THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACING THE CHALLENGE OF SKIN PENETRATION

– A STUDY ON LIPIDIC LIQUID CRYSTALLINE PHASES FOR DERMAL DRUG DELIVERY

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Cover: Photograph of the gel-like cubic phase formulation (left), two-photon microscopy image of human *stratum corneum* with the autofluorescence in green and the added protein in red (middle), the cubic phase structure (right).

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ABSTRACT

The skin is a barrier which protects us against daily attacks of foreign substances. From a pharmaceutical perspective it is also an interesting delivery route with its large surface and possibility to reach the blood stream circumventing first passage metabolism. With the varied size, structure etc. of active pharmaceutical substances, as well as the structure and function of the tissue, skin penetration is challenging.

The work included in this thesis mainly focus on exploring the lipid based cubic liquid crystalline phases as dermal drug delivery vehicles. Of particular interest has been systems based on glyceryl monooleyl ether (GME), while cubic phase containing glyceryl monooleate (GMO) and water was used as reference system. These lipids and cubic phases are very similar but an important difference is that GME requires a solvent to form a cubic phase in excess water while GMO does not. The cubic phases made of GME, are less studied but have potential to be effective dermal delivery vehicles. The phase behavior of the GME systems has been thoroughly investigated and the dermal delivery of active compounds from the cubic formulations evaluated. Examples of the methods used are small angle X-ray diffraction, *in vivo* fluorescence spectrophotometry, two photon microscopy, and high performance liquid chromatography.

The GME-based formulations showed to be as good as the GMO-based cubic phase for dermal delivery of small molecules. The cubic formulations significantly enhanced the delivery, both in amount and depth, compared to a commercial product. For larger molecules and nanoparticles, however, skin penetration was found only when a GME-based cubic formulation was used as delivery vehicle.

Through combining all obtained results, a hypothesis of the dermal delivery mechanism was formed. The skin adherence and the occluding and hydrating effects of the formulations are important for effective delivery. However, the phase behavior of the formulations is also important to explain the mechanism. The GME-based cubic phase turns into a reversed hexagonal phase in excess water, whereas the GMO-based formulation maintains its cubic structure. This small but significant difference may be the reason for the difference in ability to deliver larger molecules and particles into skin.

Keywords: *liquid crystalline phases; cubic phase; reversed hexagonal phase; dermal drug delivery; glyceryl monooleat; glyceryl monooleyl ether; pentane-1,5-diol; propylene glycol; N-Methyl-2-pyrrolidone*

LIST OF PUBLICATIONS

This thesis is based on the following scientific papers, referred to by their Roman numerals in the text. The papers are appended at the end of the thesis.

Paper I. **Effect of pentane-1,5-diol and propane-1,2-diol on percutaneous absorption of terbinafine**
Hanne Evenbratt, Jan Faergemann, Acta Dermato-Venereologica, 2009, 89, 126-129

Paper II. **Cubic and sponge phases formed in ternary ether lipid-solvent-water systems: Phase behavior and NMR characterization**
Hanne Evenbratt, Lars Nordstierna, Marica B. Ericson, Sven Engström, Langmuir, 2013, (Submitted)

Paper III. **In vivo study of an instantly formed lipid-water cubic phase formulation for efficient topical delivery of aminolevulinic acid and methyl-aminolevulinate**
Hanne Evenbratt, Charlotte Jonsson, Jan Faergemann, Sven Engström, Marica B. Ericson, International Journal of Pharmaceutics, 2013, 452, 270-275

Paper IV. **Delivery of serum albumin from a cubic phase into viable epidermis studied by two-photon microscopy**
Hanne Evenbratt, Sven Engström, Marica B. Ericson, Pharmaceutical Research, 2013 (Submitted)

CONTRIBUTION REPORT

Paper I. Performed main part of the experimental work and analyzed the results. Main author of the paper.

Paper II. Shared responsibility with Engström for planning the experimental work and for analysis of the results. Main author of the paper.

Paper III. Planned the experiments with Ericson and Engström. Performed the experimental work and data analysis. Main author of the paper.

Paper IV. Planned the experiments with Ericson. Responsible for preparing all formulations and skin samples. Performed the microscopy work, the data analysis, and interpreted the results. Main author of the paper.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Published:

Design and characterization of a novel amphiphilic chitosan nanocapsule-based thermo-gelling biogel with sustained in vivo release of the hydrophilic anti-epilepsy drug ethosuximide
Hsiao MH, Larsson M, Larsson A, Evenbratt H, Chen YY, Chen YY, Liu DM, J Control Release. 2012, 161(3), 942-948.

Effects of calcium, pH, and blockiness on kinetic rheological behavior and microstructure of HM pectin gels
Löfgren C, Guillotin S, Evenbratt H, Schols H, Hermansson AM, Biomacromolecules, 2005, 6(2), 646-652

Relevant manuscripts:

Multiphoton induced luminescence from 10 nm gold nanoparticles – the effect of interparticle distance and aggregation
Guldbrand S, Evenbratt H, Borglin J, Kirejev V, Ericson MB, 2013, (Manuscript)

ABBREVIATIONS

ALA	δ-aminolevulinic acid
AuNP	gold nanoparticles
BSA	bovine serum albumin
CARS	coherent anti-Stokes Raman Spectroscopy
CER	ceramides
CHOL	cholesterol
DP	dermal papillae
FFA	free fatty acids
GME	glyceryl monooleyl ether
GMO	glyceryl monooleate
HPLC	high-performance liquid cromatography
HSA	human serum albumin
MAL	methylaminolevulinate
NADPH	nicotinamide adenine dinucleotide phosphate
NMP	N-Methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
NP	nanoparticles
PEG	polyethylene glycol
PG	propylene glycol
POL	pentane-1,5-diol
QD	quantum dots
SA	serum albumin
SAXD	small angle X-ray diffusion
SB	stratum basale
SC	stratum corneum
SPR	surface plasmon resonance
SS	stratum spinosum
TMR-BSA	tetramethyl rhodamine conjugated bovine serum albumin
TPM	two-photon microscopy

TABLE OF CONTENTS

1 INTRODUCTION

In this thesis, I strive to put the research into a wider context and to create a knowledge platform for a discussion of my results. However, the reason for the project and the main research questions are most easily explained through a specific story.

The story begins at the department of dermatology at Sahlgrenska University Hospital, where Professor Jan Faergemann found out that an interesting antimycotic diol (pentane-1,5-diol, POL) was able to enhance dermal drug delivery. POL had previously not been used for skin formulations and Jan wanted to investigate the enhancing qualities of the substance further.

Meanwhile, at pharmaceutical technology, Chalmers University of Technology, Professor Sven Engström and his PhD -students were working with liquid crystalline phases, both characterizing them and investigating their abilities as drug delivery vehicles. During this research they came in contact with Associate Professor Marica Ericson who has extensive knowledge of advanced imaging techniques and their use for investigating skin diseases such as cancer. In a joint project they examined the use of the cubic phase as a drug delivery vehicle in photodynamic therapy (PDT), a treatment method for superficial forms of skin cancer. They found that the cubic phase significantly enhanced the diffusion of active substance into the cells. However, there were problems encountered, for example both the drug and the formulation had stability issues.

Through the center for skin research (SkinResQU) in Gothenburg, Jan, Sven and Marica met one another. By combining their separate questions and competences a new research project was born and I started my PhD studies.

My work started with comparing POL with the known permeation enhancer propylene glycol (PG) for increased delivery of terbinafine to the upper skin layers (paper I). POL showed to be an efficient permeation enhancer in the hydrogel used as a delivery vehicle, and as POL is an apolar diol it was also of interest as a component in liquid crystalline phases. Liquid crystalline phases

are molecularly ordered structures which can be formed by polar lipids, such as, glyceryl monooleate (GMO) and glyceryl monooleyl ether (GME) in presence of water. GMO forms a cubic phase in excess water while GME and water results in the reversed hexagonal phase. An apolar solvent can be used to reach specific liquid crystalline phases to, for example, form the cubic phase in the GME-water system. Included in this work were two different types of cubic formulations in excess water, the well-known GMO-water based system and the less investigated GME-solvent-water based system. The phase behavior of two systems composed of the lipid GME, water, and one of the solvents POL or N-Methyl-2-pyrrolidone (NMP) was thoroughly investigated (paper II).

With a commercial interest, improved versions of the delivery system for use in PDT were sought and found in the combination of GME, water, and POL or PG. By exchanging the lipid of the cubic phase formerly used and including one of the solvents, the stability issues of the previous system were solved and an on demand cubic formulation was obtained. The diffusion of the active substances from the formulation into skin tissue was investigated *in vivo* and showed good results (paper III). Despite the good results the commercialization process was terminated so that publication of the work was enabled.

In order to find out how effective this formulation is for dermal delivery, protein (bovine serum albumin, BSA) and nano-particles (quantum dots, QD, and gold nanoparticles, AuNP) were tested as model drugs. When AuNP in cubic formulation were applied to the skin and analyzed using two-photon microscopy (TPM), the particles could not be detected due to optical phenomena. This led to a digression into technical and physical aspects of the occurrences. However, other nano-sized particles and protein could be found within the skin when the solvent containing on demand formulations were used (paper IV).

As can be seen from the contents of the research described, there has been a clear goal to enhance dermal drug delivery by exploring cubic formulations as a delivery system. However, it is of course also important to understand the underlying mechanisms of the interaction between formulation and skin. In order to find even more clues to this puzzle, an attempt was made to locate the lipids of the formulation among the lipids of the skin and thus coherent anti-

Stokes Rahman spectroscopy (CARS) was employed. The results showed that GME penetrated around the cells and was in part found as droplet-shaped depots within the tissue.

The results obtained in the different studies all contributed to the formation of a hypothesis regarding the dermal drug delivery mechanism. The cubic phase formulations are very similar in most aspects. However, the phase behavior and the ability to deliver larger molecules and particles differ. The postulated hypothesis takes both similarities and differences into account and will be further discussed in Chapter 3.4.

However, prior to this, the research field is described through the description of, for example, the skin structure and function and dermal drug delivery in Chapter 2. With these facts in mind the reasons for choosing the nano-structured cubic phase as a delivery vehicle are revealed. The configurations of the liquid crystalline phases are presented, as well as their relation to each other. Further, the phases as carriers of active substance are described, where after the combination of cubic formulation, pharmaceuticals and skin are tied together (Chapter 3). Throughout the work a number of techniques have been used for assessment. These are described in Chapter 4. Lastly, the thesis is concluded by presenting the results of the performed studies and the impact of them on the hypothesis, followed by future research ideas (Chapter 5-7).

2 DERMAL DRUG DELIVERY

This chapter deals with skin structure and functions as well as the delivery of active pharmaceutical ingredients (API) into the skin tissue. In order to be able to suggest a delivery mechanism these are important areas to have knowledge about.

2.1. DRUG DELIVERY TODAY

2.1.1. DELIVERY ROUTES

Anyone in our modern society who has ever had a cold or any kind of illness has used pharmaceuticals of some sort. The most common administration routes for drug delivery, depicted in Figure 1, are oral, pulmonary (via the lungs), parenteral (piercing skin or mucus membranes), and topical (via body surfaces; skin or mucus membranes such as vagina, anus, throat, eyes, nose, ears). There are a number of reasons why a certain route and a certain formulation is chosen for a specific drug. Firstly, it must be decided if the drug should have a local or a systemic effect. Secondly, physical and chemical properties of both the drug and the additives must be understood and addressed as well as factors influencing drug absorption and release [1, 2].

Here the focus lies on dermal drug delivery which is a part of the fourth route mentioned – the topical route. The term dermal delivery is often confused with transdermal delivery. However, throughout this text dermal delivery will be referred to for delivery into the skin tissue while transdermal delivery enables penetration through the skin and into the blood stream.

Dermal delivery typically has a very high patient compliance [3]. The few occurring side effects can normally be circumvented simply by changing administration site [4, 5]. Although the delivery may at times be both poor and erratic, it can be controlled and sustained over a prolonged period of time [6] and with more research the drug delivery is improving. The large variability in

tissue thickness and permeability between individuals, and more importantly between healthy and diseased skin can cause problems with regard to drug delivery [7]. Topical delivery is often used to achieve a local effect, but it is also possible to aim for systemic uptake. As with injections and inhalation, the first passage effect is circumvented and, additionally, the drug application is easy and pain free [1].

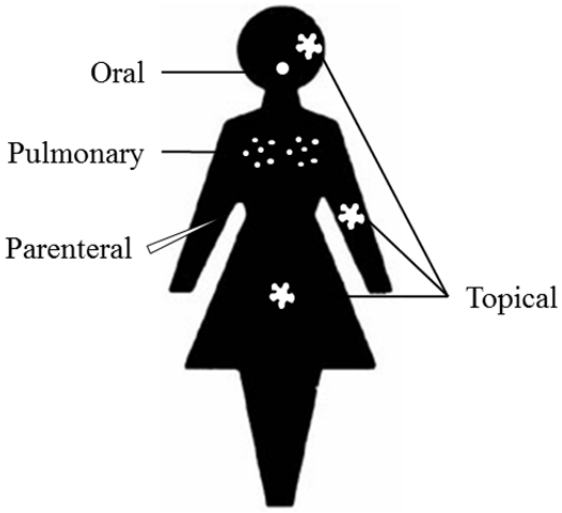


Figure 1 Schematic image showing examples of drug delivery routes.

2.1.2. ACTIVE INGREDIENTS IN THE FORMULATIONS

Current active pharmaceutical ingredients (API) tend to have larger, more complex, and less soluble molecular structures. This, of course, must be dealt with. One method is to turn the drug into a salt. This can also help stabilizing an easily degradable drug, for example a protein [8].

The formulations holding the drug must be able to contain and deliver the API, regardless of their respective difficulties and chosen delivery route. If the galenic approach is chosen wisely the drug can be aided to work as efficiently as possible. In topical treatment this is particularly difficult, as the main focus is to get the drug through the tissue. In paper III-IV dermal delivery of a highly degradable substance and large molecules are handled. This will also be discussed further in Chapter 3.2.

When comparing and interpreting dermal delivery data obtained in different studies it is important to be aware of the possible variations in methodology,

including type of equipment used, skin temperature, and skin treatment [9]. Caution is of great importance as is a substantial knowledge about skin structure and function. The skin structure and function also comes in to play when designing a well working topical drug delivery system.

2.2. THE LAYERED STRUCTURE OF THE SKIN

If you look down at the hands holding this thesis you will see skin. Depending on your size, you are covered with around 1.5-2 m^2 of the tissue. For long the skin was regarded as nothing more than what keeps our insides in place, but as research took off, our largest organ proved to have fascinating features. The skin is a barrier against environmental attacks such as UV radiation, chemicals and microbiological factors. Additionally, one of our five senses is located in the skin: the ability to feel pain or the slightest touch both protects us and increases the quality of life. Furthermore, controlling the amount of internal water and body temperature is maintained by the skin. Also worth mentioning, the essential vitamin D is here produced in a photo-driven reaction [10].

