International Journal of Pharmaceutics 384 (2010) 100–108

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Surface charged temoporfin-loaded flexible vesicles: *In vitro* skin penetration studies and stability

Nina Dragicevic-Curic[a,*], Susanna Gräfe[b], Burkhard Gitter[b], Sven Winter[c], Alfred Fahr[a]

[a] *Department of Pharmaceutical Technology, Friedrich Schiller University Jena, Lessingstrasse 8, 07743 Jena, Germany*
[b] *biolitec AG, Winzerlaer Strasse 2, 07745 Jena, Germany*
[c] *ratiopharm GmbH, Graf-Arco-Strasse 3, 89079 Ulm, Germany*

ARTICLE INFO

Article history:
Received 23 July 2009
Received in revised form
27 September 2009
Accepted 1 October 2009
Available online 9 October 2009

Keywords:
Temoporfin
Liposomes
Flexosomes
Skin
Percutaneous penetration

ABSTRACT

In order to increase topical delivery of temoporfin (mTHPC), a highly hydrophobic photosensitizer with low percutaneous penetration, neutral, anionic and cationic flexible liposomes (i.e. flexosomes) were prepared and investigated for their penetration enhancing ability. The *in vitro* skin penetration study was performed using human abdominal skin mounted in Franz diffusion cells. Besides the effect of surface charge of flexosomes on skin penetration of mTHPC, also its effect on physical properties (particle size, polydispersity index, lamellarity) and physicochemical stability of vesicles was investigated. Photon-correlation spectroscopy revealed that vesicles had after preparation a small particle size and low polydispersity index, while cryo-electron microscopy confirmed that these vesicles were mostly unilamellar and of a spherical shape. Regarding stability, contrasting to anionic flexosomes showing lack of long-term stability, neutral and cationic flexosomes were stable during 9 months storage at 4 °C. As to the penetration enhancing ability, cationic flexosomes possessed the highest, i.e. they delivered the highest mTHPC-amount to stratum corneum and deeper skin layers compared to conventional liposomes, neutral and anionic flexosomes.

In conclusion, mTHPC-loaded cationic flexosomes could be a promising tool for delivering mTHPC to the skin, which would be beneficial for the photodynamic therapy of cutaneous malignant or non-malignant diseases.

1. Introduction

In recent years liposomes have been intensively studied as drug carrier systems for topical delivery, since they have the potential to enhance drug penetration into the skin (El Maghraby et al., 2001; Verma et al., 2003a,b; Betz et al., 2005), improve therapeutic effectiveness (Seth et al., 2004; Mura et al., 2007), decrease side-effects (Seth et al., 2004) and act as a local depot for sustained release of dermally active components (Schreier and Bouwstra, 1994). The theory that intact conventional vesicles could penetrate into and even through stratum corneum (SC), acting as drug carrier systems has been rejected by several authors, who found no evidence of intact vesicles in the deeper skin layers (Lasch et al., 1991; Zellmer et al., 1995; van Kuijk-Meuwissen et al., 1998). It was suggested that most likely conventional vesicles disintegrate at the skin surface and that their components penetrate molecularly dispersed into the intercellular lipid matrix, where they mix with the SC lipids modifying the lipid lamellae (Hofland et al., 1994; Zellmer et al., 1995; Kirjavainen et al., 1996) and inducing new vesicle-like structures (Hofland et al., 1994). These conformational changes of SC lipids are strongly correlated with the enhanced percutaneous drug penetration (Coderch et al., 1999).

Regarding enhanced drug penetration, conventional liquid-state vesicles have proven to be superior to gel-state vesicles (van Kuijk-Meuwissen et al., 1998; El Maghraby et al., 1999, 2001), whereas elastic vesicles have shown to be superior to conventional gel-state and even liquid-state vesicles in terms of interactions with human skin (van den Bergh et al., 1999) and enhanced drug penetration (El Maghraby et al., 1999). Therefore, a series of liquid-state vesicles with elastic membranes were developed. These include vesicles containing phosphatidylcholine (PC) and edge activators (sodium cholate, polysorbate 80 or polysorbate 20), i.e. the so-called Transfersomes® (Cevc et al., 2002; Cevc and Blume, 2003, 2004), vesicles composed of the bilayer-forming surfactant L-595 (sucrose laurate ester) and the micelle-forming surfactant PEG-8-L (octaoxyethylene laurate ester) (van den Bergh et al., 1999), ethosomes containing phospholipids and high amounts of ethanol (Touitou et al., 2000) and invasomes composed of phosphatidylcholine, ethanol and a mixture of terpenes as penetration enhancers

* Corresponding author. Present address: Apoteka "Beograd", Bojanska 16/IV, 11 000 Belgrade, Serbia. Tel.: +381 65 6655330; fax: +381 11 3910347.
E-mail address: ninadragicevic@hotmail.com (N. Dragicevic-Curic).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.10.006

(Verma et al., 2004; Dragicevic-Curic et al., 2008a,b). One proposed mechanisms of the penetration enhancement of elastic vesicles is that vesicles or vesicle materials can disorganise and disrupt intercellular lipid lamellae thereby forming channel-like penetration pathways, through which drug molecules penetrate (van den Bergh et al., 1999), whereas another mechanism is that intact vesicles are penetrating into SC through pre-existing channels with low penetration resistance (Schätzlein and Cevc, 1998).

In addition to the thermodynamical state of the liposomes' membranes, many researchers have outlined that drug penetration can be influenced by modifying the surface charge of liposomes (Katahira et al., 1999; Ogiso et al., 2001; Manosroi et al., 2002). Montenegro et al. (1996) demonstrated *in vitro* a higher skin permeation of retinoic acid from positively charged liposomes compared to negatively charged liposomes, which showed a similar permeation to that obtained from neutral liposomes. Katahira et al. (1999) also found *in vitro* a higher skin permeability of rhodamine B from positively charged liposomes compared to negatively charged liposomes. In addition, Puglia et al. (2005) demonstrated *in vivo* a higher sustained release of methyl nicotinate from neutral and negatively charged liposomes with respect to positively charged liposomes, which was in accordance to results from Katahira et al. (1999), who found that negatively charged liposomes provided better drug retention in the skin compared to the positive ones. However, Ogiso et al. (2001) demonstrated *in vitro* and *in vivo* the capability of negatively charged liposomes to increase the permeation rate of different model drugs through the skin. Manosroi et al. (2004) evaluated the effect of liposomes' surface charge on the permeation of amphotericin B and showed sustained skin absorption of drugs entrapped in charged liposomes. Positive liposomes provided the highest amount of amphotericin B in the stratum corneum, while the negative liposomes delivered the highest amount of amphotericin B in the viable epidermis. Song and Kim (2006) showed that the *in vitro* skin permeation and *in vivo* penetration into the deeper skin layers of the low-molecular-weight heparin were significantly higher from cationic flexible liposomes than from anionic and neutral flexible liposomes.

Temoporfin (mTHPC) is a very potent second-generation synthetic photosensitizer. It is activated at 652 nm wavelength, with a depth of light penetration of at least 1 cm, and residual photosensitivity of only 2 weeks (Ris et al., 1991, 1993; Hopper, 2000). It was reported to be effective in the photodynamic therapy (PDT) of primary non-melanomatous tumours of the skin of the head and neck (Kübler et al., 1999). These treatments were performed by intravenous administration of mTHPC, since there are no topical formulations of mTHPC on the market. Topical application of mTHPC would be of great interest for the PDT of cutaneous malignant (basal-cell carcinoma) or non-malignant diseases (psoriasis). It would simplify the therapy, since the skin is readily accessible for topical treatment, increase the drug concentration in the skin, enhance patient compliance and restrict the residual photosensitivity only to the site of application. However, mTHPC, whose structure is given in Fig. 1, is a highly hydrophobic drug with a large molecular weight of 680 Da, which makes its percutaneous penetration very low.

