

MASTER THESIS FOR THE DEGREE MASTER OF PHARMACY

HYDROGELS OF NATURAL ORIGIN IN  
WOUND HEALING: FORMULATION  
DEVOLPMENT

BY

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## **Abstract**

Hydrogels made of chitosan has a well-established place in drug delivery for the skin. Our particular interest were hydrogels for wound healing. Hydrogels from low, medium and high molecular weight were prepared in different concentrations for texture and release characterization incorporating liposomes and chloramphenicol as a model drug.

A method for comparing viscosity between gels was established with a Texture analyser and back-extrusion method. The method proved to be able to distinguish differences between gels with standard deviations varying with less than 2%.

Different liposomal chitosan hydrogels were prepared with or release studies with the model drug chloramphenicol. The release study proved that liposomal hydrogels could act as vehicles for antibiotics in depot formulations.

Chitosan preparations were also tested under both accelerated stability testing and freeze-thaw test. Stability was improved with glycerine in the hydrogels. Stability seemed to be dependant on molecular weight and concentration of chitosan. The low molecular weight chitosan gels were the least stable, and higher concentrations can give more stable gels.



## 1. General introduction

Wounds are injury to tissue in which the skin is torn, punctured or cut. Blunt force trauma can also cause a contusion. Burn wounds can affect several skin layers and can be hard to treat. Chitosan gels can act as dressing for wounds keeping it moist and remove unwanted extrudates from the damaged skin. Hydrogels made of chitosan polymers can function as a scaffold, helping degenerate skin structure. The chitosan molecule has positively charged deacetylated aminogroups that can have an antimicrobial effect. Drugs can be incorporated in the gel matrix, or inside liposomes dispersed into the hydrogel for controlled localized delivery.

Hydrogels are cross-linked polymers with many hydrophilic side groups. This feature gives polymer the capability to bind water in larger quantities than its own weight. The forces between the polymer strains keep the gel from dissolving.

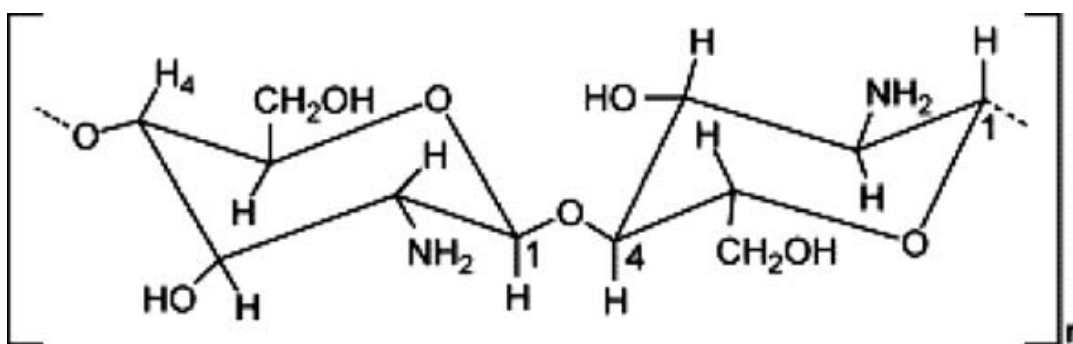
Chitin is the bricks of chitosan and is the second most abundant carbohydrate found in nature. Chitosan is generally safe for human use because of its biodegradability and non-toxic behaviour *in vivo*.

## 2. Introduction

### 2.1. Chitosan

#### 2.1.1. Properties

The term chitosan is used to describe a series of polymers of different degree of deacetylation (DD), defined as percentage of primary aminogroups in the polymer backbone and average molecular weight (Figure 1; George and Abraham, 2006). Chitosan is a semi synthetic copolymer made by deacetylation of chitin. Chitin, a component in the exoskeleton of crustaceans, crabs and some insects, is a natural carbohydrate obtained from shellfish. The copolymer polysaccharide is consisting of  $\beta$ -(1-4)-linked 2-amino-2-deoxy-D-glucose and 2-acetamidoamino-deoxy-D-glucose (Berger *et al.*, 2004). The main parameters influencing the chemical characteristics of chitosan are its molecular weight and degree of acetylation.



**FIGURE 1:** CHEMICAL STRUCTURE OF CHITOSAN (GEORGE AND ABRAHAM, 2006)

According to Takahashi *et al.* (2005) commercially available chitosans, namely low molecular weight (LMW), medium molecular weight (MMW), high molecular weight (HMW) vary in the degree of deacetylation from 83 – 90 % (Table 1). The degree of deacetylation is an important feature when using chitosan as a dressing for wound treatment (Berger *et al.*, 2004).

**TABLE 1:** MEAN MOLECULAR WEIGHT AND DEGREE OF ACETYLATION (TAKAHASHI *ET AL.*, 2005)

<b>Chitosan</b>	<b>Mean M<sub>w</sub> (g/mol)</b>	<b>DD (%)</b>
Low molecular	3,89*10 <sup>5</sup>	83,4
Medium molecular	5,59*10 <sup>5</sup>	83,1
High molecular	1,24*10 <sup>6</sup>	86,6
Chitin	3,96*10 <sup>5</sup>	1,1

Chitosan molecules are quite large polymers. High molecular weight (HMW) chitosan can hold more water in a hydrogel than a low molecular weight (LMW). The higher weights can also be prepared at lower concentrations than lower molecular weight chitosan and obtain the same viscosity. Even the chitosans from the same manufacturer have been reported to vary in molecular weight from batch-to-batch (Alsarra, 2009).

Degree of deacetylation (DD) is a property important when anticipating to what extent the hydrogel will be accelerating wound healing. Deacetylating of a chitosan molecule results in appearance of amino groups, and the substance achieves the ability to form bonds or interacts with other molecules. Amino groups can have an antimicrobial effect. High DD can therefore be a desired feature when preparing gels for wound treatment (Berger *et al.*, 2004).

Chitosan is soluble in weak acids such as acetic acid and insoluble in organic solvents and at neutral conditions (Huang and Fu, 2010).

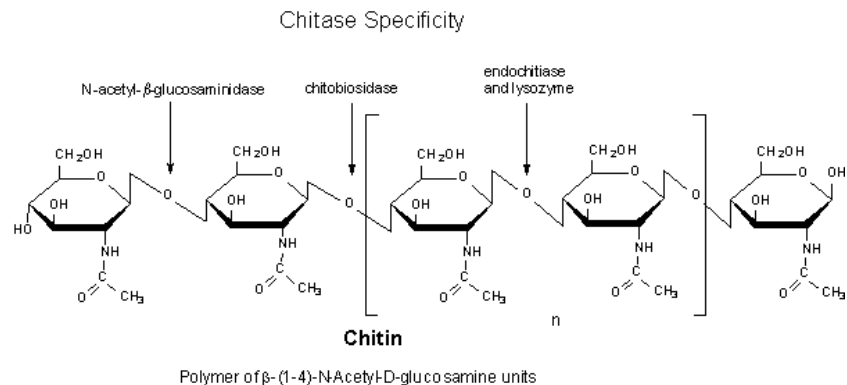
### **2.1.2. Biodegradation, biodistribution and toxicity**

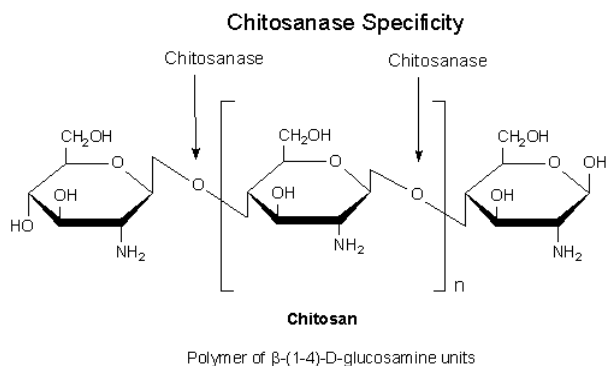
#### **Chitosan and biodegradation**

Chitosan is generally considered safe for use in pharmaceutical preparations. The chitosan gel has been used as the carrier for various drugs and route of drug administration (Kean and Thanou, 2010). When applying chitosan *in vivo* it is important to determine its rate of distribution. For that purpose, radio or fluorescent labelling can be applied (Kean and Thanou, 2010)

Metabolism should be considered when assessing potential toxicity of chitosan. If chitosan undergoes systemic absorption, high molecular weight can possibly rule out renal clearance. Molecules with molecular weight above 20000 g/mol can be filtered unhindered by kidneys (Rowland and Tozer, 2010) If Mw is larger than the kidneys can filtrate, the chitosan polymer should undergo enzymatic metabolism or some form of chemical degradation. The most common degradation of chitosan *in vivo* is considered to be lysozymal degradation, and by the bacterial enzymes in the colon (Kean and Thanou, 2010).

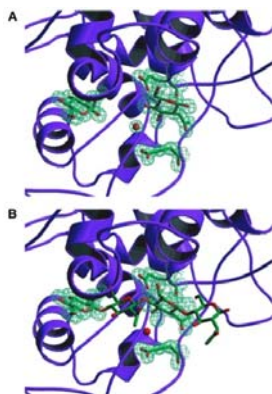
In humans, specific chitinases can hydrolyse chitin derivatives. Kean and Thanou (2010) suggested that eight of these have been identified and three have shown activity. All of these enzymes are in the glycoside hydrolase 18-family. The three enzymes that are active against chitin derivatives are acidic mammalian chitinase (AMCase), di-N-acetylchitobiase and chitotriosidase. The three enzymes are metabolizing different kinds of chitin structures and are found in lungs, liver and plasma, respectively. It has not yet been proven that these enzymes are active when metabolising chitosan polymers. Chitinases are more abundant in microorganisms than in humans. Most of the chitinases hydrolyse N-acetyl- $\beta$ -1,4-glucosaminide bonds. This suggests some kind of defence mechanism against microbes and insects, which have chitin structure. The microbacterial flora in the colon is metabolizing chitin in humans (Kean and Thanou, 2010).





**FIGURE 2:** ENZYME SPECIFICITY (WWW.SIGMAALDRICH.COM)

Chitases are chitinases that degrade chitin (Figure 2). They can act both as endo- and exo-chitinases. Hydrolysis of the glycosamine-glucosamine-glycosamine-N-acetyl-glycosamine and N-acetyl-glycosamine-N-Acetyl-glycosamine bonds are one form of enzymatic degradation (Figure 3).



**FIGURE 3:** N-ACETYL-GLUCOSAMINE (GLCNAC) (KEAN AND THANOU, 2010)

The acidic environment in the stomach will cause an acidic degradation. Other common chemical degradation will not contribute significantly to the elimination of chitosan (Kean and Thanou, 2010).

Studies *in vitro* have shown that lysozyme can degrade chitosan. The studies were conducted at 37 °C with a phosphate buffer at pH 5.5. The tested chitosan preparation had a 66% loss in viscosity after 4 hours. The degree of acetylation had a great influence on the loss of viscosity. The more chitosan resemble chitin the more rapid the degradation was observed. Covalent bonds and thiol bonds contribute to a more stable conformation and hence a slower degradation (Kean and Thanou, 2010).

After four different formulations of chitosan containing a pituitary adenylate cyclase activating polypeptide were administered to pigs, limited degradation of chitosan was observed. These specific formulations might have the capacity of replacing insulin treatment with diabetes patients. The formulations were chitosan with Mw at 400 kDa or a modified derivative, chitosan-4thiobutylamine. The chitosan formulation was also given by buccal administration and the polymer tolerated 6 hours without disintegrating (Kean and Thanou, 2010).

When assessing biodegradation of chitosan there are some important features to be evaluated. That is molecular weight, degree of acetylation, enzyme's affinity for chitin groups and chemical structure with focus on substitution of the N-groups. The amine in chitosan has a pKa of 6,3. The cationic part of chitosan needs to be protonated by a weak acid like: formate, acetate, lactate, malate, citrate, glyoxylate, pyruvate, glycolate or ascorbate (Bhatterai *et al*, 2010). When preparing formulations of chitosan as a carrier in drug delivery systems or chitosan as a topical skin delivery system, these parameters can predict how stable and effective the formulation will be (Muzzarelli *et al.*, 2007).

### **Biodistribution of chitosan**

The total molecular size and the charge of the side groups will decide chitosan kinetics fate *in vivo*. This is crucial when planning a specific release rate of drugs from chitosan formulations by per oral administration. Release can be predicted to some extent when anticipating how the chitosan formulations will degrade *in vivo*. Even when administering chitosan preparations via epicutaneous route, chitosan can be expected to undergo partial systemic absorption after application to target tissue. This is more likely when applying to damaged skin such as after burns or tissue injury. Chitosan tend to dry out after application and some decomposition will occur. This administration route is not widely studied in regard to biodistribution (Kean and Thanou, 2010).

Intracellular distribution of chitosan has been studied by using chitosan derivate. A chitosan/DNA formulation showed in *in vitro* conditions that the uptake was three times higher at 37 °C than at 4 °C (Kean and Thanou, 2010).



## **Toxicity of chitosan**

Chitosan is regarded as biodegradable and non-toxic. In Finland, Italy and Japan the government has accepted chitosan for dietary use. The FDA in the USA has approved chitosan for use in wound dressings (Kean and Thanou, 2010).

Most studies on chitosan toxicity showed little toxicity of chitosan and many of its derivatives. However, not all formulations are non-toxic. Kean and Thanou (2010) described chitosan HCl salt-derivatives as being quite toxic. Keong and Halim (2009) indicated that additives and impurities when preparing chitosan gels can contribute to its toxicity.

When the DD is high the toxicity is dependent on molecular weight and with low DD the molecular weight does not influence the toxicity. The trimethyl derivate (oligomer at 3-6 kDa) of chitosan showed increasing toxicity with higher degree of trimethylation and increasing molecular weights. Relative charge and density will decide chitosan toxicity *in vivo* (Kean and Thanou, 2010).

Chitosan preparations can be toxic to bacteria, fungi and parasites. Bacterial inhibition can be utilized in wound healing. DD at 87% and Mw at 87kDa were more effective against *Pseudomonas aeruginosa* and *Staphylococcus aureus* than DD at 73% and Mw at 532 kDa. The two chitosans in a form of an emulsion had effect on *Candida albicans* and *Aspergillus niger*. A chitosan excipient (meglumine antimoiate) showed anti parasitic effect against *Leishmania infantum* (Kean and Thanou, 2010).

One 65 days study indicated that there was no toxicity from injected chitosan oligosaccharides. The doses were 7,1-8,6 mg/kg over 5 days. Lysozyme activity increased as expected. This indicated that lysozymes are indeed effective in chitosan degradation. Lethal dose when was found to be 50 mg/kg. Injection of chitosan-<sup>166</sup>Holmium proved to be safe in treatment of cancer (Kean and Thanou, 2010).

Chitosan have been used for fat chelation at dose of 4,5g/day and no toxicity for humans was reported. However, the influence on weight loss is debatable. Administration of trimethyl chitosan/pDNA caused light diarrhea at high doses. Chitosan have little

cytototoxicity against human lymphoblastic leukaemia or human embryonic lung cell. (Kean and Thanou, 2010).

### **2.1.3. Applications in pharmaceuticals**

Hydrogels can act as artificial extracellular matrix (ECM) for tissue rebuilding. Original ECM can be regarded as a natural hydrogel. Synthetic hydrogels cannot readily function as a support and interact with cells *in vivo*. This enables hydrogels to serve as building blocks for tissue rebuilding in wound healing (Jia and Kiick, 2009).

Hydrogels can act as vehicle for drugs in several ways. With direct addition of drugs the active substance can be encapsulated during the polymers cross-linking. Active ingredients can also be diffused into the pores of the hydrogel after swelling. These methods are the simplest but the release rate is hard to control (Bhatterai *et al.*, 2010).

Release from hydrogels can be categorized as diffusion-controlled, swelling-controlled or chemically-controlled. Diffusion-controlled is release from the cross-linked matrix. The matrix can be from 5-100 nm. Drugs will often be small molecules. Peptide drugs will have therefore a more retarded and prolonged release. Chemically-controlled release is characterized by the drugs detached from the gel by some kind of chemical reaction within the gel (Bhatterai *et al.*, 2010; Jagur-Grodzinski, 2010).

Micro and nano capsules can be incorporated into the hydrogel for a more controlled and retarded release. Growth factors (example: EGF, TGF- $\beta$ 1) release can be regulated with gelatine particles. This can give a controlled release (Bhatterai *et al.*, 2010, Huang and Fu, 2010).

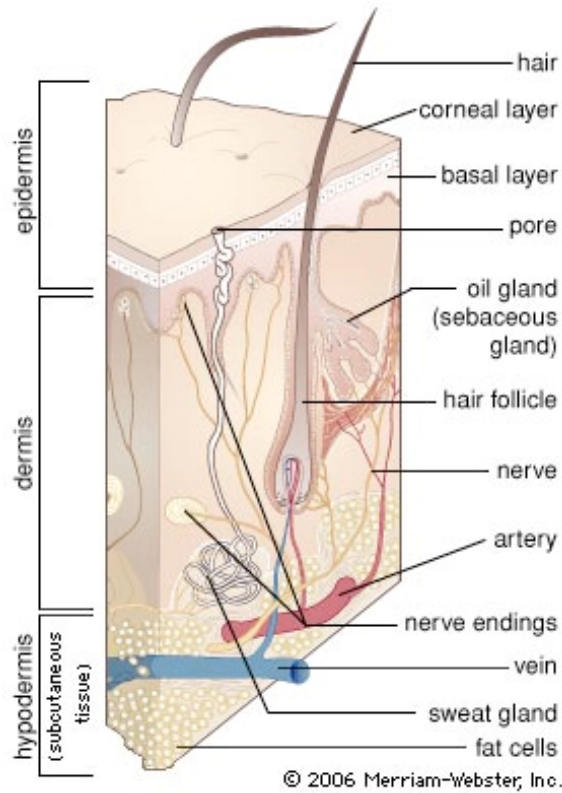
Small covalently attached molecules can have a fairly controlled release since the release is controlled by the disintegration of the hydrogel or hydrolysis. Paclitaxel (chemotherapeutic), dexamethasone (steroid) and fluvastatin (cholesterol lowering drug) are quite small and have successfully been covalently attached to hydrogel polymers. The release is not controlled but retarded with this method (Bhatterai *et al.*, 2010).

Chitosan hydrogels have been used in liquid gels, powders, beads, films, tablets, capsules, microspheres, microparticles, sponges, nanofibrils, textile fibers and even inorganic composites (Denkbaş and Ottenbrite 2006; Bhattarai *et al.*, 2010).

