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Chitosan-modified liposomes for delivery of membrane-active antimicrobials – exploring the role of the polymer

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CHITOSAN-MODIFIED LIPOSOMES FOR DELIVERY OF MEMBRANE-ACTIVE ANTIMICROBIAL – EXPLORING THE ROLE OF THE POLYMER

BY
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Abstract

Antimicrobial resistance in skin injury infections is becoming a rising problem as more conventional antimicrobials become ineffective in resistant infections. Therefore, developing novel effective antimicrobials while promoting wound healing is urgently needed. Membrane-active antimicrobials, such as antimicrobial peptides (AMPs), display promising antimicrobial activity, and could potentially solve parts of the antimicrobial resistance problems. Furthermore, the use of drug delivery systems, such as liposomes, could help overcome the challenges of AMP or other membrane-active antimicrobials, such as low stability, cytotoxicity and improve their activity. In addition, modification of liposomes with chitosan could provide appealing properties to the system, such as stability, anti-inflammatory activity, and antimicrobial activity.

In this study, chlorhexidine (CHX) was used as a model compound for AMPs and incorporated in plain liposomes, chitosomes and chitosan-coated liposomes. Liposomal formulations were characterised for their size, zeta potential, entrapment efficiency and *in vitro* drug release. Furthermore, chitosan and CHX were assessed for their antimicrobial and anti-inflammatory activity. The majority of chitosomes and chitosan-coated liposomes with CHX had a size of 167 ± 43 nm and 398 ± 39 nm, respectively. The average zeta potential of the formulations was 94.4 ± 2.2 mV for chitosomes with CHX and 83.3 ± 3.1 mV for chitosan-coated liposomes with CHX. The percentage of entrapment efficiency of chitosomes was superior to the liposomes without chitosan. Moreover, both chitosan formulations displayed a prolonged release of CHX. The antimicrobial activities of different formulations were evaluated on *S. aureus.* The results demonstrated a trend of higher antimicrobial activities from vesicles with CHX compared to the other formulations. The reduction of NO-production in LPS-induced murine macrophages was used to indicate the anti-inflammatory activity of formulations. The chitosan-coated liposomes displayed an inhibitory effect on NO production independent of CHX, which confirmed the anti-inflammatory activity of chitosan. The findings suggested chitosan formulations as a potential drug delivery system for membrane-active antimicrobials in wound therapy.

Keywords: antimicrobial peptides, chitosan, liposomes, membrane active antimicrobials, skin infections

Sammendrag

Antimikrobiell resistens ved infeksjoner i hudskader er et økende problem ettersom konvensjonelle antimikrobielle midler stadig blir mer ineffektive i behandling av resistente infeksjoner. Derfor er det et presserende behov for å utvikle nye effektive antimikrobielle midler som fremmer sårheling. Membranaktive antimikrobielle midler, som antimikrobielle peptider (AMP-er), viser lovende antimikrobiell aktivitet og kan potensielt løse deler av problemene knyttet til antimikrobiell resistens. Videre kan bruken av legemiddelleveringssystemer, som liposomer, bidra til å overvinne utfordringene knyttet til AMP-er og andre membranaktive antimikrobielle midler, som lav stabilitet, cytotoksisitet, og forbedre deres aktivitet. I tillegg kan modifikasjon av liposomer med kitosan gi fordelaktige egenskaper til systemet, som for eksempel økt stabilitet, antiinflammatorisk og antimikrobiell aktivitet.

I denne studien ble klorheksidin (CHX) brukt som en modellforbindelse for AMP-er og inkorporert i vanlige liposomer, kitosomer og kitosanbelagte liposomer. De liposomale formuleringer ble karakterisert for deres størrelse, zeta-potensiale, innfangningseffektivitet og *in vitro* legemiddelfrigjøring. Videre ble antimikrobielle og antiinflammatoriske aktiviteter av kitosan og CHX også undersøkt. Majoriteten av kitosomer og kitosanbelagte liposomer med CHX hadde en størrelse på henholdsvis 167 ± 43 nm og 398 ± 39 nm. Det gjennomsnittlige zeta-potensialet til formuleringene var 94,4 ± 2,2 mV for kitosomer med CHX og 83,3 ± 3,1 mV for kitosanbelagte liposomer med CHX. Prosentandelen av innfangningseffektivitet for kitosomer var overlegen liposomene uten kitosan. Dessuten viste begge kitosanformuleringene en forlenget frigjøring av CHX. De antimikrobielle aktivitetene til de ulike formuleringene ble evaluert i *S. aureus*. Resultatene viser en trend med høyere antimikrobiell aktivitet for liposomene med CHX sammenlignet med de andre formuleringene. Reduksjonen av NO-produksjon i LPS-induserte murine makrofager ble brukt for å indikere den antiinflammatoriske aktiviteten til formuleringene. De kitosanbelagte liposomene viste en hemmende effekt på NO-produksjon uavhengig av CHX, noe som bekreftet den antiinflammatoriske aktiviteten til kitosan. Funnene antydet at kitosanformuleringer potensielt kan fungere godt som et legemiddelleveringssystem for membranaktive antimikrobielle midler i sårbehandling.

Nøkkelord: antimikrobielle peptider, kitosan, liposomer, membranaktive antimikrobielle midler, hudinfeksjoner

List of abbreviations

AMP	Antimicrobial peptides
CHX	Chlorhexidine
CFU	Colony forming per unit
DD	Degree of deacetylation
DLS	Dynamic light scattering
EE	Entrapment efficiency
HMW	High molecular weight
IM	Inner membrane
LMW	Low molecular weight
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LUV	Large unilamellar vesicle
MLV	Multilamellar vesicle
MMW	Medium molecular weight
MSSA	Methicillin-susceptible *staphylococcus aureus*
MW	Molecular weight
MWCO	Molecular weight cut-off
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic
NO	Nitric oxide
OM	Outer membrane
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PE	Phosphatidylethanolamine
PG	Phosphatidyl glycol
PI	Polydispersity index
SC	Stratum corneum
SD	Standard deviation
TA	Teichoic acid
TEM	Transmission electron microscopy

1. Introduction

1.1 skin

Skin is the largest organ of the human body and act as the first line of protection from the environment. It plays a vital role in maintaining homeostasis, preventing water loss, protect the internal organ from mechanical injuries, microorganisms, and radiation present in the environment (6).

1.1.1 Skin structure

Skin is composed of many distinct layers, including main layers; epidermis, dermis, and hypodermis (Figure 1) (7). The epidermis is a continually renewing epithelium, divided into several layers. The stratum corneum (SC) is the outermost layer of the epidermis, followed by granular layers (Stratum granulosum), spinous layer (Stratum spinosum), and basal layer (Stratum Basale), the innermost layer just above the dermis (6).

The stratum corneum is an outermost layer of the epidermis and has a thickness of 10-15 µm (7). The stratum corneum has simple two compartments structural organization, often described with brick-and-mortar structure, where the corneocytes (brick) are embedded in a lipid matrix (mortar). The corneocytes can be up to 18-20 layers, depending on location in the body. The major lipid components of the lipid matrix are cholesterol, fatty acids, and ceramide. The skin protection is provided mainly by the outermost layer, the SC. Corneocytes in the SC provide physical and permeability barriers, while lipid matrices are essential for counteracting water loss and penetration of water-soluble substances into the skin. These components in SC provide barriers for the skin, and deficiencies in any of these result in barrier abnormalities (8).

The dermis is the layer underneath the epidermis and composed of collagen and elastic fibres (elastin), which provide supple, compact, and good mechanical support to the skin. The dermis contains blood vessels (capillaries), which supply nourishment to the epidermis, nerve endings, hair follicles, sebaceous glands, and sweat glands (6). Hair follicles and sweat glands have openings on the skin surface and open directly into the environment at the surface. This also provides a route of skin permeation for external substances (7;9).

The hypodermis or subcutaneous fat is the innermost layer of skin which providing mechanical support for skin structure. This layer act like s bridge between underlying the bones and muscles and the dermis layer of the skin (10).

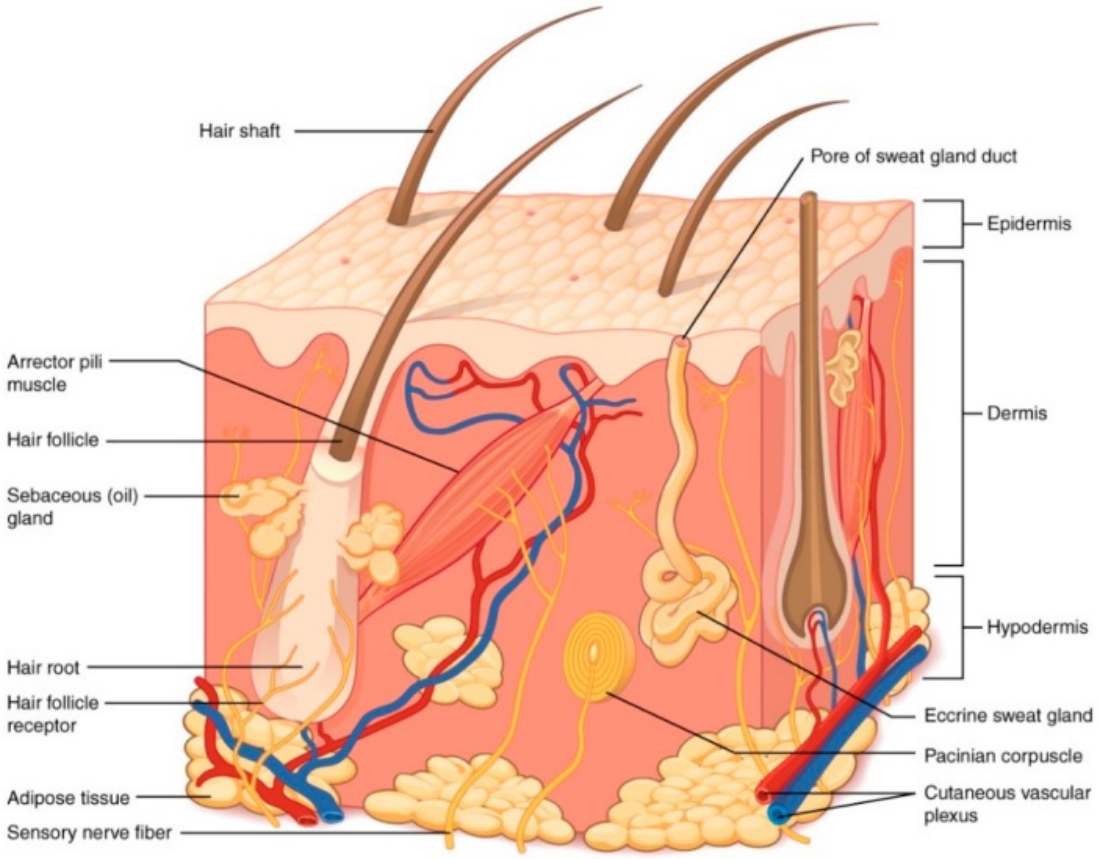

Figure 1 Skin structure. The main layers are epidermis, dermis, and hypodermis (11).

1.2 Wound healing

Wound healing is an essential process for the restoration of the function of skin and tissue after injury (12). The healing process is divided into 4 phases: coagulation and haemostasis phase, inflammation, proliferation, and remodelling phase. Immediately after injury, coagulation and haemostasis phases take place to stop bleeding. This phase helps seal wounds and limit blood loss. During the first phase, several factors and cytokines are released, attract inflammatory cells to the wound site, and start the inflammatory phase. Inflammation phases involve phagocytosis to remove foreign particles, bacteria, and damaged tissue from the wound site. Bacterial removal is essential because wounds with a bacterial imbalance will not heal. However, the excessive activity of inflammatory cells in the wound bed may cause more harm and hamper wound healing. This is because the inflammatory cells, such as neutrophils, secrete numerous toxic mediators to destroy bacteria, but they are also harmful to surrounding tissue.

After the inflammatory phase resolves, the wound shifts to the proliferation phase of tissue repair. The proliferation phase involves the formation of new blood vessels (angiogenesis) and the formation of granulation tissue and then allows the re-epithelization to take place. The keratinocytes migrate into the wound area and cover it with a new epidermis. Lastly, the remodelling phase takes place several weeks after wounding and may last up to 1 or 2 years. This phase is responsible for scar tissue formation (13).

1.3 Bacteria

1.3.1 Bacteria structure

Bacterial cells live in a harsh environment, and to survive, they must develop extra protection that shields them from these environmental factors, a cell wall. In 1884, Christian Gram discovered a Gram staining technique, a method to classify bacteria into two large groups based on their cell envelope difference, gram-positive and gram-negative bacteria. Their difference lies mainly in their outer membrane structure, as gram-negative bacteria have an outer membrane (OM) while gram-positive bacteria lack this organelle (14).

Gram-negative bacterial envelopes are composed of 3 layers, the OM, the peptidoglycan layer, and the inner membrane (IM). There is an aqueous compartment periplasm between OM and IM. The OM is essential for bacteria to survive because it provides a protective barrier and has additional stabilising properties.

The OM is composed of phospholipid in the inner leaflet of the OM, and the lipopolysaccharide (LPS) as the outer leaflet. The LPS possesses a negative charge attributed to the phosphate groups present on the LPS. The barrier for hydrophobic molecules of the bacterial membrane is contributed to LPS, and membrane proteins, such as porins, act as good barriers for hydrophilic molecules. These make OM a very effective barrier (14).

A gram-positive bacterial envelope is composed of 2 layers, the peptidoglycan layer and the IM. Peptidoglycan is found in both the gram-negative and gram-positive bacteria; however, they differ in thickness as the peptidoglycan layer of gram-negative bacteria is thinner. Peptidoglycan is composed of repeating N-acetyl glucosamine (NAG) and N-acetyl muramic (NAM) units. Furthermore, peptidoglycan is essential for bacteria, especially the gram-positive bacteria, as they do not have the OM; due to this, it is their only protective barrier. Disruption of the peptidoglycan layer will cause cell lysis because of build-up pressure in the cell (14).

The peptidoglycan layer of gram-positive bacteria contains two types of anionic polymers, teichoic acids (TA) and lipoteichoic acids (LTA). The TA are attached to peptidoglycan, while LTA are threaded through the layer and anchored to the head group of cytoplasmic membrane lipids (14).

The IM or cytoplasmic membrane of gram-positive and gram-negative bacteria is composed of phospholipid bilayer like human cells. Still, unlike eukaryotic cells, the phospholipid composition in bacteria IM is mainly anionic phospholipids, while the eukaryotic cell

membrane is primarily composed of zwitterionic phospholipids as phosphatidylcholine (PC). The IM of bacteria is composed of the zwitterionic lipid phosphatidylethanolamine (PE), and anionic lipids phosphatidyl glycol (PG) and cardiolipin. The proportion of PE, PG, and cardiolipin varies among gram-positive and gram-negative bacteria. Due to higher proportion of anionic lipid in IM of bacteria, the bacterial membrane is more negatively charged than human cell membrane (14;15).

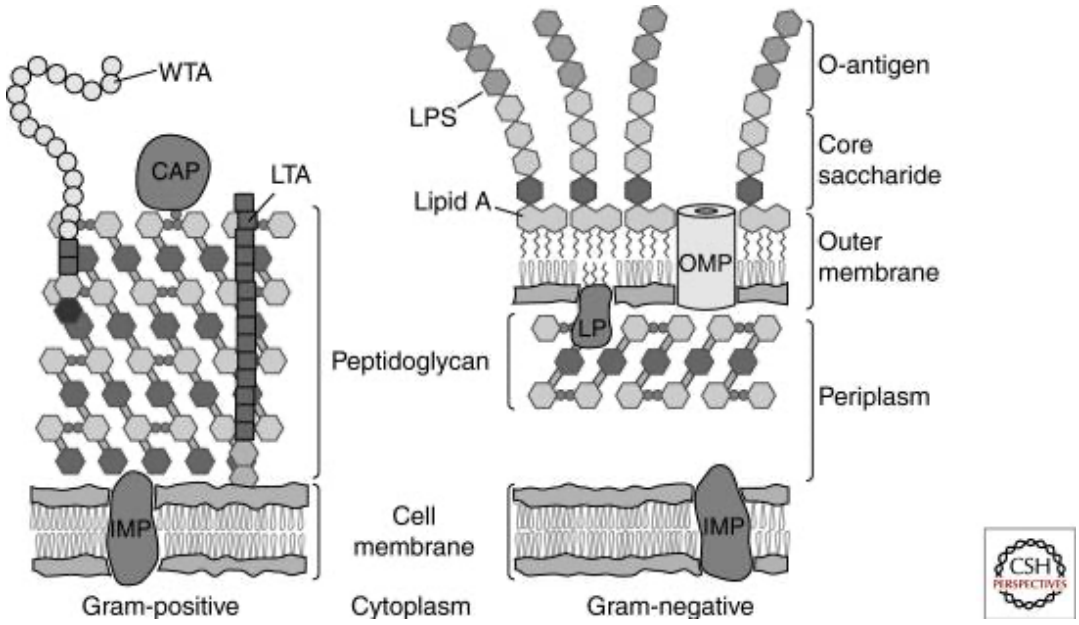

Figure 2 Structure of gram-positive and gram-negative cell membrane. Gram-positive cell membrane composed of 2 layers, thick peptidoglycan layers with teichoic acids (TA) and lipoteichoic acid (LTA) and the IM, Gram-negative cell membrane are composted of 3 layers, LPS as the outer layer, peptidoglycan and the IM (14)

1.3.2 Skin/wound infection

Skin is home to numerous microorganisms that compose the skin *microbiota,* and they play essential role in skin protection. Commensal microorganisms found on the skin are bacteria, fungi, and viruses. Microbial communities vary depending on the physiology of skin site and individual-specific characteristics. The most common microbes found on the skin are *Staphylococcus, Corynebacterium,* and *Propionibacterium* (16).
Staphylococcus epidermidis and *Propionibacterium acnes* are the most common microbes found across body sites. They provide some benefits as to prevent the colonization of pathogens by competing for space and nutrients. Some commensal microbes can also secret antimicrobial compounds that inhibit the growth of pathogens (16).

