Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin

by

Danford Mhule

(B. Pharm – St. John's University of Tanzania)

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UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Supervisor: Professor Thirumala Govender

(PhD, University of Nottingham, Nottingham, United Kingdom)

Co-supervisor: Professor Valence M.K. Ndesendo

(PhD, University of Witwatersrand, Johannesburg, South Africa)

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“Look deep into nature, and then you will understand everything better.’’

- Albert Einstein-

This work is dedicated to my parents, a wonderful wife Epifania, my daughters and sons for their unceasing support, encouragement, patience and understanding during my long absence from home.

Declaration 1 – Plagiarism

I, Mr. Danford Mhule, declare that

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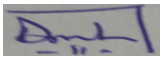
Signed:  Date: December 4, 2017

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Declaration 2 –Publications

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

Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin.

Danford Mhule, Rahul S. Kalhapure, Mahantesh Jadhav, Sanjeev Rambharose, Calvin A. Omolo, Chunderika Mocktar, Sanil Singh, Ayman Waddad, Valence M.K Ndesendo, Thirumala Govender. International Journal of Pharmaceutics, SUBMITTED MANUSCRIPT. Confirmation email can be found in appendix A.

Mr. Danford Mhule contributed to the design of the project, modification and optimization of methods and preparation and characterization of N-(2-morpholinoethyl) oleamide (NMEO) in terms of synthesis, structural elucidation techniques such as Fourier-transform infrared (FT-IR) spectroscopy, Proton and ^{13}Carbon nuclear magnetic resonance spectroscopy (^{1}H NMR and C^{13} NMR) and the bio-safety of the synthesized NMEO. He also contributed to the formulation and characterization of the vancomycin loaded pH-responsive SLNs that were formulated from the synthesized NMEO in terms of; particle size, polydispersity index, zeta potential, surface morphology, entrapment efficiency, *in vitro* drug release, differential scanning calorimetry (DSC), *in vitro* and *in vivo* antimicrobial activity, the mathematical modelling in terms of the *in vitro* release kinetics data and stability studies. He wrote the first draft of the paper and undertook all revisions.

Dr. R.S. Kalhapure guided the overall design of the project, assisted with technical problems as well as editing of the paper and abstract, and supervising the study.

Dr. M. Jadhav assisted in synthesis and characterization of NMEO lipid and the mathematical modelling of the *in vitro* drug release kinetics data.

Mr. S. Rambharose performed the cytotoxicity studies of the synthesized NMEO lipid, cell viability of vancomycin loaded NMEO SLNs against MRSA and assisted in the *in vivo* antibacterial activity studies.

Dr. A. Waddad performed molecular modelling studies while **Mr. C. Omolo** assisted in *in vitro* antibacterial studies.

Dr. C. Mocktar supervised the *in vitro* whereas **Dr. Singh** supervised the *in vivo* antibacterial activity studies of the vancomycin loaded NMEO SLNs performed against and MRSA.

Prof. V.M.K. Ndesendo served as a co-supervisor responsible for project problem solving, editing of paper and abstract, and general supervision of the study.

Prof. T. Govender served as supervisor and was responsible for overall project conceptualization, problem solving, editing of paper and abstract, and general supervision of the study.

Research output from the dissertation

Submitted manuscript

The following manuscript has been submitted to the International Journal of Pharmaceutics (IF = 3.649) based on work done during this study.

Danford Mhule, Rahul S. Kalhapure, Mahantesh Jadhav, Sanjeev Rambharose, Calvin A. Omolo, Chunderika Mocktar, Sanil Singh, Ayman Waddad, Valence M.K Ndesendo, Thirumala Govender.

* The manuscript can be found in Chapter three.

Conference Presentations

The following conference presentation was produced from data generated during this study:

Danford Mhule, Rahul S Kalhapure, Mahantesh Jadhav, Sanjeev Rambharose, Calvin .A. Omolo, Chunderika Mocktar, Sanil Singh, Valence. M. K. Ndesendo, Thirumala Govender. Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin. PSSA Conference, 6-8 July 2017, Johannesburg, South Africa.

*The poster can be found in Appendix B

Abstract

Antibiotic resistance is a health challenge that can make the most useful antibiotics ineffective against bacterial infections. Stimuli-responsive nano-drug delivery systems can optimize antibiotic delivery to infection sites. Identifying novel lipids for pH responsive delivery to acidic conditions of infection sites will enhance the performance of nano-drug delivery systems. The aim of this investigation was to synthesize and characterize a novel pH-responsive lipid for vancomycin delivery to acidic conditions of infection sites. A pH-responsive solid lipid, N-(2-morpholinoethyl) oleamide (NMEO), was synthesized and used to prepare vancomycin (VCM)-loaded solid lipid nanoparticles (VCM_NMEO SLNs). The particle size (PS), polydispersity index (PDI), zeta potential (ZP) and entrapment efficiency (EE) of the formulation were 302.8 ± 0.12 nm, 0.23 ± 0.03, -6.27 ± 0.017 mV and 81.18 ± 0.57 % respectively. The study findings also revealed that drug release and antibacterial activity were significantly greater at a pH 6.0 than at pH 7.4. Moreover, the reduction of MRSA load was 4.14 times greater ($p < 0.05$) in the skin of VCM_NMEO SLNs treated mice than that were bare VCM treated. Thus, this study confirmed that NMEO can successfully be used to formulate pH-responsive SLNs, and have the potential to enhance treatment of bacterial infections.

Key words: NMEO, pH responsive SLNs, antibiotic, nanotechnology, antibiotic resistance, methicillin-resistant *S. aureus.*

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I would like to extend my gratitude to the following individuals and organizations whose valuable contributions crowned this project with success.

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List of Abbreviations

3D	Three dimensional	**MSSA**	Methicillin sensitive *Staphylococcus aureus*
4-AEM	4-(2-Aminoethyl) morpholine	**MTT**	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
AIDS	Acquired Immune Deficiency Syndrome	**NMEO**	N-(2-morpholinoethyl) oleamide
BRU	Biomedical Resource Unit	**NMR**	Nuclear magnetic resonance
CFU	Colon forming units	**OA**	Oleic acid
CHEMS	Cholesteryly hemisuccinate	**PDB**	Protein data bank
CHPC	Center for High Performance Computing	**PDI**	Polydispersive index
DCC	*N,N'*-dicyclohexyl carbodiimide	**PE**	Phosphatidylethanolamine
DCM	Dichloromethane	**PN**	Polymeric nanoparticles
DLS	Dynamic light scattering	**PS**	Particle size
DMAP	*p*-dimethylamino pyridine	**PSSA**	Pharmaceutical Society of South Africa
DOPE	Dioleoyl phosphatidyl ethanolamine	**RMSE**	Root mean square error
DSC	Differential Scanning Calorimetry	**RT**	Room temperature
EE	Entrapment efficiency	**RTI**	Respiratory tract infections
FTIR	Fourier transform infra-red	**SA**	Stearic acid
HPH	High pressure homogenization	**SD**	Standard deviation
HIV	Human Immunodeficiency Virus (HIV)	**SDDS**	Smart Drug Delivery Systems

IFDS	Infectious diseases	**SEM**	Scanning electron microscopy
LC	Loading capacity	**SLN**	Solid lipid nanoparticles
LPHNS	Lipid-polymer hybrid nanoparticles	**TEM**	Transmission electron microscopy
MD	Molecular dynamics	**UK**	United Kingdom
MHA	Mueller Hinton Agar	**UKZN**	University of KwaZulu Natal
MHB	Mueller- Hinton Broth	**USA**	United States of America
MIC	Minimum inhibitory concentration	**VRSA**	Vancomycin resistant *Staphylococcus aureus*
MMU	Microscopy and Microanalysis Unit	**WHO**	World Health Organization
MRSA	Methicillin resistant *Staphylococcus aureus*	**XRD**	X-Ray Diffraction
MS	Material studio	**ZP**	Zeta potential

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CHAPTER 1. INTRODUCTION

1.1 Introduction

This chapter provides the background to the present study, covering the status of infectious diseases and antibiotic therapy, and its limitations. It also briefly describes nanotechnology as a solution to antimicrobial resistance crisis with emphasis on pH responsive systems. The aims and objectives, significance and novelty of the study are also presented.

1.2 Background

Despite the use of preventive interventions, such as improved hygiene, and curative interventions, such as use of antibiotics, microbial infections continue to cause a significant number of premature deaths worldwide. Killing at least 15 million people per annum, infectious diseases (IFDS) present one of the biggest challenges of global healthcare (Doolan et al., 2014). The situation is more serious in sub-Saharan Africa, where IFDS, particularly respiratory tract infections and diarrhea, accounts for more than 50% of deaths (Khan et al., 2016). Although the problem is bigger in developing countries, factors such as international migration facilitate the rapid spread of infections, making them a threat to both developed and developing countries (Becker et al., 2006).

The introduction of antibiotics into clinical practice in the 1940's played a significant role in combating microbial infections by decreasing their associated morbidity and morbidity (Ray et al., 2012; Xiong et al., 2014). However, the emergence of antibiotic resistance, which has been increasing over the last few decades, threatens to send the world back to a pre-antibiotic era by making even the most effective antibiotics ineffective. Increased patient suffering from prolonged

or recurrent infections, longer periods of hospitalization, loss of productivity at work, and higher healthcare cost are just a few adverse consequences of resistant infections (Cosgrove et al., 2014). Resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA), are frequent causes of infections in community and hospital settings (Chessa et al., 2015; Hiramatsu, 1998). Unless the rising trend of antimicrobial resistance is reversed, some important hospital procedures, such as surgery and organ transplantation, will have to be avoided due to the possibility of post-procedure infection with resistant bacterial infections that may have fatal consequences to patients.

One of the major factors that contribute to the development of antibiotic resistance is the shortcomings related to conventional dosage forms of antibiotic drugs. These shortcomings include inadequate drug concentrations at sites of infection, exposure to normal flora due to high drug concentrations, increased frequency of administration and the occurrence of side effects, which decrease patient compliance (Hess et al., 2016). In most cases, these problems are related to the main problem of traditional dosage forms, which is lack of targetability to disease site.

Various strategies, such as using natural materials with antimicrobial properties, combinational therapy, new antibiotic discovery and developing novel nano-drug delivery systems, have been proposed and tried to solve the problems related to conventional carriers of antibiotics (Khameneh et al., 2016). Of these strategies, developing nanobased drug carriers using nanotechnology is regarded as a more viable solution. The small size of nanocarriers gives them unique advantages, such as the ability to permeate through barriers and interact specifically with microorganisms, and the capability for structural and functional modification. More important, nanocarriers can be

specifically designed to deliver drugs to a targeted tissue with controlled drug release over a prolonged period (Huang et al., 2016). By maximizing drug only where it is needed, the targeted delivery of antibiotics ensures adequate concentration of drugs at infection sites for extended periods while avoiding expose of non-intended tissues. As a result, the need for large doses or frequent administration, which is common with traditional dosage forms, is avoided, while toxic and side effects are minimized, and patient compliance is promoted. Nanodelivery systems are reported to have the ability to overcome bacterial resistance mechanisms, which they do by ensuring that bacteria at the infection sites are exposed to drug concentrations well above the minimum inhibitory concentration (MIC) for an extended period. This leads to their eradication before they have a chance to develop resistance.

New generation nanodelivery systems include several strategies to potentiate their targeting and activity at disease sites. “Smart” nanodelivery systems are being designed to respond specifically to unique conditions at a disease site or to external stimuli, such as pH, temperature and enzymes, which will optimize targeting and or increase the drug release at a disease site where it is required (Kullberg et al., 2009; Schmaljohann, 2006; Ulijn, 2006). Specifically, the presence of varying pH conditions between different compartments of the body, and between normal and pathological tissues, such as tumors, infected and ischemic tissues, is well known. These pathological conditions are known to have a lower pH than the normal physiological pH, which creates a unique opportunity to fabricate nanocarriers that release drug in response to pH changes (Zhu and Chen, 2014). For years, the focus of pH responsive nanocarrier research has been in the field of non-communicable diseases. pH Responsive nanocarriers for delivering drugs, for diseases such as crohn's disease, diabetes, ischemic heart diseases and tumors, has been reported (Basak and

Adhikari, 2009; Bastings et al., 2014; Gao et al., 2014; She et al., 2013). Infection sites are acidic due to pH reduction caused by organic acids (e.g. lactic acid) that are produced by both bacterial activity (anaerobic fermentation) and the body's immune system response (phagocytosis) (Radovic-Moreno et al., 2012). This can be exploited to formulate nanodelivery systems with an acid-triggered mechanism of drug release. However, in the case of infectious diseases, only four studies have reported on the delivery of antibiotics to the acidic environment of infection sites, three of which report polymeric nanoparticles (Kalhapure et al., 2017; Pichavant et al., 2011; Radovic-Moreno et al., 2012) and only one reports Solid Lipid Nanoparticles (SLNs)-based antibiotic delivery (Kalhapure et al., 2017).

SLNs are nanosized solid lipid particles that are dispersed in water or aqueous surfactant solution. Their use is gaining ground among formulation scientists as they have a number of advantages, such as avoidance of organic solvents, stability on storage and the ability to control drug release (Hu et al., 2002; Muller et al., 2000). In addition, SLNS are prepared using physiological lipids, making them biocompatible (Wong et al., 2007), with large-scale production of these nanosystems being possible (Muller et al., 2002).

With the threat of antimicrobial resistance increasing, there is a need to identify novel pH responsive lipids that can be formulated into SLNs, thereby increasing the pool of available materials for the site-specific delivery of antimicrobial agents. Two types of pH sensitive materials, mostly polymers, have been widely used to deliver drugs, especially for cancer; i) acid cleavable materials, and ii) ionizable materials (Kanamala et al., 2016). Acid cleavable materials contain acid labile bonds, such as hydrozone, imine, acetal/ketal, amides ethers and orthoesters (Edson and Kwon, 2016). Ionizable materials may be anionic or cationic, depending on whether they possess acidic groups (e.g. carboxylic acid, phosphoric acid or sulfonic acid) or basic groups (e.g. amine

bearing groups such as imidazole, pyridine, morpholine groups) (Liu et al., 2013). Using knowledge gained from cancer research, it is possible to design acid cleavable or ionizable lipids that deliver antibiotics to acidic microenvironments of infections. The only antibiotic SLNs study mentioned above used SA-3M, a stearic acid-based pH responsive novel lipid that contains an acid cleavable acetal bond. The bond remained intact at pH 7.4 but cleaved at pH 6.5, with enhanced release and antibacterial activity. Compared to acid cleavable lipids, the use of cationic ionizable lipids has the potential to become more successful due to their ability to protonate and acquire a positive charge at low pH. This positive charge is important, as it not only makes the system more hydrophilic, which helps to increase drug release at the disease site, but it also facilitates attachment of the nanocarriers to the negatively charged bacterial cells by electrostatic interactions. Thus, this study proposes the design of a ionizable novel lipid N-(2-morpholinoethyl) oleamide (NMEO) and explores its applicability in formulating pH responsive SLNs for the targeted delivery of vancomycin to manage MRSA infections.

1.3 Aims and Objectives

Considering the potential of SLNs for targeted delivery of antibiotics, the development of new pH responsive solid lipids for their preparation was considered in this study to open the doors for novel strategies to overcome bacterial resistance. The aim of this study was therefore to explore the potential of a novel oleic acid based solid lipid for formulation into VCM loaded SLNs for enhanced therapy.

To realize this aim, the objectives of the study were to:

1. Synthesize a novel pH responsive lipid by conjugating oleic acid with 4-(2-Aminoethyl) morpholine (4-AEM) using steglich esterification approach.
2. Characterize the synthesized lipid using structural elucidation techniques such as
3. FT-IR, 1H NMR and C13 NMR.
4. Determine the toxicity of the synthesized solid lipid to confirm its biosafety.
5. Prepare vancomycin loaded pH-responsive SLNs with NMEO.
6. Evaluate the formulated SLNs in terms of size, PDI, zeta potential, morphology, entrapment efficiency, thermal properties, *in vitro* drug release, *in vitro* and in vivo antibacterial activity.
7. Undertake *in silico* studies to understand molecular interactions between VCM and NMEO.

1.4 Novelty of the study

The research undertaken is novel for the following reasons:

- The pH responsive lipid (NMEO) that was synthesized is a novel material not reported before. It has not been used been used as a formulation component for a nanodelivery system of any drug class. Synthesized from oleic acid and 4-(2-Aminoethyl) morpholine (4-AEM), NMEO form SLNs that are expected to remain negatively charged at pH 7.4, while at pH 6.0 they acquire a positive charge due to protonation of nitrogen atoms of 4-AEM. NMEO is anticipated to enhance antibacterial activity of vancomycin due to: i) increased drug release at the sites of infection due to acid induced-dissociation of the SLNs caused by increased hydrophilicity of the system at low pH.; ii) improved targetability of SLNs as they facilitate targeting of both the infection site and the bacteria due to the positive charge of

SLNs, which will help them bind to bacterial cells that are negatively charged.; iii) further potentiation of antimicrobial activity due to inherent antimicrobial activity of oleic acid.

- Only one study has reported antibiotic (vancomycin) delivery from a pH responsive SLNs and it used an acid cleavable lipid for preparing SLNs, which increased drug release (Kalhapure et al., 2017). None has reported the delivery of antibiotics using pH-responsive SLNs that employ surface-charge switching lipids. This is the first study reporting the formulation and evaluation of a surface-charge switching pH responsive SLNs for sustained release and enhanced antibacterial activity of an antibiotic.

1.5 Significance of the study

Synthesis of a new pH responsive lipid and its application in antimicrobial nano-delivery systems presents a novel and promising approach to combating antimicrobial resistance, which threatens to nullify even the most effective antibiotics currently in clinical practice. The nanosystem formulated in this study is anticipated to have the following benefits:

New Pharmaceutical Product

This study proposes a new pharmaceutical product i.e. pH responsive vancomycin loaded NMEO SLNs. This can stimulate the pharmaceutical industry into manufacturing medicines that are superior and cost effective.

Improved patient therapy and disease treatment

The proposed pH responsive formulation is expected to improve patient therapy and management of bacterial infections by improving antibacterial activity, while at the same time reducing effective dose, side effects and treatment costs.

Creation of new scientific knowledge

New knowledge on the antibacterial properties and applicability of NMEO SLNs could be obtained that can contribute to the body of knowledge available in the pharmaceutical field.

Stimulation of new research

pH-responsive NMEO SLNs may offer a novel platform for devising nanocarriers for delivery of drugs for other diseases characterized by acidification of pathological tissues/ cells such as cancer, ischemic heart diseases and inflammatory diseases. The formulated nanosystem may also help to understand the mechanism of formation of SLNs.

1.6 overview of the thesis

This study will be presented in the following three chapters:

Chapter 2. Infectious diseases and novel pH responsive nanodelivery systems for drug delivery: This chapter reviews the worldwide status of infectious diseases and current antibiotic therapy, as well as the limitation and strategies that have been used to overcome them. Smart nanodelivery systems are also described, with special emphasis on pH responsive nanodelivery systems. Moreover, a description of SLNs in terms of characteristics, preparation and characterization methods is presented. Lastly, vancomycin as a model drug is discussed.

Chapter 3. Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin: is a first author manuscript submitted to the International Journal of Pharmaceutics (Impact Factor = 3.649). The chapter is outlined in accordance with the mandatory format of the journal. The manuscript describes the synthesis and characterization of a novel NMEO lipid, formulation and physico-chemical characterization

of its vancomycin- loaded pH responsive SLNs and finally it evaluates *in vitro, in silico* and *in vivo* performance of the formulation against MRSA.

Chapter 4. Conclusion and Recommendations: It provides the conclusions from research findings in the study, highlights the potential significance of the findings and provides recommendations for future work that can be derived from this study.

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CHAPTER 2. INFECTIUOS DISEASES AND NOVEL pH RESPONSIVE NANODELIVERY SYSTEMS FOR DRUG DELIVERY

2.1 Introduction

This chapter presents a review of the literature covering the status of infectious diseases, current antibiotic therapies and their limitations, with a focus on antibacterial resistance and nanotechnology as a solution. In addition, the different types of pH responsive systems, including the potential of pH responsive lipids to improve antibiotic delivery are discussed. Finally, the characteristics, preparation and characterization methods of SLNs are reviewed, followed by an overview of vancomycin as a model drug.

2.2 Status of Infectious diseases

Infectious diseases (IFDs) are among the chief causes of human suffering in terms of morbidity and mortality globally (Liu et al., 2015). Although IFDs burden is highest in developing countries, due to factors such as inadequate sanitation and limited resources, they are also a public health challenge in developed countries (Figure 2.1), where HIV/AIDS and respiratory tract infections (RTIs) are the major causes of mortality. In resource limited countries, mortality from IFDs is mainly due to diarrhea, HIV/AIDS, RTIs (e.g. tuberculosis) and sexually transmitted diseases (Winters and Gelband, 2011). In Africa, mortality due to IFDs is still very high, and accounts for approximately 70%, 28% and nearly 90% of all global cases of HIV/AIDS, tuberculosis and malaria respectively (Chaudhry et al., 2016). The danger posed by IFDs is made more serious by the fact that bacterial infections contribute to the pathogenesis of non-infectious diseases, such as peptic ulcers, cancer and cardiovascular diseases (Ogoina and Onyemelukwe, 2009). In people with non-communicable diseases, such as diabetes, not only are the infections more common, but

they make the disease more severe (Crevel et al., 2016). Reports point to an alarming increase in IFDs that is fueled by phenomena such as global warming, human migration and the emergence of drug resistant bacterial strains (Waldvogel, 2004).

