. Preparation of albumin nanoparticles in water-in-ionic liquid microemulsions. Journal of Molecular Liquids. 2019;295:111713.
88. Asgari S, Saberi AH, McClements DJ, Lin M. Microemulsions as nanoreactors for synthesis of biopolymer nanoparticles. Trends in Food Science and Technology. 2019;86:118-30.
89. Vladisavljević GT. Preparation of microemulsions and nanoemulsions by membrane emulsification. Colloids and Surfaces A: Physicochemical and Engineering Aspects 2019;579:123709.

90. Bleger F, Murthy AK, Pla F, Kaler EW. Particle nucleation during microemulsion polymerization of methyl methacrylate. Macromolecules. 1994;27(9):2559-65.
91. Chen Z, Zhao Y, Zhu X, Möller M. Formation of Monodisperse Polymer@ SiO2 Core–Shell Nanoparticles via Polymerization in Emulsions Stabilized by Amphiphilic Silica Precursor Polymers: HLB Dictates the Reaction Mechanism and Particle Size. Macromolecules. 2019;52(15):5670-8.
92. Zori MH. Synthesis of TiO 2 nanoparticles by microemulsion/heat treated method and photodegradation of methylene blue. Journal of Inorganic and Organometallic Polymers and Materials 2011;21(1):81-90.

93. Kulkarni SA, Feng S-S. Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. Pharmaceutical research. 2013;30(10):2512-22.
94. Smyth P, Gibson TJ, Irvine G, Black G, Lavery D, Semsarilar M, et al. pH-Responsive benzaldehyde-functionalized PEG-based polymeric nanoparticles for drug delivery: Effect of preparation method on morphology, dye encapsulation and attachment. European Polymer Journal. 2020;124:109471.
95. Bhattacharjee S. DLS and zeta potential–what they are and what they are not? Journal of Controlled Release. 2016;235:337-51.
96. Cho Y-H, Yang J-E, Lee J-S. Size control of polymeric nanoparticles from polystyrene-b-poly (2-vinylpyridine). Materials Science and Engineering: C. 2004;24(1-2):293-5.
97. Chountoulesi M, Perinelli DR, Pippa N, Chrysostomou V, Forys A, Otulakowski L, et al. Physicochemical, morphological and thermal evaluation of lyotropic lipidic liquid crystalline nanoparticles: The effect of stimuli-responsive polymeric stabilizer. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2020;595:124678.
98. Wang W, Hao X, Chen S, Yang Z, Wang C, Yan R, et al. pH-responsive Capsaicin@ chitosan nanocapsules for antibiofouling in marine applications. Polymer. 2018;158:223-30.
99. Borse VB, Konwar AN, Jayant RD, Patil PO. Perspectives of characterization and bioconjugation of gold nanoparticles and their application in lateral flow immunosensing. Drug Delivery and Translational Research. 2020;10:1-25.
100. Lv J, Qiu L, Qu B. Controlled growth of three morphological structures of magnesium hydroxide nanoparticles by wet precipitation method. Journal of Crystal Growth. 2004;267(3-4):676-84.
101. Souza TG, Ciminelli VS, Mohallem NDS, editors. A comparison of TEM and DLS methods to characterize size distribution of ceramic nanoparticles. Journal of Physics: Conference Series; 2016.
102. Vladár AE, Hodoroaba V-D. Characterization of nanoparticles by scanning electron microscopy. Characterization of Nanoparticles: Elsevier; 2020. p. 7-27.

103. Lv J, Qiu L, Qu B. Controlled synthesis of magnesium hydroxide nanoparticles with different morphological structures and related properties in flame retardant ethylene–vinyl acetate blends. Nanotechnology. 2004;15(11):1576.
104. Bohrey S, Chourasiya V, Pandey A. Polymeric nanoparticles containing diazepam: preparation, optimization, characterization, in-vitro drug release and release kinetic study. Nano Convergence. 2016;3(1):1-7.
105. O'neill M. Measurement of Specific Heat Functions by Differential Scanning Calorimetry. Analytical chemistry. 1966;38(10):1331-6.
106. Tailor G, Chaudhay J, Verma D, Sarma BK. Microscopic study of zinc nanoparticles synthesised using thermosetting polymer. Applied Microscopy. 2020;50(1):1-5.
107. Dinarvand R, Mirfattahi S, Atyabi F. Preparation, characterization and in vitro drug release of isosorbide dinitrate microspheres. Journal of Microencapsulation. 2002;19(1):73-81.
108. Zhang Z, Feng S-S. The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly (lactide)–tocopheryl polyethylene glycol succinate nanoparticles. Biomaterials. 2006;27(21):4025-33.
109. Hibbitts A, Lucía A, Serrano-Sevilla I, De Matteis L, McArthur M, De La Fuente JM, et al. Co-delivery of free vancomycin and transcription factor decoy-nanostructured lipid carriers can enhance inhibition of methicillin resistant Staphylococcus aureus (MRSA). PloS one. 2019;14(9):e0220684.
110. Yu Y-H, Lee D, Hsu Y-H, Chou Y-C, Ueng SW, Chen C-K, et al. A Three-Dimensional Printed Polycaprolactone Scaffold Combined with Co-Axially Electrospun Vancomycin/Ceftazidime/Bone Morphological Protein-2 Sheath-Core Nanofibers for the Repair of Segmental Bone Defects During the Masquelet Procedure. International Journal of Nanomedicine. 2020;15:913.
111. Cheow WS, Chang MW, Hadinoto K. Antibacterial efficacy of inhalable levofloxacin-loaded polymeric nanoparticles against E. coli biofilm cells: the effect of antibiotic release profile. Pharmaceutical research. 2010;27(8):1597-609.
112. Suk JS, Lai SK, Wang Y-Y, Ensign LM, Zeitlin PL, Boyle MP, et al. The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles. Biomaterials. 2009;30(13):2591-7.
113. Sanders NN, De Smedt SC, Van Rompaey E, Simoens P, De Baets F, Demeester J. Cystic fibrosis sputum: a barrier to the transport of nanospheres. American Journal of Respiratory and Critical Care Medicine. 2000;162(5):1905-11.
114. Bennet D, Kim S. Polymer nanoparticles for smart drug delivery. Application of Nanotechnology in Drug Delivery. 2014:257-310.
115. Traini D, Young PM. Delivery of antibiotics to the respiratory tract: an update. Expert Opinion on Drug Delivery. 2009;6(9):897-905.
116. Banik BL, Fattahi P, Brown JL. Polymeric nanoparticles: the future of nanomedicine. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology. 2016;8(2):271-99.
117. Martinez JO, Brown BS, Quattrocchi N, Evangelopoulos M, Ferrari M, Tasciotti E. Multifunctional to multistage delivery systems: The evolution of nanoparticles for biomedical applications. Chinese Science Bulletin. 2012;57(31):3961-71.
118. Cheng W, Gu L, Ren W, Liu Y. Stimuli-responsive polymers for anti-cancer drug delivery. Materials Science and Engineering: C. 2014;45:600-8.
119. Hayashi K, Ono K, Suzuki H, Sawada M, Moriya M, Sakamoto W, et al. High-frequency, magnetic-field-responsive drug release from magnetic nanoparticle/organic hybrid based on hyperthermic effect. ACS Applied Materials and Interfaces. 2010;2(7):1903-11.
120. Liu J, Huang Y, Kumar A, Tan A, Jin S, Mozhi A, et al. pH-sensitive nano-systems for drug delivery in cancer therapy. Biotechnology Advances. 2014;32(4):693-710.

121. Liu R, Zhang Y, Zhao X, Agarwal A, Mueller LJ, Feng P. pH-responsive nanogated ensemble based on gold-capped mesoporous silica through an acid-labile acetal linker. Journal of the American Chemical Society. 2010;132(5):1500-1.
122. Sun JT, Yu ZQ, Hong CY, Pan CY. Biocompatible zwitterionic sulfobetaine copolymer-coated mesoporous silica nanoparticles for temperature-responsive drug release. Macromolecular Rapid Communications. 2012;33(9):811-8.
123. Oshiro-Júnior JA, Rodero C, Hanck-Silva G, Sato MR, Alves RC, Eloy JO, et al. Stimuli-responsive Drug Delivery Nanocarriers in the Treatment of Breast Cancer. Current Medicinal Chemistry. 2020;27(15):2494-513.
124. Luo L, Xu F, Peng H, Luo Y, Tian X, Battaglia G, et al. Stimuli-responsive polymeric prodrug-based nanomedicine delivering nifuroxazide and doxorubicin against primary breast cancer and pulmonary metastasis. Journal of Controlled Release. 2020;318:124-35.
125. Berenguer D, Alcover M, Sessa M, Halbaut L, Guillén C, Boix-Montañés A, et al. Topical amphotericin B semisolid dosage form for cutaneous leishmaniasis: Physicochemical characterization, ex vivo skin permeation and biological activity. Pharmaceutics. 2020;12(2):149.
126. Jain AK, Swarnakar NK, Godugu C, Singh RP, Jain S. The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen. Biomaterials. 2011;32(2):503-15.
127. Zhang Z, Tsai PC, Ramezanli T, Michniak-Kohn BB. Polymeric nanoparticles-based topical delivery systems for the treatment of dermatological diseases. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology. 2013;5(3):205-18.
128. Li Y, Liu G, Wang X, Hu J, Liu S. Enzyme-responsive polymeric vesicles for bacterial-strain-selective delivery of antimicrobial agents. Angewandte Chemie International Edition. 2016;55(5):1760-4.
129. Radovic-Moreno AF, Lu TK, Puscasu VA, Yoon CJ, Langer R, Farokhzad OC. Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics. ACS nano. 2012;6(5):4279-87.
130. Michalak G, Głuszek K, Piktel E, Deptuła P, Puszkarz I, Niemirowicz K, et al. Polymeric nanoparticles–a novel solution for delivery of antimicrobial agents. Medical Studies/Studia Medyczne. 2016;32(1):56-62.
131. Wang A, Gao H, Sun Y, Sun Y-l, Yang Y-W, Wu G, et al. Temperature-and pH-responsive nanoparticles of biocompatible polyurethanes for doxorubicin delivery. International Journal of Pharmaceutics. 2013;441(1-2):30-9.
132. Bawa P, Pillay V, Choonara YE, Du Toit LC. Stimuli-responsive polymers and their applications in drug delivery. Biomedical Materials. 2009;4(2):022001.
133. Hwang AA, Lee BY, Clemens DL, Dillon BJ, Zink JI, Horwitz MA. pH-Responsive Isoniazid-Loaded Nanoparticles Markedly Improve Tuberculosis Treatment in Mice. Small. 2015;11(38):5066-78.
134. Liang W, Chow MY, Lau PN, Zhou QT, Kwok PC, Leung GP, et al. Inhalable dry powder formulations of siRNA and pH-responsive peptides with antiviral activity against H1N1 influenza virus. Molecular Pharmaceutics. 2015;12(3):910-21.
135. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. Nature reviews Molecular Cell Biology. 2002;3(10):753-66.
136. Datz S, Illes B, Gößl D, Schirnding Cv, Engelke H, Bein T. Biocompatible crosslinked β-cyclodextrin nanoparticles as multifunctional carriers for cellular delivery. Nanoscale. 2018;10(34):16284-92.
137. Insel PA, Sriram K, Salmerón C, Wiley SZ. Proton-sensing G protein-coupled receptors: detectors of tumor acidosis and candidate drug targets. Future Medicinal Chemistry. 2020;12(6):523-32.

138. Pizzorno J. Acidosis: An old idea validated by new research. Integrative Medicine: A Clinician's Journal. 2015;14(1):8.
139. Cumhur CM, Cure E. Comment: Sodium-Glucose Cotransporters as Potential Therapeutic Targets in Patients With Type 1 Diabetes Mellitus: An Update on Phase 3 Clinical Trial Data. The Annals of Pharmacotherapy. 2020;54(9):939-40.
140. Cure E, Cure C. Comment on Sodium-Glucose Co-Transporter 2 Inhibitors and Heart Failure. The American Journal of Cardiology. 2020;125(10):1602.
141. Sadhukhan P, Kundu M, Chatterjee S, Ghosh N, Manna P, Das J, et al. Targeted delivery of quercetin via pH-responsive zinc oxide nanoparticles for breast cancer therapy. Materials Science and Engineering: C. 2019;100:129-40.
142. Thambi T, Lee DS. Stimuli-responsive polymersomes for cancer therapy. Stimuli Responsive Polymeric Nanocarriers for Drug Delivery Applications: Elsevier; 2019. p. 413-38.
143. Kanamala M, Wilson WR, Yang M, Palmer BD, Wu Z. Mechanisms and biomaterials in pH-responsive tumour targeted drug delivery: a review. Biomaterials. 2016;85:152-67.
144. Duan F, Feng X, Jin Y, Liu D, Yang X, Zhou G, et al. Metal–carbenicillin framework-based nanoantibiotics with enhanced penetration and highly efficient inhibition of MRSA. Biomaterials. 2017;144:155-65.
145. Meeker DG, Jenkins SV, Miller EK, Beenken KE, Loughran AJ, Powless A, et al. Synergistic photothermal and antibiotic killing of biofilm-associated Staphylococcus aureus using targeted antibiotic-loaded gold nanoconstructs. ACS Infectious Diseases. 2016;2(4):241-50.
146. Betancourt T, Brown B, Brannon-Peppas L. Doxorubicin-loaded PLGA nanoparticles by nanoprecipitation: preparation, characterization and in vitro evaluation. Nanomedicine. 2007;2(2):219-32.
147. Dave V, Yadav RB, Kushwaha K, Yadav S, Sharma S, Agrawal U. Lipid-polymer hybrid nanoparticles: Development & statistical optimization of norfloxacin for topical drug delivery system. Bioactive Materials. 2017;2(4):269-80.
148. Seedat N, Kalhapure RS, Mocktar C, Vepuri S, Jadhav M, Soliman M, et al. Co-encapsulation of multi-lipids and polymers enhances the performance of vancomycin in lipid–polymer hybrid nanoparticles: In vitro and in silico studies. Materials Science and Engineering: C. 2016;61:616-30.
149. Wang T, Luo Y. Chitosan hydrogel beads functionalized with thymol-loaded solid lipid–polymer hybrid nanoparticles. International Journal of Molecular Sciences. 2018;19(10):3112.
150. Thakur K, Sharma G, Singh B, Chhibber S, Katare OP. Nano-engineered lipid-polymer hybrid nanoparticles of fusidic acid: an investigative study on dermatokinetics profile and MRSA-infected burn wound model. Drug Delivery and Translational Research. 2019;9(4):748-63.
151. Lodise Jr TP, Rosenkranz SL, Finnemeyer M, Evans S, Sims M, Zervos MJ, et al. The Emperor's New Clothes: PRospective Observational Evaluation of the Association Between Initial VancomycIn Exposure and Failure Rates Among ADult HospitalizEd Patients With Methicillin-resistant Staphylococcus aureus Bloodstream Infections (PROVIDE). Clinical Infectious Diseases. 2020;70(8):1536-45.
152. Mainardi J-L, Villet R, Bugg TD, Mayer C, Arthur M. Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS Microbiology Reviews. 2008;32(2):386-408.
153. Mashaly M, El-Mashad N, El-deeb H. Detection of VanA type vancomycin resistance among MRSA isolates from an emergency hospital in Egypt. Comparative Clinical Pathology. 2019;28(4):971-6.

