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# CHITOSAN-BASED DRUG DELIVERY SYSTEM FOR A NEW CHEMICAL ENTITY FOR PHOTODYNAMIC WOUND THERAPY

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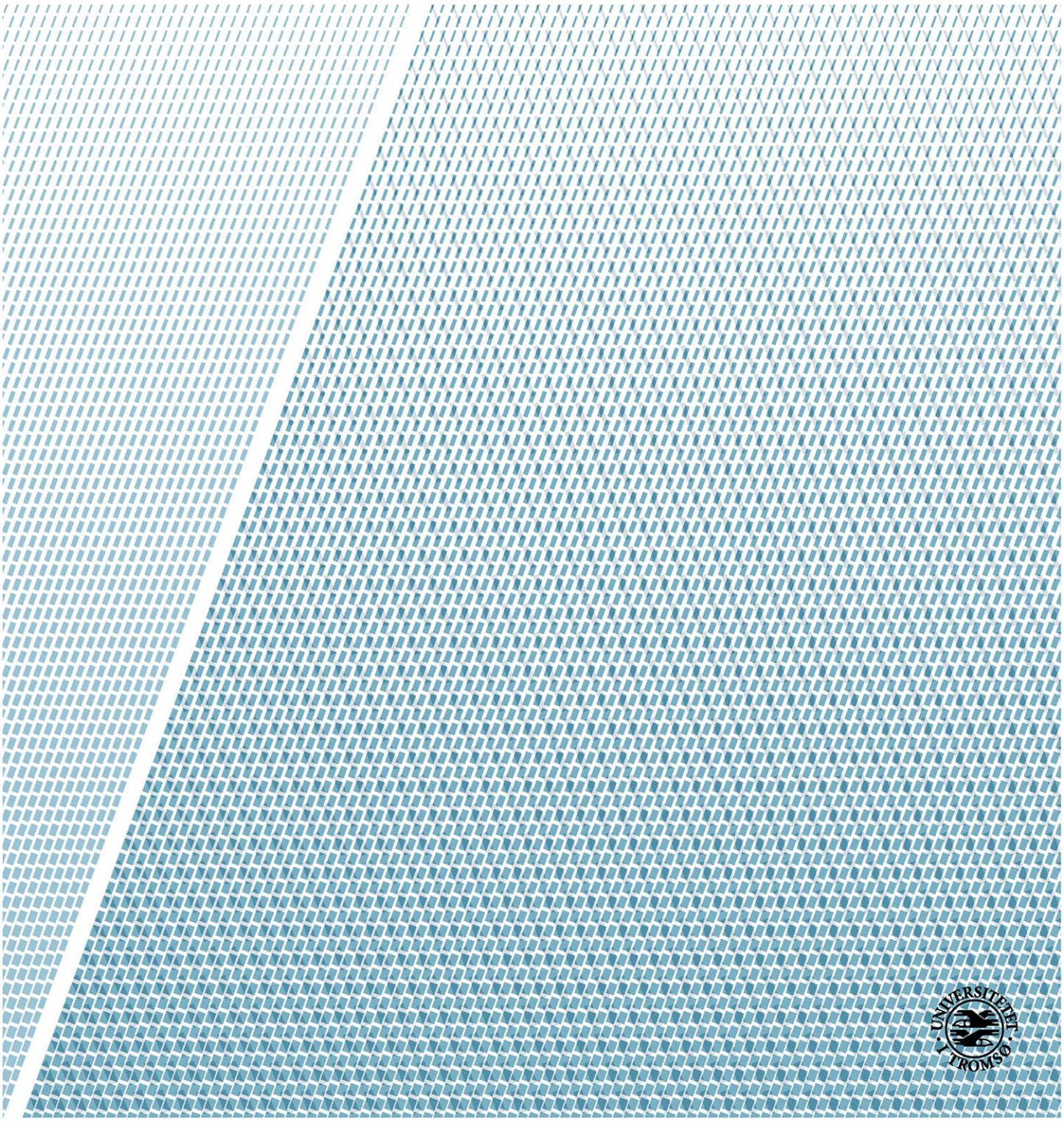
**Muna Abukar Hadafow**

*Master's thesis for the degree master of pharmacy … Mai 2014*



MASTER THESIS FOR THE DEGREE OF MASTER IN PHARMACY

# CHITOSAN-BASED DRUG DELIVERY SYSTEM FOR A NEW CHEMICAL ENTITY FOR PHOTODYNAMIC WOUND THERAPY

By

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Muna Abukar Hadafow

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## Abbreviation

| | |
|---|---|
| AK | Actinic keratosis |
| BBC | Basal cell carcinoma |
| HPLC | High performance liquid chromatography |
| HCL | Hydrochloride |
| NCE | New Chemical Entity |
| NP | Nanoparticles |
| PDT | Photodynamic treatment |
| PD | Polydispersity index |
| PS | Photosensitizers |
| SC | Stratum corneum |

## Abstract

Due to inappropriate or excessive use of antibiotics, multi resistance has become a global problem which threatens the public health. Photodynamic therapy has emerged as a promising and important approach in many fields of drug therapy. In recent years, many studies on photodynamic therapy for infections have shown promising results. One of the advantages of using PDT in treatment of infections is the mechanism of drug action which differs from that of antibiotics. This opens the door for the possibilities of treating multidrug resistant bacteria. PDT requires photosensitizer (PS), which are stable, nontoxic and effective. To improve the delivery of PS usually a delivery system which provides better stability and control of the PS. Polymer-based nanoparticles have been proposed as a suitable delivery system based on their advantages (e.g. biodegradable, less toxicity, microbial and hemostatic properties).

A New Chemical Entity (NCE) recently developed and patented by Photocure ASA is expected to exhibit superior antimicrobial properties and improved wound healing.

Chitosan lecithin nanoparticles were selected and developed as a carrier system for this new entity. At this stage of development, the delivery system was found to have many stability issues which need to be optimized in the future. The sizes of the carrier were found to be suitable but their entrapment efficiency was found to be rather low (less than 22 %).

Keywords: Chitosan; Infections; Nanoparticles; Photodynamic therapy; Polymer

## Abstract (Norwegian)

På grunn av upassende eller overdreven bruk av antibiotika, har multiresistents blitt et globalt problem som truer folkehelsen. Fotodynamisk terapi har dukket opp som en lovende og viktig tilnærming på mange felt. I de senere år har mange studier på fotodynamisk terapi for infeksjoner vist lovende resultater. En av fordelene ved PDT benyttet i behandlingen av infeksjoner, er virkningsmekanisme som er forskjellig fra den for antibiotika. Dette åpner mulighetene for behandling av multiresistente bakterier. PDT krever fotosensibiliserende (PS), som er stabile, ikke-toksisk og effektivt. For å forbedre levering av PS vanligvis et leveringssystem som gir bedre stabilitet og frigjøring av PS blir brukt. Polymer - baserte nanopartikler har blitt brukt i mange studier for sine fordeler (for eksempel biologisk nedbrytbarhet, mindre toksisitet, antimikrobiall og hemostatik egenskaper).

En ny kjemisk entitet (NCE) er ventet å vise overlegne antimikrobielle egenskaper og forbedret sårtilheling. Dette NCE er utviklet og nylig patentert av Photocure ASA

Kitosan lecithin nanopartikler ble valgt ut til å ble vurdert for dette nye system. leveringssystemet ble funnet å ha mange problemer, stabiliteten av det utviklet systemet trenger å optimaliseres i fremtiden eller eventuelt kitosan lecithin nanopartikler utviklet med annen metode. Størrelsene på leveringssystemet ble funnet å være egnet, men deres omsluttende effektivitet (entrapment) ble funnet å være mindre enn 22 %.

Nøkkel ord: Fotodynamisk terapi; Kitosan; Infeksjoner; Nanoprtiklers; Polymer

# 1. General Introduction

Skin injuries are susceptible to infection due to the loss of the original skin barrier (Percival et al., 2012c). Wound injuries, especially chronic wounds, are usually infected with bacteria, and such contamination causes delayed wound healing and also prolongs the treatment period which increases the chance of development of resistance (Jori et al., 2006). One of the mechanisms that microorganisms use to attach to the skin injuries and survive is biofilm. The role of biofilms is not only to assure the attachment to the wound, but also to provide the organisms a source of nutrition's and a way to tolerant removal and eradication (Percival et al., 2012c). This increases the resistance to antibiotics and, immunological and chemical attack (Percival et al., 2012c). Due to this the antibiotics resistance is reported to be one of the major health problems in the world (Jori et al., 2006).

Photodynamic therapy (PDT) have been showing a promising future in cancer treatment, and lately the interest in microbial photodynamic increased (Dai et al., 2010, Kharkwal et al., 2011). In cancer treatment the photosensitizer, for example, is injected to the bloodstream and in infection, the photosensitizer is delivered directly to the infected area (Dai et al., 2009, Tanaka et al., 2012). For the photosensitizer to accumulate in the tumor it may take some time and the concentration may be different than in the infection therapy were the photosensitizers is directly delivered to the infected area. The last part of the treatment is the same for both; the area will be exposed to light which results in a damaging chemical reaction to photogenes/cancer cells (Tanaka et al., 2012).

A New Chemical Entity (NCE) is expected to show superior antimicrobial properties and improved wound healing. This NCE was developed and recently patented by Photocure ASA and is expected to assure lack of resistance development in repeated therapy. Moreover, as it is destined for topical therapy onto the wounded area, the therapy is expected to be more efficient and accessible than to other inner parts of the body.

When choosing the most suitable carrier system for NCE, chitosan has been our first choice due to its reported wound healing properties. Chitosan has been used extensively as a drug delivery device and also as a vehicle to wound injuries (Dai et al., 2011). Chitosan has various properties which make it very interesting polymer of natural origin; such as its natural antimicrobial effect (Rabea et al., 2003), less toxicity, biocompatible properties, and also the

ability to stimulate the wound healing processes (Dai et al., 2011). Therefore using chitosan as a drug delivery system may enhance antimicrobial and healings effects of the drug, in our case NCE (Dai et al., 2011).

# 2. Introduction

## 2.1. Skin

The skin is the largest, self-renewing organ in the human body (Baroni et al., 2012, Wickett and Visscher, 2006), representing approximately 16 percent of total body weight (Wickett and Visscher, 2006). Skin plays an important role in protecting the internal organs from external environment (e.g. microbial, mechanically and chemical insults). It provides protection from harmful light exposure, and it plays other roles such as regulating the body temperature, limits passive water loss and synthesizing vitamin D (Wickett and Visscher, 2006, Baroni et al., 2012). The skin contains blood vessels, sweat glands, hair follicles, fat lobules, sweat duct, muscles and it's structured in layers. Skin is divided in two main layers, epidermis and dermis (Baroni et al., 2012). The structure of the skin is demonstrated in Figure 1.

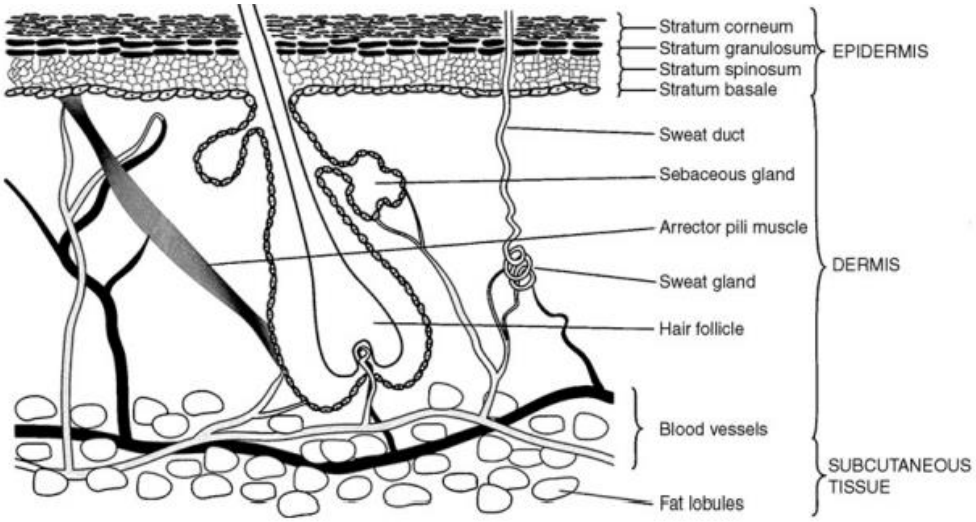


**Figure 1:** Structure of the skin (El Maghraby et al., 2008)

### 2.1.1. Epidermis

The epidermis is divided as demonstrated in Figure 2 into 4 layers; *stratum corneum* (SC), *stratum granulosum, stratum spionosum* and *stratum basale*. Each one of those layers plays an important role together in providing a protective barrier, specially the outer layer (SC) which is often modeled as brickwall structure (Wickett and Visscher, 2006).