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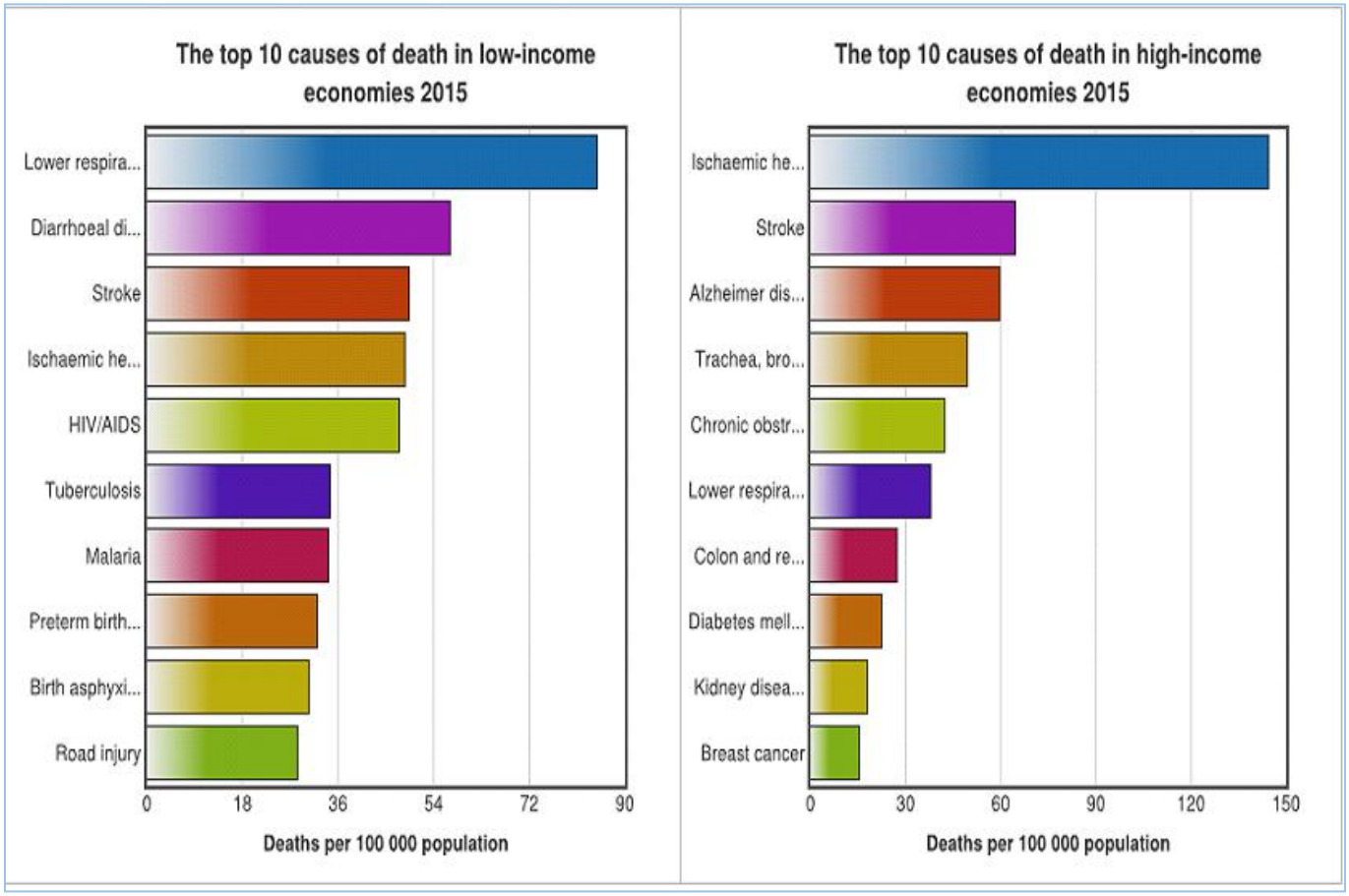


Figure 2.1. Leading causes of mortality by economy income. (World Health Organization, 2017. Fact sheet — Top 10 causes of death worldwide)

2.3 Current antibiotic therapy and its limitations

Antibiotics were first introduced into clinical practice in 1940s and have since been the main drugs to manage infectious diseases (Boucher et al., 2011). Millions of lives have been saved due to the ability of antibiotics to cure potentially fatal infections by either killing or inhibiting the growth of

microbes. This special class of drugs has also made it possible to carry out invasive procedures that are prone to post-procedure infections, such as routine surgery, and complex surgeries such as heart surgery, organ transplantation and joint replacement (Gould and Bal, 2013). By preventing or curing infections in patients with chronic diseases, such as diabetes and cancer, antibiotics play a significant role in minimizing human suffering, improving workplace productivity and increasing life expectancy (Ventola, 2015). However, the potential of antibiotics to treat infections is restricted by several shortcomings of their traditional dosage forms, which include tablets, capsules, suspensions for oral administration, creams for topical use and injections for parenteral administration. These shortcomings include sub-therapeutic drug concentration at the infection site, exposure of normal flora, more-than-once frequency of administration, poor compliance and the occurrence of side effects, some of which are fatal. Moreover, the use of high drug doses, a common way of compensating for poor pharmacokinetic characteristics, such as short half-life, complicates antibiotic therapy by increasing the chances of toxicity to not only mammalian cells but also normal flora whose presence contribute to the wellbeing of the body by limiting the growth of pathogens.

Higher doses also translate into higher production cost to manufacturing industries and higher treatment cost to end users i.e. patients. Regardless of the route of administration, side effects and inadequate drug concentration are also a consequence of lack of targetability, which is another common problem related to traditional dosage forms of antibiotics. Instead of delivering the drug at specific sites, these carriers take the drug to both infected and non-infected tissues. This unnecessary exposure of healthy tissues is one of the reasons why conventional dosage forms are associated with many unwanted effects, which, in addition to the high frequency of administration, decreases patient compliance to antibiotic treatment regimens (Gao et al., 2011).

In addition to the above-mentioned problems, insensible use of antibiotics has led to the emergence of bacteria strains that are resistant to commonly used antibiotics (Fridkin et al., 2014; Kardas et al., 2017). Since it was first reported in Japan in 1997, bacterial resistance due to MRSA has been growing over the decades to become one of the most challenging health problems. A recent study found that the prevalence of MRSA was 2.4% in Europe, 4.8% in North America, 5.4% in South America, 2.5% in Asia, 3.1% in Africa and 3.1% in Oceania among patients undergoing microbial tests (Reyes et al., 2016). When resistance develops to a particular antibiotic, its ability to treat infections is compromised, leading to treatment failure that is manifested by continuation, recurrence or worsening of the disease (Rather et al., 2017).

This forces prescribers to treat the recurring infection using alternative (second line) drugs, which are usually more expensive and associated with side effects (Lushniak, 2014). Dosage regimens of such alternative drugs are sometimes more complicated, requiring closer medical attention. Longer hospital stays are common for patients with resistant infections, and are costly to both individuals and health care systems. In the USA for example, is reported that approximately $20 billion is used to treat resistant infections, with an additional $35 billion being lost in productivity. In the same country, more than 20,000 people die each year due to resistant infections (Golkar et al., 2014; Ventola, 2015).

The problem of antibiotic resistance is complicated, as can be seen in Figure 2.2, by a decline in the development of new antibiotics. The production of new antibiotics has slowed over years due to a number of factors. First, unlike drugs for treating non-communicable diseases such has diabetes, antibiotics are used for short-time regimens, making them commercially unprofitable, which has resulted in manufacturing companies investing less in new antibiotics discovery (Chaudhary, 2016). Second, the future of new antibiotic drugs is unpredictable due to emergence

of resistance. As new antibiotics are usually reserved for severe infections, there is a greater chance of resistant to the drug developing before it starts being used (Gould and Bal, 2013). These factors, plus the stagnant approval of new drugs, have contributed to a decline to the number of new antibiotics that enter the market. Despite the slow pace of new drug development, there is also a problem in the approach used to develop them, which generally entails modifying the structures of existing antibiotics (Walsh, 2003). Although this approach is useful for safety reasons, it is not effective for curbing the problem of resistance. This is because drugs developed in this approach have a mechanism of action similar to old drugs from which they are derived, making development of cross resistance by bacteria easy. This underlies the need for new drugs with completely new mechanism of action. However, the path to such new antibiotics is not easy, taking into consideration the high costs and length of time that are usually associated with new drug development (Tiwari et al., 2012).

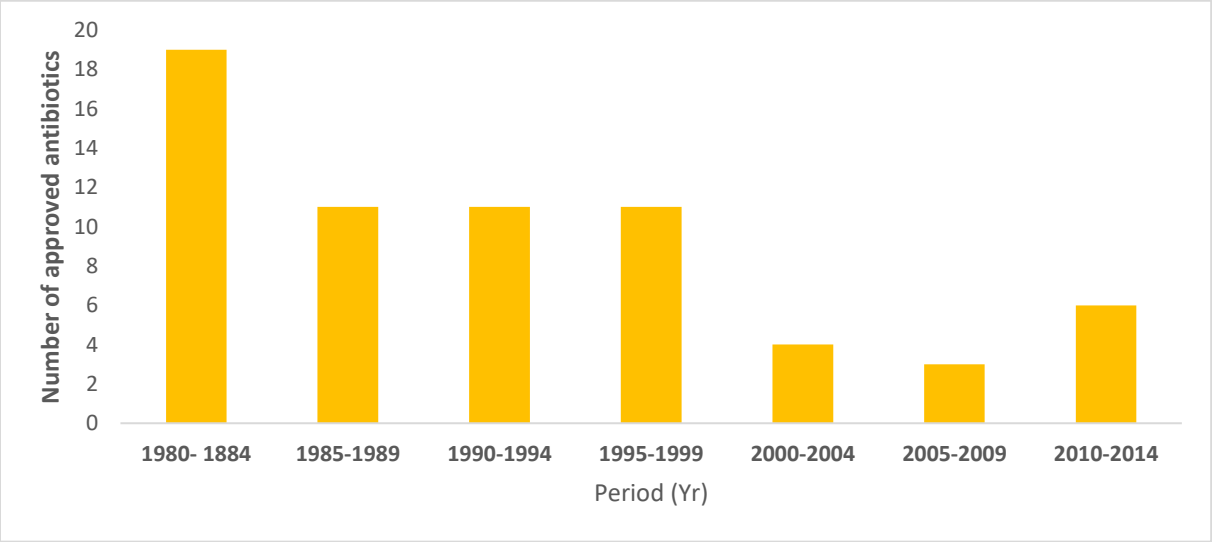


Figure 2.2 Trend of new antibiotics introduced to the market from 1980 to 2014 (Ventola, 2015).

2.4 Nanotechnology as a solution

The increasing costs, morbidity and mortality associated with the growing antibiotic resistance crisis have raised concern all over the world, with various health stakeholders calling for an urgent solution to this problem (Boucher et al., 2009; Spellberg et al., 2008). Different strategies that have been suggested including finding approaches to preserve existing antibiotics or promoting the discovery of new ones.

Several approaches have been pursued to reverse the increasing challenge of microbial resistance (Figure 2.3). Traditional medicines of plant origin, developing new antimicrobial drugs, combinational therapy and nanotechnology are among approaches that are commonly advocated (Khameneh et al., 2016). An interest in plant materials as a solution to antibiotic resistance is based on the observation that plant-based compounds, such as tannins, alkaloids, flavonoids and terpernoids, possess some activity against various microbes, namely bacteria and fungi (Cushnie and Lamb, 2005). Some of them e.g. farnesol, have shown activity against both sensitive and resistant strains of bacteria, such as *S. aureus* (Kuroda et al., 2007). However, the use of traditional medicines in treating infections is limited by several drawbacks, including an undefinable mechanism of action, non-fixed composition and hardships related to the identification, isolation and purification of bioactive compounds from plant sources. Lack of rigorously evaluated reports regarding antimicrobial activity of natural compounds makes their efficacy and safety questionable (Taylor, 2013). Combinational therapy offers advantages, such as boosting drug efficacy through synergism and delaying development of resistance by exerting inhibitory effects on multiple targets (Cottarel and Wierzbowski, 2007). However, this approach poses a danger of promoting emergence of multi-drug resistant strains (Brooks and Brooks, 2014).

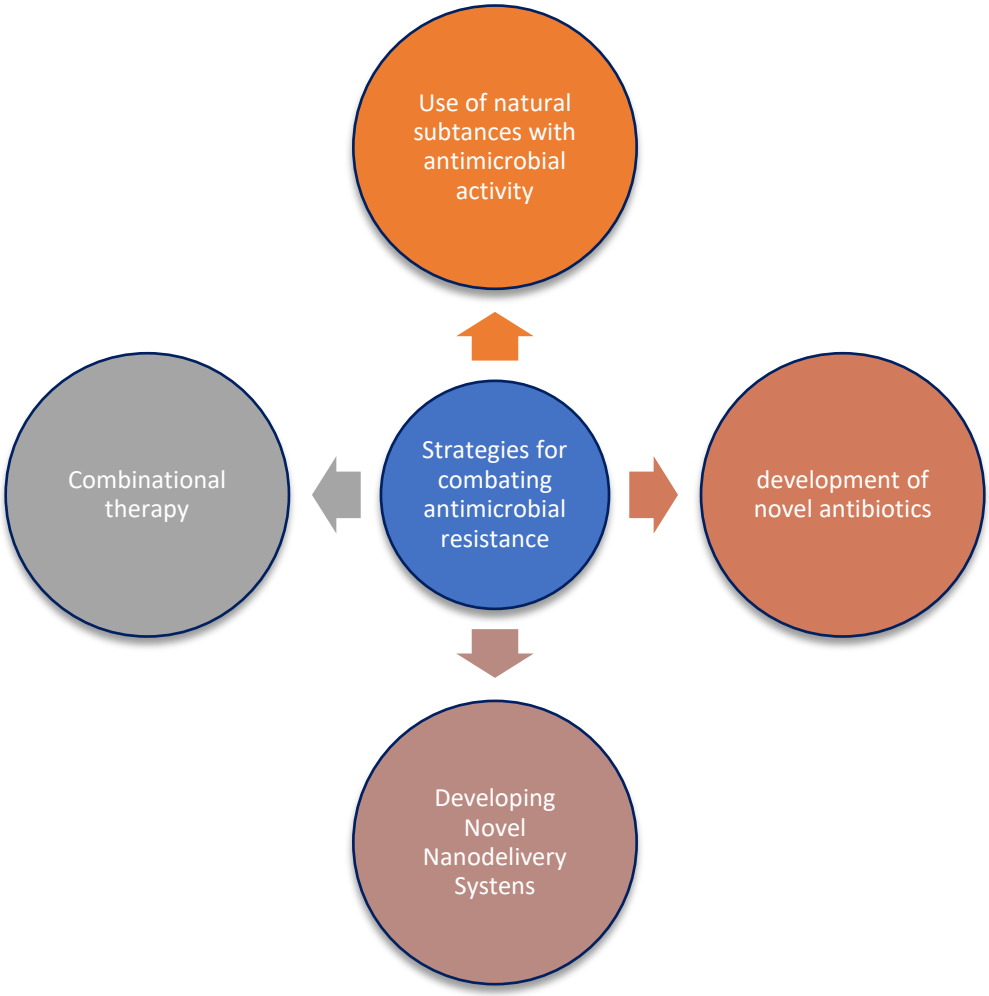


Figure 2.3. Different approaches that are employed to combat antibiotic resistance.

Development of novel antibiotics is therefore stagnant as manufacturers have shifted their attention to drugs for which they can charge more.

Conversely, use of nanotechnology in drug delivery is a more attractive approach due to its cost-effectiveness, versatility of nanomaterials and their ability to positively influence the physicochemical characteristics of drugs (Zhang et al., 2010). As the discovery of antibiotics revolutionized medicine, so nanotechnology is showing a considerable potential for taking medicine to a new level by overcoming the limitations of current therapy (Ranghar et al., 2014).

Nanotechology refers to the manipulation of materials at a nano scale, and is regarded as offering a bright future for drug delivery technology. Through a carefully designed methodology it is possible to obtain nanoparticles at sizes ranging from 10 to 1000 nm with physicochemical characteristics that suit different purposes (Marslin et al., 2015). The superiority of nanocarriers, which makes them better than conventional therapeutic agents, is derived from their unique mix of characteristics. Their small size and large surface to mass ratio makes them highly reactive, giving them the ability to be modified structurally and functionally, and to uniquely interact with bacteria and mammalian cells.

Nanobased drug carriers avoids many of the pitfalls of traditional drug carriers by delivering drugs to specific sites, improving the bioavailability of poorly soluble drugs, sustaining their release, decreasing the frequency of administration, reducing drug-inherent toxicity and boosting patient compliance (Davis et al., 2008; Peer et al., 2007; L. Zhang et al., 2008).

The application of nanotechnology to deliver antibiotic drugs is gaining the attention of researchers, as nanodelivery systems for antibiotics have been shown to be effective in overcoming drug resistance through different mechanisms, such as inhibiting efflux pumps, disrupting the cell membrane (Christena et al., 2015), damaging macromolecules by generating reactive oxygen species (Vatansever et al., 2013), inhibing biofilm formation and enabling intracellular penetration of drugs (Hetrick et al., 2009). Moreover, some classes of nanoparticle, such as inorganic nanoparticles, are usually not prone to antimicrobial resistance. Apart from being able to overcome resistance, some nanomaterials have inherent antimicrobial activity. Encapsulation of antibiotics in these nanomaterials may promote synergism while suppressing the emergence of resistance. To this end, silver nanoparticles were combined with amoxicillin by Li

et al, who found that the antimicrobial effect of the combination was greater than that of individual agents against *Escherichia coli* (Li et al., 2005).

The same results were obtained by another group of researchers who tested silver nanoparticles and cephalexin separately and in combination against *S.aureus* (Salarian et al., 2017). Antimicrobial activity of nanoparticles has been attributed to their small size, which enables them to penetrate cell walls easily and damage the bacterial cell membrane, killing the cell (Khan et al., 2016). These features have the potential to give nanomaterials an upper hand in mitigating the limitations associated with conventional antibiotic therapy, including the global problem of antibiotic resistance. While extensive research has been done in relation to nanodelivery of drugs for non-communicable diseases, such as cancer and cardiovascular systems, little has been done in the field of bacterial infections, making it a relatively new area of research. Table 2.1 summarizes some of the nanodelivery systems with the main findings that have been reported. Several nanoformulations of antibiotics have been reported and include liposomes, polymeric nanoparticles (PN), polymersomes, solid lipid nanoparticles (SLNs), lipid-polymer hybrid nanoparticles (LPHN) and carbon nanotubes (CNTs). Nanocarriers are showing the potential to improve antibiotic therapy by enhancing drug bioavailability and potency, and their sustaining drug release, with researchers continuing to explore strategies to potentiate the performance of these systems. A recent development in the field of nanodelivery of antibiotics is the recognition that targeted therapy using smart nanocarriers (stimuli responsive systems) can play an effective role in the war against resistant microbes (Edson and Kwon, 2016; Gao et al., 2010).

2.5 Smart nanodelivery systems

Despite the advantages of nanodelivery systems, challenges remain in reducing drug loss and increasing the targetability and drug release at infection sites (Ruenraroengsak et al., 2010).

Table 2.1. Examples of nanodelivery systems reported for antibiotics.

Antibiotic	Nanosystem	Main finding	Ref
azithromycin	PN	Nanoformulation exhibited high entrapment efficiency, sustained drug release and had a higher antibacterial activity than the free drug against *S. typhi.*	(Mohammadi et al., 2010)
Ampicillin	PN	*In vivo* antibacterial activity studies showed that the encapsulated drug was more potent than free drug against *S. typhi.*	(Fattal et al., 1989)
Cefuroxime axetil	SLNs	The entrapment efficiency of SLNs prepared using binary lipids was higher than those prepared using a single lipid. Also, the drug loaded SLNs showed enhanced inhibitory activity against biofilms produced by *S. aureus.*	(Sing et al., 2014)
Enrofloxacin	SLNs	Encapsulation of enrofloxacin in SLNs led to a sustained release profile and an increase in bioavailability	(Xie et al., 2011)
Amikacin	Liposomes	*In vivo* studies showed that encapsulation of the drug into liposomes prolonged its antibacterial activity.	(Leitzke et al., 1998)

Levofloxacin	LPHNs	The antibacterial activity of the nanosystem was higher against *P. aureginosa* biolfilm cells but not the planktonic cells.	(Cheow et al., 2011)
Vancomycin	Polymersomes	Drug loaded polymersomes showed sustained drug release and their *in vitro* and *in vivo* antibacterial activity against MRSA was superior to that of drug alone.	(Omolo et al., 2016)
Cefalexin	CNTs	Cefalexin - carbon nanotube composites showed stronger antimicrobial activity than drug free nanotubes against *S. aureus* and *B. subtilis*.	Qi et al., 2012

The desire to potentiate the advantages of nanocarriers has fueled the need to develop smart drug delivery systems (SDDS) that will ensure that payloads are delivered at high doses only to targeted sites that are responsive to unique conditions at the disease site e.g. pH or external stimuli such as light and temperature. Managing diseases using smart delivery carriers is more advantageous than conventional nanoparticles due to their potential to improve disease outcomes with fewer side effects (Balamurali et al., 2011). In the case of bacterial infections, SDDS are capable of minimizing chances of drug resistance development by ensuring that high local drug concentrations are achieved and sustained, thereby overwhelming the resistance mechanisms (Radovic-Moreno et al., 2012; Zhang et al., 2010). pH-Responsive drug-delivery systems have attracted a growing interest as "smart" drug-delivery systems for overcoming the shortcomings of conventional drug formulations, as they are able to deliver drugs in a controlled manner at a

specific site and time, which results in high therapeutic efficacy (Zhu et al., 2014). These nanosystems are explored further in the following sections.