154. Wormser GP, Barbour AG. Evaluation of the clinical relevance of vancomycin for the treatment of Lyme disease. Wiener klinische Wochenschrift. 2019:1-5.
155. Bartash R, Cowman K, Szymczak W, Guo Y, Ostrowsky B, Binder A, et al. Multidisciplinary Tool Kit for Febrile Neutropenia: Stewardship Guidelines, Staphylococcus aureus Epidemiology, and Antibiotic Use Ratios. Journal of Oncology Practice. 2020:JOP. 19.00492.
156. Sharma R, Hammerschlag MR. Treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections in children: a reappraisal of vancomycin. Current Infectious Disease Reports. 2019;21(10):37.
157. Mader JT, Reinarz JA. Treatment of bacterial osteomyelitis and Staphylococcus aureus endocarditis and single-dose therapy for uncomplicated Neisseria gonorrhoeae infections: an overview. Reviews of Infectious Diseases. 1984;6(Supplement_4):S857-S64.
158. Stogios PJ, Savchenko A. Molecular mechanisms of vancomycin resistance. Protein Science. 2020;29(3):654-69.
159. Naha A, Miryala SK, Debroy R, Ramaiah S, Anbarasu A. Elucidating the multi-drug resistance mechanism of Enterococcus faecalis V583: A gene interaction network analysis. Gene. 2020;748:144704.
160. Dowd F, Johnson B, Mariotti A, Kumar P. Pharmacology of specific drug groups: antibiotic therapy. Pharmacology and Dental Therapeutics. 2017:457-87.
161. Saeed A, Ahsan F, Nawaz M, Iqbal K, Rehman KU, Ijaz T. Incidence of vancomycin resistant phenotype of the methicillin resistant Staphylococcus aureus isolated from a tertiary care hospital in Lahore. Antibiotics. 2020;9(1):3.
162. Pumerantz A, Muppidi K, Agnihotri S, Guerra C, Venketaraman V, Wang J, et al. Preparation of liposomal vancomycin and intracellular killing of meticillin-resistant Staphylococcus aureus (MRSA). International Journal of Antimicrobial Agents. 2011;37(2):140-4.
163. Yang Z, Liu J, Gao J, Chen S, Huang G. Chitosan coated vancomycin hydrochloride liposomes: characterizations and evaluation. International Journal of Pharmaceutics. 2015;495(1):508-15.
164. Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Singh S, Renukuntla J, et al. pH-responsive chitosan nanoparticles from a novel twin-chain anionic amphiphile for controlled and targeted delivery of vancomycin. Colloids and Surfaces B: Biointerfaces. 2017;158:650-7.
165. Kalhapure RS, Sikwal DR, Rambharose S, Mocktar C, Singh S, Bester L, et al. Enhancing targeted antibiotic therapy via pH responsive solid lipid nanoparticles from an acid cleavable lipid. Nanomedicine. 2017;13(6):2067-77.
166. Makhathini SS, Omolo CA, Gannimani R, Mocktar C, Govender T. pH-Responsive Micelles from an Oleic Acid Tail and Propionic Acid Heads Dendritic Amphiphile for the Delivery of Antibiotics. Journal of Pharmaceutical Sciences. 2020;109(8):2594-606.
167. Wu Y, van der Mei HC, Busscher HJ, Ren Y. Enhanced bacterial killing by vancomycin in staphylococcal biofilms disrupted by novel, DMMA-modified carbon dots depends on EPS production. Colloids and Surfaces B: Biointerfaces. 2020;193:111114.
168. Zhang W, Taheri-Ledari R, Hajizadeh Z, Zolfaghari E, Ahghari MR, Maleki A, et al. Enhanced activity of vancomycin by encapsulation in hybrid magnetic nanoparticles conjugated to a cell-penetrating peptide. Nanoscale. 2020;12(6):3855-70.
169. Xie X, Sun T, Xue J, Miao Z, Yan X, Fang W, et al. Ag Nanoparticles Cluster with pH-Triggered Reassembly in Targeting Antimicrobial Applications. Advanced Functional Materials. 2020;30(17):2000511.
170. Salih M, Omolo CA, Agrawal N, Walvekar P, Waddad AY, Mocktar C, et al. Supramolecular amphiphiles of Beta-cyclodextrin and Oleylamine for enhancement of vancomycin delivery. International Journal of Pharmaceutics. 2020;574:118881.

Chapter Three

Submitted manuscript

Chapter Three – Submitted manuscript

3.1 Introduction

The following research paper was submitted to the International Journal of Pharmaceutics which has an impact factor of 4.845 from work done during this study.

Miss Yajna Jaglal contributed to the project design and characterization of the novel nanoformulation. In addition, Miss Jaglal was responsible for the analysis and interpretation of all data, wrote the first draft of the paper and undertook all corrections. Mrs Nawras Osman assisted with formula optimization and characterization and *in vitro* drug release studies. Dr Calvin Omolo contributed to conceptualisation, design and formulation of the drug delivery system and supervision of the characterization and application studies and thesis and abstract editing. Dr Chunderika Mocktar supervised the *in vitro* antibacterial activity studies. Dr Nikita Devnarain conducted the cytotoxicity studies, *in silico* studies and editing. Prof Thirumala Govender served as supervisor and was responsible for project conceptualization, thesis and abstract editing and overall supervision of the study.

3.2 Submitted manuscript

Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhancement of the antibacterial activity of vancomycin and 18β-glycyrrhetinic acid

Yajna Jaglal [1], Nawras Osman [1,2], Calvin A. Omolo [1,3], Chunderika Mocktar [1], Nikita Devnarain [1] and Thirumala Govender [1]*

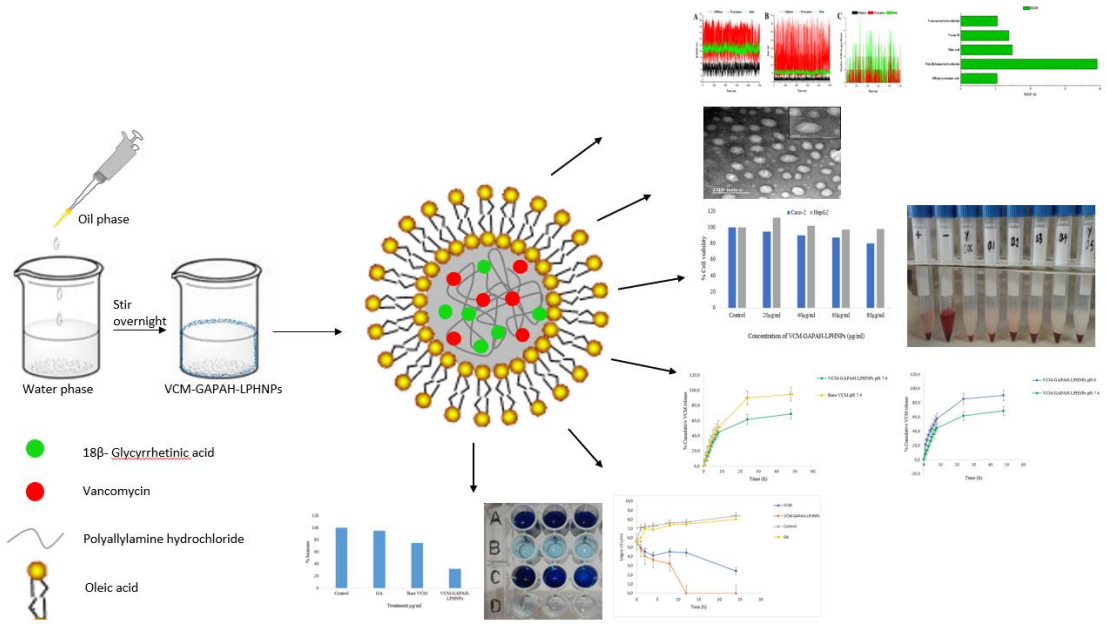
[1] Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal, Private Bag X 54001, Durban, 4000, South Africa.

[2] Department of Pharmaceutics, Faculty of Pharmacy, University of Gezira, Wad Medani, Sudan

[3] United States International University-Africa, School of Pharmacy and Health Sciences, Department of Pharmaceutics and Pharmacy Practice, P. O. Box 14634-00800, Nairobi, Kenya.

* Correspondence: govenderth@ukzn.ac.za; Tel.: +27 31 260 7357, Fax: +27 31 260 7792.

Graphical abstract



Abstract: Despite advancement in the control and therapeutics of infectious diseases, antimicrobial resistance remains a global burden. Hence there is a need for novel strategies that improve and potentiate available antibiotics to prevent a regress to a pre-antibiotic era. This study aimed to co-deliver vancomycin (VCM) and 18β-glycyrrhetinic acid (GA) via pH-responsive lipid-polymer hybrid nanoparticles (VCM-GAPAH-LPHNPs) to explore its potential for enhanced activity and targeted delivery of VCM. The stability of VCM-GAPAH-LPHNPs were supported by *in silico* studies. Biosafe VCM-GAPAH-LPHNPs were prepared using the microemulsion technique. VCM-GAPAH-LPHNPs demonstrated size, polydispersity index, zeta potential and encapsulation efficiency of 198.4 ± 0.302 nm, 0.255 ± 0.003, -3.8 ± 0.335 mV and 69.46 ± 2.52 %, respectively. *In vitro* drug release studies revealed that VCM-GAPAH-LPHNPs had sustained and faster release at acidic conditions compared to bare VCM. VCM-GAPAH-LPHNPs also demonstrated greater *in vitro* antibacterial potential against methicillin-resistant *Staphylococcus aureus* by 16-fold, when compared to the bare drug. Additionally, the time-killing assay indicated the ability of VCM-GAPAH-LPHNPs to eliminate 75 % of MRSA in less than 12 h. Furthermore, crystal violet assay confirmed VCM-GAPAH-LPHNPs potential to eliminate biofilms. Therefore, these novel LPHNPs may serve as promising nanocarriers for enhancing antibiotic drug delivery and antibacterial activity.

Keywords: pH-responsive; lipid-polymer hybrid nanoparticles; vancomycin; methicillin-resistant *Staphylococcus aureus*; 18β- glycyrrhetinic acid

1. Introduction

Globally, infectious diseases primarily due to bacterial infections, are considered one of the main contributors towards increasing morbidity and mortality rates (1, 2). The WHO reported that more than 17 million deaths are caused by infectious diseases per year (3). Recent data indicate that by 2050, more individuals will die from bacterial diseases than any other disease, including cancer (4). Moreover, by then, approximately 4 million deaths are predicted in Africa due to antimicrobial resistance, with a possibility of higher death rates if appropriate measures are not undertaken (5). Hence, urgent interventions are required for the development of novel and innovative strategies to curtail current and emerging antibiotic-resistant pathogenic bacterial strains that are no longer sensitive to conventional antibiotics (6, 7).

Current traditional antibiotic dosage forms possess various limitations, including suboptimal drug concentration at infection sites, high exposure to healthy cells, frequent administration of high doses and prolonged therapy (8-10). This results in sub-optimal drug delivery and activity, increased adverse effects, the toxicity of cells/tissues and poor patient compliance, thus contributing to poor outcomes and development of antibiotic resistance (11, 12). Hence, the engineering, formulation and application of novel drug delivery systems (NDDS) are explored (9) as an advanced strategy to suppress the limitations related to conventional dosage forms that contribute to antimicrobial resistance (13, 14).

Novel drug delivery systems have showcased their potential in addressing the disadvantages related to conventional antibiotics by enhancing drug delivery at target sites of infection due to their subcellular size, biocompatibility with host cells/tissues and sizeable surface area to mass ratio (9, 15, 16). Lipid-polymer hybrid nanoparticles (LPHNPs) are one of the promising systems that are currently being employed to deliver drugs efficiently (17, 18). LPHNPs combine the mechanical advantages of polymeric nanoparticles and prowess of lipidic systems (17, 19). They are core-shell self-assembled nanosystems composed of a hydrophobic lipid core and a polymeric shell (20). Flexibility in the formulation of LPHNPs allows the formulation of the system that has programmable ability to respond to stimuli (21, 22). This can be achieved by focusing on the biofunctional property of the nanoparticle matrix in order to introduce targeted drug delivery and enhanced activity (23). The advancement in the field of nanotherapeutics has encouraged the development of pH-responsive nanosystems for effective and efficient antibiotic delivery (24-26). However, there are few reports on employing pH-responsive LPHNPs for antibiotic delivery. Therefore using this strategy, systems can be

designed to have programmable destabilization and release the drug due to changes in pH that are synonymous with bacterial infection sites (27, 28).

Plants contain rich sources of bioactive phytochemical compounds that are active against a wide spectrum of activity, 18β-glycyrrhetinic acid (GA) is such a compound (29, 30). It is a pentacyclic triterpenoid metabolite of a hydrolyzed product of glycyrrhizic acid from the *Glycyrrhiza glabra* (liquorice) plant (31). This bioactive compound has been reported to have anti-allergic (32), antibacterial, antiviral, antitumor and anti-inflammatory properties (33, 34). Due to the antibacterial activity of GA, it can be explored by co-delivery with other antibiotics in the market for synergistic action and augmentation of antibacterial drugs in the market (33, 35).

Surveys from the literature show combinational therapy of antibiotics in the market and bioactive compounds from natural sources have shown to enhance antibacterial activity (36). However, currently, there are no reports of GA being co-delivered with antimicrobial agents in the market in a nanosystem to target bacterial infectious diseases. Therefore, the combination of GA and antibiotics can further be explored by co-loading in a stimuli-responsive nanosystem for targeted and further enhancement of antimicrobial activity (37). Such a system will also contribute to the advancement of the pharmaceutical field.

There are several reports of co-delivering GA with cancer and inflammatory drugs loaded in nanosystems for the treatment of cancer diseases (37-40). However, from our search of the literature, such a formulation for co-delivery of GA and antibiotics has not been reported before. We herein report vancomycin (VCM) and GA loaded LPHNPs that was coined from PAH and OA. This is the first report of LPHNPs for co-delivery of a hydrophilic drug and hydrophobic antibacterial agent in a pH-responsive drug delivery system for targeting bacteria. Therefore, the aim of this study was to formulate a multifunctional LPHNP that is pH-responsive and co-loaded with two antibacterial agents. We envisage enhanced antibacterial activity due to the synergistic activity of the two loaded antibacterial agents, in addition to pH-responsive targeted delivery of VCM and GA as a result of protonation and deprotonation of the OA and PAH. In basic media, OA will deprotonate becoming negatively charged and it will electrostatically combine with the positive amines of PAH, while in acidic media both OA and PAH protonate and both molecules become positively charged thus repelling each other. Hence, the disintegration of the LPHNPs system will lead to an increase in drug release. Moreover, protonation and deprotonation will lead to surface switching LPHNPs from negative

in basic media to positive in acidic media. To the best of our knowledge, such a multifunctional LPHNP system has never been reported before.

Furthermore, no study has reported the pH-responsive co-delivery of VCM and GA nanoparticles to target bacteria. The advantages of the co-delivery of both hydrophilic and hydrophobic agents for combinational therapy, pH-responsive release for targeted delivery of VCM and GA and surface charge switching of the systems to target the negatively charged bacterial cell membrane can potentiate antibacterial effects of the system by acting in different mechanisms. The formulation and evaluation of this novel multifunctional LPHNP are reported in this paper.

2. Materials and Methods

2.1 Materials

18β- Glycyrrhetinic acid, polyallylamine hydrochloride (PAH), Tween 80, VCM hydrochloride, oleic acid (OA), dialysis tubing cellulose membrane, Mueller Hinton broth 2 (MHB) were obtained from Sigma-Aldrich (USA). Mueller Hinton Agar (MHA) and Nutrient Broth were obtained from Biolab Inc. (South Africa). Milli-Q purified water was obtained from an Elix® water purification system Millipore Corp. (USA). Sheep blood was purchased from United Scientific SA cc. (South Africa). Bacterial strains used were *Staphylococcus aureus* (Rosenbach) (ATCC®BAA-1683) (MRSA) and *Staphylococcus aureus* (ATCC 25922) (SA). The CACO-2 and HepG2 cell lines were purchased from ATCC.

2.2 Molecular dynamics of components of oil phase, water phase and both phases

To study the stability of the oil phase and water phase independently and then the assembly of the oil and water phase to form a complex**,** the AMBER suite was used to simulate the oil phase components, GA and OA, then separately the water phase components including PAH, VCM and Tween 80 and finally both oil and water phase components. Molecular dynamic simulations represent an all-encompassing toolkit that delves into the atomic arrangement within molecules, thus providing novel perspectives on the structural movements of molecular systems. The molecular dynamics simulations were carried out using the PMEMD engine of the AMBER software package with GPU acceleration (41). Gasteiger charges were used to charge compounds and ANTECHAMBER was used to create atomic partial charges using the General AMBER Force Field and Restrained Electrostatic Potential (RESP) methods. The LEAP module of AMBER 14 was employed to combine, neutralize and solvate all systems via

the addition of hydrogen atoms and chloride and sodium ions and suspended the systems in an orthorhombic box of TIP3P water molecules such that all atoms were within 10 Å of the box edges. The protein residues were renumbered due to missing residues in the initial crystal structure. An initial minimization was performed for 2500 steps with a restraint potential of 10 $kcal.mol^{-1}Å^{-2}$ to the solutes and for 500 steps of steepest descent followed by 500 steps of the conjugate gradient. This was followed by 1000 steps of full minimization Langevin thermostat, with a collision frequency of 1.0 ps^{-1} with harmonic restraint of 5 $kcal.mol^{-1}$ $Å^{-2}$ on the solutes, applied during the gradual heating up of the systems to a temperature of 300 K in the canonical ensemble for 50 ps. This was followed by 50 ps of density equilibration in isothermal–isobaric ensemble and a final 500 ps equilibration at 300 K, 1 bar pressure and a coupling constant of 2 ps. The simulations were performed for 500 ns using classical molecular dynamics with a time step of 2 fs, with the frame being recorded at every 500 steps of simulation. All the bond lengths involving hydrogen atoms were constrained using the SHAKE algorithm (42). All the MD simulations were carried out using the GPU Amber 14 software package (43).

2.3 Preparation of VCM-GAPAH-LPHNPs

A previously reported micro-emulsion technique was used to prepare the VCM and GA loaded pH-responsive LPHNPs (44). Briefly, the oil phase, consisting of OA (40 mg) and GA (10 mg) was dissolved in ethanol (2 ml). While the aqueous phase was prepared by dissolving PAH (10 mg), VCM (10 mg) and Tween 80 (20 mg) in 20 ml distilled water. The oil phase was added in a dropwise manner to the aqueous phase under stirring overnight at 500 rpm.

2.4 Size, Polydispersity Index (PDI), Zeta Potential (ZP) and Morphology

The size, PDI and ZP of VCM-GAPAH-LPHNPs were determined by the dynamic light scattering (DLS) technique using a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., UK) at 25 °C. An aliquot of LPHNPs was appropriately diluted with phosphate buffers (pH 7.4, 6 and 4.5) to obtain concentrations that were within the system's sensitivity range, the experiments were performed in triplicate (44, 45).

The morphology of VCM-GAPAH-LPHNPs was determined by transmission electron microscopy (TEM). Samples were suitably diluted, stained with 1 % uranyl acetate (UA) solution, air-dried and visualized using a TEM (JOEL JEM-1010, Japan) operating at an accelerating voltage of 100 kV (46).

