FORMULATION AND EVALUATION OF NOVEL VANCOMYCIN LOADED LIPID-POLYMER HYBRID NANOPARTICLES FOR EFFECTIVE ANTIBIOTIC THERAPY

by

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Submitted in part fulfilment of the requirements for the degree of Master of Pharmacy in the discipline of Pharmaceutical Sciences of the School of Health Sciences at the University of KwaZulu-Natal

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Co-supervisor: Dr. Chunderika Mocktar

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“Education is our passport to the future, for tomorrow belongs to the people who prepare for it today.’’

-Malcolm X-

"***This dissertation is dedicated to my Dad.***

Education has always been a priority you instilled in me.

I hope that I have made you proud"

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

1. Seedat, N., Kalhapure, R.S., Mocktar C., Vepuri S., Jadhav M., Soliman M., and Govender T. 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.* SUBMITTED MANUSCRIPT. Reference Number: MSEC-D-15-01196R3.

   Ms. N. Seedat contributed to the design of the project, modification and optimisation of methods and preparation and characterisation of all LPN formulations in terms of particle size, PDI, zeta potential, encapsulation efficiency, in vitro drug release study, antibacterial activity, gel electrophoresis, X-ray diffraction and differential scanning calorimetry. Dr. R.S Kalhapure assisted with the overall design of the study and the methods of preparation and characterisation as well as editing. Dr. S. Vepuri and Prof M. Soliman were collaborators on the project and performed the molecular modelling studies. Dr. M. Jadhav performed the mathematical modelling in terms of the in vitro release kinetics data. The remaining authors served as supervisor and co-supervisor and were responsible for project conceptualisation, problem solving, co-writing of papers and abstracts and general supervision of the study.

2. Kalhapure, R. S., Suleman, N., Mocktar, C., Seedat, N., & Govender, T. (2015). Nanoengineered drug delivery systems for enhancing antibiotic therapy. *Journal of Pharmaceutical Sciences*, *104*(3), 872-905.

   Ms. N Seedat contributed by performing a literature review of the Lipid polymer hybrid nanoparticles (LPN) section. In addition, she constructed the relevant summary of literature table for the LPN section as well as contributed to the writing of this section. The remaining authors were co-authors on the paper.

Research output from the dissertation

1. Publication

a. The following review article was published in an international ISI Journal (Impact Factor: 2.59) from work done during this study.

Kalhapure, R. S., Suleman, N., Mocktar, C., Seedat, N., & Govender, T. (2015). Nanoengineered drug delivery systems for enhancing antibiotic therapy. *Journal of Pharmaceutical Sciences*, *104*(3), 872-905.

* The published article can be found in Chapter four.

2. Submitted Manuscript

b. The following paper was submitted to an international ISI journal (Impact Factor: 3.088) from data generated during this study:

Seedat, N., Kalhapure, R.S., Mocktar C., Vepuri S., Jadhav M., Soliman M., and Govender T. 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.* SUBMITTED MANUSCRIPT. Reference Number: MSEC-D-15-01196R3.

* The submitted manuscript can be found in Chapter three.

3. Conference Presentations

The following conference presentations were produced from data generated during this study:

International:

- Seedat N, Kalhapure R, Mocktar C, Govender T. Enhancing vancomycin delivery via Lipid-polymer hybrid nanoparticles. 1st European Conference on Pharmaceutics, Reims, France, 13-14 April 2015.
- Vepuri SB, Seedat N, Kalhapure R, Mocktar C, Soliman M, Govender T. Atomistic binding energy and coarse grained simulation studies to understand the structure and drug release activity of Vancomycin loaded Lipid polymer nanoparticles (LPNs). The International Nanotech and Nanoscience conference and exhibition, Paris, France, 15-17 June 2015.

Local:

- Seedat N, Kalhapure R, Mocktar C, Govender T. Optimisation of formulation variables of drug free Lipid-polymer hybrid nanoparticles. 35th Conference of the Academy of Pharmaceutical Sciences. Port Elizabeth, South Africa, 14-16 September 2014.

*The posters can be found in Appendix A & B.

ABSTRACT

Infectious Diseases remains a major cause of morbidity and mortality globally and are exacerbated by the ongoing crisis of antibiotic resistance. Vancomycin (VCM) is an antibiotic used for the treatment of serious infections that do not respond well to other antibiotics; however, resistance to VCM has also developed. Nano drug delivery systems are being widely explored to overcome the challenges with existing antibiotic dosage forms to treat bacterial infections. Lipid-Polymer Nanoparticles (LPNs) display unique advantages of both liposomes and polymeric nanoparticles while excluding some of their limitations. This is a hybrid particulate system, as it has the structural integrity of the polymeric particles and the biomimetic properties of the liposome. LPNs have several advantages that make them a superior drug delivery system compared to conventional antibiotics. As an emerging nanoparticulate delivery system, there is limited data on antibiotic loaded LPNs in the literature, especially with regard to formulation optimisation and enhancement of critical performance properties. The use of helper lipids and polymers in LPNs have further not been investigated for their potential to simultaneously improve drug encapsulation, antibacterial activity and drug release profiles. The aim of this study was therefore to explore a new lipid-polymer combination in the formulation development of an antibiotic loaded LPN using VCM as a drug, as well as to co-encapsulate helper polymers and lipids in order to simultaneously enhance important properties, such as drug encapsulation, antibacterial activity and drug release profiles. In addition to *in vitro* characterisation, extensive *in silico* modelling was undertaken to obtain a molecular understanding of the effect of the helper polymers and lipid on the VCM loaded LPNs.

LPNs were prepared using vancomycin (VCM), glyceryl triplamitate and Eudragit RS100 as the drug, lipid and polymer respectively. Oleic acid (OA), Chitosan (CHT) and Sodium alginate (ALG) were explored as helper excipients in the formulation. LPNs were prepared by a modified hot homogenisation method followed by ultrasonication. The LPNs were characterised in terms of size, PDI, zeta potential, encapsulation efficiency, morphology, *in vitro* drug release and kinetics, *in vitro* antibacterial activity, thermal profile, crystallinity as well as structural configuration using molecular modelling.

Rod-shaped LPNs with suitable size (202.5 ± 3.81 to 250.9 ± 9.04), PDI (0.251 ± 0.01 to 0.386 ± 0.02) and zeta potential (-32.8 ± 4.54 to +17.4 ± 2.84) were successfully prepared. Drug encapsulation efficiency (%EE) increased from 27.8% to 41.5%, 54.3% and 69.3% with the

addition of OA, CHT and ALG respectively. Drug release data showed that VCM-CHT had the slowest drug release of 36.1 ± 5.35%, while VCM-ALG had the fastest drug release rate of 54.4 ± 3.24% at the end of 24 h, with all formulations indicating a sustained release profile. The EE and drug release data was further corroborated by *in silico* and release kinetics data. The drug release kinetics data indicated that the drug release demonstrates controlled release with polymer swelling with water absorption and polymer chain relaxation. *In silico* studies showed that the binding free energy of the complexes correlated with the EE and drug release data. *In vitro* antibacterial studies of all formulations exhibited better activity against bare VCM and sustained activity up to day 5 against both *S.aureus* and MRSA, with VCM-OA and VCM-CHT showing better activity against MRSA. VCM-OA LPNs showed the best activity with an MIC value of 1.2µg/ml against MRSA on day 2. Gel electrophoresis confirmed the *in vitro* antibacterial activity as it showed degradation of the bacterial proteins of all the formulations. The formulations were stable at both room temperature and 4°C over a period of 3 months.

Therefore, the developed VCN LPN formulation proves to be a promising nanoantibiotic system for the delivery of VCM. It serves as a platform for further formulation and development to improve its properties as a drug delivery system. This study will contribute to the improvement in patient therapy and disease outcomes, creation of new knowledge on LPN drug delivery systems for antibiotics and generate interest for future research to be conducted.

Key words: lipid-polymer, antibiotic, nanotechnology, antibacterial, *in silico*, co-encapsulation, *in vitro*.

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List of Abbreviations

3D	Three-dimensional	OA	Oleic acid
AIC	Akaike's information criterion	OLV	Oligolamellar vesicles
AIDS	Acquired Immunodeficiency Syndrome	PAMAM	Polyamidoamine
ALG	Sodium Alginate	PEG	Polyethylene glycol
ANOVA	Analysis of variance	PC	Phosphatidylcholine
BBB	Blood brain barrier	PDI	Polydispersity index
CHT	Chitosan	PLA	Poly-lactic acid
DESE	Double-emulsification-solvent evaporation	PLGA	Poly-lactic-co-glycolic acid
DLS	Dynamic light scattering	PNP	Polymeric nanoparticle
DNA	Deoxyribonucleic acid	PVP	Polyvinylpyrollidone
DSC	Differential scanning calorimetry	RMSE	Root mean square error
EE	Encapsulation efficiency	ROS	Reactive oxygen species
ESE	Emulsificaion-solvent-evaporation	SD	Standard deviation
EUD	Eudragit RS100	SDS	Sodium dodecyl sulphate
FIC	Fractional inhibitory concentration	SEM	Scanning electron microscopy
GA	Genetic algorithm	SLN	Solid lipid nanoparticle
GTP	Glyceryl triplamitate	SUV	Small unilamellar vesicles
HCl	Hydrochloric acid	TB	Tuberculosis
HIV	Human Immunodeficiency Virus	TEM	Tranmission electron microscopy
HPLC	High pressure liquid chromotography	UFF	Universal force field
LC	Loading capacity	USA	United States of America
LPN	Lipid polymer nanoparticle	UV	Ultraviolet
LUV	Large unilamellar vesicles	VCM	Vancomycin
MDT	Mean dissolution time	VRE	Vancomycin resistant enterococci
MHB	Mueller Hinton broth	VRSA	Vancomycin resistant staphylococcus aureus
MIC	Minimum inhibitory concentration	WHO	World health organisation
MLV	Multilamellar vesicles	XRD	X-ray diffraction
MRSA	Methicillin resistant staphylococcus aureus	ZP	Zeta potential

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CHAPTER 1. INTRODUCTION

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1.1 Introduction

This chapter outlines the background to the study, indicates the problem being addressed and the resulting aim and objectives. It explores on the current status of infectious diseases, the limitations associated with antibiotic therapy, and the novelty and significance of the study. It concludes with the structure and content of the remaining objectives.

1.2 Background

Infectious diseases are causing millions of deaths a year around the world, especially in developing regions (Bhutta et al., 2014). Deaths due to infectious diseases in low-income countries account for 40% and continue to rise in the absence of effective treatment options (World Health organisation. World health report 2002). Although the use of antibiotics has decreased morbidity and mortality rates, several limitations related to their use have compromised their ability to treat infectious diseases (Huh and Kwon, 2011; Walsh, 2000; Wood et al., 1996).

There are several disadvantages associated with the currently available conventional dosage forms of antibiotics. These include inadequate antibiotic concentration at the target infection site, increased frequency of administration (Baker-Austin et al., 2006; Kardas, 2002), and others such as low water-solubility, cytotoxicity to healthy tissues, fast degradation and clearance in the bloodstream (Zhang et al., 2010). In addition, extensive use and misuse has led to the most prominent problem of antimicrobial resistance.

Antimicrobial drug resistance has had a major impact on mortality rates worldwide and is now recognised as a major burden in healthcare settings (De Kraker et al., 2011; Livermore, 2009). Together with a decline in research and development of new antibiotics, this resistance has the potential to cause a threat similar to that of the pre-antibiotic era. As a result, the major advances made in modern medicine that rely on antibiotics, such as surgery, organ transplantation and cancer chemotherapy, are at risk (Cars et al., 2011). Statistics show that an estimated 19 000 deaths per year in the U.S. are caused by methicillin-resistant *Staphylococcus aureus* (MRSA), which can only be treated by vancomycin (VCM). VCM is a glycopeptide antibiotic used in the treatment and prophylaxis of serious infections caused by Gram positive bacteria, such as *S. aureus*, that do not respond well to other antibiotics

(Zakeri-Milani et al., 2013). VCM resistance and the rising prevalence of MRSA increases the possibility of VCM resistant *S.aureus* (VRSA), which is just as deadly as MRSA but harder to treat (Klevens et al., 2007; Weigel et al., 2003). MRSA, *S.aureus* and VRSA are organisms of current concern in developing and developed countries (Zaidi et al., 2005).

Nanotechnology is being explored as a promising alternative to current dosage forms of antibiotics for immunization, drug design and delivery, controlling cross infections and overcoming resistance (Zhu et al., 2014). Nano-systems can facilitate targeted delivery of the antibiotic at a specific infection site, provide sustained release profiles (Huh and Kwon, 2011) and inherently overcome existing drug resistance mechanisms (Pelgrift and Friedman, 2013). Nano-drug delivery systems can increase the efficacy of antibiotics by improving their solubility and pulmonary accumulation, reducing dosing frequency and side effects, and improving intracellular delivery that allows a higher concentration of a drug at the site of action (Garcia-Contreras et al., 2007; Pandey and Khuller, 2005).

According to Zhu et al., there are at least 10 nanoparticle-based products on the market to diagnose infections, antibiotic drug delivery and medical devices (Zhu et al., 2014). Nanoparticles that have been explored to effectively deliver antibiotics include liposomes, solid lipid nanoparticles (SLNs), polymeric nanoparticles and dendrimers (Huh and Kwon, 2011). The antimicrobial properties of nanoparticles can be attributed to their high surface to volume ratio that allows for drug penetration by attacking the bacterial cell wall, their distinctive chemico-physical properties, the versatility of their formulation, and their biocompatibility with tissues and cells (Panyam and Labhasetwar, 2003; Weir et al., 2008). Compared to other medical conditions, such as cardiovascular disease and cancer, nano-drug delivery systems for antibiotic therapy is still in its infancy (Huh and Kwon, 2011). Therefore, to overcome the limitations with current antibiotic dosage forms and combat the ongoing crisis of bacterial resistance, applying nanotechnology to deliver antibiotics is of utmost importance (Ranghar et al., 2014).

Lipid-based nanocarriers, such as liposomes (Gregoriadis, 1995), SLNs (Pinto-Alphandary et al., 2000), nanostructured lipid carriers (Li et al., 2006) and lipid drug conjugates (Sharma and Sharma, 1997) are an attractive dosage form due to their submicron sized particles and solid state of physiological lipid carriers (Cavalli et al., 2002). Liposomes have advantageous properties, such as biocompatibility, biodegradability, non-immunogenicity, flexibility

(Gregoriadis, 1995), the ability to closely interact with host cells, and to deliver both water and oil soluble drugs (Pinto-Alphandary et al., 2000). However, their drawbacks, such as low drug loading capacity, high initial burst kinetics, drug leakage during storage, batch to batch reproducibility issues, and manufacturing and scale up issues, need serious attention (Gregoriadis, 1995; Lee et al., 2007; Li et al., 2006; Sharma and Sharma, 1997).

Polymeric nanoparticles are also a widely used nano-drug delivery system due to their high structural integrity, storage stability, sustained release and ease of preparation (Peer et al., 2007). The rigidity of the polymer matrix in a polymeric nanoparticle makes them more stable than liposomes (Pinto-Alphandary et al., 2000). However, some of their limitations are: poor encapsulation of water soluble drugs due to leakage from the nanoparticles during the emulsification process in the preparation (Cheow and Hadinoto, 2010); polymer cytotoxicity and degradation; use of toxic organic solvents and scale-up issues (Allemann et al., 1993; Pinto Reis et al., 2006).

To overcome the limitations associated with both liposomes and polymeric nanoparticles, a relatively new nano-drug delivery system, popularly termed the lipid-polymer hybrid nanoparticles (LPNs), has been developed (Zhang et al., 2008). LPNs display unique advantages of both the liposomes and polymeric nanoparticles, while excluding some of their limitations. The LPN is a hybrid nano particulate system, as it has the structural integrity of the polymeric particles and the biomimetic properties of the liposome (Hadinoto et al., 2013). The LPNs consist of: i) a biodegradable polymeric core suitable for carrying poorly water-soluble drugs and releasing them at a controlled rate; ii) a hydrophilic shell that allows particles to evade recognition by the immune system, thereby increasing the half-life of the drug; and (iii) a lipid monolayer that prevents carried agents from freely diffusing out of the nanoparticles and reduces the water penetration rate into the nanoparticles, which slows the drug release from the nanoparticles (Zhang et al., 2008). LPNs have the advantages of high structural integrity, stability, sustained release from the polymer core, high biocompatibility and bioavailability, tuneable size and surface charge, high drug loading and targeted drug delivery (Chan et al., 2009; Zhang et al., 2008).

To the best of our knowledge, despite numerous advantages offered by LPNs, their utilization in the delivery of antibiotics is very limited, with only five papers reported in the literature to date. The delivery of three fluoroqinolone antibiotics (levofloxacin, ofloxacin, ciprofloxacin),

as well as calcein (Cheow et al., 2011; Cheow and Hadinoto, 2011, 2012; Wang et al., 2012) and clindamycin phosphate (Abbaspour et al., 2013) have been studied using LPNs (Mandal et al., 2013). Furthermore, the most explored polymer for antibiotic loaded LPN synthesis is Poly Lactic-co-Glycolic Acid (PLGA) (Cheow et al., 2011; Cheow and Hadinoto, 2011, 2012; Wang et al., 2012), with sodium alginate (ALG) and dextran sulphate having been reported in one paper (Abbaspour et al., 2013), while the lipids that have been investigated include stearic acid, lecithin and phospahtidylcholine (PC) (Abbaspour et al., 2013; Cheow et al., 2011; Cheow and Hadinoto, 2010, 2012; Wang et al., 2012).

The limited antibiotic LPN studies have highlighted the need for formulation optimisation and characterization of LPNs by exploring other polymers and lipids with potent antibiotics, such as VCM. Identifying strategies to simultaneously enhance the critical properties of drug entrapment, antibacterial activity against sensitive and resistant strains, and controlled release profiles has also not been previously reported for any antibiotic LPN system. The development of antibiotic LPNs by co-encapsulating multiple lipids and polymers within its configuration could be an effective approach for simultaneously enhancing the above properties, and remains to be explored.

1.3 Aims and Objectives

The aim of this study was to formulate and evaluate novel vancomycin loaded lipid-polymer nanoparticles to enhance antibiotic therapy.

In order to achieve this aim, the objectives of the study were to:

1. Prepare VCM loaded LPNs containing a new lipid-polymer combination of Eudragit RS100 as the polymer and Glyceryl tripalmitate as the lipid.
2. Simultaneously enhance the encapsulation efficiency and antibacterial activity of the nanoparticles by incorporation of various co-excipients such as oleic acid, chitosan and sodium alginate.
3. Evaluate the lipid-polymer nanoparticles in terms of particle size, surface charge, morphology, drug release, antimicrobial activity, thermal behaviour and crystallinity and corroborate the data with *in silico* modelling.

1.4 Novelty

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

- This study uses a new lipid and polymer combination, which has not been reported previously for any antibiotic LPN system, and comprises of Eudragit RS100 as the polymer, glyceryl triplamitate as the lipid and hydrophilic VCM as the drug. This LPN system could be explored for other antibiotic drugs. It is anticipated that this study will identify novel formulation strategies to optimally encapsulate hydrophilic drugs into LPNs.
- A recent review article on lipid-polymer hybrid nanoparticles reported that the only antibiotics that have been explored for lipid-polymer nanoparticles was that of flouroquinolone antibiotics (Mandal et al., 2013). Vancomycin, which is a glycopeptide antibiotic, is used to treat serious infections that do not respond well to first line antibiotics. However, resistance to vancomycin is steadily increasing, and it is believed that incorporating vancomycin into novel nanoparticle systems will overcome the resistance issues and many of the drug delivery problems.
- To date, no studies have reported on the co-encapsulation of various excipients to simultaneously increase antibacterial activity, drug release and encapsulation efficiency of lipid-polymer nanoparticles. This study will be the first to report on this proposed method and will serve as a platform for incorporating a number of drugs to treat a variety of diseases.

1.5 Significance

Formulating vancomycin loaded lipid-polymer hybrid nanoparticles is a novel antibiotic drug delivery system that can enhance the antibiotic efficacy and overcome the limitations associated with the drug. The potential benefits of the proposed formulation in this study include the following:

- Vancomycin resistance is a major problem in antibiotic therapy, and by formulating the drug into a novel drug delivery system, such as lipid-polymer hybrid nanoparticles, it can enhance the efficacy of the drug, contribute to a decrease in antibiotic resistance and increase the therapeutic efficacy of the drug. Cost effective dosage forms can be developed to treat a range of diseases caused by bacterial infections, thereby improving patient treatment, disease outcomes and the economy of the country.
- This type of drug delivery system can benefit a wide variety of diseases, such as cancer, HIV/AIDS, cardiovascular conditions, and will enable many other drug delivery routes to be explored. Progress in nanotechnology and the development of this particular system could lend itself to enhancing many drug therapies.
- The co-encapsulation of different excipients proposed in this study could potentiate the antibacterial activity as well as the encapsulation of drugs, thereby enhancing the efficacy of the drug and decreasing manufacturing costs.
- *In silico* and *in vitro* kinetics studies can corroborate the results obtained and explain the mechanism by which the LPN formulation can achieve enhanced properties, such as encapsulation efficiency, drug release and antibacterial activity. Therefore, new knowledge about the mechanism in which these co-excipients interact with the formulation excipients can be generated.
- As data on antibacterial studies of antibiotic loaded LPNs is limited, with only one report on biofilm susceptibility testing (Cheow et al., 2011), the antibacterial data generated in this study could serve as a basis for future LPN formulations with other antibiotics.

1.6 Overview of Dissertation

The research is presented in the following chapters:

CHAPTER 2. Literature Review: This chapter focuses on the status of infectious diseases, current antibiotic therapy and the strategies that are used to overcome limitations. It focuses on nanotechnology and in particular, nano-drug delivery systems for antibiotic therapy. The emphasis is on lipid-polymer nanoparticles (LPNs) for antibiotic therapy and the various preparation methods and characterisation techniques. Finally, vancomycin as a model antibiotic is described.

CHAPTER 3. Submitted manuscript: This chapter is a first author article that was submitted in an ISI international journal. It is presented in the required format of the journal and is a report on novel work. It describes the formulation of novel vancomycin loaded LPNs that show enhanced antibacterial activity, drug release and encapsulation efficiency with the addition of helper excipients.

CHAPTER 4. Co-author review paper: This chapter is a co-author review paper published in an ISI international journal. It reviews the different nano-drug delivery systems that have been reported for antibiotic therapy.

CHAPTER 5. Conclusions: This chapter describes the conclusions reached in achieving the study aim, outlines the significance of the findings and makes recommendations for further research into antibiotic loaded LPNs.

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CHAPTER 2

LITERATURE REVIEW: NANOTECHNOLOGY FOR ANTIBIOTIC DRUG DELIVERY SYSTEMS

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

This chapter provides a summary of the literature and concepts on infectious diseases and nano-antibiotic drug delivery systems. It focuses on the emergence of bacterial resistance, as well as the currently available antibiotic therapy and its limitations. An overview of nanotechnology is presented, as well as the different types of nano-drug delivery systems that are used in antibiotic therapy, with the focus being on Lipid-Polymer Hybrid nanoparticles (LPNs). In addition, the various methods of preparation and characterization techniques are outlined for LPN formulation. Lastly, the rationale for the use of vancomycin as a model drug is outlined.

2.2 Introduction to Infectious diseases

Infectious diseases caused by a microorganism such as bacteria can spread from one person to another, either directly or indirectly (World health Organisation. Infectious diseases). The World Health Organisation reports that infectious diseases, a large proportion which are of bacterial origin, continue to be one of the leading causes of morbidity and mortality worldwide (World Health Organisation. The top 10 causes of death). They are a serious health problem in developing and developed countries, and are causing millions of avoidable and premature deaths a year, especially in developing regions (Bell et al., 2013). In 2002, deaths due to infectious diseases in developing countries account for 40% and are still on the rise (World health Organisation. World health report). Statistics show that of the 6.3 million children who died in Africa in 2013, 51.8% did so of infections, mainly pneumonia, diarrhoea and malaria (Liu et al., 2015).

One of the major causes of the rising incidence of infectious diseases is the increasing occurrence and spread of bacterial resistance. The problem of antimicrobial resistance is particularly pressing in Africa, including South Africa, due to its considerable burden of infectious diseases and the high cost of the newer antibiotics to replace the older, ineffective ones. The leading causes of death and disease in many developing countries are gastro-intestinal, respiratory, sexually transmitted and hospital-acquired infections, many of which no longer respond to the currently available antibiotics (Kalhapure et al., 2014b; Winters and Gelband, 2011). Issues such as global trade, international travel, poverty and war, as well as emerging and re-emerging infectious diseases, has exacerbated the growing problem of infectious diseases (Kalhapure et al., 2014b). In addition, infections are now playing a key role in the incidence and underlying cause/risk factor of non-communicable diseases, such as asthma, cancers, cardiovascular disease and gastrointestinal diseases (Ogoina and Onyemelukwe, 2009). A global view of infectious diseases is depicted in Figure 1 below.

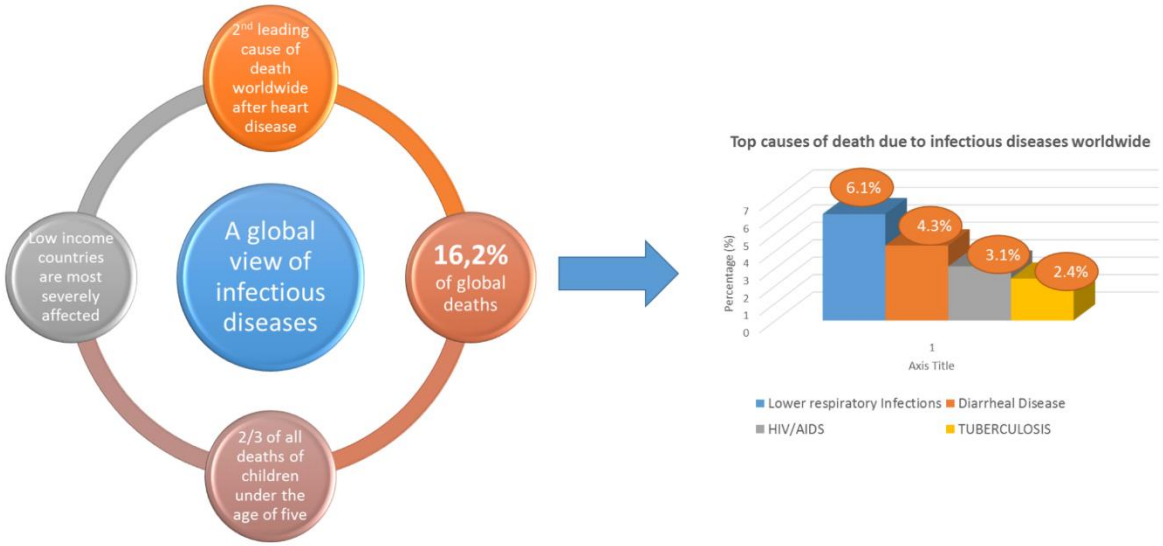


Figure 1. A global view of infectious diseases. Adapted from Clinipace Infographics: A global view of infectious diseases.

The existence of bacteria can be dated back more than 3 billion years and during this time they have come into contact with a wide range of naturally occurring antibiotics, which has resulted in them developing several antibiotic resistance mechanisms in order to survive. Resistance can be due to the innate property of the bacteria or a result of gene mutation. (Wood et al., 1996). The main mechanisms of bacterial resistance are: i) inactivation of the drug, ii) modification of the site of action, iii) modification of the permeability of the cell wall, and iv) overproduction of the target enzyme (Opal et al., 2000; Sefton, 2002; Walsh, 2000; Williams, 1996), these mechanisms being depicted in Figure 2.

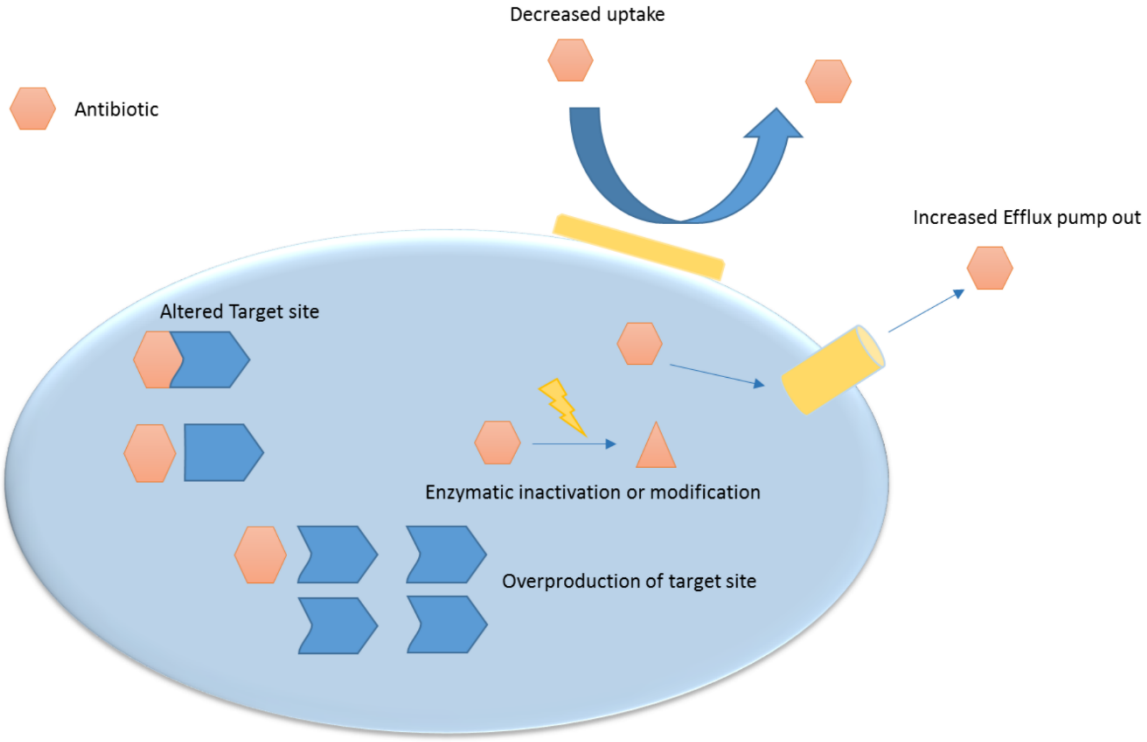


Figure 2. Mechanisms of resistance to antibacterial agents. Adapted from Coates et al. (Coates et al., 2002)

2.3 Current antibiotic therapy and limitations

The definition of an antimicrobial is a substance that can kill or deter the growth of bacteria, and since the advent of antimicrobial drugs in the 1960s, many infectious diseases have been cured (Coates et al., 2002). The current progress and health gains of clinical medicine would not be possible without the use of antibiotics. Organ transplants, surgery and cancer chemotherapy are just some of the medical procedures that would not be possible without preventing and treating bacterial infections. (Cars et al., 2011)

An antimicrobial agent works by targeting the components of bacterial metabolism, thereby inactivating the bacteria (Mandell et al., 2009). Some antibiotics have a broad spectrum of activity and inhibit a wide range of Gram-positive and Gram-negative bacteria, such as ampicillin, while others are only active against a narrow spectrum of bacteria, such as penicillin. Antibiotics also differ in their mechanism of action against bacteria. Some antimicrobials are bacteriostatic and inhibit cell growth, whereas others are bactericidal and kill bacteria. The use of a combination of antibiotics can therefore lead to increased activity, compared to each antibiotic being used alone (Coates et al., 2002; Walker, 1996).

Antibiotic use began in the late 1940s with the discovery and production of penicillin, and was a great success and since then even newer and stronger antibiotics have been developed over the years (Taubes, 2008). The benefits of antibiotics has proven to be life changing with regards to the survival rates of children, improving productivity in the workplace and longevity (Piddock, 2012). There are several conventional dosage forms of antibiotics that include oral tablets, liquid suspensions and intravenous injections, all of which have a number of limitations associated with their use. The limitations associated with current drug therapy include inadequate concentration at the target site, poor patient compliance and increased frequency of administration. In addition, widespread use and misuse of antibiotics have led to the most severe limitation, antibiotic drug resistance.

Despite the emergence of resistance, very few new drugs have been developed (Taubes, 2008), with the fast pace of bacterial resistance having far exceeded the rate of drug development (Huh and Kwon, 2011). Even the most potent antibiotics have been invalidated by the increasing rates of bacterial resistance, which results in higher mortality rates as well as increased health care costs (Brooks and Brooks, 2014). According to Fishback et al., since

the early 1960s, only four classes of new antibiotics that have entered the market, as the rest are dominated by modifications of antibiotics that were discovered half a century ago (Fischbach and Walsh, 2009). There has been a loss of interest in developing new antibiotics by pharmaceutical companies, which have focussed on chronic medications that are taken for much longer than the standard week-long antibiotic does, thereby generating greater revenue for them, and for which they can charge much higher prices (Nathan, 2004; von Nussbaum et al., 2006).

Poor patient compliance and increased dosing frequency is a major limitation associated with the use of antibiotics. Kardas reported that missed doses, change in the frequency of dosing and time interval delays are some of the major problems that are recognised with regard to patient compliance (Kardas, 2002). Besides the development of resistance, adverse side effects, such as toxicity due to high dosing of antibiotics, are also a limitation of current antibiotic therapy (Baker-Austin et al., 2006). Treatment of chronic conditions, such as cystic fibrosis and chronic obstructive pulmonary disease, are therefore hard to treat due to the high frequency dosing regimen (Beaulac et al., 1996). Antimicrobial drugs are also hard to administer due to their low water-solubility, fast degradation and clearance in the blood stream and cytotoxicity to healthy issues, (Zhang et al., 2010a).

Antimicrobial resistance can be defined as the phenomenon where pathogenic microorganisms multiply beyond the critical mass in the presence of antibiotics, resulting in treatment of the infection being compromised (Zhang et al., 2006). Antimicrobial agents have caused a significant decrease in morbidity and mortality rates globally, however, resistance to antibiotics has been reaching an alarming level worldwide, invalidating major antimicrobials that are currently used in treating infectious diseases (Brooks and Brooks, 2014; Huh and Kwon, 2011; Kalhapure et al., 2014b). Society and technological developments have caused a shift towards the unrestrained spread of resistance. This shift over the decades that led to the globalisation of antimicrobial resistance can be attributed to local and international travel, trade immigration and adoption (Stenhem et al., 2010).

The indiscriminate, inappropriate and incomplete use of antibiotics is also a major cause of antibiotic resistance. This is a result of antibiotics being available ‘over the counter’, given unnecessarily, failure to comply with the regimen, and patients not completing the course. (Hinman, 1998). Resistance in turn has an effect on morbidity and mortality, the cost of

treatment, the spread of disease and the duration of illness (Laxminarayan, 2010). The resistance phenomenon has caused a serious decline in research and development of new antibiotics, and is a threat of the pre-antibiotic era. As a result, major advances made in modern medicine are at risk, such as surgery, organ transplants and cancer chemotherapy (Cars et al., 2011).

Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus species, *Acinetobacter* species, *Pseudomonas* species, and *Klebsiella* are some of the bacteria associated with a high incidence of infection, and which also have developed resistance to treatment by many antibiotics (Falagas and Karveli, 2006). Statistics show that an estimate of 19 000 deaths per year in the USA are caused by methicillin-resistant *Staphylococcus aureus* (MRSA), which can only be treated by vancomycin. However, vancomycin resistance has developed, as has the prevalence of MRSA increased the possibility of vancomycin resistant *S.aureus* (VRSA), which is just as deadly as MRSA but harder to treat (Klevens et al., 2007; Weigel et al., 2003). The mechanisms of resistance of these drug resistant bacteria can be explained further in Table 1.

Table 1. Mechanism of resistance of drug resistant bacteria. Adapted from Ranghar et al. (Ranghar et al., 2014).

Bacterial Microorganism	**Drug use to treat infection**	**Mechanism of drug resistance**
Gonocci	Quinolone	Mutation in target site
Enterococcus	Sulfonamide Vancomycin	Changes in the target site Overproduction of the target site Development of alternate growth requirement
Enterobacteriaceae (e.g. E.coli)	β- lactams (carbapenem)	Drug degrading enzyme
Streptococcus Pneumoniae	Macrolide	Active efflux, drug efflux pump
Pseudomonas aeruginosa	Multiple drugs	Several factors including loss of porin, drug efflux pump and drug modifying enzyme
Staphylococcus Aureus	β- lactam (methicillin) Vancomycin	Production of an extra enzyme that avoids binding Thickening of cell wall changes in target

Nosocomial infections will affect the ability of hospitals to prevent deaths and will affect cures globally, with MRSA, *S. aureus* and VRSA being organisms of current concern in developing and developed countries (Zaidi et al., 2005). Major antibiotics used to treat

MRSA infections are presented in Table 2. The failure of an antibiotic to treat a resistant infection will result in the recurrence of the infection, which will need to be treated by second line drugs that are often more costly. The second line drug regimens are also more complicated in terms of dosing, side effects and need more medical attention. The patients who have resistant infections are likely to have a longer duration of illness and in some cases do not recover. These patients are also infectious for a longer duration and carry pathogens to others (Carmeli et al., 2002; Corea et al., 2003). According to Zaidi et al. 70% of hospital acquired neonatal infections could not be treated by WHO's first line drug regimen, due to the development of resistance (Zaidi et al., 2005).

Several measures that need to be taken to overcome antimicrobial resistance, such as early detection of resistance, prevention and control measures to curb the spread of infections, improved patient and healthcare education, awareness regarding the correct use of antibiotics, and developing new and novel antibiotics for effective drug therapy (Paphitou, 2013). However, over the past 20 years, the number of new drugs being introduced into the market has decreased by less than half the previous period (Ranghar et al., 2014), resulting in an increased need for antibiotics with new technology to improve their efficacy and safety, and to avoid resistance (Huh and Kwon, 2011).

Table 2 Major antibiotics for treatment of infections caused by MRSA. Adapted from J.G Bartlett (Bartlett, 2006).

	CLASS	**YEAR**	**ROUTE**	**INDICATION**	**DOSE (ADULT)**	**MAJOR ADVERSE EFFECTS**
Vancomycin	Glycopeptide	1956	IV	Pneumonia Bacteremia Bone/joint endocarditis	1g q12h	Red man syndrome
Quinupristin-dalfopristin	Streptogramin	1999	IV	Skin/soft tissue	7.5mg/kg q8-12h	Arthralgias/myalgias Injection site reactions
Linezolid	Oxazolidinone	2000	IV,PO	Pneumonia Skin/soft tissue	600mg q12h	Marrow suppression
Daptomycin	Cyclic lipopetide	2003	IV	Skin/soft tissue	4mg/kg QD	Myopathy

2.4 Approaches to overcome limitations with current antibiotic drugs

Statistics in the USA have shown that in 2002, more than 70% of bacteria that caused hospital acquired infections were resistant to at least one common antimicrobial (Zhang et al., 2010a). According to the British National Formulary, there are 63 antibiotics available to treat bacterial infections, of which half are structurally related and are directed to only a few biochemical targets in the bacterial cell (Taylor et al., 2002).

Pharmaceutical companies and researchers have explored various avenues in order develop new and novel drugs to combat the rise of resistance. For example, studies have shown that the frequency of infections in children has been significantly reduced by the *Haemophilus influenza B* vaccine (Peltola, 2000). However, treating certain infections by vaccination has proven to be more difficult and it is unlikely that their use will reduce the need to treat infections with antimicrobials (Coates et al., 2002). Other approaches that have gained interest are the discovery of naturally occurring antimicrobial peptides, as well as a new route for discovering natural products from soil (MacNeil et al., 2001). Another method of eradicating resistance is the synthesis of derivatives from existing antibiotics in the hope that some will be effective against resistant strains (Knowles, 1997). Pharmaceutical companies have adopted this short-term response by structurally altering existing molecules and testing them to see if they can overcome bacterial resistance (Bax et al., 2000). For example, antibiotics such as penicillin, cephalosporins or carbapenems are all chemically modified natural compounds (Hajipour et al., 2012). New targets for antimicrobial agents must be explored to avoid resistance, such as proteins, which are essential for bacteria to survive (McDevitt and Rosenberg, 2001). Although the modified compounds of existing antibiotics prolong the life span of each family of antibiotics for a number of decades, these resources will eventually run out. Novel compounds derived from bacteriophages, genomics, non-multiplying bacteria and non-culturable bacteria are also being explored as part of the current antibiotic therapy development initiatives (Coates and Hu, 2007).

