MASTER THESIS FOR THE DEGREE MASTER OF PHARMACY

ADVANCED DELIVERY SYSTEM FOR SKIN AND BURNS THERAPY: MUPIROCIN AS AN ANTIBACTERIAL MODEL DRUG

BY

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Abstract

Trauma to the skin in the form of severe wound, particularly burns, can facilitate colonization of potentially life threatening bacterial infections. To prevent infections of the wounded area, antimicrobial agents are recommended as standard treatment. Topical administration of antimicrobial agents, such as mupirocin, can provide local therapy, while avoiding the risks of systemic administration. Mupirocin-in-liposomes-in hydrogels were proposed as advanced delivery system for this purpose. Up to now, no liposomal mupirocin for topical administration has been reported. Chitosan was selected as hydrogel matrix due to its biodegradability and in-built antimicrobial and wound healing potentials.

Phosphatidylcholine liposomes containing mupirocin, namely non-sonicated and sonicated liposomes, were characterized for vesicle size and size distributions. Non-sonicated vesicles entrapped in average 74 and sonicated 49 % of mupirocin calcium, respectively. Sonication reduced the original vesicle size from around 1 micron down to 135 nm. Liposomes (10 %, w/w) were incorporated in chitosan hydrogels and liposomal hydrogels evaluated for their textural properties. Hydrogels were found to exhibit satisfactory adhesiveness and cohesiveness, with corresponding stability profile. Microbiological assessment confirmed antibacterial properties of liposomally entrapped mupirocin incorporated in hydrogels. *In vitro* and *ex vivo* (on pig skin) drug release profiles of various formulations containing mupirocin were performed on Franz diffusion cells. Liposomal hydrogels were compared with marketed mupirocin product, Bactroban® cream. The release studies showed that liposomal size affects the release of the incorporated drug. Liposomal hydrogels were shown to provide sustained release of incorporated mupirocin.

In conclusion, liposomal hydrogels developed for mupirocin offer the potential to increase retention time and provide sustained release of a drug, which are important parameters for improved treatment of wounds, including burns.

Key words: mupirocin calcium; liposomes; hydrogels; chitosan; burns

List of Abbreviations

Bac-cream 2 %	Bactroban® cream 2 %
d Bac-cream	Diluted Bactroban® cream
DD	Degree of deacetylation (applies to chitosan)
FDC	Franz Diffusion Cells
gel-N-SL	Non-sonicated liposomes incorporated in chitosan hydrogel
gel-SL	Sonicated liposomes incorporated in chitosan hydrogel
gel-N-SL (f+e)	Non-sonicated liposomal suspension containing both free and liposomally entrapped MC incorporated in chitosan hydrogel
HPLC	High performance liquid chromatography
LUV	Large unilamellar vesicles
MC	Mupirocin Calcium, referred in text as mupirocin
MIC	Minimum inhibitory concentration
MLV	Multilamellar vesicles
MRSA	Methicillin-resistant *S. aureus*
N-SL	Non-sonicated liposomes
OLV	Oligolamellar vesicles
PC	Phosphatidylcholine
pg	Mupirocin dissolved in propylene glycol
PI	Polydispersity index
SC	Stratum corneum
SD	Standard deviation
SL	Sonicated liposomes
SUV	Small unilamellar vesicles
ZP	Zeta potential

1. General introduction

Skin wounds are injuries to the underlying tissue in which the skin is cut, punctured or torn. When trauma appears in the form of a burn wound, it can affect several skin layers. Sever burn wounds can be a tremendously hard task to treat in burn units as they include increased risk of fluid loss, hypothermia, infections and impaired scarring (Alemdaroglu *et al*., 2006). Burn trauma to the skin creates a local immuno-compromised area, leading to potentially life-threatening microbial infections. In spite of the advances in treatment of skin wounds, wound infections are still the major cause of wound-related morbidity and mortality (Dai *et al*., 2009).

Successful burn therapy represents a specific challenge in respect to therapeutic outcome, scaring, functional and cosmetic consequences. Several promising lines in development of burn therapy were proposed, among which hydrogels appear to fulfill many of the criteria for ideal wound dressing. The ideal dressing should achieve permanent skin regeneration, have good functional and esthetic characteristics, optimal mechanical properties, be bioadhesive and possibly provide controlled release of active ingredients (Boateng *et al*., 2008). Hydrogels are water-swollen polymeric gels, which possess a three dimensional structure, with an ability to retain large amount of water. Chitosan is a natural biodegradable polymer with wound healing properties on its own (Bhattarai *et al*., 2010). Chitosan hydrogels provide a moist environment at wound site and exhibit bioadhesive properties. In order to ensure controlled release of active ingredient, liposomes bearing mupirocin were incorporated in chitosan hydrogels.

Mupirocin calcium was selected as model antimicrobial drug due to its activity against various bacteria, commonly infecting wounded areas of skin (Bageshwar *et al*., 2010). Its additional advantage is the fact that it shows low activity against microorganisms in the normal skin flora. This can be seen as an advantage due to the skins normal defences against pathogens, will not be interfered by the selected drug (Echevarria *et al*., 2003; Thomas *et al*., 2010).

2. Introduction

2.1. Skin and wounds

2.1.1. Skin structure

The largest organ of the body is the skin. Human skin consists of three layers, namely the epidermis, dermis and hypodermis, respectively (Sherwood, 2007). The epidermis consists of several layers of epithelial cells. These layers (Figure 1), from the inside to the outside, are the *stratum germinativum*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* and *stratum corneum* (El Maghraby *et al*., 2008)

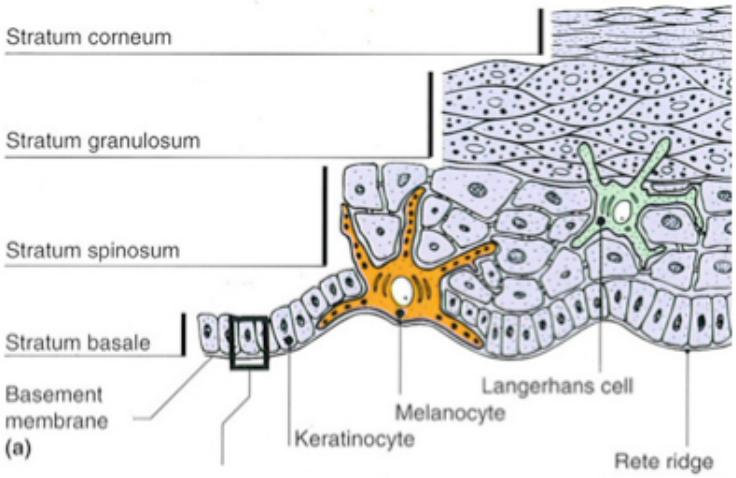

Figure 1: Different layers of the epidermis (Grawkrodger, 2008)

The innermost epidermal layer consists of cells that are promptly dividing, in contrary to the outer layer of the epidermis, comprising of dead cells, flat in appearance. This latter layer is referred to as the *stratum corneum* (SC) or the horny layer. SC varies greatly in thickness, ranging from 0.8 to 0.006 mm on the palm, soles and eyelids, respectively. The SC may be merely 10 µm thick when it is dry, but will swell several-fold when in contact with water (El Maghraby *et al*., 2008). The epidermis is dependent on diffusion of nutrients from the dermis, as there is no blood supply in the epidermis (Sherwood, 2007).

Beneath the epidermis is the dermis. This layer is ranging from 3-5 mm in thickness (Sherwood, 2007). The dermis functions as a connective tissue layer that contains elastin and collagen fibres, providing the skin with ability to stretch and strength, respectively. Blood vessels, skin appendages, lymphatic's, and specialized nerve endings are abundant in this layer. Not only is the dermis providing nutrition to the epidermis, it also plays a key role in temperature control. Due to the specialized nerve endings in the dermis, more accurately the afferent nerve fibres, dermis perceives pressure, pain, temperature and other somato-sensory inputs. The hair erection and discharge by the skins exocrine glands are controlled by the efferent nerve endings based in the dermal stratum (Sherwood, 2007).

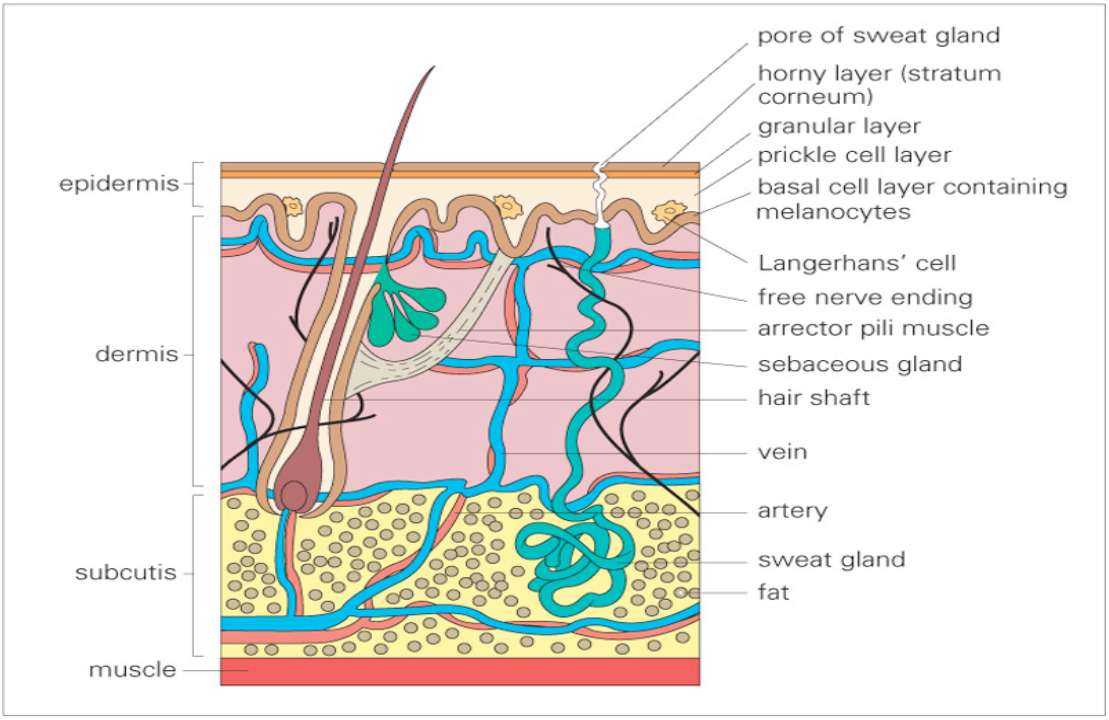

Figure 2: Anatomy of the skin (Goering *et al*., 2008)

The subcutis (Figure 2) is also known as the hypodermis. The hypodermis is a loose layer of connective tissue that anchors the underlying tissue. Adipose tissue makes up most of the hypodermis (Sherwood, 2007).

2.1.1.1. Intact skin

Human skin serves as a protective barrier against chemicals and ultraviolet light. The skin also serves as a shield against patogenic microorganisms. Synthesis of vitamin D in the epidermis, and storage of adipose tissue in the hypodermis are additional important features of intact skin. Healthy and intact skin is important for our health and well-being (Sherwood, 2007).

The surface of intact skin has a pH value in the range of 5.4-5.9. The protetcing acid barrier varies in relation to both endogenous and exogenous factors. Due to the acidic nature of skin, several bacterial strains are normally present, which makes up the natural microbial flora for healthy skin (Schmid and Korting, 2006). *Staphylococcus epidermitis*, *Staphylococcus aureus*, and *Micrococcus luteus* are the most abundant bacteria on healthy human skin (Baron, 1996).

Passage of external molecules through, across and via human skin can be facilitated by three potential pathways (Figure 3).

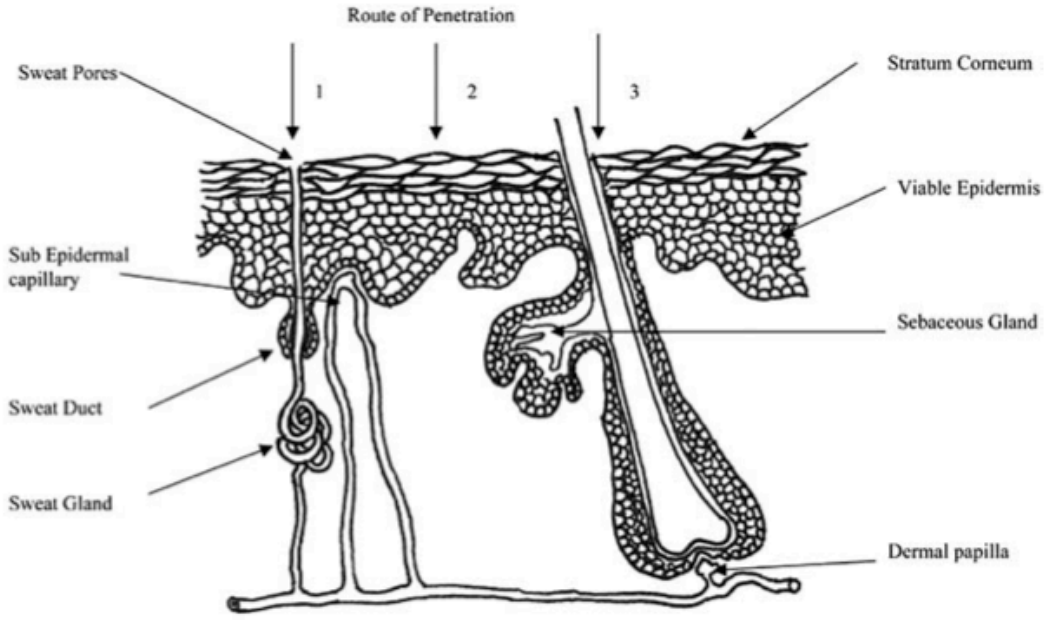

Figure 3: Illustrating routes of penetration: 1. Through sweat glands; 2. Across *stratum corneum*; 3. Via the hair follicles (Benson, 2005)

They include the pathways through the sweat ducts, via the hair follicles and the sebaceous glands (collectively called the appendageal route) or the passage directly

across the SC. It is generally accepted that the appendageal route represents approximately 0.1 % of the passage of drugs through the skin. The SC is considered to be the rate limiting step in transdermal penetration of nearly all molecules (Bouwstra and Ponec, 2006).

The structure of the SC can be seen as a so-called bricks and mortar arrangement, were the bricks represent the keratin-rich corneocytes and the mortar represents the intracellular lipid-rich matrix (Benson, 2005; El Maghraby *et al*., 2008).

External molecules can pass through the "brick and mortar" structure by either intracellular or trans cellular pathway (Figure 4).

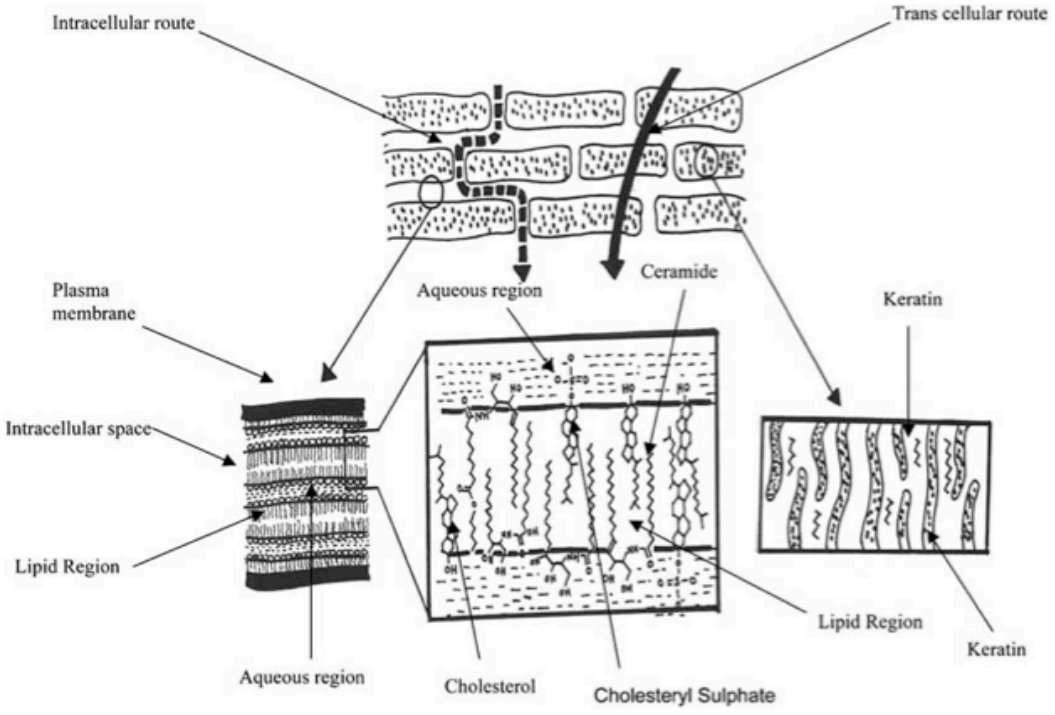

Figure 4: Illustration of the intracellular and the trans cellular route (Benson, 2005)

The intracellular route (Figure 4) is now considered to be the foremost route for permeation of most of the drugs applied to the skin. The degree of the lipophilicity and the size of the molecule will play an important role for molecules passing through the lipid domains (Benson, 2005).

2.1.1.2. Injured skin and its barrier properties

Skin injuries and chronic skin wounds, such as skin ulcers, are very serious health issues. It is estimated that over 1.25 million people experience burn injuries each year, and over 6.5 million people suffer from chronic skin wounds just in USA alone (Chaby *et al*., 2007). These injuries impair the quality of life and take up substantial health care resources. Improved wound therapy become the ultimate goal in wound therapy. There is consensus on wound therapy that for an optimal wound healing the wound environment should be kept moist. At the same time, the changed skin barrier function should be taken into account when developing dressings/drug delivery systems for injured skin. The lipophilic and hydrophilic properties of the skin are most likely to be altered when skin is injured. The pH of the skin may also be changed. Depending on the severity and the complexity of the damaged skin, the permeability of skin for various drugs might be very high (Bouwstra and Ponec, 2006; Chaby *et al*., 2007; Boateng *et al.*, 2008).

The protective role of the skin in holding in body fluids comes under the serious treath after sever burn injuries. Bacterial infections can easily get mainstay in the defenseless underlying tissue. Moreover, the systemic consequences of loss of water and plasma proteins which escape from the uncovered burned exterior are even more serious. The disturbances in the circulation can be life threatening (Sherwood, 2007).

2.1.2. Wounds

Skin wounds are injuries to the underlaying tissue in which the skin is cut, punctured or torn. Skin wounds can be divided into two classes based upon their apperance and ability to heal, as acute or chronic wounds, respectively. An acute wound is tissue injury that heals within 8-12 weeks (Boateng *et al*., 2008). The definition of a chronic wound is the injured tissue that has an impaired capability to heal up. The underlaying contidions for a chronic wound may be due to chemotherapy, steorid use, infections, arterial insufficiency, diabetes mellitus, radiation, pressure and venous insufficiency (Bao *et al*., 2009). Chronic wounds can exhibit complex microbiological consistency that can affect the healing process without showing any signs of underlaying infection (Frankel *et al*., 2009). Frankel *et al*. (2009) investigated chronic wounds and their

microbial flora and found a high percentage of methicillin-resistent *S. aureus* (MRSA), *Pseudomonas aeruginosa* and group *B Streptococcus* (45, 28, and 21%, respectively). The microbial study confirmed that wounds are prone to infections and that incorporation of antimicrobial agents in wound dressings is therefore recommended (Frankel *et al*., 2009).

Burns are specific types of wounds which represent a dynamic living environment that will alter depending on both intrinsic factors (release of inflammatory mediators, bacterial proliferation) and extrinsic factors (dehydration, cooling, systemic hypotension). When epitelisation is delayed beyond three weeks, the healed wound will leave hypertrophic scarring (Papini, 2004).

Burn wounds can be divided into several classes depending on the skin layers affected by the injury (Figure 5) (Hettiaratchy and Papini, 2004).

- Epidermal burns; only affecting the epidermis, often result of sunburns. Blistering can occur with this type of burn, however, it is not commonly seen. Healing of the skin usually takes five to seven days (Papini, 2004).

- Superficial partial thickness burns; these burns affect the epidermis and the underlaying upper dermis. In this type, blistering is commonly seen. Due to the impact on the dermal layer of the skin and the uncovered superficial nerves, this type of burns are painful. Healing of the skin depends on the compactness of skin appendages. Hairy skin heals more rapidly than hairless skin. Healing of the skin usually takes fourteen days (Papini, 2004).

- Deep partial thickness burns; these burns affect the epidermis, and deeper into the dermis. Healing of the skin usually takes two to four weeks, and is often related to substantial scarring (Moss, 2010).

- Full thickness burns; in these burns, the entire form of regenerative elements have been shattered. The healing of these burns occurs from the edges and is oftenly associated with contraction. The process of healing will take several weeks and the scarring will be visible (Papini, 2004).

- Fourth-degree burns; extend further than the skin layers, down to the subcutaneous fat, mucles and bone (Moss, 2010).

