Skin Drug Delivery
From Different Lipid Vesicular Systems

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Contents

Part one: General Introduction .................................................................................1
  1.1 The Aim of research ..................................................................................2
  1.2 The Structure of Skin ...............................................................................3
  1.3 Skin Penetration Routes ...........................................................................9
  1.4 Liposomal Systems for Skin Drug Delivery...........................................10

Part Two: Publication Overview..............................................................................13

Part Three: Publications ..........................................................................................16
  3.1 Publication 1.............................................................................................16
  3.2 Publication 2.............................................................................................29
  3.3 Publication 3.............................................................................................39

Part Four: Final Discussion .....................................................................................97
  4.1 Preparation and Characterization of Lipid vesicular systems............98
  4.2 Human Skin Preparation.......................................................................100
  4.3 The Interaction among conventional liposomes, Model drugs and
    Skin..........................................................................................................101
  4.4 The Interaction among Deformable Liposomes/Invasomes, Model
    drugs and Skin........................................................................................102
  4.5 The Interaction between Ethosomes, Model drugs and Skin.............104
  4.6 The Effect of Application mode on Skin Drug Delivery.....................106

Part Five: Summary/ Zusammfassung...............................................................108

Part Six: Reference...................................................................................................110

Acknowledgement....................................................................................................114

Curriculum Vitae.....................................................................................................115

Statement...................................................................................................................117
Part One

General Introduction

Skin is the largest organ of the human body, which interfaces with the environment and covers a surface area between 1.5 and 2.0 m². From a pharmaceutical point of view, skin offers a glorious route for delivering drugs because skin drug delivery brings forth many attractive advantages over other routes of administration, including avoidance of first-pass metabolism, sustained and controlled delivery over a prolonged period of time, reduction in side effects associated with systemic toxicity, improved patient acceptance and compliance, direct access to target or diseased site, convenient and painless administration and so on (Brown et al., 2006).

On the other hand, however, skin is also an excellent biological barrier, which is as much as 10²-10⁴ times less permeable than a blood capillary wall (Cevc and Vierl, 2010) and imposes physicochemical limitations to the type of permeants which can traverse it. For a drug to be delivered successfully and passively into or through the skin, small molecular size (ideally a molecular weight less than 500Da) and sufficient aqueous and lipid solubility (a LogP_{octanol/water} between 1 and 3) are essential and required (Yano et al., 1986). In addition, the presence of enzymes in the skin such as peptidases and esterases might metabolize the drug into a form which could be therapeutically inactive, thereby reducing the efficacy of the drug (Steinstrasser and Merkle, 1995). Moreover, skin irritation and sensitization provoked by exposure to certain stimuli such as drugs, excipients or any other component of topical formulations could also be a limitation to the product on market (Carmichael, 1994; Hogan and Maibach, 1990; Toole et al., 2002). Thus, all of these requirements mentioned above, at least, have limited the number of commercially available products based on skin delivery.

Over the past decades, numerous studies have been performed to overcome the problems associated with skin delivery and also a number of novel skin delivery systems and approaches have been developed, including the use of chemical penetration enhancers (Goodman and Barry, 1988; Williams and Barry, 2004), modifying chemical potential of the drug (Megrab, 1995), the application of liposomes and other colloidal drug carrier systems, electrically driving molecules into...
or through the tissue employing iontophoresis (Miller et al., 1990), or physically
disrupting the skin structure by electroporation or sonophoresis (Prausnitz et al.,
1993a; Prausnitz et al., 1993b) and others (Nanda A, 2006). Among these novel
techniques, lipid vesicular systems such as conventional liposomes, deformable
liposomes (transfersomes), and ethosomes may offer a promising strategy for
successfully improving skin drug delivery.

1 The Aim of Research

As mentioned, skin is known to exhibit selective permeability with respect to the
type of diffusing molecules. In fact, the ability of each drug to penetrate into the skin
and the diffusion route it takes are dependent on its own physicochemical properties
and the interactions it has with the skin’s various conduit regimes (Lu and Flynn,
2009). When it comes to liposomal drug carrier systems, the situation could be more
complicated. Firstly, encapsulated drug could be located at different position
according to their lipophilicity. For instance, lipophilic drugs could be incorporated
into the bilayers; while the hydrophilic one could be loaded within the water phase
inside the vesicles and could be also found outside the liposomes in high amounts.
This could affect the interaction between drugs and lipid carrier systems and in turn
influence the penetration and deposition behavior of drugs. Moreover, the function of
liposomal systems varies with type and composition. For instance, liposomal systems
may act as drug carriers controlling release of the medicinal agent. They may also
provide a localized depot in the skin so minimizing systemic. Alternatively, liposomes
may enhance transdermal drug delivery, increasing systemic drug concentrations. In
addition, liposomal systems can also be used for targeting delivery to skin appendages.

Thus, how the fate of drugs could be changed by different liposomal systems?
More specifically, do the liposomal systems enhance drug deposition into the skin? Or
do they enhance drug transport across the skin? Or do they enhance both dermal and
transdermal delivery? Moreover, the compositions of liposomal systems could change
due to penetration into the skin or evaporation of volatile components. These changes
depend on the amount of liposome applied and occlusion conditions. And these
differences may result in variations in the effects of liposomal systems as skin drug
delivery tools. Hence, do application mode and the amount of liposomes applied have
an impact on their effectiveness as skin drug delivery tools? At last but not the least,
what are reasonable mechanisms of different liposomal systems to deliver therapeutic
agents to and through the skin at each specific situation? Therefore, the aims of the whole study are as following:

1) Development of different lipid vesicular systems containing different model drugs, including conventional liposomes, deformable liposomes (invasomes) and ethosomes.
2) Characterization of lipid vesicular systems, including particle size distribution, zeta potential, lamellarity and particle shape.
3) Investigate the percutaneous absorption of model drugs via different lipid vesicular systems as well as the effect of application mode on this process.
4) Analysis of results and discussion on the reasonable mechanisms of action of different lipid vesicular systems when topically applied.

Furthermore, in order to better understand all these questions mentioned above, one must be knowledgeable about the anatomical structure and chemical compositions of skin, the skin barrier function and penetration routes, formulation considerations for skin drug delivery, the classification and potential compositions of different liposomal systems as well as their topical application, and so on. Therefore, all of these would be the subjects for discussion in this chapter. Rational considerations to skin drug delivery via liposomal systems rest on having such insights in advance.

2 The Structure of Skin

Skin is anatomically divided into three principal and distinct layers, from the outside of skin inward, including stratum corneum (10–20 µm thick), viable epidermis (50–100 µm thick), and dermis (1000-2000 µm thick). A fatty subcutaneous layer resides beneath the dermis. It should be pointed out that all the thickness specified here are representative only, since the actual thickness of each layer varies several fold from place to place on the body. Adnexal appendages, including hair follicles, associated sebaceous glands and pili muscles, apocrine and eccrine sweat glands, can be found dispersing throughout of the skin, varying in number and size depending on body site. The cross section of skin structure is shown is Figure 1.

2.1 Stratum Corneum

Stratum corneum is the outmost superficial layer of the skin and also the
principal barrier element of the skin. SC consists of several layers of completely keratinized flattened dead cells, corneocytes, each of which is about 30 µm in diameter with a hexagonal shape and 0.5-0.8 µm in thickness (Holbrook and Odland, 1974). These acutely flattened corneocytes are highly organized and stacked vertically 15 to 25 cell layers, which are embedded into a specialized and well structured intercellular lipid matrix (Wertz et al., 1989).

The most simplistic organizational description of SC is advocated by Elias (Elias, 1987, 1989; Williams and Elias, 1993), which is the classic “brick-and-mortar” assembly (Figure 2). The intracellular space of corneocytes is literally packed with structural protein, semi crystal line α keratin intermixed with more amorphous β keratin. The intracellular space is dense, offering little freedom of movement to drug molecules. Thus, the corneocytes work as “brick” being thermodynamically impenetrable. While the intercellular space of corneocytes is filled with a lipid “mortar” formed of cholesterol, free fatty acids, and ceramides, which seals horny structure (Figure 2).

However, the brick-and-mortar skin model is not enough to describe the panorama of the SC. In fact, the cells from basal layer of epidermis, which we describe further in the text, to the SC are built up in clusters, which represent the basic skin permeation resistance unit (Cevc, 2004). It is these clusters that are separated by surface corrugations (wrinkle line), which often reach several micrometers into the basal layer of the epidermis (Figure 3).

Fig.1 A cross sectional view of human skin, Source: From Ref. (Lu and Flynn, 2009)
Fig. 2 Brick-and-mortar model of Stratum corneum and penetration routes through it, Source from Ref. (Elias, 1981)

Fig. 3 Epidermis with Stratum corneum, including a corneocytes cluster, Source from Ref. (Cevc and Vierl, 2010)

In addition, the basolateral side of stratum corneum is in direct contact with the living epidermal mass, where the corneocytes contain water at high thermodynamic activity of the physiological milieu. On the other hand, its external surface interfaces the environment, where air tends to have a far lower water activity. Consequently a water gradient is established and water diffuses out through the stratum corneum. Under such a normal hydration situation, the stratum corneum takes up moisture to the extent of 15% to 20% of its dry weight (Lu and Flynn, 2009). It should be pointed out here that the hydration condition of SC plays an important role on the drug molecules skin penetration, which would be discussed further in the text.

2.2 Viable Epidermis

The viable epidermis is underlying the stratum corneum. It is multilayered when viewed under microscope, including, from bottom to top, basal layer (stratum germinativum), spinous layer (stratum spinosum), granular layer (stratum granulosum) and lucid layer (stratum lucidum). Each layer is defined by position, shape, and morphology and also reflects the progressive differentiation of keratinocytes which eventuates into their death and placement as chemically and physically resistant “brick” in stratum corneum. However, when physicochemically considered, the viable epidermis is just a group of tightly massed live cells, which results in a singular diffusion area or resistance in percutaneous absorption process. Water found in this live epidermis has an activity equivalent to that of 0.9% NaCl (Lu and Flynn, 2009).
While the interface between stratum corneum and epidermis is flat, the one between epidermis and dermis is papillose, which increases their contact surface area and then allows for the diffusion of nutrients or other biological or medicated molecules between dermis and epidermis (Figure 1). The epidermis itself is avascular.

Besides keratinocytes, Langerhans cells also can be found in viable epidermis. They are antigen presenting cells in the skin’s immunological responses. Moreover, another kind of cells, melanocytes, are strategically placed in the epidermis just above the epidermis and dermis junction. When influenced by melanocyte-stimulating hormone or ultraviolet radiation, melanocytes synthesize and deposit the pigment granules into skin, which gives rise to the skin coloration.

2.3 Dermis

Dermis is directly adjacent to the epidermis and extends from the epidermal-dermal junction to the subcutaneous tissue (Figure 1). Dermis consists of a network of irregular connective tissue, which provides the mechanical support for the skin. The matrix of this connective tissue consists of structural fibers, such as collagen, reticulum, and elastin. These fibers are embedded in an amorphous mucopolysaccharidic gel called the ground substance (Lu and Flynn, 2009). The dermis can be arbitrarily divided into a superficial papillary layer and a deep reticular layer. The upper papillary layer is thin, one fifth of thickness of the dermis, and protrudes into the epidermis giving rise to the dermal papilla, and also provides the support of the delicate capillary plexus which nurtures the epidermis. The deepest layer of the skin is a far coarser fibrous matrix, the reticular dermis, which is the main structural element of the skin. Equally importantly, the microcirculation which subserves the skin is entirely housed in the dermis. Blood flow through skin can vary by a factor of 100 fold depending on environmental conditions. The dermis is also penetrated by sensory nerve endings and an extensive lymphatic network. Moreover, skin appendages such as sweat gland, sebaceous glands, hair follicles, and arrector pili muscles are anchored within the dermis.

The main cell inhabitants of the dermis are fibroblasts, mast cells and macrophages. Fibroblasts synthesize the structural fibers, while mast cells are thought to synthesize the ground substance. Macrophages work as immune response. In addition, plasma cells, chromatophores, fat cells, nerve cells and endings can also be found along with blood vessels, nerves and lymphatics.
2.4 Skin Appendages

Skin appendages include hair follicles and their associated sebaceous glands, eccrine glands, apocrine glands, and arrector pili muscles.

The hair follicle unit is composed of the hair, hair follicle, associated sebaceous gland, and pili muscles. Hair is a compact of keratinized structures, which consist of three layers, including an outermost cuticle, a cortex of densely packed keratinized cells, and a medulla of loose flattened cells. Hairs can be found mostly everywhere on the body except for the soles of the feet, the palms of the hand, and mucocutaneous junctions. There are 100 follicles per square centimeter, representing one thousandth of the skin’s surface. A hair emerges from a follicle, which is set within the dermis at a slight angle. The hair follicle consists of three major components, including internal root sheath, external root sheath, and dermal papilla (Figure 4). This arrangement results in a solid implantation of the hair root in the hair follicle. In addition, each follicle is anchored to the surrounding connective tissue by an individual strand of arrector pili muscle. Furthermore, each follicle is associated with one or more flask like sebaceous glands, which secret an oily secretion, sebum. Then the sebum is forced upward around the hair shaft and onto the skin surface. Sebum mainly consists of squalene, cholesterol esters, wax esters, and triglycerides. It has several biological functions including the regulation of steroidogenesis and androgen synthesis, and providing antibacterial and water resistance to skin.

Fig.4 Skin appendages: (a) Structure of the skin (b) Structure of the hair follicle (c) Cross-section of the hair, source from Ref. (Wosicka and Cal, 2010)
Eccrine glands (sweat glands) are distributed over the entire body except the genitalia and lips. These simple tubular glands open directly on the skin surfaces and extend to the footings of the dermis. There are between 150 and 600 glands per square centimeter of body surface depending on body site (Lu and Flynn, 2009). However, the estimated number of actual sweating glands is much less than that value, since many of these glands remain dormant. Thus, these glandular openings occupy approximately one ten thousandth of the skin surface. Eccrine sweat is slightly acidic (pH=5) due to traces of lactic acid, which is moderately bacteriostatic.

### 2.5 Skin circulatory system

Skin circulatory system not only provides nutrients to the skin, hair follicles, the glandular appendages as well as the subcutaneous fat, but also participates in other biological functions, such as heat exchange, repair, and immune responses. Skin circulatory system is an extensive vascular network. The blood supply to the skin arises from substantial arteries which are located in the subcutaneous connective tissue. The terminal branches of these arteries in turn give rise to three plexuses: (i) subcutaneous plexus, which is located just beneath the dermis and gives off the branches to the (J du Plessis) cutaneous plexus, which brings a blood supply into dermis with reaching the upper dermis and then further branches to form (iii) the shallower subpapillary plexus of capillary loops which bring a blood supply up into the papillae at the dermal epidermal interface. The transport of nutrients into epidermis or substance exchange between epidermis and dermis is achieved via passive diffusion. The epidermis itself is avascular. The veins into skin are organized along the same lines as the arteries (Lu and Flynn, 2009), in which there are also three plexus including subcutaneous plexus, cutaneous plexus and subpapillary plexus. It should be pointed out that arterioles belonged to cutaneous plexus and subpapillary plexus exhibit arteriovenous anastomoses, which are shunt like connections that link the arterioles directly to corresponding venules (Lu and Flynn, 2009).

The vascular surface available for exchange of substances including drugs between the blood and the local tissue has been estimated to be of the same magnitude of that of the skin. The blood flow through the skin is reported from 0.01 up to 1 ml per minute per gram of tissue, which depends on the body site and temperature. Sufficient blood to within 150μm of the skin’s surface efficiently draws chemicals into the body that has gained access to this depth (Lu and Flynn, 2009).
3 Skin Penetration Routes

When a skin drug delivery system is applied topically, drug-containing carriers or free drug in the system could interact with either the stratum corneum or the sebum filled ducts of the pilosebaceous glands. Thus, two principle absorption routes are involved, including the transepidermal route, where the drug delivery system interacts with or diffuses through stratum corneum, and transfollicular route, where they interact with or diffuse through the follicles.

In the case of the transepidermal route, since the impermeable character of the corneocytes, the intercellular space of corneocytes provides the only continuous phase, which is also the predominant penetration pathway (intercellular route or intercorneocyte pathway) from the skin surface to the viable epidermis. However, the tortuous zigzag bestowed by staggered corneocytes arrangement (typically 18–21), corneocyte layers (Menon, 2002), as well as the highly organized crystalline lamellae structures of the mortar lead to an outstanding barrier property of the labyrinthine intercellular route. The transportation of molecules across this layer is primarily passive diffusion, in accordance with Fick’s law, and no active transport processes have been identified to date (Brown et al., 2006). Thus the permeability of stratum corneum as a penetration resistor is proportional to the diffusive mobility of drug molecules within it (diffusion coefficient, \(D_{sc}\), also proportional to the capacity of the SC to solubilize the drug molecules relative to vehicle (partition coefficient, \(K_{sc}\)) but inversely proportional to the thickness of stratum corneum (\(h_{sc}\)). Consequently, at the steady state and sink condition, drug permeation can be described as following:

\[
J_{sc} = \frac{K_{sc}D_{sc}C}{h_{sc}}
\]

(1)

where \(J_{sc}\) (\(\mu g\ cm^{-2}\ h^{-1}\)) is the steady state flux through stratum corneum. \(C\) is the concentration of drug in the topical drug delivery system.

When considering transfollicular route, initially it was not considered to be a significant skin penetration route, as evidence suggested that they accounted for only approximately 0.1% of the skin surface area (H.Schaefer and Redelmeier, 1996). Recently, it has been demonstrated that hair follicles may act as a significant penetration pathway and/or potential reservoirs for topically applied compound (Blume-Peytavi et al., 2010; Grice et al., 2010; Lademann et al., 2010; Lademann et al., 2006). As mentioned before, owing to the presence of sebum in follicles, the
Part One                                                    General Introduction

permeation through follicular route can be described as following:

\[ J_{\text{sebum}} = \frac{K_{\text{sebum}}D_{\text{sebum}}C}{h_{\text{sebum}}} \]  

(2)

where \( J_{\text{sebum}} (\mu g \text{ cm}^{-2} \text{ h}^{-1}) \) is the steady state flux through sebum/hair follicle. \( C \) is the concentration of drug in the topical drug delivery system. \( K_{\text{sebum}} \) and \( D_{\text{sebum}} \) are diffusion coefficient through sebum and drug partition coefficient in sebum/water, respectively.

In short, either or both routes can be important depending on the physicochemical properties of a drug as well as the condition of the skin, since the percutaneous absorption is a spontaneous passive diffusion process which takes the path of least resistance.

4 Liposomal Systems for Skin Drug Delivery

Liposomes were introduced in 1965 by Bangham et al (Bangham et al., 1965). Initially, they were used as a model for membrane system studies. However, the topical application of liposomal systems has attracted increasing attention in dermatology since the first liposomal product, which was an econazole preparation for topical therapy of dermatomycosis, introduced into the market in 1988.

Essentially, liposomal vesicles are colloidal particles in which one or more lipid bilayers entrap an aqueous volume. Their major components are usually phospholipids, with or without some additives. There are a wide variety of lipids and additives that can be used to prepare these vesicles. Phospholipids could spontaneously aggregate into vesicular structure after addition of water, which results from their amphipathic character due to the presence of a polar or hydrophilic (water-attracting) head-group region and a non-polar, lipophilic (water-repellent) tail. The hydrophilic head groups orientate toward the aqueous phase and the lipophilic tails orientate to each other in the presence of water (Fig. 5) (Hope and Kitson, 1993). Therefore, liposomes contain a lipophilic compartment within the bi-layer membranes and hydrophilic compartments between the membranes. Correspondingly, lipophilic drugs could be incorporated into the bilayers; while the hydrophilic one could be loaded within the water phase inside the vesicles and could be also found outside the liposomes in high amounts (Weiner et al., 1994).

Liposomes can be classified according to their particle size. Small unilamellar vesicles (SUV) have dimensions of 20 up to about 50 nm. Large unilamellar vesicles
(LUV) are between 50 and 500 nm, and multilamellar vesicles (MLV) have dimensions exceeding 500 nm but below 10,000 nm in diameter. SUVs are less suitable for drug delivery because they lack stability and their volume is too small for entrapping drugs. And generally, the penetration of liposomes through the stratum corneum decreases with increasing diameters. Therefore, the preferred structures for drug delivery are liposomes that are 50–500 nm in diameter (Hope and Kitson, 1993).

Another essential characteristic of liposomes for penetration through the stratum corneum is their state in a liquid crystal phase. The lipid bilayer passes from a gel into a liquid crystal phase at a critical phase transition temperature (cptT). At the cptT, the head groups become fully hydrated and the lipid chains become freely mobile in the membrane. Phosphatidylcholine can form bi-layers at cptT below room temperature. This liquid crystal state is essential for liposomes to interact simultaneously with the lipid and the aqueous compartments of the stratum corneum, and for delivering entrapped drugs into the skin (de Leeuw et al., 2009).