## **2.2. Skin and wounds**

### **2.2.1. Skin structure**

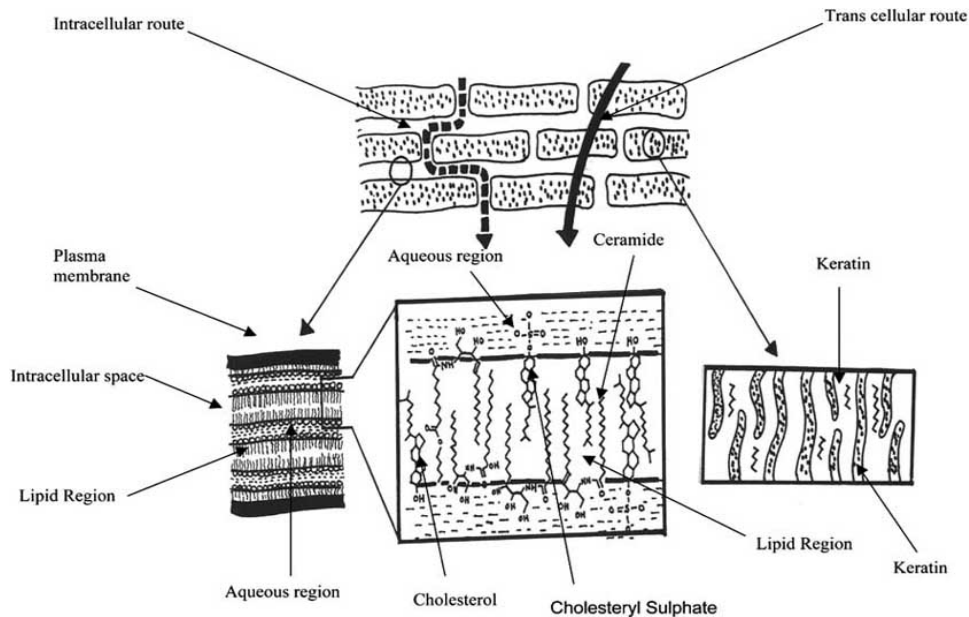
The skin is covering most of the outer body varying in its thickness and structure. The top layer of skin is the epidermis. Cells in epidermis proliferate and renew the layer regularly. This is important since the skin is the main barrier protecting the body from damaging factors. The underlying layer is the dermis. The dermis is tough for support and nourish the skin. Fibroelastic tissue is providing the skin its form. Dermis can be regarded as two zones. The upper layer is the thin papillary dermis, and the lower layer the reticular dermis. The deepest layer is the hypodermis. Hypodermis is varying the most, with mainly adipose tissue. Sweat glands, hair follicles, sebaceous glands and nerve fibers intersect all skin layers (Figure 4). Epidermis is considered avascular but the dermis is vascular. This means that epidermis is highly dependent on proper blood flow for its normal function (Young and Heath, 2000; Stevens *et al.*, 2002, Sherwood, 2007).



**FIGURE 4: SKIN STRUCTURE (BRITANNICA CONCISE ENCYCLOPAEDIA, 2010)**

### **2.2.1.1. Intact skin**

Covering the body as barrier for chemicals and ultraviolet light, skin also serves as a shield for microorganisms and protects from mechanical tear. Thermoregulation is highly dependant on the blood flow to the skin. Vitamin D is synthesised in the epidermis. Adipose tissue is metabolized into an energy source when needed. The skin is also important for our appearance and communication. Healthy skin has numerous properties of importance to our health and well-being and has a surface pH in the range of 4.2. - 5.6 (Sherwood, 2007).



**FIGURE 5:** DIFFERENT ROUTES OF PENETRATION THROUGH THE SKIN (BENSON, 2005)

Intracellular route is considered to be the most significant route for permeation of most drugs administered to the skin. Most molecules will penetrate the skin via the lipid domains and the degree of lipophilicity will play the dominant role. Small portions of drugs (about 0.1%) will possibly penetrate via appendages (Figure 5; Benson, 2005).

### 2.2.1.2. Damaged skin and barrier properties

Damaged skin can severely reduce quality of life and cause unwanted health problems when left untreated. Burns, diabetic ulcers, arterial and venous ulcers can all be a challenge to treat. When circulation is reduced, blood flow lowered or dermis damaged, the wound healing takes longer time and the wound might evolve into a chronic one (Bao *et al.*, 2009).

In regard of development of drug delivery system for damaged skin, the changed barrier function needs to be taken into consideration. pH of the skin is altered. Lipophilic and hydrophilic properties of the skin are most likely different from healthy skin. Permeability of drugs might be unexpectedly high or even low. Atrophy due to

degeneration of cells will make drug therapy regimes more difficult to design because of the reduced thickness of the skin (Boateng *et al.*, 2008).

### **2.2.2. Wounds and treatment**

Wounds can be classified into chronic wounds and acute wounds. Chronic wounds take longer than 8-12 weeks to heal. Examples include diabetic leg ulcers, arterial and venous leg ulcers and pressure sores. Acute wounds can be burn wounds, surgical wound or wounds from trauma (Chaby *et al.*, 2009; Frankel *et al.*, 2009).

Skin wounds can be also classified according to the number of skin layers that are affected. Superficial wounds are the damage to the epidermis alone. Partial thickness wounds are the damage to the epidermis and deeper layers, blood vessels, hair follicles and sweat glands. Full thickness wounds are the damage to fat or deeper tissue as well (Helms *et al.*, 2006).

Wounds require good blood flow and good access to rich blood with oxygen in order to heal. Dietary nourishment is important for rapid recovery. Healing can be impaired by low oxygen flow, infection or malnutrition (Chaby *et al.*, 2009).

Our particular interest was burns. The healing of burn wounds is a complex physiological process that involves migration, proliferation and differentiation of a variety of cell types as well as synthesis of matrix components and regulatory factors (Sidhu *et al.*, 1998).



area to renew damaged tissue. Cells grow over the wound, under the dried scab and gradually thicken the epithelial layer. This step lasts 2-3 days (Helms *et al.*, 2006).

Proliferation starts more or less with migration or just after the migration phase. In this phase granulation tissue is formed when new capillaries are transporting blood and nutrients to the wound. Collagen network are synthesized by fibroblasts. When this network is formed, the skin regains its tensile strength. With further proliferation epithelial cells migrate through the wound and the wound contracts and closes. In the proliferation phase the wound is red and can be moist but not exuding. Proliferation can last 5-20 days (Helms *et al.*, 2006).

Maturation is the last step of wound healing. Collagen fibers are strengthening the skin and more capillaries are increasing the blood flow to the wound. This phase can take from 3 weeks to 2 years. The final scar is commonly not as strong as the skin was before injury, but 70-90% of tensile strength can be expected (Keong and Halim, 2009). These processes are regulated by growth factors. Accelerated wound healing can be dependent on inducing and activating cytokines and growth factors (Bao *et al.*, 2009).

Frankel *et al.* (2009) conducted a microbial study evaluating a presence of microorganisms in the wound and found that methicillin resistant *S aureus* (MRSA) strains were very common. *B Streptococcus* was frequently found, and especially in diabetic elderly patients. Other pathogenic bacteria were also found (*Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Serratia marcescens*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*). The study confirmed that wounds are highly susceptible to infections and that inclusion of antimicrobial agent in wound dressing is recommended.

Larger wounds need some kind of cover to help healing. Bandages are widely used for wound cover. Hydrogels can function as a film on wounds and in addition keep the wound moist. Hydrogels can be washed off by water when change is needed. This will



keep the newly made granulation tissue more intact than when removing a dry bandage (Chaby *et al.*, 2007).

The meta-analysis study regarding the use of dressing for acute and chronic wounds including hydrocolloids, hydrocellular foams, polyurethane foams, alginate gels, hydrogels, hydrofibers, dextranomers, paraffin gauze, non-adherent, hyaluronic acid-impregnated, silver coated, activated charcoal and protease-modulating matrix (Promogran) showed that there were no difference in efficacy among foam dressing, paraffin gauze dressing, polyethane film or polyurethane film when using split-thickness skin grafts. Time to complete healing was lower with foam dressing than silver coated dressing. For hydrofiber dressing time was lower than for paraffin gauze. There were no difference in complete healing rates between hydrofiber dressing and wet-to-dry gauze for surgical wounds. Hyaluronic acid impregnated dressings induced slower healing when compared to glycerin-impregnated dressing. The foam dressings were more effective than silver coated dressing when evaluating the complete healing of acute wounds. Hydrofiber dressings were more effective than paraffin gauzes. Alginate and paraffin dressing was compared in regard to pain as outcome. Alginate was found to be the superior (Chaby *et al.*, 2007).

Growth factors can be incorporated into hydrogels for controlled delivery when wound healing is the focus. Vascular endothelial growth factor (VEGF) has been reviewed for use in wound healing. VEGF stimulates wound healing by angiogenic cascade (angiogenesis: the formation of new blood vessels). Matrix metalloproteinases (MMP's) are enzymes that active when tissue in wounds is broken down for wound healing. VEGF increases endothelial cell secretion of interstitial collagenase, MMP1 (matrix metalloproteinase) and MMP2 (gelatinase A). VEGF stimulates an expression of MMP-1, MMP-3 and MMP-9 in vascular smooth muscle cells to promote degradation of collagen. In general, VEGF provides endothelial movement in the extracellular space. Migration is induced by vasodilation and chemotaxis. Chemotaxis is movement of a cell as a response to a chemical gradient. Proliferation is stimulated partially by VEGF's ability to lengthen the lifespan to endothelial cells. Wound healing is dependent on

forming of granulation tissue. VEGF is stimulating angiogenesis and thereby giving increased blood flow with nutrients to the wound (Bao *et al.*, 2009).

### **2.3. Hydrogels in wound therapy**

Hydrogels are water-swollen polymeric materials that maintain a distinct three-dimensional structure. As dosage form, they are not greasy or oily and are water-washable. They are considered to be nontoxic and can function as a vehicle for drugs in various forms (Kopecek, 2009). Due to their high water content, hydrogels possess excellent biocompatibility. There is a wide variety of design options for the preparation of hydrogels of different structures and properties. The usual classification relies on the origin of gelling material (Kopecek and Yang, 2007).

Yang *et al.*, (2008) proved that chitosan hydrogels are effective against *E. coli* when compared to control or other hydrogels.

#### **2.3.1. Hydrogels of natural origin**

Chitosan has been utilized for many medical and pharmaceutical preparations. Properties such as being biocompatible, non-toxic and soluble in weak acids make it an excellent wound dressing. It is positively charged, is strong tissue adhesive and forms gel easily. It has proven to enhance function of leukocytes, macrophages and fibroblasts to enhance granulation and rebuilding tissue (Huang and Fu, 2010).

Hydrogels form stabilizing linkages between polymer chains. Covalent, hydrogen, ionic and van der Waals bonding link water and polymer strains to a gel (Bhatterai *et al.*, 2010). Polymer-polymer crosslinking between hyaluronic acid and chitosan has been used in hydrogels. Schiff's bases were formed within a fast forming hydrogel (Bhatterai *et al.*, 2010).

Collagen is a biocompatible scaffold. Cells utilize collagen as a major component in the ECM (extracellular matrix). Collagen can be used to rebuild skin after wounding. Collagen has been used in tissue engineering and in delivery systems. Collagen can be combined with other materials for tissue regeneration. Growth factors have been incorporated into collagen to enhance healing. Biocompatibility and low antigenicity make collagen a good natural polymer. Its weak feature is mechanical weakness. Collagen can be extracted from animal tissue. Human collagen can be made as a replacement for animal origin collagen that can be potentially harmful because of microbial contamination (Huang and Fu, 2010).

Galantine is widely used in pharmaceutical industry because of biodegradability and biocompatibility. Gelatine is normally denatured and has a low antigenicity. Isoelectric point can be altered to make galantine suitable for a wide array of uses with different charges and pH. Growth factors have been incorporated into galantine vehicles and used as controlled release systems for tissue like skin in wound regeneration. Galantine expresses structural differences that can be utilized to fit a fair selection of medical uses. Galantine can also be used as a vehicle for cells for skin regeneration (Huang and Fu, 2010).

Fibrin and fibronectin are components in the ECM with many roles. They induce attachments of cells. Fibrinogen can be isolated from plasma of patients. Fibrin is a network of polymerized fibrinogen. Thrombin is an enzyme that polymerizes fibrin and acts as ECM fibrin glue. Fibrin is biocompatible and has high tissue like water content. Fibrin's mechanical properties are somewhat like soft tissue. Fibrin has the ability to be injected as a liquid *in vivo* and forms to a gel *in situ*. Fibrin can also be used as a cover to stop bleeding and skin graft fixation. Fibrin undergoes rapid degradation *in vivo* and formulations will have stability problems (Huang and Fu, 2010).

Alginate is obtained from brown algae and has a long history in drug delivery and tissue engineering. Alginate is almost non-toxic, biocompatible, with non-immunogenicity, low

cost and simple gelation procedure. It is used as a wound dressing, vehicle for proteins and growth factors, and liposomes (Huang and Fu, 2010).

### **2.3.2. Hydrogels of synthetic origin**

Carbopol polymers have the ability to thicken, suspend and stabilize aquatic solutions. With more than forty years on the market, Carbopol is well tested and have many uses. Cosmetic formulations are among the most abundant. Carbopol has excellent characteristics as a vehicle for drug delivery (Islam *et al.*, 2004; Desai *et al.*, 2006) Carbopol gels are widely used because they are more stable and mechanically durable than natural hydrogels like chitosan. Biocompatibility of synthetic hydrogels is on the other hand not as good as for some hydrogels of natural origin, for example chitosan gels (Keong and Halim, 2009).

### **2.3.3. Chitosan hydrogels**

Chitosan hydrogels have been widely studied as topical formulations. Among others, Alsarra (2009) evaluated chitosan hydrogels in topical formulations for burn wounds. Different molecular weight and different degree of deacetylation ranges were compared. Chitosan formulations were compared also with fucidin ointment and placebo formulation for treatment of rats. Although high molecular weight (HMW) chitosans will be more viscous than low molecular weight (LMW) one and the medium molecular weight (MMW) at the same concentration, Alsarra proved that HMW chitosan was more effective than fucidin ointment when measuring wound contraction over time. Fucidin gave approximately the same results as MMW chitosan. The treatment with HMW chitosan gave no visible scarring after 12 days, and treatment with fucidin gave visible scarring. In conclusion, all formulations with chitosan gave better wound healing results than the placebo (Alsarra, 2009).

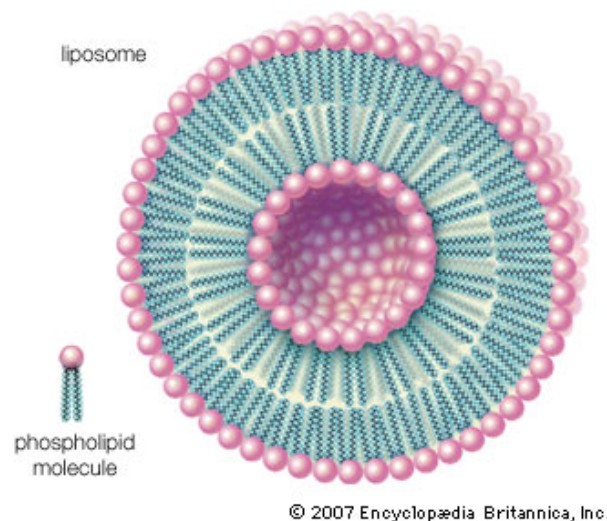
Ribeiro *et al.* (2009) also studied chitosan hydrogels in wound healing. They found that chitosan was able to promote adhesion and proliferation in wounds. Chitosan was not found to be cytotoxic in this study. Burn wounds of 3<sup>rd</sup> degree were treated with chitosan hydrogel and phosphate buffered saline, respectively. The healing was measured through histological studies and evaluation of wound size. Chitosan gave better results than the control.

Murakami *et al.* (2010) studied wound healing in healing-impaired wounds. After inducing controlled wounds in rats, they used mitomycin C that inhibits cell proliferation in fibroblasts, keratinocytes and endothelium in the wound. They found that the blend of chitin/chitosan and fucoidan powders showed better wound healing capabilities than calcium alginate dressing and control.

### 2.3.4. Liposomal hydrogels

#### 2.3.4.1. Liposomes as topical drug delivery systems

Liposomes have a promising future in drug transport and delivery. They can be prepared from natural or synthetic origin lipids (Škalko *et al.*, 1998). According to the method of preparation, liposomes may vary in their size and lamellarity. They can be characterized as small unilamellar vesicles (SUVs; 25-50 nm in diameter), large unilamellar vesicles (LUVs; 50-500 nm) or large multilamellar vesicles (LMVs; 500-10000 nm) (New, 1990).



**FIGURE 7: LIPOSOMAL STRUCTURE** ([HTTP://WWW.BRITANNICA.COM](http://www.britannica.com))

The size of liposomes will affect the entrapment efficiency for both lipophilic drugs, which will accommodate themselves in lipid bilayers of the vesicle and hydrophilic drugs, which will be entrapped in the liposomal core. Phosphatidylcholine made liposomes can be stabilized with cholesterol. Span 80 and Tween 80 are surfactants that can enhance permeation through the stratum corneum and epidermis when combined with liposomes. Regarding the topical application, large liposomes will not readily penetrate the stratum corneum (Cevc, 2004).

Liposomes can penetrate the skin by three mechanisms: Lateral diffusion, trans-epidermal osmotic gradient or pilosebaceous units. Lateral diffusion is lipid exchange between membranes. Phospholipids with less than 16 carbons exchange membranes in minutes to hours and with long-chain require hours to days. Trans-epidermal osmotic gradient is a hydration force that sucks liposomes into the epidermis (El Maghraby *et al.*, 2008; de Leeuw *et al.*, 2009).

Liposomes have a tendency to accumulate in the skin. That can be preferable when topical administration is desired as in wound healing. When transdermal delivery is the goal, various types of vesicles can be prepared such as niosomes, vesicles with non-ionic surfactants. The liposomes express enhanced penetration ability and elastic properties. Ethosomes are vesicles containing ethanol. Ethanol enhances skin penetration and can be used to deliver drugs deeper to the skin. Transfersomes are even more elastic and deformable. Transfersomes can move through channels one tenth of their diameter (Benson, 2009).

Liposomal delivery systems have been widely studied in topical administration and there are dozens of marketed cosmetic and pharmaceutical products on the market or in clinical trials right now (Benson, 2005). Several clinical trials confirmed the applicability of liposomes in the treatment of skin diseases, such as clinical trial proving that liposomes with clindamycin were superior to lotions containing the same drug in non-vesicle form (Škalko *et al.*, 1992).

#### 2.3.4.2. Liposome-based chitosan hydrogels

Liposomal chitosan gel formulations were of particular interest when preparing formulation in this study. Hydrogels as vehicles for liposomal dispersions are well established and studied for various routes of drug administration. Hydrogels preserve the original structure of liposomes and make the preparations more user friendly, resulting in better patient acceptability and compliance (Pavelić *et al.*, 2001). It is especially important for topical administration where the retention of the formulation at the administration site affects the efficiency of the therapy, such as with hydrogels as vehicles for liposomes with metronidazole for treatment of *Rosacea* (Škalko *et al.*, 1998).

Mourtas *et al.* (2008) studied the rheological properties of hydrogels prepared with liposomes. The mixtures of Carbopol 974 and hydroxyethylcellulose were made with glycerine, citrate buffer and preservatives. Liposomes made of phosphatidylcholine (PC) and hydrogenated phosphatidylcholine (HPC) were used, varying in the degree of lamellarity of the membranes. Although Mourtas *et al.* (2008) used quite low concentrations of liposomes in their gels, they found that liposomal composition has the potential to alter hydrogel viscosity. The hydrogenated liposomes showed to have a higher transition state (50 °C) than the non-hydrogenated liposomes. PC was at a liquid state under test conditions, whereas HPC was not. This can explain why HPC can alter viscosity to a higher extent than PC. Size and lamellar types of liposomes showed approximately the same rheological effects on the gels (Mourtas *et al.*, 2008).

Gabrijeličić and Šentjurc (1994) studied liposome stability and liposome transport from hydrogels into pig skin. They found that hydrogels of carboxymethylcellulose and xanthan did not hinder soya lecithin-cholesterol liposomes transport to the skin. Hydrophilic polymers can change the stability to liposomes, like xanthan polymers (Gabrijeličić and Šentjurc, 1994).

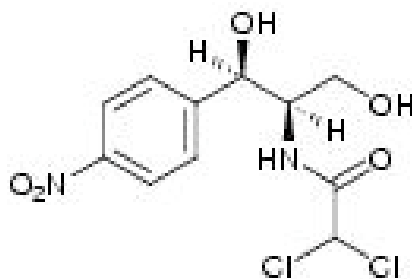
Pavelić *et al.* (2001) prepared liposomes by the polyol dilution and proliposome methods. They proved that hydrogels prepared from carbopol polymers increase the stability and enhance the release time of the liposomally entrapped drugs. Liposome-based hydrogels

were evaluated in the simulated vaginal conditions and the findings confirmed that hydrogels were good vehicles for liposomal delivery of drugs (Pavelić *et al.*, 2001).

