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Optimization of antimicrobial wound dressings:

Liposomal hydrogels with mupirocin

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Abstract

Skin injuries represent an important health problem that needs to be managed properly in order to avoid serious consequences in terms of morbidity, disability and life quality. The current focus in wound therapy is on upholding the moisture balance in the wound bed and protection against pathogenic invasion. Wound dressings in a form of hydrogels incorporating antimicrobials, such as the antibiotic mupirocin, can be applied to control dermal infections and thereby ensure optimal wound healing. Drug-in-liposomes-in-hydrogel may additionally provide prolonged contact between drug and wounded area, reducing the need for frequent application of wound dressing.

Mupirocin calcium was incorporated in nonsonicated and sonicated liposomes made of soybean lecithin. We have focused on optimization of both vesicle and hydrogel characteristics in order to optimize the combined delivery system. The first step was determination of maximum amount of mupirocin that can be entrapped in liposomes. For that purpose 3 and 4 mg/ml mupirocin suspensions were used. It seems that 3 mg/ml is the optimal amount of drug which can be used for liposome preparation, under these preparation conditions and liposomal composition. Size analysis of sonicated liposomes indicated that the particles were smaller than 300 nm, optimal for topical administration onto the skin. The stability testing suggested that liposomes were sensitive to heat and pH change. *In vitro* evaluation of the cytotoxicity of liposomal and free mupirocin in HaCaT cells proved that the drug and delivery system are nontoxic in a concentration range 1 to 100 µg/ml. Assessment of anti-biofilm effect of liposomally entrapped and free mupirocin showed that both formulations had an impact on biofilm formation and planktonic bacteria, however no clear advantage of liposomal formulation over free drug was seen.

Chitosan and Carbopol hydrogels, both empty and liposomal (10 % (w/w)), were evaluated for their affinities to bind extra liquid, such as wound exudates. Chitosan hydrogels were found to exhibit satisfactory absorbing properties, in contrast to the Carbopol hydrogels. Regarding moisturizing properties, both types of hydrogels were found to be able to donate liquid, assuring a moist wound environment.

In conclusion, liposomal hydrogels in which mupirocin is incorporated in liposomes, are safe and have potential to be used as wound dressing assuring maintenance of a moist wound environment. Chitosan liposomal hydrogels would be recommended for wounds with exudates, as their absorbing properties were better than for Carbopol-based hydrogels.

Key words: wound; infection; wound dressing; liposomes; hydrogels; mupirocin calcium

Sammendrag

Hudskader representerer et viktig helseproblem som må håndteres riktig for å unngå alvorlige konsekvenser når det gjelder sykelighet, uførhet og livskvalitet. Gjeldende fokus for sårbehandling er opprettholdelse av fuktbalanse i såret samt beskyttelse mot sykdomsfremkallende organismer. Sårforbindinger i form av hydrogeler med antimikrobielle elementer som antibiotikumet mupirosin kan brukes for å kontrollere hudinfeksjoner og dermed sørge for optimal sårtilheling. Legemiddel-i-liposom-i-hydrogel kan i tillegg forlenge kontakten mellom legemiddel og det skadede området, og dermed redusere behovet for hyppig påføring av sårforbinding.

Mupirosin kalsium ble inkorporert i usonikerte og sonikerte liposomer laget av soyalecitin. Vi har fokusert på optimalisering av karakteristika til både vesikkel og hydrogel for å optimalisere det kombinerte legemiddelleveringssystemet. Det første steget var bestemmelse av maksimal mengde av mupirosin som kan bli inkorporert i liposomer. Det ble brukt suspensjoner av mupirosin med konsentrasjonene 3 og 4 mg/ml. Det virker som at 3 mg/ml er den optimale mengden legemiddel som kan brukes for fremstilling av liposomer, under disse fremstillingsforholdene og denne liposomsammensetningen. Størrelsesanalyse viste at partiklene var mindre enn 300 nm, noe som er optimalt for topikal administrering på hud. Stabilitetstesting tydet på at liposomer var følsomme for varme og pH-forandring. *In vitro* evaluering av cytotoksisiteten av liposomal og fri mupirosin i HaCaT celler viste at legemiddel- og leveringssystemet ikke er toksisk i et konsentrasjonsområde fra 1 til 100 µg/ml. Vurdering av antibiofilmeffekten til liposomal mupirosin og fritt legemiddel viste at begge formuleringene hadde en innvirkning på dannelsen av biofilm, men ingen klar fordel av liposomal formulering fremfor fritt legemiddel ble observert.

Chitosan- og Carbopolhydrogeler, både tomme og liposomale (10 % (w/w)), ble undersøkt for deres affinitet til å binde ekstra væske, som sårvæske. Chitosanhydrogeler ble funnet å inneha tilfredsstillende absorberende egenskaper, i motsetning til Carbopolhydrogelene. Når det gjaldt fuktighetsgivende egenskaper ble begge typer hydrogeler funnet å ha evnen til å gi bort væske, og sikre et fuktig sårmiljø.

Som konklusjon er liposomale hydrogeler der mupirosin er inkorporert i liposomer trygge og har potensiale til å bli brukt som sårforbinding for å sikre opprettholdelse av et fuktig sårmiljø. Liposomale chitosanhydrogeler kan anbefales for sår med sårvæske ettersom deres absorberende egenskaper var bedre enn hydrogeler basert på Carbopol.

Nøkkelord: sår; infeksjon; sårforbinding; liposomer; hydrogeler; mupirosin kalsium

List of Abbreviations

ATCC	American type culture collection
CFU	Colony Forming Units
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
EL	Empty liposomes
EPS	Extracellular polymeric substances
FCS	Fetal Calf Serum
HaCaT	Immortalized human keratinocytes
LM	Mupirocin loaded in liposomes
M	Free mupirocin in propylene glycol
MC	Mupirocin calcium, referred in text as mupirocin
NSL	Nonsonicated liposomes
PCS	Photon correlation spectroscopy
PI	Polydispersity index
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Standard error of the mean
SL	Sonicated liposomes
TSA	Tryptic soy agar
TSB	Tryptic soy broth

1. General introduction

Each year millions of people around the world experience skin injuries of both acute and chronic nature. The estimated number of people suffering from chronic wounds annually is 37 million (Wild et al., 2010). This does not only represent disadvantages for the individual patient regarding morbidity, disability and life quality, but it also affects the economics of the countries. It is believed that the United States alone spends about one third of their health budget related to skin therapy on management of non-healing chronic wounds (Schreml et al., 2010).

Over the past fifty years, wound therapy has moved from the aim to dry out the wound bed to realizing that maintenance of a balanced humid environment is essential for an optimal healing process (Harding et al., 2000; Bowler, 2002). Traditional dressings which had their main function as absorbing wound exudate, and generally led to formation of crust on the wound surface and remarkable scarring have been widely replaced by modern dressings that aim to improve healing by handling wound fluid in a way that prevents accumulation of excess exudate while maintaining a certain degree of moisture, and thereby enhancing the chance of obtaining new skin tissue without scarring (Cutting, 2010).

Although several types of wound dressings are available, including products which contain active pharmaceutical ingredients, the ideal wound dressing does not exist as no single dressing have all the desired properties that are needed to support wound healing. These properties include the ability to maintain moisture balance, act as a barrier against pathogens, prevent infection, exchange water, vapor and gases, be easy to handle with regard to application and removal, not require frequent changes, provide pain relief and odor control as well as being biocompatible (Seaman, 2002). In order to choose a suitable wound dressing it is necessary to consider the wound type and the state of the tissue as well as the activity level and personal needs of the patient (Ovington, 2001). The performance of the chosen dressing will be affected by several factors such as the presence of underlying diseases, nutrition state, amount of wound exudate and the microflora of the wound (Fonder et al., 2008).

Injured skin is susceptible to infection due to the breakage in the natural cutaneous barrier which normally protects the underlying tissue against foreign invasion. The outcome of an

infection depends on the degree of pathogenicity of the invading organisms and the ability of the host to defend itself (Ryan, 2007). Skin infections are defined as complicated and uncomplicated depending on the type of treatment that is necessary (May, 2009). Wound infections are mainly caused by microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus epidermis* and streptococci either in their free-floating state or in biofilms where they are attached to a surface and protected by surrounding extracellular polymeric substances (EPS) produced by them. Infected skin that is left untreated may delay healing and even lead to death (Percival et al., 2012).

Wound dressings containing antimicrobials, either antiseptics or antibiotics, can be applied in order to control wound infections while reducing the risk of unwanted effects as they act locally more than systemically. The ideal topical antimicrobial drug should have broad activity, be microbicidal and safe to use without being allergenic (Spann et al., 2003). Liposomal hydrogels incorporating mupirocin has been proposed as a promising wound dressing due to their potential to retain at the site of treatment for a longer time and the ability to provide controlled release of the drug (Berg, 2011) which in this case is an antibiotic considered to be appropriate for application in topical infections since it is active against a broad range of microorganisms without affecting the normal skin flora to the same extent (Williford, 1999).

During the process of optimization of an antimicrobial wound dressing it is necessary to establish the storage stability of the product as loss of original properties due to instability may lead to decreased or lost performance (Shi and Schofield, 2004). Another issue that needs to be evaluated is the toxicity and safety of the formulation. It is important to confirm that the antimicrobial will affect only the unwanted microorganisms and not the human skin cells (Kempf et al., 2011). As wound infections can be caused by both planktonic bacteria and biofilms (Percival et al., 2012) it is therefore essential to determine whether the wound dressing has an effect on these species. Finally, the ability of wound dressing to absorb wound exudate and provide moist environment need to be evaluated (Thomas et al., 2005).

2. Introduction

2.1. Wound dressings

Skin injuries represent an important medical problem. Every year several millions of people are affected and in need of proper treatment in order to avoid morbidity, disability and resulting impaired life quality, costing the health system a prominent amount of resources (Singer and Clark, 1999; Fan et al., 2011; Peck, 2011). Skin lesions that require care include both acute and chronic wounds. Acute wounds include mechanical injuries resulting from external factors such as trauma and surgical treatment, and skin damages caused by burn or chemical exposure (Schultz et al., 2003; Li et al., 2007; Boateng et al., 2008). The most common causes of chronic wounds are diabetic, arterial, venous, and pressure ulcers (Pieper et al., 1999; Fan et al., 2011). In the United States alone, the estimated number of people in need of medical attention every year due to burns is more than 1 million (Rafla and Tredget, 2011; Rnjak et al., 2011) and over 6 million are receiving treatment for chronic wounds (Xue et al., 2009; Wild et al., 2010). Adequate wound therapy is therefore essential.

Until the 1960s the main focus in wound therapy was to absorb wound exudate and keep the wound dry as a moist environment was considered to impair the healing process. Wound management was mostly based on the application of traditional dressings that would allow evaporation of moisture. Today, there is a general consensus on that good hydration and moisture balance in the wound bed is most important for optimal wound healing. Wound surfaces exposed to air may dry and result in a hard crust where the collagen matrix underneath and the wound edge tissue are desiccated. In these cases keratinocytes have to burrow beneath the crust surface and matrix in order to migrate into the wound so reepithelization can take place. However, if the wound is kept moist, the healing process will be improved as a result of physiological promotion of migration, matrix formation and autolytic debridement. As a result of these findings, modern dressings concentrate on retaining and creating a moist environment by various types of occlusion (Schultz et al., 2003; Schultz et al., 2004; Boateng et al., 2008; Fan et al., 2011; Korting et al., 2011).

2.1.1. Classification of wound dressings

Dressings can be, in general, divided into traditional and modern dressings. Cotton wool, natural or synthetic bandages and gauzes are referred to as traditional dressings, while modern dressings include hydrocolloids, alginates, hydrogels, semipermeable adhesive film dressings, foam dressings, biological dressings and tissue engineered skin substitutes. Further categorization can be based on the functionality of the dressing (occlusive, absorbent etc.), the type of material (hydrogel, collagen etc.), and the physical form of the product (gel, ointment etc.). Dressings can also be classified as primary dressings which are in physical contact with the wound surface, secondary dressings that cover the primary dressing, and island dressings made up of an absorbent region in the middle and a surrounding adhesive part (Boateng et al., 2008).

2.1.2. Wound dressings available on the market

Several types of wound dressings are available, each having their advantages and disadvantages, making them suitable for different types of wounds (Ovington, 2001; Fonder et al., 2008).

Gauzes have been widely used in wound care up through the history due to their ability to offer good absorption and the fact that they are affordable and easily accessible. However, as later research has shown the importance of moist wound bed, traditional cotton gauze was found to be inappropriate for wounds that produce little wound exudate. Their indication nowadays is mainly for packing of deep wounds. In addition, the drying behaviour of gauzes can potentially lead to discomfort and trauma at removal. Other than drying, traditional gauzes also have the disadvantage of being a poor barrier against bacterial invasion once the dressing surface is moistened, and require frequent changing (Boateng et al., 2008; Fonder et al., 2008).

Films are thin adhesive and semiocclusive membranes that can be used as both primary and secondary dressings. They manage moisture by vapor transmission and are good barriers against foreign liquid and bacteria. As film dressings are non-absorbent and hence may lead to trapping of fluid and subsequently maceration of wound tissue, they are recommended for wounds with minimal wound exudate or as secondary dressings. Film dressings have the

advantage of only adhering to the dry periwound area which reduces the risk of pain during changing of dressing. Patients using films can also benefit from the fact that the dressings can be left in place without being changed for up to 7 days (Schultz et al., 2003; Schultz et al., 2004; Fonder et al., 2008).

