The development and characterization of a vancomycin nanostructured lipid carrier and evaluation of their antimicrobial properties on *Staphylococcus aureus*

By

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Contents

Abstract

Vancomycin is a glycopeptide antibiotic which acts upon the cell wall of Gram-positive bacteria, causing cell death by cell wall damage and compounding cell membrane leakage. Nanostructured lipid carriers (NLCs) are second-generation lipidic drug delivery vehicles made from solid and liquid lipids and stabilised with surfactants and co-surfactants. NLCs provide better clinical outcomes by increasing cell permeation and tissue absorption and allow drugs to bypass biological barriers. Blank and vancomycin loaded NLCs were formulated by hot homogenisation and probe sonication and optimised for hydrodynamic size (PS), polydispersity index (PDI), zeta potential. Entrapment efficiency (%EE) was investigated by HPLC, and drug-lipid interactions were investigated by differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). This project achieved an optimised vancomycin NLC formulation with HDS of 153.5nm ± 22.5 a PDI of 0.162 ± 0.058 and a zeta potential of -47mV ± 3.3, and drug percentage entrapment efficiency (%EE) of 84.04% ± 3.41. DSC and FTIR did show some potential for drug-lipid interaction due to the presence of a C=C bond and an increase in melting point for drug encapsulated NLCs compared against blank NLCs. Bacterial testing was conducted on *S. aureus* ATCC 29213 a vancomycin sensitive strain of *Staphylococcus aureus.* Antimicrobial susceptibility testing highlighted that vancomycin encapsulated in NLCs showed similar inhibitory effect as compared to free vancomycin on *S. aureus* culture, however, the inhibitory effect of vancomycin was prolonged from two to four hours showing potential for a local and consistent prolonged release at target sites, potentially alleviating rapid elimination. This project demonstrates the potential for vancomycin embedded NLCs to effectively treat *S. aureus* infections showing the possibility of maintaining the drug levels within therapeutic window for a longer period of time.

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List of abbreviations

AST	Antimicrobial susceptibility testing
ATCC	American type culture collection
AU	Arbitrary units
cfu	Colony forming units
ci-VCM	Continuous infusion vancomycin
CLSI	Clinical and laboratory Standards Institute
CQA	Critical quality attributes
DA	Drug Added
DSC	Differential scanning calorimetry
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FD	Free drug
FTIR	Fourier transform infrared spectroscopy
HDS	Hydrodynamic Size
HPLC	High performance liquid chromatography
ICH	International convention of harmonisation
ii-VCM	Intermittent infusion vancomycin
MBC	Minimum bactericidal concentration
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
NLC	Nanostructured lipid carrier
OD	Optical Density
SC	Sodium Cholate
SEM	Scanning electron microscopy

TD	Total Drug
THF	Tetrahydrofuran
UV	Ultraviolet
VRE	vancomycin resistant *Enterococcus*
XRD	X-Ray Diffraction

Chapter 1: Introduction to Nanoparticles and their application for antibacterial treatment

1.1 Bacterial infections

Bacteria are prokaryotic cells that replicate rapidly, infect human cells, tissues and cause disease. Human pathogenic bacteria are separated into multiple categories however, the primary categories are Gram-positive and Gram-negative. Gram positive and Gram negative relates to the colour produced from Gram staining (positive – purple, negative – pink/red). Which is a technique created by Hans Christian Gram (1884) that allows the identification of the thickness of a bacterial cell wall by retention of crystal violet. Bacterial cell walls consist of peptidoglycan molecules fixed by peptide bonds (Tanaka and Shimakawa, 1996). Gram-positive bacteria have high retention of crystal violet due to thick peptidoglycan structures, where Gram-negative have thinner cell walls with lipidic envelopes. A safranin counterstain is used to provide colour to Gram-negative bacteria which did not retain crystal violet.

Morphology is the allocation of bacteria into various groups, the primary groups being cocci (circular) and bacilli (rod) (Tanaka and Shimakawa, 1996). Classification of bacteria has evolved with more biochemical tests and advanced technology allowing better identification. Bacterial taxonomy is now based on genetic analysis comprising of polymerase chain reaction (PCR) and whole genome sequencing. Bridwell *et al.,* (2022) investigated the impact of wound swab PCR on the de-escalation of skin and soft tissue methicillin resistant *Staphylococcus aureus* infections occurring in hospitals. Finding the use of PCR in MRSA positive patients decreased the sustained overuse of antibiotics as a PCR negative result led to faster de-escalation of drugs used. Modern investigatory genetic procedures involve whole genome sequencing and 16S rRNA sequencing (Ranjan *et al.,* 2016). While Whole genome sequencing allows the genera in the sample to be identified, 16S rRNA allows the dominant species to be identified. Alternatively, Cunningham *et al.,* (2020) investigated the premise of using whole genome sequencing for clinical predictions of *Staphylococcus aureus* resistance occurring in 102 blood isolates, finding 71 isolates were methicillin resistant and successfully predicted the potential for vancomycin susceptibility in all samples.

Staphylococcus aureus is a commensal bacterium found commonly on the skin and nasal canal, with 30% of people exhibiting some level of colonisation (Tong *et al.,* 2015). It has high prevalence in skin and soft-tissue infections however is known to also cause a wider range of infections such as meningitis, endocarditis, bacteriemia and septicaemia (Tong *et al.,* 2015). When cultured *Staphylococcus aureus* forms small golden pigmented round colonies approximately 2-3mm in diameter. Age is the most prominent determinant of risk when considering *S. aureus* infections where the highest rates are seen in the first year of life and increase gradually with advancing age (Tong *et al.,* 2015). In the industrialised world every 26.1 people in every 100,000 person-years were diagnosed with non-resistant staphylococcus strains, where 1 per 100,000 person-years were diagnosed with methicillin-resistant strains.

Bacterial infections are treated clinically by antibiotics. Antibiotics have different effects on bacteria but aim to prevent certain processes causing bactericidal effects and inhibition of growth. Antibiotics can target various sites within bacterial cells. The sites are cell wall and membrane synthesis, nucleic acid replication (DNA, RNA), protein synthesis, metabolic pathway disruption and ATP synthase interference (Kohanski *et al.,* 2010).

Quinolones are a class of antibiotics which target bacterial DNA-topoisomerase complexes (Kohanski *et al.,* 2010). DNA-topoisomerase controls the ability to supercoil DNA, transcription of messenger-RNA (mRNA) and bacterial cell division. Fluoroquinolones are more commonly used and clinically relevant variants of quinolones comparable by the quantity of bacterial cell death caused, examples of these are levofloxacin, ciprofloxacin and oxalinic acid. A study completed by Gade and Qazi (2013) found that of 107 MRSA isolates taken 92.5% presented with ciprofloxacin resistance with 65.4% presenting with point mutations within the *grlA* gene. This causes the binding site to change preventing the antibiotic action (Wang, Tanaka and Sato, 1998).

Rifamycins are semi-synthetic antibiotics which disrupt DNA-dependant transcription of mRNA molecules by stable binding to actively transcribing RNA polymerase enzymes. Rifamycin's are

commonly used synergistically with vancomycin for deep-seated infections such as endocarditis where bacteria (*Enterococci, Staphylococci*) colonize heart valves (Zhou *et al.,* 2012). The synergy is utilised due to the toxic side effects and high elimination of vancomycin (Zhou *et al.,* 2012). Rifamycins were isolated from the Gram-positive bacterium *Amycolatopsis mediterranei* where mutational changes caused protective production of the molecule (Kohanski *et al.,* 2010). Recently more mutations have occurred in the bacterium, and clinically relevant rifampicin was discovered which was used for the treatment of *Mycobacterium* infections. This class of antibiotic is bactericidal against Gram-positives but biostatic against Gram-negatives attributed to lower cell uptake in Gram-negatives, this could be potentially increased with the use of lipidic delivery systems (Jammal *et al.,* 2015).

Protein synthesis interfering antibiotics are separated into to two groups 50S and 30S inhibitors. Macrolide, lincosamide and streptogramin are 50S inhibitors they bind to the 50S subunit and either physically block the channel or translocate tRNAs (Kohanski *et al.,* 2010). Tetracycline and aminocyclitol are 30S inhibitors these drugs bind to the 16S ribosomal-RNA (rRNA) component of the 30S subunit. Once bound these drugs hamper elongation of peptide chains and impact tRNA binding preventing amino acid - amino acid bonding. Once the ribosomes have been inactivated by binding of either 30S or 50S inhibitors, vital proteins for cell metabolism and stability fall below survivable limits (Kohanski *et al.,* 2010). The introduction of these inhibitors can also decrease the rate at which bacteria produce symptom causing toxins (Johnson & LaRock, 2021). As ribosomes are intracellular targets, increased cellular uptake could improve antibiotic outcomes.

Bacteria possess two physical barriers to external molecules. One being a phospholipid bilayer cell surface membrane and another a peptidoglycan cell wall. The cell wall consists of strong covalent crosslinked peptide bonds which provide protection against oxidative and osmotic stress (Silhavy *et al.,* 2010). Cell wall disruption increases the rate at which cell membrane damage occurs, leading to cell leakage, lysis and eventually cell death. Cell wall precursors are synthesized internally by bacteria and are either inserted along external helical paths, which elongate cell walls laterally, or are deposited

at septum sites where bonding occurs (Scheffers & Pinho, 2005). ß-lactams and glycopeptides are antibiotics which exploit bacterial reliance on cell wall protection against environmental pressures. ß-lactam antibiotics block cross-linking peptide bonds by binding to transpeptidase enzymes, referred to as penicillin binding proteins (PBP) and prevent cell wall synthesis (Kohanski *et al.,* 2010). Glycopeptides break D-Ala-D-Ala bonds within bacterial cell walls causing cell wall destabilisation and cell leakage (Koyama *et al.,* 2012). ß-Lactam antibiotics can act on Gram-positive and Gram-negative bacteria alike whereas, due to the generous size of glycopeptide antibiotics (approximately three times larger than amoxicillin) they cannot cross Gram-negative outer cell envelopes therefore are not active on Gram negative bacteria (Hazardous substances database (HSDB), 2023) (Koyama *et al.,* 2012).

Uniquely isoniazid acts on preventing cell wall precursor production preventing cell wall synthesis within bacteria caused granulomas. In context, *Mycobacterium* infections in the lungs are defeated by macrophages digesting the infecting cells and becoming apoptotic. The apoptotic cells are then engulfed by neutrophils and carried to lymph nodes where the apoptotic material is destroyed (Delogu *et al.,* 2013). However, tuberculosis (TB) infections can block the action of macrophages so when apoptosis occurs the cells clump and form granulomas. The TB causing bacteria then grow on the surface of the granuloma and excrete toxins which become active TB (Delogu *et al.,* 2013). Isoniazid can penetrate the granuloma and TB cells allowing a decrease in the rate at which cell wall precursors can be produced interfering with cell wall synthesis. The drug also increases the rate at which the hosts immune system produces neutrophils and monocytes causing a compounding immune response against TB infections; however, this course of antibiotics is long due to it being reliant on the immune response of the host (Khan *et al.,* 2019). Isoniazid achieves the same goal as vancomycin in cell wall destabilisation. Although, it achieves this by decreasing cell wall access to precursors opposed to direct cell wall damage.

Bacteria evolve according to natural selection and genetic drift. Due to the rapid replication rate; this evolution happens quicker than within mammalian species. The rapid replication leads to

opportunities for bacteria to mutate and create mechanisms to resist antibiotic action (Culyba and van Tyne, 2021). The main modes of antibiotic resistance are changes in uptake, modification of drug or target and efflux (Reygaert, 2018).

Antibiotics are taken up by two routes dependant on the polarity of the molecule. This is done by a lipid-mediated pathway for hydrophobic molecules and diffusion through porins for hydrophilic antibiotics (Delcour, 2009). Bacteria decrease uptake by changing the outer envelopes sensitivity to certain molecules blocking antibiotic access to cells. For example, ß-Lactam antibiotics bind to PBP within cells preventing cell wall synthesis. ß-lactamase is a naturally produced protein which was developed due to the over-use of penicillin antibiotics. Bacteria mutated to produce ß-lactamase enzymes to digest beta-lactams preventing binding. Vancomycin resistance is common in *Enterococcus* species (VRE) in which the bacteria have evolved to form D-Lactate (D-Ala-D-Lac) bonds within the cell wall (Stogios and Savchenko, 2020). VRE has a prevalence of 40.7% in the UK across all nursing related facilities (Davis *et al.,* 2020). Due to the lack of hydrogen bonding within D-Lactate peptides, vancomycin cannot bind to the peptide bond becoming ineffective due to target site modification (Stogios and Savchenko, 2020). Efflux pumps are chromosomally encoded cell surface membrane transport channels which rid the cells of toxic materials. Antibiotics are considered toxic to bacteria in this context due to the bacterial cell damage caused. Bacteria can mutate to overexpress these efflux pumps, increasing antibiotic offloading or mutate to produce efflux pumps which remove molecule families the antibiotic belongs to (Reygaert, 2018).

Due to the decreasing amount of antibiotics available on the market synergistic effects have been researched; for example, the suicide preventative clavulanic acid is used in unison with amoxicillin to overcome beta-lactamase resistance (Evans *et al.,* 2022). However, non-antibiotic-based antimicrobials are also utilized to effect bacterial growth. Linoleic acid is a polyunsaturated fatty acid that has been found to increase bacterial membrane permeability increasing the rate at which antibiotics are absorbed and increasing the rate at which cell leakage occurs causing cell death

(Greenway & Dyke, 1979). Research has shown this fatty acid synergises with antibiotics that cause cell wall disruption such as vancomycin (Kalhapure *et al.,* 2014). The investigations conducted showed vancomycin had molecular changes when bonded with linoleic acid and produced positive antimicrobial effects on *S. aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) strains (Kalhapure *et al.,* 2014). The study by Kalhapure showed a decreased MIC in methicillin resistant *S. aureus* (MRSA)(15.62µg/ml) when compared against non-resistant (62.5µg/ml).

Of known healthcare associated infections (HCAI) (infections that patient's contract whilst hospitalised) 55% are antibiotic resistant strains that are acquired by medical intervention or surgical means (Choi *et al.,* 2021). In 2017 Julian *et al* (2020) estimated that 653,000 patients contracted hospital acquired infections and 22,800 died across all NHS teaching and general hospitals costing an estimated £2.1 billion. A common causative infection associated with HCAIs is MRSA which is a resistant *S. aureus* strain. A study completed in Africa found that of all *S. aureus* HCAIs 56% were MRSA infections (Irek *et al.,* 2018). Hence, the effective treatment of these infections is vital. According to Loveday *et al.,* (2014) the NHS follow five interventions which aim to stop HCAI's these are: hospital environment hygiene, hand hygiene, use of personal protective equipment (PPE), safe use and disposal of sharps and the principles of asepsis. However, effective treatments which aim to decrease the prevalence of these resistant infections compounded by the aim to decrease re-occurrence in nearby patients could provide more promising outcomes.

Broad spectrum antibiotics act on the two main groups of human pathogenic bacteria in their entirety (Gram-positive and Gram-negative) or act on a wide range of species across both groups (Venes *et al.,* 2001). The use of empirical therapy to prescribe broad spectrum antibiotics can aid patients in critical scenarios however, when used for common or unidentified infections it creates resistances in non-disease-causing bacteria (Llor and Bjerrum, 2014). Narrow spectrum antibiotics affect a small percentage of species in the main groups, this is controlled by a specific site of action and administration (Melander *et al.,* 2018). Hence, shown in practice the application of narrow-spectrum

antibiotics in relevant infections leads to the decrease of antibiotic resistance and an increase in patient welfare (Alm & Lahiri, 2020). This means when 1st and 2nd choice treatments are prescribed, they may not be effective. To counteract this the World Health Organisation (WHO) created the access, watch, reserve and classification of antibiotics for evaluation and monitoring of use (AWaRe) (WHO, 2021). This informs healthcare providers as to the categories assigned to antibiotics prescribed to patients and relevant monitoring to be completed. Vancomycin is in the 'watch' category which applies to antimicrobial agents that present high antimicrobial properties on a select number of species. Antibiotics in the watch category also have high probability of developing resistances and are in an advantageous position for stewardship programs.

1.2 Nanoparticles

Nanoparticles (NPs) are nano scale particles prepared using a variety of materials such as metals (gold, silver, Iron), polymers and lipids. They have been employed for various clinical and biomedical applications within mammalian tissues, barriers and membranes. They are utilised to control the flow of molecules and blockage of unwanted molecules. Nanoparticles can circumvent biological barriers due to their small size (<200nm), enormous surface area and unique surface properties (Azhar *et al.* 2016). This allows the delivery of drugs to targets in the body that otherwise would be unreachable. Surface characteristics of the nanoparticles can be tailored to by appending small molecules, antibodies, peptides, aptamers and ligands to the particle surface to augment their ability to target particular disease site or tissue. Apart from active targeting using the ligands, non-specific physical targeting such as heat, radiation and magnetic field have also been reported to enable targeting of nanoparticles (Matos *et al.* 2018). NPs may present with challenges in medical use due to their toxicity and stability. Some metal nanoparticles (gold, silver and carbon) have been reported to cause cell death when used in high concentrations due to oxidative stress. This creates difficulties in the delivery of medicines which require high dosage for example, for systemic diseases (Zhang *et al.,* 2022).

1.2.1 Lipid Nanoparticles

1.2.1.1 Liposomes

Liposomes are artificial vesicles prepared from non-toxic phospholipids and cholesterol which form spherical particles and are used as drug delivery systems. They provide the ability to entrap both hydrophilic and hydrophobic molecules releasing these drugs at designated sites (Akbarzadeh *et al.,* 2013). Liposomal drug delivery vehicles excel in slow and controlled drug release with experimentation showing increased efficacy of the drug on slower release (Wang *et al.,* 2009). Liposomes encounter problems with drug leakage and stability, attributable to the phospholipid membrane dynamics causing slow release of drug by diffusion (Russell *et al.,* 2018). This is dependent on the formulation and drugs affinity to the excipients utilised, meaning drug leakage can occur at differing rates and quantities making longer infusion of these medicines difficult (Daraee *et al.,* 2016). Current research is investigating the premise of activated liposomes as a targeting technique for diseases (De Matos *et al.,* 2018). Contrasting with biological targeting techniques the active targeting provided by activated liposomes allows locational delivery. An example of this is thermosensitive liposomes, when a temperature increase is applied, the lipid bilayer undergoes a phase transition increasing permeability of the drug. Arikayce™ is an inhaled amikacin loaded liposome used for the treatment of *Mycobacterium avium* complex infections in the lungs. It was found liposome aerosolization increased amikacin levels in lung tissues 274-fold when compared against intravenous routes. It showed superior absorption compared to amikacin aerosolization alone (Khan & Chaudary, 2020).

1.2.1.2 Solid lipid nanoparticles (SLNs)

SLNs are the first-generation lipid nanoparticle where melted lipids solidify around drugs forming a protective layer. High pressure homogenisation is used to shear particles and decrease particle size. Solid lipid NPs differ to future generations of NPs due to the absence of liquid lipid counterparts. This decreases drug absorption into lipid matrixes (Nguyen *et al.,* 2022). Utilising SLNs Taheri *et al.,* (2023) found co-loading of vancomycin and ampicillin in lecithin SLNs decreased *S. aureus* growth over a sustained period but did not provide increased inhibition above free drug controls. The affordability and commercial scalability of SLNs makes them a promising vehicle for drug delivery. Although, due to

the highly ordered and crystalline lattice of solid lipids that forms within the SLN, drug loading tends to be low, exacerbated by larger molecules (Mukherjee *et al.,* 2009).

1.2.1.3 Nanostructured lipid carriers (NLCs)

NLCs are second generation lipid nanoparticles which aim to increase drug entrapment and provide a prolonged and sustained release of drug into biological systems (Naseri *et al.,* 2015). This is achieved by the formation of a solid-liquid matrix which is amorphous thus prevents drug leakage due to the crystalline nature of solid lipid when used alone. This matrix also improves the biocompatibility of drugs disguising drug properties with lipids. Due to the preparation of NLCs consisting of lipid excipients and surfactants, NLCs are stable at ambient temperature. Once the matrix is formed high heat (<80°C), pH change, and lipid affinity destabilise the particles, drugs then slowly release by diffusion out of the solid-liquid matrix.

The biopharmaceutical performance of nanoparticles is dependent on their physicochemical properties. During development of nanoparticles key physicochemical properties such as particle size (PS), polydispersity index (PDI), zeta potential and percentage entrapment efficiency (%EE) are identified as critical quality attributes (CQAs). The output values needed for optimal particles are a HDS of 80-200nm, PDI <0.300 (Danaei *et al*. 2018) and a zeta potential between -30mV or +30mV dependant on the ionic properties of the particles (Azhar *et al.,* 2016). The excipients added to the formulation maintain the hydrophilic-lipophilic balance of the nanoparticles this ratio must be controlled as an imbalanced ratio would lead to CQAs exceeding limits. Surfactants and co-surfactants are molecules which align at interfaces of water and lipids aiding in maintaining hydrophilic-lipophilic balance. The presence of surfactants prevents coalescence of particles increasing stability. Phospholipids are polar lipids with hydrophilic heads containing a phosphate group and two hydrophobic tails derived from fatty acids, these form the outer layer of the particles thus stabilising the NPS. Solubilisers are utilised to increase the solubility of non-lipophilic drugs which allows entrapment to occur in the lipid matrix.

Nanostructured lipid carriers are a promising way to allow a more controlled drug release and lower toxicity of antibiotics (de Barros *et al.,* 2021). The application of this delivery system to allow a more prolonged release could be used to maintain the drug levels in the therapeutic range for longer period of time in the body. This would be especially useful for antibiotics as steady concentration of the antibiotic in the blood would maintain an inhibitory effect on the target bacteria (Cooney *et al.,* 2017) Antibiotics are dosed to maintain minimum inhibitory concentrations of antibiotics in the patient. Using a delivery system which allows a sustained release of drug over a period would allow patients to be given fewer doses of drug but allow biological systems to maintain a consistent therapeutic range.

NLCs have gained attention as tools for dermal application of antibiotics treating skin and soft tissue infections (Jaiswal *et al.,* 2016). Chato-Astrain *et al.,* (2020) investigated the use of sodium colistimethate and amikacin NLCs on infected skin models. Concluding that even though no increase in bacterial inhibition was observed with drug loaded-NLCs as compared to free drug, they showed increased cellular absorption of drug. Common infective skin pathogens tend to be commensal flora which naturally grow on the skins surface such as *Staphylococcus aureus, Streptococcus pyogenes and* coryneform species (Aly, 1996). Biological barriers such as skin collagen and the immune system prevent infections in these areas but when barriers are broken, or the immune system is weakened, opportunistic pathogens exploit the bodies weakened defences. Due to the excipients of NLCs having low toxicity and exhibiting high colloidal properties. They provide high permeation and drug release into skin cells allowing greater access to infected cells and greater antibiotic efficacy (Pardeike *et al.,* 2009). The increased skin permeation is due to the small size of particles and the ability to bypass the pores of the stratum corneum the upper layer of the epidermis (Li & Ge, 2012).

1.2.2 **Nanostructured Lipid Carrier Preparation**

NLC preparation methods aim to force immiscible phases to mix and form nanoscopic particles. The mixing occurs due to surfactants and co-surfactants which maintain the hydrophilic-lipophilic balance.

Different methods are also utilised to decrease particle size this is done by physical means using either high-pressure or sound waves.

There are varying methods which can be applied when creating an optimised nanoparticle formulation. A common method utilised is hot homogenisation which consists of heating two phases separately one organic and one aqueous and mixing for an extended period before undergoing ultrasonication or high-pressure homogenisation to decrease particle size (Zwain *et al.,* 2021).

Table 1. 1: The advantages and disadvantages of Hot homogenisation as a preparation method of NLCs. Sources: (Khosa, Reddi and Saha, 2018), (Elmowafy and Al-Sanea, 2021), (Sharma and Baldi, 2018)

Advantages	Disadvantages
Well established and easily scaled	Dispersion can be affected by micro-particles
Does not utilise organic solvents	Can encourage drug escaping into aqueous phase
Decreases microbiological interference	

Cold homogenisation which has the lipid phase heated and then rapidly cooled by liquid nitrogen to prevent thermal degradation (Jain *et al.,* 2017). The solid is then ground in a ball mill producing a fine dry powder 50-100µm in size (Sastri *et al.,* 2020). The aqueous phase is then chilled before the lipid powder is dispersed within it. The droplet particles are then subjected to high pressure homogenisation or ultrasonication to decrease particle size (Sastri *et al.,* 2020).

Table 1. 2: the advantages and disadvantages of cold homogenisation as a preparation method of NLCs. Sources: (Khosa, Reddi and Saha, 2018), (Elmowafy and Al-Sanea, 2021)

Advantages	Disadvantages
Organic solvent avoidance	Can produce greater particle size
Allows process of a thermolabile drug	Increases particle heterogeneity

High-pressure homogenisation has both phases heated and the lipid phase dispersed in the aqueous before mixing to initiate emulsification. The particles are then sheared at high pressure in a compression chamber with a controllable exit diameter to decrease particle size to optimum size (Lødeng & Bergem, 2017). Cold homogenisation can be utilised prior to shearing, though formulations undergo rapid cooling before shearing to reduce thermal stress (Li *et al.,* 2017).

Table 1. 3: the advantages and disadvantages of High-pressure homogenisation as a preparation method of NLCs. Sources: (Khosa, Reddi and Saha, 2018), (Sharma and Baldi, 2018), (Li *et al.,* 2017)

Advantages	Disadvantages
Does not require the addition of organic solvents	Unavoidable increase in temperature during preparation
Decreases Microbiological interference	Not entirely suitable for hydrophilic drugs

Solvent-emulsification evaporation is where drug and lipids are dissolved in a water immiscible solvent, dispersed into the aqueous phase containing suitable surfactants and the solvent is then evaporated under reduced pressure 40-60mbar (Naseri *et al.,* 2015). This process allows production of formulations utilising heat sensitive drugs, due to using low pressure instead of high temperature to evaporate solvents. Although, the process is limited by the presence of residual solvents in the final formulation which could cause toxic side effects dependant on concentration (Haider *et al.,* 2020).