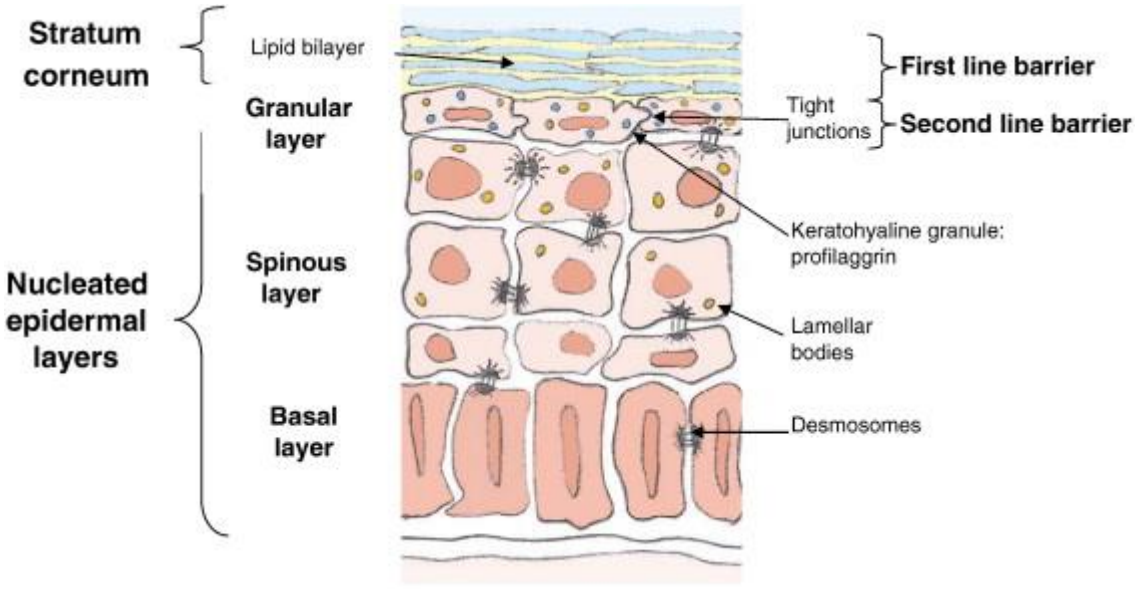


**Figure 2:** Structure of the epidermis (Baroni et al., 2012)

The cells in the basal layer are responsible for the renewal of the epidermis. The majority of these cells are in quiescent state, while only 15 % of the cells are constantly active. The cells in quiescent stat intervene when proliferation is required, for example when an injury occur (Baroni et al., 2012).

In the epidermis layer, two types of granules are formed, keratohylain granules (contains protein) and lamellar bodies (contains lipids) (Wickett and Visscher, 2006).
Keratinocytes are the predominant cell type in the epidermis which exist in the basal layer to the granular layer before they transformed to corneocytes (anucleate) in the SC (Wickett and Visscher, 2006). During maturation of keratinocytes, various structural proteins and lipids are synthesized and expressed. Keratinocytes are nucleated, but in their final steps they undergo changes in their structure, resulting in a transformation into flat anucleate cells of SC (Baroni et al., 2012). After the nucleus is digested and the cytoplasm disappears, the lipids are released into the intracellular space. The keratin filaments aggregate to form microfibrils and the cell membrane are replaced by cross-linked protein with lipids covalently bonded to its surface (Wickett and Visscher, 2006)

In the epidermis, beside the keratinocytes, melanocytes and Langerhans cells are two other important cell types. Melanocytes are pigment producing cells found in the basal layer of the skin. They make melanosomes, which are melatonin-containing granules. To provide some protection to the nucleus from ultraviolet light, melanosomes are transferred from the melanocytes to the epidermal keratinocytes. Besides providing protection from the light, this also gives the skin its color. The process of melatonin synthesis occurs continuously as the epidermis renews, but by exposure to the ultraviolet light this process can be enhanced, to produce tanning (Wickett and Visscher, 2006)

Langerhans cell are dendritic immune cells which plays an important role to the immune function of the epidermis and also participate in contact allergy (Wickett and Visscher, 2006).

Approximately every 28 days, our skin completely renews, by a balanced process between proliferation of the skin and desquamation in a healthy epidermis. When the balance of this system is disturbed, this may lead to skin disorders (e.g. psoriasis, ichthyoses) (Baroni et al., 2012).

### 2.1.2. Dermis

The dermis is highly vascularized layer that provides nutrition; not only to itself but also to the epidermis layer that lacks blood vessels. It also contains a layer of connective tissue that includes collagen and elastic fibers which provides the strength and smoothness. In the dermis, sweat glands, hair follicles, sebaceous glands are found (Baroni et al., 2012, Jensen and Proksch, 2009). Other components found in the dermis are fibroblasts, mast cells, plasma cells, and lymphocytes, and sensory nerves. The thickness of the dermis various with the body site, and are between 0.5-5 mm. The dermis is divided into two main layers (Lai-Cheong and McGrath, 2013);

- A thin papillary layer which is richly supplied by blood vessels and sensory nerve endings.
- Reticular dermis, which is in contact with the subcutis

Subcutis is the innermost layer of the skin. It is composed of lipocytes which are arranged into fat lobules and are separated from each other by fibrous septae (Lai-Cheong and McGrath, 2013).

### 2.1.3. Routes of penetration in the skin

Two routes of penetration through the intact skin is known the transepidermal route (through the intact SCC) and transappendageal (through the sweet gland and hair follicles) (Scheuplein, 1965). The transepidermal pathway divides into two micropathways; the intercellular route and the trancellular pathway. In the transepidermal pathway, penetrations occur through the lipid domains, while in trancellular penetration occurs first through the keratinocytes and then in across the intracellular lipids (Barry, 1991).

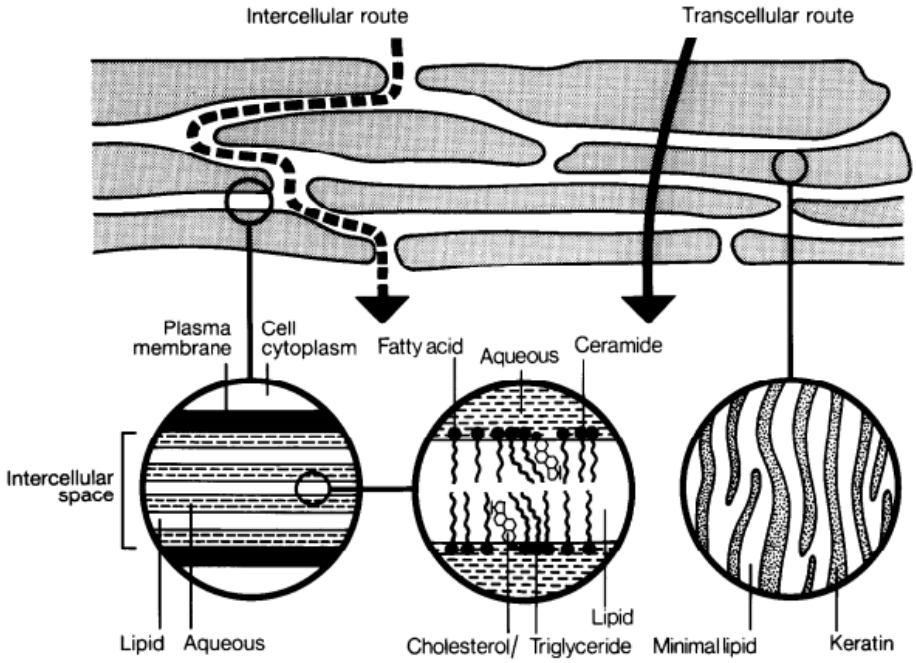


**Figure 3:** Routes of penetration through the skin (Barry, 1991)

## 2.2. Wound

A wound is a rupture or damage in the epithelial integrity of the skin. It may be accompanied by the disruption of the structure and function of the normal skin. When an injury to the skin occurs, it triggers a complex series of events (e.g. cell division, chemotaxis, neovascularization) which are regulated by many mediators (e.g. inflammatory cells, growth factors, cytokines). There are two types of wound, acute wound and chronic wound. Chronic wounds occur when the normal process of healing is disrupted at one or more stages. This leads into delay in the healing. (Enoch and Leaper, 2008). Chronic and acute wound, both are susceptible to infections due to the loss of sterility.

*Staphylococcus aureus* is found in the majority of wounds, but there are other common wound bacteria such as *Pseudomonas aeruginosa, Staphylococcus epidermidis, Serratia marcescens* (Percival et al., 2012b). *Staphylococcus aureus* is a colonizer of the skin where 30-60 % of the human population carrying the microorganism. *S.aureus* is a precipitating agent of minor self-limited skin infection. *S. epidermidis* is most prevalent bacterium of skin, representing 90 % of the microflora and therefore is a skin contaminant isolated during infections. (Percival et al., 2012a). *Pseudomonas aeruginosa* is a gram negative bacterium and is rarely found in acute wound, but is found in chronic wound. (Bjarnsholt et al., 2008).

### 2.2.1. Wound healing

Wound healing is a survival mechanism which occurs immediately after the body is injured. During the healing process, the damaged or destroyed tissues in this process are removed and the integrity of the tissues is restored (Beldon, 2010). Wound healing can be classified into three types; primary, delayed primary and secondary healing.

Primary healing occurs where the wound edges are close together, which occurs within 12-24 hours of its creation. Usually it's a clean incision from a surgical wound; where the wound edges are closed by using the sutures, tissue glue taps or a mechanical device. (Enoch and Leaper, 2008).

Delayed primary healing occurs when wound is not closed immediately and becomes contaminated.(Enoch and Leaper, 2008)

Secondary healing is seen after the surgical procedures, in major trauma, and severe burns. It occurs in wounds with extensive loss of tissue (Enoch and Leaper, 2008). Such wounds are vulnerable to infections and their healing process is slow. Vascular and diabetic ulcer are examples of such wounds (Beldon, 2010).

The mechanism of wound healing is divided into 4 overlapping stages; haemostasis, inflammation, proliferation migration, remodeling and scar maturation.

**Haemostasis:** to reduce blood loss when an injury occurs, the first response to an injury is vasoconstriction. The platelets within the blood adhere to the wall of the blood vessels and the collagen exposed, which triggers the release of numbers of inflammatory mediators, cytokines and growth factors. This leads to more aggregation of the platelets and triggers coagulation by leading to fibrin clot formation. The healing cascade initiated by the growth factors, initiates further an chemotaxis reaction involving neutrophils, macrophages muscle cells and fibroblasts (Beldon, 2010).

**Inflammation:** when the coagulation cascade is initiated and as a response to an injury, several vasoactive cytokines such as histamine and prostaglandins are released. In response to these releases, a local vasodilation and capillary permeability occurs. Monocytes along with serous fluid leaks/migrate into the wound, resulting edema. During the inflammatory period, neutrophils which are responsible to clean the wound accumulate in the wounded area and ingest via phagocytosis the bacteria. The cleaning continues after the neutrophil filled with bacteria undergoes apoptosis. To attract more fibroblast and smooth muscle cells to the wound, macrophages release some growth factors. This process takes 2-3 days in an acute wound, but if contamination occur, the process will be prolonged (e.g. chronic wound) (Beldon, 2010).