Hydrocolloids are adhesive, occlusive and absorbent dressings. Too heavy exudate may lead to fluid trapping causing tissue maceration when hydrocolloids are used as wound dressing. Therefore, they are recommended for wounds with low to moderate amounts of exudate. Hydrocolloid dressings can be worn for a long time before change is needed, an important feature as frequent removal can lead to skin stripping due to the strong adherence of the dressing to the skin (Schultz et al., 2003; Schultz et al., 2004; Boateng et al., 2008; Fonder et al., 2008).

Alginates are fibrous dressings which form into gels upon contact with the moisture in wounds and are able to absorb high amounts of fluid, suitable for management of moderately and heavily exudating wounds. They can be used to control minor bleeding due to their hemostatic properties. Additionally, alginates may provide protection against bacterial contamination, and an optimal environment for wound healing by regulation of moist and temperature in the wound bed. A downside with alginate dressings is that they may leave fibrous debris upon removal of the dressing (Paul and Sharma, 2004; Schultz et al., 2004; Boateng et al., 2008; Fonder et al., 2008).

Foams are dressings which have the ability to absorb moderately amounts of fluid making them useful for management of wounds with light and moderate levels of exudate. They are semiocclusive, and they can provide thermal insulation of the wound and protect it against shear. A disadvantage with foams is their potential for development of malodorous drainage which requires their frequent changes (Schultz et al., 2003; Boateng et al., 2008; Fonder et al., 2008).

Biological dressings are dressings containing biomaterials that support wound healing. Examples of biomaterials used in wound dressings are collagen, elastin and chitosan. Their biocompatibility and generally low toxicity make them attractive for wound care (Boateng et al., 2008; Fonder et al., 2008).

Hydrogels were first developed in the 1950s, and applied to wound therapy about 30 years later (Kennedy-Evans and Lutz, 2010). They are semipermeable and have the ability to transmit vapor and water, provide moisture to the wound, and obtain relief by their cooling effect (Fan et al., 2011). Studies showing the importance of having a moist environment when treating chronic wounds have accelerated development of hydrogel-based wound dressings (Korting et al., 2011). Other reasons for the great attention given to hydrogels are their potential to provide controlled release of drugs to the specific wound site, and the fact that they can be easily removed when the desired effect is achieved (Boateng et al., 2008).

Hydrogels promote wound healing by donating liquid and hydrating the injured tissue, thereby improving the environment for effective wound healing. Their high ability to moisturize makes hydrogels a preferable choice when the aim is to facilitate autolytic debridement in necrotic wounds and when a moist environment is needed in nonsloughy wounds. In addition to reduced pain and tenderness, and less chance for wound infection, healing of wounds in a moist environment has been shown to give better cosmetic outcomes (Schultz et al., 2003).

Depending on the dressing and the degree of hydration of the wounded tissue, the hydrogels are also able to take up a certain amount of moisture (Fonder et al., 2008; Kennedy-Evans and Lutz, 2010). However, hydrogel dressings do not have very high capability to absorb exudates from wounds compared to other types of dressings such as hydrofibers (Schultz et al., 2003; Korting et al., 2011). Application of hydrogels as wound dressings is therefore recommended for dry to minimally to moderately draining, sloughy or clean wounds, where the main focus is on providing a moist environment, and were wound fluid control comes as a secondary concern (Kennedy-Evans and Lutz, 2010). They are also a good choice for when pain relief is one of the aims of treatment (Fonder et al., 2008). Hydrogel wound dressing are mostly used for shallow wounds, skin tears, second-degree burns, partial- and full-thickness wounds, chronically damaged epithelium and dermatitis caused by radiation (Kennedy-Evans and Lutz, 2010).

2.1.3. The ideal wound dressing

The desired wound dressing should promote rapid healing by maintaining a moist environment, absorbing exudate without drying out the wound surface, protecting from

microbial invasion, preventing infection, and allowing for gaseous exchange. It should also be easy to apply, be adhesive enough to achieve adequate residence time, but easy to remove without leading to discomfort or trauma, and not require frequent changes (Lin et al., 2001; Seaman, 2002; Schultz et al., 2003; Brett, 2006). The ideal wound dressing should not contain particulate contaminants that may be left in the wound and lead to infection (Vermeulen et al., 2005). A wound dressing having analgesic properties and ability to control odor is also beneficial (Lait and Smith, 1998). Novel dressings are based on drug delivery systems for which the biocompatibility is a major concern when designing the formulation of a drug carrier. A wound dressing should not be toxic or allergenic (Jayakumar et al., 2011).

No single dressing fulfills all of the preferences mentioned above, and the choice of dressing depends on the wound type and the state of the tissue, which can vary at different stages of the healing process. The activity level and personal needs of the patient should also be considered when choosing the most suitable dressing (Schultz et al., 2003; Korting et al., 2011).

2.1.4. Wound dressings incorporating active pharmaceutical ingredients

So-called active dressings or medicated dressings have been developed by incorporating antimicrobials, growth factors, or supplements such as minerals and vitamins into the system. Cleansing or debriding agents can be incorporated for the purpose of removing necrotic tissue, and antimicrobials, growth factors and supplements will act against infection and aid regeneration of tissue, respectively (Boateng et al., 2008).

2.2. Characteristics of wounds affecting performance of wound dressing

When developing treatment for damaged skin, it is crucial to take the changed properties of the skin into account. Wounded skin no longer has normal anatomic structure and function, and the penetration barrier is less efficient than in healthy and intact skin (Lazarus et al., 1994; Korting and Schäfer-Korting, 2010).

The time frame and outcome of wound repair is influenced by many factors such as the size and depth of the wound, possible pathological changes due to underlying diseases, moisture and exudate content, flow of oxygenated blood, presence and amount of microorganisms, and

nutritional status (MacKay and Miller, 2003; Brett, 2006; Li et al., 2007; Boateng et al., 2008; Korting et al., 2011).

2.2.2. Wound healing

Wound healing is a complex process consisting of four steps: haemostasis, inflammatory reaction, proliferation and remodeling, all of which are regulated by cytokines and growth factors released by cells in the wounded area. The phases are overlapping and linear for acute wounds, whereas the chronic wounds can be found at different stages of the healing process and do not heal in orderly manner (Li et al., 2007).

Haemostasis occurs within a few minutes after a tissue is injured. The disruption of blood vessels and the resulting leakage of blood into the wound are followed by platelet activation and aggregation. This will then lead to the formation of a fibrin clot which causes the bleeding to stop and plugs the defect and seals off the exposed tissue. Drying of the clot forms a scab that provides a temporary protection to the damaged skin in addition to serving as a provisional matrix for cell migration and as a source for cytokines and growth factors (Martin, 1997; Li et al., 2007; Heng, 2011; Korting et al., 2011).

The inflammatory reaction takes place soon after haemostasis and can last for more than 72 hours. This phase consists of attraction of neutrophils and monocytes from the circulating blood to the wounded area leading to cleansing and elimination of germs and debris. The infiltration of immune cells is a result from chemotactic signals from growth factors, epitopes of invading microorganisms, and byproducts of proteolysis of fibrin and other matrix components (Singer and Clark, 1999; Shaw and Martin, 2009; Heng, 2011; Korting et al., 2011).

The proliferative phase of the wounded skin starts 4-5 days after injury and lasts for about 2-3 days. It consists of reepithelization and wound contraction. Reepithelization involves migration of keratinocytes into the wound, proliferation of keratinocytes, regeneration of the basal cells that connects the epidermis and the dermis, and reconstitution of the dermis. The latter is carried out by formation of new blood vessels, fibroblast proliferation and formation of extracellular matrix such as collagen. Wound contraction is achieved by the differentiation

of fibroblasts to myofibroblasts which have the ability to extend and retract, and the attachment of fibroblasts to collagen leading to the foundation of a scar tissue (Boateng et al., 2008; Heng, 2011; Korting et al., 2011).

Remodelling of wounds is an equilibrium between formation of new cellular connective tissue and its degradation by proteases. This stage, which may continue for months, is characterized by modification of the structural integrity of the tissue with the aim of restoring normal architecture of the skin. Depending on the regulation of this maturation process the final result may either be a scar that is indistinguishable from the healthy skin, which is the goal, or scar tissue that elevates above the surrounding unwounded skin, indicating a deficient regulation of the process (Martin, 1997; Li et al., 2007; Shaw and Martin, 2009; Heng, 2011).

2.2.3. Wound exudate

Wound healing often leads to the production of wound exudate which plays an important role in all the stages of wound healing. The volume and content of the wound fluid is associated with the healing potential of the wound. A high amount of wound exudate may give rise to microbial growth and hence infection. Excess wound exudate may also lead to maceration of the skin. The physicochemical properties of the exudate indicate whether the wound is chronic or acute, and also suggest the level of bacterial burden. Wound drainage from acute wounds are characterized by normal levels of pro-inflammatory cytokines, matrix metalloproteinases, tissue inhibitors of metalloproteinases and growth factors, whereas chronic wounds produce exudates with higher amount of pro-inflammatory cytokines and matrix metalloproteinases, and lower levels of tissue inhibitors of metalloproteinases and growth factors. Infected wounds will exude purulent and odorous fluids, in which the quality can imply the causing organism. Proper management of wound exuduate is crucial for successful wound healing (Brett, 2006).

2.2.4. Microbiology of wound

The microflora of wounds appears to change over time. Hence, the microbiology of wounds is different for acute and for chronic wounds. While the microflora of acute wounds is similar to that of intact skin, chronic wounds are colonized by bacteria to a larger extent. This increases

the risk of pathogenic invasion into viable tissue and subsequently infection which will delay the wound healing process (Mertz and Ovington, 1993).

2.3. Wound infection

Wound infection is an important infectious complication in patients with skin wounds since microbial colonization and following infection can occur when the natural cutaneous barrier is broken, exposing the underlying tissue (Weinstein and Mayhall, 2003). A burn wound will comprise of an immunocompromised skin area, and the loss of natural protection that is normally provided by the immune system makes the skin vulnerable and prone to infection (Dai et al., 2009; Percival et al., 2012).

The progress of microorganisms in a wound from colonization to infection depends on the concentration of pathogens, the virulence factors they produce and the resistance of the host to infection (Edwards and Harding, 2004). The risk of infection is increased by different factors such as presence of vascular disease, edema, malnutrition, diabetes and corticosteroids. The patient is also more susceptible to infection if the tissue is necrotic and if the wound is deep or over a large area (Dow et al., 1999; Schultz et al., 2003; Fan et al., 2011).

Wound infections can be termed as uncomplicated and complicated. Uncomplicated infections refer to conditions that can be handled with simple antibiotical treatment and without surgical intervention, such as infections in minor trauma-related wounds, while complicated infections are infections that go into deeper tissues or require surgical intervention. Infections that occur in the presence of an underlying disease state are also termed as complicated. Examples of complicated wound infections are infections in burn wounds, ulcers and diabetic wounds (May, 2009).

Typical features of wound infections are increased wound fluid, increased swelling of the affected area, increased erythema and pain, odor and raise in temperature. A change in granulation tissue may also indicate an infection of the skin (Hutchinson and Lawrence, 1991; Fan et al., 2011).

The majority of secondary skin infections are caused by *Staphylococcus aureus* and streptococci (Williford, 1999). The microorganisms *Pseudonomas aeruginosa*, *Escherichia coli,* and *Staphylococcus epidermis* are also known to contribute to skin infections (Percival et al., 2012). Pathogenic bacteria resistant to multiple drugs are an increasing problem in the treatment of contaminated burns. Current therapy needs to be improved in order to surmount this holdback (Dai et al., 2009).

Whereas planktonic, free-floating bacteria are mainly the cause of acute infections, the phenomenon of biofilms is primarily associated with chronic infections (Wolcott and Dowd, 2011). Biofilms may be defined as sessile communities of bacteria living together on a surface either by attachment to a substratum, interface or direct contact between the bacterial cells. The biofilm cells are surrounded by a mucilaginous matrix of extracellular polymeric substances (EPS) which are produced by the bacteria themselves. Microorganisms known to form biofilms are *Staphylococcus epidermis*, *Staphylococcus aureus*, *S. lugdunensis*, *Propionbacterium acnes*, *S. pyogenes* and *C. jeikeium*, *Pseudomonas aeruginosa* and *Escherichia coli* (Kennedy et al., 2010; Percival et al., 2012). Biofilms can give rise to chronic wound infections due the protective role of the EPS. This protection gives the microorganisms enhanced virulence and subsequently production of sustained host hyperinflammation. In addition, persistent infections caused by biofilms are known to have a high degree of resistance against antibiotic treatment (Martineau and Dosch, 2007; James et al., 2008; Wolcott and Dowd, 2011).

Wound infections are associated with significant morbidity and mortality. Inadequate care of wound infections may lead to a reduced healing response, loss of soft tissue, limb amputation and death (Edwards and Harding, 2004; May, 2009). Recent reports suggest that microbial infection is the cause of death for at least 10 000 people for every million wound patients (Percival et al., 2012).

2.4. Wound dressings with antimicrobials

Antimicrobial wound dressings provide local treatment and the therapeutic effect is most effective in the dermis and the superficial dermis as the active ingredient is concentrated in the skin surface and less is reaching the subcutaneous fat. The ideal antimicrobial drug for

topical treatment should have broad activity, be microbicidal, safe (nontoxic) and not leading to allergic reactions (Kaye, 2000).

Evidence suggests that topical treatment with antimicrobial agents can limit the risk of infections in wounds while reducing the risk of adverse effects (Diehr et al., 2007). Antimicrobial wound dressings may contribute in reestablishment of the normal relationship between the bioburden and the host defense by reducing the bacterial load and thereby preventing bacterial byproducts from damaging the wound bed (White et al., 2006).

By incorporating antibiotics into hydrogels, they can be applied in the management of infected wounds. Hydrogels containing antibiotics have been shown to be able to control the odor from wound exudate. Hydrogels loaded with antibiotics for local treatment reduce the risk of unintended patient exposure and adverse effects, as the drug is allowed to exert its effect locally while systemic absorption is avoided (Boateng et al., 2008).

