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Research paper

Skin penetration behaviour of liposomes as a function of their composition

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ABSTRACT

Deformable liposomes have been developed and evaluated as a novel topical and transdermal delivery system. Their mechanism of drug transport into and through the skin has been investigated but remains a much debated question. The present study concerns *ex vivo* diffusion experiments using pig ear skin in order to explain the penetration mechanism of classical and deformable liposomes. Classical and deformable vesicles containing betamethasone in the aqueous compartment through the use of cyclodextrin inclusion complexes were compared to vesicles encapsulating betamethasone in their lipid bilayer. Deformable liposomes contained sodium deoxycholate as the edge activator. Liposomes were characterised by their diameter, encapsulation efficiency, deformability, stability (in terms of change in diameter) and release of encapsulated drug. *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 that liposomes do not stay intact when they penetrate the deepest layers of the skin. Betamethasone is released from the vesicles after which free drug molecules can diffuse through the stratum corneum and partition into the viable skin tissue.

1. Introduction

A major obstacle to cutaneous drug delivery is the permeation characteristics of the *stratum corneum*, which limits drug transport, making this route of administration frequently insufficient for medical use. During the past few decades, there has been a wide interest in exploring new techniques for increasing drug absorption through the skin [1,2]. Topical delivery of drug by liposomes has aroused considerable interest.

Recently, it became evident that, in most cases, classical liposomes are of little or no value as carriers for transdermal drug delivery, as they do not penetrate skin deeply, but rather remain confined to the upper layers of the stratum corneum [3]. In order to target deeper underlying skin tissue, intensive research led to the introduction and development of a new class of lipid vesicle, the highly deformable (elastic or ultraflexible) liposomes, which were named Transfersomes® [4]. Several studies have reported that deformable liposomes are able to improve *in vitro* skin delivery of various drugs [5] and to stay intact when they penetrate the skin, *in vivo*, transferring therapeutic amounts of drugs [6]. According to Cevc and Blume, the improved drug delivery by deformable liposomes is due to the driving force provided by the osmotic gradient between the outer and inner layers of the stratum corneum [4]. The important difference between deformable liposomes and traditional liposomes is the high and stress-dependent adaptability of such deformable vesicles, which enables them to squeeze between the cells in the stratum corneum, despite the large average vesicle size [7]. Thus, they can pass through the intact skin spontaneously, under the influence of the naturally occurring, *in vivo* transcutaneous hydration gradient [8]. These vesicles consist of phospholipids and an edge activator. An edge activator is often a single-chain surfactant, with a high radius of curvature, which destabilises the lipid bilayers of the vesicles and increases their deformability [3].

Following topical application, structural changes in the *stratum corneum* have been identified and intact vesicles have been visualised within the *stratum corneum* lipid lamellar regions, but no intact vesicles have been identified in the deepest viable tissues [9]. There is still considerable debate as to whether deformable vesicles behave as a true carrier system by penetrating the skin intact or whether they act as a permeation enhancer [10].

Depending on the composition and method of preparation, vesicles can vary with respect to size, lamellarity, charge, membrane fluidity or elasticity and drug entrapment. This variability, in addition to the skin model used (man or animal, *in vitro* or *in vivo*), probably accounts for the lack of understanding of the penetration mechanism of vesicles. In this context, systematic physicochemical and pharmacokinetic studies are still needed to define the mode of action of these vesicles [10,11].

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The present study concerns *ex vivo* diffusion experiments using pig ear skin in order to compare the penetration mechanism of classical and deformable liposomes. Betamethasone was chosen as a model drug only for *ex vivo* studies.

When incorporated into phospholipid films or bilayers, betamethasone, because of its hydrophobic nature, would be expected to be associated with the hydrocarbon chain region of lipid molecules. Betamethasone is also known to form inclusion complexes with various cyclodextrins. Cyclodextrins are cyclic (β-1,4)-linked oligosaccharides of d-glucopyranose containing a relatively hydrophobic central cavity and a hydrophilic outer surface. Cyclodextrins are able to form inclusion complexes with poorly water-soluble drugs. This inclusion allows the increase of the "hydrophilic" character of betamethasone and for the preparation of aqueous solutions. This cyclodextrin inclusion allows the incorporation of betamethasone–cyclodextrin complexes in the aqueous cavity of liposomes instead of in the lipid bilayer compartment. In a previous work, we studied the effect of cyclodextrins on the encapsulation efficiency and released kinetics of betamethasone from liposomes. We showed the feasibility and the advantages of the betamethasone-in-cyclodextrin-in-liposome formulation [12]. The influence of drug incorporation in liposomes on skin penetration is studied by comparing either liposomes encapsulating betamethasone into their lipid bilayer or liposomes encapsulating betamethasone into their aqueous cavity by using betamethasone–cyclodextrin complexes.

Classical and deformable vesicles containing betamethasone (BMS) in the aqueous compartment through the use of cyclodextrin inclusion complexes (PC–BMS–HPγCD and PC–Na Deoxy–BMS–HPγCD) were compared to vesicles encapsulating betamethasone in their lipid bilayer (PC–BMS and PC–Na Deoxy–BMS). Confocal microscopy was used in order to visualise the penetration of fluorescently labelled liposomes.

The aim of this study is to compare physicochemical characteristics and the skin penetration of classical and deformable liposomes encapsulating a hydrophobic drug in the lipid bilayer or in the aqueous cavity by the use of cyclodextrins.

2. Materials and methods

2.1. Chemicals and reagents

Betamethasone (E.P.) was purchased from Medeva (Braine L'Alleud, Belgium). Soybean phosphatidylcholine (PC) was purchased from Lipoïd (Ludwigshafen, Germany). 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti Polar lipids (Alabaster, AL, USA). Hydroxypropylated-γ-cyclodextrin (HPγCD, D.S. 0.7, 3.41% H_2O) was obtained from Wacker-Chemie GmbH (Munich, Germany). Sodium deoxycholate and calcein were purchased from Sigma–Aldrich (Bornem, Belgium). Rhodamine B and acetonitrile were obtained from Merck (Darmstadt, Germany). 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 (Race: Piétrain, age: 2-months-old) were purchased from the Faculty of Veterinary Medicine at the University of Liège, Belgium.

2.2. Preparation of inclusion complexes

Cyclodextrins were dissolved in Hepes buffer pH 7.4 in order to obtain a 10 mM HPγCD solution. Betamethasone was added in excess to the cyclodextrin solution. Water-soluble inclusion complexes were formed after stirring the mixture for 48 h at 25 °C. The milky solutions were then filtered through a 0.22-μm filter. The betamethasone concentration obtained for HPγCD inclusion complexes was 2.02 mg/mL. Solubility of betamethasone in Hepes buffer without cyclodextrin was evaluated to 65.2 μg/mL.

