International Journal of Pharmaceutics 380 (2009) 174–180

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Development of a new topical system: Drug-in-cyclodextrin-in-deformable liposome

A. Gillet[a,*], A. Grammenos[b], P. Compère[c], 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 Biomedical Spectroscopy, Department of Physics, University of Liège, B5, Sart-Tilman, 4000 Liège, Belgium
[c] Laboratory of Functional and Evolutive Morphology, Department of Sciences and Environmental Management, University of Liège, Bat B6, Sart-Tilman, 4000 Liège, Belgium

ARTICLE INFO

Article history:
Received 23 April 2009
Received in revised form 19 June 2009
Accepted 23 June 2009
Available online 2 July 2009

Keywords:
Deformable liposome
Cyclodextrin
Betamethasone
Franz cells

ABSTRACT

A new delivery system for cutaneous administration combining the advantages of cyclodextrin inclusion complexes and those of deformable liposomes was developed, leading to a new concept: drug-in-cyclodextrin-in-deformable liposomes. Deformable liposomes made of soybean phosphatidylcholine (PC) or dimyristoylphosphatidylcholine (DMPC) and sodium deoxycholate as edge activator were compared to classical non-deformable liposomes. Liposomes were prepared by the film evaporation method. Betamethasone, chosen as the model drug, was encapsulated in the aqueous cavity of liposomes by the use of cyclodextrins. Cyclodextrins allow an increase in the aqueous solubility of betamethasone and thus, the encapsulation efficiency in liposome vesicles. Liposome size, deformability and encapsulation efficiency were calculated. The best results were obtained with deformable liposomes made of PC in comparison with DMPC. The stability of PC vesicles was evaluated by measuring the leakage of encapsulated calcein on the one hand and the leakage of encapsulated betamethasone on the other hand. *In vitro* diffusion studies were carried out on Franz type diffusion cells through polycarbonate membranes. In comparison with non-deformable liposomes, these new vesicles showed improved encapsulation efficiency, good stability and higher *in vitro* diffusion percentages of encapsulated drug. They are therefore promising for future use in *ex vivo* and *in vivo* experiments.

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 wide interest in exploring new techniques for increasing drug absorption through the skin (Barry, 2001; Honeywell-Nguyen and Bouwstra, 2005). Topical delivery of drug by liposomes has aroused a 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 upper layers of the stratum corneum (Elsayed et al., 2007). In order to target deeper underlying skin tissue, intensive research led to the introduction and development of a new class of lipid vesicles, the highly deformable (elastic or ultraflexible) liposomes, which have been called Transfersomes® (Cevc and Blume, 1992). Several studies have reported that deformable liposomes are able to improve *in vitro* skin delivery of various drugs (Trotta et al., 2004) and to penetrate intact skin, *in vivo*, transferring therapeutic amounts of drugs (Cevc and Blume, 2004). According to Cevc and Blume (1992), 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. The important difference between deformable liposomes and traditional liposomes is the high and stress-dependent adaptability of such deformable vesicles, which enables them alone to squeeze between the cells in the stratum corneum, despite the large average vesicle size (Cevc et al., 2002). Thus, they can pass through the intact skin spontaneously, under the influence of the naturally occurring, *in vivo* transcutaneous hydration gradient (Cevc and Blume, 2001). 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 destabilizes lipid bilayers of the vesicles and increases their deformability (Elsayed et al., 2007).

Liposomes are able to encapsulate hydrophilic drugs in their aqueous compartment, while lipophilic drugs are encapsulated in their lipid bilayer. However the accommodation of lipophilic compounds in the lipid phase can be problematic as some drugs can interfere with bilayer formation and stability. This accommodation is often limited in terms of drug to lipid mass ratio

* 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 © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.06.027

(McCormack and Gregoriadis, 1994; Maestrelli et al., 2005). In the case of the encapsulation of betamethasone into liposomes, the use of cyclodextrins was shown to increase the drug to lipid mass ratio (Piel et al., 2006). Entrapping water-soluble drug–cyclodextrin inclusion complexes in the aqueous compartment of liposomes have been proposed in order to avoid such drawbacks (McCormack and Gregoriadis, 1994; Maestrelli et al., 2005). 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. In a previous piece of research, we studied the effect of cyclodextrins on the encapsulation efficiency and release kinetics of betamethasone from liposomes. We showed the feasibility and the advantages of the betamethasone-in-cyclodextrin-in-liposome formulation (Piel et al., 2006). Hydroxypropylated γ cyclodextrin (HPγCD) and Crysmeb®, a methylated β cyclodextrin, increased the aqueous solubility of betamethasone at the highest level when compared to other cyclodextrins. The affinity constants ($K_{1\text{-}1}$) of betamethasone for HPγCD and Crysmeb® were 12,606 and 10,011 M^{-1}, respectively. These two cyclodextrins were shown to be particularly effective for obtaining high betamethasone concentrations and were consequently selected for this study. Piel et al. (2006) also demonstrated that these cyclodextrins had a higher affinity for betamethasone than for membrane lipids, allowing the entrapment of highly concentrated betamethasone inclusion complex solutions without or with minimal interaction with lipid components of the liposome membrane.