2.4.1. Different dressings with antimicrobials

Antimicrobial wound dressings may contain either antiseptics or antibiotics. Antiseptics can be applied to kill or inhibit microorganisms and have the potential to target multiple microbials. They have a broad antimicrobial spectrum, but do not have an optimal safety profile as they are often toxic to the human skin tissue, including fibroblasts and keratinocytes. Examples of antimicrobial wound dressings with antiseptics are cadexomer iodine dressing, chlorhexidine gluconate foam, povidone iodine hydrogels and silver dressings. Antibiotics, on the other hand, are generally nontoxic, but they often act against a narrower spectrum of bacteria, and their effect may be reduced or even lost due to development of bacterial resistance. Examples of topical antibiotics used for wound management are bacitracin, fusidic acid, gentamicin and mupirocin (Lipsky and Hoey, 2009).

2.4.2. Antimicrobials in liposomes for wound treatment

In order to ensure controlled release of active ingredient incorporated in wound dressing, novel drug delivery systems, such as liposomes, have been proposed (Berg, 2011). Because liposomes can function as a sustained release system for drugs, releasing the drug over longer period of time, liposomes can be applied to control the rate of drug release (Allen, 1998;

Pjanovic et al., 2010). Studies have shown that incorporating drugs into liposomes can increase drug's skin penetration (Korting and Schäfer-Korting, 2010). Regarding wound management, the potential of liposomes to deliver moisture and lipid molecules to the horny layer, even without incorporating any drug, is useful for promoting wound healing (Lasic, 1992). Liposomes have several properties that make them suitable as drug carrier; they can entrap both lipophilic and hydrophilic drugs, are easy to prepare, biodegradable and nontoxic. Their size can be manipulated through choice of preparation method (Torchilin, 2005).

Liposomes are small artificial vesicles of globular shape composed of aquatic pores encapsulated with amphiphilic phospholipid and- or cholesterol bilayer that are able to encapsulate the active drug (Figure 1) (Mishra et al., 2010). When the lipids are dispersed in aqueous media, the liposomes form spontaneously. The vesicles can range in size from several nanometres to a few microns in diameter (New, 1990). They can be classified according to their size and lamellarity; small unilamellar vesicles, large unilamellar vesicles, oligolamellar vesicles and multilamellar vesicles (Brandl, 2001).

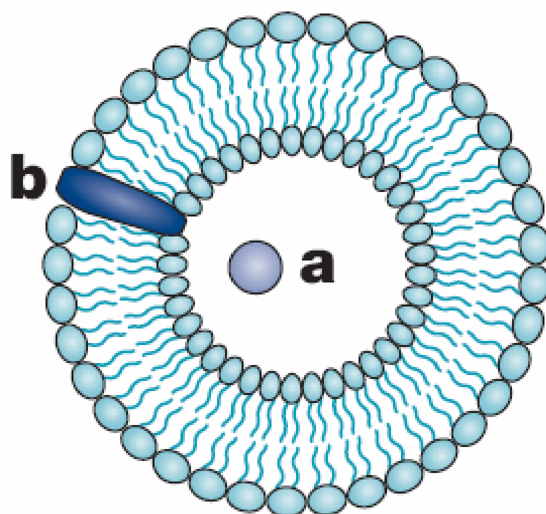

Figure 1: Structure of a liposome where the drug is localized in the aqueous core (a) and in the lipid membrane (b). Reprinted from Torchilin (2005).

Depending on the drug properties and the encapsulation process, the active pharmaceutical ingredient (API) will either be incorporated in the aqueous core or into the lipid membrane (Figure 1). By making the API take on the pharmacokinetic characteristics of the carrier, liposomes have the ability to alter both the tissue distribution and the rate of clearance of the drug (Zamboni, 2008). The pharmacokinetic parameters of the liposomes depend on their

physicochemical characteristics, such as size, surface charge, membrane lipid packing, steric stabilization, dose, and route of administration (Brandl, 2001). Problems of poor drug solubility, instability and rapid degradation can be solved by using liposomes as drug carriers (Allen, 1998). This applies to both hydrophilic and hydrophobic active substances. Lipophilic substances can be entrapped in the liposomal membrane, while hydrophilic compounds can be dissolved in the inner liquid core (Martin et al., 2006).

A study done by Price et al. (1990), comparing the antimicrobial efficacy of liposomal drug in polyurethane sponge with the efficacy of free drug in a form of solution, showed that one single application of liposomal antimicrobial resulted in the same effect in tissue bacterial counts as observed after multiple doses of free drug. This indicates the potential advantage of liposomal wound dressings to decrease bacterial counts without requiring frequent dressing changes as would be the case for free drug in solution. The reduced total amount of medication required to reach wanted therapeutic effect would also be beneficial when using dressings incorporating liposomal drugs in wound treatment (Price et al., 1990).

Pjanovic et al. (2010) performed a study in which they compared drug diffusion from liposome-based hydrogels, hydrogels containing free drug and drug in a form of solution. They found that the release of drug from hydrogels with liposomal drug and hydrogels incorporating free drug was prolonged in comparison to solution. Moreover, the diffusion rate from liposomal hydrogels was shown to be slower than from hydrogels containing free drug. The authors used two different drugs in the study and found that the drug diffusion from liposomes were similar for both drugs, indicating that the phospholipid bilayer is the main reason for sustained drug diffusion. This indicates that controlled release can be achieved by modifying the composition of the phospholipid bilayer or by optimizing the method for preparation of the liposomes (Pjanovic et al., 2010).

2.4.2.1. Mupirocin calcium in wound dressings

Wound dressings containing antimicrobials, such as mupirocin, can be used in the treatment of wounds to prevent infection. Mupirocin calcium (Figure 2) is the calcium salt of pseudomonic acid, an antibiotic produced by fermentation of *Pseudomonas fluorescens*. Its antibacterial mechanism is through inhibition of bacterial protein synthesis by binding to the

enzyme, isoleucyl-transfer-RNA synthase (Winkelman and Gratton, 1989; Lamb, 1991). This binding results in blocking of incorporation of isoleucine into proteins (Winkelman and Gratton, 1989).

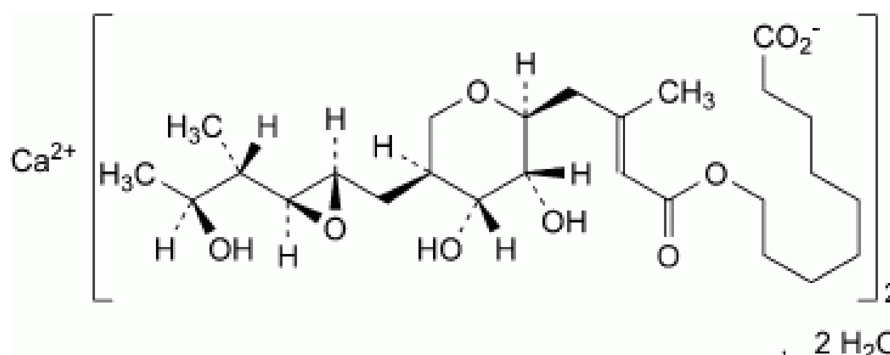

Figure 2: Structure of mupirocin calcium (European Pharmacopoeia online database 7th Edition (7.3).)

Due to its unique chemical structure and mechanism of action, cross resistance with other antibacterial drugs is not a concern (Lamb, 1991; Williford, 1999). Mupirocin has low affinity for the mammalian enzyme, and does not lead to toxicity in humans. It does not show toxicity to human fibroblasts or keratinocytes, or to cultured human skin grafts. Microccus, corynebacterium and propionibacterium which are part of the normal skin flora are not affected, meaning that mupirocin does not inhibit the natural defense of the body (Williford, 1999).

At low concentrations the drug acts as bacteriostatic, but at higher concentrations mupirocin will be bactericidal. Mupirocin has activity against most gram-positive organisms, including staphylococci and streptococci. In addition to the gram-positive bacteria, the drug also acts against certain gram-negative organisms including *Haemophilus influenza*, *Neisseria gonorrhoeae* and *meningitidis*, *Branhamella catarrhalis*, and *Pasteurella multocida*. The fungi *Candida albicans* is also affected (Williford, 1999).

When administered onto the skin, the systemic absorption of the drug is minimal, promoting mupirocin as an ideal drug for topical treatment (Williford, 1999; Echevarría et al., 2003). When applied to skin with damaged barrier properties more mupirocin is expected to penetrate through deeper layers potentially leading to being absorbed into systemic circulation. However, this is not a major concern as mupirocin is not effective systemically

due to its rapid conversion into an inactive metabolite, monic acid, which is rapidly cleared out of the kidneys (Winkelman and Gratton, 1989; Echevarría et al., 2003). The fact that mupirocin shows the ability to reside in the skin for a longer period of time, up to several days, is beneficial for topical administration as dermal treatment depends on a sufficient residence time of the pharmaceutical formulation on the area to be treated. Studies have also shown that mupirocin is able to accelerate wound healing (Williford, 1999).

2.5. Optimization of antimicrobial wound dressing

2.5.1. Stability upon storage

Pharmaceutical products stored over time may undergo different changes that will affect their properties and functionality. These changes are generally referred to as loss of originally attributed properties or even degradation. When a product loses original properties, the change may lead to decreased or lost performance. Establishment of the shelf life of a product before marketing is therefore essential. For this purpose stability tests can be applied. The stability of a product may be defined as the length of time that the product is able to resist degradation, and is dependent on the storage conditions such as temperature and humidity. Stability testing can be carried out under normal storage conditions or under stress conditions. These methods are known as real-time stability tests and accelerated stability tests, respectively. The latter is usually preferred in industry as it requires shorter time to perform than needed for testing under normal conditions. Products stored in elevated stress conditions, such as at a higher temperature, are expected to degrade faster (Magari, 2002; Magari et al., 2004; Magari and Afonina, 2011).

Liposomes can undergo different changes that will influence their structure, size and distribution. Chemical degradation of the phospholipids may lead to leakage of originally entrapped drug, and aggregation or fusion of the liposome particles. As this will have an impact of the ultimate performance of the liposomes it is necessary to determine their stability (Van Bommel and Crommelin, 1984; Amselem et al., 1989; Grit and Crommelin, 1993). By exposing liposomes to different temperatures and comparing their physical properties before and after exposure, it is possible to predict the liposome stability in relationship to the impact heat will have on their characteristics (Grit and Crommelin, 1993; Pavelić et al., 2005).

2.5.2. Toxicity and safety of wound dressings

When developing an antimicrobial wound dressing it is important to keep in mind the safety of the dressings. If skin cells themselves are affected by the antimicrobial or any other drug present in the dressing , the process of wound healing may be impaired and delayed (Kempf et al., 2011). Any possible toxicity issue needs to be addressed before animal and potential human studies. Safety to the patient needs to be confirmed and should accompany drug efficacy data (Vinardell and Mitjans, 2008).

There are several different approaches to preclinically determine the skin toxicity of products destined for topical application, including *in vitro* testing based on human cell cultures and human skin models, and *in vivo* methods based on animal models (Osborne and Perkins, 1994; Botham et al., 1998; Vinardell and Mitjans, 2008). Animal studies for cutaneous toxicity have the disadvantage of potentially causing the experimental animals discomfort and pain which raises ethical concerns and require specific approvals. They are also time consuming and expensive and are gradually being replaced by improved *in vitro* models. Another drawback in using animal experiments to determine the formulation's safety is the morphological difference between animal and human skin. *In vitro* assessment of cytotoxicity has been found to be a good predictor of formulation's skin toxicity (Osborne and Perkins, 1994; Lee et al., 2000; Eisenbrand et al., 2002; Bhogal et al., 2005; Vinardell and Mitjans, 2008).

2.5.2.1. *In vitro* cytotoxicity testing on cultured human skin cells

One of the most used non-animal models for predicting the effect of new products on human skin tissue is the cultured human skin cells model, commonly consisting of keratinocytes and fibroblasts. Testing on human skin cultures is regarded as safer, more efficient and more cost-effective practice as compared to the use of laboratory animals (Osborne and Perkins, 1991; Ponec, 1992; Korting et al., 1994; Lee et al., 2000; Vinardell and Mitjans, 2008). *In vitro* models based on submerged cultures of human skin cells are useful as they allow for the production of a large number of cells which makes it possible to apply substances in a broad concentration range for screening of toxicity (Ponec, 1992).

HaCaT cells, which are immortalized human keratinocytes, have been used for the evaluation of skin cytotoxicity of different substances (Boukamp et al., 1988; Korting et al., 1994;

Wilhelm et al., 2001; Kempf et al., 2011). To determine the potential cytotoxicity of a wound dressing, HaCaT cells can be applied in cell death analysis by exposing them to the wound dressing and subsequent addition of a specific dye. If the product being tested shows to be toxic to human skin cells, the HaCaT cells will die and become permeable to the dye, thereby making it possible to distinguish between the dead cells and the surviving cells. HaCaT cells are the most commonly used model and were used in cytotoxicity testing for evaluation of adsorbent pads, impregnated gauze dressings, foam pads, antimicrobial barrier dressings, adhesive removers, creams and ointments (Kempf et al., 2011).

2.5.3. Effect on biofilms

Microbiological evaluation of wound dressing is inevitable as antimicrobial treatment of wounds aims to prevent or treat infection by reducing the bacterial load in the wound (Bowler et al., 2001). When establishing the antimicrobial effect of a wound dressing it is important to investigate specific types of microorganisms that can be part of the microflora in wounds. As the majority of wounds contain biofilms, rather than isolated strains of bacteria, it is not adequate to test formulation only on free bacterial strains. It has been suggested that bacteria living within biofilm communities are more protected and resistant against antibiotics than single free species (Edwards and Harding, 2004; Percival et al., 2012). Hence, the antimicrobial effect of the wound dressing needs to be determined for biofilms.

