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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, ζ-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/cm^2) and infinite dose application (160 μl/cm^2), 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.

1. Introduction

Skin covers a surface area from 1.5 to 2.0 m^2 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 (Cevc, 1996; Cevc 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

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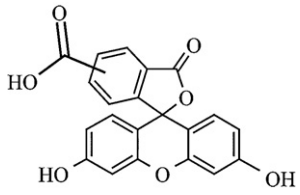

Fig. 1. Structure of 5(6)-carboxyfluorescein.

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, Fig. 1, hydrophilic molecule, $\log P_{o/w} = -1.5$; Nicole et al., 1989) and temoporfin (mTHPC, Fig. 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 available. 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 10 μl/cm^2 (Dragicevic-Curic et al., 2008; Verma and Fahr, 2004) to 1.5 ml/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 purchased 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).

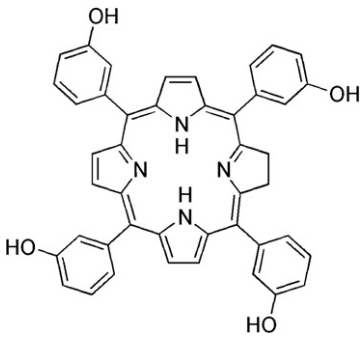

Fig. 2. Structure of temoporfin.

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 (Rotavapor R-114, BÜCHI, Flawil, Switzerland). 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 min 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).

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, 10 mM), which were also filtered throughpolycarbonate 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 and Khan Ghilzai, 2006). Calculation of ζ-potential (mV) was done by the instrument from electrophoretic mobility (Fang et al., 2008).

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 2 s between two pieces of filter-paper-strips. Shortly after that, the samples were frozen by rapidly plunged in liquid ethane (cooled to −170 to −180 °C) in a cryo-box (Carl Zeiss NTS GmbH, Germany). Excess ethane was removed with a piece of

Table 1
Composition of different lipid vesicular systems and control groups.

Code	Lipids and other components	Drug	Solvent system
CF-CL[a]	Lipoid-S100, 40.0 mg/mlCholesterol, 3.0 mg/ml	CF 5.0 mg/ml	PBS (pH 7.4)
CF-INS[b]	NAT8539, 133.0 mg/ml Terpenes[k], 10.0 mg/ml	CF 5.0 mg/ml	PBS (pH 7.4)Ethanol (10%, v/v)
CF-ETS[c]	Lipoid-S100, 40.0 mg/ml DPPG, 4.0 mg/ml	CF 5.0 mg/ml	PBS (pH 7.4)Ethanol (45%, v/v)
mTHPC-CL[d]	Lipoid-S100, 40.0 mg/mlCholesterol, 3.0 mg/ml	mTHPC 1.5 mg/ml	PBS (pH 7.4)
mTHPC-INS[e]	NAT8539, 133.0 mg/ml Terpenes[k], 10.0 mg/ml	mTHPC 1.5 mg/ml	PBS (pH 7.4)Ethanol (10%, v/v)
mTHPC-ETS[f]	Lipoid-S100, 40.0 mg/ml DPPG, 4.0 mg/ml	mTHPC 1.5 mg/ml	PBS (pH 7.4)Ethanol (45%, v/v)
CF-PBS[g]	–	CF 5.0 mg/ml	PBS (pH 7.4)
CF-HE[h]	–	CF 5.0 mg/ml	PBS (pH 7.4)Ethanol (45%, v/v)
mTHPC-ET[i]	–	mTHPC 1.5 mg/ml	Ethanol
mTHPC-HE[j]	–	mTHPC 1.5 mg/ml	PBS (pH 7.4)Ethanol (60%, v/v)

[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] PBS (pH 7.4) 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).

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 36 mm were punched out, cleaned with PBS (pH 7.4, 50 mM) 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 cm^2 and 15.0 ml, respectively. The acceptor compartment was filled with PBS buffer (pH 7.4, 50 mM) 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 h 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/cm^2 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, 160 μl/cm^2 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 (CristallKlarTesa®, 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 SC_{1-5}, while vial 2 contained strip 6–10 and named after SC_{6-10}. 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, 2 ml, 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,

Table 2
Characterization of different lipid vesicular systems.