The purpose of this study is to develop a new delivery system for cutaneous administration combining the advantages of drug–cyclodextrin inclusion complexes and those of deformable liposomes, leading to a new concept: drug-in-cyclodextrin-in-deformable liposomes, first developed by Jain et al. (2008). This present study deals with the characterization and the *in vitro* diffusion properties of these vesicles.

Betamethasone was chosen as the model drug, because of its hydrophobic nature and its well known ability to form inclusion complexes with various cyclodextrins (Flood et al., 2000).

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (PC) was purchased from Lipoïd (Ludwigshafen, Germany). 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) came from Avanti®Polar Lipids (Pelham, AL, USA). Hydroxypropylated γ cyclodextrin (HPγCD, D.S. 0.7, 3.41% H_2O) was obtained from Wacker-Chemie GmbH (Munich, Germany) and Kleptose® Crysmeb (Crysmeb, D.S 0.5, 4.29% H_2O) from Roquette Frères (Lestrem, France). Sodium deoxycholate and calcein were purchased from Sigma–Aldrich (Bornem, Belgium). Betamethasone (E.P.) was purchased from Medeva (Braine L'Alleud, Belgium). All other reagents and solvents were of analytical grade.

All experiments were performed using a 0.22 μm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Sigma–Aldrich, Bornem, Belgique), containing 145 mM NaCl and adjusted to pH 7.4 with 0.1 N NaOH solution.

2.2. Formation of inclusion complexes

Cyclodextrins were dissolved in HEPES buffer pH 7.4 in order to obtain a 10 mM HPγCD or Crysmeb 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. Betamethasone concentrations obtained for HPγCD and Crysmeb inclusion complexes were 2.02 mg/mL and 2.16 mg/mL respectively.

2.3. Liposome preparation

Classical liposomes were made of PC or DMPC with (70:30 mol%) or without cholesterol, while PC or DMPC and sodium deoxycholate at 13% m/m as "edge activator" were used for deformable liposomes. Liposomes were prepared by hydration of lipid films. In practice, the required amount of lipids was dissolved in ethanol in a round-bottomed flask, using a rotary evaporator and was dried under vacuum. The resulting lipid film was hydrated using 3 mL of the corresponding betamethasone–cyclodextrin complex solution. Suspensions were then extruded 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 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 resuspended in HEPES buffer. Betamethasone and PC were assayed in purified liposomes. The same preparation method was used to make liposomes encapsulating calcein in order to study liposome stability. A 3 mL calcein solution was used to hydrate the lipid film. In order to detect a leakage-induced effect, the self-quenching fluorescent dye calcein was entrapped in these vesicles at a concentration of 40 mM. At this concentration, calcein shows minimal fluorescence, owing to the formation of ground state dimers. Any fluorescence measured will be due to calcein leakage and dilution in the exterior aqueous media. After extrusion, external calcein was removed by a first ultracentrifugation at 35,000 rpm for 3 h at 4 °C followed by four successive ultracentrifugations at 35,000 rpm for 45 min at 4 °C.

2.4. Liposome characterization

2.4.1. Measurement of liposome size

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

2.4.2. Freeze-fracture electron microscopy

Freeze-fracture replicas of liposome suspensions were examined in transmission electron microscopy. A drop of liposome suspension, containing 20% glycerol as a cryoprotectant, 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 shadowing equipment (Balzers® BAF-400) fitted with a freeze-fracture and etching unit. The replicas were examined with a JEOL (JEM-100SX) transmission electron microscope, operating at 80 kV accelerating voltage.

2.4.3. Encapsulation efficiency

Encapsulation efficiency was calculated by two different ways. The first encapsulation efficiency ($EE_{B/l}$) corresponds to the drug to lipid mass ratio or the amount of betamethasone in purified liposomes (C_B) compared to the total lipid concentration (C_l) in purified liposomes:

$$EE_{B/l}(\%) = \left(\frac{C_B}{C_l}\right) \times 100$$

The second encapsulation efficiency ($EE_{B/Bt}$) was the yield obtained. It 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 the liposomes preparation:

$$EE_{B/Bt}(\%) = \frac{(C_B/C_l)}{(C_{Bt}/C_{lt})} \times 100$$

C_l is the lipid concentration in purified liposomes and C_{lt} is the lipid concentration first introduced.

2.4.3.1. Betamethasone determination. Betamethasone was determined using a validated HPLC method. HPLC was performed using a system consisting of a LaChrom® Merck Hitashi system L-7100 pump, an L-4000 UV detector, a L-7200 autosampler and a D-2500 chromato integrator. Twenty microliter samples were injected onto a LiChroCART® column (250 mm × 4 mm i.d.) prepared with an octadecylsilane (C18) phase Superspher® (Merck) and maintained at 30 °C. The mobile phase consisted of a 50:50 (v:v) mixture of HPLC grade acetonitrile and water. The flow rate was 0.8 mL/min. Betamethasone was detected at 240 nm.

2.4.3.2. Quantification of lipids. Total lipid concentrations were calculated by measuring PC or DMPC by 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 PerkinElmer Lambda 11).