2.5 Entrapment efficiency (EE%) and drug loading (DL%)

The EE% and DL% of VCM-GAPAH-LPHNPs were determined by an ultrafiltration method. Briefly, LPHNPs emulsion (3 ml) was inserted into Amicon® Ultra-4 centrifugal filter tubes (Millipore Corp., USA) with 10 kDa pore size and centrifuged at 2000 rpm at 25 °C for 20 min (14). The unentrapped VCM was detected using a validated High-Pressure Liquid Chromatograph (Shimadzu, Japan) with UV detection at λ 280 nm. The mobile phase, consisting of water with 0.1 % trifluoroacetic acid (TFA) and acetonitrile (85/15 v/v), was pumped through a Nucleosil 100-5C18 column (150 mm X 4.6 mm in diameter) at a flow rate of 1 ml per min and an injection volume of 100 µl, with a regression equation of Y = 0.0044 x – 0.0013 and linearity coefficient (R^2) of 0.9997. EE% and DL% were calculated using equations (1) and (2) below (27, 47).

$$\mathbf{EE}\ (\%) = \left(\frac{\textbf{Weight of VCM/GA in LPHNPs}}{\textbf{Weight of VCM/GA added}}\right) \times \mathbf{100} \quad (1)$$

$$\mathbf{DL}\ (\%) = \left(\frac{\textbf{Weight of VCM/GA in LPHNPs}}{\textbf{Total weight of nanoparticles}}\right) \times \mathbf{100} \quad (2)$$

2.6 Thermal profiles

Differential scanning calorimetry (DSC) using a DSC-60 (Shimadzu, Japan) was used to determine the thermal profiles of the bare VCM, GA, physical composition and lyophilized drug-loaded LPHNPs. Briefly, each sample (2 mg) was placed in an aluminium pan and sealed. The scanning was done at a temperature range of 30 °C - 300 °C at a constant rate of 10 °C/min under a constant nitrogen flow of 10 ml/min, an empty pan was used as a reference (48).

2.7 *In vitro* drug release and release kinetics

The *in vitro* release studies were performed using a dialysis bag technique to investigate the release profile and mechanism of VCM-loaded GAPAH-LPHNPs. Dialysis bags with a pore size of 8000–14,400 Da containing the VCM-GAPAH-LPHNPs (VCM/GA 0.5 mg/ml each) were placed in PBS (40 ml) of pH 6 and 7.4 at 37 °C in a shaking incubator at 100 rpm. At 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 h, the samples (3 ml) were withdrawn and replaced with equal quantities of the fresh PBS solutions of pH 6 and 7.4 to maintain a constant volume. The amount of VCM released per time interval was determined by HPLC (Shimadzu, Japan) with UV detection set at λ 280 nm, with a regression equation of Y = 14129 x + 115582 and linearity coefficient (R^2) of 0.9071, all experiments were performed in triplicate (1, 2, 49).

Drug release kinetics were analysed using the DDSolver software program [60]. Six mathematical models, namely zero order, first order, Higuchi, Hixson-Crowel, Korsmeyer-Peppas and Weibull were used to calculate and compare the correlation coefficient (R^2) and root-mean-square error (RMSE) values.

2.8 *In vitro* cytotoxicity

The biocompatibility of VCM-GAPAH-LPHNPs was determined using the MTT assay following a previously reported method (50) on two cell lines, which included human intestinal epithelial cancer cells and human liver adenocarcinoma cells (CACO-2 and HepG2, respectively). Briefly, the cell lines were cultured and supplemented with 10 % foetal bovine serum (FBS), 1 % streptomycin and penicillin solution and 1 % L-glutamine, which were incubated at 37 ℃ in a humidified atmosphere of 5% carbon dioxide (CO_2). Following 80 % confluency, the cell lines were seeded into a 96-well plate. After incubation for 24 h, the culture medium containing the treatment samples (20, 40, 60 and 80 µg/ml) were replaced with fresh culture medium (100 µl per well) and MTT solution (20 µl per well). After 4 h of incubation, the culture media and MTT assay solutions were immediately removed and dimethyl sulfoxide (100 µl) was added to each well to solubilize the MTT formazan crystals. The absorbance corresponding to each well was measured at λ 570 nm (Spectrostar Nano, Germany); all experiments were performed in triplicate. Equation (3) was used to determine the percentage cell viability:

$$\textbf{Cell viability}\ (\%) = \left(\frac{\textbf{A570nm treated cells}}{\textbf{A570nm untreated cells}}\right) \times \mathbf{100} \quad (3)$$

2.9 Haemolysis

Haemolytic toxicity of VCM-GAPAH-LPHNPs was determined at different concentrations (15). Briefly, sheep blood was washed thrice with an isotonic 0.1 M PBS solution (pH 7.4) by centrifugation at 3000 rpm for 5 min. For each sample, VCM-GAPAH-LPHNPs formulation was diluted with 0.1 M PBS to obtain concentrations ranging from 0.05 to 0.5 mg/ml. The red blood cell (RBC) suspension (0.2 ml) was added to 1.8 ml of each sample and incubated at 37 º C for 30 min. Thereafter the samples were centrifuged at 3000 rpm for 10 min. The supernatant of each sample was then collected and analysed for haemoglobin released by UV spectrophotometry at λ 416 nm. To obtain 0 % and 100 % haemolysis, 0.2 ml of RBC

suspension was added to 1.8 ml of PBS and distilled water, respectively, as a control. The degree of haemolysis was calculated using equation (4):

$$\textbf{Haemolysis}\ (\%) = \left(\frac{\mathbf{ABS-ABS0}}{\mathbf{ABS100-ABS0}}\right) \times \mathbf{100}\ \%\ (4)$$

where ABS100 and ABS_0 are the absorbances of the samples at 100% and 0% haemolysis, respectively.

2.10 Stability studies

The stability of VCM-GAPAH-LPHNPs was tested using DLS for 0,30,60 and 90 days of storage, at 4 °C and room temperature (51).

2.11 *In vitro* antibacterial activity and fractional inhibitory concentration (FIC) index

The minimum inhibitory concentration (MIC) values of bare VCM, GA, VCM-GAPAH-LPHNPs and blank-GAPAH-LPHNPs were determined against SA and MRSA (pH 6 and 7.4) using the broth dilution method. Briefly, bacteria culture was grown in Nutrient Broth at 37°C in a shaking incubator (Labcon, USA) at 100 rpm for 18 h. Bacterial cultures were suitably diluted to achieve a concentration equivalent to 0.5 McFarland's Standard using a DEN-1B McFarland densitometer (Latvia). This was again diluted to 1:150 with sterile distilled water to achieve colony forming units per ml (CFU/ml) of 5×10^5. All tested samples were serially diluted in MHB (pH 6 and 7.4) and incubated with the diluted bacterial cultures at 37°C in the shaking incubator at 100 rpm for a total of 96 h. At 24, 48 and 72 h, tested samples (5 µl) were spotted onto MHA and incubated at 37°C for 24 h. The bare drug formulation was used as the positive control while the blank formulation of LPHNPs was used as a negative control. All experiments were performed in triplicate (52-54).

The antibacterial effect of bare VCM and GA in combination with VCM-GAPAH-LPHNPs against SA and MRSA was determined using the cumulative FIC of bare VCM and GA based on the Loewe additivity zero-interaction theory. The FIC index was calculated using equations 5 and 6 and the FIC index is shown in (Table 1).

$$\textbf{FIC formula}: \boldsymbol{\Sigma}\ \textbf{FIC} = \textbf{FIC of agent A} + \textbf{FIC of agent B}$$

$$\textbf{FIC of agent A} = \frac{\textbf{MIC of agent A in combination with agent B}}{\textbf{MIC of agent A alone}} \quad (5)$$

$$\textbf{FIC of agent B} = \frac{\textbf{MIC of agent B in combination with agent A}}{\textbf{MIC of agent B alone}} \quad (6)$$

Agent A: Bare VCM

Agent B: GA

Agents (A-B) in combination: VCM-GAPAH-LPHNPs

Table 1. FIC index.

FIC index	Interpretation
Σ FIC ≤ 0.5	Synergism
> 0.5 Σ FIC ≤ 1	Additive
> 1 Σ FIC < 2	Indifference
Σ FIC ≥ 2	Antagonism

Synergism combination indicates that a lower concentration of agents A and B is required in order to produce the same effect as agent A and agent B alone. While additive combination indicates that the same concentration of agents A and B are required in order to produce the same effect as agent A and agent B alone, the indifference combination indicates agent B is inactive since there was no effect of it to be added but only the effect of the active agent, which is agent A. However, antagonism combination indicates that higher concentration of agents A and B are required in order to produce the same effect as agent A and agent B alone (55-58).

2.12 Time-killing assay

The time-killing analysis of VCM-GAPAH-LPHNPs was conducted using the plate colony count method, as previously reported (59, 60). MRSA was cultured for 48 h at 37 °C in nutrient broth and a shaking incubator followed by bacterial dilution in sterile PBS to obtain

concentrations of 10^5 – 10^6 CFU/ml (61). Thereafter bare VCM, GA, sterile water and VCM-GAPAH-LPHNPs were added to the PBS containing MRSA at a concentration of 5 times greater than MIC. The samples were placed in the shaking incubator at 37 °C. At 1, 2, 4, 8, 12 and 24 h intervals, 0.1 ml of each sample was sub-cultured on nutrient agar plates for 24 h. The number of colonies counted were converted to $\log_{10}$ values and plotted on a graph. The experiment was performed in triplicate.

2.13 MRSA biofilm reduction

The eradication of MRSA biofilms by VCM-GAPAH-LPHNPs was determined using crystal violet (CV) assay (62). Briefly, 100 µl of MRSA suspensions (1.5×10^8 CFU/ml) in nutrient broth was inserted into a 96-well plate and incubated for 3 days at 37 °C to form a mature biofilm. Prior to treatment, the media was removed from the wells and the wells were thoroughly washed with PBS (pH 7.4) to remove non-adherent bacterial cells. Precisely, 100 µl of bare VCM solution (390 µg/ml), GA solution (3,125 µg/ml) and VCM-GAPAH-LPHNPs (390 µg/ml) formulation were added to the wells at a concentration of 100 times greater than MIC and incubated for 12 h at 37 °C. The wells were washed with PBS for the removal of the treatments and non-adherent bacterial cells. Thereafter, the wells were fixed with methanol for 15 mins. The plate was air-dried for 0.5 h, followed by the wells being stained with 0.1 % CV solution and kept in total darkness at room temperature (25 °C) for 20 mins. After washing with distilled water, 30 % of acetic acid was added to each well. The absorbance per well was measured at λ 570 nm using equation (7) (Spectrostar Nano, Germany); all experiments were performed in triplicate.

$$\textbf{Percentage biomass}\ (\%) = \left(\frac{\textbf{A570nm untreated cells} - \textbf{A570nm treated cells}}{\textbf{A570nm untreated cells}}\right) \times \mathbf{100} \quad (7)$$

3. Results and Discussion

3.1 Structural Dynamics

Molecular dynamics simulations were applied to three systems, i.e. components of the oil phase, water phase and both phases together for 500 ns to reveal the stability of the nanosystem using virtual conditions that mimic experimental conditions, expounding on the structural dynamics of each system.

Root-mean-square deviation (RMSD) calculations confirmed that with both phases separately and together, all components of each system remained stably bound to each other, without losing interaction (Figure 1A). The oil phase simulation showed slight changes in conformation

as OA wraps around GA, while the water phase simulation showed the convergence of PAH, Tween 80 and VCM to form a complex where Tween 80 held the complex together. However, the components of the water phase merged with gaps in the complex, which were accommodated by OA and GA when all components of both phases were simulated together. Altogether, the oil and water phase together formed a stable complex that remained intact throughout the simulation.

The stability and convergence of each system were confirmed by analyzing the RMSD during the simulation (Figure 1A). While the RMSD values of the oil phase system (black) remained with a 2 Å range, that of the water phase system (red) were much higher and went beyond a 12 Å range with a deviation toward the end of the simulation. The combined oil and water phase system (green) corrected for these values as the system remained within a 2 Å range and did not have any extreme deviation throughout the simulation, although higher than the oil phase system owing to a larger complex size. In addition, the radii of gyration (RoG) of all three systems (Figure 1B) indicate that when the oil phase is combined with the water phase, the system becomes more compact than that of the water phase system alone, which correlates with the small size of the nanoparticles observed in section 3.1. These data are substantiated by results of hydrogen bond analysis (Figure 1C), which confirmed a greater number of hydrogen bonds in the complex with both phases combined (green), as opposed to either of the phases separately. This may have been a contributing factor to the compactness of the entire system.

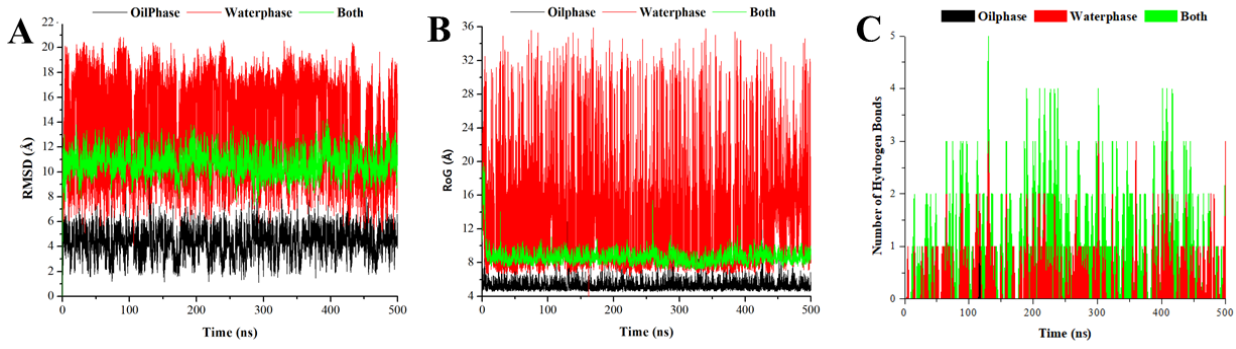


Figure 1. Post analyses of simulations of oil phase, water phase and both phases combined. Graph A. indicates RMSD analysis, B. RoG analysis and C. hydrogen bond analysis.

Root-mean-square fluctuation (RMSF) analysis revealed energy fluctuations of each component of the 500 ns simulation of the combined oil and water phase. Figure 2 indicated that PAH fluctuated more than any other molecule in the simulation, while VCM and GA had the least amount of movement.

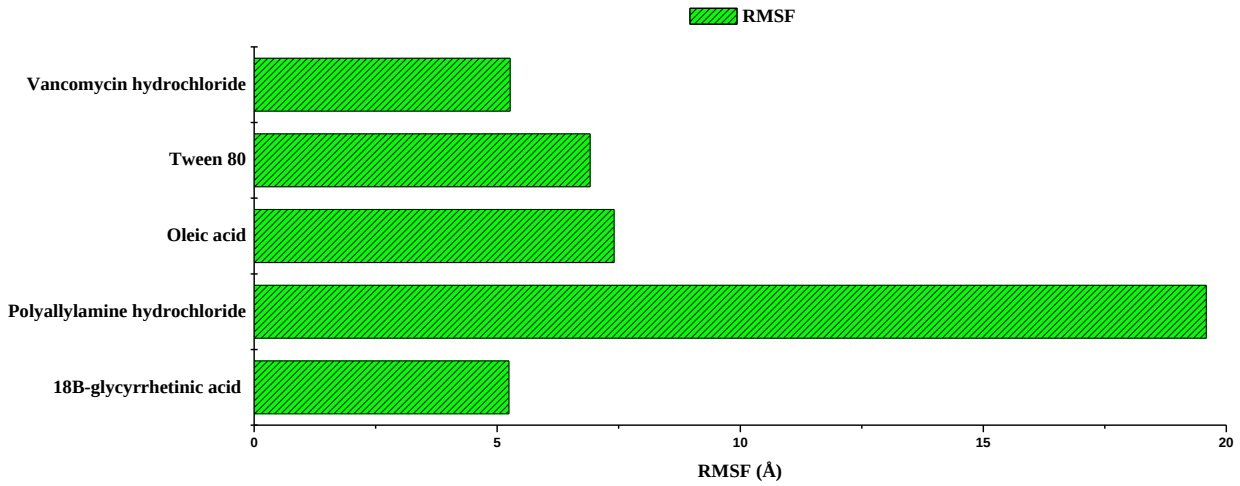


Figure 2. RMSF analysis of 500 ns simulation of all components of the combined oil and water phase system.

From these molecular dynamics analyses, it may be hypothesized that all five components of the oil and water phases bind stably and compactly to support the small size obtained from experimental data. Thus, this nanosystem has potential in the delivery of VCM to MRSA for the improvement of antibacterial activity.

3.2 Preparation of VCM-GAPAH-LPHNPs

VCM-GAPAH-LPHNPs were prepared using GA, PAH (polymer), OA (lipid), Tween 80 (surfactant) and VCM hydrochloride by micro-dilution as previously reported (63). Preliminary studies were conducted using different ratios of lipid and polymer to obtain a formulation with optimum size, PDI and ZP according to pH 7.4, 6 and 4.5 conditions with maximum VCM/GA entrapment efficiency (Table 2).

Table 2. Particle size, PDI and ZP of VCM-GAPAH-LPHNPs.