**Proliferation:** The inflammation and proliferation phase overlaps with each other, the last may last for 2-4 weeks. The proliferation phase is divided into two parts, fibroblast migration and formation of the extracellular matrix. Fibroblasts are attracted to the wound area by numbers of growth factors, resulting in a production of various components (e.g. proteins, collagen). This leads to formation of extracellular matrix.(Enoch and Leaper, 2008)

**Remodeling and scar maturation:** The extracellular matrix in this phase is remodeled and synthesized. The balance between synthesizing and breaking down collagen as the extracellular matrix is remodeled leads to equilibrating to steady state about 21 days after wounding. (Enoch and Leaper, 2008). An interaction between fibroblasts and the surrounding extra cellular matrix leads to a wound contraction which is influenced by numbers of growth factors, cytokines, platelet-derived. As the scar matures, fibronectin and hyaluronan are broken down, while collagen bundles increases in diameter, however these collagen fibers never regain maximum strength.

Factors that impede wound healing are divided into two, local factors and systemic factors. Local factors can be an infection, increased skin tension, inadequate blood supply and poor venous drainage. Systemic factors can be; obesity, malnutrition, advancing age and general immobility and systemic disease (e.g. diabetes mellitus, metabolic disease) (Enoch and Leaper, 2008)

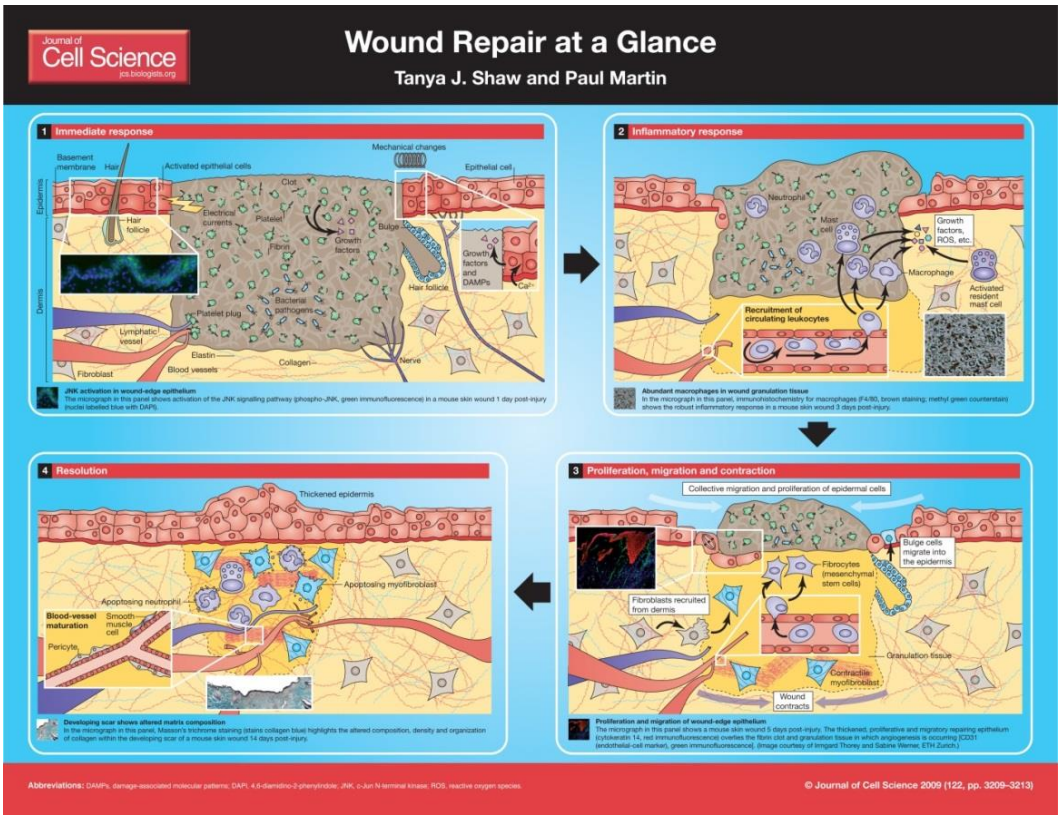


**Figure 4:** Wound repair (Shaw and Martin, 2009)

## 2.3. Biofilms

Biofilms are major concern do to their ability to tolerate antibiotics and to gain resistance and are main cause of limitations in current chronic wound therapies. It was not before the 1980s and 1990s that general opinion was that bacteria are organized in such sophisticated manner (Costerton et al., 1999). Before treating some types of infections, such as chronic wound infections, it is important to have an understanding of biofilm and how they function to be able to treat its causes properly. The mechanism for resistance to antibacterial agents can be one or more of the following mechanisms: (1) delayed penetration of the antimicrobial agent

through the biofilm matrix, (2) altered growth rate of biofilm organisms, and (3) other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002). Biofilms differ from floating bacteria or planktonic bacteria in many ways, for example, their structures, interaction with the host, gene expression and their antibiotic resistance is different (Zhao et al., 2013). For example *Pseudomonas aeruginosa* which is one of the microorganisms found in chronic wound have been observed in many studies to play a role in delaying wound healing process. Their ability to form biofilms (extracellular polymeric substance (EPS)-encapsulated biofilms) is thought to be the main reason. (Bjarnsholt et al., 2008). The EPS contains polysaccharides, alginate, extracellular DNA, and other components such as proteins and lipids. Alginates prevents phagocytosis, binds many cationic antibiotics and enhance the structure of the biofilm (Bjarnsholt et al., 2008).

## 2.4. Chitosan

Chitosan is a derivate of natural polymer chitin, which has gained a huge interest in different fields, such as biomedicine, pharmaceutical and food industry. What makes chitosan interesting is its properties and natural source. Chitin, the starting material in isolation of chitosan is found predominantly in the shells of crustaceans, such as crabs and shrimps, which are easily available at waste from seafood processing industries and raw material suppliers such as Chitinor AS, in our case. Also there are other sources which may be used for chitin production such as krill, crayfish, insects, clams and fungi (Liu et al., 2011, Rinaudo, 2006, Jayakumar et al., 2010).

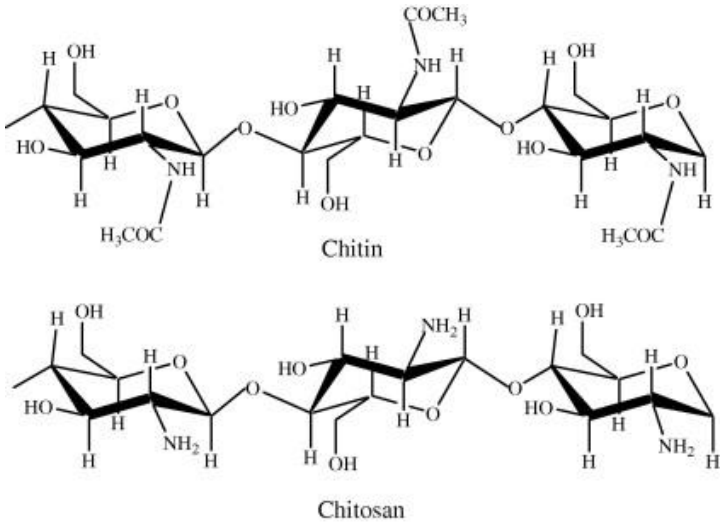


**Figure 5:** Structure of chitin and chitosan (Jayakumar et al., 2010)

### 2.4.1. Properties of chitosan

The properties of chitosan such as the solubility, biodegradability, biological activity and chemical reactivity are influenced by the degree of acetylation (DD), its molecular weight and the degree of substitution (Jayakumar et al., 2010).

Solubility: the solubility of chitosan in acidic acid (pH<6.5) can be explained by its chemical structure. The chemical structure of chitin and chitosan is quite similar, but the amino group which is present in chitosan is different than the one present in chitin. This amino group, present in chitosan, can be protonated which explains its solubility in diluted acetic acid (pH<6.5) in contrast to chitin (Liu et al., 2011, Leedy et al., 2011).

Complex formation**:** chitosan is used as purifying agent of waste waters, due to its ability to form complexes.

Microbial activity: The antibacterial and antifungal properties which these cationic polymers have can be explained by two mechanisms; a mechanism that involves the charge and the second mechanism that involves the ability of the amino groups to bind to DNA in the cells. The positively charged polymer interacts with negatively charged groups which may be present on the surface of the cell wall. This interaction prevents important and essential materials to enter the cell. While the protonated amino group binds to the DNA, which leads to inhibition of microbial RNA synthesis (Liu et al., 2011)

Mucoadhesion: the glycoprotein which is negatively charged and the positively charged chitosan interact in acidic conditions, which lead to adhesion (Aider, 2010, Liu et al., 2011). This can for example prolong the retention time of drugs at the mucosal site such as oral or vaginal cavities, as well as wounded site (Wang et al., 2011).

Biodegradable: beside the amino group, it also contains a polysaccharide forming nontoxic oligosaccharides (Muzzarelli, 2009, Liu et al., 2011)

Haemostatic: some articles have explained that the interaction between the negatively charged red blood cells, and positively charged chitosan (in acidic condition) is the mechanism behind its haemostatic properties (Rao and Sharma, 1997, Liu et al., 2011). This effect makes

chitosan a good choose when a drug delivery system is designed for wound dressing purposes. There are many chitosan-based bandages and wound dressings available on the marked. Chitoflex® Hemcon is an example of chitosan-based wound dressing (Muzzarelli, 2009)

### 2.4.2. Potential and limitations of chitosan:

As discussed so far, chitosan has many properties which explain the increased interest in many different fields. Because of its film forming properties chitosan is used to prepare films, fibers and hydrogels (Rinaudo, 2006, Liu et al., 2011). The biodegradable properties if chitosan and also their antibacterial, antifungal activities make the polymer interesting material in both food and pharmaceutical industry.

Chitosan can be modified chemically to make it more soluble in water, for example by introducing different functional groups**.** A well-known derivate of chitosan are O-and N-carboxymethlchitosans (Rinaudo, 2006, Jayakumar et al., 2010, Liu et al., 2011). O-and N-carboxymethlchitosans are water soluble in wide pH range. By chemically modifying the original structure it is possible to control its cationic character which leads to an improvement in the stability of ionic complexes (Liu et al., 2011). Because of the opportunity to modify its structure this cationic polymer is interesting for many applications; however, the toxicity issue should not be overlooked when new modifications are made.

The cationic character that this naturally occurring polymer has made it a good choice for gene delivery. However, the instability and its low buffering capacity limit its wider use (Liu et al., 2011).

Two of the major limitations of chitosan as drug delivery systems are:

- The solubility (Dai et al., 2011) which is pH-dependent. For instance in physiological environment the amino groups will be partially protonated which leads to poor solubility (Liu et al., 2011). This limits the possibly applications areas where chitosan could be used in.
- Another limitation of chitosan is the poor mechanical strength of chitosan-based formulations when chitosan is used as a single gel-forming agent. This limitation can be overcome by using different delivery systems incorporated in chitosan gel matrix or by combining chitosan with other material, such as lecithin in our case.

## 2.5. Aminolevulinic acid (5-ALA)

### 2.5.1. General

Aminolevulinic acid is a natural compound that plays role in heme biosynthesis. The photosensitive compound which forms in heme synthesis, protoporphyrin IX (PpIX), is used in various therapies such in bladder cancer and in some topical treatments. For therapeutic purposes ALA can be administrated both systemically and topically. As shown in Figure 6 ALA is a polar molecule and in natural pH occurs mainly as charged zwitterion, the fact that bears several limitations, such as reducing bioavailability and lipid solubility. To overcome these limitations, along with reduce the light sensitivity after intravenous treatment, many attempts have been conducted to modify the structure to improve the bioavailability. Two of the most successful ALA derivatives are methyl aminlolevulinate (MAL) and hexyl aminlolevulinate (HAL). (Wachowska et al., 2011)

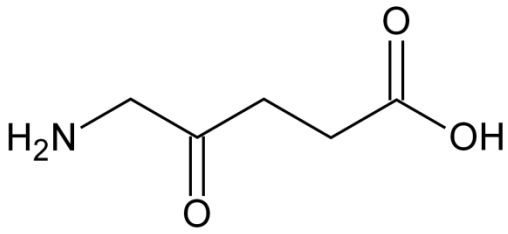


**Figure 6: Aminolevulinic acid (5-ALA)**

Metvix ® is the first 5-ALA derivate approved to be used to treat actinic keratosis (AK), when other treatments do not provide good results and to treat basal cell carcinoma (BBC). Another 5-ALA derivate, HAL (Hexcix®) is also approved for detection of bladder cancer. A study to evaluate 4 treatments with different MAL concentration (80 versus 160 mg $g^{-1}$) and application duration (1 versus 3 hours) to treat AK were performed. Scales and crust were removed from lesions before using light (600-700 nm. 75 J [cm $^{2]-1}$). The results showed that treatment with 160 mg/g MAL cream for 3 hours is slightly better (67 % response) than the other treatments (57-66 % response). A further treatment after 3 months produced an even better response for treatments with 160 mg/g MAL (90 %) compared with the other treatments (80-87 %). Another study investigated if the treatment efficacy of 160 mg/g MAL well be improved if the application time (1, 3, 5 or 18 h) of the drug is increased. The study showed the highest response is achieved when the application time was 3 h. There was no

significant improvement observed after 3 months which indicated that durations longer than 3 hours did not improve the clinical response (Fotinos et al., 2006).