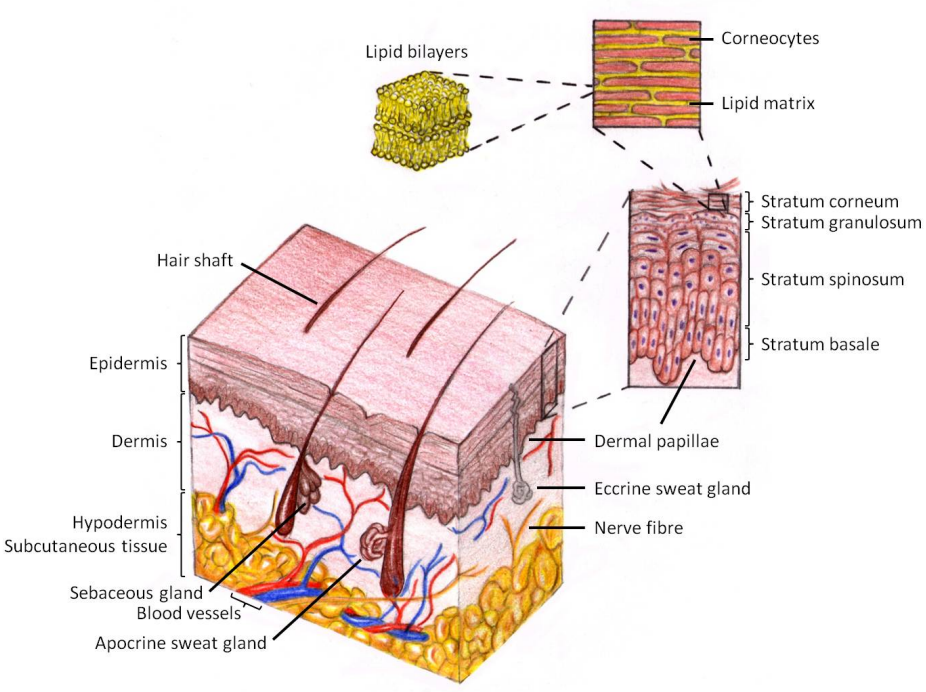


Figure 2 A schematic illustration of the skin and its layers.

The skin is divided into three main layers: epidermis, dermis, and subcutis (Figure 2). Each layer has functions of its own. For warmth and comfort, the fatty tissue of the subcutaneous layer provides insulation and padding. Subcutis is located closest to the muscle tissue with dermis on the outside. Dermis provides elasticity to the skin through collagen and elastic fibers, and contains for example, nerve cells, hair follicles, and glands (see Figure 2). Epidermis forms the skin surface and prevents water loss. Because of this vital function it is also the most prominent obstacle in transdermal drug delivery. Thus, it is the most important to understand in order to solve the obstacles encountered when administering APIs via the skin.

Epidermis is divided into layers based on keratinocyte cell differentiation: stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). Figure 3 [11] illustrates the different skin layers both as a schematic drawing and as two-photon microscopy (TPM) images (further detail regarding the methodology will presented in Chapter 4.3.). In SB the keratinocyte cells are formed and they migrate towards the skin surface becoming flatter the further out they get. When the keratinocyte cells have lost their nucleus, once they reach SC, they are referred to as corneocytes. At the surface these cells are dead and eventually peel off leaving room for the cells following in a slow but never-ending migration of cells [10].

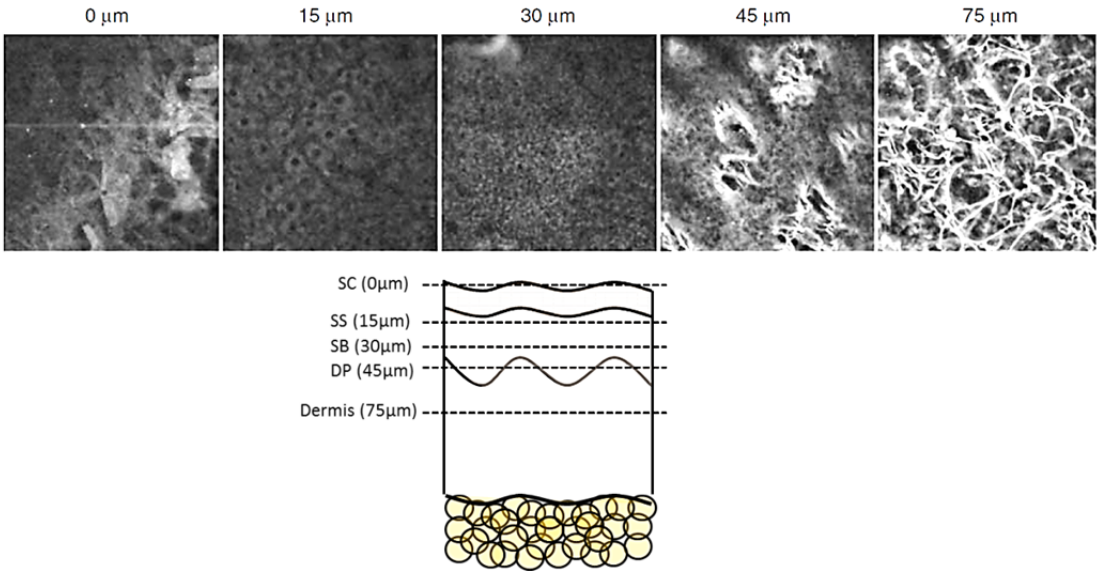


Figure 3 Two-photon microscopy images of normal skin. SC (0 mm), SS (15 mm), SB (30 mm), dermal papillae (DP) (45 mm), and dermis (75 mm) are visualized (images published with permission [11]). Below figure: a schematic illustration of depth location of the respective layers.

The structure of SC is often compared to a brick wall, where the corneocytes are the bricks and the lipids surrounding them the mortar [12]. The main lipids forming the mortar are ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA) (Figure 3b), composed in such a defined way that alterations can affect membrane integrity [13-19]. This composition is not found in many other biological membranes, especially as it practically lacks phospholipids [20]. The complexity of the lipid matrix is indicated in the fact that more than 300 species of ceramides alone have been identified therein [21]. How the lipids are organized is a complex matter, but in the late 1980s and early 1990s, it was concluded that there is an ordered structure [22-24] and that different fractions of the lipids can be found in both a liquid and solid state [19, 23, 25-29].

In 1994, Forslind presented the "domain mosaic model" which suggests that the lipid matrix is composed of lipid moieties mainly in the crystalline/gel state with a small amount of fluid state lipids between these domains [25]. This model is an interesting starting point for the hypothesis presented later (Chapter 3.4.). It has been shown that the main part of the SC lipids is rigid at 32°C with a small amount of fluid lipids present [30]. Lamellar and reversed hexagonal liquid crystalline phases have been found to exist [31], and by influencing the borders of the rigid parts, for instance by hydration or diffusion of solvents, other phase changes may occur. However, it is important to stress that the lipid matrix is an important part of the skin barrier properties with a structure hindering substance migration [19, 32].

Recently the group of Norlén and coworkers published a study of the SC matrix structure using mainly cryo-electron microscopy. They found that the lipid bilayers are a stacked structure of fully extended ceramides with cholesterol associated to specific parts [15]. The presence of alternating hydrophilic head groups and hydrophobic alkyl chains in this crystalline-like (gel-like) pattern would result in a matrix principally impermeable to water, as well as hydrophobic and hydrophilic substances [19, 32]. It would also result in the possibility for layers to slide against each other to some extent, thus maintaining the barrier while providing flexibility [15].

RELATED FACTS BOX – SKIN DIMENSIONS

It can be hard to relate to the tiny sizes and dimensions of the skin, so let us put them into perspective!

The upper part of the skin consists of, in average, 10 layers of cells which are roughly 1 µm high and 40-50 µm long [33]. Seen from the side, in cross section, it looks like the bricks in a brick wall (see Figure 2 above), though much longer and flatter. All around the cells, like mortar, we have the lipid matrix which is about 35 nm thick [15].

Figure 4 One km of road E20 surrounded by refrigerators envisions the dimensions of one cell and the lipid matrix in SC.

Let us stop there and imagine the following: Pretend that the cells are as wide (~20 m) and long as one km of road E20 (of the Europe international E-road network). Then fancy the lipid matrix as a lot of refrigerators lined up on the edge of the road (See Figure 4). Now, stack the part of road, with refrigerators around, like a brick wall and you may find it easier to relate to the dimensions of the SC skin structure… hopefully ☺

2.2.1. THE LIPID MATRIX

As this lipid matrix plays such an important role in the dermal drug delivery and possibly in the interaction between the cubic formulations and skin (paper IV and Chapter 3.3.), I wish to further discuss the sizes and structure of this barrier.

In Figure 5 we see some of the results from the work of Iwai et al. [15]. From the top down: the lipid matrix between cells in SC, the distances of the repeating units within the matrix, and a schematic model of how the CER, CHOL and FFAs are situated to build this structure.

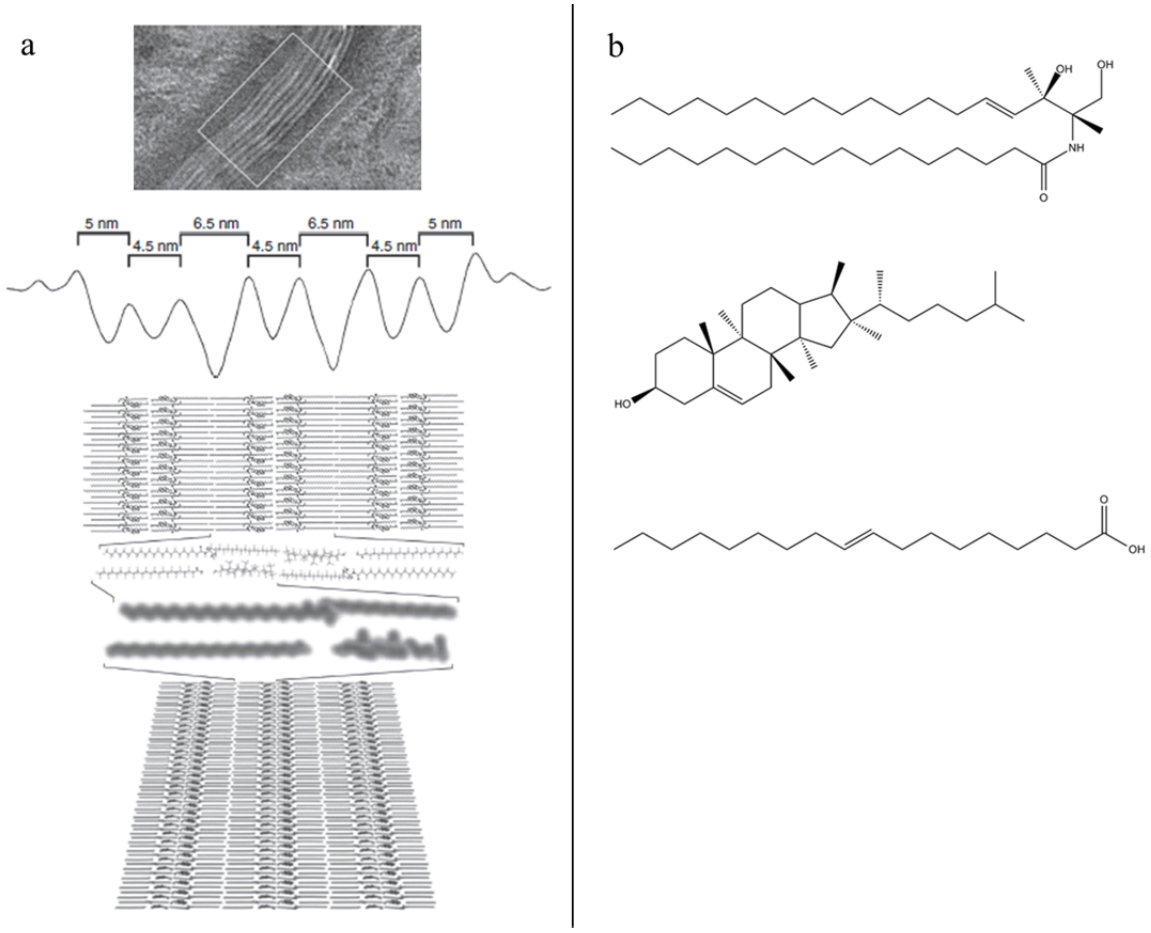


Figure 5 The SC lipid matrix as depicted by Iwai et al. (a, published with permission [15]) and examples of the molecular structure of a CER, CHOL and FFA (b, from the top down).

The lipid matrix is shown as lamellar regions with 2-12 dark lines, 6 lines on average. This would imply that the thickness (or height) of the matrix would be between ~10-60 nm. As anything passing in or out of the skin must pass this lipidic continuum, the dimension of it is important. Moreover, the bilayered

organization of the lipid matrix helps to reduce the permeability over the membrane [34], in other words this polar - non-polar - polar molecular composition in gel state forms an efficient barrier structure.

The schematic model depicted in Figure 5 is based on a bare minimum of molecules, indicating that if all the CER, CHOL and FFAs existing in SC were included, the structure of the lipid matrix would most likely be less compact and more adaptable. One interesting aspect of this is that only a small amount of fluid lipid added to gel-like parts of a bilayer alters the permeability [34].

The water transport through our skin is vital and the trans-epidermal water loss is ~250 ml/day for adults. At normally hydrated conditions the lipid matrix is winding between the cells. At increased hydration, however, water causes the cells to swell and the lipid matrix is thus flattened. The thickness of the lipid continuum seems unaffected indicating no increase of water therein [15].

2.2.2. CELLS IN EPIDERMIS

The second most abundant cells in epidermis, after keratinocytes/corneocytes, are the Langerhans cells. The Langerhans cells are potent antigen-presenting cells that are part of the immunological surveillance and effector system in our bodies. These cells can be of importance for the work presented in paper IV, as proteins delivered into the skin may be further transported to the immune system by the Langerhans cells. The cells that produce the pigment melanin are called melanocytes. Melanocytes protect the nucleus of basal keratinocytes from UV radiation. Merkel cells are sensory receptors that are in contact with nerve fibers in the dermal skin layer [1, 10].

2.3. DERMAL DELIVERY METHODS

Historically, skin diseases have been treated using herbal medicines with different outcomes. Some of the ancient knowledge has been forgotten and some reinvented, but when it comes to modern formulations for systemic treatment it took until 1979 to get a product out on the market. It started with a patch for motion sickness (Transderm Scop) and continued with pain relief,

hormone treatments and nicotine cessation. Recently, the focus has shifted more towards treating, for example, Alzheimer's, Parkinson's and dementia using the transdermal administration route [35, 36].

Drugs within the classical dermal delivery boundaries (see Table 1) are most often incorporated into hydrophobic ointments or semi-solid emulsions such as creams and gels [1]. The latter examples are just as often used in cosmetic applications making the penetrative abilities of the active ingredient somewhat questionable. Cosmetic products can moisturize the skin and treat superficial problems, for example, making the line between pharmaceutics and cosmetics unclear in some cases. Also, formulation ingredients used as penetration enhancers in a pharmaceutical system can be used as moisturizer in a cosmetic product. One interesting example, which is relevant for the work presented here, is propylene glycol (PG). PG is frequently used in both cosmetics and pharmaceuticals. It has even been used on its own to treat dandruff and various other skin issues such as fungi infections and inflammations due to its humectant and antibacterial properties [37]. However, it is the final delivery result that decides if the product will be approved and not the included components (as long as they are within regulations).

Dermal drug delivery can appear to be a simple form of treatment at first glance. Though a closer assessment reveals that a proper dermatological design is one of the most challenging tasks to tackle within the galenic formulation field [1]. The complex nature of the skin and the fact that it is a barrier protecting our bodies against any outer attack ensures that there will be numerous difficulties to conquer in order to achieve a well-functioning formulation.

2.3.1. LIMITATIONS AND POSSIBILITIES

With regards to the properties of the substance to be delivered, there are some traditional guidelines that are recommended to follow in order to successfully deliver the drug into the skin. These boundaries, summarized in Table 1, might not always be valid [35] but can at least provide a good starting point.

Table 1 Traditional limitations and boundaries for transdermal drug delivery

Partition coefficient	$1 < \log K_{o/w} < 3$
Molecular weight	< 500 Da
Melting point	< 200 °C
Daily dose	< 10 mg
Solubility (H_2O)	> 1 mg/ml
pH	5-9

Research is important in order to increase the possibilities for transdermal delivery. For example, one of the limitations mentioned in Table 1 is the size of the molecules that can be delivered transdermally. This is a difficult obstacle to address [36]. However, in our research we found that by using the instantly formed lipid-water based cubic formulation (presented in detail in Chapter 3.3.2.) as a delivery vehicle, larger APIs (5 – 20 nm) may be transported into the tissue (paper IV). Both nanoparticles and a model protein (bovine serum albumin, BSA) were delivered into viable epidermis (paper IV and Chapter 5.5.). Thus, despite the very real barriers and boundaries, a suitable formulation may be a solution to the problem.