2.5.4. Stability of wound dressing against wound exudate

Absorbent dressings are applied to many different types of surface wounds. The efficacy of these absorbent dressings needs to be tested by simulating their use on a real-life wound. Testing ensures that the characteristics of the dressings are standardized and that the product is fit for purpose. Methods for testing based upon standards such as British Standard European Norms can be used for this purpose and are usually applied by pharmaceutical industries as suitable. The fluid affinity of a hydrogel dressing indicates how the dressing will behave once exposed to different levels of wound exudates. Therefore, it is necessary to determine the absorption and moisture-donating properties of the dressing in laboratory conditions. This can be done by imitating the situation where the wound dressing is applied on exudating wound and the situation where the wound requires moisture for proper healing. The model used to test the absorbent properties of the hydrogel will hence consist of the

hydrogel applied to a material that mimics a moist wound, while the ability to provide moisture will be tested in a model consisting of the hydrogel on a material mimicking a dry wound (Thomas et al., 2005; Boateng et al., 2008). This model was applied by Thomas et al. (2005) who compared the fluid affinity of two different hydrogel dressings by using a method based upon the European norm, "BS EN 13726-1:2002 Test methods for primary wound dressings. Part 1 Aspects of absorbency, Section 3.4, Fluid affinity of amorphous hydrogel wound dressings." (Thomas et al., 2005).

2.6. Evaluation of factors affecting wound dressing performance

The performance of a wound dressing is affected by several interrelated factors which will determine the types of wounds the dressing will be applicable for. If the wound dressing is found to be safe and nontoxic to human cells while depriving unwanted microbes the product is suitable for treatment of wounds were there is a risk of developing an infection or in already infected wounds. An antimicrobial wound dressing should be effective against the specific types of pathogens present in the wound. Whether the wound dressing will affect free-floating bacteria or bacteria in biofilms or both is relevant as this will affect the applicability of the wound dressing. The ability of dressing to absorb exudate or donate moisture will determine whether the dressing is suitable for exudating or for dry types of wounds, respectively. If the dressing is found to be both absorbing and moisturizing, it can be used on both wounds with high amount of fluid and wounds which are of drier nature. The frequency of application of wound dressing is correlated to the degree of controlled and prolonged release of the active ingredient from the wound dressing. If the dressing is able to release the drug in a controlled manner over a longer period of time it would imply that the product can be used without need for frequent changing, and the patient would feel more comfortable during the treatment.

3. Aims of the study

The main aim of this project was optimization of antimicrobial wound dressing for improved wound therapy. Liposomal hydrogels with mupirocin have been proposed as delivery system providing prolonged and controlled release of the incorporated drug. We have focused on optimization of vesicle and hydrogel systems in respect to drug load, vesicle characteristics, toxicity and hydrogels properties.

More specific aims were:

- Optimization of liposomal formulation in regard to mupirocin entrapment efficiency and vesicle size and size distributions
- Evaluation of liposomal stability under stress conditions and storage at room temperature
- Determination of *in vitro* safety and toxicity in keratinocytes model
- Evaluation of anti-biofilm potency of liposomal delivery system in biofilm skin model
- Evaluation of the potential of liposomal hydrogels to absorb wound exudates and maintain a moist environment for dry wound

4. Materials and Methods

4.1.1. Materials

Acetonitrile, Sigma-Aldrich®, Steinheim, Germany

Agar, Sigma-Aldrich®, Steinheim, Germany

Ammonium acetate, VWR International, Leuven, Belgium

Calcium chloride 6-hydrate, Analar®, Poole, UK

Carbopol Ultrez 10 NF, Noveon Inc., Cleveland, USA

Cells; HaCaT, a generous gift from Research Group of Pharmacology, Department of Pharmacy, University of Tromsø, Tromsø, Norway

Chitosan, high molecular weight, Sigma-Aldrich®, Steinheim, Germany

Crystal violet*

Dialysis tubing, , Size 1 Inf Dia 8/32" – 6,3 mm: 30 M (Approx), M W C O – 12-14000 Daltons, Medicell International Ltd, London, UK

Distilled water

Dimethyl sulfoxide (DMSO), Sigma-Aldrich®, Steinheim, Germany

Ethanol (96 %, v/v), Sigma-Aldrich®, Steinheim, Germany

Fetal Calf Serum (FCS), a generous gift from Research Group of Pharmacology, Department of Pharmacy, University of Tromsø, Tromsø, Norway

Gelatin, from porcine skin, Type A, Sigma-Aldrich®, St. Louis, USA

Glycerol (86-88 % w/w), Sigma-Aldrich®, Steinheim, Germany

Lipoid S 100 (soybean lecithin; over 94 % phosphatidylcholine), Lipoid GMBH, Ludwigshafen, Germany

Methanol CHROMASOLV ®, Sigma-Aldrich®, Steinheim, Germany

Mueller-Hinton media*

Mupirocin calcium dihydrate, a generous gift from Pliva, Zagreb, Croatia

Penicillin G*

Microtiter 96-polystyrene well plates, Nunclon™ Δ surface, Nunc, Denmark

Propylene glycol, Norwegian Medicinal Depot, Oslo, Norway

Resazurin*

RPMI-1640 MEDIUM with L-glutamine and $NaHCO_3$, Sigma-Aldrich®, Steinheim, Germany

Sodium chloride, Sigma-Aldrich®, Steinheim, Germany

Staphylococcus aureus (ATCC 25923)*

Triethylamine, Merck, Honenbrunn, Germany

Trypan blue solution (0.4 %, w/v), Sigma-Aldrich®, Steinheim, Germany

Trypsin, Sigma-Aldrich®, Steinheim, Germany

Tryptic soy agar, Fluka Biochemika, Buchs, Switzerland

Tryptic soy broth, Fluka Biochemika, Buchs, Switzerland

*Used in experiments at The Pharmaceutical Sciences Laboratory, Department of Biosciences in the Division of Natural Sciences and Technology, Åbo Akademi University, Åbo, Finland

4.1.2. Instruments

Axioskop 40 Pol, Carl Zeiss Light Microscopy, Göttingen, Germany

Biofuge pico, Heraeus Instruments, Osterode, Germany

Biomek 3000 liquid handling workstation*

Branson 1510 Bath Sonicator, Branson ultrasonics, Danbury, USA

Branson 5510 Bath Sonicator, Branson ultrasonics, Danbury, USA

Büchi Rotavapor R-124, Büchi labortechnik, Flawil, Switzerland
Büchi Vacuum Controller B-721, Büchi labortechnik, Flawil, Switzerland
Büchi Waterbath B-480, Büchi labortechnik, Flawil, Switzerland

Combi multidrop dispenser*

Distillation unit Distinction D4000, Bibby Sterilin LDT, Stone, UK

NICOMP Submicron particle sizer model 370, Particle Sizing Systems, Inc, Santa Barbara, USA

pH Meter 744, Metrohm, Metrohm AG, Herisau, Switzerland

Varioskan Multimode Plate Reader, Thermo Fischer Scientific Oy, Vantaa, Finland

Ultrasonics VibraCell VC 754, 750 watt ultrasonic processor, CVR 234 converter, probe 19 mm (diameter), Sonics and Materials, Newtown, USA

Waters 996 Photodiode Array Detector, Milford, USA

Waters 2690 Separations Module, Milford, USA

Xterra® RP18 5µm 3.9x150mm Column, Waters, Dublin, Ireland

*Used in experiments at The Pharmaceutical Sciences Laboratory, Department of Biosciences in the Division of Natural Sciences and Technology, Åbo Akademi University, Åbo, Finland

4.1.3. Computer programs

Windows CW370 Software, NICOMP Particle Sizing Systems – CW370 Version 1.33

Millenium^{32} Version 3.20

GraphPad software, Prism 5.0c for Mac OS X, USA (2011)

4.2. Preparation and characterization of liposomes

4.2.1. Preparation of empty liposomes

Empty liposomes were prepared by the modified film hydration method described by New (1990). Lipoid S 100 (200 mg) was dissolved in approximately 20-25 ml of methanol in a 50 ml round bottom flask. Methanol was evaporated in a rotavapor for at least 1 hour at 50 mmBar at 45 ºC. The dry film was hydrated by adding 10 ml of distilled water followed by hand shaking till the phospholipid film became dislodged. The formed liposomes were refrigerated overnight before further use and characterization.

4.2.2. Preparation of liposomes containing mupirocin calcium

Liposomes containing mupirocin calcium were prepared by the film hydration method (New, 1990) with modifications by Berg (2011). In brief, mupirocin calcium (MC; 20, 30 and 40 mg, respectively) was dissolved together with Lipoid S 100 (200 mg) in approximately 20-25 ml of methanol in a 50 ml round bottom flask. Methanol was evaporated in a rotavapor for at least 1 hour at 50 mmBar at 45 ºC. The dry film was hydrated by adding 10 ml of distilled water, followed by hand shaking to dislodge the film. The formed liposomes were refrigerated overnight before further use and characterization.

4.2.3. Size reduction of liposomes

The size of liposomes was reduced by applying probe sonication as described by New (1990) with slight modification. A 10 ml beaker with liposomal suspension was placed on ice bath, and the needle probe tip of a sonicator immersed and positioned in the middle of the suspension. The liposomes were exposed to ultrasonic irradiation with amplitude set to 40 for a continuous cycle of 2 minutes. The total sonication time was 2 minutes for all samples. After sonication the samples were refrigerated overnight before further use and characterization.

4.2.4. Particle size analysis

The particle size analysis was performed by photon correlation spectroscopy (PCS), also known as dynamic light scattering (DLS) on a NICOMP Submicron particle sizer model 370. The instrument was equipped with a fixed 90 ºC external fiber angle and a 638.8 nm helium-neon laser. Sample preparation and all handling were carried out in a laminar airflow bench, and particle-free equipment was used to avoid environmental contamination. Prior to use, the test tubes were filled with distilled water and sonicated in an ultrasonic bath for 10 minutes, before further rinsing with filtered distilled water (using 0.2 μm pore size syringe filter). The vesicle suspensions were diluted empirically with filtered distilled water until an intensity of 250-350 kHz was obtained. Analysis was carried out at 23-25 ºC and corresponding viscosity. The refractive index was set to 1.333, and the channel width was set to automatical adjustment (Hupfeld et al., 2006). All analyses were performed in triplicates with a run time of 10 minutes for each cycle in vesicle mode, where the intensity weighted distribution of vesicles was evaluated.

Acquisition and treatment of data was performed using the software CW370 Version 1.33.

4.2.5. Entrapment efficiency determination

The entrapment efficiency was determined by separating free drug from liposomally-associated drug by applying dialysis tubing (Pavelic et al., 1999; Skalko-Basnet et al., 2000). Dialysis membranes were first submerged and soaked in water for 20 minutes. Samples of liposomal suspension were then placed in the tubes and dialysed against distilled water in the ratio 1:217 (v/v). After 24 hours, the samples were taken out, and liposomal suspension and dialyzate further diluted. Liposomal suspension was first dissolved in methanol in the ratio 1:100 (v/v) and this solution further diluted in acetonitrile and water (1:10, v/v) in the ratio 1:1 (v/v). The dialyzate was diluted in acetonitrile and water (1:10, v/v) in the ratio 1:1 (v/v). The diluted liposomal suspension and dialyzate were then subjected to HPLC analysis.

For the entrapment efficiency determination of liposomal suspensions after stability testing, the degree of dilution was adjusted to fit within the concentrations of the MC standard curve. The liposomal suspension was dissolved in methanol in the ratio 1:10 (v/v) and this solution

diluted in acetonitrile and water (1:10, v/v) in the ratio 1:1 (v/v). The diluted liposomal suspension and the undiluted dialyzate were then subjected to HPLC analysis.

4.2.6. HPLC analysis

The method previously described by Echevarria et al. (2003) with modifications by Berg (2011) was applied in HPLC analysis. Standard curves based on seven standard solutions, namely 0.2, 0.5, 0.8, 2, 6, 10 and 20 µg/ml of MC in water and in methanol, respectively, were obtained by preparing the stock solutions of MC (40 µg/ml) and diluting these with appropriate volumes of acetonitrile and water (1:10; v/v). Acetonitrile and ammonium acetate in the ratio 27.5:72.5 (v/v) made up the mobile phase. Ammonium acetate (0.05 M) was filtered through a 0.2 µm pore size filter before mixing with acetonitrile. Hydrochloric acid was used to adjust the mobile phase to pH 6.3. The separation process was performed with a flow rate set to 1 ml/min, and a photodiode array detector set to monitor compounds at 228 nm. The temperature of the column was set to 30 ºC, and the sample temperature was set to 25 ºC. Volume injected was 20 µl and the run time was 9 minutes. All analyses were performed in triplicates, respectively.

Acquisition and treatment of data were performed using Millennium 32 Chromatography Manager (4.0).

4.3. Preparation and characterization of hydrogels

4.3.1. Preparation of empty chitosan hydrogels

Dispersion of chitosan in weak acids is known to result in a formation of hydrogel (Alsarra, 2009; Cao et al., 2009). The addition of glycerol have been shown to provide a more stable hydrogel (Hurler et al., 2012). Preparation of chitosan hydrogels was carried out as previously described by Hurler et al. (2012). In brief, chitosan hydrogels in a concentration of 2.5 % (w/w) were prepared by dispersing chitosan in a mixture of 2.5 % (w/w) acetic solution and glycerol (10 % w/w). The concentration of the glycerol was 86-88 % (w/w). The mixture was manually stirred for approximately 10 minutes before it was left to swell for 48 hours at room temperature.