### 2.5.2. Heme, biosynthesis and function

Heme is an essential molecule to many organisms such as bacteria, archaea and plants. Their growth relays on heme which makes it attractive target for antibacterial drug and herbicide treatments. The heme molecules play many roles in a living organism for example many enzymes relay on heme as prosthetic group, or serves as sensors for diatomic gasses in signal transduction pathways. The heme biosynthetic pathways differ in some ways depending which living organism is represented but mostly. ALA acts as an intermediate in the heme biosynthesis. ALA is normally synthesized in mitochondria by a reaction catalyzed by ALA synthase (ALAS), between succinly-CoA and glycine. ALA reaches to cytosol, where it leads to formation of prophobilinogen (PBG). Further reaction involving prophobilinogem deaminase (PBDG) leads to formation of urophorphyrinogen III. The product forms coproporphyrinogen, transports to mitochondria to further conversion to protoporporphyrinogen IX. Next step in the heme synthesis is formation of protoporphyrin IX (PpIX) and further reaction with ferrochelatase (FECH) leads to formation of heme molecule (Wachowska et al., 2011). The heme synthesis is demonstrated in **Error! Reference source not found.**

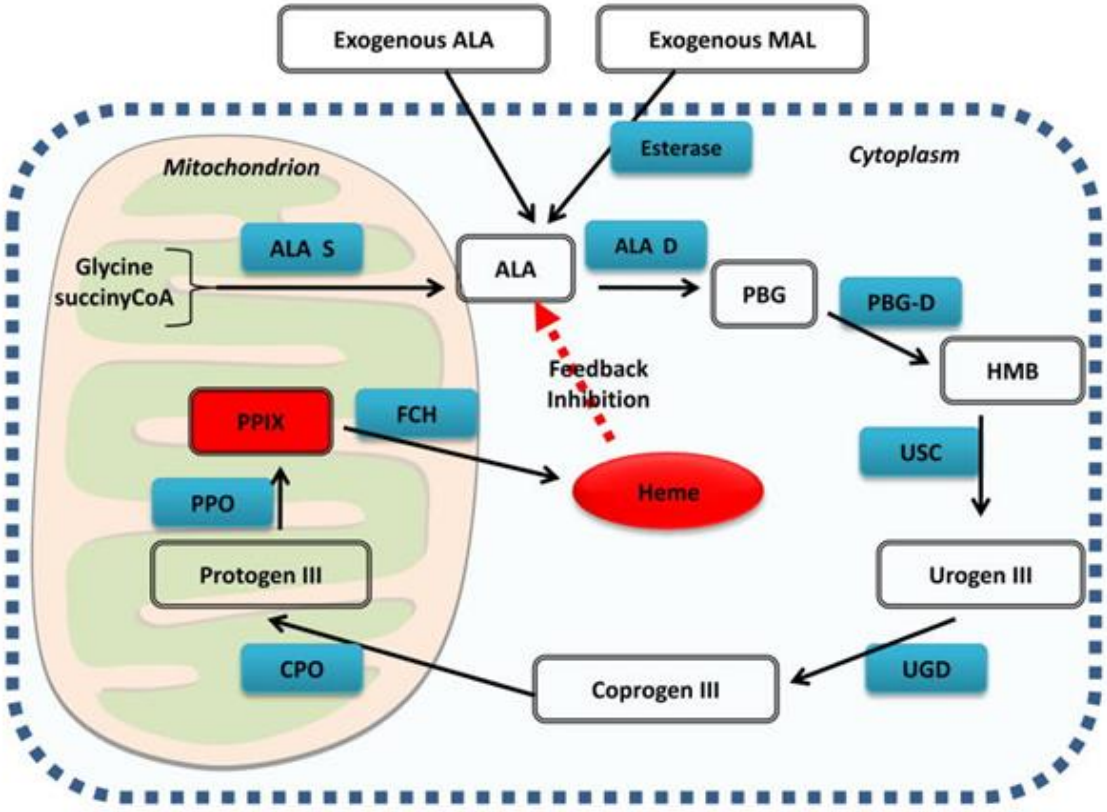


**Figure 7:** Schematic illustrating the interaction of the heme biosynthesis pathway with exogenous ALA or MAL to give intracellular PPIX Heme biosynthesis (Dai et al., 2009)

PpIX is a strong photosentiziser which leads to damage the cell after light exposure (Wachowska et al., 2011)

## 2.6. Photodynamic therapy

### 2.6.1. Introduction

Photodynamic therapy (PDT) has become an important therapeutic option which has been used in the past several years as a treatment for various conditions (e.g. infections, bladder, skin, and lung cancers) (Chatterjee et al., 2008). The principle of PDT involves the use of non-toxic photosensitizers (PS) in combination with the right wavelength visible light to excite the PS. This combination in presence of oxygen, produce cytotoxic species that are able to kill cells (Hamblin and Hasan, 2004, Dai et al., 2009). The ideal PS should be easily synthesized, stable composition, non-toxic, photostable and have minimal self-aggregation tendency. PDT in general has many advantages such as;

- Their safety for human tissue

- Do not require patient compliance
- Inexpensive
- Provides instant results
- Eliminates development of resistance
- Eradicates pathogens in biofilms

For antimicrobial PDT, the advantages of using PS containing nanoparticles include;

- Improves selectivity of the treatment by localizing delivery systems.
- limit the ability of the cell to pump the PS out (Sharma et al., 2012)

### 2.6.2. Photosensitizer (PS)

Porphyrins, chlorins, bacterichlorins and phthalocyanin are the main groups used in PDT (Benov, 2014) .

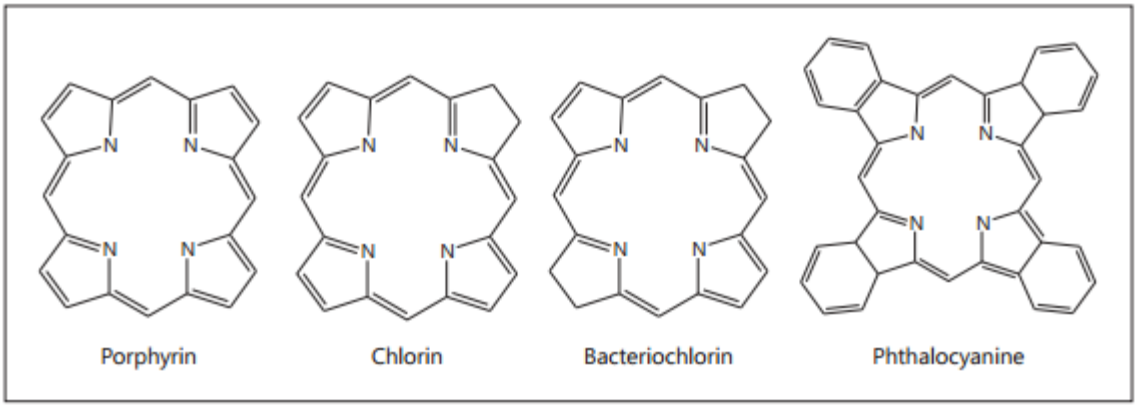


**Figure 8:** The main groups used in PDT (Benov, 2014).

There are other types PS such as, hematoporphyrin (HPD) which is water-soluble compound. benzoporphyrin (BPD). (Sharma et al., 2012). In general an ideal photosensitizer should be well-defined chemical compound with a strong selective phototoxic effect and have an ability to form active oxygen. Four types of interactions between the PS and nanoparticles have been reported: (1) PS embedded in polymeric nanoparticles, (2) PS covalent bonded to the surface of NP, (3) PS non covalent bound to nanoparticles and (4) the nanoparticles is itself a PS (Gupta et al., 2013).

### 2.6.3. Mechanism of action

The mechanism of action of PDT can divides into three phases; (1) the PS is localized in the targeted cells, (2) visible light is applied to activate the PS and the end (3) results in cell damage. The chemically reaction that occurs is divided into two types:

Type I: through electron transfer reactions, ROS is generated by a reaction between $H_2O_2$ and pictogram concentration of ferrous iron, cuprous copper or transition metals in a Fenton – like reactions yield a hydroxide ion and OH. Generating the OH in the cytoplasm leads to destruction of necessary proteins for homeostasis (Sharma et al., 2012).

Type II: occur when singlet oxygen, $^1O_2$, reacts with double bonds or sulfur atoms resulting in an oxidation of macromolecules (Sharma et al., 2012).

### 2.6.4. Drug delivery and routes of administration in microbial cells

In infections the PS is delivered to the infected area by topical application installation, interstitial injection or aerosol for airways-based infections(Sharma et al., 2012). Bacteria cells differ from cancer cells in their membranes which are more negative charged. This leads to a possibility to achieve a selective antimicrobial PS accumulation after local delivery by using positively charged PS.

Gram positive bacteria have a cytoplasmic membrane which makes it venerable to PDT. They are killed by cationic, anionic or neutral PS. Gram negative bacteria can only be killed by cationic PS or by method that allows the nonionic PS to be transported in the cell. What makes gram negative hard to kill can be explained by their membrane that differs from gram positive bacteria where the PS cannot inter through the cell wall (Sharma et al., 2012)

### 2.6.5. Nanotechnology in PDT

In the past years, the interest of nanotechnology in various fields have increased (e.g. medicine, diagnostic, imaging and drug delivery) (Barratt, 2000, Freitas Jr, 2005, Sonvico et al., 2005). Nanotechnology can be defined as a technology that permits manipulation, manufacture, control and study of structures and devices in nanometer size range (e.g.

nanoparticles) (Singh and Lillard Jr, 2009). One of the main problems to overcome in PDT is the drug delivery to the target cells. It's critical that the PS is delivered to the treatment site (e.g. infected or tumor cells) in a therapeutic concentrations with none or little uptake by other cells. The goal is a safe and effective use of PDT. Many PS agents are highly hydrophobic and insoluble in water(Gupta et al., 2013). In our case we have a hydrophilic PS, but due to low capacity to cross biological barriers a carrier is needed(Fotinos et al., 2008). This increase the need for a protecting carrier that provides protection from forming aggregates in aqueous environments (Gupta et al., 2013). By encapsulation of the PS molecule in an appropriated carrier, these issues may be overcome. The delivery system needs to assure the controllable delivery of incorporated PS. There are different nanocarriers used by PDT and they are summarized below (Gupta et al., 2013):

- Lipid-based nanoparticles (e.g. liposomes, lipoplexes and nanoemulsions)
- Polymeric nanocomposites; (chitosan, alginate, collagen)
- Carbon nanomaterial's (carbon)
- Silica-based nanoparticles (e.g. silica)
- Metal nanoparticles (e.g. gold and silver nanoparticles)
- Semiconductor nanomaterial's and quantum Dots (e.g. $TiO_2$ and ZnO)
- Upconverstion nanoparticles (e.g. sodium yttrium fluoride)
- Magnetic nanoparticles ($FeCl_2$ which is a magnetic core)
- Lipoprotein nanoparticles
- Self- illuminating nanoparticles (e.g. $BafBr/Eu^{2+}$)

Using nanotechnology is a promising approach, but the lack of information on the effect of the nanoparticles on human health and environment raises concern to some scientist, despite waste majority being supportive of the use.