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

Values represent mean ± S.D. ($n = 3$).
[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.

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 30 s. Afterwards the dispersions were ultra-centrifuged (10 min, 13,000 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. 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 to 0.25 μg/ml ($r = 0.9998$) and from 0.015 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 ± 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 ζ-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 ($n = 3$) and 77.8 ± 0.5 nm ($n = 3$) 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) is the reasoned explanation forthe 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 steric stabilization and in turn lead to a decrease in mean vesicle size (Jain et al., 2007). All of these mechanisms suggest 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 in reasonable 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 in 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). 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_{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 pK_a 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.

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 similar 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 (10 $\mu l/cm^2$) for CF or mTHPC after 12 h by a range of formulation vehicles are shown in Tables 3 and 4, respectively, with their distribution in different skin layers profiles shown in Figs. 4 and 5, respectively.

In the case of CF, the highest CF accumulation from all the test formulations (Table 3 and Fig. 4) was found in the SC superficial layer (Stratum Corneum tape stripping layer No. 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 (pH 7.4) 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 No. 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 did not 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.

In the case of mTHPC, the highest mTHPC accumulation from all the test formulations (Table 4 and Fig. 5) 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.

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) (Fig. 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 L 1-5, the drug skin distribution differed a lot according to the lipid vesicles applied (Fig. 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). First, 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