The formulation can have different delivery aims depending on what API will be administered. An active substance treating the skin itself should stay in the outer layers of the skin. It is possible to achieve a depot effect and thus provide a prolonged release, which has been seen for the cubic phase [38]. Such a depot can also induce a systemic effect over time.

In transdermal delivery the drug must reach deeper into the tissue. The pharmaceutical concentration is very high at the application site on the skin surface and very low or non-existing further in. There is much blood present deep in the skin acting as sink for the diffusing molecules. The effect of this is a high epidermal concentration gradient driving a percutaneous absorption [1].

The possible ways for the drug to move into the skin is limited, as well as debated. One pathway is through the lipid matrix surrounding the cells [39]. Here the fluid parts of the lipid matrix are crucial for enabling transport of lipophilic and amphiphilic substances, as discussed above. A second route is

more resistant but possible and leads through lipidic parts of cell membranes. Further, a transcellular pathway is also considered but has been questioned and regarded unlikely by some as the transported substance must pass in and out of water and lipid rich regions [40, 41]. Finally, a combination of these pathways is also likely depending on the drug, the formulation and the skin tissue [42]. In other words, restricting the route to, for instance, only the lipid domains might be unreasonable due to the very small space [43]. The water rich skin cells, corneocytes, are enclosed within so-called cornified envelopes consisting of cross-linked proteins with a monolayer of lipids linked to it [44]. Between the cells the lipid matrix is the only continuum and hence, beyond question, all molecules passing in or out of the skin must pass this lipid domain sooner or later [42, 45-47]. To some extent, hair follicles can also be a possible way in to the skin [48] but from the bottom of the follicle the drug must still be transported further to be of therapeutic use elsewhere.

2.3.2. FORMULATION APPROACHES

The historically most common dermal formulation approach is to use oil, emulsifier and water (o/w, w/o, etc.), and thus obtain emulsions such as creams and lotions [1, 49]. Interestingly there are often no differences between formulations with a pharmaceutical aim and cosmetic products. As an example, liposomes and lipid nanoparticles are used in cosmetic products and were actually first found on the cosmetic market before they were used aiming to dermally deliver drugs [50]. This is quite a contradiction because the medical formulations need to deliver the drug into the skin tissue while cosmetics are not allowed to enter the body [1, 49]. An effective dermal delivery, based on the same systems as are present in a cosmetic product, thus seems unlikely.

A lot of research focuses on enhancing the skin permeation in a number of different ways. Table 2 lists some of the more widely used methods of which a few (Figure 6) will be explained below in order to paint a clearer picture of the field of dermal drug delivery.

Table 2 Methods for dermal drug delivery enhancement.

Methods	Examples
Electrical	Iontophoresis, Electroporation
Mechanical	Microneedles, suction, stretching
Other device-dependent	Ultrasound, temperature changes, laser, patches
Chemical	Enhancers, lipid-based vehicles, water gradient changes

2.3.2.1 MECHANICAL METHODS

The effects of electric current applied to skin for treating diseases or relieving pain have been recognized for at least 5,000 years when electrical eels were used for this purpose [51]. The techniques have been developed since then and used in a wide variety of pharmaceutical applications. For dermal delivery of drugs iontophoresis is one recent example. In iontophoresis a power source and two electrode compartments drive ionized drug molecules into the skin, as shown in Figure 6a. The device is connected to the skin, and once the current is applied the positive charges in the anodal compartment move towards the cathode and the anions move in the opposite direction. As the only way to move is through the skin the drugs are forced into the tissue [52].

Microneedles are considered a mechanical method of administration. They are minimally invasive and are being developed in different materials and with different delivery strategies. The main application has been to make microscopic holes in the skin and then apply a transdermal patch or other drug container to the perforated area. Sometimes iontophoresis are used in combination with microneedles. The drug can be located in cavities within the needles, the tips can be dipped in drug solution, or the drug can be incorporated in biodegradable needles so that when piercing the skin or when the needles break down the drug is released (see Figure 6c) [53].

Sonophoresis uses ultrasound to enhance transdermal transport of various drugs through, for example, heating and skin disruption (Figure 6b). Ultrasound has been used in different frequency ranges, but it has been found that low frequencies (<100kHz) are more effective in drug delivery than higher frequencies [54].

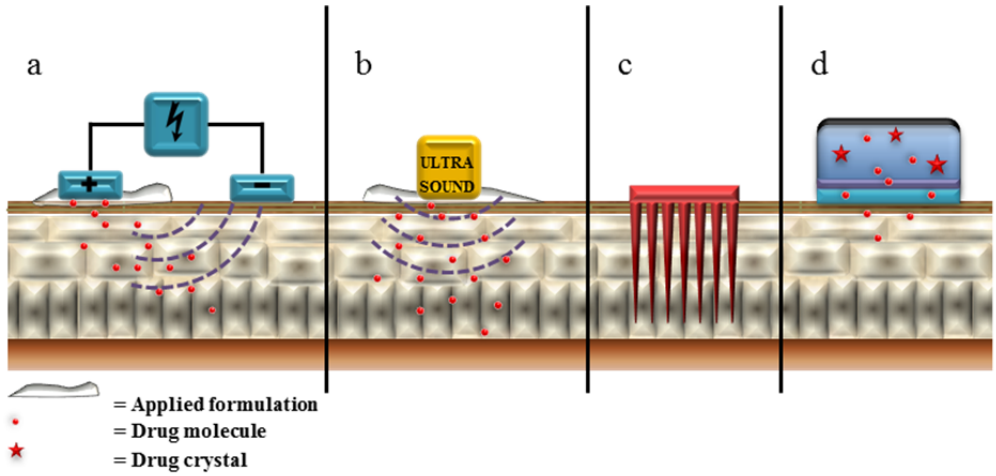


Figure 6 Schematic illustration of device driven dermal drug delivery showing (a) iontophoresis with delivery electrode and counter electrode, (b) sonification, (c) microneedles, (d) and a drug delivery patch.

2.3.2.2. NON-INVASIVE METHODS

Patches are often combined with other techniques such as those used after iontophoresis or as a micro-needle array patch [55, 56], but a lot of resources are spent on improving the patch itself. Patches are becoming more and more important in transdermal drug delivery. The traditional components in a patch are a liner, drug reservoir of some sort, adhesive, membrane, and backing (Figure 6d). Some patches include all of these parts and some do not, but either way all are developed to hold and deliver the API and to make the skin more susceptible to the it [56]. Today, besides the previously mentioned patch for motion sickness, there are patches for nicotine delivery and pain relief, for instance. Moreover, specific diseases are being targeted such as Alzheimer's [57] and Parkinson's [58], additionally patches are developed for vaccine delivery [55].

There are a number of classical non-invasive formulations such as ointments, creams, pastes, gels, suspensions, and solutions, which merely work for molecules within the boundaries presented in Table 1. When aiming for increased dermal delivery of anything else high demands are placed on the formulation. In the previous section invasive methods were presented. However, there are a number of down-sides of using these methods, including mechanically disrupting the skin surface, high manufacturing expenses, drug

degradation during storage or production [52, 59]. Therefore, it would be desirable to find less invasive delivery methods that are still effective.

One interesting group of formulations is the nanostructured lipid based liquid crystalline systems (further discussed in Chapter 3), which can have, for example, adhesive features, be engineered to provide sustained delivery, and can contain a wide variety of drugs [60-63]. Thus, these formulations can be good candidates for dermal drug delivery [61, 62, 64-67].

The consistency of a dermal formulation should be acceptable to the user and stay in place in order to interact with the skin and efficiently deliver the API. Some liquid crystalline phases fill these criteria better than others. The gel-like phases such as cubic and reversed hexagonal have better application qualities than the less viscous formulations such as lamellar and sponge phase. Furthermore, from a drug release view the cubic phase has a faster release rate than the reversed hexagonal one.

Moreover, because of the skin structure, any vesicle able to penetrate must be deformable and fluid in the lipid bilayers [68]. Delivery vehicle interaction with the skin lipids can influence a phase change so that more of the solid lipid sections of the intercellular matrix become fluid, thus enabling enhanced molecular diffusion [69].

The liquid crystalline phases can be formed into small, nanosized particles: liposomes, hexosomes and cubosomes [64, 70]. The drugs are incorporated into these vesicles. Still not many reach a sufficient administration level via the dermal delivery route [71-75], inducing a call for continued research and development thereof. There are a number of reviews written that discuss these types of drug delivery systems [17, 68, 76, 77].

Liposomes are the most frequently used of the ones mentioned and a lot of research concerns this formulation. It is often made of phospholipid bilayers shaped as closed spherical shells with a hydrophilic center where pharmaceuticals can be kept during drug delivery and storage. The liposomes are being developed to suit a wide variety of drug delivery pathways, with more or less efficient results.

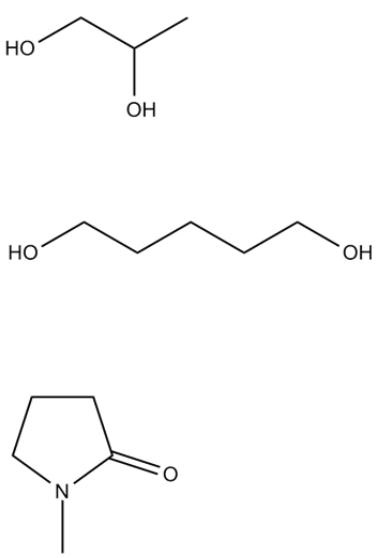
An example of a type of liposome that has received a lot of attention is the so-called transfersomes. Transfersomes have a high elasticity and can endure deformation [78-82]. The patent holders claim that the flexibility of the particles makes them superior to other nano-particulate formulations. The transfersomes have been claimed to move deeper into the partly hydrated skin layers than similar vesicles [78]. However, it has been considered unlikely that the vesicles stay intact during transdermal drug delivery. It was recently shown that they do rupture [83], though a depot effect in the upper skin layers may be possible [17]. Examples of what these vesicles have been tested for are malaria treatment [84], cancer treatment [85], and as vaccine delivery vehicles [86], with varied results.

2.3.2.3. PENETRATION ENHANCEMENT

Most dermal delivery formulations and patches include penetration enhancers. There are a number of possible different ones to use. Many of these enhancers disrupt the intercellular lipid matrix of SC, either by increasing the fluidity and thus facilitating diffusion of the drug or by forming alternate areas within the bilayer structure [87]. The penetration enhancers should be non-toxic and non-irritating, as well as without any pharmacological activity in itself. They should work rapidly and in a predictable way, as well as being a shield against loss of endogenous material while delivering the drug. When it is removed from the skin the barrier properties should rapidly return to the original structure.

No perfect enhancer has yet been discovered but some fulfill more of these criterion than others. Examples of enhancers or groups of enhancers are water, sulphoxides and similar chemicals, azone, pyrrolidones, fatty acids, alcohols, fatty alcohols and glycols, surfactants, urea, essential oils, terpenes and terpenoids, phospholipids, and solvents at high concentrations [88].

Three different penetration enhancers (not counting water) have been included in our studies: propylene glycol (PG), pentane-1,5-diol (POL), and N-Methyl-2-pyrrolidone (NMP) (molecular structures are shown in Figure 7). Depending on the use of the substances, which here are mentioned as penetration enhancers, different epithets can be used to present them.



Diols, such as PG and POL, are generally used as solvents or as anti-freezing agents but some have also been utilized as vehicles in pharmaceutical preparations [37, 89-92]. PG is the only diol widely used in dermatology [37, 91], as it reduces the drug – tissue binding and has keratolytic properties, thus promoting permeation of the drug [49, 87, 93].

Figure 7 Molecular structure of PG, POL and NMP from top down.

POL is not commonly used in pharmaceuticals, although it has been shown to be cosmetically acceptable and presents little risk for toxicity or skin and eye irritation relative to other diols [94, 95]. It is also an effective solvent, enhancer, water binding substance, antimicrobial agent, and preservative [91, 95] and may therefore replace several ingredients in a skin composition. Today, POL is mainly used as a plasticizer in cellulose products and adhesives, in dental composites and in brake fluid compositions, and as a preservative for grain [90]. However, in recent years POL has been investigated as a dermal delivery enhancer with positive results (paper I, [95]) and can be used in dermal formulations.

NMP is a somewhat questioned solvent used pharmaceutically to solubilize drug substances [96] and when studying *in situ* implant systems [97]. There have been uncertainties whether NMP causes irritations of the upper airways and eyes. A recent study of the chemosensory effects of NMP in human males found no evidence that this was the case [97]. Industrial uses include polyvinyl pyrrolidone production and use as a paint remover.

2.3.2.4. OCCLUSION

Another way of increasing the delivery is to apply an occlusive dressing covering the treated area. For example, in paper I the treated mice were covered

with impermeable plastic film over the area applied with formulation. Occlusion decreases the water gradient across SC, not allowing the water migrating out to leave the skin surface. Thus, the hydration of the outermost skin layers increase and dermal drug delivery is facilitated.

According to Björklund et al. [46], the skin barrier can be temporarily opened by controlling the water gradient across the skin; the hydration of the skin can rearrange the structure within the SC lipid matrix as the structure depends on the surroundings [98]. In this way the concentration gradient can induce a change in the barrier properties causing phase changes and thus affecting the transport into the skin, that is the chemical potential can drive the diffusion motion [34, 99]. Moreover, increasing both the hydration and temperature of the membrane induces lipid mobility and results in increased amount of fluid state lipids, enhancing permeability further [30, 31, 34]. These alterations in lipid organization and barrier properties are also influenced by the skin pH gradient. The inside of our body consists of neutral pH while the skin surface is acidic, providing additional protection from permeation [100].

2.3.2.5. DIFFUSION

For all non-mechanical delivery methods the delivery capacity of the formulation depends on the affinity for the SC environment for formulation and drug, and of the concentration gradient. The formulations and drug can be altered to enhance the partition into SC to some extent with the aid of solvents as previously discussed. However, further transport depends on the concentration gradient, in other words the passive diffusion, which is the rate-limiting step. Fick's law (equation 1) can also be used to calculate the diffusion parameters in these systems. The law states that the rate of diffusion across a membrane is inversely related to the thickness of the membrane and directly proportional to the concentration gradient of the substance on the two sides of the membrane.

$$\frac{dm}{dt} \cdot \frac{1}{A} = \frac{KDC_0}{h} \quad (1)$$

where m is the cumulative mass of diffusing drug, t is the time, A the area applied with formulation, K the partition coefficient of the API between formulation and skin, D the diffusion coefficient of the API in the membrane, C_0 the constant concentration of drug in the formulation (i.e., the amount of API is so much higher in the formulation compared to the diffusing amount that it can be considered constant), and h the thickness of the skin. For skin and other complex biological membranes it can be difficult to separate D and K. In such cases the permeability coefficient (P) may be more useful (see equation 2)

$$P = KD \tag{2}$$

[1]

Calculating the diffusion parameters can be a good way to, for example compare the delivery capacity of different formulations, the SC affinity of different drugs, or to compare different membranes (e.g. animal, human, and artificial skin).

3 LIQUID CRYSTALLINE PHASES IN DERMAL DRUG DELIVERY

3.1. LIQUID CRYSTALLINE PHASES

Lipids and other amphiphilic molecules self-assemble [101, 102] into compositions of various kinds of micellar structures or phases in an aqueous environment when they reach a certain critical micellization concentration [103]. At higher concentrations liquid crystals are formed by polar lipids, such as GMO or GME, and water, where both components diffuse freely [104]. What structure is formed depends on the polar/non-polar shape of the lipid, and on the amount of water present. Water hydrates the polar head groups of the molecules through hydrogen bonding while the tails uses van der Waals forces to stay together [105].

