International Journal of Pharmaceutics 411 (2011) 223–231

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Liposome surface charge influence on skin penetration behaviour

A. Gillet[a,*], P. Compère[b], F. Lecomte[c], P. Hubert[d], E. Ducat[a], B. Evrard[a], G. Piel[a]

[a] Laboratory of Pharmaceutical Technology, Department of Pharmacy, CIRM, University of Liège, CHU, Tour 4, Bat B36, 1 Avenue de l'Hôpital, 4000 Liège, Belgium
[b] Laboratory of Functional and Evolutive Morphology, Department of Environmental Sciences and Management, University of Liège, Bat B6, Sart-Tilman, 4000 Liège, Belgium
[c] Laboratory of Analytical Chemistry, Department of Pharmacy, CIRM, University of Liège, CHU, Tour 4, Bat B36, 1 Avenue de l'Hôpital, 4000 Liège, Belgium
[d] Laboratory of Experimental Pathology, Giga-Cancer, University of Liège, 1 Avenue de l'Hôpital, 4000 Liège, Belgium

ARTICLE INFO

Article history:
Received 12 January 2011
Received in revised form 21 March 2011
Accepted 22 March 2011
Available online 31 March 2011

Keywords:
Liposomes
Charge
Skin penetration
Betamethasone
Franz cells

ABSTRACT

Vesicular systems have shown their ability to increase dermal and transdermal drug delivery. Their mechanism of drug transport into and through the skin has been investigated but remains a much debated question. Several researchers have outlined that drug penetration can be influenced by modifying the surface charge of liposomes. In the present work we study the influence of particle surface charge on skin penetration. The final purpose is the development of a carrier system which is able to enhance the skin delivery of two model drugs, betamethasone and betamethasone dipropionate. Liposomes were characterised by their size, morphology, zeta potential, encapsulation efficiency and stability. *Ex vivo* diffusion studies using Franz diffusion cells were performed. Confocal microscopy was performed to visualise the penetration of fluorescently labelled liposomes into the skin. This study showed the potential of negatively charged liposomes to enhance the skin penetration of betamethasone and betamethasone dipropionate.

1. Introduction

Transdermal drug delivery has many potential advantages over other routes of administration. It allows the avoidance of gastrointestinal tract problems and hepatic first-pass effects, and improvement in patient compliance (El Maghraby and Williams, 2009). However, a major obstacle to cutaneous drug delivery is the permeation characteristics of the *stratum corneum*, which limits its drug transport, making this route of administration frequently insufficient for medical use. During the past few decades, there has been wide interest in exploring new techniques for increasing drug absorption through the skin. These include physical permeation enhancement techniques like iontophoresis by electrically driving molecules into and through the skin (Balaguer-Fernández et al., 2010; Kolli et al., 2010; Krishnan et al., 2011), electroporation by application of high-voltage pulses to the skin (Charoo et al., 2010; Escobar-Chávez et al., 2009) and sonophoresis by application of ultrasound (Escobar-Chavez et al., 2009). Passive penetration enhancement techniques include, for example, use of supersaturated solutions (Iervolino et al., 2001; Leveque et al., 2006), penetration enhancers (Williams and Barry, 1992; Williams and Barry, 2004) or microemulsions (Kogan and Garti, 2006). A combination of these strategies is also studied (Balaguer-Fernández et al., 2010; Nair et al., 2011).

Vesicular systems provide an alternative to improve drug delivery into and through the skin. Classical and more recently deformable liposomes have shown their ability to increase dermal and transdermal drug delivery.

Several researchers have outlined that drug penetration can be influenced by modifying the surface charge of liposomes. The lipid layer in the *stratum corneum* contains a high ratio of negatively charged lipids and it is well known that the skin may act as a negatively charged membrane (Sinico et al., 2005; Yoo et al., 2008). It has been reported that the presence of charges at the vesicle surface may affect the transcutaneous diffusion of drugs. Negatively charged vesicles generally give a higher flux than positively charge counterparts, which in turn can improve drug accumulation in the superficial skin strata (Sinico et al., 2005). However, results in the literature are contradictory.

The most efficient composition tested by Carrer et al. (2008) contained the highest proportion of charged edge activators and the authors suggested that the presence of negative charge in the membrane may allow for a better efficiency of penetration. Manosroi et al. (2004) showed that the transdermal absorption of amphotericine B was higher when entrapped in charged liposomes than in non-charged ones and that positive liposomes produced a higher absorption through the *stratum corneum* than the negatively charged ones. This result was explained by the fact that the skin surface bears a net negative charge. However, negative liposomes exhibited higher absorption through the viable epidermis and dermis than the positively charged liposomes. Ogiso et al. (2001) showed that the percutaneous absorption of betahistine from

* Corresponding author. Tel.: +32 4 366 43 06; fax: +32 4 366 43 02.
E-mail address: aline.gillet@ulg.ac.be (A. Gillet).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.03.049

a gel formulation containing negatively charged liposomes was much higher than that in the formulation with positively charged liposomes. Histological studies confirmed their observation. In contrast, Katahira et al. (1999) found that negative dicetylphosphate liposomes provided better rhodamine B retention in the skin with lower skin permeability compared with positive and neutral multilamellar liposomes. Sinico et al. (2005) also showed that negatively charged vesicles provided higher skin accumulation values of entrapped tretinoin with lower skin permeation in comparison with positively charged vesicles. Hasanovic et al. (2010) studied the influence of adding cationic polymers (chitosan or Eudragit EPO) on the stability and skin penetration of DPPC liposomes encapsulating aciclovir or minoxidil. They showed an increased stability by the addition of the two different cationic polymers and an increased skin permeation of drugs from coated liposomes. This permeation increase was explained as a tendency of positively charged liposomes to interact stronger with the skin surface or as an interaction of the polymers with skin lipids, the polymers going deeper and disrupting the tight junctions in lower epidermis layers.

In a previous paper we studied the influence of betamethasone encapsulation in liposomes. The drug was encapsulated either alone into the lipid bilayer or in the aqueous compartment of liposomes by the help of betamethasone–cyclodextrin complexes. We showed that the encapsulation into the lipid bilayer significantly enhanced the accumulation of betamethasone in the epidermis of pig ear skin (Gillet et al., in press). In the present work we would like to study the influence of the addition of a charge on the skin penetration. The aim of this study is to develop a carrier system that able to enhance the skin delivery of a model drug, betamethasone. This corticoid is too hydrophilic for good skin penetration behaviour by itself. We studied the influence of adding a charge into the lipid bilayers on the penetration efficiency of encapsulated betamethasone. The ester of betamethasone, betamethasone dipropionate, was also used in order to evaluate the properties of the drug encapsulated on the skin penetration behaviour. The penetration enhancing ability of these charged vesicles was tested *ex vivo* using pig ear skin as the model membrane. Confocal microscopy was made to visualise the penetration of fluorescently labelled liposomes.