The aim of our study was to develop a mTHPC-loaded carrier system able to enhance the skin delivery of mTHPC. In order to achieve this, neutral, positively and negatively charged mTHPC-liposomes with flexible (i.e. elastic) membranes (i.e. flexosomes) were prepared. Flexosomes contained PC as the main component, an edge activator (polysorbate 20), which provides the deformability of these vesicles, and positively or negatively charged lipids. The flexosomes were investigated *in vitro* for their penetration enhancing ability, using human abdominal skin as a model membrane, and for their long-term stability by storing them for 9 months at 4 °C.

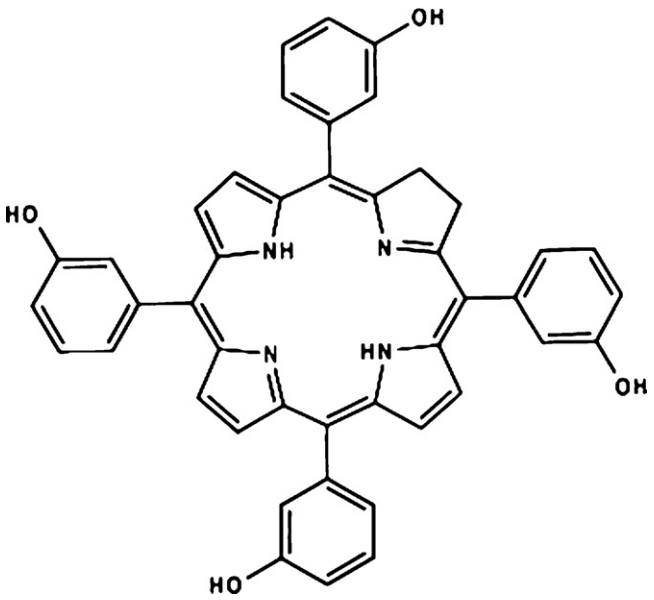

Fig. 1. Chemical structure of mTHPC.

2. Materials and methods

2.1. Materials

Unsaturated soybean phosphatidylcholine Lipoid S PC (PC, purity 98.8%) was a gift from Lipoid GmbH (Germany). Dicetyl phosphate (DCP) and stearylamine (SA) were purchased from Sigma (Germany). Unsaturated soybean PC dissolved in ethanol NAT 8539 (PC:ethanol = 75:25, w/w) was a gift from Phospholipid GmbH (Germany), and its dry residue contained PC 77.3%, lyso-phosphatidylcholine 5.0%, cephaline 3.0% and phosphatic acid 1.1%. 7,8-dihydro-5,10,15,20-tetrakis-(3-hydroxyphenyl) porphyrin (temoporfin, mTHPC) was a gift from biolitec AG (Jena, Germany). Polyoxyethylene (20) sorbitan monolaurate (polysorbate 20, Tween® 20) was obtained from Uniqema (Germany). Bovine serum albumine (BSA), potassium dihydrogenphosphate and sodium hydroxide were purchased from Sigma–Aldrich (Germany). Methanol (HPLC grade) was purchased from Carl Roth GmbH&Co. (Germany). All other chemicals were of analytical grade.

2.2. Liposome preparation

Conventional liposomes were composed only of PC, while neutral flexible liposomes (i.e. flexosomes) contained in addition to phosphatidylcholine (PC) also polysorbate 20. Cationic flexosomes were composed of PC and stearylamine (SA), approximately 9:1 molar ratio, and contained polysorbate 20. Anionic flexosomes were composed of PC and dicetyl phosphate (DCP), approximately 9:1 molar ratio, and contained polysorbate 20. The ratio of lipid to polysorbate 20 was in all flexosomes 84.6:15.4 (w/w). The amount of lipid together with polysorbate 20 was in all liposome dispersions 10% (w/w), while the mTHPC-amount was 1.5 mg/ml. The composition of the aforementioned liposome dispersions is represented in Table 1.

The different liposomes were prepared by the conventional film method. mTHPC was dissolved in solution of lipids in methanol:chloroform (2:1, v/v). This mixture was dried to a thin film by slowly reducing the pressure from 500 to 1 mbar at 50 °C using the rotary evaporator (BÜCHI Vacobox B-177, BÜCHI, Switzerland). The obtained film was kept under vacuum (1 mbar) for 2 h at room temperature and afterwards flushed with nitrogen.

Table 1
Composition of different mTHPC-loaded liposome formulations.

Formulation	mTHPC [mg/ml]	SPC [mg/ml]	SA [mg/ml]	DCP [mg/ml]	Tween 20 [mg/ml]	PBS pH 7.4
Conventional liposomes	1.5	100	–	–	–	ad 1 ml
Neutral flexosomes	1.5	84.60	–	–	15.40	ad 1 ml
Cationic flexosomes	1.5	81.35	3.25	–	15.40	ad 1 ml
Anionic flexosomes	1.5	78.26	–	6.34	15.40	ad 1 ml

The film was then hydrated with phosphate buffer saline (PBS) pH 7.4 for 30 min at 50 °C. After that, all liposome dispersions, except the anionic flexosomes, were extruded through polycarbonate membranes of different pore sizes (400 nm, 200 nm, 100 nm and 50 nm), through each 21 times, with the help of the LiposoFast® mini-extruder (Avestin, Canada). The anionic flexosomes were sonicated (15 min, duty cycle 50%, 10 W) with the probe sonicator (Soniprep 150 MSE, Zitec AG, Switzerland).

In order to enable the comparison of the penetration enhancing ability of flexosomes with that obtained for mTHPC-loaded invasomes (Dragicevic-Curic et al., 2008a), conventional liposomes used as the control in the invasomes' study (labeled as liposomes 0% ethanol) were also prepared in this study (labeled as liposomes-control to invasomes). They were prepared by the film method (Dragicevic-Curic et al., 2008a) as the flexosomes, but without polysorbate 20 and using the ethanolic phospholipid solution (NAT 8539) instead of pure PC (Lipoid SP C). The PC-amount in liposomes was 10% (w/w) and the mTHPC-amount was 1.5 mg/ml.

2.3. Physical characterization of liposome dispersions

2.3.1. Particle size, size distribution and zeta potential of liposomes

The particle size, polydispersity index (PDI) and the zeta potential of the prepared liposomes were determined by means of photon-correlation spectroscopy (PCS) using the Zetasizer NS (Malvern Instrument, Germany). The particle size was calculated from the autocorrelation function of the intensity of light scattered from particles, assuming a spherical form of particles, a medium viscosity of 0.89 mPa s and a refractory index of 1.33. Samples were diluted with phosphate buffer saline (PBS) pH 7.4 prior to the measurements. For the particle size measurements 5 μl of the vesicle dispersions were diluted with 495 μl of PBS (pH 7.4). To obtain the zeta potential values of vesicles, 10 μl of vesicle dispersions were diluted with 990 μl of PBS pH 7.4.

2.3.2. Shape and type of liposomes

Cryo-electron microscopy was used to evaluate the shape and lamellarity of liposomes. Five microliters of dispersions were put onto a perforated coated net of copper (Quantifoil R 1.2/1.3, 400 mesh). Excess was removed from the samples with a sheet of filter paper. The samples were quickly frozen with liquid ethane (−170 to −180 °C) in a cryo-box (Carl Zeiss NTS GmbH). Excess ethane was removed by blotting the samples in the cold and the samples were placed with the help of a cryo-transfer device (Gatan 626 Single Tilt Cryotransfer System) in a pre-cooled transmission-cryo-electron microscope (Philips CM 120). Microscopy was performed at 120 kV.

2.3.3. Stability of liposomes

Liposomes were stored at 4 °C for 9 months under light protection. At predetermined time intervals the particle size and polydispersity index (PDI) of liposomes were measured by photon-correlation spectroscopy (PCS), and the mTHPC-content in samples was determined spectrophotometrically (Beckman DU 640 Spectrophotometer) after diluting the samples with methanol. Measurements were performed at λ = 649 nm using methanol as the blank. The measuring range (calibration curve) was from 2 μg/μl to 10 μg/μl (r = 0.9985).