## 2.4. Chloramphenicol as model drug

Chloramphenicol is an antibiotic drug mostly used for bacterial conjunctivitis. Chloramphenicol has a broad spectrum of activity against both Gram positive and Gram negative bacteria, *rickettsias* and *Chlamydia*. The specific effect is inhibition of protein synthesis in microorganisms. Chloramphenicol has effect on a wide array of bacteria, among them, one present in wounds as well (Helms *et al.*, 2006).

Although the applicability of chloramphenicol in the treatment of wounds remain to be confirmed through broader clinical evaluation, preliminary results by Heal *et al.* (2009) indicate that single administration of chloramphenicol ointment to saturated wounds after minor surgery procedure resulted in relative reduction in infection rate of about 40 %.



**FIGURE 8:** STRUCTURE OF CHLORAMPHENICOL

We have selected chloramphenicol as a model antibacterial drug. Moreover, the formulation choice for chloramphenicol topical dosage forms is limited by its solubility and represents pharmaceutical challenge.



### **3. Aims of the study**

The main objective of the project was the development of topical formulation to be applied in treatment of skin wounds, namely burns. Hydrogels are one of the most popular types of wound dressings, and hydrogels of natural origin are known to have several advantages over synthetic origin hydrogels. Chitosan based hydrogels were selected as delivery system for wound treatment. Chloramphenicol was used a model antibacterial agents for the prevention or treatment of wound infections.

More specific aims were:

- Optimize chitosan hydrogels in regard to the effects of polymer concentration, type of chitosan used (low versus high molecular weight) and additives, on the texture properties of formed gels
- Incorporate liposomes carrying chloramphenicol in hydrogel and evaluate the effect of incorporated liposomes on hydrogel properties.
- In parallel, develop a rapid and reproducible method to analyze gel properties and compare batch-to-batch variations and stability.
- Test the stability of hydrogels and liposome-based hydrogels in accelerated stability conditions
- Evaluate the release of drug from liposomal hydrogels

## 4. Materials and Methods

### 4.1.1. Materials

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

Acetonitrile, isocratic grade for liquid chromatography, Merck, Darmstadt, Germany (I483791 918, 1.14291.2500)

Alginic acid, sodium salt, (Brookfield viscosity 20000-40000 cps) Sigma-Aldrich Chemistry, St Luis, USA (61496MJ/MKBB8171, 180947-100G)

Carbopol Ultrez® 10 NF, Noveon, Cleveland, USA (LOT #: CC73RZG554)

Chitosan, low molecular weight, (Brookfield viscosity 20000 cps) Sigma-Aldrich Chemistry, St Luis, USA (61496MJ/MKBB4232, 448869-250G/448869-50G)

Chitosan, medium molecular weight, (Brookfield viscosity 200000 cps) Sigma-Aldrich Chemistry, St Luis, USA (MKBC0060, 448877-250G)

Chitosan, high molecular weight, (Brookfield viscosity 800000 cps) Sigma-Aldrich Chemistry, St Luis, USA (MKBB0585 419419-250G/448869-50G)

Chloramphenicol micronisated MBK, Norsk Medisinal Depot, Oslo Norway (30 50 94, Anr 2N005/2)

Chloroform (HPLC grade) Merck, Darmstadt, Germany, (K38551444 812 1.02444.1000)

Distilled water

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

Lipoid S 100 (soybean lecithin with 100% phosphatidylcholine), generous gift from Lipoid GMBH, Ludwigshafen, Germany (790611-03/911)

Methanol (HPLC grade) Merck Darmstadt, Germany (1515007 950, 1.06007.2500)

Polyamide membrane, Sartorius AG, Göttingen, Germany

Triethylamine (for synthesis) Merck, Darmstadt, Germany (S3801652, 8.08352.1000)

Triglycerides (middle chain) Fagron GmbH & Co. KG, Barsbüttel, Germany

#### **4.1.2. Instruments**

Agilent technologies UV/Visible spectrophotometer, G1103A (Santa Clara, CA/USA).

Beckmann L8-70M Ultracentrifuge, Beckmann Instruments Inc, Palo Alto, USA

Biofuge Stratos, Heraeus instruments, Dipl.Ing Holm AS, Oslo Norway

MS2 Minishaker, Chiron AS, Trondheim Norway

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

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

Distillation unit Distinction D4000, Bibby Sterlin LTD. Staffordshire UK

“Freiburger schlange schnecke”: Ismatec IPC, Dan Maszansky AS, Laboratorieutstyr, Oslo Norway

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

Waters 2695, Separations Module, Waters 2487 Dual  $\lambda$  Absorbance Detector, Waters, Milford USA; with XTerra<sup>TM</sup> RP<sub>18</sub> 5 $\mu$ m (3.9\*150 mm) W01671T 004 column from Waters S.A.S. (Massachusetts, USA)

### **4.1.3. Computer programs**

Texture analyser: Millennium 32 Chromatography Manager (4.0)

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

## **4.2. Preparation of hydrogels**

### **4.2.1. Preparation of carbopol hydrogels**

Carbopol Ultrez® forms gels easily and quite rapidly. Distilled water (DW) and Carbopol Ultrez® (CU) were gently mixed in the ratios from 99:1 – 99.8-0.2 (w/w). Appropriate amount of triethylamine was added under gentle stirring to reach a pH of 6 (measured with indicator paper). In the cases when air bubbles were observed, bath sonication for up to 30 minutes was applied. The hydrogel was allowed to swell for 24 hours at room temperature.

### **4.2.2. Preparation of chitosan hydrogels**

Chitosan forms gels when dispersed in a weak acid. Low (LMWC), medium (MMWC) or high (HMWC) molecular weight chitosans were dispersed in 2.5 % acetic acid solution. Alternatively, 0.25, 1, 1.5, 2 and 4% acetic acid solutions were used when appropriate. The concentration of chitosan in the acetic acid was in the range of 1-6% (w/w). The mixture was stirred manually for 10 minutes and sonicated for 30 minutes. Chitosan hydrogels were initially allowed to swell in a refrigerator to keep the gels stable, but the time necessary for gel formation was long, therefore in optimized procedure the hydrogel was allowed to swell for 48 hours in a sealed container at room temperature. The choice of acid and preparation protocol was based on Alasarra (2009) and Cao *et al.* (2009).

Attention was given to the stirring conditions as it was found that the use of magnetic stirrer in a steel bowl trapped more air, similar to when the mixing was performed in mortar.

#### **4.2.3. Determination of the effect of additives on gel properties**

Glycerine (1, 2 and 9% (w/w), respectively) was mixed with acetic acid before adding the chitosan in predetermined amount. Glycerine content of 1 % (w/w) was preferred since it did not alter the original gel viscosity but increased the stability.

In preliminary testing of the effect of sodium alginate on gel properties, the addition of 2% (w/w) alginate resulted in too low viscosity of the gel, and was not further evaluated.

### **4.3. Texture analysis**

Texture analyser can be applied in evaluation of formulation properties of hydrogels. The instrument provides options to measure backward extrusion, forward extrusion and multiple extrusions. The recorded forces represent responses to tension, compression, penetration or bending. The selected probe will move at a programmed speed and until specified force, distance or strain is reached, which will be indicated in the record.

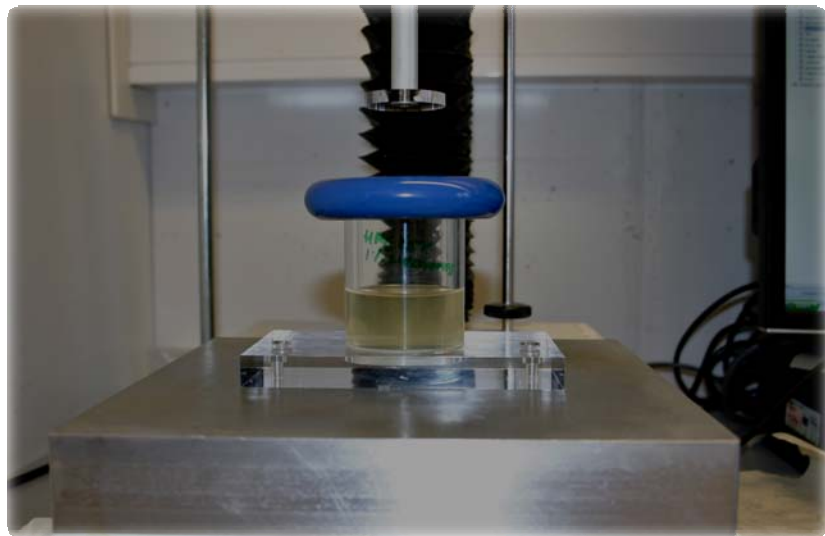
For measuring the backward extrusion force on chitosan gels, at first the A/BE-d35 probe with back extrusion rig and 35 mm disc and a torus weight was applied. As chitosan gels are sticky and dehydrate at the walls of the container, a submerged probe was found to be more suitable. The submerged disc proved to better resulting in with variations in the readings.

Force and height were calibrated at the start of each measurement. Fifty grams of gel were used in all measurements. The disc was moved 1 mm from the bottom of gel and rested for 30 seconds to relieve air-bubbles under the disc. The probe was then moved to

15 mm (submerged) and rested for 15 seconds (Figure 9). Five measurements were run with 15 seconds rest between every run. Two sets of conditions for testing were found to be equally good, namely

1: Pre-test speed: 1 mm/sec; test speed: 1 mm/sec; post-test speed: 1 mm/s; distance 10 mm; return to the start point

2: Pre-test speed: 4 mm/sec; test speed: 4mm/sec; post-test speed: 4 mm/s; distance 10 mm; return to the start point.



**FIGURE 9:** MEASUREMENT SET UP TEXTURE ANALYSER



**A**

**B**

**FIGURE 10:** MEASURING POSITION WITH PROBES

A: Cylinder probe B: Disk probe (35 mm in diameter)

Starting point for the cylinder probe was above the gel. Starting point for the 35 mm disc was submerged as see in the Figure 10B.

The force and area were recorded for total back extrusion with respect to cohesiveness and adhesiveness.

## **4.4. Liposomal hydrogels**

### **4.4.1. Empty liposomes**

Lipoid S100 (200 mg) was dissolved in methanol in the ratio 1:10 (w/v). Methanol was allowed to evaporate in a rotavapor for at least 2 hours at 100 mmHg at 30 °C and 60 rpm, and then 30 minutes at 45 mmHg at 30 °C and 60 rpm. The dry phospholipid film was re-suspended by the addition of 10 ml of distilled water. Vortex was used to help to

dislocate the film when needed. Liposomes were refrigerated for at least 24 hours before further characterization.

#### **4.4.2. Liposomes with chloramphenicol**

Chloramphenicol (20 mg) was dissolved together with Lipoid S100 (200 mg) in excess methanol and the solvent evaporated under the same conditions as described for empty liposomes. Liposomes were refrigerated for at least 24 hours before further characterization.

#### **4.4.3. Entrapment efficiency determination**

In order to separate liposomally entrapped chloramphenicol from untrapped chloramphenicol, ultracentrifugation was applied. Liposomes were centrifuged in Beckman-L8-70M ultracentrifuge (Brea, CA/USA) at 10 °C, for 25 min period at 32000 rpm. Upon centrifugation, the pellet was resuspended in 1500 µl distilled water, and an aliquot (10 µL) further diluted and used in spectrophotometrical and HPLC analyses. An aliquot (30 µL) of the supernatant was also further diluted with methanol and the chloramphenicol content determined both spectrophotometrically and by the HPLC analysis.

**Spectrophotometrical analysis:** A stock solution of chloramphenicol was made by dissolving 51.7 g of chloramphenicol in 200 mL of methanol. Working solutions were prepared by diluting the stock solution to desired concentration with methanol and calibration curve prepared using Agilent technologies UV/Visible spectrophotometer, G1103A (Santa Carla, CA/USA) at 268 nm wavelength.

**HPLC analysis:** HPLC system consisted of a Water separation module 2695 and Waters 2487 UV-spectrophotometer detector. Column used was a XTerra™ RP<sub>18</sub> 5µm (3.9\*150 mm) W01671T 004 column from Waters S.A.S. (Massachusetts, USA). The mobile phase consisted of 45% methanol, 55% filtered H<sub>2</sub>O and 0,1% acetic acid (glacial). The temperature of column was maintained at 35°C±5°C and the temperature of samples was



maintained at 35°C±2°C during the chromatographic separation. The flow rate was 1 ml/min and running time for each sample was 5 min monitored at UV 270 nm.

#### **4.4.4. Particle size analysis**

Dynamic light scattering (DLS) on the Nicomp™ model 380 particle sizing system with software version C-370 V-1.51a, and equipped with a fixed 90° external fiber angle and a 632.8 nm, 5 mW He–Ne laser was used to determine average particle size and size distribution of prepared liposomes. In order to avoid any contamination with dust, sample preparation was carried out in a clean area using particle-free equipment: all handling was done in a laminar air-flow bench, test tubes were submersed in particle-free water and sonicated for 15 min in an ultrasonic bath and rinsed with freshly filtered (0.2 µm pore size syringe filter) water prior to use. The vesicle-dispersion was diluted empirically with freshly filtrated medium until an intensity of 250–350 kHz was achieved (Hupfeld *et al.*, 2006).

#### **4.4.5. Preparation of chitosan gels with liposomes**

Liposomal preparations (empty liposomes or liposomes containing chloramphenicol) were incorporated in prepared chitosan hydrogels (concentration varying from 1 to 6%, w/w) by hand stirring (Skalko *et al.*, 1998) and allowed to stabilize for 2 hours. The final concentration of liposomes in hydrogels was 5, 10 and 15% (w/w, liposomal suspension/total), respectively.

## 4.5. Stability testing

### 4.5.1. Chitosan gels and liposomal chitosan gels in accelerated stability testing

Accelerated stability testing was applied to evaluate the stability of prepared hydrogels. The gels (50 g) were examined by the help of texture analyser (method 4.3) before and after one month of storage in an airtight container at 40 °C (thermostat).

**TABLE 2:** ACCELERATED STABILITY TESTING

Chitosan type (concentration; % w/w)	Liposomal concentration (w/w, liposomal suspension per total)
Low molecular weight (6%)	0%
Low molecular weight (6%)	1%
Low molecular weight (6%)	5%
Low molecular weight (6%)	10%
Low molecular weight (6%)	15%
Medium molecular weight (3.5%)	0%
Medium molecular weight (3.5%)	1%
Medium molecular weight (3.5%)	5%
Medium molecular weight (3.5%)	10%
Medium molecular weight (3.5%)	15%
High molecular weight (2.5%)	0%
High molecular weight (2.5%)	1%
High molecular weight (2.5%)	5%
High molecular weight (2.5%)	10%
High molecular weight (2.5%)	15%

### 4.5.2. Accelerated stability test of liposomal HMWC gels containing glycerine

Liposomal chitosan gels containing 1% glycerine, were stored for one month period in an airtight container at 40°C. The properties of gels were measured before and after on texture analyser.

**TABLE 3: ACCELERATED STABILITY TESTING OF LIPOSOMAL HYDROGELS CONTAINING GLYCERINE**

Chitosan (concentration; w/w)	Liposomal concentration (w/w, liposomal suspension per total)	Glycerine (w/w)
High molecular weight (2.5%)	0%	1%
High molecular weight (2.5%)	1%	1%
High molecular weight (2.5%)	5%	1%
High molecular weight (2.%)	10%	1%

#### 4.5.3. Stability testing under freezing conditions

HMWC gels, HMWC liposomal gels, with and without glycerine were evaluated on texture analyser before and after storage at -22 °C for 48 hours. The frozen gels were allowed to thaw at room temperature prior to the measurement.

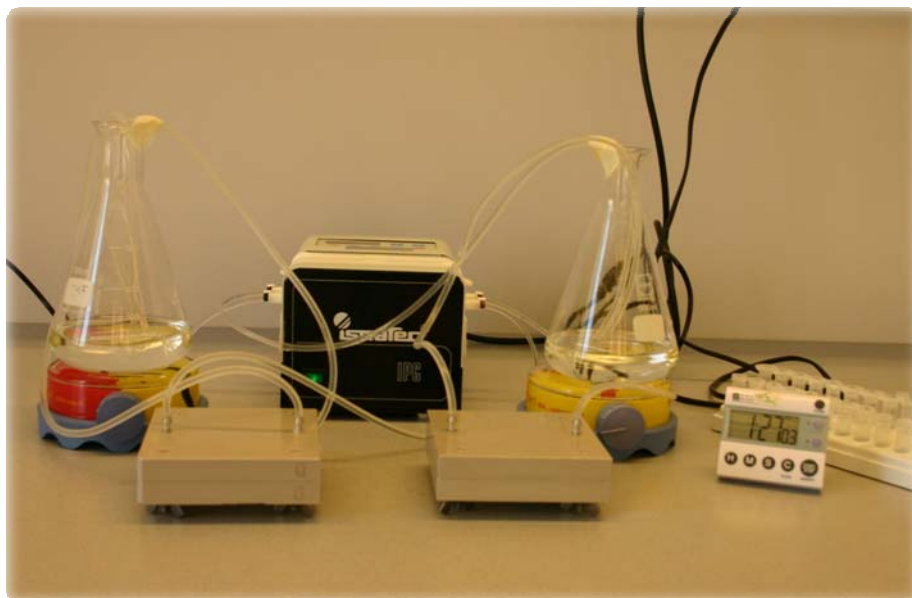
**TABLE 4: STABILITY TESTING UNDER FREEZING CONDITIONS**

Chitosan (concentration; w/w)	Liposomal concentration (w/w, liposomal suspension per total)	Glycerine (w/w)
High molecular weight (2.5%)	0%	0%
High molecular weight (2.5%)	1%	0%
High molecular weight (2.5%)	5%	0%
High molecular weight (2.5%)	10%	0%
High molecular weight (2.5%)	0%	1%
High molecular weight (2.5%)	1%	1%
High molecular weight (2.5%)	5%	1%
High molecular weight (2.5%)	10%	1%

## 4.6. *In vitro* release rate

### 4.6.1. Release from spiral system and collection of samples

Twenty five grams of HMWC (2.5%, w/w) hydrogel with liposomes (10%, w/w) containing chloramphenicol (both entrapped and unentrapped) were applied to the spiral in vitro model (Figure 10). The *in vitro* release model is also called “Freiburger schlange schnecke”. The acceptor consisted of 50 ml of medium chain triglycerides (MCT) and was pumped through the spiral (100 rpm). A polyamide membrane separated the acceptor phase (MCT) from the gel in the spiral.



**FIGURE 11:** THE *IN VITRO* RELEASE SET UP

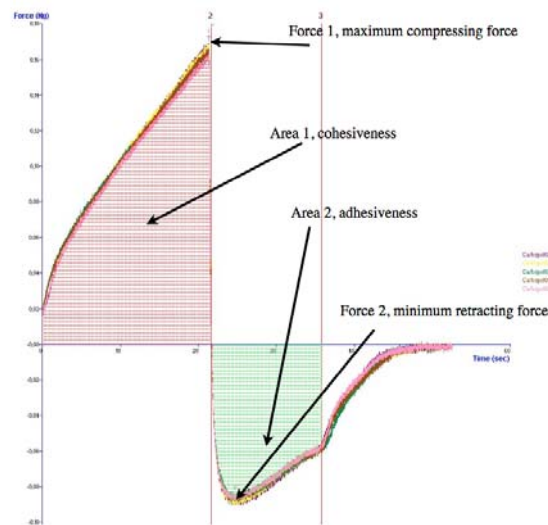
One ml samples were taken from the flow medium after 5, 10, 15, 25, 35, 45, 60, 75, 90, 180 and 300 min. The concentration of chloramphenicol in each sample was determined by HPLC (for details refer to 4.4.3.).