2. Materials and methods

2.1. Chemicals and reagents

Betamethasone (Ph. Eur.) was purchased from Medeva (Braine L'Alleud, Belgium), betamethasone dipropionate (Ph. Eur.) from Abc Chemicals (Wauthier-Braine, Belgium), Soybean phosphatidylcholine (PC) from Lipoïd (Ludwigshafen, Germany), 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) (DMPA) and 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecan}-sn-glycero-3-phosphocholine (NBD-PC) from Avanti Polar lipids (Alabaster, AL, USA), dicetylphosphate (DCP) from Santa Cruz Biotechnology (Santa Cruz, USA), hydroxypropylated-γ-cyclodextrin (HPγCD, D.S. 0.7, 3.41% H_2O) was obtained from Wacker-Chemie GmbH (Munich, Germany), stearylamine from Sigma Aldrich (Bornem, Belgium), and rhodamine B and acetonitrile from Merck (Darmstadt, Germany). Diprosone® lotion (containing 0.05% of corticoid expressed in betamethasone) was purchased from Schering-Plough Labo (Heist-op-den-Berg, Belgium). Pure water was generated from the Milli-Q system (Millipore, Bredford, MA, USA). All experiments were performed using a 0.22 μm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer (Sigma Aldrich), containing 145 mM NaCl and adjusted to pH 7.4 with 0.1 M NaOH solution. All other reagents and solvents were of analytical grade.

Pig ears came from two sources. For the first experiments with betamethasone, pig ears (Race: Piétrain, age: 2 months old) were obtained from the Faculty of Veterinary Medicine at the University of Liège, Belgium. Because of a lack of supply, experiments with betamethasone dipropionate were made with pig ears (Race: Landras, age: 6 months old) obtained from the local slaughterhouse prior to scald.

2.2. Liposome preparation

Non charged liposomes were made of PC. Negatively charged liposomes contained in addition DMPA (12.5% m/m or 15.3% mol/mol) or DCP (5.6% m/m or 7.7% mol/mol), while positively charged liposomes contained SA (3.8% m/m or 1.0% mol/mol). Liposomes were prepared by hydration of lipid films. In practice, the required amounts of lipids and drug (6 mg of betamethasone or 7.7 mg of betamethasone dipropionate) were dissolved in a 2:1 chloroform/ethanol mixture in a round-bottomed flask. The solution was then dried under vacuum using a rotary evaporator. The resulting lipid film was hydrated using 3 mL of Hepes buffer. Suspensions were then extruded three times through Nucleopore® polycarbonate membranes of successive 0.4 and 0.2 μm pore diameters (Whatman, Maidstone, UK). Free drug was separated from liposome-encapsulated drug by three successive ultracentrifugations at 165,052 × *g* (35,000 rpm). The first cycle lasted 3 h followed by two cycles of 1 h 30 at 4 °C. The supernatant was removed and the pellet was re-suspended in Hepes buffer. Betamethasone or betamethasone dipropionate and PC were assayed in purified liposomes.

2.3. Non liposomal dispersion preparation

Dispersions were made by mixing the required amount of PC with or without DMPA (12.5%) in Hepes buffer containing betamethasone or betamethasone dipropionate without further preparation.

2.4. Liposome characterisation

2.4.1. Measurement of liposome diameter and zeta potential

Liposome suspensions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments Ltd., Worcestershire, UK). Measurements were made at 25 °C with a fixed angle of 180° and results were expressed as the average liposomal hydrodynamic diameter (nm). The surface charge of the particles was determined using a Zetasizer® 2000 (Malvern Instruments Ltd., Worcestershire, UK).

2.4.2. Freeze-fracture electron microscopy

Freeze-fracture replicas of liposome suspensions were examined by transmission electron microscopy (TEM). A drop of liposome suspension, added with 20% glycerol as freeze-protectant, was deposited in a small gold cup and rapidly frozen in liquid nitrogen. Fracturing, freeze etching and shadowing with Pt-C were performed at −100 °C in a shadowing equipment (Balzers® BAF-400) fitted with a freeze-fracture and etching unit. The replicas were examined in a JEOL (JEM-100SX) transmission electron microscope, operating at 80 kV accelerating voltage.

2.4.3. Encapsulation efficiency

The encapsulation efficiency ($EE_{D/Dt}$) was the yield obtained. This corresponds to the concentration of drug encapsulated in liposomes (C_D) compared to the total drug concentration first introduced (C_{Dt}). This $EE_{D/Dt}$ was corrected to the concentration of PC

in order to take into account the loss of liposomes during their preparation:

$$EE_{D/Dt}\ (\%) = \frac{C_D/C_{PC}}{C_{Dt}/C_{PCt}} \times 100$$

C_{PC} is the PC concentration in purified liposomes and C_{PCt} is the PC concentration first introduced.

2.4.3.1. Drug chromatographic determination. The HPLC used was a LaChrom Merck-Hitachi (Darmstadt, Germany) consisting of an L-7100 pump, an L-7200 autosampler, an L-7400 UV detector, an L-7350 column oven and a D-7000 interface. The system was controlled by "D-7000 HPLC System Manager" software. The analytical column was a LiChroCART (250 × 4 mm, i.d.) packed with Superspher 100 RP-18 (particle size: 5 μm) and preceded by a guard column LiChroCART (4 mm × 4 mm, i.d.) packed with LiChrospher 100 RP-18 (particle size: 5 μm). Isocratic separation was performed at a temperature of 35 °C using a mobile phase consisting of a mixture of acetonitrile and water (50:50, v/v) for betamethasone and (55:45, v/v) for betamethasone dipropionate. The flow rate was settled at 0.8 mL/min for betamethasone and at 1 mL/min for the ester, and the sample injection volume was 20 μL. Betamethasone was monitored at 240 nm while betamethasone dipropionate was monitored at 254 nm.

2.4.3.2. Quantification of PC. PC concentration was measured through an enzymatic method (LabAssay™ Phospholipid, Wako, Osaka, Japan). The principle of this enzymatic assay consists in the cleavage of PC in choline by phospholipase D followed by the oxidation of choline into betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, couples 4-aminoantipyrine and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS). Peroxidation results in the generation of a coloured compound quantified by spectrophotometry at 600 nm (spectrophotometer Perkin-Elmer Lambda 11).

2.4.4. Liposome stability

The stability of liposomes was evaluated by measuring the particle mean diameter and polydispersity indexes by photon correlation spectroscopy after one month of storage at 4 °C. The stability of the different suspensions was also evaluated visually.

2.5. Ex vivo penetration study

2.5.1. Skin preparation

Full-thickness skin was removed from the dorsal side of freshly excised pig ear, stored at −20 °C and used within 6 months. On the day of the experiment, punches were cut out and hairs cut with scissors.

2.5.2. Permeation experiments

Diffusion studies were carried out using Franz type glass diffusion cells. The study design used in the present study was similar to that described previously for betamethasone containing formulations (Gillet et al., in press). To assure sink conditions in the case of betamethasone dipropionate, HPγCD 5 mM was added in the receptor medium giving a solubility of 54 μg/mL. 350 μL of liposome suspension or ethanolic solution or non liposomal dispersion at 150 μg/mL betamethasone or 193 μg/mL betamethasone dipropionate concentrations were placed in the donor chamber onto the *stratum corneum* of the skin, in non occlusive conditions. In order to test the same amount of betamethasone dipropionate from the Diprosone® lotion (0.05%), 100 mg of lotion was used. At the end of the experiment (24 h), the receptor phases were removed and the diffusion cells were dismantled. The skin surface was washed with 3 mL Hepes buffer on each side to remove the residual donor sample and was thawed. The surface of the skin exposed to the donor compartment was punched out. The *stratum corneum* was removed by the stripping method using 15 strips of Corneofix® tape (CKelectronic, Germany) successively. Histological studies confirmed that all the *stratum corneum* is removed after 15 strips. The tape strips were of a sufficient size to cover the surface of the skin punched out. Each strip was firmly pressed on the skin surface and rapidly removed. Only the first, fifth, tenth and fifteenth strips were kept and analysed for drug content. The piece of skin was then separated into the epidermis and dermis by pressing the skin surface against a hot plate (65 °C) for 90 s and peeling off the epidermis. The four strips, the epidermis and dermis cut into small pieces, were each soaked separately in a flask with 4 mL of Hepes (containing 5 mM HPγCD for betamethasone dipropionate) for 24 h. Samples were then shaken for 30 min in an ultrasound bath, in order to extract the entire drug accumulated in the skin pieces.