2.4. In vitro skin penetration studies

2.4.1. Skin preparation

For the penetration studies human abdominal skin, obtained after plastic surgery, was used. The subcutaneous fatty tissue was removed from the skin using a scalpel and surgical scissors. The skin surface was cleaned with Ringers' solution and allowed to dry. The skin was packed in aluminum foil and stored in a polyethylene bag at −20 °C. Under these conditions the skin is, with respect to the penetration studies, stable for 3–6 months (Harrison et al., 1984). All investigations were conducted in full compliance with ethical principles, i.e. the studies were reviewed and approved by the Ethics Committee of the Friedrich Schiller University Jena.

2.4.2. Preparation of Franz diffusion cells

The nominal surface of the Franz diffusion cells (FDC) was 3.14 cm^2 and acceptor compartments had a capacity of approximately 13 ml. Skin discs of 36 mm were punched out, allowed to thaw, cleaned with Ringers' solution and transferred onto the FDC. The acceptor compartment was filled with phosphate buffer saline (PBS) pH 7.4. Bovine serum albumine (BSA, 1%, w/w) was added in order to ensure the sink condition (i.e. the dissolution of mTHPC), since mTHPC is practically insoluble in PBS. The prepared FDC were stored in the refrigerator (4 °C) overnight in order to hydrate the skin. The next day, magnetic bars were put into the acceptor compartments of the FDC. The skin was kept at 32 °C using a water bath during the experiment. After an equilibration time of 15 min at 32 °C, formulations were applied onto the skin. In order to simulate an *in vivo* application, finite doses of formulations (10 μl/cm^2) were applied under non-occlusion. The experiment was carried out under light protection for 6 h. Incubation time of 6 h was used because it was shown in preliminary studies that the amount of mTHPC delivered into the skin significantly increased in the first 4 h, while between the 4th and 6th hour the mTHPC-amount increased to a lesser extent, indicating that a steady state was achieved and that further increase of the incubation time would not significantly increase the mTHPC-amount in the skin. Hence, after 6 h a sample of 1 ml was withdrawn from the acceptor solution and analyzed for the mTHPC-content and the formulations were removed from the skin by wiping the skin with cotton balls soaked with Ringers' solution. After cleaning, the skin was transferred onto a device for tape-stripping the stratum corneum (SC).

2.4.3. Tape-stripping of the stratum corneum (SC)

Tape-stripping of SC, i.e. the successive removal of cell layers of SC from the same skin area using adhesive tapes, is a common technique in cutaneous studies (Coderch et al., 1996; Surber, 1999). It presents a useful, efficient and minimally invasive method to study: (a) drugs' penetration into the skin, (b) drugs' permeation through the skin, (c) drugs' distribution within the SC, (d) dermatopharmacokinetics of topically applied cosmetic products and drugs (Weigmann et al., 1999; Surber et al., 2001), as well as, (e) SC physiology (Reed et al., 1993; Ohman and Vahlquist, 1994).

Among a variety of models (e.g. microdialysis, confocal laser scanning microscopy or transepidermal water loss), the tape-stripping method, which assesses drug amount in SC, seems to have the greatest potential for dermatopharmacokinetic characterization of topical drug products, which may provide an alternative approach to clinical trials for the determination of bioequivalence of topical dermatological products (Shah et al., 1998). An important advantage is also the applicability to nonradiolabeled drugs. However, this method shows also following disadvantages. The vehicle components of a product may influence the adhesive properties of the tape as well as the cohesion of the corneocytes, which makes the removal of corneocytes dissimilar (Surber, 1999). Therefore, it is essential for the comparison of the penetration of different formulations that the amount of formulation detected on the single tape-strip is related to its standardized real position in the stratum corneum (Lademann et al., 2009). In addition, tape-stripping may yield cell layers that originate from various depths also because of furrows in the skin (van der Molen et al., 1997). Another disadvantage is that depending on the skin origin and source, skin storing conditions and duration of the experiment, the integrity of the skin may vary (Surber, 1999).

The protocol of tape-stripping experiments of different research groups (Wagner et al., 2002; Verma et al., 2003a,b; Jacobi et al., 2006) is almost the same: after topical application and penetration of formulations, the cell layers of the stratum corneum are successively removed using adhesive films. In order to avoid furrows, which could be a reason for false results of the stripping procedure, the skin is stretched and mounted with pins on cork discs (Wagner et al., 2002; Verma et al., 2003a,b; Dragicevic-Curic et al., 2008a) resulting in SC layers of nearly the same thickness (Borchert, 1994; Pershing et al., 1994; Theobald, 1998) or the adhesive tapes are pressed onto the skin using rollers providing a constant overall pressure (Jacobi et al., 2006). The relative amount of SC removed with 20–30 tape-strips can be estimated with the use of transepidermal water loss and an overall SC density of 1 g/cm^3 (Kalia et al., 2001), weight of the removed adhesive tapes containing SC layers and an overall SC density of 1 g/cm^3 (Verma et al., 2003a,b; Dragicevic-Curic et al., 2008a) or following another developed standard protocol (Jacobi et al., 2005). The weight of SC layers is used to calculate the SC thickness in order to obtain a drug concentration-SC (and also skin) depth profile. The SC thickness in a cross-section also can be determined by a light microscope, and it is afterwards divided by the number of strips giving the thickness of SC layers per one strip (Wagner et al., 2002).

In this study the skin was after its cleaning stretched and mounted with pins on cork discs, in the previously mentioned special device (Section 2.4.2). The skin was covered with a Teflon mask with a central hole of 15 mm in diameter. For the purposes of removing the SC by tape-stripping, 20 pieces of adhesive tape (Cristall-klar Tesa, Beiersdorf AG, Hamburg, Germany) were used. The tape-strips were of a sufficient size to cover the hole, i.e. the area of the skin intended for stripping. In a standardized procedure, each tape-strip, previously weighed, was put onto the skin and charged for 10 s with a weight of 2 kg. Afterwards the tape-strips were rapidly removed with forceps and weighed. Using Eq. (1) the weight of each SC layer was used to calculate the thickness of each SC layer (Michel et al., 1992):

$$T = \frac{d}{a \cdot p} \quad (1)$$

where T is the thickness of removed SC (μm), d is the difference in the weight of tape-strips before and after stripping (μg), a is the stripping area (μm^2), and p is the density of SC (10^{-6} μg/μm^3). Using this equation the cumulative SC thickness could be estimated.

Due to analytical saving reasons, the stripped tapes were collected in vials according the following scheme: vial 1 = strip 1, vial 2 = strip 2–3, vial 3 = strips 4–5, vial 4 = strips 6–8, vial 5 = strips 9–12, vial 6 = 13–16, and vial 7 = 17–20.

2.4.4. Cryo-sectioning of deeper layers of the skin

After tape-stripping the skin was frozen in liquid nitrogen and a disc of 10 mm diameter was punched out of the stripped area. The skin disc was mounted with Tissue-tec O.C.T.® (Sakura Finetechnical, Tokyo, Japan) onto a metal sample holder, which was transferred into the cryomicrotome (Reichert-Jung 2800 Frigocut, Leica Instruments GmbH, Nussloch, Germany). The skin was cut parallel to the surface into 25 μm thick layers. The skin sheets were put together in the following scheme: vial 1 = incomplete cuts, vial 2 = 10 μm × 25 μm complete cuts, vial 3 = 10 μm × 25 μm complete cuts, and vial 4 = skin rest. Vials were weighed before collecting skin cuts and afterwards. The mean value of the thickness per mg (μm/mg) was calculated using Eq. (2):

$$\text{mean thickness per mg}\,(\mu\text{m/mg}) = \frac{\sum \text{thickness of the cuts}}{\text{weight of the cuts}} \quad (2)$$

Knowing the mean value of the thickness per mg (μm/mg) and the weight of the incomplete cuts and the skin rest, the thickness of the incomplete cuts and the skin rest can be calculated.