2.6 pH responsive Nanocarriers

The pH that is lower than the physiological value is common in pathological states, such as cancer, inflammation and infections (Rofstad et al., 2006; Yoshida et al., 2013). The existence in pathological tissues of a pH that is different from the physiological pH of 7.4 has provided researchers with a unique opportunity to formulate nanodelivery systems that are pH responsive (Balamurali et al., 2011; Ferreira et al., 2013). pH-responsive nanosystems are usually formulated by adding pH sensitive components into the system. In pH responsive liposomes, these components are either added to the dispersion of formed vesicles or by mixing them with polymers during vesicle preparation. In other formulations, such as polymeric nanoparticles or polymeric micelles, pH sensitive units are basic parts of the polymer structure (Chul et al., 2009; Fleige et al., 2012). There are currently two main types of approaches that have been successfully used fabricate pH responsive nanosystems, namely, the use of acid cleavable linkages and ionizable materials (Figure 2.4).

2.6.1 pH responsive nanofomulations based on acid cleavable bonds

Employment of acid cleavable linkers is an important strategy of delivering drug to the acidic environment of pathological tissues (Mahato et al., 2011). In this approach, the drug is loaded into the system by covalently linking it to the nanocarrier via an acid labile bond or by encapsulation into a nanocarrier that contain an acid labile bond (Kim et al., 2010; Shunmugam, 2012). Regardless of the strategy, the bond remains intact at physiological pH but breaks at low pH triggering release of the drug.

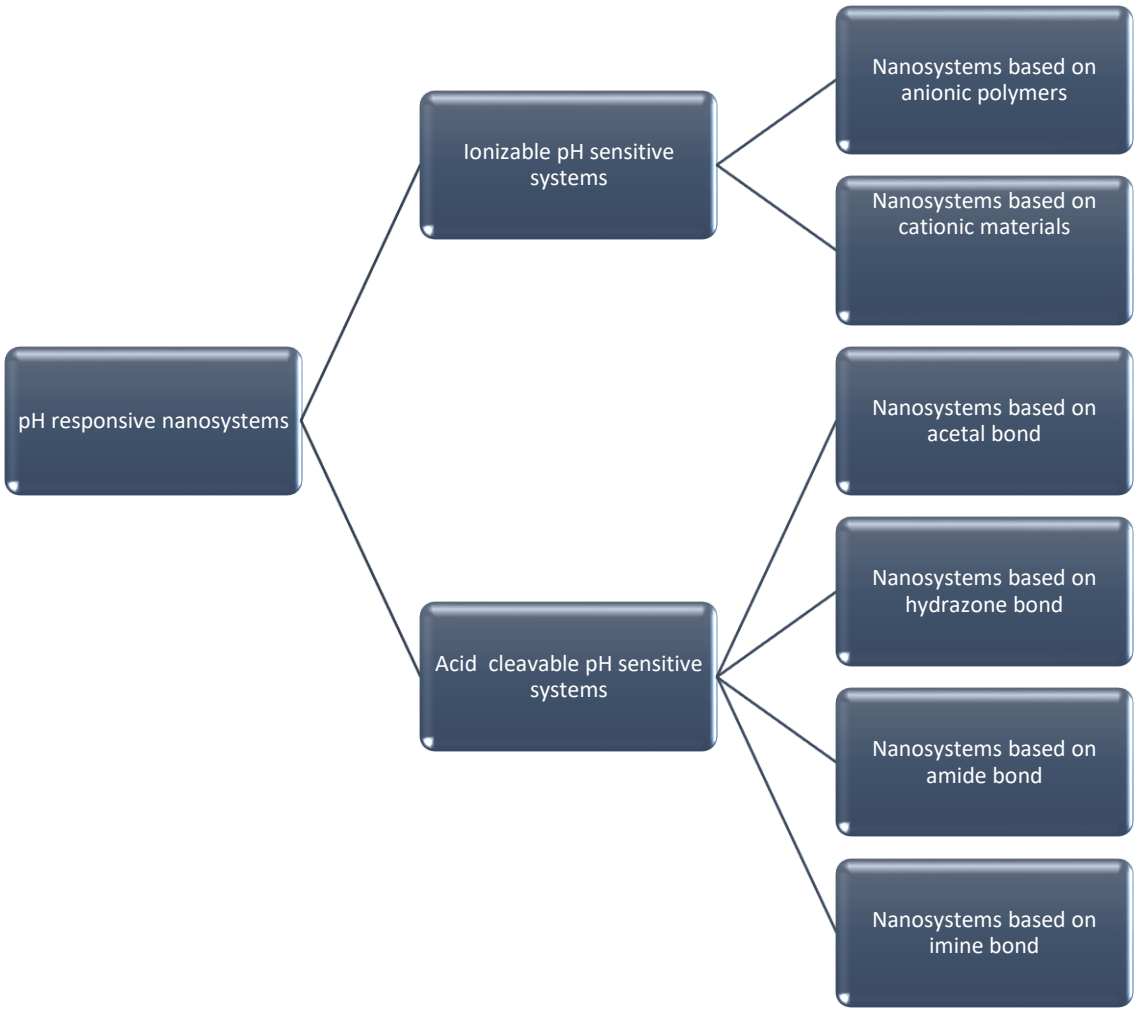


Figure 2.4. Types of pH responsive Nanocarriers.

In case of covalently linked drugs, this bond breakage disconnects the drug from its carrier enabling its release. But for carrier-encapsulated drugs, cleavage of the labile bond of the carrier cause destabilization or degradation of the system thereby facilitating drug release. As shown in Table 2.2, examples of acid-cleavable linkers that are commonly used in drug delivery are acetal/ketal, amide, hydrazone, imine, oxime and ether/orthoester bonds (Edson and Kwon, 2016).

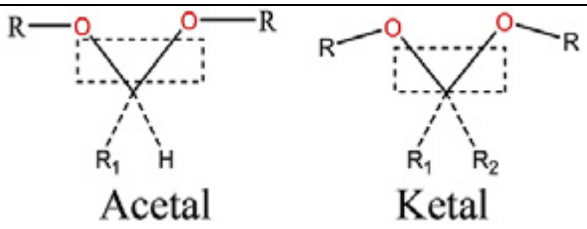
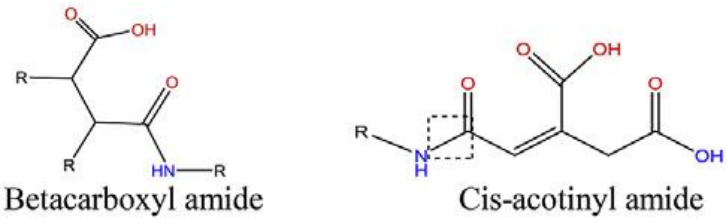
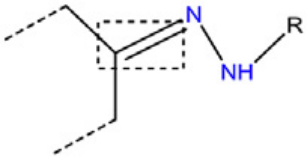
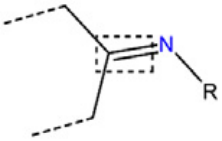
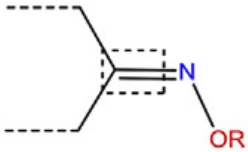
Despite being an effective strategy for site specific delivery of drugs, this approach has a number of limitations like instability of the acid -labile bond at neutral pH or its stability at low pH. Moreover, cleavage of the acid-labile bonds like amides and esters at low pH may be associated with release of acidic by-products that may induce inflammatory reactions (Kanamala et al., 2016).

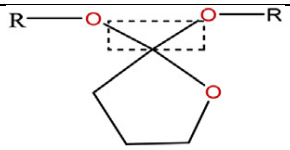
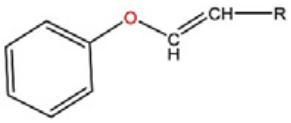
2.6.2 pH-responsive nanofomulations based on ionizable materials

These are nanosystems made from materials that contain ionizable chemical moieties which may be basic in nature like amines or acidic functional groups like carboxylic acid, sulphonic acid and phosphoric acid (Barba et al., 2009). When subjected to pH changes these chemical entities can undergo protonation/deprotonation which brings about changes in their physical characteristics such as solubility, size, shape or degradation rate leading to drug release.

By introducing weakly acidic or basic function groups, various researchers have successfully synthesized pH responsive polysaccharides, peptides, polymers and lipids which release their cargo based on their ability to protonate/deprotonate in response to changes in pH (Fei et al., 2014; Xu et al., 2015; Chiang et al., 2013; Zhang et al., 2011). The pH responsive nanofomulations based on ionizable materials are stable at physiological pH but at low pH they exhibit protonation-induced destabilization which facilitate drug release via different mechanisms like dissociation and precipitation. Although less frequently used as compared to other materials like polymers, pH responsive lipids present an interesting strategy to deliver drugs because commonly used lipids are less toxic compared to other types of materials.

Table 2.2. Examples of Acid cleavable bonds employed in pH-responsive nanodelivery systems (Kanamala et al., 2016).

Acid cleavable bond	Chemical structure
Acetal and ketal	
Amides	
hydrazone	
Imine	
Oxime	

Orthoester	
Phenyl vinyl ether	

2.7. pH-responsive lipids

The most extensively researched pH responsive lipids are cationic lipids and particularly phosphatidylethanolamine (PE) and its derivatives such as dioleoyl phosphatidyl ethanolamine (DOPE) (Sánchez et al., 2011; Shi et al., 2002). Based on literature, the application of pH responsive lipids has been confined to the preparation of liposomes intended for gene and drug delivery whereby pH responsiveness is achieved by acid induced change in lipid geometry (Fonseca and Du, 2004; Schroeder et al., 2009; Zhang et al., 2012). As described by Ferreira and coworkers, lipid geometry plays an important role in formation of stable liposomes (Ferreira et al., 2013). The cylindrical shape, in which the area occupied by the polar head is approximately similar to that of the acyl chains like in natural phospholipids such as *N*-acylated phosphoethanolamines favors formation of bilayer structures. Lipids like PE are characterized by a small molecular area in the headgroup relative to its acyl chains and thus, when used alone they form non-bilayer structures at physiological pH. But when combined with weakly acidic stabilizers e.g cholesteryl hemisuccinate (CHEMS) they rearrange into bilayers (Liu et al., 2013). This is because at high pH, acidic groups of these stabilizers ionize, and the resultant negative charge help to increase the polar headgroup volume via electrostatic repulsions with the phosphate group. However, at low

pH the carboxylate group of CHEMs protonate, electrostatic repulsion is lost, and PE molecules revert to inverted hexagonal phase destabilizing the liposomes and promoting release of entrapped drug (Sánchez et al., 2011).

Recently our group introduced another type of pH responsive lipid containing an acid cleavable bond (Kalhapure et al., 2017). The synthesized solid lipid (2-(2,4,6-trimethoxyphenyl)-1,3-dioxane-5,5-diyl) bis(methylene) distearate (SA-3M) contained an acetal bond which was stable at neutral pH but cleaved at acidic pH. *In vitro* antibacterial activity against methicillin-susceptible and resistant *Staphylococcus aureus* (MSSA and MRSA) showed that the pH responsive SLNs made from SA-3M had a greater activity at pH 6.5 than pH 7.4. Also, an *in vivo* study showed that the bacterial load (MRSA) remaining in the skin treated by pH responsive SLNs was 22 times lower than the one in the skin treated by plain vancomycin.

However, when compared to acid cleavable lipids, the use of ionizable lipids is a more promising pH responsive nano-drug delivery strategy because in addition to facilitating drug delivery at acidic environments, they can also help to attach nanocarriers to the negatively charged bacteria (Forier et al., 2014).

2.8 SLNs as drug carriers

This section provides a brief account of characteristics of SLNs as drug carriers their preparation methods and techniques used in their characterization.

2.8.1 Characteristics of SLNs as drug carriers

Apart from liposomes, which have already been widely investigated using pH responsive lipids, these lipids can also be used to formulate other lipid-based nanosystems, such as nanoemulsions (NE) and solid lipid nanoparticles. Of the two nanosystems, SLNs are increasingly catching the attention of formulation scientist due to a number of advantages, which include the possibility for

targeted delivery of drugs (Spada et al., 2012). In addition, a recent study by Xie et al showed that SLNs can augment antibacterial activity of enrofloxacin by delivering the drug intracellularly (Xie et al., 2017). This is a clear indication that fabrication of SLNs using pH responsive SLNs has the potential to improve antibiotic therapy. Regardless of having several good qualities, very few studies have reported on formulating pH responsive SLNs. One of the studies documented acid triggered release of doxorubicin from lauric acid SLNs due to a decrease of drug-lipid electrostatic attraction (Hsu and Chiu, 2015), while another reported the pH-responsive release of curcumin from SLNs via a meso–macrostructured silica matrix template (Kim et al., 2014). The vancomycin loaded SA-3M SLNs mentioned above are the only pH responsive SLNs reported so far for improving antibiotic therapy.

The effort to circumvent problems related to traditional nanocarriers, such as liposomes and polymeric nanoparticles, was rewarded in early 1990s when SLNs were first developed (Muller and Keck, 2004). These colloidal nanocarriers, which are composed of solid lipid particles dispersed in water or aqueous surfactant solution, bring together the best qualities of several other nanosystems, while avoiding some of their shortcomings (Hu et al., 2002). Like liposomes and fat emulsions, SLNs are made from physiological lipids, which makes them less toxic to body cells. Like polymeric nanoparticles, SLNs have a solid matrix, which gives them physical stability, the ability to protect the incorporated drug and control its release (Das et al., 2011). Their ability to be produced at industrial scale by cost-effective methods and to avoid toxic organic solvents in their production contrast them from polymeric nanoparticles, which lack suitable means of producing them at a large scale. SLNs have several other advantages, such as long-term stability on storage, enhancing the biovailability of encapsulated drug, using inexpensive excipients, and their ability to be sterilized by autoclaving (Harde et al., 2011). Non-toxicity of their excipients makes it

possible for SLNs to be administered via all routes of administration. SLNs are usually prepared using lipids that are solid at room and body temperature (Mu et al., 1998; Wissing et al., 2004; Muller et al., 2000), with the solid lipid forming the core of the SLN, which is stabilized by a coating with a suitable surfactant, as shown in Figure 2.5.

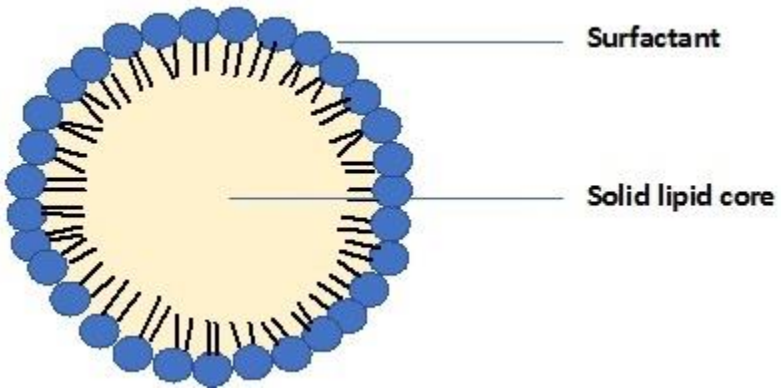


Figure 2.5. Structure of solid lipid nanoparticle.

2.8.2 Preparation Methods of SLNs

Preparation of SLNs can be performed using various methods, such as high-pressure homogenization, solvent displacement, precipitation, microemulsion, ultrasonification, membrane contractor and the spraying drying method (Almeida and Souto, 2007). Currently, high pressure homogenization (HPH) is widely regarded as the most suitable method for fabricating SLNs.

HPH is a well-known method as it has been used for several decades to prepare parenteral emulsions by the pharmaceutical industries. Homogenizers of various sizes at reasonable prices are already available, making scaling up possible (Müller et al., 2011). High pressure homogenizers break particles to submicron size by creating high shear and cavitation forces, as fluid is forced via a few microns sized gap. The fabrication of SLNs using HPH employs two

approaches, hot and cold homogenization methods (Mu et al., 1998) (Figure 2.6), both of which are described below.

Hot homogenization is performed using high pressure homogenizers or high intensity ultrasound while maintaining a temperature that is well above the lipid's melting point (Silva et al., 2012). The oily (lipid melt containing drug) and aqueous phases (containing surfactant) are heated to the same temperature, and are mixed using a high shear stirring device to attain a course oil-in-water pre-emulsion (Seedat et al., 2016). The quality of pre-emulsion is critical in SLNs formation, as it dictates the size and thus the quality of the final product. Small-sized SLNs are obtained using an elevated processing temperature, which significantly reduces the viscosity of the oily phase, thereby making mass transfer easy (Jenning et al., 2002). Owing to the high temperature used, the initial product of hot homogenization is a nanoemulsion, after which cooling it to room temperature or below solidify the liquid lipid particles into solid lipid nanoparticles (Prombutara et al., 2012). The retardation of lipid crystallization by the surfactant and small size of the particles may assist the product to remain as a supercooled melt for a long time. As exposure to high temperatures is of short duration, this technique can also be used for heat sensitive drugs, but may not be suitable for drugs that are highly sensitive to heat. The major advantage of this technique is that it can be performed using the existing production lines of emulsions for parenteral nutrition. These lines are fitted with devices that can control the temperature, which results in achieving the high temperatures used in hot homogenization and producing SLNs in large scale not being a problem (Dingler and Gohla, 2002).

Cold homogenization involves cooling the drug loaded lipid melt and grinding the solid lipid to microparticles, which are thereafter dispersed in a cold surfactant phase to produce a pre-suspension (Fathi et al., 2012).

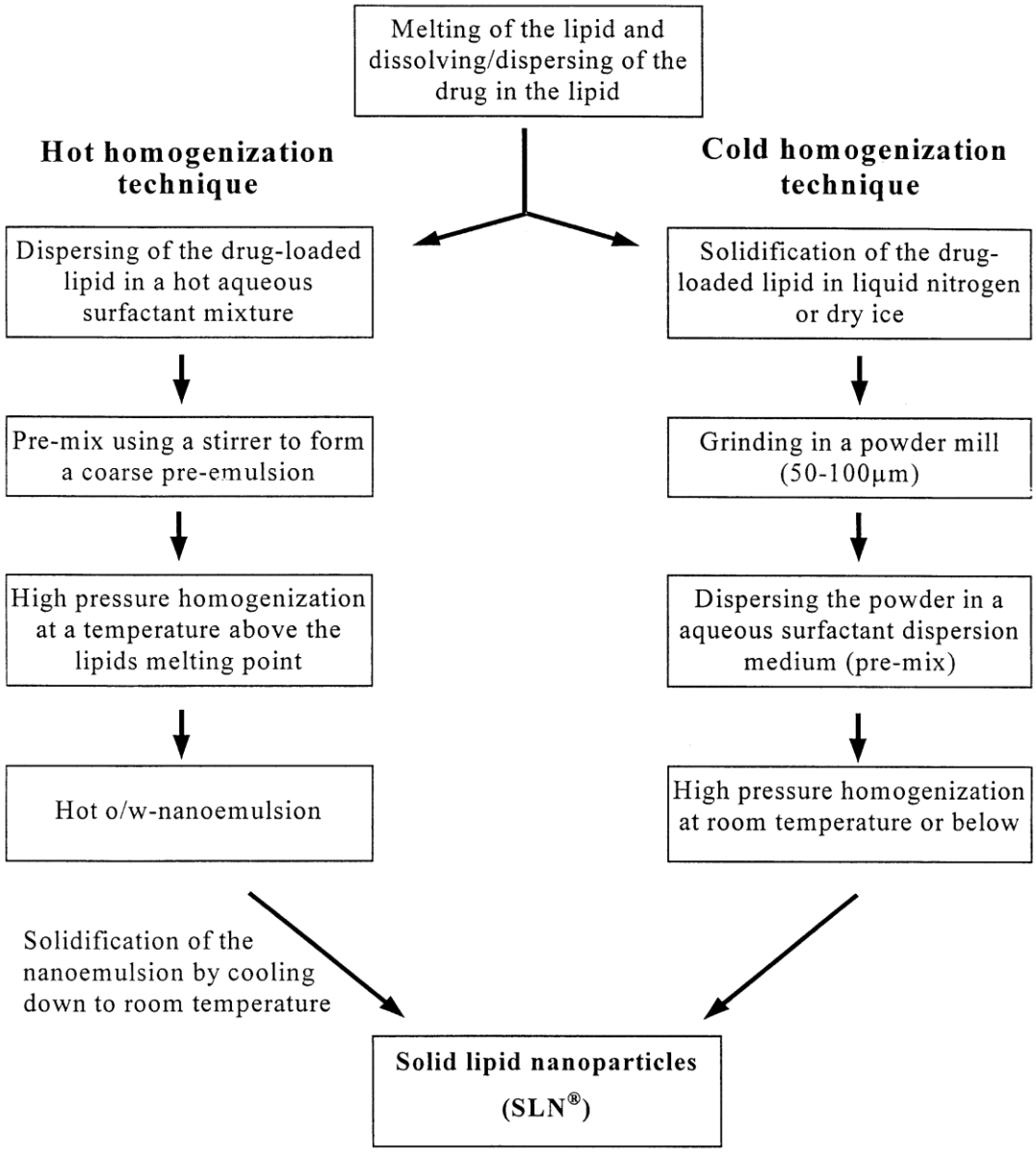


Figure 2. 6. Schematic procedure of hot and cold homogenization methods for SLN production (W. Mehnert, K. Mader, 2001).