2.5.3. Drug determination

2.5.3.1. Solid phase extraction (SPE) prior to chromatographic analysis. SPE was needed to clean up the samples before HPLC injection. The extraction procedure was previously described (Gillet et al., in press).

2.5.3.2. SPE-HPLC-UV method validation. The method for betamethasone determination was previously validated (Gillet et al., in press). The validation procedure was the same for betamethasone dipropionate. The validation was based on an accuracy profile approach (Hubert et al., 2004). For betamethasone dipropionate determination in pig ear skin, the acceptance limits were set at 30% from 40.92 to 100 ng/mL and 15% from 100 to 10,000 ng/mL, respectively and the risk level was fixed at 10% (FDA, 2001; Viswanathan et al., 2007). The most appropriate calibration model was a linear regression. For betamethasone dipropionate determination from the tape stripping method, the acceptance limits were set at 30% from 20.23 to 100 ng/mL and 10% from 100 to 10,115 ng/mL, respectively and the risk level was fixed at 10%. The most appropriate calibration model was a linear regression. The e-noval software v3.0 (Arlenda, Liège, Belgium) was used to compute the validation results as well as to obtain the accuracy profiles.

2.6. Confocal laser scanning microscopy study

2.6.1. Liposome preparation

Liposomes were prepared and characterised as described in 2.2 and 2.4 with some modifications. Liposomes were made fluorescent in two ways. First, rhodamine B ($\log P = 1.95$) (0.01% m/m) was incorporated into the lipid bilayer in order to mimic the encapsulation of betamethasone ($\log P = 1.94$) (Takegami et al., 2008). Secondly, the lipid bilayer was made fluorescent by incorporation of NBD-PC (1.33% m/m). After extrusion, non-encapsulated rhodamine B and NBD-PC were separated from liposome-encapsulated rhodamine B and NBD-PC by successive ultracentrifugations at 35,000 rpm.

2.6.2. Confocal laser scanning microscopy (CLSM)

Diffusion studies were carried out as described in Section 2.5.2. After 24 h, the remaining liposome formulation was washed and the diffusion area punched out. The diffusion area was then incorporated into OCT compound (Tissue-Tek®, Sakura, The Netherlands) and frozen at −20 °C. The frozen skin was then sectioned with a cryostat into 7 μm slices. These tissues were counterstained with TOTO-3 iodide dye (Molecular Probes, Leiden, The Netherlands). The penetration of the fluorescent probes was assessed by confocal laser scanning microscopy (Leica TCS SP2, Heidelberg GmBH,

Table 1
Diameter ± S.D. (nm), polydispersity index (PI) ± S.D., zeta potential ± S.D. (mV) and encapsulation efficiency ($EE_{D/Dt}$) ± S.D. (%) of liposomes containing betamethasone (BM) or betamethasone dipropionate (BMD) ($n = 3$).

Composition	Diameter (nm)	PI	Zeta potential (mV)	$EE_{D/Dt}$ (%)
PC-BM	178 ± 12	0.110 ± 0.007	−1.6 ± 2.8	97.8 ± 5.4
PC-SA-BM	141 ± 6	0.092 ± 0.016	+13.2 ± 2.2	92.2 ± 1.2
PC-DMPA-BM	153 ± 2	0.085 ± 0.020	−26.6 ± 3.5	96.9 ± 1.7
PC-DCP-BM	153 ± 3	0.074 ± 0.021	−19.9 ± 4.5	90.1 ± 2.8
PC-BMD	171 ± 3	0.137 ± 0.015	−3.6 ± 0.9	96.0 ± 1.0
PC-DMPA-BMD	155 ± 7	0.068 ± 0.009	−27.8 ± 5.3	94.7 ± 3.8

Germany), using sequential acquisition. NBD-PC was excited with the 488 nm laser line from an argon laser and the fluorescent emission signals are represented by a green colour. Rhodamine B was excited with the 568 nm line from a Kr laser and the fluorescent emission signals are represented by a red colour. TOTO-3-stained cell nuclei were excited with the 633 nm line from a He/Ne laser and are shown by a blue colour. Images were acquired using a 40× objective lens immersed in oil.

2.7. Statistical analysis

The significance of the differences between formulations was tested using the Student *t*-test (Graph Pad Prism, Version 4). The differences are considered statistically significant when $p < 0.05$. Correlation was evaluated by the Pearson correlation test (Graph Pad Prism, Version 4). Correlation significance is considered when $p < 0.05$.

3. Results and discussion

3.1. Liposome characterisation

Classical, non charged liposomes contained PC as phospholipids. In addition, positive liposomes contained stearylamine (SA), while negatively charged liposomes contained DMPA or DCP. Two model drugs were encapsulated, betamethasone and betamethasone dipropionate in order to evaluate the influence of the properties of the drug encapsulated on the efficiency of skin penetration. Non liposomal dispersions of PC and betamethasone or betamethasone dipropionate in Hepes with or without DMPA were also studied and compared with the liposome formulations. PCS and freeze-fracture electron microscopy were performed for diameter analysis and morphology characterisation. As shown in Table 1, liposomes are characterised by mean hydrodynamic diameters between 141 ± 6 nm and 178 ± 12 nm. Polydispersity indexes (PI) are always lower than 0.2, indicating that liposomes are homogeneous in size. Whatever the drug encapsulated, positively and negatively charged liposomes show significantly smaller sizes than non charged classical liposomes ($p < 0.05$). This fact was also observed by Namdeo and Jain (1999) where the incorporation of DCP in niosomes reduced the mean size. They explained that the presence of charge in the bilayer due to DCP increases its tendency to become curved, thereby reducing the size of the vesicles. Liposomes show very good size reproducibility from batch to batch. These results are in good agreement with the TEM-imaging of the freeze fracture replica as shown in Fig. 1. Indeed, the picture of classical non charged liposomes encapsulating betamethasone (Fig. 1A) and those of negatively charged DMPA liposomes encapsulating either betamethasone (Fig. 1B) or betamethasone dipropionate (Fig. 1C) show very similar unilamellar vesicles with a homogeneous size of about ±200 nm. In contrast, non liposomal dispersions of PC and betamethasone (Fig. 1D) or of PC, DMPA and betamethasone (Fig. 1E) in Hepes buffer appear as polymorphic vesicles in shape and size probably resulting from the self-assembling in suspension. The vesicle sizes range from a few nm up to 1 μm. In Fig. 1D, we observe the presence of different types of vesicles such as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and also multilamellar vesicles (MLV). The term "non liposomal dispersion" is therefore conflicting, as vesicles are formed in these dispersions. However, we maintain this term in order to easily differentiate it from the liposome formulations which consist of small unilamellar vesicles of the same diameter. In non liposomal dispersions, drug crystals (DC) are also found outside the vesicles (Fig. 1D).