Various factors can affect balance and alter the composition of skin microorganism communities and the skin barrier function. Disruption in the skin barrier, as in a wound, might change the diversity and abundance of microbes as the wound can provide favourable environment for bacterial colonization (11).
Infection occurs when virulence factors of microorganisms found in a wound outcompete the host immune system, followed by invasion and spreading of microorganisms that induce various host responses (17). Bacteria commonly found in skin infection are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococci*, including *S. epidermidis* and *Staphylococcus lugdunensis* (9;18). *S. aureus* and *P. aeruginosa* are the most frequent pathogens isolated from chronic wounds. Furthermore, *S. aureus* has also been reported in many acute wound infections such as burn wound infections, bite wound infections, and acute soft tissue infections (17). *S. aureus* can secret toxins and virulent factors causing prolonged inflammation and delayed wound healing (9). *S. epidermidis* does not have aggressive virulence factors as *S. aureus* but the primary virulence factor of *S. epidermidis* is the ability to form a biofilm (19).
A chronic wound infection is one of the infectious diseases which is often associated to staphylococcal biofilms. The biofilm-producing pathogens, such as *S. aureus* and *S. epidermidis*, are well-known for causing chronic infection due to its ability to resist antimicrobial treatment (20). It is estimated that bacteria in biofilms are involved in more than 60% of all chronic wound infections (21)

1.3.3 Biofilm

Biofilms are described as a microbial community that attach to biotic or abiotic surfaces such as wound surfaces, bones, lung tissues, implants, or various medical devices. The biofilm is composed of single or multi-species microbial communities which are embedded in a biofilm matrix. The matrix of biofilm consists primary of polysaccharides, protein and extracellular DNA. Theses extracellular polymeric substances which build the biofilm matrix, are produced by the bacteria to protect them self from harmful environmental factors (20). Biofilms do not only protect bacteria from hostile environment such as alter pH, osmolarity or mechanical force but also protect microbial communities from antimicrobials and host immune cells (22). Bacterial biofilm infections are serious health concerns due to biofilms ability to increase resistance against antimicrobials and host defence systems. They can be up to 1000 times more tolerant to antimicrobials than their corresponding planktonic cells, and increased concentrations of antimicrobials are often needed to kill these biofilm-embedded bacteria (20). Various explanations have been proposed for the tolerance of biofilm-embedded bacteria to antimicrobials. One of them was the ability to reduce antimicrobial penetration into biofilm, attributed to the physical barrier provided by the biofilm extracellular matrix. In addition, the biofilm-embedded bacteria can reduce their metabolic activity due to restrict in nutrient and oxygen in the biofilm, especially in the inner parts of the matrix. As the majority of conventional antimicrobials are effective against metabolically active bacteria, targeting activities, such as DNA replication and protein synthesis, biofilms limit the effectiveness of these compounds and may promote antibiotic resistance (11). Furthermore, due to the heterogeneity of bacterial cells in biofilms, transference of resistance gene is more effective in biofilm matrices (20).

1.4 Membrane-active antimicrobials

Membrane-active antimicrobials is a class of compound which exhibit an ability to interact with bacterial membranes and disrupt their function and physical integrity. Due to their non-specific mechanism of action on bacterial membrane, membrane-active antimicrobials are less susceptible to resistance, making them an attractive approach to solve the current antimicrobial resistance challenge (15;23).

1.4.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are membrane-active antimicrobials and promising compounds to combat bacterial infections. The AMPs are small peptides known for a decade as part of the immunity of many living organisms: bacteria, insects, plants, and animal (24). Unlike conventional antimicrobials that target the biosynthesis process, such as protein and RNA synthesis, AMPs interact with bacterial membranes causing membrane disruption (15).

This study aimed to develop chitosan modified liposomes as a drug delivery system for 8b, a novel antimicrobial discovered at UiT. The 8b is a peptidomimetic compound generated from existing peptides that mimic the characteristics and biological activity of an AMP (25). This study uses chlorhexidine (CHX) as a model compound for 8b.

1.4.1.1 Characteristic and structure

The AMPs are short-chain peptides comprised of a varying number of amino acids. They are broad-spectrum antimicrobials and divided into many subgroups based on their amino acid composition, structure, and other properties (26). Based on their secondary structure, most AMPs can be characterized as one of the following three types: i) α-helix, ii) β-sheet, and iii) extended structure (27). The α-helix peptides are most common in nature. Figure 3a shows an example of α-helical AMP structure (27;28). β-sheet AMPs in Figure 3b, are composed of at least two β-strands bound to each other with a disulphide bond (29). One of the β-sheet AMPs subgroups, is shown to have an important role in structural stability and degradation of AMPs as the bond helps minimize protease degradation and stabilizes the structure. Most extended AMPs have characteristics of consisting proline residues which prevent secondary α-helical or β-sheet structure. (Figure 3c) (27).

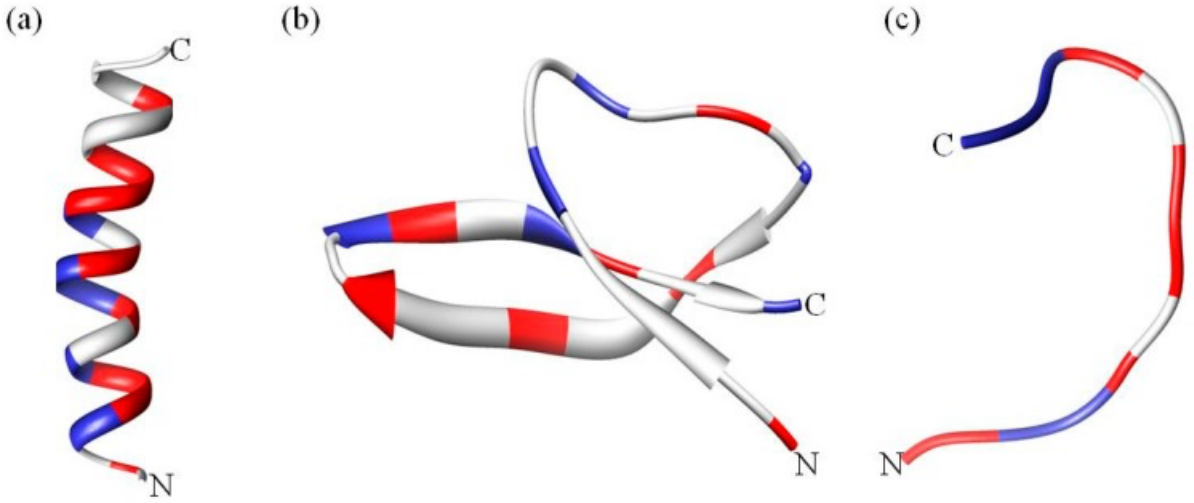

Figure 3 Three types of secondary structure of AMPs a) Alpha-helical b) Beta-sheets and c) extended. The common features which most of AMPs possess are the positive net charge and the hydrophobicity. The positive charged are colored blue and hydrophobic parts colored red. These parts are essential for AMPs' interaction with bacterial membranes (4) .

Although AMPs are varied in their sequence, structure, and source, there are several features almost all AMPs shares. Most AMPs possess a positive net charge, a cationic character (9;27). This property of AMPs is important, involving electrostatic interaction of AMPs with the anionic portions of the bacterial membrane. The cationic sections can selectively interact with anionic molecules, which contribute to AMPs' selectivity toward bacterial membrane that are more negatively charged (anionic) than human cell membrane (zwitterionic) (15;30). Hydrophobicity of AMPs is essential to drive the AMP molecules into the hydrophobic region of the membrane (30). This characteristic enables AMPs to partition the membrane lipid bilayer (4). Nevertheless, high hydrophobicity and high positive charge can lead to toxicity in human cells (30). The last common feature of AMPs is amphiphilicity. Amphiphaticity refers to a feature in which AMPs consist of both hydrophobic and hydrophilic amino acid residues (4), (27). This feature ensures electrostatic interaction of AMPs with bacterial membrane head group and ensures the insertion of AMPs into membrane interior (4;27;29).

1.4.1.2 Mechanism of action

It was initially thought that membrane targeting was the only mechanism of AMPs, but there is increasing evidence that AMPs display other modes of action (27). The mechanisms of AMPs can be divided into two main classes: membrane disruptive mechanisms and non-membrane disruptive mechanisms with intracellular targets (9;27;29).

a) Membrane disruptive

For AMPs to exert their activity on bacteria, AMPs must first attach to the bacterial cell membrane, which is enabled by their cationic and hydrophobic properties. Bacterial membranes are anionic due to their lipid bilayer composition, so the electrostatic interaction between the cationic part of AMPs and the anionic part of the membrane is essential. After attachment of AMPs, three models of bacterial membrane disruption have been proposed to describe the membrane disruptive action of AMPs: barrel-stave model, toroidal model, and carpet model (Figure 4, 5 and 6, respectively). The first two models are transmembrane pores formation, while the last model works slightly differently (4).

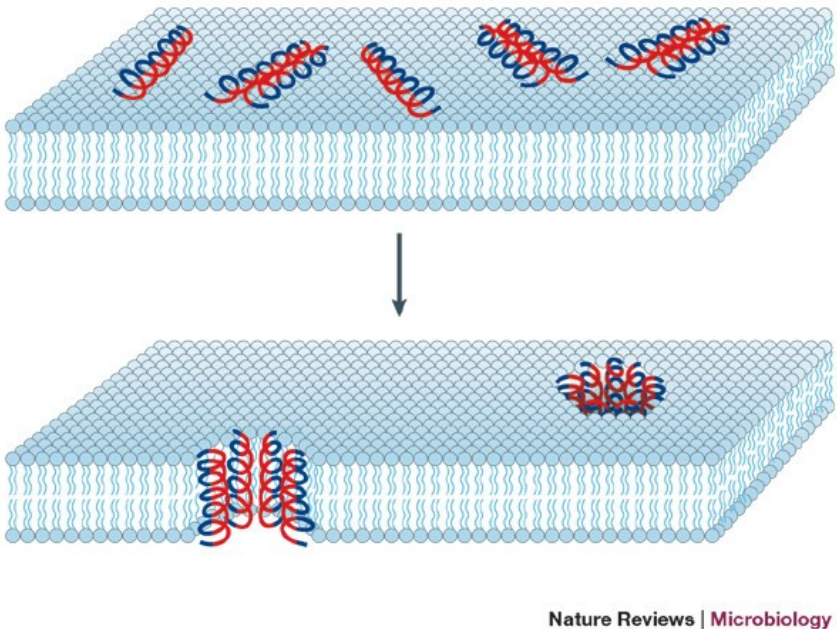

Figure 4 Barrel-stave model for transmembrane pores formation of AMPs. Peptides are oriented parallel to the membrane at low concentration and start to insert into the membrane when the concentration increase. Hydrophobic regions of AMPs interact with hydrophobic regions of the membrane, and hydrophilic parts form a hydrophilic channel. Hydrophilic regions in red and hydrophobic regions in blue (4).

In the barrel-stave model (Figure 4), peptides are bound and oriented parallel to the membrane at low concentrations. When the concentration increases, the peptides orientate and insert perpendicularly into the membrane bilayer. After insertion, peptides form a bundle with a central lumen, a transmembrane pore. In this model, amphipathic structure of AMPs is essential in pore formation as the hydrophobic region of AMPs interacts with the hydrophobic part of the bilayer, and the hydrophilic region of peptides faces inward and forms a hydrophilic pore (4;27).

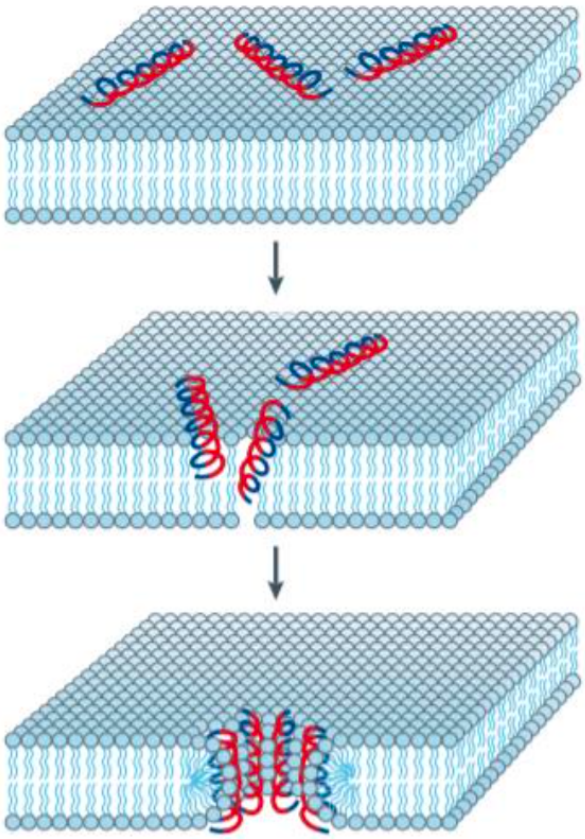

Figure 5 Toroidal model for transmembrane pores formation of AMPs, peptides inserted and induced the bend of lipids leads to hydrophilic pores, hydrophilic regions in red and hydrophobic regions in blue (4)

In the toroidal model, the peptides are also inserted perpendicularly into the membrane bilayer and induce the bend of lipid monolayers, as shown in Figure 5. The bending of the lipid monolayer leads to a transmembrane pore that the hydrophilic core is formed by both the AMPs and the lipid head groups (4).

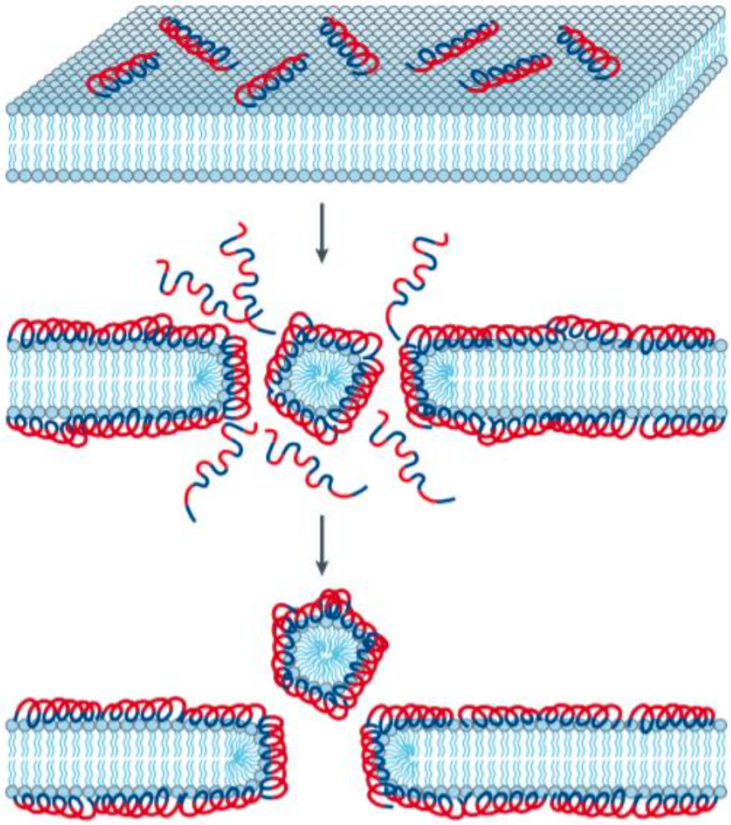

Figure 6 Carpet model, peptides are oriented parallel to the bilayer surface of the bacterial membrane. At high concentrations, peptides form a carpet. When a critical threshold concentration of peptides is achieved, membranes are disrupted in a detergent-like manner by forming micelles. The formation of micelles leads to membrane disintegration (4)

In the carpet model, peptides are accumulated on and oriented parallel to the bilayer surface of the bacterial membrane. At high concentrations, peptides can cover the surface of the membrane and form a "carpet". When a critical threshold concentration of peptides is achieved, this carpet-like manner leads to loss of membrane integrity, and membranes are eventually disrupted in a detergent-like manner by forming micelles. The formation of micelles leaves

holes behind, allowing more peptides to access the membrane, and finally, the membrane disintegrates, as shown in Figure 6 (4;27).

b) non-membrane disruptive

Membrane disruption was thought of as the primary mechanism of AMPs in the early studies, but there is increasing evidence indicating that AMPs also have other modes of action. Several studies discovered that many AMPs could translocate across the cell membrane. Once in the cytoplasm, they can inhibit bacteria's cellular processes, such as inhibiting nucleic acid synthesis, inhibiting protein synthesis or its activity, or inhibiting enzymatic activity (4;27;29). Not only direct killing, but AMPs can also modulate immune responses. The mechanism is highly complex, but some AMPs can trigger immune responses, such as attraction and activation of immune cells or regulating inflammation (27;31).

1.4.2 Chlorhexidine

Chlorhexidine (CHX) is a broad-spectrum antimicrobial compound active against gram-negative and gram-positive bacteria. It has been widely used in the healthcare sector for disinfection of skin before surgery and in impregnated medical devices. It is also commonly used in topical antimicrobial products, such as wound dressings, and oral antiseptics, such as mouthwashes (32;33). CHX (Figure 7) is a cationic biguanide exist as gluconate, acetate and hydrochloride salts. It acts as a strong base and protonate at physiological pH (34).

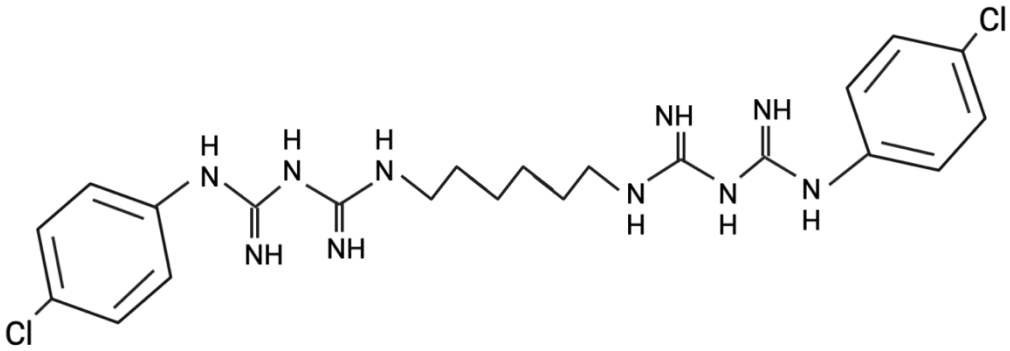

Figure 7 Structure of chlorhexidine (CHX)

The antimicrobial activity of CHX is attributed to the cationic biguanide groups of CHX which able to bind to the negatively charged bacterial cell wall (34). The interaction of cationic CHX with the negatively charged bacterial membrane disrupts membrane integrity, allowing more CHX to attract to the inner membrane. With the inner membrane, CHX form bridges between adjacent phospholipid head groups, interrupt binding of membrane and the divalent cations such as Mg^{2+} and Ca^{2+}. This result in increased permeability of the cell membrane and causes potassium ion and proton to leak out (34;35). At higher concentration, the interaction is more severe, causing membrane integrity to be disturbed which result in leakage of cellular material and eventually cell death (34;36)

CHX was used as a model for 8b as it exhibits similar mechanism of action. They also possess similar properties such as cationic character and hydrophobic regions. It was postulated that CHX would behave in the same way as 8b during the optimization of the delivery system.

1.5 Nanocarriers

Nanoparticles as drug delivery systems have gained increasing recognition due to their beneficial properties. Taking advantage of their nano size and different material and structure, they can help drugs overcome certain hindrances such as poor solubility, drug degradation in the body, and modification can provide sustained release (37).

1.5.1 Liposomes

Liposomes are vesicles composed of phospholipids, which are self-enclosed to form spheres of lipid bilayers and have an aqueous core within the bilayers. Phospholipids have a hydrophilic head group and two long hydrophobic tails. In an aqueous medium, phospholipids self-assemble into bilayers with hydrophobic tails facing toward each other and hydrophilic head groups facing the aqueous core and aqueous surroundings (Figure 8). Because of this amphiphilic property, liposomes can incorporate both hydrophilic and lipophilic compounds. Hydrophilic compounds dissolve in the aqueous core, and lipophilic compounds are incorporated in the lipid bilayer (38). Liposomes can be formed by natural or synthetic phospholipids, such as PC and PE. Furthermore, they can possess different characteristics, such as charge or bilayer rigidity, depending on their composition.