2.4.5. HPLC assay of mTHPC in the skin samples

For the extraction of mTHPC from the samples methanol was used. The tape-strips were extracted with 3 ml methanol and the skin cuts with 1 ml methanol. The samples were shaken for 4 h at 60 °C under light protection. After that, 1–2 ml of the sample was centrifuged for 10 min at 13,000 rpm (Eppendorf miniSpin, Eppendorf AG, Hamburg, Germany). The supernatant was analyzed afterwards for the mTHPC-content by HPLC. The samples removed from the acceptor compartment after 6 h incubation time, were analyzed by HPLC directly. The HPLC system consisted of the solvent module "System Gold 126" (Beckman Coulter), autosampler "Triathlon" (Spark), fluorescence detector "RF-10A XL" (Shamdzu) with SS420x interface set for excitation wavelength at 410 nm and for emission wavelength at 654 nm, online degasser (ERC3415 alpha, ERC), column thermostat Jet-Stream Plus set at 30 °C (Thermotechnic Products), column LiChroCART250-4 with Purospher STAR RP-18 endcapped and guard column LiChroCART4-4 with Purospher STAR RP-18e endcapped (Merck). The mobile phase was composed of acetonitrile: 0.1% trifluoroacetic acid (TFA) in water (57.5:42.5, v/v) and the flow rate was set at 1 ml/min. The retention time for mTHPC was about 10 min and the injection volume was 50 μl. The measuring range was from 0.25 pg/μl to 100 pg/μl (r = 0.9998) and the detection limit 0.05 pg/μl. The software used was 32 Karat Software, Version 5.0, Build 1021 (Beckman Coulter). The tissue concentration of mTHPC was determined from a calibration curve constructed by plotting the peak height of mTHPC standard solutions. The calibration was linear within this range.

2.5. Statistical analysis

All measurements in the study were performed in triplicate and reported data present mean values ± S.D. Statistical analysis was carried out using the Student's t-test. A $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Physical characterization of liposome dispersions

3.1.1. Particle size, size distribution and zeta potential of liposomes

The particle size analysis revealed that the size of all investigated vesicles was in the range of 88.40 ± 0.20 nm to 97.34 ± 0.12 nm

Table 2
Characterization parameters of different mTHPC-liposome formulations.

Liposome dispersion	Particle size (nm)	Polydispersity index	Zeta potential (mV)
Conventional liposomes	88.40 ± 0.20	0.073 ± 0.015	−2.99 ± 0.52
Neutral flexosomes	97.34 ± 0.12	0.062 ± 0.004	−5.50 ± 0.32
Cationic flexosomes	96.00 ± 0.17	0.129 ± 0.005	39.10 ± 0.78
Anionic flexosomes	94.12 ± 0.20	0.264 ± 0.017	−39.28 ± 1.06

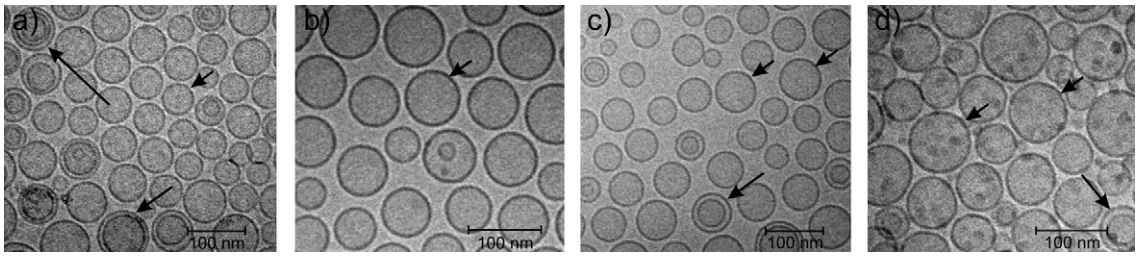

Fig. 2. Cryo-electron microscopy of mTHPC-loaded conventional and surface-charged flexible liposomes. (a) Conventional liposomes, (b) neutral flexosomes, (c) anionic flexosomes, and (d) cationic flexosomes. Short arrows represent unilamellar vesicles, medium length arrows represent bilamellar vesicles and long arrows represent oligolamellar vesicles.

(Table 2), indicating that vesicles were of a small size. Small particle size is a highly desirable property of vesicles, since it was shown that by decreasing the vesicles' particle size, the penetration of encapsulated drugs into deeper skin layers increases (Verma et al., 2003a). In addition, there was no marked difference in the particle size between the differently surface-charged flexible liposomes, i.e. the incorporation of dicetyl phosphate (DCP) and stearylamine (SA) into the flexosomes' bilayers did not have an influence on the particle size. However, the incorporation of polysorbate 20 into conventional phosphatidylcholine (PC) vesicles in order to obtain neutral flexible vesicles had an influence on the particle size, i.e. polysorbate 20 increased slightly the vesicles' particle size. The polydispersity index (PDI) of the investigated vesicles had values from 0.062 ± 0.004 (high homogeneity) to 0.264 ± 0.017 (lower, but acceptable homogeneity, since PDI < 0.3), indicating homogenous populations of vesicles (Table 2). Conventional liposomes and neutral flexosomes possessed smaller PDI values than the surface-charged flexosomes (i.e. cationic and anionic flexosomes). Hence, the incorporation of surface charged lipids (SA and especially DCP) into the flexosomes decreased their homogeneity. However, as mentioned before, the homogeneity of all vesicles was satisfactory.

Regarding the zeta potential, conventional liposomes and neutral flexosomes possessed only a small negative surface charge and were therefore considered as neutral. Cationic flexosomes possessed a high positive surface charge, while anionic flexosomes possessed a high negative surface charge. The absolute values of the zeta potential of cationic and anionic flexible liposomes were almost the same. SA and DCP were mixed with PC at the same molar ratio of 1:9 in order to obtain vesicles of similar absolute value of zeta potential, but of opposite charge. In this way the influence of vesicles' surface charge on the penetration of the incorporated drug could investigated.

3.1.2. Shape and type of liposomes

Cryo-electron microscopy was used to study the vesicles' shape and lamellarity of different vesicle dispersions. Vesicles of conventional liposome dispersion seemed to be mostly unilamellar and of a spherical shape (Fig. 2a, short arrows). However, bilamellar (Fig. 2a, medium length arrows) and oligolamellar (Fig. 2a, long arrow) vesicles were also seen in the dispersion. Vesicles of the neutral, cationic and anionic flexosome dispersion were mostly unilamellar and of a spherical shape (Fig. 2b–d, short arrows), but also bilamellar vesicles were seen (Fig. 2c and d). Hence, cryo-electron microscopy did not show structural differences between different vesicles indi-

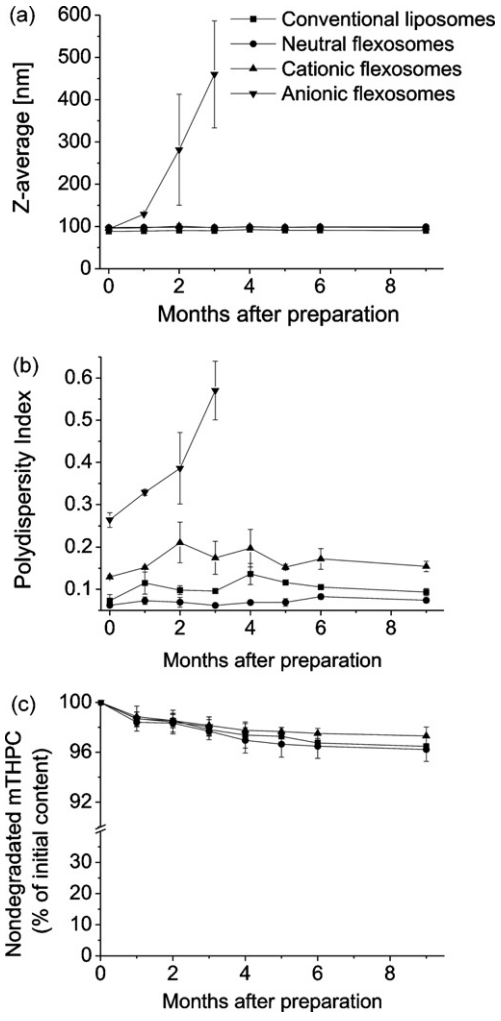

Fig. 3. Stability study of mTHPC-loaded conventional and surface-charged flexible liposomes during storage at 4 °C. (a) Change of the particle size of vesicles. (b) Change of the PDI of vesicles. (c) Change of the mTHPC-content in the different vesicles. Each data point represents the mean ± S.D. (n = 3).

cating that flexibility and surface charge did not have a marked influence on the vesicles' structure.