Regarding zeta potential values (Table 1), classical non charged liposomes possess a small negative charge and were therefore considered as neutral (Dragicevic-Curic et al., 2010). The amount of stearylamine incorporated into liposomes was selected according to Piel et al. (2006), giving positively charged vesicles, while the amount of DMPA was selected according to Yoo et al. (2008), giving negatively charged liposomes. DCP containing vesicles were used to confirm the effect of a negative charge on skin penetration but the same molar ratio as used for DMPA could not be incorporated. The maximum amount of DCP that could be incorporated into the lipid bilayer gives thus a smaller negative charge than obtained with DMPA.

Drug encapsulation efficiencies are reported in Table 1. $EE_{D/Dt}$ expresses the encapsulation efficiency as a function of the total drug concentration. Results are over 90% for each formulation.

Stability of formulation was first evaluated by visual observations (results not shown). We observed that neutral and charged liposomes appeared in homogeneous white suspensions without sedimentation after 24 h, indicating that the suspensions are physically stable due to the presence of small and uniform distributed vesicles obtained after the extrusion process. The non liposomal dispersions, however, showed high sedimentation levels with time, already visible after 2 h. The stability of liposomes was also evaluated by measuring their diameter after one month of storage at 4 °C. Results are shown in Table 2. No significant change in diameter is observed indicating the stability of the formulations. Polydispersity indexes (not shown) remain under 0.2.

3.2. Ex vivo penetration studies

Franz type diffusion cells were used to evaluate the *ex vivo* penetration of betamethasone or betamethasone dipropionate from liposomes in pig ear skin. Three reference samples were used: a

Table 2
Diameter ± S.D. (nm) at the day of preparation (T_0) and after a minimum of one month of storage at 4 °C for liposomes containing betamethasone (BM) or betamethasone dipropionate (BMD) ($n = 3$).

Composition	Diameter T_0 (nm)	Diameter after one month 4 °C (nm)
PC-BM	178 ± 12	172 ± 5
PC-SA-BM	141 ± 6	141 ± 5
PC-DMPA-BM	153 ± 2	155 ± 4
PC-DCP-BM	153 ± 3	151 ± 4
PC-BMD	171 ± 3	171 ± 1
PC-DMPA-BMD	155 ± 7	157 ± 5

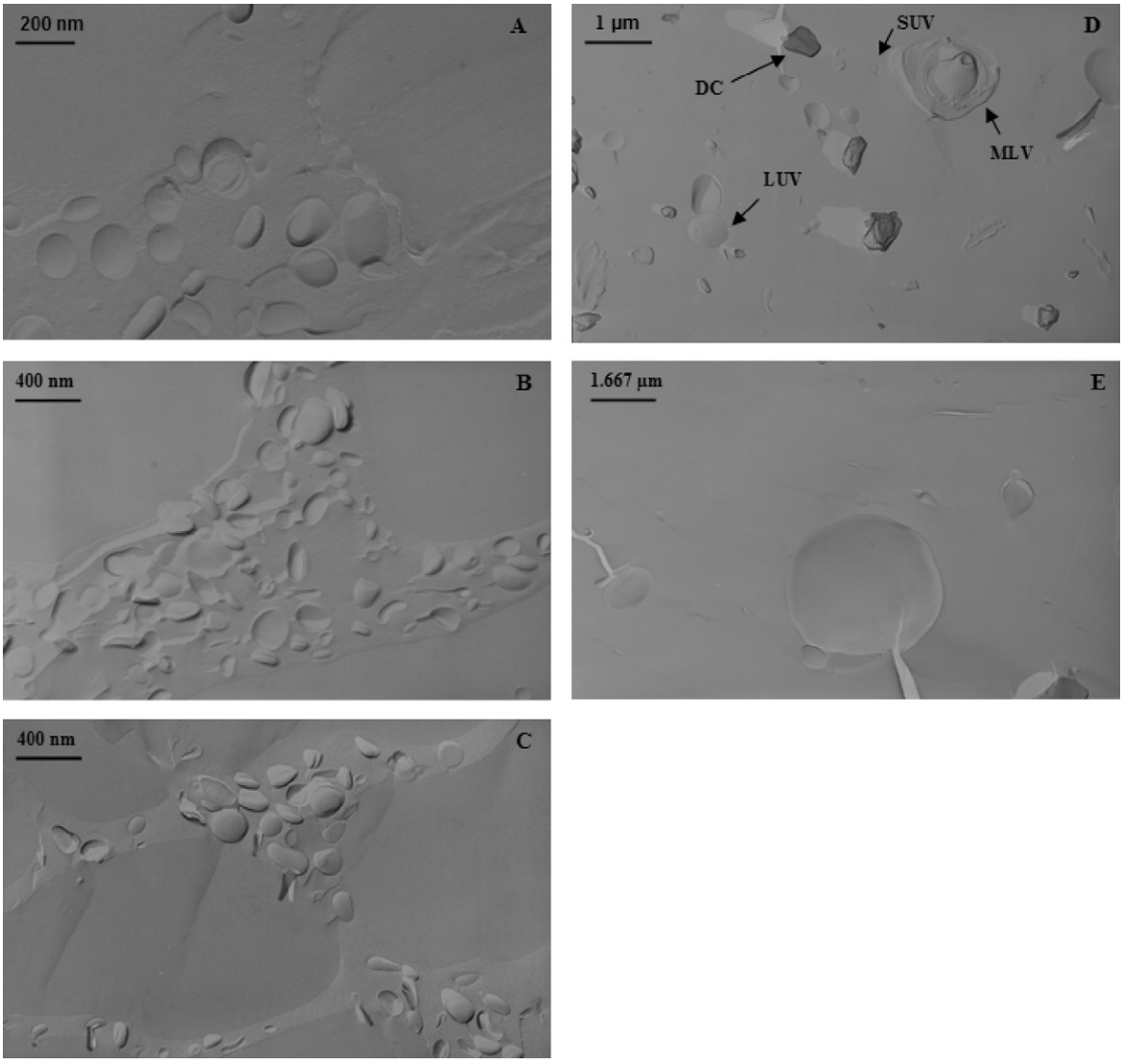

Fig. 1. Transmission electron micrographs of freeze-fracture replica of liposomes and non liposomal dispersions. Classical non charged liposomes encapsulating betamethasone (A), negatively charged PC-DMPA liposomes encapsulating betamethasone (B), negatively charged PC-DMPA liposomes encapsulating betamethasone dipropionate (C), non liposomal dispersion PC-betamethasone (D with SUV = small unilamellar vesicle, LUV = large unilamellar vesicle, MLV = multilamellar vesicle and DC = drug crystal), and non liposomal dispersion PC-DMPA-betamethasone (E).

solution of drug in absolute ethanol and the commercially available lotion Diprosone® (containing 0.05% of corticoid expressed in betamethasone).