Synthesising new antibiotics is therefore not an option due to the likelihood of resistance developing to these antibiotics. In order to control infections by VRE, VRSA, MRSA and other multi-drug resistant bacteria, the search for natural product derived antibiotics are therefore an option (Hemaiswarya et al., 2008), as are novel drug delivery systems that improve the delivery of existing antibiotics. Kim et al. also reported an approach using

selective photothermal therapy for in vivo antimicrobial treatment using a pulsed laser that causes physical damage to antimicrobial resistant strains (Kim et al., 2007). With progress made as a result of screening over 40 microbial genomes, as well as advances in screening technology and combinatorial synthesis, the future is set for the discovery of new antibiotics (Taylor et al., 2002). Future developments of antibiotics need to focus on inventing drugs with improved efficacy, that prevent resistance and protect the natural host microbiome (Brooks and Brooks, 2014). Future research and development needs to focus on smart cutting edge technology and innovative drug delivery systems that will improve the safety and efficacy as well as avoiding resistance of existing antibiotics (Hindi et al., 2009; Turos et al., 2007).

2.5 Nanotechnology and its emergence to overcome limitations with antibiotics

Nanotechnology has been referred to as the science and engineering that result in the design, synthesis, characterisation and application of nanometer scale materials and devices (Emerich and Thanos, 2003; Sahoo and Labhasetwar, 2003), and is regarded as the future of drug technology. Nanostructures are materials that have a size in the 1-100nm range, with the physical and chemical properties of these molecular scale structures being controlled through the design methodology (Safari and Zarnegar, 2014). Among the wide variety of nanosized drug delivery systems that are being explored are liposomes, polymeric nanoparticles, solid lipid nanoparticles, nanosuspensions, nanospheres, nanocapsules, nanotubes, nanowires, nanoemulsions, micellar systems and dendrimers (Kalhapure et al., 2014b; Karunaratne, 2007; Zhang et al., 2010a). These drug delivery systems have been explored to overcome a number of limitations in the diagnosis, treatment, prevention and immunization of a variety of diseases (Andrade et al., 2013). The unique physiochemical properties of nanomaterials, such as large surface area to mass ratio, small size, their high reactivity and their ability to be structurally and functionally be modified make them superior to traditional therapeutic and diagnostic agents (Zhang et al., 2007; Zhang et al., 2010a).

Nanotechnology can address many areas of the conventional drug delivery systems by improving water-soluble drug delivery, enable drug combinations and the transfer of large macromolecules to intracellular cites, target drug delivery, lower toxicity, provide more convenient routes of administration and sustained release, reduce health costs, and improve drugs therapeutic efficacy (Kalhapure et al., 2014b; Safari and Zarnegar, 2014). It has been widely explored with protein, peptides and nucleic acid drugs (Moghimi et al., 2001; Panyam and Labhasetwar, 2003) and to treat a variety of diseases, such as cancer, AIDS and hypertension (Gerson et al., 2014; Kalhapure and Akamanchi, 2012; Park, 2002). Many drug formulations cannot be taken orally because of their poor bioavailability, which can be addressed by nanotechnology due to their smaller particle size (El-Shabouri, 2002; Hu et al., 2004). Over the years, nanotechnology has shown to be effective in diseases such as Alzheimer's , diabetes, asthma, cancer, pain, allergy, and general infections (Brannon-Peppas and Blanchette, 2012; Kawasaki and Player, 2005), with more than two dozen therapeutic products have been approved for clinical use (Wagner et al., 2006).

Nanotechnology delivery systems have been explored as a promising alternative to current antibiotics in immunization, design and delivery of antibiotics, controlling cross infections and overcoming resistance (Brooks and Brooks, 2014; Zhu et al., 2014). Due to the continued emergence of bacterial resistance, nanotechnology is urgently needed in the field of antibiotics to combat this ongoing crisis (Blecher et al., 2011). When compared to other conditions, such as cardiovascular disease and cancer, nanodrug delivery systems for antibiotic therapy is still in the early stages (Huh and Kwon, 2011; Kalhapure et al., 2014b). These novel drug delivery systems allows for fast, accurate and cost effective treatment of infectious diseases, and offers a promising alternative to current antibiotic drugs (Jain, 2007; Taylor et al., 2002). The advantages of nanodrug delivery systems for antibiotic drug delivery include enhanced solubility and cellular internalisation, targeted delivery, decreased side effects, uniform tissue distribution, sustained release and increased patient compliance (Mansour et al., 2009; Sosnik et al., 2010). In addition, these nanosystems are able to overcome resistance mechanisms and create synergistic activity themselves (Zhang et al., 2010a).

The immense advantages of nanodrug delivery systems has caused an increased interest in this type of drug delivery, as is evident from the literature (Kalhapure et al., 2014b). Nanodrug delivery systems can therefore overcome limitations with many conventional antibiotics and can combat the global concern of bacterial resistance. During the next few years, nanotechnology will continue to grow and improve drug delivery, especially in the field of antibiotics.

2.6 Nano-drug delivery systems for antibiotic therapy

Nanomedicine has created an increase in the therapeutic efficacy of many drugs as well as in technological and medical breakthroughs (Couvreur, 2013). The use of nanotechnology in antibiotic therapy has proven to have many benefits, and the field continues to grow. There are several nano-delivery systems for antibiotics that include liposomes, solid lipid nanoparticles (SLNs), polymeric nanoparticles (PNPs), dendrimers, lipid polymer hybrid nanoparticles (LPNs), nanoemulsions, micellar systems, carbon nanotubes, nanosheets and nanorods (Kalhapure et al., 2014b).

2.6.1 Overview of nano-drug delivery systems and their advantages for antibiotic therapy

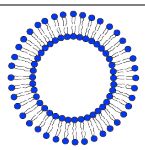
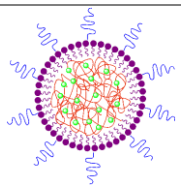
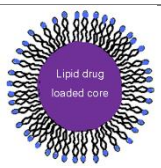
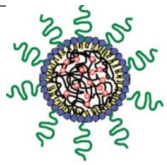
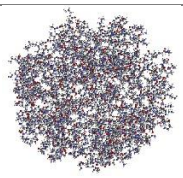
Nano-drug delivery systems are a promising alternative to conventional antibiotics, as their mechanisms of antibacterial activity are very different and they allow for targeted drug delivery and reduce bacterial resistance (Blecher et al., 2011; Seil and Webster, 2012). According to Zhu et al., there are at least 10 nano-based products on the market to diagnose infections, enable antibiotic drug delivery and medical devices (Zhu et al., 2014). A variety of antimicrobial agents can be incorporated into a number of different nanosystems. This includes lipophilic and water soluble antibiotics that exhibit improved solubility, a sustained release profile and targeted delivery when incorporated into a nanosystem (Abeylath and Turos, 2008; Allaker and Ren, 2008). The antimicrobial properties of nano-drug delivery systems can be attributed to their high surface to volume ratio that allows for drug penetration by attacking the bacterial cell wall, to their distinctive chemico-physical properties, their versatility of the formulation, and their biocompatibility with tissues and cells (Panyam and Labhasetwar, 2003; Weir et al., 2008). For example, Muhling et al. reported that bacteria that occur naturally did not develop bacterial resistance to metal nanoparticles (Mühling et al., 2009). The small size of these nanosystems also enable them to penetrate bacterial cells effectively and disrupt cell membranes, with a positive zeta potential allowing for electrostatic attraction of the negatively charged bacterial surfaces to the nanoparticles, enabling successful penetration (Seil and Webster, 2012).

The materials that make up a nanosystem and their size determine the effectiveness of the formulation, while the bactericidal and bacteriostatic effect of the system can predict the dose that is needed to inhibit bacterial growth (Seil and Webster, 2012). Nano-drug delivery

systems have also been proven to eradicate biofilms and intracellular microbes, these being the most common reason for chronic infections that cannot be treated with conventional antibiotic therapy (Zhu et al., 2014). The unique properties of antimicrobial nanoparticles allow them to attack a variety of biological pathways found in a range of bacteria that make the number of mutations necessary for them to overcome resistance very difficult. Nanoantibiotics can also be prepared and administered in cost effective ways through various routes with lower frequency of administration that make them stable enough for prolonged shelf life and long-term storage (Weir et al., 2008). They can also ensure protection from severe and harsh conditions, such as high heat sterilization, which would normally inactivate conventional antibiotics (Mansour et al., 2009; Sosnik et al., 2010). The mechanism of action of nanoantibiotics against bacterial cells is depicted in figure 2.3.

The most popular nano drug delivery systems that are being explored for antibiotic therapy include liposomes, polymeric nanoparticles, solid lipid nanoparticles, lipid-polymer nanoparticles, dendrimers, nanoemulsions, polymeric micelles, nanohybrids, carbon nanotubes, nanohorns and nanorods. These 10 main nanosystems for antibiotics are presented in Table 2.3. Extensive studies on these nanoantibiotic systems have shown enhanced activity against both sensitive and resistant bacterial strains. In addition, these nanosytems have shown enhanced solubility, drug entrapment, stability, targeted delivery, sustained drug release, penetration of the BBB and enhanced antibiotic therapy. Nanoparticles in particular are proving to be a superior drug delivery system in antibiotic therapy due to their exclusive physiochemical properties, such as controllable small size, large surface area to mass ratio, interactions with the bacteria and host cells, as well as its versatility in structure and function (Zhang et al., 2008; Zhang et al., 2010a).

Many challenges are associated with treating infections, and nanoparticles can assist in overcoming these limitations such as toxic side effects, decreased uptake and increased efflux of the drug, formation of biofilms and intracellular microbial infections. The targeted delivery of antibiotics to these infection sites, which creates increased efficacy and reduced side effects, can be attained by modifying the surface of the ligands or by microenvironment responsiveness (Huh and Kwon, 2011; Zhang et al., 2010a; Zhu et al., 2014). Nanoantibiotics are therefore a promising drug delivery system, and research indicates that the number of commercially available nano-therapeutics has significantly increased and will continue to rise, especially in the emerging field of nanoantibiotics.

TYPE OF NANOSYSTEM	STRUCTURE	ADVANTAGES IN ANTIBIOTIC THERAPY	REFERENCES
Liposomes		- They promote targeted delivery - Reduce toxicity - Improve pharmacokinetics - Enhance antibiotic activity - Effective against a wide range of microorganisms - Sustained release	(http://www.chemguideforcie.co.uk/section113/learningb; Kalhapure et al., 2014b; Schiffelers et al., 2001)
Polymeric nanoparticles		- Structural stability - Sharper size distribution of particles - Tuneable size, surface charge and drug release - Overcomes resistance - Modification of functional groups	(https://labofnano.gmu.edu/research/; Zhang et al., 2010a)
Solid lipid nanoparticles		- Enhanced Stability - High entrapment - Protection of drugs against degradation - Ease of scale up - Sustained release - Prolonged antibacterial activity	(Fadwa Odeh, 2014; Jain and Banerjee, 2008; MuÈller et al., 2000)
Lipid-polymer nanoparticles		- Improved stability - Enhanced encapsulation - Targeted delivery - Sustained release - Prolonged antibacterial activity - Tuneable size and surface charge	(Hadinoto et al., 2013; Mandal et al., 2013; Wang et al., 2012a)
Dendrimers		- Targeted intracellular delivery - Tuneable inner cavities - Enhanced solubility - Sustained drug release - Increased antimicrobial activity - Biocompatibility	(Agarwal et al., 2008; Cagin et al., 2000; Felczak et al., 2013; Kalhapure et al., 2014b)

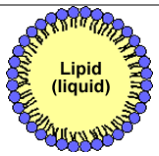
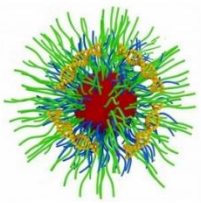
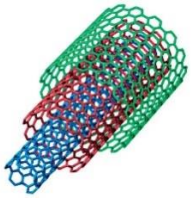
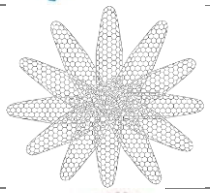
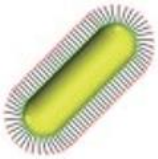
Nanoemulsions		- Biodegradability - Biocompatibility - Ease of preparation - Enhanced stability - Enhanced bactericidal activity - Sustained release	(http://soft-matter.seas.harvard.edu/index.php/Emulsions; Kalhapure et al., 2014b; Santos-Magalhães et al., 2000)
Polymeric micelles		- High kinetic and thermodynamic stability - Sustained release - Absorption promoter - Effective inhibition of bacterial growth	(Liu et al., 2013; Torchilin, 2001; Yuan et al., 2012)
Carbon nanotubes		- Good antimicrobial activity - Good chemical stability	(http://www.compositesworld.com/articles/the-key-to-cnts-functionalization; Kang et al., 2007)
Nanohorns		- Controlled release - Improved dispersability of carrier system	(Guldi, 2007; Kalhapure et al., 2014b; Xu et al., 2008)
Nanorods		- Sustained release - Antimicrobial activity against a variety of bacteria - Increased surface area - Suitable hardness	(http://www.spectroscopynow.com/details/ezine/sepspec26509ezine/Gold-nanorods-Non-toxic-coating-aids-anticancer-agents; Joshy et al., 2011; Kalhapure et al., 2014b)

Table 3. The 10 main nano-drug delivery systems explored for antibiotic therapy.

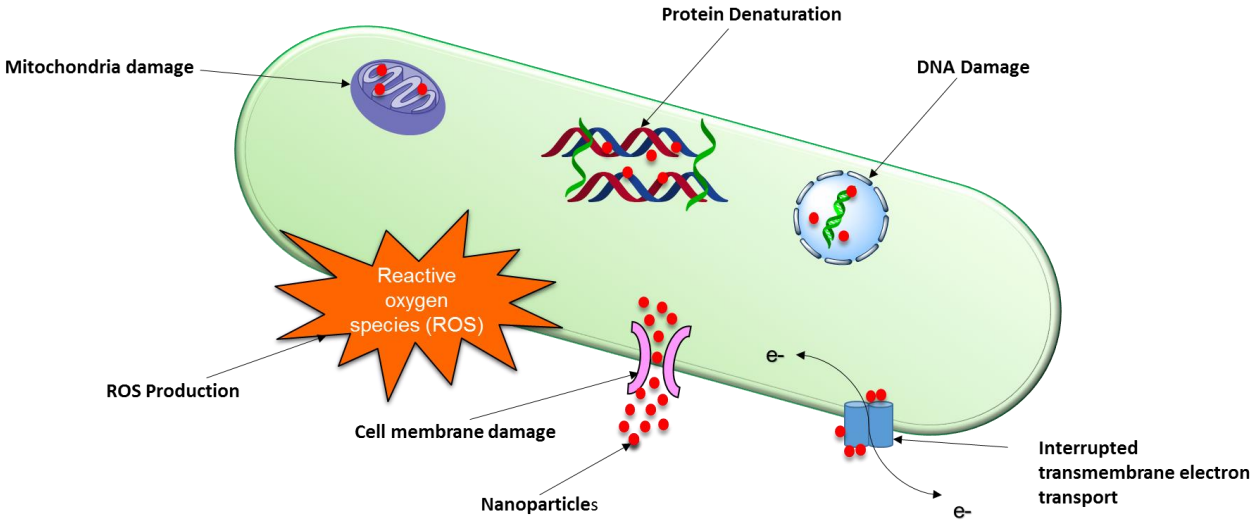


Figure 3. Mechanism of action of nanoantibiotics against bacterial cells. Adapted from Huh and Kwon (Huh and Kwon, 2011).

2.6.3 Disadvantages of nano-drug delivery systems used in antibiotic therapy

While nano-drug delivery systems are potentially life-changing, there are concerns over some of the limitations of nanocarriers, such as their size, charge of particles, purity of the formulation, solubility of substances, in vitro and in vivo stability, antigenicity and biocompatibility issues. These formulation issues can cause an increase in the costs of synthesizing the systems and of manufacturing (Blecher et al., 2011). Although the harm of nanosystems to humans is questionable, there is a concern over their toxicity to human tissues, due to their capability to infiltrate vital organs as a result of the inclusion of specific materials, such as heavy metals, which can be harmful (Kim et al., 2010).

The use of nanoantibiotics for clinical purposes has some challenges that need to be addressed before they can be approved, including the interactions of the drug delivery system with cells, organs and tissues of the body, which will determine the route of administration that can deliver the desired therapeutic effect (Sandhiya et al., 2009; Suri et al., 2007). For example, the literature has shown that nanoparticles given intravenously can collect in the colon, bone marrow, spleen, lungs and lymphatics (Hagens et al., 2007), while inhaled nanoparticles can reach the systemic circulation and spread to such organs including the brain (El-Ansary and Al-Daihan, 2008; Poma and Di Giorgio, 2008). This is facilitated by the small size of the nanoparticles that enable it to be efficiently taken up by the cells, which allows transocytosis into the blood and lymphatic circulation via the endothelial and epithelial cells (Rabea et al., 2003).

Hu et al. reported that the toxicity of antimicrobial nanosystems on the central nervous system to be inconclusive, while other non-antibiotic nanomaterials have shown toxicity, which means that it can therefore not be ruled out (Hu and Gao, 2010). It has been reported that nanosystems have toxic effects on the circulatory system by causing fluctuation in the heart rate (Chalupa et al., 2004) and on the reproductive system resulting in spermatotoxicity, and a rise in the detachment of the seminiferous epithelium (El-Ansary and Al-Daihan, 2008; Yoshida et al., 2010). Emerging technologies, together with toxicogenomics, could rectify some of the limitations associated with nanomaterials by revealing the mechanisms of toxicity (Poma and Di Giorgio, 2008). The advantages and disadvantages of antimicrobial nanosystems over free antibiotics is explained further in Table 4. Although the literature suggests that more toxicity studies on nanosystems need to be conducted, the potential

benefits of this emerging technology far outweighs its disadvantages, and should not hinder the discovery of these new types of nano-drug delivery systems.

Antimicrobial nanosytems		**Free antibiotics**	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Targeted Delivery	Accumulation of nanomaterials in tissues and organs	Absence of nanomaterials in the whole body	High side effects
Lower side effects	High systemic exposure to drugs administered locally	Absence of nanotoxixity	High antimicrobial resistance
Low antimicrobial resistance	Nanotoxicity	Well established characterisation techniques	Short half-life
Increase in half-life of drug	Lack of characterisation techniques	Low systemic exposure to drugs administered locally	Usual pharmacokinetics of free drugs
Controlled drug release			Narrow therapeutic index
Increased solubility			Poor solubility of some drugs
Wide therapeutic index			Fast elimination
Improved solubility			
Low immunosuppression			
Low cost			

Table 4 Advantages and disadvantages of antimicrobial nanosystems over free antibiotics. Adapted from Huh and Kwon (Huh and Kwon, 2011)

2.6.4 Types of nano-drug delivery systems for antibiotic therapy

As mentioned above, several main nano-drug delivery systems have been explored for antibiotic therapy and include liposomes, solid lipid nanoparticles, polymeric nanoparticles, lipid polymer nanoparticles, nanoemulsions, dendrimers, micellar systems, nanorods, carbon nanotubes and nanohorns. However, for the purpose of this study, lipid and polymer based nano-drug delivery systems are described, as they constitute the main components of the lipid-polymer hybrid nanoparticle that was synthesized and characterised in this study.

2.6.4.1 Solid Lipid Nanoparticles (SLNs)

Solid lipid nanoparticles (SLNs) were first discovered in the early 90's and are described as colloidal carriers that range in size between 50 to 1000nm (MuÈller et al., 2000). They are more advantageous than other colloidal carriers such as emulsions, liposomes and polymeric nanoparticles due to their small size, large surface area, higher drug loading capacity and better phase interaction at the interface (Li et al., 2009; Üner and Yener, 2007). SLNs have unique properties that enable them to have good biocompatibility, greater body or tissue tolerance, increased bioavailability, encapsulation of both hydrophobic and hydrophilic drugs, be administered via various routes and be manufactured on a large scale (Mehnert and Mäder, 2001; Panyam and Labhasetwar, 2003). In addition, the use of biodegradable materials in the synthesis of SLNs allows for controlled release of formulations at the site of action, which in turn reduces the frequency of administration (Vasir et al., 2003).

Other colloidal nanoparticles have several negative attributes, such as the cost of expensive polymers and phospholipids in the production of polymeric nanoparticles and liposomes, leakage of water soluble drugs, and poor storage and stability (Brewer et al., 2011; Soppimath et al., 2001). SLNs are different from liposomes as they do not possess a bilayer structure and are amorphous in nature. The particles consist of a solid lipid core that is made stable by the addition of surfactants (Mehnert and Mäder, 2001). According to the literature, antibiotics that have been incorporated into SLNs include Tilmicosin (Wang et al., 2012b), Gatifloxacin (Kalam et al., 2010), Amikacin (Ghaffari et al., 2011), Nisin (Prombutara et al., 2012), Vancomycin (Kalhapure et al., 2014a), Enrofloxacin (Xie et al., 2011), Tobramycin (Cavalli et al., 2000) and Norfloxacin (Wang et al., 2012d).

SLNs have been formulated for many routes of administration, such as parenteral, topical, oral and pulmonary routes (Bargoni et al., 2001; Videira et al., 2002). SLNs adhere to the surface of the skin and form a hydrophobic film that increases the contact time of the drug with the skin, which in turn allows for greater absorption (Müller et al., 2008; Souto et al., 2004). Jain et al. reported that the release of ciprofloxacin was controlled via local delivery for both ocular and skin infections (Jain and Banerjee, 2008). Cavalli et al. reported that the pharmacokinetics of tobramycin loaded SLNs were improved in several ways, including by intravenous administration, during which low amounts were taken up by the kidneys and a high lung concentration was noted (Cavalli et al., 2000). Studies have shown that compared to other drug classes, there have been much fewer antibiotic drug loaded SLNs (Kalhapure et al., 2014a). Inhalable SLNs are more stable, can encapsulate a high quantity of drug, and reduce the risk of absorbing residual organic solvents (MuÈller et al., 2000). Many SLN formulations can be given via various administration routes, such as parenteral, topical, oral, ocular and pulmonary (Bargoni et al., 2001; Videira et al., 2002).

The two most common methods used to prepare SLNs are high pressure homogenisation and the micro-emulsion technique (Kalhapure et al., 2014b). However, many less popular methods are used in industry, such as ultrasound and solvent based techniques, as they are more cost effective (Silva et al., 2011). Several excipients have been studied in SLN formation and include lipids such as stearic acid (Cavalli et al., 1999), Compritol 888 ATO (Schwarz and Mehnert, 1997), palmitic acid (Stancampiano et al., 2006) and glyceryl monostearate (Luo et al., 2006). Surfactants that have been studied in the formulation of SLNs include poloxamer 188, 182, 407, 908 (Göppert and Müller, 2005; Müller et al., 1996), Tween 20, 80 (Zhang et al., 2010b) and Solutol HS15 (Vighi et al., 2007). Despite its success as a drug delivery system, SLNs have limited use in antibiotic therapy due to the nature of the hydrophobic lipids used, which poorly entrap the hydrophilic antibiotics (Kalhapure et al., 2014b). Xie et al. have found that ion pairing the SLN with a fatty acid can improve the encapsulation efficiency of enrofloxacin (Xie et al., 2011), with a similar study being conducted by Kalhapure et al. who reported that the encapsulation and antibacterial activities of vancomycin loaded SLNs were increased by incorporating linoleic acid as an ion pairing agent (Kalhapure et al., 2014a).

2.6.4.2 Polymeric Nanoparticles (PNPs)

Polymeric nanoparticles range in size from 10 to 1000nm, and consist of biodegradable polymers and co-polymers (Kuo and Chen, 2006). They are superior to liposomes as they are able to improve the drug loading and stability of the nanoparticle (Abed and Couvreur, 2014). The nanoparticles have a core-shell structure that consists of a dense polymer matrix for drug entrapment, and a shell comprising of a hydrophilic polymer, such as PEG or PVP, that offers steric stability and stealth properties to the nanoparticle, which makes them good candidates for drug delivery applications (Costantino and Boraschi, 2012; Discher and Eisenberg, 2002). Drugs can be entrapped either within the particles, adsorbed on the surface, or chemically linked on the surface of the particle (Parveen et al., 2012; Zensi et al., 2009). Different types of polymers are used to synthesise nanoparticles, and include natural polymers such as albumin, gelatin, chitosan, alginate and haemoglobin (Kim et al., 2014), as well as synthetic polymers such as polyamides, poly(alkyl-cyanoacrylates), poly(amino acids), poly(ortho esters) and poly(esters) (Jain, 2000). Poly lactic co-glycolic acid (PLGA) is a popular polymer used to synthesise nanoparticles, and is widely used as it has the ability to breakdown the molecules that are normally removed from the body via normal metabolic pathways (Lü et al., 2009).

Their advantages include biocompatibility, biodegradability, high drug payload, zero-order pharmacokinetic profile and a steady drug level at the site of delivery (Hughes, 2005). Polymeric nanoparticles have been explored for a variety of diseases, such as cancer (Verderio et al., 2014), diabetes (Vijayan et al., 2013), HIV and AIDS (Zhang et al., 2011),and most importantly, it seems to be the most widely studied nano drug delivery system for antibiotic drug delivery. There are two significant polymeric nanoparticles that have been studied to deliver antibiotics, namely linear polymers and ampiphilic block co-polymers (Huh and Kwon, 2011). PNPs have been widely used to deliver a variety of antibiotics and to treat various infectious diseases. For example, gentamycin entrapped in PLA/PLGA nanoparticles has shown good antibacterial activity against the Brucella infection (Prior et al., 2000). In addition, penicillin was entrapped in polyacrylate nanoparticles and was able to retain its full antibacterial activity against MRSA (Abeylath and Turos, 2008).

PLGA nanoparticles have also been successfully synthesized to deliver ciprofloxacin (Dillen et al., 2004), azithromycin and rifampicin, and has enhanced the delivery of these drugs (Toti et al., 2011).

The delivery of antibiotics via PNPs has many advantages, such as stability in biological fluids and harsh conditions of preparation, tuneable size, zeta potential and drug release, and adaptable surface functionalization for the conjugation of drugs (Abeylath and Turos, 2008; Santos-Magalhães and Mosqueira, 2010; Zhang et al., 2010a). However, their major drawback of poor encapsulation efficiencies, especially with water soluble drugs (Kalhapure et al., 2014b), as well as their formulation, drug loading, scale up, and toxicology issues, need to be resolved (Abed and Couvreur, 2014). The methods of preparation of the PNPs include polymerization of the monomers and dispersion of the polymers (Soppimath et al., 2001).

The field of PNPs has endless opportunities, and there is a need for novel polymers that are biocompatible and biodegradable, as the natural and synthetic polymers have already been researched extensively in this field. In addition, *in vivo* studies for newly developed PNPs needs to be undertaken (Kalhapure et al., 2014b).

2.6.4.3 Liposomes

Liposomes are vesicles ranging from the nano to micro size, and comprise of a phospholipid bilayer with an aqueous core (Huh and Kwon, 2011). Liposomes were first sought out as drug delivery systems due to their vesicular structure and the presence of the lipid bilayers that are able to interact with living cells via endocytosis, adsorption, fusion and lipid exchange (Gregoriadis, 2006; Vemuri and Rhodes, 1995). Liposomes can be categorised into 3 classes based on the number of lamella : small unilamellar vesicles (SUVs) or oligolamellar (OLVs); large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs) (Pinto-Alphandary et al., 2000). The drug can be incorporated either in the aqueous spaces if it is a water soluble drug, or the lipid membrane if the drug is lipid soluble (Pinto-Alphandary et al., 2000). Liposomes have been studied as a vehicle of drug delivery for enzymes, proteins and drugs, and are used in treating a variety of diseases (Torchilin, 2005). For example, liposomal formulations containing the anticancer drug doxorubicin and antifungal amphotericin B are available on the market (Allen and Martin, 2004; Bakker-Woudenberg et al., 1995).

The most commonly used lipid in the preparation of liposomes is phosphatidylcholine, which contains fatty acyl chains (Du Plessis et al., 1996), while the methods of preparation include thin film hydration (Bangham, 1978), reversed phase evaporation (Szoka and Papahadjopoulos, 1978), solvent injection methods (Stano et al., 2004) and detergent analysis (Zumbuehl and Weder, 1981). Liposomes are most widely used to treat bacterial infections,

as their bilayer structure allows them to readily fuse with the infectious bacteria (Zhang et al., 2010a). They are able to incorporate both hydrophilic and hydrophobic drugs either in the lipid shell or the aqueous core (Lasic, 1998; Sosnik et al., 2010), and appear to be one of the first drug delivery systems explored for improving antibiotic drug delivery (Kalhapure et al., 2014b).

The advantages of liposomes as drug delivery systems include decreased toxicity, improved pharmacokinetics and bio-distribution, targeted selectivity, higher activity against intracellular pathogens and enhanced activity against extracellular pathogens in particular in overcoming drug resistance in bacteria (Pinto-Alphandary et al., 2000). However, their disadvantages include short-term stability, drug leakage, low encapsulation, high cost, scale up issues and sterility (Pinto-Alphandary et al., 2000). The use of liposomes as drug delivery systems has been studied, and has proven to significantly extend the half-life of the drug amikacin as well as alter the distribution of the drug in tissues (Gangadharam et al., 1991). It has also been successful in treating Mycobacterium avium infected mice by liposomal streptomycin (Fielding et al., 1998), prolonged blood circulation and improved localisation at the infection site of liposomal gentamicin and ceftazidime (Bakker-Woudenberg et al., 1995), and increased antibacterial activity against MRSA via vancomycin and teicoplanin encapsulated liposomes (Onyeji et al., 1994).

Liposome research has already advanced to such a level that it is now possible to modify the surface of a liposome and attach other agents or nanoparticles to obtain targeted delivery (Kalhapure et al., 2014b). However, over the last few years, the use of liposomes as a drug delivery system has declined as there is already an extensive amount of literature on the synthesis and the application of liposomes, as well as about some of its disadvantages that are now being overcome by novel nano-drug delivery systems.

2.6.5 Lipid-Polymer Hybrid nanoparticle (LPNs)

The following section describes LPNs and their advantages and disadvantages in various nano-drug systems and more specifically their use as drug delivery systems for antibiotics.

2.6.5.1 Lipid polymer hybrid nanoparticles for nano-drug systems

Lipid-based nanocarriers, such as solid lipid nanoparticles, are an attractive alternative dosage form due to their submicron sized particles and solid state of physiological lipid carriers. Many hydrophilic and hydrophobic drugs have been incorporated into SLNs, such as nifedipine, diazepam, doxorubicin, paclitaxel, tobramycin and timolol, to name a few, and their administration via different routes has been investigated. However, their drawbacks namely high initial burst kinetics, low drug loading capacity and drug leakage during storage, need serious attention (Cavalli et al., 2002; Li et al., 2006).

There is a need for new and novel nanocarrier systems that can enhance the effect of drugs, with increasing benefits being made to merge the benefits of the two most predominant nanocarriers, these being liposomes and polymeric nanoparticles (Cheow and Hadinoto, 2011). Both these classes have advantages and limitations in terms of their biological and physiochemical properties (Mandal et al., 2013). Liposomes, which are biocompatible, biodegradable, non-toxic or mildly toxic and flexible (Gregoriadis, 1995), are suitable as drug delivery vehicles due to their ability to closely interact with host cells, and delivering both water and oil soluble drugs (Pinto-Alphandary et al., 2000). However, although they are highly biocompatible, they lack structural integrity, and have several limitations in terms of physical and chemical stability, batch-to-batch reproducibility, sterilisation and drug entrapment (Sharma and Sharma, 1997). In the case of polymeric nanoparticles, the rigidity of the polymer matrix makes them more stable than liposomes (Pinto-Alphandary et al., 2000), and they are advantageous in terms of their tissue penetrating ability, small particle size, variety in preparation methods, greater stability in biologic fluids, and versatile drug loading and release profiles (Panyam and Labhasetwar, 2003; Pinto Reis et al., 2006). The disadvantages are that they poorly encapsulate water soluble drugs due to their leakage of the drug from the nanoparticles during the emulsification process in preparation (Cheow and Hadinoto, 2010), as well as their polymer cytotoxicity, polymer degradation and scale up issues (Pinto Reis et al., 2006).

Therefore, lipid-polymer hybrid nanoparticles have been introduced to overcome some of the limitations associated with liposomes and polymeric nanoparticles. Hybrid nanoparticles consist of: (i) a biodegradable polymeric core, which is suitable for carrying poorly water-soluble drugs and releasing them at a controlled rate; (ii) a hydrophilic shell that allows particles to evade recognition by the immune system and increases the half-life of the particles; and (iii) a lipid monolayer that prevents the carried agents from freely diffusing out of the nanoparticles and reducing the water penetration rate into the nanoparticles, which slows drug release from the nanoparticles (Zhang et al., 2008). The structure of the LPN is depicted in Figure 4.

The use of lipid-polymer hybrid nanoparticles has been widely explored in treating cancer, as they are able to deliver multiple drugs at the same time from a single platform (Mandal et al., 2013). They have also been prepared for targeted delivery of antibiotics to the bacterial biofilm-infested lungs of patients suffering from chronic lung infections (Pauwels and Rabe, 2004). Hybrid nanoparticles have demonstrated tuneable size and surface charge, high drug loading yield, sustained drug release profile, good stability in the serum and cellular targeting ability, and their easy synthesis method make them favourable for further scale up. These advantages make lipid-polymer hybrid nanoparticles a promising drug delivery platform for further investigation. (Zhang et al., 2008).

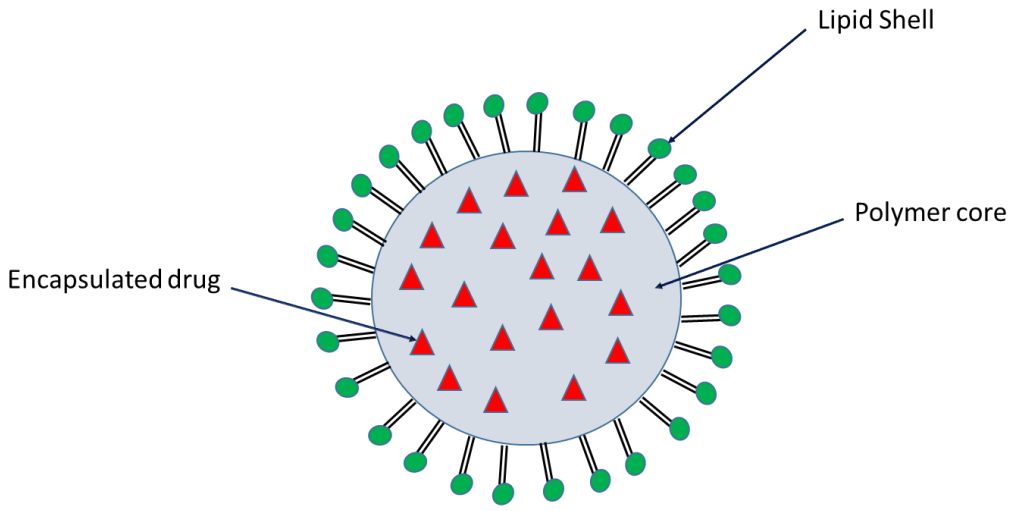


Figure 4. Structure of LPN comprising of the lipid shell and polymer core which show characteristics of both liposomes and polymeric nanoparticles. Adapted from Hadinoto et al. (Hadinoto et al., 2013)

2.6.5.2 Lipid polymer hybrid nanoparticles for nano-antibiotics

Nano drug delivery systems are being widely explored to overcome the challenges with existing antibiotics to treat bacterial infections (Hadinoto et al., 2013). Lipid-Polymer Nanoparticles (LPNs) display distinctive advantages of both liposomes and polymeric nanoparticles, while excluding some of their limitations. This is a hybrid particulate system as it has the structural integrity of the polymeric particles and the biomimetic properties of the liposome (Hadinoto et al., 2013).

The field of lipid polymer hybrid antibiotic based nanoparticles is in its infancy compared to cancer, cardiovascular and other diseases. To the best of our knowledge, despite numerous advantages offered by LPNs, their utilization in the delivery of antibiotics is very limited, with only five papers reported in the literature so far. The delivery of three fluoroqinolone antibiotics (levofloxacin, ofloxacin, ciprofloxacin), as well as calcein (Cheow et al., 2011; Cheow and Hadinoto, 2011, 2012; Wang et al., 2012c) and clindamycin phosphate (Abbaspour et al., 2013) has been studied using LPNs (Mandal et al., 2013). Furthermore, the polymer that has been explored the most for antibiotic loaded LPN synthesis is Poly Lactic-co-Glycolic Acid (PLGA) (Cheow et al., 2011; Cheow and Hadinoto, 2011, 2012; Wang et al., 2012c), with sodium alginate (ALG) and dextran sulphate being studied in one paper (Abbaspour et al., 2013), while the lipids that have been investigated include stearic acid, lecithin and phospahtidylcholine (PC) (Abbaspour et al., 2013; Cheow et al., 2011; Cheow and Hadinoto, 2010, 2012; Wang et al., 2012c). The studies done with antibiotic loaded LPNs show that there is need for formulation optimisation and characterization of LPNs by exploring other polymers and lipids with other potent antibiotics to achieve high drug entrapment, enhanced antibacterial activity against sensitive and resistant strains, controlled release and improved stability. Resistance to antibiotics warrants the need for developing new and novel antibiotics, hence the formulation of nanoparticles as a drug delivery system, in particularl, lipid-polymer nanoparticles promises to be an exciting and advantageous alternative to the conventional antibiotics. Table 5 summarises all the antibiotic loaded LPNs that have been studied to date.

ANTIBIOTICS	NATURE	EXCIPIENT	MAIN FINDINGS	CHARACTERISATION STUDIES	REF
Levofloxacin **Ciprofloxacin** **Ofloxacin** Tobramycin	Hydrophobic Hydrophilic Hydrophobic Hydrophilic	Poly (lactic-co-glycolic acid) (PLGA) Phosphatidylcholine (PC)	• Ionicity of the drug and lipid is important with regards to nanoparticle preparation • Drug lipophilicity and aqueous solubility affects drug loading and drug release, more lipohillic drug has higher drug loading and sustained release profile • Hybrid nanoparticles are larger in size, zeta potential, encapsulation and drug loading compared to its non-hybrid counterpart. • Hybrid nanoparticles are unstable in salt solution so TPGS stabiliser is incorporated into the formulation • Sizes between 120nm and 420nm with the highest encapsulation of 25% with Ofloxacin.	• Particle Size • Zeta Potential • Entrapment Efficiency • Drug Loading • In-vitro drug release • Scanning Electron Microscopy (SEM)	(Cheow and Hadinoto, 2011)
Levofloxcin	Hydrophobic	PLGA and Lecithin	• Hybrid nanoparticles exhibited a size of ≈420 ± 30 nm with zeta potential in the range of (–) 25–30 mV, encapsulation efficiency of ≈19% and drug loading of ≈2.0% (w/w). • Spray drying produced dimpled hollow spherical nano-aggregates whereas spray freeze drying produced large spherical porous nano-aggregates • PVA is better than manitol in facilitating nano-aggregate reconstitution • Nano-aggregates produced by SFD is superior to those produced by SD.	• Particle Size and Distribution • Zeta Potential • Entrapment Efficiency • Drug Loading • Powder characterisations	(Wang et al., 2012c)
Levofloxacin **Ciprofloxacin** **Ofloxacin** **Calcein**	Hydrophobic Hydrophilic Hydrophobic Hydrophilic	PLGA, rhamnolipid and PC	• Particle size ranged from 280nm - 400nm with a zeta potential range of (-) 30mV – (+) 10mV and a drug loading of 0.5 – 2.3 (% w/w). • Encapsulation ranged from 5% to 55% depending on the BCS (biopharmaceutical classification system) of the drug. • A rhamnolipid triggered release is observed with calcein however not with BCS class I drugs due to their high lipid membrane permeability. • The rhamnolipid triggered release capability of hybrid nanoparticles will enable targeted drug release in the vicinity of biofilm colonies therefore improved antibacterial efficacy is expected which will be studies further.	• Particle Size • Zeta Potential • Entrapment Efficiency • In-vitro drug Release • SEM	(Cheow and Hadinoto, 2012)

Levofloxacin	Hydrophobic	PLGA and PC	• Particle size of hybrid nanoparticles ranged from 240nm to 420nm with a zeta potential of ≈ 26mV, encapsulation efficiency ranging from 19% - 21% and drug loading of 2.3 – 2.4 (%w/w). • Hybrid nanoparticles exhibit a higher antibacterial efficacy against *P.aeruginosa* biofilm cells, however not against planktonic cells. • Possibly the presence of lipid may have enhanced the antibiotic diffusion into the biofilm matrix resulting in more effective biofilm cell eradication. Other possibilities relating to the hybrid nanoparticles have been ruled out.	• Particle Size and Zeta Potential • Entrapment Efficiency • Drug loading • In vitro release studies • SEM • Biofilm susceptibility testing	(Cheow et al., 2011)
Clindamycin phosphate	Hydrophilic	Dextran sulphate, sodium alginate and stearic acid	• Particles ranged from 400nm – 900nm. • Particle size was not affected by polymer type or the amount of drug, polymer and surfactant. • Polymer dextran sulphate had higher degree loading and drug release than sodium alginate.	• Particle size • Entrapment Efficiency • Drug loading • In vitro drug release studies • SEM	(Abbaspour et al., 2013)

Table 5. Summary of studies done on antibiotic loaded LPNs

2.6.5.3 Preparation of Lipid polymer hybrid nanoparticles

The two most commonly used methods for preparation of LPNs are the two step method and the single-step method.