Table 1. 4: the advantages and disadvantages of solvent-emulsification evaporation as a preparation method of NLCs. Sources: (Khosa, Reddi and Saha, 2018), (Elmowafy and Al-Sanea, 2021)

Advantages	Disadvantages
Avoids thermal stress	Uses organic solvents
	Inadequacies in lipid solubility in solvents

Supercritical fluid extraction uses supercritical fluids such as carbon dioxide to solubilise excipients and drugs, which are then rapidly depressurised through a nozzle into the aqueous surfactants (Chakravarty *et al.,* 2019). This rapid depressurisation leads to supersaturation and a limit on crystal growth, thereby limiting particle size (Chakravarty *et al.,* 2019) (Chattopadhyay *et al.,* 2007).

Table 1. 5: the advantages and disadvantages of supercritical fluid extraction as a preparation method of NLCs. Sources: (Li *et al.,* 2017), (Chakravarty *et al.,* 2019)

Advantages	Disadvantages
Forms minute particles	Utilises organic solvents
Environmentally friendly	Poor solubility of solutes in supercritical fluid CO_2

Hot homogenisation utilising probe ultrasonication was the preparation method chosen. The choice was made due to it avoiding the use of organic solvents and decreasing the microbial load of formulations. The microbial load was decreased due to the elevated temperature during manufacture and sonication, this would not however affect the quantity of spores, hence further controls were utilised during experimentation.

1.2.2 Vancomycin-loaded nanostructured lipid carriers

Vancomycin is a glycopeptide antibiotic acting upon the D-Ala-D-Ala bonds within the cell wall of Gram-positive bacteria (Figure 1.1). When the drug was discovered, it was initially reserved for treatments of resistant bacterial strains due to initial strains showing no premise of developing resistances (Rubinstein & Keynan, 2014). A common use is to treat MRSA bacterial infections due to the drug still having high efficacy on the methicillin-resistant strain. Vancomycin is known to have high elimination at 80-90% of administered drug excreted unchanged in the urine (M. Rybak *et al.,* 2009). In human plasma 55% of vancomycin elimination occurs due to glomerular filtration by blood protein binding (FDA, 2010). This binding causes vancomycin associated nephrotoxicity in patients receiving high dose

vancomycin for systemic infections, as reabsorption by renal cells causes mitochondrial degradation and renal failure in extreme cases (Stidham *et al.,* 2011).

Vancomycin hydrochloride is a highly fragile drug due to a low pKa (2.66) and being highly temperature sensitive (stored at -20°C) (Settimo *et al.,* 2014). In clinic, a powdered form of the drug is frozen and reconstituted into sterile saline before intravenous infusion. Research shows when reconstituted in water clinically relevant degradation occurs when infused for longer than 120 minutes (Peterlini *et al.,* 2015).

In 2002 the first VRSA (Vancomycin resistant *Staphylococcus aureus)* strain was isolated in a diabetic patient with chronic renal failure and multiple ulcerations (Quirk, 2002). After multiple treatments consisting heavily of continuous infusion vancomycin (ci-VCM) VRSA isolates were taken from a catheter and later a foot ulcer. This vancomycin resistant infection was successfully treated with a systemic treatment of co-trimoxazole. Although, data from acquired immune deficiency syndrome patients in Tanzania where isolates from common infections such as urinary tract and meningitis were taken and tested for susceptibility. They found 16.1% of isolates were *S. aureus* and 75% were resistant to co-trimoxazole showing the possibility of resistance occurring rapidly in patients under long term treatment (Marwa *et al.,* 2015).

Enterococcus faecalis is a vancomycin resistant Gram-positive coccus due to a vanA gene within its plasmid (Tn1546) that changes the D-Ala-D-Ala bond within the cell wall to D-Ala-D-Lac, this change in the target site neutralises vancomycin's bactericidal effects (De Niederhäusern *et al.,* 2011). Due to vancomycin resistant *Enterococcus* (VRE) and VRSA being isolated in the same patient's consistently, research was completed into the reason. It was discovered that lateral gene transfer of the Tn1546 transposon containing the VanA gene to coinfecting *Staphylococcus aureus* strains is achievable *in vitro* without laboratory animals. This shows the possibility of lateral gene transfer occurring creating VRSA strains in already compromised patients (De Niederhäusern *et al.,* 2011). Compounding this, the global presence of VRSA infections has increased in the last two decades with 2% pre-2006 and 7% between 2015-2020 (Li *et al.,* 2023).

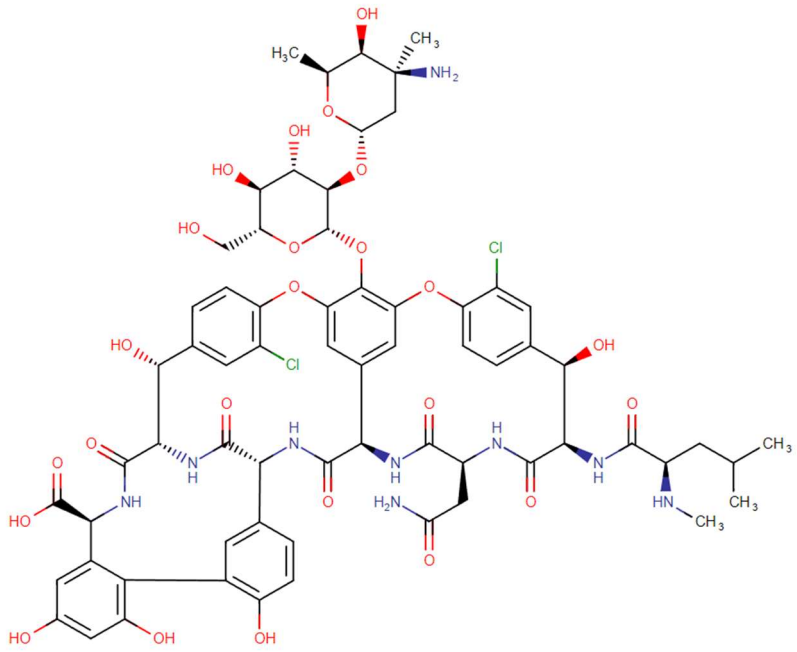


Figure 1.1: The chemical structure of vancomycin (Drug Bank, 2023)

NLCs were chosen as a potential alternate drug delivery system to improve the efficacy, storage and side effects of vancomycin treatment. NLCs have been shown to aid in protecting drugs whilst increasing the uptake by tissues and changing pharmacokinetic properties (Dumont, 2022). An increase in circulation time attributed to the blocking of blood protein binding (FDA, 2010), could prevent high dosage. A lower dosage could decrease development of resistance mechanisms in commensal flora by lowering the exposure these bacteria have to the drug. Due to the associated nephrotoxicity a decrease in elimination would also reduce reabsorption by the kidneys (Stidham *et al.,* 2011). The embedding provided by NLCs matrix would protect the drug from external forces such as heat, pH and protein binding (Chauhan *et al.,* 2020). Meaning more molecules would reach target sites in biological systems and preserve antibacterial properties in storage. This is vital as a common use for ci-VCM is the treatment of resistant bacteraemia and septicaemia which if not treated immediately and effectively is fatal (NICE - BNF, 2023).

1.2.3 Current research into vancomycin-loaded nanoparticles

Table 1. 6: A review of vancomycin loaded nanoparticles. Type, method, characteristics and EE%/DL is provided for each study. EE% and DL refers to entrapment efficiency percent and drug loading, respectively. PS, PDI and ZP refer to particle size, poly dispersity index and zeta potential, respectively. The EE%/DL section data is provided as entrapment efficiency / drug loading if only one number is given that is EE%.

Type	Preparation method	Characteristics	EE%/DL	Reference
Metal Silver nanoparticles	Chemical reduction	PS - 16-25nm	19.6% *bound*	(Esmaeillou *et al.,* 2017)
Aragonite nanoparticles	Hydrothermal Hot Homogenisation	PS – 36±6nm ZP – -21.2±5.7 mV	54.05%/95.99%	(Saidykhan *et al.,* 2016)
Polymer nanoparticles	Double emulsion evaporation	PS – 320.5±35nm PDI – 0.270±0.012 ZP – -19.5±1.3mV	94.62%/16.75%	(Nouruzi *et al.,* 2023)
Solid lipid nanoparticles	Hot homogenisation	*In acidic conditions (pH 5.5)* PS – 220.5±1.9nm PDI – 0.380±0.005 ZP – +9.39±0.046mV	81.18%/8.1%	(Mhule *et al.,* 2018)
Cationic NLCs	Cold Homogenisation	PS – 96.40±0.71nm PDI – 0.352±0.011 ZP – +29.7±0.47mV	74.80%	(Jounaki *et al.,* 2021)
Acid responsive NLCs	Hot homogenisation	PS – 225.2±9.1nm PDI – 0.258±0.02 ZP – -9.2±2.7mV	88.7%/3.55%	(Osman *et al.,* 2019)

A brief review was conducted on vancomycin loaded nanoparticles to highlight the novelty of this study and investigate already researched formulations, this is shown in table 1.6. A study by Esmaeillou (2017) investigated vancomycin tipped silver nanoparticles and their effect on multi-drug resistant strains of bacteria. Nanoparticles were prepared using a simple chemical reduction technique binding

19.6% of vancomycin added by conjugation. Bacterial strains utilised were isolates from patients, where a wide range of bacterial were utilised. It was found in this study that the conjugation of vancomycin to silver nanoparticles increased the efficacy and decreased MICs in Gram-positive tested. Studies highlighted that the MIC for *S. aureus* decreased from 3.2µg/ml to 0.05µg/ml and that the MIC for methicillin resistant *Staphylococcus epidermis* decreased from 3.2µg/ml to less than 0.02µg/ml. However, metal nanoparticles have been linked to cytotoxicity when used in high dosage which would be needed for systemic infections (Zhang et al., 2022). Another study by Saidykhan (2016) investigated the premise of cockle-shell derived nanoparticles as a biodegradable delivery system for treating osteomyelitis. Nanoparticles were manufactured by taking a hydrothermal approach to hot homogenisation and characterised using transmission electron microscopy and a Zetasisor, where FTIR and X-ray powder diffraction was utilised for interaction diagnosis. High drug loading with 54% entrapment was achieved with a weak non-covalent bond found between vancomycin and the nanoparticles. Cell viability studies were completed on human fetal osteoblast cell lines finding good absorption and 80% viability post 120hr treatment. During bacterial sensitivity testing a MRSA strain was utilised and tested against both vancomycin and the vancomycin loaded nanoparticles, finding the MIC increased from 2.83µg/ml to 19.5µg/ml, respectively. Although an increased MIC was found in the nanoparticle treatment Saidykhan determined it would effectively eliminate MRSA infections in MRSA-induced osteomyelitis.

A study by Nouruzi (2023) investigated the premise of vancomycin loaded polymer nanoparticles comprised of poly lactic-co-glycolic acid (PLGA) against *S. aureus* biofilms. Double emulsion-solvent evaporation was used to formulate nanoparticles using chloroform as a solvent. Spectroscopy was used to discover a high entrapment, but low drug loading was achieved. MTT cell line studies showed no cytotoxicity to any formulations or excipient. Biofilm sensitivity testing highlighted a slow release of vancomycin from PLGA nanoparticles shown by the continued inhibition of biofilm growth at 72hrs compared to vancomycin where decreased efficacy was observed at this time point. Agar well diffusion investigations showed decreased MICs for all nanoparticle formulations when compared to free

vancomycin at 24, 48 and 72hrs. Mhule (2018) completed a mice study into the premise of oleamide SLNs loaded with vancomycin to see if any increased efficacy could be achieved in acidic conditions. Oleamide was synthesized from esterification of oleic acid and used in hot homogenisation to formulate SLNs. A low drug loading but high entrapment was achieved measured by ultrafiltration. A cell viability of above 75% was achieved when testing synthesised oleamide and formulations showing low cytotoxicity. *In vitro* sensitivity studies revealed loaded SLNs were not more effective than free vancomycin at a neutral pH, although at an acidic pH SLNs showed an MIC of 0.244µg/ml compared to free vancomycin at 15.6µg/ml. *In vivo* studies showed a 4.14-fold increase in the reduction of MRSA in the skin of colonised mice.

Jounaki (2021) investigated the benefits that NLC encapsulation could provide to endophthalmitis infections of the eye. The aim being to create a 'depot of drug' to be slowly released at the corneal barrier to then diffuse across to the site of infection. An indirect method was used to identify entrapment which was high for a formulation containing a hydrophilic drug such as vancomycin. *In vivo* rabbit studies were completed where one of the eyes of rabbit was inoculated with MRSA and a bacterial count was completed over 5 days. It showed an injection of free-VCM caused an immediate decrease in bacterial count from 311.50 to 7.50 cfu and had a final count of 0.5 cfu on the fifth day. Whereas VCM-loaded NLCs had a smaller progressive decrease in cfu from day zero to day five ending on 1.75 cfu. Irritation studies were also conducted finding the optimized formulation had low to no effect on ocular sensitivity and had little to no change to irritation. Another NLC study completed by Osman (2019) investigated the benefits NLC encapsulation could provide to acidic environments, similar to that of Mhule (2018) but utilising the drug loading benefits of NLCs. Cell viability studies were completed showing a non-toxic formulation with greater than 75% viability after 48hrs. The optimised formulation produced a lower MIC at pH 6 when compared against pH 7.4, compounding this the MIC values produced were also lower than free vancomycin against resistant and non-resistant strains. Furthering these results *In vivo* studies were completed where a mouse skin model was utilised

showing the formulation had a superior reduction in bacterial count when compared to free vancomycin.

The studies explained show that current NLC, and nanoparticle research is primarily investigating the effect nano-delivery systems have on fighting infections in acidic conditions. The studies also show that the lipidic encapsulation of vancomycin increases efficacy and has the potential to increase absorption into tissues and cells.

1.3 NLCs Characterization

1.3.1 High performance Liquid Chromatography (HPLC)

Reverse phase HPLC (RP-HPLC) is the reverse of normal phase HPLC achieved by a stationary phase which is more non-polar that the eluting solvent. Reverse phase HPLC involves the binding of an organic molecule to a stationary phase in a non-polar environment provided by the stationary phase. It uses a column size with around 4.6mm diameter and lengths varying from 150 to 250mm. The columns are filled with silica particles which are modified with long hydrocarbon chains to make them polar. A polar solvent is then used to carry the chosen compound through a column with polar molecules passing through quicker due to the strong interaction between the solvent and the molecules (Sadaphal & Dhamak, 2022). When the molecule leaves the column, it is detected by an ultraviolet detector set to a specific wavelength of light, this generates a peak on the chromatogram where data is recorded. The mixture of solvents is referred to as the mobile phase, the constituent parts of the mobile phase are established by the properties of the molecule being analysed. For example, vancomycin is a hydrophilic molecule with a pKa of 2.66 and moderately soluble in methanol (Settimo *et al.,* 2014). RP-HPLC was utilised to quantify the entrapment efficiency of the drug encapsulated within NLCs. The mobile phase ratio, flow rate and wavelength are critical attributes that affect the quality of the peak and are optimised during development of the HPLC method.

1.3.2 Differential scanning calorimetry (DSC)

DSC is a sensitive thermal analysis method which enables the study of the thermodynamic behaviour of NLCs, by measuring the heat flow associated with endothermic and exothermic transitions as a function of temperature. In practice DSC is an investigatory test which allows identification of melting points of samples and allows comparison of any state or phase changes in excipients prior and post addition to formulation. DSC allows to differentiate between a crystalline and amorphous state of the drug and excipients. Crystallization occurs when polymers and molecules gain enough kinetic energy from the heating process to form more ordered structures and begin releasing heat (Leyva-Porras *et al.,* 2020). As the heating element is attempting to maintain a temperature it decreases the release of heat in correlation with the heat released from crystallisation, affecting heat flow. The amorphous state is described as a static structural order with temperature fluctuations similar to that of a frozen substance (Edina Vrani *et al.,* 2004). Melting points are identified by a sharp endothermic peak showing a rapid change in heat flow attributed to the crystallisation and then re-crystallisation of the sample (Fang *et al.,* 2012). This data can provide hints as to the quality of the NLC as a drug delivery system by revealing the presence of disordered lipid phases, crystallinity and drug-carrier interactions by the presence of drug within the NLCs (Pezeshki *et al.,* 2014) (Fadlelmoula *et al.,* 2022).

1.3.3 Fourier transform infrared spectroscopy (FTIR)

FTIR measures the rate at which different molecules absorb infrared radiation at different frequencies allowing the creation of unique fingerprints of their chemical structure. In the context of NLC formulations this allows identification of the molecular vibrations occurring within the NLC structure, allowing observation of lipid-drug interactions as well as excipient interactions. The infrared light is captured by a detector which generates a graph with peaks and troughs corresponding to specific frequencies within the sample. Each peak can be compared to a database and identifies the proportion of certain bonds within the structure and identifies the presence of individual components within the NLC.

Aims and objectives

The aim of this project was to investigate a nanostructured lipid carrier (NLCs) for efficient delivery of vancomycin.

The specific objectives of the project are

1. Development of vancomycin NLCs and optimisation of process and product parameters to meet the critical quality attributes of the product.
2. Physiochemical characterisation of NLCs by particles size, poly dispersity index, zeta potential and entrapment efficiency.
3. Validation of a High-performance liquid chromatography assay to quantify vancomycin concentration in vancomycin loaded NLC.
4. Microbiology investigations into Bacterial susceptibility testing to compare vancomycin NLCs with that of free vancomycin. The *in vitro* sensitivity tests comprised of determination of minimum inhibitory concentration (MIC) panels and a time-kill assay.
5. An alteration of VCM pharmacokinetics with the premise of increasing blood circulation and decreasing elimination and toxicity.

Hypothesis

The hypothesis was that encapsulated vancomycin would have equivalent bactericidal effect as free drug on the growth of *S. aureus* reference strain ATCC 29213. The encapsulated vancomycin would provide an increased circulation time and show enhanced stability in an aqueous environment.

Chapter 2: Materials and Methods

2.1 Nanostructured lipid carrier (NLCs) Preparation and Characterization

2.1.1 Hot homogenisation

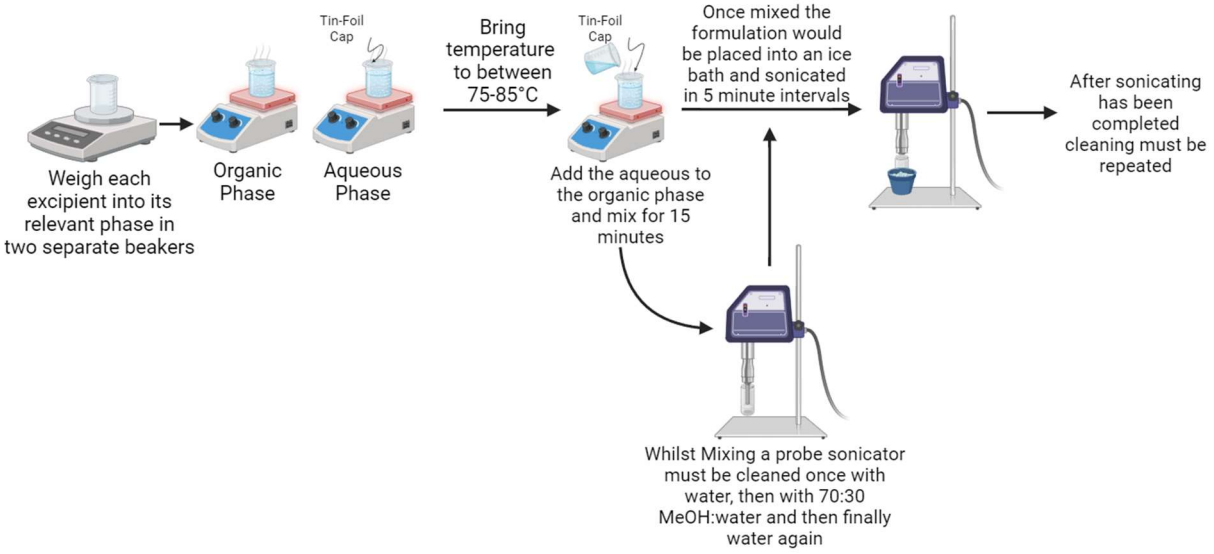


Figure 2. 1: A flow scheme which demonstrates hot homogenisation and how it was completed within this project

NLCs were prepared using the hot homogenisation technique. Two phases were prepared separately one being an organic lipid phase and another aqueous phase. Excipients were accurately weighed into beakers and heated to 80°C ± 5. The lipid phase constituted of solid lipid, liquid lipid and lipid surfactants, whilst the aqueous phase comprised of hydrophilic surfactant and water. The aqueous phase was continuously mixed using a magnetic stirrer at 150-200rpm. Once this temperature was reached the aqueous phase was maintained, and the lipid phase allowed to fully melt. Once the lipid phase had melted the two phases were combined in one beaker and mixed continuously for 15 minutes using a magnetic stirrer at 150-200rpm. The two phases must be kept at the same temperature to prevent separation and avoid particle aggregation. Meanwhile a probe-sonicator (Vibra-Cell, Newton CT, Sonics) was cleaned by sonicating HPLC-grade water for five-minutes at 40% amplification, diluted methanol (70:30 MeOH:Water, v/v) for five-minutes at 40% amplification and then water again to ensure the probe was fully clean prior to sonication of the test formulation. Formulations were sonicated within thick glass containers, held by clamps and placed within an ice

bath. After 15 minutes of mixing the formulation was sonicated at five-minute intervals at 40% amplification. A graphical schematic of hot homogenisation is shown in figure 2.1.

The ice bath allows cooling (<55°C) in between sonication intervals to dissipate the heat generated from sonication and prevent destabilisation of NLCs. For each new formulation, a sample was taken at each five-minute cooling period during sonication and identified CQAs were recorded as output values (HDS, PDI and zeta potential). The sonication time for the formulation was optimised to achieve the desired hydrodynamic size (<200nm) and PDI (<0.300). Post sonication the formulation was characterised according to the method described by Zwain *et al.,* (2021). A graphical representation of an NLC is shown in Figure 2.2.

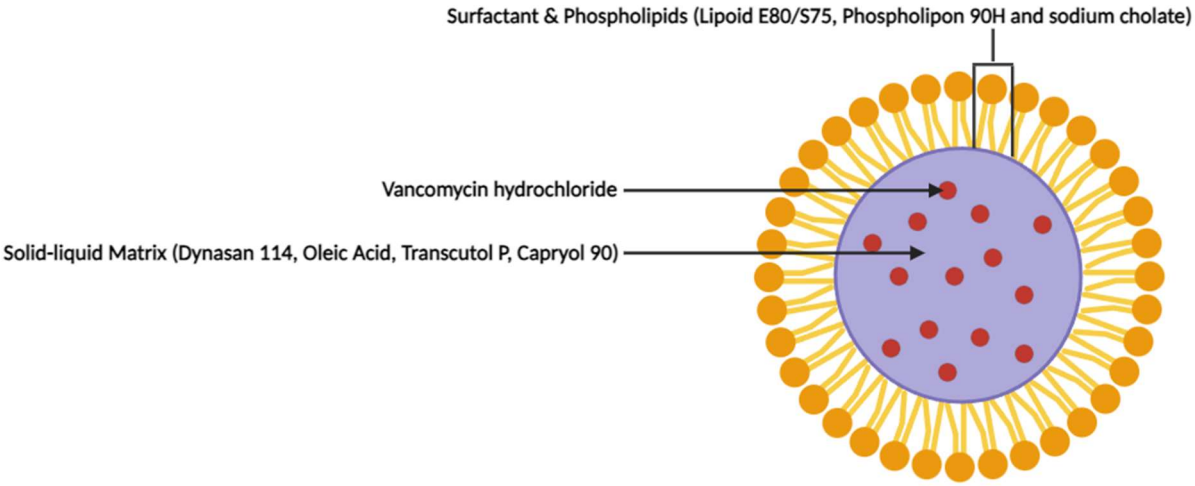
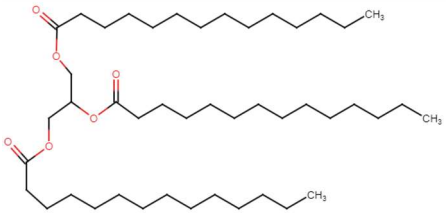
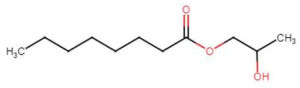
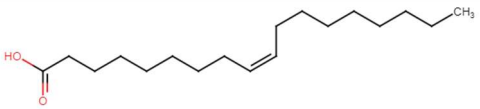
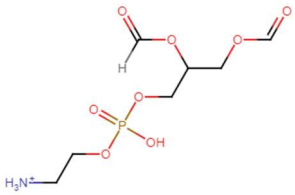
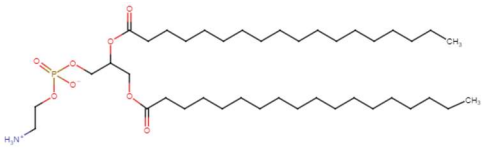
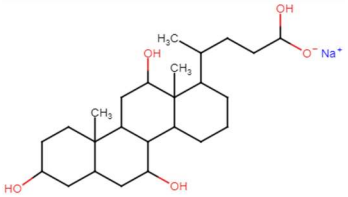


Figure 2. 2: A diagram illustrating the structure of a nanostructured lipid carrier with the location of each excipient labelled

2.1.2 **Formulation Optimisation**

During optimisation key aspects of the formulation were evaluated. These were product parameters which investigated excipients and their concentrations, processing parameters where sonication and preparation processes were observed and critical quality attributes which were the characterisation values (output values) PS, PDI, Zeta potential and entrapment efficiency (%EE).