3.2. Storage stability

The stability of different liposome dispersions was investigated during their storage at 4 °C. The results of the particle size and polydispersity index (PDI) analysis are represented in Fig. 3a and b. The particle size values of conventional and flexible liposome dispersions, except of the anionic flexosomes, did not show any marked differences compared to their initial values after 9 months' storage at 4 °C. The particle size of anionic flexosomes started to increase already in the first month after their preparation, and reached a value of 460 nm after only 3 months, which was 490% higher than its initial value. The storage of these vesicles was aborted after 3 months. The PDI values of all vesicles, except of anionic flexosomes, showed some small fluctuations during the storage time. However, the PDI values of vesicles did not differ markedly from their initial values after 9 months' storage, indicating that the homogeneity did not decrease during storage. Only the PDI value of anionic flexosomes showed an extremely high increase even during the first month of storage. In contrast to the other mentioned liposomes, which could be considered physically stable during 9 months' storage at 4 °C, anionic liposomes do not possess a long-term stability, indicating the need of preparation just before their application. This extremely high increase of particle size and PDI of anionic flexosomes can be explained by fusion or aggregation of vesicles.

In order to assess the long-term chemical stability of the investigated different liposomes, it was also necessary to determine the mTHPC-content in liposome dispersions during their storage at 4 °C for 9 months. The anionic flexosomes were analyzed for the mTHPC-content only for 3 months, since after that time their particle size and PDI value increased extremely which resulted in abortion of the further stability investigation. The results of the mTHPC-determination (Fig. 3c), revealed that mTHPC was stable in all formulations during 9 months' or 3 months' storage (in the case of anionic flexosomes). At the end of the investigation period the mTHPC-content was in all formulations in the range of 96.22–97.59% of its initial content. The obtained data revealed that the mTHPC-content did not show a marked decrease during storage.

3.3. In vitro skin penetration studies

The diffusional barrier of the skin is localized in the stratum corneum (SC) and prevents entry of molecules greater than 350 Da (Delgado-Charro and Guy, 2001). mTHPC generally penetrates the skin very poorly due to its large molecular weight of 680 Da and extremely high hydrophobicity. Therefore, mTHPC has been till now applied only intravenously in the photodynamic therapy (PDT) of skin tumours and other tumours, and there are no topical mTHPC-formulations on the market. The strategy of using vesicles is gaining interest to overcome the difficulty related to low skin penetration of drugs. Flexible mTHPC-loaded liposomes, i.e. flexosomes, of different surface charge were developed and investigated for their penetration enhancing ability. In addition to flexible liposomes also conventional phosphatidylcholine (PC) liposomes were investigated. Conventional liposomes delivered only 0.66% of the applied mTHPC-dose to the SC (Table 3 and Fig. 4a). Neutral flexosomes delivered a significantly higher ($p < 0.05$) mTHPC-amount to the SC compared to conventional liposomes, i.e. they provided 1.53% of the applied mTHPC-dose in the SC (Table 3 and Fig. 4a).

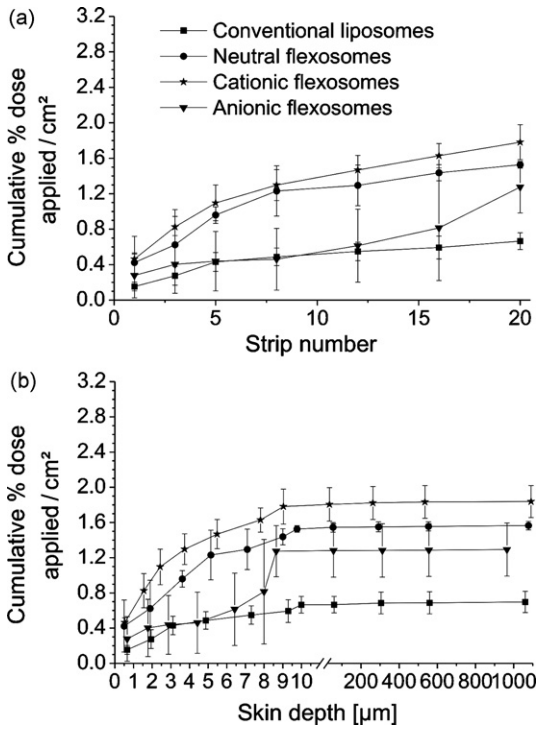

Fig. 4. Skin penetration of mTHPC after 6 h non-occlusive application of different mTHPC-formulations onto human abdominal skin (expressed as cumulative % of dose applied/cm² ± S.D., $n = 3$). (a) SC strip profile of mTHPC and (b) Skin depth profile of mTHPC.

Table 3
Amounts of mTHPC delivered from different formulations to different layers of human skin after 6 h non-occlusive application ($n = 3$) and the enhancement ratios (ER) of different formulations.

Formulation	SC		Deeper skin[a]		SC + deeper skin		ER1[b]	ER2[c]	SC	Deeper skin
	[% dose/cm²]	[ng/cm²]	[% dose/cm²]	[ng/cm²]	[% dose/cm²]	[ng/cm²]			[ng/mg tissue]	[ng/mg tissue]
Conventional liposomes-control	0.67 ± 0.09	88 ± 9	0.032 ± 0.027	4.12 ± 3.34	0.70 ± 0.12	92 ± 12	–	–	82 ± 7	0.044 ± 0.036
Neutral flexosomes	1.53 ± 0.04	221 ± 7	0.038 ± 0.023	5.61 ± 3.45	1.57 ± 0.05	226 ± 10	2.2	1.8	228 ± 23	0.056 ± 0.033
Cationic flexosomes	1.78 ± 0.20	267 ± 30	0.062 ± 0.016	9.28 ± 2.36	1.84 ± 0.18	277 ± 28	2.6	2.1	297 ± 30	0.095 ± 0.025
Anionic flexosomes	1.27 ± 0.29	184 ± 34	0.033 ± 0.013	4.78 ± 1.93	1.29 ± 0.30	189 ± 33	1.9	1.5	223 ± 49	0.054 ± 0.022
Liposomes-control to invasomes[d]	0.84 ± 0.12	131 ± 17	0.025 ± 0.017	3.92 ± 2.70	0.86 ± 0.13	135 ± 19	1.2	–	145 ± 25	0.042 ± 0.030

[a] Deeper skin = viable epidermis and dermis.
[b] ER1 = mTHPC-amount delivered to the whole skin by flexible liposomes [cumulative % of dose applied/cm²]/mTHPC-amount delivered to the whole skin by conventional liposomes used as a control [cumulative % of dose applied/cm²].
[c] ER2 = mTHPC-amount delivered to the whole skin by flexible liposomes [cumulative % of dose applied/cm²]/mTHPC-amount delivered to the whole skin by conventional liposomes used in the study on invasomes as a control [cumulative % of dose applied/cm²].
[d] These conventional liposomes were used as a control (labeled as liposomes 0% ethanol) in the study on invasomes (Dragicevic-Curic et al., 2008a) and served in this study to enable a comparison of flexosomes to invasomes.