2.4.4. Deformability

Electron spin resonance (ESR) is currently used to investigate the microenvironment in membrane liposome. The relative anisotropy observed in an ESR spectrum of a nitroxide spin probe is directly related to the rotational mobility of the probe, a term that can be correlated with the probe's microviscosity. Here, the microviscosity is defined as homogenous solution viscosity, which results in the same spectrum as that recorded in the microenvironment. Standard curves of microviscosity values have been established by calibration of the ESR spectra of three n-doxyl stearic acids (n-DSA: $n = 5$, 12, 16) probes in glycerol–ethanol mixtures of known viscosities (Bahri et al., 2005). These curves allow the quantifying of the effective microviscosity at different depths inside liposomes by measuring the order parameter (S) and the correlation time (τ_c) on n-DSA ESR spectra. S and τ_c were calculated according to the method of McConnel (Hubbell and McConnel, 1971).

In this study, we used a 5-doxyl stearic acid (5-DSA) probe to measure the microviscosity of the liposomes. All ESR measurements were performed at 9.5 GHz using a Brucker ESR 300E spectrometer (Brucker, Germany) equipped with a variable temperature controller accessory and operating at a centre field strength of 3360 G with 120 G as the scan range. The measurements were made at 32 °C. Each measurement was repeated at least four times and the microviscosity standard deviation was calculated to be 2%.

2.4.5. Liposome permeability

The membrane integrity of liposomes at three temperatures (4 °C, 25 °C, and 37 °C) was evaluated by measuring the leakage of encapsulated calcein on the one hand and the leakage of encapsulated betamethasone–cyclodextrin inclusion complexes on the other hand. The leakage of liposomal content into the medium is indicative of changes in the membrane permeability.

2.4.5.1. Calcein leakage. In practice, 100 μL liposome suspension were added to 100 μL Hepes buffered solution or 100 μL of a 2% Triton X-100 solution, for complete liposome destruction, in a 96-well plate. Calcein release from liposomes was measured fluorometrically (SpectraMax Gemini XS); excitation and emission wavelengths were 490 and 520 nm, respectively. The amount of calcein released was calculated by the following equation:

$$\%\,\text{calcein released} = \frac{I}{I_T} \times 100$$

where I is the fluorescence intensity at 520 nm and I_T is the fluorescence intensity at 520 nm after complete destruction of the liposomes by Triton X-100.

2.4.5.2. Betamethasone release. In practice, every day, a sample stored at each temperature was centrifuged at 35,000 rpm for 45 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:

$$\%\,\text{betamethasone released} = \frac{C_{Bt}}{C_{B0}} \times 100$$

where, C_{Bt} is the concentration of betamethasone in the supernatant after t days of storage and C_{B0} is the concentration of betamethasone encapsulated in liposomes at day 0.

2.5. In vitro diffusion studies

Diffusion studies were carried out using Franz type glass diffusion cells. These consist of two compartments with a polycarbonate membrane (pore size: 50 nm) clamped between the donor and receiver chambers. The cell body was filled with 7.5 mL of a receptor phase consisting of HEPES buffer solution pH 7.4 which was constantly stirred with a small magnetic bar and thermostated at 37 °C throughout the experiments. 350 μL of liposome suspension at 150 μg/mL betamethasone concentration, were placed in the donor chamber onto the polycarbonate membrane, in non-occlusive conditions. The diffusion area was 1.767 cm^2. Samples of receptor phase (<0.5 mL) were withdrawn after predetermined time intervals (0.5, 1, 2, 3, 4, and 6 h) and the betamethasone concentration was measured by HPLC. Each sample removed was replaced by an equal volume of fresh receptor phase. The calculated betamethasone was plotted as a function of time. All the permeation studies obtained were determined in triplicate in three independent experiments and the mean values ± standard deviation were calculated.

3. Results and discussion

3.1. Liposome characterization

Deformable liposomes containing betamethasone–HPγCD or betamethasone–Crysmeb inclusion complexes with sodium deoxycholate as the edge activator were always compared to the corresponding formulation of non-deformable liposomes. Two lipid compositions were tested: PC or DMPC. PCS and freeze-fracture electron microscopy were performed for size analysis and morphology determination. As shown in Table 1, liposomes are characterized by mean hydrodynamic diameters between 206 nm (±2.5) and 280 nm (±26.1). The polydispersity indexes (not shown) were always lower than 0.2, indicating that liposomes were homogeneous in size, except for deformable liposomes made of DMPC, whatever the cyclodextrin used. In the latter case, different populations with sizes ranging from a few nm up to more than 500 nm were observed. These observations were confirmed by freeze-fracture electron microscopy and could not be explained at the present time. This phenomenon was not observed either for classical liposomes made of DMPC or for deformable liposomes

Table 1
Size ± S.D. (nm) and encapsulation efficiencies ($EE_{B/l}$) ± S.D. (%) and ($EE_{B/Bt}$) ± S.D. (%) of liposomes containing betamethasone (BMS)–cyclodextrin complexes as a function of their composition (n = number of batches, ND = not determined).