PAH: OA (mg)	pH	Particle size (nm)	PDI	ZP (mV)	EE %
1:6	7.4	323.8 ± 0.5588	0.291 ± 0.007	- 7.4 ± 1.36	
	6	353.1 ± 9.824	0.728 ± 0.035	- 0.084 ± 0.035	38.60 ± 2.84
	4.5	360.1 ± 2.967	0.288 ± 0.003	0.639 ± 0.484	
1:5	7.4	209.7 ± 1.244	0.277 ± 0.002	- 6.48 ± 0.25	
	6	280.1 ± 9.223	0.311 ± 0.0032	0. 169 ± 0.4	43.06 ± 0.55
	4.5	280 ± 1.45	0.243 ± 0.01	0.708 ± 0.383	
1:4	7.4	198.4 ± 0.302	0.255 ± 0.003	- 3.8 ± 0.335	
	6	374.8 ± 5.435	0.279 ± 0.054	0.44 ± 0.325	69.46 ± 2.52
	4.5	450.4 ± 1.33	0.377 ± 0.012	0.693 ± 0.44	
p value = 0.037					

At pH 7.4, as the polymer: lipid ratio decreased from 1:6 to 1:5, the particle size and PDI of the LPHNPs decreased from 323.8 nm to 209.7 nm and 0.29 to 0.27, respectively, whereas the ZP values changed from – 7.4 mV to – 6.48 mV, while the EE% improved from 38% to 43%. The increase in particle size relative to the increase in lipid content, may have resulted in an increased viscosity, thus causing the LPHNPs to swell (72). Alternatively, the lower polymer: lipid ratios formed more stable nanoparticles with homogenous size distributions and enhanced EE.

The VCM-GAPAH-LPHNPs optimal formulation displayed size of 198.4 ± 0.302 nm, PDI of 0.255 ± 0.003 and ZP of -3.8 ± 0.335 mV. In addition, the surface charge switched from negative at pH 7.4 (physiological pH) to positive at 6 (acidic pH). This may be due to the deprotonation of the carboxylic group (COOH) in OA, which may form an electrostatic bond with the primary amine groups of PAH, thereby neutralising the cationic effect (64). The positive charge of VCM-GAPAH-LPHNPs at acidic pH can be advantageous for the adhesion to the negatively charged bacterial membrane (65, 66).

The VCM-GAPAH-LPHNPs EE% and DL% were found to be 69.46 ± 2.52% and 13.45 ± 0.68%, respectively. This encapsulation was comparable to other VCM-loaded LPHNPs (28) and VCM-loaded nanoparticles (67, 68). Thus, enhanced entrapment efficiency may be due to the high separation of the hydrophobic VCM hydrochloride within the lipid-polymer matrix

(69). The results suggest that VCM-GAPAH-LPHNPs can effectively deliver drugs to acidic sites of infection and were further characterized.

3.3 Characterization of optimal VCM-GAPAH-LPHNPs

The TEM study of VCM-GAPAH-PNPs displayed a distinct circular shape with a size of 198 nm, which corresponded with results of the DLS technique (Figure 3). To determine pH-responsiveness, VCM-GAPAH-LPHNPs were placed in pH 7.4, 6 and 4.5 buffer solutions. As the pH changed from 7.4 to 6 and then to 4.5, the size of VCM-GAPAH-LPHNPs increased from 198.4 nm to 374.8 nm to 450.4 nm, the PDI increased from 0.255 to 0.279 to 0.377 and the ZP switched from -3.8 mV to 0.44 mV to 0.693 mV.

At pH 7.4 conditions, LPHNPs were negatively charged due to the presence of OA on the LPHNPs matrix. At low pH values, the amine group and carboxylic group remained protonated, resulting in cleavage of the ionic bonds between PAH and OA and this was displayed by the positive ZP at acidic pH conditions (70). The cleavage of ionic bonds decreased the affinity between the polymer and lipid resulting in the deconstruction of LPHNPs dispersion, thus causing an increase in their particle size. The size change observed relates to the structural transformation of LPHNPs and the negative to positive charge switching was predicted to positively affect the drug release and promote binding to the negatively (anionic) charged bacterial cell wall, thus improving antibacterial activity (69).

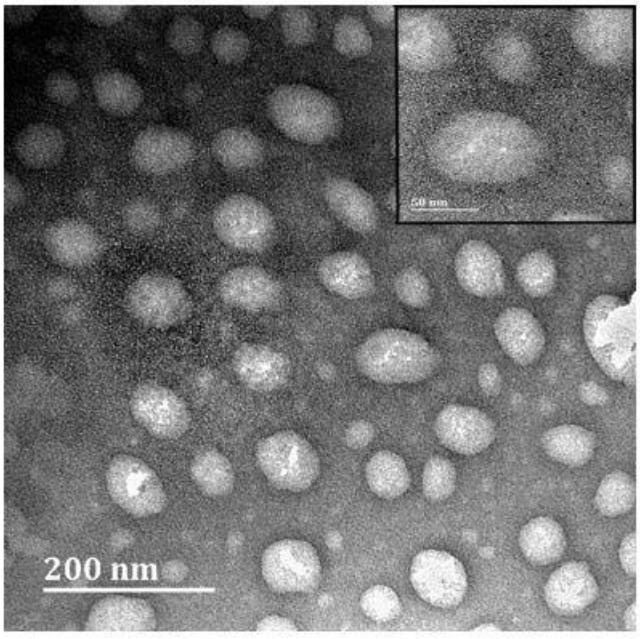


Figure 3. TEM image of VCM-GAPAH-LPHNPs.

3.4 Thermal profiles of VCM-GAPAH-LPHNPs

Thermal analysis was performed to investigate the thermal behaviour of the components of LPHNPs. The thermal profiles of the bare VCM, PAH, GA, physical composition and lyophilized VCM-GAPAH-LPHNPs were compared (Figure 4). VCM, PAH and GA peaks of were observed at 103.40 °C, 262.99 °C and 265.72 °C, respectively. The physical mixture demonstrated similar behaviour to the individual materials. The lyophilized VCM-GAPAH-LPHNPs formulation showed the disappearance of the VCM thermal peak, confirming the transformation from crystallization into its amorphous form, as it was encapsulated within the LPHNPs matrix (71).

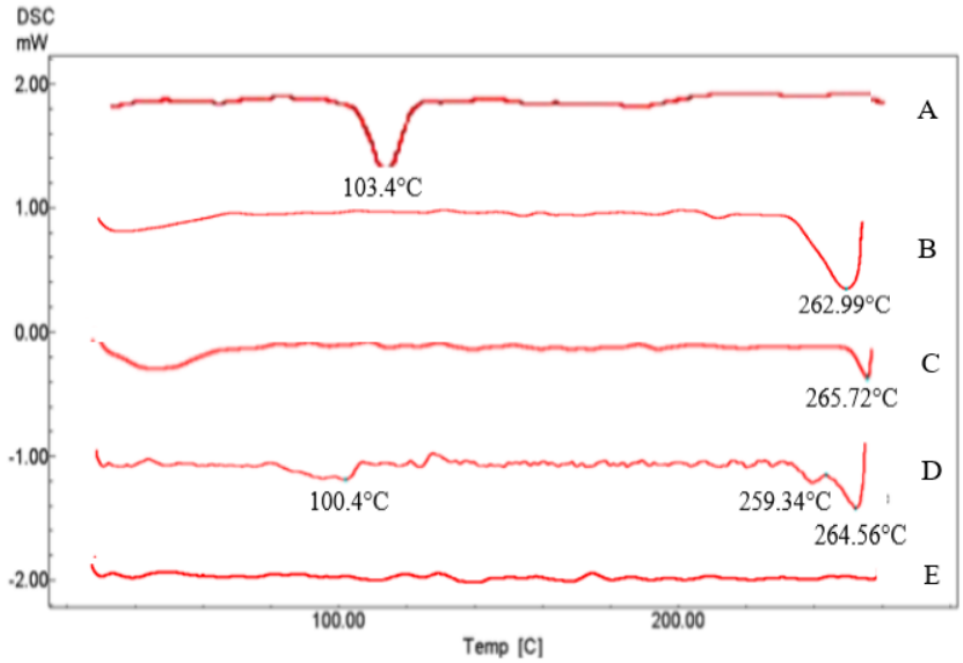


Figure 4. DSC thermogram of (A) VCM (B) PAH (C) GA (D) physical composition of VCM, PAH and GA (E) lyophilized VCM-GAPAH-LPHNPs.

3.5 *In vitro* drug release of VCM-GAPAH-LPHNPs

The *in vitro* release profile of pH-responsive VCM/GA loaded LPHNPs was investigated using the dialysis bag method at pH 7.4 and pH 6. Compared to the release of bare VCM, the release of VCM-GAPAH-LPHNPs was slower, indicating controlled release (Figure 5). The controlled release of VCM-GAPAH-LPHNPs could be attributed to the lipid-polymer matrix of VCM-GAPAH-LPHNPs, which entrap the drug for a more extended period (72). In addition, the long carbon chain length of OA due to branching could have decelerated the rate of diffusion of

VCM, resulting in slower drug release (73), which is advantageous for extended and sustained antibacterial activity (74).

From the 1st hour, VCM-GAPAH-LPHNPs illustrated a significantly faster release ($p < 0.05$) at pH 6 than at pH 7.4 (Figure 6), for up to 48 h. An elevated release at acidic pH occurred due to the protonation of OA and PAH at acidic pH, which resulted in the cleavage of the ion pair bond and an increase in the size of nanoparticles due to electrostatic repulsion between PAH and OA. This resulted in the swelling of LPHNPs, causing them to burst leading to an enhanced and faster VCM release (75, 76). This pH-sensitive release activity is crucial for enhanced VCM protection at pH 7.4, enhanced drug release and bioavailability at the acidic infection sites, thus enhancing antibacterial activity (75, 77).

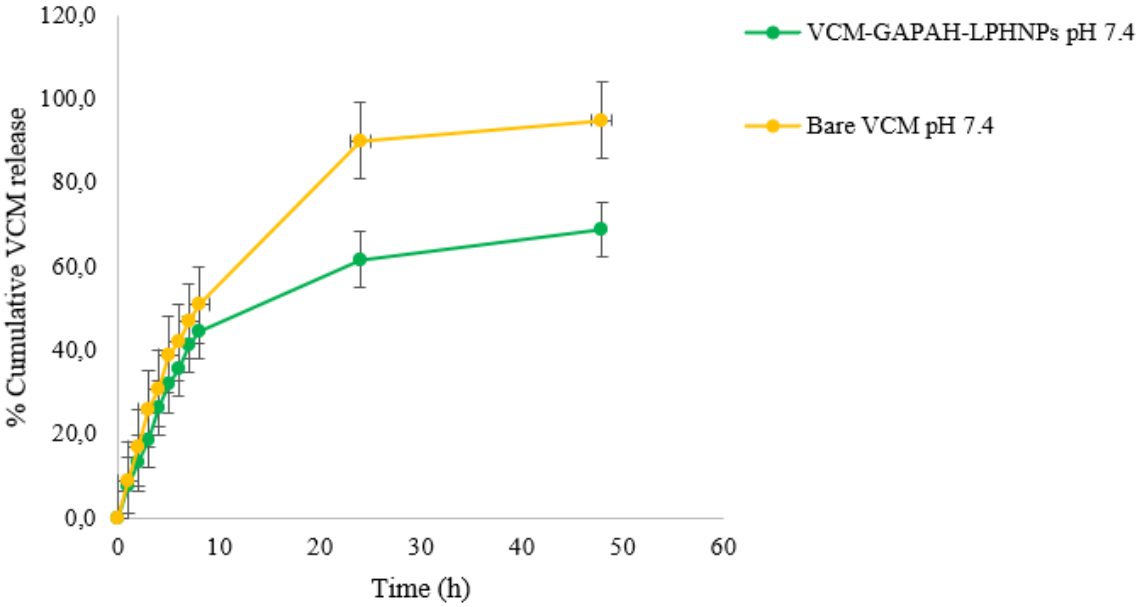


Figure 5. Drug release profiles of bare VCM and VCM-GAPAH-LPHNPs at pH 7.4 (n=3).

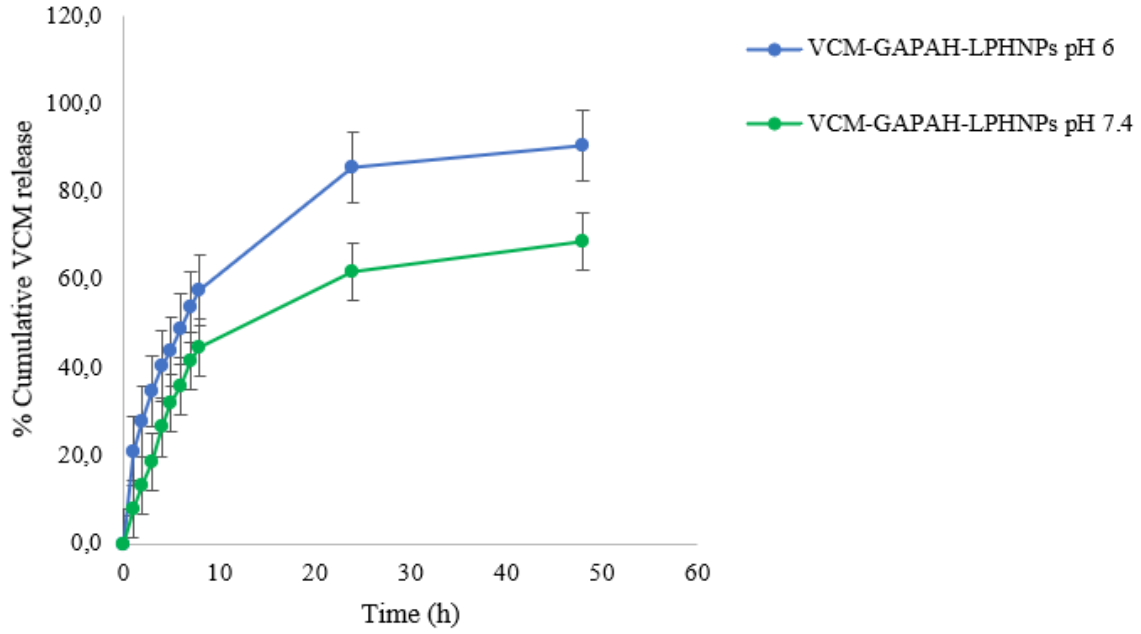


Figure 6. Effect of pH on drug release profiles of VCM-GAPAH-LPHNPs (n=3).

Regarding kinetic analysis, optimized VCM-GAPAH-LPHNPs were input into zero-order, first-order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas and Weibull models (Table 3). Although the release of the VCM-GAPAH-LPHNPs was higher at pH 6, it had similar kinetic behaviour to the release at pH 7.4. The highest R^2 values were 0.945 and 0.991, while the lowest RMSE values were 4.853 and 2.189 at pH 7.4 and pH 6, respectively. Thus, the Weibull model was considered the best fit model for VCM-GAPAH-LPHNPs release at pH 7.4 and pH 6.

The Weibull release exponent (β) value is used to determine the drug release mechanism, with $\beta \leq 0.75$ indicating a Fickian diffusion and $0.75 < \beta < 1$ indicating a combined mechanism, whereas a $\beta > 1$ is associated with a collapse release mechanism. The β values for VCM-GAPAH-LPHNPs were 0.553 and 0.741 at pH 7.4 and pH 6, respectively, indicating Fickian diffusion as the release mechanism.

The Korsmeyer-Peppas release exponent (n) value is also used to determine the drug release mechanism, with $n \leq 0.43$ indicating a Fickian diffusion, $0.43 < n < 0.85$ indicating a combination of diffusion and erosion, whereas $n \geq 0.85$ suggests an erosion release mechanism. The (β) values of Weibull model were consistent with the (n) values of Korsmeyer-Peppas

model, whereas (n) values were 0.414 and 0.351 at pH 7.4 and pH 6 respectively, confirming the Fickian diffusion mechanism of VCM release from the VCM-GAPAH-LPHNPs (78, 79).

Table 3. Drug release kinetics data for VCM-GAPAH-LPHNPs.

Model	**R^2**		**RMSE**		**Release exponent**	
pH	**7.4**	**6**	**7.4**	**6**	**7.4**	**6**
Zero order	0.028	-0.764	20.578	30.870	-	-
First order	0.746	0.895	10.470	7.069	-	-
Higuchi	0.867	0.745	22.754	11.325	-	-
Hixson-Crowell	0.614	0.735	19.4	11.224	-	-
Korsmeyer-Peppas	0.893	0.945	6.8	5.582	0.414	0.351
Weibull	0.945	0.991	4.853	2.189	0.553	0.741

3.6 *In vitro* cytotoxicity

A cytotoxicity study was conducted to determine the biosafety of the formulation. The cytotoxicity of VCM-GAPAH-LPHNPs was determined using the MTT assay, performed on CACO-2 and HepG2 cells. The results showed that VCM-GAPAH-LPHNPs maintained over 75% cell viability across concentrations of 20-80 µg/ml after 24 h, indicating non-cytotoxicity (80) (Figure 7). The results from the MTT assay using VCM-GAPAH-LPHNPs indicated a high percentage of cell viability and dose-dependent trends for all cell lines. Since VCM-GAPAH-LPHNPs showed no significant cytotoxic effect on any of the cell lines, it is thus safe for use in biomedical applications.