2.3. Liposome preparation

2.3.1. Entrapment of inclusion complexes into liposomes

Classical liposomes were made from PC, while PC and sodium deoxycholate (Na Deoxy) (87:13; m/m) as the "edge activator" were used for deformable liposomes. Liposomes were prepared by the hydration of lipid films. In practice, the required amount of lipids was dissolved in ethanol 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 betamethasone–HPγCD complex solution. Suspensions were then extruded three times through Nucleopore® polycarbonate membranes of successive 0.4 and 0.2 μm pore diameters (Whatman, Maidstone, UK). Free betamethasone complexes were separated from liposome-encapsulated betamethasone complexes 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 min at 4 °C. The supernatant was removed, and the pellet was resuspended in Hepes buffer. Liposome diameter was measured after this preparation. Betamethasone and PC were assayed in purified liposomes.

2.3.2. Entrapment of betamethasone into liposomal bilayers

Liposomes were prepared as described in Section 2.3.1 by hydration of lipid films. However, in this case, 3 mL of betamethasone solution (2 mg/mL in absolute ethanol) was added at the same time as the lipids before evaporation. The resulting lipid film was hydrated using 3 mL of Hepes buffer. Suspensions were then extruded, and free betamethasone was removed by ultracentrifugation, as described earlier.

2.4. Liposome characterisation

2.4.1. Measurement of liposome diameter

Liposome dispersions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments Ltd., Worcestershire, UK). Measurements were made at 25 °C with a fixed angle of 90°, and the results were expressed as an average liposomal hydrodynamic diameter (nm).

2.4.2. Encapsulation efficiency

The encapsulation efficiency ($EE_{B/Bt}$) corresponds to the concentration of betamethasone encapsulated in liposomes (C_B) compared to the total drug concentration first introduced (C_{Bt}). This $EE_{B/Bt}$ was corrected to the concentration of lipids in order to take into account the loss of liposomes during their preparation:

$$EE_{B/Bt}(\%) = (C_B/C_L)/(C_{Bt}/C_{Lt}) \times 100$$

C_L is the lipid concentration in purified liposomes, and C_{Lt} is the lipid concentration first introduced.

2.4.2.1. Betamethasone 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 × 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). The flow rate settled at 0.8 mL/min, and the sample injection volume was 20 μL. Betamethasone was monitored at 240 nm.

2.4.2.2. Quantification of lipids. Total lipid concentrations were calculated by measuring PC through an enzymatic method (LabAssay™ Phospholipid, Wako, Osaka, Japan). The principle of this enzymatic assay consists of the cleavage of PC in choline by phospholipase D and 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).

2.4.3. Deformability

Comparative measurements of elasticity of the bilayers of the different liposome formulations were carried out by extrusion measurements [13]. Briefly, the vesicles were extruded through a polycarbonate membrane with a pore size of 50 nm (Nucleopore, The Netherlands) at a constant pressure of 5 bars. The elasticity was expressed in terms of a deformability index D, which is proportional to $j(r_v/r_p)^2$, where j is the weight of the suspension, which was extruded in 5 min through the polycarbonate membrane, r_v the diameter of the vesicles after extrusion and r_p the pore size of the membrane. Liposome suspensions were diluted at the same lipid concentration. The loss of phosphatidylcholine during the extrusion was calculated. A corrected deformability index D_{cor} to the maximum deformability index for each formulation was calculated too.

$$D_{cor} = [(j(r_v/r_p)^2)/(j_0(r_{v0}/r_p)^2)] \times 100$$

where r_{v0} is the liposomes diameter before extrusion and j_0 is the weight of suspension used for extrusion.

2.4.4. Leakage after ultracentrifugation

Liposome resistance to ultracentrifugation was evaluated by measuring the leakage of the encapsulated drug. In practice, a sample was centrifuged at 35,000 rpm for 1 h 30 min at 4 °C. The supernatant was then assayed to determine the released betamethasone. The amount of betamethasone released was calculated by the following equation:

$$\%\ \text{Betamethasone released} = C_{Bsup}/C_B \times 100$$

where C_{Bsup} is the concentration of betamethasone in the supernatant and C_B is the concentration of betamethasone encapsulated in liposomes.

2.4.5. Liposome stability

The stability of liposomes was evaluated by measuring the particle mean diameter and polydispersity indexes by PCS after one month of storage at 4 °C.

2.4.6. Release of the encapsulated drug

The release of the encapsulated betamethasone was evaluated at three temperatures (4 °C, 25 °C, and 37 °C) for a period of one month. In practice, every week, a sample stored at each temperature was centrifuged at 35,000 rpm for 1 h 30 min at 4 °C. The supernatant was then assayed to determine the released betamethasone. The amount of betamethasone released was calculated by the following equation:

$$\%\ \text{betamethasone released}_t = [(C_{Bsup\ t}/C_{Bt})/(C_{Bsup\ t0}/C_{B\ t0})] \times 100$$

where C_{Bsup} is the concentration of betamethasone in the supernatant at the day of preparation ($C_{Bsup\ t0}$) or after t days of storage($C_{Bsup\ t}$) and C_B is the concentration of betamethasone encapsulated in liposomes at the day of preparation ($C_{B\ t0}$) or after t days (C_{Bt}) [14]. Results are calculated in order to remove the leakage due to ultracentrifugation (t_0), this leakage being reproducible.

2.5. Ex vivo diffusion study

2.5.1. Skin preparation

Full-thickness skin was removed from the dorsal side of the 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. These cells consist of two compartments with the skin clamped between the donor and receiver chambers, dermal side down. The cell body was filled with 7.5 mL of a receptor phase consisting of Hepes buffer solution pH 7.4 containing 0.01% NaN_3 as the preservative. Test conditions were chosen in order to respect sink conditions in the receiver compartment. Solubility of betamethasone in the Hepes buffer was evaluated to 65.2 μg/mL, corresponding to around 10 times the maximum concentration of betamethasone that can be found in the receiver compartment. The receptor medium was constantly stirred with a small magnetic bar and thermostated at 37 °C throughout the experiments. A volume of 350 μL of liposome suspension at 150 μg/ml betamethasone concentration was placed in the donor chamber onto the *stratum corneum* of the skin, in non-occlusive conditions. The diffusion area was 1.767 cm^2. 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 with 15 successive strips of Corneofix® tape (CKelectronic, Germany). Only the 1st, 5th, 10th and 15th strips were kept and analysed for betamethasone 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 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. Each formulation was tested in triplicate, and each batch was tested on three Franz cells (n = 9).

2.5.3. Betamethasone 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 carried out on Isolute® C18, 50 mg, 1 mL (Biotage, Uppsala, Sweden) disposable extractive cartridges (DEC). After conditioning with 1 mL acetonitrile and 1 mL Hepes buffer, 1 mL sample was loaded onto the DEC and then washed with 1 mL water. Betamethasone was eluted with 500 μL acetonitrile and 500 μL water was added before the HPLC injection. The chromatographic conditions were described in Section 2.4.2.1.

2.5.3.2. SPE–HPLC–UV method validation. For the validation, two types of standards were prepared. The first were calibration standards, prepared in the mobile phase at seven concentration levels ranging from 20 to 10,000 ng/mL. The others were validation standards, prepared at seven concentration levels ranging from 20 to 10,000 ng/mL. Validation standards were made in the skin extract

in the Hepes buffer so as to mimic real samples obtained after permeation experiments. The validation was performed in three series. For each series, all the calibration standards were analysed in duplicate, while each validation standard was analysed in triplicate.