Two step method

This method involves preparing the core and lipid shell separately, which are then combined (Zhang and Zhang, 2010). This approach involves formation of the polymer core by emulsification (Sengupta et al., 2005), high pressure homogenisation (De Miguel et al., 2000), or nanoprecipitation (Zhang et al., 2008). Thereafter, the lipid vesicles are prepared by sonication or extrusion method (Zhang and Granick, 2006). The polymeric nanoparticles and the cationic lipid vesicles are combined and drawn together via electrostatic interactions (Troutier et al., 2005b). Several methods can be used to combine the lipid vesicles with the polymeric core, such as simple vortexing, needle extrusion or high pressure homogenisation (Zhang and Zhang, 2010). There are various factors that can affect the final size of an LPN, such as the method used to prepare the lipid vesicles, the method of combining the lipid vesicle and the PNP, the surface charge of the lipid vesicles, the strength and pH of the buffers used, the temperature and incubation period, as well as the vesicle to particle ratio (Troutier et al., 2005a; Troutier and Ladavière, 2007). By using this method, LPNs with the desired size, drug loading and release characteristics can be prepared, as it allows for the these variables to be controlled (Sengupta et al., 2005; Troutier et al., 2005a).

There have been various reports in the literature about this two-step method (Hasan et al., 2011; Sengupta et al., 2005; Willem, 2012). However, there are several limitations associated with the use of this method such as the low encapsulation efficiency of the drug in the incubation step, as the molecules of the drug may leak from the core before being coated by the lipid layer (Cheow and Hadinoto, 2011). In addition, the complexity of the technical processes involved and the process of preparing the polymeric core and lipid vesicles separately are challenges that need to be overcome (Mandal et al., 2012).

Single-step method

To overcome the problems associated with the two-step method, a simple single step approach has been developed that combines the dual steps that are associated with the two-step method (Hadinoto et al., 2013). The one-step method does not require the lipid vesicles and polymer core to be synthesized separately. The LPNS are synthesized by self-assembly

after mixing the lipid and polymer solutions. The most critical factor involved in preparing the LPNs is the amount of lipid that is required to successfully coat the polymer core (Mandal et al., 2012). Self-assembly of these hybrid nanoparticles is achieved by nanoprecipitation or emulsification-solvent-evaporation method (Mandal et al., 2012).

Emulsification-solvent-evaporation (ESE) method

The two approaches of the ESE method are the single and double emulsification methods. The single emulsification method is used when the drug to be encapsulated is soluble in a solvent that is water-immiscible. This process involves adding the oil phase, which contains the polymer and drug, to the aqueous phase containing the lipid under stirring or ultrasonication to form an oil in water emulsion (o/w). The lipid can alternatively be added to the oil phase. The oil phase is then evaporated and self-assembly of the lipid around the polymer core occurs forming an LPN (Bershteyn et al., 2008; Cheow and Hadinoto, 2011; Hadinoto et al., 2013). For example, this method of preparation has been used in the literature for preparing flouroquinolone antibiotics (Cheow and Hadinoto, 2011), paclitaxel (Liu et al., 2010), doxorubicin (Chu et al., 2011) and DNA containing LPNs (Li et al., 2010). In contrast, the double ESE method can be used when the drug to be encapsulated cannot be dissolved together with the polymer in any organic solvent. The drug is therefore dissolved in the aqueous phase and thereafter emulsified with the oil phase. The oil phase will contain the polymer and the lipid. This emulsion is further emulsified for the second time with the aqueous phase (w/o/w) and after evaporation of the oil phase the LPNs are formed (Cheow and Hadinoto, 2011). The double ESE method has been reported for DNA (Zhong et al., 2010), siRNA (Shi et al., 2011) and some flouroquinolone antibiotics (Cheow and Hadinoto, 2011).

It should be noted that the majority of studies have used the single ESE method to prepare LPNS, with the double ESE method only being introduced recently. To prepare LPNs by the ESE method, certain factors that need to be considered, such as the lipid to polymer ratio and the drug, polymer and lipid interactions with each other, which determines the amount of drug encapsulated in the LPNs (Hadinoto et al., 2013).

Nanoprecipitation method

This method involves dissolving the polymer and hydrophobic drug in a water miscible organic solvent, such as acetone or acetonitrile, and the resultant solution being added

dropwise to the aqueous phase containing the lipid. The mixture is vortexed, homogenised and then sonicated to produce suitable nanoparticles (Hadinoto et al., 2013). The factors that need to be optimised to formulate LPNs via this method are particle size, zeta potential, PDI, lipid to polymer ratio and viscosity of the polymer (Maurer et al., 2001; Prabaharan et al., 2009; Wang et al., 2010). This method of preparation of LPNs has been widely used to encapsulate substances such as docetaxel (Zhang et al., 2008), paclitaxel (Chan et al., 2010) and DNA (Yang et al., 2012) to name a few. However, it has been noted that the ESE method is more popular and preferred over the nanoprecipitation method, as it creates nanoparticles with higher encapsulation efficiency (Hadinoto et al., 2013). Therefore, although the nanoprecipitation method has proven to be effective and capable of large scale manufacture, there are still limitations, such as low encapsulation due to leakage of the drug in the aqueous phase (Cheow and Hadinoto, 2011).

2.6.5.4 Characterisation of Lipid polymer hybrid nanoparticles

The main methods reported so far to characterize the LPNs, in terms of their physiochemical properties, have included size, zeta potential and morphology. Particle size is of significance to assess the systemic circulation of the nanoparticles as well as their capability to accumulate at sites of infection (Zhang and Zhang, 2010). Dynamic light scattering (DLS) is a fast and uncomplicated method to determine the size and distribution of nanoparticles. The zeta potential of the nanoparticle is a measure of the electrokinetic potential between the surface of the particle and the bulk solution (Alexis et al., 2008). The zeta potential will determine both the *in vitro* and *in vivo* stability of the nanoparticles, and can also be measured using DLS. The morphology of the nanoparticles can be determined either by Scanning electron microscopy (SEM) or Transmission electron microscopy (TEM), and is used to measure the physical dimensions and structure of the particle (Zhang and Zhang, 2010). The above methods have been reported in the literature, specifically with LPNs (Cheow and Hadinoto, 2010, 2011, 2012; Wong et al., 2006).

To determine the amount of drug encapsulated in the LPN, as well as drug loading, the drug concentration is measured using a UV sperctrophotometer or alternatively, can be measured by using High pressure liquid chromatography (HPLC). Drug release from the LPN is performed using the dialysis method, with samples being collected at a series of time intervals and measured using HPLC or UV method. Details of these methods used can be found in the literature (Cheow and Hadinoto, 2011; Venkateswarlu and Manjunath, 2004; Wong et al., 2006). In order to corroborate the results obtained for drug release and encapsulation efficiency, analysis of drug release kinetics and mechanism, as well as molecular modelling, can be performed, which have not been reported before for antibiotic loaded LPNs.

With LPNs loaded with antibiotics, the *in vitro* antibacterial activity can be measured. To determine the antibacterial activity, the minimum inhibitory concentration (MIC) is measured, this method having been used extensively in the literature (Kalhapure et al., 2014a; Qi et al., 2004; Suleman et al., 2015), however, it has not been reported for LPNs. The only method of antibacterial testing was biofilm susceptibility testing reported by Cheow et al. for LPNs (Cheow and Hadinoto, 2012). Another method that can be used to confirm the antibacterial activity data of the nanoparticles is gel electrophoresis. This method has not

been reported before for LPNs, but it has been studied for other systems, as reported in the literature (Sitohy et al., 2012). In this method, damage to the cell wall of *S.aureus* and MRSA can be determined by a breakdown of the bacterial cell wall proteins, and has been previously discussed in the literature (Sitohy et al., 2012).

In addition to the above studies, X-Ray Diffraction (XRD) and differential scanning calorimetry (DSC) can be used to determine the changes in crystallinty and thermal behaviour of the drug and excipients used in the formulation. These methods have not been reported for LPNs but have been reported for other nanoparticles (Das et al., 2012; Motwani et al., 2008).

2.7 Vancomycin as a model drug for antibiotic therapy

Vancomycin is a glycopeptide antibiotic used in the treatment and prophylaxis of serious infections caused by gram positive bacteria, such as Staphylococcus aureus, that do not respond well to other antibiotics (Zakeri-Milani et al., 2012). It acts by preventing the critical steps in the biosynthesis of peptidoglycan and the assembly of NAMNAG-polypeptide into the growing peptidoglycan chain (Chakraborty et al., 2010). Owing to its large size and hydrophilic nature, it diffuses poorly across the gastrointestinal mucosa, and therefore requires intravenous administration for systemic therapy as it is not absorbed from the intestine (Chakraborty et al., 2010; Pogue et al., 2009; Rao et al., 2011). However, resistance to vancomycin is steadily increasing (Huh and Kwon, 2011), and it is therefore contended that the incorporation of vancomycin into novel nanoparticle systems will overcome the resistance issues and the many drug delivery problems associated with it.

Vancomycin HCl has a molecular formula of $C_{66}H_{75}Cl_2N_90_{24}HCL$ with a molecular weight of 1485.71 g/mol, and its chemical structure is shown in Figure 5 below. Vancomycin has been incorporated into other nanosystems, such as gold nanoparticles (Gu et al., 2003), liposomes (Onyeji et al., 1994), polymeric nanoparticles (Zakeri-Milani et al., 2013), solid lipid nanoparticles (Kalhapure et al., 2014a), dendrimers (Choi et al., 2012) and nanoemulsions (Palamoor and Jablonski, 2014), however, it has not been reported in an LPN system.

Many factors need to be considered during the development of vancomycin HCl in a LPN drug delivery system, such as the solubility of the drug, melting point, the ultraviolet absorption, as well as its pharmacological properties.

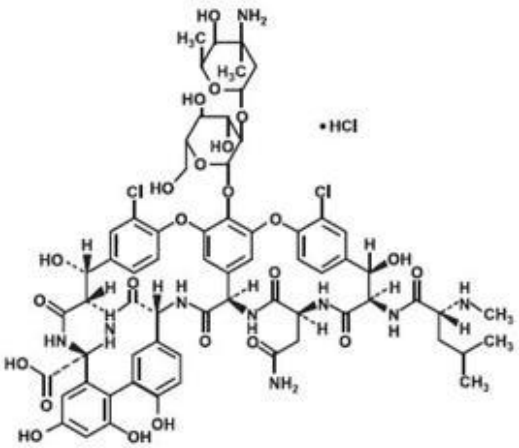


Figure 5. Chemical structure of vancomycin HCl

2.8 Conclusion

This chapter has highlighted the current state of infectious diseases, the available drug therapies and their limitations, as well as the strategies to overcome these limitations, including the use of nano drug delivery systems, in particular Lipid polymer hybrid nanoparticles (LPNs). The review has shown the potential advantages of nano drug delivery systems in treating infectious diseases and the lack of data available on antibiotic loaded LPNs. Therefore, extensive formulation and characterisation of LPNs has to be undertaken to contribute to this developing field. Vancomycin is identified as the model drug, as drug resistance has caused a major problem worldwide and it is used as the last line drug in treating serious infections. It is hydrophilic in nature and has a short half-life making it the ideal candidate for a controlled delivery system such as LPNs.

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CHAPTER 3. SUBMITTED MANUSCRIPT

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3.1 Introduction

The following paper was submitted to Materials, Science and Engineering C (Impact factor: 3.088) which is an international ISI peer reviewed journal and reports on original research:

Ms. N. Seedat contributed to the design of the project, modification and optimisation of methods and preparation and characterisation of all LPN formulations in terms of particle size, PDI, zeta potential, encapsulation efficiency, in vitro drug release study, antibacterial activity, gel electrophoresis, X-ray diffraction and differential scanning calorimetry. Mr. R.S Kalhapure assisted with the overall design of the study and the methods of preparation and characterisation as well as editing. Dr. S. Vepuri and Prof M. Soliman were collaborators on the project and performed the molecular modelling studies. Mr. M. Jadhav performed the mathematical modelling in terms of the in vitro release kinetics data. The remaining authors served as supervisor and co-supervisor and were responsible for project conceptualisation, problem solving, cowriting of papers and abstracts and general supervision of the study.

This chapter is presented in the required format of the journal and is the final revised version. SUMBITTED MANUSCRIPT: Reference Number: MSEC-D-15-01196R3 (Please refer to Appendix C)

Tile: Co-encapsulation of multi-lipids and polymers enhances the performance of vancomycin in lipid polymer hybrid nanoparticles: *in vitro* and *in silico* studies.

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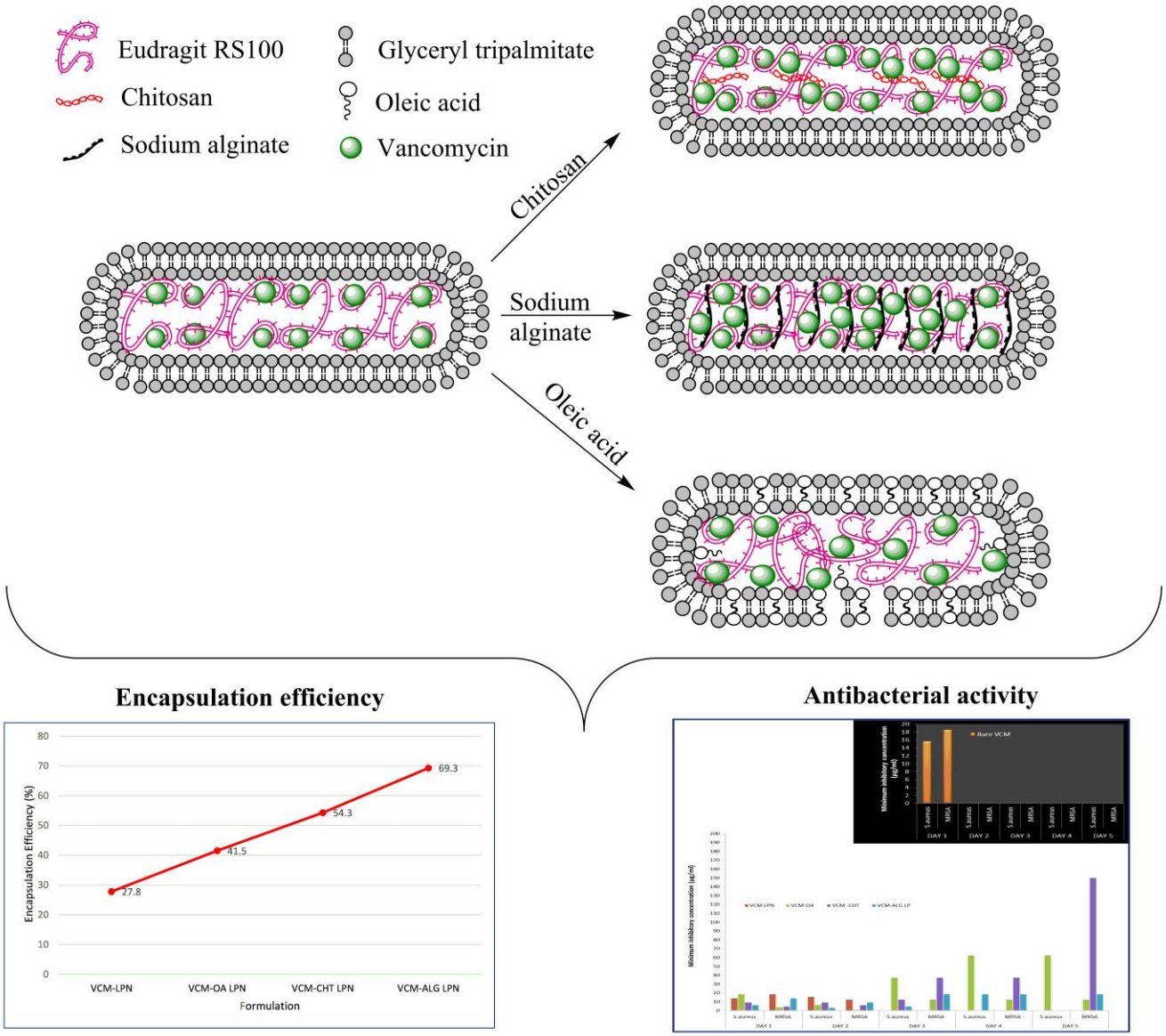
GRAPHICAL ABSTRACT



Vancomycin loaded LPNs were successfully formulated and the addition of helper excipients oleic acid, chitosan and sodium alginate enhanced the encapsulation efficiency, sustained drug release and antibacterial activity.

ABSTRACT

Nano drug delivery systems are being widely explored to overcome the challenges with existing antibiotics to treat bacterial infections [1]. Lipid-Polymer Nanoparticles (LPNs) display unique advantages of both liposomes and polymeric nanoparticles while excluding some of their limitations, particularly the structural integrity of the polymeric particles and the biomimetic properties of the liposome [1]. The use of helper lipids and polymers in LPNs have not been investigated, but have shown potential in other nano-drug delivery systems to improve drug encapsulation, antibacterial activity and drug release. Therefore, LPNs using co-excipients were prepared using vancomycin (VCM), glyceryl triplamitate and Eudragit RS100 as the drug, lipid and polymer respectively. Oleic acid (OA), Chitosan (CHT) and Sodium alginate (ALG) were explored as co-excipients. Results indicated rod-shaped LPNs with suitable size, PDI and zeta potential, while encapsulation efficiency (%EE) increased from 27.8% to 41.5%, 54.3% and 69.3% with the addition of OA, CHT and ALG respectively. Drug release indicated that VCM-CHT had the best performance in sustained drug release of 36.1 ± 5.35% after 24h. The EE and drug release was further corroborated by *in silico* and release kinetics data. *In vitro* antibacterial studies of all formulations exhibited better activity against bare VCM and sustained activity up to day 5 against both *S.aureus* and MRSA, with VCM-OA and VCM-CHT showing better activity against MRSA. Therefore, this LPN proves to be a promising system for delivery of VCM as well as other antibiotics.

Keywords:

Vancomycin, Lipid- polymer; nanoparticle, MRSA, antibacterial, *in silico*

1. INTRODUCTION

The ongoing crisis of infectious diseases caused by a range of bacteria has resulted in an exponential increase in deaths globally [2]. Although the use of antibiotics decreased morbidity and mortality rates, antimicrobial resistance (AMR) is causing a serious issue in treating infectious diseases [3-5]and is now recognised as a major burden in healthcare settings [6, 7]. The AMR and a serious decline in research and development of new antibiotics, have caused a threat similar to that of the pre-antibiotic era. As a result, the major advances made in modern medicine such as surgery, organ transplantation and cancer chemotherapy are at risk of being compromised [8].

Statistics show that an estimated 19 000 deaths per year in the U.S. are caused by methicillin-resistant *Staphylococcus aureus* (MRSA), which can only be treated by vancomycin (VCM), a glycopeptide antibiotic. However, VCM resistance has developed, and the rising prevalence of MRSA increases the possibility of VCM resistant *S. aureus* (VRSA), which is just as deadly as MRSA but more difficult to treat [9, 10]. MRSA, *S. aureus* and VRSA are organisms of current concern in developing regions as well as in developed countries [11].

In addition to antibiotic resistance there are several disadvantages associated with conventional dosage forms of antibiotics. These include inadequate antibiotic concentration at target infection site, increased frequency of administration [12, 13], low water-solubility, cytotoxicity, and fast degradation and clearance in the bloodstream [14]. These disadvantages can be overcome by the use of nano drug delivery systems by improving antibiotics' solubility, pulmonary accumulation, intracellular delivery, concentration at the target site, release profile, and reducing dosing frequency and side effects [3, 15, 16]. In addition, nano delivery systems have inherent ability to overcome existing drug resistance mechanisms [16].

There are at least 10 nanoparticle-based products on the market for infection diagnosis, antibiotic drug delivery and medical devices [17]. Nanoparticles that have been explored for effective antibiotic delivery include liposomes, solid lipid nanoparticles (SLNs), polymeric nanoparticles and dendrimers [3]. The antimicrobial properties of nanoparticles can be attributed to their high surface to volume ratio allowing for drug penetration in the bacterial cell wall, distinctive chemico-physical properties, versatility of the formulation and biocompatibility with tissues and cells [18, 19]. Compared to other medical conditions, such as cardiovascular disease and cancer, nano-drug delivery systems for antibiotic therapy is still in its infancy [3]. Therefore, to combat the ongoing crisis of AMR, applying nanotechnology to deliver antibiotics is of the utmost importance [20].

Lipid-based nanocarriers, such as liposomes [21], SLNs [22], nanostructured lipid carriers [23] and lipid drug conjugates [24] are an attractive dosage form due to their submicron sized particles and solid state of physiological lipid carriers [25]. To overcome the limitations such as low drug loading capacity, high initial burst kinetics, drug leakage during storage, batch to batch reproducibility issues, poor encapsulation of water soluble drugs, polymer cytotoxicity and degradation, use of toxic organic solvents, and scale up issues associated with both liposomes and polymeric nanoparticles, a relatively new nano-drug delivery system popularly termed lipid-polymer hybrid nanoparticles (LPNs) has been developed [26]. The LPN, which is a hybrid nano particulate system with structural integrity of the polymeric particles and the biomimetic properties of the liposome displays unique advantages of both nanoparticles while excluding some of their limitations [1]. LPNs have the advantages of high structural integrity, stability, sustained release from the polymer core, high biocompatibility and bioavailability, tuneable size and surface charge, high drug loading and targeted drug delivery [26, 27]. Despite numerous advantages offered by LPNs, their utilization in the delivery of antibiotics is very limited, with only five papers being reported thus far in the literature. The

delivery of three fluoroqinolone antibiotics (levofloxacin, ofloxacin, ciprofloxacin), calcein [28-31] and clindamycin phosphate [32] has been studied to date using LPNs [33]. Furthermore, the polymer that has been explored the most for antibiotic loaded LPN synthesis is Poly Lactic-co-Glycolic Acid (PLGA) [28-31], with sodium alginate (ALG) and dextran sulphate being studied in one paper [32], and lipids that have been investigated include stearic acid, lecithin and phospahtidylcholine (PC) [29-32, 34]. The limited antibiotic LPN studies highlight the need for formulation optimisation and characterization of LPNs by exploring other polymers and lipids with other potent antibiotics, such as VCM. The identification of strategies to simultaneously enhance the critical properties of drug entrapment, antibacterial activity against sensitive and resistant strains and controlled release profiles has not been previously reported for any antibiotic LPN system. The development of antibiotic LPNs by co-encapsulation of multiple lipids and polymers within its configuration could be an effective approach for simultaneously enhancing the above properties and remains to be explored. The aim of this study was therefore to explore a new lipid-polymer combination in the formulation development of an antibiotic loaded LPN using VCM as a drug, as well as to co-encapsulate helper polymers and lipids in order to simultaneously enhance important properties, such as drug encapsulation, antibacterial activity and drug release profiles. In addition to *in vitro* characterisation, extensive *in silico* modelling was undertaken to obtain a molecular understanding of the effect of the helper polymers and lipid on the VCM loaded LPNs.

2. MATERIALS

Glyceryl tripalmitate (GTP), oleic acid (OA), Solutol HS15 (Kolliphor HS15), ALG, CHT (medium molecular weight), and dialysis membrane (MWCO 12271) were purchased from Sigma-Aldrich (USA). Eudragit RS100 was generously provided by Evonik Industries (Germany), while Vancomycin hydrochloride (VCM) was purchased from Sinobright Import and Export Co., LTD (China). Nutrient Broth, Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) were obtained from Biolab (Midrand, South Africa). The bacterial cultures used were *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) (*S. aureus* Rosenbach ATCC BAA 1683). Purified water used throughout the studies was produced in the laboratory with a Milli-Q purification system (Millipore corp., USA). All other chemicals and solvents were of analytical grade and used without further purification.

3. METHODS

3.1. Preparation of LPNs

Both drug loaded and drug free LPNs were produced by hot high pressure homogenisation followed by ultrasonication [35]. Briefly, GTP (0.5g) (oil phase) was heated at 80° C, and a solution of the Eudragit RS100 (1% w/v) and surfactant Solutol HS15 (1% w/v) in 80% (v/v) ethanol were heated separately to 80 °C and added to the lipid. The mixture was homogenised for approximately 45 min until the solvent evaporated, and distilled water was added to adjust the volume to 25 ml and then homogenised at 6000 rpm for 10 min with an Ultra Turrax T-25 homogenizer (IKA Labortechnik, Germany). The resultant emulsion was immediately subjected to high intensity probe sonication at 30% amplitude for 30 min using the Omni sonic ruptor 400 Ultrasonic Homogenizer (Kennesaw, GA 30144, USA) at the same temperature, and cooled immediately to 20 °C. The final volume of LPN dispersion was maintained at 25 ml. For drug loaded LPN, VCM (20mg) was added to the polymer and

surfactant solution, and the same procedure was followed. For co-encapsulation with OA and helper polymers, the OA (1:10 drug to fatty acid molar ratio), CHT (0.5:1 helper polymer to polymer ratio) and ALG (0.5:1 helper polymer to polymer ratio) were mixed with the drug, polymer and surfactant solution, and then added to the melted lipid. The procedure that followed thereafter was the same as above.

3.2. Characterisation

3.2.1. Particle size, Polydispersity Index (PDI) and zeta potential(ZP)

The particle size, PDI and ZP were determined by using photon correlation spectroscopy (PCS) (Nano ZS Zetasizer, Malvern Instruments Corp, UK) at 25° C in polystyrene cuvettes with a path length of 10 mm. Measurements were performed by diluting 40 µl of nanoparticle suspension to 10 ml milli-Q water. All measurements were performed in triplicate.

3.2.2. Determination of Encapsulation Efficiency (% EE) and drug loading capacity (LC)

To determine the concentration of VCM in the LPNs, an ultrafiltration method using Amicon® Ultra-4, centrifugal filter tubes (Millipore Corp., USA) of 10 kDa molecular weight cut-off was used [36]. Briefly, the 25 ml LPN suspension was made up to 100 ml volume with milli-Q water. Thereafter, 1 ml of this diluted suspension was placed into a centrifugal filter tube and centrifuged at 500 x *g* at 25° C for 15 min, 200 µl of filtrate was withdrawn and diluted to 10 ml with distilled water, and the amount of free drug was detected by a validated High Pressure Liquid Chromatography (HPLC) (Shimadzu, Japan) method at 230 nm. The mobile phase consisting of ammonium dihydrogen phosphate and acetonitrile (92/8 v/v) was pumped through Hichrome Nucleosil 120-5C18 column (15cm x 4.0mm internal diameter) at a flow rate of 1 ml/min. The injection volume was 20µl [37]. The regression equation and linearity (r^2) were y = 39924x – 132005 and 0.9972 respectively.

The % EE and % LC was calculated using the following equations [35]:

$$EE\ (\%) = \left[\frac{Mi - Mfree\ VCM}{Mi}\right] x\ 100 \qquad \text{(Equation 1)}$$

$$LC\ (\%) = \left[\frac{Mdrug\ in\ LPN}{M(LPN)}\right] x\ 100 \qquad \text{(Equation 2)}$$

Where '*Mi*' is the initial mass of VCM used, *'Mfree VCM'* is the mass of free VCM detected in the filtrate after ultrafiltration, 'M*drug in LPN'* is the mass of VCM in the formulation and 'M (*LPN)*' is the mass of the LPN formulation.

3.2.3. Morphology

The morphology of the LPNs was examined using a Scanning Electron Microscope (SEM) technique. A few drops of the LPN suspension were placed on a cover slip placed on carbon tape, dried thoroughly and sputter coated by gold. The image was captured by field-emission gun SEM (ZEISS FEGSEM Ultra Plus, Germany) at an accelerated voltage of 5 kV for the drug free and VCM LPNs, and 10 kV for the VCM-CHT LPNs.

3.3. *In vitro* drug release studies

Drug release studies were performed using a dialysis-bag method under a sink condition at 37° C in an incubator at 100 rpm. A dialysis bag containing a dilution of 1 ml LPN suspension and 1ml PBS (pH 7.4) was placed in a 50 ml capacity bottle containing 40 ml PBS (pH 7.4) as the release medium. To determine the amount of drug diffused through the dialysis tube, 2 ml of the release medium was withdrawn at predetermined time intervals and equal volumes of PBS was added to maintain sink conditions. The amount of drug released at each time interval was measured by HPLC (Shimadzu, Japan) at 280 nm as described above in 3.2.2. The measurement was performed in triplicate. The regression equation and linearity (r^2) were y = 39924x – 132005 and 0.9972 respectively.

In vitro drug release kinetics and mechanism

The *in vitro* drug release data of VCM, VCM-OA, VCM-CHT and VCM-ALG LPNs were analysed to determine the drug release kinetics by using various mathematical models shown below [38].

a) Kinetic models

Zero-order model	:	$Q = k.t + Q_0$	(Equation 3)
First-order model	:	$Q = Q_0 e^{kt}$	(Equation 4)
b) Higuchi model	:	$Q = k.t^{1/2}$	(Equation 5)
c) Hixson–Crowell model	:	$Q^{1/3} = kt + Q_0^{1/3}$	(Equation 6)
d) Weibull model	:	$Q = 1\exp[-(t)^{b/a}]$	(Equation 7)
e) Korsmeyer–Peppas model	:	$Q = k.t^n$	(Equation 8)

where:

Q represents the amount of drug released in time t,

Q_0 is the start value of Q,

k is the rate constant,

a is the time constant, and b is the shape parameter,

n is the diffusional exponent, an indicative of drug release mechanism.

To understand the release kinetics (best fit model) and dissolution enhancement (model independent parameter), the drug release data were used to calculate the squared correlation coefficient (R^2) and mean dissolution time (MDT) using KinetDS 3.0 Rev. 2010 software [39]. Furthermore, the Korsmeyer–Peppas model was employed in the *in*

vitro drug release behaviour analysis of LPNs to distinguish between competing release mechanisms (Table 1) [40-42].

Table 1. Exponent n of the Korsmeyer-Peppas model and drug release mechanism from LPN controlled delivery system.

Entry	n Value	Drug release mechanism
1	Less than 0.43	Fickian release (diffusion-controlled release)
2	0.43 to 0.85	non-Fickian release (anomalous transport)
3	0.85 to 1.00	case-II transport (relaxation-controlled release)
4	More than 1	Super case-II transport mechanism (Swelling and polymer chain relaxation controlled release)

3.4. In vitro antibacterial activity

The minimum inhibitory concentration (MIC) values for drug free and VCM loaded LPN formulations (VCM-LPNs) were determined against *S. aureus* and MRSA using a broth dilution method. Dilutions of VCM-LPNs, VCM-OA-LPNs, VCM-CHT LPNs and VCM-ALG LPNs were prepared in MHB and incubated with the bacterial cultures at 37° C. Thereafter, at specified time intervals, 10 µl was spotted on MHA plates and incubated for 24 h at 37° C to determine the MIC values. Experiments were performed in triplicate and drug free LPNs, and the different excipients alone were used as controls.

In order to determine the effects of the helper excipients in combination with VCM on antibacterial activity, the fractional inhibitory concentration (FIC) values were determined. The FIC can be described as the method used to quantify the MIC results using the FIC index, as described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [43]. The equations used to calculate the ΣFIC is shown below:

For two antibacterials A and B alone and in combination:

$$FIC_A = \frac{\text{MIC (A in presence of B)}}{\text{MIC (A alone)}} \quad \text{(Equation 9)}$$

$$FIC_B = \frac{\text{MIC (B in presence of A)}}{\text{MIC (B alone)}} \quad \text{(Equation 10)}$$

$$\Sigma FIC = FIC_A + FIC_B \quad \text{(Equation 11)}$$

The FIC index is shown in Table 2. Indifference can be described as the combination of drug LPN and excipient is equal to that of the most active compound. An additive effect is when the effect of combining drug LPN and the excipient is equal to the sum of effects of the individual components. Synergistic action is present if the effect of the combination of drug LPN and excipient exceeds the additive effect of the individual components.

Table 2. FIC Index

Index	**Result**
≤ 0.5	Synergy
>0.5-1	Additive
>1 to <2	Indifference
≥ 2	Antagonism

3.5. Gel Electrophoresis

To determine the cell membrane damage to S.*aureus* and MRSA, the SDS-Page study similar to that reported in the literature [44] of the bacterial proteins, was carried out after the bacterial cells were incubated and treated with the VCM, VCM-OA, VCM-CHT and VCM-ALG LPNs. Briefly, S.*aureus* and MRSA cultures were grown overnight and incubated at 37° C. Thereafter, 200 µl of the grown bacterial suspension (1 x 10^9 CFU/ml) was inoculated into 10 ml of freshly prepared MHB and incubated for 24 h at 37° C. The bacterial cells were then separated by centrifugation at 8000 rpm for 5 min and then re-suspended in 10 ml of sterile saline solution (8.5g NaCl/L). Thereafter, 400 µl of LPN

sample was added to the sterile suspensions of S.*aureus* and MRSA respectively. Untreated suspensions of S.*aureus* and MRSA were used as controls. An aliquot of 50 µl of the bacterial suspension was heated at 100 ^{o}C for 10 min after combining with 25 µl of the sample buffer pH-6.8 (1 M Tris–HCl, 50% glycerol, 10% SDS, 10% β-mercaptoethanol, 0.1% Bromophenol blue). Thereafter, for the stacking and resolving gel, this treated aliquot was loaded in 3 and 12% SDS-PAGE respectively. After running at 10 mA and 20 mA on the stacking gel and resolving gel respectively, protein bands were visualized on the gels by Coomassie Brilliant Blue R250.

3.6. X-ray Diffraction (XRD)

The XRD patterns of the excipients alone, as well as VCM, VCM-OA, VCM-CHT and VCM-ALG LPNs, were obtained using a Bruker D8 Advance Diffractometer (Germany) equipped with a graphite monochromator operated at 40 kV and 40 mA. The radiation source was a CuKα X-ray source with λ = 1.5406 Å. Data was collected at a step of 0.021° and at a scanning speed of 0.454 ° s^{-1}, while the 2θ range covered was between 10 ° to 90°.

3.7. Differential Scanning Calorimetry (DSC)

The thermal profile of the excipients alone as well as VCM, VCM-OA, VCM-CHT and VCM-ALG LPNs was determined by DSC (Shimadzu DSC-60, Japan). Briefly, 2 mg of the sample was placed in an aluminium pan and sealed using a crimper, which was heated to 300° C at a constant rate of 10° C/min under the constant nitrogen flow of 20 ml/min.

3.8. Stability Studies

In the present investigation, the standard protocol in terms of storage conditions and physical parameters evaluated for stability evaluation of lipid nanoparticles was followed [45-49]. Samples were stored at 4 °C and room temperature for 3 months. Physical appearance, particle size, PDI, and ZP were evaluated.

3.9. Molecular Modelling

The 3D model for the drug VCM was developed from its stable crystal structure coordinates (PDB ID: 1SHO), as reported in the Protein Data Bank (PDB) [50]. The 3D structure of the polymer Eudragit RS100 (EUD) was constructed using ChemBio3D Ultra in its syndiotactic stereochemistry. The structure of CHT (CSID:64870) was obtained from the chemspider database [51], while the structures of sodium alginate (ALG) (CID:6850754), OA (CID:445639) and GTP (CID:11147) were obtained from the PUBCHEM database [52-54]. All the structures were optimized to their lowest energy conformations using Universal Force Field (UFF) [55]. Binding affinity studies were performed on various complexes of the drug-polymer systems to comprehend the % EE and drug release profiles demonstrated by the various formulations.

The Flexible binding simulation study was performed using ArgusLab 4.0.1 [56]. The Argus Lab molecular modelling program 4.0.1, installed on a local windows operating system (Windows 7), was used to calculate the binding free energy of optimal polymer-drug/drug-auxiliary agent/polymer-drug-helper agent/polymer-drug-helper agent-lipid complexes. A Genetic algorithm (GA) based binding energy calculation protocol was followed using the scoring method Ascore from the ArgusLab 4.0.1 suite [56]. Ascore is based on the decomposition of the total host–guest binding free energy (Equation 4), in terms of the van der Waals interaction, the hydrophobic effect, the hydrogen bonding, the hydrogen bonding involving charged donor and/or acceptor groups, the deformation effect, the effects of the translational, and rotational entropy loss in the binding process, respectively [56].

$$\Delta G_{bind} = \Delta G_{vdw} + \Delta G_{hydrophobic} + \Delta G_{H\text{-}bond} + \Delta G_{H\text{-}bond\ (chg)} + \Delta G_{deformation} + \Delta G_0 \quad \text{(Equation 12)}$$

Each binding calculation was repeated five times to get the best results. A complex of one polymer molecule with drug/helper agent/lipid was assembled by screening several configurations, and the energy-minimization was repeated to generate the final models, as described in Table 3. Complete geometrical optimization was conducted using UFF in vacuum, and by employing the steepest descent method until a RMS (root mean squared) gradient of 0.001 kcal/mol was reached [55]. A maximum of 150 poses for the molecular complexes were allowed to be analysed. Accelrys Discovery Studio Visualizer 63 [57] was used to visualize the interactions in the complex structures.

Table 3: The studied molecular models and their representation

Model	**Description**
EUD -VCM	Binding of vancomycin with the polymer eudragit
VCM-CHT	Binding of vancomycin with the helper polymer chitosan
VCM-ALG	Binding of vancomycin with the helper polymer alginate
VCM-OA	Complex formation between vancomycin and oleic acid
EUD-VCM-CHT	Binding of vancomycin to chitosan-eudragit complex
EUD-VCM-ALG	Binding of vancomycin to alginate-eudragit complex
EUD-VCM-OA	Binding of vancomycin to oleic acid-eudragit complex
EUD-VCM-GTP	Binding of vancomycin to glyceryl tripalmitate-eudragit complex
EUD-VCM-CHT-GTP	Binding of glyceryl tripalmitate with the vancomycin attached chitosan-eudragit complex to form the final lipid-polymer-drug assembly.
EUD-VCM-ALG-GTP	Binding of glyceryl tripalmitate with the vancomycin attached alginate-eudragit complex to form the final lipid-polymer-drug assembly.
EUD-VCM-OA-GTP	Binding of glyceryl tripalmitate with the vancomycin attached oleic acid-eudragit complex to form the final lipid-polymer-drug assembly.

3.10. Statistical analysis

The results obtained were expressed as a mean ± SD and analysis of the data was performed using GraphPad Prism®5 (Graphpad Software Inc, USA). One way ANOVA (Kruskal-Wallis test) followed by a t-test (non-parametric Mann Whitney test) were performed, and the difference was considered statistically significant when $p < 0.05$.