Depicted in Figure 8 is a three component phase diagram. Each point in this diagram represents a certain weight percentage of the three components. A vial containing such a mixture may show one, two, or three phases (see further Chapter 4.1.). The one-phase areas that can be formed are marked in the figure. Those are hexagonal (H_2), lamellar (L_α), cubic (I_2), sponge (L_3) and liquid (L) (see Figure 8), though the so-called sponge and liquid phases are not liquid crystals. Additionally, reversed forms of the hexagonal and cubic phases can form. These exist when the polar head groups face towards the enclosed water. In the phase diagrams, published within the scope of this thesis, the reversed forms are present (paper II). The transition between phases is driven by either enthalpy or entropy [106, 107].

The hexagonal phase consists of lipid tubes with water within or around, depending if it is a reversed structure or not. Lipid bilayer sheets with water between them define the lamellar phase. The sponge phase is not a true liquid crystalline phase but is best described as a swelled, melted cubic phase. The cubic phase is quite complex and consists of a ~3-4 nm thick lipid bilayer continuum traversed by two independent water channel networks of

approximately 5 nm diameter [108-111]. Adding to this, there can be different space groups formed within the cubic phase area based on minimal surfaces. In these systems the gyroid (g), diamond (d) and Schwartz's Primitive surface (p) can form. The two which can form in the systems included in this thesis, d and g, are shown in Figure 8.

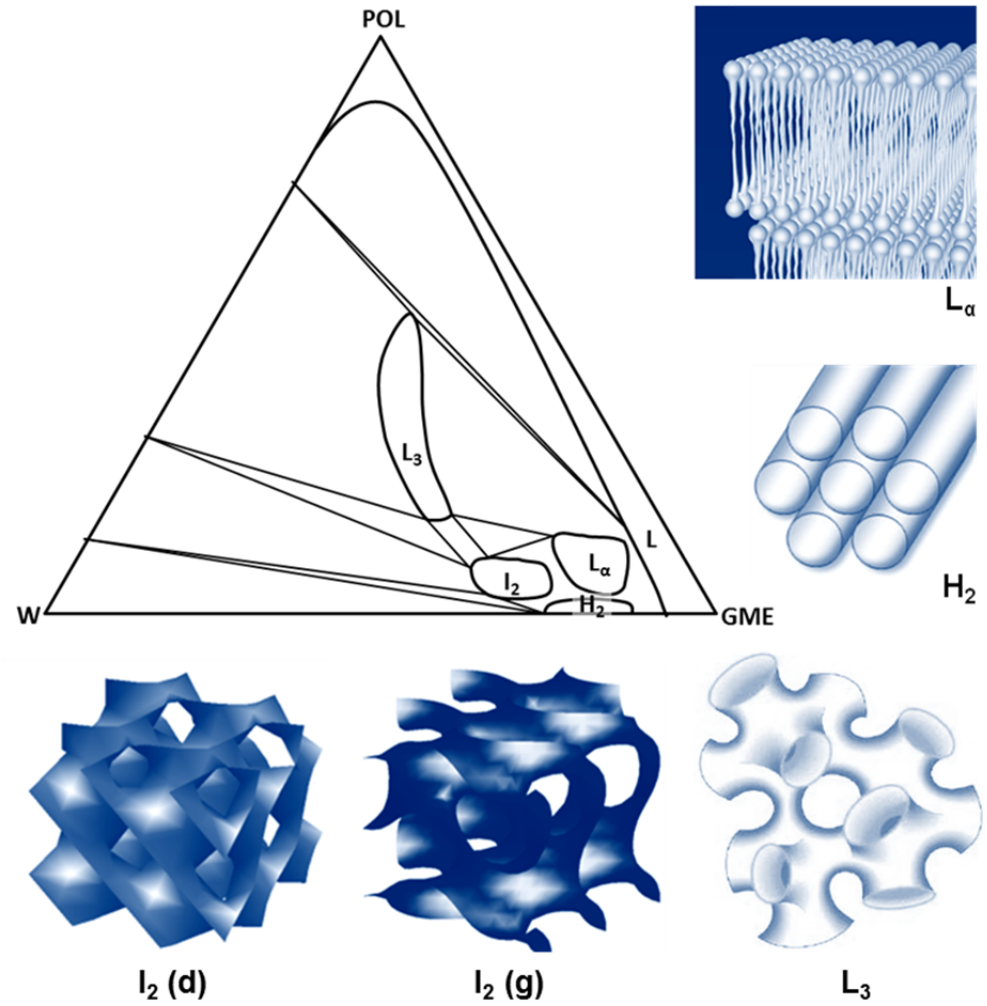


Figure 8 The GME-POL-water phase diagram and examples of liquid crystalline phase structures. Letters denotes: liquid phase, L; lamellar phase, L_α; reversed hexagonal phase, H_2; cubic phase, I_2; and sponge phase, L_3.

The cubic phases, which are the main focus in this thesis, were discovered in the early 1960s by three groups independently [112-114]. Much were still to be clarified though and a decade later, with the use of nuclear magnetic resonance (NMR) and small angle X-ray diffraction (SAXD), new results were combined with the old so that the true structure of the cubic phase could be identified (for a comprehensive review see [115]).

The cubic phase structure provides some interesting and noteworthy properties that come in handy for many applications, as we shall see later: (i) it forms spontaneously, (ii) has a gel like texture, and (iii) has a high lipid bilayer/water interfacial area of ~400-600 m^2/g lipid [108, 110, 116].

Sometimes a solvent, aprotic preferably, is needed for certain phases to form [101, 117, 118]. Depending on how effective the solvating capacity of the solvent is, the phases can be formed from a narrow or wide range of component ratios and the phase diagrams alter accordingly.

3.1.1. LIQUID CRYSTALLINE PHASES IN NATURE

In nature, lipid bilayers (and liquid crystalline phases) are found in abundance, as it is present in every cell. Focusing merely on cubic structures, important examples in nature are: the golgi apparatus, the endoplasmic reticulum, infolding of plasma membranes, and mitochondrial membranes during inactivity [103, 119, 120]. The lipid processes and phase shifting are believed to be very important for the cells. Signal transduction and inter- and intra-cellular trafficking may be governed by lateral segregation of lipids and switching between phases [103, 119-123].

Moreover, lipid crystalline structures have been found within the lipid matrix of the skin [31, 124]. The lipids in SC are uncharged and relatively non-polar, contrary to the lipids forming the flexible liquid crystalline phases. Nonetheless, the liquid crystalline phases are very similar to the lipid matrix in SC [125]. The natural occurrence of the liquid crystalline phases and the shown efficiency when used as drug delivery vehicles makes it an interesting formulation to use for topical, and other, applications.

3.1.2. COMPOSING THE LIQUID CRYSTALLINE FORMULATIONS

It is possible to form the liquid crystalline phases from a number of lipids, with or without solvent, in the presence of water. In the scope of this thesis, however, the materials of interest for forming the cubic phases are the polar lipids, glyceryl monooleate (GMO), and glyceryl monooleyl ether (GME) together with water, and sometimes an aprotic solvent, PG, POL or NMP (for

details see paper II). GMO has been widely used in studies of these systems. It is derived through esterification of oleic acid, glycerol or refined vegetable oils [126] but are also formed endogenously in small amounts in the intestine during oil digestion [127]. GME (or selachyl alcohol) is the ether analogue of GMO and is found in nature in, shark-liver oil, for example. This lipid has several medical effects such as antibacterial and antifungal, as well as being an immunological stimulus [128].

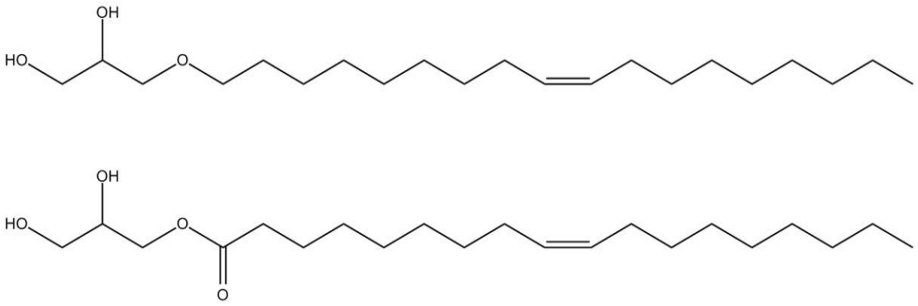


Figure 9 Molecular structures of GME (top) and GMO (bottom).

The molecular structures of GME and GMO are very similar, as illustrated in Figure 9. The carbonyl oxygen differentiates the lipids, which alters the phase behavior when in excess water: GMO forms a cubic phase and GME a reversed hexagonal phase [117, 129]. Aprotic solvents such as PG, POL or NMP, when mixed with GME and water, are able to transform the reversed hexagonal phase into a cubic phase [117] (paper II). The addition of the solvent has other positive effects on the formulation since it increases the water swelling capacity considerably, which in turn decreases the viscosity and makes the formulation easier to handle. Additionally, it is possible to form a cubic structure within minutes using GME, which was discovered by our research group and is of importance from a drug delivery point of view (see Chapter 3.3.2. and paper III-IV).

RELATED FACTS BOX

WHAT DO CUBIC PHASES AND OUTER SPACE HAVE IN COMMON?

Polar lipids and water molecules arrange themselves so that every molecule is as comfortable as possible with its surroundings. That is why the mixture in the formulations, given time, self-assembles into the cubic structure. At that point the system is in its most favorable state in terms of energy. This also leads to the formation of so-called minimal surfaces, in other words an area of average zero curvature. One example of such an area is a saddle point. Look at the red lines in Figure 10. Where the lines meet, the saddle goes up in two directions and down in two, that is it is neither a top nor a bottom. If you look at the cubic structure next to it you can see similar surfaces there.

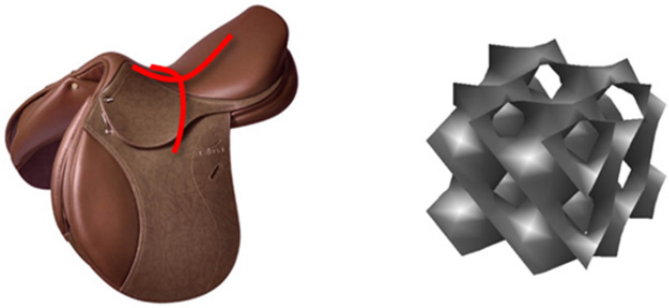


Figure 10 An example of a saddle point to the left, and a cubic phase structure to the right.

After considering the cubic structure in nature, it is interesting to take an even bigger step back to see the whole picture. The minimal surfaces in my gel-like goo can in fact be found in outer space (Figure 11)! Mathematicians and theoretical physicists use them in calculations regarding, for example, black holes and in string theory. For the theoretical physicist, *strings* is another name for minimal surfaces [130, 131].

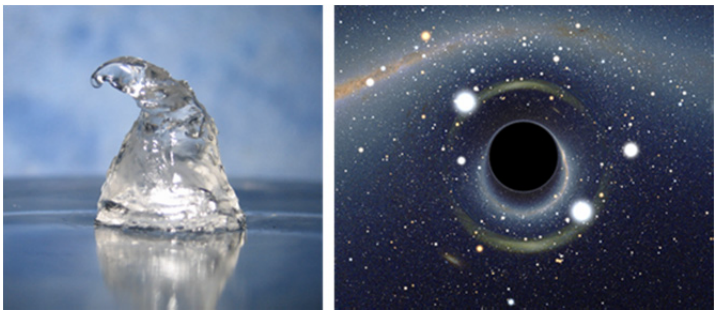


Figure 11 Minimal surfaces is the link between cubic structures and black holes.

The same basic principles can be found everywhere in our universe. We just have to broaden our horizon to see it. ☺

3.2. ACTIVE SUBSTANCES IN LIQUID CRYSTALLINE PHASES

Liquid crystalline phases have the ability to dissolve/disperse hydrophobic, hydrophilic and amphiphilic compounds. The cubic phase, and the reversed hexagonal phase, has gained a lot of interest as a drug delivery vehicle and has been tried for oral, buccal, transdermal, vaginal, nasal, and intramuscular, administration [61, 132-137], for example. Examples of what has been incorporated within the different phases so far are small molecular APIs, proteins, peptides, vitamins, and DNA [61, 64, 109, 111, 138-148]. In our research, we used small molecules, proteins and additionally nano-particles (papers III-IV and Chapter 5.3.-5.5.).

Adding these substances to the liquid crystalline formulation can of course affect the structure and phase behavior [139, 141-144, 149, 150] but it does not necessarily mean that alterations will take place [139, 151]. Knowing the phase behavior of the system at hand and preparing for potential changes due to the addition of molecules provides a dynamic drug delivery tool.

Large bio-molecules in cubic phases have, to my knowledge, been studied for over 40 years since Gulik-Krzywicki et al. [152] was first with their phosphatidylinositol-lyosozyme-H_2O system. Such bulky structures cannot be confined solely within the aqueous channels or the lipid bilayer but might be encapsulated in pockets or be partially in both water and lipid, thus possibly altering parts of the structure without destabilizing it [111, 153]. The active substance itself could also be affected in terms of solubility [154] and drug release. For example, something as big as DNA molecules cannot within reasonable time limits diffuse out of the cubic network [141].

Proteins incorporated in the cubic phase do not seem to undergo significant structural changes [139, 141]. Thus, the cubic phase shows an ability to protect the proteins within from degradation and prolong their half-life [104, 108, 155]. One reason for this is that the activity of water in the channel systems is reduced [108, 156] resulting in protection of proteins and peptides [157]. These facts have been put to use in, for instance, membrane protein crystallization [158].

There are a number of reviews that discuss different aspects of the areas mentioned above [70, 103, 108, 109, 153, 159] and Rizwan et al. [159] show a list of compounds of different molecular size that have been incorporated into the cubic phase. A rough size evaluation of such compounds is summarized in Figure 12.

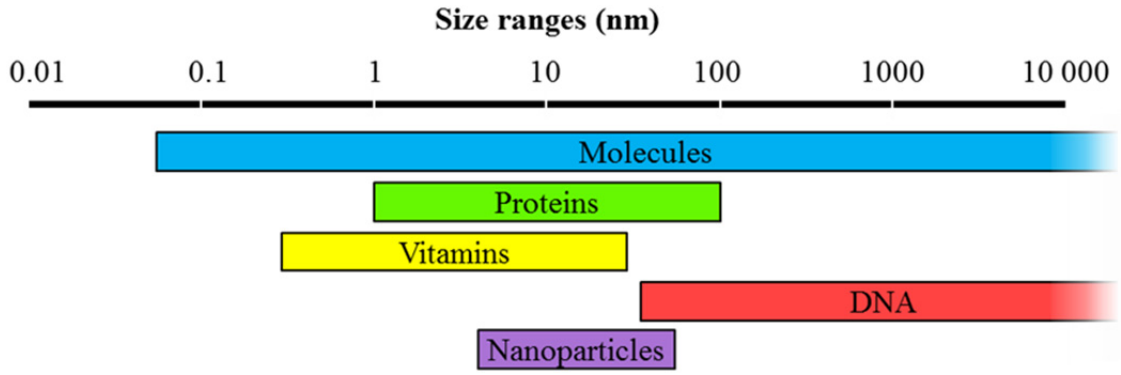


Figure 12 Approximate size ranges of various substances incorporated into the cubic phase.

One advantage of using liquid crystalline phases as a drug delivery vehicle is the ability to achieve sustained release. Many studies of sustained release from the cubic phase have been performed [108, 109, 141, 142, 145, 157, 160-164], showing release of the APIs that follows the square root of time according to Higuchi [165, 166].

The formulation can be modified to some extent in order to allow for a faster or slower diffusion of the API. Altering the diameter of the water channels is one very direct way to modulate the release [141]. Examples of ways to do such modulations include using different charged lipids and surfactants [167, 168]. The viscosity of the system is another factor that seems to have an effect on the release rate; the higher the viscosity the slower the release [164, 169]. Additionally, the continuous nature of the lipid bilayers provides the possibility for vast migration. Investigations of the migration of oligonucleotide within the cubic phase compared to the migration in a hydrogel showed this property to be of importance [170].