Homogenization of this suspension at or below room temperature produce strong cavitation forces that break the suspended particles into solid lipid nanoparticles, this technique being developed to

address problems related to hot homogenization technique (Mehnert and Mader, 2001). This technique minimizes thermal exposure thereby preserving the quality of the thermo-labile drugs, reducing the loss of hydrophilic drugs to the aqueous phase and preventing/reducing the crystallization-triggered polymorphic transitions of lipid common in hot homogenization (Muller et al., 2000).

2.8.3 Characterization of SLNs

Satisfactory characterization of nanocarriers is a useful aspect of their quality control, with SLNs being no exception. The important parameters that are used to evaluate the quality of SLNs include particle size, particle size distribution, zeta potential surface morphology (shape), encapsulation efficiency and drug release profile. Antibiotic loaded SLNs can further be characterized for their *in vitro* and *in vivo* antimicrobial activity, with the parameters and methods used to evaluate them being briefly explained below.

Particle size and its distribution is an important characteristic of nanoparticles, as they influence other formulation characteristics, such as entrapment efficiency, release pattern, stability, biodistribution and targetability. Several methods exist for characterizing particle size, including scanning electron microscopy (SEM), transmission electron microscopy (TEM) and dynamic Light Scattering (DLS), with the latter being a popular method as it is fast, uncomplicated and sensitive to submicron particles (Ebrahimi et al., 2015; S. Zhang et al., 2008).

Zeta potential measurements are essential during the characterizing SLNs, as with other formulations, the zeta potential value of SLNs may help to predict stability of the formulation on storage. Generally, particles with high zeta values are expected to repel each other, thereby preventing coagulation. However, nanosystems comprising surfactants that produce stabilization

through steric hindrance do not strictly follow this rule. Zeta potential can also be measured using DLS (Manoj et al., 2010).

Entrapment Efficiency (EE) is another important parameter of SLNs that is significant due to its ability to influence the release profile of the formulation. The EE of the SLNs are determined by ultracentrifugation of the formulation, with the amount of free drug in the supernatant being calculated by methods such as High Performance liquid chromatography or Ultraviolet (UV) spectrophotometry (Saraf, 2009; Kalhapure et al., 2015).

The morphology of the SLNs can be evaluated by using SEM or TEM, and is used to measure the physical dimensions and structure of the formulation particles (Zhang and Zhang, 2010), with both methods having been reported in the literature in relation to SLNs. Determining the drug release from the SLNs is usually performed using the dialysis method, whereby samples are collected at specific time intervals and measured using HPLC or UV method, with details of these methods being reported in the literature (Cheow and Hadinoto, 2011; Venkateswarlu and Manjunath, 2004; Wong et al., 2006).

For antibiotic loaded SLNs, the antibacterial activity both *in vitro* and *in vivo* is established by measuring the minimum inhibitory concentration (MIC) of the formulation against a particular species of the microorganism. Other methods, such as X-Ray Diffraction (XRD) and differential scanning calorimetry (DSC), are also commonly used to determine thermal properties, as well as the changes related to crystallinity of the drug and additives used in the SLN formulation (Gonçalves et al., 2016; Noack et al., 2012).

2.9 Vancomycin as a model drug for antibiotic therapy

Vancomycin is a last line drug for treating several serious infections caused by Gram positive bacteria, such as MRSA. This glycopeptide antibiotic acts by binding to the terminal d-Ala-d-Ala sequence of nascent cell wall mucopeptides, thereby weakening the cell wall (Fayaz et al., 2011). Owing to its low oral absorption potential, vancomycin must be administered intravenously to treat systemic infection. Besides being inconvenient, the parenteral administration of vancomycin is associated with several limitations, such as poor tissue penetrations and severe side effects, namely nephrotoxicity and ototoxicity (Argenziano et al., 2017; Yousry et al., 2016). In addition, the emergence of MRSA strains that are also resistant to vancomycin has raised concern about the future of this antibiotic. It is thus widely acknowledged that encapsulating vancomycin into novel nanodelivery systems will overcome the issues related to resistance and other drug delivery problems associated with it.

Vancomycin has been successfully loaded into various nanocariers, such as liposomes, SLNs (Kalhapure et al., 2014), nanobubbles (Argenziano et al., 2017), dendrimers (Choi et al.,2013), polymersomes (Omolo et al.,2016) and gold nanoparticles (Fayaz et al., 2011). Vancomycin has been delivered using pH responsive polymers (Kalhapure et al., 2017a; Pichavant et al., 2011; Radovic-Moreno et al., 2012) and acid cleavable SLNs (Kalhapure et al., 2017), but not by using surface charge switching SLNs. Formulating surface charge switching SLNs to deliver vancomycin, which is the focus of this study, will not only widen the pool of available nanocarriers for antibiotic delivery but also set a platform for futures studies of its kind.

As discussed in this chapter, nano-drug delivery systems show considerable potential to restore the efficacy of available antibiotics by circumventing the limitations of their current dosage forms. Considering the large costs, toxicity and lengthy of time needed to develop new antibiotics, all of which can be avoided through nanotechnology, the importance of nanocarriers, and particularly those capable of site specific delivery of antibiotics as solution to the problems of the current antibiotic therapy, cannot be ignored. While much work has been done in fields such as oncology, research related to nanosystems for the targeted delivery of antibiotics is still in its early stages. The knowledge and skills gained in cancer research will make it possible to design nanocarriers that specifically deliver antibiotics directly to infection sites in response to stimuli like pH.

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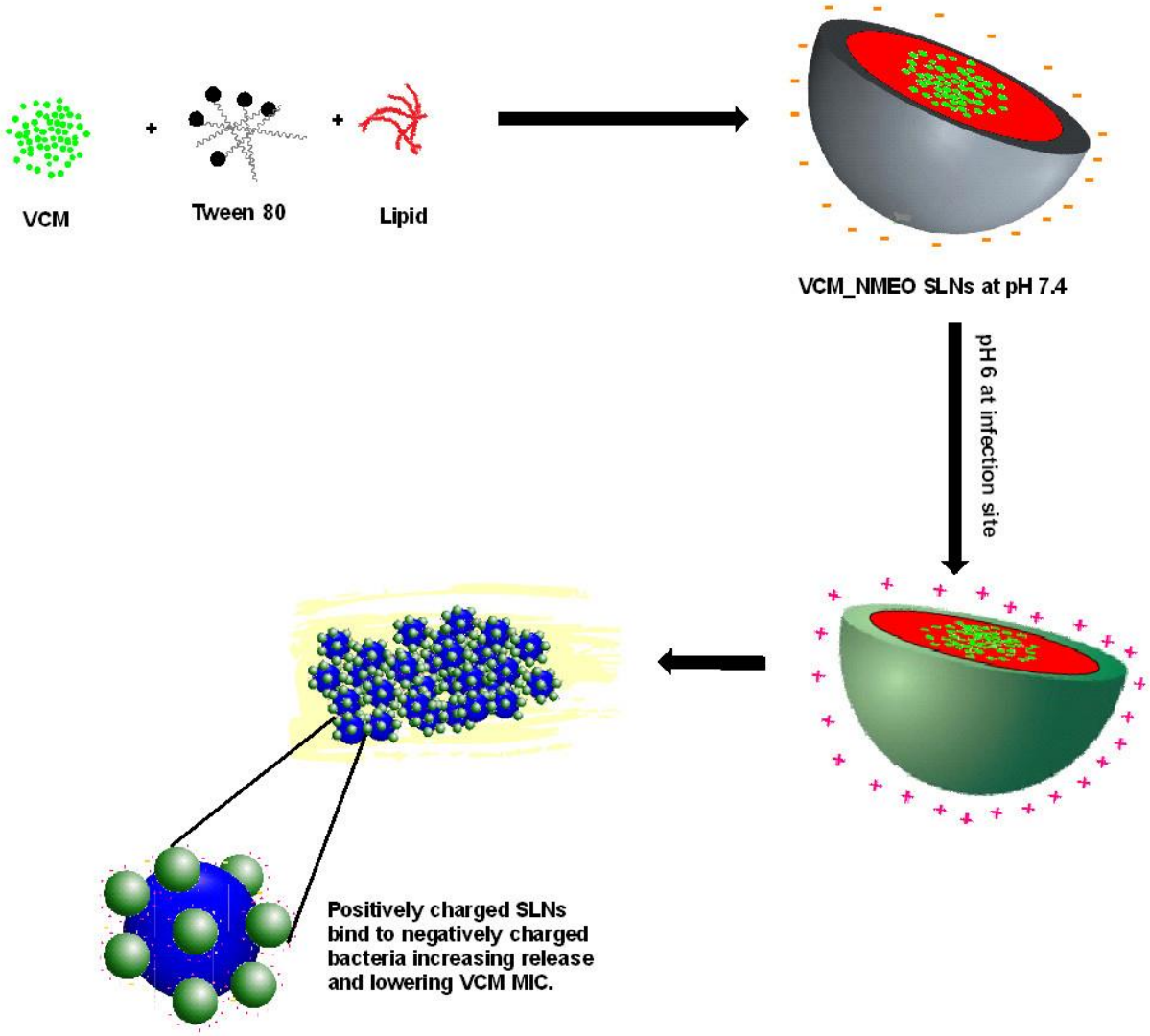
CHAPTER 3. SUBMITTED MANUSCRIPT

3.1 Introduction

This chapter is presents a first author manuscript submitted to the International Journal of Pharmaceutics (Impact Factor = 3.649). The manuscript is presented in the required format of the journal. The manuscript describes the synthesis and characterization of a novel NMEO lipid, formulation and physico-chemical characterization of its vancomycin- loaded pH responsive SLNs and finally it evaluates *in vitro, in silico* and *in vivo* performance of the formulation against MRSA.

Proof of submission can be found in Appendix A.

3.2 Graphical abstract



3.3 Submitted manuscript

Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin

Danford Mhule[1], Rahul S. Kalhapure[1, *, †], Mahantesh Jadhav[1], Sanjeev Rambharose[1], Calvin A. Omolo[1], Chunderika Mocktar[1], Sanil Singh[2], Ayman Waddad[1], Valence M.K Ndesendo[3], Thirumala Govender[1, *].

[1]Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa.

[2]Biomedical Resource Unit, University of KwaZulu-Natal, Westville Campus, Durban, 4000, South Africa.

[3]School of Pharmacy and Pharmaceutical Sciences, St. John's University of Tanzania, P.O. Box 47, Dodoma, Tanzania.

* Corresponding Author:

Email address: govenderth@ukzn.ac.za (T. Govender), kalhapure@ukzn.ac.za (R.S. Kalhapure).

†Author's present address: School of Pharmacy, The University of Texas at El Paso, 500 W. University, El Paso Texas, 79968, USA. Email address: rkalhapure@utep.edu

Abstract

Stimuli-responsive nano-drug delivery systems can optimize antibiotic delivery to infection sites. Identifying novel lipids for pH responsive delivery to acidic conditions of infection sites will enhance the performance of nano-drug delivery systems. The aim of the present investigation was to synthesize and characterize a novel pH-responsive lipid for vancomycin delivery to acidic conditions of infection sites. A pH-responsive solid lipid, N-(2-morpholinoethyl) oleamide (NMEO) was synthesized and used to prepare vancomycin (VCM)-loaded solid lipid nanoparticles (VCM_NMEO SLNs). The particle size (PS), polydispersity index (PDI), zeta potential (ZP) and entrapment efficiency (EE) of the formulation were 302.8 ± 0.12 nm, 0.23 ± 0.03, -6.27 ± 0.017 mV and 81.18 ± 0.57 % respectively. The study revealed that drug release and antibacterial activity were significantly greater at pH 6.0 than at pH 7.4, while the *in silico* studies exposed the molecular mechanisms for improved stability and drug release. Moreover, the reduction of MRSA load was 4.14 times greater ($p < 0.05$) in the skin of VCM_NMEO SLNs treated mice than that of bare VCM treated specimens. Thus, this study confirmed that NMEO can successfully be used to formulate pH-responsive SLNs with potential to enhance the treatment of bacterial infections.

Introduction

The emergence of resistance against antibiotics has given rise to many problematic strains of bacteria, one of them being methicillin-resistant *Staphylococcus aureus* (MRSA) (Sonawane et al., 2016), a bacterium responsible for a wide range of infections in community and hospital settings (R. A. Ma et al., 2014; Song et al., 2011). It is resistant to several antibiotics and is also threatening vancomycin, which is considered a drug of last resort for combating MRSA infections (Kali et al., 2013; Mandal et al., 2017; Zetola et al., 2005). This makes its infections difficult and costly to treat, and associated with high mortality globally (Boucher and Corey, 2008).

A major contributory factor for resistance development is the limitations associated with the current dosage forms of antibiotics (Sharma et al., 2012), which include inadequate concentrations of the drug at infections sites and increased drug exposure to normal flora (Priya et al., 2009). In addition, their high doses and frequency of administration lead to toxicity, which affects patients' adherence to treatment regimens (Xiong et al., 2014). Nanosized drug delivery systems are a strategy receiving increasing attention for their enhancing antibiotic therapy (Andrade et al., 2013). Nanocarriers are considered suitable for managing antimicrobial resistance as they are capable of ensuring both targeted and controlled /sustained delivery of antibiotics at infection sites as well as decreased exposure to normal tissues (Zazo et al., 2016). In this way, a constant and effective drug concentration is maintained above the minimum inhibitory concentration (MIC) at the site of infection. Consequently, the frequency of administration is lowered, side effects are decreased, and patient compliance is increased (Gao et al., 2011; Huh and Kwon, 2011). Advances in nanoscience have enabled researchers to design delivery systems that target biomarkers associated with specific pathological conditions/ tissues, and include the over expression of specific molecules or changes in physiological conditions, such as pH and temperature (Kullberg et al., 2009; Timko et al., 2010; Wang et al., 2008). Of these biomarkers, pH is useful for targeting bacterial infections, having been reported by various researchers that the pH level at infection sites is usually lower than the physiological value (7.4). (Dubos, 1955; Poschet et al., 2002; Tate et al., 2002). Arming nanoparticles with moieties that respond to pH changes has proved successfully in binding them to bacteria and increasing the drug release at sites of infection and bacteria (Radovic-Moreno et al., 2012). However, literature shows that most of the pH responsive systems reported so far are for cancer therapy whilst very few papers report pH-based nanoparticles for antibiotic delivery (Kalhapure et al., 2017a; Kalhapure et al., 2017b; Pichavant et al., 2011; Radovic-Moreno

et al., 2012). There is therefore a need to identify novel materials and nanodelovery systems of antibiotics with pH responsiveness to maximize their delivery to infection sites (Radovic-Moreno et al., 2012).

Various nano-based delivery systems, such as nanoemulsions (Kumar et al., 2008; Rapoport et al., 2009), liposomes, polymeric nanoparticles (PNs) (Chawla and Amiji, 2002; Dhar et al., 2008; Shenoy and Amiji, 2005), and solid lipid nanoparticles (SLNs) (Gupta et al., 2007; Spada et al., 2012) have been reported for targeted delivery. The fabrication of nanoemulsions needs liquid oils while SLNs use solid lipids, which make them more stable (Geszke-Moritz and Moritz, 2016). In addition, both SLNs and liposomes are made using physiological lipids, but unlike liposomes and PNs, SLNs avoids the use of organic solvents (Briones et al., 2008). These features, their ability to encapsulate both hydrophobic and hydrophilic drugs, and their ease of scaling up and sterilization has led to a growing interest by researchers in SLNs (Gastaldi et al., 2014). SLNs are usually fabricated using solid lipids such as fatty acids, glycerides and waxes which lack pH responsiveness, and results in researchers including pH sensitive materials such as phospholipids in the lipid matrix to achieve pH-responsive delivery (Kashanian et al., 2011; Rostami et al., 2014). Our group recently reported (Kalhapure et al., 2017b) a pH responsive SLN formulation for antibiotic delivery using a stearic acid based acid cleavable lipid for targeted delivery of vancomycin, this being the only study to do so. There is therefore a need to widen the pool of available responsive lipids for targeting antibiotics to infection sites. In this project, a novel pH responsive solid lipid, N-(2-morpholinoethyl) oleamide (NMEO), was synthesized using oleic acid and 4-(2-Aminoethyl) morpholine (4-AEM). This material was first synthesized by our group as an intermediate for synthesizing heterocyclic quaternary ammonium surfactants (Jadhav et al., 2016). To the best of our knowledge, it has not been reported as a formulation component for a

nanodelivery system of any drug class and disease condition. Oleic acid is a biocompatible, unsaturated fatty acid that finds wide application in drug delivery (Srisuk et al., 2012). It has been used as a penetration enhancer in transdermal delivery systems (Touitou et al., 2002), a stabilizer in liposomes (Bergstrand et al., 2003; Drummond et al., 2000) and magnetic nanoparticles (Darwish, 2017; Soares et al., 2016) and a liquid lipid in nanostructed lipid carriers (Zhao et al., 2016). It also has antibacterial activity, which may enable the fabrication of nanocarriers that can synergize antimicrobial activity of the encapsulated drugs (Huang et al., 2011). 4-AEM is a ligand commonly used to synthesize Schiff bases for application in biological systems (Zhu, 2009). Based on its ability to protonate at low pH, this moiety is used to target acidic microenviroments, such as lysosomes (Wang et al., 2015; Yang et al., 2014; Yu et al., 2012). Hence, we hypothesize that a lipid formed from these two compounds would be biocompatible and display pH responsiveness that could be helpful in targeting bacterial infection sites. Specifically, this study aimed at synthesizing an oleic acid based lipid and subsequently exploring it to formulate pH responsive SLNs for the targeted delivery of vancomycin to manage MRSA infections. In vitro, in silico and in vivo results obtained by loading vancomycin in NMEO based SLNs are presented in this paper.

Materials and methods

Analytical grade 4-(2-Aminoethyl) morpholine (4-AEM) was procured from Sigma-Aldrich Co. Ltd., (UK). *p*-dimethylamino pyridine (DMAP) and oleic acid (OA) were purchased from Sigma-Aldrich Co. Ltd. (USA), and *N, N'*-dicyclohexyl carbodiimide (DCC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Merck Co. Ltd., (Germany). Mueller- Hinton Broth (MHB) was obtained from Sigma-Aldrich (USA), while Mueller Hinton Agar (MHA) and Nutrient Broth were bought from Biolab Inc., (South Africa). The water used

was purified by an Elix® system from Millipore Corp., (USA), and the bacterial strain used was *S. aureus* Rosenbach ATCC®BAA-1683 (MRSA).

Synthesis and characterization of NMEO, (Figure 3.1)

NMEO was synthesized via steglich esterification (Ha et al., 2008, 2009), based on a previously reported procedure (Jadhav et al., 2016). In brief, 4.25 g (35.3 mmol) of 4-AEM was slowly added into 250 ml of dry dichloromethane (DCM) containing a mixture of oleic acid 10 g (35.4 mmol), DMAP 0.5 g (4 mmol) and DCC 7.125 g (34.5 mmol) under stirring at room temperature (RT). This mixture was kept under stirring at the same temperature for 24 h, after which the precipitated dicyclohexylurea was removed by filtration. The filtrate was evaporated under reduced pressure to obtain the compound of interest, which was purified by column chromatography.

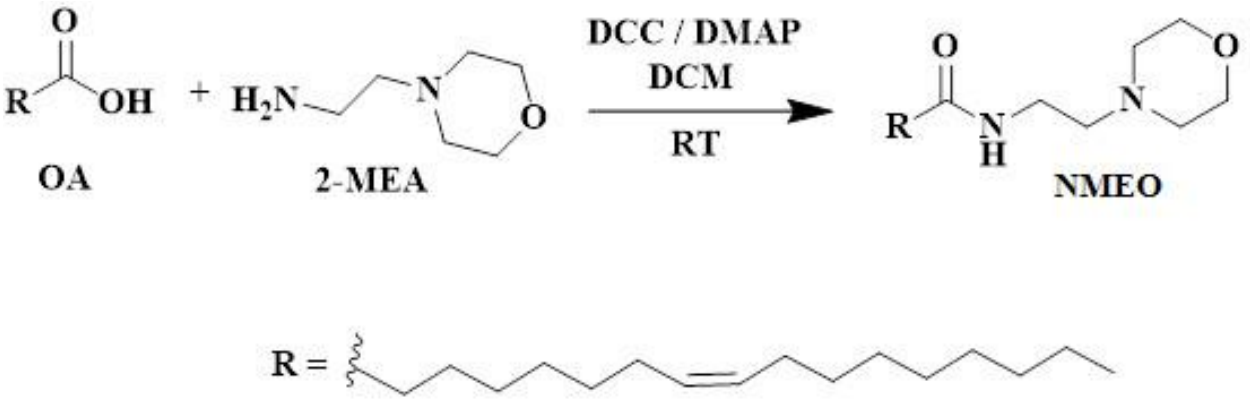


Figure 3.1. Synthesis scheme for NMEO.

