THESIS FOR THE DEGREE MASTER OF PHARMACY

CARBOPOL HYDROGELS FOR TOPICAL ADMINISTRATION: TREATEMENT OF WOUNDS

BY

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2010

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Table of contents

List of tables

List of figures

Acknowledgement

This study was carried out in the period between October 2009 and June 2010 at the Department of Pharmacy, section of Drug Transport and Delivery Research Group, under the supervision of Professor Doctor Natasa Skalko-Basnet at the University of Tromsø.

I would like to express my gratitude to my supervisor, Prof Dr. Natasa Skalko-Basnet, for her outstanding contribution, valuable advice, support, caring, kindness and encouragement. I will always remember and admire the combination of a high professional career and humility that characterizes your personality.

I would like to thank Ph.D. student Julia Hurler and Master student André Engesland for their fantastic cooperation and patient help all the way. I wish you both a brilliant life ahead.

I am grateful to Merete L. Skar for always taking the time to help me, and patiently assisting me with the laboratory equipments. And warm thanks to all my colleagues in the Drug Transport and Delivery Research Group.

I would like to thank class 2005 for the five unforgettable years that we have spent together. Special thanks go to the Ladies: Meera Satish Thanki and Toril Andersen, and to the gentlemen: Yashar Ammari, Shwan Hiwa, Herem Mahmoud and Haider Hussain.

I have always failed to thank my father for all he has done for me. Thank you for all the times you got up in the dead of night, working to make a better future for me. From that time on I have always tried to make you proud, and I hope you are happy with me.

It is my pleasure to thank my mother for always being there and believing in me. I would not be where I am if it was not for you. Thank you, mom, for being my greatest supporter during the hardest times of my life. Thank you for working so hard because of me for all these years.

I am grateful to my parents-in-law for the security they have given to me for the last three years. Thank you for doing my duties for me and supporting me in this way. Thank you, my only dear sister Shokouh and my good brother-in-law, Moein, for all you have done for me.

It is my honor to thank Mrs. Åse Helene Berntsen for all the kindness she has shown me. I learned a lot from you.

Finally, it is my greatest honor and pleasure to thank my wonderful wife, the soul of my whole being, Samira. My dearest Samira, thank you for your patience, kindness, tolerance, grace, support and compassion. My heart is awfully sorry for the loneliness and sadness you have gone through during my studies. I have thought of you every day and I appreciate all the sacrifices you have made because of me. I cherish and honor you more than you can imagine, and it is an honor and privilege to have you as my wife. Words can not express my gratitude toward you. I love you beyond words.

May God bless us all,

Bahador Poorahmary Kermany

Abstract

The very positive effect of hydrogels on wounds and enhanced wound healing process has been proven. Hydrogels provide a warm, moist environment for wound that makes it heal faster in addition to their useful mucoadhesive properties. Moreover, hydrogels can be used as carriers for liposomes containing variety of drugs, such as antimicrobial drugs. This will provide a depot release of drug to the wound bed. In order to optimize the liposomal hydrogel formulation, texture properties responsible for the retention of gel on the wound, need to be well characterized. Characterizing the behaviour of Carbopol hydrogels by texture analyser as described in this Thesis is a new method. As a first step, we evaluated the effects of each parameter used in texture analysis in order to establish reproducible measuring conditions. Behaviour of Carbopol Ultrez 10 hydrogels was studied in regard to the polymer concentration and pH of the gel. Accelerated stability tests were conducted for both Carbopol gels and liposomal Carbopol gels. Chloramphenicol was used as a drug model to be entrapped in liposomes and its release from liposomal hydrogels was studied. Carbopol hydrogels are stable formulations and easy to produce. Their texture properties are affected by the pH values of the gel. Accelerated stability indicated that the attention needs to be given to the gel composition. When incorporating liposomal dispersions, there is a limit of proportion of the dispersion which could be incorporated without affecting the original properties of the gel. Liposomal Carbopol hydrogels show potential to be used as drug delivery system.

1. Introduction

1.1. Skin

The human body has two systems that protect it from the harmful organisms existing in the environment. The internal defense system destroys microorganisms and bacteria that have already attacked the body. The external defense system prevents microbial microorganisms to enter the body. Skin is biggest external defense system. Skin covers the outside of the body but has other functions beside the defense mechanism. It serves as a mechanical barrier between the inner part of the body and the external world (Sherwood, 2007). Temperature of skin varies in a range of 30 to 40 °C degree depending on the environmental conditions (Noble, 1993).

1.1.1. Anatomy of skin

Skin is the largest organ in the body. It consists of three layers. The outer layer is called epidermis, the middle layer is dermis and the inner most layer is hypodermis (Figure 1).

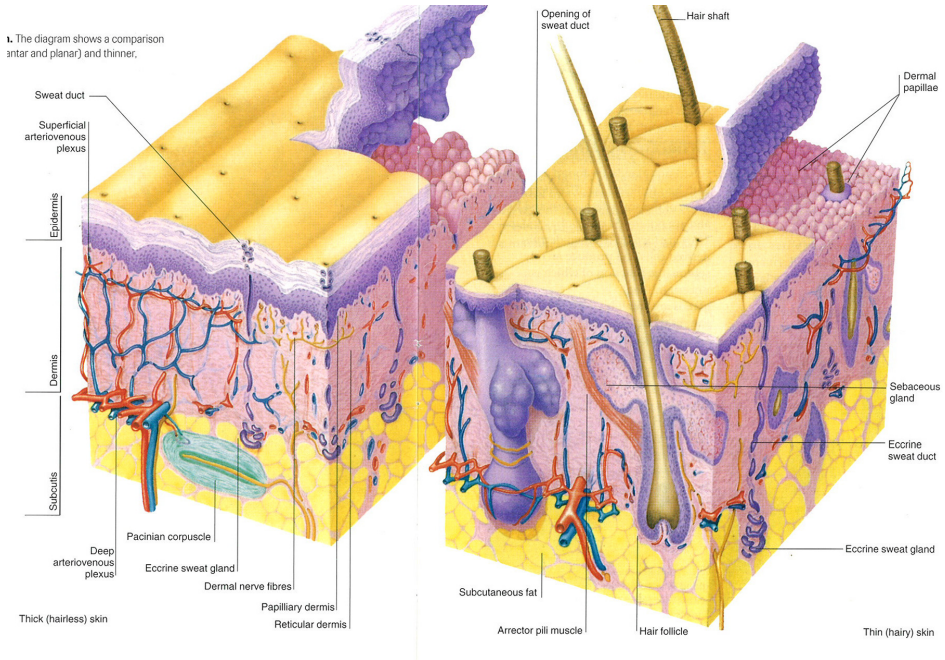


Figure 1: Structure of skin (Gawkrodger, 2002).

Epidermis: Consists of epithelial cells. Among these cells, both living cells and dead cells can be found. These new cells at the bottom of epidermis divide fast and push the older cells upward. The epidermis does not have any direct source of blood veins to provide nutrition for it. It takes its nutrients from the diffusion of necessary molecules from a rich vascular network in the underlying dermis. Epidermal cells are connected very strongly by desmosomes. Desmosomes are in contact with the intracellular keratin filmates. Keratin filmates produce keratin. Keratin cells accumulate and crosslink with the other keratin cells in the cytosol during their maturation. Afterward when the older cells die, this network of keratin fibroses remains and provides a tough and hard protective layer in epidermis, called protective keratinized layer. This layer is waterproof and airtight. It prevents most substances to enter the body or leave from the body. In diseased skin, particularly burns, epidermis is destroyed causing potential loss of body fluid and an increase in susceptibility to microbial infections, leading to fatal consequences untreated (Sherwood 2007).

Cell types that exist in the epidermis are:

- Keratinocytes; these are the main cell types in epidermis (95% of cells).
- Melanocytes; these are the pigment producer cells and found in the basal layers of epidermis
- Langerhans cells; these cells are important immunological cells and can be found in the mid dermis as well
- Merkel cells; these cells are found in the basal layer of epidermis and are one part of amine precursor and decarboxylation system (Mackie, 2002).

Epidermis consists of five layers, namely from inside to outside; stratum germinativum (basal layer), stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Figure 2). Stratum corneum is the outer most layer of epidermis and has a thickness of 10-20 μm when it is dry and 40 μm when it is hydrated and becomes swollen (El Maghraby et al., 2008).

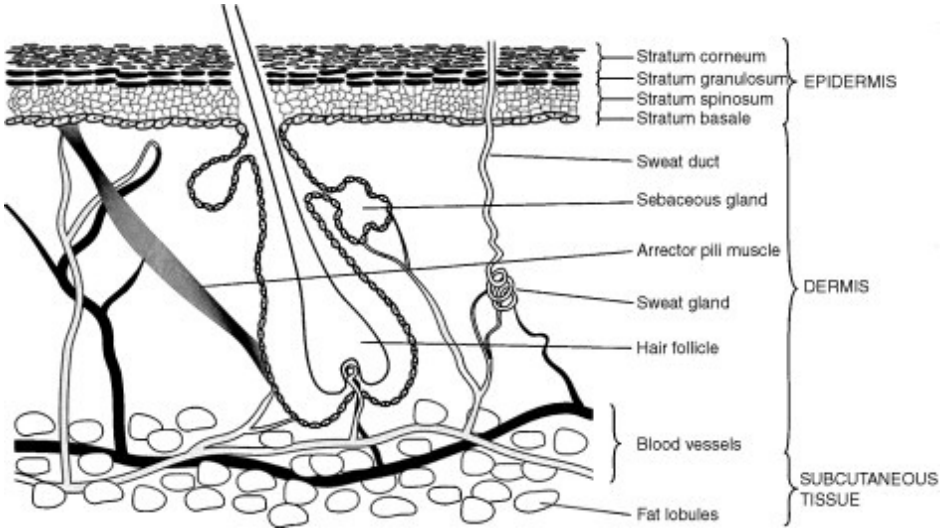


Figure 2: Epidermal and skin layers (El Maghraby et al., 2008).

Stratum corneum has a structure of "bricks and mortar" arrangement (Figure 4). In this model the keratin rich corneocytes (bricks) are sitting in the intracellular lipid rich matrix (mortar) (El Maghraby et al., 2008).

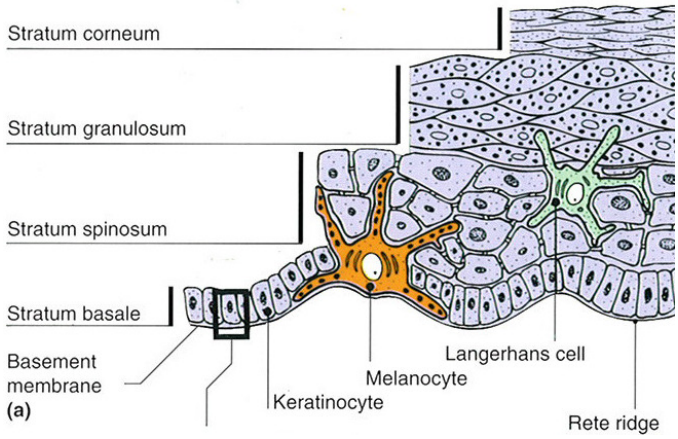


Figure 3: Layers of epidermis (Gawkrodger, 2002).

Corneocytes (the bricks) create 85 % of stratum corneum and intracellular lipids (15%) are arranged in 15-20 layers. Statum corneum consists of 70% proteins, 15% lipids and only 15% water (Nino et al., 2010).

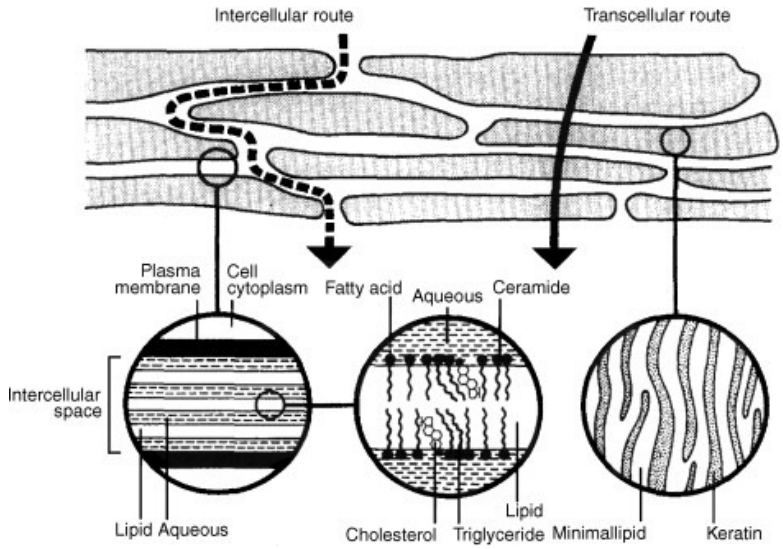


Figure 4: Structure of stratum corneum and penetration pathways (El Maghraby et al., 2008).

Molecules can basically permeate through skin by two different pathways. The first pathway is called the transappendegeal route. In this route the molecules should permeate through skin by permeation through sweat glands and across the hair follicles. The number of molecules which can penetrate through this pathway is very limited. The second pathway of penetration through skin is the transepidermal pathway. In this pathway molecules should pass through stratum corneum as multilayered barrier. This pathway has two micropathways; the intracellular micropathway and the transcellular micropathway (Figure 4; El Maghraby et al., 2008)

Dermis: Dermis is positioned under epidermis and is characterized by lots of elastin fibers that provide the stretching ability as well as lots of collagen that provides the strength to the skin. Blood vessels found in dermis provide nutrients for both dermis and epidermis. Dermis also plays a major role in temperature regulation. Nerves present there are responsible for pressure and pain sensations (Sherwood, 2007). Dermis has a thickness of 3-5 mm. In addition to elastin fibers, blood vessels and nerves, an interfibrillar gel of glycosaminoglycan, salt, water, lymphatic cells and sweet glands are parts of dermis also (El Maghraby et al., 2008).

