Novel fatty acid based pH-responsive nanostructured lipid carriers for enhancing vancomycin activity

by

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

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“Education is the most powerful weapon which you can use to change the world.”

- Nelson Mandela-

"***This dissertation is dedicated to my grandmother, parents, teachers, and friends who have always taught me how to see the vitality in myself, conquer fears and overcome challenges.***

I hope that I have made you proud"

Declaration 1 – Plagiarism

I, Miss Nawras Osman declare that

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

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Declaration 2 – Publications

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

Nawras Osman, Calvin A. Omolo, Ramesh Gannimani, Ayman Y. Waddad, Sanjeev Rambharose, Chunderika Mocktar, Sanil Singh, Raveen Parboosing, Thirumala Govender. Novel fatty acid based pH-responsive nanostructured lipid carriers for enhancing vancomycin activity. SUBMITTED MANUSCRIPT. Reference Number: EJPB_2017_1184

Miss Nawras Osman contributed to the design of the project, synthesis, and characterization of the novel lipids, formulation, and characterization of the nanostructured lipid carriers. In addition, Miss Nawras Osman was responsible for analysis and interpretation of all data, wrote the first draft of the paper and undertook all revisions. Mr. Calvin A. Omolo assisted with lipid synthesis and performed cell viability studies and *in vivo* antibacterial studies. Dr. Sanjeev Rambharose performed the cytotoxicity studies. Dr. Ramesh Gannimani supervised the lipids synthesis and characterization methods. Dr. Ayman Y. Waddad performed the molecular modeling studies. Dr. Chunderika Mocktar supervised the *in vitro* and *in vivo* antibacterial activity studies. Dr. Sanil Singh supervised the *in vivo* antibacterial activity studies. Dr. Raveen Parboosing was a collaborator on the project. Prof. Thirumala Govender served as supervisor and was responsible for project conceptualization, problem-solving, paper and abstract editing and general supervision of the study.

Research output from the dissertation

Submitted Manuscript

The following research paper was submitted to the European Journal of Pharmaceutics and Biopharmaceutics which has an impact factor of 4.389 from work done during this study.

Nawras Osman, Calvin A. Omolo, Ramesh Gannimani, Ayman Y. Waddad, Sanjeev Rambharose, Chunderika Mocktar, Sanil Singh, Raveen Parboosing, Thirumala Govender. Novel fatty acid based pH-responsive nanostructured lipid carriers for enhancing vancomycin activity. SUBMITTED MANUSCRIPT. Reference Number: EJPB_2017_1184

* The submitted manuscript can be found in Chapter three.

Conference Presentations

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

- N Osman, Omolo C, R Gannamani, A Waddad, C Mocktar, S Singh and T Govender. Synthesis of fatty acid-based novel lipids for formulating pH-responsive NLCs for targeted delivery of vancomycin against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. 38th Annual Conference of the Academy of Pharmaceutical Sciences,06-08 July 2017, Johannesburg, South Africa.

- Osman N, Omolo C A, Gannimani R, Ayman Y Waddad, Rambharose S, Mocktar C, Singh S, Parboosing R, Govender T. Novel fatty acid based pH-responsive nanostructured lipid carriers for enhancing vancomycin activity. College of Health Sciences Annual Research Symposium, University of KwaZulu Natal, 05-06 October 2017, Durban, South Africa.

- N Osman, Omolo C, R Gannamani, A Waddad, C Mocktar, S Singh and T Govender. Synthesis of fatty acid-based novel lipids for formulating pH-responsive NLCs for targeted delivery of vancomycin against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. University of KwaZulu-Natal Nanotechnology Platform Workshop, 22nd November 2017.

*The poster can be found in Appendix A and B.

Abstract

Targeted delivery of antibiotics via pH-responsive nano-formulations can combat the limitations of conventional dosage forms and reduce resistance development. Identification of new lipids for the preparation of nanocarriers is required for the development of pH-responsive systems. The aims of this study were to synthesize and characterize novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)] and to explore their potential for the preparation of vancomycin pH-responsive nanostructured lipid carriers (VCM-SAOA-NLCs). Biosafe lipids were synthesized and characterized using FTIR, ^{1}H NMR, ^{13}C NMR, and cytotoxicity. VCM-SAOA-NLCs were prepared using hot homogenization followed by ultrasonication technique and characterized in terms of size, polydispersity index (PDI), zeta potential (ZP), surface morphology, encapsulation efficiency (EE), *in vitro* drug release, *in silico* studies, *in vitro* and *in vivo* antibacterial activity in mice as well as bacterial cell viability studies.

Biosafe lipids were successfully synthesized and characterized. Spherically shaped VCM-SAOA-NLCs were prepared. VCM-SAOA-NLCs formulation size, PDI, and ZP were 225.9 ± 9.1 nm, 0.258 ± 0.02 and -6.69 ± 1.1 mV respectively. EE was found to be 88.7 ± 13.12 %. *In silico* studies confirmed that the higher EE of VCM at a solid lipid to liquid lipid ratio of 2:1 (1603.991 kcal/mol) than 1:1 (1367.44 kcal/mol) was due to the higher affinity of VCM with the solid lipid than the liquid lipid. *In vitro* drug release studies revealed that VCM-SAOA-NLCs had pH-sensitive sustained drug release. *In vitro* antibacterial activity against methicillin-susceptible and resistant *Staphylococcus aureus* revealed that VCM-SAOA-NLCs had superior antibacterial activity compared to bare VCM against both bacterial strains at both pH conditions. Moreover, at pH 6 VCM-SAOA-NLCs activity was four times and two times better against SA and MRSA respectively than at pH 7.4. MRSA bacterial cell viability study showed enhanced uptake and antibacterial activity of VCM-SAOA-NLCs compared to bare VCM. Furthermore, the *in vivo* study revealed that MRSA CFU load in mice skin treated with VCM-SAOA-NLCs was 37-fold lower than bare VCM ($P < 0.05$).

Therefore, this novel pH-responsive NLCs may serve as a promising nanocarrier for enhancing antibiotic delivery and activity.

Keywords: nanostructured lipid carriers; pH-responsive; vancomycin; methicillin-resistant *Staphylococcus aureus*; antibacterial activity.

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List of acronyms

ANOVA	One-way analysis of variance	NDDS	Nano drug delivery systems
CFU	Colony forming units	NLCs	Nanostructured lipid carriers
DCC	Dicyclohexyl carbodiimide	NMR	Nuclear magnetic resonance
DL	Drug loading	OA	Oleic acid
DLS	Dynamic light scattering	OALL	Oleic acid derived liquid lipid
DMAP	4-Dimethylaminopyridine	PBS	Phosphate-buffered saline
DSC	Differential scanning calorimetry	PI	Propidium iodide
EE	Encapsulation efficiency	RMSE	Root mean square error
FSC	Forward scatter	SA	Staphylococcus aureus
FT-IR	Fourier-transform infrared spectroscopy	SASL	Stearic acid derived solid lipid
HIV	Human immunodeficiency virus	SEM	Scanning electron microscopy
HPH	High-pressure homogenization	SSC	Side scatter
HPLC	High-performance liquid chromatography	TBA	Tertiary butyl acrylate
HRMS	High-resolution mass spectrometry	TEM	Transmission electron microscopy
ID	Infectious diseases	TFA	Trifluoroacetic acid
MD	Molecular dynamics	TIPS	Triisopropylsilane
MDR	Multidrug resistance	TLC	Thin-layer chromatography
MHA	Mueller Hinton Agar	UA	Uranyl acetate
MHB	Mueller Hinton Broth	VCM	Vancomycin
MIC	Minimum inhibitory concentration	ZP	Zeta potential
MRSA	Methicillin-resistant Staphylococcus aureus		
MS	Materials Studio		

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CHAPTER 1. INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Introduction

This chapter outlines a brief background of the study including the status of infectious diseases, limitations of current antibiotic therapy and the growing threat of bacterial resistance. It further discusses the role of nanotechnology strategy based pH-responsive systems for combating the challenges encountered with antibiotic resistance. It further provides the aims, objectives, novelty, and significance of the study, and concludes with the structure and content of the thesis.

1.2 Background

Infectious diseases (ID) are considered one of the top ten leading causes of death globally and remain a major threat despite significant advances in research [1]. Emerging and re-emerging ID are associated with high mortality and morbidity rates and treatment costs, which affects the economic stability of societies [2]. The advent of antimicrobials in1940s revolutionized the treatment of ID and saved millions of lives. However, the current antibiotics dosage forms require optimal antimicrobial therapy administration of frequent high doses for a prolonged period to maintain sufficient concentration at infection sites [3], and to prevent therapy failure and infection recurrences [4, 5]. Exposure to high concentration of antibiotic is therefore associated with higher toxic and adverse effects, prolonged hospital residency, increased health care cost and poor patient compliance [6, 7].

These limitations, coupled with the overuse and misuse of antibiotics, have contributed to bacterial resistance development [8, 9]. The bacterial resistance crisis continues to spread, for multiple classes of antibiotics [10], and multidrug resistance (MDR) organisms are on the rise [11]. Concern has been heightened by methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria emergence, which causes significant diseases, such as skin abscess, cellulitis, endocarditis and septic arthritis, and invasive bacteremia. The slow discovery and development of new antibiotics, the high costs of production and long approval times by regulatory bodies highlights the urgency to adopt new strategies to overcome the alarming increase of bacterial resistance [12] and limitations with conventional antibiotics.

Novel drug delivery systems, such as nanocarriers, have been investigated as a promising approach to address the limitations of antibiotic treatment and bacterial resistance [13-15]. Nano drug delivery systems (NDDS) are biocompatible small particles with large surface areas [16, 17] that hold several advantages including improving antibiotic solubility and biodistribution profiles [18-20], thus enhancing antibiotic bioavailability at the target sites [21, 22]. In addition, nanocarriers can enhance pharmaceutical stability [23] and release antibiotic at the site of infection in a controlled manner [24, 25], consequently reducing side and toxic effects [26] and improving patient compliance [27], which can lead to improving antibiotic efficacy and prevent the emergence of resistance.

Several nanosystems have been reported for antibiotics delivery, including polymeric nanoparticles [28], liposomes [29], dendrimers [30], micelles [25] and solid lipid nanoparticles [31]. Of these nanosystems, nanostructured lipid carriers (NLCs) were proposed in the 1990s as a promising drug delivery system. NLCs are composed of solid lipids with a specified content of liquid lipids [32], this combination, offering several advantages, including biocompatibility, suitability for most routes of administration and feasible scale up production. In addition, NLCs improve drug loading capacity [33], protect loaded drugs against degradation, can incorporate both lipid and non-lipid soluble drugs [34] and control drug release [32], therefore, NLCs can be an efficient system for antibiotic delivery.

Recently, nanotechnology research advances have focused on developing stimuli-responsive nanocarriers to potentiate the advantages of NDDS [35, 36]. Stimuli-responsive drug delivery systems are able to respond to a specific stimulus at a disease site, such as enzymes [37], pH [38], and temperature changes [39] to potentiate targeted drug release, thus enhancing drug accumulation and bioavailability at the site of action [36]. Certain disease conditions, such as cancer and infectious diseases, exhibit pH that differs from the physiological pH of 7.4 [40, 41]. This property can serve as a potential trigger for pH-responsive nanocarriers in targeting acidic pH conditions [42, 43]. pH-Responsive delivery systems have been efficiently explored for targeted delivery of anti-cancer drugs to tumor sites [44]. However, limited studies have been conducted on antibiotic delivery to the acidic infection site, which can greatly impact the treatment of ID through enhancing targeted antibiotic delivery, thereby minimizing systemic

antibiotic exposure, reducing adverse effects and overcoming conventional antibiotic dosage form limitations [28, 31, 38, 45, 46].

Currently, vancomycin (VCM) is considered the last effective resort against infections caused by MRSA [47]. It is a tricyclic glycopeptide that binds D-alanyl-D-alanine moieties, thus preventing bacterial cell wall synthesis [48]. VCM has low bioavailability and is mainly administrated parenterally as it a large hydrophilic molecule besides its hydrophilicity [49]. However, the intravenous administration of VCM causes severe adverse effects, such as neutropenia, nephrotoxicity [50], ototoxicity and red man syndrome [51]. Additionally, the emergence and spread of VCM resistance is a growing health sector problem that is leading to increased mortality and morbidity rates [52], making it important to find strategies to improve VCM delivery and restore drug efficacy.

Nanocarriers for VCM delivery have proven to be effective and can overcome the conventional dosage form limitations. The liposomal VCM formulation has prolonged circulation time and enhanced treatment efficiency [6, 53-55], while the VCM-SLNs formulation has improved entrapment efficiency and antimicrobial activity [12]. In addition, the lipid-polymer hybrid nanoparticles formulation has shown a sustained VCM release and antibacterial activity [23, 56], and Omolo et al reported on VCM-loaded polymersomes that potentially enhanced the anti-MRSA activity [57]. However, to best of our knowledge, there is no literature reporting on the preparation of NLCs system for VCM delivery, despite their advantages.

The development of pH-responsive nanocarriers requires identification of new materials such as polymers, lipids, polysaccharides, and peptides [58]. At acidic conditions, these materials achieve pH-responsive drug delivery via two mechanisms: i) protonation followed by pH-triggered conformational structural change and ii) hydrolysis of the acid labile bonds resulting in targeted drug release [58-61]. To the best of our knowledge, the only pH-responsive lipid for antibiotic delivery so far has been reported by Kalhapure et al in our group, who noted a pH-responsive acid cleavable lipid for a pH-responsive VCM loaded solid lipid nanoparticles preparation [31]. In addition, pH-responsive VCM polymeric nanoparticles that can protonate at acidic condition have been reported for enhancing VCM targeted delivery and antibacterial activity [28, 45]. No study to date has reported pH-responsive VCM loaded NLCs, highlighting the need to identify novel pH-responsive lipids for a pH-responsive VCM NLCs preparation.

In this study, we propose the synthesis of novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)] to formulate pH-responsive NLCs. The solid lipid carboxylic acid group can form an ion-pair electrostatic interaction with the liquid lipid primary amino group. Consequently, at low pH, NLCs will protonate, resulting in NLCs surface charge switching from negative to positive, followed by ion-pair bond breakage, which can result in NLCs swelling and enhance drug release. Moreover, the positive charge of NLCs can promote binding to the anionic bacterial membrane, thus enhancing antimicrobial activity [12, 46, 57, 62, 63], with no such lipids having been reported for any pH-responsive delivery of any drug class before.

1.3 Problem statement

Infectious diseases, particularly bacterial infections, have been recognized as a major cause of deaths globally due to the limitations of current antibiotic conventional dosage forms, including suboptimal concentrations at the target site of infection, poor pharmacokinetic profiles, higher frequent doses, potential side effects and poor patient adherence to treatment. These limitations have led to the development of an antibiotic resistance crisis and increased mortality and morbidity rates. The development of new antimicrobial agents is declining, highlighting the need to develop new strategies to improve current antibiotic therapy. The introduction of nano antibiotic delivery systems, primarily smart pH-responsive nano antibiotic delivery systems, can potentiate targeted antibiotic release, therefore enhancing antibiotic therapy. Despite the advantages of pH-responsive nano antibiotic delivery systems, to best of our knowledge, no study has been conducted on pH-responsive NLCs for antibiotic delivery to date.

1.4 Hypothesis or research question

We hypothesized that the synthesis of novel pH-responsive fatty acids based lipids to formulate VCM loaded NLCs can enhance its targeted delivery. It can also increase its localization at acidic bacterial infection site and enhance its antibacterial efficacy. This can lead to the protection of the antibiotic during systemic circulation and it can lead to the reduction of the dosage required for optimal therapy. Subsequently, it reduces the dose dependent-adverse effects and improves patient’s compliance.

1.5 Aims and Objectives

The aim of this study was to explore the potential of novel fatty acids based lipids to formulate VCM loaded NLCs to enhance its antibacterial efficacy.

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

1. To synthesize pH-responsive novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)].
2. To characterize SASL and OALL synthesized lipid using FT-IR, ^{1}H NMR and C^{13} NMR and cytotoxicity studies.
3. To explore the potential of SASL and OALL novel lipids to prepare vancomycin loaded pH-responsive nanostructured lipid carriers from (VCM-SAOA-NLCs).
4. To optimize and characterize VCM-SAOA-NLCs in terms of size, polydispersity index, zeta potential, pH responsiveness, morphology, entrapment efficiency, *in vitro* drug release, *in vitro* and *in vivo* antibacterial activity.
5. To undertake *in silico* studies to understand the interactions between VCM and lipids carriers (SASL and OALL) as well as the effect of lipid ratio on entrapment efficiency

1.5 Novelty of the study

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

- In this study, pH-responsive NLCs were formulated from a newly designed and synthesized solid lipid (SASL) and liquid lipid (OALL). These lipids are novel and have not been reported for any pH-responsive nano-formulations.
- Although pH-responsive solid lipid nanoparticles [31] and polymeric nanoparticles [28, 45] have been reported for enhancing VCM antibacterial efficiency at acidic infection sites, no study has reported pH-responsive NLCs loaded with VCM. This is the first study on pH-responsive NLCs for targeting VCM release and enhancing antibacterial activity at acidic infection sites.
- While several nano delivery systems have been developed for VCM, such as polymersomes [57], liposomes [53, 64], dendrimers [23, 65], lipid-polymer hybrid nanoparticles [56], solid lipid nanoparticles [12] and nanoplexes [66], to best of our knowledge, this is the first study that reports a VCM-loaded NLC formulation.

1.6 Significance of the study

This reported pH-responsive NLCs carrier presents a novel and promising approach for targeting poorly soluble antibiotics into acidic infection sites, therefore enhancing drug activity and preventing resistance development. The potential significance of the formulation explored in this study are:

New Pharmaceutical Products

This study proposes novel pH-responsive lipids as pharmaceutical materials and a new pH-responsive NLCs formulation as a medicine. This novel pharmaceutical material and medicine can represent a basic platform for the pharmaceutical industry to develop new pH-responsive delivery systems that could be superior to other conventional dosage forms.

Improved patient therapy and disease treatment

This novel pH-responsive nanocarrier can improve bacterial infection treatment by protecting the antibiotics during systemic circulation, improve targeted and sustained antibiotic release, and enhance antibiotic localization and bioavailability at the acidic infection sites. This can contribute to improving the antibacterial activity, and reduce dosing frequency and adverse effects. This can lead to improving patient compliance, enhancing antibacterial therapy and combating antimicrobial resistance threats.

Stimulation of new research

This study can provide new potential research directions for:

- The synthesis and characterization of pH-responsive lipids and other materials for their potential applications in pH-responsive nanosystems formulation for various drug classes.
- Further optimization of NLCs formulation and process variables to be suitable for eventual patients use.

1.7 overview of the Dissertation

The research is presented in the following chapters:

CHAPTER 1. Introduction:

This chapter provides a brief background of the study including the status of infectious diseases, limitations of current antibiotic therapy and the growing threat of bacterial resistance. It further discusses the role of nanotechnology strategy based pH-responsive systems for combating the challenges encountered with antibiotic resistance. Additionally, it provides the aims, objectives, novelty, and significance of the study, and concludes with the structure and content of the thesis.

CHAPTER 2. Literature Review:

This chapter outlines an overview of the current status of infectious diseases and antibiotic therapy limitations which have led to the development of bacterial resistance. It outlines the use of pH-responsive nano drug delivery as a strategy to influence the treatment of infectious diseases. It also describes nanostructured lipid carrier features, preparation, and characterization methods and concludes with an overview of vancomycin as a model drug.

CHAPTER 3. Submitted manuscript:

This chapter is a first author article that was submitted to an international ISI journal i.e. European Journal of Pharmaceutics and Biopharmaceutics (Impact factor of 4.389). The chapter is presented in the required format of the journal, it describes the formulation of novel vancomycin pH-responsive loaded nanostructured lipid carriers that showed enhanced antibacterial activity.

CHAPTER 4. Conclusion:

This chapter describes the conclusions reached in achieving the study aim, outlines the significance of the findings and makes recommendations for further research into antibiotic-loaded pH-responsive nanostructured lipid carriers.

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CHAPTER 2. LITERATURE REVIEW

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2.1 introduction

This chapter provides an overview of the current status of infectious diseases and antibiotic therapy limitations that have led to the development of bacterial resistance. It outlines the use of pH-responsive nano drug delivery as a strategy to influence the treatment of infectious diseases. It also describes nanostructured lipid carrier features, and preparation and characterization methods, and concludes with an overview of vancomycin as a model drug.

2.2 Current infectious diseases burden and antibiotic therapy limitations

Infectious diseases (ID) are a major public health problem globally, despite the success of antimicrobial therapy [67-71]. ID epidemiology is affected by several factors, such as changes in human behavior and ecology, economic growth, industrial development, human migration, trade and genomics of bacterial pathogens [72]. The ongoing crisis of ID is associated with high morbidity and mortality rates in developing and developed countries (Fig. 1) [2], with diarrhea, tuberculosis and lower respiratory tract infections being among the top ten leading causes of death [73]. In 2015, bacterial infections were the main cause of death among children under age of five in Africa [74], whereas in South Africa, tuberculosis infection accounted for 12% of deaths in 2010 and remained the leading cause of death until 2015 (Fig. 2) [75].

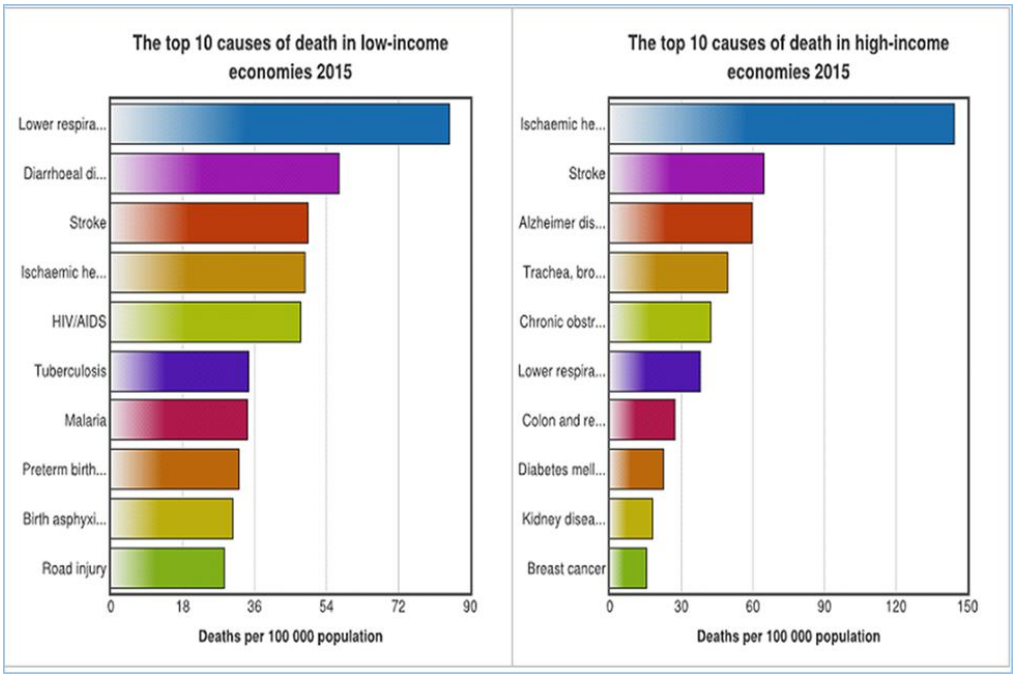

Fig. 1. Leading causes of death in developing and developed countries [73].

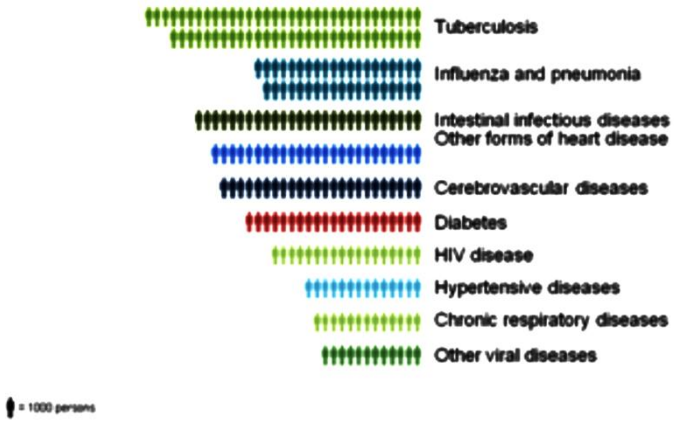

Fig. 2. Cause of death in South Africa [76].

Since the discovery of antibiotics in the 1940s, ID therapy changed dramatically, with their introduction significantly contributing to infection prevention, treatment, and control [77, 78]. Antibiotics have different classes according to their mechanism of actions, such as cell wall synthesis inhibitors, protein synthesis inhibitors, cell membrane disrupters, nucleic acid synthesis inhibitors and antimetabolites (Fig. 3) [79]. Antibiotics are available in number of dosage forms including tablets, capsules [80], suspensions [81], injections [82], creams and ointments [83, 84].

Optimum antibiotic therapy should provide an effective concentration at the infection site with low systemic adverse effects. However, these current conventional antibiotic dosage forms are associated with suboptimal antibiotic concentration at the infection site and poor stability. In addition, poor pharmacokinetic properties, such as insufficient absorption, rapid metabolism, and excretion from the body, lead to reduced tissue penetration and low bioavailability at the infection site [4, 85, 86]. Subsequently, high frequent antibiotic doses are required to achieve sustained therapeutic concentration, these often being associated with increased systemic adverse effects, high costs and poor patient adherence to therapy [6]. The conventional antibiotics dosage form limitations, together with irrational antibiotic use, are the key issues in ID therapy failure, infection re-emergence and the development of bacterial resistance (Fig. 4) [5].