Composition	Size ± S.D. (nm)	n	$EE_{B/l}$ ± S.D. (%)	n	$EE_{B/Bt}$ ± S.D. (%)	n
DMPC–BMS-HPγCD	280 ± 26.1	4	0.98 ± 0.14	4	24.1 ± 3.4	4
DMPC–BMS-HPγCD–deoxycholate Na	ND		0.89 ± 0.13	4	19.2 ± 2.9	4
DMPC–BMS-Crysmeb	265 ± 27.6	3	0.74 ± 0.04	3	17.2 ± 1.0	3
DMPC–BMS-Crysmeb–deoxycholate Na	ND		0.73 ± 0.03	3	14.5 ± 0.8	3
PC–BMS-HPγCD	269 ± 7.9	3	1.63 ± 0.09	7	40.6 ± 2.1	7
PC–BMS-HPγCD–deoxycholate Na	209 ± 4.9	3	2.11 ± 0.17	7	45.3 ± 3.7	7
PC–BMS-Crysmeb	261 ± 6.7	3	1.31 ± 0.01	3	30.4 ± 0.1	3
PC–BMS-Crysmeb–deoxycholate Na	206 ± 2.5	3	2.29 ± 0.21	3	46.0 ± 4.3	3

composed of PC. These deformable PC liposomes showed a significantly smaller size than that of the corresponding non-deformable liposome, whatever the cyclodextrin used (206 and 209 nm for PC deformable liposomes with Crysmeb or HPγCD betamethasone complexes versus 261 and 269 nm for PC non-deformable liposomes with Crysmeb or HPγCD betamethasone complexes; $p < 0.05$). This reduction of the particle size for deformable PC 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. (2009). Betamethasone in cyclodextrin in deformable PC liposomes showed very good size reproducibility from batch to batch. The choice of cyclodextrins, HPγCD or Crysmeb, did not influence the size of PC deformable liposomes containing inclusion complexes. To confirm the results by an imaging method, a freeze-fracture technique with subsequent transmission electron microscopy was used. This technique was only applied on liposomes containing betamethasone–HPγCD inclusion complexes. Pictures obtained are shown in Fig. 1. The bar represents 200 nm. Fig. 1(A) and (C) represents classical liposomes made of PC and DMPC, respectively. These two pictures show the presence of ±200 nm small unilamellar vesicles of homogeneous size. Deformable liposomes made of PC are shown in Fig. 1(B). We can observe that these vesicles showed a more flattened shape in comparison with the shape of the corresponding classical liposomes, maybe due to their elastic properties. Fig. 1(D) shows deformable liposomes made of DMPC. This picture shows the presence of big vesicles with a size greater than 1 μm. The results from PCS and freeze-fracture electron microscopy measurements were in good agreement.

Betamethasone encapsulation efficiencies are reported in Table 1. It must be noted that encapsulation efficiency $EE_{B/l}$ represents the drug to lipid ratio, which explains the relatively low values obtained in comparison with the $EE_{B/Bt}$ expressing the encapsulation efficiency as a function of the total drug concentration. The addition of sodium deoxycholate significantly enhanced the $EE_{B/l}$ from 1.63% (±0.09) to 2.11% (±0.17) for PC liposomes containing betamethasone–HPγCD complexes, and from 1.31% (±0.01) to 2.29% (±0.21) for PC liposomes containing betamethasone–Crysmeb complexes ($p < 0.05$). Concerning the calculation of the yield, the addition of sodium deoxycholate significantly enhanced the $EE_{B/Bt}$ from 40.6 ± 2.1 to 45.3 ± 3.7 for PC liposomes containing betamethasone–HPγCD complexes,