The validation was based on an accuracy profile approach [15]. For betamethasone determination in the pig ear skin, the acceptance limits were set at 30% from 24.2 to 60 ng/mL and 15% from 60 to 10,000 ng/mL respectively, and the risk level was fixed at 10%. For betamethasone determination from the tape stripping method, the acceptance limits were set at 10% from 20.02 to 10,000 ng/mL and the risk level was fixed at 5% [16,17]. The most appropriate calibration model was a weighted ($1/x^2$) 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 (CLSM) study

2.6.1. Liposome preparation

Liposomes were prepared and characterised as described in Sections 2.3 and 2.4 with some modifications. Liposomes were made fluorescent in two ways. First, the aqueous cavity was made fluorescent by the incorporation of 16 mM calcein solution in order to avoid the self quenching phenomenon that occurs at higher concentration. Calcein ($\log P = -5.02$) is a hydrophilic dye, which was expected to be encapsulated in the aqueous compartment of liposomes like the betamethasone–cyclodextrin inclusion complexes [18]. Secondly, rhodamine B ($\log P = 1.95$) (0.01% m/m) was incorporated into the lipid bilayer in order to mimic the encapsulation of betamethasone ($\log P = 1.94$) [19]. In each case, the lipid bilayer became fluorescent through the incorporation of NBD-PC (1.33% m/m). After extrusion, non-encapsulated calcein, rhodamine B or NBD-PC were separated from liposomes by successive ultracentrifugations at 35,000 rpm.

It must be noted that calcein and NBD-PC are fluorescent in the same range of wavelengths, so they could not be encapsulated in the same formulation; two formulations were needed.

2.6.2. Confocal laser scanning microscopy

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 an 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, Germany). All optical sections were recorded with the same settings. Calcein and NBD-PC were 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 in a blue colour. Images were acquired using a 40× objective lens immersed in oil.

2.7. Statistical analysis

The significance of the differences between different 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 and deformable liposomes containing betamethasone–HPγCD inclusion complexes were compared to the corresponding formulation of liposomes with betamethasone included in their lipid bilayer. PCS was performed for diameter analysis. As shown in Table 1, liposomes are characterised by a mean hydrodynamic diameter of between 128 ± 6 nm and 178 ± 12 nm. The polydispersity indexes (not shown) were always lower than 0.2, indicating that the liposomes were homogeneous in size. Deformable liposomes were significantly smaller in size than the corresponding non-deformable liposomes (140 and 128 nm for deformable liposomes encapsulating betamethasone–HPγCD complexes or betamethasone respectively, *versus* 169 and 178 nm for classical liposomes encapsulating betamethasone–HPγCD complexes or betamethasone respectively; $p < 0.05$). This reduction of the particle diameter for deformable liposomes may be ascribed to increased flexibility and reduced surface tension of the vesicles due to the presence of sodium deoxycholate, as observed by Chen et al. [20].

Betamethasone encapsulation efficiency is reported in Table 1. Encapsulation of betamethasone into the lipid bilayer significantly enhances the encapsulation efficiency from 39.1 ± 1.9% to 97.8 ± 5.4% for classical liposomes and from 42.5 ± 2.0% to 74.6 ± 2.5% for deformable liposomes. The addition of sodium deoxycholate significantly decreased the encapsulation efficiency from 97.8 ± 5.4% to 74.6 ± 2.5% for liposomes containing betamethasone in their lipid bilayer ($p < 0.05$). This can be explained by a competition phenomenon between betamethasone and sodium deoxycholate in the lipid bilayer. The absence of drug crystals in the case of liposomes encapsulating betamethasone in their lipid bilayer was confirmed by the transmission electron microscopy (TEM) of freeze-fracture replica. Thus, betamethasone is rightly associated with the lipid bilayer.

Prepared formulations were subjected to the deformability test by extrusion measurements. Results are expressed in terms of deformability index *D* (Table 1). As expected, deformability is higher when liposomes contain sodium deoxycholate ($D = 8.2 \pm 0.6$ when betamethasone is encapsulated by inclusion complexes and 8.0 ± 0.4 when encapsulated alone in the lipid bilayer) than that of classical liposomes ($D = 3.4 \pm 0.3$ and 5.2 ± 1.0, respectively) ($p < 0.05$). The deformability index varies between a minimum value and a maximum value. The minimum value is 0 for all formulations, as no suspension passes through the polycarbonate membrane ($j = 0$). The maximum value depends on the formulation as liposome diameter varies with the formulation. Thus, a corrected deformability index to the maximum value was calculated (D_{cor} in Table 1). However, this correction does not change the aforesaid conclusions. The percentage of phosphatidylcholine recovered after the extrusion was calculated to ensure that the concentration of liposomes remains constant. For deformable liposomes, the percentage of phosphatidylcholine recovered is 89.5 ± 12.9% when betamethasone is encapsulated into the aqueous compartment and 84.1 ± 4.2% when betamethasone is encapsulated into the lipid bilayer. However, for classical liposomes, the percentage of phosphatidylcholine recovered is only 11.9 ± 1.7% when betamethasone is encapsulated into the aqueous compartment and 3.5 ± 2.8% when encapsulated into the lipid bilayer. In the case of classical liposomes, only low amounts of liposomes were extruded through the 50-nm-pore-size membrane.

Table 1
Diameter ± S.D. (nm), encapsulation efficiency ($EE_{B/Bt}$) ± S.D. (%), deformability index D ± S.D., corrected deformability index D_{cor} ± S.D. and leakage of betamethasone after ultracentrifugation ± S.D. (%) of classical or deformable liposomes containing betamethasone–cyclodextrin complexes (BMS–HPγCD) or betamethasone (BMS) ($n = 3$).

Composition	Diameter (nm)	$EE_{B/Bt}$ (%)	Deformability index D	Corrected deformability index D_{cor}	Leakage after ultracentrifugation
PC–BMS–HPγCD	169 ± 4	39.1 ± 1.9	3.4 ± 0.3	11.3 ± 1.2	20.9 ± 1.6
PC–Na deoxycholate–BMS–HPγCD	140 ± 2	42.5 ± 2.0	8.2 ± 0.6	30.6 ± 1.6	44.8 ± 3.2
PC–BMS	178 ± 12	97.8 ± 5.4	5.2 ± 1.0	17.1 ± 2.7	76.2 ± 1.6
PC–Na deoxycholate–BMS	128 ± 6	74.6 ± 2.5	8.0 ± 0.4	32.5 ± 2.4	82.7 ± 4.1

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 classical or deformable liposomes containing betamethasone–cyclodextrin complexes (BMS–HPγCD) or betamethasone (BMS) ($n = 3$).

Composition	Diameter T_0 (nm)	Diameter after 1 month 4 °C (nm)
PC–BMS–HPγCD	169 ± 4	169 ± 1
PC–Na deoxycholate–BMS–HPγCD	140 ± 2	141 ± 1
PC–BMS	178 ± 12	172 ± 5
PC–Na deoxycholate–BMS	128 ± 6	128 ± 6

This observation confirms that classical liposomes are more rigid and less deformable than those containing the edge activator.