## 5. Results and discussion

### 5.1. Texture analysis

#### 5.1.1. Optimisation of measurement conditions

Although texture analyser is widely used in pharmaceutical and cosmetic industry to evaluate gels and gel like structures, the scientific literature on the use of texture analysis for that purpose is rather limited. At the beginning of the optimization of the measurements, we used two types of hydrogels, namely Carbopol Ultrez and chitosan hydrogels to exclude the effect of the type of gel on the method set up. Carbopol Ultrez based gels are stiff but can be characterized as visco-elastic systems with pseudo plastic and shear thinning behaviour (Fresno-Contreras *et al.*, 2001). Non-Newtonian fluids have typical shear thinning behaviour. Hydrogels that are pseudo plastic would be suitable to be characterized by the texture analysis because of lower mechanical stress in the characterization process than in the methods with more kinetic energy put on the gels.

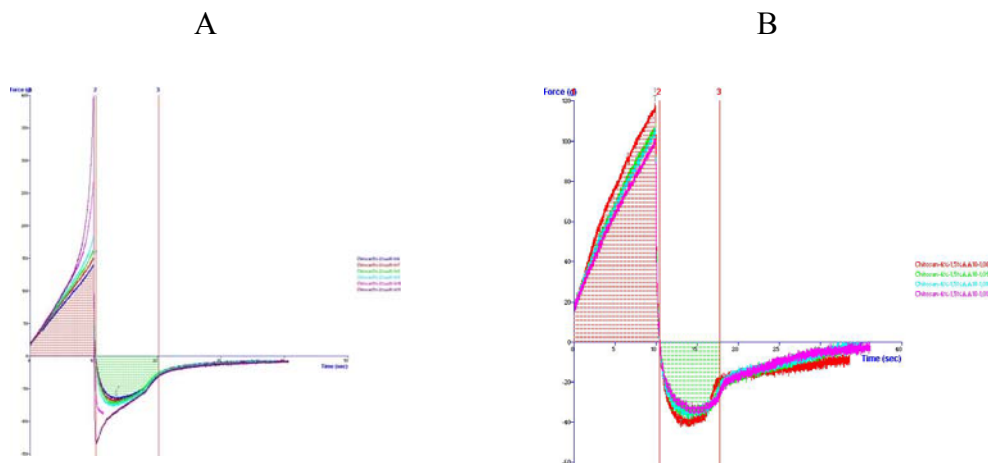


**FIGURE 12:** REPRESENTATIVE EXAMPLE OF MEASUREMENT READING FOR CARBOPOL GEL

The four factors are recorded: Force 1, Area 1, Force 2, and Area 2. Standard deviations from 5 separate readings were all below 2%. The probe used was cylinder type.

By comparing the maximum force, the minimum force, and area under the curve for downward forces on the probe and area for the retraction forces (Figure 12), the insight on the texture properties of the gel can be obtained. Initially the readings were found to vary within 5-10%. We observed that the different beakers used in the measurement, varying the placement for the beaker and the surface of the gel (smoothness) had the direct effect on measurements. Therefore, it was important to fix the beaker to a rack and mark the placement position. Under these more controllable conditions, characterization of Carbopol hydrogels gave reproducible results with standard deviations below 2 %.

Chitosan hydrogels on the other hand were not giving the same measurement reproducibility under the same measurement conditions. Chitosan gels were more fluid like, almost consistence of honey. As a result, the gel started to flow when put under kinetic stress.



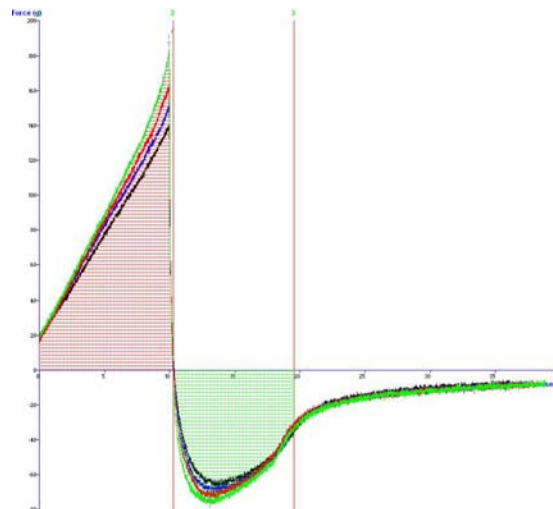
**FIGURE 13:** TYPICAL READING FOR LOW MOLECULAR WEIGHT CHITOSAN

LMWC gel (5%, w/w) Speed: 1 mm/sec, distance 10 mm, 35 mm cylinder probe.

Another important finding was related to the loss of incorporated water. Water evaporates readily from the gel when left in the air. So when thin layers of gel are left on the beaker walls, it dries out in minutes and sticks to the walls. This adherence interferes with

characterization. A thin film will change the surface adherence of the probe and interfere with the results. As seen in Figure 13, forces recorded increased with every run. The left graph (A) shows what happened when the probe was used for repeated runs without any washing or drying during the process. The right graph (B) shows what happened when the probe was washed, but the beaker was not washed or dried during the measurement.

Results improved with thorough washing during the measurement, but the measurement become very time-consuming. Even when the beaker was dried with a tissue paper, and probe washed with water and soap between every run, the forces recorded increased with every run.

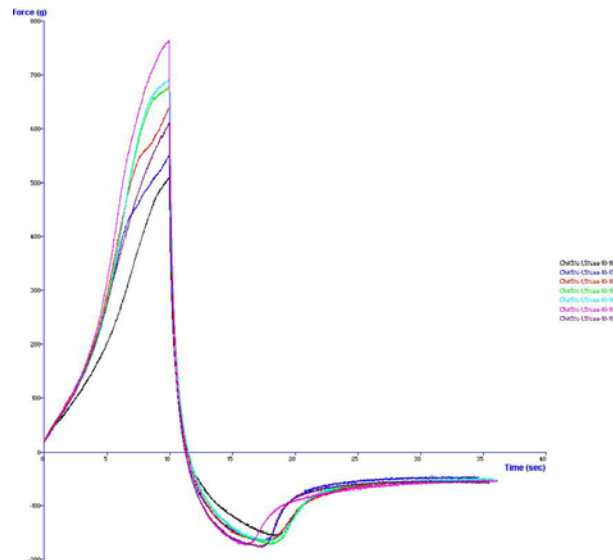


**FIGURE 14:** TYPICAL READING FOR LOW MOLECULAR WEIGHT CHITOSAN

LMWC gel (5%, w/w), Speed: 1 mm/sec, distance 10 mm, 35 mm cylinder probe. The probe was washed and beaker dried between the runs.

In order to further evaluate the effect of water loss on the gel characterization, chitosan gels were put in a thermostat (40 °C) to study the changes in the gel. When left uncovered, water evaporated gradually and the gel turned into more rubber-like form.

Figures 15 and 16 show the same chitosan hydrogel (the same concentration) after one and two weeks in the oven. The adherence-factor with the gel sticking to the walls also increased. The force needed to compress the gel was approximately 5 times more as compared to freshly prepared gel. By comparison, chitosan gels left airtight in the oven became gradually more fluid-like.

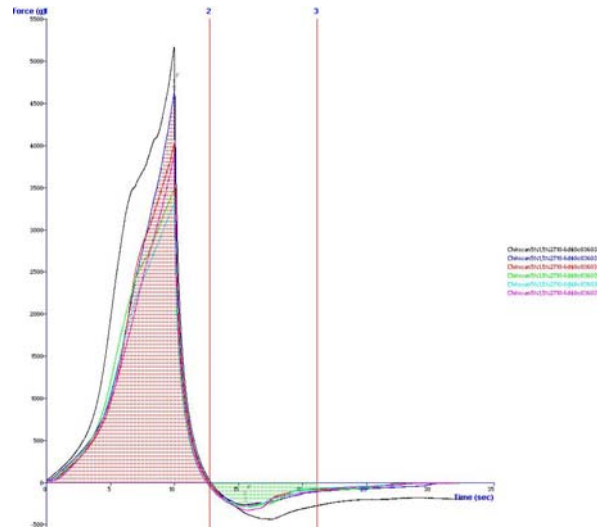


**FIGURE 15:** TYPICAL READING FOR LOW MOLECULAR WEIGHT CHITOSAN

LMWC gel (5%, w/w) stored uncovered at 40 °C for one week. Speed: 1 mm/sec, distance 10 mm, 35 mm cylinder probe.

When the gel was left for 2 weeks in the oven, the force needed to compress the gel was over 4000 g, approximately 25-30 times the force needed before it was put in the oven. Chitosan gels become more rubber-like when water vaporizes.

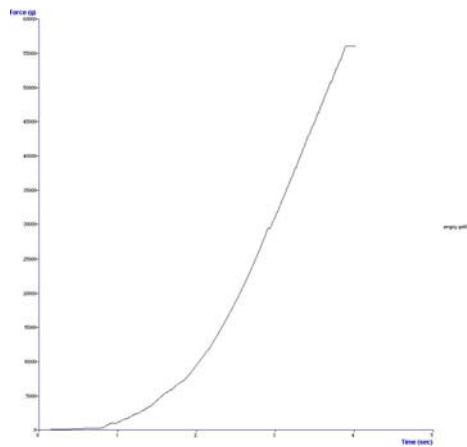




**FIGURE 16:** TYPICAL READING FOR LOW MOLECULAR WEIGHT CHITOSAN

LMWC gel (5%, w/w) stored uncovered at 40 °C for two weeks. Speed: 1 mm/sec, distance 10 mm, 35 mm cylinder probe.

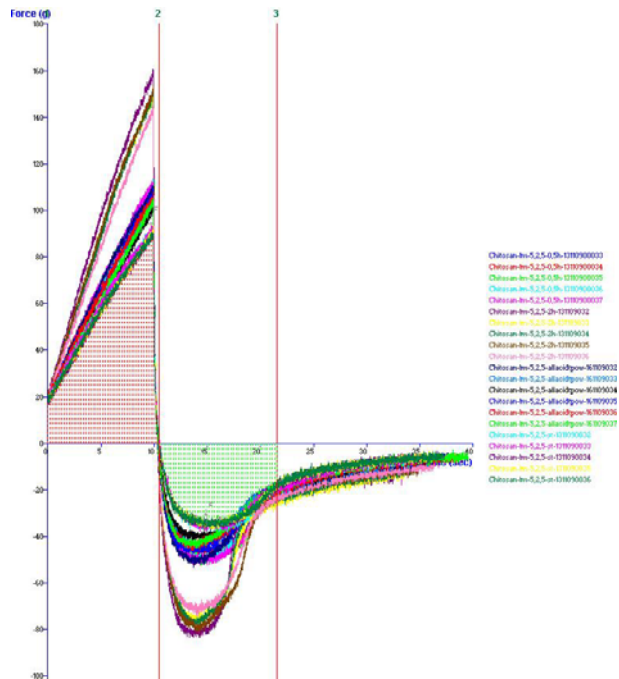
If the chitosan gel was left even longer in the oven, the polymer can transform and obtain plastic properties. Figure 17 shows dried out chitosan. The machine stopped at 55 kg force.



**FIGURE 17:** TYPICAL READING FOR LOW MOLECULAR WEIGHT CHITOSAN UPON DRYING OUT

LMWC gel (6%, w/w) stored uncovered at 40 °C until drying out. Speed: 1 mm/sec, distance 10 mm, 35 mm cylinder probe.

The same concentration of chitosan (LMWC; 5% w/w) was also exposed to air. Although the results are not statistically significant, they can be used as an indication that the exposure to air can increase cohesiveness of the gel (Figure 18 and Figure 19).



**FIGURE 18:** THE EFFECTS OF EXPOSURE TO AIR ON TEXTURE CHARACTERISTICS

LMWC (5%, w/w) were exposed to air for 0, 0.5 and 2 hours. Speed: 1 mm/sec, distance 10 mm, 35 mm cylinder probe.

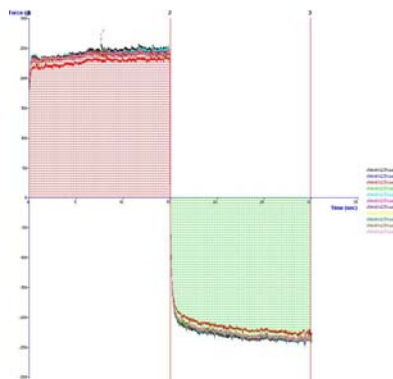
The results with the largest forces (absolute values) represent gels exposed for two hours, the middle forces gels exposed for 0.5 hours, and the lowest forces were not exposed to air more than during preparation of gel (Figure 18).

Chitosan formulations are fluid at low concentrations and get thick and firm when prepared at concentrations close to maximum soluble amount. Considering that the gels were destined to be applied onto skin, we focused on the spreadability of gels as well. The personal evaluation and testing revealed that preferable concentration of chitosan gel for LMWC would be around 6%, MMWC 3.5% and HMWC 2.5% (w/w).

Different concentrations of acetic acid were used for gel preparation during the process of gel optimization. Addition of acetic acid in concentration of 2.5% (w/w) provided the gel pH value of approximately 4.5 (Figure 19). Any lower pH would not be suitable for skin formulations. Yang *et al.* (2009) showed that swelling of chitosan is best at pH right below 4 and is decreasing when gels are approaching neutral conditions. Yang *et al.* (2009) also propose that washing of the prepared gel can neutralize the gel.



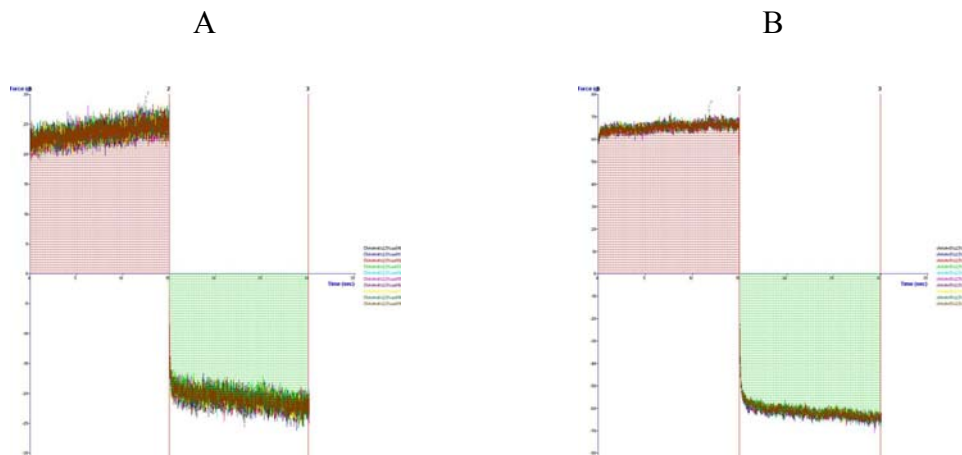
**FIGURE 19:** LMWC (FROM LEFT TO RIGHT: 2.5-7%, W/W) CONTAINING 2.5% (W/W) ACETIC ACID



**FIGURE 20:** TYPICAL READING FOR LMWC (6%, W/W) GEL WITH 2.5% (W/W) ACETIC ACID

The sample was measured with back-extrusion equipment and 40 mm disc. Speed: 1 mm/sec, distance: 15 mm. The beaker was moved between every run.

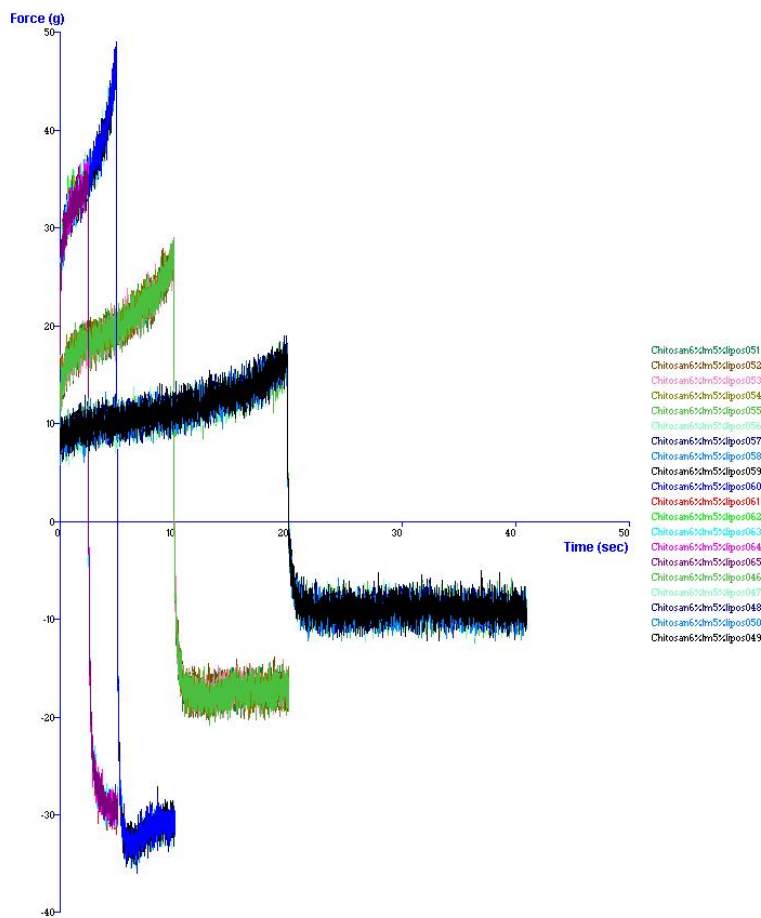
At this stage of method development, the reproducibility of measuring was improving. Back-extrusion disc with special beaker was placed in a mounted track. The disc was submerged into the gel. This minimized adherence to the walls and decreased the elapsed time for testing. Initially 40 mm disc was used. Even if the track for the beaker was controlling the placement, there was some slack. This slack is detectable as seen in Figure 21. The solution was to use 35 mm disc instead. With the smaller disc, decreased force 1 and force 2 was inevitable.



**FIGURE 21:** THE EFFECT OF GEL CONCENTRATION ON TEXTURE MEASUREMENT

LMWC gel (4%; w/w) containing 2.5% (w/w) acetic acid (A) and LMWC gel (5%, w/w) containing 2.5% (w/w) acetic acid (B) were measured. Speed: 1 mm/sec, distance: 15 mm and 40 mm disc (back extrusion).

The next step in development of texture analysis method to be used in characterization of chitosan gels was to determine the optimal speed and distance the probe should travel into the gel. The method should be carried out as fast as possible without jeopardizing the accuracy and variation. The method should also be applicable to various kinds of chitosan gels. Figure 22 represents the effect of different forces and different probe speeds on measurements.



**FIGURE 22:** THE EFFECT OF DIFFERENT FORCES AND DIFFERENT PROBE SPEEDS ON MEASUREMENTS

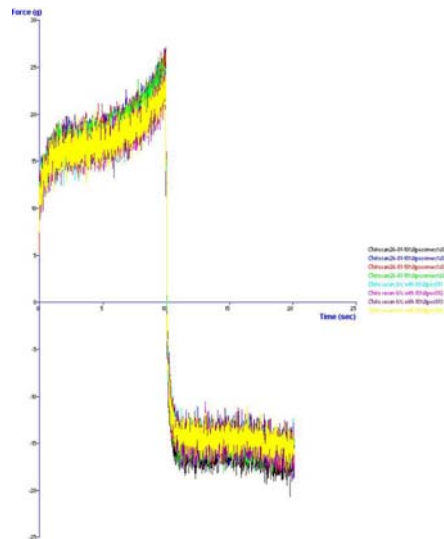
The gel used was LMWC gel (6%; w/w) containing 2.5% (w/w) acetic acid and 5% liposomal dispersion (w/w of total). Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm. Speed: Black: 0.5 mm/sec, green: 1 mm/sec, blue: 2 mm/sec and purple: 4 mm/sec.