Liposomes also can be classified according to their components. Most commonly,
the vesicles only composed of phospholipids, with or without cholesterol, can be called conventional liposomes. Since the first liposome based topical formulation available in the market, shortly afterwards, several other conventional liposome formulations for dermal delivery of local anesthetic, antifungal, anti-inflammatory and anticancer drugs were developed and produced (Sinico and Fadda, 2009). All these conventional liposomes carry drugs mainly targeted to the dermal region of the skin (Sinico and Fadda, 2009). In 1992, Cevc and Blume (Cevc and Blume, 1992) introduced the deformable liposomes, referred to as Transfersomes® (IDEA AG, Munich, Germany). And recently Fahr and colleagues developed invasomes (Dragicevic-Curic et al., 2008; Verma, 2002), composed of phosphatidylcholine, ethanol and a mixture of terpenes as penetration enhancers. All these vesicles contain additional edge activators such as surfactants and lysophosphatidylcholine (LPC) to modify the bilayer elasticity and to increase deformability. Because of their elasticity and deformability, they seem to be a useful carrier for transdermal delivery of some drugs (Elsayed et al., 2007b). The effectiveness of deformable liposomes as both dermal and transdermal delivery systems has been successfully demonstrated by using numerous chemical agents of different molecular sizes and lipophilicity, such as lidocaine, tetracaine, cyclosporin, hydrocortisone, dexamethasone, triamcinolone acetonide, diclofenac, ibuprofen, tamoxifen, testosterone, etc. (Cevc, 1997). Another kind of liposomal vesicle are ethosomes, which are also composed of phospholipids but embodying a high concentration (20–45%) of alcohol (ethanol or isopropyl alcohol). Ethosomes too can penetrate the skin and improve dermal, transdermal delivery of a lot of drugs, including highly lipophilic molecules and cationic drugs such as acyclovir, trihexyphenidyl, minoxidil, cannabinoids, zidovudine, and testosterone through the skin (Godin and Touitou, 2003; Nanda A, 2006; Touitou et al., 2000). Pharmaceutical formulations based on ethosomes technology have been developed by Novel Therapeutic Technology, Inc., for the treatment of several diseases (i.e., alopecia, erectile dysfunction, dermatitis) by topical application of drugs (Sinico and Fadda, 2009).

In short, it is believed that the topical use of lipid vesicles can support various medical applications because their delivery enhancing properties could be modulated by changes in composition and structure. Moreover, lipid vesicles also provide a promising approach aiming at both increasing efficiency and reducing toxicity of drugs with topical application.

**Abstract:** Application of lipid vesicular formulations as transdermal delivery systems in order to enhance drug transport across the skin barrier is one of the most controversial technologies, which was expounded in this review, with a special emphasis on the effectiveness of these liposomal systems as skin drug delivery systems. On the other hand, the significance of the skin and fascinating functions it performs cannot be overstated. Thus, any breach in the integrity of the skin or any compromised function will have a profound effect so that the protection of damaged skin cannot be neglected, which will be discussed at length in this review.

**Own contribution to the manuscript:**
Review of liposomal vesicles as transdermal drug delivery, including conventional liposomes, deformable liposomes and ethosomes.

Abstract: Different lipid vesicular systems were designed and prepared in order to improve skin delivery of ferulic acid which is a traditional Chinese medicine (TCM) exhibiting a wide range of therapeutic effects against various diseases. All test formulations were characterized for particle size distribution, $\zeta$-potential, vesicular shape and surface morphology. Furthermore, the skin delivery capabilities of different vesicular systems were investigated with in vitro human skin permeation and skin deposition study, indicating that well developed lipid vesicles are promising vesicular carriers for delivering ferulic acid into or across the skin.

Own contribution to the manuscript:
1) Preparation and characterization of different lipid vesicular systems, including conventional liposomes, Tween 80-based deformable liposomes, invasomes and ethosomes
2) Preparation of stratum corneum/epidermis (SCE) membranes and establishment of fluorescent assay of ferulic acid
3) Performance of in vitro skin permeation and deposition studies of all lipid vesicular formulations
4) Data evaluation, and interpretation and representation of the results

Abstract: Different lipid vesicular systems containing Carboxyfluorescein (hydrophilic model drug) or Temoporfin (lipophilic model drug) were prepared in order to evaluate the influence of different lipid vesicular systems on their skin penetration and deposition behaviors. All test formulations were characterized for particle size distribution, \(\zeta\)-potential, vesicular shape and surface morphology. Furthermore, the effect of different application modes, including application of finite dosage and infinite dosage, on skin penetration and deposition were investigated. All the results revealed that the factors influencing the drug skin distribution concern the physicochemical characteristics of the drug, the choice of the vehicle formulation and the application mode applied.

Own contribution to the manuscript:
1) Preparation and characterization of different lipid vesicular systems containing Carboxyfluorescein or Temoporfin, including conventional liposomes, invasomes and ethosomes
2) Preparation of full thickness human skin and establishment of extraction method and fluorescent assay of Carboxyfluorescein and Temoporfin in different skin layers
3) Performance of in vitro skin penetration and deposition studies of all lipid vesicular formulations
4) Data evaluation, and interpretation and representation of the results
Part Three

Publications

Publication 1

Liposomes as Promising Tools for the Skin Delivery and Protection of Damaged Skin

Alfred Fahr and Ming Chen


Pages in the dissertation: 16-28
INTRODUCTION

The significance of the skin and fascinating functions it performs, such as protection, sensation, heat regulation, control of evaporation, absorption, storage and synthesis, water resistance, and so on, cannot be overstated. Because of limited space, this chapter will not provide detailed information on all aspects of skin function. However, more information may be accessible from other chapters in this book (see chap. 1) and from other literature (1–3). Furthermore, on account of the demonstrated importance of skin, any breach in the integrity of the skin or any compromised function will have a profound effect so that the protection of damaged skin cannot be neglected, which will be discussed at length in this chapter.

On the other hand, skin covers a surface area between 1.5 and 2.0 m² and is often regarded as the largest organ of the human body. From a pharmaceutical point of view, it offers a glorious route for delivering drugs because transdermal drug delivery brings forth many attractive advantages over other routes of administration, including avoidance of first-pass metabolism, lower fluctuations in plasma drug levels, and good patient compliance (4). According to a report by Jain PharmaBiotech, the value of the global market for transdermal drug delivery was $12.7 billion in 2005 and is expected to increase to $21.5 billion in 2010 and $31.5 billion in 2015. The transdermal drug delivery market is about to soar and become an indispensable contributor within the pharmaceutical industry.

However, every coin has two sides, for the pharmaceuticals, the skin is also a tough barrier for delivering drugs. The first commercial transdermal product was made available in the global transdermal market in the early 1980s, and there are only 11 approved molecules utilizing transdermal technology introduced over past two decades that includes scopolamine, nitroglycerin, clonidine, estradiol, fentanyl, nicotine, norethisterone, lidocaine, oxybutynin, and methylphenidate (5). The major reason for so few drugs is the low permeability of drugs in the stratum corneum (SC), the outermost layer of the skin that acts as the primary barrier (6). Numerous techniques have been employed to overcome the barrier posed by the SC to improve transdermal drug delivery. One of the most controversial technologies is the employment of lipid vesicular formulations as transdermal delivery systems to enhance drug transport across the skin barrier. This will be expounded in this chapter, with a special emphasis on the effectiveness of these liposomal systems as skin drug delivery systems.
LIPOSOMAL SYSTEMS AS A TOOL FOR TRANSDERMAL DRUG DELIVERY

Skin Barrier and Penetration Pathways

Anatomically, the skin consists of three distinct layers, including SC (10–20 μm thick), viable epidermis (50–100 μm thick), and dermis (1–2 mm thick). As mentioned above, the skin’s barrier function is accomplished by the outermost layer of the skin, the SC (see chap. 1).

The most simplistic organizational description of SC is advocated by Elias (7) (see chap. 2), which is the classic “brick-and-mortar” assembly. This structure is analogous to a wall that is composed of corneocytes (brick, because of the cornified envelope around each corneocyte) embedded in a bilayer lipid membranes matrix (mortar) formed of cholesterol, free fatty acids, and ceramides (6).

Importantly, the “mortar” provides the only continuous phase, which is also the predominant penetration pathway (intercellular route or intercorneocyte pathway), from the skin surface to the viable epidermis. However, the impermeable character of the “brick” and the tortuous zigzag bestowed by staggered corneocyte arrangement (typically 18–21), corneocyte layers (8), as well as the highly organized crystalline lamellae structures of the mortar lead to an outstanding barrier property of the labyrinthine intercellular route. The transportation of molecules across this layer is primarily passive diffusion, in accordance with Fick’s law, and no active transport processes have been identified (4) to date. Moreover, the SC is very selective with respect to the type of diffusing molecules, which means the barrier nature of the skin imposes physicochemical limitations to the type of permeants that can traverse the skin, including hydrophilicity, size, and hydrogen-bonding ability (9–11). As a consequence, all of these factors are responsible for the high transport resistance of the intercellular penetration route.

On the other hand, the brick-and-mortar skin model is not enough to describe the panorama of the SC. In fact, the cells from basal layer of epidermis to the SC are built up in clusters, which represent the basic skin permeation resistance unit (12). It is these clusters that are separated by surface corrugations, which often reach several micrometers into the basal layer of the epidermis and are “hot spots” for transdermal drug delivery because these pathways between the clusters of corneocytes in the SC offer a lower transport resistance. However, comparing with intercellular pathway, these intercluster pathways are sparse (12).

More specifically, the intercellular pathway width ranges from 0.4 to 36 nm, on average, with a peak around 20 nm, while the intercluster pathways are much wider than 30 nm and almost permanently open (12). Moreover, the total skin porosity is estimated to be no more than 0.25%, and a two-cell contact equals to one-path approximation (12). In short, from the penetrant point of view, the skin behaves as a mechanical, nanoporous barrier perforated by a number of short, discontinuous, and gap-like penetration pathways (8).

Formulation Considerations for Transdermal Drug Delivery

Comparing other administration routes, the benefits offered by transdermal drug delivery, as mentioned before, are attractive and impressive; however, it is noteworthy that transdermal drug delivery is not able to achieve rapid and immediate drug inputs, rather it is usually applied to hold a slow sustained drug delivery or controlled drug input. Although there still remains a large pool
of transdermal candidates that can benefit from this route of delivery, there are only a handful of marketed transdermal agents that can be formulated to be delivered across the skin passively. The key problem stems from the daily drug dose that can be systemically delivered within a reasonable “patch-sized” area less than 10 mg range due to the excellent diffusion resistance caused by the SC (5). Consequently, this limitation imposes two criterions for a successful transdermal candidate. The first one is that the pharmacological potency of transdermal candidates must be high enough, usually requiring therapeutic blood concentrations in the ng/mL range or less, and the second one is that these candidates are expected to possess acceptable physicochemical parameters related to molecular hydrophobicity, molecular weight (size), and the ability to interact with the other molecules, for example, via hydrogen bond formation (13–15).

**Liposomal Vesicles as Transdermal Drug Delivery**

It can be easily understood that not all drugs are suitable or feasible for transdermal drug delivery. Hence, there exists a clear and pressing expectation by the pharmaceutical scientists that the skin’s barrier could be technically breached to enhance the transdermal flux across the skin and to expand the range of transdermally delivered drugs. In fact, during the past decades, there has been great interest in exploring new approaches to target this aim. These approaches include the use of chemical penetration enhancers (16,17), chemical potential of the drug (18), electrically driving molecules into or through the tissue employing iontophoresis (19), or physically disrupting the skin structure by electroporation or sonophoresis (20,21), and others (22,23). Colloidal drug delivery systems such as liposomes, ethosomes, and transfersomes may offer a promising strategy for approaching the purpose of improved skin drug delivery.

Essentially, liposomal vesicles are colloidal particles in which one or more lipid bilayers entrap an aqueous volume. Their major components are usually phospholipids, with or without some additives. There are a wide variety of lipids and additives that can be used to prepare these vesicles. In light of their changeable composition, liposomal systems can be classified into several types. Most commonly, the vesicles only composed of phospholipids, with or without cholesterol, can be called liposomes or conventional (traditional) liposomes. The vesicles, which are structurally similar to liposomes but contain an additional edge activator such as surfactants to modify the bilayer elasticity and to increase deformability, are named ultraflexible liposomes, ultradeformable liposomes, or transfersomes. Another kind of vesicle are ethosomes, which are also composed of phospholipids but embodying a high concentration (20–45%) of alcohol (ethanol or isopropyl alcohol). It is very possible that future research will lead to the introduction and development of novel liposomal vesicles systems.

Moreover, it has to be pointed out that the diversity of potential composition of these liposomal systems are influenced by their physicochemical characteristics such as particle size, charge, thermodynamic phase, and bilayer elasticity, etc., which in turn have a significant effect on the interaction between vesicles and the skin and hence on the effectiveness of these vesicles as transdermal delivery systems.

**Conventional Liposomes as Transdermal Delivery Systems**

General and the effect of conventional liposomes on transdermal drug delivery. Several studies have reported that conventional liposomes only enhanced the drug
deposition in the skin, suggesting that they are only useful for dermal delivery (24–28). However, others have suggested conventional liposomes being suitable for transdermal delivery of some drugs (29–31). These inconsistent results can, at least in part, be attributed to several possible discrepancies in different studies, including different liposomal composition and physicochemical characteristics, different vesicle preparation methods, different types of drugs loaded, different assessment methods applied, different skin samples adopted, and different application procedures administered. Although it is controversial whether conventional liposomes are useful to enhance drugs for transdermal delivery, but there is an agreement by specialists in this field that in most cases, conventional liposomes are of little or no value as carriers for transdermal drug delivery studies, because they do not penetrate the skin but remain confined to upper layers of the SC (23) or form a deposit on the surface of the skin. Furthermore, it was found that the thermodynamic state of the membranes (a liquid state or a gel state) plays a vital role in its effectiveness as a skin delivery vehicle, revealing that gel-state vesicles are less effective in increasing drug permeation across the skin than liquid-state vesicles (32–35). Gel-state vesicles can even inhibit drug permeation across the skin. However, other physicochemical properties of the vesicles, such as size, lamellarity, and charge, have a less pronounced effect on the drug permeation (36,37).

**Mechanisms of action of conventional liposomes.** The specific mechanism can fall into one of three categories, including the intact vesicular skin penetration, the penetration enhancing effect, and vesicle adsorption to and/or fusion with the SC (38).

The use of vesicles for transdermal drug delivery has been introduced in 1980 (39). The statement that intact conventional liposomes can penetrate across the skin was received with skepticism. Some studies supported that intact vesicular penetration may be a possible mechanism for improved skin accumulation (27,40), while others indicated that intact liposomes did not penetrate the skin (36,41,42). Realistically, conventional liposomes such as colloids from an aqueous suspension can cross the skin barrier only through hydrophilic pathways (intercellular route or intercorneocyte pathway). However, intact skin contains only an insignificant number of pathways of sufficient width to allow passage of even small colloids. Hence, any colloids that are trying to penetrate through narrow pores of fixed size in the skin have to possess two capabilities: the colloid-induced opening of the very narrow (~0.4 nm) gaps between cells in the barrier to pores with a diameter greater than 30 nm, and self-adapting to the size of 20 to 30 nm without destruction (12). Obviously, conventional liposomes fall short of these prerequisites. As a result, it is almost impossible for large conventional liposomes to penetrate the densely packed SC in great numbers (38).

The penetration enhancing effect was the second possible mode of action that was described in 1987 (43) and supported by others (42,44,45). They suggested that liposome lipids may act as penetration enhancers, thereby loosening the lipid structure of the SC and promoting an impaired barrier function (46). However, negative findings have also been reported (47–49), claiming that the penetration enhancing effect was invalid and liposomes must be applied concomitantly with the drug or the drug must be encapsulated within them (48).

The mechanism of vesicle adsorption to and/or fusion with the SC also was reported by others (44,50,51), suggesting that liposome lipids penetrated...
into the SC by adhering onto the surface of the skin and subsequently destabilizing and fusing or mixing with the lipid matrix (51). However, the collapse of vesicles on skin surface may form an additional barrier, reducing the permeation of hydrophilic molecules encapsulated in the vesicular aqueous core (52).

In summary, it can be concluded that conventional liposomes do not enter the SC as intact entities, and the vesicle-skin interactions could occur either at the skin surface or in the deeper layers of the SC, which will depend on the compositions and the physicochemical properties of the liposomes.

**Ultraflexible Liposomes as Transdermal Delivery Systems**

*General and the effect of ultraflexible liposomes on transdermal drug delivery.* The ultraflexible liposomes, first introduced in 1992, are a novel type of liquid-state modified liposomes named transfersomes and consist of phospholipids and an edge activator (53,54). At the same time, a new physical parameter was introduced, namely the elasticity of the vesicles bilayers (53). The presence of an edge activator destabilizes the lipid bilayer of the lipid vesicles and increases the deformability of the bilayer by lowering its interfacial tension. Hence, both the type and concentration of the surfactants are most important in the formulation of transfersomes. These edge activators employed include sodium cholate, sodium deoxycholate, Span 60, Span 65, Span 80, Tween 20, Tween 60, Tween 80, and others. Moreover, for the ultraflexible liposomes, the mode of application also plays a crucial role in the vesicles-skin interactions. A few studies compared the difference between occlusive and nonocclusive application of ultraflexible liposomes, revealing that occlusive application of these vesicles was less effective than the nonocclusive one (53,55). However, these results were a little bit of unexpected because water has been reported to be an effective permeation enhancer (56). According to the literature (53), the water gradient has been suggested to be an important driving force for the ultraflexible liposomes. As a result, driven by the osmotic gradient across the skin, these elastic vesicles nonocclusively applied could follow the local hydration gradient and “squeeze” through the SC. But contrarily, occlusion would dispel this osmotic gradient (53,57).

The effectiveness of ultraflexible liposomes as transdermal delivery systems has been successfully demonstrated by using numerous chemical agents of different molecular sizes and lipophilicity, such as lidocaine, tetraacaine, cyclosporin, hydrocortisone, dexamethasone, triamcinolone acetonide, diclofenac, ibuprofen, tamoxifen, testosterone, etc. (58). Furthermore, it has also been reported that ultraflexible liposomes were able to deliver macromolecules and proteins through the skin in preclinical experiments with a significant systemic biological activity of these biomolecules, including calcitonin (58), insulin (59), interferon (60,61), gap junction protein (62), and others (63-66). Hence, it was even suggested that ultraflexible liposomes can be used as possible carriers for noninvasive gene delivery and transcutaneous immunization.

Although most of these results indicated that ultraflexible liposomes can improve transdermal drug delivery, some studies suggested that ultraflexible liposomes were more useful for dermal than for transdermal delivery in the case of ketotifen (67) and that the use of ultraflexible liposomes would even be restricted for dermal delivery of a few drugs (68,69). Furthermore, it is notable that ultraflexible liposomes are quite effective under in vivo conditions because in vitro transport rates are much lower than in vivo (52,70).
Mechanisms of action of ultraflexible liposomes. There are two possible mechanisms responsible for the enhanced skin drug delivery via ultraflexible liposomes (71,72). First, ultraflexible liposomes may act as drug carrier systems by which intact vesicles enter the SC carrying vesicle-bound drug into or across the skin (52). Direct supporting results have been published (57,73). Additional studies have shown that pretreatment of skin membranes with empty deformable liposomes did not enhance estradiol flux, while application of estradiol entrapped within vesicles resulted in a 14- to 17-fold increase in estradiol flux relative to control (74). A reduction in vesicle size improved the deposition and penetration of two fluorescently labeled model substances with large structures improving the deposition only (75), and epidermal permeation enhancement via the vesicular components in a lipid solution in 90% propylene glycol in water was inferior (49), suggesting that ultraflexible liposomes may act as drug carriers and are better for lipid components to be applied in the form of vesicles (49). However, in case of accepting this mechanism, as mentioned before, two prerequisites are necessary: the sufficient carrier stability on the skin and hydrophilic pore opening in the skin by such vesicles (12). This is why only well-designed ultraflexible liposomes can overcome the skin barrier posed by the SC. On the other hand, the water gradient was regarded as the driving force for ultraflexible liposomes entering the skin (53). However, the water gradient across the skin may not be linear, consequently, as a result of the osmotic force such vesicles will not penetrate beyond the level of the lowest layers in the SC (76). Hence, the drugs will be released first from such vesicles and then penetrate alone to reach the systemic circulation (72).