Table 2.1: B1 excipient list where excipient name, group, chemical structure and properties are given. Sources: Dynasan114 – (IOI Oleochemical, 2024), Transcutol P - (Osborne *et al.*, 2018), Capryol 90 – (Abou Assi *et al.*, 2020), Lipoid E80/S75 – (Kanno *et al.*, 2007), Phospholipon 90H – (Kolbina *et al.*, 2019), Sodium Cholate – (Miatmoko *et al.*, 2022), Oleic acid – (Ruiz *et al.*, 2010)

Excipient	Excipient Group	Chemical Structure	Properties
Dynasan 114	Solid Lipid		- High melting point - Effective lubricant for oral drugs
Transcutol P	Liquid Lipid		- Clear liquid - Protic solvent - High Boiling point
Capryol 90	Liquid Lipid		- Non-ionic surfactant - Lipid solvent
Oleic acid	Liquid Lipid		- Low melting point - Insoluble in water
Lipoid E80	Lipid surfactant	 *Lipoid E80 composes of 80% phosphatidylcholine hence is shown above (Lipoid GmbH, 2022) *Lipoid S75 composes of 70% phosphatidylcholine hence is shown above (Lipoid GmbH, 2022)	- Low monomeric solubility - increases membrane transfer rate - one of four glycerol-phospholipids which build eukaryotic lipid bilayers
Lipoid S75	Lipid surfactant		
Phospholipon 90H	Lipid surfactant	 *Phospholipon consists of 90% hydrogenated phosphatidylcholine (Lipoid GmbH, 2022)	- Non irritating - Non sensitising - increases uptake and entrapment in lipid matrices of water-soluble drugs
Sodium Cholate	Aqueous Surfactant		- Anionic surfactant - soluble in water and not in lipid

During experimentation different individual excipient concentrations were changed to see the effect on output values. The concentration of liquid lipids oleic acid, Transcutol P and Capryol 90 were optimised to increase %EE of drug within NLCs. The range used was 4mg/ml and 12mg/ml. The surfactants sodium cholate and Kolliphor HS15 was optimised to increase stability and decrease coalescence. The range used here was 4mg/ml and 10mg/ml. The optimisation steps were completed in 25ml formulations if volume was decreased concentrations reflected this. These changes informed the decision making on the product parameters, and how changing these concentrations can affect the final optimised formulation. The formulation components used are shown in table 2.1.

2.1.3 NLC Hydrodynamic size, Polydispersity Index and Zeta potential

Characterization was conducted on a Malvern Panalytical Zetasizer Nano ZS (Malvern Panalytical, UK). Samples were characterised according to manufacturer's instructions. This was done by filling a clear polystyrene cuvette with the test formulation and placing within the cuvette Zetasizer port (orientated appropriately to the cuvette used) and analysed at 25°C using water as the medium and the Malvern Zetasizer software (Ver 8.02) provided by Malvern Panalytical. The machine utilises dynamic light scattering (DLS) analysis in conjunction with Brownian motion to ascertain the HDS and PDI. Brownian motion utilises the latent kinetic energy of particles to convert natural translocation and velocity into size, where velocity is inversely proportional to size (Nienhaus & Nienhaus, 2020). DLS shines lasers on particles, the laser is then reflected back at a detector and intensity is recorded, the intensity is converted to velocity and allows quantification of Brownian motion and therefore size and distribution (Wang *et al.,* 2021). Post testing, cuvettes and formulations are disposed of accordingly.

For zeta potential analysis copper probed polystyrene cuvettes are used (Malvern Panalytical, UK). The copper probes seen in the zeta potential cuvette allow a measurement of the electrophoretic light scattering (ELS) and doppler effect the magnetic induced movement has on particles. Particles move through the capillary channel induced to move from one electrode to another and ELS allows the rate

at which these particles move to be converted into a value by using the Smoluchowski/Hückel approximation (Ramaye *et al.,* 2021).

2.2 Optimisation and Validation of High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was carried out on an Agilent technology (California, USA) 1200 series HPLC equipped with a degasser, autosampler and ultraviolet (UV) detector. The method was optimised and analysed using a reverse phase Kinetex 5 µ C18 100 Å, 150x4.6 mm column (PhenomeX Inc., Emeryville, USA).

2.2.1 Stock solution preparation

Stock solutions and working standards were prepared for vancomycin in water. This would allow testing of vancomycin in solution for validation and then free-drug vancomycin (FD-VCM) from NLC formulations. A stock solution was prepared by weighing 5mg of vancomycin hydrochloride (VCM) powder (Sigma Aldritch, UK) into a 5ml volumetric flask and dissolving in 5ml of HPLC grade water, producing a 1mg/ml solution. This was then pipetted into 0.5ml aliquots and frozen at -20°C, each aliquot was dated and after 5 days disposed of accordingly. On each day of HPLC an aliquot was taken out, thawed and the working concentrations were prepared. The range of concentrations used were 0.5, 1, 2, 4, 8, 16, 20µg/ml, all validation was completed within this range.

2.2.2 Method optimisation

The optimum method was established by changing individual key aspects of the method used by Milla *et al* (2020) to best suit the analysis of VCM in water rather than plasma. The chromatographic conditions were established to be mobile phase ratio, flow rate, pH and wavelength. The testing range for optimisation was a flow rate of between 1ml/min and 0.7ml/min and a pH range of between 2.6 and 3.15. Mobile phase ratio was adjusted from 60% pH adjusted water to 100%. Wavelength was optimised from 190 to 290nm.

2.2.3 Validation

The method was validated following the International Council for Harmonisations (ICH) guidelines of Technical Requirements for Pharmaceuticals for Human Use Q2(R1) (ICH, 1995) (Shabir, 2004). Calibration curves were obtained by thawing a vancomycin stock solution and creating a 100µg/ml solution by pipetting 100µl of stock into a microcentrifuge tube and making up to 1ml with HPLC grade water. A retention time study was completed by injecting one concentration seven times and comparing the standard deviation of the retention time. Secondly, a range of seven concentrations was created ranging from 0.5µg/ml to 20µg/ml. Using the optimised method each concentration was tested six times and the area of the peak recorded. Precision was completed as to the repeatability and reproducibility of the method across differing conditions achieved by different days and time of day. An intra-day study (repeatability) was completed by using a high and a low concentration within the range, testing morning and afternoon on the same day. An inter-day study (reproducibility) was completed by using the same high and low concentration but across three different days.

2.2.4 VCM-HCL Free drug (FD)

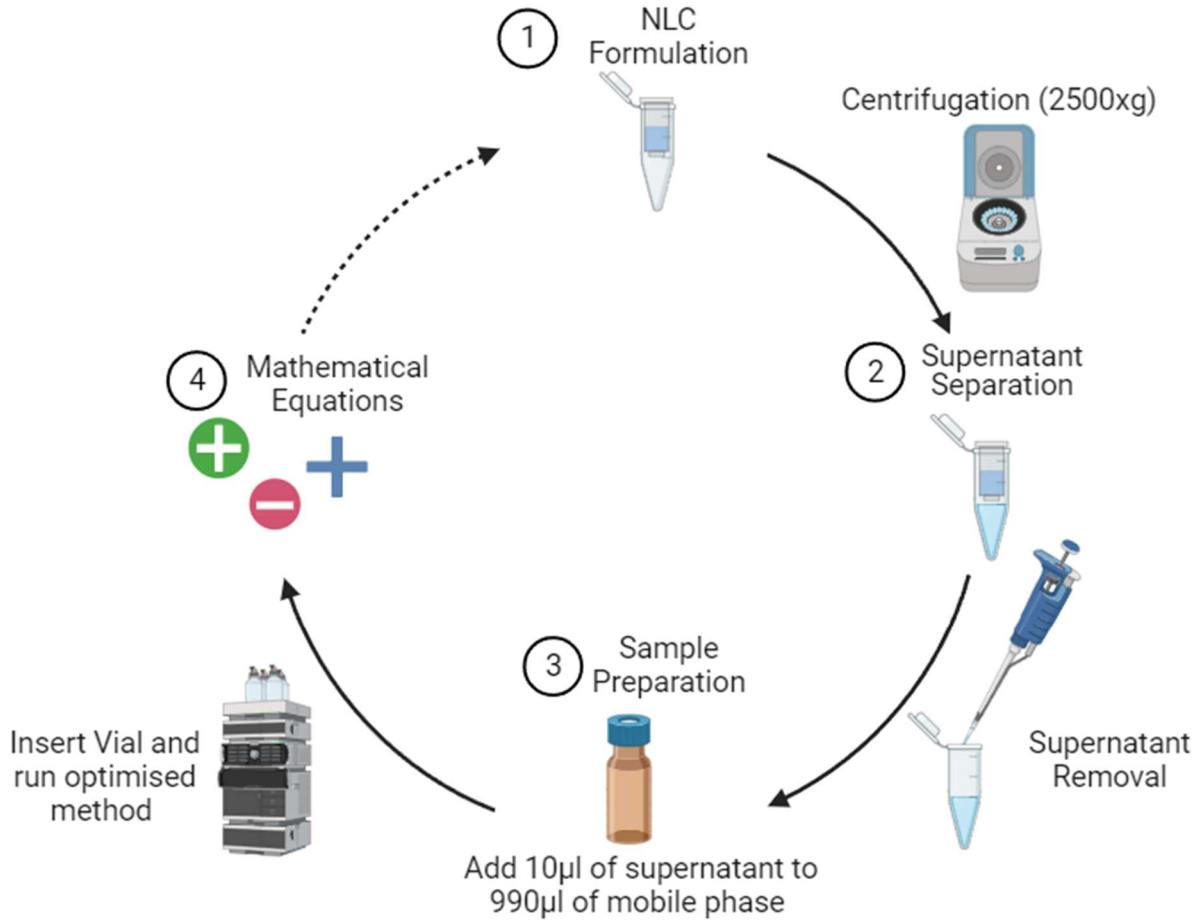


Figure 2. 3: A cyclic flow demonstrating the indirect method to quantify drug entrapment utilising 3kDa Amicon molecular cut off filters and reverse phase high performance liquid chromatography

Free drug was calculated by pipetting 0.5ml of formulation into a 3kDa Amicon molecular cut-off centrifuge filter (Merk Millipore, UK) and centrifuging at 2500 x g for 30 mins. This separates the formulation into solid lipid particles and a supernatant which contains free drug. 10µl of the supernatant was pipetted into a HPLC vial and made up to 1ml with mobile phase before being analysed by HPLC. Encapsulated drug was calculated by subtracting the calculated free drug from drug added (DA), divided by drug added and multiplying by one hundred to produce a percentage. This is shown in equation 1 below and illustrated in figure 2.3.

$$\frac{DA - FD}{DA} x100$$

DA – Drug Added
FD – Free Drug

Equation 1: Formula used for calculating percentage entrapment efficiency (%EE)

2.3 Differential Scanning Calorimetry (DSC)

All samples were run on a TA DSC Q2000 (Thermo-analysis, Elstree-Hertfordshire, UK) on a heat cool method. Tzero hermetic aluminium pans (Thermo-analysis, Elstree-Hertfordshire, UK) were used for sample preparation. Each sample was accurately weighed to between 3-7mg. The sample was heated to 25°C at a rate of 10°C a minute, heated to 70°C at 10°C per minute and held isothermal for 60 minutes, cooled to 0°C at 10°C per minute and held for 2 minutes and then heated to 250°C at 10°C per minute. DSC analysis was completed on free drug, solid lipids, liquid lipids and physical mixture alongside blank and loaded nanoparticles. The range of samples completed provided insights into stability and crystal lattice morphology by measuring the heat flow and how it changed when samples underwent crystallisation, melting and then re-crystallisation (Chauhan *et al.,* 2020). This data was analysed within the TA analysis software.

2.4 Scanning electron microscopy (SEM)

SEM allows visual confirmation of NLC morphology, size and surface topography. Due to the high resolution and magnification provided by the SEM platform, data can be generated to visualise the morphology of the particle and advise on its capability of bypassing biological systems (Patel *et al.,* 2012) (Abdolahpour *et al.,* 2017). A 1 in 50 dilution was performed on the final optimised formulation, samples were then transferred to a gold-plated mesh grid and allowed to dry for 24 hours. Once dry, samples were transferred to a Thermo Scientific™ Quattro SEM for imaging at 35000x magnification.

2.5 Fourier-transform infrared spectroscopy (FTIR)

FTIR was completed by a Nicolet iS 10 Fourier transform infra-red spectroscopy analyser (Thermo Scientific, UK). Samples were dropped on the crystal analyser and analysis was completed in the range of 400-4000cm^{-1} transmittance. The data was analysed aided by an analytical chemistry sheet created by Brunning (2015). Data was analysed using the OMNIC® software (ThermoFisher Scientific, UK).

2.6 **Bacterial Characterisation**

Staphylococcus aureus ATCC strain 29213 (Kwik-Stik, BioConnections, UK) was selected for testing NLCs as this organism is the EUCAST quality control reference strain for vancomycin MIC testing (EUCAST, 2023). Antimicrobial susceptibility data for this strain is available for a wide range of antibiotics and this allowed for antibiogram profiling before and during the study to characterise the strain and confirm the authenticity of the culture. For example, ATCC strain 29213 was utilised by Campanini-Salinas *et al.,* (2021) to investigate quinolone antibiotics as a reference for methicillin resistant *S. aureus* (ATCC 43300). According to EUCAST data this strain of *Staphylococcus aureus* (ATCC 29213) has an MIC for vancomycin of 1µg/ml.

Characterisation was completed on morphology by light microscopy covering shape and Gram-stain to identify the genera of the organism subcultured. A latex agglutination test was then conducted to determine the species subcultured and an antimicrobial susceptibility test (AST) was completed to provide an antibiogram of the antibiotics the organism reacts with, and to what extent. Finally, a growth curve was completed to provide context for kill-time assays.

2.6.1 **Culture Preparation and Storage**

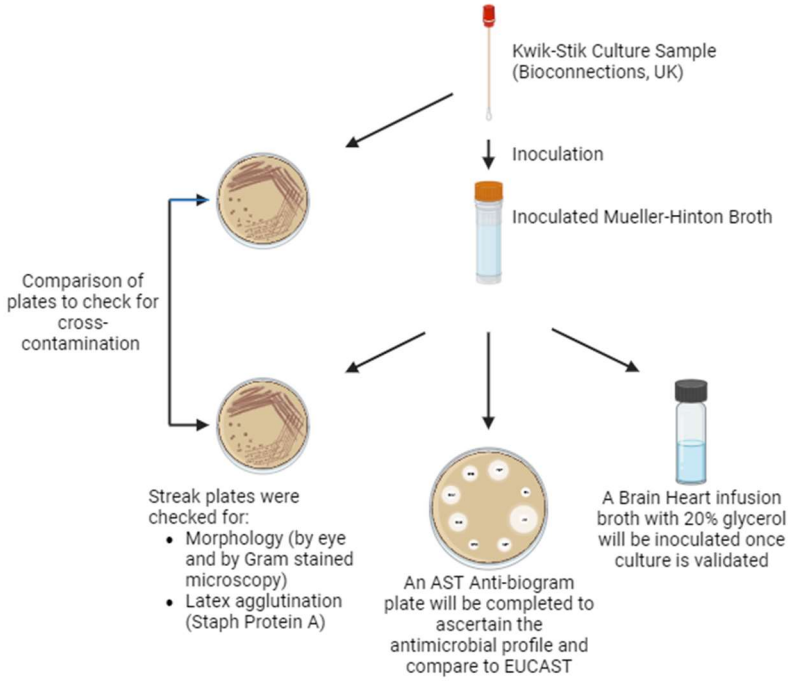


Figure 2. 4: An Illustration of the Steps taken to prepare and characterise cultures.

Cultures were reconstituted from Kwik-Stik (BioConnections, UK) primary cultures according to manufacturer's instructions and subcultured into Mueller-Hinton broth (MHB) (Oxoid, Thermofisher Scientific, UK) and streaked onto Nutrient agar in sterile 90mm polystyrene petri dishes (Oxoid, Thermofisher Scientific, UK), before both were incubated aerobically for 24 hours at 37°C. After incubation, cultures were again streaked to check for purity and compared to prior plate to ensure to contamination had occurred. Once purity was confirmed 100µl was pipetted into 900µl of 20% glycerol in brain heart infusion broth (Oxoid, Thermofisher Scientific, UK) and stored at -80°C. To aid in preventing mutational changes streak plates were created from an original -80°C stock once every three to four weeks (figure 2.4). If contamination was suspected, a -80°C stock vial allowed for a new working plate to be created and experiments repeated.

2.6.2 *Staphylococcus aureus* characterisation investigations

The organism was characterised via light microscopy with a Gram stain to verify morphology, and a *Staphylococcus aureus* protein A latex agglutination test (Prolex™ Blue Staph Latex kit, Pro-Lab

diagnostics, UK) was completed to verify it was a *Staphylococcus aureus,* shown in figure 2.3. This was completed in accordance with manufacturer instructions. The negative Control reagent was utilised according to manufacturer's instructions confirming the positive result from test organisms. Latex agglutination refers to latex particles conjugated to the protein A antigen only found in *S. aureus*. The particles bind to the bacteria and create dark coloured aggregates in the faintly blue coloured solution.

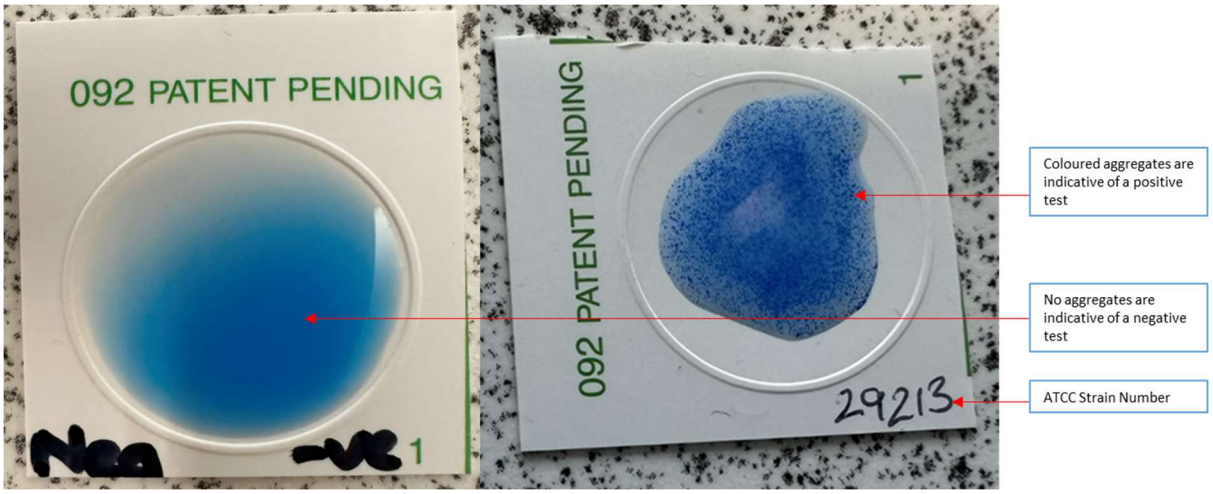


Figure 2. 5: Staphylococcus aureus latex agglutination testing for protein A. Strain tested is shown and labelled by ATCC number. A negative test is shown on the left with the test organism result on the right.

An antimicrobial susceptibility testing (AST) antibiogram was generated (figure 2.4) according to the EUCAST method for disc diffusion assay (EUCAST, 2023) using a wide range of different antibiotics with different target sites to investigate the sensitivity of the strain. The antibiotics used were ciprofloxacin (5µg), gentamicin (10µg), meropenem (10µg), vancomycin (30µg), ampicillin (10µg), tetracycline (30µg), ceftazidime (10µg) and trimethoprim (5µg) (Oxoid, UK). The antibiogram allowed simultaneous screening of the strain's susceptibility to different antimicrobials. This was completed by fully inoculating two Mueller-Hinton agar plates with a 0.5 McFarland standard of the *S. aureus* (ATCC 29213) strain from a fast-growing overnight culture. The eight antibiotics were then placed equidistant from each other, four on each plate. The two plates were cultured for 18-24 hours at 37°C aerobically. Following incubation, zones of inhibition were measured in millimetres and compared to the EUCAST Quality Control tables (version 13.1) (EUCAST, 2023).

2.6.3 Total viable count

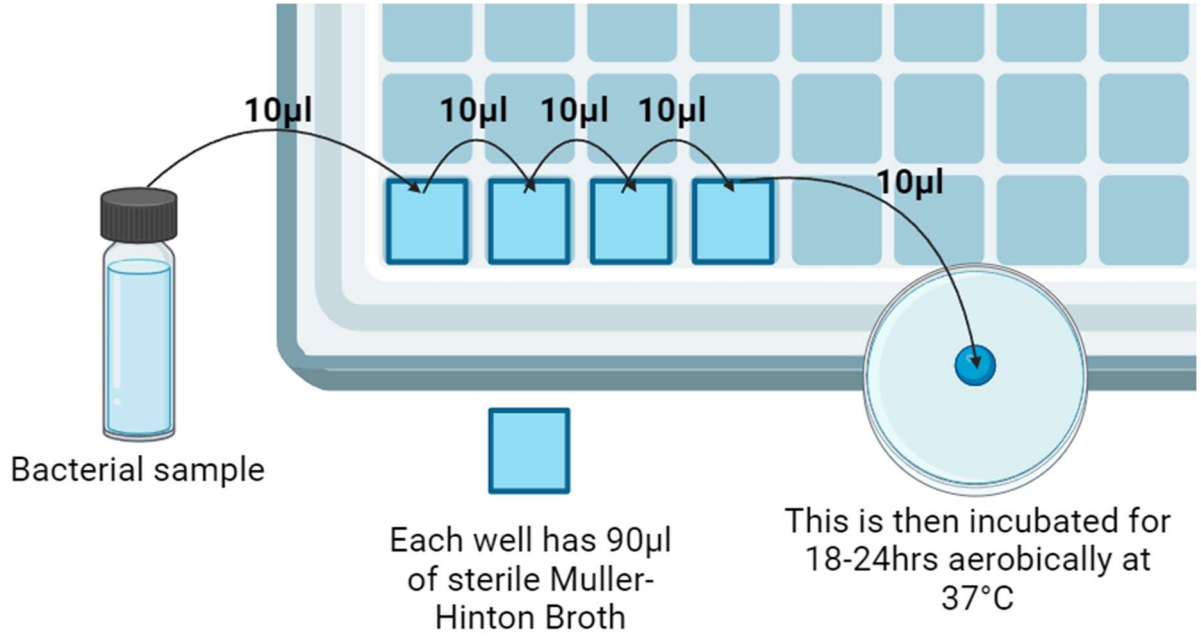


Figure 2. 6: An illustration of how a total viable count was completed. Utilising a flat bottom 96-well plate bacterial count was serially decreased allowing quantification.

Figure 2.6 illustrates how cell counting was completed, 10µl of bacterial broth was pipetted into 90µl of sterile MHB, this was repeated three times in a 96-well microtiter plate achieving a 4-log serial dilution. Then, 10µl of the final dilution would be pipetted onto a nutrient agar plate and incubated for 24 hours in an aerobic environment at 37°C. This resulted in a 5-log dilution in the bacterial count as shown by Miles *et al.,* (1938). Miles *et al.,* (1938) estimated a dilution of this size allowed a maximum of $5x10^7$cfu/ml in undiluted cultured to be accurately counted.

2.6.4 Growth Curve of *S. aureus* strain to determine logarithmic growth

A growth curve was conducted to establish a time frame for rapid cell division indicative of a logarithmic culture, prior to testing the time-kill assay. A logarithmic culture is a culture at which bacteria are in rapid cell division shown by exponential growth.

A MHB was inoculated with the *Staphylococcus aureus* ATCC 29213 reference strain and cultured aerobically for 24 hours at 37°C. A standardised 0.5 MacFarland standard suspension was prepared using a Densilameter (ERBA, UK) and 2ml was taken and added to 18ml of pre-sterilized MHB and

cultured at 37°C aerobically without shaking. Every hour, the sample was mixed thoroughly, and a total viable count was conducted (2.6.3), after 10 samples were collected a Nutrient agar plate was streaked from the experimental broth to check for final purity.

2.7 **Bacterial Susceptibility testing**

Antimicrobial susceptibility testing of *Staphylococcus aureus* ATCC 29213 was completed on VCM free drug, loaded NLCs and blank NLCs. This was to provide insight into which aspect of the loaded formulation was having an inhibitory effect on bacterial growth. Investigations completed were minimum inhibitory concentration (MIC) according to ISO 20776-1 (2019) and time-kill assays (Foerster *et al.*, 2016).

2.7.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

An MIC by microbroth dilution is the reference method for antimicrobial susceptibility of fast-growing aerobic bacteria (EUCAST, 2022). It provides data with high specificity and insight into therapeutic doses for bacterial infections. The MIC is the lowest concentration at which the test organism is inhibited by an antibiotic.

MICs were completed by the microdilution method (ISO 20776-1:2019). A MHB was inoculated with *Staphylococcus aureus* ATCC 29213 and cultured at 37°C for 18-24 hours aerobically. After incubation, a Densilameter (ERBA, BioConnections, UK) was used to create a 0.5 MacFarland standard suspension of the organism and 45µl was pipetted into 9ml of MHB to produce an inoculum with a bacterial concentration of approximately $5.5x10^5$ cfu/ml, this was verified by a total viable count (2.6.2). Using a flat bottom 96-well microtiter plate 100µl of inoculum was placed in ten of the twelve wells in three rows, with a row to separate. In the remaining two columns 100µl of uncultured MHB was pipetted and in the final column 100µl of pure antimicrobial standard was added to the uncultured broth, this encompassed the negative control and antibiotic control. An antibiotic control was utilised to ensure no bacterial contaminants were introduced during NLC preparation. Therefore, to exclude unwanted bacterial interference from data, an antibiotic negative control was adopted. Serial doubling dilutions

of vancomycin from 32µg/ml to 0.125µg/ml were then prepared in sterile saline and 100µl were pipetted into the relevant wells. The microtiter plate was then incubated for 24 hours at 37°C in an aerobic environment. Results were analysed according to EUCAST (2022) by observation of turbidity. After incubation, the turbidity was observed, and the relevant wells diluted and subcultured for a total viable count (2.6.3).