Different speeds yield different areas and forces. Faster probe speed gives increased force, but also decreased elapsed time. Total run time at 5 seconds and 20 seconds were found equally good, but 5 seconds was preferable because the measurement was faster. The initial method was using percentage of minimum force in the macro for calculating area. The new method for chitosan gels involved elapsed time instead. Method was now

found to be reproducible. Standard deviation was varying within less than 2%, and typically less than 1%.

One precaution to be considered in regard to the newly established method was the fact that when the disc approaches the bottom, the force rises. This effect also increases with higher probe speeds. Disc further away from the surface gives a flatter curve response, especially with thicker (more viscous) gels. The working scope of the disc should be with good clearance to the gel surface and the bottom. The amount of gel used in measurement was found to be optimal if around 50-60 g was used.

### 5.1.2. Established method (summary)



**FIGURE 23:** TYPICAL MEASUREMENT PROFILE AFTER METHOD ESTABLISHMENT

Comparison of 3+3 runs in two batches of LMWC gel (6%; w/w) with 2.5% (w/w) acetic acid and 10% liposomal dispersion (w/w per total) measured with Texture analyser, back-extrusion equipment and 35 mm disc. Speed: 1 mm/sec, distance: 10 mm.

Figure 23 and Table 5 show two batches of LMWC gel (6%, w/w) with 10% (w/w) added liposomal dispersion. They seem quite similar at first glance (Figure 23). But p-values shown in Table 5 reveal that the two batches are statistically different when comparing force 1, area 1 or area 2 with p-values between 0.00003-0.0014. Force 2 has a p-value of 0.241. P-values less than 0.05 indicate that there is less than 5% probability that the measured difference between the sample-sets are caused by coincidences. Even with small forces recorded, the method is capable of distinguishing between small variations within the tested gels. The method can maintain this reproducibility only when the same amount of gel is measured in each sample. Only then can the method differentiate between different structures and viscosity. The method can be used to examine differences between parameters involved in preparation methods and various raw materials (composition).

**TABLE 5:** COMPARISON OF TWO BATCHES OF LIPOSOMAL LMWC GELS

<b>Batch 1/run</b>	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2 (g)</b>	<b>Area 2 (g·sec)</b>
1	25.074	175.286	17,802	140.111
2	25.509	174.781	18.670	141.736
3	25.291	174.590	18.019	141.716
Ave.	25.291	174,885	18,164	141.188
SD	0.177	0.294	0.369	0.761
CV	0.701	0.168	2.031	0.539
<b>Batch 2/run</b>	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2 (g)</b>	<b>Area 2 (g·sec)</b>
1	27.137	187.314	18.779	147.147
2	27.028	186.965	18.562	147.874
3	26.594	186.407	18.344	148.827
Ave.	26.920	186.895	18.562	147.949
SD	0.234	0.374	0.177	0.688
CV	0.871	0.200	0.955	0.465
P-value	0.0014	0.00003	0.241	0.0007

Comparison of 3+3 runs in two batches of LMWC gel (6%, w/w) containing 2.5% (w/w) acetic acid and 10% liposomal dispersion (w/w of total) measured with back-extrusion equipment and 35 mm disc. Speed: 1 mm/sec, distance: 10 mm. The standard deviation (SD) from the 3 runs was calculated as well as the variation coefficient (VC). VC value represents the percentage of how much SD varies from the average values. P-values are calculated for comparison.

## 5.2. Stability testing

### 5.2.1. Accelerated stability testing

#### Low molecular weight chitosan hydrogels prior to testing

**TABLE 6: LMWC GELS PRIOR TO ACCELERATED STABILITY TESTING**

<b>A: LMWC gel (6.0%, w/w) without liposomes</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	170.267	337.991	123.136	268.044
SD	0.802	2.401	0.271	1.422
VC	0.471	0.710	0.220	0.531
<b>B: LMWC gel (6.0%, w/w) with 1% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	165.903	332.501	119.966	260.935
SD	0.483	2.138	0.715	1.192
VC	0.291	0.643	0.596	0.457
<b>C: LMWC gel (6.0%; w/w) with 5% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	123.005	246.709	89.074	200.165
SD	0.612	0.907	0.427	0.691
VC	0.497	0.368	0.479	0.345
<b>D: LMWC gel (6.0%, w/w) with 10% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	96.996	202.006	75.243	165.778
SD	0.646	0.910	0.359	0.610
VC	0.666	0.451	0.478	0.368
<b>D: LMWC gel (6.0%, w/w) with 15% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	74.00	161.068	61.784	135.415
SD	0.572	0.899	0.187	0.571
VC	0.766	0.558	0.302	0.422

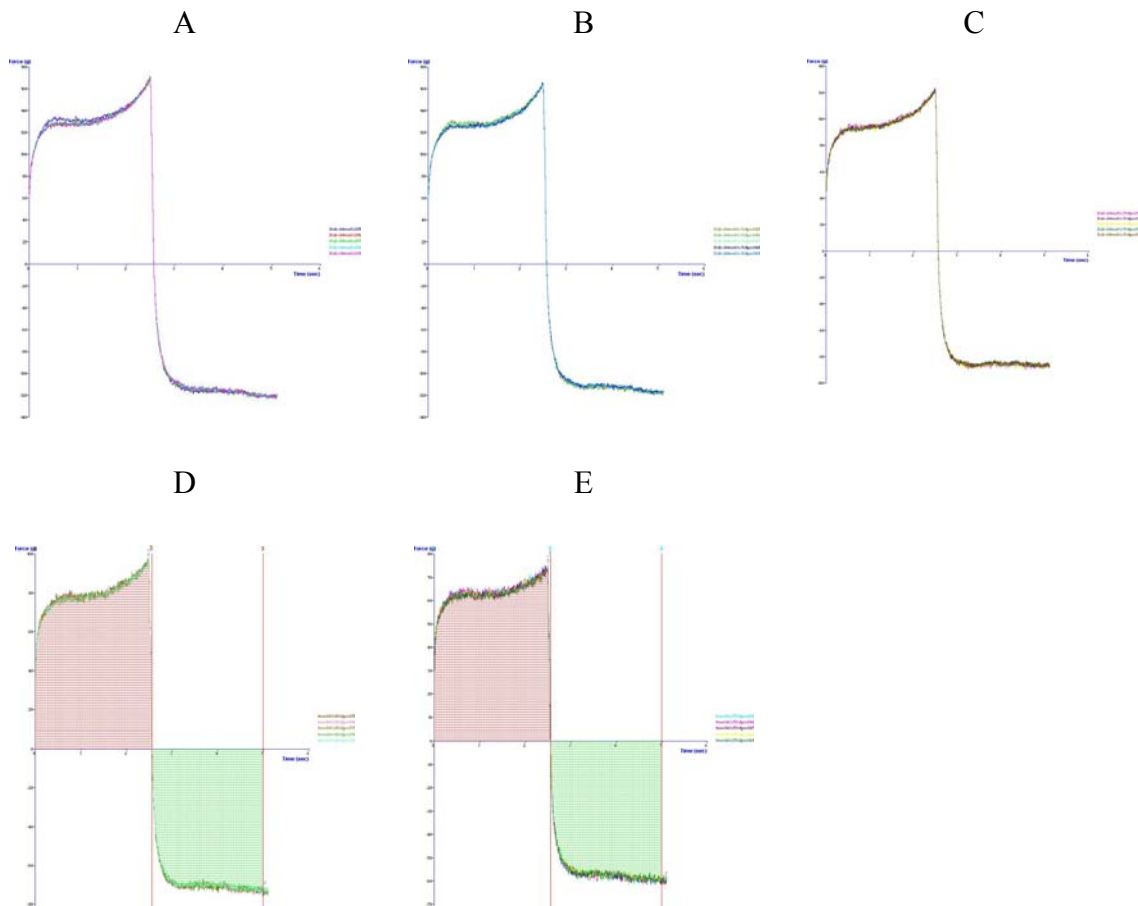
The Table 6 shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. The standard deviation (SD) from the 5 runs is calculated as well as the variation coefficient (VC). Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed: 4 mm/sec.

All chitosan preparations were found to be less cohesive after one month storage in the oven (40 °C). MMWC gels were found to remain of similar viscous properties after one month storage at elevated temperature. However, the MMWC were also the most cohesive before the test started. The HMWC are expected to be more stable. However,



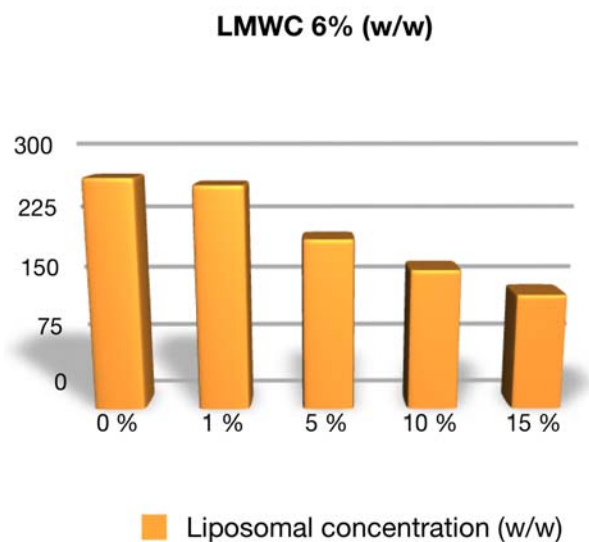
we need to mention that the degree of deacetylation was not considered when evaluating the stability in this study.

As shown in Figure 24, the texture property of chitosan gels is highly dependent on the concentration of chitosan used and the dilution of original gel by incorporation of liposomal suspension has direct effect on the measurement.



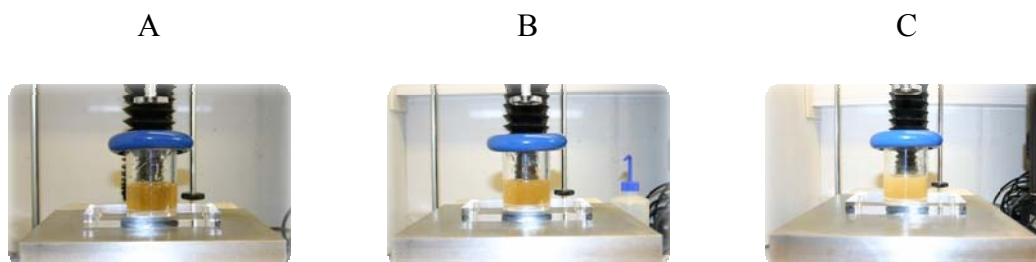
**FIGURE 24:** THE EFFECT OF THE ADDITION OF LIPOSOMAL DISPERSION TO LMWC GEL

LMWC gel (6%, w/w) containing 2.5% (w/w) acetic acid and 0-15% liposomal dispersion (w/w of total). Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec. A: No liposomes; B: 1% liposomes (w/total), C: 5% liposomes (w/total); D: 10% liposomes(w/total); E: 15% liposomes (w/total).



**FIGURE 25:** THE EFFECT OF LIPOSOMES ON THE AREA 2 VALUES

Moreover, we could visually observe the effect of added liposomes on gel property. The colour and the appearance changed as can be seen in Figure 26.



**FIGURE 26:** THE CHANGE IN LMWC GEL APPEARANCE IN RELATION TO INCORPORATION OF LIPOSOMES.

A: No liposomes; B: 1% liposomes (w/total), C: 5% liposomes (w/total)

The LMWC gels have a more brownish colour than the MMWC and HMWC gels. With incorporated liposomes the gels get increasingly more clouded and lighter in colour.

With the increase in liposomal concentration incorporated in hydrogels, the area 2 decreases in an approximate linear manner. Table 7 and Figure 25 indicate that the ratio

between force 2 and area 2 is roughly half in comparison between 15% liposomal gel and gel without liposomes (empty). The ratio between area 1 and force 1 is less than half for 15% liposomal gel versus empty gel. This can indicate that cohesive effect is more affected by liposomes than adhesive effect. The consequence of adding liposomes is the lowering of gel viscosity.

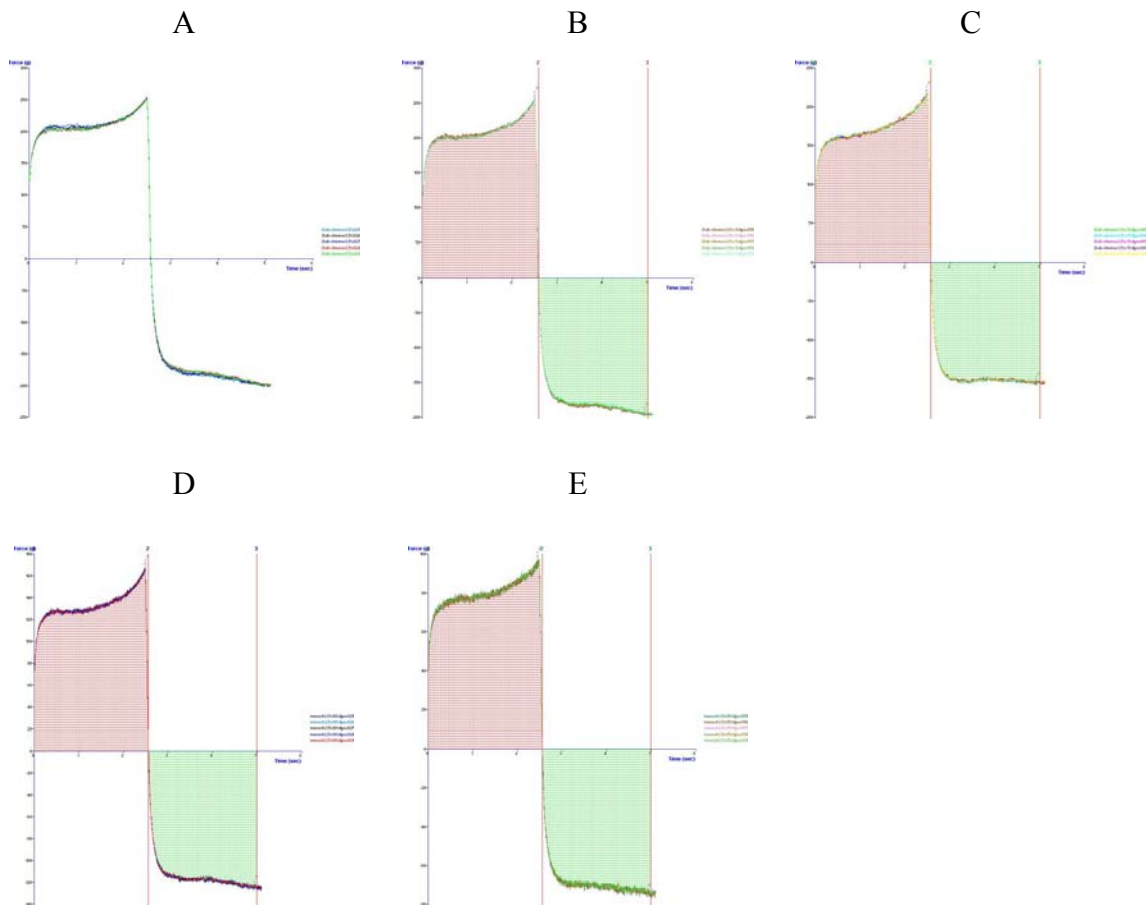
### Medium molecular weight chitosan hydrogels prior to testing

**TABLE 7: MMWC BEFORE ACCELERATED STABILITY TEST**

<b>A: MMWC gel (3.5%, w/w) without liposomes</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	253.132	534.868	201.767	427.788
SD	1.141	2.863	0.649	3.796
VC	0.451	0.535	0.322	0.887
<b>B: MMWC gel (3.5%, w/w) with 1% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	256.149	531.208	197.490	426.571
SD	1.303	1.589	0.488	2.730
VC	0.509	0.299	0.247	0.640
<b>C: MMWC gel (3.5%, w/w) with 5% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	216.942	436.780	157.762	355.567
SD	0.624	0.610	0.702	1.060
VC	0.288	0.140	0.445	0.298
<b>D: MMWC gel (3.5%, w/w) with 10% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	166.963	337.789	127.041	276.850
SD	0.701	0.417	0.521	0.638
VC	0.420	0.123	0.410	0.230
<b>D: MMWC gel (3.5%, w/w) with 15% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	97.169	202.584	76.589	165.777
SD	0.242	0.185	0.189	0.380
VC	0.249	0.091	0.247	0.229

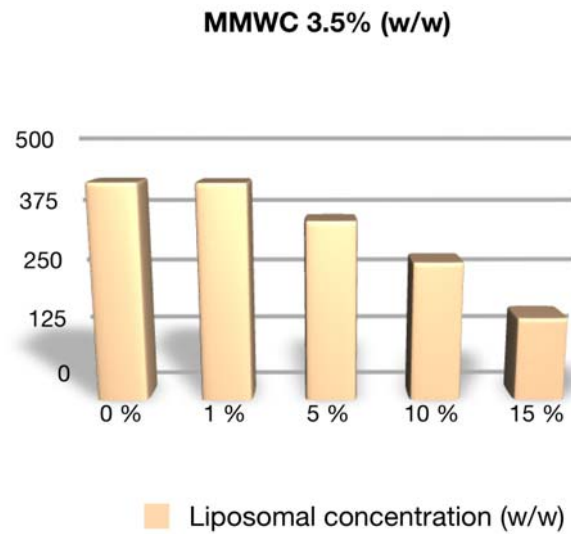
The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.

If LMWC and MMWC gels are compared, the values for empty gels are higher for MMWC. With 15% liposomes (w/total) added to the gel, the values are still higher but the ratio is smaller. The area 2 for empty LMWC gel is 62% of the area 2 for MMWC gel. But with 15% liposomes added to both types of gel, the area 2 for LMWC gel is 81% of area 2 for MMWC gel. This indicates that LMWC can withstand higher concentrations of liposome dispersion added. But then again it needs to be prepared at higher concentrations to obtain desired texture properties.



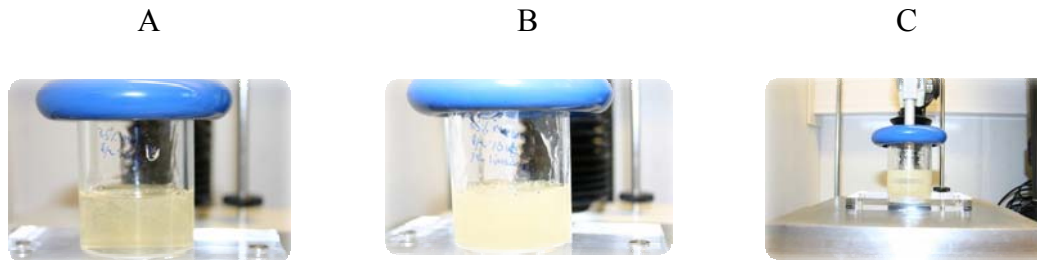
**FIGURE 27:** THE EFFECT OF THE ADDITION OF LIPOSOMAL DISPERSION TO MMWC GEL

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 10% (w/total) liposomes; E: 15% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.



**FIGURE 28:** THE EFFECT OF INCORPORATED LIPOSOMES ON THE AREA 2 VALUES

The equation  $y = -17,555x + 439.64$  with  $R^2 = 0.99089$  can be used to prove that the addition of liposomes to MMWC gels decreases the viscosity in a linear manner.