This observation is also confirmed by the leakage of betamethasone after ultracentrifugation (Table 1). The resistance of liposomes to ultracentrifugation was evaluated. The percentage of betamethasone released is 20.9 ± 1.6% for classical liposomes encapsulating betamethasone–cyclodextrin inclusion complexes *versus* 44.7 ± 3.1% for deformable ones. The loss of the entrapped drug is probably due to the deformation of lipid membrane that occurs during the ultracentrifugation process, as observed by Lopez-Pinto et al. [21]. Deformable liposomes are certainly more sensitive to ultracentrifugation than classical liposomes because of the presence of sodium deoxycholate, which destabilises the membrane and makes it more permeable. The percentage of betamethasone released is 76.5 ± 2.1% for classical liposomes containing betamethasone in the lipid bilayer and 82.7 ± 4.1% for deformable ones. These results show that the use of cyclodextrins improves the retention of betamethasone inside the liposome cavity. This high drug release was also observed by Bhardwaj et al. with DMPC liposomes encapsulating dexamethasone in their lipid bilayer. They observed that dexamethasone release from extruded liposomes was fast and that most of the content was released within 48 h at 37 °C. Based on differential scanning calorimetry, they showed that dexamethasone destabilized the liposome membranes as indicated by a decrease in enthalpy and an increase in the peak width of the main transition [22].

The results show that the incorporation of sodium deoxycholate and/or betamethasone in the lipid bilayer enhances the permeability of the membrane.

The stability of liposomes was evaluated by measuring their diameter after 1 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.

The release of encapsulated betamethasone was evaluated as a function of time. Fig. 1 shows the percentage of betamethasone released from classical and deformable liposomes encapsulating betamethasone–HPγCD complexes for a period of one month. The release of the encapsulated drug does not increase significantly after one month when liposomes are stored at 4 °C or 25 °C ($p > 0.05$). However, when liposomes are stored at 37 °C, the release of betamethasone increases ($p < 0.05$). The difference in drug release between classical and deformable liposomes encapsulating

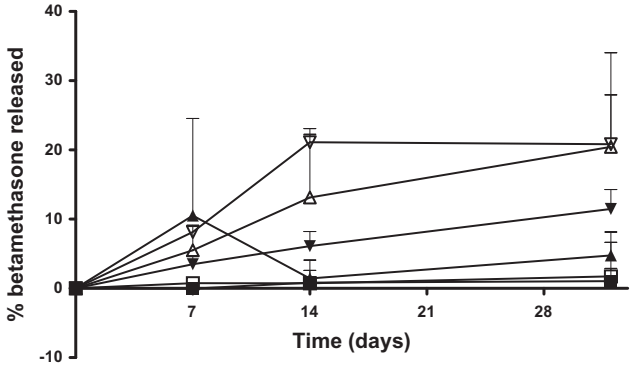

Fig. 1. Percentage of betamethasone released from liposomes encapsulating betamethasone–HPγCD complexes as a function of time. Classical (dark symbol) and deformable (open symbol) liposomes are stored at three different temperatures: 4 °C (■, □); 25 °C (▲, Δ); and 37 °C (▼, ▽).

betamethasone–cyclodextrin complexes after one month of storage at each temperature is not significant. Fig. 2 shows the percentage of betamethasone released from classical and deformable liposomes encapsulating betamethasone in their lipid bilayer as a function of time. The release of the encapsulated drug does not increase significantly after one month when liposomes are stored at 4 °C or 25 °C ($p > 0.05$). The release of betamethasone increases when liposomes are stored one month at 37 °C. However, due to high standard deviations, results are not significantly different ($p > 0.05$). The difference in drug release between classical and deformable liposomes encapsulating betamethasone alone after one month of storage at each temperature is not significant.

These results on characterisation show that the liposome diameter decreases when sodium deoxycholate is added. The encapsulation efficiency is higher for liposomes encapsulating betamethasone in their lipid bilayer compared with liposomes

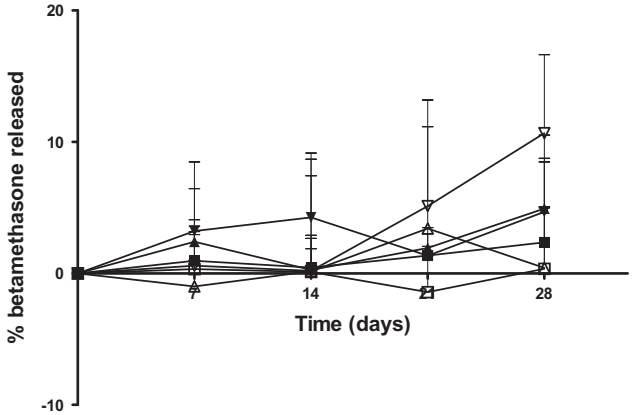

Fig. 2. Percentage of betamethasone released from liposomes encapsulating betamethasone in the lipid bilayer as a function of time. Classical (dark symbol) and deformable (open symbol) liposomes are stored at three different temperatures: 4 °C (■, □); 25 °C (▲, Δ); and 37 °C (▼, ▽).

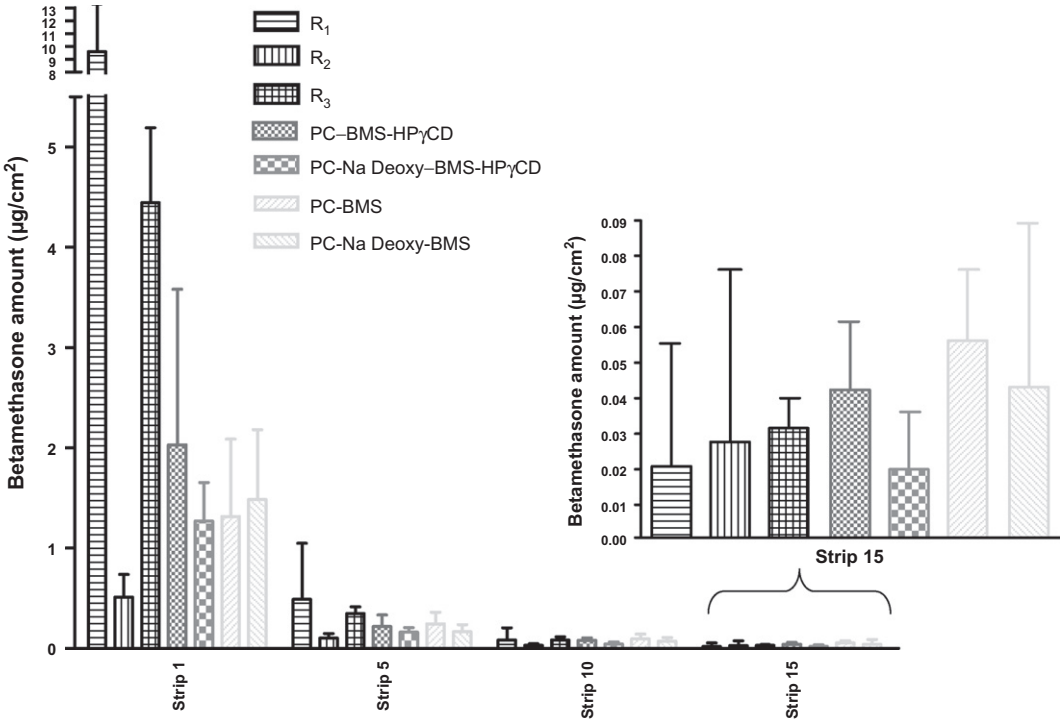

Fig. 3. Amount of betamethasone as a function of the strip number (µg/cm^2). R_1: solution of betamethasone in absolute ethanol, R_2: aqueous solution of betamethasone–HPγCD inclusion complexes and R_3: dispersion of phosphatidylcholine and betamethasone in Hepes buffer ($n = 9$).

encapsulating betamethasone–cyclodextrin complexes. Concerning the deformability study, the addition of the edge activator increases the deformability of the vesicles. The use of betamethasone–cyclodextrines complexes enhances the drug retention in liposomes. Liposome diameter did not change after one month at 4 °C. The release of the encapsulated drug increases with temperature.