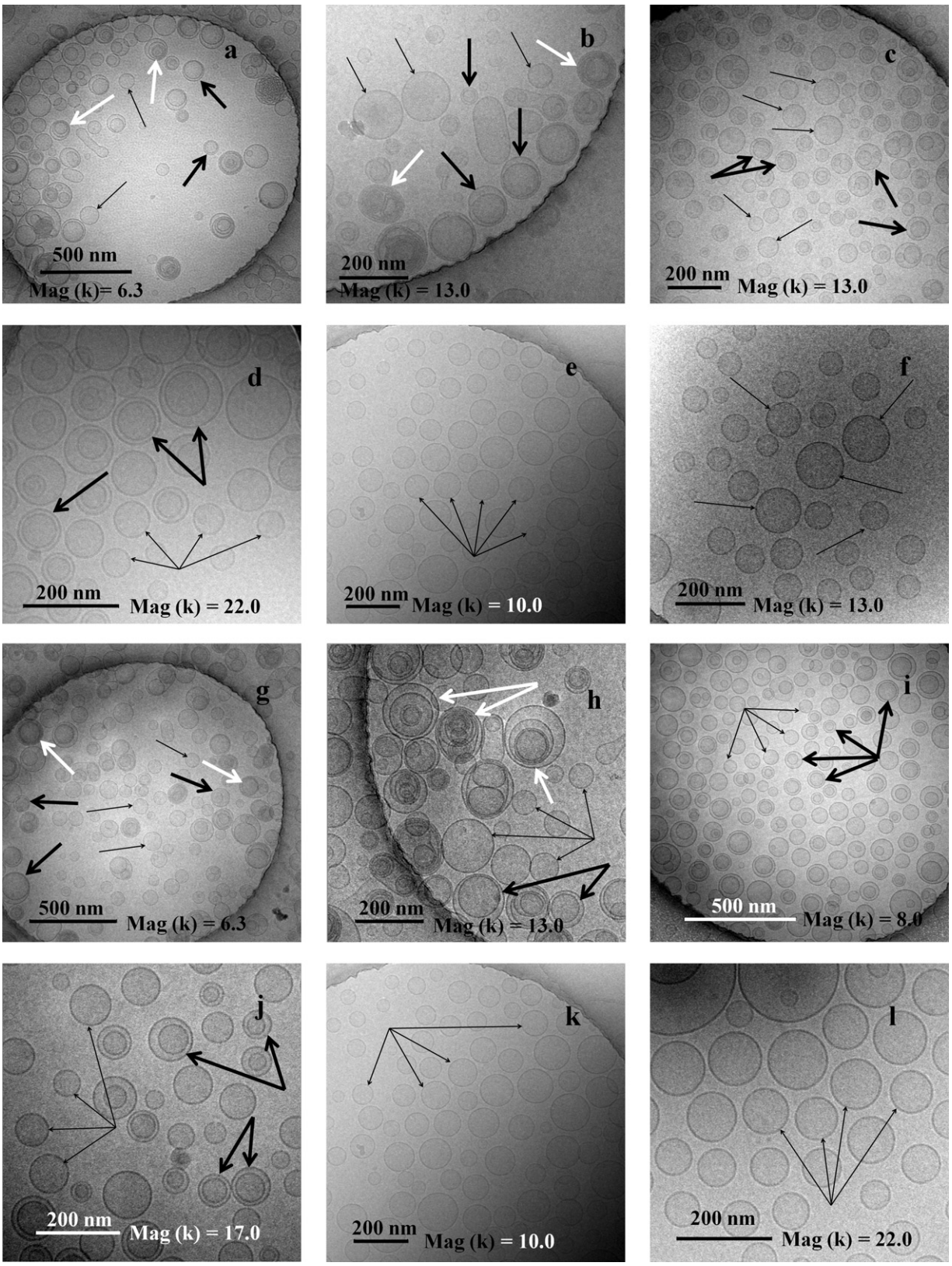

Fig. 3. (A) Visualization of different liposomal systems containing hydrophilic model drug (CF) bycryo-transmission electron microscopy: (a and b) conventional liposomes containing 5.0 mg/ml of CF; (c and d) invasomes containing 5.0 mg/ml of CF; (e and f) ethosomes containing 5.0 mg/ml of CF. Black light arrows represent unilamellar vesicles; black thick arrows represent bilamellar vesicles, while white thick arrows represent oligolamellar vesicles. (B) Visualization of different liposomal systems containing lipophilic model drug (mTHPC) by cryo-transmission electron microscopy: (g and h) conventional liposomes containing 1.5 mg/ml of mTHPC; (i and j) invasomes containing 1.5 mg/ml of mTHPC; (k and l) ethosomes containing 1.5 mg/ml of mTHPC. Black light arrows represent unilamellar vesicles; black thick arrows represent bilamellar vesicles, while white thick arrows represent oligolamellar vesicles.

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 h.

Formulations	Dose CF delivered (%)					
	SC1-5[g]	SC6-10[h]	Epidermis	Dermis	Receptor	Total
CF-PBS[a]	0.048 ± 0.010	0.001 ± 0.001	0.013 ± 0.006	0.032 ± 0.007	n.d.[f]	0.094 ± 0.020
CF-HE[b]	0.388 ± 0.005	0.039 ± 0.005	0.024 ± 0.005	0.020 ± 0.004	n.d.	0.471 ± 0.010
CF-CL[c]	0.070 ± 0.009	0.009 ± 0.005	0.029 ± 0.014	0.029 ± 0.011	n.d.	0.137 ± 0.018
CF-INS[d]	0.042 ± 0.003	0.023 ± 0.001	0.045 ± 0.012	0.027 ± 0.003	n.d.	0.136 ± 0.006
CF-ETS[e]	0.434 ± 0.026	0.253 ± 0.063	0.044 ± 0.011	0.071 ± 0.030	n.d.	0.802 ± 0.090

Values represent mean ± S.D. (n = 3).
[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.
[g] Stripped tapes of stratum corneum containing from 1st to 5th tapes.
[h] Stripped tapes of stratum corneum containing from 6th to 10th tapes.

Table 4
Results of skin penetration and deposition of mTHPC into full-thickness human skin from different formulations with finite dosage application (10 μl/cm²) after 12 h.

Formulations	Dose mTHPC delivered (%)					
	SC1-5[g]	SC6-10[h]	Epidermis	Dermis	Receptor	Total
mTHPC-ET[a]	1.500 ± 0.113	0.040 ± 0.013	0.047 ± 0.007	n.d.[f]	n.d.	1.580 ± 0.120
mTHPC-HE[b]	2.893 ± 0.480	0.200 ± 0.053	0.133 ± 0.027	n.d.	n.d.	3.220 ± 0.487
mTHPC-CL[c]	1.673 ± 0.167	0.073 ± 0.013	0.033 ± 0.007	n.d.	n.d.	1.780 ± 0.180
mTHPC-INS[d]	0.447 ± 0.180	0.033 ± 0.020	0.047 ± 0.020	n.d.	n.d.	0.527 ± 0.160
mTHPC-ETS[e]	1.253 ± 0.053	0.040 ± 0.020	0.060 ± 0.020	n.d.	n.d.	1.353 ± 0.073