Second, vesicles may act as penetration enhancers, whereby membrane bilayers as vesicles interact with the SC and subsequently modify the intercellular lipid lamellae. It has been shown that deformable liposomes were able to carry both the entrapped and the nonentrapped carboxyfluorescein (albeit to a lesser extent) into the SC and possibly to deeper layers (77), enoxacin permeation across lecithine-treated skin was higher than that across nontreated skin after 12 hours of pretreatment (78), indicating that this mechanism is also reasonable.

Hence, most possibly, both mechanisms play a role in the enhanced transdermal delivery of drugs by ultraflexible liposomes under nonocclusive conditions. It is possible that one of the two mechanisms might predominate according to the physicochemical properties of the drug considered (52).

Ethosomes as Transdermal Delivery Systems
General and the effect of ethosomes on transdermal drug delivery. Ethosomes are another novel permeation-enhancing lipid vesicles (79) that are composed of phospholipid and a high concentration (20–45%) of ethanol and water. The effect of ethanol and its concentration on the physicochemical characteristics of the ethosomes have been investigated (67,79,80). As reported, ethanol can confer a surface negative net charge to the liposome, which in turn causes the size of vesicles to decrease (79,81). The decrease of ethanol concentration in the range of 20% to 45% can result in the increase in the size of ethosomes (79). Moreover, due to the multilamellarity of ethosomes (79), the presence of ethanol in ethosomes, and the solubility of many drugs, ethosomes can exhibit high encapsulation efficiency and drug loading.
Because of their unique structure, ethosomes can entrap and effectively deliver highly lipophilic molecules and cationic drugs such as acyclovir, trihexyphenidyl, minoxidil, cannabinoids, zidovudine, and testosterone through the skin (22,79,82). More interestingly, unlike ultraflexible liposomes, ethosomes are able to improve the skin delivery of drugs both under occlusive and non-occlusive conditions (67,79,81,83), depicting a different mechanism of action for ethosomes.

Mechanisms of action of ethosomes. Although the exact process of skin drug delivery by ethosomes remains unclear, it appears that a synergistic mechanism between ethanol, lipid vesicles, and skin lipids facilitates drug delivery to the deeper skin layers or across the skin (79,80,84).

At first, ethanol must be responsible for the enhanced skin drug delivery described. Ethanol is a well-known permeation enhancer. It can interact with intercellular lipid molecules in the polar head group region, thereby increasing their fluidity and decreasing the density of the lipid multilayer, which results in an increase in membrane permeability. Ethanol is also supposed to extract the SC lipids and these penetration enhancement effects may be referred to as “pull-effects” (85). In addition, ethanol imparts flexibility to the ethosomal vesicles, which in turn facilitates skin permeation. Furthermore, ethanol can act as a “blending” agent (86) for lipid vesicles, increasing their distribution in various skin layers.

The ethanol effects are followed by the interaction between ethosome vesicles and the skin. The interdigitated, malleable, ethosomal vesicles behave as ultraflexible liposomes and can interact with the skin barrier to “forge” penetration or permeation pathways by itself in the highly organized SC and finally release drug at various points along the penetration pathway as well as in deep skin layers (52,82).

Liposomes for the Protection of Damaged Skin

The barrier function of damaged skin is diminished in comparison to healthy skin. Therapeutic agents may have an easier access to damaged skin. Damaged skin could use liposomes consisting of skin lipids to fill the damaged skin area.

Higher drug concentrations can be established in this damaged skin by liposomes, either by penetrating deeper into the damaged skin or by depositing drug on the top of the damaged area, thereby protecting the damaged area by spreading the liposomal lipids on top. These synergizing effects may explain the beneficial effects of liposomes in reducing the toxicity of herbal and drug compounds encapsulated in liposomes (87).

One of the first drugs in a commercialized liposomal formulation for topical administration was an econazole liposome gel. A comparative study revealed that, in contrast to the commercial cream formulation, the liposome gel formulation had less toxic effects and no candidosis was detected in a reconstructed human epidermis model of human cutaneous candidosis (88).

Substances that can be degraded on the way to the target cells in the damaged skin can be protected by encapsulating them in liposomes, which is important for proteins and nucleic acids. A recent study on xeroderma pigmentosum, where a DNA repair enzyme mechanism (endonuclease) was applied in a liposomal formulation, demonstrated protection (89,90).
effect of superoxide dismutase as racidal-capturing agent was enhanced when encapsulated in liposomes and spread onto the wounds in an animal model (91). IL-13 antisense oligonucleotides were formulated with cationic flexible liposomes, and in a murine model a significant improvement was seen in atopic dermatitis skin (92). By the additional use of DMSO as a penetration enhancer, liposomes served as vectors in gene therapy through intact chicken skin (93). In addition, the general protection of the skin by antioxidants, such as α-tocopherol, has enhanced using liposomes in an in vitro model (94).

Doxorubicin liposomal formulations administered intravenously did not cause as much skin lesions as the free form of doxorubicin because the liposomal administered doxorubicin did not extravasate—and did not go into the skin—as strongly as the free doxorubicin (95). The same findings were reported for vincristine (96). In both formulations less skin irritation was seen at the site of injection.

In summary, whatever mechanism may be involved in the interaction or penetration of liposomes into the skin, beneficial effects were seen in some compounds. It is recommended that for each new drug intended for topical application, the liposomal formulations should be screened for better efficacy and/or a reduced toxicity.

REFERENCES

Skin Delivery of Ferulic Acid from Different Vesicular Systems

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Skin Delivery of Ferulic Acid from Different Vesicular Systems

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The aim of the present research is to evaluate the skin delivery capabilities of different vesicular systems, including conventional liposomes (CL), Tween 80-based deformable liposomes (DL), invasomes (INS) and ethosomes bearing ferulic acid (FA) being an antioxidant exhibiting a wide range of therapeutic effects against various diseases. All of the test formulations were characterized for particle size distribution, \( \zeta \)-potential, vesicular shape and surface morphology, \textit{in vitro} human skin permeation and skin deposition. Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) defined that all of liposomal vesicles were almost spherical, displaying unilamellar structures with low polydispersity (PDI < 0.2) and nanometric size range (\( z \)-average no more than 150 nm). In addition, all the vesicular systems except conventional liposomes were negatively charged to a certain extent. \textit{In vitro} skin permeation and skin deposition experiments demonstrated that the permeation profile of ferulic acid through human stratum corneum epidermis membrane (SCE) and the drug deposition in skin were both improved significantly using these vesicular liposomal systems. Permeation and skin deposition enhancing effect was highlighted by the ethosomal system containing 18.0 mg/ml of ferulic acid with a significantly (\( P < 0.01 \)) enhanced skin flux (267.8 \( \pm \) 16.77 \( \mu \)g/cm²/h) and skin drug deposition (51.67 \( \pm \) 1.94 \( \mu \)g/cm²), which was 75 times and 7.3 times higher than those of ferulic acid from saturated PBS (pH 7.4) solution, respectively. This study demonstrated that ethosomes are promising vesicular carriers for delivering ferulic acid into or across the skin.

Keywords: Ferulic Acid, Skin Delivery, Liposomes, Invasomes, Ethosomes, \textit{In Vitro} Skin Permeation, Skin Deposition.

1. INTRODUCTION

There is always a lurking but reasonable expectation from both public and scientific community that many human diseases could be treated by phytochemicals derived from dietary components. Ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-Propenoic acid, \( C_{10}H_{10}O_4 \), FA) (Fig. 1), is present in the cell walls of numerous plants, including rice, wheat, barley, oat, vegetables, citrus fruits and leaves, and many other plants.\(^1\) Nowadays, Ferulic acid is receiving greater attention in science because it has an established record of therapeutic effects against various diseases such as anti-skin disorders, anti-cancer, anti-ageing, anti-diabetes, anti-cardiovascular disease, neuroprotection, anti-inflammatory and others.\(^2\) This arises mainly from its antioxidant property by virtue of its phenolic nucleus and unsaturated side chain forming a resonance-stabilized phenoxy radical (Fig. 2). The therapeutic concentration varies with the diseases under consideration; for anti-oxidant effective concentration.

Ferulic acid (FA) is the main effective constituent in a few of traditional Chinese medicinal herbs, such as \textit{Dang gui} (Radix Angelica sinensis), \textit{Chuan xiong} (Ligusticum chuanxiong Hort.) and \textit{A wei} (Ferula sinkiangensis K. M. Shen). These Traditional Chinese herbs have been widely used for the prevention and treatment of many diseases since antiquity in China, such as headaches, abdominal ache, chest pain, muscle pain, boils, difficulty in menses, amenorrhea and rids of blood stasis.\(^3\) In recent years FA has been already formulated into injection, infusion and tablets in Chinese market to treat cardiovascular diseases. But, due to its short elimination half-life (\( t_{1/2} < 15 \) min) after intravenous or intragastric administration, it needs to be administered quite frequently resulting in reduction of patients’ compliance.\(^3\) Hence, transdermal delivery of FA is expected to be a better alternative to traditional administration routes. This assumption is supported by the fact, that because of its imparting cutaneous benefits,\(^4\) FA is
also found as an active ingredient in a variety of cosmetics products such as facial moisturizer/treatment, anti-aging, skin fading/lightener, facial cleanser. Together with its satisfying physico-chemical properties for skin delivery (small molecular weight: 194.2 Dalton; its amphiphilic nature as having a carboxyl group attached via a short hydrocarbon group to a lipophilic phenolic nucleus; a logPoct of 1.51 and a solubility of FA in PBS (pH 7.4) 7.36 ± 0.23 mg/mL), it was tempting for us to try to develop a delivery system for FA.

Despite many attempts to deliver drugs via topical administration, only a few cases were successful. The key problem is the formidable barrier function by the stratum corneum (SC), the outermost layer of the skin. During the past decades there has been wide interest in exploring new approaches to overcome this situation. These approaches include the use of chemical penetration enhancers, adjustment of chemical potential of the drug,7 driving molecules into or through the tissue employing iontophoresis or physically disrupting the skin structure, for example by electroporation or sonophoresis10 and other means.11,12 Vesicular drug delivery systems such as conventional liposomes,13 ethosomes14 and transfersomes also offer a promising strategy for achieving the purpose of improved skin drug delivery. It should be pointed out that the great diversity of potential composition of these vesicles influences their physicochemical characteristics such as particle size, charge, thermodynamic phase and bilayer elasticity, which in turn have a significant effect on the interaction between vesicles and the skin and hence on the effectiveness of these vesicles as transdermal delivery systems.

The present work focuses on developing a suitable vesicular system for the skin delivery of ferulic acid and investigating the influence of different vesicular systems on the percutaneous absorption of FA.

2. MATERIALS AND METHODS

2.1. Chemicals and Instruments

Lipoid S 100 (Phosphatidylcholine from soybean lecithin) was purchased from Lipoid GmbH, Germany. NAT 8539 (Soybean phosphatidylcholine (PC) dissolved in ethanol containing: 77.3% phosphatidylcholine, 5% lysophosphatidylcholine, 3% cephaline and 1.1% phosphatidic acid of the dry residue) was purchased from Nattermann Phospholipid GmbH, (Hermersberg, Germany). Ferulic acid was purchased from Sigma-Aldrich, (Steinheim am Albuch, Germany). DPPG (1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol) was purchased from Genzyme Pharmaceuticals, Sygena Facility, (Liestal, Switzerland). Limonene, Citral and Cineole were purchased from Sigma-Aldrich, (Steinheim am Albuch, Germany). All other solvents used in this study were of analytical grade and were purchased from Merck, (Darmstadt, Germany).

2.2. Preparation of Vesicular Formulations

All vesicular systems were prepared by a conventional rotary evaporation method.17,18 Briefly, the appropriate weight of lipid or lipids and FA (the compositions of different vesicular systems are shown in Table I) were dissolved in methanol/chloroform solution (1:2, v/v) in round bottom flask. Thin lipid films were obtained by removing the organic solvents under vacuum condition (500 mbar 10 min, 200 mbar 10 min, 100 mbar 10 min, 35 mbar 1 hr) at a temperature of 43 °C with a rotary evaporator. The resulted dry lipid films on the inside wall of round bottom flask were hydrated and dispersed with different hydration systems corresponding to all formulations (Table I) at room temperature. The obtained macroscopically homogenous solution was sonicated for totally 15 minutes in 3 cycles (5 min for each cycle and 5 min pause among these cycles) with a sonication ice-water bath. The obtained suspensions were extruded through polycarbonate membrane (diameter: 19 mm, pore diameter: 100 nm, Armatis, Germany) 21 times to produce liposomes of the desired size with the help of a Hamilton-Bonaduz extruder (GASTIGHT#1001, Switzerland).
2.3. Characterization of Vesicular Systems

2.3.1. Particle Size Distribution and ζ-Potential Measurement

The particle size, polydispersity index (PDI) and ζ-potential of the vesicular systems were determined by photon correlation spectroscopy (Zetasizer Nano series, Malvern Instruments Ltd., Worcestershire, UK). Before the size and ζ-potential measurements, the samples were diluted 50-times with different solvent systems corresponding to their compositions (Table I), which were also filtered through polycarbonate membrane (Minisart® syringe end filters 0.2 μm, Sartorius AG, Goettingen, Germany). Measurements were made at 25 °C with a fixed angle of 137°. Sizes quoted are the z-average mean for the hydrodynamic diameter. Calculation of ζ-potential (mV) was done by the instrument software from the measured electrophoretic mobility.

2.3.2. Visualization by Cryo-Transmission Electron Microscopy

The different vesicular systems (CL, DL, INS and ETS-2) were visualized by cryo-electron microscopy, which allowed beside shape investigation also the measurement of the lipid lamellarity. 5 μL of test formulation were placed on a perforated copper grid (Quantifoil R 1.2/1.3) and excess liquid was blotted automatically for two seconds between two pieces of filter-paper-strips. Shortly after that, the samples were frozen by rapidly plunging in liquid ethane (cooled to 170−180 °C) in a cryo box (Carl Zeiss NTS GmbH, Germany). Excess ethane was removed with a piece of filter paper in the cryo box. The sample was transferred with the liquid nitrogen cooled holder (Gatan 626, USA) into the pre-cooled cryo-electron microscope (Phillips CM 120, Netherlands) operated at 120 kV and viewed under low dose conditions. The Images were recorded with a 1 k CCD Camera (FastScan F114, TVIPS, Gauting, Germany).

2.4. Skin Preparation

Female human abdominal skin tissue from plastic surgery was used. The stratum corneum/epidermis (SCE) membranes were prepared by a heat separation technique. After removal of excess fat and connective tissue, the skin was soaked for 2 min in a water bath at 60 °C. Then the epidermis was carefully peeled away from the dermis. The SCE membranes were dried in desiccators at ~25% relative humidity and frozen at −20 °C for later use up to 3 months after storing. Before use, the epidermis membranes were floated with the stratum corneum side up on receptor medium and the upper surface was left open to the atmosphere overnight at 4 °C. After verifying of the integrity by transepidermal water loss (TEWL) measurements (VapoMeter, Delfin Technology Ltd., Kuopio, Finland) it was also ensured, that samples were free from any surface irregularity such as tiny holes or crevices in the portion that was used for the permeation studies.

2.5. In Vitro Skin Permeation and Deposition Studies

In Vitro skin permeation and deposition experiments of the vesicular systems containing FA were run in Franz diffusion cells with the formulations applied in a non-occlusive way; the skin surface temperature was kept at 32 °C throughout the experiment. The effective permeation area and receptor cell volume was 0.64 cm² and 5.4 ml, respectively.

The design in the present study was the same as described by El Maghraby et al. Experiments were performed in two stages. The first stage was for determination of the drug permeating across the skin. In this stage, PBS buffer pH 6.0 was used as the receptor medium. Skin membranes were mounted, with the stratum corneum side...
up and the donor compartment dry and open to atmosphere for 0.5 hr. 100 μl of the test formulations were applied to skin surface. 0.5 ml of the samples were removed from receptor cells at appropriate intervals (at 0, 1, 2, 3, 4, 5, 6 and 8 hr) for the concentration measurement by fluorescence assay using a Fluostar (Optima Microplate Reader, BMG LABTECH GmbH. Offenburg, Germany) and immediately replaced with fresh medium. At the end of this stage (8 h), the donor compartment and the skin surface were washed five times with warm (45 °C) receptor medium.

The second stage was employed to determine the deposited drug in skin. The skin samples were transferred into special tubes loaded with 0.55 gm of ceramic pellets. For the extraction of FA 1 ml of ethanolic solution (50%, v/v) was added to each tube. The skin was homogenized by a Precellys S24 device (Berlin Technology, Bad Wildbad, Germany) with the following settings: 6000 rpm, 3 cycles each 30 s. The dispersions were ultra-centrifugated (10 min, 10000 rpm, Minispin®, Eppendorf, Germany) to remove remaining skin debris. The supernatant was collected, diluted with ethanol solution (50%, v/v) and analyzed by fluorescence measurement. Each vesicular system was investigated in triplicate.

FA flux through the skin was calculated by plotting the cumulative amount of drug permeating across the skin against time and determining the slope of the linear portion of the curve. The x-axis-intercept values (lag time) by linear regression analysis. Drug flux (μg/(cm²*h)), at steady-state, was calculated by dividing the slope of the linear portion of the curve by the area of the skin surface covered with formulation. Statistical analysis of data was performed using Student’s t-test.

The effectiveness of different vesicular systems to deliver FA across or into the skin was determined by comparing FA flux and skin deposition between the different test formulations and PBS (pH 7.4) saturated solutions of FA as standard, these ratios were defined as the enhancement ratio of flux (ERflux) or as the enhancement ratio of skin deposition (ERdeposition):

\[ \text{ER}_{\text{flux}} = \frac{\text{ferulic acid flux at steady-state from test formulation}}{\text{ferulic acid flux at steady-state from PBS (pH 7.4) saturated solution}} \] (1)

\[ \text{ER}_{\text{deposition}} = \frac{\text{ferulic acid skin deposition from test formulation}}{\text{ferulic acid skin deposition from PBS (pH 7.4) saturated solution}} \] (2)

2.6. Fluorescent Assay of FA

The concentration of FA was determined by fluorescence spectroscopy method. Fluorescence detection was performed at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The method was validated for linearity, accuracy and precision.

2.7. Data Analysis

All reported data are mean ± S.E.M. Statistical significance was checked by Student’s t-test and considered to be significant at \( P < 0.05 \), unless indicated using Minitab® Ver.14 software.

3. RESULTS

3.1. Particle Size Distribution and ζ-Potential

The results of particle size distribution and ζ-potential of the investigated vesicular systems are summarized in Table II. Regarding vesicle size, the largest vesicles of 130.3 ± 4.7 nm (n = 3) were present in conventional liposome formulations, while Tween 80-based deformable liposomes (93.4 ± 1.6 nm), ethosomes (88.9 ± 0.7 and 92.1 ± 3.0 nm (n = 3)) in the case of ethosomes containing 12.0 mg/ml and 18.0 mg/ml of FA, respectively) had significantly (\( P < 0.01 \)) lower mean vesicle sizes relative to conventional liposomes. All vesicular systems exhibited low polydispersity index (PDI) values (PDI < 0.2), indicating that all of them were highly homogeneous suspensions.

The ζ-potential is related to the charge on the surface of the vesicle influencing vesicular properties such as stability and skin–vesicle interactions. Conventional liposomes as prepared in this study were found to possess a ζ-Potential of 2.9 ± 0.4 mV (n = 3). Tween 80-based deformable liposomes exhibited a ζ-Potential of −6.3 ± 0.1 mV (n = 3). Invasones also exhibited a negative ζ-Potential of −39.7 ± 2.0 mV (n = 3). This result is in reasonable agreement with other data on invasones from our group.25,26 Ethosomes containing 12 and 18 mg/ml of FA showed similar ζ-Potentials of −37.3 ± 1.6 and −30.2 ± 0.9 mV (n = 3), respectively. In this case, incorporation of ethanol, as well as DPPG, a negatively-charged phospholipid,27 is expected to produce negatively charged vesicles.

3.2. Morphology of Different Liposomal Vesicles

Cryo-transmission electron microscopy was used to visualize vesicles, and to study the shape and lamellarity of

Table II. Characterization of different vesicular systems containing ferulic acid.