3.2. Ex vivo penetration study

Franz-type diffusion cells were used to evaluate the *ex vivo* penetration of betamethasone encapsulated in liposomes in pig ear skin. Despite their advantages, human skin samples are rarely used due to limited resources, high logistic effort and regulatory issues. Therefore, porcine (ear) skin, bovine (udder) skin or rat skin is usually employed as an alternative [23]. Pig skin is considered as a good model for human skin [24–27]. All samples were adjusted at a final betamethasone concentration of 150 µg/mL. Penetration studies were carried out in non-occlusive conditions to allow the driving force provided by the osmotic gradient. Three reference samples were used for comparison: a solution of betamethasone in absolute ethanol (R_1), an aqueous solution of betamethasone–HPγCD inclusion complexes (R_2) and a dispersion of phosphatidylcholine and betamethasone in Hepes buffer (R_3).

3.2.1. Penetration in the stratum corneum

Fig. 3 shows the amount of betamethasone determined on strips 1, 5, 10 and 15. The other strips were discarded to reduce the number of samples. Histological studies confirmed that all the *stratum corneum* is removed after 15 strips. We can observe that the amount of betamethasone decreases with the number of strips. As shown in Fig. 3, for the first strip, we observe a high difference in betamethasone content between the betamethasone ethanolic solution (R_1) and the aqueous solution containing the betamethasone–HPγCD inclusion complexes (R_2). The reason for this difference is the effectiveness of skin washing by Hepes, which can more easily remove betamethasone from the aqueous solution than from the ethanolic solution. Betamethasone included in cyclodextrin is water soluble and is easily removed by the washing procedure. Ethanolic solution, when evaporated, leaves betamethasone not soluble in water and thus not easily washed.

3.2.2. Penetration in the epidermis, dermis and receptor medium

The amount of betamethasone accumulated in the epidermis, dermis and in the receptor medium of the Franz cells is shown in Fig. 4.

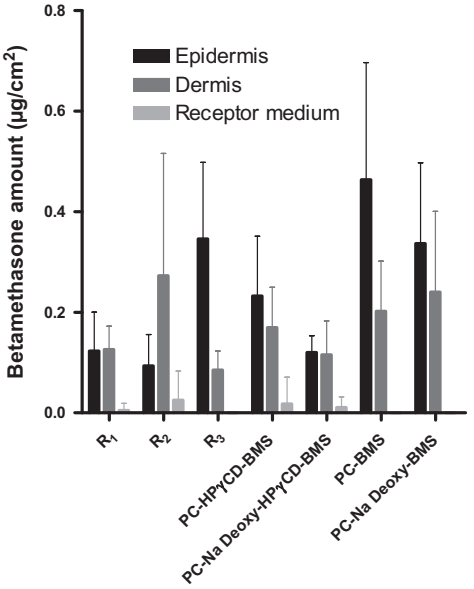

Fig. 4. Amount of betamethasone in the epidermis, dermis and receptor medium of Franz cells (µg/cm^2). R_1: solution of betamethasone in absolute ethanol, R_2: aqueous solution of betamethasone–HPγCD inclusion complexes and R_3: dispersion of phosphatidylcholine and betamethasone in Hepes buffer ($n = 9$).

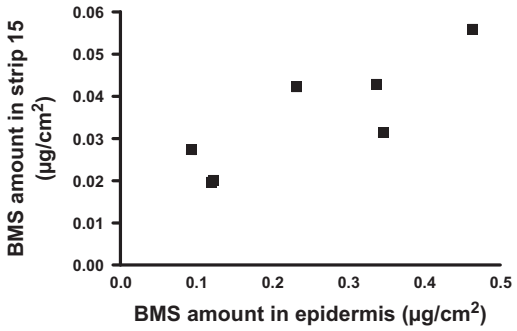

Fig. 5. Correlation between the amount of betamethasone (BMS) determined in strip 15 (µg/cm²) and the amount of betamethasone in the epidermis (µg/cm²).

Concerning the references, we observed that the dispersion of betamethasone and phosphatidylcholine in Hepes (R_3) penetrates the epidermis better than the ethanolic and the cyclodextrin complex solution (R_1 and R_2, respectively) ($p < 0.001$). The aqueous solution of betamethasone–HPγCD inclusion complexes (R_2) seems to penetrate the dermis better than the ethanolic solution (R_1) and the dispersion (R_3), but the standard deviation is too high and the difference is not significant. The better epidermis penetration of betamethasone from the dispersion (R_3), compared with other references, confirmed that phosphatidylcholine acts as a penetration enhancer.

Concerning liposome formulations, liposomes encapsulating betamethasone–cyclodextrin complexes systematically show a lesser cutaneous penetration than the corresponding formulation containing betamethasone alone. Cyclodextrin complexes reduce the amount of betamethasone in the epidermis from 0.46 ± 0.23 µg/cm² to 0.23 ± 0.12 µg/cm² for classical liposomes and from 0.34 ± 0.16 µg/cm² to 0.12 ± 0.03 µg/cm² for deformable liposomes ($p < 0.05$). The reservoir effect of liposomes containing cyclodextrins contributes to the reduced penetration. Regarding the high release of betamethasone from liposomes containing the drug in their lipid bilayer (Table 1) and the better epidermis penetration of this formulation, betamethasone would not penetrate the skin under an encapsulated form. Betamethasone is released from vesicles after which the free drug can penetrate through the *stratum corneum* and partition into the viable skin tissue. Maestrelli et al. also observed that ketoprofen permeated faster from liposomes containing plain drugs than from those containing drug–cyclodextrin complexes. This finding was attributed to the different preparation method of the liposomes. The drug alone, incorporated into the phospholipidic membrane bilayer, can be released rapidly, whereas the complexed drug, entrapped in the internal aqueous core, permeated more slowly, since it had to overcome the lipidic barrier of the vesicle membrane [28]. These results are in agreement with those observed in the present study.