Cell types found in dermis are:

- Fibroblasts: collagen producing cells
- Macrophages: scavenger cells
- Mast cells: responsible for immunological reactions and interactions with eosinophils (Mackie, 2002).

Dermis plays an important role as connection to other skin layers also. Changes in the metabolism in dermis can influence growth integrity of the epidermis, hair follicles and skin glands (Noble, 1993).

Hypodermis: Hypodermis is the inner layer of skin. It is the contact layer between skin and the underlying tissues in body such as muscles and bone (Sherwood, 2007).

Skin exocrine glands and hair follicles: Sweet glands, sebaceous glands and hair follicles enfold in epidermis but they stem from dermis. Sweat glands release a dilute salt solution into the surface of skin. The evaporation of this solution makes skin cool and this is important for temperature regulation of both body and skin. Sweet glands are present all over the body. The amount of dilutions (sweet) that gets produced depends on environmental temperature, the amount of heat generating skeletal muscle activity and various emotional factors (Sherwood, 2007). The sebaceous glands produce sebum. Sebum is an oily liquid released into hair follicles and from there onto the skin surface. Sebum protects both hair and skin from drying out and provides waterproof layer (Sherwood, 2007).

1.1.2. Wound

A wound is defined as a defect or break in the skin, resulting from physical or thermal damage or as a result of the presence of an underlying medical or physical condition. Based on the nature and repair process of wounds, they can be classified as chronic wounds and acute wounds (Boateng et al., 2008).

Acute wounds are tissue injuries that heal within 8-12 weeks. The primary causes of acute wounds are mechanical injuries (friction contact between skin and hard surfaces), burns and chemical injuries. In the case of burns, the temperature of the

source and time of exposure is important to decide the degree of wound. Burn wounds need normally specialist care because of associated trauma (Boateng et al., 2008).

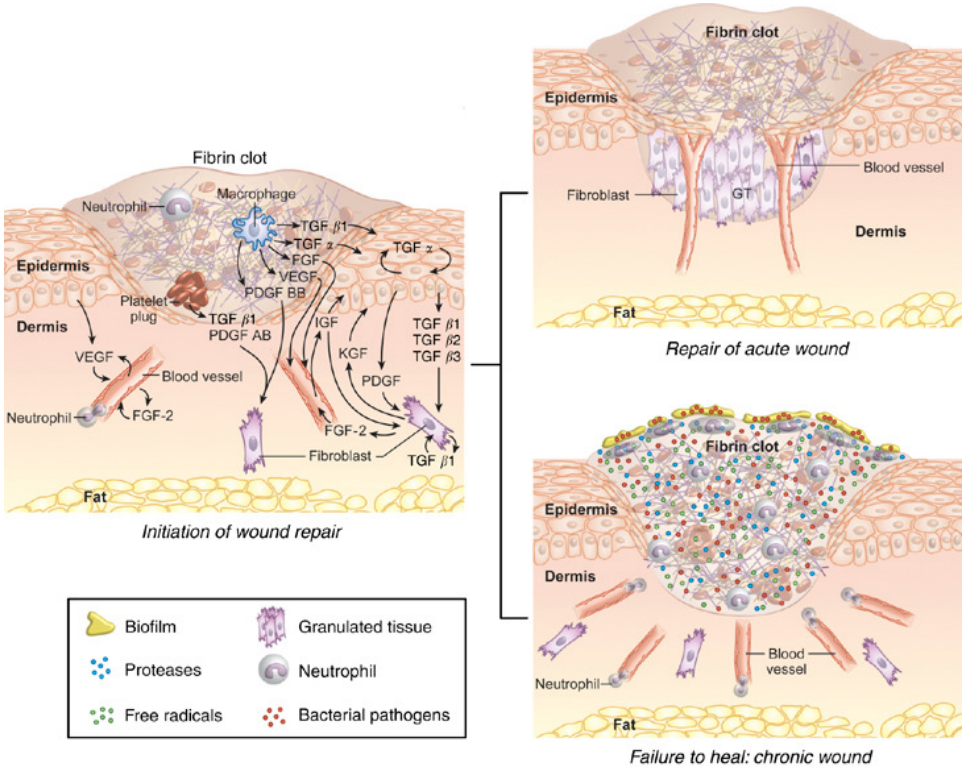


Figure 5: Differences between acute and chronic wound (www.nature.com).

Chronic wounds heal slowly and leave serious scars. There can be different reasons that chronic wound do not heal as fast as acute wounds. Among most common are diabetes, infections and poor primary treatment (Boateng et al., 2008).

The common chronic wounds are:

- Venous ulcers
- Arterial ulcers
- Diabetic foot ulcers
- Pressure ulcers
- Vasculitis
- Pyoderma gangrenosum (Fonder et al., 2008).

Both acute and chronic wounds can be classified as a complex wound if the wound has these characterizes:

- Extensive loss of the integument which comprises skin, hair and associated glands
- Infection which can result tissue loss
- Tissue death or signs of circulations
- Presence of pathology

A wound is colonized when growth and death of bacterial in the wound is balanced by the host. If the host is not able to keep the bacterial growth in balance, the wound will enter the infection phase (bacterial load in excess of 10^{15}). Symptoms for an infected wound are erythema, edema, warmth, pain and exudate. Infections of chronic wounds are often polybacterial with *Staphylococcus aureus* and anaerobs being the most common in chronic wound (Fonder et al., 2008).

1.1.3. Wound healing process

The wound healing process is a series of independent and overlapping stages. In these stages will both cellular and matrix compounds work to reestablish the integrity of damaged tissue and replacement of lost tissue. These overlapping series can be classified in five stages (Boateng et al., 2008):

- Haemostasis
- Inflammation
- Migration
- Proliferation
- Maturation

Haemostasis: the first response to injury is bleeding. Bleeding is an effective way to wash out bacteria that are on the surface of skin. Afterwards, bleeding activates haemostasis stage that is initiated by clotting factors. The clot dries out and creates a hard surface over the wound that protects tissues underlying (Boateng et al., 2008).

Inflammation: this stage starts almost at same time as haemostasis. It occurs from between few minutes to up to 24 minutes after injury. In this stage histamine and

serotonin are released into wound area and activate phagocytes to enter the wound area and engulf dead cells (Boateng et al., 2008).

Migration: in this stage the reestablishment of wound begins. The epithelial cells and fibroblasts move into the injured area and grow rapidly under the hard scab to replace the damaged tissue (Boateng et al., 2008).

Proliferation: this stage has three characteristics. First, the granulation tissue is formed by growth of capillaries. Second, lymphatic vessels enter into wound and the third one, synthesis of collagen starts providing form and strength to the injured tissue (Boateng et al., 2008).

Maturation: in this stage, the shape of the final scar is determined by formation of cellular connective tissue and strengthening of the new epithelium (Boateng et al., 2008).

The different stages in wound healing process can be defined in simplified form as

- The cleansing phase (exsudative phase)
- The granulation phase (proliferation phase)
- The epithelialisation phase (differentiation phase) (Goossens and Cleenewerck, 2010).

Or as:

- Inflammation
- Proliferation
- Remodeling (Figure 6; Fonder at al., 2008).

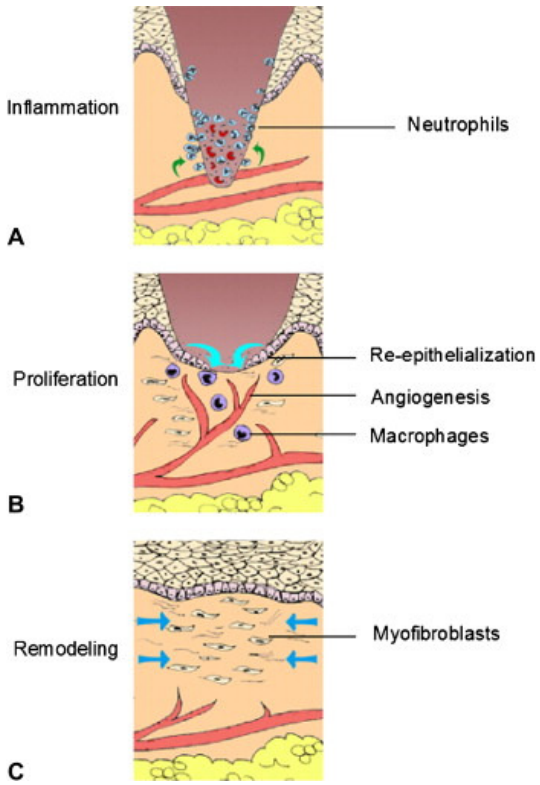


Figure 6: Different stages in wound healing process (Fonder et al., 2008).

One factor that can slow the wound healing process is excessive inflammation, especially in burn wounds. This kind of inflammation is not a response to infection and is known as aseptic inflammation. Excessive inflammation is therapeutical challenge in wounds such as burns and chronic wounds (Beukelman et al., 2008).

Debridement is the process during which slough, eschar and exudate, bacterial biofilms and callus get removed from the wound bed to enhance the healing process. Drastic methods of debridement can be painful, therefore use of moisture-donating wound dressings will rehydrate desiccated and devitalized tissue and then, separation from healthy tissue will be easier (Fonder et al., 2008).

1.1.4. Wound exudate

Wound exudate can be described as generic term given to liquid produced from chronic wounds, fistulae or other more acute injuries once the haemostasis has been achieved. Exudate is actually blood free from red blood cells and components. Existence of exudate is very important in wound healing process. Exudate keeps the wound moist as wound healing process gets faster in a moist environment. Exudate provides nutrition for the injured tissue and enhances the migration of epithelial cells. Exudate supplies the wound with leucocytes, which control bacterial growth and reduce the incidence of infection (Boateng et al., 2008).

In chronic wounds, an excess production of exudate can create problems. This excess can be a result of oedema caused by inflammation. One of the aims of the new types of dressings is to remove the excess of exudate, but at the same time maintain a moist environment for the wound to get healed faster (Boateng et al., 2008).

In chronic wounds there are complex microbiological communities. Bacterial load can affect the healing process without showing the signs of infection (Frankel et al., 2009).

1.2. Wound dressing

From old times people tried to heal wounds. They used crude drug extracts (mostly of plant origin), animal fat and honey to heal wounds. For example in Senegal, the people used the leaves of *Guiera senegalensis* to put on wound. In Ghana the people used extracts of *Commelina diffusa* herb and *Spathodea campanulata* bark to put on wound and heal it. The interesting point is that the researches have shown that some of these extracts and herbs have indeed antibacterial and antioxidant effect (Boateng et al., 2008). In order to specify optimal features of ideal wound dressing, several characteristics should be addressed such as:

An ideal material to be applied to wound should be

- Nontoxic
- Biocompatible
- Enhance cellular interaction and tissue development
- Be biodegradble and bioresorbable (Huang and Fu, 2010).

Moreover, the ideal properties of a wound dressing are:

- Providing a moist environment
- Creating a protective mechanical barrier and thermal isolation
- Protecting against secondary infections
- Keeping the wound environment moist
- Absorbing the exudate and bacteria
- Promoting debridement
- Contributing to simple gas exchange
- Decreasing or removing trauma in the defected area
- Being acceptable for patient
- Not possessing any toxic, irritant or allergic properties
- Cost-effectiveness (Goossens and Cleenewerck, 2010).

Some of wound dressings can cause allergic reactions when applied to the defected area. There are three kind of allergic reactions that can appear via wound dressings:

- Irritant reactions that originally have mechanical reasons. These reactions can happen because of occlusion or strong adhesion of dressing to the wound
- Immediate allergic reaction (contact urticaria)
- Delayed allergic reactions (contact eczema) (Goossens and Cleenewerck, 2010).

1.2.1. Classification of dressings

Dressings can be classified in a number of ways. They can be classified based on their function in the wound (antibacterial, absorbent), type of material employed to produce the dressing (collagen, hydrocolloid), physical form of the dressing (ointment, film and gel), traditional and modern dressings. Some dressings can be placed in several classifications because they fit criteria in several groups. The simplest classification is as traditional and modern dressings and particular focus will be given to hydrogels, one of the most common modern dressings (Boateng et al, 2008).

Traditional wound dressings can be classified as topical pharmaceutical formulations and traditional dressings (Boateng et al., 2008).

Topical pharmaceutical formulations: these formulations can be liquids such as solutions and suspensions or semi liquid materials such as ointments and creams. These formulations can be used in the initial stages of wound healing, for example as antibacterials (Boateng et al., 2008).

Traditional dressings: these are, unlike topical formulations, dry materials as cotton wool and natural or synthetic gauzes. These dressings are more used in chronic wounds and burn wounds because liquid and semi liquid dressings do not remain on the wound over optimal time (Boateng et al., 2008).

While some clinicians insist that gauzes are as effective as new dressings, some studies show that moisture retentive dressings are associated with faster healing time. Gauzes have been one of the most popular wound dressings. There are several disadvantages with use of gauzes. They can promote desiccation of the wound base, they bind to the wound bed and it causes pain and trauma for patients while dressing change. They do not provide a good barrier against bacterial growth because they are susceptible to full thickness saturation with wound fluid (Fonder et al., 2008).