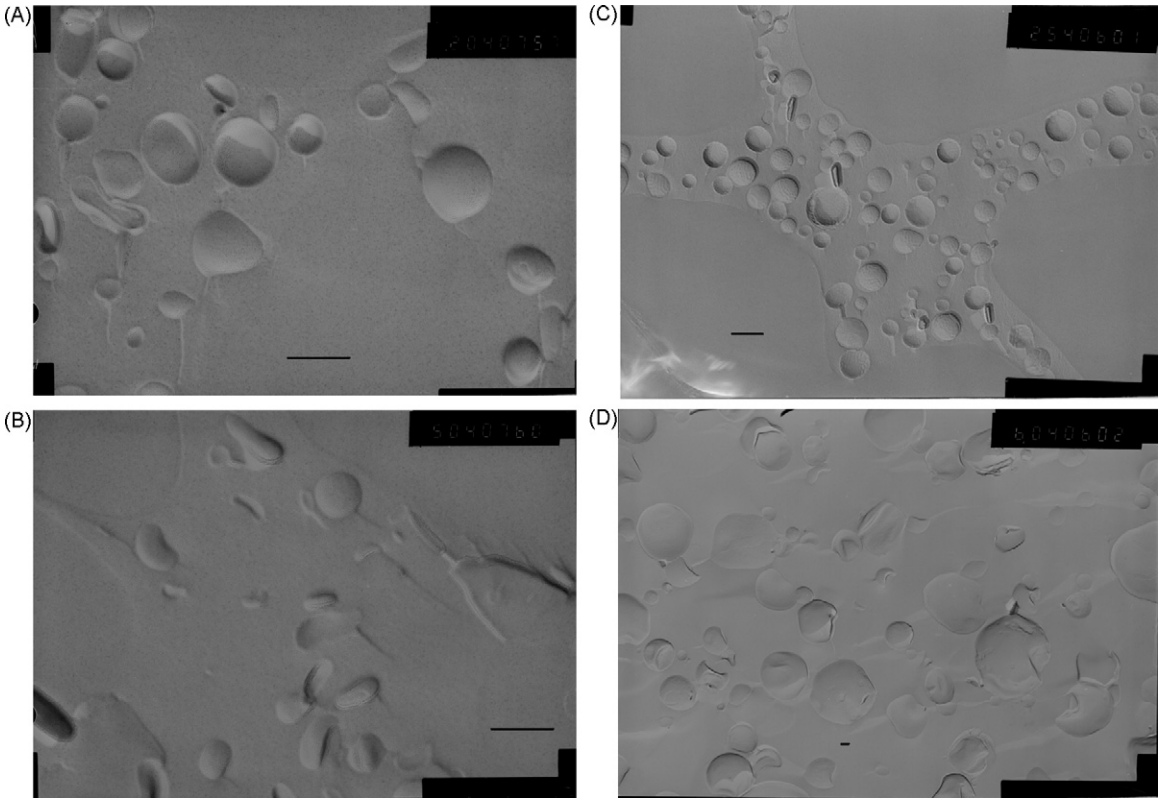

Fig. 1. Transmission electron micrographs of freeze-fractured liposomes containing betamethasone—HPγCD inclusion complexes. Classical PC liposomes (A), deformable PC liposomes (B), classical DMPC liposomes (C), and deformable DMPC liposomes (D). The bar represents 200 nm.

Table 2
Microviscosity as a function of the liposome composition ($n > 4$; S.D. <2%).

Composition	Microviscosity
PC–cholesterol	226.0 mPa s
PC	175.9 mPa s
PC–deoxycholate Na	169.2 mPa s
PC–deoxycholate Na–BMS-Crysmeb	166.2 mPa s
PC–deoxycholate Na–BMS-HPγCD	166.2 mPa s

and from 30.4 ± 0.1 to 46.0 ± 4.3 for PC liposomes containing betamethasone–Crysmeb complexes ($p < 0.05$). This increase in encapsulation efficiency could be explained by the presence of sodium deoxycholate in the bilayer, which could be supposed to be able to "solubilise" and "hold" the free betamethasone (betamethasone–cyclodextrin complex is in equilibrium with free betamethasone and cyclodextrin) in the lipid bilayer and therefore enhance the encapsulation efficiency for the deformable liposomes, as observed by Chen et al. (2009) with fenofibrate. Concerning DMPC liposomes, the encapsulation efficiency was always lower than that of corresponding PC liposomes. In addition, no difference was observed between deformable DMPC liposomes and DMPC liposomes without sodium deoxycholate, whatever the cyclodextrin used ($p > 0.05$). The results obtained for size and encapsulation efficiency led us to abandon the formulation of liposomes made of DMPC.

The deformability was evaluated by measuring the microviscosity of the liposome membrane by electron spin resonance spectroscopy. These experiments were only carried out on PC liposomes. ESR spectra were collected for the 5-DSA probe. The *S* parameter was measured at 32 °C, to evaluate the microviscosity of vesicles at skin temperature. Results are summarised in Table 2. Measurements showed that the microviscosity of empty PC liposomes is higher in the presence of cholesterol (70:30 mol%) and in the absence of cholesterol, respectively 226 mPa s and 175.9 mPa s, than the PC deformable ones (169.2 mPa s). In fact, cholesterol is known to increase the stability and thus, the rigidity of liposome membranes. This explains the difference observed for classical liposomes with or without cholesterol (226 mPa s versus 176 mPa s). The addition of the edge activator allowed a decrease in the microviscosity of the membrane to around 169 mPa s. This decrease in membrane microviscosity may reflect a deformation increase in these vesicles. Moreover, in comparison with the non-deformable liposomes, the incorporation of betamethasone–cyclodextrin complexes allowed a reduction of the microviscosity to 166 mPa s, whatever the cyclodextrin used. The difference in microviscosity between empty deformable liposomes and deformable liposomes containing the inclusion complexes is not significant. Thus, the addition of inclusion complexes did not significantly influence the viscosity of the membrane of the deformable liposomes. EPR spectroscopy seemed to show a tendency towards higher deformability of the new deformable vesicles.

The stability of PC liposomes was first evaluated by measuring the leakage of encapsulated calcein. When calcein was able to leak out from the vesicles into the surrounding buffer, the fluorescence increased dramatically, due to the relief from the self-quenching that occurs. Control wells contained the detergent Triton X-100® to rupture the vesicles and cause complete calcein release. This allowed us to determine maximum fluorescence for the proper normalization of results. The leakage of calcein from the interior of liposomes was measured every day for 8 days. The results are shown in Fig. 2(A) and (B). No significant increase in calcein leakage was observed when liposomes were stored at 4 °C, under nitrogen ($p > 0.05$). However, Fig. 2(B) shows a rise in calcein leakage with temperature, up to around 32% in comparison with day 0, after 8 days of storage at 37 °C for deformable liposomes. Loss of calcein