Bacterial growth was indicated by turbidity, in the test well. Where the culture medium was clear and without turbidity, this indicated either inhibition or a bactericidal effect. The MIC was the lowest concentration of antibiotic that showed no turbidity after incubation. All test wells without turbidity were subcultured. If no growth was observed after subculture this was considered a bactericidal effect. The MBC was the lowest concentration of antibiotic that showed no growth after subculture. Generally, the MBC comes at a higher concentration to the MIC due to many antibiotics inhibiting growth before causing cell death (Honda *et al.,* 2011). The MBC for *S. aureus* (ATCC 29213) vancomycin is 1µg/ml to 32µg/ml encompassing the MIC.

However, it was observed that in high concentrations of NLC formulation turbidity occurred in the test system on addition of the NLC without bacterial growth. Therefore, in NLC MICs after incubation, all wells were diluted and subcultured to determine the relevant MIC and MBC.

2.7.2 Time-kill assay

A time-kill assay experiment was adapted from a similar assay reported by Foerster *et al.,* (2016) on *Neisseria gonorrhoea.* Initially *S. aureus* ATCC 29213 was subcultured from Kwik-Stiks (Bioconnections, UK) into MHB and incubated for 18-24 hours aerobically at 37°C. A 0.5 MacFarland suspension of the organisms was prepared and 18µl was pipetted into 9ml of MHB to create an inoculum of approximately $2x10^5$cfu/ml. It was established from the growth curve that the organism would be in log phase after 3 hours of aerobic incubation. Hence, the inoculated MHB was pre-incubated for three hours before addition of the test material. The concentrations of vancomycin or VCM-NLC were then created ranging from 0.16 x MIC to 16 x MIC (0.32µg/ml to 32µg/ml). The MIC target for *S. aureus* was

1µg/ml in the range 0.5-2µg/ml (EUCAST, 2023), to account for the range 2µg/ml was utilised as the MIC. After the preincubation period of 3 hours, antibiotic and VCM-NLC concentrations were added where each row was its own concentration and each column a timepoint from one to six hours (Figure 2.4). The final row would be inoculated without antimicrobial and would act as positive control. Every hour post antibiotic introduction each well, in the relevant time point, would have 10µl removed and a total viable count performed (2.6.3). Each agar plate would then be incubated aerobically at 37°C for 24 hours before colony counting. This procedure was repeated for VCM-NLCs and free-VCM.

Due to the dilutions completed creating variance within the bacterial culture, the range of quantifiable bacteria was a range of $1x10^6$cfu/ml to $1x10^8$cfu/ml. Hence any samples with zero colonies were considered to have less than $1x10^6$cfu/ml and confluent growth was greater than $1x10^8$cfu/ml.

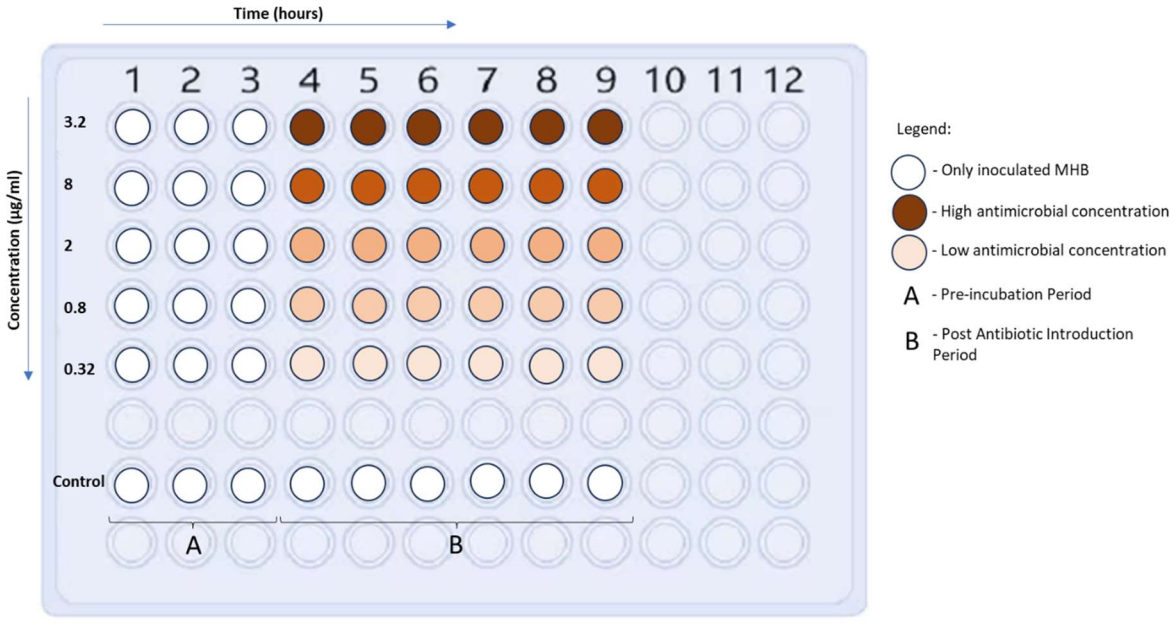


Figure 2. 7: A graphical representation of the layout of the 96-well plate in time-kill assays prepared.

2.8 Statistical analysis

Collected data was expressed as mean ± standard deviation (S.D) (n=3) using Microsoft Excel software for Microsoft office 360 (Microsoft Corporation, USA). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software (Ver. 28.0.1.1) (IBM Company, USA). To determine the statistical significance of the results, one way analysis of variance (ANOVA) was carried out, whereas the post-hoc was conducted by Tukey's test. Where relevant paired and single t-tests were conducted to highlight significance. The level of significance value was defined as $p<0.05$, the null hypothesis being there is no significant difference between the samples.

Chapter 3: Results

3.1 Blank Formulation Optimisation

Initially, optimisation was carried out for the blank non-drug loaded formulation to select and optimise the concentration of key excipients for the formulation of NLCs. Allowing the optimisation of the process parameters. Two liquid lipids; Capryol 90 and Transcutol P, and two surfactants; Kolliphor HS15 and sodium cholate were selected and NLC compositions with various concentrations of these excipients were investigated. Probe sonication was used for the reduction of hydrodynamic size in NLCs. The length of sonication time has an impact on the hydrodynamic size of the final NLCs, sonication time was also considered as one of the optimisation parameters. The output values considered for optimisation were HDS, PDI and Zeta potential as these values critically impacted the performance of the NLCs. The target parameters for the output values were HDS 80-200nm, PDI <0.300 and zeta potential >30mV ±, respectively (Matos *et al.,* 2018).

3.1.1 **Investigations into liquid lipid solubility**

Solubility was investigated to ascertain the liquid lipids which could solubilise VCM. A solubility study was conducted on VCM to determine the volume of liquid lipid needed to fully solubilise 10mg of VCM. Investigations showed that Transcutol P and Capryol 90 had good solubility for VCM, with Capryol 90 needing the lowest volume to fully dissolve 10mg of VCM (Table 3.1).

Table 3.1: Solubility study completed on vancomycin in liquid lipids measured in volume (ml) of liquid lipid needed to fully dissolve 10mg of vancomycin.

	Capryol 90	Transcutol P
Volume (ml)	13	19

3.1.2 Investigations into the effect of sonication time

Sonication time was investigated as a process parameter. Critical quality attributes: hydrodynamic size and PDI were used as the parameters to inform experiments. Hydrodynamic size was found to decrease from the initial 1643nm to below 200nm and remained stable. Investigations showed as sonication time increased PDI decreased with the lowest PDI at 15 minutes where sonication beyond

this significantly (p<0.05) increased PDI. Figure 3.1 shows this with an increase in PDI observed after 15 minutes. The increase in PDI observed in figure 3.2 was the indicator that the optimum sonication time had been achieved.

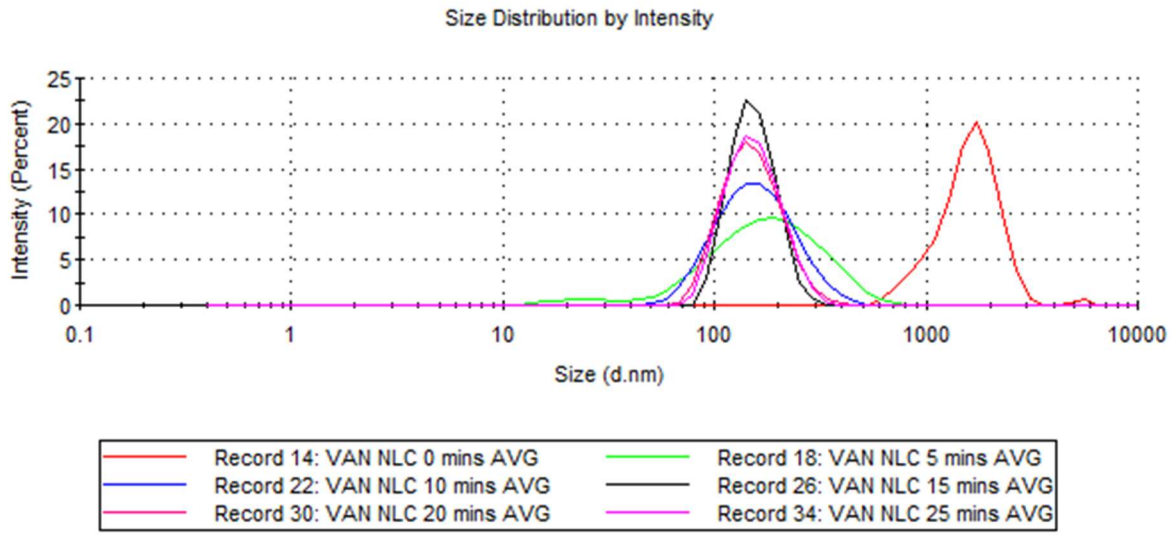


Figure 3.1: A Zetasizer trace of formulation one demonstrating the change in output values (PS, PDI) associated with increasing sonication time

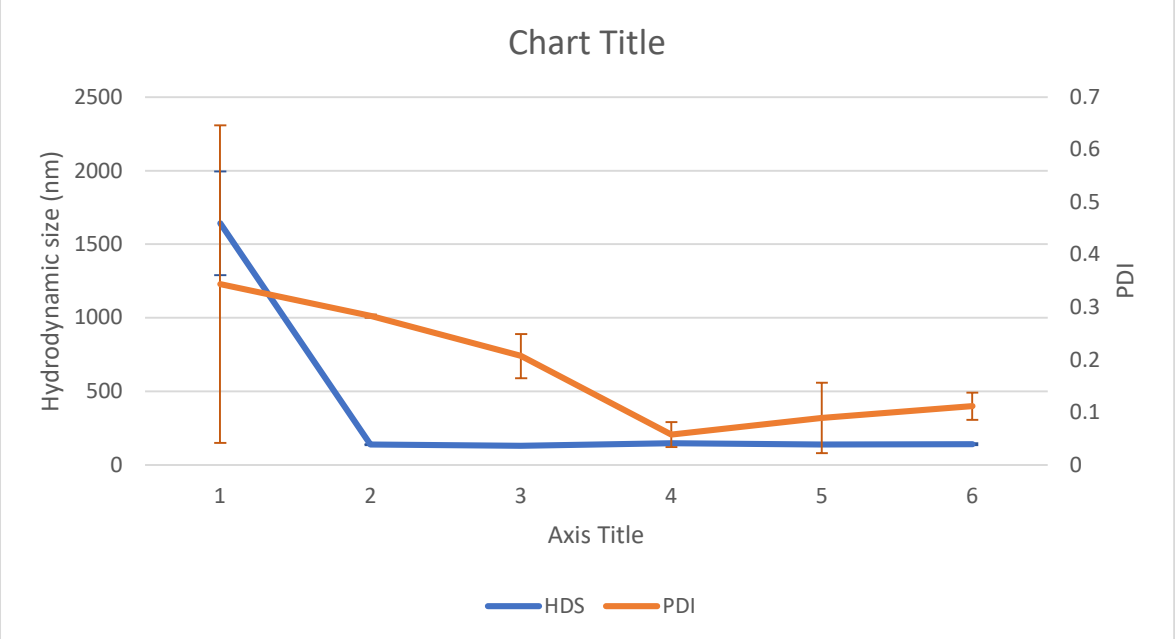


Figure 3.2: A graphical representation of the effect changing sonication time on B1had on output values (HDS, PDI, Zeta potential)

3.1.3 **Optimisation of liquid lipid excipients in blank formulations**

Liquid lipid product parameters investigated were Transcutol P and Capryol 90. These parameters were investigated due to the prior solubility study showing affinity towards vancomycin emulsification. These excipients were critical due to the hydrophilic nature of vancomycin possibly causing problems with entrapment. Blank optimisation took place initially to view the effect changing excipients and excipient concentrations had on output values (HDS, PDI and zeta).

Capryol 90

Capryol 90 was initially investigated in isolation in the NLCs resulting in a hydrodynamic size of 127.2nm, a PDI of 0.277 and a zeta potential of -53.2mV (Table 3.2). Although PDI values were within parameters (>0.300) they were high at 0.277, with HDS being significantly (p<0.05) different. When observing Zetasizer graphs Capryol 90 formed smaller peaks to the left of the main peak showing the presence of micelles formed from the free surfactant (Figure 3.3). Micelles are particles that form from excess surfactant, they are created when nanoparticles are too large or with high concentrations of surfactant.

Table 3.2: Tables demonstrating the use of Capryol 90 on the change to output values (PS, PDI and Zeta). A - shows formulation excipient concentrations and B - shows output values

A

Formulation	Dynasan 114 (mg)	Capryol 90 (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)	Sonication time (min)
B2	1000	300	300	300	80	100	25	15

B

Formulation	Hydrodynamic size (nm)	PDI	Zeta Potential (mV)
B2	127.2±6.2	0.277±0.015	-53.2±1.1

Transcutol P

NLCs containing Transcutol P in isolation were then investigated (Figure 3.3) showing a HDS of 208nm, a PDI of 0.168 and a zeta potential of -53.7mV (Table 3.3). Although PDI values were significantly (p<0.05) decreased from Capryol 90, HDS increased to above parameters set (80-200nm).

Table 3.3: Tables demonstrating the use of Transcutol P on the change to output values (PS, PDI and Zeta). A - shows formulation excipient concentrations and B - shows output values.

A

Formulation	Dynasan 114 (mg)	Transcutol P (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)	Sonication time (min)
B3	1000	300	300	300	80	100	25	15

B

Formulation	Hydrodynamic Size (nm)	PDI	Zeta Potential (mV)
B3	208±83	0.168±0.200	-53.7±4.7

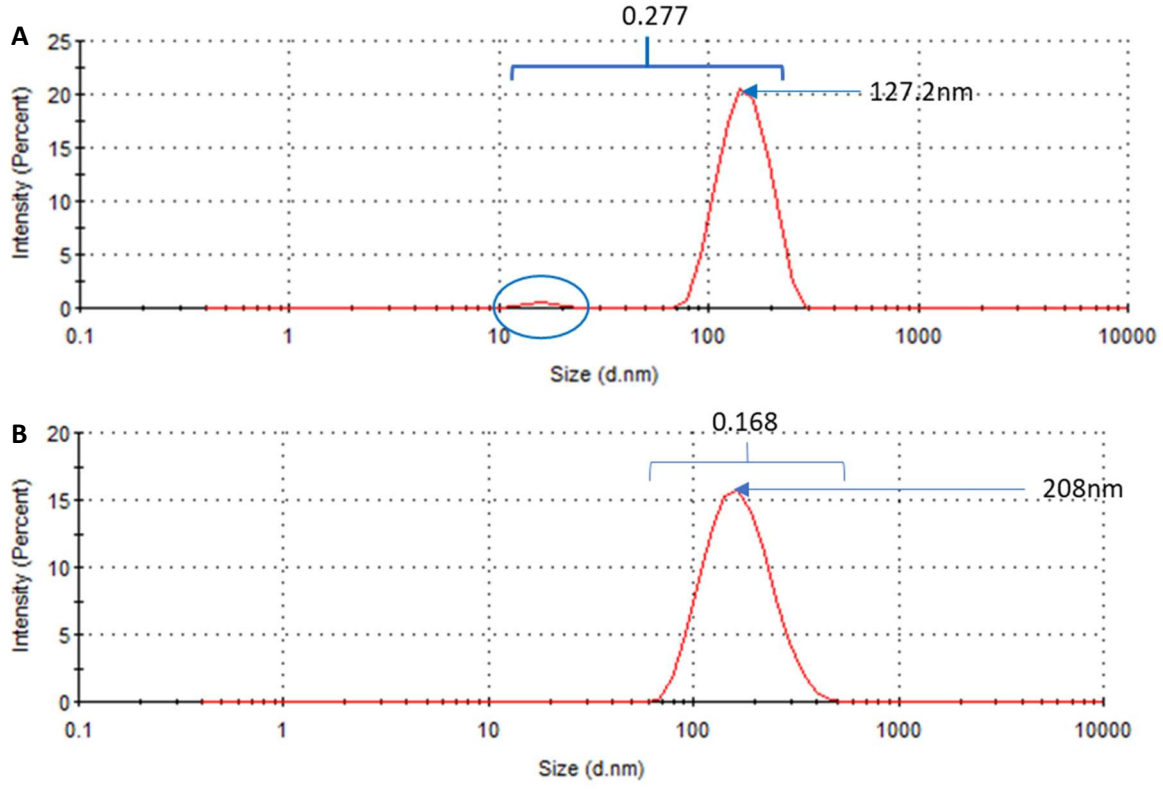


Figure 3.3: A is a Zetasizer trace demonstrating the appearance of free surfactant in Capryol 90 (formulation 3), with a circle highlighting a surfactant peak. B is a Zetasizer trace demonstrating the increasing CQAs of B3 when using Transcutol P. HDS is indicated to by an arrow and PDI by a bracket.

Transcutol P and Capryol 90

Due to the opposing CQAs observed from liquid lipids, combinations of Capryol 90 and Transcutol P were investigated. Various concentrations of Capryol 90 were utilised due to the PDI increase observed, but Transcutol P remained at 200mg. Initially using 100mg of Capryol 90 showed a HDS of 147.1nm, a PDI of 0.058 and a zeta potential of -60.1mV (Table 3.4). Secondarily 200mg of Capryol 90 showed a HDS of 144.0nm, a PDI of 0.202 and a zeta potential of -42.3 (Table 3.4). From these investigations the addition of Transcutol P to formulations in conjunction with Capryol 90 showed a significant (p<0.05) decrease in PDI and zeta potential, but once Capryol 90 was increased an increase in PDI was seen again, however lower than when used in isolation. Further optimisation was therefore needed due to the non-ionic surfactant properties of Capryol 90 (Shakeel *et al.,* 2013), other surfactants (Kolliphor HS15 and Sodium cholate) were optimised.

Table 3.4: Shows results from the process of optimising Transcutol P and Capryol 90 in combination (A) is the formulation excipients and concentrations (B) is the Zetasizer data.

A

Formulation	Dynasan 114 (mg)	Capryol 90 (mg)	Transcutol P (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)	Sonication time (min)
B1	1000	100	200	300	300	80	100	25	15
B4	1000	200	200	300	300	80	100	25	10

B

Formulation	Hydrodynamic Size (nm)	PDI	Zeta Potential (mV)
B1	147.1±13.5	0.058±0.043	-60.1±3.7
B4	144.0±10.8	0.202±0.027	-42.3±4.7

3.1.4 Optimisation of surfactants and surfactant concentration

Sodium Cholate

Some of the prior mentioned concerns were alleviated by using differing concentrations of sodium cholate, a hydrophilic surfactant, which acts on the external surface of the nanoparticles preventing coalescence (Danaei *et al*. 2018).

With Transcutol P and Capryol 90 showing the greatest solubility for VCM, all NLC formulations from this point forward used these liquid lipids. Due to Transcutol P showing an increase in HDS when used in isolation (208.0nm ± 83) and Capryol 90 producing an increased PDI (0.277 ± 0.015) (Table 3.3 & 3.2). The synergistic effect seen in B1 was further optimised with hydrophilic surfactant sodium cholate. Due to Capryol 90 having greater solubility of VCM an increase of this liquid lipid was also investigated.

Hydrodynamic size and zeta potential showed insignificant (p<0.05) changes throughout investigations (Table 3.5). PDI was the primary parameter investigated. When comparing a constant sodium cholate concentration but an increase in Capryol 90 (100-200mg), PDI significantly (p<0.05) increased from 0.040 to 0.202 (Table 3.5). When Capryol 90 concentration was 100mg, but now with 150mg of sodium cholate PDI values were more controlled at 0.183, showing an insignificant (p<0.05) change (B6 and B8 Table 3.5) but showing optimisation of sodium cholate could improve outcomes. When Capryol 90 was increased again to 200mg PDI values further decreased to 0.139 now showing a significant (p<0.05) decrease. From these investigations 150mg was chosen as the optimum concentration of sodium cholate, due to it facilitating an increase in Capryol 90 whilst maintaining CQAs.

Table 3.5: Shows results from the process of optimising sodium cholate (A) is the formulation excipients and concentrations (B) is the Zetasizer data.

A

Formulation	Dynasan 114 (mg)	Capryol 90 (mg)	Transcutol P (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)	Sonication time (min)
B5	1000	100	200	300	300	80	100	25	15
B6	1000	100	200	300	300	80	150	25	10
B7	1000	200	200	300	300	80	100	25	10
B8	1000	200	200	300	300	80	150	25	5

B

Formulation	Hydrodynamic Size (nm)	PDI	Zeta Potential (mV)
B5	147.5±14.2	0.040±0.077	-48.6±4
B6	158.9±5.1	0.183±0.012	-52.4±2.3
B7	144.0±10.8	0.202±0.027	-42.3±4.7
B8	132.4±14.7	0.139±0.062	-43.6±2

Kolliphor HS15

Kolliphor HS15 was optimised as a surfactant again attempting to achieve an increase in Capryol 90. Initially Capryol 90 concentration was increased from 100mg to 200mg, whilst keeping Kolliphor HS15 concentration at 200mg. Observing an insignificant (p<0.05) change in HDS of 164.3nm to 163.4nm, the PDI insignificantly (p>0.05) increased from 0.213 to 0.252 and an insignificant (p<0.05*)* zeta potential increase from -23.2mV to -29.8mV (Table 3.6). Zeta potential values were both below the ->30mV parameter and PDI values although within range, were higher compared to non Kolliphor HS15 formulations. At this stage, an increase of Kolliphor HS15 from 200mg to 300mg was used whilst Capryol 90 was maintained at 200mg. This saw a HDS decrease of 163.4nm to 147.5nm, a significant (p<0.05) PDI decrease of 0.252 to 0.04 and a zeta potential decrease of -29.8mV to -24.7mV (Table

3.6). An increased concentration of Kolliphor HS15 significantly ($p<0.05$) improved PDI outcomes but still showed zeta potential values below the parameters set (-30mV).

Table 3.6: Shows results from the process of optimising Kolliphor HS15 (A) is the formulation excipients and concentrations (B) is the Zetasizer data.

A

Formulation	Dynasan 114 (mg)	Capryol 90 (mg)	Kolliphor HS15 (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)	Sonication time (min)
B9	1000	100	200	300	300	80	150	25	5
B10	1000	100	200	300	300	80	200	25	5
B11	1000	200	200	300	300	80	150	25	5
B12	1000	200	300	300	300	80	150	25	10
B13	1000	200	300	300	300	80	200	25	5

B

Formulation	Hydrodynamic Size (nm)	PDI	Zeta Potential (mV)
B9	145.4±12.3	0.152±0.102	-27.0±4.1
B10	92.61±11.11	0.303±0.006	-29.3±3
B11	164.3±0.002	0.213±0.034	-29.8±4.5
B12	131.1±10.6	0.272±0.007	-29.5±3.1
B13	163.4±2.3	0.252±0.033	-18.2±1.9

Kolliphor HS15 and Sodium Cholate

Due to the prior observed beneficial effects of optimising sodium cholate the same was done in formulations containing Kolliphor HS15. Initially a comparison was made in a formulation where 100mg of Capryol 90 and 200mg of Kolliphor HS15 was used, whilst sodium cholate was increased from 150mg to 200mg. This observed a significant ($p<0.05$) HDS decrease from 164.3nm to 131.1nm, a significant ($p<0.05$) PDI increase of 0.213 to 0.272 and a significant ($p<0.05$) zeta potential increase from -23.2mV to -29.3mV (Table 3.6). The increased PDI and persistent insignificant ($p<0.05$) change in zeta potential values below parameters (>30mV) continued with increased sodium cholate concentrations, 300mg of Kolliphor HS15 was next investigated with sodium cholate again increased to 200mg. Where a significant ($p<0.05$) change occurred for HDS and PDI values in the form of a HDS

increase of 131.1nm to 163.4nm, a PDI decrease of 0.272 to 0.252 and a significant (p<0.05) zeta potential decrease of -29.5mV to -18.2mV (Table 3.6). This formulation showed CQAs all nearing to, or elapsing parameters set (HDS 80-200nm, PDI <0.300 and zeta potential >-30mV).

Due to the zeta potential of all formulations containing Kolliphor HS15 having zeta potential values outside of parameters set, Kolliphor HS15 was excluded from all further experimentation. Formulation B8 was therefore taken forward for further investigation due to product parameters being met and process parameters optimised.

3.1.1 Blank NLC formulation stability

Table 3. 7: The excipient list and concentrations used within the optimised blank formulation B8. This formulation was taken for further investigation with vancomycin and stability.

Formulation	Dynasan 114 (mg)	Capryol 90 (mg)	Transcutol P (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)	Sonication time (min)
B8	1000	200	200	300	300	80	150	25	5

Due to formulation B6 producing output values within parameters set and showing the premise for high encapsulation this formulation was taken forward for vancomycin investigations. In this formulation sodium cholate was optimised as a surfactant product parameter and sonication time was optimised as a process parameter. Capryol 90 was used synergistically with Transcutol P to decrease the potential for micelle formation, seen in formulations where Capryol 90 was used in isolation (Figure 3.3) (Table 3.2). With B6 established as the optimum formulation a stability study was conducted to ascertain the stability of the formulation over a period of 30 days at regulation temperatures (15-25°c).

A one-way ANOVA was conducted on data showing a significant (p<0.05) increase in PDI at 21 days when compared against 0 days showing NLCs had become less monodispersed at this stage. Indicating a less stable formulation at 21 days. An insignificant (p<0.05*)* increase in HDS was observed across the 30-days assessed. Therefore, the formulation remained stable over 21 days with a deviation in HDS of 9.9nm and PDI of 0.082 (Figure 3.4).