<table>
<thead>
<tr>
<th>Code</th>
<th>Particle size (nm)</th>
<th>PDI*</th>
<th>ζ-Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL†</td>
<td>130.3 ± 4.7</td>
<td>0.110 ± 0.008</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>DL‡</td>
<td>93.4 ± 1.6</td>
<td>0.044 ± 0.013</td>
<td>−6.3 ± 0.1</td>
</tr>
<tr>
<td>INS†</td>
<td>129.1 ± 1.3</td>
<td>0.112 ± 0.007</td>
<td>−39.7 ± 2.0</td>
</tr>
<tr>
<td>ETS-1†</td>
<td>88.9 ± 0.7</td>
<td>0.082 ± 0.025</td>
<td>−37.3 ± 1.6</td>
</tr>
<tr>
<td>ETS-2†</td>
<td>92.1 ± 3.0</td>
<td>0.088 ± 0.022</td>
<td>−30.2 ± 0.9</td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n = 3). *Conventional liposomes; †Tween 80-based deformable liposomes; ‡Invasones; §Ethosomes containing ferulic acid (12.0 mg/ml); ¶Ethosomes containing ferulic acid (18.0 mg/ml). Polydispersity index; * \( p < 0.01 \) in comparison with Conventional liposomes (CL);
all vesicular systems, including CL, DL, INS, and ETS-2. From the results (Fig. 3), the vesicles of the conventional liposomes seemed to be mostly unilamellar (Figs. 3(a and b), black light arrows) with rare occasions of bilamellarity (Figs. 3(a and b), black thick arrows). In the case of Tween 80-based deformable liposomes, the vesicles were also mostly unilamellar (Figs. 3(c and d), black light arrows) and bilamellar (Figs. 3(c and d), black thick arrows) and a small percentage of oligolamellar vesicles (Fig. 3(c), black long length arrow). Regarding Invasomes, the vesicles seemed to be also unilamellar (Fig. 3(f), black short length arrow) and some bilamellar (Fig. 3(f), black thick arrows), but the percentage of deformed vesicles increased dramatically in comparison to other liposomal structures (Figs. 3(e and f), white light arrows). In comparison to other vesicles, ethosomal vesicles appeared to be homogenously unilamellar (Figs. 3(g and h), black light arrows).

### 3.3. In Vitro Skin Permeation and Skin Deposition Studies

The abilities of different liposomal systems and related control groups to deliver FA were investigated by determining the flux of FA across SCE ($J_{ss}$), lag time (LT) and the quantity of skin deposition of the drug (QSD). The results are summarized in Table III. The cumulative amount of FA permeated per unit area across SCE via various formulations was plotted as a function of time (Fig. 4). From the results, all the elastic vesicular systems including Tween 80-based deformable liposomes (DL), Invasomes (INS) and ethosomes (ETS-1 and ETS-2) showed a better permeation profile than the PBS saturated solution group (CLT-3). Furthermore, it could be clearly demonstrated that the amount of FA permeated through the human SCE skin in vitro from ethosomes containing 18 mg/ml of FA (ETS-2) was significantly ($P < 0.01$) higher than that from all other formulations. The flux from ethosomes containing 18 mg/ml of FA (ETS-2, 267.8 ± 16.77 µg/cm²/h) was 7-fold higher than that obtained after application of 4% phospholipid in ethanol solution (CTL-2, 33.20 ± 9.17 µg/cm²/h), 11-fold higher than that of the ethanol solution of drug (CTL-1, 20.62 ± 1.28 µg/cm²/h), 75-fold higher than that of the drug saturated solution in PBS (pH 7.4) (CTL-3, 3.58 ± 2.69 µg/cm²/h), 9-fold higher than that of conventional liposomes (CL, 29.34 ± 9.78 µg/cm²/h), 5-fold higher than that of Tween 80-based deformable liposomes and invasomes (DL, 52.00 ± 12.90 µg/cm²/h and INS, 54.57 ± 13.86 µg/cm²/h) and 2.5-fold higher than that of ethosomes containing 12 mg/ml of FA (ETS-1, 115.0 ± 11.21 µg/cm²/h). All the profiles were suitable to be fitted by a zero-order equation, and all showed the typical time lag of skin delivery, which represents the time required to establish steady state diffusion and corresponds

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**Fig. 3.** Visualization of different liposomal systems containing ferulic acid by cryo-transmission electron microscopy. (a, b) conventional liposomes containing 12 mg/ml of ferulic acid; (c, d) Tween 80-based deformable liposomes containing 12 mg/ml of ferulic acid; (e, f) invasomes containing 12.0 mg/ml of ferulic acid; (g, h) ethosomes containing 18.0 mg/ml of ferulic acid. Black light arrows represent unilamellar vesicles; black thick arrows represent bilamellar vesicles, while white thick arrows represent oligolamellar vesicles, white small arrows represent deformed vesicles.
to the nonlinear portion of a permeation profile. Among all formulations, the elastic liposomes showed a significantly smaller \((P < 0.01)\) time lag, ranging from 0.9 hr to 1.8 hr, as compared with the lag time (2.9 hr) of PBS saturated solution (CTL-3), indicating that elastic liposomes could reduce the needed time for delivery systems to reach the steady state. Moreover, the ethanol solution of drug (CTL-1) also showed relatively smaller \((P < 0.05)\) time lag, suggesting that the presence of ethanol in delivery system reduces the equilibration time in the skin layer investigated.

Regarding skin deposition studies, both conventional liposomes (CL) and elastic liposomal systems (DL, INS, ETS-1 and ETS-2) led to better drug deposition in the skin (epidermis) layer. The enhanced skin depositions of different formulations were clearly illustrated in Table III and Figure 5. As shown, the ethosomes containing 18 mg/ml of FA (ETS-2) also induced the highest skin deposition (51.67 ± 1.94 μg/cm²) of FA. In contrast, the skin deposition of ferulic acid from PBS saturated solution group (CL-T3) was only 7.08 ± 0.27 μg/cm². Among other investigated formulations, the conventional liposomes showed the second highest skin deposition, 34.61 ± 0.67 μg/cm².

### Table III. Permeation and skin deposition parameters of ferulic acid via different formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>IJ(\text{s}^\text{b} \quad \mu\text{g/cm}^2\text{/h})</th>
<th>LT(\text{c}^\text{b} \quad \text{hours})</th>
<th>Pm(\text{d}^\text{b} \quad \text{cm}^3/\text{h} \times 10^{-3})</th>
<th>ER(\text{e}^\text{b} \quad % \text{ deposition})</th>
<th>QSD(\text{f}^\text{b} \quad \mu\text{g/cm}^2 \quad \text{steady state})</th>
<th>ER(\text{f}^\text{b} \quad % \text{ deposition})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL(\text{g}^\text{b} \quad \text{control group})</td>
<td>29.34 ± 9.78</td>
<td>2.8</td>
<td>0.3</td>
<td>8.2</td>
<td>34.61 ± 0.67</td>
<td>4.2</td>
</tr>
<tr>
<td>DL(\text{h}^\text{b} \quad \text{Tween 80-based deformable liposomes})</td>
<td>52.00 ± 12.90</td>
<td>1.2*</td>
<td>0.5</td>
<td>14.5</td>
<td>15.77 ± 0.40</td>
<td>2.2</td>
</tr>
<tr>
<td>INS(\text{i}^\text{b} \quad \text{Invasomes})</td>
<td>54.57 ± 13.86</td>
<td>1.8*</td>
<td>0.6</td>
<td>15.2</td>
<td>21.31 ± 0.46</td>
<td>3.0</td>
</tr>
<tr>
<td>ETS-1(\text{j}^\text{b} \quad \text{ethosomes containing ferulic acid (12.0 mg/ml))}</td>
<td>115.0 ± 11.21</td>
<td>0.9*</td>
<td>1.2</td>
<td>32.1</td>
<td>23.23 ± 1.04</td>
<td>3.3</td>
</tr>
<tr>
<td>ETS-2(\text{k}^\text{b} \quad \text{ethosomes containing ferulic acid (18.0 mg/ml))}</td>
<td>267.8 ± 16.77*</td>
<td>1.7*</td>
<td>1.9</td>
<td>74.7</td>
<td>51.67 ± 1.94*</td>
<td>7.3</td>
</tr>
<tr>
<td>CTL-1(\text{l}^\text{b} \quad \text{ethosomes containing ferulic acid (12.0 mg/ml))}</td>
<td>20.62 ± 1.28</td>
<td>2.2*</td>
<td>0.2</td>
<td>5.8</td>
<td>5.98 ± 0.17</td>
<td>0.8</td>
</tr>
<tr>
<td>CTL-2(\text{m}^\text{b} \quad \text{ethosomes containing ferulic acid (12.0 mg/ml))}</td>
<td>33.20 ± 9.17</td>
<td>2.7</td>
<td>0.3</td>
<td>9.3</td>
<td>17.84 ± 0.34</td>
<td>2.5</td>
</tr>
<tr>
<td>CTL-3(\text{n}^\text{b} \quad \text{ethosomes containing ferulic acid (18.0 mg/ml))}</td>
<td>3.58 ± 2.69</td>
<td>2.9</td>
<td>0.1</td>
<td>1.7</td>
<td>7.08 ± 0.27</td>
<td>—</td>
</tr>
</tbody>
</table>

Values represent mean ± SD \((n = 3)\). *Conventional liposomes; *Twine 80-based deformable liposomes; *Invasomes; *Ethosomes containing ferulic acid (12.0 mg/ml); *Ethosomes containing ferulic acid (18.0 mg/ml); \(P < 0.01\) in comparison with all other formulations; *Steady state transdermal flux; *Lag time; *Permeability coefficient; *Enhancement ratio of skin flux from formulation to PBS saturated drug solution; *Quantity of skin deposition; *Enhancement ratio of amount of drug deposition from formulation to PBS saturated drug solution; *Control group; *\(p < 0.01\) in comparison with control group 3; *\(p < 0.05\) in comparison with control group 3.

4. DISCUSSION

4.1. Particle Size Distribution and ζ-Potential

One of the reasonable explanations about the reduced size of elastic liposomal systems is the presence of ethanol in these formulations. Ethanol could probably reduce the membrane thickness due to the formation of a phase with interpenetrating hydrocarbon chains,28,29 Furthermore, the addition of ethanol in phospholipid vesicles imparts negative charge to the formulation,14 which also explains there net change flip over from conventional liposomes to other vesicles with fluid bilayers with the presence of ethanol. This modification of net charge of liposomal systems confers lipid vesicles some degree of steric stabilization and in turn lead to decrease in mean vesicle size.30 All of these mechanisms suggested that the ethanol possess some condensing ability for lipid vesicles. Moreover, in case of Tween 80-based deformable liposomes, reduction of particle size could also be explained by inclusion...
of the surfactant.31 While in case of invasomes, lysophosphatidylcholine (LPC), as one of the components of the NAT 8539, has a single hydrophobic chain and a polar head group and integrates spontaneously into membrane bilayers, is acting as a surfactant and creating a high positive curvature in membranes.32 However, the inclusion of 1% (w/v) terpene mixture could lead to the increase of particle size distribution 129.1 ± 1.3 nm (n = 3).26 The effect of surface charge of liposomes on the drug penetration has not been fully understood so far. Some researchers support the theory that the positive charges on the surface of liposomes could bind to negative charges of the SC enhancing the drug penetration/permeation through the skin.33,34 However, other studies found that permeation of drugs through the skin is promoted by negatively charged vesicles.35,36 In our previous study (data not shown here), the flux of FA from negatively charged ethosomes is somewhat higher than from positively charged ethosomes, but without any significant difference. In case of amphiphilic drug such as FA, we suggested that the surface charge of the vesicles will not only play a role on the interaction between skin and vesicles but also might contribute to the drug release from the vesicles. The drug release from vesicles in the stratum corneum is an important step which will affect transdermal flux.37 The rate and amount of released drug is a balance between two factors: (1) drug affinity to vesicles, and (2) drug solubility in lipids of the stratum corneum.37 Since the pK\textsubscript{a} of Ferulic acid\textsuperscript{38} is 4.52, it is negatively ionized at pH = 7.4 and therefore it could be retained in the positively charged vesicles, which in turn influences the skin penetration or permeation.

4.2. Morphology of Vesicular Systems

Since all of the vesicular systems were prepared with an extrusion procedure as described, the vesicles from different liposomal systems are supposed to be mostly unilamellar, which already have been confirmed from the results in this study. Furthermore, the increase of deformed vesicles in invasomes suspensions can be explained as the incorporation of 1% of terpenes into the vesicles, which had an influence on the ultraflexibility of these vesicles. This finding is in accordance with the results published in other research of our department.25,26

4.3. In Vitro Skin Permeation and Skin Deposition Studies

Due to the barrier function of the skin, the development of an appropriate drug skin delivery system is particularly important to overcome this situation. In spite of extensive studies conducted concerning the effect of various liposomal systems on percutaneous absorption, their mechanism of action still remains unclear. However, it has to be pointed out that the diversity of potential composition of these liposomal systems could influence their physicochemical characteristics such as particle size, charge, thermodynamic phase, bilayer elasticity, etc., which in turn have a significant effect on the interaction between the particles and the skin and hence on the effectiveness of these structures as transdermal delivery systems.

Conventional liposomes were expected to be effective at delivering drugs into the upper layers of the skin. It is agreed in the recent literature, that in most cases conventional liposomes are of little or no value as carriers for transdermal drug delivery, as they are not penetrating the skin but remain confined to upper layers of the SC\textsuperscript{39} or form a deposit on the surface of the skin. The result in this study supports this notion. The penetration properties of conventional liposomes may fall into one of two possible categories, including the penetration enhancing effect and vesicle adsorption to and/or fusion with the SC.\textsuperscript{40} The first possible mode of action that was described firstly in 1987\textsuperscript{41} and supported by others.\textsuperscript{42-44} This mode suggests that liposome lipids may act as penetration enhancers, thereby loosening the lipid structure of the SC and promoting an impaired barrier function.\textsuperscript{44} The second possible mode for conventional liposomes is adsorption to and/or fusion with the SC,\textsuperscript{31,45,46} suggesting that the liposomal lipids penetrate into the SC by adhering onto the surface of the skin and subsequently destabilizing and fusing or mixing with the lipid matrix.\textsuperscript{46} However, the collapse of vesicles on skin surface may form an additional barrier, reducing the permeation of hydrophilic molecules encapsulated in the vesicular aqueous core.\textsuperscript{47}

Tween 80-based deformable liposomes and Invasomes showed better permeation profiles and enhanced skin deposition of FA compared to PBS saturated solution group (CLT-3). There are two possible mechanisms responsible for the enhanced skin drug delivery via deformable liposomes.\textsuperscript{37,48} First, deformable liposomes may act as drug carrier systems by which intact vesicles enter the SC carrying vesicle-bound drug into or across the skin. Second, vesicles may act as penetration enhancers, whereby the vesicle lipid bilayers interact with the SC and subsequently modify the intercellular lipid lamellae. Most possibly, both mechanisms play a role in the enhanced transdermal delivery of drugs by deformable liposomes under non-occlusive conditions. It is possible that one of the two mechanisms might predominate according to the physicochemical properties of the drug considered.\textsuperscript{47} The improved permeation profile and skin deposition of FA from ethosomal formulations (ETS-2) could be attributed to the lipid vesicle’s characteristics and some kind of synergistic mechanism between ethanol, lipid vesicles and skin lipids.\textsuperscript{14,49,50} Firstly, the high concentration of ethanol within ethosomes demonstrated some ability to condense the size of the bilayer vesicles and induced a charge transition from positive to negative, which was a favorable physicochemical change for transdermal penetration.\textsuperscript{51} Moreover, ethanol is a well-known
permeation enhancer. The penetration enhancing effect of ethanol can be attributed to two effects: (a) ‘Push effect:’ increased thermodynamic activity due to evaporation of ethanol and improved solubility of solute such as that of FA in this study; (b) ‘Pull effect:’ ethanol can interact with intercellular lipid molecules in the polar head group region, thereby increasing their fluidity and decreasing the density of the lipid multilayer, which results in an increase in membrane permeability. Ethanol is also supposed to extract the SC lipids\textsuperscript{52} lowering thereby the barrier function of the SC.

In addition, ethanol imparts fluidity to the vesicle’s bilayers, which in turn facilitates skin permeation. Furthermore, ethanol can act as a “blending” agent\textsuperscript{53} for lipid vesicles, increasing their distribution in various skin layers. The ethanol effects can be followed by the interaction between ethosomal vesicles and the skin. The ethosomal vesicles behave as deformable liposomes and can interact with the skin barrier to “forge” penetration or permeation pathways by itself in the highly organized SC and finally release drug at various points along the penetration pathway as well as in deep skin layers.\textsuperscript{47,54}

On the other hand, the high flux of ethosomes (ETS-2) could be also due to high dosage of FA (18.0 mg/ml) in this formulation, which arise from the enhanced solubility of FA in the hydroethanolic system (27.51 ± 0.21 mg/ml (n = 3), ethanol 45%, v/v, at 25 °C) as compared with that of PBS saturated solution (7.36 ± 0.23 mg/mL (n = 6)).

The deposition of FA in the skin attains for ethosomal delivery values of 51.67 μg/cm², which can be related (assuming a thickness of the heat-separated epidermis of 200 μm) to a concentration of FA in the epidermis of about 13 mmol/L. This concentration is far above the minimal effective concentration for e.g., antioxidant action of FA as inhibiting lipid peroxidation.\textsuperscript{55} The obtained flux through the skin model may be sufficient for systemic action especially in the light of the favorable pharmacokinetics (see Introduction), but this needs to be evaluated in forthcoming animal experiments, as many other parameters besides the skin area treated determine the amount of drug reaching the systemic circulation from the place of application.

5. CONCLUSION

In this study, different liposomal systems containing FA have shown different abilities to deliver the drug across or into skin. Moreover, the ethosomes containing relatively high concentration of FA (ETS-2) delivered a significantly higher amount of FA across the skin and also lead to a better epidermal skin deposition of the drug, which would be sufficiently high for e.g., antioxidant effects. Therefore ethosomes could be a promising carrier for enhanced skin delivery of FA.

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References and Notes

Skin Delivery of Ferulic Acid from Different Vesicular Systems


Skin penetration and deposition of Carboxyfluorescein and Temoporfin from different lipid vesicular systems: In vitro study with finite and infinite dosage application

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Pages in the dissertation: 39-101
Skin penetration and deposition of Carboxyfluorescein and Temoporfin from different lipid vesicular systems:

In vitro study with finite and infinite dosage application

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Abstract: The aim of the present research is to evaluate the influence of different lipid vesicular systems as well as the effect of application mode on skin penetration and deposition behaviors of carboxyfluorescein (hydrophilic model drug) and Temoporfin (lipophilic model drug). All of the lipid vesicular systems, including conventional liposomes, invasomes and ethosomes, were prepared by film hydration method and characterized for particle size distribution, $\zeta$-potential, vesicular shape and surface morphology, in vitro human skin penetration and skin deposition. Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) defined that all of lipid vesicles had almost spherical structures with low polydispersity (PDI<0.2) and nanometric size range (z-average no more than 150 nm). In addition, all lipid vesicular systems exhibited a negative zeta potential. In vitro skin penetration and deposition experiments demonstrated that, in the case of CF with finite dose application (10 µL/cm²) and infinite dose application (160 µL/cm²), lipid vesicular systems, especially ethosomes and invasomes, compared with non-vesicular systems, can significantly improve the delivery of hydrophilic drug such as carboxyfluorescein into skin deep layers or across the skin. While in the case of mTHPC with finite and infinite dose application, most of drug accumulation was observed in the skin superficial layer for both lipid vesicular systems and non-vesicular systems. The results also revealed that the factors influencing the drug skin distribution concern the physicochemical characteristics of the drug, the choice of the vehicle formulation and the application mode applied.

Keywords: Lipid vesicular systems, in vitro skin penetration and deposition, skin distribution, finite dose, infinite dose
1. Introduction:

Skin covers a surface area from 1.5 to 2.0 m² and is regarded as the largest organ of the human body. From a pharmaceutical point of view, it offers advantages over other routes of administration, including avoidance of first-pass metabolism, smaller fluctuations in plasma drug levels for repeated dosing, good patient compliance (Brown et al., 2006). However, although skin delivery systems may have the described advantages, most drugs are not amenable to this mode of administration because of the barrier function of the skin. Anatomically, the skin consists of three distinct layers, including stratum corneum (SC) having a thickness of 10–20 µm, viable epidermis (50–100 µm), and dermis (1–2 mm). The most simplistic organizational description of SC is suggested as the classic “brick-and-mortar” assembly with the corneocytes as the bricks and the intercellular lipids as the mortar (Elias, 1983). It is the “brick and mortar” architecture and lipophilic nature of the SC, which primarily accounts for the barrier properties of the skin (Elias, 1983).