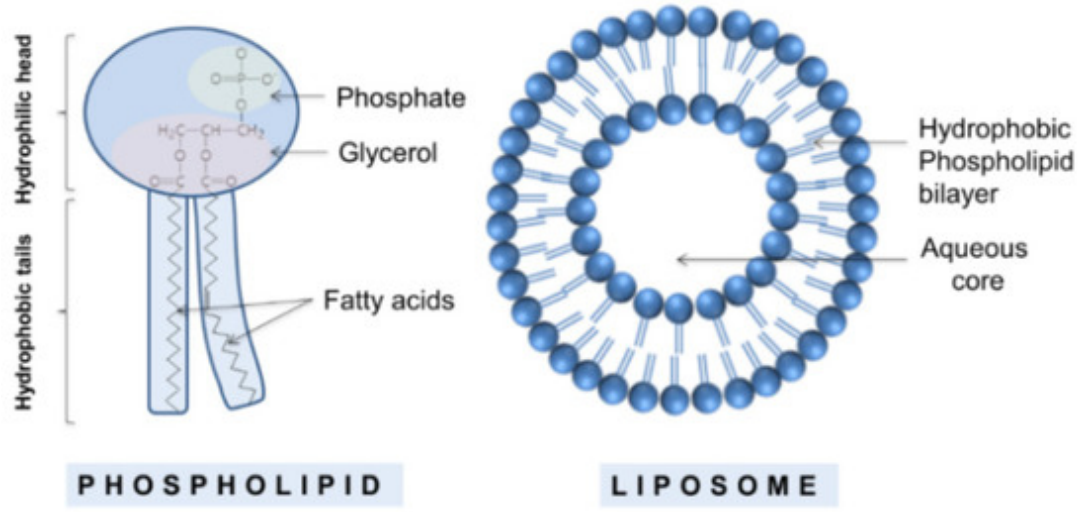

Figure 8 Structure of small unilamellar vesicle (SUV) or liposomes and phospholipid (2).

1.5.2 Characteristic of liposomes

1.5.2.1 Size

Liposomes are most frequently classified according to size and lamellarity. The size is categorized into small, large, and giant vesicles. Based on lamellarity, they can be classified into two categories: more bilayers present, multilamellar vesicles (MLV), or single bilayer, unilamellar vesicles (ULV). Unilamellar vesicles can be classified into small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) (Figure 9).

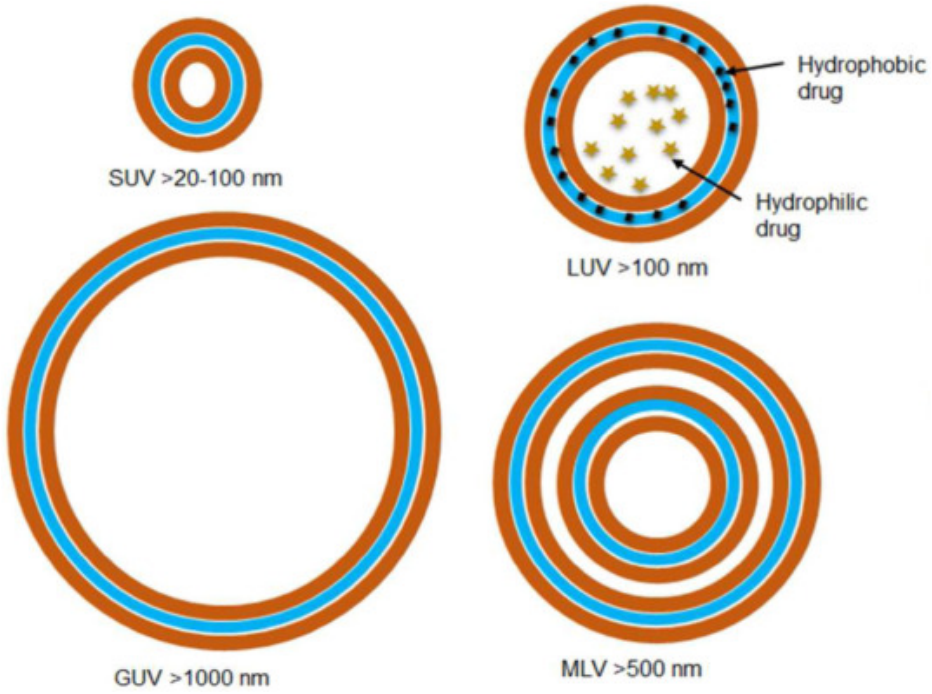

Figure 9 Overview of liposomes size and lamellar classification (39), (permission requested)

The SUVs have a size range of 20-100 nm, LUV larger than 100 nm, and MLV larger than 500 nm (40). The size of liposomes is considered a critical factor affecting encapsulation efficiency, stability, drug release profile, and cellular uptake of liposomes (41). Due to this, size of liposomes is an important parameter to be considered when preparing formulation for any route of administration. For drug delivery into the skin, vesicles of 600 nm or larger showed not to penetrate deeper layers of skin and would stay on the stratum corneum. Vesicles of 300 nm or lower can deliver drugs to some extent into the deeper layers of the skin, while vesicles of 70 nm or lower show to be superior in delivering drugs in the deeper layers (42). Stratum corneum, as mentioned earlier, is a barrier for external substance; in its absence, absorption of drugs into the skin increases (42). This project aims to develop formulations used in wound and skin

infections. Based on our aim, the appropriate size of our liposomes lays between 250-300 nm, which can deliver the drug to some extent into the skin and at the same time limit the absorption into the systemic circulation.

1.5.2.2 Charge

Liposomes can be classified into three categories based on their surface charge. Depending on their composition, they can have positive (cationic), negative (anionic), or neutral surface charges. The surface charge properties of liposomes contribute to their biological behaviour, such as stability, the interaction of liposomes with another surface, and skin penetration of liposomes (43). The surface charge of liposomes can be evaluated by measuring zeta potential.

1.5.2.3 Zeta potential

Zeta potential gives an indication of the stability of nanoparticle systems. It is a measure of the charge on the surface of liposomes in an aqueous phase. The liquid layer surrounding the liposomes exist as two parts: the inner part called the stern layer and the outer part called diffuse layer (Figure 10). The stern layer and the diffuse layer are part of an electric double layer which exist on the surface of particles. The stern layer consists of ions with opposite charge to that of the particle. The diffuse layer consists of both same and opposite charged ions which grow beyond the stern layer. When applied an electric field to the dispersion, the particles will move toward the electrode of the opposite charge. Within the diffuse layer there is a boundary calls the slipping plane which acts as the interface between the moving particle and the surrounding liquid. The potential at the slipping plane is known as the zeta potential (3).

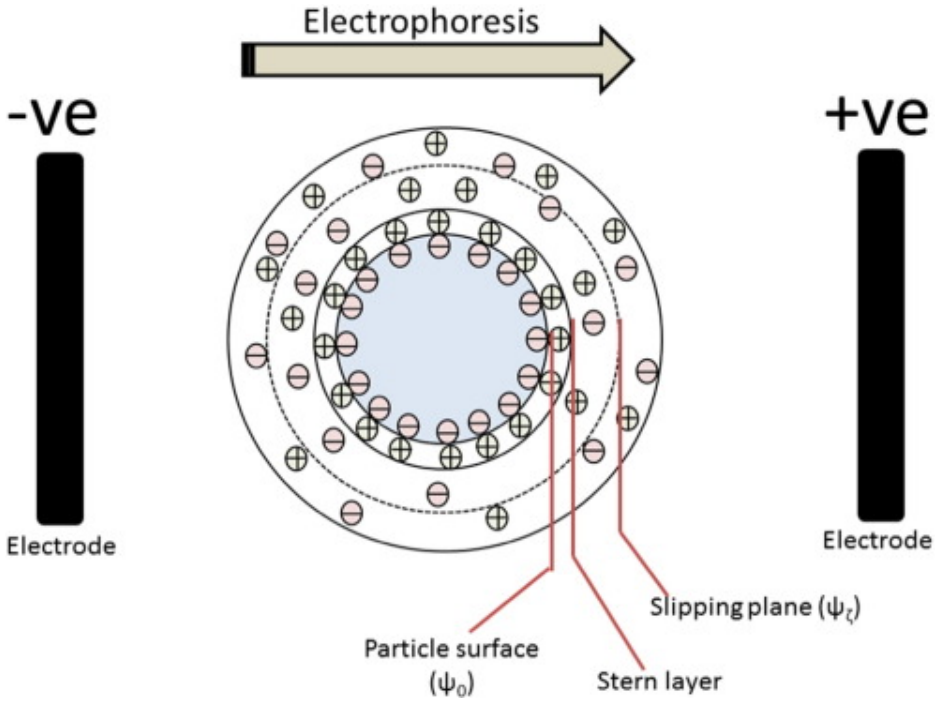

Figure 10 Illustration show the electric double layer surround particle in aqueous phase (3), (permission requested)

Liposomes that are uncharged or have a low surface charge tend to aggregate, while liposomes with higher zeta potential, either negative or positive, are less likely to aggregate due to repulsive forces. It was reported that zeta potential outside the range of +30 mV and -30 mV indicates good stability (44). In this project, we are tailoring liposomes with positive zeta potential, as this could be beneficial for interaction between the liposomes and bacterial cell membranes. Moreover, this could potentially also assure improved stability.

1.5.3 Advantages of liposomes as an antimicrobial carrier

Liposomes have proved to be good candidates for the delivery of antimicrobials. Antimicrobials encapsulated in liposomes have demonstrated increased accumulation at the infection site, reduced toxicity, and improved protection of the antimicrobials from degradation in the body. These properties of liposomes may potentially improve the efficacy of antimicrobials and help overcome issues around antimicrobial resistance (45).

Liposomes are vesicles with amphiphilic property allowing them to incorporate both hydrophilic and lipophilic compounds (38). This advantage gives them the potential to carrier

a diversity of drugs or multiple drugs within the same formulation. Furthermore, they offer several appealing features, such as being biocompatible, biodegradable and low toxicity, as they are composed of phospholipids that are similar to those present in the human cell membrane. This means they are generally safe to use on skin (45). Depending on the composition, liposomes can be designed to release the incorporated drug in a controlled manner, giving them the possibility to achieve a sustained release or controlled release system (44). Sustained release is advantageous for wound treatment because it reduces the requirement for medication application and dressing change as well as bacterial regrowth.
Moreover, liposomes can be modified with polymer to attain positive charged on the surface and that could improve delivery of drugs to the infection sites since liposomes with positively charged surface can target the negatively charged bacteria by electrostatic interactions (45).

1.6 Polymers

Many natural or synthetic polymers are frequently used as excipients in medical applications intended for the antimicrobial therapy (46). Some of them can help stabilize liposomes or possess inherent antimicrobial activity (39). Despite all advantages liposomes, conventional liposomes often display low stability, leading to rapid leakage of the drug before reaching the target which limited the use of liposomes. Several studies have proposed surface modification of liposomes with biopolymer to overcome these limitations of the liposomes (39).

1.6.1 Chitosan

Chitosan is a natural-derived cationic polysaccharide that has a wide range of applications in biomedical purposes due to its biocompatibility and biodegradable (1).
Chitosan is obtained from the deacetylation of chitin (Figure 11), which is found naturally in the crustacean shells such as shrimp or crabs, insect exoskeletons, and fungi (1;47). The polymer is composed of (1→ 4) -2- amino -2- deoxy-β-D-glucan, which can be partial- to entirely -deacetylated form of chitin.

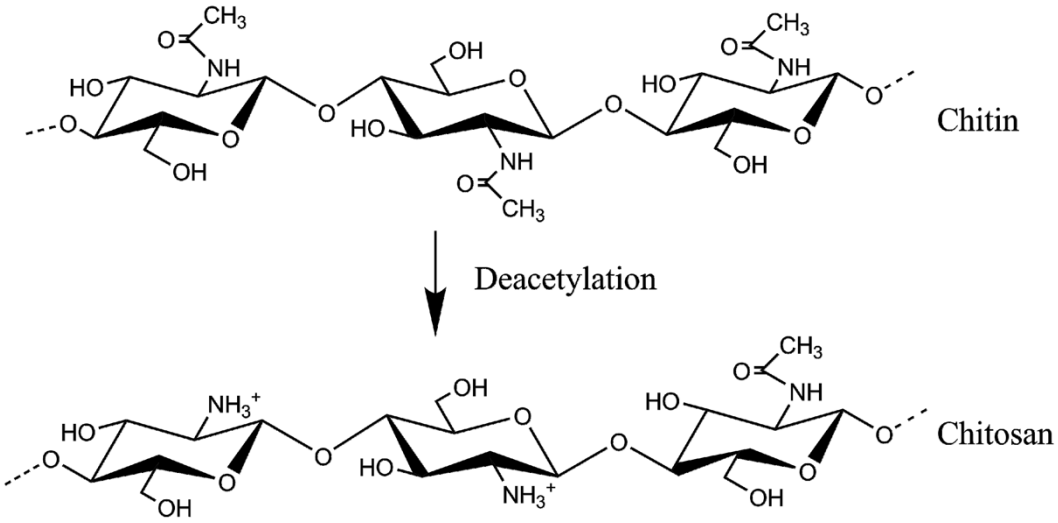

Figure 11 Structure of chitosan and chitin (5)

The amine group in chitosan can protonate under acidic conditions (pH lower than its pKa value of 6.3), giving positive charge to the molecule and is an essential factor that affects the physical properties of chitosan (Figure 12). The protonation of amine makes chitosan soluble in aqueous acidic solution (47).

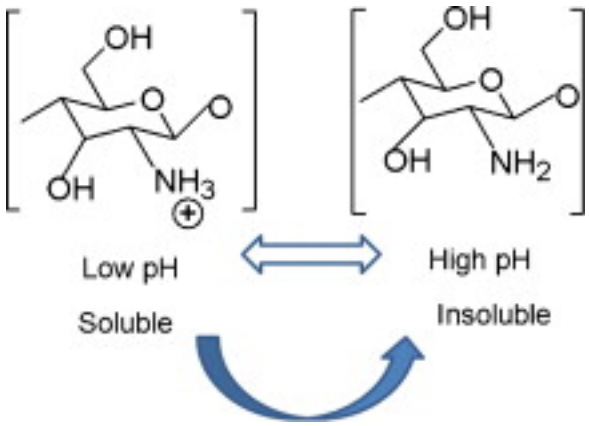

Figure 12 Structure of chitosan protonated at low pH and deprotonated in high pH (1)

Chitosan can be classified in to different group based on degree of deacetylation (DD) and molecular weight (Mw) (1). Chitosan is often classified into three grades depending on their Mw, high, medium, and low Mw. However, the range of each grade is not well defined. High grades are characterized by high molecular weight (HMW), usually referring to 190 -375 kDa,

medium molecular weight (MMW) is often including a range between 190-310 kDa, and low grades are characterized by low molecular weight (LMW) often between 20 -190 kDa (47).

1.6.1.1 Application

Chitosan has many beneficial properties in medical applications, such as biocompatibility, biodegradability, and low toxicity (46;47). The polymer exhibits antimicrobial, antifungal and anti-inflammatory activity, antioxidative effects, bioadhesive properties, and promotes wound healing (46;48). These properties make chitosan an interesting and attractive excipient in drug delivery systems intended to deliver antimicrobials for wound treatment (46). In addition, the use of chitosan in drug delivery systems has demonstrated to enable prolonged release of antimicrobials and thus prolonged the antimicrobial activity (49).
There are various applications of chitosan in pharmaceuticals; they can be used as the carrier of the drug, coating material, and excipients (40).

1.6.1.1.1 Antimicrobial activity: mechanism of action

One of the most attractive properties of chitosan in antimicrobial therapy is its inherent antimicrobial properties. The mechanism of the antimicrobial properties of chitosan is not clear. However, four main mechanisms are most known and accepted:

i) Disruption of cell membrane due to electrostatic interaction: Electrostatic interaction of chitosan and cell membrane alters the cell membrane permeability and leads to bacterial cell lysis. This interaction occurs due to the interaction between the positively charged chitosan and negatively charged LPS or teichoic acids on the bacterial cell membrane (46;47).

ii) Interaction with DNA: chitosan has shown to interact with the DNA of bacteria. The interaction is mainly observed in the case of LMW chitosan, and affects the protein synthesis of bacteria and hinders protein synthesis, which will cause bacterial cell death (46).

iii) Formation of an envelope on the bacterial surface: HMW chitosan can deposit on the bacterial membrane and form an envelope on the surface of the bacterial cell. The envelope prevents bacterial cell uptake of nutrients and excretion of metabolic products, leading to cell death (46;47).

iv) Chelation of nutrients: Both gram-negative and gram-positive bacteria attract divalent metal cations (Mg^{2+}, Ca^{2+}), which are important to its enzymatic functions and integrity of membranes. Divalent cations will bind to the negatively charged part of the bacterial cell membrane, LPS in gram-negative and teichoic acids in gram-positive. Chitosan possesses chelating properties and competes with divalent cations in binding with negatively charged bacteria. The lack of these metal cations makes bacteria weak and cell walls more permeable (47)

In this project, the cationic character and antimicrobial activity of chitosan are of interest. The aim was to couple the antimicrobial activity of chitosan and the membrane-active antimicrobial to achieve a synergic antimicrobial effect.

1.7 Conventional strategies for the treatment of skin infections

Treatment of skin infections is based on the grade of severity of the condition. For superficial infection, burn damage and wound infection with mild to moderate grade, non-medical treatment or/and local antimicrobials are recommended. Non-medical treatment involves maintaining the clean and moist environment of the wound site to promote wound healing. However, the susceptibility to infection complicates wound care and often involve medical treatment. Medical treatment of wound and skin infections can involve the application of topical or systemic antimicrobials (11). There are two classes of topical antimicrobials for topical treatment: antiseptics and antibiotics. The topical antiseptics most commonly used are CHX, triclosan and hydrogen peroxide. The current commonly used topical antibiotics are mupirocin, bacitracin, retapamulin, fusidic acid, neomycin and polymyxin. Not all of them are used in antimicrobial therapy of wounds or skin infections, as some are commonly used as wound cleansing or to reduce the microbial burden on the skin surface (11).

For more severe infections, systemic antibiotics are recommended. The choice of systemic antimicrobials for infection therapy is broad, spanning almost all primary classes of antibiotics. In contrast, the option of topical antimicrobials is far less than the systemic. Furthermore, perspectives on the therapeutic usefulness of topical antibiotics are conflicting, and alternative systemic therapies are available for most indications (11).

1.8 Challenges of current therapy for skin infection

Systemic treatment with antibiotics is often required to treat skin or wound infections. This route of administration could introduce various systemic adverse events in patient and higher possibility of interaction with other drugs. When systemic antibiotics are used, higher blood concentrations are required to achieve desired or appropriate concentrations in the skin tissue, resulting in a greater possibility of severe adverse effects. In addition, inadequate local antimicrobial concentrations within wounds from a systemic dosage may lead to the development of antimicrobial resistance in bacteria. Moreover, there is insufficient evidence to support the use of systemic antibiotics in some types of wounds, such as diabetic ulcers, as a wide variation of antibiotic concentrations in tissue has been observed (11).

Topical antimicrobial therapy offers several advantages over systemic therapy. It offers reduced toxicity or adverse effect introduced by the systemic exposure, and higher probability of adherence owning to a more straightforward application for patients. Local treatment can offer a high accumulation of antimicrobials at the infected site, thus reducing the concentration of the antimicrobial required for treatment (11;50). Nevertheless, guidelines for wound or skin infection therapy primarily focuses on the use of systemic antibiotics. In addition, the options for topical antimicrobials are far less than the systemic antibiotics. Despite many advantages topical treatment possesses over systemic, delivery of topical antimicrobials in wound infections is still challenging. The wound environment is one factor contributing to the challenges of topical application. For optimal healing, the wound should be kept with appropriate moisture. The moist environment will help expedite the healing process as it allows the epithelia to resurface the damage, enhanced angiogenesis and collagen synthesis (11). However, moist environments present a challenge for topical antimicrobial. Delivery of drugs from the product may be difficult because the moisture in the wound that can affect the bioadhesion, stability, and rate of drug delivery from the cream or ointment and thus can change the properties and efficacy of products. Another problem with the topical application is that application of topical products on open wounds, such as in burn injury, can be very painful. The pain may reduce the adherence as the patient may want to avoid experiencing pain (11).