The structure of the synthesized lipid (NMEO) was confirmed by Fourier transform infra-red (FT-IR) spectroscopy, Nuclear magnetic resonance (NMR) imaging (^{1}H and ^{13}C) and mass analysis. Bruker Alpha spectrophotometer (German) and Waters Micromass LCT Premier/TOF-MS instrument (United Kingdom) were used to record the FT-IR and Electrospray ionization mass spectra respectively. ^{1}H NMR and ^{13}C NMR spectra were obtained using Bruker 400/600 Ultrashield™ NMR spectrometer (United Kingdom) at 25 °C.

In vitro cytotoxicity

The biosafety of the synthesized NMEO was assessed using an MTT assay following a literature reported procedure (Omolo et al., 2016). The study used human lung epithelial (A549), human embryonic kidney (HEK-293) and human liver (HEP G2) cell lines. The cells (2.2×10^{3} / cell) were seeded into 96-well plates, incubated for 24 h and treated with NMEO concentrations of 20, 40, 60, 80 and 100 μg/ml for 48 h. At the end of the 48 h incubation period, the culture medium containing the test material was removed from the wells into which fresh culture medium and MTT solution (100 μl each) were added. These two solutions (MTT solution and culture medium) were removed after 4 h of incubation at 37 ºC, and the formed MTT formazan was dissolved by adding dimethylsulfoxide. The absorbance corresponding to each well was measured using a microplate spectrophotometer (Spectrostar nano, Germany) at 540 nm. The culture medium with cells was used as the positive and those without cells as a negative control, with all experiments being run in six replicates. The following equation was used to determine percentage cell viability:

% Cell viability = A_{540nm}treated cells/A_{540nm} untreated cells) x 100%

Preparation of SLNs

SLNs were formulated using a hot homogenization and ultrasonification method (Kalhapure et al., 2014), with the oily phase consisting of NMEO (500 mg) as a solid lipid and vancomycin free base (50mg) and the aqueous phase comprised of Tween 80 (250 mg) in milli Q-water (125 ml). Tween 80 was selected based on the results of a prior surfactant screening study. Both phases were heated separately to 80 ºC, this being followed by the addition of the aqueous to oily phase once the lipid had melted. The resultant mixture was homogenized at 10000 rpm for 15 min and sonicated (30% amplitude) for 15 min using an Ultra Turrax T-25 homogenizer (IKA Labortechnik, Germany) and the Omni sonic ruptor 400 Ultrasonic Homogenizer (Kennesaw, GA 30144, USA) respectively.

The obtained nanoemulsion was immediately cooled to 20 ºC for the lipid to crystallize and form VCM NMEO_SLNs (Souto and Müller, 2006), with the final volume being adjusted to 125 ml using milli Q-water. The same procedure was used for blank (drug-free) NMEO SLNs, while stearic acid SLNs (SA SLNs), as a non-pH responsive nanosystem, were also prepared using the same procedure for comparison to the pH responsive VCM NMEO_SLNs in antibacterial studies.

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

The average PS, PDI and ZP of formulated SLNs were determined by a dynamic light scattering technique. Dilutions were made using milli-Q water or appropriate phosphate buffer solutions, and the parameters were measured at room temperature (25 ºC) using a Zetasizer Nano ZS90 (Malvern Instruments, UK), with all parameters being analyzed in triplicate to ensure reliability. The morphological features of the nanoparticles were investigated by transmission electron microscopy (TEM) analysis (Gonçalves et al., 2016). The samples were appropriately diluted, stained with phosphotungstic acid and fixed on a copper grid for drying. The dried samples were analyzed on a JEOL Microscopy (JEM 1010, Japan) with images being acquired at 100 kV.

Entrapment efficiency (EE) and Loading Capacity (LC)

The amount of drug encapsulated in the SLNs was determined by an ultrafiltration method using Amicon® Ultra-4 centrifugal filter tubes (10 kDA molecular weight cut-off). Samples were placed in the centrifugal filter tube and centrifuged at 3000 rpm at 25 °C for 30 min. After separating the supernatant, the amount of drug in it was analyzed by a UV-Visible spectrophotometer (Shimadzu UV- 1650 PC) at 280 nm (Kalhapure et al., 2014). The determination of % EE and % LC was based on equations (1) and (2) respectively (Ma et al., 2014).

% EE = (Mass of drug in nanoparticles / Mass of drug added) x 100% (1)

LC = (Mass of drug in nanoparticles / Mass of nanoparticles) x 100%. (2)

Differential Scanning Calorimetry (DSC)

The DSC measurements of formulation components (individually), physical mixture and the drug were performed by DSC (Shimadzu DSC-60, Japan), and the weighed samples (2 mg) were placed in aluminum pans that were then sealed and scanned from 30 °C to 300 °C under a nitrogen stream at 10 ^{o}C/min.

In-vitro drug release study

A dialysis tube diffusion technique was used to investigate the *in-vitro* release behavior of drug loaded formulations (pH responsive and non-pH responsive SLNs) that were prepared in triplicate. Briefly, 2 ml of the drug loaded SLNs and their respective blanks were loaded into dialysis tubes (MWCO 14 kDa), sealed, and dialysed against 40 ml phosphate buffer solutions (PBS) (7.4, and 6.0) at 37 °C in an incubator maintained at 100 rpm. At predetermined time intervals, 3 ml samples were withdrawn from the receiver and the amount of vancomycin was determined by a spectrophotometric method (UV-1650PC, Shimadzu, Japan) at 280 nm. To keep the volume of release medium constant and maintain sink conditions, an equal amount of fresh PBS was added after each sampling.

In vitro drug release kinetics

Drug release kinetics were evaluated using a DDsolver Add- In program (Zhang et al., 2010). Six mathematical models were used whereby the correlation coefficient (R^2) and root mean square error (RMSE) were calculated and compared, the models being zero order, first order, Weibull, Korsmeyer-Peppas, Higuchi and Hixson-Crowel.

Stability

The short-term physical stability of the VCM_NMEO SLNs was evaluated at 4 °C and at room temperature (RT) for 90 days. The evaluation of the physical appearance of the formulations, as well as their particle size, PDI and ZP, was performed at the end of 30, 60 and 90 days, with the study being performed in triplicate.

Molecular modelling

Molecular modelling was performed to understand the type of interactions that occurred between VCM and NMEO based on a previously reported method with modifications (Sonawane et al., 2016). All molecular modeling techniques were performed using Bovia Materials Studio (MS) 2016 that was installed on the remote server at the Center for High Performance Computing (CHPC) (Cape Town, South Africa). The structure of VCM (PDB:1SHO) was obtained from the RCSB website, while NMEO was drawn using ChemBioDraw Ultra 14. All the structures were cleaned, and hydrogen atoms were added, while the smart minimizer algorithm in geometry optimization option available in forcite module of MS software was used to optimize all the structures to their lowest energy conformations. A universal energy force field was applied, and the convergence tolerance criteria set to 0.001 kcal/mol during the geometrical optimization study. The molecular dynamics (MD) study was performed in vacuum to obtain a stable complex of VCM and NMEO, both being initially placed inside the cubic cell (10x10x10 nm), with the crystal builder and amorphous cell module of MS 2016 being used to construct this model. Geometry optimization of the whole system was performed prior to MD simulation using the same protocol, as mentioned above, and optimization of the cell parameters was allowed during energy minimization. The stabilized system was then subjected to MD simulation under periodic boundary conditions, which was performed at room temperature and 4°C over 50 ps. The final complex

structure was then studied for the intermolecular interactions to understand the various non-covalent forces that were responsible for the complex formation, with the Biovia Discovery Studio Visualizer being used to depict the interactions in the drug-lipid complex.

In vitro antibacterial activity

The minimum inhibitory concentration (MIC) of vancomycin loaded SLNs were determined using a broth dilution method (Mohammed Fayaz et al., 2011). After an overnight growth in Nutrient Broth at 37 °C in a shaking incubator (Labcon, USA), the MRSA cultures were diluted with sterile de-ionised water using a densitometer (DEN-1B McFarland, Latvia) to achieve a turbidity of 0.5 McFarland. The bacterial cultures were further diluted 1:150 with sterile de-ionised water to obtain a concentration of 2×10^5 colony forming units (CFUs)/ml. The minimum inhibitory concentration (MIC) of bare VCM, drug-free (blank) NMEO SLNs, VCM loaded SA SLNs (VCM_SA SLNS) and VCM loaded NMEO SLNs (VCM_NMEO SLNS) were determined using the broth dilution method. The test compounds were serially diluted with Mueller-Hinton Broth 2 in 96-well plates at pH 6.0 and pH 7.4. The plates were incubated in a shaking incubator at 37 °C for 18 h, after which, 10 µl of each dilution was spotted onto Mueller-Hinton Agar (MHA) plates after 24 h of further incubation. The MHA plates were incubated at 37 °C for 18 h, with the studies being performed in triplicate. The blank formulation of NMEO SLNs was used as a negative control, while vancomycin loaded SA SLNs and bare VCM were used as positive controls.

In vivo antibacterial activity

The *in vivo* antibacterial activity of bare VCM and VCM_NMEO SLNS against MRSA was investigated using a procedure approved by the Animal Research Committee of the University of KwaZulu-Natal (UKZN) (Approval no. AREC/104/015PD). BALB/c mice (18-20 g in weight)

were purchased from the University's Biomedical Research Unit and divided into three groups (n = 4) categorized as treatment , positive and negative control groups. 50 µl of MRSA saline suspension (1.5 x 10^8 CFU/ml) was injected intradermally to the three groups of with their back hair removed and disinfected with 70% ethanol one day before. Half an hour after infection, bare VCM and VCM_NMEO SLNS were injected at the infection sites of the mice categorized as the positive control and treatment groups respectively, while nothing was given to the third group that was used as a negative control. After 48 h, the infected skin was harvested from the sacrificed mice and homogenized in 5ml of phosphate buffer solution (pH 7.4). The serial dilutions of tissue homogenates were plated on nutrient agar plates (Biolab, South Africa) and incubated at 37 °C, with the number of CFUs being counted after 24 h of incubation. The histological investigation was performed according to a previously reported procedure (Omolo et al., 2016). Briefly, skin samples were fixed in formaldehyde at 25°C for seven days, dehydrated using ethanol, implanted in paraffin wax. The tissue wax blocks were sectioned using a microtome (Leica RM2235, Leica Biosystems, Germany) and sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). Sections were examined and captured with a Leica Microscope DM 500, fitted with a Leica ICC50 HD camera (Leica Biosystems, Germany).

Statistical analysis

Statistical analysis of data was performed using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test using GraphPad Prism® 6 (GraphPad Software Inc., USA). Statistical significance was based on a p-value <0.05, with the data being expressed as mean ± standard deviation (SD).

Results and discussion

Characterization of NMEO

M.p. 42-44 °C; FT-IR:3285.91, 2918.5, 2848.71, 1646.85, 1559.86, 1462.07, 1117.96, 777.97, 715.29 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.836-0.869 (t; 3H; -*CH_3*), 1.241-1.276 (m; 20H; -*CH_2*-), 1.581-1.616 (q; 2H;-*CH_2*CH_2COO-), 1.958-1.972 (m; 4H; -*CH_2*-CH=CH-*CH_2*-), 2.00-2.163 (t; 2H; *CH_2*COO-), 2.521-2.550(m;6H; -*CH_2*-N(*CH_2)$_2$*-), 3.351-3.379 (t; 2H; -NH*CH_2*CH_2-), 3.718-3.740 (t; 4H; -*CH_2*O*CH_2*-), 5.290-5.328 (m;72H; -*CH=CH*-), 6.25 (s; 1H; -*NH*-). ^{13}C-NMR ($CDCl_3$) δ (ppm): 14.09, 22.86, 25.72,27.16, 29.15, 29.27, 29.48, 29.70, 31.88, 35.24, 36.68, 53.23, 57.23, 66.45, 129.70,129.98, 173.31; ESI-TOF MS *m/z*: $[M + H]^+$ - calculated 395.3638 ($C_{24}H_{46}N_2O + H^+$),actual mass 395.3633.

In vitro cytotoxicity

Exposure of NMEO to the cell lines for a period of 48 h displayed that the novel lipid exhibited a high percentage cell viability across the concentration range studied (**Figure 3.2**). NMEO displayed a cell viability between 79.42 to 88.12%, 78.42 to 81.75% and 76.96 to 79.70% for A549, HEK-293 and HEP G2 cells respectively (**Figure 3.2**). The results displayed no dose dependent trends and is consistent with previous studies that report on materials that exhibit cell viability that is independent of the concentration (Romic et al., 2016; Di Gioia et al., 2015; Sikwal et al., 2017).

Literature reports that materials with a cell viability of greater than 75% can be considered as low toxicity in the framework of safety use (Cao et al. 2010; Omolo et al., 2017). The findings of the MTT study therefore suggest that the use of NMEO will be safe for biological/pharmaceutical applications.

Preparation of drug-free NMEO SLNs

Initially drug-free SLNs were prepared from the newly synthesized NMEO using several surfactants, the one with acceptable characteristics being selected and its quantity optimized.

Screening and selection of surfactant

Surfactants of different types were screened at a fixed concentration to identify the most suitable one for formulating blank NMEO SLNs with desirable physicochemical characteristics.

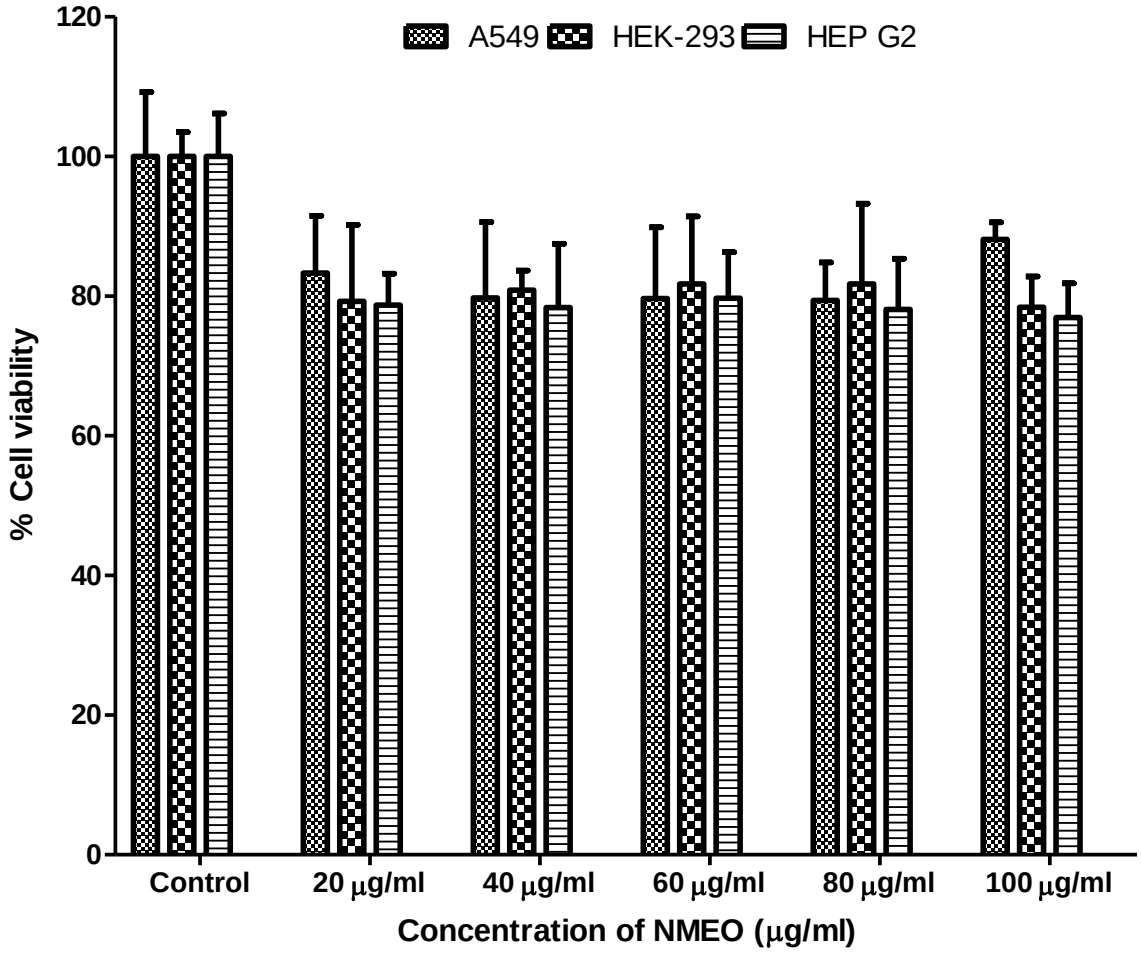


Figure 3.2. Cytotoxicity evaluation of various concentrations of NMEO against A549, HEK-293 and Hep G2 cells.

The surfactants used were Cremophor RH 40, Lutrol F-68, Solutol HS 15 and Tween 80. Of these, Tween 80 displayed the best results in terms of size and PDI (**Figure 3.3**). Similar results were obtained by Ebrahimi and coworkers who studied the effect of different stabilizers (polyvinly alcohol, Pluronic F 127, polyvinyl pyrrolidone, Tween 80 and phosphatidylcholine) on the behavior of SLNs. According to these authors, surfactants of low molecular weight, such as Tween 80, produce small sized particles due to their ability to be quickly adsorb into interfacial surfaces (Ebrahimi et al., 2015), which resulted in it being selected for further studies.

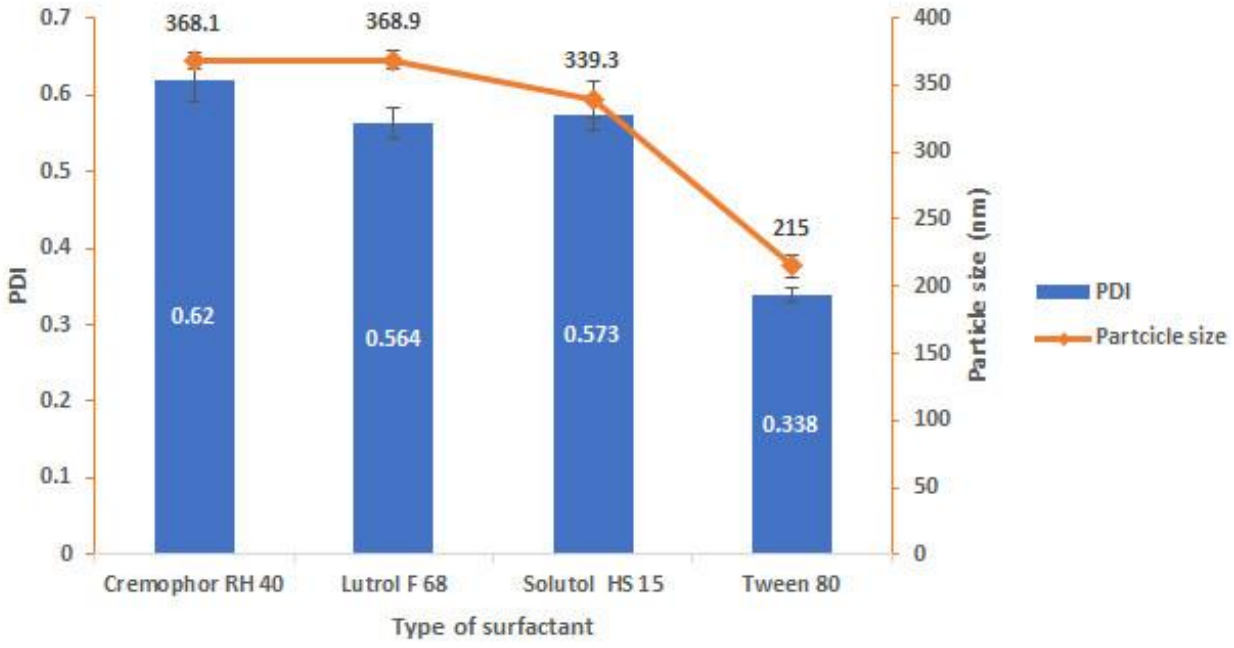


Figure 3.3. Effect of surfactant type on size and PDI of SLNs.

3.3.2. Effect of surfactant concentration

The surfactant (Tween 80) concentration was varied from 0.1 % (w/v) to 0.24 % (w/v) to determine the optimal concentration. As depicted in **Table 3.1**, PS and PDI of the blank formulations decreased with increasing surfactant concentration, with the particle size increasing beyond 0.2%.