4.3.2. Preparation of empty Carbopol hydrogels

The dispersion of Carbopol in water is acidic and very fluid. However, the addition of neutralizing agents is known to increase the consistency of the polymer dispersion and form a high-viscosity transparent gel (Hernandez et al., 1998; Skalko et al., 1998). A procedure described by Skalko et al. (1998) was slightly modified and used for preparing Carbopol hydrogels. Carbopol hydrogels in a concentration of 0.5 % (w/w) were prepared by dispersing determined amount of Carbopol in distilled water, followed by adding of triethylamine drop-wise under gentle hand stirring until pH 6 was reached. After mixing, the hydrogels were left to swell for 48 hours at room temperature before further use.

4.3.3. Preparation of liposomal hydrogels

Suspensions with empty liposomes (20 mg/ml) were mixed into chitosan hydrogels and Carbopol hydrogels prepared and described in section 4.3.1. and 4.3.3. applying hand stirring (Skalko et al., 1998). The final concentration of liposome suspension in the hydrogels was 10 % (w/w). When a smooth hydrogel texture was obtained, the hydrogel was left to stabilize at room temperature for at least 2 hours before further use.

4.4. Stability testing

4.4.1. Accelerated stability testing of nonsonicated and sonicated liposomes

Accelerated stability testing was carried out by incubating multilamellar and sonicated liposomal suspensions free of unentrapped mupirocin at 40 ºC for 30-days. The vesicle suspensions were kept in sealed containers. Changes in liposomal size and drug entrapment were determined by comparing the original size and drug entrapment of liposomes with the particle size and amount of drug still associated with the liposomes after accelerated stability testing (Basnet et al., 2012). The pH of the liposomal suspension was measured before and after completion of stability testing.

4.4.2. Real-time stability testing of nonsonicated and sonicated liposomes

Real time stability testing was carried out by storing multilamellar and sonicated liposomal suspensions free of unentrapped mupirocin at 23 ºC for 90 days. Changes in liposomal size and drug entrapment were determined by comparing the original size and drug entrapment of liposomes with the particle size and amount of drug still associated with the liposomes after room temperature stability testing. The pH of the liposomal suspension was measured before and after completion of stability testing.

4.5. Cell culture preparation

The cell cultures were prepared based on the procedure described by Basnet et al. (2012).

4.5.1. Preparation of cell growth medium

For the preparation of cell growth medium, a bottle of 500 ml of Roswell Park Memorial Institute (RPMI) medium and a container with 50 ml of Fetal Calf Serum (FCS) were stored for 2 hours at 37 ºC, prior of addition of FCS to the RPMI medium. The mixture was left to stabilize in refrigerator overnight. All preparations were carried out using aseptic technique in a safety cabinet (Basnet et al., 2012).

4.5.2. Cell seeding

Cell seeding was performed by leaving a 25 cm^2 flask with 6 ml of the cell growth medium for 1 hour at 37 ºC before adding 1 ml of thawed HaCaT cell suspension into the flask. The cell culture was kept in incubator at 37 ºC in an atmosphere of 5 % CO_2. The cell growth medium was changed after 24 hours and then after 48 hours until a confluent monolayer of cells was obtained. Changing of culture media was done by first removing the old media from the flask and replacing it with fresh culture media (pre-warmed for 1 hour at 37 ºC). Whilst growing, the cells were checked under microscope every day to make sure they were healthy and growing as expected. All cell handling was carried out using aseptic technique in a safety cabinet (Basnet et al., 2012).

4.5.3. Cells plating

The HaCaT cells in a confluent monolayer were washed with 10 ml of RPMI medium without FCS, in order to washing away the FCS, known inhibitor of trypsin. The FCS-media was then removed prior to adding 5 ml of pre-warmed trypsin. Addition of trypsin led to trypsinization of cells. The flask was kept in incubator at 37 ºC for 3 minutes before shaking and detaching the cells. The base of the flask was inspected under microscope to confirm detachment. The content was transferred into a 50 ml test tube. To neutralize trypsin, 10 ml of fresh pre-warmed cell growth medium containing FCS was added, and samples centrifuged at 1000 rpm for 2 minutes. The medium and trypsin were then removed, and 30 ml of cell growth medium containing FCS was added. Three ml of the mixture was transferred into a flask containing 10 ml of pre-warmed RPMI medium for growing new cells. The cells were grown as previously described. Preparation was carried out using aseptic technique in a safety cabinet (Basnet et al., 2012).

4.6. Cell toxicity testing

The testing of cell toxicity was performed based on the method by Kempf et al. (2011). A solution of MC dissolved in DMSO in a concentration of 10100 µg/ml and a liposomal suspension of MC in a concentration of 1192.4 µg/ml were diluted to 1, 5, 10, 50, 100 µg/ml MC, respectively, using appropriate volumes of cell growth medium. The diluted solutions and suspensions were added to the cell culture plates described in previous section (Figure 3). Three out of the 24 wells in each plate were left as controls. Cell growth medium served as control. Each concentration was tested in triplicates. Empty liposomes and DMSO (solvent) were also tested in concentrations of 5 and 100 µg/ml to exclude potential toxicity of empty liposomes and DMSO, respectively. The plates were left in incubator at 37 ºC and 5 % CO_2 for 24 hours.

The medium in the wells was removed and the wells were washed with 0.5 ml RPMI medium (without FCS) before adding 150 µl of trypsin, and plates left at 37 ºC for 2 minutes. The content in the wells was then transferred to eppendorf tubes. Two hundred µl of cell growth medium with FCS was added to stop trypsinization before centrifugation at 1000 rpm for 2 minutes. The trypsin supernatant was then removed and the cell pellets were resuspended in

0.5 ml of prewarmed medium. A mixture of 100 µl cell suspension and 100 µl trypan blue was kept in 37 ºC for 2 minutes before the cells were counted under microscope. Each plate was divided in three parts and counted respectively. Dead cells could be distinguished from living cells as they would appear blue due to the trypan blue dye. Alive cells were not permeable to trypan blue dye. The cell viability was calculated by subtracting the amount of dead cells from the total number of cells and expressed as percentage.

Control	1 µg/ml	5 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml
Control	1 µg/ml	5 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml
Control	1 µg/ml	5 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml
5 µg/ml	5 µg/ml	5 µg/ml	100 µg/ml	100 µg/ml	100 µg/ml

Figure 3: Schematic representation of the 24-well plate containing either liposomal MC or MC solution (depicted in yellow), and either empty liposomes or DMSO (depicted in grey), respectively.

4.7. Biofilm testing

The testing on liposomal formulations on biofilms was performed in cooperation with the group of Professor Pia Vuorela at Åbo Akademi University in Finland. Samples described in section 4.7.1. were prepared at Drug Transport and Delivery Research Group, and the rest of the work was executed in Finland.

4.7.1. Preparation of samples

Empty liposomes and liposomes containing MC were prepared as described under section 4.2.1 and 4.2.2. Entrapped drug was separated from free drug as described under section

4.2.5. The amount of liposomal drug was determined by HPLC. Empty liposomes, liposomal MC and MC dissolved in propylene glycol (10 %, w/w) were sent to the group of Professor Pia Vuorela at Åbo Akademi University in Finland for testing on biofilms. The concentration of MC was 1.729 mg/ml in all samples containing drug.

All the following descriptions, including the rest of this section and section 4.7.2., 4.7.3., 4.7.4 and 4.7.5., are citations of the report received from Docent Adyary Fallarero in Finland.

Before starting the experiments the samples were left for 30 minutes at room temperature and sonicated for 5 minutes in a bath sonicator. Penicillin G was used as positive control in all experiments. A stock solution of penicillin G in a concentration 20 mM in Mueller-Hinton media was prepared prior to the experiments.

4.7.2. Preparation of bacteria cultures

The bacterial strain chosen as model bacteria was *Staphylococcus aureus*, bacteria known of producing biofilm. Preparation of bacteria cultures were done according to Sandberg et al. (2008). Tryptic soy broth (TSB) was used for culturing bacteria. The bacteria were cultured under aerobic conditions at 37 ºC and 200 rpm until exponential growth up to a concentration of 10^8 Colony Forming Units (CFU) per ml was achieved. Spectrophotometric measurement of turbidity at 595 nm with the aid of a Varioskan Multimode Plate Reader, and colony counts on tryptic soy agar (TSA) plates were used to determine the bacterial concentration (Sandberg et al., 2008).

4.7.3. Planktonic and biofilm experiments

For the planktonic experiments, sterile, flat-bottomed, 96-polystyrene microtiter well plates were used for incubation of bacterial growth in the presence of samples for 18 hours at 37 ºC. Automatic measurement of the absorbance at 620 nm was done every 15 min by a Varioskan multimode plate reader aided by a kinetic loop. An automatic plate shaking step (240 rpm, 5 s) was made before each measurement. Biofilm formation was promoted by adding bacterial suspension grown to exponential phase. The bacterial concentration of the suspensions was 10^6 CFU/ml, and each of the wells in the 96-polystyrene microtiter well plates would contain

200 µl of suspension. The plates were incubated for 18 h under aerobic conditions at 37 ºC and 200 rpm in an incubator shaker. Compounds were added to the wells simultaneously with the bacterial suspensions for preventive anti-biofilm screenings. For measurement of the effects on mature biofilms, the biofilms were formed 18 hours before the planktonic solution in each well was replaced with the compound in fresh TSB. The plates were incubated for further 24 hours at 37 ºC and 200 rpm.

4.7.4. Biofilm quantification assays

The protocols described in Sandberg et al. (2008) and Sandberg et al. (2009) were used for evaluation of the viability of cells and the total biomass in the wells. Staining of biofilms with 20 µM resazurin for 20 minutes at room temperature and 200 rpm, and measuring of fluorescence at $\lambda_{excitation}$ = 560 nm and $\lambda_{emission}$ = 590 by a Varioskan multimode plate reader was followed by replacement of resazurin by crystal violet and staining for 5 minutes at room temperature by a Combi multidrop dispenser. Subsequently, the washing of the biofilms was done twice with milli Q-water with a Biomek 3000 liquid handling workstation. Finally, a Varioskan multimode plate reader was used to measure absorbance at λ = 595 nm after solubilizing the remaining dye in 96 % ethanol.

4.7.5. Data processing

Determination of the potency of the different formulations was done by a non-linear regression curve fitting with variable slope using GraphPad software, Prism 5.0 c for Mac OS X, USA (2011). The potencies were expressed as half-inhibitory concentrations, IC_{50} values. At least 8 concentrations were tested, each with 8 replicates in 3 independent experiments. Calculations of statistical parameters for evaluation of the performance of the anti-biofilm assays were done according to Sandberg et al. (2008) by the aid of the untreated biofilms and media control samples. The statistical parameters calculated were signal to window coefficient (Z'), signal-to-noise (S/N) and signal-to-background (S/B). Equations for these parameters are described in Sandberg et al. (2008).

4.8. Fluid affinity testing

The ability of hydrogels to donate moisture, or absorb liquid, was tested in the method described in the European norm, "BS EN 13726-1:2002 Test methods for primary wound dressings. Part 1 Aspects of absorbency, Section 3.4, Fluid affinity of amorphous hydrogel wound dressings" (Thomas et al., 2005).

In brief, gelatin (35 %, w/w) was prepared by adding gelatin powder to a solution of sodium chloride (142 mmol/L) and calcium chloride (2.5 mmol/L) under magnetic and manual stirring at 70 ºC. When gelatin became fully dissolved, the solution was kept in a thermostat at 100 ºC for 24 hours to obtain a gelatin solution free of air bubbles. Barrels of 60 ml syringes from which the nozzle ends have been removed and closed with rubber plugs (to form smooth-sided cylinders), were filled with 10 ± 0.1 g of gelatin (35 %, w/w). The syringes with gelatin plugs were left to stiffen for 2 hours before 10 ± 0.1 g chitosan and Carbopol hydrogels, both empty and liposomal, were placed onto the surface of the gelatin plugs, respectively (Figure 4). After incubation for 48 hours ± 30 minutes at 25 ± 2 ºC, the hydrogels were gently removed from the gelatin plugs, and re-weighed.

Agar (2 %, w/w) was prepared by adding agar to a solution of sodium chloride and calcium chloride containing 142 mmol/L of sodium ions and 2.5 mmol/L of calcium ions at 70 ºC under magnetic and manual stirring. Barrels of 60 ml syringes from which the nozzle ends have been removed and closed with rubber plugs, were filled with 10 g ± 0.1 g of agar (2 % w/w). The syringes with agar plugs were left to stiffen overnight before 10 g ± 0.1 g of empty and liposomal chitosan and Carbopol hydrogels were placed onto the surface of the agar plugs, respectively (Figure 4). After incubation for 48 hours ± 30 minutes at 25 ± 2 ºC, the hydrogels were gently removed from the agar plugs, and re-weighed.

For each experiment the pH of the hydrogels was measured before and after the incubation. The pH of the sodium chloride and calcium chloride solution was measured before incubation.