During the past decades, numerous techniques have been employed to overcome the barrier posed by the SC to improve transdermal drug delivery, one of which is the employment of lipid vesicular formulations as skin drug delivery systems to enhance drug transport across or into the skin barrier. Lipid vesicular systems such as conventional liposomes (Lasch and Wohlrab, 1986), transfersomes (Ceve, 1996; Ceve and Blume, 1992) and ethosomes (Touitou et al., 2000) offer a promising strategy for achieving the purpose of improved skin drug delivery. It should be pointed out that the great diversity of potential composition of these vesicles influences their physicochemical characteristics such as particle size, charge, thermodynamic phase and bilayer elasticity, which in turn have a significant effect on the interaction between vesicles and the skin and hence on the effectiveness of these vesicles as skin delivery systems.
Moreover, the SC is also known to exhibit selective permeability with respect to the type of diffusing molecules, meaning that the barrier nature of the skin imposes physicochemical limitations to the type of permeants that can traverse the skin, including hydrophilicity, size, hydrogen-bonding ability and so on (Akomeah et al., 2007; Lian et al., 2008; Potts and Guy, 1992). On the other hand, drugs can be encapsulated and located at different positions in the lipid vesicular system according to their lipophilicity. Hydrophilic drugs can be entrapped into the internal aqueous compartment, whereas amphiphilic, lipophilic and charged hydrophilic drugs can be associated with the vesicle bilayer. For these reasons, two model drugs, including Carboxyfluorescein (CF, Figure 1, hydrophilic molecule, $\log P_{o/w} = -1.5$ (Nicole et al., 1989)) and Temoporfin (mTHPC, Figure 2, lipophilic one, $\log P_{o/w} = 9.24$ (unpublished experimental data from our department)), were selected to assess and compare the skin penetration enhancing effect of different lipid vesicular systems on them.

Furthermore, the enhancement effects and mechanisms of action of these lipid vesicular systems differ among the reports. The compositions of these formulation change due to penetration into the skin or evaporation of volatile components. These changes depend on the amount of liposome applied and occlusion conditions. Many studies have employed non-occluded conditions, but the application amount was varied. The amounts of formulation have ranged from 10µL/cm$^2$ (Dragicevic-Curic et al., 2008; Verma and Fahr, 2004) to 1.5mL/cm$^2$ (Elsayed et al., 2007a). These differences may be related to variations in the observed effects of lipid vesicular systems, but there have been few reports aiming to clarify the effects of dose on lipid vesicular systems penetration.

In this study, the influence of different lipid vesicular systems (including conventional liposomes, invasomes and ethosomes) as well as the effect of application mode (including finite
dose application and infinite dose application) on skin penetration and deposition behaviors of CF
and mTHPC has been investigated. This should provide an insight into the mechanisms of action of
different lipid vesicular systems for model drugs with different physicochemical properties when
different application modes are used.

2. Materials and Methods

2.1 Chemicals

Lipoid S 100 (Phosphatidylcholine from soybean lecithin) was a gift from Lipoid GmbH,
(Ludwigshafen, Germany). NAT 8539 (Soybean phosphatidylcholine (SPC) dissolved in ethanol
containing: 77.3% phosphatidylcholine, 5% lysophosphatidylcholine, 3% cephaline and 1.1%
phosphatidic acid of the dry residue) was purchased from Nattermann Phospholipid GmbH,
(Hermersberg, Germany). 5(6)-Carboxyfluorescein was a gift from Sigma-Aldrich, (Steinheim,
Germany). Temoporfin (7,8-dihidro-5,10,15,20-tetrakis-(3-hydroxyphenyl) porphyrin, mTHPC)
was a gift from biolitec AG, (Jena, Germany). Methanol (HPLC grade) was purchased from Carl
Roth GmbH &Co. (Karlsruhe, Germany). DPPG (1, 2-dipalmitoylsn-glycero-3-
phosphatidylglycerol) was purchased from Genzyme Pharmaceuticals, Sygena Facility, (Liestal,
Switzerland). Bovine albumin serum (BSA), Limonene, Citral and Cineole were purchased from
Sigma-Aldrich, (Steinheim am Albuch, Germany). And all other solvents used in this study were of
analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2 Preparation of lipid vesicles

All the lipid vesicles were prepared by a conventional rotary evaporation method (Bendas and
Tadros, 2007; Ita et al., 2007). Briefly, the appropriate weights of lipid or lipids (and with mTHPC in
the case of lipid vesicles containing mTHPC) (the compositions of different vesicular systems
shown in Table 1) were dissolved in methanol/chloroform solution (1:2, v/v) in round bottom flask. Thin lipid films were obtained by removing the organic solvents under vacuum condition (500 mbar 10 min, 200 mbar 10 min, 100 mbar 10 min, 35 mbar 1 hr) at a temperature of 43 °C with a rotary evaporator Type???. The resulted dry lipid films on the inside wall of round bottom flask were hydrated and dispersed with different hydration systems (and also containing CF in the case of lipid vesicles containing CF) corresponding to all formulations (Table 1) at room temperature. The obtained macroscopically homogenous solution was sonicated for totally 15 minutes in 3 cycles (5 min for each cycle and 5 min pause among these cycles) with a sonication ice-water bath. Then these suspensions were extruded through polycarbonate membrane (diameter: 19 mm, pore diameter: 100 nm, Armatis, Schriesheim, Germany) 21 times to produce liposomes of the desired size with the help of a Hamilton-Bonaduz extruder (GASTIGHT#1001, Bonaduz, Switzerland) (MacDonald et al., 1991).

Table 1 should be inserted here

2.3 Characterization of lipid vesicular systems

2.3.1 Particle size distribution and ζ-potential measurement

The particle size, polydispersity index (PDI) and ζ-potential of all the lipid vesicles were determined by photon correlation spectroscopy (Zetasizer Nano series, Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK). Before the size and ζ-potential measurements, different liposomes samples were diluted 100-times with PBS (pH 7.4, 10mM), which were also filtered through polycarbonate membrane (Minisart® syringe end filters 0.2 μm, Sartorius AG, Goettingen, Germany). Measurements were made at 25°C with a fixed angle of 137°. Sizes quoted are the z-average mean for the liposomal hydrodynamic diameter (Nanda A, 2006). Calculation of
2.3.2 Visualization by Cryo-Transmission Electron Microscopy

The different lipid vesicles (CL, INS and ETS) were visualized by cryo-electron microscopy and their shape and lamellarity was investigated. 5 µL of test formulation were placed on a perforated copper grid (Quantifoil R 1.2/1.3) and excess liquid was blotted automatically for two seconds between two pieces of filter-paper-strips. Shortly after that, the samples were frozen by rapidly plunged in liquid ethane (cooled to -170 °C to -180°C) in a cryo-box (Carl Zeiss NTS GmbH, Germany). Excess ethane was removed with a piece of filter paper in the cryo-box. The sample was transferred with the liquid nitrogen cooled holder (Gatan 626, USA) into the pre-cooled cryo-electron microscope (Philips CM 120, Netherlands) operated at 120 kV and viewed under low dose conditions. The Images were recorded with a 1k CCD Camera (FastScan F114, TVIPS, Gauting, Germany).

2.4 In vitro skin penetration and deposition studies

2.4.1 Skin preparation

Female human abdominal skin from plastic surgery was used. The subcutaneous fatty tissue was removed from the skin by using a scalpel and surgical scissors and then frozen at -20 °C with aluminum foil packed for later use in 3 months at most. Before use, the skin disks of 36mm were punched out, cleaned with PBS (pH 7.4, 50mM) and allowed to thaw with the stratum corneum (SC) side up open to the atmosphere and the dermal side bathed with receptor medium for overnight at 4°C. After that the integrity of skin disks were checked with trans-epidermal water loss (TEWL) measurement (VapoMeter, Delfin Technology Ltd., Kuopio, Finland) to ensure that samples were free from any surface irregularity such as tiny holes or crevices in the portion that was used for skin
penetration and deposition studies.

2.4.2 Franz diffusion cell preparation

In vitro skin penetration and deposition experiments of different liposomal systems containing mTHPC or CF were run in Franz diffusion cells non-occlusively and maintained at 37±1°C throughout experiments, in order to maintain the skin surface at 32 °C. The effective penetration area and receptor cell volume are 3.14 cm² and 15.0 ml, respectively. The acceptor compartment was filled with PBS buffer (pH 7.4, 50mM) as the receptor medium. Each test formulation was investigated in triplicate. Skin disks were mounted, with the SC side up and the donor compartment left dry and open to atmosphere for 0.5 hr before application of test formulation. Caution was taken to remove all air bubbles between the underside of the skin (dermis) and the acceptor solution. Also the skin was stretched in all directions to avoid the presence of furrows. In the case of finite dosage application, 10 µL/cm² of the test formulation was applied to skin surface by a pipette and homogenously distributed by an inoculating loop (1 µL, VWR ® International, GmbH. Darmstadt, Germany). While in the case of infinite dosage application, 160µL/cm² of the test formulation was applied using the same procedure. The experiments were carried out under non-occlusion with light protection. The incubation time of the skin with different test formulations was 12 h. At the end of experiment, a sample of 1 ml was withdrawn from the acceptor solution for the CF or mTHPC concentration measurement by fluorescence assay using a Fluostar (Optima Microplate Reader, BMG LABTECH GmbH. Offenburg, Germany). Then the formulations were removed from the skin by being washed five times with warm (45 °C) receptor medium. After cleaning, the skin was transferred for tape-stripping the SC.

2.4.3 Stripping of the SC
The stratum corneum (SC) was removed by striping with an adhesive tape (Cristall Klar Tesa®, Beiersdorf AG, Hamburg, Germany). In order to avoid any furrows, which could be a reason for false results of the stripping procedure, the skin was stretched and mounted with pins on cork discs mentioned previously. The skin was covered with a Teflon mask with a central hole of 15 mm in diameter. Each tape was put onto the skin and a weight of 2 kg was placed on the tape for 10 s. Afterwards the tape was rapidly removed with forceps and transferred into a glass vial of suitable size. Ten stripping procedures were performed consecutively. For analytical reasons, the stripped tapes were collected in vials according the following scheme: vial 1 included strip 1–5 with a code name as SC1–5, while vial 2 contained strip 6–10 and named after SC6–10. For extraction of CF from the adhesive tapes, 2 mL of a mixture solution of ethanol and PBS pH 7.4 (1:1, v/v) was added to each vial. In the case of mTHPC, 2 mL of methanol was used. Then these vials were shaken for 2 h followed by vortex for 2 min and sonication for another 2 min. The supernatant were withdrawn, diluted if necessary with methanol or the mixture solution of ethanol and PBS pH 7.4 (1:1, v/v) for mTHPC or CF, respectively, and analyzed by fluorescence measurement.

2.4.4 Extraction of mTHPC or CF from deeper layers of the skin

After the tape-stripping, the epidermis sheet was separated from the dermis with a surgical sterile scalpel. Afterwards dermis was cut into small pieces. Then the epidermis sheet and dermis pieces were transferred into special vials (Micro packaging Vials, 2mL, PeQLab Biotechnology GmbH, Erlangen, Germany). One vial was used for epidermis and nine vials for dermis, each of which was loaded with 0.55 g of ceramic beads inside (Zirconium oxide beads, 1.4 mm in diameter, Bertin Technology, Bad Wildbad, Germany). For extraction of mTHPC or CF from epidermis or dermis, 1 ml of methanol or the mixture solution of ethanol and PBS pH 7.4 (1:1, v/v) was added to
each vial, respectively. The epidermis sheet and dermis pieces were micronized by Precellys S24 (Bertin Technology, Bad Wildbad, Germany) with the following settings: 6000 rpm, 3 cycles, each one cycle for 30s. Afterwards the dispersions were ultra-centrifuged (10min., 13000 rpm, Minispin, Eppendorf, Germany) to subside skin tissue pieces at the bottom. The supernatant were withdrawn, diluted if necessary with the mixture solution of ethanol and PBS pH 7.4 (1:1, v/v) (in the case of mTHPC, methanol was used) and analyzed by fluorescence measurement.

2.4.5 Fluorescent assay of CF and mTHPC

The concentration of CF and mTHPC were determined by fluorescence spectroscopy method. Fluorescence detection was performed at an excitation of 485 nm and an emission of 520 nm in the case of CF while at an excitation of 390 nm and an emission of 645 nm in the case of mTHPC. The method was validated for linearity, accuracy and precision. The linear range during the measurements for CF and mTHPC was from 0.0005 µg/mL to 0.25 µg/mL \((r=0.9998)\) and from 0.015 µg/mL to 0.75 µg/mL \((r=0.9997)\), respectively. The software used was Optima, version 2.10, BMG Lab Tech.

2.5 Data analysis

All reported data are mean \pm S.E.M. Statistical significance was checked by Student’s t-test and considered to be significant at \(P < 0.05\), unless indicated.

3. Results and discussion

3.1 Particle size distribution and \(\zeta\)-potential of lipid vesicular systems

In this study, different lipid vesicular systems, including conventional liposomes, invasomes and ethosomes, were prepared and characterized in order to evaluate their capability to improve skin
delivery of two model drugs, hydrophilic model drug CF and lipophilic model drug mTHPC. The compositions of these different lipid vesicular systems are reported in Table 1 and their corresponding results of particle size distribution and ζ-potential are summarized in Table 2.

Conventional liposomes showed the largest mean vesicle size of 121.7±0.8 nm (n=3) and 114.6±2.0 nm (n=3) for CF and mTHPC, respectively. Invasomes (115.3±1.4 (n=3) and 109.9±0.2 nm (n=3) in the case of invasomes containing CF and mTHPC, respectively) and ethosomes (81.6±5.8 nm (n=3) and 77.8±0.5nm (n=3) in the case of ethosomes containing CF and mTHPC, respectively) had significantly (P<0.01) lower mean vesicle size relative to corresponding conventional liposomes. In the case of ethosomes, the presence of high concentration of ethanol (45%, v/v) could be the reasonable explanation about the reduced particle size of ethosomes in comparison with conventional liposomes. Ethanol could probably reduce the membrane thickness due to the formation of a phase with interpenetrating hydrocarbon chains (Barry and Gawrisch, 1994; Dubey et al., 2007). Furthermore, the addition of ethanol in phospholipid vesicles imparts negative charge to the formulation and this modification of net charge of the system confers lipid vesicles some degree of stearic stabilization and in turn lead to decrease in mean vesicle size (Jain et al., 2007). All of these mechanisms suggested that ethanol possesses some condensing ability for lipid vesicles. While, in the case of invasomes, the incorporation of 10% of ethanol could also reduce the particle size distribution with the same mechanism. Moreover, another important component, lysophosphatidylcholine (LPC) which was one of the components of NAT 8539 works as a surfactant creating a high positive curvature in membranes (Fuller and Rand, 2001). However, the inclusion of 1% (w/v) terpenes mixture could lead to the increase of particle size distribution (Dragicevic-Curic et al., 2008). Regarding the polydispersity index (PDI), all the lipid vesicular
systems showed low values (PDI<0.2), indicating that all of them were highly homogeneous suspensions.

The ζ-potential is related to the charge on the surface of the vesicle which influences both vesicular properties such as stability, as well as skin–vesicle interactions. Conventional liposomes containing CF or mTHPC prepared in this study were found to possess a ζ-potential of -12.3±0.7 mV (n=3) and -6.2±1.4 mV (n=3), respectively. Invasomes containing CF or mTHPC exhibited a negative ζ-Potential of -41.1±1.5 mV (n=3) and -39.4±1.2 mV (n=3), respectively. This result is unreasonable agreement with the recent research data on invasomes containing mTHPC from our department (Dragicevic-Curic et al., 2008, 2009). Ethosomes containing CF or mTHPC also showed a negative ζ-Potential of -79.7±1.4 mV (n=3) and -84.1±1.4 mV (n=3), respectively. In the case of ethosomes, incorporation of DPPG (Samad et al., 2007), is expected to produce highly negatively charged vesicles. The effect of surface charge of liposomes on the drug penetration has not been fully understood so far. Some researchers support the theory that the positive charges on the surface of liposomes could bind to negative charges of the SC enhancing thereby the drug penetration/permeation through the skin (Katahira et al., 1999; Song and Kim, 2006). However, other studies found that permeation of drugs through the skin is promoted by negatively charged vesicles (Ogiso et al., 2001; Sinico et al., 2005). According to the best of our knowledge, the surface charge of the vesicles will not only play a role on the interaction between skin and vesicles but also might contribute to the drug release from the vesicles. The drug release from vesicles in the stratum corneum is an important step which will affect transdermal flux (Honeywell-Nguyen and Bouwstra, 2003). The rate and amount of released drug is a balance between two factors: (1) drug affinity to vesicles, and (2) drug solubility in lipids of the stratum corneum (Honeywell-Nguyen and Bouwstra,
In our previous study, another amphiphilic model drug, ferulic acid (FA), was used to investigate the effect of surface charge of liposomes on the drug skin permeation. This study (Chen et al., 2010) revealed that the flux of FA from negatively charged ethosomes is somewhat higher than from positively charged ethosomes, but without any significant difference. Since the pK\textsubscript{a1} of FA is 4.52 (Erdemgil et al., 2007), it is negatively charged at pH 7.4. Therefore we suggested that FA could be retained in the positively charged vesicles, which in turn influences the skin penetration or permeation. In the case of CF, since it is also negatively charged at pH 7.4 (CF has a pKa of 6.3 (Nicole et al., 1989)), we preferred to prepare the negatively charged lipid vesicles containing CF for comparison. For the effect of positively charged liposomes and neutralized liposomes on the skin penetration and deposition of CF and mTHPC is investigated in a forthcoming study.

Table 2 should be inserted here

3.2 Morphology of different lipid vesicles

Cryo-Transmission electron microscopy was used to visualize vesicles, and to study the shape and lamellarity of different lipid vesicles containing CF (Fig.3A) or mTHPC (Fig.3B). From the results, no matter if CF or mTHPC was encapsulated, the lipid vesicles had almost same shapes and structures. The vesicles of the conventional liposomes seemed to be unilamellar (Fig. 3a, b, g and h, black light arrows) and rarely bilamellar (Fig. 3a, b, g and h, black thick arrows), almost spherical and oval in shape, and some detected oligolamellar vesicles (Fig. 3a, b, g and h, white arrows). In the case of invasomes, the vesicles seemed to be almost unilamellar (Fig. 3c, d, i and j, black light arrow) and bilamellar (Fig. 3c, d, I and j black thick arrow). Regarding ethosomes, the vesicles appeared to be homogenously unilamellar (Fig. 3e, f, k and l, black light arrow).

3.3 In vitro skin penetration and skin deposition studies
3.3.1 Finite dose application for CF and mTHPC in vitro study

Penetration and deposition data across full-thickness human skin with non-occlusive application of a finite dose (10µL/cm²) for CF or mTHPC after 12 hr by a range of formulation vehicles are shown in Table 3 and Table 4, respectively, with their distribution in different skin layers profiles shown in Figure 4 and Figure 5, respectively.

| Table 3 should be inserted here |
| Table 4 should be inserted here |

In the case of CF, the highest CF accumulation from all the test formulations (Table 3) was found in the SC superficial layer (Stratum Corneum tape stripping layer Nr.1-5, SC L1-5) where ethosomes containing CF (CF-ETS) and hydroethanolic solution containing CF (the mixture solution of ethanol and PBS pH 7.4 (9:11, v/v), CF-HE) significantly enhanced CF accumulation in comparison with PBS (pH7.4) solution containing CF (CF-PBS) group (by a factor of 8.9 and 8.1, respectively, p < 0.01). Conventional liposomes containing CF (CF-CL) slightly improved CF accumulation in SC L1-5 in comparison with CF-PBS (by a factor of 1.5; p < 0.05). CF accumulations in SC deep layer (Stratum Corneum tape stripping layer Nr. 6-10, SC L6-10) and in epidermis were all improved when using all the lipid vesicular systems as well as CF-HE in comparison with CF-PBS, but with different magnitude. In the case of CF accumulation in SC L6-10, CF-ETS showed the highest potential, followed by CF-HE > CF-INS > CF-CL> CF-PBS.

For CF accumulation in epidermis, both CF-ETS and CF-INS showed the highest CF accumulation in epidermis, followed by CF-CL > CF-HE > CF-PBS. CF accumulation in dermis was only significantly improved by CF-ETS in comparison with CF-PBS (by a factor of 2.2) and other formulations didn’t show any significant enhancement. Furthermore, no permeation of CF through
the full thickness human skin was detected with this application condition from all the test formulation vehicles.

Figure 4 should be inserted here

In the case of mTHPC, the highest mTHPC accumulation from all the test formulations (Table 4) was also found in the SC L1-5 where mTHPC-HE (hydroethanolic solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC) showed the highest mTHPC accumulation, followed by mTHPC-CL> mTHPC-ET> mTHPC-ETS> mTHPC-INS. Regarding mTHPC accumulation in SC L6-10 and in epidermis, mTHPC-HE also showed the highest potential, but followed by different orders: mTHPC-CL> mTHPC-ET>mTHPC-ETS≥ mTHPC-INS and mTHPC-ETS> mTHPC-ET≥ mTHPC-INS≥ mTHPC-CL, respectively. The comparison of these five formulations showed that significant differences existed between mTHPC-HE and others (p< 0.01) regarding the mTHPC accumulation in these three different skin layers. However, the permeation of mTHPC through the full thickness human skin and mTHPC accumulation in dermis were not detected with this application condition for all test formulations.