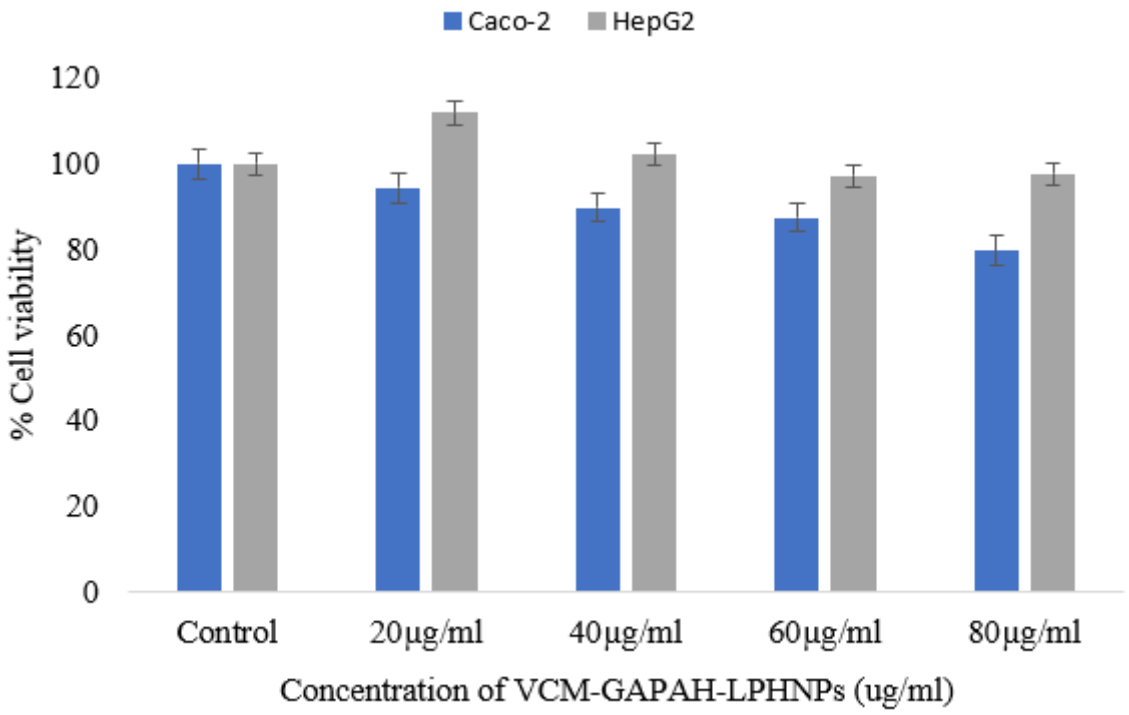


Figure 7. MTT assay of VCM-GAPAH-LPHNPs.

3.7 Haemolysis

Haemolysis of RBCs due to lipid-polymer hybrid systems may be detrimental; therefore, it is important to evaluate the safety of formulation. Haemolytic properties of VCM-GAPAH-LPHNPs were evaluated on sheep RBCs (Figure 8). VCM-GAPAH-LPHNPs were found to be non-haemolytic to RBCs in the concentration range tested (0.05 to 0.5 mg/ml). At 0.5 mg/ml, VCM-GAPAH-LPHNPs showed <1% haemolysis, indicating the non-haemolytic property to RBCs.

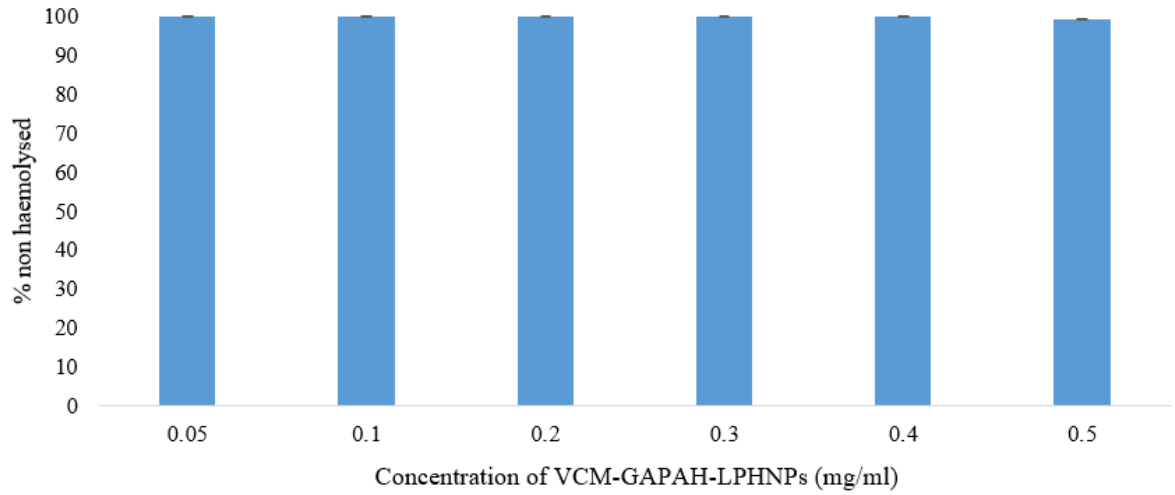


Figure 8. Haemolysis assay of VCM-GAPAH-LPHNPs.

3.8 Stability studies

The experimental results show no significant changes in particle size, PDI and ZP of VCM-GAPAH-LPHNPs throughout the 90 days of storage, at 4 °C and at room temperature – the system remained stable (Table 4).

Table 4. Effect of storage conditions and time on particle size, PDI and ZP of VCM-GAPAH-LPHNPs.

Time (days)	4 °C			Room temperature (25 °C)		
	Particle size (nm)	PDI	ZP (mV)	Particle size (nm)	PDI	ZP (mV)
0	198.4 ± 0.302	0.255 ± 0.003	-3.8 ± 0.335	198.4 ± 0.302	0.255 ± 0.003	-3.8 ± 0.335
30	198.5 ± 0.402	0.155 ± 0.002	-3.63 ± 0.88	200.5 ± 1.124	0.257 ± 0.004	-1.17 ± 0.02
60	199 ± 0.452	0.251 ± 0.041	-2.8 ± 0.55	205.2 ± 8.25	0.38 ± 0.028	-4.18 ± 2.26
90	199.9 ± 0.40	0.26 ± 0.005	-2.82 ± 0.35	206.4 ± 3.2	0.46 ± 1.33	-5.1 ± 3.14

3.9 *In vitro* antibacterial activity and FIC index

The micro broth dilution method was used to determine the MIC of VCM-GAPAH-LPHNPs at pH 7.4 and 6 against SA and MRSA (Table 5). The MIC values for bare VCM against SA and MRSA at pH 7.4 was 1.95 µg/ml and 3.9 µg/ml, respectively and increased to 3.9 µg/ml and 7.8 µg/ml, respectively, at pH 6.

The MIC values of VCM-GAPAH-LPHNPs against SA and MRSA at pH 7.4 was 7.8 µg/ml and 3.9 µg/ml respectively and decreased to 3.9 µg/ml and 0.48 µg/ml at pH 6. Thus, VCM-

GAPAH-LPHNPs had improved and sustained antibacterial activity compared to bare VCM activity against MRSA only at both pH levels. The enhanced extended antibacterial activity of VCM-GAPAH-LPHNPs, in comparison to the bare VCM, could be attributed to its subcellular size with a large surface area and the presence of GA acid for its known antibacterial properties (81, 82). Additionally, the lipophilic nature of VCM-GAPAH-LPHNPs could allow for enhanced uptake into the bacterial cell wall, thus enhancing VCM activity (83). Furthermore, the presence of OA could have enhanced VCM-GAPAH-LPHNPs activity (84).

On the other hand, at pH 7.4, GA activity against SA and MRSA was extended up to 48 h and 72 h respectively, while at pH 6, GA had activity for only 24 h against SA and MRSA. More importantly, VCM-GAPAH-LPHNPs demonstrated lower MIC values against MRSA at pH 6 than pH 7.4. At 24 h, VCM-GAPAH-LPHNPs activity was 8 times greater against MRSA at pH 6 than at pH 7.4, while at 48 h and 72 h, VCM-GAPAH-LPHNPs was 4 times greater against MRSA at pH 6 than at pH 7.4. At 96 h, VCM-GAPAH-LPHNPs was 2 times greater against MRSA at pH 6 compared to pH 7.4.

The enhanced antibacterial activity of VCM-GAPAH-LPHNPs against MRSA at acidic pH conditions may be due to VCM-GAPAH-LPHNPs protonation and cleavage of ionic bonds between PAH and OA, thus resulting in an enhanced VCM release. Furthermore, VCM-GAPAH-LPHNPs charge switching to positive in acidic conditions can enhance the binding of VCM-GAPAH-LPHNPs with the negatively charged bacterial cell wall, leading to a higher release and localization of VCM at acidic infection sites, thereby increasing its uptake into the bacterial cell wall (85, 86).

The formation of ion pairs between OA and PAH could have enhanced the diffusion of VCM and GA across the thicker peptidoglycan layer of MRSA compared to SA (87). In addition, the enhanced penetration of VCM and GA, along with OA into the lipophilic bacterial cell membrane could have contributed to the enhanced antibacterial activity of MRSA compared to SA (88).

Table 5. *In vitro* antibacterial activity at pH 7.4 and pH 6.

	SA				MRSA			
Time (h)	**24**	**48**	**72**	**96**	**24**	**48**	**72**	**96**
***In vitro* antibacterial activity at pH 7.4**								
Bare VCM	1.95	3.9	3.9	7.8	3.9	7.8	15.6	15.6
VCM-GAPAH-LPHNPs	7.8	3.9	3.9	7.8	3.9	3.9	3.9	7.8
GA	125	125	NA	NA	31.25	62.5	62.5	NA
Blank GAPAH-LPHNPs	NA	NA	NA	NA	NA	NA	NA	NA
***In vitro* antibacterial activity at pH 6**								
Bare VCM	3.9	3.9	7.8	15.6	7.8	15.6	15.6	31.25
VCM-GAPAH-LPHNPs	3.9	3.9	7.8	7.8	0.48	0.97	0.97	3.9
GA	125	NA	NA	NA	31.25	NA	NA	NA
Blank GAPAH-LPHNPs	NA	NA	NA	NA	NA	NA	NA	NA

NA = No activity. The values are expressed as mean (n=3).

FIC index was calculated using equations 5 and 6, to determine the combined impact of VCM and GA against SA and MRSA at pH 7.4 and 6 (Table 6). For 24, 48, 72 and 96 h against SA at pH 7.4, Σ FIC was 4.062, 1.031, 1 and 1, respectively, indicating antagonistic, indifferent and additive combinations, respectively. Whereas at pH 6, Σ FIC was 1.031, 1, 1 and 0.5 respectively, indicating indifferent, additive and synergistic combinations, respectively.

Furthermore, at pH 7.4, Σ FIC against MRSA was 1.124, 0.562, 0.312 and 0.5 respectively, indicating indifferent, additive and synergistic combinations, respectively. Lastly, Σ FIC against MRSA at pH 6, was 0.076, 0.062, 0.062 and 0.012 respectively, indicating synergistic combinations at all time intervals.

Synergy indicates that a lower concentration of VCM-GAPAH-LPHNPs is required in order to produce the same effect as VCM and GA alone. Synergistic interactions can potentially increase antibacterial efficacy and decrease toxicity. Whereas additivity indicates that the same concentration of VCM-GAPAH-LPHNPs is required in order to produce the same effect as VCM and GA alone. However, indifference is when one compound, which is GA, is inactive because there was no effect of it to be added but only the effect of the active drug, which was VCM. Lastly, antagonism indicates that a higher concentration of VCM-GAPAH-LPHNPs is required in order to produce the same effect as bare VCM and GA alone. Antagonistic

interactions can potentially decrease antibacterial efficacy and increase toxicity (55-58). Significantly, VCM-GAPAH-LPHNPs works best against MRSA at pH 6, confirming that GA and VCM had a synergistic effect at the acidic conditions.

VCM hinders bacterial cell wall synthesis by binding to the D-alanyl-D-alanine moiety of the growing peptide chain (89), whereas GA hinders bacterial cell membrane synthesis via the inhibition of genes involved in carbohydrate, amino acid and nucleic acid metabolism (35, 90). Moreover, studies have shown that GA inhibits bacterial DNA replication which could result in the inhibition of the production of bacterial toxins and enzymes (91). GA also has the ability to regulate the production of haemolysins, leukotoxins and adhesins (92, 93). Long *et al.* (2013) reported a decrease in the expression of saeR, hla, mecA and sbi genes after SA incubation with GA (94). In addition, Li *et al*. (2012) reported the downregulation of RNAIII transcript after MRSA incubation with GA (95). Hence GA can modulate virulence by synergistically inducing antibacterial effects. However, the precise mechanism underlying its activity remains unknown (94, 95).

On the other hand, the administration and exposure of VCM to human cells has several harmful effects such as nephrotoxicity, neutropenia and ototoxicity (96). However, GA can cause alkalosis, muscular paralysis and hyperkalaemia (97). By co-encapsulating VCM and GA within LPHNPs, their pharmacokinetic profiles and therapeutic indices are remarkably enhanced (98). Therefore, the synergistic combination of VCM and GA against MRSA at acidic pH can potentially increase the antibacterial efficacy, reduce cytotoxicity and prevent the emergence of antimicrobial resistance as compared to monotherapy regimens (55-57).

Table 6. FIC index against SA and MRSA at pH 7.4 and 6.

Time (h)	Bare VCM	GA	Σ FIC (Bare VCM + GA)	Interpretation
SA (pH 7.4)				
24	4	0.062	4.062	Antagonism
48	1	0.031	1.031	Indifference
72	1	-	1	Additive
96	1	-	1	Additive
SA (pH 6)				
24	1	0.031	1.031	Indifference
48	1	-	1	Additive
72	1	-	1	Additive
96	0.5	-	0.5	Synergy
MRSA (pH 7.4)				
24	1	0.124	1.124	Indifference
48	0.5	0.062	0.562	Additive
72	0.25	0.062	0.312	Synergy
96	0.5	-	0.5	Synergy
MRSA (pH 6)				
24	0.061	0.015	0.076	Synergy
48	0.062	-	0.062	Synergy
72	0.062	-	0.062	Synergy
96	0.012	-	0.012	Synergy

3.10 Time-killing assay

Time-kill analysis results of bare VCM, GA, control and VCM-GAPAH-LPHNPs at 5 times greater MIC against MRSA are illustrated in Figure 9. At 8 h interval GA eliminated 26% of bacteria, bare VCM eliminated 55% of bacteria and ultimately, VCM-GAPAH-LPHNPs eliminated 68% of bacteria. VCM-GAPAH-LPHNPs displayed spontaneous bacterial elimination with nearly 75% clearance of MRSA in <12 h with an equal concentration of bare VCM. At 12 h VCM-GAPAH-LPHNPs demonstrated 100% bacterial elimination, whereas bare VCM demonstrated 66% bacterial elimination. Hence VCM-GAPAH-LPHNPs could effectively treat MRSA infections at a faster rate.

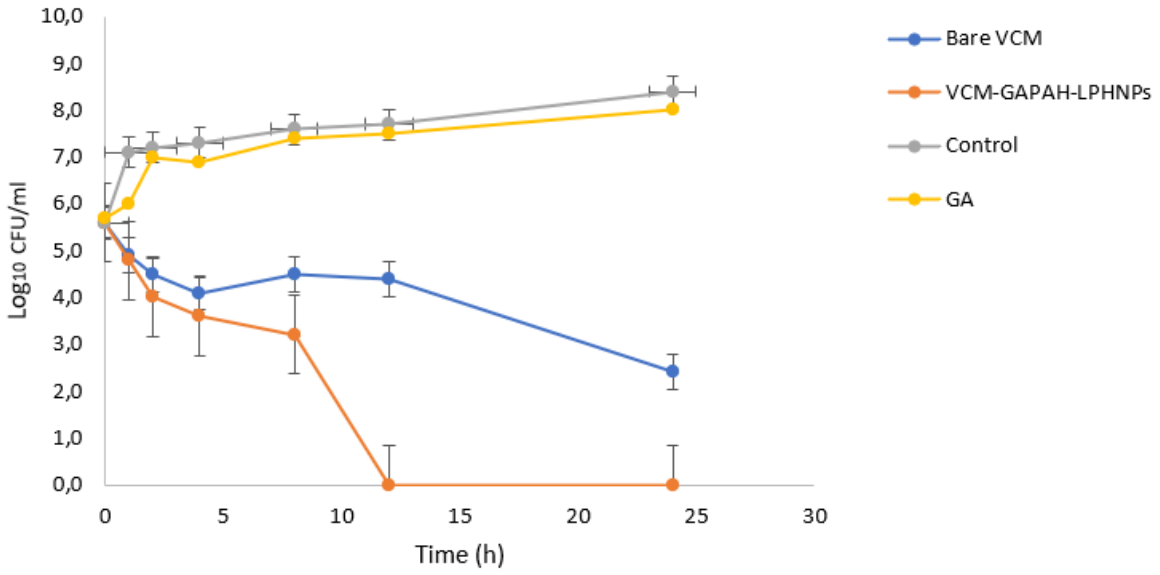


Figure 9. The killing kinetics of MRSA exposed to 5x MIC of bare VCM, VCM-GAPAH-LPHNPs, GA and sterile water (control).

3.11 MRSA biofilm elimination

The CV assay was performed to analyse the ability of VCM-GAPAH-LPHNPs to decrease MRSA biofilms by calculating the biomass percentage (Figure 10). The control (untreated biofilms) demonstrated high purple intensity (Figure 11A) resulting in a high percentage biomass of 100 %. The percentage biomass of GA, bare VCM and VCM-GAPAH-LPHNPs present were 95 %, 74 % and 31 %, respectively. Hence GA, bare VCM and VCM-GAPAH-LPHNPs eliminated 5 %, 26 % and 69 % of MRSA biofilms, respectively.