We have also observed that sodium deoxycholate systematically show a lesser epidermis penetration than the corresponding formulation containing only PC. Sodium deoxycholate reduces the amount of betamethasone in the epidermis from 0.23 ± 0.12 µg/cm² to 0.12 ± 0.03 µg/cm² for liposomes encapsulating betamethasone–cyclodextrin complexes ($p < 0.05$) and from 0.46 ± 0.23 µg/cm² to 0.34 ± 0.16 µg/cm² for liposomes encapsulating betamethasone alone (however, $p > 0.05$).

No significant difference was found in the betamethasone content of the dermis between the different formulations. However, deformable liposomes encapsulating betamethasone in their lipid bilayer accumulate better in the dermis in comparison with deformable liposomes encapsulating betamethasone–cyclodextrin complexes ($p < 0.05$).

Only very small amounts of betamethasone were found in the receptor medium of Franz diffusion cells in some cases. This is certainly due to the use of full-thickness skin instead of dermatomed skin or a heat-separated epidermis. Thus, we were not able to observe any transdermal delivery of betamethasone.

As a conclusion of *ex-vivo* penetration study, we can observe that encapsulation of betamethasone–cyclodextrin complexes systematically decreases the betamethasone cutaneous penetration compared to the formulation containing betamethasone into lipid bilayers. We can also observe that surprisingly, deformable liposomes do not improve the penetration of betamethasone in the skin compared to classical liposomes. This observation cannot be explained at the present time. *Ex vivo* studies showed that the best formulation seems to be liposomes encapsulating betamethasone in the lipid bilayer.

It must be noted that the difference of accumulation in the epidermis between the dispersion of phosphatidylcholine and betamethasone (R_3) and the corresponding (PC–BMS) liposomes was not significant, indicating that the incorporation of betamethasone in liposomes would not be an advantage.

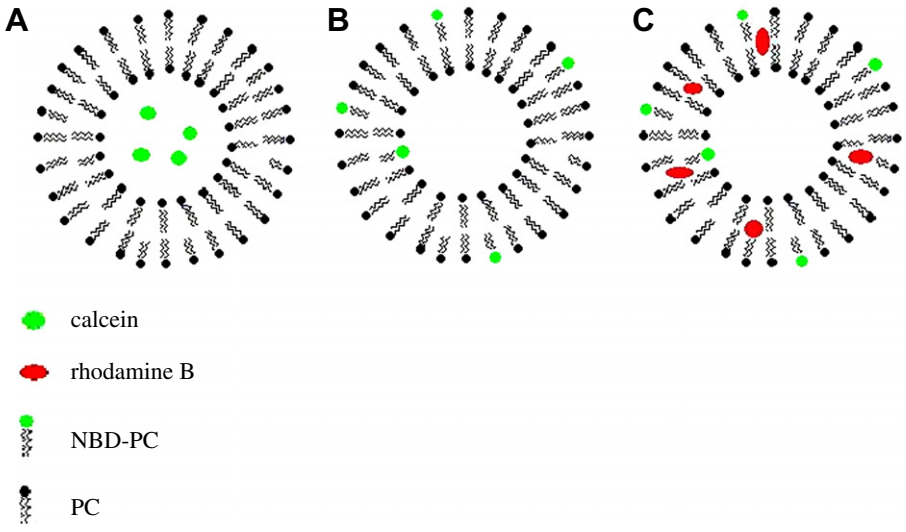

Fig. 6. Schematic representation of liposomes encapsulating calcein in their inner cavity (A), liposomes encapsulating NBD-PC in their lipid bilayer (B) and liposomes encapsulating rhodamine B and NBD-PC in their lipid bilayer (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

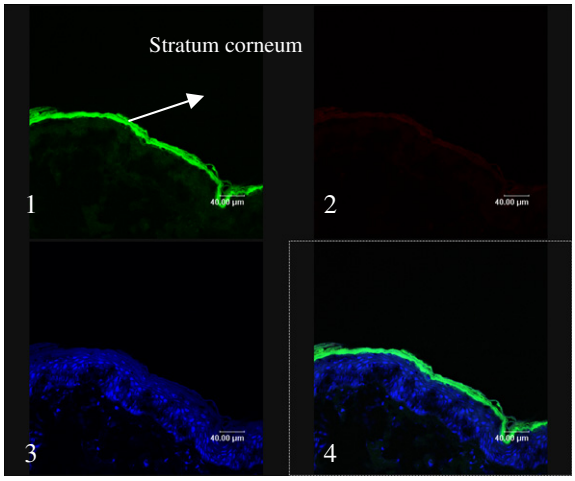

Fig. 7. CLSM images of skin autofluorescence (skin treated with TOTO-3 alone) divided into four parts with 1: green autofluorescence, 2: red autofluorescence, 3: fluorescence of cell nuclei and 4: overlay of 1, 2 and 3. Scale bar represents 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.3. Correlation between stratum corneum and epidemis penetration

Fig. 5 shows the relation between the betamethasone content in strip 15 (see enlargement in Fig. 3) and the amount of betamethasone in the epidermis. A good correlation could be shown between the betamethasone amount in strip 15 and the betamethasone amount determined in the epidermis (Pearson test, $p < 0.05$, $r^2 = 0.8436$). The betamethasone amount in the last strip, close to the viable epidermis, is reflective of the amount penetrated more deeply in the epidermis.

3.3. Confocal microscopic observations

In order to visualise the skin delivery of the different formulations tested, classical liposomes are made fluorescent in two ways. In the first experiment, the aqueous cavity of classical liposomes is made fluorescent by the inclusion of calcein, a hydrophilic dye ($\log P = -5.02$) supposedly encapsulated in the same compartment as the betamethasone–cyclodextrin inclusion complexes (Fig. 6A). In the second experiment, rhodamine B ($\log P = 1.95$) was encapsulated into the lipid bilayers of classical liposomes (Fig. 6C). This lipophilic dye is supposedly encapsulated into the lipid bilayer like free betamethasone ($\log P = 1.94$). In the two cases, the lipid bilayer is made fluorescent by the incorporation of NBD-PC (Fig. 6B and C). It must be noted that calcein and NBD-PC are fluorescent in the same range of wavelengths, so they could not be encapsulated in the same formulation but two formulations were needed (Fig. 6A and B). Skin penetration of fluorescently labelled deformable liposomes was also realised. Deformable liposomes showed the same characteristics as classical liposomes with no visible difference. Thus, these results are not shown.

3.3.1. Skin autofluorescence

TOTO-3 iodide dye-treated skins show an autofluorescence of the *stratum corneum* when excited with the 488-nm laser line (Fig. 7, part 1 in green colour) and a weak autofluorescence when excited with the 568-nm laser line (Fig. 7, part 2 in red colour). All measurements are made in sequential mode, so the autofluorescence observed is due to the skin and not to an overlapping of the TOTO-3 iodide staining (Fig. 7, part 3 in blue colour).

3.3.2. Experiments with calcein

The penetration of an aqueous solution of calcein in Hepes buffer was evaluated, and the results are shown in Fig. 8A and B. We can observe that calcein does not penetrate the epidermis. In addition, there is no accumulation in the hair follicles (Fig. 8B).