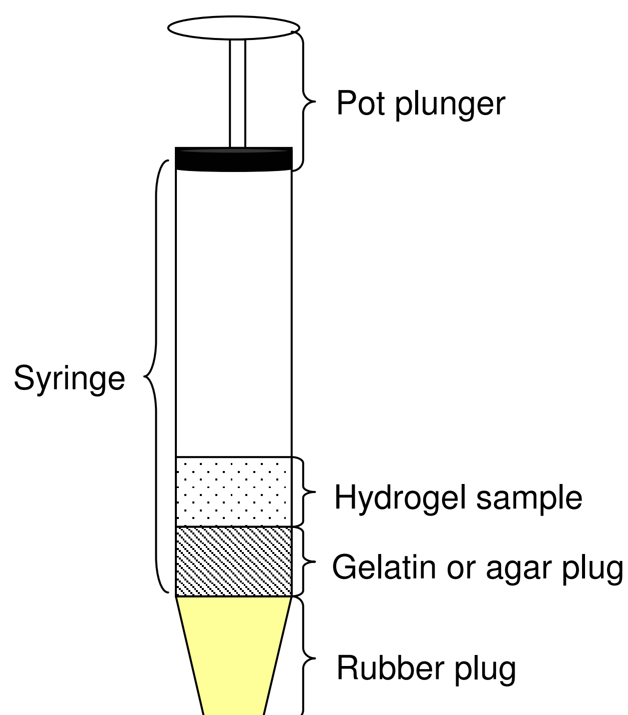

Figure 4: Schematic drawing of the fluid affinity setup

The following preparations were evaluated:

i) empty chitosan gel
ii) chitosan gel with empty nonsonicated liposomes
iii) empty Carbopol gel
iv) Carbopol gel with empty nonsonicated liposomes

All experiments were performed in triplicates.

4.9. Statistical evaluations

Statistical evaluations were performed by using *Student's t-test* to determine the level of significance.

5. Results and Discussion

5.1. Liposome characterization

The mupirocin entrapment efficiency was determined for liposomes prepared with 30 and 40 mg of MC (Table 1).

Table 1: Drug entrapment in liposomes

Type of liposomes	Amount of drug (mg)	Entrapment efficiency (%)	Drug/lipid ratio (µg/mg)	Drug recovery (%)
NSL	30	62.39 ± 8.80*	84.4 ± 11.4	89.66 ± 8.85
NSL	40	47.82 ± 6.85*	81.8 ± 12.0	90.47 ± 8.67
SL	30	32.10 ± 2.55	45.5 ± 2.0	78.76 ± 5.39
SL	40	29.30 ± 2.39	50.6 ± 3.6	90.25 ± 3.42

NSL; nonsonicated liposomes, SL; sonicated liposomes. All preparations contained 200 mg of lipid. The values denote the mean of 4 separate experiments ± SD. $^*p<0.05$

The first step of liposome characterization involved determination of the amount of drug that could effectively be incorporated into liposomes. Liposomes prepared with starting amount of 30 and 40 mg of MC were compared. Earlier, Berg (2011) prepared liposomes with 5, 10 and 20 mg of MC and we wanted to establish the maximum amount of MC which can be entrapped in liposomes without precipitation of unincorporated MC. As indicated in Table 1, the entrapment efficiency (expressed as percentage) was significantly higher ($p<0.05$) for the nonsonicated liposomes prepared with 30 mg of drug than for liposomes for which the starting amount of drug was increased to 40 mg. This trend was not seen for the sonicated liposomes as the difference was not found to be significantly different. However, when comparing the drug to lipid ratio no significant difference could be found between the 30 mg and 40 mg liposomal preparations, neither for the nonsonicated or the sonicated samples (Table 1). This would indicate the 30 mg as starting amount of MC is maximum amount. In addition, precipitates of drug were observed in liposomal suspension containing higher amount of MC.

When comparing the results from the current work with the findings from Berg (2011) there are in general agreement.

Drug to lipid ratio is the important factor in optimization of liposomal characteristics, and the results in Table 1 indicate that 30 mg as starting amount of MC is optimal amount of MC for liposomes prepared by this method and liposomal composition. The lower drug to lipid ratio determined for sonicated vesicles prepared from 30 mg as starting amount as compared to those with 40 mg can be explained by rather low drug recovery for those liposomes.

Table 2: Size characteristics of sonicated liposomes

Amount of drug (mg)	**1st peak (nm)**	***Intensity (%)***	**2nd peak (nm)**	***Intensity (%)***	**Polydispersity index**
30	40.6 ± 12.1	*24*	178.5 ± 60.6	*76*	0.41 ± 0.04
40	42.1 ± 11.3	*19*	169.6 ± 65.6	*81*	0.46 ± 0.03

The values denote the mean of 4 separate experiments ± SD.

Based on experiments with liposomal preparations for which 5, 10 and 20 mg of MC was used, Berg (2011) suggested that the size of vesicles, sonicated under the same conditions, is affected by the drug/lipid ratio, namely that liposomes tend to increase in size with more entrapped drug inside liposomes. It was suggested that liposomes may be resisting particle size reduction upon sonication as a result of incorporation of drug molecules in the liposomal bilayer, which contributed to form a more rigid membrane. Up to now, we do not have evidence on exact positioning of mupirocin within liposome, assuming that based on its log P (2.77), it would position itself in lipophilic part of vesicle (Berg, 2011). In the case of liposomes prepared from 30 and 40 mg of MC, no significant difference in particle size of sonicated liposomes could be observed and there was no obvious correlation between the liposomal size and the amount of drug incorporated into liposomes (Tables 1 and 2).

The particle sizes and size distributions of nonsonicated liposomes were not determined as the samples were to polydispersed (PI over 0.70) and too big in size to be measured accurately with the NICOMP measuring device (Berg, 2011). Similar liposomes prepared from same phospholipids and same preparation method, but containing metronidazole were found to

have mean diameter of 850 nm (data not included). In general, liposomes prepared by the film hydration method and hand shaking are known to be multilamellar with a heterogenous size distribution ranging from 500 to 5000 nm (Torchilin, 2005).

5.2. Stability testing

Real time stability testing was executed to determine the shelf life of the formulations during long term storage at normal product use conditions which in this case was at room temperature. The accelerated stability testing was performed to assess the stability of the formulations when exposed to stress conditions, expected to increase the degradation rate over a shorter time period at elevated temperature. In the current work the real time stability testing was performed over 3 months period and the accelerated stability testing at 40 °C for one month (Basnet et al., 2012). The reality of pharmaceutical products is that they are expected to be stable for several years, but stability testing for such long time periods would be too time consuming and is generally conducted only in pharmaceutical industries and regulatory (Aulton, 2007). In order to predict the shelf life under normal storage conditions, at proposed storage temperatures by using results from stability testing at higher temperatures (accelerated stability testing), the Arrhenius equation (Eq. 1),

$$k = Ae^{-E/RT} \quad \text{(Eq. 1)}$$

where k is the rate constant, A is an Arrhenius factor, E is the energy of activation, R is the gas constant and T is temperature in Kelvin, can be applied (Kirkwood, 1977; Magari et al., 2002).

The stability of nonsonicated and sonicated liposomes prepared with 30 and 40 mg drug was determined in respect to drug entrapment values after the storage at 23 ºC for three months for real time stability testing and at 40 ºC for one month for accelerated stability testing. The amounts of drug associated with liposomes before and after stability testing are compared in Figure 5.

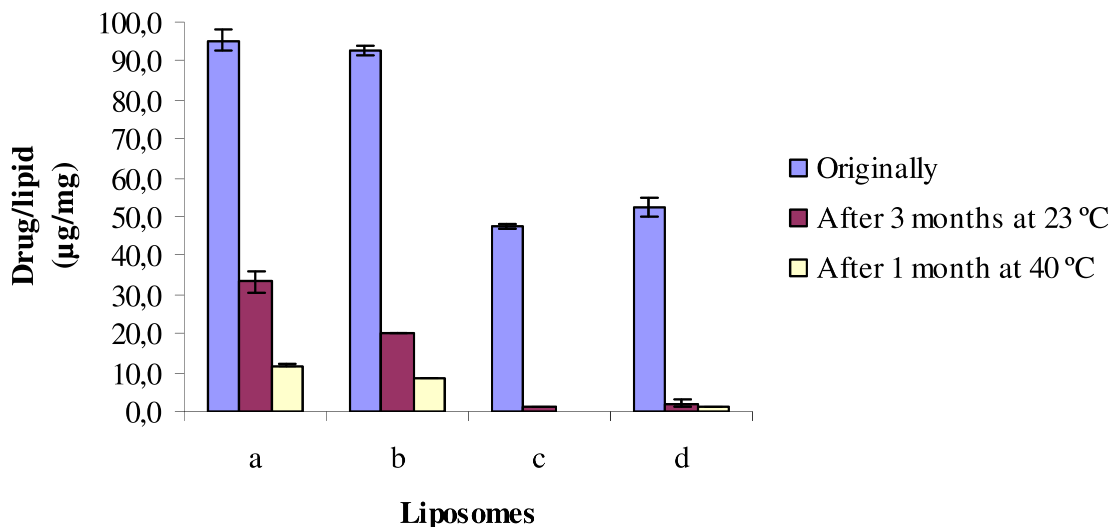

Figure 5: Drug/lipid ratio before and after stability testing: a; nonsonicated liposomes with 30 mg drug, b; nonsonicated liposomes with 40 mg, c; sonicated liposomes with 30 mg drug, d; sonicated liposomes with 40 mg drug. The values denote the mean of 2 separate experiments ± SEM.

Comparison of the original drug entrapment and the amount of drug remaining in liposomes after stability testing is as expected (Figure 5). Higher temperature induced stronger leaking of originally entrapped drug. Sonicated liposomes showed a higher leakage than the nonsonicated vesicles. This was also observed by Basnet et al. (2012) for liposomes containing curcumin, a highly lipophilic drug. The reason for this leakage may be that sonicated liposomes have fewer lipid bilayers in their structure. In regard to their size, our sonicated liposomes are expected to be mixtures of unilamellar and oligolamellar vesicles. Assuming that the drug is accommodated in lipid bilayers, multilamellar vesicles have more bilayers presenting stronger barriers for the drug to leak out (Van Bommel and Crommelin, 1984). Measurements of pH of the lipsomal suspensions after stability testing showed that nonsonicated liposomal suspensions had a pH around 5 and sonicated liposomal suspensions had a pH around 4. The pH before storage was 6.8. Liposomes of phospholipids stored at temperatures above 4-6 ºC are known to be vulnerable to degradation by hydrolysis. In addition, phospholipids are at their most stable point at pH 6.5 (Grit and Crommelin, 1993; Thompson et al., 2006). This indicates that the liposomes were degraded due to the effect of temperature resulting also in decreased pH.

Sonicated liposomes were also evaluated in respect to change in vesicle size and polydispersity. Liposomal size before and after exposure to different is presented in Figures 6 and 7 for sonicated liposomes prepared with 30 and 40 mg MC, respectively. Figure 8 shows the changes in polydispersity of sonicated liposomal suspensions.

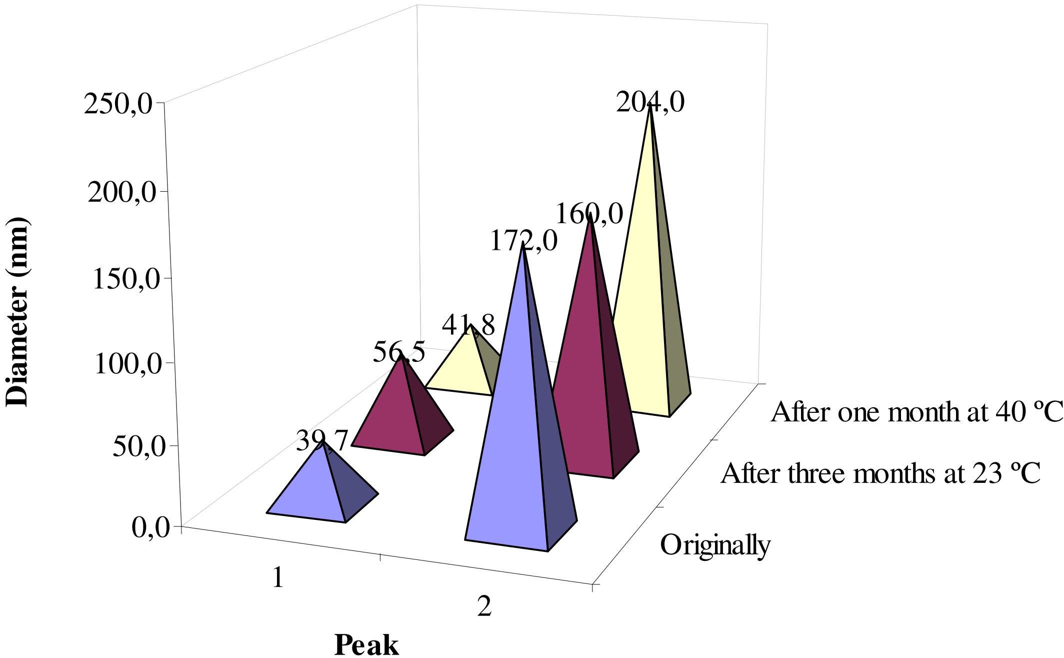

Figure 6: Size distribution of sonicated liposomes with 30 mg of drug taken in preparation before and after stability testing. The values denote the mean of 2 separate experiments. The size distribution is based on intensity.