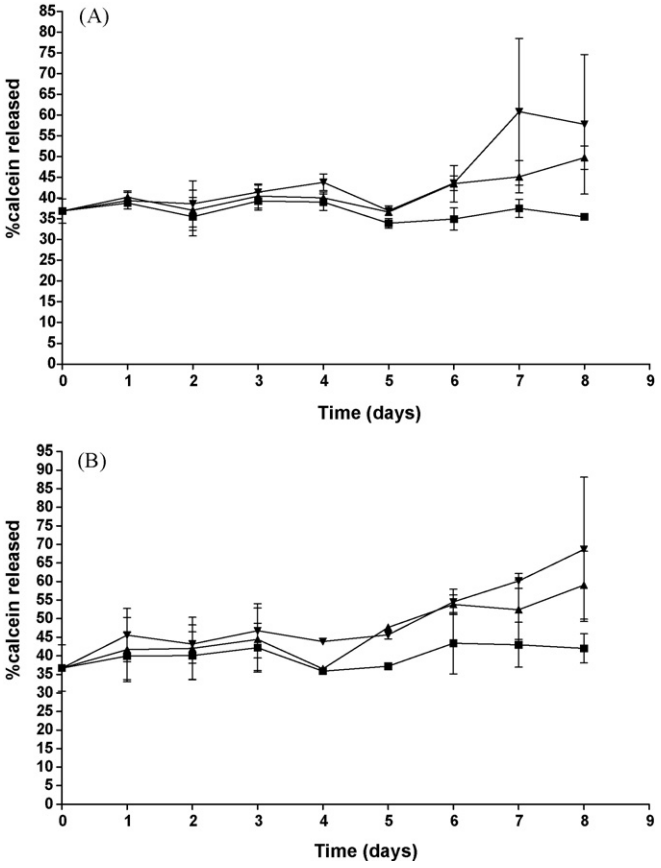

Fig. 2. Liposome membrane permeability, expressed as a percentage of calcein released from PC liposomes as a function of time. (A) Classical liposomes and (B) deformable liposomes are stored at three different temperatures: 4 °C (■); 25 °C (▲); 37 °C (▼).

from the vesicles stored at elevated temperatures may be attributed to the effect of temperature on the gel to liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in the membrane packing, as observed by Dubey et al. (2006).

After 1 month of storage at 4 °C, the increase in the percentage of calcein released compared to day 0 was 9.43% (±5.38) and 23.75% (±4.36) for classical and deformable liposomes respectively (results not shown). It must be noted that the basal fluorescence, at day 0, was around 37% for both classical and deformable PC liposomes.

In a second experiment, the stability of PC liposomes containing betamethasone–cyclodextrin inclusion complexes was evaluated by measuring the leakage of encapsulated betamethasone. Fig. 3(A) and (B) shows the results obtained for liposomes containing betamethasone–HPγCD and betamethasone–Crysmeb inclusion complexes respectively. We can observe that, whatever the cyclodextrin used, PC classical and deformable liposomes were stable for 1 month at 4 °C ($p > 0.05$). As observed for the release of calcein, the leakage of betamethasone increased with an increased temperature. However this increase occurred more slowly than was observed with calcein. The higher release of calcein compared to betamethasone could be explained by the concentration gradient that appears through the lipid bilayer. Betamethasone–cyclodextrin inclusion complexes are in equilibrium with free betamethasone and cyclodextrin. Because only free betamethasone can diffuse through the liposome membrane, the gradient is smaller in the case of betamethasone than for calcein, which is not complexed with cyclodextrin. According to Fick's law, higher is the gradient of concentration higher is the flux through the membrane. The percentage

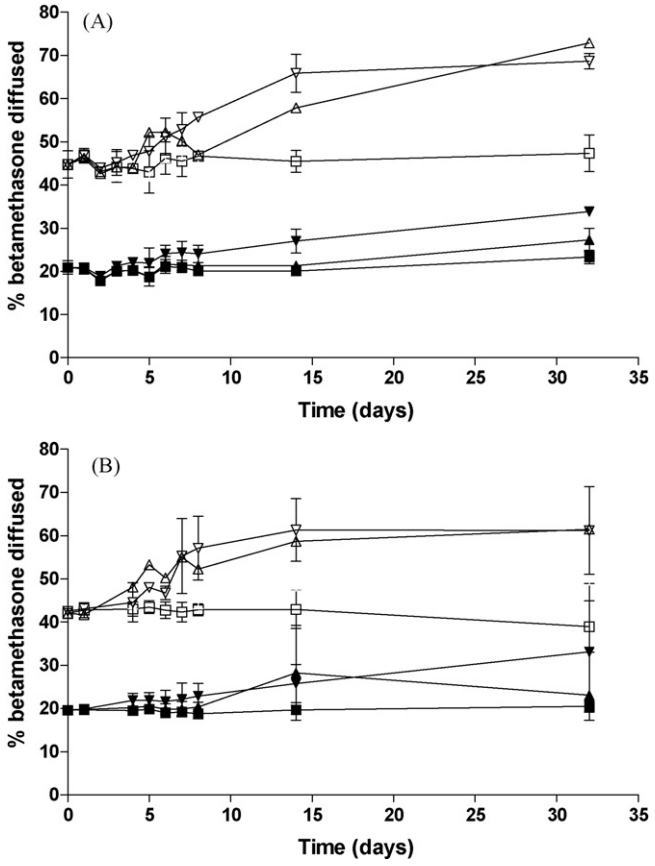

Fig. 3. Liposome membrane permeability, expressed as a percentage of betamethasone released from PC liposomes as a function of time. (A) Liposomes containing betamethasone–HPγCD inclusion complexes, and (B) liposomes containing betamethasone–Crysmeb inclusion complexes. Classical (dark symbol) and deformable (open symbol) liposomes are stored at three different temperatures: 4 °C (■, □); 25 °C (▲, △); 37 °C (▼, ▽).