The main aim of modern wound dressing is to create a moist environment for wound to make the healing process facilitated. Modern wound dressings are often classified as hydrocolloid dressings, alginate dressings, hydrogel dressings, dressings in form of gels, foams and films, etc. The modern dressings do not enhance the re-epithelialization, but stimulate collagen synthesis and promote the angiogenesis. They can provide a pain relief felling to patient. The modern dressings can inhibit bacterial growth by maintaining a barrier against external contamination and some of them by decreasing pH at the wound surface. They provide a moist environment for the wound bed to enhance the healing process. Dry wound healing process would not only delay the wound healing process, but can cause further tissue death (Fonder et al., 2008).

Five key points that technology and methodology of biomaterial design should consider when formulating biomaterials are as follows:

The first key is to produce cell scaffolds for enhanced repair, proliferation and differentiation process. In a big area of tissue defect, both cells and ECM are missing. Therefore, it is important to make a three dimensional scaffolds "ECM" that provides a suitable environment for the healing process and helps the attachment of cells while the natural ECM is produced. ECM is not only a physical support for cells, but provides also a natural environment for cell proliferation and differentiation. This artificial scaffold should be porous, because cells can infiltrate into scaffold. The porosity provides oxygen and nutrition for cells and cell wastes can be washed out through the porosity. This artificial cell scaffold is a temporary ECM for the cells. Cells begin to produce ECM naturally after the tissue regeneration is initiated (Tabata, 2009).

The second key is to provide the space for cell based tissue regeneration and supply nutrients and oxygen to cells by angiogenesis. When a defect happens in a body tissue, fibroblasts produce fibrous which occupies the defected area immediately. This is a process that fills and repairs the defected area. Once the fibroblasts have occupied the area, repair of the target tissue area will be hard. To enable the transplanted cells to survive, there should be a cell scaffold in the defected area that provides oxygen and supply for the transplanted cells. This artificial scaffold should be a barrier membrane that in addition to cells and space providing membranes, contains also the signaling molecules. Signaling molecules can accelerate the tissue regeneration (Tabata, 2009).

The third key in the technology of tissue engineering is use of growth factor. In some cases growth factor is required to promote tissue regeneration. Growth factor can not be injected as a solution to the regeneration site. It diffuses out from the defected area and becomes digested or deactivated fast. Therefore, it is necessary to incorporate the growth factor in a biomaterial carrier. It will be then possible to make a controlled release of growth factor at the regeneration site over a longer period of time (Tabata, 2009).

Presenting signaling cells as growth factor and vascular endothelial growth factor (VEGF) to the wound is important since a wound which matrix is lost does not have ability to regenerate itself just by presented cells (Huang and Fu, 2010).

Introducing VEGF to the wound can stimulate angiogenesis, collagen deposition and epithelialization. The molecule will accelerate healing of chronic wounds in particular (Bao et al., 2008).

The forth technology refers to finding the right type and quality of cell culture that have the ability to survive and regenerate in the defected site. For this purpose, isolation, induction and *in vitro* culture technologies are needed (Tabata, 2009).

The fifth and last technological key in the way to successful tissue engineering is to use genetic engineering to create cells that are suitable for the defected area. The stem cells should have properties that fit the base cells in that area. These cells should function in a same way as the base cells. It requires development of carriers for gene transfection and for efficient gene expression. The technology of providing carriers for gene expression is an important step in the tissue engineering (Tabata, 2009).

Hydrocolloid dressings:

This group of dressings is a combination of hydrocolloid materials (gel forming agents) and other materials such as elastomers and adhesives. They are widely clinically used because they can adhere to both dry and moist surface. They are used mostly in light to moderately exuding wounds (Boateng et al., 2008).

In contrast to hydrogels, hydrocolloids have the absorbent ability. They absorb wound exudate and form a hydrophilic gel that helps to maintain a moist environment. These types of dressings are adhesive, occlusive and comfortable dressings. They have less moisture vapour transmission compared to films and manage absorb exudate well (Fonder et al., 2008).

Figure 7: A typical hydrocolloid dressing (www.carepathway.com).

Alginate dressings:

This type of dressing is produced from calcium and sodium salts of alginic acid, a polysaccharide comprising mannuronic and guluronic acid units. When alginate dressings are applied to the wound, ions present in the alginate fibers are exchanged with those present in exudates and blood. This makes it possible to maintain an optimal moist environment and an optimal temperature for the wound during healing process. They can be used for moderate to heavily exuding wounds (Boateng et al., 2008). Alginates can hold water up to 20 times of their weight. Alginates contain calcium and sodium salts of alginic acid. An ion exchange occurs between calcium from alginate and sodium from wound fluid, forming sodium-calcium alginate which gives a gelatinous mass and can keep the moist environment (Fonder et al., 2008).

Semi-permeable adhesive film dressings:

These wound dressings are made from nylon derivatives that can be applied for moist wound healing. They have also several disadvantages that make their use limited. For example they cannot absorb much of exudates and therefore they should be changed quite often. If not they can cause maceration of the skin. They are also very thin and cannot be applied for deep wounds. They can be applied mostly for shallow wounds (Boateng et al., 2008)

Films:

Films can be used as an adhesive dressing directly on the wound or as a secondary dressing for a non adhesive dressing. Films are semi-occlusive. It means they permit exchange of oxygen and water between wound bed and outside and at the same time they are impermeable to bacterial and liquid contaminants. Films can not be used for wounds that contain lots of exudate since they are not absorbent. They provide a moist environment by vapour transmission only. If they are applied to wounds with heavy exudate, they can cause fluid trapping (Fonder et al., 2009).

Foam dressings:

These dressings are porous polyurethane foams or polyurethane foam films. They are highly absorbent and preferred to gauze in term of pain reduction, patient acceptability and nursing time (Boateng et al., 2008).

Hydrogel dressings:

Hydrogels are swellable hydrophilic materials. They are made from synthetic polymers such as polymethacrylate or polyvinylpyrrolidine. Hydrogels can be produced in two shapes, amorphous or solid sheet/films. If hydrogels are applied to the wound as gels, they need a second cover such as gauze. On the other hand if they are applied as films to the wound, they can be used both as a primary and secondary dressing (Boateng et al. 2008). Hydrogels fit most criteria for a suitable wound dressing as they:

- Help to the rehydration of dead tissues and increase the healing of debridement
- Are suitable for cleansing of dry, sloughy or necrotic wounds
- Do not react with biological reacts
- Are permeable to metabolites
- Are nonirritant
- Promote moist healing
- Are non-adherent
- Cool the surface of the wound

Hydrogels should be used for dry or low level of exudate wounds. The excess moisture can lead to maceration of skin. Hydrogels can be applied and removed with minimal trauma and pain from wound bed. Because of the cooling effect that hydrogels have on wound bed, they can give a relief feeling to patients (Fonder et al., 2008). However, hydrogels have some disadvantages also. Due to the higher amount of water (70-90%) hydrogels do not have the ability of absorption of exudates. Accumulation of fluid in hydrogels provides a suitable environment for bacterial growth and can produce infected smell afterward. Therefore, hydrogels should be changed quite often. Hydrogels have low mechanical strength (Boateng et al., 2008).

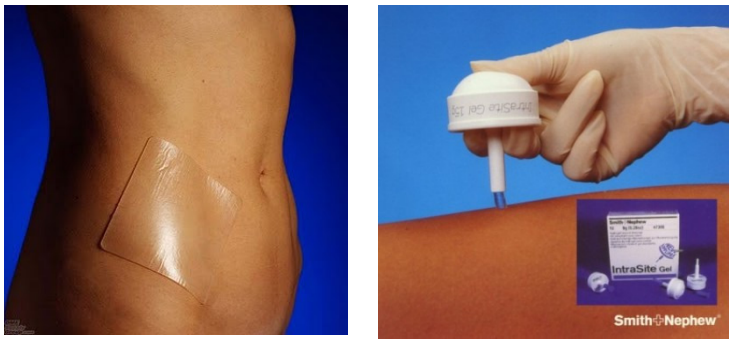


Figure 8: Hydrogels as sheet dressing and in form of gel (www.pacmedhawaii.com).

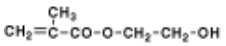
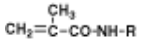
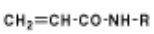
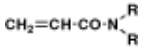
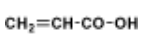
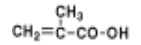
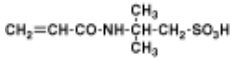
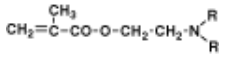
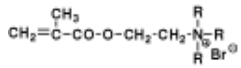
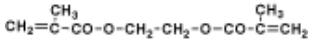
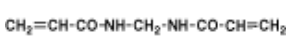
1.3. Hydrogels

In general, hydrogels can be prepared from either synthetic polymers or natural polymers. The synthetic polymers are hydrophobic in nature and chemically stronger compared to natural polymers. Their mechanical strength results in slow degradation rate, but on the other hand mechanical strength provides the durability as well. These two opposite properties should be balanced through optimal design (Tabata, 2009).

Polymeric gels are the liquid-solid systems. It means that they have a solid matrix that swells in water and forms a three dimensional network. These polymers do not dissolve in the liquid. Creating this three dimensional network is a result of cross-linking that is again a result of chemical bindings.

There are a numerous monomers that have been used to prepare hydrogels (Table 1; Kopecek and Yang, 2007).

Table 1. Monomers for hydrogel synthesis

Monomers	Neutral		2-Hydroxyethyl methacrylate
			*N*-alkylmethacrylamides
			*N*-alkylacrylamides
			*N*,*N*-dialkylacrylamides
	Acidic		Acrylic acid
			Methacrylic acid
			2-Acrylamido-2-methyl propane sulfonic acid
	Basic		*N*,*N*-dialkylaminoethyl methacrylate
			Methacryloyloxyethyltrialkylammonium bromide
Crosslinking agents			Ethylene dimethacrylate
			Methylenebisacrylamide

The main features of hydrogels influencing their use in wound treatment are:

- Shape stability and softness similar to that of the soft surrounding tissues
- Chemical and biochemical stability
- Absence of extractable
- High permeability for water-soluble nutrients and metabolites across the biomaterial Tissue-interface (Kopecek, 2009).

Hydrogels can be the most suitable dressing in debridement stage of a chronic wound (Vaneau et al., 2007).

To improve the mechanical properties of hydrogels several manufacturing methods were proposed, among which three resulted in significant improvements in the mechanical properties. Namely,

- Double network hydrogels
- Hydrogels containing sliding cross-linking agents
- Nanocomposite hydrogels (Kopecek, 2009).

Double network hydrogels: in this method, two hydrogels are combined together. One of them is a highly cross-linked polyelectrolyte and the other one is a loosely cross-linked or maybe uncross-linked natural hydrogel. This combination will result in an effective relaxation of locally applied stress and dissipation of crack energy (Kopecek, 2009).

Hydrogels containing sliding cross-linking agents: in this method two cyclodextrin molecules get cross-linked. These molecules will create double rings that can move slightly along the PEG chains. This will result in an excellent mechanical property for hydrogel. It will provide a hydrogel with a high degree of swelling and a high stretching ratio without fracture (Kopecek, 2009).

Nanocomposite hydrogels: in this method polymer *N*-isopropylacrylamide (NIPAAm) that is clay-contained is combined with hectorite $[Mg_{5.34}Li_{0,66}Si_8O_{20}(OH)_4]Na0,66$ as a multifunctional cross-linker. The mechanical property of the hydrogel was enhanced and the tensile module and strength were proportional with clay content (Kopecek, 2009).

Some additional advantages of hydrogels as wound dressings are:

- Suitable rheological properties
- Good tissue compatibility
- Convenience in handling
- Ease of application
- Excellent biocompatibility due to their high water content (Kopecek, 2009).

Because of the water medium that is filled in the capillary space of hydrogels, they have good electrical conductivity that can be a benefit using in a combination with iontophoresis penetration method (Liu et al., 2008).
Polymeric systems such as hydrophilic gels, express non-Newtonian pseudoplastic behaviour, which contributes to their spreadability when applied on a biologic surface. As the degree of pseudoplasticity increases, easiness of spreadability augments (das Neves et al., 2009).

1.3.1. Classification of hydrogels

Chemically cross-linked hydrogels: Radical polymerization is usually applied to make these polymers. When these types of hydrogels come in contact with H_2O molecules, they begin to swell up and spread their network (Jagur-Grodzinski, 2009).
Physically cross-linked hydrogels: Physically cross-linked hydrogels do not need introduction of an external cross-linking agent. Cross-linking agents are usually non-degradable and can be toxic and a removal of their residuals may be needed before they can be used in biomedical or pharmaceutical purpose. The physically cross-linked hydrogels are usually biodegradable. Their amorphous hydrophilic phase is held together by highly ordered aggregated chain segments held together by secondary molecular forces such as hydrogen bonding, Van der Waals forces or hydrophobic interaction (Jagur-Grodzinski, 2009).

There are several other classifications for hydrogels. They can also be classified based on the nature of the network: homopolymer, copolymer, interpenetrating, or double networks; physical structure: homogeneous (optically transparent), microporous, and macroporous hydrogels; or in relation to their fate in the organism: degradable and nondegradable hydrogels (Kopecek, 2009).