Bare VCM demonstrated a reduced purple intensity (Figure 11B), indicating a lower percentage of MRSA biomass, compared to the control. Hence a decrease in biofilms was observed when treated with bare VCM, demonstrating its considerable antibacterial ability. Notably, GA treated biofilms exhibited higher purple intensity (Figure 11C) than VCM treated biofilms, which indicated a higher percentage of MRSA biomass.

Interestingly, a substantial decrease in biofilms treated with VCM-GAPAH-LPHNPs formulation was observed, as shown in the reduction of purple intensity (Figure 11D), when compared to all other treated and untreated samples. This confirms the superior potential of VCM-GAPAH-LPHNPs to reduce MRSA biofilms.

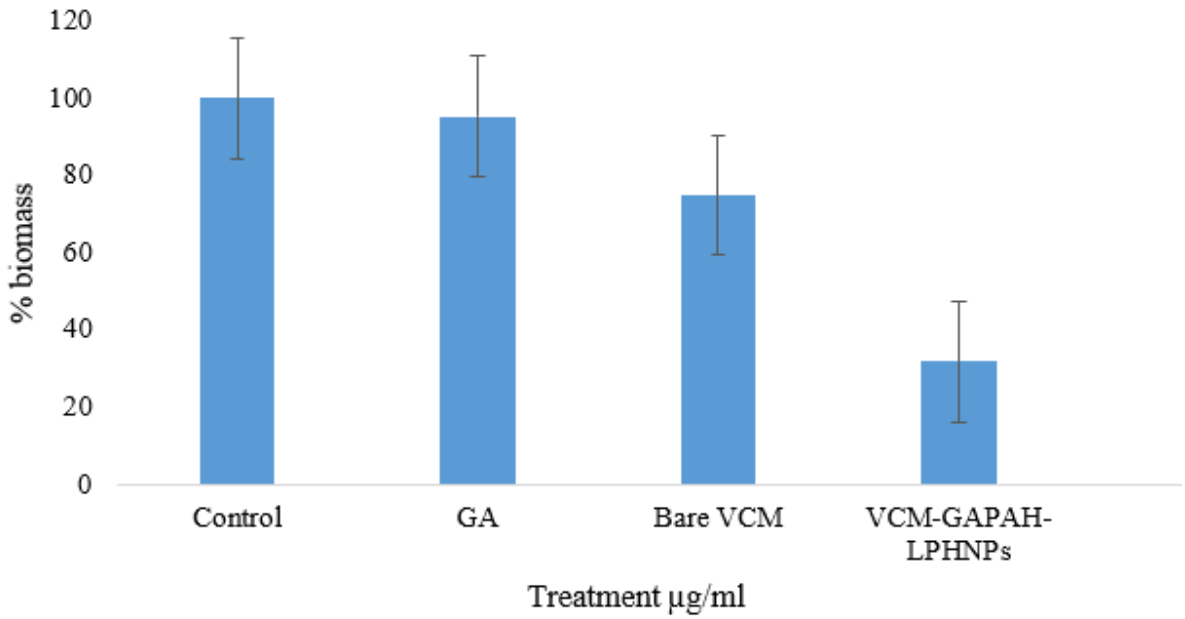


Figure 10. Percentage biomass of MRSA exposed to 100x MIC of control (untreated biofilms), GA, bare VCM and VCM-GAPAH-LPHNPs.

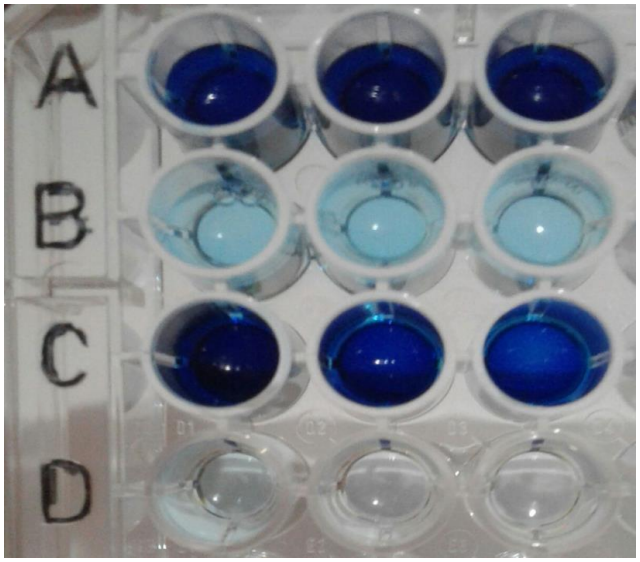


Figure 11. Crystal violet assay of A) MRSA untreated biofilms; B) MRSA biofilms treated with bare VCM C) MRSA biofilms treated with GA D) MRSA biofilms treated with VCM-GAPAH-LPHNPs.

4. Conclusions

There has been an increasing interest in the development of novel nanoantibiotic drug delivery systems due to the global health threat posed by antimicrobial resistance. In this study, the potential to prepare novel pH-responsive lipid-polymer hybrid nanoparticles with co-loaded GA and VCM (VCM-GAPAH-LPHNPs) was explored. A stable VCM-GAPAH-LPHNPs formulation was successfully prepared with favourable particle size, PDI, ZP, morphology, EE% and DL%. The stability of the nanosystems were demonstrated via the analyses of *in silico* studies. *In vitro* biocompatibility and haemolytic studies confirmed the biosafety of VCM-GAPAH-LPHNPs. The release of VCM from VCM-GAPAH-LPHNPs was higher at pH 6 compared to physiological pH 7.4. The *in vitro* antibacterial studies of the VCM-GAPAH-LPHNPs against MRSA revealed an enhanced antibacterial effect at acidic medium compared to pH 7.4, with 75% bacterial elimination in <12 h. The crystal violet assay further validated the antibacterial potential of VCM-GAPAH-LPHNPs by displaying a significantly higher percentage of biofilm eradication when compared to bare VCM, GA and untreated biofilms. Our findings suggest that novel VCM-GAPAH-LPHNPS can potentially be effective in drug delivery to the target site of bacterial infection with low pH and effectively prevent the progression of antimicrobial resistance. In addition, the proposed nanosystem can be used to co-deliver GA with other antimicrobial agents to further explore the antimicrobial potential against other species of bacteria and microorganisms.

Author Contributions: Conceptualization, Y.J., C.A.O. and T.G.; methodology, Y.J., N.O., C.M. and N.D.; formal analysis, Y.J. and C.A.O.; investigation, Y.J. and N.O.; writing—original draft preparation, Y.J.; writing—review and editing, C.A.O., C.M., N.D. and T.G.; supervision, C.A.O and T.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation, South African Medical Research Council, UKZN Nanotechnology Platform and UKZN College of Health Sciences.

Acknowledgements: The authors acknowledge the Microscopy and Microanalysis Unit, Biomedical Resource Unit and Department of Pharmaceutical Chemistry for their assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rahimzadeh G, Gill P, Rezai MS. Cysteine/Histidine-Dependent Amidohydrolase/Peptidase (CHAP)-Displayed Nano Phages: Antimicrobial Function against Methicillin-Resistant Staphylococcus aureus (MRSA). Avicenna Journal of Medical Biotechnology. 2020;12(2):85-90.
2. Zhen X, Chudal L, Pandey NK, Phan J, Ran X, Amador E, et al. A powerful combination of copper-cysteamine nanoparticles with potassium iodide for bacterial destruction. Materials Science and Engineering C. 2020;110:110659.
3. WHO. The top 10 causes of death 2018 [Available from: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death.] (accessed: 21:03:2020).
4. Medina Cruz D, Mi G, Webster TJ. Synthesis and characterization of biogenic selenium nanoparticles with antimicrobial properties made by Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), Escherichia coli, and Pseudomonas aeruginosa. Journal of Biomedical Materials Research Part A. 2018;106(5):1400-12.
5. Govender K, Sharma S, Jessee W, Nagaraju K, Pearse NJ, Chhetri P, et al. Leadership and Task Shifting to Address the Challenges of Antimicrobial Resistance in South Africa. Asian Journal of Pharmaceutical and Clinical Research. 2018;10(1):10-20.
6. Casadei MA, Cerreto F, Cesa S, Giannuzzo M, Feeney M, Marianecci C, et al. Solid lipid nanoparticles incorporated in dextran hydrogels: a new drug delivery system for oral formulations. International Journal of Pharmaceutics. 2006;325(1-2):140-6.
7. Sur S, Rathore A, Dave V, Reddy KR, Chouhan RS, Sadhu V. Recent developments in functionalized polymer nanoparticles for efficient drug delivery system. Nano-Struct Nano-Objects. 2019;20:100397.
8. Gulías Ò, McKenzie G, Bayó M, Agut M, Nonell S. Effective Photodynamic Inactivation of 26 Escherichia coli Strains with Different Antibiotic Susceptibility Profiles: A Planktonic and Biofilm Study. Antibiotics. 2020;9(3):98.
9. Kalhapure RS, Suleman N, Mocktar C, Seedat N, Govender T. Nanoengineered drug delivery systems for enhancing antibiotic therapy. Journal of Pharmaceutical Sciences. 2015;104(3):872-905.
10. Sonawane SJ, Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Govender T. AB2-type amphiphilic block copolymer containing a pH-cleavable hydrazone linkage for targeted antibiotic delivery. International Journal of Pharmaceutics. 2020;575:118948.
11. Dancer S. How can antibiotics make us sick and what to do about it. Pathology. 2016;48:S52.
12. Hidayat LK, Hsu DI, Quist R, Shriner KA, Wong-Beringer A. High-dose vancomycin therapy for methicillin-resistant Staphylococcus aureus infections: efficacy and toxicity. JAMA Internal Medicine. 2006;166(19):2138-44.
13. Chakraborty SP, Sahu SK, Mahapatra SK, Santra S, Bal M, Roy S, et al. Nanoconjugated vancomycin: new opportunities for the development of anti-VRSA agents. Nanotechnology. 2010;21(10):105103.
14. Fraix A, Conte C, Gazzano E, Riganti C, Quaglia F, Sortino S. Overcoming Doxorubicin Resistance with Lipid–Polymer Hybrid Nanoparticles Photoreleasing Nitric Oxide. Molecular Pharmaceutics. 2020;17(6):2135-44.
15. Khattabi AM, Alqdeimat DA, editors. The effect of cyclodextrin on both the agglomeration and the in vitro characteristics of drug loaded and targeted silica nanoparticles. IOP Conference Series: Materials Science and Engineering; 2018.
16. Wang H, Xu Y, Zhou X. Docetaxel-loaded chitosan microspheres as a lung targeted drug delivery system: in vitro and in vivo evaluation. International Journal of Molecular Sciences. 2014;15(3):3519-32.

17. Mandal B, Bhattacharjee H, Mittal N, Sah H, Balabathula P, Thoma LA, et al. Core–shell-type lipid–polymer hybrid nanoparticles as a drug delivery platform. Nanomedicine: Nanotechnology, Biology and Medicine. 2013;9(4):474-91.
18. Yan J, Wang Y, Zhang X, Liu S, Tian C, Wang H. Targeted nanomedicine for prostate cancer therapy: docetaxel and curcumin co-encapsulated lipid–polymer hybrid nanoparticles for the enhanced anti-tumor activity in vitro and in vivo. Drug Delivery. 2016;23(5):1757-62.
19. Cheow WS, Hadinoto K. Factors affecting drug encapsulation and stability of lipid–polymer hybrid nanoparticles. Colloids and Surfaces B: Biointerfaces. 2011;85(2):214-20.
20. Zhang L, Zhu D, Dong X, Sun H, Song C, Wang C, et al. Folate-modified lipid–polymer hybrid nanoparticles for targeted paclitaxel delivery. International Journal of Nanomedicine. 2015;10:2101.
21. Bose RJ, Ravikumar R, Karuppagounder V, Bennet D, Rangasamy S, Thandavarayan RA. Lipid–polymer hybrid nanoparticle-mediated therapeutics delivery: advances and challenges. Drug Discovery Today. 2017;22(8):1258-65.
22. Hadinoto K, Sundaresan A, Cheow WS. Lipid–polymer hybrid nanoparticles as a new generation therapeutic delivery platform: a review. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(3):427-43.
23. Crucho CI. Stimuli-responsive polymeric nanoparticles for nanomedicine. ChemMedChem. 2015;10(1):24-38.
24. Abbasian M, Roudi M-M, Mahmoodzadeh F, Eskandani M, Jaymand M. Chitosan-grafted-poly (methacrylic acid)/graphene oxide nanocomposite as a pH-responsive de novo cancer chemotherapy nanosystem. Internationa Journal of Biological Macromolecules. 2018;118:1871-9.
25. Joglekar M, Trewyn BG. Polymer-based stimuli-responsive nanosystems for biomedical applications. Biotechnology. 2013;8(8):931-45.
26. Ling D, Li H, Xi W, Wang Z, Bednarkiewicz A, Dibaba ST, et al. Heterodimers made of metal–organic frameworks and upconversion nanoparticles for bioimaging and pH-responsive dual-drug delivery. Journal of Materials Chemistry B. 2020;8(6):1316-25.
27. Radovic-Moreno AF, Lu TK, Puscasu VA, Yoon CJ, Langer R, Farokhzad OC. Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics. ACS nano. 2012;6(5):4279-87.
28. Seedat N, Kalhapure RS, Mocktar C, Vepuri S, Jadhav M, Soliman M, et al. Co-encapsulation of multi-lipids and polymers enhances the performance of vancomycin in lipid–polymer hybrid nanoparticles: In vitro and in silico studies. Materials Science and Engineering C. 2016;61:616-30.
29. Jutooru I, Chadalapaka G, Chintharlapalli S, Papineni S, Safe S. Induction of apoptosis and nonsteroidal anti-inflammatory drug-activated gene 1 in pancreatic cancer cells by a glycyrrhetinic acid derivative. Molecular Carcinogenesis. 2009;48(8):692-702.
30. Lefaki M, Papaevgeniou N, Tur JA, Vorgias CE, Sykiotis GP, Chondrogianni N. The dietary triterpenoid 18α–Glycyrrhetinic acid protects from MMC-induced genotoxicity through the ERK/Nrf2 pathway. Redox Biology. 2020;28:101317.
31. Ming LJ, Yin ACY. Therapeutic effects of glycyrrhizic acid. Natural Product Communications. 2013;8(3):415-8.
32. Kowalska A, Kalinowska-Lis U. 18β-Glycyrrhetinic acid: its core biological properties and dermatological applications. International Journal of Cosmetic Sciences. 2019;41(4):325-31.
33. Wang L, Yang R, Yuan B, Liu Y, Liu C. The antiviral and antimicrobial activities of licorice, a widely-used Chinese herb. Acta Pharmaceutica Sinica B. 2015;5(4):310-5.

34. Wang L-J, Geng C-A, Ma Y-B, Huang X-Y, Luo J, Chen H, et al. Synthesis, biological evaluation and structure–activity relationships of glycyrrhetinic acid derivatives as novel anti-hepatitis B virus agents. Bioorganic & Medicinal Chemistry Letters. 2012;22(10):3473-9.
35. Oyama K, Kawada-Matsuo M, Oogai Y, Hayashi T, Nakamura N, Komatsuzawa H. Antibacterial effects of glycyrrhetinic acid and its derivatives on Staphylococcus aureus. PLoS One. 2016;11(11):e0165831.
36. Madhumathi K, Rubaiya Y, Doble M, Venkateswari R, Kumar TS. Antibacterial, anti-inflammatory, and bone-regenerative dual-drug-loaded calcium phosphate nanocarriers—in vitro and in vivo studies. Drug Delivery and Translational Research. 2018;8(5):1066-77.
37. Wu J-L, Tian G-X, Yu W-J, Jia G-T, Sun T-Y, Gao Z-Q. pH-responsive hyaluronic acid-based mixed micelles for the hepatoma-targeting delivery of doxorubicin. International Journal of Molecular Sciences. 2016;17(4):364.
38. Hefnawy A, Khalil IH, Arafa K, Emara M, El-Sherbiny IM. Dual-Ligand Functionalized Core-Shell Chitosan-Based Nanocarrier for Hepatocellular Carcinoma-Targeted Drug Delivery. International Journal of Nanomedicine. 2020;15:821-37.
39. Sun Y, Lu J, Yan D, Shen L, Hu H, Chen D. Cellular uptake mechanism and clearance kinetics of fluorescence-labeled glycyrrhetinic acid and glycyrrhetinic acid–modified liposome in hepatocellular carcinoma cells. Environmental Toxicology and Pharmacology. 2017;53:46-56.
40. Wang C-Y, Kao T-C, Lo W-H, Yen G-C. Glycyrrhizic acid and 18β-glycyrrhetinic acid modulate lipopolysaccharide-induced inflammatory response by suppression of NF-κB through PI3K p110δ and p110γ inhibitions. Journal of Agricultural and Food Chemistry. 2011;59(14):7726-33.
41. Case D, Ben-Shalom I, Brozell S, Cerutti D, Cheatham III T, Cruzeiro V, et al. AMBER 2018; University of California: San Francisco, CA, USA, 2018.© 2019 by the authors. 2018.
42. Ryckaert J-P, Ciccotti G, Berendsen HJ. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. Journal of Computational Physics. 1977;23(3):327-41.
43. Case DA, Cheatham III TE, Darden T, Gohlke H, Luo R, Merz Jr KM, et al. The Amber biomolecular simulation programs. Journal of Computational Chemistry. 2005;26(16):1668-88.
44. Nagavarma B, Yadav HK, Ayaz A, Vasudha L, Shivakumar H. Different techniques for preparation of polymeric nanoparticles-a review. Asian Journal of Pharmaceutical and Clinical Research. 2012;5(3):16-23.
45. Mukherjee A, Waters AK, Kalyan P, Achrol AS, Kesari S, Yenugonda VM. Lipid–polymer hybrid nanoparticles as a next-generation drug delivery platform: state of the art, emerging technologies, and perspectives. International Journal of Nanomedicine. 2019;14:1937.
46. Mhango EK, Kalhapure RS, Jadhav M, Sonawane SJ, Mocktar C, Vepuri S, et al. Preparation and optimization of meropenem-loaded solid lipid nanoparticles: in vitro evaluation and molecular modeling. AAPS PharmSciTech. 2017;18(6):2011-25.
47. Khan AU, Khan M, Cho MH, Khan MM. Selected nanotechnologies and nanostructures for drug delivery, nanomedicine and cure. Bioprocess and Biosystems Engineering. 2020:1-19.
48. Abdou EM, Fayed MA, Helal D, Ahmed KA. Assessment of the hepatoprotective effect of developed lipid-polymer hybrid nanoparticles (LPHNPs) encapsulating naturally extracted β-Sitosterol against CCl 4 induced hepatotoxicity in rats. Science Reports. 2019;9(1):1-14.
49. Makhathini SS, Omolo CA, Gannimani R, Mocktar C, Govender T. pH-Responsive Micelles from an Oleic Acid Tail and Propionic Acid Heads Dendritic Amphiphile for the Delivery of Antibiotics. Journal of Pharmaceutical Sciences. 2020;109(8):2594-606.