It is possible to shift phases from one to another by changing, for example, amount of water, temperature and pressure. This can be used for drug release

purposes if the phase shifting occurs under controlled circumstances. During the transformation between two phases caution must be observed as drug release may increase temporarily [162]. The spontaneous formation of a cubic phase, when in a more water rich environment, from a lamellar starting point is one example [109, 171, 172]. The researchers investigated this idea with the hope of commercialization. However, the researchers soon realized that there was a risk of burst release [171], and the idea had to be revised to find a functioning approach to the same solution [173, 174]. Going from a cubic to a reversed hexagonal liquid crystalline structure can be a way of suppressing drug release [147]. There are examples of using pressure or, even better, temperature to achieve such a shift [147, 175, 176].

3.3. CUBIC PHASE FOR DERMAL DRUG DELIVERY

Depending on the intended delivery route, the cubic phases have more or less attractive features. The highly viscous phase might be suitable for oral delivery [126, 135, 162]. It is also mucoadhesive with the ability to stick to the skin surface [60, 61, 70, 132, 133]. However, to some extent it is a question of taste as some researchers find the high viscosity an advantage in dermal delivery [61, 62] while another finds it to be a disadvantage and prefers the less viscous sponge phase [177].

Liquid crystalline phases and the cubic phase in particular have shown to be good candidates for transdermal drug delivery (paper III-IV) [61, 62, 64-67]. The reasons for this are numerous: it has, as mentioned, adhesive features, it can be engineered to provide sustained delivery, it is non-toxic, and it can contain a wide variety of active substances [60-63]. Moreover, because of the skin construction any vesicle able to penetrate must be deformable and fluid in the lipid bilayers [68]. Delivery vehicle interaction with the skin lipids can influence a phase change so that more of the solid lipid sections of the intercellular matrix become fluid, thus enabling enhanced molecular diffusion [69].

3.3.1. THE CUBIC PHASE IMPROVING DERMAL DRUG DELIVERY

There are examples of studies using cubic phase systems as a transdermal drug delivery vehicle for treating both dermal diseases and internal problems where systemic delivery is needed. With the aim of treating atopic dermatitis, GMO-based formulations were studied on excised rat skin resulting in effective skin delivery [178]. In another study enhanced systemic effect was observed both *in vivo* and *ex vivo* when using a cubic formulation compared to two microemulsion gels [179]. Yet another study showed that the cubic phase were more efficient for transdermal drug delivery than the reversed hexagonal and lamellar phases [180]. However, it must be pointed out that some of these results were obtained from studies performed on shaved rats. The shaving disturbs and affects the skin resulting in a less than optimal set-up. However, the dermal delivery studies included in this thesis (papers III-IV) concur with the observation that the cubic phase increases the extent of drug diffusion.

A relevant example of improving dermal drug delivery by using the cubic phase is within the skin cancer treatment termed photo dynamic therapy (PDT) ([61], paper III). In short, this treatment is based on making cells sensitive to light so that when illuminated the cells die. The use of δ-aminolevulinic acid (ALA) and ALA-esters in PDT is the object of many studies even though the more specific area of interest can differ from cancer treatment to reducing acne, for example. There are examples of clinical studies reporting positive effects when using methyl aminolevulinate (MAL) in PDT treatment compared to other methods such as surgery and cryosurgery [181, 182]. Experiments of the permeation of ALA across skin [183, 184] and the stability of the drug in formulated drug delivery vehicles [61] have also been studied in detail. Studies using different drug delivery vehicles arrive at different conclusions with regards to the transdermal absorption of ALA and MAL. When comparing the results presented in these references with the results obtained in our study (paper III) it is evident that the cubic formulations we present have a significantly enhancing effect on absorption of the drug.

3.3.2. THE ON DEMAND CUBIC FORMULATION

A protocol for "on demand" preparation of cubic formulations suitable for dermal drug delivery was developed, building on previous work within the research group. The cubic formulations previously used needed to be allowed to equilibrate at least over night before use. The new protocol is especially suitable for drugs that have limited stability in solution, and therefore should be prepared as close to the administration occasion as possible. Thus, it was evaluated in the aforementioned *in vivo* PDT study (paper III).

The preparation of the formulation includes one syringe filled with GME and solvent, and one syringe containing water. The syringes are joined with a connector that holds the API in dry form. By using the syringe pistons to push the content back and forth through the connector until thoroughly mixed, the cubic phase formulation is produced in less than one minute (see Figure 13).

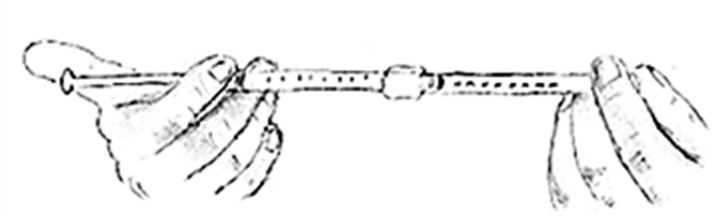


Figure 13 The mixing of components to obtain an on demand cubic formulation.

This formulation solves the stability issue of the API stored in the connector piece. Moreover, by merely using GME instead of GMO it circumvents the stability issues otherwise present in a standard GMO-based cubic formulation due to hydrolysis of the extra carbonyl oxygen. The solvents used to prepare the on demand cubic phase were PG and POL, but other solvents could also be used.

As the formulation proved to be an effective delivery vehicle for small molecules, it was also tested for proteins and nano-particles (NP) (BSA, gold nano-particles (AuNP), and quantum dots (QD)), discussed further in paper IV and in Chapter 5.4.-5.5.) with positive results. This on demand formulation is a vital part of the research presented in this thesis.

3.4. THE HYPOTHESIS: INTERACTION BETWEEN FORMULATION AND SKIN

When working with the cubic formulations as a dermal drug delivery vehicle one thing is abundantly clear: the phase is virtually impossible to wash of. This has to do with the fact that it swells in water to a certain extent. However, it is also due to an interaction with the skin that is not completely explained as of yet. Through the use of different techniques and methods (Chapter 4) further information could be gathered about the formulations and their skin penetration, as well as dermal drug delivery abilities (results in Chapter 5). Combining these results with information about the lipid matrix (Chapter 2.2.1.) and the phase behavior of the system (paper II and Chapter 5.2.) makes it possible to hypothesize over what happens when we add the formulation to the skin surface.

The formulations applied to the skin samples in the included studies were the GMO-water based and the GME-water-solvent based cubic phases. These molecules and formulations are very similar but the systems differ in a few key points. In excess water GMO forms a cubic phase while GME forms a reversed hexagonal phase and needs the solvent to form a cubic phase. In the dermal delivery studies no significant difference could be seen when small molecules were tested; however, for large molecules and nanoparticles the only efficient delivery vehicle was the GME-water-solvent based cubic formulation. The change of lipid, decided on due to possible commercialization reasons, proved to be an interesting choice.

The applied formulation provides hydration to the tissue, both from the formulation itself and through occlusion of the area. The hydration results in swelling of the cells and straightening of the lipid matrix [15]. The samples treated with the cubic formulation swell more than water treated samples. This is not unlikely as the lipids in SC are uncharged and relatively nonpolar, thus less prone to swelling in a hydrating environment compared to the polar lipids of the cubic formulation [15, 185]. However, the liquid crystalline phases are similar to the lipid matrix in SC [125]. Introducing the formulation to the skin lipid layers should thus induce an increased swelling of the tissue due to increased uptake of water. Moreover, the high relative humidity at the

boundary between phase and skin will result in increased membrane permeability [34]. Adding to this is the fact that the solvent/penetration enhancer included in the GME-based formulation has keratolytic properties [49, 93, 186]; therefore, the following hypothesis may be a plausible explanation to the increased delivery:

The applied GME-based cubic formulation adheres to the surface, occludes and hydrates the skin. When the cubic phase comes in contact with the water present within the skin the solvent is released. The hydration from occlusion in combination with the released solvent generates swelling of the cells and detachment from each other. When the cubic phase loses POL it will transition into a reversed hexagonal phase, in other words it goes from a continuous to a discrete system, according to the phase diagram in paper II. The influenced skin lipid matrix meets the reversed cylindrical micelles budding of the applied formulation, enabling migration between the cell layers. The release of the solvent continues during the skin penetration, as water will always be present.

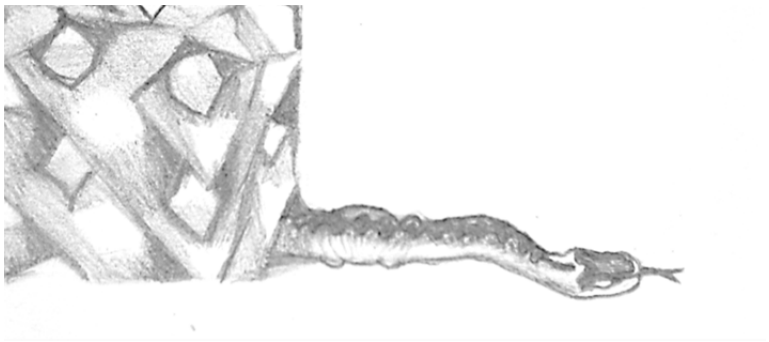


Figure 14 An illustration representing the GME cubic phase from which a reversed cylindrical micelle emerges, represented by a snake, preceded by its cloven POL tongue and carrying the loaded API as a swallowed prey.

Moreover, the fluid lipids of the formulation come in contact with the lipids of the quite rigid matrix and increase the mobility of the lipids therein, possibly altering the local liquid crystalline structure. This combination of events work its way downward, opening pathways and dragging with it what was first loaded into the cubic phase. This process takes time and droplet-like depots can form (see Chapter 5.5.) from where the progression continues. Smaller molecules may be delivered into the cells from the depots while larger molecules and particles most likely follow the formulation until deposited.

RELATED FACTS BOX – ANCIENT SKIN CARE

Today skin care and cleanliness is a given, but also lucrative business. This has not always been the case. In the cold northern countries washing and skin care was not high priority, though it was never as bad as during the middle ages when bathing was more or less unheard of. Women could possibly rinse their skin with wine to get a smooth, silky touch. Other than that, Europeans in general used perfume to cover the body odors instead of washing [187, 188].

The situation further south was quite different, where both culture and religion requested cleanliness. For example, documents from Egypt, Greece, and Roman lands, as well as the Bible as historical documentation from the same time period, describes washing procedures and lubrication with oils as a daily ritual in many cases. Cleopatra is well-known for bathing in sour milk to stay young. The lactic acid is favorable for skin exfoliation, so why not give it a try? The Bible also mentions milk baths, as well as both men and women putting oil on their faces and hair. The most common choice was olive oil, which sometimes was infused with herbs or flowers and sometimes in combination with honey as a further moisturizer [187-189].

The combination honey and oil was also commonly used by men and women in Greece for health and looks. The athletes in ancient Greece covered their bodies with olive oil and fine sand to regulate body temperature and for protection from sunburns [187, 188].

We should learn from these ancient ideas and take good care of the skin; it is our largest organ and must last a lifetime. Watch out though! The etheric oils which were frequently used are now known contact allergens, and many a vain lady succumbed to arsenic or led poisoning which were common ingredients in cosmetic products. We can all be thankful for lifesaving research results! ☺

4 RESEARCH METHODS AND ANALYSIS

Over the years of work leading up to this thesis a large number of techniques were used. These techniques will be described briefly and, to some extent, compared to other possible techniques that were disregarded from use for different reasons. For further details of the methods and equipment used, readers are referred to the included papers. All techniques were chosen in the attempt to find the best way to answer our various questions.

4.1. ANALYZING LIQUID CRYSTALLINE PHASES

The most common techniques used to analyze liquid crystalline phases are crossed polarizers, SAXD and NMR. The combination of these techniques gives a clear image of the phases and phase behavior. Thus, they are valuable tools when constructing a phase diagram. For a complete phase diagram, a vast number of samples have to be prepared, analyzed, and composed. The mentioned methods have been used for sample analysis in the papers included in this thesis and will all be presented here.

4.1.1. CROSSED POLARIZERS

Crossed polarizers (Figure 15) are very useful for determining if there is birefringence in the liquid crystalline samples. After ocularly determining the number of phases present in the vial and the relative amount of each phase, it is possible to obtain a first indication of which phase is which and its position in the vial by holding the sample between crossed polarizers. Both lamellar and reversed hexagonal phases alter the direction of the light making the sample look like it is glowing (anisotropic phases). Cubic, sponge and liquid phases do not glow, thus looking completely black (isotropic phases).

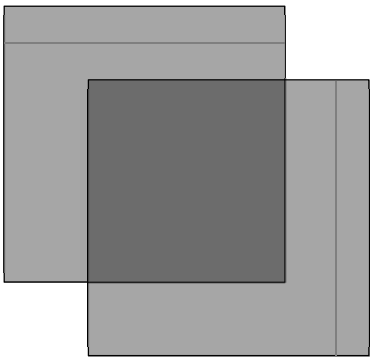


Figure 15 Schematic image of crossed polarizers where the light is blocked in all but one direction, thus if one of the plates is turned 90° no light will pass through.

For more detailed information about the texture of the birefringent phases or of phase shifts due to, for example, temperature alterations, an optical microscope equipped with crossed polarizers can be used. A cubic sample can, for instance, be studied under the microscope and stepwise heated until it turns into a reversed hexagonal phase. This gives an indication of the heat tolerance of the phase (studied in paper II). The reversed hexagonal phase can be distinguished from the lamellar phase, as they show a colorful image and a black and white structure, respectively. Any further details must be found using other techniques.

4.1.2. SMALL ANGLE X-RAY DIFFRACTION

The small angle X-ray diffraction (SAXD) technique is applied to study structures that are approximately 1-100 nm in size. An X-ray photon primary beam is directed at the sample at a small angle causing photons to be emitted in all directions as scatter. The photons can sum up and give rise to diffraction. Diffraction is obtained when the difference in distance between the planes in the sample are equal to the wavelength of the radiation, stipulated by Bragg's law (equation 3). Only the diffracted photons that travel parallel to a specified direction are allowed into the detector which is divided into 1024 channels, in other words angles or scattering vectors [190].

When data is collected Bragg's law [191] is used to calculate the distance between layers within the sample.

$$n\lambda = 2d \sin\theta \quad (3)$$

where n is an integer, λ is the wavelength of the incoming beam, d the distance between two molecular layers, and θ the angle between incoming or reflected beams and the molecular layer (see Figure 16). This equation shows that for a small d, such as within a molecular or atomic lattice, the incoming primary beam angle must be wide. For a larger d, such as in the liquid crystalline phases, the angle must be small in which case SAXD is the suitable technique to use [190].

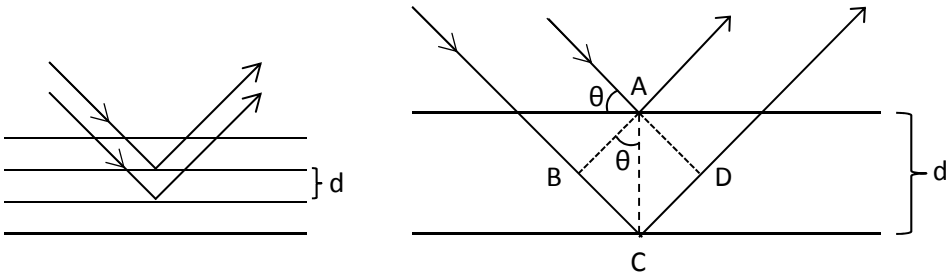


Figure 16 Schematic image of Bragg diffraction, where two beams of identical wavelengths hit a surface and are reflected by atoms in two different planes. The lower beam travels a distance of *2d*sin*θ*. Constructive interference occurs when this length corresponds to an integer multiplied by the wavelength of the beam.