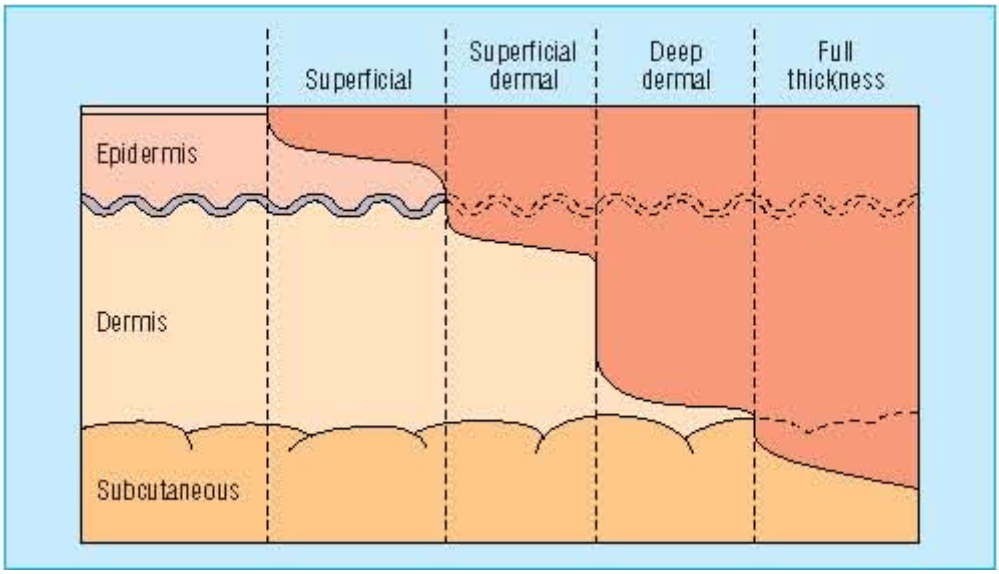

Figure 5: Classification of burns based on burn depth (Hettiratchy and Papini, 2004)

2.1.2.1. Wound healing

Wound healing is a complicated biological process which comprises of four basic steps, namely (i) the immediate response, (ii) the inflamatory response, (iii) the proliferation, migration, contraction and (iv) the final wound resolution, respectively (Shaw and Martin, 2009).

Immediate response: starts with a burst of damage signals (Figure 6). Wounded and stressed cells respond by activating signaling pathways, that leads to a phosphorylation cascade ending with alterations in gene expression, metabolism, and cell survival. With platelet activation and aggregation, the resulting formation of a insoluable nettwork of fibrin fibers acts not only as a plug, but also as a source of growth factors (Shaw and Martin, 2009).

Inflammatory response: starts within a couple of minutes after the damage has occurred. The damaged blod vessels will leak out leukocytes (Figure 6). These speciallized immune cells will further enroll neutrophiles and macrophages from neighboring vessels. This process is further enhanced by nitric oxide, histamine and other factors as a consequence of vessel dilation. (Shaw and Martin, 2009).

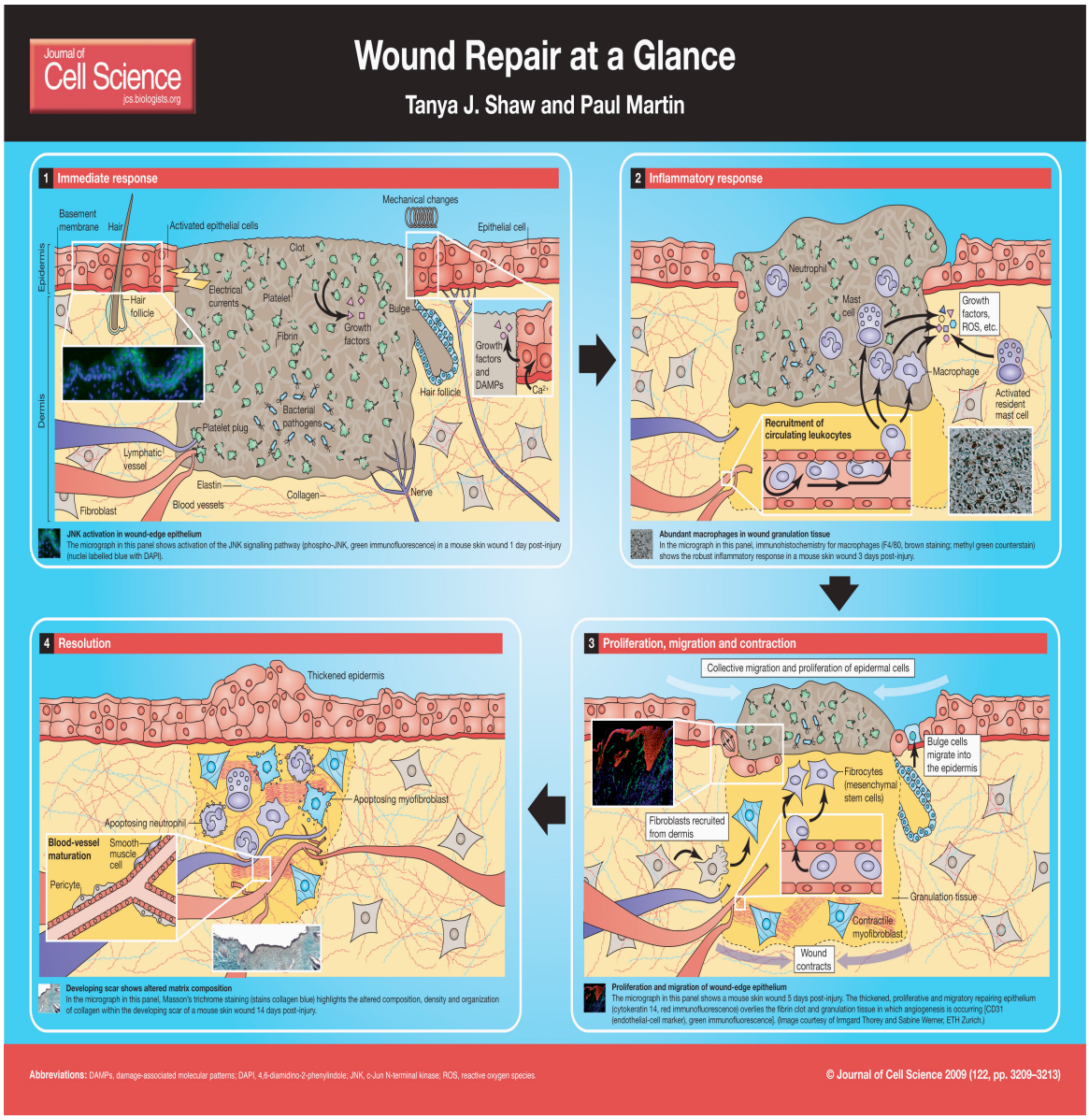

Figure 6: Wound repair at a glance (Shaw and Martin, 2009, *with permission*)

Proliferation, migration and contraction phase: fibroblast and epithelial cells will move into the area and grow to replace damaged tissue (Figure 6). Angiogenesis, the formation of new blod vessels, will provide the area with nutrition and assist the formation of granulation tissue. Lymphatic vessels will also enter the wound area. Myofibroblasts and fibroblasts will help to contract the wound, thus supporting the collagen fibre synthesis (Shaw and Martin, 2009).

Resolution phase: is vital for renewal of the wounded tissue. Here the blood vessels are refined. Remodelling of the extracellular matrix is due to the equilibrium of collagen synthesis, processing and degradation (Figure 6). In this phase, the

myofibroblasts will undergo apoptosis. An imperfection of the resolution phase can eventually result in excessive scar tissue formation (Shaw and Martin, 2009).

For a wound to heal properly a good blod flow and access to oxygenated blod is required. The nutritionus status is also an important factor affecting wound recovery. Infections of the recovering wound can increase the healing time and seriously affect the scarring (Chaby *et al*., 2007).

2.1.2.2. Wound exudate

Normally healing wounds have, to some extent, wound exudate containing endogenous materials, namely enzymes, growth factors and cytokines, that collaborate and play an important role in the wound healing cascade. In chronic wounds, due to the inflamation or infections, there is an increase in wound exudate formation. If wounds become infected, a change in the apperance of the exudate is recognizable. Exudate becomes more dense and purulent in apperance. If the wound is infected with strains of *Pseudomonas* or *Proteus*, a thick, green and smelly exudate will be produced, and for the latter, a typical ammonia smell is evident (Sussman and Bates-Jensen, 2007).

2.2. Wound dressings

In the past, traditional dressings such as natural and synthetic bandages, cotton wool and similar, were primarily used to keep the wound dry by allowing evaporation of wound exudates and preventing bacterial infections (Boateng *et al*., 2008). Nowadays, it is agreed among medical and pharmaceutical experts that a warm and moist wound environment enables faster and more successful wound healing (Harding *et al*., 2007).

No single dressing is suitable for all wound types, and in addition, wound healing process has several different phases that cannot be targeted by any particular dressing, meaning that dressing types can also vary in different phases of wound healing (Boateng *et al*., 2008).

Dressings can be classified depending on their function in the wound (debridement, occlusive, etc), type of material used to produce the dressing (hydrocolloid, collagen, chitosan, etc) and the physical form (ointment, gels, etc) (Harding *et al*., 2007). The dressings can be further classified as the primary dressings, which make physical contact with the wound surface, the secondary, covering primary dressing and the so called island dressings (central absorbent region is surrounded by the adhesive portion (Boateng *et al*., 2008).

Table 1: Type and corresponding function of most common dressings (Harding *et al*., 2007)

Type of dressing	Function
Films	Superficial protection
Foams	Protection, absorbency
Hydrogels	Debridement, rehydration
Hydrocolloids	Debridement, protection
Alginates	Absorbency, haemostasis
Medicated dressings	Exudate handling, infection control

However, many of dressing types fit all the criteria, therefore the more suitable classification would be into traditional and modern (providing moist wound environment) dressings (Boateng *et al*., 2008).

An ideal wound dressing or wound covering should perform or mimic numerous actions of human skin, such as being not permeable to bacteria, being adhesive, occlusive, lasting and elastic enough for patient not to feel discomfort (Alsarra, 2009).

Managing moisture in the wound is particularly challenging. The optimal dressing should be able to draw away components of wound fluid by providing good fluid-

handling capacity while, at the same time, maintaining a moist environment. Fluid-handling capacity is essential feature of dressings (Harding *et al*., 2007).

Modern dressings (Table 1) offer a possibility to retain and create a moist environment around the wound and offer improved wound healing. They are mainly classified according to the material they are made of, as:

- Hydrocolloid dressings
- Alginate dressings
- Hydrogel dressings
- Semi-permeable adhesive film dressings
- Foam dressings
- Biological dressings
- Tissue engineered skin substitutes (Boateng *et al*., 2008).

New generations of medicated dressings incorporate various active ingredients, which possess therapeutic values, such as antimicrobials, growth factors, and various supplements (vitamin C for example). The most advanced dressings include systems able to provide controlled delivery of active substances at wound site (Boateng *et al*., 2008).

We focused on hydrogels as wound dressings, particularly hydrogels incorporating liposomal delivery systems, incorporating antimicrobial agent.

2.3. Hydrogels

2.3.1. Hydrogels and their classification

Hydrogels are water-swollen polymeric gels, which possess a distinct three-dimensional structure. They were the first biomaterials designed for clinical use. Due to their high water content, most hydrogels exhibit excellent biocompatibility (Kopecek, 2009). Hydrogels enable high retention of water, due to cross-linking with many hydrophilic side groups, providing the moist environment for wound healing and therefore, appear to be an ideal media to enhance healing of wounds (Bhattarai *et al*., 2010).

Hydrogels can be classified on the basis of their network, namely as entangled networks, covalently cross-linked networks and networks obtained by secondary interactions (Berger *et al*., 2004a, b).

For the preparation of chemically cross-linked hydrogels, radical polymerization is generally used, through the cross-linking of the polymeric chains in aqueous media, and subsequent formation of hydrogel. Physically cross-linked hydrogels do not require the presence of a cross-linking agent, and are formed spontaneously under optimal conditions. This type of hydrogels is biodegradable. Their amorphous hydrophilic phase is held together by hydrophobic interactions, hydrogen bonding and/or van der Waals forces (Jagur-Grodzinski, 2010).

Hydrogels can also be classified according to:

- Their source: synthetic, natural or hybrid hydrogels
- The nature of the network: homopolymer, copolymer, interpenetrating, or double networks
- Their physical structure: homogeneous, microporous and macroporous hydrogels
- Their degradability: degradable and non-degradable hydrogels (Kopecek, 2009).

2.3.2. Characteristics of hydrogels

Hydrogels can be characterized by their rheological properties, their viscosity and viscoelastic strength, respectively (Boateng *et al*., 2008). Hydrogels, composed of hydrophilic polymers exhibit non-Newtonian pseudo plastic behavior. If the grade of pseudo plasticity decreases, the ability to spread on organic surfaces declines gradually (das Neves *et al*., 2009).

In addition, more advanced hydrogels may also be divided into several categories depending on their sensitivity to different stimuli (bioreponsive hydrogels) or the way they responds to the change in the swelling state (Kopecek, 2009; Jagur-Grodzinski, 2010).

Temperature responsive hydrogels: are characterized by their temperature dependent sol-gel transition T_{gel} that relate to the lower critical solution temperature, meaning that the polymer network collapse when temperature increases, and by the upper critical solution temperature T_P that relate to precipitation or dissipation of a gel. Polymers such as poly N-isopropylacrylamide, methylcellulose and pluronics display these properties (Kopecek, 2009; Jagur-Grodzinski, 2010).

pH responsive hydrogels: are prepared by introducing weak acids or weak bases, such as acrylic acid and amines to the functional groups of the polymer. The swelling of the polymer is due to the changes in pK_A or pK_B values. Hydrogel composed of both weak basic and weak acidic polymer can display lowest swelling in the pH region around 4-5, but the swellability increases markedly in low and higher pH values environment (Jagur-Grodzinski, 2010).

Analyte responsive hydrogels: are gels sensitive to the stimuli, for instance, mono and di-saccharides, enzymes, antigens and a variety of ions. They should be able to function under physiological temperature, pH and ionic strength (Jagur-Grodzinski, 2010).

Ion responsive hydrogels: can be made by fixing ethers, ligands of alkali and alkaline earth metal ions to a poly *N*-isopropylacrylamide based hydrogel. The T_{gel} are somewhat shifted in the preparation due to the complexation of ions by the ligands. The significance of the shift depends on the gel concentration and on the ions used in the solution (Jagur-Grodzinski, 2010).

2.3.3. Drug release from hydrogels

Several factors will influence the drug release from polymer-based formulations:

- Hydration of the polymer by liquids, particularly water
- Swelling of the polymer to form a gel
- Diffusion of the drug through the swollen material
- The erosion of the gel

These points will also play an important part in the controlled delivery of drugs when the dressing come in close contact with wound exudate (Boateng *et al*., 2008).

Diffusion controlled release through the mesh of the hydrogel is one of the main release mechanism for many of the drugs incorporated in hydrogels. If the diffusion of the drug is considerably faster than the hydrogel expansion, then the swelling is considered to be the main mechanism of the drug release. Chemically controlled release of the drug is dependent on chemical reactions within the gel matrix, either by hydrolytic or enzymatic metabolism (Bhattarai *et al*, 2010).

2.3.4. Hydrogels for wound management

Among the potential advanced delivery systems serving as wound dressings, hydrogels appear to be among the most promising (Bhattarai *et al*., 2010). Hydrogels dressings contain significant amount of water (up to 90%) and are therefore recommended for moderately exuding wounds. They posses most of the characteristics of ideal wound dressing and are suitable for cleansing of dry, sloughy or necrotic wounds. In addition, they are non-reactive with biological tissue, permeable to metabolites, and non-irritant (Boateng *et al.,* 2008). Hydrogels also promote moist healing, are non-adherent and cool the surface of the wound, resulting in pain reduction and high patient acceptability. They are particularly suitable to treat wounds in patients who cannot tolerate even reduced compression due to pain, such as burn patients. Moreover, they leave no reside, are malleable and enhance wound

re-epithelialisation. However, hydrogels should be avoided in treatment of heavily exuding wounds (Boateng *et al*., 2008).

Although various polymeric materials are used in hydrogel formation, chitosan, natural origin polymer, is the main focus of many research groups. Due to its biocompatibility, its ability to absorb wound exudate, its film forming properties, and wound healing potentials, this naturally derived polysaccharide is a good candidate for burns therapy as well (Alsarra, 2009).

By incorporating antimicrobial agents in hydrogels, wound infections can be prevented or combat. Several hydrogel formulations have been reported to be effective in this line. Even in early nineties Sawada *et al*. (1990) proposed an antimicrobial gel sheet as improved treatment for dermal burn wounds. The formulation caused enhanced epithelialisation, in comparison to conventional ointment-impregnated gauze dressing. The use of hydrogel formulations with antimicrobial activity may provide a lower resistance, and also a reduced hindrance to the wound healing process. Moreover, local drug delivery directly to the site of action may possibly prevail over unsuccessful systemic antibacterial treatment, due to the reduction in blood circulation in burn injuries (Boateng *et al*., 2008).

Important advantage of the sustained drug delivery systems incorporated in hydrogels is the fact that the need for frequent change of the dressing can be significantly reduced. In addition, due to the nature of the polymer, and its biodegradability, hydrogel can be removed from the wound surface by easy washing if necessary. Especially in the case of chronic wounds with associated pain, this will ease the therapy and increase the patient compliance. Topical delivery of active substances using wound dressings can also prevent patient exposure to unwanted high systemic doses, thereby reducing the unnecessary drug load outside the wound site (Boateng *et al*., 2008).

2.4. Chitosan

2.4.1. Structure of chitosan

Chitosan (Figure 7) is a linear hydrophilic polymer made of copolymers of N-acetyl glucosamine linked by β(1-4) glycosidic bonds and glucosamine. Chitosan is the deacetylated form of chitin, a natural polysaccharide found in exoskeleton of insects, crustaceans, and some fungi (Montembault *et al*., 2005; Bhattarai *et al*., 2010).

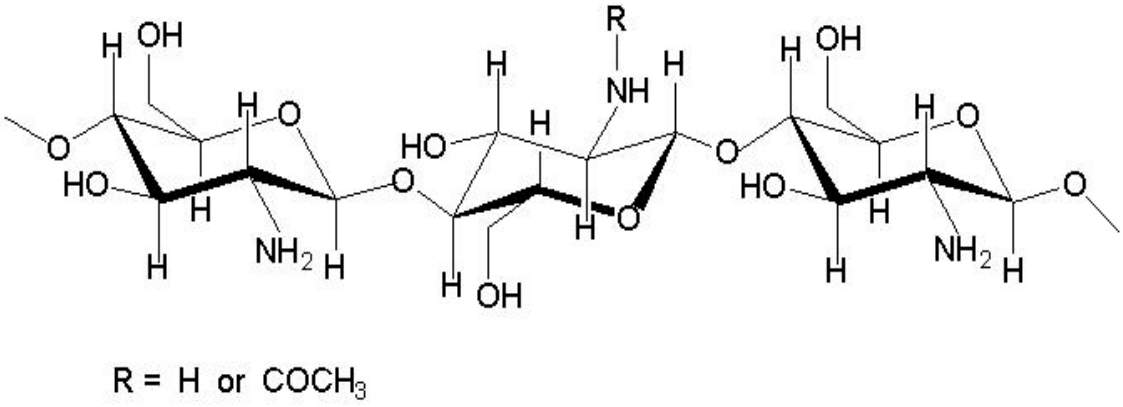

Figure 7: Structure of chitosan (Ph Eur)

The main parameters influencing the characteristics of chitosan are its molecular weight and degree of acetylation, representing the proportion of deacetylated units. These parameters are dependent on the conditions applied during the chitosan preparation, but can also be modified at later stage (Berger *et al*., 2004b).

Chitosans of different degree of deacetylation (DD) and different molecular weight display several advantages as gelling polymers in respect to their biological properties. These properties include homeostasis, stimulation of wound healing, potential to serve as tissue engineering scaffolds, and potential in drug delivery, especially controlled drug delivery (Ueno *et al*., 2001; Dai *et al*., 2009). Chitosan possesses positively charged amino groups and, as a result, chitosan is reported to have antimicrobial properties as well (Dai *et al*., 2009). Moreover, chitosan exhibits bioadhesiveness at the site of application resulting in increased retention time at the administration site, due to the charge at physiologic pH. The skin is known to exhibit negative charge and chitosan, being a cationic polymer, can bind electrostatically to

the skin and assure closer contact of delivery system, resulting in improved therapy (Berger et al., 2004a,b; Ribeiro *et al*, 2009).

2.4.2. Biodegradability of chitosan

Various enzymes, able to hydrolyze the linkages N-acetyl-glucosamine-N-acetyl glucosamine, glucosamine-glucosamine, and glucosamine-N-acetyl-glucosamine, can degrade chitosan. In the glycoside hydrolase18 family, there have been identified 8 human chitinases. Three of them, namely the acidic mammalian chitinase, di-N-acetylchitobiase and chitotriosidase exhibit enzymatic action. Chitosan given orally is most likely to be metabolized by the bacterial enzymes and lysozymes present in the large intestine. There is also possibility of diverse oxidation-reduction depolymerization and free radical degradation. The rate and degree of degradation are dependent on the DD; with lower values of deacetylation, an increase in the rate of biodegradation can be seen. The milieu of the gastrointestinal tract may also affect the degradation (Kim *et al*., 2008; Kean and Thanou, 2010). After intravenous administration it is believed that chitosan degradation profile is directly dependent on its molecular weight (Kean and Thanou, 2010).

2.4.3. Biodistribution of chitosan

The biodistribution of chitosan will be affected by its molecular size and charge of the side groups. After administering chitosan via the oral route, and the consequent absorption, the tissue distribution will be, for the most part, affected by the M_w. It is reported that increasing the M_w of chitosan, results in a decreased plasma concentration (Kean and Thanou, 2010). Intracellular uptake and distribution in *in vitro* conditions from a chitosan/DNA complex resulted in a 3-fold increased uptake at 37 ºC when compare to the uptake at 4 ºC (Kean and Thanou, 2010).