Table 3.1. Effect of surfactant concentration on physicochemical characteristics of drug free NMEO SLNs. The values given are expressed as mean ± SD, n = 3.

pH	PS	PDI	ZP
		0.1%	
7.4	215.0 ± 8.3	0.34 ± 0.031	-6.03 ± 0.49
6.0	227.6 ± 1.5	0.340 ± 0.025	5.1 ± 0.250
5.5	211 ± 1.51	0.369 ± 0.07	10.4 ± 1.60
		0.12%	
7.4	162.8 ± 1.6	0.209 ± 0.03	-5.04 ± 0.471
6.0	162.0 ± 5.21	0.171 ± 0.013	4.4 ± 1.16
5.5	145.5 ± 2.4	0.179 ± 0.01	9.7 ± 1.9
		0.16%	
7.4	146.2 ± 3.0	0.174 ± 0.04	-4.7 ± 0.1
6.0	167.7 ± 1.8	0.259 ± 0.022	4.01 ± 0.82
5.5	168.0 ± 7.4	0.26 ± 0.07	9.06 ± 1.0
		0.2%	
7.4	122.2 ± 5.54	0.129 ± 0.02	-4.41 ± 0.88
6.0	124.3 ± 7.4	0.137 ± 0.012	3.46 ± 0.48
5.5	122.6 ± 3.1	0.152 ± 0.023	10.4 ± 0.21
		0.24%	
7.4	130.2 ± 0.74	0.119 ± 0.026	-4.17 ± 0.6
6.0	129.1 ± 1.35	0.095 ± 0.01	3.28 ± 0.48
5.5	140.9 ± 3.24	0.146 ± 0.037	7.45 ± 1.77

These results are in line with the findings of other studies related to SLNs (Das et al., 2011) and other types of nanoparticles (Dora et al., 2010; Singh et al., 2010). The ability of a surfactant to produce small particles depends on how fast it gets adsorbed to the particle surfaces before the particles grow through collisions. At higher concentrations, this adsorption process is faster, with the particles being maintained at smaller sizes (Helgason et al., 2009). Therefore, the drug-free formulation composed of 0.2%w/v surfactant was considered suitable to proceed with drug loading.

Preparation and characterization of drug loaded SLNs

Drug loaded pH responsive SLNs were prepared using VCM, Tween 80 and NMEO as drug, surfactant and pH responsive lipid respectively. Hot homogenization, a method that combines high pressure, thermal and mechanical forces to attain consistent nanosized formulations was used in addition to ultrasonification. The prepared SLNs were characterized for pH responsiveness and other physicochemical characteristics by dispersion in different buffer solutions. PS, PDI and ZP of VCM_NMEO SLNS and VCM_SA SLNs are presented in **Table 3.2** and **Figure 3.4**. The zeta potential of VCM_NMEO SLNS changed from -6.27 at pH 7.4 to +9.39 at pH 5.5, and there was a decrease in particle size from 302.8 at pH 7.4 to 220.5 at pH 5.5. The switch from negative to positive zeta potential was not observed for VCM_SA SLNs, which also did not show any major change in size confirmed that the pH-responsiveness of SLNs was due to NMEO. The ability to switch from negative at basic to positive charge at acidic pH is critical for pH-responsive systems for antibiotic delivery as it serves two purposes: i) it helps the system to become more hydrophilic and release higher quantities of the drug in the acidic conditions of infection sites (Mura et al., 2013), and ii) it enables the carrier to bind easily to the negatively charged bacterial cells, enhancing the targeting potential of the system (Chakraborty et al., 2012). Morphological analysis

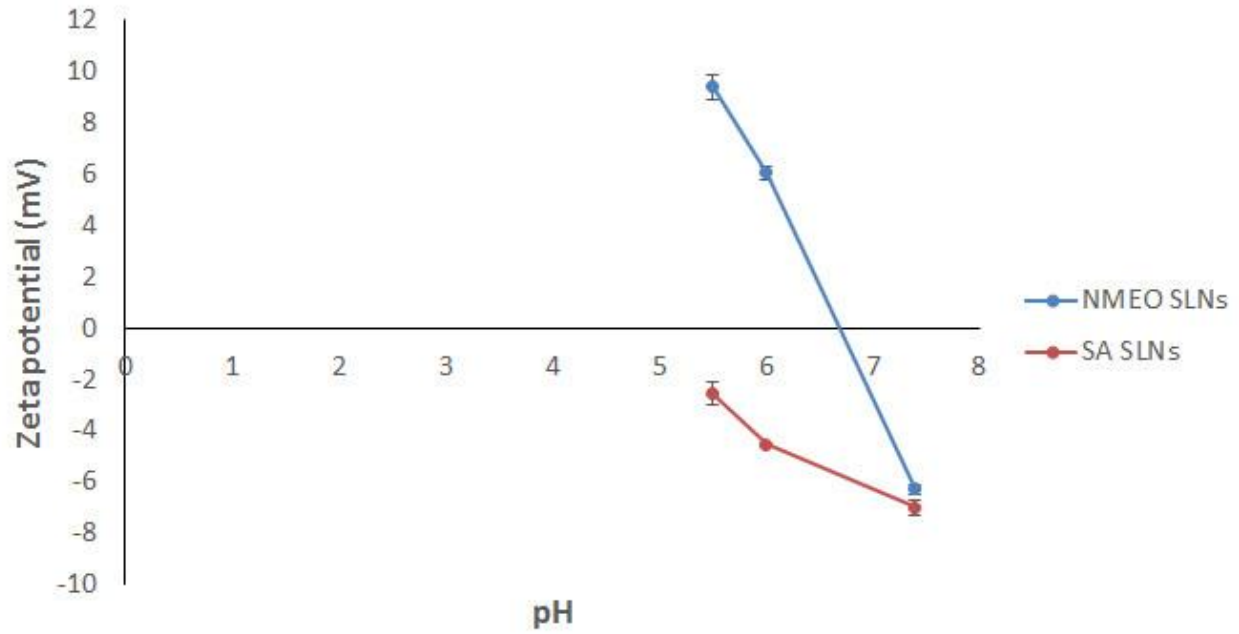


Figure 3.4. Effect of pH on zeta potential of VCM_NMEO SLNs.

Table 3.2. Effect of pH on VCM loaded formulations. The values given are expressed as mean ± SD, n = 3.

SLNS	VCM_NMEO SLNS			VCM_SA SLNs		
pH	PS	PDI	ZP	PS	PDI	ZP
7.4	302.8± 0.12	0.292 ± 0.025	-6.27 ± 0.017	365.6 ± 17.8	0.143 ± 0.021	-7.0 ± 0.11
6.0	260.7 ± 9.5	0.261 ± 0.018	6.05 ± 0.013	363.8 ± 15	0.316 ± 0.022	-4.12 ± 0.27
5.5	220.5 ± 1.9	0.380 ± 0.005	9.39 ± 0.046	368.1 ± 12.9	0.209 ± 0.025	-2.57 ± 0.09

using TEM showed that VCM_NMEO SLNS were discrete and had an almost spherical shape (**Figure 3.5**). The % EE and DL for drug loaded NMEO SLNs were 81.18 ± 0.57 % and 8.1% respectively. While the entrapment efficiency is often a challenge in drug delivery with nano-drug systems, the % EE obtained in this study was higher than several non-pH responsive SLNs reported (Liu et al., 2014; Seedat et al., 2016; Yousry et al., 2016), will be beneficial for reducing drug loss and costs, and will significantly reduce the amount of the drug to be administered. This data therefore confirmed that the NMEO lipid can be used to prepare VCM solid lipid nanoparticles with desirable properties.

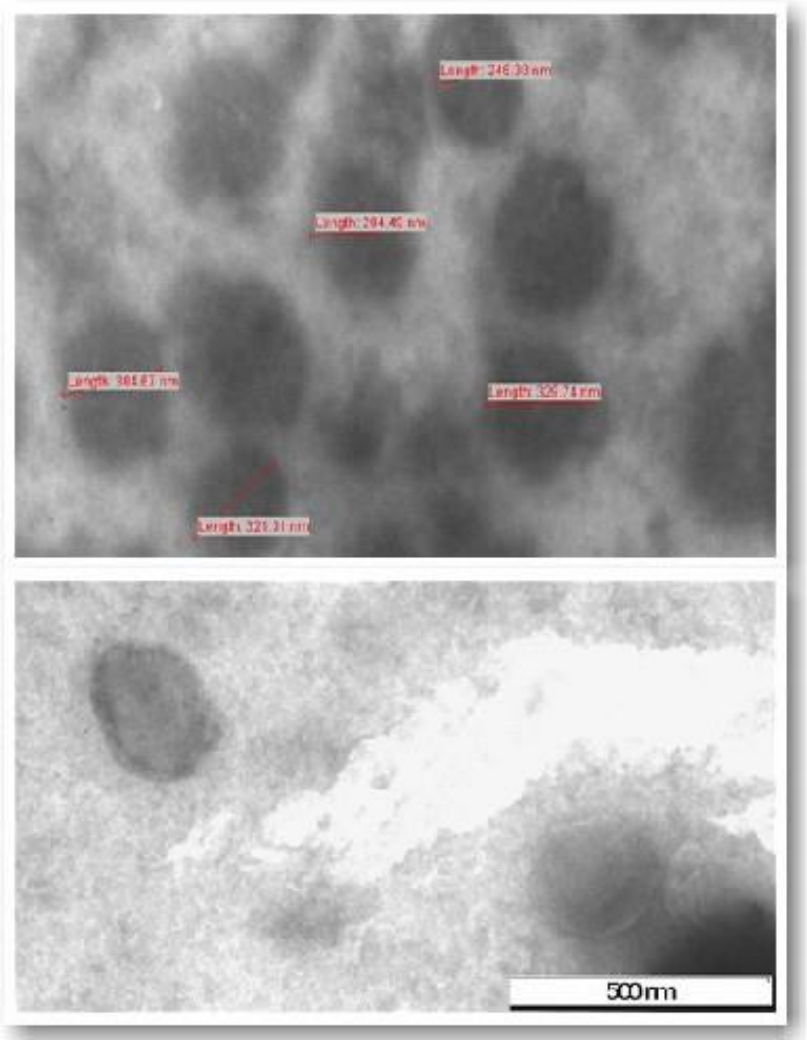


Figure 3.5. Morphology of VM_NMEO SLNs.

DSC studies

The aim of the DSC studies was to confirm entrapment of VCM in the NMEO SLNS, with the thermograms of SLNs, VCM and NMEO being shown in **Figure 3.6**, the thermal peaks of the latter two being observed at 112.14 °C and 41.22 °C respectively. Analysis of their physical mixture revealed no major shifts in the thermal peaks of the formulation excipients.

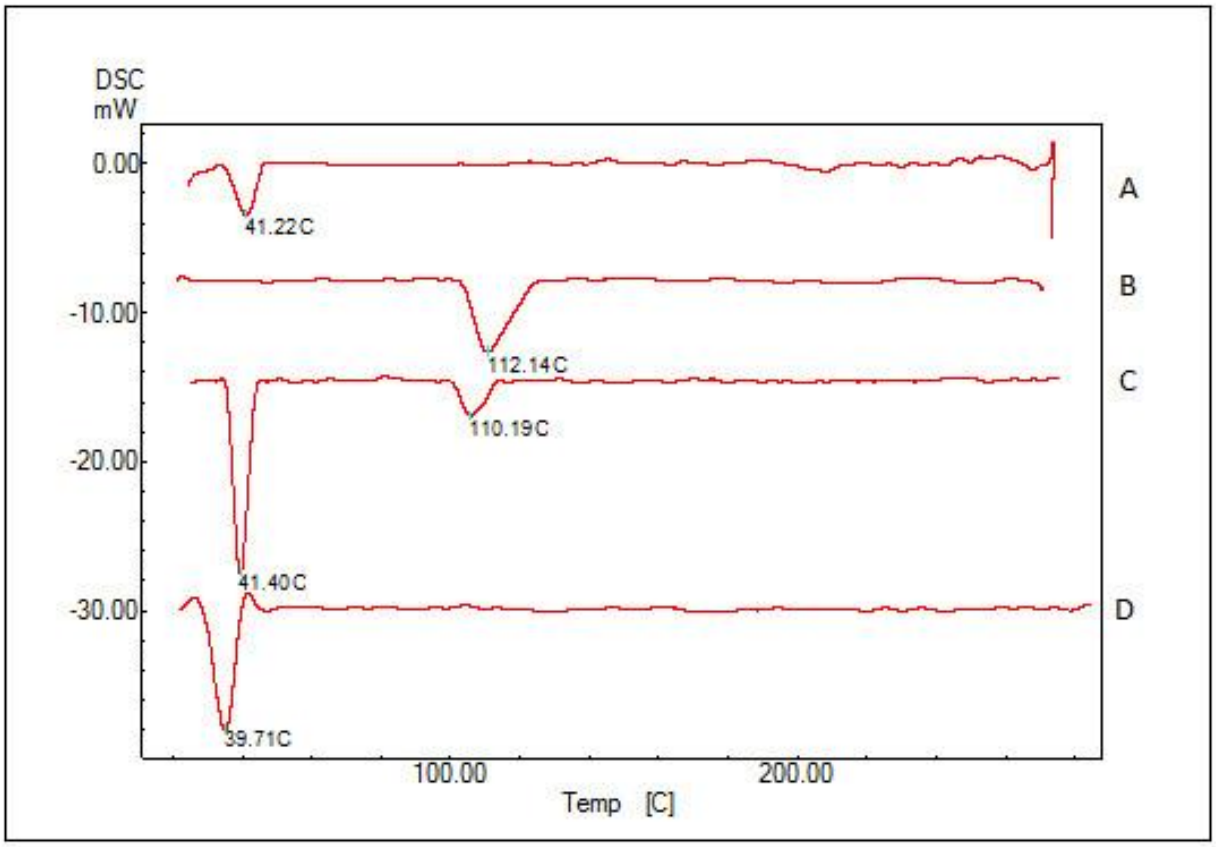


Figure 3.6. DSC thermogram of (A) NMEO, (B) VCM (C) physical mixture of VCM and NMEO and (D) lyophilized VCM_ NMEO SLNs.

The lyophilized SLNs exhibited an endothermic peak at 39.71 °C, which can be associated with the NMEO layer. The VCM peak was absent in the SLN's thermogram, indicating that the drug was entrapped in an amorphous form in the lipid matrix (Seedat et al., 2016b).

In vitro drug release profiles

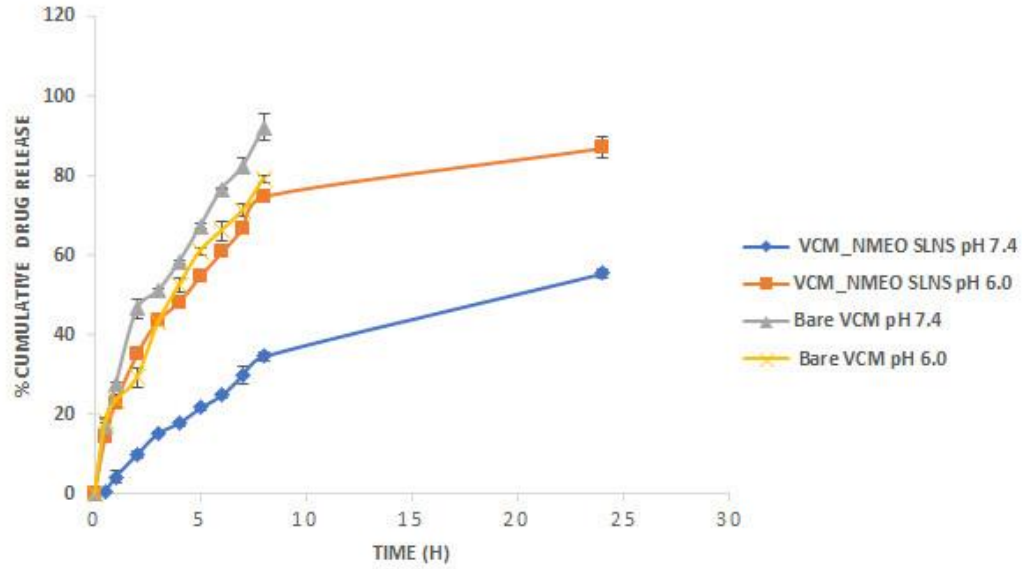
The in vitro release profiles of bare VCM, VCM_NMEO SLNs and VCM_SA SLNs are shown in **Figures 3.7 A and 3.7 B**, with the release of bare VCM at pH 7.4 and pH 6.0 being almost complete within the first 8 hours. Throughout the study, the amount of VCM released from the VCM_NMEO SLNs was higher at pH 6.0 than pH 7.4. After 8 h, the release of VCM from the VCM_NMEO SLNs was 34.66% at pH 7.4 and 74.89% at pH 6.0. At the end of the study (24 h), drug release was 1.58 times higher at acidic pH than at physiological pH. This increased drug release at pH 6.0 can be due to the pH responsiveness of NMEO, with the protonation of nitrogen atoms of its morpholinoethyl moiety at acidic pH increasing the hydrophilicity of the formulation, promoting faster drug release. Pu et al. (2014) obtained similar results, whereby protonation of the imidazole moiety conjugated to the pendant groups of poly (L-aspartate) triggered release of doxorubicin at acidic conditions from the pH-sensitive poly (L-aspartate)-b-poly (ethylene glycol) micelles.

The release of VCM from the VCM_SA SLNs was sustained at both pH values and did not show any pH dependence, with drug release after 24 h being 87.63% at pH 7.4 and 85.07% at pH 6.0. It can thus be concluded that the pH dependent drug release observed for VCM_NMEO SLNs was linked to NMEO.

In vitro drug release kinetics

The drug release kinetics data of the drug loaded formulations are presented in **Table 3.3**. At pH 7.4, both stearic acid and NMEO formulations released vancomycin according to the Weibull model, which is among the kinetic models that have a wide application in describing drug release processes (Koester et al., 2004).

A



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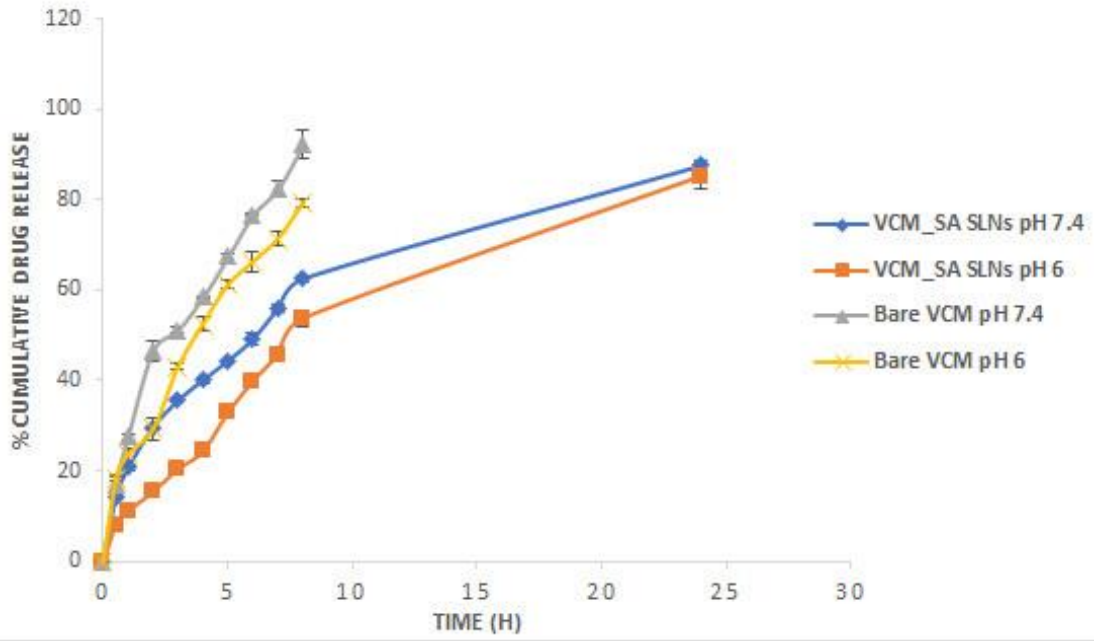

Figure 3.7. Effect of pH on drug release profiles of (A)bare VCM and VCM_NMEO SLNs And (B) VCM and VCM_SA SLNs (n = 3).

The values of R^2 and RMSE were 0.9848 and 2.2925 for the VCM_SA SLNs respective, and 0.9918 and 2.1057 for the VCM_NMEO SLNs.

Table 3.3. Drug release kinetics data for VCM NMEO_SLNS.

Model	Equation	R^2		RMSE		Release exponent (n)	
pH		7.4	6.0	7.4	6.0	7.4	6.0
Zero order	$Q = k_* t + Q_0$	0.6286	0.8626	12.8061	7.8804	-	-
First order	$Q = Q_0 * e^{kt}$	0.9032	0.9608	6.5348	4.2060	-	-
Higuchi	$Q = k * t^{½}$	0.9584	0.9924	4.2870	1.8305	-	-
Korsmeyer-Peppas	$Q = k * t^n$	0.9604	0.9976	4.3857	1.1054	0.527	0.566
Hixson-Crowell	$Q^{⅓} = k * t + Q_0{}^{⅓}$	0.8344	0.9374	8.5487	5.3143	-	-
Weibull	$Q = 1 \exp [-(t)^{a/b}]$	0.9918	0.9974	2.1057	1.2530	-	-

R2 = linear regression coefficient, RMSE = Root mean square error.