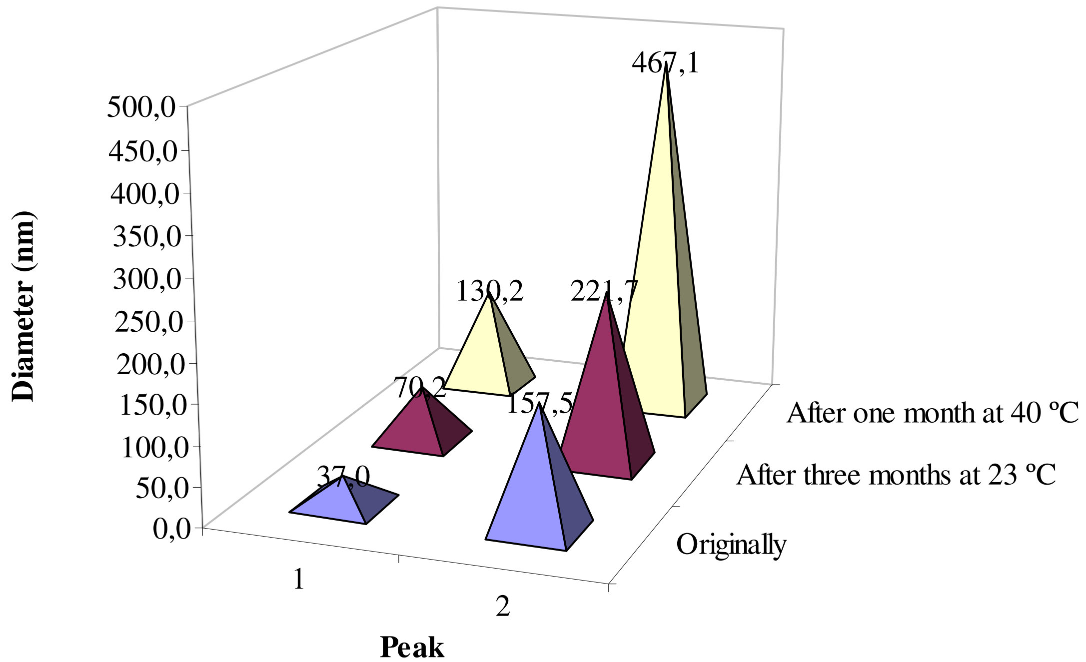

Figure 7: Size distribution of sonicated liposomes with 40 mg of drug taken in preparation before and after stability testing. The values denote the mean of 2 separate experiments. The size distribution is based on intensity.

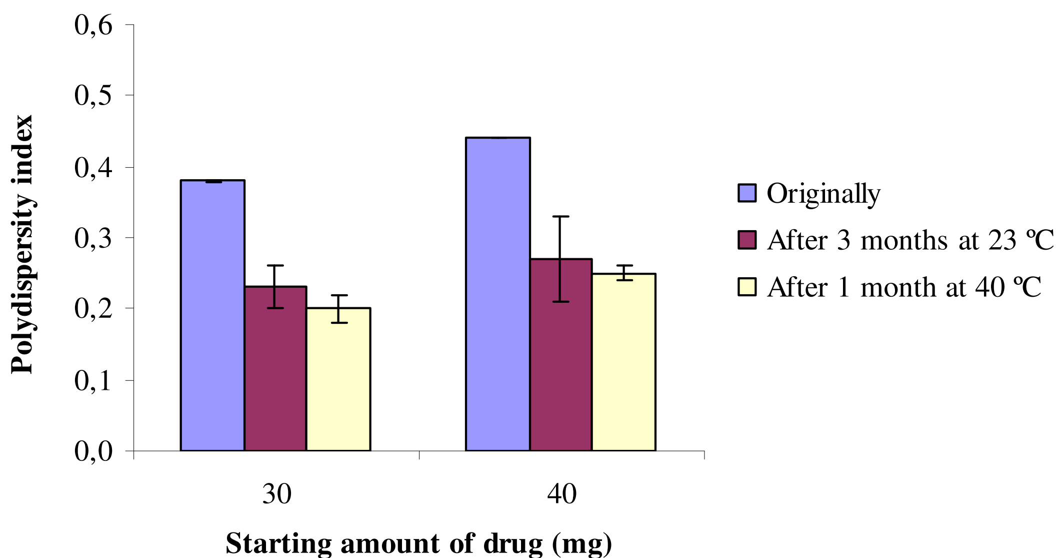

Figure 8: Polydispersity of sonicated liposomes before and after stability testing. The values denote the mean of 2 separate experiments ± SEM.

Comparison of the particle size of liposomes before and after stability testing showed that the particle size was increasing with temperature (Figure 6 and 7). However, the polydispersity indexes appear to be decreasing by an increase in temperature. Although contradictory to the fact that original particle size increased during the storage, reduced polydispersity index for size distribution is possibly a result of particles forming aggregates and thereby be detected as fewer and larger particles, expressed as narrower size distributions. The increase of original particle size was also observed by Berg (2011) after stability testing at 40 ºC for one month.

The liposomal suspensions, both the nonsonicated and the sonicated samples, appeared to contain precipitates after as detected by examining the post-stability samples visually. This was also seen in experiments previously performed by our group (Basnet et al., 2012). This kind of sedimentation may result from fusion and agglomeration of the vesicles (Casals et al., 2003) or, more probably, precipitation of the leaked drug. The precipitation was not observed in stability testing by Berg (2011) who used lower starting amount of drug.

5.3. Cell toxicity testing

The toxicity profile of liposomal and free mupirocin was established by testing the formulations on immortalized human keratinocytes, HaCaT cells, type of cells often used in toxicity testing of formulations destined for skin therapy (Kempf et al., 2011). After 24 hours incubation, the cell viability was determined by counting the cells after their staining with trypan blue dye, which is exclusively dyeing dead cells (Figure 10). Testing was performed for liposomal mupirocin and free mupirocin in the same concentration. In order to assure that neither lipid nor DMSO, which was used as a solvent for MC, had influence on the cell viability, the test was also performed with empty liposomes and DMSO. Cell growth medium without any addition served as negative control. The results for two formulations containing mupirocin are presented in Figure 9.

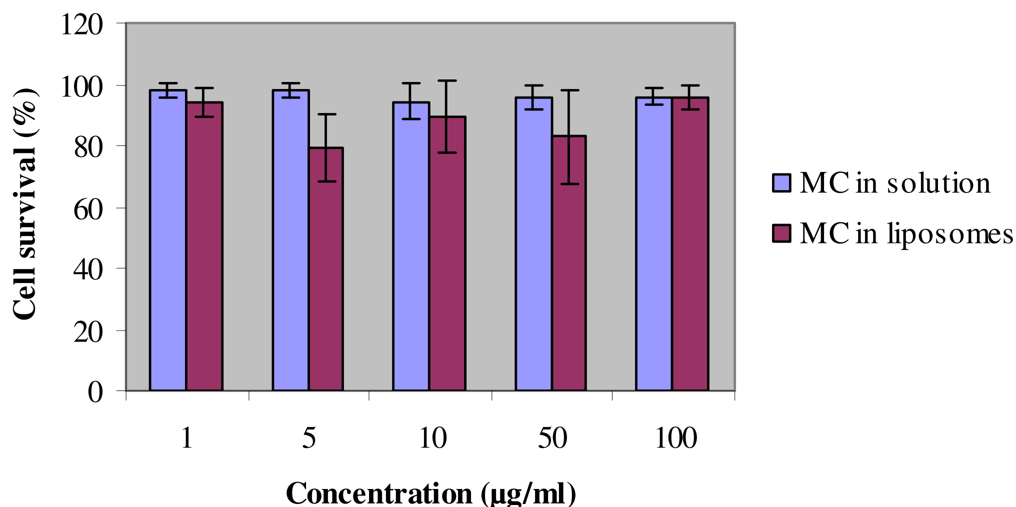

Figure 9: Viability of HaCaT cells exposed to free MC in solution and liposomal MC after 24 hours of incubation. The values denote the mean of 3 separate experiments ± SD.

As Figure 9 indicates, the free mupirocin had almost no effect on the cells' viability. Liposomal mupirocin seemed to slightly reduce the number of surviving cells, although the standard deviations for liposomal samples are higher than SD for DMSO formulation. A small amount of cells did die in the case of both formulations, but the reason behind can be related to exposure time. Trypan blue itself is known to kill certain amount of cells if it is incubated with the cells for more than 5 minutes (Strober, 2001). When carrying out this experiment the cells were generally counted within 5 minutes, but the possibility that longer incubation periods may have occurred can not be ruled out. Another reason for reduced viability counts may be due to apoptosis. However, there was no statistically significant difference between free mupirocin and liposomal mupirocin in respect to cell viability.

Empty liposomes and DMSO alone affected the cells in a similar manner as the formulations containing drug (data not shown). A very small number of cells were also found dead. This indicates that MC did not affect cell viability in the tested concentrations. The control containing only cells in growth medium showed the cell viability similar as observed for the rest of the samples (data not shown). Hence the reduced viability counts may be attributed to too long incubation time with trypan blue dye and occurrence of apoptosis as mentioned in previous paragraph.

These findings suggest that mupirocin calcium, both in its free form and formulated in liposomes, is safe and nontoxic to human skin cells in an concentration range from 1 to 100 μg/ml.

Typical photograph of cell staining is given in Figure 10.

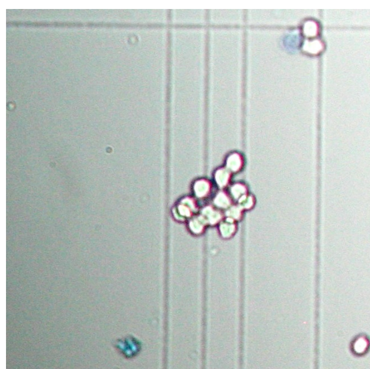

Figure 10: Cells after staining with trypan blue dye. The blue cells are dead cells, and the nocolored cells are viable.

5.4. Anti-biofilm potential

The effect of liposomal formulations with and without MC, and free drug on planktonic bacteria and biofilm was evaluated by exposing the free bacterial cells and the bacterial cells in biofilms to the different formulations prior to their staining with resazurin and crystal violet. The anti-bacterial and anti-biofilm activities are presented in Table 3, and Figure 11 compares their impact on planktonic growth.

Table 3: Potency of mupirocin formulations against planktonic bacteria and biofilm (as reported by Docent Adyaro Fallalero)

	Formulation	Potency IC_{50} (µM)	95 % confidence interval
Planktonic bacteria	M	0.19	0.42 – 0.67
	LM	0.53	0.14 – 0.26
Biofilm prevention	M	0.27	0.48 – 0.70
	LM	0.58	0.16 – 0.45
	Penicillin G	0.13	0.12 – 0.14
Biofilm destruction	M	> 405	-
	LM	> 405	-

M; free mupirocin in propylene glycol, LM; mupirocin loaded in liposomes. Compound concentrations were within the range 0.01-70µM for the prevention trials and within the range 1-405µM for the destruction trials.

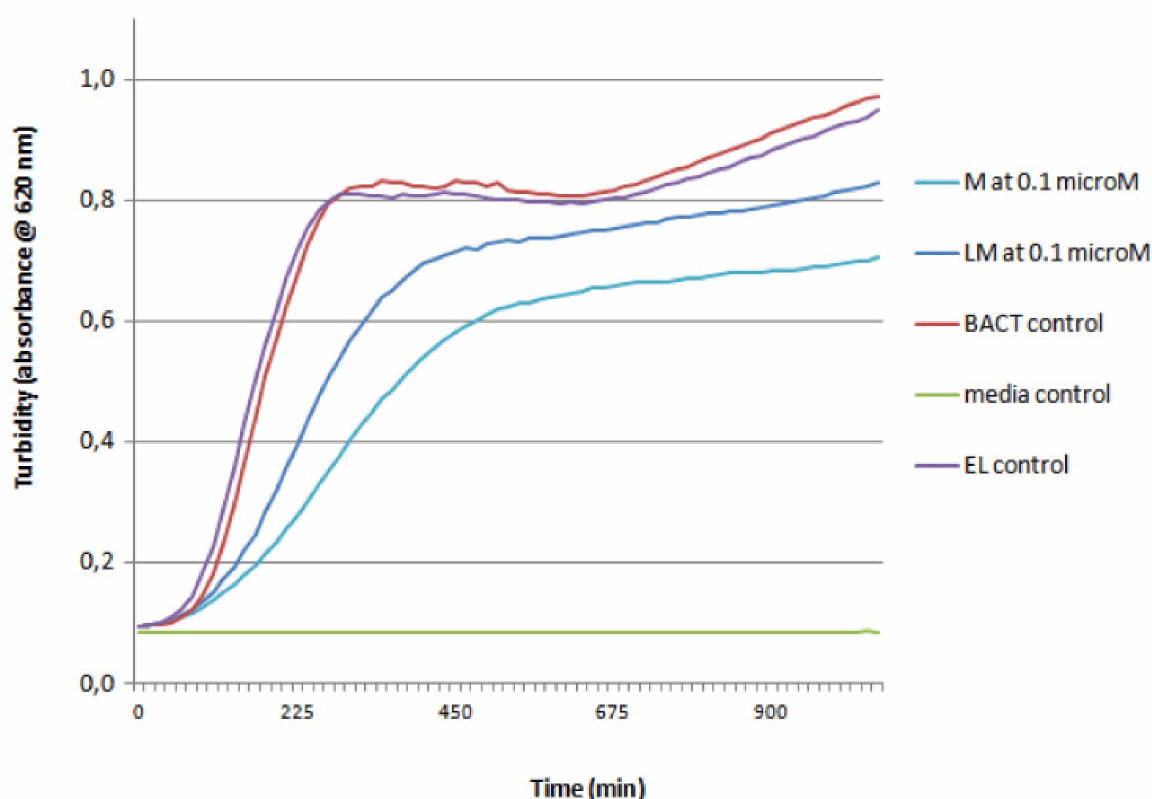

Figure 11: Kinetic growth curves of planktonic *S. aureus* exposed to M; free mupirocin in propylene glycol, LM; mupirocin in liposomes, and EL; empty liposomes. (Reprinted from report by Docent Adyaro Fallalero).

End point measuring, after 18 hours of planktonic bacterial growth, showed that liposomal mupirocin was less active against planktonic bacteria as compared to free mupirocin (Table 3). Free mupirocin was found to be able to delay the growth of bacteria at a larger extent than the mupirocin loaded in liposomes (Figure 11). The reason for the lower effectiveness of the liposomal formulation compared to the effect of free drug may be that the lipid around the drug in liposomes containing mupirocin creates a barrier that makes it more difficult for the drug to interact with the bacteria, hence limiting its potential to exert antibiotic effect. The growth of planktonic bacterial was not affected by empty liposomes. This phenomena needs to be further evaluated, as it would be very important to determine whether the same would be seen in the case of liposomes containing more hydrophilic antibiotic.