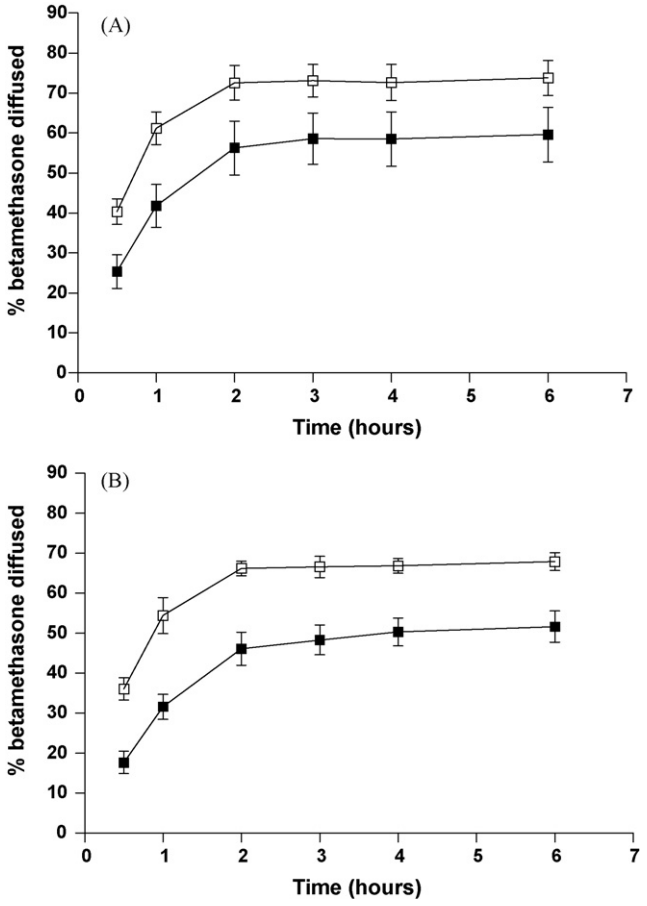

Fig. 4. Comparison of betamethasone release kinetics from (■) classical PC liposomes containing betamethasone–cyclodextrin inclusion complexes and from (□) deformable PC liposomes containing betamethasone–cyclodextrin inclusion complexes: (A) HPγCD and (B) Crysmeb.

of betamethasone diffused at day 0 was about 20% for classical liposome and more than 40% for deformable ones, whatever the cyclodextrin used. This high level of difference was not observed for liposomes encapsulating calcein. The ultracentrifugation cycle, needed before each measurement in the betamethasone release study, could explain this difference. The loss of entrapped drug was probably due to the deformation phenomenon of lipid membrane that occurs during the ultracentrifugation process, as observed by Lopez-Pinto et al. (2005). Deformable liposomes are certainly more sensitive to ultracentrifugation than classical liposomes because of the presence of sodium deoxycholate, which destabilizes the membrane and makes it more permeable.

3.2. *In vitro diffusion studies*

Franz type diffusion cells were used to evaluate the *in vitro* diffusion of betamethasone from liposomes. Test conditions were chosen in order to respect sink conditions in the receiver compartment. Saturation concentration of betamethasone in HEPES buffer was evaluated at 65.2 μg/mL, corresponding to around 10 times the maximum concentration of betamethasone that could be found in the receiver compartment. All samples were adjusted at a final betamethasone concentration of 150 μg/mL.

The polycarbonate membrane used had a pore size of 50 nm, a smaller size than the mean diameter of liposomes. The diffusion studies were carried out in non-occlusive conditions to allow the driving force provided by the osmotic gradient. Fig. 4(A) and (B) shows the cumulative percentage of betamethasone released from classical and deformable liposomes containing betamethasone–HPγCD and betamethasone–Crysmeb inclusion complexes respectively, during 6 h of diffusion. The presence of sodium deoxycholate enhanced the percentage of betamethasone diffused from 59.63% (±7.03) to 73.79% (±4.61), when HPγCD was used ($p < 0.001$) (Fig. 4 (A)). When Crysmeb was used, the presence of sodium deoxycholate enhanced the percentage of betamethasone diffused from 51.64% (±4.25) to 67.92% (±0.26) ($p < 0.001$) (Fig. 4(B)). The type of cyclodextrin did not influence significantly the percentage of betamethasone diffused (for classical liposome, $p = 0.1676$ and for deformable liposomes, $p = 0.0925$). The release profiles exhibited a plateau effect after about 3 h diffusion. This plateau was due to donor depletion, which occurs where the proportion of permeant entering the membrane is large, relative to the amount applied (finite dose technique). The percentage of betamethasone diffused did not rise up to 100%, even after 24 h of diffusion (results not shown) and even if sink conditions were respected. This observation could be explained by the fact that the liposomal formulation did not completely dry up after the diffusion time; a part of sample remaining in the donor chamber. The amount of phospholipids that had permeated through the membrane was determined in the receptor phase by the enzymatic assay. At the end of the experiments, 4 mL of receptor phase was lyophilized and dispersed in 200 μL water in order to concentrate this receptor phase. Even then, no phospholipids were found in the receiver compartment. The first explanation was that the membrane used is hydrophilic and may not appropriate for studying the diffusion of liposomes, but only that of the aqueous