The Weibull release model, as applied to drug release, can best be explained by considering its exponent parameter (β), as it was described by Papadopoulou et al. (Papadopoulou et al., 2006). A value of β between 0.39 and 0.69 suggest that the release mechanism is diffusion in fractal or disordered substrate, but the $0.69 < \beta < 0.75$ suggests that the drug release was by diffusion in normal Euclidian space. The value of exponent parameter (β) for VCM_NMEO SLNs was 0.629, which suggests that the mechanism of VCM release at pH 7.4 was by diffusion in fractal or disordered substrate.

At pH 6.0, Korsmeyer-Peppas was the best fit model, and the R^2 and RMSE values were 0.9922 and 1.8138 respectively for VCM_SA SLN, and 0.9976 and 1.1054 respectively for VCM_ NMEO SLN. The value of the release exponent (n) of the Korsmeyer -Peppas model at pH 6.0 was above 0.5, suggesting a non Fickian mechanism of release (Waddad et al., 2013).

Stability

To determine the physical stability of the product, the samples of NMEO SLNs were evaluated at 4 ºC and at RT for 90 days to understand how the stability related parameters were affected by time and storage conditions. The absence of visual signs of instability, and the lack of significant difference in values of particle size, PDI and ZP ($p > 0.05$), proved that the formulation was stable at 4 ºC throughout the study period (**Table 3.4**). Unlike those at 4 ºC, the samples kept at RT were not stable, as revealed by their significant particle growth ($p < 0.05$) and high PDIs.

Table 3.4. Effect of storage on physicochemical characteristics of vancomycin loaded NMEO SLNs. The values are expressed as mean ± SD, n = 3.

Storage condition	Particle size		PI		ZP	
Time (days)	4 ºC	RT	4 ºC	RT	4 ºC	RT
0	302.8 ± 0.12	302.8 ± 0.12	0.23 ± 0.03	0.23 ± 0.03	-6.27 ± 0.01	-6.27 ± 0.01
30	303.4 ± 5.2	585 ± 1.7	0.253 ± 0.07	0.395 ± 0.02	-6.4 ± 0.4	-6.0 ± 0.6
60	303.7 ± 1.03	588.9 ± 12.3	0.251 ± 0.01	0.422 ± 0.09	-6.50 ± 1.0	-6.2 ± 1.2
90	305.3 ± 3.8	596 ± 22.01	0.343 ± 0.04	0.455 ± 0.01	-6.30 ± 0.7	-6.21± 2.2

Molecular modelling

Molecular modelling was used to investigate and identify the stability of the VCM NMEO SLN system, and the interaction between VCM and NMEO, as this understanding will also explain the release behavior of VCM from the NMEO SLNs. The initial energy of VCM and NMEO were found to be 3894.83 and 274.09 kcal/mol respectively. Geometry optimization using the smart minimizer algorithm in forcite module resulted in producing more stable molecules with final energy of 289.54 kcal/mol for VCM and 41.59Kcal/mol for NMEO (**Figures 3.8 A** and **3.8 B**). The MD study revealed the formation of VCM-NMEO complex, with the potential energy, the mass of the complex, increasing from 546.585 kcal/mol to 854.939 kcal/mol by the end of MD study (**Figure 3.9 A**). This confirms the successful formation of the VCM NELO SLN system ((Florence and Attwood, 2015), while the rise in potential energy also explains the increase in particle size from 122.2 nm for blank to 302.8 nm when VCM was loaded into NMEO SLNs. The stability of the VCM_NMEO SLNs was also studied using MD simulation by monitoring the kinetic energy at room temperature and 4°C. The kinetic energy, which reflects the entropy of the molecules during the simulation, was reduced by the end of the MD study at both temperatures. At room temperature, the kinetic energy was reduced from 685.88 kcal/mol to 386.34 kcal/mol, while at 4°C it was reduced from 633.64 kcal/mol to 370.61 kcal/mol. The kinetic energy is known to be reduced at lower temperatures, resulting in reduced free energy and hence a more stable complex (Vyas et al. 2017). These results were in line with those obtained from the stability study, which showed that the VCM_NMEO SLNs were more stable when stored at 4 °C in terms of PS, PDI and ZP (Table 4). The presence of hydrogen bonds and hydrophobic interaction were visualized using Discovery studio, and contributed to the stability of the VCM_NMEO SLNs (**Figure 3.9 B**). The hydrogen bonds would have a major role in the stability of the VCM_NMEO

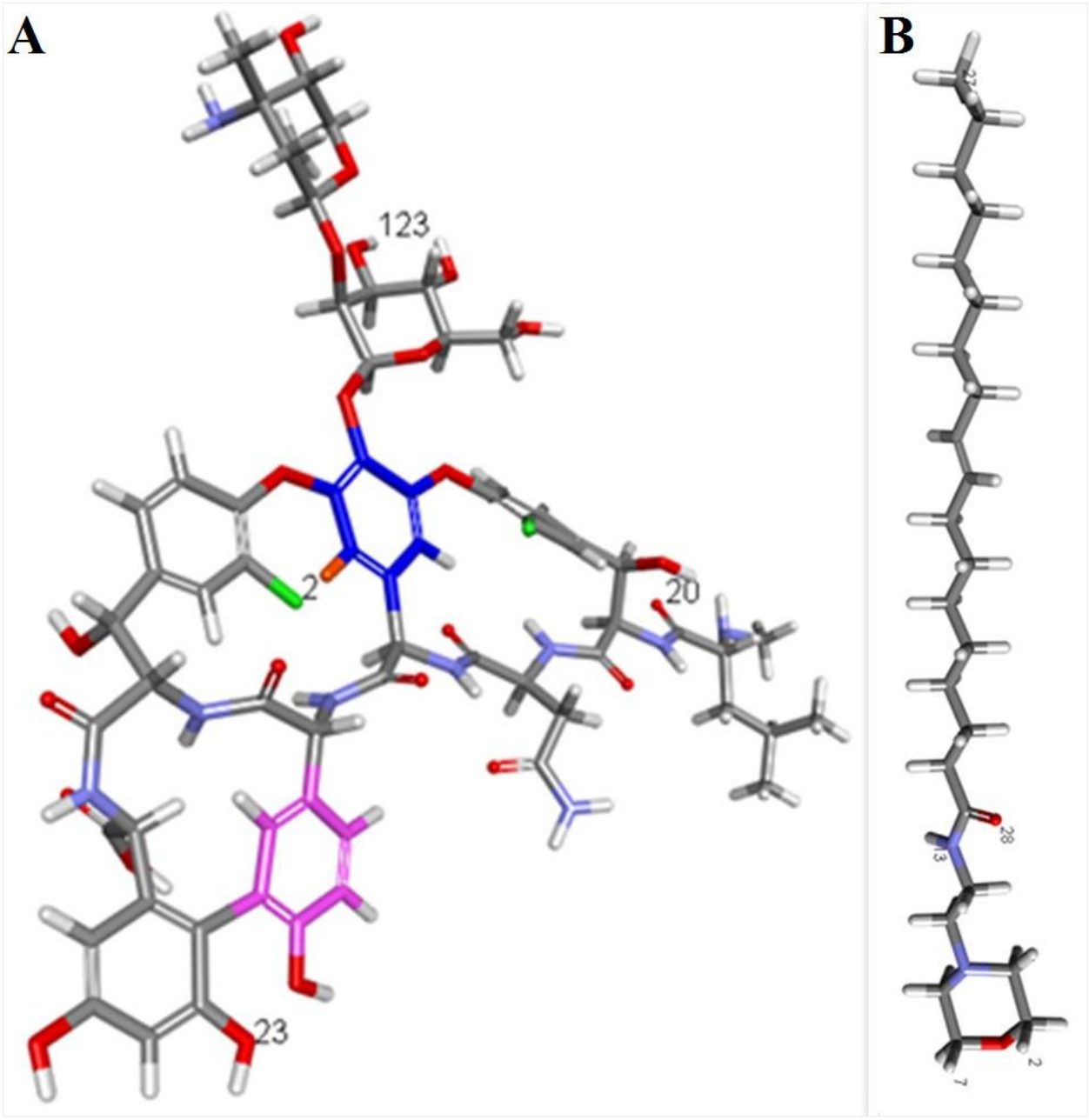

Figure 3.8. 3D structures of NMEO (A) and vancomycin (B) interaction sites are labelled with atom numbers.

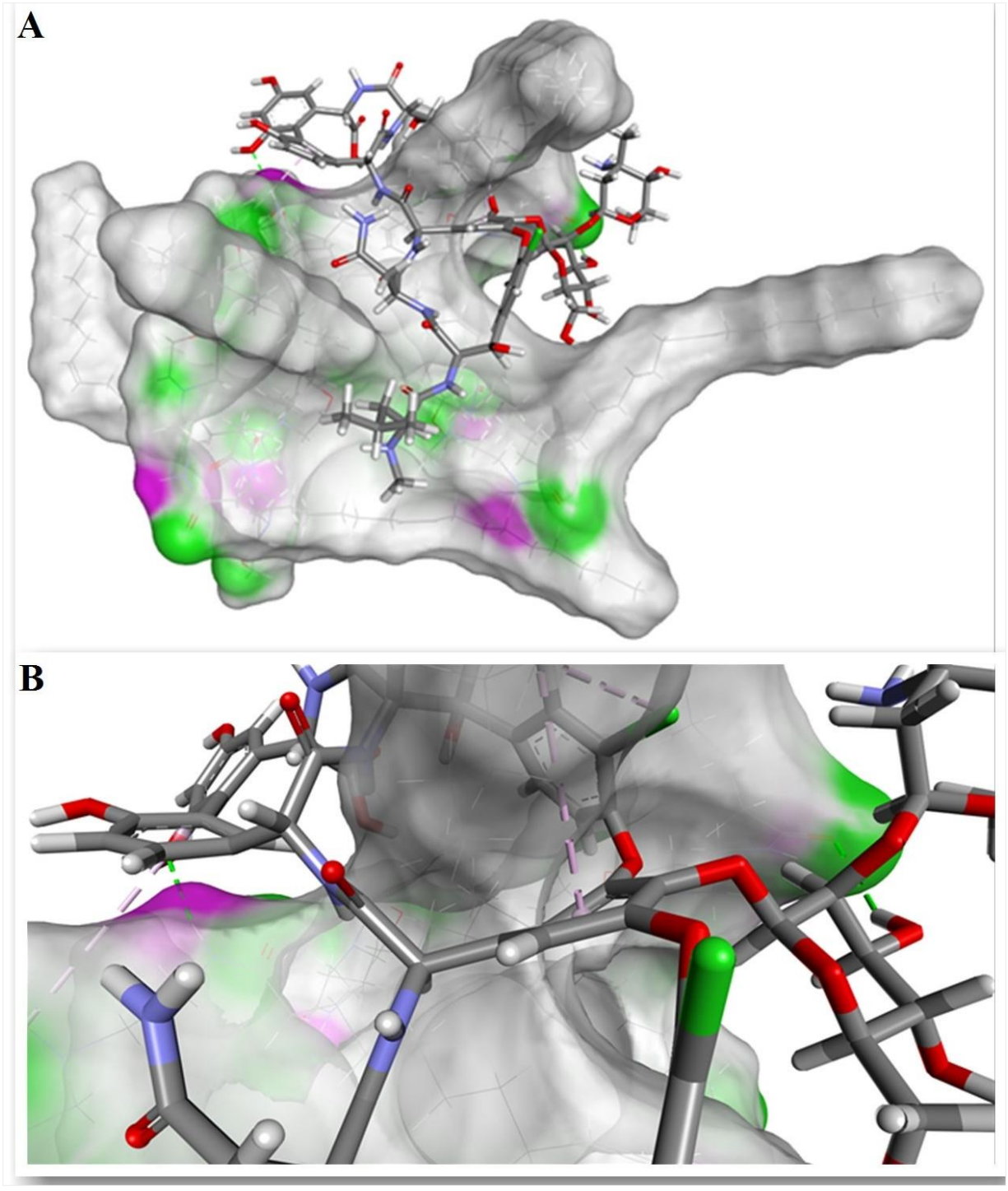

Figure 3.9: Illustration of vancomycin (stick model) (A) entrapment inside the lipid network (hydrogen bond surface model) by means of hydrogen bond (green lines) and hydrophobic interaction (purple line) (B).

SLNs, as these bonds are more stable at lower temperatures (Teo et al., 1997), which was confirmed by the SLNs being more stable at 4 °C over a three-month period (Table 4). The drug release profiles at pH 7.4 and pH 6 (Figures 6A and 6B) may also be explained by these MD studies. The slower release of VCM from the NMEO SLNs at pH 7.4 can be attributed to not only hindrance by the hydrophobic SLNs, but also to the presence of hydrogen bond interactions between VCM and NMEO. Conversely, at pH 6, due to protonation of the nitrogen atom of NMEO, hydrogen bonding may no longer occur, leading to the faster release profile displayed (Hao and Li, 2011).

In vitro antibacterial Activity

Table 3.5 summarizes the results of the in vitro antibacterial activity of bare VCM, drug free and drug loaded pH responsive NMEO SLNs and VCM SA _SLNs against MRSA. The MIC value for bare VCM was 7.8 µg/ml at physiological pH (7.4), and increased to 15.65 µg/ml at acidic pH (6.0). The observed 2-fold loss of activity of bare VCM at acidic pH is consistent with what has been reported by other researchers (Mercier et al., 2002; Radovic-Moreno et al., 2012), and might be due to decreased solubility of vancomycin (Faustino, 2008). Bare VCM had a lower MIC (7.8 µg/ml) than VCM NMEO_SLNs (15.65), an observation consistent with other nanoantibiotic studies of VCM (Kadry et al., 2004; Kalhapure et al., 2014). It should be noted that under in vivo conditions, higher concentrations of VCM would still reach the infection site in the SLN compared to a conventional VCM formulation for better activity. In addition, considering the toxicity of VCM, its encapsulation in NMEO SLNs could be useful in mitigating the drugs toxic effects, which includes nephrotoxicity (Dong et al., 2015). At pH 6, VCM NMEO SLNs had a MIC value (0.244 µg/ml) that was lower than that of bare VCM (15.65 µg/ml), which indicated that the

NMEO SLNs were capable of protecting VCM against loss of its efficacy, which occurs at low pH (Radovic-Moreno et al., 2013).

The MIC value of the VCM_NMEO SLNs decreased from 15.6 µg/ml at pH 7.4 to 0.244 µg/ml at pH 6.0, which is equivalent to an almost 64-fold increase in their activity at acidic compared to neutral pH. These results were comparable to those from a study previously reported by our group, whereby VCM-encapsulated pH responsive SLNs (VCM-FB_SA-3M_SLNs) also had an activity against MRSA that was better at acidic than at physiological pH (Kalhapure et al., 2017b). Interestingly, the previous study reported a 4-fold improvement of activity against MRSA at acidic pH, which is lower than the 64-fold increase in activity reported in this study.

Table 3.5. In vitro antibacterial activity of the formulations against MRSA at pH7.4 and 6.0. The values are expressed as mean ±SD, n=3.

Formulation	(MIC µg/ml)	
pH	7.4	6.0
Bare VCM	7.8	15.65
NMEO SLNs (drug free)	*NA*	*NA*
VCM_NMEO SLNs	15.65	0.244
VCM_SA_SLNs	31.5	31.5

NA = No activity.

In our previous study, the VCM-FB_SA-3M_SLNs were prepared by an acid-cleavable lipid that was meant to facilitate higher drug release only by cleavage of an acid labile link (acetal bond) at the acidic sites. VCM_NMEO SLNs, on the other hand, were made from a lipid that protonates and acquires positive charge at acidic medium. Superiority of the VCM_NMEO SLNs over the

VCM-FB_SA-3M_SLNs can be because the latter influences only VCM release, while the former influences both release of drug from formulation and its interaction with bacteria. Therefore, apart from facilitating the release of the drug by making the system hydrophilic, the positive charge also helps to adhere VCM_NMEO SLNs to bacterial cells, thereby enhancing their activity (Forier et al., 2014).

Conversely, the MIC value of VCM_SA_SLNs against MRSA was 31.5 µg/ml at pH 7.4, and remained the same at pH 6. This indicated their lack of pH dependent antibacterial activity and confirmed that enhanced antibacterial activity of the formulation (VCM_NMEO SLNs) might be due to the pH responsive NMEO lipid.

In vivo antibacterial activity

In *vivo* studies in mice using a skin infection model was thereafter performed to confirm the antibacterial activity of VCM_NMEO SLNS, whereby MRSA were delivered intradermally to localize temporarily at the dermal layer, without gaining a deeper entry into the systemic circulation. For each treatment group, the number of colony-forming units (CFUs) were determined and presented as $\log_{10,}$ as shown in **Figure 3.10**. When compared to untreated skin samples, both the VCM_NMEO SLNS and bare VCM significantly reduced the MRSA load in the skin samples treated by them. The mean MRSA load ($\log_{10}$ CFU) recovered from untreated skin samples was 6.58 ± 0.01, while the values for the VCM_NMEO SLNS and bare VCM treated samples were 4.36 ± 0.10 and 4.97 ± 0.12 respectively. The $\log_{10}$ CFU values for the formulation was statistically lower than both the untreated and VCM only treated ($p<0.05$). In terms of CFU/ml, the results showed that the bacterial load in mice treated with the VCM_NMEO SLNS was 162.4 times lower than the one in the untreated mice ($p <0.05$). Bacterial load reduction by VCM_NMEO SLNS was also significantly greater (4.14 times) than that of bare VCM ($p <0.05$).

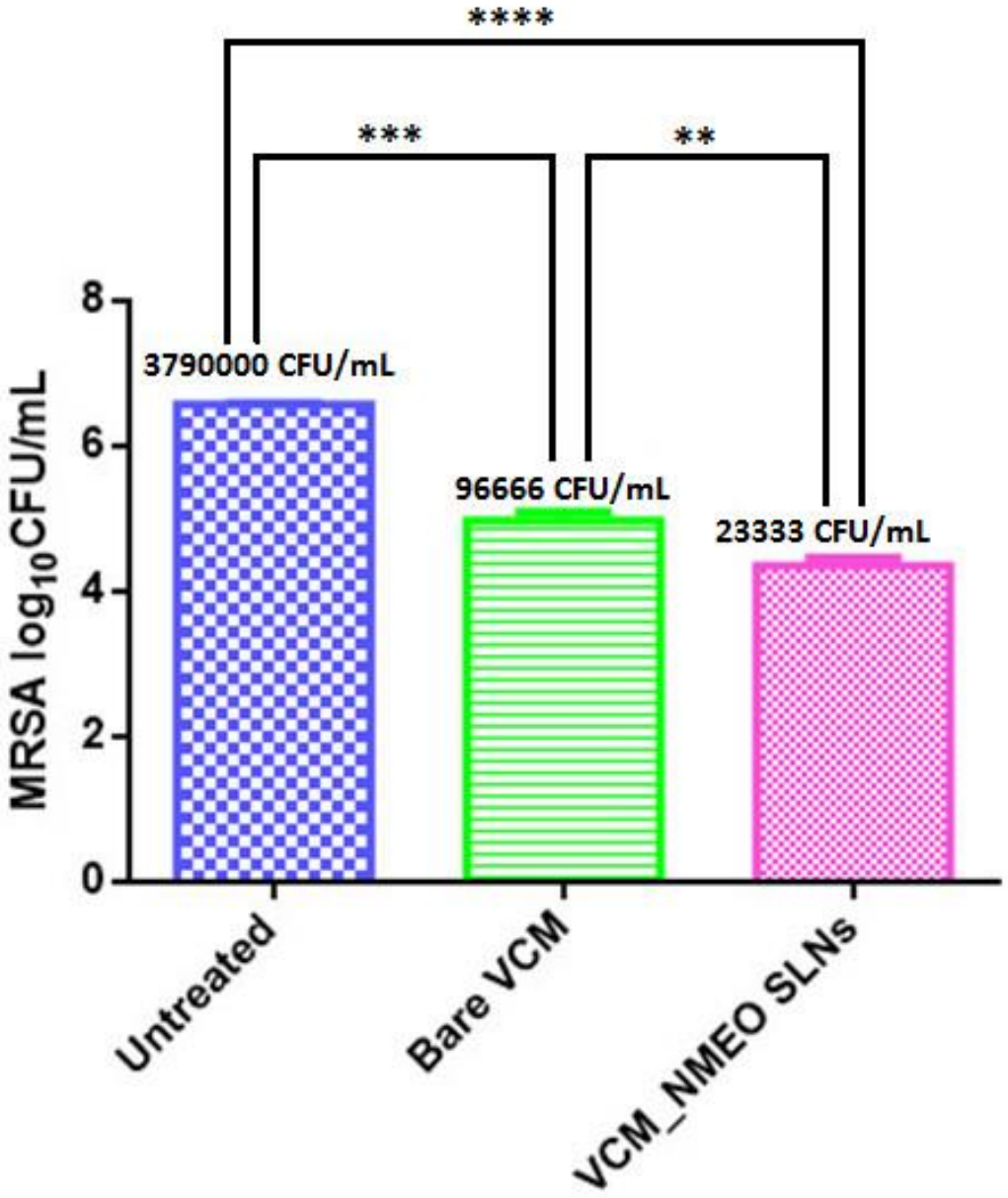

Figure 3.10. MRSA burden after 48 h treatment period. Data is presented as mean ± SD (n=3).