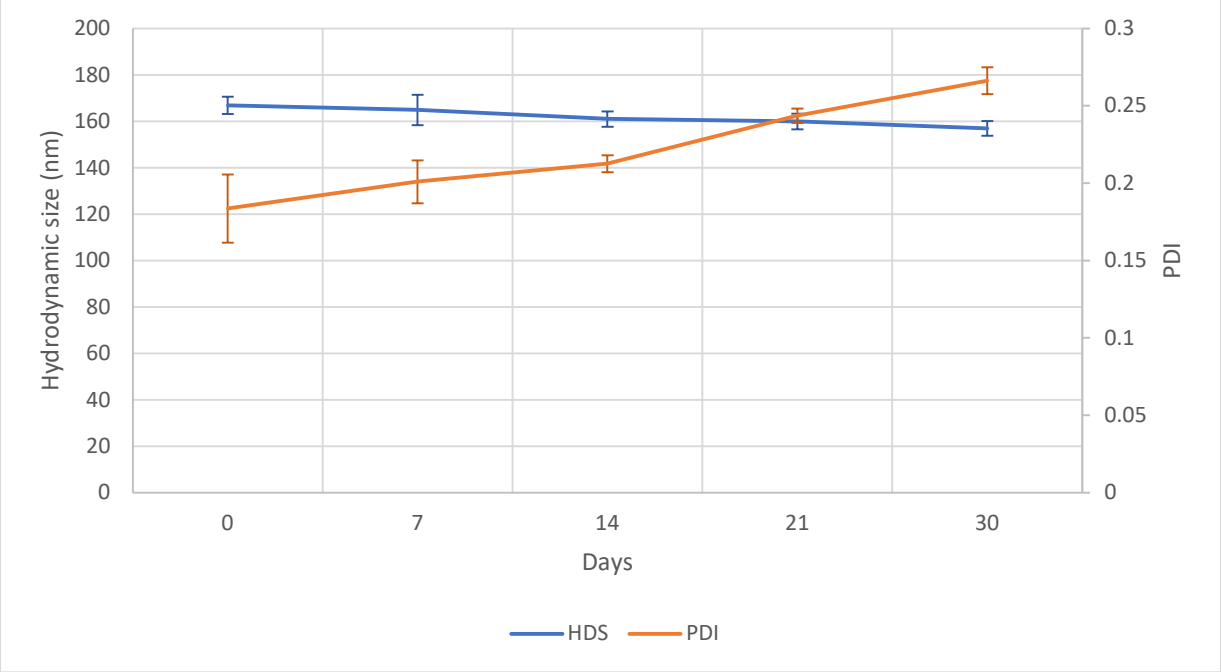


Figure 3.4: stability study of the optimised NLC at ambient temperature over 30 days.

3.2 **Optimisation and Validation of High-performance Liquid chromatography**

Before proceeding to drug loading, investigations were conducted as to a UV-HPLC method for quantification of vancomycin in NLCs. HPLC was validated according to the international conference of harmonisation (ICH) guidelines Q2(R1). The chromatographic conditions considered were flow rate, mobile phase ratio and wavelength. The output values considered were peak area, retention time and symmetry. Symmetry was maintained throughout with all experiments where it was kept above 0.7. Peak area was optimised considering the UV wavelength used by the detector. Retention time was optimized by changing chromatographic conditions associated with mobile phase and ratio. The mobile phase chosen was HPLC grade water pH adjusted to 2.7 and HPLC grade methanol.

3.2.1 Optimisation of Wavelength

Optimisation was completed into wavelength to investigate the optimal absorbance for vancomycin detection. The wavelength which produced the highest area with the lowest concentration of contamination peaks was 220nm (figure 3.5). The contamination seen with an un-optimised wavelength is demonstrated in figure 3.6.

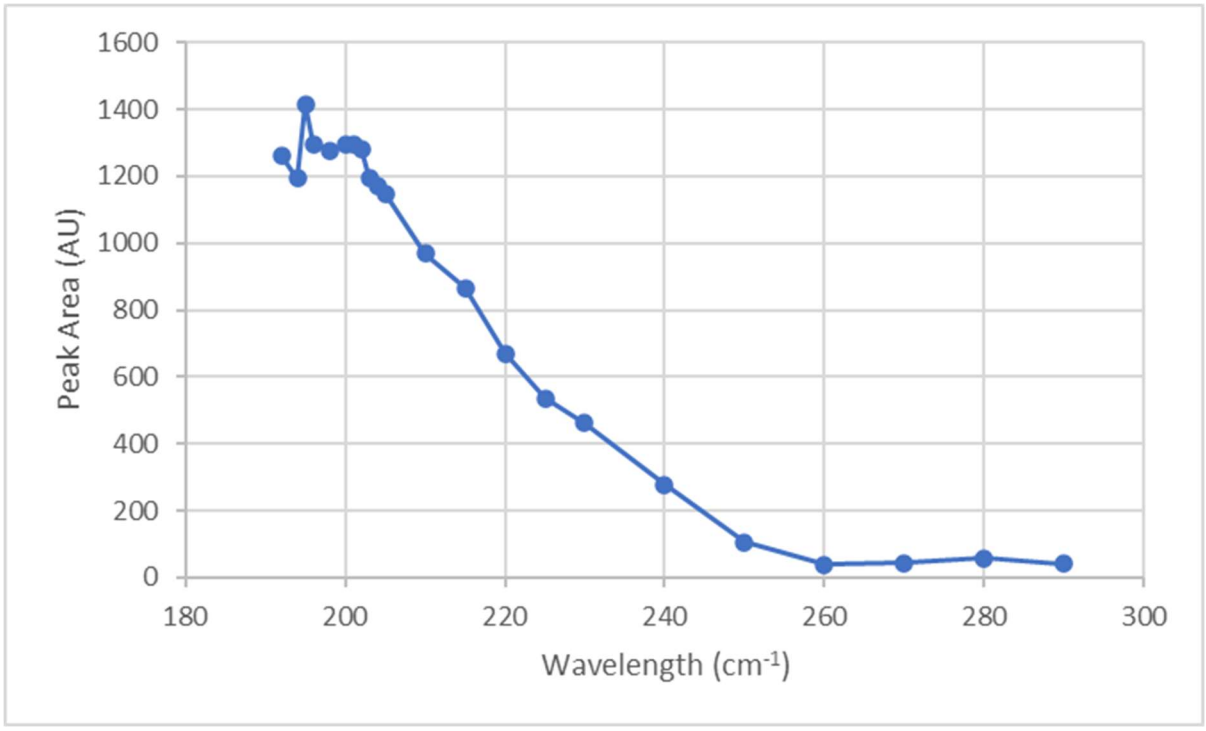


Figure 3.5: A comparison of Peak area collected on HPLC testing and a varying absorbance wavelength.

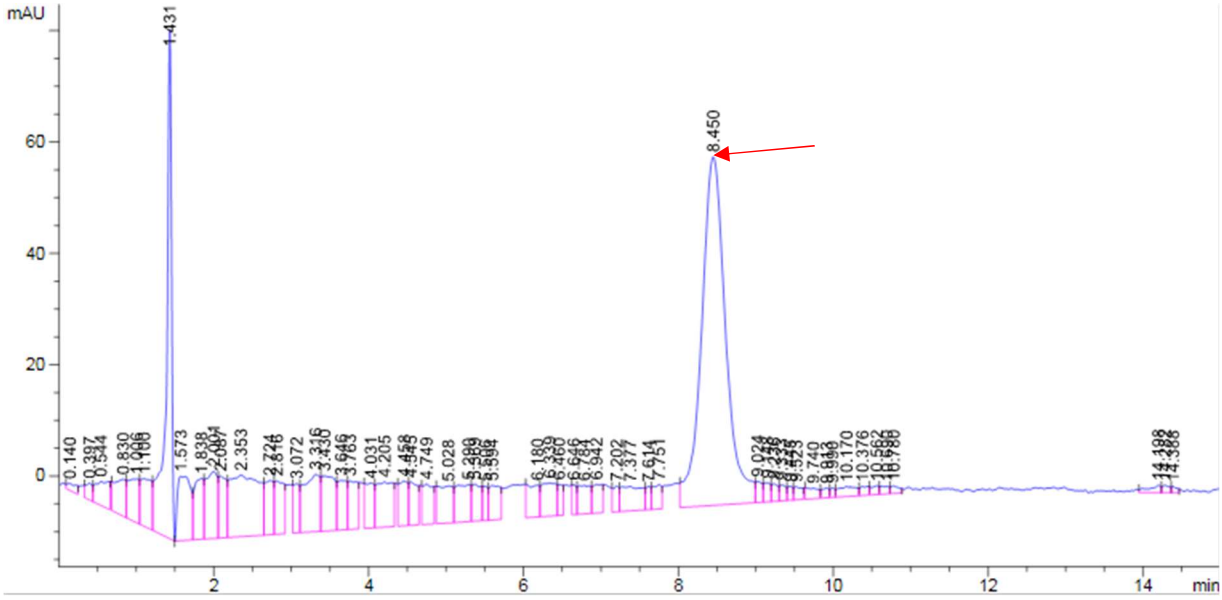


Figure 3.6: A HPLC chromatogram trace demonstrating the effect of 200cm-1 absorbance on the VCM peak. The VCM peak is indicated by a red arrow at 8.450 elute time.

3.2.2 Retention time

Following the method 10µg/ml was injected seven times consecutively resulting of a mean retention time of 6.15min ± 0.12. As the SD% for the retention time is 1.95% it is within ICH guidelines (ICH, 1995) (Shabir, 2004).

3.2.3 **Investigations into Mobile Phase**

Figure 3.6 was compared to figure 3.7, no peaks were seen in the same region as the VCM peak indicating no interfering peaks occurred, meaning that the area seen in the chromatogram is true to that of VCM. This reflects within VCM-NLC formulations tested within HPLC, ensuring the mobile phase did not contaminate the peak of interest.

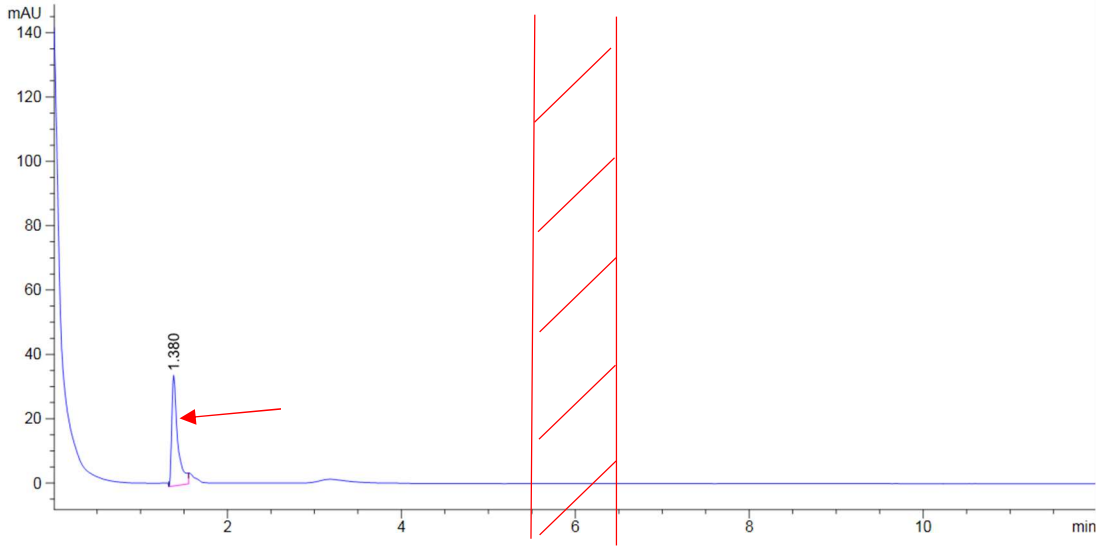


Figure 3.7: A HPLC chromatogram tracing the Mobile Phase showing the positioning of the solvent front, indicated by a red arrow. The peak of interest at 1.380 retention time. The red hashed zone is the retention time (±SD) of the vancomycin method.

3.2.4 Optimisation of HPLC Mobile Phase ratio

HPLC optimisation was conducted to provide the optimum chromatographic parameters. During optimisation two concentrations of vancomycin was used to observe the change in peak area. This was used to confirm the peak seen was vancomycin, as an increased area would suggest an increase in concentration. This is seen in figures 3.8 and 3.9 where a 4-16µg/ml concentration change was adopted, and the area increases from 181 to 736, showing a 4.02-fold increase in peak area which directly corresponds to the 4-fold change in concentration.

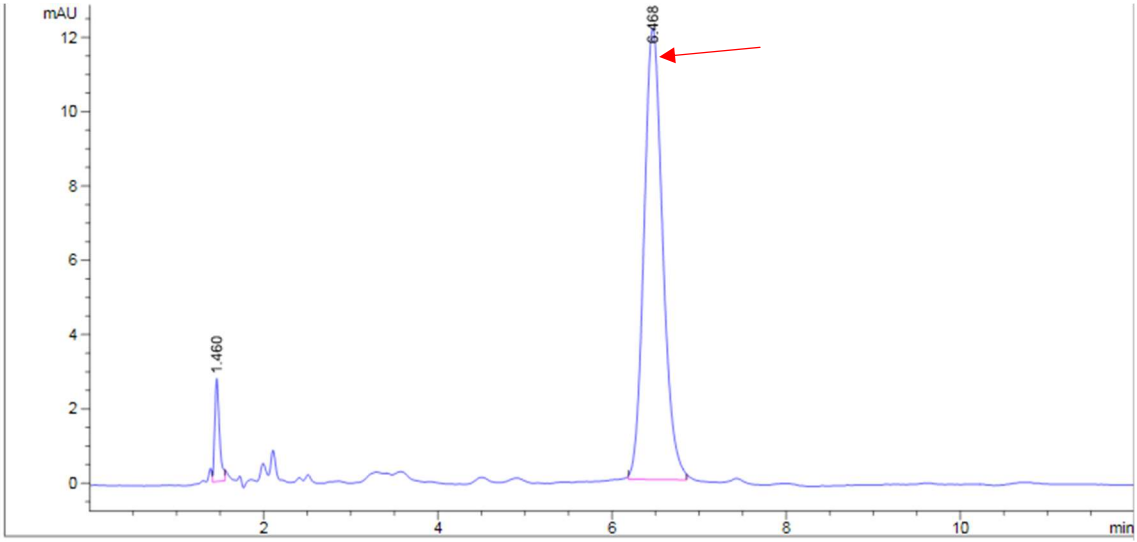


Figure 3.8: A HPLC chromatogram showing 4µg/ml of FD-VCM in water. The VCM peak is indicated by a red arrow. The peak of interest at 6.468 minutes retention time.

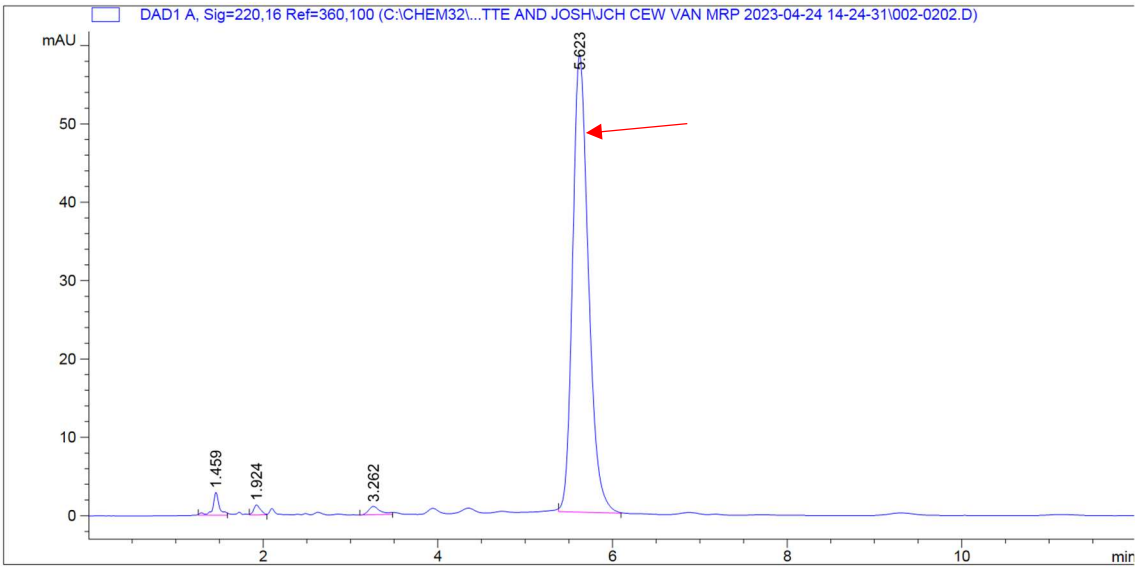


Figure 3.9: A HPLC chromatogram showing 16µg/ml of FD-VCM in water. The VCM peak is indicated by a red arrow The peak of interest at 6.623 minutes retention time.

Optimisation also saw individual aspects of the method changed to ascertain the best parameters for reliably identifying and quantifying VCM. These were highlighted to be pH due to VCMs pKa (2.66) (Settimo *et al.,* 2014), mobile phase ratio and flow rate. A pH of 2.7 was chosen due to it being similar to that of the pKa and mobile phase ratio was changed to provide a symmetrical peak with retention time in the range 4-8 minutes. It was observed a higher water (pH 2.7) to methanol ratio increased the retention time (Figure 3.10) where increasing methanol concentration (0-40%) decreased retention time (figure 3.11).

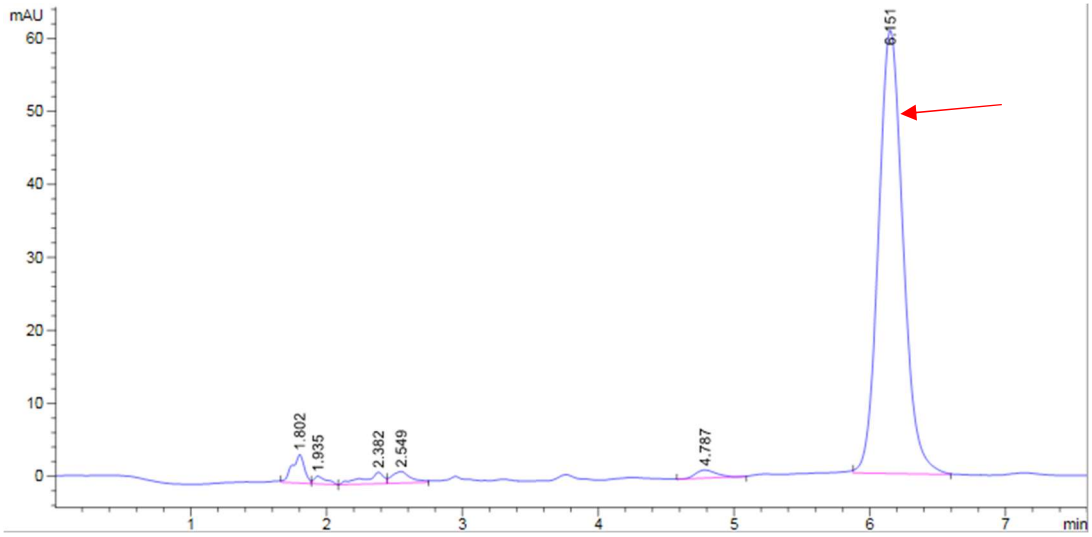


Figure 3.10: A HPLC chromatogram showing a higher pH adjusted water to methanol in the mobile phase. The VCM peak is indicated by a red arrow. The peak of interest at 6.151 retention time.

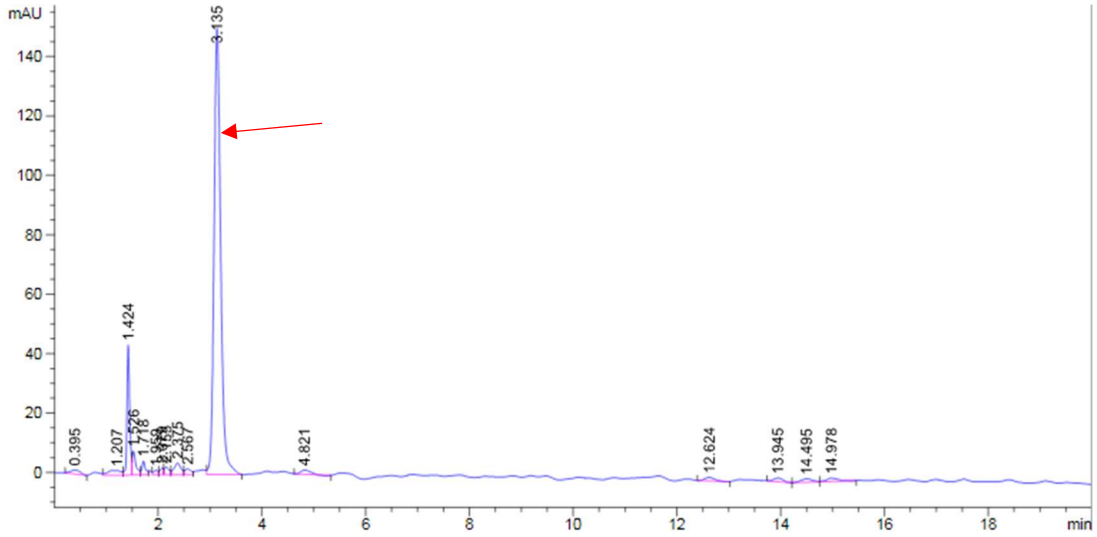


Figure 3.11: A HPLC chromatogram showing a higher methanol to pH adjusted water ratio in the mobile phase. The VCM peak is indicated by a red arrow. The peak of interest at 3.135 retention time.

3.2.5 **Optimisation of Flow rate**

Flow rate was optimised simultaneously throughout all experimentation. The range used was 0.7ml/min and 1ml/min. Whilst maintaining chromatographic conditions and only changing flow rate, 0.7ml/min produced a retention time of 10.98 minutes and symmetry of 0.576. Where a flow rate of 1ml/min produced a retention time of 6.15 minutes and a symmetry of 0.838.

3.2.6 Calibration Curve

Post optimisation, validation was conducted to verify the effectiveness of the method. The optimised parameters were a flow rate of 1ml/min, an isocratic mobile phase of pH 2.7 HPLC-grade water and HPLC grade methanol (84:16, H2O: MeOH, v/v), 20µl injection and a wavelength of 220nm.

Calibration curves were constructed where a range of concentrations (0.5µg/ml to 20µg/ml) were injected twice following the method. The peak area from two injections for each of the concentrations was averaged and taken as the value for plotting the calibration curve. This was replicated three times and again an average was taken of the three values with a scatter graph as depicted in figure 3.12. The R^2 value shown in figure 3.12 is 0.9997 showing high linearity and is above the ICH guideline 0.997 (ICH, 1995) (Shabir, 2004).

When SD percentage was calculated only 1µg/ml exceeded 10% (Table 3.8), which is the cut-off criteria. All other concentrations were accepted (ICH, 1995) (Shabir, 2004). False negatives were also considered (Log Q) and no calculated values were below the p-value, meaning the null hypothesis was accepted and no false negatives were collected (Table 3.8).

The reliability of the curve accuracy calculation was determined for all concentrations, and all concentrations above 2µg/ml showed a high accuracy (80%<x>120), concentrations below 2µg/ml would be considered less accurate compared to higher concentrations (Table 3.9). Limit of detection and Limit of quantification was calculated, this was shown at 0.26µg/ml to be the lowest detectable value and 0.31µg/ml to be the lowest quantifiable value. All symmetry values collected for each concentration did not decrease below 0.700 the exclusion point set by ICH (1995)

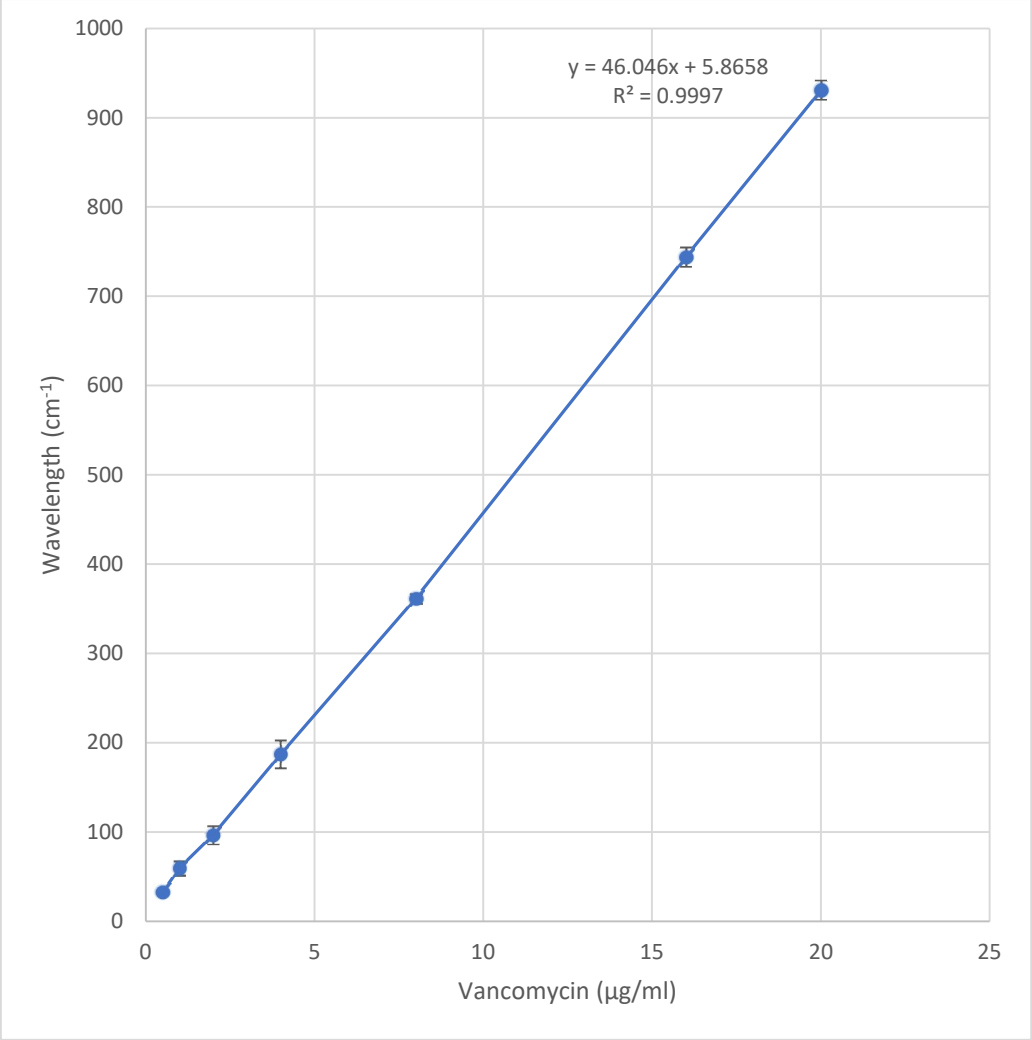


Figure 3.12: The Calibration curve created for VCM in water using the HPLC method optimised, (n=6).