Hence, neutral flexosomes showed a 2.2-fold higher mTHPC-accumulation in the SC than conventional liposomes, indicating a positive effect of the surfactant presence in the liposomes' bilayers on the penetration of mTHPC. In order to further enhance the mTHPC-penetration, the effect of surface charge of flexosomes on the drug penetration was investigated. The incorporation of dicetyl phosphate (DCP) into the flexosomes which resulted in the formation of negatively charged flexosomes did not further increase the mTHPC-penetration into the SC compared to neutral flexosomes. These anionic flexosomes delivered 1.27% of the applied mTHPC-dose to the SC (Table 3 and Fig. 4a), which was less than the amount delivered by neutral flexosomes, but 1.9-fold higher ($p < 0.05$) than the mTHPC-amount provided by conventional liposomes. However, the incorporation of stearylamine (SA) into flexosomes resulting in the formation of positively charged flexosomes increased further the mTHPC-penetration. Cationic flexosomes delivered 1.78% of the applied mTHPC-dose to the SC, which was 2.6-fold higher ($p < 0.05$) than the mTHPC-amount provided by conventional liposomes (Table 3 and Fig. 4a). The mTHPC-amount delivered by cationic flexosomes was even higher than the amount delivered by neutral and negatively charged flexosomes. This finding was in agreement with the findings of other authors (Manosroi et al., 2004; Song and Kim, 2006).

Summarizing, the SC deposition of mTHPC decreased in the following order: cationic flexosomes > neutral flexosomes > anionic flexosomes > conventional liposomes.

The skin depth profile of mTHPC (Table 3 and Fig. 4b) was almost the same as the SC strip profile, i.e. cationic flexosomes provided the highest accumulation of mTHPC in the deeper skin layers, followed by neutral flexosomes and anionic flexosomes. The smallest amount of mTHPC was delivered to the deeper skin layers by conventional liposomes.

The highest total penetration enhancing effect was ascribed to cationic flexosomes, since they delivered the highest mTHPC-amount to the whole skin (expressed as cumulative % of the dose applied/cm^2 or cumulative absolute amount ng/cm^2), which was shown by the highest enhancement ratio (ER1) (Table 3). As to the permeation through skin, mTHPC was not found in the acceptor compartment regardless of the applied vesicle formulation, indicating no risk of systemic side-effects (i.e. photosensitivity).

Regarding the effect of surface charge of liposomes on the drug penetration, the results obtained are in agreement with those of Katahira et al. (1999) and Montenegro et al. (1996), who found that the skin permeation of drugs incorporated in positively charged liposomes was higher compared to negatively charged liposomes. Since it was suggested that the skin could act as a negatively charged membrane (Burnette and Ongpipattanakul, 1987), the aforementioned authors 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. In addition, Puglia et al. (2005) showed that neutral and negatively charged liposomes enabled a higher sustained release of the drug with respect to positively charged liposomes. They proposed that the electrostatic interaction between the negatively charged skin surface and the positively charged liposomes could promote the drug permeation and its consequent rapid depletion by the bloodstream in the vascularized section of the skin. Since our results are in agreement with the aforementioned findings, we support their theory that probably positive charges on the surface of cationic liposomes could bind to negative charges of the SC leading to an enhanced drug penetration into/through the skin. Song and Kim, 2006 demonstrated *in vitro* and *in vivo* a higher drug penetration from positively charged flexible liposomes compared to neutral and negatively charged flexible liposomes. On the contrary, Ogiso et al. (2001) observed higher drug permeation from negatively charged liposomes. However, it should be outlined that the effect of the inclusion of surface charged phospholipids into flexosomes on the drug penetration was smaller in comparison to the effect of the incorporation of the surfactant polysorbate 20 into conventional liposomes. The difference in the penetration enhancing ability between differently surface charged flexosomes was not as pronounced as between flexosomes and conventional liposomes, i.e. the addition of polysorbate to conventional liposomes showed a higher impact on the penetration enhancing ability of vesicles. The higher penetration enhancing ability of deformable vesicles compared to conventional liposomes is in accordance with the findings of other authors (El Maghraby et al., 1999, 2001; Trotta et al., 2002; Honeywell-Nguyen et al., 2003; Jain et al., 2003a, b, 2004; Mishra et al., 2007), who also demonstrated the superiority of deformable liposomes. The mechanism of the penetration enhancing ability of deformable vesicles was in detail investigated by Cevc (2004), who introduced the first passive, drug concentration insensitive vesicular system for non-diffusive transcutaneous drug delivery, the so-called Transfersomes® (Cevc, 1995). Due to the inclusion of membrane softeners (bile salts or polyoxyethylene surfactants) into the PC membrane, these vesicles possess high adaptability, allowing the vesicles to adjust their shape to fit into pores smaller than their size, and surface hydrophilicity (Cevc and Gebauer, 2003; Cevc, 2004), which makes such vesicles capable to cross the skin (acting as a nano-porous membrane) following the hydration gradient across the skin. According to Cevc (2004), after the non-occlusive application of the aqueous vesicle dispersion onto the skin, water starts to evaporate and when the vesicles reach their solubility limit, their very deformable and hydrophilic aggregates start to experience an attractive hydration gradient ("hydrational driving force" or hydrotaxis) across the skin barrier. This allows the vesicles to be "pulled" from the relatively dry skin surface through the stratum corneum into relatively water-rich viable skin regions (Cevc et al., 2003). The process continues until the vesicles reach the water-rich viable epidermis, where the osmotic "pull" ceases (Schätzlein and Cevc, 1998). The vesicles' bulkiness hampers further vesicle motion, as the vesicles' concentration gradient and their diffusivity are comparably small in a living tissue (Cevc, 2004).

The intention of this study was not to investigate the mechanism of the penetration enhancing ability of flexible vesicles. However, since our vesicles are composed of soya PC and contain a polyoxyethylene surfactant as in the case of deformable vesicles investigated by Cevc et al. (2008a,b), we support the theory of Cevc (2004). This concept of hydration-driven transcutaneous transport proposed already in 1992 (Cevc and Blume, 1992) was later confirmed by others (El Maghraby et al., 1999; Honeywell-Nguyen et al., 2003).

Flexosomes used in this study could also have been compared to invasomes investigated in our previous study (Dragicevic-Curic et al., 2008a), since in this study also conventional liposomes (liposomes-control to invasomes), used as a control in the invasomes' study, were prepared and tested (Table 3). From the data represented in Table 3 and from the study on invasomes (Dragicevic-Curic et al., 2008a) it can be concluded that cationic flexosomes were regarding their penetration enhancing ability comparable with invasomes containing 0.5% (w/w) of the standard terpene mixture, i.e. they were less efficient than invasomes containing 1% (w/w) of the terpene mixture.

In addition, the results showed that mTHPC reached upon the application of all flexosomes doses in the SC and deeper skin layers, which would be required in the PDT to induce tumour necrosis. Namely, it was shown in mice bearing the subcutaneously (s.c.) located human colorectal carcinoma HT-29 that the required mTHPC-amount in the s.c. located tumour was between 0.105 and 0.050 ng mTHPC/mg wet tissue weight, depending on the light dose used in PDT, i.e. the amount decreased with increasing the light

dose from 10 to 50 J/cm^2 (unpublished data from biolitec AG, Germany).

4. Conclusion

Neutral and cationic flexosomes appeared to be physically stable during storage at 4 °C for 9 months in contrast to anionic flexosomes. mTHPC could be delivered into the stratum corneum (SC) and deeper skin layers in sufficient amounts required for a topical photodynamic therapy (PDT) by dermal application of neutral and surface charged flexosomes. Flexosomes provided significantly enhanced mTHPC-amount in the skin compared to conventional liposomes. Cationic flexosomes delivered the highest mTHPC-amount to SC and deeper skin layers. In addition, mTHPC was not found in the acceptor compartment regardless of the applied vesicle formulation, indicating no risk of systemic side-effects (i.e. photosensitivity).