** denotes significant difference between VCM NMEO_SLNs and bare VCM, *** denotes significant difference when bare VCM is compared to untreated samples and **** denotes significant difference between VCM NMEO_SLNs and untreated samples.

This clearly suggests that encapsulation of VCM in the NMEO SLNs improved its antibacterial activity.

Vancomycin exerts its antibacterial activity by inhibiting bacterial cell wall synthesis, which it does by diffusing through the cell wall and reaching its target site (terminal D-Ala-d-Ala residues) located on the cell membrane (Meng et al., 2017; Pereira et al., 2007). The thickened cell wall displayed by the MRSA helps the bacteria to resist vancomycin action by trapping the drug and reducing the number of drug molecules that reach the cytoplasmic membrane (Van Bambeke et al., 2007). In our study, the enhanced activity of VCM in NMEO SLNs was probably be due to several factors, such as: i) a faster drug release (Subedi et al., 2009) and improved binding to bacterial cells due to the ability of the NMEO SLNs to acquire positive charge at pathological acidic conditions; ii) improved permeation of VCM through the MRSA cell wall to its target site due to the ability of oleamide portion of the NMEO to act as a permeation enhancer (Lane, 2013), and iii) synergism between VCM and its oleic acid based carrier, which is thought to have inherent anti-MRSA activity also due to its membrane disruption ability (Engelbrecht et al., 2011; Huang et al., 2011). By displaying the possibility of having multiple mechanisms of actions against MRSA, the VCM_NMEO SLNs developed in this study have the potential to be effective in combating the growing threat of bacterial resistance against vancomycin. Data regarding in vivo antibacterial studies of antibiotic loaded pH responsive SLNs is limited, and this study could therefore provide a foundation for future studies related to delivering antibiotics using surface switching pH responsive SLNs.

The histomorphological evaluations were performed on excised skin from the untreated, bare VCM and VCM_NMEO SLN group to assess the morphological changes and skin integrity after MRSA infection. The H&E stained slides revealed that the untreated skin samples displayed

evidence of tissue inflammation and abscess formation (**Figure 3.11 A**). The bare VCM group also showed evidence of swelling and abscess formation, however to a lesser extent than the untreated group (**Figure 3.11 B**). The VCM_NMEO SLN group displayed minimal signs of inflammation and no evidence of abscess formation (**Figure 3.11 C**). Both the untreated and bare VCM group presented with large quantities of white blood cells at the infection site, however this was evidently lower in the VCM_NMEO SLN group (**Figure 3.11C**). The findings of the histomorphological analysis correlate with the CFUs calculated in the in vivo antibacterial study

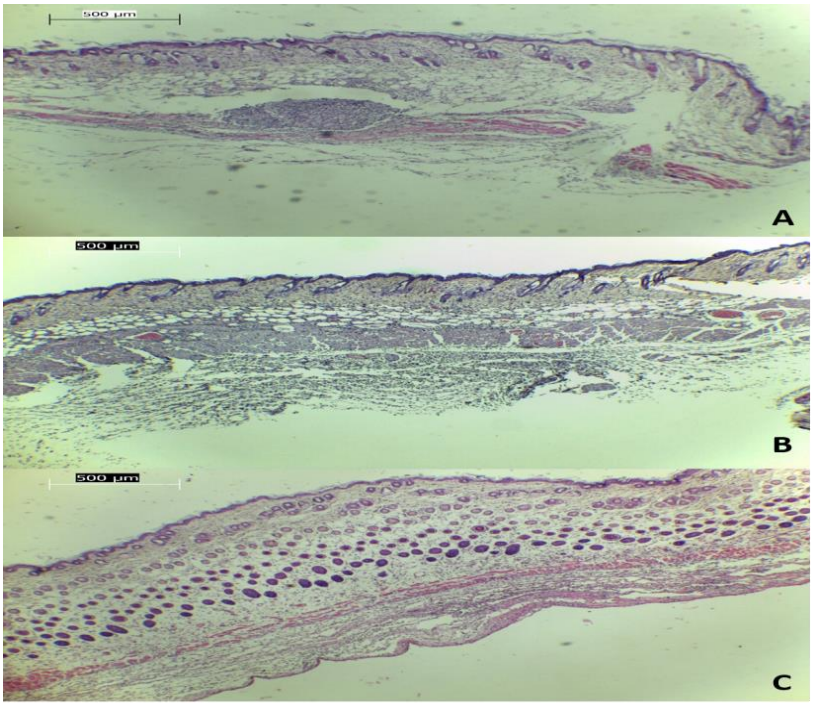

Figure 3.11. Photomicrographs of the control and the treated skin samples H&E stained (4X): (A) untreated (B) bare VCM treated and (C) VCM_NMEO SLN treated.

as the skin sample with the most number of recovered bacteria also presents with the highest degree of inflammation, abscess formation and presence of white blood cells. This is due to the greater immune response to the larger number of bacteria present at the infection site of the untreated

group. The VCM_NMEO SLN group which displayed the lowest number of isolated bacteria presented with minimal signs of inflammation and no abscess formation, this could be due to a reduced immune response to a statistically lower number of isolated bacteria at the infection site. These findings further exhibit the antimicrobial superiority of the VCM_NMEO SLNs.

Conclusion

Nanobased antibiotic carriers are increasingly being recognized as a potential approach to improve the activity of existing drugs in an era when bacterial resistance is rising while the discovery of new antibiotic drugs is declining. In this project, an oleic acid based pH-responsive-lipid, NMEO, was synthesized, characterized and successfully formulated into pH responsive SLNs. The *in vitro* release showed that the release of vancomycin from the NMEO SLNs was faster at pH 6 than at pH 7.4, with the MIC value of the formulation against MRSA decreasing by 64-fold at acidic pH. The potential of the NMEO SLNs was also confirmed by their *in vivo* antibacterial activity. Mean bacterial load of samples treated by NMEO SLNs were 4-fold lower than those treated by bare VCM, and 162-fold lower than untreated samples. Thus, the developed pH responsive NMEO SLNs could be an effective delivery system to improve the performance of vancomycin and other antibiotics against resistant bacteria infections.

Conflict of interest

Authors declare that they have no conflict of interest.

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CHAPTER 4. GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1 General Conclusions

Limitations inherent to traditional dosage forms of antibiotics have given rise to challenging resistant strains of bacteria like MRSA which pose a danger of making even the most effective antibiotics like vancomycin useless. Recently pH-responsive nanoformulations have shown significant potential in their ability to address antibiotic resistance problems by maximizing targeted delivery of an antibiotic to acidic conditions at infection sites created by some bacteria such as MRSA. The identification of novel lipids for fabrication of lipid-based pH responsive antibiotic carriers like solid lipid nanoparticles (SLNs) will improve antibiotic therapy because SLNs are less toxic, stable and can be produced at industrial scale. Despite their potential, studies on pH responsive SLNs for antibiotic delivery are still limited, a clear indication of the need to focus in this area of research. The aim of this study was therefore to synthesize, formulate and evaluate novel vancomycin loaded N-(2-morpholinoethyl) oleamide (NMEO) SLNs activity against MRSA.

The objectives of this study were to:

(i) Synthesize a novel lipid, NMEO, using steglich esterification approach.

(ii) Characterize the synthesized NMEO using structural elucidation techniques such as FT-IR, 1H NMR and C13 NMR.

(iii) Determine the toxicity of the synthesized NMEO to confirm its safety for use in biological systems.

(iv) Formulate vancomycin loaded pH responsive SLNs using the solid lipid NMEO.

(v) Assess the fabricated SLNs in terms of particle size, PDI, zeta potential, morphology, entrapment efficiency, thermal properties, *in-vitro* drug release, *in vitro* and in vivo antibacterial activity.

(vi) Undertake *in silico* studies to understand molecular interactions between VCM and NMEO

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

- A novel lipid NMEO was successfully synthesized by conjugating oleic acid with 4-(2-Aminoethyl) morpholine (4-AEM).
- FT-IR, ^{1}H NMR and ^{13}C NMR analyses, confirmed the successful synthesis and structure of the lipid NMEO.
- Cytotoxicity studies carried out using an MTT assay on mammalian cell lines A549, HEK-293 and HEP G2 cells showed that NMEO is a biosafe lipid.
- It was observed that NMEO could form SLNs capable of responding to changes in pH by changing their surface charge and size. The NMEO SLNs' zeta potential switched from -6.39 at pH 7.4 to + 6.05 at pH 6.0 and their size decreased from 302.8 nm at pH 7.4 to 260.7 nm at pH 6.0. The formulated pH responsive NMEO SLNs had high entrapment efficiency of vancomycin (81.18 %) and a narrow size distribution (PDI <0.3).
- Differential scanning calorimetry studies confirmed the entrapment of vancomycin in the NMEO SLNs. TEM images showed that the formulated vancomycin- loaded NMEO SLNs were discrete and spherical in shape.
- *In vitro* drug release studies revealed that vancomycin was released from SLNs in a pH-dependent fashion and the release was higher at acidic pH. The value of release exponent

(n) of Korsmeyer-Peppas model at pH 6.0 was above 0.5 suggesting a non Fickian mechanism of release.

- The *In vitro* antibacterial activity against MRSA further confirmed the pH-responsive nature of the SLNs as activity against this bacterial strain was higher at acidic (pH 6.0) than at physiological conditions (pH 7.4). NMEO SLNs also proved superior to over bare vancomycin since encapsulation of the drug into NMEO decreased its MIC value by 64-fold against MRSA at pH 6. This confirmed that encapsulation of VCM into NMEO SLNs helped to protect the drug against acid-induced loss of activity which was evident when VCM was used alone.
- *In vivo* antibacterial activity using infected mouse skin model showed that as compared to bare vancomycin, the pH- responsive SLNs could decrease the microbial load at the infection site more effectively. The reduction of MRSA load was 4.14 times greater in the skin of VCM_NMEO SLNs treated mice than that of bare VCM treated mice. These findings were supported by Histomorphological studies which showed that mice skin treated with VCM_NMEO SLNs had less signs of inflammation compared to bare vancomycin treated and untreated skin.
- *In silico* studies revealed that both hydrophobic and hydrogen bond interactions were responsible for formation of a stable VCM- NMEO complex. The presence of hydrogen bond interactions between VCM and NNEO slowed release at pH 7.4 while their absence at pH 6.0 due to protonation of nitrogen atoms of NMEO contributed to the observed higher drug release at acidic pH.

The findings of this study, therefore, confirmed: (i) the practicability of using a novel lipid NMEO in formulating pH responsive SLNs and (ii) the potential of vancomycin-loaded pH responsive NMEO SLNs to enhance activity of vancomycin against infections caused by MRSA.

4.2 Significance of the findings in the study

Synthesis of a new pH responsive lipid and its application in antimicrobial nano-delivery systems presents a novel and promising approach to combating antimicrobial resistance which is threatening to nullify even the most effective antibiotics currently in clinical practice. The nanosystem formulated in this study is anticipated to have the following benefits:

New Pharmaceutical Products

The pH responsive vancomycin loaded NMEO SLNs successfully developed in this study is a new product with potential to improve treatment of infections. It can stimulate the pharmaceutical industry into manufacturing medicines that are superior and cost effective.

Improved patient therapy and disease treatment

The formulated NMEO SLNS had high entrapment efficiency and its drug release and antibacterial activity against MRSA were higher at acidic than physiological pH. Therefore, it has the potential for improving treatment of bacterial infections by delivering effective doses specifically to acidic conditions of infection sites while minimizing drug release at other sites. The site-specific delivery and high entrapment efficiency shown by NMEO SLNs will help to reduce the dose amount and frequency which may translate into decreased side effects, improved patience compliance and lowered treatment costs. Therefore, this product has potential to improve quality of lives and save lives of patients with diseases associated with infections.

Creation of new scientific knowledge

The study identified formulation and process variables that affect the quality of NMEO SLNs. It also provided knowledge on how VCM interacts with NMEO and an understanding on the formation of SLNs from the novel lipid NMEO.

Stimulation of new research

Other research can be performed based on data obtained in this study. NMEO SLNs can be used for devising nanocarriers for delivery of drugs for other diseases conditions characterized by acidification of pathological tissues/ cells like cancer, ischaemic heart diseases and inflammatory diseases. Based on this study it is possible to design other lipids that are pH responsive based on their ability to switch surface charge from negative at physiological pH to positive at acidic pH. Mechanistic analysis of SLNs can also be performed using other models.

4.3 Recommendations

This study has formed the foundation for formulating antibiotic loaded surface charge switching SLNs into a suitable nano-drug delivery system. Further studies to optimize the formulation may include the following:

- NMEO was synthesized by conjugating 4-AEM with oleic acid. In the next phase of this study 4-AEM should be conjugated to other unsaturated fatty acids like linoleic acid or saturated fatty acids stearic acid and analyzed to understand how factors like lipid type can possibly be manipulated to further improve the antibacterial activity, drug encapsulation and drug release.

- Additional studies should be conducted to understand the effect of the formulated system on morphological changes that occur on the bacterial cell wall when treated with vancomycin loaded NMEO SLNs.
- More extensive *in vitro* and *in vivo* antibacterial studies should be performed to evaluate the spectrum of activity of vancomycin loaded NMEO SLNs against other gram positive and gram-negative bacteria.
- *In silico* modelling simulations to explain mechanistically how vancomycin loaded NMEO SLNs interact with the bacteria should be performed on these systems.
- Further *In vivo* studies could be performed in both animals and human to obtain more information in respect of the bioavailability and the pharmacokinetic properties that will guide improvement of the formulation.
- A large-scale production method could be established to make the formulation feasible in the pharmaceutical industry. While large scale production has been long been established for fatty emulsions, a protocol for nanoparticles needs to be established

4.4 Conclusion

The findings of this study confirm the potential of the newly developed nanofomulation in managing infections due to resistant bacteria. The contribution made by this study is significant in addressing the limitations related to the current antibiotic therapy using nano-carrier based approaches. However, to fully realize the potential of nanotechnology to solve the current wide spread antibiotic therapy crisis, more studies are needed which must also be multidisciplinary. Research involving scientists from different disciplines will help shed more light by addressing the problem from different angles thereby coming forth with more effective solutions that will prevent the world from entering a post-antibiotic era.

Appendix A: Confirmation email for submitted manuscript.

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Corresponding Author: Rahul Kalhapure
Co-Authors: Danford Mhule, M. Pharm; Mahantesh Jadhav, Ph.D; Sanjeev Rambharose, Ph.D; Calvin A Omolo, M. Pharm; Chunderika Mocktar, Ph.D; Sanil Singh, Ph.D; Ayman Y Waddad, Ph.D; Valence Ndesendo, Ph.D; Thirumala Govender, Ph.D.
Title: Synthesis of an oleic acid based pH-responsive lipid and its application in nanodelivery of vancomycin

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International Journal of Pharmaceutics

Appendix B: Poster presentation for PSSA Conference.

SYNTHESIS OF AN OLEIC ACID BASED pH RESPONSIVE LIPID AND ITS APPLICATION IN NANODELIVERY OF VANCOMYCIN

Danford Mhule[1], Rahul S Kalhapure[1], Mahantesh Jadhav[1], Sanjeev Rambharose[1], Calvin .A. Omolo[1], Chunderika Mocktar[1], Sanil Singh[2], Valence. M. K. Ndesendo[3], Thirumala Govender[1]

[1]Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa.
[2]Biomedical Research Unit, University of KwaZulu-Natal, Westville Campus, Durban, 4000, South Africa.
[3]School of Pharmacy and Pharmaceutical Sciences, St John's University of Tanzania, P. O. Box 47,Dodoma,Tanzania.

INTRODUCTION AND AIMS

- Antibiotic resistance, a problem associated with traditional dosage forms of antibiotics, is a health challenge that has made once useful antibiotics useless.[1]
- It has been shown recently that pH responsive nanoformulations are able to address antibiotic resistance problems by maximizing targeted delivery of antibiotics to acidic conditions at infection sites created by some bacteria like *Staphylococcus aureus*.[2]
- The identification of novel pH responsive lipids will improve the development of solid lipid nanoparticles (SLNs) for antibiotic delivery.[3]
- The purpose of this study was to synthesize a novel lipid N-(2-morpholinoethyl)oleamide (NMEO) and employ it to formulate vancomycin pH responsive SLNs (VM_NMEO_SLNs) for managing *Staphylococcus aureus* (SA) infections.

METHODS

Synthesis and characterization of the novel lipid NMEO

NMEO was synthesized based on scheme 1 and characterized using FTIR, ^{1}H, and ^{13}C NMR.

Scheme 1: synthesis of NMEO

Preparation of SLNs

- VFB_NMEO SLNs were prepared by hot homogenization technique using Ultra Turrax T-25 homogenizer (IKA Labortechnik,Germany) and ulrasonification method using a probe sonicator (Kennesaw, GA 30144, USA). Blank (drug free) SLNs and non pH responsive stearic acid SLNs (for comparison in antibacterial studies) were prepared using the same procedure.

Determination of particle size (PS) ,polydispersive index (PDI) and zeta potential (ZP)of VM_NMEO_SLNs

- Prepared SLNs were characterized for particle size, polydispersity index (PDI) and zeta potential (ZP) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK).

***In vitro* drug release**

- Drug release was performed in PBS of pH 7.4 and pH 6.0 at 37 ºC.

***In vitro* antibacterial activity**

- The minimum inhibitory concentration (MIC) values for SLNs were determined against SA and Methicilin resistant SA (MRSA) by broth dilution method.

***In vivo* antibacterial activity**

- In vivo antibacterial activity was performed using a mouse skin infection model with BALB/c mice (Ethical Clearence Approval Number: AREC/ 104 / 015PD)

RESULTS AND DISCUSSION

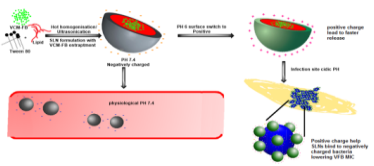

Fig. 1. Mechanism of formation and action of VM_NMEO_SLNs

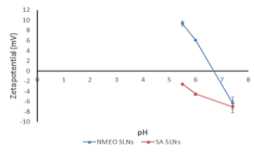

Fig. 2. pH responsiveness of NMEO SLNs at pH 7.4, 6.5 and 5.5.

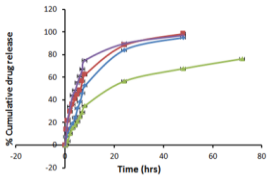

Fig. 3. In vitro release profile of VM_NMEO_SLNs

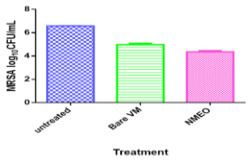

Fig. 4. MRSA burden in mice after 48h of treatment (n=3)

- PS, PDI and ZP of VM_NMEO_SLNs were 302.8 ± 0.12nm, 0.23 ± 0.03 and -6.27 ± 1.2mV respectively at pH 7.4 and 260.7 ± 9.5nm, 0.261 ± 0.018 and 6.05 ± 0.013 mV respectively at pH 6.0.
- Entrapment efficiency of the formulation was 81.18 %.

Table 1A. *In vitro* antibacterial activity of NMEO SLNs at pH 7.4 (n = 3).

Time (Hrs)	24	48	72	24	48	72
	S aureus (MIC ug/mL)			MRSA (MIC ug/mL)		
Bare VM	3.9	250	NA	7.8	500	NA
NMEO SLNs	0.988	0.988	62.5	15.6	0.488	0.488
Stearic acid SLNs	3.9	62.5	62.5	31.5	NA	NA
Blank	NA	NA	NA	NA	NA	NA

Table 1B. *In vitro* antibacterial activity of NMEO SLNs at pH 6.0 (n=3)

Time (Hrs)	24	48	72	24	48	72
	S aureus (MIC ug/mL)			MRSA (MIC ug/mL)		
Bare VM	7.8	250	NA	15.65	500	NA
NMEO SLNs	0.244	0.488	62.5	0.244	0.98	3.9
Stearic acid SLNs	1.95	62.5	NA	31.5	250	NA
Blank	NA	NA	NA	NA	NA	NA

NA= no activity

- A novel lipid NMEO was successfully synthesized, characterized and used to prepare vancomycin loaded SLNs with acceptable pH responsiveness, size and PDI.
- As shown in Fig. 3, the *in vitro* release studies revealed that drug release from SLNs at pH 6.0 was significantly higher than at pH 7.4 ($p = 0.0068$). This can be due to the swelling of SLNs at acidic pH caused by protonation of nitrogen atoms in NMEO.
- Also, the *in vitro* antibacterial activity of SLNs against methicillin sensitive SA (MSSA) and MRSA (Table 1A & 1B) was higher at acidic than physiological pH. This might be due to electrostatic attraction between positively charged VM_NMEO_SLNs and negatively charged bacterial cells.
- Reduction of MRSA load in the skin of VM_NMEO_SLNs treated mice was 4.14 times greater than that of bare VM treated mice ($p = 0.0317$)

CONCLUSION

- This study confirmed that NMEO can successfully be used to formulate pH responsive SLNs which could deliver antibiotics to acidic infection sites for enhanced efficacy.

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