Table 3.8: The formatted area data associated with the calibration curve showing the area average calculated from each of the curves, Standard deviation, standard deviation percentage and the Log Q values.

Concentration (µg/ml)	Area Average (AU) (Mean ± SD, n=3)	SD%, n=3	Log Q
0.5	32.83 ± 0.29	0.87	0.19
1	59.52 ± 6.03	10.13	2.20
2	96.48 ± 8.15	8.45	1.84
4	186.93 ± 12.64	6.76	1.47
8	361.5 ± 4.29	1.18	0.26
16	744.12 ± 8.78	1.18	0.26
20	931.12 ± 8.59	0.92	0.20

Table 3.9: The Accuracy data calculated from the three calibration curves (n=3).

Concentration (µg/ml)	Accuracy % (Mean ± SD, n=3)
0.5	143.32 ± 0.82
1	128.98 ± 13.08
2	103.6 ± 8.10
4	97.09 ± 1.57
8	97.44 ± 0.65
16	100.62 ± 1.29
20	100.29 ± 0.14

3.2.4 Precision

Intra and interday testing was investigated following guidelines from ICH. A standard of 4µg/ml and 16µg/ml was injected, this when analysed showed 3.82µg/ml and 15.85µg/ml, respectively. All standard deviation percentages were below 2% which is in line with ICH guidelines (Table 3.10 and 3.11) (ICH, 1995) (Shabir, 2004).

Table 3.10: The SD and SD% calculated from the intraday studies. A is 4µg/ml B is 16µg/ ml standard deviation (n=4)

A

Intra-day	AM	PM	Mean	SD (n=4)	SD%
1	3.82	3.89	3.86	0.05	1.19
2	3.89	3.86	3.87	0.02	0.59
3	3.86	3.82	3.84	0.02	0.60

B

Intra-day	AM	PM	Mean	SD (n=4)	SD%
1	15.85	15.82	15.84	0.03	0.90
2	15.63	15.66	15.65	0.02	0.098
3	15.83	15.81	15.82	0.02	0.097

Table 3.11: The SD and SD% calculated from the interday studies. A is 4µg/ml B is 16µg/ml standard deviation (n=4)

A

Inter-day	Day 1	Day 2	Mean	SD (n=4)	SD%
1	3.82	3.87	3.84	0.04	0.91
2	3.82	3.89	3.86	0.05	1.20
3	3.88	3.82	3.86	0.04	1.10

B

Inter-day	Day 1	Day 2	Mean	SD (n=4)	SD%
1	15.85	15.64	15.74	0.15	0.97
2	15.83	15.66	15.74	0.12	0.78
3	15.85	15.83	15.84	0.02	0.10

3.3 Optimisation of VCM-NLCs

3.3.1 Optimisation of vancomycin concentration

When optimising vancomycin concentration, the total volume of formulation was reduced to 10mL to reduce waste and antibiotic usage. An insignificant (p<0.05*)* change was seen between B1 and B8 output values when volume was decreased from 25ml to 10ml. Observations showed increased Capryol 90, and sodium cholate providing more promising values within parameters (132.4nm and 0.139). Hence, formulation B8 was used for investigating vancomycin drug loading.

Vancomycin was optimised within the range 0.5-1.5mg/ml. When vancomycin was added to B8 (Table 3.12) an insignificant (p<0.05) decrease in zeta potential was observed from -43.6mV to -30.8mV (Table 3.12). However, HDS and PDI was maintained within the parameter range similar to that of non-loaded formulations. When vancomycin concentration was increased to 15mg insignificant (p<0.05) changes were observed in PS and zeta potential, but PDI significantly (p< 0.05) increased from 0.146 to 0.273 (Table 3.12).

At this stage, an increase in Capryol 90 concentration was used to view the change this had on output values. Capryol 90 concentration was increased due to the solubility study showing potential

to emulsify vancomycin. This was conducted in formulations containing 10 and 15mg of vancomycin. Observing significant (p<0.05) changes in all values showing an increase in vancomycin concentration positively changes zeta potential likely due to the bonded HCl group, but negatively effects HDS and PDI (Table 3.12). Considering this, a decrease in vancomycin concentration was used to observe the change. Insignificant (p<0.05) changes were observed in hydrodynamic size but saw a significant (p<0.05) decrease in PDI 0.245 to 0.168 and an insignificant (p<0.05) increase zeta potential of -41.8 mV to -48.8mV (Table 3.12). Taking this into account a lower vancomycin concentration (VCM5) was used for entrapment efficiency investigations.

Table 3.12: Shows results from the process of optimising vancomycin concentration (A) is the formulation excipients and concentrations (B) is the Zetasizer data.

A

Formulation	Vancomycin (mg)	Dynasan 114 (mg)	Capryol 90 (mg)	Transcutol P (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)
VCM1	10	400	40	80	120	120	32	60	10
VCM2	15	400	40	80	120	120	32	60	10
VCM3	10	400	80	80	120	120	32	60	10
VCM4	15	400	80	80	120	120	32	60	10
VCM5	5	400	80	80	120	120	32	60	10

B

Formulation	Hydrodynamic Size (nm)	PDI	Zeta Potential (mV)
VCM1	137.4±14.1	0.146±0.138	-30.8±1.1
VCM2	140.8±3.1	0.273±0.161	-41.8±4.9
VCM3	130.2±16.6	0.235±0.105	-35.5±5.3
VCM4	141.9±0.4	0.245±0.033	-41.8±7.6
VCM5	140.6±20.6	0.168±0.081	-48.8±8.2

3.3.1 Entrapment Efficiency optimisation

An indirect method was used for entrapment efficiency investigations due to difficulties with discovering a direct method which allowed reliable quantification. A HPLC method was investigated

using tetrahydrofuran as a universal solvent to breakdown formulations and solubilise vancomycin, however this could not be reliably quantified using HPLC following ICH Q2(R1) (1995) guidelines. Due to vancomycin's moderate solubility in methanol a centrifugal method was attempted but without the ability to know if all vancomycin had been extracted this method was also dismissed.

Initial investigations into entrapment efficiency (%EE) presented a low entrapment of vancomycin (49.1%) in the NLCs (Table 3.13). Further investigations were conducted to determine the effect of changing the concentration of liquid lipid on %EE with the potential to increase entrapment of vancomycin in the NLCs. Due to investigations by Kalhapure (2014) a synergistic observation was made between oleic acid and vancomycin. Due to this oleic acid was introduced as a new excipient acting as a liquid lipid solubiliser.

Table 3.13: Shows results from the process of optimising entrapment efficiency (A) is the formulation excipients and concentrations (B) is the Zetasizer data.

A

Formulation	Vancomycin (mg)	Dynasan 114 (mg)	Capryol 90 (mg)	Transcutol P (mg)	Oleic acid (mg)	Lipoid E80 (mg)	Lipoid S75 (mg)	Phospholipon 90H (mg)	Sodium Cholate (mg)	Volume (ml)
VCM6	5	400	80	80	0	120	120	32	60	10
VCM7	5	400	120	80	0	120	120	32	60	10
VCM8	5	400	120	80	100	120	120	32	60	10

B

Formulation	Hydrodynamic Size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)
VCM6	152.4±1.7	0.230±0.008	-64.9±4.6	49.076
VCM7	161.4±2.4	0.225±0.031	-34.8±1.6	63.38
VCM8	153.5±11.46	0.163±0.031	-47.3±3.3	84.04

Hydrodynamic size insignificantly ($p>0.05$) changes zeta potential significantly ($p<0.05$) changes, but stayed within parameters set (80-200nm, >30mV), where PDI significantly ($p<0.05$) decreased showing oleic acid improved PDI and %EE outcomes. Transcutol P remained fixed as prior experiments informed

an increase in HDS is associated with increasing Transcutol P. The increase in PDI associated with Capryol 90 was not observed in these formulations where Capryol 90 concentration was increased from 200mg to 300mg observing an insignificant (p<0.05) change in PDI. A significant (p<0.05) increase in entrapment efficiency of 49.08% to 63.38% was however observed (Table 3.13).

The addition of 100mg of oleic acid saw a significant (p<0.05) PDI decrease of 0.225 to 0.163 and a significant (p<0.05*)* increase in entrapment efficiency of 63.38% to 84.04% (Table 3.13). Considering the increase in entrapment efficiency VCM8 was chosen as the final optimised formulation and taken for microbiology investigations. The HPLC trace associated with this formulation is shown in figure 3.13.

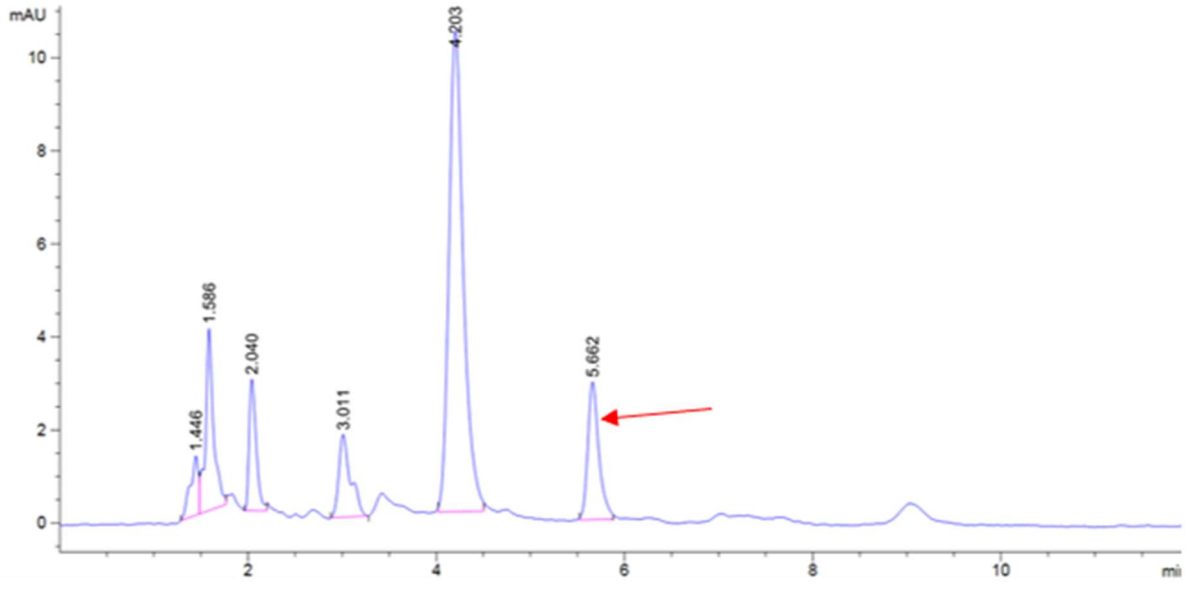


Figure 3.13: A HPLC chromatogram trace demonstrating the FD-VCM separate from formulation by centrifugation. The VCM peak is indicated by a red arrow. Peak of interest at 5.662 retention time.

3.3.2 Scanning Electron Microscopy

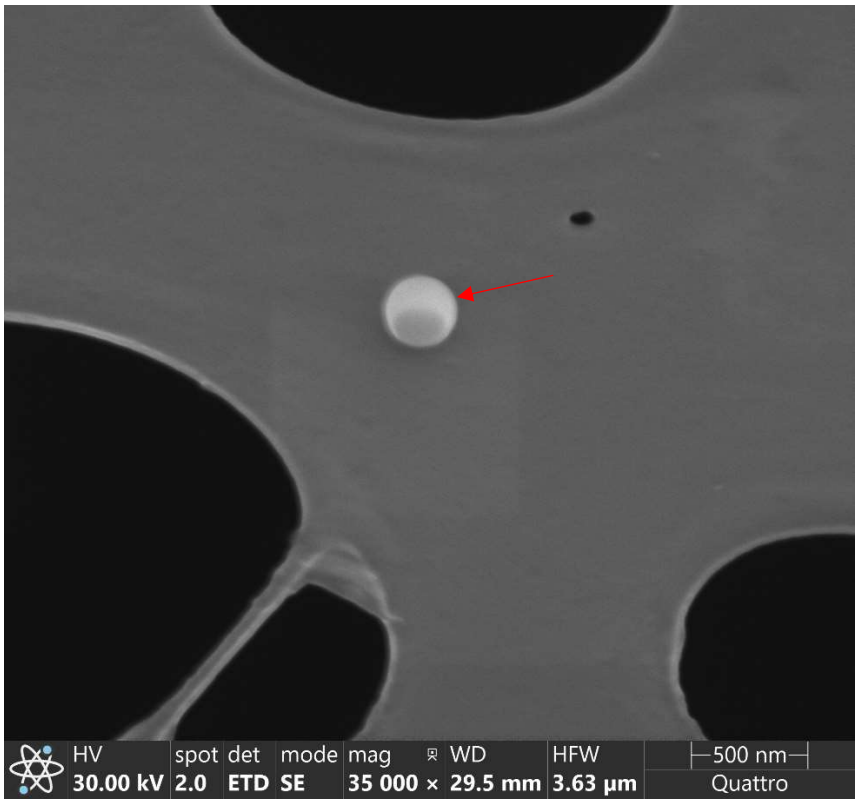


Figure 3.14: SEM images of optimised VCM-NLC formulation where the nanoparticle seen is labelled with a red arrow. Magnification was performed at 35000x.

Initially, there was difficulty focussing the microscope due to artifacts created by unincorporated excipients of the formulation. Therefore, a 1 in 50 dilution was completed on the formulation in HPLC grade water, the image produced is shown in figure 3.14. The image portrays good spherical morphology attributed to NLC particles.

3.3.3 Stability of Optimised VCM-NLC

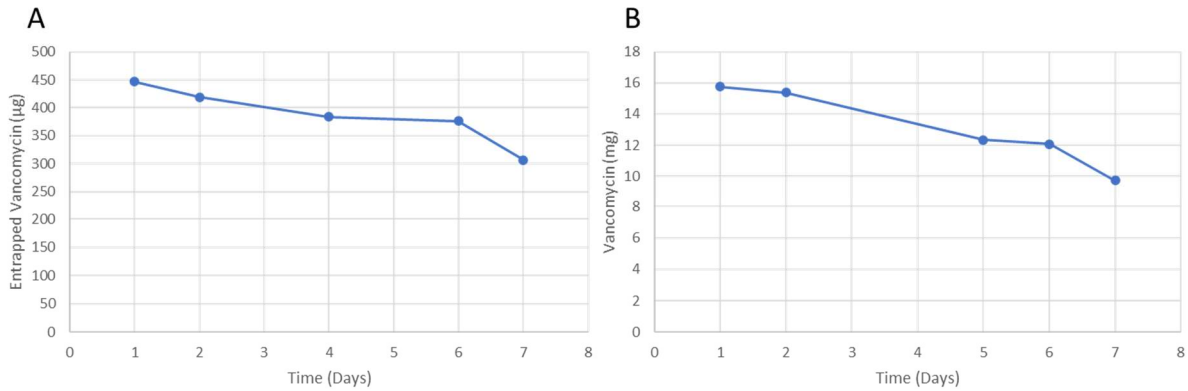


Figure 3.15: A comparison of the formulation VCM leakage over 7 days against VCM degradation across 7 days. A is VCM-NLC B is VCM in solution.

The output values of VCM-NLC compared against Blank-NLC showed insignificant (p<0.05) increases meaning the stability found in Blank-NLCs can be attributed to loaded formulations. However, with the addition of vancomycin drug diffusion and leakage out of the NLC now needed considerations taken. A 7-day study was therefore conducted to investigate the VCM leakage and degradation of VCM by UV-purity.

Free VCM degradation was measured from UV purity using HPLC, an area decrease was attributed to a degradation of drug. This data showed VCM began to degrade from day two but did so gradually decreasing from 15.7 to 9.7mg/ml showing a 37.5% decrease in UV purity (Figure 3.15).

The NLC delivery vehicle maintained low drug leakage over the seven days. At day seven VCM-NLC shows a significant increase in FD-VCM. Meaning after nine days 3.33% of VCM within the formulation would have undergone approximately 37.5% degradation. Data collected by Vigneron *et al.,* (2019) showed a similar 25% decrease in UV purity at 16 hours when kept at ambient room temperature (20°C-25°C).

When comparing percentage degradation vancomycin in solution decreased by 37.5% over the seven-day study where only 3.33% of vancomycin leaked out of VCM-NLCs in the same time period. It showed that significant (p<0.05) VCM leakage occurs after seven days within the NLC formulation (Figure 3.15).

The increase seen after seven days accompanied by no split peaks observed on chromatograms suggests whilst the VCM-NLC was encapsulated, vancomycin did not undergo major degradation.

3.3.4 Differential scanning calorimetry

DSC data in figure 3.16 shows peaks at temperatures 253.67°C for vancomycin hydrochloride, 59.41°C for Dynasan 114, 54.86°C for the Blank formulation and 53.35°C for physical mixture. The VCM-NLCs showed one primary and a secondary melting point at 104.14°C and 110.46°C, respectively. Liquid lipids did not show a peak due to being liquid at room temperature.

3.3.5 Fourier transform infrared spectroscopy.

FTIR was performed using a Nicolet iS50 Fourier-transform infrared spectrometer. The peak seen at 3326.33 (Figure 3.17) for both the blank and loaded VCM-NLC is water known by the O-H stretch bonding occurring within this region (Brunning, 2015). When comparing blank VCM-NLC and loaded VCM-NLC the only differing peak is at 2174.96 (Figure 3.17).

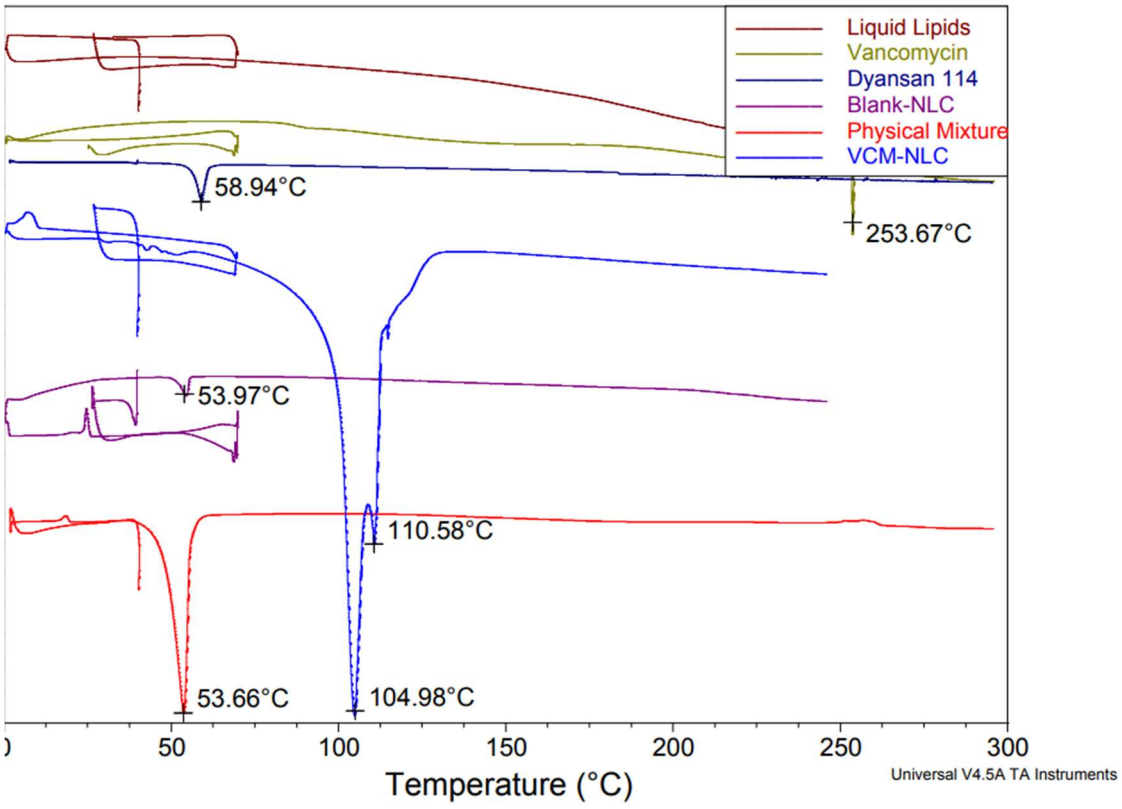


Figure 3.16: Differential scanning calorimetry data output comparing heat flow against temperature of individual NLC components. Using the TA analytical suite. Graphs representing Dynasan 114, Blank VCM-NLC. Liquid Lipids, Vancomycin, Loaded VCM-NLC and physical mixture.

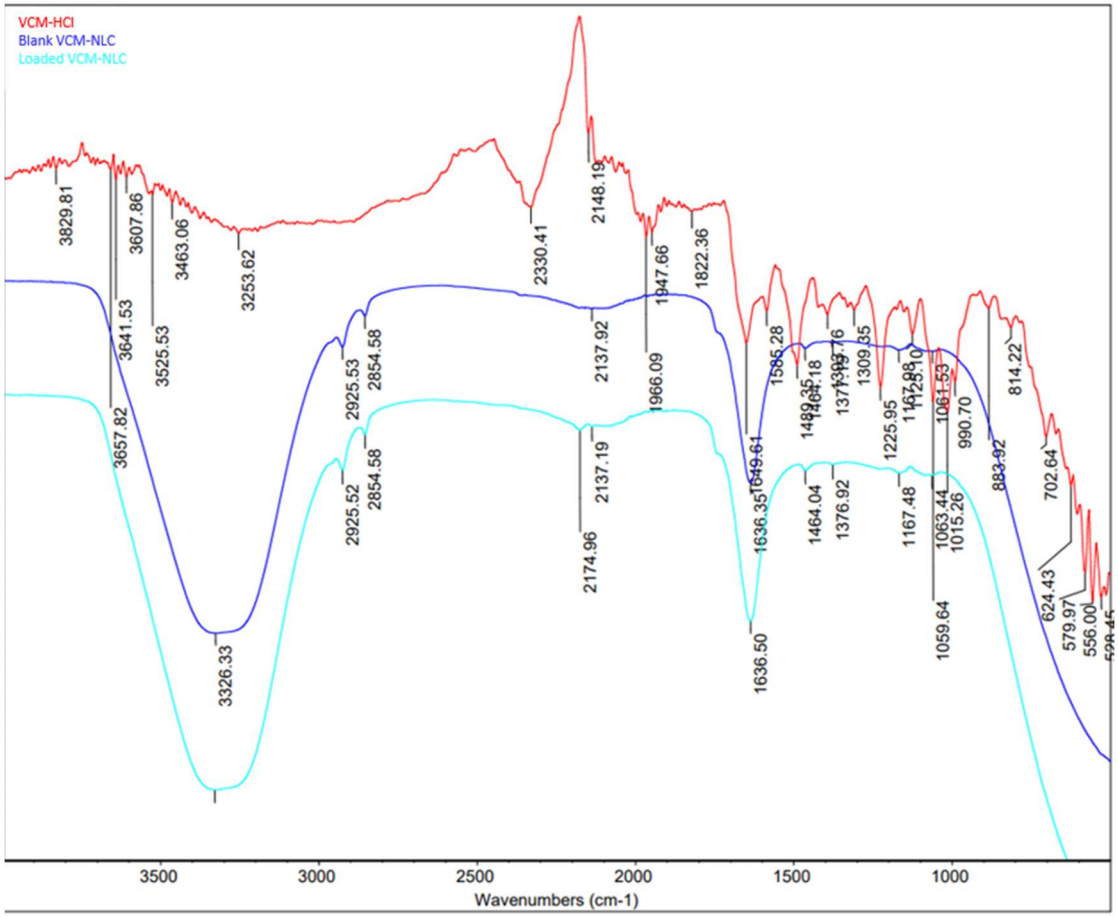


Figure 3.17: Fourier-transform infrared spectroscopy data output comparing transmittance against wavenumbers creating using the Nicolet FTIR analytical suite. Graph presenting VCM-HCl, Blank VCM-NLC and Loaded VCM-NLC.