Hence, cationic flexosomes could be used as an efficient drug delivery system for the highly hydrophobic photosensitizer mTHPC, which would be advantageous for the PDT of cutaneous malignant (basal-cell carcinoma) or non-malignant diseases (psoriasis, acne, etc.).

Acknowledgements

We would like to express our gratitude to biolitec AG and the DAAD for their financial support, to Dr. Richter and Dipl. Ing. Steiniger for their help during the electron-microscopic investigations, to Ms Brabetz for her help in the *in vitro* penetration studies and to Dr. Gruhl for providing the human abdominal skin.

References

Betz, G., Aeppli, A., Menshutina, N., Leuenberger, H., 2005. In vivo comparison of various liposome formulations for cosmetic application. Int. J. Pharm. 296, 44–54.

Borchert, D., 1994. Methoden zur Untersuchung der simultanen Penetration von Arzneistoffen und Vehikelbestandteilen aus Salben in exzidierter Humanhaut. Dissertation University Saarbruecken.

Burnette, R.R., Ongpipattanakul, B., 1987. Characterization of the permselective properties of excised human skin during iontophoresis. J. Pharm. Sci. 76, 765–773.

Cevc, G., Blume, G., 1992. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim. Biophys. Acta 1104, 226–232.

Cevc, G., 1995. Material transport across permeability barriers by means of lipid vesicles. In: Hoff, A.V. (series Eds.), Lipowsky, R., Sackmann, E. (volume Eds.), Handbook of Biological Physics, vol. I, North-Holland, pp. 465–490.

Cevc, G., Schätzlein, A., Richardsen, H., 2002. Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. Biochim. Biophys. Acta 1564, 21–30.

Cevc, G., Blume, G., 2003. Biological activity and characteristics of triamcinolon-acetonide formulated with the self-regulating drug carriers, Transfersomes®. Biochim. Biophys. Acta 1614, 156–164.

Cevc, G., Gebauer, D., 2003. Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. Biophys. J. 84, 1010–1024.

Cevc, G., Schätzlein, A.G., Richardsen, H., Vierl, U., 2003. Overcoming semipermeable barriers, such as the skin, with ultradeformable mixed lipid vesicles, transfersomes, liposomes, or mixed lipid micelles. Langmuir 19, 10753–10763.

Cevc, G., 2004. Lipid vesicles and other colloids as drug carriers on the skin. Adv. Drug Deliv. Rev. 56, 675–711.

Cevc, G., Blume, G., 2004. Hydrocortisone and dexamethasone in very deformable drug carriers have increased biological potency, prolonged effect, and reduced therapeutic dosage. Biochim. Biophys. Acta 1663, 61–73.

Cevc, G., Vierl, U., Mazgareanu, S., 2008a. Functional characterisation of novel analgesic product based on self-regulating drug carriers. Int. J. Pharm. 360, 18–28.

Cevc, G., Mazgareanu, S., Rother, M., Vierl, U., 2008b. Occlusion effect on transcutaneous NSAID delivery from conventional and carrier-based formulations. Int. J. Pharm. 359, 190–197.

Coderch, L., Oliva, M., Pons, M., de la Maza, A., Manich, A.M., Parra, J.L., 1996. Percutaneous penetration of liposomes using the tape stripping technique. Int. J. Pharm. 139, 197–203.

Coderch, L., de Pera, M., Perez-Cullell, N., Estelrich, J., de la Maza, A., Parra, J.L., 1999. The effect of liposomes on skin barrier structure. Skin Pharmacol. Appl. Skin Physiol. 12, 235–246.

Delgado-Charro, M., Guy, R.H., 2001. Transdermal drug delivery. In: Hillery, A.M., Lloyd, A.W., Swarbrick, J. (Eds.), Drug Delivering and Targeting for Pharmacists and Pharmaceutical Scientists. Taylor and Francis, London, New York, pp. 207–236.

Dragicevic-Curic, N., Scheglmann, D., Albrecht, V., Fahr, A., 2008a. Temoporfin-loaded invasomes: development, characterization and in vitro skin penetration studies. J. Control. Release 127, 59–69.

Dragicevic-Curic, N., Gräfe, S., Albrecht, V., Fahr, A., 2008b. Topical application of temoporfin-loaded invasomes for photodynamic therapy of subcutaneously implanted tumours in mice: a pilot study. J. Photochem. Photobiol. B 91, 41–50.

El Maghraby, G.M.M., Williams, A.C., Barry, B.W., 1999. Skin delivery of oestradiol from deformable and traditional liposomes: mechanistic studies. J. Pharm. Pharmacol. 51, 1123–1134.

El Maghraby, G.M.M., Williams, A.C., Barry, B.W., 2001. Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in vitro. J. Pharm. Pharmacol. 53, 1069–1077.

Harrison, S.M., Barry, B.W., Dugard, P.H., 1984. Effects of freezing on human skin permeability. J. Pharm. Pharmacol. 36, 261–262.

Hofland, H.E., van der Geest, R., Bodde, H.E., Junginger, H.E., Bouwstra, J.A., 1994. Estradiol permeation from nonionic surfactant vesicles through human stratum corneum in vitro. Pharm. Res. 11, 659–664.

Honeywell-Nguyen, P.I., Frederik, P.M., Bomans, P.H.H., Junginger, H.E., Bouwstra, J.A., 2003. The in vitro transport of pergolide from surfactant based elastic vesicles though human skin: a suggested mechanism of action. J. Control. Release 86, 145–156.

Hopper, C., 2000. Photodynamic therapy: a clinical reality in the treatment of cancer. Lancet Oncol. 1, 212–219.

Jacobi, U., Weigmann, H.J., Ulrich, J., Sterry, W., Lademann, J., 2005. Estimation of the relative stratum corneum amount removed by tape stripping. Skin Res. Technol. 11, 91–96.

Jacobi, U., Tassopoulos, T., Surber, C., Lademann, J., 2006. Cutaneous distribution and localization of dyes affected by vehicles all with different lipophilicity. Arch. Dermatol. Res. 297, 303–310.

Jain, S., Jain, P., Jain, N.K., 2003a. Transfersomes: a novel vesicular carrier for enhanced transdermal delivery: development, characterization and performance evaluation. Drug Dev. Ind. Pharm. 29, 1013–1026.

Jain, S., Sapre, R., Umamaheshwari, R.B., Jain, N.K., 2003b. Protransfersomes for effective transdermal delivery of norgestrel preparation and in vitro characterization. Ind. J. Pharm. Sci. 65, 153–161.

Jain, S., Umamaheshwari, R.B., Bhadra, D., Jain, N.K., 2004. Ethosomes: a novel vesicular carrier for enhanced transdermal delivery of an anti-HIV agent. Ind. J. Pharm. Sci. 66, 72–81.

Katahira, N., Murakami, T., Kugai, S., Yata, N., Takano, M., 1999. Enhancement of topical delivery of a lipophilic drug from charged multilamellar liposomes. J. Drug Target. 6, 405–414.

Kalia, Y.N., Alberti, I., Naik, A., Guy, R.H., 2001. Assessment of topical bioavailability in vivo: the importance of stratum corneum thickness. Skin Pharmacol. Appl. Skin Physiol. 14, 82–86.

Kirjavainen, M., Urti, A., Jääskeläinen, L., Suhonen, T.M., Paronen, P., Valjakka-Koskela, R., Kiesvaara, J., Mönkkönen, J., 1996. Interaction of liposomes with human skin in vitro-the influence of lipid composition and structure. Biochim. Biophys. Acta 1304, 179–189.

Kübler, A.C., Haase, T., Staff, C., 1999. Photodynamic therapy of primary non-melanomatous skin tumors of the head and neck. Laser Surg. Med. 25, 60–68.

Lademann, J., Jacobi, U., Surber, C., Weigmann, H.J., Fluhr, J.W., 2009. The tape stripping procedure—evaluation of some critical parameters. Eur. J. Pharm. Biopharm. 72, 317–323.