To calculate the distance between the planes, d, from the SAXD measurements equation 4 is used.

$$d = 2\pi/q \quad (4)$$

where q is the scattering vector obtained during measurements. In this way information such as bilayer structure and thickness of the sample can be obtained [190, 192, 193]. The diffraction pattern and q position of the obtained spectra gives the sample space group and (nano)size, respectively.

4.1.3. NUCLEAR MAGNETIC RESONANCE

NMR is a versatile analytical technique used in many disciplines of scientific research, medicine, and various industries, for example, for determining the content and purity of a sample and its molecular structure. It can be used to

quantitatively analyze known mixtures or solve the basic structure of unknown compounds. NMR can further be used to study solubility, diffusion, phase changes, and other physical properties on a molecular level. To achieve all of these applications a number of different NMR techniques have been developed [192].

To describe NMR in its most basic terms, I cite Joseph P. Hornak: "Nuclear magnetic resonance is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess the property called spin." [194]

Accordingly, these electrically charged nuclei may transfer energy between energy levels when the external magnetic field is applied. When the spin returns to its base level the obtained energy is emitted at the same frequency as was sent in. The transfer signal is measured and processed, yielding an NMR spectrum for each such nucleus [194].

Different nuclei, even within the same molecule, come into resonance at different frequencies. This can be used to provide information about the molecular structure and position of the different parts of the molecules in the media. The so-called chemical shift shows the difference between the resonance frequency of each nuclei and a reference standard [192, 194]. This was used in our work (paper II) where the obtained single ^{1}H resonances from the α, β and γ hydrogen's of POL provided chemical shifts in lipid and water domains of the cubic and sponge phases. From these results the distribution of the POL molecules in its surroundings was determined. Moreover, the ^{2}H NMR signal pattern of some phases was recorded to confirm the presence of a sponge phase in the GME-POL-W phase diagram. The ^{2}H NMR signal originates from deuterated samples, in this case water replaced with D_2O.

4.2. MODEL SYSTEMS

The techniques presented below were used *in vivo* or *in vitro/ex vivo*. The *in vivo* methods were executed using hairless mice in search of a pharmacological response. *In vitro/ex vivo* measurements were performed on excised human skin tissue in order to screen a number of formulations and diffusing molecules and particles.

4.2.1. *IN VITRO* AND *EX VIVO*: DIFFUSION CELLS

Franz cells are very common equipment to use for penetration studies through human or animal skin, artificially grown skin, or other membranes. The simple layout is presented in Figure 17 with the skin sandwiched between the formulation and the receptor fluid, allowing for a set-up as similar as possible to the *in vivo* situation with regard to temperature and so on (it is also possible to use these cells for iontophoresis, for example). This technique was used for the majority of samples within the research presented here. No other *in vitro*/*ex vivo* method can, to my knowledge, mimic the natural dermal application situation better.

However, some may argue that techniques such as tape stripping, which can be used directly on the patient, are preferable. In tape stripping a tape adheres to the skin surface and is then pulled off, taking some of the skin layers with it. This is not too painful and such multiple procedures can be performed before the area is too damp for the tape to adhere. Each sample can then be analyzed in search of, for example, the amount of API. However, this method has a number of inherent difficulties such as: i) by disrupting the layers artifacts can be introduced, thus it is not possible to remove specific homogenous layers making the location in the skin somewhat uncertain [195]; ii) the test subject is subjected to the drug with the possibility of unwanted systemic effect; and iii) the tissue must be clean and dry before the technique can be used. The latter would create obstacles in our case, as the cubic formulations are hard to remove without leaving an oily residue.

Figure 17 A Franz diffusion cell with donor chamber, receptor chamber and clamp, seen in the top image. The receptor chamber is filled with fluid and the skin sample is placed over the opening with the surface facing upward. The donor chamber is placed on top of the skin and the whole diffusion cell is clamped together, enabling penetration studies of the substance applied to the skin via the donor chamber.

The skin was treated with the formulations in the diffusion cells before they were analyzed using different methods depending on the research question. The diffusion always had to be monitored, and thus there was a need to investigate the amount API present in the receptor fluid and to enable looking deep into the skin tissue.

4.2.2. *IN VIVO*: TRIALS FOR PHARMACOLOGICAL RESPONSE

In order for an API to reach the market it must go through animal and human trials. Animal testing is a proof-of-concept method used for APIs and techniques prior to clinical trials. The desire is to do as little animal trials as possible and each study must have an ethical approval before the experiments can commence. Well known guideline principles for more ethical animal testing are the Three Rs: i) Replacement – whenever possible, replace animal trials with another method to achieve the same scientific aim; ii) Reduction – use as few animals as possible and/or obtain more information from the same

number of animals; and iii) Refinement – enhance animal welfare and minimize pain, suffering or distress for the animals used [196].

There are a number of different animal models to choose from that are more or less similar to humans. Pros and cons must be carefully weighed with regards to factors such as costs, housing opportunities, ease of handling, animal robustness, and immunological status. Mice are a common choice of animal that are easy to maintain and acquire at a relatively low cost. Mice are thus ideal to use when a high number of animals are needed for statistical assessment [197].

In the *in vivo* trials performed for paper III, hairless mice (see Figure 18) were chosen in order for us to be able to apply the formulation on intact, unshaven skin. The PDT process must be studied on living organisms in search of a pharmacological response. ALA enters the heme biosynthesis cycle, which only functions in living cells, thus the need for live animals in this case. For our need for statistical data, mice were a good choice. They are small animals that are easy to handle, have short life-cycles and the housing facilities have a vast experience of these creatures.

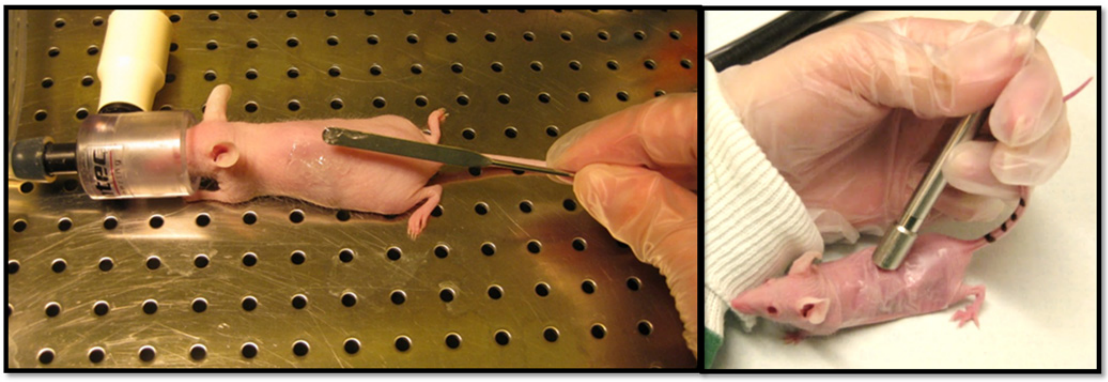


Figure 18 Photographs of an anesthetized mouse during application of cubic formulation (to the left) and spectroscopic measurements performed on the mouse through the occluding dressing (to the right).

For assessment we used a non-invasive method: fluorescence spectrophotometry (fluorescence will be further explained in Chapter 4.3.1.3.). An excitation wavelength appropriate for the PpIX signal was sent into the tissue and the emission was detected through a fiber optic coupler accessory.

Fluorescence readings were simply performed by holding the optical probe against the skin of the treated mice. This was the easiest way to detect the changes in PpIX formation over time without injuring the animals.

4.3. ANALYTICAL METHODS

4.3.1. LASER SCANNING MICROSCOPY

When facing the problem of clear imaging deep into skin tissue without histopathological or other invasive preparations, a number of issues arise. Firstly, the skin scatters light, making a clear image hard to obtain. Red near-infrared (NIR) light are spread the least, thus to achieve deep light penetration, wavelengths ranging from approximately 700-1000 nm are preferred. NIR light not only penetrates deeper into scattering tissue but is also generally less phototoxic [198]. Secondly, the skin consists of a number of layers with different structures. In order to visualize this, the technique used must be able to provide a detailed 3D view.

Optical microscopy is commonly used with histopathological samples. However, the resolution and details of the obtained images are often insufficient. A better choice would be to use confocal microscopy. This fluorescence microscopy technique use linear (one-photon) absorption (see Figure 19) processes for contrast generation. It achieves 3D resolution and optical sectioning through the use of a detection pinhole that rejects all light that does not originate from the focus. Due to the scattering effect of the tissue this limits the use on the skin surface for high-resolution imaging.

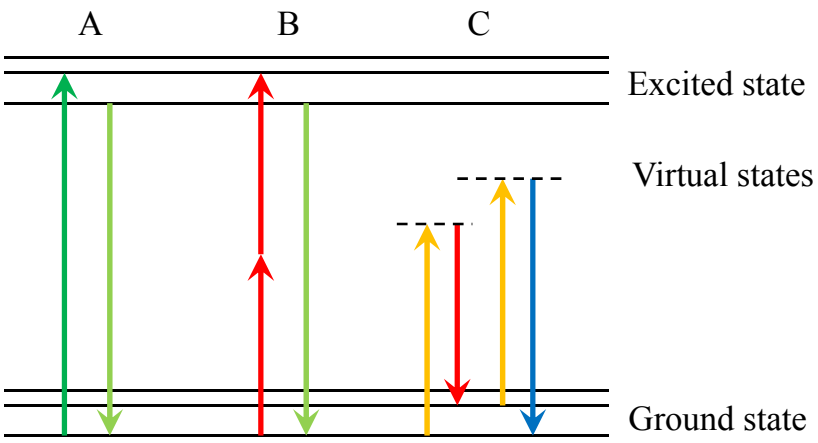


Figure 19 Jablonski diagram showing energy levels for one-photon absorption (A), two-photon absorption (B) and coherent anti-Stokes Raman scattering (C).

A further improvement of the microscopy technique was necessary in order to perform non-invasive imaging. To avoid the effects of scattering and to take advantage of the low absorbing near-infrared excitation light, a non-linear process was employed. One example of a non-linear process is multi-photon excitation. For such an unlikely process to occur at least two photons need to combine their energies to excite the fluorescent molecule simultaneously (within ~0.5 fs) (see Figure 19). The emission will occur in the visible spectral range, same as for one-photon excitation, but with the aid of pulsed lasers achieving high peak intensities the multi-photon excitation can take place using NIR light [198]. Moreover, the excitation only occurs at the focal point, hence the technique is more specific even without the use of a pinhole [199]. Two techniques that employ this are Two-photon microscopy and Coherent anti-Stokes Raman spectroscopy, both presented below.

4.3.1.1. TWO-PHOTON MICROSCOPY

Two-photon excited fluorescence laser scanning microscopy (TPM) was used in paper IV for acquiring images deep within the light spreading skin tissue. As mentioned above, it makes use of low scattering, low absorbing NIR excitation light, and more effective emission detection compared to confocal microscopy. Moreover, the skin auto-fluorescence has an emission wavelength within the green area, which enables detection of added red fluorescing molecules or particles. This can be used so that the auto-fluorescence shows where in the skin layers the image is taken while the red fluorescence depicts the location of

the added substance. The auto-fluorescence originates from a number of different substances within the skin, which are presented in Table 3. Our work is based on the knowledge provided by a number of interesting studies and reviews that have been published on the use of two-photon microscopy on human skin *in vivo* and *ex vivo* [199-204].

Table 3 The location of auto-fluorescing substances found in skin tissue.

Skin layer	Source of auto-fluorescence
SC	Keratin
SG	NADPH
SB	Melanin
Dermis	Elastin, Collagen

With the aid of TPM, diffusion of nanoparticles, as well as fluorescently labeled proteins, have been visualized in our studies. TPM is becoming more and more used for skin imaging due to it being an efficient tool when aiming for visualization of events taking place deep into light spreading tissue [38, 205-207].

It is noteworthy that the auto-fluorescence signal in TPM increased when applying a cubic formulation to the skin sample. This may be due to optical clearing [208].

4.3.1.2. COHERENT ANTI-STOKES RAMAN SPECTROSCOPY

Coherent anti-Stokes Raman spectroscopy (CARS) has its background in Raman spectroscopy, as the name suggests. In Raman spectroscopy the electrons are pumped by a single continuous wave to an imaginary energy state. A way of explaining this, perhaps a somewhat peculiar statement, is that the photons that are sent into the electron clouds influence the electrons without affecting them. When the electrons then return to a vibrational state the energy difference is detected. This can be a decrease in energy called Stokes, but it can also be an increase in energy called anti-Stokes. The latter is used in CARS.

The CARS process uses the fact that different chemical bonds have different vibrational spectra, much like a fingerprint [209, 210]. The CH bonds, which are present in a high amount in lipids and thus of interest in my research, are one example of such bonds. In order to see these fingerprints two laser pulses are needed, similar to TPM. The difference in frequency between the two pulses must be set to match the vibration of the molecule aimed to study. By using the two pulses, called pump and Stokes, the system can contain both the ground state and an excited state at the same time (see Figure 19). If the corresponding vibrational spectra is present in the sample this will result in the anti-Stokes signal [211].

CARS can be combined with other microscopy techniques, such as TPM, for obtaining an even more complete image of the tissue at hand [212]. In this way it is possible to selectively image biomolecules or other complex structures without any labeling. The CARS signal is detected on the blue side of the incoming radiation, which is free from fluorescence (compared to the Raman signal which is detected on the red side where it might have to compete with other fluorescent processes).

4.3.1.3. FLUORESCENT SIGNAL

In order for the TPM technique to be useful there must be a fluorescent signal in the sample. In a linear process the emitted fluorescent light has a longer wavelength than the absorbed light, though when the two-photon process is used the emission of radiation has a shorter wavelength than the absorbed radiation.

Fluorophores

A fluorophore is a fluorescent chemical compound (typically containing aromatic groups), that after excitation re-emits light. The fluorophore is excited by a light source and emits light when relaxing back to its ground state (in the same way as shown in Figure 19). As mentioned, the emitted fluorescence light has a longer wavelength than the absorbed light. This is due to a loss of energy, between excitation and emission, known as Stokes shift. Fluorophores are, for biological applications, ideally small molecules that can be linked to larger molecules, such as proteins, in order to study the complex. Pioneering work in

the context of TPM, studied 3D distributions of fluorescent probes in excised full-thickness human skin [33].

Semiconductor nanocrystals

Quantum dots (QDs) are luminescent semiconductor nanocrystals. The mechanism behind the luminescence differs between fluorophores and QDs. As previously explained, fluorescence from molecules is due to the excited electrons that fall back into their ground state. However, in semiconductor nano-crystals the movements are a bit more restricted. When an electron in a semiconductor is excited from the valence band to the conduction band, a hole in the valence band is created. This electron hole pair is called an exciton, and can be compared to the state between the proton and the electron of the hydrogen atom. The size-related optical and electronic phenomena are also known as "quantum size effect", hence the name Quantum dots [213].

QDs are characterized as having sharp and symmetrical emission spectra, broad absorption spectra, size dependent emission wavelength tuneability, high quantum yields, and good chemical stability and photo-stability [214]. The particle surface is often covered with an organic coating that is necessary during synthesis to control, for example, the growth, solubility and crystal morphology (Figure 20). Depending on application this can later be of use to couple other coatings to the particle [213], for instance to create stealth effect (see further Chapter 5.5.).

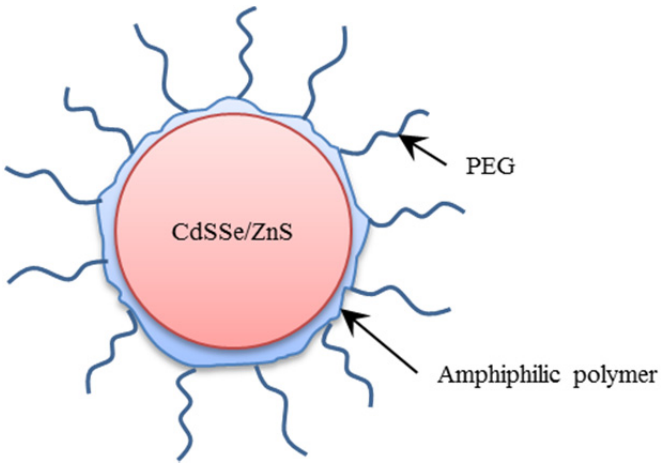


Figure 20 Schematic image showing a possible structure of QDs, with a cadmium and Zink-based core covered with an amphiphilic polymer to which polyethylene glycol (PEG) is coupled in order to achieve stealth effect.