3.6 Bacterial Characterisation

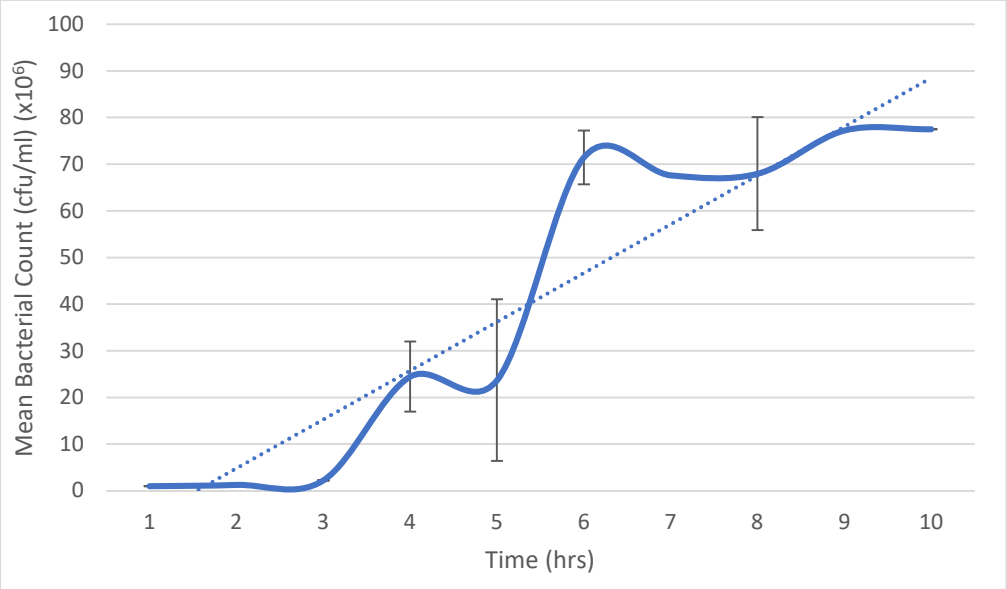
Bacterial characterisation was completed on the *S. aureus* reference strain ATCC 29213. This was done to confirm the bacteria cultured and further testing completed upon, was the strain purchased. Typical morphology showed a *S. aureus* which is round and domed with golden pigmented colonies 1-2mm in diameter. Under high power microscopy (x1000) Gram-positive cocci were seen in grape like clusters.

3.6.1 Growth Curve

Growth curve data was generated to characterise the rate at which the reference strain replicated and to provide a point of fast growth applicable for use within time-kill assays. Figure 3.18 shows *S. aureus* leaves the lag phase at three hours entering the logarithmic growth phase and enters the stationary phase at nine hours. A longer experimental period would show the decline phase however that was

not needed for this experiment. The three-hour log phase was adopted as at three hours *S. aureus* growth showed the beginning of rapid replication.

A



B

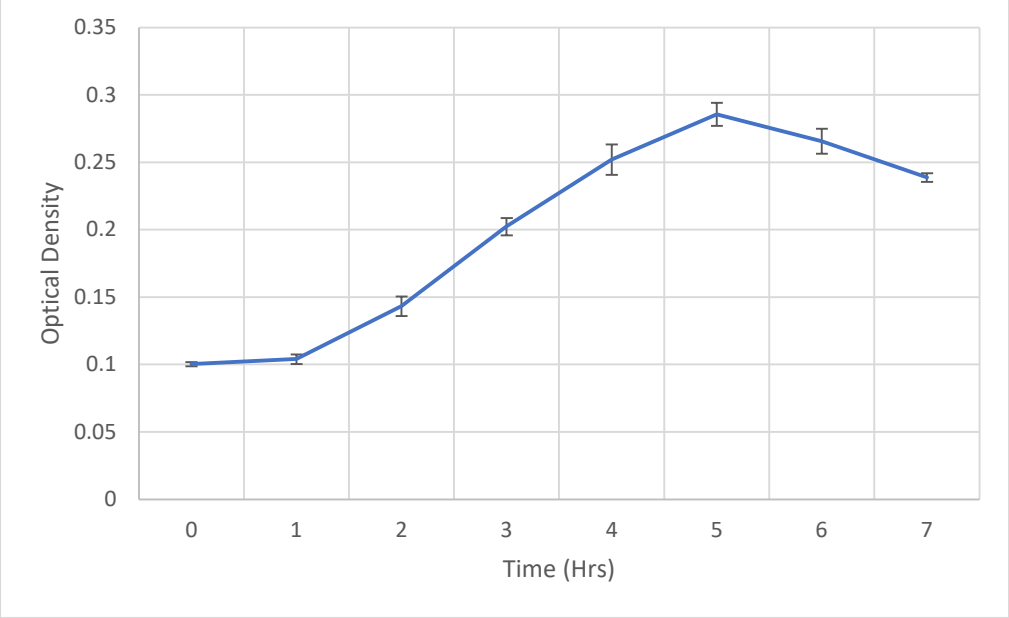


Figure 3.18: A growth curve shown by comparing bacterial count of a *S. aureus* culture over time. Standard deviation (n=3) A = a growth curve by colony count B = a growth curve by OD_{600}

3.6.2 AST Antibiogram

Table 3.14 shows the EUCAST breakpoints and zones of inhibition generated when the AST antibiogram was completed. The reference strain showed susceptibility to ampicillin, meropenem, tetracycline, trimethoprim and gentamicin. No breakpoints are given for vancomycin due to difficulty differentiating

breakpoints from reference strains due to wild types causing transfer of the vanA gene. However, a zone of inhibition is observed hence, the vanA gene is assumed not to be present. Insignificant (p<0.05) difference was observed between the antibiogram completed prior and post sensitivity testing, indicating the same organism was present.

Table 3.14: Shows EUCAST breakpoints for zone of inhibition (mm) of different antibiotics against *S. aureus* ATCC 29213 (EUCAST, 2023). N/A representing data that was not applicable to the test strain. VA susceptibility is not given due to the difficulty EUCAST found with differentiating from wild types.

Antimicrobial	Mean (n=2)	Susceptibility Target	Susceptibility Range	Susceptible
AMP10	16	18	15-21	Yes
MEM10	30.5	30	27-33	Yes
TE30	22.5	27	23-31	Yes
W5	25	25	22-28	Yes
CAZ10	0	N/A	N/A	N/A
CN10	20.5	22	19-25	Yes
VA30	16	N/A	N/A	N/A
CIP5	27.5	24	21-27	No

3.7 Bacterial Susceptibility Testing

3.7.3 Minimum Inhibitory Concentration (MIC)

MIC investigations were conducted on VCM in solution, VCM-NLC and blank NLC on an overnight culture of *S. aureus*. MIC values for free-VCM and VCM-NLC were 1µg/ml this followed EUCAST guidelines (EUCAST, 2023). Blank-NLC formulations were not inhibitory at any concentration.

VCM in solution was frozen at -20°C and is stable for 2 weeks as stated by the HSDB (Hazardous substances database (HSDB), 2023). The antimicrobial effect was therefore investigated after the two-weeks of storage. After two weeks of storage at -20°C the MICs of FD VCM showed pellets of bacteria forming in 1µg/ml wells. According to EUCAST (2023) pellets or 'tiny buttons' of bacterial growth

should be disregarded and considered biostatic. After 4 weeks the MIC showed turbid growth in 1µg/ml and a MIC of 4µg/ml.

3.7.4 Time-Kill Assay

Time-kill investigations were conducted on *S. aureus* (ATCC 29213) with VCM-NLCs and free VCM in solution as a positive control. A three-hour pre-incubation was shown as negative numbers plotted and post antibiotic introduction (PAI) growth was recorded. As shown in figure 3.19, both VCM-NLCs and VCM in solution had the same bacterial growth inhibition seen at 0.8µg/ml and above, but this inhibitory activity occurred at different time points. Free-drug VCM showed a decrease in viable count between 1 and 2 hours and VCM-NLCs showed a decrease in viable count below the limit of detection after 5 hours. The inhibitory activity seen is in line with the MIC of vancomycin at 1µg/ml.

However, in VCM-NLCs a concentration of 0.2µg/ml showed a colony count of 6.8×10^7 consistently over the six-hour test period while higher concentrations were bactericidal, this was not saw in vancomycin in solution.

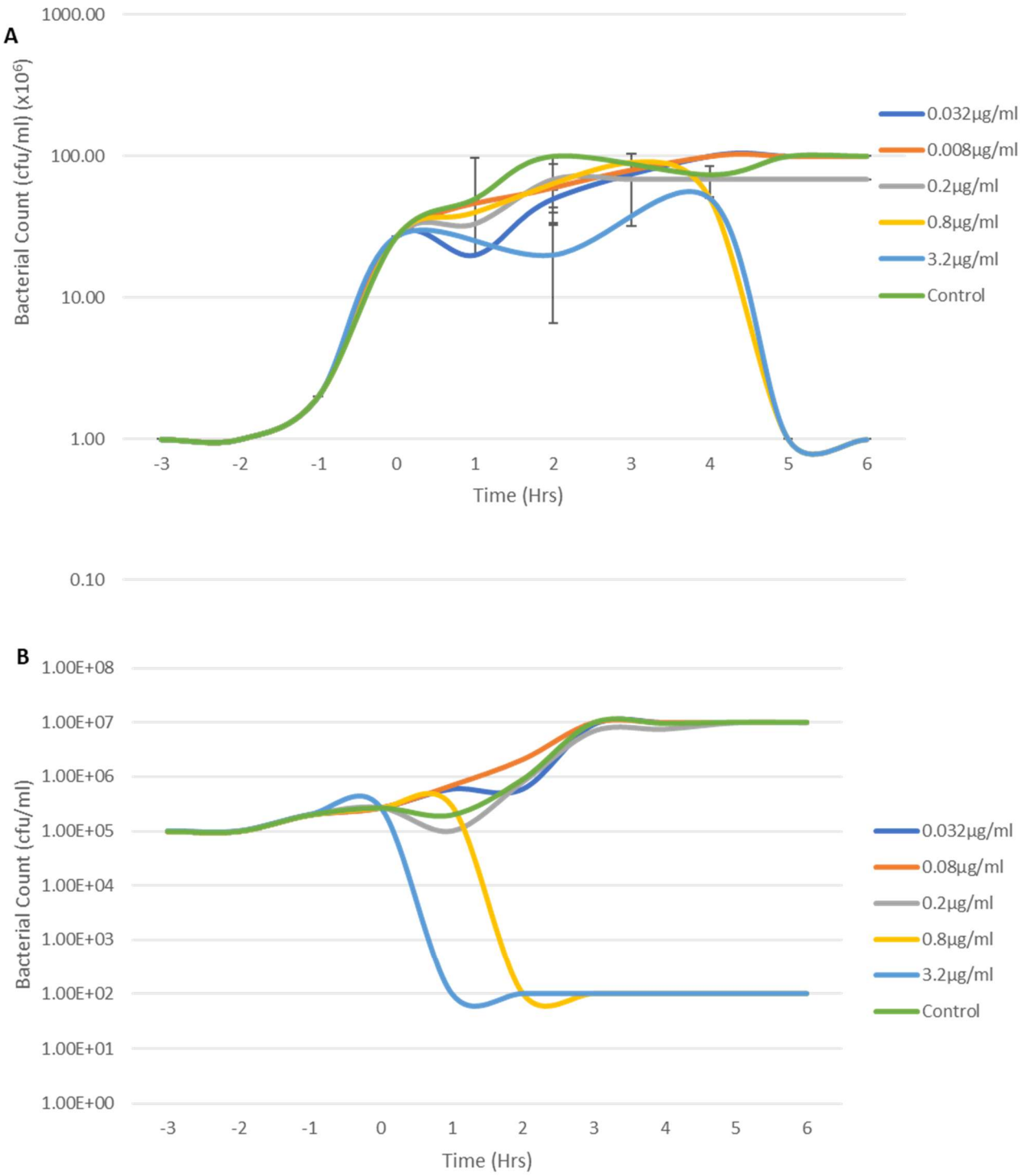


Figure 3.19: A graphical representation of time-kill analysis completed showing the change in bacterial growth observed over 9 hours. Where antibiotics were added at T=0 and T=-3 to T=0 was pre incubation. A VCM-NLC. B VCM only. *1.00E+6 is the lower quantifiable range, 1.00E+08 is the upper.

Chapter 4: Discussion

Due to the use of empirical antibiotic prescription and overuse of antibiotics in healthcare and community settings *S. aureus* has developed a wide range of resistance to multiple antibiotics commonly used for treatment of staphylococcal infections such as methicillin (Chambers *et al.,* 2009). An antibiotic reserved specifically for treatments of these resistant infections is vancomycin, a glycopeptide antibiotic which disrupts peptide crosslinking within bacterial cells walls (Koyama *et al.,* 2012). Due to the high hydrophilicity and its ability to bind to blood plasma proteins vancomycin exhibits high glomerular excretion. With this elimination occurring in tissues residing in the kidneys, it is also associated with increased nephrotoxicity due to reabsorption causing mitochondrial oxidative stress (Stidham *et al.,* 2011).

Vancomycin is administered intravenously by long term continuous infusion or intermittent (pulsed) infusion (ii-VCM). Continuous infusion requires patients to spend time in hospital and have VCM administered over 8-12 hours (VANCOMYCIN IV Monograph (Part of York Teaching Hospital NHS Foundation Trust IV Guide), 2017). This administration is preferred for deep seated severe systemic infections. The intermittent infusion-VCM dose is administered every 12-48 hours and patients spend 1 hour for every dose in hospital and are monitored for creatine levels and renal function. A study completed comparing continuous and intermittent infusion of vancomycin found, when using continuous infusion significantly ($p>0.05$) less subtherapeutic ranges were attained with 54% patients attaining the therapeutic target in ci-VCM and 22% attaining in ii-VCM. Therefore, if a VCM-NLC could be administered on an intermittent basis but provide the therapeutic range needed for deep seated infections patients would be able to potentially spend less time in hospital and reduce the toxic renal side effects which is attributable to ci-VCM (VANCOMYCIN IV Monograph (Part of York Teaching Hospital NHS Foundation Trust IV Guide), 2017).

Nanostructured lipid carriers (NLCs) are second generation lipid nanoparticles which have shown to protect and improve the stability of drugs and enhance the biopharmaceutical properties of encapsulated drugs. This study therefore sought to investigate vancomycin encapsulated NLCs *in vitro*

for their potential of improved bactericidal effects. Vancomycin is a large glycopeptide molecule and to achieve high enough entrapped concentration the target hydrodynamic size of 80-200nm was considered suitable. The solid lipid used was Dynasan 114, trimyristin, a glycerol triester of myristic acid. It was used due to its high melting point and its common use within oral and dermal pharmaceuticals, indicating low toxicity (Devi *et al.,* 2019). Liquid lipids used were Capryol 90, Transcutol P and oleic acid. During the blank-NLC formulation stage of the project, optimisation was completed on excipients and excipient concentrations to attain a formulation which provided sufficient output values (HDS, PDI, Zeta potential). Post optimisation vancomycin was added to emulsions and further optimised for entrapment efficiency percent (EE%).

NLC optimisation was conducted on hydrodynamic size (HDS) to ensure that when NLCs were used on early log phase *S. aureus* the HDS did not exceed that of the cell (two microns), but also was small enough that it could pass cell surface membranes of mammalian tissue cells to access the internalised infection site (biofilm). Considerations were also made as to the size of the vancomycin molecule so particles could not be too small as this would affect drug loading and %EE. Smaller hydrodynamic size could result in lower retention of vancomycin and greater loss during preparation means, the resultant effect would cause lower amounts of drug to be utilised in *in vitro* testing (Akanda *et al.,* 2021). The size of the optimised NLC in this study was 153.5 ± 11.46nm which would provide particles which a high probability of particles diffusing across eukaryotic membranes, as shown by Rawl *et al.,* (2023). Data showed hydrodynamic size decreased with increased sonication time however, excessive sonication showed the occurrence of micelles causing a non-uniform formulation, which is consistent with Zwain *et al.,* (2023).

SEM was conducted to visualise the morphology of the VCM-NLC. The SEM data exhibited a spherical formulation with a smooth surface. No VCM was detected on the surface on the NLC, this suggests the lipid matrix had encapsulated the drug in the production process. These finding were in line with Shimojo *et al.,* (2019), who saw a spherical morphology and suggested Encapsulation of their drug.

Polydispersity index (PDI) is the measure of the dispersity of size within any given population (Danaei *et al*., 2018). In context of this study, it is a measure of the dispersity of hydrodynamic size of particles measured by DLS. PDI provides insight into to the quality of formulations and the ability of DLS analysis to accurately measure formulations. Mozafari *et al.,* (2017) stated that PDI is a dimensionless scale with a value of 0.7 or above showing a broad range of particle size and values of 0.05 are near to monodisperse however, also stating that values exceeding the values 0.7 and 0.05 (0.05>x>0.7) are unsuitable for DLS analysis. Furthering this the FDA emphasized the importance of particle size and size distribution in devices intended for biological use, highlighting optimum values must be attained to avoid unwanted biological effects and accurate dosing (FDA, 2006). Although, the FDA did not provide an optimum PDI.

PDI was optimised within this study to maintain the aforementioned benefits of HDS and ensure beneficial accumulation occurred in target tissues (Khosa *et al.,* 2018). Azhar *et al.,* (2016) states a large oil to water ratio presented a lower PDI, but greater hydrodynamic size. Hence, a balance would need to be maintained within formulations especially with hydrophilic drugs such as VCM. As a greater lipid concentration could lead to VCM prioritising the aqueous phase over lipid solubilisers (Rybak, 2006). PDI was optimised by sonication studies which observed a consistent decrease in PDI and hydrodynamic size with an increase in sonication time, until the decrease in hydrodynamic size became non uniform causing an increase in PDI demonstrated by figure 3.1 and corroborated by Ruiz *et al.,* (2022). The PDI achieved within this study was 0.163 ±0.031 which would provide a formulation close to monodispersity therefore, would provide better entrapment outcomes and maintain the benefits of a low HDS for absorption and accumulation by tissues (Danaei *et al.,* 2018).

Zeta potential is the measure of the polarity of particles and is an indicator of the likelihood of aggregation. Zeta potentials greater than -30mV would increase repulsion occurring between particles preventing aggregation (Patel *et al.,* 2012). Gordillo-Galeano & Mora-Huertas, (2021) found during investigations in NLC optimisation zeta potential also affects aspects such as pH and ionic strength.

They stated it was attributed to a larger zeta potential causing ions to cling to the NLC surface causing effects to pH and increasing ionic strength. This information is important to consider as the optimum growth range for *S. aureus* is a pH of 6-7 hence values outside this range would cause a decrease in bacterial growth irrelevant of vancomycin (Food standards of Australia and New Zealand, 2013). Compounding this, due to the low pKa of VCM it could also affect %EE (Settimo *et al.,* 2014). The zeta potential in this study was -47.3 ±3.3mV with the high negative value increasing repulsion between particles therefore being indicative of a stable formulation as shown by Coc *et al.,* (2021).

Vancomycin entrapped NLCs (VCM-NLCs) were investigated to determine their physiochemical properties and compared to blank NLC formulations. This included optimisation of VCM concentration, optimisation of liquid lipid concentration, and optimisation of hydrophilic surfactant with VCM-NLCs. This optimisation was required to compare the effects of free drug VCM on bacteria and the benefits provided from VCM Encapsulation in the NLCs. To obtain the final VCM-NLC formulation the product parameters: solubilisers and surfactants and the process parameters sonication time were considered and optimised. The critical quality attributes (HDS, PDI and Zeta potential, entrapment efficiency) were used as guidance to optimise the product and process variables. Optimisation was initially carried out in non-drug loaded formulations (blank) to investigate basic lipid and surfactant interactions. Optimisation included individual excipients and their concentrations (Table 2.1) adjusted individually to access impact on critical quality attributes. When comparing blank and loaded formulations data showed HDS and PDI were insignificantly (p>0.05) different and Zeta potential was significantly (p<0.05) different. Showing encapsulation caused negligible change to HDS and PDI but changed the charge of the particle. The irrelevant change in HDS and PDI would be attributed to the solubilisers in use effectively entrapping drug. This would most likely be due to the HCl group bound to VCM changing the surface charge and pH of the solution (Gordillo-Galeano and Mora-Huertas, 2021).

Total drug (TD) is the concentration of VCM loaded into formulations. Total drug is not equal to amount added to the formulation, as drug is lost in the preparation process due to adhesion to surfaces and

evaporation in water (Akanda *et al.,* 2021). Experiments and investigations were completed into total drug and drug loading. However, difficulties were had with finding a solvent which fit the following parameters: NLC breakdown, drug solubility and ability provide a clear chromatogram peak for HPLC analysis. Tetrahydrofuran (THF) is a common solvent for NLC breakdown as it acts as a universal solvent. When experiments were conducted using THF, VCM degradation was observed; when analysed on HPLC, split peaks were seen with fronting and lagging tails. Better HPLC outcomes were found when lower concentrations of THF was utilised due to it lowering the concentration of drug dissolved in THF. This reduces the number of peaks overlapped by the solvent front. This were exhibited by a decrease in split peaks and more defined peaks shown by greater symmetry. However, concentrations could not be decreased drastically as investigations found a ratio of 3:2 (THF: NLC, v/v) was needed to have complete NLC breakdown. Hence, THF could not be used for further investigations. Due to VCMs moderate solubility in methanol (MeOH), a centrifugal method was adopted where NLCs were spun at high g-force (13000 x g) to precipitate VCM out of NLCs and into MeOH. This method provided sufficient drug solubility and a clear chromatographic peak on HPLC analysis however as it did not cause NLC breakdown, due to it being a weak solvent, meaning there was no way to confirm all the VCM has been extracted from NLCs; so, this method was also not utilised further.

Entrapment efficiency (%EE) is the measure of drug Encapsulation within NLCs. Entrapment efficiency is dependent on many factors within the development of NLCs such as the solubility of the drug in lipids, the nature of the crystalline matrix and preparation method of NLCs. The choice of liquid lipid and solubilising agents like Caproyl 90, Transcutol and oleic acid in VCM-NLCs, consequently resulted in increasing entrapment efficiency (Tiwari, Gatto and Wilkinson, 2018) (Osman et al., 2019). Entrapment efficiency was considered within the optimisation process of the NLCs to ensure when bacterial sensitivity testing was conducted encapsulated drug and labelled drug information could be used. Due to FD-VCM still causing bacterial inhibition, bacterial access to FD-VCM needed to be limited. This was achieved by decreasing vancomycin concentration to 0.5mg/ml and increasing solubilisers to limit FD-VCM to below 10-20% of drug added.

The indirect method utilised within this study produced an entrapment efficiency of 84.04 ±3.41%. With vancomycin being a hydrophilic drug, high EE% is hard to achieve and EE% is higher than other similar vancomycin loaded lipid nanoparticles reported (Jounaki *et al.,* 2021) (Mhule *et al.,* 2018) (Saidykhan *et al.,* 2016). Addition of oleic acid at 4mg/mL and Capryol 90 at 12mg/mL to the formulation increased the Encapsulation of vancomycin at 0.5mg/mL from 49% to 84%.

Oleic acid, an unsaturated fatty acid with eighteen chain carbon length was utilised due to an investigation completed by Kalhapure *et al.,* (2014) which reported synergistic interactions between vancomycin and linoleic acid (another unsaturated fatty acid with 18 carbon chain length) was discovered showing inhibitory effects towards *Staphylococcus aureus* (ATCC 25922) and an increase in %EE. The synergistic interaction was found due to the application of ion-pairing in the study by Kalhapure where they investigated the potential of increasing vancomycin lipid solubility by removing the HCl group and replacing with linoleic acid. The ion-pairing achieved an increase in entrapment from 16.81% to 70.73%, showing the potential for ion-pairing and oleic fatty acids improving vancomycin entrapment in NLCs.

Sodium cholate is a hydrophilic surfactant added in the aqueous phase. Sodium cholate was optimised to counteract an increase in PDI produced by Capryol 90. Capryol 90 is an organic non-ionic surfactant utilised due to its ability to solubilise vancomycin, therefore increasing %EE of drug in the NLCs. Optimisation of these two excipients allowed the hydrophilic-lipophilic balance to be maintained, whilst increasing entrapment efficiency. This was shown in formulation B8 with a HDS of 132.4nm ± 14.7 and a PDI of 0.139 ± 0.0062 using 150mg of sodium cholate, whereas 100mg of sodium cholate concentration showed a HDS of 158.9nm and a PDI of 0.183 ± 0.012, indicating the importance of maintaining the hydrophilic-lipophilic balance especially between surfactants. Severino *et al.,* (2012) reported studies onto specific lipid excipients and the contributions they make to the hydrophilic-lipophilic balance in nanoparticle preparation. The hydrophilic-lipophilic balance (HLB) depicts the value assigned to surfactant and lipid excipients. If the same or similar value is applied to lipid and

surfactant excipients it results in a more suitable formulation *in vitro* and is more likely to result in positive output values. As Capryol 90 is has a HLB value of 15 and sodium cholate a value of 18 these were investigated and utilised within this study (Rao *et al.,* 2014). Liu *et al.,* (2007) also investigated the effect of sodium cholate on nanoparticle preparation finding an increase of up to 0.05% showed a significant reduction in hydrodynamic size (109.7nm) and an increased entrapment efficiency (99%).

Transcutol P and Capryol 90 were utilized due to a solubility study conducted in this project, showing both lipids had potential to solubilise vancomycin (Table 3.1). Phospholipon 90H, lipoid S75 and Lipoid E80 are phospholipids which formed the outermost layer of the particles (Khurana *et al.,* 2016). Consisting primarily of phosphatidylcholine (Table 2.1) which is a vital component of eukaryotic membranes, facilitating phosphatidylcholine-mediated diffusion of particles into cells (Lipoid GmbH, 2022) (Conde-Alvarez *et al*., 2006) (Akopian *et al*., 2015). The concentration change for these lipids and output values was not investigated as previous studies in our lab have optimised their concentrations and were used as is in this project.

Vancomycin was first quantified via an optimized HPLC method following ICH guidelines (1994). Initially, a calibration curve was constructed by analysing a range of known VCM concentrations (0.5,1, 2, 4, 8, 16 and 20 µg/ml), two times with three repeats (n=6). This achieved a calibration curve with an R^2 value of 0.9997 (Shabrir, 2004). This indicated that the method had good linearity and was repeatable. Accuracy was calculated at seven concentrations (0.5,1, 2, 4, 8, 16 and 20 µg/ml). While the higher concentrations were within the recommend 80-120% by the ICH the lower concentrations 0.5 and 1µg/ml exceeded the recommend 80-120% by the ICH (1995) with values of 143.32% ± 0.82 and 128.98% ± 13.08, respectively. Therefore, the assays with this method were restricted to concentrations above 1µg/ml.

Inter and intraday studies were also completed using vancomycin standards at a concentration of 4µg/ml and 16µg/ml, with all concentrations tested showing a SD% below the 10% recommended by ICH guidelines (1994) (Shabrir, 2004). This also indicated that VCM concentration quantified within the

VCM NLCs was precise. Repeatability of the HPLC assay was investigated with a vancomycin standard at 10µg/ml injected seven times, which produced a SD% below 2% in line with ICH guidelines (ICH 1994) (Shabrir, 2004).