Figure 5 should be inserted here

As can be seen, significant differences can be found between lipid vesicular systems containing CF and mTHPC with respect to drug skin distribution profile of CF or mTHPC. In the case of mTHPC, most of mTHPC can be found in SC superficial layers (SC L1-5) (Figure 5 and Table 4). The percentage of mTHPC present in SC L1-5 was 94.0%, 84.8% and 92.6% of the total mTHPC delivered for conventional liposomes (mTHPC-CL), invasomes (mTHPC-INS) and ethosomes (mTHPC-ETS), respectively. While, in the case of CF, even though the highest drug accumulation was also found in SC L1-5, the drug skin distribution differed a lot according to the lipid vesicles.
applied (Figure 4 and Table 3). The percentage of CF present in SC L1-5 was 51.4%, 30.9% and 54.1% of the total CF delivered for conventional liposomes (CF-CL), invasomes (CF-INS) and ethosomes (CF-ETS), respectively.

In order to explain this drug skin distribution difference, it is necessary to consider possible mechanisms of action of different lipid vesicular systems. Conventional liposomes were expected to be effective at delivering drugs into the upper layers of the skin. It is agreed in the recent literature that in most cases conventional liposomes are not penetrating the skin but remain confined to upper layers of the SC or form a deposit on the surface of the skin (Tanner and Marks, 2008). The penetration properties of conventional liposomes may fall into one of two possible categories, including the penetration enhancing effect and vesicle adsorption to and/or fusion with the SC (El Maghraby et al., 2006). The first possible mode of action that was described firstly in 1987 (Kato et al., 1987) and supported by others (Hofland et al., 1995; Kirjavainen et al., 1999; Zellmer et al., 1995). This mode suggests that liposomal lipids may act as penetration enhancers, thereby loosening the lipid structure of the SC and promoting an impaired barrier function (Kirjavainen et al., 1999). The second possible mode for conventional liposomes is adsorption to and/or fusion with the SC (Abraham and Downing, 1990; Hofland et al., 1995; Kirjavainen et al., 1996), suggesting that the liposomal lipids penetrate into the SC by adhering onto the surface of the skin and subsequently destabilizing and fusing or mixing with the lipid matrix (Kirjavainen et al., 1996). However, the collapse of vesicles on skin surface may form an additional barrier, reducing the permeation of hydrophilic molecules encapsulated in the vesicular aqueous core (Elsayed et al., 2007b).

Regarding invasomes (belonging to the class of deformable liposomes) due to the presence of lysophosphatidylcholine (LPC) and ethanol and terpenes (Dragicevic-Curic et al., 2008; Verma and
Fahr, 2004). Hence, there are two possible mechanisms responsible for its enhanced skin drug delivery (Dragicevic-Curic et al., 2008). First, invasomes may act as drug carrier systems by which intact vesicles can enter the SC carrying vesicle-bound drug into or across the skin. Second, invasomes may act as penetration enhancers, whereby the vesicle lipid bilayers interact with the SC and subsequently modify the intercellular lipid lamellae. It may also possible that one of the two mechanisms might predominate according to the physicochemical properties of the drug considered (Elsayed et al., 2007b).

The enhancing effect of ethosomes could be attributed to the synergistic mechanism between ethanol, lipid vesicles and skin lipids (Dayan and Touitou, 2000; Elsayed et al., 2006; Touitou et al., 2000). Firstly, ethanol is a well-known permeation enhancer. The penetration enhancing effect of ethanol can be attributed to two effects: (a) ‘Push effect’: increased thermodynamic activity due to evaporation of ethanol and improved solubility of solute in this study; (b) ‘Pull effect’: ethanol can interact with intercellular lipid molecules in the polar head group region, thereby increasing their fluidity and decreasing the density of the lipid multilayer, which results in an increase in membrane permeability. Ethanol is also supposed to extract the SC lipids (Bach and Lippold, 1998) lowering thereby the barrier function of the SC. In addition, ethanol imparts fluidity to the vesicle's bilayers, which in turn facilitates vesicles skin permeation. Furthermore, ethanol can act as a “blending” agent for lipid vesicles with increasing their distribution in various skin layers (Panchagnula et al., 2005).

The ethanol effects can be followed by the interaction between ethosomal vesicles and the skin. The ethosomal vesicles may also behave as deformable liposomes and can interact with the skin barrier to “forge” penetration or permeation pathways by itself in the highly organized SC and finally release drug at various points along the penetration pathway as well as in deep skin layers (Elsayed...
Moreover, the different molecular mechanisms by which the diffusion through the stratum corneum of hydrophilic molecule (CF) and lipophilic molecule (mTHPC) should be also taken into account, because drug skin penetration and deposition via lipid vesicular systems involves several processes, including interaction between SC and lipid vesicles, partitioning of the drug from its lipid vesicular system to the skin and the following drug diffusion in the skin. Drugs are considered to penetrate through the skin by one of three pathways: the polar, non-polar, or polar/non-polar route depending on their physicochemical properties, in which logP<sub>o/w</sub> of drugs is thought to be the key factor (Verma and Fahr, 2004). The logP<sub>o/w</sub> value, which is a measure of how well a substance partitions between a lipid and water, determines the route of drug penetration through the skin. Temoporfin (mTHPC), which is highly lipophilic, is expected to penetrate the skin by non-polar pathways, whereas CF, which is hydrophilic, should utilize the polar pathways. The intrinsic permeability of both hydrophilic and lipophilic penetrants is governed by the composition of the skin, with the former limited by their partitioning into the lipophilic SC and the latter, by partitioning from the SC into the less lipophilic epidermis (Nicoli et al., 2008; Zhang et al., 2010).

Consequently, the logP<sub>o/w</sub> value of drug molecule has an effect on the enhancement efficacy of penetration enhancers. Hydrophilic molecules such as CF, owing to their low partition coefficient and high hydrogen-bonding potential, would show a dramatic increase in permeation with suitable enhancers, however, lipophilic molecules which move with relative ease through the SC do not have the same opportunity to act as indicators of enhancement (Barry and Bennett, 1987; Verma and Fahr, 2004; Zhang et al., 2010).

From all the discussion above, lipid vesicular systems including conventional liposomes,
invosomes (deformable liposomes) and ethosomes can act as penetration enhancers to improve the skin drug delivery by their vesicle lipid bilayers or their additives such as ethanol and terpenes interacting with the SC and subsequently modifying the SC intercellular lipid lamellae. However, this penetration enhancing effect of lipid vesicular systems could play a much more important role in the enhanced skin delivery of hydrophilic drug such as CF than in the case of lipophilic drug such as mTHPC because this penetration enhancing effect just increases the partitioning of CF into the lipophilic SC but does not really increase the partitioning of mTHPC from the SC into the less lipophilic epidermis. Hence, for a lipophilic drug such as mTHPC, the entrapment of the drug in vesicular lipid bilayers and intact vesicles penetration could be crucial for optimum skin deposition and transdermal permeation. From this point of view, it is almost impossible for conventional liposomes to reach this aim. For deformable liposomes such as invosomes and ethosomes, it is possible for both of them to achieve this purpose because they somehow can act as drug carrier systems, whereby intact vesicles enter the SC carrying vesicle-bound drug molecules into the skin. However, for deformable liposomes such as invosomes, it should be pointed out that the driving force for them entering the skin is xerophobia which is the tendency to avoid dry surroundings of water-“loving” phospholipids (Cevc and Blume, 1992) and recent evidence showed that the water gradient across the skin may not be linear and there may be a relatively ‘dry’ region within the stratum corneum. It was also noticed that even in fully hydrated state, the water content in the lowest stratum corneum layers close to the viable epidermis is much lower than in central regions of the stratum corneum (Williams, 2003). Therefore, it was expected that, as a result of the osmotic force, deformable liposomes will not penetrate beyond the level of the lowest layers in stratum corneum. Regarding ethosomes, from the results of this study, it is also not very successful to deliver
mTHPC into deeper layers of skin. Therefore, other or better designed carrier systems for mTHPC should be developed.

Another important technology which can also improve significantly the skin delivery for both CF and mTHPC is the application of hydroethanolic solution (Table 1, CF-HE and mTHPC-HE). From the results, in the case of CF, CF-HE significantly increased the CF accumulation in SC layers (SC L1-5 and SC L6-10) compared with all other formulations containing CF except CF-ETS. While, in the case of mTHPC, mTHPC-HE showed the highest mTHPC accumulation in both SC layers and epidermis compared with all other formulation containing mTHPC. There are two reasonable explanations responsible for its enhanced drug skin delivery effect. The first one is the penetration enhancing effect by ethanol, which has been already described in detail above. The second one is the increased thermodynamic activity of drugs due to the incorporation of ethanol or water. In the case of CF, because it is hydrophilic and has a lower solubility in ethanol than in water, the incorporation of ethanol in water will increase the thermodynamic activity of CF compared with aqueous solution containing the same concentration of CF such as CF-PBS, CF-CL and CF-INS. Both CF-ETS and CF-HE could significantly (p<0.01) increase the CF accumulation in SC compared to the other formulations. Moreover, because the synergistic penetration enhancing effect between ethanol, lipid vesicles and the possible intact vesicle penetration mechanism of ethosomes, CF-ETS also showed the highest CF accumulation in epidermis and dermis compared with all others. In the case of mTHPC, the situation is different. Because mTHPC is highly lipophilic and its low solubility in water, the incorporation of water in ethanol will increase its thermodynamic activity compared with ethanol solution containing the same concentration of mTHPC such as mTHPC-ET. For the lipid vesicular systems containing mTHPC in this study, even
though high water amounts are involved (see Table 1), entrapment of mTHPC in the lipid bilayers, in fact, solubilizes mTHPC. Hence, the thermodynamic activity of mTHPC of these systems is not equally increased compared with mTHPC-HE, which explains why mTHPC-HE showed the best potential of improving mTHPC skin delivery.

3.3.2 Infinite dose application for CF and mTHPC in vitro study

Full-thickness human skin penetration and deposition data for application of an infinite dose (160µL/cm²) for CF or mTHPC after 12 hr non-occlusive treatment with a range of formulation vehicles are shown in Table 5 and Table 6, respectively, with their distribution in different skin layers profiles shown in Figure 6 and Figure 7, respectively.

Table 5 should be inserted here

Table 6 should be inserted here

In the case of CF, the highest CF accumulation from all the test formulations (Table 5) was found in the SC L1-5 where CF accumulation decreased in the following order: CF-ETS > CF-HE >> CF-CL > CF-INS > CF-PBS. CF accumulation in SC L1-5 was significantly improved by CF-ETS and CF-HE compared with CF-PBS (by a factor 33.9 and 17.9, respectively, p <0.01), while CF-CL and CF-INS also significantly improved CF accumulation in SC L1-5 compared with CF-PBS, but to a smaller extent (by a factor of 3.8 and 1.9, respectively, p <0.01). CF accumulation in SC L6-10 was significantly improved by CF-ETS compared with CF-PBS (by a factor 63.3, p <0.01), while CF-HE, CF-INS and CF-CL also significantly improved CF accumulation in SC L6-10 compared with CF-PBS, but to a smaller extent (by a factor of 7.0, 3.5 and 5.0, respectively, p <0.01). For epidermis and dermis, CF accumulation decreased in the same following orders: CF-ETS >> CF-INS > CF-HE > CF-CL > CF-PBS. When compared with CF-PBS, CF accumulation both in
epidermis and in dermis were significantly improved by CF-ETS (by a factor 26.7 and 13.8, respectively, p <0.01) and by CF-INS (by a factor 3.0 and 2.1, respectively, p <0.01). While CF accumulation both in epidermis and in dermis were also improved, but to a small extent, by CF-HE (by a factor of 2.1 and 1.7, respectively, 0.05<p<0.1) and by CF-CL (by a factor of 1.7 and 1.5, respectively, 0.05<p<0.1). CF permeated through the full thickness skin (CF in receptor fluid) can be found from all the tested formulations. However, the concentration of CF in receptor fluid from CF-PBS and CF-HE were too low and out of the linear range of CF for the fluorescent detector. For this reason, the CF in receptor fluid were only calculated from lipid vesicular systems and had a following order: CF-ETS > CF-INS > CF-CL. Moreover, from the results, CF across the full-thickness skin from CF-ETS, CF-INS and CF-CL accounted for quite a small percentage of the total CF delivered into skin, only 1.5%, 2.1% and 1.1%, respectively.

Figure 6 should be inserted here

In the case of mTHPC, the highest mTHPC accumulation from all the test formulations (Table 6) was still found in the SC L1-5 where mTHPC-HE showed the highest mTHPC accumulation, followed by mTHPC-ETS> mTHPC-CL> mTHPC-ET> mTHPC-INS. However, regarding mTHPC accumulation in SC L6-10 and in epidermis, mTHPC-ET showed the highest potentials, but followed by different orders: mTHPC-HE> mTHPC-CL=mTHPC-ETS≥ mTHPC-INS and mTHPC-ETS> mTHPC-CL=mTHPC-HE≥ mTHPC-INS for SC L6-10 and epidermis, respectively.

Both the permeation of mTHPC through the full thickness human skin and mTHPC accumulation in dermis were still not detected with this application condition from all the tested formulations.

Figure 7 should be inserted here

From these results, with an infinite dose application, most of mTHPC delivered into skin was
still inclined to be deposited in superficial layers of SC (Table 6 and Figure 7), while much more percentage of CF could be delivered into deeper layers of skin, especially by lipid vesicular systems, but with different extents (Table 5 and Figure 6). In the case of CF-CL, with an infinite dose applied, the ratio between CF accumulation in SC L1-5 and in SC L6-10 significantly decreased compared with a finite dose applied, which means more percentage of CF was delivered into SC deeper layers. The reason for this could be that the application of an infinite dose in donor compartment will form a thick liquid formulation layer covering on the skin surface with a height of 1.6mm (calculated value), while a finite dose just can form a thin layer of only 0.1mm (calculated value). This will in turn result in much higher hydration condition of the skin with an infinite dose than with a finite dose. This increased skin hydration facilitated the interaction between conventional liposomes and skin, which in turn increased the CF penetration into SC deeper layers. Moreover, in the case of CF-CL, with a finite dose applied, the CF delivered into the skin deep layer (including CF accumulated in epidermis, dermis and receptor phase) was 42.6% from the amount delivered into the skin; while, with an infinite dose applied, it decreased to 18.4%. However, in the case of mTHPC-CL, with a finite dose applied, the mTHPC delivered into the skin deep layer was only 1.9%; while, with an infinite dose applied, it increased to 4.4%. As mentioned before, according to the mechanism of action of conventional liposomes, this reason could be that the collapse of vesicles on skin surface may form an additional barrier, reducing the permeation and penetration of hydrophilic molecules such as CF encapsulated in the vesicular aqueous core (Elsayed et al., 2007b).

In the case of CF-INS, with an infinite dose application, the ratio between CF accumulation in SC and in skin deep layers significantly increased compared with a finite dose applied, which means
less percentage of CF was delivered into skin deep layers. With a finite dose applied, the CF delivered into the skin deep layer (including CF accumulated in epidermis, dermis and receptor phase) was 52.9%; while, with an infinite dose applied, it decreased to 37.8%. In the case of mTHPC-INS, there was a similar trend. With a finite dose applied, the mTHPC delivered into the skin deep layer was 8.8%; while, with an infinite dose applied, it decreased to 6.1%. Considering one of possible mechanisms of action of invasomes, with a thick formulation liquid layer covering on the skin surface formed by an infinite dose, invasomes which are regarded as deformable liposomes could lose its penetration driving force because of the disappearance of the trans-epidermal osmotic gradient in this application mode. Hence, less percentage of CF and mTHPC could be delivered into skin deep layers with infinite dose application. On the other hand, in the case of CF-INS, with a finite dose applied, the CF skin delivery enhancement ratio between CF-INS and CF-PBS was a factor of 1.4; while, with an infinite dose applied, it increased to a factor of 2.4. This result suggested another possible the mechanism of action of invasomes: one part of the vesicles is fragmented during their penetration into the upper skin layers, the released terpenes, as well as phospholipids, act also as penetration enhancers fluidizing the intercellular lipids (Dragicevic-Curic et al., 2008), which improves the drug skin delivery. Therefore, all these results from this study supported the hypothesis, our department suggested before, on mechanism of action of invasomes: some of the invasomes were fragmented during their penetration through the SC, while some of the small and deformable invasomes could have penetrated to the deeper SC layers intact (Dragicevic-Curic et al., 2008).

In the case of CF-ETS, with an infinite dose application, the ratio between CF accumulation in SC and in deeper skin layers (including in epidermis and in dermis as well as in receptor fluid)
significantly decreased compared with a finite dose applied, which means much more percentage of CF was delivered into skin deeper layers and across the skin. With a finite dose applied, the CF delivered into the skin deep layer (including CF accumulated in epidermis, dermis and receptor phase) was 11.7%; while, with an infinite dose applied, it increased to 20.7%. In the case of mTHPC-INS, there was also a similar trend. With a finite dose applied, the mTHPC delivered into the skin deep layer was 4.4%; while, with an infinite dose applied, it increased to 6.1%. Increased skin hydration could also be responsible for this. Another important reason could be different amount of ethanol in this two application mode. With a finite dose application, because of the small amount of ethanol applied, the evaporation of ethanol is much quicker than with an infinite dose applied. This means that more ethanol could interact with skin and lipid vesicles with an infinite dose applied, which in turn improved the CF penetration into deeper skin layers or across the skin.

Furthermore, with a finite dose application, a low CF accumulation in SC layers for CF-INS compared with CF-ETS can be found, however, a similar CF accumulation in epidermis. The reason might be that CF-INS penetrates faster through the deeper layers in the SC than CF-ETS due to different mechanisms of action between them. There is another thing should be pointed out that an infinite dose of mTHPC-ET resulted in a significant increase of percentage of mTHPC delivered into SC L6-10 and epidermis. This could be explained by the long contact time (12 hrs) with ethanol, impairing the skin structure.

Moreover, a comparison of an infinite dose and a finite dose under non-occlusive application for CF and mTHPC revealed that the total amount of both of them delivered into skin were significantly increased when an infinite dose applied, but to a significantly different extent. In the case of CF,
total amount of CF delivered into skin with a finite dose (10µl/cm²) ranged from 0.047±0.010 µg/cm² (CF-PBS) to 0.401±0.045 µg/cm² (CF-ETS), while with an infinite dose (160µl/cm²) from 0.327±0.107 µg/cm² (CF-PBS) to 10.837±1.210 µg/cm² (CF-ETS). Therefore, with increasing dosage of formulation from a finite dose to an infinite dose, total amount of CF delivered into skin increased by from 6.9 times (CF-PBS) to 27.0 times (CF-ETS). However, in the case of mTHPC, total amount of mTHPC delivered into skin with a finite dose (10µl/cm²) ranged from 0.079±0.024 µg/cm² (mTHPC -INS) to 0.483±0.073 µg/cm² (mTHPC -HE), while with an infinite dose (160µl/cm²) from 0.312±0.035 µg/cm² (mTHPC -INS) to 0.956±0.003 µg/cm² (mTHPC -HE).

Hence, with the same situation, total amount of mTHPC delivered into skin only increased by from 1.9 times (mTHPC -HE) to 3.9 times (mTHPC -INS). There could be two possible reasons responsible for this. The first one is that the increased skin hydration effect rooted from a thick liquid formulation layer covering on the skin surface formed by an infinite dose applied in donor compartment. Generally, increased tissue hydration appears to increase transdermal delivery of both hydrophilic and low lipophilic compounds due to an increase in partition into the skin of drugs (Williams and Barry, 2004). It is proposed that the hydration effect of them on the stratum corneum could make the penetration of hydrophilic drugs easier. However, for the high lipophilic compounds (log P<sub>ow</sub>＞2), partition into the “hydrated” stratum corneum are made difficult, consequently, which results in a reduction in their permeation capacity through the skin (Zhang et al., 2010). Another possible reason could be the different molecular mechanisms by which the diffusion through the stratum corneum of CF and mTHPC, which has already been discussed above. With an infinite dose of formulation applied on the skin, the modification of the micro-structure of SC could be reinforced compared with a finite dose applied. This will facilitate the skin penetration and deposition of CF
because the limited step for skin penetration of CF is its partitioning into SC from formulation.

However, due to the limited step for mTHPC is their partitioning from the SC into the less lipophilic epidermis, the augmented modification of the micro-structure of SC by an infinite dose could not result in the same enhancing effect for mTHPC as for CF.