Values represent mean ± S.D. (n = 3).
[a] Ethanol solution containing mTHPC (1.5 mg/ml).
[b] Hydroethanolic solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC (1.5 mg/ml).
[c] Conventional liposomes containing mTHPC (1.5 mg/ml).
[d] Invasomes containing mTHPC (1.5 mg/ml).
[e] Ethosomes containing mTHPC (1.5 mg/ml).
[f] Not detected.
[g] Stripped tapes of stratum corneum containing from 1st to 5th tapes.
[h] Stripped tapes of stratum corneum containing from 6th to 10th tapes.

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

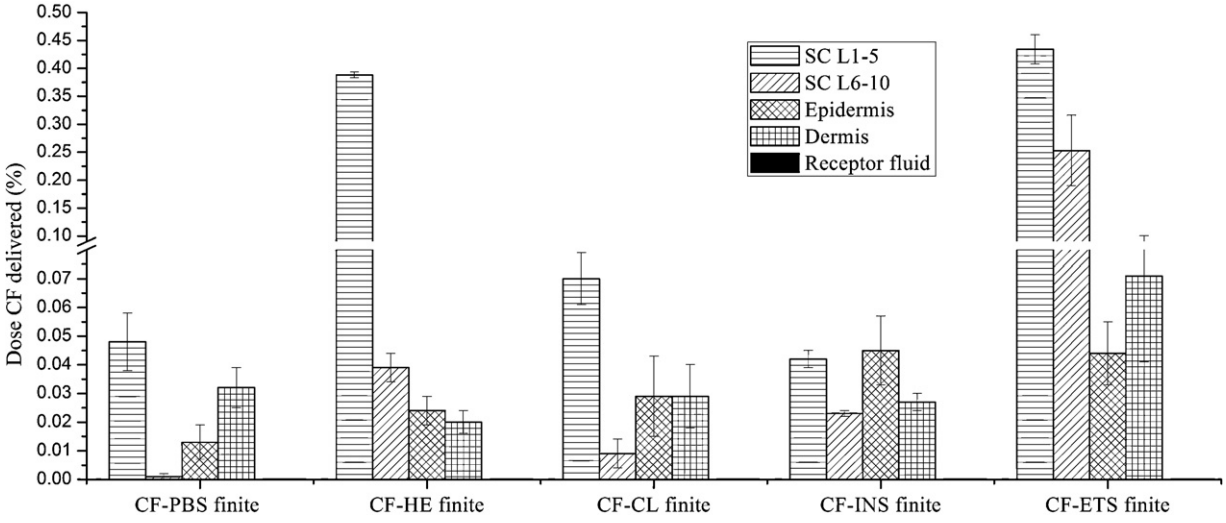

Fig. 4. Skin penetration and deposition of CF in different skin layer via full-thickness human skin from different formulations with finite dosage application (10 μl/cm²) after 12 h. *Note*: CF-CL: conventional liposomes containing CF (5.0 mg/ml); CF-INS: invasomes containing CF (5.0 mg/ml); CF-ETS: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