2.4.4. Toxicity of chitosan

Chitosan is, according to the Food and Drug Administration (FDA), generally recognized as safe (GRAS) (Weng *et al*., 2008). The toxicity profile is dependent on its M_w and its DD. When the DD is high, the toxicity is correlated with the M_w, and the chitosan concentration. When the DD is lower, it expresses a lower toxicity profile, and thereby is less affected by the M_w. Chitosan and its derivatives show potential in having antimicrobial activity against bacteria, fungi and parasites. Some of the bacteria include *P. aeruginosa* and *S. aureus*. This is also an additional advantage when applying chitosan drug delivery systems in skin injuries and burns. However, one has to consider the factors that may affect the chitosan toxicity, such as its purity, source, the salt form and polydispersity (Kean and Thanou, 2010). Due to its source of origin, chitosan may not be recommended orally to those people who are allergic to shellfish.

2.4.5. Chitosan-based delivery systems in skin and burns therapy

Due to its superior features in respect to wound healing potentials, chitosan has been prepared and evaluated in various drug delivery systems.

Alsarra (2009) studied the wound healing properties of chitosan expressing different DD and M_w in respect to healing in dermal burn wounds. The chitosan formulations were compared with Fucidin® ointment (conventional wound treatment) and non-treatment as a negative control. The wound contraction, the formation of epithelial and granulation tissue were found to be superior for the high M_w and high DD preparations ($p < 0.05$) when compared to the other treatment in respect to wound healing in rats. This demonstrates that chitosan possesses advantages as polymeric material when formulating drug delivery systems for burns therapy (Alsarra, 2009).

Minocycline incorporated in chitosan-polyurethane film dressing showed to be promising in the treatment of burn wounds in rats. Chitosan with different DD (67, 83, and 93 % (mol/mol), respectively) were prepared and evaluated. The most effective formulation in respect to wound healing was found to be the one consisting of 83 % DD (Aoyagi *et al*, 2007).

Ishihara *et al*. (2001) studied the wound healing properties of a photo-cross-linked chitosan hydrogel on full-thickness skin incision in mice. The hydrogel expressed enhanced wound contraction, closure and healing properties when compared to untreated (controls) mice (Ishihara *et al*., 2001).

When considering the application of chitosan hydrogels in the skin and burn injuries, it is also important to evaluate its tolerability and the tissue acceptability by the treated species. Boucard *et al*. (2007) studied the third degree burns on pig skin. Chitosan (3 %; w/w) was dispersed in water and hydrochloric acid (37 %; w/w) was added to protonate the amine groups. The formulations showed full acceptability by the host organism. Potential induction of inflammatory cells migration and angiogenetic activity was also evaluated. After 100 days, the new tissue was found to be analogous to native (undamaged) skin, with acceptable aesthetic feature and great scar flexibility (Boucard *et al*., 2007).

Deng *et al*. (2007) studied the biological properties of chitosan-gelatin sponge dressing in healing of wounds. In respect to different dryness of the formulation, the antibacterial properties were evaluated compared to cefradine, ciprofioxacin and penicillin (conventional antimicrobial treatment). The formulation showed stronger antimicrobial properties against *Escherichia coli* K88 than penicillin. The effect against *Streptococcus* was superior with the dressing, than to cefradine. In addition, the authors observed a shorter wound healing time for chitosan-gelatin sponge than with the control (sterile vaseline gauze) (Deng *et al*., 2007).

Chitosan wound dressings with polyphosphate and silver (procoagulant and antimicrobial, respectively) were prepared in order to evaluate the hemostatic and antimicrobial properties of the dressing. The formulation showed accelerated blood clotting, augmented platelet adhesion, quicker thrombin generation, and improved blood absorption, ($p=0.001$, $p=0.002$, $p=0.002$, $p< 0.001$, respectively) as compared to the control, chitosan. Incorporating silver into the optimized chitosan-polyphosphate dressing resulted in superior bactericidal activity against *P. aeruginosa* and *S. aureus* in *in vitro* models. The same optimized dressing reduced the mortality in *P. aeruginosa* infected mice wounds from 90.0 to 14.3 % (Ong *et al*., 2008).

Dai *et al*. (2009) demonstrated the efficiency of chitosan acetate dressing in treating *P. aeruginosa* and *P. mirabilis* infected third degree burns in mice. The topical

chitosan dressing, compared to nanocrystalline dressings and untreated (control) mice expressed survival rate of 73.3, 27.3 and 13.3 %, respectively for the *P. aeruginosa* infected group. For the *P. mirabilis* infected group the dressing showed survival rates of 66.7 in comparison to 62.5 % for the nanocrystalline dressing and 23.1 % for the control group. This demonstrated that chitosan wound dressings could be effective in preventing possibly lethal burn wound infection (Dai *et al*., 2009).

In order to assure prolonged and controlled delivery of incorporated active ingredient, such as antimicrobials, the concept of delivery-system in chitosan-based vehicle is proposed.

2.5. Liposomes as delivery systems

2.5.1. Lipids

Over the past fifty years there have been numerous research papers and patents on liposomes as drug delivery systems. Liposomes are spherical particles consisting of phospholipid bilayers. The choice of phospholipid used in the preparation will influence the properties of vesicles. Phosphatidylcholine (PC; Figure 8) is the most commonly used phospholipid. PC has a polar head group, represented by the quaternary ammonium moiety choline that is linked to the glycerol backbone by a phosphoric ester. The other two hydroxyl groups on the glycerol are further esterified with fatty acids. The phospholipid charge is neutral at physiological pH (Brandl, 2001).

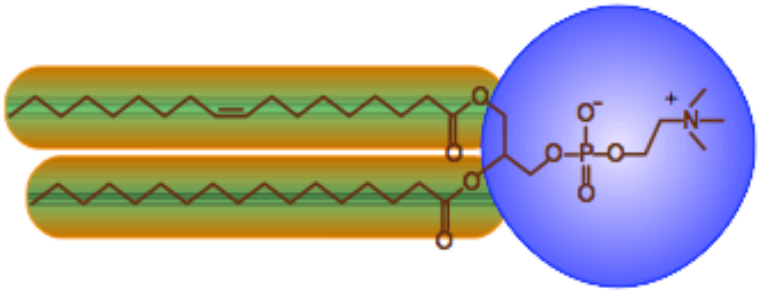

Figure 8: Structure of phosphatidylcholine (Brandl, 2001; *with permission*)

2.5.2. Classification of liposomes

Liposomes can be classified based on their structure, the method of preparation, their composition and application, etc (Samad *et al.*, 2007).

In respect to vesicle lamellarity and size, liposomes can be classified accordingly:

- Unilamellar vesicles: all size range (UV)
- Small unilamellar vesicles: 20-100 nm in diameter (SUV)
- Medium unilamellar vesicles and Large unilamellar vesicles: more than 100 nm in diameter (MUL, LUV)
- Giant unilamellar vesicle: more than 1000 nm in diameter (GUV)
- Oligolamellar vesicles: 100-1000 nm in diameter (OLV)
- Multilamellar vesicles: more than several hundred nm in diameter (MLV)
- Multi vesicular vesicles: more than 1000 nm in diameter (MV) (modified from New, 1990; Samad *et al.*, 2007)

However, this and similar classification should not be taken as absolute as many of liposome types may be classified in in-between categories. Moreover, without electron microscopy or small angle X-ray scattering evaluation, it is not possible to confirm the exact number of lamellas within the vehicle (Škalko *et al*., 1998b). In addition, one should consider the polydispersity of the vesicle population as well, as not all of the vesicles will express monodispersity.

Based on the method of preparation, liposomes can be classified as (Samad *et al.*, 2007):

1. Mechanical methods:

   A. Film method: here the liposomes are prepared by dissolving the phospholipid in an organic solvent, which is then removed under the vacuum.

When the organic solvent is evaporated, the film formed is then hydrated with appropriate aqueous medium. Following hydrating and swelling, the liposomes will be formed. The structure and the size of liposomes prepared by this method are the MLVs with a corresponding size around and over 1 μm in diameter.

B. Methods applying sonication: performing ultrasonic irradiation on aqueous lipid dispersion of phospholipid vesicles will results in vesicle size reduction and ultimately unilamellar vesicles. Sizes (diameter) of the liposomes are dependent on the time and amplitude of sonication, respectively.

2. Methods based on replacement of organic solvents:

A. Reverse phase evaporation: aqueous phase containing material to be entrapped into the liposome is dispersed into an organic solution were the lipids are co-solvated. Further, rotary evaporator is used to remove the organic solvent. The system is then purged with nitrogen, and the lipids are again dissolved in the organic phase usually by using ether (diethyl or isopropyl) as solvent. An emulsion is obtained, and the solvent is then evaporated forming a semisolid gel under diminished pressure. The non-encapsulated material is then removed from the mixture. The liposomes formed by this method are called reverse phase evaporation vesicles (REV), and the size and lamellarity corresponds to LUVs and OLVs.

B. Solvent vaporization method: can be performed by either the use of the ethanol or the ether injection method. In both methods the dissolved lipids are injected through a fine needle into a surplus of saline or other aqueous medium. In the ethanol injection method the injection is done promptly, while in the ether injection method, the injection is performed in slow manner. The size and lamellarity of vesicles vary.

3. Methods based on size transformation or fusion of performed vesicles:

   A. Freeze-thawing extrusion method: Liposomes containing entrapped drug, prepared by the conventional film method, are frozen in water medium, left to thaw and stirred. Upon completing the desired number of freeze thawing cycles, the sample is finally extruded through desired filter. The vesicle size and lamellarity will depend on the number of cycles and the size of filter pores.

   B. Dehydration-rehydration method: Pre-manufactured liposomes are frozen and freeze-dried in the presence of the material to be entrapped. Rehydration with the appropriate media but with reduced volume results in high entrapment and MLVs. The vesicle size can be further reduced by various size reduction methods (Škalko *et al*., 1998b).

Based on the phospholipid composition, liposomes can be classified into conventional liposomes, made of neutral or negatively charge phospholipids and cholesterol, or cationic liposomes, made of cationic lipids such as dioleoyl phosphotidylethanolamine. Other types may include immunoliposomes with antibody sequences or other recognition markers attached on their surface, and also long circulating liposomes, bearing polyethylene glycol (PEG) designed to avoid reticuloendothelial system (Samad *et al.,* 2007).

Both hydrophilic and lipophilic drugs can be incorporated in liposomes (Figure 9) and the drug entrapment efficiency will be dependent on the preparation method used, vesicle size, lipid composition and the properties of the drug (New, 1990; Škalko *et al*, 1992). Hydrophilic drugs can be entrapped into the interior aqueous part, while lipophilic and charged hydrophilic drugs can be incorporated within the phospholipid bilayer(s) by electrostatic or hydrophobic forces, or both (Honeywell-Nguyen and Bouwstra, 2005). Moreover, various ligands or active molecules can be attached to liposomal surface, enabling receptor mediated targeting for example (Škalko *et al*., 1998c).

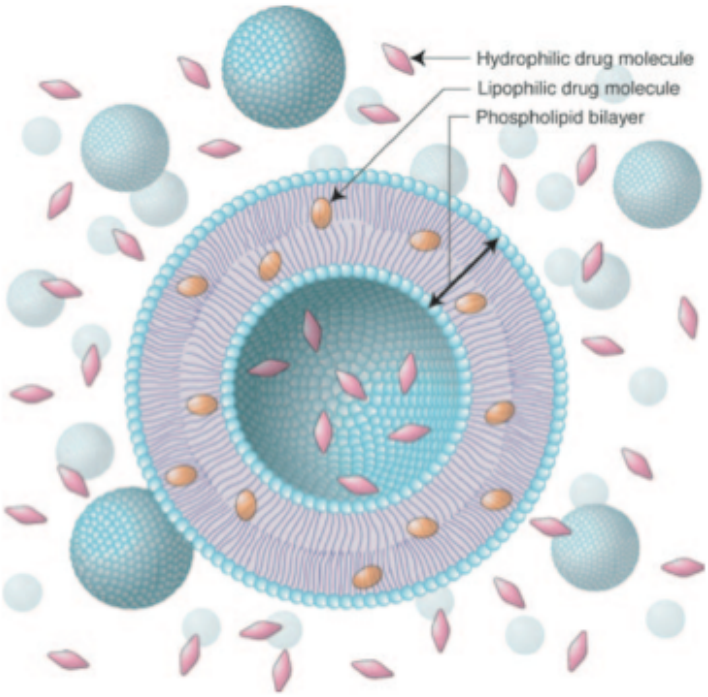

Figure 9: SUV with lipophilic drug incorporated into the phospholipid bilayer, and hydrophilic drug in the aqueous compartments (Hupfeld *et al*, 2006, *with permission*)

2.5.3. Liposomes for topical application onto skin

Liposomes can be administered by various routes of drug administration, and as this project focuses on the skin as administration site, skin application of liposomes will be discussed in more details.

Targeting of the active ingredients for a localized effect requires full understanding of the skin barrier function, and in the case of skin wounds, damaged skin barrier (Bouwstra and Ponec, 2006).

Although the consensus whether intact liposomes can penetrate into the skin remains to be a challenge, recent review proposed the following mechanisms:

The three possible mechanisms described for liposomal penetration of the skin

- Lateral diffusion; involving lipid exchange via molecular diffusion
- Passage via trans-epidermal osmotic gradient, related to hydration force where liposomes are sucked into the epidermis

- Passage via the pilosebaceous units (hair follicles with their associated sebaceous glands) (de Leeuw *et al.*, 2009).

The vesicles applied topically onto the skin provide several potential advantages. The liposomes can overcome some of the limitations related to oral route, such as the pH, food intake and the motility of the GI tract, as well as bypassing the hepatic metabolism (Honeywell-Nguyen and Bouwstra, 2005).

However, our aim is to assure localized delivery of active ingredient, avoiding the systemic absorption. Liposomes than provide the advantage of reducing skin irritation by sustaining the drug release at the application site, and hydrating the epidermal layer (Honeywell-Nguyen and Bouwstra, 2005).

Liposomes have also a tendency to accumulate on the *stratum corneum*, upper skin strata and in the sweat ducts, hair follicles and sebaceous glands following a negligible entry to deeper tissues or to the systemic circulation when applied to the skin exterior (El Maghraby *et al*., 2008; Benson, 2009; de Leeuw *et al.*, 2009). As a consequence, potential reduction in serious side effects, and potential incompatibilities that might arise from unwanted high systemic absorption of the selected drug are reduced (Egbaria and Weiner, 1990).

For drug delivery systems containing liposomes with entrapped or incorporated drug to be applied topically onto skin, it has been shown that a mean particle size of around 300 nm in diameter is preferable, due to the high drug concentration and reservoir in deeper skin layers (Škalko *et al.*, 1998a). Very important advantage of liposomes as skin delivery system lies in their ability to enhance the skin permeation of hydrophilic drugs, which then can increase therapeutic outcome (Škalko *et al*., 1998a). This is also true for the hydrophobic drugs, due to the amphipathic character shown by the liposomal carrier. Additional advantage is in stabilizing unstable drugs, which then have the opportunity to permeate across the skin strata undamaged (de Leeuw *et al*, 2009).

Several antimicrobial drugs for liposomal skin delivery have been studied, including clindamycin hydrochloride, metronidazole and amphotericin B (Škalko *et al*., 1992; Škalko *et al*., 1998a; Gupta *et al*., 2010).

Up to best of our knowledge, mupirocin has not been studied in topical liposomal drug delivery system.

2.5.4. Liposomal hydrogels for topical administration onto skin

Liposomal suspensions/dispersion are liquid in nature and will not remain at the administration site over longer period of time. Therefore, in order to assure the prolonged and controlled release of incorporated drug destined for topical application and mode of action, liposomal hydrogels have been proposed as promising drug delivery system (Pavelic *et al*., 2001).

By using the right vehicle in the preparation of liposomal hydrogels, it is possible to maintain the original size distribution of the liposomal formulation. When applying formulations topically, it is important to assure proper retention time of the drug at the site of action, to increase the therapeutic outcome and patient compliance and adequacy (Škalko *et al*., 1998a).

Various research groups have been studying liposomal hydrogels for topical administration onto vagina, eye, skin etc (Pavelic *et al*., 2001; Pavelic *et al*., 2004; Hosny, 2009; de Leeuw *et al*., 2009).

Liposomal hydrogels and their potentials in wound therapy were studied by Engesland (2010) and Poorahmary (2010). They evaluated the potential of both chitosan and carbopol hydrogels as vehicles for liposomes incorporating chloramphenicol, respectively.

In respect to *in vivo* evaluation of liposomal hydrogels aiming at treatment of skin wounds, particularly interesting work is discussed below:

Homann *et al*. (2007) conducted a randomized clinical trial with a liposomal hydrogel in the treatment of partial-thickness burn wounds in patients (n= 43). The preparation was a liposomal formulation with 3 % polyvinyl-pyrrolidone iodine in a Carbomer 940 (crosslinked acrylic acid polymer) hydrogel. The phospholipid used for the preparation of liposomes was hydrogenated soybean PC (3 %). The formulation was directly compared to conventional silver-sulfadiazine cream (Flammazine®) in clinical evaluation, resulting in significant reduction in healing time, 9.9 ± 4.5 versus

11.3 ± 4.9 days, respectively. The liposomal formulation also exhibited improved smoothness, elasticity and appearance of the wounded area, as compared to the conventional cream (Homann *et al*., 2007).

Vogt *et al*. (2001) studied liposomal polyvinyl-pyrrolidone iodine hydrogel (acrylic acid polymer) in comparison to conventional chlorhexidine gauze dressing when addressing the rate of epithelialisation and wound healing characteristic in the patients receiving skin grafts after suffering from burn trauma. The postulated formulation displayed an improved re-epithelialisation after day eleven (96.3 versus 75.9 %) and day thirteen (100 versus 82.3 %), respectively. The hydrogel formulation was also superior to the control dressing when considering the wound-healing characteristics ($p = 0.004$).

2.6. Mupirocin calcium as a model antimicrobial drug

Mupirocin calcium (MC) (Figure 10) is a calcium salt of the antibiotic produced by *Pseudomonas fluorescens* (Sutherland *et al*., 1985; Goering *et al*., 2008). Its mechanism of action is by inhibiting bacterial isoleucyl transfer RNA synthetase, which results in blocking protein synthesis and indirectly inhibiting RNA synthesis (Bageshwar *et al*., 2010).

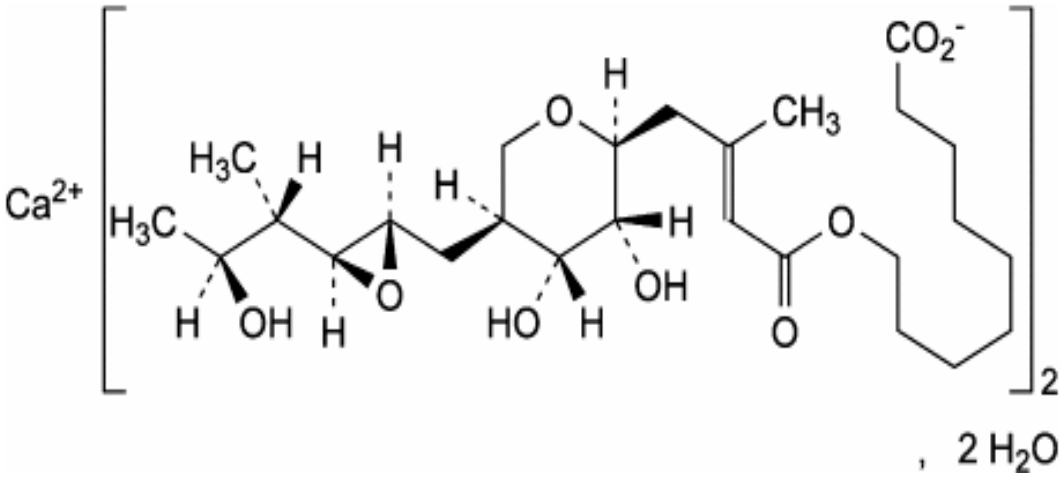

Figure 10: Structure of mupirocin calcium (Ph Eur)

Mupirocin expresses a broad activity against various bacteria. Those include *Staphylococci*, together with methicillin-resistant strains (MRSA) and *Streptococci*. *Haemophilus influenzae, Moraxella catarrhalis*, *Neisseria gonorrhoeae, Neisseria meningitides* and *Bordetella pertussis* are some of the gram- negative organisms that mupirocin shows activity against (Sutherland *et al*., 1985; Echevarria *et al*., 2003; Bageshwar *et al*., 2010).

The drug will undergo hydrolysis *in vivo*, which results in its inactivation, and the drug will also bind resiliently to serum (95%), thereby reducing its bioavailability. Due to these limitations, it is often used as a topically applied antibacterial drug. Mupirocin is also used nasally in infection-control programs to eradicate nasal colonization by MRSA. It shows low activity against members of the normal skin flora, including corynebacteria, micrococci and *Propionibacterium* spp. This can be seen as an advantage due to the skin normal defences against pathogens, which remains unaffected by mupirocin. The potent antibacterial activity of MC will be further enhanced in an acidic environment, and can thus be an advantage in relation to the acidic pH associated with the skin and its surroundings (Sutherland *et al*., 1985; Thomas *et al*., 2010).

When skin is injured or traumatized in any form, mupirocin can potentially penetrate to deeper layers. This is also true when occlusive dressings are used, resulting in higher permeation. However, the skin gradually metabolises MC to the inactive major metabolite monic acid. The use of MC ointment has shown well acceptability and related side effect were reported to be negligible (Echevarria *et al*., 2003)

MC is slightly soluble in water, sparingly soluble in anhydrous ethanol and in methylene chloride (Ph. Eur). It has a log P value at 2.7 (o/w), and a pk_a value of 4.7 (www.gsk.com)

Patients suffering from burn injury are at high risk of attracting pathogens and developing infections. Burn wounds colonization with *S. aureus* vary to a great extent, as well as with the severity of the burn wound, the patients age, the patients own nasal and pharyngeal *S. auerus* colonization, the health care workers and the type of care given by the health care professional at the centre of treatment. Burn wounds infected by *Staphylococcus aureus* have been associated with a delay in the wound healing process, an increased demand for surgery, and a longer hospital residence.