1.3.2. Characteristics of hydrogels

Hydrogels can be divided into several groups based on their stimuli-sensitivity. Stimuli-sensitivity is related to how different groups of hydrogels express varying degrees of response (continuous or discontinuous changes in swelling) to minor changes in environment conditions, such as pH, temperature, ionic strength, quality of solvent, or biorecognition (Kopecek, 2009).

Temperature responsive hydrogels: examples of temperature responsible hydrogels can be hydrogels containing polymers such as chitosan PEG-poly, *N*-isopropylacrylamide hydrogel (PNIPAA), methyl cellulose and tetronics. These hydrogels are characterized by temperature dependent sol-gel transition T_{gel}, which corresponds to the lower critical solution temperature (LCST), and by the gel-sol transition temperature T_p (upper critical solution temperature, UCST), which corresponds to dissipation or precipitation of a gel (Jagur-Grodzinski, 2009). When the temperature is below LCST, the H_2O molecules make hydrogen bonds with the polar groups of the polymer. These bonds shape kind of hydrophobic groups as iceberg water. When the temperature increases above the LCST, these hydrogen bonds are released to the bulk with a large gain in entropy resulting in collapse of the polymer network. They can be used in sustained drugs, gene delivery and tissue engineering (Kopecek, 2009).

pH responsive hydrogels: in pH responsive hydrogels, the functional group of the polymer gets introduced to a week acidic group such as acrylic acid or week basic groups such as amines. Changes in pK_A and pH value of these polymers make sudden swelling. Some polymers have carboxylic acids as their functional groups. These polymers accept hydrogen at low pH but exchange it for other cations above the pK_A value. They become ionized at higher pH. The hydrodynamic volume and swelling capacity of these polymers increase sharply when these carboxylic groups become ionized and the highest plateau approaches near pH 7 (Jagur-Grodzinski, 2009).

Analyte responsive hydrogels: the analyte responsive hydrogels should function under physiologically relevant temperature, pH and ionic strength. Mono and disaccharides, enzymes, antigens and various ions are among the stimulus for analytic responsive hydrogels (Jagur-Grodzinski, 2009).

1.3.3. Biomedical applications of hydrogles

Hydrogels that are used for biomedical purposes should be biocompatible and often biodegradable.

Drug delivery: right after hydrogels were discovered, their use as anticancer and antibiotic deliver systems was studied.

Hydrogels have a porous network. Often we can control the porosity of hydrogels by controlling the density of cross-links or by changing the swell affinity of hydrogels in the environment. This porosity property of hydrogels helps the release of drugs from hydrogels. The release of drug from hydrogels can be controlled by controlling the diffusion coefficient of drugs through hydrogel matrix. We can also make a depot formulation of hydrogel-drug. The depot formulation can be made by trapping drugs into liposomes and incorporating liposomes in the hydrogel (Kopecek, 2009).

Hydrogels for tissue engineering and regenerative medicine: hydrogels enable the incorporation of growth factors and control over their release. The release rate is controlled by degree of cross-linking of hydrogel. The protein will diffuse out of hydrogel through the water pathways. Gelatin hydrogel was able to release the incorporated growth factor for up to 3 months (Tabata, 2009).

There are many kinds of tissue engineering scaffolds. Among all of these hydrogels are the most popular candidates due to:

- Their structure is similar to the natural ECM
- Their good biocompatibility
- Tunable viscoelasticity
- High water content
- High permeability of oxygen
- Essential nutrients (Jia and Kiick, 2009).

1.4. Carbopol hydrogels

1.4.1. Carbopol

Caropol is made of carbomers. Carbomer polymers are cross-linked together and make a microgel structure that makes them optimal to be used as a drug vehicle for dermatological purposes. They can be used in cases when drug delivery in a controlled manner is desired. The microgel structure makes it possible for these systems to tolerate the physical movement of the body and shape themselves after the application area movement (Islam et al., 2004).

Carbopol polymers are acrylic acid cross-linked with polyalkenyl ethers or divinyl glycol.

These polymers are anionic polymers that need naturalization to become gellified. Organic amines like triethylamine can be used to naturalize these polymers in liquids (Islam et al., 2004).

Carbopol polymers have been used in the personal care industry for forty years. They have been used in producing gels, creams, lotions and suntan products. Among the most commonly used are: Carbopol ETD2001, Carbopol ETD 2020 and Carbopol ETD 2050 (Desai et al., 2006). Carbopol gels have been applied as drug vehicles in several routes of administration.

Advantages with use of carbopol gels as vehicles are:

- Good rheological properties resulting in long statement on the administration site
- Good alternative to oil-based ointment formulations
- Anionic hydrogels with good buffering capacity which contributes to maintenance of the desired pH (Liu et al., 2008)
- High viscosity already at low concentrations
- Wide concentration interval and characteristic flow behavior
- Compatibility with many active ingredients
- Bioadhesive properties
- Good thermal stability
- Excellent organoleptic characteristics
- Good patient acceptability (Islam et al., 2004).

Islam et al. (2004) studied the rheological behavior of topical carbopol gels in a pH range between 5.0 and 8.0. Propylene glycol and glycerol were used as co-solvents. The gels were showing the behavior of elastic systems. The elasticity of these networks can be explained by that triethylamine as a swelling agent enabling closely packing and the tight binding between the long chains and the side chains.

Carbopol gels show a pseudoplastic behaviour at high rate. The polymer chain segments deform and adjust themselves in the direction of flow. The pesudoplastic and non-Newtonian features of gels can be explained by the solvent-solvent and solvent -polymer interaction and higher viscosity of glycerol as a co-solvent. The viscosity of Carbopol gels increases with pH as well as by addition of glycerol (Islam et al. 2004).

1.4.2. Carbopol Ultrez 10

Carbopol Ultrez 10 polymer is one of the newest Carbopol polymers with very good ability of dispersing and gels could be prepared without extensive mixing (Desai et al., 2006). Carbopol Ultrez 10 polymer has a very low dispersion viscosity and unlike other traditional Carbopols, the dispersing and swelling process will accelerate with

an increase in temperature. The temperature can be increased up to 60 ºC without having influence on the quality of gel. Triethylamine is an appropriate neutralizing agent that can be used in gel manufacturing (Desai et al., 2006).

Beside easy dispersing ability and low termosensitivity, Carbopol Ultrez 10, has added advantages as; high viscosity already at low concentrations, wide viscosity interval and characteristic flow behavior, compatibility with many active ingredients, good bioadhesive properties, good organoleptic characteristics and good patient acceptance (Fresno Contreras et al., 2001).

1.5. Liposomes

Liposomes are vesicles made of phospholipid bilayers. These phospholipid bilayers surround an aqueous core. Liposomal size is directly related to the method of preparation and can range from 50 nm to several microns. They form spontaneously when these lipids are dispersed in aqueous media. Vesicles can be constructed of natural constituents such that the vesicle membrane forms a bilayer structure which is principal identical to the lipid portion of natural cell membrane. Their ability to mimic the behaviour of natural membranes and also to be degraded by the same pathways, makes vesicles a very safe and efficacious vehicle for medical applications. Vesicles can be composed even of entirely artificial components, chosen for their improved chemical properties (e.g. fatty acids, double chain secondary amines, cholesterol derivates). Moreover, liposomes may entrap both hydrophilic and lipophilic molecules; and be used as drug carrier for both types of drug molecules (Cevc, 2004).

Vesicle membranes are semi-permeable membranes, in that the rate of diffusion of molecules and ions across the membrane varies considerably. For molecules with high solubility in both organic and aqueous media, a phospholipid membrane clearly constitutes a very tenuous barrier, but polar solutes and higher molecular weight compounds pass across the membrane only very slowly. Release rate of different

types of drug molecules from liposomes is dependent on the type of drug applied (New, 1990).

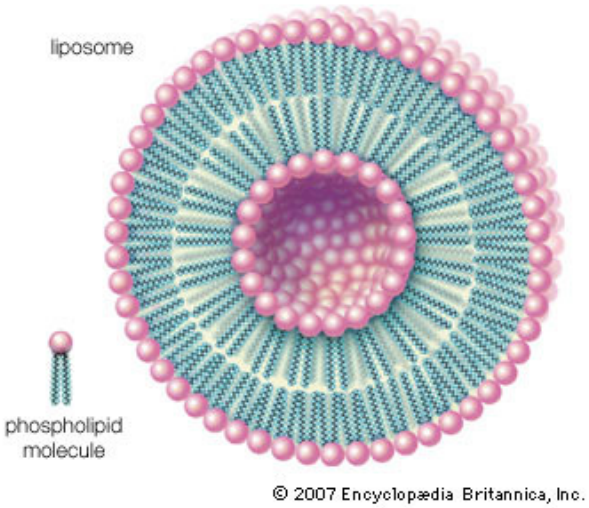


Figure 9: Liposomal structure (www.britannica.com).

Cholesterol may be included to improve bilayer characteristics of vesicles, increase microviscosity of the bilayers, reduce permeability of the membrane to water soluble molecules, stabilize the membrane and increase the rigidity of the vesicles (New, 1990).

Classification of vesicles according to size is the most common index of characterization in current use:

- Multilamellar vesicles (MLVs). Vesicles cover a wide range of sizes (100 – 1000 nm) and consist of five or more concentric lamellae.
- Small unilamellar vesicles (SUVs). These vesicles are at or close to the lower size limit (15 -25 nm), so they will be a relatively homogeneous population in terms of size.
- Intermediate-sized unilamellar vesicles (IUVs). Vesicles with diameter of the order of magnitude of 100 nm.
- Large unilamellar vesicles (LUVs). Vesicles with diameter of the order of 1000 nm (New, 1990).

Very often the preparations are meta-stable. That means that the state of free enthalpy is not in equilibrium with the environment. As a result, the vesicles change their lamellarity, size, size distribution and shape with time. For example small vesicles

tend to form larger ones and large vesicles smaller ones. Nevertheless, the stability seems to be the optimal in a range of about 100-300 nm (Lautenschlager, 2006).

1.5.1. Topical applications of liposomes

The major obstacle for topical drug delivery is the low diffusion rate of drugs across the stratum corneum. Several methods have been proposed to increase the permeation rate of drugs temporarily. One of the most promising approaches is the application of drugs in vesicle-based formulations (Bouwstra and Honeywell-Nguyen, 2002).
Visicles in dermal and transdermal delivery systems can be used as:

- Deliver the entrapped drug into or through skin
- Act as penetration enhancers
- Deliver dermal active compounds in form of a depot system
- Be as a rate limiting membrane barrier and modulate the systemic absorption (Honeywell-Nguyen and Bouwstra, 2005).

Liposomes can be employed as a drug carrier, or they can act as penetration enhancers that increase the transport rate of drug across the skin (Figure 10; Honeywell-Nguyen and Bouwstra, 2005).

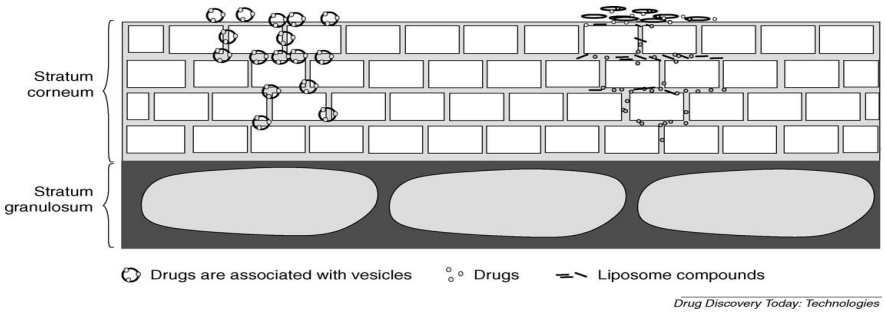


Figure 10: Different ways that liposomes can release drug into the skin (Honeywell-Nguyen and Bouwstra, 2005).

The exact mechanism of liposomal action as penetration enhancer is still not agreed on, namely some of the authors believe that when liposomes containing drugs are applied onto the skin, they disrupt the skin lipids which causes skin partitioning and

the drug dissolves in skin cells (Fang, 2006). Another possibility would be that liposomes become one part of skin layer and release drug into the skin (Fang, 2006). Recently, more flexible types of vesicles were proposed to be able, through transdermal osmotic gradients and hydration force, to reach not only stratum corneum but the systemic circulation as well (Fang, 2006).

In brief, three ways that liposomes can penetrate through skin can be described as:

- Lateral diffusion of liposomes in the stratum corneum
- Via a trans-epidermal osmotic gradient (liposomes get sucked into the epidermis)
- Via the pilosebaceous units (Leeuw et al., 2009).

Ferderber et al. (2009) studied and compared the ability of liposomes, transfersomes, microemulsions and micellar solutions as drug delivery systems applied onto skin. Skin permeation decreased for tested drugs by increasing the content of phosphatidylcholine in the formulations. The permeation rate was increasing in this range: micelles>transfersomes>liposomes>microemulsion. The hydrophilicity and lipophilicity of drug influence the drug permeability through skin. Lipophilic drugs penetrate faster through skin especially when the carrier system is lipophilic as well (Ferderber et al., 2009).

1.5.2. Liposomal hydrogels

Liposomal gels have the advantages that they enhance the skin retention of drugs, provide higher and sustained concentrations of drug in skin and at the same time do not enhance the systemic absorption of drugs. They can also serve as a drug reservoir that provides a localized and controlled drug delivery and it is also possible to deliver sufficient amount of drugs into skin by using liposomal gels (Dragicevic-Curic et al., 2009).