4. Conclusion

In this study different lipid vesicular systems including conventional liposomes, invasomes and ethosomes containing CF or mTHPC were developed and characterized. In vitro human full-thickness skin penetration studies revealed that, in the case of CF with finite dose application (10 µL/cm²), the highest drug accumulation in epidermis was observed from CF-INS as well as CF-ETS. The drug accumulation in dermis was only significantly improved by CF-ETS in comparison with CF-PBS. There was no CF detected in receptor phase. While in the case of CF with infinite dose application (160 µL/cm²), the highest drug accumulation both in epidermis and in dermis was observed from CF-ETS. CF also could be detected in the receptor phase from all lipid vesicular systems with such an order: CF-ETS > CF-INS > CF-CL. On the other hand, in the case of mTHPC with finite and infinite dose application, most of drug accumulation was observed in skin superficial layer from both lipid vesicular systems and non-vesicular systems, but with different orders. There was no mTHPC detected in dermis and in receptor phase from all the tested formulations. The results suggested that lipid vesicular systems are more effective for improving the penetration and deposition of hydrophilic drugs such as CF than for lipophilic drugs such as mTHPC. In order to confirm this point, further studies with more different model drugs and different kind of lipid vesicular systems involved are needed. Moreover, the composition of lipid vesicular systems also
played a significant role on drug skin distribution for the hydrophilic drugs such as CF, but not for lipophilic drugs such as mTHPC. From the results, we also found that the application mode (finite or infinite dose application) not only had a direct action on the drug skin penetration and deposition, but also could affect the degree of hydration of the SC and the possible mechanism of some lipid vesicular system which in turn influence the drug skin distribution.

**Acknowledgement**

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References


Panchagnula, R., Desu, H., Jain, A., Khandavilli, S., 2005. Feasibility studies of dermal delivery of paclitaxel with binary combinations of ethanol and isopropyl myristate: role of solubility, partitioning...
and lipid bilayer perturbation. Farmaco 60, 894-899.


Legends to Figures

Fig.1. Structure of 5(6)-Carboxyfluorescein

Fig.2. Structure of Temoporfin

Fig.3A. Visualization of different liposomal systems containing CF by cryo-transmission electron microscopy.
(a, b) conventional liposomes containing 5.0mg/ml of CF; (c, d) invasomes containing 5.0mg/ml of CF; (e, f) ethosomes containing 5.0mg/ml of CF. Black light arrows represent unilamellar vesicles; black thick arrows represent bilamellar vesicles, while white thick arrows represent oligolamellar vesicles.

Fig.3B. Visualization of different liposomal systems containing mTHPC by cryo-transmission electron microscopy. (g, h) conventional liposomes containing 1.5mg/ml of mTHPC; (i, j) invasomes containing 1.5mg/ml of mTHPC; (k, l) ethosomes containing 1.5mg/ml of mTHPC. Black light arrows represent unilamellar vesicles; black thick arrows represent bilamellar vesicles, while white thick arrows represent oligolamellar vesicles.

Fig.4. Skin penetration and deposition of CF in different skin layer via full-thickness human skin from different formulations with finite dosage application (10µl/cm²) after 12hr. Note: CF-CL: Conventional liposomes containing CF (5.0mg/ml); CF-INS: Invasomes containing CF (5.0mg/ml); CF-ETS: ethosomes containing CF (5.0mg/ml); CF-PBS: PBS (pH7.4) containing CF (5.0mg/ml); CF-HE: Hydroethanolic mixture solution of ethanol and PBS pH 7.4 (9:11, v/v) containing CF (5.0mg/ml). Values represent mean ± SD (n=3)

Fig.5. Skin penetration and deposition of mTHPC in different skin layer via full-thickness human skin from different formulations with finite dosage application (10µl/cm²) after 12hr. Note: mTHPC-CL: Conventional liposomes containing mTHPC (1.5mg/ml); mTHPC-INS: Invasomes containing mTHPC (1.5mg/ml); mTHPC-ETS: Ethosomes containing mTHPC (1.5mg/ml); mTHPC-ET: Ethanol solution containing mTHPC (1.5mg/ml). mTHPC-HE: Hydroethanolic mixture solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC (1.5mg/ml). Values represent mean ± SD (n=3)

Fig.6. Skin penetration and deposition of CF in different skin layer via full-thickness human skin from different formulations with infinite dosage application (160µl/cm²) after 12hr. Note: CF-CL: Conventional liposomes containing CF (5.0mg/ml); CF-INS: Invasomes containing CF (5.0mg/ml); CF-ETS: ethosomes containing CF (5.0mg/ml); CF-PBS: PBS (pH7.4) containing CF (5.0mg/ml); CF-HE: Hydroethanolic mixture solution of ethanol and PBS pH 7.4 (9:11, v/v) containing CF (5.0mg/ml).
solution of ethanol and PBS pH 7.4 (9:11, v/v) containing CF (5.0mg/ml). Values represent mean ± SD (n=3)

Fig.7. Skin penetration and deposition of mTHPC in different skin layer via full-thickness human skin from different formulations with infinite dosage application (160µl/cm²) after 12hr. Note, CL: Conventional liposomes containing mTHPC (1.5mg/ml); INS: Invasomes containing mTHPC (1.5mg/ml); ETS: Ethosomes containing mTHPC (1.5mg/ml); CTL-1: Control group 1 Ethanol solution containing mTHPC (1.5mg/ml). Values represent mean ± SD (n=3)
Table 1: Composition of different lipid vesicular systems and control groups

<table>
<thead>
<tr>
<th>Code</th>
<th>Lipids and other components</th>
<th>Drug</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-CLa</td>
<td>Lipoid-S100, 40.0 mg/ml</td>
<td>CF</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>Cholesterol, 3.0 mg/ml</td>
<td>5.0 mg/ml</td>
<td></td>
</tr>
<tr>
<td>CF-INSb</td>
<td>NAT8539, 133.0 mg/ml</td>
<td>CF</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>Terpenes, 10.0 mg/ml</td>
<td>5.0 mg/ml</td>
<td>Ethanol (10%, v/v)</td>
</tr>
<tr>
<td>CF-ETSf</td>
<td>Lipoid-S100, 40.0 mg/ml</td>
<td>CF</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>DPPG, 4.0 mg/ml</td>
<td>5.0 mg/ml</td>
<td>Ethanol (45%, v/v)</td>
</tr>
<tr>
<td>mTHPC-CLd</td>
<td>Lipoid-S100, 40.0 mg/ml</td>
<td>mTHPC</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>Cholesterol, 3.0 mg/ml</td>
<td>1.5 mg/ml</td>
<td></td>
</tr>
<tr>
<td>mTHPC-INSf</td>
<td>NAT8539, 133.0 mg/ml</td>
<td>mTHPC</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
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<td>Terpenes, 10.0 mg/ml</td>
<td>1.5 mg/ml</td>
<td>Ethanol (10%, v/v)</td>
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<tr>
<td>mTHPC-ETSf</td>
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<td>mTHPC</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>DPPG, 4.0 mg/ml</td>
<td>1.5 mg/ml</td>
<td>Ethanol (45%, v/v)</td>
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<tr>
<td>CF-PBSg</td>
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<td>CF</td>
<td>PBS (pH 7.4)</td>
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<tr>
<td></td>
<td>5.0 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF-HEh</td>
<td>-</td>
<td>CF</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>5.0 mg/ml</td>
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<td>Ethanol (45%, v/v)</td>
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<tr>
<td>mTHPC-ETi</td>
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<td>mTHPC</td>
<td>Ethanol</td>
</tr>
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<td>1.5 mg/ml</td>
<td></td>
<td></td>
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<tr>
<td>mTHPC-HEi</td>
<td>-</td>
<td>mTHPC</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>1.5 mg/ml</td>
<td></td>
<td>Ethanol (60%, v/v)</td>
</tr>
</tbody>
</table>

Note: a Conventional liposomes containing CF; b Invasomes containing CF; c Ethosomes containing CF;

d Conventional liposomes containing mTHPC; e Invasomes containing mTHPC; f Ethosomes containing mTHPC;

8 PBS (pH7.4) containing CF; h Hydroethanolic solution containing CF (the mixture solution of ethanol and PBS pH 7.4 (9:11, v/v)); i Ethanol solution containing mTHPC; j Hydroethanolic solution containing mTHPC (the mixture solution of ethanol and PBS pH 7.4 (6:4, v/v)); k Terpenes mixture (Limonene: citral: cineole=1:4.5:4.5, v/v)
Table 2: Characterization of different lipid vesicular systems

<table>
<thead>
<tr>
<th>Code</th>
<th>Particle size (nm)</th>
<th>PDI$^g$</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-CL$^a$</td>
<td>121.7±0.8</td>
<td>0.159±0.015</td>
<td>-12.3±0.7</td>
</tr>
<tr>
<td>CF-INS$^b$</td>
<td>115.3±1.4</td>
<td>0.110±0.011</td>
<td>-41.1±1.5</td>
</tr>
<tr>
<td>CF-ETS$^c$</td>
<td>81.6±5.8</td>
<td>0.161±0.007</td>
<td>-79.7±1.4</td>
</tr>
<tr>
<td>mTHPC-CL$^d$</td>
<td>114.6±2.0</td>
<td>0.149±0.008</td>
<td>-6.2±1.4</td>
</tr>
<tr>
<td>mTHPC-INS$^e$</td>
<td>109.9±0.2</td>
<td>0.076±0.005</td>
<td>-39.4±1.2</td>
</tr>
<tr>
<td>mTHPC-ETS$^f$</td>
<td>77.8±0.5</td>
<td>0.130±0.012</td>
<td>-84.1±1.4</td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n=3)

Note: a Conventional liposomes containing CF; b Invasomes containing CF; c Ethosomes containing CF;

d Conventional liposomes containing mTHPC; e Invasomes containing mTHPC; f Ethosomes containing mTHPC;

$^g$ Polydispersity index
Table 3: Results of skin penetration and deposition of CF into full-thickness human skin from different formulations with finite dosage application (10 µl/cm²) after 12 hr

<table>
<thead>
<tr>
<th>Formulations</th>
<th>SC1-5</th>
<th>SC6-10</th>
<th>Epidermis</th>
<th>Dermis</th>
<th>Receptor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-PBSa</td>
<td>0.048±0.010</td>
<td>0.001±0.001</td>
<td>0.013±0.006</td>
<td>0.032±0.007</td>
<td>n.d. f</td>
<td>0.094±0.020</td>
</tr>
<tr>
<td>CF-HEb</td>
<td>0.388±0.005</td>
<td>0.039±0.005</td>
<td>0.024±0.005</td>
<td>0.020±0.004</td>
<td>n.d.</td>
<td>0.471±0.010</td>
</tr>
<tr>
<td>CF-CLc</td>
<td>0.070±0.009</td>
<td>0.009±0.005</td>
<td>0.029±0.014</td>
<td>0.029±0.011</td>
<td>n.d.</td>
<td>0.137±0.018</td>
</tr>
<tr>
<td>CF-INSd</td>
<td>0.042±0.003</td>
<td>0.023±0.001</td>
<td>0.045±0.012</td>
<td>0.027±0.003</td>
<td>n.d.</td>
<td>0.136±0.006</td>
</tr>
<tr>
<td>CF-ETSd</td>
<td>0.434±0.026</td>
<td>0.253±0.063</td>
<td>0.044±0.011</td>
<td>0.071±0.030</td>
<td>n.d.</td>
<td>0.802±0.090</td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n=3)

Note: a PBS (pH 7.4) containing CF (5.0 mg/ml); b Hydroethanolic solution of ethanol and PBS pH 7.4 (9:11, v/v) containing CF (5.0 mg/ml); c Conventional liposomes containing CF; d Invasomes containing CF; e Ethosomes containing CF; f Not detected
Table 4: Results of skin penetration and deposition of mTHPC into full-thickness human skin from different formulations with finite dosage application (10µl/cm²) after 12hr

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Dose mTHPC delivered (%)</th>
<th>SC1-5</th>
<th>SC6-10</th>
<th>Epidermis</th>
<th>Dermis</th>
<th>Receptor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTHPC-ET&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.500±0.113</td>
<td>0.040±0.013</td>
<td>0.047±0.007</td>
<td>n.d.&lt;sup&gt;f&lt;/sup&gt;</td>
<td>n.d.</td>
<td>1.580±0.120</td>
<td></td>
</tr>
<tr>
<td>mTHPC-HE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.893±0.480</td>
<td>0.200±0.053</td>
<td>0.133±0.027</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.220±0.487</td>
<td></td>
</tr>
<tr>
<td>mTHPC-CL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.673±0.167</td>
<td>0.073±0.013</td>
<td>0.033±0.007</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.780±0.180</td>
<td></td>
</tr>
<tr>
<td>mTHPC-INS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.447±0.180</td>
<td>0.033±0.020</td>
<td>0.047±0.020</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.527±0.160</td>
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</tr>
<tr>
<td>mTHPC-ETS&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.253±0.053</td>
<td>0.040±0.020</td>
<td>0.060±0.020</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.353±0.073</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n=3)

Note: <sup>a</sup>Ethanol solution containing mTHPC (1.5mg/ml); <sup>b</sup>Hydroethanolic solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC (1.5mg/ml); <sup>c</sup>Conventional liposomes containing mTHPC (1.5mg/ml); <sup>d</sup>Invasomes containing mTHPC (1.5mg/ml); <sup>e</sup>Ethosomes containing mTHPC (1.5mg/ml); <sup>f</sup>Not detected
Table 5: Results of skin penetration and deposition of CF into full-thickness human skin from different formulations with infinite dosage application (160 µl/cm²) after 12hr

<table>
<thead>
<tr>
<th>Formulation</th>
<th>SC1-5</th>
<th>SC6-10</th>
<th>Epidermis</th>
<th>Dermis</th>
<th>Receptor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-PBSa</td>
<td>0.020±0.007</td>
<td>0.006±0.003</td>
<td>0.005±0.001</td>
<td>0.009±0.002</td>
<td>n.d.⁷</td>
<td>0.041±0.013</td>
</tr>
<tr>
<td>CF-HEb</td>
<td>0.361±0.050</td>
<td>0.044±0.005</td>
<td>0.011±0.001</td>
<td>0.015±0.005</td>
<td>n.d.</td>
<td>0.431±0.051</td>
</tr>
<tr>
<td>CF-CLc</td>
<td>0.077±0.002</td>
<td>0.031±0.003</td>
<td>0.010±0.001</td>
<td>0.013±0.001</td>
<td>0.001±0.001</td>
<td>0.133±0.001</td>
</tr>
<tr>
<td>CF-INSd</td>
<td>0.038±0.010</td>
<td>0.022±0.006</td>
<td>0.016±0.003</td>
<td>0.019±0.002</td>
<td>0.002±0.001</td>
<td>0.097±0.007</td>
</tr>
<tr>
<td>CF-ETSf</td>
<td>0.682±0.041</td>
<td>0.397±0.100</td>
<td>0.138±0.012</td>
<td>0.123±0.037</td>
<td>0.021±0.004</td>
<td>1.361±0.152</td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n=3)

Note: ⁶ PBS (pH7.4) containing CF (5.0mg/ml); ⁷ Hydroethanolic solution of ethanol and PBS pH 7.4 (9:11, v/v) containing CF (5.0mg/ml); ⁸ Conventional liposomes containing CF; ⁹ Invasomes containing CF; ¹⁰ Ethosomes containing CF; ¹¹ Not detected
Table 6: Results of skin penetration and deposition of mTHPC into full-thickness human skin from different formulations with infinite dosage application (160µl/cm²) after 12hr

<table>
<thead>
<tr>
<th>Formulations</th>
<th>SC1-5</th>
<th>SC6-10</th>
<th>Epidermis</th>
<th>Dermis</th>
<th>Receptor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTHPC-ET^a</td>
<td>0.200±0.017</td>
<td>0.043±0.013</td>
<td>0.091±0.020</td>
<td>n.d.*</td>
<td>n.d.</td>
<td>0.334±0.030</td>
</tr>
<tr>
<td>mTHPC-HE^b</td>
<td>0.364±0.013</td>
<td>0.026±0.009</td>
<td>0.010±0.006</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.400±0.001</td>
</tr>
<tr>
<td>mTHPC-CL^c</td>
<td>0.228±0.044</td>
<td>0.016±0.005</td>
<td>0.011±0.002</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.255±0.048</td>
</tr>
<tr>
<td>mTHPC-INS^d</td>
<td>0.113±0.009</td>
<td>0.010±0.003</td>
<td>0.008±0.003</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.131±0.015</td>
</tr>
<tr>
<td>mTHPC-ETS^e</td>
<td>0.288±0.018</td>
<td>0.013±0.004</td>
<td>0.020±0.004</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.322±0.012</td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n=3)

Note: ^a Ethanol solution containing mTHPC (1.5mg/ml); ^b Hydroethanolic solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC (1.5mg/ml); ^c Conventional liposomes containing mTHPC (1.5mg/ml); ^d Invasomes containing mTHPC (1.5mg/ml); ^e Ethosomes containing mTHPC (1.5mg/ml); *Not detected
Part Four

Final Discussion

As mentioned in the introduction section, the function of liposomes as skin drug delivery system varies with their type and composition, which has also been confirmed from all the results in this dissertation. Several kinds of liposomal systems were involved in this doctoral study, including conventional liposomes, deformable liposomes, invasomes, and ethosomes. As can be seen, for any given model drug, the composition diversity of its different carriers, lipid vesicles, could influence their physicochemical characteristics such as particle size, charge, morphology and bilayer elasticity, which in turn have a significant effect on the interaction between these vesicles and the skin and hence on their effectiveness as skin delivery systems. In addition, three model drugs with different lipophilicity were also involved in this dissertation, including Ferulic acid (logP_{o/w}=1.51), Carboxyfluorescein (logP_{o/w}=-1.5) and Temoporfin (logP_{o/w}=9.24). Thus, for a specific lipid vesicular system, the skin permeation or penetration and deposition behavior of its “cargo”, different model drugs, were significantly different. Hence, although the skin delivery of an active ingredient can be optimized by well-designed liposomal systems, it could be unfeasible to develop a universal liposomal formulation which would result in optimized skin delivery for all the active ingredients. In other words, the optimized skin drug delivery via liposomal systems should be designed and tested on a case by case basis.

Furthermore, skin drug delivery via liposomal systems could involve several processes, including interaction between SC and lipid vesicles, partitioning of the drug from its lipid vesicular system to the skin and the following drug diffusion in the skin. Thus, the process of percutaneous absorption of model drug via liposomal systems as a whole could be influenced by not only the interaction between the liposomal system and skin but also the interactions between the liposome and drugs and ones between skin and drugs. And it is likely that these interactions might coincide since the lipid vesicles could interact with skin as well as the drug encapsulated.
1 Preparation and Characterization of Lipid vesicular systems

All vesicular systems developed in publication 2 and 3 were prepared by a conventional rotary evaporation method combined with extrusion method. More detailed information could be found in the publication section. From the results on particle size distribution, the particle size of different lipid vesicular systems ranged from \(~80\text{nm}\) to \(~130\text{nm}\), with all PDI < 0.2, indicating that all of them were highly homogeneous suspension systems. More specially, regardless of the model drug encapsulated, conventional liposomes showed the largest mean vesicle size. Ethosomes always showed the smallest mean particle size, which could be attributed to the incorporation of high amount of ethanol. Ethanol could probably reduce the membrane thickness due to the formation of a phase with interpenetrating hydrocarbon chains (Dubey et al., 2007). Moreover, the addition of ethanol in phospholipid vesicles imparts negative charge to the formulation (Touitou et al., 2000), which also explains there a net change flip over from conventional liposomes to other vesicles with fluid bilayers with the presence of ethanol. This modification of net charge of liposomal systems confers lipid vesicles some degree of steric stabilization and in turn lead to decrease in mean vesicle size (Jain et al., 2007). The reduction of particle size of Tween 80-based deformable liposomes when compared conventional liposomes when compared conventional liposomes could also be explained by inclusion of the surfactant involved (Elsayed et al., 2007a). In case of invasomes, lysophosphatidylcholine (LPC), as one of the components of NAT 8539, has a single hydrophobic chain and a polar head group and integrates spontaneously into membrane bilayers, is acting as a surfactant and creating a high positive curvature in membranes (Fuller and Rand, 2001). However, the inclusion of 1% (w/v) terpene mixture could lead to the increase of particle size distribution (Dragicevic-Curic et al., 2008), when compared with ethosomes and Tween 80-based deformable liposomes.

From the results of Cryo-Transmission electron microscopy, regardless of model drugs encapsulated, the lipid vesicles had similar shapes and structures. The vesicles of the conventional liposomes seemed to be unilamellar and rarely bilamellar, almost spherical and oval in shape, and some detected oligolamellar vesicles. In the case of Tween 80-based deformable liposomes, the vesicles were also mostly unilamellar. In the case of invasomes, the vesicles seemed to be almost unilamellar and bilamellar, but the percentage of deformed vesicles increased in comparison to other liposomal
structures. Regarding ethosomes, the vesicles appeared to be homogenously unilamellar.