Plasmon resonance

Gold nanoparticles (AuNPs) are somewhat more difficult than QDs and fluorophores to use in optical microscopy such as TPM. The effect, which enables visualization of AuNPs, is called surface plasmon resonance (SPR). The SPR in AuNPs is the coherent excitation of all the free electrons within the conduction band, leading to oscillation. The surface of the particle plays an important role for SPR through altering the polarizing conditions for the metal, which shifts the resonance to optical frequencies. Globular nanoparticles give fewer signals than nanorods, for example. Thus, the surface plasmon absorption is a small particle effect and not a quantum size effect [215]. What complicates the matter is that the spherical particles cannot be too far apart if they are to be detected using TPM. (This we learned the hard way.)

AuNPs have been used in cell studies using TPM for assessment. The conclusion drawn by the authors, quite logically, was that if AuNPs were detected they were attached to or incorporated within the cells, and if they could not see any particles, there were none present [216]. As this seemed straight forward, our idea was to incorporate AuNPs in the formulations, apply them to the skin and study the penetration into skin tissue (in the same way as later was performed using QDs, see Chapter 5.5.). However, the 5, 20 or 40 nm spherical AuNPs were not detectable using TPM in water solution, the cubic phase, or the skin tissue. Only aggregates could be detected. This of course raised questions about the measurements. When drying AuNP solution on a glass slip it was clear that the obtained signal only originated from aggregates. The problems to obtain a two-photon signal lead to a more in depth study of the physical origin of the luminescence signal (work to be published) [217]. The results showed that for 10 nm particles the longest separating distance possible was <10 nm. These findings imply that particles can be present though too far apart to be detectable using TPM.

In my opinion this is an important issue that can cause problems, for example, for cell studies such as those mentioned above. In our case it led to performing some initial experiments with SEM and TEM to detect the particles in the skin samples (ongoing work) and to replace the AuNP formulations with systems containing QDs in order to follow the diffusion using TPM.

4.3.2. CHEMICAL ANALYSIS

4.3.2.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC (High Performance Liquid Chromatography) is a very commonly used technique for chemical analysis. HPLC can be used to separate, quantify and identify different compounds. A suitable mobile phase is pumped at a steady pace, pushing solubilized samples through an analytical column, resulting in separation of the components of the sample. The sample components elute at the other side of the column at different times depending on how much the composition of the column delays specific substances. This separation is affected by choice of column and composition of the mobile phase. Variations of the latter can include different solvents, pH, ion strength, and so on. During the first study performed (paper I), the amount of terbinafine was detected in receptor fluid samples. As terbinafine absorbs within the UV range it could be analyzed using a UV detector, where the eluted fractions continue through a flow cell and energy changes are detected.

4.3.2.2. TECHNIQUES USED WITHIN PROTEOMICS

The analysis of proteins and particles in skin tissue originates from the scientific question whether or not the cubic phases are able to enhance dermal delivery of large molecules and particles. Actual proteins can interact with both the formulation and the skin, thus the nanoparticles were chosen to be easily traced inert models of protein drugs. However, as the penetration of QDs into viable epidermis was successful, the diffusion of fluorescently marked BSA was also tested as a model of a bulky protein drug.

The TPM results showed a deep penetration of BSA into *ex vivo* skin. With knowledge of dermal delivery of large molecules this was quite spectacular; therefore, we felt the need to make certain that the obtained signal originated solely from the TMR-BSA complex. Although unlikely, it might be possible that the fluorophore leaves the protein and is separately transferred deeper into the skin (TMR is covalently bound to BSA and it should thus take active cleaving to separate them), or that free TMR could be present in the sample despite the cleaning made by the suppliers. With this in mind, the need to find a

way to distinguish BSA from HSA in skin samples arose. Our work turned towards the scientific branch of Proteomics.

Proteomics studies the structure and function of proteins. The word “proteome” is derived from PROTEins expressed by a genOME, and it refers to all the proteins produced by an organism, much like the genome is the entire set of genes. Proteomic technologies play an important role into finding the link between genes, proteins and disease. Often the findings make it possible to discover defective proteins that cause particular diseases, enabling the development of new drugs that either alter the shape of a defective protein or mimic a missing one [218].

Proteomics is a complex field with a number of available techniques depending on the research question. The techniques focus on revealing structure and conformation, as well as measuring protein concentrations in varying conditions. Within each step, for instance structural analysis, purification, protein concentration, and separation a number of techniques can be used.

With the aid of experts in the field, the choice of techniques fell on 2D gel electrophoresis combined with LC-MS/MS analysis. This combination enables separation of the proteins on size and thereafter the ability to distinguish BSA from HSA.

A few alternative methods were discussed but ruled out. One example is albumin depletion, which is normally used to remove serum albumin (SA) from blood samples, for example. The high amount of SA can disturb the analysis of low abundant proteins, thus enriching columns, and antibody-based methods have been developed to remove SA [219]. This method would in our case be used to single out the SAs and then distinguish BSA from HSA. However, compared to the approach we chose, this would be more costly reaching the same goal, and was thus ruled out.

Another idea was to use immunoprecipitation. This technique uses a specific antibody that binds the particular protein searched for in order to isolate it from the other proteins in the sample. The protein is immobilized on solid substrates and later eluted and identified [220]. However, this is an extremely time

consuming procedure which can take up to half a year to perfect, as a specific antibody only working for BSA would have to be located for the technique to be usable.

As the techniques chosen achieved the set goals, no other methods were tried.

5 RESULTS

The research results included in this thesis are here summarized and further discussed.

5.1. PAPER I

EFFECT OF PENTANE-1,5-DIOL AND PROPANE-1,2-DIOL ON PERCUTANEOUS ABSORPTION OF TERBINAFINE

Two penetration enhancers were here compared in order to achieve effective delivery of Terbinafine into the skin. Terbinafine is an allylamine with antimycotic properties. In this case (5 or 20 %) PG or POL was added to a hydrogel containing the API and applied to excised breast skin in Franz diffusion cells. The amount API that diffused into the receptor fluid was measured using HPLC and related to which penetration enhancer was present, in what amount, and how much of the formulation that was left on the skin surface.

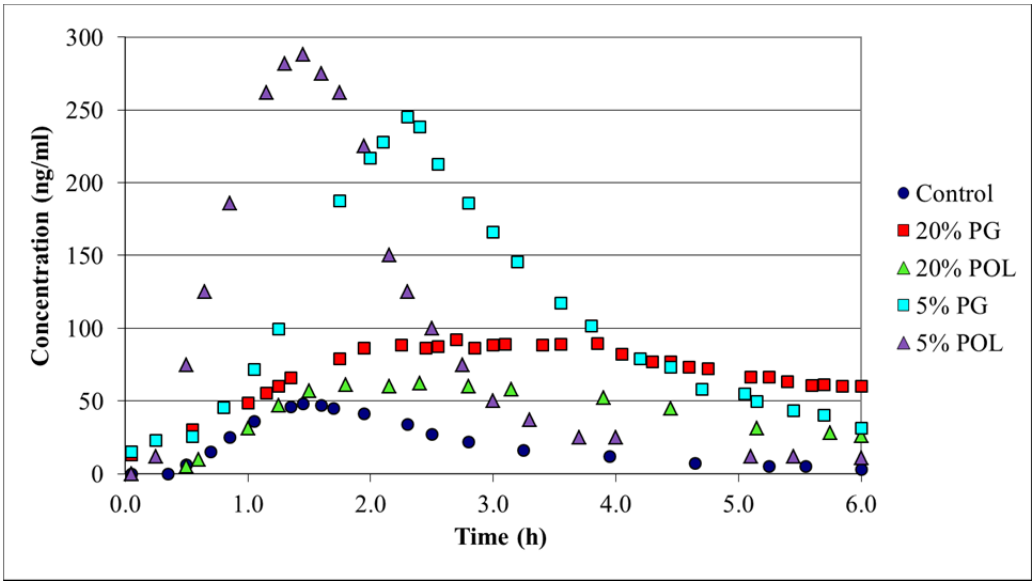


Figure 21 Results from HPLC analysis of Terbinafine concentration in the receptor fluid over time. The gel formulations contained 1 % Terbinafine and a varied amount of either enhancer.

As POL was unheard of as a dermal delivery formulation component, this investigation gave new insights into its use and comparability to a known penetration enhancer, PG. It was found that both PG and POL increased the percutaneous absorption of Terbinafine and that 5% POL resulted in the highest absorption Figure 21. Moreover, a very small amount of formulation was left on top of the skin.

The higher lipophilicity of POL compared to PG can be a reason why POL was the most efficient enhancer in this case, as the API is lipophilic. The low amount of POL present in the hydrogel gave a better drug diffusion than the higher amount, that is, less is more. This may be due to the low amount of POL facilitating interaction between the API and both the enhancer and the lipophilic parts of the skin

5.2. PAPER II

CUBIC AND SPONGE PHASES FORMED IN TERNARY ETHER LIPID-SOLVENT-WATER SYSTEMS: PHASE BEHAVIOR AND NMR CHARACTERIZATION

Liquid crystalline systems are fascinating in their predictability combined with ability to surprise. Their uses are far from completely mapped, which we touch upon in the introduction of paper II.

The aim of this work was to thoroughly investigate the two GME-POL-water and GME-NMP-water systems which are applicable for use in biological fields such as protein crystallization, API-delivery etc.

NMP behaved as predicted compared to a previously published diagram with PG as solvent [117], that is as an effective solvent with the one-phase areas lining up parallel to the GME-NMP base line, except that no lamellar phase was found at ambient temperatures. POL, however, showed interesting and unanticipated results, regarding the shape of the sponge one phase area, for example (see Figure 22).

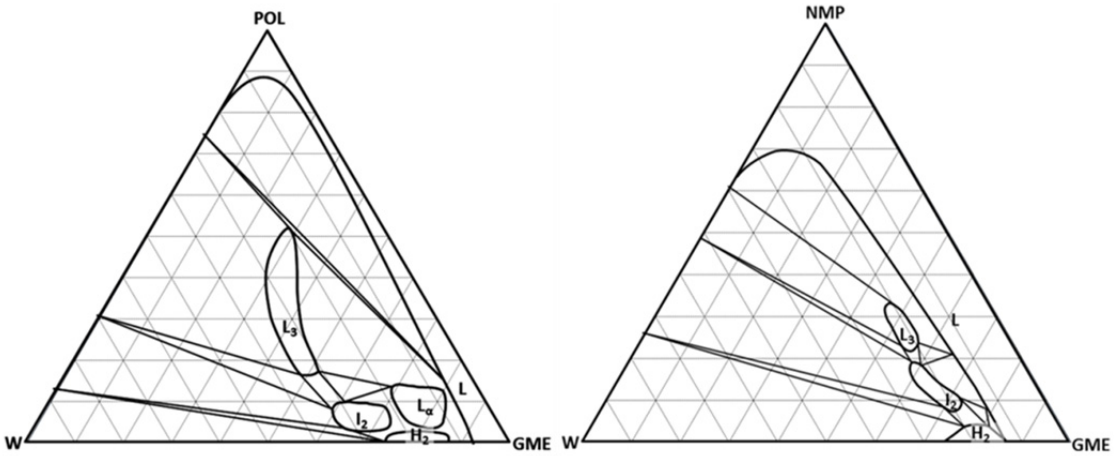


Figure 22 The GME-POL-water and GME-NMP-water phase diagrams. The letters in the one-phase areas denotes: liquid phase, L; lamellar phase, L_α; reversed hexagonal phase, H_2; cubic phase, I_2; and sponge phase, L_3.

When looking deeper into this conundrum using NMR, we found that the bola-shaped POL reside to a slightly higher extent in the water domains of the cubic and sponge phases, though prefers the interface between water and lipid. This preference is part of the explanation why there is such a large sponge phase area compared to comparable phase diagrams, as the presence of POL flattens the bilayers. Moreover, the bola shape of the POL molecule may enable POL to behave as two solvent molecules through bending around the hydrophilic head group of the lipid. This combination makes POL act as an efficient solvent but still allows for large ordered liquid crystalline-like bilayered structures.

The presented phase diagrams show two GME-based systems which are temperature stable and easy to handle. New applications should be possible to find for whoever puts their mind to it.

5.3. PAPER III

IN VIVO STUDY OF AN INSTANTLY FORMED LIPID-WATER CUBIC PHASE FORMULATION FOR EFFICIENT TOPICAL DELIVERY OF AMINOLEVULINIC ACID AND METHYL-AMINOLEVULINATE

The aim of this study was to improve dermal delivery of ALA and MAL in order to enhance the efficacy of PDT. This work builds on a previous study

performed in our group [61]. The cubic formulation was in the mentioned study found to be very efficient compared to other delivery systems. However, a solution to a few problems had to be found in order to obtain a usable system.

Thus, we set out to solve the main issues while maintaining the previous good results. First of all; the APIs used in PDT is extremely prone to hydrolysis and should be stored in dry form. This would imply preferred mixing on site before application. With this conclusion another issue arose; the GMO based cubic phase takes time and strenuous mixing to form. Also, GMO, which was used in the original cubic formulation, hydrolyzes with time, and even faster in the presence of the API salts. However, the solution to the mentioned problems lay in replacing GMO with GME. The GME cubic phase can be formed instantly and is also less viscous and thus easier to apply than the GMO cubic phase.

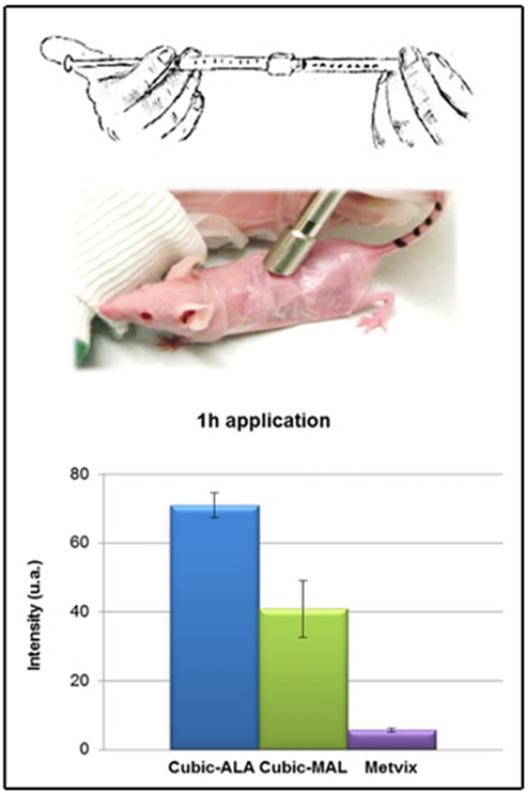


Figure 23 Mixing the formulation, measuring *in vivo*, and some of the subsequent results.

To obtain a cubic phase using GME, a solvent is required; here either PG or POL. An effective solvent flattens the lipid bilayer by organizing itself in the border between lipid and water, pushing the lipid head groups apart. This favors the cubic structure because of the flatter less strained lipid bilayer. When the lipid chains are organized in this way it is possible for the cubic phase to hold a higher amount of water [221]. More water and wider channels are desirable for topical drug delivery of hydrophilic compounds [109].

After mixing the on demand formulation and applying it to the skin of the mice the subsequent formation of PpIX was monitored. In Figure 23 we see a small fraction of the results, which show that the cubic formulations are significantly

better at delivering the API into the skin cells. It is noteworthy that Metvix contains five times more API than the on demand cubic system tested here.