Lipid composition of liposomes and the lipid concentration of liposomes incorporated into hydrogels are two most important factors that influence the rheological

properties of gels. In the case of hydrophilic drugs, release is not affected by the amount of lipid loaded in gels, but can be affected by the amount of rigid membrane liposomes that are used. For lipophilic drugs, lipid concentration added in the gel has a strong affect on the drug release and the rigidity of membranes is not important (Mourtas et al., 2008).

Carbopol hydrogels as vehicles for liposomes have ability to enhance local delivery of drugs in the vagina (Pavelic et al., 2005).

The release of drug is controlled by degradation of hydrogel matrix. One can control release rate of drug by tailoring the hydrogel degradation (Huang and Fu, 2010).

Beukelman et al. (2008) developed liposomal hydrogels containing PVP-ILH (povidone iodine). The hydrogel had both the moistening effect and an anti-infection effect. Moistening effect was contributed to carbomer based hydrogels and anti-infective effect was a result of PVP-ILH.

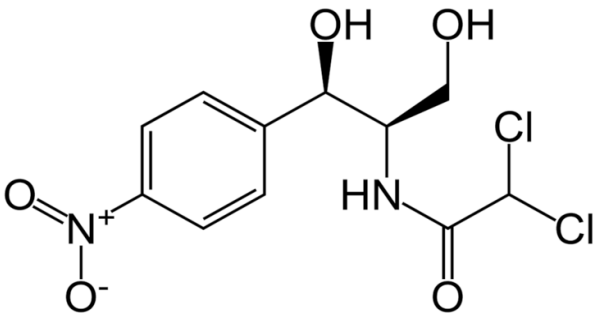
Shishu and Aggarwal (2006) developed stable hydrogels as vehicle for griseofulvin. The hydrogels were transparent, non-sensitizing, non-irritating and non-gritting. All the formulations were easily spreadable and non-dripping in nature.

Methotrexate (MTX) that can be used for treatment of psoriasis has been administrated mainly via oral route. Mohamed Ali et al. (2008) prepared photosensitive liposomes containing MTX for treatment of localized psoriasis via topical administration. Liposomal MTX was incorporated in 2% Carbomer 974 NF gel. Gel was applied once a day and followed by 80-J laser session 3 times weekly. This regime improved the treatment in comparison to control (Mohamed Ali et al., 2008).

1.6. Chloramphenicol

Chloraphemicol was used as model antimicrobial drug. This antimicrobial agent inhibits bacterial growth by inhibiting protein synthesis in bacteria. Chloramphenicol has mainly bacteriostatic effect on Gram negative, Gram positive organisms and rickettsiae, but has a strong bactercid effect on *H. influenzae*. Chloramphenicol can be administered via oral, parenteral and topical route. It has a half-life of only 2 hours.

The adverse effects of chloramphenicol are bone marrow suppression, leukemia and gray baby syndrome (Rang et al., 2007).



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 sutureted wounds after minor surgery procedure resulted in relative reduction in infection rate of about 40 %.

Moreover, the formulation choice for chloramphenicol topical dosage forms is limited by its solubility and represents pharmaceutical challenge.

2. Aim of the study

Burn wounds are complicated wounds that influence first and foremost the functional activity of the skin and above all the vital activity of skin cells. In burn wounds a large area of skin can be affected, and the dressings which can be applied to the affected area should provide the protection and also improve the natural healing process, as well as be skin-friendly.

Among the formulations to be applied on damaged skin, hydrogels have shown the superiority as they can provide a moist environment for the wound and at the same time deliver the incorporated drug to the wound. Carbopol hydrogels have proven good bioadhesive properties on the mucosal surface and skin surface. Moreover, by incorporating liposomes into Carbopol hydrogels the release of the incorporated liposomal drugs will be prolonged and in a more controlled manner.

The aim of the study was to develop liposomal hydrogel to be applied onto wounded skin. For that purpose:

- The properties of Carbopol gels were evaluated on texture analyzer to optimize the formulation.
- Selected gel composition was used as vehicle for liposomal suspensions incorporating model antimicrobial drug, chloramphenicol.
- The effect of liposomal suspension on gel properties was determined by texture analysis.
- The stability of Carbopol hydrogels as well as liposomal hydrogels were studied at accelerated conditions.
- Based on the results, final conclusions and comments are drawn.

3. Materials and methods

3.1. Materials

Gel: Caropol Ultrez 10 NF was purchased from Noveon (Cleveland, USA); Triethylamine (TEA) was the product of Merck Schuchardt (Hohenbrunn, Germany); Acetic acid (glacial) was purchased from Merck KGaA (Darmstadt, Germany)

Liposomes: Methanol (HPLC grade) was the product of Merck KGaA (Darmstadt, Germany); Lipoid S 100 was a generous gift from Lipoid GMBH (Ludwigshafen, Germany); Chloramphenicol was the product of Sigma Aldrich (Steinheim, Germany)

Others**:** Triglicerids were obtained from Fagron (Barsbuttel, Germany)

3.2. Preparation of hydrogels

Carbopol Ultrez 10 has very good dispersion ability and forms gels rapidly. In brief, the gels (0.2, 0.5 and 1% w/w, respectively) were prepared by the following procedure (Skalko et al., 1998): Carbopol resin (weight in grams) was dispersed in distilled water (volume according to the desired concentration of gel). The mixture was stirred until thickening occurred and then neutralized by drop-wise addition of 50% (w/w) triethylamine, until a transparent gel appeared. Quantity of triethylamine was adjusted to achieve gel with desired pH. Gels were stored for 24 hours at the room temperature to stabilize.

3.3. Gel characterization

The texture characterization of gels was performed on Texture analyzer TA.XT plus Texture Analyzer (Stable Micro Systems Ltd., UK). The following was studied:

- The correlation between the gel concentration and its texture
- Gel consistence and changes in texture as results of change in pH of a gel
- Changes in gel texture in relationship to gel stability

Texture analyzer:

Texture analyzer is an instrument that measures the response of a sample to:

- Tension
- Compression
- Penetration
- Bend

When the probe comes in contact with the sample, the instrument begins to measure the triggered force by test speed. The probe travels into the sample at the test speed until the specified force, distance or stain is reached. After that, the probe returns to the start at the post test speed. The probe continues moving upward and measures stickiness of the sample. The probe continues moving until it returns to the starting position.

The texture analyser can measure tension, compression, penetration and bend in three different ways.

- Backward extrusion
- Forward extrusion
- Multiple extrusion

In backward extrusion, the probe is forced through sample and the product extrudes around the disc. Backward extrusion can be used for viscous liquids and sauces, gels, pastes, etc. In forward extrusion the sample is forced through an orifice in bottom of pot. Forward extrusion can be used for viscous liquids and sauces, gels, pastes and processed fruit and vegetables. Multiple extrusion measures the structural breakdown of semi-solid materials e.g fats (Stable Micro Sytems, TA.XT plus, Texture analyser).

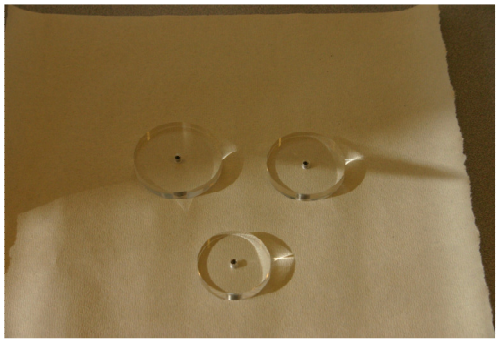
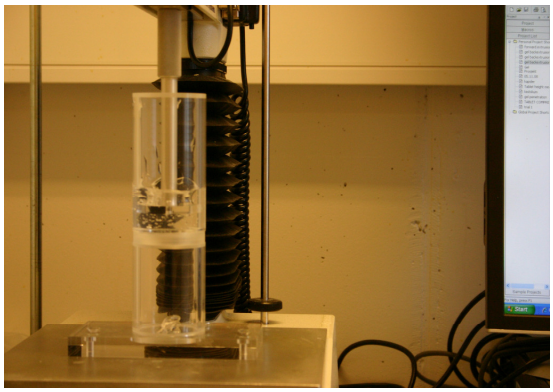
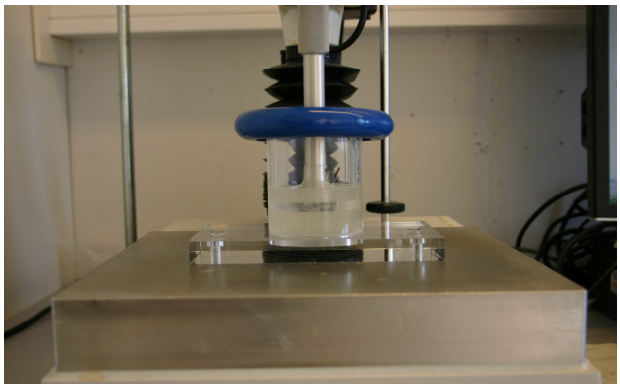


Figure 11: Probes with 35, 40 and 45 mm in diameter.





A **B**

Figure 12: Forward (A) and backward extrusion (B).

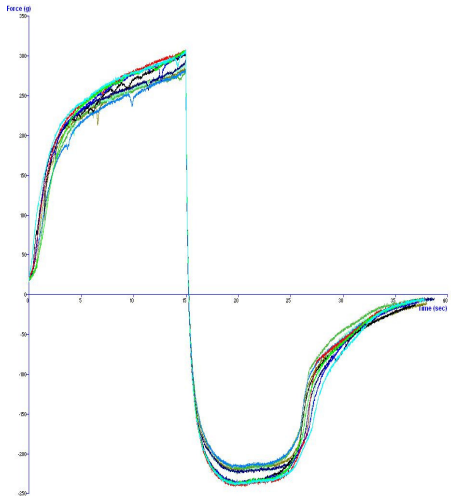


Figure 13: Typical profile of backwards extrusion.

Comment: when a surface trigger is attained (i.e the point at which the disc's lower surface is in full contact with the product) the disc proceeds to penetrate to a depth of 15 mm (or other specified distance). At this point (most likely the maximum force), the probe returns to its original position (Figure 13). The peak or maximum force is taken as a measurement for firmness/hardness. The higher the value the firmer is the sample. The area under the curve up to this point is taken as a measurement of consistency. The higher the value is the thicker consistency of the sample. The negative region of the graph, produced on probe return, is a result of stickiness that sucks the probe downwards. Due to back extrusion it gives again an indication of

consistency/resistance. The maximum force is taken as an indication of the cohesiveness of the sample the more negative the value the more cohesive is the sample. The area of the negative region of the curve may be referred to as the work of cohesion, the higher the value the more resistant to withdrawal the sample is which is an indication of the cohesiveness and also consistency/viscosity of the sample. The positive area in the graph is a measure of the total forces required to extrude (consistency). The negative area is a measure of the total resistance to withdrawal force indicating sample consistency and index of viscosity. Maximum peak in the positive area shows the maximum sample firmness. Maximum peak in the negative area shows the maximum sample cohesiveness (Figure 13; Stable Micro Sytems, TA.XT plus, Texture analyser).

3.3.1. Development of reproducible method for determination of hydrogel texture

As the texture analyzer was not previously employed to characterize texture properties of hydrogles in this laboratory, we needed to establish the reproducible method of measurement. The first focus of the method development was the measurement of gel hardness. All measurements were performed by three independent researchers and in triplicates. As the start, 0.5 % Carbopol gel was used. The measurements were performed in two different ways (Table 2):

Table 2: Experimental set up for gel characterization

First method		Second method	
Pre-test speed	1 mm/s	Pre-test speed	1 mm/s
Test speed	0.7 mm/s	Test speed	1 mm/s
Post-test speed	0.7 mm/s	Post-test speed	1 mm/s
Distance	15 mm	Distance	15 mm

Based on preliminary results, all future measurements were done under the following conditions:

TA settings:

- Mode: measure force in compression
- Option: return to start
- Pre-test speed: 1.0 mm/s
- Test speed: 1.0 mm/s
- Post-test speed: 1.0 mm/s
- Distance: 15 mm
- Trigger force: 20 g

Accessories: 35 and 40 mm disc on extension bar, 5 kg load cell

3.3.2. Influence of polymer concentration on gel properties

Three different gel concentrations, namely 0.2, 0.5 and 1% were prepared and their texture properties measured by using 40 mm diameter compression disc.

3.3.3. Influence of pH on gel viscosity

Three batches of two different gel concentrations, namely 0.2 and 0.5 % were prepared and analyzed on texture analyzer. Each sample was measured 5 times. Triethylamine was used to neutralize the gel and to prepare gels with different pH values given below:

0.2% Carbopol gel	pH values: 4.6	5.4	5.8	6.9	10.1	10.8	11.1
0.5 % Crabopol gel	pH values: 4.2	4.8	5.9	6.4	7.0	8.3	10.2

3.4. Stability testing of hydrogels

Accelerated stability testing was used to study the influence of temperature on the gel stability. For that purpose, 0.5% Carbopol hydrogel with pH values of 4.2, 4.7, 5.8, 6.9, 8.2 and 10.4, respectively were kept in a thermostat at 40 °C. After one month period, the gels were analyzed on texture analyzer as previously described.

3.5. Preparation of liposomal hydrogels

3.5.1. Preparation of liposomes

Empty: Liposomes were prepared by the conventional film method (New, 1990). Firstly, phospholipids (200 mg) were dissolved in concentrated methanol (approx 100 ml) in a round bottomed flask. The solvent was then completely removed on a rotary vacuum evaporator (Buchi waterbath B-480, Buchi vacuum controller B-721, Buch rotavapor R-124, Switzerland) and the lipid film deposited on the flask wall was hydrated by the addition of 10 ml of distilled water. Dispersion was hand-shaken for 20 min and left overnight prior to the characterization.