Finally, another critical point is that in wound conditions, skin is not intact, and there is a higher chance of systemic absorption than in normal skin. If the wound is of significant size and depth, systemic absorption may occur, introducing systemic adverse effects from a local administration (11).

2. Aim of study

This study aimed to develop, optimize, and characterize chitosan-modified lipid-based drug delivery systems for membrane-active antimicrobials targeting wound and skin infections. The project focuses on developing delivery systems that enhance antimicrobial activity and anti-inflammatory properties while reducing the toxicity of the antimicrobial compound.

Specific aims:

- To develop a liposomal formulation for membrane-active antimicrobial by optimization of preparation method and vesicle size and size distribution.
- To prepare chitosomes and chitosan-coated liposomes with chlorhexidine as model compound and compare their characteristics: size, charge, and entrapment efficiency.
- To evaluate the *in vitro* release of liposomes and effect of chitosan on the release profile.
- To evaluate the antimicrobial activity of formulations with and without chitosan to assess polymer effect on antimicrobial activity of formulations.
- To evaluate anti-inflammatory activity of chitosan and chlorhexidine, which will be used in formulation intended to treat wound infections.

3. Method and Material

3.1 Materials

Acetic acid glacial, VWR international, Paris, France

Blood agar plate delivered by University Hospital of North Norway, Tromsø, Norway

Chitopharm S (chitosan), LMW (50 – 1000 kDa), DD >70 %, Chitinor, Tromsø, Norway

Chlorhexidine ≥99.5%, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Cibacron Brilliant Red 3B-A, Santa Cruz Biotechnology, Dallas, USA

Distilled water

Ethanol 96%, Sigma Aldrich® Productions GmbH, Steinheim, Germany

Fetal bovine serum (FBS) Sigma-Aldrich, Steinheim, Germany

Glycine, ≥99.0% (HPLC), Sigma-Aldrich, St. Louis, USA

Hydrochloric acid, Sigma-Aldrich, St. Louis, USA

Kollisolv PEG E 400, Sigma-Aldrich, Steinheim, Germany

Lipoid S 100, (soybean lecithin, >94 % phosphatidylcholine), Lipoid GmbH, Ludwigshafen, Germany

Lipopolysaccharide (from *Escherichia coli* 055:B5), Sigma Life Science Norway AS, Oslo, Norway

Methanol, CHROMANORM for HPLC, Pennsylvania, USA

Mueller-Hinton broth, University Hospital of North Norway, Tromsø, Norway

n-(1-naphthyl)ethylenediamine dihydrochloride, Sigma Aldrich Chemie GmBH, Steinheim, Germany

Ortho-phosphoric acid ≥85%, Kebo Lab Ab, Oslo, Norway

Penicillin–streptomycin (10 000 units/10 mg), Sigma-Aldrich, Steinheim, Germany

Phosphate-buffered saline (PBS) tablet, VWR Chemicals, Ohio, USA

Roswell Park Memorial Institute (RPMI) medium 1640 with L-glutamine and sodium bicarbonate, Sigma-Aldrich, Steinheim, Germany

Sodium chloride, Sigma-Aldrich, St. Louis, USA

Sodium nitrate, Sigma-Aldrich, St. Louis, USA

Sulfanilamide, Sigma-Aldrich, St. Louis, USA

3.2 Biological material

Murine macrophage RAW 264.7 cells, ATCC, Virginia, USA

Staphylococcus aureus (ATCC® BAA-1721TM) MSSA 476, LGC standards AB, Borås, Sweden

3.3 Utensils

Cell scraper, 25 cm 20 mm, VWR, Paris, France

Cellophane foil, Bringmann folia, Wendelstein, Germany

Cuvettes, polystyrene, 10 x 10 x 45 mm, Sarstedt AG & Co. KG, Numbrecht, Germany

Dialysis membrane, Spectra/PorTM 4, standard RC tubing, MWCO: 12-14 kD, Fischer Scientific, Gothenburg, Sweden

Falcon ® 24-well plate, sterile-R, non-pyrogenic, Corning incorporation, Life Sciences, Durham, USA

Folded Capillary Zeta Cell, Malvern Instruments Limited, Engima Buisness Park, Grovewood Road, Malvern, Worcestershire, UK

Nunc™ EasYFlask™ Cell Culture Flasks, T75, Thermo Scientific™, Scientific, Roskilde, Denmark

UV plate: Corning®, 96 well, no lid with UV-transparent flat bottom, Corning, New York, USA

3.4 Instruments

Branson 5510 Ultrasonic cleaner, Marshall Scientific, Hampton, USA

Büchi Waterbath B480, Büchi Vac V-500, Büchi vacuum controlled B-721, Büchi rotavapor R-124, Büchi Labortechnik, Flawil, Switzerland

Dual Action Shaker, Model KL-2, Edmund Bühler GmbH, Bodelshausen, Germany

Franz Diffusion cell 15 mm with 12 mL receptor volume, flat ground joint clear glass, clamp and stir bar, Permgear, Hellertown, Pennsylvania, USA

Julabo heating circulator, Julabo CORIO CD-200F, JULABO GmBH, Seelbach, Germany

McFarland densitomter, model DEN-1, BIOSAN, by BioNordica, Oslo, Norway

NICOMP Submicron Particle Sizer, model 370, Nicomp Particle Sizing Systems, Santa Barbara, California, USA

Sartorius BP211D, scale, Sartorius AG, Göttingen, Germany

sensION+ PH31 meter, HACH, Colorado, USA

SONICS high-intensity ultrasonic processor, 500-watt model with temperature controller, probe diameter 13 mm, Sonics & Materials Inc., Newtown, USA.

SPARK® multimode microplate reader, Tecan Trading AG, Männedorf, Switzerland

Vertical laminar airflow Erlab Captair Biocap PCR Workstation, Rowley, USA

Zetasizer Nano Zen 2600, Malvern Instruments Limited, Engima Buisness Park, Grovewood Road, Malvern, Worcestershire, UK

3.5 Software

Photon Correlation Spectroscopy, CW 388 version 1.68, NICOMP Particle Sizing Systems, Santa Barbara, California, USA

SparkControl, version 2.3, Tecan Trading AG, Männedorf, Switzerland

Zeta Potential, Zeta potential report version 2.2, Malvern Instruments Limited, Malvern, UK.

3.6 Methods

3.6.1 Preparation of liposomes

Liposomes were prepared by the thin-film hydration method (51). The method involved creating a thin lipid film and rehydrating the film to form liposomes. Lipoid S 100 (SPC, 200 mg) was weighed directly in a round bottom flask and dissolved in methanol (an appropriate amount). For drug-contained liposomes, CHX (10 mg) was directly weighed in a round bottom flask with lipid. A rotary evaporator was used to evaporate the solvent, and a lipid film was formed. Rotary-evaporator was set to 45 °C and 60 rpm rotation. The pressure was gradually reduced to 60 mbar. Once the desired pressure was achieved, the flask was lowered into the water bath and left rotating for 1 hour. The resulting lipid film was rehydrated with different media to form liposomes. For liposomes without polymer, lipid film was rehydrated with distilled water (10 ml) and ensured that all lipid was dislodged and that we obtained homogeneous dispersion. The dispersion is then stored in the refrigerator overnight.

3.6.2 Preparation of chitosomes

Lipid film for chitosomes was prepared with the same method as described above. The resulting lipid films were rehydrated with 10 ml of 0.2 % (w/v) chitosan solution under shaking for 45 min with Edmund Buhler shaker (52). The dispersions were kept in the refrigerator overnight before further experiments. For chitosan solution, chitosan was dissolved in 0.1 M acetic acid. The solutions were left under magnet stirring overnight before rehydrating lipid films.

3.6.3 Preparation of chitosan-coated liposomes

Liposome dispersions with chitosan-coated liposomes were prepared with the same method for empty liposome (without drug) or plain liposome (with drug). Size reduction and size measurement of liposomes was performed before coating with the polymer. For liposomes with CHX, the unencapsulated drug had to be removed before coating. The 0.2 % (w/v) chitosan solution for coating of liposomes were prepared as described for chitosomes (chitosan was dissolved in 0.1 M acetic acid). Coated liposomes were prepared by adding an equal amount of chitosan solutions to liposomal dispersions under magnet stirring (250 rpm). The polymer solutions were added in a drop-wise manner (1.22 min/ml). After the addition of polymer solutions was completed, the dispersions were left under stirring for 1 hour and stored in a refrigerator for further experiment (53).

Table 1 Overview of formulations produced in this project

Formulation	Description
LIPemp	Empty liposomes
CHIemp	Empty chitosomes
CHI-emp	Empty chitosan-coated liposomes
LIP_CHX	Liposomes + chlorhexidine
CHI_CHX	Chitosomes + chlorhexidine
CHI-CHX	Chitosan-coated liposome + chlorhexidine

3.6.4 Liposome size-reduction

The size of vesicles was reduced by probe-sonication. The liposome dispersions were placed on the ice bath to prevent overheating during sonication. The needle probe tip is inserted just beneath the surface of the sample. The sonicator was set to 40% amplitude, and samples were sonicated with different sonication times to achieve the desired size. The sonication time of each formulation is presented in Table 2. For liposome dispersions that were sonicated more than one interval, were sonicated with 45 seconds rest between each interval on ice bath to prevent overheating. Furthermore, some samples were extruded with a 0.4 mm polycarbonate membrane filter (Nucleopore, Whatman). Liposomes were then stored in the refrigerator.

Table 2 Sonication time and interval of different formulations

Formulation	Sonication time (second)	Sonication interval	Extrusion (round)
LIPemp	10	10	3
CHIemp	10	10	3
CHI-emp	10	18	-
LIP_CHX	5	1	-
CHI_CHX	5	2	-
CHI-CHX	5	1	-

LIPemp: empty liposomes, CHIemp: empty chitosomes, CHI-emp: chitosan-coated empty liposome, LIP_CHX: liposome with CHX, CHI_CHX: chitosome with CHX and CHI-CHX: chitosan-coated liposomes with CHX

3.6.5 Particle size analysis

Photon correlation spectroscopy (PCS) was utilized to determine particle size and size distribution of vesicles. Sample tubes were bath sonicated 10 min beforehand. Dilution of the samples was prepared in a laminar airflow bench. Test tubes were rinsed three times with filtrated distilled water (0.2 µm syringe filter). The test tubes were filled with filtrated distilled water up to 2/3 of total volume and then added one drop of sample to the test tubes. Mixed samples in the test tubes with the help of a 1 ml syringe by drawing the samples into the syringe and emptying the syringe into the test tubes three times. Samples were diluted until the intensity was within the range of 250-350 kHz (54).

Table 3 Overview of photon correlation spectroscopy (PCS) settings used for measurement of particle size

Settings	value
Channel width	Auto set
Temperature	23°C
Liquid viscosity	0.933 CP
Intensity setpoint	300 kHz
Laser wavelength	632.8 nm
Scattering angle	90 degrees

The samples were first measured for about 45-60 min for a new formulation to ensure sufficient data for an appropriate run time. Afterward, samples were measured in 3 cycles with suitable run time. Run times of each formulation are presented in Table 4.

Table 4 Run time used to measure the size of vesicles of different formulations.

Formulations	Run time (min)
LIP_emp	15
CHI_emp	15
CHI-emp	20
LIP_CHX	15
CHI_CHX	15
CHI-CHX	20

LIPemp: empty liposomes, CHIemp: empty chitosome, CHI-emp: chitosan-coated empty liposome, LIP_CHX: liposome with CHX, CHI_CHX: chitosome with CHX and CHI-CHX: chitosan-coated liposomes with CHX

3.6.6 Determination of zeta potential

The zeta potential of liposomes surface was determined by using the ZetaSizer Nano ZS. Samples were diluted (1:14) prior to measurement. An aliquot of 70 µl of samples diluted with 930 µl of filtrated (0.2 µm syringe filter) tap water. Diluted samples were prepared in duplicate, one for the rinsing of the zeta cell and the other for zeta potential measurement. Samples were measured once, and all measurements were performed in three cycles at 25 °C.

3.6.7 Determination of pH

pH values of different formulations were determined by using pH meter. The pH meter calibrated prior measurement of the samples and the liposomal formulations were measured at room temperature with three replicates.

3.6.8 Entrapment efficiency (EE%)

To separate unentrapped CHX from an encapsulated drug in liposomes, dialysis was used. The amount of entrapped drugs was determined by UV-absorbance with the SPARK plate reader. Dialysis tubes with a membrane with MWCO at 12-14 kDa were utilized. The membranes were conditioned for 30 min prior to use, then 1 ml of samples were dialyzed against 1 L of medium (distilled water) under magnet stirring for 4 hours at room temperature.

After dialysis, non-dialyzed liposomes and dialyzed liposomes were diluted with methanol (1:40) to break the liposomes, and 100 µl of dilutions was transferred to a 96-wells plate for measuring the amount of chlorhexidine with a SPARK plate reader at 261 nm.

A standard curve was prepared from a stock solution of 500 µg/ml, diluted to concentrations of 40, 20, 10, 5, 2.5, and 1.25 µg/ml with methanol. Measurement of absorbance was performed by the SPARK plate reader at 261 nm (52).

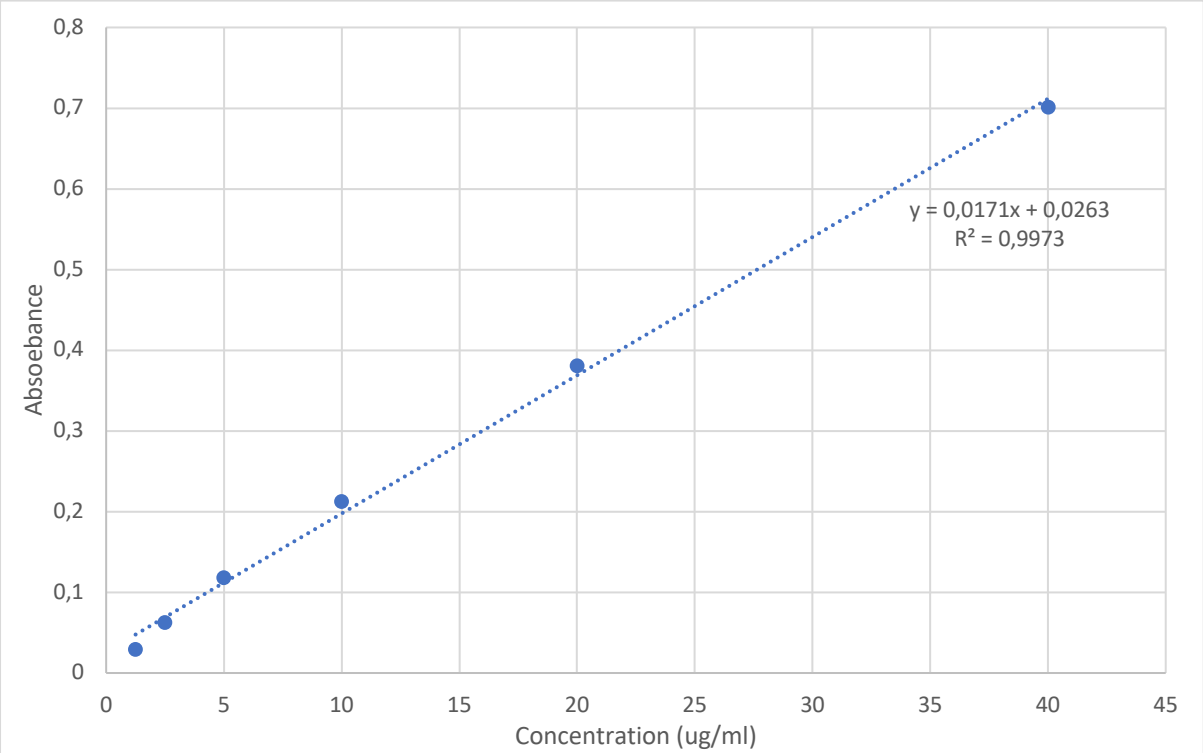

Figure 13 Standard curve of chlorhexidine in methanol for determination of entrapment efficiency

3.6.9 *In vitro* drug release

Prior to testing of *in vitro* release, a standard curve of CHX in the medium (PEG 400 10% (v/v) in distilled water) was prepared before the experiment.

3.6.9.1 Standard curve

The standard curve was prepared from a stock solution of 500 µg/ml in 10% (v/v) PEG E 400 (55). The stock solution was diluted to concentrations of 40, 20, 10, 5, 2.5, and 1.25 µg/ml. Measurement of absorbance was performed by a SPARK plate reader at 261 nm with three replicates.

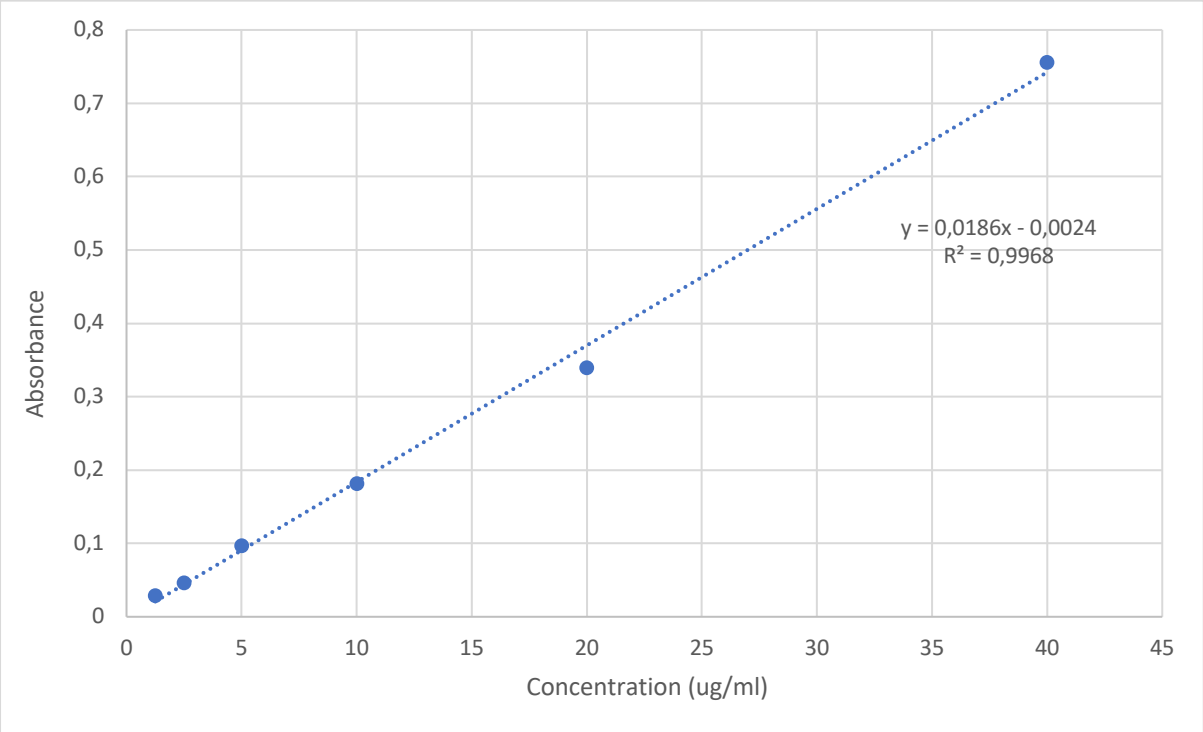

Figure 14 Standard curve of chlorhexidine in 10% PEG E 400 for determination of drug release

3.6.9.2 Franz cell diffusion

The *in vitro* release of CHX was performed by utilizing Franz diffusion cells as described in Jøraholmen *et al*. (49), with minor adjustments. The acceptor chambers (12 ml) were filled with a medium, 10 % PEG E 400, and the temperature of the Franz cell system was set to 32 °C by circulating water. Cellophane membranes were cut into appropriate size and conditioned in the medium for 30 min before use. Liposome dispersions (600 µl) were added into the donor chambers, and the systems were sealed properly. An aliquot of 500 µl of the samples was collected from the acceptor chambers after 1, 2, 3, 4, 5, 8, and 24 hours. After collecting samples from the acceptor chambers, an equal volume of fresh medium was filled into the acceptor chambers to ensure sink conditions. Finally, 100 µl of collected samples were used to measure UV-absorbance by SPARK at 261 nm (without dilution). Control of CHX release (0.65 mg/ml) was prepared by dissolving CHX with 10% (v/v) PEG E 400 and under magnet stirring for 1 hour (55).