Biofilm formation was affected by both liposomal formulation of mupirocin and free mupirocin (Table 3). The ability of the two formulations to prevent formation of biofilm was similar to the potency determined in planktonic bacteria. The anti-biofilm effect may be due to the liposomal and free drug inhibiting planktonic bacteria before they have reached the surface of the microtiter well plates to start the process of producing biofilm. A liposomal formulation was not found to be superior in prevention of biofilm formation as compared to free drug under the tested conditions. The results presented in Table 3 are based on measurements performed with the resazurin reduction assay. This method is based on the ability of metabolically active bacteria residing on the inner core of biofilms to convert the resazurin redox dye to resorufin, a fluorescent product, which makes it possible to measure the fluorescence in the biofilm sample and hence translate these signals to determine the bacterial or biofilm load (Sandberg et al., 2009). The results from measurements with crystal violet dye are not presented, but they were found to give similar trends as for the resazurin readings. This supports the view that both free and liposomal mupirocin had inhibiting effect on the viability and biomass of the biofilms.

Destruction of biofilms that had matured for 18 hours did not exceed 50 % neither for free mupirocin or the mupirocin formulated in liposomes even for the samples with the highest concentration (Table 3). This made it impossible to give an estimation of the IC_{50} values other than that they must be more than 405 µM as this was the highest concentration tested.

5.5. Fluid affinity testing for liposomal hydrogels

The absorbing and moisturizing properties of chitosan and Carbopol hydrogels, both liposomal and empty, were evaluated by placing hydrogel samples on material mimicking the environment of an exudating and a dry wound (Figure 12), respectively. Tables 4 and 5 show the effects, expressed as either negative or positive percentage in original weight change after 24 hours incubation.

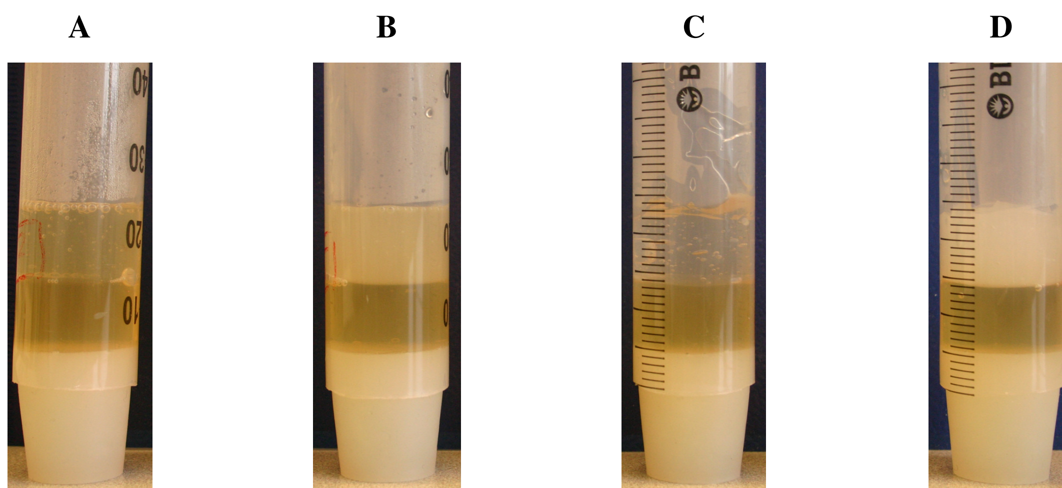

Figure 12: Syringes with empty chitosan hydrogel (A), liposomal chitosan hydrogel (B), empty Carbopol hydrogel (C) and liposomal Carbopol hydrogel (D) before 48 hours incubation. In these pictures all the hydrogels were placed on gelatin. The syringes containing hydrogels on agar looked the same and are therefore not presented here with pictures.

Table 4: Fluid uptake from agar by hydrogels and change in original pH-values

Hydrogel	Change in hydrogel weight (%)	pH before incubation	pH after incubation
Empty chitosan hydrogel	64.27 ± 1.37	3.90	4.05
Liposomal chitosan hydrogel	61.71 ± 0.06	3.96	4.06
Empty Carbopol hydrogel	1.38 ± 0.57	6.84	5.12
Liposomal Carbopol hydrogel	-0.27 ± 1.07	6.63	5.35

The values denote the mean of 3 separate experiments ± SD.

The values in Table 4 show that the chitosan hydrogels, both in the form of liposomal and the empty hydrogel, increased their original weight after incubation. This indicates the ability of the chitosan hydrogels to absorb fluid, as suggested by several authors (Alsarra, 2009; Ribeiro

et al., 2009; Dash et al., 2011; Tsao et al., 2011). The agar plug appeared to have shrunk and was about half of its original size (Figure 13). This additionally supports reports on ability of chitosan hydrogels to take up fluid, and indicates that wounds with exudates can be managed with wound dressings based on chitosan hydrogels (Alsarra, 2009; Ribeiro et al., 2009; Dash et al., 2011; Tsao et al., 2011).

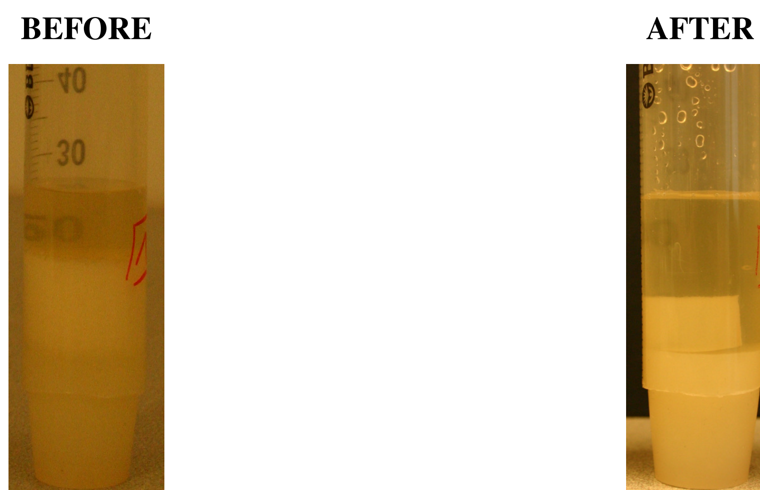

Figure 13: Syringes with empty chitosan hydrogel and agar before (left) and after (right) 24 hours incubation.

For the Carbopol hydrogels, both empty and liposomal hydrogels, the fluid uptake from agar was not as obvious as seen for the chitosan hydrogels. They seem to have less or almost no absorbing capability compared to the chitosan hydrogels. In this case, the agar plug was not reduced as compared to the size before incubation (data not shown), which also indicates that Carbopol hydrogels are less absorbent than chitosan hydrogels. Hence, wound dressings based on Carbopol hydrogels seem to be inappropriate in treatment of exduating wounds.

Table 5: Fluid donation to gelatin by hydrogels and change in original pH-values

Hydrogel	Change in hydrogel weight (%)	pH before incubation	pH after incubation
Empty chitosan hydrogel	-4.68 ± 3.80	3.78	4.16
Liposomal chitosan hydrogel	-9.76 ± 2.67	3.88	4.16
Empty Carbopol hydrogel	2.33 ± 2.58	5.87	4.89
Liposomal Carbopol hydrogel	-6.08 ± 6.92	6.25	5.43

The values denote the mean of 3 separate experiments ± SD.

The pH of solution A used for preparation of agar and gelatin was 5.70

The values in Table 5 show a decrease in hydrogel weight after incubation, with the exception of the empty Carbopol hydrogel. After incubation, the gelatin appeared to be softer and more liquid in nature than originally. This supports the theory that the hydrogels are able to donate fluid to gelatin (Alsarra, 2009; Ribeiro et al., 2009; Dash et al., 2011; Tsao et al., 2011). However, when hydrogel samples, both chitosan hydrogels and Carbopol hydrogels, were going to be removed from the tube after incubation, the difficulties in separating hydrogel from gelatin were encountered. The hydrogel and gelatin was blended to a certain degree and could not be separated effectively. The hydrogel sample seemed to contain some kind of residues from gelatin (Figure 14).

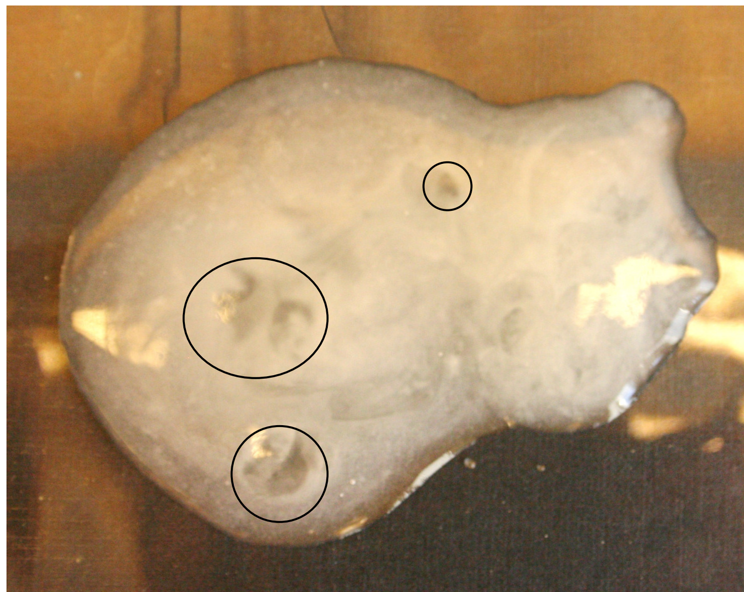

Figure 14: Carbopol hydrogel after 24 hours incubation on gelatin plugs. The species assumed to be gelatin are highlighted by the circles.

This may have contributed to a smaller change in hydrogel weight than would be expected if the separation between hydrogel and gelatin could be complete. This theory is supported by the fact that the gelatin plugs seemed to have decreased in size and became thinner than originally.

Based on the findings presented in Table 5, it is hard to determine with certainty the degree of moisture that chitosan and Carbopol hydrogels are able to provide to wounds as the experiment did not proceed as planned. However, although less than expected, hydrogels have

shown moisturizing properties up to certain degree. Moisturizing effect and, at the same time, ability to handle wound exudate, are vital properties in optimization of wound dressings.

After incubation the viscosity of Carbopol hydrogels seemed to have decreased, resulting in a more liquid gel. At first thought this was assumed to be a result of the fluid absorbed by hydrogels, resulting in a decrease in polymer concentration which led to a less rigid gel. However, the change in viscosity was observed in both the fluid uptake experiments and the fluid donation experiments. Before incubation, the pH of the Carbopol hydrogels was closer to physiologic pH than what was measured after 48 hours (Table 4 and Table 5). Literature states that the swelling behaviour of Carbopol is highly dependent on pH (Hagerstrom et al., 2000; Hurler et al., 2012) and ionic strength, and that if a sufficiently high amount of Carbopol is dispersed in an aqueous media in physiological pH, the polymer exists in a form of a swollen gel. In contrast, Carbopol dispersed in a media with ions is known to produce gels with lower viscosity and elasticity due to less repulsion between the polymer chains due to the shielding of the negatively charged carboxylic groups (Hagerstrom et al., 2000). Hence the more liquid hydrogel after incubation is most likely a result from decrease in pH and addition of sodium ions and calcium ions from the sodium chloride and calcium chloride solution in the agar and gelatin plugs.

The findings from the fluid affinity testing (Tables 4 and 5) suggest that chitosan hydrogels can be used as wound dressings for wounds that need control of drainage and most probably also for treatment of dry wounds. Wounds that contain a high amount of fluid will probably not heal better if treated with Carbopol-based hydrogel dressings, however, this type of wound dressing might be more suitable for dry wounds. Additional insight could be provided by employing more sensitive methods such as texture analysis or rheological characterization prior and after incubation experiments.

6. Conclusions

As the first step in optimization of liposomal hydrogels with mupirocin, we evaluated the improvement in the entrapment efficiency for mupirocin in liposomes, in order to obtain higher drug concentration in the final delivery system. The optimal drug concentration used for liposomal formulation was found to be 3 mg/ml when 20 mg/ml of lipid was used. Those liposomes were found to be smaller than 300 nm, a size distribution suitable for topical administration onto skin. *In vitro* evaluation of the potential cytotoxicity proved that both drug and liposomal delivery system are safe and nontoxic in a concentration range of 1 to 100 µg/ml. Mupirocin and liposomal mupirocin were evaluated for anti-biofilm potential and results indicate that both mupirocin and liposomal mupirocin act on both biofilm and planktonic bacteria, very important when considering that biofilm formation is common cause of impaired wound healing. In addition, since liposomal hydrogels with mupirocin will be used as wound dressings, we tested two types of hydrogels and found that chitosan hydrogels exhibited better absorbing properties compared to Carbopol hydrogels, hence being more appropriate for wound treatment where control of wound exudate is required.

7. Perspectives

The findings from the current project can serve as base for further optimization and evaluation of the drug delivery system as actual wound dressing. *In vivo* studies in mice burn model are currently being conducted in our research group. This will give us an indication of the preclinical safety profile of the formulation as well as information about the efficiency of the system in animal model. The next step would involve clinical testing on human skin, both healthy and wounded skin, in order to gain knowledge about the possibility for skin irritancy and for evaluation of the clinical efficiency, respectively. Moreover, the long term stability of the drug delivery system should be determined before finally establishing the potential for large-scale production.

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