Administering nasal mupirocin to patients with high risk of developing the infections, may contribute to reduced risk of wound colonization with *S. aureus* (Kooistra-Smid *et al*., 2008).

Evaluation of the efficacy of mupirocin ointment in MRSA burn wound infection, showed total elimination of MRSA in all wounds treated (59 patients), moreover, the treatment was well tolerated by all patients. The study also recommended mupirocin ointment for patients suffering from burns (< 20 % of total body surface) when other conventional therapy has failed to eradicate the infection, however the time period for treatment was recommended not to exceed 5 days (Rode *et al*., 1989).

3. Aims of the study

The main aim of the study was the development of topical formulation for mupirocin to be applied in treatment of wounds, particularly burns. There are no literature data available on liposomal mupirocin for topical administration. Hydrogels are one of the most promising wound dressings, and chitosan hydrogels offer additional advantage of chitosan itself having wound healing and antimicrobial properties. Mupirocin-containing liposomes incorporated in hydrogels were expected to provide sustained release of incorporated drug, very important feature in improved wound therapy.

Specific aims, in more details, were:

- Development of liposomal formulation for mupirocin though optimization of preparation method, entrapment efficiency and vesicle size
- Development of liposomes-in-hydrogels delivery system and its optimization through evaluation of its textural properties
- Antimicrobial evaluation of liposomal hydrogels based on the antimicrobial activity of incorporated mupirocin
- Evaluation of liposomal hydrogels based on the *in vitro* release profile (Franz diffusion cells) of incorporated mupirocin
- Evaluation of liposomal hydrogels based on the *ex vivo* release profile on pig skin
- Comparison of antimicrobial and drug release characteristics between marketed product containing mupirocin (Bactroban® cream) and liposomal hydrogels with mupirocin

4. Materials and Methods

4.1.1. Materials

Acetic acid (glacial) GR for analysis, Merck, Darmstadt, Germany (K25892763 846 1.00063.1000)

Acetonitrile, CHROMASOLV® for HPLC, gradient grade, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany (Lot SZBA119B 34851)

Ammonium acetate, UWR BDH PROLABO, Leuven, Belgium (Product 21200.264, Batch 09C100004)

Bacteria; *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), Generous gift from Research Group in Microbiology, Molecular and Pharmaco-epidemiology, Department of Pharmacy, University of Tromsø, Norway

Bactroban 2 % (w/w) cream, (base containing benzyl alcohol cetomacrogol 1000, cetyl alcohol, liquid paraffin, phenoxy ethanol, stearyl alcohol, xanthan gum and purified water), GlaxoSmithKline, Barnard Castle, UK (Part. no 000701)

Chitosan, high molecular weight, Sigma-Aldrich Chemistry, St Luis, USA (MKBD1916V 419419-250G)

Cuprophan sheets, dimensions: 250 mm x 250 mm, molecular weight cut-off 10000 Daltons, Medicell International Ltd, London, UK (Code CUP.03.001)

Dialysis tubing, Molecular weight cut-off 12-14000 Daltons, Medicell International Ltd, London, UK (Visking code DTV12000.01.000)

Diluted Bactroban (*ex tempore*), Excipients: cetomacrogol 1000, Apotekproduksjon AS, Oslo, Norway (322156 ANR: 4D018/1), cetyl alcohol Apotekproduksjon AS, Oslo, Norway (304824 ANR: 2D107/7), liquid paraffin, Norwegian Medicinal Depot, Oslo, Norway (ANR: 3H066/3), stearyl alcohol, Merck Darmstadt, Germany (K502377802 8.07680.1000) and purified water

Distilled water

E-test MU MUPIROCIN (0.064-1024 µg/ml) Biomèrieux SA, Lyon, France (Lot 516308430, Ref 516308)

Folded capillary cells, Malvern Instruments Limited, Malvern, UK (DTS 1060 09/10/09/314)

Glycerin, anhydrous pure, Merck Darmstadt, Germany, (K29746193 142, 1.04093.1000)

Lipoid S 100 (soybean lecithin, 100 % phosphatidylcholine), a generous gift from Lipoid GmbH, Ludwigshafen, Germany (Lot 790631-03)

Methanol CHROMASOLV® for HPLC, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany (Lot SZBA119S 34860)

MICROLITER^{TM} Syringe 250 µl with RN Needle, Hamilton Company, Nevada, USA (P/N: 7657-01/00, P/N: 7806-01/00)

Muller Hinton agar plates, Section for microbiology and infection control (SUMP), University hospital, Tromsø, Norway

Mupirocin Calcium dehydrate micronized, a generous gift from GlaxoSmithKline, Zagreb, Croatia (Lot 41156047)

Pig ear skin, obtained from Nortura, Målselv, Norway

Propylene glycol, Norwegian Medicinal Depot, Oslo, Norway (331405 ANR: 1K083/3)

Sartorius polyamide membrane 0.2 µm, Sartorius AG, Göttingen, Germany (Lot 0510 25007 0940583)

4.1.2. Instruments

Beckmann L8-70M Ultracentrifuge, Beckmann Instruments Inc., Palo Alto, USA (MJM-1184-45-6L)

Beckmann SW 60 Ti rotor, Beckmann Instruments Inc., Palo Alto, USA (Serial nr: 05V2693)

Branson 5510E-MT, Bransonic ultrasonic cleaner, Danbury, USA

B. Braun Labsonic® U, B. Braun 2000 U with needle probe tip 40 T, B. Braun Biotech International GmbH, Melsungen, Germany

Büchi Waterbath B480, Büchi Vac V-500, Büchi vacuum controller B-721, Büchi rotavapor R-124, Büchi labortechnik, Flawil, Schwitzerland

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

Franz Diffusion Cell 9 mm with 5 ml receptor volume, flat ground joint, clear glass, clamp and stirbar, PermeGear, Hellertown, USA

Franz Diffusion Cell 15 mm with 12 ml receptor volume, flat ground joint, clear glass, clamp and stirbar, PermeGear, Hellertown, USA

Julabo heating circulator F12-ED, JULABO Labortechnik GmbH, Seelbach, Germany (10155866)

MS2 Mini shaker, Chiron AS, Trondheim, Norway

NICOMP Submicron particle sizer, model 370, Nicomp Particle Sizing system, Langhorne, USA

PermeGear V6A Stirrer, PermeGear, Inc., Hellertown, USA

Sonics High Intensity Ultrasonic Processor, 500 Watt model with temperature controller, probe horn 13 mm (diameter), Sigma-Aldrich, Chemie GmbH, Steinheim, Germany (Z513253-1EA, MW09055, Batch 3110)

TA.XT.Plus Texture Analyser, Stable Microsystems, Surrey UK. Backward Extrusion Rig A/BE, Stable Microsystems, Surrey, UK

Waters 2690, Separation module, Waters 996 Photodiode Array Detector, Waters Milford, USA; with XTerra^{TM} RP_{18} 5 µm (3.9*150 mm) and XTerra^{TM} RP_{18} 5 µm Guard Column (3.9*20 mm) from Waters, Dublin, Ireland

Zetasizer Nano Z 2600, Malvern Instruments Limited, Malvern, UK (MAL 1037062)

4.1.3. Computer programs

High Performance Liquid Chromatography: Millennium 32 Chromatography Manager (4.0)

Photon correlation spectroscopy: CW 388 version 1.68

Texture Analyser: Texture Exponent, 32 (3.0.5.0) Stable Microsystems, Surrey, UK

Zeta potential: Zeta potential report version 2.2, Malvern Instruments Limited, Malvern, UK

4.2. Liposomal characterisation

4.2.1. Preparation of liposomes with mupirocin (MC)

Mupirocin (5, 10 and 20 mg, respectively) was dissolved together with Lipoid S 100 (200 mg) in excess methanol. The organic solvent was evaporated in a rotavapor for at least 20 min at 100 mmHg (45 °C and 50 rpm), and then for additional 45 min at 45 mmHg (45 °C and 50 rpm). The obtained film was then re-suspended in 10 ml of distilled water. Votex was used to equally dislodge the film when needed. The liposomes were stored in a refrigerator for at least 24 hours before further use and characterization.

4.2.2. Entrapment efficiency determination

To determine the entrapment efficiency (EE) for MC, the unentrapped MC was separated from liposomes by ultracentrifugation and dialysis.

Ultracentrifugation: Liposomes were centrifuged in Beckman-L8-70M ultracentrifuge for 90 min at 10 °C and 32000 rpm. The pellet formed upon ultracentrifugation was re-suspended in 1 ml of distilled water. From both the supernatant and the pellet, a sample of 100 µl was further dissolved/diluted in 10 ml of methanol (total volume) and used in HPLC analysis.

Dialysis: Dialysis was performed in a dialysis tubing. One ml of sample was dialysed against 200 and 400 ml of distilled water for 24 hours, respectively. One hundred µl, of both liposomal suspension and dialyzate, were further dissolved/diluted in 10 ml of methanol (total volume) and used in HPLC analysis.

4.2.3. HPLC analysis

The HPLC method applied was based on the method described by Echevarria *et al*. (2003), with slight modification. To obtain standard curves in both water and methanol, the stock solution of MC was prepared in a concentration of 40 µg/ml. Thirteen standard solutions of 0.2, 0.4, 0.5, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 15 and 20 µg/ml MC were prepared by diluting the stock solution with appropriate volumes of acetonitrile and water (1:10; v/v). The mobile phase consisted of acetonitrile and ammonium acetate (0.05 M) in ratio of 27.5:72.5 (v/v), adjusted to pH 6.3 with hydrochloric acid. The mobile phase was filtered through a 0.2 µm pore size filter prior to analysis. The flow rate was set to 1 ml/min, and photodiode array detector set at 228 nm. The column temperature was set at 30 °C, and the sample temperature was set at 25 °C during the separation process. The run time was 9 min and the volume injected was 20 µl. All analyses were performed in triplicates, respectively.

Data asset and management were performed by the help of a Compaq computer using Millennium 32 Chromatography Manager (4.0).

4.2.4. Particle size analysis

Photon correlation spectroscopy, also called dynamic light scattering, was used to determine the particle size distribution. Determination was performed on the NICOMP Submicron Particle Sizer model 370, operational by a helium-neon laser (632.8 nm) at an angle of 90 degrees and a temperature at 24 °C ± 1 °C. To circumvent any contamination with particles from the surrounding milieu, sample preparation was carried out in an uncontaminated area using particle-free equipment. Preparation and all samples handling were performed in a laminar airflow bench. Test tubes were filled with distilled water and sonicated for 20 minutes using an ultrasonic

bath, and further rinsed with filtered water (0.2 μm pore size syringe filter) before use. The sample was diluted empirically with freshly filtered water until an intensity of 250-350 kHz was obtained (Hupfeld *et al*., 2006).

4.2.5. Size reduction of liposomes

Liposomes were transferred to a 2 ml round bottom vial and placed on ice bath. The needle probe tip of sonicator was fixed in a vial, and positioned in the centre of the volume. Vesicle suspensions were exposed to ultrasonic irradiation with an output of 50 Watt for a continuous cycle of 5 min to obtain desired particle size.

Due to the technical problems, we needed to change the type of sonicator used in particle size reduction. Therefore, for preparation of liposomes incorporated in chitosan gels for *in vitro* and *ex vivo* release study on Franz diffusion cells (FDC), the optimized sonication time on a new sonicator (Sonics High Intensity Ultrasonic Processor) was set to a continuous cycle of 25 s with amplitude at 40 %, respectively. These conditions corresponded, in respect to the particle size of obtained vesicles, to the conditions of previously applied sonicator.

4.2.6. Zeta potential

Zeta potential (ZP) was measured on Zetasizer Nano Z 2600 to assess stability of liposomes.

Before performing the measurements, the cell was thoroughly rinsed with distilled water and ethanol using an appropriate syringe, to ensure totally wetting of the cell. An aliquot of 100 μl of liposomes was resuspended in freshly distilled water (total volume 10 ml) and added to the cell via a syringe. The number of runs for each sample was set to 10 cycles.

4.3. Hydrogels preparation and characterization

4.3.1. Preparation of chitosan hydrogels (empty gel)

It is known that the gelation of chitosan occurs when it is dispersed in week acid (Cao *et al*., 2009). The method used in hydrogel preparation was based on Alsarra (2009) and Cao *et al*. (2009). Chitosan was dispersed in 2.5 % acetic acid solution (w/w) and glycerol (10 %, w/w) added into the mixture. Glycerol was added into mixture to stabilize hydrogel (Engesland, 2010). The final concentration of chitosan in acetic acid was 2.5 % (w/w). The mixture was manually stirred for approximately 10 min. The preparation was allowed to swell for 48 hours in a sealed container before further use.

4.3.2. Preparation of liposomal chitosan hydrogels (gel-N-SL; gel-SL; gel-N-SL (f+e))

Hydrogels were prepared as mention under section 4.3.1. Liposomal chitosan hydrogels were prepared as follows, 10 % liposomal preparations (w/w) were incorporated in pre-prepared chitosan (2.5 %; w/w) hydrogels, containing 10 % (w/w) glycerol. Liposomal suspensions used for incorporation into hydrogels were of three types:

a) non-sonicated liposomes free from unentrapped MC

b) sonicated liposomes free from unentrapped MC

c) non-sonicated liposomal suspension containing both free and liposomally entrapped MC

Hand stirring was applied to fully disperse liposomes within (Škalko *et al.,* 1998a) the hydrogel. The prepared hydrogels were allowed to set for 2 hours before further use.

4.3.3. Preparation of chitosan hydrogels containing MC dissolved in propylene glycol (gel-pg)

Chitosan hydrogels were prepared as mention under section 4.3.1. MC dissolved in propylene glycol (pg) (10 %, w/w) was incorporated in hydrogel by hand stirring. The prepared hydrogels were allowed to set for 2 hours before further use.

4.3.4. Textural properties of chitosan hydrogels

Textural properties of hydrogels, namely gel cohesiveness, adhesiveness, and hardness, are important features of gels, in respect to gel optimization (Hurler *et al*., 2010). Texture analyser TA.XT. Plus was employed to determine the textural properties of gels. Based on the previously recommended optimization of experimental set up for chitosan hydrogels (Engesland, 2010), a submerge probe disk (40 mm in diameter) was used and measurements conducted by the backward extrusion. All three types of hydrogels, namely empty chitosan hydrogel, liposomal chitosan hydrogel and chitosan hydrogel incorporating propylene glycol were evaluated.

Before starting the experiment, the force applied and the height of the probe were calibrated. Sixty grams of various gels were used in the experiments. The probe was immersed (30 mm from the top) and left unmoved for 30 seconds. Five consecutive measurements with 30 seconds resting interval between each run were performed (Engesland, 2010).

The experimental conditions were as followed:

pre-test speed: 4 mm/sec;

test speed: 4 mm/sec;

post-test speed: 4 mm/s;

distance 10 mm; return to the start point.

The areas and the forces (Figure 11) were measured (Engesland, 2010).

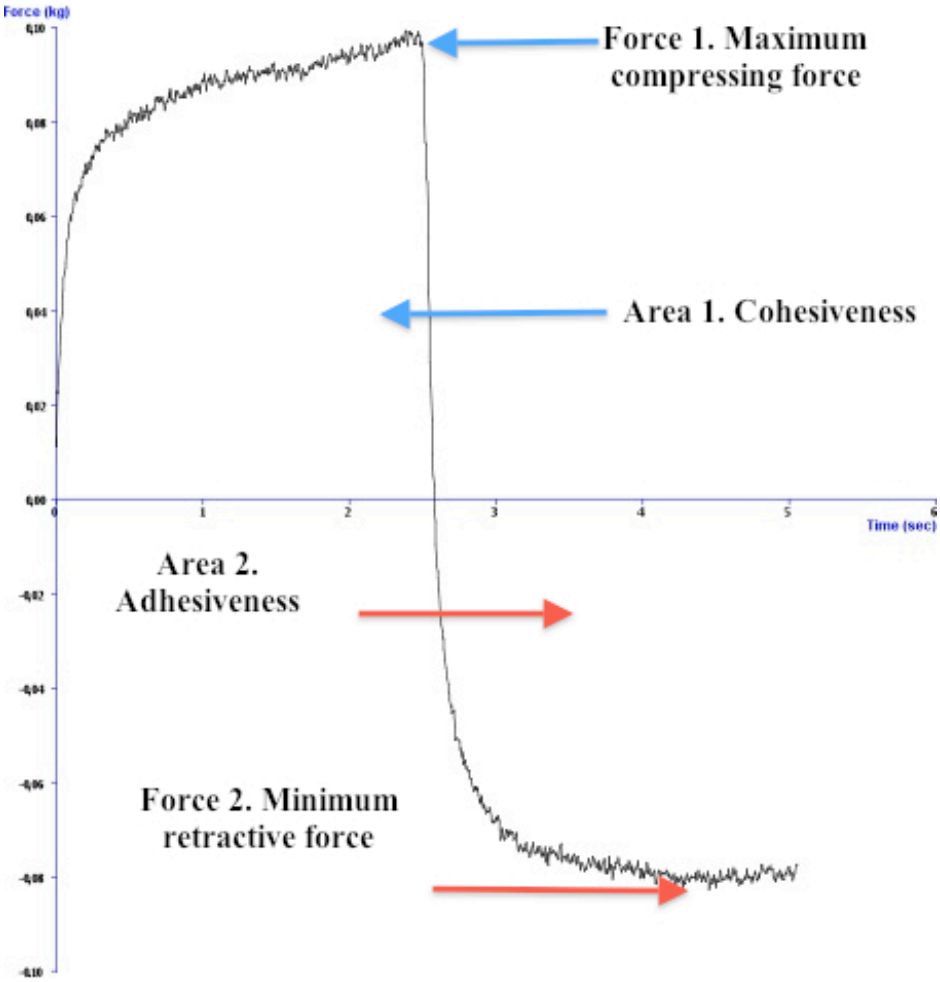

Figure 11: Parameters measured in texture analysis

Four parameters were recorded, namely the maximum compressing force (Force 1), the cohesiveness (Area 1), the minimum retracting force (Force 2) and the adhesiveness (Area 2) (Figure 11).

4.4. Stability testing

4.4.1. Accelerated stability testing of non-sonicated (N-SL) and sonicated liposomes (SL)

N-SL suspension was stored for a 30-days period in an airtight container at 40 °C. SL suspension was treated in the identical manner. The original vesicle size and size distribution was compared to the vesicle size and size distribution after the accelerated stability testing.

4.4.2. Accelerated stability testing of empty chitosan hydrogels

The gels were stored in an airtight container at 40 °C for a 30-days period. The textural properties (see 4.3.4) of freshly prepared hydrogels and hydrogels after the accelerated stability testing were compared and evaluated.

4.4.3. Accelerated stability testing of liposomal chitosan hydrogels

a) Non-sonicated liposomal chitosan hydrogels (gel-N-SL) were stored in an airtight container at 40 °C for a 30-days period. The textural properties (see 4.3.4) of freshly prepared hydrogels and hydrogels after the accelerated stability testing were compared and evaluated.

b) Sonicated liposomal chitosan hydrogels (gel-SL) were stored in an airtight container at 40 °C for a 30-days period. The textural properties (see 4.3.4) of freshly prepared hydrogels and hydrogels after the accelerated stability testing were compared and evaluated.

4.5. Microbiological evaluation of liposomal preparations

4.5.1. Determination of minimum inhibitory concentration (MIC) values for MC on selected bacterial strains

Prior to selecting the bacterial strains, the E- test was applied to determine the MIC values. Final selection of microorganisms was as follows: *B. subtilis, S. epidermidis*, *E. coli*, *S. aureus*, *E. faecalis*, and *M. luteus*.

For each strain, bacterial suspensions were prepared with turbidity corresponding to that of 0.5 McFarland in a sterile 0.9 % (w/w) saline, and further inoculated on an agar plate (Mueller-Hinton) by the help of sterile cotton tip dipped in the suspension and brushed in three different directions (Simpson *et al*., 1995). The E- test strip was put on the agar with the help of a tweezer, assuring that no air was trapped underneath the test strip. The inoculated plates were then incubated at 37 °C for 24 hours (Simpson *et al*., 1995). The experiments were performed in triplicates, respectively.

4.5.2. Zones of inhibition for selected bacterial strains

Plates containing bacterial suspension were prepared as explained under section 4.5.1, but without further incubation step. The plates were divided into five different areas where different preparations could be tested (Figure 12). The following formulations were evaluated:

i) gel-N-SL

ii) gel-SL

iii) Diluted Bactroban® cream (d Bac-cream) (mupirocin concentration corresponding to the concentration in liposomal hydrogels).

Water and empty chitosan gel served as negative controls, respectively. The same procedure was performed for pg and gel-pg (the drug concentration was kept identical as in other samples).

Aliquot (10 µl) of each sample was inoculated on the bacteria to be tested (*B. subtilis* and *S. aureus,* respectively). In all tested samples, the concentration of mupirocin was

set to be 505 µg/ml. The plates were then incubated at 37 °C for 24 hours. The experiments were done in triplicates, respectively.