Liposomes containing chloramphenicol: The procedure was identical to the previously described one, except that chloramphenicol (20 mg) dissolved in methanol was mixed with methanol solution of lipid and solvent removed on a rotary vacuum evaporator. All other steps were identical to the procedure for empty liposomes.

3.5.2. Entrapment efficiency determination

Ultracentrifugation was applied as separation method. Liposomes were centrifuged in Beckman-L8-70M ultracentrifuge (CA/USA) at 10 °C, for 25 min period at 32000 rpm in order to separate unentrapped chloramphenicol from liposomally entrapped drug. Upon centrifugation, the pellet was resuspended in 1500 μl distillated water, and an aliquot (10 μL) further diluted and used in spectrophotometrical and HPLC analyses. An aliquot (30 μL) of the supernatant was further diluted with methanol and the chorlamphenicol 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 (CA/USA) at 268 nm wavelength.

HPLC analysis: HPLC system consisted of a Water separation module 2695 and Water 2487 UV-spectrophotometer detector. Column used was a XTerra^{TM} RP_{18} 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_2O and 0.1% acetic acid (glacial). The temperature of column was maintained at 35 ºC±5 ºC and the temperature of samples was maintained at 35 ºC±2 ºC 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.

3.5.3. Particle size analysis

Diameters of vesicles were measured by dynamic light scattering (DLS) on the NicompTM model 380 particle sizing system (USA) with software version C-370 V-1.51a, and equipped with a fixed 90^{o} external fiber angle and a 632.8 nm, 5 mW He–Ne laser. 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 nm 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).

3.5.4. Incorporation of vesicles in hydrogels

Based on the evaluation of texture properties, 0.5 % Carbopol gel was selected as the most suitable vehicle for liposome incorporation. Liposomes (either empty or in the presence of unentrapped drug) were incorporated into Carbopol gels following the procedure described by Skalko et al. (1998). Briefly, liposomes were mixed into hydrogels by the help of hand mixing, with the concentration of liposomes in the gel being 5, 10 and 15 % (w/w, liposomal suspension/total), respectively.

3.5.5. Characterization of liposomal hydrogels by texture analysis

Empty 0.5% Carbopol hydrogel was examined again to assure that the measuring conditions are indeed the same. Liposomal hydrogels were examined in the following sequence:

- empty 0.5% gel
- 0.5% gel with 5% empty liposomes
- 0.5% gel with 10% empty liposomes
- 0.5% gel with 15% empty liposomes

All measurements were performed five times.

3.5.6. tability testing of liposomal hydrogels

The procedure and the conditions were identical to the procedure described for stability testing of hydrogels (**3.4**).

3.6. In vitro release studies

To study the release of chloramphenicol from liposomal hydrogels, the *in vitro* release model for topical dosage forms (“Freiburger SchlangeSchnecke”) was used (Figure 14). Liposomal hydrogels (25 g) containing liposomally-unentrapped chloramphenicol as well, were spread on the donor side of the release cells. Triglycerides (50 mL) served as an acceptor phase in the experiment.

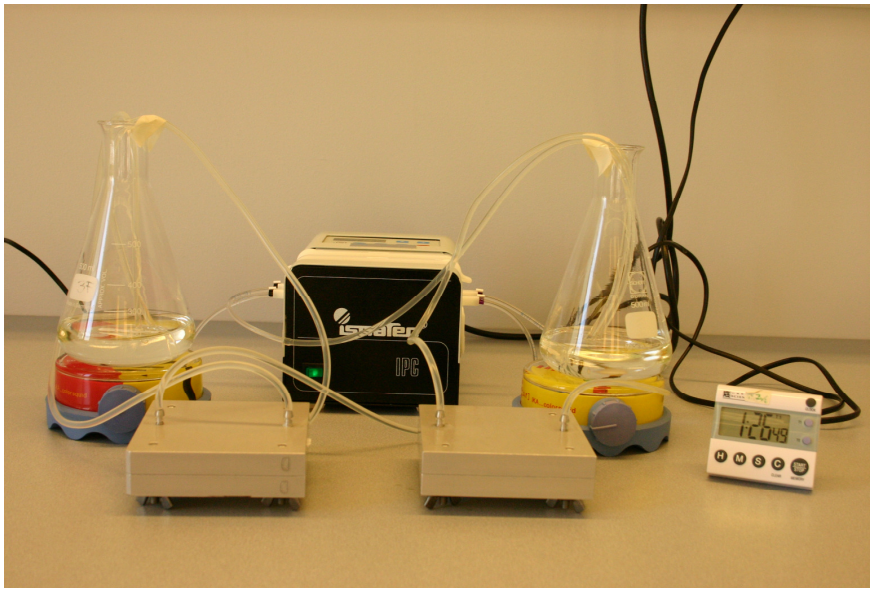


Figure 14: *In vitro* release study set up.

The release was studied during 300 minutes time period. Samples were taken at predetermined time intervals, namely at 5, 10, 15, 25, 35, 45, 60, 75, 90, 180 and 300 min. The concentration of chloramphenicol was determined by HPLC method. The procedure and the conditions were identical to the procedure described in **3.5.2.**

4. Results and Discussion

The first step in optimizing the hydrogel formulation destined for wound treatment is to have a reproducible and simple method for determination of the texture properties of the gel, as bioadhesivness and viscosity affect the retention time at the applications site, and the retention time is directly correlated to the efficiency of the therapy. Insight on the texture properties would shorten the optimization of formulation process.

4.1. Development of reproducible method for determination of hydrogel texture

Table 3: Trial measurements for Carbopol hydrogel

Measurement	Force 1 (g)	Area 1 (g sec)	Force 2 (g)	Area 2 (g sec)
1. Average	308.5	4051.8	-200.1	-1700.5
SD	8.7	61.6	4.2	20.3
CV	2.8	1.5	-2.1	-1.1
2. Average	316.7	4044	-211.2	-1727.2
SD	9.4	117.6	4.3	66.7
CV	2.9	2.9	-2	-3.8
3. Average	306.2	3728.2	-218.8	-1603
SD	19.4	119.7	8.9	23
CV	6.3	3.2	-4.1	-1.4
Average mean	310.5	3941.3	-210	-1676.9
SD average	14.1	182.7	9.8	68.1
CV average	4.5	4.6	-4.7	-4

The gel concentration used was 0.5% (w/w) and the pH value 6.0

Measurement conditions: distance 15 mm, speed 0.7 mm/s, 35 mm probe, 5 runs.

The measurements were performed by three independent researchers to gain insight on the effect of experimenter on the measurements.

Table 4: The effect of measurement speed on measurements

Measurement	Force 1 (g)	Area 1 (g sec)	Force 2 (g)	Area 2 (g sec)
Average	337	3085	-200.2	-1991
SD	1.2	26.3	1.4	51.8
CV	0.3	0.8	-0.7	-2.6
Average	337	3085	-200.2	-1991
SD	1.2	26.3	1.4	51.8
CV	0.3	0.8	-0.7	-2.6
Average	312.6	2984.4	-194.9	-1672.7
SD	9.9	71.1	7.6	53.8
CV	3.1	2.4	-3.9	-3.2
Average mean	328.9	3039.5	-198.4	-1884.9
SD average	12.8	79.3	5.2	158.9
CV average	3.9	2.6	-2.6	-8.4

The gel concentration used was 0.5% (w/w) and the pH value 6.0
Measurement conditions: distance 15 mm, speed 1.0 mm/s, 35 mm probe, 5 runs.
The measurements were performed by three independent researchers to gain insight on the effect of experimenter on the measurements.

We could observe the clear difference in the values in regard to the changed speed of the measurement. Although is the texture analyzer widely used in pharmaceutical applications, particularly pharmaceutical industry, not much scientific information is available on the effect of measurement set up on the measurement values.
Most of the literature available is on use of texture analyser to optimise adhesiveness and cohesiveness of water-in-oil emulsions (Lemaitre-Aghazarian et al., 2004),

evaluate mucoadhesive properties of various polymers (Cilurzo et al., 2005), test tablet disintegration from fast-dissolving preparations (El-Arini and Clas, 2002) and correlation between drug dissolution and polymer hydration (Li and Gu, 2007).

Moreover, texture analyzer has been used widely in food industry as well Zhuang et al., 2007; Brighenti et al., 2008; St-Gelais et al., 2009.

The main advantage of using this instrument in pharmaceutical applications is the possibility that by changing probes or measurement parameters, this instrument can be used for multiple pharmaceutical drug dosage forms, from solid to liquid-like (Li and Gu, 2007).

Since use of texture analyzer to measure gels texture was not reported before, finding a reproducible method to measure the viscosity of gels with texture analyzer was important.

The decision was to measure the viscosity with 1mm/s speed. This condition resulted in a faster method.

4.1.1. Influence of polymer concentration on gel properties

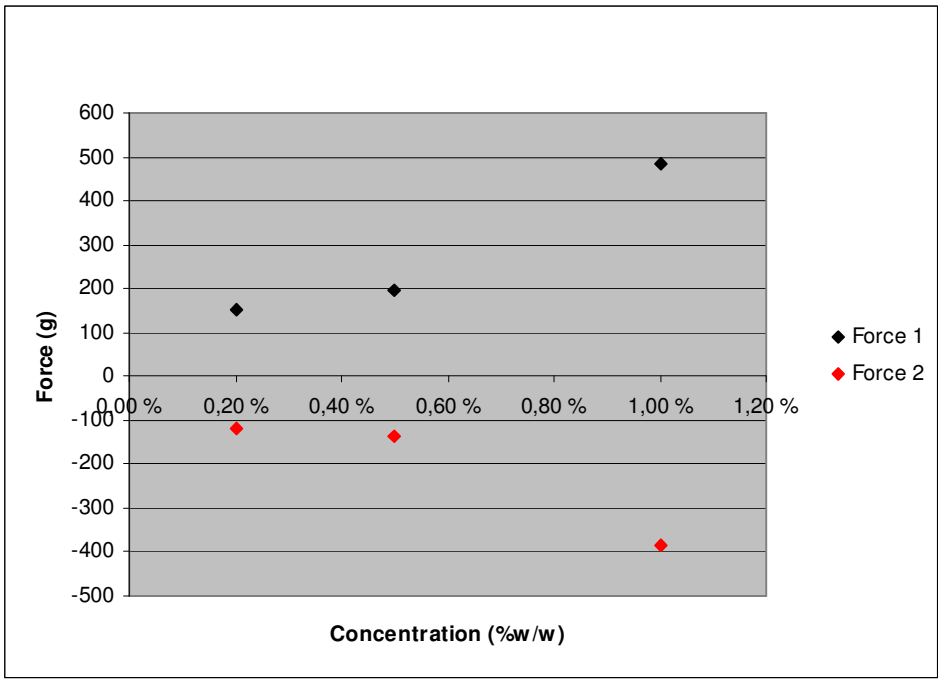


Figure 15: The effect of gel concentration on force.

The gels used were 0.2, 0.5 and 1.0 % (w/w) Carbopol hydrogel and the pH of gels varied between 5 to 6. Measurement conditions: distance 15 mm, speed 1.0 mm/s, probe: 40 mm, 5 runs.

As can be seen from Figure 15, by increasing the concentration of Carbopol polymers in the hydrogel, the forces increase. Obviously the force that texture analyzer uses to move in 1% Carbopol hydrogel is almost 5 times more than force that is used for 0.2% Carbopol hydrogel.

Studying the rheological and mechanical properties of polymers is important since through these studies it can be possible to design vehicles that have the optimal properties. Studying mechanical behaviour of Carbopol hydrogels was one of the first points that we focused on in our work.

When poly acryl acid (PAA) polymers come in contact with an aquaous environment, they behave as anionic polyelectrolytes and form week hydrogel structures. When the neutralisation agent is added, a stable three dimensional viscoelastic network is formed. When the base is added to the polymer in the aqueous environment, the repulsion of negative charges along the polymer backbone gets enhanced and the osmotic pressure inside the swelling polymer increases and cause formation of a three dimensional structure (Tamburic and Craig, 1996). In other words, Carbopol macromolecules associate together, get mixed together and make a flock in solutions and this determines the Carbopol gel structure (Fresno Contreras et al., 2001).

By increasing a concentration of polymer in the aqueous environment, there will be larger amounts of polymer chains that get cross-linked together after adding the neutralization agent. Higher concentration of polymers in the aqueous environment, produces more packed and compact network of gels that is harder to move when applying the force to it (Fresno Contreras et al., 2001).