Fig. 9A–D shows the first experiments with calcein and NBD-PC. Fig. 9A shows the penetration of classical liposomes encapsulating calcein. Fig. 9B shows the penetration of classical liposomes containing NBD-PC as a marker of the lipid constituting the membrane. Each confocal image is divided into three parts in which 1 corresponds to the fluorescence of calcein (Fig. 9A) or NBD-PC (Fig. 9B), 2 corresponds to the fluorescence of cell nuclei and 3 is the overlay of image 1 and image 2. Fig. 9A shows that only a very low amount of the components of the liposome inner cavity (calcein) penetrates the epidermis. On the contrary, Fig. 9B shows that NBD-PC penetrates the epidermis and dermis deeply, which means that membrane components may penetrate more deeply. We can observe that calcein and NBD-PC penetrate the hair follicles of pig ear skin (Fig. 9C and D, respectively). These results confirm a penetration mechanism of liposomes already described in the literature that is a penetration of the hair follicles [29,30]. From these confocal images, we can conclude that calcein in solution or in the inner cavity of liposomes does not penetrate the epidermis, while NBD-PC penetrates the epidermis more deeply.

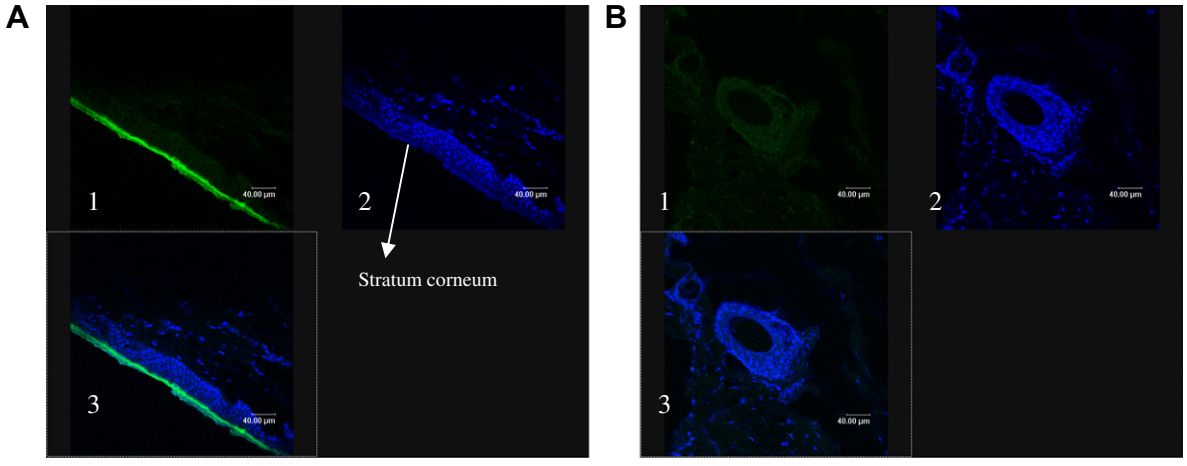

Fig. 8. (A) and (B): CLSM images of the penetration of a solution of calcein in Hepes buffer, each image divided into three parts with 1: fluorescence of calcein, 2: fluorescence of cell nuclei and 3: overlay of 1 and 2. Scale bar represents 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

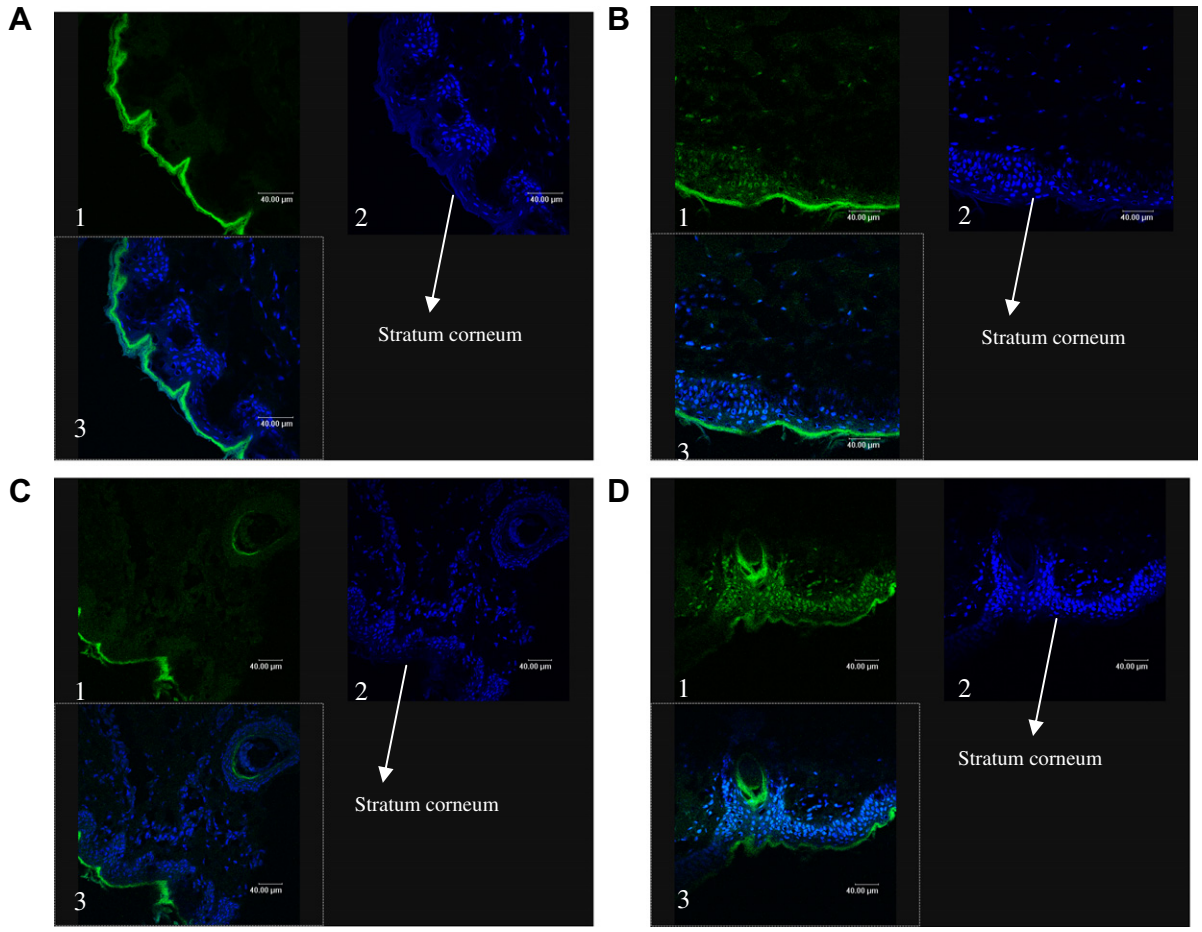

Fig. 9. (A–D): CLSM images, each image divided into three parts with 1: fluorescence of calcein or NBD-PC, 2: fluorescence of cell nuclei and 3: overlay of 1 and 2. (A) and (C): classical liposomes encapsulating calcein; (B) and (D): classical liposomes encapsulating NBD-PC. Scale bar represents 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3.3. *Experiments with rhodamine B*

Fig. 10 shows the penetration of an aqueous solution of rhodamine B and NBD-PC. We can observe that rhodamine B accumulates in the *stratum corneum* and that a small amount penetrates the epidermis (Fig. 10A). Unlike the calcein solution, rhodamine B in the Hepes solution does penetrate the hair follicles (Fig. 10B).