3.6.10 Stability

The stability of liposomes is important regarding maintaining its efficacy after production, under distribution, and storage. Size and zeta potential play an important role in the activity and stability of liposomes; hence liposomes should maintain constant size and zeta potential under storage. The stability of liposomes was monitored for 3 months stored at 4 °C, and the samples were measured after 0, 2, 4, and 12 weeks for evaluating the particles size, zeta potential, polydispersity index (PI), and pH as described in sections 3.6.5, 3.6.6 and 3.6.7.

3.6.11 Chitosan quantification

The determination of surface-available chitosan was based on a method described by Jøraholmen *et al*. with some adjustment (49). Glycine buffer (pH 3.2) was prepared by dissolved glycine (1.87 g) and sodium chloride (1.46 g) with distilled water (250 ml). Next, 81 ml of the glycine solution was further diluted with 0.1 M HCl to final volume of 100 ml. Solution of 0.1 M HCl (50 ml) was prepared in advance by diluted HCl (182.25 mg) with distilled water to final volume of 50 ml. Next step was to prepared dye solution by dissolved Cibacron Brilliant Red 3B-A (150 mg) in 100 ml of distilled water. Finally, 5 ml of dye solution was further diluted to 100 ml with glycine buffer to get the final dye solution (100 ml). Used brown bottle to protect the dye solution and final dye solution from light.

Standard curve of chitosan was prepared with 0.05 % (w/v) chitosan standard solution in 0.05 % (v/v) acetic acid, diluted with glycine buffer to desired concentrations. 0.05 % (w/v) chitosan standard solution was prepared by dissolved chitosan (50 mg) in 10 ml of 0.05% (v/v) acetic acid (750 µl acetic acid diluted to total volume of 15 ml with distilled water) and left under magnet stirring overnight. Concentrations of chitosan solutions to form standard curve were 5 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, 130 ug/ml, and 160 µg/ml.

Liposomal formulations were diluted with distilled water to desirable concentration (1:20 v/v). Diluted standard solutions and samples (300 µl) were mixed with 3 ml of final dye solution. Finally, the samples and standards were measured in duplicate. UV-vis absorbance of samples was measured at 575 nm with SPARK microplate reader.

The percentage of surface-available chitosan was calculated using the following equation:

$$Percentage\ of\ surface\ available\ chitosan = \frac{Cs}{Cc}\ x\ 100$$

Cs: concentration of chitosan in samples

Cc: concentration of chitosan used to prepare formulations

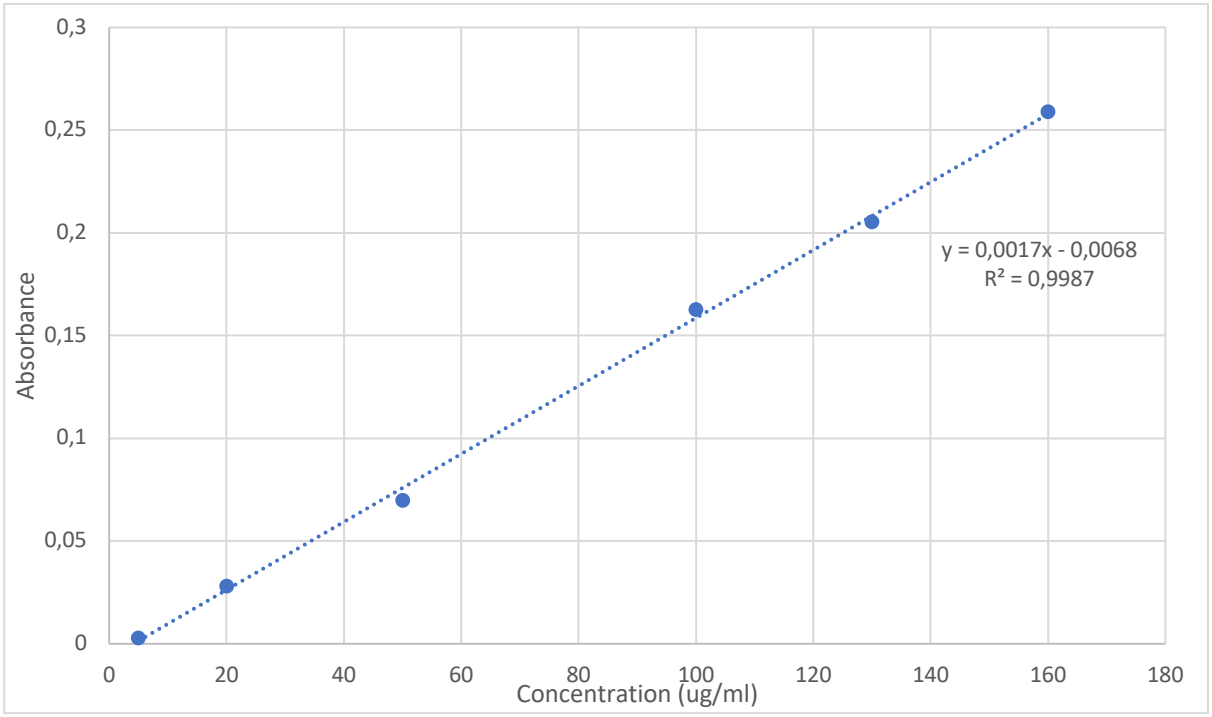

Figure 15 Standard curve of Chitosan in 0.05 % (v/v) acetic acid for determination of surface-available chitosan on liposomes

3.6.12 Antibacterial activity

For *in vitro* evaluation of antibacterial activities of liposome dispersions, the modified broth microdilution method was used (56). The activities of formulations were test against a gram-positive strain, methicillin-susceptible *Staphylococcus aureus* (MSSA). First step was to prepare a 0.5 McFarland bacterial suspension in 0.85 NaCl, which correspond to $\sim 1\ x\ 10^8$colony forming unit (CFU)/ml. The 0.5 McFarland suspensions were further diluted with Mueller Hinton broth (1:150), to correspond approximately $1\ x\ 10^6$ CFU/ml. Second step was to prepare 2-fold serial dilutions of test formulations with Mueller Hinton broth in 96-well plates (125 µl). Diluted bacterial suspension (125 µl) was added into each well of the plates. The plates were then incubated at 37°C under shaking (100 rpm) for 24 hours (56). For control of bacterial growth, bacterial suspension was added into the well with only Mueller Hinton broth (125 µl).

Bacterial survival was evaluated by 10-fold serial dilution of bacterial suspension (20 µl) from the plate incubated the day before with PBS (180 µl), and each dilution were then plate on

blood agar following overnight incubation at 37°C. Colonies of bacteria were counted and percentage of bacterial survival was determined by comparing the surviving bacteria at lipid concentration of 0.3 mg/ml to the control where no antibacterial compound was added (100%).

3.6.13 Anti-inflammatory activity

The anti-inflammatory activity of formulations was evaluated by measure the ability of liposomes to inhibit nitric oxide (NO) production in LPS-induced murine macrophages (RAW 264.7).

Standard curve

Standard curve of NO_2 was prepared by using sodium nitrite ($NaNO_2$) as standard. $NaNO_2$ (10 mM) is diluted to 100 µM with water (total volume 1.5 ml). From this stock solution, concentrations of 20, 16, 12, 8, 6, 4, 2 and 1 µM were prepared. The diluted solutions were further diluted in Griess reagent (1:1) by adding 300 µl of the dilution and 300 µl of Griess reagent. The concentrations after dilution were 10, 8, 6, 2, 1 and 0.5 µM. All concentrations were measured spectrophotometrically at 560 nm in triplicates to create standard curve (55).

Griess reagent was prepared by dissolve 2 g sulphanilamide and 200 mg N-(1-Naphthyl) ethylenediamine (NEDA) in 200 ml 2.5% (v/v) phosphoric acid (H_3PO_4) in water.

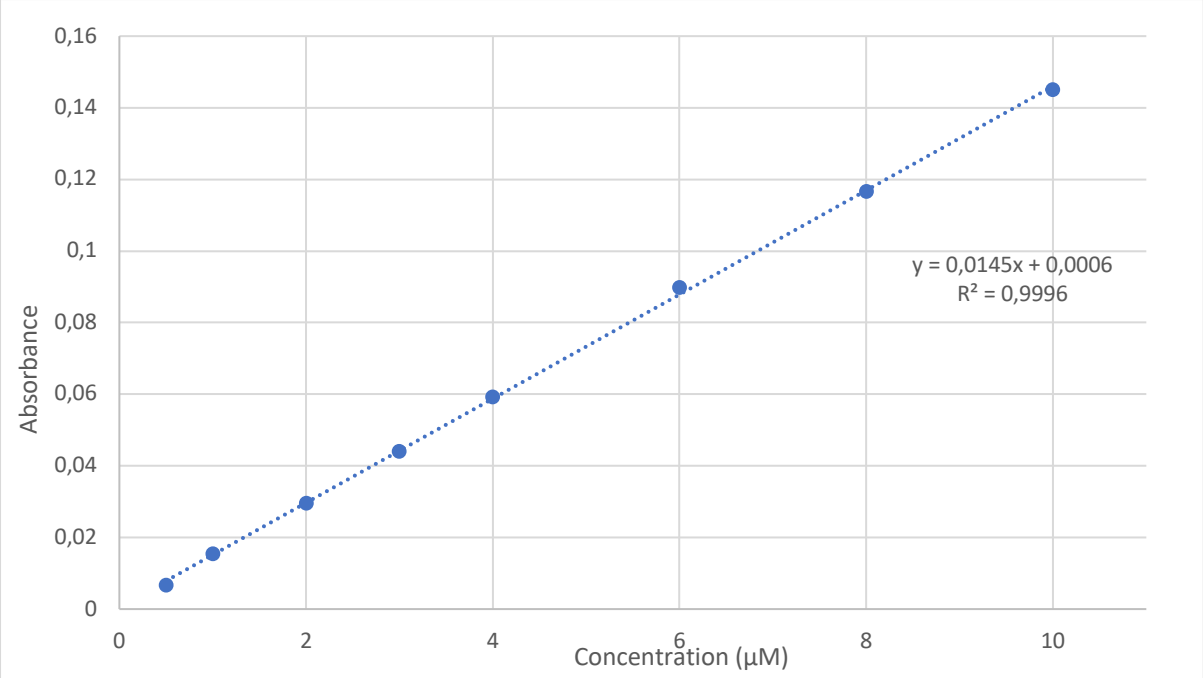

Figure 16 Standard curve of nitric oxide (NO) for assessment of anti-inflammatory activity. The standard curve was obtained from standard solution of sodium nitrite ($NaNO_2$) dissolve in water and mixed with Griess reagent to measure spectrophotometrically at 560 nm

The murine macrophage cell suspension (5 x 10^5 cell/ml in RPMI, 10% FBS, 1% penicillin and streptomycin) was seeded in 24-well plates and incubated for 24 hours in 5% CO_2 at 37°C. The cells were evaluated under microspore prior to further testing. The old medium was removed, and new medium or treatment (1 ml) were added in each well as show in Figure 17. The complete RPMI medium (1 ml) were added in well A1-A3, LPS solution (1 µg/ml, 1 ml) were added in wells A4-A6, and the rest were added with 990 µl LPS solution + 10 µl of diluted formulations (0.1 mg/ml, 1 mg/ml, and 5 mg/ml). The plate was then incubated for 24 hours in 5% CO_2 at 37°C.

For measurement of nitric oxide production, Griess reagent was used. Equal amount of Griess reagent (300 µl) was added into the samples (300 µl) from each well. The mixed solutions (100 µl) were plated in 96-wells plate and measured spectrophotometrically at 560 nm.

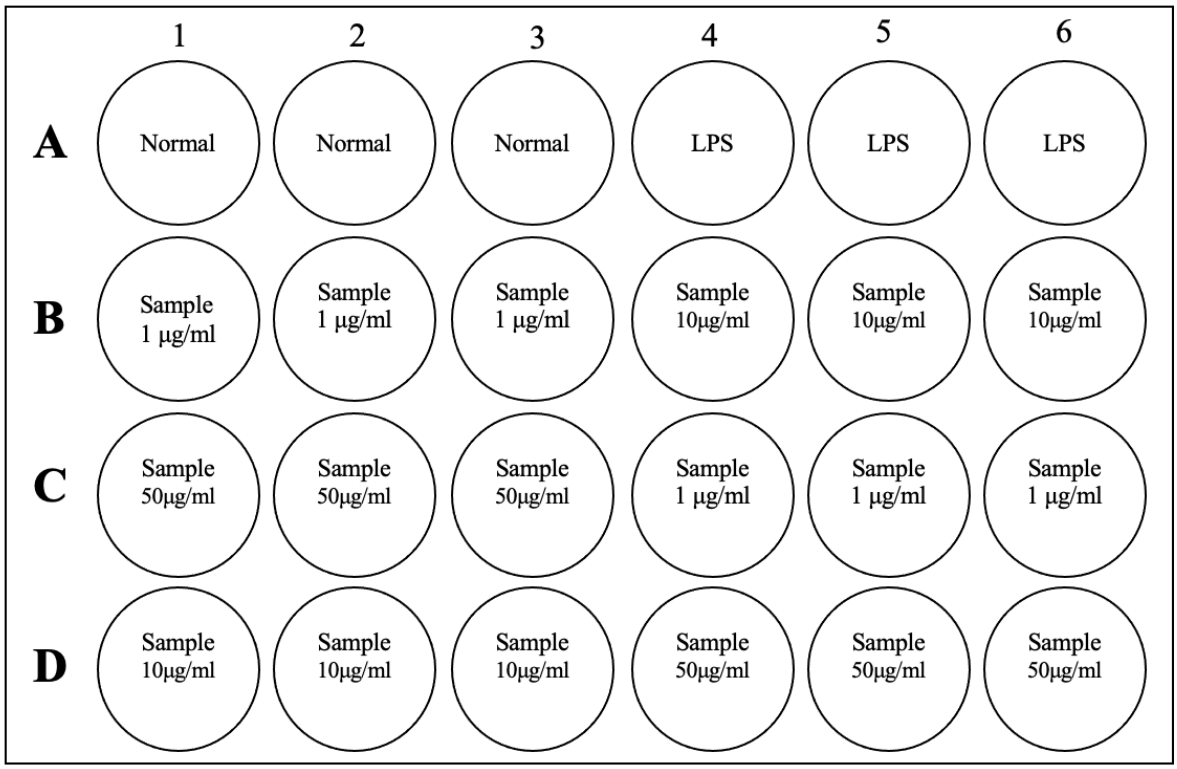

Figure 17 The overview of the 24-well plate for anti-inflammatory evaluation. Normal: cells and complete medium, LPS: cells and LPS solution, sample: cells with LPS (990 µl) and sample (10 µl) with different concentrations (final concentrations are 1 µg/ml, 10 µg/ml, and 50 µg/ml)

3.6.14 Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical significance was calculated by using student´s t-test. A $p < 0.05$ or less was considered statistically significant.

4. Results and discussions

4.1 Vesicle characteristics

4.1.1 Vesicle size

When developing nanoparticles as a pharmaceutical product, particle size and size distribution (polydispersity index or PI) are important physical characteristics to consider. This is because particle size affects stability, skin penetration and other properties of liposomes.

This project aimed to develop a topical antimicrobial therapy. Consequently, liposomes' size should be appropriate for topical application. The preferred size range of liposomes for a topical administration route was suggested to be between 250-350 nm, as mentioned in section 1.5.2.1. Liposomes were prepared with thin-film hydration know to generate giant liposomes with multiple lamellar structure (38). To reduce liposome size to the targeted size range, probe-sonication was utilised.

The size distributions of PCS measurements can be expressed as one of these two modes: Gaussian or NICOMP distributions. Gaussian distribution represents the mono-modal size distribution whereas the NICOMP represent the bimodal or multimodal size distribution.
Chi-square describes how well the size distribution fits the gaussian distribution. The value between 0-2 is ideal and indicates good fits while value > 3 indicates poor fit. When Chi-square excess 3, the NICOMP distribution should be used (54).

Table 5 presents the size and PI of various formulations after size reduction at production. As seen in the table, the liposomes are fitted to a multimodal size distribution (NIOMP), and the size of vesicles is expressed as three peaks size distribution.

Table 5 Vesicle characteristics: size and size distribution.

	Size (nm) ± SD			
	Peak 1 %	**Peak 2 %**	**Peak 3 %**	**PI**
LIPemp	25 ± 13 2 ± 2	133 ± 43 38 ± 9	431 ± 9 60 ± 10	0.37 ± 0.04
CHIemp	25 ± 14 3 ± 2	135 ± 46 44 ± 21	436 ± 145 53 ± 23	0.32 ± 0.01
CHI-emp	17 ± 3 2 ± 1	82 ± 16 26 ± 12	299 ± 54 72 ± 13	0.35 ± 0.01
LIP_CHX	22 ± 6 2 ± 2	111 ± 31 35 ± 14	409 ± 87 63 ± 16	0.38 ± 0.03
CHI_CHX	19 ± 11 5 ± 2	86 ± 60 35 ± 25	167 ± 43 61 ± 25	0.34 ± 0.07
CHI-CHX	27 ± 14 3 ± 3	118 ± 40 29 ± 11	398 ± 39 68 ± 13	0.39 ± 0.02

LIPemp: empty liposome, CHIemp: empty chitosome, CHI-emp: empty chitosan-coated liposome, LIP_CHX: liposome med chlorhexidine (plain liposome), CHI_CHX: chitosome with chlorhexidine, CHI-CHX: chitosan-coated with CHX

Table 5 shows that most of the formulations have the main peak in the desired size range, except chitosomes with CHX, which are smaller than 200 nm. However, these size distributions obtained from PCS are expressed as a mean of size within the individual peak and based on mathematical calculations. The PCS underestimates the small particles if bigger particles are present, which might explain the high mean size in the main peaks (3). Moreover, this distribution analysis represents a simplified version of the actual size distribution of our samples and the resolution of DLS is not sufficient to provide more detail on the distribution (3). In Figure 18 from section 4.2, vesicles' size is present differently and expressed as cumulative size ≤80%. The result shows that 80% of liposomes in nearly all formulations (at production) are in the intended size range, except for the chitosan-coated liposomes with CHX.

This means that the majority of liposomes in all formulations were within the desired size range.