4.1.2. Influence of pH on gel texture properties

4.1.2.1. Influence of pH on viscosity of 0.2% (w/w) Carbopol gel

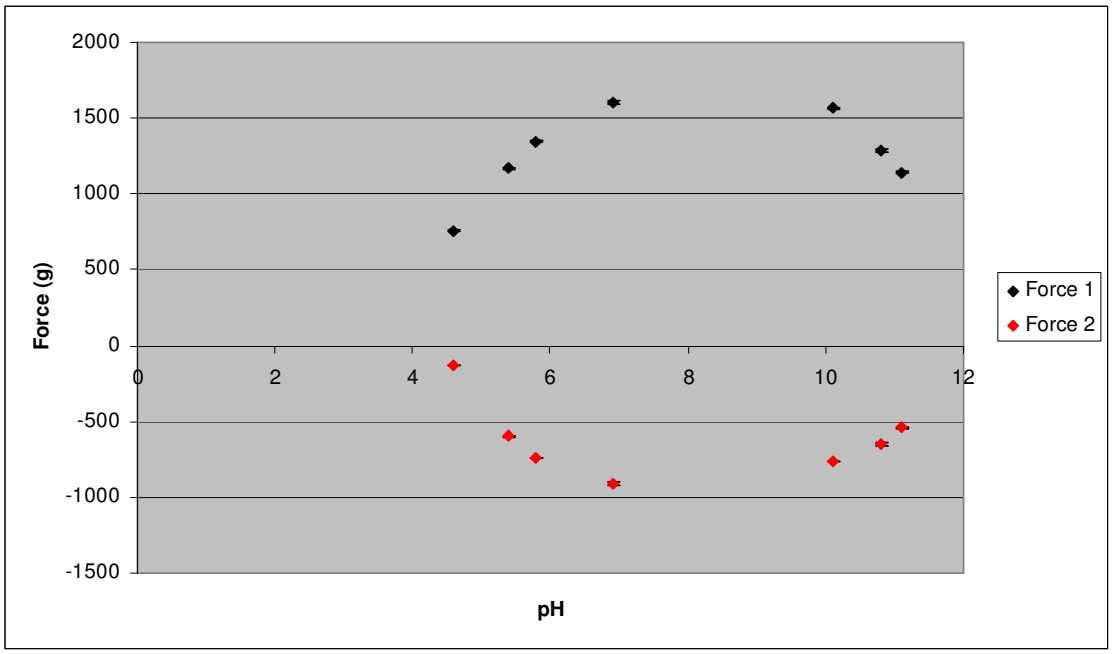


Figure 16: The effect of pH of 0.2% Carbopol hydrogel on force.
The gel used was 0.2 % (w/w) Carbopol hydrogel. Measurement conditions: distance 15 mm, speed 1.0 mm/s, probe 35 mm, 5 runs.

The Figure 16 indicates that from pH 4 and up to pH 7, the cohesivness increases. From pH 7 to pH 10, not much change in cohesiveness was observed, from pH value of 10 and upward the cohesiveness decreases.

Although we could clearly observe that the changes in pH values of hydrogels resulted in changes in forces, one has to keep in mind that the formulation is intended to be applied onto wounded skin, therefore the pH values need to be in a range acceptable for skin application.

4.1.2.2. Influence of pH on viscosity of 0.5% (w/w) Carbopol gel

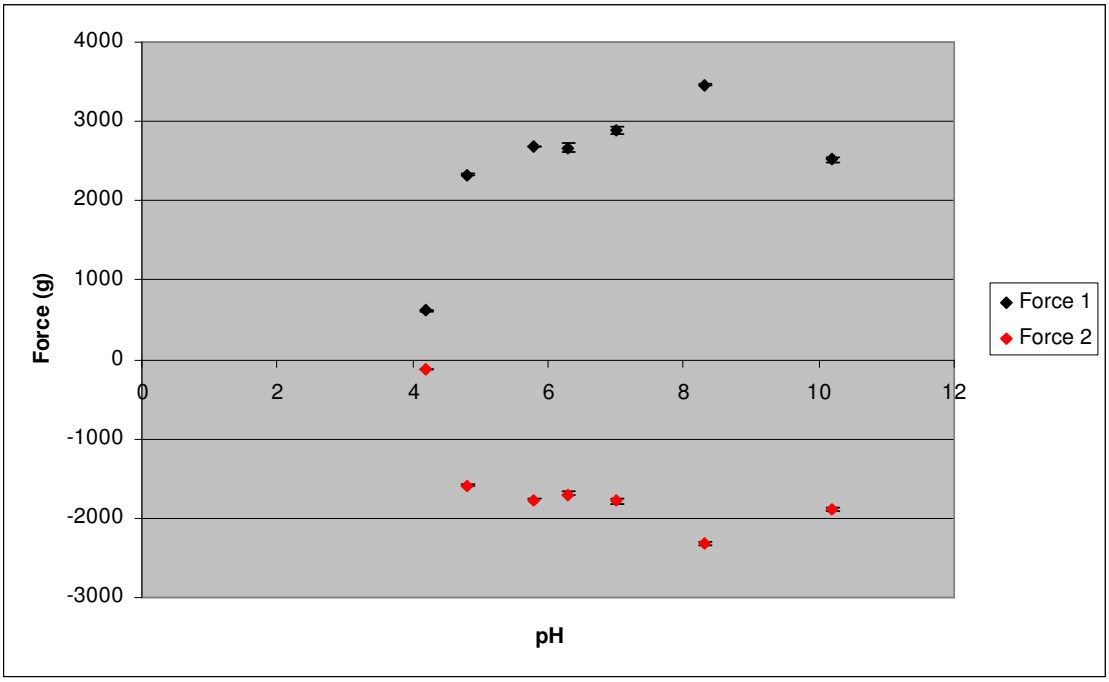


Figure 17: The effect of pH of 0.5% Carbopol hydrogel on force.
The gel used was 0.2 % (w/w) Carbopol hydrogel. Measurement conditions: distance 15 mm, speed 1.0 mm/s, probe 35 mm, 5 runs.

The Figure 17 is almost identical to Figure 16. The cohesiveness increases from pH 4 and up to pH 9. From pH 9 and upward, the cohesiveness decreases.

In concord with some other researchers, increase of pH at constant polymer concentration gave us a decrease of flow index. In other words, increasing the pH gives the gel an increase in pseudoplastic character (Fresno Contreras et al., 2001).

The manufacturer Noveon (Lubrizol Corporation) showed in one of their rapports about Carbopol polymer Ultrez 10, the relation between viscosity and pH. In their graphs the cohesivness increased up to pH 6, remained stable from pH 6 to 10 and decreased from pH 10 and upwards.

The manufacturer used in gel formulations the 18% NaOH as neutralizer while our neutralizer was triethylamine (TEA). TEA is a better neutralisation agent than NaOH and Tromethamins (TRIS). There are a greater charge barrier in TEA layer than NaOH and TRIS. Binding ability of TEA cations to PAA is better than TRIS and

NaOH and it gives a higher degree of polymer chain expension (Tamburic and Craig, 1996).

4.1.3. Stability testing of hydrogels

The measurement as presented in Figure 17 was done after the gel was tested for accelerated stability at 40 °C (1 month period). The results are presented in Figure 18.

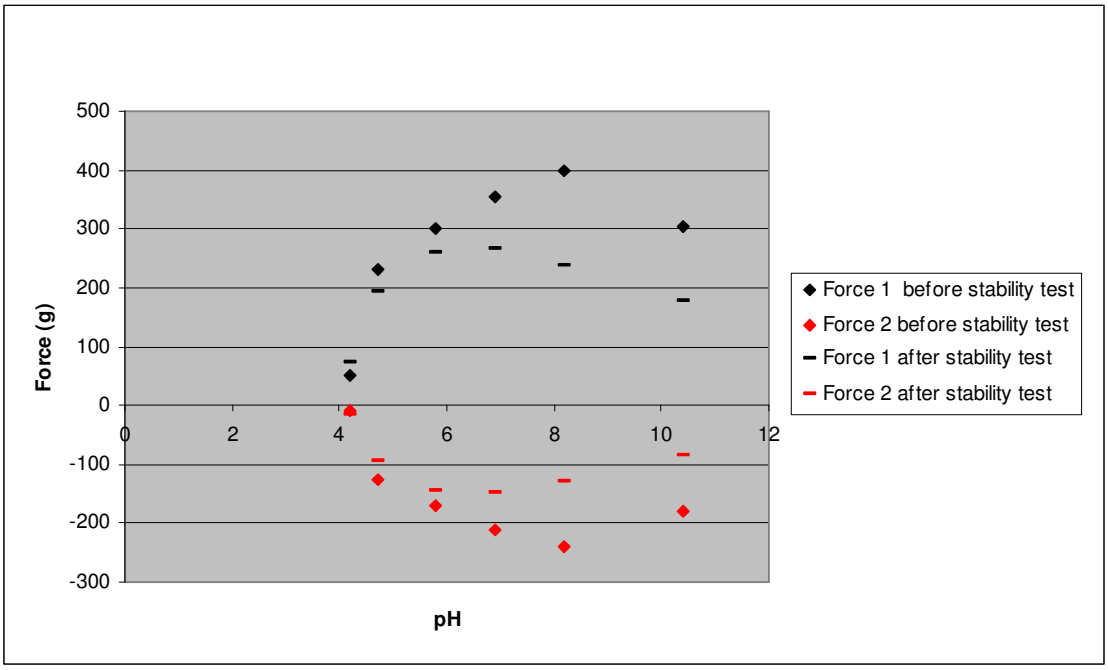


Figure 18: Changes in gel cohesiveness in relationship to pH after storage at 40 °C. Gel (0.5% Carbopol hydrogel, varying in pH) was measured before and after the stability testing. Measurement conditions: distance 15 mm, speed 1.0 mm/s, probe 35 mm, 5 runs.

As can be seen for Figure 18, the cohesiveness for almost all gels after they were stored at 40 °C was found to be lower as compared to freshly prepared gel. The differences were more pronounced for pH values from 7 to 10.

By increasing the temperature, the mobility of polymer chains increase and we can see a fall in the gel cohesiveness. Increase mobility of polymer chains, gives a shorter chain entanglement lifetime and it gives a more Newtonian flow to the gels (Fresno

Contreras et al., 2001). It is known from literature that PAA hydrogels do show changes in original structure after storage tests. This can be because of catalytic degradation of polymer chains and changes in the distribution of neutralising ions within the gel. The ions within the gel can get released from the immediate vicinity of the polymers gels into the bulk of aqueous phase (Tamburic and Craig, 1995).
However, more recent publications on Carbopol Ultrez 10 gels suggest a little viscosity change under the normal storage and use temperatures. One of the advantages of using Carbopol Ultrez 10 should be the fact that the possibility of unaccepted changes in the product's characteristic will be minimised (Fresno Contreras et al., 2001).

4.2. Liposomal characterization

Table 5: The characteristics of liposomes containing chloraphenicol

Amount of lipid (mg)	Particle size ± SD (nm)	Polydispersity Index (PI)	Entrapment efficiency (%)	Drug to lipid ratio (µg/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 vesicles, resulting in higher PI values as well. Due to this fact, for preparation of liposomal hydrogles we used liposomal dispersion containing both liposomal and unentrapped (free) chloramphenicol as entrapment was found to be rather high (Table 5).

4.3. Characterization of liposomal hydrogels by texture analysis

4.3.1. The effect of added liposomes on texture properties of gel

Liposomal suspesnions containing both entrapped and free drug were incorporated in the hydrogels because liposomes can be used as a drug vehicle for both hydrophilic and lipophilic drugs. Liposomes can be seen as an "organic solvent" for the solubility of poorly soluble drugs and a local depot for dermally active compounds (Lee et al., 2007).

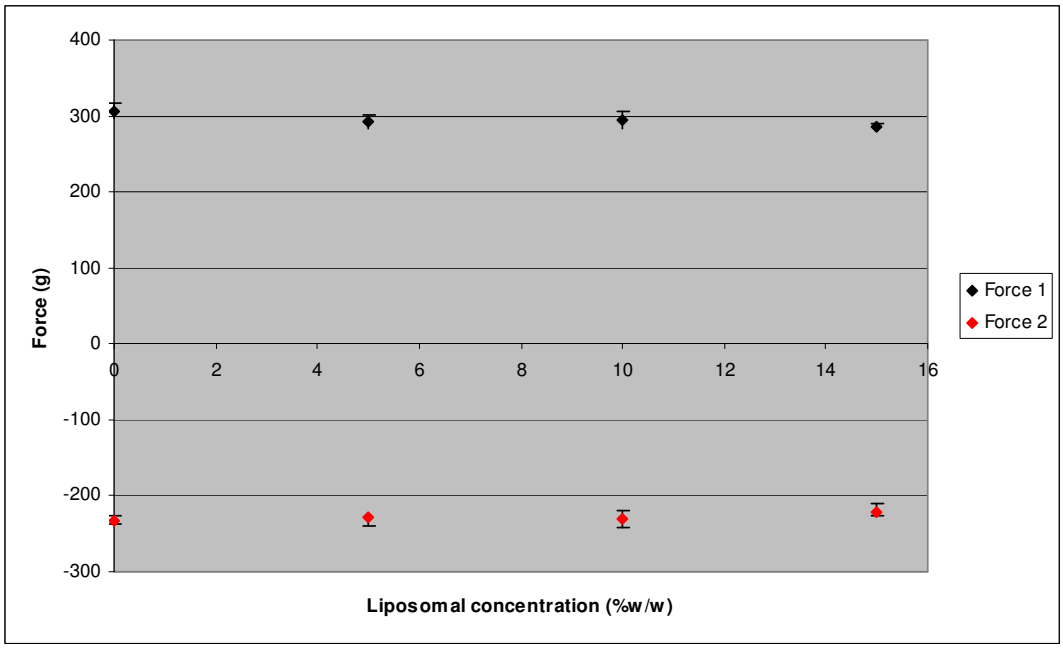


Figure 19: The effect of liposomal dispersion on texture properties of hydrogles. Liposomes in concentration of 0, 5, 10and 15% (weight per total weight) were incorporated in 0.5% Crabopol hydrogel. Measurement conditions: distance 15 mm, speed 1.0 mm/s, probe 40 mm, 5 runs.