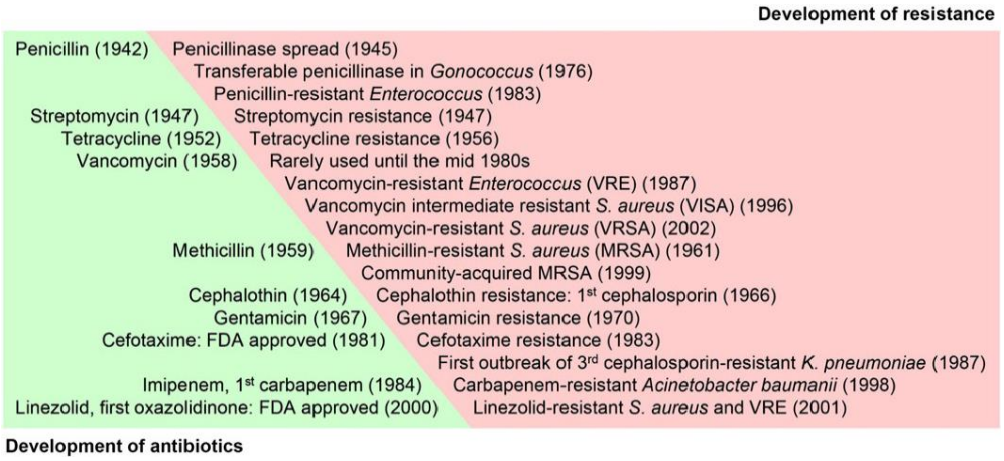

Fig. 3. History of antimicrobial agent development and resistance by microorganisms [27].

Bacteria can acquire antibiotic resistance intrinsically or by horizontal gene transfer [87]. The main resistance mechanisms include minimizing antibiotic uptake [88], genetic modification of the antibiotic target site [89], activation of antibiotic efflux pump [90] and antibiotic enzymatic inactivation (Fig. 4) [87, 91]. Bacteria strains that are resistant to multiple classes of antibiotics represent a major persistent health challenge, with multidrug resistance (MDR) bacterial infection being untreatable and associated with ineffective, more toxic and expensive antibiotic therapy [10, 11, 92].

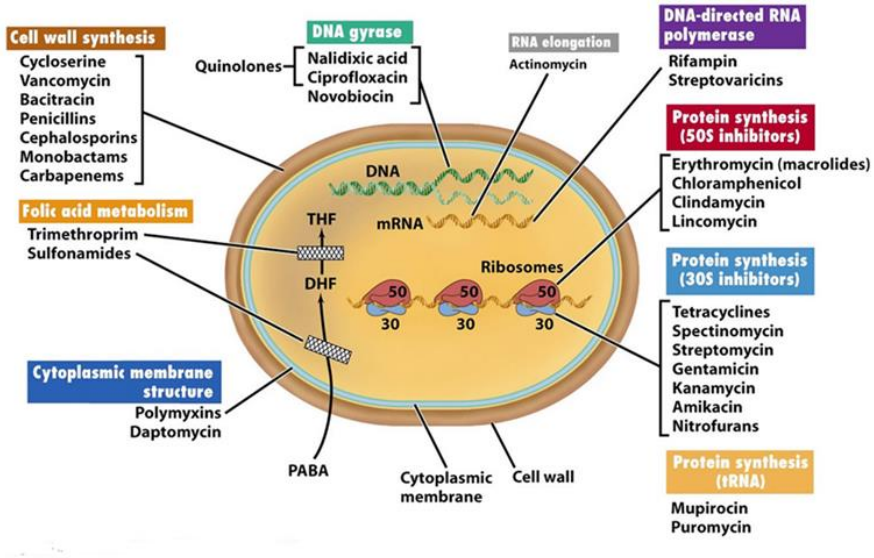

Fig. 4. Commonly used antibiotics mechanism of action [79].

Among the MDR bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) was identified in 1961 [93], the causative organism causing serious infection such as skin and soft tissue infection, endocarditis and bacteremia, which are associated with raised morbidity and mortality rates, increased therapy cost and prolonged hospital stay [94-97].

The antibiotic resistance crisis requires the intervention of health sectors experts, policy makers, health authorities, pharmaceutical companies and community members in order to undertake collaborative efforts and approaches to combat bacterial resistance [15, 98]. While bacterial resistance is growing, the development rate of new antimicrobials against MDR organisms is declining [99], due to high production costs, long drug approval processes period, poor return on investment compared to other drug classes and regulatory body barriers (Fig. 5) [12, 72, 94, 100]. Thus, new strategies have been investigated to improve conventional antibiotic dosage forms delivery and restore efficacy [14, 15] such as individualizing antibiotic therapy [101], antibiotic therapeutic drug monitoring [102, 103] and delivering antibiotics in controlled and targeted delivery [104, 105].

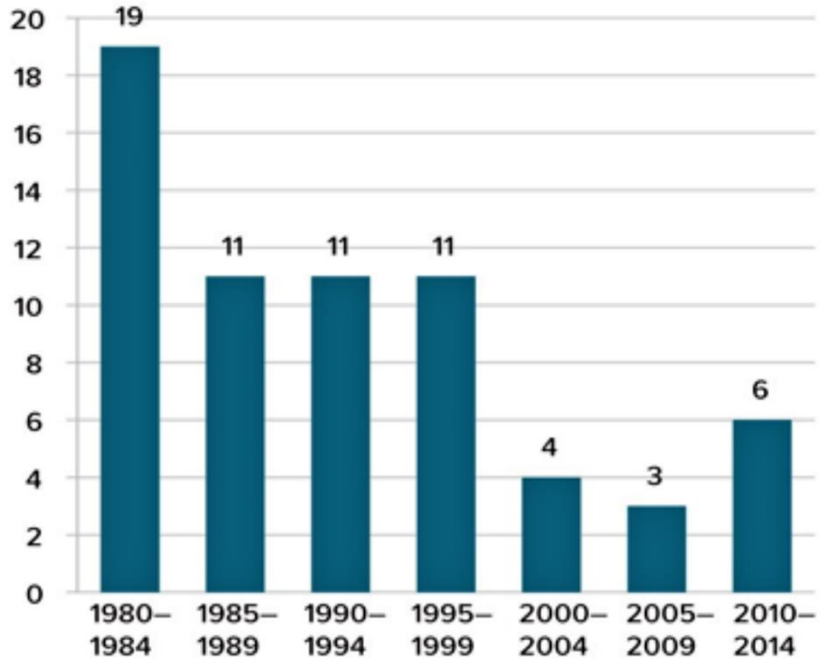

Fig. 5. Declining number of new antibiotics [9].

2.3 Nanoengineered antibiotic delivery systems

Nanotechnology is the science that engineers designs and characterizes nanometer-scale materials for various applications [106], with nanoparticles having a small particle size with a large surface area. In addition, nanoparticle surface can be modified and functionalized for their potential applications, which results in nano drug delivery systems (NDDS) being a promising carrier for enhancing antibiotics delivery and improving efficacy at the site of infection (Fig. 6). Several NDDS have being investigated for encapsulating various classes of antibiotic against a wide range of bacteria (Table 1), such as: polymeric nanoparticles [107], liposomes [29], dendrimers [30], micelles [25] and solid lipid nanoparticles [4, 12], nanoemulsions [108], carbon nanotubes [109], polymersomes [57] and nanostructured lipid carriers [110, 111]. These nanosystems have been widely applied for different routes of antibiotics administration, such as: oral [112], parenteral [113], topical [114], inhalation [114] and transdermal [115].

NDDS have several advantages over the conventional antibiotic dosage forms, such as: improving antibiotic solubility and bio-distribution profiles [18-20], enhancing tissue penetration and bioavailability at infection sites [21, 22], enhancing antibiotic pharmaceutical stability [23], targeting and controlling antibiotic release at infection site [24, 116] improving antibiotic localization at intracellular sites [117, 118] and achieving synergistic effects via co-delivery of antibiotic and antimicrobials [119-122]. In addition, NDDS can potentiate antibacterial therapy due to its lower frequent dosing and systemic side effects [26], which promotes patient compliance [27]. Therefore, NDDS can overcome conventional antibiotics dosage forms limitations, and offer a promising approach that can improve antibiotic therapy and consequently prevent bacterial resistance development [14, 27].

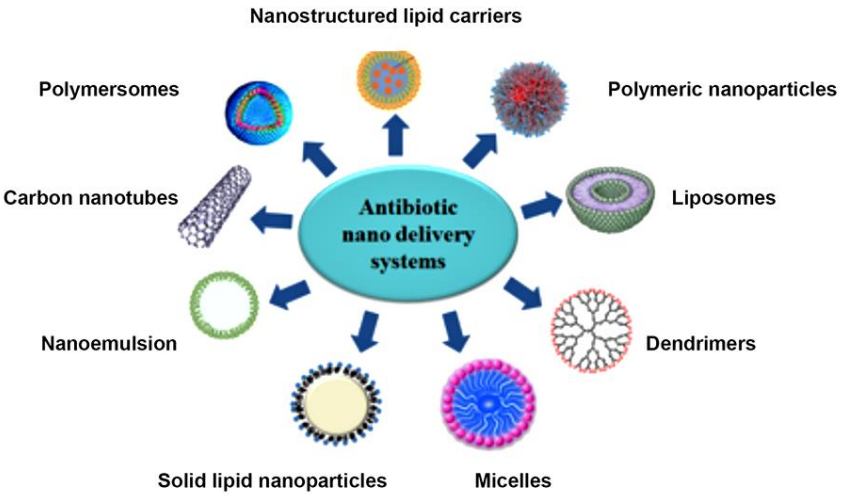

Fig. 6. Examples of nano-delivery systems reported for antibiotics

Table 1. Examples of nanosystems reported for antibiotics.

Nanosystem	Encapsulated antibiotic	Targeted bacteria	Mechanism for improved antibiotic efficacy	Reference
Polymeric nanoparticles	Levofloxacin	*Escherichia coli*	- Sustained drug release. - Enhanced *the* biofilm inhibitory *activity.*	[123]
Liposomes	Polymyxin B	*Pseudomonas aeruginosa*	- Sustained drug release. - Enhanced lung delivery.	[124]
Dendrimers	Ciprofloxacin	*Staphylococcus aureus* and *Escherichia coli*	- Co-administration of dendrimers reduced the effective required dose of the drug. - Synergistic antibacterial activity.	[125]
Micelles	Ciprofloxacin	*Bacillus subtilius* and *Escherichia coli*	- Enhanced drug uptake across the Blood-Brain Barrier.	[116]
Solid lipid nanoparticles	Meropenem	*Escherichia coli*	- Controlled release and enhanced antibacterial activity.	[4]
Nanoemulsions	Amoxicillin	*Helicobacter pylori*	- Controlled drug release. - Enhanced drug localization at the site of infection.	[126]
Polymersomes	Vancomycin	*Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*	- Sustained drug release and *in vitro* and *in vivo* antibacterial activity	[57]

2.4 Nanostructured lipid carriers

Nanostructured lipid carriers (NLCs) were developed in the 1990s as the second generation of solid lipid nanoparticles (SLNs), and are composed of a matrix of biodegradable/biocompatible solid and liquid lipids coated with surfactant (Fig. 7) [127]. NLCs lipid matrix is less ordered due to the presence of the liquid lipid, which results in the drug loading capacity being superior to SLNs [128]. In addition, NLCs have several advantages, including biocompatibility, suitability for drug delivery via different routes of administration [33], high storage stability [129], simple scale up production [32]. Also, NLCs can protect the loaded drug against degradation [34], incorporate lipophilic and hydrophilic drugs [34, 130], control drug release [32, 131] and enhance drug bioavailability [132]. These advantages make NLCs an efficient nanocarrier for antibiotic

delivery in order to improve ID therapy via targeted antibiotic release, enhance antibiotic bioavailability at infection site and prevent bacterial resistance.

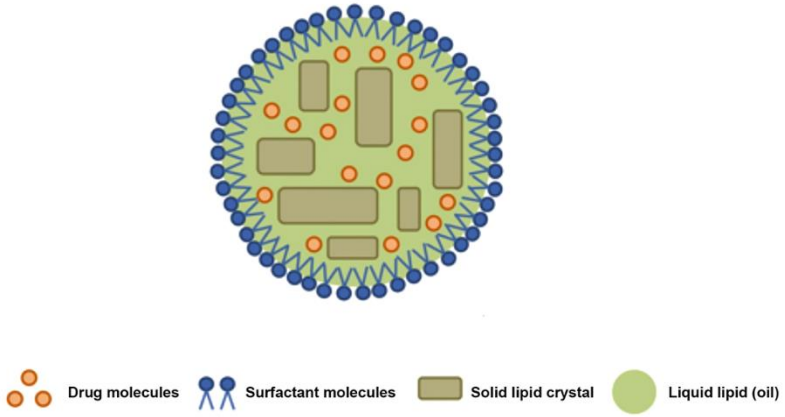

Fig. 7. Nanostructured lipid carriers structure [133]**.**

2.4.1 NLCs composition

NLCs are mainly composed of biodegradable/biocompatible solid and liquid lipids and emulsifier**.** The most common solid lipid used for NLCs preparation are cetyl palmitate, glyceryl monostearate, stearic acid, glyceryl palmitostearate, cholesterol, glycerol behenate and glyceryl tripalmitate. The most commonly used liquid lipids are oleic acid, miglyol 812, castor oil, and medium chain triglycerides. Poloxamer 188/407, polysorbate 80/40/20 and phosphatidylcholines are often employed as the emulsifier [7, 134, 135].

2.4.2 Types of NLCs

NLCs are classified based on the structure, lipid composition, and ratios into 3 types (Fig. 8)

1) Imperfect type: These NLCs are composed of different ratios of solid and liquid lipids matrix that have imperfect crystalline lipid order, which results in them having high drug loading capacity due to the imperfect lipid matrix.
2) Amorphous type: This type of NLCs is produced by mixing the solid lipid with specific liquid lipids, such as hydroxy octacosanylhydroxystearate, isopropyl myristate and medium chain triglycerides that can form amorphous lipid matrix. This type has high storage stability due to the amorpho.us lipid matrix
3) Multiple type: In this type, a higher amount of liquid lipid is added to the lipid phase in order to enhance the hydrophobic drug solubilization [92, 136].

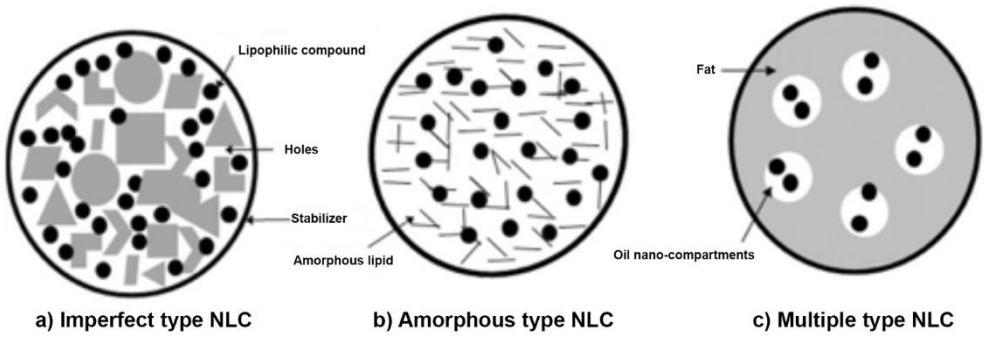

Fig. 8. Types of Nanostructured lipid carriers [136].

2.4.3 Methods applied for NLCs production

Several methods have been applied to produce NLCs, including high pressure homogenization (HPH) [33], solvent emulsification-evaporation [137], solvent diffusion method [138], solvent injection [139], phase inversion [140], emulsification sonication and membrane contractor techniques [34]. HPH is the most commonly used method for NLCs preparation as it has several advantages, such as the ability to produce small particle sizes, simple scale up production and short production time compared to the other methods [141, 142].

HPH can be done at different temperature ranges, the hot method being done at a temperature degree above lipid melting point, the drug is mixed with lipid melt, then the drug-containing lipid is dispersed in the hot aqueous surfactants phase. Subsequently, both phases are homogenized using high shear mixing device resulting in emulsion formation, which is subjected to cooling to obtain NLCs (Fig. 9 A) [135, 143, 144]. The cold HPH is done by dissolving the drug in the lipid melt, followed by solidifying it using dry ice or liquid nitrogen. Subsequently, the drug-lipid mixture is milled and dispersed in a cold surfactant solution (Fig. 9 B) [136, 145]. The hot HPH technique can result in smaller particle size than the cold HPH due to lipid viscosity reduction at high temperature. However, increasing the homogenization temperature can accelerate the drug and lipid degradation rate [135, 136].

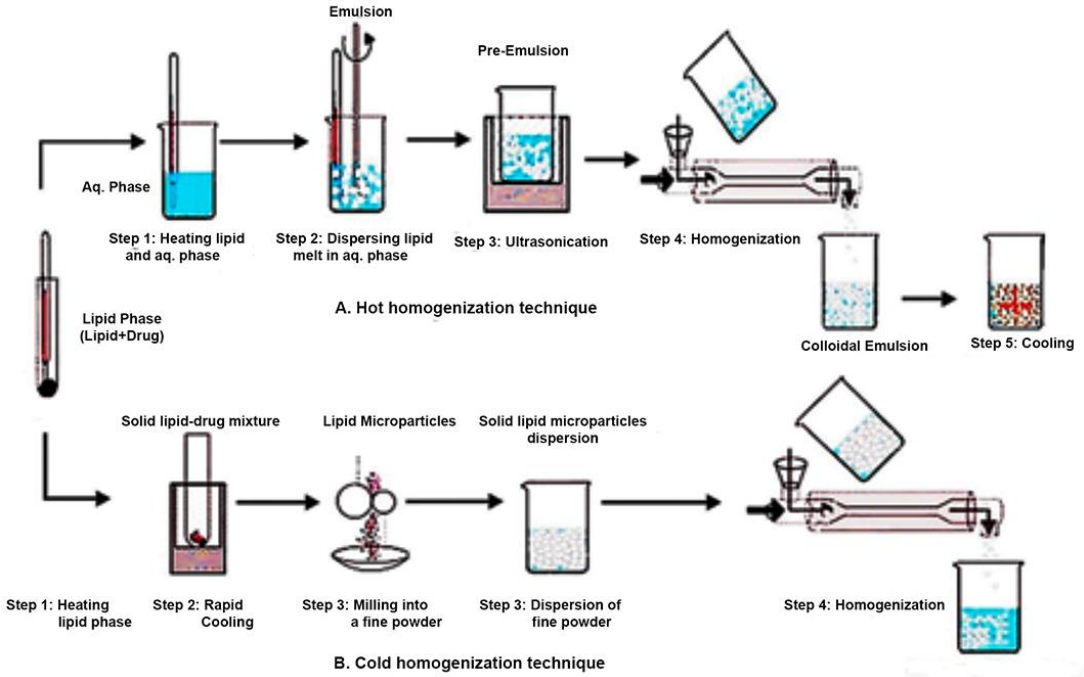

Fig. 9. Homogenization technique. A) Hot homogenization technique B) Cold homogenization technique [146].

2.4.4 NLCs characterization

Various methods are used for NLCs characterization in order to explore their potential application and quality evaluation. NLCs are characterized in terms of particle size, particle size distribution, surface charge, morphology and other parameters, these being critical indicators of NLCs quality, stability, and biological kinetics profiles. Moreover, these parameters can significantly influence biological membrane penetration, distribution, excretion and drug release [131].

Particle size and size distribution

Analytical instruments based on dynamic light scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques are commonly used to determine the particle size. The DLS technique is the most frequently used for particle size and size distribution (expressed as the polydispersity index (PDI)) determination as it is practical and simple [131, 142, 147, 148].

Surface charge

Nanoparticles surface charge, expressed as zeta potential (ZP), is a critical measure for NLCs stability assessment during storage. High positive or negative ZP values indicate high electrostatic repulsion, thus, NLCs particles avoid aggregation and better system stability is maintained. Analytical instruments based on the DLS technique is used for ZP measurement [131, 142, 148, 149].

Morphology

SEM and TEM techniques are additionally used for NLCs shape and morphology studies [150, 151].

Entrapment efficiency (EE)

Drug entrapment efficiency (EE) is the amount of drug that has been entrapped in the nanoparticle lipid matrix, hence lipophilic drugs have higher EE than hydrophilic drugs. The free (unentrapped) drug is separated from the NLCs using ultra-centrifugation, ultra-filtration and gel permeation methods, after which the free drug quantity is analyzed using the HPLC or UV method [32, 137, 152].

Thermal profiles

Analytical instruments based on X-ray spectroscopy and differential scanning calorimetry technique are widely used to investigate NLCs crystallization state and thermal behavior [136].

In vitro drug release

Drug release studies are commonly carried out using dynamic dialysis bag method to investigate the release behavior of drug loaded NLCs. The samples are analyzed for the drug content using an analytical method, such as HPLC or UV [153]. Several factors can influence NLCs drug releases, such as NLCs particles size, surface area, and lipid matrix viscosity. Additional NLC characterization studies can be performed depending on the application of NLCs.

2.4.5 NLCs for antibiotic delivery

Despite the successful outcomes of NLCs application for several drug classes delivery via different routes of administration such as oral [130, 131, 154], topical [155, 156], parenteral [153, 157] and pulmonary [158, 159]**,** few studies have been conducted on NLCs antibiotics delivery. Moreno-Sastre et al reporting tobramycin loaded NLCs against *Pseudomonas aeruginosa* infections, the results showing improved EE, sustained release beside increased pulmonary

distribution of the drug [111]. Similar results were reported by Pastor et al., where NLCs enhanced sodium colistimethate target release and antibacterial activity against *Pseudomonas aeruginosa* infection [160]. Rifampicin loaded NLCs showed improved pharmacokinetic profiles that enhanced the pulmonary targeted delivery [161].

However, there is a need for a nano antibiotic carrier that efficiently protects and stabilize the antibiotic during systemic circulation, and enhances targeted antibiotic release and localization at infection sites. Thus, researchers have recently explored "smart" nanosystems to further improve the first generation of nano antibiotic delivery systems [7, 35, 162, 163].

2.5 pH-Responsive drug delivery system

Recent research advances focus on developing nanocarriers that release the drug in response to various stimuli, such as pH, enzymes, ultrasound, electric field, magnetic field and temperature [37, 39, 164-166]. Stimuli-responsive drug delivery strategy can improve drug protection against degradation over systemic circulation [44], target drug release, increase intracellular uptake and drug accumulation at the site of action [36]. This can lead to enhanced drug stability, bioavailability and therapeutic effectiveness [35]. Hence, "smart" nanosystems can be more promising drug delivery carriers than the conventional NDDS.

Current investigations of pH-responsive drug delivery system that can target and release the drug in response to a pH-change stimulus have received much attention [167]. Certain body compartments, such as gastrointestinal tract and disease pathophysiological conditions (e.g. tumors and infections) exhibit pH different from the physiological pH of 7.4. This property can potentially trigger the design of pH-responsive nanosystems for targeted drug release [35, 40, 41]. Table 2 summarize studies reported on pH-responsive drug delivery nanosystems. The development of pH-responsive nanocarriers requires the identification of new materials, with the literature reporting various pH-responsive materials, such as polymers, lipids, polysaccharides, and peptides [58].

2.5.1 pH-responsiveness mechanisms

At acidic conditions, these materials achieve pH-responsiveness via two mechanisms:

1) Protonation: pH-Responsive materials are designed to have ionizable groups that remain unprotonated, while in acidic pH these groups undergo protonation and a surface charge reversal, resulting in structural and conformational changes followed by drug release.

2) Acid-labile bonds hydrolysis :pH-responsive materials can form an acid-labile bond with a drug that undergoes hydrolysis at the acidic condition, resulting in drug release [58-61].

2.5.2 pH-Responsive nanocarriers application

One of the most promising applications of pH-responsive nanocarriers is pH-responsive tumor-targeted drug delivery, which can protect cytotoxic drug at the physiologic pH of 7.4, target the drug release, and enhance drug localization and uptake at the acidic tumor site. They can, therefore, enhance the cytotoxic activity and prevent resistance development [38, 42, 43]. Additionally, pH-sensitive nanocarriers can impact *Helicobacter pylori* therapy by improving drug protection at the acidic gastric site, enhancing targeted release and uptake at infection site[168-170].

Some bacterial infections are acidic, as bacteria produce acetic acid and lactic acid under anaerobic conditions. Therefore, the pH-responsive strategy can be applied to enhance antibiotic target delivery, reduce healthy site exposure to antibiotic, target and control antibiotic release and increase antibiotic localization at the acidic infection site, which can enhance antibacterial activity and prevent bacterial resistance development [28].

Table 2. Examples of pH-responsive nano delivery systems

Drug	pH-responsive nanosystem	Main findings	Reference
Amoxicillin	Polymeric nanoparticles	Drug protection against gastric acid degradation. Enhanced antibacterial activity against *Helicobacter pylori* in the infected gastric mucosa.	[168]
Heparin	Polymeric nanoparticles	Enhanced drug interaction with *Helicobacter pylori* infection site thereby increasing gastric mucosal cells regeneration.	[169]
Tenofovir	Polymeric nanoparticles	Enhanced intravaginal drug delivery for HIV transmission prevention.	[40]
Cisplatin	Polymeric nanoparticles	Improved targeted drug delivery at the acidic lysosome environment in cancer cells led to improved drug efficacy.	[42]
Paclitaxel	Polymeric nanoparticles	Improved drug loading capacity and targeted drug release at the acidic tumor site.	[171]
Doxorubicin	Polymeric Nanoparticles	Improved drug intracellular bioavailability and enhanced its cytotoxic activity.	[172]
Vancomycin	SLNs	Enhanced encapsulation efficiency and release at acidic pH. *In vitro* and *in vivo* studies showed better anti-MRSA activity.	[46]
Curcumin	SLNs	Sustained drug release with higher stability and bioavailability at acidic conditions.	[173]
Doxorubicin	SLNs	Superior proliferative inhibition of multidrug-resistant cancer cells.	[62]
Vancomycin	Polymeric Nanoparticles	Stronger binding to the bacterial surface in acidic media compared to the basic one, led to better antimicrobial efficacy against SA compared to the free drug.	[28]

2.6 Vancomycin as a model drug for antibiotic therapy

Vancomycin (VCM) is a tricyclic glycopeptide bactericidal antibiotic (Fig. 10), and acts by making a complex with the D-alanyl-D-alanine moieties, thereby inhibiting the peptidoglycan synthetase enzyme, and subsequently preventing peptidoglycan formation and cell wall biosynthesis [48].

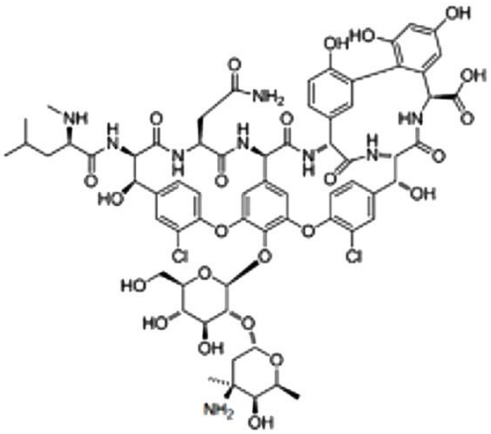

Fig. 10. VCM structure [12].