3.2.1. Penetration into the stratum corneum

Fig. 2A shows the amount of betamethasone determined on strips one, five, ten and fifteen. The other strips were discarded to reduce the number of samples. We can observe that the amount of drug decreases with the number of strips. As shown in Fig. 2A, for the first strip we observe a high betamethasone content from the betamethasone ethanolic solution. The reason is the effectiveness of skin washing by Hepes, which can more easily remove betamethasone from aqueous medium than from the ethanolic solution. A higher amount of the drug is also determined on the first strip in the case of non liposomal dispersions in comparison with liposomes. It can be explained by sedimentation of the drug on the skin surface due to the instability of the non liposomal dispersions. Fig. 2B shows the amount of betamethasone dipropionate determined on strips one, five, ten and fifteen. We also observe that the amount of the drug decreases with the number of strips. As shown in Fig. 2B, for the first strip we observe a high betamethasone dipropionate content from the betamethasone dipropionate ethanolic solution and from Diprosone®.

3.2.2. Penetration into the epidermis, dermis and receptor medium

Fig. 3 shows the amount of betamethasone (BM; left part of the graph) and betamethasone dipropionate (BMD; right part) in the epidermis, dermis and receptor medium of Franz type diffusion cells.

Regarding the betamethasone amount in the epidermis (left part in Fig. 3), the encapsulation in liposomes significantly enhances the penetration of betamethasone compared with the ethanolic solution ($p < 0.05$). No significant difference in penetration is observed between neutral liposomes (PC-BM), positively charged liposomes (PC-SA-BM), and the non liposomal dispersions (Dispersion PC-BM, Dispersion PC-DMPA-BM) ($p > 0.05$). However, negatively charged PC-DMPA-BM liposomes enhance the penetration of betamethasone 9.3 times compared with the ethanolic solution, 2.5 times compared with neutral liposomes and 2.7 times compared with

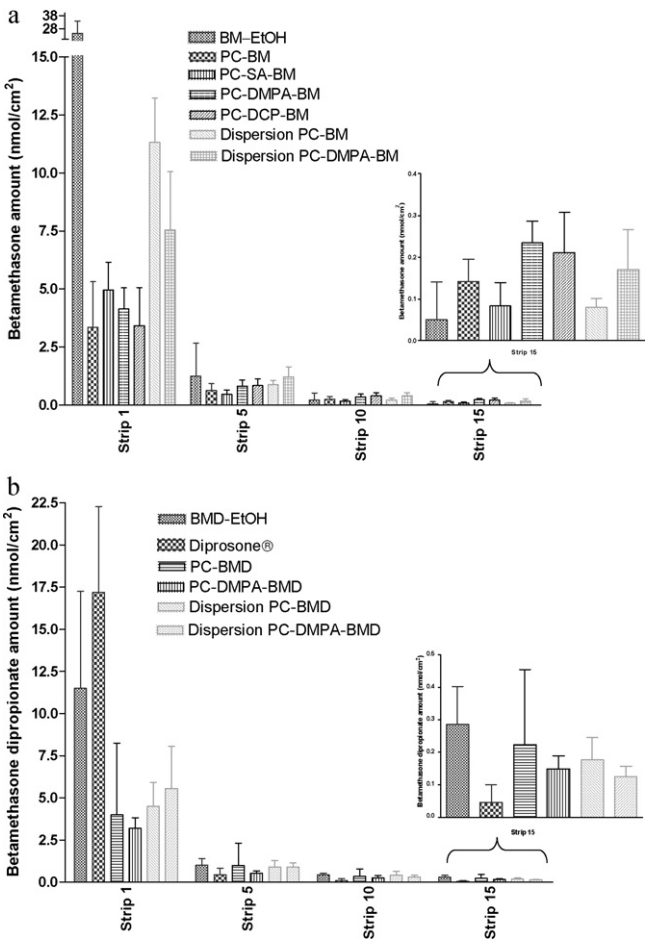

Fig. 2. Amount of betamethasone (A) or betamethasone dipropionate (B) as a function of the strip number (nmol/cm^2) ($n = 9$).

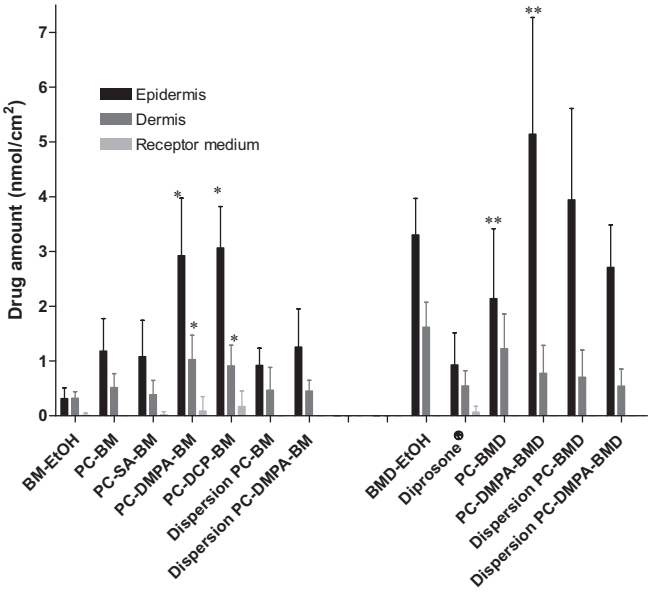

Fig. 3. Amount of betamethasone (BM, left part of the graph) or betamethasone dipropionate (BMD, right part of the graph) in the epidermis, dermis and receptor medium of Franz cells (nmol/cm^2) ($n = 9$) (* $p < 0.05$ compared with other BM formulations; ** $p < 0.05$ compared with Diprosone® lotion).

positively charged vesicles. In order to understand if this increase is due to the incorporation of DMPA or to the presence of a negative charge, DMPA was replaced by DCP. As shown in Fig. 3, the incorporation of DCP increases the penetration of betamethasone at the same level as DMPA ($p > 0.05$), indicating that the presence of a negative charge in the lipid bilayer of liposomes is enough to enhance the penetration of the encapsulated drug. For neutral liposomes, there is no significant difference between liposomes and the non liposomal dispersion, indicating that the vesicle formulation is not necessary ($p > 0.05$). However, in the case of negatively charged liposomes, the vesicle formulation is of high importance for the enhanced penetration. These differences between charged and uncharged liposomes and non liposomal dispersions could not be explained at this time and further investigations are needed. Regarding the betamethasone accumulation in the dermis, the penetration of betamethasone is also higher for negatively charged liposomes compared with the other formulations ($p < 0.05$).

The right part of Fig. 3 shows the penetration of betamethasone dipropionate from the different formulations. Differences with betamethasone are obvious. Regarding the accumulation in the epidermis, betamethasone dipropionate ethanolic solution penetrates well. The more lipophilic properties of the ester of betamethasone ($\log P = 4.07$) compared with betamethasone ($\log P = 1.94$) could explain this difference in the penetration behaviour (Takegami et al., 2008). Neutral liposomes (PC-BMD) and negatively charged liposomes (PC-DMPA-BMD) enhance the penetration in comparison with the commercially available lotion Diprosone® ($p < 0.05$). Negatively charged liposomes enhance the penetration of betamethasone dipropionate 1.6 times compared with the ethanolic solution, and 2.4 times compared with neutral liposomes. Negative liposomes also penetrate the epidermis better than the dispersion PC-DMPA-BMD ($p < 0.05$). However, the enhanced epidermis accumulation of the negative liposomes compared with the dispersion PC-BMD is not significant ($p > 0.05$).