Jounaki (2021) and Osman (2019) both studied vancomycin loaded NLCs manufactured by hot homogenisation. Jounaki *et al*., (2021) investigated vancomycin encapsulated NLCs for ocular application achieving output values; particle size of 96.40nm ± 0.71, PDI of 0.352 ± 0.011 and an indirect entrapment efficiency of 74.80% ± 4.30. Finding that the liquid lipid and liquid lipid ratio had the greatest effect on particle size and entrapment, similar to that of this study. The finalised NLCs found within this study produced a lower PDI and increased %EE than those found in Jounaki *et al.,* (2021). Reasoning being that Jounaki *et al*., did not utilise solubilisers hence decreasing entrapment and causing a more dispersed emulsion due to a greater proportion of free vancomycin. However, the study completed by Jounaki (2021) aimed to create a 'depot of drug' at the junction of the vitreous humour and corneal surface, hence uniform particles may have not been optimal. Another study conducted by Osman (2019) into the effect of vancomycin loaded nanoparticles on MRSA achieved a PS of 225.9nm ± 9.1, a PDI of 0.258 ± 0.02 and zeta potential of -6.69mV ± 1.1. The PS (32% increase) and PDI (38% increase), obtain by Osman exceeded the optimum parameters set by this study (PS 80-200nm, PDI <0.300) The large decrease seen in zeta potential when compared to this study (86%) would potentially increase coalescence and therefore reduce long-term stability. A decrease in long term stability could decrease the efficacy of drug in shipment time Osman (2019) also utilised oleic acid as a liquid lipid which also showed an increased entrapment efficiency as seen in this project. The reason for differing output values could be attributed to the choice of target being acidic environments, hence the decreased zeta potential would increase coalescence in a neutral environment but may not in acidic. This however was not investigated.

Mhule *et al.,* (2018) also investigated the promise of vancomycin loaded nanoparticles but instead by utilising the solid lipid nanoparticle format and cold homogenisation. The characteristics attained in

the finalised formulation was a PS of 220.5nm, a PDI of 0.380, a zeta potential of +9.39mV and direct entrapment of 81.18%. The particle size and PDI achieved was attained by utilising Tween 80 surfactant which can decrease particle size and increase storage time of particles (Upadhyay *et al*., 2012) (Ebrahimi *et al*., 2015). However, Tween 80 also has a toxicology profile which needs to be considered with it causing a range of toxic side effects in animal subjects tested, examples are an antagonistic effect on the immune system and a hypotensive effect on the blood pressure (Varma *et al*., 1985). Varma (1985) determined it should only be used in neuropsychopharmacology application in doses of no larger than 1ml/kg. Considering this, this project did not utilise Tween 80 instead optimised sodium cholate as the aqueous surfactant which does not produce any toxic effects, proven by human cell line studies utilising SLNs and NLCs (Tekade, Mittha and Pisal, 2022) (Sharma *et al*., 2023) (Liu *et al*., 2007).

To ensure the stability of the blank NLCs over a period of time, a short-term stability study was conducted over 30 days. The blank NLC observed a significant (p<0.05*)* increase in PDI at 21 days, with an increase of 0.09 to 0.243. The PDI remaining within the optimum product parameters (Danaei *et al*. 2018), indicating the blank NLC remained stable past the 30-day period. This is due to the low PDI indicating a well mono-dispersed formulation which would lead to greater stability over an extended time (Coc *et al.,* 2021). Another key indicator into stability is the increased zeta potential produced by this study. A study by Shirisha *et al.,* (2020) completed a stability study at room temperature into NLCs consisting of primarily Dynasan 114. Insignificant changes were observed in particle size and slightly increased PDI values as seen in this study.

The short-term blank stability study conducted highlights the potential for this NLC delivery system to be used as a tool to possibly increase aqueous stability of vancomycin. Conversely, this improved short-term blank stability was not observed in loaded formulations, therefore a seven-day entrapment efficiency study was conducted. After seven days the FD-VCM had significantly increased (p<0.05). Meaning that the formulation would increase stability of vancomycin at room temperature for an additional seven days (20°C-25°C) (Hazardous substances database (HSDB), 2023). Free drug increased

as vancomycin will slowly diffuse out of the liquid lipid solution it is in and into the water of the aqueous phase, this is attributable to vancomycin's hydrophilicity and affinity for water (Hazardous substances database (HSDB), 2023).

When stored at below 4°C aqueous vancomycin is stable for two weeks and 48 hours at room temperature (Hazardous substances database (HSDB), 2023) (Dodd et al., 2006). As the DSC data showed all boiling points were above 50°C. Indicating if the VCM-NLC was stored at below 4°C solidification would begin to occur; this was observed in experimentation. Determining the VCM-NLC would not provide better stability outcomes compared against frozen-powdered VCM. Meaning freeze-dry storage would be vital for this formulation to maintain characteristics and stability of vancomycin over a longer period of two weeks. Further research would need to be completed on colloidal studies into the NLC in cryoprotectants to ascertain NLC stability in long-term freeze-dried storage.

Mhule *et al.,* (2018) completed a 90-day study into the physical stability of formulations at room temperature finding immediate significant changes in stability of particle size and PDI showing high instability, although a similar study was completed at 4°C where values did not significantly ($p<0.05$) change over the 90-days. This could be attributable to the use of Tween 80 a surfactant which provides improved results when used at lower temperatures (4-8°C), as at above these temperatures it increases aggregation and begins undergoing thermal degradation when in solution (Agarkhed et al., 2013). This study improved upon Mhule (2018) by maintaining a stable formulation for seven-days at room temperature and avoided the use of Tween 80 a possibly toxic surfactant. Jounaki *et al.,* (2021) completed a 12-week stability study into their optimized formulation finding after 12-weeks insignificant leakage of vancomycin had occurred with a near constant particle size and PDI. Creating superior stability than when compared to this study mainly attributed to the differing use of excipients as again Tween 80 was used alongside cholesterol and stearic acid. Indicating greater optimisation

would need to be conducted within this study to attain stability similar to that of Jounaki *et al.,* (2021) without utilising Tween 80.

DSC was used to measure the melting point of VCM, VCM-NLCs, Blank-NLCs, individual excipients Dynasan 114, Capryol 90, Transcutol P, oleic acid and combination samples liquid lipids and physical mixture. DSC is a tool which can be utilised to identify drug-lipid interactions and indicate encapsulation. The Dynasan peak found can be corroborated by Zwain *et al.,* (2021) who found a peak of 52°C. The primary excipient in the blank VCM-NLC and physical mixture samples was Dynasan 114, with melting points being slightly lower than that of only Dynasan 114. Therefore, it could be assumed that as the liquid lipid and phospholipid excipients were the next largest in concentration, the lipid interactions of these excipients with Dynasan 114 decreased the melting point of the blank VCM-NLC. The increased melting point of the VCM-NLC compared against the blank VCM-NLC shows vancomycin's presence is changing the heat fluctuations occurring within the formulation which could indicate that some drug-lipid interactions are taking place with VCM, and that encapsulation has occurred. Mohamed *et al.* (2017) investigated the benefits of VCM loaded noisomes completing DSC data upon VCM and excipients. VCM did not show a consequential peak in this study however Mohamed (2017) did note a heat fluctuation at 60°C, suggesting his vancomycin sample was within its amorphous phase.

FTIR was conducted to investigate into potential lipid-drug interactions within NLCs. FTIR highlights vibrational changes within the bonding of the formulation and vancomycin. The vancomycin fingerprint region shown in figure 19 is concurrent with a study completed by Hagbani *et al.,* (2022) where similar peaks were seen. No peaks from the vancomycin fingerprint region are seen within the loaded NLC formulation which could be indicative of strong encapsulation. The only differing peak observed within FTIR investigations was at 2174.96. This is most likely the lipid-VCM interaction seen in DSC analysis. This is due to this bonding being outside the fingerprint region and when compared to Brunning (2015) shows carbon-carbon double bonding (C=C), strong covalent bonds which would

increase melting point as shown in DSC analysis. Any drug-lipid interactions would need to be conclusively proven by X-ray diffraction analysis.

Due to *S. aureus* being a commensal bacterium, treatment which can decrease bacterial count whilst maintaining AMR stewardship is vital for clinical practice. Bacterial sensitivity testing was completed on *S. aureus* ATCC 29213 by MIC and time-kill with prior testing completed to inform these tests. EUCAST disc diffusion characterisation data showed susceptibility to a wide range of antibiotics tested. Vancomycin is not marked as susceptible as EUCAST in disc diffusion assay, as EUCAST could not distinguish between wild type isolates and non-*vanA* mediated resistance' (EUCAST, 2023). Bauer *et al.,* (1966) stated MIC and zones of inhibition are inversely proportional, with a larger zone of inhibition suggesting a lower MIC. This is due to a zone of inhibition being produced by the diffusion of drug into the agar medium and inhibiting bacterial growth. As drug diffuses through the agar it will have an inhibitory effect and concentration of drug will decrease, this decrease means at a point inhibition will stop and a zone around the drug without bacterial growth will appear (Tenover, 2019). Hence the zone of inhibition recorded suggests an inhibitory effect, confirmed by MICs. EUCAST states the MIC of *S. aureus* ATCC 29213 has a target of 1µg/ml and a range of 0.5-2µg/ml, this project achieved the target of 1µg/ml with free-VCM due to the lack of *vanA* wild types in the test strain suggesting vancomycin exposure will have a non-limited inhibitory effect on bacteria tested. VCM-NLC had an identical MIC of 1µg/ml showing encapsulation did not inhibit the effect of VCM.

Minimum inhibitory concentration investigations were completed to ascertain the minimum concentration which causes an inhibitory effect in VCM and VCM-NLCs. This is shown by the range of concentrations used within the time-kill assays encompassing the MIC. Blank-NLCs were tested to investigate the inhibitory properties of excipients in the concentrations used in loaded formulations and to prove it was the presence of vancomycin within loaded formulation which caused an inhibitory effect. Blank-NLCs formed pellicle structures which were an interesting point as upon subculture, confluent growth matching typical *S. aureus* growth (golden colonies 1-2mm in diameter, verified by

protein A latex agglutination) was seen even under dilution. As pellicles were seen at all concentrations it is unlikely aggregation was caused by NLCs and rather a constant concentration of bacteria, showing the potential of *S. aureus* causing NLC breakdown by lipase action. Sargison *et al.,* (2021) commented on the lipase activity of *S. aureus* and the inability the lipase activity has on preventing immunological killing mechanisms. She concluded *S. aureus* excreted two lipase enzymes lipase 1 and lipase 2. Lipase 1 has affinity for short-chain triglycerides where lipase 2 has no bias. This study therefore could corroborate the statement of *S. aureus* breaking down NLC lipids with two isoforms digesting a range of fatty acids and triglycerides. Lipid excipients from NLC formulations could be the aggregates observed on the surface of the water. Although, this was not confirmed. Osman (2019) conducted an MIC with a Blank-NLC. Their study was in line with research done within this project, which found Blank-NLC also containing oleic acid, caused no inhibition to growth when tested against *S. aureus* but did not state any observation towards any pellicle structures. This could possibly be due to the acidic condition's experiments were completed in preventing lipase activity, as *S. aureus* lipase enzymes work optimally at neutral to basic pH (Mates, Sudakevitz, 1973).

Kill-time assays exposed the *S. aureus* reference strain (ATCC 29213) to VCM and VCM-NLC for six hours with hourly total viable counts to ascertain an exposed growth curve. Blank-NLC was not included within this experiment due to MIC investigations revealing no inhibitory action. An inhibitory concentration of between 0.8µg/ml and 3.2µg/ml was observed at two hours for VCM and four hours for VCM-NLC. There is no study the VCM-NLC time-kill results can be compared against due to the method being created specifically for this project. When diluting samples for kill-time investigations difficulties with bacterial variance were encountered. Due to early samples or samples with high antibiotic load showing zero colonies upon dilution and subculture, a lower quantifiable limit was set. This was done to consider variance within samples as to fully confirm a result, samples would need to be subcultured in their entirety. Hence, to account for this variance $1x10^6$cfu/ml was assigned to a result of zero colonies as zero would be less than 1 colony. Singh *et al.,* (2009) investigated time-kill assays for free vancomycin finding that when $5x10^5$cfu/ml *S. aureus* (hospital obtained MRSA isolates)

was exposed to 0.5µg/ml of vancomycin a similar reduction in bacterial count was observed as in this study.

With the biocidal concentration of 0.8µg/ml being evident in both VCM and VCM-NLC time-kill assays, encapsulation did not change MBCs similar to that of MIC assays. If comparisons are to be made between the MIC and time-kill assays the primary variable would be contact time. Samples in MIC assays underwent a 24-hour contact time culture and samples in time-kill assays underwent a six-hour contact time. An increased contact time as shown, could therefore be causing a sub-lethal effect in bacterial growth.

In MIC investigations all values below 1µg/ml for VCM-NLC showed no inhibition where in time-kill assays 0.2µg/ml was shown to have an inhibitory effect over a lower contact time (six compared to 24 hours) (figure 3.19). As the study by Singh *et al.,* (2009) shows a 3-log reduction in *S. aureus* growth over 6 hours it is assumed if a biocidal effect were to be observed a reduction in bacterial count would have been seen in the period used. As confluent growth outside of the range of detection is seen at four-hours for both VCM and VCM-NLC it is difficult to determine if growth was halted or decreased after this time, within the range evaluated. Conclusions can be made that in the time frame used both VCM and VCM-NLC at the same concentration caused a similar four-log reduction of *S. aureus* cfu/ml over 4 hours, when compared to the positive control.

The inhibitory effect (0.2µg/ml) observed in VCM-NLC time-kills could be attributable to a synergistic effect seen between oleic acid and vancomycin. Initial experiments investigated oleic acid purely as a solubiliser to increase entrapment. However, MICs generated by Kalhapure (2014) showed oleic acid had an inhibitory effect at a concentration of 1250µg/ml. Although, 1250µg/ml seems high in comparison to the MIC of 1µg/ml seen in vancomycin, the synergistic potential due to modes of action could prove beneficial. Vancomycin causes cell wall break down by peptide crosslinking destruction, where oleic acid causes the formation of permanent pores in bacterial cell membranes of variable sizes (Desbois & Smith, 2010). The simultaneous breakdown of both bacterial physical barriers would cause

increased bacterial inhibition at lower concentrations of NLCs, consequently also increasing absorption of drug and decreasing the chance of efflux in *S. aureus* and resistant (MRSA) strains (Sharma, Gupta and Pathania, 2019). This inhibitory effect could therefore be due to a sustained release of drug and antimicrobial excipients. In this study an extension of two hours was observed with drug diffusing into media over 4 hours, this slow release would prolong the possible synergistic effect preventing further growth. As Tanaka *et al.,* (2018) showed *S. aureus* lipase activity occurred within 15 minutes the prolonged release is majorly going to be due to drug diffusion out of the NLC matrix. However, the longer contact time observed by MICs provided a sub-lethal effect on *S. aureus*. The inhibitory effect seen within this time limit could be an interesting clinical point as it could be used for intermittent infusion at low concentrations but allow clinicians to maintain an inhibitory effect over 6 hours. Possibly allowing the alleviation of toxic side effects. (Stidham *et al.,* 2011). The premise of intermittent infusion NLCs would need greater data to ascertain if it could be developed as a competitive treatment for existing continuous infusion of free vancomycin, this would come in the form of release assays and a narrower range of values used for time-kill investigations.

The VCM-NLCs hydrodynamic size produced in this project (80-200nm) would not be able to permeate bacterial cells through porins which are 3nm in diameter (Nikaido *et al.,* 2003) however, there are other mechanisms reported whereby the nanoparticles interact with bacterial cells (Rassouli 2018). The nanoparticles could either fuse with microbial cell wall or membrane and release the encapsulated drugs and serve as a drug depot to continuously release drug molecules. There would, however, be potential for mammalian cell permeation through various mechanisms like micropinocytosis and endocytosis. Rawal *et al.,* (2023) investigated into docetaxel loaded NLCs sharing a common excipient within this study (Phospholipon 90H). Generating a HDS of 188.41nm ± 2.13 and found after 7 hours had high mammalian lung cell uptake, showing particles with PS similar to that of this study (153.5nm) had high uptake into mammalian tissues.

Endocytosis is the uptake of molecules or metabolites into cells and tissues by the creation of membrane bound vesicles when the cell surface membrane engulfs the external material. The rate of endocytosis can be increased by the use of the ubiquitination network, the addition of surface markers and the use of lipids similar to that of the membrane such as phosphatidylcholine (found in Lipoid E80/S75 and Phospholipon 90H) (Conte & Sigismund, 2016) (Akopian, Kawashima and Medh, 2015). In the context of NLCs the lipidic nature of the phospholipids used on the outer layer allows easier access to mammalian cells by endocytic uptake by cells. A study by Mohanty & Sahoo (2010) found by the use of curcumin nanoparticulate formulations and fluorescent microscopy, 10-30µM of the formulation was taken up by endocytic routes in PANC-1 epithelial cells. Meaning by the hydrodynamic size generated and excipients chosen in this project (Lipoid E80/E75, Phospholipon 90H) would facilitate the VCM-NLCs produced to bypass mammalian cells and access the infection site by either cell pore permeation or endocytosis of cells.

S. aureus is a biofilm forming organism meaning it forms layers of bacteria within a biofilm matrix secreted by the bacteria which interact by quorum sensing (Archer et al., 2011). In the context of bacterial interactions NLCs would be used as drug delivery vehicles which would bypass mammalian barriers to access the site of action. Once NLCs had interacted with *S. aureus* biofilms VCM would slowly diffuse out of the liquid-lipid core and produce a sustained release of drug. This was shown *in vitro* by the time-kill assays completed extending VCMs biocidal action by two to three hours. *In vivo* NLCs would prevent blood protein binding and decrease elimination, potentially decreasing the dosage required for a therapeutic effect and alter the toxic side effects.

Recent research studies completed into vancomycin loaded nanoparticles aimed to change the pharmacokinetics or increase efficacy in non-ideal environments. Due to this, investigations produced nanoparticle MIC values similar to that of free vancomycin but improved other aspects. Kalhapure *et al.,* (2014) first investigated MIC values for excipients used in formulations, finding oleic acid and linoleic acid had a high MICs of 1250µg/ml and 625µg/ml against *S. aureus*. Establishing although

physiochemical characteristics were out of parameter range the NLCs were successful in the sense they caused a 2-log reduction in bacterial colony count after 5 days in samples taken from inoculated rabbit eyes. MIC investigations showed identical MICs (15.6µg/ml) in free VCM and VCM-SLNs after 18 hours, where VCM-Linoleic acid SLNs produced an MIC of 62.5µg/ml. However, when compared to a resistant MRSA strain a MIC of 15.6µg/ml was maintained over a 72-hour period where free VCM produced a lower MIC of 3.19µg/ml but only lasted 18 hours. The conjugation of linoleic acid to VCM therefore increased entrapment to similar of what was achieved in this study it produced MICs which were higher showing vancomycin efficacy was decreased in non-resistant strains. Conversely, the study by Osman (2019) showed a decreased MIC of 0.488µg/ml. Osman (2019) also investigated pH and this decreased MIC was achieved at a pH of six which is out of optimum growth parameters for S. aureus, meaning an inhibitory effect would be seen irrespective of antimicrobial concentration (Sharma et al., 2021). As no controls which grew *S. aureus* in isolation of antimicrobials at pH 6 were utilised this comparison couldn't be seen. Mhule *et al.,* 2018 also investigated the premise of vancomycin loaded SLNs and their effect on MRSA species. Observing a reduction in MIC value from 15.6µg/ml in free-VCM to 0.244µg/ml when VCM was encapsulated which was a lower MIC than achieved in this study. An acidic-responsive NMEO lipid was utilised with Mhule *et al.,* (2018) concluding this NMEO lipid was the cause for the increased efficacy. As in non-acidic environments the MIC of VCM and encapsulated VCM increased to 31.5µg/ml which was considerably increased when compared to this study.

4.1 **Future and other Current Research**

Future research for this study would primarily lie with visualising and documenting interactions with VCM-NLC and utilising various reference strains such as MRSA, *Enterococci* and Gram-negatives to see if an inhibitory effect could be stimulated due to NLCs lipidic properties. Fluorescent microscopy could provide visualisation to the interactions taking place between NLCs and bacteria. This experiment could consist of using logarithmic *S. aureus* exposed to double MIC VCM-NLCs and observe the morphology changes that occur within the test strain and observe activity of NLCs and how they

interact with bacterial cells. It would also provide insight into accumulation and cell wall disruption caused by vancomycin. However, as posed in the hypothesis the potential to increase VCMs aqueous stability is a vital component. Investigations could be completed to ascertain the lipase activity of *S. aureus* on NLCs and NLC excipients. A dissolution release assay could provide insightful data to the rate at which drug leaks from formulations, this data could then be used to corroborate the data collected from the time-kill assays. This would add confirmatory data towards the possible observed prolonged release. The application of colloidal studies into cryoprotectants could potentially increase stability over longer periods of time. Further enhancing the minor increase to VCM stability observed in this project. A study completed by Khan *et al.,* (2019) found that freeze drying did not significantly (p<0.05) change %EE for formulations when tested upon a range of cryoprotectants over 6 months. Hence, if this could be applied to VCM-NLC it would increase storage time whilst maintaining the benefits provided by NLCs. DSC and FTIR investigations highlighted the possibility of a drug-lipid interaction shown by the increased melting point and the presence of a C=C bond. X-ray diffraction analysis (XRD) is a tool which allows crystallographic analysis of materials, exposing physical and chemical structural properties (Sharma *et al*. 2021). XRD would allow confirmation of the state of different excipients used within the formulation. It would also allow further insight into the potential drug-lipid interactions.

Overall when considering other lipid nanoparticle formulations greater interest seems to be held in a lower particle size compared to the dispersity of particles. Due to NLCs primarily aiming to increase cell uptake whilst decreasing the rate at which drugs are released into cells, a smaller particle size could increase cell uptake by accommodating the mammalian cell pore size (Zhou *et al.,* 2009). However, a large PDI accompanied by a small PS would not equate to an emulsion of smaller particles overall as a larger dispersity of PS would be seen. Meaning, the particles on the lower part of the spectrum would be freely absorbed, but larger particles will struggle to endocytose. Meaning, drug will release externally and NLC benefits will be irrelevant. Hence, a balance between PDI and HDS would be vital to be found in any further optimisation completed. Researchers need to optimise for protection through biological barriers and for controlled targeted release. Aiding in this, the phospholipid layer

that forms the external section of the particles is highly receptive to targeting molecules such as glycolipids, antigens/antibodies and ligands that increase personalised medicine properties of the NLC structure. The liquid lipid core can also be enhanced to have further effect on the site of action with more synergistic molecules, these include fatty acids, solid lipids and siRNA.

When considering the occurrence of AMR susceptibility in lipase producing *S. aureus,* investigations would need to be completed as to the potential for *S. aureus* to develop enzymes that did not digest NLC excipients, potentially preventing the increased release of drug. In prokaryotic cells fatty acids are utilised to increase membrane fluidity, in maintenance of this carotenoids are produced to decrease fatty acid concentrations. Due to the antibacterial activity of oleic fatty acids being unknown researchers began investigations and found through increased exposure, bacterial generations began to increase carotenoid production to the specific fatty acids exposed to (Tiwari *et al.,* 2018), this same experiment could be utilised to investigate the possibility of resistance to fatty acid excipients. In future research a more expansive contact time could also be utilised in kill-time testing to ascertain the actual time to kill for all concentrations. This would also provide more information as to the inhibitory action seen by VCM-NLCs (0.2µg/ml) but not VCM in solution (Figure 3.19).

As a result of the blood protein binding causing increased elimination and nephrotoxicity, investigations into NLC stability and drug leakage in human plasma could provide interesting insight into the change in affinity for VCM when encapsulated. NLCs could aid in the blockage of blood protein binding, whilst simultaneously increasing absorption into tissues (Drug Bank, 2023). Considering this, the increased absorption could lead to potential problems with the development of AMR in species previously inaccessible by VCM. An example could be VCM gaining access to the blood brain barrier, while this would be beneficial to *S. aureus* infections in the brain it could also cause the development of resistances within the brain's commensal flora (Eleraky *et al.,* 2020).

As an alternative to NLCs Zhang *et al.,* (2019) found that pegylated liposomal nanoparticles containing penicillin eliminated 99.9998% of $10^{8.5}$ methicillin sensitive *S. aureus.* Experimentation was completed

primarily on A549 lung epithelial cells to investigate intracellular activity of the LNPs. This study could potentially be applied to vancomycin as the encapsulated antimicrobial, further compounding the conclusions met from the VCM-NLC utilised. As release concerns could be alleviated by LNPs destabilising within tissues but still maintaining the increased absorption. However, the possible prior observed lipase action of *S. aureus* species could cause disruptions to the charge specific release. This was not observed in this study most likely due to the excipients used.

Chapter 6: Conclusion

Vancomycin encapsulated nanostructured lipid carriers are a promising strategy to maintain efficacy whilst providing the potential to increase circulation time. As shown, the use of solubilisers in vancomycin entrapment is vital, however this must be balanced against surfactants and lipid content. Similar *S. aureus* inhibition was observed in vancomycin in solution compared against VCM-NLCs, meaning NLCs acted as delivery systems in susceptibility testing. Though a possible prolonged response was observed in time-kill assays, where NLCs were found to extend the biocidal response from two to four hours.

Blank NLCs were prepared by hot homogenization optimized and characterized for physiochemical properties using a Malvern Panalytical Zetasizer. HPLC was validated according to ICH guidelines Q2(R1) (1994) to assay vancomycin in solution and in VCM-NLC formulations. VCM-NLCs were optimized to maximize VCM entrapment efficiency to limit free drug. *S. aureus* reference strain ATCC 29213 was characterized and tested for susceptibility of VCM, VCM-NLCs and Blank-NLCs by MIC assay and time-kill assay. A growth curve was used to select the optimum challenge time for the time-kill assay which found VCM-NLCs extended the time to kill by 2 hours when treated with a concentration of higher than 0.8µg/ml.

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