We observed that the cohesiveness of hydrogels was not affected by the incorporation of liposomal dispersions, even at higher concentrations, which was in a way unexpected (Figure 19). Carbopol hydrogels appear to be stable formulations that can incorporate liposomes in their network without changing their original texture properties. One possible explanation can be that liposomes accommodate themselves in the empty spaces inside the gel's three dimensional structure. Some of the earlier

reports on the stability of liposomal creams indicate that the creams are not stable formulations, thereof, incorporating liposomes into gels is a good solution for administration of drugs in topical form (Gabrijelcic and Sentjurc, 1995). Moreover, gels are patient and skin-friendly formulations (Skalko et al, 1998).

4.3.2. Stability testing of liposomal hydrogels

After storage of liposomal hydrogels at 40 °C, it was observed that liposomal hydrogels are not stable at higher temperature. The liposomal dispersion within gels precipitated down and on the top of the gel we could observe the gel "sweating" phenomena (Figure 20).

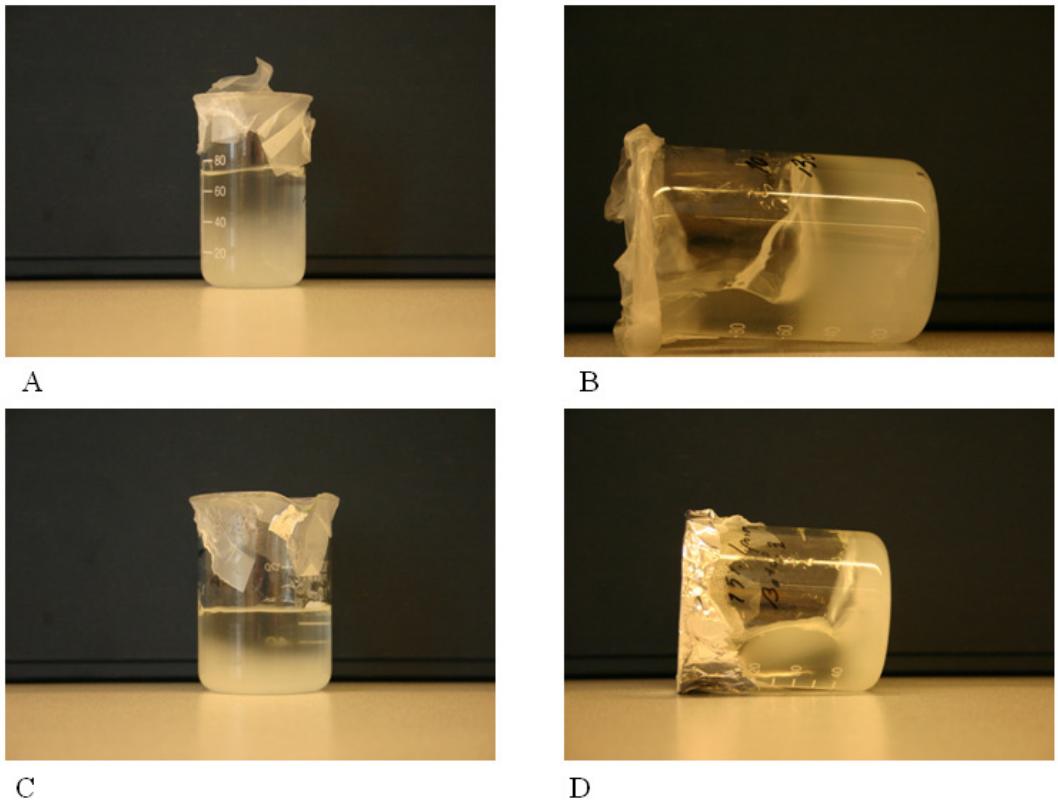


Figure 20: Accelerated stability testing of liposomal hydrogels.

The testing was performed at 40 °C for 1 month period. A and B represent the liposomal hydrogels containing 10 % liposomal dispersion (w/w), C and D the liposomal hydrogels containing 15 % liposomal dispersion after the testing.

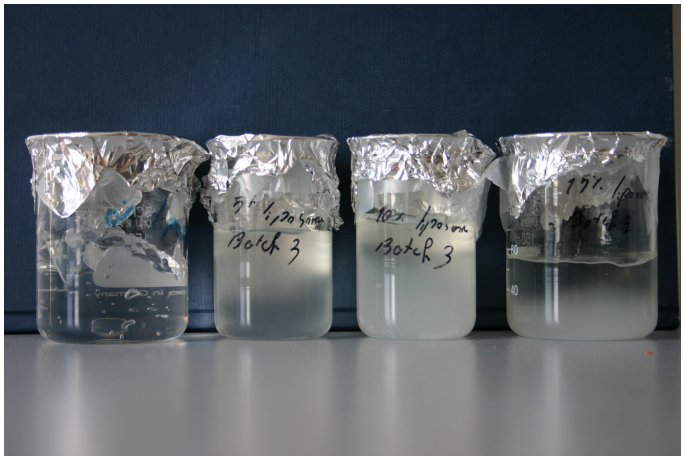


Figure 21: The appearance of liposomal hydrogels containing 0, 5, 10 and 15 % (w/ total weight) liposomal dispersions after the stability testing.

What indeed happens when liposomal hydrogels are stored at 40 °C for one month? One explanation can be that the cross-linked bounds inside the gel can get weak due to temperature increase and that the polymers chain can get partially become degraded. When the strong bounds become weaker, liposomes that are incorporated between these cross-linked networks can sediment onto the bottom of gel. In regard to stability of liposomal hydrogles, the research literature shows that the stability depends on the type of gelling polymer and the composition of gel used. Some hydrophilic polymers like for example carboxymethylcellulose are not affected by the incorporation of liposomes in hydrogel. However, in some hydrogels like Xanthan hydrogels, leak of drugs from liposomes can be observed already after 10 days. Pavelic et al. (2001) reported that liposomal Carbopol hydrogels containing glycerine perform well in the stability testing.

4.4. *In vitro* release studies

We chose chloramphenicol as a drug model to study the release of drug from hydrogels. Although there is not really an agreement about the use of topical antibiotics in wound healing, as for example the Australian and British guidelines suggest that one should be restricted in use of topical antibiotics, there are evidences in clinical trials that antibiotic reduce the incidence of wound infections (Heal et al., 2009). Nevertheless, chloramphenicol release from liposomal hydrogels has not been reported in literature up to now.

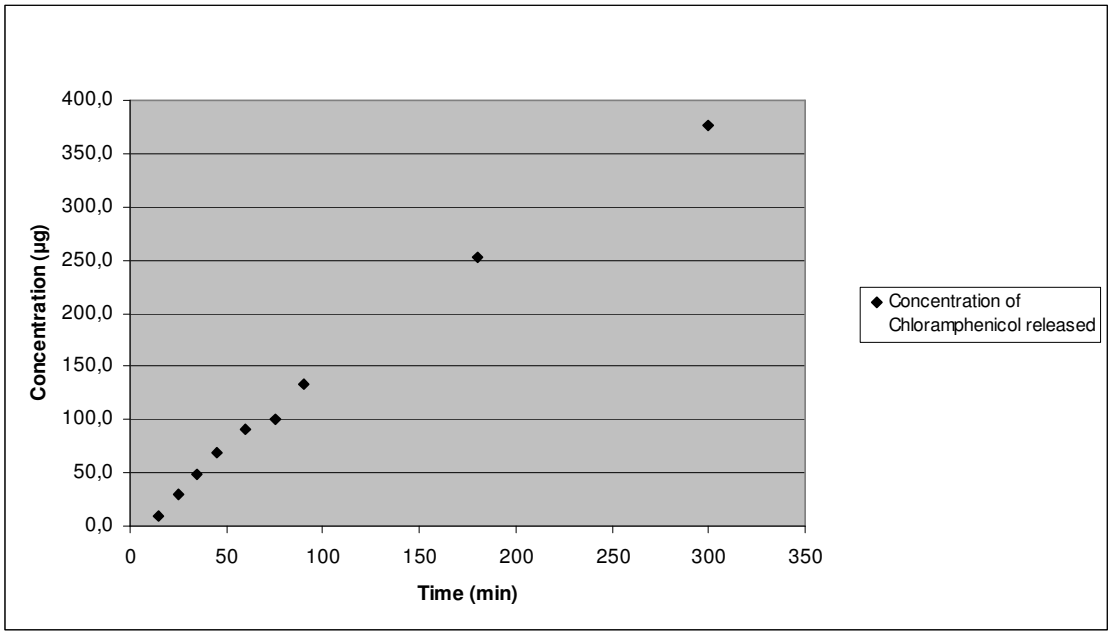


Figure 22: Chloramphenicol release from liposomal hydrogels.

The drug concentration was determined by the HPLC analysis. The measurement conditions were: Mobile phase was 45% methanol, 55% filtered H_2O 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.

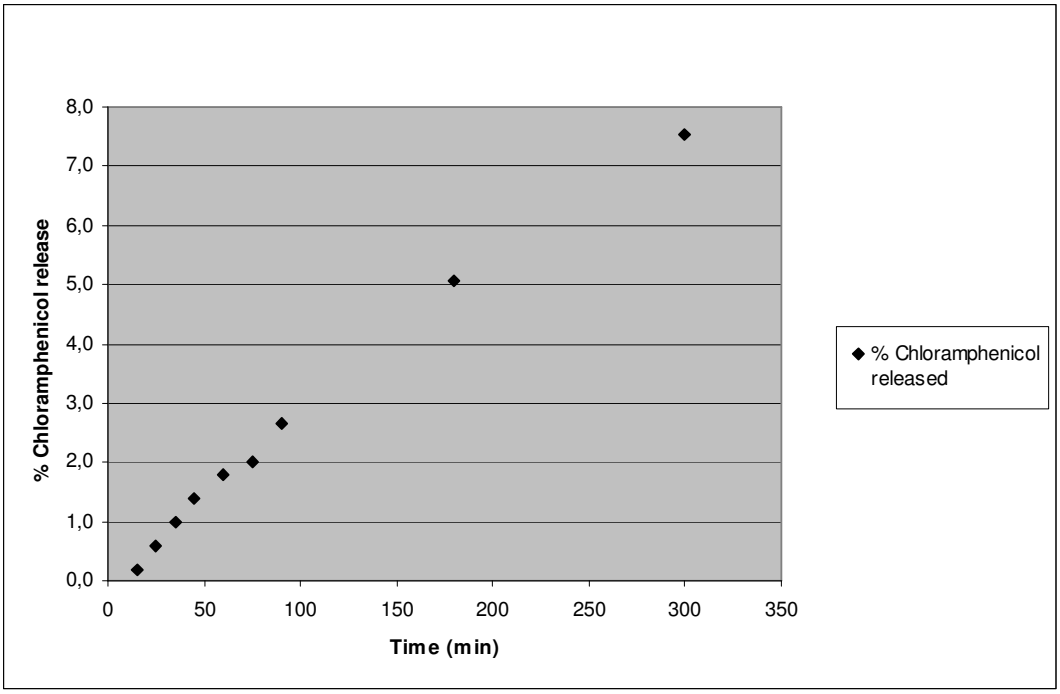


Figure 23: Percentage release of Chloramphenicol from Carbopol hydrogel over tested period of time.

The drug concentration was determined by the HPLC analysis. The measurement conditions were: Mobile phase was 45% methanol, 55% filtered H_2O 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.

The percentage of chloramphenicol release from Carbopol hydrogel was found to be between 8 to 9% during the tested period of time. We observed that the release studies were performed in too short interval, so that the future experiments needed to be performed for at least 24 hours period. As the release was rather slow, one of the options to accelerate the release would be to adjust the composition of hydrogles.

One can improve the properties of a gel, by combining more than one polymer together. These polymers can either be two synthetic polymers or one synthetic and one natural polymer. In last case the interaction between two chains of polymers can give a good synergistic effect. This synergistic effect can be very useful in designing hydrogels with controlled drug release. By varying the composition of components,

one can adjust the kinetics of drug release from gels. Another way to improve the mechanical property of gels is by combining a biological macromolecule like collagen or elastin with synthetic polymers. As an example, combining Hyaluronic acid with Carbopol hydrogels has direct effect on the ease and efficiency of the application of the gel on the skin (Jimenez et al., 2007). Moreover, it is known that the combination of two polymers can produce gels that have a higher degree of rigidity as well which is expected to affect their stability at the same time (Jimenez et al., 2007).

5. Conclusion

Adhesiveness, stability and release of incorporated drugs are the main features that influence the applicability of hydrogels for topical treatment, including the wound healing process. Adjustment of the textural properties of hydrogel should be conducted routineosly. The texture analyzer measurements can provide deeper insight on gel adhesivness. The gel properties are dependent on the polymer concentration and the pH. Carbopol hydrogels can take up to 15% (w/w) of liposomal dispersions, however, the stability of the liposomal gels need to be evaluated as well. Carbopol hydrogels can be used as advanced drug delivery systems.

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7. Appendix

7.1. Abstract submitted for Conference presentation

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 bioadhesivness of hydrogels. Carbopol hydrogels remained stable at accelerated stability conditions, whereas chitosan hydrogels did not resistant 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.

7.2. Abstract submitted for Conference presentation

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 considerating 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 bioadhesivness of hydrogels. Carbopol hydrogels remained stable at accelerated stability conditions, whereas chitosan hydrogels did not resistant 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 gelforming 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.

7.3. Chromatographic profile of release test