Properties of the encapsulated drug seem to be of high importance for skin penetration behaviour. The entrapment in negatively charged liposomes enhances the epidermis absorption more than 9 times for betamethasone while the entrapment of betamethasone dipropionate enhances it only 1.6 times compared with their respective ethanolic solution. The penetration of a more lipophilic drug, with a high intrinsic penetration in ethanolic solution such as betamethasone dipropionate, is less enhanceable when incorporated into liposomes. The encapsulation in liposomes is therefore more interesting to improve the penetration of a drug with poor intrinsic penetration such as betamethasone.

Only very small amounts of the drug are found in the receptor medium of Franz diffusion cells in some cases. Concerning Diprosone® lotion, a small amount of betamethasone dipropionate was found in the receptor medium of 3 diffusion cells ($n = 9$). However, this potential transdermal delivery need to be confirmed on dermatomed skin or heat separated epidermis in order to control the thickness of the skin.

As a conclusion of *ex vivo* diffusion study, we can observe that the encapsulation of betamethasone and its ester in negatively charged liposomes significantly enhances skin penetration. These results are in agreement with those of Sinico et al. (2005) where the use of negatively charged DCP liposomes increased skin accumulation of entrapped tretinoin in comparison with positively charged SA liposomes.

3.2.3. Correlation between stratum corneum and viable skin penetration

Fig. 4A–C shows the relation between the betamethasone content in strip 15 (see enlargement in Fig. 2A) and the amount of betamethasone in the epidermis, dermis and total skin (epidermis and dermis), respectively. A good correlation can be shown

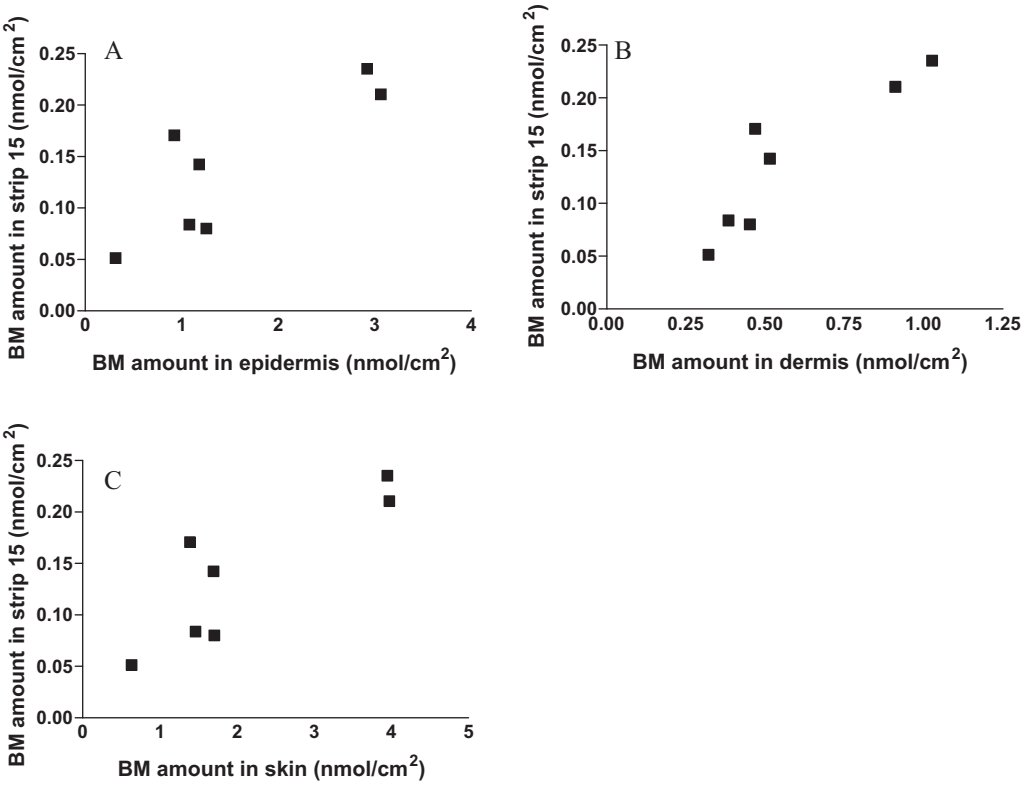

Fig. 4. Correlation between the amount of betamethasone (BM) determined in strip 15 (nmol/cm^2) and the amount of betamethasone in the epidermis (A), dermis (B) and total skin (epidermis + dermis) (C) (nmol/cm^2).

between the betamethasone content in strip 15 and the betamethasone amount determined in the epidermis (Fig. 4A, Pearson test, $p < 0.05$, $r = 0.8436$), in the dermis (Fig. 4B, Person test, $p < 0.05$, $r = 0.9035$) and in the total skin (Fig. 4C, Pearson test, $p < 0.05$, $r = 0.8603$). The betamethasone amount in the last strip, next to the viable epidermis is reflective of the amount penetrated deeper in the epidermis and dermis. Fig. 5A–C shows the relation between betamethasone dipropionate content in strip 15 (see

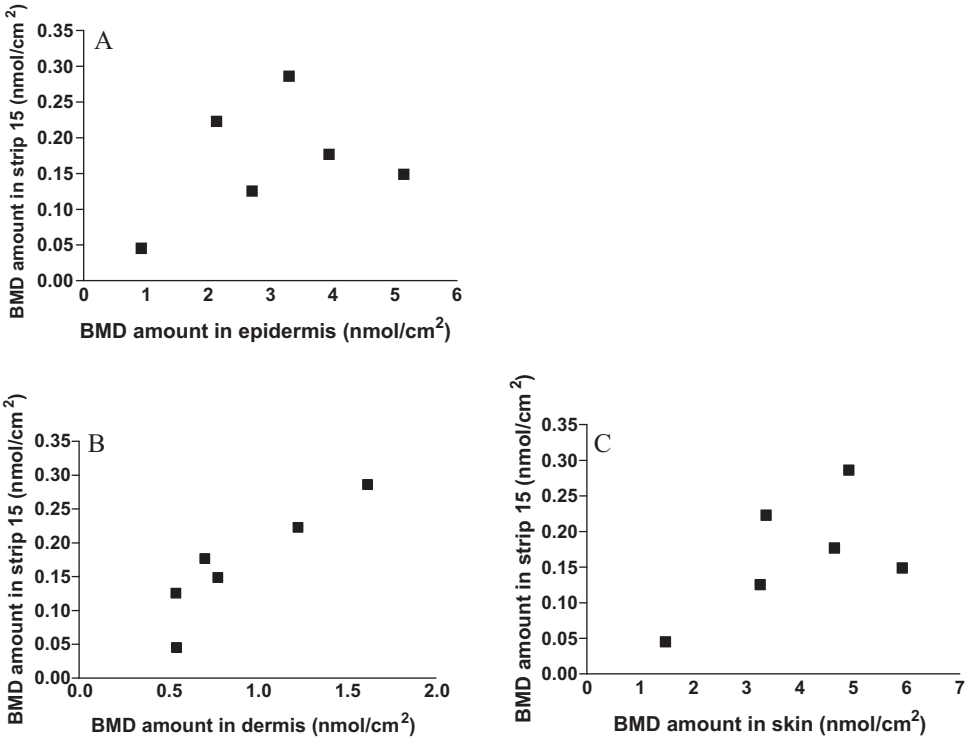

Fig. 5. Correlation between the amount of betamethasone dipropionate (BMD) determined in strip 15 (nmol/cm^2) and the amount of betamethasone dipropionate in the epidermis (A), dermis (B) and total skin (epidermis + dermis) (C) (nmol/cm^2).