2.6.1 VCM activity

VCM is administered orally to treat pseudomembranous colitis infections, which is caused by *Clostridium difficile* bacteria and is administrated parenterally for MRSA infections. It has been considered as a standard effective resort for treatment and prophylaxis of the life-threatening Gram-positive bacterial infections, such as skin infections, bacteremia, and endocarditis that are unresponsive to other antibiotic classes [47, 49, 174]

2.6.2 VCM adverse effects

Parenteral administration of VCM is associated with severe adverse effects, such as neutropenia, nephrotoxicity [50], ototoxicity and most commonly red man syndrome [51, 175].

2.6.3 VCM nano delivery systems

VCM is a hydrophilic large molecule, thus it has low absorption, bioavailability and tissue penetration [49]. In addition, the emergence and spread of VCM resistance is a growing health sector problem causing VCM treatment failure and increased minimum inhibitory concentration [52]. VCM nano delivery systems have proven to be very effective compared to VCM conventional dosage forms. Liposomal VCM formulation had prolonged circulation time and

enhanced antibacterial efficiency [6, 53, 55], while SLNs were a suitable carrier to improve VCM entrapment efficiency and antimicrobial activity [12]. Lipid polymer hybrid nanoparticles formulation showed a sustained VCM release and antibacterial activity [56]. Omolo et al reported VCM-loaded polymersomes that potentially enhanced its anti-MRSA activity [57].

2.6.4 VCM pH-Responsive nanosystems

Smart nano delivery systems have been applied for enhanced targeted VCM delivery [28, 31, 45, 176], with Kalhapure et al having reported pH-responsive acid cleavable lipid for pH-responsive vancomycin loaded SLNs preparation, their results showing enhanced antibacterial activity against methicillin-susceptible and resistant *Staphylococcus aureus* [31]. pH-responsive vancomycin polymeric nanoparticles have also been reported for enhancing drug targeted delivery and activity [28, 45]. Thus, pH-responsive nanosystems for VCM targeted delivery can contribute to enhancing VCM efficacy, overcome VCM conventional dosage forms limitations, and combat bacterial infections and bacterial resistance. However, despite the unique adva ,ntage ,of NLCs there is no literature data reported on the preparation of pH-responsive NLCs system for VCM delivery yet.

2.7 Conclusion

The chapter highlighted the literature findings of the current state of infectious diseases, lack of effective antibiotic therapy and the emergence of antibiotic resistance. It also outlined the role of nano drug delivery systems, mainly pH-responsive nanosystems, in overcoming antibiotic therapy limitations. Although extensive research has been conducted on NLCs potential applications, no study has been reported on pH-responsive NLCs for antibiotic delivery yet, such system possibly being the new “magic bullets” to restore the efficacy of the antibiotic and prevent the post-antibiotic era evolution.

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CHAPTER 3. SUBMITTED MANUSCRIPT

3.1 Introduction

The following research paper was submitted to the European Journal of Pharmaceutics and Biopharmaceutics which has an impact factor of 4.389 from work done during this study.

Miss Nawras Osman contributed to the design of the project, synthesis, and characterization of the novel lipids, formulation and characterization of the nanostructured lipid carriers. In addition, Miss Nawras Osman was responsible for analysis and interpretation of all data, wrote the first draft of the paper and undertook all revisions. M. Calvin A. Omolo assisted with lipid synthesis and performed cell viability studies and in vivo antibacterial studies. Dr. Sanjeev Rambharose performed the cytotoxicity studies. Dr. Ramesh Gannimani supervised the lipids synthesis and characterization methods. Dr. Ayman Y. Waddad performed the molecular modeling studies. Dr. Chunderika Mocktar supervised the in vitro and in vivo antibacterial activity studies. Dr. Sanil Singh supervised the in vivo antibacterial activity studies. Dr. Raveen Parboosing was a collaborator on the project. Prof. Thirumala Govender served as supervisor and was responsible for project conceptualization, problem-solving, paper and abstract editing and general supervision of the study.

3.2 Submitted manuscript

Novel fatty acid based pH-responsive nanostructured lipid carriers for enhancing vancomycin activity

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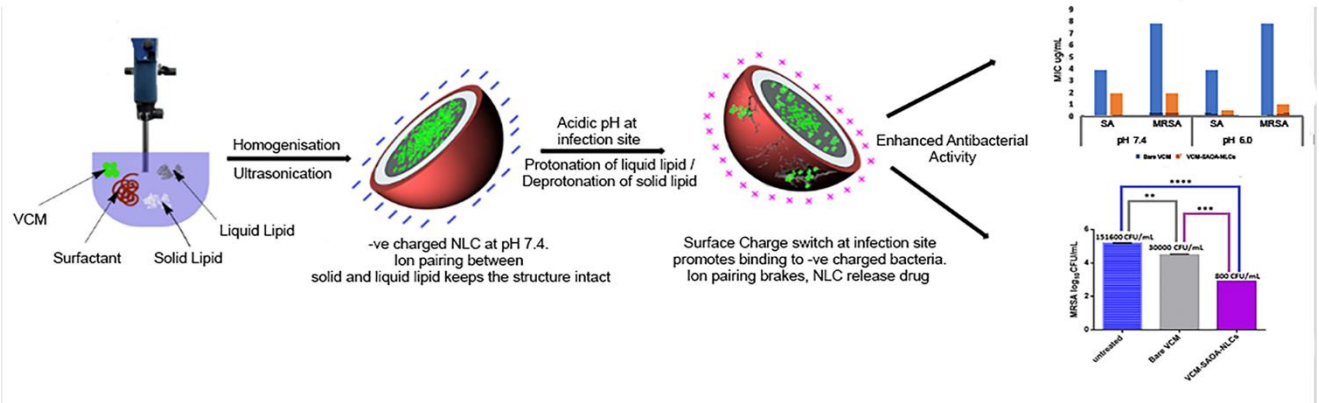
Graphical abstract



Abstract

The aim of the present study was to synthesize and characterize novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)] and explore their potential for preparing vancomycin pH-responsive nanostructured lipid carriers (VCM-SAOA-NLCs). Biosafe SASL and OALL were successfully synthesized and VCM-SAOA-NLCs were prepared using the hot homogenization technique. VCM-SAOA-NLCs size, polydispersity index, zeta potential and encapsulation efficiency were found to be 225.9 ± 9.1 nm, 0.258 ± 0.02, -6.69 ± 1.1 mV and 88.7 ± 13.12 % respectively. *In silico* studies showed higher VCM encapsulation efficiency at 2:1 than 1:1 solid lipid to liquid lipid ratio. *In vitro* drug release studies revealed that VCM-SAOA-NLCs had pH-sensitive sustained drug release. *In vitro* antibacterial activity against methicillin-susceptible and resistant *Staphylococcus aureus* revealed that VCM-SAOA-NLCs had superior antibacterial activity compared to bare VCM against both bacterial strains at both pH conditions. MRSA bacterial cell viability study showed enhanced uptake and antibacterial activity. Furthermore, the *in vivo* study revealed that MRSA CFU load in mice skin treated with VCM-SAOA-NLCs was 37-fold lower than bare VCM ($P < 0.05$). Therefore, this novel pH-responsive NLCs may serve as a promising nanocarrier for enhancing antibiotic delivery and activity.

Keywords: pH-responsive; nanostructured lipid carriers; vancomycin; methicillin-resistant *Staphylococcus aureus*; fatty acid

1. Introduction

The golden era of antibiotic discovery was followed by the emergence of drug resistance, which has become a considerable health threat globally and is leading to higher medical costs for individuals and healthcare sectors [177-179]. Infectious organisms have developed resistance to multiple classes of antibiotics [180]. Of concern are community and hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infections, which pose a major therapeutic challenge, and are associated with raised risks of treatment failure and mortality rates [181-183]. Although vancomycin (VCM) has been the most effective antibiotic against MRSA [184], there are several reports of resistance and therapy failure, highlighting the need for new strategies to enhance its activity and to prevent the spread of resistance [174, 185, 186].

Current conventional antibiotics dosage forms have several disadvantages, including insufficient therapeutic concentration at infection sites [187], poor intracellular uptake, and exposure to noninfectious/healthy sites [188]. In addition, effective therapy for infectious disease is achieved via high antibiotic doses and frequent administration [179]. This leads to increased incidence of adverse drug reactions, toxicity and poor patient's adherence [189, 190], which contribute significantly to resistance development [191]. Novel drug delivery systems for antibiotics delivery is therefore explored as a strategy to overcome limitations with conventional antibiotics dosage forms [14].

Nano drug delivery systems (NDDS) are a promising approach to enhance antibiotics delivery due to their small particle size, large surface area and biocompatibility [192]. These unique properties offer several advantages, including increased therapeutic concentration at the infection site [193], and sustained and targeted release of antibiotics [185], therefore enhancing bioavailability [179] and reducing systemic exposure of antibiotics [194, 195]. These systems enhance the efficacy of conventional antibiotics, reduce their side effects and can overcome resistance [31, 196].

Various types of nano-carriers are currently used to deliver antimicrobial drugs, including polymeric nanoparticles [28, 123, 197], liposomes [29, 193], dendrimers [23], polymersomes [57] micelles [116] and solid lipid nanoparticles (SLNs) [198, 199]. Nanostructured lipid carriers (NLCs) have attracted major attention recently as a drug delivery system. NLCs are composed of liquid and solid lipids, this combination offers

prime advantages of improved drug loading capacity [200, 201], incorporating both hydrophilic and hydrophobic drugs, enabling simple large-scale production, biocompatibility [154, 202] and enhanced storage stability [147, 203]. Moreover, NLCs protect the loaded drug against degradation, enable improved drug bioavailability [149, 159] and achieved drug release in a controlled and targeted manner [204, 205]. These features have made NLCs highly competent in comparison to other nanoparticle carriers [206], and can, therefore, be a promising nanocarrier for antibiotic delivery.

More recently, a new approach to enhance therapeutic outcomes with NDDS has focused on the development of nanosystems that respond to stimuli, such as light, pH, electric fields, magnetic field and temperature changes [39, 207]. Stimuli-responsive nanosystems offer enhanced drug protection against degradation during systemic circulation and increase the release of the loaded drug at the target site in response to the stimuli [44]. One of the most promising smart nanocarriers is pH-responsive nanosystem, which ideally releases the drug in response to pH changes at its target site [44, 208]. Several diseases, such as cancer, gastric and duodenal ulcers, besides some bacterial infections, exhibit acidic pathophysiological states that can provide a suitable stimulus for pH-responsive systems [38, 167, 209, 210]. These systems for antibiotic delivery can be used to decrease drug loss from nanoparticles prior to reaching the infection sites [31, 38, 44], increase drug release at the infection site and promote interaction with the bacteria [28].

Although numerous studies have been reported for pH-responsive systems in tumor targeting, there have been very few on developing pH-responsive nanoparticles for antibiotic delivery [171, 211]. At low oxygen concentration, certain bacteria initiate anaerobic fermentation that produces lactic and acetic acids. This acidic infection site serves as an ideal platform for pH-responsive nanocarriers to possess acid triggered targeted antibiotic release [28]. Developing nanoparticles with surface charge switching property at acidic media can enhance cationic fusion with the anionic bacterial cell wall and therefore facilitate the availability of higher antibiotic concentration at the bacterial site [31]. Thus, pH-responsive targeted delivery systems can enhance antibiotic delivery, minimize systemic antibiotic exposure, reduce adverse effects and help to overcome antibiotic resistance mechanisms [28, 31, 45].

Despite the advantages of NLCs, no study has reported pH-responsive NLCs for antibiotic delivery, highlighting the need to identify novel lipids to prepare them. We hypothesize that pH responsiveness can be accomplished via NLCs via a combination of free fatty acid derived solid lipid with a carboxylic acid group and a liquid lipid with a primary amino group that can form an ion pair by electrostatic interaction. These interactions are mainly influenced by the pH-dependent ionization property of both acid and base in aqueous solution. The self-assembling properties of NLCs formed by this combination of the solid and liquid lipid can be pH sensitive. In addition, free fatty acid lipids increase bacterial membrane permeability and hold antimicrobial activity against wide ranges of bacteria including MRSA [12, 31, 57, 62, 63]. These properties could represent a potential solution to overcome bacterial resistance via engineering the free fatty acids lipid nanocarriers to develop pH-sensitive NLCs system that can enhance antibacterial activity [28, 31, 45]. No such lipids have been reported for any class of drug.

The aim of this study was, therefore, to synthesize and characterize novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)], and to explore the potential for preparing vancomycin pH-responsive nanostructured lipid carriers (VCM-SAOA-NLCs). The synthesis and characterization of the lipids are described followed by their use for the preparation and characterization of VCM loaded NLCs.

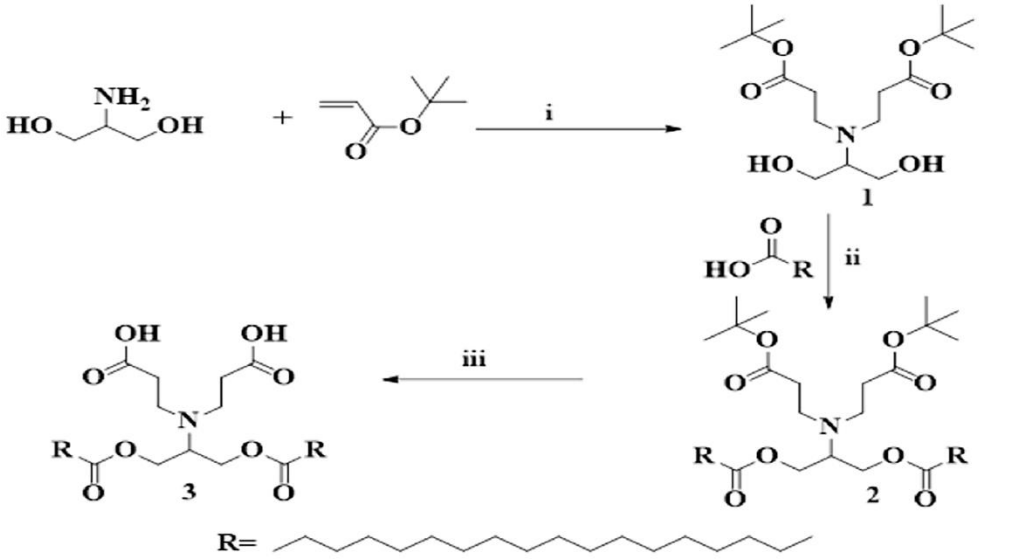
2. Materials and methods

2.1 Materials

Stearic acid and serinol were purchased from Sigma-Aldrich (UK), while Trifluoroacetic acid (TFA), N, N'-dicyclohexyl carbodiimide (DCC) and di-tert-butyl dicarbonate (t-Boc) were purchased from Merck (Germany). Triisopropylsilane (TIPS) and tertiary butyl acrylate (TBA) were purchased from Sigma-Aldrich (Germany). Mueller Hinton Agar (MHA) and Nutrient Broth were obtained from Biolab Inc. (South Africa), and Vancomycin hydrochloride, 4-(Dimethylamino)pyridine (DMAP), oleic acid (OA), Mueller Hinton broth 2 (MHB), dialysis tubing cellulose membrane and all other reagents were procured from Sigma-Aldrich (USA). Vancomycin hydrochloride was converted to

the vancomycin free base (VCM), according to a previously reported method [12]. Milli-Q purified water was obtained from an Elix® water purification system Millipore Corp. (USA). Bacterial strains used were *Staphylococcus aureus* Rosenbach (ATCC®BAA-1683) (MRSA) and *Staphylococcus aureus* (ATCC 25922) (SA).

2.2. Synthesis and characterization of the lipids



i)MeOH, 100 °C, 12hours, ii)DCC/DMAP, 24hours vi)TFA/TIPS/DCM, 4hours

Scheme 1 Synthesis of stearic acid derived solid lipid (SASL).

2.2.1. di-tert-butyl 3,3'-((1,3-dihydroxypropan-2-yl) azanediyl) dipropionate **1**

Compound 1 was synthesized via Michael's addition of the amino group of serinol to tert-Butyl methacrylate (TBA) [212]. Briefly, to a stirred solution of 3.55 g (0.039 mol) of serinol in methanol, 15 g (0.117 mol) of TBA was added and refluxed for 12 hours. After completing the reaction by TLC, methanol was removed under reduced pressure and the crude purified by column chromatography on silica gel (hexane/ethyl acetate, 6:4) to give compound 1 as a clear viscous liquid with a high yield of above 87%. Characterization data is as follows: IR (ATR): 3315, 1682, 1160 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37 (s, 18H), 2.29 – 2.34(t, 4H), 2.79 – 2.82 (m, 5H), 3.48 – 3.58 (m, 4H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 28.0, 35.0, 45.7, 59.7, 63.0, 80.6, 172.2; HRMS (ES-TOF): $[M + H]^+$

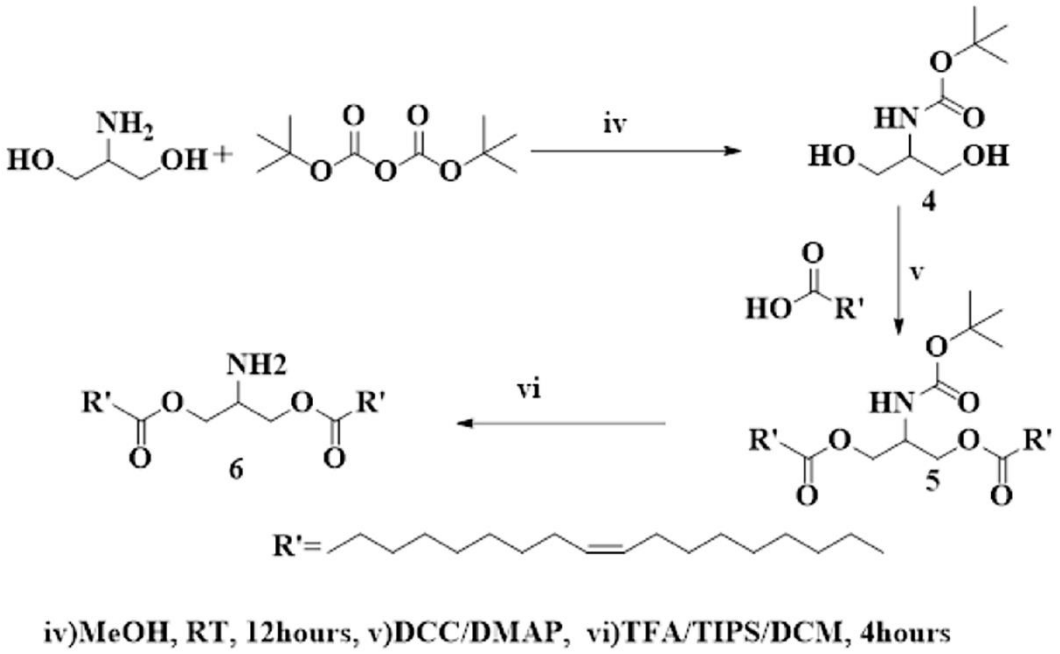
calculated for $C_{17}H_{33}NO_6+H^+$: 348.2387, found 348.2393 (Figure S3; Supplementary material).

2.2.2. 2-(bis(3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl distearate **2**

To the solution of stearic acid (8.5 g, 0.02995 mol) in dry DCM, N, N'-Dicyclohexylcarbodiimide (7.09 g), DMAP (0.9 g) and compound 1 (5 g, 0.0149 mol) were added [213], with the resulting mixture being stirred for 24 hours. DCC urea was filtered off, and the filtrate was concentrated and purified by column chromatography on silica gel (hexane/EtOAc, 8: 2) to obtain a white solid lipid (compound 2) with yield of 76.3%. Characterization data are as follows: IR (ATR): 2913, 2849, 1725, 1469, 1392-1366, 1149 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.792- 0.826 (m, 6H), 1.18 (m, 57H), 1.37 (m, 18H) 1.51 – 1.55 (m, 4H), 2.21 – 2.25(m, 7H), 2.8 (m, 4H), 4.12 – 4.18 (m, 4H). ^{13}C NMR (400 MHz, $CDCl_3$): δ14.11, 22.69, 24.89, 28.11, 29.19 – 29.7, 31.92, 34.27, 36.00, 47.19, 57.7, 62.5, 76.69, 77.01, 77.33, 80.37, 171.6, 173.5; HRMS (ESI m/z) $[M + H]^+$ calculated for $C_{53}H_{101}NO_8+H^+$: 880.7606, found 880.7630 (Figure; Supplementary material).

2.2.3. 3,3'-((1,3-bis(stearoyloxy)propan-2-yl)azanediyl)dipropionic acid **3**

For the hydrolysis of tertiary butyl esters of compound 2, a previously reported method was adopted with slight modifications [214]. To the solution of compound 2 (7 g) in dry DCM, a mixture of TFA and TIPS (as scavenger) in the ratio 9:1 was added at room temperature and stirred for six hours. The excess TFA was removed under reduced pressure at room temperature, after which the crude mixture was purified by column chromatography (Chloroform/MeOH, 9: 1) to obtain a white solid, with the yield being 92%. Characterization data are as follows: IR (ATR): 3450, 2915, 2850, 1728, 1462, 1359, 1182 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 0.791- 0.825 (m, 6H), 1.18 (m, 57H), 1.49 – 1.52 (m, 4H) ,2.25 – 2.28 (t, 4H), 2.84 (m, 4H), 3.47 (m, 4H), 3.9 (m, 1H), 4.32 – 4.48 (m, 4H);^{13}C NMR (400 MHz, CDCl3): δ 14.10, 22.69, 24.60, 27.93, 28.72 – 29.75, 31.93, 33.6 – 33.77, 48.36, 58.73, 59.41, 82.6, 173.81,173.05; HRMS (ESI m/z) $[M+H]^+$ calculated for $C_{45}H_{85}NO_8+H^+$:768.6354; found 768.6348 (Figure S3;Supplementary material).



Scheme 2 Synthesis of oleic acid derived liquid lipid (OALL).

2.2.4. tert-butyl (1,3-dihydroxypropan-2-yl)carbamate **4**

To a stirred solution of serinol 3 g (0.0329 mol) in methanol, di-tert-butyl dicarbonate (9.3 g, 0.043 mol) was added and stirred overnight at room temperature [33]. Methanol was removed by evaporation to form a white crystalline compound 4 with a yield of 95.85%. Characterization data are as follows: IR (ATR): 2923, 1721, 1160 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.34 (s, 9H), 3.56 – 3.68(m, 5H), 3.8 (s, 1H), 5.43 (s, 1H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 28.3, 53.1, 62.3, 79.87, 156.4; HRMS (ESI m/z) $[M + H]^+$ calculated for $C_8H_{17}NO_4+Na^+$: 214.1056; found 214.1055 (Figure S3;Supplementary material).

2.2.5. 2-((tert-butoxycarbonyl)amino)propane-1,3-diyl dioleate **5**

To a solution of oleic acid (6.4 g, 0.022 mol) in dry DCM, DCC (5.1 g, 0.0247 mol), DMAP (0.7 g, 0.0057 mol) and compound 4 (2 g , 0.0105 mol) were added and stirred for 24 hours [213]. The DCC urea formed was filtered off and the solvent removed under reduced pressure. Isolation of the pure product from the reaction mass was done via column chromatography on silica gel (hexane/EtOAc, 8: 2) to obtain a yellow viscous lipid, with yield of 88.3%. Data from characterization are as follows: IR (ATR): 3345,

2850-2975 1724, 1467, 1366,1252, 1149 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.8 – 0.82 (m, 6H), 1.2 – 1.24 (m, 40H), 1.38 (s, 9H), 1.53 – 1.57 (m, 4H), 1.91 – 1.96 (m, 8H), 2.23 – 2.27 (m, 4H), 3.99 – 4.14 (m, 4H), 4.73 (m, 1H), 5.26 – 5.29 (m, 4H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 14.0, 22.6 ,24.8, 27.1, 28.3, 29.0, 29.1, 31.8, 34.0, 53.3, 62.8, 129-130, 156.4, 173.3; HRMS (ESI m/z) $[M + H]^+$ calculated for $C_{44}H_{81}NO_6+Na^+$: 742.5962; found 742.5953 (Figure S3;Supplementary material).

2.2.6. 2-aminopropane-1,3-diyl dioleate **6**

Compound 5 obtained from the earlier step was dissolved in dry DCM, and a mixture of TFA and TIPS in the ratio of 9:1 was added at room temperature and stirred for four hours [214]. After completing the reaction, the excess TFA was carefully removed under reduce pressure and the crude product purified via column chromatography (silica, mesh size 60–100) (Chloroform/MeOH, 9: 1) to obtain a yellow brownish viscous oil with a yield of 94%. Characterization was as follows: IR (ATR): 2923, 1708, 1672, 1461, 1165, 800, 722 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 – 0.90 (m, 9H), 1.27 – 1.3 (m, 43H), 1.58 – 1.61 (m, 4H), 1.92 – 1.95 (m, 7H), 2.28 – 2.29 (m, 4H), 4.21 – 4.33 (m, 4H), 5.24 – 5.32 (m, 4H); ^{13}C NMR (400 MHz, CDCl3) δ 14.0, 20.9, 22.54, 22.6, ,24.8, 25.6, 27.2, , 29.0, 29.1, 29.7, 31.9, 33.6, 33.9 , 48.5, 62.8, 127.88, 129.67, 129.96, 130.15, 155 173.61; HRMS (ESI m/z) $[M + H]^+$ calculated for $C_{39}H_{73}NO_4+H^+$: 620.5634; found 620.5618 (Figure S3;Supplementary material).

2.3. *In vitro* cytotoxicity

MTT assay was performed to evaluate the biosafety of SASL and OALL following a previously reported method [57, 215]. Human lung epithelial (A549), human embryonic kidney (HEK-293) and human liver (HEP G2) cell lines were cultured in a medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solution and incubated for 24 hours under a humidified atmosphere of 5% CO_2 at 37 ℃. Thereafter, SASL and OALL samples were dissolved in dimethyl sulfoxide and distilled water diluted in the culture medium to give final concentrations of 20, 40, 60, 80 and 100 µg/ml.