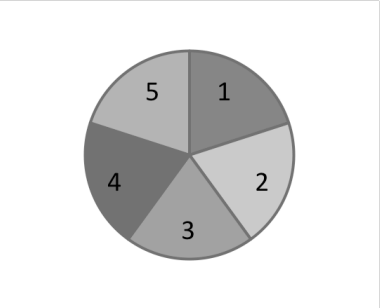

Figure 12: Dividing zones on agar plate

4.6. *In vitro* and *ex vivo* mupirocin release studies

4.6.1. *In vitro* mupirocin release as determined on Franz diffusion cell system (FDC)

FDC was applied to determine the release of the incorporated drug from vesicles and liposomal hydrogels (Gonzàlez-Paredes *et al*., 2010) Prior to any diffusion experiments, both the receptor and the donor chamber (Figure 13) were filled and washed with deionised water, followed by methanol, for approximately one hour. The FDC were dried sufficiently before the receptor medium was applied to the cells.

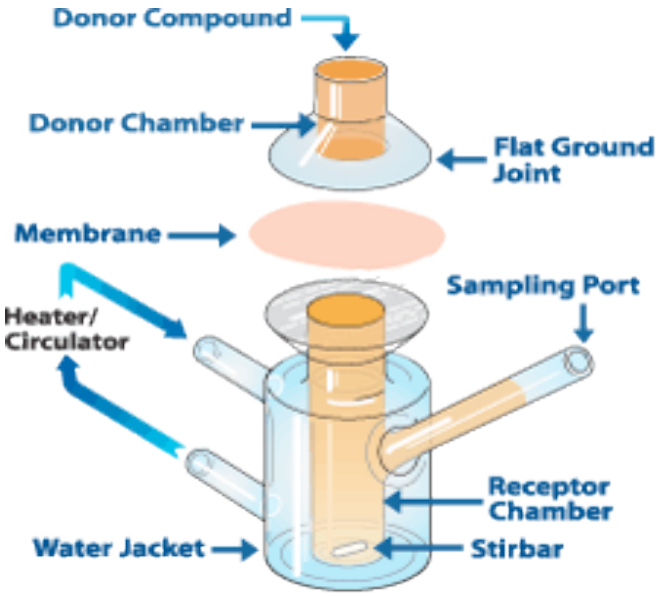

Figure 13: Schematic presentation of Franz Diffusion Cell (www.permegear.com)

To assure the uniform stirring during the dialysis process, the automatic stirrers were applied as shown in Figure 14.

Figure 14: PermeGear V6A stirrer (www.permegear.com)

In addition, to assure that the temperature remained to be constant and corresponding to physiological temperature (37 °C), the cells were connected to a heater circulator as illustrated in Figure 15.

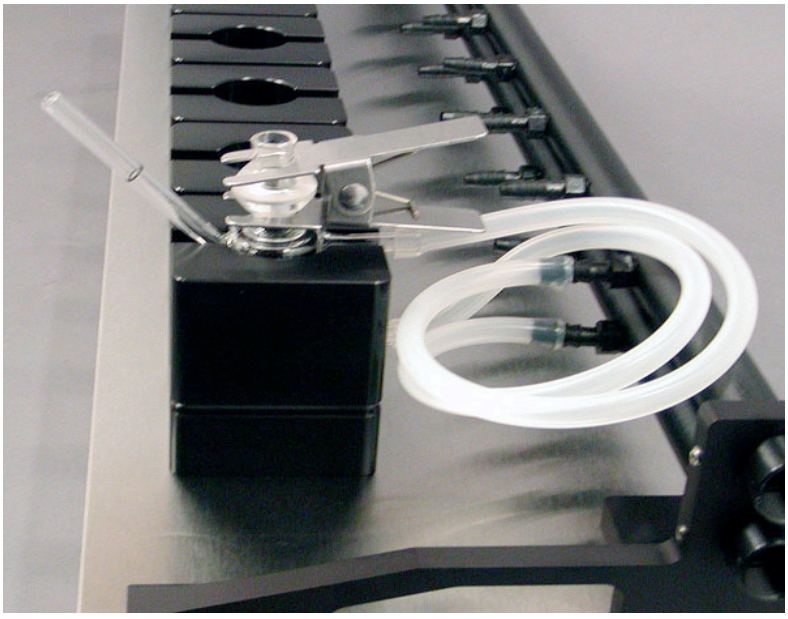

Figure 15: Circulator connection to Franz Diffusion Cell (www.permegear.com)

The membranes used in the experiments, namely Sartolon polyamid (0.2 µm) and Cuprophan (MWCO 10.000 Daltons) were evenly cut to fit the top of the receptor chamber. The donor chamber was placed on top, with the joint packing in between. A metal clamp (Figure 15) was used to hinder any interference of air in the FDC. If air-bobbles were detected, the cell was flipped to manage release of entrapped air.

Samples (300 µl aliquot) of different formulations were put into the donor chamber with a help of plastic syringe. The receptor medium (12 ml) used was the mobile phase previously mentioned under section 4.2.3. At different time intervals (10, 20, 30, 45, 60, 120, 180, 240, 420 and 1440 min) sample aliquots of 200 µl were taken out with a Hamilton microliter syringe, and if needed, further diluted with sufficient amount of receptor medium, to be analyzed in HPLC analysis. The volume of medium taken out of the cell was replaced with fresh receptor medium after each sampling point. The sampling ports were covered with quadruplicate layers of

parafilm, whereas the donor chambers were sealed with a rubber plug to prevent any contamination and evaporation of both sample and receptor medium.

The following preparations were tested to determine the release profile of mupirocin:

i) Bactroban® 2 % cream (Bac-cream 2 %)

ii) d Bac-cream

iii) gel-N-SL

iv) gel- SL

v) gel N-SL (f+e)

vi) gel- pg

vii) N-SL

viii) SL

ix) pg

All experiments were performed in quadruplicates.

4.6.2. *Ex vivo* release studies

The skin slices for *ex vivo* release studies were prepared by scalpel sectioning, after removing all underlying connective and fat tissues. The prepared samples of pig ear skin were frozen in saline and let to thaw at room temperature prior to diffusion experiments.

For *ex vivo* release studies on pig ear skin, FDC (9 mm) with 5 ml receptor phase volume were used.

The following preparations were tested to determine the release profile for MC:

i) d Bac-cream

ii) gel-N-SL

iii) gel-SL

iv) N-SL

v) SL

vi) pg

The mupirocin concentration in all formulations was set to be 505 μg/ml.

Based on prior experiments and in order to assure sink conditions, we decided to use Bac-cream as diluted sample. The diluted cream contained the same amount of mupirocin as equivalent to liposomal concentration of entrapped drug. In addition, to avoid the possible effect of diluent on release profiles, a base containing the same excipients as Bac-cream 2 % (60 %, w/w water, 5 %, w/w cetomacrogol 1000, 5 % w/w, cetyl alcohol, 5 %, w/w stearyl alcohol and 25 % w/w liquid paraffin) was used for dilution.

The sink conditions were assured in all experiments.

All experiments were performed in duplicates.

4.7. Statistical evaluations

When applicable, student *t test* was used to determine the level of significance.

5. Results and Discussion

5.1. Liposome characterization

The results of entrapment efficiencies and particle size distributions for liposomes prepared by the film method are presented in Tables 2A and 2B. The separations of unentrapped drug were performed by either the ultracentrifugation method (A) or the dialysis (B).

Table 2A: Drug entrapment in N-SL as determined by ultracentrifugation

Drug (mg)	Entrapment efficiency (%)	Mean diameter (nm)	Polydispersity index	Drug recovery (%)
5	73.40 ± 1.54	905.3*	1.54	104.08 ± 3.81
10	74.79 ± 0.66	905.3*	1.60	76.89 ± 13.68
20	68.47 ± 3.17	905.3*	1.83	76.34 ± 2.39

* The estimated size as vesicles are too polydispersed

All preparations contained 200 mg of lipid. The values denote the mean of 3 separate experiments ± SD.

The drug recovery was rather low for liposomes prepared with higher starting amounts of mupirocin (Table 2A). Although the entrapment efficiency values were found to be comparable for liposomes which were centrifuged to remove unentrapped drug to values for liposomes dialyzed to separate the unentrapped drug (Tables 2A and 2B), the drug recovery were found to be lower in experiments with dialyzed samples. The determination of the drug content in dialyzate involves multiplication with dilution factor based on the total volume (several hundreds millilitres), which often results in lower recovery.

Table 2B: Drug entrapment in N-SL as determined by dialysis

Drug (mg)	Entrapment efficiency (%)	Mean diameter (nm)	Polydispersity index	Drug recovery (%)
5	79.20 ± 4.03	905.3*	1.53	92.15 ± 6.56
10	77.14 ± 1.68	905.3*	1.60	68.68 ± 15.21
20	72.47 ± 2.29	905.3*	1.83	62.95 ± 2.68

*The estimated size as vesicles are too polydispersed

All preparations contained 200 mg of lipid. The values denote the mean of 3 separate experiments ± SD.

The determined particle sizes and size distributions indicated that the liposomal suspensions were very polydispersed, which would affect the controlled release of the incorporated drug, therefore, the next step in optimizing the liposomal formulations was to prepare liposomes of more uniform size. We applied probe sonication as a method to reduce the originally large particle size of vesicles. Probe sonication is known to reduce the size and narrow the size distribution of heterogeneous populations (New, 1990). Important parameter that needs to be considered related to sonication is the loss of the originally entrapped drug. The loss needs to be monitored throughout the process.

We tried to optimize the sonication conditions in order to prepare vesicles of more uniform size distributions, with most of the originally entrapped material retained in liposomes (data not shown). The dialysis method was applied as the separation method for all sonicated liposomes.

The optimized sonication conditions resulted in liposomal populations presented in Table 3.

Table 3: Loss of the originally entrapped mupirocin in SL

Drug (mg)	Entrapment efficiency (%)	Loss of originally incorporated drug (%)	Drug/lipid ratio (µg/mg)	Drug recovery (%)
5	57.33 ± 0.14	27.66 ± 3.70	8.9	85.15 ± 6.85
10	54.31 ± 2.44	29.58 ± 2.79	18.9	63.42 ± 9.35
20	34.96 ± 0.74	51.72 ± 1.56	16.8	62.18 ± 3.05

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

As indicated in Table 3, the loss of originally entrapped mupirocin was higher for liposomes for which higher amounts of drug (20 mg) were taken in preparation. For those liposomes, the loss was over 50 % as compared to liposomes for which 5 or 10 mg were taken in preparation. However, one should consider the drug/lipid ratio, as real indicator of how much of the drug is associated with liposomes, therefore final ratios are included in Table 3.

At the same time, it was expected that the polydispersity indexes and mean particle size would be significantly reduced upon sonication. The polydisersity indexes for sonicated liposomes were between 0.42 and 0.44 for all preparations, which is acceptable and indicates more uniform vesicle size distributions.

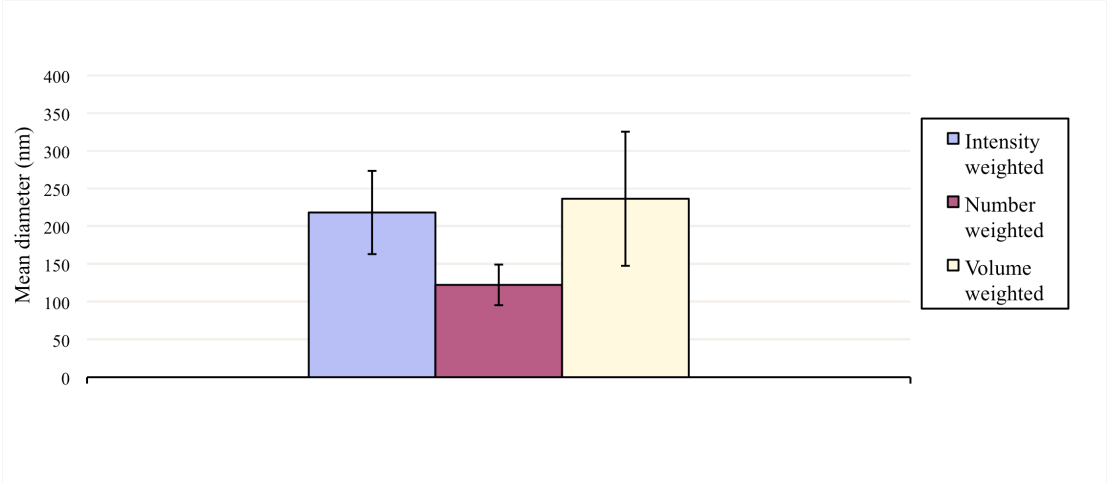

Figure 16: Particle size of sonicated liposomes (5 mg drug taken in preparation)
The values denote the mean of 3 separate experiments ± SD.

As can be seen in Figure 16, the mean diameter of vesicles was significantly reduced upon sonication and was around 200 nm (expressed as intensity and volume weighted), which represents the optimal vesicle size for skin applications (Cevc, 2004).

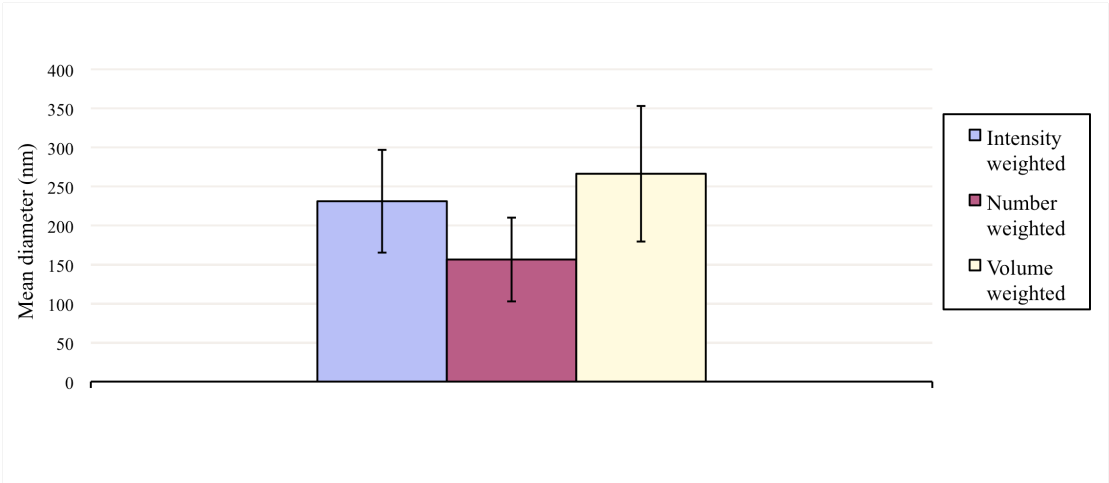

Figure 17: Particle size of sonicated liposomes (10 mg drug taken in preparation)
The values denote the mean of 3 separate experiments ± SD.

Under the same conditions of sonication and same lipid amount used in liposome preparation, the particle size of vesicles for which preparation 10 mg of drug was used was found to be larger (Figure 17) than for liposomes shown in Figure 16. It is probably the consequence of higher drug per lipid ratio (Table 3).

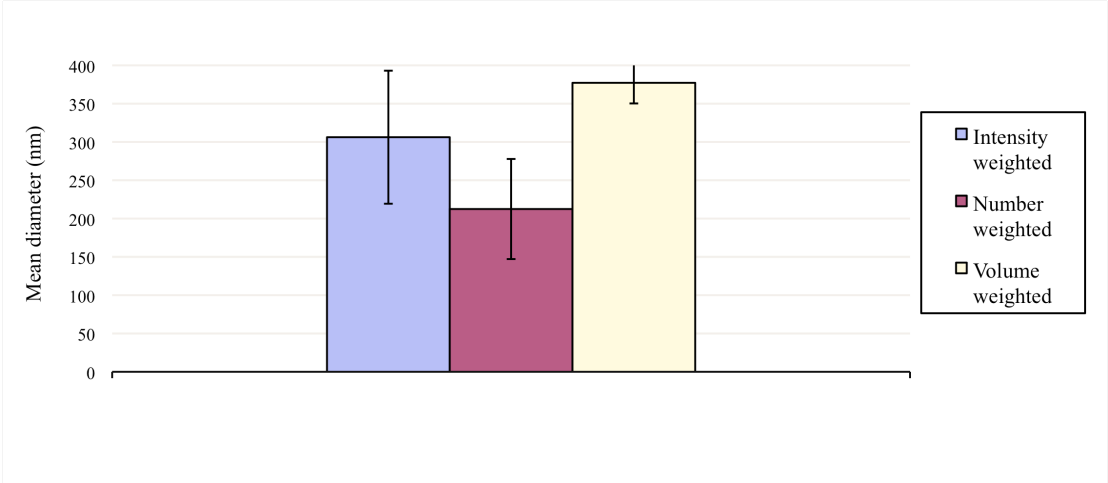

Figure 18: Particle size of sonicated liposomes (20 mg drug taken in preparation) The values denote the mean of 3 separate experiments ± SD.

The effect of the starting amount of the drug taken into the preparation on liposomal size was even more pronounced for liposomes for which preparation the highest amount of drug was used (Figure 18). It appears that drug incorporated in the vesicle bilayers represents a kind of the barrier, providing the rigidity to the bilayers and resisting further particle size reduction. Similar findings were reported on the effect of cholesterol or lipophilic compounds incorporated in lipid bilayers of liposomes, through an increase in membrane rigidity and resistance of vesicles to sonication (di Cagno *et al*., 2011). However, mupirocin, based on its solubility profile, is expected to accommodate itself in both lipophilic and hydrophilic part of liposomes. We cannot, at this stage, confirm the exact positioning of mupirocin within liposomal vesicle.

Table 4: Zeta-potential of non-sonicated and sonicated liposomes

Drug (mg)	Lipid (mg)	ZP (mV)	
		Non-sonicated vesicles	Sonicated vesicles
20	200	-30.33 ± 3.73	6.27 ± 0.58

The values denote the mean of 3 separate experiments ± SD

Zeta potential is an important indicator for repulsion forces between colloidal particles, and thereby may postulate the stability of liposomal preparations. If the ZP is highly negative or positive, the vesicles may repel each other, and thereby show suspension stability, whereas particles with low values for ZP often tend to aggregate due to diminutive repulsive forces. The ZP for liposomes is depending on the lipid composition used in the preparation (Gonzàlez-Paredes *et al*., 2010). As illustrated in Table 4, non-sonicated large multilamellar vesicles exhibited ZP of -30.33. This value can indicate rather stable liposomal preparation. However, liposomes were intended to be incorporated into hydrogels, in which case the protective hydrogel network can additionally stabilize liposomal suspensions (Pavelic *et al*., 2001).

Interestingly, ZP values for sonicated liposomes, made of the same phospholipid composition, and expected to have similar ZP, seem to be more prone to aggregation as seen from lower ZP values (Table 4). This phenomenon is difficult to explain, one of the possible explanations could be that during sonication some of mupirocin released from vesicles becomes associated/attached to outer liposomal membranes, affecting the change in surface charge. Whether it is indeed so, remains to be further evaluated.

Stability of liposomes is discussed in part 5.3.1.

5.2. Hydrogel characterization

5.2.1.Textural properties of empty chitosan gel and gel-pg

As our aim was to develop the delivery system for improved therapy, we wanted to assure that textural properties of hydrogels correspond to the expected properties (Engesland, 2010). It is important for the delivery system to provide increased association with the compromised area (retention time at wound site), namely to prolong the contact time between the drug and the wounded site (Jones *et al*., 1997; Alsarra, 2009; Akomeah, 2010). Moreover, as chitosan itself exhibits the wound healing potentials, longer residence time for chitosan hydrogels at the site enables improved therapy (Bhattarai *et al.*, 2010). It was therefore important to investigate gel cohesiveness and adhesiveness in particular (Area 1 and Area 2, respectively) in order to correlate those two properties to the applicability of the system as wound dressing.

Table 5A: Characteristics of empty chitosan hydrogel

Run		Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
	1	98.57	234.94	91.41	191.82
	2	98.90	235.37	90.97	191.53
	3	100.53	234.50	91.62	191.07
	4	99.22	234.54	91.08	190.92
	5	100.53	235.37	90.86	190.85
Average		99.55	234.94	91.19	191.24
SD		0.82	0.38	0.28	0.37
CV		0.83	0.16	0.31	0.20

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

Both Area 1 (cohesiveness) and Area 2 (adhesiveness) provide an indication of hydrogel potential to residue at the site over longer period of time. Both were in range suitable for the application onto the skin (Engesland, 2010).

However, we are not aiming at using empty hydrogels, and in order to assure that incorporation of different additives is not changing the original textural properties of

hydrogel to a greater extent, we evaluated the hydrogel into which propylene glycol was added. Mupirocin was dissolved in propylene glycol prior to its incorporation into hydrogel.

Table 5B: Characteristics of chitosan hydrogel incorporating pg

Run	Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
1	81.31	192.18	73.17	152.40
2	81.42	193.00	72.84	151.39
3	80.98	192.03	74.47	153.04
4	80.98	191.66	72.08	150.04
5	81.96	193.70	71.75	148.99
Average	81.33	192.52	72.84	151.17
SD	0.36	0.73	0.98	1.49
CV	0.44	0.38	1.34	0.98

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

Figure 19, along with Table 5B, illustrates the changes in original hydrogel properties as a result of incorporation of propylene glycol. The texture properties of hydrogel, namely its cohesiveness and adhesiveness, declined to some extent. The decreased values of the cohesiveness and adhesiveness were still in the range of acceptable values for hydrogels destined for topical treatment (Engesland, 2010).