Chitosan-coated liposomes with CHX were lager than the preferred size range (approximately 400 nm). This could be due to the addition of chitosan after the size reduction of liposomes. It is worth mentioning that larger particles than 300 nm are acceptable in topical administration. Generally, particles larger than 600 nm are not able to deliver their content into the deeper layer of skin, but sizes of 300 or lower can deliver content into the deeper layer of skin. This means that sizes between 300- 500 nm can still deliver their content to some extent (41). It is also important to remember that in wound conditions, the skin barrier is impaired, which can increase the penetration of the drug into the skin (11).

Furthermore, it is more critical to have liposomes with too small size. Small liposomes with a diameter of 70 nm or less have the ability to deliver their content into the deeper layers of the skin, increasing the risk of systemic absorption and adverse effects, which is not preferred (41).

The multimodal distribution obtained from the result indicates that liposomes have a polydispersed size distribution. The PI also defines the size distribution of liposomes. It is used to describe the degree of spreading of size distribution, and a PI of 0.3 or less is considered a homogenous population, and a PI bigger than 0.7 indicates a broad particle size distribution (41). The PI of all liposomal formulations in this project was higher than 0.3. The explanation for this could be due to the technique used to reduce the size of liposomes, as probe-sonication was reported to generate a polydisperse vesicle suspension (57). Even though the PI of all formulations was larger than 0.3, the PI in this project was still in the accepted range, considering that liposomes were developed intended for topical administration.

4.1.2 Surface charge

Zeta potential of liposomes was determined by using Zetasizer as described in section 3.6.6 and the results of the zeta potential measurements of all formulations are presented in Table 6. Empty liposomes were the initial formulation and serves as a control.

Table 6 Zeta potential of different formulations presented as mean ± SD.

Formulation	**Zeta potential (mV) ± SD**			
	Production	**2 weeks**	**4 weeks**	**12 weeks**
LIPemp	-1.6 ± 1.4	-2.6 ± 0.6	-3.9 ± 0.1	-6.7 ± 0.7
CHIemp	12.4 ± 0.4	11.1 ± 0.9	11.1 ± 0.9	11.5 ± 0.4
CHI-emp	13.0 ± 0.5	12.5 ± 1.0	11.8 ± 0.6	45.6 ± 7.1
LIP_CHX	42.9 ± 5.9	40.2 ± 7.6	42.2 ± 10.5	25.4 ± 2.7
CHI_CHX	94.9 ± 2.2	92.2 ± 7.8	91.9 ± 3.3	86.7 ± 1.8
CHI-CHX	83.3 ± 3.1	83.4 ± 3.1	78.6 ± 0.9	77.1 ± 2.0

LIPemp: empty liposome, CHIemp: empty chitosomes, CHI-emp: empty chitosan-coated liposomes, LIP_CHX: liposome med CHX (plain liposome), CHI_CHX: chitosomes with CHX. CHI-CHX: chitosan-coated with CHX

The zeta potential of empty liposomes is expected to be near zero since the phospholipid utilized to prepare liposomes is neutral. The empty liposomes exhibited a zeta potential close to zero (-1.6 ± 1.4 mV). A zeta potential between +10 and -10 mV is considered as neutral (58) and as a result, we consider our empty liposomes to be neutral. The empty chitosomes exhibited a positive zeta potential (12.4 ± 0.4 mV) reflecting the positive charge of surface-available chitosan. Chitosan can be protonated to produce a positive charge, its presence on the surface of liposomes will increase the zeta potential of the liposomes to a positive value. The zeta potential of chitosan-coated liposomes also exhibited a positive value (13.0 ± 0.5 mV). The

rise in zeta potential indicated that chitosan was available on the liposome surface and that the chitosan coating of liposomes was successful. This result is similar to the results of Ahsan, who also reported a rise in zeta potential to positive values of chitosomes (55). Liposomes with CHX exhibited high positive zeta potential. The CHX was expected to be embedded in the lipid bilayer due to its physical and chemical properties. However, the positive zeta potential of liposomes with CHX, suggests that CHX also present on the surface of liposomes as well as in the lipid bilayer. The chitosomes with CHX formulation had the highest zeta potential (94.9 ± 2.2 mV). Since the zeta potential of chitosomes with CHX was higher than that of chitosomes without CHX, this could indicate that both chitosan and CHX were present on the surface of liposomes. Moreover, the zeta potential of chitosomes with CHX was higher than the combined zeta potential of CHX-loaded liposomes and chitosomes without CHX. This finding might lead us to believe that CHX and chitosan have a synergistic effect on the surface charge of liposomes. This finding is in agreement with Ashan (2021), who reported that chitosomes with CHX had the highest zeta potential of any formulations (55).

Chitosan-coated liposomes with CHX also exhibited a high zeta potential (83.3 ± 3.1 mV). This might mean that we successfully coated liposomes containing CHX with chitosan, and that both were available on the surface of liposomes.

Positive zeta potential is beneficial since bacterial membranes are known to have a negative charge and would interact well with positively charged liposomes (14). Both CHX and chitosan are positively charged and can interact with bacterial membranes (34;46). Thus, the synergistic effect of CHX and chitosan on the surface of liposomes would produce a strong interaction with bacteria, potentially enhancing the formulation's antimicrobial activity. In addition, cationic surface makes liposomes more selective to bacterial membrane because of higher content of negatively charged cell wall components. Whereas human cell membrane is composed mainly of neutral phospholipid (15).

Although a positive zeta potential provides a beneficial interaction with the bacterial membrane, a high positive zeta potential can potentially be cytotoxic (55). Our chitosan formulations with CHX have a relatively high zeta potential. To assess the safety of the formulations, the toxicity of the formulation should be examined.

4.1.3 pH

The pH values of various formulations throughout 12 weeks are presented in Table 7. Empty liposomes had a pH value of around 5-6, which is similar to the pH of normal skin (59). The pH value of liposomes with CHX was the highest of all the formulations (8.5 ± 0.1). The high pH of CHX-loaded liposomes could be explained by the cationic characteristic of CHX. CHX can protonate at physiological pH and behave as a strong base, increasing the liposomal formulation's pH value.

Table 7 pH values of different formulation over 12 weeks presented as mean ± SD.

Formulations	**pH ± SD**			
	Production	**2 weeks**	**4 weeks**	**12 weeks**
LIPemp	5.8 ± 0.5	6.0 ± 0.3	5.7 ± 0.2	5.3 ± 0.1
CHIemp	3.6 ± 0.0	3.6 ± 0.3	3.7 ± 0.0	3.7 ± 0.0
CHI-emp	3.7 ± 0.0	3.7 ± 0.0	3.7 ± 0.0	3.7 ± 0.0
LIP_CHX	8.5 ± 0.1	7.7 ± 0.2	7.8 ± 0.4	7.3 8 ± 0.3
CHI_CHX	3.7 ± 0.0	3.8 ± 0.0	3.8 ± 0.0	3.8 ± 0.0
CHI-CHX	3.8 ± 0.0	3.8 ± 0.0	3.8 ± 0.0	3.8 ± 0.0

LIPemp: empty liposome, CHIemp: empty chitosome, CHI-emp: empty chitosan-coated liposome, LIP_CHX: liposome med chlorhexidine (plain liposome), CHI_CHX: chitosome with chlorhexidine, CHI-CHX: chitosan coated with CHX

In comparison to empty liposomes, formulations containing chitosan had lower pH values. As chitosan was only soluble in acidic solution, the acidic solution used to prepare liposomes contributed to the decrease in pH values of chitosan formulations. The pH value of chitosan formulations including CHX and those not including CHX differs slightly. The CHX-loaded formulation had a slightly higher pH than the formulation without CHX. This might be attributed to CHX's alkaline characteristic in the CHX-loaded formulation.

The effect of pH on wound healing was studied by Schneider L. *et al* in 2007. The underlying tissue of a wound is exposed to the external environment and has a pH value of 7.4. Bacterial growth is optimal at this pH because most human pathogenic bacteria grow best at pH values over 6, and lower pH could inhibit their growth (59). The finding suggests that a low pH value might be favourable for a formulation meant to treat a skin infection since bacterial growth can be prevented at such a low pH. As a result, the chitosan formulations with a pH of 3.8 could have the potential to be an ideal formulation for treating wound infections.

The application of a liquid liposomal formulation directly on a wound is not appropriate. The duration of time the formulation remain on the wound is also important for successful wound treatment. Liquid formulations are known to have low viscosity and can rapidly adsorb wound fluid and become more mobile resulting in poor adhesion and low retention time at wound site. A secondary formulation, such as solid bioadhesive polymeric wound dressing which able to incorporate liposomes could potential be a promising secondary formulation (60). Therefore, the pH of the final formulation is of higher importance.

4.2 Vesicle stability

It is important to consider that particles tend to agglomerate during storage and will behave in different manner than the single particles. Therefore, size, PI and zeta potential of liposomes were monitored for 12 weeks in order to evaluate the stability of liposomes after storage in the refrigerator at 4°C. The results of particles size and PI are presented in Figure 18 (and for more detail in Table A1, A2, A3 in Appendix I). In Figure 18, size is presented as cumulative size of 80% of the liposomes to make it easier to assess the potential size and PI change over time.

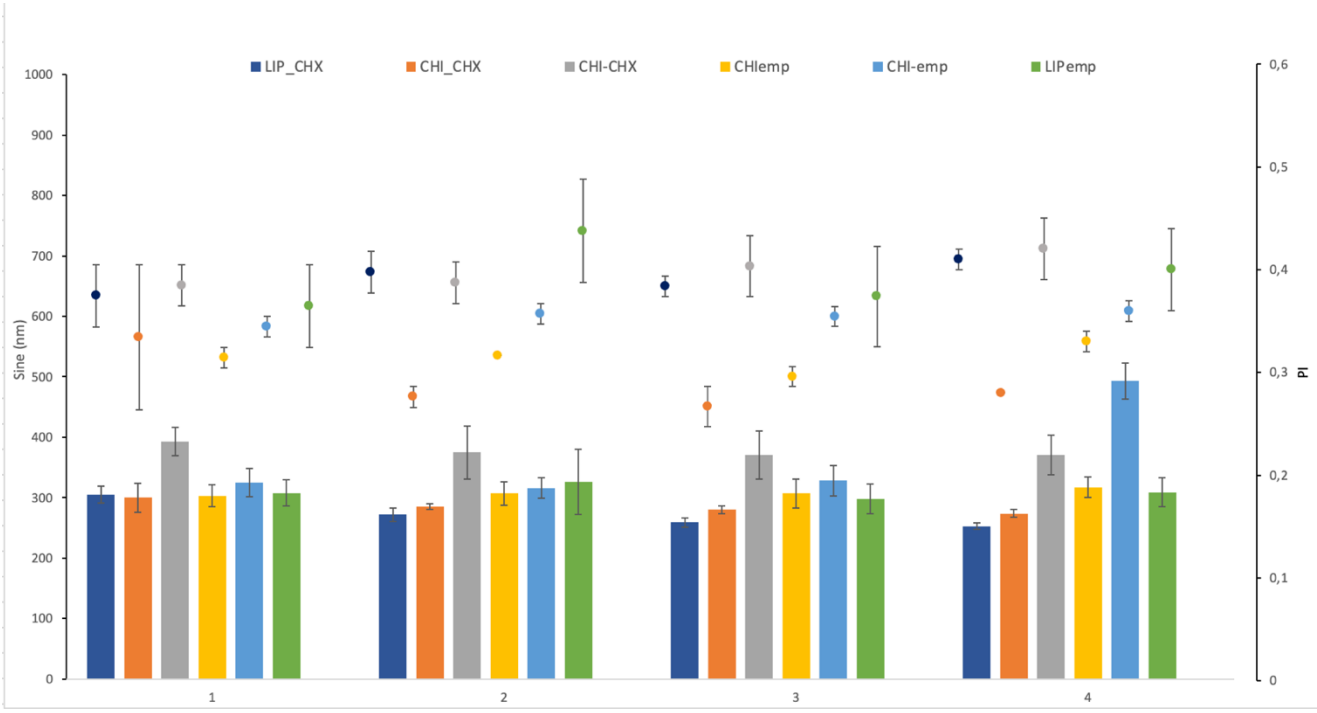

Figure 18 Size and PI (mean ± SD) of different formulations throughout 12 weeks, presented as cumulative size (bars) and PI (scatter plot). The samples were measured after production (1), 2 weeks (2), 4 weeks (3) and 12 weeks (4). LIPemp: empty liposomes, CHI-emp: empty chitosan coated liposomes, CHIemp: empty chitosomes, LIP_CHX: liposomes with CHX, CHI-CHX: chitosan coated liposomes with CHX and CHI_CHX: chitosomes with CHX

Zeta potential of liposomes can be used as an indicator for stability of liposomal system (Figure 19). A high zeta potential either positive or negative more than 30 mV indicates good stability (44).

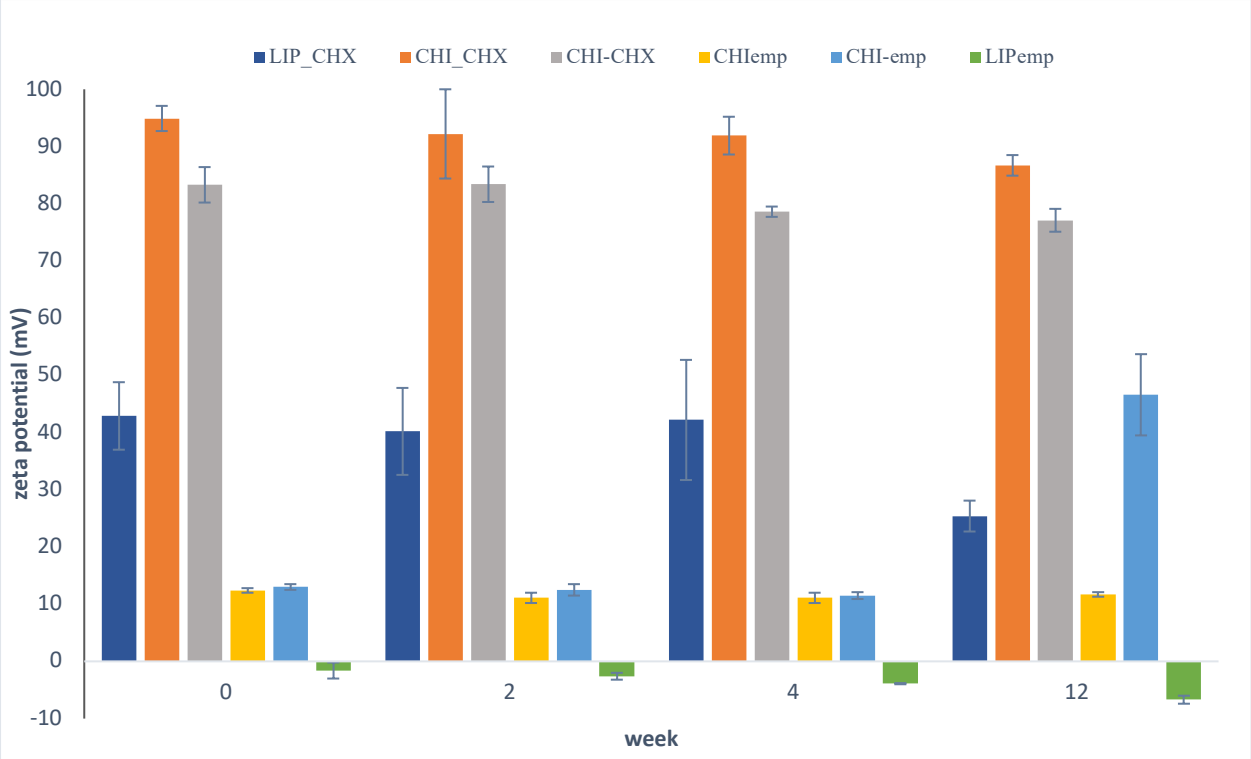

Figure 19 Zeta potential of different liposomal formulations throughout 12 weeks (mean ± SD). LIPemp: empty liposomes, CHI-emp: empty chitosan-coated liposomes, CHIemp: empty chitosomes, LIP_CHX: liposomes with CHX, CHI-CHX: chitosan-coated liposomes with CHX and CHI_CHX: chitosomes with CHX

Most of liposomes showed to be in targeted size range throughout 12 weeks, which indicates good stability. Unexpectedly, empty liposomes show to be stable and have little change in size and PI. Low surface charge of nanoparticles tend to generate lowered stability and the particles could therefore aggregate (61). Since the empty liposomes in this project had a low surface charge, they could potentially have increased in size and PI. The zeta potential of empty liposomes showed that after 2 and 4 weeks, the zeta potential of empty liposomes became more negative, and the zeta potential was significantly different between the first and twelfth week. Although the size of chitosan-coated liposomes with CHX was larger than the targeted size, the formulation was able to maintain the same size range and PI with no significant change for 12 weeks, indicating that the system was stable. For size and PI of chitosomes with CHX, there was no significant change in size and PI from the first week to the twelfth week, indicating their good stability. The high zeta potential can explain the good stability of both chitosan formulations. Both chitosomes with CHX and chitosan-coated liposomes with CHX had zeta potential over 30 mV, indicating high electrostatic stabilisation of liposomes (44). The strong

charge on the surface of liposomes could provide a repulsion effect and thus prevent aggregation, as mentioned previously. The zeta potential of these formulations was maintained at a high value for 12 weeks, which could explain the good stability of the formulations.

Chitosomes with and without CHX seemed to be the most stable formulations in this project. Both maintained in the targeted size range and low PI throughout 12 weeks with no significant change. This supports our postulation in using chitosan to help stabilize the liposome systems. As other chitosan formulations remained stable for 12 weeks, we were expecting the chitosan-coated liposomes without CHX to do the same. However, chitosan-coated liposomes without CHX maintained stable for four weeks, and the size significantly increased (+167 nm) from the first week. The zeta potential of the formulation also significantly increased in the twelfth week. The chitosan coated liposomes without CHX should be examined more thoroughly with transmission electron microscopy (TEM) in order to confirm this result obtained from the PCS.

4.3 Entrapment efficiency

The EE described the amount of drug encapsulated in liposomes and was determined using the dialysis method. The amount of entrapped drugs was determined by UV-absorbance readings. The result is expressed as a percentage of CHX encapsulated in liposomes relative to the theoretically total amount of CHX utilised to prepare liposomes (Figure 20).