4. RESULTS

4.1 Particle size, PDI, ZP, % EE, LC and morphology of LPNs

The mean diameter, ZP, EE and LC of VCM, VCM-OA, VCM-CHT and VCM-ALG LPNs are presented in Table 4. The data shows that particle size varied from 202.5 ± 3.81 to 250.9 ± 9.04, with the highest particle size being the VCM-CHT LPNs. The PDI, ZP and EE increased with the addition of the helper excipients, except in the case of VCM-OA LPNs, where both the PDI and ZP decreased. The EE increased significantly from 27.8% to 41.5% ($p = 0.0048$), 54.3% ($p = 0.0048$) and 69.3% ($p = 0.0048$) with the addition of OA, CHT and ALG respectively. SEM images of LPNs showed particles that were rod shaped, discrete and homogeneous (Figure 1). There were no distinct morphological differences in the various formulations, and the particle sizes were slightly smaller than those obtained using the zetasizer. A similar trend in morphology was exhibited by all the LPN formulations, with only the drug free, VCM and VCM-CHT LPN images being shown below. One-way ANOVA of particle size, PDI, ZP and EE showed statistical significance, with p values of 0.0004, 0.0013, 0.0005 and 0.0156 respectively.

Table 4. LPN formulations characterisation in terms of size, PDI, Zeta potential and EE (n =3).

LPN	Particle Size (d.nm)	PDI	ZP (mV)	EE (%)	LC (%)
Drug Free	214.1 ± 6.86	0.251 ± 0.01	+28.9 ± 1.98	----------	---------
VCM	216.4 ± 9.98	0.284 ± 0.03	+29.7 ± 4.91	27.8 ± 1.84	0.74
VCM-OA	202.5 ± 3.81*	0.261 ± 0.02	+17.4 ± 2.84*	41.5 ± 2.89*	1.05
VCM-CHT	250.9 ± 9.04*	0.296 ± 0.04	+30.6 ± 1.38	54.3 ± 0.44*	1.24
VCM-ALG	205.2 ± 9.86*	0.386 ± 0.02*	-32.8 ± 4.54*	69.3 ± 0.71*	1.58

*p<0.05 when compared to VCM LPN

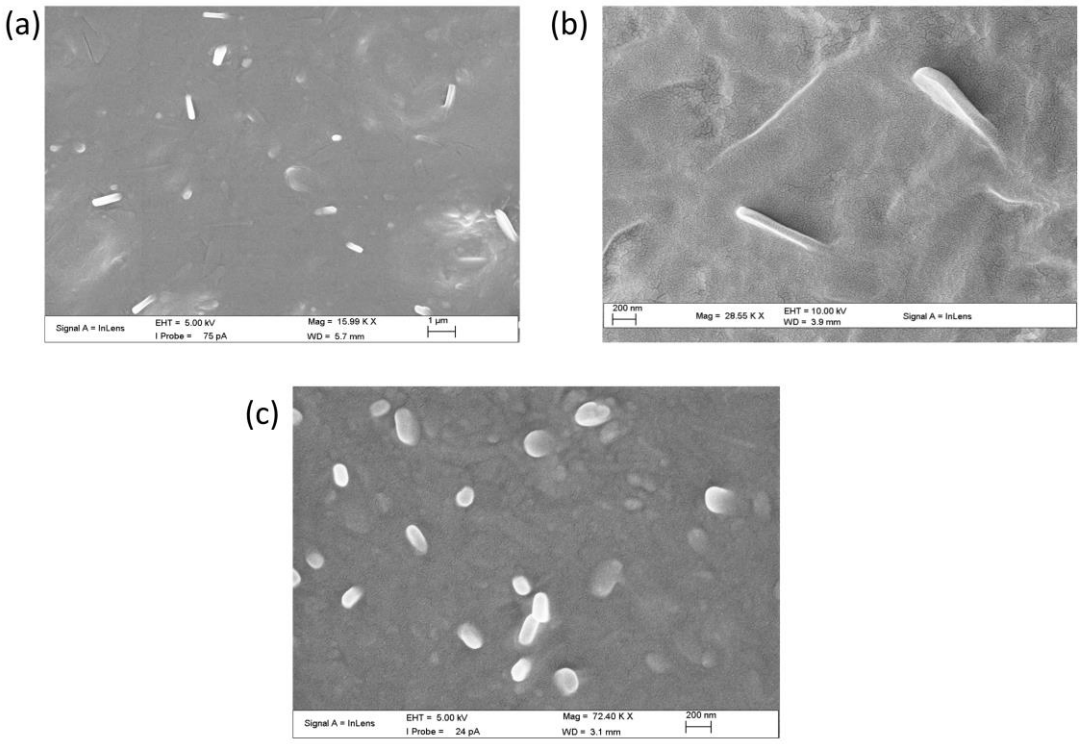


Figure 1. SEM images of (a) VCM –LPNs, (b) VCM-CHT LPN and (c) LPN (drug free).

4.2. In vitro drug release studies

Figure 2 illustrates the drug release profiles of bare VCM as well as VCM, VCM-OA, VCM – CHT and VCM-ALG LPNs over 24 hours. The results indicate that all formulations showed a sustained release profile when compared to the release rate of bare VCM (100% after 7 hours). The data shows that VCM-CHT had the slowest drug release of 36.1 ± 5.35 %, while VCM-ALG had the fastest drug release rate of 54.4 ± 3.24 % at the end of 24 h.

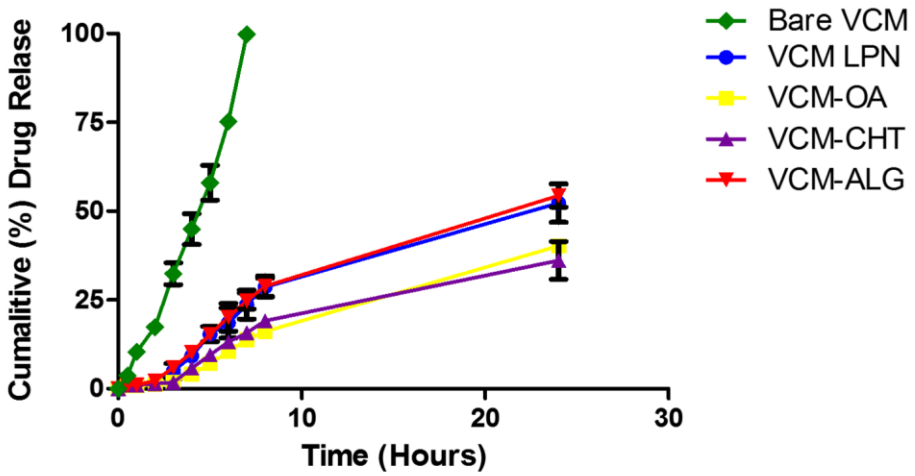


Figure 2. Drug release profiles of different LPN formulations containing VCM (n=2).

In vitro drug release kinetics and mechanism

The *in vitro* drug release data from various LPNs was evaluated kinetically using a number of mathematical models such as zero order, first order, Hixson–Crowell, Weibull, Higuchi, and Korsmeyer–Peppas. The correlation coefficient (R^2), Root mean square error (RMSE) and Akaike's information criterion (AIC) values of these models were determined using KinetDS 3.0 Rev. 2010 software to understand the best fit model for VCM release from LPNs. The results of the curve fitting into various mathematical models are given in Table 5. The calculated highest R^2 value for VCM, VCM-OA, VCM-CHT, and VCM-ALG LPNs were 0.947, 0.977, 0.922 and 0.9463 respectively. The lowest RMSE values determined for VCM,

VCM-OA, VCM-CHT, and VCM-ALG LPNs were 1.735, 2.962, 3.134 and 2.930 whereas lowest AIC values were 48.74, 38.04, 49.87 and 48.54 respectively.

The value of the release exponent (n) and rate constant (k) derived from Korsmeyer Peppas equation were in between 1.136 – 1.267 and 1.036 – 1.6959 respectively (Table-5). The mean dissolution time (MDT) values calculated for 50% VCM release from VCM, VCM-OA, VCM-CHT, and VCM-ALG LPNs were 9.482, 14.422, 14.050, and 9.213 hours respectively (Table 6).

Table 5. Results of curve fitting of the *in vitro* VCM release data from the various LPN formulations.

Sr. No	Name of release model	VCM-LPN (A)			VCM-OA LPN (B)			VCM-CHT LPN (C)			VCM-ALG LPN (D)		
		R^2	RMSE	AIC	R^2	RMSE	AIC	R^2	RMSE	AIC	R^2	RMSE	AIC
1	Zero order	0.9197	4.348	56.520	0.9772	1.735	38.040	0.9220	2.962	48.740	0.9262	42.990	56.190
2	First order	0.5587	27.829	93.540	0.6777	15.632	82.010	0.6013	16.179	82.700	0.5583	27.700	93.450
3	Higuchi	0.2963	12.877	78.140	0.2566	9.900	72.880	0.3204	8.752	70.410	0.3254	13.000	78.320
4	Korsmeyer-Peppas	0.9394	3.662	52.980	0.8940	2.247	43.210	0.8800	4.098	55.240	0.9373	35.250	52.220
5	Weibull	0.9474	3.134	49.870	0.8955	2.254	43.280	0.8838	3.150	50.020	0.9463	2.930	48.540
6	Hixson-Crowell	0.7019	10.648	74.330	0.8169	5.657	61.690	0.7233	6.877	65.590	0.7043	10.740	74.510

Table 6. Calculated MDT values for various LPN formulations using the Korsmeyer-Peppas model.

Code	LPNs	Korsmeyer-Peppas		$MDT_{50\%}$
		K	n	
A	VCM	1.486	1.267	09.482
B	VCM-OA	1.036	1.152	14.422
C	VCM-CHT	1.221	1.136	14.050
D	VCM-ALG	1.659	1.240	09.213

4.3. In vitro antibacterial studies

The MIC values for the different formulations, as well as the controls are presented in Table 7. All the formulations showed better activity than bare VCM, and exhibited sustained activity over a period of five days. Interestingly, the formulations VCM-OA and VCM-CHT LPNs

showed better activity against MRSA compared to *S. aureus*. VCM-OA LPNs showed the best activity with an MIC value of 1.2 µg/ml against MRSA on day 2. The calculated ΣFIC values are given in Table 8.

Table 7. *In vitro* antibacterial activity of LPN formulations containing VCM for 5 days.

	MIC (µg/ml)									
Formulation	**DAY 1**		**DAY 2**		**DAY 3**		**DAY 4**		**DAY 5**	
	S.*aureus*	**MRSA**	**S.*aureus***	**MRSA**	**S.*aureus***	**MRSA**	**S.*aureus***	**MRSA**	**S.*aureus***	**MRSA**
Bare VCM	15.6	18.5	300	NA	400	NA	400	NA	400	NA
VCM LPN	14.06	18.75	15.62	12.5	NA	NA	NA	NA	NA	NA
OA	400	400	200	200	400	400	400	400	NA	NA
VCM-OA LPNs	18.75	3.9	6.6	1.2	37.5	12.5	62.5	12.5	62.5	12.5
CHT	400	400	37.5	37.5	NA	NA	NA	NA	NA	NA
VCM -CHT LPNs	9.4	4.7	9.4	6.25	12.5	37.5	NA	37.5	NA	150
ALG	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VCM-ALG LPNs	6.25	14.1	3.5	9.4	4.7	18.75	18.75	18.75	NA	18.75

Table 8. ΣFIC for *in vitro* antimicrobial activity of VCM-OA and VCM-CHT LPNs on Day 1. VCM-ALG ΣFIC could not be calculated as ALG did not show any antimicrobial activity.

Sample	ΣFIC		Results	
	S.*aureus*	MRSA	S.*aureus*	MRSA
VCM-OA LPN	1.247	0.211	Indifference	Synergy
VCM-CHT LPN	0.626	0.266	Additive	Synergy

4.4. Gel Electrophoresis

Degradation of the bacterial cell wall proteins after treatment with an antibacterial agent can be detected by using gel electrophoresis technique [58]. The effect of the different LPN formulations on *S.aureus* and MRSA cell proteins was therefore studied using this technique, with the results being depicted in Figure 3. The results of all the *S.aureus* treated LPNs after 24 h showed the presence of faded protein bands when compared to the strong and clear bands in the *S.aureus* control. The VCM-CHT LPN showed the greatest difference, with an

almost complete absence of the proteins of different molecular weights present in the control. Similarly, with the LPN treated MRSA samples, there was a visible difference between the molecular proteins present in the control and the bacterial cells treated with the various LPN formulations, as the bands appeared lighter than the control sample.

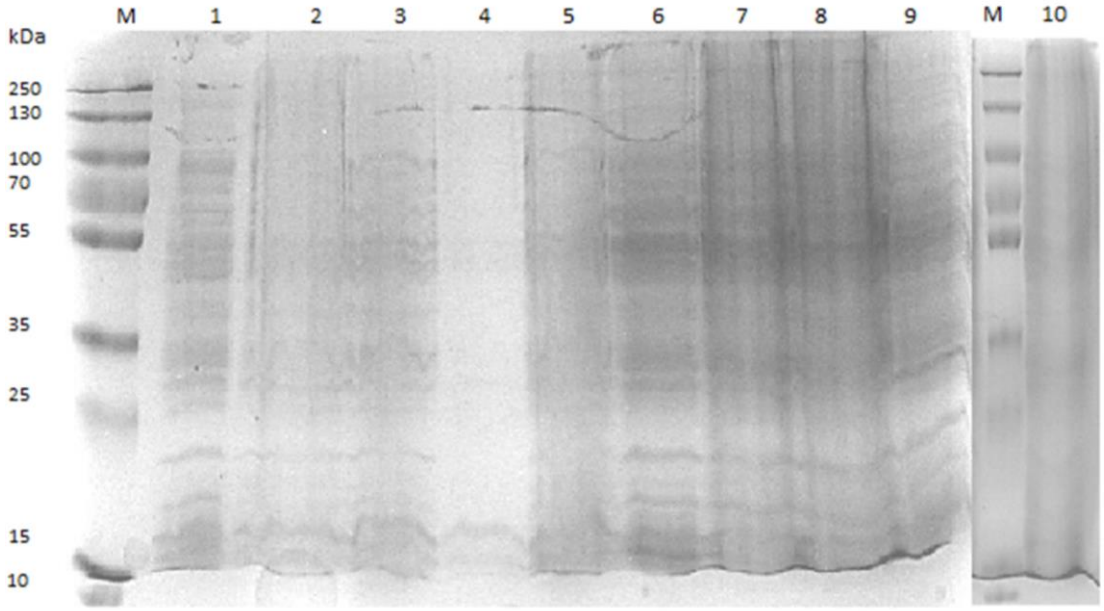


Figure 3. SDS Page patterns of (1) *S.aureus* control, (2) VCM LPN treated *S.aureus*, (3) VCM-OA treated *S.aureus*, (4) VCM-CHT treated *S.aureus*, (5) VCM-ALG treated *S.aureus*, (6) MRSA control, (7) VCM LPN treated MRSA, (8) VCM-OA treated MRSA, (9) VCM-CHT treated MRSA and (10) VCM-ALG treated MRSA. (M = Marker).

4.5. X-Ray Diffraction

The diffractograms were in good agreement with the results of the DSC thermal analysis (Figure 4). GTP was in the crystalline state with the strong diffractions, while Eudragit RS100, VCM and ALG showed an amorphous state with no diffractions. CHT showed a relatively small diffraction, which is characteristic of a partial crystalline polymer [59]. The x-ray diffraction pattern of GTP gave noticeable peaks around 2θ values of 24 º, 26.5 º, 30.5 º and 40º which can be correlated to 100, 110 and 111 diffraction planes. Chitosan exhibited two distinct crystalline peaks at 2θ-scattered angles of 10º from (020) planes and 20º from

(110) planes which is consistent with previous results [60, 61]. VCM, VCM-OA, VCM CHT and VCM ALG LPNs showed mainly a reflection of the crystalline GTP, with different intensities in the diffractions.

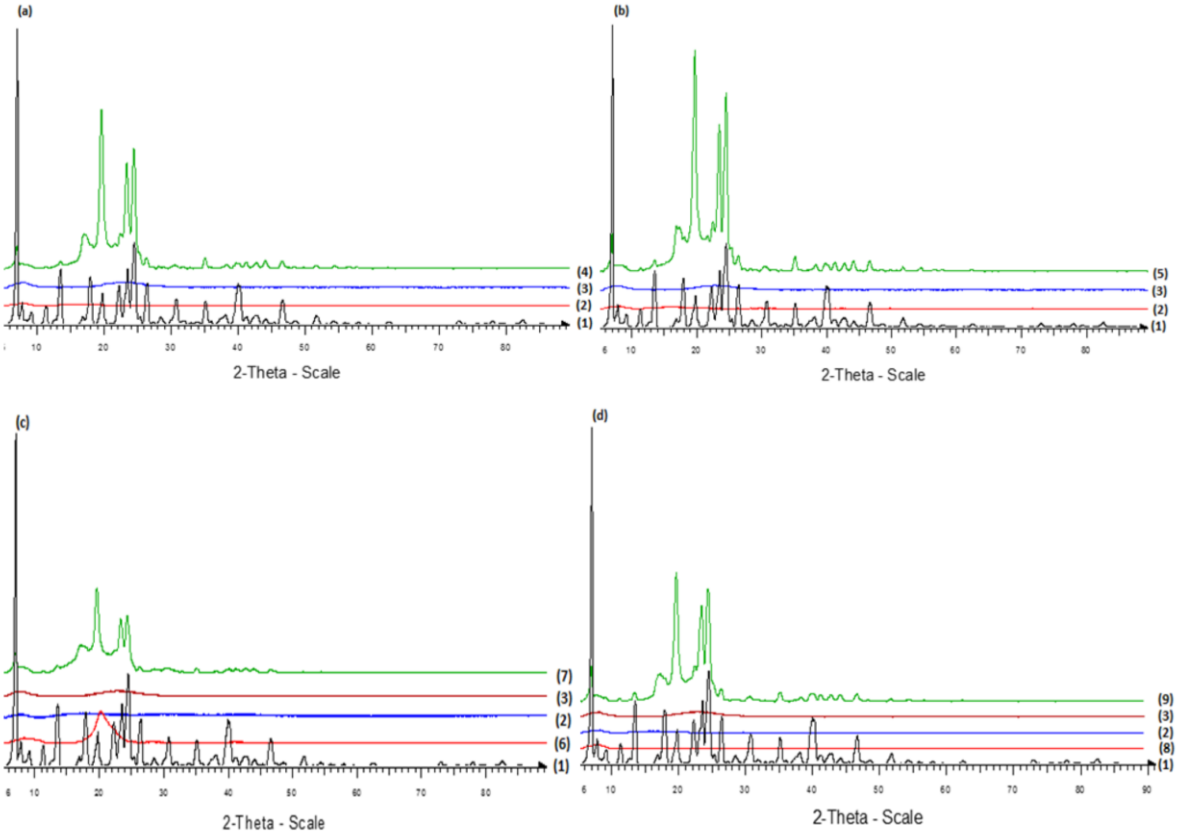


Figure 4. Overlaid XRD crystallographs of (1) GTP, (2) Eudragit RS100, (3) VCM, (4) VCM LPN, (5) VCM-OA LPN, (6) CHT, (7) VCM-CHT LPN, (8) ALG and (9) VCM-ALG LPN.

4.6. Differential Scanning Calorimetry

The DSC study was performed to investigate the melting and crystallization behaviour of materials in LPN, with the thermograms obtained from DSC being depicted in Figure 5. The thermal behaviour of all different excipients, as well as the formulations with and without helper excipients were studied. Any influential or sudden change in the drug, polymer, helper excipients or lipid thermal behaviour can suggest possible interactions [62]. The endothermic peak of VCM can be observed at 110.77° C, ALG showed a prominent endothermic peak at

132.58° C and chitosan showed a peak at 165.08° C. There was no noticeable endotherm in Eudragit RS100 over the studied temperature range. The lipid GTP exhibited a sharp endothermic peak at 73.31 °C, while the peak was observed at 65.43, 64.06, 69.64, 68.4 and 66.58 °C in VCM LPNs, VCM-CHT LPNs, VCM-OA LPNs, VCM-ALG LPNs and drug free LPNs respectively. Additional broad endothermic peaks at 203.8 ºC and 135.95 ºC were observed for VCM-CHT and VCM-OA respectively.

The VCM-OA LPNs showed an additional broad peak at 135.9 °C, and VCM ALG LPN showed a shift in the endothermic ALG peak from 132.58 °C to 118.91 °C.

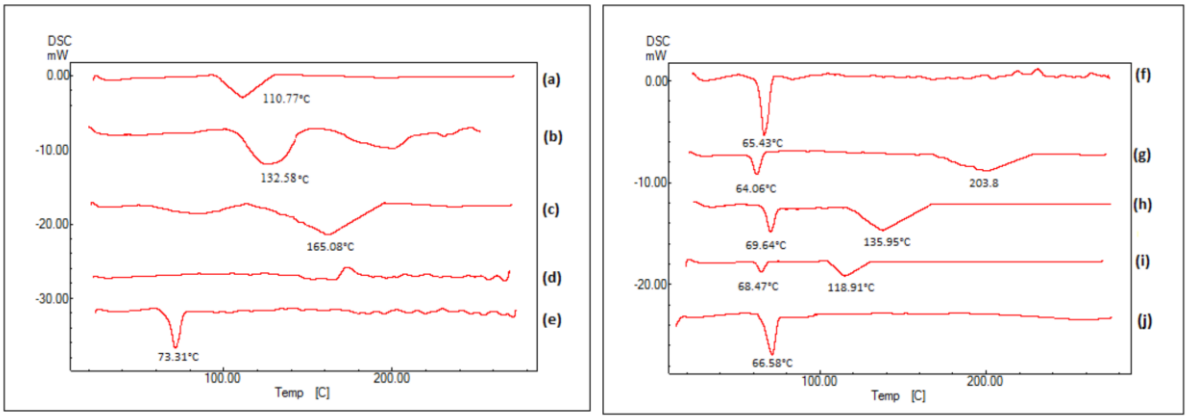


Figure 5. Overlaid DSC thermograms of (a) VCM, (b) ALG, (c) CHT, (d) Eudragit RS100, (e) GTP, (f) VCM LPNs, (g) VCM-CHT LPNs, (h) VCM-OA LPNs, (i) VCM-ALG LPNs and (j) drug free LPNs.

4.7. Stability Studies

Stability studies were performed on aqueous dispersions of the LPN formulations over a period of three months at 4 °C and room temperature and the results are depicted in Tables 9-13. The results show that all formulations were stable at both 4° C and room temperature.

Table 9. Effect of temperature and storage period on drug free LPNs (n=3).

	SIZE (d.nm)		PDI		ZP (mV)	
	4 °C	**RT**	**4 ° C**	**RT**	**4 ° C**	**RT**
Day 1	214.1 ± 6.86	214.1 ± 6.86	0.251 ± 0.01	0.251 ± 0.01	28.9 ± 1.98	28.9 ± 1.98
1 month	208.9 ± 1.35	223.0 ± 1.55	0.262 ± 0.01	0.260 ± 0.01	22.8 ± 3.25	26.2 ± 1.11
2 months	210.2 ± 4.07	219.3 ± 6.01	0.258 ± 0.01	0.252 ± 0.03	23.4 ± 0.71	29.8 ± 3.82
3 months	211.8 ± 4.12	218.6 ± 6.15	0.260 ± 0.01	0.256 ± 0.01	21.9 ± 1.70	25.7 ± 1.20

Table 10. Effect of temperature and storage period on VCM LPN (n=3).

	SIZE (d.nm)		PDI		ZP (mV)	
	4 ° C	**RT**	**4 ° C**	**RT**	**4 ° C**	**RT**
Day 1	192.3 ± 6.29	192.3 ± 6.29	0.267 ± 0.01	0.267 ± 0.01	24.8 ± 1.83	24.8 ± 1.83
1 month	182.6 ± 2.05	185.4 ± 0.28	0.259 ± 0.02	0.239 ± 0.02	22.03 ± 3.18	24.2 ± 0.07
2 months	187.1 ± 0.35	183.9 ± 1.98	0.299 ± 0.04	0.289 ± 0.03	25.56 ± 0.99	28.8 ± 2.40
3 months	187.4 ± 0.71	186.3 ± 3.65	0.260 ± 0.02	0.385 ± 0.01	22.6 ± 4.52	26.2 ± 1.18

Table 11. Effect of temperature and storage period on VCM-OA LPN (n=3).

	SIZE (d.nm)		PDI		ZP (mV)	
	4 ° C	**RT**	**4 ° C**	**RT**	**4 ° C**	**RT**
Day 1	186.7 ± 4.17	186.7 ± 4.17	0.271 ± 0.03	0.271 ± 0.03	24.8 ± 1.41	24.8 ± 1.41
1 month	179.5 ± 1.84	183.6 ± 1.87	0.241 ± 0.01	0.262 ± 0.01	24.3 ± 5.44	23.9 ± 1.82
2 months	178.6 ± 4.28	180.3 ± 2.68	0.262 ± 0.01	0.268 ± 0.01	28.1 ± 6.15	24.5 ± 1.58
3 months	173.4 ± 3.74	184.3 ± 3.13	0.257 ± 0.01	0.272 ± 0.04	22.0 ± 3.82	22.1 ± 1.62

Table 12. Effect of temperature and storage period on VCM-CHT LPN (n=3).

	SIZE (d.nm)		**PDI**		**ZP (mV)**	
	4 °c	**RT**	**4 °C**	**RT**	**4 °C**	**RT**
Day 1	228.9 ± 9.04	228.9 ± 9.04	0.296 ± 0.04	0.296 ± 0.04	45.8 ± 1.38	45.8 ± 1.38
1 month	225.6 ± 1.84	212.5 ± 3.23	0.300 ±0.01	0.287 ± 0.01	42.9 ± 0.35	42.5 ± 2.48
2 months	230.8 ± 1.34	216.8 ± 2.33	0.292 ± 0.01	0.285 ± 0.01	41.8 ± 7.99	41.8 ± 7.99
3 months	217.7 ± 4.24	215.7 ± 2.62	0.289 ± 0.01	0.291 ± 0.01	46.3 ± 2.26	42.8 ± 2.13

Table 13. Effect of temperature and storage period on VCM-ALG LPN (n=3).

	SIZE (d.nm)		PDI		ZP (mV)	
	4 ° C	RT	4 °C	RT	4 ° C	RT
Day 1	212.4 ± 9.76	212.4 ± 9.76	0.391 ± 0.01	0.391 ± 0.01	-33.7 ± 2.76	-33.7 ± 2.76
1 month	220.7 ± 7.57	213.1 ± 0.57	0.396 ± 0.01	0.385 ± 0.01	-31.3 ± 4.60	-38.4 ± 4.10
2 months	218.8 ± 4.67	212.1 ±5.38	0.391 ± 0.03	0.390 ± 0.01	-38.7 ± 0.28	-33.6 ±2.05
3 months	222.8 ± 5.02	215.5 ± 3.38	0.399 ± 0.01	0.388 ± 0.01	-32.9 ± 2.19	-35.4 ± 2.00

4.8. Molecular Modelling

In *silico* binding studies were performed to explore the binding themes, affinities and drug release profile of the studied complexes. Binding free energies at each stage of complex formation were calculated, and molecular stability was estimated by comparing the free energy of binding. A negative binding score indicated that the complex formation is more favourable [63]. Molecular complexes with their binding score value and potential intermolecular forces are shown in Table 14, and the 3D models are depicted in Figures 6-9.

Table 14. Binding Energy data for the various VCM-Polymer assemblies.

Complex	Binding Energy (Kcal/mol)	Binding forces	Number of Hydrogen Bonds
VCM-CHT	-0.57	ES	1
VCM-ALG	-3.32	ES	5
VCM-OA	-2.9	VdW	0
EUD-VCM-CHT	-2.53	ES & VdW	4
EUD-VCM-ALG	-2.62	ES & VdW	3
EUD-VCM-OA*	-	None	-
EUD-VCM-GTP	-3.09	ES & VdW	0
EUD-CHT-VCM-GTP	-4.11	ES & VdW	4 + 2 ES
EUD-ALG-VCM-GTP	-3.23	ES & VdW	2
OA-VCM-GTP	-3.48	ES & VdW	0

* No stable configuration was obtained. ES- Electrostatic and VdW- Van der Waals force

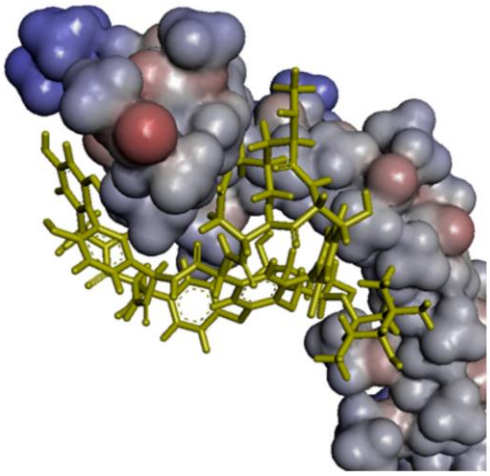


Figure 6: 3D representation of VCM (stick model) binding with the polymer EUD (solid surface model)

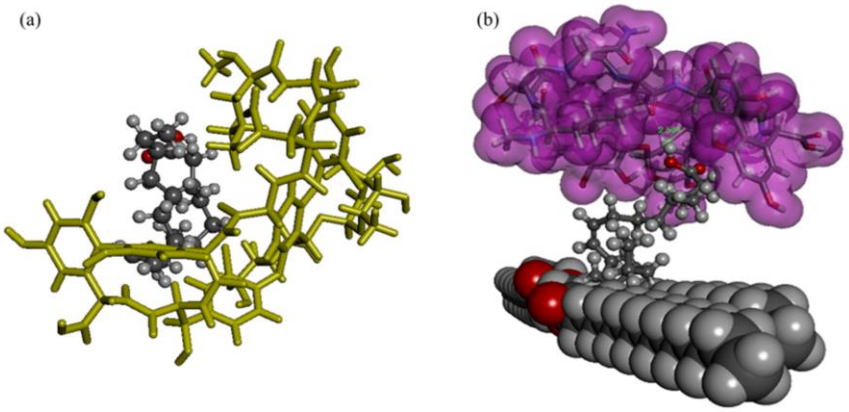


Figure 7. 3D representation of VCM (stick model) interacting with OA (ball and stick model) via Van der Waals interactions (a) and interaction of VCM (stick model with transparent violet surface) with GPT (CPK model) bound OA (ball and stick model) via hydrogen bond formation (b)

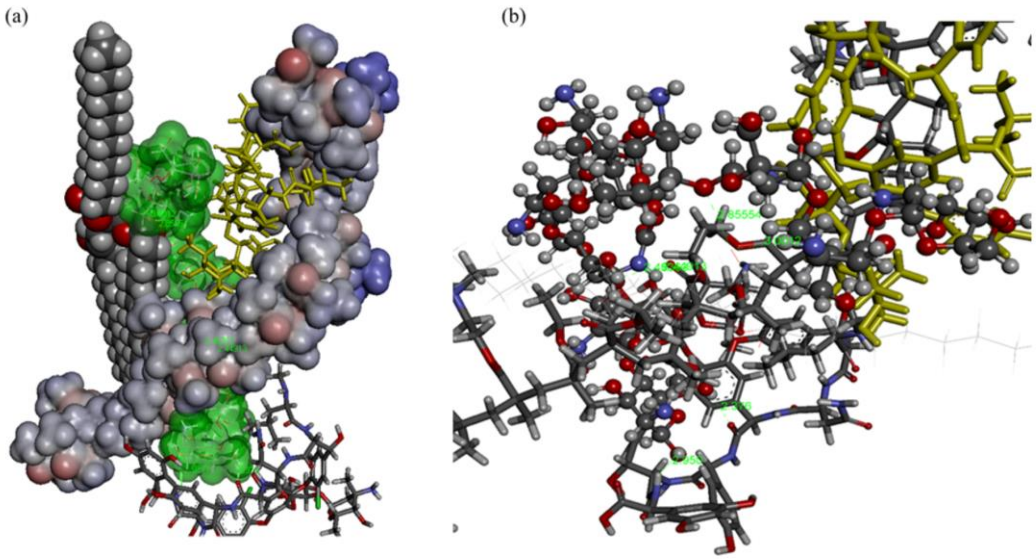


Figure 8. 3D representation of pentamolecular assembly showing Van der Waals interactions between a VCM (stick model) molecule and EUD (solid surface model), hydrogen bonds between CHT and another VCM (stick model) molecule and close electro static contacts between GTP (CPK model) and CHT (a). Close view of electro static interactions between CHT (ball and stick model) and EUD (stick model), CHT (ball and stick model) and VCM (stick model) and CHT (ball and stick model) and GTP (line model) (b).

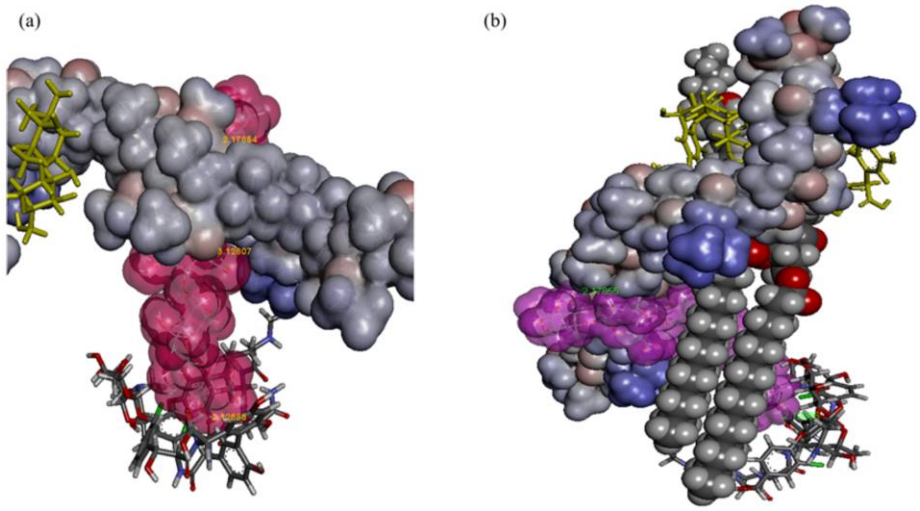


Figure 9. 3D representation of tetramolecular assembly of ALG (line model with red surface) with EUD (solid surface model) and VCM (stick model) showing three electro static contacts (a). 3D representation of pentamolecular assembly of ALG (line model with violet surface) with EUD (solid surface model) and VCM (stick model) showing two electro static contacts with GTP (CPK model) (b).

5. DISCUSSION

5.1 Particle size, PDI, ZP, EE, DL and morphology of LPNs

The results obtained (Table 4) were consistent with other LPNs that have been reported, and are within the range and indicate particles that are stable [28, 30, 64]. The data shows that with the addition of OA, the particle size, PDI and ZP decreased slightly, but the EE increased from 27.8% to 41.5%. The increase in entrapment efficiency may be due to the complex that could have formed between the GTP bound OA and VCM [45], which can be confirmed by the molecular modelling in 5.8. With the addition of CHT, the size, PDI, ZP and LC increased, while the EE increased by almost three fold, as CHT is able to easily form nanoparticles in an aqueous medium and therefore encapsulate drug molecules [65]. The increase in EE is due to the complex that is formed between VCM and CHT and can be further explained in section 5.8. The increase in size of CHT co-encapsulated LPNs may

confirm the incorporation of the helper polymer in LPNs [66]. In addition, a positive zeta potential of VCM-CHT can be attributed to the addition of CHT. It has been reported that the presence of CHT as a helper polymer will increase the size of the nanoparticles because CHT molecules will adhere and get adsorbed onto the surface of the particles [66, 67]. CHT has also been proven to increase drug bioavailability and has superior biocompatibility [68]. The negative charge of VCM-ALG can corroborate the addition of ALG [66]. The use of ALG as a helper polymer resulted in the formulation with the highest % EE (69.3 %) (p=0.0048) of VCM (Table 3), with an increase in the PDI, ZP and LC. This increase in EE with ALG could be attributed to the ionic interaction of the anionic ALG with the cationic VCM, which in turn increases the encapsulation efficiency [65]. ALG has also been reported to improve the encapsulation efficiency of hydrophilic drugs [69]. From the results obtained, it can be confirmed that the addition of helper excipients is beneficial in increasing the encapsulation efficiency of hydrophilic VCM into LPNs.

The SEM studies revealed the presence of rod shaped, homogenous and discrete particles, which differs from the spherical LPNs reported in the literature [28, 30, 64]. However, rod shaped nanoparticles have been reported for other nano systems, such as nanocrystals, elemental nanoparticles and silver nanoparticles [70-73]. The size of various VCM LPNs by DLS measurement was 212-226 nm, compared to the SEM size measurement of 190-205nm. The drug free LPNs similarly had a size measurement of 210-219nm by DLS compared to those obtained by SEM of 180-200 nm. In the case of VCM-CHT LPNs, the size obtained by DLS was 237-261 nm, while those obtained by SEM were 100-150 nm, this being a similar difference in size measurements to that observed in the literature [74, 75]. The size measured by SEM may be smaller than those obtained by DLS, as the SEM describes the size of the particles in a dried state, and DLS measures it in a hydrated state. The particle size measured by DLS therefore had a larger hydrodynamic diameter and a larger size value [75]. In

addition, it must be noted that for the size measurement of non-spherical particles the diameter needs to be redefined, as a rod shaped particle may have two different length scales, therefore, depending on the orientation of the particle, one length scale may be dominating the other [76].

It has been reported that the conditions and method of preparation will determine the shape of nanoparticles produced [77]. However, there is limited data available on the exact mechanism in which a rod shaped nanoparticle forms. An example of the effect of shape can be demonstrated in cancer studies, where it has been reported that the size, shape and chemistry of the nanoparticles will influence the concentration that can be accumulated maximally in the tumour [78]. Therefore, the manipulation of shape serves to be an important tool and can be beneficial in treating a wide variety of diseases. In terms of antibacterial activity, it has been reported that the size and shape of the nanoparticles plays an important role in its interaction with the bacteria. Rod-shaped nanoparticles have a larger surface area that can come into contact with the bacterial cell wall compared to spherical nanoparticles, hence potentiating a greater interaction between the nanoparticle and the bacterial cell wall [79]. A recent study by Sadeghi et al. showed the effect of different nano silver shapes on the antibacterial activity, and it was found that the antibacterial activity is dependent on the surface area of the nanoparticle. Rod shaped nanoparticles had a greater contact surface area than spherical nanoparticles with the bacterial cell wall, and hence exhibited increased antibacterial activity [72]. The plausible mechanism of action by which a rod shaped particle interacts with a bacterial cell involves the interaction of long axis of the rod shape with the receptors at the bacterial cell and subsequent uptake inside the cell [78]. Therefore, the shape and size of LPNs needs to be further developed and analysed in future studies in order to understand exactly how they are formed, and to improve their interaction with bacterial cells.

5.2. In vitro drug release studies

The drug release profiles (Figure 2) of all formulations showed sustained drug release from the LPNs when compared to bare VCM. In previous studies with antibiotic loaded LPNs, >80% of the drug was released after 24 h [28, 30, 34]. In contrast, all the developed VCM loaded LPNs showed a more sustained release profile with up to only 50% drug being released after a period of 24 h. This sustained release profile can be attributed to the inclusion of the helper excipients, which permits the controlled release of drug from the nanoparticles [32, 45, 80] . Co-encapsulation with CHT as a helper polymer allowed for more slow release (36.1 %) of the drug, as the CHT layer serves as an additional barrier to release VCM [81]. Similar results were reported by Dudhani et al. where, after 24 h, only 32% of drug was released from catechin encapsulated CHT nanoparticles [82]. We postulate that the addition of a co-polymer will provide a more rigid polymer matrix that will only permit a small amount of drug to diffuse out of the polymer core at regular time intervals as well as the lipid shell, which controls the release of the drug out of the nanoparticle. The addition of ALG to the LPN showed a faster release rate of 54.4% after 24 h compared to the other formulations, while VCM-ALG still showed sustained release. Similarly, the addition of the hydrophilic polymer ALG can result in sustained drug release, as the ionic polymer can enable the drug to partition in the lipid phase, which can create a more controlled release [32]. The release of VCM from VCM-OA LPNs was similar to that observed for the CHT containing LPNs (40.3%). The prolonged release is characteristic of unsaturated long chain fatty acids such as OA. The long carbon chain length of OA facilitates a slower release of the drug, due to the enhanced lipophilicity that results in better drug retaining capacity [83, 84]. In order to understand the release of VCM from the different formulations, analysis of the drug release kinetics and mechanism is explained below.