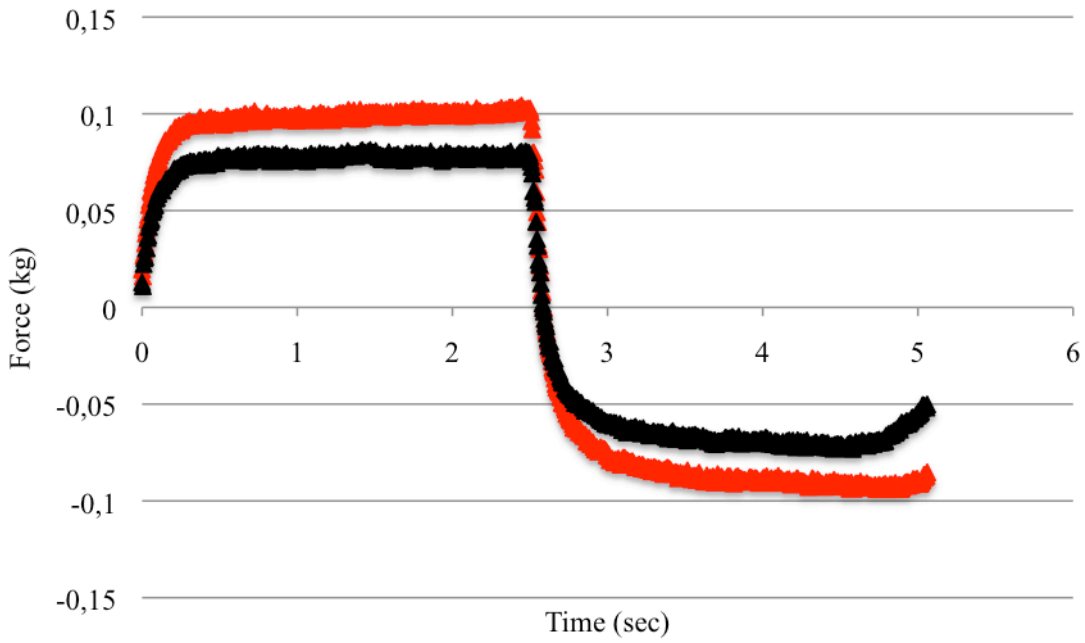

Figure 19: Texture properties of chitosan hydrogel before incorporating pg (red) and after incorporating pg (black)

5.3. Stability testing

5.3.1. Liposomal stability testing

Important parameter in characterizing liposomal formulations, particularly for smaller vesicle size populations, is their ability to overcome stability problems. Particularly interesting are the leakage of originally entrapped drug, and the aggregation, fusion of particles resulting in vesicle size increase (Pavelic *et al*., 2001). To evaluate the stability we applied the accelerated stability testing (one-month storage at 40 ºC). The liposomal characteristics are presented in Table 6.

Table 6: Characteristics of N-SL and SL prior to accelerated stability testing

Vesicle type	Entrapment efficiency (%)	Mean diameter (nm)	Polydispersity index	Drug recovery (%)
N-SL	66.54 ± 1.30	905.3*	1.01	88.33 ± 2.78
SL	31.50 ± 5.87	133.77 ± 24.32	0.35	81.06 ± 3.46

*The estimated size as vesicles are too polydispersed

All preparations contained 200 mg of lipid and the starting amount of drug was 20 mg. The values denote the mean of 3 measurements ± SD.

Due to the high polydispersity index (PI) for non-sonicated vesicles, which were multilammelar in structure and with average diameter of around 1 μm, we could not detect the changes in vesicle size after the stability testing, as the PI value for non-sonicated vesicles was over 1 (1.09). This is a clear limitation of the NICOM measuring device, as for the particles bigger than several hundreds nanometers and polydisperse in nature, the machine is not suitable. Characterization on Malvern instrument, such as Zetasizer, would be recommendable. However, it is expected that the vesicle size of non-sonicated vesicles is increasing upon storage at higher temperatures (Skalko *et al.*, 1998a).

However, when assessing the size of sonicated vesicles (Figure 20), the increase in the vesicle size, due to the aggregation of vesicles, was observed as expected. After one-month incubation at 40 °C the mean diameter was found to be 178 nm (as compared to 134 nm prior to testing) and the PI was measured to be 0.39, respectively.

Our aim was to develop liposomal hydrogels for mupirocin, therefore, liposomes containing mupirocin will be incorporated within the hydrogel network, and the increase in vesicle size seen for sonicated vesicles will not be relevant once liposomes

are incorporated in hydrogel. Hydrogels are known to protect the original structure of incorporated liposomes (Pavelic *et al*., 2001).

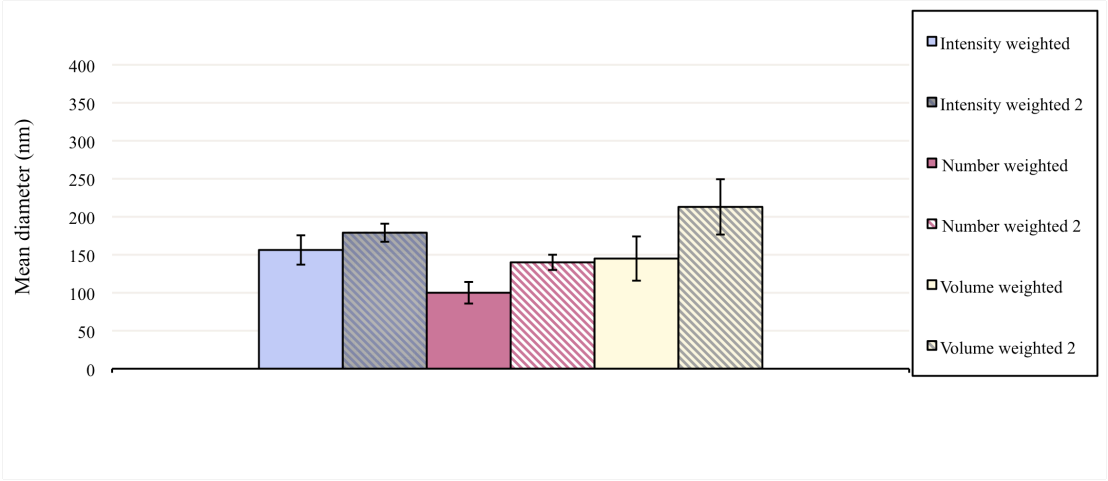

Figure 20: Particle size on sonicated liposomal preparations before (colour) and after accelerated stability testing (patterned). The values denote the mean of 3 separate measurements ± SD.

5.3.2. Stability testing of hydrogels

5.3.2.1. Accelerated stability testing of empty chitosan hydrogels

To further evaluate and investigate textural properties of chitosan hydrogels, we performed stability testing under the accelerated stability conditions (one-month at 40 ºC) and the corresponding results are presented in Tables 7A and 7B.

Table 7A: Characteristics of empty chitosan hydrogels before stability testing

Run	Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
1	103.58	246.33	95.98	199.88
2	104.23	245.75	93.91	199.01
3	105.10	246.28	94.56	198.34
4	104.55	245.30	94.56	198.81
5	104.66	245.28	94.78	199.12
Average	104.42	245.79	94.76	199.03
SD	0.51	0.45	0.67	0.50
CV	0.49	0.18	0.71	0.25

SD= standard deviaiton, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

As can be seen from Table 7A, the measured values corresponded well to values in Table 5A, for the same type of hydrogels, studied earlier to determine the effect of additive on textural properties of hydrogels.

Table 7B: Characteristics of empty chitosan hydrogels after stability testing

Run		Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
	1	36.37	83.54	34.96	67.15
	2	36.69	85.15	35.28	65.88
	3	37.02	86.08	33.54	63.92
	4	36.80	84.76	34.20	66.10
	5	36.91	85.74	33.54	64.67
Average		36.76	85.05	34.30	65.54
SD		0.22	0.88	0.71	1.13
CV		0.61	1.04	2.08	1.73

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

The values in Table 7B indicate stability problem related to empty chitosan hydrogel. After one month storage at 40 °C, the hydrogel lost its original cohesiveness and adhesiveness. It would be interesting to identify when (in time frame) the gels textural properties began to decline. Although the hydrogels for wound treatment are expected to be stored refrigerated and the accelerated stability testing is conducted under the extreme conditions, the findings represent serious concern and require explanation. One can suspect that physiochemical properties of chitosan were affected by the increased temperature. The hydrogels consist of series of chemical bonds holding the identical side chain molecules together (Jagur-Grodzinski, 2009). One may suspect that some form of partial breakdown of chitosan network matrix occurred, which resulted in decreased viscosity of hydrogel, thereby decreasing the adhesive and cohesive properties of original hydrogel (Figure 21).

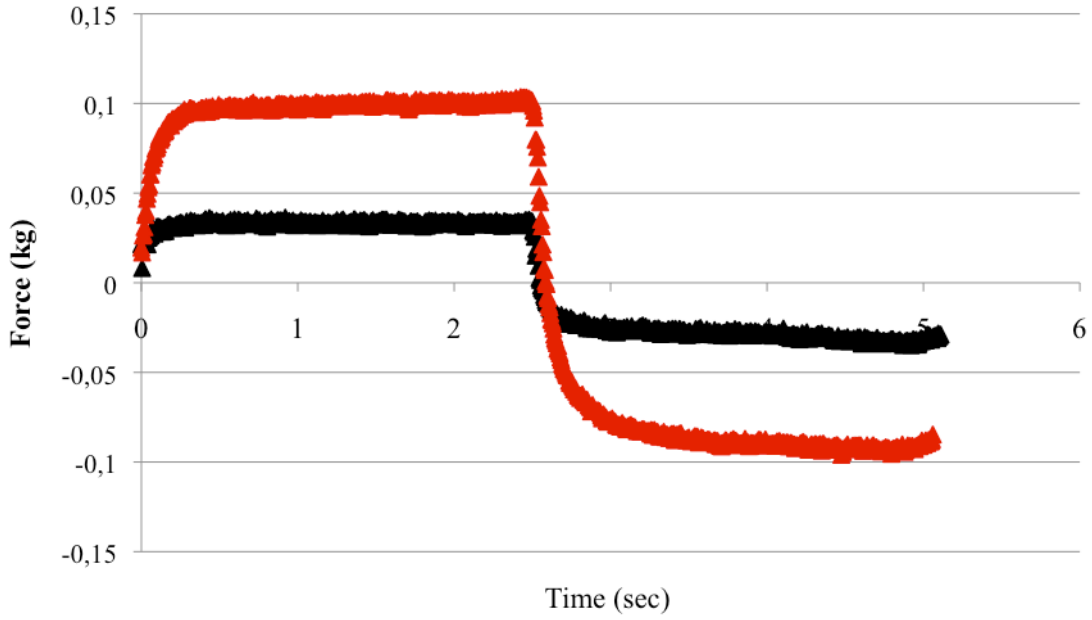

Figure 21: Texture properties of empty chitosan hydrogel before (red) and after (black) accelerated stability testing

Although the stability results for empty chitosan hydrogels were not satisfactory, an important information was determined, namely that empty chitosan hydrogels, without any preservative present in the composition, remained to be microbiologically acceptable, without any traces of microbial contamination (Figure 22). The observation (visual) confirms self-preservative properties of chitosan, which is an additional advantage when optimizing chitosan hydrogel. This is also a clearly benefit for wound dressings, as preservatives included in wound dressings may cause wound irritation and lead to toxicity (Alsarra, 2009).

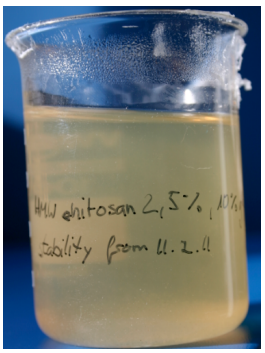

Figure 22: Empty chitosan gel after stability testing

In previous work by Engesland (2010) and Poorahmary (2010) an interesting hypothesis was proposed, namely that liposomes incorporated in hydrogels improve the stability of hydrogel networking. Therefore, our next step was to investigate the impact of both N-SL and SL on chitosan hydrogel matrix.

5.3.2.2. Accelerated stability testing of chitosan hydrogels containing non-sonicated liposomes (gel-N-SL)

The experiments were performed with two basic purposes, one to evaluate the effect of incorporated liposomes on the texture properties of hydrogels, and second to evaluate the effect of incorporated liposomes on hydrogel stability.

Table 8A: Characteristics of gel-N-SL before stability testing

Run		Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
	1	69.92	165.66	65.58	133.75
	2	69.59	165.32	64.27	133.18
	3	71.01	165.70	64.38	132.67
	4	70.46	165.09	64.27	133.58
	5	70.25	164.97	63.84	133.47
Average		70.25	165.35	64.47	133.25
SD		0.48	0.29	0.58	0.39
CV		0.68	0.18	0.91	0.30

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

Table 8A and Figure 23 indicate the changes in original textural properties of hydrogel, the original values for Area 1 being 246, and Area 2 being 199, respectively (Table 7A) into decreased adhesive and cohesive properties. The findings are in correspondence to changes in textural properties upon addition of mupirocin-in-propylene glycol to the prepared gel (Table 5A and 5B, respectively). The changes are acceptable, but need to be monitored.

Table 8B: Characteristics of gel-N-SL after stability testing

Run	Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
1	45.59	102.55	41.25	76.51
2	44.40	102.10	41.25	76.26
3	45.27	102.60	40.28	75.61
4	44.73	103.06	40.28	75.70
5	45.05	102.39	40.28	75.65
Average	45.01	102.54	40.67	75.95
SD	0.42	0.31	0.48	0.37
CV	0.92	0.30	1.18	0.48

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

However when comparing the values in Table 7B with those in 8B, interesting phenomenon can be seen. Gel-N-SL resisted the changes due to increased temperature more than the empty gels under the same conditions. This can be attributed to ability of liposomes to accommodate them self within the polymeric network and in a way stabilize the network. Although the measured values for adhesiveness and cohesiveness are still lower than what would be desirable, the finding is promising, and in line with previous reported data by Engesland (2010) and Poorahmary (2010). This line of research needs to be further elaborated, as it appears that liposomal suspensions have ability to interact with the original polymer network by preserving the network in certain way. It would be interesting to define whether the effect is dependent on the amount of liposomes added into the hydrogel (10 % w/w in our case) or on liposomal composition and characteristics, such as surface charge and similar. As the aim of our study was development of formulation for mupirocin, due to time constraint we did not elaborate further on those findings.

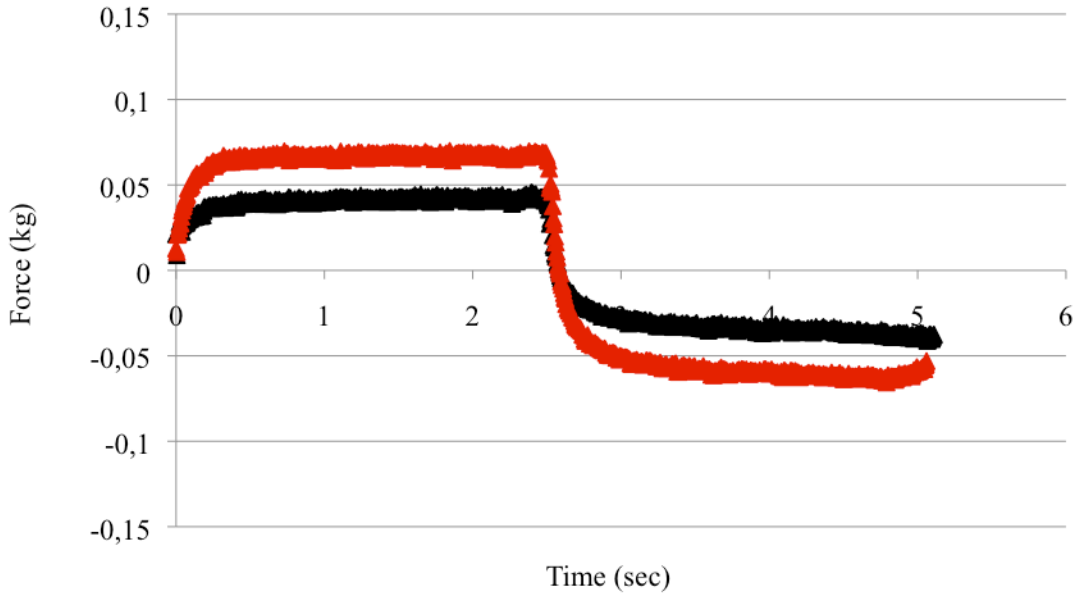

Figure 23: Texture properties of gel-N-SL before (red) and after (black) stability testing

Figure 24 confirms again that no contamination of hydrogels could be visually observed. However, one can argue that in this case mupirocin is also present in vesicles-incorporated in hydrogels.

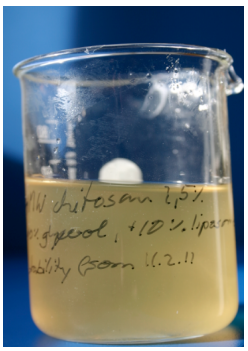

Figure 24: gel-N-SL after stability testing

5.3.2.3. Accelerated stability testing of chitosan hydrogels containing sonicated liposomes (gel-SL)

To determine whether the size of incorporated vesicles affects the changes in texture properties of hydrogels, we performed the same experiments with hydrogels containing sonicated vesicle (around 135 nm in size) as previously described for non-sonicated vesicles (size over micron).

Table 9A: Characteristics of gel-SL before stability testing

Run		Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
	1	73.60	173.86	67.85	140.30
	2	73.60	173.56	67.85	140.37
	3	73.93	173.70	67.63	139.30
	4	74.25	174.87	66.76	138.73
	5	73.28	174.15	67.09	139.57
Average		73.73	174.03	67.44	139.65
SD		0.33	0.46	0.44	0.62
CV		0.45	0.27	0.65	0.44

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

Table 9B: Characteristics of gel-SL after stability testing

Run		Force 1 (g)	Area 1 (g.sec)	Force 2 (g)	Area 2 (g.sec)
	1	55.26	122.02	43.10	82.23
	2	57.10	122.26	43.86	81.52
	3	54.93	120.42	44.51	82.96
	4	54.82	121.64	43.97	81.93
	5	55.26	121.78	43.75	82.15
Average		55.47	121.62	43.84	82.16
SD		0.83	0.64	0.45	0.47
CV		1.50	0.53	1.03	0.57

SD= standard deviation, CV= % coefficient of variation. The values are the average of 5 runs and are absolute.

Tables 9A, 9B and Figure 25 confirmed previous findings. Incorporation of sonicated vesicles into chitosan hydrogel seems to interfere to some degree with the gels texture properties, however, it appears that the effect of smaller vesicles is less pronounced (Table 9A) than the effect of bigger vesicles (Table 8A). In addition, smaller vesicles seemed to stabilize the polymer network even more than bigger vesicles (Table 8B) as the decrease in Area 1 and Area 2 upon stability testing is less (Table 9B). It could be explained by the ability of smaller vesicles to accommodate themselves better within the polymer network, than what would be possible for larger vesicles. This fact needs to be further explored.

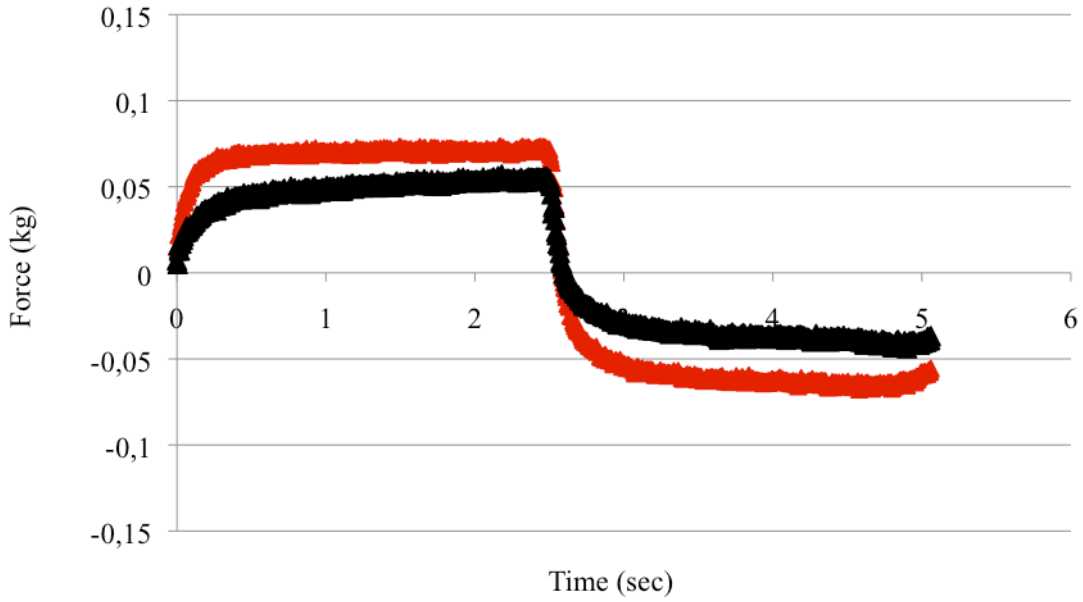

Figure 25: Texture properties of gel-SL before (red) and after (black) stability testing

As observed earlier, hydrogel did not show any microbial contaiminations upon stability testing (Figure 26)

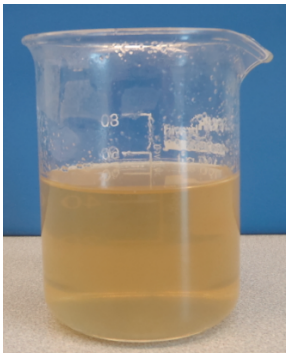

Figure 26: gel-SL after accelerated stability testing

5.4. Microbiological evaluation of liposomal preparations

5.4.1. Determination of MIC- values for mupirocin on selected bacterial strains

Prior to preparing mupirocin-in-liposome-in-hydrogel formulation, able to provide sustained release of mupirocin in contact with wounded skin, and therefore prevent bacterial infections, we wanted to assure that liposomally entrapped mupirocin retains its antimicrobial potentials. For that purpose, we firstly determined the MIC values for mupirocin against predetermined bacterial strains. The selection of bacteria was among the most commonly tested bacteria in evaluation of antibacterial potentials of mupirocin (Sutherland *et al*., 1985; Simpson *et al*., 1995). The MIC values are presented in Table 10.