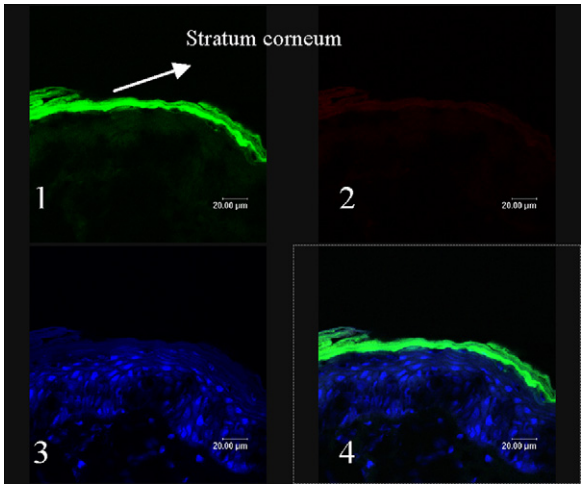

Fig. 6. CLSM images of skin autofluorescence (skin treated with TOTO-3 alone) divided into four parts with 1: green autofluorescence, 2: red autofluorescence, 3: fluorescence of cell nuclei; 4: overlay of Images 1–3. Scale bar represents 20 μm.

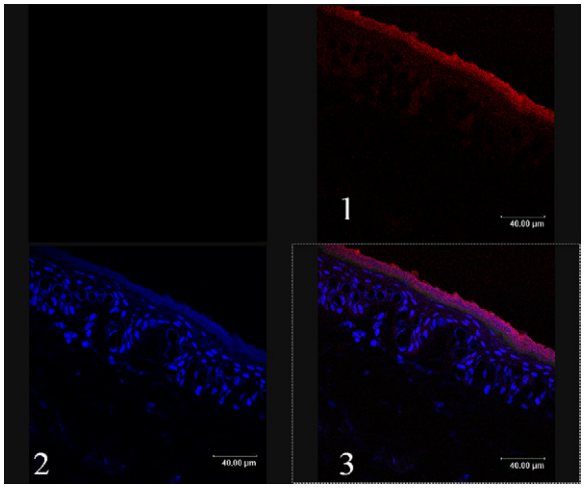

Fig. 7. CLSM images of the penetration of a solution of rhodamine B in Hepes buffer. The confocal image is divided into three parts with 1: the fluorescence of rhodamine B, 2: fluorescence of cell nuclei and 3: overlay of Images 1 and 2. Scale bar represents 40 μm.

enlargement in Fig. 2B) and the amount of betamethasone dipropionate in the epidermis, dermis and total skin (epidermis and dermis), respectively. Regrettably, the only significant correlation for betamethasone dipropionate is between the drug content in strip 15 and in the dermis (Fig. 5B, Pearson test, $p < 0.05$, $r = 0.9086$).

3.3. Confocal microscope observations

In order to visualise the skin delivery of the different formulations tested, liposomes were made fluorescent by two ways. Rhodamine B was encapsulated in the lipid bilayers of neutral and negatively charged liposomes, this lipophilic dye being supposed to mimic the encapsulation of betamethasone (similar log *P* values). Otherwise, the lipid bilayer was also made fluorescent by the incorporation of NBD-PC. This double labelling allows visualising the penetration of the lipid bilayer materials and the encapsulated drug simultaneously.

As explained previously, Fig. 6 shows the autofluorescence of TOTO-3 iodide dye treated skin (Gillet et al., in press). As a control, Fig. 7 shows the weak penetration in the epidermis of a solution of rhodamine B alone in Hepes buffer.

Fig. 8A and B reveals the penetration of neutral liposomes and negatively charged PC-DMPA liposomes, respectively. These images are divided into four parts in which 1 corresponds to the fluorescence of NBD-PC, 2 corresponds to the fluorescence of rhodamine B, 3 corresponds to the fluorescence of cell nuclei and 4 is the overlay of the three first images. Rhodamine B is seen to penetrate the epidermis deeply as well as NBD-PC and to follow the penetration of NBD-PC. Compared with the solution of rhodamine B in Hepes (Fig. 7), the encapsulation into neutral or negatively charged liposomes clearly enhances the penetration of rhodamine B (Fig. 8A and B). Similar results were obtained by Mura et al. (2007), where the encapsulation of rhodamine 6G in multilamellar liposomes increased its skin penetration in comparison with a rhodamine 6G solution. It must however be noted that rhodamine 6G (log *P* = 4.02) is more lipophilic than rhodamine B (Cheruvu and Kompella, 2006). In addition, compared with neutral liposomes (Fig. 8A), negatively charged liposomes appear to enhance the NBD-PC and rhodamine B fluorescence in the epidermis (Fig. 8B).

The present observations in confocal microscopy show that negatively charged vesicles do enhance the penetration of rhodamine B in the epidermis. They are in good agreement with the analytical results with betamethasone. In addition, owing that rhodamine B is supposed to mimic an encapsulated drug into liposomes and since it is strongly associated to the lipid bilayer, the results strongly suggest that negative charges at the surface of liposomes enhance the penetration of the vesicles together with their encapsulated drug. However, as only the fluorescent dyes are visible, this statement

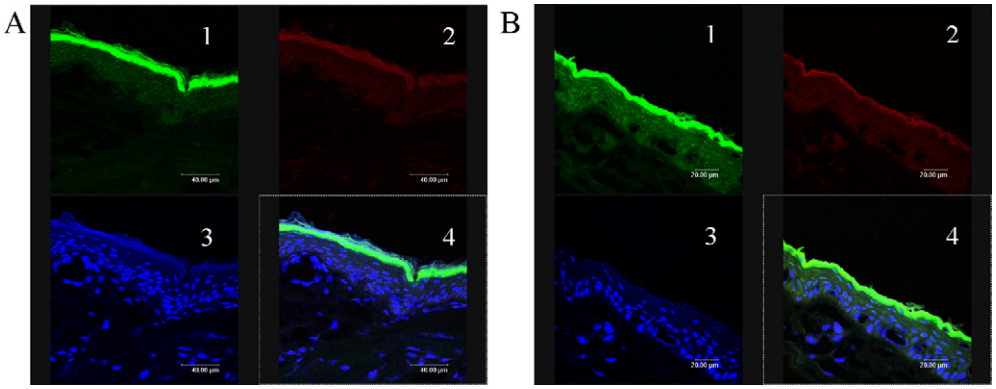

Fig. 8. CLSM images of the penetration of neutral liposomes encapsulating rhodamine B and NBD-PC (A) and negatively charged liposomes encapsulating rhodamine B and NBD-PC (B). Each confocal image is divided into four parts with 1: fluorescence of NBD-PC, 2: fluorescence of rhodamine B, 3: fluorescence of cell nuclei and 4: overlay of Image 1, Image 2 and Image 3. Scale bar represents 40 μm (A) or 20 μm (B).

needs to be confirmed. Indeed, the liposomes physical integrity during the penetration remains to be demonstrated.