Analysis of Drug release kinetics and mechanism

VCM release from VCM and VCM-ALG LPNs followed the Weibul model with the respective higher R^2value (0.9474 and 0.9463), whereas VCM-OA and VCM-CHT LPNs follow the zero order model with higher R^2 value (0.9772 and 0.9220 respectively) as the best fit models over a period of 24 h. In addition, it was observed that Korsmeyer-Peppas (R^2 0.880 – 0.9394) model was found to be closer to the best-fit Weibul and zero-order models. The best fitting Weibul and zero order models were confirmed by comparing the calculated RMSE and AIC values for each applied models. The minimum RMSE and AIC values for Weibul and zero order models ranged from 2.930 - 3.134; 1.735 - 2.9620 and 48.54 – 49.87; 38.04 - 48.74 respectively. The best fit of Weibul and zero-order models indicate that the drug release from LPNs followed controlled-release pattern [85, 86].

The value of the release exponent (n) determined from *in vitro* VCM release data of LPNs ranged from 1.136 – 1.267 (Table 6), indicating super case-II transport mechanism for drug release. This indicates that the drug release demonstrates controlled release with polymer swelling with water absorption and polymer chain relaxation [85].

The model independent parameter mean dissolution time (MDT) is the arithmetic mean value of dissolution profile, and provides an accurate drug release rate. A lower MDT value indicates a faster dissolution rate [87, 88]. The $MDT_{50\%}$ values were calculated from *in vitro* drug release data (Table 6). The LPNs without helper lipid (VCM LPN, $MDT_{50\%}$ = 9.482) and with ALG (VCM-ALG, $MDT_{50\%}$ = 9.213) showed faster release than LPNs with OA (VCM-OA, $MDT_{50\%}$ = 14.422) and CHT (VCM-CHT, $MDT_{50\%}$ = 14.050) (Fig 2).

These findings are in good agreement with the k value (Table 6), determined according to the Korsmeyer-Peppas model, as LPNs with higher k values indicate faster release rate [89].

5.3. In vitro antibacterial studies

The MIC values for all the LPN formulations and the excipients alone are indicated in Table 8. The data on antibacterial studies of antibiotic loaded LPNs is limited, with only one report on biofilm susceptibility testing [29], therefore antibacterial data given in this study could serve as a basis for future antibiotic LPN work and can validate the potential effects of incorporating an existing antibiotic, such as VCM in a LPN delivery system. VCM itself had good activity against *S. aureus* and MRSA on day 1 (15.6 µg/ml and 18.5 µg/ml respectively), however, the activity decreased drastically from day two onwards for *S. aureus* and showed no activity against MRSA. In comparison, VCM LPNs showed better activity against *S. aureus* and MRSA up to day 2 (15.62 µg/ml and 12.5 µg/ml respectively). This shows that the LPN delivery system itself potentiated antibacterial activity. This could be due to the correlation of controlled release of the drug from the LPN shown in Figure 2, as the drug is entrapped in the polymer core, which allows for controlled release, hence sustained antibacterial activity. VCM LPNs only showed activity up to day 2, while the other formulations showed sustained antibacterial activity up to day 5, indicating the effect of the different helper excipients on the antibacterial activity. The formulation that showed the best antibacterial activity was VCM OA LPNs, with a MIC of 1.2 µg/ml against MRSA on day 2. VCM inhibits the biosynthesis of peptidoglycan and the assembly of NAM-NAG-polypeptide into the peptidoglycan chain [75]. OA is an unsaturated fatty acid that showed antibacterial activity up to day 4 in our study (400 µg/ml against *S. aureus* and MRSA). Unsaturated fatty acids such as OA are more active against Gram positive bacteria, and a correlation exists between the number of carbon atoms and antibacterial activity [90]. OA, having 18 carbon atoms, acts by inhibiting the bacterial cell attachment, and therefore has a natural protective effect against primary adhesion [91]. In combination with the VCM in the LPN, the antibacterial activity is potentiated, which could be due to the combination of antibacterial effects of the VCM and OA that acts by different mechanisms of action [45]. Similarly, CHT

itself showed antibacterial activity against S.*aureus* and MRSA up to day 2 (37.5 µg/ml). Chitosan has been reported to increase membrane permeability and cause leakage of cellular proteins, as well as inhibit the activity of enzymes [92, 93]. In combination with the LPN, the antibacterial effect was potentiated as explained above. Therefore, the development of resistance with VCM-OA and VCM-CHT LPNs could be difficult, as it will require a number of different mechanisms in the same bacterial cell at the same time [45]. The ΣFIC values in Table 8 indicate that the addition of OA to the formulation caused a synergistic action against MRSA, while CHT addition created an additive effect against *S. aureus* and a synergistic effect against MRSA. ALG showed no antibacterial activity itself, however, in combination with the LPN, it increased activity against both S.*aureus* and MRSA and showed sustained activity. We postulate that the sodium alginate creates a very tight gel polymer matrix that controls the release of VCM, and that the lipid shell sustains the diffusion of the drug out of the nanoparticle, hence a sustained release as explained in the mechanisms of drug release in section 5.2. Interestingly, VCM-OA and VCM-CHT LPNs showed better activity against MRSA than S.*aureus*, which could be attributed to the differences in the structure and composition of the bacteria. It has been reported that the most widely used mechanism of bacterial resistance in *S. aureus* is the growth of a modified penicillin binding protein (PBP), termed PBP 2a, found in MRSA [94, 95]. The outermost layer of Gram positive bacteria is peptidoglycan, and can be synthesised by membrane bound enzymes PBP [94]. In MRSA, the PBP 2a is intrinsically resistant to the inhibition by ß-lactams, and will remain active even when an antibiotic that would normally inhibit PBP enzymes is present. This will cause a change in the role of PBP enzymes in the cell wall synthesis, thereby allowing the growth in the presence of ß-lactam inhibitors, such as methicillin [94, 96]. The increase in activity of VCM-OA and VCM-CHT LPNs observed against MRSA could be due to the higher valency of the VCM-OA and VCM-CHT nanoparticle, which could result in better binding of PBP 2a

of MRSA than PBP of *S. aureus* [96]. We postulate that the VCM OA and VCM chitosan binding to the PBP 2a might be greater compared to PBP enzyme, thereby resulting in more activity against MRSA. A paper by Choi et al. explains the mechanistic method in which vancomycin-conjugated G5 PAMAM dendrimers act against S.*aureus* and MRSA [97], however, there is no data available on novel drug delivery systems against S. *aureus* and MRSA. Therefore, further studies using molecular modelling and other methods need to be carried out in order to confirm this hypothesis and explain the interaction of LPNs with the bacterial cell wall.

5.4. Gel Electrophoresis

Based on the results shown in Figure 3, it can be seen that *S. aureus* and MRSA LPN treated bacterial cells showed a difference in appearance in the bands of all molecular weight proteins when compared to the control. This indicates the disruption of the bacterial cell, and suggests that the LPN formulations were able to permeate bacterial cell membranes by reducing the content of cellular soluble proteins [58] with VCM-CHT as the most active formulation.

5.5. X-Ray Diffraction

XRD is an important method used to detect any changes in the crystalline nature of the drug [98]. The results in Figure 4 indicate that all the formulations showed the peaks of the lipid with a change in the intensities of the peaks, suggesting that the crystalline lipid changed slightly after the formation of nanoparticles [47]. Raw VCM was in an amorphous state, as seen from the absence of diffraction peaks and a broad spectrum, and therefore no changes in the drug were observed in the LPN formulations [99].

5.6. Differential Scanning Calorimetry (DSC)

The thermograms obtained from the DSC (Figure 5) showed the presence of a GTP peak in VCM LPNs, which was indicative of presence of the lipid in the LPN. The absence of the

VCM peak revealed that the drug was entrapped within the LPN [100], and was in the amorphous state. The amorphous form is expected to have increased surface area, high energy, solubility, dissolution rate and bioavailability [101, 102]. CHT showed an endothermic peak at 165.08 °C and this was due to the influence of strong inter- and intra- molecular hydrogen bonds. This strong intermolecular hydrogen bonds is characteristic of the insoluble nature of the polymer in water [103]. The shift in endothermic peak of plain GTP from 73 ^{o}C to 66 ^{o}C in LPNs could be due to increase in surface area and a reduction in particle size which lead to a decrease in melting enthalpy [48, 104]. The loading of VCM and helper lipid and polymers did not affect the melting behaviour of GTP. The VCM-OA LPN exhibited an endotherm at 135.95° C, which could be due to the product of ionic interaction between the carboxylic acid function of OA and amine function of VCM. VCM-ALG LPN showed a second peak at 118.91° C, which is characteristic of the peak that is shown in the ALG thermogram with a slight shift. Although, there were no broad endothermic peaks at higher temperature in the individual components, VCM-OA and VCM-CHT LPNs did show these peaks. These additional broad endothermic peaks might have appeared due to the phase transition of drug-polymer system (VCM-EUD in case of VCM-OA LPNs and VCM-CHT-EUD in case of VCM-CHT LPNs). However, the phase behaviour of a drug-polymer combination can be extremely complicated since the drug can be present in any of the polymeric forms such as crystalline, partially amorphous or completely amorphous form [105]. Therefore, in addition to phase transition [105] one of the possibilities could be co-crystallization of different ingredients of the formulation in the presence of ethanol used in the manufacturing process. The co-crystalline forms have been reported to have different melting behaviours than their individual components [106, 107]. Similar kind of peaks at a higher temperature were observed in DSC thermograms of co-crystals of low density polyethylene and high density polyethylene [108].

5.7. Stability studies

Stability studies at a developmental stage of formulation development provides data which may be of value to determine shelf-life, storage conditions and container closure system of a proposed new product. It therefore provides preliminary data for confirming the potential of the product to eventually meet full stability requirements as per regulatory approval requirements [109, 110]. No change in the physical appearance and colour as well as absence of agglomeration was observed in all the developed formulations (VCM, VCM-CHT, VCM-OA and VCM-ALG LPNs) upon storage at 4 ^{o}C and room temperature. Further no significant differences ($p > 0.05$) in size, PDI and ZP was observed at all the time periods tested (0-90 days) at specified storage conditions. These results confirmed the stability of developed LPNs under the prescribed conditions.

5.8 Molecular Modelling

To correlate the complexes stability with their EE and the drug release, the intermolecular interactions governing the formation of lipid-polymer-drug assemblies were analysed. The EE and drug release were correlated with the binding free energy of the complexes based on hydrogen bonding, electrostatic and Van der waals forces.

VCM LPNs

The binding pose of VCM with EUD implies that the drug preferably binds with the polymer by means of Van der Waals forces, as no hydrogen bonds are seen between EUD and VCM (Figure 6). Due to the weak intermolecular forces, the molecular complex could be easily dissociated, and hence the drug is released instantly, as observed in the drug release kinetics study (Figure 2)

VCM-OA LPNs

As a bimolecular complex, OA is completely trapped by Van der Waal's forces inside the hydrophobic pocket of VCM, as its structure is relatively smaller (Figure 7a). This prevents further conjugation of OA with EUD to form trimolecular complex and hence, no stable configuration was obtained for the EUD-VCM-OA complex. This suggests that OA-VCM conjugate may not be compatible inside the polymer network. However, the GTP bound OA was able to bind with VCM by forming a hydrogen bond between its carboxyl group and carbonyl oxygen of the glycopeptide residue of VCM molecule (Figure 7b). This indicates that VCM might be encapsulated preferably inside the fatty acid network in the presence of OA, resulting in increased encapsulation of VCM in VCM-OA LPNs. As per the drug release kinetics the VCM, release was slower in OA system, which might be due to entropy driven aggregation of lipid encapsulated VCM in aqueous medium that could direct the system to release the drug at slower rate. Furthermore, the high free binding energy for the OA system (ΔG_{bind} = -3.48 Kcal/mol) compared to ALG (ΔG_{bind} = -3.23 Kcal/mol) and native EUD systems (ΔG_{bind} = -3.09 Kcal/mol) imparts more stability for the complex, and releases the drug at slower rate.

VCM-CHT LPN

CHT and ALG are able to interact with EUD by hydrogen bonding, and additionally incorporate VCM molecule for subsequent binding. The simultaneous binding of CHT/ALG with EUD and VCM explains higher entrapment of VCM in the polymer network. Overall, VCM-EUD complex can thus entrap more VCM molecules in the presence of helper polymers and form a stable supramolecular complex, as seen in Figure 8. The encapsulation efficiency of EUD is therefore increased by the supramolecular linking of the helper polymers. Comparison of binding affinities among the helper polymer complexes revealed that CHT binding mode is relatively tighter than ALG due to greater number of electrostatic bonds in the tetra and

pentamolecular complexes. The carbonyl oxygen of EUD methacrylate functional group accepts two hydrogen bonds instantaneously from *N*-acetyl functional group (d_{A---H} = 2.44913 A^o) and hydroxyl group (d_{A---H} = 2.48363 A^o) of CHT. Supported by this bifurcated hydrogen bond, the EUD bound CHT further binds to VCM molecule by forming two hydrogen bonds with carbonyl (d_{A---H} = 2.376 A^o) and amine (d_{A---H} = 2.950 A^o) functional groups in the glycopeptide chain of VCM. The binding of GTP with the above tetramolecular complex is facilitated by two close electrostatic interactions that are observed among the carbonyl functional group of the GTP and the glycosidic oxygen atoms of the CHT (d_{o---o} = 2.85554 and 3.0012 A^o). Thus, with a total of six electrostatic intermolecular bonds, CHT in the pentamolecular complex (ΔG_{bind} = -4.11 Kcal/mol) is more stable and supports controlled break down of the complex, which ultimately results in sustained drug release.

VCM-ALG LPN

A very strong affinity for VCM with ALG in the absence of EUD and GTP was found. Howver, as a tetramolecular complex, the binding affinity of ALG with VCM is altered due to the conformational change brought by the EUD interaction. In the tetramolecular complex (Figure 9a), ALG binds to VCM by forming a hydrogen bond (d_{A---H} = 2.12685 A^o) between carbonyl functional group in the glycopeptide chain of VCM and the hydroxyl group of ALG. A strong hydrogen bond (d_{A---H} = 2.17854 A^o) between the carbonyl oxygen of EUD methacrylate functional group and hydroxyl functional group of ALG was observed. A weak hydrogen bond (d_{A---H} = 3.12807 A^o) was also observed between the carboxylate group of ALG and the carbonyl oxygen of EUD methacrylate function. Interestingly, this weak hydrogen bond is not seen in the pentamolecular complex (Figure 9b). The altered binding affinity due to conformational changes and a reduction in hydrogen bonds produced a less stable ALG system at high level molecular complexes. Though the ALG facilitates the EE as similar to CHT, the lower free energy of binding for the ALG system (ΔG_{bind} = -3.23 Kcal/mol) at high molecular

level allows the components to dissociate faster than CHT, and hence the drug is released much faster than that of CHT and OA system. No major difference in drug release rate was observed among ALG and the native EUD system, as the helper polymer is not tightly entangled with the EUD. The loose binding of ALG with EUD allows ALG to interact more freely with solvent medium and releases the drug at much faster rate.

6. CONCLUSION

LPNs with suitable size, PDI and ZP were successfully formulated to deliver the antibiotic VCM. Furthermore, critical properties of the LPN system such as drug encapsulation, drug release and antibacterial activity, was further enhanced by the addition of the helper lipid OA and helper polymers, CHT and ALG. Compared to VCM LPNs, the LPN systems with the addition of helper lipid and polymers, exhibited a controlled release profile, higher drug encapsulation, sustained and enhanced antibacterial activity against both sensitive and resistant strains of bacteria. The EE and drug release was corroborated by the release kinetics data. In addition, *in silico* modelling also revealed an understanding of the EE and drug release of the VCM LPN systems. This LPN system demonstrates the potential for future studies incorporating other antibiotics, as well as further formulation development to improve its properties as a drug delivery system.

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CHAPTER 4. CO-AUTHOR REVIEW PAPER

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4.1 Introduction

The following paper was published in Journal of Pharmaceutical Sciences (Impact Factor: 2.59) which is an international ISI peer reviewed journal and reviews all the existing nanoengineered drug delivery sytems for antibiotic therapy:

Ms. N Seedat contributed by performing a literature review of the Lipid polymer hybrid nanoparticles (LPN) section. In addition, she constructed the relevant summary of literature table for the LPN section as well as contributed to the writing of this section. The remaining authors were co-authors on the paper.

Nanoengineered Drug Delivery Systems for Enhancing Antibiotic Therapy

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ABSTRACT: Formulation scientists are recognizing nanoengineered drug delivery systems as an effective strategy to overcome limitations associated with antibiotic drug therapy. Antibiotics encapsulated into nanodelivery systems will contribute to improved management of patients with various infectious diseases and to overcoming the serious global burden of antibiotic resistance. An extensive review of several antibiotic-loaded nanocarriers that have been formulated to target drugs to infectious sites, achieve controlled drug release profiles, and address formulation challenges, such as low-drug entrapment efficiencies, poor solubility and stability is presented in this paper. The physicochemical properties and the *in vitro*/*in vivo* performances of various antibiotic-loaded delivery systems, such as polymeric nanoparticles, micelles, dendrimers, liposomes, solid lipid nanoparticles, lipid–polymer hybrid nanoparticles, nanohybirds, nanofibers/scaffolds, nanosheets, nanoplexes, and nanotubes/horn/rods and nanoemulsions, are highlighted and evaluated. Future studies that will be essential to optimize formulation and commercialization of these antibiotic-loaded nanosystems are also identified. The review presented emphasizes the significant formulation progress achieved and potential that novel nanoengineered antibiotic drug delivery systems have for enhancing the treatment of patients with a range of infections. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: infectious diseases; nanoantibiotics; antibiotic resistance; nanodrug delivery systems; nanotechnology; polymeric drug carrier; polymeric drug delivery systems; controlled release; targeted drug delivery

INTRODUCTION

Infectious diseases continue to be one of the main reasons for death globally for both adults and children, and is recognized as a signi cant public health challenge.[1] Africa and South Africa in particular have a high burden of infectious diseases, including speci cally a large portion that is of bacterial origin. As a result of this, gastrointestinal, respiratory, sexually transmitted, and hospital acquired infections are leading causes of death in the developing world.[2] In addition, emerging and re-emerging infectious diseases,[3] together with issues such as the growing global trade and international travel and the probability of bioterrorist attacks in several countries, have compounded the seriousness of infectious diseases. Importantly, there is a recent growing acknowledgement that infections also play an important role in facilitating the occurrence of noncommunicable diseases. For example, diseases such as certain cardiovascular disorders, cancers, asthma, and gastrointestinal diseases have been reported to be linked to infectious diseases (including bacterial infections) as an underlying cause/risk factor.[4] The consequent adverse economic, social, and political impact of the global burden of infectious diseases therefore warrants novel and effective treatment strategies to overcome these challenges.

The advent of antibiotics, which was initiated with the introduction of penicillin more than 70 years ago and the more advanced compounds in later years, revolutionized the treatment of infectious diseases, and contributed signi cantly to decreasing the associated morbidity and mortality.[3] Antibiotics are considered pivotal in virtually all critical therapeutic areas, for example, general surgery including organ transplant procedures, treatment of premature babies, and chemotherapy in cancer patients cannot be achieved without effectively treating and preventing bacterial infections.[5] However, there are numerous limitations associated with the current antibiotic drug therapies. Several available dosage forms of antibiotics are compromised by inadequate drug concentrations at target infection sites, severe side effects, increased frequency of administration, and poor patient compliance that compromise drug therapy.[3,6] These limitations, together with the widespread use and abuse of antibiotics, have led to their most serious limitation, resistance to bacterial microorganisms. Microbial resistance nulli es the use of even the most potent antibiotics, which leads to patient suffering and/or mortality because of infection control failure and escalated health care costs.[3] Among these resistant pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA),[7] vancomycin-resistant *Enterococcus* (VRE),[8] vancomycin-resistant *S. aureus* (VRSA),[9] and penicillin-resistant *Streptococcus pneumonia*[10] have become major clinical threats. The antibiotic resistance crisis has also been further aggravated by pan drug-resistant and extensively drug-resistant organisms to antibiotics, which has reached alarming levels globally.[5,11]

According to a recent report released by the WHO on April 30, 2014, antibiotic resistance can no longer be regarded as an issue for the future but rather a current crisis that requires urgent interventions.[12] Although new antibiotics are being investigated to overcome antibiotic resistance, a steady and gradual decline in the introduction of new drugs have been reported by the US Food and Drug Administration (FDA).[13] This is because of exorbitant costs and lengthy times for eventual regulatory approval of new compounds, as well as low returns on

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investment, which compounds the current crisis.[14] Two systemic antibacterial agents were approved for use in humans by the US FDA from 2008 to 2012, compared with 16 approved from 1983 to 1987.[15] It is clear that the pace of drug development and registration has not been timeously responsive to the rapid development of resistance by microbial pathogens. This escalating emergence of antibiotic resistance to currently used antibiotics and decline in introduction of new antibiotic drugs is clearly a threat to human health globally. The search for new and effective strategies to enhance drug therapy with current antibiotics is therefore recognized globally as a major focus area of research priority.

The signi cant bene ts of using nanotechnology for treating various diseases such as cancer,[16 19] AIDS,[20 24] in ammation,[25 27] and hypertension[28 30] by improving the solubility, bioavailability, ef cacy, and speci city of drugs are widely documented in the literature. Nanotechnology, which refers to the design, production, and application of nanosized materials, is regarded as a new paradigm for optimizing the outcomes in infectious diseases treatment.[3]

Novel nanosized drug delivery systems could be a promising strategy to overcome the current challenges associated with antibiotic therapy because of their unique physicochemical properties. These include their large ratio of surface area to mass, small size, and unique interactions with microorganisms and cells of the host, as well as their ability to be structurally and functionally modi ed.[31,32] The advantages of a nanosized antibiotic drug delivery system include targeted delivery, relatively uniform distribution in the identi ed tissue, enhanced cellular internalization and solubility, sustained drug release and minimized side effects, and improved patient compliance.[33,34] Furthermore, nanosystems themselves have been found to inherently overcome existing speci c drug-resistance mechanisms by microbes.[35] In addition, the codelivery of multiple antibiotics into these nanosystems that are capable of having antimicrobial activity and overcoming resistance mechanisms themselves can promote synergistic activities and resistance overcoming effects.[31] These advantages are recognized as major contributors to overcoming bacterial resistance associated with poor delivery of antibiotics.[36]

Nanodrug delivery systems therefore offer an advanced and superior approach to overcoming several limitations associated with antibiotic drug therapy, including the serious global threat of antibiotic resistance. Compared with cancer and cardiovascular disease conditions, use of nanodrug delivery systems for speci cally encapsulating and delivering antibiotic drugs is still in its infancy.[3] Because of its potential advantages, there has been a surge of data in the literature on a range of differently engineered antibiotics-loaded nanodrug delivery systems. A perusal of the literature highlights the need for a review paper that speci cally focuses on the various reported nanodrug delivery systems to date that have been used for antibiotics. A comprehensive review of the various nanoengineered drug delivery systems that have emerged for antibiotic drugs is presented. The paper will therefore identify the technological progress that has been achieved regarding the development of these delivery systems and their potential for addressing the various formulation and therapeutic challenges with current antibiotic therapy. Future studies that need to be conducted for optimization and commercialization of these antibiotic-loaded nanosystems will be identi ed.

NANOENGINEERED ANTIBIOTIC DELIVERY SYSTEMS

The development of nanomedicines has facilitated an increase in the therapeutic index of many components. With changes in size from tens of micrometers to tens or hundreds of nanometers having been a signi cant technological and medical breakthrough.[37] A comprehensive literature search on several databases from 1960 to 2014 identi ed a range of nanodelivery systems for antibiotics that include liposomes, polymeric nanoparticles (PNPs), solid lipid nanoparticles (SLNs), lipid polymer hybrid nanoparticles (LPHNs), dendrimers, nanoemulsions (NEs), micellar systems, nanostructures made of pure carbon [carbon nanotubes (CNTs), nanosheets, and nanorods], nanohybrids, and others. As the 10 main nanodelivery systems that are used for antibiotic delivery, these will be discussed and evaluated in detail.

Liposomes

Liposomes, the rst closed bilayer systems, were described in 1965 and were soon proposed as drug delivery systems[38] using natural or synthetic lipids. Phosphatidylcholine (PC), which is a neutral phospholipid that contains fatty acyl chains, is one of the most commonly used lipids in liposome preparation. Adjustment of membrane rigidity and stability can be achieved by incorporating cholesterol into the preparation.[39] The two main classes of liposomes are multilamellar vesicles that comprise multiple phospholipid bilayer membranes, and unilamellar vesicles (ULVs) comprising a single lipid bilayer. ULVs can be further divided into large ULVs and small ULVs.[40] Since their inception, the most commonly applied methods used for preparing liposomes include thin- lm hydration,[41] reversed-phase evaporation,[42] solvent injection techniques,[43,44] and detergent dialysis.[45] Materials used for preparation, classi cation, and different techniques for the preparation of liposomes can be found elsewhere in the literature.[40,46 57]

Liposomes, consisting of phospholipid bilayers, are spherical lipid vesicles that can provide an improvement in the solubility of compounds and promote fusion with biological membranes and the subsequent release of their entrapped compounds into the target site.[58 60] In addition, it is possible to incorporate both hydrophilic and hydrophobic antimicrobial drugs in the aqueous core and in phospholipid bilayer, respectively.[33,61] Liposomes appear to be the earliest reported nanodrug delivery systems studied for antibiotic delivery in the literature, and clearly provided a platform for conceptualizing and developing other antibiotic nanodelivery systems. They have emerged as nanodelivery vehicles for antibacterial therapy, speci cally as they promote targeted delivery to the infection site, improve pharmacokinetics, reduce toxicity, and enhance antibacterial activity of antibiotics.[62]

Historically, the use of liposomes for antibiotic entrapment can be traced back to the early 1970s, after which this eld has expanded signi cantly to include various antibiotics in liposomes to effectively treat infections. A summary of various reported liposomal systems for antibiotic therapy with their rationale for formulation development is provided chronologically in Table 1. This overview clearly shows that liposomes have diverse applications for addressing various challenges with antibiotic therapy. Their potential for treating numerous disease conditions, being effective against a wide range of microorganisms, reducing toxicity, enhancing stability, and achieving sustained drug release and activity have been con rmed. More

Table 1. A Chronological Overview of Antibiotic Liposomal Development

Formulation	Active Ingredient	Targeted Microorganism	Rationale for Formulation	Reference
PC, diacetyl phosphate, and cholesterol	Filipin	None	Removal of the haemolytic activity of Filipin.	Ref. 63
Egg lecithin, cholesterol, phosphatidic acid, dipalmitoyl lecithin, and stearylamine	Potassium benzyl penicillin	None	Lysosomal localization of liposome-entrapped drugs in the liver and spleen.	Ref. 64
Egg PC, cholesterol, and phosphatidic acid	Dihydrostreptomycin	*S. aureus*	Killing of intraphagocytic *S. aureus*.	Ref. 65
Egg PC, cholesterol, diacetylphosphate, and total lipid extract of rat intestinal mucosa	Ampicillin, amoxicillin, cephalexin, sodium cepfazolin, sodium ceftezol, sodium cephalothin, cephaloridine, and cephradine	None	Study of the liposomal membrane permeability to antibiotics.	Ref. 66
PC, cholesterol, and phosphatidylserine	Cephalothin	*S. typhimurium*	Intracellular killing of the microorganisms.	Ref. 67
PC, cholesterol, and phosphatidylserine	Penicillin-G	*Listeria monocytogenes*	Treatment of intracellular infections	Ref. 68
Proprietary formulation prepared by Fountain Pharmaceuticals, Inc. (Knoxville, Tennessee)	Tobramycin and silver sulfadiazine	*P. aeruginosa*	Topical delivery for treatment of soft tissue wounds.	Ref. 69
Egg PC, cholesterol, and diacetylphosphate	Vancomycin and teicoplanin	MRSA	Treatment of intracellular MRSA.	Ref. 70
Egg lecithin and cholesterol	Amikacin, netilmicin, tobramycin	*P. aeruginosa, Xanthomonasmaltophilia E. Coli, Streptococcus faecalis,* and *S. aureus*	Enhancement of antibacterial activity.	Ref. 71
Soybean PC and cholesterol	Ampicillin	*Micrococcus Luteus*	Improvement of drug stability and retention of antibacterial activity.	Ref. 72
Hydrogenated PC and cholesterol	Gentamycin	None	Modi cation of drug release pro le to achieve sustained drug release.	Ref. 73
Proprietary formulation prepared by Bristol	Amikacin	*Mycobacterium Avium*	Prolongation of antibacterial activity in *in vivo* studies.	Ref. 74
Dipalmitoyl-DL-α-phosphatidyl-L-serine, cholesterol, lipopolysaccharide, L-α-phosphatidyl-DL-glycerol, dihexadecyl hydrogen phosphate, dihexadecyl hydrogen phosphate, dihexadecyl hydrogen phosphate, and 1,2-dipalmitoyl-*sn*-glycero-phosphatidic acid sodium salt	O oxacin	*Enterococcus faecalis, Escherichia Coli, S. aureus,* and *P. aeruginosa*	Enhancement of the activity of uoroquinolone antibacterials.	Ref. 75
Dipalmitoylphosphatidylcholine, cholesterol, and dimethylammonium ethane carbamoyl cholesterol	Penicillin-G	*S. aureus*	Enhancement of the effectiveness of penicillin-G at low concentration and short exposure time.	Ref. 76
Cationic, anionic, and neutral liposomes lecithin (egg PC), stearylamine and cholesterol, L-α-phosphatidyl-DL-glycerol and cholesterol, and lecithin and cholesterol	Cipro oxacin and vancomycin	*S. aureus*	• Treatment of chronic *staphylococcal osteomyelitis* by combination therapy • Reduction in nephrotoxicity, enhancement of antibacterial activity depending on charge and sonication time.	Ref. 77

Continued

Table 1. Continued

Formulation	Active Ingredient	Targeted Microorganism	Rationale for Formulation	Reference
1,2-Dioleoyloxy-3-trimethylammonium-propane, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, PC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	Meropenem	*P. aeruginosa*	Enhancement of antibiotic activity against sensitive and resistant strains.	Ref. 78
1,2-Dimyristoyl-*sn*-glycero-3 phosphocholine and cholesterol	Gentamicin	*P. aeruginosa*	Improvement of killing time and prolongation of antimicrobial activity to treat chronic respiratory infections associated with cystic brosis.	Ref. 79
PC, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-3000] (ammonium salt), L-α-phosphatidyl ethanolamine-*N*-(lissaminerhodamine B sulfonyl) (ammonium salt) and 1,2-distearoyl-3-trimethylammonium-propane (chloride salt), poly(ethylene glycol)-α-disteroylphosphatidyl-ethanolamine,-ω-benzotriazole carbonate MW 3400	Rifampicin	*S. epidermidis*	Development of an antimicrobial barrier on polymer surface of interest for medical applications.	Ref. 80
1,2-Distearoyl-*sn*-glycero-3-phosphocholine, methylpolyethyleneglycol 1, 2-distearoyl-phosphatidyl ethanolamine conjugate	Vancomycin	None	Increasing lung tissue concentration of vancomycin for effective treatment of pneumonia caused by MRSA by surface modi cation of liposomes with PEG.	Ref. 81
Egg PC and cholesterol	Vancomycin	MRSA	Selective delivery of antimicrobials to the sites of bacterial infections by utilizing bacterial toxins to activate drug release from gold nanoparticle-stabilized phospholipid liposomes.	Ref. 82

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recent studies are focusing on exploiting the bene ts of surface modi cation and responsive drug delivery to further enhance the effectiveness of liposomal systems. Some of these studies are brie y discussed further.

One of the rst applications for liposomes in antibiotic drug delivery is reported for lipin, a polyene macrolide antibiotic known for its haemolytic activity.[63] It should however be noted that the liposomes in this study were not explored as a carrier, but rather as a model to test the sterol receptor hypothesis of polyene action. Similarly, a few years later, liposomes were also used as a model to investigate the intestinal absorption mechanisms of several antibiotics.[66] Although not used as a delivery system itself, liposomes have proved useful in providing the necessary information for optimizing therapy with polyene macrolide antibiotics.

The potential of liposomes as an antibiotic carrier probably began with Gregoriadis.[64] He entrapped potassium benzyl penicillin in liposomes composed of egg lecithin, cholesterol, phosphatidic acid, dipalmitoyl lecithin, and stearylamine to overcome the failure of penicillin to penetrate cells of the reticuloendothelial system (RES). These *in vivo* studies with rats showed lysozymal localization of penicillin-entrapped liposomes into the liver and spleen.[64] This early study did not focus on antibacterial activity against microorganisms, as researchers at that stage were attempting to prove its targeting potential. The intracellular residence of bacteria may complicate effective treatment of bacterial infections. In subsequent studies, other research expanded this area, and reported specifically on intracellular killing of various classes of sensitive and resistant bacteria by liposomal formulations using drugs such as dihydrostreptomycin,[65] cephalothin,[67] penicillin-G,[68] vancomycin, and teicoplanin.[70] In addition to intracellular targeting, liposomes have been studied for topical applications, with reports indicating that topical infections of soft tissues by *Pseudomonas aeruginosa* can be effectively treated by liposomal tobramycin silver sulfadiazine.[69]

Several other liposome-based antimicrobial drug delivery systems have also been recently developed for various applications and for reducing antibiotic toxicity,[58] and have found applications in vaccine technology. Zhao et al.[83] genetically linked the urease linear epitope with cholera toxin B subunit to obtain a novel fusion peptide CtUBE and expressed it in *Escherichia coli*, and formulated an oral liposome vaccine against *H. pylori*. The sizes of the liposomes were between 100 and 500 nm, and almost 71.4% CtUBE was entrapped in liposomes. The study demonstrated that after oral immunization, liposomal CtUBE was able to protect BALB/c mice from *H. pylori* infections.[83] Another unique study emphasized the diverse applications of liposomal antibiotic formulations. Surface coating of polystyrene by cationic rifampicin-loaded liposomes was performed in order to develop an antimicrobial barrier on a polymer surface to be exploited for medical uses.[80] The rifampicin-loaded liposomes as an antimicrobial barrier reduced bacterial growth on polystyrene, with activity being dependent on the charge of the liposomes with the polystyrene surface. Effective activity against various organisms for other disease conditions, such as gentamicin liposomes[79] and meropenem liposomes[78] against *P. aeruginosa*, ampicillin liposomes against *Micrococcus luteus*,[72] and penicillin liposomes against *S. aureus*[76] have also been reported.

Another research goal by liposomal researchers has been to achieve prolonged release and/or enhanced activity of antibiotics. In early studies, Omri and Ravaoarinoro[71] entrapped various antibiotics (amikacin, netilmicin, and tobramycin) into liposomes. Although netilmicin had lower liposomal encapsulation ef ciencies than tobramycin and amikacin, it had reduced minimum inhibitory concentrations (MICs) against Gram-positive and Gram-negative bacteria compared with free drug, whereas liposomal tobramycin and amikacin antibacterial activity was not improved as compared with the free solution. In this study, only encapsulation ef ciencies and antimicrobial activities were reported. Being initial liposomal antibiotic formulation studies in this eld, other critical data such as size, polydispersity index, surface charge, morphology, and stability were not reported, unlike more recent papers where this is essential. Prolonged and/or enhanced activity has also been reported for liposomal formulations, such as gentamycin,[73] amikacin,[74] o oxacin,[75] penicillin-G,[76] meropenem,[78] and gentamicin[79] against a wide range of microorganisms. The prolonged antibacterial activity has been attributed to the sustained release of drugs from liposomes, which have also been shown to enhance the stability of antibiotics. For example, it has been shown that free ampicillin lost 50% initial activity after 5 weeks of storage at 4°C, whereas some of the liposomal ampicillin formulations lost only 17% activity.[72] On the basis of the differences between liposomal formulations, it would be useful in future to investigate how variables such as drug encapsulation ef ciencies and lipid content affect stability as well as antimicrobial activity.

Liposome size and surface charge can be modi ed and optimized depending on its therapeutic application.[84] Liposomes encompassing surface modi cation with materials such as glycolipids or sialic acid have been prepared.[85] Thus, cationic or anionic liposomes can be prepared by using cationic or anionic ingredients in the liposomal formulations. In one such study, to establish a new antibiotic therapeutic approach against chronic staphylococcal osteomyelitis infections presenting in rabbits, two antibiotics, namely, cipro oxacin and vancomycin were encapsulated alone and in combination in liposomes. The study was undertaken to: (1) lower nephrotoxicity, (2) overcome poor antibiotic accumulation in bone tissue, (3) completely sterilize bone tissue by combination therapy, and (4) most importantly to facilitate optimal liposome bacterium interaction via evaluation of cationic, anionic, and neutral liposomes.[77] The results showed a greater percentage of drugs being entrapped in charged liposomes than neutral, and among all the three formulations, enhanced antibacterial activity against *S. aureus* was observed for cationic liposomal formulation. This proved the concept that interaction between the cationic liposomes and negatively charged bacterial cell surface can occur.[77,86] Reduction in nephrotoxicity was also reported with animal studies using rabbits.

Another active area of research is surface modi cation of liposomes, which is used for various purposes, such as stabilizing liposomes against fusion[87] and controlling liposome blood clearance.[88] The incorporation of poly-(ethylene glycol) (PEG) in the liposome composition represented a major step in the development of liposomes with increased circulation and half-life.[85] Pneumonia caused by MRSA is dif cult to treat with vancomycin because of low lung tissue and intracellular penetration of vancomycin, leading to MRSA evading phagocytic killing. Muppidi et al.[81] proved that MRSA pneumonia can be effectively treated by using PEGylated liposomal vancomycin as compared with conventional and non-PEGylated

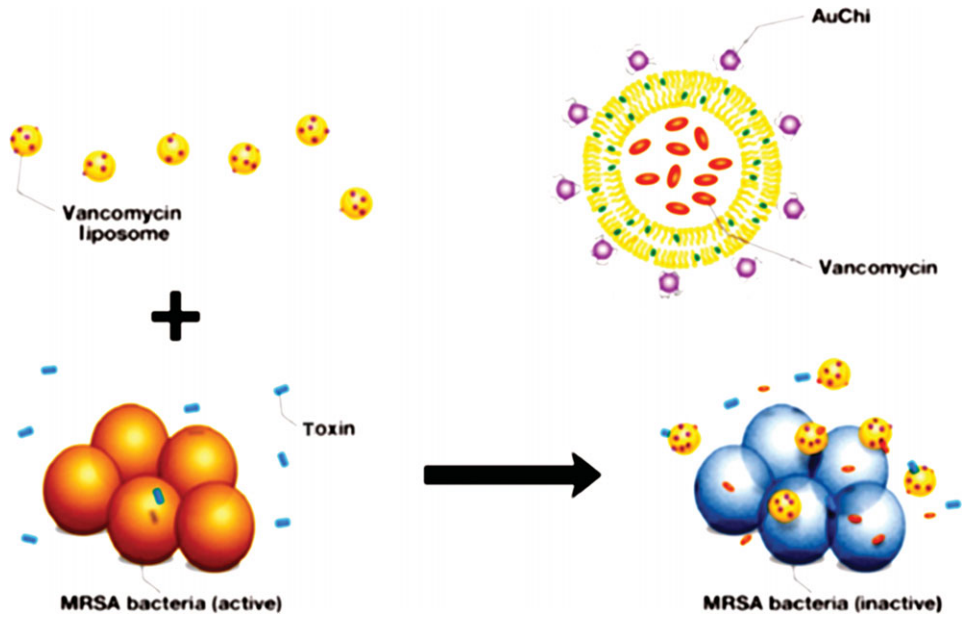

Figure 1. Schematic principle of bacterial toxin-triggered antibiotic release from gold nanoparticle-stabilized liposomes to treat toxin-secreting bacteria. Reproduced from Pornpattananangkul et al.[82] with permission from American Chemical Society.

preparations. This was possible because of the ability of PEGylated liposomal vancomycin to signi cantly extend circulation time in the blood, and increase lung, liver, and spleen deposition while also reducing accumulation in the kidney tissue. It has been suggested that administration of PEGylated liposomal vancomycin may enhance the effective treatment of MRSA pneumonia and simultaneously reduce the nephrotoxicity risk. This study was purely an *in vivo* study, and the promising results with these surface modi cation studies should be followed up with formulation optimization and characterization investigations to con rm stability and activity. It would also be interesting to investigate how the PEGylation affects antibacterial activity in terms of interaction with bacterial cell membranes.