The ζ-potential is related to the charge on the surface of the lipid vesicle which influences both vesicular properties such as physical stability and skin vesicle interactions. The electrostatic repulsive forces can prevent the dispersed particles having surface charges of the same sign from approaching each other. For dispersed particles below of 1µm, moderate physical stability is achieved when the ζ-potential is between ±30 and ±60mV, and good to excellent physical stability is achieved when the ζ-potential is between ±60 and ±100mV (Rungseevijitprapa et al., 2009). Invasomes, regardless of model drug encapsulated, showed a ζ-potential about -40mV. Ethosomes containing ferulic acid (Publication 2) showed a ζ-potential about -30mV, while ethosomes containing CF or mTHPC showed a ζ-potential about -80mV. Thus, invasomes and ethosomes developed in this doctoral study were well stabilized against interparticle attraction or coagulation. On the other hand, conventional liposomes and Tween 80 based deformable liposomes had low ζ-potentials and tend to be more sensitive to coagulation. However, all liposomal formulations for in-vitro penetration/permeation study were freshly prepared in order to avoid instability of lipid vesicular systems. The effect of surface charge of liposomes on the drug penetration has not been fully understood so far. Some researchers support the theory that the positive charges on the surface of liposomes could bind to negative charges of the SC enhancing thereby the drug penetration/permeation through the skin (Katahira et al., 1999; Song and Kim, 2006). However, other studies found that permeation of drugs through the skin is promoted by negatively charged vesicles (Ogiso et al., 2001; Sinico et al., 2005). According to the best of our knowledge, the surface charge of the vesicles will not only play a role on the interaction between skin and vesicles but also might contribute to the drug release from the vesicles. The drug release from vesicles in the stratum corneum is an important step which will affect transdermal flux(Honeywell-Nguyen and Bouwstra, 2003). The rate and amount of released drug is a balance between two factors: (1) drug affinity to vesicles, and (2) drug solubility in lipids of the stratum corneum (Honeywell-Nguyen and Bouwstra, 2003). The results of the pilot experiment of publication 2 revealed that the flux of FA from negatively charged ethosomes is somewhat higher than from positively charged ethosomes, but without any significant difference. Since both the pKa1 of FA (4.52 (Erdemgil et al., 2007)) and pKa of CF (6.3 (Nicole et al., 1989)) are below 7.4, they
are negatively charged at pH 7.4. Therefore we suggested that both of them could be retained in the positively charged vesicles, which in turn influences their skin penetration or permeation. Thus, we also preferred to prepare the negatively charged lipid vesicles containing mTHPC for comparison. For the effect of positively charged liposomes and neutralized liposomes on the skin penetration and deposition of CF and mTHPC is investigated in future.

2 Human Skin Preparation

As mentioned in the introduction section, when a skin drug delivery system is applied topically, two principle absorption routes are involved, including the transepidermal route, where the drug delivery system interacts with or diffuses through stratum corneum, and transfollicular route, where they interact with or diffuse through the follicles. Female human abdominal skin tissue from plastic surgery was used in publication 3. From literature (Starcher et al., 2005), the hair follicles of human are surrounded by an elastic fiber network, which connects the follicles to each other. The fibers not only surround the hair follicles but also lie as parallel fibers throughout the entire dermis. This different pattern of elastic fibers suggests an important role in the elasticity of skin. If the skin is excised, the elastic fibers surrounding the hair follicles contract, which possibly leads to a significant reduction of the follicular penetration pathway (Patzelt et al., 2008). Moreover, other factors concerning the reduced follicular penetration must also be taken into consideration, such as loss of humidity and the absence of blood flow in excised skin. These aspects presumably contribute additionally to the differences in the penetration pathways to a certain extent (Patzelt et al., 2008). Furthermore, in publication 2, the stratum corneum/epidermis (SCE) membranes were prepared by a heat separation technique and applied with in vitro permeation study. In this skin model, intrafollicular route could be involved. However, at in vivo situation of human skin as mentioned in the introduction section, hair follicular route could be highly lipophilic because of presence of sebum, while the follicle route in this skin model could be a hydrophilic one due to the absence of sebum. Moreover, it remains unexplored and unclear that how different lipid vesicular systems can influence the follicular route contribution of drug permeation and deposition.

All of these problems mentioned could be investigated in future study. In this dissertation, only transepidermal route would be discussed at length.
3 The Interaction among conventional liposomes, Model drugs and skin

From the results presented in publication 2 and publication 3, when compared with deformable liposomes or ethosomes, conventional liposomes cannot deliver considerable much amount of model drug into skin deep layer, such as epidermis, dermis as well as the receptor phase (subcutaneous layer), they can significantly enhance model drugs deposition in the stratum corneum layer. This suggested that conventional liposomes could not be a suitable approach for transdermal delivery of drugs but could be relatively useful for dermal delivery. The involved mechanism of action of conventional liposomes could fall into one of three categories (reviewed in publication 1), including the intact vesicular skin penetration, the penetration enhancing effect, and vesicle adsorption to and/or fusion with the stratum corneum (El Maghraby et al., 2006).

The first possible mechanism that intact conventional liposomes can penetrate across the skin was received with skepticism. Realistically, any phospholipid vesicles can cross the skin barrier only through hydrophilic pathways (intercellular route) and they have to possess two capabilities: the colloid-induced opening of the very narrow (~0.4 nm) gaps between cells in the barrier to pores with a diameter greater than 30 nm, and self-adapting to the size of 20 to 30 nm without destruction (Cevc, 2004). Obviously, conventional liposomes fall short of these prerequisites. As a result, it is almost impossible for large conventional liposomes to penetrate the densely packed SC in great numbers. The skin permeation or penetration results in publication section also suggested that the penetration of intact conventional liposomal vesicles may be not a reasonable, or at least not a predominant, mechanism for improved skin drug delivery.

The second mechanism, penetration enhancing effect, is that conventional liposomal lipids may act as penetration enhancer, thereby loosening the lipid structure of the SC and promoting an impaired barrier function (Kirjavainen et al., 1999). From the results of publication 3, it can be recognized that the effectiveness of this penetration enhancing effect of conventional liposomes could depend on the lipophilicity of drug molecule. From results of publication 3, with same conventional liposomal formulation, most of mTHPC (both finite and infinite dose application) was found in SC top layer, while relatively much more percentage of CF (also both finite
and infinite dose application) could be delivered into skin deep layers compared with their control group, respectively. There three reasonable explanation. First, for hydrophilic molecules such as CF owing to their low partition coefficient and relatively slow diffusion through stratum corneum, they tend to be more enhance-able and hence would show a dramatic increase in permeation or penetration as well as deposition in skin deep layers via conventional liposomes. In addition, this penetration enhancing effect just increases the partitioning of model drugs into the lipophilic SC but does not really increase the partitioning of model drugs from the SC into the less lipophilic epidermis. This means that permeability of lipophilic penetrants such as mTHPC is limited by partitioning from lipophilic SC into hydrophilic epidermis. Another possible reason why most of mTHPC (both finite and infinite dose application) was found in SC top layer is that mTHPC possibly could interact with SC, specifically with keratin or lipids in corneocytes. Thus, mTHPC were bottled up in SC. This phenomenon is referred to as the Stratum Corneum reservoir effect (Vickers, 1972). Other drugs such as corticosteroids also have this phenomenon (Lu and Flynn, 2009).

Another mechanism, vesicle adsorption to and/or fusion with the stratum corneum, is that topical application of conventional liposome could result in the formation of lamellae and rough structures on skin surface, which could increase the skin hydration and in turn increase the driving force for permeation of liberated molecules. However, the collapse of vesicles on skin surface may form an additional barrier, reducing the permeation of hydrophilic molecules encapsulated in the vesicular aqueous core (Elsayed et al., 2007b). This could be responsible for the difference between CF and mTHPC on their skin distribution ratio shift and change when comparing an infinite dose with a finite dose application in the case of conventional liposome (publication 3).

In addition, one thing could be important for hydrophobic drug such as mTHPC, which is that the solubility of mTHPC in conventional liposomes was increased definitely. In publication 3, the concentration of mTHPC in all liposomal systems is 1.5mg/ml, while the aqua solution of mTHPC is not available since its extremely low solubility in water. When liposomal systems interact with skin, mTHPC encapsulated would be released into stratum corneum. This point could contribute quite a lot to skin delivery of mTHPC via conventional liposomes.
4 The Interaction among Deformable Liposomes/Invasomes, Model drugs and Skin

When compared with conventional liposomes, deformable liposomes, including Tween 80-based deformable liposomes and invasomes showed a better permeation ferulic acid across the stratum corneum and epidermis membrane (Publication 2), or invasomes led to relatively higher drug accumulations in skin deep layers (including epidermis and dermis as well as the receptor phase) in the case of both CF and mTHPC (Publication 3). Thus we suggested that deformable liposomes, compared with conventional liposomes, could be more effective as a transdermal drug delivery system (Reviewed in publication 1).

There are two possible mechanisms responsible for the enhanced skin drug delivery via deformable liposomes (Honeywell-Nguyen et al., 2003; Honeywell-Nguyen and Bouwstra, 2003). First, invasomes may act as drug carrier systems by which intact vesicles can enter the SC carrying vesicle-bound drug into or across the skin. Water gradient or xerophobia, which is the tendency to avoid dry surroundings of water-“loving” phospholipids, was regarded as the driving force for deformable liposomes entering the skin by this mechanism (Cevec and Blume, 1992). However, the water gradient across the skin may not be linear, consequently, as a result of the osmotic force such vesicles will not penetrate beyond the level of the lowest layers in the SC (Honeywell-Nguyen and Bouwstra, 2003). Consequently, the drugs will be released first from such vesicles and then penetrate alone to skin deep layers or to reach the systemic circulation (Bouwstra and Honeywell-Nguyen, 2002). Therefore, non-occlusive application is necessary for the enhanced skin drug delivery based on deformable liposomes by this mechanism. As mentioned above, when infinite dose applied, with a thick formulation liquid layer formed covering on the skin surface, deformable liposomes such as invasomes could lose its penetration driving force because of the disappearance of the trans-epidermal osmotic gradient in this application mode. Hence, less percentage of CF and mTHPC could be delivered into skin deep layers with infinite dose application (Publication 3). Second, deformable liposomes may also act as penetration enhancers, whereby the vesicle lipid bilayers interact with the SC and subsequently modify the intercellular lipid lamellae. Similarly, from the results presented in publication 3, penetration enhancement of model drugs by this mechanism could be influenced by the lipophilicity of model...
drug. Specially, for hydrophilic drugs such as CF, the penetration enhancing effect could play a more important role in the enhanced skin delivery than in the case of lipophilic drugs. The possible reasons have already been discussed above. In fact, one of the two mechanisms might predominate according to the physicochemical properties of the drug considered (Elsayed et al., 2007b).

In addition, Tween 80-based deformable liposomes (10% of ethanol involved inside) showed a better permeation profile and skin deposition of ferulic acid than hydroethanolic solution (Publication 2). This could be explained by the synergistic effect of phospholipids and ethanol, which is that ethanol and phospholipids applied together have a synergistic effect on fluidizing the intercellular SC lipids, which results in an enhanced penetration of chemicals (Verma and Fahr, 2004). Moreover, the enhanced skin delivery of ferulic acid via invasomes when compared with hydroethanolic solution (Publication 2) as well as the enhanced skin delivery of CF via invasomes when compared with hydroethanolic solution (Publication 3) could also support a similar synergistic effect of phospholipids, terpenes and ethanol. However, improved skin delivery of mTHPC via invasomes was not observed when compared with hydroethanolic solution. The main possible reason is that mTHPC is highly hydrophobic, the incorporation of water in ethanol will increase its thermodynamic activity compared with pure ethanol solution containing the same concentration of mTHPC. For the lipid vesicular systems containing mTHPC such as invasomes prepared in publication 3, even though high water amounts are involved, entrapment of mTHPC in the lipid bilayers, in fact, solubilizes mTHPC. Hence, the thermodynamic activity of mTHPC of these systems is not equally increased compared with mTHPC hydroethanolic solution, which explains why mTHPC hydroethanolic solution showed the better potential of improving mTHPC skin delivery than all lipid vesicles including invasomes. From all these results presented in publication 2 and publication 3, we support the hypothesis that some of the invasomes were fragmented during their penetration through the SC, while some of the small and deformable invasomes could have penetrated to the deeper SC layers intact.

In short, the mechanism of action of deformable liposomes or invasomes is not clearly and completely understood, and it should be further investigated and confirmed with involving more model drugs and novel technologies.
5 The Interaction between Ethosomes, Model drugs and Skin

As we can see from the results presented in publication section, Ethosomes showed a better permeation profile and a higher skin epidermis deposition of ferulic acid than all other lipid vesicular systems (Publication 2). Ethosomes also delivered more percentage of CF into skin deep layers (epidermis and dermis, as well as receptor phase) than all other lipid vesicular systems (Publication 3). Interestingly, unlike deformable liposomes, ethosomes are able to improve the skin delivery of drugs both under occlusive and non-occlusive conditions (Elsayed et al., 2007a; Lopez-Pinto et al., 2005; Paolino et al., 2005; Touitou et al., 2000), depicting a different mechanism of action for ethosomes (Reviewed in publication 1).

High concentration of ethanol (45%, v/v) involved in the ethosomal system (Publication 2 and 3) must be responsible for the enhanced skin drug delivery described. Ethanol is a well-known permeation enhancer (Williams and Barry, 2004). The penetration enhancing effect of ethanol can be attributed to two effects: (a) “Push effect”: increased thermodynamic activity due to evaporation of ethanol and improved solubility of solute in this study; (b) “Pull effect”: ethanol can interact with intercellular lipid molecules in the polar head group region, thereby increasing their fluidity and decreasing the density of the lipid multilayer, which results in an increase in membrane permeability. Ethanol is also supposed to extract the SC lipids (Bach and Lippold, 1998) lowering thereby the barrier function of the SC.

Furthermore, ethosomes showed a better permeation profile and skin deposition of ferulic acid than both ethanol solution and phospholipid ethanol solution (Publication 2). Also, ethosomes delivered much more CF into skin deep layers, including epidermis, dermis and receptor phase than hydroethanolic solution (Publication 3). These results support that a synergistic mechanism between ethanol and lipid vesicles could facilitate drug delivery to the deeper skin layers or across the skin (Dayan and Touitou, 2000; Elsayed et al., 2006; Touitou et al., 2000). More, ethanol could impart fluidity to the vesicle's bilayers, which in turn facilitates vesicles skin permeation. Furthermore, ethanol has a tendency to mix with lipids as well as water (Panchagnula et al., 2005), with increasing the partition of liposomal particles into skin. Thus it can act as a “blending” agent for lipid vesicles with increasing their distribution in skin (Panchagnula et al., 2005). The ethanol effects can be followed by the interaction between ethosomal vesicles and the skin. The ethosomal vesicles may
also behave as deformable liposomes and can interact with the skin barrier to “forge” penetration or permeation pathways by itself in the highly organized SC and finally release drug at various points along the penetration pathway as well as in deep skin layers (Elsayed et al., 2007b).

From the discussion above, we suggest that ethanol and its concentration are key points which influence the physicochemical characteristics of the ethosomes and in turn affect the ability of ethosomes as skin delivery systems. In fact, the effect of concentration of ethanol on skin drug delivery ability of ethosomes has been investigated in pilot experiments of publication 2 (data not involved in this dissertation). From the results of this pilot experiment, 45% of ethanol in the ethosomal system showed the best potential for enhanced skin delivery of ferulic acid. For comparison, this concentration was also applied in publication 3. However, for the purpose of optimum skin delivery of different model drugs, optimal concentration of ethanol in ethosomes could be varied depending on the physicochemical property of model drug. Thus, effective ethosomes for different model drugs could be also designed and test on a case by case basis. For instance, from the results presented in publication 3, ethosomes didn’t show a significantly enhanced skin delivery of mTHPC compared with ethanol solution and hydroethanolic solution. Two possible reasons have been discussed above. Briefly, first, since mTHPC can be encapsulated in the ethosomal vesicles bilayers, ethosomes could also be the solubilization matrix of mTHPC. Thus the thermodynamic activity of mTHPC in ethosomes is not equally increased compared with mTHPC hydroethanolic solution. Second, the ethosomes containing mTHPC developed in publication 3 didn’t show a satisfying and promising skin penetration enhancing effect for mTHPC compared with its hydroethanolic group. From this point of view, the composition of ethosomes need be optimized further. Moreover, even with same composition, different vesicular structure could be developed by different preparation method. Therefore, better designed lipid carrier systems especially for mTHPC could be developed in future.

6 The Effect of Application mode on Skin Drug Delivery

The compositions of these formulation change due to penetration into the skin or evaporation of volatile components. These changes depend on the amount of liposome applied and occlusion conditions. Many studies have employed non-occluded conditions, but the application amount was varied. The amounts of formulation have
ranged from 10μL/cm² (Dragicevic-Curic et al., 2008; Verma and Fahr, 2004) to 1.5mL/cm² (Elsayed et al., 2007a). These differences may be related to variations in the observed effects of lipid vesicular systems, but there have been few reports aiming to clarify the effects of dose on lipid vesicular systems penetration.

In publication 3, a comparison of an infinite dose and a finite dose under non-occlusive application for both CF and mTHPC revealed that the total amount of both of them delivered into skin were significantly increased when an infinite dose applied, but to a significantly different extent depending on the lipophilicity of model drugs. There could be two possible reasons responsible for this. The first one is that the increased skin hydration effect rooted from a thick liquid formulation layer covering on the skin surface formed by an infinite dose applied in donor compartment. This increased tissue hydration appears to increase transdermal delivery of both hydrophilic and low lipophilic compounds such as CF due to an increase in partition into the skin of drugs but not for highly lipophilic drug such as mTHPC. Another possible reason could be the different molecular mechanisms by which the diffusion through the stratum corneum of CF and mTHPC is acting. More specifically, with an infinite dose of formulation applied on the skin, the modification of the micro-structure of SC could be increased compared with a finite dose applied. This will facilitate the skin penetration and deposition of CF because the limiting step for skin penetration of CF is its partitioning into SC from the formulation. However, due to the limiting step for mTHPC being the partitioning from the SC into the less lipophilic epidermis, the augmented modification of the micro-structure of SC by an infinite dose could not result in the same enhancing effect for mTHPC as for CF.

In short, application mode, including infinite dose application and finite dose application, not only had a direct action on the drug skin penetration and deposition, but also could affect the degree of hydration of the SC and the possible mechanism of some lipid vesicular system which in turn influence the drug skin distribution.
Part Five

Summary

Skin is the largest organ of the human body and offers a glorious route for delivering drugs because skin drug delivery brings forth many attractive advantages over other routes of administration. On the other hand, however, skin is also an excellent biological barrier, which imposes physicochemical limitations to the type of permeants which can traverse it. Over the past decades, numerous studies have been performed to overcome the problems associated with skin delivery and also a number of novel skin delivery systems and approaches have been developed. Among these novel techniques, lipid vesicular systems may offer a promising strategy for improving skin drug delivery.

In this doctoral dissertation, different lipid vesicular systems including conventional liposomes, Tween 80-based deformable liposomes, invasomes and ethosomes were prepared and characterized in order to improve the skin delivery of ferulic acid, which is a traditional Chinese medicine exhibiting a wide range of therapeutic effects against various diseases. As can be seen from in vitro permeation and deposition study, skin permeation flux and skin deposition of ferulic acid could be significantly improved by well developed lipid vesicles. Moreover, different lipid vesicular systems containing another two model drugs, Carboxyfluorescein and Temoporfin, were developed and characterized in order to evaluate and compare the influence of different lipid vesicular systems on their skin penetration and deposition behaviors. In addition, different application modes, including application of finite dosage and infinite dosage, were involved and investigated. In vitro penetration and deposition studies revealed that different lipid vesicular systems could possibly provide variable skin drug delivery effects according to their composition, entrapped drug as well as application mode applied.

Also, a variety of possible mechanisms have been discussed in details for the enhanced skin delivery of drugs from different lipid vesicles, implying that the skin drug delivery via lipid vesicular systems could be influenced by the interaction not only between the liposomal system and skin but also between the liposome and drugs, and between skin and drugs.
Zusammenfassung


Part Six

References


Lademann, J., Richter, H., Meinke, M., Sterry, W., Patzelt, A., 2010. Which skin model is the most appropriate for the investigation of topically applied substances into the hair follicles? Skin Pharmacol Physiol 23, 47-52.


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1. Ming Chen, Khaled Shalaby Ahmed, Xiangli Liu, Alfred Fahr. Skin penetration and deposition of mannitol and Corticosterone from different lipid vesicular systems.

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Jena, 12th of December, 2010

Ming Chen