Following the exponential growth phase, the cell lines were collected and seeded equivalently (2.4×10^3) into a 96-well plate and incubated for 24 hours to allow cell adherence. Thereafter, the culture medium was removed and replaced with the fresh medium (100 ml per well) containing the test samples. Culture medium with cells only was used as a negative control, while culture medium without cells was used as a blank.

After incubation, the culture medium containing the test samples were removed and replaced with fresh culture medium and MTT solution (100 µl each). After four hours of incubation, the media and MTT solution were removed and 100 µl of dimethyl sulfoxide was added to each well to solubilize the MTT formazan. The absorbance corresponding to each well was measured using a microplate spectrophotometer (Spectrostar nano, Germany) at 540 nm, with all the experiments being run in six replicates. The following equation was used to determine percentage cell viability:

$$\% \text{ Cell viability} = \left(\frac{\text{A540nm treated cells}}{\text{A540nm untreated cells}}\right) \times 100 \qquad (1)$$

2.4. Preparation of VCM-SAOA-NLCs

A previously reported hot homogenization and ultrasonication method were employed to prepare the VCM loaded SASL and OALL pH-responsive NLCs (VCM-SAOA-NLCs) [216]. Briefly, the lipid phase, consisting of a total of 500 mg of OALL and SASL lipids in varying ratios and VCM (20 mg) were mixed and melted at a temperature above the melting point of the lipid. Meanwhile, the aqueous phase was prepared by dissolving 250 mg of Tween 80 in 20 ml distilled water and heated separately to the same temperature. Both phases were mixed and homogenized for 15 min at 6000 rpm with an Ultra Turrax T-25 homogenizer (IKA Labortechnik, Germany), the resultant emulsion being subjected to sonication (30% amplitude) for 10 min and subsequently cooled to 20 ºC using an ice bath, with the final volume being made up to 20 ml by adding distilled water. Blank (drug-free)-SAOA-NLCs was prepared using the same technique, with batches being prepared in triplicate.

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

The size, PDI, and ZP of VCM-SAOA-NLCs were measured by dynamic light scattering technique using a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., UK) at 25 °C. NLCs sample was appropriately diluted with appropriate phosphate buffer solutions (PBS) to obtain concentrations that were within the instrument's sensitivity range, the measurements being performed in triplicate [4, 31, 217].

The morphology of VCM-SAOA-NLCs was determined by transmission electron microscopy (TEM). The samples diluted and negatively stained with 1% uranyl acetate (UA) solution, then samples mixed with UA were deposited on a copper grid and air-dried before visualizing using a transmission electron microscope (JOEL JEM-1010, Japan) operated at an accelerating voltage of 100 kV [217].

2.6. Entrapment efficiency (EE) and drug loading (DL)

The EE and DL of VCM-SAOA-NLCs were determined by an ultrafiltration method. Briefly, NLCs suspension (3 ml) was placed in Amicon® Ultra-4 centrifugal filter tubes (Millipore Corp., USA) of 10 kDa pore size and centrifuged at 2000 rpm at 25 °C for 45 min [12]. The amount of the unentrapped VCM in the filtrate was detected using a validated High-Pressure Liquid Chromatography (HPLC) (Shimadzu, Japan) method, with UV detection at a wavelength of 280 nm. The mobile phase consisting of a mixture of water with 0.1 % TFA and acetonitrile (85/15 v/v), which was pumped through Nucleosil 100-5C18 column (150 mm X 4.6 mm internal diameter) at a flow rate of 1 ml/min. with an injection volume of 100 µl. The regression equation and linearity (r^2) were y = 32376 x − 149,208 and 0.9915 respectively. The EE (%) and DL (%) were calculated using the following equations [56, 193, 218, 219].

$$\text{EE (\%)} = \left(\frac{\text{Weight of VCM in NLCs}}{\text{Weight of VCM added}}\right) \times 100 \qquad (2)$$

$$\text{DL (\%)} = \left(\frac{\text{Weight of VCM in NLCs}}{\text{Total weight of nanoparticles}}\right) \times 100 \qquad (3)$$

2.7. Thermal profiles

The thermal profiles of the VCM, SASL, physical mixture and lyophilized drug loaded NLCs were determined by differential scanning calorimetry (DSC) using a DSC-60 differential scanning calorimeter (Shimadzu, Japan). Briefly, appropriate quantities of the samples were placed in aluminum pans and sealed with lids. The scanning was performed in a temperature range of 30 °C to 300 °C at a rate of 10 °C/min with nitrogen flow rate of 10 ml/min, with an empty pan being used as a reference [156].

2.8. *In vitro* drug release

The *in vitro* release studies were carried out using a dialysis bag technique to investigate the release behavior of drug loaded pH-responsive NLCs and blank-SAOA-NLCs. Dialysis bags (pore size: 8000–14,400 Da) containing 2 ml of the drug-loaded NLCs and its respective blank were placed in 40 ml PBS solutions of pH 6 and 7.4 at 37 °C in a shaking incubator and stirred at 100 rpm.

At predetermined time intervals, 3 ml of the samples were withdrawn and replaced with equivalent quantities of the fresh PBS solutions of pH 6 and 7.4 to maintain a constant volume. The amount of VCM released at each time interval was determined by HPLC (Shimadzu, Japan) with UV detection at a wavelength of 280 nm, as described above. The regression equation and linearity (r^2) were y = 32376 x - 149,208 and 0.9915 respectively, with each experiment being performed in triplicate [56, 156, 219, 220].

2.9. *In vitro* drug release kinetics and mechanism

Drug release kinetics of VCM-SAOA-NLCs were investigated using various mathematical models, including zero order, first order, Higuchi, Weibull, Hixson-Crowell, and Korsmeyer–Peppas. The correlation coefficient (R^2) and root mean square error (RMSE) were calculated to obtain the best fit model. Moreover, the Korsmeyer–Peppas model release exponent (n) was calculated to determine the release mechanism. These *in vitro* drug release kinetics parameters were calculated using DDSolver software [219, 221].

2.10. Molecular Modelling

Molecular modeling was undertaken to understand the interactions that occurred between the VCM and lipids carriers (SASL and OALL), as well as the effect of lipid ratio on the EE of VCM in the presence of a constant ratio of Tween 80 as a stabilizer [23]. All molecular modeling techniques were performed using Biovia Materials Studio (MS) 2016 that was installed on the remote server at the Centre for High-Performance Computing (CHPC) (Cape Town South Africa). The structure of VCM (PDB:1SHO) was obtained from RCSB website, SASL and OALL were drawn using ChemBioDraw Ultra 14, and the Tween 80 structure was obtained from PubChem website (CID 443315), after which all the structures were cleaned and the hydrogen atoms were added.

The smart minimizer algorithm in the geometry optimization option available in the forcite module of MS software was used to optimize all the structures to their lowest energy conformations. COMPASS force field was applied and the convergence tolerance criteria were set to 0.001 kcal/mol during the geometrical optimization study. The molecular dynamics (MD) study was performed in a vacuum to obtain a stable complex of VCM-SAOA-NLCs. All VCM, SASL, OALL and Tween 80 structures were initially placed inside the cubic cell (10 x 10 x 10 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 over 50 ps. In order to understand the effect of the lipid ratio on EE all the MD, studies were conducted in two different ratios of 2:1 and 1:1 for SASL and OALL, respectively. The final complex structures were then studied for the intermolecular interactions to understand the various non-covalent forces that were responsible for the complex formation. Biovia Discovery Studio Visualizer was used to depict the interactions in the drug-lipid complex.

2.11. *In vitro* antibacterial activity

The minimum inhibitory concentration (MIC) values for bare VCM, VCM-SAOA-NLCs and the blank (drug-free)-SAOA-NLCs were determined against SA and MRSA at pH 6 and 7.4 using a broth dilution method. Bacterial cultures were grown overnight in Nutrient Broth at 37°C for 18 hours in a shaking incubator (Labcon, USA) at 100 rpm. The bacterial cultures were diluted with sterile distilled water to achieve a concentration equivalent to 0.5 McFarland's Standard using a DEN-1B McFarland densitometer (Latvia). This was further diluted to 1:150 with sterile distilled water to achieve a final concentration of 2×10^5 colony forming units (CFU)/ml. Serial dilutions of bare VCM, VCM-SAOA-NLCs, and blank-SAOA-NLCs were prepared in MHB (pH 6 and 7.4), inoculated with the diluted bacterial cultures and incubated at 37°C in the shaking incubator at 100 rpm for 18 hours. After incubation, the test samples (10 µl) were spotted onto MHA and incubated at 37°C for 18 hours, this process was repeated every day for five days, with the studies being performed in triplicate. The blank formulation of NLCs was used as a negative control while the bare VCM was used as a positive control [57, 222, 223].

2.12. Bacterial cell viability assay

Cell viability studies on the MRSA cells were performed following a flow cytometry assay method. A pure culture of MRSA was grown overnight in Nutrient Broth at 37°C in a shaking incubator (Labcon, USA) at 100 rpm. Thereafter, the bacteria were diluted with sterile deionized water to achieve a concentration equivalent to 0.5 McFarland standard using a DEN-1B McFarland densitometer (Latvia). The bacterial suspension was further diluted to 1:150 with sterile deionized water to obtain a final concentration of 5×10^5 colony forming units (CFU)/ml. Thereafter, 15 µl of the bacterial suspension was added to a 96 well plate containing 135 µl of pure VCM (positive control), and VCM-SAOA-NLCs at the MIC concentrations (7.8 µg/ml and 0.98 µg/ml respectively), and incubated at 37 °C in a shaking incubator (100 rpm). The untreated MRSA cells were used as a negative control. The percentage cell viability being determined after six hours of incubation [224]. VCM and VCM-SAOA-NLCs broths (50 µl) were added to flow cytometry tubes each containing 350 µl of the sheath fluid and vortexed for 5 min. The mixture was incubated for 30 min with 5 µl of a non-cell wall permeant propidium iodide

(PI) fluorescent nucleic acid dye that binds to DNA by intercalating between the bases. PI has an excitation and emission spectrum spectra of 488/636 nm, and once DNA binds, its fluorescence is enhanced 20- to 30-fold to the blue wavelength [225-227]. The BD FACSCANTO II (USA) instrument was used for flow cytometry, with its settings including a sheath fluid flow rate of 16 ml/min and a sample flow rate of 0.1 ml/min. Data with the fixed cells were collected using a flow cytometer software (BD FACSDIVA V8.0.1 software [USA]), after which the captured data were analyzed using Kaluza-1.5.20 (Beckman Coulter USA) flow cytometer software. The voltage settings used for fluorescence-activated cell sorting (FACS) analysis were: 731 (forward scatter [FSC]), 538 (side scatter [SSC]), 424 (Fluorescein isothiocyanate [FITC]) and 444 for PI. Bacteria were initially gated using forward scatter. Subsequently, cells of the appropriate size were then gated with at least 10,000 cells being collected for each sample in triplicate, then the position of the 'live' and 'dead' cells gates was determined. To avoid background signal from particles smaller than bacteria, the detection threshold was set to 1,000 in SSC analyses [228].

2.13. *In vivo* antibacterial activity and histological evaluation

For further evaluation of the anti-MRSA activity of the optimal VCM-SAOA-NLCs formulation, a mouse skin infection model was used for *in vivo* antibacterial activity assessment, as per the protocol described in other papers [31, 45, 57]. BALB/c mice weighing 18-20 g were obtained from the University of KwaZulu-Natal's Biomedical Research Unit. A day before the experiment, the back hair of the mice was shaved off and the mice skin was disinfected with 70% ethanol. The next day, the mice were divided into three groups of four mice each: positive control, treatment, and negative control. Then, 50 µl of MRSA was diluted in saline solution to achieve a concentration of 1.5 x 10^8 CFU/ml and administered intradermally. Thirty minutes later the positive control group and treatment group were injected at the same infected sites of the mice with bare VCM and VCM-SAOA-NLCs respectively, while the negative control group was injected with the MRSA only. The mice were kept under observation for 48 hours under the normal condition of 12 hours light and 12 hours darkness at 19–23°C, 55 ± 10 % relative humidity with adequate ventilation. Thereafter, the mice were sacrificed and the infected skin was

harvested and homogenized in 5 ml of PBS (pH 7.4). The tissue homogenates were serially diluted with PBS (pH 7.4), after which 20 µl were spotted onto on nutrient agar plates and incubated at 37 °C for 24 hours and the CFU was determined [4, 229].

Histological evaluations were conducted on the excised skin from the injection site, with the skin samples being transferred directly after harvesting and excision from normal saline into 10% buffered formalin. After seven days, the skin samples were embedded in paraffin wax after dehydration in an ethanol gradient. 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). The sections were examined and captured with a Leica Microscope DM 500 that was fitted with a Leica ICC50 HD camera (Leica Biosystems, Germany) [57]. All the animal experiments were performed in accordance with the protocol approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (Approval number. AREC/104/015PD).

2.14. Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests using GraphPad Prism® 6 (GraphPad Software Inc., USA). Individual groups were compared against each other and the difference considered significant when the *P* value was < 0.05, with the results being expressed as a mean ± standard deviation (SD).

3. Results and discussion

3.1. Synthesis and characterization of the lipids

The solid lipid SASL was synthesized in three steps. The first step involved the bis-aza-Michael addition of tertiary butyl acrylate to serinol to form compound 1 which was confirmed by both ^{1}H NMR and ^{13}C NMR. The appearance of a strong multiplet at δ 1.37 ppm integrating for 18 protons in ^{1}H NMR, and the appearance of carbon peaks at δ 28, 35, 80 and 172 in ^{13}C NMR, representing isobutane carbons, -CH2C=O-, $C(CH_3)_3$-COO - and C=O, confirmed the formation of compound 1. Secondly, the two free hydroxyl groups on compound 1 were coupled with stearic acid using DCC/DMAP chemistry to obtain compound 2. The product was confirmed by the appearance of aliphatic peaks at δ 0.808 (multiplet), δ 1.18 (multiplet) and δ 1.53 (multiplet) on ^{1}H NMR. Lastly, tertiary butyl esters of compound 2 were hydrolyzed to form SASL, using TFA and TIIPS as scavengers to avoid any possible side reactions. The successful synthesis of SASL was confirmed by the disappearance of isobutane peaks at 1.4 ppm in ^{1}H NMR and at 28 ppm in ^{13}C NMR.

For the OALL synthesis, firstly, the amine group on serinol was protected using di-ter-butyl dicarbonate (t-Boc) and the product confirmed by both ^{1}H NMR and ^{13}C NMR. A peak appeared at δ 1.34 ppm in ^{1}H NMR, which integrated to 9 protons, while the peak at δ 28.3 ppm in ^{13}C NMR confirmed the isobutane chain. In addition, a peak at δ 156. 4 ppm confirmed the presence of carbonyl group of boc amide which confirmed the t-Boc protection of an amino group of serinol (compound 4). Compound 4 was coupled with oleic acid using DCC and DMAP as catalysts to yield compound 5. In ^{1}H NMR, the characteristic peaks at δ 0.803 to 0.837 ppm and peaks at δ 5.28 to 5.299 represented terminal -CH_3, and olefinic protons of oleic acid respectively, confirming the synthesis of compound 5, these peaks integrating to their expected theoretical calculated protons (supplementary material). Compound 5 was subjected to Boc deprotection using TFA and TIIPS to obtain compound 6 as OALL. The disappearance of isobutane peaks at δ 1.4 ppm in ^{1}H NMR and at δ 28 ppm in ^{13}C NMR indicated the successful cleavage of boc deprotection and confirmed the successful synthesis of OALL.

3.2. *In vitro* cytotoxicity

A cytotoxicity study was performed in order to determine the biosafety of the newly synthesized lipids. The MTT results revealed that the synthesized novel lipids SASL and OALL displayed a high percentage (> 75%) cell viability after 48-hour exposure across the concentration range studied (Fig. 1). SASL and OALL displayed a cell viability between 77.55 to 94.83 % and 75.70 to 87.20 % respectively (Fig. 1 A and B respectively). Although the % cell viability decreased slightly at the higher concentrations, there were no clear dose-dependent trends observed for either SASL or OALL across all the cell lines studied (Fig. 1). Studies report that cell viabilities greater than 75% are considered as low toxicity in the framework of safety use [57, 230]. SASL and OALL can, therefore, be considered to be biologically safe and nontoxic to mammalian cells.

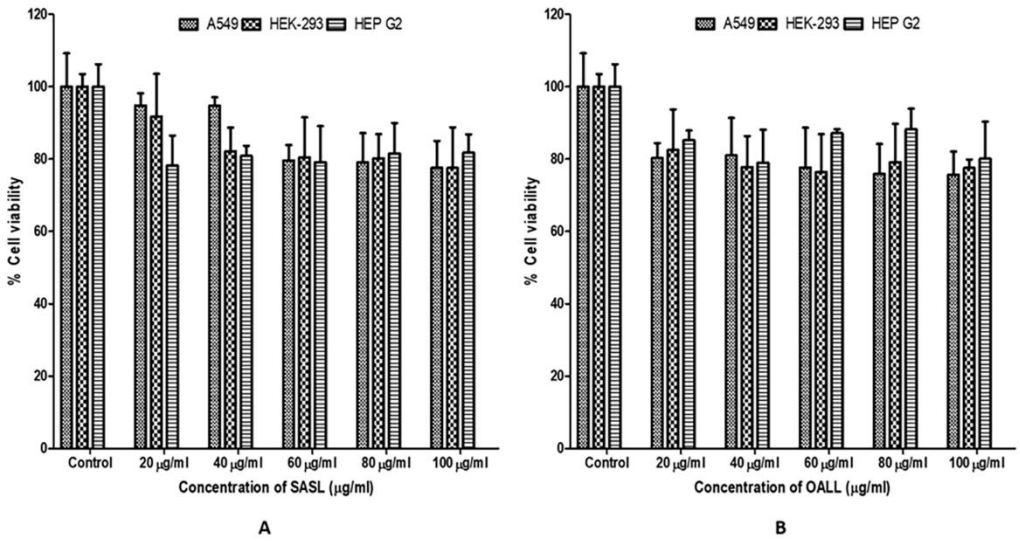


Fig. 1. Cytotoxicity evaluation of various concentrations of A) SASL B) OALL against A 549, HEK-293, and HEP G2 cells.

3.3. Preparation of drug loaded NLCs

Having confirmed the structure of SASL and OALL and their biosafety, their potential for NLCs formation was then explored. VCM-SAOA-NLCs were prepared from the newly synthesized lipids using OALL as liquid lipid and SASL as a solid lipid, a Tween 80 surfactant, and VCM by hot homogenization followed by ultrasonication. Preliminary studies were performed using different ratios of lipids to obtain a formulation with

optimal size, PDI and acceptable switch from negative to the positive charge in different pH with maximum entrapment efficiency of the VCM (Table 1).

Table 1 Particle size, PDI, and ZP of VCM-SAOA-NLCs

SASL: OALL ratio	**pH**	**Particle size (nm)**	**PDI**	**ZP (mV)**	**EE%**
0.5:1	7.4	555.7 ± 76.3	0.70 ± 0.27	-4.7± 0.7	57.23± 3.0
	6.0	798.6 ± 318	0.72 ± 0.28	-2.8 ± 0.9	
	4.5	854,7 ± 125	0.75 ± 0.20	11.0 ± 1.3	
1:1	7.4	290.3 ± 20	0.36 ± 0.08	-7. 4 ± 1.3	69.25±2
	6.0	358.3 ± 38	0.49 ± 0.06	0.2 ± 0.3	
	4.5	442.8 ± 80	0.49 ± 0.40	12.1 ± 0.4	
2:1	7.4	225.2 ± 9.1	0.258 ± 0.02	-9.2 ± 2.7	88.7±13.12
	6.0	322.4 ± 37.7	0.386 ± 0.02	3.37 ± 0.8	
	4.5	458.7 ± 6	0.431 ± 0.04	9.8 ± 0.4	

At pH 7.4, as the solid lipid: liquid lipid ratio increased from 0.5:1 to 2:1, the particle size and PDI of the NLCs decreased from 555.7 nm to 225.2 nm and 0.7 to 0.2 respectively, whereas the ZP values shifted from -4.7 mV to -9.2 mV, while the EE percentage improved from 57% to 88%. The increase in particle size, in accordance with an increase in liquid lipid content, may have led to an increase in the viscosity and consequently NLCs swelling [231, 232].

Alternatively, the higher solid lipid to liquid lipid ratios formed more stable nanoparticles with homogeneous size distributions and enhanced EE. The size, PDI, ZP, and EE of VCM-SAOA-NLCs formulations were similar to other reported NLCs using fatty acids lipids [147, 233, 234] and confirmed the ability of the newly synthesized lipids to form VCM loaded NLCs.

The VCM-SAOA-NLCs formulation with a solid lipid: liquid lipid ratio of 2:1 specifically displayed a size, PDI and ZP of 225.2 ± 9.1 nm, 0.258 ± 0.02, -9.2 ± 2.7 mV respectively. The EE (%) and DL (%) for this VCM-SAOA-NLCs were found to be 88.7 ± 13.12 % and 3.55 ± 0.52 % respectively. This enhanced EE (%), compared to the NLCs with lower solid lipid, could be due to the higher partitioning of the VCM free base in the lipid matrix, due to its hydrophobic nature. In addition, SASL has two carboxylic arms, which increase the ion pairing between the solid lipid and VCM, leading to higher entrapment. Similar results were reported where ion-pairing mechanism enhanced the EE (%) of poorly soluble drugs in solid lipid nanoparticles [12, 235, 236] and polymeric nanoparticles [237]. Thus, the VCM-SAOA-NLCs system consisting of a solid lipid: liquid lipid ratio of 2:1 was considered the optimal formulation, as it had the lowest size, smallest PDI and highest stability with highest EE (%), and was characterized further.

3.4. Characterization of the optimal VCM-SAOA-NLCs

To obtain more information about the VCM-SAOA-NLCs size and morphology, a TEM investigation was performed. The TEM study demonstrated that VCM-SAOA-NLCs had a well-defined spherical shape with an average size of 235.6 nm, which correlated well with the results of the dynamic light scattering technique (Fig. 2), these results being comparable to other NLCs reported [127, 238].

To determine the pH responsiveness of the VCM-SAOA-NLCs, they were dispersed in buffer solutions with different pH ranges (7.4, 6 and 4.5). The optimal formulation, as shown in Table 1, shows pH responsiveness in terms of size, PDI, and ZP. At pH 7.4, 6 and 4.5, the VCM-SAOA-NLCs size increased from 225.2 nm to 322.4 nm and 458.7 nm respectively, the PDI increased from 0.258 to 0.386 and 0.431 respectively, and the ZP also switched from -9.2 ± 2.7 mV to +3.37 mV and +9.8 mV respectively.

In the VCM-SAOA-NLCs, the primary amine group of OALL binds to the carboxylic acid group of SALL by proton exchange ionic interaction [239]. At pH 7.4, the NLCs are negatively charged due to the carboxylate ions of the SASL on the NLCs surface. At low pH, due to the high concentration of H^+ ions, both the amine group of OALL and the carboxylate group of SASL remain protonated, which results in a breakage of the ionic

interaction between the two lipids [240-242], this phenomenon was demonstrated by the positive ZP at low pH. The breakage of the ion pair bridge between the two lipids minimizes their affinity to each other, causing disassembly of the NLCs-dispersion and an eventual increase in their hydrodynamic diameter. The above change in size signifies a change in the conformational structure of the NLCs, while the switch from negative to a positive charge is expected to have an influence on increasing drug release and promoting interactions with the negatively charged bacterial cell wall for enhancing antibacterial activity [28, 31, 45, 243].

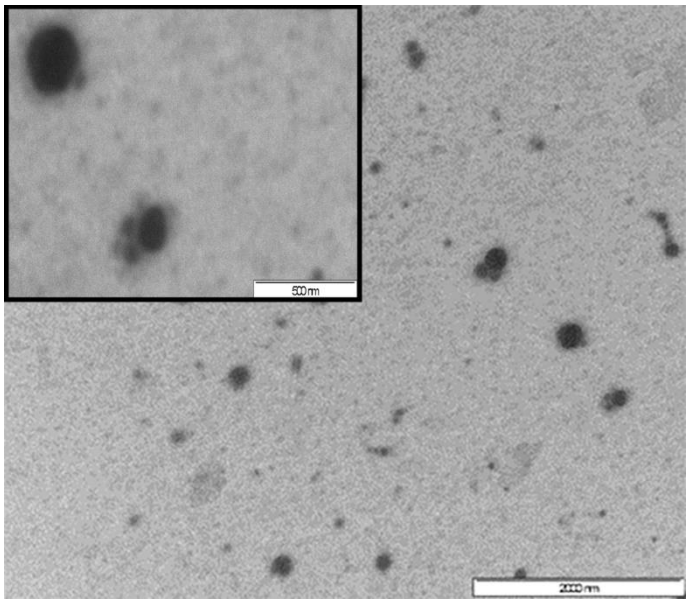


Fig. 2. Morphology of VCM-SAOA-NLCs.

3.5. Thermal profiles

The purpose of DSC study was to confirm the entrapment of VCM in the NLCs through investigating the crystallization state and change of thermal behavior of the NLCs components. The thermal profiles of SASL, VCM, lyophilized VCM-SAOA-NLCs and the physical mixture of VCM and SASL were compared (Fig. 3). The endothermic peaks of SASL and VCM were observed at 71.53ºC and 103.40ºC respectively, with the physical mixture showing nearly the same thermal behavior as the individual components. While the peak for the lyophilized VCM-SASL-NLCs appeared at 62.57ºC, the VCM

peak disappeared, which could be attributed to its transformation from the crystalline form into the amorphous form, as it was entrapped within the NLCs matrix [66].

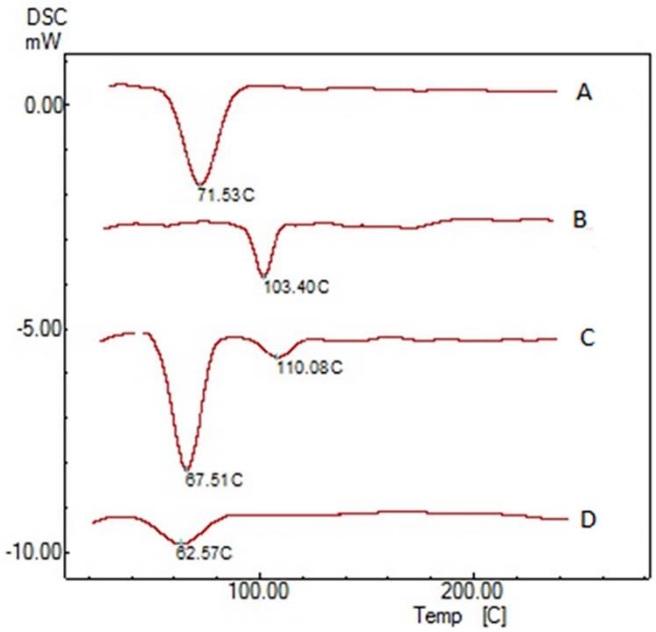


Fig. 3. DSC thermogram of (A) SASL (B) VCM (C) physical mixture of VCM and SASL and (D) lyophilized VCM-SAOA-NLCs.