**FIGURE 29:** THE CHANGE IN MMWC GEL APPEARANCE IN RELATION TO INCORPORATION OF LIPOSOMES.

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes

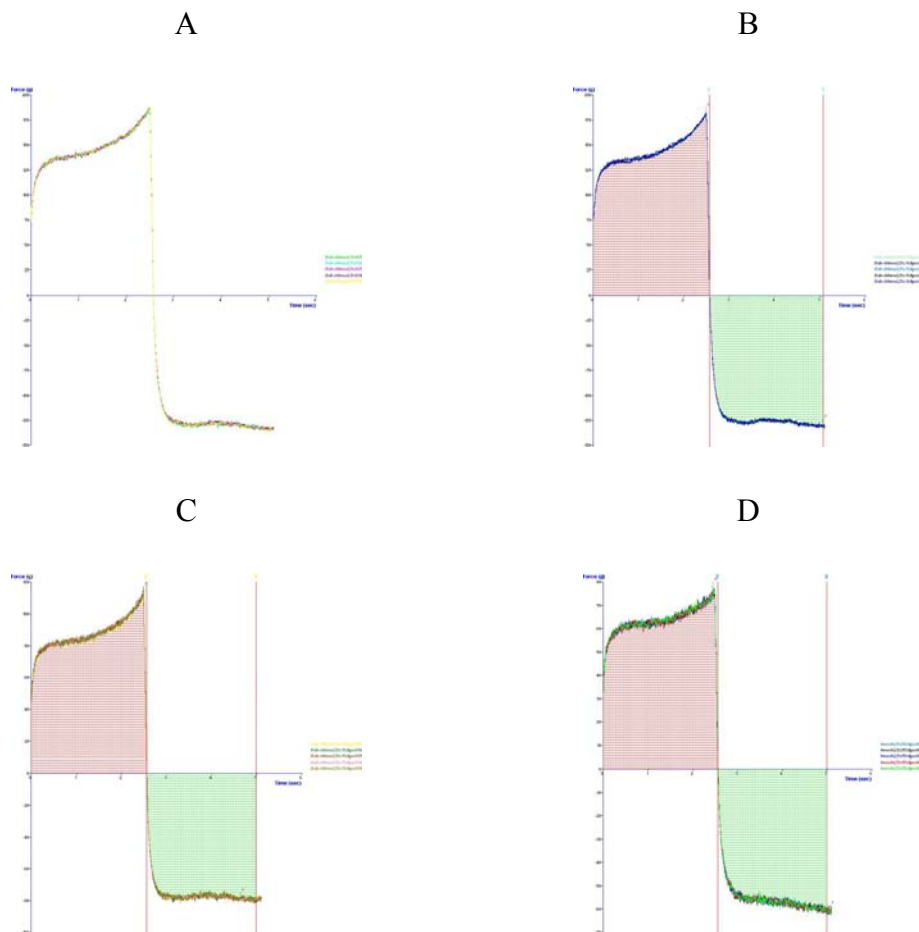
## High molecular weight chitosan hydrogels prior to testing

Empty HMWC gel (2.5%, w/w) appears to be more viscous than empty LMWC gel (6%, w/w). When both gels contain 15% (w/w) liposomal dispersion the consistency appears to be the same. As HMWC gel can be prepared at lower concentrations than both LMWC and MMWC gels, its acceptance limit for incorporation of liposomes can be higher.

**TABLE 8: HMWC GELS BEFORE ACCELERATED STABILITY TESTING**

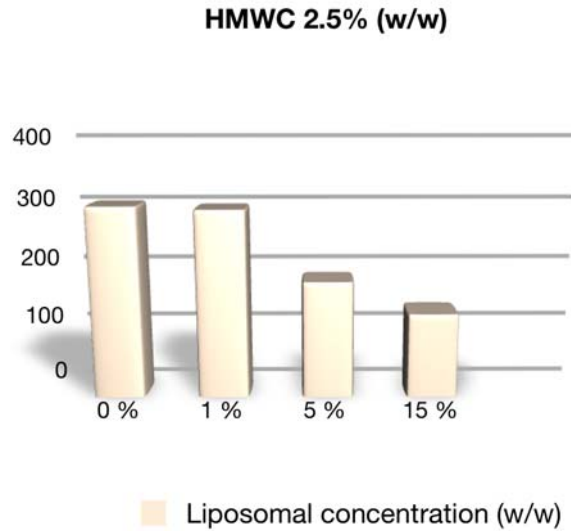
<b>A: HMWC gel (2.5%, w/w) without liposomes</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	188.155	373.192	135.640	301.060
SD	0.958	0.154	0.552	0.411
VC	0.509	0.041	0.407	0.136
<b>B: HMWC gel (2.5%, w/w) with 1% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	181.643	363.914	132.775	295.666
SD	1.411	0.973	0.174	0.294
VC	0.777	0.267	0.131	0.099
<b>C: HMWC gel (2.5%, w/w) with 5% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	113.301	221.628	81.714	183.522
SD	1.766	1.229	0.511	0.220
VC	1.558	0.554	0.625	0.120
<b>D: HMWC gel (2.5%, w/w) with 15% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	76.437	161.893	62.478	133.320
SD	0.653	0.451	0.053	0.388
VC	0.854	0.278	0.085	0.291

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.

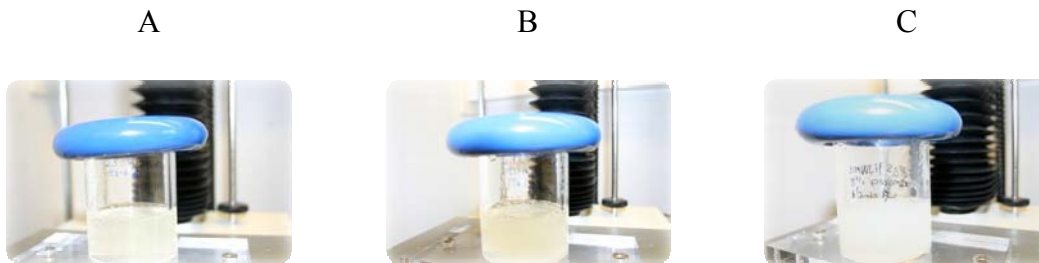


**FIGURE 30:** THE EFFECT OF THE ADDITION OF LIPOSOMAL DISPERSION TO HMWC GEL

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 15% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.



**FIGURE 31:** THE EFFECT OF LIPOSOMES ON THE AREA 2 VALUES



**FIGURE 32:** THE CHANGE IN HMWC GEL APPEARANCE IN RELATION TO INCORPORATION OF LIPOSOMES.

A: No liposomes; B: 1% (w/total) liposomes; C: 5% (w/total) liposomes

The HMWC gel is clear in appearance when empty, but got more clouded when liposomes were incorporated. With 5% (w/w) or higher liposomal dispersion concentrations, the HMWC gel appeared white and opaque.



### **Low molecular weight chitosan hydrogels after one month storage at 40 °C**

There are no literature data available (up to the best of our knowledge) on the stability of liposomal chitosan gels. Several research groups studied to chitosan coated liposomes and their stability.

Wang *et al.* (2010) studied the stability of chitosan anchored liposomes (CALs) and chitosan coated liposomes (CCLs). They proved that plain liposomes, CALs and CCLs are stable for 30 days when stored at 4 °C. When stored for 30 days at 25 °C, the plain liposomes showed fusion of liposomal particles, while the CALs and CCLs showed no changes in the sizes. Both the sizes of CALs and CCLs increased after 30 days at 37 °C. This proved that chitosan coated liposomes can be stored at room temperature or at 4 °C for a month, but not necessarily at 40 °C.

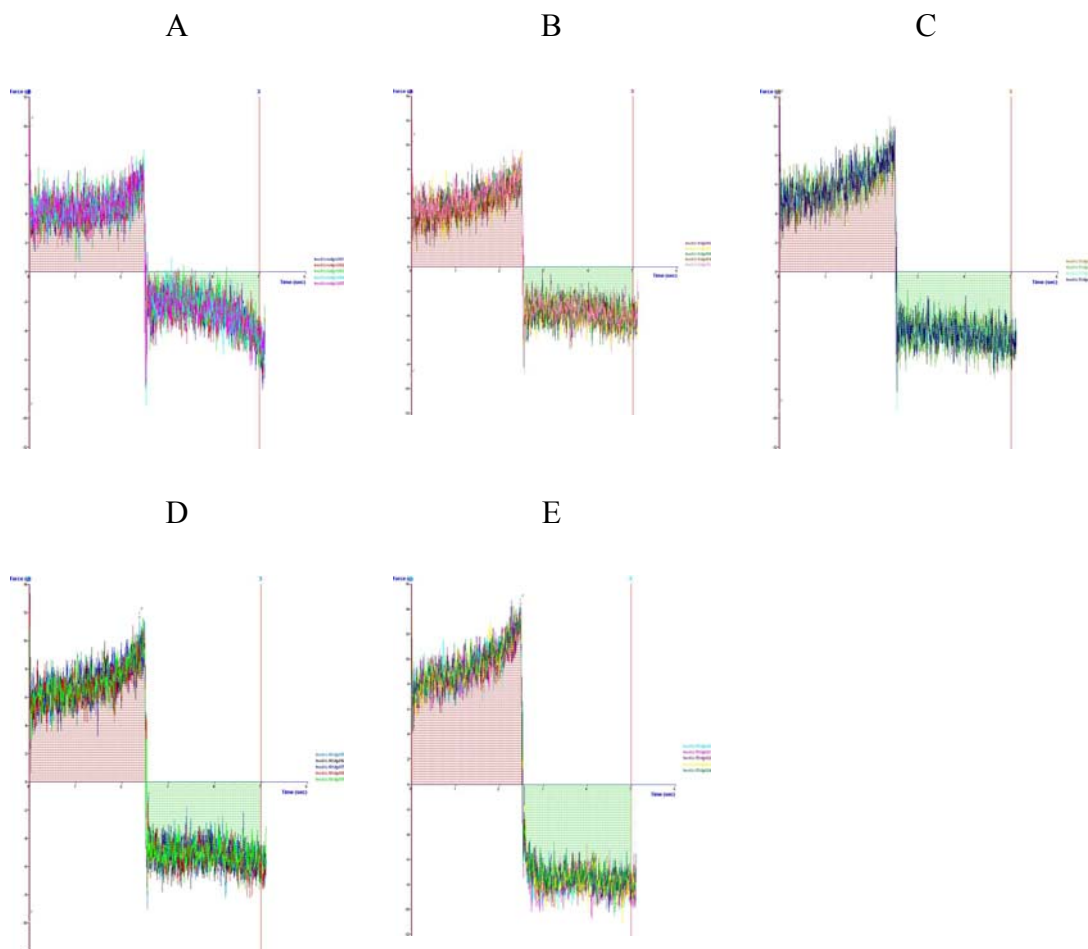
Hafner *et al.* (2009) studied melatonin-loaded lecetin/chitosan nanoparticles. They examined the changes of drug loading, size, polydispersity and zeta-potential over a two months period at 4 °C. They proved that the most stable preparations were the largest nanoparticles with the highest chitosan concentrations.

**TABLE 9: LMWC GELS AFTER ACCELERATED STABILITY TEST**

<b>A: LMWC gel (6.0%, w/w) without liposomes</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	9.878	0.049	10.030	4.744
SD	0.400	0.009	1.121	0.238
VC	4.053	18.561	11.176	5.007
<b>B: LMWC gel (6.0%, w/w) with 1% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	10.269	0.046	10.334	4.271
SD	1.134	0.005	0.506	0.668
VC	11.038	11.230	4.900	15.650
<b>C: LMWC gel (6.0%, w/w) with 5% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	11.181	0.044	10.340	4.359
SD	0.837	0.006	0.835	0.373
VC	7.489	14.754	8.077	8.553
<b>D: LMWC gel (6.0%, w/w) with 10% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	12.071	0.033	9.183	4.702
SD	0.688	0.009	0.695	0.379
VC	5.699	26.784	7.572	8.050
<b>D: LMWC gel (6.0%, w/w) with 15% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	14.242	0.024	10.508	5.237
SD	0.286	0.007	0.385	0.490
VC	2.011	29.910	3.661	9.363

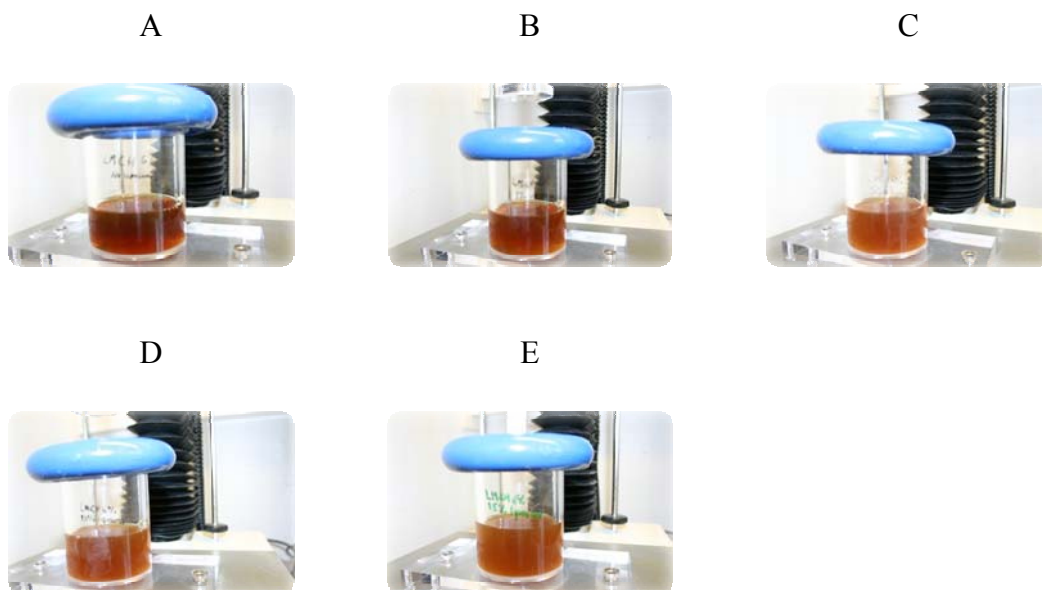
The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.

LMWC liposomal gels failed the accelerated stability test. The cohesiveness was very low. The results presented in Table 9 cannot be scientifically evaluated. Next step would be to find out actually the time point when the gels start to degrade. As both the Table 9 and Figure 33 shows the HMWC gels were almost water like in consistence. All gels were destroyed and appeared as liquid formulations.



**FIGURE 33:** LIPOSOMAL LMWC GELS AFTER ACCELERATED STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 10% (w/total) liposomes; E: 15% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.



**FIGURE 34:** THE APPEARANCE OF LIPOSOMAL LMWC GELS AFTER STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w7total) liposomes; D: 10% (w7total) liposomes; E: 15% (w/total) liposomes

There were little differences between the appearance of liposomal hydrogels and the empty gel. The colour went from light brown to red-brown and gel became completely unclear.

## Medium molecular weight chitosan hydrogels after one month storage at 40 °C

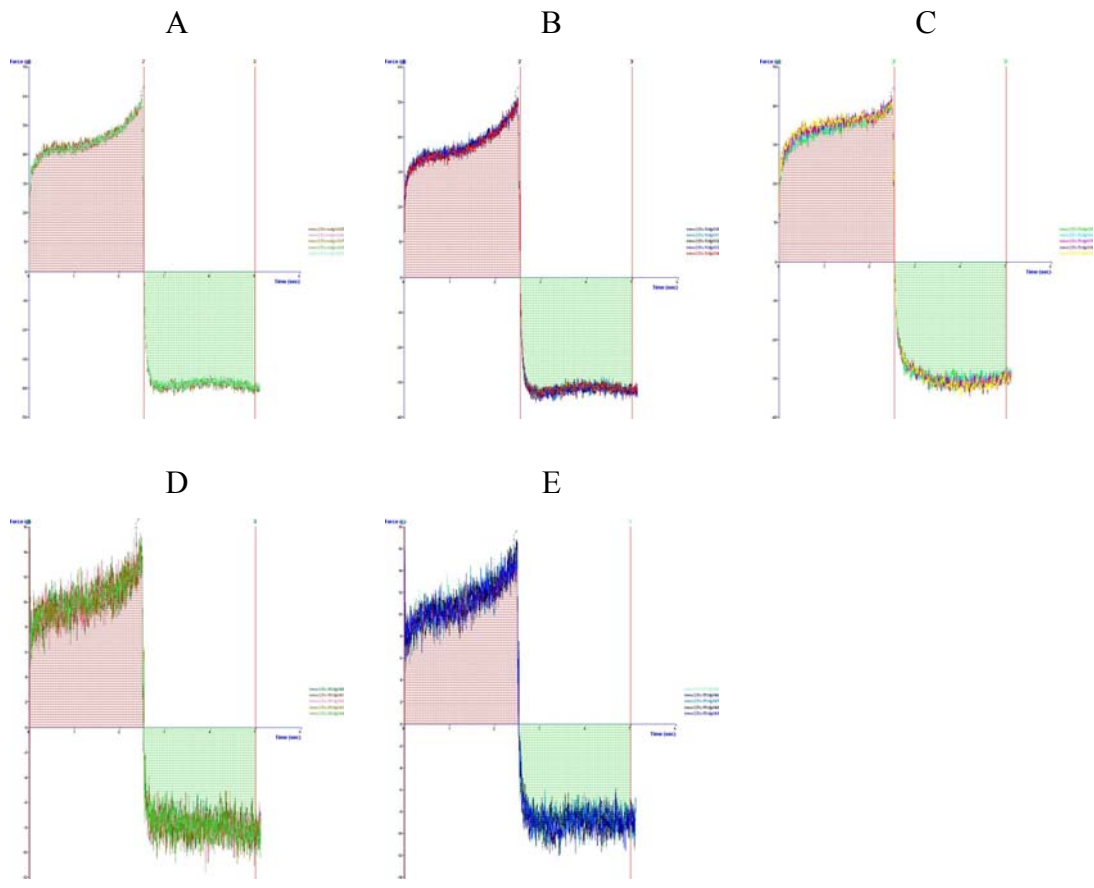
**TABLE 10: MMWC GELS AFTER ACCELERATED STABILITY TEST**

<b>A: MMWC gel (3.5%, w/w) without liposomes</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	59.226	113.041	42.075	93.310
SD	0.593	0.721	0.288	0.530
VC	1.001	0.638	0.685	0.568
<b>B: MMWC gel (3.5%, w/w) with 1% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	51.019	95.132	35.149	77.191
SD	0.275	0.690	0.379	0,566
VC	0.538	0.725	1.077	0,734
<b>C: MMWC gel (3.5%, w/w) with 5% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	4.836	87.554	33.977	71.479
SD	0.427	1.209	0.697	0.628
VC	1.020	1.380	2.051	0.878
<b>D: MMWC gel (3.5%, w/w) with 10% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	15.219	0.022	10.964	6.578
SD	0.346	0.012	0.418	0.436
VC	2.274	56.416	3.809	6.627
<b>D: MMWC gel (3.5%; w/w) with 15% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g-sec)</b>	<b>Force 2</b>	<b>Area 2 (g-sec)</b>
Ave	16.413	0.001	11.810	6.585
SD	0.397	0.020	0.221	0.316
VC	2.417	3657.321	1.875	4.806

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.