betamethasone–cyclodextrin complexes. However, this fact was also observed by Sinico et al. (2005). They studied the diffusion of liposomes encapsulating tretinoin through a silicone membrane, which is a polymeric hydrophobic membrane, and they did not find any phospholipids in the receiver compartment either; even though about 16% of tretinoin permeated after 24 hours from their liposomal formulation. The improvement of betamethasone release from deformable liposomes could be explained by a higher bilayer permeability because of the presence of sodium deoxycholate. We could not show a diffusion of intact deformable liposomes through the polycarbonate membrane. *Ex vivo* diffusion studies using pig skin would help us to confirm these results and to understand the diffusion mechanism.

4. Conclusion

This study proved the feasibility of a new topical delivery system: drug-in-cyclodextrin-in deformable liposomes and showed its promising characteristics for future experiments using pig ear skin and human skin. The use of sodium deoxycholate as the edge activator increased up to 1.8 times the encapsulation efficiency and up to 1.3 times the percentage of betamethasone diffused in comparison with classical liposomes. These new vesicles showed higher deformability and a good stability, when stored at 4 °C.

Ex vivo diffusion studies using pig skin and *in vivo* studies would help us to confirm these *in vitro* results.

Acknowledgement

A. Gillet is a Ph.D. student supported by the FNRS, Brussels, Belgium.

References

Bahri, M., Heyne, B., Hans, P., Seret, A., Mouithys-Mickalad, A., Hoebeke, M., 2005. Quantification of lipid bilayer effective microviscosity and fluidity effect induced by propofol. Biochem. Chem. 114, 53–61.

Barry, B.W., 2001. Novel mechanism and devices to enable successful transdermal drug delivery. Eur. J. Pharm. Sci. 14, 101–114.

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

Cevc, G., Blume, G., 2001. New, highly efficient formulation of diclofenac for topical, transdermal administration in ultradeformable drug carriers, transfersomes. Biochem. Biophys. Acta 1514, 191–205.

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

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

Chen, Y., Lu, Y., Chen, J., Lai, J., Sun, J., Hu, F., Wu, W., 2009. Enhanced bioavailability of the poorly water-soluble drug fenofibrate by using liposomes containing a bile salt. Int. J. Pharm., doi:10.1016/j.ijpharm.2009.04.022.

Dubey, V., Mishra, D., Asrhana, A., Jain, N.K., 2006. Transdermal delivery of a pineal hormone: melatonin via elastic liposomes. Biomaterials 27, 3491–3496.

Elsayed, M.M.A., Abdallah, O.Y., Naggar, V.F., Khalafallah, N.M., 2007. Lipid vesicles for skin delivery of drugs: reviewing three decades of research. Int. J. Pharm. 332, 1–16.

Flood, K., Reynolds, E., Snow, N., 2000. Characterization of inclusion complexes of betamethasone-related steroids with cyclodextrins using highperformance liquid chromatography. J. Chromatogr. A 903, 49–65.

Honeywell-Nguyen, P.L., Bouwstra, J.A., 2005. Vesicles as a tool for transdermal and dermal delivery. Drug Discov. Today: Technol. 2, 67–74.

Hubbell, W.L., McConnel, H.M., 1971. Molecular motion in spin-labeled phospholipids and membranes. J. Am. Chem. Soc. 93, 314–326.

Jain, S.K., Gupta, Y., Jain, A., Amin, S., 2008. Elastic liposomes bearing meloxicam-β-cyclodextrin for transdermal delivery. Curr. Drug Deliv. 5, 207–214.

Lopez-Pinto, J.M., Gonzales-Rodrigez, M.L., Rabasco, A.M., 2005. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int. J. Pharm. 298, 1–12.

Maestrelli, F., Gonzales-Rodriguez, M.L., Rabasco, A.M., Mura, P., 2005. Preparation and characterisation of liposomes encapsulating ketoprofen–cyclodextrin complexes for transdermal drug delivery. Int. J. Pharm. 298, 55–67.

McCormack, B., Gregoriadis, G., 1994. Drugs-in-cyclodextrins-in-liposomes: a novel concept in drug delivery. Int. J. Pharm. 112, 249–258.

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.

Trotta, M., Peira, E., Carlotti, M.E., Gallarate, M., 2004. Deformable liposomes for dermal administration of methotrexate. Int. J. Pharm. 270, 119–125.