3.6. *In vitro* release profile

The *in vitro* release profile of the bare VCM and VCM-SAOA-NLCs were investigated using the dialysis bag diffusion technique at pH 7.4 and pH 6. At pH 7.4, the bare VCM release was almost complete within the first 24 hours, while VCM-SAOA-NLCs released only 47 % at the same time point (Fig. 4). In comparison with bare VCM, the VCM-SAOA-NLCs release was slower, indicating sustained release profiles. This prolonged-release could be attributed to the lipid matrix of the VCM-SAOA-NLCs, which retain the hydrophobic VCM free base for a longer time [12]. In addition, fatty acids long carbon chain length slows the diffusion rate of the drug, resulting in slower drug release [22, 147, 154], which could be beneficial for prolonged and sustained antibacterial activity [12, 48, 244].

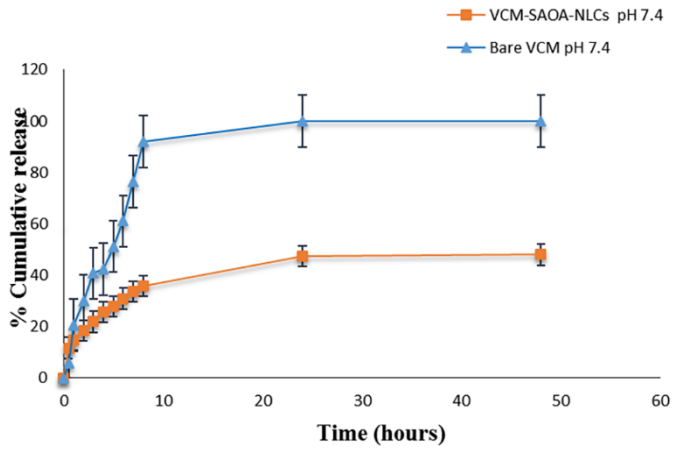


Fig. 4. *In vitro* drug release profiles of bare VCM and VCM-SAOA-NLCs at pH 7.4 (n= 3).

Whilst the VCM-SAOA-NLCs release was similar up to the 4th hour at both pH conditions (Fig. 5) thereafter, it had a statistically significant ($P < 0.05$) faster release at pH 6 compared to pH 7.4 up to 48 hours. This higher release at lower pH was attributed to the protonation of OALL and SASL at acidic media, leading to a breakage of the ion pair between the two lipids and the nanoparticles hydrodynamic diameter increasing due to electrostatic repulsion between the lipids. This resulted in the NLCs swelling and breaking and subsequently leading to higher and faster drug release. This pH-sensitive release behavior is significant for enhanced VCM protection at physiologic pH, improved targeted release, enhanced localization, and bioavailability at acidic infection sites, thus improving the antibacterial activity [28, 31, 45].

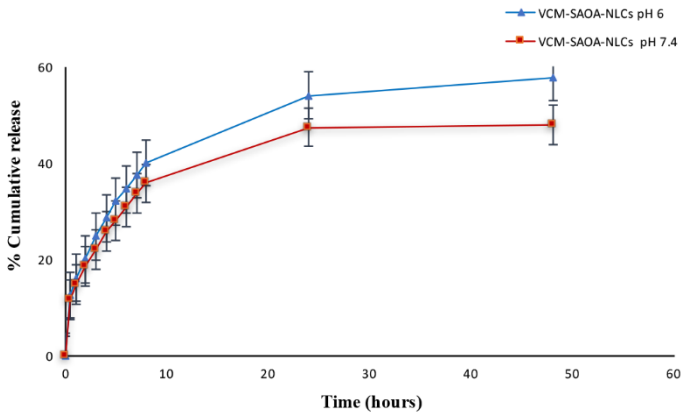


Fig. 5. Effect of pH on drug release profiles of VCM-SAOA-NLCs (n= 3).

3.7. *In vitro* drug release kinetics

The release data for kinetic analysis of the optimized VCM-SAOA-NLCs was fitted into zero order, first order, Higuchi, Weibull, Hixson-Crowell, and Korsmeyer–Peppas models (Table 2). Although the VCM-SAOA-NLCs release was higher at pH 6, it had similar kinetic behavior to the release at pH 7.4. The highest R^2 values were 0.94 and 0.961, while the lowest root means square error (RMSE) values were 3.04 and 2.89 at pH 7.4 and pH 6 respectively. Thus, the Weibull model was considered the best fit model for the VCM-SAOA-NLCs release at both pH conditions. The Weibull exponent parameter (β) value is used to interpret the drug release mechanism, with $\beta \leq 0.75$ indicating a Fickian diffusion, and $0.75 < \beta < 1$ indicating a combined mechanism, whereas a β value higher than 1 is associated with the complex release mechanism. The values of exponent parameter (β) for VCM-SAOA-NLCs were 0.37 and 0.418 at pH 7.4 and pH 6 respectively, indicating Fickian diffusion as the release mechanism. These results were consistent with the (n) values of Korsmeyer-Peppas model, whereas (n) values at pH 7.4 and pH 6 and were 0.289 and 0.308 respectively, confirming the Fickian mechanism of VCM release from the VCM-SAOA-NLCs, as (n) the value was less than 0.5 [219, 221, 245, 246].

Table 2 Release kinetics data from different models.

Model	Equation	R^2		RMSE		Release exponent (n)	
		pH 6	pH 7.4	pH 6	pH 7.4	pH 6	pH 7.4
Zero Order	$Q = k.\ t + Q_0$	1.460	-1.783	22.218	20.075		
First Order	$Q = Q_0 . e^{kt}$	-0.018	-0.600	14.185	15.219		
Higuchi	$Q = k.\ t^{½}$	0.625	0.495	8.667	8.542		
Korsmeyer-Peppas	$Q = k\ .\ t^n$	0.936	0.913	3.601	3.531	0.308	0.289
Hixson-Crowell	$Q^{⅓} = kt + Q_0^{⅓}$	-0.377	-1.003	16.517	17.025		
Weibull	$Q = 1 \exp [-(t)a/b]$	0.961	0.935	2.801	3.043		

R^2 = linear regression coefficient, RMSE = Root mean square error

3.8. Molecular Modeling

Molecular modeling was used to investigate the interaction of VCM with the SAOA-NLCs system, and the effect of the solid and liquid lipid ratio on drug entrapment efficiency. The initial energy of VCM free base, SASL, OALL and Tween 80 were found to be 1509.249, 590.150, 354.144 and 213.780 kcal/mol, respectively. Geometry optimization using the smart minimizer algorithm in the forcite module led to producing more stable molecules, with final energies of 123.318, -164.488, -47.506, and -1.317 kcal/mol for VCM, SASL, OALL and Tween 80, respectively. The molecular dynamics (MD) study for the 2:1 lipid ratio revealed the formation of VCM-SAOA-NLCs. The potential energy (mass of the complex) increased from 866.075 kcal/mol to 1367.44 kcal/mol by the end of MD study (Fig. 6 A), this energy increase confirming the successful formation of the VCM-SAOA-NLCs system, as a phase transformation of the individual components of the NLCs occurred and produced one stable NLC system [247]. When the ratio of the solid lipid to liquid lipid was 1:1, the potential energy was found to be 1603.991 kcal/mol by the end of the MD study, and the VCM was observed to interact with Tween 80 rather than the SASL or OALL (Fig. 6 B). The lower potential energy at a 2:1 ratio (1367.44 kcal/mol) indicated better stability of the system. Furthermore, the VCM was incorporated into the SASL (Fig. 6 A) rather than being repelled out, as in the

case of 1:1 ratio. This observation may explain the higher EE of VCM at solid lipid: liquid lipid of 2:1 compared to the lower EE when the solid lipid: liquid lipid ratio was 1:1 (Table 1), and can be explained by the competition between the liquid lipid and the VCM to interact with the solid lipid. Increasing the amount of solid lipid led to the higher partitioning of the VCM free base in the lipid, as the SASL has two carboxylic arms, which increases the ion pairing between the solid lipid and VCM, subsequently improving the EE [12]. These studies, therefore, confirmed the stability of the NLCs system and identified the mechanism behind the higher EE with a higher ratio of the solid lipid (SASL) than the liquid lipid (OALL).

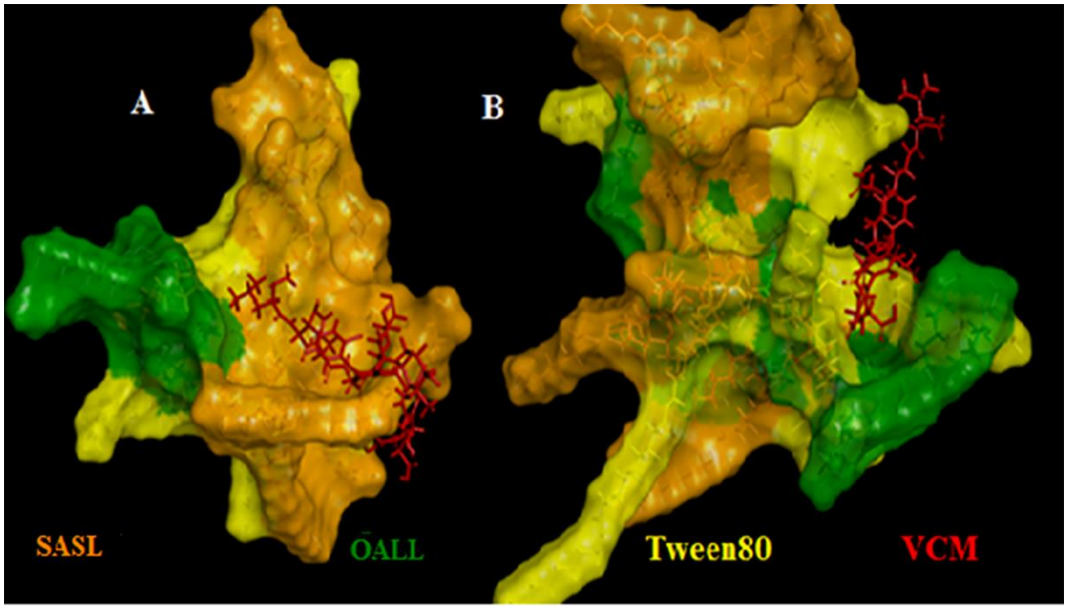


Fig. 6. The multivalent binding phenomenon of SASL, OALL and Tween 80 with VCM. (A) Solid lipid: liquid lipid is 2:1 (B) Solid lipid: liquid lipid is 1:1

3.9. *In vitro* antibacterial activity

The *in vitro* antibacterial activities of bare VCM, VCM-SAOA-NLCs and the blank-SAOA-NLCs were investigated against SA and MRSA at pH 6 and 7.4 using a broth dilution method (Table 3). The MIC value of bare VCM against SA was 3.9 µg/ml at both pH conditions and increased to 7.8 µg/ml against MRSA at both pH conditions. MIC values of VCM-SAOA-NLCs against SA were 1.95 µg/ml and 0.488 µg/ml, whereas the

MIC values of VCM-SAOA-NLCs against MRSA were 1.95 µg/ml and 0.98 µg/ml at pH 7.4 and pH 6 respectively. These results showed that the VCM-SAOA-NLCs had lower MIC values and thus superior antibacterial activity compared to the bare VCM against both bacterial strains at both pH conditions. Furthermore, the VCM-SAOA-NLCs formulation activity was extended up to 96 hours, while the bare VCM had an activity for only 48 hours.

The enhanced extended anti-microbial activity, in comparison to the bare VCM, could be attributed to the small size with the large surface area as well as the lipophilic nature of VCM-SAOA-NLCs, which could enable better penetration and uptake into the bacterial cell wall, thus enhancing the VCM activity [4, 57, 199]. In addition, the VCM-SAOA-NLCs release over a prolonged period of time (Fig. 5) could have contributed to the extended activity of up to 96 hours compared to bare VCM [12, 248]. Additionally, the antibacterial activity of oleic acid can enhance the VCM-SAOA-NLCs efficacy [12, 249, 250].

Importantly, the VCM-SAOA-NLCs had lower MIC values at pH 6 than at pH 7.4 against both bacterial strains. At pH 6, the VCM-SAOA-NLCs activity was four and two times better against SA and MRSA respectively than at pH 7.4. The superior antibacterial activity of the VCM-SAOA-NLCs at acidic pH compared to pH 7.4 may be due to the protonation of the VCM-SAOA-NLCs and ion pair bridge breakage, and hence a higher VCM release at the acidic site. In addition, the VCM-SAOA-NLCs surface charge switching to positive in acidic media can enhance the binding of the VCM-SAOA-NLCs with the anionic bacterial cell wall, and therefore release a higher VCM concentrations at the acidic infection site, subsequently increasing its uptake by the bacteria cell wall and reducing the total number recovered. Therefore, targeting the acidic bacterial site via pH-responsive nanoparticles represents a very promising platform to develop delivery systems that enhance drug localization at infections site to improve the antibacterial activity. This strategy will, therefore, go a long way in reducing the emergence of bacterial resistance [28, 31, 45].

Table 3 *In vitro* antibacterial activity of the formulations at pH 7.4 and pH 6

***In vitro* antibacterial activity at pH 7.4**								
Time (hours)	**24**	**48**	**72**	**96**	**24**	**48**	**72**	**96**
	SA (MIC µg/ml)				**MRSA (MIC µg/ml)**			
Bare VCM	3.9	250	NA	NA	7.8	NA	NA	NA
VCM-SAOA-NLCs	1.95	1.95	1.95	3.9	1.95	1.95	1.95	1.95
Blank- SAOA-NLCs	NA	NA	NA	NA	NA	NA	NA	NA
***In vitro* antibacterial activity at pH 6**								
Time (hours)	**24**	**48**	**72**	**96**	**24**	**48**	**72**	**96**
	SA (MIC µg/ml)				**MRSA (MIC µg/ml)**			
Bare VCM	3.9	500	NA	NA	7.8	NA	NA	NA
VCM-SAOA-NLCs	0.488	0.488	1.95	3.9	0.98	0.98	1.95	7.8
Blank-SAOA-NLCs	NA	NA	NA	NA	NA	NA	NA	NA

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

3.10. Bacterial cell viability assay

Having confirmed the antibacterial activity of VCM-SAOA-NLCs, a flow cytometry method was used to quantify the viable MRSA bacteria cells after exposure to bare VCM and VCM-SAOA-NLCs **,**and to investigate the VCM uptake [224, 251]. Following incubation of MRSA cells with bare VCM and VCM-SAOA-NLCs medium for six hours, live/dead bacterial cells in the population were discriminated by detection of the changes in bacterial morphology and the internal complexity using PI fluorescent dye. As PI cannot cross the membrane of live cells, it is generally used to detect dead cells in the population [226, 252].

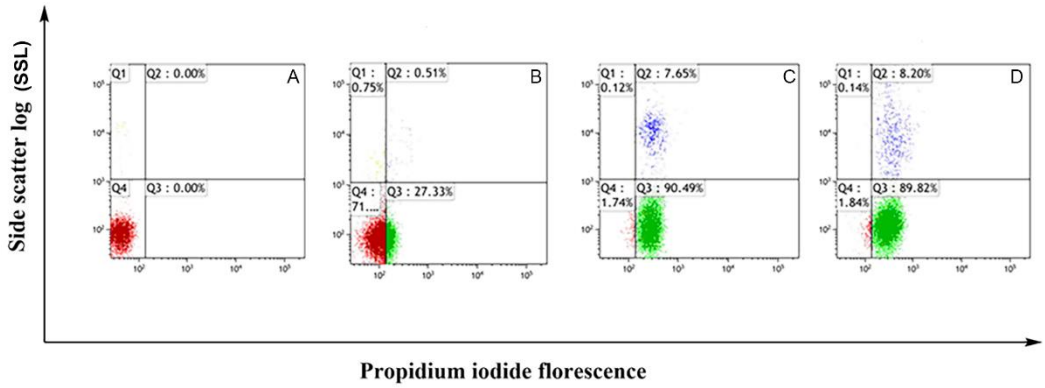


Fig. 7. Dot plots of flow cytometry data of subpopulation phenotypes of MRSA cells after treatment A) MRSA cells (negative control), B) MRSA cells treated with bare VCM at 0.488 µg/ml, C) MRSA cells treated with bare VCM at its MIC (7.8 µg/ml) and D) MRSA treated VCM-SAOA-NLCs at its respective MIC (0.98 µg/ml).

The phenotypic changes in MRSA cells before and after treatment with bare VCM and VCM-SAOA-NLCs at their respective MICs (7.8 µg/ml and 0.98 µg/ml respectively) were investigated by plotting PI fluorescence log versus SSC log. MRSA cells treated with bare VCM at lower concentration (0.488 µg/ml) was investigated in order to study the effect of bare VCM on MRSA at a concentration lower than the MICs of both bare VCM and VCM-SAOA-NLCs which will help to explain the role of SAOA-NLCs to enhance the activity of VCM (Fig. 7) [226].

Live MRSA cells (negative events) are represented in dot plots with the quadrants 1 and 4 (Q1 and Q4). Dead MRSA cells (positive events) showed fluorescence shifts as represented in the quadrants 2 and 3 (Q2 and Q3). These shifts indicating the PI uptake and intercalation with the DNA of the dead bacterial cells, confirming the changes in cell morphology and complexity as result of VCM bactericidal activity.

MRSA cells showed two distinct subpopulations after treatment with bare VCM and VCM-SAOA-NLCs. The first population displayed a shift in PI fluorescence with no shift in the SSC scale as shown by Q3 (Fig. 7 B, C, and D). For negative control cell, there was 0 % of dead cells in Q3 (Fig. 7 A). However, MRSA cells treated with bare VCM and VCM-SAOA-NLCs at their respective MICs (7.8 µg/ml and 0.98 µg/ml respectively) had 90.49 ± 1.23 % and 89.82 ± 1.83 % of dead cells respectively in Q3 (Fig. 7 C and D). Interestingly, MRSA cells treated with bare VCM at concentration half

of VCM-SAOA-NLCs MIC had a lower percentage of dead cells (27.33 %) as shown in Q3 (Fig. 7 B).

The second population showed a shift in PI fluorescence and a shift on SSC scale (Fig. 8 B, C, and D – Q2). Cells in quadrant (Q2) represented PI cell penetration and intercalation with DNA, therefore, resulting in a shift in fluorescence. Cells treated with bare VCM and VCM-SAOA-NLCs at their respective MICs had PI uptake of 7.65 ± 1.49 % and 8.20 ± 0.83% respectively as shown in Q2 (Fig. 7 C and D), while bare VCM at the concentration half of VCM-SAOA-NLCs MIC had PI uptake of 0.51 % (Fig. 7 B)

These results clearly demonstrate that VCM-SAOA-NLCs produced the same killing effect and slightly better penetrating capabilities although the concentration was 8-fold lower compared to bare VCM MIC. Therefore, VCM-SAOA-NLCs had higher killing effects compared to bare VCM .This confirms that VCM-SAOA-NLCs had a greater impact on the cell wall in comparison to bare VCM and hence resulted in higher uptake of PI leading to the change in the phenotype of cells as shown in Q2 [253].

3.11. *In vivo* antibacterial activity

Having confirmed the *in vitro* antibacterial activity of the VCM-SAOA-NLCs formulation, we then assessed its *in vivo* efficacy to confirm its performance. The BALB/c mouse skin infection models were used to further evaluate the VCM-SAOA-NLCs activity against MRSA, its counts being 151600, 30000 and 800 CFU/ml for untreated, bare the VCM and VCM-SAOA-NLCs groups respectively. These results further reflect the enhanced activity of the VCM-SAOA-NLCs against MRSA compared to the bare VCM.

There was a statistically significant ($P < 0.05$) reduction in MRSA counts that remained in the skin of the mice treated with VCM-SAOA-NLCs and bare VCM compared to the untreated group. The bacterial burdens were 189- and 5-fold less in the mice skin treated with VCM-SAOA-NLCs and bare VCM respectively than the untreated group. However, there was a significant ($P < 0.05$) 37-fold reduction in the bacterial load treated with VCM-SAOA-NLCs than bare VCM (Fig. 8).

These results confirmed VCM-SAOA-NLCs superior anti-MRSA activity to the bare VCM, which could be attributed to the small nanoparticle size, large surface area and hydrophobicity of the NLCs, which enhance the MRSA bacterial membrane binding capacity and permeability. Moreover, oleic acid is able to enhance penetration across bacteria cell wall in addition to its inherent anti-MRSA activity [12, 249, 254], thereby possibly contributing to enhanced activity.

The NLCs as a nanocarrier can also protect VCM and enhance its delivery in a sustained manner, thus ensuring higher concentrations at the site of action [48]. At acidic infection sites, the VCM-SAOA-NLCs protonation led to the surface charge switching to positive, which can improve binding to the negatively charged MRSA cell wall and enhance VCM uptake. Subsequently, increasing VCM binding to the terminal D-Ala-D-Ala residues targeted site, resulting in bacterial cell wall synthesis inhibition and MRSA colonization reduction [28, 31, 45]. Thus, pH-responsive VCM-SAOA-NLCs can be an effective multifunctional delivery system that can be used to improve the efficacy of VCM and other antibiotics against resistant bacteria, with the added advantage of reducing the high dose-dependent toxicity of antibiotics, thereby improving treatment outcomes and patient compliance.

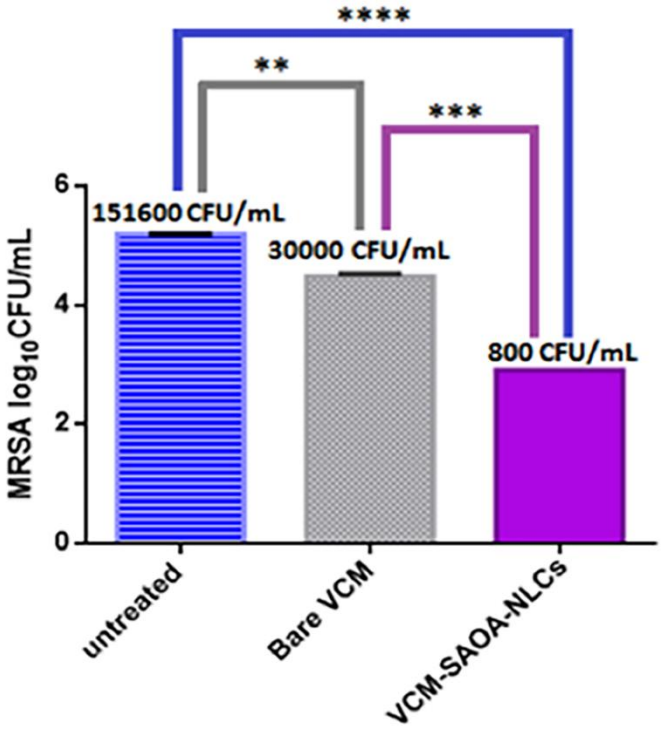


Fig. 8. *In vivo* anti-MRSA activity. Data are presented as mean ± SD (n=3).

To further evaluate the skin integrity and morphological changes, a histological analysis was performed on all skin samples. Light microscopy analysis of the H&E stained slides showed evidence of inflammation and the formation of an abscess at the infection sites of the untreated and bare VCM groups (Fig. 9 A and B respectively). The skin samples of these groups presented with swelling of the dermal layer and the presence of white blood cells at the inflammation site. However, there were no signs of abscess formation and only minimal evidence of tissue inflammation in the skin samples of the VCM-SAOA-NLCs treated group (Fig. 9 C).

In the untreated group, a large portion of the tissue at the infection site was infiltrated by the bacteria, as evidenced by the degree of the inflammatory response to the infection site (Fig. 9 A). This inflammatory response was also seen in the bare VCM treated samples, but to a lesser extent, these findings correlating directly with the MRSA counts from the infection sites. The untreated group displayed the largest number of recovered bacteria and displayed the greatest degree of inflammation and abscess formation. The VCM-SAOA-NLCs treated group displayed the least signs of inflammation and abscess

formation, which correlated with its statistically significant lower bacterial load. However, the bare VCM group presented a statistically significantly larger number of isolated bacteria than the VCM-SAOA-NLCs treated group, displayed more histomorphological signs, suggestions of tissue inflammation and abscess formation. These histological assessments further confirm the antimicrobial advantage of the VCM-SAOA-NLCs.

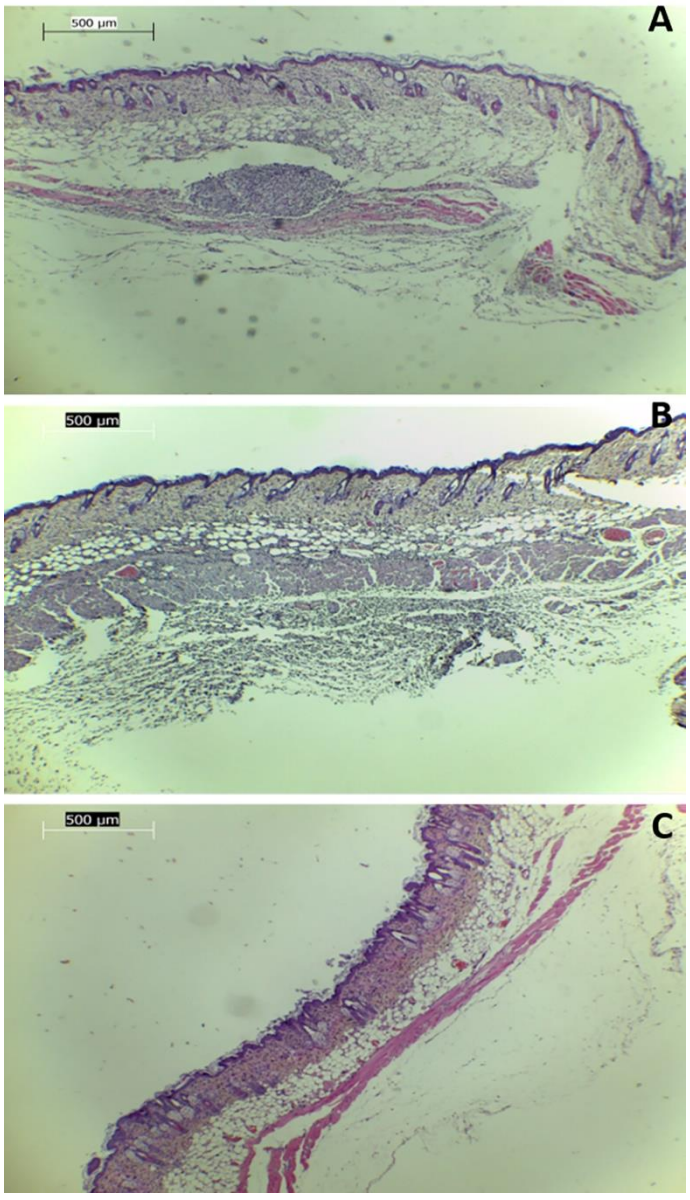


Fig. 9. Light microscopy images of H&E stained samples of (A) untreated (B) bare VCM and (C) VCM-SAOA-NLCs treated skin selections.