Using nanotechnology in a combination with PDT has many advantages (Chatterjee et al., 2008). Improving solubility is one of the advantages which can be reached by using nanotechnology to improve solubility of a drug. This can be obtained by encapsulating the PS which also improves their stability. Another advantage which this technology provides is the possibility to modify the surface by adding functional groups that gives it additional properties.

### 2.6.5. Clinical evaluation of PDI in wound treatment

To determine if PDT can reduce bacterial load in a chronic leg ulcer and potentially lead to accelerated wound healing a randomized placebo-controlled study were performed. Patients with 16 venous le ulcers and 16 diabetic ulcers were enrolled into this study. For each ulcer type two groups were formed, were one group received a PDT treatment with the active ingredient (PPA904) and the other they received a PDT treatment with placebo. The results showed a broad and significant bacteria cell kill and a promising trend towards wound healing. (Morley et al., 2013)

Another study based in using 5-ALA and it's derivate to determine their effect on gram positive and negative bacteria was published in 2007. This study showed that 5-ALA and its derivate have a significant effect on gram positive and gram negative bacteria, but gram negative bacteria shows more resistance in contrast with the gram positive. This issue may be overcome by using polycationic polymers that binds to the gram negative membrane (Fotinos et al., 2008).

## 2.7. Lecithin-chitosan based nanoparticles

Lecithin chitosan based nanoparticles have become an alternative for colloidal carrier system to polymeric nanoparticles, liposomes, nanoemulsions and solid nanoparticles (Hafner et al., 2011). These nanoparticles are obtained from the supramolecular self-organizing interaction of negative lipid material in presence of the positively charged polysaccharide. They are prepared by injecting the soybean lecithin alcoholic solution directly into chitosan water solution. The sizes obtained from this method along with the surface charge depended on the content of the lecithin. (Sonvico et al., 2006).

The potential of lecithin-chitosan nanoparticles as a colloidal nanosystem for transdermal melatonin delivery were investigated by Hafner (Hafner et al., 2011). The study concluded that lecithin chitosan nanoparticles have proved to show a promising colloidal nanocarrier for transdermal delivery of melatonin. (Hafner et al., 2011)

## 2.8. A new chemical entity

The new chemical entity which will be studied in this project is thought to have antibacterial effect. The substance is a derivate of 5-aminolevulinic acid and has a good solubility in water (3.6 g/g water). The pH measured in 10% and 30 % solution is 1.3 and 0.8 respectively. Main degradation pattern of NCE is hydrolyses to ALA 4, or dimerization to dyhdropyraz

## 3. Aims of the study

The main aim of this project was the development of a suitable carrier for the NCE which may have microbial properties which makes it suitable for topical treatment. In this project we well develop and optimize chitosan-lecithin-based nanoparticles, and investigate the particle size, size distribution, and zeta potential and entrapment efficiencies.

The specific aims in more details were:

- Development of nanoparticle formulations by optimization of preparation method in regard to NCE entrapment efficiency and size distribution
- Evaluation of nanoparticles stability under two different storage conditions.
- Evaluation of nanoparticles stability by using zeta potential as an indicator.
- Evaluation of the HPLC method

# 4. Materials and Methods

### 4.1.1. Materials

Acetonitrile, CHROMASOLV® for HPLC, gradient grade, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Ammonium acetate, UWR BDH PROLABO, Leuven, Belgium

Chitosan S, Chitopharm M, Chitinor AS, Haugesund, Norway

Dialysis tubing, Molecular weight cut-off 12-14000 Daltons, Medicell international Ltd, London, UK

Distilled water

International Disposable culture tubes borosilicate glass, Kimble Chase, USA

Ethanol Sigma Aldrich, St. Louis, USA

Hydrochloride acid 37 %, VWR international BUBA, Leuven, Belgium

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

Methanol CHROMASOLV® for HPLC, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

Buffer solution, pH 4 and 7, AVS Titrinorm®, VWR BDH PROLABO, Leuven, Belgium

### 4.1.2. Instruments

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

Beckman SW 60 Ti rotor, Beckman coulter, Beckman Instruments, Palo Alto, USA

Biocap™ RNA DNA dynamic enclosure, NO 189 2000, Cedex, France

Branson 5510-.MT, Branson Ultrasonic cleaner, Danbury, USA

Distilled water, Milli.Q Biocel, 0.22µm, Bergman AS, Tromheim, Norway

Magnetic stirrer from IKA, type RCT basic, Stefen, Germany

NICOMP Submicron particle sizer, model 370, Nicomp Particle Sizing systems, Santa Barbara, USA

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

Sequant® ZIC®-HILIC PEEK Column 150x4.6mm 3.5µm 200Å, from Merck, Darmstadt, Germany Waters e2795, separation module, Waters Corporation, Milford, USA

Waters UV/VISBLE detector, Waters 2489, Waters Corporation, Milford, USA

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

### 4.1.3. Computer programs

High Performance Liquid Chromatography: Empower 3 Chromatography software (Build 3471), Milford, USA

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

Windows CW370 Software, NICOMP Particle sizing Systems, CW388 Version 1.68, Santa Barbara, USA

## 4.2. Methods

### 4.2.1. Preparation and characterization of nanoparticles

#### *4.2.1.1. Preparation of empty nanoparticles*

Nanoparticles were prepared by the modified method of Sonvico et al (2006). Empty nanoparticles (50 ml) were prepared by adding 2 ml of chitosan solution in hydrochloride solution (1 %, w/v) or 0.5 % acetic acid (1 %, w/v) to 44 ml of distilled water, followed by injection of 4 ml lecithin ethanol solution (2.5 %, w/v). Two different injection rate were used, namely 34 and 2 ml/min. The mixture was stirred for approximately 1 hour and left in the refrigerator at least 24 hours before further investigation.

#### *4.2.1.2. Preparation of nanoparticles containing new chemical entity (NCE)*

Lecithin/chitosan nanoparticles (NP) containing NCE were prepared according to the method previously reported by Sonvico et al (2006). The only modification was that we dissolved chitosan in acetic acid rather than in hydrochloride acid. *Ten*, 30 and 100 mg of NCE respectively were dissolved in 4 ml of lecithin ethanol solution (resulting in 2.5, 5 and 10 % solutions) before injecting the solution (injection rate 34 ml/min) into chitosan solution in acetic acid under continuous magnetic stirring. The mixture was stirred for approximately 1 hour and left in the refrigerator for at least 24 hours before further investigation.

#### *4.2.1.3. HPLC analysis*

The HPLC analysis was based on the method described by the company Photocure AS with a slight modification on the injection volume (20µl). A metal free HPLC column (Sequant® ZIC®-HILIC PEEK Column 150x4.6mm 3.5µm 200Å, Darmstadt, Germany) were used in the measurements. The mobile phases were prepared by dissolving 1.5 g ammonium acetate in the ratio of acetonitrile and water for 900:200 ml, and 100:900 ml

The HPLC measurements setting were as followed:

- Temperature of the column: 40 ºC
- Samples temperature 8 ºC
- Injection volume: 20 µl
- Flow rate: 1 ml/min
- Run time: 12 minutes
- Detection wavelength: 270 nm
- Injection sequence Blank-standard-Test samples-standard

#### *4.4.1.4. Calibration curve*

To obtain a standard curve, stock solution of NCE was prepared in a concentration of 5 mg/ml. Six standard solutions of 4, 2, 1, 0.5, 0.1 and 0.05 mg/ml, respectively were prepared by diluting the stock solution (5 mg/ml) with appropriate volumes of dilution solvent.

#### *4.2.1.5. Entrapment efficiency determination*

To determine entrapment efficiency for NCE, the unentrapped NCE was separated from loaded nanoparticles by ultracentrifugation and dialysis.

Dialysis was performed in dialysis tubing. Five ml of sample was dialyzed against 100, 200 and 500 ml of distilled water, respectively for 6 hours. One hundred µl of nanoparticle suspension (NPS) was dissolved in1.9 ml of methanol (total volume 2 ml) and used in HPLC analysis. Two ml of the dialysis medium was also analyzed along with the 2 ml diluted nanoparticle suspension.

Ultracentrifugation: NPS was centrifuged in Beckman-L8-70M ultracentrifuge for 2 hours at 10 °C and 165000 g. The pellet formed upon ultracentrifugation was dissolved in 1 ml of methanol and used in HPLC analysis along with the supernatant (undiluted).

#### *4.2.1.6. Particle size analysis*

To determine particle size distribution, NICOMP Submicron Particle Sizer model 370 was used at an angle of 90 degrees and a temperature at 24 °C ± 1 °C. All preparations were carried out in an uncontaminated era using particle-free equipment (Biocap™ RNA DNA dynamic enclosure, NO 189 2000, Cedex, France). The test tubes were prepared beforehand by filling them with distilled water and sonicated for 5 minutes using an ultrasonic bath to remove attached dust particles. The tubes were further rinsed with filtered distillated water

with 0.2 µm size syringe filter. All samples were diluted empirically with filtered distillated water until intensity between 250 – 350 KHZ was obtained. All analyses were performed in triplicates with run time of 10 minutes for each cycle.

#### *4.2.1.7. Zeta potential*

To assess stability of nanoparticles, zeta potential of all samples were measured on Zetasizer Nano Z2600

Before performing the measurements the cell was thoroughly washed with ethanol and water using an appropriate syringe, to ensure totally wetting of the cell. The number of runs was set to 12, each sample measured for 3 runs.

### 4.2.2. Stability testing

4.5.1. Real time stability testing of nanoparticles containing NCE

The test was carried out by storing the nanoparticles at 23 and 4˚C, respectively for 30 days. The changes in the nanoparticles size distributions and drug entrapment were followed. The zeta potential of the nanoparticles was also measured before the stability testing and after 30 days

### 4.2.3. Statistics

All data shown are the average of at least three different experiments, except for some entrapment efficiency and optimization of particle size. Student t-*tests* were applied to compare results. The $p$ value of $\leq 0.05$ was considered statistically significant.

### 4.2.4. Evaluation of data

Student t-test was performed on all the results. The particle sizes were evaluated using NICOMP distribution.

There are some data which are not triplicated, such as some of the entrapment efficiency results. These results are marked with *.

# 5. Results and discussion

## 5.1. HPLC

### *5.1.1. Method validation*

In this project, the HPLC method development of Photocure ASA Company was used with a slight modification in the injection volume. To evaluate the method, 6 different concentrations were used to establish a calibration curve (Figure 9).

The concentration range of 0.05 - 4 mg/ml was found to result in a linear relationship between drug concentration and peak area. The correlation coefficient ($R^2$) was found to exhibit a good linearity (0.9997).

For the method validation, the limit of detection (LOD) and the limit of quantitation (LOQ) were determined. LOD is the lowest concentrations which can be determined as statistically different from a blank while LOQ represents the smallest amount that can be consider reliable(Armbruster and Pry, 2008). The SD is the standard deviation of the response and the slop. The LOD was found to be 0.07 and LOQ 0.23, respectively.