In a recent paper, surface modi cation of liposomal surface was explored not only for altering distribution, but also for achieving triggered drug release at an infection site. A new approach to differentially release vancomycin to the site of infection to inhibit the growth of *S. aureus* for topical treatment of skin bacterial infections was developed by attaching chitosan-modi ed gold nanoparticles (AuChi) onto the surface of negatively charged liposomes.[82] This strategy was based on the fact that few bacteria release a toxin, and this toxin can be used to activate drug release from AuChi-stabilized liposomes. In nature, *S. aureus* secretes alpha haemolysin (α-toxin) as a water-soluble 34 kDa protein monomer.[89] A heptameric structure with a central 2 nm size pore is formed when the α-toxin spontaneously incorporates into the lipid membranes and self-oligomerizes. This pore permits the passive diffusion of small molecules of up to 3 kDa through the membranes.[90,91] Figure 1 illustrates the principle involved in the selective release of vancomycin at the site of infection.[82] The mechanism involves binding of AuChi to the negatively charged surface of liposomes via electrostatic attractions, which stabilizes liposomes by preventing fusion with one another and also prevents unwanted drug leakage. When the stabilized liposomes have reached the vicinity of *S. aureus*, the α-toxin secreted by bacteria inserts into the liposome membrane and forms pores that allow the encapsulated vancomycin to be released. The vancomycin that has been released close to the bacteria will then be allowed to exert its rapid and local antibacterial activity.

Incubation studies with MRSA con rmed 48% and 100% release within 0.5 and 24 h, respectively, and no drug release in the absence of MRSA. Vancomycin release in the presence of MRSA therefore con rmed the drug release in the presence of the bacterial toxin only. The study did not report release data on unmodi ed vancomycin liposomes, which could have provided additional supportive con rmation of the principle of triggered release with the AuChi modi cation. Antibacterial studies showed that the AuChi vancomycin liposomes inhibited microbial growth to the same level as vancomycin liposomes. Therefore, the triggered release only on exposure to the toxin with retention of antibacterial activity was considered an improved approach for enhancing therapy with vancomycin. This approach will certainly provide a new paradigm for the treatment of infections, by speci cally releasing antibiotics at infection target sites while minimizing possible nontarget adverse effects.[82]

The overview in Table 1 indicates a decrease in the last few years of the use of liposomes for antibiotic delivery. This could be because of the already extensive body of literature available for its application in other disease states, as well as to some disadvantages that are being overcome by newer technologically advanced systems, as discussed later in this paper. In the present scenario, liposome nanotechnology has nevertheless advanced to such an extent that it is possible to modify their surface, attach other nanoparticles (NPs) or targeting moieties on their surface in order to obtain site-speci c/targeted delivery and to control the release of antibiotics. Ongoing research regarding the delivery of antibiotics via liposomes using advanced nanotechnological aspects will certainly be fruitful if some challenges such as stability (*in vitro* and *in vivo*) are addressed, which will expedite several potential liposome-based antibiotic clinical products in the 21st century.

Polymeric Nanoparticles

Polymeric nanoparticles are solid colloidal particles, ranging in size from 1 to 1000 nm. They comprise several biocompatible polymeric matrices in which the therapeutic moiety is either entrapped, adsorbed, or covalently attached.[92] Because of their polymeric composition, PNPs may have greater stability than liposomes in biological uids and under storage.[93] The main aim of preparing NPs using polymers is to increase therapeutic bene ts, minimize side effects of conventional drugs, and to ef ciently deliver drug to a target site.[94,95] Natural polymers, such as chitosan, gelatin, and alginate as well as synthetic polymers, such as poly(lactic-co-glycolic)acid (PLGA), poly-*n*-(cyanoacrylate), and polycaprolactone (PCL) are widely used to fabricate PNPs.[96]

Poor therapeutic ef cacy because of rapid clearance by RES, the initial drawback of PNPs, has been overcome using strategies such as modi cation with hydrophilic excipients.[97] PNPs have been widely studied for various disease states, such as in ammatory bowel diseases,[98] cancer,[99] hypertension and angina,[100] airway in ammatory diseases,[101] diabetes,[102] and AIDS.[103] Although nanotechnology for antibiotics is still in its infancy, PNPs appear to be one of the most extensively studied nanosystems for antibiotic delivery. Their unique characteristics for antibiotic delivery include: (1) structural stability; (2) possibility of synthesis with a sharper size distribution; (3) precise tuning of properties such as particle size, surface charge, and drug release pro les via selection of appropriate polymers, surfactants, and organic solvents during preparation; and (4) the option of modifying the functional groups at the surface of PNPs by either drug moieties or targeting ligands.[104] The active moiety can be encapsulated, entrapped, dissolved, or attached to a polymeric matrix to generate either NPs, nanospheres, or nanocapsules depending on the method of preparation employed. Dispersion of preformed polymers and polymerization of the monomers have been mainly used for the preparation of NPs.[105] Other methods of PNP preparation can be found elsewhere.[106 108]

Polymeric nanoparticles have been explored for delivering a wide range of antibiotics for the treatment of diverse infections caused by different bacteria and have shown enhanced therapeutic ef cacy. Table 2 depicts a chronological summary of antibiotic-loaded PNP systems reported in the literature. The polymers and antibiotics used, method of PNPs preparation, characterization study performed, and the main ndings achieved are extracted, summarized, and presented. As can be seen in Table 3, in initial studies, polyalkylcyanoacrylates (PACA) were the materials of choice for preparing antibiotic-loaded PNPs.[109 111] To address the problem of resistance of intracellular infections to chemotherapy because of low intracellular uptake of commonly used antibiotics or their decreased activity at the acidic pH of lysosomes,[110] several studies have been conducted to deliver antibiotic intracellularly using PNPs. In early studies, ampicillin was bound to polyisohexylcyanoacrylate (PIHCA) to form PNPs, with an average size of 187 ± 13 nm for intracellular targeting of antibiotic. *In vivo* studies in experimentally infected C57BL/6 mice revealed that the therapeutic index of ampicillin against *Salmonella typhimurium* was increased by 120-fold when bound to PIHCA NPs.[109] Furthermore, in *in vivo* studies on PIHCA, bound ampicillin PNPs showed that 0.8 mg of ampicillin incorporated into NPs had a greater therapeutic effect as compared with 48 mg of free ampicillin against *S. typhimurium*. Furthermore, the ampicillin NPs were rapidly taken up by the liver and spleen, leading to a subsequent higher concentration of the drug in these organs.[110]

Formulation development of polyethylcyanoacrylate (PECA) NPs containing pe oxacin and o oxacin quinolone antibiotics using the incorporation or adsorption method was reported by Fresta et al.[111] These PECA NPs exhibited twofold to 50-fold more antimicrobial activity against *P. aeruginosa*, *S. aureus*, *E. coli*, and *Enterococcus faecalis*, with *in vivo* proof that the delivery system was preferentially captured by the mononuclear phagocyte system. In another experiment using PACA, cipro oxacin-loaded polyethylbutylcyanoacrylate (PEBCA) nanoparticlulate formulation with adequate drug loading and release properties was developed by an emulsion polymerization technique. It should be noted that MIC or minimum bactericidal concentration (MBC) against *S. Typhimurium* was not changed by the binding of cipro oxacin to PEBCA NPs. MIC and MBC values were same (0.062 and 0.5 μg/mL, respectively), irrespective of the form used.[112] Several years later in 2007, *N*-thiolated and acrylated β-lactam antibiotics were also loaded onto polyacrylate nanoparticles by conjugation onto its framework to protect it from the β-lactamase enzyme.[117,118] NP formulations of *N*-acrylated β-lactam antibiotic were found to be more potent compared with NP formulations of *N*-thiolated one. It should be noted that these early studies were mainly focused on studying the antimicrobial activity (*in vitro* and *in vivo*) of antibiotic-loaded PACA NPs, with few attempts only at formulation optimization, in depth characterization of PNPs, and surface modi cation for targeted delivery.

Table 2 also reveals a recent decrease in the use of PACAs for synthesizing PNPs. As from the 21st century scientists are clearly switching to more biocompatible and biodegradable natural and synthetic polymers, such as PLGA, chitosan, lecithin, and PCL. Furthermore, the synthesis of novel biocompatible and biodegradable materials to formulate nanosystems for infection control is also an emerging research area in the literature,[132 134] and polymers with multifunctional properties for antibiotic delivery is no exception to this trend. These studies are described in the section hereunder.

Poly(lactic-co-glycolic)acid appears to be one of the most widely studied polymers for antibiotic delivery. Initially, Dillen et al.[114] attempted the formulation development of cipro oxacin PLGA NPs using a factorial design to study the effect of different parameters on particle size, zeta potential, drug entrapment, and release. Their ndings showed that homogenization had a marked effect on particle size, release rate, and entrapment ef ciency. Homogenization decreased the particle size and drug release, but also increased the drug entrapment ef ciencies. In this study, antibacterial activity of the PNPs was found to be comparable to free drugs against *P. aeruginosa* and *S. aureus*.[114] However, it should be noted that although 100% of the drug was not released after 24 h, it nevertheless had equivalent activity. These researchers recognized that PLGA, being negative, might have low interactions with the anionic mucus for ocular infections. They then extended this study and incorporated cationic polymers into this PLGA formulation. In a subsequent study, they investigated the effect of including cationic polymers, namely, Eudragit® RS100 or RL100 on physicochemical properties, the release pro le, and antibacterial activity of cipro oxacin-loaded PLGA-containing PNPs.[115] They found that the zeta potential of all formulations

Table 2. Polymeric Nanoparticulate Systems Used for Antibiotic Therapy

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
Polyisohexylcyanoacrylate	Ampicillin	Emulsion polymerization	• Laser light scattering • *In vivo* antibacterial activity	Increased potency of ampicillin-bound NPs than free ampicillin assessed by *in vivo* treatment of salmonellosis infection of *S. typhimurium*.	Ref. 109
Polyisohexylcyanoacrylate	Ampicillin	Emulsion polymerization	• *In vitro* drug release studies in fetal calf serum • *In vitro* antibacterial activity using *B. subtilis* spores • *In vivo* experiments on *S. typhimurium*- and *L. monocytogenes*-infected mice	Greater therapeutic ef cacy of ampicillin-bound NPs than free ampicillin con rmed by experimental *Listeria monocytogenes* infection.	Ref. 110
Polyethylcyanoacrylate	Pe oxacin mesilate and o oxacin	Incorporation or adsorption method	• Size and molecular weight • Morphology • MIC by broth dilution • Drug accumulation studies in bacteria	Enhancement of antimicrobial activity against *P. aeruginosa, E. coli, S. aureus*, and *E. faecalis* from twofold to 50-fold.	Ref. 111
Polyethylbutylcyanoacrylate	Cipro oxacin	Emulsion polymerization	• Size by light scattering • Zeta potential by zeta sizer • Molecular weight by gel permeation • Loading ef ciency using HPLC and agar diffusion method • Release kinetics • *In vitro* antibacterial activity using microdilution method	• Ef cient loading of drug, controlled release, and suitable size PNPs for intravenous administration. • The presence of cipro oxacin in polymerization medium strongly in uenced the NP size and molecular weight because of the formation of tight chemical bond between cipro oxacin and ethyl cyanoacrylate.	Ref. 112
Lectin and gliadin	Acetohydroxamic acid	Desolvation method	• Size and zeta potential • Morphology by SEM • Drug entrapment • Drug release by dialysis cell membrane method • *In vitro* activity on pig gastric mucin • NP binding to *H. pylori* using agglutination assay • *In vitro* growth inhibition assay • *In situ* adherence assay on adult human esophagus, stomach, and duodenum	Targeted antibiotic delivery onto carbohydrate receptors of *H. pylori* bacteria, enhanced antibacterial activity as compared with free drug.	Ref. 113

Continued

DOI 10.1002/jps.24298

Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
PLGA	Cipro oxacin	Emulsi cation solvent evaporation method	• Size and zeta potential • Drug loading • *In vitro* release • Differential scanning calorimetry (DSC) • X-ray diffraction (XRD) • *In vitro* antibacterial activity	• Enhanced drug entrapment • Decreased particle size and release rate of cipro oxacin • Faster drug release after gamma sterilization of PNPs	Ref. 114
PLGA, Eudragit RS® 100, or RL 100	Cipro oxacin	Emulsi cation solvent evaporation method	• Size and zeta potential • DSC • Drug loading • *In vitro* release • *In vitro* antimicrobial activity • Evaluation of NP adhesion to *P. aeruginosa* and *S. aureus*	Prolonged drug release, positively charged NPs for prolonged residence time in anionic mucus for effective management of *P. aeruginosa*, and *S. aureus* infections.	Refs. 115 and 116.
Butyl acrylate and styrene	Acrylated penicillins	Free radical emulsion polymerization	• Size, zeta potential, and morphology using DLS, TEM, and atomic force microscopy (AFM) • Stability • *In vitro* antibacterial activity	Enhanced activity against β-lactamase producing MRSA.	Ref. 117
Butyl acrylate and styrene	N-thiolated β-lactam derivatives	Free radical emulsion polymerization	• Size, zeta potential, and morphology using DLS, SEM, TEM, and AFM • *In vitro* cytotoxicity • *In vitro* antibacterial activity	Novel β-lactam antibiotics and polymeric NPs thereof for enhanced anti-MRSA activity.	Ref. 118
PLGA	Cipro oxacin	Multiple emulsion solvent evaporation method	• Drug content and loading ef ciency • XRD • TEM • Size • Drug release studies • *In vitro* and *in vivo* susceptibility testing of NPs • *In vitro* and *in vivo* antibacterial activity	Effective *in vivo* growth inhibition of pathogenic *E. coli* because of sustained-release characteristics of NPs.	Ref. 119

Continued

Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
PLGA and PCL	Doxycycline	Solvent evaporation	• Size and zeta potential • SEM • Fourier transform infrared spectra (FT-IR) • DSC • Entrapment ef ciency • *In vitro* release • *In vitro* antibacterial activity	Increased entrapment of drug, sustained release with enhanced activity against DH5α strain of *E. coli*.	Ref. 120
PLGA	Azithromycin	Nanoprecipitation	• Size and zeta potential • SEM • Encapsulation ef ciency • DSC • FT-IR • *In vitro* dissolution study • *In vitro* antibacterial activity	Ef cient drug loading, sustained release, increased ef ciency against *S. typhi* than free drug with the targeting of drug to phagocytic cells.	Ref. 121
PLGA	Levo oxacin	Modi ed standard methods	• Size and zeta potential • SEM • In vitro drug release • *In vitro* antibacterial activity • Drug encapsulation and loading	Enhanced encapsulation of highly water-soluble antibiotic by modi cation of preparation method.	Ref. 122
Chitosan tagged with folic acid	Vancomycin	Emulsi cation	• FT-IR • Size • TEM • *In vitro* cytotoxicity • *In vitro* antibacterial activity	Effective drug delivery system for VRSA. Transport of drug-loaded NPs through endocytosis across the plasma membrane into cytoplasm.	Ref. 123
PLGA	Rifampin and azithromycin	Emulsion solvent evaporation	• Size and zeta potential • Drug loading and encapsulation ef ciency • *In vitro* release • Study of NP traf cking to infection	Enhanced effectiveness of the antibiotics in microbial burden in chlamydia infections by intracellular targeting.	Ref. 124
α-ω-Functionalized poly(ethylene oxide)	Gentamicin	Ring-opening metathesis copolymerization	• Size • *In vitro* cytotoxicity • *In vitro* antibacterial activity	pH-sensitive NPs for local delivery of antibiotics	Ref. 125

Continued

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Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
O-carboxymethyl chitosan	Tetracycline	Ionic cross-linking	• Size • SEM • FT-IR • *In vitro* drug release • Bacterial binding study • *In vitro* antibacterial activity • *In vitro* cytotoxicity • Hemolysis assay • Coagulation assay • Platelet aggregation assay • Confocal microscopy	Sustained release, improved bioavailability, and intracellular targeting of *S. aureus*.	Ref. 126
PLGA, PVA, chitosan, and alginate	Tobramycin	Modi ed emulsion/solvent diffusion technique	• Size and zeta potential • TEM • Drug encapsulation • *In vitro* assessment of NP interaction with mucus • *In vitro* release kinetics • *In vitro* antimicrobial susceptibility testing	• PVA and chitosan optimize the size and modulate the surface properties of NPs. • Ef cient entrapment of antibiotic into NPs because of alginate. • Good *in vitro* antibacterial activity of NP formulation against *P. aeruginosa* planktonic cells.	Ref. 127
PLGA PLH PEG	Vancomycin	Double emulsion/solvent evaporation method	• Size and zeta potential • TEM • pH-dependent characterization of NPs • NP bacterium binding using ow cytometry and uorescence confocal imaging • Drug encapsulation and release • *In vitro* antibacterial study	PLGA PLH PEG NPs as systemically administered drug carriers that can target and potentially treat Gram-positive, Gram-negative, or polymicrobial infections associated with acidity	Ref. 128

Continued

Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
Chitosan and heparin	Amoxicillin	Emulsi cation	• Size and zeta potential • TEM • Encapsulation and loading capacity • Drug release • *In vitro* cellular uptake and confocal laser scanning microscopy • Western blotting and immuno uorescence staining • *In vitro* cytotoxicity study • *In vitro* and *in vivo* antibacterial activity • Histological examinations and immunohistochemistry staining analysis • *In vitro* and *in vivo*	A multifunctional NP system for targeting *H. pylori*	Ref. 129
PLGA	Vancomycin	Modi ed solvent evaporation method	• Size and zeta potential • Drug loading and loading ef ciency • FT-IR • DSC • XRD • *In vitro* release • *In situ* intestinal permeation	Oral biodegradable vancomycin NPs with improved intestinal permeability	Ref. 130
PCL	Roxithromycin	Emulsion solvent evaporation technique	• Size and zeta potential • SEM • Encapsulation ef ciency and drug loading • Short-term stability study • *In vitro* drug release • *Ex vivo* human skin penetration study	Development of organogel containing roxithromycin NPs for delivery to hair follicles	Ref. 131

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Table 3. Summary of SLNs Investigated for Antibiotic Delivery

Lipid	Antibacterial Agent	Size (nm)	Zeta Potential (mV)	Targeted Microorganism	Main Findings	Reference
Stearic acid	Tobramycin	85 ± 5	−20.3	None	Gastrointestinal absorption of tobramycin, prolonged circulation time than i.v.-administered tobramycin solution.	Ref. 170
Stearic acid	Tobramycin	85 ± 5	−20.3	None	Increased passive transport of tobramycin incorporated in SLN to cross the BBB.	Ref. 171
Stearic acid	Cipro oxacin	73 ± 2 to 98 ± 44	−28 ± 1	None	Prolonged antibiotic release in a controlled manner.	Ref. 172
Tetradecanoic acid	Enro oxacin	116.7 ± 15.5	−29.03 ± 0.64	*S. aureus*	Sustained and prolonged drug release, increased bioavailability, and extended mean residence time in combination with fatty acid.	Ref. 173
Palmitic acid		111 ± 7.2	−31.57 ± 3.76			
Stearic acid		217.3 ± 20.1	−40.03 ± 0.67			
Hydrogenated castor oil	Tilmicosin	343 ± 26	−7.9 ± 0.4	*S. aureus*	Sustained drug release, sustained and enhanced antibacterial activity, and decreased degree of in ammation.	Ref. 174
Stearic acid	Nor oxacin	250 ± 5	−31.1 ± 1.85	*E. coli*	Sustained drug release and enhanced antibacterial activity.	Ref. 175
Compritol 888® ATO	Vancomycin	102.7 ± 1.01	−38.8 ± 2.1	*S. aureus,* MRSA	Ion pairing of vancomycin with antibacterial fatty acid (linoleic acid) enhanced encapsulation ef ciency and antibacterial activity of vancomycin in SLNs.	Ref. 135

containing Eudragit was positive and sustained release of cipro oxacin was achieved. All formulations were comparable to the free drug solution, con rming no loss of activity on encapsulation into a sustained-release formulation. It was also noted that drugs in this formulation were less active in killing *S. aureus* compared with *P. aeruginosa*. To understand the activity demonstrated, a further paper with ow cytometry studies on these PNPs presented the nding that Eudragit NPs showed more bacterial adhesion with test organisms (*P. aeruginosa* and *S. aureus*) compared with PLGA-only NPs, and can thus reside for prolonged time in anionic mucus membrane to effectively manage infections.[116] This opened a new research area of targeted delivery of antibiotics based on surface charge difference between bacteria and PNP formulation. The ndings of this study also emphasized the importance of polymer choice, not only for NP formation, but also for antibacterial activity.

Poly(lactic-co-glycolic)acid NPs containing cipro oxacin with particle sizes of 100 300 nm were also formulated and evaluated for their antibacterial potential (*in vitro* and *in vivo*) against pathogenic *E. coli* by Jeong et al.[119] These NPs displayed lower *in vitro* antibacterial activity as compared with free cipro oxacin and was attributed to their sustained drug release pro les. Cipro oxacin was released from NPs over a period of 14 days. However, *in vivo* antibacterial activity of NPs was greater than the free drug, showing the superiority of the formulation. Although these authors did not explain the differences in *in vitro* and *in vivo* behavior of the PNPs against the free solution, this may clearly be because of the fact that the *in vitro* studies were carried out after 24 h and for a single time period only, whereas in the *in vivo* study, mice were sacri ced after 3 days.[119] This suggests that sustained-release antibiotic formulations should undertake *in vitro* activity studies over a prolonged period, as has been performed is several studies for nanoantibiotic formulations other than PNPs.[135,136] In other studies, NPs formulated using PLGA polymer have been shown to enhance the delivery of azithromycin and rifampicin to intracellular chlamydial infections caused by chlamydia trachomatis and chlamydia pneumonia.[124] Using detailed micrometric, crystallographic (Fourier transform infrared, X-ray diffraction, and differential scanning calorimetry), mathematical modeling of drug release data, and *in situ* permeability evaluations, an improvement in intestinal permeability of vancomycin in male Wistar rats was observed by delivering it via PLGA NPs.[130] The researchers attributed this nding from less than 500 nm size NPs to the large surface area, improved paracellular passage, and their endocytic uptake.

Poor incorporation ef ciencies of the drug into NPs are a well-recognized challenge, especially with water soluble drugs. To this end, several groups working with PLGA polymers have investigated varying approaches to enhance

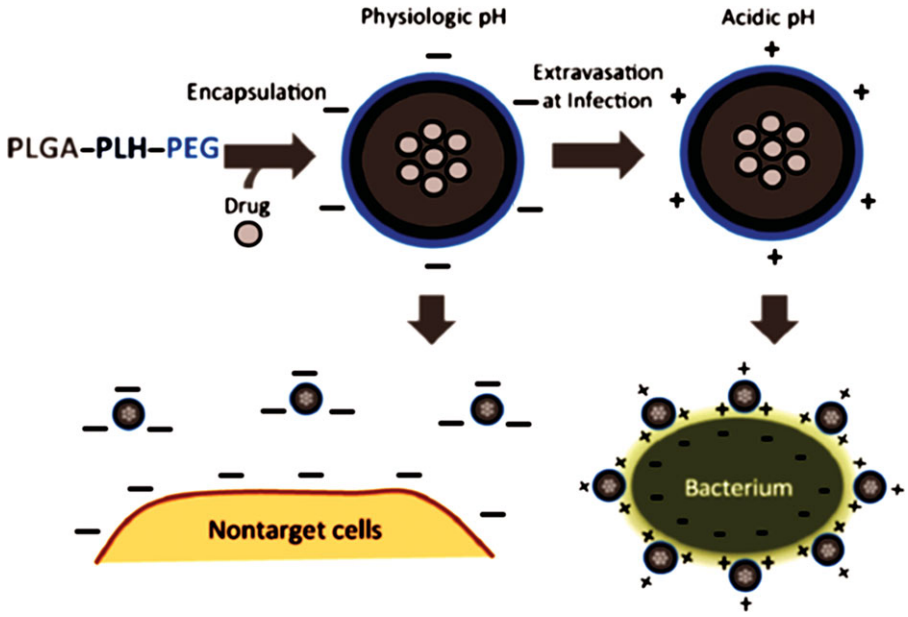

Figure 2. Schematic representation of the designed surface charge-switching PNPs-mediated drug targeting to bacterial cell walls. Reproduced from Radovic-Moreno et al.[128] with permission from American Chemical Society.

encapsulation ef ciencies.[120 122,127,137] Cheow and Hadinoto,[122] in their study with levo oxacin, modi ed the standard NP preparation techniques, single- and double-emulsi cation solvent evaporation, and nanoprecipitation. They found that encapsulation ef ciency of highly water-soluble drugs in PLGA NPs can be enhanced by these modi ed methods by taking levo oxacin as a model drug.[122] The inclusion of lecithin into the aqueous phase, and modifying the water miscibility level of the oil phase, were found to be particularly useful. In another study, the drug and polymer ratio was particularly investigated to prepare azithromycin PLGA NPs for optimum encapsulation and biological properties. A drug to polymer ratio of 1:3 was found to be optimal in enhancing encapsulation ef ciency to 78.5%. The optimized formulation was more effective against *S. typhi* by displaying equivalent antibacterial effect at 1/8th the concentration of the free drug,[121] As combining PCL with PLGA was found to increase the doxycycline entrapment ef ciency, selecting appropriate polymeric core composition can be a useful strategy for enhancing drug encapsulation. A PLGA PCL ratio of 80:20 was found to be optimal to increase entrapment ef ciency to 32% from 25% at a PLGA PCL ratio of 60:40. Altering the aqueous phase pH from 7.4 to 4 additionally increased entrapment to 70%.[120] A study by Ungaro et al.,[127] who formulated a PLGA NP dry powder formulation as a pulmonary delivery system or tobramycin, also highlighted the importance of helper hydrophilic polymers, for example, chitosan, alginate, and polyvinyl alcohol (PVA) for achieving optimal drug entrapment, size, and release pro les.

A recent development in the eld of PLGA NPs for antibiotic delivery has been its modi cation to synthesize a polymer that is particularly responsive to infection sites. Vancomycin-encapsulated, pH-responsive, surface charge-switching PLGA-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) (PLGA-PLH-PEG) NPs have been synthesized (mean size = 196.0 ± 7.8 nm). A lack of interaction of NPs with bacteria at pH 7.4 and at acidic pH strong af nity of NPs toward bacteria was observed. PLH gets protonated because of the acidic pH at the infection site and activates a surface charge-switching mechanism that leads to binding of the NPs to the negatively charged bacteria (Fig. 2).[128] This was con rmed by NP-binding studies using confocal imaging and ow cytometry. Studies demonstrated pH-sensitive NP binding to bacteria, that is, a 3.5 ± 0.2- to 5.8 ± 0.1-fold increase in bacterial binding at pH 6.0 as compared with 7.4 was reported. It was also observed that upon reduction in pH, the PLGA-PLH-PEG NPs switched their surface charge from a negative zeta potential at pH 7.4 (−3.9 ± 0.4 mV) to a positive one. They also showed that the surface charge transition occurred, as early as pH 7.0 (2.3 ± 1.0). The results obtained using PLGA-PLH-PEG NPs are promising, and pave the way for synthesizing other responsive PLGA-based polymers. These studies have therefore clearly con rmed PLGA as a suitable material for antibiotic-loaded PNP formulations.

Among the natural polymers, chitosan has attracted considerable interest for the use against microbial growth because of its antimicrobial and antifungal activity.[138 140] Its antimicrobial action may be because of ef cient binding to negatively charged bacterial cell walls that destabilizes the cell envelope altering permeability, followed by attachment to DNA and inhibition of its replication.[141] Several approaches have been used to exploit chitosan as a polymer for antibiotic delivery. Folic acid tagged noncytotoxic chitosan NPs have been employed as Trojan horses to target vancomycin into the bacterial cell by synthesizing a new carboxymethyl chitosan-2,2′-(ethylenedioxy)-bis-(ethylamine)-folic acid (CMC-EDBE-FA) polymer. This experiment was performed to address the problem associated with VRSA treatment, which is a serious issue in medical practice.[123] FA, an essential nutrient required for nucleotide synthesis for bacteria helps to transport the NPs loaded with drug through endocytosis, across the plasma membrane, and into the cytoplasm.[142,143] The prepared nanoconjugated vancomycin decreased both the MIC and MBC values of VRSA to a signi cant level (Fig. 3).

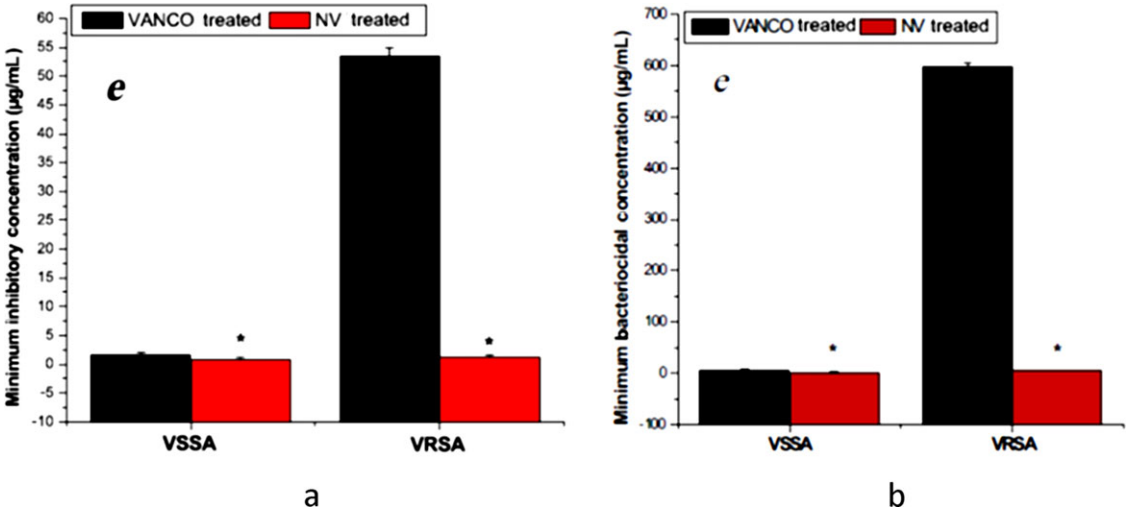

Figure 3. (a) Minimum inhibitory concentration and (b) MBC of vancomycin (vanco) and nanoconjugated vancomycin (NV) against vancomycin susceptible and resistant S. *aureus* (VSSA and VRSA). Reproduced from Chakraborty et al.[123] with permission from IOP Publishing.

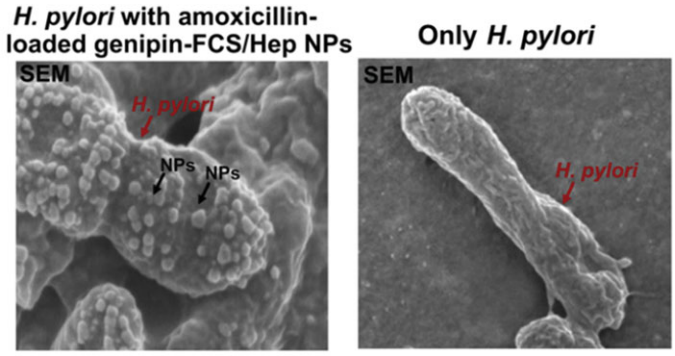

Figure 4. Scanning electron microscope images showing strategy and observation for eradicating H. *pylori* by amoxicillin-loaded genipin-FCS/Hep NPs. Reproduced from Lin et al.[129] with permission from Elsevier Science Ltd.

Using ionic cross-gelation technique, biocompatible, 200 nm-sized tetracycline (TC) encapsulated *O*-carboxymethyl chitosan NPs have also been prepared to eradicate intracellular *S. aureus* infections effectively.[126] Recently, amoxicillin entrapped genipin cross-linked fucose chitosan/heparin NPs (genipin FCS/Hep NPs) in the size range of 150 210 nm have been shown to eradicate *H. pylori*, a Gram-negative microorganism causing gastric infections. Via in-depth studies on this multi-functional responsive polymeric PNP including encapsulation, release, *in vitro* cellular uptake and confocal laser scanning microscopy, *in vitro* growth inhibition, *in vivo* animal studies, histology and immunochemistry, and uorescent bacteria binding, this formulation was shown to decrease drug release at gastric acids and increased release at an *H. Pylori* survival situation (Fig. 4). In addition, a more complete *H. pylori* clearance effect and ability in decreasing gastric in ammation associated with *H. pylori* was reported.[129]

Other polymers have also been randomly used in the literature to encapsulate antibiotics, and are highlighted hereunder. As NPs may accumulate in hair follicle openings, drug delivery through this mechanism, with the use of NPs, is gaining more importance. Roxithromycin NPs (size 300 nm), using PCL as a polymer, were prepared using an emulsion solvent evaporation method and were embedded in pluronic-lecithin organogel (PLO). *In vitro* human skin penetration studies revealed that it is possible to preferentially target the pilosebaceous unit with the polymeric NPs, whereas the PLO formulation did not promote follicular penetration more ef ciently than suspension of NPs.[131] Therefore, antibiotic-loaded PNPs can now also be entrapped into a gel for facilitating transdermal delivery.

The synthesis of pH-sensitive functionalized NPs by ring-opening metathesis copolymerization (ROMP) has also been disclosed by Pichavant et al.[125] For this purpose, a pH-sensitive α-norbornenyl-poly(ethylene oxide) macromonomer was used to synthesize different polymeric derivatives. The plurifunctionalization of NPs containing prodrugs and reactive chemical groups as carboxylic acids was explored in the study using macromonomer route. Gentamycin was linked via a pH-sensitive imine bond to a polymer, and the NPs prepared using ROMP were found to be noncytotoxic by neutral red and MTT assays. The MIC measurements performed at different pH values (4 7) on *S. epidermidis* revealed that for gentamycin-functionalized macromonomer, there was no signi cant inhibition of growth at pH 7, whereas a decrease at conditions of pH 4 and 5 was observed.[125] For targeted delivery, lectin-conjugated gliadin NPs speci cally binding to carbohydrate receptors on *H. pylori* cell walls with release of the antimicrobial agents into the bacteria were found to have an inhibitory effect twofold higher than gliadin NPs.[113]

Thus, the section on PNPs can be summarized as: rst, PNPs are extensively studied nanodelivery systems for antibiotics and have advantages over liposomes; second, it is possible to achieve site-speci c and targeted delivery of antibiotics by surface modi cation of PNPs with targeting moieties, and by using pH-responsive materials for synthesis or by formation of covalent bonds, which can be degraded at acidic environment at infection site. Third, the eld of antibiotic PNPs seems to be growing, and there are opportunities for scientists to develop novel-biocompatible and biodegradable-responsive polymers for antibiotic PNPs formulation, as conventionally used natural and synthetic polymers have been exploited extensively and have some limitations. Lastly, the literature indicates that

Table 4. Antibacterial Activity of VCM-HCl, VCM-LA2, VCM-HCl_SLNs, and VCM-LA2_SLNs

Formulation	MIC (μg/mL)[a]							
Bacteria	*S. Aureus*				MRSA			
Time (h)	18	36	54	72	18	36	54	72
Blank SLNs	NA	NA	NA	NA	NA	NA	NA	NA
VCM-HCl	15.62	NA	NA	NA	3.91	NA	NA	NA
VCM-LA2	218.75	437.5	109.35	218.75	1750	850	1750	1750
VCM-HCl_SLNs	15.62	250	500	NA	15.62	500	500	NA
VCM-LA2_SLNs	62.5	31.25	31.25	31.25	15.62	15.62	15.62	15.62

[a] $n = 3$.
NA, no activity.
Reproduced from Kalhapure et al.[135] with permission from Elsevier Science Ltd.

most of the antibacterial studies are carried out *in vitro* and therefore, in future, there is a need to focus studies on *in vivo* performance of reported and newly developed antibiotic PNPs.

Solid Lipid Nanoparticles

Solid lipid nanoparticles, introduced in the early 1990s, have gained signi cant popularity as an alternative drug delivery colloidal system[144] because of their advantages. These include using biocompatible materials, being easy to scale up preparation techniques, stability during storage,[145,146] high entrapment of lipophilic drugs into their lipophilic core,[147,148] protection of labile drugs against degradation,[149 152] improved body/tissue tolerance, and less stringent regulatory requirements because of utilization of physiologically acceptable lipids.[145,146] SLNs typically have mean diameters ranging in size from 50 to 1000 nm[148] and can be delivered by almost all routes for various disease conditions.[153] Avoiding organic solvents and the feasibility of production on a larger scale are two main advantages of SLNs. They are uniquely attractive in that they display the advantages of conventional NPs while simultaneously eliminating some of their reported drawbacks, such as the high cost of polymers and phospholipids used for producing PNPs and liposomes, the need to maintain drug bioactivity throughout the conjugation scheme if the drug is being conjugated to PNPs,[154] rapid leakage of water-soluble drugs, and poor storage stability.[105]

A high melting point lipid composition forms the core of SLNs. The core remains in the solid state at room and body temperature and is coated with amphiphilic surfactants that form the outer shell.[148] Many solid lipids, such as stearic acid,[155] palmitic acid,[156] glycerol behenate (Compritol 888 ATO),[157] and glyceryl monostearate[158] have been used in preparing SLNs. Similarly, various surfactants, such as poloxamer 188, 182, 407, 908,[159 161] tween 20, 80,[162,163] and solutol HS 15[164] have been reported to stabilize the SLN formulation. Recently, novel surfactants, such as polyhydroxy surfactants[165] and an oleic acid based bicephalous dianionic surfactant,[166] have also been found as potential stabilizers for SLN preparations. A comprehensive list of lipids and surfactants used in SLN formulation development can be found elsewhere in the literature.[167,168] High-pressure homogenization and microemulsion technique are the two main techniques employed for the production of SLNs. However, many other methods such as the ultrasound and solvent-based techniques have been used to promote cost-effective and simpler ways of production.[169]

Although SLNs have shown great therapeutic potential for delivering drugs with diverse pharmacological activities, the development history of their antibiotic delivery system is shorter. A literature search for this paper revealed that there are fewer SLN-based antibiotic delivery systems compared with other drug classes.[135] SLN-based antibiotic formulations with their properties (size and zeta potential), microorganism/s used to assess antibacterial activity, and main outcomes of the study are summarized chronologically in Table 4. The data indicate that SLNs are being exploited for overcoming absorption inhibitors, facilitating transport across membrane barriers, modifying drug release pro les, increasing bioavailability, and enhancing and prolonging antibacterial activity.

Tobramycin, which is administered via the oral route, is used against *P. aeruginosa* infections.[176] Its poor absorption rate is because of active exportation of the drug from the cells via P-glycoproteins (P-gp) and ATP-dependent drug ef ux pumps. This poor intestinal absorption was overcome by formulating tobramycin-loaded SLNs, which signi cantly suppressed the P-gp ef ux pump by penetrating the intestinal linings through endocytosis rather than passive diffusion. SLNs removed from drug ef ux pumps released the drug inside the cells after being internalized through endocytosis. Achievements of tobramycin-loaded SLNs were modi ed pharmacokinetics, low amounts taken up by the kidneys and high lung concentration following intravenous administration by the duodenal and intravenous route.[170] They reported that aminoglycosides have low permeability across the blood brain barrier (BBB) when administered via the parenteral route. In a subsequent paper, these authors showed that in tissue distribution studies, no tobramycin could be detected in the brain after an i.v. solution, whereas it was detected in the brain, with SLN indicating passage through the BBB.[171] This important study with an antibiotic, although not having antibacterial activity studies, con rmed the use of SLNs to overcome the P-gp ef ux pump and pass through the BBB when loaded with an antibiotic.