Table 10: MIC values for mupirocin against selected bacteria

Bacterial strain*	ATCC	MIC values µg/ml
B. subtilis (Gram positive)	6633	0.19
S. epidermidis (Gram positive)	12228	0.13
E. coli (Gram negative)	25922	192
S. aureus (Gram positive)	25923	0.38
E. faecalis (Gram positive)	29212	48
M. luteus (Gram positive)	9341	> 1064

* (Sutherland *et al*., 1985; Simpson *et al*., 1995).

The MIC values obtained (Table 10) directly corresponded to the published data (Sutherland *et al*., 1985; Simpson *et al*., 1995).

The photographs in Figure 27 are the examples of representative samples indicating the MIC values determined for *S. aureus* and *B. subtilis*, respectively.

After assuring that our experimental set up is correct, we proceeded with the determination of the zones of inhibition for various formulations containing mupirocin (Table 11).

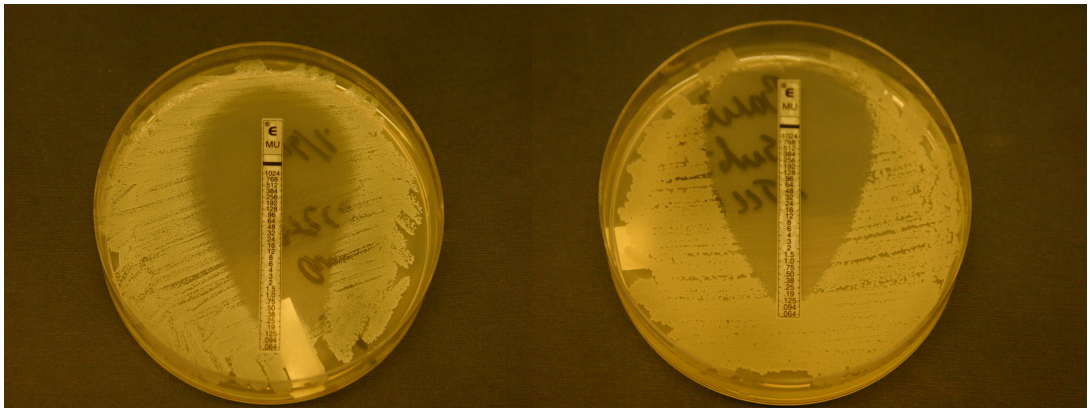

Figure 27: MIC values for mupirocin against *S. aureus* (left) and *B. subtilis* (right)

5.4.2. Zones of inhibition for selected bacterial strains

Table 11: Zones of inhibition as determined after 24 h incubation (n=3)

Bacterial strain	**Type of mupirocin formulation**			**Controls**	
	gel-N-SL (mm)	**gel-SL (mm)**	**d Bac-cream (mm)**	**Empty gel (mm)**	**Water (mm)**
S. aureus	17.13 ± 1.96	15.23 ± 1.05**	21.57 ± 0.42	6.37 ± 0.47	-
B. subtilis	22.23 ± 1.07*	19.03 ± 0.15*	22.97 ± 0.15	5.80 ± 0.10	-

- indicates that no growth inhibition was observed. Mupirocin concentration in all formulations was 505 µg/ml. * $p<0.05$; ** $p<0.01$

The zones of inhibition (Table, 11; Figure 28) varied greatly for the tested mupirocin-containing samples. Several interesting findings need to be addressed. First important finding was the fact that empty hydrogel (not containing mupirocin) also inhibited the bacterial growth to certain degree. This again confirms previous data on chitosan hydrogel stability (see section 5.3.2.1.), where no microbial contamination was observed (1 month at 40 °C) even though hydrogels did not contain any preservative. Secondly, the hydrogels containing non-sonicated liposomes induced stronger

antibacterial response (Table 11; $p<0.05$) than the hydrogels containing sonicated vesicles. As the amount of drug in all formulations was constant, this finding was rather difficult to explain at this stage of research. The possible explanation can be related to the release of liposomally entrapped drug from liposomes, followed by the release from hydrogel. More comment on this proposed explanation is given in part 5.5. As a third important finding, we confirmed that liposomal hydrogels with mupirocin induced similar antimicrobial effect as diluted marketed product, d Bac-cream. Against *B. subtilis*, the activity of gel-N-SL was identical to diluted marketed sample, however gel-SL was significantly less effective than diluted Bactroban ($p< 0.05$). In the case of *S. aureus,* the inhibition was less effective, with similar tendency, with the exception that gel-SL was significantly less active at the level of $p< 0.01$.

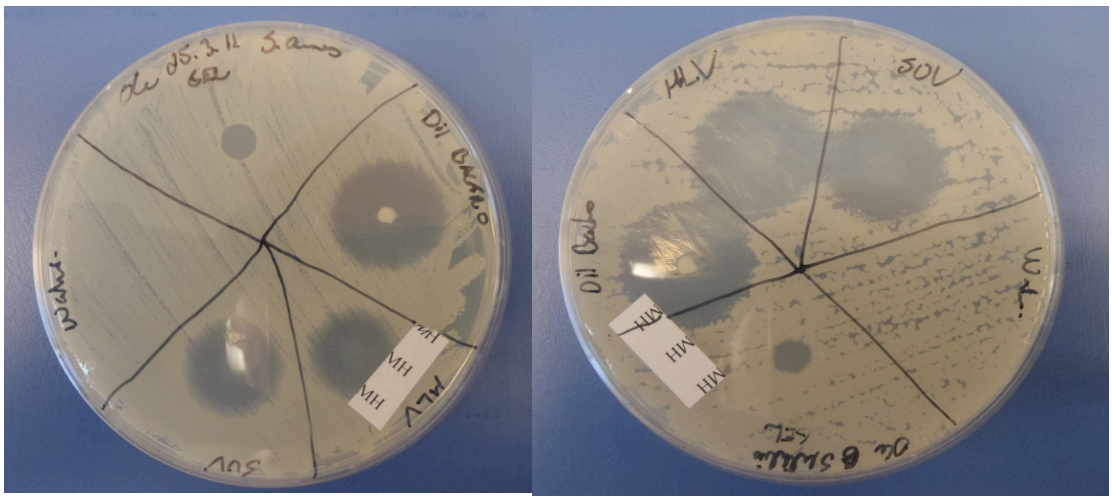

Figure 28: Zones of inhibition for different mupirocin formulations (*S. aureus* on the left*; B. subtilis* on the right)

We also wanted to compare the antibacterial activity of mupirocin incorporated in different vehicles not containing liposomes, to determine whether liposomes as drug carrier have additional beneficial effect in improving antibacterial properties of mupirocin. Therefore, mupirocin-in-propylene glycol, mupirocin-in-propylene glycol-in hydrogel and empty hydrogel were evaluated (Table 12 and Figure 29).

Table 12: Zones of inhibition obtained after 24 h incubation (n=3)

Bacterial strain	pg (mm)	gel-pg (mm)	empty gel (mm)
S. aureus	17.37 ± 0.41	17.20 ± 0.22	6.13 ± 0.12
B. subtilis	18.80 ± 0.10	17.40 ± 0.5*	5.50 ± 0.3

Mupirocin concentration in all formulations was 505 µg/ml. * p< 0.05

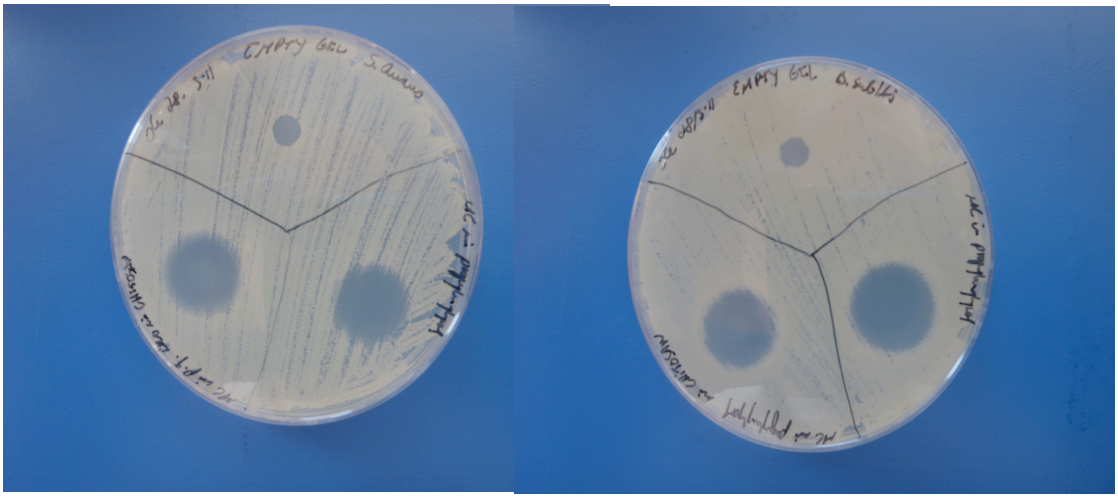

Figure 29: Zones of inhibition for different formulations (*S. aureus,* left; *B. subtilis* right)

The zones of inhibition detected in these set of experiments can be compared to the zones measured for liposomal formulations (Table 11). Mupirocin-in-propylene glycol-in-hydrogel was found to be less active against *B. subtilis* than mupirocin-in-propylene glycol ($p<0.05$).

Liposomes, incorporated in hydrogels, as drug carrier system did neither improve nor hinder the antibacterial activity of mupirocin against tested bacteria. However, it seems that preferable type of liposomes to be incorporated in hydrogels, at least based on antimicrobial profile of entrapped mupirocin, are larger vesicles.

5.5. *In vitro* and *ex vivo* mupirocin release studies

5.5.1. *In vitro* mupirocin release as determined on FDC system

The main objective of these studies was to evaluate release profiles of various formulations containing mupirocin. We wanted to evaluate the potentials of liposomes as drug carrier, as well as liposomal hydrogels as delivery system able to ensure sustained release of mupirocin. All formulation were compared to marketed product, in either original or diluted form (Tables 13 and 14 and Figures 30-32).

Table 13: Cumulative release profile for different mupirocin formulations through polyamide membrane (n=4)

Type of formulation	Released after 30 min (%)	Released after 7 h (%)	Released after 24 h (%)
gel- N-SL	12.45 ± 2.47	37.65 ± 8.02	43.14 ± 7.41
gel-SL	3.85 ± 0.07	8.89 ± 0.70	9.33 ± 1.23
Bac-cream 2 %	5.28 ± 0.89	21.40 ± 3.08	28.62 ± 5.00
d Bac-cream	10.48 ± 1.47	28.94 ± 2.43	42.89 ± 2.66
gel-pg	38.82 ± 1.90	90.46 ± 4.25	90.91 ± 4.57
pg	97.27 ± 1.27	97.33 ± 2.40	100.03 ± 2.34
N-SL	68.79 ± 3.09	103.17 ± 4.11	nd
SL	79.81 ± 5.38	102.09 ± 6.37	nd
gel-N-SL (f+e)	11.04 ± 1.56	32.48 ± 3.00	33.11 ± 2.78

nd = not determined

Table 14: Cumulative release profile for different mupirocin formulations through cuprophan membrane (n=4)

Type of formulation	Release after 30 min (%)	Release after 7 h (%)	Release after 24 h (%)
gel-N-SL	5.61 ± 0.29	15.70 ± 0.36	17.25 ± 0.61
gel-SL	3.92 ± 0.04	10.76 ± 0.17	12.09 ± 0.57
d Bac-cream	9.48 ± 0.19	32.11 ± 1.29	58.99 ± 3.49

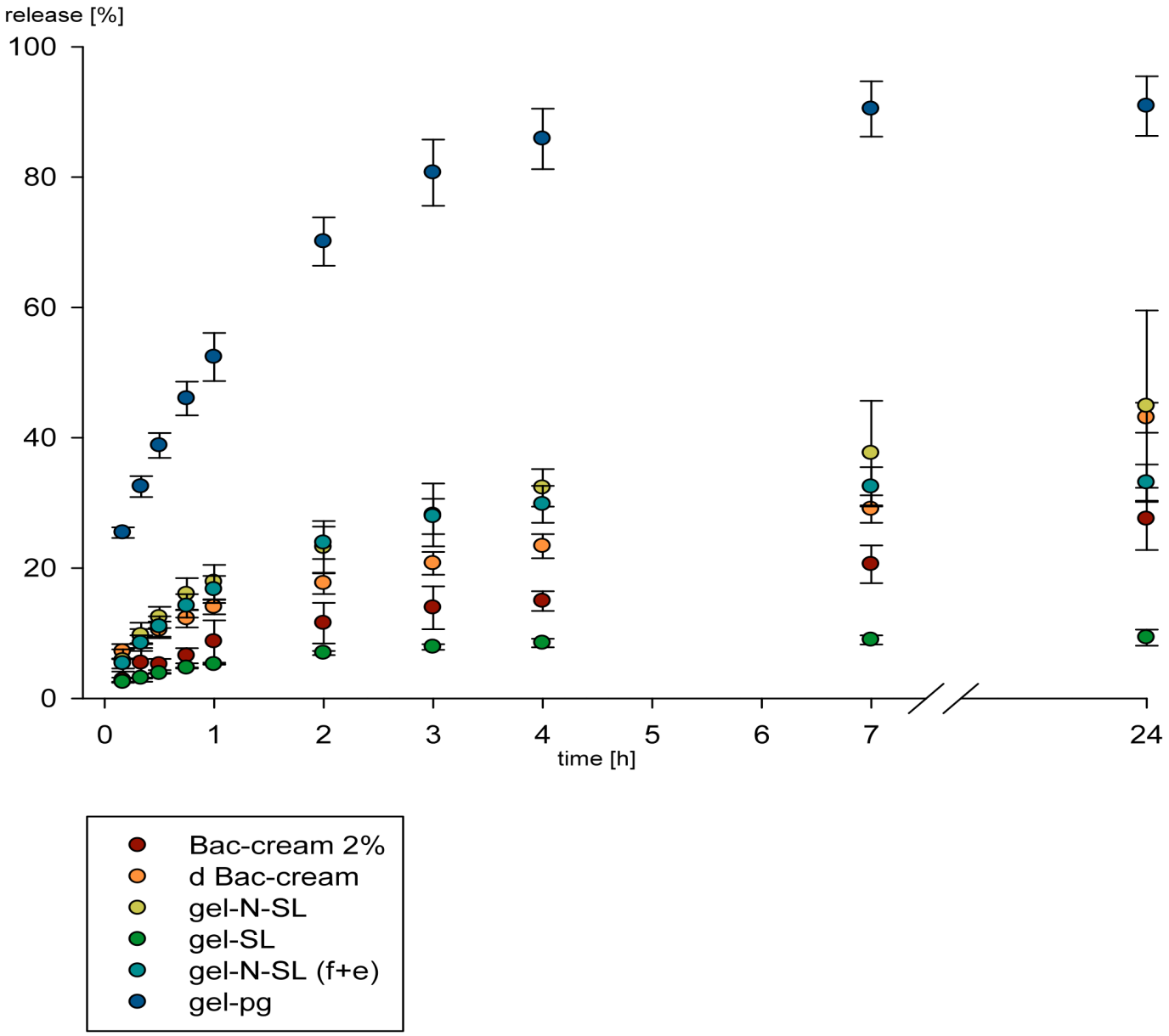

Figure 30: *In vitro* release profiles of different mupirocin formulations through polyamide membrane (n=4) Mupirocin concentration in all formulations was 505 µg/ml.

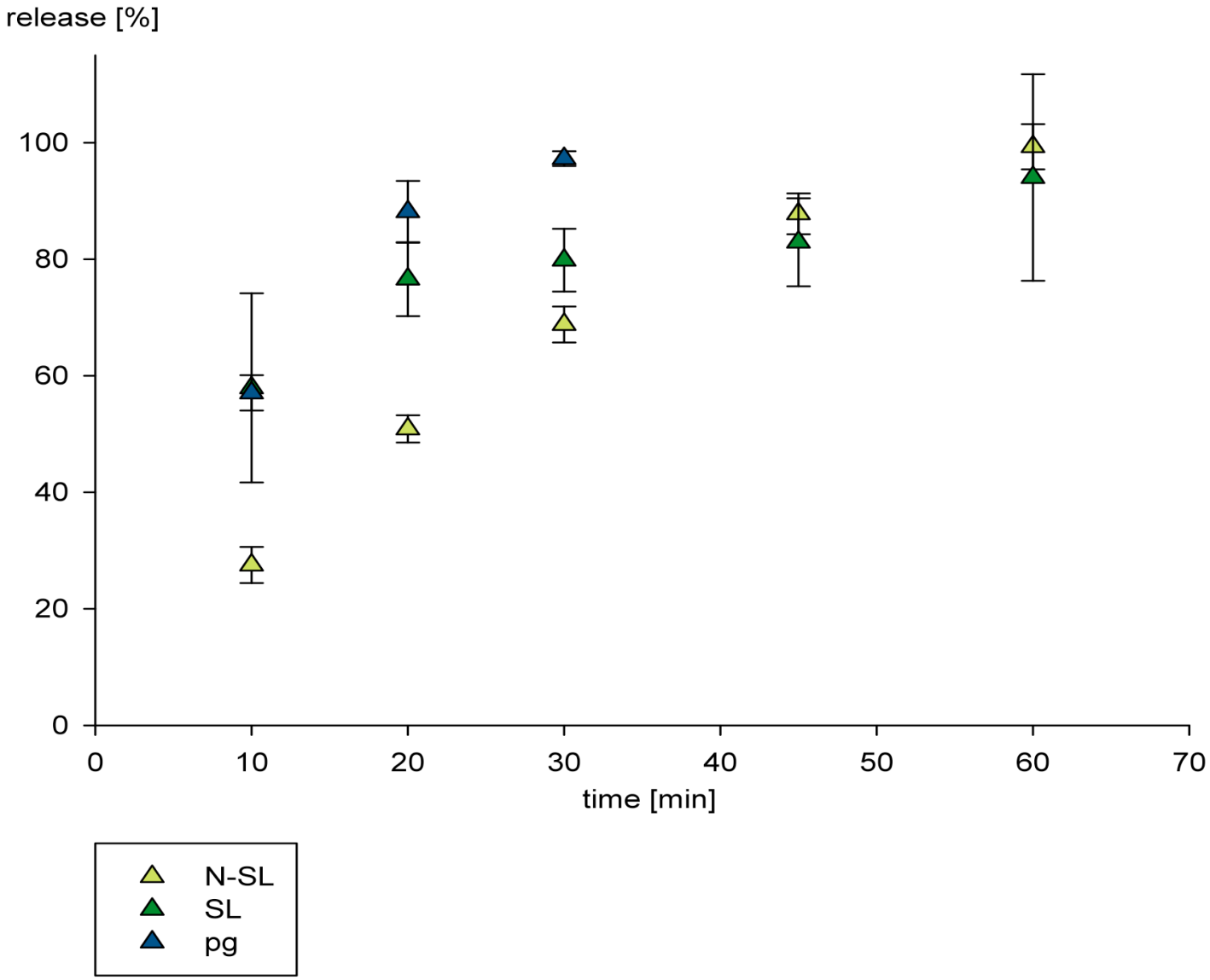

Figure 31: *In vitro* release profiles of liposomally-entrapped and propylene glycol-dissolved mupirocin through polyamide membrane (n = 4) Mupirocin concentration in all formulations was 505 µg/ml.

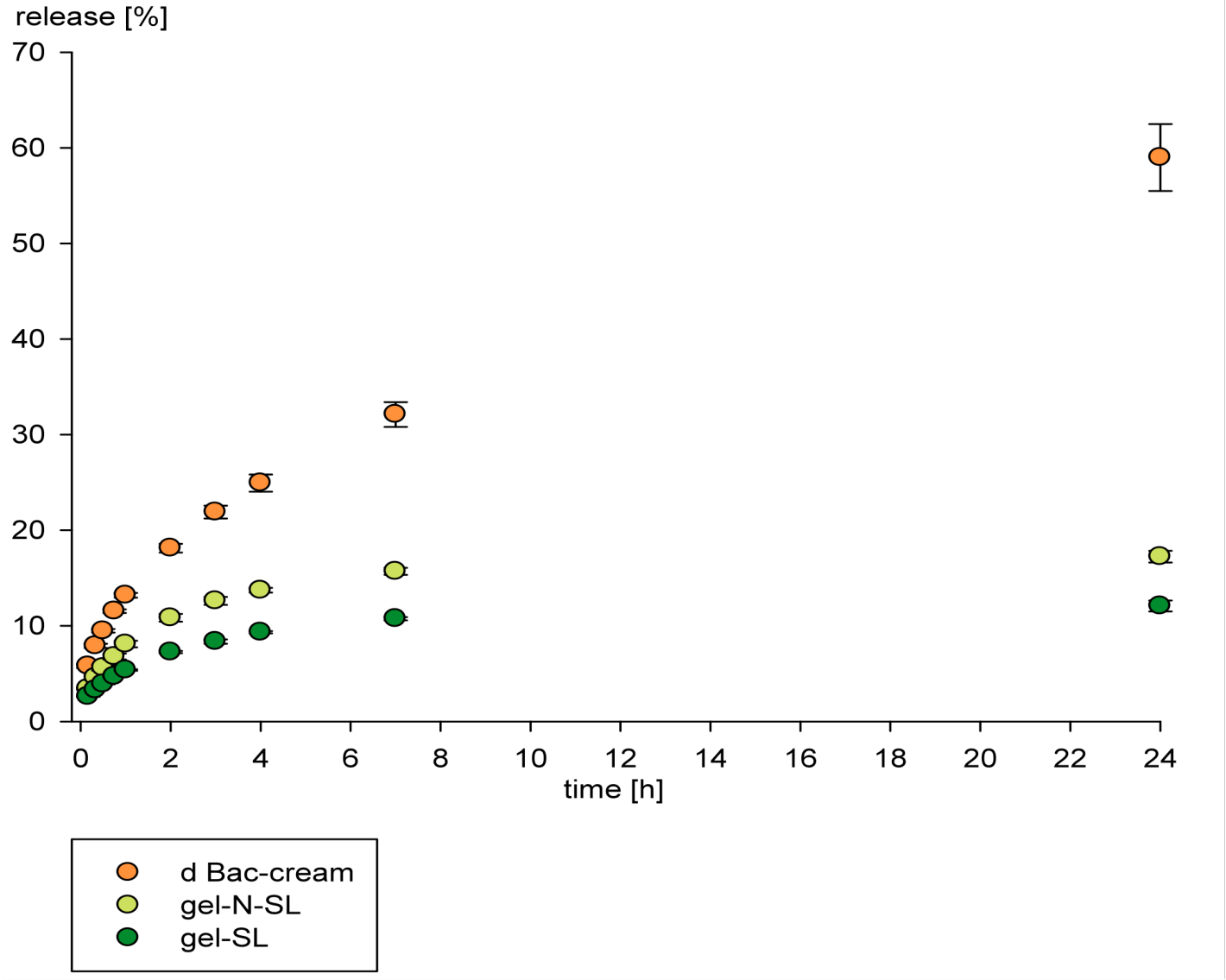

Figure 32: *In vitro* release profiles of different mupirocin formulations through cuprophane membrane (n=4) Mupirocin concentration in all formulations was 505 µg/ml.