50. Ahmad N, Ahmad R, Alam MA, Ahmad FJ, Amir M, Pottoo FH, et al. Daunorubicin oral bioavailability enhancement by surface coated natural biodegradable macromolecule chitosan based polymeric nanoparticles. International Journal of Biological Macromolecules. 2019;128:825-38.
51. Hassan D, Omolo CA, Fasiku VO, Mocktar C, Govender T. Novel chitosan-based pH-responsive lipid-polymer hybrid nanovesicles (OLA-LPHVs) for delivery of vancomycin against methicillin-resistant Staphylococcus aureus infections. International Journal of Biological Macromolecules. 2020;147:385-98.
52. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 2016;6(2):71-9.
53. García I, del Carmen Conejo M, Ojeda A, Rodríguez-Baño J, Pascual A. A dynamic in vitro model for evaluating antimicrobial activity against bacterial biofilms using a new device and clinical-used catheters. Journal of Microbiological Methods. 2010;83(3):307-11.
54. Omolo CA, Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Ndesendo VM, et al. Pegylated oleic acid: A promising amphiphilic polymer for nano-antibiotic delivery. European Journal of Pharmaceutics and Biopharmaceutics. 2017;112:96-108.
55. Berenbaum MC. What is synergy? Pharmacology Reviews. 1989;41(2):93-141.
56. Greco WR. The search for synergy: a critical review from a response surface perspective. Pharmacology Reviews. 1995;47:331-85.
57. Meletiadis J, Pournaras S, Roilides E, Walsh TJ. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against Aspergillus fumigatus. Antimicrobial Agents and Chemotherapy. 2010;54(2):602-9.
58. Microbiology ECfASTotESoC, Diseases I. Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clinical Microbiology and Infection. 2000;6(9):503-8.
59. Jain J, Arora S, Rajwade JM, Omray P, Khandelwal S, Paknikar KM. Silver nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical use. Molecular Pharmaceutics. 2009;6(5):1388-401.
60. Seil JT, Webster TJ. Antimicrobial applications of nanotechnology: methods and literature. International Journal of Nanomedicine. 2012;7:2767-81.
61. David MZ, Dryden M, Gottlieb T, Tattevin P, Gould IM. Recently approved antibacterials for methicillin-resistant Staphylococcus aureus (MRSA) and other Gram-positive pathogens: the shock of the new. International Journal of Antimicrobial Agents. 2017;50(3):303-7.
62. Liu H, Zhao Y, Zhao D, Gong T, Wu Y, Han H, et al. Antibacterial and anti-biofilm activities of thiazolidione derivatives against clinical staphylococcus strains. Emerging Microbes & Infections. 2015;4(1):1-6.
63. Gharbavi M, Danafar H, Sharafi A. Microemulsion and bovine serum albumin nanoparticles as a novel hybrid nanocarrier system for efficient multifunctional drug delivery. Journal of Biomedical Materials Research Part A A. 2020;108(8):1688-702.
64. Uskoković V, Desai TA. In vitro analysis of nanoparticulate hydroxyapatite/chitosan composites as potential drug delivery platforms for the sustained release of antibiotics in the treatment of osteomyelitis. Journal of Pharmaceutical Sciences. 2014;103(2):567-79.
65. Gumustas M, Sengel-Turk CT, Gumustas A, Ozkan SA, Uslu B. Effect of polymer-based nanoparticles on the assay of antimicrobial drug delivery systems. In: Grumezescu A, editor. Multifunctional systems for combined delivery, biosensing and diagnostics. 1st ed: Elsevier; 2017. p. 67-108.

66. Wang Y, Yang C, Hu R, Toh HT, Liu X, Lin G, et al. Assembling Mn: ZnSe quantum dots-siRNA nanoplexes for gene silencing in tumor cells. Biomaterial Sciences. 2015;3(1):192-202.
67. Honary S, Ebrahimi P, Hadianamrei R. Optimization of particle size and encapsulation efficiency of vancomycin nanoparticles by response surface methodology. Pharmaceutical Development and Technology. 2014;19(8):987-98.
68. Zakeri-Milani P, Loveymi BD, Jelvehgari M, Valizadeh H. The characteristics and improved intestinal permeability of vancomycin PLGA-nanoparticles as colloidal drug delivery system. Colloids and Surfaces B: Biointerfaces. 2013;103:174-81.
69. Liu Y, Busscher HJ, Zhao B, Li Y, Zhang Z, van der Mei HC, et al. Surface-adaptive, antimicrobially loaded, micellar nanocarriers with enhanced penetration and killing efficiency in staphylococcal biofilms. ACS nano. 2016;10(4):4779-89.
70. Severin FF, Severina II, Antonenko YN, Rokitskaya TI, Cherepanov DA, Mokhova EN, et al. Penetrating cation/fatty acid anion pair as a mitochondria-targeted protonophore. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(2):663-8.
71. Pupe CG, Villardi M, Rodrigues CR, Rocha HVA, Maia LC, de Sousa VP, et al. Preparation and evaluation of antimicrobial activity of nanosystems for the control of oral pathogens Streptococcus mutans and Candida albicans. International Journal of Nanomedicine 2011;6:2581-90.
72. Kalhapure RS, Mocktar C, Sikwal DR, Sonawane SJ, Kathiravan MK, Skelton A, et al. Ion pairing with linoleic acid simultaneously enhances encapsulation efficiency and antibacterial activity of vancomycin in solid lipid nanoparticles. Colloids and Surfaces B: Biointerfaces. 2014;117:303-11.
73. Xie S, Zhu L, Dong Z, Wang X, Wang Y, Li X, et al. Preparation, characterization and pharmacokinetics of enrofloxacin-loaded solid lipid nanoparticles: influences of fatty acids. Colloids and Surfaces B: Biointerfaces. 2011;83(2):382-7.
74. Esmaeili A, Ghobadianpour S. Vancomycin loaded superparamagnetic MnFe2O4 nanoparticles coated with PEGylated chitosan to enhance antibacterial activity. International Journal of Pharmaceutics. 2016;501(1-2):326-30.
75. Aldawsari HM, Hosny KM. Solid lipid nanoparticles of Vancomycin loaded with Ellagic acid as a tool for overcoming nephrotoxic side effects: preparation, characterization, and nephrotoxicity evaluation. Journal of Drug Delivery Science and Technology. 2018;45:76-80.
76. Unagolla JM, Jayasuriya AC. Drug transport mechanisms and in vitro release kinetics of vancomycin encapsulated chitosan-alginate polyelectrolyte microparticles as a controlled drug delivery system. European Journal of Pharmaceutical Sciences. 2018;114:199-209.
77. Farrokhi-Rad M, Fateh A, Shahrabi T. Electrophoretic deposition of vancomycin loaded halloysite nanotubes-chitosan nanocomposite coatings. Surface and Coatings Technology. 2018;349:144-56.
78. Gao Y, Zuo J, Bou-Chacra N, Pinto TdJA, Clas S-D, Walker RB, et al. In vitro release kinetics of antituberculosis drugs from nanoparticles assessed using a modified dissolution apparatus. BioMed Research International. 2013;2013:136590.
79. Papadopoulou V, Kosmidis K, Vlachou M, Macheras P. On the use of the Weibull function for the discernment of drug release mechanisms. International Journal of Pharmaceutics. 2006;309(1-2):44-50.
80. Riss T, Hook B, Duellman S. Evaluation of real time cell viability assays multiplexed with other methods. Toxicology Letters. 2015;238:S179-S80.

81. Ghaffari S, Varshosaz J, Saadat A, Atyabi F. Stability and antimicrobial effect of amikacin-loaded solid lipid nanoparticles. International Journal od Nanomedicine. 2011;6:35-43.
82. Yang Y, Zhu Q, Zhong Y, Cui X, Jiang Z, Wu P, et al. Synthesis, anti-microbial and anti-inflammatory activities of 18β-glycyrrhetinic acid derivatives. Bioorganic Chemistry. 2020;101:103985.
83. Huang C-M, Chen C-H, Pornpattananangkul D, Zhang L, Chan M, Hsieh M-F, et al. Eradication of drug resistant Staphylococcus aureus by liposomal oleic acids. Biomaterials. 2011;32(1):214-21.
84. Wang X, Zhang S, Zhu L, Xie S, Dong Z, Wang Y, et al. Enhancement of antibacterial activity of tilmicosin against Staphylococcus aureus by solid lipid nanoparticles in vitro and in vivo. Veterinary Journal. 2012;191(1):115-20.
85. Chen C-H, Wang Y, Nakatsuji T, Liu Y-T, Zouboulis CC, Gallo RL, et al. An innate bactericidal oleic acid effective against skin infection of methicillin-resistant Staphylococcus aureus: a therapy concordant with evolutionary medicine. Journal of Microbiology and Biotechnology. 2011;21(4):391-9.
86. Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Singh S, Renukuntla J, et al. pH-responsive chitosan nanoparticles from a novel twin-chain anionic amphiphile for controlled and targeted delivery of vancomycin. Colloids and Surfaces B: Biointerfaces. 2017;158:650-7.
87. Sarveiya V, Templeton JF, Benson H. Ion-pairs of ibuprofen: increased membrane diffusion. Journal of Pharmacy and Pharmacology. 2004;56(6):717-24.
88. Sonawane SJ, Kalhapure RS, Jadhav M, Rambharose S, Mocktar C, Govender T. Transforming linoleic acid into a nanoemulsion for enhanced activity against methicillin susceptible and resistant Staphylococcus aureus. RSC Advances. 2015;5(110):90482-92.
89. Kim SJ, Cegelski L, Preobrazhenskaya M, Schaefer J. Structures of Staphylococcus aureus cell-wall complexes with vancomycin, eremomycin, and chloroeremomycin derivatives by 13C {19F} and 15N {19F} rotational-echo double resonance. Biochemistry. 2006;45(16):5235-50.
90. De León L, López M, Moujir L. Antibacterial properties of zeylasterone, a triterpenoid isolated from Maytenus blepharodes, against Staphylococcus aureus. Microbiological Research. 2010;165(8):617-26.
91. Salari MH, Eshraghi S, Noroozi M. Antibacterial effect of glycyrrhetinic acid on 55 hospital strains of staphylococcus aureus and 32 actinobacillus actinomycetemcomitans. DARU Journal of Pharmaceutical Sciences. 2001;9(3-4):37-9.
92. Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. Journal of Infectious Diseases. 2010;201(2):241-54.
93. Giraudo AT, Mansilla C, Chan A, Raspanti C, Nagel R. Studies on the expression of regulatory locus sae in Staphylococcus aureus. Current Microbiology. 2003;46(4):0246-50.
94. Long DR, Mead J, Hendricks JM, Hardy ME, Voyich JM. 18β-Glycyrrhetinic acid inhibits methicillin-resistant Staphylococcus aureus survival and attenuates virulence gene expression. Antimicrobial Agents and Chemotherapy. 2013;57(1):241-7.
95. Li H-e, Qiu J-z, Yang Z-q, Dong J, Wang J-f, Luo M-j, et al. Glycyrrhetinic acid protects mice from Staphylococcus aureus pneumonia. Fitoterapia. 2012;83(1):241-8.
96. Rocha JLL, Kondo W, Baptista MIDK, Cunha CAd, Martins LTF. Uncommon vancomycin: induced side effects. Brazilian Journal of Infectious Diseases. 2002;6(4):196-200.
97. Darvishi B, Manoochehri S, Kamalinia G, Samadi N, Amini M, Mostafavi SH, et al. Preparation and antibacterial activity evaluation of 18-β-glycyrrhetinic acid loaded PLGA nanoparticles. Iran Journal of Pharmaceutical Sciences. 2015;14(2):373.

98. Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr H. Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. Lancet. 2003;361(9374):2045-6.

Chapter Four

General conclusions and future recommendations

Chapter Four – General conclusions and future recommendations

4.1 Introduction

Globally, infectious diseases are one of the top ten leading causes of death. The discovery of antibiotics greatly improved the prevention and control of infections, however, the disadvantages and overuse of conventional antibiotics led to the development of therapy complications and the antimicrobial resistance crisis. One of the major health risks is MRSA infection, which has increased mortality and morbidity rates worldwide. VCM is the last effective resort against MRSA, with the spread of resistance being a serious concern, urging the need to develop strategies to restore VCM efficiency. Although novel nano drug delivery systems have shown enhanced VCM therapy, there is still a need to optimize nano antibiotic carriers to protect and localize VCM during systemic circulation, target VCM release and improve its bioavailability at the infection site. Therefore, current research advances focus on developing pH-responsive nanosystems to improve VCM targeted delivery to the acidic infection sites. LPHNPs are an efficient nanosystem for antibiotic delivery and enhancing antibacterial activity. As per the study aim, the results of the objectives outlined the potential of novel pH-responsive lipid-polymer hybrid nanoparticles co-delivery with VCM and GA to enhance its antibacterial efficacy.

The results pertaining to the study aim and objectives were as follows:
1. To formulate and optimize novel pH-responsive GAPAH lipid-polymer hybrid nanoparticles encapsulating VCM and GA.

2. To optimize and characterize VCM-GAPAH-LPHNPs in terms of particle size, PDI, ZP, pH-responsiveness, morphology, entrapment efficiency and *in vitro* drug release.

3. To assess *in vitro* antibacterial activity of VCM-GAPAH-LPHNPs against MRSA.

4. To identify time-killing kinetics of VCM-GAPAH-LPHNPs against MRSA.

5. To evaluate antibiofilm activity of the novel VCM-GAPAH-LPHNPs.

The main conclusions generated from the research data are summarized below:

- VCM-loaded GA and polyallylamine hydrochloride lipid-polymer hybrid nanoparticles (VCM-GAPAH-LPHNPs) were successfully synthesized.

- The cytotoxicity studies performed by MTT assay on mammalian cell lines (CACO-2 and HEPG2) revealed that VCM-GAPAH-LPHNPs was a biologically safe formulation.
- Spherically shaped VCM-GAPAH-LPHNPs were successfully prepared using micro-emulsion technique. The optimal formulation showed pH-responsiveness in terms of size, PDI and ZP. VCM-GAPAH-LPHNPs size increased from 198.4 ± 0.302 nm at pH 7.4 to 374.8 ± 5.435 nm at pH 6 and 450.4 ± 1.33 nm at pH ,4.5 respectively. PDI increased from 0.255 ± 0.003 at pH 7.4 to 0.279 ± 0.054 at pH 6 and 0.377 ± 0.012 at pH 4.5, respectively. Also, ZP switched from – 3.8 ± 0.335 mV at pH 7.4 to + 0.44 ± 0.325 mV at pH 6 and + 0.693 ± 0.44 mV at pH 4.5, respectively. The EE % was found to be 69.46 ± 2.52.
- *In vitro* drug release studies showed a controlled and pH-dependent VCM release over a period of 48 hours. VCM-GAPAH-LPHNPs had faster drug release at pH 6 compared to pH 7.4.
- *In vitro* antibacterial activity against SA and MRSA confirmed the superiority of VCM-GAPAH-LPHNPs over bare VCM, GA and blank-LPHNPs as VCM-GAPAH-LPHNPs had enhanced and prolonged activity against MRSA at pH 6 and pH 7.4. Moreover, VCM-GAPAH-LPHNPs activity was eight times better against MRSA at pH 6 than at pH 7.4.
- The time-killing assay study showed that VCM-GAPAH-LPHNPs could effectively treat MRSA infections by showing 75% bacterial cell death in less than 12 hours.
- VCM-GAPAH-LPHNPs showed a decrease in crystal violet intensity and were able to eradicate 69 % of MRSA biofilms.
- The findings of this study, therefore, confirmed the potential of the novel GA and polyallylamine hydrochloride for preparation of pH-responsive LPHNPs for enhancing VCM efficacy. In addition, these findings can serve as a basis for future scientific research on the synthesis of novel natural compounds to develop pH-responsive nanocarriers for targeted drug delivery.