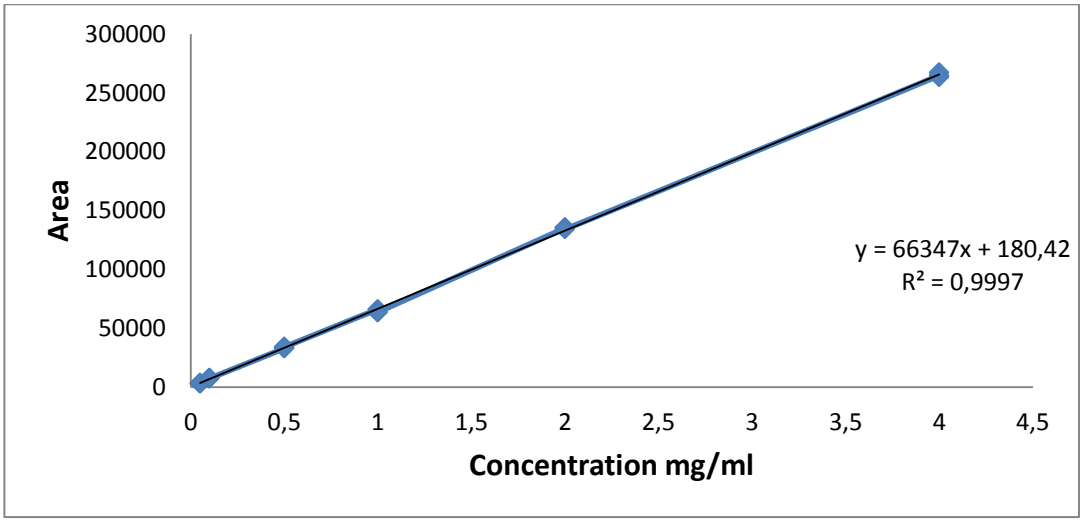


**Figure 9: Calibration curve of NCE**

Our results for the determination of NCE concentrations were within a range of 0.09 to 0.44 mg/ml, which is above the LOD value (0.7), indicating that our data are highly reliable. Some of the results were below LOQ and needed to be considered with precautions.

### *5.1.2. Comments on the HPLC results*

To assure that we indeed were able to separate NCE and the byproduct (ALA-4), we tested a mixture of both substances in a 90:10 weight ratio. The mixture was dissolved in dilution solvent and the analysis resulted in two peaks with a very close peak time (Figure 10).

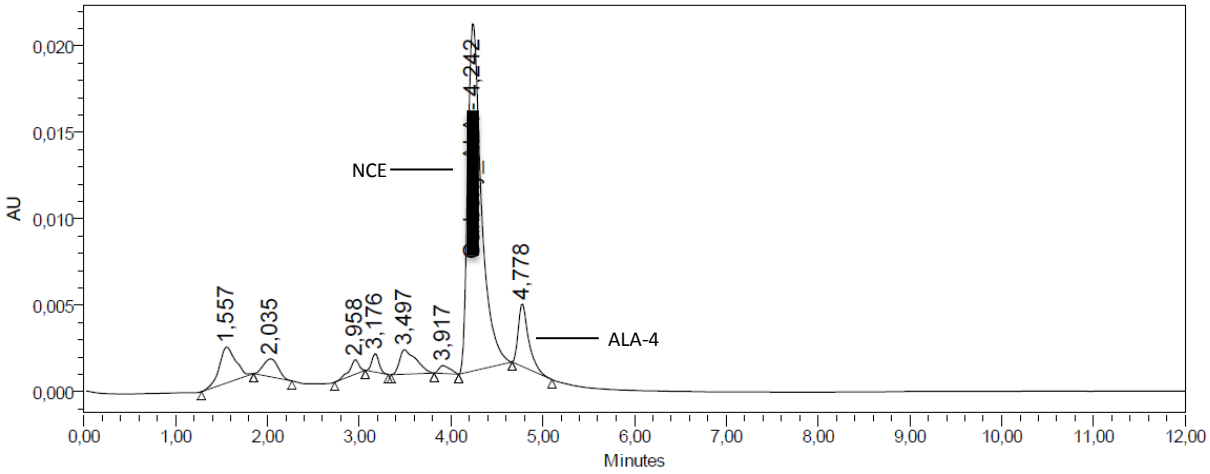


**Figure 10:** The NCE and primary reference standard (ALA-4) mixed in 90:10 weight ratios

As shown in Figure 10, the ALA-4 elute at approximately 4.6 minutes, while the NCE elute at 4.1 minutes. The two substances are separated but a better separation method is needed in the future in respect to stability studies. The information given from the company, addressed that the ALA-4 and NCE have indeed two different retention times, 4 and 7 minutes, but that was not confirmed in this project even after repeated testing.

As a second step, empty and NCE containing nanoparticles were investigated by HPLC to exclude any interference in the measured results. Results demonstrated in Figure 11 and Figure 12 clearly shows that the excipients have a chromatographic peak, with a retention time shorter than that of native NCE. This confirms that the ingredients present in the formulations do not interfere with the peak of NCE.

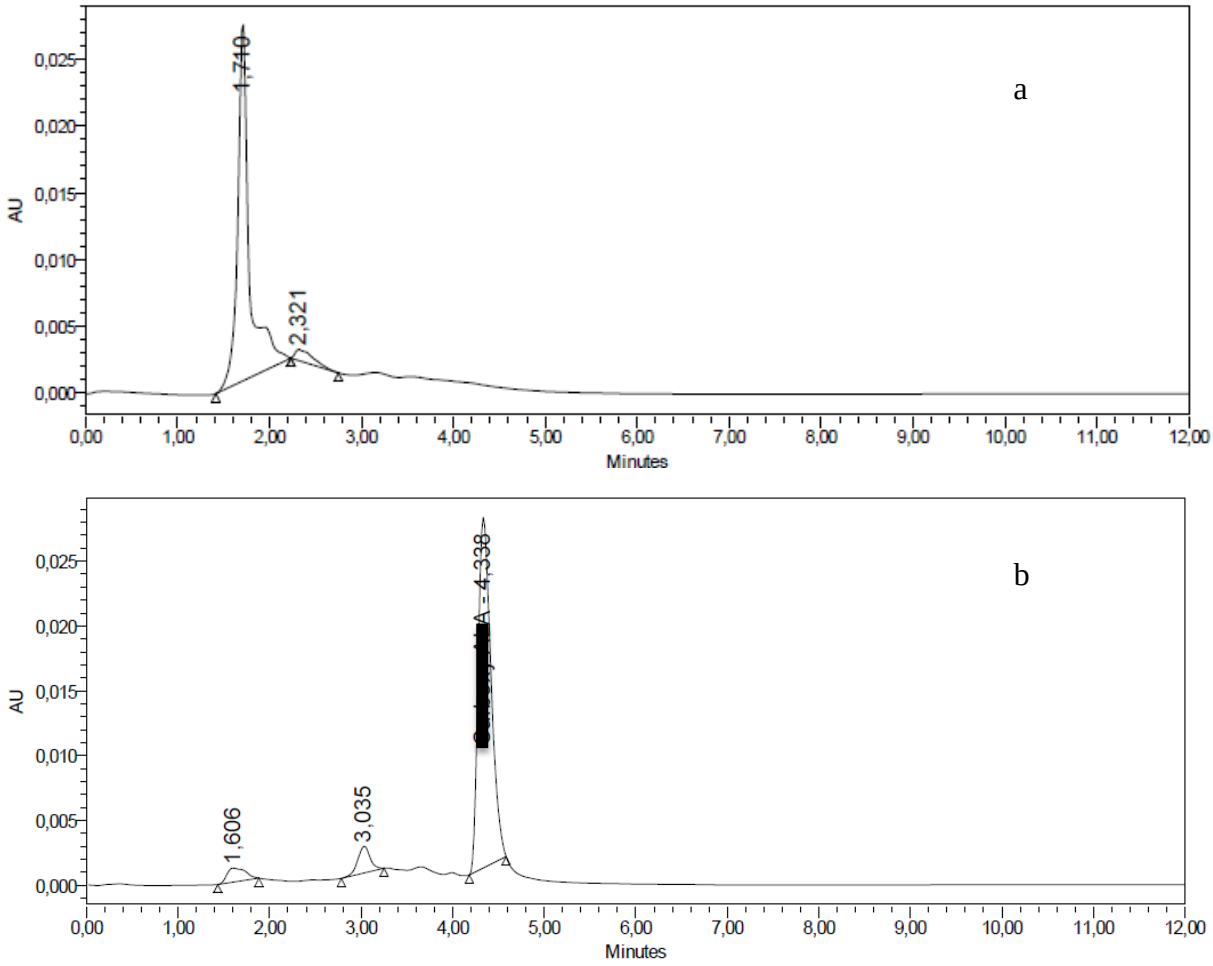


**Figure 11:** Chromatogram of empty nanoparticles (a) and NCE dissolved in dilution solvent (b)

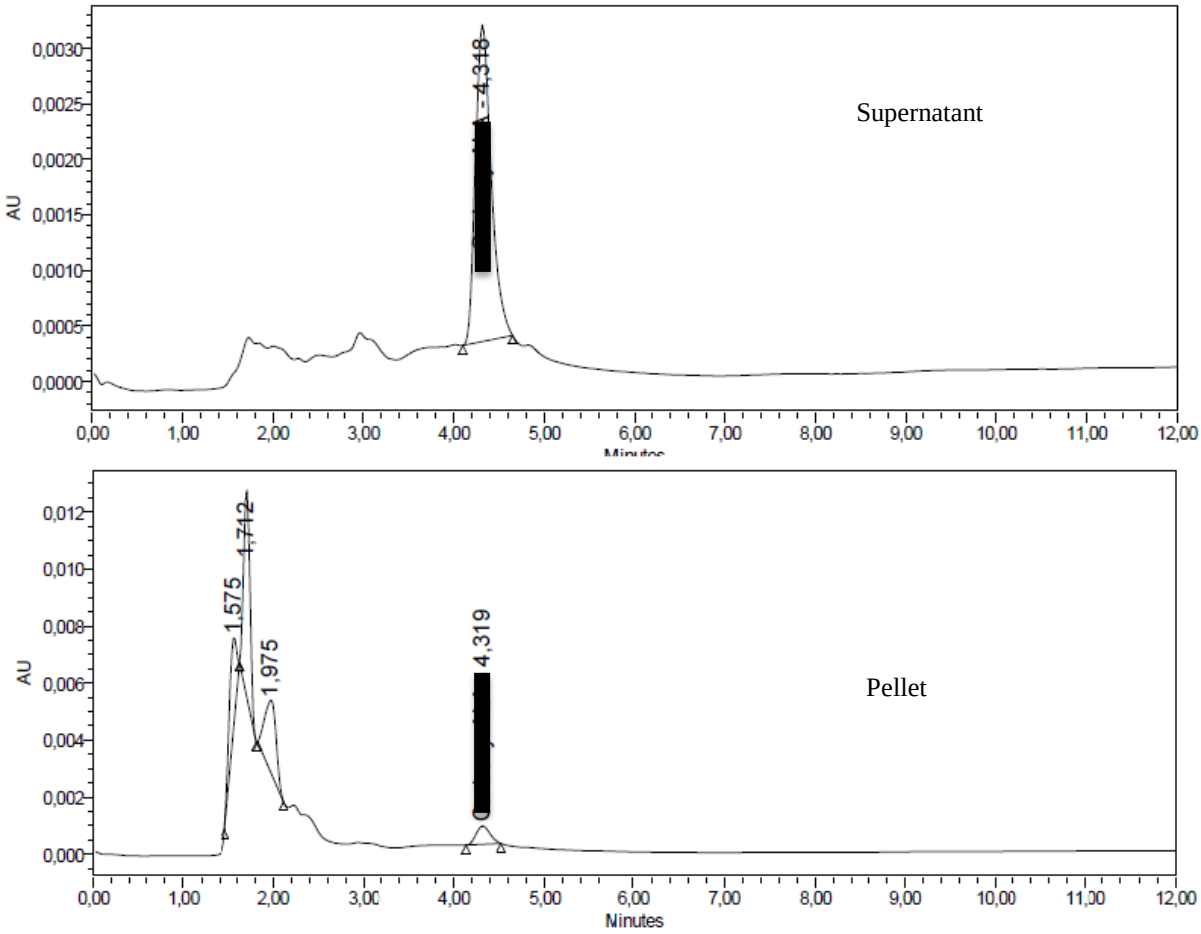


**Figure 12:** Chromatogram of NCE containing nanoparticles centrifuged (pellet and supernatant were both analyzed)

The last step was to investigate the stability of the NCE in dilution solvent and in water. The stability of NCE in dilution solvent after 3, 5, 20 and 59.6 hours from the preparation time is demonstrated in Figure 13. The chromatogram at 3 hours and after 59 hours shows a growing peak in the same retention time. This indicates that the NCE is degraded to another substance. The main degradation pattern for NCE is hydrolysis to ALA-4 and dimerization to dihydropyazine followed with oxidation (information from the Photocure ASA). The suspected degraded product elutes earlier than ALA-4 received from the company. This chromatogram indicates that the degradation product in dilution solvent is not ALA-4. Unfortunately, the stability in water was not determined to evaluate which degradation product is present, due to instrument failure. Wrong storage of the NCE may have affected the stability of the substance causing degradation. The substance need to be stored at a cold temperature. This may have affected our results leading to error and bias.