4. Conclusion

In this study, novel fatty acid based lipids were synthesized and formulated into pH-responsive NLCs for enhanced targeted delivery of VCM to the acidic infectious site. Negatively charged VCM-SAOA-NLCs with a small size, low PDI and acceptable EE from novel lipids were successfully prepared and showed responsiveness in terms of size and surface charge switching at acidic pH. Drug release was higher at pH 6 due to the protonation of both OALL and SASL components of NLCs, leading to breakage of the ion pair between them, and the subsequent swelling of NLCs and a burst drug release. *In silico* studies confirmed that the higher EE of VCM at a solid lipid to liquid lipid ratio of 2:1 (1603.991 kcal/mol) than 1:1 (1367.44 kcal/mol) is due to the higher affinity of VCM with the solid lipid than the liquid lipid. The *in vitro* antibacterial activity against SA and MRSA revealed that NLCs activity was enhanced and prolonged at pH 6 as compared to pH 7.4. Furthermore, the *in vivo* BALB/c mouse skin infection models revealed that there was a 37-fold reduction in the MRSA colony forming units load in mice skin treated with VCM-SAOA-NLCs compared to that treated with bare VCM. Histological investigations further confirmed the antimicrobial efficiency of VCM-SAOA-NLCs, as it showed minimal signs of tissue inflammation and abscess formation. Therefore, from the present work, it could be concluded that OALL and SASL as lipids show potential for preparing pH-responsive VCM loaded NLCs with enhanced activity against SA and MRSA. These materials may, therefore, contribute to optimizing lipid-based nanoformulations for improving the treatment of patients with bacterial infections.

Conflict of interest

The authors declare that they have no conflict of interest in this work.

Acknowledgement

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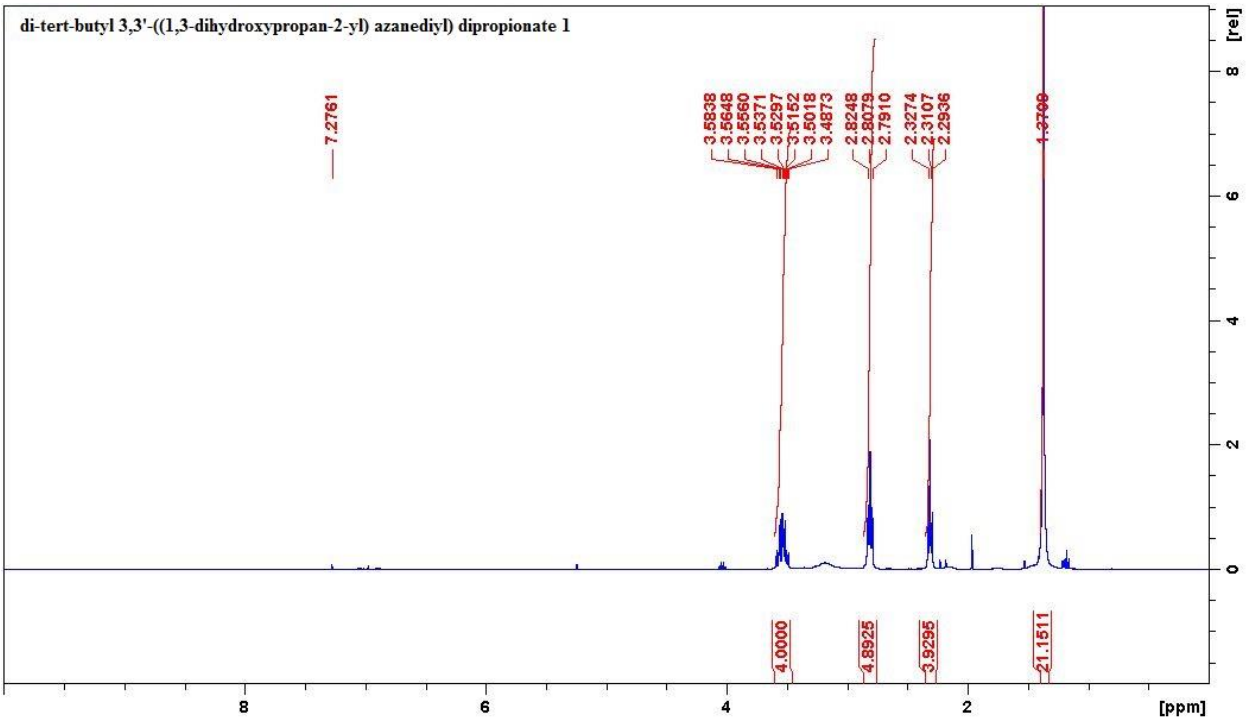
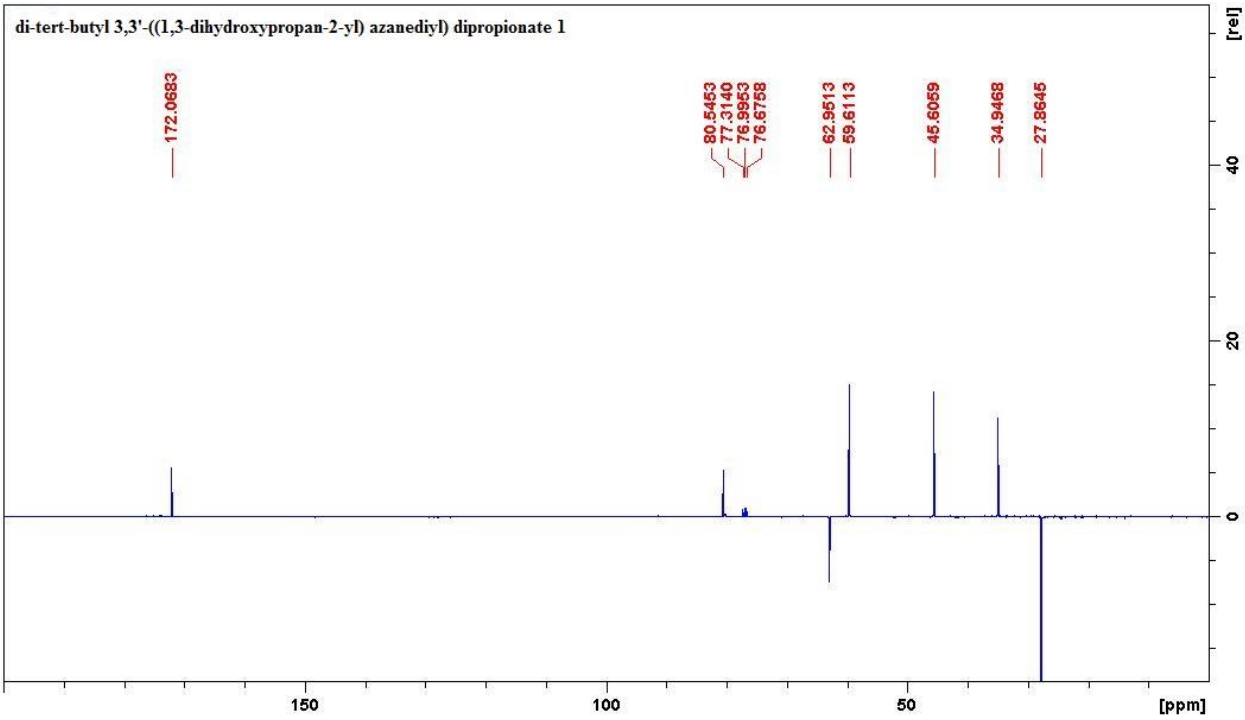
Supplementary Materials

^{1}H NMR characterization of compound 1



^{13}C NMR characterization of compound 1

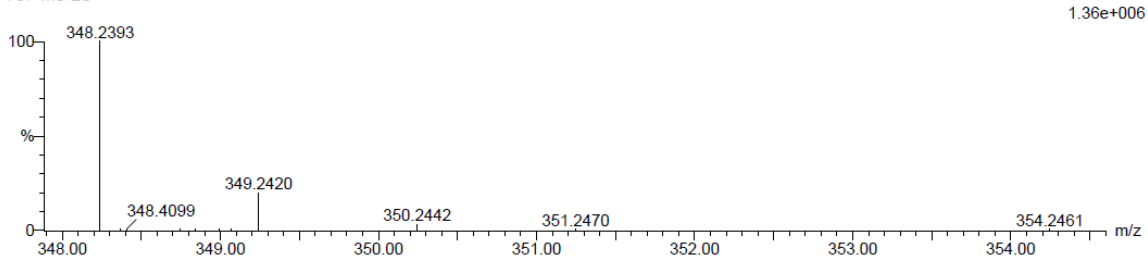


HRMS characterization of compound 1

Elemental Composition Report di-tert-butyl 3,3'-((1,3-dihydroxypropan-2-yl) azanediyl) dipropionate 1 **Page 1**

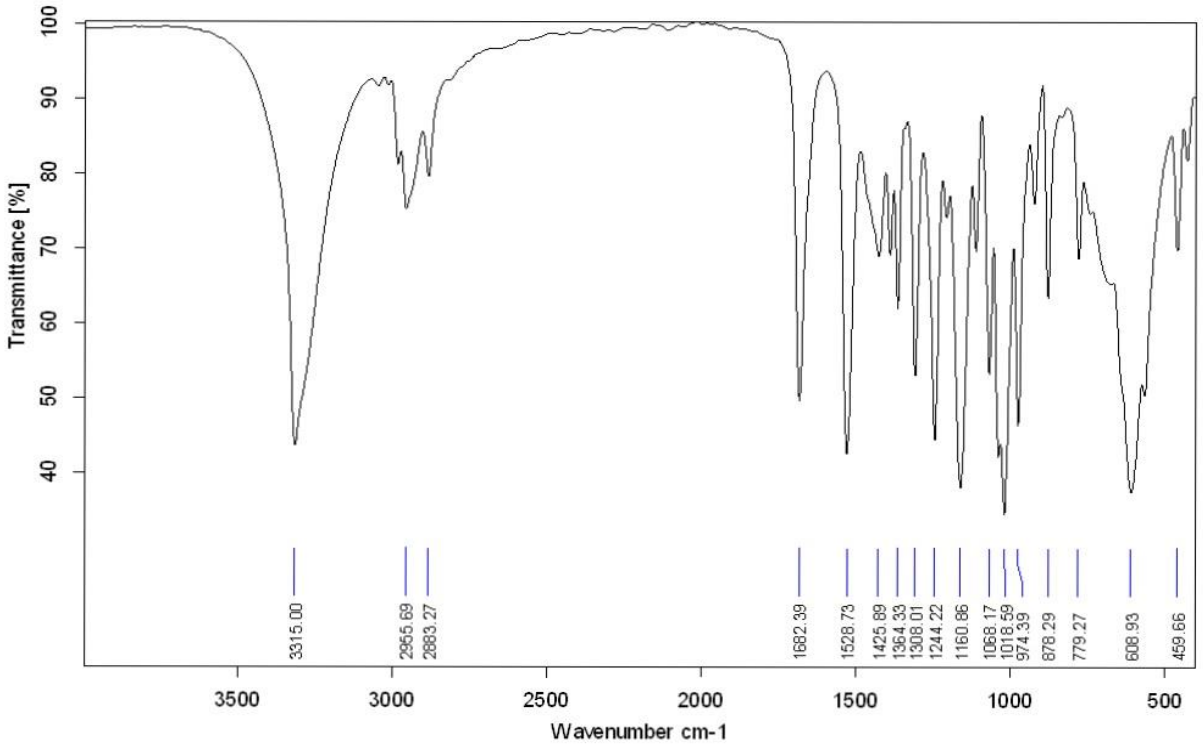
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
17 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 15-20 H: 30-35 N: 0-5 O: 5-10
Di-tertbutyl Serinol 50 (1.652) Cm (1:61)
TOF MS ES+



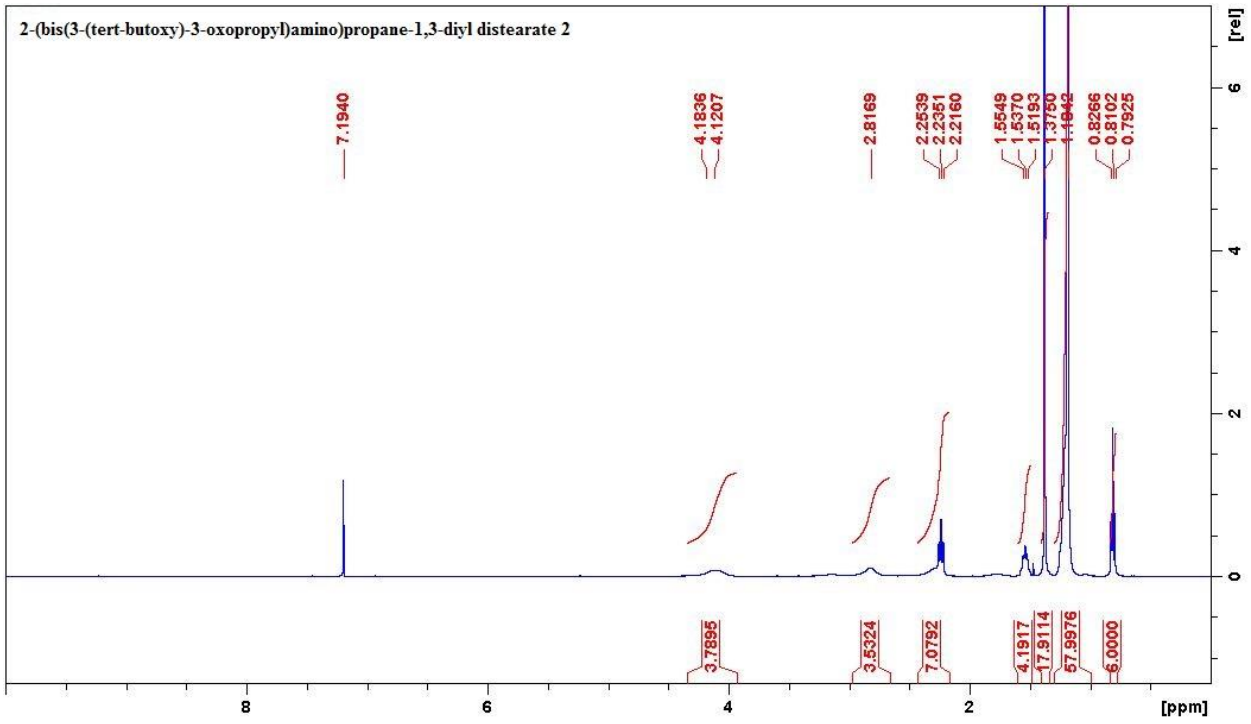
Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
348.2393	348.2386	0.7	2.0	1.5	116.3	0.0	C17 H34 N O6

FTIR characterization of compound 1

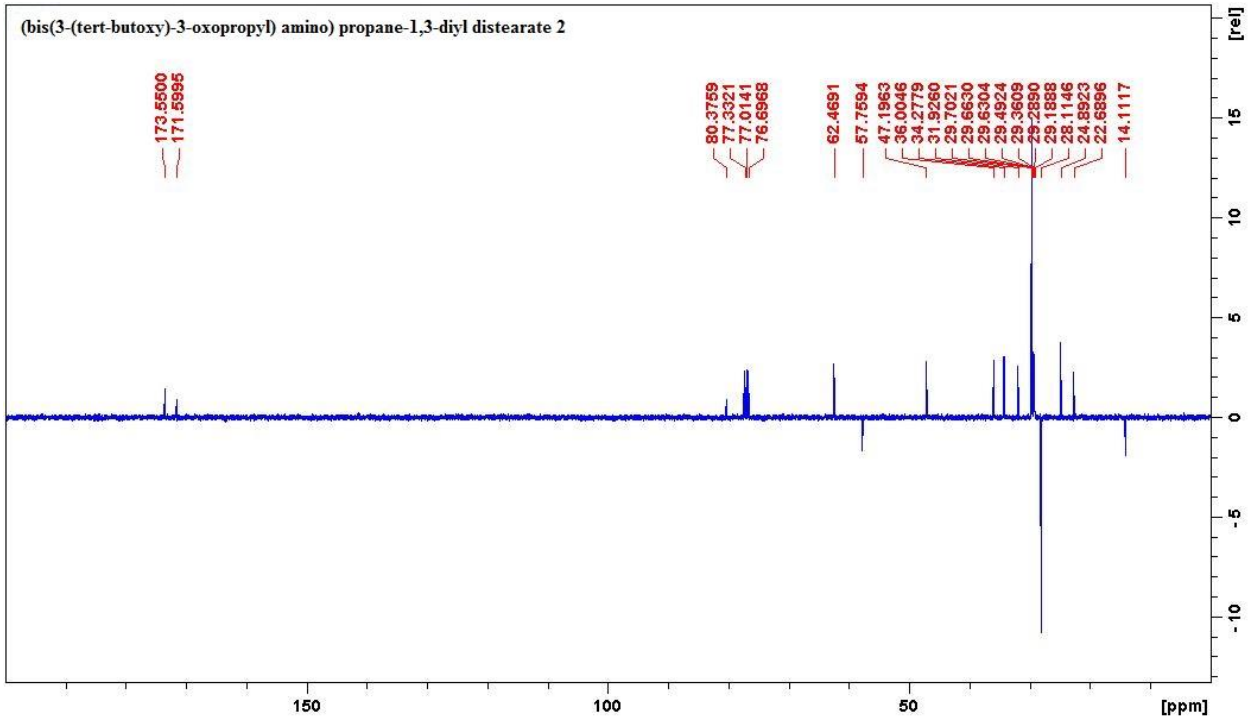


C:\nawras\.9 SERINOL T BAC ATR platinum Diamond 1 Refl 27/07/2017
di-tert-butyl 3,3'-((1,3-dihydroxypropan-2-yl) azanediyl) dipropionate 1

^{1}H NMR characterization of compound 2



^{13}C NMR characterization of compound 2

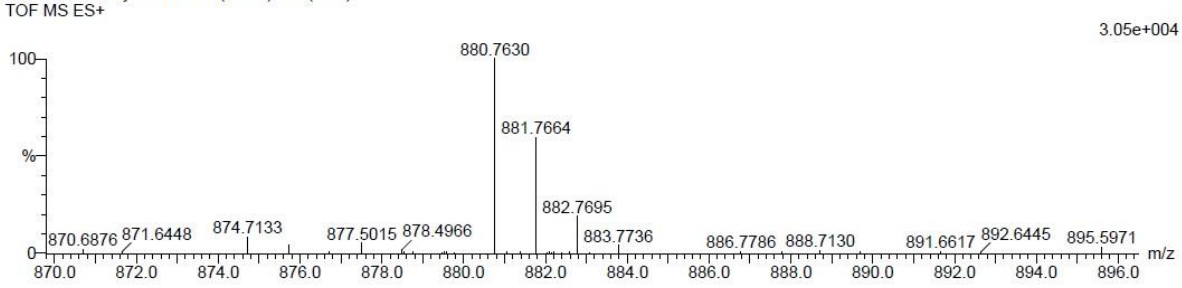


HRMS characterization of compound 2

Elemental Composition Report 2-(bis(3-(tert-butoxy)-3-oxopropyl)amino)propane-1,3-diyl distearate 2 **Page 1**

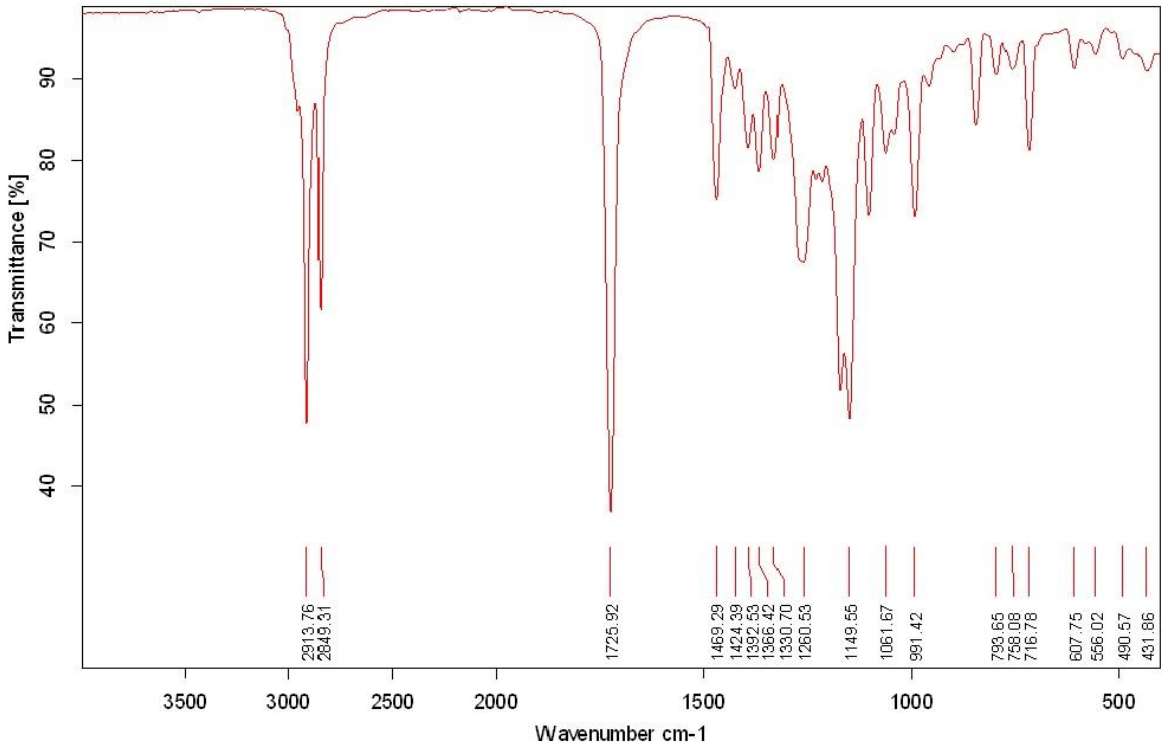
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
9 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 50-55 H: 100-105 N: 0-5 O: 5-10
Bis-SA-ditertbutyl Serinol 42 (1.383) Cm (1:61)
TOF MS ES+



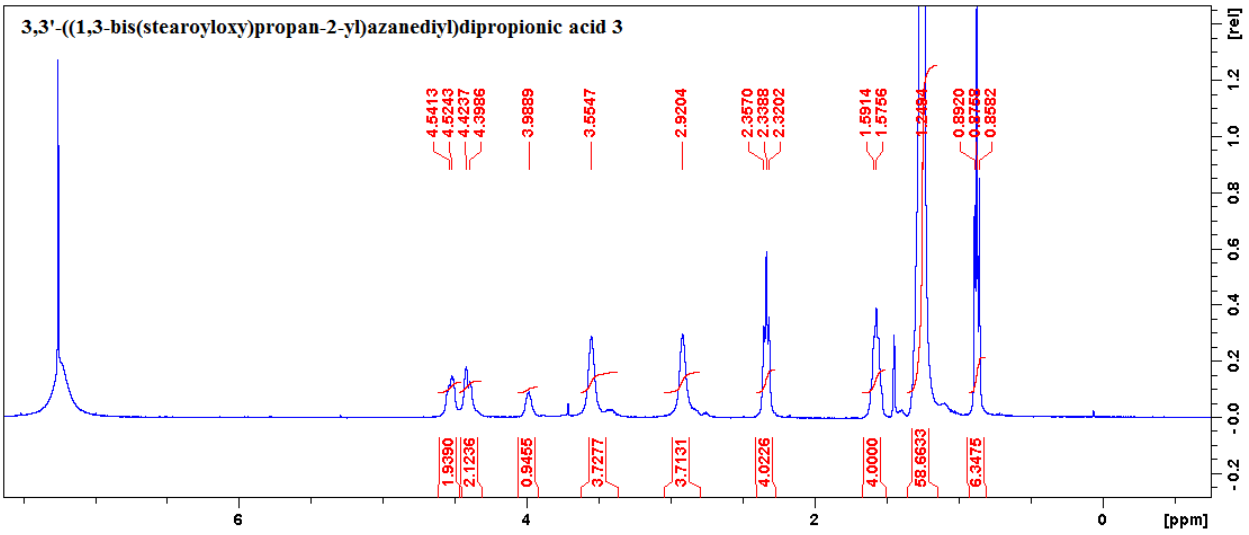
Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
880.7630	880.7605	2.5	2.8	3.5	68.3	0.0	C53 H102 N O8