4.2 Significance of the findings in the study

The pH-responsive VCM-GAPAH-LPHNPs were designed to improve VCM targeted delivery to acidic target sites of infection for enhancing antibacterial activity. The significance of the findings of the study are as follows:

New Pharmaceutical Products

Novel pH-responsive VCM-GAPAH-LPHNPs formulation were successfully developed in this study, which can stimulate the pharmaceutical industry to develop new pH-responsive materials and medicines to improve antibiotic delivery.

Improved patient therapy and disease treatment

The *in vitro* studies of the developed pH-responsive nanosystem showed enhanced antibiotic activity and sustained VCM release in acidic medium. This nanosystem has the potential to improve bacterial infection therapy by protecting the antibiotic during systemic circulation, target the delivery of optimal antibiotic concentration to infection sites, decrease healthy sites exposure to the antibiotic and enhance bacterial antibiotic uptake. These benefits can result in reducing antibiotic dosing frequency, adverse drug reactions and toxicity. This can lead to improving patient compliance, enhancing antibacterial therapy and combating antimicrobial resistance threats.

Creation of new scientific knowledge

This study can identify new scientific advancements in the preparation and characterization of combinational delivery of antibiotics and natural plant compounds of pH-responsive lipid-polymer hybrid nanoparticles. This can serve as a basis for smart nano delivery systems development and enhance their potential pharmaceutical applications.

Stimulation of new research

The findings can provide potential research advancements to explore the pH-responsive natural compounds i.e., GA, for potential applications in pH-responsive LPHNPs for the co-delivery of antibiotics and natural compounds nanosystems formulation for various drug classes, such as anticancer, antiviral and anti-inflammatory drugs.

4.3 Recommendations for future studies

The present study concluded that the synthesis of novel pH-responsive GA and polyallylamine hydrochloride formulation for VCM and GA targeted co-delivery can enhance treatment of bacterial infections. The following studies are recommended to improve drug targeted delivery via pH-responsive VCM-LPHNPs:

- Other natural compounds, lipids and polymers can be investigated to analyze the effect of lipid-polymer type and ratios on pH-responsiveness, drug entrapment, drug release and antibacterial activity.
- Additional *in vitro* and *in vivo* antimicrobial activity screening against other Gram-positive and Gram-negative bacteria should be done to further assess VCM-GAPAH-LPHNPs spectrum of activity.
- Further *in silico* modeling and simulation studies are required to better understand molecular interactions of VCM-GAPAH-LPHNPs against SA and MRSA bacteria.
- *In vivo* pharmacokinetic profiling could be conducted to provide more information regarding pH-responsive targeted drug delivery, bioavailability and bio-distribution profiles.
- A large-scale production method could be established to influence the development and optimization of the nano antibiotic formulation by local pharmaceutical industries.

4.4 Conclusion

The findings of this study confirmed the potential of the synthesized novel pH-responsive nanoformulation in targeting antibiotic delivery to improve bacterial infection therapy. This study has contributed to the advancement of drug delivery strategies to address the limitations of conventional antibiotic dosage forms and antimicrobial resistance.

Appendix

Appendix A: Submitted manuscript to the International Journal of Pharmaceutics (Impact factor: 4.845). Reference number: IJP-S-20-04690

Elsevier Editorial System(tm) for
International Journal of Pharmaceutics
Manuscript Draft

Manuscript Number:

Title: Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhancement of the antibacterial activity of vancomycin and 18β-glycyrrhetinic acid

Article Type: Research Paper

Section/Category: Pharmaceutical Nanotechnology

Keywords: pH-responsive; lipid-polymer hybrid nanoparticles; vancomycin; methicillin-resistant Staphylococcus aureus; 18β- glycyrrhetinic acid

Corresponding Author: Professor Thirumala Govender, Ph.D.

Corresponding Author's Institution: University of KwaZulu-Natal

First Author: Yajna Jaglal

Order of Authors: Yajna Jaglal; Nawras Osman; Calvin A Omolo; Chunderika Mocktar; Nikita Devnarain; Thirumala Govender, Ph.D.

Abstract: Despite advancement in the control and therapeutics of infectious diseases, antimicrobial resistance remains a global burden. Hence there is a need for novel strategies that improve and potentiate available antibiotics to prevent a regress to a pre-antibiotic era. This study aimed to co-deliver vancomycin (VCM) and 18β-glycyrrhetinic acid (GA) via pH-responsive lipid-polymer hybrid nanoparticles (VCM-GAPAH-LPHNPs) to explore its potential for enhanced activity and targeted delivery of VCM. The stability of VCM-GAPAH-LPHNPs were supported by in silico studies. Biosafe VCM-GAPAH-LPHNPs were prepared using the microemulsion technique. VCM-GAPAH-LPHNPs demonstrated size, polydispersity index, zeta potential and encapsulation efficiency of 198.4 ± 0.302 nm, 0.255 ± 0.003, -3.8 ± 0.335 mV and 69.46 ± 2.52 %, respectively. In vitro drug release studies revealed that VCM-GAPAH-LPHNPs had sustained and faster release at acidic conditions compared to bare VCM. VCM-GAPAH-LPHNPs also demonstrated greater in vitro antibacterial potential against methicillin-resistant Staphylococcus aureus by 16-fold, when compared to the bare drug. Additionally, the time-killing assay indicated the ability of VCM-GAPAH-LPHNPs to eliminate 75 % of MRSA in less than 12 h. Furthermore, crystal violet assay confirmed VCM-GAPAH-LPHNPs potential to eliminate biofilms. Therefore, these novel LPHNPs may serve as promising nanocarriers for enhancing antibiotic drug delivery and antibacterial activity.

Suggested Reviewers: Yashwanth Pathak
ypathak1@health.usf.edu
Nano delivery, polymer science, molecular modelling

Aliasger Salem
aliasger-salem@uiowa.edu
Nano delivery, polymer science, molecular modelling

Appendix B: Turnitin report

ORIGINALITY REPORT

13%	%	13%	%
SIMILARITY INDEX PAPERS	INTERNET SOURCES	PUBLICATIONS	STUDENT

PRIMARY SOURCES

1	Nawras Osman, Calvin A. Omolo, Ramesh Gannimani, Ayman Y. Waddad et al. "Novel fatty acid-based pH-responsive nanostructured lipid carriers for enhancing antibacterial delivery", Journal of Drug Delivery Science and Technology, 2019 Publication	3%
2	Mohammed Salih, Calvin A. Omolo, Nikita Devnarain, Ahmed A. Elrashedy et al. " Supramolecular self-assembled drug delivery system (SADDs) of vancomycin and tocopherol succinate as an antibacterial agent: , and evaluations ", Pharmaceutical Development and Technology, 2020 Publication	2%

3 Ruma Maji, Calvin A. Omolo, Nikhil Agrawal, Kaminee Maduray et al. "pH-Responsive Lipid–Dendrimer Hybrid Nanoparticles: An Approach To Target and Eliminate Intracellular Pathogens", Molecular Pharmaceutics, 2019 — 2%
Publication

4 Calvin A. Omolo, Rahul S. Kalhapure, Nikhil Agrawal, Mahantesh Jadhav et al. "A hybrid of mPEG-b-PCL and G1-PEA dendrimer for enhancing delivery of antibiotics", Journal of Controlled Release, 2018 — 1%
Publication

5 Danford Mhule, Rahul S. Kalhapure, Mahantesh Jadhav, Calvin A. Omolo et al. "Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin", International Journal of Pharmaceutics, 2018 — 1%
Publication

6 J. Meletiadis, S. Pournaras, E. Roilides, T. J. Walsh. "Defining Fractional Inhibitory Concentration Index Cutoffs for Additive Interactions Based on Self-Drug Additive Combinations, Monte Carlo Simulation Analysis, and In Vitro-In Vivo Correlation Data for Antifungal Drug Combinations against Aspergillus fumigatus", Antimicrobial Agents and Chemotherapy, 2009 1%
Publication

7 Sifiso S. Makhathini, Calvin A. Omolo, Ramesh Gannimani, Chunderika Mocktar, Thirumala Govender. "pH-Responsive Micelles from an Oleic Acid Tail and Propionic Acid Heads Dendritic Amphiphile for the Delivery of Antibiotics", Journal of Pharmaceutical Sciences, 2020 <1%
Publication

Calvin A. Omolo, Rahul S. Kalhapure, 8
Mahantesh Jadhav, Sanjeev Rambharose et al. "Pegylated oleic acid: A promising amphiphilic polymer for nano-antibiotic delivery", European Journal of Pharmaceutics and Biopharmaceutics, 2017
Publication

<1%

Mbuso Faya, Heba A. Hazzah, Calvin A. Omolo, 9
Nikhi Agrawal et al. "Novel formulation of antimicrobial peptides enhances antimicrobial activity against methicillin-resistant Staphylococcus aureus (MRSA)", Amino Acids, 2020
Publication

<1%

Calvin A. Omolo, Nagia A. Megrab, Rahul S. 10
Kalhapure, Nikhil Agrawal et al. "Liposomes with pH responsive ‘on and off’ switches for targeted and intracellular delivery of antibiotics", Journal of Liposome Research, 2019
Publication

<1%

Sandeep J. Sonawane, Rahul S. Kalhapure,

11 Sanjeev Rambharose, Chunderika Mocktar et <1% al. "Ultra-small lipid-dendrimer hybrid nanoparticles as a promising strategy for antibiotic delivery: In vitro and in silico studies",

International Journal of Pharmaceutics, 2016

Publication

---

12 Sandeep J. Sonawane, Rahul S. Kalhapure, <1%

Mahantesh Jadhav, Sanjeev Rambharose, Chunderika Mocktar, Thirumala Govender. "AB2-type amphiphilic block copolymer containing a pH-cleavable hydrazone linkage for targeted antibiotic delivery", International Journal of Pharmaceutics, 2020

Publication

---

13 Mahantesh Jadhav, Rahul S. Kalhapure, <1%

Sanjeev Rambharose, Chunderika Mocktar, Sanil Singh, Tetsuya Kodama, Thirumala Govender. "Novel lipids with three C18-fatty acid chains and an amino acid head group for pHresponsive and sustained antibiotic delivery",

Chemistry and Physics of Lipids, 2018

Publication

14	Rao, J.P.. "Polymer nanoparticles: Preparation techniques and size-control parameters", Progress in Polymer Science, 201107 Publication	<1%
15	Daniel Hassan, Calvin A. Omolo, Ramesh Gannimani, Ayman Y. Waddad et al. "Delivery of Novel Vancomycin Nanoplexes for Combating Methicillin Resistant staphylococcus aureus (MRSA) Infections", International Journal of Pharmaceutics, 2019 Publication	<1%
16	Tushar Date, Vaishnavi Nimbalkar, Jyostna Kamat, Anupama Mittal, Ram I. Mahato, Deepak Chitkara. "Lipid-polymer hybrid nanocarriers for delivering cancer therapeutics", Journal of Controlled Release, 2018 Publication	<1%

17	Pavan Walvekar, Ramesh Gannimani, Mohammed Salih, Sifiso Makhathini, Chunderika Mocktar, Thirumala Govender. "Self-assembled oleylamine grafted hyaluronic acid polymersomes for delivery of vancomycin against methicillin resistant Staphylococcus aureus (MRSA)", Colloids and Surfaces B: Biointerfaces, 2019 Publication	<1%
18	Sifiso S. Makhathini, Rahul S. Kalhapure, Mahantesh Jadhav, Ayman Y. Waddad et al. " Novel two-chain fatty acid-based lipids for development of vancomycin pH-responsive liposomes against and methicillin-resistant (MRSA) ", Journal of Drug Targeting, 2019 Publication	<1%
19	Rahul S. Kalhapure, Nadia Suleman, Chunderika Mocktar, Nasreen Seedat,	<1%

Thirumala Govender. "Nanoengineered Drug Delivery Systems for Enhancing Antibiotic Therapy", Journal of Pharmaceutical Sciences, 2015
Publication

---

20 Rahul S. Kalhapure, Dhiraj R. Sikwal, Sanjeev Rambharose, Chunderika Mocktar et al. "Enhancing targeted antibiotic therapy via pH responsive solid lipid nanoparticles from an acid cleavable lipid", Nanomedicine: Nanotechnology, Biology and Medicine, 2017
Publication

<1%

---

21 Mohammed Salih, Calvin A. Omolo, Nikhil Agrawal, Pavan Walvekar et al. "Supramolecular amphiphiles of Betacyclodextrin and Oleylamine for enhancement of vancomycin delivery", International Journal of Pharmaceutics, 2020
Publication

<1%

---

22 Pavan Walvekar, Ramesh Gannimani, Sanjeev Rambharose, Chunderika Mocktar, Thirumala

<1%

Govender. "Fatty acid conjugated pyridinium cationic amphiphiles as antibacterial agents and self-assembling nano carriers", Chemistry and Physics of Lipids, 2018
Publication

---

23 Nikita Devnarain, Nawras Osman, Victoria Oluwaseun Fasiku, Sifiso Makhathini et al. " Intrinsic nanocarriers for smart drug delivery of antibacterial agents—An review of the last two decades ", WIREs Nanomedicine and Nanobiotechnology, 2020
Publication

<1%

---

24 Nermin E. Eleraky, Ayat Allam, Sahar B. Hassan, Mahmoud M. Omar. "Nanomedicine Fight against Antibacterial Resistance: An Overview of the Recent Pharmaceutical Innovations", Pharmaceutics, 2020
Publication

<1%

Nayab Tahir, Muhammad Tahir Haseeb, 25 Asadullah Madni, Farzana Parveen et al. "Chapter 3 Lipid Polymer Hybrid Nanoparticles: A Novel Approach for Drug Delivery", IntechOpen, 2020

<1%

Publication

26 Rahul S. Kalhapure, Mahantesh Jadhav, Sanjeev Rambharose, Chunderika Mocktar, Sanil Singh, Jwala Renukuntla, Thirumala Govender. "pH-responsive chitosan nanoparticles from a novel twin-chain anionic amphiphile for controlled and targeted delivery of vancomycin", Colloids and Surfaces B: Biointerfaces, 2017 <1%

Publication

27 Susana Martins. "Drug Delivery Across Blood Brain-Barrier by Means of I.V. Administration of Lipid Nanoparticles.", Repositório Aberto da Universidade do Porto, 2014. <1%

Publication

28	Nasreen Seedat, Rahul S. Kalhapure, Chunderika Mocktar, Suresh Vepuri, Mahantesh Jadhav, Mahmoud Soliman, Thirumala Govender. "Co-encapsulation of multi-lipids and polymers enhances the performance of vancomycin in lipid–polymer hybrid nanoparticles: In vitro and in silico studies", Materials Science and Engineering: C, 2016 Publication	<1%
29	Calvin Andeve Omolo, Rahul S Kalhapure, Nikhil Agrawal, Sanjeev Rambharose, Chunderika Mocktar, Thirumala Govender. "Formulation and Molecular Dynamics Simulations of a Fusidic Acid Nanosuspension for Simultaneously Enhancing Solubility and Antibacterial activity.", Molecular Pharmaceutics, 2018 Publication	<1%

Bhushan S. Pattni, Vladimir V. Chupin, Vladimir

30 P. Torchilin. "New Developments in Liposomal <1%

Drug Delivery", Chemical Reviews, 2015

Publication

Ceyda T. Sengel-Turk, Mehmet Gumustas, <1%

31

Bengi Uslu, Sibel A. Ozkan. "A novel approach for drug targeting", Elsevier BV, 2018

Publication

Jana Ghitman, Raluca Stan, Adi Ghebaur, <1%

32

Sergiu Cecoltan, Eugeniu Vasile, Horia Iovu. "Novel PEG-Modified Hybrid PLGA-Vegetable

Oils Nanostructured Carriers for Improving Performances of Indomethacin Delivery", Polymers, 2018

Publication

---

33 Danyelle R. Long, Julia Mead, Jay M. Hendricks, Michele E. Hardy, Jovanka M. Voyich. "18β-Glycyrrhetinic Acid Inhibits Methicillin-Resistant Staphylococcus aureus Survival and Attenuates Virulence Gene Expression", Antimicrobial Agents and Chemotherapy, 2013 <1%
Publication

34 Advances in Delivery Science and Technology, 2015. <1%
Publication

35 Shaimaa Ahmed, Suresh B. Vepuri, Muthusamy Ramesh, Rahul Kalhapure, Nadia Suleman, Thirumala Govender. "In Silico Characterization of the Binding Affinity of Dendrimers to Penicillin-Binding Proteins (PBPs): Can PBPs be Potential Targets for Antibacterial Dendrimers?", Applied Biochemistry and Biotechnology, 2016 <1%
Publication

36	Juho Lee, Dongmin Kwak, Hyunwoo Kim, Jihyun Kim, Shwe Phyu Hlaing, Nurhasni Hasan, Jiafu Cao, Jin-Wook Yoo. "Nitric Oxide-Releasing S-Nitrosoglutathione-Conjugated Poly(Lactic-Co-Glycolic Acid) Nanoparticles for the Treatment of MRSA-Infected Cutaneous Wounds", Pharmaceutics, 2020 Publication	<1%

Exclude quotes	On	Exclude matches	< 15 words
Exclude bibliography	On		