4. Conclusions

In this study, we show that negatively charged liposomes significantly enhance betamethasone penetration in the epidermis compared to positively charged and neutral liposomes. This is observed with DMPA and DCP negative liposomes encapsulating betamethasone or betamethasone dipropionate. Negative non liposomal dispersions of PC, DMPA and betamethasone are unable to enhance skin penetration at the same level. Observations in confocal microscopy study seem to confirm the potential of the negatively charged vesicles. Further studies will be made in order to understand the mechanisms by which negatively charged liposomes enhance penetration. TEM observation of freeze fracture replica of skin samples will probably help us to see any effect on the *stratum corneum* ultra-structure and to see if intact vesicles are present.

Acknowledgments

We are very thankful to the Giga Cell Imaging and Flow Cytometry Platform for their technical help in the confocal study. A. Gillet is a PhD student supported by the FNRS, Brussels, Belgium.

References

Balaguer-Fernández, C., Femenía-Font, A., Muedra, V., Merino, V., López-Castellano, A., 2010. Combined strategies for enhancing the transdermal absorption of midazolam through human skin. J. Pharm. Pharmacol. 62, 1096–1102.

Carrer, D.C., Vermehren, C., Bagatolli, L.A., 2008. Pig skin structure and transdermal delivery of liposomes: a two photon microscopy study. J. Control. Release 132, 12–20.

Charoo, N.A., Rahman, Z., Repka, M.A., Murthy, S.N., 2010. Electroporation: an avenue for transdermal drug delivery. Curr. Drug Deliv. 7, 125–136.

Cheruvu, N.P., Kompella, U.B., 2006. Bovine and porcine transscleral solute transport: influence of lipophilicity and the Choroid-Bruch's layer. Invest. Ophthalmol. Vis. Sci. 47, 4513–4522.

Dragicevic-Curic, N., Grafe, S., Gitter, B., Winter, S., Fahr, A., 2010. Surface charged temoporfin-loaded flexible vesicles: in vitro skin penetration studies and stability. Int. J. Pharm. 384, 100–108.

El Maghraby, G.M., Williams, A.C., 2009. Vesicular systems for delivering conventional small organic molecules and larger macromolecules to and through human skin. Expert. Opin. Drug Deliv. 6, 149–163.

Escobar-Chávez, J.J., Bonilla-Martínez, D., Villegas-González, M.A., Revilla-Vázquez, A.L., 2009. Electroporation as an efficient physical enhancer for skin drug delivery. J. Clin. Pharmacol. 49, 1262–1283.

Escobar-Chavez, J.J., Bonilla-Martinez, D., Villegas-Gonzalez, M.A., Rodriguez-Cruz, I.M., Dominguez-Delgado, C.L., 2009. The use of sonophoresis in the administration of drugs throughout the skin. J. Pharm. Pharm. Sci. 12, 88–115.

FDA, U., 2001. Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER).

Gillet, A., Lecomte, F., Hubert, P., Ducat, E., Evrard, B., Piel, G. Skin penetration behaviour of liposomes as a function of their composition. Eur. J. Pharm. Biopharm., in press.

Hasanovic, A., Hollick, C., Fischinger, K., Valenta, C., 2010. Improvement in physicochemical parameters of DPPC liposomes and increase in skin permeation of aciclovir and minoxidil by the addition of cationic polymers. Eur. J. Pharm. Biopharm. 75, 148–153.

Hubert, P., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., Compagnon, P.A., Dewé, W., Feinberg, M., Lallier, M., Laurentie, M., Mercier, N., Muzard, G., Nivet, C., Valat, L., 2004. Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal—part I. J. Pharm. Biomed. Anal. 36, 579–586.

Iervolino, M., Cappello, B., Raghavan, S.L., Hadgraft, J., 2001. Penetration enhancement of ibuprofen from supersaturated solutions through human skin. Int. J. Pharm. 212, 131–141.

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.

Kogan, A., Garti, N., 2006. Microemulsions as transdermal drug delivery vehicles. Adv. Colloid Interface Sci. 123–126, 369–385.

Kolli, C.S., Chadha, G., Xiao, J., Parsons, D.L., Babu, R.J., 2010. Transdermal iontophoretic delivery of selegiline hydrochloride, in vitro. J. Drug Target. 18, 657–664.

Krishnan, G., Roberts, M.S., Grice, J., Anissimov, Y.G., Benson, H.A.E., 2011. Enhanced transdermal delivery of 5-aminolevulinic acid and a dipeptide by iontophoresis. Pept. Sci. 96, 166–171.

Leveque, N., Raghavan, S.L., Lane, M.E., Hadgraft, J., 2006. Use of a molecular form technique for the penetration of supersaturated solutions of salicylic acid across silicone membranes and human skin in vitro. Int. J. Pharm. 318, 49–54.

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

Mura, P., Maestrelli, F., González-Rodríguez, 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.

Nair, A., Vyas, H., Shah, J., Kumar, A., 2011. Effect of permeation enhancers on the iontophoretic transport of metoprolol tartrate and the drug retention in skin. Drug Deliv. 18, 19–25.

Namdeo, A., Jain, N.K., 1999. Niosomal delivery of 5-fluorouracil. J. Microencapsul. 16, 731–740.

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.

Piel, G., Piette, M., Barillaro, V., Castagne, D., Evrard, B., Delattre, L., 2006. Betamethasone-in-cyclodextrin-in-liposome: the effect of cyclodextrins on encapsulation efficiency and release kinetics. Int. J. Pharm. 312, 75–82.

Sinico, C., Manconi, M., Peppi, M., Lai, F., Valenti, D., Fadda, A.M., 2005. Liposomes as carriers for dermal delivery of tretinoin: in vitro evaluation of drug permeation and vesicle–skin interaction. J. Control. Release 103, 123–136.

Takegami, S., Kitamura, K., Funakoshi, T., Kitade, T., 2008. Partitioning of anti-inflammatory steroid drugs into phosphatidylcholine and phosphatidylcholine-cholesterol small unilamellar vesicles as studied by second-derivative spectrophotometry. Chem. Pharm. Bull. (Tokyo) 56, 663–667.

Viswanathan, C., Bansal, S., Booth, B., DeStefano, A., Rose, M., Sailstad, J., Shah, V., Skelly, J., Swann, P., Weiner, R., 2007. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. Pharm. Res. 24, 1962–1973.

Williams, A.C., Barry, B.W., 1992. Skin absorption enhancers. Crit. Rev. Ther. Drug Carrier Syst. 9, 305–353.

Williams, A.C., Barry, B.W., 2004. Penetration enhancers. Adv. Drug Deliv. Rev. 56, 603–618.

Yoo, J., Shanmugam, S., Song, C.K., Kim, D.D., Choi, H.G., Yong, C.S., Woo, J.S., Yoo, B.K., 2008. Skin penetration and retention of L-ascorbic acid 2-phosphate using multilamellar vesicles. Arch. Pharm. Res. 31, 1652–1658.