Lasch, J., Laub, R., Wohlrab, W., 1991. How deep do intact liposomes penetrate into human skin? J. Control. Release 18, 55–58.

Manosroi, A., Podjanasoonthon, K., Manosroi, J., 2002. Development of novel topical tranexamic acid liposome formulations. Int. J. Pharm. 235, 61–70.

Manosroi, A., Kongkaneramit, L., Manosroi, J., 2004. Stability and transdermal absorption of topical amphotericin B liposome formulations. Int. J. Pharm. 270, 279–286.

Michel, C., Purmann, T., Mentrup, E., Seiller, E., Kreuter, J., 1992. Effect of liposomes on percutaneous penetration of lipophilic materials. Int. J. Pharm. 84, 93–105.

Mishra, D., Garg, M., Dubey, V., Jain, S., Jain, N.K., 2007. Elastic liposomes mediated transdermal delivery of an anti-hypertensive agent: propranolol hydrochloride. J. Pharm. Sci. 96, 145–154.

Montenegro, L., Panico, A.M., Ventimiglia, A., Bonina, F.P., 1996. In vitro retinoic acid release and skin permeation from different liposome formulations. Int. J. Pharm. 113, 89–96.

Mura, P., Maestrelli, F., Gonzalez-Rodrıguez, M.L., Michelacci, I., Ghelardini, C., Rabasco, A.M., 2007. Development, characterization and in vivo evaluation of benzocaine-loaded liposomes. Eur. J. Pharm. Biopharm. 67, 86–95.

Ogiso, T., Yamaguchi, T., Iwaki, M., Tanino, T., Miyake, Y., 2001. Effect of positively and negatively charged liposomes on skin permeation of drugs. J. Drug Target. 9, 49–59.

Ohman, H., Vahlquist, A., 1994. In vivo studies concerning the pH gradient in stratum corneum and upper epidermis. Acta Derm. Venereol. 74, 375–379.

Pershing, L.K., Corlett, J., Jorgensen, C., 1994. In vivo pharmacokinetics and pharmacodynamics of topical ketoconazole and miconazole in human stratum corneum. Antimicrob. Agents Chemother. 38, 90–95.

Puglia, C., Esposito, E., Menegatti, E., Nastruzzi, C., Rizza, L., Cortesi, R., Bonina, F., 2005. Effect of charge and lipid concentration on in-vivo percutaneous absorp-

tion of methyl nicotinate from liposomal vesicles. J. Pharm. Pharmacol. 57, 1169–1176.
Reed, J.T., Ghadially, R., Elias, P.M., 1993. Skin type, but neither race nor gender influence epidermal permeability barrier function. Arch Dermatol. 131, 1134–1138.
Ris, H.-B., Altermatt, J.H., Interbitzi, R., Hess, R., Nachbur, B., Stewart, J.C., Wang, Q., Lim, C.K., Bonnett, R., Berenbaum, M.C., et al., 1991. Photodynamic therapy with chlorins for diffuse malignant mesothelioma: initial clinical results. Br. J. Cancer 64, 1116–1120.
Ris, H.-B., Altermatt, J.H., Stewart, C.M., Schaffner, T., Wang, Q., Lim, C.K., Bonnett, R., Althaus, U., 1993. Photodynamic therapy with m-tetrahydroxyphenylchlorine in vivo: optimization of the therapeutic index. Int. J. Cancer 55, 245–249.
Seth, A.K., Misra, A., Umrigar, D., 2004. Topical liposomal gel of idoxuridine for the treatment of herpes simplex: pharmaceutical and clinical implications. Pharm. Dev. Technol. 9, 277–289.
Schätzlein, A., Cevc, G., 1998. Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: a high-resolution confocal laser scanning microscopy study using highly deformable vesicles (Transfersomes). Br. J. Dermatol. 138, 583–592.
Schreier, H., Bouwstra, J., 1994. Liposomes and niosomes as drug carriers: dermal and transdermal drug delivery. J. Control. Release 30, 1–15.
Shah, V.P., Flynn, G.L., Yacobi, A., Maibach, H.I., Bon, C., Fleischer, N.M., Franz, T.J., Kaplan, S.A., Kawamoto, J., Lesko, L.J., Marty, J.P., Pershing, L.K., Schaefer, H., Sequeira, J.A., Shrivastava, S.P., Wilkin, J., Williams, R.L., 1998. Bioequivalence of topical dermatological dosage forms—methods of evaluation of bioequivalence. Pharm. Res. 15, 167–171.
Song, Y.-K., Kim, C.-K., 2006. Topical delivery of low-molecular-weight heparin with surface-charged flexible liposomes. Biomaterials 27, 271–280.
Surber, C., 1999. Tape-stripping technique. In: Bronaugh, R.L., Maibach, H.I. (Eds.), Percutaneous Absorption: Drugs–Cosmetics–Mechanisms-Methodology. Marcel Dekker, Inc., New York, Basel, pp. 395–409.
Surber, C., Schwarb, F., Smith, E.W., 2001. Tape-stripping technique. J. Toxicol. Cutan. Ocul. Toxicol. 20, 461–474.
Theobald, F., 1998. In vitro Methoden zur biopharmazeutischen Qualitaetspruefung von Dermatika unter Beruecksichtigung der Lipidzusammensetzung des Stratum corneum. Dissertation, University Saarbruecken.
Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M., 2000. Ethosomes-novel vesicular, carriers for enhanced delivery: characterization and skin penetration properties. J. Control. Release 65, 403–418.
Trotta, M., Peira, E., Debernadi, F., Gallarate, M., 2002. Elastic liposomes for skin delivery of potassium glycyrrhizinate. Int. J. Pharm. 241, 319–327.
van den Bergh, B.A.I., Bouwstra, J.A., Junginger, H.E., Wertz, P.W., 1999. Elasticity of vesicles affects hairless mouse skin structure and permeability. J. Control. Release 62, 367–379.
van der Molen, R.G., Spies, F., van Noordende, J.M., Boelsma, E., Mommaas, A.M., Koerten, H.K., 1997. Tape stripping of human stratum corneum yields cell layers that originate from various depths because of furrows in the skin. Arch. Dermatol. Res. 289, 514–518.
van Kuijk-Meuwissen, M.E.M.J., Junginger, H.E., Bouwstra, J.A., 1998. Interactions between liposomes and human skin in vitro, confocal laser scanning microscopy study. Biochim. Biophys. Acta 1371, 31–39.
Verma, D.D., Verma, S., Blume, G., Fahr, A., 2003a. Particle size of liposomes influences dermal delivery of substances into skin. Int. J. Pharm. 258, 141–151.
Verma, D.D., Verma, S., Blume, G., Fahr, A., 2003b. Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: a skin penetration and confocal laser scanning microscopy study. Eur. J. Pharm. Biopharm. 55, 271–277.
Verma, D.D., Verma, S., McElwee, K.J., Freyschmidt-Paul, P., Hoffman, R., Fahr, A., 2004. Treatment of alopecia areata in the DEBR model using cyclosporin A lipid vesicles. Eur. J. Dermatol. 14, 332–338.
Wagner, H., Kostka, K.H., Lehr, C.M., Schaefer, U.F., 2002. Human skin penetration of flufenamic acid: in vivo/in vitro correlation (deeper skin layers) for skin samples from the same subject. J. Invest. Dermatol. 11, 540–544.
Weigmann, H.-J., Lademann, J., von Pelchrzim, R., Sterry, W., Hagemeister, T., Molzahn, R., Schaefer, M., Linscheid, M., Schaefer, H., Shah, V.P., 1999. Bioavailability of clobetasol propionate—quantification of drug concentrations in the stratum corneum by dermatopharmacokinetics using tape stripping. Skin Pharmacol. Appl. Skin Physiol. 12, 46–53.
Zellmer, S., Pfeil, W., Lasch, J., 1995. Interaction of phosphatidylcholine liposomes with the human stratum corneum. Biochim. Biophys. Acta 1237, 176–182.