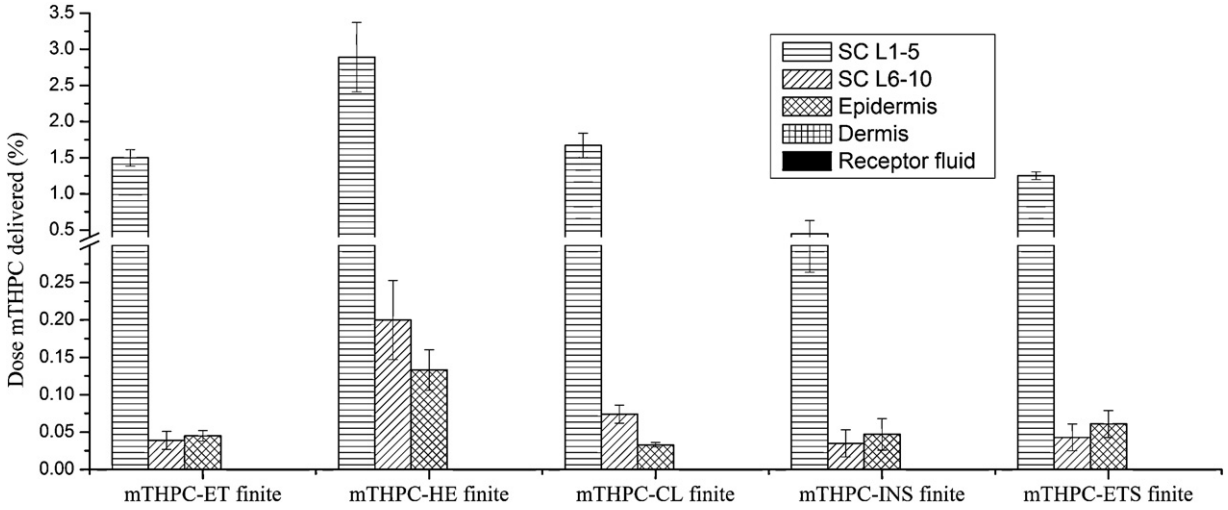

Fig. 5. Skin penetration and deposition of mTHPC in different skin layer via full-thickness human skin from different formulations with finite dosage application (10 μl/cm^2) after 12 h. *Note*: mTHPC-CL: conventional liposomes containing mTHPC (1.5 mg/ml); mTHPC-INS: invasomes containing mTHPC (1.5 mg/ml); mTHPC-ETS: ethosomes containing mTHPC (1.5 mg/ml); mTHPC-ET: ethanol solution containing mTHPC (1.5 mg/ml). mTHPC-HE: hydroethanolic mixture solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC (1.5 mg/ml). Values represent mean ± S.D. (n = 3).

SC and finally release drug at various points along the penetration pathway as well as in deep skin layers (Elsayed et al., 2007b; Godin and Touitou, 2003).

Moreover, the different possible 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 $\log P_{o/w}$ of drugs is thought to be the key factor (Verma and Fahr, 2004). The $\log P_{o/w}$ 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 $\log P_{o/w}$ 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, invasomes (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 invasomes 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 invasomes, 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 was 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 solutions (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 (160 $\mu l/cm^2$) for CF or mTHPC after 12-h non-occlusive treatment with a range of formulation vehicles are shown in Tables 5 and 6, respectively, with their distribution in different skin layers profiles shown in Figs. 6 and 7, respectively.

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 $\gg$ 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 $\gg$ 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 fluorescent detection. 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.

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 $\approx$ mTHPC-ETS $\geq$ mTHPC-INS and mTHPC-ETS > mTHPC-CL $\approx$ mTHPC-HE $\geq$ 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.

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 Fig. 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 Fig. 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 1.6 mm (calculated value), while a finite dose just can form a thin layer of only 0.1 mm (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 support 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

Table 5
Results of skin penetration and deposition of CF into full-thickness human skin from different formulations with infinite dosage application (160 μl/cm^2) after 12 h.

Formulation	Dose CF delivered (%)					
	SC1-5[g]	SC6-10[h]	Epidermis	Dermis	Receptor	Total
CF-PBS[a]	0.020 ± 0.007	0.006 ± 0.003	0.005 ± 0.001	0.009 ± 0.002	n.d.[f]	0.041 ± 0.013
CF-HE[b]	0.361 ± 0.050	0.044 ± 0.005	0.011 ± 0.001	0.015 ± 0.005	n.d.	0.431 ± 0.051
CF-CL[c]	0.077 ± 0.002	0.031 ± 0.003	0.010 ± 0.001	0.013 ± 0.001	0.001 ± 0.001	0.133 ± 0.001
CF-INS[d]	0.038 ± 0.010	0.022 ± 0.006	0.016 ± 0.003	0.019 ± 0.002	0.002 ± 0.001	0.097 ± 0.007
CF-ETS[e]	0.682 ± 0.041	0.397 ± 0.100	0.138 ± 0.012	0.123 ± 0.037	0.021 ± 0.004	1.361 ± 0.152

Values represent mean ± S.D. (n = 3).
[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.
[g] Stripped tapes of stratum corneum containing from 1st to 5th tapes.
[h] Stripped tapes of stratum corneum containing from 6th to 10th tapes.