Other studies have con rmed their abilities to provide sustained drug release and prolonged antibacterial activity. Jain and Banerjee[172] developed a SLN-based single dose nanodelivery system for cipro oxacin that provided a prolonged release of the antibiotic in a controlled manner. Their study revealed that SLNs of cipro oxacin were more promising than other cipro oxacin nanodelivery systems that have been formulated.[172] Similarly, enhancement of *in vitro* and *in vivo* antimicrobial activity of tilmicosin against *S. aureus* was achieved by encapsulating it into SLNs that were formulated

using hydrogenated castor oil.[174] This research group also prepared nor oxacin-loaded SLNs as a novel formulation and studied different aspects of the formulation such as stability, *in vitro* release, *in vitro* antibacterial activity, and *in vivo* ef cacy in mice against *E. coli*. SLNs were found to be stable for up to 9 months at 4°C, and the drug release was slower, lasting for 48 h. Although the SLN formulation was initially less effective within 24 h, it was interestingly much more effective than the bare nor oxacin during *in vitro* antibacterial evaluations at all other time points up to 144 h, con rming sustained drug release. For *in vivo* therapeutic ef cacy, treatment was performed 2 h postintraperitoneal infection of mice with *E. coli*. Enhanced ef cacy was observed for SLNs, which was indicated by decreased bacteria in the spleen and kidney homogenates and a high proportion of survivors, which was probably because of the high bioavailability of drugs.[175]

The role of fatty acids in enhancing SLN preparations with antibiotics is being increasingly recognized. Saturated carbon fatty acids are commonly used as a lipid matrix to prepare SLNs. As they vary in terms of carbon chain length and properties, Xie and coworkers[173] investigated the in uence of fatty acids on the properties and pharmacokinetics of enro oxacin-loaded SLNs. It was found that stearic acid produced SLNs with the highest encapsulation and had a greater zeta potential but larger particle size and polydispersity index than palmitic acid and tetradecanoic acid. Although in *in vitro* studies the three developed formulations exhibited similar antibacterial activity as that of native enro oxacin, in *in vivo* studies, it was found that the bioavailability of tetradecanoic, palmitic, and stearic acid SLNs increased 6.79-, 3.56-, and 2.39-fold, whereas the mean residence time of the drug was extended from 10.60 to 180.36, 46.26, and 19.09 h, respectively.[173] This study therefore highlighted the signi cant effects of the fatty acid properties as the lipid matrix on the performance of SLNs. In a more recent study, our group exploited the diverse advantages of fatty acids by including them as a counter ion to form an ion pair with vancomycin, instead of being the lipid core itself, as was performed in the previous study. A Compritol-based SLN formulation (VCM-LA2_SLNs) of vancomycin and linoleic acid using an ion pairing mechanism[135] was prepared. Our goal was to develop a nanoantibiotic system acting by multiple simultaneous mechanisms of actions, as it would be dif cult for bacteria to develop resistance to such a system, this requiring multiple simultaneous mutations in the same microbial cell.[35,177] Linoleic acid served two purposes in the formulation; (1) it acted as a contra ion for vancomycin to form an ion pair, and (2) being an antibacterial, it served as a nondrug antibacterial agent in the formulation. The particle size and polydispersity index of the formulated VCM-LA2_SLNs were 102.7 ± 1.01 nm and 0.225 ± 0.02, respectively. Zeta potential was -38.8 ± 2.1 mV, con rming the high stability of VCM-LA2_SLNs. The study revealed greater encapsulation of vancomycin in SLNs, and enhanced and extended period of antibacterial activity of the novel formulation against MRSA and *S. aureus*. Encapsulation ef ciencies were 16.81 ± 3.64 and 70.73 ± 5.96 for vancomycin SLN and the developed VCM-LA2_SLNs, respectively. Although at the initial 18 h testing time, bare vancomycin showed highest activity (low MIC) against both *S. Aureus* and MRSA (15.62 and 3.91 μg/mL, respectively), at subsequent time intervals (36, 54, and 72 h), VCM-LA2_SLNs was the only active formulation against both the strains exhibiting MICs of 31.25 and 15.62 μg/mL, respectively, against *S. aureus* and MRSA (Table 5).[135] The strategy of coencapsulation of a fatty acid with an antibiotic in SLNs therefore proved successful in enhancing activity against sensitive and resistant strains. Investigating the effect of other fatty acids of different carbon chain lengths on drug loading and antibacterial activity, as well as on molecular modeling to explain their association with the SLN, will be an interesting study to guide their selection for future optimal formulations.

Although SLNs are emerging as a lipidic delivery system of choice for nanodrug delivery, this review shows that despite its advantages, this nanodelivery system has not been exploited to a great extent for antibiotics. One of the reasons might be the hydrophilic nature of most antibiotics used clinically, which will have low entrapment ef ciency and loading capacity in the hydrophobic lipids. Recent studies do indicate that this problem could be surpassed by the use of techniques such as ion pairing and/or conjugation mechanisms. Detailed characterization using techniques such as atomic force microscopy, confocal laser scanning microscopy, and ow cytometry to elucidate the mechanisms involved in antibacterial activity with these systems should also be considered.

Lipid–Polymer Hybrid Nanoparticles

Liposomes and PNPs appear to be the most explored nanoparticulate system for antibiotics thus far. To overcome some of the reported limitations associated with these systems though, LPHNs have been more recently introduced.[32] LPHNs are novel integrated systems in which the structural and architectural advantages of a polymer core and the biomimetic properties of lipids are combined to generate a delivery system that is superior. LPHNs are therefore solid, nanosized particles composed of at least two components: lipid and polymer.[178] In a well-designed LPHN, the polymeric core serves to entrap either water- or oil-soluble drugs and to provide a robust structure, whereas the external lipid coat serves as a biocompatible shield. The latter also functions as a template for surface modi cation and further acts as a barrier to minimize the burst release of water-soluble drugs.[179]

A number of methods have been reported to produce LPHNs, namely, multiple step procedure involving coincubation of separately prepared NPs and lipid vesicles[180,181]; a single-step nanoprecipitation technique[32,182]; a method using emulsi cation with lipids replacing traditional surfactants[183]; a sonication method[182]; and a double-emulsi cation-solvent-evaporation technique.[184] A recent review on LPHNs provides details on materials and methods used for preparing, identifying the physicochemical characteristics, immunocompatibility, and their applications in drug delivery. LPHNs have to date been studied most extensively for delivering anticancer drugs.[178] It is only recently since 2011 that these LPHNs possessing characteristics of both liposomes and PNPs being explored for their bene ts in antibiotic delivery.

Table 5 provides a summary of research undertaken so far on the preparation of antibiotic-loaded LPHNs, with four of the ve papers emanate from the same research group. In the earliest reported antibiotic-loaded LPHN study, three uoroquinolone antibiotics, cipro oxacin, levo oxacin, and o oxacin were entrapped in LPHNs using PLGA as a polymer and PC as a lipid component by a double-emulsi cation-solvent-evaporation method in pursuit of developing nanodrug delivery system for treating pulmonary infections. The study also explored the factors affecting encapsulation ef ciency and

Table 5. Summary of Studies Undertaken to Date with LPHNs and Antibiotics

Antibiotic	Nature of Antibiotic	Polymer and Lipid	Main Findings	Characterization Studies	Reference
Levo oxacin O oxacin Cipro oxacin Tobramycin	Hydrophobic Hydrophobic Hydrophilic Hydrophilic	PLGA and PC	• Ionicity of the drug and lipid is important with regard to LPHNs preparation. • Drug lipophilicity and aqueous solubility affect drug loading and drug release; more lipophilic drug has higher drug loading and sustained release pro le. • LPHNs are larger in size, zeta potential, encapsulation, and drug loading compared with its nonhybrid counterpart. • Incorporation of D-α-tocopheryl polyethylene glycol 1000 succinate stabilized the formulation. • Sizes between 120 and 420 nm with the highest encapsulation of 25% with o oxacin.	• Particle size • Zeta potential • Entrapment ef ciency • Drug loading • *In vitro* drug release • SEM	Ref. 179
Levo oxacin	Hydrophobic	PLGA and PC	• Particle size of LPHNs ranged from 240 to 420 nm with a zeta potential of approximately 26 mV, encapsulation ef ciency ranging from 19% to 21% and drug loading of 2.3% 2.4% (w/w). • LPHNs exhibited a higher antibacterial ef cacy against *P. aeruginosa* bio lm cells, however, not against planktonic cells. • Possibly, the presence of lipid may have enhanced the antibiotic diffusion into the bio lm matrix resulting in more effective bio lm cell eradication.	• Particle size and zeta potential • Entrapment ef ciency • Drug loading • *In vitro* release studies • SEM • Bio lm susceptibility testing	Ref. 184
Levo oxacin Cipro oxacin O oxacin Calcein	Hydrophobic Hydrophilic Hydrophobic Hydrophilic	PLGA, rhamnolipid and PC	• Particle size ranged from 280 to 400 nm with a zeta potential range of (−)30 (+)10 mV and a drug loading of 0.5% 2.3% (w/w) • Encapsulation ranged from 5% to 55% depending on the BCS class of the drug. • A rhamnolipid-triggered release was observed with calcein, however, not with BCS class I drugs because of their high lipid membrane permeability. • The rhamnolipid-triggered release capability of LPHNs will enable targeted drug release in the vicinity of bio lm colonies and therefore improved antibacterial ef cacy is expected.	• Particle size • Zeta potential • Entrapment ef ciency • *In vitro* drug release • SEM	Ref. 185
Levo oxacin	Hydrophobic	PLGA and lecithin	• LPHNs exhibited a size of ≈420 ± 30 nm with zeta potential in the range of (−) 25 30 mV, encapsulation ef ciency of ≈19% and drug loading of ≈2.0% (w/w). • Spray drying produced dimpled hollow spherical nano-aggregates whereas spray freeze drying produced large spherical porous nano-aggregates. • PVA was better than mannitol in facilitating nano-aggregate reconstitution. • Nano-aggregates produced by spray freeze drying were superior to those produced by spray drying.	• Particle Size and distribution • Zeta potential • Entrapment ef ciency • Drug loading • Powder characterizations	Ref. 186
Clindamycin phosphate	Hydrophilic	Stearic acid, dextran sulfate and sodium alginate	• LPHNs ranged from 400 to 900 nm. • Particle size was not affected by polymer type or the amount of drug, polymer, and surfactant. • Polymer dextran sulfate had higher degree loading and drug release than sodium alginate.	• Particle size and distribution • Entrapment Ef ciency • Drug loading • *In vitro* drug release studies • SEM	Ref. 187

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stability of LPHNs.[179] This paper clearly formed the foundation for subsequent antibiotic-loaded LPHN systems, as it highlighted the importance of lipid and drug ionicity for forming the NPs and drug lipophilicity, as well as aqueous solubility on drug entrapment and release pro les. The poor stability of the LPHNs in this study was overcome by the addition of d-α-tocopheryl PEG 1000 succinate as a solubilizer. The low drug encapsulation and inadequate stability reported in this paper re ect the challenges with this delivery system during their preparation. Strategies such as choice of solvents, pH of aqueous phase, and counter ion complexation can be considered for enhancing drug incorporation, whereas other hydrophilic substances can be considered to modify the surface to promote stability during storage and *in vivo*. Having established critical factors for successfully forming LPHNs, these authors then proceeded to investigate the antibio lm ef cacy of the LPHNs against *P. aeruginosa* preparing LPHNs containing PLGA, PC, and levo oxacin. LPHNs, both in suspension and powder form, displayed higher antimicrobial activity against 1-day-old *P. aeruginosa* bio lm cells than nonhybrid NPs, but were less effective against planktonic cells.[184] To further enhance the performance of these LPHNs as antibio lm drug carriers, the target release of the encapsulated drug at bio lm colonies needed to be demonstrated. In another study, they investigated the trigger release properties of the LPHNs in response to rhamnolipids that are present in bio lm colonies of *P. aeruginosa* by using various biopharmaceutical classi cation system (BCS) antibiotic drugs as a model.[185] In the absence of the triggering agent (rhamnolipid), both levo oxacin and o oxacin (BCS class I model drugs) were readily released from the LPHNs at rapid rates. The percentage of levo oxacin and o oxacin released in 6 h were 70% and 90%, respectively. These fast release rates were attributed to their free solubility in water and high lipid membrane permeabilities, con rming that the presence of the lipid coat did not deter their outward diffusion. In the absence of the triggering agent, calcein (BCS class III model drug) was eventually released, but only in minimal amount from the LPHNs, which was indicated by a 20% release of the encapsulated calcein after 2.5 h. This initial calcein release was likely because of the dissolution of nonencapsulated calcein present on the NP surfaces. Upon the addition of rhamnolipid, calcein was immediately released, with almost 60% being released within the rst 5 min. This study therefore showed that rhamnolipid-triggered release may enable targeted release in the vicinity of bio lm colonies. Although previous studies mainly focused on formulation variables, the focus of another paper by this group was on optimizing manufacturing technologies for these LPHNs. They compared spray-drying (hollow dimpled spherical nanoaggregates) and spray freeze-drying (large spherical porous nanoaggregates) techniques to produce inhalable dry powder forms of LPHNs. It was found that both methods were able to produce inhalable dry powders of the LPHNs in the form of microscale aggregates.[186] Nanoaggregates produced by the spray freeze-drying technique was superior to those produced by spray drying.

The most recent paper by Abbaspour et al.[187] used sodium alginate and dextran sulfate as polymers and stearic acid as the lipid to prepare clindamycin-loaded LPHNs. They used a multilevel factorial design to nd a mathematical relationship between the amount of polymers and the amount of surfactants on drug-loading ef ciencies. They attributed higher drug-loading ef ciencies with dextran sulfate, rather than to sodium alginate to ionic interactions between the anion in dextran sulfate and the cationic clindamycin. Although it is clearly useful to use an experimental design, this study could have been strengthened if the generated mathematical model had been validated. Furthermore, although the authors indicate the undertaking of scanning electron microscope (SEM) analysis of the LPHNs, which con rmed their morphology, no SEM images were provided in the paper.

These studies with antibiotic-loaded LPHNs clearly con rm their potential as an effective nanosystem for antibiotics. Table 5 shows that to date, PLGAs have been mainly used as the polymer, with the basic characterization in terms of size, polydispersity index, *in vitro* release, and surface morphology having been studied. Only antibacterial activity for bio lm susceptibility testing has been assessed. In-depth physicochemical/mechanical characterization studies, including *in vitro* and *in vivo* bacterial activities against a range of organisms, are therefore essential for formulation optimization. The reported advantages of this delivery system necessitate investigating various classes of antibiotics with different polymers and lipids to identify optimal formulation excipients. In addition to antibio lm therapy, other applications that can be studied include antibacterial activity against sensitive and resistant bacterial strains for infections as well as macrophages infection studies. Mechanistic studies to understand the complex self-assembly of the drug, lipid, and polymer into these LPHN constructs will also be useful. These studies, together with tuning the lipid and polymer composition and employing surface strategies, will certainly result in LPHNs emerging as novel effective hybrid nanodelivery systems. This will provide new platform for developing nanoantibiotics with enhanced performance in terms of high drug (both hydrophilic and lipophilic) loading, targeted delivery, as well as sustained and prolonged activity.

Dendrimeric Nanostructures

Dendrimers are homogenous, well-de ned monodisperse structures. They consists of tree-like structures in nanosized form and are radially symmetric molecules.[188] These monodisperse nanosized polymers are shaped like the head of a tree, and exploit two traits, that is, globular structure and polyvalency, which is found in many naturally occurring systems.[189 194] Tomalia et al.[195] disclosed the synthesis of rst family of dendrimers, known as poly(amido amine) (PAMAM), resulting in PAMAM becoming one of the most popular dendrimers. Since their disclosure, a variety of dendrimers have been synthesized and evaluated for various applications in chemistry, nanotechnology, biomedicine, and pharmaceutical sciences.[17,196 201] Depending on the chemical moieties and types of linkages present, dendrimers are classi ed into four types: glycodendrimers,[202] peptide dendrimers,[203] janus dendrimers,[204,205] and metallodendrimers.[206] Dendrimers have gained increasing interest among drug delivery scientists because of their nanosize, globular shape, derivatizable peripheral functionality, multivalency, tunable inner cavities, and physicochemical properties that resemble those of biomolecules. Their applications in drug delivery technology include: as vehicles,[207] solubility enhancers for poorly soluble drugs,[208] controlled release,[209] targeted delivery,[210,211] prodrug preparation,[212 214] HIV prophylaxis,[215] gene therapy,[216,217] as vaccines,[218] in diagnostics,[219] and as drugs.[220]

Table 6. Dendrimers with Their Role in Antibiotic Drug Delivery

Dendrimer	Drug	Role of Dendrimer	Reference
PAMAM	Nadi oxacin and pruli oxacin	Drug carrier to enhance solubility without affecting antibacterial activity.	Ref. 224
PPO-PAMAM	Triclosan	Micellar carrier with high drug loading and controlled release for hydrophobic drug.	Ref. 225
PAMAM	Sulfamethoxazole	Solubility enhancer to obtain increased antimicrobial activity with sustained release.	Ref. 226
PAMAM	Erythromycin	Conjugation with a drug to act as a carrier for sustained and targeted intracellular delivery in periprosthetic in ammation.	Ref. 227
PAMAM	Azithromycin	Conjugation with a drug to act as a carrier for ef cient intracellular delivery to address chlamydia infections.	Ref. 228
PAMAM	Erythromycin and tobramycin	No speci c role. Study was conducted to investigate effect of dendrimers on antibacterial activity of two drugs with different solubility pro le.	Ref. 229
PAMAM	Silver sulfadiazine	Solubility enhancer forming a NP system with enhanced antimicrobial properties for the topical treatment of burn-wound infections.	Ref. 230
PAMAM	Vancomycin	Scaffold for vancomycin to form drug dendrimer conjugate with high-binding avidity to bacterial cell wall.	Ref. 231
PPI	Nadi oxacin	Coadministration with antibiotic for enhancement of antibacterial activity.	Ref. 232
PPI	Cipro oxacin	Coadministration with antibiotic for reducing the required dose of drug for antibacterial activity.	Ref. 233
HPO hexadentate-based dendrimeric chelators	Nor oxacin	Combination agent with antibiotic for synergistic bactericidal effect.	Ref. 234

The literature reveals that dendrimers themselves have been found to be effective antibacterials, which prompted many scientists to focus on synthesizing antibacterial dendrimers. The details of these antibacterial dendrimers are out of the scope of this review and can be found elsewhere.[221 223] The following sections, therefore, only highlights the use of dendrimers to enhance the properties of antibiotics via nanostructures. Table 6 is a chronological summary of studies where dendrimeric materials have been used to prepare antibiotic-loaded nanostructures. These antibiotic-loaded dendrimeric nanostructures have been exploited for enhancing drug solubility and antibacterial activity, for prolonging sustained drug release, and to prepare various nanostructures, such as micelles and conjugates, for antibiotic delivery.

Because of poor aqueous solubility of quinolone antibacterials, there are dif culties in formulating their liquid dosage forms, consequently restricting their use in topical formulations. To overcome this problem, Cheng et al.[224] investigated the potential of G3-G5 PAMAM dendrimers as biocompatible carriers for an improvement in the aqueous solubility of nadi oxacin and pruli oxacin. They observed that the solubility of quinolones was greater in higher generation dendrimers than in lower ones. Encapsulation/complexation of quinolones into/with dendrimers resulted in excellent solubility enhancement and a similar antibacterial activity as that of pure drugs.[224] Similarly, sulfamethoxazole, which causes problems in its clinical applications because of its poor solubility, has been investigated for its solubility, *in vitro* drug release, and antibacterial activity using PAMAM dendrimers with ethylenediamine core.[226] The results of this investigation revealed that there was a 40-fold solubility increase in G3 PAMAM dendrimer solutions (10 mg/mL) as compared with the solubility in double-distilled water. The release of drug from dendrimer was also sustained, with the dendrimer drug being more potent against *E. coli* than free sulfamethoxazole (almost fourfold to eightfold increase in antibacterial activity).[226] A recent study indicated that PAMAM dendrimer complexes with silver sulfadiazine, a poorly soluble drug, and silver could be employed to achieve a bottom-up approach to synthesize and enhance the solubility of highly soluble silver sulfadiazine NPs and create a nanosystem with enhanced antimicrobial properties.[230]

The amphiphilic linear dendritic block copolymer composed of poly(propylene oxide) (hydrophobic core), and PAMAM dendrimer (outer corona), was prepared and triclosan, a hydrophobic drug, encapsulated in layer-by-layer lms formed from micelles of the dendritic polymer showed release times over a period of several weeks. Furthermore, a Kirby Bauer test on *S. aureus* con rmed that the released drug was still active to ensure growth inhibition of *S. aureus*.[225]

Targeted intracellular delivery has also been a goal for dendrimeric nanostructures of antibiotics, with erythromycin, a macrolide antibiotic, being conjugated with bifunctional PAMAM dendrimer (G4-OH-Link-NH_2), which resulted in its sustained release. This study further focused on intracellular delivery studies for erythromycin as an anti-in ammatory agent to manage periprosthetic in ammation. It has been also observed that the synthesized conjugate retained its antibacterial activity, its antibacterial activity being similar to free erythromycin against *S. aureus* at different concentrations.[227] The lack of detailed studies on antibacterial activity of conjugate was

addressed in 2011 by Mishra et al.,[228] who synthesized conjugate of azithromycin, a macrolide antibiotic, with G4-PAMAM dendrimer, to obtain dendrimer drug conjugate nanodevice for treating *Chlamydia trachomatis* infections. This study explored the potential of G4 PAMAM dendrimers as intracellular drug delivery vehicles into chlamydial inclusions. Approximately 90% of the drug was released from the azithromycin PAMAM conjugate over a 16 h period and azithromycin readily entered the *Chlamydia*-infected HEp-2 cells and inclusions. When added at the time of infection, the conjugate was significantly superior to free drugs in the prevention of productive infections in cells. In addition, the conjugate was found to be better in decreasing the size and number of inclusions after adding the conjugate at either 24 or 48 h post infection. This study emphasized the nding that even if the organism is in the persistent form, dendrimers can ef ciently deliver drugs to growing intracellular *C. trachomatis*.[228]

Recent ndings suggest that even coadministration of antibiotic with a dendrimer results in lowering the dose of drug required for antibacterial action.[232,233] This was proved by coadministering nadi oxacin[232] and cipro oxacin[233] with G4 PPI dendrimer. G4 PPI dendrimers and their maltose-modi ed derivatives exhibited enhanced antibacterial activity of nadi oxacin against Gram-negative *E. coli* ATCC 25922, *P. aeruginosa* ATCC, 15442 and *Proteus hauseri* ATCC 13315 without any harmful effect on eukaryotic cells.[232] Similarly, coadministration of cipro oxacin with PPI dendrimers resulted in a formulation with improved antibacterial properties of a cipro oxacin at lower concentrations against Gram-positive *S. aureus* ATCC 6538 and Gram-negative *E. coli* ATCC 25922. These ndings are signi cant because of drug resistance as a result of the extensive use of antibiotics.[233] However, a study on the effect of G2 and G3 PAMAM dendrimers on the antibacterial activity of poorly water-soluble erythromycin and freely water-soluble tobramycin disclosed that though solubility of erythromycin was increased by seven to eightfold in PAMAM dendrimers, there was only a minimal effect on its antimicrobial activity.[229] A twofold and fourfold decrease in MBC values of erythromycin was observed for hydroxyl-terminated and amine-terminated G3 PAMAM, respectively. Furthermore, it was found that there was no in uence of PAMAM on the antimicrobial activity of tobramycin. Antibacterial activity studies in this investigation were performed on *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 700603, *E. cloacae* ATCC 700323, *Acinetobacter baumannii* LMG 1025, and clinical strains of *S. aureus* and *E. Faecalis*. [229] The differences among these studies show the in uence of dendrimer type in terms of core, branching element, and dendrimer generation on antibiotic activity.

A dendrimer was recently used to conjugate vancomycin to increase the drug cell wall avidity,[231] this being active against Gram-positive bacteria because of its strong attraction to a cell wall precursor terminated with a $_{(D)}$-Ala-$_{(D)}$-Ala peptide residue (Ala-alanine).[235 237] However, it is not active against VRE, as it displays a weak af nity for the $_{(D)}$-Ala-$_{(D)}$-Lac (Lac-lactate) residue present on its surface.[238] Vancomycin-conjugated G5 PAMAM dendrimer series have been synthesized and their avidity to $_{(D)}$-Ala-$_{(D)}$-Ala or $_{(D)}$-Ala-$_{(D)}$-Lac cell wall precursor was established using surface plasmon resonance studies. The nanoconjugates exhibited signi cant enhancement in avidity in the tested cell wall models. As compared with free vancomycin, the nanoconjugate showed a greater increase in binding by four to ve orders of magnitude. As a synthetic polymer, NP, with a size of 5.4 nm G5 PAMAM dendrimer, served as a platform for conjugating multiple copies of vancomycin on its structure, resulting in high-avidity binding on the bacterial surface. Iron oxide magnetic nanodevices were prepared using the conjugates with high af nity to the bacterial surface to investigate the possibility of combining the bacteria-targeting strategy with the speed and convenience delivered by magnetic isolation technology. These dendrimer-covered iron oxide magnetic NPs demonstrated a more rapid sequesteration of bacterial cell walls compared with iron oxide NPs. The study proved the concept that bacteria-targeted dendrimers might be used for fabrication of magnetic NPs, with the resulting formulation opening a convenient route for bacterial magnetic isolation and enumeration.[231]

Most recently, synergistic *in vitro* bactericidal effect against Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria has been reported for nor- oxacin in combination with 3-hydroxypyridin-4-one (HPO) hexadentate-based dendrimeric chelator. Owing to their large molecular weight, dendrimeric chelators penetrate membranes slowly and have the bene t of low toxicity compared to smaller molecules. The authors therefore proposed that a combined formulation of HPO hexadentate-based dendrimeric chelator and quinolone antibiotic can have medical potential, principally in treating external infections including wounds and ulcers.[234]

The studies on dendrimer-mediated nanodelivery of antibiotics are limited, although drugs from several therapeutic categories have been studied for their delivery, either by conjugation, entrapment, or encapsulation to enhance their performance in terms of release pattern, solubility, and pharmacological action. This lack in dendrimer-mediated delivery of antibiotics may be attributed to the fact that the research focused mainly on inventing new dendrimers with their antibacterial activity. Although it is interesting to obtain novel dendritic antibacterial dendrimers that may evolve as potential drug candidates in future, it should be noted that US FDA approval of these new chemical entities as antibiotics is a long process. In the present situation, there is an urgent need developing novel nanoformulations using currently existing biocompatible dendrimers and antibiotic drugs in order to combat emerging resistant strains. The review also revealed that PAMAMs are the mostly studied dendrimers for antibiotic delivery, and that most of the studies have focused on *in vitro* antibacterial activity. Therefore, other novel biocompatible dendrimers that have already been reported in the literature should also be exploited for effective nanodelivery of antibiotics, and more emphasis should be given to *in vivo* performances of these nanosystems in order to introduce a dendrimeric nanoantibiotic in clinical trials.

Nanoemulsions

Nanoemulsions can be described as heterogeneous systems comprising dispersed oil droplets stabilized by surfactant molecules in an aqueous media. Their nanometer size makes them kinetically stable during storage over long-term periods.[239,240] NEs display many attractive biological and pharmaceutical characteristics including biodegradability, biocompatibility, ease of preparation, and physical stability.[241] Because of their interesting properties,

recently, increasing attention has been focused on NE-based drug delivery systems.[242] NEs can be effectively produced by high-pressure homogenization,[243] micro uidization,[244] ultrasonication,[245] and phase inversion.[246]

Nanoemulsions containing antibiotics have been investigated by several researchers for their bactericidal activity, with Penicillin G containing injectable NE being developed and studied for its properties.[241] NE has been proven to be a stable formulation for intravenous delivering rifampicin.[247] A water-in-oil emulsion technique has been established for preparing NE particles of chitosan/heparin with better encapsulation of amoxicillin. The formulated amoxicillin NE showed controlled release and localization at intracellular spaces and in the cell cytoplasm to the site of *H. pylori* infections, with a signi cant increase in the growth inhibition.[248] An oil-in-water submicron emulsion, with globule size of 278 ± 12 nm and prepared by incorporating hydrophobic ion-pair complexes of cipro oxacin with sodium deoxycholate in the core, showed high entrapment ef ciency, noncytotoxicity to J774 macrophage cells, and enhancement in antimicrobial ef cacy against *E. coli*, *S. aureus*, and *P. aeruginosa in vitro*.[249] Studies so far have focused on the role of NEs to enhance antibiotic activity, indicating that their applications as a delivery system to site-speci c delivery, sustained, and prolonged release could be further exploited. Besides these, NEs that have been formulated using different oils and are devoid of any antibiotic drug have also been found to be effective antibacterials, for example, peppermint oil NE,[250] cinnamon oil NE,[245] eucalyptus oil NE.[251] Overall, results of these studies suggest that antibacterial activity of bio-based oils could be enhanced by dispensing them into nano form.

Polymeric Micelles

Self-assembling colloidal systems possessing a core/shell structure (size < 200 nm) formed by assembly of block or graft amphiphilic block copolymers are known as polymeric micelles (PMs)[252,253] and are frequently based on copolymers having an AB diblock structure.[254,255] The hydrophobic core facilitates the solubilization of hydrophobic drugs via hydrogen bonding and/or hydrophobic interaction and the hydrophilic shell remains exposed to the external environment. This kind of arrangement helps in protecting the bioactive against degradation and also facilitates escape from the RES, thereby exhibiting prolonged systemic circulation.[256,257]

A few studies have been reported so far for antibiotic delivery via PMs. In one such report, cloxacillin sodium, an anionic drug, was incorporated into a protonated polyvinyl pyridine (PVP) block of polystyrene-*b*-2-vinyl pyridine-*b*-ethylene oxide (PS-PVP-PEO) micelles. The experiment was designed to investigate the possibility of the micelle being an antibiotic drug carrier. This study used zeta potential measurements, dynamic light scattering, and uorescence spectroscopy speci cally, and proved that cloxacillin could be ef ciently incorporated into 69 nm-sized micelles prepared from PS-PVP-PEO because of electrostatic interaction between the protonated PVP block and anionic drug.[258] Although the release kinetics were identi ed, this study would have been strengthened by including at least transmission electron microscope image to con rm the appearance and morphology of the micelles, drug encapsulation ef ciencies, as well as antibacterial activity, as encapsulation of the drug molecule was not unexpected. PMs appear to be very promising ocular drug delivery systems because of their properties, such as high kinetic and thermodynamic stability, sustained drug release pro les, and the ability to act as an absorption promoter in order to enhance drug permeability across ocular epithelia.[253,259] Considering this fact, ocular delivery of netilmicin sulfate was studied by three copolymers of polyhydroxyethyl aspartamide. *In vitro* permeability studies with primary cultured rabbit conjuctival and corneal epithelial cells demonstrated that micelles of two of the polymers provided greater drug permeation across the latter compared with a simple drug solution or suspension.[260] Dif culty in transporting antibiotics through the BBB has also been overcome by PMs prepared from cholesterol-conjugated PEG and anchored with transcript or activator TAT peptide (TAT-PEG-b-Col). The cipro oxacin-loaded TAT-PEG-*b*-Col micelles smaller than 180 nm showed sustained antibacterial activity against *B. Subtilis* and *E. Coli*, and *in vivo* animal tests con rmed that the formulation can pass the BBB. This study therefore highlighted the applicability of these micelles for developing nanodelivery systems to treat brain infections.[261] The extensive *in vitro* and *in vivo* characterization of this PM formulation, in terms of size, zeta potential, morphology, *in vitro* release, antibacterial activity, cellular uptake, cytoxicity, and *in vivo* animal studies with male rats, is in contrast to the inadequately characterized system of PS-PVP-PEO micelles[258] mentioned earlier.

Increasing attention is being focused on polymers that are inherently antimicrobial because of their wide applications in the health care of both humans and animals.[262–265] The advantages of antimicrobial polymers are their effective inhibition of bacterial growth without the low-molecular-weight toxic chemicals being released to the environment,[265] as well as no resistance development by common bacterial strains such as *E. coli* and *S. aureus*.[266] This has stimulated researchers to develop PMs devoid of any drug as antibacterial agents, such as PMs containing quaternary ammonium compound poly[2-(tert-butylamino)ethyl methacrylate] (PTBAEMA or PTA).[267] On the basis of these ndings about PTA, Yuan et al.[265] reported synthesis of two triblock antibacterial polymers consisting of poly(ethylene oxide (PEO)-PCL **1** and PTA (PEO-*b*-PCL-*b*-PTA) **2** polymers. PEO was used to enhance the biocompatibilty and colloidal stability of the self-assembled micelles in aqueous solution, whereas PTA was used for interacting with the microbial cell wall/membrane. Both these polymers were able to form micelles in THF/water, with a mean diameter of 18 ± 3 nm for polymer **1** and 25 ± 4 nm for polymer **2**. The MBC for polymer **1** was 0.30 mM and 0.15 mM against *E. Coli* and *S. aureus*, respectively, whereas for polymer **2,** it was reported to be 0.20 mM and 0.08 mM in micellar form.[265] Thus, it can be concluded that these PEO-*b*-PCL-*b*-PTA polymers can be used as promising sterilizing agents or as antimicrobial drugs in future. The promising properties of the drug-loaded and drug-free antimicrobial PMs highlighted in this section indicates an opportunity for researchers to encapsulate current antibiotic drugs into the antimicrobial PMs to achieve a multifunctional delivery system with synergistic antibiotic effects.

CNTs, Nanohorns, and Nanorods

Carbon nanotubes, nanohorns, and nanorods have also been reported as nanosystems for antibiotics. Cylindrical nanostructures of pure carbon atoms covalently bonded in a hexagonal array are called CNTs,[268] produced either by arc

discharge, chemical vapor deposition, or laser ablation methods. The details on the methods of CNT production can be found elsewhere.[269] CNTs with a single pipe (1 5 nm diameter) are single-walled CNTs (SWCNTs), and those having many nested tubes (lengths from 100 nm to micrometers) are known as multiwalled CNTs (MWCNTs).[270] Both SWCNTs and MWCNTs possess antimicrobial activity, with the former exhibiting much stronger antimicrobial properties[271] than the latter. Although ease of functionalization together with its good chemical stability makes SWCNTs additionally attractive as antimicrobial biomaterials,[272] its synthesis cost are high.[273] Qi et al.,[274] in an attempt to exploit the lower costs with MWCNT and to overcome its reduced antibacterial activity, used covalent immobilization of cefalexin on MWCNTs via PEG as a linker to enhance the antimicrobial and antiadhesive characteristics of MWCNTs against *S. aureus* and *B. Subtilis* (Gram positive), and *E. Coli* and *P. aeruginosa* (Gram negative). Confocal laser scanning microscopy studies of attached MWCNTs and MWCNT cefalexin revealed that most of the *P. aeruginosa* and *S. aureus* cells were stained with propidium iodide dye (dead cells) on MWCNT cefalexin deposited lm, and with SYTO 9 dye (live cells) on the MWCNT deposited lm. This nding revealed that MWCNT cefalexin deposited lm has superior antimicrobial property than the drug-free MWCNTs deposited lm.[274]

Kang et al.[271] prepared low metal content, narrowly distributed and highly puri ed SWCNT with strong antibacterial activity. As with the study by Qi et al.,[274] such a SWCNT system could be used for encapsulating an antibiotic drug for enhanced activity. Aslan et al.[272] reported an interesting strategy to overcome the high cost and limited range of material properties with SWCNTs. They investigated the concept of combining SWCNTs (as a minority component) with a biomedical polymer, that is, PLGA, to obtain a material that would be antimicrobial and provide a broad range of structural, mechanical, and degradation properties. The SWCNT PLGA polymer was found to be far superior in antibacterial activity than the PLGA only. The possibility of antibiotic loading into biomedical polymers containing SWCNT being an effective strategy for a superior antimicrobial nonintegrated implant needs to be investigated further.

Although antimicrobial activity of CNTs has been reported, cytotoxicity associated with them is a major concern, as reported by a number of studies.[275 277] Future studies with drug-free and drug-loaded CNTs should therefore also focus on approaches to overcome the cytoxicity of these promising delivery systems.

Nanohorns are similar to fullerenes and SWCNTs, and consist of a seamlessly closed one-atom-thick wall of carbon that separates the exterior from the hollow interior. The body of a nanohorn is more or less tubular, with an irregularly varying diameter along its length. Representative nanohorn diameters are between 2 and 5 nm with one end being cone-shaped, the horn, whereas the opposite end is at or rounded.[278 280] Unlike nanotubes, nanohorns assembling into cylindrical bundles with their long axes parallel to each other form spherical aggregates.[278 281] A new type of graphene tubules with a diameter of 2 5 nm and a length of 40 50 nm is known as a single wall nanohorn (SWNH). A spherical aggregate with a narrow diameter distribution of 80 100 nm is formed by an assembly of approximately 2000 SWNHs.[280] The potential of nanohorns in drug delivery has been demonstrated.[281 283] SWNH aggregates have been reported as potential promising drug carriers having some advantages over other carriers. Oxidized SWNH (SWNHox) have been reported for providing controlled release of vancomycin hydrochloride (Fig. 5) to address the problems associated with the drug, such as severe side effects while blood concentration is too high. Controlled release was obtained by exploring the bene t of interaction between vancomycin hydrochloride and SWNHox. Additionally, to improve the dispersibility of this carrier system in aqueous systems, the

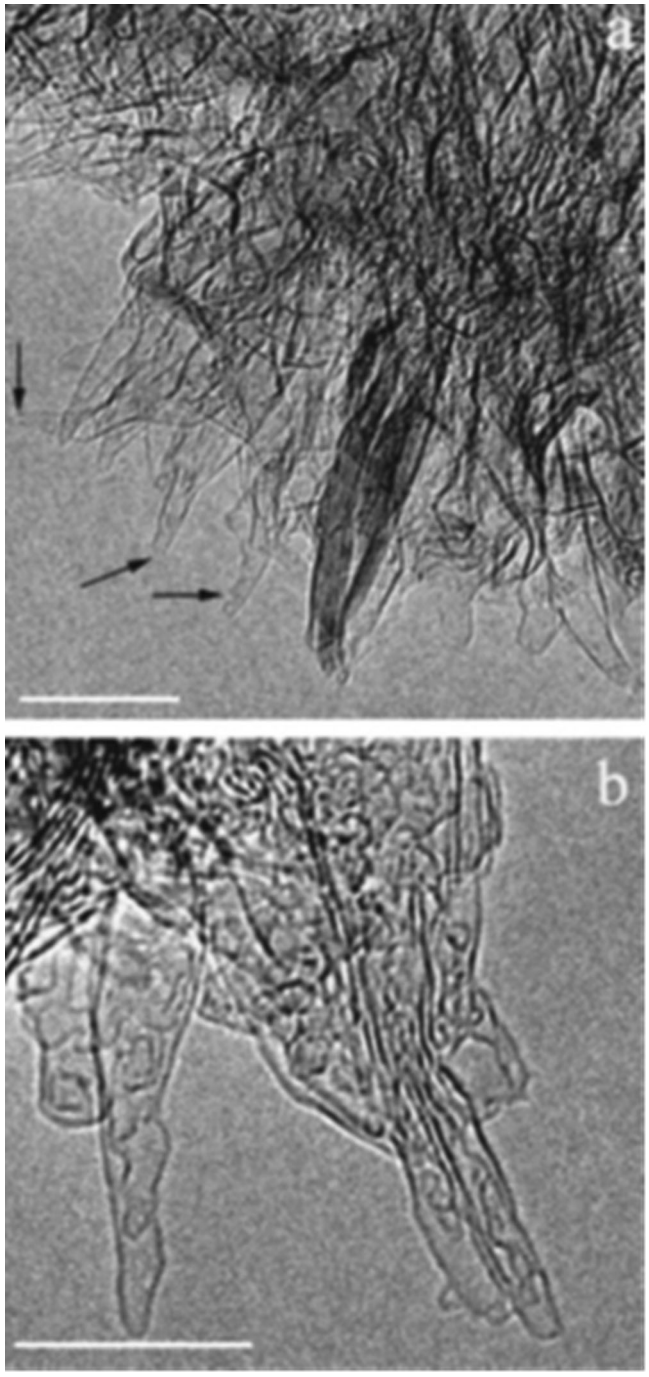

Figure 5. Transmission electron microscopy images of (a) SWNHox (scale bar = 20 nm) and (b) VCM SWNHox (scale bar = 10 nm). Reproduced from Xu et al.[284] with permission from Elsevier Science Ltd.

hydrophobic surface of SWNHox was modified by phospholipid PEG.[284]

Nanorods are rod-shaped NPs, with different kinds having been reported in the literature depending on the material used, for example, silver,[285] zinc oxide,[286] stannous oxide,[287] barium carbonate,[288] and gold,[289] the latter being an attractive vehicle for drug delivery applications.[290 292] Nanorods of lanthanum hydroxyapatite have been used for sustained amoxicillin release, specifically those that showed antimicrobial activity against *bacillus*, *pseudomonas*, *E. coli*, and *S. aureus*. In addition to the antimicrobial and drug release studies, this nanorod system was extensively characterized for its physical properties. The increased surface area and suitable hardness, crystallinity, and crystallite size led the authors to propose this nanorod system as potential implants in the biomedical field.[293]

Nanohybrids

Bioactive molecules incorporated in layered double hydroxide (LDH) forming nanohybrids (NHs) have gained attention in drug delivery, being normally referred to as hydrotalcites or anionic clays.[294] LDHs represent a family of synthetic or natural materials designated by the formula $[M_{(1-x)}^{II} M_x^{III}(OH)_2][A^{n-}]_{x/n} \cdot 2H_2O$, where M^{II} and M^{III} are divalent and trivalent metal, respectively, and A^{n-} is the interlayer anion.[295] The first delivery system based on magnesium aluminum LDHs was reported in 2005.[296] LDHs form successive positively charged metal hydroxide layers and negatively charged anionic layers. Amid the various properties, the anion-exchange property of LDHs provides a simple method enabling replacement of the interlayer anion, thus permitting the synthesis of a various layered materials.[297] Using this ion-exchange reaction, bioactives have been incorporated/intercalated into LDHs to generate NHs with a slow release of the active.[298,299] Intercalation of two hydrophobic drugs, namely, gramicidin and amphotericin B and two hydrophilic drugs, namely, ampicillin and nalidixic acid, with LDHs was studied using a simple ion-exchange reaction. All four drugs intercalated successfully and the release studies showed that the synthesized NHs can function as controlled-release drug delivery systems for various antibiotics.[294] A new polymeric composite material has been prepared and characterized by incorporating chloramphenicol succinate-NH into a biocompatible, biodegradable polymer matrix, PCL. In the NH consisting of a LDH of Mg Al hydrotalcite type, simple ion-exchange reaction was used to replace the nitrate anions present in the host galleries with chloramphenicol succinate anions. The objective of the study was to develop a controlled-release formulation for topical application.[298] From the unique biphasic release profiles of chloramphenicol, the authors concluded that the structural design of this hybrid offers several ways to modify drug release properties. These consist of the ionic force present in the outside solution, drug concentration inside the inorganic lamellae, inorganic component concentrations into the polymer matrix, type of polymeric matrix, and the sample form and thickness. LDH NHs intercalated with amoxicillin by coprecipitation method have also been encapsulated into PCL electrospun fibers. This NH-integrated system provided sustained release of the drug, although initial rapid release was found.[300] This study highlights the applicability of this NH system to be integrated into other novel delivery systems for further enhancing drug therapy.