Fig. 11 shows the second experiments with liposomes encapsulating rhodamine B. Confocal 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 image 1, 2 and 3. Rhodamine B penetrates the epidermis and the dermis deeply as well as

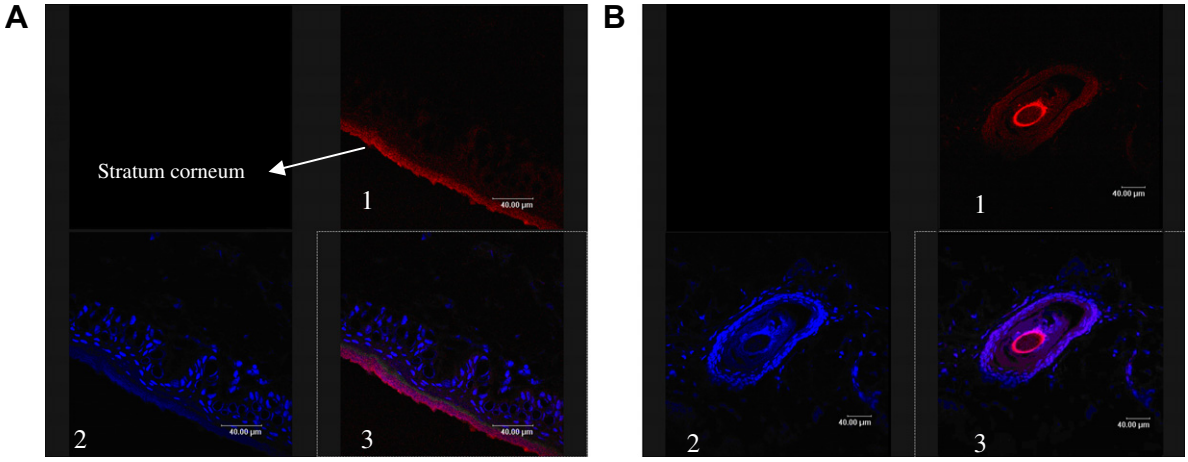

Fig. 10. CLSM images of the penetration of a solution of rhodamine B in Hepes buffer. The confocal image is divided into three parts with 1: fluorescence of rhodamine B, 2: fluorescence of cell nuclei and 3: overlay of 1 and 2. Scale bar represents 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

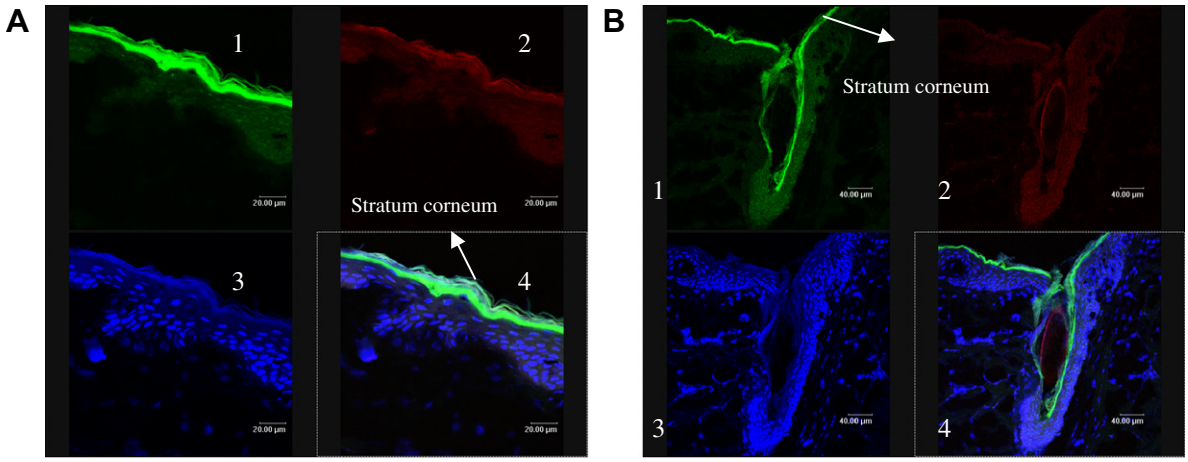

Fig. 11. CLSM images of the penetration of classical liposomes encapsulating rhodamine B and NBD-PC. The confocal image is divided into four parts with 1: fluorescence of NBD-PC, 2: fluorescence of rhodamine B, 3: fluorescence of cell nuclei and 4: overlay of 1, 2 and 3. Scale bar represents 20 μm (A) or 40 μm (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NBD-PC and follows the penetration of NBD-PC (Fig 11A). Compared to the penetration of the rhodamine B aqueous solution, the encapsulation of rhodamine B into the liposome bilayer seems to enhance its penetration. Rhodamine B encapsulated in liposomes also penetrates the hair follicles (Fig. 11B).

The confocal microscopic observations are in agreement with the analytical results. Calcein encapsulated in liposomes, which mimics the encapsulation of the betamethasone–cyclodextrin complexes, does not penetrate the epidermis, while rhodamine B encapsulated in liposomes, which mimics the encapsulation of betamethasone alone in the lipid bilayer, penetrates the epidermis.

The results also suggest that liposomes would not remain intact when penetrating the skin. Calcein and the aqueous inner cavity content are released and remain in the upper layers of the skin, in the *stratum corneum*. When betamethasone is encapsulated as a cyclodextrin complex, betamethasone will remain in the upper layers of the skin. These results are in agreement with those of Bahia et al. [9]. They studied the penetration of deformable liposomes encapsulating calcein through full-thickness hairless mouse skin. They concluded that the high membrane permeability to calcein of deformable vesicles and the non-encapsulated state of calcein after *in vitro* skin permeation are contradictory to a passage of calcein through the stratum corneum under the encapsulated form.

However, rhodamine B encapsulated in liposomes and NBD-PC penetrate the epidermis and the dermis deeply. Thus, when betamethasone is encapsulated in lipid bilayers, a deeper penetration may be obtained. Rhodamine B penetrates the epidermis better when encapsulated in liposomes than in the Hepes solution. Thus, PC could act as a penetration enhancer.

An accumulation of fluorescent dyes was observed in the hair follicles, confirming penetration of these cutaneous appendages. However, we could not confirm a diffusion of the fluorescent dye from these hair follicles.

4. Conclusion

This study shows that classical and deformable liposomes do not remain intact when penetrating the deepest layers of the skin and that phosphatidylcholine acts as a penetration enhancer. Betamethasone is released from the vesicles after which free drug molecules can diffuse through the *stratum corneum* and partition into the viable skin tissue. Within the framework of our study, deformable liposomes were not able to enhance the penetration of betamethasone compared to classical liposomes. The use of drug–cyclodextrin inclusion complexes enhanced the stability of the formulation but did not improve the penetration of betamethasone.

Acknowledgement

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