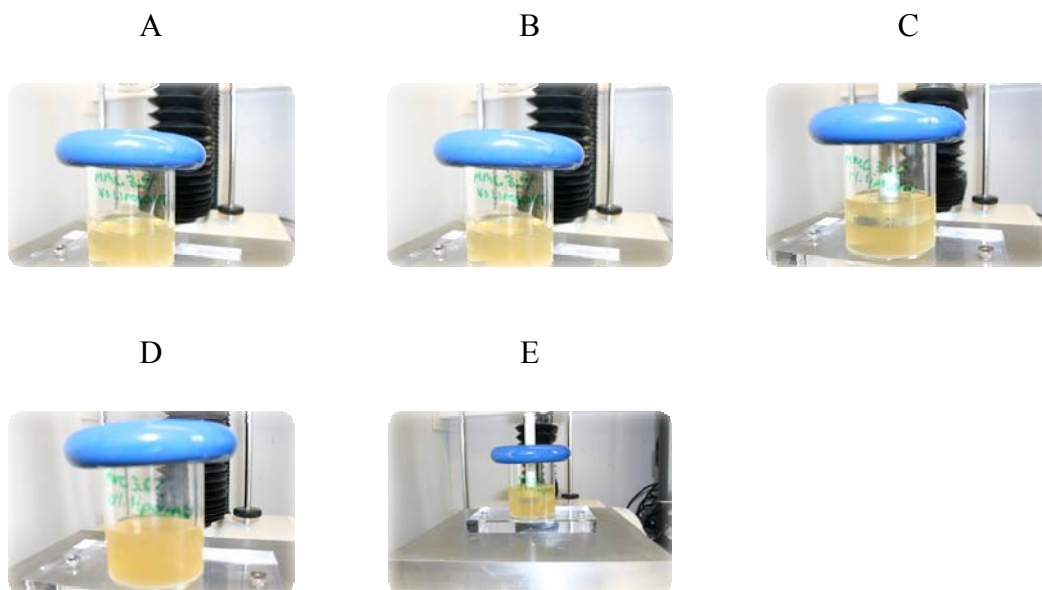
The MMWC liposomal gels performed better during the accelerated stability test. The empty gel, 1% (w/w) liposomal gel and the 5% (w/w) liposomal gel were firm and maintained the original consistency during the accelerated stability testing.

The Figure 35 also shows that first three gels maintain most of the original degree of cohesiveness and adherence.



**FIGURE 35:** LIPOSOMAL MMWC GELS AFTER ACCELERATED STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 10% (w/total) liposomes; E: 15% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.



**FIGURE 36:** THE APPEARANCE OF LIPOSOMAL MMWC GELS AFTER STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 10% (w/total) liposomes; E: 15% (w/total) liposomes

The MMWC gels appeared to be slightly darker yellow in colour than prior to the stability testing. Two possible suggestions for the decomposition of the hydrogels can be liposomal disintegration or chitosan breakdown. It would be interesting to compare the 10% and 15% MMWC liposomal hydrogels with adjusted chitosan concentration after added liposomes. If gels were prepared at a higher concentrations adjusting for the liposomal concentration, lesser decomposition might occur. The colour after stability test was a little closer to the LMWC gels before the test. When considering that the colour of the MMWC gels appeared more like the colour of LMWC gels, one might suggest that a degradation of the medium chain chitosan molecules.

## High molecular weight chitosan hydrogels after one month storage at 40 °C

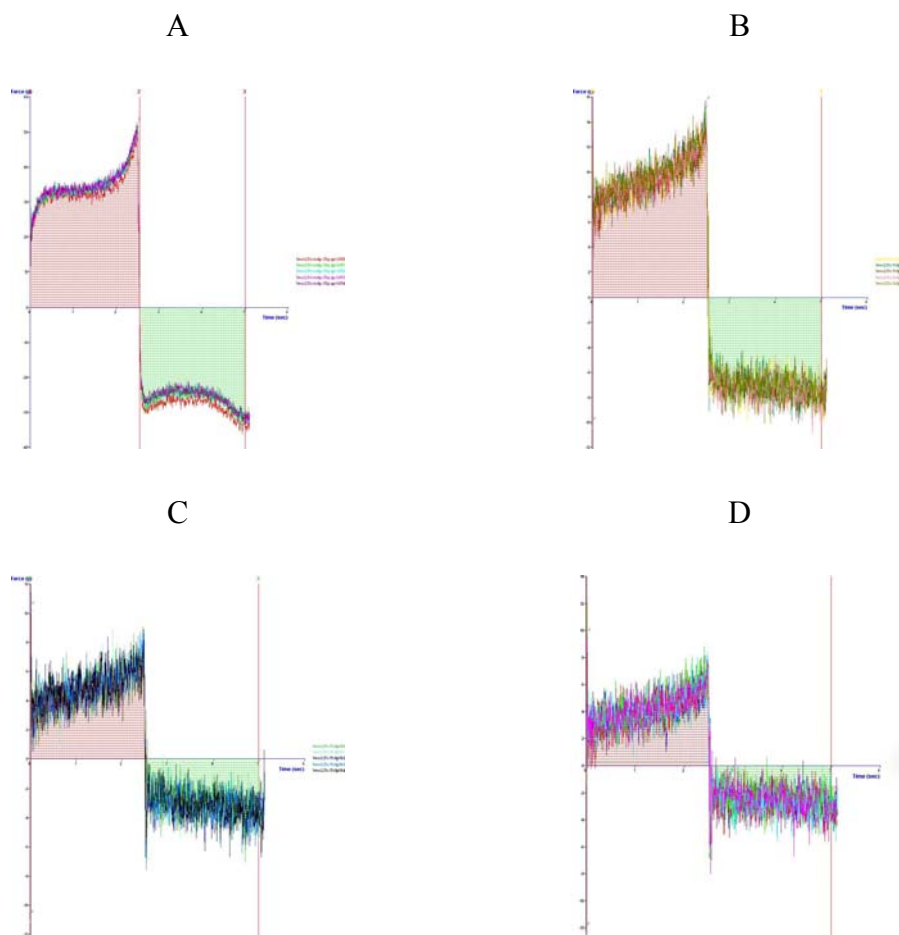
**TABLE 11: HMWC GELS AFTER ACCELERATED STABILITY TEST**

<b>A: HMWC gel (2.5%, w/w) without liposomes</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	51.844	87.586	33.933	63.484
SD	0.745	1.602	1.170	2.688
VC	1.436	1.829	3.449	4.234
<b>B: HMWC gel (2.5%, w/w) with 1% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	15.024	0.024	10.312	6.610
SD	0.312	0.007	0.370	0.353
VC	2.074	28.418	3.585	5.347
<b>C: HMWC gel (2.5%, w/w) with 5% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	9.987	0.052	10.920	4.393
SD	0.783	0.005	0.769	0.339
VC	7.838	9.080	7.046	7.725
<b>D: HMWC gel (2.5%, w/w) with 15% (w/w) liposomes in gel</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	9.813	0.062	11.767	4.040
SD	1.286	0.006	0.577	0.512
VC	13.100	9.669	4.902	12.677

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.

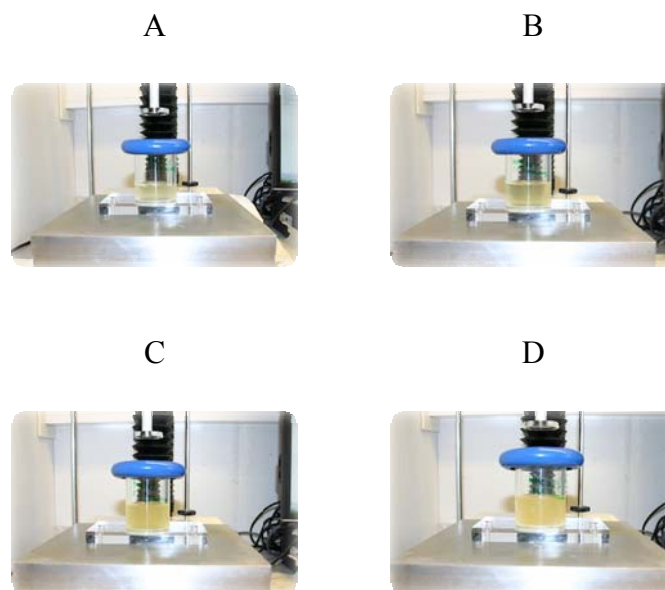
As can be seen in Table 11 and Figure 37, the empty HMWC gels remain to keep some of the gel matrix intact after the stability test. Moreover, HMWC gels can be prepared at higher concentrations than 2.5% (w/w) so that this type of hydrogels have the potential to be even more stable and more widely applied.





**FIGURE 37:** LIPOSOMAL MMWC GELS AFTER ACCELERATED STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 15% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.



**FIGURE 38:** THE APPEARANCE OF LIPOSOMAL HMWC GELS AFTER STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes; D: 15% (w/total) liposomes

HMWC liposomal gels become more yellow after the stability test. However, empty gel (without liposomes) hardly changed its original colour.

### **5.2.2. Accelerated stability testing of chitosan hydrogels containing glycerine**

It is well known fact that glycerine can prevent dehydration of various gel types (Mourtas *et al.*, 2008). In our case as well, glycerine was added to prevent water evaporation seen on the (para-film) cover used to prevent dehydration of the gel. Glycerine is also known to function as a co-solvent for incorporated active ingredients. HMWC was found to be the most suitable gelling agent among the tested chitosan types. HMWC gel was prepared with 1% glycerine and liposomes incorporated as can be seen in Table 12. In the case of

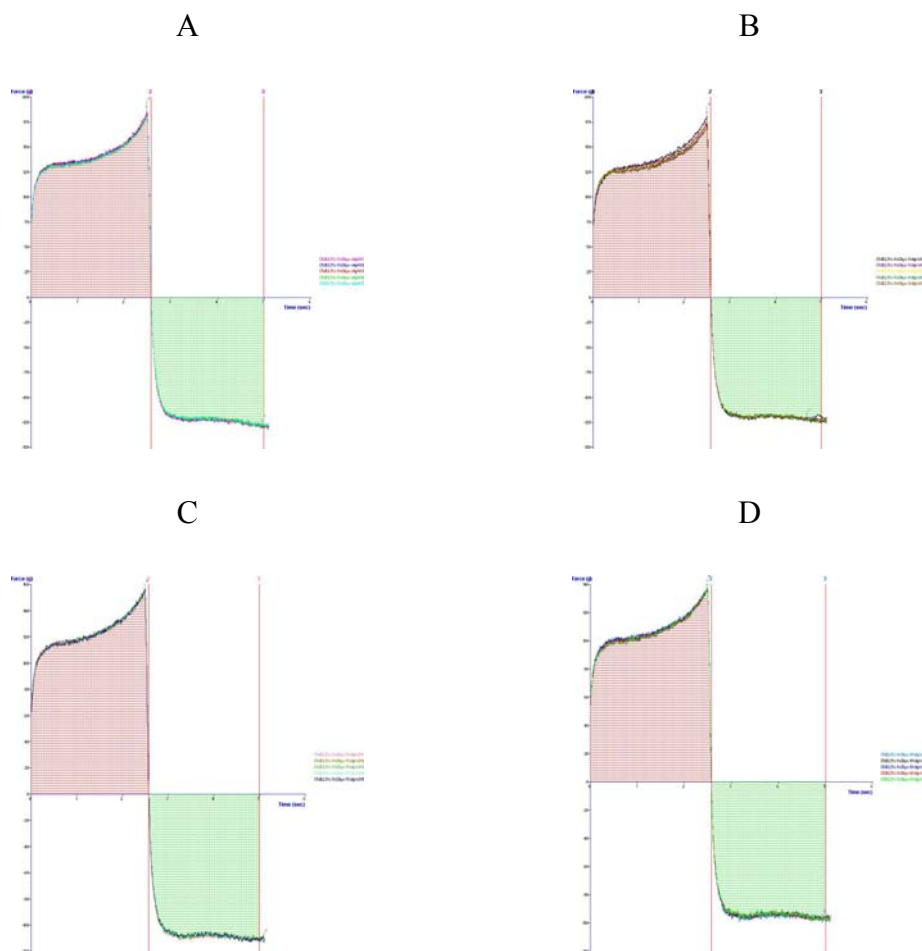
empty gel, the glycerine slightly lowered the gel cohesiveness. When comparing Table 12 and Table 13, the effect of added glycerine is apparent.

**TABLE 12: HMWC GELS CONTAINING GLYCERINE BEFORE ACCELERATED STABILITY TESTING**

<b>A: HMWC gel (2.5%, w/w) without liposomes, with 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	183.170	359.149	130.892	285.028
SD	1.893	1.979	0.674	1.392
VC	1.034	0.551	0.515	0.488
<b>B: HMWC gel (2.5%, w/w) with 1% (w/w) liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	174.725	345.655	125.312	278.140
SD	3.432	4.101	0.564	0.378
VC	1.964	1.186	0.450	0.136
<b>C: HMWC gel (2.5%, w/w) with 5% (w/w) liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	155.902	311.944	113.241	252.189
SD	1.294	0.209	0.359	0.897
VC	0.830	0.067	0.317	0.356
<b>D: HMWC gel (2.5%, w/w) with 10% (w/w) liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	136.971	271.979	99.368	221.577
SD	0.909	1.317	0.757	1.257
VC	0.664	0.484	0.762	0.567

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.

The incorporation of glycerine also affected the difference between liposomal HMWC gels and the empty gel HMWC in a way that the difference became less pronounced.



**FIGURE 39:** HMWC LIPOSOMAL HYDROGELS WITH ADDED 1% GLYCERINE BEFORE STABILITY TESTING

A: No liposomes; B: 1% (w/total) liposomes, C: 5% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.

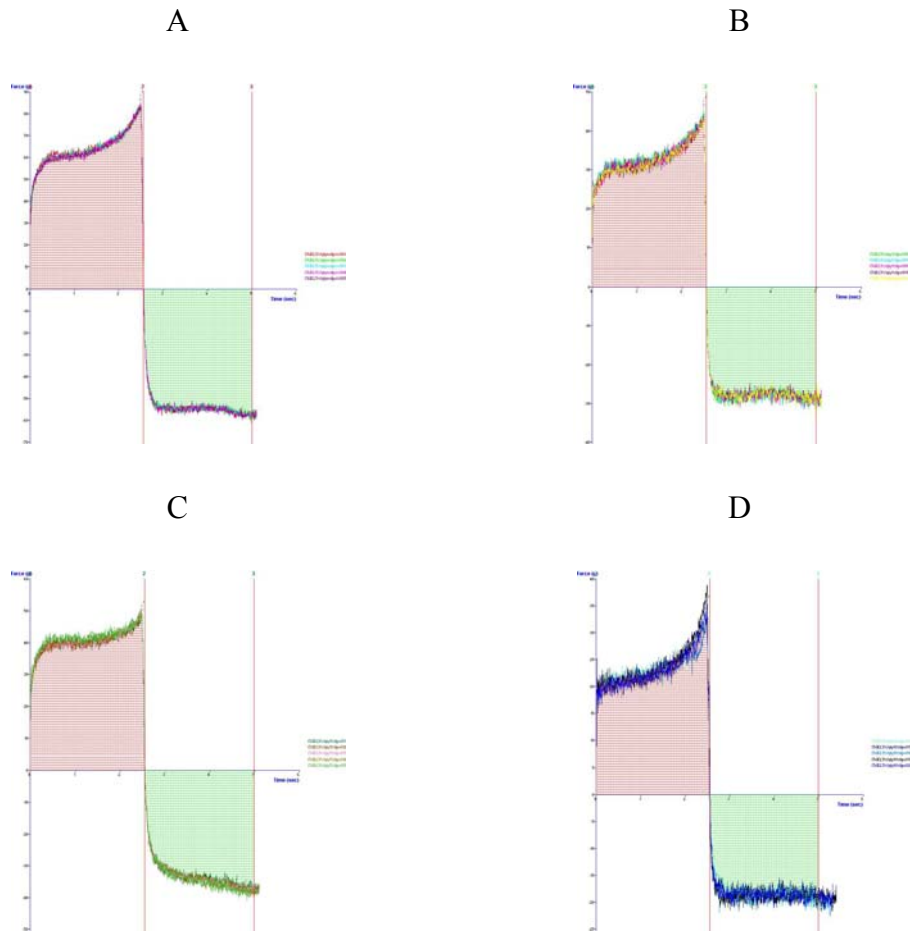
Table 13 reveals that glycerine indeed stabilized HMWC gels but especially strong impact was seen on the liposomal gels. The gels maintained some of the original texture

properties. A stability test at room temperature to get more accurate insight on how stable the gels are at expected storage conditions would be the next step.

**TABLE 13: HMWC GELS CONTAINING GLYCERINE AFTER ACCELERATED STABILITY TEST**

<b>A: HMWC 2.5% (w/w) with no liposomes and 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	84.647	162.179	59.984	130,816
SD	0.359	0.861	0.709	0.413
VC	0.425	0.531	1.182	0.316
<b>B: HMWC 2.5% (w/w) with 1% (w/w) liposomes and 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	44.397	83.296	31.740	67.281
SD	0.743	0.826	1.076	0.481
VC	1.673	0.992	3.390	0.715
<b>C: HMWC 2.5% (w/w) with 5% liposomes and 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	49.846	103.251	39.772	80.925
SD	0.525	1.110	0.754	0.929
VC	1.053	1.075	1.896	1.147
<b>D: HMWC 2.5% (w/w) with 10% (w/w) liposomes and 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	35.279	59.484	21.688	44.481
SD	1.890	1.059	0.584	0.937
VC	5.358	1.781	2.693	2.107

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. The liposomal dispersion was containing 0.2% (w/w) chloramphenicol. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.



**FIGURE 40:** HMWC LIPOSOMAL GELS CONTAINING GLYCERINE AFTER ACCELERATED STABILITY TESTING

A: No glycerine, no liposomes; B: No glycerine, 10% (w/total) liposomes; C: 1% (w/w) glycerine, 10% (w/total) liposomes; D: 1% (w/w) glycerine, 10% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.

### 5.2.3. Stability testing in freezing conditions

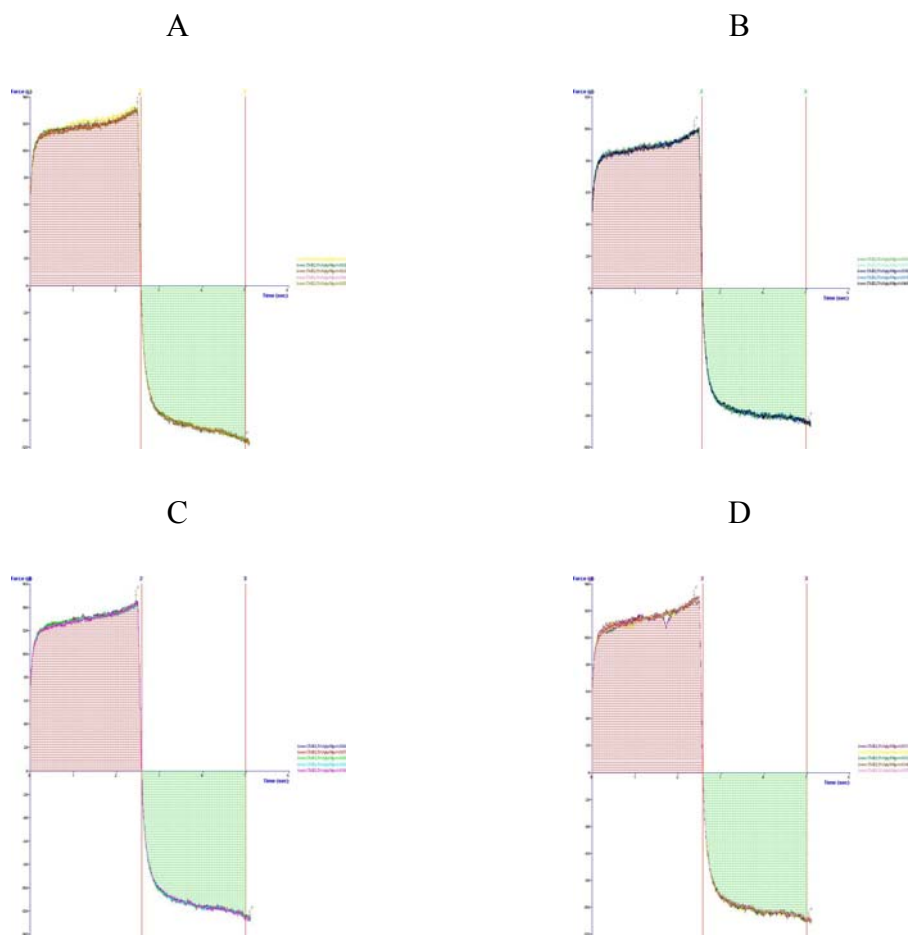
HMWC hydrogels (2.5% w/w) with or without added glycerine and with and without incorporated liposomes were evaluated in freezing conditions. The stock gels were bath sonicated over two hours prior to the testing, which might explain why the texture property is different (Table 14) as compared to previous results with the same preparation. The cohesiveness of the empty gel without glycerine was found to be higher after freezing and thawing. With the liposomal hydrogel without glycerine the cohesive forces (area 1) went up and adhesive forces (area 2) went down. Even though the results are near. This showed that something happened to the gel matrix. Both the empty hydrogel with glycerine and the liposomal hydrogel with glycerine showed increased area 1 and area 2 (Table 15). That indicates that freezing combined with glycerine in the formulation increases the stability. Further analyses of the gel structure needs to be done to confirm that the gels can tolerate freezing. However, the freezing did not lower the viscosity in the gels tested, rather the opposite. Yang *et al.* (2009) describes that freeze-thaw method and irradiation method in combination is suitable to yield stable gels. Stauffer and Peppas (1992) proved that freeze-thawing reinforced poly(vinyl alcohol) hydrogels.