The decoration of MWCNTs with metal NPs, such as Fe_3O_4, results in the formation of MWCNTs NHs. This exercise of decorating MWCNTs with metal NPs is executed to overcome toxic effects and dispersibility problems associated with MWCNTs, and confer unique features to the NH system. They have a prolific effect on microbicidal and biofilm inhibition activity, biocompatibility, and drug targeting.[301] Hyperbranched polyurethane (HBPU) is a well-known wound healing material and potent drug carrier.[301,302] Its application, along with Fe_3O_4 MWCNT NH to form Fe_3O_4 MWCNT NH/HBPU nanocomposites (NNCs), has been explored in the development of effective wound healing material. *In vitro* antibacterial activity of gentamicin sulfate-loaded NNCs against *K. pneumonia* and *S. aureus* MTCC96, using the agar well diffusion method, showed best performance along with good hemo compatibility and nonimmunogenicity because of controlled-release profiles. *In vivo* wound healing experiments performed on albino mice showed significant acceleration in wound healing process. Furthermore, the fluid handling capacity and moisture vapor permeability of these NNCs suggested its immense potential to provide an optimal moist environment to accelerate the wound healing process. The findings of this study prove that this novel Fe_3O_4 MWCNT NH/HBPU NNC is a potential wound healing material with the ability to deliver antibiotics to the wound site.[301] The incorporation of antibiotics either into NHs alone, intercalated with NHs for coencapsulation into fibers, or loaded into NNCs comprising metal-coated CNT NHs and wound healing material, is evident of the diverse potential of NHs for antibiotic delivery.

Other Nanosystems for Antibiotic Delivery

In addition to the aforementioned more widely published nanoantibiotic systems, researchers have reported on a number of other nanodelivery systems for antibiotics, which are reviewed below.

Nanofibers

Nanofibers are defined as fibers with a diameter of 100 nm or less, but in general, all fibers with a diameter below 1 μm are considered as nanofibers.[303] Nanofibers are being studied for wound healing purposes in antibacterial therapy. Electrospun nanofibers have shown great ability for wound dressing as a result of properties, such as their high-surface area that enables them to effectively absorb exudates and adjust the wound moisture.[304]

Electrospun drug-loaded nanofibrous membranes are advantageous over conventional nanofibers. Electrospun sandwitch-structured PLGA/collagen nanofibrous membranes containing vancomycin and gentamicin were found to be effective wound dressing materials.[305] These authors successfully confirmed the antibacterial efficacy, cytocompatibility, and sustained drug release properties of these antibiotic-loaded nanofibers. Kataria et al.[306] recently reported the development of ciprofloxacin-loaded transdermal patch prepared from PVA and sodium alginate (NaAlg) electrospun composite nanofibers for local delivery of antibiotic. In their experiments, they prepared PVA, PVA NaAlg, ciprofloxacin loaded PVA, and ciprofloxacin-loaded PVA NaAlg nanofibers, and performed comparative studies in terms of morphology, drug release, and *in vivo* wound healing efficacy. All nanofibers with average diameter in the range of 300 400 nm showed nonwoven mat-like structures

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and smooth surfaces. In *in vitro* drug release experiments, the drug release from PVA NaAlg nano bers was slower compared with PVA nano bers. Furthermore, higher hydroxyproline content in animal studies with cipro oxacin-loaded PVA NaAlg nano bers indicated their superior wound healing capability compared with the drug-loaded PVA nano bers, and in less time.[306] This study opens the opportunity of nano brous transdermal patches as an alternative and superior delivery system for local delivery of antibiotics and even other classes of drugs.

Nanofibrous Scaffolds

Regeneration of natural bone tissue or the creation of biological substitutes for defective bone tissues is possible through the use of scaffolds.[307] Nano brous scaffolds, as the terminology suggests, refers to scaffolds composed of nano bers. The advantages of a nano brous scaffold are its high surface-to-volume ratio, high porosity, changeable pore-size distribution, and similarity to the natural extracellular matrix in terms of morphology.[308] Nano brous scaffolds fall under the category of polymer-based drug carriers that are of synthetic origin, are biodegradable,[309] and are mainly used for tissue engineering purposes.[310] The advantages of electrospun nano brous scaffolds can be summarized as: (1) they can be used as carriers for both hydrophilic and lipophilic drugs, (2) ne control over the drug release pro le can be achieved by controlling the scaffold s porosity, morphology and composition, and (3) it is possible to achieve site-speci c delivery into the body for any number of drugs from the scaffold.[309] As a result of these advantages, nano brous scaffolds are being studied for delivering antibiotics such as (1) novel nano brous scaffolds of doxicycline to obtain high local bioavailability, low systemic side effects, and controlled delivery to treat dental, periodontal and bone infections[311]; (2) gentamicin-loaded novel PLGA/lecithin scaffolds for bone-repairing therapeutics[312]; (3) PLGA-based nano brous scaffolds with lidocaine, an anesthetic and mupirocin, an antibiotic having controlled-release mechanism for wound dressing[313]; and (4) cefoxitin sodium-incorporated PLGA-based nano brous scaffolds with sustained drug release for preventing postsurgical adhesion and infections.[309] Although one of the earliest antibiotic-loaded nano brous scaffold appears to have been reported 10 years ago in 2004, there have been very few studies since then addressing the necessity of surgery for implantation.

Nanosheets

Recent developments in nanotechnology have made it possible to fabricate quasi, two-dimensional, freestanding polymeric ultrathin lms (polymer nanosheets or simply nanosheets) with remarkable properties, such as high exibility, minimum surface roughness, and noncovalent adhesive properties.[314 319] The polysaccharide nanosheet forms a stable platform for facilitating drug loading, with nanosheets loaded with TC for treating gastrointestinal defects, such as gastric peritonitis and other surgical defects, having been reported in the literature.[319] TC was compressed between polyvinyl acetate (PVAc) and polysaccharide nanosheet to form a PVAc TC nanosheet of 177 nm thickness. *In vivo* studies on mice revealed that therapy with the PVAc TC nanosheet signi cantly increased survival rate of mice after cecal puncture, and an increase in intraperitoneal bacterial and leukocyte count was also suppressed.[319] In a separate paper, these authors found the same nanosheet to be an effective nanoantibiotic system to treat full-thickness burn wound infections by *P. aeruginosa in vivo*.[320] It would have been interesting for the researchers to have included bioadhesivity and textural analysis, as optimal bioadhesion and mechanical properties are critical aspects of this delivery system. These are preliminary studies on nanosheets, and formulation optimization and characterization appear to be in its infancy.

Nanoplexes

Nanoplexes are complexes of a drug and oppositely charged polyelectrolyte forming stable amorphous NPs, and are manufactured by mixing two aqueous salt solutions, one containing the former and the other the latter.[321] Cheow and Hadinoto[321] recognized that the amphiphilicity and solubility in acid or basic solutions of antibiotics can be exploited for preparing antibiotic NPs via a process known as self-assembly amphiphilie polyelectrolyte complexation. Higher drug-loading capabilities can therefore be achieved compared with conventional NPs. The authors synthesized drug polyelectrolyte complexes (nanoplexes) of o oxacin and levo oxacin by self-assembly complexation within dextran sulfate with an antibiotic loading of 60% 80% (w/w) and sizes less than 400 nm. The optimal preparation conditions based on its size, stability, and drug loading by varying the pH, polyelectrolyte charge ratio, drug, and salt concentration were identi ed. These nanoplexes were examined *in vitro* against *P. aeruginosa* planktonic cells and the activities were found to be comparable to native antibiotics. The main advantages of these nanoplexes were salt-promoted drug release and rapid antibiotic release, rendering it suitable for antibio lm treatment, which needs high doses of antibiotic in order to eliminate the appearance of antibiotic-resistant strains.[322] Nanoplexes certainly have promising potential for diverse applications and growth as it can facilitate high drug encapsulation, unlike polymeric and liposomal nanosystems, offers greener and simpler methods of preparation for various antibiotics, and the charged surface makes them readily functionalized.

CONCLUSIONS AND FUTURE PERSPECTIVES

Factors such as poor targeting of antibiotics to infection sites, increased dosing frequencies and side effects, the spread of resistance to currently used antibiotic medicines, slow development rate of newer antibacterials, and the possibility of resistance to future new antimicrobial drugs all highlight the need to follow novel approaches for managing microbial infections. In the last four to ve decades, considerable research has been undertaken on nanodelivery systems, resulting in revolutionary changes to drug delivery technology for various disease conditions. More recently, an explosion of interest in the use of nanotechnology to overcome the signi cant challenges associated with antibiotic drug therapy is evident in the literature.

This review indicated that a range of diverse nanoengineered drug delivery systems, such as liposomes, PNPs, SLNs, dendrimers, NEs, LPHNs, PMs, CNTs, nanorods, nanohorns, NHs, nano bers, nano brous scaffolds, nanosheets, and nanoplexes are being investigated for antibiotic delivery. Studies on these antibiotic-loaded nanosystems have con rmed enhanced activity against sensitive and resistant bacteria. The ability of these

nanosystems to improve solubility, stability, and drug entrapment provides sustained drug release, target infection sites, penetrate the BBB, improve antibio lm therapy, and overcome bacterial resistance have been amply demonstrated. It is also clear that researchers are moving toward antibiotic nanosystems with multifunctional properties and multiple mechanisms of action to enhance antimicrobial action and prevent drug resistance.

Although signi cant progress has been achieved in the eld of nanoantibiotics, much remains to be accomplished to optimize these systems for eventual regulatory approval and commercialization. This review has speci cally identi ed a number of areas that need to be investigated and prioritized. Formulation optimization technologies and in-depth physicochemical/mechanical characterization for newly emerging and promising antibiotic nanosystems, such as LPHNs, PMs, SLNs, nanorods/plexes/sheets, and dendrimers need to be prioritized, as these are less investigated in the literature compared with liposomes and PNPs. Several lipid- and polymer-based nanosystems can be enhanced by identifying and synthesizing new lipidic and polymeric materials with responsive properties to promote targeting to infection sites. For example, lipids and polymers responsive to speci c pH, bacterial toxin, and enzymatic changes at infection sites can be considered. Identifying these novel materials will widen the pool of superior materials for developing nanoantibiotics. The coencapsulation of antibiotics with other antibiotics, as well as nondrug antimicrobial agents, offers the opportunity of developing nanosystems with multiple mechanisms of action against bacteria that can enhance activity and also overcome resistance mechanisms. A goal should therefore be nanosystems comprising responsive antimicrobial materials with multiple antimicrobial agents. Such a multidimensional integrative nanodelivery system will give rise to a generation of smart nanoantibiotics. There is also a lack of data that offers a mechanistic and molecular understanding of these nanosystems in terms of their antimicrobial activity against various organisms, drug entrapment, and drug release properties. Such studies will guide formulation scientists in designing optimal antimicrobial materials and nanosystems. More formulation studies also need to focus on *in vivo* antimicrobial investigations for both widely and less studied antibiotic nanosystems. Scale-up and strategies and studies on these systems should also be a focus.

It is evident that a multidisciplinary collaborative relationship among researchers in academia and the pharmaceutical industry will be essential to successfully develop smart nanoantibiotics, which are clearly showing potential for saving millions of lives globally from serious life-threatening infections by microorganisms.

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CHAPTER 5. CONCLUSION

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

CHAPTER 5. CONCLUSION

5.1 General Conclusions

Infectious diseases are a growing concern globally, with the limitations of current antibiotic dosage forms and the increasing problem of antibiotic resistance having resulted in rising morbidity and mortality deaths rates worldwide. Novel nano drug delivery systems that offer an alternative strategy to deliver antibiotics are being explored to overcome the limitations associated with existing dosage forms of antibiotics. Lipid polymer hybrid nanoparticles (LPNs) are a relatively new type of nano drug delivery system with several advantages that make them suitable for antibiotics. However, to date, there is limited data available on LPN loaded antibiotics and formulation optimisation needs to be studied, as they have not been extensively investigated or characterised in the field of antibiotics.

The aim of this study was therefore to formulate and evaluate novel vancomycin loaded lipid-polymer nanoparticles to enhance antibiotic therapy. The objectives of this study were therefore to i) to prepare VCM loaded LPNs containing a new lipid-polymer combination of Eudragit RS100 as the polymer and Glyceryl tripalmitate as the lipid., ii) to simultaneously enhance the encapsulation efficiency and antibacterial activity of the nanoparticles by incorporation of various co-excipients such as oleic acid, chitosan and sodium alginate and iii) to evaluate the lipid-polymer nanoparticles in terms of particle size, surface charge, morphology, drug release, antimicrobial activity, thermal behaviour and crystallinity and corroborate the data with *in silico* modelling.

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

- The first step was to screen different lipids, polymers and surfactants that would ultimately constitute the best LPN in terms of particle size, PDI, zeta potential and encapsulation efficiency. LPNs were successfully prepared by hot homogenisation method followed by ultrasonication. To achieve the best results, the variables in the method of preparation used was also changed to optimise the formulation. The optimal formulation comprised of Glycerly tripalmitate (0.5g) as the lipid, Eudragit RS100 (0.25g) as the polymer, and Solutol HS 15 (1%w/v) as the surfactant, and was reached at a homogenisation speed of 6000 rpm for 10 minutes followed by ultrasonication at 30% amplitude for 30 minutes. This formulation achieved a sutiable particle size of 214.1 ± 6.86 nm, PDI of 0.251 ± 0.01 and zeta potential of +28.9 ± 1.98 mV.

- The next step of the process was to add the drug vancomycin (VCM), and to determine the optimal amount to be used in the formulation. Different concentrations of drug were used with 0.02g being the optimal quantity. In addition, the lipid to polymer ratio was an important variable to achieve the highest encapsulation efficiency, with an optimal ratio of 2:1 being determined. The combination of optimal drug and lipid to polymer ratio revealed rod shaped particles with a size of 216.4 ± 9.98 nm, a PDI of 0.284 ± 0.03, a zeta potential of +29.7 ± 4.91 mV, encapsulation efficiency of 27.8 ± 1.84% and drug release of 52.3 % after 24 hours.
- The final step was to incorporate different helper excipients to enhance critical properties such as the encapsulation efficiency, drug release and antibacterial activity. Two polymers, chitosan (CHT) and sodium alginate (ALG), and one fatty acid, oleic acid (OA), were studied as helper excipients. The results showed that the EE increased from 27.8% to 41.5%, 54.3% and 69.3% with the addition of OA, CHT and ALG respectively. Drug release data showed that VCM-CHT had the slowest drug release of 36.1 ± 5.35%, while VCM-ALG had the fastest drug release rate of 54.4 ± 3.24% at the end of 24 h, with all formulations indicating a sustained release profile. *In vitro* antibacterial studies of all formulations exhibited better activity against bare VCM, and sustained their activity up to day 5 against both *S.aureus* and MRSA, with VCM-OA and VCM-CHT specifically showing better activity against MRSA. VCM-OA LPNs showed the best activity with an MIC value of 1.2µg/ml against MRSA on day 2. All formulations were evaluated in terms of particle size, PDI, zeta potential, EE, morphology, drug release, antibacterial activity, X-ray diffraction studies (XRD), differential scanning calorimetry (DSC) and stability studies. XRD showed an amorphous state of the drug, and no changes in crystallinity of the drug was observed in the LPN formulation. The DSC results revealed that the VCM was entrapped within the LPN, as depicted from the absence of the VCM peak in the LPN formulation. Stability studies indicated that all formulations were stable at both 4°C and room temperature for 3 months.
- The *in vitro* release kinetics and *in silico* studies were performed to corroborate the *in vitro* data obtained. The *in silico* results explained the binding complexes between the VCM, the polymer and the helper excipients, which justified the increase in entrapment of the LPNs and the sustained drug release. The *in vitro* release kinetics data also supported the controlled release of the drug from all formulations. These studies

provided a mechanistic understanding of the molecular interactions involved in the LPN formation, and corroborated the EE and drug release data which indicated the highest entrapment of 69.3% and the fastest release of 54.4% with the addition of alginate.

The findings in this study serve as a basis for future antibiotic loaded LPNs in the field of novel drug delivery systems. The above data confirms the potential of the newly developed VCM LPN as a promising nanoantibioic. The strategies developed in this study for formulating and optimising will be useful to other scientists, and further studies in this developing field will require new approaches to achieve the best results.

5.2 Significance of the findings in the study

The formulation of vancomycin in an LPN formulation was designed to overcome the limitations associated with the drug and to enhance the antibiotic efficacy. The significant findings of the study are as follows:

New drug delivery system for Vancomycin

- A novel nano-drug delivery system not yet reported for vancomycin was developed in this study, and widens its pool of available nano-drug delivery systems that can be explored for further development.

Improvement in patient therapy and disease outcomes

- A nano-drug delivery system of vancomycin with sustained drug release and enhanced antibacterial activity against both sensitive and resistant strains was developed. It has the potential for improving patient therapy and disease outcomes by targeting effective doses to infection sites, reducing dosing frequency, decreasing side effects and enhancing antibacterial performance. The above contributes to optimal outcomes of various disease conditions that are due to antibiotic infections.

Creation of new knowledge on LPN drug delivery systems for antibiotic therapy

- This study utilised *in silico* and *in vitro* kinetics study, and explained mechanistically the interaction of the excipients and co-excipients that achieved enhanced properties, such as encapsulation efficiency, drug release and antibacterial activity. New knowledge explaining the mechanism in which different excipients interact to dictate drug release and encapsulation efficiency was generated.
- In addition, new characterisation studies on antibiotic loaded LPNs, such as antibacterial activity, gel electrophoresis, XRD and DSC were performed in this study, and provided knowledge on their in vitro performance and structural properties, thereby serving as a basis for future LPN studies.

Impact of this study on future research

- The findings of this study can stimulate further research with LPNs. For example, from the unique rod shaped particle generated, it would be interesting to study the effect of nanoparticle shape on antibacterial activity. The effective use of co-excipients can stimulate research into the systems and identify novel materials not yet reported to enhance encapsulation efficiency, drug release and antibacterial activity.
- The differences in antibacterial activity between the formulations can be further studied using additional characterisation methods to show the effect that the co-excipients have

on the system that enhance it. In addition, further molecular modelling studies would be interesting to explain the effect of the different formulations against sensitive and resistant strains.

5.3 Recommendations

This study has provided the basis for antibiotic loaded LPN formulations into a suitable nano drug delivery system. Further studies are essential to improve and enhance the delivery of antibiotics via LPNs such as:

- The next phase of this study would be to incorporate two or more of the helper excipients in the LPN, and to analyse the effects that it could potentially show by working together and possibly creating a further enhancement in the antibacterial activity, drug encapsulation and drug release.
- Additional characterisation studies can be conducted, such as morphological changes in the bacterial cell wall after treatment with the LPNs, more extensive in vitro antibacterial studies against gram positive and gram negative bacteria, and additional molecular modelling studies to understand the mechanism of LPN antibacterial activity against *S.aureus* and MRSA.
- In vivo studies using both animals and human subjects could be performed to test the formulation. This will provide information regarding the bioavailability and the pharamacokinetic properties that will be valuable for formulation modification.
- A large scale production method could be established in order to make the formulation feasible in the pharmaceutical industry. While large scale production has been established with microparticles, a protocol for nanoparticles needs to be established.
- Antibiotics other than vancomycin can be incorporated into the LPN and tested against different organisms in order to assess its advantages over a wide range of antibiotics.

APPENDIX

ENHANCING VANCOMYCIN DELIVERY VIA LIPID–POLYMER HYBRID NANOPARTICLES

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INTRODUCTION AND AIM

Nano drug delivery systems are being widely explored to overcome challenges with antibiotics for optimising the treatment of bacterial infections. Lipid polymer nanoparticles (LPNs), a kind of hybrid nanoparticlulate system having structural integrity of polymeric nanoparticles and the biomimetic properties of liposomes display advantages of both the systems whilst excluding some of their limitations (Hadinoto et al., 2013). Research focusing on antibiotic loaded LPNs is recent and mostly involves PLGA as a polymer to incorporate hydrophobic drugs (Huh & Kwon, 2011). The increased emergence of resistant bacterial strains necessitates further exploration of this hybrid delivery system using other polymers and antibacterial drugs such as Vancomycin. Therefore, the aim of the present study was to formulate and evaluate LPNs for antibiotic delivery using Vancomycin, a glycopeptide antibiotic active against MRSA.

MATERIALS AND METHODS

➢Materials

Vancomycin hydrochloride (VCM) was purchased from Sinobright Import and Export Co., Ltd. (China). Glyceryl Tripalmitate (GTP) and Solutol HS 15 were purchased from Sigma-Aldrich Co., Ltd. (USA), and Eudragit® RS 100 was kindly donated by Evonik Industries (Germany). Nutrient Broth, Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) were obtained from Biolab Inc., (South Africa). Purified water was obtained through a Milli-Q water purification system by Millipore Corp., (USA). *Staphylococcus aureus* (*S.aureus)* (ATCC 25922) and *S.aureus* Rosenbach (ATCC ® BAA-1683™) (MRSA) were used in antibacterial studies. All other chemicals and solvents used were of analytical grade and used without further purification

➢Preparation of LPNs

Drug loaded and drug free LPN's were produced by hot high pressure homogenisation followed by ultrasonication. Briefly, Glyceryl Monostearate (0,5g) was heated at 80 °C and different concentrations of Eudragit® RS100 polymer solution (80 °C) was added to the lipid and homogenised (oil phase). Solutol HS15 solution (1% w/v) was heated separately to 80 °C (aqueous phase) and added to the oil phase and homogenised (6000 rpm, 10 minutes) with an Ultra Turrax T-25 homogenizer (IKA Labortechnik, Germany). The resultant emulsion was subjected to high intensity probe sonication (30% amplitude. 30 min) using the Omni sonic ruptor 400 Ultrasonic Homogenizer (Kennesaw, GA 30144, United States) and cooled to 20 °C. The same procedure was followed for the preparation of drug loaded LPNs by adding VCM to the aqueous surfactant solution.

➢ Particle size, Polydispersity index (PI) and Zeta potential (ZP)

These parameters were determined at 25 °C by Photon Correlation Spectroscopy using a Nano ZS Zetasizer (Malvern Instruments Corp, UK).

➢ Morphology

Morphology of the LPNs was examined by scanning electron microscopy (SEM) (ZEISS FEGSEM Ultra Plus, Germany).

➢ In Vitro Antimicrobial activity

The MIC (minimum inhibitory concentration) values for drug free LPN and VCM-LPN formulation were determined against *S. aureus* and MRSA using a micro-broth dilution method. Experiments were performed in duplicate

➢ Determination of Encapsulation Efficiency (EE)

To determine the concentration of VCM in the LPNs, an ultrafiltration method using Amicon® Ultra-4, centrifugal filter tubes (Millipore Corp., USA) was used. Drug was detected by a validated spectrophotometric method at 280.4 nm using Shimadzu UV 1601 (Japan) spectrophotometer

RESULTS AND DISCUSSION

LIPID: POLYMER RATIO	SIZE (d.nm) (PDI)	ZP (mV)
0.4:1	165.47 ± 4.61 (0.293 ± 0.01)	+14.2 ± 1.61
1: 1	180.87 ± 45.07 (0.293 ± 0.04)	+19.7 ± 5.01
2: 1	208.78 ± 18.18 (0.263 ± 0.01)	+26.3 ± 2.21
4: 1	238.41 ± 31.35 (0.269 ± 0.02)	+12.5 ± 3.88

Table 1. Effect of Lipid:Polymer ratio on particle size, PDI and ZP

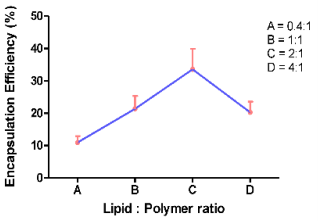

Figure 1. Effect of Lipid:Polymer ratio on Encapsulation Efficiency

Varying the lipid: polymer ratio from 0.4 to 2:1 led to an increase in EE from 14.2 % to 33.6 % (Figure 1) and a particle size from 165.47 ± 4.61 to 208.78 ± 18.18 nm (Table 1). Varying the polymer ratio led to no significant change in the particle size and EE. Therefore, the lipid shell is important in preventing diffusion of small drug molecules out of the polymer core into the aqueous phase.

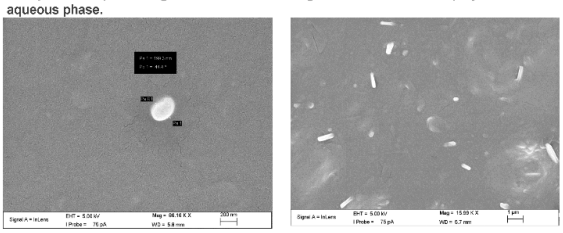

Figure 2. SEM image displaying surface morphology of VCM LPNs

Morphological evaluation revealed particles that were discrete and rod shaped (Figure 2). In a recent study, rod shaped nanoparticles were found to have greater antibacterial activity than spherical nanoparticles due to their larger surface area that is in contact with the surface of the endothelial cells making them more effective (Sadeghi et al., 2012). Therefore, VCM LPNs prepared in this study could be an effective nanoantibiotic system against both susceptible and resistant bacteria.

FORMULATION	DAY	MIC (µg/ml)	
		S. aureus	MRSA
CONTROL (VCM-HCl)	1	15.62	3.91
	5	NA	NA
DRUG FREE LIPID POLYMER NANOPARTICLES	1	NA	NA
	5	NA	NA
VCM LOADED LIPID POLYMER NANOPARTICLES	1	12.5	12.5
	5	12.5	12.5

*NA = No Activity

Table 2. Antibacterial Activity of VCM LPNs

Antibacterial studies show that the control showed antibacterial activity against *S.aureus* and MRSA on day 1 only, whilst VCM loaded LPNs showed sustained antibacterial activity against both *S.aureus* and MRSA.

CONCLUSION

The optimal formulation of VCM loaded LPNs was identified to be the 2:1 lipid-polymer (Glyceryl triplamitate: Eudragit®RS100) ratio achieving a maximum encapsulation of 33.6% and sustained activity against both sensitive and resistant bacterial strains. Further in vitro and in vivo studies are under progress.

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Atomistic binding energy and Coarse grained simulation studies to understand the structure and drug release activity of Vancomycin loaded Lipid Polymer Nanoparticles (LPNs)

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INTRODUCTION

- Nanotechnology in pharmaceutics has the advantages like target delivery, enhancement of bioavailability and potency.
- Our laboratory is engaged in design and development of new drug products with the aid of nanotechnology and molecular modeling to combat antibacterial drug resistance [1,2].
- The stability of nano drug delivery system is strictly governed by its free energy whilst, the encapsulation efficiency (EE) and drug release (DR) are influenced by the intermolecular forces.

OBJECTIVES

- To reveal the supramolecular architecture of the VCM conjugated lipid-polymer complexes
- To relate the binding energy estimate of various complexes with the EE and DR
- To investigate the structural organization of lipid-polymer assemblies in building the nanosystem.

METHODS

Binding Energy Calculations

- Genetic algorithm (GA) protocol
- Flexible docking with Universal Force Field (UFF) calculations

Coarse Grained Simulation studies

- Optimization of LPN mesosystems
- Energy calculations using modified MS Martini force field.

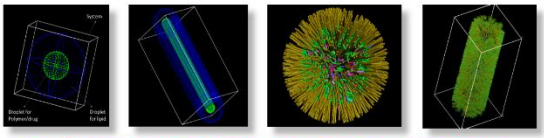

Fig. 1. Construction of mesostructure- Templates for sphere (a) and Rod (b) shape. Packing of the mesomolecules into respective shapes (c) and (d).

RESULTS AND DISCUSSION

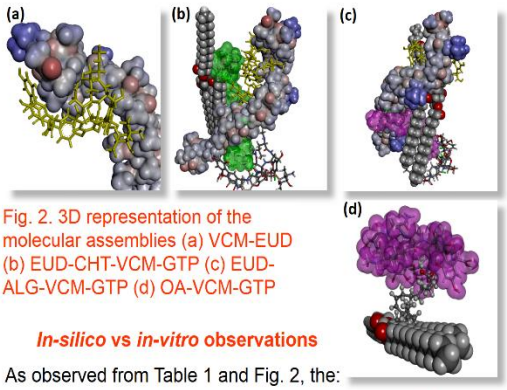

Fig. 2. 3D representation of the molecular assemblies (a) VCM-EUD (b) EUD-CHT-VCM-GTP (c) EUD-ALG-VCM-GTP (d) OA-VCM-GTP

In-silico vs *in-vitro* observations

As observed from Table 1 and Fig. 2, the:

- Weak binding forces and less stable native VCM system hence, poor EE and faster DR
- High binding affinity with VCM and relatively less stable final ALG system hence, High EE and faster DR
- CHT-EUD complex facilitated more entrapment of VCM and high stability of final system controlled the drug release.
- Improvement in EE and slower DR in the OA system is due to VCM-lipid interactions and the highly stable entropy favoured final system.
- As observed from Table 2 and Fig. 3 (a and b), Rod shape is favoured than sphere shape for the LPNs, which correlated with the SEM images of the LPNs (Fig. 3c)

Table 1. Binding energy and supramolecular interactions of various complexes with their experimental EE and DR data

Complex	Binding Energy (Kcal/mol)	Binding forces	Number of Hydrogen Bonds	Encapsulation Efficiency [3]	MDT50% [3]
VCM-CHT	-0.57	ES	1		
VCM-ALG	-3.32	ES	5		
VCM-OA	-2.9	VdW	0		
EUD-VCM-CHT	-2.53	ES & VdW	4		
EUD-VCM-ALG	-2.62	ES & VdW	3		
EUD-VCM-OA	-	None	-		
EUD-VCM-GTP	-3.09	VdW	0	**27.8 ± 1.84**	**09.482**
EUD-CHT-VCM-GTP	-4.11	ES & VdW	4 + 2 ES	**54.3 ± 0.44***	**14.050**
EUD-ALG-VCM-GTP	-3.23	ES & VdW	2	**69.3 ± 0.71***	**09.213**
OA-VCM-GTP	-3.48	ES & VdW	0	**41.5 ± 2.89***	**14.422**

*$p<0.05$ when compared to VCM LPNs; ES- Electrostatic; vdW- Van der Waal

Table 2. Energy values for the optimized LPN mesostructures

LPN shape	Total Enthalpy (Kcal/mol)	Valence energy	Van der waals energy	Electrostatic energy
Sphere	-9454.2148	1322.150	-10446.212	-670.742
Rod	-62662.3616	2617.081	-63310.266	1713.493

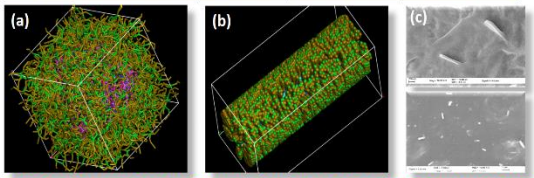

Fig. 3. LPN models (a) Sphere shape (b) Rod shape and (c) SEM images

CONCLUSIONS

- Binding energy calculations explained the variations in EE and DR
- Mesoscale simulation study supported the formation of rod shaped LPNs.

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OPTIMISATION OF FORMULATION VARIABLES FOR DRUG FREE LIPID–POLYMER HYBRID NANOPARTICLES

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INTRODUCTION AND AIM

Nanoparticulate drug delivery can overcome several disadvantages associated with various classes of drugs and is a powerful tool in the field of medicine.[1] Lipid-polymer hybrid nanoparticles (LPHNs) are an attractive alternative dosage form which can overcome the limitations associated with polymeric nanoparticles and liposomes. It simultaneously displays the high structural integrity of polymeric nanoparticles as well as the superior biomimetic characteristics of liposomes.[2] Since LPHN's are a recent and emerging drug delivery system in the literature, there is a need to identify optimal lipid-polymer formulations that can be used for various classes of drugs. Eudragit RS100 has not been studied as a polymer in LPHN's. Therefore, The purpose of this study was to identify an optimal drug free LPHN formulation containing Eudragit RS100 as the polymer, for future drug incorporation studies.

MATERIALS AND METHODS

➢Materials

The polymer Eudragit RS100 was kindly donated by Evonik Industries (Germany) and Compritol AT880 was a gift from Gattefossé (France). The Solid lipids glyceryl triplamitate as well as stearic acid and the surfactants Poloxamer 188, Solutol HS15, Lutrol F68 and Tween 80 were purchased from Sigma-Aldrich (USA). The solid lipid Glyerol Monostearate (GMS) was purchased from Alfa Aesar(Germany). Purified water used throughout the studies was produced in the laboratory with a Milli-Q purification system (Millipore corp., USA). All other chemicals and solvents used were of analytical grade and used without further purification.

➢Preparation of LPHNs

Drug free LPHN's were prepared by hot high pressure homogenisation followed by ultra-sonication. The excipients that were investigated included polymer, lipid and surfactant. The formulation variables such as concentration, lipid to polymer ratio and process variables such as homogenisation speed and time, sonication amplitude and time were also investigated.

➢ Particle size, polydispersity index (PI) and Zeta potential (ZP)

These parameters were determined at 25 °C by Photon Correlation Spectroscopy using a Nano ZS Zetasizer (Malvern Instruments Corp, UK).

➢TEM

Morphology was determined by Transmission Electron Microscopy (JEM-1010, JEOL, UK) at an accelerating voltage of 100 kV.

CONCLUSION

Optimising the formulation for synthesis of LPHN's is crucial in obtaining suitable and acceptable results in terms of size, PDI and ZP. We have successfully optimised the LPHN formulation containing Eudragit RS100 as a polymer for the first time. Further drug loading studies are in progress in our laboratories.

ACKNOWLEDGEMENTS

NRF and UKZN for financial support.

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RESULTS AND DISCUSSION

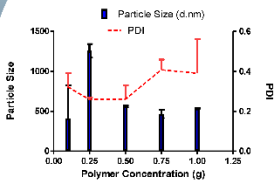


Fig.1. Effect of Eudragit RS100 concentration on the particle size and PDI.

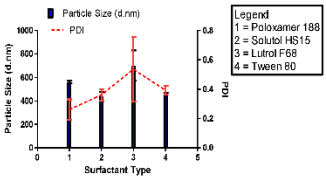


Fig.2. Effect of surfactant type on the particle size and PDI

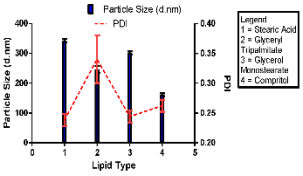


Fig.3. Effect of lipid type on the particle size and PDI

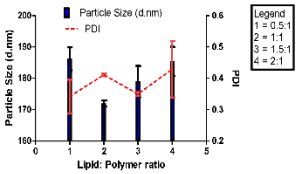
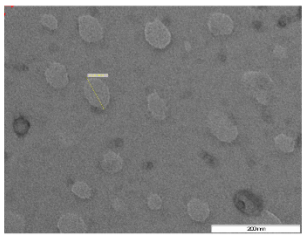


Fig.4. Effect of lipid : polymer ratio on the particle size and PDI

	Z AVE (d.nm)	PDI	ZP (mv)
10 minutes homogenisation @ 6000rpm 20 minutes sonication @ 30% amp	246.2 ± 11.78	0.340 ± 0.04	+16.52 ± 1.41
10 minutes homogenisation @ 6000rpm 30 minutes sonication @ 30% amp	217.3 ± 3.81	0.259 ± 0.004	+18.23 ± 0.21
10 minutes homogenisation @ 6000rpm 20 minutes sonication @ 40% amp	211.9 ± 9.05	0.280 ± 0.005	+19.96 ± 0.07
20 minutes homogenisation @ 6000rpm 20 minutes sonication @ 30% amp	212.1 ± 5.23	0.277 ± 0.004	14.37 ± 4.24

Table 1. Effect of Process variables on the particle size, PDI and zeta potential.



Particles are discrete, homogenous and spherical in shape (Fig. 1).

Fig. 5. TEM image of drug free LPHNs.

Excipient	Quantity (%w/v)
Eudragit RS100	1
Glyceryl tripalmitate	2
Solutol HS15	1

Table 2. Optimal Formulation Excipients

Formulation Variable	Optimised time/speed
Homogenisation Time	10 minutes
Homogenisation Speed	6000 rpm
Sonication Time	30 minutes
Sonication Speed	30% amplitude

Table 3. Process Variables

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Title: Co-encapsulation of multi-lipids and polymers enhances the performance of vancomycin in lipid polymer hybrid nanoparticles: in vitro and in silico studies.

Article Type: Research Paper

Keywords: Vancomycin; Lipid- polymer; nanoparticle; MRSA; antibacterial; in silico

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Abstract: Nano drug delivery systems are being widely explored to overcome the challenges with existing antibiotics to treat bacterial infections [1]. Lipid-Polymer Nanoparticles (LPNs) display unique advantages of both liposomes and polymeric nanoparticles while excluding some of their limitations, particularly the structural integrity of the polymeric particles and the biomimetic properties of the liposome [1]. The use of helper lipids and polymers in LPNs have not been investigated, but have shown potential in other nano-drug delivery systems to improve drug encapsulation, antibacterial activity and drug release. Therefore, LPNs using co-excipients were prepared using vancomycin (VCM), glyceryl triplamitate and Eudragit RS100 as the drug, lipid and polymer respectively. Oleic acid (OA), Chitosan (CHT) and Sodium alginate (ALG) were explored as co-excipients. Results indicated rod-shaped LPNs with suitable size, PDI and zeta potential, while encapsulation efficiency (%EE) increased from 27.8% to 41.5%, 54.3% and 69.3% with the addition of OA, CHT and ALG respectively. Drug release indicated that VCM-CHT had the best performance in sustained drug release of 36.1 ± 5.35% after 24h. The EE and drug release was further corroborated by in silico and release kinetics data. In vitro antibacterial studies of all formulations exhibited better activity against bare VCM and sustained activity up to day 5 against both S.aureus and MRSA, with VCM-OA and VCM-CHT showing better activity against MRSA. Therefore, this LPN proves to be a promising system for delivery of VCM as well as other antibiotics.