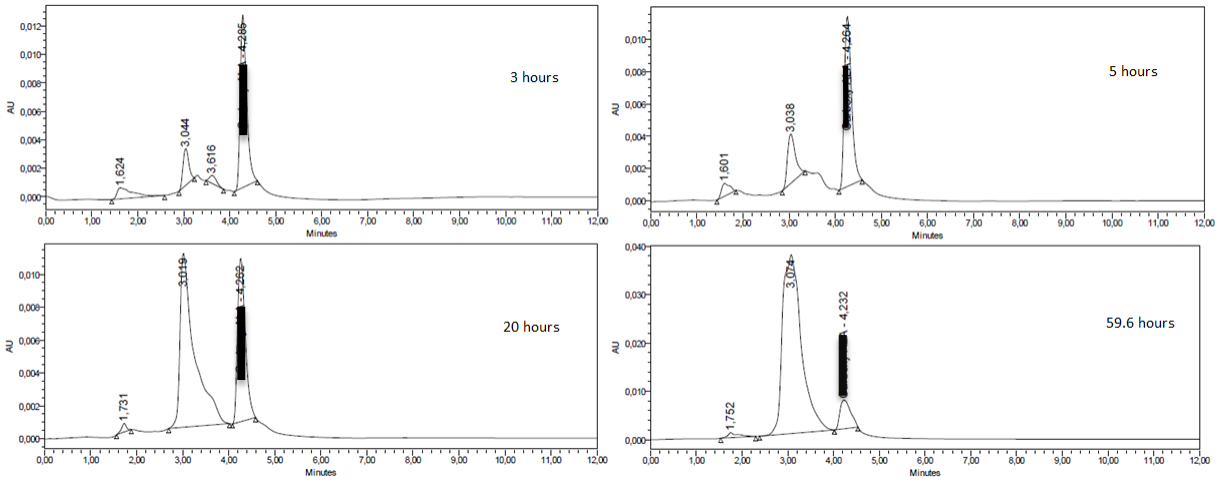


**Figure 13:** Chromatogram of NCE dissolved in dilution solvent at 3, 5, 20 and 59.6 hours from preparation. The samples were stored at 5 ±1 ℃

## 5.2. Nanoparticles characterization

The focus in this study was the development of a new delivery system containing NCE destined for topical application. A formulation based on solid nanoparticles, namely lecithin/chitosan nanoparticles, was selected for this proposes (Sonvico et al., 2006). As a first step, we followed the effects of the lecithin injection rate and the acid used to dissolve chitosan on the particle size distributions in order to optimize these two factors. The results from mixing lecithin/chitosan in 5:1 weight ratios are presented in Table 1.

**Table 1:** The effect of injection rate and HCl and acetic acid on size distribution

| | | **Particle size** | | | | |
|---|---|---|---|---|---|---|
| **Acid type** | **Injection rate ml/min** | Peak 1 (nm) | Intensity (nm) | Peak 2 (nm) | Intensity (%) | **PDI** |
| HCL | 2 | 27.9 ± 4.9 | 4.6 ± 1.5 | 112.6 ± 1.3 | 95.4 ± 1.5 | 0.22 |
| | 34 | 32.9 ± 25.3 | 9.6 ± 12.4 | 137.8 ± 24.6 | 90.4 ±12.4 | 0.16 |
| Acetic | 2 | 51.3 ± 8.0 | 9.2 ±2.2 | 184.8 ± 8.0 | 90.0 ±2.26 | 0.23 |
| acid | 34 | 58.3 ± 7.6 | 12.6 ±5.2 | 205.3 ± 22.2 | 87.0 ± 5.5 | 0.24 |

Chitosan was dissolved in hydrochloric acid, as reported in the method established for lecithin-chitosan preparation (Sonvico et al., 2006), and also in acetic acid (Tan et al., 2011).

The injection rate used along with the choice of the acid has shown an effect on the particle size and the polydispersity (PD) of nanoparticles. A slight increase in their size was observed when the injection rate increased from 2-34 ml/min. However, the differences were not on significant level ($p>0.05$).

Polydispersity is an indicator of the width of the particle size distribution (Rogošić et al., 1996). A significant reduction of the PD was observed when chitosan/HCL solution was used (Table 1). This corresponds to the finding in the previous reports (Sonvico et al., 2006) where the reported PD was less than 0.2 (injection rate 40 ml/min). This indicates that the particles were more homogeneous in sizes then those prepared with the slow injection rate. On the other hand, there were no significant differences in the PD when chitosan/acetic acid was used.

Based on these findings, we have chosen to continue using chitosan/acetic acid for their optimal particle size (205.3 ± 22.2 nm) and applied 34 ml/min injection rate in further experiments.

The second step in this study was to develop a delivery system containing NCE. The separations of the entrapment drug were performed by using ultracentrifugation, when dialysis method (Skalko-Basnet et al., 2000) was found to be unsuitable for our samples. The results are represented in Table 2.

**Table 2:** Influence of the concentration of NCE on entrapment efficiency

| **NCE (mg)** | **Lecithin (mg)** | **Chitosan (mg)** | **Entrapment efficiency (%)** |
|---|---|---|---|
| 0 | 100 | 20 | n.d. |
| 10 | 100 | 20 | n.d |
| 30 | 100 | 20 | * |
| 100 | 100 | 20 | 4.15 ± 0.23 |

n.d. not determined
* not triplicated

The NCE is a hydrophilic drug which explains its low entrapment efficiency (Sonvico et al., 2006). Provisional experiments showed that encapsulation of metoclopramide HCl, a hydrophilic drug, in lecithin/chitosan nanoparticles were nearly impossible (Calvo et al., 1997). We managed to achieve entrapment efficiency of less than 5 % when 100 mg drug concentrations were used.

The determined size distributions of lecithin/chitosan nanoparticles were found to be influenced by the amount of NCE incorporated in nanoparticles as demonstrated in Table 3

**Table 3:** Representative size distribution

| | **Particle size** | | | | |
|---|---|---|---|---|---|
| **NCE** | Peak 1 | Intensity | Peak 2 | Intensity | **PD** |
| **(mg)** | (nm) | (nm) | (nm) | (%) | |
| 0 | 58.3 ± 7.6 | 12.6 ±5.2 | 205.3 ± 22.2 | 87.0 ± 5.5 | 0.23 |
| 10 | 38.4 ± 8.3 | 6.7 ±1.5 | 177.9 ±18.6 | 92.9 ±1.8 | 0.25 |
| 30 | 58.8 ± 2.1 | 13.9 ± 1.4 | 214.2 ± 8.8 | 85.4 ±1.3 | 0.27 |
| 100 | 54.3 ± 19.3 | 15.1 ± 10.4 | 188.3 ±35.6 | 84.6 ± 10.4 | 0.26 |

Nanoparticles prepared with 10 and 100 mg NCE have shown a decrease in size and an increase in PD. These findings correspond with to findings by Barbieri et al. (2013). However, when 30 mg of NCE was used in the preparation of nanoparticles, an increase in size distribution and the PD were observed. These results indicate possibly that the NCE may have an influence on the structure of nanoparticles (Barbieri et al., 2013).

Another two parameters which were affected by the concentration of NCE used for the preparation of nanoparticles are the zeta potential and the pH.

**Table 4:** The influence of the concentration of NCE on zeta potential and pH

| Drug (mg) | Zeta potential (mV) | pH |
|---|---|---|
| **0** | **-** | 4,6 |
| 10 | 2.4 ± 0.4 | 4.1 |
| 30 | 3.1 ± 1.2 | 3.5 |
| 100 | 17.5 ± 5.8 | 2.6 |

As demonstrated in Table 4 , the zeta potential increased with the increased NCE concentration from 2.4 ± 0.4 to 17.5 ± 5.8 mV (Barbieri et al., 2013). The zeta potential was observed to be rather unstable while measuring, especially with the increased NCE concentration.

The NCE has an acidic functional group which explains the decrease of pH with the amount of NCE added. When the amount of NCE is increased in the suspension the acidity also increased, which resulted in decrease in pH values from 4.6 for empty nanoparticles to 2.6 in nanoparticles for which preparation 100 mg NCE was used.

To optimize the new delivery system, in order to increase the entrapment efficiency, various factors were investigated. In this study we investigated the effect of the polymer and lecithin concentration on the particle size and entrapment efficiency.

A preliminary analysis, multiple linear regressions, was performed on the data to see if chitosan and lecithin have any significant effect on the size. The results are represented in Figure 14. (The data used in the analysis are not shown)

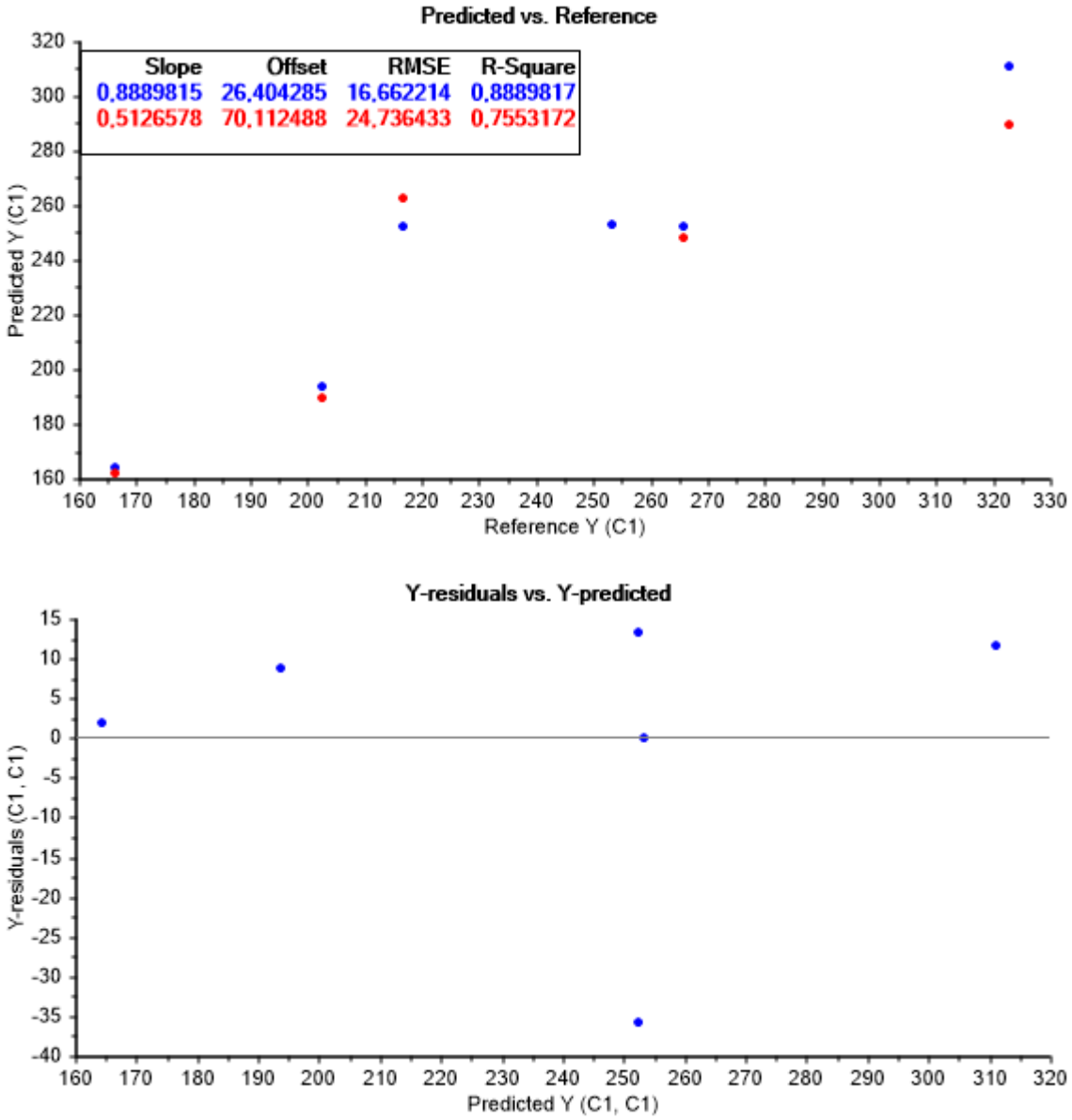


**Figure 14:** Multiple linear regressions of empty nanoparticles with different concentration of lecithin and chitosan

Lecithin and chitosan were found to significantly contribute to the increase in the size, however, due to the limited number of experiments the findings need to be taken with precaution. The findings provided an idea in which direction should the further optimization proceed.