The release profiles of mupirocin (through polyamid membrane) from different formulations are presented in Figure 30. How important is the assurance of sink conditions through out the experiment can be observed on example of original Bactroban (2 %, w/w) cream and diluted Bactroban cream (diluted to the corresponding amount of drug in liposomal formulations). The slower release of mupirocin from marketed product as seen in Figure 30 is the consequence of the absence of sink conditions. This is very important observation for various bioequivalence studies, as the relationship between solubility of drug and assured sink conditions needs to be carefully monitored. Regarding different liposomal hydrogels, it is evident that the liposomal size affects the release of entrapped drug, in agreement with data obtained in microbiological studies (Table 11). The highest percentage of released mupirocin was determined for hydrogels containing non-sonicated liposomes. The release was significantly more than from liposomal hydrogels containing sonicated liposomes ($p<0.01$). Sonicated liposomes-in hydrogels provided sustained release of incorporated mupirocin, as only 9.33 % of mupirocin was released after 24 hours. Although from the technological point of view, it is impressive sustained release profile, in respect to antimicrobial potentials, the release is too slow and needs to be modified. The same liposomes, not incorporated in hydrogels, released in the same time frame all of the entrapped mupirocin (Table 13). We did not see significant difference between total amount of mupirocin released from non-sonicated and sonicated liposomes (Table 13, Figure 31). Interestingly, the release profiles became different once the vesicles were incorporated in hydrogles. The release profile from non-sonicated vesicles was almost linear with time (Figure 31). Even more interesting is the finding that the release profiles for hydrogels incorporating liposomes free from unetrapped mupirocin, and the same hydrogel incorporating liposomally entrapped and free mupirocin differed (Figure 31). This indicates that liposomes, incorporated in hydrogels, play important role in defining the release profile of entrapped drug. The explanation of why mulitlamellar in structure and larger in size vesicles, incorporated in hydrogels, released the entrapped drug faster than smaller vesicles incorporated in hydrogels, could be related to the partition preferences of mupirocin. It seems that mupirocin preferably partitions itself (logP 2.7) from mulitilamellar vesicles into the hydrogel, from where it is released faster. One can suspect that MC diffuses, partitions itself, in a step-wise mode, so that the concentration gradient is maintained through out bilayered structure of liposomes.

Smaller vesicles, many of them unilamellar, could provide some form of equilibrium, slowing the release of a drug. The fastest and maximum release was seen for mupirocin-dissolved-in-propylene glycol-incorporated in hydrogel (Table 13).

By manipulating the size and properties of liposomes intended for incorporation into hydrogels, it is possible to achieve desired release profile for drug entrapped in liposomes, incorporated in hydrogels.

Hydrogels did not only protect the original structure of liposomes and prevent the leakage of liposomally entrapped drug (Skalko *et al*., 1998a; Pavelic *et al.,* 2001), but in our case, clearly provided sustained release of liposomally entrapped drug (Table 13, Figure 30).

Kang *et al*. (2010) studied a dispersion of cationic liposomes, which were loaded with amphotericin B (polyene type antibiotic antifungal drug), incorporated into a thermosensitive gel (poloxamer 407 and poloxamer 188 at various ratio). FDC permeation study (0.4 μm membrane filter) revealed that the gel prolonged the drug release from liposomes, compared to liposomal suspension and free amphotericin incorporated in the gel, however the study did not indicate that liposomal gels sustained the release of amphotericin B, rather the opposite were observed (Kang *et al*., 2010). It is important to note that amphotericin and mupirocin vary in their properties, and that the hydrogels used in our experiments vary from poloxamer hydrogels, therefore, the experiments cannot be directly compared.

Mulik *et al*. (2009) reported the superior sustained release properties of cytatabine-in-liposomes-in-gel, in comparison to gel containing free cytarabine. The group attributed these observations to the sustained diffusion of drug through the gel matrix after the incorporated cytarabine was released from the phospholipid vesicles, and secondly as a consequence of the lipid bilayer stiffness.

Paavola *et al*. (2000) studied liposomal ibuprofen release from poloxamer 407 gels through a two-compartment *in vitro* method, using cellulose membrane (12-14000 MWCO) Experiments revealed cumulative release of ibuprofen, with enhanced prolonged release from both liposomal suspension and liposomal gel, in comparison to ibuprofen in solution and ibuprofen-in-solution-in-gel, respectively (Paavola *et al*., 2000).

The ability of delivery system to prolong the release of incorporated drug provides not only the advantage of sustained release, and consequent reduction in frequency of drug administration, but reduces the side effects as rapid release of drug at the wound site can cause local irritancy and pain (Mulik *et al*., 2009).

How difficult is to compare the literature data on release profiles of different drugs in different delivery system, and different experimental set ups, is indicated in comparison between release profile of mupirocin through polyamide membrane (Figure 30) and cuprophane membrane (Figure 32). It is evident that the type of the membrane used affects the release profile. However, again the tendency of sonicated liposomes, incorporated in hydrogels, to prolong the release more than non-sonicated ones is clearly seen (Figure 32).

5.5.2. *Ex vivo* release studies

In order to evaluate the mupirocin release profile from various formulations under the conditions closer to *in vivo* conditions, we performed diffusion experiments on pig ear skin (Table 15 and Figure 33).

Table 15: Cumulative release profile of mupirocin from different formulations through pig ear skin (n= 2)

Type of formulation	Release after 30 min (%)	Release after 7 h (%)	Release after 24 h (%)
gel-N-SL	1.15 ± 0.22	8.46 ± 0.10	14.54 ± 0.31
gel-SL	1.22 ± 0.28	4.88 ± 1.41	7.98 ± 1.35
N-SL	2.20 ± 0.48	39.31 ± 4.40	71.08 ± 16.82
SL	2.16 ± 0.38	32.48 ± 3.85	57.61 ± 17.88
d Bac-cream	0*	7.76 ± 1.49	18.86 ± 2.09
pg	31.73 ± 1.59	78.33 ± 0.73	86.12 ± 4.03

* No detectable permeation at given time

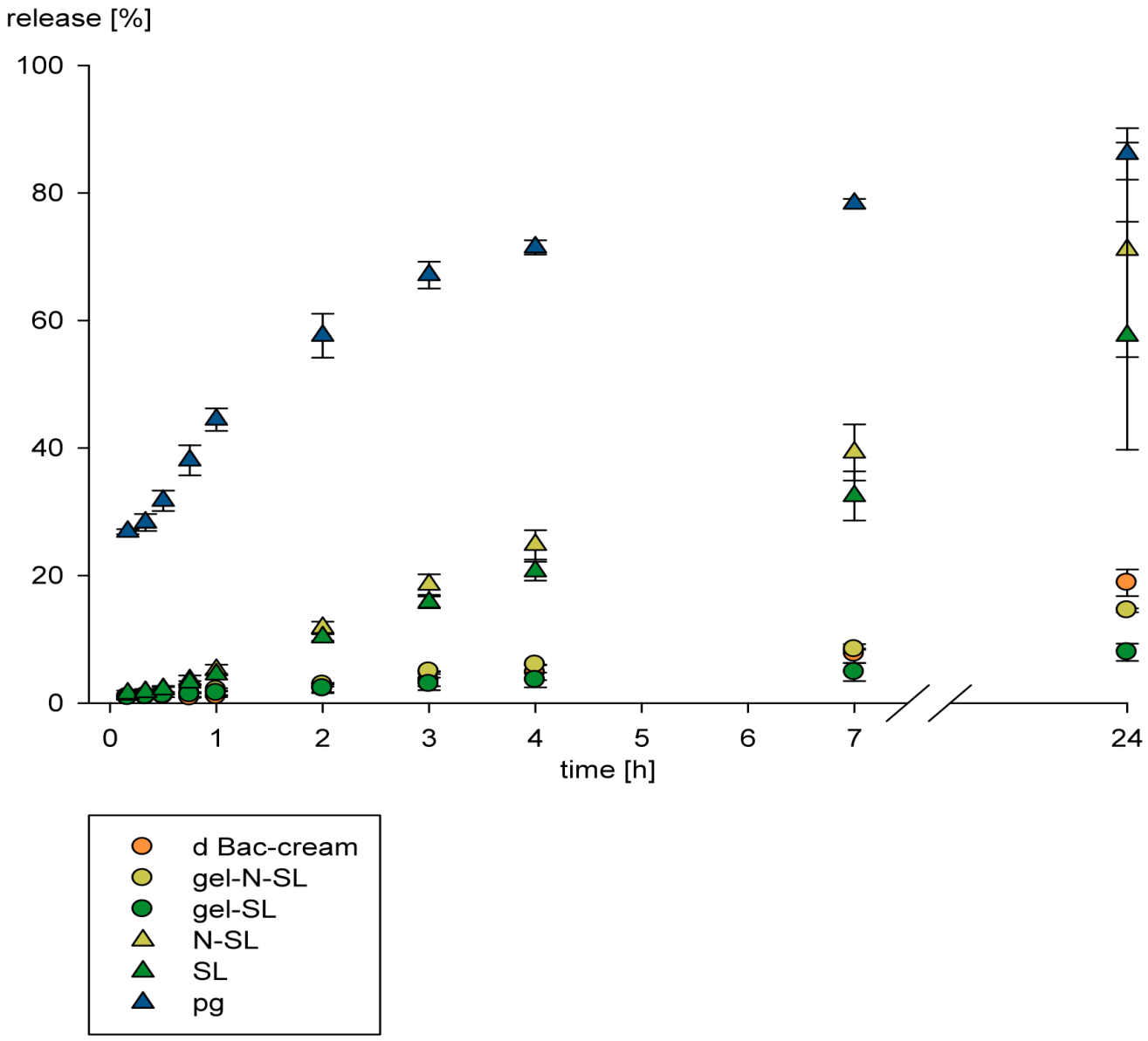

Figure 33: *Ex vivo* release profiles of mupirocin from different formulations through pig skin (n=2) Mupirocin concentration in all formulations was 505 µg/ml.

The release profiles for mupirocin from different formulation through pig skin (Figure 33) followed similar pattern observed for the release through polyamide membrane (Figure 30). The maximum release was achieved from formulation in which mupirocin was dissolved in propylene glycol. However, from the therapy point of view, such a formulation would be rapidly removed from the administration site, and has therefore no therapuetical relevance. Similarly, liposomal suspensions, particularly non-sonicated ones, released mupirocin faster and in higher amount as compared to liposomes-in-hydrogels formulation. The release profiles for liposomal hydrogels incorporating non-sonicated vesicles and diluted Bactroban were similar, with slowest release being again observed for sonicated liposomes-in-hydrogels (Figure 33).

Liposomal hydrogels provided a mean to prolong the release of incorporated mupirocin. Similar observation was reported by Paavola *et al*. (2000) on ibuprofen gels tested for permeation through pig lumbar dural membrane.

In vitro and *ex vivo* release studies performed on Franz diffusion cells provide a valuable information on the potentials of liposomal hydrogels to provide sustained, and to certain degree, controlled release of mupirocin. Moreover, liposomes can be design in a manner to adjust and optimize the entrapped drug release profile, which, together with the right choice of hydrogels, opens broad possibilities for optimization of antibacterial wound therapy.

6. Conclusions

We were able to develop liposomal delivery system for mupirocin destined for wound therapy, which is, to the best of our knowledge, the first report on liposomal mupirocin for topical administration. By incorporating liposomes-containing mupirocin in chitosan hydrogels, we were able to develop advanced delivery system, with acceptable hydrogel adhesiveness and cohesiveness, and satisfactory stability profile. Moreover, liposomal mupirocin retained its antimicrobial potentials. *In vitro* and *ex vivo* release studies revealed that by manipulating the liposomal characteristics such as vesicle size, it is possible to achieve sustained release of incorporated mupirocin. Moreover, the comparison with marketed product Bactrocan® cream confirmed the potentials of liposomal hydrogels for mupirocin as promising advanced delivery system in burn therapy.

7. Perspectives

Short-term perspectives on developed formulation

- Deeper insight on location of mupirocin within liposomes by more advanced characterization methods such as confocal microscopy for example, combined with X-ray diffraction methods
- Evaluating the effect of liposomal composition, such as surface charge and membrane rigidity, on release profile of mupirocin
- Performing time-kill assay to investigate in more details the antibacterial efficacy for liposomal mupirocin hydrogels
- Comparing skin adhesion of liposomal mupirocin hydrogels to Bactroban® cream in pig skin bioadhesion studies

Long-term perspective on developed formulation

- Evaluation of safety and efficiency in animal burn model
- Preliminary testing on healthy human skin for potential irritancy

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9. Appendix

1. ABSTRACT Accepted

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Liposomal mupirocin: on the way to improved wound and burns therapy

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Trauma to the skin in form of severe burns gives rise to potentially life threatening bacterial infections. Mupirocin was reported to be a promising antibiotic in topical treatment of skin infections [1]. Liposomal mupirocin is expected to provide prolonged and controlled release of the drug. In order to increase the bioadhesiveness of the system, liposomes were incorporated in chitosan hydrogels.

Liposomes were prepared by the film method and were either used as prepared or reduced in size through sonication. Vesicle size was determined by dynamic light scattering and entrapment efficiency of mupirocin by HPLC. Liposomal hydrogels were compared to Bactroban® cream in respect to drug release profiles by using Franz Diffusion Cells. Antibacterial efficacy was tested against several strains of bacteria (*S. aureus, S. subtilis*). Bioadhesiveness was determined on Texture Analyzer.

MLV liposomes (1 μm) entrapped 74 % of mupirocin, whereas sonicated liposomes (200 nm) entrapped 49 %. The drug release profile was depended on the formulation characteristics. Microbiological evaluation confirmed antibacterial properties of liposomal systems for mupirocin. Liposomal chitosan hydrogel showed increased bioadhession in comparison to the commercially available product.

Liposomal mupirocin shows strong potential for improved wound therapy.

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2. ABSTRACT Submitted

ANTIBACTERIAL DRUG THERAPY FOR IMPROVED BURN TREATMENT: LIPOSOMAL HYDROGELS FOR MUPIROCIN

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INTRODUCTION

Trauma to the skin in the form of severe wound, particularly burns, can facilitate colonization of potentially life threatening bacterial infections. To prevent infections of the wounded area, antimicrobial agents are recommended as standard treatment (1). Topical administration of antimicrobial agents, such as mupirocin, can provide local therapy, while avoiding the risks of systemic administration. Mupirocin-in-liposomes-in hydrogels was proposed as advanced delivery system for this purpose. Up to now, no liposomal mupirocin for topical administration has been reported. Hydrogels are one of the most promising wound dressings, and chitosan hydrogels offer additional advantage of chitosan itself having wound healing and antimicrobial properties (2). Mupirocin-containing liposomes incorporated in hydrogels were expected to provide sustained release of incorporated drug, very important feature in improved wound therapy. Moreover, we compared the antimicrobial and drug release characteristics of our newly developed system to marketed product containing mupirocin, namely Bactroban® cream.

MATERIALS AND METHODS

MATERIALS

Mupirocin calcium was kindly provided by GlaxoSmithKline, Zagreb, Croatia and phosphatidylcholine (S-100) was a generous gift from Lipoid, Germany. Chitosan, high molecular weight, was from Sigma-Aldrich Chemistry, St Luis, USA. Bactroban 2 % (w/w) cream was a product by GlaxoSmithKline, Barnard Castle, UK. All chemicals used in experiments were of analytical grade. Pig ear skin, was purchased from Nortura, Målselv, Norway.

METHODS

Liposomes containing mupirocin were prepared by the modified film method, followed by sonication, and characterized for particle size, polydispersity and encapsulation efficiency (3). Chitosan hydrogels were prepared by the modified method of Cao et al. (4) and liposomes incorporated into hydrogels by hand stirring (5). Liposomal hydrogels were characterized for their textural properties and stability profile on Texture Analyzer TA.XT. (Stable Microsystems, Surrey, UK) as shown in Figure 1.

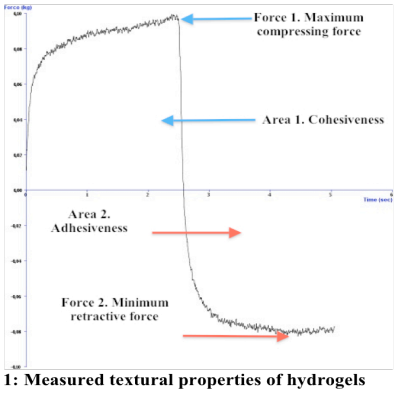

Figure 1: Measured textural properties of hydrogels

All mupirocin formulations were characterized for their antimicrobial properties against *B. subtilis* and *S. aureus* strains.

In vitro and *ex vivo* (pig ear skin) studies were performed on Franz diffusion cells.

RESULTS AND DISCUSSION

MLV liposomes (1 µm) entrapped 74 % of mupirocin, whereas sonicated liposomes (130 nm) contained 49 % of mupirocin, respectively.

Incorporation of liposomes in hydrogels affected original gel cohesiveness and adhesiveness to an acceptable extent. Interestingly, liposomes incorporated in hydrogels were shown to improve the stability of hydrogels, particularly small liposomes.

Microbiological evaluation confirmed antibacterial properties of liposomal hydrogels for mupirocin. The antimicrobial activity was found to correspond to the activity of marketed product.

The drug release profile, in *in vitro* (Table 1) conditions, was found to be depended on the formulation characteristics.

Table 1: Cumulative release profile of mupirocin (n=4)

Type of formulation	Released after 30 min (%)	Released after 7 h (%)	Released after 24 h (%)
gel- N-SL	12.45 ± 2.47	37.65 ± 8.02	43.14 ± 7.41
gel-SL	3.85 ± 0.07	8.89 ± 0.70	9.33 ± 1.23
Bac-cream	5.28 ± 0.89	21.40 ± 3.08	28.62 ± 5.00
d Bac-cream	10.48 ± 1.47	28.94 ± 2.43	42.89 ± 2.66
gel-pg	38.82 ± 1.90	90.46 ± 4.25	90.91 ± 4.57
pg	97.27 ± 1.27	97.33 ± 2.40	100.03 ± 2.34
N-SL	68.79 ± 3.09	103.17 ± 4.11	nd
SL	79.81 ± 5.38	102.09 ± 6.37	nd
gel-N-SL (f+e)	11.04 ± 1.56	32.48 ± 3.00	33.11 ± 2.78

nd= not determined; gels-N-SL= hydrogel with incorporated non-sonicated vesicles; gel-SL= hydrogel with incorporated sonicated vesicles; Bac-cream= Bactroban® cream; d Bac cream= diluted Bactroban cream to the concentration corresponding to liposomal hydrogel concentration of mupirocin; gel-pg= hydrogel containing mupirocin in propylene glycol; pg= mupirocin dissolved in propylene glycol; N-SL= non-sonicated liposomes containing mupirocin; SL= sonicated liposmes containing mupirocin; gel-N-SL (f+e)= hydrogel containing non-sonicated liposomes with unentrapped mupirocin also present in hydrogel.

Mupirocin concentration in all formulations was 505 µg/ml.

Hydrogels clearly prolonged the release of liposomally entrapped mupirocin. The liposomal size was found to affect the release profile of mupirocin (Table 1).

Similar release profiles as observed in the *in vitro* experiments were seen in the experiments on pig ear skin (*ex vivo*).

CONCLUSIONS

We were able to optimize liposomal delivery system for mupirocin destined for wound therapy. By incorporating liposomes-containing mupirocin in chitosan hydrogels, we developed advanced delivery system, with acceptable hydrogel adhesiveness and cohesiveness, and satisfactory stability profile. Microbiological, *in vitro* and *ex vivo* release studies revealed that by manipulating the liposomal characteristics such as vesicle size, it is possible to achieve sustained release of incorporated mupirocin. The comparison with marketed product Bactrocan® cream confirmed the potentials of liposomal hydrogels for mupirocin as promising advanced delivery system in burn therapy**.**

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