FTIR characterization of compound 2

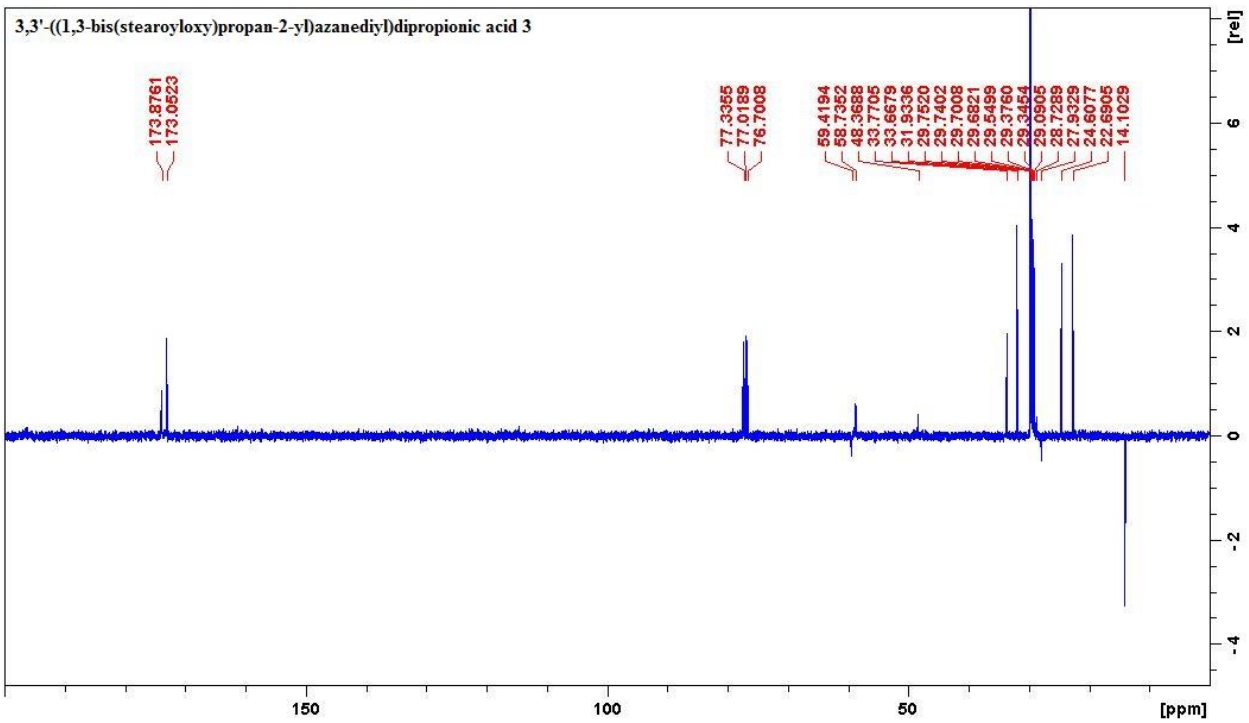


C:\UKZN\CALVIN\ESTER.0 ESTER ATR platinum Diamond 1 Refl 04/08/2017
2-(bis(3-(tert-butoxy)-3-oxopropyl)amino)propane-1,3-diyl distearate 2

^{1}H NMR characterization of compound 3



^{13}C NMR characterization of compound 3

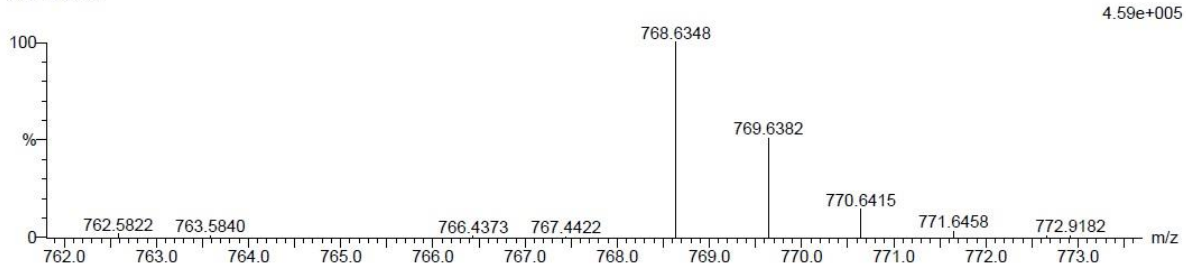


HRMS characterisation of compound 3

Elemental Composition Report 3,3'-((1,3-bis(stearoyloxy)propan-2-yl)azanediyl)dipropionic acid 3 **Page 1**

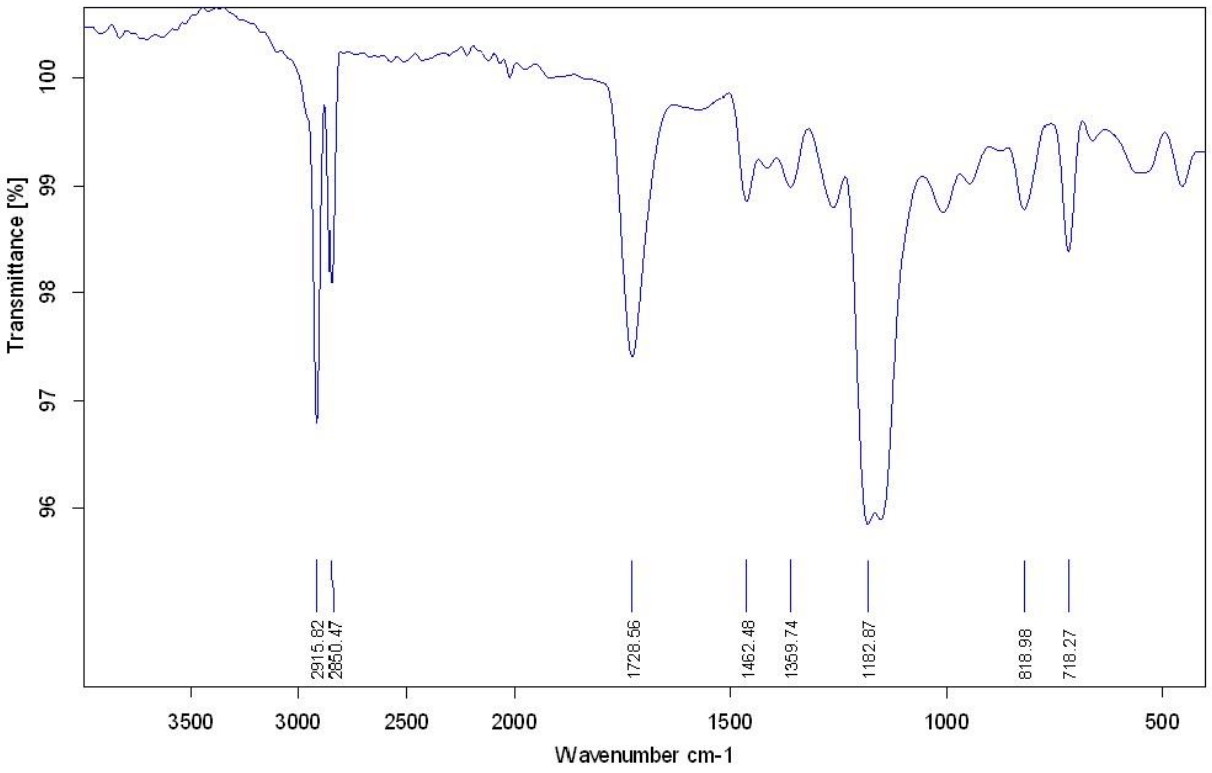
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
10 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 40-45 H: 85-90 N: 0-5 O: 5-9
Bis-SA-dipropanoic acid Serinol 2 (0.034) Cm (1:61)
TOF MS ES+



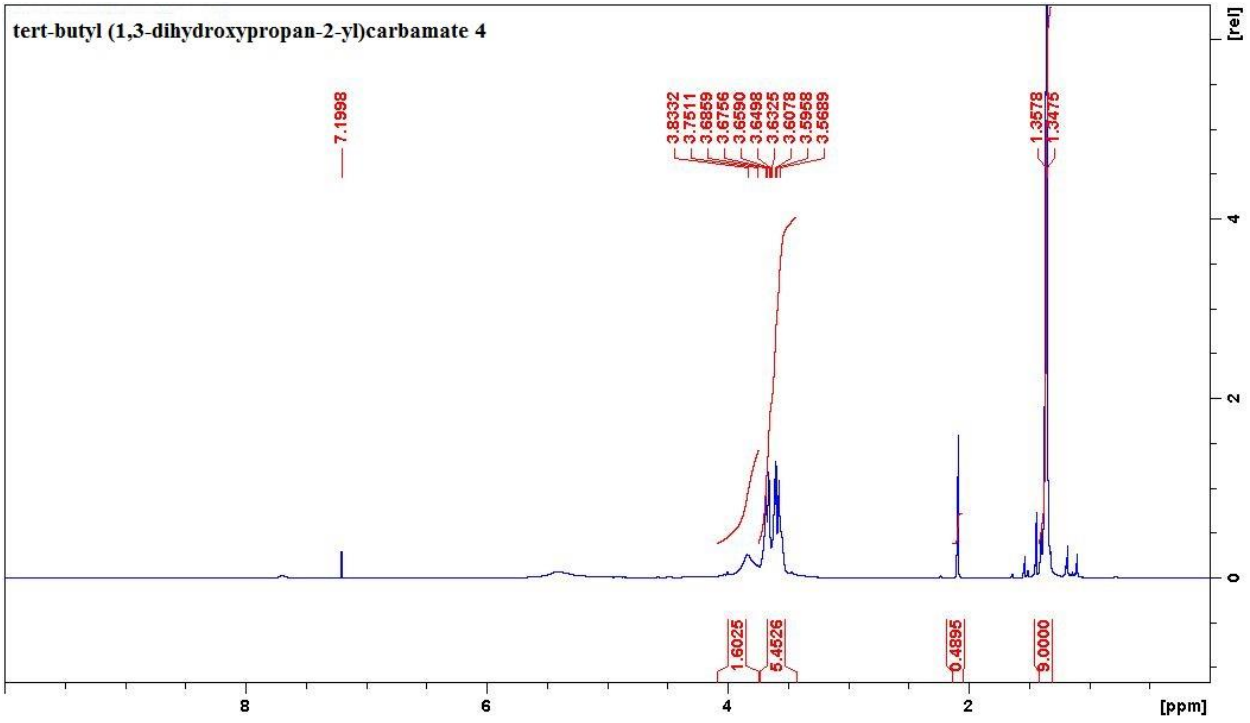
Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
768.6348	768.6353	-0.5	-0.7	3.5	14.8	0.0	C45 H86 N O8

FTIR characterization of compound 3

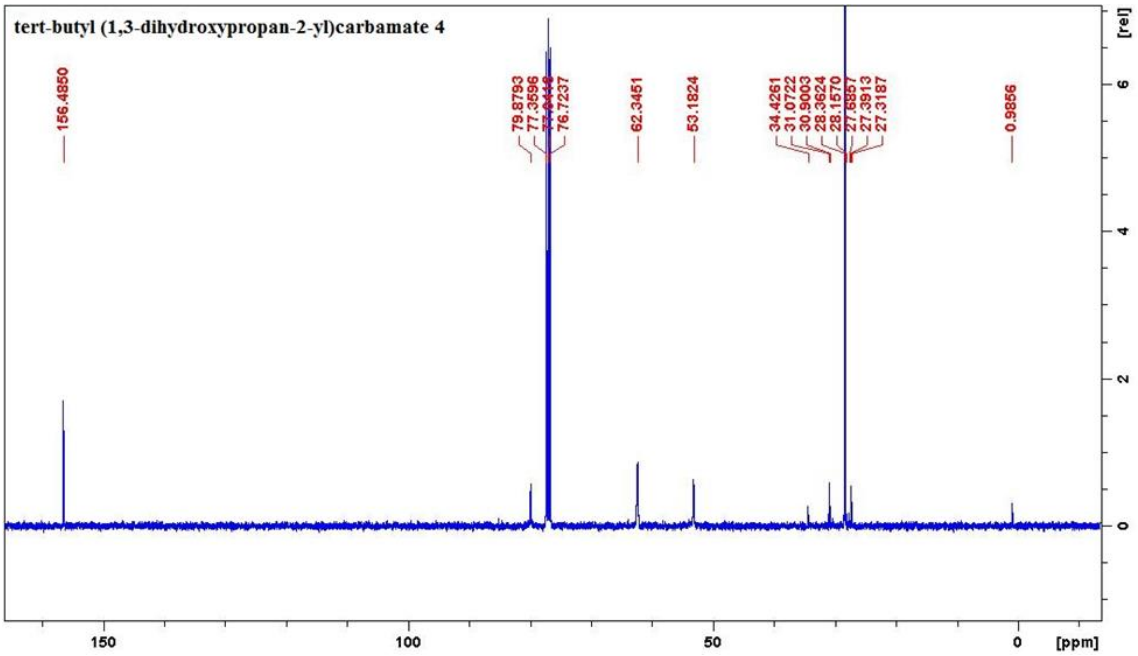


C:\UKZN\CALVIN\ACID.0 ACID ATR platinum Diamond 1 Refl 04/08/2017
3,3'-((1,3-bis(stearoyloxy)propan-2-yl)azanediyl)dipropionic acid 3

^{1}H NMR characterization of compound 4



^{3}C NMR characterization of compound 4

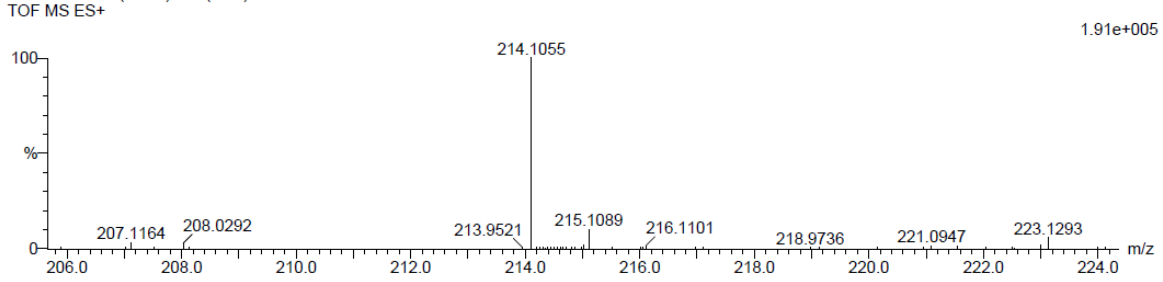


HRMS characterization of compound 4

Elemental Composition Report tert-butyl (1,3-dihydroxypropan-2-yl)carbamate 4 **Page 1**

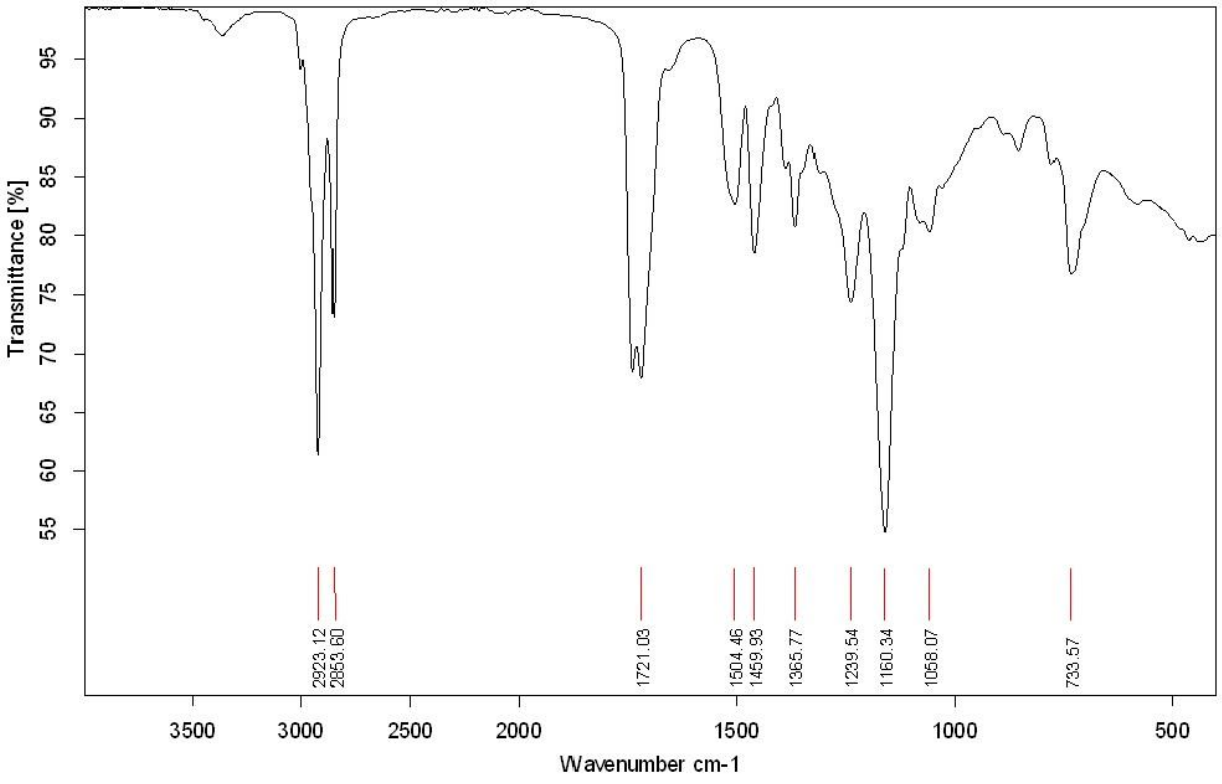
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
35 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 5-10 H: 15-20 N: 0-5 O: 0-5 Na: 0-1
Serinol Boc 23 (0.742) Cm (1:61)
TOF MS ES+



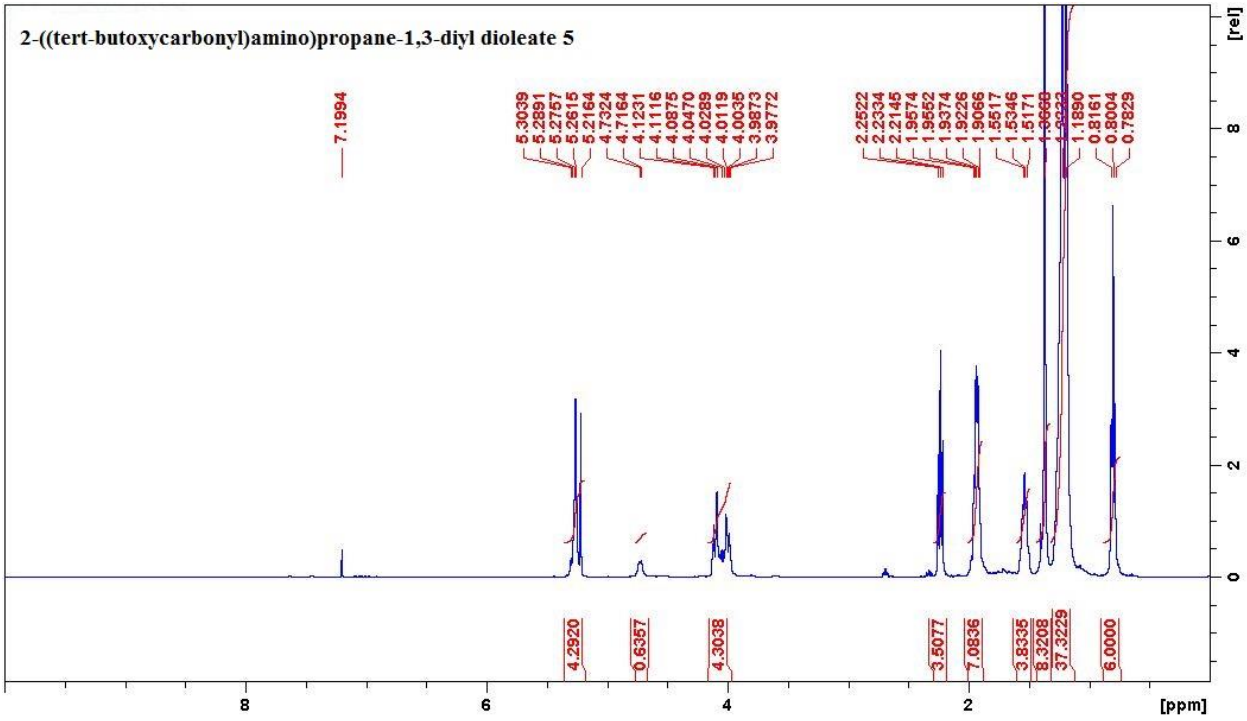
Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
214.1055	214.1055	0.0	0.0	0.5	233.8	0.0	C8 H17 N O4 Na

FTIR characterization of compound 4

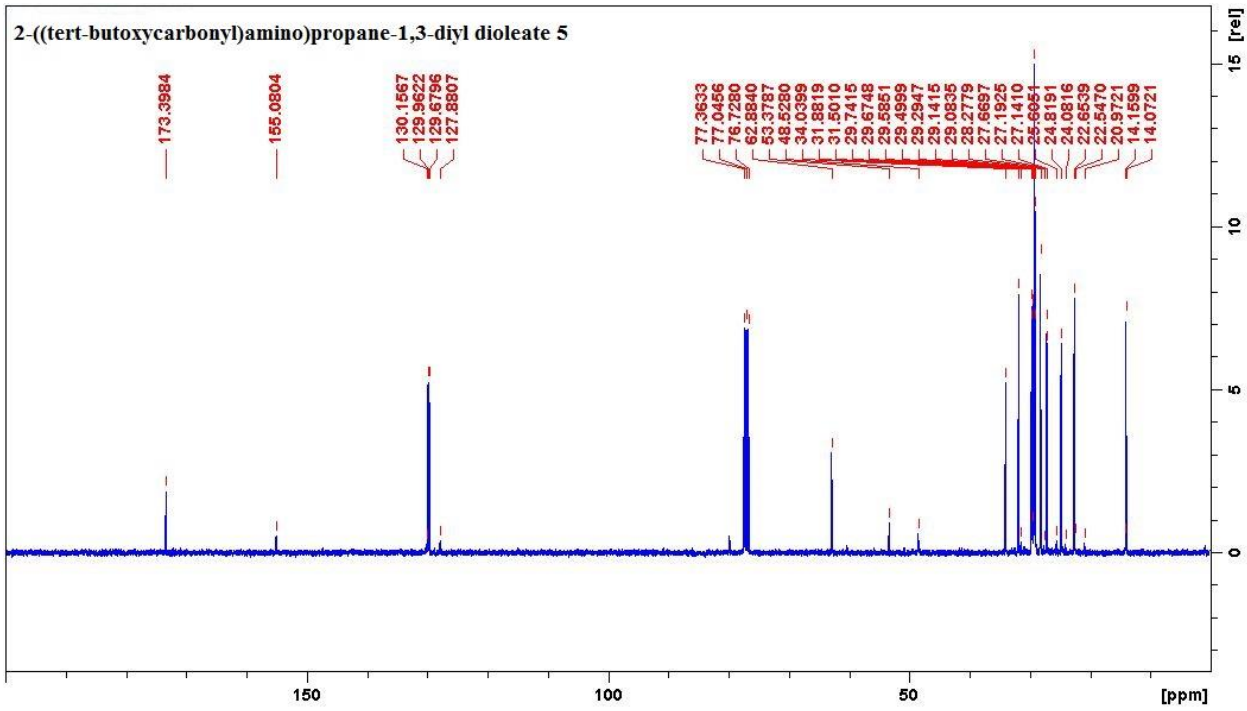


C:\nawras\.10 LL LINKER ATR platinum Diamond 1 Refl tert-butyl (1,3-dihydroxypropan-2-yl)carbamate 4	27/07/2017

1H NMR characterization of compound 5



^{13}C NMR characterization of compound 5

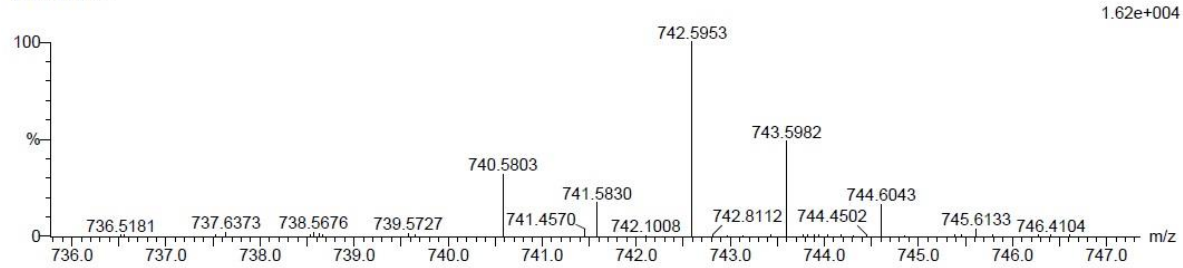


HRMS characterization of compound 5

Elemental Composition Report 2-((tert-butoxycarbonyl)amino)propane-1,3-diyl dioleate 5 **Page 1**

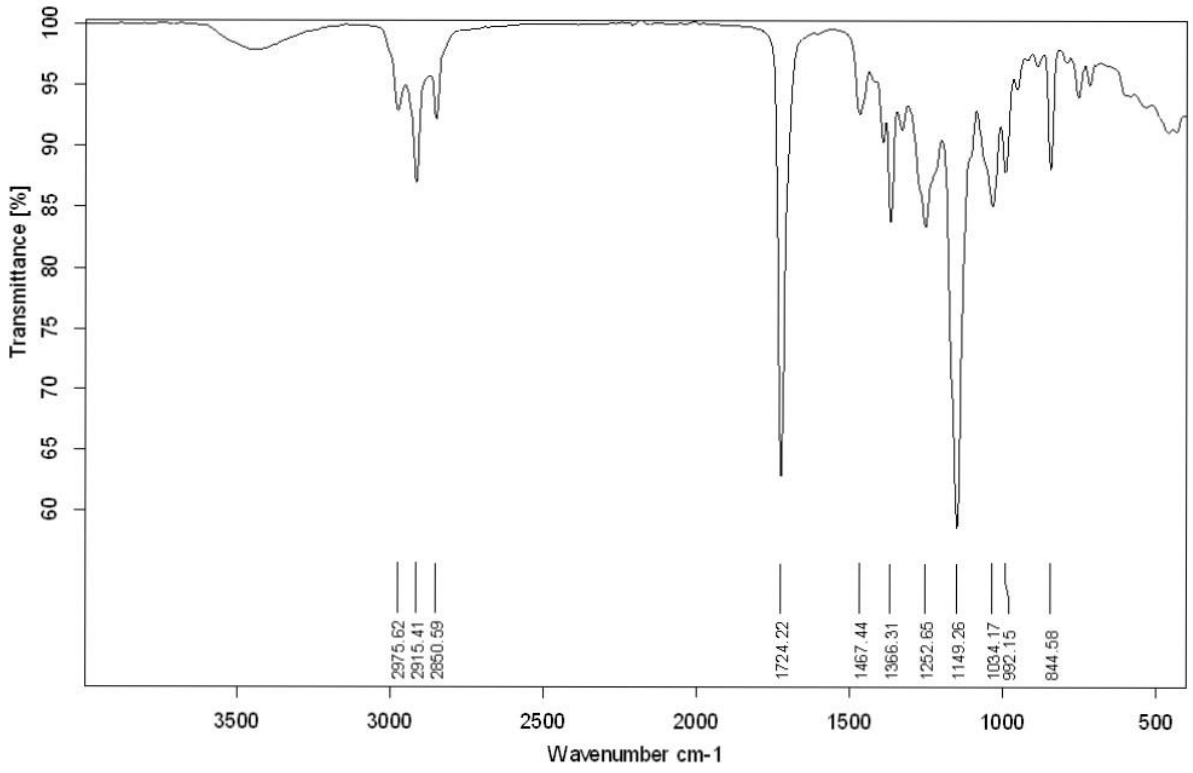
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
10 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 40-45 H: 80-85 N: 0-5 O: 5-10 Na: 1-1
DiOA-serinol Boc 2 (0.034) Cm (1:61)
TOF MS ES+