**TABLE 14: STABILITY OF HMWC GELS PRIOR TO FREEZING**

<b>A: HMWC gel (2.5%, w/w) without liposomes, without glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	132.148	299.767	118.232	241.694
SD	1.119	2.773	0.957	0.580
VC	0.847	0.925	0.810	0.240
<b>B: HMWC gel (2.5%, w/w) with 10% (w/w) liposomes, without glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	100.191	223.835	86.492	182.788
SD	0.602	0.638	0.463	0.604
VC	0.601	0.285	0.535	0.330
<b>C: HMWC gel (2.5%, w/w) without liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	144.457	328.682	128.349	262.303
SD	1.337	1.580	0.808	0.507
VC	0.926	0.481	0.630	0.193
<b>D: HMWC gel (2.5%, w/w) with 10% (w/w) liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	130.563	290.056	111.654	233.375
SD	1.086	0.563	0.709	1.133
VC	0.832	0.194	0.635	0.485

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.





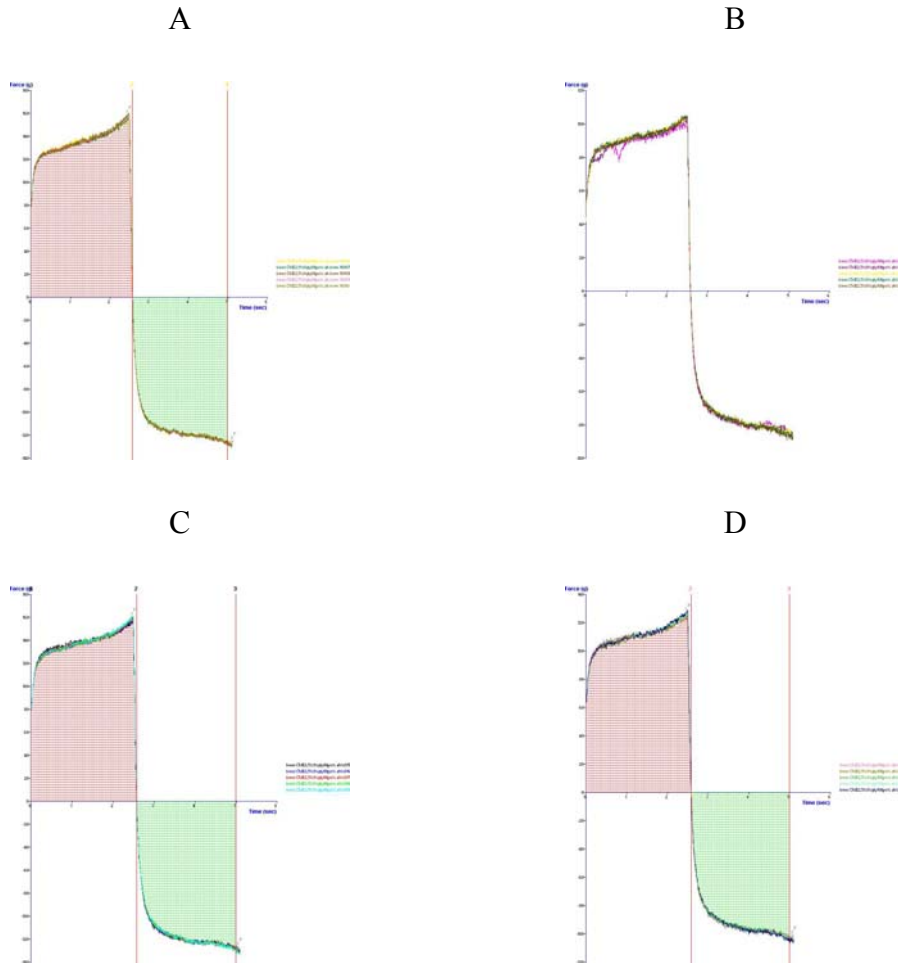
**FIGURE 41:** HMWC GELS PRIOR TO FREEZING

A: No glycerine, no liposomes; B: No glycerine, 10% (w/total) liposomes; C: 1% (w/w) glycerine, 10% (w/total) liposomes; D: 1% (w/w) glycerine, 10% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.

**TABLE 15: STABILITY UPON FREEZING AND THAWING**

<b>E: HMWC gel (2.5%, w/w) without liposomes, without glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	184.560	365.799	131.196	291.932
SD	2.140	1.457	1.438	0.989
VC	0.847	0.398	1.096	0.339
<b>F: HMWC gel (2.5%, w/w) with 10% (w/w) liposomes, without glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	104.383	230.994	88.035	179.954
SD	1.465	2.229	1.092	0.521
VC	1.404	0.965	1.240	0.289
<b>G: HMWC gel (2.5%, w/w) without liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	159.267	350.517	132.477	274.519
SD	2.097	0.872	0.776	0.422
VC	1.317	0.249	0.585	0.154
<b>H: HMWC gel (2.5%, w/w) with 10% (w/w) liposomes, 1% (w/w) glycerine</b>				
	<b>Force 1 (g)</b>	<b>Area 1 (g·sec)</b>	<b>Force 2</b>	<b>Area 2 (g·sec)</b>
Ave	126.354	280.418	106.207	218.198
SD	1.886	0.919	0.912	0.637
VC	1.493	0.328	0.859	0.292

The table shows maximum force (force 1), minimum force (force 2), area 1 and area 2. The values are the average (Ave) of 5 runs and are absolute. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec.



**FIGURE 42:** STABILITY UPON FREEZING AND THAWING

A: No glycerine, no liposomes; B: No glycerine, 10% (w/total) liposomes; C: 1% (w/w) glycerine, 10% (w/total) liposomes; D: 1% (w/w) glycerine, 10% (w/total) liposomes. Measured with back-extrusion equipment and 35 mm disc. Distance: 10 mm, speed 4 mm/sec, 5 runs.

The freeze-thawing experiment showed that the cohesiveness of the gel increased. It is possible that the reinforcement of the hydrogel matrix can have occurred (Yang *et al.*, 2009). It is known that liposomes are sensitive to the freezing process, however, as liposomes were incorporated in gels, it is expected that the gel provide a protection to liposomes from outer conditions (Pavelic *et al.*, 2001).

### 5.3. *In vitro* release rate

#### 5.3.1. Entrapment efficiency determination and particle size analysis

Prior to incorporation of liposomes in hydrogels, liposomal size, size distributions and chloramphenicol entrapment values were determined. The results are presented in Table 16.

**TABLE 16:** THE CHARACTERISTICS OF LIPOSOMES CONTAINING CHLORAMPHENICOL

Lipid (mg)	Particle size $\pm$ SD (nm)	Polydispersity Index (PI)	Entrapment efficiency (%)	Drug to lipid ratio ( $\mu\text{g}/\text{mg}$ )
200	906	0.637	81 %	80

During the process of separation of liposomally entrapped drug and free drug (ultracentrifugation), we observed that the supernatant, which would normally not contain liposomes as we prepared MLVs by the film method, also contained very small particles. This was further confirmed by the PCS measurements, where clear distinctions could be seen between very small and much larger vesicle, resulting in higher PI (0.637) values as well. Due to this fact, for preparation of liposomal hydrogels we used liposomal dispersion containing both liposomal and unentrapped (free) chloramphenicol as entrapment was found to be rather high. This high entrapment is in agreement with Škalko *et al.* (1998) who proved that encapsulation values are higher for larger liposomes. In their study of liposomes containing metronidazole, the median size of 300 nm liposomes gave the highest drug entrapment values, providing the reservoir for drug in the deeper skin layers.

#### 5.3.2. Standard curve HPLC

The standard curve was made by the concentrations: 2, 5, 8, 25 and 50  $\mu\text{g}/\text{ml}$  chloramphenicol in methanol.

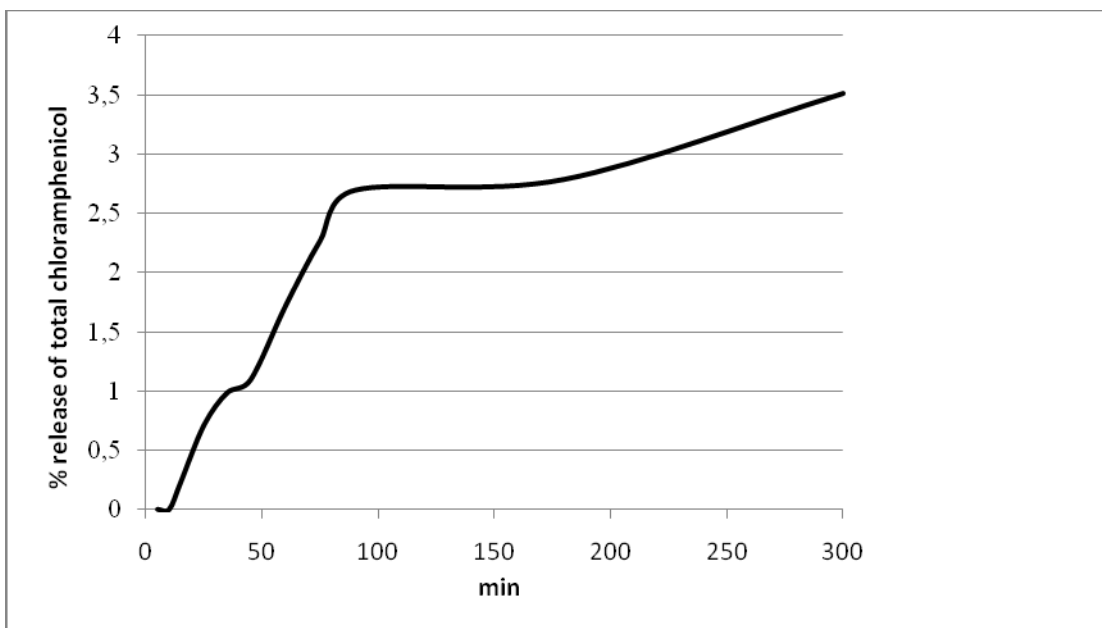
Equation for standard curve:  $Y = X \cdot 1.57 \cdot 10^4 + 6.93 \cdot 10^3$

### 5.3.3. HPLC analysis

TABLE 17: *IN VITRO* RELEASE STUDY

Sample	Time (min)	Area	Amount of drug in acceptor medium ( $\mu\text{g}$ )	% release
1	5	3094.0	-	-
2	10	6632.5	-	-
3	15	10822.0	11.636	0.232
4	25	18849.5	35.667	0.714
5	35	23517.5	49.344	0.986
6	45	25369.5	54.652	1.094
7	60	36495.5	85.831	1.716
8	75	46635.5	113.605	2.272
9	90	54575.0	134.845	2.697
10	180	56337.5	139.448	2.788
11	300	70560.5	175.685	3.514

HMWC gel 2.5% (w/w) with 10% (w/w) of added liposomal dispersion containing chloramphenicol was used in testing. The results are an average of two determinations. The drug concentration was determined by the HPLC analysis. The measurement conditions were: Mobile phase was 45% methanol, 55% filtered H<sub>2</sub>O and 0.1% acetic acid (glacial). Temperature of samples and column was 35 °C. Flow rate was 1 ml/min. Running time for each sample was 5 minute and picks were monitored at UV 270 nm. Total amount of chloramphenicol in gel was 5 mg, acceptor medium: middle chain triglycerids, polyamid membrane and 1 ml sample size. The table shows the amount of chloramphenicol in the acceptor medium after given time intervals. The values are cumulative and adjusted for the decreasing amount of acceptor medium during sampling.



**FIGURE 43:** PERCENTAGE RELEASE (CUMULATIVE) OF CHLORAMPHENICOL

Data from release study. X-axis is showing the time elapsed. The Y-axis is showing the release in percent of total amount (cumulative).

The results are an average of two determinations. The drug concentration was determined by the HPLC analysis. The measurement conditions were: Mobile phase was 45% methanol, 55% filtered H<sub>2</sub>O and 0.1% acetic acid (glacial). Temperature of samples and column was 35 °C. Flow rate was 1 ml/min. Running time for each sample was 5 minute and peaks were monitored at UV 270 nm. The values are cumulative and adjusted for the decreasing amount of acceptor medium during sampling.

Figure 43 and Table 17 show that the release of chloramphenicol was quite linear up to 90 minutes during the release study. The release was continuing up to 300 minutes when the last sample was taken. We realized that the experimental set up should include minimum 24 hours sampling and the current measurement continues with sampling for 24 hours. Chloramphenicol in our case was both distributed in liposomes and liposomal dispersion medium. The initial release can be a combination of release from both. Liposomes are known for prolonging the drug release. Panduranga Rao and Alamelu

(1992) studied the release of an aqueous marker from liposomes and liposomes in chitosan. They found that liposomes prolonged the release compared to release from hydrogel alone. And adding increasing amounts of a cross-linking agent even further prolonged the release. Drugs entrapped into liposomes function as depot formulations (Jagur-Grodzinski, 2009). Around 19 percent of the chloramphenicol was entrapped in the small liposomes or dissolved in the liposomal dispersion phase. The release was expected to be faster from chloramphenicol dissolved in the dispersion phase. Therefore, faster release initially was to be expected. Although chloramphenicol could be dissolved into hydrogels alone, liposomes containing chloramphenicol can be preferable. Drugs incorporated into liposomal hydrogels give a retarded release compared to drugs dissolved in a hydrogel (Ruel-Gariépy *et al.*, 2002). Since chitosan preparations for wound healing should be left on the skin for hours and maybe up to 24 hours or more, a depot is preferred for a prolonged release to the wound.

## 6. Conclusions

Chitosan hydrogels for wound healing were prepared from low, medium and high molecular weight chitosan. The preferred concentrations of chitosan in the gels were 6% (w/w) for low molecular weight, 3.5% (w/w) for medium molecular weight and 2.5% (w/w) for high molecular weight. The high molecular weight chitosan was the optimal because it could be prepared at lower concentrations and still maintain an acceptable viscosity.

A method for evaluating and comparing texture properties of hydrogels was established with the Texture analyser and back-extrusion equipment. Gels can be analysed in minutes and the method was reproducible with standard deviations varying with less than 2%. This makes it applicable in comparing variations between different batches of gels as well as the stability.

Stability was tested with the preferred concentrations of chitosan gels as well as liposomal gels. Accelerated stability tests proved that chitosan gels are not stable for 30 days at 40 °C. The viscosity increased after one freeze-thaw cycle for high molecular weight chitosan gel. Liposomal dispersion added to the gels made them less viscous and less stable. The addition of 1% (w/w) glycerine prepared with high molecular weight chitosan made the gels more stable. Especially liposomal hydrogels proved to be more stable when glycerine was in the gel.

Liposomal chitosan gels were prepared with chloramphenicol as a model drug. Release was tested on in vitro release model and quantified by HPLC. Liposomal hydrogels prepared from high molecular weight chitosans gave a small burst of chloramphenicol initially and more prolonged release from the liposomes.



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# Appendix

## A. Abstract submitted

### HYDROGELS FOR IMPROVED WOUND HEALING: THE CHOICE OF GELLING MATERIAL

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**Purpose:** In respect to an increasing market in the wound care it is of vital interest to improve topical wound formulations, both in terms of shortening the time of therapy and considering financial aspects. Properties of hydrogels applied locally to wounds have the direct impact on therapy efficiency. By the right choice of gelling material, improvement of healing process can be achieved.

**Methods:** Two types of hydrogels, namely synthetic Carbopol Ultrez 10 and natural polymer chitosan, were evaluated for their texture properties on TA.XT plus Texture Analyser (Stable Micro Systems Ltd., UK). Accelerated stability testing was applied to evaluate gel stability. The effect of gel type on release pattern of liposomally entrapped model drug, chloramphenicol, was studied in an *in vitro* model for topical release.

**Results:** Both gel concentration and pH have direct influence on the viscosity and bioadhesiveness of hydrogels. Carbopol hydrogels remained stable at accelerated stability conditions, whereas chitosan hydrogels did not resist temperature increase. However, glycerol improved the stability of chitosan gels to great extent. Release of liposomally entrapped drug was found to be slower from chitosan hydrogels as compared to Carbopol hydrogels.

**Conclusions:** Gel adhesiveness, stability and the release of incorporated drug are recommended as the main features influencing the choice of gel-forming material in regard to topical treatment of wounds. However, the compatibility of gel forming material and incorporated drug should be taken into consideration, as well as cost-effectiveness of the formulation.

## B. Abstract submitted

### Improved wound therapy: characterization of hydrogel texture

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**Aim:** Retention time of wound dressing at the application site will determine the efficacy of the wound treatment. The choice of a hydrogel matrix and a drug carrier system has direct influence on the effectiveness of a topical gel formulation. To adjust properties of a formulation to the desired application, it is important to be able to characterize the hydrogel and its properties such as viscosity, adhesiveness and drug release behaviour.

**Methods:** Two types of hydrogels, namely synthetic Carbopol Ultrez 10 and natural polymer chitosan, were evaluated for their texture properties on TA.XT plus Texture Analyser (Stable Micro Systems Ltd., UK). Texture properties, including bioadhesiveness were evaluated and compared. Accelerated stability testing was applied to give insight on gel stability. A liposomal gel formulation with chloramphenicol as a model drug was used to study drug release properties in an *in vitro* model for topical release.

**Results and Discussion:** Both gel concentration and pH have direct influence on the viscosity and bioadhesiveness of hydrogels. Carbopol hydrogels remained stable at accelerated stability conditions, whereas chitosan hydrogels did not resist temperature increase. However, glycerol improved the stability of chitosan gels to great extent. Release of liposomally entrapped drug was found to be slower from chitosan hydrogels as compared to Carbopol hydrogels.

**Conclusion:** The choice of a certain gel matrix and the adjustment of the technological characteristics affects the properties of a topical gel formulation to a great extent. Adhesiveness and drug release are two of the main properties which can be influenced by that. Chitosan and carbopol are suitable model matrices to analyse the effect of different impacts towards the abilities of the hydrogel formulation.