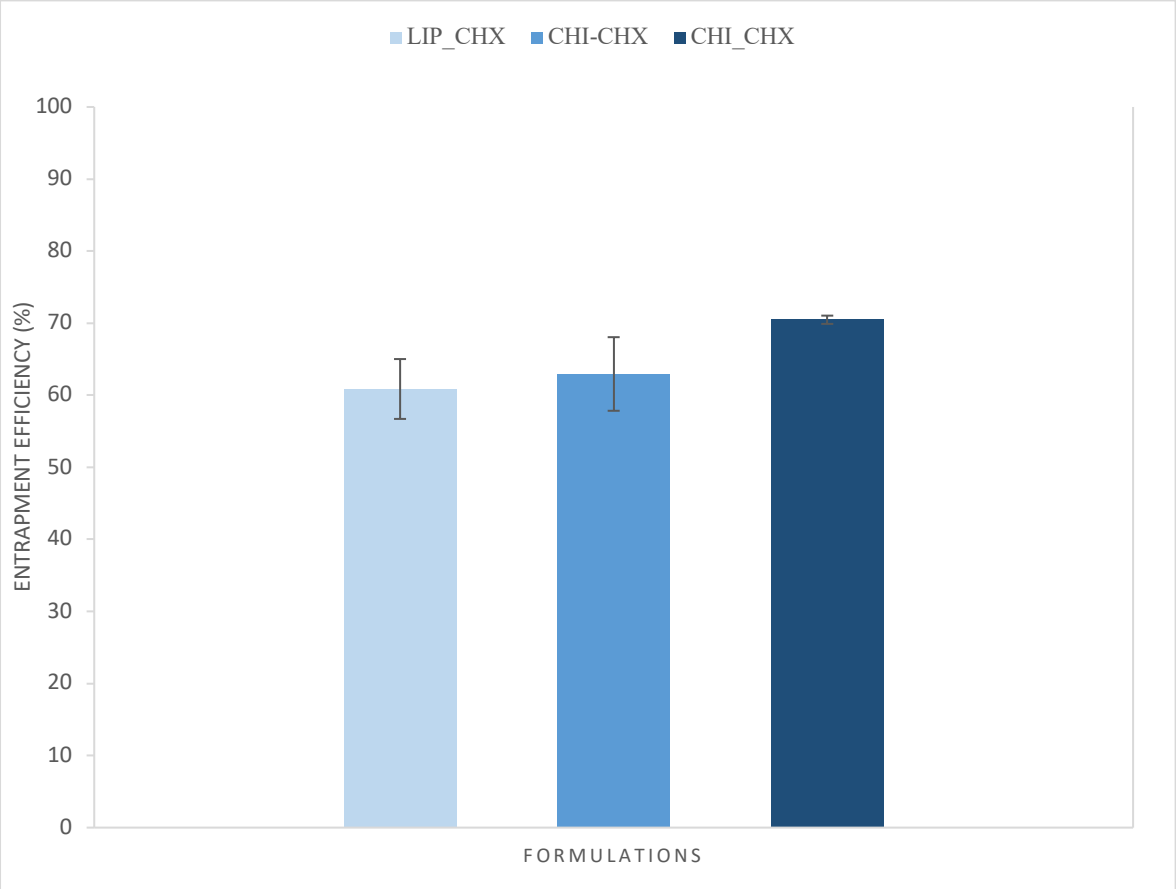

Figure 20 Entrapment efficiency of CHX presented as percentage (%) for different formulations ± SD. LIP_CHX: liposomes with CHX, CHI-CHX: chitosan-coated liposomes with CHX and CHI_CHX: chitosomes with CHX

The EE of the three formulations was found to be relatively high. The EE of chitosan-coated liposomes is the same as that of non-coated liposomes. The chitosomes with CHX were found to have the highest EE, and the EE was significantly higher ($p<0.05$) than the other formulations. This finding differs from the finding of Ahsan, which reported that the EE did not differ between chitosomes and plain liposomes (55).

Nonetheless, in both studies, the EE of liposomes was relatively high. The high EE might be attributed to CHX's characteristics. CHX is a poorly soluble compound that is predicted to be found in the lipid bilayer of liposomes. Nonetheless, CHX has a logP of 0.08, indicating that CHX has an affinity to both aqueous and lipid phases. This amphiphilic characteristic of CHX allows it to be within the lipid bilayer and interact with the aqueous phase of liposomes, resulting in high loading of CHX in the liposomes (55).

In the development of antimicrobial formulations, a high EE is desirable. This is because high entrapment ensures that a sufficient amount of drug is delivered to the infection site. This can assure effective bacterial eradication (62).

In addition to the high EE of the formulations, the results show a low variation of EE within each formulation (small SD), indicating good batch reproducibility. The preparation procedure of liposomes consistently produced the same level of EE from batch to batch, suggests that the method is reliable and reproducible.

4.4 Chitosan surface-availability

The presence of chitosan on the surface of liposomes is indicated by the increase zeta potential of chitosan formulations. Additionally, it was of interest to investigate how much chitosan was available on the surface of liposomes in comparison to the initial concentration, and whether adding CHX to the formulation would impact the concentration of surface-available chitosan. The colorimetric assay was performed to determine the surface-available chitosan. The percentage of surface-available chitosan is presented in Table 8. In all formulations, the percentage of surface-available chitosan is relatively high. This confirms our assumption that chitosan is present on the surface of liposomes based on the zeta potential.

Table 8 Chitosan surface available presented as a percentage of chitosan available on the surface in relative to the initial concentration ± SD.

Formulations	Surface-available chitosan (%) ± SD
CHIemp	84 ± 16
CHI-emp	53 ± 7
CHI_CHX	90 ± 3
CHI-CHX	82 ± 4

CHIemp: chitosomes without CHX, CHI-emp: chitosan coated liposomes without CHX, CHI-CHX: chitosan coated liposomes with CHX and CHI_CHX: chitosomes with CHX

Interestingly, there was a trend in the results indicating that chitosan formulations with CHX seemed to have a higher percentage of surface-available chitosan than chitosan formulations without CHX (Figure 21). These findings suggest that chitosan and CHX may interact within liposomal bilayer, affecting the amount of chitosan present on the surface. However, there was no significant difference in the percentage of chitosan on the surface of chitosomes with and without CHX. Additionally, chitosomes without CHX have a larger SD. As a result, it is difficult to tell with certainty how CHX affect the surface availability of chitosan. Apparently, more testing is required to confirm the hypothesis. This finding was contrary to Hemmingsen *et al.*, who reported that the percentage of surface-available chitosan was the same for chitosomes with and without CHX (62). Furthermore, the percentage of surface-available chitosan on chitosomes with or without CHX this study was higher than in Hemmingsen´s study.

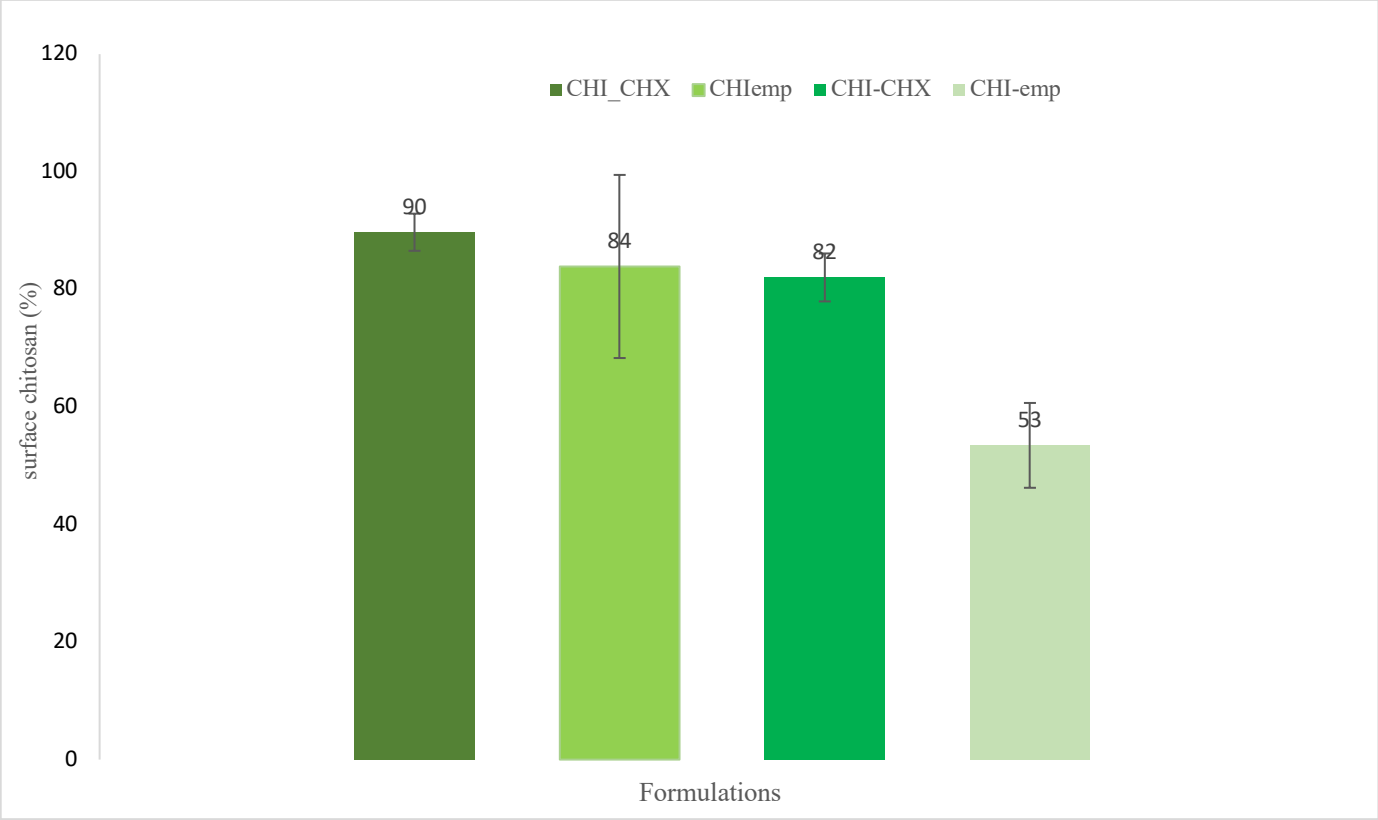

Figure 21 Representation of surface-available chitosan presented as a percentage of chitosan available on the surface relative to the initial concentration (mean ± SD). CHIemp: chitosomes without CHX, CHI-emp: chitosan coated liposomes without CHX, CHI-CHX: chitosan coated liposomes with CHX and CHI_CHX: chitosomes with CHX

For the chitosan-coated formulations, the percentage of surface-available chitosan for formulation with CHX was significantly different from the chitosan-coated formulations without CHX ($p < 0.05$). In comparison to the studies of Jøraholmen *et al.* (2015), the percentage of surface-available chitosan for chitosan-coated liposomes without CHX in this study was similar to the liposomes coated with 0.03% (w/v) chitosan concentration in Jøraholmen's study. Reports show that lower chitosan concentrations result in more chitosan available on the surface of liposomes as coating could reach a saturation state when the concentration exceeded 0.1%. The concentration of chitosan used in this project was 0.2% (w/v) which exceeded 0.1% and might explain the low percentage of surface-available chitosan for chitosan-coated liposomes without CHX in this study (49).

All formulations show to have had more than 50% of the initial chitosan concentration available on the surface of liposomes. Since the aim in this project was to preserve chitosan on

the surface, this finding suggests that the approach used to prepare these liposomes might be a promising procedure for producing chitosan-containing liposomes encapsulated membrane-active antimicrobials. The reason for this is that the presence of chitosan on the surface allows chitosan to interact with bacteria and disturb their membrane functions, resulting in cell death (46).

4.5 *In vitro* release

The *in vitro* release study was performed using the Franz cell diffusion method. The results for the release profile of different CHX-containing formulations are presented in Figure 22. The release profile is depicted as cumulative percentage release of CHX and assessed over a period of 24 hours. Free CHX in the release medium was used as a control. All liposomal formulations provided a more prolonged release than free CHX. This confirms that liposomes formulation could prolong the release of CHX.

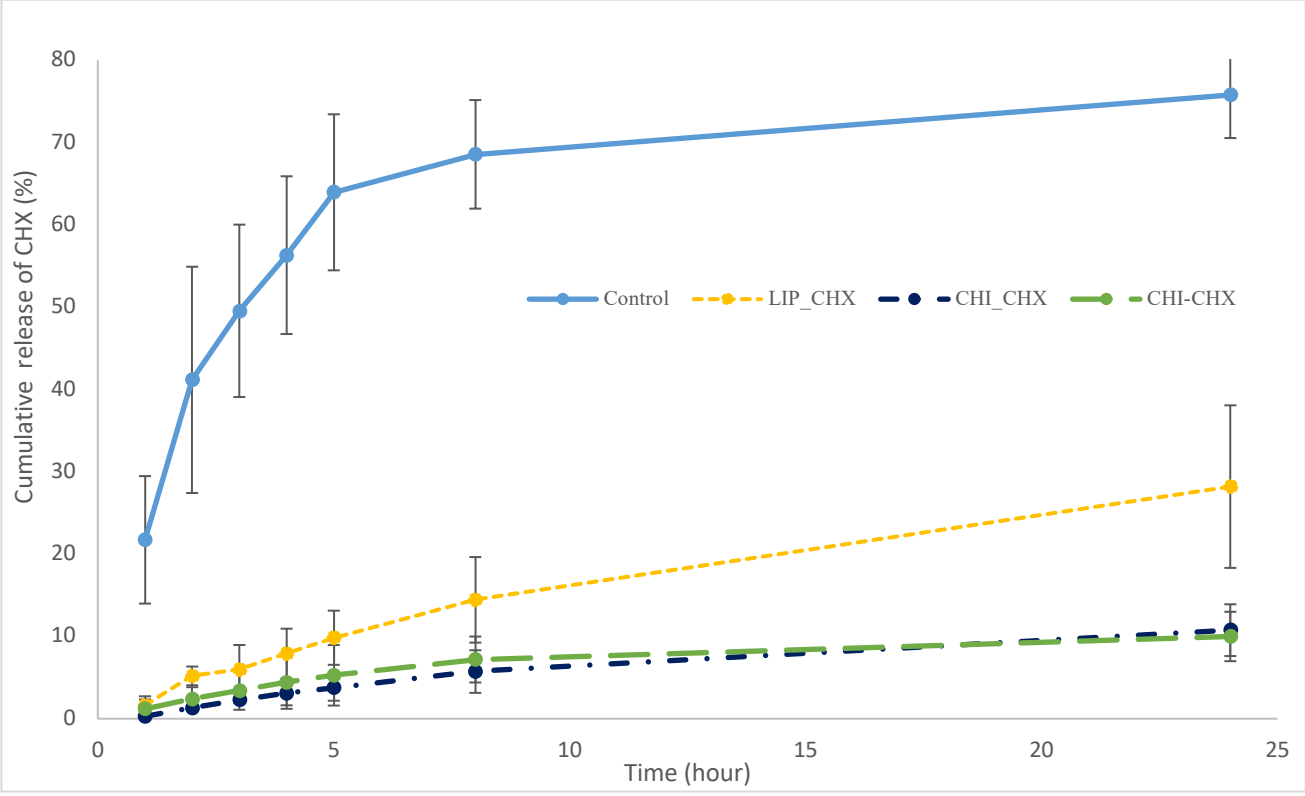

Figure 22 Cumulative *in vitro* release (%) of CHX in PEG 400 10% or from formulations for 24 hours (mean ± SD). Control: free drug, LIP_CHX: liposomes with CHX, CHI_CHX: chitosomes with CHX and CHI-CHX: chitosan-coated with CHX

Furthermore, chitosan-coated liposomes and chitosomes released CHX at a slower rate than non-coated liposomes and was significant at 24 hours. This indicates that liposomes combined with chitosan have the ability to prolong the release of CHX.
The possible explanation for prolonged release of CHX from chitosan formulations can be due to the features of chitosan and CHX. Chitosan has hydrophilic features, which make the surface of liposomes less hydrophobic and might cause a diffusion barrier for drug release from the surface of liposomes (63). CHX is reported to be amphiphilic (logP 0.08), meaning it exhibits affinity for both the lipophilic and aqueous phases. As a result, it is assumed that CHX would be incorporated in the lipid bilayer and would have an affinity for the aqueous core (55). The CHX that had the affinity to the lipid bilayer might have difficulties diffusing through the hydrophilic layers of chitosan, whereas CHX with an aqueous core affinity might have difficulty diffusing through the lipid bilayers of liposomes.

These results are comparable with Jøraholmen´s findings (49). They observed that the release of resveratrol from plain liposomes and chitosan-coated liposomes was more sustained than the release of free resveratrol in the medium. In addition, the release of the compound from the chitosan-coated formulations was likewise slower than the non-coated formulations (49). Although this finding cannot directly compare to the result from this project because different compounds were used, these findings still indicate that chitosan may be able to provide a more prolonged release from liposomal formulations.

Chitosan-containing liposomes were superior to non-coated liposomes in terms of prolonged CHX release, supporting our hypothesis that chitosan-containing liposomal formulations might provide better prolonged drug release. Prolonged drug release is preferable in the treatment of wound infection. This is because prolonged release can reduce the frequency of product applications onto wounds. As the application of topical products on wounds could be uncomfortable and painful, repeatedly applying products may reduce patient´s compliance (11). Another reason to aim for prolonged release is that it ensures the drug's presence at the infection site for a longer period of time, minimizing the chances of an antimicrobial-free periods at the infection site (11). Periods without the presence of antimicrobial at the infection site can lead to bacterial regrowth and cause persistent bacterial infection (11). The solution could be to apply products regularly, however, as previously mentioned, frequent application may not be appropriate. As a result, chitosan-containing liposomal formulations might be a potential option for prolonging antimicrobial release.

4.6 Antimicrobial activity

Antimicrobial activity of chitosan has shown to be effective against *S. aureus*, one of the most common skin pathogens (46). The mechanism of action is still not fully understood, but the most common explanation is thought to be the electrostatic interaction between the positively charged chitosan and the negatively charged bacterial membrane (46). Based on this, the hypothesis was proposed, stating that the antimicrobial property of chitosan would act in synergy with antimicrobial and enhance the antimicrobial activity of the formulation. Therefore, the antimicrobial activity of different formulations was investigated to assess their efficacy in the eradication of *S. aureus*.

The modified broth microdilution technique was used to determine antibacterial activity, and the results are shown in Figure 23. The results are expressed as percentage of *S. aureus* viability compared to the control group (untreated bacteria).

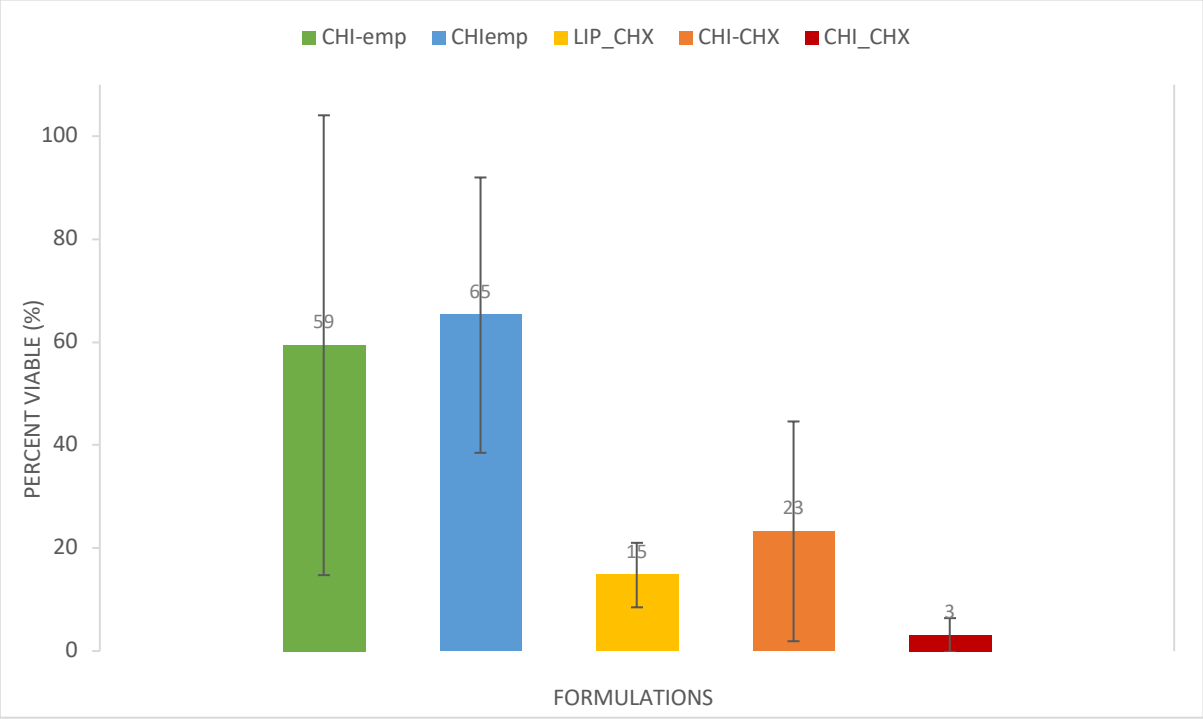

Figure 23 Antimicrobial activity of different formulations presented as percentage of bacterial viability (%, mean ± SD) at a lipid concentration of 0.3 mg/ml. CHI-emp: empty chitosan-coated liposomes, CHIemp: empty chitosomes, LIP_CHX: liposomes with CHX, CHI-CHX: chitosan-coated liposomes with CHX and CHI_CHX: chitosomes with CHX

According to the finding, all formulations displayed higher antimicrobial activity than the plain, empty liposomes (data not shown). This was expected given that the plain, empty liposomes previously showed to possess low antimicrobial activity (56).