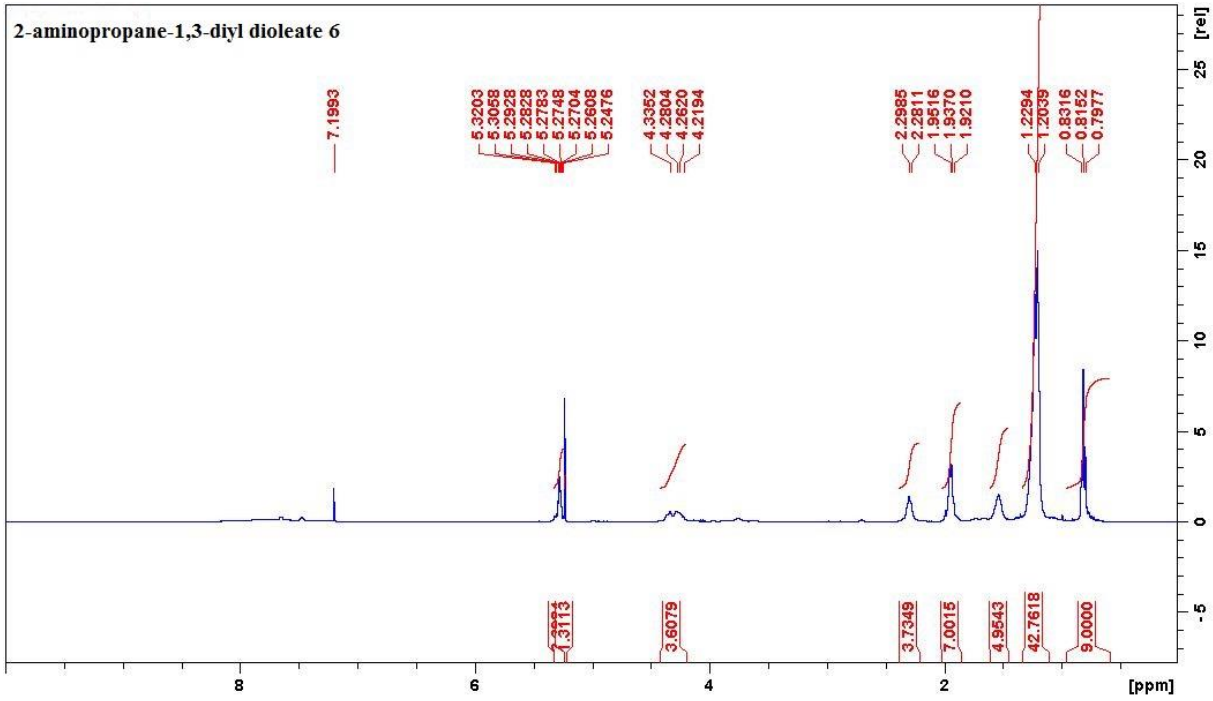
Minimum:		5.0	5.0	-1.5			
Maximum:				100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
742.5953	742.5962	-0.9	-1.2	4.5	86.9	0.0	C44 H81 N O6 Na

FTIR characterization of compound 5

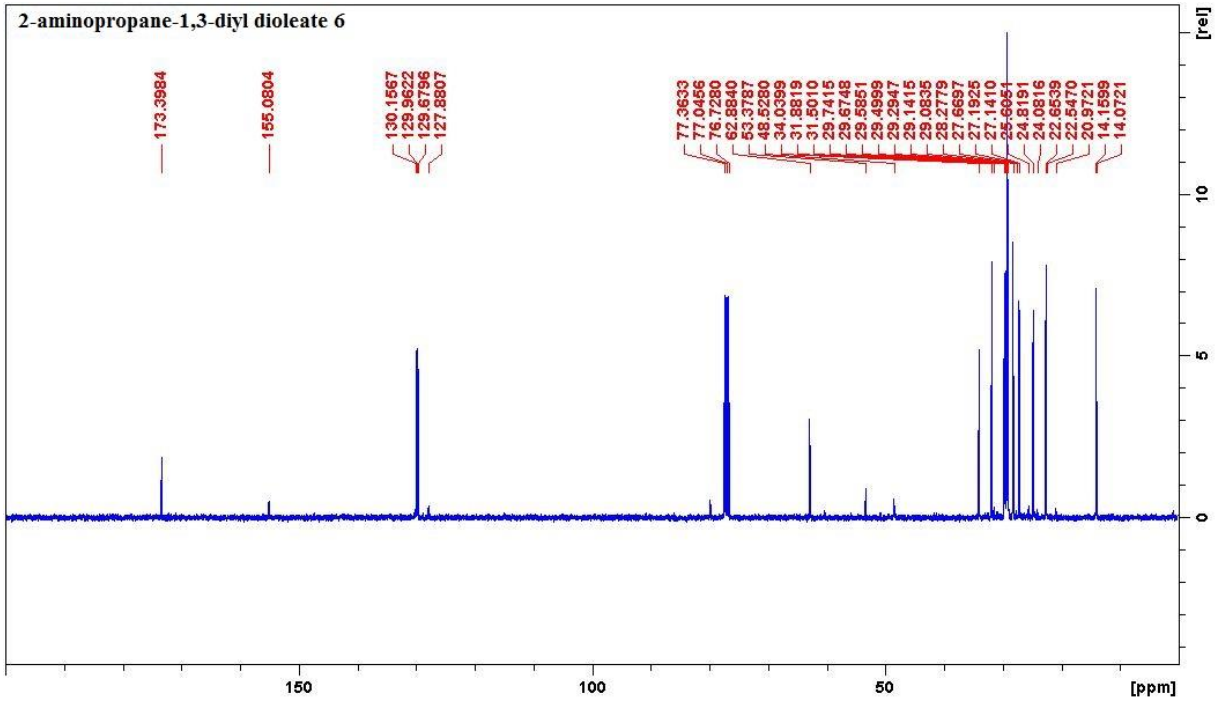


C:\nawras\.12 O A SERINOL ATR platinum Diamond 1 Refl	27/07/2017
2-((tert-butoxycarbonyl)amino)propane-1,3-diyl dioleate 5	

^{1}H NMR characterization of compound 6



^{13}C NMR characterization of compound 6

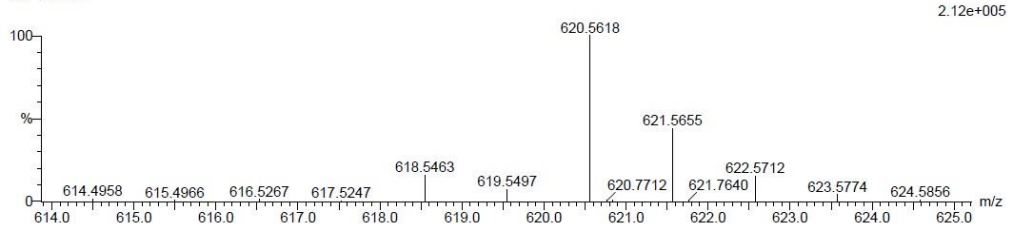


HRMS characterization of compound 6

Elemental Composition Report 2-aminopropane-1,3-diyl dioleate 6 **Page 1**

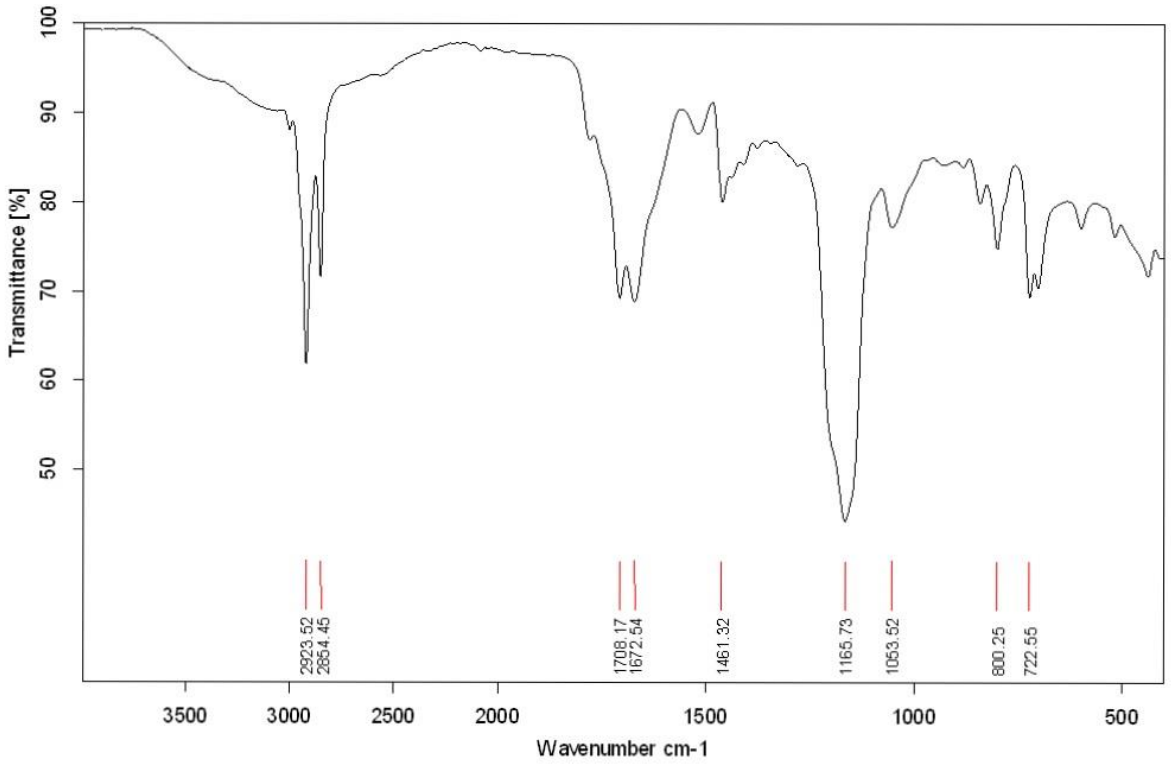
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
7 formula(e) evaluated with 1 results within limits (up to 20 best isotopic matches for each mass)
Elements Used:
C: 35-40 H: 70-75 N: 0-4 O: 0-5
DiOA-serinol 43 (1.417) Cm (1:61)
TOF MS ES+



Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
620.5618	620.5618	0.0	0.0	3.5	52.7	0.0	C39 H74 N O4

FTIR characterization of compound 6



C:\nawras\.8 LIQUID LIPID FINAL ATR platinum Diamond 1 Refl 27/07/2017
2-aminopropane-1,3-diyl dioleate 6

CHAPTER 4. CONCLUSIONS

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

CHAPTER 4

CONCLUSION

4.1 Introduction

Infectious diseases are one of the top ten leading causes of death globally. The discovery of antibiotics greatly improved the prevention and control infections, however, limitations of conventional antibiotic dosage forms, coupled with their irrational use, have contributed to the development of therapy complications and a bacterial resistance crisis. One of the major health risks is methicillin-resistant *Staphylococcus aureus* (MRSA) infection, which has increased morbidity and mortality rates globally. Vancomycin (VCM) is considered the last effective resort against MRSA bacteria, with the spread of resistance being a growing concern, highlighting the need to develop strategies to restore VCM efficiency. Although novel nano drug delivery systems have shown improved VCM therapy, however, there is still a need to optimize a nano antibiotic carrier to protect and stabilize VCM during systemic circulation, target VCM release and improve its bioavailability at the infection site. Therefore, current research advances focus on developing pH-responsive nanosystems to improve VCM targeted delivery to the acidic infection site. Nanostructured lipid carriers (NLCs) is an efficient nanocarrier for antibiotic delivery and enhancing antibacterial activity. However, no study in the literature has reported VCM-loaded NLCs yet. Therefore, there is a need for identification of novel pH-responsive lipids for development of pH-responsive VCM-loaded NLCs formulation. As per the study aim, the results of the Objectives outlined the potential of novel fatty acids based lipids to formulate VCM loaded NLCs to enhance its antibacterial efficacy.

The results of the study objectives are as follows as they relate to the Aim are as follow:
Objective 1. To synthesize pH-responsive novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)].

Objective 2. To characterize the synthesized SASL and OALL novel lipids using FT-IR, ^{1}H NMR and ^{13}C NMR and cytotoxicity studies.

Objective 3. To explore the potential of SASL and OALL novel lipids for vancomycin loaded pH-responsive nanostructured lipid carriers (VCM-SAOA-NLCs) preparation.

Objective 4. To optimize and characterize VCM-SAOA-NLCs in terms of size, polydispersity index (PDI), zeta potential (ZP), pH responsiveness, morphology, entrapment efficiency (EE), *in vitro* drug release, *in vitro* and *in vivo* antibacterial activity.

Objective 5. To undertake *in silico* studies in order to understand the interactions between VCM and the novel lipids as well as the effect of lipids ratio on entrapment efficiency.

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

- Stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL) were successfully synthesized.
- FT-IR, ^{1}H NMR, and ^{13}C NMR analysis confirmed the successful synthesis and structures of SASL and OALL lipids.
- The cytotoxicity studies performed by MTT assay on mammalian cell lines A549, HEK-293 and HEP G2 revealed that SASL and OALL were biologically safe lipids.
- Spherically shaped VCM-SAOA-NLCs were successfully prepared using hot homogenization followed by ultrasonication method. The optimal formulation showed pH-responsiveness in terms of size, PDI, and ZP. VCM-SAOA-NLCs size increased from 225.2 nm at pH 7.4 to 322.4 nm at pH 6 and 458.7 nm at pH 4.5 respectively. PDI increased from 0.258 at pH 7.4 to 0.386 at pH 6 and 0.431 at pH 4.5 respectively. Also, ZP switched from -9.2 ± 2.7 mV at pH 7.4 to +3.37 mV at pH 6 and +9.8 mV at pH 4.5 respectively. The EE % was found to be 88.7 ± 13.12.
- *In vitro* drug release studies showed a controlled and pH-dependent VCM release over a period of 48 hours. VCM-SAOA-NLCs had faster drug release at pH 6 compared to pH 7.4.
- *In vitro* antibacterial activity against SA and MRSA confirmed the superiority of VCM-SAOA-NLCs over bare VCM as VCM-SAOA-NLCs had enhanced and prolonged activity against both bacterial strains at pH 6 and pH 7.4. Moreover, VCM-SAOA-NLCs activity was four times and two times better against SA and MRSA respectively at pH 6 than at pH 7.4.
- *In vivo* antibacterial activity showed that the MRSA burden in mice treated with VCM-SAOA-NLCs was reduced by189- and 4-fold compared to untreated and bare VCM respectively. Histomorphological studies revealed that the skin of VCM-SAOA-NLCs treated group showed the least signs of inflammation and abscess formation, confirming the superior activity of VCM-SAOA-NLCs compared to the skin of bare VCM and untreated groups.
- *In silico* studies confirmed that the higher EE of VCM was at a solid lipid to liquid lipid ratio of 2:1 (1603.991 kcal/mol) than 1:1 (1367.44 kcal/mol) due to the higher affinity of VCM with the solid lipid than the liquid lipid.

The findings of this study, therefore, confirmed the potential of the novel pH-responsive fatty acid based lipids for preparation of pH-responsive NLCs for enhancing VCM efficacy .In addition , these findings can serve as a basis for future research studies on the synthesis of novel pH-responsive lipids to develop pH-responsive nanocarriers for targeted drug delivery.

4.2 Significance of the findings in the study

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

New Pharmaceutical Products

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

Improved patient therapy and disease treatment

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

Creation of new scientific knowledge

New knowledge was generated by identifying methods of synthesis and characterization of pH-responsive lipids, as well as antibiotic NLCs formulation and characterization. This can serve as a basis for smart nano delivery systems development and enhance their potential pharmaceutical applications.

Stimulation of new research

The findings can provide potential research directions to explore the pH-responsive lipids i.e. SASL and OALL, for potential applications in pH-responsive nanosystems formulation for various drug classes, such as anticancer and anti-ulcer drugs.

4.3 Recommendations for future studies

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

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

4.4 Conclusion

The findings of this study confirmed the potential of the synthesized novel lipids and pH-responsive nanoformulation in targeting antibiotic delivery to improve bacterial infection therapy. This study has contributed a “smart bullet” as an alternative drug delivery approach to address the limitations of conventional antibiotic dosage forms and bacterial resistance.

APPENDIX

Appendix A: Poster presentation for the 38th Annual Conference of the Academy of Pharmaceutical Sciences and University of KwaZulu-Natal Nanotechnology Platform Workshop

SYNTHESIS OF FATTY ACID BASED NOVEL LIPIDS FOR FORMULATING PH RESPONSIVE NLCS FOR TARGETED DELIVERY OF VANCOMYCIN AGAINST *STAPHYLOCOCCUS AUREUS* AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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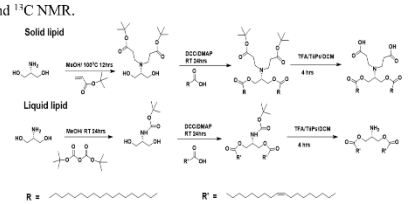
INTRODUCTION AND AIMS

- Bacterial infections are a significant threat due to the emergence of antibiotic resistance.[1] pH-Responsive nano-formulations are able to maximize the targeted delivery of antibiotics to acidic infection sites that are acidic, created by some bacteria such as *Staphylococcus aureus* (SA), thereby improving efficacy and decreasing development of resistance.[2]
- Identification of novel lipids will improve the development of pH-responsive antibiotic nanostructured lipid carriers (NLCs).[2]
- The aim of the present study was to synthesize and characterize novel fatty acid based lipids [stearic acid derived solid lipid (SASL) and oleic acid derived liquid lipid (OALL)] and explore their potential for preparing vancomycin pH-responsive nanostructured lipid carriers (VCM-SAOA-NLCs).

METHODS

Synthesis of the lipids

SASL and OALL were synthesized and characterized for structure using FTIR, ^{1}H and ^{13}C NMR.



NLCs preparation

- VCM-NLCs were prepared using a hot homogenization and ultrasonication. The lipid phase and the aqueous phase were heated separately and homogenized for 15 min at 6000 rpm with an Ultra Turrax T-25homogenizer (IKA Labortechnik, Germany).
- The resultant emulsion was sonicated for 15 min (30% amplitude) using a probe sonicator (Kennesaw, GA 30144, USA) and then cooled immediately to 20 °C using an ice bath.

Particle size, Polydispersity index (PDI) and Zeta Potential (ZP)

Prepared VCM-NLCs 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 using dialysis membrane in PBS of pH 6 and pH 7.4 at 37 °C.

***In vitro* antibacterial activity**

The minimum inhibitory concentration (MIC) values for VCM-NLCs were determined against S A and MRSA at pH 6 and 7.4.

***In vivo* antibacterial activity**

The *in vivo* antibacterial activity was evaluated against MRSA using BALB/c mice (Protocol approval number. AREC/104/015PD).

Molecular modeling

All molecular modeling techniques were performed using Biovia Materials Studio (MS) 2016 software and Drug Discovery Studio visualizer 2016, Biovia through the CHPC, South Africa.

RESULTS AND DISCUSSION

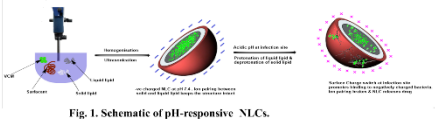


Fig. 1. Schematic of pH-responsive NLCs.

Table 1: Particle size, PDI and ZP of VCM-NLCs.

pH	Size (nm)	PDI	ZP (mV)	EE %
7.4	285 ± 5.6	0.31±0.03	-9.21 ± 2.7	88.7±13.12
6.0	298.1 ± 2.1	0.7 ± 0.02	3.4 ± 0.42	-

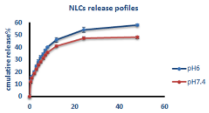


Fig. 2. *In vitro* pH-dependent release profile of *VCM-NLCs*(n =3).

Table 2: *in vitro* antibacterial activity results of VCM-NLCs formulation (n = 3).

In vitro antibacterial activity at pH 6								
Time (hours)	24	48	72	96	24	48	72	96
	SA (MIC µg/mL)				MRSA (MIC µg/mL)			
Bare VCM	3.9	250	NA	NA	7.8	NA	NA	NA
VCM- NLCs	1.95	1.95	1.95	3.9	1.95	1.95	1.95	1.95
Blank	NA	NA	NA	NA	NA	NA	NA	NA
In vitro antibacterial activity at pH 7.4								
Time (hours)	24	48	72	96	24	48	72	96
	SA (MIC µg/mL)				MRSA (MIC µg/mL)			
Bare VCM	3.9	500	NA	NA	7.8	NA	NA	NA
VCM-NLCs	0.488	0.488	1.95	3.9	0.98	0.98	1.95	7.8
Blank	NA	NA	NA	NA	NA	NA	NA	NA

NA = No activity. The values are expressed as mean ±SD, n=3.

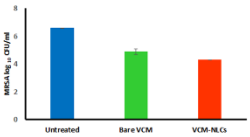


Fig. 3. *In vivo* anti-MRSA activity

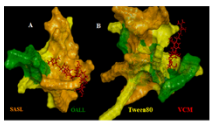


Fig. 4. Multivalent binding phenomenon of SASL, OALL & Tween 80 with VCM
A. solid lipid : liquid lipid is 2:1
B. solid lipid : liquid lipid is 1:1

- Negatively charged VCM-NLCs with low PDI and acceptable EE from novel lipids were successfully prepared.
- Drug release was higher at pH 6 due to the protonation of the tow lipids leading to breakage of the iron pair between the tow lipids, NLCs breakage and drug release[2].
- *in vitro* antibacterial activity against SA and MRSA revealed that NLCs activity was enhanced and prolonged at pH 6 as compared to pH 7.4 .
- An *in vivo* BALB/c mouse, skin infection models revealed that there was a 189 and a 5-fold reduction in the MRSA colony forming units load in mice skin treated with VCM-NLCs compared to that of untreated and VCM respectively.
- Molecular modeling showed that higher entrapment efficiency of VCM at solid lipid to liquid lipid ratio of 2:1 is due to the higher affinity of VCM with the solid lipid, therefore increasing the amount of liquid lipid will compete with VCM and reduce its entrapment efficiency. The binding energy was optimized from 1509.24 to 123.31 kcal/mol.

REFERENCES & ACKNOWLEDGMENT

References

1. Zhuang,C.et al. Int. J. Pharm.2010,394, 179–185.
2. Kalhapure, R.S.et al. Nanomedicine: NBM.2017, doi: 10.1016/j.nano.2017.04.010.

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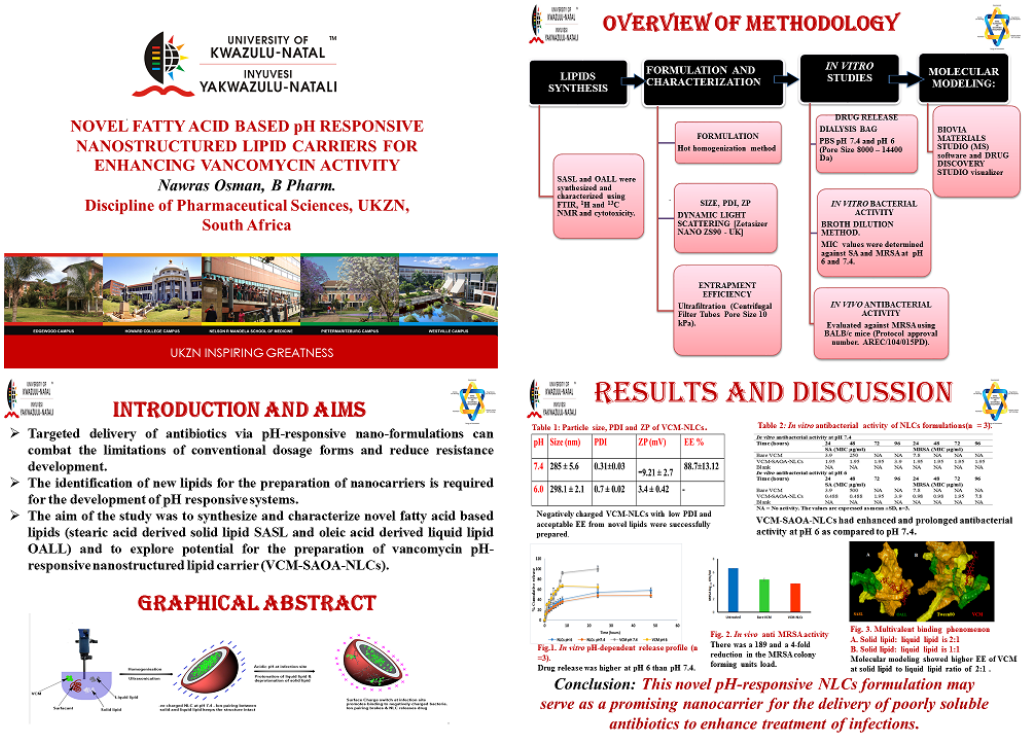
CONCLUSION

The novel pH-responsive VCM-NLCs was successfully prepared and provided sustained but higher drug release and enhanced the antibacterial activity of VCM against sensitive and resistant SA. Therefore, this novel pH-responsive NLCs formulation may serve as a promising nanocarrier for the delivery of poorly soluble antibiotics to enhance treatment of infections.

Appendix B: Poster presentation for the College of Health Sciences Annual Research Symposium



Appendix C: Submitted Manuscript

Submission no: EJPB_2017_1184
Submission title: Novel fatty acid based pH-responsive nanostructured lipid carriers for enhancing vancomycin activity
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Listed co-author(s): Dr Sanjeev Rambharose, Miss Nawras Osman, Dr Raveen Parboosing, Dr Ayman Y. Waddad, Dr Ramesh Gannimani, Mr Calvin Omolo, Dr Chunderika Mocktar, Dr Sanil Singh