Table 6
Results of skin penetration and deposition of mTHPC into full-thickness human skin from different formulations with infinite dosage application (160 μl/cm^2) after 12 h.

Formulations	Dose mTHPC delivered (%)					
	SC1-5[g]	SC6-10[h]	Epidermis	Dermis	Receptor	Total
mTHPC-ET[a]	0.200 ± 0.017	0.043 ± 0.013	0.091 ± 0.020	n.d.[f]	n.d.	0.334 ± 0.030
mTHPC-HE[b]	0.364 ± 0.013	0.026 ± 0.009	0.010 ± 0.006	n.d.	n.d.	0.400 ± 0.001
mTHPC-CL[c]	0.228 ± 0.044	0.016 ± 0.005	0.011 ± 0.002	n.d.	n.d.	0.255 ± 0.048
mTHPC-INS[d]	0.113 ± 0.009	0.010 ± 0.003	0.008 ± 0.003	n.d.	n.d.	0.131 ± 0.015
mTHPC-ETS[e]	0.288 ± 0.018	0.013 ± 0.004	0.020 ± 0.004	n.d.	n.d.	0.322 ± 0.012

Values represent mean ± S.D. (n = 3).
[a] Ethanol solution containing mTHPC (1.5 mg/ml).
[b] Hydroethanolic solution of ethanol and PBS pH 7.4 (6:4, v/v) containing mTHPC (1.5 mg/ml).
[c] Conventional liposomes containing mTHPC (1.5 mg/ml).
[d] Invasomes containing mTHPC (1.5 mg/ml).
[e] Ethosomes containing mTHPC (1.5 mg/ml).
[f] Not detected.
[g] Stripped tapes of stratum corneum containing from 1st to 5th tapes.
[h] Stripped tapes of stratum corneum containing from 6th to 10th tapes.

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 deliv-

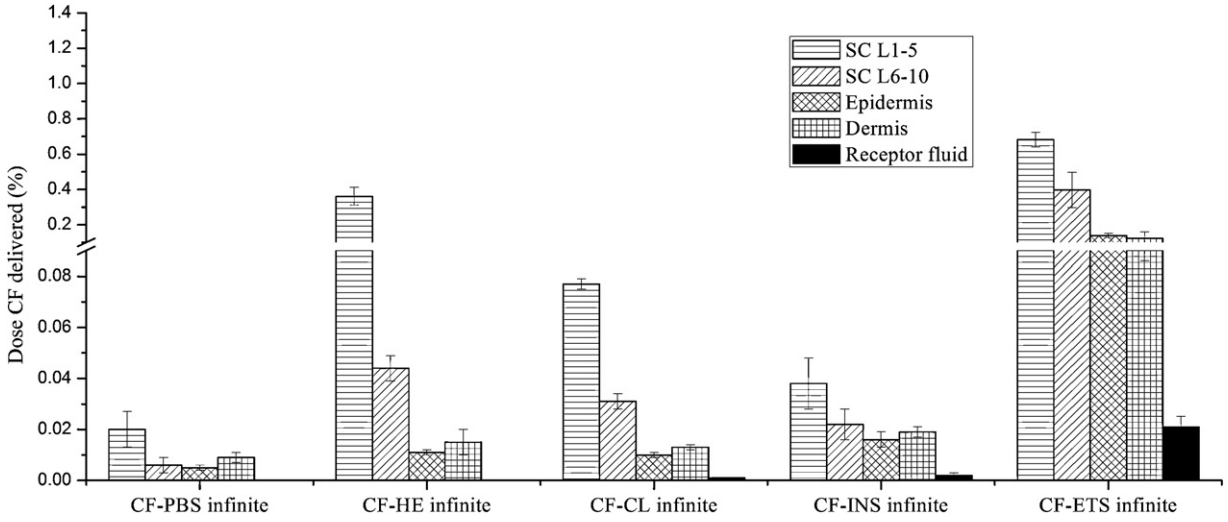

Fig. 6. Skin penetration and deposition of CF in different skin layer via full-thickness human skin from different formulations with infinite dosage application (160 μl/cm^2) after 12 h. *Note*: CF-CL: conventional liposomes containing CF (5.0 mg/ml); CF-INS: invasomes containing CF (5.0 mg/ml); CF-ETS: ethosomes containing CF (5.0 mg/ml); CF-PBS: PBS (pH 7.4) containing CF (5.0 mg/ml); CF-HE: hydroethanolic mixture solution of ethanol and PBS pH 7.4 (9:11, v/v) containing CF (5.0 mg/ml). Values represent mean ± S.D. (n = 3).