The effect of different chitosan lecithin concentration on entrapment efficiency, size and zeta potential were investigated. The aim was to increase the size, entrapment efficiency, and to investigate the stability of those new formulations.

**Table 5:** Influence of lecithin and chitosan concentration on NCE entrapment efficiency and pH

| NCE (mg) | Chitosan (mg) | Lecithin (mg) | Entrapment efficiency (%) | pH |
|---|---|---|---|---|
| 100 | 20 | 100 | 4.6 ± 0.2 | 2.6 |
| 100 | 20 | 200 | 12.2 ± 1.2 | 2.6 |
| 100 | 20 | 400 | 18.4 ± 0.9 | 2.6 |
| 100 | 40 | 400 | 21.2 ± 0.2 | 2.6 |
| 100 | 120 | 100 | 5.8 ± 0.2 | 4.1 |
| 100 | 120 | 200 | 12.0* | 4.1 |

* The entrapment efficiency was determined in duplicate

As shown in Table 5, the determined entrapment efficiency increased in all formulation from 4.6 ± 0.2 up to 21.2 ± 0.2 %. Lecithin concentration showed the most significant effect on the entrapment efficiency. By increasing the lecithin amount from 100 – 400 mg we achieved the entrapment efficiency of 4.6 ± 0.2 to 18.4 ± 0.9 %. Doubling the chitosan concentration alone from 20 – 40 mg has resulted in entrapment efficiency of 4.6 ± 0.2 to 5.8 ± 0.2 %. The combination effect of lecithin and chitosan were also investigated by preparing chitosan and lecithin in 40:400 weight ratios. This combination gave the best entrapment achieved (21.2 ± 0.2 %).

The zeta potential on NCE-containing nanoparticles varied, even when we tried to control the pH of the water used for measuring and for preparation of formulations. The results were unfortunately not representative and are therefore not included in this thesis. To investigate the source of this instability we suspected other factors such as the pH of the suspension, light exposure and temperature changes. The last two factors were investigated and their effects presented later in the results.

The pH remained fairly constant in all formulations except in those where chitosan amount increased from 20 to 120 mg. This can be explained by chitosan's cationic character (Liu et al., 2011).

Last was the effect of lecithin and chitosan concentrations on particle size distribution as demonstrated in Table 6. Both lecithin and chitosan exhibited an effect on size distribution.

**Table 6:** Representative size distribution

| | Particle size | | | | |
|---|---|---|---|---|---|
| **C:L weight ratio (mg : mg)** | Peak 1 (nm) | Intensity (%) | Peak 2 (nm) | Intensity (%) | **PD** |
| 20:100 | 54.3 ± 19.3 | 15.1 ± 10.4 | 188.3 ±35.6 | 84.6 ± 10.4 | 0.26 |
| 20:200 | 118.9 ± 34.5 | 24.4 ± 13.0 | 305.2 ± 21.1 | 84.3 ± 4.4 | 0.31 |
| 20:400 | 159.7 ± 24.2 | 18.9 ± 6.1 | 679.9 ± 153.6 | 81.1 ± 6.1 | 0.41 |
| 40:400 | 157.3 ± 74.6 | 16.1 ± 10.3 | 677.0 ± 170.7 | 83.9 ± 10.3 | 0.47 |
| 120:100 | 174.2 ± 21.2 | 31.8 ± 7.8 | 767.7 ± 195.0 | 66.3 ± 8.0 | 0.46 |
| 120:200 | 162.0 ± 43.4 | 21.0 ± 5.0 | 661.6 ± 286.8 | 76.8 ± 5.2 | 0.52 |

Notes: C:L, chitosan to lecithin weight ratio; PD, Polydispersity

As demonstrated Table 6 the PD increased in all formulations (from 0.26 to 0.52); chitosan and lecithin in the 120:200 weight ratios formed the most heterogeneous populations of nanoparticles (PD value 0.52). The particle sizes have increased in all formulations. However, the sizes are optimal for application to the skin and wound.

### 5.3. Comments on the instability of the zeta potential

The instability of the zeta potential was one of the challenges we faced already in the early stages of the project. The instability was first suspected to be due to the pH fluctuation of the water supply in Drug Transport and Delivery Research Group facilities. We tried using water with the same pH value for each measurement. However, the instability in values measured for zeta potential continued. This implied that other factors such as the effect of the suspensions pH (2.6), light exposure and temperature exposure can also contribute to the changeable zeta potential values. To investigate the effect of temperature and light exposure, two formulations (20:400 chitosan to lecithin weight ratio) were stored at two different temperatures (5 ± and 23 ±1 °C) and were well protected from light. The zeta potential was measured every week for five weeks. The results are presented in Figure 15.

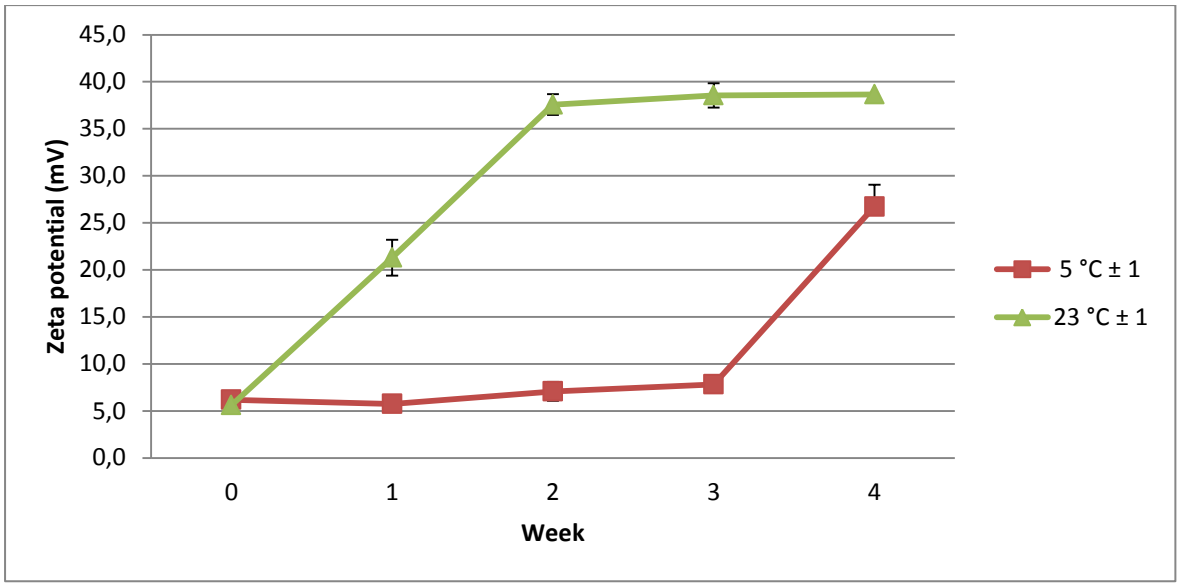


**Figure 15:** The change of the zeta potential stored in two different storage conditions over 4 weeks

Temperature is shown to have significant effect on zeta potential, during 4 weeks of testing the zeta increased from 5.6 to 38 mV. This means that the higher temperature results in more repulsive particles in contrast with the low temperature. Nanoparticles stored at low temperature were stable for 4 weeks and by the end of the 4th week, an increase was observed. Chitosan and the degradation product of NCE are suspected to have an influence on these instabilities. Chitosan is a cationic polymer, which contains an amino group that can be protonated at pH<6.5. The NCE may form a degradation product which may have another charge in pH 2.6. Electrostatic interaction between positively charged amino group and negatively charged lecithin forms nanoparticles. (Sonvico et al., 2006).

The effect of light exposure has been observed in earlier formulations. In formulations which were stored at 5 ±1°C, without protection from light exposure, a change in the zeta potential was more rapid than when protected from light (data not shown). In the next step of the project, more experiments need to be performed to elaborate on this finding.

## 5.4. Stability testing

To evaluate the stability of the formulation, we selected 20:400 mg chitosan lecithin nanoparticles (due to their sizes) and performed a stability test in two storage conditions, 23 ± 1 and 5 ± 1˚C, respectively. The samples were protected from light throughout the testing and the results are presented in Table 7 and Table 8

**Table 7:** Size distrubustion of NP stored in 23±1˚C and 5 ± 1˚C for 1 month

| | | Particle size | | | | |
|---|---|---|---|---|---|---|
| **Temperature (˚C)** | **Day** | Peak 1 (nm) | Intensity (nm) | Peak 2 (nm) | Intensity (%) | **PD** |
| | 1 | 220.9 ± 100.0 | 32.0 ± 18.3 | 741.8 ± 226.5 | 66.1 ± 19.8 | 0.40 |
| 23 ± 1 | 30 | 167.3 ± 78.0 | 16.2 ± 11.0 | 903.9 ± 0.9 | 83.4 ± 11.2 | 0.44 |
| | 1 | 136.8 ± 43.4 | 16.4 ± 9.6 | 517.0 ± 62.0 | 68.45 ± 13.5 | 0.39 |
| 5 ± 1 | 30 | 189.1 ± 27.1 | 22.3 ± 4.2 | 673.5 ± 163.5 | 79.1± 3.5 | 0.41 |

The size and the PD in both storage conditions increased as compared with the freshly prepared formulations. This increase however was found to be not on the significant level.

**Table 8:** The entrapment efficiency and zeta potential changes during stability testing

| **Temperature (˚C)** | **Day** | **Entrapment efficiency (%)** | **Zeta potential (mV)** |
|---|---|---|---|
| | 1 | 18.3 ± 0.2 | 5.7 ± 0,0 |
| **23 ± 1** | 30 | - | 38.7 ± 1.2 |
| | 1 | 18.0 ± 0.2 | 6.2 ± 0.4 |
| **4 ± 1** | 30 | - | 26.7 ± 2.2 |

The zeta potential has increased dramatically during stability testing at room temperature compared with formulation stored at 5 ±1 ˚C (Figure 15). This indicates that the temperature is affecting the stability of the suspension. The entrapment efficiency after 30 days was not determined due to the technical problems, but the changes in zeta potential indirectly suggest that NCE was more exposed on the out surfaces of nanoparticles.
Another possibility for the zeta instability is that the degraded product has another charge which affects the zeta potential. Chitosan and NCE in low pH (2.9) may undergo more protonation which affects the zeta charge.

The findings in stability testing along with previous results indicate a need to address the stability issue of both NCE as substance and nanoparticles containing NCE. The effect of degradation product on the stability also needs to be investigated in the future.

## Conclusions

Our aim in this project was to develop chitosan lecithin carrier for the NCE destined for topical treatment. The selected carrier was found not to be optimal with respect to the entrapment efficiency. The vesicles we have developed might be more appropriate for more lipophilic substances; however, there is a room for improvement and optimization of a system. We confirmed that both chitosan and lecithin have effect on the vesicle characteristics but other factors such as stirring rate, pH, the choice of the acid and the type of chitosan can be also utilized to optimize the carrier system. We have developed a reliable HPLC method for NCE; however, the method should be optimized in respect to the separation of intact NCE and the byproduct ALA-4. More experiments in long term stability are needed.

## 7. Perspectives

- Chitosan lecithin nanoparticles developed through this project need to be optimized due to their instability. The effect of these factors needs to be further investigated and optimized
    - pH
    - temperature
    - storage conditions
    - stability

- In the long term perspective, more experiments in long term stability are needed.

- Chitosan nanoparticles prepared by other method should be evaluated as potential alternative.

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