Compared to the control, both chitosan-based formulations without CHX showed a decrease in bacterial viability, indicating their efficacy against *S. aureus*. The chitosome with CHX showed the lowest bacterial viability, indicating that this formulations' antimicrobial activity is higher in comparison to other formulations. This result supports our hypothesis that chitosan can enhance the antimicrobial activity of the antimicrobial formulation. However, the difference between formulations with chitosan and without chitosan was not statistically significant. As the SD of the chitosan formulations with CHX is relatively large, we cannot conclude that their antimicrobial activity is superior to CHX-loaded liposomes without chitosan.

Although we were unable to confirm the difference in the efficiency of these formulations due to the large SD, we are still able to see a trend that indicates that chitosan formulations with CHX exhibited higher antimicrobial activity than the other formulations, and liposomes with CHX had better activity than the liposomes containing only chitosan. Hemmingsen and colleagues discovered a similar finding. They found that chitosomes with CHX had the strongest antibacterial activity and liposomes with CHX had stronger antimicrobial activity than liposomes containing only chitosan. Even though the antimicrobial activity is reported as a minimum bactericidal concentration in this finding, and thus cannot be compared directly to our results, both studies demonstrated a similar pattern in antimicrobial activity (62).

Despite the large SD and lack of statistical significance, the results still give some promising indications. The experiment should be repeated in order to acquire more reliable results.

4.7 Anti-inflammatory activity

The anti-inflammatory activity of chitosan-coated liposomes was evaluated by assessing the reduction in nitric oxide (NO) production in LPS-induced murine macrophages. This is because NO is known to plays a very important role in the inflammation process and as an immunomodulator (49). Production of NO in LPS-induced murine macrophages without treatment was used as control (100% NO production) and the results were expressed as a percentage of NO production relative to the control. The results are presented in Figure 24. Three different lipid concentrations were tested to assess how various concentrations affected the anti-inflammatory activity of the formulations.

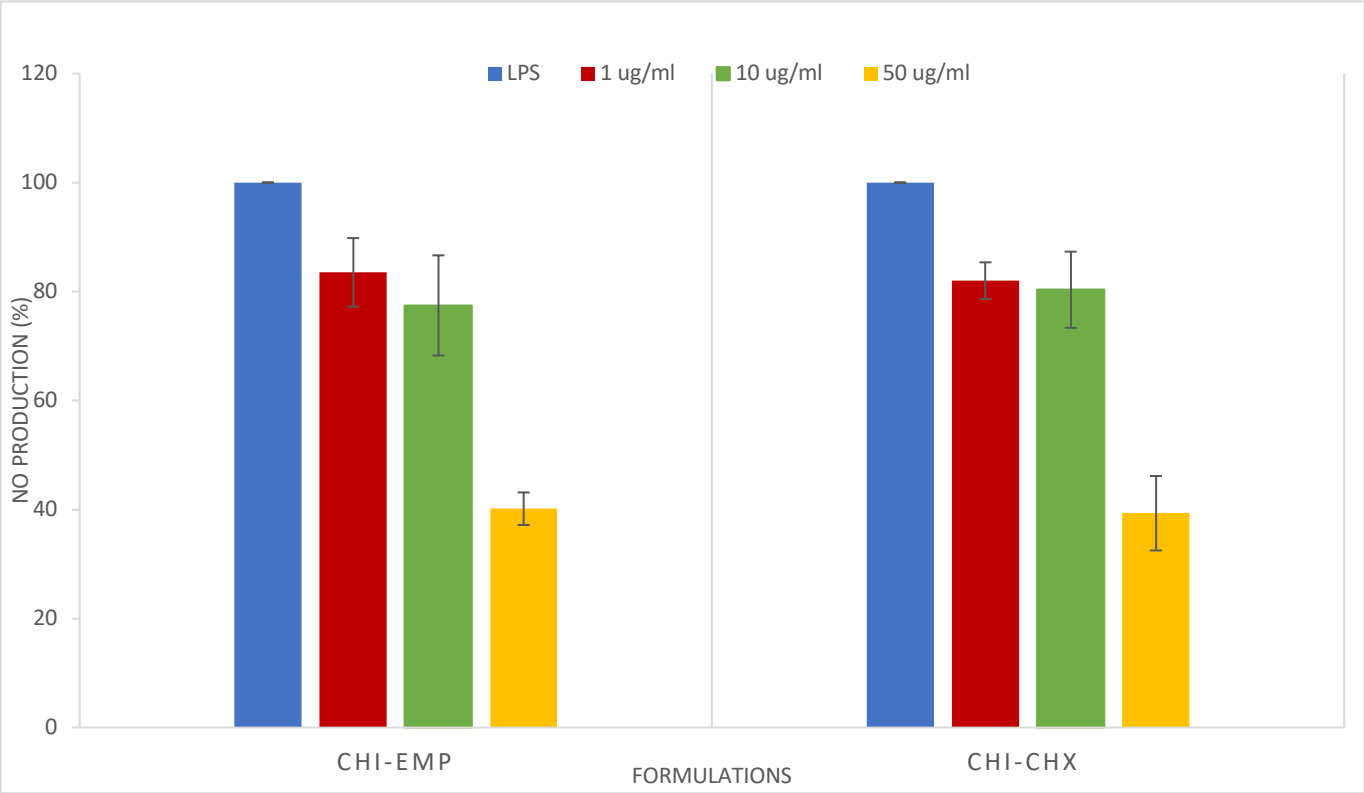

Figure 24 Anti-inflammatory activity of chitosan-coated liposomes with and without CHX. The anti-inflammatory activity is shown as production of nitric oxide (NO) in different formulations compared to control (LPS-induced, non-treated cells): LPS: LPS-induced murine macrophages without formulations, 1 µg/ml: LPS-induced murine macrophages with 1 µg/ml of formulations, 10 µg/ml: LPS-induced murine macrophages with 10 µg/ml of formulations, 50 µg/ml: LPS-induced murine macrophages with 50 µg/ml of formulations. CHI-CHX: chitosan-coated liposomes with CHX and CHI-emp: chitosan-coated liposomes without CHX

When compared to the control, both formulations demonstrated a significant reduction in NO production at all concentrations ($p < 0.05$). However, there was no statistically significant difference in NO production between 1 and 10 µg/ml. Both formulations provide the greatest decrease in NO production at 50 µg/ml. This data suggests that both formulations have anti-inflammation properties, and that the formulations' anti-inflammatory activity might be concentration dependent, with higher concentrations (between 10 and 50 µg/ml) increasing the anti-inflammatory activity. This finding is similar to Ahsan's findings. He also found that higher lipid concentrations (50 µg/ml), cause the most pronounced reduction in NO production. Chitosan-based formulations have been reported to possess anti-inflammatory properties, which might make it useful in the treatment of chronic wound infections (62). The liposomes used in this project and those used by Ahsan have very similar compositions, and Ahsan observed a difference in NO production between liposomes with and without chitosan (55). Chitosomes demonstrated higher anti-inflammatory activity than liposomes without chitosan in Ahsan´s project. This indirectly supports our hypothesis about the anti-inflammatory properties of chitosan formulations.

In addition, it would be interesting to assess how CHX affects inflammatory activity of the formulation. At every concentration, there was no significant difference in NO production between liposomes with and without CHX. This finding suggests that CHX has no influence on the anti-inflammatory activity of the formulations, thus the effect is attribute to chitosan. Although CHX does not contribute to the reduction in inflammation, it worth mentioning that CHX does not induce inflammation. This is important because induction of inflammation from CHX is not preferred in treatment of chronic wounds.

Inflammation is an essential part of wound healing because it promotes the removal of pathogens and death cells from the wound area. However, in many chronic wounds, the inflammation phase is prolonged, and wound fails to progress through the inflammation stage. A constant state of inflammation in wounds contribute to the wounds non-healing status (12).

Therefore, the development of a formulation capable of reducing inflammation might be advantageous in the treatment of wound infections. Due to the lowering of inflammation, the chitosan formulation would be more beneficial for chronic wounds than acute wounds since excess levels of inflammation mediators were reported in chronic wounds (12). Nonetheless, the antimicrobial activity evaluation of the formulations in section 4.6 indicates that when

modified with chitosan, the formulations had enhanced antibacterial activity which would be favourable for both acute and chronic wounds. According to the findings, CHX-loaded chitosan formulations appear to be promising formulations for treating wound infections as they were able to minimize inflammation and enhanced antimicrobial activity. Despite the promising results derived from these two formulations, additional testing of the other formulations is required to confirm the postulation.

5. Conclusion

In this project, the chitosan-modified lipid-based drug delivery system was developed for membrane-active antimicrobials primarily intended to treat skin injuries prone to infections. CHX was used as a model compound for peptidomimetic, 8b.

Two chitosan-modified drug delivery systems were prepared, namely chitosomes and chitosan-coated liposomes. To evaluate the influence of chitosan and CHX on vesicle properties, six formulations were tailored. The formulations were investigated in terms of size and size distribution, zeta potential, entrapment efficiency, *in vitro* release, chitosan surface-availability, vesicle stability, antimicrobial activity, and anti-inflammatory activity.

The size and size distribution of all formulations are in the accepted range indicating that probe-sonication is an effective technique to reduce the size of liposomes. Chitosan increased the zeta potential of vesicles to a positive value, which is desirable for interaction with bacterial membranes. Furthermore, the high zeta potential could indicate stability, and the stability study confirmed good stability of chitosan formulations. The CHX entrapment efficiency was relatively high in all formulations and superior in chitosomes. The chitosan surface availability study confirmed the presence of chitosan on the surface and that the methods applied for coating and adding chitosan to liposomes with CHX were successful. Together with zeta potential, these findings confirmed that both chitosan and CHX were available on the surface, which is advantageous for interaction with bacteria. The chitosan formulations displayed a prolonged release that could provide a long-term effect on bacteria in the wound site. Moreover, the chitosan formulations containing CHX showed a tendency of enhanced antimicrobial activity against *S. aureus*, however, the SD were large, and the study is inconclusive. Additionally, the chitosan-coated liposomes were able to decrease inflammation in LPS-induced macrophages in a concentration-dependent manner.

Based on the findings in this thesis, we believe that the approach used to prepare these liposomes is a promising method. In addition, the results support our hypothesis about the potential of chitosan-modified liposomes as a promising drug delivery system for membrane-active antimicrobials destined to treat wound infections.

6. Perspectives

Short-term perspectives

- Complete the planned experiments
    - Toxicity evaluation
    - Repeat antimicrobial susceptibility testing
    - Lipid quantification
    - Anti-inflammatory activity of all formulations
- Evaluate the antimicrobial activity in other common skin pathogens, such as *S. epidermis*
- Evaluate the effect of chitosan formulations on biofilm formation and biofilm eradication
- Used TEM to examine the chitosan-coated liposomes to find out the effect of chitosan on liposomes size and zeta potential after the twelfth week
- Examine the effects and interactions of CHX and chitosan on liposomes, as well as their accommodation

Long term perspectives

- Prepare liposomes modified by other polymers such as hyaluronic acid and compare their properties to chitosan formulations.
- Develop secondary formulation for liposomes
- *In vivo* studies

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Appendix I

The size and PI of different liposomal formulations from the second week to the twelfth week (Table A1, A2 and A3).

Table A1 The stability evaluation of different liposomal formulation at second week after production.

Stability **2 weeks**	**Size (nm) ± SD**			
	Peak 1 %	**Peak 2 %**	**Peak 3 %**	**PI**
CHIemp	33 ± 21 4 ± 5	158 ± 86 53 ± 36	430 ± 262 43 ± 41	0.32 ± 0.00
LIPemp	40 ± 15 8 ± 3	172 ± 59 60 ± 15	687 ± 269 32 ± 18	0.44 ± 0.05
CHI-emp	17 ± 5 2 ± 1	75 ± 14 18 ± 4	312 ± 33 80 ± 4	0.36 ± 0.01
LIP_CHX	23 ± 3 3 ± 2	109 ± 15 50 ± 18	341 ± 112 47 ± 19	0.40 ± 0.02
CHI_CHX	18 ± 7 6 ± 6	97 ± 23 35 ± 15	212 ± 76 60 ± 17	0.28 ± 0.01
CHI-CHX	23 ± 10 2 ± 1	119 ± 46 29 ±11	382 ± 46 69 ± 13	0.39 ± 0.02

Results are presented as mean size of each peak ± SD, and mean PI ± SD

Table A2 The stability evaluation of different liposomal formulation at fourth week after production.

Stability **4 weeks**	**Size (nm) ± SD**			
	Peak 1 %	**Peak 2 %**	**Peak 3 %**	**PI**
CHIemp	39 ± 5 7 ± 3	177 ± 17 53 ± 17	373 ± 53 41 ± 16	0.31 ± 0.01
LIPemp	36 ± 20 18 ± 15	138 ± 78 48 ± 32	502 ± 227 35 ± 19	0.39 ± 0.05
CHI-emp	20 ± 7 2 ± 1	93 ± 30 24 ± 7	355 ± 105 74 ± 8	0.37 ± 0.01
LIP_CHX	38 ± 3 10 ± 2	163 ± 5 70 ± 3	362 ± 37 21 ± 2	0.40 ± 0.01
CHI_CHX	15 ± 5 2 ± 1	71 ± 40 29 ± 16	226 ± 124 69 ± 16	0.28 ± 0.02
CHI-CHX	23 ± 14 2 ± 2	120 ± 66 31 ± 18	388 ± 214 67 ± 20	0.42 ± 0.03

Results are presented as mean size of each peak ± SD, and mean PI ± SD.

Table A3 The stability evaluation of different liposomal formulation at twelfth week after production.

Stability **12 weeks**	**Size (nm) ± SD**			
	Peak 1 %	Peak 2 %	Peak 3 %	PI
CHIemp	42 ± 8 6 ± 3	199 ± 33 64 ± 24	544 ± 77 30 ± 26	0.33 ± 0.01
LIPemp	42 ± 18 8 ± 6	204 ± 95 62± 8	540 ± 317 30 ± 12	0.40 ± 0.04
CHI-emp	22 ± 0 2 ± 1	125 ± 20 24 ± 5	535 ± 77 74 ± 4	0.36 ± 0.01
LIP_CHX	24 ± 7 5 ± 3	85 ± 18 35 ± 8	225 ± 65 60 ± 11	0.41 ± 0.01
CHI_CHX	21 ± 7 3 ± 1	115 ± 35 41 ± 18	327 ± 68 56 ± 18	0.28 ± 0.00
CHI-CHX	22 ± 14 2 ± 1	98 ± 65 33 ± 19	293 ± 154 65 ± 21	0.42 ± 0.03

Results are presented as mean size of each peak ± SD, and mean PI ± SD.

Appendix II

The initial plan of this project was to investigate effect of different polymer on properties of liposomal formulation. One of the polymers that was supposed to be used to make a liposomal formulation was pectin. The original idea was to create pectosomes with and without CHX to compare it with chitosomes. The pectosomes without CHX were made successfully and negative zeta potential could indicate the presence of pectin on liposomes (Table A6). However, rehydrating of liposomes with CHX led to precipitation. Due to this, pectin-coated formulation was suggested as an alternate formulation. Unfortunately, adding pectin solution to the liposome suspension during the coating process also resulted in precipitation. As a result, the pectin formulations were excluded from further testing.Procedures for developing of pectin formulation are described below.

Method and materials

Pectin from citrus peel, Galacturonic acid ≥74.0%, Sigma-Aldrich, Denmark

Method

Preparation of pectosomes

Lipid film for pectosomes was prepared with lipid film hydration method as described for chitosomes with or without CHX. The resulting lipid films were rehydrated with 10 ml of 0.2 % (w/v) pectin solution under shaking for 45 min with Edmund Buhler shaker. The dispersions were kept in the refrigerator overnight before further experiments. Pectin solution was prepared by dissolved pectin in distilled water. The solution was left under magnet stirring overnight before rehydrating of lipid films (64).

Preparation of pectin-coated liposomes

Liposome dispersions for pectin-coated liposomes were prepared with the same method for empty liposome (without CHX) or plain liposome with CHX. Size reduction of liposomes was performed before coating with pectin. For liposomes with CHX, the unencapsulated CHX had to be removed before coating. 0.2 % (w/v) pectin solution for coating liposomes were prepared as described for pectosomes. Coated liposomes were prepared by adding an equal amount of pectin solutions to liposomal dispersions under magnet stirring (250 rpm). The polymer

solution was added in a drop-wise manner (1.22 min/ml). After the addition of polymer solutions was completed, the dispersions were left under stirring for 1 hour.

Size reduction

The liposome dispersions were placed on the ice bath to prevent overheating under sonication. The needle probe tip is inserted just beneath the surface of the sample. The sonicator was set to 40% amplitude and were sonicated with different times to achieve the desired size. Pectosomes were sonicated for 10 seconds with 10 intervals and then were extruded with 0,4 mm membrane filter for 3 times. For pectin coated liposomes, the empty liposomes were sonicated with 10 seconds for 18 times. For formulation with CHX, the dispersions were precipitated after adding pectin solutions. Liposomes for pectin coated were sonicated with the same procedure as the plain liposomes with CHX (LIP_CHX) before coating.

Table A4 Overview of sonication time used for size reduction of pectin formulations.

Formulation	Sonication time (second)	Sonication interval	Extrusion (round)
PECemp	10	10	3
PEC-emp	10	18	-
PEC_CHX*	*	*	*
PEC-CHX	5	1	-

*PEC_CHX were precipitated after adding pectin solutions

The size and zeta potential of pectin formulations are displayed in Table A5 and A6, respectively.

Table A5 Size and PI for pectin formulations

	Size (nm) ± SD			
	Peak 1 %	**Peak 2 %**	**Peak 3 %**	**PI**
PECemp	47 ± 8 6 ± 2	163 ± 79 48 ± 30	517 ± 214 46 ± 31	0.38 ± 0.03
PEC-emp	22 ± 8 3 ± 2	111 ± 38 39 ± 24	374 ± 141 62 ± 29	0.43 ± 0.13
PEC_CHX			precipitation	
PEC-CHX			precipitation	

Results are presented as mean size of each peak ± SD, and mean PI ± SD.

Table A6 Zeta potential of pectin formulations

Formulation	**Zeta potential (mV) ± SD**			
	Production	**2 weeks**	**4 weeks**	**12 weeks**
PEC-emp	-10.7 ± 12.7	-	-	-
PECemp	-3.0 ± 0.1	-	-	-
PEC_CHX		precipitation		
PEC-CHX		precipitation		

Results are presented as mean ± SD.