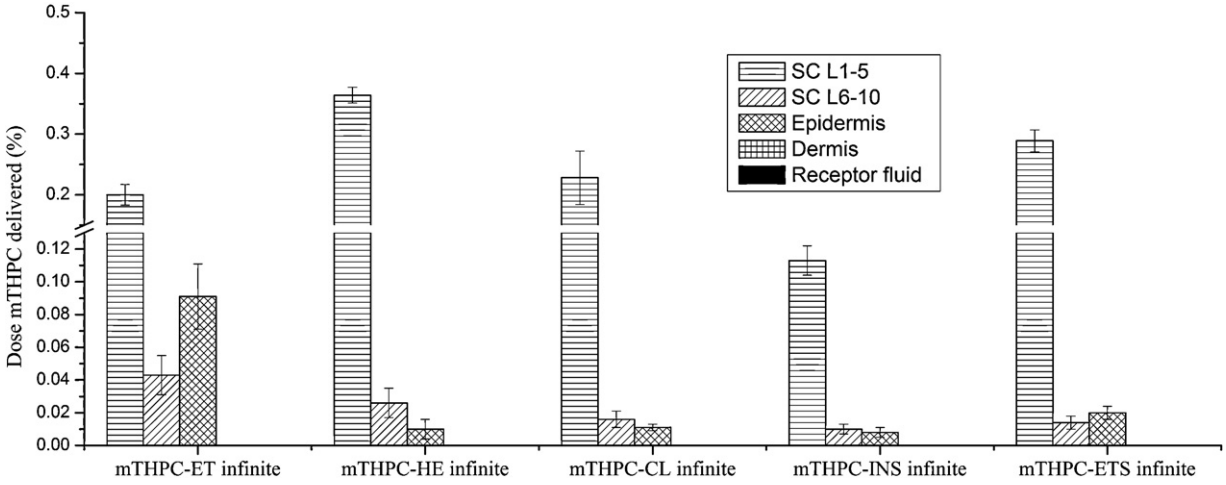

Fig. 7. Skin penetration and deposition of mTHPC in different skin layer via full-thickness human skin from different formulations with infinite dosage application (160 μl/cm^2) after 12 h. *Note*: CL: conventional liposomes containing mTHPC (1.5 mg/ml); INS: invasomes containing mTHPC (1.5 mg/ml); ETS: ethosomes containing mTHPC (1.5 mg/ml); CTL-1: control group 1 ethanol solution containing mTHPC (1.5 mg/ml). Values represent mean ± S.D. (n = 3).

ered 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 with 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. It should also 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 h) with ethanol, impairing 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, the total amount of CF delivered into skin with a finite dose (10 μl/cm^2) ranged from 0.047 ± 0.010 μg/cm^2 (CF-PBS) to 0.401 ± 0.045 μg/cm^2 (CF-ETS), while with an infinite dose (160 μl/cm^2) from 0.327 ± 0.107 μg/cm^2 (CF-PBS) to 10.837 ± 1.210 μg/cm^2 (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 (10 μl/cm^2) ranged from 0.079 ± 0.024 μg/cm^2 (mTHPC-INS) to 0.483 ± 0.073 μg/cm^2 (mTHPC-HE), while with an infinite dose (160 μl/cm^2) from 0.312 ± 0.035 μg/cm^2 (mTHPC-INS) to 0.956 ± 0.003 μg/cm^2 (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 caused by a thick liquid formulation layer-covering formed on the skin by an infinite dose applied in the 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 ($\log P_{o/w} > 2$), 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 happens, 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/cm^2), 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/cm^2), 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 detectable 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 com-

position 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.

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