The results of this paper show an interesting formulation for use in PDT. However, a clinical trial has to be performed before human use is possible. As the formulation excelled the commercial reference product, we wanted to know how efficient the formulation actually is. Thus the work in the following paper investigates this further.

5.4. PAPER IV

DELIVERY OF SERUM ALBUMIN FROM A CUBIC PHASE INTO VIABLE EPIDERMIS STUDIED BY TWO-PHOTON MICROSCOPY

The aim was to evaluate the ability of the on demand cubic formulation as a dermal delivery vehicle for, in this context, large substances. The TPM analysis showed penetration of BSA at least 20-30 µm into the skin tissue, but only when using the GME-PG-water on demand cubic formulation.

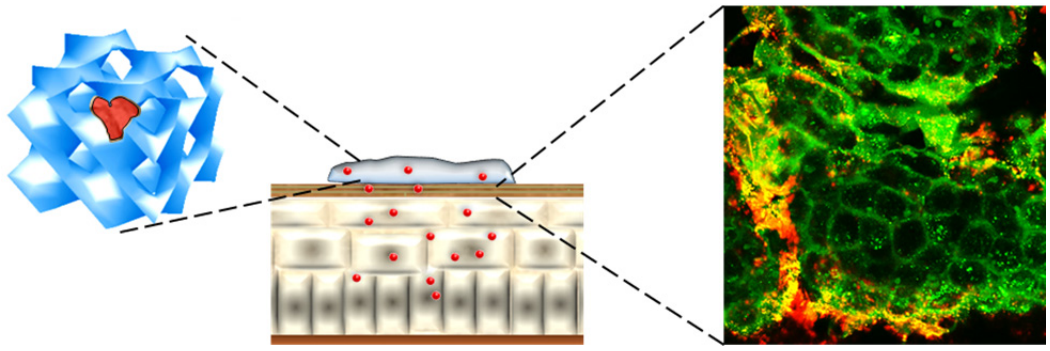


Figure 24 The BSA containing cubic phase formulation was applied to the skin surface and the permeation was observed using TPM.

For confirmation a second technique was employed. Proteomic methods confirmed the results obtained using TPM and revealed that no free fluorophore (not attached to BSA) was present in the skin samples.

Enabling diffusion of larger molecules into the skin is beneficial for, e.g., needle free vaccination. The depth of diffusion may only be necessary far

enough for the Langerhans cells to reach the delivered API, i.e. deeper tissue concentrations may occur earlier and be greater for live skin than predicted by passive dermal diffusion *ex vivo* [222].

5.5. WORK IN PROGRESS

OVERCOMING THE SKIN BARRIER FOR NANOPARTICLES USING NANO-STRUCTURED CUBIC LIPID SYSTEMS

The same methodology as for investigating skin delivery of TMR-BSA (paper IV and Chapter 5.4.), was performed for QDs of ~17 nm size. The results obtained confirmed the results presented in paper IV, and further strengthened our theory of how the enhanced penetration came to pass.

TPM was used to envision the diffusion of these large particles into human skin after 22 h (34°C) topical application. The formulations compared, all containing the same concentration of QDs, was water, water-PG solution, the on demand GME-PG-water cubic formulation, GMO-based cubic formulation, and hexagonal GME-water phase.

The only case when particles were found within the skin tissue was when the GME-based cubic phase was used as delivery vehicle. For the water-PG solution a slight penetration at the top few μm gave an indication of its importance. Though, most dermal delivery products on the market contain penetration enhancers without providing sufficient API absorption. Therefore, a combination of the proved effective cubic dermal delivery system ([61] and paper III-IV) and an effective penetration enhancer seemed the way to go.

In the same way as the proteomics analysis ensured that BSA was present in the samples in paper IV, initial experiments were performed with TEM and SEM to detect nanoparticles in the samples. This is ongoing work however and no conclusive results has been obtained as of yet.

Besides just wanting to find out how large molecules and particles might be deliverable using the cubic formulations, there were other reasons why to

choose nanoparticles and in particular PEGylated ones. Nanoparticles administered into the body are taken up and accumulated within e.g. cancer cells resulting in increased therapeutic effect [223], though when delivered intravenously they have been shown to be cleared from the body within minutes depending on size, shape, surface charge etc. [224-226]. Thus measures have to be taken to hide the particles from the effective immune system. Covering the particles with an electrically neutral hydrophilic surface layer is a strategy which can extend the circulation half-life with more than 40 h [227]. This shielding is known as the stealth effect, referring to stealth planes [224]. Polyethylene glycol (PEG) is commonly applied to achieve stealth effect and additionally reduces the tendency for particles to aggregate by steric hindrance [224].

PEGylated QDs were detected using TPM within viable epidermis when the GME-based cubic formulation was used as delivery vehicle. AuNPs could not be detected using TPM, thus alternative techniques has to be employed in order to detect these particles. Thus, this project continues into the future.

VISUALIZING DERMAL ABSORPTION OF LIPID CUBIC PHASE FORMULATION FOR UNDERSTANDING DERMAL DRUG DELIVERY: A COHERENT ANTI-STOKES RAMAN SPECTROSCOPY STUDY

After finding that the cubic phase was effective as a dermal drug delivery vehicle also for large molecules and particles, new questions were raised. Why does it work better than the formulations used for comparison? How does the cubic phase penetrate into the skin? Do the lipids in the phase migrate into the lipid matrix of the SC, or is it the liquid crystalline structure that resembles the ordered arrangement of the lipids in the skin that enables interaction?

To answer some of these questions a technique had to be found that could provide the opportunity to look into skin and distinguish between the formulation, which was added to the surface, and what was already present in the skin. In this case fluorescence would not help as the phase itself does not fluoresce, thus TPM was ruled out. The samples should still be intact, making a number of other techniques inadequate.

A technique that works well with lipids and was accessible through another research group at the university was coherent anti-Stokes Raman spectroscopy (CARS). Looking into published data there were indications that it might be possible to distinguish between the lipids in the formulation and the lipids in the skin [212]. Not much similar research had been done and the possible outcomes were intriguing.

Thus, initial experiments were performed to measure the CARS signal from a GME-based cubic phase applied to skin. The difference in CARS spectra between GME and the skin was determined (Figure 25), and the lipid signal of the applied formulation was followed into the skin (Figure 26). A skin sample treated only with water was used for comparison. The most interesting stretch vibration was believed to be CH_2 (2845 cm^{-1}) [212, 228], as this is found a lot in lipids, but measurements were performed to cover the frequency range from 2835 to 2955 cm^{-1} (corresponding to pump/probe beam between 817.4 and 809.5 nm). The experimental set-up has been described before [229].

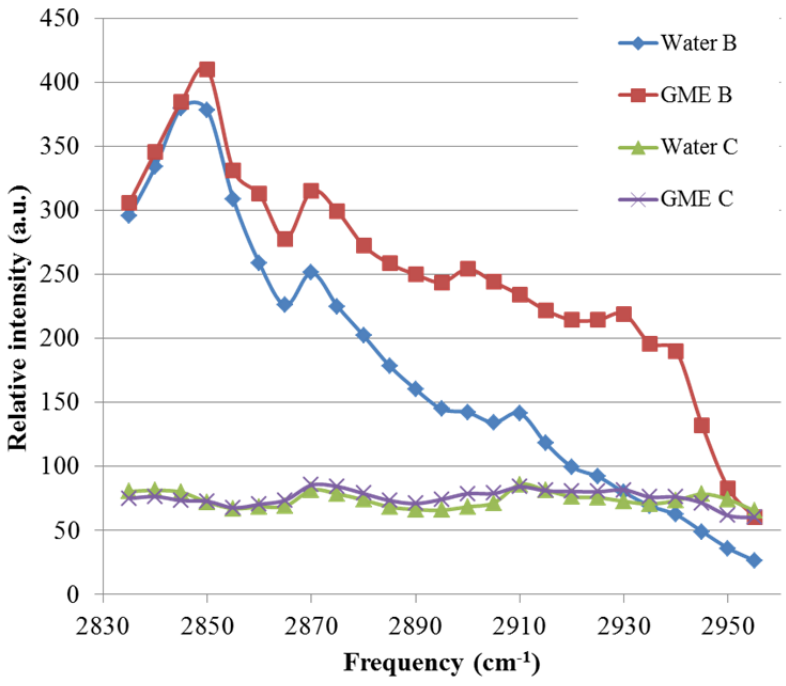


Figure 25 CARS spectra measured at the skin surface of samples treated with GME-based cubic formulation or water. Measurements were conducted between cells (B) and within cells (C).

What we report has to my knowledge not been shown previously and were of importance in the understanding of the GME-based cubic formulation as an

efficient dermal drug delivery system. Both Figure 25 and Figure 26 show that the signal from the lipids originates from around the cells, which of course is no surprise, and that the signal is strong in skin wrinkles. The intensity of the signal was overall considerably higher with GME present.

Looking closer at the top line of images in Figure 26 it seems as if the lipid formulation diffuses downward and is found in part as droplets or depots deep into the tissue. The added lipids also continue to enhance the signal in images acquired deep within the tissue and spectral examinations showed that the signal originated from the added lipids.

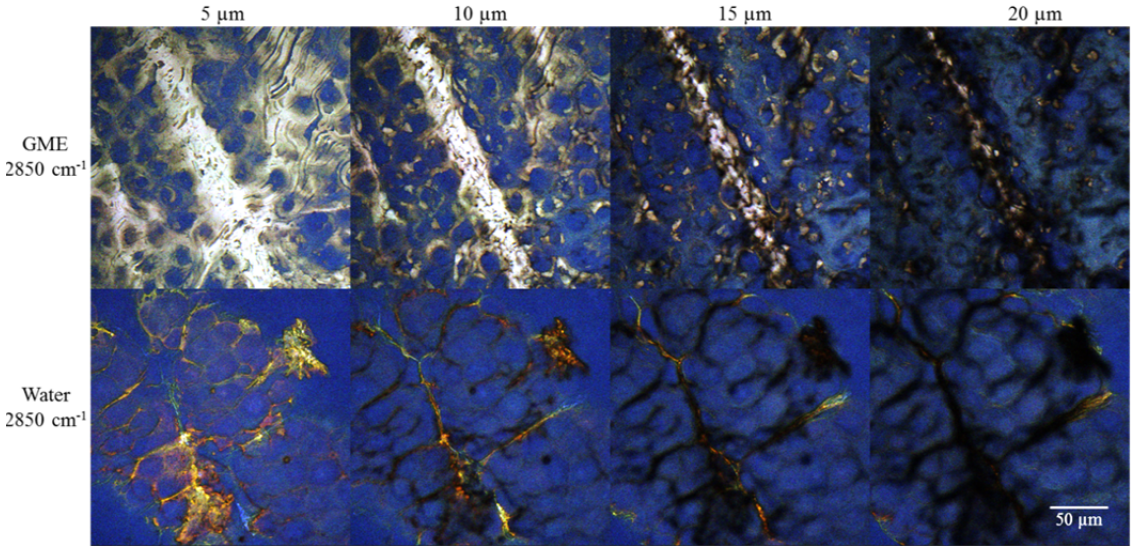


Figure 26 The top line shows CARS images of skin treated with GME-based cubic formulation at various depths. Below are comparative images of skin treated with water. The images are color coded: red (2845 cm^{-1}), green (2870 cm^{-1}), blue (2945 cm^{-1}), and the color was scaled so that white equals the cubic lipids. The images were retrieved using 20x dry objective, 240x240 µm, 512x512 pixels.

The results agree with our hypothesis with regards to formulation penetration. However, more investigations are needed to thoroughly answer the questions at hand. This data must be repeated and compared with the GMO-based cubic formulation and other lipid-based formulations. We do not know if this diffusion pattern is specific for the GME-based cubic formulation, if it defines the diffusion of cubic phases in general, or if other lipid formulations give similar results. The latter is unlikely though, as published results of omega-3 oil applied to skin did not give a similar result [212]. To visually show the

formulation penetrating deep into the tissue without influencing the system with fluorophores or other imaging aids are in itself quite remarkable. The results presented here are in manuscript form aiming for publication in the near future.

6 CONCLUSION

The results of each study included in this thesis have contributed to the postulated hypothesis. The key facts from each study, on which we built the hypothesis, are here presented.

Paper I: POL and PG were both found to be efficient percutaneous absorption enhancers.
Paper II: Without the addition of a solvent, GME forms a reversed hexagonal phase in excess water. The solvent POL was found to prefer the interface between water and lipid, though ends up to a higher extent in the water rich domains of the cubic phase compared to the lipid domains.
Paper III: An effective on demand dermal drug delivery formulation can be formed using GME water and PG or POL. The cubic formulations, based on GME or GMO, were significantly better at delivering the API into the skin cells, compared to the commercial product Metvix. It is noteworthy that Metvix contains five times more API than the tested cubic systems.
Paper IV: TPM and proteomic methods showed that delivery of BSA into viable epidermis was possible only when using the GME based cubic formulation as delivery vehicle.
Work in progress: TPM studies of QD penetration into skin tissue confirmed the results presented in paper IV, with regard to both penetration and delivery vehicle. The preliminary CARS measurements, aiming to distinguish the lipids of the formulation (starting in its cubic composition) from the lipids of the skin, indicated GME penetration around the cells, in part as droplet-like depots.

From the concluded results and facts previously presented in this thesis, our hypothesis can be presented in short: the on demand GME-water-solvent cubic formulation adheres well to the skin surface and increases the hydration of the tissue. Upon contact with water the solvent is released and the enhancing properties act. Simultaneously, due to the loss of solvent, this cubic phase transitioned into a reversed hexagonal phase. The increased mobility gained enables movement within the skin lipid matrix, further assisted by decreased

rigidity due to the fluid lipids of the formulation. Thus, this process continues downward and brings the loaded API with it.

Also for the GMO-based cubic phase much of the above is true. However, this cubic phase does not transition into a reversed hexagonal phase and is thus less mobile. This may be a reason why we did not see increased absorption for BSA and nanoparticles while the delivery of the small molecules ALA and MAL was as effective.

7 FUTURE OUTLOOK

The research field in which my projects are included is broad and there are a number of ways to go from here. However, first of all the aim should be to challenge the postulated hypothesis. Information is needed of how the delivery occurs, that is the diffusion of the API and the formulation components into skin. One way to elucidate this may be to study the dermal delivery of API from the GME-based cubic formulations in the same way as Brewer et al. did when finding that transfersomes do not stay intact during dermal delivery [83]. Moreover, solvents should be included in the GMO-based cubic formulation and the reversed hexagonal GME-based phase in order to elucidate if this would enable drug delivery to the same extent as shown for the GME-based cubic formulations.

Furthermore, the ongoing work has to be finished. The nanoparticle studies only lack the finishing TEM and SEM measurements, which has commenced but not been completed. Regarding the CARS study, data must first of all be repeated. Thereafter, it should be compared with the GMO-based cubic formulation and with other lipid-based formulations in order to visualize similarities and differences from which further conclusions can be drawn.

There are a number of research questions related to my performed work that would be interesting to look deeper into, for example:

- Test the on demand cubic system *in vivo* both through clinical trials of ALA-delivery and through animal studies of protein and nanoparticle skin penetration and effect.
- Study the long term effect on the skin barrier after repeated exposure of cubic lipid systems. So far most studies are restricted to single exposure for maximum 24 hours. As the results of this thesis points towards a significant interaction between the lipid constituents of the phase and the skin lipids, thorough investigations of this interaction should be undertaken not only from an efficacy, but also a safety point of view.
- Continue the work with sponge phases to provide the protein crystallization field with a better understanding of the occurring

processes and a more efficient tool to work with. Also, to find other new uses for this interesting phase.

- Combine TPM of, for example, fluorescent proteins with, for example, FCS to find an easier way to detect if the fluorophore stays attached to the molecule of interest and to follow the diffusion in more detail.
- Continue the work with TPM and AuNP gradient plates for increased understanding and for new applications within, for example, the pharmaceutical field.

I hope there will be more people interested in working with these issues in the future and that my contribution to the field, however small, may aid in some way to an increased understanding.

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